Arabidopsis WRKY33 Is a Key Transcriptional Regulator of Hormonal and Metabolic Responses toward *Botrytis cinerea* Infection[1][W]

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The Arabidopsis (*Arabidopsis thaliana*) transcription factor WRKY33 is essential for defense toward the necrotrophic fungus *Botrytis cinerea*. Here, we aimed at identifying early transcriptional responses mediated by WRKY33. Global expression profiling on susceptible *wrky33* and resistant wild-type plants uncovered massive differential transcriptional reprogramming upon *B. cinerea* infection. Subsequent detailed kinetic analyses revealed that loss of WRKY33 function results in inappropriate activation of the salicylic acid (SA)-related host response and elevated SA levels post infection and in the down-regulation of jasmonic acid (JA)-associated responses at later stages. This down-regulation appears to involve direct activation of several *jasmonate ZIM-domain* genes, encoding repressors of the JA-response pathway, by loss of WRKY33 function and by additional SA-dependent WRKY factors. Moreover, genes involved in redox homeostasis, SA signaling, ethylene-JA-mediated cross-communication, and camalexin biosynthesis were identified as direct targets of WRKY33. Genetic studies indicate that although SA-mediated repression of the JA pathway may contribute to the susceptibility of *wrky33* plants to *B. cinerea*, it is insufficient for WRKY33-mediated resistance. Thus, WRKY33 apparently directly targets other still unidentified components that are also critical for establishing full resistance toward this necrotroph.

Plants are constantly challenged by pathogens, but only a few are capable of successfully colonizing a specific host, suggesting the existence of potent recognition and defense mechanisms. In general, there are two types of microbial pathogens that grossly differ in their lifestyles (Glazebrook, 2005). Biotrophic pathogens require living plant cells for growth and reproduction. They tend to keep the colonized plant tissue widely intact for long-term nutritional benefits. By contrast, necrotrophic pathogens need to kill plant cells to make nutrients accessible for growth and for completion of their life cycles. Whereas one general defense strategy of plants against biotrophic pathogens is to sacrifice infected cells by triggering cell death, the maintenance of plant cell viability is generally the defense strategy against necrotrophic pathogens (Spoel et al., 2007).

To discriminate between these distinctive pathogens and to activate appropriate responses, plants use phytohormones for signaling (Pieterse et al., 2009). Studies using transgenic and mutant lines impaired in hormone biosynthesis, perception, or signaling revealed that responses against biotrophic pathogens are in general regulated by salicylic acid (SA; Vlot et al., 2009), while responses to necrotrophs are mediated by jasmonic acid (JA) and ethylene (ET; Farmer et al., 2003). In case of multiple infections or when plants were treated simultaneously with methyl jasmonate and SA, substantial cross talk between the SA and JA-response pathways was observed (Spoel et al., 2007; Koornneef et al., 2008). Often, the SA pathway was shown to be dominant and to suppress the JA pathway. However, synergistic effects between both pathways have been reported when low concentrations of SA and JA were applied, indicating that the concentration and the balance of these hormones as well as their temporal activation kinetics may be decisive (Koornneef and Pieterse, 2008; Leon-Reyes et al., 2010). A recent comprehensive study revealed that synergistic and compensatory relationships exist among the response pathways and that they are important for optimal resistance to different pathogens (Tsuda et al., 2009).

Nectrotrophic fungi including *Alternaria brassicicola*, *Botrytis cinerea*, *Fusarium oxysporum*, and *Sclerotinia sclerotiorum* are the largest class of fungal plant pathogens and cause serious crop losses worldwide (Łaźniewska et al., 2010). In contrast to numerous biotrophic pathogens, specific recognition of necrotrophic pathogens by products of major plant resistance (*R*) genes is currently unknown. Indeed, the resistance of Arabidopsis

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(*Arabidopsis thaliana*) to *B. cinerea* appears to be under complex genetic control (Rowe and Kliebenstein, 2008). Numerous genetic and pharmacological studies have uncovered plant genes and products that influence the outcome of host-*B. cinerea* interactions. These include enzymes of cutin biosynthesis and secondary cell wall formation (Hernández-Blanco et al., 2007; Tang et al., 2007; Voisin et al., 2009; Raiola et al., 2011), the receptor-like kinase BIK1 (Veronese et al., 2006), the TIR domain-encoding gene *RLM3* (Staal et al., 2008), the mitogen-activated protein kinase MPK3 (Ren et al., 2008), the RING E3 ligases HUB1 and BOI (Dhawan et al., 2009; Luo et al., 2010), and several autophagy genes (Lai et al., 2011; Lenz et al., 2011). Certainly, one critical component appears to be the phytoalexin camalexin, because plants deficient in *CYP71B15* (*PAD3*), which catalyzes the last step in camalexin biosynthesis, are highly susceptible to *B. cinerea* (Ferrari et al., 2007).

Global transcriptional profiling studies also revealed that *B. cinerea* infection of Arabidopsis leaves results in massive transcriptional reprogramming in the host, indicating the involvement of key regulators in this process (AbuQamar et al., 2006; Ferrari et al., 2007; Rowe et al., 2010). As expected, several transcription factors (TFs) have been identified that positively or negatively affect the outcome of such an interaction. Loss of function of the helix-loop-helix Leu zipper TF MYC2, which antagonistically regulates two distinct branches of the JA pathway, rendered plants more resistant toward *B. cinerea* (Lorenzo et al., 2004). A similar resistant phenotype was observed in mutant plants defective in MYB46 function and in the double mutant *anac019 anac055* encoding two NAC family proteins (Bu et al., 2008; Ramírez et al., 2011). In contrast, loss-of-function mutants of *MYB108/BOS1* and of *EIN3*, encoding an AP2-type TF involved in ET signaling, showed enhanced susceptibility to *B. cinerea* infection (Mengiste et al., 2003; Van Baarlen et al., 2007). Moreover, overexpression of the AP2-type TF genes *ERF1* and *ORA59* rendered plants more resistant toward several necrotrophs, including *B. cinerea* (Berrocal-Lobo et al., 2002; Pré et al., 2008). ERF1 and ORA59 both appear to be key integrators of the JA and ET signaling pathways (Pieterse et al., 2009; Leon-Reyes et al., 2010; Zarei et al., 2011). In some cases, however, contradictory phenotypic results have been reported. This can be partly explained by the different experimental setups used, but recent reports also draw attention to the fact that they may also be due to isolate-specific differences of this pathogen (Rowe and Kliebenstein, 2010; Rowe et al., 2010).

The WRKY family of TFs also modulates host defenses toward various phytopathogens (Pandey and Somssich, 2009). In particular, WRKY33 was shown to be required for resistance toward the necrotrophs *A. brassicicola* and *B. cinerea* (Zheng et al., 2006). In that previous study, negative regulation of the SA pathway and positive regulation of the JA pathway by *WRKY33* were concluded based on the higher expression of *PR* genes and the lower expression of JA-response genes in *wrky33* compared with wild-type plants upon *B. cinerea* challenge. Clues as to how WRKY33 may transduce pathogen-triggered host signals to the nucleus were provided by work showing that WRKY33 interacts with MPK4 and the protein MKS1 within the nucleus and that, upon challenge with the hemibiotrophic pathogen *Pseudomonas syringae* or upon elicitation by the microbe-associated molecular pattern flg22, WRKY33 is released from the trimeric complex and subsequently binds to the *PAD3* promoter (Andreasson et al., 2005; Qiu et al., 2008). Whether the same signal transduction cascade is utilized during infections with necrotrophic pathogens remains uncertain, since recent studies imply that WRKY33 is phosphorylated by the mitogen-activated protein kinases MPK3 and MPK6 in vivo upon *B. cinerea* infection (Mao et al., 2011). Finally, ATG18a, a critical autophagy protein, was also shown to interact with WRKY33 within the cell nucleus (Lai et al., 2011).

In this study, we performed a detailed transcriptomic analysis in order to obtain a deeper view of the transcriptional responses mediated by WRKY33 upon challenge with *B. cinerea*. We identified numerous genes encoding TFs, components of the SA, JA, and ET signaling pathways, and redox regulators that were misexpressed in the *wrky33* mutant. By chromatin immunoprecipitation (ChIP), several direct in vivo target genes of WRKY33 were identified. In infected *wrky33* plants, the SA response pathway was inappropriately activated, which later during infection resulted in SA-mediated repression of JA responses. This repression involved deregulated expression of the WRKY33 direct target genes *TRX-H5*, *jasmonate ZIM-domain1* (*JAZ1*), *JAZ5*, *ORA59*, and *GLIP1*. Additional genetic studies, however, showed that down-regulation of JA responses by the premature activation of SA-mediated defenses on its own was insufficient to lead to susceptibility in *wrky33* plants. Rather, WRKY33 appears to regulate the expression of various distinct components of defense pathways that are crucial in activating appropriate host responses toward *B. cinerea*.

RESULTS

Transition from Nonaggressive to Aggressive Growth of *B. cinerea* on the *wrky33* Mutant

WRKY33 is involved in the regulation of defense against the necrotrophic fungi *A. brassicicola* and *B. cinerea* (Zheng et al., 2006). To analyze differences in the development of *B. cinerea* isolate 2100 on *wrky33* compared with wild-type Columbia-0 plants, we collected spray-inoculated leaves at time points 16, 24, 40, 48, and 64 h post inoculation (hpi) and stained them either with 3,3-diaminobenzidine (DAB) to visualize hydrogen peroxide (H_2O_2) accumulation, a major component of reactive oxygen species (ROS), or with trypan blue to detect the fungus and dying host cells. Mock-treated leaves revealed no cell death or H_2O_2

production for both genotypes (Supplemental Fig. S1). On infected leaves, spotty trypan blue staining, indicative of dying cells, was clearly visible at 40 hpi predominantly in mesophyll cells of the wild type and more pronounced in *wrky33*. Microscopically, we could not detect significant differences in *B. cinerea* development between the two genotypes up to 40 hpi (Fig. 1, A–D). The spores germinated with the same high efficiency (nearly 100%), and hyphal growth was indistinguishable. Later than 40 hpi, the fungus switched from nonaggressive to aggressive growth only in infected *wrky33* leaves, manifested by a boost of hyphal growth that gave rise to rapidly expanding lesions on the leaves. In wild-type plants during the same period, necroses became visible, which were associated with fungal growth arrest (Fig. 1, E and F). Conidiophores appeared 3 dpi only on the susceptible *wrky33* plants.

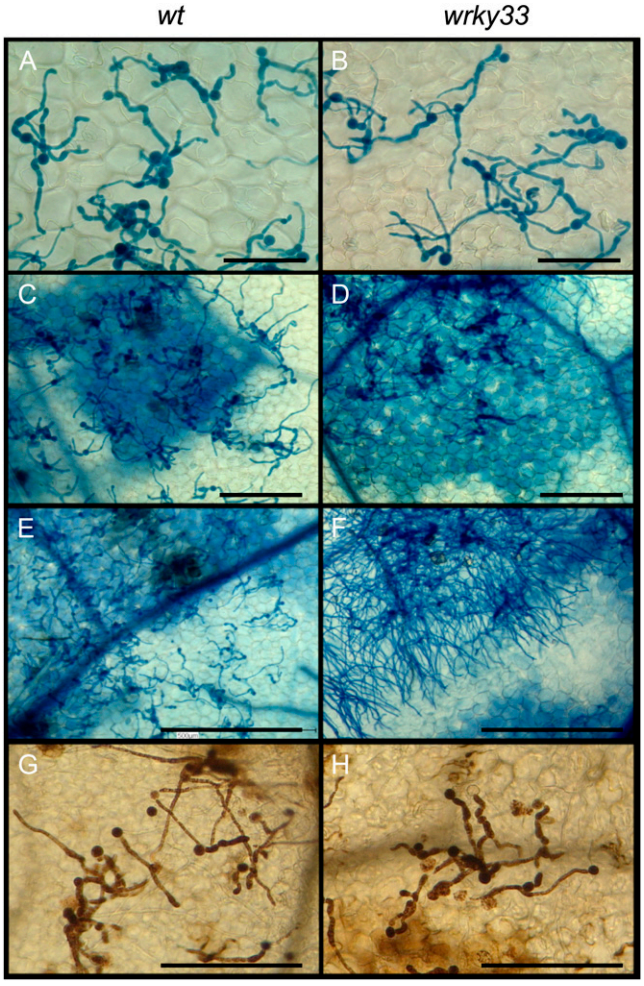

Figure 1. Fungal development and ROS production on wild-type (wt) and *wrky33* plants. Leaves from 4-week-old wild-type and *wrky33* plants were spray inoculated with *B. cinerea* and analyzed for fungal growth and the development of cell death by staining with trypan blue at 24 hpi (A and B), 40 hpi (C and D), and 64 hpi (E and F). To visualize H_2O_2, leaves were stained with DAB at 16 hpi (G and H). Bars = 100 μm in A, B, G, and H and 500 μm in C to F.

