The Arabidopsis Mediator Subunit MED25 Differentially Regulates Jasmonate and Abscisic Acid Signaling through Interacting with the MYC2 and ABI5 Transcription Factors

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Transcriptional regulation plays a central role in plant hormone signaling. At the core of transcriptional regulation is the Mediator, an evolutionarily conserved, multisubunit complex that serves as a bridge between gene-specific transcription factors and the RNA polymerase machinery to regulate transcription. Here, we report the action mechanisms of the Mediator25 (MED25) subunit of the Arabidopsis thaliana Mediator in regulating jasmonate- and abscisic acid (ABA)-triggered gene transcription. We show that during jasmonate signaling, MED25 physically associates with the basic helix-loop-helix transcription factor MYC2 in promoter regions of MYC2 target genes and exerts a positive effect on MYC2-regulated gene transcription. We also show that MED25 physically associates with the basic Leu zipper transcription factor ABA-INSENSITIVE5 (ABI5) in promoter regions of ABI5 target genes and shows a negative effect on ABI5-regulated gene transcription. Our results reveal that underlying the distinct effects of MED25 on jasmonate and ABA signaling, the interaction mechanisms of MED25 with MYC2 and ABI5 are different. These results highlight that the MED25 subunit of the Arabidopsis Mediator regulates a wide range of signaling pathways through selectively interacting with specific transcription factors.

INTRODUCTION

The jasmonate family of oxylipins, including jasmonic acid (JA) and its bioactive derivatives, which are collectively referred to as JAs, regulates diverse aspects of plant immunity and development. It is generally believed that, in addition to promoting plant defense responses to herbivore attack, pathogen infection, and mechanical wounding, JAs also inhibit growth-related processes such as cell division and photosynthesis (Creelman and Mullet, 1997; Turner et al., 2002; Browse, 2005, 2009; Wasternack, 2007; Howe and Jander, 2008; Kazan and Manners, 2008; Chung et al., 2009; Pauwels et al., 2009; Sun et al., 2011). Underlying these important physiological effects, JAs orchestrate genome-wide transcriptional reprogramming of plant cells to coordinate growth- and defense-related processes.

Much of our understanding of the JA signal transduction pathway has come from the recent elucidation of the molecular details of JA-regulated gene transcription. Conventional genetic evidence together with recent biochemical and structural studies support the view that the F-box protein CORONATINE-SENSITIVE1 (COI1) is the receptor of jasmonoyl-L-Ile (JA-Ile), a molecularly active form of JA (Xie et al., 1998; Katsir et al., 2008; Fonseca et al., 2009; Yan et al., 2009; Sheard et al., 2010; Suza et al., 2010; Wasternack and Kombrink, 2010). Significant advancement in our understanding of how the JA signaling operates came from the discovery that the JASMONATE ZIM DOMAIN (JAZ) proteins, which serve as transcriptional repressors of JA-induced gene expression, are substrates of the E3 ubiquitin ligase SCFCOI1 (Chini et al., 2007; Thines et al., 2007; Yan et al., 2007; Chung et al., 2009). Recent structure-function studies indicated that the jasmonate receptor is a three-molecule complex consisting of COI1, JAZ transcriptional repressors, and inositol pentakisphosphate (Sheard et al., 2010). These studies together revealed that jasmonate and auxin show a similar signal perception and transduction paradigm, in which F-box proteins (receptors) mediate the degradation of negative regulators of gene transcription cascades (Mockaitis and Estelle, 2008). At low JA levels, JAZ proteins interact with NOVEL INTERACTOR OF JAZ (NINJA) to recruit TOPLESS (TPL) as a coexpressor to repress the activity of JAZ-targeted transcription factors, including the basic helix-loop-helix (bHLH) transcription factor MYC2, which regulates diverse aspects of JA-mediated gene expression (Boter et al., 2004; Lorenzo et al., 2004; Dombrecht et al., 2007; Pauwels et al., 2010). In response to internal or external cues, JA-Ile is quickly synthesized by JASMONATE RESISTANT1 (JAR1) (Staswick and Tiryaki, 2004; Fonseca et al., 2009).
transcription factor MYC2 and executes a positive effect on MYC2-regulated expression of JA-responsive genes. We also show that MED25 interacts with the basic domain/Leu zipper (bZIP) transcription factorABA-INSENSITIVE5 (ABI5) and exerts a negative effect on ABI5-regulated expression of ABA-responsive genes. We provide evidence showing that the conserved activator-interacting domain (ACID) of MED25 is sufficient for its interaction with ABI5 but not sufficient for its interaction with MYC2. In addition, we reveal that while the putative transcriptional activation domain (TAD) of MYC2 is involved in its interaction with MED25, the TAD domain of ABI5 is not involved in its interaction with MED25. These findings highlight that, in response to different hormonal signals, a single plant Mediator subunit can regulate distinct transcriptional programs via interactions with relevant gene-specific transcription factors.

RESULTS

The ber6 Mutant Contains a Mutation in the MED25 Gene and Affects Diverse Aspects of JA Responses

The bestatin-resistant6 (ber6) line of Arabidopsis was identified as a JA-insensitive mutant in JA-induced root growth inhibition (Zheng et al., 2006). Our further characterization of ber6 was performed with a homozygous line obtained from successive backcrosses of the original mutant to the wild type Columbia-0 (Col-0). JA response assays with this line showed that ber6 was generally less sensitive than the wild type to the inhibition effect of a range of concentrations of JA on root growth (Figure 1A). We then examined whether the expression of JA-responsive genes is affected in ber6 plants. Two distinct branches of JA-responsive genes that are differentially regulated by the transcription factor MYC2 were identified in Arabidopsis (Boter et al., 2004; Lorenzo et al., 2004). One of these branches, including the VEGETATIVE STORAGE PROTEIN1 (VSP1) gene (Berger et al., 1995), is believed to be involved in plant responses to wounding. The other branch, including the plant defensin gene PDF1.2 (Penninckx et al., 1998), is considered to be associated with plant responses to pathogen infection (Boter et al., 2004; Lorenzo et al., 2004). Promoter-reporter fusion assays revealed that the ber6 mutation substantially impaired JA-induced expression of both VSP1pro:GUS (for β-glucuronidase) and PDF1.2pro:LUC (for luciferase) (Figures 1B and 1C). Consistent with this, quantitative real-time PCR (qRT-PCR) assays showed that the JA-induced expression levels of VSP1 (Figure 1D) and PDF1.2 (Figure 1E) were markedly reduced in ber6 compared with those in the wild type. Next, we examined the response of ber6 plants to infection by Pseudomonas syringae pv tomato (Pst) DC3000. As measured by leaf symptoms (Figure 1F) and pathogen growth (Figure 1G) of the infected plants, the effects of Pst DC3000 infection in ber6 are largely similar to those in the coi1-2 mutant, which harbors a point mutation of the JA receptor geneCOI1 and therefore is resistant to Pst DC3000 (Kloek et al., 2001; Xu et al., 2002; Laurie-Berry et al., 2006). Together, our results support that ber6 is a JA signaling mutant that is defective in JA-induced defense gene expression.
Figure 1. The Defective JA Responses of the ber6 Mutant Are Caused by a Mutation of the MED25 Gene.

(A) Root growth inhibition assay of 10-d-old seedlings from Col-0 and ber6 grown in MS medium containing indicated concentrations of JA. Results shown are the mean ± SD of measurements from 90 seedlings. For each JA concentration, the data were analyzed by Student’s t test using SPSS. Asterisks denote t test significance compared with JA-grown wild type plants: **P < 0.01.

(B) Photographs of representative control and 50 µM MeJA (6-h treatments) treated 10-d-old seedlings of VSP1pro::GUS/Col-0 and VSP1pro::GUS/ber6.

