농 학 박 사 학 위 논 문

AtMYB44 modulates crosstalk between jasmonate- and salicylate-mediated plant defense responses

자스몬산과 살리실산에 의한 식물의 방어 반응을 길항적으로 조절하는 ***AtMYB44*** 전사인자의 기능 분석

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Thesis for the Degree of Doctor of Philosophy

AtMYB44 modulates crosstalk between jasmonate- and salicylate-mediated plant defense responses

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ABSTRACT

Transcription factors, which can interact with *cis*-element and regulates series of responsive genes, are critical components in a complex signal network. AtMYB44, an R2R3 MYB transcription factor, is associated with various defense signal cascades. *AtMYB44* is induced by jasmonic acid (JA) through CORONATINE INSENSITIVE 1 (COI1) and by salicylic acid (SA) through the NONEXPRESSOR OF PR1 (NPR1) independent pathway. *AtMYB44* overexpression plants showed lower defense responses against a necrotrophic pathogen *Alternaria brassicicola* (*A. brassicicola*). It coincides with the suppression of JA-mediated defense responsive genes by *AtMYB44* overexpression. *AtMYB44* overexpression resulted in elevated expression of *WRKY70* and *PR* genes, leading to enhanced resistance to the biotrophic pathogen *Pseudomonas syringae* pv tomato *DC3000*. The knockout mutant *atmyb44* showed opposite effects. To test whether the effects of *AtMYB44* overexpression are mediated by SA, *AtMYB44* overexpression plants were crossed with the SA depleting plant *NahG*. Overexpression phenotype of *AtMYB44* such as growth retardation, up-regulation of *PR* genes and down-regulation of *PDF1.2* were reversed by SA depletion. It means that SA is required for function of *AtMYB44*. ß-estradiol-induced expression of *AtMYB44* resulted in hierarchical activation of *WRKY70* and *PR1*. Induction of *WRKY70* by SA is reduced in the *atmyb44* and *npr1-1*, and is totally abolished in *atmyb44 npr1*-1 double mutant. These data showed that *WRKY70* is also regulated by *AtMYB44* through the *NPR1* independent pathway. Effect of *AtMYB44*

overexpression such as activation of *PR1* and suppression of *PDF1.2* are abolished in the *wrky70* knockout mutant. It suggests that activation of *PR* genes and suppression of *PDF1.2* by *AtMYB44* are mediated by *WRKY70*. AtMYB44 directly binds to at least two *WRKY70* promoter regions containing the *cis*-element (5'-CNGTTA-3'), and the C-terminal region of AtMYB44 has transcriptional activation activity. It indicates that AtMYB44 act a transcriptional activator of *WRKY70* through direct binding to a *cis*-element in the *WRKY70* promoter. These results demonstrate that AtMYB44 regulates antagonistic interaction between JA and SA by activating SA-mediated defense responses and suppressing JA-mediated defense responses through direct control of *WRKY70* expression.

Keywords; *Arabidopsis*, *AtMYB44*, Jasmonate, R2R3 MYB transcription factor, Salicylate, *WRKY70*

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LIST OF ABBREVIATIONS

35S	cauliflower mosaic virus promoter
bHLH	basic helix-loop-helix
ChIP	chromatin immunoprecipitation
COI1	coronatine insensitive1
COR	coronatine
EMSA	electrophoretic mobility shift assay
ET	ethylene
ETI	effector triggered immunity
GST	Glutathion-S-transferase
GUS	beta-glucuronidase
JA	jasmonic acid
JA-Ile	jasmonoyl-isoleucine
JAs	jasmonates
JAR1	jasmonate resistant1
JAZ	jasmonate ZIM-domain
MeJA	methyl jasmonate
MeSA	methyl salicylate
MS	Murashige and Skoog
NB-LRR	nucleotide-binding site-leucine rich repeat
NINJA	novel interactor of JAZ

NPR1	nonexpressor of *PR1*
PCD	programmed cell death
PAMP	pathogen associated molecular pattern
PR	pathogenesis related
PRRs	pattern-recognition receptors
PTI	pattern triggered immunity
Pst DC3000	*Pseudomonas syringae* DC3000
qPCR	quantitative polymerase chain reaction
RT-PCR	reverse transcription-polymerase chain reaction
SA	salicylic acid
SAR	systemic acquired resistance
SD	synthetic dropout
TGA	TGACG motif binding
TPL	TOPLESS
Y1H	yeast one-hybrid

INTRODUCTION

In nature, plants live in complex environment in which interact with a broad range of microbial pathogens. To protect themselves against various pathogens which have different infection strategies, plants have elaborated defense strategies to perceive their attacker and to develop this perception into an effective immune responses. Two types of recognition mechanism have been defined. The first branch is pattern-triggered immunity (PTI) which triggered by the recognition of pathogen associated molecular patterns (PAMP) by host surface pattern-recognition receptors (PRRs) (Jones and Dangl, 2006). This activates sufficient defense responses against nonpathogenic microbes and some pathogenic microbe. To cope with PTI of plants, evolved pathogens have acquired the ability to deliver effectors into the plant cell to suppress plant defense responses. The second branch recognizes pathogen effectors which introduced in plant cell by nucleotide-binding site-leucine rich repeat (NB-LRR) resistance (R) protein. It results in activation of effector-triggered immunity (ETI) (Jones and Dangl, 2006; Robert-seilaniantz *et al*., 2011).

The regulation of defense responses is mediated by accumulation of plant hormones. These hormones is required for the regulation of development, growth and survival to abiotic and biotic stresses. Upon pathogen attack, plant produced tailored blend of phytohormones according to infection strategy and life style of challenged pathogen. This activates distinct but overlapping sets of defense-mediated genes. In this process, regulation of quantity and composition of SA, JA and ET is important to

develop tailored defense response according to specific purpose (Reymond and Farmer, 1998; Kunkel and Brooks, 2002; Spoel *et al*., 2003; Robert-Seilaniantz *et al*., 2011).

1. Salicylic acid signaling

Salicylic acid is a monohydroxybenzoic acid and is produced in wide range of organism as a secondary metabolite. In plants, SA functions as an important hormone required for innate immunity against biotrophic pathogen and hemibiotrophic pathogen (Feys and Parker, 2000; Durrant and Dong, 2004).

Most of induced SA is produced by isochorismate synthease (ICS). In Arabidopsis, The ICS1 was characterized through loss-of-function mutants salicylic acid induction deficient 2 (*sid2*) and *enhanced disease susceptibility 16* (*eds16*) (Dewdney *et al*., 2000; Wildermuth et al., 2001). The major route for the biosynthesis of SA takes place in the chloroplast. The SA produced in chloroplast is exported into the cytoplasm by EDS5. The lipase-like protein ENHANCED DISEASE SUSCEPTIBILITY 1 (EDS1) and its interacting partner PHYTOALEXIN DEFICIENT 4 (PAD4) act upstream of SA in PAMP as well as ETI (Parker *et al*., 1996; Fey *et al*., 2001) The enhanced susceptibility of their mutants can be rescued by exogenous SA, placing these genes upstream of SA accumulation.

1.1. NPR1 dependent pathway

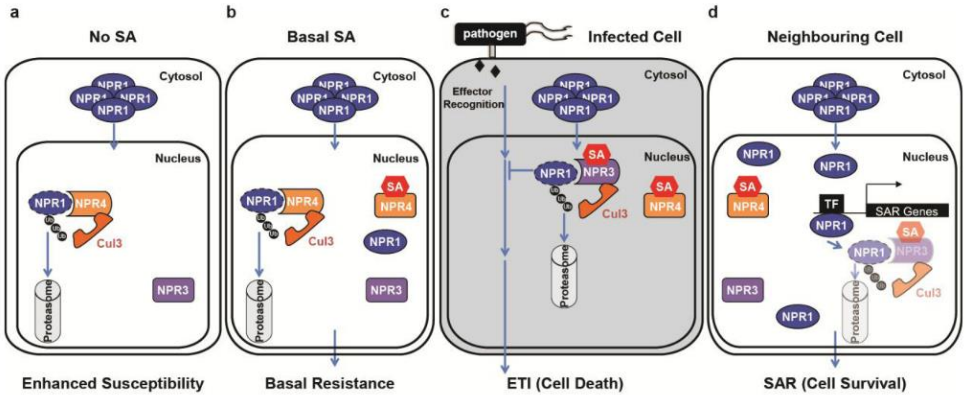
In SA-mediated defense responses, *NPR1* is a central regulator (Cao *et al*., 1997; Spoel *et al*., 2003; Durrant and Dong, 2004). It was isolated in a genetic screening for mutants that failed to express *PR1* after Systemic Acquired resistance (SAR) induction (Cao *et al*., 1994). Mutation in the *NPR1* gene block SA-mediated transcriptional reprogramming and compromises their ability to mount SAR. NPR1 contains an ankyrin-repeat motif which is involved in protein-protein interaction. Although lacking a DNA binding domain, NPR1 regulates expression of *PR* gene through interaction with TGACG motif binding (TGA) transcription factors (Zhang *et al*., 1999).

In the absence of SA, NPR1 is sequestered in cytoplasm as a multimers through intermolecular disulfide bond via S-nitrosylation (Spoel *et al*., 2003; Tada *et al*., 2008). SA accumulation by perception of pathogen induces a redox change in the cytosol leading to the monomerization of NPR1 through thioredoxins and migration of NPR1 monomer into the nucleus. In the nucleus, NPR1 interacts with TGA transcription factors and enhances their binding to promoter of SA-responsive genes. This initiates the SA-mediated global transcriptional regulation (Mou *et al*., 2003; Spoel *et al*., 2003; Tada *et al*., 2008; Robert-Seilaniantz *et al*., 2011).

The downstream regulation of SA regulates defense responses were well-

documented but the perception and initiation of signal cascade of SA are recently illuminated by the discovery of NPR3 and NPR4 (Fu *et al*., 2012). NPR3 and NPR4 encode adaptors protein of Cullin 3 (CUL3) ligase and each of them is interact with NPR1 in different nuclear condition. NPR3 has low SA-binding affinity and requires high levels of SA for its interaction with NPR1. However, NPR4 has higher SA-binding affinity than NRP3 and its interaction with NPR1 is disrupted by SA. In healthy plants where SA is existed in very low level, NPR4 constantly interact with NPR1 and removes most of NPR1 via CUL3-NPR4 mediated protein degradation. This regulation is required to prevent abnormal activation of energy-consuming defense responses. In infected plants where SA is existed in high level, interaction of NPR4 and NPR1 is disrupted by SA and it allows accumulation of NPR1 in nucleus. At the same time, NPR3 is interact with NPR1 and its consequence in degradation of NPR1. Proteasome-mediated turnover of NPR1 is required for ETI, characterized by rapid programed cell death (PCD). Thus, CUL3-NPR3 mediated fast NPR1 turnover in infected tissue leading to strong defense response such as PCD (Figure 1; Fu *et al*., 2012; Moreau *et al*., 2012).

Figure 1. Current model of SA signal transduction. (a) In SA-deficient background, NPR1 is depleted due to $CUL3^{NPR4}$-mediated degradation by the proteasome, leading to enhanced disease susceptibility. (b) In WT plants, basal SA may bind to the NPR4 complex to reduce tis accessibility to NPR1 to allow some NPR1 to accumulate to confer basal resistance. (c) , (d) In response to pathogen challenge, SA levels are increased both locally and systemically. The highest level of SA inside the lesion may lead to $CUL3^{NPR3}$-mediated degradation of NPR1 to allow effector-triggered PCD and resistance. (c) In the neighboring cells, the lower level of SA limits NPR1-NPR3 interaction, enabling NPR1 to accumulate to inhibit PCD and establish SAR. (d) The $CUL3^{NPR3}$-mediated degradation in these cells facilitates target gene expression probably through enhanced transcription reinitiation. Ub, Ubiquitin; TF, transcription factor.



Fu *et al*., 2012, *Nature*, **486**:228-232.

1.2. NPR1 independent pathway

Salicylic acid (SA) is an important signal molecule regulating plant defense responses. The Arabidopsis NPR1 is a key component of the SA signal transduction pathway. However, loss-of-function mutations in NPR1 do not confer complete loss of SA-mediated resistance. The SA-depleted *NahG* plants are more susceptible to *Pst* DC3000 than the *npr1* mutant. Moreover, resistance against turnip crinkle virus in *Arabidopsis* has been shown to be SA dependent, but NPR1-independent pathway (Kachroo *et al*., 2000). It means that SA-mediated defense responses also can be activated via an NPR1-independent pathway. The existence of an NPR1-independent pathway is further supported by several studies of various *Arabidopsis* mutants which constitutively accumulate SA and the transcript of *PR* genes even in the absence of NPR1. A putative negative regulator of SAR, SNI1, was identified through screening for suppressors of the *npr1-1* mutant (Li *et al*., 1999). In the *npr1-1* mutant background, the *sni1* mutation restores *PR* gene induction by SA and pathogen infection. More components in the NPR1-independent pathway were identified through screening of EMS mutagenized *npr1-5* for constitutive *PR* gene expression. The *ssi* mutants show constitutive expression of *PR* genes and accumulation of SA (Shah *et al*., 2001; Shirano *et al*., 2002). Moreover, WHIRLY (WHY) and MYB transcription factor are also reported as NPR1 independent components. *AtWHY1* is induced in *npr1-1* mutants by SA treatment, thus activates expression of *PR* genes

(Desveaux *et al*., 2004). *AtMYB30* positively regulates the pathogen-induced HR in an SA-dependent, NPR1 independent manner (Raffaele *et al*., 2006).

1.3. SA in systemic acquired resistance

Both PTI and ETI are associated with the activation of defense responses including accumulation of SA and expression of *PR* genes in local infected tissues (Jones and Dangl, 2006; Robert-seilaniantz *et al*., 2011). In addition to these local responses, PTI and ETI also trigger defense responses in the systemic tissues (uninfected distal part of plants) known as SAR (Durrant and Dong, 2004; Dempsey and Klessig, 2012). For SAR to be worked in the systemic tissues, a signal produced in infected tissues is transported to systemically via the vasculature such as phloem. Continued efforts to identify the putative phloem mobile signal have implicated a methylated SA (MeSA), G3P, lipid transfer protein (LTP) DIR1, dicarboxylic acid AzA, abietane diteroenoid DA and JA (Park *et al*., 2007; Maldonado *et al*., 2002; Jung *et al*., 2009; Dempsey and Klessig, 2012).

MeSA, methylated derivative of SA, is one of the mobil signal which required in systemic tissue for SAR development. To illuminate mechanism of SAR development, several SA-binding proteins have been found such as SABP2 (Kumar and Klessig, 2003). SABP2 is a methyl salicylate esterase and its activity is inhibited by binding of SA (Forouhar *et al*., 2005). MeSA is synthesized from SA by SA methyltransferase such as NtSAMT1 (SA METHYLTRANSFERASE 1), AtBSMT1 (BENZOIC ACID/

SA CARBOXYL METHYL TRANSFERASE 1) and OsBSMT1 (Koo *et al*., 2007; Park *et al*., 2007; Liu *et al*., 2010). The hypothesis that conversion between SA and MeSA is required for SAR development was validated by graft and transgenic plants research. SAR is suppressed by altered expression of SA methyltransferase and salicylate esterase (Koo *et al*., 2007; Park *et al*., 2007; Dempsey and Klessig, 2012).

NtSAMT1/AtBSMT1 activity in the pathogen inoculated leaves converts some of the SA into MeSA. At the same time, MeSA is converted to SA by MES (MeSA esterase) such as SABP2, and leading to maintain absent or low level of MeSA in plant tissues. When the level of SA is increased in infected tissues, activity of SABP2 is inhibited by binding of SA to SAPB2. It allows accumulation of MeSA and accumulated SA travels to the systemic tissue through phloem. Once MeSA is introduced in systemic tissues, MeSA is converted to SA by SABP2, and leading to accumulation of SA in systemic tissues. In systemic tissues, SA is existed in intermediate level and it results in weak interaction of both NRP4-NPR1 and NPR3-NPR1. This allows accumulation of NPR1 and establishment of systemic acquired resistance in systemic tissues without PCD (Robert-Seilaniantz *et al*., 2011; Moreau *et al*., 2012).

2. Jasmonic acid signaling

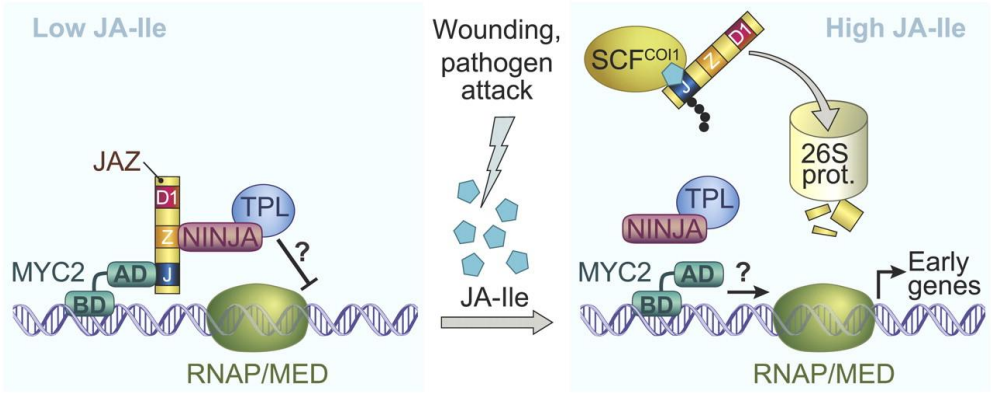
Jasmonic acid and its derivatives, collectively referred to as jasmonates (JAs), are

derived from the linolenic acid and act important regulators in plant defense responses. JA is central regulator to modulating defense response against herbivore and necrotrophic pathogens. Most of JA-mediated responses are mediated through COI1 (Fey *et al*., 1994; Xie *et al*., 1998; Devoto *et al*., 2002). The COI1 is characterized by mutant screening on the purpose of interconnecting between coronatine- and JA-mediated signaling because they showed similar growth inhibiting effects on Arabidiosis (Fey *et al*., 1994). All of 14 independent coronatine-insensitive mutants were alleles at the *coi1* locus. COI1 encodes F-box protein and it is participated in formation of SCF^{COI1} complex (Xie *et al*., 1998; Devoto *et al*., 2002; Xu *et al*., 2002). F-box proteins associates with Cullin, Skp1 and Rbx1 proteins to form an E3 ubiquitin ligase as known Skp/Cullin/F-box (SCF) complex. Other components of SCF complex such as AXR1, COP9 and SGT1b also required for JA-mediated signaling cascade (Lorenzo and Solano, 2005). In SCF complex, F-box protein has a role to determine target proteins for ubiquitin-mediated degradation by the 26S proteasome. Subsequently, identification of COI1 target proteins is one of the major quests in the JA field. Almost a decade after characterization of F-box protein COI1, JAZ proteins were characterized as substrate of COI1 (Chini *et al*., 2007; Thines *et al*., 2007; Yan *et al*., 2007). JAZ protein interact with the positive regulator of JA signaling, MYC2, and degraded by the SCF^{COI1} complex response to JA (Chini *et al*., 2007; Thines *et al*., 2007). It interacts with Novel Interactor of JAZ (NINJA) which containing EAR motif, and recruits corepressor TOPLESS (TPL). This JAZ-NINJA-TPL complex act a negative regulator in JA signaling (Szemenyei *et al*., 2008;

Pauwels et al., 2010).

