The Arabidopsis Mediator Complex Subunit16 Is a Key Component of Basal Resistance against the Necrotrophic Fungal Pathogen *Sclerotinia sclerotiorum*[1][OPEN]

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Although *Sclerotinia sclerotiorum* is a devastating necrotrophic fungal plant pathogen in agriculture, the virulence mechanisms utilized by *S. sclerotiorum* and the host defense mechanisms against this pathogen have not been fully understood. Here, we report that the Arabidopsis (*Arabidopsis thaliana*) Mediator complex subunit MED16 is a key component of basal resistance against *S. sclerotiorum*. Mutants of *MED16* are markedly more susceptible to *S. sclerotiorum* than mutants of 13 other Mediator subunits, and *med16* has a much stronger effect on *S. sclerotiorum*-induced transcriptome changes compared with *med8*, a mutation not altering susceptibility to *S. sclerotiorum*. Interestingly, *med16* is also more susceptible to *S. sclerotiorum* than *coronatine-insensitive1-1* (*coi1-1*), which is the most susceptible mutant reported so far. Although the jasmonic acid (JA)/ethylene (ET) defense pathway marker gene *PLANT DEFENSIN1.2* (*PDF1.2*) cannot be induced in either *med16* or *coi1-1*, basal transcript levels of *PDF1.2* in *med16* are significantly lower than in *coi1-1*. Furthermore, ET-induced suppression of JA-activated wound responses is compromised in *med16*, suggesting a role for MED16 in JA-ET cross talk. Additionally, MED16 is required for the recruitment of RNA polymerase II to *PDF1.2* and *OCTADECANOID-RESPONSIVE ARABIDOPSIS ETHYLENE/ETHYLENE-RESPONSIVE FACTOR59* (*ORA59*), two target genes of both JA/ET-mediated and the transcription factor WRKY33-activated defense pathways. Finally, MED16 is physically associated with WRKY33 in yeast and in planta, and WRKY33-activated transcription of *PDF1.2* and *ORA59* as well as resistance to *S. sclerotiorum* depends on MED16. Taken together, these results indicate that MED16 regulates resistance to *S. sclerotiorum* by governing both JA/ET-mediated and WRKY33-activated defense signaling in Arabidopsis.

Sclerotinia sclerotiorum is one of the most devastating necrotrophic fungal plant pathogens in agriculture. It infects over 400 plant species worldwide and causes annual losses of more than $200 million in the United States (Boland and Hall, 1994; Bolton et al., 2006). *S. sclerotiorum* depends on several virulence mechanisms to successfully attack the broad range of host plants. One mechanism is to produce the non-host-selective toxin oxalic acid, which inhibits plant defense responses, modulates host redox environment, suppresses autophagy, and activates cell wall-degrading enzymes (Marciano et al., 1983; Godoy et al., 1990; Cessna et al., 2000; Rollins and Dickman, 2001; Kim et al., 2008; Williams et al., 2011; Kabbage et al., 2013). Secretion of cell wall-degrading enzymes is another virulence mechanism of *S. sclerotiorum*, which facilitates penetration, tissue maceration, and plant cell wall depolymerization (Lumsden, 1979; Riou et al., 1991, 1992). *S. sclerotiorum* may also secrete effector proteins, such as *S. sclerotiorum* INTEGRIN-LIKE and *S. sclerotiorum* CHORISMATE MUTASE1, to diminish plant defense responses (Kabbage et al., 2013; Zhu et al., 2013). A recent bioinformatic study revealed that the *S. sclerotiorum* genome encodes a large set of candidate effector proteins (Guyon et al., 2014), which may have functions in *Sclerotinia* spp. pathogenesis.

Compared with the virulence mechanisms, resistance in host plants against *S. sclerotiorum* is less well understood. Microarray results indicate that *S. sclerotiorum* infection induces the expression of genes encoding components of diverse biological processes, including the jasmonic acid (JA) and ethylene (ET) signaling pathways

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C.W., J.A.R., and Z.M. conceived the project; C.W. and Z.M. designed the experiments; C.W. performed most of the experiments; X.D. provided technical assistance to C.W.; Y.Z. performed the microarray experiment; J.Y. and Y.S. analyzed the microarray data; Z.M. wrote the article with contributions from C.W., J.A.R., and J.Y.

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(Zhao et al., 2007, 2009). Recent studies of Arabidopsis (*Arabidopsis thaliana*) mutants revealed that JA, ET, auxin, abscisic acid, nitric oxide, and reactive oxygen species all contribute to basal resistance against *S. sclerotiorum* (Guo and Stotz, 2007; Perchepied et al., 2010; Stotz et al., 2011). Conclusions in these studies about the role of the salicylic acid (SA) signaling pathway in resistance to *S. sclerotiorum* are contradictory (Guo and Stotz, 2007; Perchepied et al., 2010; Wang et al., 2012; Nováková et al., 2014). Nevertheless, these results suggest that the Arabidopsis basal resistance against *S. sclerotiorum* is complex and involves multiple signaling pathways.

JA and ET are well known to cooperate in resistance against necrotrophic fungal pathogens (Thomma et al., 2001; Kunkel and Brooks, 2002; Glazebrook, 2005). They synergistically induce the pathogen-defense gene *PLANT DEFENSIN1.2* (*PDF1.2*) and antagonize each other's specific responses (Penninckx et al., 1998; Schenk et al., 2000). For instance, JA reduces ET-induced expression of the apical hook-regulating gene *HOOKLESS1* (*HLS1*; Turner et al., 2002), whereas ET suppresses JA-mediated activation of wound-responsive genes, including *VEGATATIVE STORAGE PROTEIN1* (*VSP1*), *VSP2*, and *JASMONATE RESPONSIVE1* (*JR1*; Rojo et al., 1999; Lorenzo et al., 2004). Two transcription factors, ETHYLENE INSENSITIVE3 (EIN3) and ETHYLENE INSENSITIVE3-LIKE1 (EIL1), which regulate most, if not all, of the ET responsiveness, are signaling hubs of JA/ET cooperation (Chao et al., 1997; Solano et al., 1998; Alonso et al., 2003b; An et al., 2010). JA and ET signaling converge at EIN3/EIL1, inducing genes encoding APETALA2/ETHYLENE-RESPONSIVE FACTORs (AP2/ERFs), such as *OCTADECANOID-RESPONSIVE ARABIDOPSIS APETALA2/ETHYLENE-RESPONSIVE FACTOR59* (*ORA59*) and *ERF1*, which in turn activate *PDF1.2* expression (Solano et al., 1998; Lorenzo et al., 2003; Pré et al., 2008; Zarei et al., 2011; Zhu et al., 2011). On the other hand, EIN3 and EIL1 interact with and repress the activity of MYC2, a transcription factor responsible for the activation of JA-mediated wound responses. Conversely, MYC2 attenuates *HLS1* expression by promoting EIN3/EIL1 proteolysis and repressing their activity (Song et al., 2014; Zhang et al., 2014a). Such cooperation between JA and ET allows plants to prioritize defense against invading necrotrophic pathogens over development and other responses.

The transcription factor WRKY33 is an important regulator of resistance to necrotrophic fungal pathogens. Expression of the *WRKY33* gene is highly inducible by *Botrytis cinerea* infection (AbuQamar et al., 2006). Mutations in *WRKY33* cause enhanced susceptibility to *B. cinerea* and *Alternaria brassicicola*, whereas overexpression of *WKRY33* leads to increased resistance to these pathogens and elevated basal expression of *PDF1.2* (Zheng et al., 2006). Moreover, expression of the *Brassica napus WRKY33* (*BnWRKY33*) gene is highly inducible by *S. sclerotiorum* infection, and overexpression of *BnWRKY33* in *B. napus* results in constitutive expression of *BnPDF1.2* and markedly enhanced resistance to *S. sclerotiorum* (Wang et al., 2014), suggesting that WRKY33 may be a positive regulator of resistance against *S. sclerotiorum*. WRKY33 has been shown to directly control the expression of *ORA59* during the later stages of pathogen infection (Birkenbihl et al., 2012), but how WRKY33 activates *ORA59* transcription is unclear.

In eukaryotes, RNA POLYMERASE II (RNAPII) catalyzes the transcription of protein-encoding genes (Woychik and Hampsey, 2002). A highly conserved multiprotein complex named Mediator plays an essential role in RNAPII-mediated transcription (Kim et al., 1994; Kornberg, 2005; Takagi and Kornberg, 2006; Conaway and Conaway, 2011a). Mediator exists in multiple functionally distinct forms and serves as either a transcriptional activator or a repressor, depending on its associated protein partners (Conaway and Conaway, 2011b). The Mediator core contains more than 20 subunits organized into head, middle, and tail modules (Guglielmi et al., 2004; Chadick and Asturias, 2005). This core associates with the RNAPII complex to form the holoenzyme, stimulating basal transcription and supporting the activation of transcription by specific transcription activators (Mittler et al., 2001; Baek et al., 2002; Zhu et al., 2006; Ansari et al., 2009). Individual Mediator subunits converge diverse signals to the RNAPII transcription complex via interaction with a particular or a class of transcription activators, leading to pathway-specific gene transcription (Balamotis et al., 2009; Kagey et al., 2010; Takahashi et al., 2011). The Mediator core also interacts with a kinase module, which prevents its binding to the RNAPII complex, resulting in transcriptional repression (Holstege et al., 1998; Akoulitchev et al., 2000; Knuesel et al., 2009). The distinct forms of Mediator thus underlie various pathway-specific transcription activation or suppression (Balamotis et al., 2009).

The Arabidopsis Mediator complex contains 27 conserved subunits and six additional subunits whose positions in the complex are unassigned (Bäckström et al., 2007; Mathur et al., 2011). A number of Arabidopsis Mediator subunits have been implicated in signaling pathways related to plant development and abiotic responses. For instance, the Arabidopsis MEDIATOR SUBUNIT14 (MED14)/STRUWWELPETER is a key regulator of cell proliferation (Autran et al., 2002). MED25/PHYTOCHROME AND FLOWERING TIME1 was first identified as a key regulator of flowering (Cerdán and Chory, 2003) and later found to regulate final organ size and light signaling (Xu and Li, 2011; Klose et al., 2012). MED18 was found to control flowering time and floral organ identity (Zheng et al., 2013). MED5a/MED33a/REDUCED EPIDERMAL FLUORESCENCE4 (REF4)-RELATED1 and MED5b/MED33b/REF4 are required for phenylpropanoid homeostasis (Bonawitz et al., 2012). The Mediator kinase module subunits CYCLIN-DEPENDENT KINASE8 (CDK8)/HUA ENHANCER3, MED12/CRYPTIC PRECOCIOUS, and MED13/MACCHI-BOU2 regulate the specification of floral organ identity, early embryo patterning/flowering, and embryo patterning/cotyledon organogenesis, respectively (Wang and Chen, 2004; Gillmor et al., 2010; Ito et al., 2011; Imura et al., 2012). MED17, MED18, and MED20a play roles in small and long noncoding RNA production (Kim et al., 2011). Moreover, MED2/MED32,

MED14, and MED16/SENSITIVE TO FREEZING6 regulate cold-responsive genes (Knight et al., 1999, 2008, 2009; Hemsley et al., 2014). MED16 also modulates iron uptake and homeostasis (Yang et al., 2014; Zhang et al., 2014b).