(C) Photographs of representative control and 50 µM MeJA (48-h treatments) treated 10-d-old seedlings of PDF1.2pro::LUC/Col-0 and PDF1.2pro::LUC/ber6.

(D) VSP1

(E) PDF1.2

(F) qRT-PCR analysis of VSP1 (6-h treatments; [D]) and PDF1.2 (48-h treatments; [E]) RNA levels in 10-d-old Col-0 and ber6 seedlings treated with 50 µM MeJA. The break ranges are shown on the y axis. Data shown are mean values of three biological repeats with SD. Asterisks denote Student’s t test significance compared with MeJA-treated Col-0 plants: **P < 0.01. Numbers on the white bars represent fold induction values in MeJA-treated plants relative to control (black bars) plants.

(F) Symptoms on rosette leaves of 4-week-old soil-grown Col-0, ber6, and coi1-2 following inoculation with Pst DC3000 bacteria for 3 d.

(G) Pst DC3000 populations, shown as colony-forming units (c.f.u.) cm$^{-2}$ of leaf area in Col-0, ber6, and coi1-2 plants at day 0 (black) and day 3 (white) after inoculation. Asterisks denote Student’s t test significance compared with Pst DC3000–treated Col-0 plants: **P < 0.01.

(H) Fine genetic and physical mapping of BER6. The target gene was mapped to a genetic interval between markers F21J9 and T24P13 on chromosome 1. Analysis of a F2 mapping population of 1800 plants delimited the gene to a region of 50 kb on the BAC clone F2J7 (red). Numbers in parentheses indicate the number of recombination events identified between markers and the target gene.

(I) MED25 gene structure, showing the mutation site of ber6/med25-4. The T-DNA insertion sites in med25-2, med25-3, pft1-1, and pft1-2 and the point mutation site of med25-1 are also indicated. Black boxes represent exons, lines represent introns, and white boxes represent 5′-untranslated region, respectively. Bar = 1 kb.

(J) Schematic representation of MED25 protein. The MED25 protein contains a vWF-A domain (blue), an MD domain (white), an ACID domain (red), and a GD domain (gray). Bar = 100 amino acids (aa).
The decreased JA-induced root growth inhibition of ber6 provides a facile assay for map-based cloning studies aimed at determining the genetic basis of this defect. Using a total of 1800 F2 plants showing the mutant phenotype, BER6 was delimited to a 50-kb region on the BAC clone F2J7 (Figure 1H). DNA sequencing revealed that ber6 contains a G-to-A transition at the junction between the fifth exon and the fifth intron of the annotated gene At1g25540 (Figure 1I), which encodes the MED25 subunit of the Arabidopsis Mediator (Bäckström et al., 2007). The G-to-A mutation in ber6 destroys an RsaI restriction site, and a cleaved amplified polymorphic sequence marker was developed to detect the ber6 mutant allele (see Supplemental Figure 1C online). In the ber6 mutant, the 5’ exon-intron boundary of intron 5 is changed from GT to AT (Figure 1I), replacing a G that is highly conserved at plant gene splice sites. The mutation in ber6 altered the splicing of At1g25540 mRNA (see Supplemental Figure 1A online), resulting in a frame shift that destroys the von Willebrand Factor A (vWF-A) domain, which has been shown to be crucial for the binding of MED25 to the Mediator complex, and the ACID domain is important for interaction with transcriptional activators, whereas the GD domain could be important for transcriptional activation (Cerdán and Chory, 2003; Mittler et al., 2003; Bäckström et al., 2007; Lee et al., 2007; Elfving et al., 2011).

**MED25 Affects the Function of MYC2 in Regulating the Transcriptional Expression of JA-Responsive Genes**

To elucidate the action mechanisms of MED25 in JA-mediated transcriptional regulation, we investigated the effect of MED25 on the function of MYC2, a master transcriptional regulator that controls diverse aspects of JA responses (Boter et al., 2004; Lorenzo et al., 2004; Dombrecht et al., 2007). Toward this goal, we generated a double mutant line med25-4 myc2-2 and compared its JA responses with those of the single mutants in defense gene expression. Consistent with the well-recognized finding that MYC2 negatively regulates the expression of the branch of pathogen-responsive genes (Boter et al., 2004; Lorenzo et al., 2004; Dombrecht et al., 2007), our qRT-PCR assays indicated that JA-induced expression levels of PDF1.2 (Figure 2A) and ETHYLENE RESPONSE FACTOR1 (ERF1; Figure 2B) were markedly increased in myc2-2 compared with those in the wild type. Importantly, JA-induced expression levels of these genes were strongly reduced in the med25-4 mutant (Figures 1E, 2A, and 2B), suggesting that MED25 is required for JA-induced expression of this group of genes. Significantly, in the med25-4 myc2-2 double mutant, JA-induced expression levels of PDF1.2 and ERF1 were essentially comparable to those in med25-4 (i.e., med25-4 suppressed the phenotype of myc2-2 in terms of JA-induced PDF1.2 and ERF1 expression) (Figures 2A and 2B), suggesting that MED25 acts genetically downstream of MYC2 in regulating the expression of these genes. Together, these results support that, in response to JA, MED25 is required for the MYC2 function to repress the expression of pathogen-responsive genes. Consistent with the positive role of MYC2 in regulating the expression of wound-responsive genes (Boter et al., 2004; Lorenzo et al., 2004; Dombrecht et al., 2007), we found that the JA-induced expression levels of VSIP1 (Figure 2C), LIPOXYGENASE2 (LOX2; Figure 2D), JAZ6 (Figure 2E), and JAZ8 (Figure 2F) were substantially reduced in myc2-2 mutants compared with those in wild-type plants (Figures 2C to 2F). JA-induced expression levels of these genes were also reduced in med25-4 (Figures 2C to 2F), suggesting that MED25 is also important for JA-induced expression of this group of genes. As expected, in the med25-4 myc2-2 double mutant, JA-induced expression levels of these genes were largely similar to those in med25-4 (Figures 2C to 2F), suggesting that MED25 is also important for the MYC2 function to activate the expression of these wound-responsive genes. Taken together, these results led us to the conclusion that, during JA response, MED25 exerts a positive effect on the function of the MYC2 transcription factor to regulate the expression of JA-responsive genes.

**MED25 Is Recruited to Promoters of MYC2 Target Genes in a JA-Dependent Manner**

It has been shown that the human MED25, together with the retinoic acid (RA)–bound retinoic acid receptor (RAR), was
recruited to the promoter regions of RA-responsive genes and therefore regulates the function of RAR (Lee et al., 2007). In this context, we speculate that the *Arabidopsis* MED25 could associate with MYC2 in chromatin regions of MYC2 target genes. To test this hypothesis, we first assessed the effect of MED25 on the expression of JA-responsive genes that are directly targeted by MYC2. It has been shown that in response to JA, MYC2 quickly activates the expression of the JAZ family genes via directly binding to the G-box (5'-CACGTG-3') sequence in the promoters of these genes (Chini et al., 2007; Hou et al., 2010). Our qRT-PCR assays revealed that the med25-4 mutation markedly reduced the JA-induced expression levels of the JAZ family genes (see Supplemental Figure 2 online). Chromatin immunoprecipitation (ChIP) assays using 35S<sub>pro</sub>:MYC2-GFP (for green fluorescent protein) plants and anti-GFP antibodies indicated that MYC2 associated with the G-box (5'-CACGTG-3') motifs in the promoters of JAZ6 and JAZ8 (Figures 3A and 3B), confirming that JAZ6 and JAZ8 are direct targets of MYC2. ChIP assays using 35S<sub>pro</sub>:MED25-GFP plants indicated that, like MYC2, MED25 was also enriched in the G-box (5'-CACGTG-3') motifs of JAZ6 and JAZ8 promoters, and, importantly, the enrichment of both MYC2 and MED25 was substantially enhanced by JA treatment (Figure 3B). These results demonstrate that MED25 associates with the transcription factor MYC2 in chromatin regions of MYC2 target genes.

