The role of *Arabidopsis WRKY33* in modulating host immunity towards the necrotroph *Botrytis cinerea*

Inaugural-Dissertation

zur

Erlangung des Doktorgrades

der Mathematisch-Naturwissenschaftlichen Fakultät

der Universität zu Köln

vorgelegt von

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Köln, November 2014

Die vorliegende Arbeit wurde angefertigt am
Max-Planck-Institut für Pflanzenzüchtungsforschung in Köln
in der Abteilung für Pflanze-Mikroben Interaktionen
(Direktor: Prof. Dr. Schulze-Lefert).

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Tag der Disputation: 19. Januar 2015

Abstract

The *Arabidopsis thaliana* transcription factor WRKY33 is a key transcriptional regulator of plant responses towards *B. cinerea* strain 2100 infection, and is essential for resistance. To elucidate the critical molecular components involved, global mapping of WRKY33-regulated target genes was performed by integrating ChIP-seq data of WRKY33 binding sites with RNA-seq results of 14h *B. cinerea*-induced and WRKY33-dependent gene expression profiles. Comparative gene expression analysis between resistant wild-type Columbia-0 and susceptible *wrky33* mutant plants revealed that *B. cinerea* 2100-induced WRKY33 has both transcriptional activator and repressor roles in regulating hundreds of target genes with distinct molecular functions, but mainly acts as a transcriptional repressor. Genome-wide analysis confirmed previously known WRKY33 targets involved in ethylene and jasmonic acid hormone signaling and phytoalexin biosynthesis, but also uncovered new vital components affecting the complex regulatory circuitry affecting resistance towards *Botrytis*. In particular, the abscisic acid (ABA) biosynthetic genes *NCED3* and *NCED5* were identified as key targets of WRKY33-mediated resistance towards this necrotroph. Further genetic, biochemical and molecular studies confirmed that WRKY33 acts upstream of *NCED3* and *NCED5* to negatively regulate ABA biosynthesis and signaling. These results reveal a novel role of WRKY33 in modulating host immunity by repressing part of the ABA hormone regulatory network.

Comparative analysis of WRKY33 function between the *B. cinerea* avirulent strain 2100 and the virulent strain B05.10 revealed that *Botrytis*-induced WRKY33 gene expression and protein accumulation are significantly lower in *B. cinerea* B05.10 infected WT plants than in plants challenged with strain 2100. As a consequence, the expression levels of WRKY33-regulated target genes involved in the biosynthesis of antifungal camalexin and JA/ET related defense signaling are clearly reduced upon B05.10 infection. However, WRKY33-overexpression lines were clearly resistant to B05.10. These data strongly suggest that WRKY33-dependent early immune responses in WT plants were negatively affected by *B. cinerea* virulent strain B05.10. Further analysis of *Arabidopsis* ABA deficiency mutants in combination with *B. cinerea* B05.10 *BcABA* mutants revealed that, as is the case for strain 2100, *Arabidopsis*-derived ABA negatively regulates plant immune responses to B05.10. Elevated JA/ET levels were observed in *nced3 nced5* double mutants, suggesting that ABA antagonizes JA/ET signaling upon B05.10 infection. This ABA antagonism of JA/ET signaling was dependent on WRKY33.

In conclusion, this thesis provides novel insights into the role of WRKY33 in modulating host immunity towards both an avirulent and a virulent strain of the necrotrph *B. cinerea*.

Zusammenfassung

Der Transkriptionsfaktor WRKY33 von *Arabidopsis thaliana* ist ein zentraler Regulator von pflanzlichen Reaktionen auf den *B. cinerea* Stamm 2100 und essentiell für die Resistenz der Pflanze gegenüber diesem Stamm. Um beteiligte wichtige molekulare Komponenten in diesem Prozess zu finden, wurden WRKY33-regulierte Gene 14 Stunden nach Behandlung mit *B. cinerea* genomweit kartiert. Dazu wurden die mittels ChIP-seq ermittelten WRKY33 Bindungsstellen im Genom mit RNA-seq Ergebnissen von *B. cinerea* induzierten und WRKY33-abhängigen Eexpressionsprofilen abgeglichen. Vergleichende Genexpressionsanalysen zwischen resistenten wildtyp Colombia-0 und anfälligen *wrky33* Mutantenpflanzen ergab, daß *B. cinerea*-induziertes WRKY33 Protein sowohl transkriptionsaktivierende als auch -hemmende Aufgaben bei der Regulation von hunderten Zielgenen mit bestimmten molekularen Funktionen hat, aber hauptsächlich als Repressor fungiert. Die genomweite Untersuchung bestätigte bereits bekannte WRKY33 Zielgene, die an den Ethylen (ET) und Jasmonsäure (JA) Hormon-Signalketten und der Phytoalexin Biosynthese beteiligt sind. Es wurden aber auch neue wesentliche Komponenten entdeckt, die die komplexe Regulation der Resistenz gegenüber *B. cinerea* beeinflussen. Im Besonderen wurden die Abscisinsäure (ABA) Biosynthesegene *NECD3* und *NECD5* als Schlüsselgene der WRKY33-vermittelten Resistenz gegenüber diesem nekrotrophen Pilz identifiziert. Eingehendere genetische, biochemische und molekulare Untersuchungen ergaben, daß WRKY33 oberhalb von *NCED3* und *NCED5* agiert um in negativer Weise die ABA Biosynthese und Signalkette zu regulieren. Damit stellen diese Ergebnisse eine neue Rolle von WRKY33 bei der Modulierung der wirtseigenen Immunität (host immunity) dar, indem ein Teil des ABA Hormon zugehörigen Regulationsnetzwerkes unterdrückt wird.

Vergleichende Untersuchungen des WRKY33 Verhaltens gegenüber dem *B. cinerea* avirulenten Stamm 2100 und dem virulenten Stamm B05.10 ergaben, daß die WRKY33 Genexpression und Proteinanreicherung in B05.10-infizierten wildtyp Pflanzen signifikant geringer war als in Pflanzen die mit dem Stamm 2100 infiziert waren. Als eine Folge davon war die Expressionsstärke der WRKY33-regulierten Zielgene, die an der Biosynthese des fungiziden Camalexins und an der JA/ET Signalkette beteiligt sind, nach Behandlung mit B05.10 klar reduziert. Allerdings waren WRKY33 überexpremierende Pflanzen nach Infizierung mit B05.10 eindeutig resistent. Diese Ergebnisse deuten stark darauf hin, daß WRKY33-abhängige frühe Immunreaktionen in wildtyp Pflanzen durch den virulenten *B. cinerea* Stamm B05.10 negativ beeinflußt waren. Eine genauere Untersuchung von

Arabidopsis ABA-defizienten Mutanten in Kombination mit B05.10 *BcABA* Mutanten ergab, daß, wie auch beim Stamm 2100, von *Arabidopsis* herrührendes ABA die Pflanzenimmunreaktionen unterdrückt. In *nced3* und *nced5* Mutanten wurden erhöhte JA/ET Werte beobachtet, was darauf hindeutet, daß ABA die JA/ET Signalketten nach B05.10 Infektion unterdrückt. Dieser Antagonismus zwischen ABA und der JA/ET Signalkette war abhängig von WRKY33.

Abschließend kann gesagt werden, daß die vorliegende Arbeit neue Einblicke in die Aufgaben von WRKY33 bei der Modulation der Wirtsimmunität gegenüber einem avirulenten und einem virulenten *B. cinerea* Stamm gewährt.

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Chapter 1: General introduction

1.1. Plant innate immunity

In nature, plants are under continuous biotic and abiotic stresses that compromise plant growth, survival and reproduction. The biotic stresses are mostly caused by different phytopathogens such as bacteria, fungi, viruses, oomycetes, and insects. Such stresses already affect global food security and thus endanger the human civilization (Dodds and Rathjen, 2010; Dangl et al., 2013). In the past, the oomycete *Phytoththora infestans* caused the late blight Irish potato famine of the 1840s, and the fungus *Fusarium oxysporum*, the causal agent of Panama disease, led to the loss of the world's first mass-cultivated banana cultivar *Gros Michel* in the 1920s. Currently, the wheat stem, leaf and yellow stripe rust epidemics are spreading from East Africa into the Indian subcontinent caused by rust fungi *Puccinia graminis* and *P. striiformis* are all testament to the recurring impact of plant diseases affecting human food supply (reviewed by Dangl, et al., 2013).

Plants have developed mechanisms to detect pathogens and to activate defense responses (Denance et al., 2013). The plant immune system consists of two interconnected branches termed PAMP-triggered immunity (PTI) and effector-triggered immunity (ETI) (Dodds and Rathjen, 2010). PTI is initiated upon the perception of common microbial compounds, named pathogen- or microbe- associated molecular patterns (PAMPs or MAMPs), such as bacterial flagellin or fungal chitin, by pattern recognition receptors (PRRs) at the cell surface (Staal et al., 2008; Zipfel, 2009). Plants also respond to endogenous plant-derived signals that arise from damage caused by pathogen invasion, called damage-associated molecular patterns (DAMPs) (Staal et al., 2008). Successful pathogens have evolved means to minimize host immune stimulation and utilize virulence effector molecules to bypass PTI, either by suppressing PTI signaling or preventing detection by the host (Pieterse et al., 2014). In turn, plants have acquired an additional line of defense in which resistance (R) NB-LRR (nucleotide-binding-leucine-rich repeat) receptor proteins mediate recognition of pathogen-specific effector molecules, resulting in ETI (Dodds and Rathjen, 2010). ETI is a manifestation of gene-for-gene resistance, which is often accompanied by a programmed cell death at the site of infection that prevents further ingress of biotrophic or hemi-biotrophic pathogens that thrive on living host tissue (Pieterse et al., 2014). Currently, specific recognition of necrotrophic pathogens by similar mechanisms has not been documented. With the exception of *Arabidopsis thaliana* RESISTANCE TO LEPTOSPHAERIA MACULANS 3 (RLM3), a Toll/interleukin 1 receptor domain R-protein implicated in broad immunity to several necrotrophs (Staal et al., 2008), no R-gene has been specially associated with

resistance to necrotrophs such as *Botrytis cinerea*. Plant immunity to *B. cinerea* appears to be under complex genetic control (Rowe and Kliebenstein, 2008).

Upon pathogen infection, or perception of pathogens in PTI and ETI, a set of downstream events are induced, including alterations in hormonal levels, activation of distinct signalling pathways, and transcriptional reprogramming. In particular, rapid induction of hormone-mediated pathways within the host have been demonstrated to play pivotal roles for immunity against pathogenic threats (Glazebrook, 2005; Tsuda et al., 2009).

1.2. Hormonal modulation of plant immunity

The plant hormones salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) are recognized as major defense hormones and have been well studied (Robert-Seilaniantz et al., 2011). However, other hormones such as abscisic acid (ABA) (Ton et al., 2009), gibberellins (GA) (Navarro et al., 2008), auxins (IAA) (Kazan and Manners, 2009), cytokinins (CK) (Walters and McRoberts, 2006), brassinosteroids (BR) (Nakashita et al., 2003), nitric oxide (NO) (Moreau et al., 2010) and strigolactones (STR) (Torres-Vera et al., 2014) can also function as modulators of plant immune signaling networks (Pieterse et al., 2012). Generally, SA signaling positively regulates plant defense against biotrophic pathogens, whereas ET/JA pathways are commonly required for resistance to necrotrophic pathogens and chewing insects (Glazebrook, 2005; Bari and Jones, 2009). However, the situation appears more complex since the SA pathway is also required for plant resistance towards specific necrotrophic pathogens, whereas ET/JA pathways were found to be essential for resistance to some biotrophic pathogens (Berrocal-Lobo et al., 2002; Robert-Seilaniantz et al., 2011).

In *Arabidopsis* the biosynthesis of the small phenolic compound SA is based on the conversion of the primary metabolite chorismate via two distinct enzymatic pathways (Vlot et al., 2009), one involving the enzyme PHENYLALANINE AMMONIA LYASE (PAL), and the other ISOCHORISMATE SYNTHASE (ICS1/SID2). Only a small fraction of SA is produced via the PAL pathway upon pathogen infection, while the main proportion of SA is converted by ICS1 to isochorismate and further processed by ISOCHORSMATE PYRUVATE LYASE (IPL) to SA. *ICS1/SID2* mutants are severely compromised in pathogen-induced SA production and resistance (Garcion et al., 2008). Major components of SA-mediated signaling during pathogenic attack are ENHANCED DISEASE SUSCEPTIBILITY1 (EDS1) and PHYTOALEXIN DEFICIENT4 (PAD4) acting upstream of SA in basal resistance to adapted biotrophic pathogens and in *R* gene mediated ETI (Gassmann et al., 1999; Jirage et al., 1999; Wiermer et al., 2005). Downstream SA signaling

is highly dependent on the function of NON-EXPRESSOR OF PATHOGENESIS-RELATED GENE 1 (NPR1), which, upon activation by SA, acts as a transcriptional co-activator of a large set of defense related genes including PATHOGENESIS-RELATED 1 (*PR1*) (Mou et al., 2003). Recent studies have demonstrated that nuclear NPR1 and its paralogs NPR3, NPR4 can act as SA receptors, respectively (Fu et al., 2012; Wu et al., 2012). Interestingly, NPR1 in the cytosol plays a role in the crosstalk between SA and JA (Vlot et al., 2009).

JA belongs to the jasmonate class of plant hormones. JA is derived from α-linolenic acid. α-linolenic acid is processed by a series of reactions involving different enzymes including lipoxygenease (LOX), allene oxide synthase (AOS) and allene oxide cyclase (AOC) to 12-oxo-phytodienoic acid (OPDA) (Wasternack and Kombrink, 2010). OPDA is further processed via several enzymatic reactions in the peroxisome including the OPDA REDUCTASE 3 (OPR3), to JA. Finally, JASMONATE RESISTANT 1 (JAR1) catalyzes the conjugation of the amino acid isoleucine (Ile) to JA thereby forming the molecularly active hormone JA-Ile (Staswick et al., 2002; Fonseca et al., 2009). The F-box protein CORONATINE INSENSITIVE 1 (COI1) is a key regulator of the JA signaling pathway. Together with JASMONATE ZIM (JAZ) domain transcriptional repressor proteins, COI1 functions as a JA-Ile receptor in the E3 ubiquitin-ligase Skp1-Cullin-F-box complex SCF^{COI1} (Sheard et al., 2010). JAZ proteins act as transcriptional repressors of JA signaling by binding to positive transcriptional regulators such as MYC2, 3 and 4 (Fernandez-Calvo et al., 2011; Niu et al., 2011). After JA (JA-Ile) accumulation and perception, the physical interaction between JAZ proteins and the transcriptional activators are disrupted due to degradation of the JAZ proteins, which results in derepression of the JA signaling pathway and activation of a large number of JA-responsive genes (Memelink, 2009).

The JA and ET pathways are thought to operate mainly synergistically during plant defense as both hormones induce similar subsets of defense-related genes upon pathogen challenge (Bari and Jones, 2009). Both JA and ET signaling can activate members of the APETALA2/ETHYLENE RESPONSE FACTOR (AP2/ERF) family of transcription factors, such as ERF1 and OCTADECANOID-RESPONSIVE ARABIDOPSIS59 (ORA59) (Lorenzo et al., 2003; Pre et al., 2008). ERF1 and ORA59 positively regulate one JA/ET sub-branch leading to the activation of downstream JA-responsive gene such as *PLANT DEFENSIN1.2* (*PDF1.2*). In contrast, MYC factors positively control the second sub-branch thereby activating genes such as *VEGETATIVE STORAGE PROTEIN2* (*VSP2*) and *THIONINE 2.1* (*Thi2.1*) (Dombrecht et al., 2007). MYC factors negatively influence the ERF1/ORA59

branch. In general, the ERF branch of the JA pathway is associated with enhanced resistance to necrotrophic pathogens, whereas the MYC branch of the JA pathway is associated with the wound-response and defense against insect herbivores, although MYC2 also plays a role in priming for enhanced pathogen defense (Pieterse et al., 2012).

The phytohormone ABA plays crucial regulation role in many aspects of plant growth, development, and responses to environmental stresses including biotic and abiotic stresses (Nambara and Marion-Poll, 2005; Raghavendra et al., 2010; Denance et al., 2013; Leng et al., 2014). In higher plants, the ABA biosynthetic pathway is well understood, and numerous mutants have been identified at each step in this pathway in *Arabidopsis*, tomato, and maize (Leng et al., 2014). The early step of ABA biosynthesis takes place in plastids (Finkelstein, 2013). All *Arabidopsis* mutants isolated on the basis of AB-deficiency affect steps downstream of zeaxanthin synthesis, for example, *aba1, aba2, aba3, aba4, nceds,* and *aaos*. The ABA deficiency mutant *aba1* produces non-dormant seeds (Koornneef et al., 1982). *ABA1* in *Arabodopsis* encodes zeaxanthin epoxidase (ZEP), the enzyme that converts zeaxanthin to violaxanthin via the intermediate antheraxanhin (Rock and Zeevaart, 1991). The next steps in ABA synthesis are conversion to trans-neoxanthin, isomerization of either (trans)-violaxanthin and trans-neoxanthin to their 9-cis-isomers, and cleavage by 9-cis-epoxycarotenoid dioxygenase (NCED) to release xanthoxin, also known as xanthoxal (Finkelstein, 2013). Overexpression experiments in transgenic plants demonstrated that xanthophyll cleavage by NCED is a key rate-limiting step in ABA biosynthesis (Nambara and Marion-Poll, 2005; Leng et al., 2014). NCEDs are encoded by multigene families in all species analyzed, with differential expression of specific family members contributing to ABA synthesis in different context (Finkelstein, 2013). Arabidopsis has 9 potential NCED genes, 5 of which actually function as *NCEDs* (Tan et al., 2003). Xanthoxin exits the plastid into the cytosol where it is oxidized in two further steps by ABA2 and abscisic aldehyde oxidase (AAO) to form ABA (Qin and Zeevaart, 1999; Schwartz et al., 2003; Taylor et al., 2005; Finkelstein, 2013). *ABA2* encodes a short chain dehydrogenase/reductase-like (SDR1) enzyme catalyzing production of abscisic aldehyde. The final step is catalyzed by abscisic aldehyde oxidase (AAO). AAO requires a molybdenum cofactor for activity and *AtABA3* encodes the sulfurase that produces a functional cofactor. Consequently, *aba3* mutants are disrupted in all possible AAO activities. ABA catabolism occurs via hydroxylation reactions. In the hydroxylation pathway, among three different methyl groups, C-8’ is the predominant position for the hydroxylation reaction, which is mediated in *Arabidopsis* by proteins encoded by the *CYP707A* gene family, including *CYP707A1, CYP707A2* and *CYP707A3* (Kushiro et

al., 2004; Saito et al., 2004).

In addition to its role in development and adaption to abiotic stress (Shinozaki and Yamaguchi-Shinozaki, 2007; Wasilewska et al., 2008; Leng et al., 2014), ABA has emerged as an important modulator of the plant immune signaling network (Asselbergh et al., 2008; Ton et al., 2009; Cao et al., 2011; Feng et al., 2012; Sanchez-Vallet et al., 2012). In plant defense, ABA can function as a positive or a negative regulator of plant defense depending on the lifestyle of the pathogen (Denancé et al., 2013). ABA positively regulate host resistance to some pathogens including *Alternaria brassicicola, Ralstonia solanacearum*, and *Pythium irregular*, as ABA-deficient and insensitive mutants were found to be more susceptible than wild-type (WT) plants to these pathogens (Adie et al., 2007; Flors et al., 2008; Jiang et al., 2009; Garcia-Andrade et al., 2011). However, ABA-impaired (in biosynthesis or signaling) mutants in tomato (*sitiens*) and *Arabidopsis* were shown to have enhanced resistance to some other pathogens such as *B. cinerea, Pseudomonas syringae, F. oxysporum, Plectosphaerella*, and *Hyaloperonospora parasitica* (Audenaert et al., 2002; Mohr and Cahill, 2003; de Torres-Zabala et al., 2007; de Torres Zabala et al., 2009; Garcia-Andrade et al., 2011; Sanchez-Vallet et al., 2012). In addition, ABA plays a direct role in regulating R protein activity. Plants treated with ABA or to high temperature both have reduced nuclear accumulation of SNC1 (Suppressor of *npr1*, constitutive 1) and RESISTANCE TO PSEUDOMONAS SYRINGAE4 (RPS4), and are compromised in disease resistance to *P. syringae* (Mang et al., 2012).

Negative interactions of ABA with the major hormones involved in plant defense (SA, JA, and ET) have been described (Robert-Seilaniantz et al., 2011; Pieterse et al., 2012; Sanchez-Vallet et al., 2012). For instance, the application of exogenous ABA or drought stress to *Arabidopsis* reduced plant tolerance to an avirulent *P. syringae* strain (Mohr and Cahill, 2003). Moreover, genetic and chemical studies in *Arabidopsis* showed that systemic acquired resistance (SAR) induction is suppressed by ABA through inhibition of the pathway both upstream and downstream of SA (Yasuda et al., 2008). In PAMP signaling, ABA is utilized by pathogens to suppress the SA mediated pathway (Boatwright and Pajerowska-Mukhtar, 2013). The bacteria toxin, coronatine, triggered ABA accumulation, resulting in the suppression of SA synthesis (de Torres Zabala et al., 2009). The suppression of SA by ABA is also observed in tomato and rice plants (McDonald and Cahill, 1999a; Audenaert et al., 2002; Asselbergh et al., 2008). Conversely, activation of SAR suppresses both ABA-biosynthetic and ABA responsive genes in an NPR1-dependent manner (Yasuda et al., 2008). Likewise, the suppressive effect of SA on abiotic stress responses has also been shown. For

example, pre-treatment of *Zea mays* with SA results in decreased drought tolerance (Nemeth et al., 2002). ABA and SA have been shown to function antagonistically, they both trigger stomata closure thereby acting together to avoid invasion by the bacterium *P. syringae* in *Arabidopsis* (Melotto et al., 2006).

Negative interaction between ABA and JA/ET signaling pathways has been demonstrated (Anderson et al., 2004; Robert-Seilaniantz et al., 2011). Exogenous application of ABA on *Arabidopsis* plants repressed expression of JA/ET-related defense genes. Consistent with this result, disruption of the ABA biosynthesis-related genes *ABA1* and *ABA2* led to enhanced expression of ET/ JA responsive genes (Anderson et al., 2004). This is also consistent with the negative interaction of ABA- and JA-signaling in the modulation of *Arabidopsis* resistance to the necrotrophic fungus *Plectosphaerella cucumerina* (Sanchez-Vallet et al., 2012). Conversely, mutants in JA and ET signaling showed up-regulation of ABA responsive genes or ABA-related phenotypes, indicating that JA/ET also act to suppress the ABA signaling pathway (Anderson et al., 2004). ABA also played a positive role in activation of JA biosynthesis. In *Arabidopsis*, ABA has been shown to be required for JA biosynthesis that is essential for resistance to *Pythium irregulare* (Adie et al., 2007). Moreover, an activation-tagged mutant that overexpresses *NCED5*, encoding an ABA biosynthetic enzyme, accumulates higher amounts of ABA and JA and had reduced SA content (Fan et al., 2009).

It is now obvious that the phytohormone pathways are all intimately connected and that substantial crosstalk between pathways exists. This complex network of different signaling pathways, probably allows plants to fine-tune their responses to different biotic stress situations (Pieterse et al., 2012; Denance et al., 2013).

1.3. Phytoalexins in defense against pathogens

Next to the plant hormones, plant secondary metabolites play important roles in plant defense. Low molecular mass secondary metabolites with antimicrobial activity that are induced by various stresses are collectively named phytoalexins (Hammerschmidt, 1999; Pedras et al., 2011a). Phytoalexins show biological activity towards a variety of pathogens and are considered as molecular markers of disease resistance (Ahuja et al., 2012).

Camalexin is the major phytoalexin of *A. thaliana*, which is induced in the leaves by a great variety of biotrophic and necrotrophic plant pathogens (Glawischnig, 2007). The camalexin biosynthetic capacity is not restricted to leaves but also is observed in roots upon infection with the *Pythium sylvaticum* (Bednarek et al., 2005). Camalexin can also be induced by

PAMPs, such as the oomycete-derived necrosis and ethylene-induced peptide 1 (Nep1)-like proteins and bacteria-derived peptidoglycans (Qutob et al., 2006; Gust et al., 2007). Other PAMPs such as flg22 were reported to induce the expression of camalexin biosynthetic genes, although triggering of camalexin biosynthesis has not been observed in all instances (reviewed by Ahuja, et al., 2012).

Camalexin is derived from tryptophan and the early biosynthetic steps are shared with other indolic compounds, such as indole glucosinolates. Although camalexin biosynthesis in Arabidopsis has not yet been fully elucidated, several of the steps in the pathway have been characterized. The enzymes in this pathway include CYP79B2/B3 (Hull et al., 2000; Mikkelsen et al., 2000), CYP71A12 (Millet et al., 2010), CYP71A13 (Nafisi et al., 2007), GSTF6 and GGT1/GGT2 (Su et al., 2011), GGP1/GGP3 (Geu-Flores et al., 2011), PCS1 (Bottcher et al., 2009) and CYP71B15 (PAD3) (Glazebrook and Ausubel, 1994; Zhou et al., 1999). CYP79B2 and CYP79B3 convert tryptophan to indole-3-acetaldoxime (IAOx), and the *cyp79b2 cyp79b3* double mutant is devoid of indole glucosinolates, has only trace amounts of camalexin, and synthesizes reduced levels of the phytohormone indole-3-acetic acid (IAA) in heat stressed seedlings and root tips (Zhao et al., 2002; Glawischnig et al., 2004; Ljung et al., 2005). Thus, IAOx is the substrate not only for the biosynthesis of camalexin, but also for indole glucosinolates and IAA. Subsequently, CYP71A13 catalyzes IAOx to indole-3-acetonitrile (IAN) in leaves (Nafisi et al., 2007). A homolog gene *CYP71A12* was shown to have an important role in camalexin synthesis in roots (Millet et al., 2010). The penultimate steps in camalexin biosynthesis is catalyzed by the multifunctional enzyme CYP71B15 (PAD3) (Schuhegger et al., 2006; Bottcher et al., 2009). The *pad3* mutant accumulates at best trace amounts of camalexin upon biotic stress.

Phytoalexins are typically synthesized locally in proximity to the site of pathogen infection (Kuc, 1995). This is also the case for camalexin based on spatial distribution analyses after infection with *B. cinerea* and *Alternaria alternate* (Schuhegger et al., 2007). High camalexin concentrations were observed at the infection site (*A. alternate*) or in proximity to the lesions induced by *B. cinerea*, while leaf areas that did not show disease symptoms were camalexin deficient (Kliebenstein et al., 2005).

The regulation of camalexin biosynthesis was reported to involve hormones, MAPKs, and microRNAs (Ahuja et al., 2012). Based on numerous genetic and chemical studies, hormone-dependancy of camalexin biosynthesis is thought to depend on the infecting pathogen. Some studies indicate that induction of camalexin is dependent on SA (Denby et al., 2005), JA

(Rowe et al., 2010) and ET (Thomma et al., 1999a; Heck et al., 2003), respectively. Other reports demonstrate that camalexin biosynthesis is independent of hormones (Nawrath and Metraux, 1999; Thomma et al., 1999b; Roetschi et al., 2001; van Wees et al., 2003; Ren et al., 2008b). In addition, several reports have shown that camalexin biosynthesis is regulated through MAPK cascades and WRKY transcription factors (TFs) (Qiu et al., 2008; Mao et al., 2011). For example, MPK3/MPK6 affected camalexin levels through transcriptional activation and phosphorylation of WRKY33 in response to *B. cinerea* infection. WRKY33 was previously shown to control camalexin levels by regulating the expression of genes, such as *PAD3* and *CYP71A13* (Mao et al., 2011). Another MAP kinase, MPK4 interacts with its substrate MAP kinase substrate 1 (MKS1), the later directly interacting with WRKY33, thereby forming a ternary protein complex. PAMP treatment or *P. syringae* infection activates MPK4, which phosphorylates MKS1 and this event releases WRKY33 from the ternary protein complexes. The WRKY33 protein is subsequently detected at the promoter of *PAD3* and presumably activates gene expression (Qiu et al., 2008). Two other WRKY TFs, WRKY18 and WRKY40, have been implicated in negatively regulating camalexin biosynthesis based on the observation that higher transcriptional activation of *CYP71A13*, and higher camalexin levels are detected in the *wrky18 wrky40* double mutants compared to WT plants (Pandey et al., 2010).

1.4. Transcriptional regulation of plant immunity

Global expression analyses suggest that transcriptional re-programming of plant cells is a crucial step to mount an efficient defense response (Ferrari et al., 2007; Pandey and Somssich, 2009; Rushton et al., 2010; Birkenbihl et al., 2012; Mathys et al., 2012; Mulema and Denby, 2012). Several transcription factor families are involved in the regulation of gene expression upon pathogen challenge. These defense-related TFs include MYB, AP2/ERF, MYC/bHLH, NAC, TGA/bZIP and WRKY family members.

For example, several MYB-type *Arabidopsis* transcription factors have been identified that regulate distinct host transcriptional outputs in response to *B. cinerea* infection. BOS1 (*BOTRYTIS SUSCEPTIBLE 1*)/MYB108 appears to restrict necrosis triggered by *B. cinerea* and *A. brassicicola*, and loss-of-BOS1 function resulted in increased plant susceptibility (Mengiste, 2012). In response to stress and *B. cinerea*, BOS1 physically interacts with and is ubiquitinated by BOI, a RING E3 ligase that contributes to defense by restricting the extent of necrosis (Luo et al., 2010). MYB51 is involved in the transcriptional activation of indole glucosinolate biosynthetic genes, which also contributes to resistance towards necrotrophs

(Kliebenstein et al., 2005; Sanchez-Vallet et al., 2010). In contrast, the MYB-related genes *ASYMMETRIC LEAVES 1 (AS1)* and *MYB46* appear to play a role in disease susceptibility as the mutants show increased disease resistance towards *B. cinerea* (Choquer et al., 2007b; Ramirez et al., 2011).

Many transcriptional activators and repressors of the ET/JA pathways also impact resistance to *B. cinerea* (Glazebrook, 2005; Bari and Jones, 2009). In particular the TFs ERF1, ORA59, ERF5, ERF6 and RAP2.2, have regulatory functions in influencing host susceptibility to *B. cinerea* (Berrocal-Lobo et al., 2002; Pre et al., 2008; Moffat et al., 2012; Zhao et al., 2012). Transgenic Arabidopsis lines over-expressing *ERF1* or *ORA59* were sufficient to confer resistance to *B. cinerea* (Kazan and Manners, 2013) whereas *RNAi-ORA59* silenced lines were more susceptible (Pre et al., 2008). Both ERF1 and ORA59 appear to be key integrators of the ET- and JA-signaling pathways (Muckenschnabel et al., 2001). In contrast, the bHLH transcription factor MYC2/JIN1 is a master regulator of diverse JA-mediated responses by antagonistically regulating two distinct branches of the JA signaling pathway in response to necrotrophs (Kazan and Manners, 2013).

NAC TFs are also involved in plant immunity. Overexpression of NAC TF *ATAF1* resulted in enhanced susceptibility of Arabidopsis plants to *B. cinerea* (Wang et al., 2009). In contrast, the ability of *Arabidopsis* to restrict penetration by the non-host barley pathogen *Blumeria graminis* was shown to be dependent on ATAF1-mediated repression of ABA biosynthesis (Jensen et al., 2008). ATAF1 was shown to directly bind to the *NCED3* promoter, which positively correlated with increased *NCED3* expression and ABA levels (Jensen et al., 2013). Like *ATAF1*, transgenic lines overexpressing *NAC019* or *NAC055* resulted in enhanced susceptibility of Arabidopsis plants to *B. cinerea.* In contrast, the *nac019 nac055* double mutant showed increased resistance to *B. cinerea* compared with WT plants (Bu et al., 2008).

Co-regulators interacting with TFs also play key roles in plant immunity (Buscaill and Rivas, 2014). For example, during the *Arabidopsis* SAR response, SA induces NPR1 activation and nuclear NPR1 interacts with members of the TGA-bZIP TF subfamily to modulate the transcriptional responses of *PR1* and *WRKY* defense-related genes (Tada et al., 2008; Pajerowska-Mukhtar et al., 2013). As already mentioned the JAZ proteins act as transcriptional repressors of JA signaling by binding to the bHLH class transcriptional regulators MYC2, 3 and 4 (Pauwels et al., 2010; Fernandez-Calvo et al., 2011). More recently, JAZ proteins are reported to additionally interact with other bHLH TFs, including bHLH003, bHLH012 and bHLH017, which function as transcriptional repressors (Fonseca et

al., 2014). These studies suggest an intricate competition between activators and repressors interacting with JAZ repressors that determine the proper output of JA-dependent transcriptional responses (Buscaill and Rivas, 2014).

1.5. WRKY transcription factors in plant immunity

In addition to other TFs, the WRKY family of transcriptional regulators have been shown to regulate various developmental processes but most prominently to regulate gene expression during plant defense responses (Pandey and Somssich, 2009).

In Arabidopsis, the WRKY family contains 74 expressed genes (Eulgem et al., 2000; Rushton et al., 2010). All WRKY TFs contain at least one highly conserved ~60 amino acid long region, designated WRKY domain, encompassing the WRKYGQK heptapeptide along with a zinc-finger forming motif that enables binding to a specific DNA elements termed the W box (5'-T/C-TGAC-T/C-3'). The slight variations of the WRKY domain and alternative binding sites have also been identified (Ciolkowski et al., 2008; Pandey and Somssich, 2009). Moreover, the nucleotides directly adjacent to the W-box sequence, either at the 5'end or at the 3'end, can determine certain binding preferences of different WRKY proteins (Ciolkowski et al., 2008). Yamasaki et al. (2012) could resolve the structure of the WRKY domain in complex with a W-box (Yamasaki et al., 2012). This analysis revealed that the WRKY domain consists of several β-strands. The WRKYGQK residues form the most N-terminal β-strand and appear to enter the major DNA groove and form contacts with the W-box. WRKY proteins contain characteristic features of TFs such as nuclear localization signals, activation/repression domains and domains associated with protein - protein interactions (Eulgem et al., 2000; Rushton et al., 2010; Chi et al., 2013; Llorca et al., 2014).

Several genetic studies have revealed the importance of various WRKY TFs in positively or negatively regulating plant immunity although due to functional redundancy, single *wrky* mutants often to not show clear phenotypes (Eulgem and Somssich, 2007). In *Arabidopsis*, WRKY18 and WRKY40 negatively modulate host defense towards the biotrophic powdery mildew fungus *Golovinomyces orontii*, as *wrky18 wrky40* double mutants render otherwise susceptible wild-type plants resistant to this pathogen (Pandey et al., 2010). Resistance in *wrky18 wrky40* double mutant plants is accompanied by massive transcriptional reprogramming, modulating expression of the defense regulator *EDS1* and camalexin biosynthetic pathway genes, fine-tuning hormones signaling. Interestingly, WRKY18 and WRKY40 were also shown to negatively regulate PTI upon infection with *P. syringae*

DC3000 but to positively impact RPS4-dependent ETI (Chen et al., 2010; Schnon et al., 2013). *Arabidopsis* WRKY11 and WRKY17 were also found to act together to negatively regulate PTI to *P. syringae* DC3000 (Journot-Catalino et al., 2006). A similar finding was observed for WRKY38 and WRKY62 (Kim et al., 2008b). Additional *Arabidopsis* WRKY TFs that appear to negatively regulate host immunity include; WRKY7, WRKY23, WRKY25, WRKY27, WRKY41, WRKY48, WRKY53, WRKY58, WRKY60 (Pandey and Somssich, 2009). In contrast, *Arabidopsis* WRKY3 and WRKY4 have redundant functions in positively modulating resistance towards *B. cinerea* (Lai et al., 2008), and WRKY53 and WRKY70 both positively modulate SAR (Wang et al., 2006). Furthermore, WRKY TFs are crucial mediators in defense-related hormone signaling crosstalk (Pieterse et al., 2012). *Arabidopsis* WRKY70 represents an important node of convergence between SA and JA signaling (Li et al., 2004; Li et al., 2006). The mutant lacking WRKY70 function is susceptible to different bacteria, fungi and the oomycete, but is not affected in the antagonistic effect of SA on the JA pathway (Ren et al., 2008a; Leon-Reyes et al., 2010). Based on genetic studies other WRKY TFs have been connected to SA-JA signaling crosstalk, including WRKY8, WRKY11, WRKY17, WRKY18, WRKY40, WRKY41, WRKY50, WRKY51, WRKY60 and WRKY62 (Journot-Catalino et al., 2006; Xu et al., 2006; Higashi et al., 2008; Chen et al., 2010; Gao et al., 2011; Pieterse et al., 2012).

In rice *OsWRKY45* expression is induced upon SA treatment and infection with the rice blast fungus *Magnaporthe grisea*. Rice contains the pair of allelic genes *OsWRKY45-1* and *OsWRKY45-2* (Tao et al., 2009). Overexpression of *OsWRKY45-1 and -2* both lead to an enhanced resistance towards *M. grisea*, whereas *OsWRKY45* knockdown plants did not reveal increased susceptibility (Shimono et al., 2007; Shimono et al., 2012). Interestingly however, *OsWRK45-1* appears to negatively regulate resistance to *Xanthomonas oryzae* whereas *OsWRKY45-2* seems to be a positive regulator. More recently, OsWRKY45 was reported to interact with a coiled-coiled-nucleotide-binding site-leucine-rich repeat (CC-NB-LRR) protein Pb1 (Inoue et al., 2013). Further analysis indicated that Pb1-mediated rice panicle blast resistance is largely compromised when *WRKY45* expression was knocked down in a Pb1-containing rice cultivar 'Modan'.

In barley, ETI to barley powdery mildew (*B. graminis f. sp. hordei*) requires recognition of the fungal AVR effector by the CC-NB-LRR type R protein MLA (Shen et al., 2007). Recognition of AVR_{A10} by MLA10 induces nuclear association between the activated MLA10 homodimer and the transcriptional repressor *Hv*WRKY1 or *Hv*WRKY2 (Shen et al., 2007;

Maekawa et al., 2011). Thus, MLA-WRKY association appears to interfere with the WRKY repressor function thereby resulting in de-repression of PTI. A recent report identified the MYB TF HvMYB6 as an additional MLA10-interacting TF that positively regulates resistance to *B. graminis* (Chang et al., 2013). HvMYB6 also interacts with the HvWRKY1 repressor. Activated MLA releases the HvMYB6 activator from HvWRKY1 repression, thereby enabling HvMYB6 binding to DNA and activating gene expression.

A recent report showed that WRKY TFs are able to act synergistically with calcium-dependent protein kinases (CPKs) proteins during ETI signaling in *Arabidopsis* (Gao et al., 2013). During ETI, a subgroup of WRKY TFs including WRKY8, 28, and 48 are phosphorylated by closely related CPK4, 5, 6 and 11, and subsequently activating expression of an ETI marker gene *WRKY46*. Activation of *WRKY46* expression is thought to be dependent on the kinase activity of CPKs and the phosphorylated WRKYs (Gao et al., 2013). A direct interaction between MAPK and WRKY proteins has also been reported in *Nicotiana benthamiana*. NbWRKY8 regulates broad-spectrum disease resistance, and is a substrate of three pathogen-responsive MAPKs, SIPK, WIPK, and NTF4 (Ishihama et al., 2011). Phosphorylation of NbWRKY8 enhanced its DNA-binding and transcriptional activities.

Based particularly on the extensive studies performed in *Arabidopsis* it appears that the WRKY TF family is firmly involved in plant immunity with numerous members engaged in controlling diverse signaling pathways and mediating signaling crosstalk. Numerous *Arabidopsis* WRKY genes are themselves strongly responsive to pathogenic stimuli and their expression appear to be often dependent on W-box elements within their promoters. This implies the existence of substantial feedback and feed-forward mechanisms (cross-regulation and auto-regulation) as has been previously hypothesized (Eulgem and Somssich, 2007).