Mediator is emerging as a master regulator of plant immune responses. MED14, MED15, MED16, and MED19a have been shown to regulate the SA-triggered immunity against biotrophic and hemibiotrophic pathogens (Canet et al., 2012; Zhang et al., 2012, 2013; Caillaud et al., 2013), whereas MED8, MED12, MED13, MED16, MED21, MED25, and CDK8 have been found to function in JA/ET-mediated immunity against necrotrophic pathogens (Dhawan et al., 2009; Kidd et al., 2009; Zhang et al., 2012; Zhu et al., 2014). MED18 also functions in resistance to necrotrophic pathogens, but the resistance is independent of the JA/ET signaling (Lai et al., 2014). Interestingly, the Arabidopsis oomycete pathogen *Hyaloperonospora arabidopsidis* RXLR effector44 (HaRxL44) interacts with MED19a and promotes its degradation, which shifts the balance of defense transcription from SA to JA/ET signaling (Caillaud et al., 2013). This result suggests that, while Mediator positively regulates plant immunity, pathogens may have evolved virulence mechanisms to interfere with Mediator function.

In this study, we identified MED16 as a central regulator of basal resistance against *S. sclerotiorum*. We show that MED16 is required not only for JA/ET-mediated defense responses but also for ET-induced suppression of JA-mediated wound responses. We demonstrate that MED16 associates with WRKY33 and mediates WRKY33-activated defense gene expression and resistance to *S. sclerotiorum*. Our results indicate that MED16 is a key regulator of both JA/ET-mediated and the transcription factor WRKY33-activated defense signaling in Arabidopsis.

RESULTS

MED16 Is a Critical Mediator Subunit for Basal Resistance against *S. sclerotiorum*

Since mutations in the Arabidopsis Mediator subunits MED8, MED16, and MED25 compromise basal resistance against the necrotrophic fungal pathogens *B. cinerea* and *A. brassicicola* (Kidd et al., 2009; Zhang et al., 2012), they may also affect resistance to *S. sclerotiorum*. To test this hypothesis, we inoculated *med8*, *med16-1*, *sensitive to freezing6-2* (*sfr6-2*), *med25-1*, *med25-2*, and wild-type plants with *S. sclerotiorum*. Interestingly, while *med16/sfr6* and *med25* mutants exhibited enhanced susceptibility to *S. sclerotiorum*, *med8* did not show altered susceptibility compared with the wild type (Fig. 1, A–D). At 48 h post inoculation (hpi), the average lesion sizes on *med8* and wild-type plants were approximately 7.8 mm, and those on *med16/sfr6* and *med25* were approximately 16.3 mm and approximately 11.9 mm, respectively (Fig. 1A). At 5 d post inoculation (dpi), approximately 27.7% of the inoculated *med8* and wild-type plants were decayed, whereas 100% and approximately 62.5% of the inoculated *med16/sfr6* and *med25* plants, respectively, were decayed (Fig. 1C). These results indicate that both MED16 and MED25, but not MED8, contribute to basal resistance against *S. sclerotiorum* and that MED16 plays a more important role than MED25 in this resistance.

As Mediator is a multisubunit complex, other Mediator subunits may also function in basal resistance against *S. sclerotiorum*. To test this, we identified transfer DNA (T-DNA) insertion homozygous lines for the Mediator subunits MED13, MED17, MED18, MED20a, MED23, MED31, MED32, MED33b, MED34, and MED36 and tested their susceptibility to *S. sclerotiorum*. The T-DNA lines for MED13, MED18, MED32, and MED33b have been characterized previously (Supplemental Table S1), and those for MED17, MED20a, MED23, MED31, MED34, and MED36 are either knockout or knockdown mutants (Supplemental Fig. S1). The wild type, *med8*, *med16-1*, *med25-1*, and the previously characterized *med14-1* T-DNA insertion mutant were also included in the experiment (Supplemental Table S1). At 48 hpi, the average lesion sizes on *med8*, *med13*, *med17*, *med18*, *med20a*, *med23*, *med31*, *med32*, *med33b*, *med34*, and *med36* were not significantly different from that on the wild type, whereas the average lesion sizes on *med14*, *med16*, and *med25* were significantly larger than that on the wild type (Fig. 1, E and F). Among all the tested Mediator subunit mutants, *med16* exhibited the highest susceptibility to *S. sclerotiorum*, indicating that MED16 is a critical Mediator subunit regulating basal resistance against *S. sclerotiorum*.

MED16 and MED8 Differentially Regulate *S. sclerotiorum*-Induced Transcriptional Reprogramming

Although Mediator is a multisubunit complex, not all subunits are required for a specific biological process (Balamotis et al., 2009; Hemsley et al., 2014). For basal resistance against *S. sclerotiorum*, MED16 appears to play a more important role than other Mediator subunits (Fig. 1, E and F). To uncover the molecular mechanisms underlying this unique requirement of MED16 for basal resistance against *S. sclerotiorum*, we performed a microarray experiment to monitor *S. sclerotiorum*-induced transcriptome changes in *med16-1*, *med8*, and the wild type (National Center for Biotechnology Information Gene Expression Omnibus series no. GSE65165). We chose *med8* over other Mediator subunit mutants because *med8* is susceptible to *B. cinerea* and *A. brassicicola* but not to *S. sclerotiorum* (Kidd et al., 2009; Fig. 1). We inoculated *med16-1*, *med8*, and wild-type plants with *S. sclerotiorum*. Leaf tissues were collected as controls at 0 dpi, the inoculated leaves were collected as local tissues at 1 dpi, and the upper uninoculated leaves were collected as systemic tissues at 2 and 4 dpi. Triplicate experiments were performed independently, and the data were analyzed to identify genes that were induced or suppressed in *med16-1*, *med8*, and the wild type. We used q values to identify induced or suppressed genes. Genes that showed a 2-fold or higher induction or suppression with a low q value ($q \leq 0.05$) in *med16-1*, *med8*, and the wild type were chosen for further analysis. We found that all three

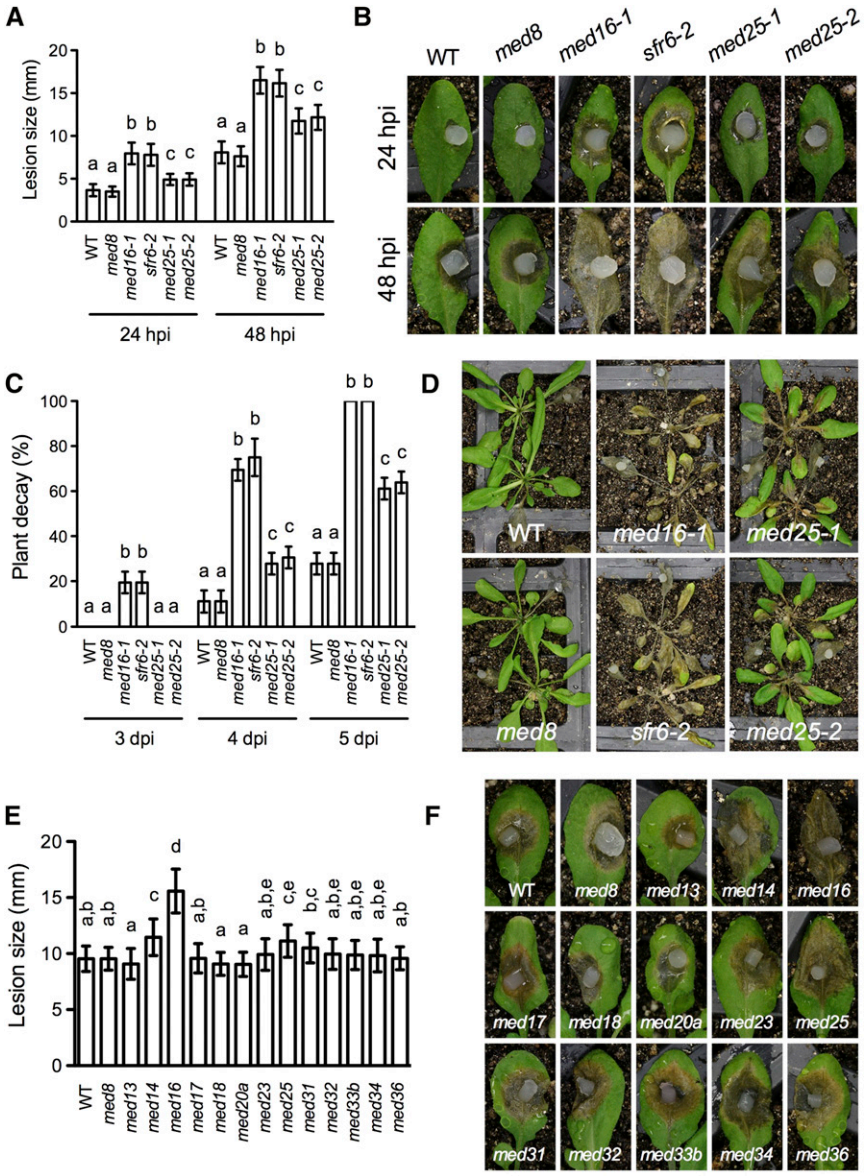

Figure 1. Susceptibility of 14 Mediator subunit mutants to *S. sclerotiorum*. A, Size of the necrotic lesions formed on the *S. sclerotiorum*-infected wild-type (WT), *med8*, *med16-1*, *sfr6-2*, *med25-1*, and *med25-2* plants at 24 and 48 hpi. Data represent means of lesion sizes on 24 leaves (on 24 plants) with SD. Different letters above the bars indicate significant differences ($P < 0.05$, one-way ANOVA). B, Disease symptoms on the *S. sclerotiorum*-infected wild-type, *med8*, *med16-1*, *sfr6-2*, *med25-1*, and *med25-2* leaves. Photographs were taken at 24 and 48 hpi. C, Decay percentages of *S. sclerotiorum*-infected wild-type, *med8*, *med16-1*, *sfr6-2*, *med25-1*, and *med25-2* plants at 3, 4, and 5 dpi. Data represent means of three groups (12 plants per group) of *S. sclerotiorum*-infected plants with SD. Different letters above the bars indicate significant differences ($P < 0.05$, one-way ANOVA). D, Disease symptoms on the *S. sclerotiorum*-infected wild-type, *med8*, *med16-1*, *sfr6-2*, *med25-1*, and *med25-2* plants. Photographs were taken at 5 dpi. E, Size of the necrotic lesions formed on the *S. sclerotiorum*-infected wild-type, *med8*, *med13*, *med14*, *med16*, *med17*, *med18*, *med20a*, *med23*, *med25*, *med31*, *med32*, *med33b*, *med34*, and *med36* plants at 48 hpi. Data represent means of lesion sizes on 24 leaves (on 24 plants) with SD. Different letters above the bars indicate significant differences ($P < 0.05$, one-way ANOVA). F, Disease symptoms on the *S. sclerotiorum*-infected wild-type, *med8*, *med13*, *med14*, *med16*, *med17*, *med18*, *med20a*, *med23*, *med25*, *med31*, *med32*, *med33b*, *med34*, and *med36* leaves. Photographs were taken at 48 hpi. The statistical comparisons in A and C were performed among the wild type, *med8*, *med16-1*, *sfr6-2*, *med25-1*, and *med25-2* for each time point. Experiments in A, C, and E were repeated three independent times with similar trends. Results from a representative experiment are presented. Photographs in B, D, and F represent typical disease symptoms on *S. sclerotiorum*-infected leaves or plants.

genotypes exhibited dramatic transcriptional reprogramming upon *S. sclerotiorum* inoculation and that both *med16-1* and *med8* significantly shifted their transcriptome profiles (Fig. 2, A and B). The numbers of genes up- or down-regulated in *med16-1* were larger than those in the wild type in local tissues at 1 dpi and systemic tissues at 4 dpi and smaller in systemic tissues at 2 dpi (Fig. 2A). In *med8*, except for the number of genes up-regulated in local tissues at 1 dpi, all others were smaller than those in the wild type (Fig. 2A).