To determine the recruitment dynamic of MYC2 and MED25 to JAZ promoters, 35S<sub>pro</sub>:MYC2-GFP and 35S<sub>pro</sub>:MED25-GFP

**Figure 2. MED25 Affects the Function of MYC2 in Regulating the Transcriptional Expression of JA-Responsive Genes.**

Expression of PDF1.2 (48-h treatments; [A]), ERF1 (1-h treatments; [B]), VSP1 (6-h treatments; [C]), LOX2 (1-h treatments; [D]), JAZ6 (1-h treatments; [E]), and JAZ8 (1-h treatments; [F]) was examined by qRT-PCR in Col-0, med25-4, myc2-2, and med25-4 myc2-2 plants following 50 μM MeJA treatment. The break ranges (for PDF1.2, VSP1, and JAZ8) are shown on the y axis. Data presented are mean values of three biological repeats with so. Numbers on the white bars represent fold induction values in MeJA-treated plants relative to control (black bars) plants for each genotype.
plants were incubated with JA for varying lengths of time. ChIP assays indicated that low levels of MYC2 was recruited to JAZ6 promoter at steady state and that JA treatment leads to a marked increase of MYC2 recruitment within 15 min; MYC2 binding to JAZ6 promoter then remains at high levels during the time course investigated (Figure 3C). Parallel experiments revealed that MED25 shows negligible association with JAZ6 promoter at steady state (Figure 3D). In the presence of the ligand, however, MED25 recruitment was quickly increased and reached a maximum within 15 min after JA treatment (Figure 3D); MED25 binding then exhibited a tendency of reduction for the duration of the experiment (Figure 3D). Next, we examined whether the JAZ6 mRNA levels follow the recruitment pattern of MED25 to the promoter of JAZ6. As shown in Figure 3E, in wild-type plants, JAZ6 mRNA levels increased markedly at 15 min, peaked at 60 min, and then showed a tendency of decrease up to 180 min upon JA treatment. In JA-treated med25-4 plants, JAZ6 expression exhibited similar induction kinetics, albeit its expression levels are substantially reduced in med25-4 compared with those in wild-type plants (Figure 3E). These results indicate that the maximum recruitment of MED25 to the JAZ6 promoter occurs generally earlier than the peaked activation of JAZ6 expression. This scenario is consistent with a previous observation in mammalian system showing that Mediator and other coactivators are

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**Figure 3.** MED25 Is Recruited to Promoter Regions of MYC2 Target Genes in a JA-Dependent Manner.

(A) Schematic diagram of the promoter region of JAZ6 and JAZ8. Black lines represent the promoter region of the two genes. Black boxes on the line represent the putative MYC2 binding G-box (5’-CACGTG-3’); numbers above indicate the distance away from the ATG. Region between the two coupled arrowheads indicates the DNA fragments used for ChIP-PCR. The translational start sites (ATG) were shown as +1. Bar = 200 bp.

(B) Binding of MYC2 and MED25 to JAZ6 and JAZ8 promoter regions. The 35Spro·MYC2-GFP and 35Spro·MED25-GFP transgenic seedlings were used in ChIP using anti-GFP antibody (Abcam). For MeJA treatment, 35Spro·MYC2-GFP and 35Spro·MED25-GFP seedlings were treated with 100 µM MeJA for 60 min before cross-linking. The “No Ab” (no antibody) immunoprecipitates served as negative controls. The ChIP signal was quantified as the percentage of total input DNA by real-time PCR. Three biological replicates were performed and identical results were obtained. Standard deviations were calculated from three technical repeats.

(C) and (D) Dynamic recruitment of MYC2 and MED25 to JAZ6 promoter. ChIP assays were performed as in (B), except that 35Spro·MYC2-GFP and 35Spro·MED25-GFP plants were treated with 100 µM MeJA for varying lengths of time (0, 15, 60, and 180 min) before cross-linking.

(E) JAZ6 mRNA level follows the recruitment pattern of MED25 to the promoter. Col-0 and med25-4 were treated with MeJA for varying lengths of time (0, 15, 60, and 180 min) as in (C) and (D). Data presented are mean values of three biological repeats with SD.

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targeted to a promoter before significant gene activation occurs (Sharma and Fondell, 2002).

**Mapping of the Domains Involved in the MED25/ MYC2 Interaction**

It has been shown that the MED25 counterparts from animals physically associate with multiple cellular transcriptional activators, including the herpes simplex viral activator VP16 (Mittler et al., 2003; Yang et al., 2004), RAR (Lee et al., 2007), hepatocyte nuclear factor4 (Rana et al., 2011), and the chondrogenesis-related transcriptional activator Sox9 (Nakamura et al., 2011). Our data showing that mutation of the *Arabidopsis* MED25 gene affects the action of MYC2 in regulating the expression of JA-responsive genes prompted us to characterize the physical interaction of the two proteins in vivo. Toward this goal, we conducted coimmunoprecipitation (Co-IP) experiments using our recently described 35S pro:Myc2-4myc plants (Chen et al., 2011) and anti-MED25 antibody. Co-IP assays revealed that, in the absence of exogenous JA, MED25 could be precipitated by MYC2 (Figure 4A), suggesting that MED25 interacts with MYC2 at steady state. Parallel Co-IP assays indicated that the precipitation efficiency of MED25 by MYC2 was not substantially changed by JA treatment for 30 min (Figure 4A), suggesting that JA shows little effect on the MED25 and MYC2 interaction at the time point investigated. Next, we tried to map the interaction domain of MED25 with MYC2 using yeast two-hybrid assays. For these experiments, we fused the full length or derivatives of MED25 to the GAL4-DNA binding domain and fused the full-length MYC2 to the GAL4-activation domain (Figure 4B). To circumvent the problem that full-length MED25 can activate the transcription of reporter genes in yeast (Ou et al., 2011), we performed our yeast two-hybrid assays in the presence of 10 mM 3-amino 1,2,4-triazol (Bharti et al., 2000), which could effectively repress the background transcription activation activity of full-length MED25 (see Supplemental Figure 3 online). Under these experimental conditions, we found that full-length MED25 showed interaction with MYC2 in yeast (Figure 4B; see Supplemental Figure 3 online). On the contrary, none of the four individual MED25 domains, including the vWF-A (MED25 vWF-A), the MD (MED25 MD), the ACID (MED25 ACID) and the GD (MED25 GD) domains, showed interaction with MYC2 in yeast (Figure 4B). In the context that the ACID domain serves as the ACID of *Arabidopsis* MED25 (Elfving et al., 2011; Ou et al., 2011) as well as its mammalian counterparts (Mittler et al., 2003; Yang et al., 2004; Lee et al., 2007) and that deletion of the ACID and MD, but not of the vWF-A and GD, destroyed MED25 interaction with MYC2 (Figure 4B), we propose that the ACID and the MD are important for MED25 interaction with MYC2. Indeed, the MD-ACID fusion (MED25 MD-ACID) showed interaction with MYC2 in yeast two-hybrid assays (Figure 4B). We then employed a yellow fluorescent protein (YFP) bimolecular fluorescence complementation (BiFC) system (Weinthal and Tzfiria, 2009) to characterize further the MYC2-interacting domain of MED25. Like the full-length MED25 (Figure 4C), the MD-ACID fusion (MED25 MD-ACID) showed interaction with MYC2 in BiFC assays (Figure 4D). On the contrary, neither MED25 MD (Figure 4E) nor MED25 ACID (Figure 4F) alone showed interaction with MYC2. Further mapping efforts using BiFC assays revealed that the smallest positive MED25 fragment (MED25 540-680) included the ACID domain plus 10 amino acids of the MD (Figure 4G). Together, these data indicate that the ACID domain alone is not sufficient for MED25 interaction with MYC2.