1.6. The necrotrophic pathogenic fungus *Botrytis cinerea*

B. cinerea is an ascomycetous fungus that causes rotting of plant material accompanied by the formation of gray conidiophores and conidia, thus the name gray mold disease. *B. cinerea* is a typical necrotroph, which co-opts programmed cell death pathways in the host to achieve colonization (van Baarlen et al., 2007). The fungus has a broad host-range capable of infecting more than 200 plant species worldwide, including a range of agronomically and economically important crops (Williamson et al., 2007). Due to its broad host-range, *B. cinerea* is the second most agriculturally important fungal plant pathogen (Dean et al., 2012). Global expenses of *Botrytis* control easily surmount € 1 billion per year (Dean et al., 2012).

B. cinerea is a genuine necrotroph: it has a broad host range, it secretes a large set of cell wall degrading enzymes and phytotoxic low molecular weight compounds, it rapidly kills host tissue, and is able to draw its nutrients exclusively from dead tissue (Tudzynski and Kokkelink, 2009). The life cycle of *B. cinerea* strain B05.10 under laboratory conditions is shown in Figure 1.1.

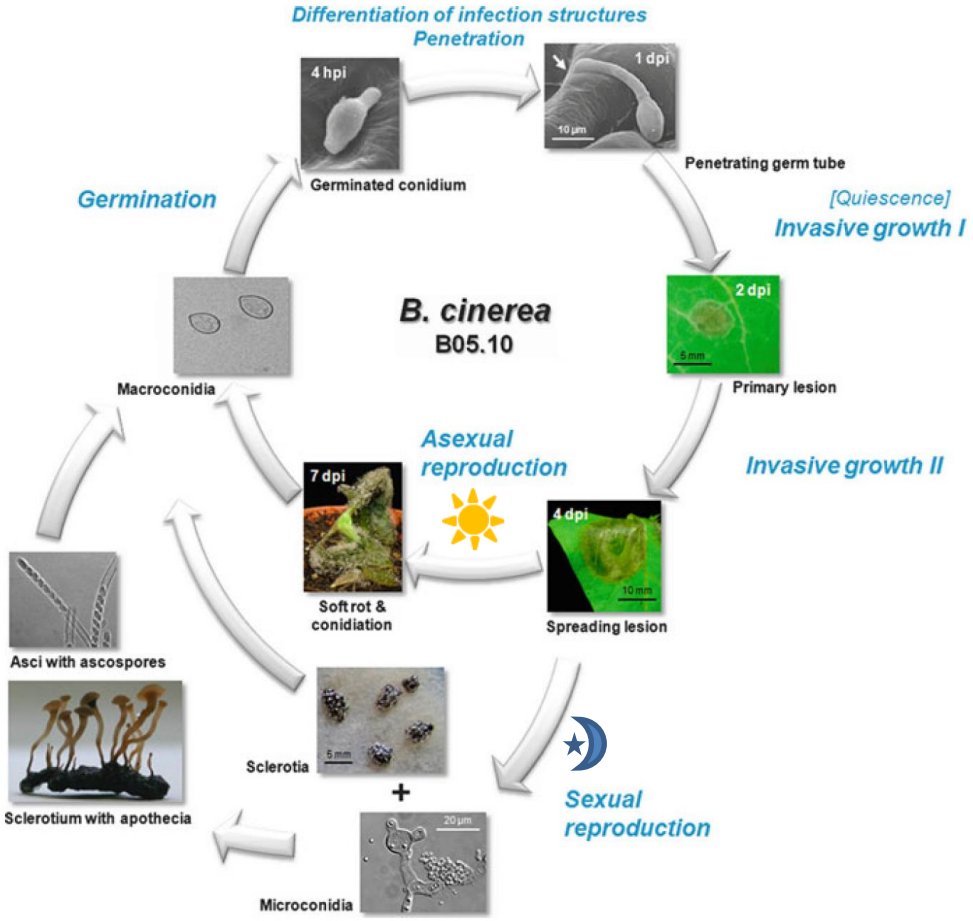


Figure 1.1: Life cycle of *B. cinerea* B05.10 (modified from Schumacher and Tudzynski, 2012). Within a few hours, the germination of conidia on primary leaves of French bean (*Phaseolus vulgaris*) starts. Quickly, the short germ tubes penetrate the epidermis and invade the plant tissue (Penetration). After two days post innoculation, the first macroscopically visible symptoms appear on the leaf including small necrotic spots (primary lesions). Subsequently, the lesion spreads quickly (Invasive growth II), reaching diameters of 25 mm after 4 days. After about 1 week the entire leaf is infected: the plant tissue collapses and becomes watery (soft rot) accompanied by formation of gray conidiophores and conidia under light condition (asexual reproduction). In the absence of light long-term survival structures such as sclerotia are formed after 3-4 weeks. In the field, these survival structures can be associated with living plants or with plant debris lying on or buried in soil. Sclerotia may germinate by forming conidiophores and conidia under appropriate conditions, forming the primary source of inoculum in the field. Additionally, the sclerotia can act as female (sclerotial) parent for fertilization with suspensions of microconidia from a male (spermatial) parent carrying the opposite mating type

(sexual reproduction). After several weeks of incubation under diurnal illumination, apothecia can be found containing asci with eight ascospores.

In the field, *B. cinerea* populations are known for high genetic variation in their aggressiveness on different plant species (Grindle, 1979). Thus, the outcome of experiments using different isolates should be carefully analyzed. Detailed molecular and comparative analysis among different isolates is becoming feasible as the genomes of two *B. cinerea* strains have recently been sequenced (Amselem et al., 2011).

The genome sequencing information is available for strain B05.10 (*Botrytis cinerea* Sequencing Project, Broad Institute of Harvard and MIT; http://www.broadinstitute.org/annotation /genome/botrytis cinerea/MultiHome.html) (Staats and van Kan, 2012) and strain T4 (Botrytis cinerea genome project, URGI, Genoscope; https://urgi.versailles.inra.fr/Species/Botrytis). The availability of the genome sequence and the feasibility of obtaining knockout mutants (van Kan et al., 1997; Schumacher et al., 2008) or achieving gene silencing (Patel et al., 2008, 2010), together with its economic relevance, have contributed to *B. cinerea* being the most extensively studied necrotrophic fungal pathogen.

Genome-wide transcriptional profiling of *B. cinerea* B05.10 gene expression during infections with different hosts has recently been reported (Blanco-Ulate et al., 2014). This study confirmed that *Botrytis* expressed most of its genes (>80%) encoding putative secreted Carbohydrate-active enZymes (CAZymes) during infection of three different hosts, including pectin backbone-modifying enzymes, hemicelluloses-modifying proteins, enzymes that potentially target pectin and hemicellulose side-branches and putative cellulose degrading enzymes (Blanco-Ulate et al., 2014). In addition, several proteomic analyses have been reported on *B. cinerea* (Shah et al., 2009b; Shah et al., 2009a; Espino et al., 2010; Fernandez-Acero et al., 2010; Cherrad et al., 2012; Li et al., 2012a; Gonzalez-Fernandez et al., 2013; Gonzalez-Fernandez et al., 2014). More recently, the mycelium and secreted proteome of six *B. cinerea* wild-type strains with different host range have been reported, including B05.10, T4, CECT2100, CECT2850, CECT2996 and CECT20518 (Gonzalez-Fernandez et al., 2014). Fungal genetic versatility among different strains was confirmed at the proteome level for both mycelium proteome and secreted proteins. In particular, some of identified proteins from the secretome have been reported as virulence factors, and play important roles in the successful colonization of a plant host. Among these studies, only 10% of the predicted secreted proteins from *B. cinerea* have been identified based on the methodology available

currently (Choi et al., 2010; Grandesso et al., 2014). Whether this is consistent with transcriptional profiling of secreted-protein related genes in *B. cinerea* is not known so far.

Some cell wall-degrading enzymes are related with *Botrytis* virulence based on molecular and genetic studies, including *Bcpme1* (Valette-Collet et al., 2003; Kars et al., 2005a), *Bcpg1* (ten Have et al., 1998), *Bcpg2* (Kars et al., 2005b) and *xyn11A* (Brito et al., 2006). However, most cell wall-degrading enzymes are encoded by multigenic families and some may have partly redundant functions (Choquer et al., 2007b). In addition, *B. cinerea* produces numerous phytotoxic compounds and proteins. Among them, the best know is botrydial (Colmenares et al., 2002). It accumulates around infection sites to high concentrations and its virulence to the host is strain dependent. Deletion of the *bcbot1* gene in some *B. cinerea* strains blocks the biosynthesis of botrydial and shows reduced virulence; however that is not the case for all tested strains (Siewers et al., 2005). *B. cinerea* can produce other toxins such as botcinolides (Reino et al., 2004; Tani et al., 2006). Thus, distinct *B. cinerea* strains may use different toxins, or combinations hereof, to kill plant tissue. Additionally, active oxygen species (AOS) production is also associated with *B. cinerea* pathogenicity (Govrin and Levine, 2000; Patykowski and Urbanek, 2003). The targeted deletion of the fungal superoxide dismutase (SOD) gene significantly reduced virulence and extracellular H_2O_2 accumulation at the host-fungus interface (Rolke et al., 2004). Moreover, *B. cinerea* is capable of producing several plant hormones in axenic cultures. So far, all tested *B. cinerea* strains produce large quantities of ethylene and low levels of IAA, while ABA is produced only by some, but not all, strains in culture (Siewers et al., 2004; Siewers et al., 2006; Cristescu et al., 2007). However, production of these plant hormones by *Botrytis in planta* has not been demonstrated, and it remains uncertain whether the fungus indeed utilizes plant substrates to produce plant hormones, and whether the fungal-produced plant hormones affect disease development.

1.7. The aims of the thesis

Previously our group and others have provided convincing evidence that *Arabidopsis* WRKY33 is a key transcriptional regulator of plant immunity towards the necrotrophic fungus *B. cinerea* (Zheng et al., 2006; Birkenbihl et al., 2012). WRKY33 positively modulates JA-dependent signaling while negatively affecting the SA pathway thereby implicating this TF in SA-JA signaling crosstalk (Birkenbihl et al., 2012). Applying ChIP-qPCR a small number of gene loci were identified that proved to be direct *in vivo* WRKY33 targets upon *B. cinerea* infection. These genes included *CYP71A13* and *PAD3* involved in camalexin biosynthesis, *JAZ1* and *JAZ5* encoding repressors of JA signaling, *ORA59*

encoding a TF positively affecting one branch of the JA-ET signaling pathway while negatively affecting the other branch (Lippok et al., 2007; Mao et al., 2011; Birkenbihl et al., 2012). However, based on the massive differential transcriptional reprogramming observed in WT and *wrky33* mutant plants upon *B. cinerea* 2100 infection (Birkenbihl et al., 2012), it was obvious that additional *in vivo* targets for WRKY33 binding are present within the Arabidopsis genome. Moreover, although genetic studies of *PAD3* clearly confirm its contribution to WRKY33-dependent resistance it cannot be the only component involved, as fungal growth *in planta* is significantly higher in the *wrky33* mutant compared to *pad3*. Hence, it was apparent that other key genes involved in WRKY33-dependent resistance towards this fungus have not yet been identified. We observed that the JA pathway was activated both in WT and *wrky33* mutant plants 14h post inoculation (14hpi) with *B. cinerea* but significantly decreased in the mutant 24hpi, whereas the SA pathway was strongly induced only in the mutant during infection (Birkenbihl et al., 2012). We therefore hypothesized that SA-mediated antagonism of the JA signaling pathway occurred at later infection stages, and that this antagonism should in part contribute to the susceptibility of *wrky33* to *B. cinerea*. However, genetic studies indicated that SA suppression of JA signaling was insufficient for WRKY33-mediated resistance (Birkenbihl et al., 2012).

Thus, the first and major aim of my study was to employ next generation sequencing technology (ChIP-seq and RNA-seq) to identify *B. cinerea* 2100-dependent WRKY33 *in vivo* binding targets on a genome-wide scale, and to associate such binding sites to neighboring genes whose expression is clearly altered during the infection process. Subsequent biochemical and genetic analyses would then be employed to determine the contribution of such candidate target genes in mediating host immunity towards this fungus.

As a second aim I plan to address the question whether WRKY33-dependent *Arabidopsis* immunity only acts against the *B. cinerea* strain 2100, or whether it also contributes to host immunity towards other *B. cinerea* strains or isolates. The interaction of *Botrytis* with numerous plants has been extensively studied in the past. Still, most studies on the molecular bases of plant-necrotroph interactions failed to consider possible pathogen variation. Recent findings however indicate that similar to what has been observed with bacterial and oomycete pathogens, plant immune responses to various *B. cinerea* strains may differ and may be mediated by distinct recognition mechanisms due to intraspecific variation in virulence gene repertoires of the fungus (Choquer et al., 2007b; Rowe and Kliebenstein, 2010). Thus, I plan to include two additional *Botrytis* isolates in my study to determine if pathogen variation has

an influence on host immunity determined by WRKY33.

In summary, the aim of the current thesis is to gain deeper insights into how *Arabidopsis* WRKY33 regulates host transcriptional outputs towards *B. cinerea* to establish plant immunity. Beyond this, this study should provide the first genome-wide view of a transcription factor modulating host target gene expression during the *in vivo* interaction of a plant with a phytopathogen.

Chapter 2: Genome-wide study of WRKY33-dependent host immunity networks towards *B. cinerea* strain 2100 involve repressing ABA signaling

2.1. Abstract

The necrotrophic fungus *Botrytis cinerea* causes serious crop losses worldwide. Plant immunity to the necrotrophic fungus *B. cinerea* is under complex genetic control. In *Arabidopsis thaliana* loss of WRKY33 function renders otherwise resistant Col-0 plants susceptible towards *B. cinerea* strain 2100. To elucidate the molecular components involved we performed global mapping of WRKY33 regulated target genes by integrating ChIP-seq data of WRKY33 binding sites with RNA-seq results of 14h *B. cinerea*-induced and WRKY33-dependent gene expression profiles. In total we identified ~1500 genes associated with WRKY33 high confidence binding sites upon pathogen challenge. The majority of the WRKY33 binding peak regions were localized to promoter and 5' intergenic sites and contained the major WRKY factor DNA binding motif, the W-box. The genome-wide expression profiles revealed that 318 out of the 2765 genes affected in their expression by WRKY33 are direct targets at 14h post infection. Interestingly, the expression of over 75% of the WRKY33 target genes is negatively affected after pathogen challenge including critical genes involved in signaling, hormone response, and defense, while expression of around 25% is positively affected including camalexin biosynthetic pathway genes. In particular, WRKY33 negatively regulates an ABA branch of the defense regulatory network by suppressing the expression of *NCED3* and *NCED5*, which are involved in ABA biosynthesis. Genetic studies showed that in the *wrky33 nced3 nced5* triple mutant resistance towards *B. cinerea* 2100 was restored to wild-type (WT). Moreover, the differential expression of many *WRKY33* regulated genes also returned to WT levels within this triple mutant post infection. Consistently, ABA and SA levels decreased to WT levels in the *wrky33 nced3 nced5* triple mutant compared to *wrky33*. Our results reveal a new role of WRKY33 in modulating host immunity by repressing part of the ABA hormone regulatory network. Furthermore, the dual activator and repressor role of WRKY33 in host defense response is discussed.

2.2. Introduction

Necrotrophic fungi including *B. cinerea*, *F. oxysporum*, *A. brassicicola* and *Sclerotinia sclerotiorum,* are the largest class of fungal plant pathogens and cause serious crop losses worldwide (Audenaert et al., 2002; Lazniewska et al., 2010). These pathogens extract nutrients from host cells killed prior to or during colonization, by producing a range of phytotoxic compounds and cell wall degrading enzymes (Williamson et al., 2007; Mengiste, 2012). *B. cinerea* has a broad host-range, causes disease both pre- and postharvest, and is the second most agriculturally important fungal plant pathogen (Dean et al., 2012).

Thus, plant immunity to *B. cinerea* appears to be under complex genetic control (Rowe and Kliebenstein, 2008). This remains broadly true since *B. cinerea* uses multiple strategies to quickly kill host cells. However, in the last two decades both forward and reverse genetic screens have identified numerous genes that influence the outcomes of host- *B. cinerea* interactions. Large-scale transcriptional reprogramming is often observed after *B. cinerea* infection, indicating the involvement of key transcription factors (TFs) in this process (AbuQamar et al., 2006; Birkenbihl et al., 2012; Windram et al., 2012). Several MYB-type *Arabidopsis* transcription factors have been identified that regulate distinct host transcriptional responses towards *B. cinerea*. BOS1 (*BOTRYTIS SUSCEPTIBLE 1*)/MYB108 appears to restrict necrosis triggered by *B. cinerea* and *A. brassicicola*, and loss-of-BOS1 function resulted in increased plant susceptibility (Mengiste et al., 2003; Veronese et al., 2004). In response to stress and *B. cinerea*, BOS1 can be stabilized by a physicaly interaction with the protein BOI, a RING E3 ligase, which ubiquitinates BOS1 (Luo et al., 2010). BOI functions similar to BOS1 and contributes to defense against *B. cinerea* by restricting the extent of necrosis (Luo et al., 2010). MYB51 is involved in the transcriptional activation of indole glucosinolate biosynthetic genes, which contribute to resistance to necrotrophic fungi (Clay et al., 2009). In contrast, the MYB-related gene *ASYMMETRIC LEAVES 1* (*AS1*) and *MYB46* appear to play a role in disease susceptibility as the mutants show increased disease resistance towards *B. cinerea* (Nurmberg et al., 2007; Ramirez et al., 2011). Many transcriptional activators and repressors in ethylene (ET) and jasmonic acid (JA) pathways impact resistance to *B. cinerea* since plant ET / JA signaling are more important for regulating resistance to necrotrophic pathogens (Thomma et al., 1998; Glazebrook, 2005; Bari and Jones, 2009). Expression of several of these, including ERF1, ORA59, ERF5, ERF6 and RAP2.2, influences host susceptibility to *B. cinerea* (Berrocal-Lobo et al., 2002; Pre et al., 2008; Moffat et al., 2012; Zhao et al., 2012). Transgenic Arabidopsis lines over-expressing *ERF1* confer resistance towards the *B. cinerea* (Berrocal-Lobo et al., 2002). Similarly, overexpression of *ORA59* increased resistance toward *B. cinerea* whereas *RNAi-ORA59* silenced lines were more susceptible (Pre et al., 2008). Both ERF1 and ORA59 appear to be key integrators of the ET and JA-signaling pathway (Pieterse et al., 2009). In contrast, *JA INSENSITIVE1* (*JIN1/MYC2*) affects host resistance by antagonizing JA-ET defense responses to necrotrophs (Lorenzo et al., 2004; Nickstadt et al., 2004). Members of the NAC TF family have also been shown to affect the outcome of such an interaction, either positively or negatively (Bu et al., 2008; Wang et al., 2009). However, very little is known about the TF

regulated target genes on a genome-wide level and about the associated regulatory networks modulating host transcriptional responses to *B. cinerea* infection.

The WRKY family of TFs also modulate host defense towards *B. cinerea* (Pandey and Somssich, 2009). In particular, WRKY33 positively regulates host defense to necrotrophs such as *A. brassicicola* and *B. cinerea* isolates since *wrky33* knockout mutants were fully susceptible to these pathogens (Zheng et al., 2006; Birkenbihl et al., 2012). WRKY33-mediated immune response appear also to involve post-translational regulation (Ishihama and Yoshioka, 2012). In one case, WRKY33 was shown to be directly phosphorylated by the MAP kinases MPK3 and MPK6 *in vivo* upon *B. cinerea* infection and thus subsequently to activate *PAD3* expression (Mao et al., 2011). *PAD3* encodes a key biosynthetic enzyme required for the production of the antifungal phytoalexin camalexin (Zhou et al., 1999). Since phosphorylation of WRKY33 does not affect its DNA-binding capability, MPK3 and MPK6 are thought to promote additional WRKY33 functions. Moreover, WRKY33 directly interacts with its own gene promoter, suggesting a potential positive feedback regulation loop thereby further enhancing *WRKY33* transcription (Mao et al., 2011). In a second case study, WRKY33 was found to interact with MAP KINASE SUBSTRATE1 (MKS1), a VQ-motif containing protein, also designated VQ21, and to form a MPK4-MKS1-WRKY33 ternary protein complex within the nucleus of resting cells (Andreasson et al., 2005; Qiu et al., 2008). Upon challenge with the hemibiotrophic pathogen *Pseudomonas syringae* or upon elicitation by the Microbe Associated Molecular Pattern (MAMP) flg22, activated MPK4 phosphorylates MKS1 thereby releasing WRKY33 from the complex. WRKY33 was subsequently found bound to the *PAD3* promoter, and this binding correlated with enhanced *PAD3* expression (Andreasson et al., 2005; Qiu et al., 2008). In other cases, WRKY33 interactions with different VQ motif-containing proteins such as MVQ1-5 (MPK3/6-targeted VQ-motif-containing protein 1-5), MVQ10, VQ8, and VQ23/16 (SIGMA FACTOR BINDING PROTEIN SIB1/SIB2) were also observed (Lai et al., 2011b; Lai et al., 2011a; Cheng et al., 2012; Pecher et al., 2014; Weyhe et al., 2014). The dynamic nature of these VQ protein interactions has been suggested to have distinct roles and regulatory functions during the plant response to either bacterial or fungal pathogens (Buscaill and Rivas, 2014).

In recent years, ChIP-on-chip and ChIP followed by sequencing (ChIP-seq) have emerged as powerful tools to profile genome-wide direct target genes of transcription factors. The methods were first applied for human transcription factors (Johnson et al., 2007; Nielsen et al., 2008; Robertson et al., 2008; Wederell et al., 2008; Sakabe et al., 2012), but more recently

this technology has also been reported for plant transcription factors. In *Arabidopsis*, genome-wide direct target gene analysis has been reported for several key TFs, including MADS domain proteins such as SEP3, AP1, FLC, SVP, AGL15 and SOC1 (Kaufmann et al., 2009; Zheng et al., 2009; Kaufmann et al., 2010; Deng et al., 2011; Immink et al., 2012; Tao et al., 2012); bHLH TFs such as PIF1, PIF3, PIF4, PIF5, HY5, FHY3 and HBI1 (Oh et al., 2009; Ouyang et al., 2011; Zhang et al., 2011a; Zhang et al., 2013; Fan et al., 2014); the ethylene response TF EIN3 (Chang et al., 2013); and the plant-specific TF LEAFY, which regulates the transition from vegetative growth to flowering (Winter et al., 2011). In *Zea mays* and *Oryza sativa*, the KNOTTED1 and IPA1 target genes have been identified, respectively (Bolduc et al., 2012; Lu et al., 2013). In addition, identification of genome-wide target genes for transcriptional regulators containing no DNA binding domains have recently been reported, such as *Arabidopsis* Phytochrome A (phyA) and its chaperone FHY1 (Chen et al., 2014a; Chen et al., 2014b). Currently, this technology has been utilized in identifying genome-wide targets of TFs associated with plant development, but no similar studies about TFs involved in plant immunity, especially responding to pathogen attack have been reported.

Our previous studies using chromatin immunoprecipitation (ChIP)-qPCR have shown that WRKY33 is a key transcriptional regulator towards *B. cinerea* strain 2100 infection by direct binding to sequences upstream of genes involved in JA signaling (*JAZ1, JAZ5*), ET-JA cross talk (*ORA59*), and camalexin biosynthesis (*CYP71A13, PAD3*) (Birkenbihl et al., 2012). In addition, we showed that the JA pathway was activated both in WT and *wrky33* mutant plants 14h post inoculation (14hpi) with *B. cinerea* but decreased in the mutant after 24hpi, whereas the SA pathway was strongly induced only in the mutant during infection (Birkenbihl et al., 2012). SA-mediated antagonism of the JA signaling at late infection stages appears to contribute to the susceptibility of *wrky33* to *B. cinerea*. However, genetic studies indicated that SA suppression of JA signaling was insufficient for WRKY33-mediated resistance (Birkenbihl et al., 2012). Therefore, WRKY33 apparently targets additional unidentified components that are critical for establishing full WRKY33-dependent resistance towards *B. cinerea*. Thus, genome-wide identification of *B. cinerea*-triggered binding of WRKY33 to target genes may help us with uncover novel aspects related to the role of this key regulator in host immunity towards this fungal nectrotroph.

Here, we performed ChIP-seq and RNA sequencing (RNA-seq) experiments to identify WKRY33 regulated direct target genes in the *A. thaliana* genome after *B. cinerea* 2100 infection. The results showed that WRKY33 has dual transcriptional activator and repressor

roles in regulating hundreds of target genes with distinct molecular functions. The data deduced from these studies and the subsequent genetic analysis of selected mutants revealed that WRKY33 regulation of the ABA branch of the defense regulatory network is critical for resistance to *B. cinerea* infection.

2.3. Results

2.3.1. Genome-wide mapping of loci bound by WRKY33

To gain a deeper insight into how WRKY33 regulates plant immunity to *B. cinerea* 2100, we performed a genome-wide analysis to identify WRKY33 *in vivo* target genes using ChIP-seq. For this, a transgenic *wrky33* mutant line expressing a HA epitope-tagged *WRKY33* construct under the control of its native promoter (*P*$_{WRKY33}$*:WRKY33-HA*) was used. This line was previously shown to complement for *B. cinerea* 2100 resistance similar to WT Col-0 plants (Birkenbihl et al., 2012). 4-week old rosette leaves of plants untreated or spray-inoculated for 14 hours with spores of *B. cinerea* 2100 were used to perform ChIP-seq based on the induced WRKY33-HA protein levels observed on western blots (Figure 2.1). No WRKY33-HA protein was detected in the absence of infection. As an additional control identical tissue was harvested from WT plants that did not express *WRKY33-HA*. Two biological replicates were analyzed for mock and *B. cinerea* 2100 treated WRKY33-HA plants. Previously identified WRKY33 *in vivo* targets, *CYP71A13* and *PAD3* (Birkenbihl et al., 2012), were used to monitor for enrichment in samples used for library construction and sequencing.

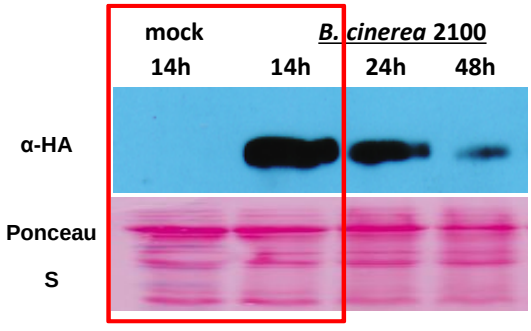


Figure 2.1 Western-blot analysis of WRKY33-HA protein levels after mock treatment or spray-inoculation of *P*$_{WRKY33}$*:WRKY33-HA* transgenic plants with *B. cinerea* 2100 spores. Plant material selected for ChIP-seq is boxed in red. Antibody:α-HA; Protein stained with Ponceau S.

From ChIP-seq experiments, we obtained ~2300 high confident peaks in each biological replicate, among which around 1700 peaks were reproducible between the two replicates (Table 2.1). The sequence reads from each two samples were pooled. We identified 1684 high confidence peaks (named WRKY33 binding sites) associated with 1576 genes common to

both replicates (designated WRKY33 candidate targets). Detection of all of these sites was dependent on infection of the plants with *B. cinerea* 2100 as nearly no sites were observed in mock treated plants.

The fidelity of the data obtained by ChIP-seq was subsequently confirmed by qPCR for numerous genes (Table 2.2). Moreover, nearly all previously reported WRKY33 *in vivo* targets including *PAD3*, *CYP71A13*, *ACS2*, *JAZ1*, *ORA59*, *TRX-h5* and *WRKY33* itself were identified in our ChIP dataset (Mao et al., 2011; Birkenbihl et al., 2012; Li et al., 2012).

Table 2.1. Summary of identified WRKY33 binding sites 14h post *B. cinerea* infection.

Peak Call	replicate 1			replicate 2			pooled		
w33_Bc_IP	total	reproducible[1)]	% reproducible [1)]	total	reproducible[1)]	% reproducible [1)]	total	consistent[3)]	% consistent [3)]
vs w33_Bc_input	2341	**1753**	*74.9*	2305	**1740**	*75.5*	2578	**1728**	*67.0*
vs w33_mock_IP	2288	**1720**	*75.2*	2302	**1708**	*74.2*	2578	**1695**	*65.7*
vs wt_Bc_IP	n.a.	**n.a.**	*n.a.*	2307	**n.a.**	*n.a.*	n.a.	**n.a.**	*n.a.*
high confidence[2)]	2269	**1710**	*75.4*	2299	**1698**	*73.9*	2577	**1684**	*65.3*

Note: This analysis was performed by Dr. Barbara Kracher.

[1)] A peak region in a replicate is counted as reproducible, if it overlaps with a peak region in the other replicate by at least 50% of the length of the smaller peak region.

[2)] A peak region is counted as high confidence region (within a sample), when it is identified against all (two/three) negative controls used for peak calling.

[3)] A peak region in the pooled sample is counted as consistent, if it overlaps with a reproducible peak region in both of the original replicates by at least 50% of the length of the smaller region.

n.a. not analyzed.

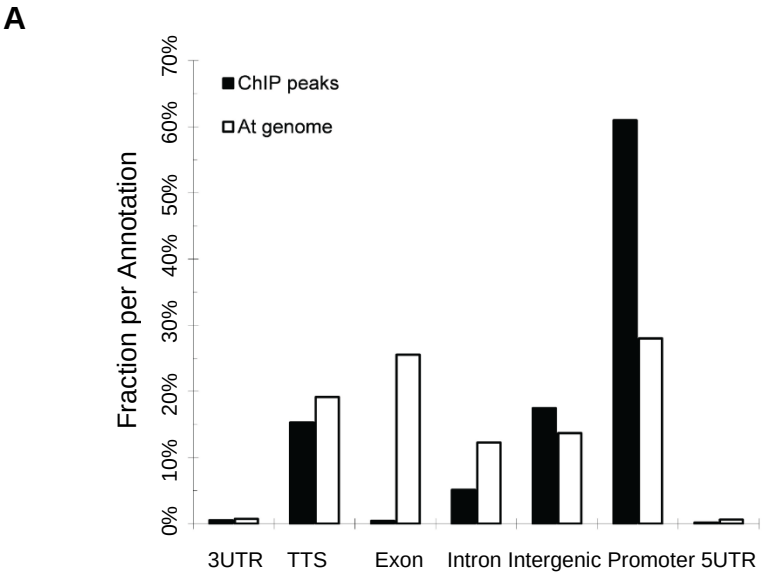
Table 2.2. List of confirmed WRKY33 targets.

Gene	Description	Test methods	
AT2G30750	CYP71A12	ChIP-qPCR	
AT2G30770	CYP71A13	ChIP-qPCR	LinDA-qPCR
AT3G26830	PAD3	ChIP-qPCR	LinDA-qPCR
AT2G38470	WRKY33	ChIP-qPCR	
AT5G22570	WRKY38	ChIP-qPCR	
AT5G26170	WRKY50	ChIP-qPCR	
AT4G23810	WRKY53	ChIP-qPCR	
AT4G11070	WRKY41	ChIP-qPCR	
AT5G49520	WRKY48	ChIP-qPCR	
AT2G40740	WRKY55	ChIP-qPCR	
AT3G44350	NAC061	ChIP-qPCR	LinDA-qPCR
AT5G22380	NAC090	ChIP-qPCR	
AT2G26650	AKT1	ChIP-qPCR	LinDA-qPCR
AT3G14440	NCED3	ChIP-qPCR	LinDA-qPCR
AT1G30100	NCED5	ChIP-qPCR	LinDA-qPCR

AT5G45340	CYP707A3	ChIP-qPCR	
AT1G16090	WAKL7		LinDA-qPCR
AT5G44280	RING1A		LinDA-qPCR
AT5G45110	NPR3		LinDA-qPCR
AT5G20960	AAO1		LinDA-qPCR
AT1G01480	ACS2		LinDA-qPCR
AT3G55970	JRG21		LinDA-qPCR
AT5G47230	ERF5		LinDA-qPCR
AT3G23240	ERF1		LinDA-qPCR
AT4G11280	ACS6	ChIP-qPCR	
AT1G72520	LOX4	ChIP-qPCR	

2.3.2. Characterization of WRKY33 binding sites

We next explored the distribution of WRKY33 binding sites over genic regions. WRKY33 binding sites were evenly distributed on the five chromosomes and >78 % were located in the – 1000 bp promoter or intergenic regions (Figure 2.2A). Furthermore, the locations of WRKY33 binding sites were found to be concentrated within a 250-bp window immediately upstream of the transcription start sites (Figure 2.2B). This distribution pattern of WRKY33 binding sites is consistent with its role as a transcriptional regulator. In addition, 15.4% of the peaks were located near transcription termination sites (TTS) of neighboring genes, and 5.1% of the peaks were detected in intronic regions (in contrast to less than 1% in exons), suggesting that these regions may also have a role in WRKY33-mediated gene transcription.



B

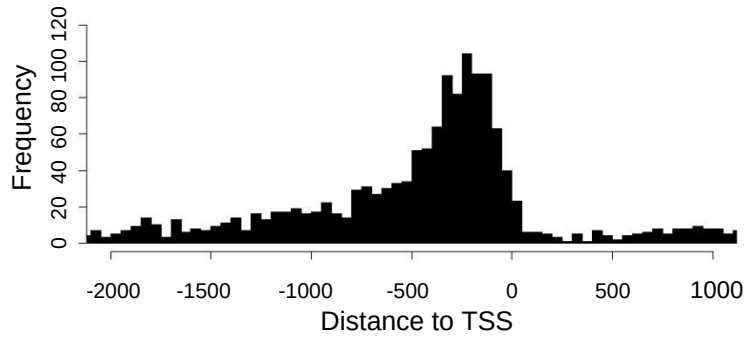


Figure 2.2 Genome-wide identification of WRKY33-binding sites. (A) Relative binding-peak distribution across genomic regions. A 1-kb region before the transcription start site (TSS) is defined as the promoter region. The fraction of each annotation type in all genomic genes is shown as a control. (B) WRKY33 binding sites are highly enriched within a 250-bp window immediately upstream of the transcription start site.

Numerous studies have shown that nearly all WRKY proteins bind to a specific DNA motif, TTGACT/C, termed the W box (Eulgem et al., 2000), although adjacent sequences (W box extended motifs) can also influence binding (Ciolkowski et al., 2008). To determine the consensus of WRKY33 binding motifs across the genome, we used MEME software to pinpoint conserved sequences within the 500bp WRKY33 binding peak summit regions. Of the 1684 WRKY33-binding sites, 76% contained the well-established W box motif with the core consensus sequence TTGACT/C (Figure 2.3A). Moreover we also found W box extended sequence motifs within the WRKY33 binding regions (Figure 2.3B). Recently, a conserved core sequence, GACTTTT, was reported to interact with *Arabidopsis* WRKY70 and to be required for WRKY70-activated gene expression (Machens et al., 2014). We also found this GACTTTT sequence within the set of W box extended sequence motifs at WRKY33 binding regions (Figure 2.3B).

Apart from the W box and extended W box variants, we found an additional sequence motif, T/GTTGAAG, that occurs in 55% of the WRKY33 binding regions (Figure 2.3A), and is often associated with the W box (5'-T/C-TGAC-T/C-3'). More than 40% of WRKY33 binding peaks contain both W box and this motif. Whether this motif can also be bound by WRKY33 or by some other transcription factor associated with WRKY33 function remains to be determined.

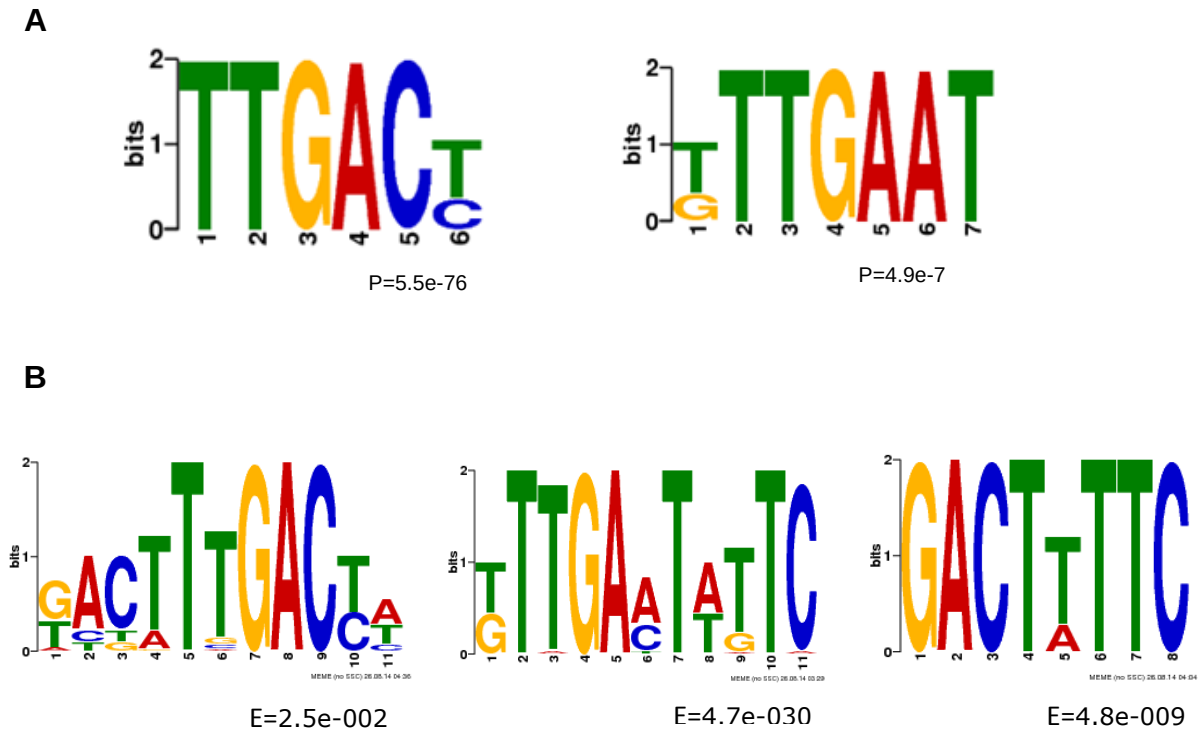


Figure 2.3 Genome-wide identification of WRKY33-binding motifs. (A) DREME motif search identified conserved DNA elements within the 500 bp WRKY33 binding peak summit regions: Left -- well established W box (TTGACT/C); Right-- newly identified motif (T/GTTGAAT). (B**)** MEME motif search identified conserved DNA elements within the 500bp WRKY33 binding peak summit regions. Left: W box extended motifs in 5' end; Middle: W box extended motifs in 3' end; Right: Conserved sequence GACTT/ATTC.

