

# CYCLIN-DEPENDENT KINASE8 Differentially Regulates Plant Immunity to Fungal Pathogens through Kinase-Dependent and -Independent Functions in *Arabidopsis*<sup>©W</sup>

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**CYCLIN-DEPENDENT KINASE8 (CDK8) is a widely studied component of eukaryotic Mediator complexes. However, the biological and molecular functions of plant CDK8 are not well understood. Here, we provide evidence for regulatory functions of *Arabidopsis thaliana* CDK8 in defense and demonstrate its functional and molecular interactions with other Mediator and non-Mediator subunits. The *cdk8* mutant exhibits enhanced resistance to *Botrytis cinerea* but susceptibility to *Alternaria brassicicola*. The contributions of CDK8 to the transcriptional activation of defensin gene *PDF1.2* and its interaction with MEDIATOR COMPLEX SUBUNIT25 (MED25) implicate CDK8 in jasmonate-mediated defense. Moreover, CDK8 associates with the promoter of *AGMATINE COUMAROYLTRANSFERASE* to promote its transcription and regulate the biosynthesis of the defense-active secondary metabolites hydroxycinnamic acid amides. CDK8 also interacts with the transcription factor WAX INDUCER1, implying its additional role in cuticle development. In addition, overlapping functions of CDK8 with MED12 and MED13 and interactions between CDK8 and C-type cyclins suggest the conserved configuration of the plant Mediator kinase module. In summary, while CDK8's positive transcriptional regulation of target genes and its phosphorylation activities underpin its defense functions, the impaired defense responses in the mutant are masked by its altered cuticle, resulting in specific resistance to *B. cinerea*.**

## INTRODUCTION

The plant transcriptome is significantly reprogrammed in response to pathogen infection (Hahlbrock et al., 2003). The expression of genes related to hormone signaling pathways, the cell wall, cuticle modification processes, and the synthesis of defense metabolites, as well as pathogenesis-related (PR) genes, increases upon attempted pathogen infection (Dixon, 2001; Ausubel, 2005; Jones and Dangl, 2006; Boller and Felix, 2009; Fu and Dong, 2013). Precise transcriptional regulation of a battery of genes encoding diverse molecules in these plant processes determines plant resistance or susceptibility (Somssich and Hahlbrock, 1998). Plant transcriptional regulators and coregulators of plant immunity have been described (Moore et al., 2011). Recent studies link Mediator to transcriptional processes underpinning plant immunity to bacterial and fungal infection (Canet et al., 2012; Chen et al., 2012;

Wathugala et al., 2012; Zhang et al., 2012; An and Mou, 2013; Lai et al., 2014). Mediator is an evolutionarily conserved eukaryotic multiple-protein complex that recruits RNA polymerase II (RNAP II) to specific promoters by either linking activators and repressors or recruiting transcription factors (TFs) to RNAP II (Kornberg, 2005; Malik and Roeder, 2005). In yeast (*Saccharomyces cerevisiae*) and animal cells, the core Mediator is subdivided into the head, middle, and tail modules (Guglielmi et al., 2004; Chadick and Asturias, 2005) and a fourth and separable kinase module consisting of CYCLIN-DEPENDENT KINASE8 (CDK8), C-type cyclin (CycC), MEDIATOR COMPLEX SUBUNIT12 (MED12), and MED13 (Borggreffe et al., 2002; Andrau et al., 2006).

Previous studies in yeast and metazoan cells suggested that CDK8 is mainly a negative regulator of transcription. First, yeast CDK8 (Srb10) was originally identified from a genetic screen for RNAP II C-terminal domain suppressors (Nonet and Young, 1989). Second, CDK8 could inhibit transcription in vitro probably because the kinase module association with core Mediator prevented the interaction between core Mediator and RNAP II (Elmlund et al., 2006). Other kinase module subunits, including CycC, MED12, and MED13, are also required for the repressive function of the kinase module, as the loss-of-function mutants of those four subunits displayed similar phenotypes, especially in response to nutrient deprivation and heat shock in yeast (Carlson, 1997; Knuesel et al., 2009a). Increasing evidence also demonstrates that CDK8 serves as a coactivator of transcription (Nemet et al., 2014) in many pathways, including p53-dependent

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transcription (Donner et al., 2007a, 2007b; Beckerman et al., 2009), the serum response network (Donner et al., 2010), the Transforming Growth Factor  $\beta$  signaling pathway (Alarcón et al., 2009; Knuesel et al., 2009b), and thyroid hormone-dependent transcription (Belakavadi and Fondell, 2010). Moreover, the kinase activity of CDK8 seems to be essential for its biological functions. For example, CDK8 positively affected TF Gal4-dependent transcription by directly phosphorylating Gal4 at the critical site Ser-699 (Hirst et al., 1999; Rohde et al., 2000). Disruption of this phosphorylation suppressed the induction of Gal4-dependent transcription (Ansari et al., 2002). Several other CDK8 phosphorylation substrates have also been identified. Recombinant CDK8 was able to phosphorylate the C-terminal domain of RNAP II and TFIIH as well as histone H3, MED13, and some general TFs (Hengartner et al., 1998; Rickert et al., 1999; Meyer et al., 2008; Knuesel et al., 2009b), but how those phosphorylation events contribute to the biological function of CDK8 is still largely unknown.

Purification of the core Mediator from *Arabidopsis thaliana* suspension cells (Bäckström et al., 2007) demonstrated that most Mediator components are present in the *Arabidopsis* complex as in other organisms. However, the kinase module was missing, in accordance with the transient and reversible association between the kinase module and core Mediator observed in other systems (Andrau et al., 2006). A number of Mediator subunits play critical roles in plant defense and development. MED21 is the first Mediator subunit implicated in resistance to necrotrophic pathogens based on the increased susceptibility of the MED21 RNA interference lines to *Botrytis cinerea* and *Alternaria brassicicola* (Dhawan et al., 2009). Subsequently, MED8, MED16, MED15, and MED25 were also implicated in resistance to necrotrophic pathogens (Canet et al., 2012; Chen et al., 2012; Wathugala et al., 2012; Zhang et al., 2012; An and Mou, 2013). Two independent studies reported that MED16 regulates both salicylic acid (SA)-mediated systemic acquired resistance and jasmonic acid (JA)/ethylene-mediated plant defense (Wathugala et al., 2012; Zhang et al., 2012). MED25 integrates signals from biotic and abiotic stresses and also contributes to the control of flowering time, hormone signaling, stress tolerance, and plant defense responses to necrotrophic pathogens. Consistent with its multiple functions, MED25 interacts with diverse TFs, including ERF1, ORA59, MYC2, MYC3, MYBs, WRKYs, and many ERF/AP2s (Çevik et al., 2012).

The kinase module has also been suggested to function in plant development. *Arabidopsis* CDK8 (CDKE1) was first identified as the mammalian and yeast CDK8 homolog (van de Peppel et al., 2005), later named HUA ENHANCER3, and is known to regulate floral organ identity (Wang and Chen, 2004). CDK8 interacts with *Arabidopsis* LEUNIG, a transcription co-repressor, which in turn interacts with HISTONE DEACETYLASE19, a regulator of JA-dependent defense responses, as well as MED14 (Gonzalez et al., 2007). More recently, studies on *Arabidopsis regulator of alternative oxidase1*, which carries a mutation in the CDK8 gene, revealed that CDK8 regulates mitochondrial retrograde signaling in response to H<sub>2</sub>O<sub>2</sub> and cold stress (Ng et al., 2013). The other kinase module components MED12 and MED13 are required for proper embryo axis development (Gillmor et al., 2010; Ito et al., 2011). The *med13* mutant enhances auxin responses (Ito et al. 2011). In addition, the *Arabidopsis* genome contains two cyclin C-type genes, but

no clear biological function has been assigned to the two cyclins (Menges et al., 2005).

In this work, we describe the functions of the *Arabidopsis* Mediator subunit CDK8 in plant defense and its underlying molecular and biochemical mechanisms. CDK8 regulates the expression of defensin genes, including *PDF1.2* and several *ETHYLENE RESPONSE TRANSCRIPTION FACTORS (ERFs)* known for their disease resistance functions. Consistent with this observation, CDK8 is required for ERF1- and OCTADECANOID-RESPONSIVE *ARABIDOPSIS* AP2/ERF59 (ORA59)-dependent activation of *PDF1.2* expression. These data, coupled with the interaction between CDK8 and MED25, implicate CDK8 in the regulation of plant immunity through a JA-dependent pathway. CDK8 also regulates resistance to *A. brassicicola* through direct transcriptional regulation of *AGMATINE COUMAROYLTRANSFERASE (AACT1)*, which is critical for the biosynthesis of hydroxycinnamic acid amides (HCAAs), secondary metabolites known for their functions in fungal resistance. Intriguingly, despite the loss of critical defense responses, the *cdk8* mutant shows enhanced resistance to another necrotrophic pathogen, *B. cinerea*, possibly attributed to the increased cuticle permeability and altered cuticle structure in the *cdk8* mutant. These features of plant cuticles have previously been associated with enhanced resistance to *B. cinerea* (Kurdyukov et al., 2006; Bessire et al., 2007; Chassot et al., 2007). CDK8 interacts with WAX INDUCER1 (WIN1), an ERF family protein known to regulate cuticular wax biosynthesis, thus implicating CDK8 in cuticle development, which is consistent with the observed changes in cuticle structure and permeability of the *cdk8* mutant. Moreover, expression of CDK8 that is defective in phosphorylation activity failed to rescue the *A. brassicicola* susceptibility of the *cdk8* mutant, while the resistance to *B. cinerea* was restored to the wild-type level, suggesting the kinase-dependent and independent functions of CDK8. CDK8 strongly interacts with two CycCs, suggesting an evolutionarily conserved structure of the kinase module in plants. Two other kinase module mutants, *med12* and *med13*, exhibit disease responses and increased cuticle permeability similar to that of the *cdk8* mutant, underlining the common functions and structural conservation of the kinase module. Taken together, our data shed light on multiple regulatory roles of the Mediator CDK8 module in plant defense and development.

## RESULTS

### CDK8 Mediates Responses to Fungal and Bacterial Pathogens

We have previously described the functions of Mediator subunits MED21 and MED18 in plant defense responses to necrotrophic pathogens (Dhawan et al., 2009; Lai et al., 2014). In a parallel reverse genetic screen covering mutants of Mediator subunits, we determined that two homozygous mutant alleles of *CDK8* from the SALK T-DNA insertion collection were susceptible to *A. brassicicola* (Figure 1). PCR analysis confirmed that both *cdk8-1* and *cdk8-2* carry homozygous T-DNA insertions in the *CDK8* gene (Figures 1A and 1B), and quantitative RT-PCR (RT-qPCR)

revealed that the expression of *CDK8* is abolished in the two mutant lines (Figure 1C). The primer sequences used to verify the T-DNA insertion in *CDK8* and to determine gene expression are listed in Supplemental Data Set 1. When inoculated with *A. brassicicola*, both *cdk8* mutant lines exhibited symptoms of increased susceptibility to the fungus (Figure 1D). At 4 d after inoculation (DAI), leaves of wild-type plants had only very restricted disease lesions at the inoculation sites characteristic of *Arabidopsis* wild-type responses to this fungus, in contrast with *cdk8* mutant plants, which had significantly larger disease lesions surrounding the inoculation sites. Fungal growth was analyzed in inoculated plants to determine whether the enhanced disease symptoms in the mutant were accompanied by increased fungal growth. Trypan blue staining to observe fungal growth patterns clearly revealed that *A. brassicicola* proliferated significantly more quickly on leaves of the *cdk8* mutant at 48 h after inoculation, with hyphal growth extending beyond the inoculation sites (Figures 1E to 1H; Supplemental Figures 1C and 1D).

We then challenged *cdk8* and wild-type plants by drop-inoculation with a conidial suspension of *B. cinerea* as described previously (Mengiste et al., 2003). *B. cinerea* infection caused obvious disease symptoms, with larger and expanding disease lesions beyond the inoculation site in the wild-type plants at 4 DAI (Figure 1I; Supplemental Figure 1A). However, in *cdk8* mutants, disease symptoms were restricted to the site of inoculation without any noticeable spread of disease symptoms. Similarly, *B. cinerea* spray inoculation confirmed that both *cdk8* mutant lines exhibited enhanced resistance compared with the wild-type plants (Supplemental Figure 1B). The contrasting responses of the *cdk8* mutants to these two closely related necrotrophic fungi were unexpected in light of previous reports that show largely similar host response mechanisms. In contrast with the responses to *A. brassicicola*, the growth of *B. cinerea* was significantly restricted on leaves of the *cdk8* mutant compared with the wild type at 36 h after inoculation (Figures 1J to 1M; Supplemental Figure 1D). Leaves of the wild type and the *cdk8* mutant were also stained with trypan blue after spray inoculation with *B. cinerea*, which also demonstrated that the growth of *B. cinerea* was attenuated on the *cdk8* mutant (Supplemental Figure 1C). The germination of conidia was not inhibited, but later stages of fungal growth were significantly restricted.

We also investigated the responses of *cdk8* mutants to the bacterial pathogen *Pseudomonas syringae* virulent strain DC3000 and the nonpathogenic strain DC3000 hrcC, which is defective in the type III secretion system. This secretion system is used by plant pathogenic bacteria to deliver effector proteins into plant cells. Three days after inoculation with *P. syringae* DC3000, both *cdk8* mutant lines supported significantly more bacterial growth than wild-type plants, revealing the susceptibility of the *cdk8* mutant (Figure 1N). No differences were observed after inoculation with the hrcC strain. These data suggest that *CDK8* is also a positive regulator of bacterial resistance similar to its function in *A. brassicicola* resistance.