We then use yeast two-hybrid assays to map the domain of MYC2 responsible for interaction with MED25. For these experiments, several MYC2 protein derivatives were fused to the GAL4-activation domain and full-length MED25 was fused to the GAL4-DNA binding domain (Figure 4H). As indicated in Figure 4I, the N-terminal part (MYC21-189) containing the putative TAD (MYC2 143-189) (Lorenzo et al., 2004; Fernández-Calvo et al., 2011) showed interaction with MED25, whereas no interaction was observed using the middle part (MYC2 189-445) or C-terminal part (MYC2 446-624). Moreover, the putative TAD of MYC2 alone was sufficient to interact with MED25 (Figure 4H).

It has been shown that in yeast and animals, the Mediator complex physically associates with both DNA-bound transcription factors and Pol II and thus serves as an integrative hub for transcriptional regulation (Malik and Roeder, 2005, 2010; Conaway and Conaway, 2011; Larivière et al., 2012). In this context, our results showing that the *Arabidopsis* MED25 physically associates with MYC2 and strongly affects its function suggest that mutation of MED25 could impair the recruitment of the general transcriptional machinery during JA-triggered gene transcription. To test this, we assessed the effect of the med25-4 mutation on JA-induced recruitment of NRPB2 (DNA-directed RNA polymerase II subunit RPB2), the second largest subunit of Pol II (Onodera et al., 2005, 2008; Zheng et al., 2009), to the promoter of MYC2 target genes. Indeed, ChIP assays revealed that JA-induced recruitment of NRPB2 to JAZ6 promoter was markedly reduced in *med25-4* compared with that in the wild type (Figure 4I).

In a current view of MYC2-directed transcription of JA-responsive genes, it is hypothesized that the JAZ-mediated repression of MYC2 function is achieved through recruitment of the corepressor TPL by NINJA, which physically associates with JAZs (Pauwels et al., 2011). This scenario predicts that during JA response, both MED25 and TPL could be recruited to the promoter of MYC2 targets. Indeed, our ChIP assays revealed that, as with MYC2 (Figures 3B and 3C) and MED25 (Figures 3B and 3D), the TPL corepressor was also recruited to the JAZ6 promoter (Figure 4J). Importantly, TPL recruitment to JAZ6 promoter was substantially reduced upon 1 h of JA treatment (Figure 4J), suggesting that TPL recruitment to MYC2 target promoters is regulated by the JA ligand. In addition, Co-IP assays using the reported 35S pro:TPL-GFP plants (Szemenyei et al., 2008) and anti-MED25 antibody indicated that MED25 and TPL could exist in a protein complex (Figure 4K), albeit yeast two-hybrid assays failed to detect their direct physical interaction (see Supplemental Figure 4 online). Taken together, these data support that MED25 may act as part of the general transcriptional machinery in regulating JA-triggered gene expression.

**MED25 Negatively Regulates ABA Response during Seed Germination and Early Seedling Growth**

Considering that MED25 regulates plant responses to a variety of abiotic stresses (Elfving et al., 2011), in which the phytohormone ABA plays an important regulatory role, we asked whether...
Figure 4. Mapping of Protein Domains Involved in the MED25/MYC2 Interaction.

(A) Co-IP assays showing that MED25 associates with MYC2 in plant cells. Protein extracts from 10-d-old 35Spro:Myc2-4myc seedlings were incubated with (+) or without (−) MeJA (100 μM) for 30 min before immunoprecipitation with myc antibody-bound agarose beads (Santa Cruz). Total and immunoprecipitated proteins were analyzed by immunoblotting using anti-MED25 and anti-myc antibodies. The experiments were repeated three times with similar results.

(B) Mapping of the domains involved in the MED25/MYC2 interaction using yeast two-hybrid assay. Based on the schematic protein structure of MED25 (top panel), full-length MED25 or its derivatives (pGBK7-MED25 or pGBK7-MED25 derivatives) were tested for interaction with MYC2 (pGADT7-MYC2) (see Methods for details). Transformed yeast was grown on selective media lacking Ade, His, Leu, and Trp (SD-4) plus X-a-Gal to test protein interactions (left panel). The empty pGAD7 vector was cotransformed with MED25 and its derivatives in parallel as negative controls (right panel).
mutation of MED25 affects plant response to ABA. In our ABA response assays (Bu et al., 2009; Li et al., 2011), the tested three mutant alleles of MED25, including med25-4, pft1-1, and pft1-2, were more sensitive than the wild type to the inhibition effect of ABA in seed germination and early seedling growth (Figures 5A to 5C). Consistent with this, ABA-induced expression levels of Em1, Em6, and RAB-RELATED GENE18 (RAB18; Läng and Palva, 1992), three ABA-responsive marker genes in Arabidopsis (Lopez-Molina et al., 2002), were highly increased in med25-4 compared with those in the wild type (Figure 5D). These results support that MED25 plays a negative role in regulating ABA responses during seed germination and early seedling growth.

**Mutation of MED25 Affects the Protein Abundance of the ABI5 Transcription Factor**

It has been shown that the bZIP transcription factor ABI5 plays a key role in ABA-triggered processes during seed germination and early seedling growth (Lopez-Molina et al., 2001). To examine the genetic interaction between MED25 and ABI5, we generated a double mutant line between med25-4 and the abi5-7 mutant, which has been shown to be insensitive to ABA (Figures 5E and 5F) (Bu et al., 2009). Surprisingly, the med25-4 abi5-7 double mutant exhibits an ABA-insensitive phenotype similar to that of abi5-7, indicating that the abi5-7 mutation suppressed the ABA-hypersensitive phenotype of med25-4 (Figures 5E and 5F). These genetic data support that the negative effect of MED25 on ABA signaling requires the function of the transcription factor ABI5.

We then examined the possible effect of MED25 on the expression of ABI5 at both transcription and protein levels. Our qRT-PCR assays revealed that, in germinating seeds (Figure 5G) and 10-d-old seedlings (Figure 5H), the ABA-induced mRNA levels of ABI5 showed slight but statistically significant decreases in med25-4 compared with those in the wild type, indicating that MED25 plays a positive role in the ABA-triggered increase of ABI5 transcripts. These results exclude the possibility that the ABA hypersensitive phenotype of med25-4 is caused by elevated ABI5 transcripts. To examine the MED25 effect on ABI5 expression at the protein level, we crossed the previously described 35Spro:ABI5-4myc (Bu et al., 2009) plants with med25-4 and generated 35Spro:ABI5-4myc/med25-4 plants. In line with our previous observations (Bu et al., 2009), 35Spro:ABI5-4myc plants contain a functional ABI5-4myc construct (Bu et al., 2009) and therefore exhibit ABA-hypersensitive phenotype (see Supplemental Figure 5 online). It is noteworthy that the ABA hypersensitivity of 35Spro:ABI5-4myc/med25-4 plants was increased compared with 35Spro:ABI5-4myc plants (see Supplemental Figure 5 online).

In our protein gel blot assays, an anti-myc antibody against protein extracts from these seedlings could detect two bands (Figures 5I and 5K), suggesting that the ABI5-4myc fusion protein could exist as different isoforms in these transgenic plants. These results are consistent with previous observations showing that the ABI5 protein mainly accumulates as two isoforms (Lopez-Molina, et al., 2001, 2003; Stone et al., 2006). As shown in Figures 5I and 5J, ABA treatment substantially increased the ABI5-4myc fusion protein levels of 35Spro:ABI5-4myc plants. In the med25-4 background, the ABI5-4myc fusion protein levels were already high in the absence of ABA, and no obvious induction was observed in response to ABA treatment (Figures 5I and 5J). These results indicate that MED25 negatively regulates the protein abundance of ABI5. Therefore, the ABA hypersensitivity of med25-4 coincides with elevated ABI5 protein levels in this mutant. These data, together with our double

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**Figure 4.** (continued).