2.3.3. Gene Ontology analysis of genes bound by WRKY33 after *B. cinerea* 2100 infection

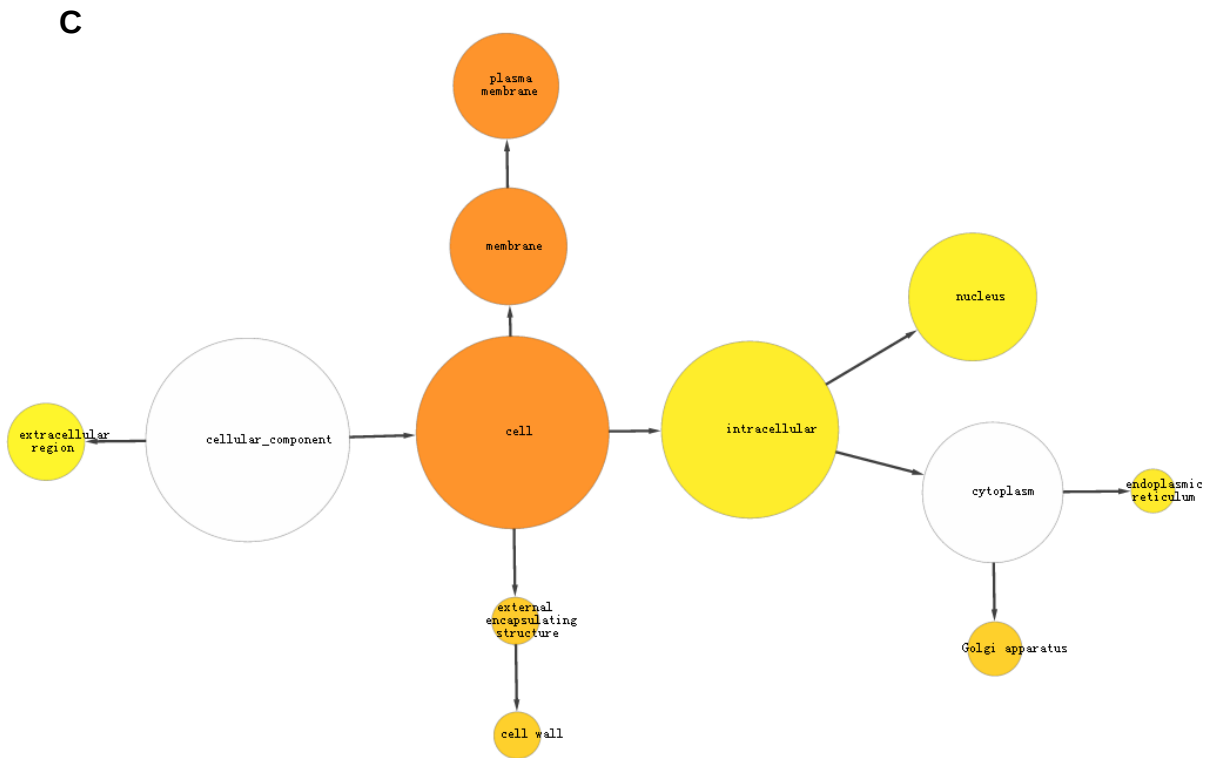
We next performed a Gene Ontology (GO) analysis using BiNGO (Maere et al., 2005) to characterize these WRKY33 target genes. The GO analysis reveals that several gene clusters are enriched: target genes in response to stress, response to biotic or abiotic stimulus, response to external or endogenous stimulus, target genes involved in metabolic and cellular processes, target genes related to transport and signal transduction are enriched in the term biological processes (Figure 2.4A). Target genes associated with transporter activity, transcriptional regulator activity, binding, and catalytic activity, especially kinase activity, are enriched in the term molecular functions (Figure 2.4B); target genes associated with membrane, intracellular and cell wall are enriched in the term cell functions (Figure 2.4C).

A

Figure 2.4. GO analysis of WRKY33 total candidate target genes. (A) category biological processes, (B) molecular functions, (C) cell functions. Each circle represents an enriched category compared with the whole genome after false discovery rate correction. The size of each circle is proportional to the number of genes annotated to the node. The yellow color of the circles represents enriched categories based on the FDR-corrected P value ranging from 0.05 (yellow) or below (darker yellow). Red arrows in (B) indicates targets enriched in WRKY33 target genes but not enriched in WRKY33-regulated target genes.

B


oxygen binding
protein binding
carbohydrate binding
binding
nucleic acid binding
molecular_function
catalytic activity
transferase activity
kinase activity
DNA binding
transcription factor activity
transcription regulator activity
transporter activity



2.3.4. Expression profile and characterization of genes associated with *B. cinerea* 2100-induced WRKY33 binding

ChIP-seq studies from both the animal and plant field have shown that the majority of binding sites bound by specific transcription factors *in vivo* do not result in altered expression levels of associated genes (MacQuarrie et al., 2011; Chang et al., 2012; Fan et al., 2014). All detected WRKY33-enriched binding sites were only observed upon pathogen infection. Thus to study the impact of WRKY33 binding on target gene expression, we performed RNA-seq and examined *WRKY33*-mediated gene expression changes in mock and *B. cinerea* 2100 (14hpi) treated *wrky33* and WT 4-week old plants.

Data from three biological replicates allowed us to identify genes altered in their expression after *B. cinerea* infection. In WT plants, the expression of 6101 genes was altered 2-fold or more ($P \leq 0.05$) compared with noninfected plants, with 3048 genes being up-regulated and 3053 genes being down-regulated (Figure 2.5A). In *wrky33*, upon infection, the expression of 7441 genes, 3583 up-regulated and 3858 down-regulated, was altered more than 2-fold, with a common set of 4686 genes showing changes upon infection in WT and *wrky33* plants (Figure

2.5A, B). While upon infection the expression of 1415 genes was altered only in the WT, differential expression of 2755 genes was triggered in the loss of WRKY33 functional mutant (Figure 2.5B). Comparing the expression profiles from noninfected plants identified 705 genes that were constitutively differentially expressed between *wrky33* and the WT, 458 of them up-regulated and 247 down-regulated (Figure 2.5A). Comparing the expression profiles of *B. cinerea*-infected *wrky33* and WT plants (w33 *B.c.* vs WT *B.c.*), we identified 2765 differentially expressed genes, of which 1675 were up-regulated and 1090 were down-regulated in a WRKY33-dependent manner, therefore being defined as WRKY33-repressed genes and WRKY33-induced genes, respectively (Figure 2.5A, C).

We then compared the WRKY33-dependent differentially expressed genes obtained from the RNA-seq data with the WRKY33 target genes revealed by ChIP-seq and thereby identified 318 WRKY33-regulated candidate target genes that were both bound by WRKY33 and exhibited WRKY33-dependent altered gene expression (Figure 2.5C), of which 240 (75.5%) are repressed by *B. cinerea* 2100 while 78 (24.5%) are induced (Figure 2.5C, D). These genes were termed WRKY33-repressed targets and WRKY33-induced targets, respectively. Thus, it appears that a key role of WRKY33 in mediating immunity to this pathogen is to repress the expression of many specific host target genes. The remaining genes that were altered in their expression (1435 repressed and 1012 induced) in the *wrky33* mutant compared to WT, but that showed no WRKY33 enrichment within the locus were defined as WRKY33-dependent non-targets (WRKY33-repressed non-targets and WRKY33-induced non-targets) (Figure 2.5C). The overlap observed between WRKY33 binding and altered expression of the associated genes upon fungal infection was around 20% (318 of 1576). Similar levels of overlap have been reported for other plant TFs such as EIN3, HBI1 and BES1 (Yu et al., 2011; Chang et al., 2013; Fan et al., 2014).

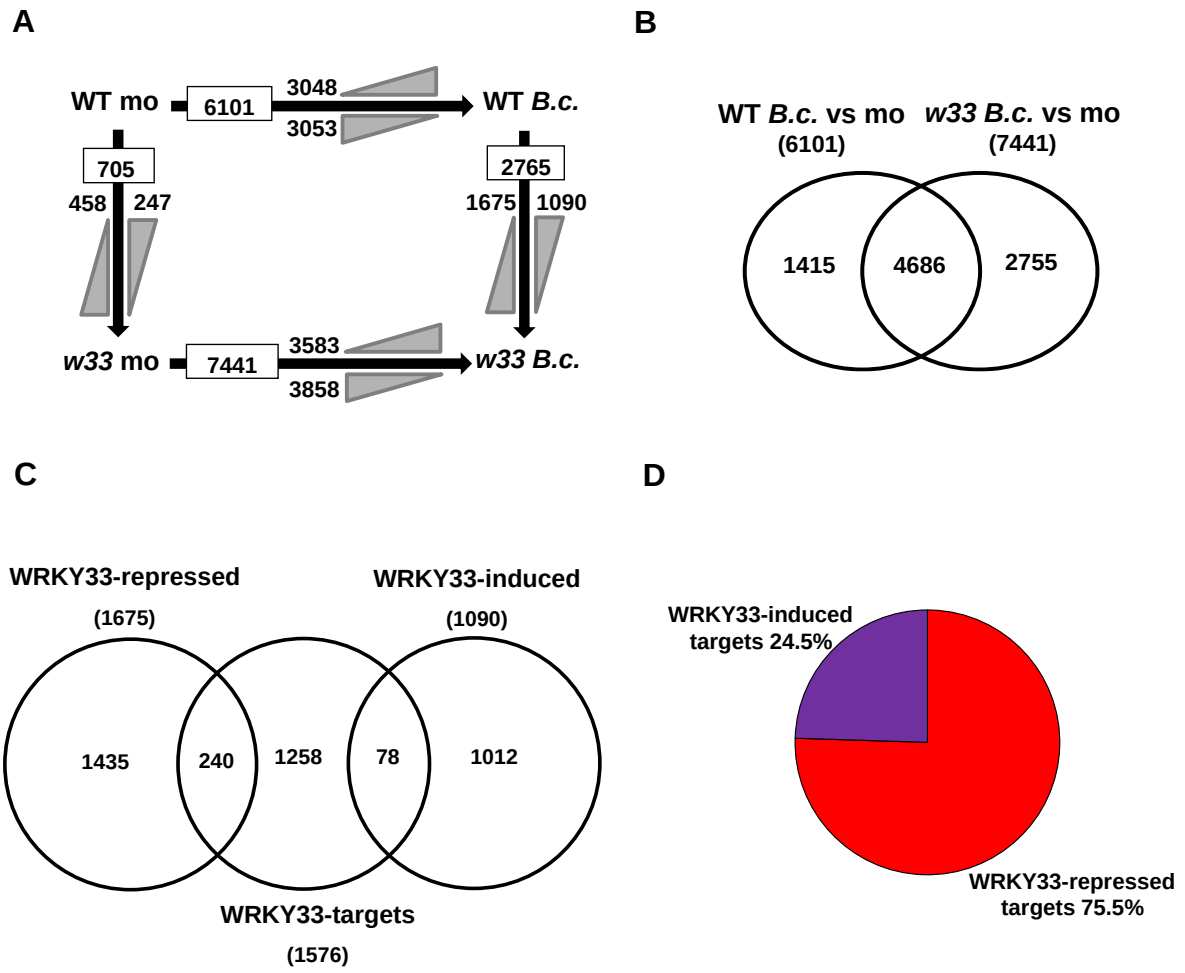


Figure 2.5. Comparative RNA-seq and ChIP-seq data identify WRKY33 regulated candidate target genes in response to *B. cinerea* infection. (A) Number of differentially expressed genes (≥2-fold; P≤0.05) between the WT and *wrky33* (*w33*) at 14h after mock treatment (mo) or *B. cinerea* spray inoculation (*B.c.*). Indicated are total number (boxes) and number of up-regulated ( ) and down-regulated ( ) genes between treatments or genotypes. (B) Venn diagram illustrating total number and overlap of genes affected in WT and *wrky33* plants at 14h post *B. cinerea* inoculation. (C) Venn diagram showing the overlap between the WRKY33-regulated genes and WRKY33 direct target genes. (D) Percentage of WRKY33-repressed targets and WRKY33-induced targets (total number of WRKY33-regulated targets is 318).

GO analysis to characterize these identified WRKY33-regulated target genes are shown in Figure 2.6, and reveal enrichment in genes related to stresses, stimulus, signal transduction, transport, metabolic process, catalytic activity and membrane when compared with the entire genome (P<0.05). These results suggest a direct involvement of WKRY33 in these processes. However, in molecular function, GO terms related to transcription factor activity and DNA binding were not significantly represented in WRKY33-regulated target genes (mentioned in bottom paragraph), although they were significantly enriched in WRKY33 total target genes (Figure 2.4). This supports previous reports that transcription factor binding does not necessarily coincide with changes in transcription, especially for master regulators targeting

other TFs or other factors involved in chromatin state regulation (Yu et al., 2011; Chang et al., 2013; Fan et al., 2014).

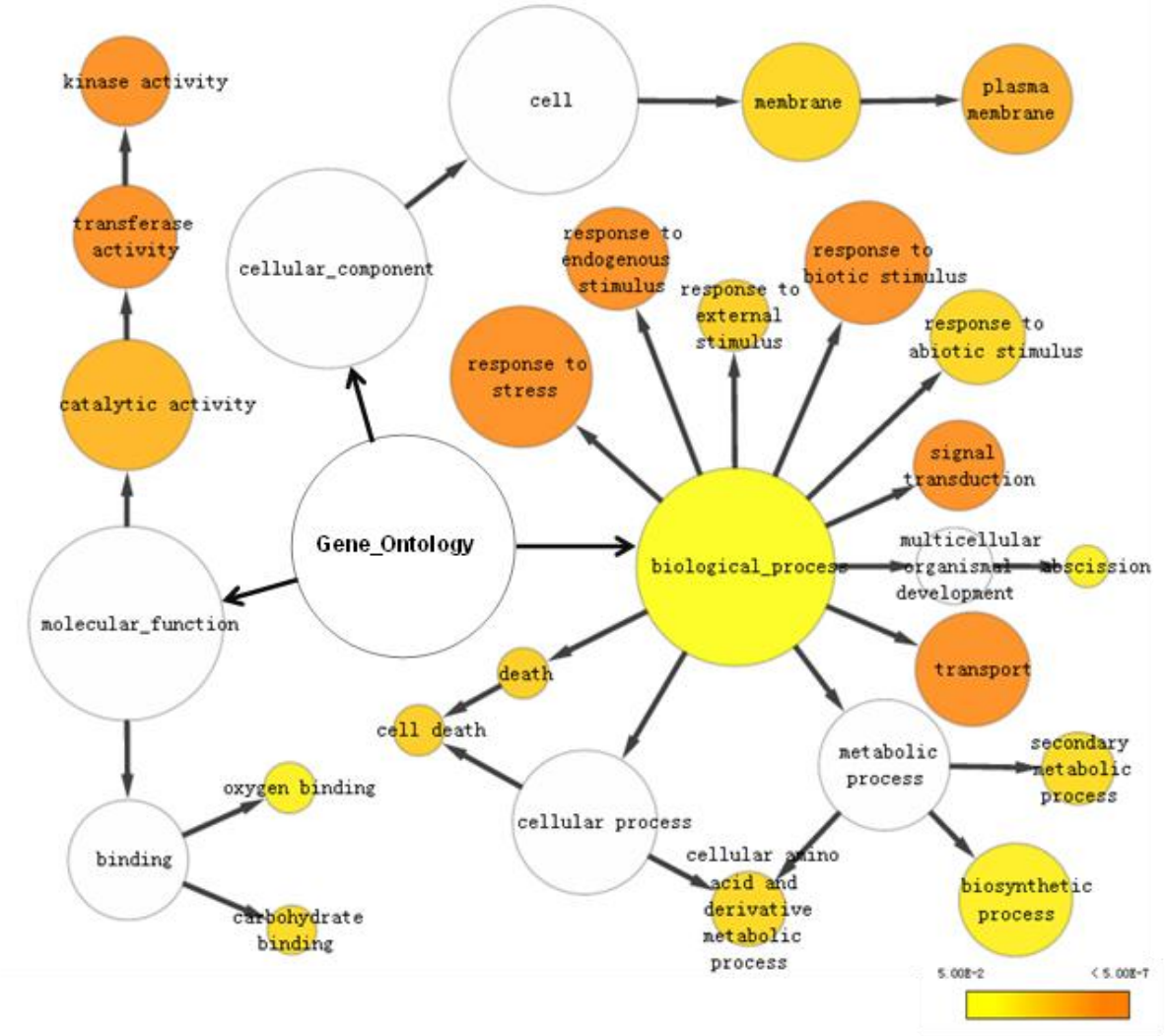


Figure 2.6. GO analysis of WRKY33-regulated target genes. Each circle represents an enriched category compared with the whole genome after false discovery rate correction. The size of each circle is proportional to the number of genes annotated to the node. The yellow color of the circles represents enriched categories based on the FDR-corrected P value ranging from 0.05 (yellow) or below (darker yellow).

2.3.5. WRKY33 function often represses expression of genes involved in plant immunity

Functional classification of the WRKY33-regulated (induced or repressed) and WRKY33 bound (targets or non-targets) genes based on GO categories showed that WRKY33 directly or indirectly regulates a range of genes involved in biological processes and molecular functions, and many of these genes are repressed by WRKY33 (Figure 2.7). For example,

genes involved in the GO term “defense response” were highly overrepresented in WRKY33 repressed targets (37.9%) and in WRKY33 repressed non-targets (17%), suggesting that WRKY33 often functions as a repressor of plant defense responses. Still, expression of nearly 18% of WRKY33 regulated targets were induced whereas only 2% of WRKY33 induced non-targets were enriched in the GO term “defense response”, indicating that WRKY33 can also function as an activator of certain defense associated genes particularly when they are direct targets.

Next to hormonal pathways discussed below, genes involved in the GO terms “cell death” or related to diverse “kinase activities” were markedly enriched in WRKY33 repressed targets. 42 out of 318 WRKY33-regulated targets are involved in cell death, and 38 appear to be repressed by WRKY33 (Table S1). This predominance of WRKY33 function to repress host responses associated with cell death may be one important feature required for resistance towards necrotrophic pathogens that complete their life styles on dead host tissue. Interestingly, more than 80% (34 out of 42) of the cell death associated targets are also enriched in the GO term “response to salicylic acid (SA)”, indicating that WRKY33 repression of host “cell death” is associated with “response to SA” (Figure 2.8A). 41 of the WRKY33 target genes encode for various kinases, including 10 leucine-rich repeat (LRR) RPKs, 8 lectin receptor protein kinases (LecRKs), 7 cysteine-rich (CR) receptor-like protein kinases, and 8 serine/threonine kinase (Table S2). Again the majority of these genes appear to be repressed by WRKY33 (Table S2). For LecRK VI.2 a critical role in resistance against hemibiotrophic *P. syringae* pv. tomato DC3000 and necrotrophic *Pectobacterium carotovorum* bacteria has been demonstrated (Singh et al., 2012; Singh et al., 2013; Huang et al., 2014).

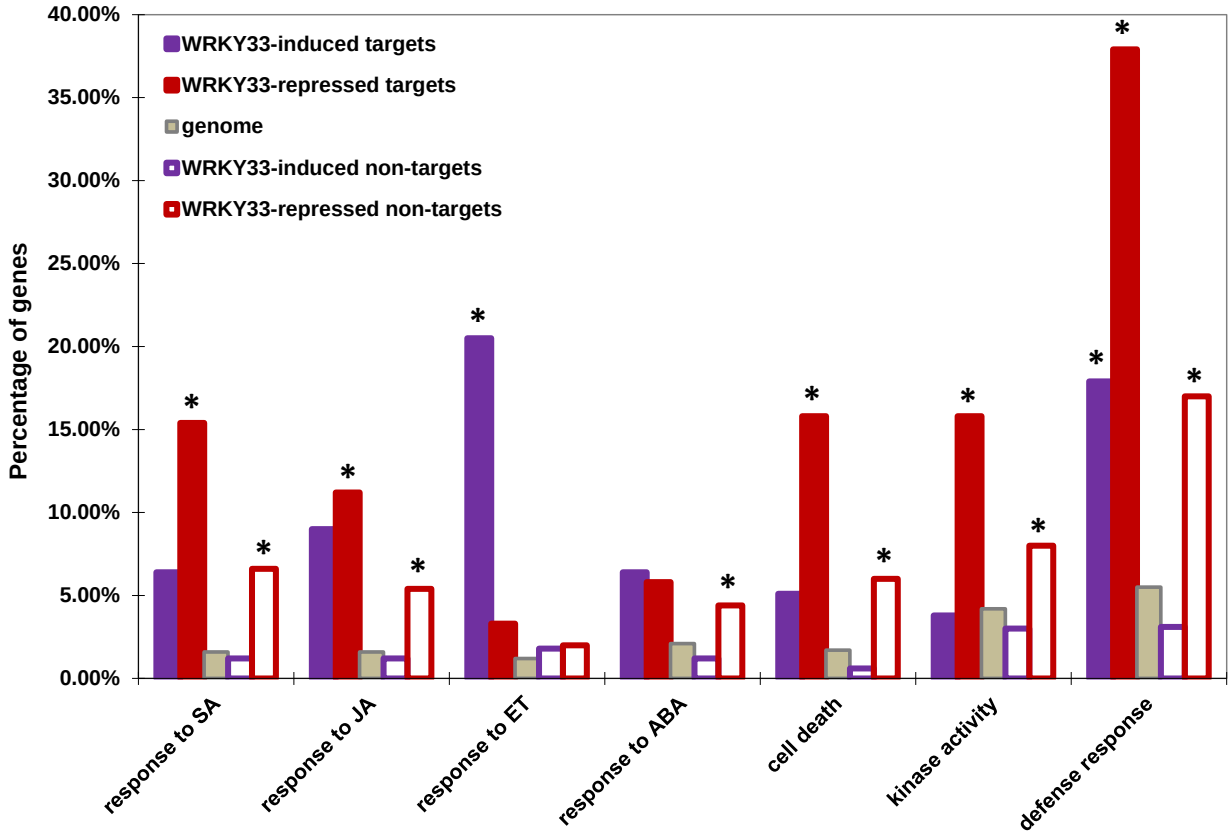


Figure 2.7. Gene Ontology analysis of WRKY33-regulated targets and WRKY33-regulated non-targets associated with defense response, kinase activity, cell death and hormone responses. Numbers indicate the percentages of genes belonging to each GO category. Asterisk indicates significant difference from genome ($P < 0.05$).

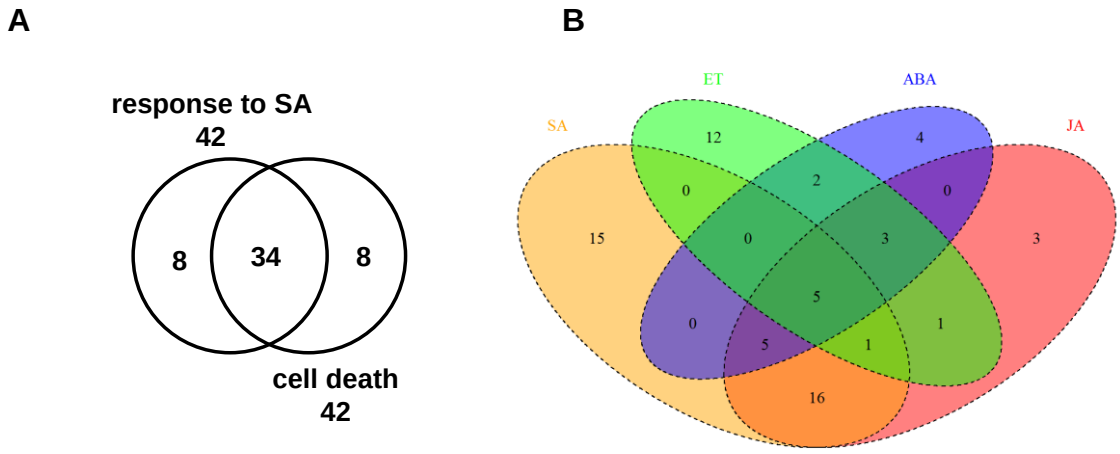
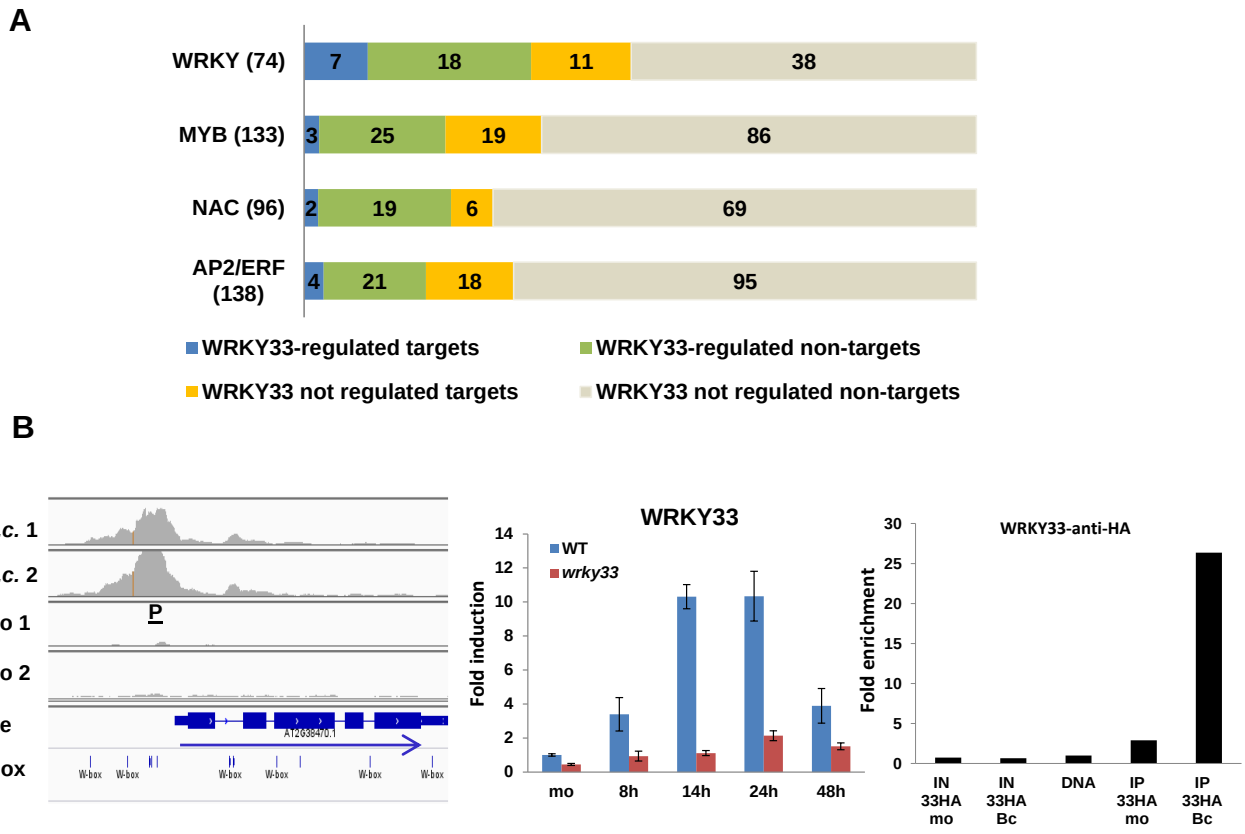
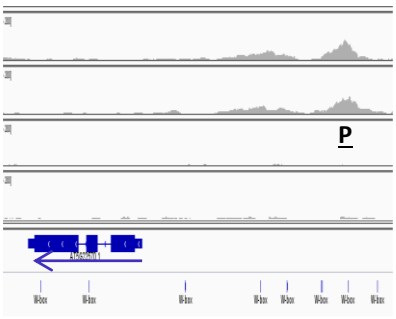
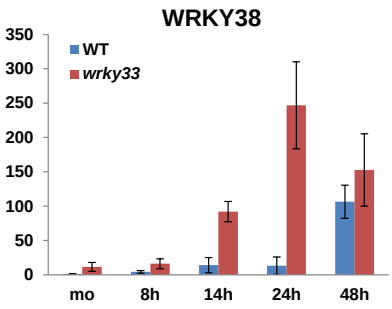


Figure 2.8 GO analysis of WRKY33-regulated target genes in hormone response and cell death. A. Venn diagram showing the overlap between the WRKY33-regulated genes responsive to SA and cell death. B. The overlap of WRKY33-regulated target genes associated with the hormone pathway SA, ET, ABA and JA.

Several TF gene families, many involved in defense response, were targeted by WRKY33. ChIP-seq data showed that WRKY33 binds to 133 transcription factor gene loci. Predominant among these are members of the AP2/ERFs, MYBs, WRKYs, and NACs families (Figure 2.9A). However, expression of only 16% (21 of 133) was directly modulated in a WRKY33-dependent manner after *B. cinerea* infection. A complete list of TF coding genes that are direct targets of WRKY33 and are part of the transcriptional network associated with defense to *B. cinerea* is given in Table S3. As an example, 18 *WRKY* genes were identified as direct targets of WRKY33 including *WRKY33* itself (Figure 2.9B). However, only 7 genes, i.e. *WRKY33, WRKY38, WRKY41, WRK48, WRKY50, WRKY53* -and *WRKY55*, showed altered expression upon WRKY33 binding post infection (Figure 2.9B, Table S3). The observed autoregulation of *WRKY33* by WRKY33 is consistent with previous reports suggesting a positive feedback regulatory loop resulting in high-level accumulation of WRKY33 in response to *B. cinerea* (Mao et al., 2011). In addition, altered transcription of 18 other *WRKY* genes was observed that was dependent of WRKY33 function after fungal infection but to which no WRKY33 binding could be detected, indicating that these WRKY genes are indirectly regulated by WRKY33 (Figure 2.9A). Consistent with our general observation, WRKY33 mainly appears to act as a repressor of most of these *WRKY* targets.




P
W-box


WRKY38
WT
wrky33
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300
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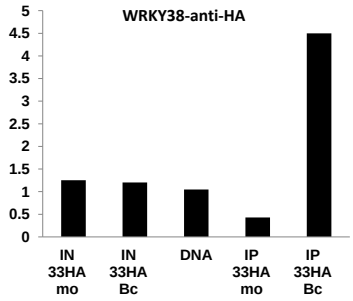
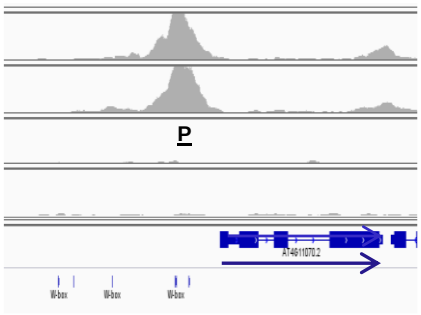
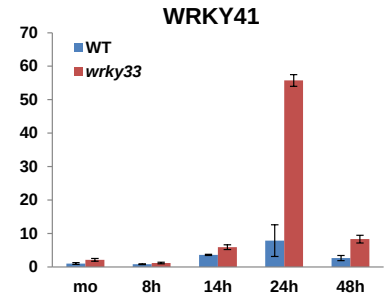

WRKY38-anti-HA
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
IN 33HA mo
IN 33HA Bc
DNA
IP 33HA mo
IP 33HA Bc


P
W-box


WRKY41
WT
wrky33
70
60
50
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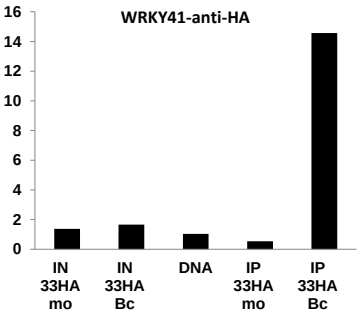
10
0
mo
8h
14h
24h
48h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IN 33HA mo
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DNA
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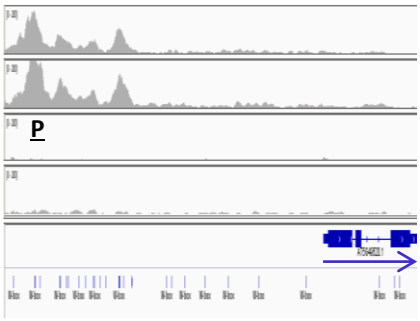
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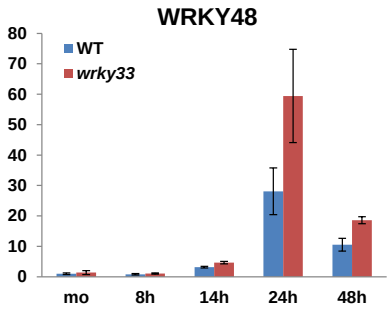
W-box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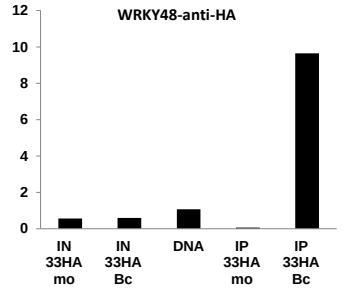
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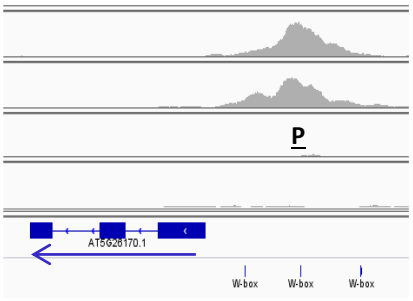
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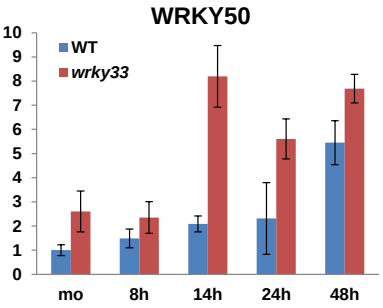
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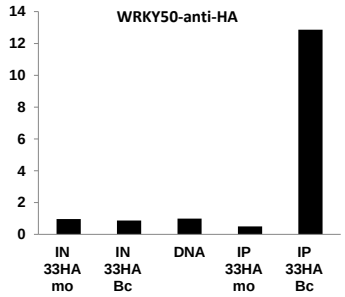
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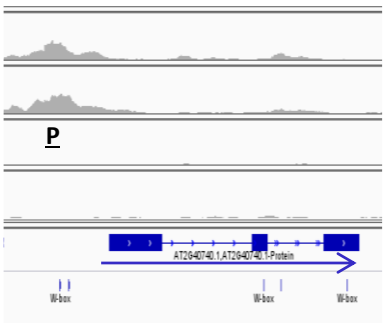
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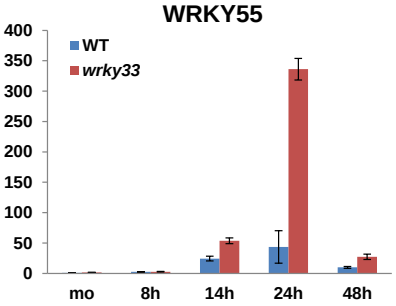
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wrky33
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300
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200
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50
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24h
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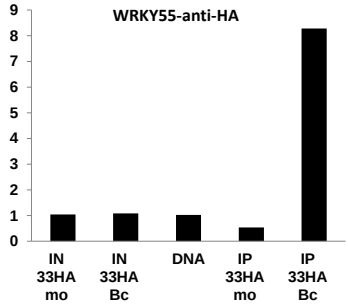
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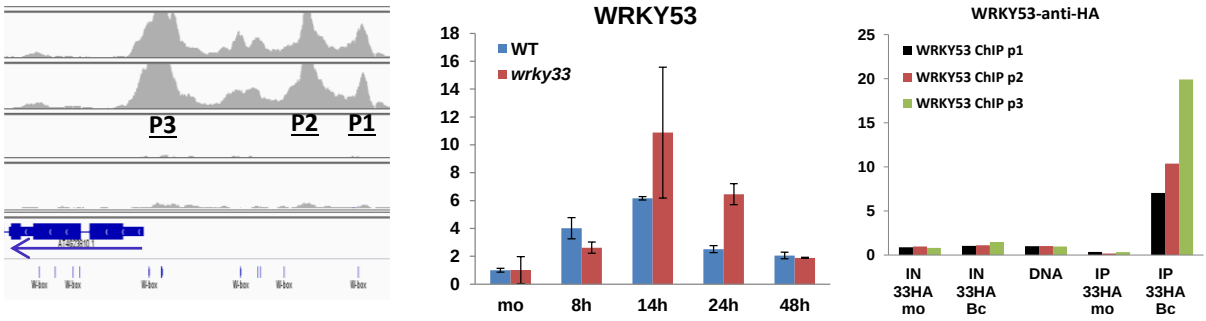


Figure 2.9 WRKY33 regulated transcription factor families commonly associated with stress responses. (A) WRKY, MYB, NAC and AP2/ERF TF families are dominant transcription factor targets of WRKY33 after *B. cinerea* infection. The total number of members for each TF family are given in parenthesis next to the name. The number of directly or indirectly WRKY33-regulated gene members is indicated. (B) Visualization (IVG; Integrative Genomic Viewer) of ChIP-seq data revealing strong infection-dependent WRKY33 enrichment at the indicated WRKY promoter (both biological repetitions, IP *B.c.* 1 and IP *B.c.* 2, are shown). Structure of the WRKY gene is indicated below along with the position of all W-box motifs. Arrows indicate direction of transcription. qRT-PCR analysis of *B. cinerea* 2100-induced expression of indicated *WRKYs* (*WRKY33, WRKY38, WRKY41, WRK48, WRKY50, WRKY53* and *WRKY55*) in WT and *wrky33* mutant plants at indicated timepoints post fungal spore application, respectively. Validation of the ChIP-seq data by ChIP-qPCR supporting WRKY33 binding to the promoter region of indicated WRKYs. For qRT-PCR all data were normalized to the expression of At4g26410 and fold induction values of all genes were calculated relative to the expression level of mock-treated (mo) WT plants set to 1. Error bars represent SD of three biological replicates (n=3). For ChIP-qPCR, leaves from WRKY33-HA (33HA) plants were spray inoculated with *B. cinerea* 2100 (Bc) or mock treated (mo) for 14h. Input DNA before immunoprecipitation (IN) and co-immunoprecipitated DNA using an anti-HA (IP) were analyzed by qPCR employing gene-specific primer pairs (P) shown in the IGV, 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 similar results.

2.3.6. WRKY33 differentially regulates genes in hormonal pathways

Genes encoding components of pathways related to the key hormone signaling molecules SA, JA, ET and abscisic acid (ABA) were highly enriched in the WRKY33 regulated gene set (Figure 2.7). For instance, genes involved in SA response were enriched in WRKY33-repressed direct targets and non-target genes, while genes responsive to ethylene were highly enriched in the WRKY33-induced direct target dataset. This is consistent with our previous report showing that WRKY33 directly or indirectly repressed the expression of genes in SA biosynthesis and SA-mediated signaling (Birkenbihl et al., 2012).

Some WRKY33 induced target genes are associated with response to ET, such as *ACS6*, *ORA59* and *ERF5* (Figure 2.7, 2.15). ACS6 was reported to be involved in *Botrytis*-induced

ethylene production, and to play an important role in plant immunity (Han et al., 2010; Li et al., 2012). ORA59 and ERF5 belong to the AP2/ERF domain TF family with ORA59 acting as the integrator of the JA and ET signaling pathways, and as a positive regulator of resistance against *B. cinerea*, while ERF5 is a key component of chitin mediated immunity (Pre et al., 2008; Moffat et al., 2012). Genes responsive to ABA and JA were also overrepresented in our GO analysis but in this case genes responsive to ABA were significantly enriched in the WRKY33-repressed non-target genes, while genes involved in JA response were enriched in WRKY33-repressed direct targets and non-target genes (Figure 2.7). Interestingly, many of the target genes were involved in more than one hormonal response (Figure 2.8B). For example, 5 of the WRKY33-regulated targets are described as being responsive to four hormones - SA, JA, ET, and ABA, indicating that WRKY33 may be involved in hormone co-regulation or crosstalk.

In addition, two *GH3* genes, *GH3.2* and *GH3.3* that were recently reported to conjugate auxin and JA with amino acids, respectively (Gonzalez-Lamothe et al., 2012; Gutierrez et al., 2012) are direct targets of WRKY33 and their expression increased highly in *wrky33* after *B. cinerea* 2100 infection (Figure 2.10A). GH3.2 was shown to be involved in plant disease development since the *gh3.2* knock out mutant increased resistance to *B. cinerea* (Gonzalez-Lamothe et al., 2012; Gutierrez et al., 2012). GH3.3 is postulated to control JA homeostasis in seedlings (Gonzalez-Lamothe et al., 2012; Gutierrez et al., 2012).

These results suggest that WRKY33 plays a role in fine-tuning various hormonal responses during the plant response to *B. cinerea* 2100. However, previous studies have shown that *wrky33 sid2-1*, *wrky33 npr1-1* mutants affected in SA biosynthesis and SA amplification do not restore WT resistance towards *B. cinerea* 2100, and mutants in ET/JA signaling such as *ora59, ein2, opr3* and *jar1* showed unaltered WT resistant (Birkenbihl et al., 2012). In this study, we also generated *wrky33 gh3.2 gh3.3* triple mutants, which may affect auxin and JA conjugation with amino acids. Although both the single *gh3.2* and *gh3.3*, as well as the *gh3.2 gh3.3* double mutant plants showed WT-like resistance towards *B. cinerea* 2100, the triple mutants were still nearly as susceptible as *wrky33* plants indicating that these *GH3* genes are not required for full resistance mediated by WRKY33 (Figure 2.10B). Taken together, these and our previous results suggest that none of the identified and further investigated potential candidate genes appeared to be critical components in WRKY33-dependent host defense towards *B. cinerea* 2100.