### CDK8-Dependent Arabidopsis Transcriptome Revealed by RNA Sequencing

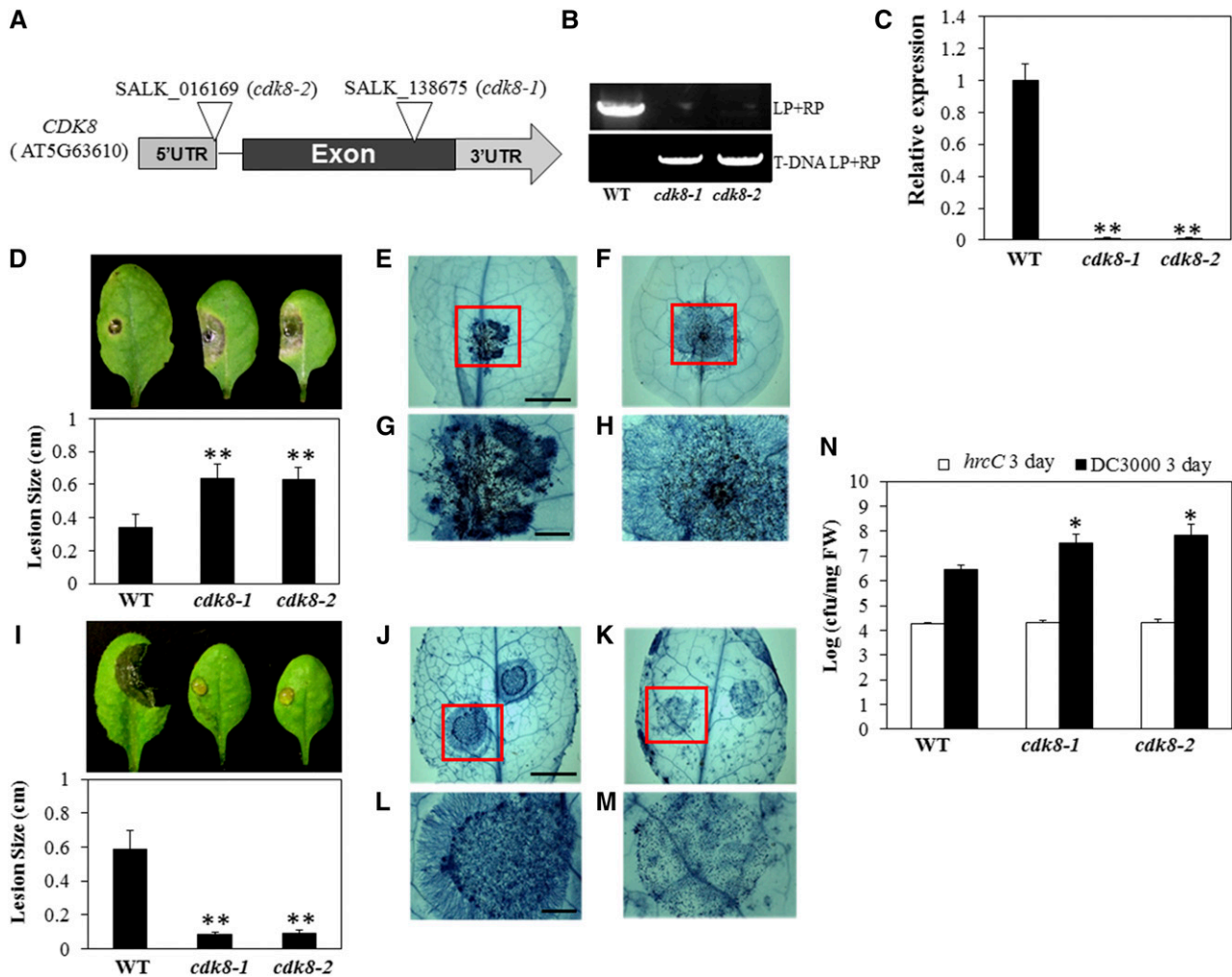
RNA sequencing (RNA-seq) analyses of mock- or *B. cinerea*-inoculated plants were conducted to further understand the

genome-wide regulatory impact of *CDK8* in pathogen-induced transcriptional changes and to identify *CDK8*-regulated target genes. *B. cinerea* and *A. brassicicola* trigger similar transcriptional changes (van Wees et al., 2003; AbuQamar et al., 2006; Lai and Mengiste, 2013); thus, we conducted the RNA-seq experiment from *B. cinerea*-inoculated samples. Reads per kilobase of exon model per million mapped reads values for each sample of the RNA-seq analysis were calculated from the count data as described recently (Lai et al., 2014). Pathogen-induced or -suppressed genes with at least 2-fold changes and false discovery rate values ( $P \leq 0.05$ ) were selected. The Venn diagram in Supplemental Figure 2A summarizes the number of genes that were differentially expressed in *CDK8*-dependent and independent manners. Moreover, genes belonging to different functional categories, including abiotic and biotic stress, transcription, lipid and secondary metabolism, hormone response, and signaling, were closely analyzed because their expression was altered in response to infection. The heat map in Supplemental Figure 2B shows differentially expressed genes in the different functional categories. Compared with wild-type plants at 36 h after *B. cinerea* infection, a number of genes encoding TFs, including *ERF/AP2*, *MYB*, *WRKY*, and *bHLH* family genes, were differentially expressed in *cdk8* in response to infection (Supplemental Data Set 2), implying that *CDK8* is a critical regulator with broader impact on gene regulation. In addition, we found that the expression of defense marker *PDF* genes was significantly reduced in the *cdk8* mutant, which is consistent with the observed susceptibility to *A. brassicicola* but does not correlate with the resistance to *B. cinerea*.

A number of studies have shown that the necrotrophic pathogens *B. cinerea* and *A. brassicicola* trigger similar defense responses (Laluk and Mengiste, 2011; Lai and Mengiste, 2013; Lai et al., 2014). Therefore, we investigated the expression of several defense genes in wild-type and *cdk8* plants after inoculation with the two necrotrophic pathogens to link the disease response phenotype with changes in gene expression. Analysis of RT-qPCR data confirmed the *CDK8*-dependent expression of *ERF1*, *ORA59*, and *ERF2*, which was significantly suppressed in the *cdk8* mutant (Figure 2A). The expression of plant defensin genes *PDF1.2*, *PDF1.2b*, and *PDF1.3*, which are also *ERF* target genes, was also significantly reduced in *cdk8* (Figure 2B). Similarly, the expression of *ORA59*, *PDF1.2*, and *PDF1.3* was compromised in the *cdk8* mutant in response to *A. brassicicola*, despite the contrasting phenotypes (Supplemental Figure 3). Thus, the differences in the responses of *cdk8* to *B. cinerea* and *A. brassicicola* are not linked to the pathogen-induced expression of these defense genes.

### CDK8 and MED25 Are Required for TF-Dependent Activation of PDF1.2

From an analysis of gene expression data, we observed a clear reduction of *PDF1.2* expression in the *cdk8* mutant. After methyl jasmonate (MeJA) treatment, *PDF1.2* was significantly induced in wild-type plants but remained at extremely low levels in *cdk8*, similar to the response of *med25* mutants (Figure 3A; Kidd et al., 2009). These data suggest that both *CDK8* and *MED25* are required for the induced expression of *PDF1.2*. The TFs *ERF1* and

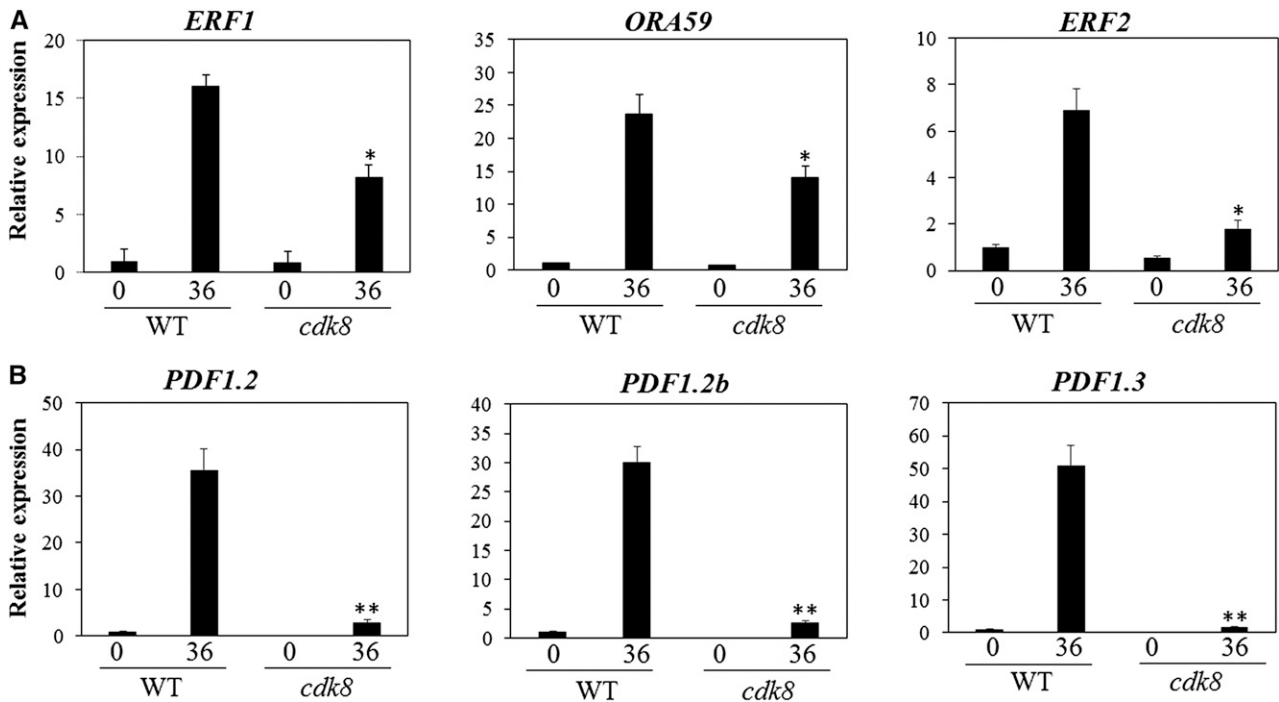


**Figure 1.** The *cdk8* Mutant Displays Increased Susceptibility to *A. brassicicola* and *P. syringae* but Resistance to *B. cinerea*.

(A) Genomic structure of the CDK8 gene and positions of the T-DNA insertion in *cdk8* mutant alleles. UTR, untranslated region. (B) Verification of *cdk8* T-DNA insertion mutants by genomic PCR. LP, T-DNA left border genomic primer; RP, T-DNA right border primer. (C) RT-qPCR data showing loss of CDK8 expression in *cdk8* mutants. Relative transcript levels were normalized with *Arabidopsis* ACT2. The normalized expression level of the wild type was set to 1. Error bars indicate  $\pm$  SE ( $n = 3$ ). Two independent biological replicates were performed. Significance between the mean values was analyzed statistically (Student's *t* test, \*\* $P < 0.01$ ). (D) *A. brassicicola* disease symptoms (top panel) and disease lesion size (bottom panel) in the wild type and the *cdk8* mutant at 4 DAI. (E) and (F) Trypan blue staining of *A. brassicicola*-inoculated leaves of the wild type (E) and the *cdk8* mutant (F) 48 h after inoculation. Bar = 4 mm. (G) and (H) Closeups of the disease lesion areas from (E) and (F). Bar = 1 mm. (I) *B. cinerea* disease symptoms (top panel) and disease lesion size (bottom panel) in the wild type and the *cdk8* mutant at 4 DAI. (J) and (K) Trypan blue staining of the wild type (J) and the *cdk8* mutant (K) 36 h after inoculation with *B. cinerea*. Bar = 4 mm. (L) and (M) Closeups of the disease lesion areas from (J) and (K). Bar = 1 mm. (N) Bacterial growth in wild-type and *cdk8* mutant plants. Leaves of 5-week-old plants were infiltrated with bacterial suspension ( $OD_{600} = 0.0005$ ). At 3 DAI, leaf discs were collected and bacterial growth was quantified and expressed in colony-forming units (cfu). FW, fresh weight. The disease assay was performed by drop-inoculation of *B. cinerea* on leaves of soil-grown plants or *A. brassicicola* on detached leaves. The average lesion sizes are mean values  $\pm$  SE from four independent replicates ( $n = 40$ ). A minimum of 10 leaves for each genotype were used for each biological replicate, and the disease assay was repeated at least four times with similar results. All of the data were statistically analyzed. Asterisks indicate significant differences (Student's *t* test, \* $P < 0.05$ , \*\* $P < 0.01$ ).

ORA59 activate *PDF1.2* expression (Lorenzo et al., 2003; Pré et al., 2008). To determine the role of CDK8 in *PDF1.2* gene expression, we performed transactivation assays. The activation of *PDF1.2* by ERF1 and ORA59 requires functional MED25 (Çevik et al., 2012). To test whether CDK8 is required for the coactivation

of *PDF1.2*, we coexpressed a *PDF1.2* promoter- $\beta$ -glucuronidase (GUS) reporter with effector construct 35S:ERF1/ORA59-MYC or the empty vector in *Arabidopsis* protoplasts with cauliflower mosaic virus 35S-driven, full-length luciferase (LUC) coexpressed as an internal control. When *PDF1.2* pro-GUS and the empty