We then queried the microarray data and identified genes that showed a 2-fold or larger difference in their expression levels with a low *q* value ($q \leq 0.05$) between *med16-1* or *med8* and the wild type. As shown in Figure 2C, considerably more genes were differentially expressed between *med16-1* and the wild type than between *med8* and the wild type. A total of 493, 2,101, 921, and 2,561 genes were differentially expressed between *med16-1* and the wild type at 0, 1, 2, and 4 dpi, respectively, whereas only 236, 97, 259, and 453 genes were differentially expressed between *med8* and the wild type (Fig. 2C; Supplemental Data Set S1). We compared the differentially expressed genes in *med16-1* and *med8* and found that approximately 43.6% to 61.9% of the genes that were differentially expressed in *med8* were also differentially expressed in the *med16-1* mutant (Fig. 2D), suggesting that MED16 and MED8 regulate some common genes during *S. sclerotiorum* infection, although these genes may not contribute to basal resistance against *S. sclerotiorum*. Taken together, these results indicate that the *med16-1* mutation has a much broader impact than *med8* on *S. sclerotiorum*-induced transcriptome changes.

Since MED16 positively regulates both SA and JA/ET signaling (Wathugala et al., 2012; Zhang et al., 2012), we wondered how SA and JA/ET pathway genes were influenced by the *med16-1* mutation during *S. sclerotiorum* infection. To this end, we further explored the microarray data and identified SA and JA/ET pathway genes that showed a 2-fold or larger difference in their expression levels with a low *q* value ($q \leq 0.05$) between *med16-1* and the wild type (Table I). Interestingly, the JA biosynthesis genes *LIPOXYGENASE3* (*LOX3*), *ALLENE OXIDE CYCLASE3* (*AOC3*), and *OPR3*, the ET biosynthesis genes *1-AMINOCYCLOPROPANE-1-CARBOXYLATE SYNTHASE2* (*ACS2*) and *ACS8*, as well as the SA biosynthesis genes *ISOCHORISMATE SYNTHASE1* (*ICS1*) and *ENHANCED DISEASE SUSCEPTIBILITY5* (*EDS5*) were up-regulated in *med16-1* in local tissues at 1 dpi

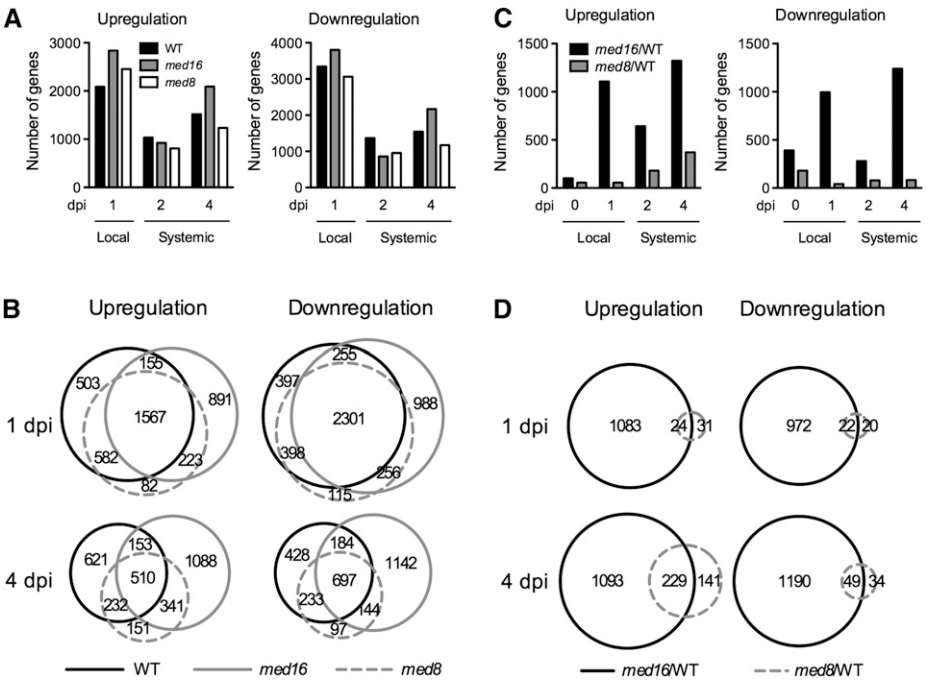

Figure 2. *S. sclerotiorum*-induced transcriptome changes in *med16* and *med8*. Three independent RNA samples per genotype at each time point were used for the microarray experiment, and data were analyzed to identify genes that showed a 2-fold or higher induction or suppression with a low *q* value ($q \leq 0.05$) in *med16-1*, *med8*, and the wild type or genes that showed a 2-fold or larger difference in their expression levels with a low *q* value ($q \leq 0.05$) between *med16-1* or *med8* and the wild type. A, Dynamic changes in the number of genes that are up- or down-regulated in the wild type (WT), *med16*, and *med8* after *S. sclerotiorum* infection. B, Overlapping circles indicating the number of genes that are commonly, partially commonly, or uniquely up- or down-regulated at 1 and 4 dpi in the wild type, *med16*, and *med8*. C, Dynamic changes in the number of genes that are differentially expressed between *med16* and the wild type and between *med8* and the wild type after *S. sclerotiorum* infection. D, Overlapping circles indicating the number of genes that are commonly or uniquely differentially expressed at 1 and 4 dpi between *med16* or *med8* and the wild type.

and/or systemic tissues at 4 dpi. Surprisingly, however, while a number of SA pathway genes (*EDS1*, *PHYTOALEXIN DEFICIENT4* [*PAD4*], *NONEXPRESSER OF PATHOGENESIS-RELATED GENES1* [*NPR1*], *WRKY18*, *WRKY38*, *WRKY53*, *PATHOGENESIS-RELATED GENE1* [*PR1*], and *PR2*) and several JA-regulated wound-responsive genes (*MYC2*, *JAR1*, *VSP1*, and *VSP2*) were up-regulated in *med16-1*, a group of JA/ET-regulated defense genes (*ORA59*, *ERF1*, *ERF14*, *PDF1.2*, *PDF1.2b*, *PDF1.2c*, *PDF1.3*, *PR4*, and *Chitinase B* [*ChiB*]) and *PR5* were down-regulated. On the other hand, only a few of these genes were differentially expressed between *med8* and the wild type (Table I). These results indicate that, during *S. sclerotiorum* infection, SA signaling and JA-mediated wound signaling were enhanced, whereas JA/ET-controlled defense signaling was inhibited in the *med16-1* mutant.

MED16 Is a Key Regulator of Basal Resistance against *S. sclerotiorum*

It has been shown that the SA, JA, and ET signaling mutants, *npr1*, *coi1*, and *ein2*, respectively, have enhanced susceptibility to *S. sclerotiorum* (Guo and Stotz, 2007). To compare the susceptibility between these mutants and *med16*, we inoculated *npr1-3*, *ein2-1*, *coi1-1*, *med16-1*, *sfr6-2*, and wild-type plants with *S. sclerotiorum*. We used the *coi1-1* null allele because it is extremely susceptible to *S. sclerotiorum* compared with other mutants (Perchepied et al., 2010). As shown in Figure 3, A to D, *npr1-3*, *ein2-1*, and *coi1-1* were all more susceptible to *S. sclerotiorum* than the wild type. We consistently observed enhanced susceptibility in the *npr1-3* mutant, supporting the previous conclusion that NPR1-mediated SA signaling plays a role in resistance to *S. sclerotiorum* (Guo and Stotz, 2007). In our experiments, the *coi1-1* mutant was more susceptible than *ein2-1*, which in turn was more susceptible than *npr1-3*. Interestingly, the *med16/sfr6* mutants exhibited even higher susceptibility than *coi1-1* to *S. sclerotiorum*. At 36 hpi, the average lesion sizes (approximately 16.6 mm) on *med16/sfr6* plants were slightly but significantly larger than that (approximately 14.9 mm) on *coi1-1* (Fig. 3A). At 5 dpi, approximately 91.6% and approximately 98.6% of inoculated *coi1-1* and *med16/sfr6* plants, respectively, were decayed, even though these percentages were not significantly different (Fig. 3D). These results together demonstrate that MED16 is a key regulator of basal resistance against *S. sclerotiorum* in Arabidopsis.

We have shown previously that mutations in *MED16* block *B. cinerea*-induced expression of *PDF1.2*, a marker gene of the JA/ET-mediated defense responses (Zhang et al., 2012). Since COI1 is known to be essential for *PDF1.2* expression (Penninckx et al., 1996; Lorenzo et al., 2003; Pré et al., 2008), we compared *S. sclerotiorum*-induced *PDF1.2* expression in the *coi1-1* and *med16/sfr6* mutants. The wild type, *npr1-3*, and *ein2-1* were included in the experiment as controls. As shown in Figure 3, E and F, and Supplemental Figure S2, *PDF1.2* was drastically induced in *npr1-3* and the wild-type plants and also significantly induced in *ein2-1*, but it was not induced in the *coi1-1* and *med16/sfr6* plants. The expression levels of *PDF1.2* in the *med16/sfr6* plants were even lower than those in *coi1-1*. Therefore, like the COI1 protein, MED16 is essential for *S. sclerotiorum*-induced *PDF1.2* expression.

MED16 Regulates ET-Activated Inhibition of JA-Mediated Wound Signaling

JA and ET signaling are well established to interact both synergistically and antagonistically (Glazebrook, 2005; Pieterse et al., 2009). JA and ET independently and synergistically induce pathogen-responsive genes, such as *PDF1.2*, to support resistance against necrotrophic pathogens, whereas ET inhibits JA-induced wound-responsive genes, such as *VSP1*, *VSP2*, and *JR1*. Since JA/ET-regulated pathogen-responsive genes and JA-mediated wound-responsive genes are differentially regulated in *med16-1* during *S. sclerotiorum* infection (Table I), MED16 may modulate the cross talk between JA and ET signaling. To test this hypothesis, we treated *med16-1*, *sfr6-2*, and wild-type plants with methyl jasmonate (MeJA), 1-aminocyclopropane-1-carboxylic acid (ACC), or their combination and tested the induction of the pathogen-responsive genes *PDF1.2* and *ORA59* as well as the wound-responsive genes *VSP1*, *VSP2*, and *JR1*. As shown in Figure 4, JA, ET, and their combination induced *PDF1.2* and *ORA59* expression in the wild-type plants but not in *med16-1* and *sfr6-2*, confirming the requirement of MED16 for induction of these two genes. On the other hand, JA-induced expression of *VSP1*, *VSP2*, and *JR1* was not altered by the *med16/sfr6* mutations, indicating that the JA-mediated wound signaling pathway is intact in these mutants. As expected, ET significantly inhibited JA-induced expression of *VSP1*, *VSP2*, and *JR1* in the wild-type plants, but the inhibition was largely alleviated in the *med16-1* and *sfr6-2* mutants, indicating that MED16 is required for ET to maximally suppress JA-mediated wound responses.