In cell containing low level of JA, JAZ proteins binds to transcription factors that promote the expression of JA-responsive genes and inhibits the transcriptional activity of them. JAZ-mediated suppression of JA responses is relieved in response to signal perception that activates biosynthesis of JA and Ile conjugates of JA, JA-Ile. Elevated JA-Ile levels stimulates interaction of COI1 and JAZ proteins, and results in SCF^{COI1}-mediated ubiquitination of JAZ proteins. Ubiquitinated JAZ proteins is degraded by the 26S proteasome and it allows transcriptional activation of JA-responsive genes such as MYC2 (Figure 2).

Figure 2. Current model of JA signal transduction. Low JA-Ile levels (left section) permit the accumulation of JAZ proteins (denoted with their Jas [J], ZIM/TIFY [Z], and Domain 1 [D1] regions) that bind to the bHLH-type transcription factor MYC2 (BD, DNA-binding domain; AD, activation domain). Repression of JA response genes involves binding of JAZ to NINJA, which contains an EAR motif that recruits the corepressor TPL. The mechanism by which TPL silences gene expression is unknown (?). In response to stress-related cues that activate JA-Ile synthesis, high levels of the hormone (right section) promote SCF^{COI1}-mediated ubiquitination and subsequent degradation of JAZs by the 26S proteasome (26S prot.). JAZ degradation relieves TPL-mediated repression of gene expression, and may also alleviate passive repression by allowing MYC2 to engage RNA polymerase II (RNAP) and/or the Mediator complex that links RNAP to MYC2.



Howe, 2010, *Plant Physiol*., **154**: 471-474.

2.1. The COI1-JAZ-JA-Ile receptor complex

Interaction of COI1 and JAZs is depends on intracellular concentration of Ile conjugates of JA, JA-Ile (Thine *et al*., 2007; Katsir *et al*., 2008). JA is converted to JA-Ile by JASMONIC ACID RESISTANCE 1 (JAR1), and (+)-7-iso-jasmonoyl-L-Ile is illuminated as endogenous active form of JA through ligand binding affinity analysis of various form of JA derivatives (Staswick *et al*, 1992; Suza and Staswick, 2008; Fonseca *et al*., 2009). Coronatine (COR) that is secreted from *Pseudomonas syringae* is a structural mimic of JA-Ile and have a function to mediate interaction between COI1 and JAZs (katsir *et al*., 2008). It is one of the example of hijacking of plant defense response by pathogens.

Direct interaction with COI1 has been shown for at least JAZ1, 2, 3, 6, 9 and 10. The C-terminal region containing Ja-associated (Jas) domain of JAZ proteins is required for interaction with COI1 and binding of COR to the complex (Katsir *et al*., 2008). The minimal amino acid sequence sufficient for COR binding (JAZ degron) was consisted with loop and an amphiphatic α-helix. ELPIARR which formed the loop in JAZ1 protein act as a lid of the JA-Ile binding pocket and binds both JA-Ile and COI1 (Sheard *et al*., 2010; Shyu *et al*., 2012). The JAZ degron is presented in JAZ protein but JAZ7 and JAZ8 lack the conserved basic amino acid which is essential for COI interaction. Recently, it is reported that JAZ7 and JAZ8 are more stable from SCF^{COI}-mediated degradation, and it contributes to maintain AtMYC2

repression in cross-talk between JA and GA (Shyu *et al*., 2012; Wild *et al* ., 2012).

2.2. The JAZ-NINJA-TOPLESS corepressor complex

A major advance in our understanding of how JAZ proteins suppress the expression of JA-responsive gene is accomplished by discovery of NINJA (Pauwels *et al*., 2010). NINJA was identified from tandem affinity purification approach to identify proteins that interact with JAZ1. NINJA contains an EAR motif (ERF-associated amphiphilic repression) which previously identified one of the representative repression motif (Kagale and Rozwadowski, 2011). In case of auxin, Aux/IAA proteins contain EAR motif that recruits the corepressor TPL (Tan *et al*., 2007; Szemenyei *et al*., 2008). NINJA also interact with TPL and these findings indicate that the TPL corepressor can be participated in multiple hormone signaling via the EAR motif containing proteins. Interaction of the NINJA/TPL complex with ZIM domain of JAZ protein is supported by previous genetic approach of Chung *et al*. (2010). They showed that repression of JA signaling by JAZ10.4 splice variant is dependent on the TIFY motif in the ZIM domain.

Among 12 JAZ proteins, four JAZ proteins (JAZ5, 6, 7 and 8) contain their own EAR motif (Kagale *et al*., 2010). As expected, direct interaction of JAZ5 and JAZ8 with TPL has been confirmed in a plant interactome project (Arabidopsis Interactome Mapping Consortium, 2011). It seems that recruitment mechanism of TPL to JAZ-regulated transcription factors could be contributed to multiple regulation of JA

signaling.

2.3. MYC2 transcription factor is a target of JAZ proteins

Exogenous application of JA induces broad range of transcriptional reprogramming and MYC2 has a significant contribution in this process (Berger *et al*., 1996; Lorenzo *et al*., 2004). MYC2 was characterized from a screening for jasmonate-insensitive root growth mutant. MYC2 is involved in differential regulation of two branch of the JA signaling. One of these branches, involved in wound responses, is positively regulated by MYC2 through activation of gene expression such as *VSP1*, *VSP2* and *LOX3*. The other branch involved in pathogenesis is negatively regulated by MYC2 through suppression of gene expression such as *PDF1.2* and *PR4*. Nevertheless, MYC2 functions as a transcriptional activator when targeted to a heterologous promoter (Pauwels and Goossens, 2008). Mutant analysis in Arabidopsis has also revealed that MYC2 act as a regulator of light, ABA and JA (Abe *et al*., 2003; Lorenzo *et al*., 2004; Yadav *et al*., 2005).

After its discovery by forward genetics, it is spotlighted by the characterization of its binding protein JAZ. It means that JAZ proteins might function as regulators of JA responses by binding and inhibiting the function of MYC2 (Chini *et al*., 2007). The fact that nearly all JAZ proteins interact with MYC2 in Y2H assays indicates relatively low contribution of biochemical specificity in MYC2-JAZs binding (Chini *et al*., 2007; Chung and Howe, 2009). It is reported that Jas domain is sufficient for

interaction of JAZ3 with MYC2 (Chini *et al*., 2007, 2009). Thus, Jas domain in JAZ proteins is required for interaction with COI1 and MYC2. However, mutation of Jas domain blocks interaction hormone dependent interaction of JAZ9 with COI1 but not MYC2 (Melotto *et al*., 2008). It suggests that the COI1 and MYC2 interaction with Jas domain is not identical.

JAZ3 interact with N-terminal region of MYC2 which contains plant specific sequences motifs conserved in a small group of bHLH proteins (Heim *et al* 2003; Chini *et al*., 2007). It means that other bHLH transcription factors have a possibility to interact with JAZ proteins. It also supported by mutant analysis that atmyc2 mutants are unaffected in some of JA responses including male fertility and trichome initiation (Lorenzo *et al*., 2004). Recently, Interaction of JAZ with other bHLH transcription factors, MYC3 and MYC4, was reported (Fernandez-Calvo *et al*., 2011; Niu *et al*., 2011).

3. Crosstalk between SA and JA in defense signaling

During their life time, plant encounters numerous pathogens and herbivore with diverse mode of attack. To maintain their lives, plants have to perceive attack of enemy and respond adequately by activation appropriate defense responses. Upon pathogen or insect attack, plants produce specific blend of plant hormones according to infected enemy and their infection strategy. Plant defense responses is

rapidly activated by the major signaling molecules such as 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 jasmonate-isoleucine receptor (Xie *et al*., 1998; Devoto *et al*., 2002; Yan *et al*., 2009; Sheard *et al*., 2010). COI1 acts as a part of the SCF^{COI1} complex to activate JA signaling by 26S proteasome-mediated degradation of Jasmonate ZIM domain proteins (JAZs). JA induces degradation of JAZs and this degradation resulted in activation of JA responsive gene expression (Chini *et al*., 2007; Thines *et al*., 2007; Chung and Howe, 2009). These JA-activated genes include 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 *PATHOGENESIS-RELATED* (*PR*) genes (*PR1*, *PR2* and *PR5*) and 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 PR 1 (NPR1) (Cao *et al*., 1997; Spoel *et al*., 2003). NPR1-independent pathways, however, 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 *npr1-1* mutation (Clarke *et al*., 1998; Shah *et al*., 2001).

Global expression analysis provided evidence that plant hormones interact each other (Riechmann *et al*., 2000; Van Wees *et al*., 2000; Glazebrook *et al*., 2003; De Vos *et al*., 2005; Eulgem, 2005). In some cases, different 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). In this process, crosstalk between JA and SA is well established. Although there are exceptions, generally it can be demonstrated that SA-mediated defense responses are required for resistance to biotrophic pathogens, whereas JA-mediated defense responses are effective for resistance to necrotrophic pathogens (Thomma *et al*., 1998; Kunkel and Brooks, 2002; Robert-Seilaniantz *et al*., 2011).

The interaction between JA- and SA-mediated defense responses appear to be complex. There is evidence for both positive and negative interactions between these pathways. However, the primary mode of interaction between JA and SA appears to

be mutual antagonism (Van Wees *et al*., 2000; Spoel et al., 2007; Koornneef and Pieterse, 2008). As a result of negative cross talk between JA and SA, activation of SA signaling make a plant more susceptible to attackers that are resisted via JA-mediated defense responses and vice versa.

Early studies of the role of SA in tomato wounding responses revealed that exogenous SA and its acetylated form, aspirin, suppressed JA-induced wound responses (Doherty *et al*., 1988). 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 *Pst* DC3000 infection (Delaney *et al*., 1994; Spoel *et al*., 2003). In Arabidopsis, exogenous SA suppresses JA-dependent gene expression and defense responses against *A. brassicicola* infection (Spoel *et al*., 2007). Plant defensing *PDF1.2*, which are associated with JA, are present in higher levels in SA insensitive and deficient mutants than wild-type (Thomma et al., 1998; Li *et al*., 2006).

JA also suppresses SA signaling (Kunkel and Brooks, 2002). Treatment with exogenous JA inhibits the expression of SA-dependent genes (Niki *et al*., 1998; Koornneef and Pieterse, 2008). 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).

Several studies have demonstrated that exogenous application of SA suppresses the expression of JA-responsive genes, especially JA biosynthesis genes. It suggests that SA may targets the JA biosynthesis pathway to suppress downstream JA signaling (Spoel *et al*., 2003; Leon-Reyes *et al*., 2010). However, it is also reported that exogenous application of SA inhibited the JA-induced expression of genes downstream of JA biosynthesis (Spoel *et al*., 2003; Li *et al*., 2006; Koornneef and Pieterse, 2008).

The antagonism between JA and SA often occurs through the NPR1 (Spoel *et al*., 2003), which is associated to both systemic acquired resistance and induced systemic resistance. In *npr1-1* mutant, it results in enhanced expression of *LOX2*, a key enzyme in octadecanoid pathway leading to the biosynthesis of JA. In addition, NPR1 is also involved in JA-mediated defense responses against necrotrophic pathogen *Alternaria brassicicola*. The activation by NPR1 of SA-mediated defense gene *PR1* requires the localization of this regulatory protein to the nucleus. In contrast, the antagonistic effect to JA that occurs through NPR1 is accomplished without localization of NPR1 to the nucleus (Spoel *et al*., 2003).

JA and SA antagonism has been reported in a total of 17 plant species. The presence of orthologs of genes known to be involved in the JA and SA antagonism are predicted such as NPR1 and COI1 (Thlaer *et al*., 2012). This suggests that many regulatory features of cross-talk between JA and SA have been conserved in plant kingdom.

3.1. WRKY transcription factors

A large set of transcription factors are 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.

WRKY transcription factors are one of the largest families of transcriptional regulator in plants. WRKY transcription factors are defined by the highly conserved amino acid sequence WRKYGQK and are important regulator of SA-mediated defense responses (Rushton *et al*., 2010) and some of them have been implicated in cross talk between JA and SA. 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). Arabidopsis *WRKY70* was identified as a node of convergence between JA and SA (Li *et al*., 2004, 2006). Expression of *WRKY70* is activated by SA but suppressed by JA. They showed that overexpression of *WRKY70* caused enhanced expression of *PR* genes and suppressed expression of *PDF1.2*, leading to enhanced resistance against biotrophic pathogens and enhanced susceptibility to necrotrophic pathogens. Moreover, expression of *WRKY70* is suppressed by COI1. Besides WRKY70, WRKY11 and WRKY17 have also implicated in cross talk between JA and SA. In a *wrky11 wrky 17* double mutants, transcripts of SA-responsive gene accumulated to higher levels, whereas those of JA-responsive genes were notably lower. Expression of *WRKY70* was elevated in this double mutants, suggesting that WRKY11 and WRKY17 functions as negative

regulator of WRKY70 (Journot-Catalino *et al*., 2006). It also supported that cross talk between JA and SA is regulated through WRKY70 transcription factor. WRKY62 was also reported as one of the WRKY transcription factors with a putative role in cross talk between JA and SA (Mao *et al*., 2007). *WRKY62* is synergistically activated by SA and JA through NPR1 dependent pathway. Furthermore, *wrky62* mutants showed enhanced expression of JA responsive genes *LOX2* and *VSP2*, whereas overexpression of *WRKY62* resulted in suppression of these genes.

3.2. Glutaredoxin GRX480

Another putative regulator in crosstalk between JA and SA is the glutaredoxin GRX480. Glutaredoxin catalize thiol disulfide reductions and have been implicated in redox dependent regulation of protein activities (Lemaire, 2004; Ndamukong *et al*., 2007). GRX480 is identified from yeast-two-hybrid screen for interactors woth TGA transcription factors. Expression of GRX480 was induced by SA and dependent on NPR1. Overexpression of GRX480 suppresses expression of PDF1.2 but VSP2. This suggests that GRX480 affects only part of the JA-responsive genes expression. Suppression of PDF1.2 by GRX480 overexpression was abolished in *tga2 tga5 tga6* triple mutant background, suggesting that interaction between GRX480 and TGA factors is essential for GRX-mediated cross talk (Ndamukong *et al*., 2007).

4. Purpose of this study

The MYB transcription factors are large, functionally diverse and represented in all eukaryotes. MYB transcription factors are characterized by the highly conserved DNA-binding domain, the MYB domain. This domain consists of four imperfect amino acid sequence repeats of about 52 amino acids, each forming three α-helix. Most plant MYB transcription factors are R2R3 type, which are thought to have evolved from R1R2R3 gene ancestor (Dubos *et al*., 2010). Arabidopsis R2R3-MYB transcription factors have been implicated in abiotic stress responses and development (Dubos *et al*., 2010).

Among the Arabidopsis 137 R2R3 MYB transcription factors, some of them have been shown to regulate plant defense responses. The *BOTRITIS-SUSCEPTIBLE1* (*BOS1*/ *AtMYB108*) was identified in a screen for mutants altered in their response to the *Botrytis cineria* (*B. cineria*). The *bos1* mutant showed reduced resistance to *B. cineria* and *A. brassicicola* but unaffected resistance in response to biotrophic pathogen (Mengiste *et al*., 2003). *AtMYB46* is reported as a negative regulator in resistance to *B. cineria* via the regulation of a cell wall-binding peroxidase (Ramirez *et al*., 2011). *AtMYB96* positively regulates accumulation of SA by activating *SID2* expression (Seo and Park, 2010). *AtMYB30* is reported as an activator of the hypersensitive responses in response to pathogen attack through the regulation of very-long-chain fatty acids synthesis. However, the contribution of R2R3-MYB transcription factors to regulate antagonistic interaction between JA

and SA remains unclear.

AtMYB44 (synonym AtMYBR1), together with AtMYB70, AtMYb73 and AtMYB77 (synonym AtMYBR2), belongs to subgroup 22 in the R2R3 MYB transcription factor family. The genes in subgroup 22 have similar gene expression pattern such as wounding and are associated in stress responses. *AtMYB44* is induced by defense signal such as *Pst DC3000* and MeJA treatment (Cheong *et al*., 2002; Fowler and Thomashow, 2002; Jung *et al*., 2007).

JA is one of the major defense hormone in plants. It mediates defense responses against necrotrophic pathogen and herbivore. The loss-of-function mutant of *COI1* showed low survival rate against attack of necrotrophic pathogen (Thomma *et al*., 1998). In our laboratory, microarray has been performed to characterize the regulatory component in JA signaling (Jung *et al*., 2007). Using this experiment, Jung identified several genes which expression are induced by MeJA treatment. *AtMYB44* is one of those genes. Because *AtMYB44* gene encodes R2R3-MYB transcription factor, which is involved in transcriptional regulation of downstream target genes, characterizing the role of the *AtMYB44* give an answer of the question about what regulates and mediates JA signaling. To elucidate the function of *AtMYB44*, its overexpression and knockout mutation plants were used. Overexpression of *AtMYB44* led to suppression of JA-responsive genes expression (Jung *et al*., 2010). It means that AtMYB44 act as a negative regulator in JA signaling. But it is also possible that AtMYB44 negatively regulates JA signaling via activation of SA signaling. Overexpression of SA-responsive genes resulted in suppression of JA-

responsive genes expression (Li *et al*., 2004; Mao *et al*., 2007). To test this hypothesis, expression of SA-responsive genes were checked in the *AtMYB44* overexpression plants. Also, expression of SA-responsive gene were verified by inducible expression of *AtMYB44*. From those experiment, I found that *AtMYB44* activates expression of *WRKY70* and *PR*s.