**Figure 2.** CDK8-Dependent Expression of *ERF* and Plant Defensin Genes.

**(A)** Induced expression of genes encoding the TFs *ERF1*, *ORA59*, and *ERF2* is reduced in the *cdk8* mutant.

**(B)** Induced expression of defensin genes is dependent on CDK8.

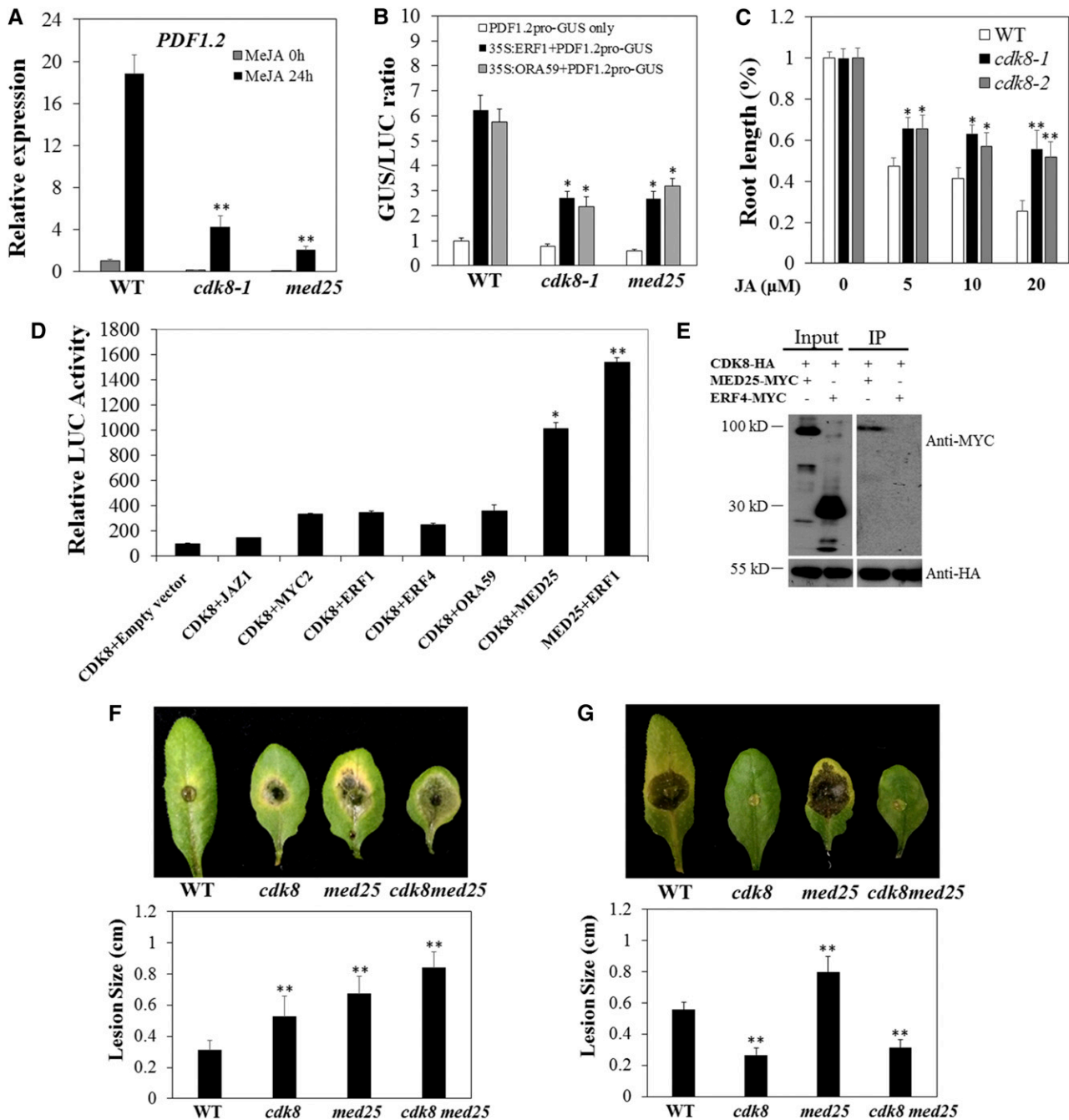
Total RNAs were extracted from 5-week-old plants grown in soil before and after *B. cinerea* inoculation. Relative transcript levels were normalized with *Arabidopsis ACT2*. The normalized expression level of the wild type at 0 h was set to 1. Error bars indicate  $\pm$  SE ( $n = 3$ ). Three independent biological replicates were performed. Significance of differences between the mean values was analyzed statistically (Student's *t* test, \* $P < 0.05$ , \*\* $P < 0.01$ ).

vector alone were coexpressed, the GUS activities from the *PDF1.2* promoter were similar in the wild type and the *cdk8* and *med25* mutants (Figure 3B). On the other hand, coexpression of the effector plasmids 35S:ERF1-MYC and 35S:ORA59-MYC, run in parallel, noticeably enhanced the *PDF1.2* pro-GUS activity in wild-type protoplasts, which confirms previously reported observations (Çevik et al., 2012). In particular, the average induction of *PDF1.2* pro-GUS activity in *med25* and *cdk8* protoplasts was significantly reduced compared with that of the wild type (Figure 3B), which is consistent with a previous report (Kidd et al., 2009). Protein gel blot analysis was performed to confirm the equivalent transformation efficiency in different protoplasts (Supplemental Figure 4). These results indicate that CDK8 and MED25 are required for the ERF1- or ORA59-dependent activation of *PDF1.2*. Thus, CDK8 is a coactivator in the regulation of *PDF1.2*. Moreover, seedling growth responses to MeJA revealed that both *cdk8* mutant lines were less sensitive to a range of JA concentrations than the wild type (Figure 3C), further suggesting that MED25 and CDK8 function in the JA response pathway.

#### Arabidopsis CDK8 Interacts with MED25

Analysis of gene expression demonstrated that the transcription of *PDF1.2* and other plant defensin genes was significantly reduced

in *cdk8* mutants in mock-, pathogen-, or MeJA-treated plants. Thus, CDK8 was hypothesized to play roles in either recruiting ERF/AP2 domain TFs to the *PDF1.2* promoter or interacting with some important factors in the JA pathway. With this rationale, we cloned the genes encoding the TFs ERF1, ORA59, ERF4, MYC2, JAZ1, and MED25 and performed a split-luciferase complementation assay to explore the potential interaction between these proteins and CDK8. CDK8 was fused to N-terminal luciferase (nLuc), and other TFs and MED25 were fused to C-terminal luciferase (cLuc). After coexpression, there was only a background level of LUC activity from coexpression of CDK8-nLuc with the empty cLuc vector, which was used as a negative control (Figure 3D). The coexpression of CDK8-nLuc with JAZ1, MYC2, ERF1, or ORA59-cLuc resulted in slightly stronger LUC activity (only ~2-fold higher) than that of the negative control. By contrast, there was significantly stronger LUC activity from the coexpression of CDK8-nLuc and MED25-cLuc (~5- to 6-fold higher), indicating that CDK8 directly interacts with MED25. Recently, MED25 was shown to physically interact with 18 TFs, including ERF1, ORA59, MYC2, and WRKYs (Çevik et al., 2012). Therefore, we also constructed MED25-nLuc and coexpressed it with ERF1-cLuc as a positive control, which exhibited strong LUC activity, confirming the efficiency of our split-luciferase complementation assays. Moreover, the interaction between CDK8 and MED25 was verified in coimmunoprecipitation (Co-IP)



**Figure 3.** Interactions between CDK8 and MED25 Promote the Expression of *PDF1.2*.

**(A)** Reduced MeJA-induced expression of *PDF1.2* in *cdk8* and *med25* mutants. Gene expression was determined by RT-qPCR with the *Arabidopsis ACT2* used for normalization. The expression level of *PDF1.2/ACT2* in the mock-treated wild type was set at 1. Error bars indicate  $\pm$  SE ( $n = 3$ ).

**(B)** CDK8 and MED25 are required for ERF1/ORA59-mediated *PDF1.2* activation. The activity of the reporter gene construct *PDF1.2* pro-GUS was normalized to the full-length *LUC* construct used as an internal control. Relative *GUS:LUC* activity ratios (fold change) are mean values from three independent biological replicates ( $n = 6$ ). Error bars indicate  $\pm$  SE.

**(C)** Root growth responses of *cdk8* to MeJA. Wild-type and *cdk8* seeds were germinated on half-strength Murashige and Skoog medium. Root lengths were measured at 7 d after transferring to medium containing MeJA at the indicated concentrations. Results are mean values  $\pm$  SE from two independent replicates ( $n = 50$ ).

**(D)** Interaction between CDK8 and MED25 revealed by split-luciferase complementation assay. Equal amounts of purified plasmids were transiently coexpressed in *Arabidopsis* protoplasts with substrate luciferin. The LUC activities are mean values from three biological replicates, and error bars indicate  $\pm$  SE ( $n = 3$ ).

assays in *Arabidopsis* protoplasts. MED25-MYC was immunoprecipitated by CDK8-HA but ERF4-MYC was absent in CDK8-HA precipitates, confirming the specific interaction between CDK8 and MED25 (Figure 3E).

To determine the genetic interaction between MED25 and CDK8, we generated a *cdk8 med25* double mutant by crossing *cdk8-1* and *med25*. The *cdk8 med25* double mutant displayed increased susceptibility to *A. brassicicola*, significantly more than that of the single mutants, at 3 DAI (Figure 3F), indicating that both CDK8 and MED25 may affect disease responses in more than one pathway. Interestingly, the double mutant exhibited increased resistance to *B. cinerea* similar to that of the *cdk8-1* single mutant, despite the susceptibility of the *med25* single mutant to *B. cinerea* (Figure 3G). These data suggest that the *cdk8* mutation is epistatic to *med25* with respect to *B. cinerea* resistance but may affect overlapping as well as distinct pathways with respect to *A. brassicicola* resistance.

### Transcriptional Regulation of *PDF1.2* by CDK8

To shed light on the molecular mechanisms of the regulation of *PDF1.2* expression by CDK8, we sought to determine whether CDK8 is required for the recruitment of RNAP II to the promoter regions of *PDF1.2*. Chromatin immunoprecipitation-quantitative PCR (ChIP-qPCR) assay was conducted using antibodies raised against the C-terminal domain of RNAP II (anti-RPB2) and quantitative PCR primers designed at different positions, including 500-bp upstream, TATA box (the RNAP II binding site), and coding and terminator regions of *PDF1.2* (Figure 4A). The recruitment of RNAP II to all four *PDF1.2* regions was significantly lower in the *cdk8* mutant than in the wild type after MeJA treatment, especially for the TATA box of the *PDF1.2* promoter region. The recruitment of RNAP II to *Arabidopsis ACTIN2* (*ACT2*), which was used as an endogenous control, was similar in the wild type and the *cdk8* mutant (Figure 4B). From these findings, we infer that CDK8 contributes to the transcription of *PDF1.2* through its role in RNAP II recruitment. In parallel, the *cdk8;35S::CDK8-MYC* transgenic plants were used for ChIP-qPCR experiments to determine the association of CDK8 with target genes. The association of CDK8-MYC with the 500-bp upstream, TATA box, and terminator sites of the *PDF1.2* promoter region were enhanced compared with wild-type plants in mock-treated plants (Figure 4C). The enrichment of CDK8-MYC was at least 2- to 5-fold higher in these regions. By contrast, there was no difference at the *PDF1.2* coding region and the endogenous *ACT2* control. We then investigated whether pathogen infection would enhance the enrichment of CDK8 at the

*PDF1.2* promoter. The recruitment of CDK8 to the four *PDF1.2* regions tested was significantly enhanced by *B. cinerea* infection, suggesting that CDK8 directly regulates the expression *PDF1.2* in response to pathogen attack (Figure 4D).

### CDK8 Is Required for the Accumulation of HCAAs

The RNA-seq analysis identified many genes that are significantly induced by *B. cinerea* in a CDK8-dependent manner. Among these, the *AACT1* gene, which catalyzes the last step of HCAA biosynthesis, was significantly induced by *B. cinerea* or *A. brassicicola* infection, but its expression remained at significantly lower levels in the *cdk8* mutant (Figure 5A; Supplemental Data Set 3). The HCAAs *p*-coumaroylagmatine, feruloylagmatine, *p*-coumaroylputrescine, and feruloylputrescine are important secondary metabolites that accumulate in response to infection and are known to contribute to resistance to *A. brassicicola* (Muroi et al., 2009). Liquid chromatography-mass spectrometry (LC-MS) analysis revealed that *p*-coumaroylputrescine and feruloylputrescine accumulated at significantly lower levels in the *cdk8* mutant (Figure 5B). Consistently, the *Arabidopsis* mutants in the *AACT1* gene exhibited increased susceptibility to *B. cinerea* (Supplemental Figure 5). Previous data show that the *aact1* mutant has reduced accumulation of HCAAs and enhanced susceptibility to *A. brassicicola* (Muroi et al., 2009).

To determine whether the transcriptional regulation of *AACT1* by CDK8 is direct or indirect, we studied the association of CDK8 with the *AACT1* gene by ChIP-qPCR using primers designed based on the regulatory and coding regions of *AACT1*. CDK8 associated with the 500-bp upstream region but not the TATA box of the *AACT1* promoter (Figure 5C). After inoculation with *B. cinerea*, the associations of CDK8 at the 500-bp upstream, TATA box, and coding and terminator regions were all significantly increased (Figure 5D), indicating that the association of CDK8 with these regions contributes to the regulation of *AACT1* transcription in response to pathogen infection.

### The *cdk8* Mutant Has Altered Cuticle Structure

Changes in cuticle composition and structure are known to alter plant defense responses (Bessire et al., 2007; Reina-Pinto and Yephremov, 2009; L'Haridon et al., 2011). We noticed that the leaves of *cdk8* mutants are particularly glossy, which is reminiscent of altered cuticle structure (Figure 6A; Supplemental Figure 6). Toluidine blue staining revealed that leaves of the *cdk8* mutant stained more quickly than leaves of the wild type (Figure 6B), suggesting increased permeability in the *cdk8* mutant. After

**Figure 3.** (continued).

**(E)** CDK8 and MED25 interact in Co-IP assays in *Arabidopsis* protoplasts. MED25-MYC, but not ERF4-MYC, was present in CDK8-HA-precipitated complex. The experiment was repeated three times.

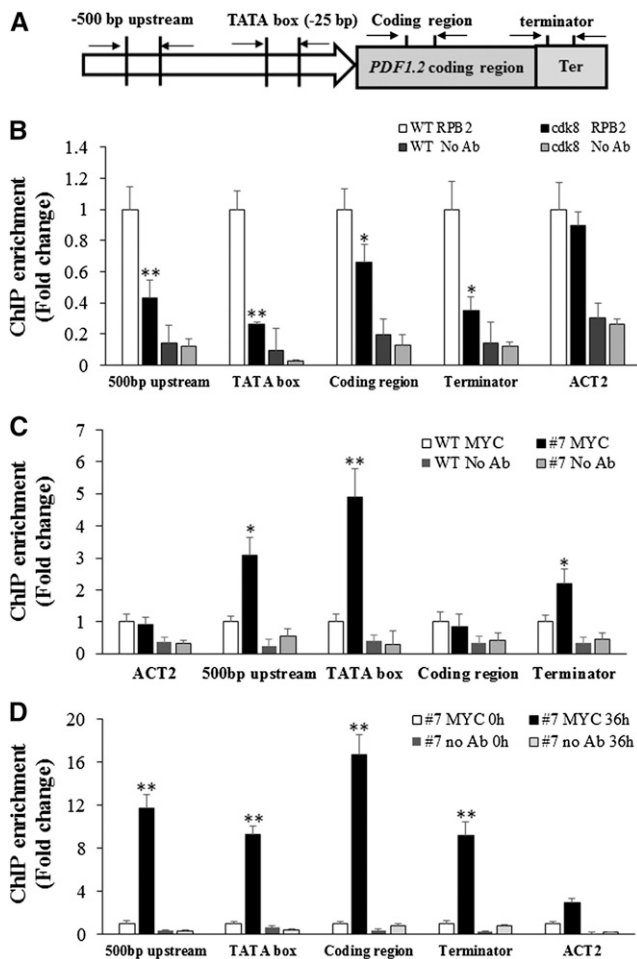
**(F)** *A. brassicicola* disease symptoms and disease lesion size in the wild type, *cdk8*, *med25*, and *cdk8 med25* double mutant.

**(G)** *B. cinerea* disease symptoms and disease lesion size in the wild type, *cdk8*, *med25*, and *cdk8 med25* double mutant.

The disease assays and lesion size measurements were performed as described in Figure 1 and were repeated at least three times. The lesion sizes are mean values  $\pm$  SE from at least 20 disease lesions. The lesion sizes and photographs are from 4 DAI. All of the data were statistically analyzed. Asterisks indicate significant differences (Student's *t* test, \**P* < 0.05, \*\**P* < 0.01).