Accumulation of H_2O_2 in leaves was observed in both the wild type and *wrky33* already at 16 hpi, mainly in the mesophyll cells. Moreover, consistent with findings by Temme and Tudzynski (2009), the fungus itself exhibited strong DAB staining in both genotypes up to 24 hpi, indicating that it produced ROS (Fig. 1, G and H).

Temporal Expression of *WRKY33* during *B. cinerea* Infection

In order to monitor the WRKY33 protein in subsequent experiments, a complementation line was created expressing a *WRKY33* genomic construct with a C-terminal hemagglutinin (HA) tag under the control of its native promoter in the *wrky33* mutant (designated WRKY33-HA). Upon droplet inoculation with *B. cinerea* spores, this line exhibited the same resistance as wild-type plants, validating its functionality (Supplemental Fig. S2).

To identify changes in the transcriptome related to WRKY33 action during infection with *B. cinerea*, we first investigated the expression profile of *WRKY33* itself. Samples from wild-type and *wrky33* spray-inoculated or mock-treated plants taken at various time points were analyzed by quantitative real-time (qRT)-PCR. In the wild type, compared with its basal levels in mock-treated plants, WRKY33 RNA levels were induced about 6-fold at 8 hpi and increased further during the course of infection (Fig. 2A). In the *wrky33* mutant, no WRKY33 transcript was detected.

The appearance and stability of the WRKY33 protein during infection were determined by western blotting using total leaf extracts of the spray-inoculated WRKY33-HA complementation line (Fig. 2B). WRKY33-HA protein was clearly detectable at 8 hpi, consistent with the onset of induction at the RNA level. Maximal protein accumulation was observed at 14 hpi and remained constant until 24 hpi, after which it decreased. Induction of WRKY33 mRNA upon *B. cinerea* inoculation after 8 h, and highest accumulation of the corresponding protein at 14 hpi, led us to set the time point for subsequent global expression profiling to 14 hpi.

B. cinerea Infection Massively Alters Gene Expression in Wild-Type and *wrky33* Plants

To identify genes involved in the *WRKY33*-dependent response to *B. cinerea* on a genome-wide level, we used Arabidopsis whole genome (ATH1) microarrays. For this, total RNAs derived from wild-type or *wrky33* plants 14 h after spray inoculation or mock treatment were compared. At this infection stage, *B. cinerea* conidia had already germinated but the hyphae were still only in the process of elongation and no infection hyphae were yet detected. In wild-type plants, the expression of 1,577 genes was altered 2-fold or more ($P \leq 0.05$) compared with noninfected plants, with 1,154 genes being up-regulated and 423 genes being down-regulated. In *wrky33*, upon infection, the expression of 2,387 genes,

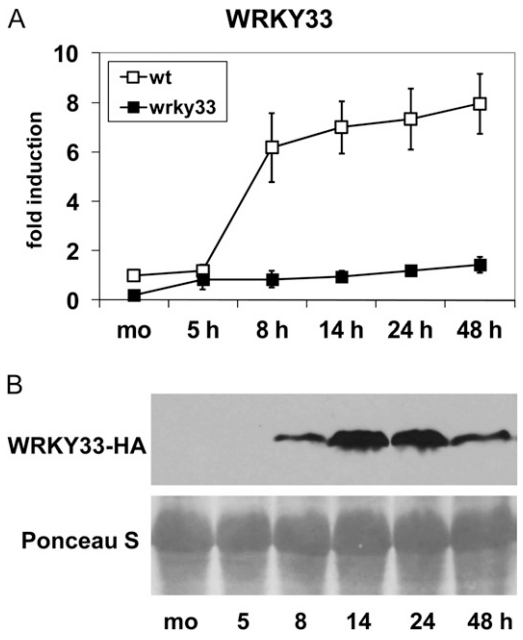

Figure 2. Kinetics of *WRKY33* expression upon *B. cinerea* infection. A, Wild-type (wt) and *wrky33* plants were spray inoculated with *B. cinerea*, and leaf samples were taken at the indicated time points to quantify the transcript levels of *WRKY33* and other genes (subsequent figures) by qRT-PCR. Data were normalized to the expression of At4g26410 and are expressed relative to mock-treated (mo) wild-type plants set to 1. Error bars represent SD of three biological replicates ($n = 3$). B, WRKY33 protein accumulation during infection was monitored by using the WRKY33-HA complementation line. Plant treatment and sample collection were as in A. Protein levels were visualized by western blotting using an anti-HA antibody. As a loading control, a section of the filter stained with Ponceau S is shown.

1,369 up-regulated and 1,018 down-regulated, was altered more than 2-fold, with a common set of 1,379 genes showing similar changes upon infection in wild-type and *wrky33* plants (Supplemental Table S1). While upon infection the expression of 198 genes was changed only in the wild type, differential expression of 1,008 genes was triggered by the loss of WRKY33 function. Comparing the expression profiles from noninfected plants identified 309 genes that were constitutively differentially expressed between the wild type and *wrky33*, 212 of them up-regulated and 97 down-regulated (Fig. 3). Based on Gene Ontology terms, genes differentially expressed in the wild type and *wrky33* upon infection were clearly overrepresented in the categories responsive to stress and responsive to abiotic or biotic stimuli (Supplemental Table S2).

The broad reprogramming of the host transcriptome at this early infection stage (14 hpi) suggested the involvement of many TFs. Indeed, *B. cinerea* infection altered the expression levels of 110 and 160 TF genes more than 2-fold in the wild type and *wrky33*, respectively, with an overlap of 97 TF genes affected in both (Supplemental Table S3). TF families described to be involved in pathogen defense (Libault et al., 2007) were similarly represented in both genotypes. Most prominent were genes encoding AP2-ethylene-responsive element-binding proteins, NAC TFs, and WRKY TFs (Supplemental Table S3). Among the 34 TF genes differentially expressed in infected *wrky33* mutants relative to wild-type plants were, in particular, the AP2-ERF TF genes ERF2, CEJ1, and ORA59, which are involved in the regulation of JA and ET defense genes (McGrath et al., 2005), and CBF1 and CBF2, involved in the cold stress response (Novillo et al., 2007). Also included were the NAC genes NAC016, NAC036, and NAC090, WRKY18, WRKY38, and WRKY54, which are positive regulators of SA-mediated responses downstream of NPR1 (Wang et al., 2006), WRKY46 and WRKY60, and MYB2, which is responsive to dehydration and abscisic acid (Abe et al., 2003). WRKY50 and WRKY51, not represented on the ATH1 microarray but recently shown to negatively influence the outcome of Arabidopsis-*B. cinerea* interactions (Gao et al., 2011), were analyzed by qRT-PCR and found to be also differentially expressed (Supplemental Fig. S3). Except for *ERF2* and *ORA59*, which appeared to be positively controlled by WRKY33, all other mentioned TF genes were up-regulated upon infection in the mutant, suggesting that they are under direct or indirect negative regulation by WRKY33 (Supplemental Table S3).

In *wrky33* the JA/ET Signaling Pathways Are Functional Early during *B. cinerea* Infection but the JA Response Is Repressed at Later Stages

Our microarray studies revealed that genes involved in the biosynthesis of JA as well as the JA-response genes were up-regulated at 14 hpi in the wild type and *wrky33* (Table I). There was no significant difference between wild-type and *wrky33* plants, with the exception of the defensin *PDF1.1* and the *LIPOXYGENASE2* genes, whose mRNA levels were higher in wild-type plants.

To verify and extend the expression data for selected genes, total RNA samples collected at different time points after inoculation were subjected to qRT-PCR analysis. The induction profiles for two genes encoding key enzymes in JA biosynthesis, *AOC3* and *OPR3*, showed no differences between the wild type and *wrky33* up to 14 hpi (Fig. 4A). However, at 24 hpi, when fungal growth was still indistinguishable between the wild type and the mutant, there was a significant increase in the expression of both genes only in the mutant. While expression of *JAR1*, encoding the enzyme that converts JA to the bioactive JA-Ile conjugate, and the regulatory gene *COI1* was similar in both genotypes and not affected by inoculation, the JA-response genes *PDF1.1* and *PDF1.2* were strongly up-regulated at 24 hpi only in the wild type. *PDF1.1* and *PDF1.2* expression levels were clearly also induced in *wrky33* at 14 hpi but returned to their basal levels thereafter (Fig. 4A). Together, these data show that up to 14 hpi, the JA pathway was similarly

Table I. *Differential expression of hormone pathway-associated genes upon B. cinerea infection*

Affymetrix ATH1 chips were hybridized with cDNAs derived from leaf total RNAs of *B. cinerea*-inoculated and mock-treated wild-type and *wrky33* plants (14 hpi). The columns show pathway, locus, gene annotation, and fold differences in transcript abundance ($P \leq 0.05$) between *B. cinerea*-infected wild-type versus mock-treated wild-type plants (wtBc/wtm), infected *wrky33* versus mock-treated *wrky33* plants (w33Bc/w33m), infected *wrky33* versus infected wild-type plants (w33Bc/wtBc), and mock-treated *wrky33* versus mock-treated wild-type plants (w33m/wtm).

Pathway	Locus	Gene Name	wtBc/wtm	w33Bc/w33m	w33Bc/wtBc	w33m/wtm
JA	AT3G45140	LOX2	7.10	3.75	−3.18	−1.68
	AT1G72520	LOX	13.41	12.95	1.16	1.20
	AT1G17420	LOX3	2.62	3.29	1.11	−1.13
	AT5G42650	AOS	1.56	1.67	1.05	−1.02
	AT3G25760	AOC1	4.97	3.45	−1.25	1.15
	AT3G25780	AOC3	6.07	10.70	1.98	1.12
	AT1G76680	OPR1	3.41	4.65	1.77	1.30
	AT2G06050	OPR3	4.91	4.67	−1.08	−1.03
	AT2G46370	JAR1	1.02	1.15	1.45	1.29
	AT2G39940	COI1	−1.13	1.06	1.02	1.03
	AT1G75830	PDF1.1	34.07	1.95	−15.74	1.11
	AT5G44420	PDF1.2a	39.05	22.06	−1.63	1.09
	AT2G26020	PDF1.2b	24.82	15.98	−1.10	1.41
	AT1G19180	JAZ1	9.43	4.36	−1.07	2.03
	AT1G17380	JAZ5	5.52	6.11	1.22	1.10
	AT5G13220	JAZ10	2.54	3.60	1.53	1.08
	AT1G70700	JAZ9	2.25	1.73	−1.14	1.14
	AT1G32640	MYC2	2.58	1.89	−1.14	1.20
SA	AT1G74710	ICS1/SID2	2.77	6.93	3.40	1.36
	AT3G48090	EDS1	2.06	2.84	1.89	1.37
	AT3G52430	PAD4	3.33	6.70	3.69	1.84
	AT4G39030	EDS5/SID1	9.91	22.02	2.65	1.19
	AT1G19250	FMO1	5.53	15.49	2.56	−1.09
	AT1G64280	NPR1	1.85	2.09	1.43	1.26
	AT5G13320	PBS3	3.29	17.92	9.73	1.79
	AT1G02450	NIMIN-1	1.77	16.67	10.01	1.06
	AT2G14610	PR1	13.16	12.21	2.24	2.36
	AT3G57260	PR2	13.09	11.55	3.15	3.57
	AT3G12500	PR3	1.45	2.65	2.36	1.29
ET	AT3G23240	ERF1	4.40	4.18	1.11	1.16
	AT5G47220	ERF2	8.83	2.34	−2.18	1.73
	AT3G15210	ERF4	8.84	6.14	1.38	1.99
	AT5G47230	ERF5	9.55	2.62	−1.54	2.37
	AT4G17490	ERF6	13.18	7.08	−1.09	1.70
	AT1G28370	ERF11	5.47	5.93	1.80	1.66
	AT2G31230	ERF15	2.68	1.80	1.13	1.68
	AT5G61600	ERF104	5.06	1.37	−1.24	2.98
	AT1G06160	ORA59	12.65	3.11	−2.61	1.55
	AT3G50260	CEJ1	3.44	6.96	2.20	1.09
	AT4G11280	ACS6	11.35	3.70	−1.41	2.17
	AT4G17500	ERF-1	9.03	4.73	−1.31	1.45
	AT3G23230	ERF put	7.93	14.44	1.75	−1.04
	AT2G22300	ERF put	2.35	2.37	−1.15	−1.16
	AT1G05710	ERF put	2.63	4.05	1.81	1.18
	AT5G40990	GLIP1	17.14	1.58	−12.09	−1.12

induced in both genotypes, while later during *B. cinerea* inoculation, the JA response was repressed in the *wrky33* mutant.