MED25regulates the protein abundance of ABI5. The ABI5 protein mainly accumulates as two isoforms (Lopez-Molina, et al., 2001, 2003; Stone et al., 2006). As shown in Figures 5I and 5J, ABA treatment substantially increased the ABI5-4myc fusion protein levels of 35Spro:ABI5-4myc plants. In the med25-4 background, the ABI5-4myc fusion protein levels were already high in the absence of ABA, and no obvious induction was observed in response to ABA treatment (Figures 5I and 5J). These results indicate that MED25 negatively regulates the protein abundance of ABI5. Therefore, the ABA hypersensitivity of med25-4 coincides with elevated ABI5 protein levels in this mutant. These data, together with our double
Figure 5. MED25 Negatively Regulates ABA Responses during Seed Germination and Early Seedling Growth.

(A) Quantification of seed germination. Seed germination percentage of the indicated genotypes grown on different concentrations of ABA was recorded at 3 d after the end of stratification. Data shown are mean ± SD of three replicates. At least 100 seeds per genotype were measured in each replicate.

(B) Quantification of cotyledon greening. Cotyledon-greening percentage of the indicated genotypes grown on medium containing 0.5 μM ABA was recorded at 5 d after the end of stratification. Data shown are mean ± SD of three replicates. At least 100 seeds per genotype were measured in each replicate. Cotyledon greening variance on ABA medium was analyzed by Fisher’s LSD mean separation test (SPSS). Samples with the different letters are significantly different at P < 0.01.

(C) Photographs showing the ABA hypersensitivity of med25 alleles in early seedling growth. Growth conditions are the same as (B), and photos were taken 7 d after stratification. Cotyledons of Col-0 all turned green at that stage.

(D) qRT-PCR analyses of ABA-responsive genes. Seeds were kept in darkness at 4°C for 3 d without ABA and then transferred to 10 μM ABA plates for 3 d. Germinating seeds were collected for RNA extraction using the RNAqueous kit (Ambion) for small-scale RNA isolation. Data shown are mean values of three biological repeats with SD. Control indicates the time immediately following transfer. Numbers on the white bars represent fold induction values in ABA-treated plants relative to control (black bars) plants.

(E) Quantification of seed germination in the med25-4 abi5-7 double mutant. Seed germination percentage of the indicated genotypes grown on different concentrations of ABA was recorded at 3 d after the end of stratification. Data shown are mean ± SD of three replicates. At least 100 seeds per genotype were measured in each replicate.

(F) Quantification of cotyledon greening in the med25-4 abi5-7 double mutant. Cotyledon greening percentage of the indicated genotypes grown on medium containing 0.5 μM ABA was recorded at 5 d after the end of stratification. Data shown are mean ± SD of three replicates. At least 100 seeds per genotype were measured in each replicate. Cotyledon greening variance on ABA medium was analyzed by Fisher’s LSD mean separation test (SPSS). Samples with the different letters are significantly different at P < 0.01.
mutant analysis between med25-4 and the abi5-7, support a hypothesis that the ABA hypersensitive phenotype of med25-4 is caused by the elevated ABI5 protein abundance.

We then asked whether 26S proteasome-dependent proteolysis is involved in the above-described MED25-mediated modulation of ABI5-myc protein abundance. Consistent with a previous report that the ABI5 protein is subject to 26S proteasome-mediated protein degradation (Lopez-Molina et al., 2001), we found that MG132 treatment led to an obvious increase of the ABI5-myc fusion protein in 35Spro:ABI5-4myc plants (Figures 5K and 5L). In 35Spro:ABI5-4myc/med25-4 plants, the ABI5-myc fusion protein levels were already high and only a mild increase was observed upon MG132 treatment (Figures 5K and 5L). These results support the fact that MED25-mediated increase of ABI5 protein abundance possibly involves the 26S proteasome-mediated proteolysis system.

**ABA Affects the Recruitment of ABI5 and MED25 to Promoters of ABI5 Targets**

We then used ChIP assays to examine whether MED25 is recruited to promoters of ABI5 target genes during ABA signaling. As shown in Figure 6A, the promoter region of Em6 contains a G-box type abscisic acid–responsive element (ABRE), which has been shown to be targeted by ABI5 (Carles et al., 2002). Time-course ChIP assays using 35Spro:ABI5-4myc plants revealed that, in the absence of ABA, a low amount of ABI5 bound to the chromatin region covering the G-box type ABRE of the Em6 promoter (Figure 6B). Binding of ABI5 to Em6 promoter was clearly enhanced 1 h after ABA treatment, and this ABA-induced enhancement showed a tendency to increase during the time course investigated (Figure 6B). In a parallel time-course ChIP experiment, we use the 35Spro:MED25-4myc plants to investigate the ABA dependency of MED25 recruitment to the same region of Em6 promoter. Significantly, we found that, in contrast with ABI5, MED25 was highly enriched in the Em6 promoter in the absence of ABA; in response to ABA treatment, MED25 recruitment to the Em6 promoter exhibited a trend of decrease for the duration of the experiment (Figure 6C).

**Mapping of the Domains Involved in the MED25/ABI5 Interaction**

BiFC assays revealed that full-length MED25 interacts with full-length ABI5 in Nicotiana benthamiana leaves (Figure 6D). Physical interaction between MED25 and ABI5 was confirmed by Co-IP experiments using 35Spro:ABI5-4myc plants and anti-MED25 antibody. As shown in Figure 6E, MED25 could be precipitated by ABI5 in the absence of exogenous ABA, suggesting that MED25 interacts with ABI5 at steady state. Significantly, Co-IP assays also revealed that, 3 h after ABA treatment, the precipitation efficiency of MED25 by ABI5 was substantially reduced, suggesting that ABA attenuates the interaction between ABI5 and MED25. These results are consistent with the above-described time-course ChIP data showing that the MED25 enrichment to the ABI5 targets was reduced by ABA (Figure 6C). We then examined the domains involved in MED25 and ABI5 interaction using yeast two-hybrid assays. As shown in Figure 6F, MED25 interacts with ABI5 in yeast, and the ACID domain of MED25 is sufficient for this interaction. It is noteworthy that even though the ACID domain itself is not sufficient for MED25 interaction with MYC2, this domain is sufficient for MED25 interaction with ABI5, suggesting that the action mechanisms of MED25 in regulating JA and ABA signaling are different. To map the MED25-interacting domain of ABI5, we generated several ABI5 derivatives based on previous functional analyses of the ABI5 protein (Bensmihen et al., 2002; Lopez-Molina et al., 2003). Interestingly, we found that the ABI5 C-terminal part, but not its N-terminal part containing the TAD (ABI51223) (Nakamura et al., 2001), showed interaction with MED25 (Figure 6G). We further delimited the MED25 interaction domain of ABI5 to amino acids 221 to 349 (ABI5221-349) (Figure 6G).

**DISCUSSION**

Upon perception by plant cells, phytohormones trigger genomewide transcriptional reprogramming. Regulated gene expression therefore plays a central role in hormone signaling. While much effort in the field of hormone signaling is devoted to gene-specific transcription factors, we know relatively little about the function of...
Figure 6. MED25 Associates with ABI5 in Promoter of ABI5 Target Genes.

(A) Schematic diagram of the promoter region of Em6. Black line represents the promoter region of the gene. Black box on the line indicates the putative ABI5 binding G-box type ABRE cis-element of Em6 promoter (Carles et al., 2002). Region between the two coupled arrowheads (red line) indicates the DNA fragments used for ChIP-PCR. The translational start sites (ATG) are shown as +1. Bar = 200 bp.