[See online article for color version of this figure.]





**Figure 4.** CDK8 Associates with the Regulatory Regions of the *PDF1.2* Gene.

**(A)** Schematic showing the positions of *PDF1.2* gene primers used for ChIP-qPCR.

**(B)** CDK8 is required for MeJA-induced RNAP II recruitment to the *PDF1.2* promoter. ChIP-qPCR results are shown with *PDF1.2* gene-specific primers. Chromatin was extracted from wild-type and *cdk8* seedlings 1 h after treatment with 100  $\mu$ M MeJA and then precipitated with anti-RPB2 antibody (Abcam) or only IgG (negative control with no antibody [No Ab]). The RNAP II recruitment at *Arabidopsis* *ACT2* was used as a control because its expression is independent of CDK8. The ChIP-qPCR data show that RNAP II recruitment to *PDF1.2* 500-bp, TATA box, and coding and terminator regions decreased compared with the wild type.

**(C)** CDK8 associates with the *PDF1.2* promoter. Chromatin was extracted from 5-week-old wild-type and *cdk8;35S:CDK8-MYC* transgenic plants and then precipitated with anti-MYC antibody (Abcam) or only IgG (No Ab).

**(D)** Recruitment of CDK8 to the *PDF1.2* promoter is significantly enhanced by *B. cinerea* infection. Chromatin was extracted from 5-week-old *cdk8;35S:CDK8-MYC* #7 transgenic plants that were mock-inoculated or *B. cinerea* spray-inoculated. ChIP was performed with anti-MYC antibody (Abcam) or only IgG (No Ab). The CDK8 recruitment to 500-bp upstream, TATA box, and coding and terminator regions of *PDF1.2* was determined by quantitative PCR using primers at different positions of the *PDF1.2* gene as shown in **(A)**.

inspection of the cuticle by transmission electron microscopy, we observed that the cuticle layer is thinner in the *cdk8* mutant than in wild-type plants (Figure 6C). Accordingly, relative to wild-type plants, the percentage water loss is higher in the *cdk8* mutant and those plants wilt more quickly, as predicted from the changes in the cuticle structure of the mutant (Figure 6D). The expression of genes implicated in cuticle and cuticular wax biosynthesis was studied to explore the potential mechanisms underlying the altered cuticle structure in the *cdk8* mutant. The expression of several cuticle wax biosynthesis genes, including *ECERIFERUM1* (*CER1*) and *CER6*, was significantly increased in the *cdk8* mutant (Supplemental Figure 7). Overexpression of *CER1* has been shown to cause increased susceptibility to bacterial and fungal pathogens (Bourdenx et al., 2011), which is consistent with the susceptibility of the *cdk8* mutant to *A. brassicicola* and *P. syringae* DC3000.

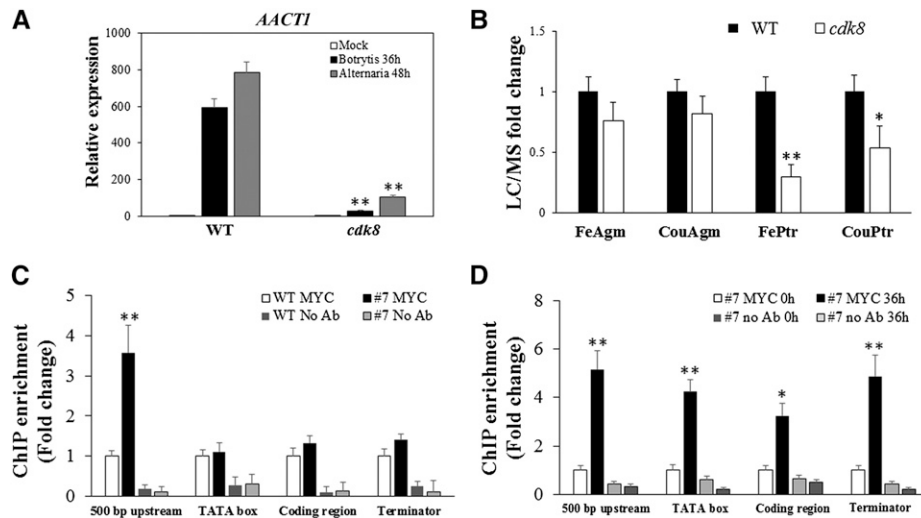
Previous studies have shown that increased cuticle permeability is associated with enhanced resistance to *B. cinerea* (Bessire et al., 2007). Thus, the increased permeability of the *cdk8* mutant may contribute to the resistance of *cdk8* to *B. cinerea*. The growth phenotype and permeability of the cuticle of the *cdk8 med25* double mutant were also analyzed (Supplemental Figure 8). The cuticle permeability of the *med25 cdk8* double mutant was significantly increased relative to the *med25* single mutant but was comparable to the *cdk8* mutant (Supplemental Figure 8B). These data, together with the *B. cinerea* resistance of the double mutant, suggest that the compromised defense responses in *med25* is overcome by the enhanced cuticle permeability, resulting in resistance to *B. cinerea*.

#### CDK8 and MED25 Interact with the Ethylene Response TF WIN1

It was previously reported that overexpression of the TF *WIN1* increases cuticle thickness (Broun et al., 2004; Kannangara et al., 2007) and results in susceptibility to *B. cinerea* (Sela et al., 2013), which contrasts with the phenotypes of the *cdk8* mutant. These observations suggest that CDK8 and WIN1 have overlapping functions. Therefore, we tested the possible interaction between CDK8 and WIN1, a protein belonging to the ERF family of TFs. The coexpression of CDK8-nLuc and WIN1-cLuc led to ~5-fold higher LUC activity than the negative control, suggesting a physical interaction (Figure 7A). Co-IP experiments in *Arabidopsis* protoplasts confirmed that WIN1-MYC but not ERF4-MYC was immunoprecipitated by CDK8-HA (Figure 7B). The interaction between CDK8 and MED25 further raised the possibility of MED25 and WIN1 being in the same complex with CDK8. Interestingly, the WIN1-MED25 interaction was stronger than WIN1-CDK8, because the coexpression of WIN1-cLuc and MED25-nLuc resulted in more than 10-fold higher LUC activity than the negative control (Figure 7A). These data show that

Error bars in **(B)** to **(D)** indicate SE ( $n = 3$ ). Two biological replicates were performed with similar results for each ChIP-qPCR experiment. The significance of differences in mean values is marked by asterisks (Student's *t* test, \* $P < 0.05$ , \*\* $P < 0.01$ ).





**Figure 5.** CDK8 Is Required for the Accumulation of *Arabidopsis* HCAAs.

(A) Expression of the *AACT1* gene in response to *B. cinerea* and *A. brassicicola*. RNA was extracted from mock-, *B. cinerea*-, or *A. brassicicola*-inoculated leaves. The experiment was repeated three times. Error bars indicate SE ( $n = 3$ ).

(B) Detection of HCAAs from rosette leaves of 5-week-old *Arabidopsis* wild-type and *cdk8* plants at 48 h after inoculation with *A. brassicicola*. The data are mean values  $\pm$  SE from two biological replicates ( $n = 4$ ). FeAgm, feruloylagmatine; CouAgm, *p*-coumaroylagmatine; FerPtr, feruloylputrescine; CouPtr, *p*-coumaroylputrescine.

(C) CDK8 is specifically recruited to a 500-bp region upstream of the *AACT1* promoter region.

(D) Recruitment of CDK8 to the *AACT1* gene is enhanced by *B. cinerea* infection.

In (C) and (D), chromatin was extracted from 5-week-old *cdk8*;35S:CDK8-MYC #7 transgenic plants that were mock- or *B. cinerea*-inoculated. Error bars indicate SE ( $n = 3$ ). ChIP-qPCR experiments were repeated two times with similar results. No Ab, negative control with no antibody.

The data were statistically analyzed. Asterisks indicate significant differences (Student's *t* test, \* $P < 0.05$ , \*\* $P < 0.01$ ).

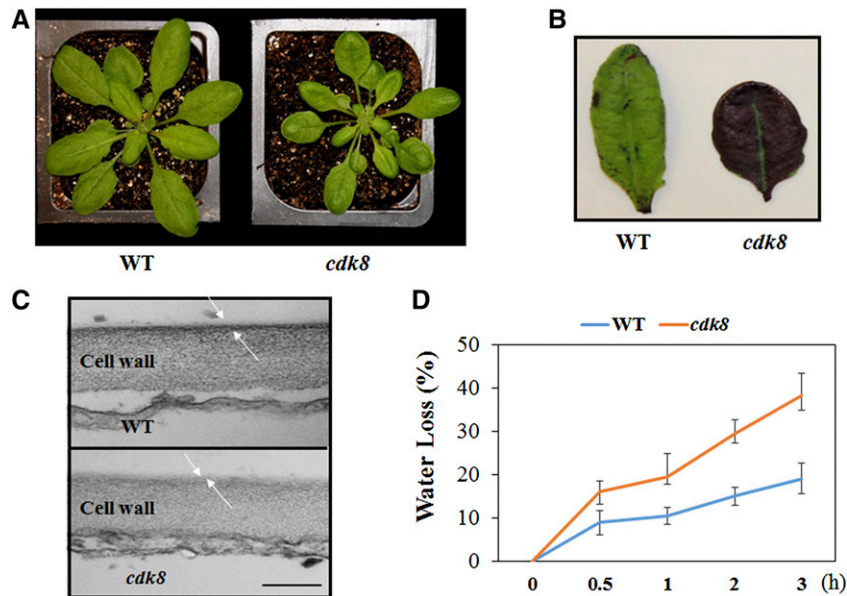
MED25 interacts with other ERF/AP2 domain TFs besides ERF1 and ORA59. Co-IP experiments also demonstrated that WIN1-MYC was precipitated by MED25-HA but not by CycCb, which was used as a control (Figure 7B).

We generated *WIN1* overexpression (*WIN1 OE*) lines by transforming 35S:WIN1-HA into wild-type plants. As confirmed by RT-qPCR, the expression of WIN1 was more than 6-fold higher in *WIN1 OE* lines 2 and 8 plants relative to the wild-type plants (Figure 7C). Next, we tested the *WIN1 OE* lines for responses to *A. brassicicola* and *B. cinerea* infection. Leaves of *WIN1 OE* lines 2 and 8 plants had larger disease lesions at 4 d after *B. cinerea* drop-inoculation, suggesting enhanced susceptibility (Figure 7D). Similarly, leaves of *WIN1 OE* lines 2 and 8 plants exhibited increased susceptibility to *A. brassicicola* at 4 DAI (Figure 7E). The enhanced susceptibility to the two fungal pathogens was accompanied by significantly reduced expression of *PDF1.2* (Figure 7F). Thus, it appears that WIN1 actually affects defense through its function in the cuticle as well as other distinct pathways. It is noteworthy that WIN1 functions in fatty acid biosynthesis and most likely has more functions than just cuticle development (Kannangara et al., 2007). Furthermore, to determine whether WIN1 is a major component of the CDK8-regulated pathway, we expressed WIN1-HA in the *cdk8* mutant background (*cdk8*;WIN1-HA). The *cdk8*;WIN1-HA plants showed disease lesions similar to wild-type plants at 4 DAI (Supplemental Figure 9). By contrast, the *cdk8* mutant exhibited resistance without detectable disease symptoms. This genetic evidence

further supports the notion that the resistance to *B. cinerea* in the *cdk8* mutant is due to the altered cuticle profile linked to the loss of CDK8 functions.

### The Phosphorylation Activity of CDK8 Is Required for the Suppression or Activation of Defense

CDK8 is the only component of Mediator with known enzymatic functions. Therefore, we sought to determine the biological functions of CDK8 enzymatic activity. Sequence comparisons show that *Arabidopsis* CDK8 shares significant sequence identity with human CDK8 (Figure 8A). From previous studies of human CDK8, the single amino acid substitution of aspartic acid to alanine (D173A) was sufficient to nullify the kinase activity of human CDK8 (Knuesel et al., 2009b). The same residues are conserved in other animal and plant species (Supplemental Figure 10). Therefore, we generated *Arabidopsis* CDK8 with alanine substitution at position 176 (CDK8<sup>D176A</sup>) corresponding to the human CDK8 173D, and the recombinant protein was produced as a glutathione S-transferase (GST) fusion. Based on in vitro kinase assays, the wild-type CDK8 displayed autophosphorylation as well as phosphorylation of myelin basic protein (MBP), a commonly used artificial kinase substrate. Nonetheless, the CDK8<sup>D176A</sup> mutant resulted in a complete loss of autophosphorylation and MBP phosphorylation activities (Figure 8B). Subsequently, we cloned CDK8 and CDK8<sup>D176A</sup> into a plant transformation vector and transformed the constructs into the



**Figure 6.** The *cdk8* Mutant Has Increased Cuticle Permeability and a Reduced Cuticular Layer.

(A) Leaves of the *cdk8* mutant (right) showing the glossy phenotype.

(B) Enhanced cuticle permeability of *cdk8* leaves (right) revealed by toluidine blue staining.

(C) Transmission electron microscopic images of wild-type and *cdk8* mutant leaf epidermal cells. The white arrows mark cutin. Bar = 200 nm.

(D) The *cdk8* mutant shows enhanced water loss. Water loss was expressed as the percentage of initial fresh weight. Values are averages from 20 leaves for each of three independent experiments. Values shown are means  $\pm$  SE.

*cdk8* mutant, generating *cdk8*;35S:CDK8-MYC and *cdk8*;35S:CDK8<sup>D176A</sup>-MYC transgenic plants (Supplemental Figure 11). Immunocomplex kinase assays with anti-MYC conjugated beads revealed that immunoprecipitates from 35S:CDK8-MYC phosphorylate histones, whereas the immunoprecipitates of 35S:CDK8<sup>D176A</sup>-MYC plants showed much weaker kinase activity, which is consistent with the data from bacterially expressed recombinant protein (Figure 8C).

To determine whether the kinase activity of CDK8 contributes to its defense functions, the transgenic plants *cdk8*;35S:CDK8-MYC and *cdk8*;35S:CDK8<sup>D176A</sup>-MYC were tested for their disease responses. At 4 DAI, larger disease lesions were observed in wild-type, *cdk8*;35S:CDK8-MYC, and *cdk8*;35S:CDK8<sup>D176A</sup>-MYC lines after inoculation with *B. cinerea*, suggesting that both CDK8 constructs restored the *B. cinerea* disease responses to wild-type levels (Figure 8D). On the other hand, *cdk8*;35S:CDK8-MYC transgenic lines showed wild-type levels of resistance to *A. brassicicola*, indicating that ectopic expression of CDK8 rescued the susceptibility of the *cdk8* mutant to this fungus. However, the 35S:CDK8<sup>D176A</sup>-MYC lines were as susceptible as the *cdk8* mutant to *A. brassicicola*, indicating that the kinase activity of CDK8 is necessary for resistance to *A. brassicicola* but not for responses to *B. cinerea* (Figure 8E).