To investigate if enhanced expression of the JA biosynthetic enzymes led to higher JA levels in the infected mutant plants, we determined the amounts of free JA and the bioactive JA-Ile conjugate in leaves from infected wild-type and *wrky33* plants (Fig. 4B). Prior to inoculation, the levels of both substances were low and nearly identical in the wild type and *wrky33*. At 24 h after inoculation, there was a 3-fold increase in JA in the wild type but a more than 6-fold increase in *wrky33*. For JA-Ile, a slight increase was observed upon infection, with similar levels reached in both genotypes. These data show that the repression of the JA response in *wrky33* occurred downstream of JA biosynthesis. The fact that in *wrky33* the JA-Ile levels were not higher than in the wild type, despite

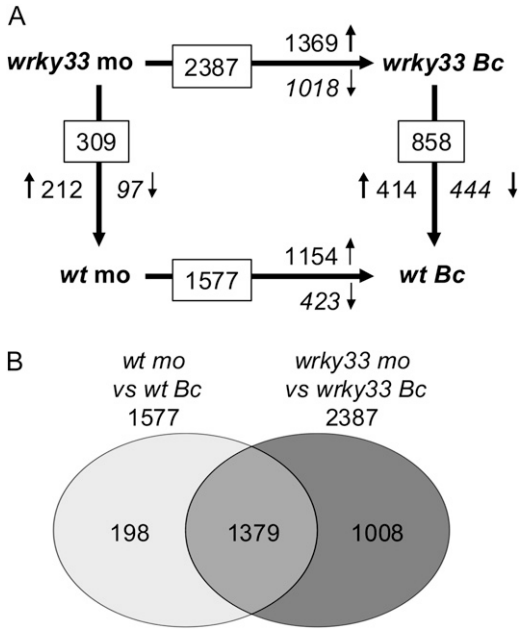

Figure 3. Transcriptional reprogramming in wild-type (wt) and *wrky33* plants upon *B. cinerea* infection. A, Numbers of differentially expressed genes (2-fold or greater; $P \leq 0.05$) between the wild type and *wrky33* at 14 h after mock treatment (mo) or *B. cinerea* spray inoculation (Bc). Indicated are total numbers (boxes) and numbers of up- and down-regulated genes (arrows) between treatments and genotypes. B, Venn diagram illustrating total numbers and overlap of genes affected in wild-type and *wrky33* plants at 14 h post *B. cinerea* inoculation.

higher JA levels, may be due to the altered turnover of JA-Ile in the mutant, as has been reported for *coi1* in wild tobacco (*Nicotiana attenuata*; Paschold et al., 2008).

Along with the down-regulation of the JA response in *wrky33*, a significant increase of transcripts encoding the jasmonate ZIM-domain proteins *JAZ1*, *JAZ5*, *JAZ7*, *JAZ8*, and *JAZ10* was observed (Fig. 5). These proteins serve as negative regulators of the JA-signaling pathway (Chini et al., 2007). We performed ChIP using the WRKY33-HA complementation line in combination with qPCR to investigate direct WRKY33 binding to the promoter regions of these *JAZ* genes. By employing primer sets, representing different regions within their promoters and in the coding regions, we could detect significant *B. cinerea*-dependent binding to distinct promoter regions of *JAZ1* and *JAZ5* that contain varying numbers of WRKY factor-binding sites (W-boxes; Fig. 6, A and B), suggesting direct negative regulation of these *JAZ* genes by WRKY33 upon infection. For *JAZ8* and *JAZ10*, no WRKY33-specific binding was observed (Fig. 6, C and D), while *JAZ7* does not contain W-boxes in its promoter region. However, ChIP qPCR performed using an antibody raised against the conserved WRKY domain (anti-all WRKY) that recognizes many different WRKY proteins (Turck et al., 2004) revealed additional occupancy of all four *JAZ* promoters by other WRKY factors. In the case of *JAZ8* and *JAZ10*, the binding of these WRKY proteins was clearly enhanced upon *B. cinerea* infection (Fig. 6, C and D). For *JAZ1* and *JAZ5*, the WRKY33-specific promoter-binding sites appear to be occupied to some extent by other WRKY factors prior to infection in both genotypes (Fig. 6, A and B), an observation also found in parsley (*Petroselinum crispum*) for the PcWRKY1-binding site within the *PcPR1* promoter and indicative of a complex and dynamic WRKY factor occupancy network (Turck et al., 2004).

Interaction between the JA and ET pathways can be synergistic (Lorenzo et al., 2003). In our infection experiments, at 14 hpi, ET responses, as indicated by up-regulation of many ET response factor (*ERF*) genes and the gene encoding the ET biosynthesis enzyme 1-aminocyclopropane-1-carboxylic acid synthase, were clearly induced, with only small differences observed for some transcript levels between the wild type and *wrky33* (Table I). Upon *B. cinerea* infection, expression of the *ERF* gene *ORA59* was induced in the wild type and *wrky33*, with a peak at 14 hpi. Its expression remained elevated in the wild type thereafter, while in *wrky33*, it returned to noninduced levels (Fig. 7A). This is consistent with the role of *ORA59* as an activator of JA-response gene expression and could be one reason for the high JA response observed later than 14 hpi only in the wild type. The *ORA59* promoter contains numerous W-boxes, and WRKY33 may prolong expression of the gene after initial induction. To test whether WRKY33 directly targets *ORA59*, we performed ChIP qPCR and observed strong *B. cinerea*-induced binding to two distinct W-box-containing promoter regions (Fig. 7C).

Another ET pathway-associated gene, *GDSL LIPASE1* (*GLIP1*), shown to be involved in defense against the necrotroph *A. brassicicola* (Oh et al., 2005), was significantly higher induced upon *B. cinerea* infection in the wild type (Fig. 7B). ChIP PCR experiments revealed strong inducible WRKY33 binding to one W-box-containing site 3.5 kb upstream from the translation start site of *GLIP1* (Fig. 7D).

In summary, the expression of several genes encoding components associated with JA and ET signaling is modulated by WRKY33 during *B. cinerea* infection. Of these, *JAZ1*, *JAZ5*, *ORA59*, and *GLIP1* were found to be direct in vivo targets of WRKY33, with the first two likely being negatively regulated and the latter two positively regulated by WRKY33.

WRKY33 Is a Negative Regulator of the SA Pathway upon *B. cinerea* Infection

Most of the genes associated with the SA signaling pathway were higher expressed in leaves of the *wrky33* mutant compared with wild-type plants at 14 hpi. This holds true for the genes encoding the key SA biosynthesis enzyme *ICS1/SID2*, the positive regulatory proteins *EDS1*, *PAD4*, *EDS5/SID1*, *FMO1*, and *PBS3*, the

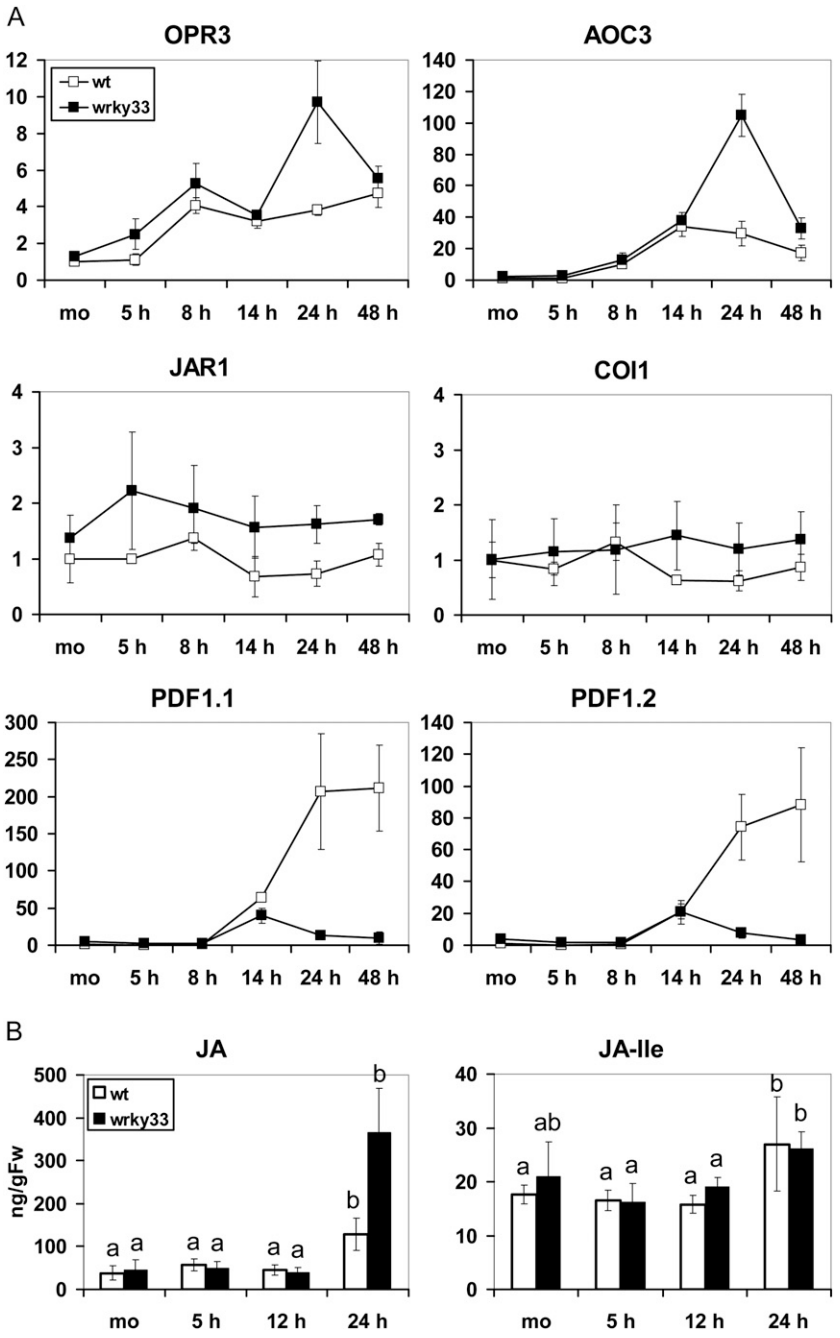

Figure 4. Expression levels of JA pathway-associated genes and JA levels during *B. cinerea* infection. A, Transcript levels of selected JA pathway-associated genes were determined by qPCR using the cDNAs described in Figure 2A. Expression of At4g26410 was used for normalization, and fold induction values of all genes were calculated relative to the expression level of mock-treated (mo) wild-type (wt) plants set to 1. Error bars represent SD ($n = 3$). B, JA and JA-Ile concentrations measured at 5, 12, and 24 hpi in wild-type and *wrky33* plants spray inoculated with *B. cinerea* or mock treated. Error bars represent SD ($n = 3$). Different letters indicate significant differences among time points within each genotype (ANOVA; $P < 0.0001$ for JA, $P < 0.05$ for JA-Ile). Fw, Fresh weight.

negative regulatory protein *NIMIN1*, and the SA response genes *PR1*, *PR2*, and *PR3* (Table I).