In this study, I 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. Modulation of antagonistic interaction between SA and JA mediated by *AtMYB44* is also demonstrated by overexpression and mutation analysis.

MATERIALS AND METHODS

1. Bacterial strains and culture media

Bacterial strains used in this study were *E. coli* DH10B [F-, *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) ϕ80*lac*ZΔM15, Δ*lac*X74, *deo*R, *rec*A1, *end*A1, *ara*D139, Δ(*ara*, *leu*)7697, *gal*U, *gal*K, λ-, *rps*L(str^r), *nup*G] for subcloning and *E. coli* BL21 (DE3) *pLys*S [F$^-$, *omp*T, *gal*, *dcm*, *lon*, *hsd*S$_B$(r_B^- m_B^-) λ(DE3) *pLys*S(cm^R)] for protein expression. *E. coli* DH10B and BL21 (DE3) *pLys*S were gown in LB (Luria-Bertani) medium (1% tryptone; 0.5% yeast extract; 0.5% NaCl). Transformed strains were cultured in LB broth media with ampicillin (50 μg/ml) or kanamycin (50 μg/ml). Solid medium was made with 1.5% (w/v) Bacto-agar.

2. Enzymes and chemicals

Klenow fragment, Restriction enzymes and T4 DNA ligase were purchased from Takara (http://www.takara.com). Taq DNA polymerase was purchased from Bioneer (http://www.bioneer.co.kr) and exTaq polymerase was purchased from Takara. Superscript III Reverse transcriptase was purchased from Invitrogen (http://www.invitrogen.com). Methyl jasmonate, salicylic acid and agarose were purchased from Sigma (http://www.sigmaaldrich.com) and Duchefa (http://www.duchefa-biochemie.nl). Amylose resin was purchased from New England Biolab (http://www.neb.com). dNTPs and [α-^{32}P]dATP were purchased from Izotope (http://izotope.com). Acrylamide and N, N'-bisacrylamide were purchased

from Sigma. Nylon membrane was purchased from PerkinElmer (http://www.perkinelmer.com). Nitrocellulose membrane was purchased from Whatman (http://www/whatman.com). MS salt was purchased from Duchefa. Other chemicals were purchased from Aldrich, Merck, Sigma, Duchefa.

3. Yeast strain

The yeast strain used for one hybrid assay was *S. cerevisiae* Y187 [*MATα, ura3-52, his3-200, ade2-101, trp1-901, leu2-3, 112, gal4Δ, met–, gal80Δ, URA3::GAL1*$_{UAS}$-*GAL1*$_{TATA}$-*lacZ*]. (Clontech).

4. Plant materials and treatments

Arabidopsis (*Arabidopsis thaliana*) ecotype Columbia (Col-0) was used as wild-type plant 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 Drs 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 Drs Ingyu Hwang and Yong Hwan Lee, respectively (Seoul National University, Seoul, Korea).

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

To examine the effect of plant hormones on gene expression, a solution of 50 μM MeJA (Sigma) or 1 mM SA (Sigma) was applied to the surface of MS-agar plates in which 2-week-old seedling were growing. For inducible expression of *AtMYB44*, an *AtMYB44* cDNA was inserted into XhoI and SpeI site under the control of a ß-estradiol-inducible promoter of pER8 vector (Zuo *et al*., 2000). A full-length *AtMYB44* cDNA (EST 119B8) was obtained from TAIR. Twelve-day-old transformants grown on MS-agar plates were transferred to 5 μM ß-estradiol containing media for induction of *AtMYB44*.

5. Plant transformation and analyses of transgenic plants

Agrobacterium tumerfaciens 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 hygromycin (Duchefa).

6. 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). Five μg of total RNA was separated on 1.3% formaldehyde agarose gels and transferred to Genescreen Plus hybridization transfer membranes (Perkin-Elmer). [α-^{32}P]-labeled cDNA probes containing gene specific

sequences were hybridized to detect signal.

For quantitative real-time PCR (qRT-PCR), the SYBR kit (JMC R&D, Seoul, Korea) was used. Forty cycles of amplification (15 seconds at 95˚C, annealing 60 seconds at 68˚C) after an initial step (10 minutes at 95˚C) were carried out in a Rotor-gene 2000 (Corbett, Sydney, Australia). Primer sequences are presented in Table S1 online. *Actin 1* was included in the assay for normalization. The qRT-PCR reactions were carried out with two or three biological and three technical repeats. The comparative $\Delta\Delta C_T$ method was used for relative quantification of each amplified product.

7. Pathogen infection assay

A. brassicicola was cultivated on potato dextrose agar (PDA) plates. After 12 days, spores were washed with sterilized distilled water from the surface of the media. Concentration of spores suspension was determined to 5×10^5 spores/ml using hematocytometer. Four-week-old soil grown plants were inoculated by placing 5 μl of suspension onto the surface of 5^{th} to 11^{th} true leaves. Inoculated plants were incubated at 23˚C at 100% relative humidity. Relative fungal DNA levels of *A. brassicicola* were determined by qPCR using primers specific for the genomic *5.8S ribosomal RNA* (GenBank accession number U05198; 5'-TCAAGCTTTGCTTGGTGTTG-3', 5'-CCTACCTGATCCGAGGTCAA-3') and primers for Arabidopsis genomic *Actin2* (5'-CTCCCGCTATGTATGTCGCC-3', 5'-CGGTTGTACGACCACTGGC-3').

The bacterial strain *Pseudomonas syringae* pv. *tomato* DC3000 was grown in

Kings B media and adjusted to 5 $\times 10^5$ cfu/ml in 10 mM $MgCl_2$ solution. Pathogen inoculation was performed by infiltration. For quantification of bacterial growth in plants, leaf discs were taken 3 days after infiltration. They were homogenized in 100 μl of 10 mM $MgCl_2$ solution. Appropriately diluted samples were plated on Kings B plates supplemented with rifampicin (50 μg/ml) and incubated at 28˚C for 2 day.

8. Quantification of SA and glucosylated SA

SA and glucosylated SA (SAG) were extracted from the leaves and quantified as described previously (Koo *et al*., 2007). Extracts were separated by Symmetry C_{18} HPLC column [4.6 mm (inside diameter) by 15 cm long; particle size, 5 μm; Waters, Milford, MA] equipped in a Shimadzu LC-6A HPLC and quantified by RF-10A XL fluorescence detector (excitation 301 nm, emission 412 nm). The amount of SAG was quantified by the difference between SA quantities with and without glucosidase treatment (160 units for 60min at 37˚C, Sigma). To monitor sensitivity and recovery, known amount of SA was added into the sample and analyzed in parallel. The measurements were averaged from 3 biological replicates.

9. 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 pGEM-TEasy vector (Promega)

and nucleotide sequences of 36 DNA fragments were determined by ABI377. The obtained sequences were aligned and visualized using the Weblogo package (Crooks *et al*., 2004; http://weblogo.berkeley.edu/logo/cgi).

10. Electrophoretic mobility shift assay (EMSA)

The R2R3 domain (amino acid residue 1 to 111) of *AtMYB44* was fused with the GST coding sequence through the BamHI and EcoRI sites of the pGEX-5x-1 expression vector. 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 the purified GST-AtMYB44 protein for 20 min at 23°C in 25 μl of binding buffer (20 mM HEPES pH 7.8, 50 mM KCl, 1 mM EDTA, 0.5 mM DTT, 5μg of BSA, 200 μg of poly[dI-dC] and 10% glycerol). The reaction mixture was separated on 6% gels by native PAGE.

11. Chromatin immunoprecipitation

Two-week-old 35S:*GFP-AtMYB44* transgenic plants grown on MS-agar plates were used for chromatin immunoprecipitation (ChIP). ChIP assays were carried out as described previously (Saleh *et al*., 2008). Fragmented chromatin was immunoprecipitated with anti-GFP antibody (Clontech). DNA extracts separated from the DNA-protein complex were used for qRT-PCR analysis. The primer sets used in this analysis amplify different regions of the *WRKY70* locus (Figure 20b and Table 1). The ChIP experiments were performed 3 times for biological replicates.

12. 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 24C 3days. Histochemical GUS assay was performed as previously described (Jung *et al*., 2008).

13. Analysis of transcript level by RT-PCR

For cycle-limited RT-PCR analysis, pairs of gene specific primers were used for PCR amplification. Twenty-five cycles of amplification were carried out. The number of cycles was optimized to be in the linear range of amplification. PCR of *ACT1* was used as an input control. cDNA was generated from 1 μg of total RNA using Superscript III (Invitrogen) according to the manufacturer's instructions.

14. Yeast-one-hybid (Y1H) and ß-galactosidase assay

The *AtMYB44* CDS recombinded with GAL4 activation domain of pGADT7-Rec2 vector (Clotech) . A DNA fragment consisting of four copies of the *WRKY70* promoter sequence (-328 to -299) containing the AtMYB44 core binding sequence was chemically synthesized (Bioneer) and ligated into the pHIS2 vector (Clontech). In the mutant version of the promoter, 6 nucleotides of the core binding sequence, CGGTTA, were replaced with ATTAAT. The yeast transformation and selection were performed according to the manufacturer's instructions. ß-Galactosidase assay was carried out according to manufacturer's instruction (Clontech, Yeast Protocol Handbook).

15. Analysis of root growth inhibition and root hair development

For root growth inhibition assay, plants were germinated on MS media supplemented with MeJA specified. Root length of fifty seedlings was measured after 5 days of germination. The mean and the standard deviation were obtained from the measurement of 50 seedlings. The average root length from each treatment was normalized as a percent of Arabidopsis Col-0 grown without MeJA.

To analyze root hair development, plants were germinated on MS agar media for 4 days and then transferred into MS agar media supplemented with 1 μM MeJA for additional 2 days. Root hairs were viewed using bright field microscopy (Nikon) and Root hair density of six days old seedling was scored as described previously (Zhu *et al*., 2006). The mean and the standard deviation were obtained from the measurement of 15 seedlings.

Table 1. Primers used in Northern blot analysis, ChIP PCR and subcloning

Primer	Usage	sequence
AtMYB44 -F	cDNA probe	5'-CGGATCCTACGACCATCGGGGTTAC-3'
AtMYB44 -R	cDNA probe	5'-GGAATTCCTACTCGATTCTCCCAAC-3'
WRKY70-F	cDNA probe	5'-CCAAGGGTGCAAGGCAACAAA-3'
WRKY70-R	cDNA probe	5'-TTCCACTCTACATGGCCTAAT-3'
PR1-F	cDNA probe	5'-TTCACAACCAGGCACGAGGAG-3'
PR1-R	cDNA probe	5'-TCGTAATCTCAGCTCTTATTT-3'
PR2-F	cDNA probe	5'-CGGCCAACATCCATCTAGACT-3'
PR2-R	cDNA probe	5'-ACACGCTGAAAGCGCATTAAT-3'
PR5-F	cDNA probe	5'-AAGATGTAACGGCGGCGGAGT-3'
PR5-R	cDNA probe	5'-GTGGAAGACGATGATTCATAG-3'
AOS-F	cDNA probe	5'-TGATCTAACCGTAGCGACACG-3'
AOS-R	cDNA probe	5'-CCAGATCCTTCTTCGCTCTAC-3'
VSP-F	cDNA probe	5'-CTACTCTTGCTCTTGGTCGCTACG-3'
VSP-R	cDNA probe	5'-CCGGGAGTCCTGGAGTTGATTC-3'
PDF1.2-F	cDNA probe	5'-GTAATAATCATCATGGCTAA-3'
PDF1.2-R	cDNA probe	5'-GCACCAAAGATTATTGGTAG-3'
NPR1-F	cDNA probe	5'-AGTCATTGCCGGAAGAGCTTG-3'
NPR1-R	cDNA probe	5'-GGCTCGAGGCTAGTCACTATG-3'
Site1-F	ChIP PCR	5'-CTCTTCCCCATTGACAGTTTCT-3'
Site1-R	ChIP PCR	5'-GGATCCAATAACCATATGATAA-3'
Site2-F	ChIP PCR	5'-TTAGGCTAGAGAGCCAACCTT-3'
Site2-R	ChIP PCR	5'-GTACCACTTAACTTCAGACCA-3'
Site3-F	ChIP PCR	5'-GAAGAGAGTGAGGCTGTGAGGGA-3'
Site3-R	ChIP PCR	5'-GTGTATGTGGTCGATCGACTCA-3'
Site4-F	ChIP PCR	5'-GGTAGTACAATCTCGAGGAACA-3'
Site4-R	ChIP PCR	5'-GAATGATGTCATTTCATCCCT-3'
Site5-F	ChIP PCR	5'-GGGAGTTTCTGCGTTGGTGTTA-3'
Site5-R	ChIP PCR	5'-GTATACCCAAGGGTGCAAGGCA-3'
Site6-F	ChIP PCR	5'-GGGAGGGATGTTTTAATCCAGCA-3'
Site6-R	ChIP PCR	5'-ATTAGGCCATGTAGAGTGGAA-3'
pER_AtMYB44-F	subcloning	5'-CCCTCGAGGAAGATTGCTAAAGTTTGAT-3'
pER_AtMYB44-R	subcloning	5'-GACTAGTCTTTGTTCACTCGATTTCCT-3'
pGADT_AtMYB44-F	subcloning	5'-GGAATTCATGGCTGATAGGATC-3'
pGADT_AtMYB44-r	subcloning	5'-GGAATTCCTACTCGATTCTCCCAA-3'
pGEX_AtMYB44R2R3-F	subcloning	5'-CGGGATCCGTTCCAGTGATTCTTCACGGCGT-3'
pGEX_AtMYB44R2R3-R	subcloning	5'-CGGAATTCATGGCTGATAGGATCAAAGGT-3'

RESULTS

1. *AtMYB44* is induced by JA through COI1

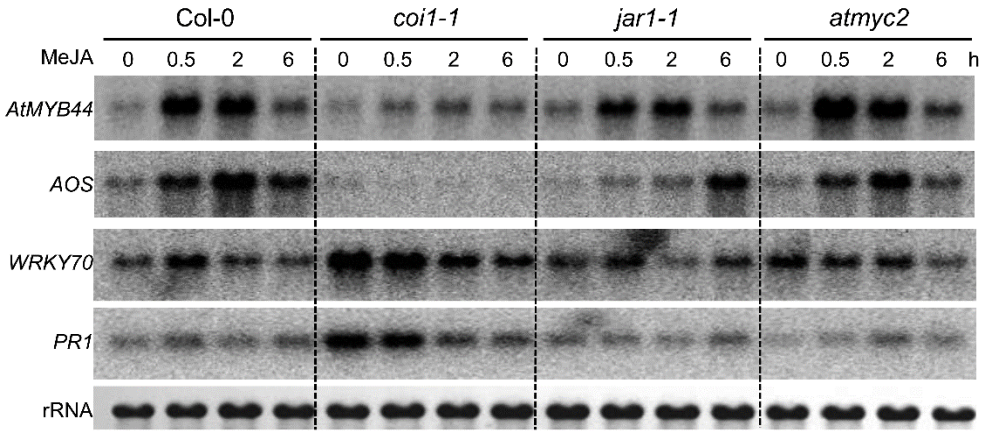
AtMYB44 is reported as one of the JA responsive transcription factor (Jung *et al*., 2007). To address the function of *AtMYB44* in JA mediated defense responses, I examined its expression in response to MeJA treatment. *AtMYB44* was rapidly induced by MeJA in control Col-0 plants (Figure 3). To establish whether the induction of *AtMYB44* by MeJA treatment requires an intact JA signaling pathway, *AtMYB44* expression was also examined in the JA signaling mutant *coi1-1* and *jar1-1*. *JAR1* encodes a jasmonate-isoleucine synthase responsible for the synthesis of JA-Ile, which acts as a JA-signaling molecule (Staswick and Tiryaki, 2004). Induction of *AtMYB44* by MeJA was slightly reduced in *jar1-1* but was strongly reduced in *coi1-1*. *AOS*, a JA signaling marker, is also slightly reduced in *jar1-1* and not induced in *coi1-1* mutants (Figure 3). However, *AtMYB44* was not affected in mutants of *atmyc2*, which encodes another transcription factor responsive to JA (Figure 3). It means that expression of *AtMYB44* is not mediated by *AtMYC2*. These results show that induction of *AtMYB44* and *AOS* by JA occurs through COI1-dependent JA signaling.

WRKY70 and *PR1* are constitutively expressed in *coi1-1* mutant. It is reported that *WRKY70* is negatively regulated by COI1 (Li *et al*., 2004). *WRKY70* positively

regulates expression of SA-responsive gene but negatively regulates expression of JA-responsive genes (Li *et al*., 2004; Koo *et al*., 2007). It is regarded as representative example of antagonistic interaction between JA and SA. Interestingly, *WRKY70* is rapidly induced by MeJA in early time point. This induction is abolished in *coi1-1* mutant. It means that COI1 is required for JA-mediated expression of *WRKY70*. Moreover, elevated *WRKY70* and *PR1* transcript is suppressed by MeJA in *coi1-1* mutant. These data show that crosstalk between JA and SA is tightly regulated through complex regulatory node.

Figure 3. Induction of *AtMYB44* expression by JA

Arabidopsis Col-0 and JA signaling mutants were treated with 50 μM of MeJA for the indicated number of hours and JA responsive genes were analyzed by Northern blot. rRNA was visualized by ethidium bromide staining as a loading control.