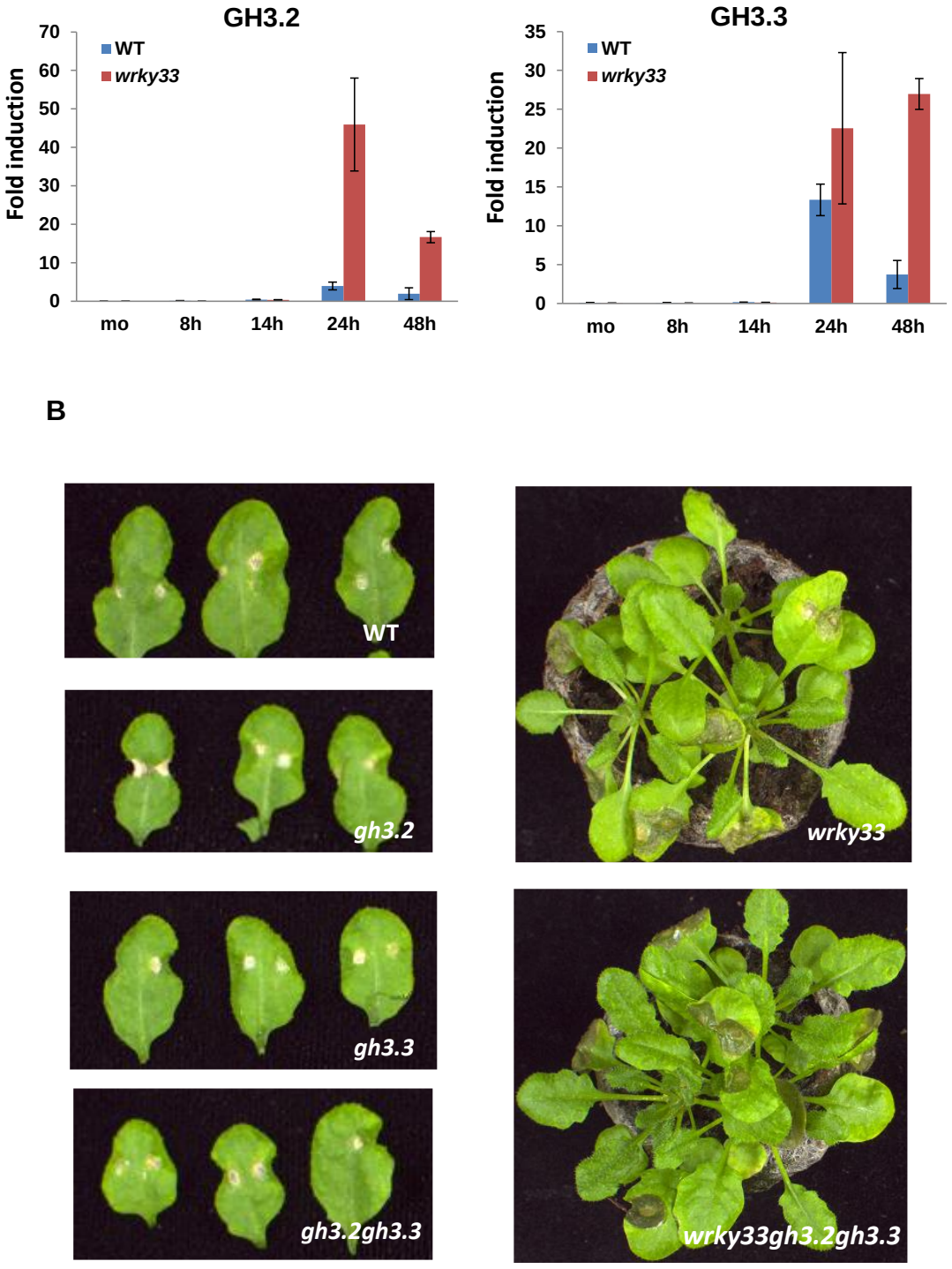
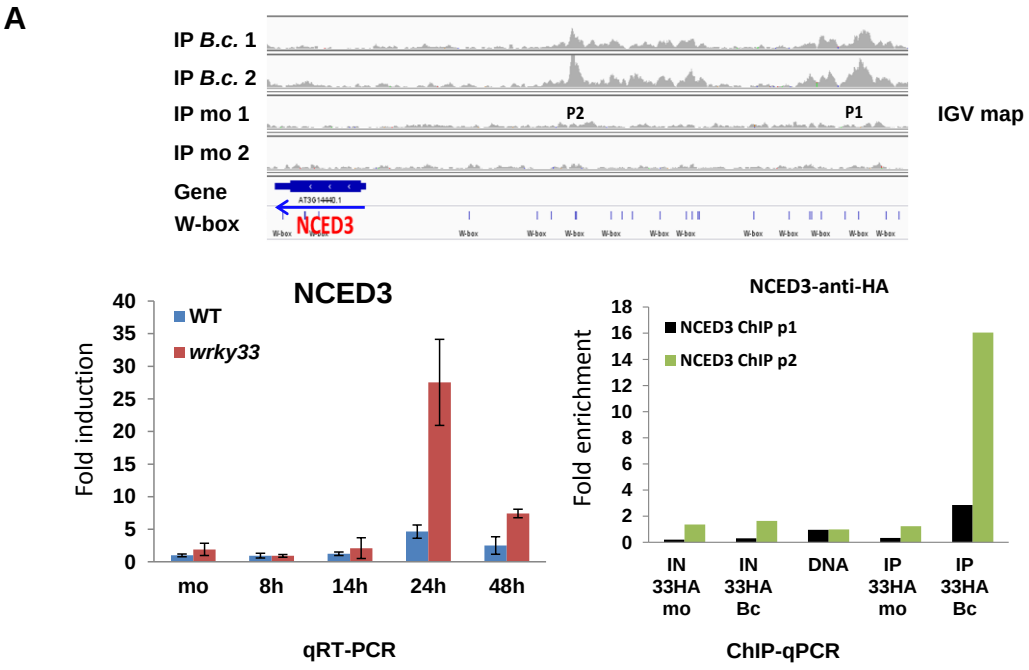


Figure 2.10 The role of GH3 genes in WRKY33-mediated resistance to *B. cinerea* 2100. (A) qRT-PCR analysis of *GH3.2* and *GH3.3* in WT and *wrky33* at the indicated time points after *B. cinerea* inoculation. (B) *B. cinerea* infection phenotypes of indicated *Arabidopsis* plants, including WT (Col-0), *wrky33*, *gh3.2*, *gh3.3*, *gh3.2 gh3.3* and *wrky33 gh3.2 gh3.3*.

2.3.7. The role of ABA in mediating WRKY33-dependent resistance to *Botrytis*

The role of SA and JA/ET in plant defense is well documented. In contrast, less is known regarding ABA in host defense although increased ABA levels can enhance pathogen susceptibility (Robert-Seilaniantz et al., 2011), and can also positively or negatively impact the outcome of plant-microbe interactions, depending on the lifestyle of the pathogen (Adie et al., 2007). Our ChIP-seq data revealed that WRKY33 binds to the promoter of *NCED3* and to the TTS region of *NCED5* (Figure 2.11A, B), two major genes encoding 9-cis-epoxycarotenoid dioxygenase, a key enzyme in the biosynthesis of ABA (Frey et al., 2012). WRKY33 binding to both gene loci was confirmed by ChIP-qPCR (Figure 2.11 A, B). Moreover, expression levels of *NCED3* and *NCED5* both increased in the *wkry33* mutant after *B. cinerea* infection (Figure 2.11 A, B), indicating that WRKY33 negatively regulates expression of these genes. This suggests that WRKY33 can very likely repress ABA biosynthesis in WT plants. In addition, WRKY33 also bound to the promoter of *CYP707A3* (Figure 2.11 C), a gene involved in ABA metabolism (Kushiro et al., 2004; Saito et al., 2004), and *CYP707A3* expression increased in WT plants but decreased in the *wrky33* mutant after *B. cinerea* infection (Figure 2.11 C). These results strongly suggest that WRKY33 is involved in repressing ABA levels after *B. cinerea* infection and that this repressor function may be an important component in host resistance to this pathogen.



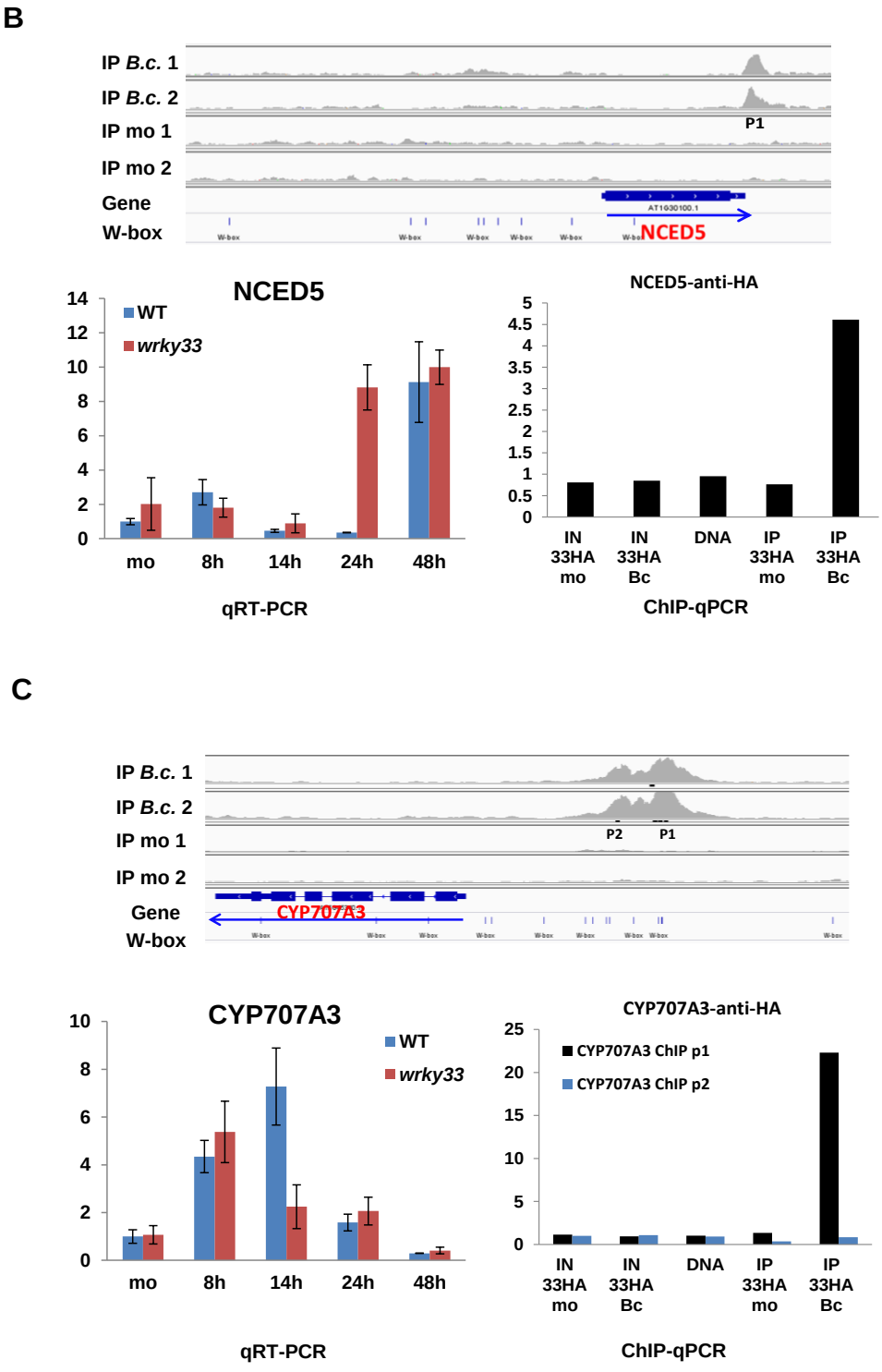


Figure 2.11 WRKY33 directly regulates target genes encoding ABA biosynthetic (*NCED3*, *NCED5*) and metabolic (*CYP707A3*) enzymes by binding to their promoters or 3'UTR after *B. cinerea* 2100 treatment. (A-C) Visualization (IVG; Integrative Genomic Viewer) of ChIP-seq data revealing strong infection-dependent WRKY33 enrichment at the *NCED3* (A), *NCED5* (B) and *CYP707A3* (C) loci (both biological repetitions, IP *B.c.*1 and IP *B.c.*2, are shown). Structure of the respective genes are indicated below along with the position of all W-box motifs. Arrows indicate direction of transcription. Transcript levels of *NCED3*, *NCED5* and

CYP707A3 at the indicated time points after inoculation were determined by qRT-PCR. Data were normalized to the expression of At4g26410 and fold induction values of all genes were calculated relative to the expression level of mock-treated (mo) WT plants set to 1. Validation of the ChIP-seq results was achieved by ChIP-qPCR. For ChIP-qPCR, the leaf material and treatment as performed as indicated in legend to Figure 2.9. Input DNA before immunoprecipitation (IN) and co-immunoprecipitated DNA using an anti-HA (IP) were analyzed by qPCR employing gene-specific primer pairs (P1, P2) shown in the IGV, 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 similar results.

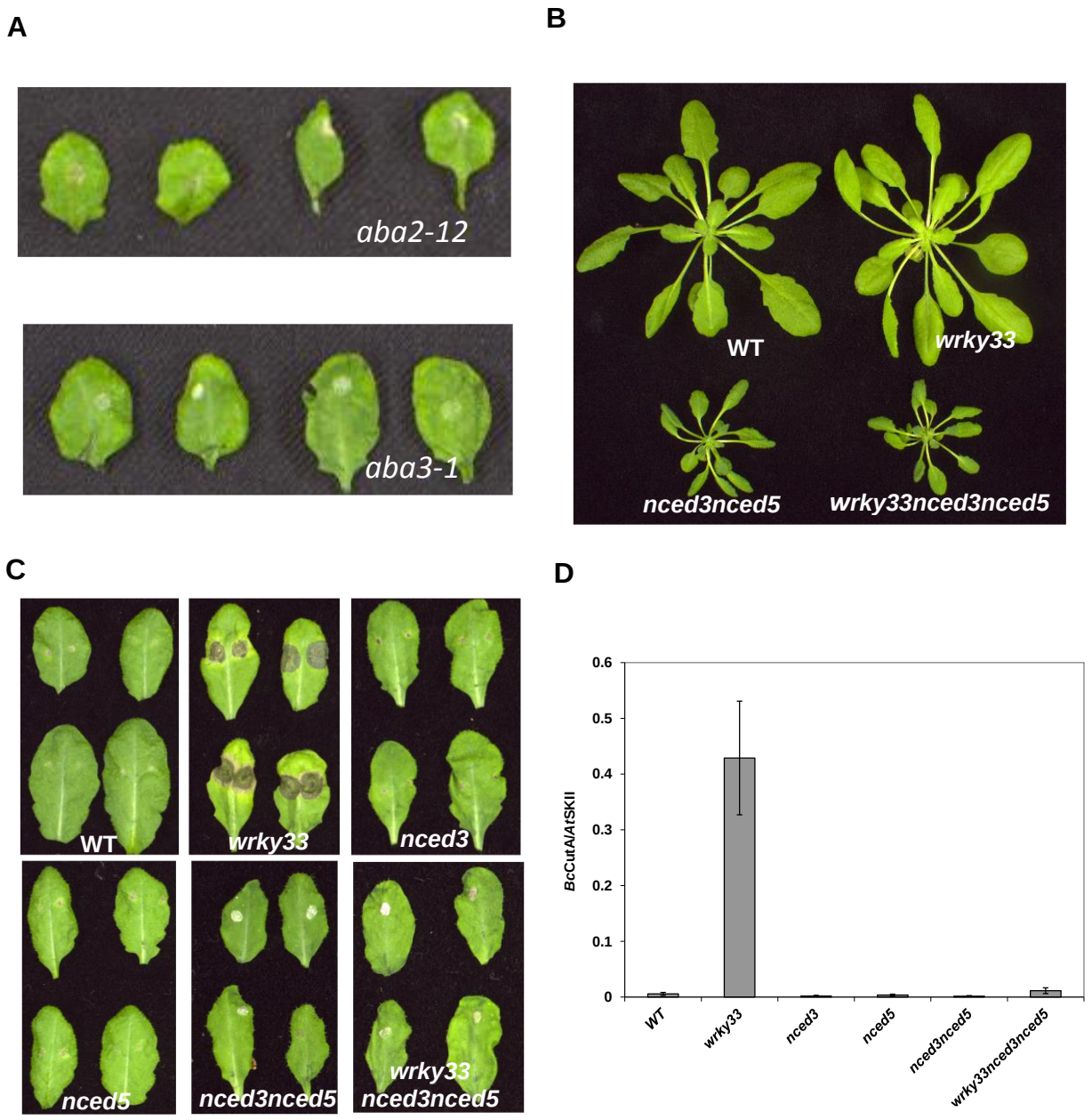
2.3.8. In *wrky33 nced3 nced5* mutants WT-like resistance to *B. cinerea* 2100 is restored

To test whether ABA is involved in WRKY33-mediated host defense to *B. cinerea* 2100, we analyzed ABA mutants with respect to their phenotypes after fungal infection. Mutants in ABA biosynthesis, *aba2-12*, *aba3-1*, *nced3-2*, *nced5-2*, and *nced3 nced5* were nearly as resistant as WT plants (Figure 2.12A, C). This was expected as previous reports showed that *aba2-12* (Adie et al., 2007), *aba3-1* (Leon-Kloosterziel et al., 1996), and *nced3 nced5* (Frey et al., 2012) accumulated much less ABA than WT plants. To test whether WT resistance *to B. cinera* 2100 could be due to WRKY33 repression of *NCED3* and *NCED5* expression we generated *wrky33 nced3*, *wrky33 nced5* double, and *wrky33 nced3 nced5* triple mutants and tested their phenotypes following infection.

ABA deficiency severely affects plant growth leading to stunted phenotypes, as observed in *nced3 nced5*, *aba2-12* and *aba3-1* mutants. A strong reduction of rosette diameter was also observed in *wrky33 nced3 nced5* mutants under short day conditions similar to that of *nced3 nced5* (Figure 2.12B). However, unlike the *wrky33* single mutant, *wrky33 nced3 nced5* plants showed clear resistance to *B. cinerea* 2100 similar to WT (Figure 2.12C). In contrast, *wrky33 nced3* and *wrky33 nced5* double mutants remained as susceptible as *wrky33* to infection by *B. cinerea* 2100 (Figure 2.12F). Supporting the observed phenotype, qPCR analysis showed strongly reduced fungal biomass (measured as abundance of fungal DNA) in *wrky33 nced3 nced5* compared to *wrky33* (Figure 2.12D). These results indicate that increased expression levels of both *NCED3* and *NCED5* in *wrky33* mutants contribute to susceptibility toward *B. cinerea* 2100, and that a key role of WRKY33 in host immunity towards this pathogen is to repress the biosynthesis of ABA in WT plants.

Since the *nced3 nced5* mutant showed reduced ABA levels, we tested whether exogenous application of ABA to such mutants could revert the resistant phenotype. Indeed, application of ABA together with the fungal spore droplet to the *wrky33 nced3 nced5* triple mutant partially rendered plants susceptible to *B. cinerea* 2100 (Figure 2.12E). We also tested the

phenotypes of transgenic plants overexpressing *NCED3* and *NCED5* driven by the 35S CaMV promoter, respectively (Fan et al., 2009). These ectopic overexpressor lines showed clear resistance towards *B. cinerea* 2100 (Figure 2.12G). It has been reported that mutants of ABA metabolism (*CYP707As*) accumulated more ABA than lines overexpressing ABA biosynthetic genes (Okamoto et al., 2006; Finkelstein, 2013). We therefore further tested the phenotypes of *cyp707a1, cyp707a2, and cyp707a3* following infection with *B. cinerea* 2100. Interestingly, all of these mutants were as resistant as WT plants (Figure 2.12H). Taken together, our genetic analysis revealed that WRKY33-mediated expression of *NCED3* and *NCED5* plays a critical role in host resistance towards *B. cinerea* 2100, very likely by altering ABA biosynthesis.



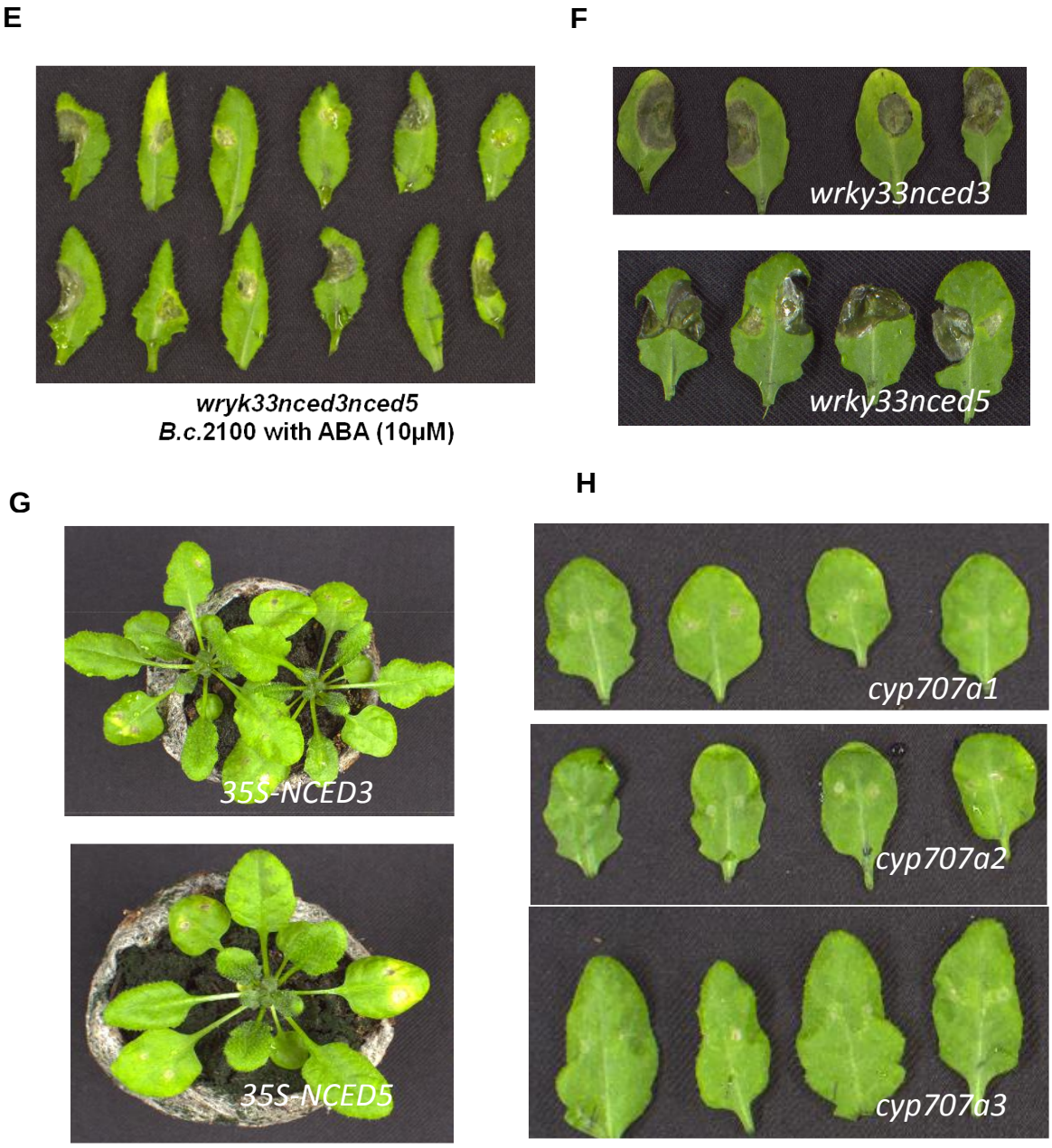
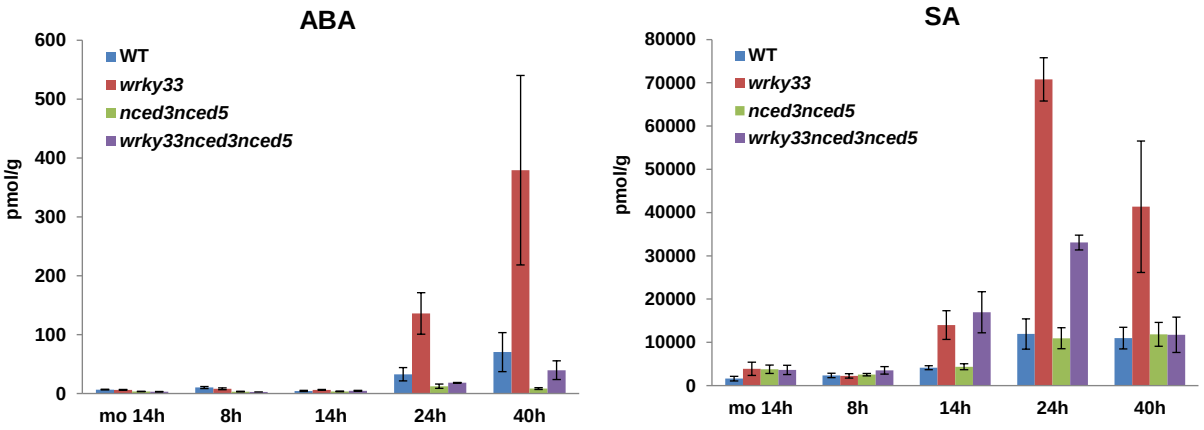


Figure 2.12 WRKY33 controls ABA-mediated plant susceptibility to *B. cinerea* 2100. (A) *B. cinerea* infection phenotypes of *aba2-12* and *aba3-1*. (B) Growth phenotypes of WT, *wrky33*, *nced3 nced5* and *wrky33 nced3 nced5* plants. (C) *B. cinerea* infection phenotypes of WT, *wrky33*, *nced3*, *nced5*, *nced3 nced5* and *wrky33 nced3 nced5* plants. (D) *B. cinerea* biomass growth analysis of indicated *Arabidopsis* genotypes. The relative abundance of *B. cinerea* and *Arabidopsis* DNA was determined by qPCR employing specific primers for *Bc*Cutinase A and *At*SKII, respectively. (E) Exogenous application of ABA (10µM) to *wrky33 nced3 nced5* triple mutants partially renders plants susceptible to *B. cinerea*. (F-G) *B. cinerea* infection phenotypes of indicated *Arabidopsis* plants, including *wrky33 nced3*, *wrky33 nced5*, *35S-NCED3*, *35S-NCED5*, *cyp707a1*, *cyp707a2* and *cyp707a3*.

2.3.9. WRKY33 controls hormone homeostasis in response to *B. cinerea*

Given that *wrky33 nced3 nced5* plants restore WT-like resistance compared with the susceptible *wrky33* mutants, we hypothesized that WRKY33 plays a critical regulatory role in controlling ABA levels. We therefore measured hormone levels in WT, *wrky33*, *nced3 nced5* and *wrky33 nced3 nced5* plants following infection with *B. cinerea* 2100 at various time points.

As shown in Figure 2.13, ABA, SA, JA, jasmonoyl-isoleucine (JA-Ile), 12-oxo-phytodienoate (OPDA) and l-aminocyclopropane-1-carboxylate (ACC; a precursor of ethylene) levels increased strongly in susceptible *wrky33* compared to resistant WT plants at later infections stages (24hpi and 40hpi). The higher levels of ABA detected in the *wrky33* mutant is consistent with our hypothesis that WRKY33 negatively regulates ABA biosynthesis. Also consistent with our assumptions we could clearly detect reduced ABA levels in resistant *wrky33 nced3 nced5* plants. Interestingly however, concomitant reduction of SA, JA, JA-Ile, OPDA and ACC levels were also observed in *wrky33 nced3 nced5* compared with *wrky33*, indicating that the elevated levels of SA, JA, JA-Ile, OPDA and ACC in w*rky33* at late infection stages are a consequence of higher ABA levels. This suggests that ABA/ABA signaling may exert a positive role on the biosynthesis of these other hormonal components. Taken together, these data indicate that a key function of WRKY33 in WT plants challenged with *B. cinerea* is to repress ABA levels. Consistent with the gene expression patterns observed, mutation of *WRKY33* affects the WT hormonal balance and results in elevated ABA activity, thereby subsequently inducing among others SA biosynthesis as possibly leading to the activation of SA responses, such as host cell death, which could be beneficial for *B. cinerea* colonization. Furthermore, the elevated ABA levels found in the *wrky33* mutant also appear to influence JA/ET levels.



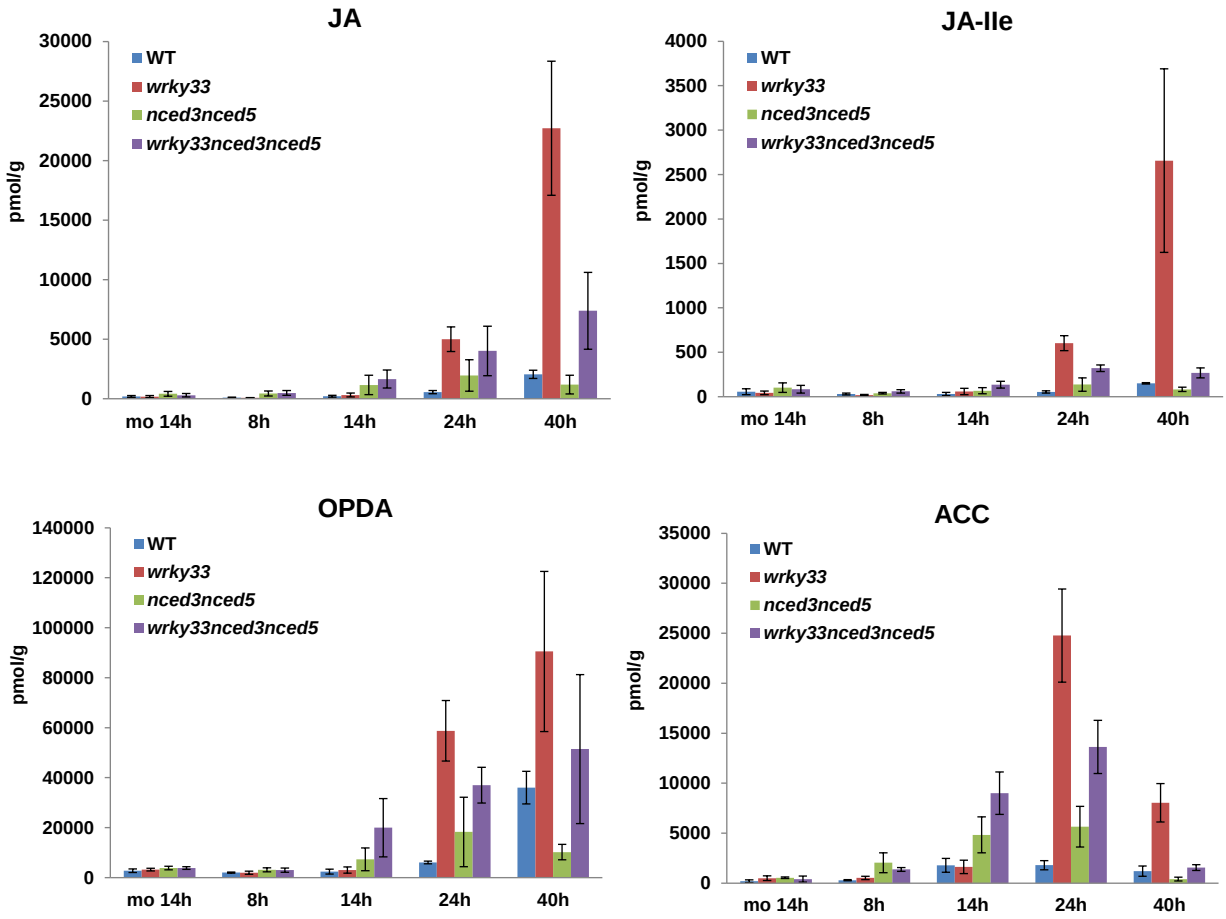


Figure 2.13 Hormone levels during *B. cinerea* 2100 infection of Arabidopsis plants. The hormone concentrations of ABA, SA, ACC, JA, JA-lle and OPDA were measured at 8, 14, 24 and 40hpi in WT, *wrky33*, *nced3nced5* and *wrky33 nced3 nced5* plants spray inoculated with *B.cinerea* spores or mock treated. Error bars represent SD (n=4). Note: These experiments were collaborated with Dr. Jörg Ziegler (IPB Halle, Germany), and he performed the hormone measurement.

2.3.10. In *wrky33 nced3 nced5* plants, expression of many up-regulated genes in *wrky33* return to WT like levels

As noted above more than 75% of WRKY33-regulated target genes showed increased expression levels in the susceptible *wrky33* mutant after *B. cinerea* infection. To gain additional insights into altered gene expression in the resistant *wrky33 nced3 nced5* plants we performed qRT-PCR analyses. The levels of many highly expressed SA-related genes observed in the *wrky33* mutant post infection decreased in the infected leaves of *wrky33 nced3 nced5* plants often reaching WT levels. These included: *ICS1, NPR1, NPR3, NPR4, TRX-H5* and *FMO1* (Figure 2.14). However, the expression levels of other SA-related genes such as *EDS1, PAD4, MININ1,* and *PR1* were not as strongly affected and their expression levels remained higher than in WT (Figure 2.14). Our results indicate that simultaneous

mutations of *NCED3* and *NCED5* in a *wrky33* genetic background partially impaired SA biosynthesis and signaling.

The expression levels of some NAC TF genes, such as *NAC019*, *NAC055*, *NAC061* and *NAC090*, were reduced in the *wrky33 nced3 nced5* mutant compared to the *wrky33* mutant at 24 hpi, indicating that ABA positively regulates their expression in *wrky33* plants (Figure 2.14). This was also the case for some WRKY genes including *WRKY41*, *WRKY48*, *WRKY53* and *WRKY55*, while expression of *WRKY38* and *WRKY50* increased in *wrky33 nced3 nced5* to even higher levels than observed in *wrky33* (Figure 2.14). These data indicate ABA has a positive effect on the expression of *WRKY41*, *WRKY48*, *WRKY53* and *WRKY55*, while negatively affecting *WRKY38* and *WRKY50* in *wrky33* plants.

The fungal-induced expression of many other ABA response genes such as *ABI1*, *CDPK1*, *MPK11*, *CRK36*, *Lectin-domain containing receptor kinase A4.2* were also restored to wild-type levels in the *wrky33 nced3 nced5* triple mutant (Figure 2.14). Interestingly, *CDPK1*, *MPK11*, *CRK36* and *NPR3* are also responsive to SA and all of these genes are also associated with the GO term involved in 'cell death', suggesting that ABA could have a positive effect on cell death responses (Figure 2.8A, Table S1, S2). Thus, WRKY33 suppresses expression of many of its target genes by negatively regulating ABA responses. Taken together, molecular analysis indicates that many, but not all, of the genes that are strongly up-regulated in the susceptible *wrky33* mutant post infection return to WT-like levels in the resistant *wrky33 nced3 nced5* triple mutant. Thus, such genes showing restoration of WT-like expression levels in this mutant may be prime candidate genes that are causal for WRKY33-mediated resistance against this necrotrophic fungus.

2.3.11. *wrky33 nced3 nced5* resistance to *B. cinerea* does not depend on ET/JA signaling

ET/JA signaling are generally assumed to be more important for regulating resistance to necrotrophic pathogens (Glazebrook, 2005). Several of our identified WRKY33 target genes associated with the ET/JA signaling pathway show reduced expression levels in *wrky33* plants upon infection (Figure 2.7). We therefore next asked the question whether resistance to *B. cinerea* of *wrky33 nced3 nced5* mutants depends on ET/JA signaling. As shown in Figure 2.15 decreased gene expression levels of *ACS6*, *ERF5*, *ORA59* and *PDF1.2* were observed in *wrky33 nced3 nced5* triple mutants compared to WT plants post infection. Both *wrky33 nced3 nced5* and WT plants are resistant to *B. cinerea*, but the expression levels of these genes involved in ET/JA signaling were much lower in the former, indicating the resistance in *wrky33 nced3 nced5* is independent on these genes. This is further supported by that fact that

expression of all of these genes is, in nearly all cases, even lower in the resistant *wrky33 nced3 nced5* plants compared to the susceptible *wrky33* mutant. In addition, the expression levels of *ORA59* (Figure 2.15) and *ERF1* (Figure 2.14) are reduced in the *wrky33 nced3 nced5* mutant compared to the *wrky33* mutant at 24 hpi indicating that ABA positively regulates their expression in *wrky33* plants. This is also consistent with the reduced JA/ET levels observed in *wrky33 nced3 nced5* plants (Figure 2.13) at later infection stages. Thus, JA/ET signaling very likely does not contribute to WRKY33-dependent *Botrytis* resistance observed in the *wrky33 nced3 nced5* plants, and possibly also not in WT Col-0 plants.

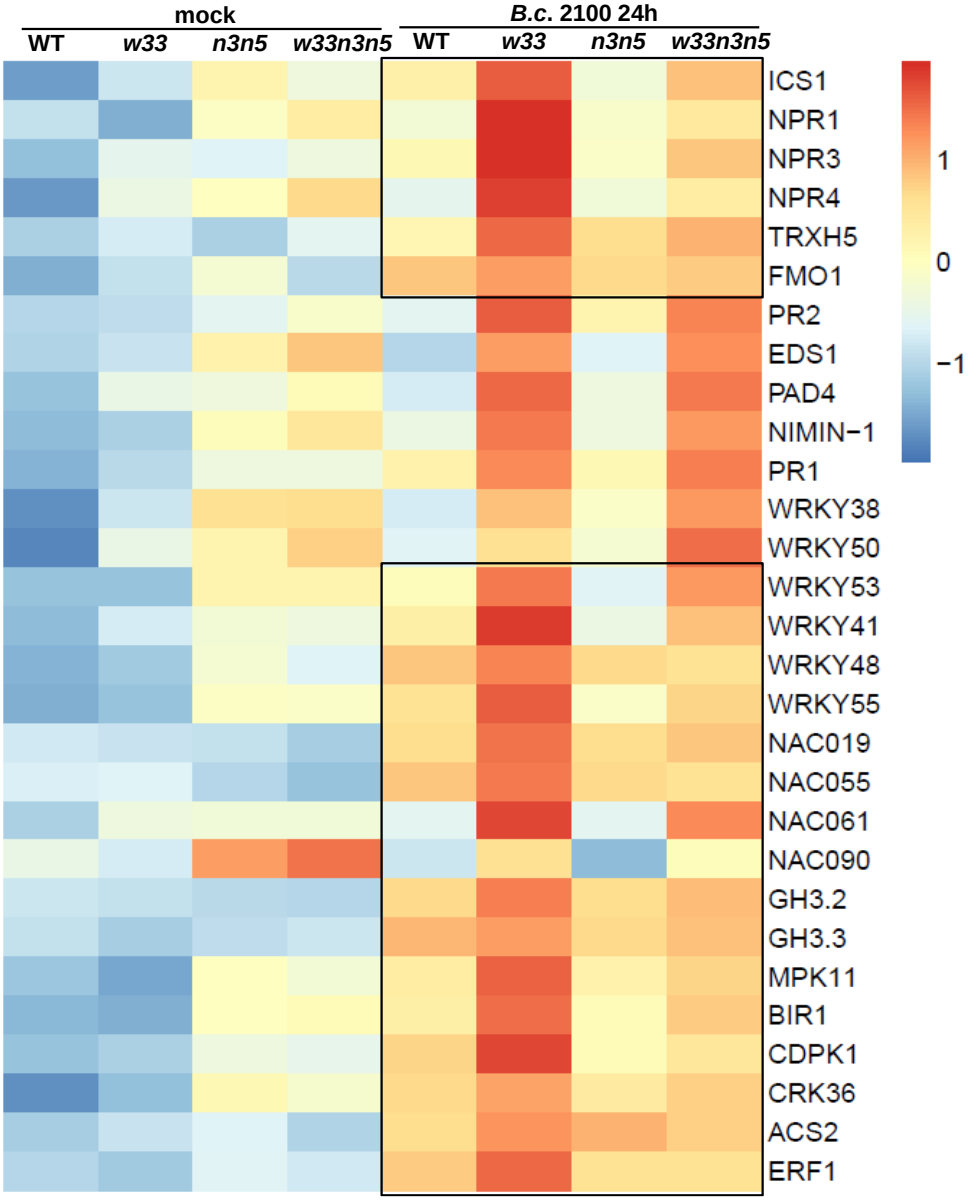


Figure 2.14 Heatmap presentation of differentially expressed genes in WT, *wrky33* (*w33*), *nced3 nced5* (*n3n5*) and *wrky33 nced3 nced5* (*w33n3n5*) after mock and *B. cinerea* (*B. c.* 2100) infection at 24h (based

on qRT-PCR data). In *wrky33 nced3 nced5* plants expression of many genes that are strongly up-regulated in *wrky33* mutants show WT-like levels (framed).