For selected genes, we performed qRT-PCR to get a better understanding of their temporal expression behavior during the infection process. The data revealed that beyond 14 hpi, the expression of *ICS1*, *PAD4*, and *PBS3* as well as *PR1* and *PR2* became strongly enhanced in *wrky33* compared with wild-type plants, indicating that the loss of *WRKY33* function abolished negative control over genes involved in the SA signaling pathway (Fig. 8A). Comparing the basal expression levels of SA-associated genes in uninfected plants revealed no significant differences between the wild type and *wrky33*.

We also determined the SA levels from infected plants of both genotypes. While prior to inoculation the levels of the bioactive free SA were nearly the same in wild-type and *wrky33* plants, a stronger accumulation of SA was observed in *wrky33* during infection, reaching three times higher levels in the mutant at 24 hpi. Similarly, four times higher levels of total SA were observed in the *wrky33* mutant at 18 hpi (Fig. 8B). These results are consistent with the high induction

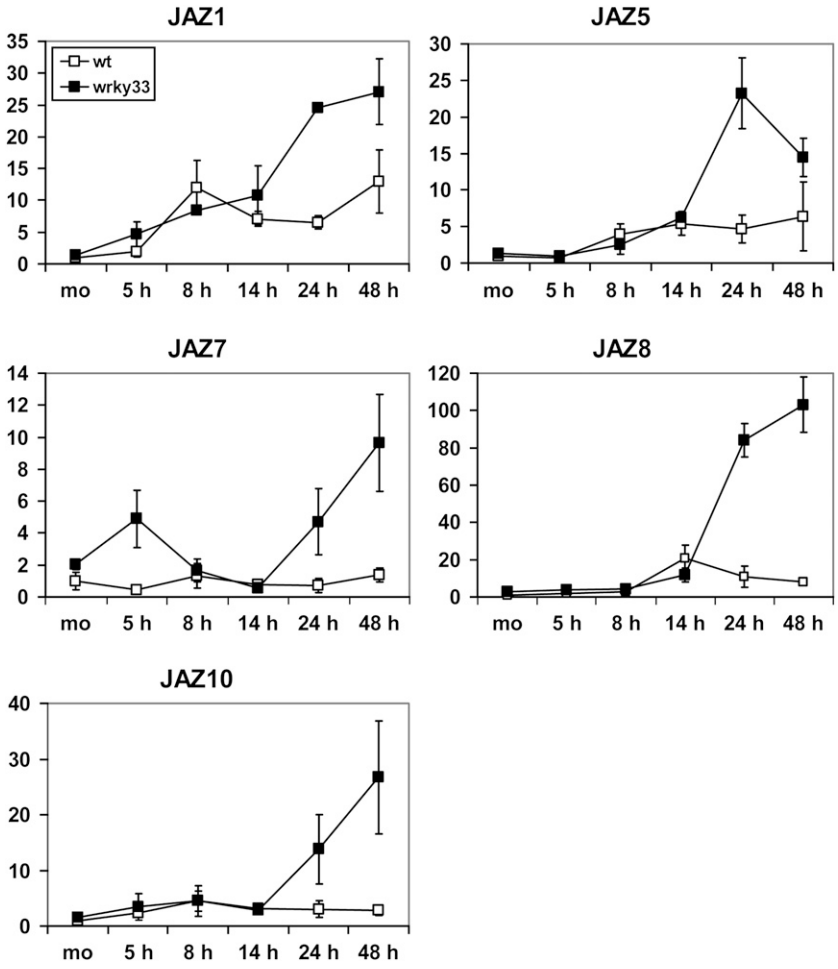

Figure 5. Differential up-regulation of *JAZ* genes upon *B. cinerea* infection in *wrky33*. The cDNAs described in Figure 2A were used to analyze by qPCR the expression kinetics of selected *JAZ* genes from *B. cinerea*-inoculated or mock-treated (mo) wild-type (wt) and *wrky33* plants. After normalization to gene At4g26410, fold induction values were calculated relative to mock-treated wild-type plants set to 1. Error bars represent SD ($n = 3$).

of *ICS1* observed in the mutant. Together, these data show that upon *B. cinerea* infection in wild-type plants, WRKY33 is involved in preventing the inappropriate induction of the SA pathway.

Production of Camalexin upon *B. cinerea* Infection Is Delayed in *wrky33*

Previous studies showed that *wrky33* plants inoculated with *B. cinerea* accumulated significantly lower levels of the phytoalexin camalexin than wild-type plants (Qiu et al., 2008; Mao et al., 2011). Since WRKY33 is a positive regulator of the camalexin biosynthetic genes *PAD3* (*CYP71B15*) and *CYP71A13*, and the *pad3* mutant is similarly susceptible to this isolate of *B. cinerea* as *wrky33* (Supplemental Fig. S4), the susceptibility of *wrky33* may be solely due to a lack of camalexin. Thus, we determined the camalexin levels of wild-type, *wrky33*, and *pad3* plants during the infection process (Fig. 9A). While in uninfected plants of all three genotypes and in infected *pad3* almost no camalexin was detectable at 24 hpi, in the wild type, about three times higher camalexin accumulation was found than in *wrky33*. At 48 hpi, a strong increase in camalexin in both genotypes was observed, but at this stage, significantly higher levels accumulated in *wrky33* plants.

The delay in camalexin biosynthesis in *wrky33* early during infection and the strong increase at later time points are reflected at the mRNA levels for *CYP71A13* and *PAD3* (Fig. 9B). At 14 hpi, transcript levels of both genes in *wrky33* were significantly lower than in the wild type, while at 24 hpi, when fungal growth was still indistinguishable in both genotypes, there was a sharp increase of both transcripts in *wrky33*. This increase very likely resulted in elevated camalexin levels, since PAD3 transcript and camalexin accumulation are highly correlated (Zhou et al., 1999).

Direct WRKY33 binding to the *PAD3* promoter at around −500 bp (relative to the translation start site) has been shown previously (Qiu et al., 2008; Mao et al., 2011). Our ChIP analyses revealed an additional stronger WRKY33-binding site within the *PAD* promoter at about −1,800 bp at 24 hpi (Fig. 9C). Binding to both sites was clearly induced by fungal infection. For *CYP71A13*, we observed strong inducible binding at −2,800 bp, again indicating direct positive regulation by WRKY33. To test if the later increase in camalexin

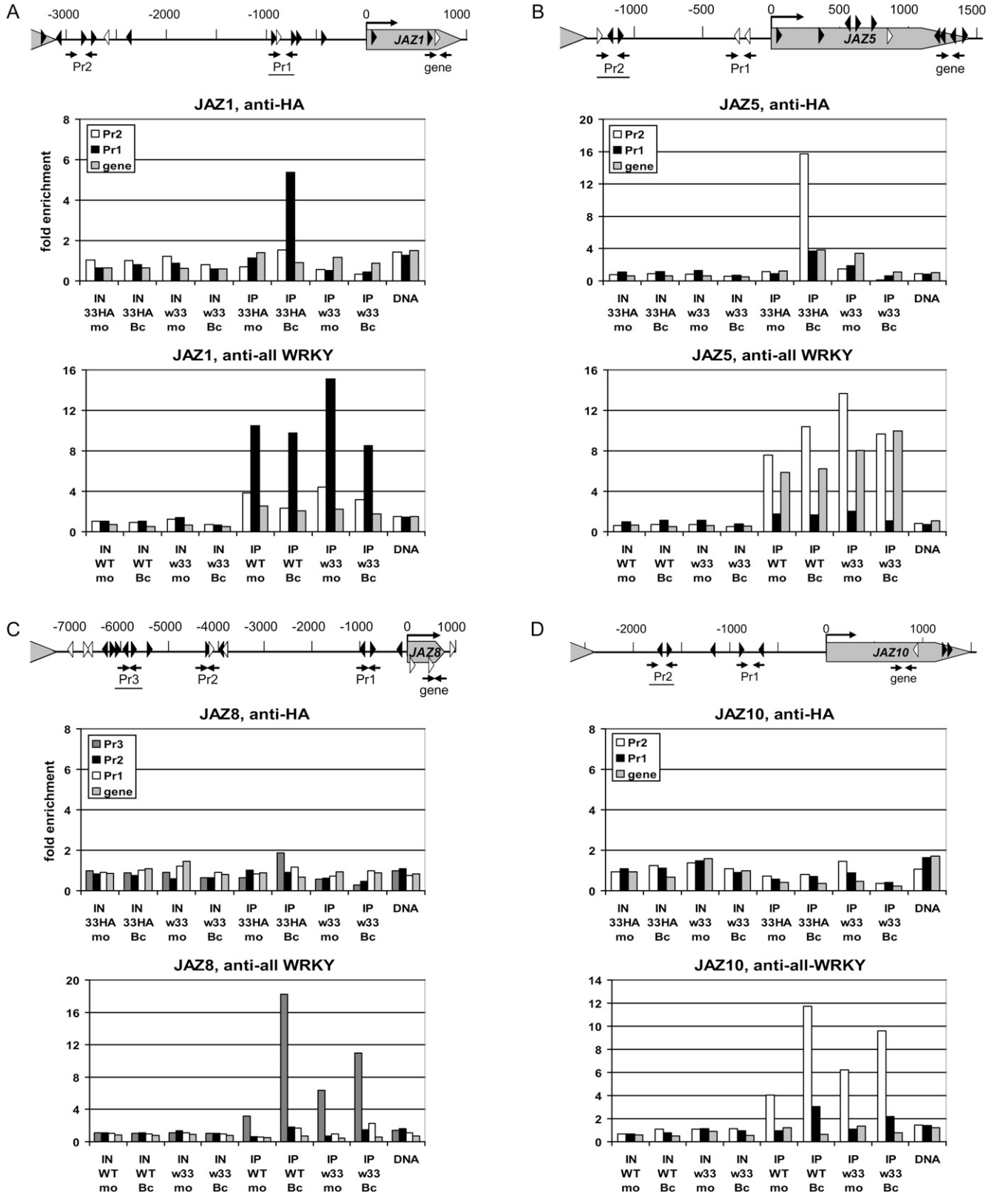

Figure 6. Direct binding of WRKY factors to *JAZ* gene promoters. ChIP qPCR was used to demonstrate WRKY factor binding to the promoters of selected *JAZ* genes. On top for each gene locus, a schematic drawing including the promoter region is shown (A, *JAZ1*; B, *JAZ5*; C, *JAZ8*; D, *JAZ10*). W-boxes (T/CTGACT/C) at the indicated positions are depicted by black triangles, and W-box-