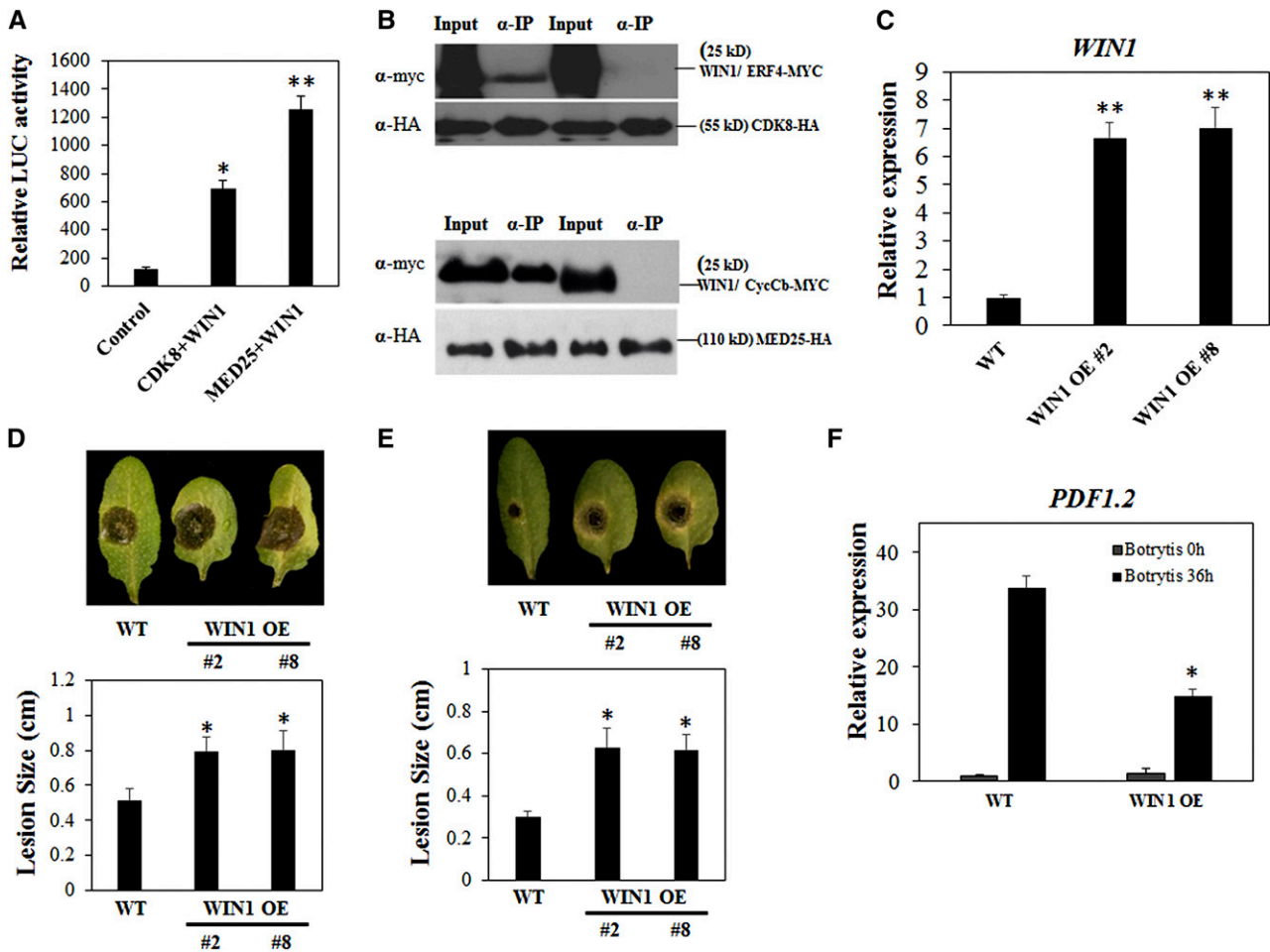
At the molecular level, the expression of *PDF1.2* and *AACT1* was rescued in the *cdk8*;35S:CDK8-MYC lines but not in the *cdk8*;35S:CDK8<sup>D176A</sup>-MYC lines (Figures 9A and 9B), indicating that the kinase activity of CDK8 is essential for the regulation of *PDF1.2* and *AACT1* by CDK8. On the other hand, the expression

of both wax biosynthesis genes *CER1* and *CER6* was restored to wild-type levels in *cdk8*;35S:CDK8-MYC and *cdk8*;35S:CDK8<sup>D176A</sup>-MYC transgenic lines (Figures 9C and 9D), indicating a CDK8 kinase-independent regulation.

The roles of the kinase activity of CDK8 in its interaction with functional partners and in RNAP II recruitment were further studied. To explore whether the kinase activity of CDK8 affects the CDK8-MED25 interaction, we cloned CDK8<sup>D176A</sup> in the nLuc vector and coexpressed it with MED25-cLuc. The LUC activities from the coexpression of CDK8-nLuc or CDK8<sup>D176A</sup> with MED25-cLuc were almost the same, implying that the CDK8-MED25 interaction is not dependent on kinase activity (Figure 9E). To better understand whether the RNAP II recruitment to the *PDF1.2* promoter is affected by its kinase activity, *cdk8*;35S:CDK8<sup>D176A</sup>-MYC and *cdk8*;35S:CDK8-MYC plants were spray-inoculated with *B. cinerea* and used for chromatin immunoprecipitation (ChIP) assays. RNAP II recruitment to the *PDF1.2* promoter regions was significantly reduced in the lines expressing kinase-dead CDK8, suggesting that kinase activity is required for RNAP II recruitment to the *PDF1.2* promoter (Figure 9F).

#### ***med12* and *med13* Mutants Display Phenotypes Similar to Those of the *cdk8* Mutant**

CDK8, CycC, MED12, and MED13, together, constitute the kinase module of Mediator in other eukaryotic organisms (Knuesel et al., 2009a). In parallel with the *cdk8* mutant, we characterized SALK T-DNA insertion alleles of *MED12* and *MED13* for plant



**Figure 7.** CDK8 and MED25 Both Interact with the TF WIN1.

(A) CDK8 and MED25 interact with WIN1 in a split-luciferase complementation assay. The LUC activities are mean values from three biological replicates, and error bars indicate SE ( $n = 3$ ).

(B) WIN1 and CDK8/MED25 interact in a Co-IP assay.

(C) The expression of *WIN1* in two independent transgenic WIN1 overexpression lines.

(D) *B. cinerea* disease symptoms and disease lesion size in the wild type and WIN1 overexpression lines at 4 dpi.

(E) *A. brassicicola* disease symptoms and disease lesion size in the wild type and WIN1 overexpression lines at 4 dpi.

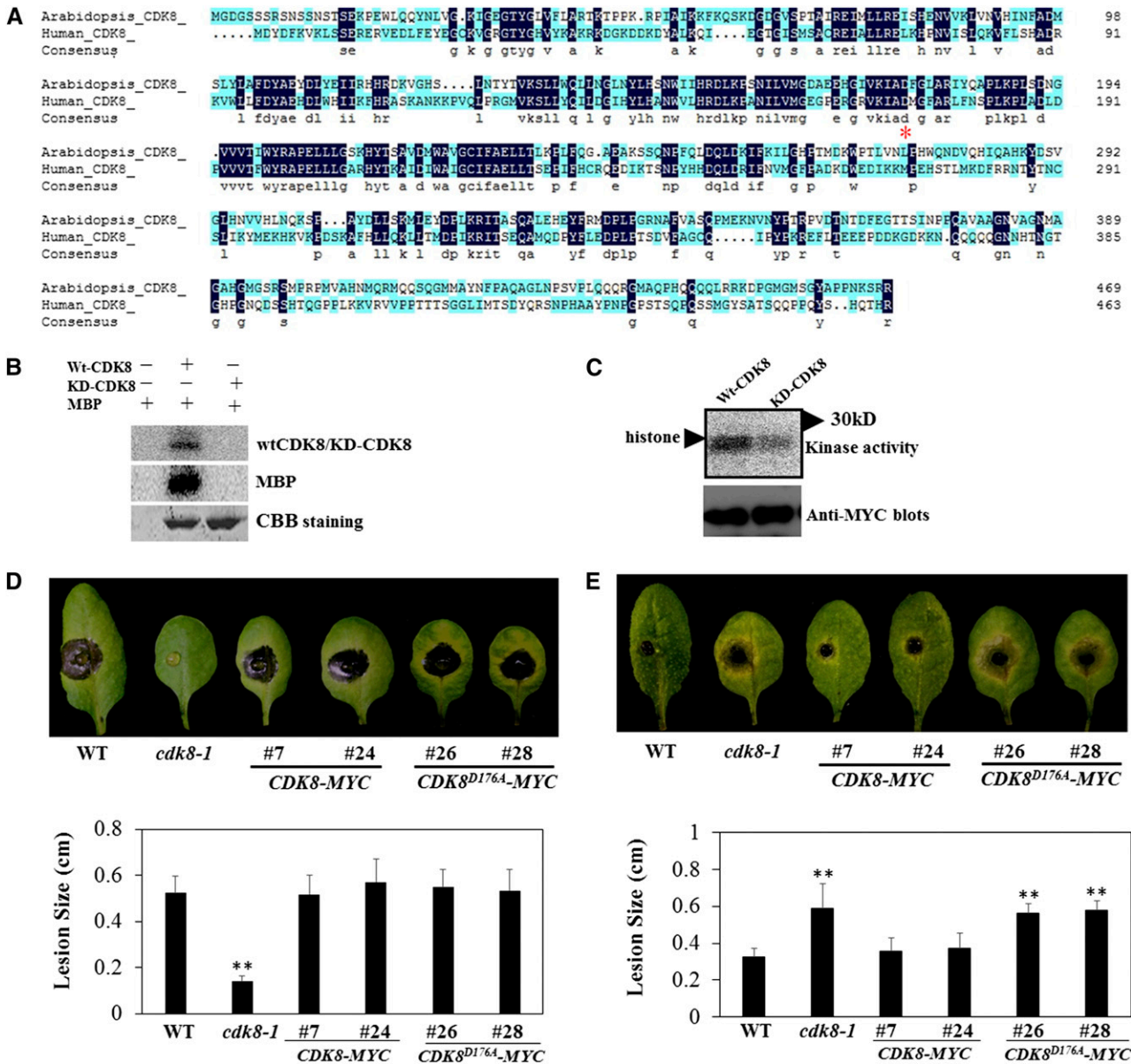
(F) Expression of the *PDF1.2* gene in the wild type and WIN1 overexpression lines after mock or *B. cinerea* inoculation.

Two independent WIN1 overexpression lines (WIN1 OE #2 and WIN1 OE #8) were used in these studies. The significance of differences between mean lesion sizes was analyzed. Values shown are means  $\pm$  SE. Asterisks indicate significant differences (Student's *t* test, \* $P < 0.05$ , \*\* $P < 0.01$ ).

growth and pathogen responses (Figure 10; Supplemental Figure 12). The *cdk8*, *med12*, and *med13* mutants exhibit reduced overall plant stature, glossy leaves, and late flowering (Figure 10A; Supplemental Figure 12B). The *med12* and *med13* mutants displayed enhanced resistance to *B. cinerea* but increased susceptibility to *A. brassicicola*, mirroring the disease response phenotypes of *cdk8* (Figures 10B and 10C), which suggested that subunits of the kinase module share similar functions in regulating plant responses to necrotrophic fungi. Moreover, toluidine blue staining of *cdk8*, *med12*, and *med13* mutants revealed enhanced permeability relative to the wild type. When 0.05% toluidine blue was applied, leaves of *cdk8*, *med12*, and *med13* mutant plants stained much more quickly

and strongly than wild-type leaves (Figure 10D). These observations suggest that the kinase module may regulate cuticle development in a coordinated manner.

In addition to the disease phenotypes and increased leaf permeability noted among kinase module mutants, gene expression data suggest that CDK8, MED12, and MED13 regulate the expression of some of the same target genes, including *PDF1.2* and *PR1* (Figure 10E). The expression of both defense-related genes was significantly reduced in *cdk8*, *med12*, and *med13* mutants. Interestingly, *CER3* was upregulated in all mutants, while *CER1* was upregulated only in *cdk8* (Figure 10E), indicating that MED12 and MED13 may also have functions different from those of CDK8.



**Figure 8.** CDK8 Functions in Kinase-Dependent and Independent Manners.

**(A)** Amino acid sequence comparison between *Arabidopsis* and human CDK8 proteins. The conserved aspartic acid at amino acid position 176 (176D) of CDK8, which is critical for its kinase activity, is marked by the red asterisk.