(B) and (C) Time-course ChIP assays showing the dynamic recruitment of ABI5 (B) and MED25 (C) to Em6 promoter. The 35Spro:ABI5-4myc and 35Spro:MED25-4myc transgenic seedlings were used in ChIP using anti-myc antibody (Millipore). 35Spro:ABI5-4myc or 35Spro:MED25-4myc seedlings were treated with 100 µM ABA for varying lengths of time (0, 1, 3, and 6 h) before cross-linking. The “No Ab” (no antibody) immunoprecipitates served as negative controls. The ChIP signal was quantified as the percentage of total input DNA by real-time PCR. Three biological replicates were performed and identical results were obtained. Standard deviations were calculated from three technical repeats.
protein complexes that interact with the RNA polymerase during gene transcription, such as the Mediator complex. The Mediator complex has only recently been isolated from plant cells, and diverse functions, including development (Atran et al., 2002; Wang and Chen, 2004; Gillmor et al., 2010; Ito et al., 2011; Xu and Li, 2011), stress responses (Cerdán and Chory, 2003; Kidd et al., 2009; Elfving et al., 2011), and non-coding RNA production (Kim et al., 2011), have been attributed to plant Mediator subunits. In this study, we provide evidence showing that MED25, a multifunctional subunit of the Arabidopsis Mediator complex, differentially regulates JA and ABA signaling through distinct interaction mechanisms with hormone-specific transcription factors.

**MED25 Plays a Positive Role in MYC2-Regulated Expression of JA-Responsive Genes**

MED25 was originally identified as PHYTOCHROME AND FLOWERING TIME1 (PFT1), which plays an important role in the regulation of flowering time in response to light quality (Cerdán and Chory, 2003). Biochemical purification of the Arabidopsis Mediator revealed that PFT1 is homologous to the MED25 subunit of the metazoan Mediator complex (Bäckström et al., 2007). Later it was reported that MED25 is also involved in plant defense responses to a variety of biotic (Kidd et al., 2009) and abiotic (Elfving et al., 2011) stresses. More recently, MED25 was shown to be important for the control of final organ size (Xu and Li, 2011). These studies suggest that, like its animal counterparts (reviewed in Borggrefe and Yue, 2011), the Arabidopsis MED25 subunit plays multiple roles in plant development, hormone signaling, and stress responses. Notably, however, the molecular mechanisms underlying the diverse functions of MED25 remain largely unknown.

Our investigation of the MED25 function in JA signaling is focused on its interplay with MYC2, a master transcription factor regulating diverse aspects of JA-responsive gene expression. In line with a recent report that MED25 is important for JA-induced defense responses to pathogen infection (Kidd et al., 2009), we provide several lines of evidence showing that MED25 facilitates the action of MYC2 in regulating JA-induced defense gene expression. First, MED25 positively influences the function of MYC2 in regulating the expression of both wound- and pathogen-responsive genes. Second, double mutant analyses reveal that MED25 acts genetically downstream from MYC2 in regulating the expression of JA-responsive genes. Third, MED25 physically interacts with the putative TAD of MYC2. Fourth, MED25 and MYC2 are recruited to promoter regions of MYC2 target genes at roughly the same time after JA treatment. The above-described action mode of Arabidopsis MED25 in regulating MYC2-mediated transcription shows high similarity to that of its human counterpart in regulating RAR-mediated transcription. It has been well established that in the presence of RA, the RAR ligand, human MED25 is recruited to promoter regions of RAR targets and, through direct interaction with RAR, imposes a positive effect on RAR transcriptional activation (Lee et al., 2007). Together, these data support that MED25 might act as a coactivator of MYC2 in regulating JA-induced defense gene expression. Significantly, we provide evidence showing that, in response to JA, a Pol II subunit is recruited to the promoter of MYC targets in a MED25-dependent manner. In addition, we show that, the TPL corepressor, which is involved in JAZ-mediated repression of MYC2 function (Pauwels et al., 2010), is associated with MED25 in the promoter of MYC2 targets. These results together support that MED25 acts as part of the general transcriptional machinery in regulating JA-triggered gene expression. It will be interesting in future studies to elucidate the molecular details of how MED25 coordinates the actions of numerous coactivators and corepressors and therefore facilitates MYC2-dependent regulation of JA-responsive genes. It was shown that the transcription factor ETHYLENE INSENSITIVE3 (EIN3) and its closest homolog EIN3-LIKE1 (EIL1) integrate ethylene and JA signaling in defense gene expression (Zhu et al., 2011). This study demonstrates that, in the absence of JA, EIN3/EIL1 are repressed by JAZs, which recruit HDA6 (for histone deacetylase6) as a corepressor. JA enhances the transcriptional activity of EIN3/EIL1 by removal of JAZ proteins to regulate gene expression (Zhu et al., 2011). It is of significance to examine the possible association of MED25 with EIN3/EIL1, JAZs, and HDA6 in the promoter of EIN3/EIL1 target genes.

Notably, a recent study found that MED25 also interacts with several other JA-responsive transcription factors, including ETHYLENE RESPONSE FACTOR1 (ERF1) (Ou et al., 2011), suggesting that MED25 integrates multiple signals from different JA-responsive transcription factors during JA-induced gene expression. In the context that ERF1 itself is a direct target of MYC2 and that the negative regulation of PDF1.2 by MYC2 is...
mediated by suppression of ERF1, which in turn directly binds to the PDF1.2 promoter (Dombret et al., 2007; Zarei et al., 2011), it is reasonable to speculate that, upon JA induction, MED25 is recruited in a sequential manner to targets of MYC2 and to targets of a spectrum of intermediate JA-responsive transcription factors. In line with this hypothesis, our time-course ChIP assays indicated that MED25 accumulation in MYC2 direct targets showed an obvious reduction at 60 min after JA treatment. We are currently aiming to understand the molecular mechanisms underlying the dynamic recruitment of MED25 to targets of different JA-responsive transcription factors.

**MED25 Plays a Negative Role in ABI5-Regulated Expression of ABA-Responsive Genes**

Our investigation of the MED25 function in ABA signaling is focused on its genetic and physical interaction with the bZIP transcription factor ABI5, an important regulator of the ABA signaling during seed germination and early seedling growth. Surprisingly, we found that the MED25 effect on ABA signaling distinguishes its effect on JA signaling in several aspects. First, in contrast with a positive effect on MYC2-dependent JA responses, MED25 negatively regulates ABI5-dependent ABA responses. Second, our double mutant analyses reveal that, whereas MED25 acts genetically downstream of MYC2 in JA signaling, this Mediator subunit acts genetically upstream of ABI5 during ABA-mediated seed germination and early seedling development. Third, whereas the putative TAD of MYC2 is involved in MED25/MYC2 interaction, the TAD of ABI5 is not involved in MED25/ABI5 interaction. Fourth, time-course ChIP assays indicate that the basal recruitment of MED25 to MYC2 target promoters is relatively low, and JA treatment substantially stimulates the binding of MED25 to chromatin of MYC2 targets. By contrast, MED25 was highly enriched in ABI5 target promoters at steady state and ABA treatment led to reduction of this enrichment. These results together suggest that, as a coregulator of transcription factors, MED25 employs distinct mechanisms to affect the function of ABI5 and MYC2.

Considering that MED25 imposes an overall negative effect on ABI5-dependent ABA responses, our investigation of the recruitment dynamics of ABI5 and MED25 to the Em6 promoter provides clues to understand how the ABI5–MED25 interaction coordinates ABI5-directed gene expression. In the absence of ABA, ABI5 accumulation at its target promoter is low, whereas MED25 accumulation at the same promoter region is high, which keeps ABI5-regulated gene expression at low levels. ABA treatment stimulates the recruitment of ABI5 to its target promoter and, at the same time, leads to reduced recruitment of MED25 to the same promoter region. Increased ABI5 and reduced MED25 both favor ABI5-regulated gene expression at high levels.