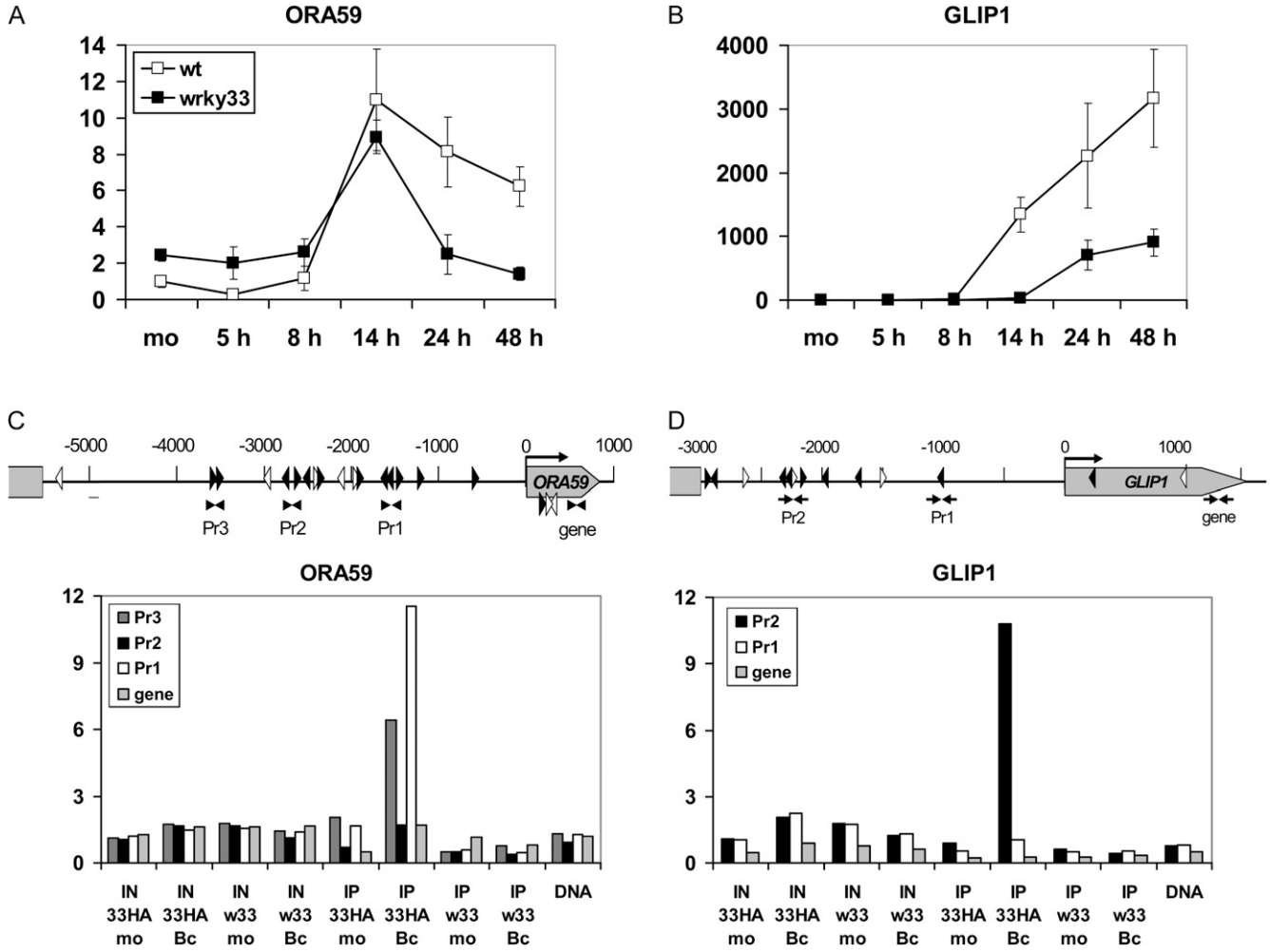

Figure 7. *ORA59* and *GLIP1* are direct targets of WRKY33. A and B, Expression levels of *ORA59* (A) and *GLIP1* (B) from *B. cinerea*-inoculated or mock-treated (mo) wild-type (wt) and *wrky33* plants were analyzed by qRT-PCR. Fold induction was calculated relative to mock-treated wild-type plants set to 1 after normalization to gene At4g26410. Error bars represent SD (n = 3). C and D, Binding of WRKY33 to the *ORA59* (C) and *GLIP1* (D) promoters. A schematic drawing for each gene locus including the promoter region is shown at the top (described in Fig. 6). For ChIP qPCR, leaves from *wrky33* (w33) and *WRKY33*-HA (33HA) were spray inoculated with *B. cinerea* (Bc) or mock treated for 24 h. Input DNA before immunoprecipitation (IN) and coimmunoprecipitated DNA using an anti-HA antibody (IP) were analyzed by qPCR employing gene-specific primer pairs (Pr1, Pr2, Pr3, gene). Values are fold enrichment relative to a DNA fragment from At4g26410. Each ChIP experiment was repeated at least twice with the same result.

levels in *wrky33* was related to the binding of other WRKY factors in the absence of WRKY33, we performed ChIP using the anti-all WRKY antibody. With this antibody, additional WRKY factors binding to the WRKY33-specific sites were detected not only in the wild type but also in *wrky33* plants (Fig. 9C, bottom panels). This suggests that other WRKY factors play a partly redundant function in regulating camalexin biosynthesis, particularly in the absence of WRKY33.

Figure 6. (*Continued.*)
like (TGACT/C) sequences are depicted by white triangles. Open reading frames are represented by large arrows in the direction of translation. The scale is in bp relative to the translation start of the respective gene. For ChIP, leaves from *wrky33* (w33), wild-type (WT), and *WRKY33*-HA (33HA) plants were spray inoculated with *B. cinerea* (Bc) or mock treated (mo) for 24 h. Input DNA before immunoprecipitation (IN) and coimmunoprecipitated DNA using an anti-HA or the anti-all WRKY antibody (IP) were analyzed by qPCR employing gene-specific primer pairs (Pr1, Pr2, Pr3, gene) and are expressed as fold enrichment relative to a DNA fragment from At4g26410. As a control for primer efficiency, purified genomic DNA was included in the analysis. Each ChIP experiment was repeated at least twice with the same result.

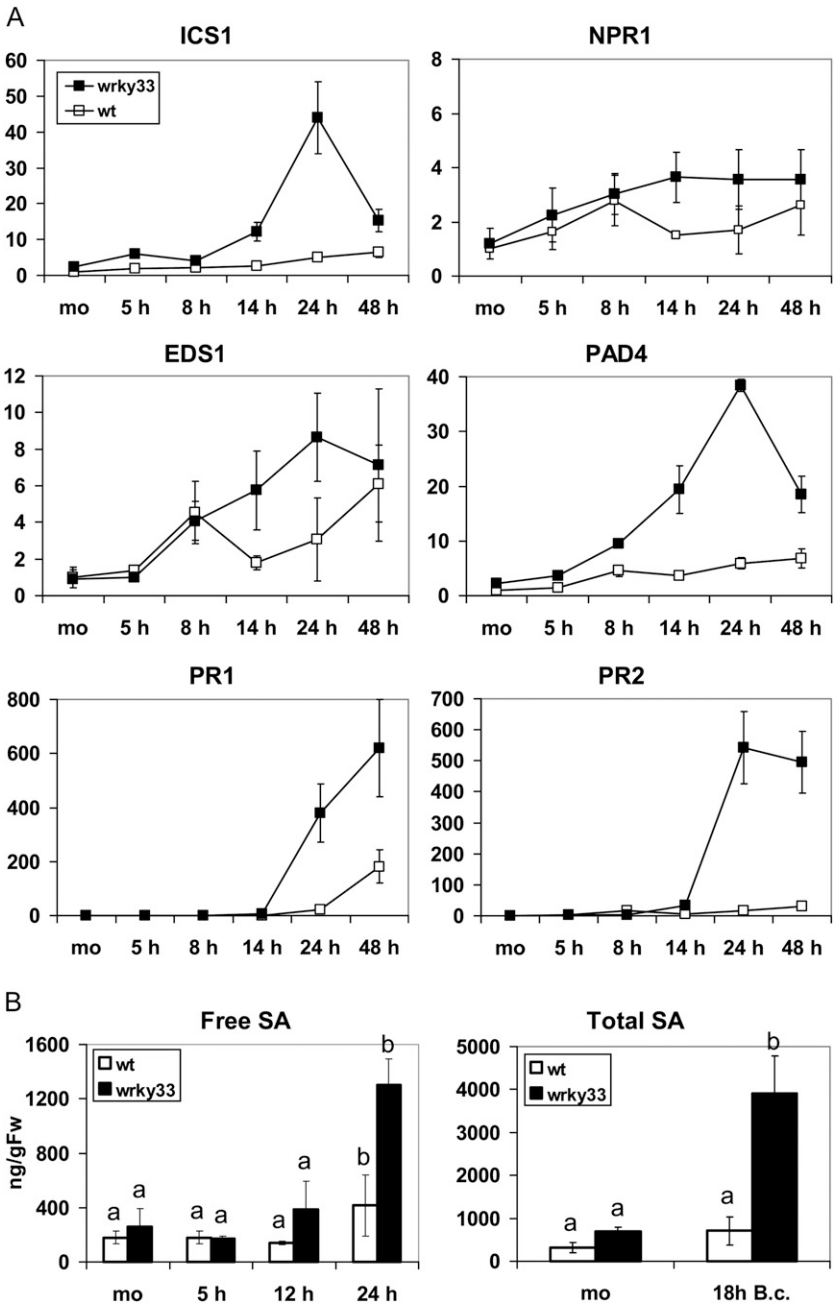

Figure 8. Expression levels of SA pathway-associated genes and SA levels after *B. cinerea* inoculation. A, Transcript levels of selected SA-associated genes were determined by qPCR using as templates the cDNAs described in Figure 2A. Fold induction values of all genes except for *PR1* were calculated relative to mock-treated (mo) wild-type (wt) plants set to 1. Due to very low expression in the mock-infected wild type, *PR1* is shown as fold change relative to the expression level of gene At4g26410, which was used in all qPCRs for normalization. Error bars represent SD ($n = 3$). B, Total SA and free SA concentrations at the indicated time points were determined in wild-type and *wrky33* leaves after mock treatment or spray inoculation with *B. cinerea* (Bc). Error bars represent SD ($n = 3$). Different letters indicate significant differences among time points within each genotype (ANOVA; $P < 0.05$ for free SA, $P < 0.0001$ for total SA). Fw, Fresh weight.

Expression of Genes Required for Redox Homeostasis upon *B. cinerea* Infection

Another group of host genes affected by *B. cinerea* infection code for proteins involved in the regulation of cellular redox homeostasis. Among them were several up-regulated glutathione *S*-transferases, FAD-binding domain proteins, redoxins, reductases, and peroxidases (Supplemental Table S1). Moreover, the expression of *RBOHD*, the main cytosolic stress-induced ROS producing NADPH oxidase, recently shown to be an essential component in rapid systemic ROS signaling (Miller et al., 2009), was 3- to 5-fold elevated in the wild type and *wrky33* upon *B. cinerea* infection. Altogether, 71 of the 81 genes previously reported to be induced in an RBOHD-dependent manner (Miller et al., 2009) were also up-regulated 14 h after *B. cinerea* inoculation in our experiments (Supplemental Table S1). With few exceptions, the induction of these genes was independent of WRKY33. This suggests that systemic ROS signaling, which is independent of SA, JA, and ET (Miller et al., 2009), is triggered upon *B. cinerea* infection, possibly also due to the observed ROS production by the fungus (Fig. 1).