Col-0
coi1-1
jar1-1
atmyc2
MeJA
0 0.5 2 6 0 0.5 2 6 0 0.5 2 6 0 0.5 2 6 h
AtMYB44
AOS
WRKY70
PR1
rRNA

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

To clarify the function of *AtMYB44* in JA-mediated defense responses, I tested two previously characterized *AtMYB44* overexpressing lines, OX18 and OX21 (Jung *et al*., 2008). To examine the JA-mediated defense responses of these plants, I challenged wild-type, *AtMYB44* overexpressing lines and *atmyb44* mutants with the necrotrophic pathogen *A. brassicicola*. Defense responses against necrotrophic pathogen is mediated by JA in plants (Spoel *et al*., 2003). When infected with the pathogen, wild type plants showed limited necrosis at inoculation sites but non-extending local legions (Figure 4a). However, OX18 and OX21 overexpressing lines showed more severe disease symptoms with extended necrosis. The average diameter of lesions in OX18 and OX21 plants caused by *A. brassicicola* infection was about six-fold larger than that of wild type plants. By contrast, *atmyb44* mutant plants showed reduced lesion size.

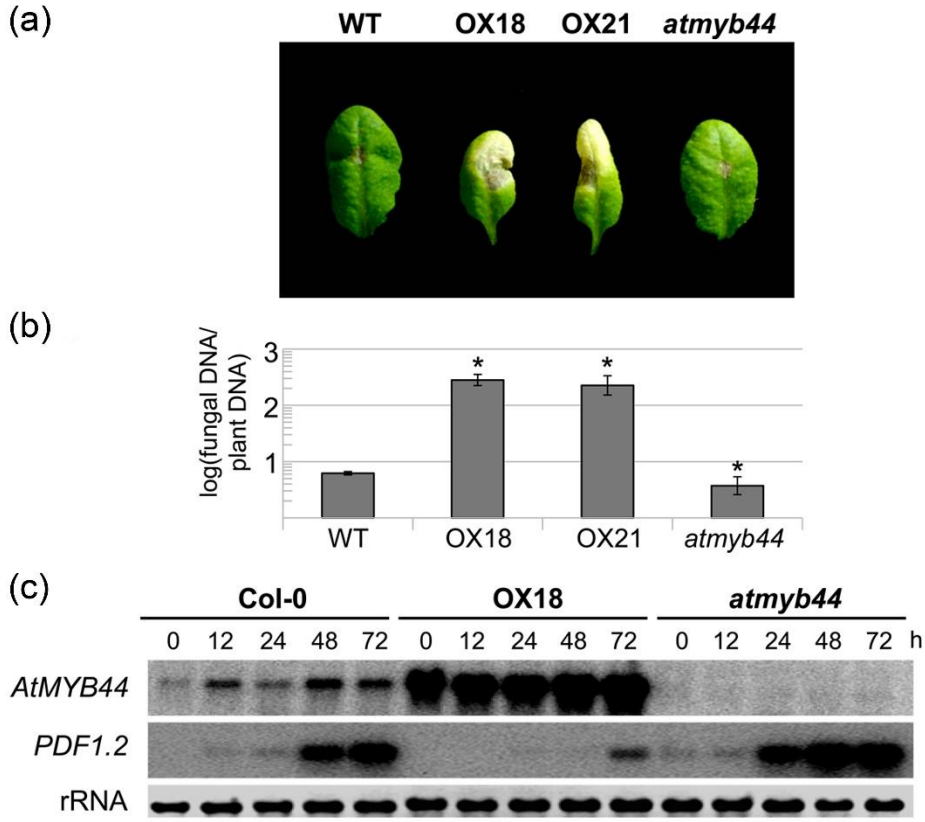
To determine whether the altered lesion size and necrosis resulted from changes in the growth of fungi in plants, the amount of fungal DNA in infected leaves was measured by quantitative PCR with *A. brassicicola* and Arabidopsis gene-specific primers. The level of *A. brassicicola* specific DNA in OX18 and OX21 plants was about 30 times higher than in wild type plants (Figure 4b). Consistent with their

reduced lesion formation phenotype, the relative amounts of fungal DNA in *atmyb44* knockout plants was lower than in wild type plants by a small but significant amount ($P<0.01$).

Enhanced susceptibility to *A. brassicicola* in OX18 and OX21 plants and increased resistance in *atmyb44* knockout mutants suggest that *AtMYB44* negatively regulates the 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 4c). The *AtMYB44* transcript was detected at 12 hours after infection in wild type plants, and expression of *PDF1.2* was induced later, 48 hours after infection (Figure 4c). *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 were inversely correlated with *AtMYB44* expression.

Figure 4. Suceptibility of *AtMYB44* overexpressing 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 different *AtMYB44* overexpressing lines (Jung *et al*., 2008). The picture was taken 10 days after inoculation. (b) Quantification of fungal growth by relative amount of fungal DNA compared with plant DNA by qPCR. Data represent mean values of three measurements and error bars represent standard deviation. The experiments were repeated twice with similar results. Statistical significance of the measurements was determined using a t-test (*P<0.01) by comparison with the value of Col-0. (c) Four-week-old Arabidopsis plants were inoculated with *A. brassicicola*. Inoculated plants were collected for RNA extraction and analyzed by Northern blot.


(a)
WT
OX18
OX21
atmyb44
(b)
log(fungal DNA/ plant DNA)
3
2
1
*
*
*
WT
OX18
OX21
atmyb44
(c)
Col-0
OX18
atmyb44
0 12 24 48 72 0 12 24 48 72 0 12 24 48 72 h
AtMYB44
PDF1.2
rRNA

3. *AtMYB44* is a negative regulator in JA signaling pathway

I next tested whether gene expression patterns induced by MeJA treatment are consistent with those produced by *A. brassicicola* infection in *AtMYB44* overexpression lines and *atmyb44* knockout mutants. Indeed, overexpression of *AtMYB44* led to delayed and reduced expression of the JA responsive genes *AOS, VSP1* and *PDF1.2* after MeJA treatment (Figure 5). In contrast to the overexpression phenotype, knockout mutation of *AtMYB44* enhances MeJA-mediated expression of these genes. Basal expression levels of *VSP1* and *PDF1.2* were constitutively up-regulated in *atmyb44* knockout plants.

To elucidate whether JA-mediated growth response were affected by *AtMYB44*, we characterized the role of *AtMYB44* in root growth, accumulation of anthocyanin and root hair formation which are regulated by JA. As shown in figure 6a, root growth of plants was significantly inhibited by MeJA. The root lengths of wild type plants in MS agar medium supplemented with over 10 nM of MeJA were significantly shorter than those of untreated seedling. Root growth inhibition of *atmyb44* knockout plants was detected in 1 nM MeJA. Delayed root growth in untreated *AtMYB44* overexpression plants is reported previously phenotype (Jung *et al*., 2008). Root growth inhibition of *AtMYB44* overexpression plants was observed over 100nM MeJA but more pronounced even without MeJA treatment (Figure 6a). This observation suggested that *AtMYB44* overexpression plants less sensitive to JA

than wild type plants in root growth inhibition. *AtMYB44* also appeared to control JA-induced root hair formation. As shown in figure 6b, the number of root hair in wild type was increased under MeJA treatment. This effect was significantly reduced in *AtMYB44* overexpression plants and increased in *atmyb44* knockout plants. These results coincide with accumulation of anthocyanin by JA treatment. Overexpression of *AtMYB44* inhibited JA induced accumulation of anthocyanin, while knock out mutation of *AtMYB44* showed opposite effect (Figure 7).

Taken together, these results demonstrate that *AtMYB44* negatively regulates various JA-mediated responses.

Figure 5. *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.

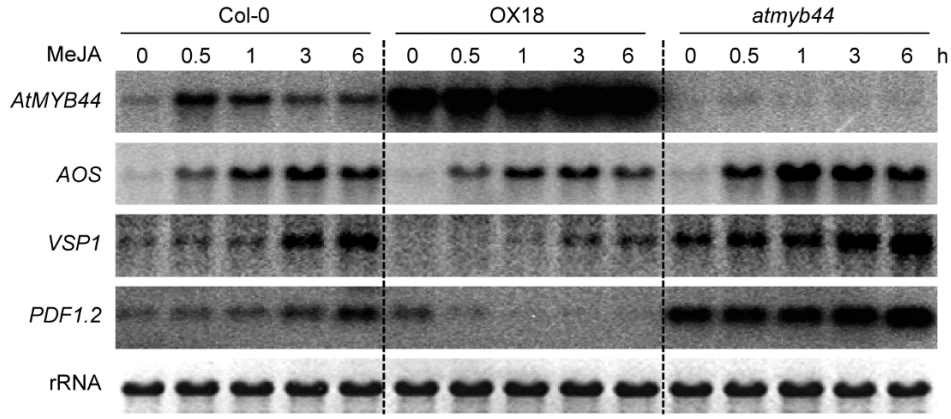

Col-0
OX18
atmyb44
MeJA
0 0.5 1 3 6 0 0.5 1 3 6 0 0.5 1 3 6 h
AtMYB44
AOS
VSP1
PDF1.2
rRNA

Figure 6. MeJA response of *AtMYB44* overexpressing and *atmyb44* knockout plants **(a)** Root lengths of Arabidopsis Col-0, *AtMYB44* overexpression plants (OX18) and *atmyb44* knockout plants were measured. Data represent the mean root length from fifty seedlings. Error bars represent standard deviation. **(b)** Root hair density was quantified from Arabidopsis Col-0, *AtMYB44* overexpression plants (OX18, OX21) and *atmyb44* knockout plants. The values represent the mean of fifteen seedlings. Error bars represent standard deviation. Statistical significance of the measurements was determined using a t-test (*$P<0.01$) by comparison with the value of Col-0.

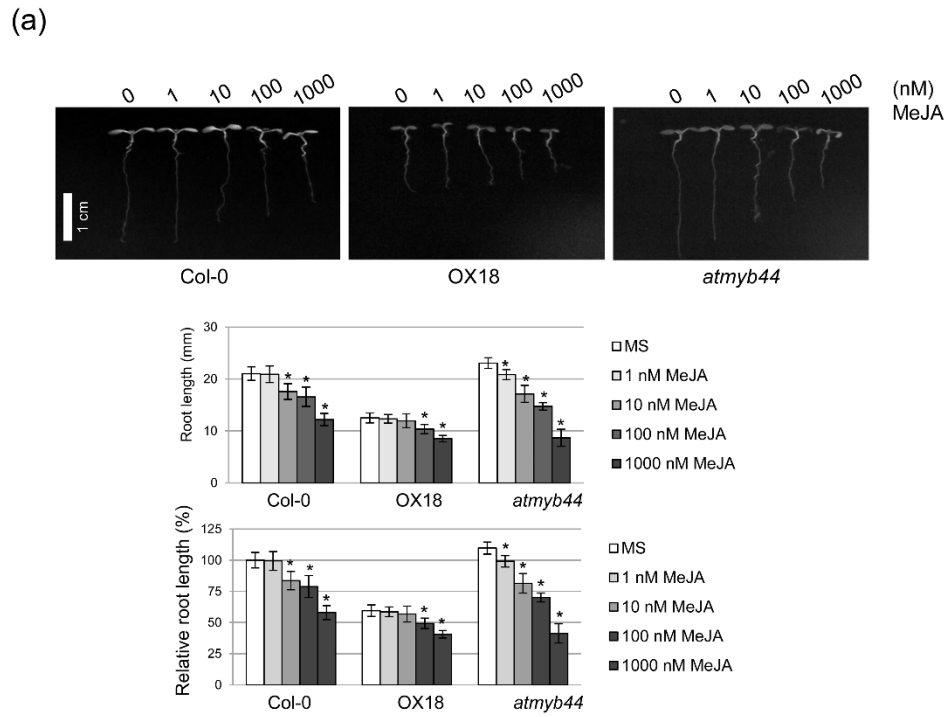
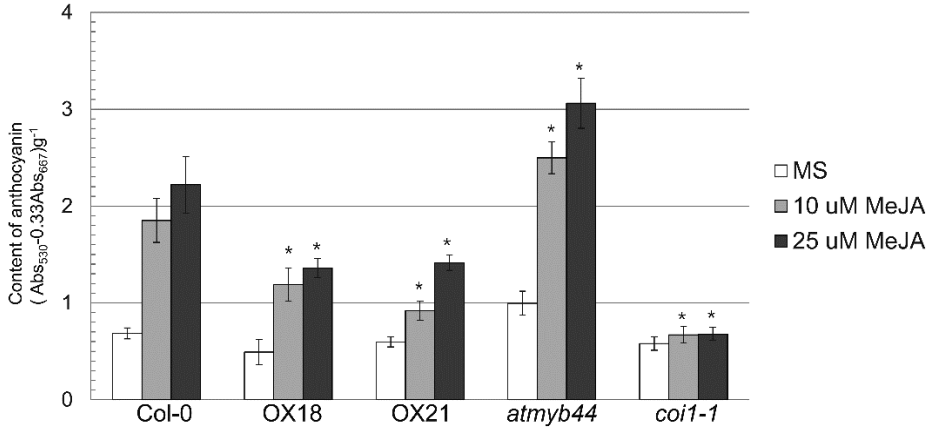

(a)
0 1 10 100 1000
(nM) MeJA
1 cm
Col-0
OX18
atmyb44
Root length (mm)
Relative root length (%)
MS
1 nM MeJA
10 nM MeJA
100 nM MeJA
1000 nM MeJA
(b)
Col-0
OX18
OX21
atmyb44
MS
1 μM MeJA
1 mm
Root hair density (number/mm)

Figure 7. Accumulation of anthocyanin in *AtMYB44* overexpression and knockout mutation plants.

Anthocyanin was extracted from 12-day-old Arabiodopsis Col-0, *AtMYB44* overexpression and knockout mutant seedlings grown on the medium supplemented with indicated concentration of MeJA. Arabidopsis *coi1-1* mutant was used for negative control. Data represent the mean of six measurement and error bars represent standard deviation. Statistical significance of the measurements was determined using a t-test (*$P<0.05$) by comparison with the value of Col-0.


Content of anthocyanin
(Abs530-0.33Abs667)g-1
4
3
2
1
0
Col-0
OX18
OX21
atmyb44
coi1-1
MS
10 uM MeJA
25 uM MeJA

4. *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 (Figure 5). Therefore, I next tested whether *AtMYB44* is involved in SA-mediated bacterial defense responses by testing *AtMYB44* mutant and overexpressing lines for resistance to the biotrophic bacterial pathogen *Pst* DC3000. *AtMYB44* is induced by *Pst* DC3000 (Figure 8). I found that overexpression of *AtMYB44* led to enhanced resistance to *Pst* DC3000 (Figure 9) 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. Indeed, I found that the titer of *Pst* DC3000 in OX18 and OX21 plants was about 10 times lower (infiltration) and 100 times lower (spray) than that in wild type plants (Figure 9b and d). In *atmyb44* knockout plants, bacterial titer was slightly increased compared to wild type plants. This result indicates that *AtMYB44* increases resistance to *Pst* DC3000.

Figure 8. Expression of *AtMYB44* by *Pst* DC3000 infection

Four-week-old Arabidopsis Col-0 plants were treated with *Pst* DC3000. rRNA is shown for checking equal loading.

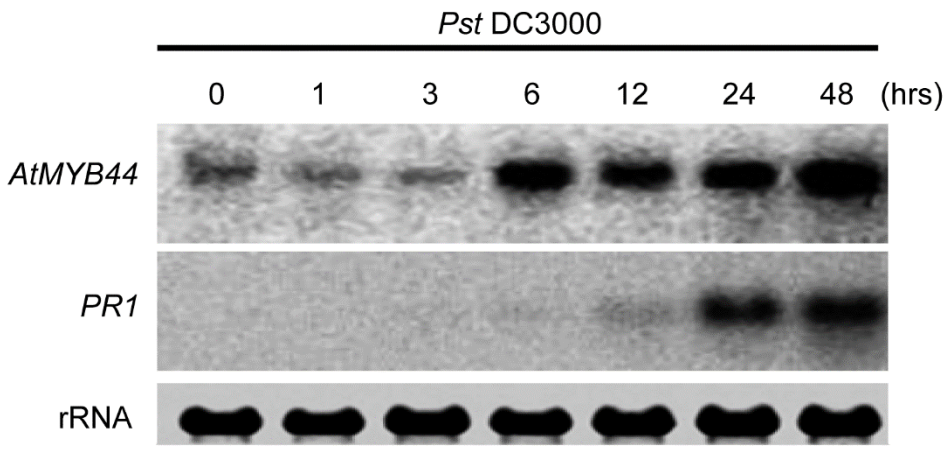
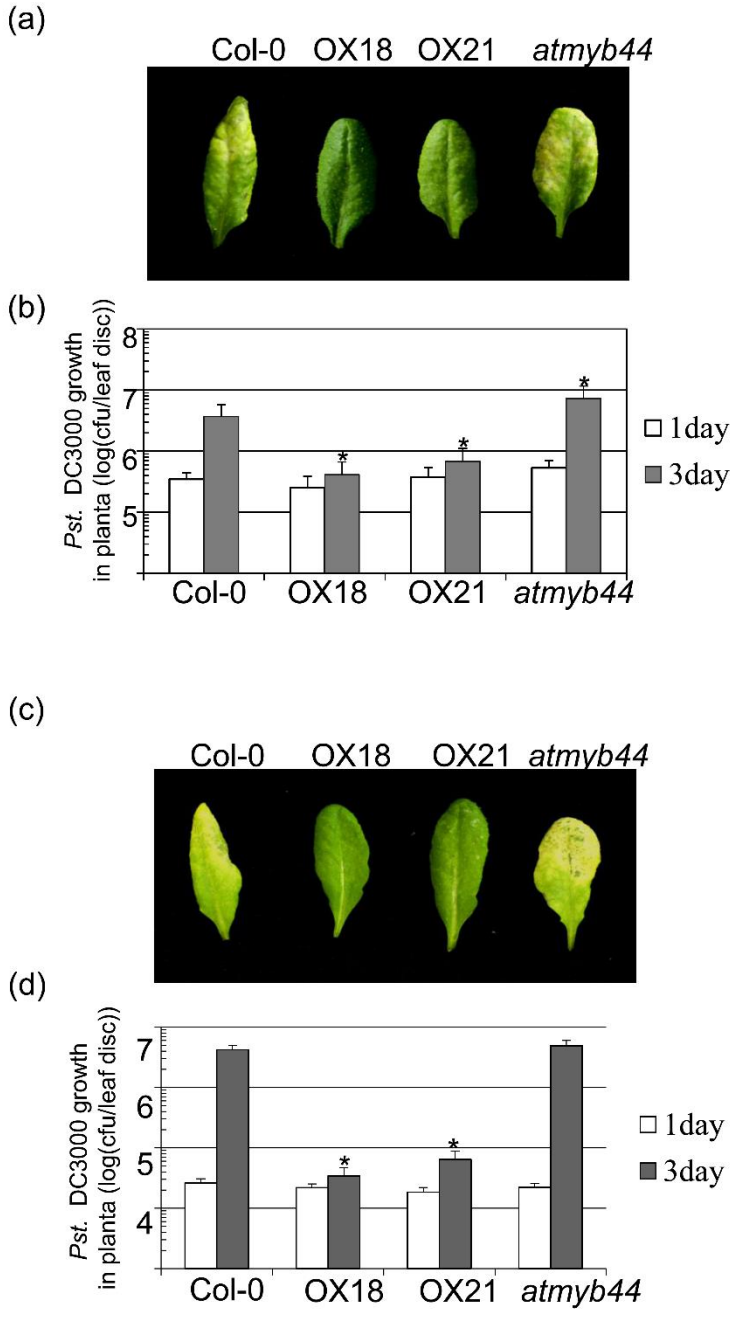

Pst DC3000
0
1
3
6
12
24
48
(hrs)
AtMYB44
PR1
rRNA

Figure 9. Resistance of *AtMYB44* overexpressing and *atmyb44* knockout mutant plants against the biotrophic pathogen *Pst* DC3000

(a)Four-week-old Arabidopsis Col-0, *AtMYB44* overexpressing plants (OX18, OX21) and *atmyb44* knockout mutant plants were inoculated with *Pst* DC3000 by infiltration. Picture was taken 3 days after inoculation. (b) Bacterial growth in leaves was determined one (open bars) or three days (closed bars) after inoculation. Statistical significance of the measurements was determined using a t-test (*P<0.01) by comparison with the value of Col-0. Data represent the mean values of eight independent plants and error bars represent standard deviation. The experiments were repeated twice and showed similar results. (c) *Pst* DC3000 solution is sprayed to four-week-old Arabidopsis Col-0, *AtMYB44* overexpressing plants (OX18, OX21) and *atmyb44* knockout mutant plants. (d) Bacterial growth in leaves was determined one (open bars) or three days (closed bars) after inoculation. Statistical significance of the measurements was determined using a t-test (*P<0.01) by comparison with the value of Col-0. Data represent the mean values of eight independent plants and error bars represent standard deviation.