MED16 Mediates the Recruitment of RNAPII to the *PDF1.2* and *ORA59* Genes

Recruitment of RNAPII is a critical step for the transcription of protein-encoding genes in eukaryotic cells (Woychik and Hampsey, 2002). Since MED16 is required for JA/ET-induced transcription of *PDF1.2* and *ORA59* (Fig. 4), it may mediate the recruitment of RNAPII to these genes. To test this hypothesis, we performed chromatin immunoprecipitation with an RNAPII-specific antibody to analyze RNAPII occupancy on the coding regions of the *PDF1.2* and *ORA59* genes in *med16-1* and wild-type plants treated with or without MeJA plus ACC. The *CYP79B3* gene was included as a control, since MeJA plus ACC treatment does not drastically induce this gene (Mikkelsen et al., 2003). As shown in Figure 5, in the wild-type plants, MeJA plus ACC treatment dramatically increased RNAPII

Table I. *Defense genes that are differentially expressed between med16-1 or med8 and the wild type during S. sclerotiorum infection*

AGI, Arabidopsis Genome Initiative; FC, fold change.

AGI Locus	Gene Name	*med16-1*/Wild Type				*med8*/Wild Type				AGI Description
		Local, 1 dpi		Systemic, 4 dpi		Local, 1 dpi		Systemic, 4 dpi		
		Log_2(FC)	*q* Value	Log_2(FC)	*q* Value	Log_2(FC)	*q* Value	Log_2(FC)	*q* Value	
JA/ET pathway genes										
At1g17420	*LOX3*	2.105	0.003					1.131	0.007	LIPOXYGENASE3
At3g25780	*AOC3*	1.846	0.007	2.49	0			2.06	0.003	ALLENE OXIDE CYCLASE3
At2g06050	*OPR3*	1.037	0.033							12-OXOPHYTODIENOATE-REDUCTASE3
At1g32640	*MYC2*			1.293	0.005					JASMONATE INSENSITIVE1
At2g46370	*JAR1*			1.069	0.008					JASMONATE RESISTANT1
At5g24780	*VSP1*	3.062	0.002	2.224	0					VEGETATIVE STORAGE PROTEIN1
At5g24770	*VSP2*	2.642	0.002	2.684	0					VEGETATIVE STORAGE PROTEIN2
At1g01480	*ACS2*	1.628	0.004	2.583	0.001					1-AMINO-CYCLOPROPANE-1-CARBOXYLATE SYNTHASE2
At4g37770	*ACS8*			1.779	0.002					1-AMINO-CYCLOPROPANE-1-CARBOXYLATE SYNTHASE8
At1G06160	*ORA59*	−3.72	0.001	−4.616	0					Ethylene-responsive factor
At3g23240	*ERF1*	−1.021	0.004							ETHYLENE-RESPONSIVE FACTOR1
At1G04370	*ERF14*	−1.823	0.004							ETHYLENE-RESPONSIVE FACTOR14
At5g44420	*PDF1.2*	−4.234	0.004	−5.712	0					PLANT DEFENSIN1.2
At2G26020	*PDF1.2b*	−7.437	0.002	−9.356	0					PLANT DEFENSIN1.2b
At5g44430	*PDF1.2c*	−6.647	0.002	−8.894	0					PLANT DEFENSIN1.2c
At2g26010	*PDF1.3*	−5.289	0.007	−7.961	0.001					PLANT DEFENSIN1.3
At3g04720	*PR4/HEL*	−2.933	0.009	−3.998	0.001					PATHOGENESIS-RELATED4
At3G12500	*ChiB*	−4.521	0.001	−2.588	0			−1.559	0.006	BASIC CHITINASE
SA pathway genes										
At3g48090	*EDS1*	1.055	0.001							ENHANCED DISEASE SUSCEPTIBILITY1
At3g52430	*PAD4*	2.989	0	2.011	0					PHYTOALEXIN DEFICIENT4
At1g74710	*ICS1*	1.912	0.001	2.551	0					ISOCHORISMATE SYNTHASE1
At4g39030	*EDS5*	2.019	0.003	2.513	0			1.171	0.006	ENHANCED DISEASE SUSCEPTIBILITY5
At1g64280	*NPR1*	1.187	0.002							NONEXPRESSOR OF PATHOGENESIS-RELATED GENES1
At4g31800	*WRKY18*	2.041	0.002							WRKY DNA-BINDING PROTEIN18
At5g22570	*WRKY38*	3.528	0.002	3.613	0			1.695	0.007	WRKY DNA-BINDING PROTEIN38
At4g23810	*WRKY53*	2.111	0.001	2.7	0			2.84	0.001	WRKY DNA-BINDING PROTEIN53
At2g14610	*PR1*			1.348	0.006	1.743	0.017	1.115	0.018	PATHOGENESIS-RELATED GENE1
At3g57260	*PR2*	2.166	0.004	3.841	0	1.099	0.036	1.499	0.005	PATHOGENESIS-RELATED GENE2
At1g75040	*PR5*	−1.88	0.001	−1.222	0.001			−1.025	0.013	PATHOGENESIS-RELATED GENE5

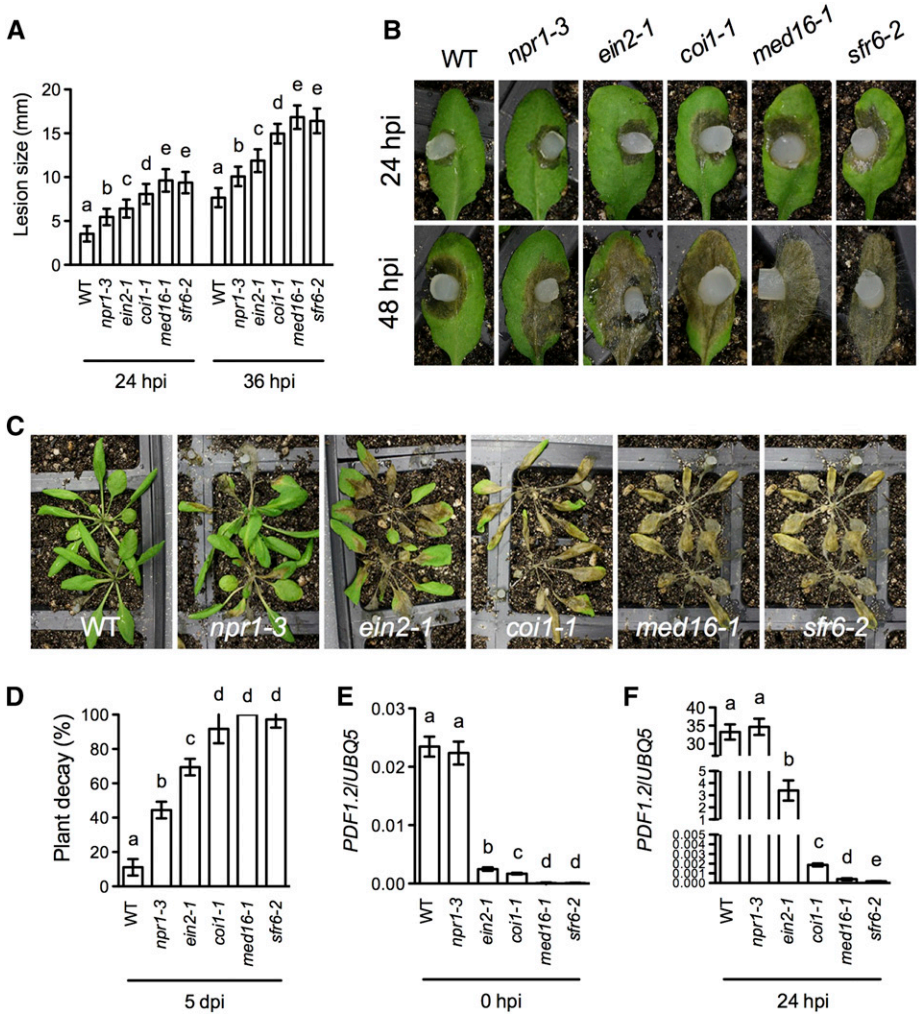

Figure 3. *S. sclerotiorum*-induced defense responses in *med16*, *npr1*, *ein2*, and *coi1*. A, Size of the necrotic lesions formed on the *S. sclerotiorum*-infected wild type (WT), *npr1-3*, *ein2-1*, *coi1-1*, *med16-1*, and *sfr6-2* at 24 and 36 hpi. Data represent means of lesion sizes on 24 leaves (on 24 plants) with SD. Different letters above the bars indicate significant differences ($P < 0.05$, one-way ANOVA). The statistical comparisons were performed among the wild type, *npr1-3*, *ein2-1*, *coi1-1*, *med16-1*, and *sfr6-2* for each time point. B, Disease symptoms on the *S. sclerotiorum*-infected wild-type, *npr1-3*, *ein2-1*, *coi1-1*, *med16-1*, and *sfr6-2* leaves. Photographs were taken at 24 and 48 hpi. C, Disease symptoms on the *S. sclerotiorum*-infected wild-type, *npr1-3*, *ein2-1*, *coi1-1*, *med16-1*, and *sfr6-2* plants. Photographs were taken at 5 dpi. D, Decay percentages of *S. sclerotiorum*-infected wild-type, *npr1-3*, *ein2-1*, *coi1-1*, *med16-1*, and *sfr6-2* plants at 5 dpi. Data represent means of three groups (12 plants per group) of *S. sclerotiorum*-infected plants with SD. Different letters above the bars indicate significant differences ($P < 0.05$, one-way ANOVA). E, *PDF1.2* expression levels in the wild type, *npr1-3*, *ein2-1*, *coi1-1*, *med16-1*, and *sfr6-2* at 0 hpi. Expression of *PDF1.2* was normalized against the constitutively expressed *UBIQUITIN5* (*UBQ5*). Data represent means of three biological replicates (samples taken from different plants during the same experiment) with SD. Different letters above the bars indicate significant differences ($P < 0.05$, one-way ANOVA). F, *PDF1.2* expression levels in the wild type, *npr1-3*, *ein2-1*, *coi1-1*, *med16-1*, and *sfr6-2* at 24 hpi. Expression of *PDF1.2* was normalized against the constitutively expressed *UBQ5*. Data represent means of three biological replicates with SD. Different letters above the bars indicate significant differences ($P < 0.05$, one-way ANOVA). Experiments in A, D, E, and F were repeated three independent times with similar trends. Results from a representative experiment are presented. Photographs in B and C represent typical disease symptoms on *S. sclerotiorum*-infected leaves or plants.

occupancy on three different sites in the coding regions of *PDF1.2* and *ORA59* but not on those of *CYP79B3*, indicating that the treatment induced gene-specific enrichment of RNAPII. In the *med16-1* mutant, on the other hand, MeJA plus ACC treatment did not induce considerable increases in RNAPII occupancy on *PDF1.2* and *ORA59*, indicating that MED16 is a key Mediator subunit for JA/ET-induced recruitment of RNAPII to *PDF1.2* and *ORA59*.