Relevant to WRKY33 function were the redox-related genes differentially expressed between *wrky33* and the

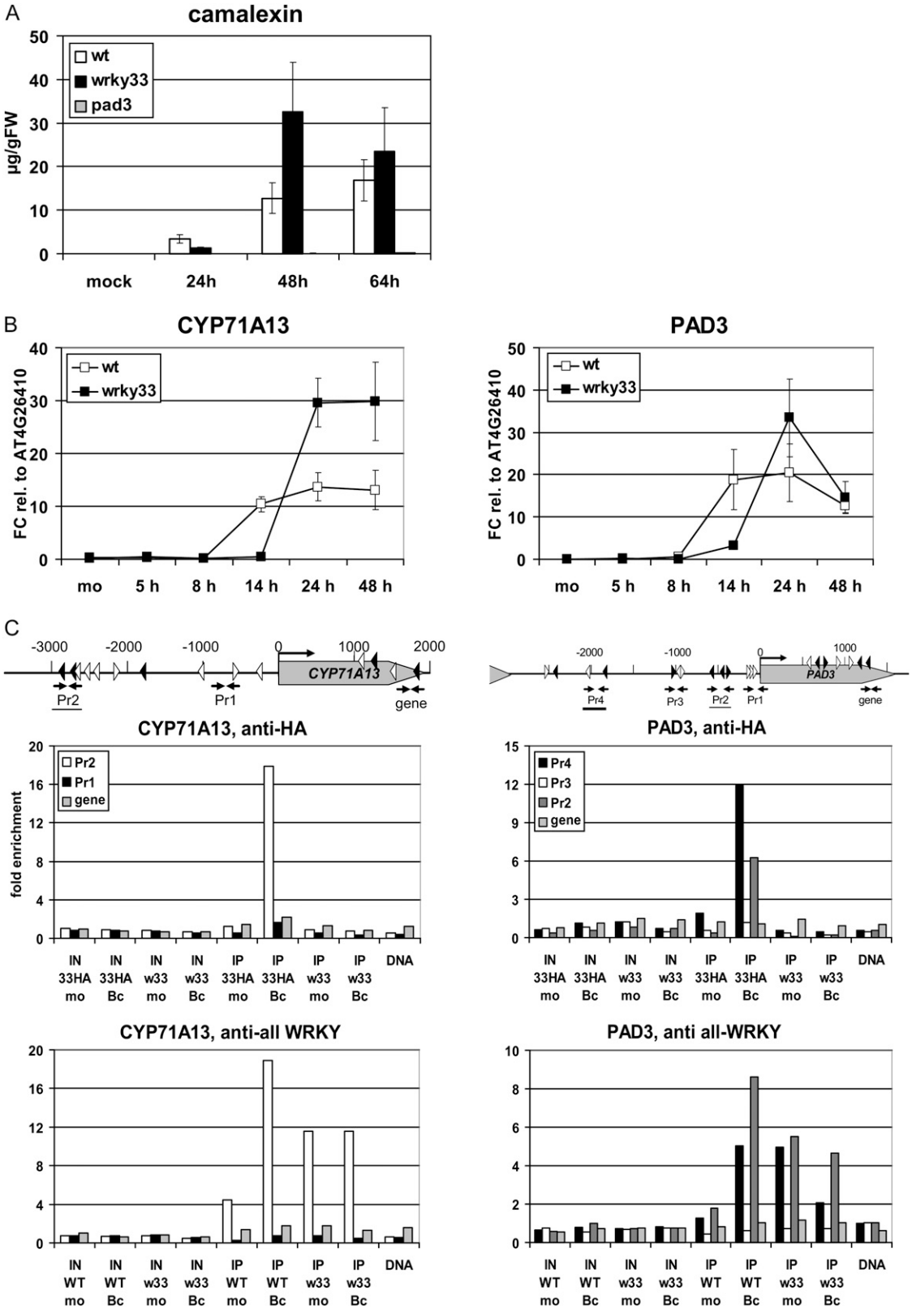

Figure 9. Direct regulation of camalexin biosynthesis by WRKY factors upon *B. cinerea* infection. A, Accumulation of camalexin in *B. cinerea*-inoculated wild-type (wt), *wrky33*, and *pad3* plants. At the indicated time points, camalexin concentrations were determined in mock-treated and inoculated wild-type, *wrky33*, and *pad3* leaves. Error bars represent SD ($n = 3$). Fw, Fresh weight. B, Transcript levels of *CYP71A13* and *PAD3*. Transcript levels of *CYP71A13* and *PAD3* at the indicated time points after

wild type. Among them were the glutaredoxins *GRX480* and *GRXS13*, thioredoxin *TRX-H5*, thioredoxin-dependent peroxidase *TPX2*, monodehydroascorbate reductase *MDAR3*, and cytokinin oxidase *CKX4*, all related to ROS signaling and scavenging (Van Breusegem et al., 2008). Upon *B. cinerea* inoculation, significantly higher expression levels of *GRX480*, *TRX-H5*, *CKX4*, and *MDAR3* were observed in *wrky33* compared with the wild type, indicating negative regulation by WRKY33 (Fig. 10A). Contrarily, *TPX2* was much less induced in *wrky33*, suggesting positive regulation by WRKY33. In *B. cinerea*-infected WRKY33-overexpressing plants, the expression of *GRX480*, *TRX-H5*, *CKX4*, and *MDAR3* was close to wild-type levels while the expression of *TPX2* was massively enhanced, consistent with negative and positive regulation by WRKY33, respectively (data not shown).

We performed ChIP using the WRKY33-HA complementation line to investigate direct WRKY33 binding to the promoter regions of these genes. While for *GRX480*, *CKX4*, *MDAR3*, and *TPX2* the data were inconclusive, significant *B. cinerea*-induced WRKY33 binding to a defined W-box-bearing *TRX-H5* promoter region was observed (Fig. 10B). This suggests direct *B. cinerea*-dependent negative regulation of *TRX-H5* by WRKY33.

Genetic Analysis of *WRKY33*-Regulated Genes

We tested mutants in hormone signaling, redox homeostasis, and antimicrobial metabolite biosynthesis with *B. cinerea* isolate 2100 and monitored susceptibility toward this fungus (Supplemental Fig. S4). Mutants in SA biosynthesis and signaling, *npr1-1*, *sid2-1*, *eds1-1*, and *pad4-1*, were nearly as resistant as the wild type, consistent with previous reports (Ferrari et al., 2003). Interestingly, the mutants *opr3*, *jar1-1*, *jrg21*, and *ein2*, corresponding to genes associated with the JA and ET pathways, were also resistant. These findings partly contrast with previous studies demonstrating the enhanced susceptibility of *jar1-1* and *ein2* toward this fungal pathogen (Thomma et al., 1999; Ferrari et al., 2003; Nickstadt et al., 2004; Van Baarlen et al., 2007) and highlight the fact that defenses against *B. cinerea* can be dependent on the fungal isolate (Rowe et al., 2010). Additionally, mutants in genes shown to act in fungal penetration resistance, *pen1-1*, *pen3-3*, and *pen4-1* (Collins et al., 2003), were resistant, as was *cyp81F2* (Pfalz et al., 2009), a key component in glucosinolate toxification. We also assayed the sensitivity of *ora59*, *glip1*, *trx-h5*, and *grx480* plants. Somewhat unexpectedly, all mutants exhibited similar resistance as wild-type plants to this fungus (Supplemental Fig. S5). Thus, apart from *wrky33*, only the *pad3-1* mutant exhibited enhanced susceptibility toward this fungal isolate. However, plants ectopically overexpressing *GRX480* (*GRX480ox*) were strongly susceptible to this fungus, resulting in large lesion formation and conidiophore development (Supplemental Fig. S5). In contrast, overexpression of *TRX-H5* (*TRX-H5ox*) did not alter the resistant phenotype. Thus, the WRKY33-dependent negative regulation of *GRX480* upon infection is likely one required mechanism for resistance toward *B. cinerea*.

To test if the inappropriately induced SA responses are causal for the enhanced susceptibility of *wrky33* toward *B. cinerea*, we generated double mutants of *wrky33* with the SA biosynthesis mutant *sid2-1*, with *npr1-1*, a key response regulator of the SA pathway, with *wrky70*, an NPR1-dependent TF known to play a role in SA-JA cross talk (Li et al., 2004), and with *rbohD* and then inoculated them with *B. cinerea* spores. Both lesion morphology (data not shown) and fungal biomass determination at 3 dpi revealed that the *sid2-1 wrky33* and *npr1-1 wrky33* double mutants were nearly as susceptible as *wrky33* (Fig. 11). This indicates that inappropriate induction of the SA pathway and early SA-mediated repression of the JA response alone are insufficient to cause the strong infection phenotype of *wrky33* plants toward this fungus. The double mutant *wrky70 wrky33* showed reduced fungal growth, indicating a possible contribution of this factor to the phenotype.

DISCUSSION

Earlier studies have shown that WRKY33 is required for resistance to *B. cinerea* and have implicated this factor to positively regulate JA- and ET-mediated defenses while negatively affecting SA-mediated signaling (Zheng et al., 2006). Our results, however, suggest that early JA signaling upon *B. cinerea* infection is independent of WRKY33 function, while WRKY33 acts as a negative regulator of SA signaling and thereby prevents subsequent SA-mediated suppression of the JA pathway. Thus, in *wrky33* mutants, inappropriate activation of SA-mediated responses leads to a suppression of JA responses downstream of JA biosynthesis at later stages of *B. cinerea* infection,

Figure 9. (*Continued.*)
inoculation were determined by qRT-PCR. Fold change was calculated relative to the expression level of gene At4g26410. C, Direct binding of WRKY factors to the *CYP71A13* and *PAD3* promoters. A schematic drawing for each gene locus including the promoter region is shown at the top (described in Fig. 6). For ChIP, leaves from wild-type, *wrky33* (w33), and *WRKY33*-HA (33HA) plants were spray inoculated with *B. cinerea* (Bc) or mock treated (mo) for 24 h. Input DNA before immunoprecipitation (IN) and coimmunoprecipitated DNA (IP) using an anti-HA or the anti-all WRKY antibody were analyzed by qPCR employing gene-specific primer pairs (Pr1, Pr2, Pr3, Pr4, gene). Fold enrichment is expressed relative to a DNA fragment from At4g26410. Each ChIP experiment was repeated twice with the same result.