Interestingly, our study reveals that the mode of action of MED25 on ABI5 is complex. On one hand, MED25 positively regulates ABA-induced ABI5 expression at the transcription level. On the other hand, MED25 negatively regulates ABI5 expression at the protein level. In addition, we could not exclude the possibility that MED25 interacts with ABI5 and, therefore, negatively affects ABI5-dependent induction of its target genes. This latter effect could be achieved through MED25-dependent recruitment of transcription cofactors to the promoter region of ABI5 targets. It has been shown that during RA signaling in human cells, CREB binding protein/p300, a histone acetyltransferase, was recruited in a MED25-dependent manner to the promoter region of RAR targets and therefore cooperates in RAR activation (Lee et al., 2007).

An interesting aspect of the MED25 function in ABA responses concerns its effect on the protein accumulation and stability of ABI5, which has been considered as a checkpoint of ABA-mediated arrest of seed germination and early seedling growth (Lopez-Molina et al., 2001; Fujita et al., 2011). In the context that the Arabidopsis Mediator complex contains a detachable kinase module, which is composed of the cyclin-dependent kinase Cdk8, Cyclin C, and two additional subunits, MED12 and MED13 (Bäckström et al., 2007; Kidd et al., 2011; Kim and Chen, 2011), it is reasonable to speculate that MED25, or other relevant Mediator subunits, regulates the phosphorylation and/or protein degradation of ABI5. It will be interesting in future studies to elucidate the molecular mechanisms concerning MED25-mediated regulation of ABI5 protein modification and/or degradation.

**MED25 Is an Interaction Node between JA and ABA Signaling Pathways**

JA and ABA are phytohormones involved in plant responses to biotic and abiotic stresses, and it is increasingly evident that significant interactions occur between these pathways. Our finding that MED25 positively regulates JA signaling whereas it negatively regulates ABA signaling highlights the existence of an antagonistic interaction between JA and ABA in regulating seed germination. In line with this, early studies indicate that several of the JA-insensitive mutants, including jar1, ja insensitive4, and coi1-16, showed increased sensitivity to low concentrations of ABA in seed germination, suggesting that JA antagonizes ABA in regulating the onset of seed germination (Staswick et al., 1992; Berger et al., 1996; Ellis and Turner, 2002). This long-observed antagonistic interaction between JA and ABA could be employed to identify molecular components of ABA signaling in seeds. For example, several ABA-related mutants named coi1-16 resistant to ABA have been recovered based on their ability to suppress the hypersensitive phenotype of coi1-16 to low concentrations of ABA in seed germination (Fernández-Arbaizar et al., 2012). Characterization of these mutants promises to shed new light on the molecular mechanisms governing JA and ABA interactions in regulating seed germination and early seedling growth.

Intriguingly, however, JA has also been shown to have a synergistic effect with ABA in regulating seed germination. For example, both hormones induce the expression of MYC2, which plays a positive role in both JA- and ABA-mediated inhibition of seed germination (Abe et al., 2003; Lorenzo et al., 2004). Considering that the induction of MYC2 by ABA depends on the JA receptor COI1, it is likely that ABA activates the expression of MYC2 by the JA signaling pathway (Lorenzo et al., 2004).

Previous studies have also observed both antagonistic and cooperative interactions between JA and ABA in regulating plant
resistance to different pathogens. For example, it was shown that ABA antagonizes JA-induced expression of defense-related genes and that Arabidopsis mutants defective in ABA biosynthesis or signaling show increased resistance to the fungal pathogen Fusarium oxysporum (Anderson et al., 2004), suggesting a negative role of ABA in plant resistance to F. oxysporum. By contrast, it was shown that ABA activates JA biosynthesis and therefore plays a positive role in resistance of Arabidopsis plants to the oomycete pathogen Pythium irregulare (Adie et al., 2007). The differential role of ABA within different plant–pathogen interactions implies that this hormone could be important for the fine-tuning of plant resistance to a particular pathogen.

METHODS

Plant Materials and Growth Conditions

Arabidopsis thaliana ecotype Col-0 was used as the wild type. Some of the plant materials used in this study were previously described: ber6/med25-4 (Zheng et al., 2006); med25-1, med25-2, and med25-3 (Xu and Li, 2011); pft1-1 (Cerdán and Chory, 2003); pft1-2 (Kidd et al., 2009); myc2-2 (Boter et al., 2004); cof1-2 (Xu et al., 2002); ab15-7 (Nambara et al., 2002; Tamura et al., 2006); VSP1pro::GUS and PDF1.2pro::LUC (Zheng et al., 2006); 35Spro::MYC2-4myc (Chen et al., 2011); 35Spro::ABI5-4myc (Bu et al., 2009); and 35Spro::MYC2-GFP (Szemenyi et al., 2008). The med25-4 myc2-2 and med25-4 ab15-7 double mutants were generated by crossing parental single homozygous lines. The resulting F2 segregating progenies were genotyped to identify plants homozygous for each locus. The med25-4 and myc2-2 (Salk_083483) mutants were identified with PCR-based markers (see Supplemental Figure 1 online). Identification of the ab15-7 mutation was previously described (Bu et al., 2009).

Arabidopsis plants were grown in Murashige and Skoog (MS) medium at 22°C with a 16-h-light/8-h-dark photoperiod (light intensity 120 μM photons m⁻² s⁻¹) as previously described (Sun et al., 2009). JA-mediated root growth inhibition assays were described (Chen et al., 2011). For Arabidopsis responses, seeds harvested at the same time were used for the germination and cotyledon greening assays as recently described (Bu et al., 2009; Li et al., 2011). Nicotiana benthamiana was grown under a 16-h-light (28°C)/8-h-dark (22°C) photoperiod.

Map-Based Cloning of BER6

The ber6/med25-4 (Col-0) mutant was crossed to Landsberg erecta. In the resulting F2 population, individuals showing the ber6 phenotype (i.e., insensitivity to JA-induced root growth inhibition) were identified and used for mapping. Rough mapping using 30 plants indicated that the target gene is linked to the marker ciw12 on chromosome 1. New markers (see Supplemental Table 1 online) in this region were designed according to the Monsanto Arabidopsis polymorphism database (http://www.Arabidopsis.org/browse/Cereon/). Using these markers, we analyzed 1800 plants showing the mutant phenotype and delimited the target gene to a 50-kb region covered by the BAC clone F2J7 (Figure 1H). Among the 15 genes in this region, sequencing analyses revealed a G-to-A mutation in the MED25 gene (At1g25540).

For complementation analysis, the 35Spro::MED25-GFP construct was introduced into the ber6 plants using Agrobacterium tumefaciens-mediated transformation.

For the allelic test, the ber6/med25-4 mutant was crossed to the previously characterized pft1-2 mutant (Kidd et al., 2009), and the resulting F1 plants were examined.

Measurement of Flowering Time

Measurements of flowering time were performed as previously described (Robson et al., 2010). Flowering time was recorded from at least 20 plants per genotype that were grown in soil under either long days (16 h white light/8 h dark) or short days (10 h white light/14 h dark). Flowering time was scored as the number of days from germination to the first appearance of buds at the apex. The total number of rosette leaves was counted after the main stem has bolted 1 cm.