(a)
Col-0
OX18
OX21
atmyb44
(b)
Pst. DC3000 growth
in planta (log(cfu/leaf disc))
8
7
6
5
*
*
*
1day
3day
Col-0
OX18
OX21
atmyb44
(c)
Col-0
OX18
OX21
atmyb44
(d)
Pst. DC3000 growth
in planta (log(cfu/leaf disc))
7
6
5
4
*
*
1day
3day
Col-0
OX18
OX21
atmyb44

5. *PR* genes are up-regulated in *AtMYB44* overexpressing plants

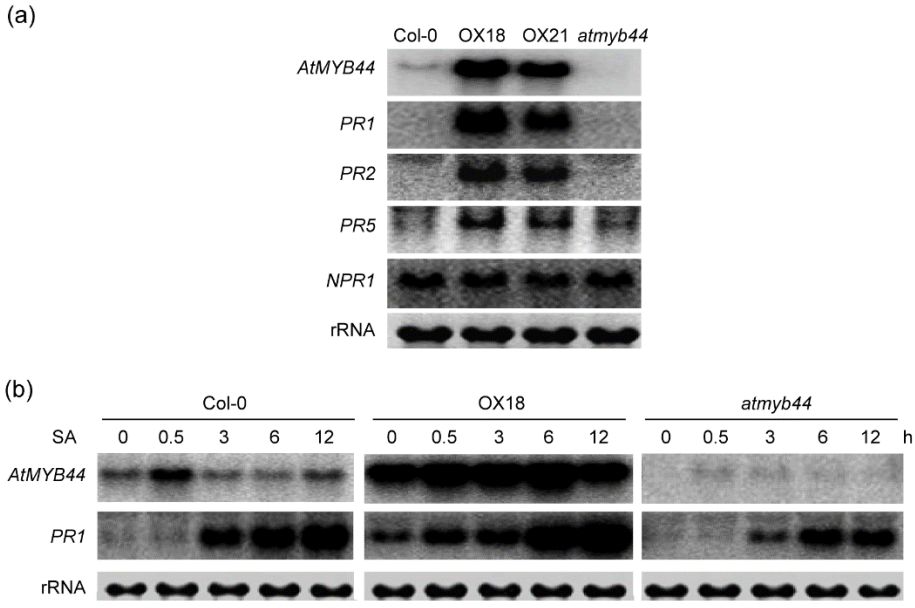
To investigate the enhanced resistance of OX18 and OX21 plants to *Pst* DC3000, I examined the expression levels of *PR* genes, which participate in SA mediated defense responses. Northern blot analysis demonstrated that *PR1*, *PR2* and *PR5* were constitutively overexpressed in *AtMYB44* overexpressing plants but were not expressed in untreated knockout and wild type plants (Figure 10a).

Gene expression patterns induced by SA treatment were consistent with the observed changes in resistance against *Pst* DC3000 infection in *AtMYB44* overexpression and *atmyb44* knockout mutant plants. In wild-type plants after SA treatment, *AtMYB44* was induced rapidly, within 30 minutes, but *PR1* was induced after approximately 3 hours (Figure 10b). Even though the level of *PR1* was already higher in OX18 plants than that in wild type plants, after SA treatment, *PR1* was induced to a much higher level**.** 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.

Figure 10. SA-mediated response in *AtMYB44* overexpressing and *atmyb44* knockout plants.

(a) Northern blot showing basal level expression of *PR* genes in Arabidoposis Col-0, *AtMYB44* overexpression (OX18, 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.


(a)
Col-0
OX18
OX21
atmyb44
AtMYB44
PR1
PR2
PR5
NPR1
rRNA
(b)
Col-0
OX18
atmyb44
SA
0
0.5
3
6
12
0
0.5
3
6
12
0
0.5
3
6
12
h
AtMYB44
PR1
rRNA

6. *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, I 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* but was significantly reduced in *NahG* plants (Figure 11a). 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*.

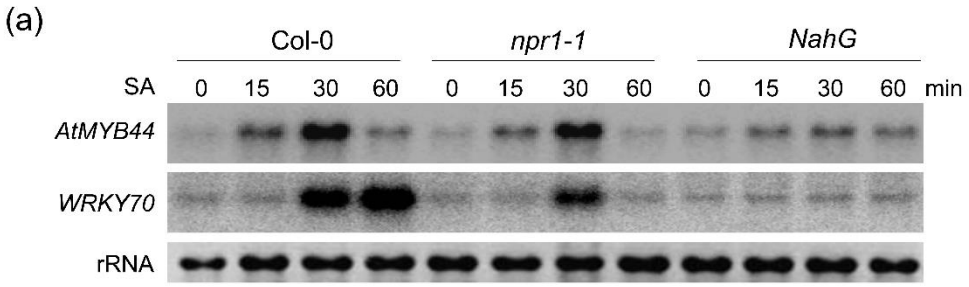
To dissect the molecular components upstream and downstream of *AtMYB44* in the SA signaling pathway leading to *PR* gene expression, I determined the epistatic relationships between various components in the SA signaling pathway. OX18 plants were crossed with *npr1-1, NahG or sid2-2* mutant plants. *SID2* encodes an isochorismate synthase acting in SA biosynthesis. Activation of *PR1* expression by *AtMYB44* overexpression was slightly reduced in OX18 *npr1-1* plants but totally abolished in OX18 *NahG and* OX18 *sid2-2* (Figure 11b). These results indicate that *AtMYB44* depends on a basal level of SA to activate *PR1* expression.

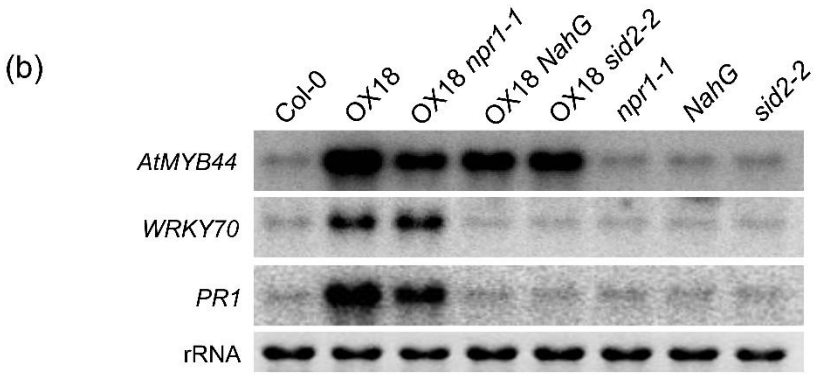
To understand the role of *NPR1* and SA in *AtMYB44* mediated disease resistance, I next challenged the OX18 *npr1-1* and OX18 *NahG* plants with *Pst* DC3000. Consistent with the expression patterns of *PR1* in Figure 11b, bacterial resistance

was enhanced in OX18 plants but completely compromised in the OX18 *NahG* plants. In OX18 *npr1-1*, resistance to *Pst* DC3000 was less than in OX18 but not as low as OX18 *NahG* and wild type (Figure 11c). Resistance of *npr1-1* and *NahG* to *Pst* DC3000 is decreased than that of Col-0 plants (Figure 12). Decreased resistance of OX18 *npr1-1* is likely due to the disruption of *NPR1*-dependent pathways. *NPR1* is required for full scale activation of *PR1* transcription mediated by *AtMYB44*. I measured the degree of resistance by quantification of bacterial growth in infected leaves (Figure 11c) and found that resistance varies with the level of *PR1* gene expression (Figure 11b).

Figure 11. 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* overexpression. The OX18 *AtMYB44* overexpression 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* overexpression. Four-week-old Arabidopsis Col-0, *AtMYB44* overexpression plants (OX18), OX18 *npr1-1* and OX18 *NahG* were inoculated with *Pst* DC3000. The picture was taken 3 days after inoculation. Bacterial growth in leaves was determined one (open bars) or three days (closed bars) after infection. Statistical significance of the measurements was determined using a t-test (*P<0.01) by comparison with the value of Col-0. Data represent the mean value of eight independent plants and error bars represent standard deviation. The experiments were repeated twice and showed similar results.


(a)
Col-0
npr1-1
NahG
SA
0 15 30 60 0 15 30 60 0 15 30 60 min
AtMYB44
WRKY70
rRNA


(b)
Col-0
OX18
OX18 npr1-1
OX18 NahG
OX18 sid2-2
npr1-1
NahG
sid2-2
AtMYB44
WRKY70
PR1
rRNA

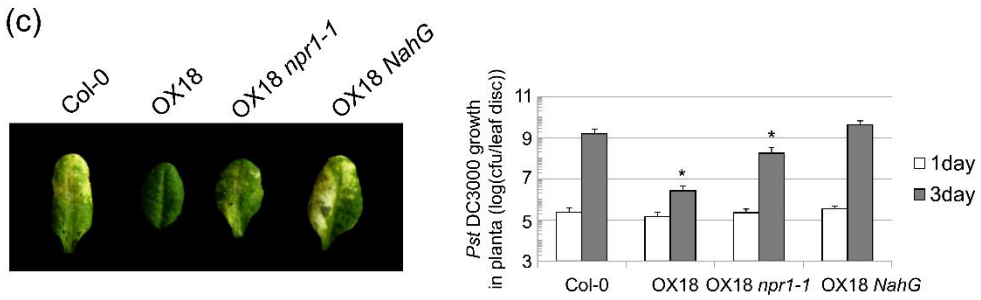
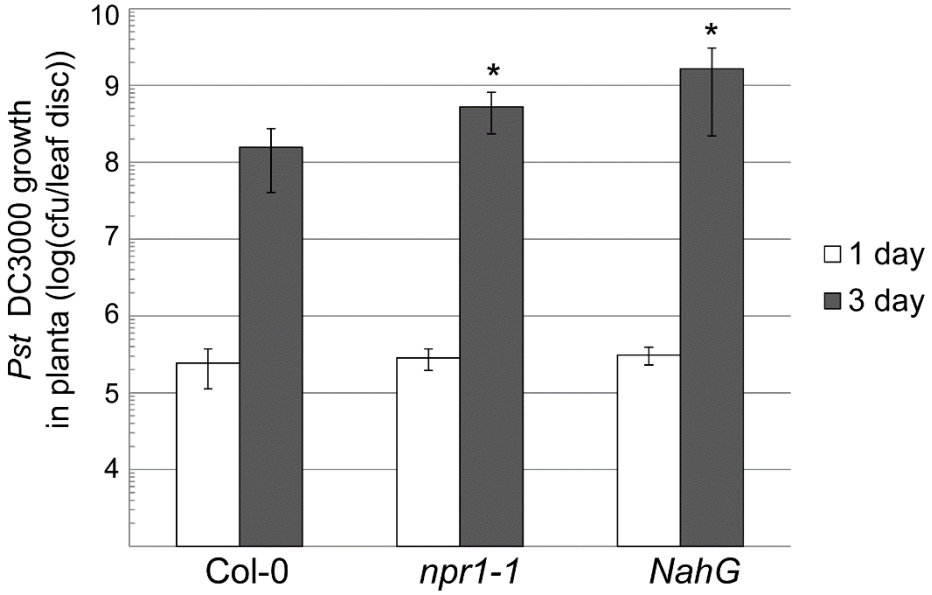

(c)
Col-0
OX18
OX18 npr1-1
OX18 NahG
Pst DC3000 growth
in planta (log(cfu/leaf disc))
11
9
7
5
3
*
*
1day
3day
Col-0
OX18
OX18 npr1-1
OX18 NahG

Figure 12. Resistance of *npr1-1* and *NahG* plants against the biotrophic pathogen *Pst* DC3000

Four-week-old Arabidopsis Col-0, *npr1-1* and *NahG* plants were inoculated with *Pst* DC3000. Bacterial growth in leaves was determined one (open bars) or three days (closed bars) after inoculation. Statistical significance of the measurements was determined using a t-test (*$P<0.05$) by comparison with the value of Col-0. Data represent the mean values of eight independent plants and error bars represent standard deviation.


10
9
8
7
6
5
4
*
*
Pst DC3000 growth
in planta (log(cfu/leaf disc))
1 day
3 day
Col-0
npr1-1
NahG

7. Overexpression phenotypes of *AtMYB44* are reversed by SA depletion

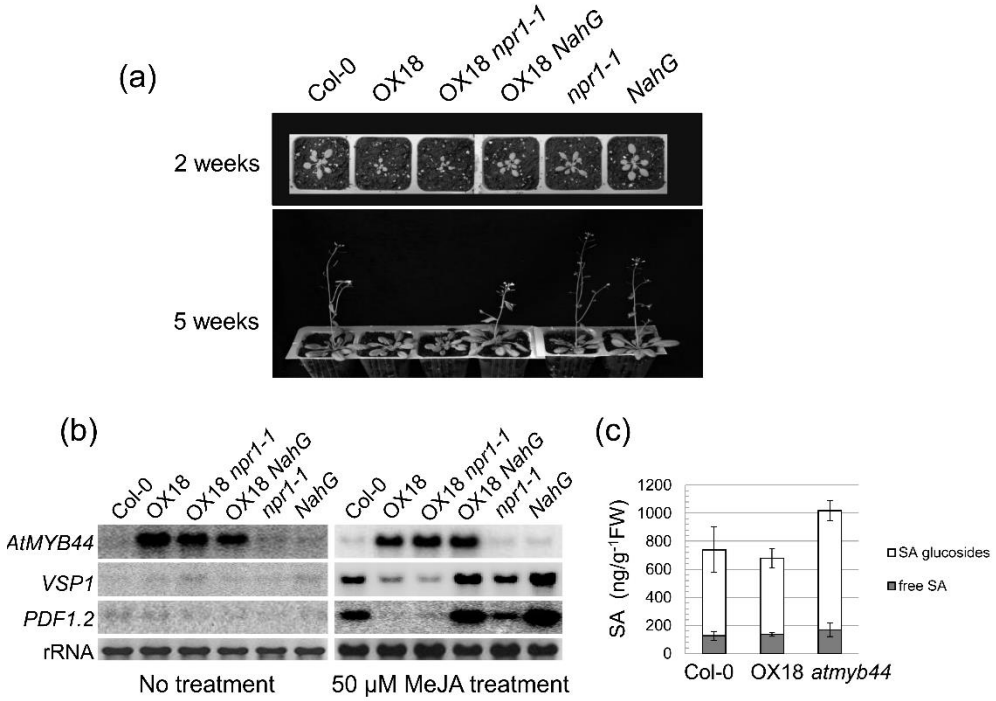
Because *PR1* activation in OX18 plants was abolished in the *NahG* background, I looked into the growth phenotypes of OX18 *NahG* plants. Growth of OX18 and OX18 *npr1-1* plants was severely retarded, similar to plants overexpressing *PR* genes (Bowling *et al*., 1997; Li *et al*., 2004). However, the OX18 growth retardation was abolished in OX18 *NahG* plants (Figure 13a).

I also examined the JA response of OX18 *NahG* plants. In OX18 plants, JA responsive genes such as *VSP1* and *PDF1.2* were not substantially induced by treatment with MeJA; however, in OX18 *NahG*, *VSP1* and *PDF1.2* were strongly induced by MeJA treatment (Figure 13b). By contrast, OX18 *npr1-1* plants showed similar growth retardation and repression of JA responsive genes as OX18 plants, because the *NPR1* independent *AtMYB44* pathway leading to *PR1* is still working as shown in Figure 11b.

Because the effects of *AtMYB44* overexpression were reversed in the *NahG* background, I next measured the levels of SA in wild type, OX18, and *atmyb44* mutant plants (Figure 13c). One-way ANOVA analysis 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 is not a result of enhanced SA biosynthesis.