MED16 Is Physically Associated with WRKY33

The transcription factor WRKY33 has been shown to bind to two distinct W box-containing regions in the

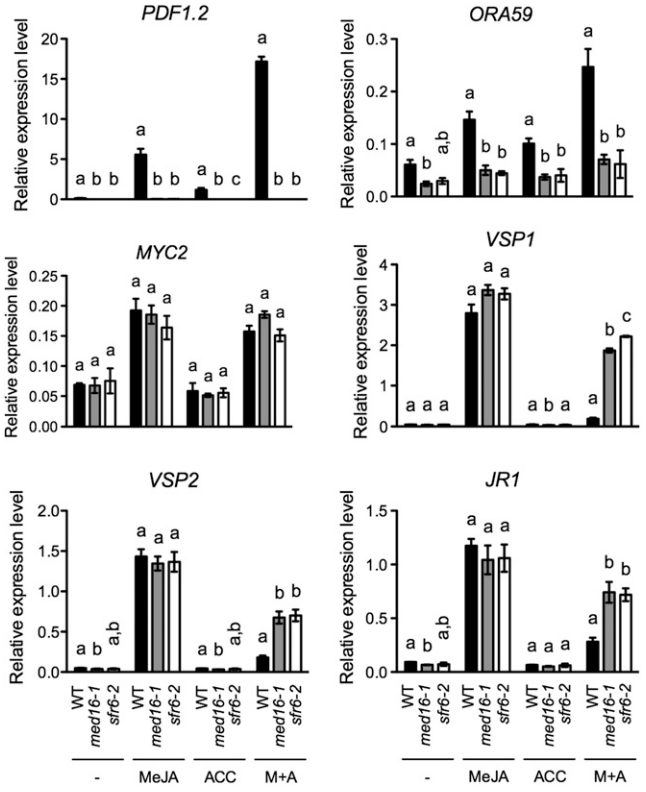

Figure 4. MeJA-, ACC-, and their combination-induced pathogen- and wound-responsive genes in *med16*. Ten-day-old wild-type (WT), *med16-1*, and *sfr6-2* seedlings grown on one-half-strength Murashige and Skoog medium were treated with 0.1 mM MeJA, 0.1 mM ACC, or 0.1 mM MeJA plus 0.1 mM ACC (M+A). Seedlings for the negative control (−) were treated with water. Total RNA was extracted from plant tissues collected 24 h after the treatment and subjected to quantitative PCR (qPCR) analysis. Expression of *ORA59*, *PDF1.2*, *MYC2*, *VSP1*, *VSP2*, and *JR1* was normalized against the constitutively expressed *UBQ5*. Data represent means of three biological replicates with SD. Different letters above the bars indicate significant differences ($P < 0.05$, one-way ANOVA). The statistical comparisons were performed among the wild type, *med16-1*, and *sfr6-2* for each treatment. The experiment was repeated three independent times with similar trends. Results from a representative experiment are presented.

ORA59 promoter and to regulate *B. cinerea*-induced prolonged expression of *ORA59* (Zheng et al., 2006; Birkenbihl et al., 2012). Since MED16 is required for the recruitment of RNAPII to the *ORA59* gene (Fig. 5), WRKY33 may activate *ORA59* transcription by physically interacting with MED16. To test this hypothesis, we subcloned the coding regions of *MED16* and *WRKY33* into the yeast two-hybrid bait vector pGBKT7 and the prey vector pGADT7, respectively, and cotransformed the resulting plasmids pGBKT7-MED16 and pGADT7-WRKY33 into the yeast (*Saccharomyces cerevisiae*) strain AH109. The empty bait and prey vectors, pGBKT7 and pGADT7, were used as negative controls and cotransformed with pGADT7-WRKY33 and pGBKT7-MED16, respectively. As shown in Figure 6A, yeast cells harboring pGBKT7-MED16 and pGADT7-WRKY33 grew on quadruple synthetic dextrose (SD) dropout medium (Ade-His-Leu-Trp) supplemented with 5-bromo-4-chloro-3-indolyl-α-D-galactopyranoside (X-α-Gal) and formed blue colonies, whereas those harboring either pGBKT7 and pGADT7-WRKY33 or pGBKT7-MED16 and pGADT7 did not grow and change color. These results indicate that MED16 and WRKY33 physically interact in yeast.

In order to test whether MED16 is physically associated with WRKY33 in planta, we created transgenic Arabidopsis plants coexpressing functional epitope-tagged MED16 and WRKY33 proteins. We first generated transgenic *med16-1* plants expressing a MED16-3×FLAG fusion driven by its native promoter. The *MED16pro*:*MED16-3×FLAG* transgene complemented all of the *med16-1* mutant phenotypes, including pale green leaf color, lack of inducible *PDF1.2* expression, and enhanced pathogen susceptibility (Fig. 6B; Supplemental Fig. S3, A–C). When the transgenic plants were subjected to immunoblot analysis with an anti-FLAG antibody, two protein bands corresponding to MED16-3×FLAG were detected (Fig. 6C). These bands were not present in *med16-1* and wild-type plants, confirming the specificity of the anti-FLAG immunoblot analysis. The *MED16pro*:*MED16-3×FLAG* transgene was then crossed into the previously characterized *35S:4×Myc-WRKY33* transgenic line (Mao et al., 2011). We chose this transgenic line because the fusion protein has been demonstrated to be functional and can be easily detected using an anti-Myc antibody (Mao et al., 2011). Additionally, overexpression of *WRKY33* has been shown to elevate basal levels of *PDF1.2* (Zheng et al., 2006), which would allow us to detect the interaction between MED16 and WRKY33 in the absence of any treatment.

The transgenic plants coexpressing MED16-3×FLAG and 4×Myc-WRKY33 were subjected to a coimmunoprecipitation experiment. The *MED16pro*:*MED16-3×FLAG* transgenic line was used as a negative control in this experiment. As shown in Figure 6D, MED16-3×FLAG was coimmunoprecipitated with 4×Myc-WRKY33 using the anti-Myc antibody from the nuclear protein extract of the *MED16pro*:*MED16-3×FLAG*/*35S:4×Myc-WRKY33* plants but not from that of the *MED16pro*:*MED16-3×FLAG* plants. Moreover, MED16-3×FLAG was not coimmunoprecipitated with 6×Myc using the anti-Myc antibody from protein extract of *Nicotiana benthamiana* leaf tissues transiently coexpressing MED16-3×FLAG and 6×Myc (Supplemental Fig. S4). These results clearly indicate that WRKY33 physically associates with MED16 in planta.

MED16 Is Required for WRKY33-Activated Transcription of *PDF1.2* and *ORA59* and Resistance to *S. sclerotiorum*

Previous work has shown that WRKY33 is required for *B. cinerea*-induced expression of *PDF1.2* and *ORA59* and that overexpression of *WRKY33* leads to elevated basal expression of *PDF1.2* (Zheng et al., 2006; Birkenbihl et al., 2012). Since WRKY33 associates with MED16 and MED16 is required for the recruitment of RNAPII to the

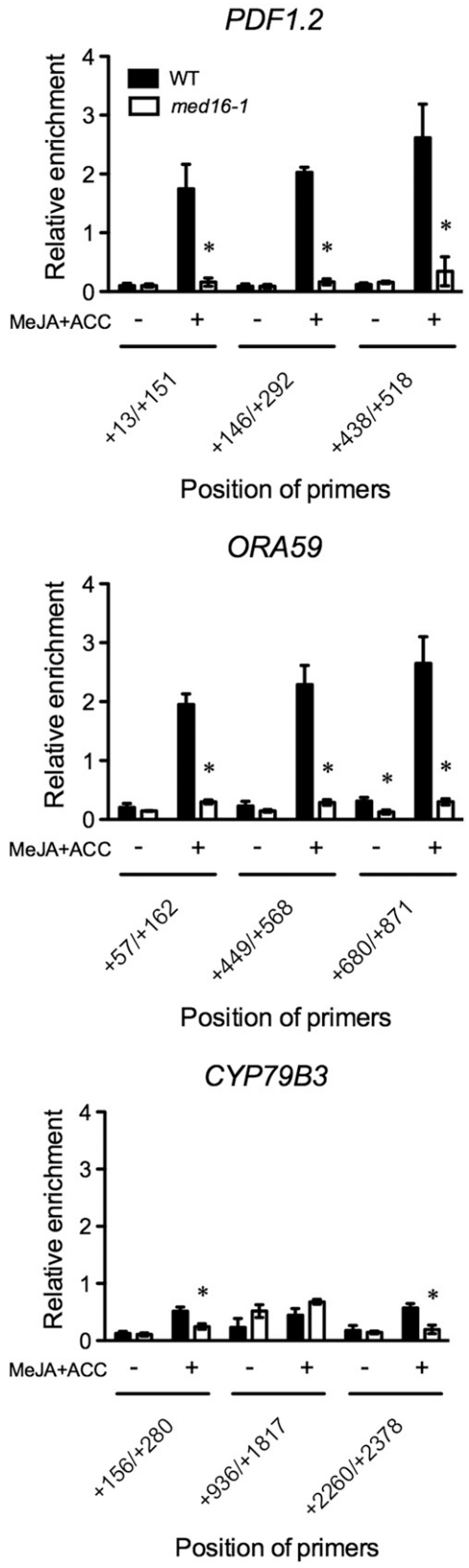

Figure 5. MeJA/ACC-induced recruitment of RNAPII to *PDF1.2* and *ORA59* in *med16*. Chromatin immunoprecipitation-qPCR results show that the *med16-1* mutation prevented MeJA/ACC-induced recruitment of NRPB2 (DNA-directed RNA polymerase II subunit RPB2) to the coding regions of *PDF1.2* and *ORA59*. The *CYP79B3* gene was included as a control. Chromatin was extracted from wild-type (WT) and *med16-1* seedlings treated with (+) or without (−) 0.1 mM MeJA plus 0.1 mM ACC (MeJA+ACC) for 24 h and then precipitated with anti-RPB2 antibody. The amount of precipitated DNA corresponding to a specific gene region was determined by qPCR and normalized to input DNA. Data represent means of three biological replicates with SD. Asterisks indicate significant differences between the wild type and *med16* ($P < 0.05$, Student's *t* test). The experiment was repeated three independent times with similar trends. Results from a representative experiment are presented.

PDF1.2 and *ORA59* genes (Figs. 5 and 6), the constitutive expression of *PDF1.2* in the *WRKY33*-overexpressing plants may depend on MED16. To test this hypothesis, we crossed the previously characterized *35S:4×Myc-WRKY33* transgene (Mao et al., 2011) into the *med16-1* mutant background and compared the basal expression levels of *PDF1.2* and *ORA59* in the wild-type, *med16-1*, *35S:4×Myc-WRKY33*, and *35S:4×Myc-WRKY33 med16-1* plants. Confirming the previous observation (Zheng et al., 2006), basal transcription levels of *PDF1.2* were elevated in the *35S:4×Myc-WRKY33* plants (Fig. 7A). Similarly, basal expression of *ORA59* was also enhanced in the *35S:4×Myc-WRKY33* plants (Fig. 7A), which supports the conclusion that the *ORA59* gene is a direct target of WRKY33 (Birkenbihl et al., 2012). Importantly, the enhancement effects of the overexpression of *WRKY33* on *PDF1.2* and *ORA59* expression were completely blocked by the *med16-1* mutation (Fig. 7A), indicating that *WRKY33*-activated transcription of *PDF1.2* and *ORA59* requires MED16.

A recent report indicates that overexpression of *BnWRKY33* in *B. napus* confers markedly enhanced resistance to *S. sclerotiorum* (Wang et al., 2014), suggesting that WRKY33 may be a positive regulator of resistance against *S. sclerotiorum*. Indeed, the *wrky33-1* mutant was slightly but significantly more susceptible than the wild type to *S. sclerotiorum* (Fig. 7, B–E), and overexpression of *WRKY33* provided marginally but significantly increased resistance, which could be seen before 36 hpi (Fig. 7D). Intriguingly, the increased resistance disappeared as disease progressed. Nevertheless, the resistance conferred by the overexpression of *WRKY33* was completed inhibited by the *med16-1* mutation, indicating that WRKY33-mediated resistance against *S. sclerotiorum* also depends on MED16.