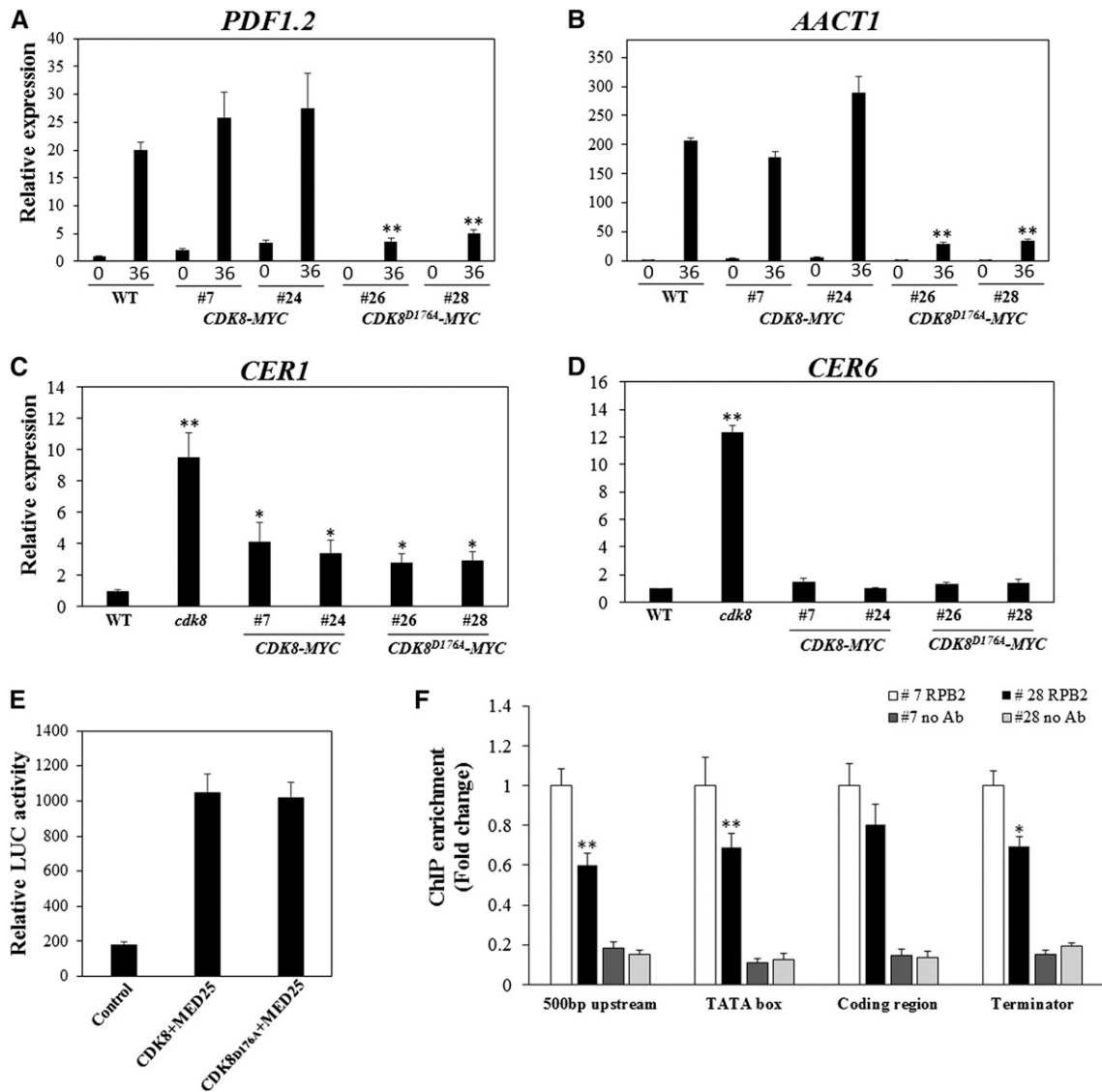
**(B)** In vitro kinase assay showing that substitution of 176D to alanine nullifies the kinase activity of *Arabidopsis* CDK8. GST-CDK8 recombinant protein displayed autophosphorylation as well as phosphorylation of substrate MBP. However, GST-CDK8<sup>D176A</sup> recombinant protein lost both autophosphorylation and MBP phosphorylation activities. The experiment was repeated two times with similar results. CBB, Coomassie Brilliant Blue.

**(C)** Immunocomplex kinase assays showing the phosphorylation of histones by CDK8. Kinase assay was performed using protein extracts from *cdk8;35S:CDK8-MYC* #7 and *cdk8;35S:CDK8<sup>D176A</sup>-MYC* #26 transgenic seedlings.

**(D)** Ectopic expression of *CDK8-MYC* or *CDK8<sup>D176A</sup>-MYC* restores wild-type levels of *B. cinerea* responses in the *cdk8* mutant.

**(E)** *CDK8-MYC* but not *CDK8<sup>D176A</sup>-MYC* plants restore wild-type responses to *A. brassicicola*.

In **(D)** and **(E)**, the disease assays were performed as described in Figure 1, and disease lesions were recorded at 4 DAI. Data are mean values  $\pm$  SE ( $n = 20$ ), and statistical analysis was conducted to determine the significant differences of values (Student's *t* test, \*\* $P < 0.01$ ).



**Figure 9.** The Kinase Activity of CDK8 Is Required for Target Gene Expression.

(A) to (D) *PDF1.2* (A), *AACT1* (B), *CER1* (C), and *CER6* (D) gene expression levels in wild-type, *cdk8*;35S:*CDK8-MYC*, and *cdk8*;35S:*CDK8<sup>D176A</sup>-MYC* transgenic plants. Lines 7 and 24 are two independent transgenic lines expressing 35S:*CDK8-MYC*, whereas lines 26 and 28 are 35S:*CDK8<sup>D176A</sup>-MYC* lines. Error bars represent SE ( $n = 3$ ).

(E) The kinase-dead CDK8 mutation does not affect the CDK8-MED25 interaction in split-luciferase complementation assays. Error bars represent SE ( $n = 3$ ).

(F) RNAP II recruitment to the *PDF1.2* promoter is dependent on the kinase activity of CDK8. Reduced RNAP II recruitment is shown in *cdk8*;35S:*CDK8<sup>D176A</sup>-MYC* transgenic plants compared with *cdk8*;35S:*CDK8-MYC* plants after *B. cinerea* infection. Error bars indicate SE ( $n = 3$ ). ChIP-qPCR experiments were repeated two times with similar results. No Ab, negative control with no antibody.

Statistical analysis was conducted to determine differences in mean values. Asterisks indicate significant differences (Student's *t* test, \* $P < 0.05$ , \*\* $P < 0.01$ ).

### CDK8 Physically Interacts with Two CycCs

In yeast and mammalian cells, Mediator kinase modules contain a CycC that interacts with CDK8 (Borggreve et al., 2002; Andrau et al., 2006). The *Arabidopsis* genome contains two CycC genes, *CycCa* (At5g48630) and *CycCb* (At5g48640) (Menges et al., 2005). In split luciferase complementation assays to

determine interactions between CDK8 and CycCa/b, the co-expression of CDK8-nLuc and CycCa/CycCb-cLuc resulted in significant LUC activities, which averaged ~7- and 10-fold higher than the negative control, respectively (Figure 11A). The interaction of CDK8 and CycCb was stronger than that of CDK8 and CycCa based on the LUC reconstitution signal. In addition, Co-IP assays confirmed the direct interactions between CDK8 and CycCa/b.

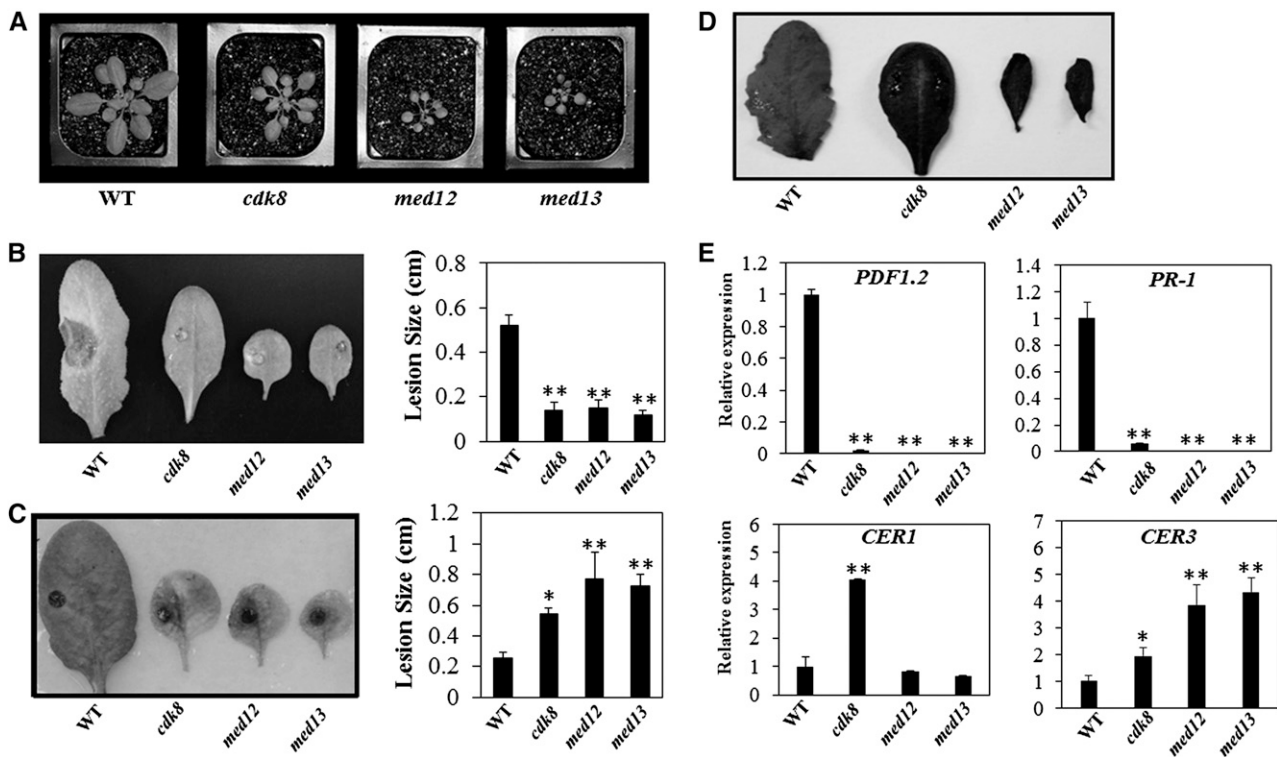


Both CycCa-MYC and CycCb-MYC were successfully precipitated by CDK8-HA, whereas the negative control ORA59-MYC was not precipitated (Figure 11B). These data suggest that the CycC-CDK8 interaction is evolutionarily conserved in *Arabidopsis*, yeast, and mammalian cells.

The disease phenotypes of *cycC* mutants were examined to determine the defense functions of the two cyclin genes. Unlike *cdk8*, *med12*, and *med13* mutants, the single *cycCa* and *cycCb* mutants showed wild-type levels of disease responses (Supplemental Figure 13), which implied redundant functions of the two CycC genes. The two genes are linked, which precluded the possibility of generating double mutants. However, we identified a T-DNA insertion homozygous line (SALK\_039400C) designated *cycCab* in which both *CycCa* and *CycCb* are downregulated due to the insertion of the T-DNA in the small intergenic region between the two genes (Figure 11C). The *cycCab* mutant showed increased susceptibility to *A. brassicicola* compared with wild-type plants, with larger disease lesions at 4 DAI, similar to the *cdk8* mutant, which is consistent with their physical interaction (Figure 11D). The *cycCab* mutants displayed no altered responses to *B. cinerea*, unlike the resistance in the *cdk8* mutant.

## DISCUSSION

In yeast and metazoan cells, CDK8 defines the kinase module of Mediator, which also contains MED12, MED13, and CycC (Borggreve et al., 2002; Andrau et al., 2006). Although increasing evidence has demonstrated that CDK8 is a critical regulator of transcription in metazoan and yeast cells, the function of plant CDK8 remains largely unknown. In this work, we show that *Arabidopsis* CDK8 has both negative and positive roles in the regulation of plant responses to pathogens, and we provide insights into the molecular and biochemical functions of CDK8 in plant immunity. Overall, we provide extensive data supporting the complex function of CDK8 and the kinase module of Mediator. These include (1) genetic data revealing the contrasting defense functions of CDK8 to related necrotrophic fungi sharing similar mechanisms of pathogenesis, virulence, and modes of nutrition; (2) epistatic interactions between resistance based on altered cuticle layer and JA-mediated immune responses; (3) the impact of the enzymatic activity of CDK8 and its biological function, particularly in response to fungal infection; (4) the molecular interactions between CDK8 and other Mediator subunits (MED25, cyclins) as well as the ERF/AP2 family TF WIN1,



**Figure 10.** Mediator Kinase Module Components MED12 and MED13 Mediate Defense Responses.

(A) Growth phenotypes of 4-week-old wild-type, *cdk8*, *med12*, and *med13* mutant plants.

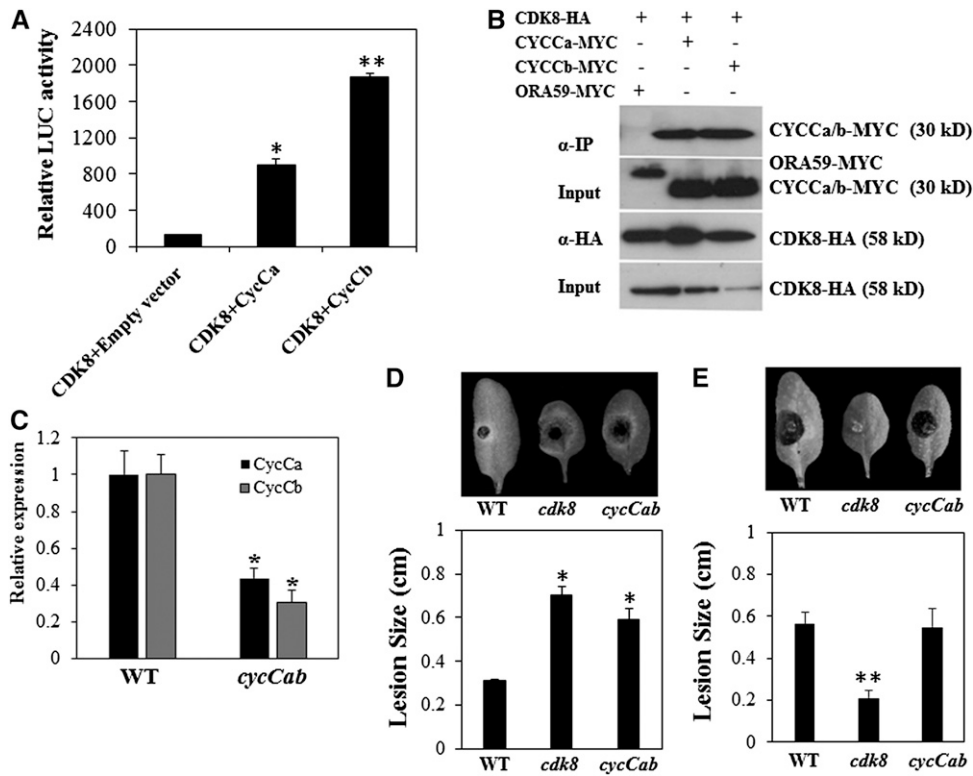
(B) *B. cinerea* disease symptoms and disease lesion sizes in the wild type and Mediator kinase module mutants.

(C) *A. brassicicola* disease symptoms and disease lesion sizes in the wild type and Mediator kinase module mutants.

(D) Enhanced cuticle permeability of the wild type and Mediator kinase module mutants revealed by toluidine blue staining.

(E) Relative expression of defense and cuticular wax genes in the wild type and Mediator kinase module mutants.

The disease assays in (B) and (C) were conducted as described in Figure 1. The lesion sizes are mean values  $\pm$  SE from at least 20 disease lesions, and the data were analyzed with Student's *t* test (\**P* < 0.05, \*\**P* < 0.01).