Figure 13. Effect of *npr1* mutation or *NahG* expression on *AtMYB44* mediated responses

(a) Growth of plants overexpressing *AtMYB44* in different mutant backgrounds. Pictures were taken 2 (upper panel) or 5 weeks (lower panel) after germination. (b) JA response of plants overexpressing *AtMYB44* in different mutant backgrounds. Two-week-old plants were treated with MeJA for 6 hours and total RNA was analyzed by Northern blot. rRNA was visualized by ethidium bromide staining as a loading control. (c) Quantification of SA (closed bars) and SA glucosides (open bars) in *AtMYB44* overexpressing plants and *atmyb44* mutant plants. The values represent the average of three replicates and error bars represent standard deviation. One way ANOVA analysis reveals no difference in SA content among control and mutant plants at a confidence level of $P<0.05$.


(a)
Col-0
OX18
OX18 npr1-1
OX18 NahG
npr1-1
NahG
2 weeks
5 weeks
(b)
Col-0
OX18
OX18 npr1-1
OX18 NahG
npr1-1
NahG
AtMYB44
VSP1
PDF1.2
rRNA
No treatment
50 μM MeJA treatment
(c)
SA (ng/g-1FW)
1200
1000
800
600
400
200
0
SA glucosides
free SA
Col-0
OX18
atmyb44

8. *AtMYB44* drives *WRKY70* and *PR1* expression

Because activation of *PR* genes by *AtMYB44* overexpression required SA but did not result from SA accumulation, I investigated the expression of various genes related to expression of *PR* genes in *AtMYB44* overexpression plants. I used RT-PCR, with a limited number of cycles, to screen the expression of 18 regulatory factors and SA-biosynthesis-related genes; by this assay, *WRKY70* was the only gene affected by *AtMYB44* overexpression or knockout mutation (Figure 14). SA biosynthesis and signaling genes, other *WRKYs*, and *TGA* genes, which are induced by SA and biotrophic pathogens, were not affected by *AtMYB44* overexpression or knockout mutation. I also used Northern blot analysis to confirm that *WRKY70* was constitutively overexpressed in OX18 and OX21 plants but was not expressed in *atmyb44* mutants (Figure 15a).

To demonstrate activation of *WRKY70* by *AtMYB44* overexpression, I produced transgenic Arabidopsis plants expressing *AtMYB44* under the control of the ß-estradiol-inducible promoter. *AtMYB44* was induced within 6 hours after ß-estradiol treatment (Figure 15b). *WRKY70* was induced about 6 hours after induction of *AtMYB44* and *PR1* was induced after another 12 hours, suggesting a hierarchical relationship among these genes. This observation supports the hypothesis that induction of *AtMYB44* mediated increased expression of *WRKY70*, which in turn mediates *PR1* gene expression. Moreover, it means that *AtMYB44* overexpression

effects such as activation of *WRKY70* and *PR1* is valid in physiological level.

To test whether the activation of *PR1* and suppression of *PDF1.2* (Figures 5 and 10) in *AtMYB44* overexpression plants required *WRKY70*, I 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, in the OX18 *wrky70* double mutant, *PDF1.2* was induced by MeJA as in wild type. These results demonstrate that expression of *PR1* and suppression of *PDF1.2* in OX18 plants is mediated by *WRKY70* (Figure 15c).

Figure 14. Expression of SA signaling genes in *AtMYB44* overexpressing and *atmyb44* knockout plants

Expression of SA signaling genes was determined by RT-PCR with a limited number of cycles (25 cycles) and PCR product was visualized by ethidium bromide staining.

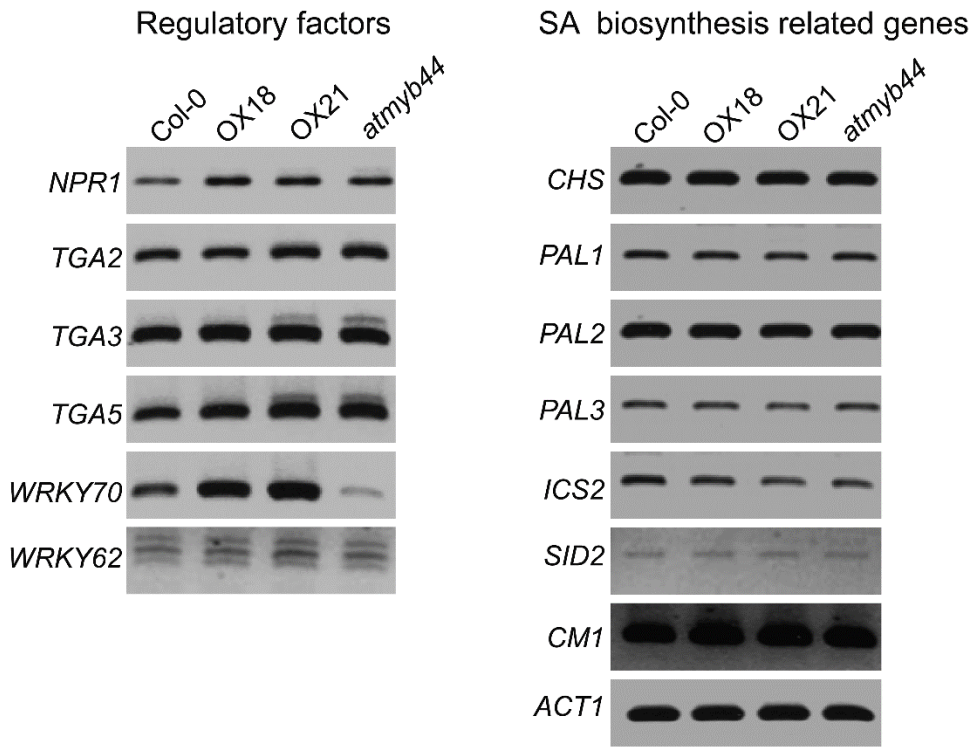
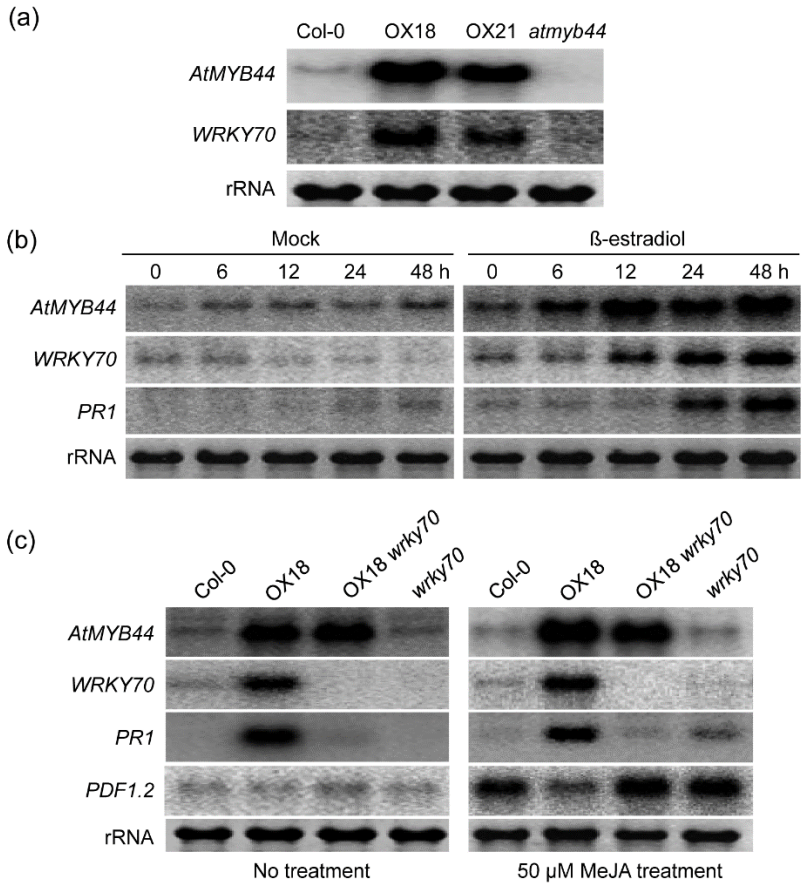

Regulatory factors
Col-0
OX18
OX21
atmyb44
NPR1
TGA2
TGA3
TGA5
WRKY70
WRKY62
SA biosynthesis related genes
Col-0
OX18
OX21
atmyb44
CHS
PAL1
PAL2
PAL3
ICS2
SID2
CM1
ACT1

Figure 15. Role of *AtMYB44* in expression of *WRKY70* and *PR1*

(a) Northern blot showing basal expression of *WRKY70* in Arabidopsis Col-0, *AtMYB44* overexpression (OX18, OX21) and *atmyb44* knockout plants. (b) Induced expression of *WRKY70* and *PR1* by *AtMYB44*. Twelve-day-old seedling 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* overexpressing plants. Two-week-old plants were treated with MeJA for 6 hours and total RNA was analyzed by Northern blot. rRNA was visualized by ethidium bromide staining as a loading control.

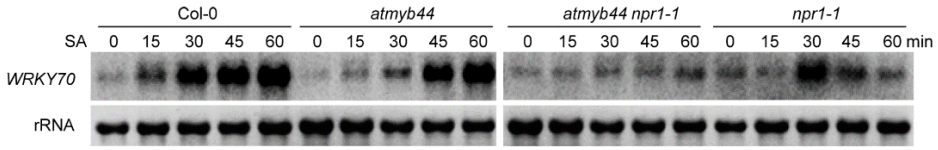

(a)
Col-0
OX18
OX21
atmyb44
AtMYB44
WRKY70
rRNA
(b)
Mock
ß-estradiol
0
6
12
24
48 h
AtMYB44
WRKY70
PR1
rRNA
(c)
Col-0
OX18
OX18 wrky70
wrky70
AtMYB44
WRKY70
PR1
PDF1.2
rRNA
No treatment
50 μM MeJA treatment

9. *AtMYB44* activates *WRKY70* independently of *NPR1*

Accumulation but initiative expression of *WRKY70* is known to be regulated by *NPR1* (Li *et al*., 2004). To define the contribution of *AtMYB44* to *WRKY70* expression, I generated an *atmyb44 npr1-1* double mutant. In wild type plants, *WRKY70* was activated 15 min after SA treatment and increased continuously (Figure 16). 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.

Figure 16. 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.

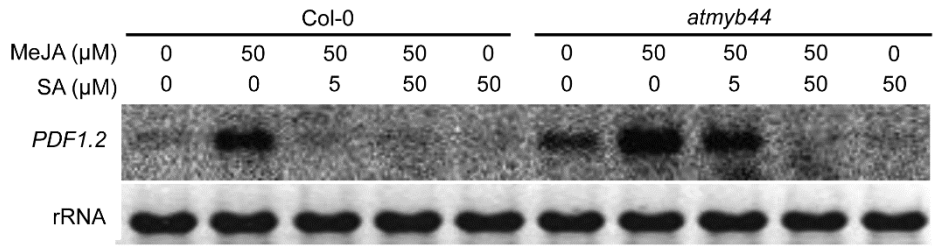

Col-0
atmyb44
atmyb44 npr1-1
npr1-1
SA
0 15 30 45 60 0 15 30 45 60 0 15 30 45 60 0 15 30 45 60 min
WRKY70
rRNA

10. *AtMYB44* modulates crosstalk between SA and JA

Antagonistic interaction between SA and JA is well addressed in plants (Spoel *et al*., 2007; Robert-seilaniantz *et al*., 2011). Exogenous application of SA repressed expression of JA-mediated genes, leading to decreased resistance to necrotrophic pathogen (Spoel *et al*., 2007). *AtMYB44* overexpression led to activation of SA-mediated defense responses but suppression of JA-mediated defense responses. Suppression of JA responses by *AtMYB44* is abolished in SA-deficient condition. It means that function of *AtMYB44* on JA-mediated responses is accomplished through SA-dependent pathway. Thus, it is possible that *AtMYB44* mediated activation of SA signaling leads to suppression of JA-mediated gene expression. To verify this hypothesis, I tested expression of JA-responsive genes *PDF1.2* in Col-0 plants and *atmyb44* knockout plants. *PDF1.2* is induced in MeJA-treated Col-0 plants. At the same time, application of 5 μM SA led to complete suppression of JA-inducible *PDF1.2* expression. However, complete suppression of *PDF1.2* expression was accomplished by application of 50 μM SA in *atmyb44* knockout mutant plants (Figure 17). This data demonstrate that *AtMYB44* participated in SA-mediated suppression of JA-mediated gene expression.

Figure 17. SA-mediated suppression of JA responses in *atmyb44* knockout mutant plants

Two-week-old Arabidopsis plants were treated with combination of SA and JA. Treated seedlings were harvested 6 hour after treatment. Total RNA was analyzed by Northern blot and rRNA was visualized by ethidium bromide staining as a loading control.


Col-0
atmyb44
MeJA (μM) 0 50 50 50 0 0 50 50 50 0
SA (μM) 0 0 5 50 50 0 0 5 50 50
PDF1.2
rRNA

11. AtMYB44 binds to the promoter region of *WRKY70*

AtMYB44 regulates transcriptional activation of *WRKY70* (Figures 15 and 16). To test the possibility that AtMYB44 acts as a direct transcriptional activator for *WRKY70*, I used a GAL4-ß-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-ß-galactosidase reporter plasmid (Figure 18a). The AtMYB44 C-terminal domain without the DNA binding R2R3 domain showed the highest transcriptional activation activity (Figure 18b). Therefore, AtMYB44 can act 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 2 different proteins might be affected by juxtaposition and thus have lost their DNA binding activities (Gourrierec *et al*., 1999: Yu *et al*., 2011).

To determine the consensus binding sequence of AtMYB44, I carried out 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 19a). 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 with GST and expressed in *E. 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 19b).

Binding of full-length AtMYB44 to the promoter region of *WRKY70* was confirmed by using a yeast one hybrid (Y1H) assay (Figure 20a). Thirty nucleotides of sequence from the *WRKY70* promoter region (-328 to -299) containing the AtMYB44 core binding sequence was repeated 4 times and placed upstream of the *HIS3* selectable marker gene. Transformation with the core binding site-*HIS3* construct and full-length AtMYB44 fused with GAL4 activation domain made auxotrophic yeast viable on his^- selectable media. However, a mutant version of the promoter fragment did not activate HIS3 in the Y1H assay.

Chromatin immunoprecipitation (ChIP) experiments were employed to test whether AtMYB44 binds directly to *WRKY70 in vivo*. Extracts from plants overexpressing AtMYB44-GFP were subjected to ChIP analysis and compared with

wild-type plants. ChIP from AtMYB44-GFP overexpressing plants with anti-GFP antibody showed enrichment of the *WRKY70* promoter region containing the AtMYB44 core binding sequence (Figure 19c). Control ChIP product from wild-type plants did not show enrichment of the *WRKY70* promoter region. AtMYB44 also bound to an upstream region of the *WRKY70* promoter containing the AtMYB44 core binding sequences (Sites1 and 2, Figure 20b). 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 a 3'-UTR region of *WRKY70* (Site 6).

I performed transient GUS assay in *Nicotiana benthamiana* to confirm transactivation of *WRKY70* through the core binding sequence. *WRKY70* promoter sequences (-328 to -299) was repeated 4 times and fused with *GUS* reporter gene (4xRE44). The reporter plasmid and effector plasmid, *35S:AtMYB44*, were co-infiltrated into *N. benthamiana*. *GUS* reporter gene was expressed by the co-infiltration of *35S:AtMYB44* with 4xRE44. However, mutant reporter containing mutated version of *WRKY70* promoter (4xmRE44) was not activated by AtMYB44 (Figure 19d).

Figure 18. Transcriptional activation domain assay of AtMYB44

(a) Various fragments of AtMYB44 were recombined with the GAL4 binding domain as shown in the diagram. (b) Transcriptional activity was measured by β–galactosidase assay. Data represent mean values of three independent measurements. Error bars represent standard deviation.

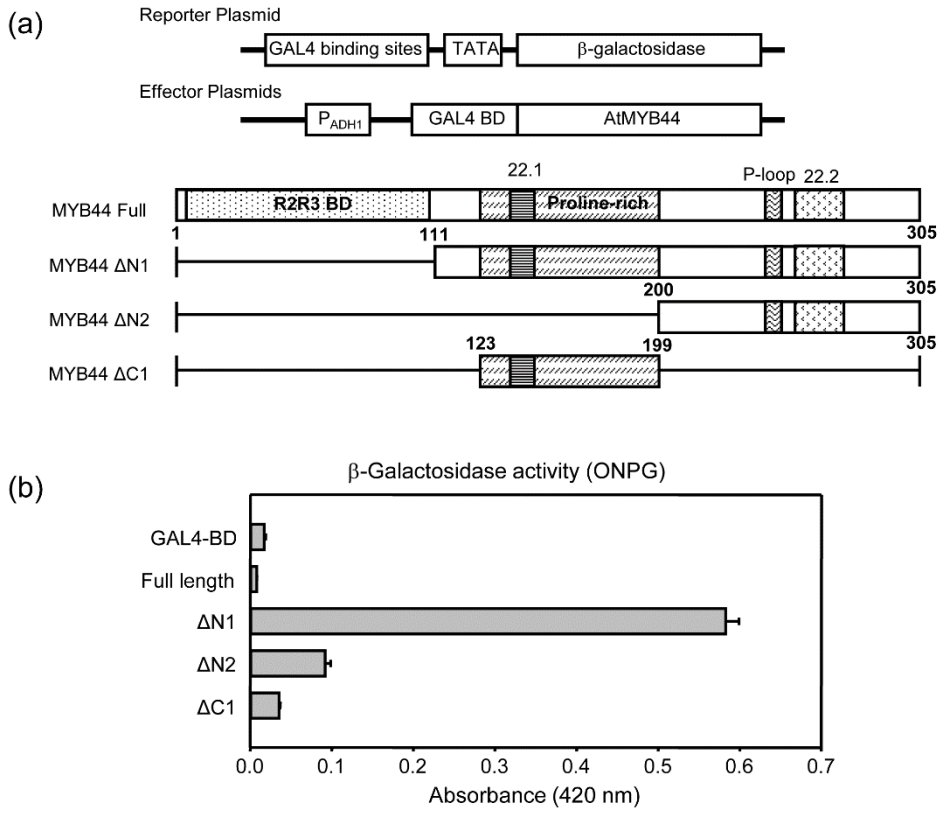

(a)
Reporter Plasmid
GAL4 binding sites
TATA
β-galactosidase
Effector Plasmids
P ADH1
GAL4 BD
AtMYB44
22.1
P-loop
22.2
MYB44 Full
R2R3 BD
Proline-rich
1
111
305
MYB44 ΔN1
200
305
MYB44 ΔN2
123
199
305
MYB44 ΔC1
(b)
β-Galactosidase activity (ONPG)
GAL4-BD
Full length
ΔN1
ΔN2
ΔC1
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
Absorbance (420 nm)

Figure 19. 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 characters 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 wild type *WRKY70* promoter region (wt) and mutant versions of the promoter (m1, m2, m3) are shown on the top. Radiolabeled probe was competed with excess 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* overexpressing plants were immunoprecipitated with anti-GFP. For quantitative analysis, PCR products amplified by the primer set containing AtMYB44 core binding sequence were analysis by qPCR. Statistical significance of the measurements was determined using a t-test (*$P<0.01$) by comparison with the value of Col-0. Three measurements were averaged for individual analysis. Error bars indicate standard error of the mean. (d) *WRKY70* promoter sequence of -328 to -299 containing the core binding sequence (RE44) or its mutant version (mRE44) was repeated 4 times and combined with *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.