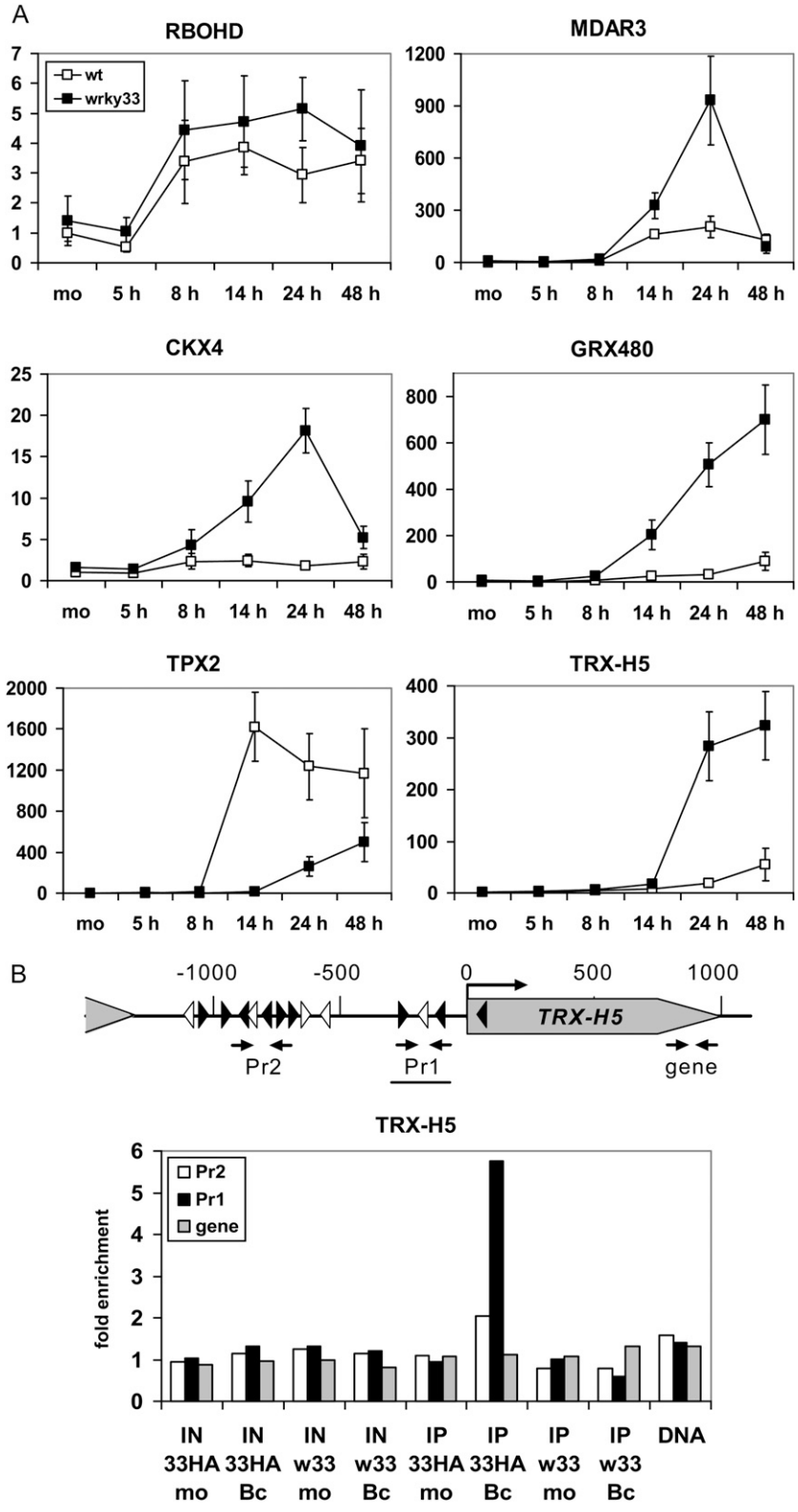

Figure 10. WRKY33 modulates the expression of redox-related genes and directly targets *TRX-H5*. A, Differentially expressed redox-related genes after *B. cinerea* infection. Transcript levels of selected redox genes at the indicated time points of *B. cinerea*-inoculated wild-type (wt) and *wrky33* leaves were quantified by qRT-PCR. After normalization to gene At4g26410, fold induction values were calculated relative to mock-treated (mo) wild-type plants set to 1. Error bars represent SD ($n = 3$). B, Direct binding of *WRKY33* to the *TXR-H5* promoter upon *B. cinerea* infection. A schematic drawing of the gene locus including the promoter region is shown at the top (described in Fig. 6). ChIP was performed using leaves from spray-inoculated (Bc) and mock-treated *wrky33* (w33) and *WRKY33*-HA (33HA) plants at 14 hpi. DNA before immunoprecipitation (IN) and after immunoprecipitation using an anti-HA antibody (IP) were quantified by qPCR employing the primer pairs Pr1, Pr2, and gene. Values are fold enrichment relative to a fragment of gene At4g26410. The ChIP experiment was repeated twice with the same result.

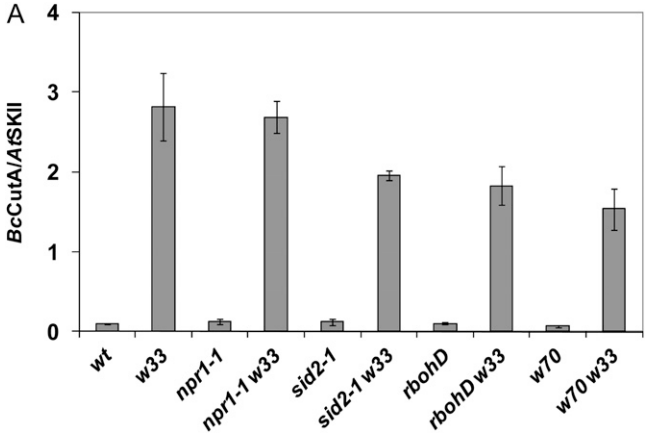

Figure 11. *B. cinerea* growth quantification on *wrky33* double mutants. The indicated Arabidopsis lines were inoculated with two droplets of *B. cinerea* spores per leaf, and after 3 d the relative abundance of *B. cinerea* and Arabidopsis DNA was determined by qPCR employing specific primers for BcCutinase A and AtSKII, respectively. Error bars represent SD ($n = 3$). wt, Wild type.

thereby contributing to the susceptibility of the plant toward this necrotroph.

Our conclusions were mainly derived from genome-wide transcript profiling obtained at 14 hpi (maximal WRKY33 protein levels) and at 24 hpi, when gene expression differences between wild-type and *wrky33* plants were the largest. At these infection stages, no obvious differences in fungal growth and development were observed between plants of both genotypes. Thus, alterations in transcript and hormone levels are likely direct consequences of the loss of WRKY33 function. The partial discrepancy between our findings and those of Zheng et al. (2006) is very likely due to differences in sampling times, since 24 hpi was the earliest time point analyzed previously.

Different from a previous report (Mao et al., 2011), we found that WRKY33 is not absolutely essential to promote *Botrytis*-induced camalexin production. Rather, we observed a delay in camalexin biosynthesis in *wrky33* that was fully restored at later stages of infection, reaching even higher camalexin levels than in the wild type. Consistent with this observation was the delayed temporal expression of the camalexin biosynthetic genes *CYP71A13* and *PAD3* in *wrky33*, which also reached higher transcript levels at later infection stages than in the wild type. Although both genes are direct targets of WRKY33, ChIP assays revealed that other WRKY factors can also bind to the same promoter regions and thus may functionally compensate in the *wrky33* mutant (Fig. 9). Camalexin, however, is not absolutely required for the resistance of Arabidopsis toward *B. cinerea*, and its contribution to WRKY33-mediated resistance remains unclear (Denby et al., 2005; Veronese et al., 2006; Walley et al., 2008; Berr et al., 2010).

High SA levels in plants can favor the growth of necrotrophic fungi by triggering programmed cell death (Govrin and Levine, 2000) and by suppressing appropriate JA defense pathways. For instance, exogenously applied SA suppressed the expression of JA-response genes induced by infection with the necrotrophs *A. brassicicola* and *B. cinerea* and by insect herbivores (Koornneef et al., 2008). Similarly, infection with a virulent strain of the biotrophic pathogen *P. syringae* that induces SA-dependent defenses resulted in increased susceptibility to *A. brassicicola* (Spoel et al., 2007).

SA-mediated suppression of JA responses is also modulated by pathogen-triggered changes in cellular redox potential and by the cytosolic function of NPR1 (Spoel et al., 2003; Ndamukong et al., 2007; Koornneef et al., 2008). We identified *TRX-H5* to be a direct target of WRKY33 and its expression to be exaggerated in *wrky33* plants. TRX-H5 reduces disulfide bonds between cytosolic NPR1 molecules for monomerization, a prerequisite for nuclear *NPR1* function (Tada et al., 2008). We hypothesize that *TRX-H5* is negatively controlled by WRKY33 and that loss of WRKY33 function results in strong pathogen-dependent *TRX-H5* expression, resulting in enhanced enzymatic activity. This may partly explain the preferential expression of NPR1-dependent genes in *wrky33* compared with the wild type (Supplemental Table S4). Moreover, the glutaredoxin gene *GRX480* was also massively induced by *B. cinerea* infection only in *wrky33* plants. Expression of *GRX480* is induced by SA, is dependent on NPR1, and interacts with TGA factors regulating SA-responsive genes. Plants overexpressing this gene suppressed the transcription of JA-responsive genes (Ndamukong et al., 2007) and resulted in the susceptibility of plants toward *B. cinerea* (Supplemental Fig. S5). Consistent with this, SA-mediated suppression of JA signaling has been shown to coincide with a cellular increase in glutathione levels, and inhibition of glutathione biosynthesis antagonized this effect (Koornneef et al., 2008). It is also in agreement with findings demonstrating that Columbia-0 plants overexpressing *ROXY1*, another GRX of the CC type, were highly susceptible to *B. cinerea* (Wang et al., 2009), whereas plants impaired in the glutaredoxin gene *GRXS13* were resistant toward this nectrotroph (La Camera et al., 2011).

In *wrky33*, simultaneous with the up-regulation of SA-associated genes, only JA-response genes were repressed, while the expression of the JA-signaling genes *JAR1* and *COI1* remained unchanged, and the JA-biosynthesis genes *OPR3* and *AOC3* were even up-regulated, resulting in higher JA accumulation. JA-response genes are mainly under the negative control of JAZ repressors (Chung and Howe, 2009; Pauwels and Goossens, 2011). The expression of several *JAZ* genes was strongly and positively influenced in *wrky33* upon pathogen challenge. For two, *JAZ1* and *JAZ5*, in vivo enrichment of WRKY33 at specific W-box-containing promoter regions could be shown, strongly indicating direct negative regulation by this TF. We hypothesize that all identified *JAZ* genes,

except *JAZ7*, are under additional positive regulation by *NPR1*-dependent WRKY factors. We deduce this from the fact that the expression peaks of these *JAZ* genes coincided with those of the differentially induced *WRKY* genes in *wrky33* following *B. cinerea* infection (Supplemental Fig. S3) and from ChIP assays using an antibody raised against the conserved WRKY domain (Turck et al., 2004) that revealed additional occupancy of all four *JAZ* promoters by such factors (Fig. 6). Prime candidate interactors may be WRKY50, WRKY51, WRKY53, WRKY54, and WRKY70. WRKY50 and WRKY51 were recently shown to repress JA signaling in a SA- and low-oleic-acid-dependent manner in *ssi2* plants (Gao et al., 2011). Mutations in the *SSI2* gene, encoding a plastid-localized stearoyl-acyl carrier protein desaturase, resulted in dwarf plants with high levels of SA and defects in JA-mediated defense responses. Such plants showed high susceptibility to *B. cinerea* but enhanced resistance to bacterial and oomycete pathogens (Nandi et al., 2005). Overexpression of *WRKY70* repressed several JA-responsive genes and compromised resistance toward the necrotrophic fungus *A. brassicicola* while enhancing SA-dependent resistance toward the biotrophic fungus *Erysiphe cichoracearum* (Li et al., 2006). Moreover, WRKY53 was demonstrated to be partly redundant to WRKY70, whereas WRKY54 and WRKY70 have dual roles in SA biosynthesis and SA signal transduction (Wang et al., 2006).

Based on the up-regulation of many ET response factor (*ERF*) genes in our study, the ET response was clearly induced in both genotypes early during *B. cinerea* infection. This was also true for *ERF1* and *ORA59*, two principal integrators of JA and ET signaling (Lorenzo et al., 2003; Pré et al., 2008). *ORA59* plays an important role in counteracting the SA-mediated repression of JA-response genes (Leon-Reyes et al., 2010). In the cases where the SA pathway is activated prior to or simultaneous with the JA pathway, SA suppresses *ORA59* expression, resulting in the repression of JA-response genes such as *PDF1.2* (Leon-Reyes et al., 2010). This is consistent with the decline in the expression of *ORA59* and the subsequent reduced expression of the JA-response genes *PDF1.1* and *PDF1.2* observed in *B. cinerea*-infected *wrky33* plants as a consequence of inappropriate early activation of the SA pathway. We identified *ORA59* to be a direct target of WRKY33. Moreover, the expression of *ORA59* and consequently PDF1.2 was further substantially reduced in *B. cinerea*-infected *wrky33 sid2-1* and *wrky33 npr1-1* double mutants, compared with *sid2-1* and *npr1-1* plants, providing additional evidence that WRKY33 directly regulates *ORA59* in a positive manner (Supplemental Fig. S6). Since pathogen-triggered early activation of *ORA59* was similar in wild-type and *wrky33* plants, the binding of WRKY33 may be essential in maintaining the prolonged expression of this gene.

Another identified direct in vivo target of WRKY33 was *GLIP1*, encoding a secreted lipase associated with ET signaling. GLIP1 targets fungal cell walls and possesses antimicrobial activity, and *glip1* mutants are susceptible to *A. brassicicola* (Oh et al., 2005; Kwon et al., 2009). *B. cinerea* infection in *wrky33* plants significantly decreased *GLIP1* transcript levels compared with wild-type plants, indicating a positive regulatory role of WRKY33 in its expression.