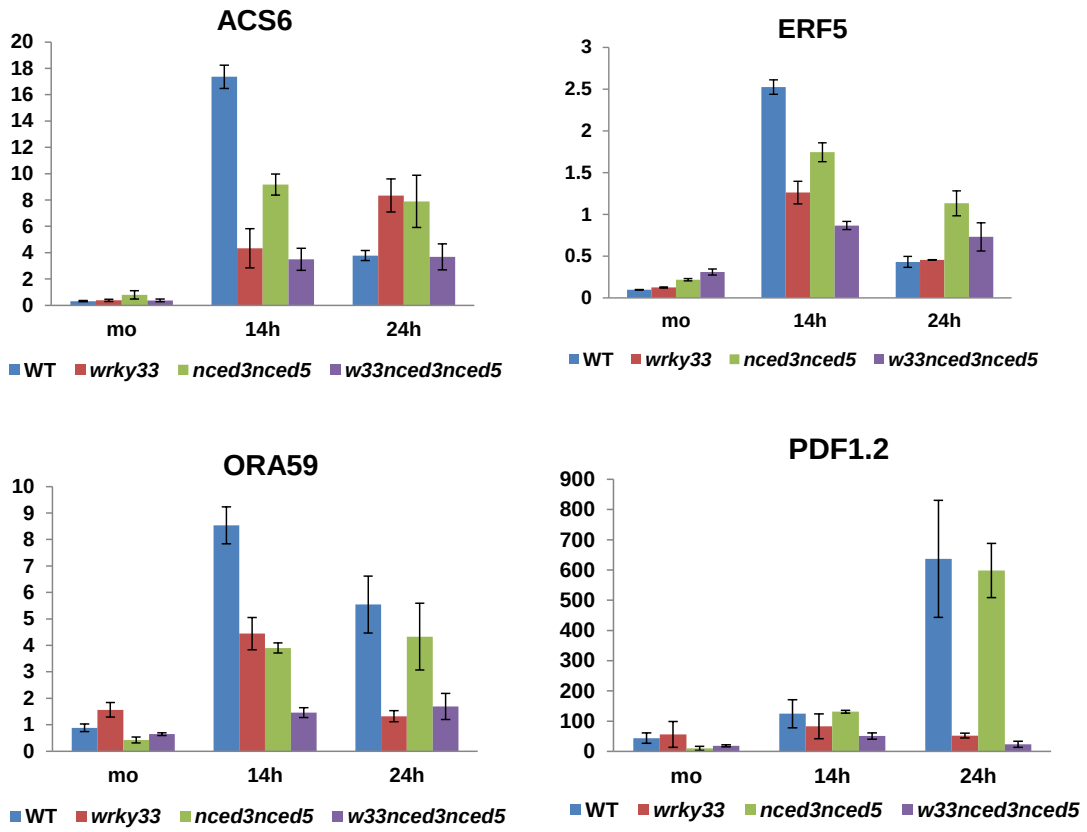


Figure 2.15 Expression levels of selected genes associated with ET/JA signaling in WT, *wrky33*, *nced3nced5* and *wrky33nced3nced5* plants determined by qRT-PCR at indicated timepoints after *B. cinerea* 2100 inoculation. Error bars represent SD of three biological replicates (n=3).

2.4. Discussion

Plant immunity is driven by complex genetic programs that result in massive transcriptional reprogramming in the host upon pathogen perception and signal transduction to the nucleus. Transcription factors are key components of terminal signaling and act by activating and repressing the expression of numerous defense-associated genes. The TF WRKY33 plays a major role in conferring resistance of *Arabidopsis* plants to the fungal necrotroph *B. cinerea*. In the present work, ChIP-seq and RNA-seq were employed to gain a global overview of all WRKY33 binding sites within the Arabidopsis genome following fungal infection and to correlate such binding to altered transcriptional outputs. By including appropriate mutants in this study we identified components of the ABA hormonal pathway that act downstream of WRKY33 to mediate host resistance.

A genome-wide analysis of *in vivo* binding sites for a selected TF expressed under the control of its native promoter in intact plant tissue following infection with a pathogen has not yet been reported. Our study shows that the number of high-affinity WRKY33 binding sites within the genome by far exceeds the number of direct target genes affected in their transcriptional response upon WRKY33 binding during *B. cinerea* infection. In fact, we found 1258 genes (80% of WRKY33 ChIP targets) that had a WRKY33-binding region in close vicinity but were not differentially expressed in the *wrky33* mutant. This excess number of gene loci bound by a given transcription factor but unaffected in their expression is consistent with previous ChIP-seq studies although the reason for this discrepancy remains to be elucidated (MacQuarrie et al., 2011). One possibility is that transcriptional activation/repression at a specific promoter is context dependent, that is, may require next to WRKY33 binding, additional diverse input signals. For example we detected strong enrichment of WRKY33 within the promoters of numerous genes coding for receptors of various MAMPs and Damage Associated Molecular Patterns (DAMPs) including *FLS2*, *ERF*, *PEPR1*, and *PEPR2*, in our ChIP-seq studies, but no altered expression of these genes in the *wrky33* mutant upon *B. cinerea* infection. WRKY33 has been shown to also be strongly and rapidly induced during MAMP-triggered immunity (Lippok et al., 2007) and thus a regulatory function of WRKY33 at these promoters may require additional co-factors only induced/activated during MAMP/DAMP signaling.

So far, several ChIP-seq experiments for TFs reported in *Arabidopsis* used seedlings as material while our ChIP-seq and RNA-seq studies were performed using intact rosette leaves from 4-week old plants under short-day growth condition and spray infection with *B. cinerea* spores. Although we do not know how synchronous the infection process is and how many plants cells within the leaf tissue are actually responding to the fungus we could observe stable accumulation of the WRKY33-HA protein already at 14hpi and up to 48hpi in the plant material. Actually, we found about 75% reproducible peaks from two biological replicates after ChIP-seq (Table 2.1). Subsequent ChIP-qPCR analysis of about 30 selected target genes confirmed to 100% enrichment of WRKY33 at these loci. Thus our experiments demonstrate the ability to use pathogen-challenged intact mature rosette leaves for ChIP-seq studies.

Using conservative criteria for selecting differentially expressed genes in *wrky33* mutants compared to WT (Col-0) plants after *B. cinerea* 2100 infection, we found that about 2600 genes were transcriptionally up- and down-regulated. The strikingly high number of modulated genes at early infection stages (14hpi) highlights the importance of WRKY33 to

initiate host responses to this pathogen. At this early stage no difference in fungal biomass, hyphal expansion or other phenotypic criteria can be observed between resistant WT plants and susceptible *wrky33* mutants.

2.4.1. WRKY33 binds to genes enriched in different pathways

WRKY33 bound to approximately 1500 genes after *B. cinerea* 2100 infection. GO analysis of these genes identified functional terms such as 'response to stress' and 'response to stimulus' as being significantly over-represented in the list of putative WRKY33 targets and WRKY33-regulated targets. Similar results was identified in EIN3, the master regulator of the ethylene signaling pathway (Chang et al., 2013). ET signaling pathway positively regulates host defense to the necrotrophic *B. cinerea*, and many genes in ET pathway are direct targets of WRKY33 including *EIN3, ACS2, ACS6, ERF1, ERF5* and *ORA59*. Here the observation of similar functional categories in WRKY33 and EIN3 indicate that both of them are involved in host defense to *B. cinerea*. Interestingly, recent reports show several key regulators in plant development processes also targeting genes enriched in 'stress' and 'stimulus'. These regulators include FLOWERING LOCUS C (FLC) (Deng et al., 2011), SHORT VEGETATIVE PHASE (SVP) (Gregis et al., 2013), Phytochrome A (phyA) (Chen et al., 2014b), and LONG HYPOCOTYL 5 (HY5) (Zhang et al., 2011b). FLC and SVP regulate *Arabidopsis* flowering, while phyA and HY5 regulate plant perception of light environment. These regulators are involved in plant responses to environmental signals during growth and development, therefore, their target genes could be enriched in 'response to stimulus' such as 'external stimulus', 'endogenous stimulus' and 'abiotic stimulus'. In this respect, plant defense related regulators such as WRKY33 might also regulate target gene functions in plant environmental signaling and development.

Our data show that WRKY33 target genes were enriched in 'transcription factor activity' but not significantly overrepresented in WRKY33-regulated target genes, i.e. WRKY33 binds to 133 TF gene loci while only 21 show altered transcription 14hours post *B. cinerea* 2100 infection. This is different to what has been reported for EIN3-, PIFs- (bHLH TF) and HY5- (bZIP TF) regulated target genes, in which transcription factors are enriched (Zhang et al., 2011a; Chang et al., 2013; Zhang et al., 2013). One must keep in mind that our study merely reflects a snapshot of the binding events occurring over time. The 14hpi timepoint was selected based on the abundance of *Botrytis*-induced WRKY33-HA protein. However, WRKY33-HA protein is detected up to 48hpi. Thus, genes not altered in their expression at 14hpi may still show altered expression at other timepoints. Indeed, in the EIN3 study (Chang

et al., 2013), the authors showed that EIN3 regulates distinct transcriptional responses in several temporal waves. The second possibility is the differences in the material used for ChIP-chip (-seq) and RNA-seq. In the PIFs, EIN3 and HY5 studies 2 to 4-day-old seedlings were used while in this work we used intact rosette leaves from 4-week old plants following spraying infection with *B. cinerea* spores. Moreover, in the case of the PIF studies seedlings ectopically expressing the TF under the strong 35S CaMV promoter were used for ChIP-seq analysis (Zhang et al., 2013). Expression of the PIFs under non-physiological concentrations may have led to numerous off-target events that could also include several TF loci. Finally, expression of some TF targets may play additional roles for example at different developmental stages or in specific tissue types.

Our data show kinases are overrepresented in WRKY33 target genes and WRKY33-regulated targets, i.e. WRKY33 binds to 155 genes encoding proteins with kinase activity while 41 displayed altered transcription 14hours post *B. cinerea* 2100 infection. This is consistent with EIN3 bound genes that are enriched in the GO term 'kinase activity'. Plant protein kinases were reported to play a central role in signaling during pathogen recognition, the subsequent activation of plant defense mechanisms, and developmental control (Afzal et al., 2008). Different to EIN3 target genes that are induced by ethylene, here most of the WRKY33-regulated targets with kinase activity were repressed by WRKY33. For example, two LecRK members of Arabidopsis LecRK-VI clade, LecRK VI.2 and LecRK VI.3, are direct targets and are negatively regulated by WRKY33 after *B. cinerea* infection. LecRK VI.2 was critical for resistance against hemibiotrophic *P. syringae* pv. tomato DC3000 and the necrotrophic *Pectobacterium carotovorum* bacteria (Singh et al., 2012; Singh et al., 2013; Huang et al., 2014). CDPK1 was another negatively regulated target gene of WKRY33. CDPK1 was reported to respond to drought stress with both T-DNA insertion and overexpression lines confirming that CDPK1 enhanced plant tolerance to drought (Zou et al., 2010). Since constitutive activation of defense responses is detrimental to plant fitness, plant resistance signaling pathways must be negatively controlled. This study suggest that WRKY33 may be involved the process by repressing transcription of selected defense signaling genes. For another example, WRKY33 directly repressed expression of *BIR1*, a LRR- receptor-like kinase interacting with BAK1 upon bacterial flagellin treatment. BIR1 was shown to negatively affect defense because *bir1* plants display constitutive long-term immune responses, including elevated SA, H_2O_2, *PR1*, *PR2* expression, and cell death (Gao et al., 2009).

Our ChIP-seq data also revealed that numerous putative direct targets of WRKY33 are involved in hormone signaling. Plant hormones are important components to modulate immune responses (Pieterse et al., 2012). In response to *B. cinerea* infection, plant hormone levels are commonly enhanced including SA, JA, ET and ABA (Mengiste, 2012). Therefore, hormone responses and hormone homeostasis are vital for immunity. However, how plants precisely control hormone homeostasis during the immune response to *B. cinerea* is not clear. WRKY33 binds to numerous genes involved in auxin, SA, JA, ET and ABA biosynthesis and signaling. Moreover, WRKY33 binding appears to affect the expression of some of these genes in response to *B. cinerea* 2100 infection, thereby resulting in altered hormone biosynthesis and hormonal responses. Indeed, out of 1576 WRKY33-target genes, 154 (9.8%, $p<0.001$) and 32 (2%, $p<0.05$) are involved in hormone-mediated signaling pathways and in the hormone biosynthetic process, respectively. Additionally, out of the 318 WRKY33-regulated target genes, 36 (11.3%, $p<0.001$) are involved in hormone-mediated signaling pathways.

As shown above, WRKY33-induced target genes are enriched in GO term ‘responsive to ET’, while WRKY33-repressed target genes are enriched in the GO terms ‘response to SA and JA’. Several of the WRKY33-regulated target genes were involved in more than one hormonal response highlighting the potential role of WRKY33 in controlling hormonal crosstalk in *Arabidopsis*. In addition, many of the WRKY33 targets with transcription factor functions also are involved in hormone signaling, such as AP2/ERFs, NACs and MYCs. Similar to WRKY33, many of the EIN3 regulated candidate targets were enriched in hormone related categorical sets, including downstream effectors of the ethylene response, key ethylene signaling players, and genes involved in other hormone pathways/responses such as ABA, IAA and methyl jasmonate (Chang et al., 2013). EIN3 candidate target genes were suggested to involve hormone co-regulation (Chang et al., 2013). Similar to EIN3, our findings suggested that *B. cinerea*-induced WRKY33 could directly control hormone co-regulation, and WRKY33 targets hormone pathways at multiple levels, thereby fine-tuning hormone homeostasis.

2.4.2. Negative regulation of the ABA network by WRKY33 is critical for host defense towards *B. cinerea* 2100

The role of ABA in biotic stress responses is complex and currently ill-defined. Recent transcriptomic studies using 4-week old detached Arabidopsis leaves infected with *B. cinerea* strain pepper revealed that genes involved in the suppression of ABA accumulation and

signaling were up-regulated at early infection stages (Windram et al., 2012). Our data clearly demonstrate that increased expression of WRKY33 target genes associated with ABA biosynthesis (*NCED3* and *NCED5*) are causal for the susceptibility of *wrky33* to *B. cinerea*, as the ABA deficient *wrky33 nced3 nced5* mutant restored WT-like resistance towards this necrotroph. Thus our findings reveal a novel role of WRKY33 in modulating host resistance to *B. cinerea* by suppressing ABA accumulation and signaling (Figure 2.16). These finding are consistent with observations showing that ABA-deficient mutants in both tomato (Audenaert et al., 2002; Asselbergh et al., 2007) and *Arabidopsis* (Adie et al., 2007) are less susceptible to this pathogen.

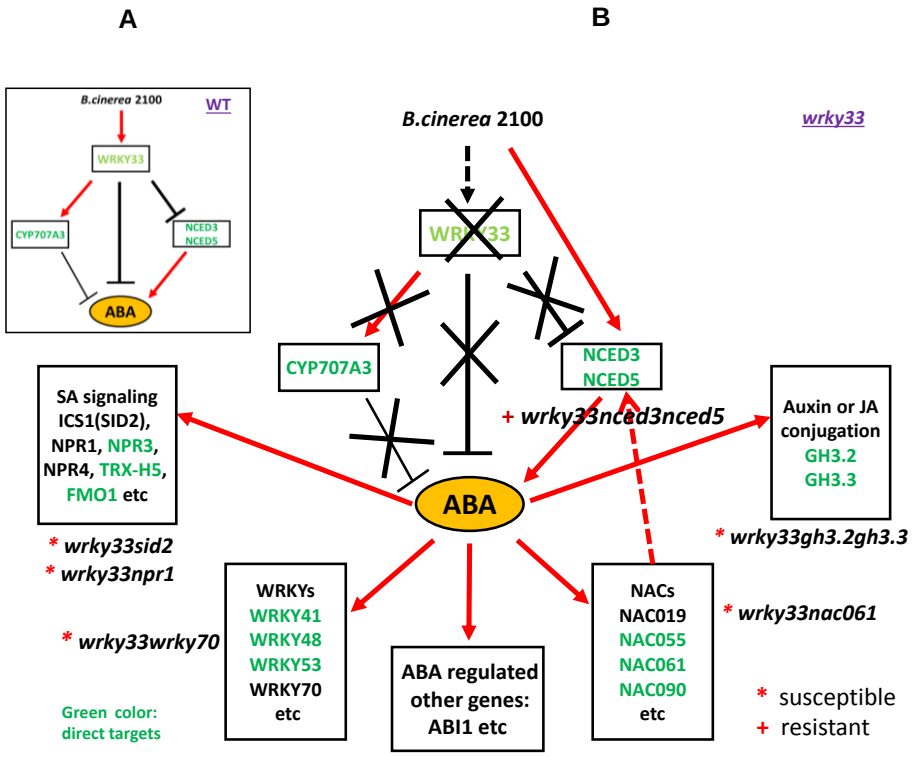


Figure 2.16 Model shows a new role of WRKY33-dependent host immunity towards the necrotroph *B. cinerea* through negative regulation of ABA signaling. (A) In WT plants (framed), *B. cinerea*-induced WRKY33 controls ABA level not only by repressing expression of *NCED3* and *NCED5*, two targets involved in ABA biosynthethesis, but also by inducing expression of *CYP707A3*, a target gene involved in ABA metabolism. (B) In *wrky33* mutants, *B. cinerea* infection increases ABA level that activates the ABA-mediated network. The *wrky33 nced3 nced5* triple mutant restored WT resistance indicates ABA acts as one of the key sub-nodes in WRKY33-dependent host immunity towards *B. cinerea* 2100. Thus, mutation of ABA downstream genes in *wrky33* mutant background could not restore WT resistance (e.g. *wrky33 sid2*, *wrky33 npr1*, *wrky33 wrky70*, *wrky33 gh3.2 gh3.3*, *wrky33 nac061* etc). Furthermore, many genes in ABA-mediated network are WRKY33-repressed targets (green color), thereby forming a more complex WRKY33-ABA regulation network.

Other reports have shown that ABA can function as a repressor of SA-, ET-, and JA/ET-dependent signaling (Asselbergh et al., 2008; Ton et al., 2009). However, our genetic and chemical studies showed that elevated ABA levels in *wrky33* mutants also resulted in concomitant increases in SA, JA and ACC (precursor of ET) levels upon *B. cinerea* 2100 infection, implying a positive effect of ABA on these hormone signaling components during this host-pathogen interaction. Furthermore, molecular analysis of *Botrytis* challenged *wrky33* and *wrky33 nced3 nced5* plants confirmed that ABA only activates the NPR1-dependent SA signaling part while not obviously affecting the EDS1-PAD4 pathway (Figure 2.14). Elevated ABA levels also appeared to activate *ERF1* and *ORA59* expression, two targets in ET/JA signaling (Figure 2.14, 2.15). Interestingly, consistent with reduced SA levels in *wrky33 nced3 nced5* plants, several WRKY33 target genes that are involved both in response to SA and in cell death responses have WT-type like expression levels, indicating a positive effect of ABA on cell death, and this activation may depend on ABA-dependent downstream SA signaling. If this were the case then WRKY33 function would suppress host cell death by repressing part of the ABA signaling network.

How elevated ABA levels activate these hormone signaling components remains to be elucidated. The receptors for ABA are now known (Miyakawa et al., 2013), but how ABA signaling downstream of ABA perception interconnects to the other hormonal pathways requires further investigation. One possibility is that the increased ABA levels triggers the activation of downstream unidentified ABA-response factors that bind to the ABA response elements (ABRE, 'ACGTGG/T') or G-box (CACGTG) present in some target gene promoters resulting in transcriptional activation. Actually, we found some genes including *NAC019, NAC061*, *GH3.2* and *GH3.3* involved in hormone signaling that contain such conserved motifs, which could respond to ABA, and be activated by ABA.

Besides hormonal signaling, TFs such as certain NAC members may also be activated by ABA in the *wrky33* mutant. More recently, it was reported that the NAC transcription factor ATAF1 regulated ABA biosynthesis by directly binding to the *NCED3* promoter (Jensen et al., 2013). In our study we observed increased expression of *NAC002 (ATAF1), NAC019, NAC055, NAC061* and *NAC090*, which may also regulate expression of *NCEDs* or other genes involved in ABA biosynthesis/accumulation in the *wrky33* mutant, and may thereby form a positive feedback regulatory loop to enhance ABA biosynthesis (Figure 2.16). We generated *wrky33 nac061* double mutants and examined their phenotype. Although *wrky33 nac061* did not restore WT resistance towards *B. cinerea*, we found that expression of *NCED3*

was somewhat decreased at 24hpi in double mutants compared with *wrky33* (Figure 2.17). However, *NCED3* expression remained higher than in WT plants, suggesting that other NAC members may be functionally redundant to NAC061 in regulating *NCED3* expression. If this were the case then WRKY33 function may regulate *NCEDs* at multiple levels. Further genetic studies on other NAC mutants (i.e. *NAC019*, *NAC055*, *NAC090*) in combination with *wrky33* may help to uncover part of the WRKY33-dependent regulation of the ABA signaling network.

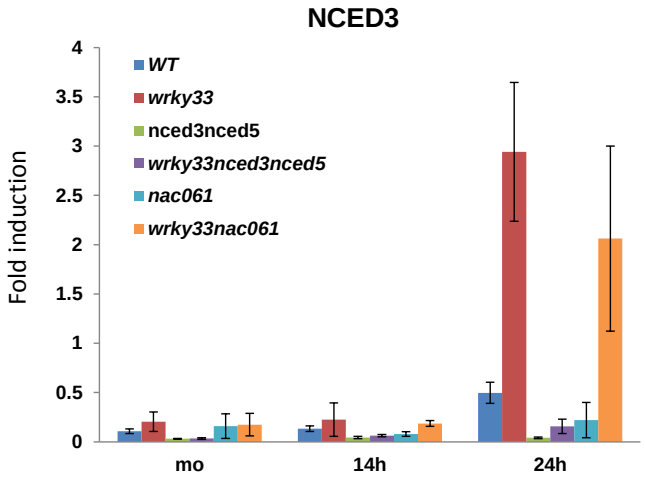


Figure 2.17 Expression levels of *NCED3* in WT, *wrky33*, *nced3 nced5*, *wrky33 nced3 nced5*, *nac061* and *wrky33 nac061* plants determined by qRT-PCR at indicated timepoints after *B. cinerea* 2100 inoculation. Error bars represent SD of three biological replicates (n=3).

2.4.3. Dual activator and repressor role of WRKY33 in regulating distinct functional categories of target genes

WRKY proteins often act either as activators or repressors, and selected members of the family in diverse plant species have been identified as key regulators in diverse plant processes (Rushton et al., 2010). Our data strongly suggest that WRKY33 is a bi-functional transcription factor that can act both as an activator and as a repressor in a promoter-context dependent manner (Figure 2.18).

Earlier studies had shown that WRKY33 positively regulates genes involved in camalexin biosynthesis such as *CYP71A13* and *PAD3* by directly binding to their promoter regions (Mao et al., 2011; Birkenbihl et al., 2012). Mutants of these genes were susceptible to the necrotrophs *A. brassicicola* and *B. cinerea* (Zhou et al., 1999; Nafisi et al., 2007). Next to *CYP71A13* and *PAD3*, we now found that two additional camalexin biosynthetic genes namely, *AMT1* and *CYP71A12*, are also positively regulated and are direct targets of

WRKY33. WRKY33 appears to be directly involved in camalexin accumulation by directly activating several genes within the biosynthetic pathway. In addition, many target genes in the ethylene biosynthesis pathway and ethylene-mediated downstream signaling pathway were activated earlier in WT compared to *wrky33* mutant plants upon *B. cinerea* inoculation implicating WRKY33 as an activator in this process (Figure 2.7, 2.15). Overall, 78 target genes were positively regulated by WRKY33, that is these genes were up-regulated in WT plants but down-regulated in *wrky33* after *B. cinerea* 2100 infection. In contrast, as noted above, WRKY33 also acts as a direct repressor of many genes. WRKY33 repressed expression of 240 target genes associated with different functional categories, e.g. response to SA and JA, involved in ABA regulation network, associated with cell death, and having kinase activity. These genes were all down-regulated in WT plants while up-regulated in *wrky33* after pathogen challenge.

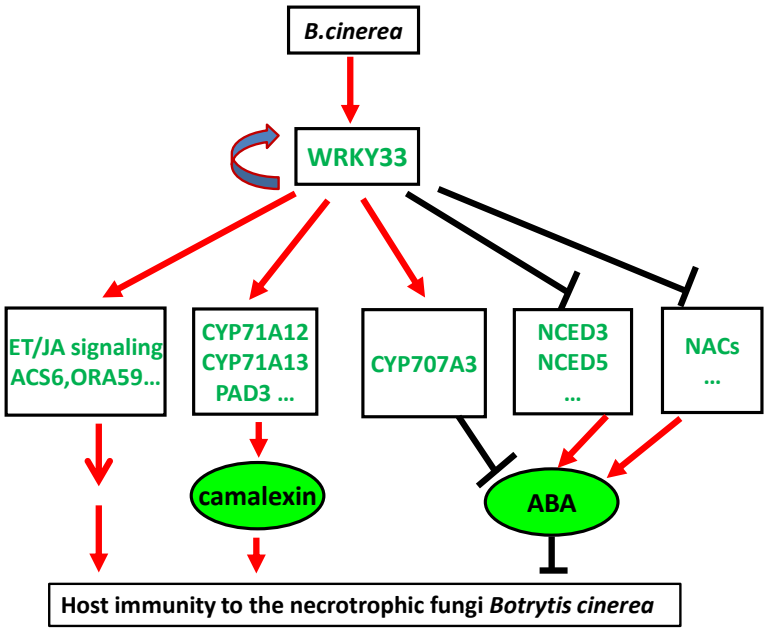


Figure 2.18 Dual activator and repressor role of WRKY33 in modulating host defense to *B. cinerea* 2100. WRKY33 positively regulates target genes involved in camalexin biosynthesis thereby contributing to host resistance towards *B. cinerea* 2100. Moreover, target genes involved in ET/JA signaling and ABA metabolism are also positively regulated by WRKY33. These data represent activator role of WRKY33. On the other hand, WRKY33 acts as a repressor to negatively regulates ABA levels by i.e. directly targeting *NCED3* and *NCED5*. Thus, both activator and repressor functions of WRKY33 appear to be dependent on the promoter context.

In animals and humans, TFs have been described that act as both transcriptional activators and repressors, depending on DNA binding sequences or additional co-activators and co-repressors (Peng and Jahroudi, 2002; Alexandre and Vincent, 2003; Adkins et al., 2006; Sakabe et al., 2012; Zhu et al., 2012). In plants, few factors with dual functions have been

unequivocally identified and characterized. In tomato, the transcriptional activator Pti4 represses the expression of *PR10-10a* by forming a complex with the SEBP repressor (Gonzalez-Lamothe et al., 2008). In *Arabidopsis*, the TF WUSCHEL (WUS) acts mainly as a repressor in stem cell regulation but can function as an activator in regulating *AGAMOUS(AG)* during floral patterning (Ikeda et al., 2009). *Arabidopsis* WRKY53 can activate or repress the expression of genes, depending on the nature of the promoter sequence of its target genes (Miao et al., 2004). Similarly, *WRKY6* activates *PR1* expression while suppressing the expression of its own gene, and that of its closely related family member *WRKY42* by an unknown mechanism (Robatzek and Somssich, 2002). How WRKY33 exerts its dual regulatory functions will be the focus of future research. For this WRKY33-containing transcriptional complexes associated with DNA need to be isolated and WRKY33-interacting proteins identified. In this respect, several WRKY33-interacting proteins containing a VQ motif have recently been discovered that influence defense gene expression (Lai et al., 2011b; Pecher et al., 2014). For two, SIB1 and SIB2, interaction with WRKY33 via their VQ motif was required to stimulate WRKY33 DNA binding activity, and are thought to be positive regulators of WRKY33-mediated resistance to necrotrophic fungi (Lai et al., 2011b). In a second study, the protein MVQ1 was thought to act as a negative regulator of WRKY transcriptional activators including WRKY33. Upon MAMP treatment, degradation of MVQ1 following MAPK-mediated phosphorylation allows WRKY factors to promote transcription of defense genes (Pecher et al., 2014). Interestingly, MVQ1 was also hypothesized to be a positive regulator whose function may be antagonized by interaction with WRKY repressors (Pecher et al., 2014).

Further studies will try to identify and characterize WRKY33 interacting proteins after *B. cinerea* 2100 infection, and to uncover additional regulatory mechanisms governed by WRKY33.

Chapter 3: Comparative analysis of WRKY33-dependent host immunity towards *B. cinerea* strains 2100 and B05.10

3.1. Abstract

WRKY transcription factors are a large family of regulatory proteins involved mainly in plant innate immunity. The *Arabidopsis* transcription factor WRKY33 is a key transcriptional regulator of hormonal and metabolic responses toward *B. cinerea* strain 2100 infection, and is essential for resistance. However, its molecular effects may differ with other *B. cinerea* strains or isolates. In contrast to *B. cinerea* strain 2100, the *B. cinerea* strain B05.10 is virulent on WT Col-0 *Arabidopsis* plants. My comparative studies revealed that *Botrytis*-induced WRKY33 gene expression and protein accumulation are significantly lower in *B. cinerea* B05.10 infected WT plants than in plants challenged with strain 2100. As a consequence of this decrease, the expression levels of the WRKY33 target genes involved in the biosynthesis of the antifungal secondary metabolite camalexin and JA/ET related defense signaling are also clearly reduced after B05.10 infection. Furthermore, the phytohormone ABA negatively influences defense to B05.10 as ABA deficient mutants are resistance to this pathogen and display increased JA/ET levels. Consistent with the results in Chapter 2 and previous work, here I show that *Arabidopsis* WRKY33 also positively modulates host resistance to *B. cinerea* strain B05.10 by positively regulating camalexin biosynthesis and by negatively regulating ABA biosynthesis. Downregulation of ABA biosynthesis positively affects JA/ET defense responses that also contribute to resistance. WRKY33 acts as a potential target for *B. cinerea* virulent strain was further discussed.

3.2. Introduction

Pathogens commonly possess naturally occurring intraspecific variation for traits associated with pathogenicity or virulence (Rowe and Kliebenstein, 2010). The well-studied phytopathogen varieties are the biotrophic pathogen species. The biotrophic pathogen such as *Pseudomonas* exhibits considerable variation in activation or repression of plant defense signaling. In response to pathogens attack, plants have developed sophisticated immune system that enables them to perceive potential invaders. Generally, plant innate immune system consists of two major branches: microbe-/pathogen-associated molecular pattern (MAMP or PAMP) triggered immunity (PTI), and pathogen effector triggered immunity (ETI) (Jones and Dangl, 2006). PTI is activated by receptor-mediated recognition of common MAMPs or PAMPs (Derckel et al., 1999; Jeandet et al., 2002). PTI effectively prevents colonization of plant tissue by most non-adapted potential pathogens (Dodds and Rathjen, 2010). Successful pathogen species such as *Pseudomonas* can deliver effector proteins to plant cells to suppress PTI and promote pathogen virulence. In turn, plants have evolved

resistance (R) proteins to recognize some effectors, and to activate ETI (Dodds and Rathjen, 2010). ETI is an accelerated and intensified defense response towards specific isolates of biotrophic and hemibiotrophic pathogens (Jones and Dangl, 2006). Thus, while the elements of plant defense signaling may be associated with resistance to particular pathogens, pathogen variation in activation, manipulation, and response to plant defense signaling may again alter these associations (Rowe et al., 2010). Currently, specific recognition of necrotrophic pathogens by similar mechanisms has not been documented. With the exception of *Arabidopsis thaliana* RESISTANCE TO LEPTOSPHAERIA MACULANS 3 (RLM3), a Toll/interleukin 1 receptor domain R-protein implicated in broad immunity to several necrotrophs (Boller and Felix, 2009), no R-gene has been specially associated with resistance to necrotrophs such as *B. cinerea*. Plant immunity to *B. cinerea* appears to be under complex genetic control (Rowe and Kliebenstein, 2008).

Large-scale transcriptional reprogramming is usually observed after *B. cinerea* infection and very likely plays a major role in plant defense (Ferrari et al., 2007; Rowe et al., 2010; Mathys et al., 2012; Mulema and Denby, 2012). During the last two decades, studies by global transcriptional profiling have identified thousands of *Arabidopsis* transcripts altered in the host following *B. cinerea* infection, indicating the involvement of key transcription factors (TFs) in this process (AbuQamar et al., 2006; Birkenbihl et al., 2012; Windram et al., 2012). Genetic studies have identified several groups of TF families that affect the outcome of such an interaction, including ERFs (Berrocal-Lobo et al., 2002; Pre et al., 2008; Moffat et al., 2012; Zhao et al., 2012), MYBs (Mengiste et al., 2003; Abuqamar et al., 2009; Ramirez et al., 2011), NACs (Bu et al., 2008; Wang et al., 2009), MYCs (Lorenzo et al., 2004), and WRKYs (Zheng et al., 2006; Pandey and Somssich, 2009; Birkenbihl et al., 2012). These TFs form integral parts of the signaling webs that modulate plant defenses either positively or negatively.

In addition, plant hormones are important components that modulate immune responses (Pieterse et al., 2012). The contribution of hormones to host immunity vary depending on the infection strategy and nutritional requirements of the pathogen (Mengiste, 2012). SA has been traditionally associated with defense against biotrophic and hemibiotrophic pathogens, whereas JA and ET signaling appear to be more important towards necrotrophic pathogens and herbivores (Thomma et al., 1998; Farmer et al., 2003; Glazebrook, 2005; Bari and Jones, 2009; Vlot et al., 2009). The effect of ABA on host immunity appears more complex and displays context-dependent functions (Bari and Jones, 2009). Furthermore, in response to

specific pathogens (microbes) plants can fine-tune the hormone balance by extensive crosstalk between hormonal pathways (Verhage et al., 2010; Pieterse et al., 2014). In response to *B. cinerea* infection, plant hormone levels are commonly enhanced for SA, JA, ET and ABA (Mengiste, 2012). Therefore, hormone responses and hormone homeostasis are vital for proper host immunity. It is therefore not surprising that pathogens can target and thereby interfere in phytohormone pathways through virulent effectors (Kazan and Lyons, 2014).

Many reports have shown that *B. cinerea* produces virulent factors required for successful colonization of the host (Choquer et al., 2007b). This fungus can kill host cells through the production of toxins, generation of reactive oxygen species and the induction of a plant-produced oxidative burst, and additionally feed on different tissue using cell wall-degrading enzymes (CWDEs). The toxins produced by *B. cinerea* during infection include botrydial (Deighton et al., 2001), botcinic acid (Reino et al., 2004; Tani et al., 2006) and their derivatives. During early *B. cinerea* infection stages, hydrogen peroxide (H_2O_2) accumulates that seems partly to be produced by the fungus as gene inactivation of H_2O_2 –generating enzymes in *B. cinerea* reduced such H_2O_2 levels (Rolke et al., 2004; Choquer et al., 2007a). For CWDEs, most are encoded by multigenic families and some may have partly redundant functions (Choquer et al., 2007b). Reported CWDEs as virulent factors include *Bcpme1* (Valette-Collet et al., 2003; Kars et al., 2005a), *Bcpg1* (ten Have et al., 1998), *Bcpg2* (Kars et al., 2005b) and *xyn11A* (Brito et al., 2006). In addition, signal transduction cascades regulating fungal development and virulence are remarkably conserved between distantly related fungi, indicating specific roles in the pathogenicity of some signaling components of *B. cinerea* (Choquer et al., 2007b). Furthermore, *B. cinerea*-derived small RNAs can silence Arabidopsis and tomato genes involved in immunity and also act as effectors to suppress host immunity (Weiberg et al., 2013a; Weiberg et al., 2013b).

However, different strains of *B. cinerea* may have different repertoires of virulence factors. Previous gene inactivation studies demonstrated that the phytotoxin botrydial is a strain-dependent virulence factor (Siewers et al., 2005). It was also reported that *B. cinerea* strains have different capacities to produce the hormone ABA, with some strains being ABA-overproducers (Siewers et al., 2004; Siewers et al., 2006). In addition, some *B. cinerea* isolates produce ethylene (Cristescu et al., 2002). Examples of such naturally occurring intraspecific pathogen variation affecting plant defense against necrotrophs include variation in toxin production by pathogens and variation in pathogen tolerance or detoxification of plant-produced defense compounds (Derckel et al., 1999; Quidde et al., 1999; Jeandet et al.,

2002; Ferrari et al., 2003; Kliebenstein et al., 2005). Thus, compared with many reports on numerous isolates of biotrophic or hemibiotrophic pathogens that show differences in pathogenicity and plant host responses, very little work in the past has focused on fungal variation within the necrotrophic fungi, including *B. cinerea*. Until recently pathogen variation in *Arabidopsis* - *B. cinerea* interaction studies was highly neglected with the majority of experiments performed only with one isolate or the declaration of the employed isolate not even stated (Rowe and Kliebenstein, 2010; Rowe et al., 2010). However, as with some biotrophic pathogens, *B. cinerea* variation recently has been reported to dramatically affect the outcome of an interaction with the plant host (Rowe et al., 2010).

Here, I first tested three *B. cinerea* strains 2100, BMM and B05.10 differed in their pathogenicity on *Arabidopsis* ecotype Col-0 plants. Different symptoms were observed on *Arabidopsis* plants using these *Botrytis* strains, Whereas WT plants were resistant to strains 2100 and BMM, they were susceptible to B05.10. In contrast, *wrky33* mutant plants were susceptible to all three strains. Further comparative analyses of the plant immune response to *Botrytis* focusing on the avirulent strain 2100 and the virulent strain B05.10 were performed.

3.3. Results

3.3.1. *Arabidopsis* Col-0 plants display different responses upon interaction with *B. cinerea* strains

I tested whether Arabidopsis plants showed a similar response to three strains of *Botrytis cinerea*. The *B. cinerea* strain 2100 was originally isolated from *Vicia faba* (CECT, Spanish Type-Culture Collection), while BMM was originally isolated from *Pelargonium zonale* (Zimmerli et al.. 2001), and B05.10 is a benomyl derivative of the strain SAS56 originally isolated from *Vitis* (Quidde et al., 1998).

3 days post droplet infection of *Arabidopsis* WT Col-0 leaves with spores of the *B. cinerea* strains 2100, BMM, and B05.10, the plants displayed a clear difference in lesion phenotypes. Whereas lesion development with strains 2100 and BMM was mild and locally restricted, large expanding lesions were observed with strain B05.10 (Figure 3.1), indicating that WT plants are rather resistant to 2100 and BMM while susceptible to B05.10. The *wrky33* mutant plants however were fully susceptible to all three tested strains as they all showed strong leaf maceration. This indicates the Col-0 resistance to strains 2100 and BMM depends on the functions of the TF WRKY33. Full resistance of WT plants to strain B05.10 could also partly depend on WRKY33 since even larger lesion development was observed on *wrky33* mutant

plants. As the behavior of strains 2100 and BMM are similar on WT plants, and strain B05.10 showed a different phenotype I chose the strains 2100 and B05.10 for further analyses.

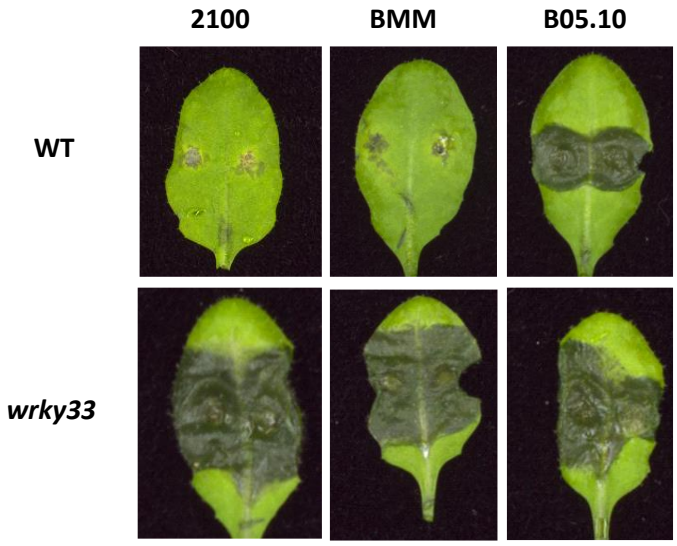


Figure 3.1. Phenotypes observed on Arabidopsis WT Col-0 and *wrky33* plants following droplet inoculations for 3 days with spores of the indicated *B. cinerea* strains.