**Figure 11.** Arabidopsis CDK8 Interacts with Two CycCs.

(A) Strong interactions between CDK8 and two CycCs in split-luciferase complementation assays. Data are mean values  $\pm$  SE from three independent experiments. Error bars indicate SE ( $n = 3$ ).  
 (B) CDK8 interacts with CycCa and CycCb in Co-IP assays. The experiment was repeated at least three times.  
 (C) Reduced expression of CycCa and CycCb genes in the cycCab mutant allele. The cycCab allele (SALK\_039400C) carries a T-DNA insertion between the CycCa and CycCb genes disrupting the expression of both genes. CycCa and CycCb are tightly linked and transcribed in the same orientation.  
 (D) *A. brassicicola* disease symptoms and disease lesion sizes in wild-type, *cdk8*, and *cycCab* mutant plants.  
 (E) *B. cinerea* disease symptoms and disease lesion sizes in wild-type, *cdk8*, and *cycCab* mutant plants.  
 The disease assays in (C) and (D) were conducted as described in Figure 1. The lesion sizes are mean values  $\pm$  SE from at least 20 disease lesions. Asterisks indicate significant differences (Student's *t* test, \* $P < 0.05$ , \*\* $P < 0.01$ ).

shedding light on the various mechanisms of CDK8 function; and (5) CDK8 regulates the accumulation of secondary metabolite HCAs through association with *AACT1*, which mediates a critical biosynthetic step.

**CDK8 Positively Regulates JA Responses and *PDF1.2* and *AACT1* Gene Expression**

Analysis of loss-of-function alleles of CDK8 revealed that CDK8 contributes to JA-mediated defense responses and regulates the synthesis of the secondary metabolite HCAs, which are essential for plant resistance to fungal infection. ChIP data demonstrated that CDK8 positively regulates *PDF1.2* and *AACT1* transcription by associating with their promoter regions. These findings suggest a positive role for CDK8 in the regulation of gene expression. Furthermore, CDK8 interacts with the multifunctional Mediator subunit MED25, which is known to regulate JA responses (Kidd et al., 2009). MED25 interacts with diverse TFs, including MYC2, ERF1, and ORA59 (Çevik et al.,

2012; Chen et al., 2012), which are all implicated in plant defense against fungi. MYC2 is known to suppress resistance to *B. cinerea*, while ERF1 and ORA59 contribute to resistance (Berrocal-Lobo et al., 2002; Lorenzo et al., 2004; Pré et al., 2008). The interaction data indicate that CDK8 functions in concert with MED25 to regulate the expression of JA-responsive genes. CDK8 may be recruited by ERF1/ORA59 through MED25 to the *PDF1.2* promoter. This is consistent with the contributions of MED25 and CDK8 to the ERF1- and ORA59-dependent activation of *PDF1.2* expression as well as the association of CDK8 with the *PDF1.2* promoter.

It was also recently suggested that plants regulate the transcription of specific genes through the combined actions of subsets of Mediator subunits (Hemsley et al., 2014). For example, RNAP II recruitment to C-repeat binding factor-responsive cold-regulated genes requires MED16, MED2, and MED14 subunits. Genes inducible by darkness also required MED16, but they required a different combination of Mediator subunits for their expression from those of genes induced by



cold. Similarly, the *Arabidopsis yellow and sensitive to iron-deficiency1* (*yid1*) mutant, which harbors a mutation in the MED16 gene, accumulates reduced amounts of iron. MED16 interacted with MED25, which implicated MED25 in the regulation of iron homeostasis. This role of MED25 was attributed to the interaction with EIN3 and EIL1, two TFs that function in ethylene signaling that were previously associated with the regulation of iron homeostasis (Yang et al., 2014). The transcriptome in *yid1* and *med25* mutants was significantly affected by iron deficiency. The transcription of marker genes for iron homeostasis and iron uptake was reduced in the *yid1* and *med25* mutants under iron-deficient conditions. Thus, MED16 and MED25 coordinately regulate iron homeostasis in *Arabidopsis*. These findings illustrate the complex functions of Mediator subunits through combinatorial interactions with other Mediator subunits and non-Mediator proteins. To date, MED16 has been implicated in defense, iron homeostasis, and abiotic stress tolerance (Wathugala et al., 2012; Yang et al., 2014). Similarly, CDK8 may also have different functions through interactions with MED25, cyclins, and WIN1 and possible interactions with other Mediator subunits and TFs.

#### The Differential Impact of CDK8 on Responses to Necrotrophic Fungal Species Is Linked to Its Function in Cuticle Development

The plant cuticle is a structured layering of long-chain soluble waxes comingled within a cutin polyester matrix that serves as the primary physical barrier against pathogens and prevents nonstomatal water loss (Bessire et al., 2007). Despite the positive regulatory role of CDK8 in the expression of *PDF1.2* and *AACT1* genes and in the accumulation of HCAAs, the *cdk8* mutant is resistant to *B. cinerea*. We attribute this to the likely loss of plant cues specifically required for the virulence of *B. cinerea*, which may have masked the defects in other pathways. We also cannot preclude the possible loss of other susceptibility factors regulated by CDK8, besides changes in the cuticle profile, which are specifically required for *B. cinerea*. Many pathogens are dependent on host susceptibility factors or virulence targets to cause disease. Alternatively, *cdk8* may accumulate compounds that have specific inhibitory effects on *B. cinerea* but not on *A. brassicicola*. However, based on current observations by many laboratories on the correlation between loss of cuticle integrity and resistance, we suggest that the resistance of *cdk8* is also linked to its altered cuticle.

The function of CDK8 in cuticle development is supported by genetic and molecular data. Leaves of the *cdk8* mutant exhibit glossy and smooth appearances reminiscent of changes in the cuticle. Furthermore, the *cdk8* mutant has enhanced cuticle permeability and reduced cuticle thickness. Importantly, CDK8 interacts with WIN1, an ERF family TF that is known to modulate the wax profile of the *Arabidopsis* cuticle by directly binding to the *LACS2* promoter (Kannangara et al., 2007). *LACS2* encodes a member of the long-chain acyl-CoA synthases involved in cutin biosynthesis in *Arabidopsis* (Schnurr et al., 2004). Interestingly, *WIN1* overexpression results in increased expression of several wax biosynthesis genes, increased alkane levels, and susceptibility to fungal infection (Broun et al., 2004; Sela

et al., 2013). Due to the lack of appropriate loss-of-function alleles of the *WIN1* gene, we could not make direct comparisons of the *win1* and *cdk8* mutants. However, overexpression of CDK8 restores susceptibility to *B. cinerea* to at least wild-type levels, and *WIN1* overexpression confers susceptibility. Similarly, *WIN1* overexpression enhances cuticle thickness (Broun et al., 2004), but the *cdk8* mutation reduced cuticle thickness. These data of contrasting phenotypes in the gain-of-function *WIN1* and loss-of-function *cdk8* suggest shared functions of *WIN1* and CDK8 consistent with their physical interactions. Intriguingly, the expression of *CER1* and *CER6*, implicated in cuticle biosynthesis pathways, is significantly high in the *cdk8* mutant, but the biological significance of this is unclear. Despite the reported correlation between loss of cuticle integrity and resistance to *B. cinerea* in many *Arabidopsis* mutants, the exact mechanisms are still unclear. This intriguing correlation is also debatable, as there are mutants that have permeable cuticles but do not show altered pathogen responses (Voisin et al., 2009).

#### CDK8 Functions in Both Kinase-Dependent and Independent Manners

*Arabidopsis* CDK8 shows phosphorylation activities consistent with previous data in yeast and mammalian cells (Nelson et al., 2003; Knuesel et al., 2009b). Interestingly, our data suggest that CDK8 could function in kinase-dependent and independent manners. The expression of kinase-dead CDK8 failed to rescue the attenuated *PDF1.2* and *AACT1* transcription as well as the *A. brassicicola* susceptibility of the *cdk8* mutant. By contrast, the negative regulation of *B. cinerea* resistance by CDK8 is independent of its kinase functions, because the kinase-dead CDK8 restored the responses to *B. cinerea* to wild-type levels. Consistent with the phenotypes, the kinase-dead CDK8 also restored the expression of *CER1* and *CER6*, two cuticular wax biosynthesis genes, to wild-type levels.

#### Overlapping Functions of CDK8 Module Subunits in Arabidopsis Defense and Development

All four subunits of the kinase module of Mediator CDK8 (i.e., MED12, MED13, and the two CycCs) share defense functions. For example, all aspects of the *cdk8* mutant appear to be less severe forms of the *med12* and *med13* mutants, thus adding credence to the common functions of the kinase module of Mediator. *cdk8*, *med12*, and *med13* mutants displayed similar morphological and disease phenotypes as well as increased cuticle permeability. Similarly, *PDF1.2*, *PR1*, and *CER3* show similar patterns of gene expression in the three kinase module mutants. *CER1* is upregulated only in *cdk8*, but not in *med12* and *med13*, suggesting that MED12 and MED13 also have some separate functions. Our study also demonstrates strong physical interactions between CDK8 and two *Arabidopsis* CycCs, suggesting the conserved partnership of CDK8-CycC throughout eukaryotes. Consistent with this physical interaction, the *cycCab* mutant, in which both *CycCa* and *CycCb* are downregulated, exhibited enhanced susceptibility to *A. brassicicola*. The detailed mechanism has yet to be determined, but these data further support the complexity of CDK8 function.

### ***cdk8* Identifies Differences in the Pathogenesis of *B. cinerea* and *A. brassicicola***

The contrasting responses of *cdk8*, *med12*, and *med13* mutants to the necrotrophic fungi *B. cinerea* and *A. brassicicola* was unexpected but suggest that the two pathogens might rely on different plant cues for activating their virulence. The two fungi are known to share related pathogenesis strategies, and plants activate similar immune responses against these pathogens (Lai and Mengiste, 2013). Most *Arabidopsis* mutants identified to be susceptible to *B. cinerea* also exhibit susceptibility to *A. brassicicola*, with the exception of some ethylene pathway mutants, which show unaltered responses to *A. brassicicola* (van Wees et al., 2003). The genetic data suggest that CDK8 is a negative regulator of immune responses to *B. cinerea*. However, resistance to *B. cinerea* in the *cdk8* mutant is linked to changes in the cuticle profile, which is epistatic to the functions of CDK8 in regulating secondary metabolites and plant defensins. Resistance due to cuticle permeability was previously attributed to faster activation of defense, faster mobility of defense compounds to the infection site (Bessire et al., 2007), efficient perception of cutin monomers in the absence of intact cuticle, and attenuated fungal virulence due to loss of plant-derived cues (Chassot et al., 2007). In a comprehensive study involving many *Arabidopsis* mutants with altered cuticles and having permeable cuticles, resistance in cuticle mutants was suggested to be independent of the cuticle composition per se but due to the overactivation of defense (Voisin et al., 2009). In *cdk8*, changes in cuticle integrity may have resulted in reduced activation of virulence in *B. cinerea* but not *A. brassicicola*. Histological observation of infected tissue through trypan blue staining also clearly showed that *B. cinerea* has attenuated virulence and fungal growth that is significantly retarded on the *cdk8* mutant. Consistent with this, many *Arabidopsis* mutants with permeable cuticles, including *long-chain acyl CoA synthase2*, are resistant to *B. cinerea* but not susceptible to *A. brassicicola*. The disease response of the *cdk8 med25* double mutant suggests that the *cdk8* mutation is epistatic to *med25* with respect to *B. cinerea* resistance but synergistic with respect to *A. brassicicola* resistance and JA responses. Despite the extreme susceptibility of *med25* to *B. cinerea*, *cdk8 med25* is resistant to *B. cinerea*, which further strengthens the observation that the altered cuticle in *cdk8* overrides defects in other plant immune responses.

### **Mediator and Plant Immunity**

Many recent reports implicate plant Mediator in the regulation of gene expression underlying various physiological processes, including hormone responses, flowering time, and biotic and abiotic stress tolerance (Canet et al., 2012; Chen et al., 2012; Wathugala et al., 2012; Zhang et al., 2012; An and Mou, 2013). Additional functions will likely be discovered as research in plant Mediator expands. The challenge, however, is to dissect the sequence of molecular and biochemical actions of Mediator as it relates to other regulators, including TFs. Also, the post-transcriptional regulation of Mediator subunits will be an interesting layer to study, as that adds complexity and functional diversity to the functions of the complex. Most Mediator subunits are implicated in defense, most likely, because pathogen

response may be an obvious phenotypic read out for Mediator functions in other fundamental cellular and molecular processes. Direct defense function was recently provided for a Mediator subunit when it was discovered that Mediator is a target of pathogen virulence. The *Hyaloperonospora arabidopsidis* nucleus-localized effector HaRxL44 interacts with MED19a, resulting in the degradation of MED19a in a proteasome-dependent manner. HaRxL44 interferes with Mediator function by degrading MED19, shifting the balance of defense transcription from SA-responsive defense to JA/ethylene signaling and enhancing susceptibility to biotrophs by attenuating SA-dependent gene expression (Caillaud et al., 2013).

In conclusion, this work demonstrates the impacts of the kinase module of the Mediator complex on the regulation of a major group of plant defense molecules, secondary metabolites, and defense-linked TFs as well as the development of the plant cuticle. The distinct responses of CDK8 in plant defense to related pathogens are the consequences of its regulatory impacts on cuticle development on the one hand and plant defensins and secondary metabolites on the other. The *cdk8* mutant displays unique immune responses that combine susceptibility to *A. brassicicola* and *P. syringae* with resistance to *B. cinerea*. Thus, CDK8 provides a powerful genetic tool for dissecting differences in plant immunity to the two related necrotrophs.

## **METHODS**

### **Plant Materials, Growth Conditions, and Disease Assays**

All mutant and transgenic *Arabidopsis thaliana* lines used in the studies described here are in the Columbia-0 (Col-0) ecotype background unless indicated otherwise. *cdk8-1* (SALK\_138675), *cdk8-2* (SALK\_016169), *med12* (SALK\_108241), *med13* (SALK\_018056), *med25* (SALK\_129555C), *aact1* (SALK\_097380), *cycCa* (SAIL\_102\_B02), *cycCb* (SALK\_053291C), and *cycCab* (SALK\_039400C) were obtained from the ABRC. The T-DNA insertions were confirmed by genomic DNA-PCR using the primers listed in Supplemental Data Set 1.

For root growth response assay to JA, *Arabidopsis* seeds were surface-sterilized and incubated at 4°C for 2 d before plating on half-strength Murashige and Skoog medium containing 0.8% agar and 1% sugar with the indicated concentration of MeJA. Then, the seeds were germinated under continuous light at 22°C for 7 to 10 d.