DISCUSSION

Using genetic, molecular, and biochemical approaches, here we demonstrate that (1) MED16 is a central regulator of basal resistance against *S. sclerotiorum* in Arabidopsis; (2) MED16 is required for ET-promoted suppression of JA-mediated wound responses; (3) MED16 is required for the recruitment of RNAPII to both *PDF1.2* and *ORA59*; and (4) MED16 is physically associated with WRKY33 and is required for WKRY33-activated transcription of *PDF1.2* and *ORA59* and resistance to *S. sclerotiorum*.

Mediator is a multisubunit complex, and the requirement for each subunit depends on specific biological

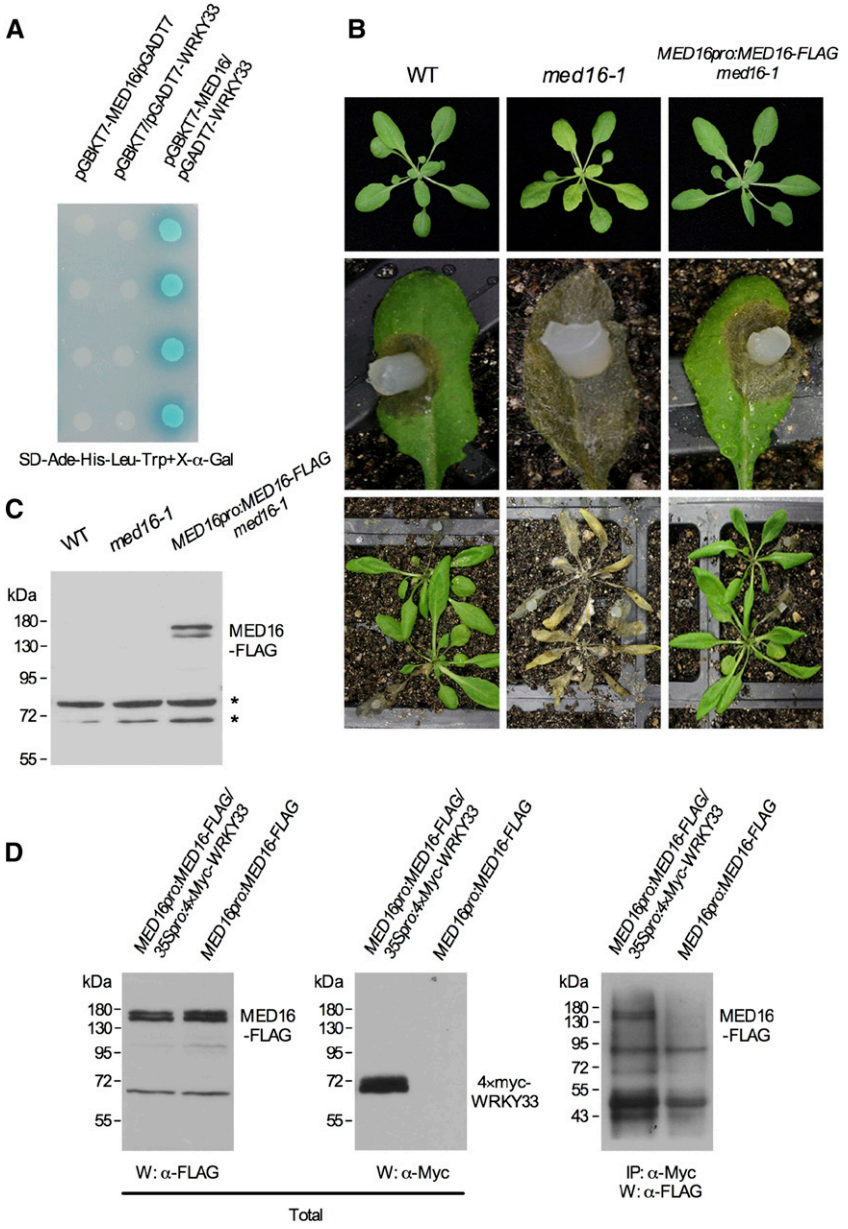

Figure 6. Physical association between MED16 and WRKY33. A, Growth and color of yeast cells carrying pGBKT7/pGADT7-WRKY33, pGBKT7-MED16/pGADT7, or pGBKT7-MED16/pGADT7-WRKY33. Yeast cells carrying pGBKT7-MED16/pGADT7-WRKY33 grew on quadruple dropout medium supplemented with X-α-Gal (SD-Ade-His-Leu-Trp+X-α-Gal) and formed blue colonies, whereas those harboring either pGBKT7/pGADT7-WRKY33 or pGBKT7-MED16/pGADT7 did not grow and change color, indicating that MED16 interacts with WRKY33 in yeast. B, Morphology (top row) of and *S. sclerotiorum* disease symptoms (middle and bottom rows) on the wild type (WT), *med16-1*, and the *MED16pro:MED16-3×FLAG med16-1* transgenic line. Representative photographs were taken 4 weeks after germination (top row), at 48 hpi (middle row), and 5 dpi (bottom row). C, The MED16-3×FLAG fusion protein was detected in *MED16pro:MED16-3×FLAG med16-1* plants but not in the wild type and *med16-1*, indicating the specificity of anti-FLAG antibody for immunoblot analysis of MED16-3×FLAG. The asterisks show two nonspecific bands present in all genotypes. D, Nuclear protein extracts of *MED16pro:MED16-3×FLAG/35Spro:4×Myc-WRKY33* and *MED16pro:MED16-3×FLAG* plants were immunoprecipitated with anti-Myc antibody. The precipitated proteins were separated by SDS-PAGE, transferred onto a nitrocellulose membrane, and probed with anti-FLAG antibody (right image). Inputs were analyzed with the anti-FLAG and anti-Myc antibodies, showing the presence or absence of MED16-3×FLAG (left image) and 4×My-WRKY33 (middle image). IP, Immunoprecipitation; W, western blotting. The experiment was repeated four independent times with the same results.

processes (Balamotis et al., 2009; Hemsley et al., 2014). Among the 14 Mediator subunit mutants tested in this study, *med14*, *med16*, and *med25* exhibited significantly enhanced susceptibility to *S. sclerotiorum* (Fig. 1). Although *med8*, *med13*, and *med18* have been shown to be more susceptible than the wild type to the necrotrophic fungal pathogens *B. cinerea* and *A. brassicicola* (Kidd et al., 2009; Lai et al., 2014; Zhu et al., 2014), they did not show enhanced susceptibility to *S. sclerotiorum*. This is probably because MED8, MED13, and MED18 do not play a major role in regulating *S. sclerotiorum*-induced transcriptome changes (Fig. 2), suggesting that the molecular mechanisms underlying susceptibility/resistance against various necrotrophic pathogens are different. On the other hand, the *med16* mutant is considerably more susceptible than *med14* and *med25* to *S. sclerotiorum* (Fig. 1, E and F), indicating that MED16 plays a more important role than MED14 and MED25 in regulating basal resistance against *S. sclerotiorum*.

Surprisingly, the *med16/sfr6* mutants are even more susceptible to *S. sclerotiorum* than the *coi1-1* null mutant, and the transcription levels of *PDF1.2* are also lower in *med16* than in *coi1-1* (Fig. 3; Supplemental Fig. S2). Since *coi1-1* has been shown to be the most susceptible Arabidopsis mutant to *S. sclerotiorum* and the *coi1-1* mutation completely blocks JA/ET-induced *PDF1.2* expression (Pré et al., 2008; Perchepied et al., 2010), the *med16* mutation may either have a stronger effect than *coi1-1* on JA/ET-mediated defense signaling or compromise both JA/ET-dependent and -independent defense pathways.

The function of MED16 in JA/ET-mediated defense signaling has recently been revealed. Zhang et al. (2012)