Whereas up-regulation of the SA pathway by *Botrytis* isolate B191 was identified as causal for susceptibility in tomato (*Solanum lycopersicum*; El Oirdi et al., 2011), our genetic studies clearly demonstrate that this was not the case in Arabidopsis *wrky33* plants infected with the *Botrytis* isolate 2100. The *wrky33* double mutants with *sid2-1* and *npr1-1* were similarly susceptible like *wrky33*. Accordingly, the JA and ET single mutants *opr3*, *coi1*, *jar1*, *ein2*, and *ora59* appeared as resistant as wild-type plants, again indicating that loss of the JA/ET responses alone is not the major cause for *wrky33* susceptibility. Rather, it seems that WRKY33 regulates various distinct components of the phytohormone and possibly other signaling pathways that, in their concerted action, are essential to maintain proper temporal/spatial coordination of defense responses toward this necrotrophic fungus. This is consistent with recent studies by Tsuda et al. (2009) demonstrating that the establishment of optimal resistance to the necrotroph *A. brassicicola* involves complex interactions and requires substantial cross talk between various signaling pathways.

In summary, our studies indicate that by regulating components of both the SA and JA pathways, WRKY33 provides a mechanistic link to how SA-mediated repression of the JA response is achieved during the course of *B. cinerea* infection. This is partly accomplished by directly regulating the expression of genes such as *TRX-H5* and *ORA59* or by WRKY-mediated transcriptional activation of *JAZ* genes leading to the de novo synthesis of JAZ repressors. Supplemental Figure S7 outlines the currently known WRKY33 interactions in Arabidopsis. It is obvious that the network is complex and will involve additional WRKY33-dependent regulatory components. In particular, one should note that loss of WRKY33 function without infection did not result in altered expression of the majority of defense genes identified. Rather, many of the differential responses that we observed were dependent on the pathogen stimulus. Such inducible plant responses may be the result of altered protein specificities/activities as a general consequence of changes in cellular redox homeostasis or of pathogen-triggered alterations of specific signaling pathways involving WRKY33 functions.

MATERIALS AND METHODS

Plant Materials

All experiments were done with Arabidopsis (*Arabidopsis thaliana*) plants of cv Columbia-0. The *wrky33* mutant line used was the GABI_324B11 insertion line. To generate the *WRKY33* HA-tagged complementation line (WRKY33-HA), the genomic region from −1,218 to 2,370 bp relative to the start codon was PCR amplified and cloned into the *Xho*I and *Not*I sites of

vector KWS-5 (B. Ülker and I.E. Somssich, unpublished data). In a second step, the *Pst*I-*Not*I fragment containing the stop codon was removed and replaced by the *Pst*I-*Not*I fragment containing an in-frame HA tag sequence. This construct was stably transformed into *wrky33* as described (Logemann et al., 2006). The single-insertion homozygous line was indistinguishable from wild-type plants with respect to growth and response to *B. cinerea*.

The overexpression line *WRKY33ox* was generated by cloning HA-tagged full-length cDNA into the pDONR201 Gateway vector (Invitrogen). This entry clone was recombined into the Gateway-compatible binary vector pAM-Kan-2x35S-intron:GW-HA, a pPAM (GenBank accession no. AY027531) derivative. The construct was transformed into Arabidopsis *wrky33* by *Agrobacterium tumefaciens*-mediated transformation as described (Logemann et al., 2006). The expression of *WRKY33* was confirmed by western-blot analyses (data not shown).

Other mutants or plant lines used in this study were as follows: *grx480* (SALK_031817), *GRX480ox* (Ndamukong et al., 2007), *ora59* (GK_061A12), *glip1* (SALK_130146), *wrky70* (GK_752F08), *pad3-1* (Zhou et al., 1999), *rbohD* (Torres et al., 2002), *sid2-1* (Wildermuth et al., 2001), *npr1-1* (Cao et al., 1997), *eds1-1* (Falk et al., 1999), *pad4-1* (Glazebrook et al., 1997), *oprs* (Stintzi et al., 2001), *jar1-1* (Staswick et al., 2002), *ein2-1* (Guzmán and Ecker, 1990), *jrg21* (Delker et al., 2007), *pen1-1* (Collins et al., 2003), *pen3-3* (Stein et al., 2006), and *cyp81F2* (SALK_123882). The double mutants *wrky33 npr1-1*, *wrky33 sid2-1*, *wrky33 rbohD*, and *wrky33 wrky70* were generated by crossing single mutants followed by PCR-based verification.

Plant Growth Conditions and Pathogen Inoculation Procedure

Plants were grown for 5 weeks under short-day conditions in closed cabinets (Schneijder chambers: 20°C, 16 h of light/8 h of darkness, 80% relative humidity) on 42-mm Jiffy-7 pots (Jiffy) to prevent pathogen infection from garden soil. Before sewing, the Jiffy pot peat pellets were soaked in water containing 0.1% liquid fertilizer Wuxal (Manna).

For inoculations, the *Botrytis cinerea* isolate 2100 (CECT2100; Spanish Type Culture Collection, Universidad de Valencia) was used, which was cultivated on potato dextrose plates at 22°C for 10 d. Spores were collected, washed, and frozen at −80°C in 0.8% NaCl at a concentration of 10^7 spores mL^{-1}. For inoculation of Arabidopsis plants, the spores were diluted in Vogelbuffer (in 1 L: 15 g of Suc, 3 g of Na-citrate, 5 g of K_2HPO_4, 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.1 g of $CaCl_2 \cdot 2H_2O$, and 2 g of NH_4NO_3) to 5×10^5 spores mL^{-1}. For droplet inoculations, 5 μL was applied to single leaves of 5-week-old plants. The same spore suspension was used for spray inoculations. For mock treatment, Vogelbuffer alone was used. One day before inoculation and during the entire infection process, plants were incubated at high humidity under a hood.

Quantification of Fungal Growth by qPCR

Quantification of fungal biomass relative to plant biomass by qPCR was basically performed as described by Gachon and Saindrenan (2004). Leaves of the indicated Arabidopsis lines were inoculated with two 4-μL droplets of *B. cinerea* spores, and after 3 d, DNA from whole leaves of similar weight was extracted following the modified protocol of Edwards et al. (1991). The relative amounts of *B. cinerea* and Arabidopsis DNA were determined by qPCR employing specific primers for cutinase A and SKII, respectively.

Microarray Analysis

For microarray analysis, 100 mg of leaf tissue was collected and total RNA was isolated using TRI reagent following the supplier's instructions (Ambion). The samples were collected from spray-infected and mock-treated wild-type and *wrky33* plants after 14 h. Leaves from three plants were pooled to make one biological replicate, and three independent biological replicates were isolated for each genotype and treatment for hybridization against a total of 12 Arabidopsis ATH1 GeneChip arrays (Affymetrix). Quality control, labeling, hybridization, and scanning were done at Integrated Functional Genomics, Interdisziplinäre Zentrum für Klinische Forschung, at the University of Muenster. Data were analyzed using the software package GeneSpring GX (Agilent Technologies). Differential expression of genes was calculated using a combination of ANOVA (Benjamini-Hochberg multiple testing) at $P \leq 0.05$ and a cutoff of 2-fold or greater.

qRT-PCR

Total RNA was isolated from leaves at 0, 5, 8, 14, 24, and 48 hpi as described above and reverse transcribed with oligo(dT) primer to produce cDNA using the SuperScript First-Strand Synthesis System for Reverse-Transcription PCR following the manufacturer's protocol (Invitrogen). cDNA corresponding to 2.5 ng of total RNA was subjected to qPCR with gene-specific primers (Supplemental Table S5) using the Brilliant SYBR Green qPCR Core Reagent Kit accordingly (Stratagene). The qPCRs were performed on the iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad) with two technical replicates in the same run and three biological replicates in different runs. The endogenous reference gene for normalization was At4g26410, which was described as being highly constant under varying stress conditions (Czechowski et al., 2005) and which also displayed very constant expression levels in our microarrays. The results were analyzed using the ΔΔCt method (Livak and Schmittgen, 2001). To simplify data interpretation, transcript abundance was expressed as a ratio relative to mock-treated wild-type plants, which was set to 1. Data shown are means ± SD from three biological replicates.

ChIP

Five-week-old plants were spray inoculated or mock treated for 14 or 24 h before they were processed following the protocol by Gendrel et al. (2005) with some modifications. Each 2-g leaf sample was cross-linked by vacuum infiltration of 1% formaldehyde solution for three subsequent 7-min treatments. Sonication of the isolated nuclei was performed on a UP50H sonicator (Hielscher) equipped with an MS1 tip four times for 30 s each with 30 s of cooling on ice in between. The sheared chromatin (25 μg) was diluted 10-fold in ChIP dilution buffer (Gendrel et al., 2005) containing protease inhibitors (Roche) and phosphatase inhibitor (Sigma) to lower the SDS concentration to 0.1%. After preclearing with protein A-agarose beads, the chromatin was incubated overnight with rabbit polyclonal antibodies to HA (ChIP grade; ABCAM) at 4°C on a nutator. Immuncomplexes were collected by incubation with protein A-agarose. After washing, the beads were resuspended in 250 μL of Tris EDTA buffer, pH 8.0, containing 0.5% SDS with 2 μg of RNase (Roche) and 2 μg of proteinase K (Invitrogen) and incubated overnight at 65°C to reverse cross-links. The phenol-chloroform-extracted DNA was precipitated with ethanol and resolved in 50 μL of Tris EDTA buffer, pH 8.0. One microliter was used for qPCR.

Quantification of Phytohormones

Analysis of phytohormone levels was conducted as described (Diezel et al., 2009) with the only difference being that as one internal standard, (±)-jasmonic acid-9,10-D2 was added. To determine total SA levels, 0.1 mL of 0.1 M NaOAc, pH 5.0, containing β-glucosidase (final concentration, 100 units mL^{-1}) was added to dried extract residues after solvent evaporation, and the samples were incubated for 3 h at 37°C. Fifty microliters of 10% trifluoroacetic acid was added, and the solution was extracted three times with 600 μL of ethyl acetate. The three ethyl acetate fractions were pooled, the solvent was evaporated in a Speed-Vac, and after adding 70% methanol, the levels of free SA were determined by HPLC.

Quantification of Camalexin

In three biological replicates, 100 mg of *B. cinerea*-inoculated or mock-treated leaves was harvested at various time points and homogenized in dimethyl sulfoxide (2.5 μL mg^{-1} fresh weight). After centrifugation (20 min, 15,000 rpm, 4°C), supernatants were collected in fresh tubes and subjected to HPLC analysis.

DAB and Trypan Blue Staining of Plant Material

To detect H_2O_2, *B. cinerea* spray-inoculated and mock-treated leaves were stained with DAB as described (Torres et al., 2002). To visualize fungal tissue and dying plant cells, the leaves were stained with trypan blue as described (Roetschi et al., 2001).

Microarray data have been deposited in the NASCArrays database under accession number NASCARRAYS-577.

Supplemental Data

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

Supplemental Figure S1. *B. cinerea* 2100-induced ROS production and cell death.

Supplemental Figure S2. *WRKY33*-HA complements *wrky33* susceptibility to *B. cinerea* 2100 infection.

Supplemental Figure S3. Differential up-regulation of *WRKY* genes after *B. cinerea* 2100 infection.

Supplemental Figure S4. Sensitivity of selected mutants from hormone and metabolite pathways toward *B. cinerea* 2100.

Supplemental Figure S5. Susceptibility of mutant and overexpression lines to *B. cinerea* 2100 infection.

Supplemental Figure S6. Expression levels upon *B. cinerea* 2100 infection of hormone-response genes in double mutants containing the *wrky33* mutation.

Supplemental Figure S7. Schematic representation of WRKY33 interactions.

Supplemental Table S1. Fold induction of all genes at 14 hpi with *B. cinerea* 2100 ($P \leq 0.05$).

Supplemental Table S2. Gene Ontology counts of differentially expressed genes upon *B. cinerea* 2100 infection.

Supplemental Table S3. Differential expression of TFs upon *B. cinerea* 2100 infection at 14 hpi.

Supplemental Table S4. Differential expression of *NPR1* target genes in *wrky33* upon *B. cinerea* 2100 infection.

Supplemental Table S5. Primers used in qPCR.

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