DNA Constructs and Plant Transformation

DNA constructs for plant transformation were generated following standard molecular biology protocols and Gateway (Invitrogen) technology. Full-length coding sequence of MED25 was amplified with Gateway-compatible primers. The PCR product was cloned by pENTR Directional TOPO cloning kits (Invitrogen) and then recombined with the binary vector pGBWB5 (35S promoter, C-GFP) to generate the 35Spro::MED25-GFP construct. Full-length coding sequence of MED25 was also cloned into the pGWB17 vector (35S promoter, C-4myc) to generate the 35Spro::MED25-4myc construct. Similarly, we generated the 35Spro::MYC2-GFP construct. All primers used for DNA construct generation are listed in Supplemental Table 2 online.

The above constructs were then transformed into Agrobacterium strain GV3101 (pMP90), which was used for transformation of Arabidopsis plants via a floral dip method. Transforms were selected based on their resistance to hygromycin. Homozygous T3 or T4 transgenic seedlings were used for phenotype and molecular characterization.

Co-IP Assays

Co-IP assays were performed according to published procedure (Spöel et al., 2009) with minor modifications. In brief, 10-d-old 35Spro::MYC2-4myc seedlings were homogenized in protein lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Triton X-100, 0.2% Nonidet P-40, 0.6 mM PMSF, and 20 μM MG132 with Roche protease inhibitor cocktail). After protein extraction, 20 μL protein G plus agarose (Santa Cruz) was added.
to the 2-mg extracts to reduce nonspecific immunoglobulin binding. After 1 h of incubation, the supernatant was transferred to a new tube. To test the effect of JA on the interaction of MED25 with MYC2, the supernatant was incubated with or without 100 μM methyl jasmonate (MeJA) (Sigma-Aldrich) for 30 min at 4°C before immunoprecipitation of the protein complexes. Myc antibody-bound agarose beads (Santa Cruz) were then incubated with or without (±) ABA (Sigma-Aldrich) at 10 μM for 3 h at 4°C before immunoprecipitation of the protein complexes. To test the interaction of MED25 with TPL in plants, 6-d-old 3SSpro::TPL-GFP plants were used in Co-IP assays, and 3 mg of protein extracts were precleared with the protein G plus agarose beads and incubated with GFP antibody (Abcam) and the protein G plus agarose beads at 4°C for 4 h.

**BIFC Assays**

Full-length coding sequences of MED25, MYC2, ABI5, and derivatives of MED25 were cloned into the binary N-terminal fragment of YFP or the C-terminal fragment of YFP vector through Gateway reaction with pENTR vector system (Invitrogen) and sequence verified. Primers for the construction are listed in Supplemental Table 2 online. The resulting constructs were then introduced into Agrobacterium strain GV3101. N. benthamiana infiltration was performed as described (Song et al., 2011). After infiltration, plants were incubated for at least 50 h before observation. The YFP fluorescence was imaged under a Leica confocal laser scanning microscope (Leica Microsystems). Leaves were infiltrated with 2 μg/mL 4,6-diamidino-2-phenylindole for nuclei staining 2 h before observation.

**ChIP-PCR Assays**

ChIP assays were performed following a published protocol (Gendrel et al., 2005) with minor modifications. Briefly, 1.5 g of 3SSpro::MED25-GFP, 3SSpro::MED25-4myc, 3SSpro::MYC2-GFP, 3SSpro::ABI5-4myc, 3SSpro::TPL-GFP, Col-0, or med25-4 seedlings were cross-linked in 1% formaldehyde and their chromatin isolated. GFP antibody (Abcam), myc antibody (Millipore), or RPB2 antibody (catalog no. ab10338; Abcam) was used to immunoprecipitate the protein-DNA complex, and the precipitated DNA was purified using a PCR purification kit (Qiagen) for qRT-PCR analysis. The ChIP experiments were performed three times. Chromatin precipitated without antibody was used as negative control, while the isolated chromatin before precipitation was used as input control. Primers used for ChIP-PCR are listed in Supplemental Table 3 online.

**RNA Extraction and Gene Expression Analyses**

For qRT-PCR analysis of JA-responsive genes, total RNA was extracted from 10-d-old seedlings treated with MeJA as indicated (using Trizol [Invitrogen] reagent). For qRT-PCR analysis of ABA-responsive genes, seeds were germinated on 10 mM ABA for 3 d after stratification. Total RNA was extracted using the RNAqueous kit (Ambion) for small-scale RNA isolation. cDNA was prepared from 2 μg of total RNA with SuperScript III reverse transcriptase (Invitrogen) and quantified with a cycler apparatus (Roche 480) with the SYBR Green kit (Takara) according to the manufacturer’s instructions. Expression levels of target genes were normalized to ACT777, and the expression levels in Col-0 without hormone treatment were arbitrarily set to 1. The statistical significance was evaluated by Student’s t test. Primers used for qRT-PCR are listed in Supplemental Table 3 online.

**Antibody Generation**

Full-length coding sequence of MED25 was PCR amplified from reverse transcription product with gene-specific primers (see Supplemental Table 2 online). The resulting PCR product was cloned into the BamHI and SalI sites of the pMAL-c2 vector (NEB) to express MBP-MED25 protein in Escherichia coli strain BL21. The recombinant fusion protein was purified with amylose resin (NEB) and used to raise polyclonal antibodies in mouse. The antibodies were used in MED25 immunoblots at a final concentration of 1:1000.

**Immunoblot Assays**

For ABI5 protein level analysis, protein extraction was performed by homogenizing 10-d-old seedlings in extraction buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.1% Nonidet P-40, 10% glycerol, 1 μM DTT, 20 μM MG132, and Roche protease inhibitor cocktail). For immunoblot analysis, SDS sample buffer was added to the protein extracts. Protein samples were boiled for 5 min, separated on SDS-PAGE gels, and transferred to polyvinylidene fluoride membranes. Immunoblots were probed with anti-myc antibody (Abmart). Ponceau S-stained membranes are shown as loading controls.

For MYC2-4myc immunoblots, an anti-myc antibody (Abmart) was used at a final dilution of 1:2000. For TPL-GFP immunoblots, an anti-GFP antibody (Abcam) was used at a final dilution of 1:1000.

**Pst DC3000 Infection**

Inoculation of Pst strain DC3000 was performed as previously described (Liang et al., 2009). To determine bacterial growth, infected leaves were collected at 0 and 3 d after inoculation. At each time point, 10 leaves were collected from each genotype.

**Supplemental Data**

The following materials are available in the online version of this article.

**Supplemental Figure 1.** Genetic Complementation of the ber6/med25-4 Mutant.

**Supplemental Figure 2.** qRT-PCR Analyses of JA-Induced Expression of the JAZ Family Genes in Col-0 and med25-4.

**Supplemental Figure 3.** Yeast Assays Showing That the Transcriptional Activity of MED25 Can Be Suppressed by 3-AT.

**Supplemental Figure 4.** Yeast Two-Hybrid Assays with MED25, ABI5, and TPL.

**Supplemental Figure 5.** MED25 Negatively Regulates the Function of ABI5 in ABA Signaling.

**Supplemental Table 1.** DNA Primers Used for Map-Based Cloning and Diagnostic PCR.

**Supplemental Table 2.** DNA Primers Used for Construct Generation.
SUPPLEMENTAL TABLE 3. DNA Primers Used for qRT-PCR and ChIP-qPCR Assays.

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AUTHOR CONTRIBUTIONS

R.C. and H.J. designed and performed the research, analyzed the data, and wrote the article. L.L., Q.Z., L.Q., W.Z., X.L, H.L., W.Z., and J.S. performed the research and analyzed the data. C.L. supervised and wrote the article. L.L., Q.Z., L.Q., W.Z., X.L, H.L., W.Z., and J.S. designed and performed the research, analyzed the data, and wrote the article.

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The Arabidopsis Mediator Subunit MED25 Differentially Regulates Jasmonate and Abscisic Acid Signaling through Interacting with the MYC2 and ABI5 Transcription Factors
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