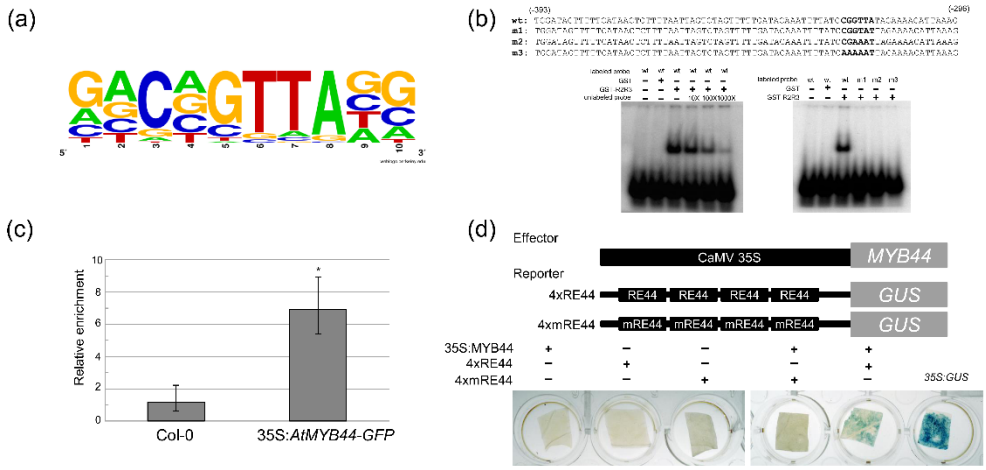
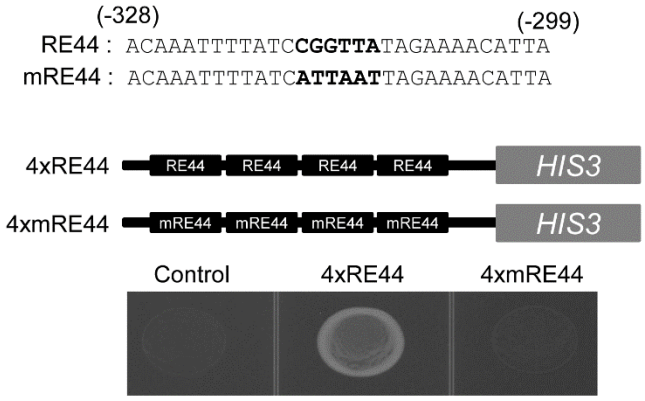

(a)
(b)
(c)
(d)
Relative enrichment
Col-0
35S:AtMYB44-GFP
Effector
CaMV 35S
MYB44
Reporter
4xRE44
RE44
GUS
4xmRE44
mRE44
35S:MYB44
35S:GUS

Figure 20. Direct binding of AtMYB44 to the *WRKY70* promoter *in vivo*

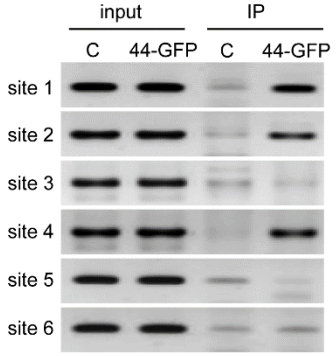
(a) Interaction of AtMYB44 with *WRKY70* promoter was tested by yeast one-hybrid (Y1H) assay. *WRKY70* promoter sequence of -328 to -299 containing the core binding sequence (RE44) or its mutant version (mRE44) was repeated 4 times and combined with HIS3 for the Y1H assay. A cDNA encoding the whole AtMYB44 protein was fused with the activation domain as bait. (b) Structure of *WRKY70* indicating regions amplified by PCR (top). Open boxes at sites 1, 2 and 4 represent the core AtMYB44 binding sequence. PCR primer sets binding to another promoter region (Site 3), CDS region (Site 5) or 3'-UTR region of *WRKY70* (Site 6) were also employed for negative controls. Fragmented chromatin DNA of Arabidopsis Col-0 and *AtMYB44-GFP* overexpressing plants were immunoprecipitated with anti-GFP and analyzed by PCR (bottom left). Chromatin DNA before immunoprecipitation was PCR amplified as an input control.

(a)


(-328)
(-299)
RE44 : ACAAATTTTATCCGGTTATAGAAAACATTA
mRE44 : ACAAATTTTATCATTAATTAGAAAACATTA
4xRE44
RE44
RE44
RE44
RE44
HIS3
4xmRE44
mRE44
mRE44
mRE44
mRE44
HIS3
Control
4xRE44
4xmRE44

(b)


site1
site 2
site 3
site 4
site 5
site 6
TAACAG
TAACGG
CGGTTA
WRKY70
-2189
-1878
-1200
-311

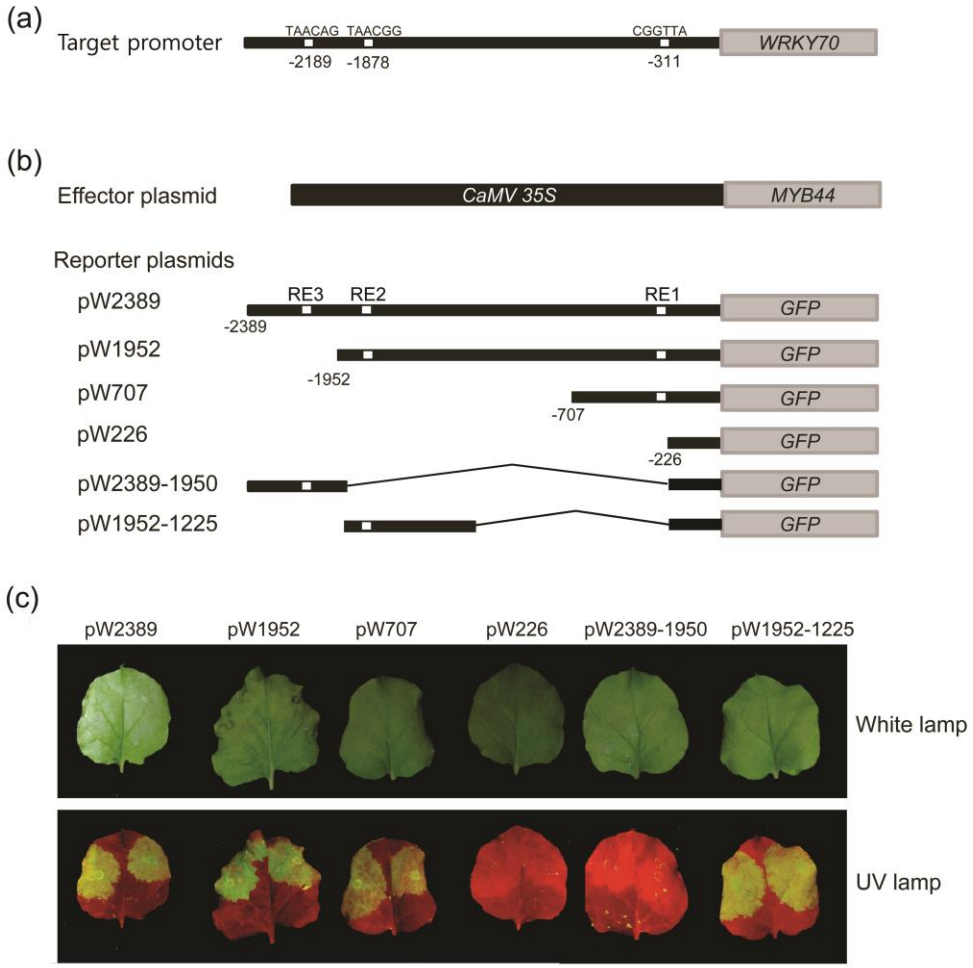

input
IP
C
44-GFP
C
44-GFP
site 1
site 2
site 3
site 4
site 5
site 6

12. Transactivation of *WRKY70* by AtMYB44

From SELEX, EMSA and ChIP experiments I demonstrated that *AtMYB44* directly binds to the CNGTTA element in the promoter region of *WRKY70*. There are three MYB binding elements (REs) in the promoter region of *WRKY70*. To test their function in transcriptional activation of *WRKY70* by *AtMYB44*, I carried out *trans*-activation analysis by using transient expression system in *Nicotiana benthamiana* (Figure 21). The *WRKY70* promoter region of 2,380 bps containing three MYB binding elements was successfully activated reporter gene expression by the effector AtMYB44. To define the contribution of each MYB binding sequence to *AtMYB44* dependent activation of *WRKY70*, I co-infiltrated various constructs of truncated *WRKY70* promoter sequences with *AtMYB44* effector plasmid. Presence of RE1 or RE2 in the reporter plasmid was sufficient to activate *GFP* gene by *AtMYB44*. However, *GFP* gene fused under *WRKY70* promoter sequence containing RE3 alone was not activated by *AtMYB44*. It means that AtMYB44 binds to RE1 and RE2, and directly activates expression of *WRKY70*. Those findings demonstrate that AtMYB44 binds to at least two regions of *WRKY70* promoter for target gene activation.

Figure 21. Transactivation of *WRKY70* by AtMYB44

(a) Structure of AtMYB44 binding sequence in *WRKY70* promoter. (b) A cDNA encoding AtMYB44 was fused to the CaMV 35S promoter as an effector and various constructs of truncated *WRKY70* promoter sequences was fused to the GFP reporter gene. (c) The reporter and effector constructs were infiltrated into *Nicotiana bethamiana*. Transactivation activity was detected by GFP fluorescence.


(a)
Target promoter
TAACAG
TAACGG
CGGTTA
-2189
-1878
-311
WRKY70
(b)
Effector plasmid
CaMV 35S
MYB44
Reporter plasmids
pW2389
RE3
RE2
RE1
-2389
GFP
pW1952
-1952
GFP
pW707
-707
GFP
pW226
-226
GFP
pW2389-1950
GFP
pW1952-1225
GFP
(c)
pW2389
pW1952
pW707
pW226
pW2389-1950
pW1952-1225
White lamp
UV lamp

DISCUSSION

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

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

Here I show that *AtMYB44* is induced by MeJA (Figure 3). JA signaling is required for disease resistance against necrotrophic pathogens such as *A. brassicicola* (Thomma *et al*., 1998; Seo *et al*., 2001); *AtMYB44* was also induced by *A. brassicicola* (Figure 4c). However, overexpression 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 4 and 5). In *atmyb44* plants, expression of *VSP1* and *PDF1.2* were also up-regulated without JA treatment (Figure 5). These data indicate that *AtMYB44* acts as negative regulator of JA-mediated defense responses. The negative effects of *AtMYB44*

overexpression on JA signaling were not limited to the defense response but also affected JA-mediated root growth inhibition, root hair development (Figure 6) and anthocyanin accumulation (Figure 7; Jung *et al*., 2010).

AtMYB44 overexpressing plants showed enhanced resistance against a biotrophic pathogen *Pst* DC3000, but *atmyb44* knockout plants showed decreased resistance compared to wild type (Figure 9). 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). Enhanced disease resistance established in overexpressing plants was accompanied by activation of SA-dependent *PR* genes (Figure 10, Li *et al*., 2004). Moreover, *PR1* was rapidly and strongly activated in *AtMYB44* overexpressing plants by exogenous SA treatment (Figure 10). This demonstrates that *AtMYB44* acts as a positive regulator of SA-mediated defense responses. Moreover, antagonistic effect of SA on JA pathway was reduced in *atmyb44* mutant (Figure 17). Mutual antagonism between JA- and SA-mediated responses is thus observed in overexpression lines and knockout mutants of *AtMYB44*.

2. *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* overexpression (Figure 14). Data in this study show that *WRKY70* was up-regulated, thus up-regulating *PR1* in *AtMYB44* overexpressing plants (Figure 14 and 15). *PR* genes are activated by *WRKY70* in SA signaling and *WRKY70* was identified as an important regulatory component in 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* overexpressing plants (Li *et al*., 2004, 2006). *AtMYB44* overexpressing plants showed a similar pattern of disease resistance to *WRKY70* overexpressing 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 of induction of *PDF1.2* expression and elimination of *PR1* expression (Figure 15c).

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, I showed that *AtMYB44* acts as a transcriptional activator (Figure 18). AtMYB44 binds to the promoter of

WRKY70, which contains the AtMYB44 core binding sequence CNGTTA (Figure 19). ChIP, Y1H and EMSA 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 by *AtMYB44* overexpression and β-estradiol induced transactivation of *AtMYB44* (Figure 15). Transient expression of the reporter gene driven by the core binding sequence of *WRKY70* promoter provides more evidence that *AtMYB44* regulates expression of *WRKY70* (Figure 19d, 20 and 21) .

Enhanced expression of *PR* genes in OX18 is not a result of increased SA content (Figure 13c). It is coincided with the data that expression of SA biosynthesis genes is not affected by *AtMYB44* overexpression and knockout mutation (Figure 14). The phenotype is reminiscent of *WRKY70* overexpression plants, in which *PR1* was constitutively overexpressed 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 13b). These results suggest that a basal level of SA may be essential to activate *AtMYB44* and *WRKY70*. The precise mechanism remains to be determined.

One possibility is that basal level of SA is essential for activation of *WRKY70*. There are reports that expression of *WRKY70* was totally abolished in *NahG* transgenic plants even with SA treatment and biotrophic pathogen infection (Li *et al*., 2004; Knoth *et al*., 2007). It is also reported that *NahG* and *sid2* mutant plants

only contain about 30% of endogenous SA compare with that of control plants (Maria and Sergi, 2009). Thus, decreased SA level in *NahG* transgenic plants could affect the expression of *WRKY70*.

It is reported that expression of *WRKY70* is controlled by *ARABIDOPSIS HOMOLOG TRITHORAX 1* (*ATX*1). *ATX1* is involved in establishing the trimethylation pattern of histone H3 tail lysine 4 residues of nucleosomes (H3K4me3) (Alvarez-Venegas *et al*., 2007). In normal state, low level of H3K4me3 is detected in *WRKY70* nucleosome. In infected condition, H3K4me3 level is increased in *WRKY70* nucleosome and it leads to activation of *WRKY70*. Above ATX, several components such as *Suppressor of NPR1, Inducible 1* and *Photoperiod independent early flowering 1* are participated in control of SA-responsive gene (Alvarez *et al*., 2010). And their regulation of SA-responsive gene expression is depended on cellular SA level. Expression of several SA-responsive genes is controlled by chromatin remodeling complex. And recruitment of chromatin remodeling complex is driven by accumulation of endogenous SA. Moreover, low level of SA leads to histone replacement (H2A.Z to H2A) of SA-responsive genes (van den Burg and Takken, 2009). Thus, it is possible that decreased SA level in *NahG* transgenic plants could affect the accessibility of transcriptional activator such as *AtMYB44* to *WRKY70* promoter.

Another possibility interpretation is that basal level of SA is essential for activation of uncharacterized factor which synergistically activate downstream gene with *AtMYB44*. Additional transcription factors or co-factors are involved in the formation

of a transcriptional complex for target gene expression (Thanos and Maniatis, 1995). MYB transcription factors are one of the largest transcription factor families in *Arabidopsis*, and they control various physiological processes (Dubos *et al*., 2010). MYB transcription factor regulates development and stress responses by cooperation with other factors. In Arabidopsis, AtMYB77 interact with ARF7 and synergistically activate auxin dependent gene (Shin *et al*., 2007). More recently, it has been reported that AtMYB30 interact with BES1 and regulated expression of BR signal pathway (Li *et al*., 2009). *AtMYB2* and *AtMYC2* act together for activation of ABA-dependent genes (Abe *et al*., 2003). They showed that overexpression of both *AtMYB2* and *AtMYC2* is required for activation of ABA-dependent genes. Thus, it is possible that basal level of SA is required for activation of AtMYB44 interacting protein which cooperatively activates *WRKY70*.

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

There is a report that *AtWhy1* 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 *npr1-1* mutant background (Clarke *et al*., 1998, Shah *et al*., 2001). Therefore, an *NPR1*-independent branch does exist in the SA signaling pathway.

WRKY70 is associated with both *NPR1*-dependent and *NPR1*-independent pathways in SA signaling (Li *et al*., 2004). For example, *WRKY70* overexpression resulted in activation of *PR* genes in the *npr1-1* mutant background. It is also 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 I showed that SA-induced expression of *AtMYB44* does not require *NPR1* (Figure 11a). By double mutant analysis, I showed that activation of *WRKY70* and *PR1* by *AtMYB44* overexpression also did not require *NPR1* (Figure 11b). Moreover, *NPR1*-independent expression of *WRKY70* is abolished in the *atmyb44 npr1* double mutant (Figure 16). These data demonstrated that *AtMYB44* is an *NPR1*-independent regulatory component that directly regulates expression of *WRKY70*. Interestingly, activation of *PR1* by *AtMYB44* overexpression plants is reduced in *npr1-1* mutant background (Figure 11b and c). It means that *AtMYB44*-mediated activation of *PR1* is partially achieved through *NPR1*. However, activation of *WRKY70* by *AtMYB44* overexpression is not affected by *npr1* mutation (Figure 11b). It means that *AtMYB44* could activate expression of *WRKY70* without NPR1. It is reported that *WRKY70* overexpression led to activation of *PR1*. And activation of *PR1* by *WRKY70* overexpression is reduced in *npr1-1* mutant background (Li *et al*., 2004). Therefore, reduction of *AtMYB44*-mediated activation of *PR1* in *npr1-1* mutant background is explained by *NPR1* dependence of *WRKY70* for *PR1* activation.