Using this droplet inoculation assay, I first tested these two strains on various Arabidopsis genotypes to gain initial insights into the potential host pathways required for resistance. As indicated in Table 3.1, WT plant resistance to *B. cinerea* 2100 depends on the production of camalexin, as mutations of genes in the camalexin biosynthetic pathway are susceptible to some necrotroph pathogens (Zhou et al., 1999; Nafisi et al., 2007; Birkenbihl et al., 2012). These mutants include *cyp79b2 cyp79b3*, *cyp71a13,* and *pad3*. Resistance does not appear to depend on the JA/ET pathway, as the selected single or double mutants in JA/ET biosynthesis/signaling are not susceptible. These include *dde2* (*aos1*), *jar1, ein2, dde2 ein2, ora59, erf5, erf6* and *erf5 erf6*. DDE2/AOS1 is involved in JA biosynthesis while JAR1 conjugates JA to the amino acid isoleucine thereby forming JA-IIe, which is the major active JA conjugate in JA signaling (Staswick et al., 2002; Fonseca et al., 2009). EIN2, ORA59, ERF5 and ERF6 are transcription factors involved in ET mediated defense signaling or ET-JA cross-talk (Berrocal-Lobo et al., 2002; Adie et al., 2007; Pre et al., 2008; Moffat et al., 2012). Resistance to 2100 also does not appear to depend on SA signaling, since the selected mutants such as *sid2, pad4, npr1,* and *sid2 pad4* do not alter the resistance phenotype (Table 3.1). SID2 is involved in SA biosynthesis (Garcion et al., 2008) while PAD4 (Jirage et al., 1999) is a key upstream regulator of SA signaling. NPR1 was another key regulator of SA signaling

(Mou et al., 2003). However, compared with *dde2 ein2,* and *sid2 pad4* double mutants, a quadruple mutant, *dde2 ein2 sid2 pad4*, was susceptible to strain 2100, indicating a co-regulation role of SA/JA/ET signaling in resistance to 2100.

Infections with strain B05.10 revealed several differential phenotypes compared to infections with 2100. Most importantly, WT plants are clearly susceptible to this strain as was the *wrky33* mutant (Figure 3.1). Interestingly, mutants in ABA biosynthesis (e.g. *aba2-12, aba3-1* and *nced3 nced5*) altered the WT susceptible phenotype resulting in resistance towards B05.10. Moreover, mutants in JA/ET and SA pathway that remained resistant towards strain 2100 displayed the WT-like susceptible phenotype towards B05.10, while the quadruple mutant *dde2 ein2 sid2 pad4* remained susceptible to B05.10. These results indicate that ABA plays a negative role in plant defense to B05.10, while JA, ET and SA may positively affect host resistance.

As already addressed in chapter 2 and also mentioned above, WRKY33 plays a key regulatory role in WT host resistance to 2100. Susceptibility to strain 2100 is strongly linked to loss-of-WRK33 function as is further illustrated in crosses of the *wrky33* mutant allele with different mutants such as *sid2, npr1, rbohd, wrky25, wrky40, wrky46* and *wrky70*. All of these single mutants are otherwise resistant to strain 2100 (data not shown) whereas all of the double mutants; *wrky33 rbohd, wrky33 sid2, wrky33 npr1, wrky33 wrky25, wrky33 wrky40, wrky33 wrky46* and *wrky33 wrky70,* are clearly susceptible (Table 3.1). All of these double mutants are also susceptible to B05.10, while a triple mutant *wrky33 nced3 nced5*, already described in detail in chapter 2, is resistant to both the strains 2100 and B05.10. The resistance of *wrky33 nced3 nced5* to B05.10 lends further support that suppression of ABA signaling is critical for WRKY33-dependent host immunity.

Table 3.1. The infection phenotypes of indicated *Arabidopsis* mutants in response to *B. cinerea* isolates 2100 and B05.10. (R: resistant; S: susceptible).

pathway	genotypes	*B. c.* 2100	*B. c.* B05.10
	WT	R	S
camalexin	*cyp79b2cyp79b3*	S	S
	cyp71a13	S	S
	pad3	S	S
JA/ET	*dde2 (aos)*	R	S
	jar1	R	S
	ein2	R	S
	dde2ein2	R	S
	erf5	R	S
	erf6	R	S
	erf5erf6	R	S
	ora59	R	S
SA	*sid2*	R	S
	pad4	R	S
	npr1	R	S
	pad4sid2	R	S
JA/ET/SA	*dde2ein2pad4sid2*	S	S
ABA	*aba2-12*	R	R
	aba3-1	R	R
	nced3nced5	R	R
wrky33 associated	*wrky33*	S	S
	wrky33sid2	S	S
	wrky33npr1-1	S	S
	wrky33rbohd	S	S
	wrky33wrky70	S	S
	wrky33wrky40	S	S
	wrky33wrky46	S	S
	wrky33nced3nced5	R	R

3.3.2. *WRKY33* expression and protein levels differ upon infection with *B. cinerea* strains 2100 and B05.10

We have shown that WRKY33 acts as a key transcriptional regulator in plant defense to *B. cinerea* strain 2100. However, WT plants are susceptible to *B. cinerea* strain B05.10 despite a functional *WRKY33* gene. However in the droplet infection assays I consistently noticed that the size of the expanded lesions were larger in the *wrky33* mutants challenged with B05.10 than in WT plants (Figure 3.1), indicating that WRKY33 may also partly contribute to host defense towards strain B05.10.

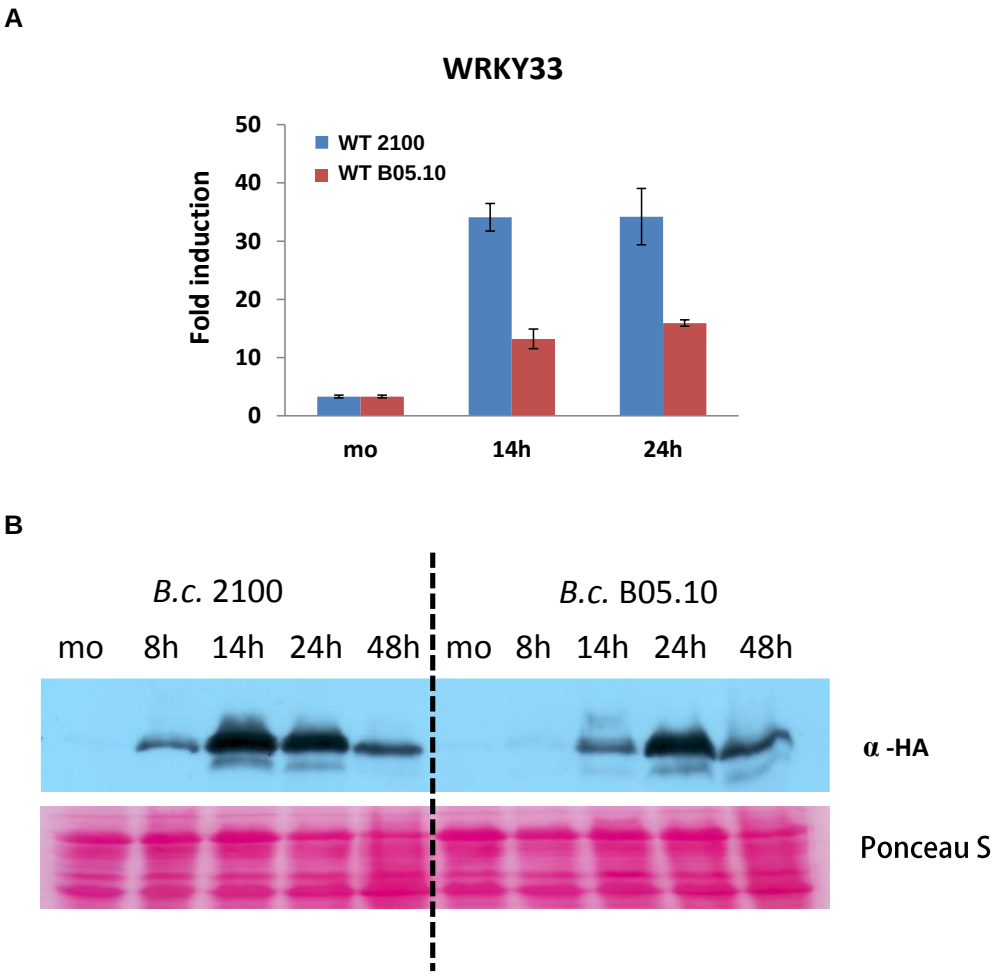


Figure 3.2. Comparative analysis of *WRKY33* transcription and protein accumulation levels after infection with *B. cinerea* strains 2100 and B05.10. (A). Endogenous WRKY33 expression levels analyzed by qRT-PCR in Arabidopsis WT plants at different timepoints after mock treatment (mo) or spray-inoculation with spores of the indicated *B. cinerea* strains. (B). Western-blot analysis of WRKY33-HA protein levels in *pWRKY33*: *WRKY33-HA* transgenic plants (in the *wrky33* background) after mock treatment or spray-inoculation with the indicated *B. cinerea* spores. The critical timepoints where a clear difference in protein levels is observed between plants inoculated with the two fungal strains is marked in red.

To address this, I first tested *WRKY33* gene expression levels after infection with *B. cinerea* strains 2100 and B05.10, respectively. As shown in Figure 3.2A, the induction of *WRKY33* gene expression was detected in WT plant leaves after infection with both strains compared with mock treated leaves. Both strains could induce *WRKY33* expression in WT plants, but there was a clear difference in WRKY33 transcript levels. Transcript levels of WRKY33 were 2- to 3-fold higher in 2100 infected plants compared with B05.10 at both post infection timepoints analyzed. Since no antibody is available to measure endogenous WRKY33 protein levels I used a previously generated WRKY33 complementation line for comparison of protein levels (Birkenbihl et al., 2012). This complementation lines was generated in the background of the *wrky33* mutant and expresses an HA-epitope tagged WRKY33 protein

driven by the WRKY33 native promoter and restored WT-like resistance towards strain 2100. Using an antibody detecting the HA tag, WRKY33 protein levels were assayed on western blots at various times after infection with the two fungal strains (Figure 3.2B). *B. cinerea* strain 2100 could strongly and rapidly induce WRKY33-HA protein accumulation, with the protein observed as early as 8 hours post inoculation. The highest levels of WRKY33-HA were detected at the 14hpi timepoint with substantial amounts still detectable at 24hpi and clearly reduced at 48hpi. In plant leaves inoculated with strain B05.10 WRKY33-HA protein is barely detectable at 8hpi, and is still significantly lower at 14hpi compared to plants infected with strain 2100 (Figure 3.2B). Highest levels of protein are detected at 24hpi (comparable to the levels observed at 14hpi after inoculation with strain 2100). However, at 48hpi reduced protein levels are comparable again between 2100- and B05.10-inoculated plants. Based on these studies the major difference at the protein level was that in 2100 infected plants WRKY33-HA protein accumulated much more rapidly than in B05.10 infected plants. One can hypothesize that *B. cinerea* strain B05.10 may somehow impair the expression of *WRKY33* and thereby partially impair WRKY33 functions at early infection stages.

3.3.3. The expression levels of WRKY33 downstream target genes are reduced in B05.10 infected plants compared to 2100 inoculated plants

Previous reports and the work described in chapter 2 showed that several genes involved in the camalexin biosynthetic pathway were direct targets of WRKY33 (Mao et al., 2011; Birkenbihl et al., 2012). These include *AMT1, CYP71A12, CYP71A13* and *PAD3.* Expression of these genes was analyzed by qRT-PCR following infection of plants with the two fungal strains. As shown in Figure 3.3, both *B. cinerea* strains can induce expression of these genes. However, inoculation with the strain 2100 resulted in higher induced gene expression levels than upon inoculation with the strain B05.10. This lower level of induction by strain B05.10 was consistently observed for all four genes tested at two different infection timepoints. As all these genes are direct WRKY33 activated downstream target genes (see chapter 2), and their reduced expression levels in B05.10-infected plants correlates well with the observed lower induced WRKY33 protein levels (Figure 3.2B).

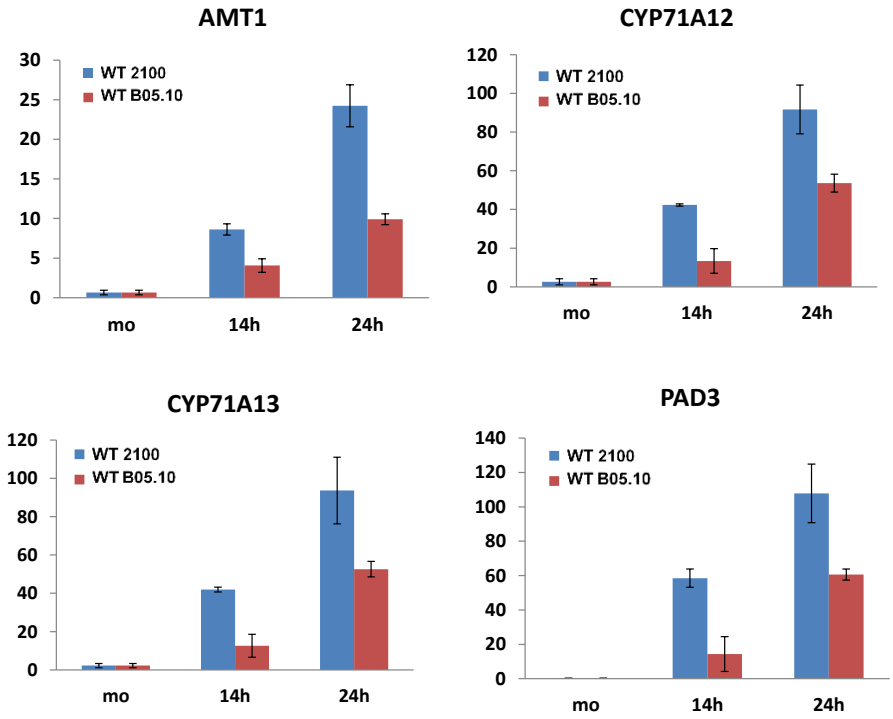
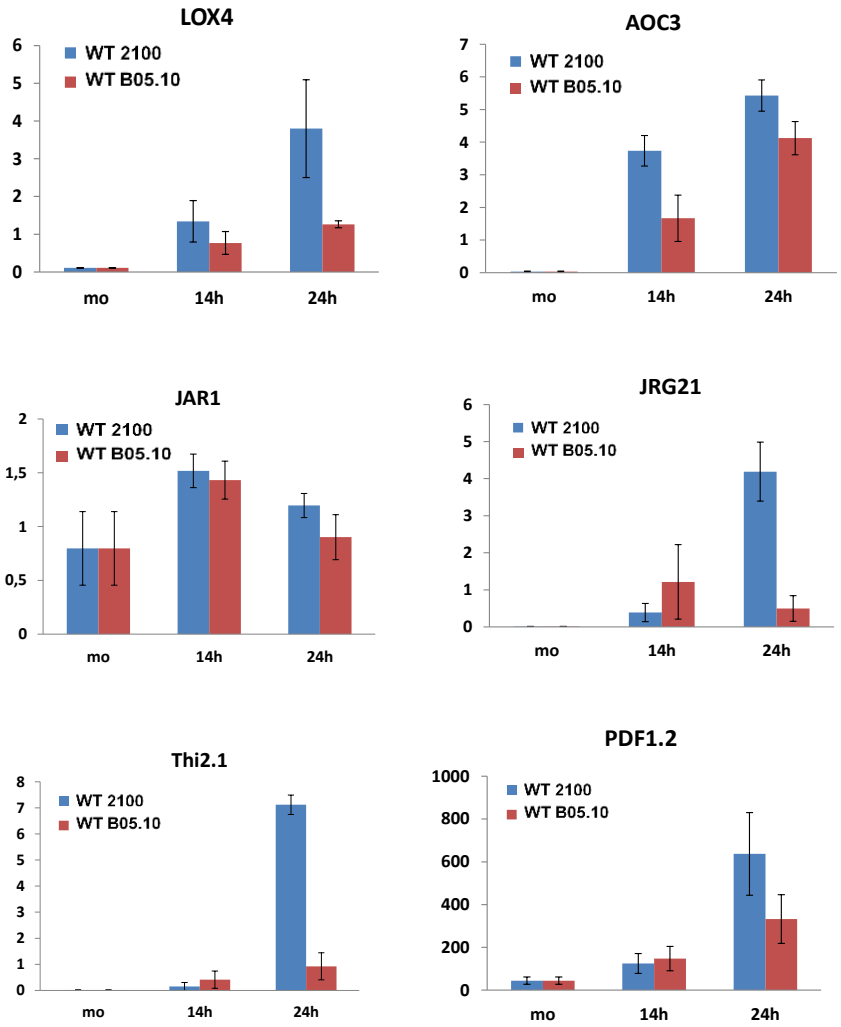


Figure 3.3. **Expression levels of genes involved in camalexin biosynthesis.** *AMT1*, *CYP71A12*, *CYP71A13* and *PAD3* expression levels following infection of Arabidopsis WT plants with *B. cinerea* strains 2100 and B05.10 or after mock treatment (mo) at the indicated timepoints were analyzed by qRT-PCR. All data were normalized to the expression of At4g26410. Error bars represent SD of three biological replicates (n=3).

JA/ET signaling pathways are required for plant resistance against the infection of necrotroph pathogens (Glazebrook, 2005). Our genetic studies indicate that JA/ET signaling may be involved in the Arabidopsis-B05.10 interaction, while JA/ET/SA all appear to be required for resistance to *B. cinerea* strain 2100. qRT-PCR was performed to test if the expression levels of various genes involved in JA or ET biosynthesis or signaling differ upon infection with strains 2100 or B05.10. As shown in Figure 3.4, the induced expression of JA-related genes was detected in Col-0 leaves after infection with both strains compared with mock treated leaves including; *LOX2, LOX4, AOC3, JAR1, JASMONATE REGULATED GENE21* (*JRG21),* the thionin gene *Thi2.1*, and the plant defensin gene *PDF1.2*, while expression levels of *DDE2* and *VSP2* were not induced compared with control plants. *LOX2, LOX4, DDE2* and *AOC3* are involved in JA biosynthesis, while *JAR1* plays an important role in JA signal transduction. *JRG21, Thi2.1, PDF1.2* and *VSP2* respond to JA stimulation and are often used as JA responsive downstream marker genes. Furthermore, *JRG21, Thi2.1* and *PDF1.2* are associated with plant defense to pathogen infections, while *VSP1 (2)* and *LOX2* genes are believed to be associated with wound response (Bu et al., 2008). However, some of these JA-related genes show a lower level of induced expression upon challenge with strain B05.10. These include *LOX4, AOC3, JRG21, Thi2.1* and *PDF1.2*. In contrast, *VSP2* and

LOX2 expression was somewhat higher in plants that were inoculated with strain B05.10 compared to 2100-infected plants. Interestingly, *LOX4*, *AOC3* and *JRG21* are direct targets of WRKY33 (see chapter 2). I also observed induced *MYC2* expression, with somewhat higher levels being observed 14hpi with B05.10 than strain 2100 (Figure 3.4). Previous studies have shown that the transcription factor MYC2 fine tunes early JA signaling by positively regulating wound responses, herbivore defense, oxidative stress tolerance and flavonoid metabolism while negatively regulating expression of pathogen defenses, and secondary metabolism (Dombrecht et al., 2007; Kazan and Manners, 2013). Whether MYC2 is involved or associated with delayed WRKY33 function in B05.10 infected plants remains to be tested.



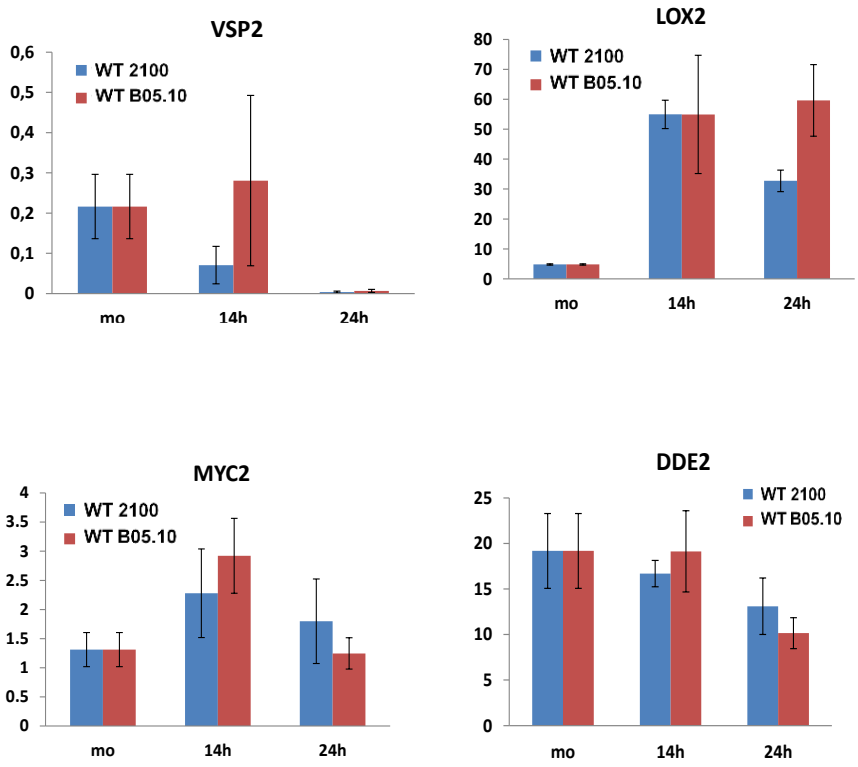


Figure 3.4. **Expression levels of selected JA related genes in Arabidopsis WT plants determined by qRT-PCR at the indicated timepoints after *B. cinerea* 2100 and B05.10 inoculations.** All data were normalized to the expression of At4g26410. Error bars represent SD of three biological replicates.

Expression of several genes in the ET pathway were also observed to be less induced by strain B05.10 compared with 2100, including *ACS2*, *ACS6*, *ERF1* and *ORA59* (Figure 3.5). *ACS2* and *ACS6* are important genes involved in ET biosynthesis after *B. cinerea* infection (Han et al., 2010; Li et al., 2012b). *ERF1* and *ORA59* are AP2/ERF transcription factors and both positively regulate host defense to *B. cinerea* (Lorenzo et al., 2003; Pre et al., 2008). All four of these genes are direct activated downstream targets of WRKY33 (chapter 2). Thus, their lower expression levels in B05.10 infected plants also correlate well with the lower observed WRKY33 protein levels at early infection stages (8-14 hpi; Figure 3.2B). Taken together, these results indicate ET/JA defense signaling is somewhat compromised during B05.10 infection as compared with plants infected with the fungal strain 2100. This reduced response may impair the overall functions of WRKY33 in such plants.

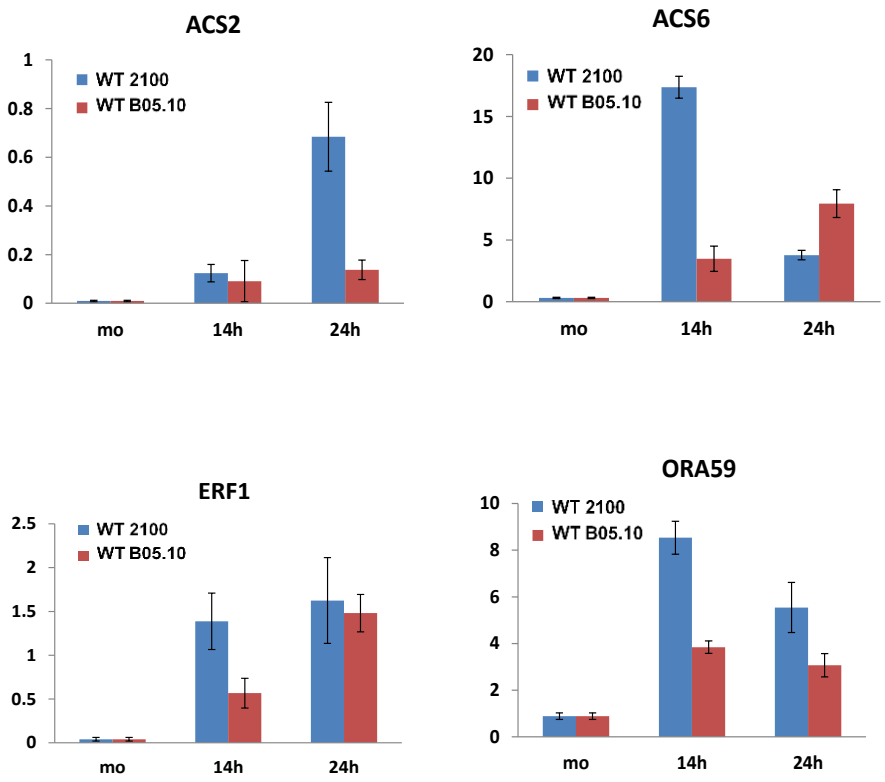


Figure 3.5. Expression analysis of selected ET related genes in Arabidopsis WT plants determined by qRT-PCR at indicated timepoints after *B. cinerea* 2100 and B05.10 inoculations. All data were normalized to the expression of At4g26410. Error bars represent SD of three biological replicates.

Apart from genes related to camalexin biosynthesis and JA/ET responses expression of several genes associated with SA responses (eg. *EDS1*, *PAD4*, *PR1*, *PR2*, *NPR1*, *NPR3* and *NPR4*) and ABA biosynthesis (*NCED3*, *NCED5*, *ABI1*) are also reduced to some degree in B05.10 infected Col-0 plants compared to plants inoculated with strain 2100 at certain timepoints (Figure 3.6, 3.7). Interestingly, *NPR3*, *NCED3* and *NCED5* were also identified as being direct in vivo targets of WRKY33 (see chapter 2) and their lower expression levels also correlate well with the lower levels of WRKY33 protein observed in B05.10 infected plants. Moreover, expression of several other WRKY33 target genes also showed decreased levels in B05.10 infected WT plants such as *HSF4A*, *GH3.2* and *GH3.3* (data not show). Thus, there seems to be a general reduction of the expression levels of numerous genes in plants that are inoculated with the *B. cinerea* strain B05.10 and this reduction appears to correlate well with the overall reduction in WRKY33 protein levels in such infected host leaves.

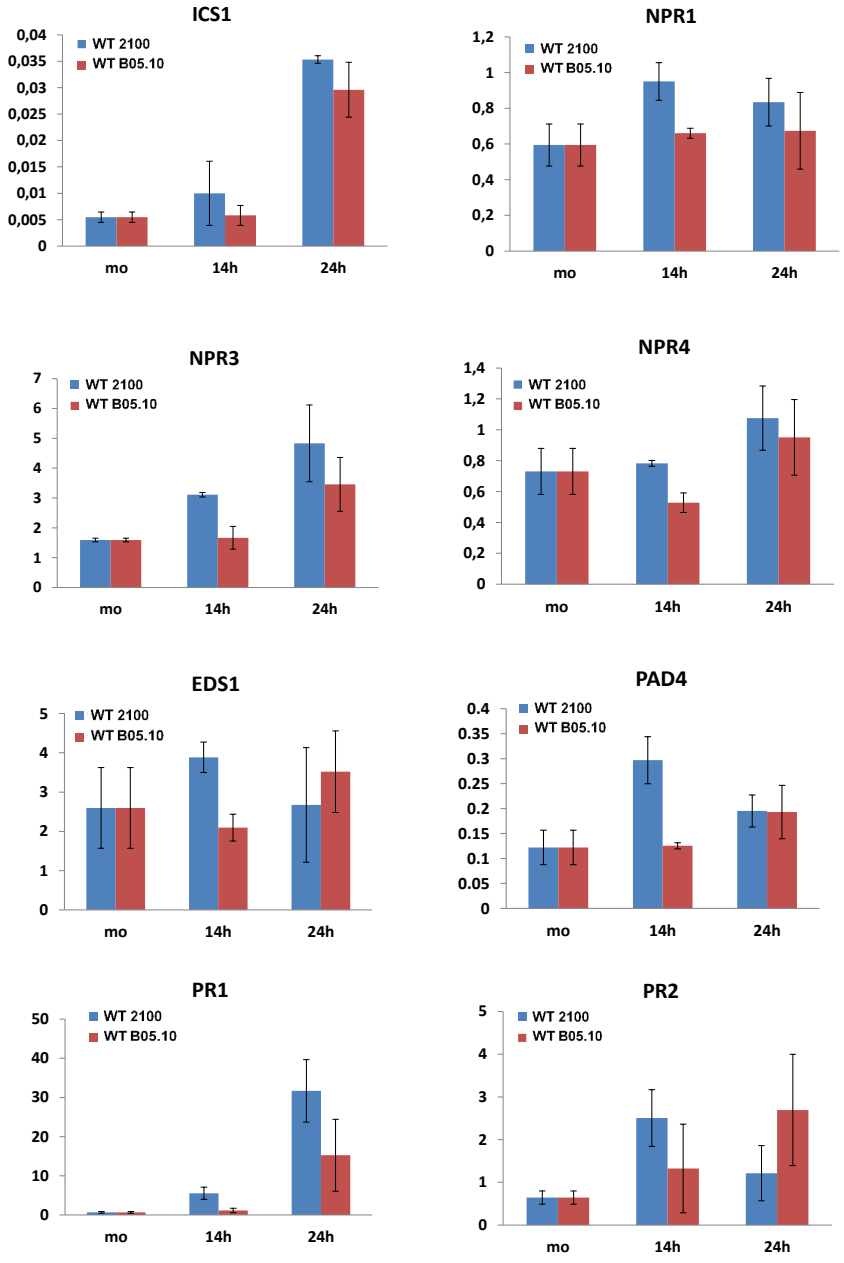
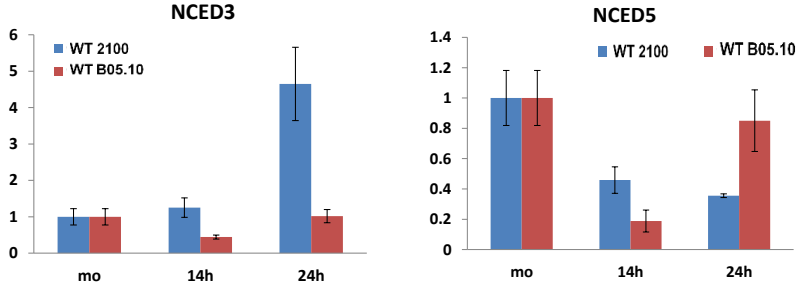


Figure 3.6. Expression analysis of selected SA related genes in Arabidopsis WT plants determined by qRT-PCR at indicated timepoints after B. cinerea 2100 and B05.10 inoculations. All data were normalized to the expression of At4g26410. Error bars represent SD of three biological replicates.



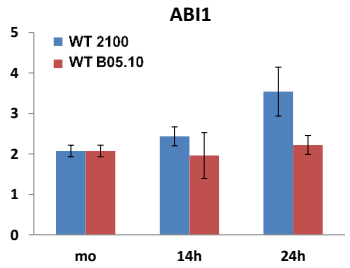
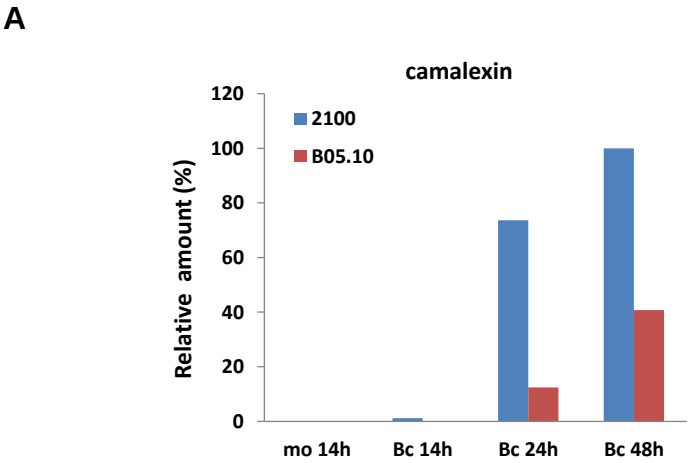


Figure 3.7. Expression analysis of selected ABA related genes and WRKY33-regulated target genes in Arabidopsis WT plants determined by qRT-PCR at indicated timepoints after *B. cinerea* 2100 and B05.10 inoculations. All data were normalized to the expression of At4g26410. Error bars represent SD of three biological replicates.

3.3.4. Plant defense-related hormones and camalexin differentially accumulate in WT plants in response to *B. cinerea* strain 2100 and B05.10 infections

Following the observation of lower expression levels of genes involved in the camalexin biosynthetic pathway in B05.10 infected Arabidopsis leaves, we next examined whether camalexin levels themselves are also affected. As shown in Figure 3.8A, *B. cinerea* strain 2100 and B05.10 challenged plants both accumulate camalexin over the course of 48hpi. However, as was anticipated the relative camalexin levels in strain 2100-infected plants was significantly higher than the levels observed following infection with strain B05.10. In fact, the relative camalexin amounts were about 15-fold higher in 2100 infected plants than in B05.10 infected plants at 14hours, 6-fold at 24, and 2.5-fold at 48 hours, respectively. Camalexin is a toxic compound to fungi including *B. cinerea*. Thus, it is conceivable that one mechanism used by strain B05.10 to achieve virulence is to perturb WRKY33 functions thereby reducing host camalexin levels during the course of infection.



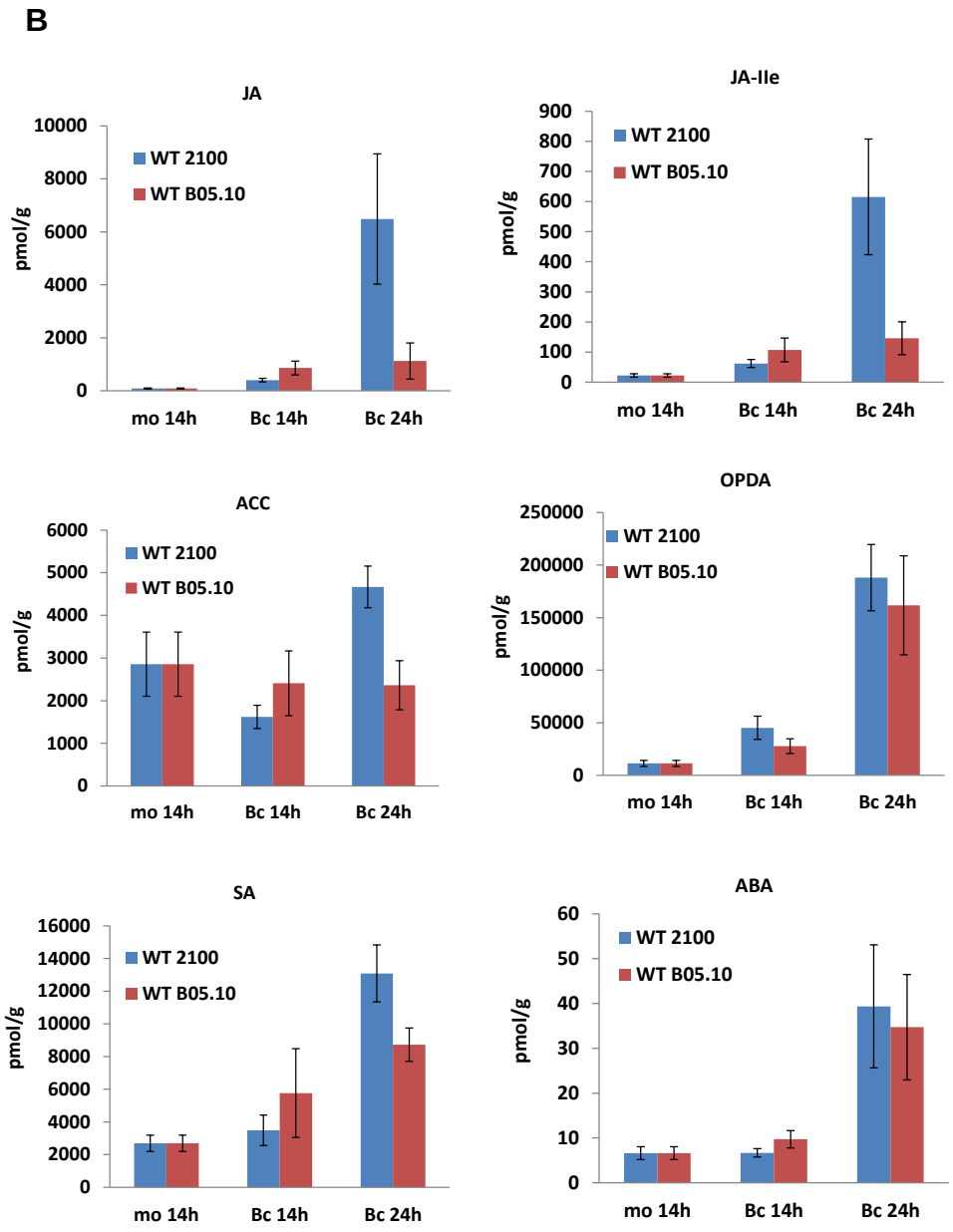
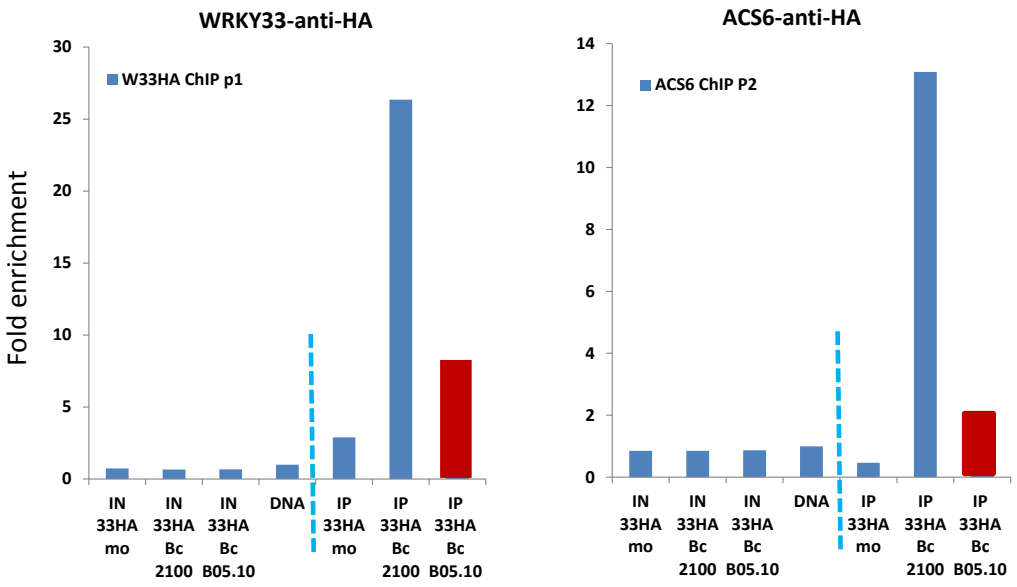


Figure 3.8. Analysis of camalexin and phytohormone levels in Arabidopsis WT plants at indicated timepoints after *B. cinerea* 2100 and B05.10 inoculations. (A). HPLC analysis of the camalexin levels either in mock treated (mo) or spray-infected plants with the indicated *B. cinerea* strains. Sample with the highest camalexin levels was set to 100%, and the relative percentage of the other samples was calculated. Bc = *B. cinerea* infected plants. (B). Altered phytohormone levels in WT plants during infections with *B. cinerea* strains 2100 and B05.10. Concentrations of the indicated phytohormones were measured at 8, 14, 24 and 40 hours after spray inoculation with spores of *B. cinerea* (Bc) or after mock (mo) treatment. Error bars represent SD (n=4). Note: These experiments were collaborated with Dr. Jörg Ziegler (IPB Halle, Germany), and he performed the hormone measurements.