Plant growth conditions, fungal growth media, and fungal disease assays were as described previously (Dhawan et al., 2009; Laluk and Mengiste, 2011). In brief, plants were grown in growth chambers at 24°C, 70% relative humidity, and a 12-h-light/12-h-dark cycle. For *Botrytis cinerea* assay, plants were grown for 5 weeks in growth chambers under the above conditions and then inoculated with a conidial suspension of 250,000 to 300,000 spores/mL in 1% Sabouraud Maltose Broth buffer. For *Alternaria brassicicola* assay, at least 10 fully expanded adult leaves were excised and placed in plastic trays lined with wet filter paper for each biological replicate. Leaves were inoculated with 500,000 spores/mL in 1% Sabouraud Maltose Broth buffer. The transparent trays with leaves were sealed to maintain high humidity. Disease lesion diameters were measured after 3 to 6 d. All disease assays were repeated at least four times.

### **Trypan Blue Staining**

Trypan blue stock solution contains 10 g of phenol, 10 mL of lactic acid, 10 mL of glycerol, 10 mL of distilled water, and 40 mg of trypan blue (Sigma-Aldrich). Trypan blue working solution is made by mixing 2

volumes of 96% ethanol and 1 volume of trypan blue stock solution. After standard inoculation with conidial suspensions, the detached leaves were incubated in boiling trypan blue working solution for 1 min. Then, the leaves were transferred to wells of cell culture plates and immersed in chloral hydrate solution (250%, w/v) until chlorophyll was removed. The stained leaves were then observed with a Leica imaging system or the photographs were taken using a 10×/0.25 numerical aperture objective on a Nikon Microphot SA wide-field fluorescence microscope equipped with a CCD camera (ORCA-ER C4742-95; Hamamatsu Photonics).

### Molecular Cloning

The full-length coding sequences of CDK8, MED25, WIN1, ERF1, ORA59, ERF4, MYC2, JAZ1, CycCa, CycCb, and other genes described in our studies were amplified by PCR from wild-type Col-0 *Arabidopsis* cDNA using the primers listed in Supplemental Data Set 1. For Gateway-based cloning, fresh PCR products were first cloned into pENTER vector (Invitrogen) and then transferred to destination vectors that contained N/C-terminal fragments of LUC via LR reactions (according to the Invitrogen manual). For Co-IP, the indicated genes were transferred to pHBT95-HA/MYC vectors from sequenced templates via transfer PCR (Erijman et al., 2011).

The CDK8<sup>D176A</sup> point mutation was generated by overlapping PCR with the primers listed in Supplemental Data Set 1. Full-length sequence of CDK8 and CDK8<sup>D176A</sup> were cloned into pGEX-4T-2 for recombinant protein expression and *in vitro* kinase assay and subsequently inserted into the binary vector pBA-MYC for generating transgenic plants. All constructs were verified by sequencing.

### Generation of *Arabidopsis* Transgenic Plants and Double Mutant

The constructs with the expression cassettes 35S:CDK8-MYC and 35S:CDK8<sup>D176A</sup>-MYC were transformed into *cdk8-1* mutant plants by *Agrobacterium tumefaciens*-mediated transformation (Clough and Bent, 1998). Primary transformants were selected for Basta resistance, and CDK8 protein expression levels were determined by protein gel blotting with anti-MYC antibody (1:5000 dilution; Abcam). Transgenic lines with a 3:1 (resistant:sensitive to Basta) segregation ratio were selected, and at least three homozygous lines were identified in the T3 generation. The *med25 cdk8* double mutant was generated by crossing, and homozygous double mutant individuals were identified by genomic DNA-PCR.

### RNA Extraction, Reverse Transcription, and RT-qPCR

Total RNA was extracted from *Arabidopsis* leaves or seedlings with Trizol reagent according to the manufacturer's instructions (Sigma-Aldrich). After RNase-free DNase I treatment (Ambion), total RNA was reverse-transcribed into cDNA (New England Biolabs). RT-qPCR was performed with a SYBR Green kit (Bio-Rad) according to the manufacturer's instructions using gene-specific primers with *Arabidopsis ACT2* as an endogenous reference for normalization. A minimum of three technical replicates of the RT-qPCR assay were used for each sample with at least three biological replicates. Primers used are listed in Supplemental Data Set 1.

### RNA-seq and Analysis

Five-week-old wild-type (Col-0) and *cdk8-1* plants grown on soil were mock-inoculated or inoculated with *B. cinerea*. The leaves were collected at 36 h after inoculation for RNA isolation. The RNA-seq data analysis and sequence quality control were performed according to Lai et al. (2014). Gene expression levels were determined by reads per kilobase per million reads. Genes with more than 2-fold changes (*P* value and false discovery rate  $\leq 0.05$ ) in *cdk8* compared with wild-type plants were selected as

differentially expressed genes. The differentially expressed gene expression patterns in the wild type and *cdk8* were presented as a heat map using the R statistical computing package.

### Split-Luciferase Complementation Assays

Transient expression in *Arabidopsis* protoplasts was conducted as described previously (Yoo et al., 2007; Zhu et al., 2010). All plasmids used in this assay were purified with the Qiagen Plasmid Maxi Kit or the Qiagen Plasmid Midi Kit. Ten micrograms of purified plasmid of each construct was used for protoplast transformation. After coexpression, the substrate LUC was added and incubated in the dark for at least 1 h. The recombinant LUC activity was measured using a Wallac VICTOR2 plate reader.

### Co-IP

Purified plasmids were transiently expressed in *Arabidopsis* protoplasts as described (Yoo et al., 2007; Zhu et al., 2010). After transformation, protoplasts were harvested and suspended in 1 mL of lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.1% [v/v] Triton X-100, and 1× protease inhibitor cocktail from Sigma-Aldrich plus 1 mM phenylmethylsulfonyl fluoride) and then centrifuged at 12,000 rpm for 10 min at 4°C. Fifty microliters of the supernatant served as input, and the rest of the supernatant was incubated with 20  $\mu$ L of prewashed monoclonal anti-HA agarose antibody (Sigma-Aldrich) at 4°C for at least 4 h with gentle rotation. The agarose was washed four times with lysis buffer and boiled in 50  $\mu$ L of 1× SDS loading buffer for 5 min. Protein samples were then subjected to protein gel blot analysis and detected with anti-HA or anti-MYC antibody (1:5000 dilution; Abcam).

### Transcriptional Activation Assay

*Arabidopsis* protoplasts were isolated as described (Yoo et al., 2007; Zhu et al., 2010). Protoplasts were cotransformed with 35S:full-length LUC for an internal control, *PDF1.2* promoter-GUS, and effector plasmid construct 35S:ERF1-MYC or 35S:ORA59-MYC, which were described previously (Çevik et al. 2012). After overnight transformation, protoplasts were suspended on ice in 20  $\mu$ L of lysis buffer containing 2.5 mM Tris-phosphate, pH 7.8, 1 mM DTT, 2 mM DACTAA, 10% glycerol, and 1% Triton X-100. Protoplast lysates mixed with 20  $\mu$ L of LUC substrate (Promega) were measured with a plate reader for LUC activity. At the same time, 5  $\mu$ L of protoplast lysate was mixed with 10  $\mu$ L of 4-methylumbelliferyl  $\beta$ -D-glucuronide substrate buffer (10 mM Tris-HCl, pH 8, 1 mM 4-methylumbelliferyl  $\beta$ -D-glucuronide, and 2 mM MgCl<sub>2</sub>) for at least 1 h at 37°C, and the reaction was stopped by the addition of 100  $\mu$ L of 0.2 M Na<sub>2</sub>CO<sub>3</sub>. GUS activity was also measured using a plate reader. GUS activities were normalized by internal LUC activities from the same sample, and at least three biological replicates were analyzed.

### In Vitro and Immunocomplex Kinase Assays

Recombinant GST-CDK8 or GST-CDK8<sup>D176A</sup> was incubated in 25  $\mu$ L of kinase reaction buffer (50 mM Tris, pH 7.5, 1 mM DTT, 10 mM MgCl<sub>2</sub>, 1  $\mu$ M ATP, and 5  $\mu$ Ci of [ $\gamma$ -<sup>32</sup>P]ATP) with 1  $\mu$ g of MBP at room temperature for 30 min. The reaction was stopped by the addition of SDS sample buffer, and the mixtures were then subjected to SDS-PAGE. Fourteen-day-old *cdk8*;35S:CDK8-MYC or *cdk8*;35S:CDK8<sup>D176A</sup>-MYC transgenic seedlings were used for the immunoprecipitation kinase assay. After immunoprecipitates were washed with 1 mL of kinase reaction buffer (50 mM Tris-Cl, pH 7.5, 10 mM MgCl<sub>2</sub>, and 1 mM DTT), kinase assays were performed at room temperature for 30 min in buffer containing 25 mM ATP, 1  $\mu$ M ATP, 5  $\mu$ Ci of [ $\gamma$ -<sup>32</sup>P]ATP, and histone. After electrophoresis on a 12% SDS gel, the phosphorylated histone was visualized by autoradiography.

### Transmission Electron Microscopy

For transmission electron microscopy analysis, the ninth leaves from 4-week-old plants were fixed by the microwave method as described previously (Dhawan et al., 2009). The primary fixation was performed with buffer containing 2% paraformaldehyde, 2.5% glutaraldehyde, and 0.1 M potassium phosphate buffer, pH 6.8. Secondary fixation was performed with buffer containing 1% OsO<sub>4</sub> and 1.5% K<sub>3</sub>Fe(CN)<sub>6</sub>. After fixation, samples were dehydrated in an ethanol series and propylene oxide. Samples were embedding in polymerized resin. Ultrathin sections were viewed with an FEI/Philips CM-100 transmission electron microscope.

### Measurement of HCAAs by LC-MS

Wild-type and *cdk8* mutant plants were inoculated with *A. brassicicola*, and at 48 h after inoculation, ~200 mg of *Arabidopsis* leaves was extracted with 2 mL of methanol overnight at 25°C. HCAAs were analyzed with a Waters 600 LC-MS instrument (Agilent QQQ 6460 system coupled to a 1200 series liquid chromatography pump and autosampler) as described (Muroi et al., 2009).

### ChIP-qPCR

ChIP assays were performed according to Saleh et al. (2008) with minor modifications. Basically, 1 g of 14-d-old *cdk8;35S:CDK8-MYC* seedlings or 4 g of 30-d-old leaves of wild-type, *cdk8;35S:CDK8-MYC*, or *cdk8;35S:CDK8<sup>D176A</sup>-MYC* transgenic plants were used for each assay. Chromatin was isolated and sonicated. One hundred microliters of immunoprecipitate was diluted 10 times with nuclear lysis buffer and incubated at 4°C overnight with anti-MYC or anti-RPB2 antibody (Abcam) or rabbit IgG as a negative control. The protein-chromatin complexes were then captured by salmon sperm DNA/protein A agarose (Millipore). After washing and reverse cross-linking, the precipitated DNA was purified with a PCR clean-up kit. RT-qPCR was performed with negative control (IgG-precipitated DNA) and input control (the chromatin before precipitation). The ChIP enrichment was normalized to the input and then converted to fold change. All primers are listed in Supplemental Data Set 1.

### Toluidine Blue Staining and Water Loss Measurement

Fully expanded adult leaves were excised and submerged in 0.05% toluidine blue solution. The stain was allowed to incubate up to 1 h at room temperature. At least eight leaves from each genotype were tested, and the staining experiments were repeated at least three times. For water loss experiments, leaves of 5-week-old plants were detached and left at room temperature, and the weight (~20 leaves) was recorded. Water loss was expressed as the percentage of initial fresh weight.

### Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL libraries under the following accession numbers: *CDK8* (AT5G63610), *MED12* (AT4G00450), *MED13* (AT1G55325), *CYCCa* (AT5G48630), *CYCCb* (AT5G48640), *MED25* (AT1G25540), *ERF1* (AT3G23240), *ORA59* (AT1G06160), *AACT1* (AT5G61160), *WIN1* (AT1G15360), *ERF2* (AT5G47220), *ERF4* (AT3G15210), *CER1* (AT1G02205), *CER2* (AT4G24510), *CER3* (AT5G57800), *CER4* (AT4G33790), *CER6* (AT1G68530), *CER7* (AT3G60500), *LTP3* (AT5G59320), and *KCS1* (AT1G01120).

### Supplemental Data

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

**Supplemental Figure 1.** Disease Symptoms and Fungal Growth Revealed by Trypan Blue Staining of *B. cinerea*-Inoculated Leaves.

**Supplemental Figure 2.** Venn Diagram and Heat Map Presenting Differential Expression of Genes in the Wild Type and *cdk8* after Mock or *B. cinerea* Inoculation.

**Supplemental Figure 3.** The Expression of *PDF1.2*, *ORA59*, and *PDF1.3* in the Wild Type and *cdk8* in Response to *A. brassicicola*.

**Supplemental Figure 4.** Protein Gel Blot Confirming Equal Protein Amounts Used for Transactivation Assays.

**Supplemental Figure 5.** The Expression of Cuticle Wax Biosynthesis Genes in the Wild Type and *cdk8* Mutant.

**Supplemental Figure 6.** The CDK8 Target Gene *AACT1* Is Required for Resistance to *B. cinerea*.

**Supplemental Figure 7.** The Glossy Leaf Surface of *cdk8* Mutant Compared with Wild-Type Plants.

**Supplemental Figure 8.** Growth and Cuticle Permeability of *cdk8* and *med25* Single and Double Mutants.

**Supplemental Figure 9.** *B. cinerea* Disease Symptoms of *cdk8*;WIN1-HA Plants.

**Supplemental Figure 10.** Amino Acid Sequence Comparisons between Full-Length Plant, Human, *Drosophila*, and Mouse CDK8 Proteins.

**Supplemental Figure 11.** Protein Gel Blot Showing Plants Expressing Wild-Type and Kinase-Dead CDK8 Protein.

**Supplemental Figure 12.** *Arabidopsis* Mutants *med12* and *med13* in CDK8 Kinase Module Have Altered Growth.

**Supplemental Figure 13.** Disease Symptoms in *cycCa* and *cycCb* Single Mutants after Inoculation with *B. cinerea* and *A. brassicicola*.

**Supplemental Data Set 1.** Primer Sequences Used in This Study.

**Supplemental Data Set 2.** Differential Expression of Transcription Factors in *cdk8* Compared with Wild-Type Plants after *B. cinerea* Infection.

**Supplemental Data Set 3.** List of Differentially Expressed Genes in *cdk8* Compared with the Wild Type from RNA-seq Analysis.

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### AUTHOR CONTRIBUTIONS

Y.Z. conducted most of the experiments. Y.Z. and T.M. designed most of the experiments and directed the project. C.M.S. performed the initial mutant screen that identified the *cdk8* and *cyclin* mutants. F.F. and J.T. performed the analyses of RNA-seq data. P.W. conducted the kinase assays. T.M., J.-K.Z., D.-J.Y., and S.Y.L. analyzed the data and wrote the article.

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