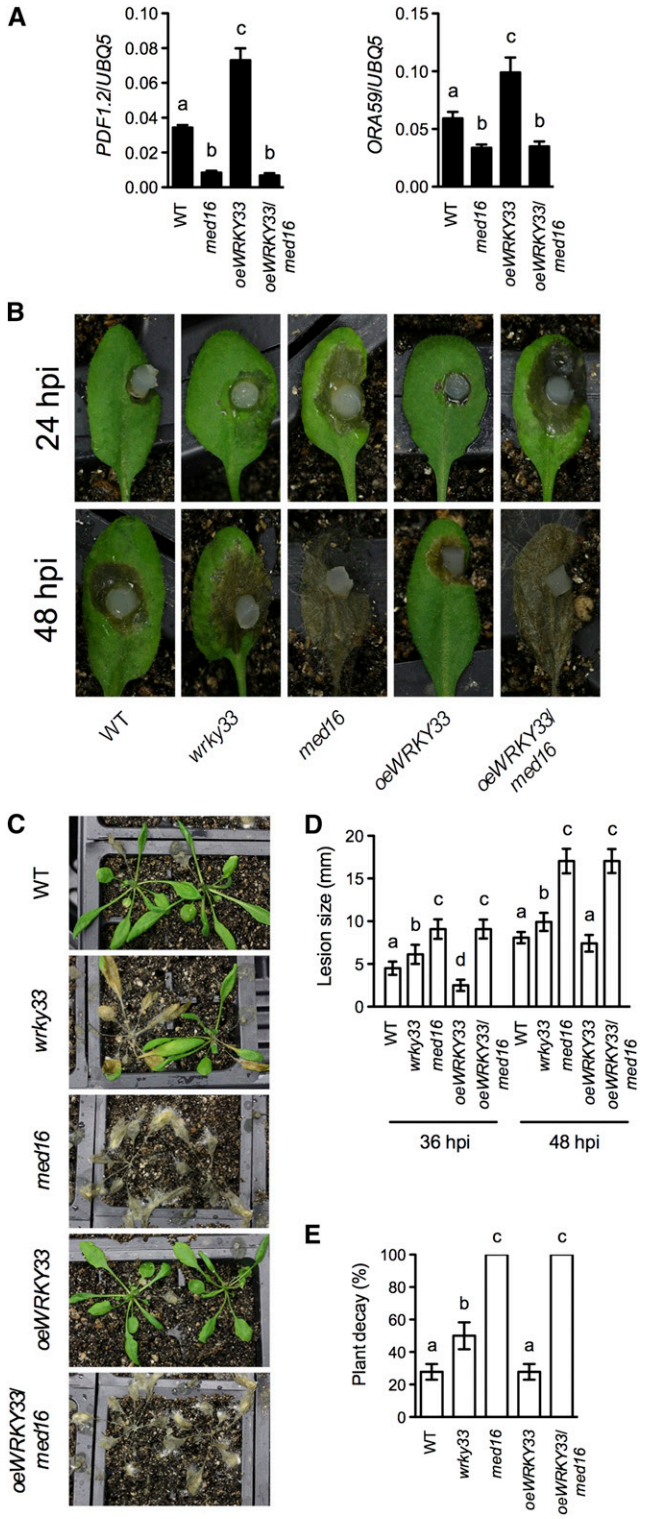

Figure 7. Dependence of WRKY33-activated defense responses on MED16. A, Expression levels of *PDF1.2* and *ORA59* in the wild type (WT), *med16-1*, *35S:4×Myc-WRKY33* (*oeWRKY33*), and *35S:4×Myc-WRKY33 med16-1* plants. Leaves of 4-week-old soil-grown plants were collected and subjected to total RNA extraction and qPCR analysis. Expression of *PDF1.2* and *ORA59* was normalized against the constitutively expressed *UBQ5*. Data represent means of three biological replicates with SD. Different letters above the bars indicate significant differences ($P < 0.05$, one-way ANOVA). B, Disease symptoms on the *S. sclerotiorum*-infected wild-type, *wrky33-1*, *med16-1*, *35S:4×Myc-WRKY33*, and *35S:4×Myc-WRKY33 med16-1* leaves. Photographs were taken at 24 and 48 hpi. C, Disease symptoms on the *S. sclerotiorum*-infected wild-type, *wrky33-1*, *med16-1*, *35S:4×Myc-WRKY33*, and *35S:4×Myc-WRKY33 med16-1* plants. Photographs were taken at 5 dpi. D, Size of the necrotic lesions formed on the *S. sclerotiorum*-infected wild type, *wrky33-1*, *med16-1*, *35S:4×Myc-WRKY33*, and *35S:4×Myc-WRKY33 med16-1* at 36 and 48 hpi. Data represent means of lesion sizes on 24 leaves with SD. Different letter above the bars indicate significant differences ($P < 0.05$, one-way ANOVA). The statistical comparisons were performed among the wild type, *wrky33-1*, *med16-1*, *35S:4×Myc-WRKY33*, and *35S:4×Myc-WRKY33 med16-1* for each time point. E, Decay percentages of *S. sclerotiorum*-infected wild-type, *wrky33-1*, *med16-1*, *35S:4×Myc-WRKY33*, and *35S:4×Myc-WRKY33 med16-1* plants at 5 dpi. Data represent means of three groups (12 plants per group) of *S. sclerotiorum*-infected plants with SD. Different letters above the bars indicate significant differences ($P < 0.05$, one-way ANOVA). Experiments in A, D, and E were repeated three independent times with similar trends. Results from a representative experiment are presented. Photographs in B and C represent typical disease symptoms on *S. sclerotiorum*-infected leaves or plants.

and Wathugala et al. (2012) have shown that mutations in *MED16* block JA/ET- and *B. cinerea*-induced defense gene expression and compromise resistance to *B. cinerea* and *A. brassicicola*. It has been well documented that expression of the defense gene *PDF1.2* is regulated by a group of AP2/ERF domain transcription factors including ORA59 and ERF1 (Lorenzo et al., 2003; Pré et al., 2008) and that genes encoding the AP2/ERF factors are, in turn, controlled by the transcription factors EIN3 and EIL1 (Solano et al., 1998; Zhu et al., 2011). Here, we show that MED16 is required for the recruitment of RNAPII to both *ORA59* and *PDF1.2* (Fig. 5), suggesting that MED16 may directly participate in both EIN3/EIL1- and AP2/ERF-mediated transcription. Consistent with this hypothesis, elevated expression of *PDF1.2* activated by the overexpression of ERF5 depends on MED16 (Wathugala et al., 2012). Furthermore, EIN3, EIL1, ORA59, and ERF1 have all been shown to interact with MED25, a Mediator subunit physically associating with MED16 (Çevik et al., 2012; Yang et al., 2014). Taken together, it could be concluded that MED16 plays an essential role in relaying defense signals of the JA/ET pathway to the RNAPII transcription machinery. Whether any of the EIN3/EIL1 and AP2/ERF transcription factors interact directly with MED16 needs further investigation.

Unlike COI1 and MED25, which are required for both branches of the JA signaling pathway, namely, the wound response branch and the defense response branch (Xie et al., 1998; Pré et al., 2008; Kidd et al., 2009; Chen et al., 2012), MED16 is only required for the defense response branch (Fig. 4). In fact, MED16 positively contributes to ET-induced suppression of JA-mediated wound responses (Fig. 4), suggesting either a direct or indirect role for MED16 in JA-ET cross talk (Dong, 1998; Pieterse et al., 2009).

MED16 is required for the transcription factor WRKY33-activated defense signaling. WRKY33 is an important regulator of defense responses against necrotrophic fungal pathogens (Zheng et al., 2006; Lai et al., 2011). Mutations in *WRKY33* compromise *B. cinerea*-induced defense gene expression and enhance susceptibility to both *B. cinerea* and *A. brassicicola*. Expression of the *WRKY33* gene is highly inducible by *B. cinerea* infection (AbuQamar et al., 2006), but the induction does not require the JA and ET signaling components COI1 and EIN2, respectively (Zheng et al., 2006), suggesting that WRKY33 may be activated by a JA/ET-independent defense pathway. Recently, Wang et al. (2014) reported that expression of the *BnWRKY33* gene is highly inducible by *S. sclerotiorum* infection and that overexpression of *BnWRKY33* in *B. napus* leads to increased basal expression of *BnPDF1.2* and resistance to *S. sclerotiorum*. Consistent with that report, we found that the Arabidopsis *wrky33-1* mutant is more susceptible to *S. sclerotiorum* than the wild type and that overexpression of *WRKY33* confers a low level of resistance to *S. sclerotiorum* during early time points of infection (Fig. 7). Based on the microarray data, *WKRY33* is also inducible by *S. sclerotiorum* in Arabidopsis and MED16 is not required for the induction. However, our further study revealed that WRKY33-activated defense responses depend on MED16. We found that MED16 physically associates with WRKY33 and is required for WRKY33-activated *PDF1.2* and *ORA59* expression and resistance to *S. sclerotiorum* (Figs. 6 and 7). These results indicate that MED16 is required not only for JA/ET-mediated defense responses but also for WRKY33-activated defense signaling. Blocking the WRKY33-mediated defense signaling in *med16* probably contributes to the reduced *PDF1.2* expression and enhanced susceptibility to *S. sclerotiorum*.

Although results from previous studies about the role of NPR1 in resistance against *S. sclerotiorum* are contradictory (Guo and Stotz, 2007; Perchepied et al., 2010), we consistently observed that the *npr1-3* mutant is more susceptible than the wild type to this pathogen (Fig. 3, A–D). The *npr1-3* mutation does not affect *S. sclerotiorum*-induced *PDF1.2* expression (Fig. 3, E and F), which is consistent with NPR1 being an SA signaling component (Cao et al., 1997). Since the SA biosynthesis mutants, *SA induction-deficient1* (*sid1*)/*eds5* and *sid2*/*eds16*, as well as *nahG* (encoding a salicylate hydroxylase) transgenic plants, which are impaired in SA accumulation, do not show enhanced susceptibility to *S. sclerotiorum* (Perchepied et al., 2010), how NPR1 plays a positive role in resistance to this necrotrophic fungal pathogen needs further investigation. Nevertheless, because we have previously shown that mutations in *MED16* reduce NPR1 protein accumulation (Zhang et al., 2012), the reduced NPR1 protein levels in the *med16* mutant might contribute to the enhanced susceptibility to *S. sclerotiorum*.

In summary, our study revealed that MED16 is a central component of basal resistance against *S. sclerotiorum*. We demonstrate that MED16 is essential not only for JA/ET-mediated defense pathways but also for the transcription factor WRKY33-activated defense signaling. Further investigations on the molecular mechanisms underlying the function of MED16 in resistance to *S. sclerotiorum* would help in the design of strategies for controlling this broad-host-range necrotrophic fungal pathogen.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

The wild-type Arabidopsis (*Arabidopsis thaliana*) Columbia ecotype was utilized. The *med* mutants used in this study are listed in Supplemental Table S1, and *wrky33-1* (SALK_006603), *npr1-3*, *ein2-1*, and *coi1-1* were described previously (Cao et al., 1997; Xie et al., 1998; Alonso et al., 1999; Zheng et al., 2006). The T-DNA insertion lines were obtained from either the Arabidopsis Biological Resource Center at Ohio State University or the European Arabidopsis Stock Centre at the University of Nottingham. Homozygous mutant plants of the T-DNA insertion lines were confirmed with primers flanking the T-DNA insertions (Supplemental Table S2) and the left border primers LBa1 and LB3 (Sessions et al., 2002; Alonso et al., 2003a). *MED16pro:MED16-3×FLAG/35Spro:4×My-WRKY33* plants were generated by crossing *MED16pro:MED16-3×FLAG med16-1* with the previously characterized *35Spro:4×My-WRKY33* transgenic line (Mao et al., 2011). The *35Spro:4×My-WRKY33* transgene was also crossed into the *med16-1* mutant background to generate *35Spro:4×My-WRKY33 med16-1* plants. The Arabidopsis seeds were sown on autoclaved soil (Sunshine MVP; Sun Gro Horticulture) and cold treated at 4°C for 3 d. Plants were germinated and grown at 23°C to 25°C under a 16-h-light/8-h-dark regime.

Pathogen Infection

Sclerotinia sclerotiorum inoculation was performed as described by Guo and Stotz (2007) with minor modifications. Briefly, sclerotia of *S. sclerotiorum* isolate 1980 were germinated at room temperature on potato dextrose agar medium (213400; Becton-Dickinson). About 3 d later, a small piece of agar containing mycelia was transferred onto minimal medium (1 g of NaOH, 3 g of DL-malic acid, 2 g of NH_4NO_3, 0.1 g of $MgSO_4 \cdot 7H_2O$, and 39 g of Bacto-agar per liter; Cruickshank, 1983) and cultured for about 3 d prior to inoculation to reduce the pathogen aggressiveness (Guo and Stotz, 2007). An agar plug (2 mm in diameter) containing the advancing edge of *S. sclerotiorum* mycelia was removed to inoculate Arabidopsis leaves. One rosette leaf per plant was inoculated for basal resistance assessment. Lengths and widths of lesions were measured with a caliper before disease symptoms expanded beyond inoculated leaves, and the average of the length and the width was used to represent the size of a lesion. The decay percentage of *S. sclerotiorum*-infected plants was scored to assess disease development. A plant was considered to be decayed when the lesion expanded beyond the inoculated leaf to the center of the plant.

RNA Analysis

RNA extraction, reverse transcription, and qPCR analyses were carried out as described by DeFraia et al. (2010). Primers used for qPCR in this study are listed in Supplemental Table S3.

Microarray Analysis

Four-week-old soil-grown plants were inoculated with the *S. sclerotiorum* isolate 1980. Total RNA samples extracted from leaf tissues collected at the indicated time points after *S. sclerotiorum* inoculation were subjected to microarray analysis. Briefly, RNA concentration was determined on a NanoDrop Spectrophotometer (Thermo Fisher Scientific), and sample quality was assessed using the 2100 Bioanalyzer (Agilent Technologies). Complementary DNA (cDNA) was synthesized from 200 ng of total RNA and used as a template for in vitro transcription in the presence of T7 RNA Polymerase and cyanine-labeled CTPs using the Quick Amp Labeling kit (Agilent Technologies) according to the manufacturer's protocol. The amplified, labeled copy RNA was purified using the RNeasy Mini kit (Qiagen). For each array, 1,650 ng of Cy3-labeled copy RNA was fragmented and hybridized with rotation at 65°C for 17 h. Samples were hybridized to Arabidopsis 4 × 44k arrays (Agilent Technologies). The arrays were washed according to the manufacturer's protocol and then scanned on a G2505B scanner

(Agilent Technologies). Data were extracted using Feature Extraction 10.1.1.1 software (Agilent Technologies).