Considering that the expression levels of numerous genes associated to several phytohormone pathways were also differentially regulated by *B. cinerea* strains 2100 and B05.10, we further measured the levels of various hormone including JA, JA-Ile, OPDA, ACC, SA and ABA

after infection with these two strains. As shown in Figure 3.8B, JA and JA-IIe levels increased following inoculation with both strains compared with mock treated plants. However, accumulation of these compounds in leaves dramatically increased at 24 hours following infection with the strain 2100, whereas significantly lower levels were observed in B05.10 infected plants. In contrast no big difference in OPDA was observed between 2100 and B05.10 infected plants. For ACC, a precursor of ET, its levels increased somewhat in 2100 infected leaves after 24hpi, whereas ACC levels remained rather constant and similar to mock-treated control plants in B05.10 infected plants over the time period tested. Host SA levels increased over the course of infections with both tested strains but again, total SA levels remained lower in B05.10 inoculated leaves compared to 2100-infected leaves (Figure 3.8B). For ABA, no significant differences were observed between strain 2100 and strain B05.10 inoculated plants. These results are in good agreement with our expression studies and demonstrate that the levels of camalexin, JA, ET and SA are all reduced in strain B05.10-infected leaves compared to leaves challenged with the strain 2100. As many genes involved in JA/ET signaling are direct WRKY33-induced targets, the virulence of strain B05.10 on Arabidopsis Col-0 plants may be mediated by active suppression of WRKY33 functions by this fungus. Alternatively, infection by B05.10 fails to strongly or rapidly trigger WRKY33-dependent host responses and thereby results in only a weak or delayed plant defense response that is insufficient to retard fungal growth. However at this stage one also cannot exclude that other host responses that are independent of WRKY33 function may be impaired by *B. cinerea* B05.10, or that B05.10 actively produces virulence components that act independently of WRKY33 in plants resulting in host susceptibility.



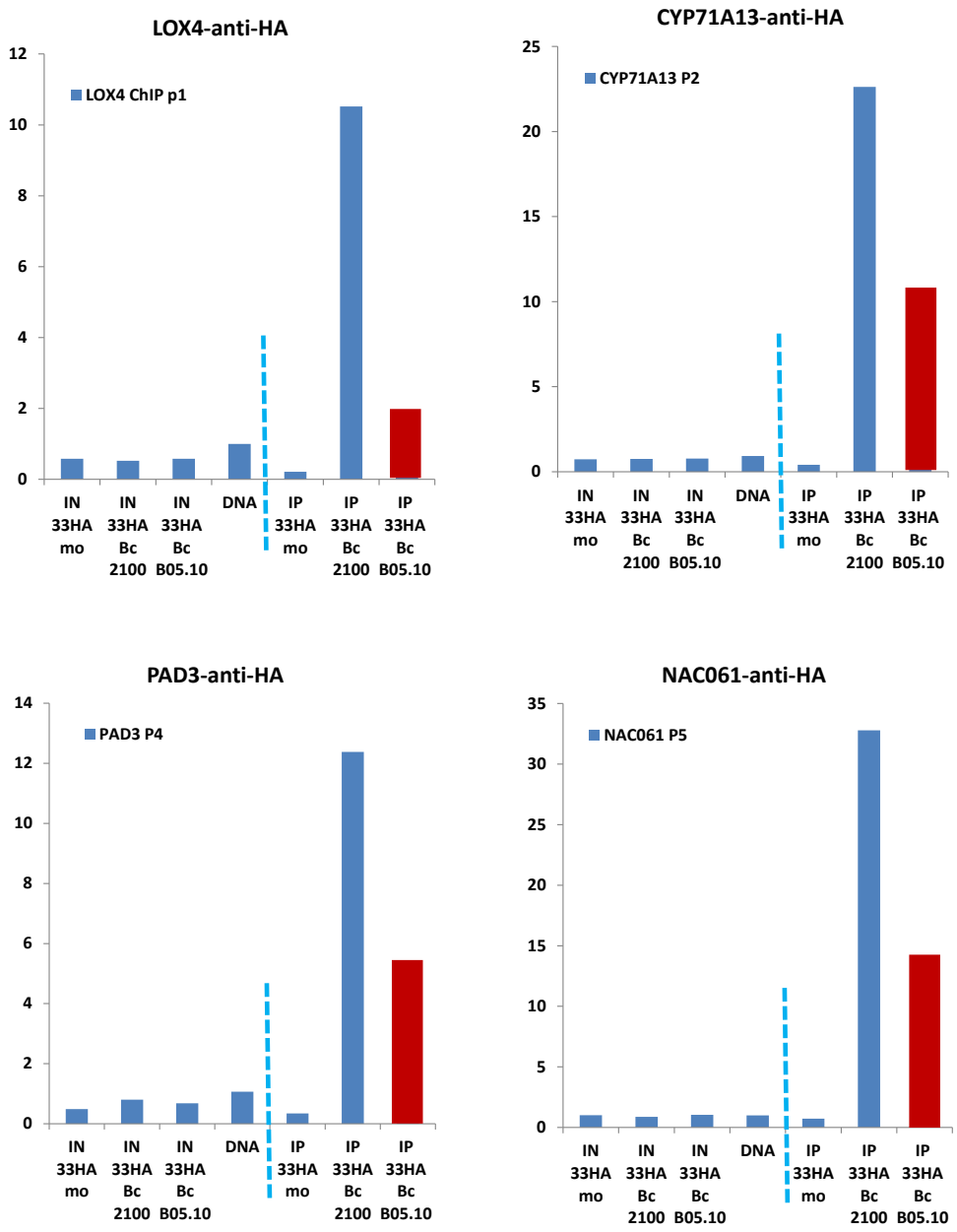


Figure 3.9. Enrichment of WRKY33-HA protein at its target loci upon *B. cinerea* infection. ChIP-qPCR analysis to detect differences in enrichment of WRKY33-HA bound to its selected targets after *B. cinerea* 2100 and B05.10 inoculations for 14hours. Leaves from WRKY33-HA (33HA) plants were spray inoculated with *B. cinerea* (Bc) strains 2100 or B05.10 or mock treated (mo) for 14h. Input DNA before immunoprecipitation (IN) and co-immunoprecipitated DNA using an anti-HA (IP) were analyzed by qPCR employing gene-specific primer pairs, and are expressed as fold enrichment relative to a DNA fragment from At4g26410. As a control for primer efficiency, purified genomic DNA (DNA) was included in the analysis. Each ChIP experiment was repeated at least twice with similar results.

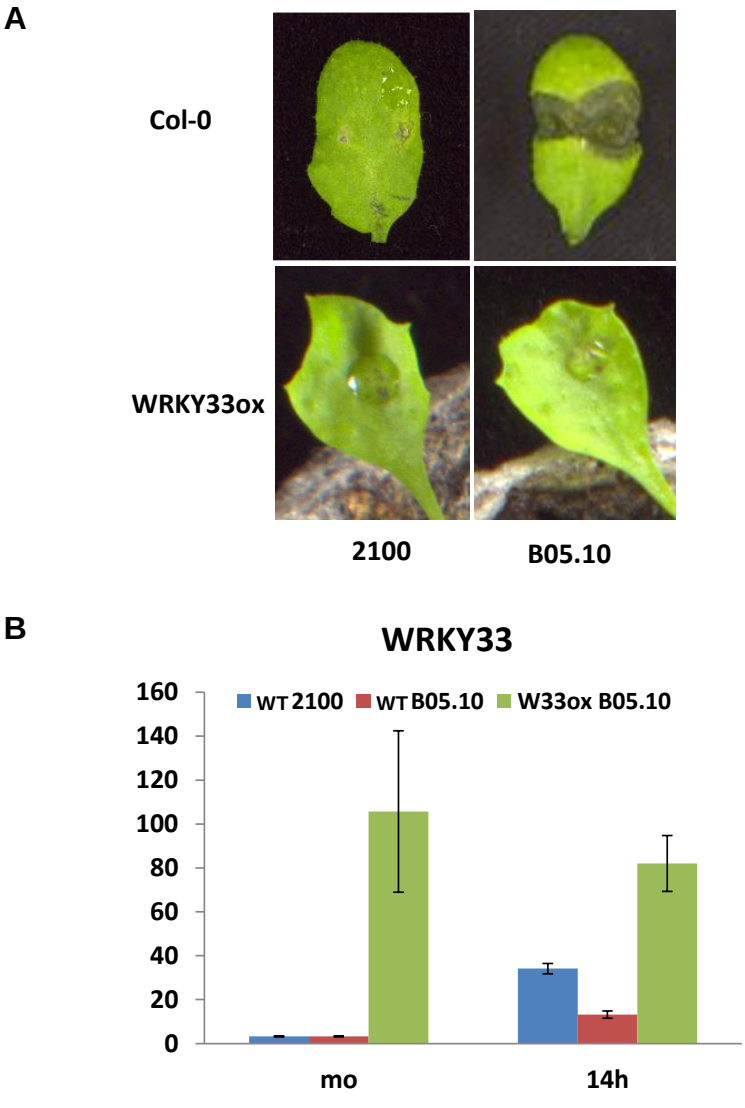
3.3.5. Enrichment of WRKY33 binding at its target genes is reduced upon infection with *B. cinerea* B05.10

As described in the first chapter WRKY33 can directly bind to around ~1500 target genes after infection with *B. cinerea* strain 2100. Since inoculation of leaves with the virulent *B. cinerea* strain B05.10 resulted in significantly lower levels of WRKY33 protein compared to that observed after inoculation with the avirulent strain 2100, I tested whether this reduced WRKY33 levels also affected their binding to the identified target genes. Thus, I performed ChIP-qPCR on the same plant material (*pWRKY33*: *WRKY33-HA* transgene in the background of *wrky33*) that was previously used for ChIP (see chapter 2), and the experiment was carried out over the same time period. Figure 3.9 shows the results of these experiments for a selected set of WRKY33 target genes, including *WRKY33, ACS6, LOX4, CYP71A13, PAD3* and *NAC061*. Consistent with my earlier results specific binding of WRKY33-HA protein was detected at the promoters of the six genes tested. Enrichment of WRKY33-HA at these sites ranged from 11-fold at the *LOX4* locus to 32-fold at the *NAC061* locus, respectively. By comparison, enrichment of WRKY33-HA at all of these loci was significantly reduced in plant material derived from *B. cinerea* strain B05.10 inoculated leaves (Figure 3.9). Whereas in the case of *CYP71A13*, *NAC061*, and *PAD3* WRKY33-HA enrichment is reduced only by about a fold of two, the reduction is more pronounced at the *WRKY33* (4-fold), *LOX4* (5.5-fold), and *ACS6* (6.5-fold) loci. This shows that the reduced levels of total WRKY33-HA observed upon infection with B05.10 (Figure 3.2) also results in the significantly lower amounts of WRKY33 protein bound to its target genes. Probably this may explain why expression of these genes are lower in B05.10 infected Col-0 plants compared with strain 2100.

3.3.6. Plants constitutively expressing *WRKY33* are resistant to *B. cinerea* strain B05.10

The comparative analysis between Arabidopsis leaves challenged by *B. cinerea* strains 2100 and B05.10 described above indicated that virulence of strain B05.10 may be the result of lower accumulation of WRKY33 protein at early infection stage thereby leading to lower WRKY33-dependent host defense responses. To test this hypothesis I analyzed plants that constitutively expressed WRKY33 under the control of the *35S CaMV* promoter (Birkenbihl et al., 2012) with respect to their resistance toward the fungal strain B05.10. We tested the phenotype of a *WRKY33-HA* overexpression line (*WRKY33ox*), in which *WRKY33* expression was driven by the 35S-promoter, and where constitutive levels of WRKY33 are expected. These plants did show some altered phenotypes namely they were smaller than WT and

showed altered leaf morphology (Figure 3.10A). Nevertheless, droplet inoculations with spores of the two *B. cinerea* strains revealed that these *WRKY33ox* plants were equally resistant towards both strains 2100 and B05.10, while the control WT plants were resistant to strain 2100 but susceptible to strain B05.10 (Figure 3.10A). Expression analysis showed that *WRKY33* was constitutively expressed in the *WRKY33ox* lines even in the absence of the pathogen (mo; Figure 3.10B). In *Botrytis* treated plants, constitutive expression levels of *WRKY33* were much higher in the *WRKY33ox* plants than in WT plants inoculated with spores of the strains B05.10 and 2100 for 14h (6-fold and 2.5-fold higher, respectively). The WRKY33 protein levels in the *WRKY33ox* plants showed no major differences in the leaves challenged by the two strains over the time period tested (Figure 3.10C), but was significantly higher than in challenged WT leaves (Figure 3.2B). One can conclude from these results that elevated expression of *WRKY33* can render Arabidopsis WT Col-0 plants resistant to the otherwise virulent *B. cinerea* strain B05.10.



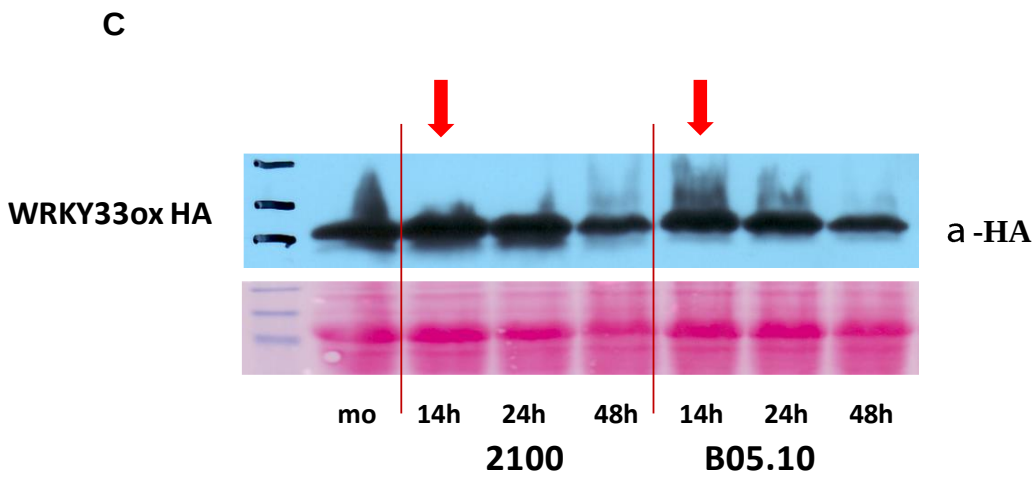
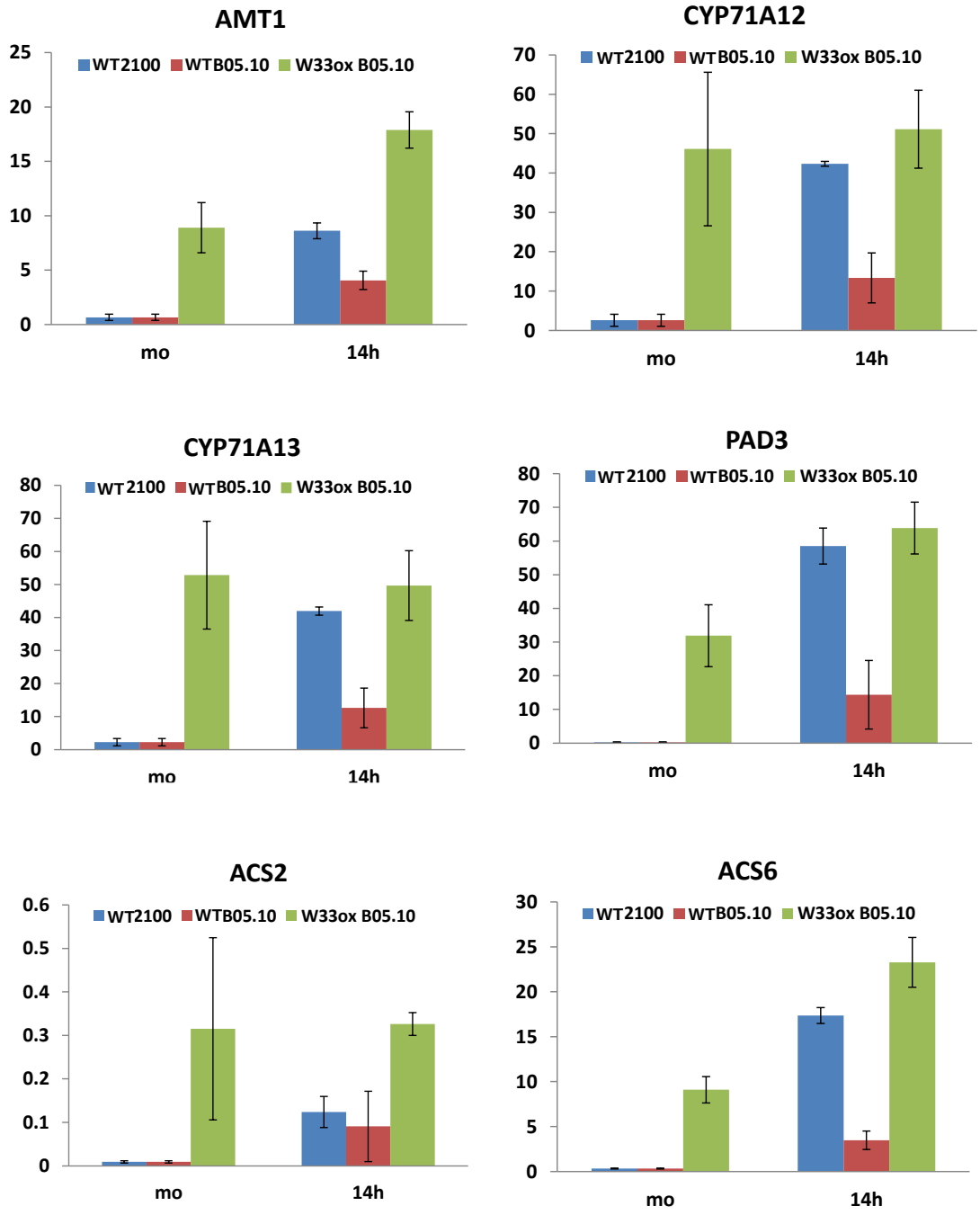


Figure 3.10. ***WRKY33ox* plants are resistant to both strains of *B. cinerea*.** (A). Phenotype of *Arabidopsis* WT and *WRKY33ox* plants to the indicated *B. cinerea* strains after droplet inoculations for 3 days. (B). *WRKY33* expression levels analyzed by q-PCR in WT plants at 14h post spray-inoculation with indicated *B. cinerea* strains or upon mock treatment (mo). (C). Western-blot analysis WRKY33-HA protein levels in *WRKY33ox* plants after mock treatment or spray-inoculation with indicated *B. cinerea* spores at indicated timepoints. Red arrows indicate WRKY33 protein level has no major difference in the *WRKY33ox* leaves challenged by the two strains at early time point (14hpi).

3.3.7. Expression of WRKY33-regulated target genes reach higher levels in the *WRKY33ox* line compared to WT plants after inoculation with B05.10

As noted above, many of the tested WKRY33-target genes showed significantly lower expression levels in B05.10 infected WT Col-0 plants compared to inoculation with strain 2100 (Figures 3.3 - 3.7). Since elevated WRKY33 expression levels lead to resistance of WT plants to strain B05.10 I tested whether expression of WRKY33 downstream targets are also affected. I performed qRT-PCR analysis on resistant *WRKY33ox* plants, at 14hpi. Indeed, many of the WRKY33-target genes showed increased expression level in B05.10 challenged *WRKY33ox* plants compared with B05.10-inoculated WT plants (Figure 3.11). Among the genes affected were: *AMT1*, *CYP71A12*, *CYP71A13*, *PAD3*, *ACS2*, *ACS6*, *AOC3* and *ERF1*. As mentioned previously, these genes are involved in the camalexin and ET/JA biosynthetic pathways, respectively, and their expression is directly activated by WRKY33 after *B. cinerea* 2100 infection. The increased expression levels of these genes in *WRKY33ox* plants correlates well with the elevated WRYK33 protein levels observed in these plants. In fact, expression levels of these genes were already found to be increased in the mock control (Figure 3.11; mo), consistent with the finding that they are directly activated WRKY33 targets, which upon ectopic WRKY33 expression, do not appear to require the pathogen infection for their induction. However, for some genes such as AMT1, PAD3 and ACS6 stimulation by the

pathogen may also be required for maximal expression. Taken together, elevated levels of WRKY33-HA in *WRKY33ox* plants results in increased expression levels of WRKY33 target genes, and this may be causal for the resistance observed in these plants towards the *B. cinerea* strain B05.10.



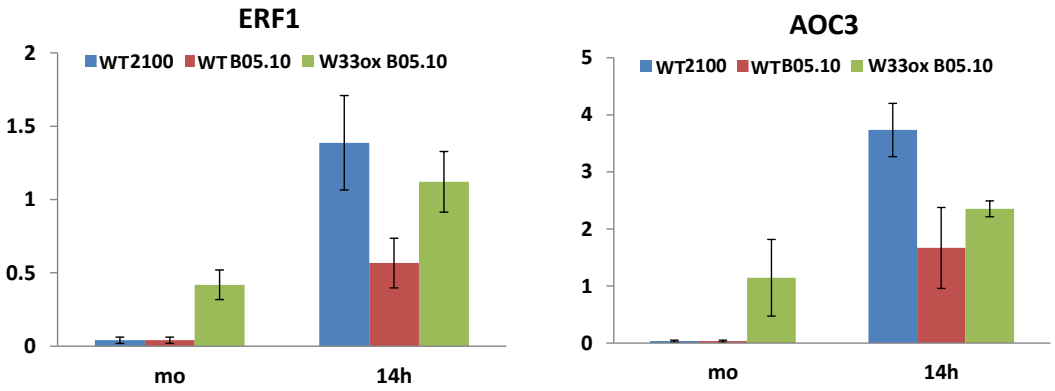


Figure 3. 11. **Elevated expression levels of WRKY33 target genes are detected in *WRKY33ox* plants.** qRT-PCR expression analysis of selected WRKY33 target genes involved in camalexin biosynthesis and JA/ET signaling in WT and *WRKY33ox* (W33ox) plants at 14hours after *B. cinerea* 2100 and B05.10 inoculations. All data were normalized to the expression of At4g26410. Error bars represent SD of three biological replicates (n=3).

3.3.8. ABA deficient mutants are resistant to *B. cinerea* strain B05.10, and elevated constitutive expression levels of *WRKY33* is observed *in nced3 nced5* compared to wild-type plants.

In the experiments described in the chapter 2, I showed that the phytohormone ABA negatively regulates WRKY33-dependent host resistance to *B. cinerea* strain 2100 (*wrky33* mutant was susceptible, while the *wrky33 nced3 nced5* triple mutant was resistant). Previous reports in Arabidopsis have indicated the existence of an antagonistic interaction between the phytohormone ABA and JA/ET (Anderson et al., 2004). Since I observed reduced JA/ET levels in B05.10 challenged WT plants (Figure 3.8B), I hypothesized that ABA might also be involved in the interaction with strain B05.10. ABA deficient mutants such as *aba2*, *aba3*, and *nced3 nced5* were used to test B05.10 infection phenotypes. As shown in Figure 3.12, restricted lesion size is observed on leaves of these mutants in contrast to the extended lesions seen on WT and *wrky33* mutant leaves. In addition, consistent with the conclusions drawn in chapter 2 that WRKY33-dependent defenses are negatively regulated via ABA signaling, the *wrky33 nced3 nced5* triple mutant also showed a strong resistance phenotype towards strain B05.10 (Figure 3.12). This strongly indicates that ABA also negatively influences resistance to *B. cinerea* B05.10.

I also monitored the expression level of *WRKY33* in *nced3 nced5* plants and observed that its expression was higher than in WT plants even without pathogen inoculation (mock; Figure

3.13), indicating that ABA signaling may be partly involved in repressing basal expression of *WRKY33*.

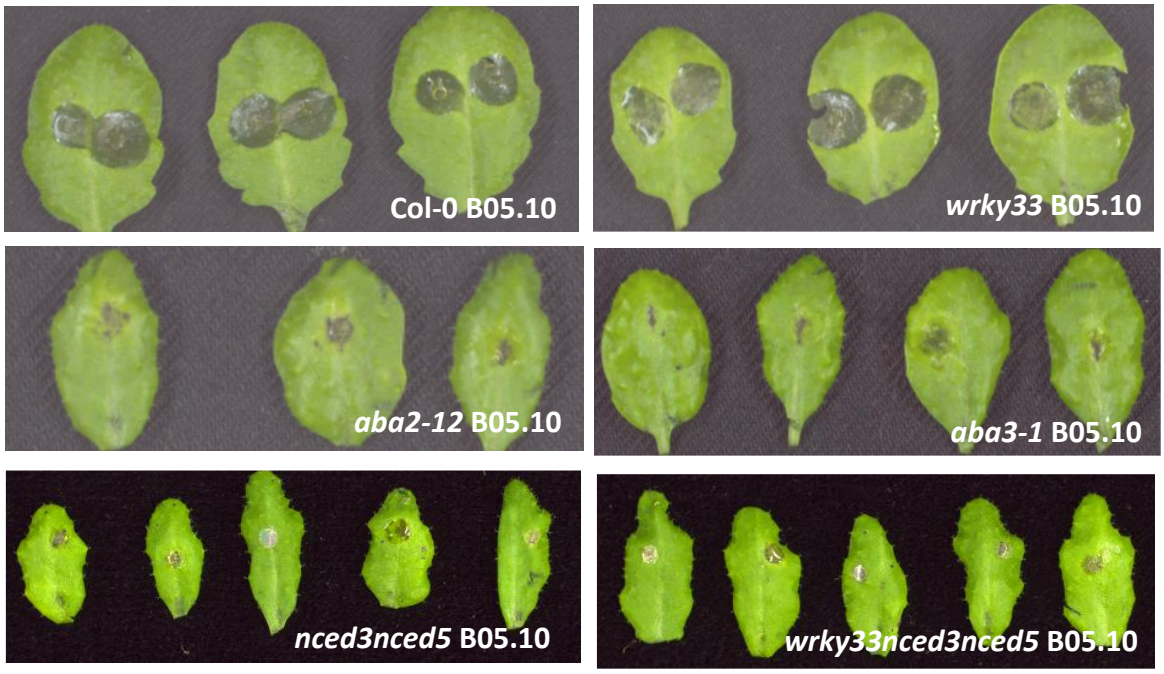


Figure 3. 12. ***B. cinerea*** **B05.10 infection phenotypes (3 days post treatment) of WT, *wrky33, aba2-12, aba3-1, nced3 nced5* and *wrky33 nced3 nced5* plants.** 2 µL droplets containing 2.5x10^5 spores were applied to leaves of intact plants of the designated genotypes.

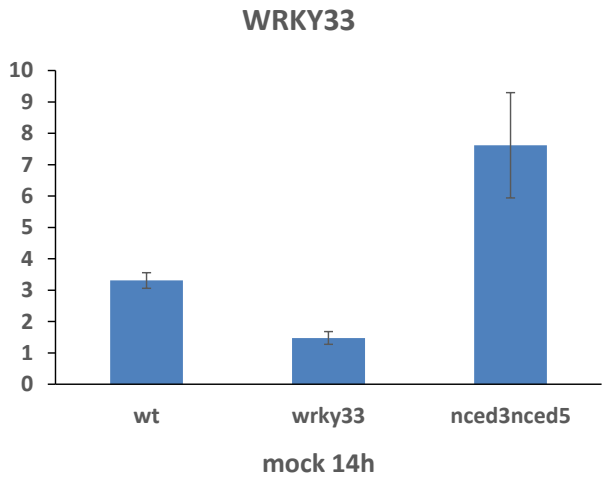


Figure 3.13. Basal expression levels of *WRKY33* are elevated in *nced3 nced5* mutant plants. qRT-PCR expression analysis of WRKY33 in WT, *wrky33, nced3nced5, aba2-12* and *aba3-1* plants (mock treated).

3.3.9. In B05.10 challenged *nced3 nced5* mutants JA/ET hormone levels are elevated compared to inoculated WT plants

Since ABA appears to negatively influence host resistance to *B. cinerea*, and *nced3 nced5* mutants are resistant to the strain B05.10, we further measured hormone levels after B05.10

infection. As shown in Figure 3.14, ABA levels increased in WT plants after B05.10 infection at 40 hours indicating that B05.10 induced ABA levels. As anticipated, little ABA accumulation was observed in *nced3 nced5* plants. In addition, accumulation of JA, JA-Ile, OPDA and ACC highly increased in B05.10 challenged *nced3 nced5* plants compared with WT. In contrast, SA accumulation did not significantly differ between B05.10 infected WT and *nced3 nced5* plants, only slightly increased at early stage (14hpi). These data are consistent with previous reports that ABA antagonizes JA/ET signaling and that B05.10 resistance of *nced3 nced5* plants may be due to increased JA/ET levels and thus enhanced JA/ET signaling.

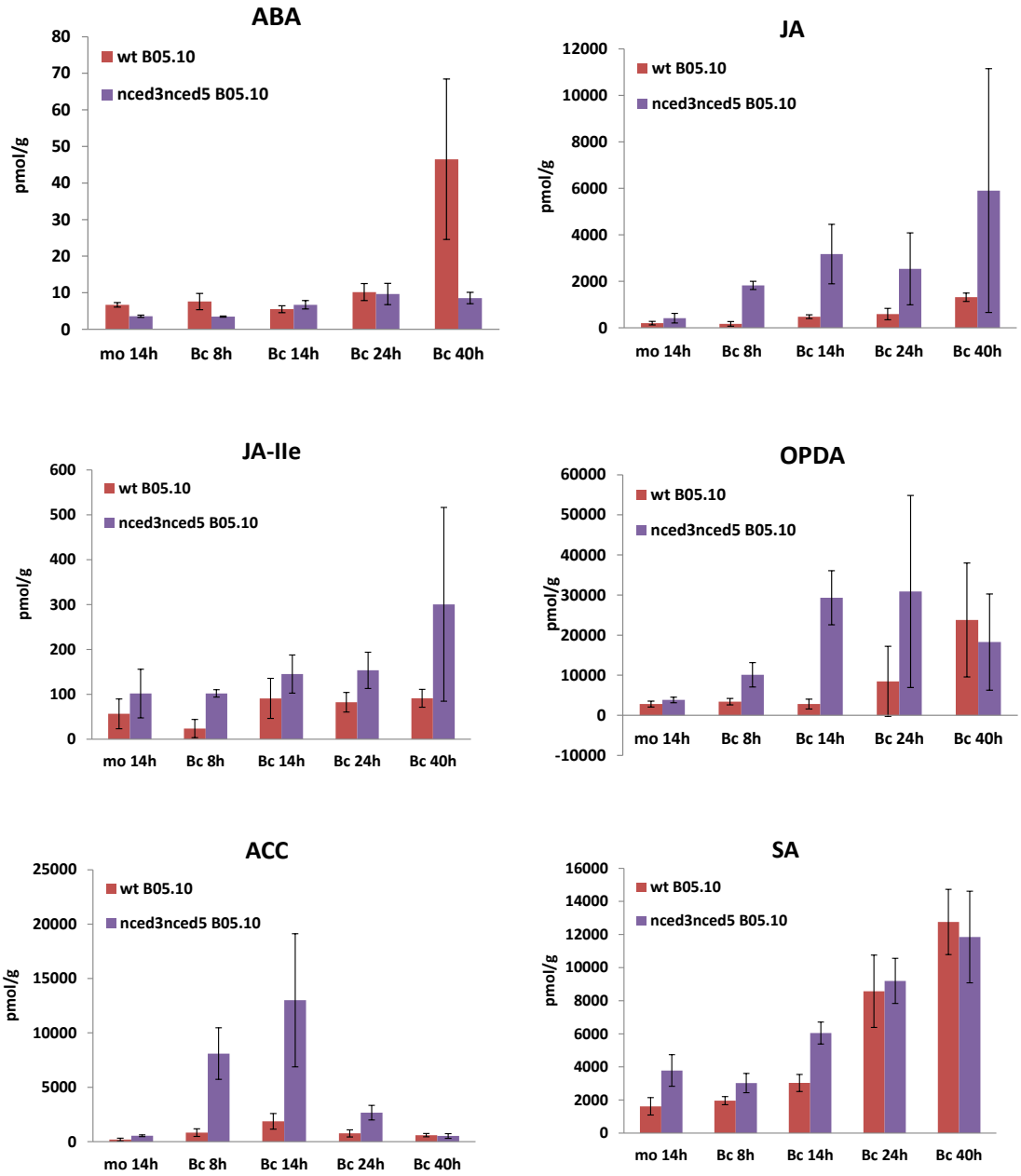
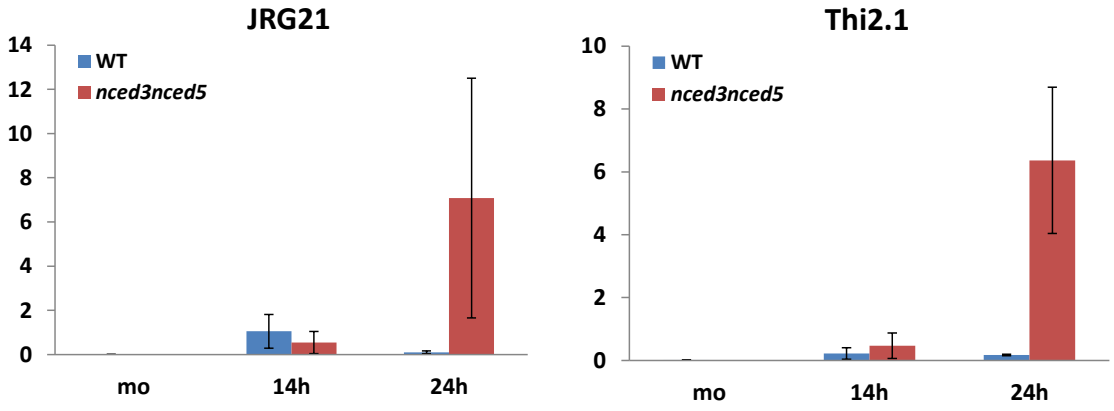


Figure 3.14. Altered hormone levels in different Arabidopsis genotypes during *B. cinerea* B05.10 infection. Concentrations of the indicated hormones were measured at 8, 14, 24 and 40hpi in WT and *nced3 nced5* plants spray-inoculated with spores of *B. cinerea* B05.10 (Bc) or mock (mo) treated. Error bars represent SD (n=4). Note: These experiments were performed by Dr. Jörg Ziegler (IPB Halle, Germany).

3.3.10. Expression of defense related genes in B05.10 challenged *nced3 nced5* plants

Since *nced3 nced5* mutant plants are resistant to *B. cinerea* strain B05.10 and plant defense related hormone levels are altered, I next examined the expression of several defense related genes. As shown in Figure 3.15, *JRG21* and *Thi2.1*, two genes acting downstream in JA signaling, as well as the ET biosynthesis-associated gene *ACS2*, are dramatically elevated in the *nced3 nced5* mutant compared to WT plants. This indicates that ABA represses downstream JA responses and ET biosynthesis. This is also consistent with increased levels of JA, JA-Ile and ACC observed in *nced3 nced5* upon pathogen infection. However, expression of *ORA59*, *ERF1* and *PDF1.2* are not dramatically increased in B05.10 challenged *nced3 nced5* plants compared with WT. In addition, expression of the SA biosynthetic gene *ICS1*, and *PR1*, a gene acting downstream in SA signaling, showed elevated levels compared to WT while *NPR1* (data not shown) expression was not altered between n*ced3 nced5* and WT plants. Increased expression of *ICS1* is in agreement with the elevated SA level detected in *nced3 nced5* plants at least at early infection stages (14hpi).

Interestingly, the elevated expression levels of *JRG21* and *Thi2.1* in *nced3 nced5* plants upon B05.10 infection appear to depend on WRKY33 function, as can be concluded from their dramatic decreases in expression in *nced3 nced5 wrky33* triple mutant plants (Figure 3.16), suggesting a role of ABA in negatively affecting WRKY33 functions to antagonize JA/ET signaling in WT plants upon B05.10 infection.



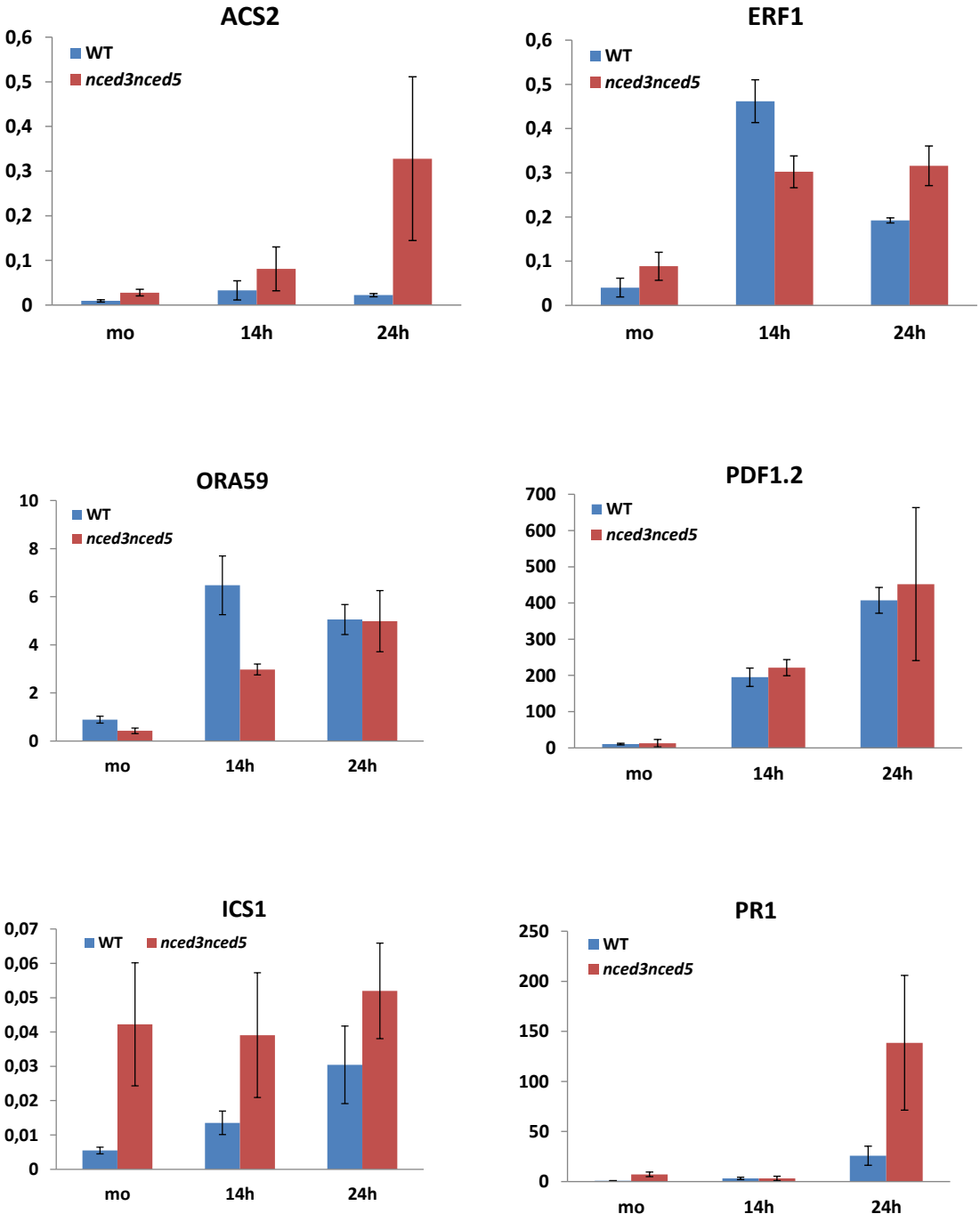


Figure 3.15. **Expression analysis of selected genes associated with phytohormone responses.** Expression of genes in WT and *nced3 nced5* plants determined by qRT-PCR at indicated timepoints after *B. cinerea* B05.10 spray inoculation. All data were normalized to the expression of At4g26410. Error bars represent SD of three biological replicates (n=3).