4. Regulation of defense responses by *AtMYB44*

The network of SA- and JA-responsive gene expression mediated by *AtMYB44* is summarized in Figure 22. Plant resistance is triggered by recognition of the invading pathogen. Against pathogen attack, plants develope effective defense response by changing the endogenous defense hormone level such as SA and JA (De Vos *et al*., 2005; Koo *et al*., 2007; Tsuda *et al*., 2008). *AtMYB44* transcript is detected at 12 hours infection of *A. brassicicola* and 6 hour after *Pst* DC3000 infection (Figure 4c and 8). *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 3). JA induces expression of JA-responsive genes such as *PDF1.2* through COI1, which confers 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 activation of JA-responsive downstream genes (Li *et al*., 2004). At the same time, JA-induced expression of *AtMYB44* activates *WRKY70* (Figure 3). 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 needs to be under tight and finely tuned regulation (Spoel *et al*., 2003; Moore *et al*., 2011). Induction of a negative regulator in response to signal molecules contributes to the fine-tuning of defense responses (Journot-Catalino *et al*., 2006; Chini *et al*., 2007). Thus, the biological role of *AtMYB44* could be a fine-tuning of 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 5). *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 can be counter-balanced by the function of the WRKY family in promoting the JA signal. For example, *WRKY7, WRKY8, WRKY11* and *WRKY17* are induced by the biotrophic pathogen *Pst* DC3000, but these *WRKY*s 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 existence of fine-tuned regulation of the defense response.

Another potential role of *AtMYB44* in the plant defense response was also described previously. In Arabidopsis, the MPK3 and its upstream regulator MKK4 initiate signal cascade to abiotic and biotic stress responses. In pathogen molecular

pattern (PAMP)-induced resistance, VirE2 interacting protein 1 (VIP1) is phosphorylated by MPK3 for nuclear transportation in response to bacterial invasion. In the nucleus, phosphorylated VIP1 activates expression of stress inducible genes including *PR1* (Djamei *et al*., 2007). *AtMYB44* is reported to be one of the targets of *VIP1* (Pitzschke et al., 2009). I showed that *AtMYB44* directly activates expression of *WRKY70* thus *PR1* (Figure 15b and 19). From those data, *AtMYB44* could mediate PAMP induced defense responses through *WRKY70* and *PR1*. Even though its effect in biotic stress response is not known, there are reports that AtMYB44 is phosphorylated by MPK3 and its phosphorylation is required for abiotic stress tolerances (Nguyen *et al*., 2012; Persak and Pitzschke, 2013). MPK3 is also participated in activation of biotic stress responses. The effect of phosphorylation of AtMYB44 by MPK3 in biotic stresses remains to be determined.

According to previous reports, *AtMYB44* is also induced by abiotic stresses such as drought, cold temperature and salt (Jung *et al*., 2008). ABA is an important component of abiotic stress resistance such as drought and salt stresses (Christmann *et al*., 2006). It is reported that ABA negatively regulates SA-mediated defense responses. Exogenous ABA prevents the accumulation of SA and suppresses resistance (Mohr and Cahill, 2007). Moreover, ABA-deficient mutants showed enhanced SA-mediated resistance (Jensen *et al*., 2008; Fan *et al*., 2009). Although antagonistic interactions have been reported between ABA and SA, recent studies documented that positive interaction between the ABA and SA. A subset of *PR* genes (*PR1*, *PR2* and *PR5*) is also induced by cold, drought and high salt (Seo *et al*.,

2008). An activation tagging line of *Activated Disease Resistance 1* (*ADR1*) showed drought tolerance and SA-mediated resistance to biotrophic pathogens (Grant *et al*., 2003). Drought tolerance of *ADR1* activation tagging line was reduced by *NahG* and *eds1* background (Chini *et al*., 2004). Activation tagging line of *AtMYB96* also showed similar phenotype. Moreover, SA biosynthesis gene *SID2* is upregulated by ABA, drought and salt treatment (Seo *et al*., 2010).

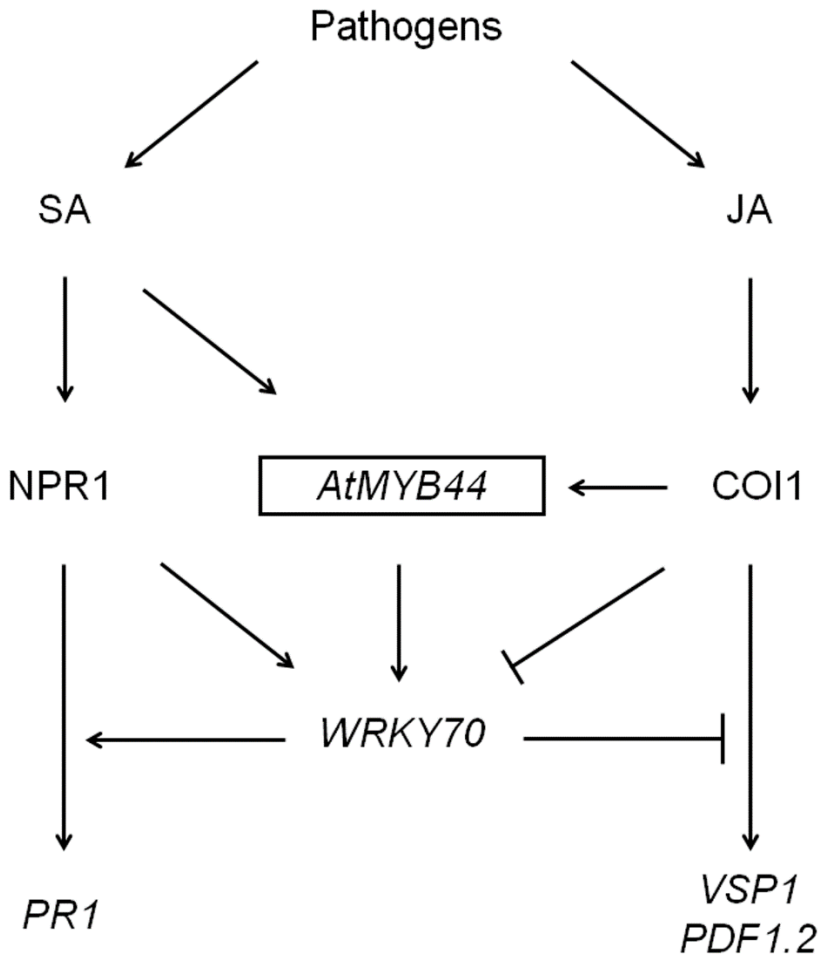
AtMYB44 overexpression plants showed tolerance to those ABA-mediated abiotic stresses by modulating stomatal closure. In fact, *AtMYB44* is highly expressed in guard cell and its overexpression resulted in rapid closure of stomata (Jung *et al*., 2008). Stomatal closure triggered by bacterial invasion is considered to be a key part in the beginning of plant innate immunity. There are reports that ABA and SA are required for stomatal closure to biotrophic pathogen and PAMPs (Melotto *et al*., 2006; Ton *et al*., 2009). Stomatal closure induced by PAMPs is abolished in ABA biosynthesis mutant *aba3-1* and ABA signaling mutant *ost1-2*. Similarly, stomata of SA-deficient mutant *NahG* and *eds16* do not response to PAMPs (Melotto *et al*., 2006). Bacterial titer of *AtMYB44* overexpression plants was about 10 times lower (infiltration; Figure 9a and b) and 100 times lower (spray; Figure 9c and d) than that in wild type plants. It means that *AtMYB44* overexpression plants showed more enhanced resistance by *Pst* DC3000 spray treatment than direct infiltration. It is therefore possible that regulation of stomatal movement and defense gene expression by *AtMYB44* together could contribute to plant innate immunity against biotrophic pathogens. Further studies on the function

and molecular mechanism of *AtMYB44* in plant innate immunity are required.

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

Figure 22. Role of *AtMYB44* in crosstalk 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, PDF1.2*) by direct transcriptional activation of *WRKY70*. JA-mediated expression of *AtMYB44* is 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).


Pathogens
SA
JA
NPR1
AtMYB44
COI1
WRKY70
PR1
VSP1
PDF1.2

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ABSTRACT IN KOREA

식물은 다양한 병원체의 공격으로부터 자신을 보호하기 위하여 유기적인 방어 기작을 가지고 있으며, 이 방어기작은 식물 호르몬에 의해서 조절된다. 이 과정은 식물 호르몬인 살리실산 (salicylic acid)와 자스몬산 (jasmonic acid)에 의해 주도적으로 조절된다. 살리실산은 바이오트로픽 병원균에 대한 방어기작을 담당하는 주요 식물 호르몬으로서 *NPR1* 및 *PR* 유전자의 발현을 조절하여 병원균을 사멸하는 역할을 한다. 자스몬산은 네크로트로픽 병원균 및 곤충에 대한 방어기작을 담당하며 *COI1, VSP, PDF* 유전자의 발현을 조절하여 병원균의 공격을 방어한다. 자스몬산과 살리실산은 길항작용을 하며 효과적인 방어기작을 유도하는 것으로 알려져 있다. *AtMYB44*는 식물의 R2R3 MYB 전사인자 그룹에 속하는 유전자로 자스몬산과 살리실산 등에 의해 발현이 유도된다. 이 유전자의 기능 분석을 위하여 *AtMYB44*의 과발현 식물체 및 삽입돌연변이 식물체 (*atmyb44*)를 이용하였다. *AtMYB44* 유전자를 과발현시킨 식물의 경우 자스몬산 신호전달을 받는 하위 유전자들의 발현이 억제되었고 외부에서 메틸자스몬산 (methyl jasmonate)을 처리하였을 때도 대조군에 비해 하위 유전자가 낮은 수준으로 발현되는 것을 확인하였다. *AtMYB44* 과발현 식물체는 자스몬산이 매개하는 뿌리 성장 저해, 안토사이아닌 생합성 및 뿌리털 발달에 있어서 대조군에 비해 적은 영향을 받는 것을 확인하였다. 또한 살리실산에 반응하는 하위 유전자의 경우 *AtMYB44* 과발현 식물체에서 그 발현이 증가하는 것으로 나타났다. *atmyb44* 삽입돌연변이체의 경우 과발현 식물체와는 반대로 자스몬산에 반응하는

하위 유전자의 발현이 증가하고 살리실산에 반응하는 하위 유전자의 발현이 감소하는 것으로 나타났다. 이와 같은 하위 유전자의 발현 양상과 사물기생 병원균인 *Alternaria brassicicola* 와 활물기생 병원균인 *Pseudomonas syringae* pv. *tomato* DC3000 을 이용한 실험을 통해 *AtMYB44* 가 자스몬산과 살리실산에 의한 식물체의 방어메카니즘을 길항적으로 조절함을 확인하였다.

AtMYB44 과발현 식물체의 특성은 살리실산을 분해하는 효소가 과발현된 식물체인 *NahG* 에 의해 회복된다. 하지만 살리실산 신호전달의 중요 조절인자인 NPR1 은 *AtMYB44* 의 발현 및 기능에 있어서 직접 작용하지 않음을 확인하였다. 따라서 *AtMYB44* 의 기능에 있어서 살리실산이 중요한 역할을 하며, 이 유전자는 *NPR1* 이 아닌 다른 조절인자에 의해 기능함을 확인하였다. *AtMYB44* 과발현 식물체에서 발현이 증가하는 유전자 중에 *WRKY70* 은 자스몬산과 살리실산의 상호작용에 있어서 중추적인 역할을 하는 유전자이다. *WRKY70* 은 *AtMYB44* 과발현 식물체에서 발현량이 증가하며 *AtMYB44* 를 유도발현한 경우에도 발현이 유도되고, *atmyb44* 돌연변이식물체에서 발현이 감소되는 것으로 보아 *AtMYB44* 의 하위에 존재함을 증명하였다. *WRKY70* 은 살리실산 반응을 매개하는 중요 조절인자로서 *NPR1* 에 의해 부분적으로 조절되는 특징을 가지고 있다. 이에 기반하여 제작한 이중돌연변이체 (*atmyb44 npr1-1*)를 이용한 실험에서 *WRKY70* 의 발현이 *AtMYB44* 와 *NPR1* 에 의해서 각각 독립적으로 조절됨을 확인하였다. *AtMYB44* 전사인자를 통한 자스몬산과 살리실산의 신호전달 조절원리를 규명하기 위하여 이 전사인자의 결합서열 (5'-CNGTTA-3')을 systematic evolution of ligands by exponential enrichment (SELEX) 실험을 통해 찾아내었다. 그리고 프로모터 분석을

통해 AtMYB44 의 조절을 받는 *WRKY70* 유전자의 프로모터 지역에 AtMYB44 의 결합서열이 존재함을 확인하였다. AtMYB44 와 *WRKY70*의 관계를 확인하기 위하여 yeast one hybrid (Y1H), electrophoretic mobility shift assay (EMSA), chromatin immunoprecipitation (ChIP)를 수행하였고 이를 통해 AtMYB44 전사인자가 식물체 내에서 *WRKY70* 유전자의 프로모터 지역에 결합함을 증명하였다. 또한 *Nicotiana benthamia*를 이용한 co-infiltration assay 및 효모를 이용한 trans-activation activity 실험을 수행하여 AtMYB44 가 *WRKY70* 유전자의 프로모터에 결합하여 발현을 증가시킴을 증명하였다. 이러한 실험 결과로부터 AtMYB44 전사인자가 *WRKY70* 유전자의 프로모터에 결합하여 *WRKY70* 유전자의 발현을 직접 조절하고, 이를 통해 자스몬산과 살리실산에 의한 식물의 방어 반응을 조절한다는 것을 밝혀내었다.

주요어: *AtMYB44*, R2R3 MYB 전사인자, 자스몬산 신호 전달 체계, *WRKY70*, 살리실산 신호 전달 체계, 애기장대

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PUBLICATIONS

1. Shim, J.S., Jung, C., Lee, S., Min, K., Lee, Y.-W., Choi, Y., Lee, J.S., Song, J.T., Kim, J.-K., and Choi, Y.D. (2013), *AtMYB44* regulates *WRKY70* expression and modulates antagonistic interaction between salicylic acid and jasmonic acid signaling. ***Plant J***., 73, 483-495.

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3. Jung, C., Kim, Y.-K., Oh, N.I., Shim, J.S., Seo, J.S., Choi, Y.D., Nahm, B.H. and Cheong, J.J. (2012), Quadruple 9-mer-based protein binding microarray analysis confirms AACnG as the consensus nucleotide sequence sufficient for the specific binding of AtMYB44. ***Mol. Cells***, 34, 531-537.

4. Jung, C., Shim, J.S., 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.

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먼저 학부생때부터 지금까지 의욕만 앞섰던 부족한 저를 학문의 길로 인도하여 주시고 많은 가르침을 주신 최양도 교수님께 고개 숙여 감사를 드립니다. 선생님께서 기회를 주시고 지켜봐주시고 또한 이끌어 주셨기에 모든 일이 가능하였습니다. 언제나 학생들을 먼저 생각하고 기다려 주신 배려 감사합니다. 교육자로서 그리고 과학자로서 선생님께서 보여주신 모습들 유념하여, 앞으로도 선생님의 가르침을 잊지 않고 기본에 충실한 과학자가 되도록 최선을 다하겠습니다. 그리고 박사학위청구논문 심사에 있어서 체계를 잡아 주시고 조언을 아끼지 않으신 이종섭 교수님, 김민균 교수님, 배의영 교수님, 하선화 교수님께 깊은 감사를 드립니다. 이 논문을 완성할 수 있게끔 도움을 주시고 조언과 격려를 아끼지 않으신 김주곤 교수님께 감사를 드립니다. 항상 관심을 가져주시고 호르몬 정량에 있어서도 도움을 주신 이인원 교수님과 김정한 교수님께 감사를 드립니다. 또한 실험을 하면서 마주한 문제를 같이 고민해 주신 송종태 교수님, 서학수 교수님, 최연희 교수님께도 감사를 드립니다. 만날 때마다

관심을 보여주시고 조언해주신 이용환 교수님께 역시 감사를 전합니다. 10년이 넘는 시간동안 부족한 저에게 전공 분야에 대한 지식을 전해주시고, 여러 가지 도움과 조언을 해주신 정진 교수님, 김수일 교수님, 김수언 교수님, 김정한 교수님, 오기봉 교수님, 노희명 교수님, 이상기 교수님, 신찬석 교수님께도 감사를 전합니다. 학위 과정에서 여러 실험 결과에 대한 조언을 해주신 정종주 교수님께도 감사를 드립니다.

이 실험을 시작하면서 선행 연구를 통해 많은 도움을 주시고, 실험에 있어 부족한 부분을 채워주신 정춘균 박사님께 감사를 드립니다. 학부생인 저를 위해 유전자 재조합부터 기계분석까지 도움을 주시고 제 옆자리에서 관심을 쏟아주신 구연종 박사님께 감사를 드립니다. 두분 모두 앞으로 건승하시기를 기대합니다. 무엇보다도 오랜 시간동안 동거동락 해온 가족 같은 우리 분자생물학 실험실원들께 감사를 드립니다. 실험 계획을 세움에 있어 도움이 되주신 주석이형, 단백질 실험에 대한 많은 조언을 해주신 송연이형, 항상 등 뒤에서 지켜보시고 같이 고민해 주신 한용이형, 언제나 먼저 신경써주시는 고마운 규필이형, 귀찮을 법도 한 질문에도 항상 같이 고민해 주신 준성이형, 실험실 일에 항상 열성이었고 형을 많이 도와준 상준이, 지금도 실험실을 위해 노고를 마다하지 않는 듬직한 태영이, 무뚝뚝한 선배들 잘 챙기며 맡은바에 소홀함이 없는 순현이에게 진심으로 고맙다는 말씀을 드립니다. 여러분들이 아껴주시고 독려해주셨기에 오늘이 있음을 잊지 않겠습니다. 그리고 지금은 각자의 길에서 최선을 다하고 계신 우리 실험실 멤버들인 가람 누나, 황배 형님, 나리 누나, 현진, 은실에게도 감사를 드립니다. 세미나를 통해 만나뵙고 도움을 받을 수 있

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