Data (individual signal intensity values) obtained from the microarray probes were background corrected using the normexp+offset method, in which a small positive offset ($k = 50$) was added to move the corrected intensities away from zero (Ritchie et al., 2007). The resulting data were log transformed (using 2 as the base) and normalized between individual samples by scaling the individual log-transformed signal intensities so that all data sets had comparable lower quartile, median, and upper quartile values (Smyth, 2005). After normalization, Student's t test was performed considering a probe-by-probe comparison between different genotypes at the same time point using wild-type Columbia as the reference sample and between different time points of the same genotype using the 0-h sample as the reference. In each comparison, a P value and fold change were computed for each gene locus. The gene expression fold changes were computed based on the normalized log-transformed signal intensity data. To control false discovery rate and correct multiple hypothesis testing, a q value was calculated and used to assess the significance of each test (Storey and Tibshirani, 2003). The comparison results were further explored to obtain numbers of overlapped genes between/among different comparisons.

Chromatin Immunoprecipitation

Chromatin immunoprecipitation was performed as described by Saleh et al. (2008) with minor modifications. Briefly, approximately 2 g of 2-week-old seedlings was submerged in 50 mL of cross-linking buffer (10 mM Tris-HCl, pH 8, 0.4 M Suc, 1 mM phenylmethylsulfonyl fluoride [PMSF], 1 mM EDTA, and 1% [v/v] formaldehyde) and vacuum infiltrated three times for 3 to 4 min each at room temperature. The cross-linking reaction was stopped by adding 2.5 mL of 2 M Gly to a final concentration of 100 mM and vacuum infiltration for 5 min. Plant tissues were washed three times with cold sterile deionized water. After removing water, plant tissues were submerged in liquid nitrogen, ground to a fine powder, and resuspended in 20 to 25 mL of cold nuclei isolation buffer (15 mM PIPES, pH 6.8, 0.25 M Suc, 5 mM $MgCl_2$, 60 mM KCl, 15 mM NaCl, 1 mM $CaCl_2$, 0.9% [v/v] Triton X-100, 1 mM PMSF, 2 μg mL^{-1} pepstatin A, and 2 μg mL^{-1} aprotinin). After brief vortex and incubation, the homogenized slurry was filtered through one layer of Miracloth. After centrifugation at 3,220g for 20 min, the pellet (nuclei) was resuspended in 1.5 mL of cold nuclei lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.1% [w/v] SDS, 0.1% [w/v] sodium deoxycholate, 1% [v/v] Triton X-100, 1 μg mL^{-1} pepstatin A, and 1 μg mL^{-1} aprotinin). DNA was sheared into approximately 500-bp (200–1,000 bp) fragments by 6 to 10 min of 3-s pause sonication at 40% to 43% amplitude using a TM-100 sonic disruptor (TekMar). After centrifugation at 13,800g for 10 min, the supernatant (200 μL) was diluted 5-fold with nuclei lysis buffer and precleared by adding 50 μL of salmon (*Oncorhynchus keta*) sperm DNA/protein A agarose beads. After removing the agarose beads, 5 μL of anti-RPB2 antibody (ab10338; Abcam) was added, and the mixture was incubated at 4°C for 5 h to overnight with gentle rotation, and then 60 to 75 μL of salmon sperm DNA/protein A agarose beads was added and the incubation was continued for 2 to 3 h. After centrifugation at 3,800g for 2 min, the agarose beads were sequentially washed with low-salt wash buffer (20 mM Tris-HCl, pH 8, 150 mM NaCl, 0.2% [w/v] SDS, 0.5% [v/v] Triton X-100, and 2 mM EDTA), high-salt wash buffer (20 mM Tris-HCl, pH 8, 500 mM NaCl, 0.2% [w/v] SDS, 0.5% [v/v] Triton X-100, and 2 mM EDTA), LiCl wash buffer (10 mM Tris-HCl, pH 8, 0.25 M LiCl, 1% [w/v] sodium deoxycholate, 1% [v/v] Nonidet P-40, and 1 mM EDTA), and TE buffer (twice; 1 mM EDTA and 10 mM Tris-HCl, pH 8). The immunocomplexes were eluted with freshly prepared elution buffer (0.1 M $NaHCO_3$ and 0.5% [w/v] SDS) and incubated at 65°C for 15 min with gentle rotation. Twenty microliters of 5 M NaCl was added to 500 μL of the immunocomplex solution, the mixture was incubated at 65°C for 4 h to overnight to reverse cross linking, then 20 μL of 1 M Tris-HCl, pH 6.5, 10 μL of 0.5 M EDTA, and 2 μL of proteinase K (10 mg mL^{-1}) were added, and the mixture was incubated at 45°C for 1.5 h to digest the proteins. Immunoprecipitated DNA was purified using a mixture of phenol: chloroform:isoamyl alcohol (25:24:1), and the resulting DNA was used for qPCR analysis. The amount of precipitated DNA corresponding to a specific gene region was determined by qPCR and normalized to input DNA (Haring et al., 2007). Primers used for chromatin immunoprecipitation-qPCR are listed in Supplemental Table S4.

Plasmid Construction and Plant Transformation

The 3×FLAG fragment was removed with *Spe*I and *Xba*I from pCR8GW-XB3New-3×FLAG (Wang et al., 2006) and ligated into the corresponding sites of pBluescript SK+. The primers *EcoRI-MED16F* and *SpeI-MED16R* (Supplemental Table S5) were used to amplify the coding region of *MED16* from Arabidopsis cDNAs. The PCR products were digested with *Eco*RI and *Spe*I and ligated into the corresponding sites of pBluescript SK+-3×FLAG, resulting in the plasmid pBluescript SK−MED16-3×FLAG. The primers *SalI-MED16PF* and *EcoRI-MED16PR* (Supplemental Table S5) were used to amplify the promoter region of *MED16* from the Arabidopsis genomic DNA. The PCR products were digested with *Sal*I and *Eco*RI and ligated into the corresponding sites of pBluescript SK+-MED16-3×FLAG, resulting in the plasmid pBluescript SK+-MED16pro:MED16-3×FLAG. The MED16pro:MED16-3×FLAG fragment was then recovered using *Sal*I and *Xba*I and subcloned into the corresponding sites of pBI101-Luc (Zhang et al., 2012). The resulting plasmid pBI101-MED16pro:MED16-3×FLAG was introduced into the *Agrobacterium tumefaciens* strain GV3101(pMP90) by electroporation and transformed into *med16-1* following the floral dip method (Clough and Bent, 1998).

Chemical Treatment

Ten-day-old seedlings grown on one-half-strength Murashige and Skoog medium were treated with 0.1 mM MeJA, 0.1 mM ACC, or their combination. Seedlings for the negative control were treated with water. Plant tissues except roots were collected and subjected to total RNA extraction.

Coimmunoprecipitation

The coimmunoprecipitation assay was performed as described by Qiu et al. (2008) with minor modifications. Briefly, nuclei were isolated according to Gendrel et al. (2002) and Nelson et al. (2006). Nuclei were resuspended in coimmunoprecipitation buffer (100 mM Tris-HCl, pH 7.5, 75 mM NaCl, 1 mM EDTA, 0.1% [v/v] Triton X-100, 0.05% [w/v] SDS, 10% [v/v] glycerol, 2.5 mM dithiothreitol, 50 μg mL^{-1} protease inhibitors tosyl-L-phenylalanyl chloromethyl keton and tosyl-L-lysyl-chloromethane hydrochloride, and 0.6 mM PMSF). Ten units of Universal Nuclease (88700; Pierce Biotechnology) was added into the suspension, and the mixture was incubated on ice for 1 h and centrifuged at 16,000g for 30 min. The supernatant was incubated with anti-Myc antibody (sc-789; Santa Cruz Biotechnology) overnight at 4°C followed by precipitation with protein G Plus-Agarose (sc-2002; Santa Cruz Biotechnology) for 4 h. After washing four times with coimmunoprecipitation buffer, proteins were eluted by boiling in 40 μL of 2× Laemmli sample buffer for 10 min. The eluates were separated by 8% (w/v) SDS-PAGE, transferred onto a nitrocellulose membrane (1215458; ME Manufacturing), and probed with anti-FLAG antibody (3165; Sigma) to detect coimmunoprecipitated MED16-3×FLAG protein.

Yeast Two-Hybrid Assay

The full-length coding sequence of *MED16* was amplified from Arabidopsis cDNAs with primers *SalI-MED16F* and *EcoRI-MED16R* (Supplemental Table S5). *Sal*I/*Eco*RI-digested PCR products were cloned into the corresponding site of the bait vector pGBKT7. Full-length *WRKY33* coding sequence was amplified from Arabidopsis cDNAs with primers *BamHI-WRKY33F* and *EcoRI-WRKY33R* (Supplemental Table S5), digested with *Bam*HI and *Eco*RI, and cloned into the prey vector pGADT7. The resulting plasmids pGBKT7-MED16 and pGADT7-WRKY33 were cotransformed into the yeast (*Saccharomyces cerevisiae*) strain AH109. The bait vector pGBKT7 and prey vector pGADT7 were cotransformed with pGADT7-WRKY33 and pGBKT7-MED16, respectively, to generate negative controls. The presence of the transgenes was confirmed by growth on an SD-Leu-Trp agar plate. To assess protein interactions, the transformed yeast cells were suspended in liquid SD-Leu-Trp medium to an optical density at 600 nm of 1. Five microliters of suspended yeast cells was dropped onto an SD-Ade-His-Leu-Trp+X-α-Gal (4 mg mL^{-1}) agar plate. The resulting agar plate was incubated at 30°C and observed for yeast growth and color changes.

Statistical Methods

Except for those used in microarray analysis, statistical analyses were performed using the one-way ANOVA in Prism 5.0b (GraphPad Software) and the data analysis tools in Excel of Microsoft Office 2004 for Macintosh (Student's t test: two samples assuming unequal variances).

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers:

MED8 (At2g03070), MED13 (At1g55325), MED14 (At3g04740), MED16 (At4g04920), MED17 (At5g20170), MED18 (At2g22370), MED20a (At3g28230), MED23 (At1g23230), MED25 (At1g25540), MED31 (At5g19910), MED32 (At1g11760), MED33b (At2g48110), MED34 (At1g31360), MED36 (AT4g25630), NPR1 (At1g64280), EIN2 (At5g03280), COI1 (At2g39940), WRKY33 (At2g38470), ORA59 (At1g06160), PDF1.2 (At5g44420), MYC2 (At1g32640), VSP1 (At5g24780), VSP2 (At5g24770), JR1 (At3g16470), and UBQ5 (At3g62250), and the National Center for Biotechnology Information Gene Expression Omnibus series number GSE65165 (microarray data).

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. *MED* gene expression in the corresponding T-DNA insertion mutants.

Supplemental Figure S2. Comparison of *PDF1.2* expression in *med16* and *coi1-1*.

Supplemental Figure S3. Complementation of *med16* with *MED16pro*:*MED16-3×FLAG*.

Supplemental Figure S4. Evidence that 6×Myc does not interact with MED16-3×FLAG.

Supplemental Table S1. Mediator mutants used in this study.

Supplemental Table S2. Primers used for the identification of homozygous T-DNA insertion mutant plants.

Supplemental Table S3. Primers used for qPCR analysis of gene expression.

Supplemental Table S4. Primers used for chromatin immunoprecipitation-qPCR analysis.

Supplemental Table S5. Primers used for plasmid construction.

Supplemental Data Set S1. Comparison of gene expression between *med16-1* and the wild type.

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