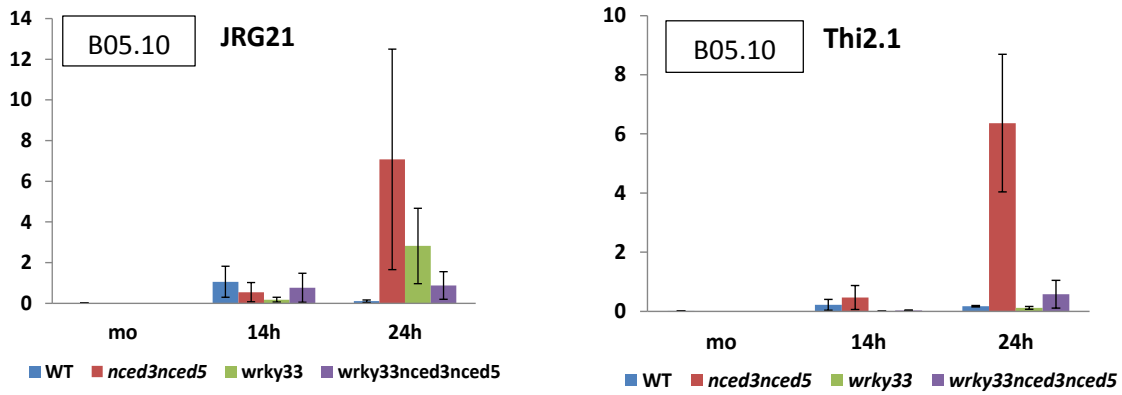


Figure 3.16. Expression levels of the JA inducible genes *JRG21* and *Thi2.1*, in WT, *wrky33*, *nced3 nced5* and *wrky33 nced3 nced5* plants determined by qRT-PCR at indicated timepoints after *B. cinerea* B05.10 spore spray-inoculation. Error bars represent SD of three biological replicates (n=3). All data were normalized to the expression of At4g26410.

3.3.11. Mutations in ABA biosynthesis genes present in *B. cinerea* strain B05.10 do not alter strain virulence

As observed above, the ABA deficient mutants of *Arabidopsis* are resistance to *B. cinerea* strain B05.10, supporting a role of this phytohormone in host susceptibility to B05.10. Previous reports indicate the existance of an ABA gene cluster in *B. cinerea*, that include the genes *bcaba1*, *bcaba2*, *baaba3* and *bcaba4* (Siewers et al., 2006). Targeted inactivation of these genes proved the involvement of BcABA1, BcABA2 and BcABA3 in ABA biosynthesis and indicated that BcABA4 may also contribute (Siewers et al., 2004; Siewers et al., 2006). The *B. cinerea* strain B05.10 contains such a gene cluster, and low expression of *bcaba1* and *bcaba2* in axenic culture was observed (Siewers et al., 2006). However, B05.10 does not produce ABA in axenic culture. Nevertheless, no information is available on whether B05.10 can produce ABA during colonization of host plants. To test if virulence of this strain on Arabidopsis Col-0 plants may be due to a contribution of additional ABA via this fungal gene cluster I infected WT and *nced3 nced5* plants with wild-type *B. cinerea* strain B05.10 or with two independent mutant lines each for the *B. cinerea ABA1* gene (B05.10:Δ*bcaba1* lines T7 and T10) or the *B. cinerea ABA3* (B05.10:Δ*bcaba3* lines T14 and T22), respectively. As shown in Figure 3.17, WT plants are resistance to strain 2100 while being susceptible to the wild-type strain B05.10. However, none of the *B. cinerea* B05.10 ABA mutant lines showed alterations in host susceptibility compared to the wild-type B05.10 strain. The *wrky33* mutant plants always showed susceptibility to all tested strains (data not show), while *nced3 nced5*

plants were resistant to all strains (Figure 3. 18). This indicates that *B. cinerea* B05.10-derived ABA does not directly contribute to virulence during infection, or that this strain does not produce ABA during the course of infection. Irrespective of these two possibilities one can conclude that resistance of *nced3 nced5* plants to B05.10 is very likely due to reduced endogenous ABA. Thus, the phytohormone ABA negatively regulates host defense to B05.10 and is sufficient to promote disease.

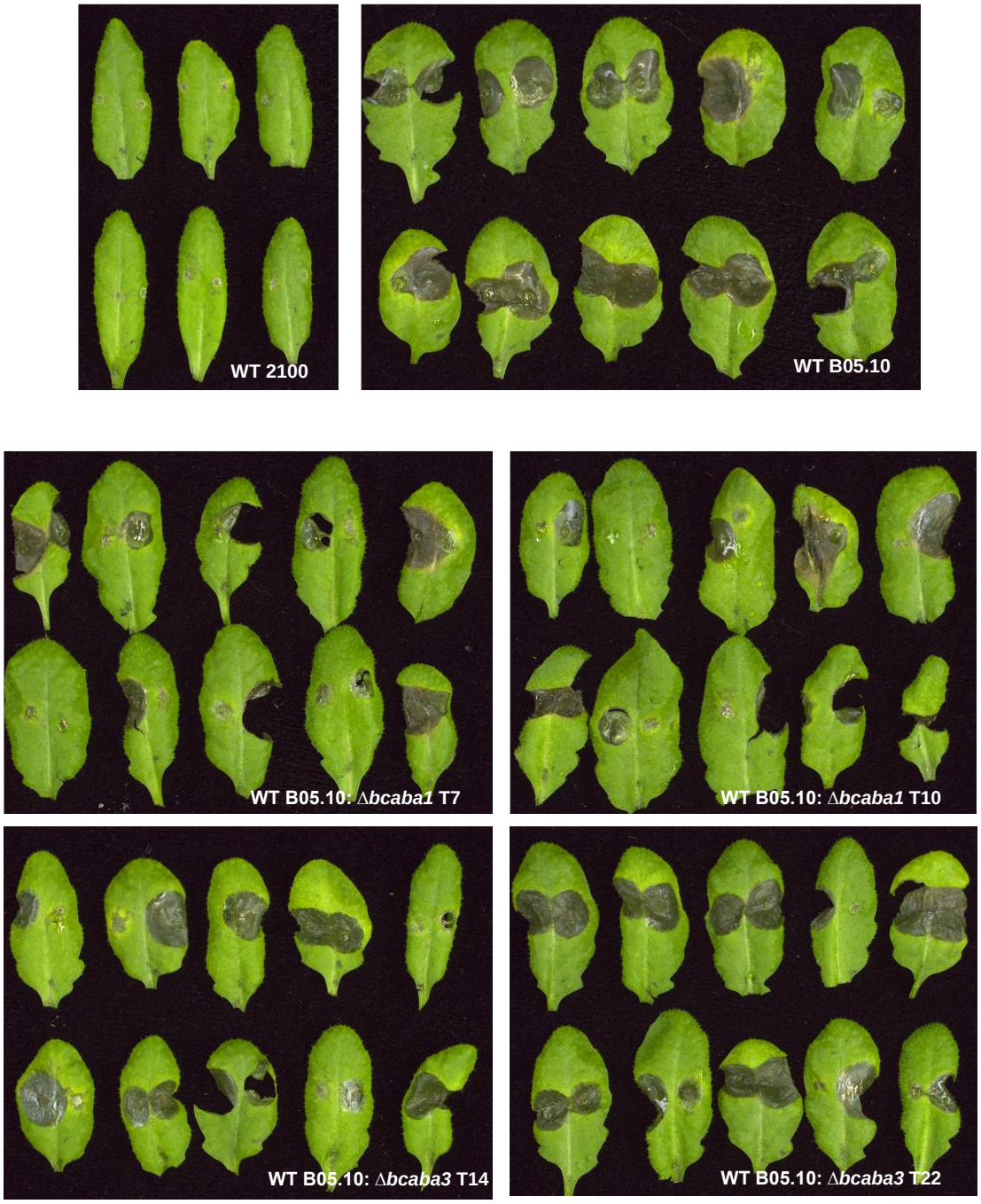


Figure 3.17. **Phenotypes of *Arabidopsis* WT plants challenged with *B. cinerea* B05.10 ABA mutants.** Pictures were taken of leaves 3 days post inoculation with *B. cinerea* strain 2100, wild-type strain B05.10, or

with ABA mutant lines of strain B05.10 (B05.10:Δ*bcaba1* lines T7 and T10, and B05.10:Δ*bcaba3* lines T14 and T22). 2 µL droplets containing $2.5x10^5$ spores of each indicated *B. cinerea* genotype were applied to leaves of intact Arabidopsis plants, respectively.

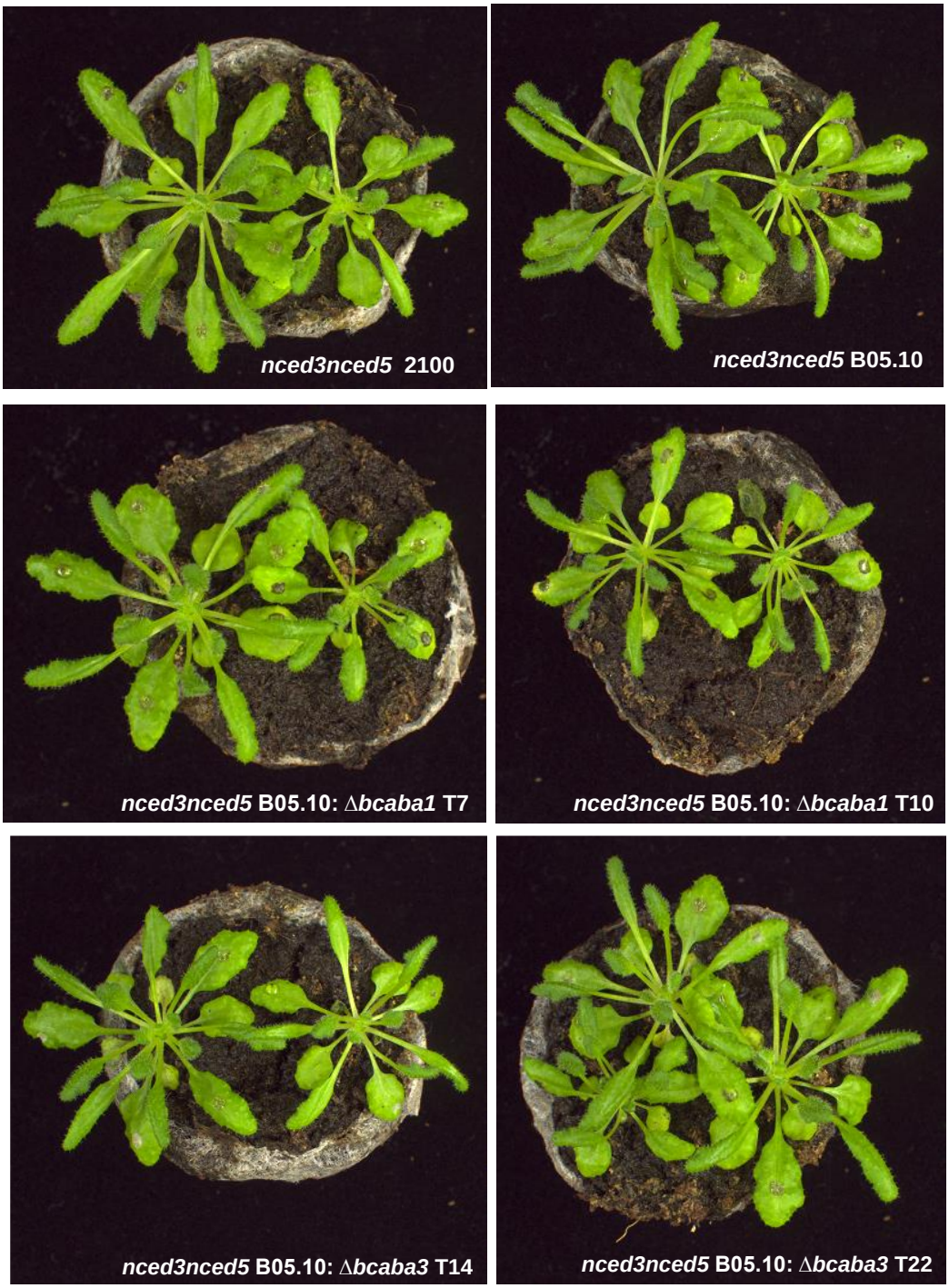


Figure 3.18. **Phenotypes of *Arabidopsis nced3 nced5* plants challenged with *B. cinerea* B05.10 ABA mutants.** Pictures were taken of leaves 3 days post inoculation with *B. cinerea* strain 2100, wild-type strain B05.10, or with ABA mutant lines of strain B05.10 (B05.10:Δ*bcaba1* lines T7 and T10, and B05.10:Δ*bcaba3*

lines T14 and T22). 2 µL droplets containing $2.5x10^5$ spores of each indicated *B. cinerea* genotype were applied to leaves of intact Arabidopsis plants, respectively.

3.4. Discussion

We previously showed that WRKY33 plays a vital role in Arabidopsis defense against the necrotrophic fungal pathogen *B. cinerea* strain 2100 (Birkenbihl et al., 2012). However, its molecular effects may differ against other strains or isolates of *B. cinerea*. Here we initially compared three *B. cinerea* strains, 2100, BMM and B05.10, and found that resistance of WT Col-0 plants to strains 2100 and BMM were dependent on WRKY33 function, whereas this was not apparently the case for strain B05.10. Subsequent more detailed comparisons between infections using strains 2100 and B05.10 revealed that the avirulent strain 2100 induced WRKY33 transcript and protein accumulation to much higher levels in host plants at early infection stages than the virulent strain B05.10 did. The difference in the strength of WRKY33 induction was also mirrored at the level of numerous tested WRKY33-dependent downstream events. This variation in the plant response was strongly correlated with differential accumulation of the *A. thaliana* defense hormones JA and ET and with the production of the antifungal compound camalexin. Analyses of *A. thaliana* ABA deficiency mutants in combination with *B. cinerea* B05.10 *BcABA* mutants revealed that *Arabidopsis* derived-ABA negatively regulates plant immune response to B05.10 similar to what was observed with strain 2100.

The Arabidopsis phytoalexin, camalexin, has been well described as playing a critical role in host defense particularly towards necrotrophic fungi and *pad3* mutants defective in the final steps of camalexin biosynthesis are highly susceptible to *B. cinerea* (Ahuja et al., 2012). Compared to plants infected with strain 2100, the camalexin levels observed in B05.10-infected plants were significantly reduced (Figure 3.8A). Several genes in the camalexin biosynthetic pathway are directly targeted and activated by WRKY33, and their expression levels too were reduced in B05.10 infected WT plants. Reports have shown that camalexin detoxification mechanisms are used by *B. cinerea*. Camalexin treatment induces *B. cinerea* apoptotic-like programmed cell death (PCD) and that a transgenic strain with enhanced anti-apoptotic capacity is less susceptible to camalexin (Shlezinger et al., 2011). In planta, camalexin might thus induce fungal PCD, limiting the spread of lesions during the early *B. cinerea* infection stage, while the fungal anti-apoptotic machinery would allow the fungus to recover and subsequently establish infection (Shlezinger et al., 2011). When *B. cinerea* is

exposed to camalexin, it also induces the expression of *BcatrB*, an ABC transporter that has an efflux function, acting as a protective mechanism against the fungitoxic effect of camalexin (Stefanato et al., 2009). In addition a camalexin detoxification mechanism acting through metabolization has also been reported resulting in the production of 3-indolecarboxylic acid by *B. cinerea* (Pedras et al., 2011b). However, *wrky33* mutants are far more susceptible to *B. cinerea* 2100 and B05.10 than are *pad3* plants. Thus, although WRKY33-dependent control of camalexin biosynthesis plays an important role in Arabidopsis defense towards these strains, additional WRKY33 functions must be acting in the host that are also impaired upon challenge with strain B05.10.

In response to pathogen attack, a universal defense response employed by plants involves, among others, activation of complex phytohormone signaling networks (Schenk et al., 2000). In particular, JA, ET and SA appear to be primary defense hormones, associated with host defense. Other phytohormones, such as ABA, IAA and CKs, which are better known for their roles in abiotic stress tolerance or plant growth and development, also can regulate plant defense, either alone or in conjunction with the primary defense hormones (Robert-Seilaniantz et al., 2011). However, pathogens have developed capabilities to manipulate or subvert plant phytohormone signaling for their own benefits (Kazan and Lyons, 2014).

Plant defense responses regulated by JA often promote resistance against necrotrophic pathogens and herbivorous insects (Glazebrook, 2005; Kazan and Manners, 2008; Mengiste, 2012). ET is a gaseous hormone and ET signaling often works synergistically with JA to promote resistance to necrotrophic pathogens such as *B. cinerea* and *Rhizoctonia solani* in *Arabidopsis* (Thomma et al., 1999b) and *Medicago* (Anderson et al., 2010), respectively. However, similar to other phytohormones, the involvement of ET in disease susceptibility, either alone or in combination with other phytohomones, has also been documented (Chen et al., 2009; Jia et al., 2013; Pantelides et al., 2013; Wang et al., 2013). Therefore, disruption or activation of JA/ET signaling by certain pathogens often promotes disease. From the comparative analysis of Arabidopsis Col-0 responses to the *B. cinerea* avirulent strain 2100 and the virulent strain B05.10 in this study, it appears that the outcome of the interaction between the host and the two fungal pathogens is mainly determined by qualitative differences in the WRKY33-dependent activation of a similar defense response. In particular WT plants inoculated with strain B05.10 were significantly impaired in the accumulation of JA, JA-IIe and ACC (a precursor of ET) compared with plants infected with strain 2100

(Figure 3.8B) and this impairment seems to be causal for disease development. Whether strain B05.10 actively suppresses host JA/ET signaling or whether this is the consequence of some other B05.10-dependent host perturbation remains to be elucidated. Other reports have shown some pathogens have evolved abilities to suppress the JA/ET pathway. For instance, SSITL (SCLEROTINA SCLEROTIORUM INTEGRIN-LIKE) protein, a secretory effector produced by the necrotrophic fungal pathogen *S. sclerotiorum*, suppresses JA-dependent defenses (Zhu et al., 2013). The mechanism by which SSITL suppresses JA-dependent defense is currently unknown. In a second case, the *Xanthomonas oryzae.* pv *oryzae* effector XopD encodes a small ubiquitin-like modifier protease with helix-loop-helix and EAR (ERF-Associated Amphiphilic Repression) DNA binding domains (Kim et al., 2008a). Mutants in *xopd* lacking either of these domains show reduced virulence (Kim et al., 2008a). The EAR motif acts as a transcriptional repressor in plants (Kazan, 2006). Further work indicates that the effector XopD from the related bacterial species *X. c.* pv *vesicatoria* targeted the tomato TF ERF4 to suppress ET mediated host defense responses (Kim et al., 2013).

Pathogens can also exploit phytohormone crosstalk during their interaction with plants. A well-known example of phytohormone crosstalk is the mutual antagonism between the SA and the JA signaling pathways (Kazan and Lyons, 2014). While the SA pathway often confers resistance to biotrophic pathogens, activation of this pathway attenuates JA signaling, thereby compromising resistance to necrotrophic pathogens. It has been shown that *B. cinerea* secretes an exopolysaccharide (EP) that enhances disease severity in tomato (El Oirdi et al., 2011). The EP activates the SA pathway, which antagonizes the JA signaling pathway, promoting enhanced disease. The SA-mediated disease development caused by the virulence function of EP is dependent on NPR1, a major regulator of SA-dependent immune responses (El Oirdi et al., 2011). In contrast, activation of JA pathway enhances resistance to some necrotrophs but inhibits the SA pathway and resistance to biotrophs (Thaler et al., 2012). In this study I observed a significantly reduced JA level in WT leaves challenged with the virulent strain B05.10 compared to infections with the avirulent strain 2100. This reduced JA levels is very likely due, in part, to the loss of WRKY33-mediated repressing SA signaling, but also to the additional role that WRKY33 plays in enhancing JA/ET signaling.

Although ABA is the major phytohormone involved in responses to abiotic stresses, it also can influence different aspects of plant immunity. ABA has been reported to mediate immune responses to necrotrophic bacteria, oomycetes, and certain fungi, contributing to disease

resistance or susceptibility (Mengiste, 2012). Elevated ABA levels lead to susceptibility to a wide range of pathogens including *B. cinerea* (Audenaert et al., 2002), *F. oxysporum* (Anderson et al., 2004), *M. grisea* (McDonald and Cahill, 1999a; Modi and McDonald, 1999), *P. sojae* (McDonald and Cahill, 1999b), and the bacterial pathogen *X. oryzae* pv *oryzae* (Xu et al., 2013), while it promotes resistance to pathogen such as the rice-infecting fungal pathogen *Cochliobolus miyabeaus* (De Vleesschauwer et al., 2010), and the oomycete pathogen *Pythium irregular* (Adie et al., 2007).

ABA-mediated resistance or susceptibility is often thought to be a consequence of crosstalk with the defense hormones JA, SA, or ET. My studies show that ABA deficiency in *Arabidopsis*, due to mutations in the ABA biosynthetic genes *NCED3* and *NCED5*, result in up-regulation of transcripts of JA/ET-related genes, increased JA and ET related hormone accumulation, and enhanced resistance to *B. cinerea* B05.10. This suggests that ABA-JA/ET crosstalk modulates defense gene expression and thereby results in disease resistance to B05.10. A negative role of ABA was further supported by the enhanced resistance to B05.10 observed in additional ABA mutants namely, *aba2-12,* and *aba3-1* (Table 3.1, Figure 3.12). Previous work has revealed an antagonistic effect between ABA and the JA/ET signaling pathways based on ABA deficient mutants leading to up-regulation of basal and induced transcription of JA/ET-responsive genes, and enhanced resistance to necrotrophs (Anderson et al., 2004; Hernandez-Blanco et al., 2007). The ABA-deficient tomato mutant *sitins* is resistant to *B. cinerea*, which is attributed to the timely production of hydrogen peroxide and cell wall modifications in the epidermis, enhanced cuticle permeability, and altered pectin composition, as well as increased basal and induced JA-ET-dependent defense gene expression (Asselbergh et al., 2007; Curvers et al., 2010).

Phytohormone responses are regulated by a large number of TFs. Therefore, a potential way for pathogens to disturb phytohormone pathways is to target TFs either directly or indirectly (Kazan and Lyons, 2014). In *Medicaga truincatula*, ERF19 is targeted by a secreted effector (SP7) from the arbuscular mycorrhizal fungus *Glomus intraradices*. SP7 interaction with ERF19 causes increased mycorrhizal colonization by *G. intrardices* and prolongs the biotrophic phase in rice roots when SP7 is expressed in the hemibiotrophic rice blast fungus *M. grisea* (Kloppholz et al., 2011). The *Xanthomonas* effector XopD interacts with ERF4 in tomato and the MYB TF MYB30 in *Arabidopsis*. MYB30 is a key regulator of multiple hormone signaling pathways, including SA, ABA, and BR signaling (Raffaele and Rivas,

2013). In addition to direct interaction with effectors, host TFs may be indirectly targeted by pathogen. For instance, The PAMP flg22 induces alternatively polyadenylated forms of ERF4 transcript in *Arabidopsis* (Lyons et al., 2013). The full length transcript encodes an AtERF4 isoform, which contains an EAR domain, and is involved in transcriptional repression, while the flg22-induced truncated transcript encoded an ERF4 isoform that does not contain the EAR domain and performs novel defense related functions (Lyons et al., 2013). Additionally, various members of the NAC family TFs involved in positively or negatively modulating host defenses, have recently been shown to be targeted by pathogen effectors (McLellan et al., 2013; Block et al., 2014; Donze et al., 2014). In the present study our data suggested that JA/ET signaling was affected by B05.10 infection, most likely by downregulating WRKY33 expression (Figure 3.2B). Unfortunately compared with bacteria, nearly nothing is known about potential effectors from necrotrophic pathogens such as *B. cinerea*. Whether virulence factors exist that are used by B05.10 to target WRKY33 remain to be revealed. Given the large number of transcriptional regulators potentially involved in plant defense, future research will also identify how WRKY33 was directly or indirectly targeted by *B. cinerea* B05.10 to promote disease susceptibility.

An additional question is whether ABA is also involved in suppressing WRKY33-dependent host immune responses? *B. cinerea* strain B05.10 appears to impair early accumulation and function of WRKY33 (Figure 3.2), whereas in the *nced3 nced5* mutant basal *WRKY33* expression levels are already high and further increases only moderately upon challenge with strain B05.10 (Figure 3.19). Thus, ABA seems in part to negatively affect *WRKY33* expression. In addition, ABA also seems to negatively impact the JA/ET pathway at least based on the elevated expression levels of *JRG21* and *Thi2.1* in *nced3 nced5* plants upon B05.10 infection (Figure 3.15). However, the activation of these JA/ET-related genes also appear to depend on WRKY33 function, as can be concluded from their dramatic decreases in expression in *nced3 nced5 wrky33* triple mutant plants (Figure 3.16), supporting a role of ABA in negatively affecting WRKY33 functions in WT plants upon B05.10 infection. However, increased *WRKY33* expression in *nced3 nced5* plants is not observed after B05.10 infection compared with WT plants (14hpi; Figure 3.19). This could indicate that ABA-mediated suppression of *WRKY33* expression acts via transcription factors that themselves are induced by ABA.

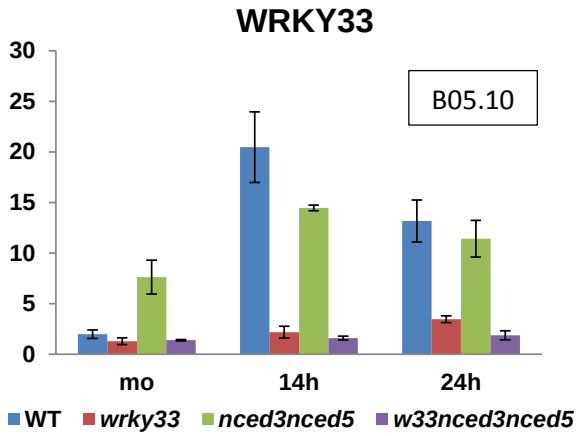


Figure 3. 19. Expression levels of *WRKY33* in WT, *wrky33*, *nced3 nced5* and *wrky33 nced3 nced5* plants determined by qRT-PCR at indicated timepoints after *B. cinerea* B05.10 spore spray-inoculation. Error bars represent SD of three biological replicates (n=3). All data were normalized to the expression of At4g26410.

Analysis of the *WRKY33* promoter for putative DNA binding sites (Table 3.2) revealed the existence of conserved abscisic acid (ABA) response elements (ABRE motifs), binding sites found in several ABA-responsive gene promoters that are bound by ABFs, a class of bZIP TFs (Sarkar and Lahiri, 2013). ABFs are themselves activated in an ABA-dependent manner upon stress through phosphorylation of their conserved domains by SNF1-related kinase2s (Yoshida et al., 2014). In addition, the *WRKY33* promoter also contains one MYC2 binding site. I tested the expression of *MYC2* in leaves of *nced3 nced5* and WT plants challenged by B05.10 and observed a significant reduction in the double mutant at 14hpi compared to WT (Figure 3.20), This is consistent with previous reports showing that ABA positively regulates *MYC2* (Kazan and Manners, 2013). Thus, one hypothesis is that ABA signaling induces ABFs and MYC2 and that these TFs are partly involved in regulating *WRKY33*. *myc2/jin1* mutants are resistant to some isolates of *B. cinerea* (Lorenzo et al., 2004), but whether *myc2* plants are resistant to *B. cinerea* strain B05.10, and whether MYC2 can directly target the *WRKY33* promoter and down-regulate its expression remain to be tested.

Table 2 List of known DNA motif in the promoter of WRKY33 (-2545 to 1).

Selected motif	DNA sequence	Number	Cluster
Evening element motif	AAATATCT	2	Yes
TGA binding site	TGACG	4	yes
ABA response element (ABRE)	ACGTGG	3	yes
HSF binding motif	GAAGAAGAA	3	yes
MYB binding site	CACATG	1	
MYC2 binding site	CATGTG	1	
G box	CACGTG	1	
W-box	TTGACT/C	5	yes
WRKY33 potential binding site	ATTCAAA	1	

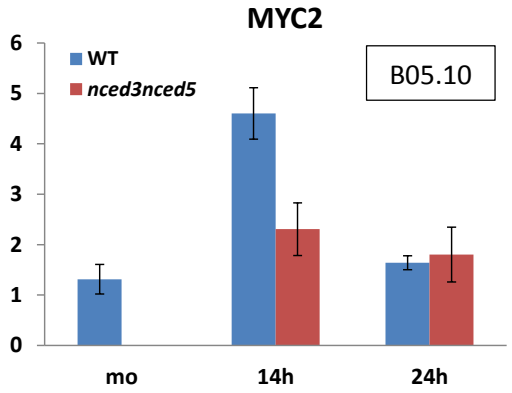


Figure 3.20. **Expression analysis of MYC2 in WT and *nced3 nced5* plants determined by qRT-PCR at indicated time points after *B. cinerea* B05.10 spore spray-inoculation.** Error bars represent SD of three biological replicates (n=3). All data were normalized to the expression of At4g26410.

Beyond ABA biosynthesis in the plant, some strains of *B. cinerea* can produce ABA (Siewers et al., 2004; Siewers et al., 2006). Thus, *Botrytis*-derived ABA can contribute to elevated ABA levels with host cells during the infection process. However, based on my analysis of *B. cinerea* B05.10 ABA mutants *BcABA1* and *BcABA3* this does not appear to be the case in this interaction. Rather, it appears that the endogenous plant-derived ABA is sufficient to result in host susceptibility. Still, pathogen-derived effectors can also modulate host ABA levels. For example, *P. syringae* infection induces ABA concentrations, and the effector AvrPtoB seems to be specifically involved in this process since transgene expression of this effector protein in *Arabidopsis* elevates ABA levels and enhances susceptibility to *P. syringae* (de Torres et al., 2006; de Torres-Zabala et al., 2007). By increasing ABA levels, *P. syringae* seems to antagonize the SA pathway required for resistance to this pathogen (Zabala et al., 2009). How AvrPtoB affects ABA levels is currently not known. The AvrBAvrC domain of the AvrXccC8004 from the bacterial pathogen *X. c.* pv *campestris* elicits ABA accumulation by

induction of the ABA biosynthesis gene *NCED5* when expressed as a transgene in *Arabidopsis*. Compared to the wildtype strain, an *X. c.* pv *campestris* mutant strain deficient in AvrXccC8004 triggers reduced ABA levels when inoculated onto *Arabidopsis*. Furthermore, exogenous ABA application allows increased growth of the AvrXccC8004-deficient strain, suggesting that *X. c.* pv *campestris* mediated ABA induction promotes virulence (Ho et al., 2013). Unfortunately nearly nothing is known about the repertoire of effectors encoded B. *cinerea* strain B05.10.

Taken together, my studies revealed that the virulent *B. cinerea* strain B05.10 represses *WRKY33* gene expression and delays WRKY33 protein accumulation thereby leading to clearly reduced early immune responses. From my results I hypothesize that several WRKY33-dependent Arabidopsis responses are required for resistance towards the fungus *B. cinerea*. On the one hand, WRKY33-mediated production of camalexin plays an important role in host-mediated resistance. Additionally however, enhancement of the JA and ET signaling pathways leading to appropriate defense responses to this necrotrophic fungus is also required, and this is achieved as a consequence of the downregulation of ABA biosynthesis by direct binding of WRKY33 to the promoters of ABA biosynthetic genes. How this negative cross-talk between ABA and JA/ET signaling is mechanistically achieved needs to be further investigated. Similarly, it remains unclear how WRKY33 function is impaired during the interaction of Arabidopsis with the virulent *B. cinerea* strain B05.10.

4. Conclusions and future perspectives

The studies in this thesis have revealed novel findings about the role of WRKY33 in regulating host defenses towards the necrotrophic fungus *B. cinerea*. In particular these studies have provided the first genome-wide *in vivo* overview of potential binding sites for a critical transcription factor during the interaction of an intact plant with a phytopathogen, and what the consequence of such binding had on host gene expression. Thus it provides the framework for the establishment of a transcriptional regulatory network model for host immunity.

The ChIP-seq and RNA-seq data provided in chapter 2 show that *B. cinerea* 2100-induced WRKY33 has a dual regulatory role by acting either as a transcriptional activator or as a repressor in modulating hundreds of target genes with distinct molecular functions. Further genetic and biochemical studies highlighted that importance of WRKY33-dependent positive regulation of camalexin biosynthetic genes, and the negative regulation of ABA signaling for Arabidopsis immunity towards *B. cinerea* 2100. WRKY33 was shown to act upstream of ABA biosynthesis as demonstrated by the re-establishment of WT-like resistance in the *wrky33 nced3 nced5* triple mutant upon *B. cinerea* 2100 challenge.

Studies described in chapter 3 of this thesis revealed that WRKY33-dependent *Arabidopsis* immunity also contributes to defense towards the *B. cinerea* virulent strain B05.10, but that during this interaction WRKY33-dependent early immune responses were clearly reduced or dampened. Genetic studies showed that ABA deficient mutants in *Arabidopsis* are also resistant to strain B05.10, and this resistance is correlated with observed elevated basal expression levels of *WRKY33* in *nced3 nced5* double mutant plants, suggesting ABA also negatively affects *WRKY33* basal expression. ABA antagonizes JA/ET signaling upon B05.10 infection, and this antagonism is dependent on WRKY33. These findings suggest that *B. cinerea* virulent strain B05.10 impairs WRKY33 function through the phytohormone ABA or via ABA signaling.

Although these studies have provided an extensive and deeper insight into the role of the transcription factor WRKY33 in modulating host immunity towards *B. cinerea*, several important questions remain to be resolved. They include:

- Next to the classical WRKY factor binding site, the W-box, an additional conserved DNA motif (5'- T/G-TTGAAG-3') was often identified within the WRKY33 binding peak regions.

This motif occurred in 55% of the WRKY33 binding regions, and was often associated with the W box (in 40% of the cases). It remains to be determined whether this new motif has functional relevance and can be bound by WRKY33 itself, or whether it represents a binding site for another type of transcription factor.

- What is the molecular basis for the observed antagonism between ABA and JA/ET signaling? My studies showed that numerous genes are misregulated in the susceptible *wrky33* mutant compared to the resistant WT plant. In the resistant *wrky33 nced3 nced5* triple mutant expression of many but not all genes are restored to WT-like levels. Thus, genes showing restoration of WT-like expression levels are prime candidates for further investigations as they may be causal for WRKY33-mediated resistance against this necrotrophic fungus.

- Why are SA levels so strongly reduced in the ABA biosynthetic double mutant *nced3 nced5* compared to *wrky33* upon 2100 infection? Where does ABA signaling interconnect with the SA signaling pathway?

- How does ABA negatively impact *WRKY33* expression and possibly also WRKY33 protein function? Are the ABA-responsive *cis*-regulatory DNA elements present in the *WRKY33* promoter relevant in this respect, and if so, which factors bind to these motifs?

- How does the virulent *B. cinerea* strain B05.10 negate WRKY33-dependent host resistance? Does this fungal strain use virulence effectors to target WRKY33 or some other molecular component(s) within the WRKY33-dependent defense pathway? With the advance of rather inexpensive next generation sequencing technologies it should be feasible to sequence the fungal strains 2100 and BMM and to compare their sequences with the already sequenced genome from strain B05.10. Bioinfomatic analyses should help to uncover differences in their potential effector repertoires along with additional unique gene sets that may provide clues why WRKY33-dependent host immunity remains effective against strains 2100 and BMM but not against B05.10.

The simplified model for the function of WRKY33 in modulating host defense towards *B. cinerea* is described in Figure 4.1.

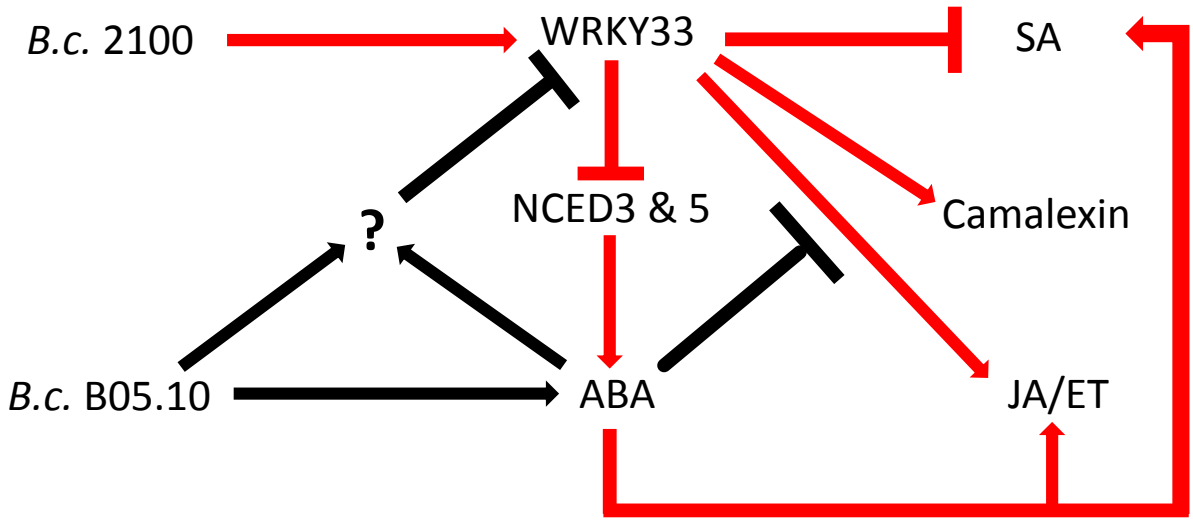


Figure 4.1. The involvement of WRKY33 in modulating host immunity towards *B. cinerea* strains 2100 and B05.10. The arrows indicate induction or positive modulation; the bar heads indicate block or suppression. The red color indicates the role of WRKY33 in host response to *B. cinerea* strain 2100, and the black color indicates the role of WRKY33 in host response to strain B05.10. SA, salicylic acid; JA, jasmonic acid; ET, ethylene; ABA, abscisic acid.

5. Materials and Methods

The material and methods section is subdivided into two parts, listing all materials used within this work in the first part. This includes the list of plant lines, oligonucleotides, pathogen strains, buffers and chemicals, media, solutions and enzymes. The second part describes the methods applied in this work.

5.1. Materials

5.1.1. Plant material

All experiments were performed using *Arabidopsis thaliana* Columbia-0 (Col-0) wildtype plants or mutants in the Col-0 background. The double or triple mutants *wrky33 nced3 nced5, wrky33 nced3, wrky33 nced5, wrky33 wrky46, gh3.2 gh3.3-1* and *wrky33 gh3.2 gh3.3* were generated by crossing the homozygous *wrky33* (GABI_324B11) with single or double mutants followed by PCR-based verification of the T-DNA insertion. Mutant plants are listed in Table 5.1, transgenic plants in Table 5.2.

Table 5.1. *Arabidopsis* mutants used in this study.

Gene	Accession	Reference/Source
cyp79b2 cyp79b3	Col-0	Zhao et al. 2002
cyp71a13	Col-0	Nafisi et al. 2007
pad3-1	Col-0	Zhou et al., 1999
dde2-2	Col-0	von Malek et al, 2002
jar1-1	Col-0	Staswick et al., 2002
ein2	Col-0	Guzman and Ecker, 1990
dde2 ein2	Col-0	Tsuda et al. 2009
erf5-1	Col-0	Son et al. 2012
erf6-1	Col-0	Son et al. 2012
erf5 erf6	Col-0	Son et al. 2012
ora59	Col-0	GK_061A12
sid2-1	Col-0	Wildermuth et al., 2001
pad4-1	Col-0	Glazebrook et al., 1997
npr1-1	Col-0	Cao et al., 1997
pad4 sid2	Col-0	Tsuda et al. 2009
dde2 ein2 pad4 sid2	Col-0	Tsuda et al. 2009
aba2-12	Col-0	González-Guzmán et al. 2002
aba3-1	Col-0	Leon-Kloosterziel et al. 1996
nced3-2	Col-0	Frey et al. 2012
nced5-2	Col-0	Frey et al. 2012
nced3 nced5	Col-0	Frey et al. 2012
wrky33	Col-0	GABI_324B11
wrky33 sid2-1	Col-0	Birkenbihl et al. 2012

wrky33 npr1-1	Col-0	Birkenbihl et al. 2012
wrky33 rbohd	Col-0	Birkenbihl et al. 2012
wrky33 wrky70	Col-0	Birkenbihl et al. 2012
wrky33wrky40	Col-0	this study
wrky33 wrky46	Col-0	this study
wrky33 nced3 nced5	Col-0	this study
wrky33 nced3	Col-0	this study
wrky33 nced5	Col-0	this study
nac019	Col-0	Bu et al. 2008
nac055	Col-0	Bu et al. 2008
nac019 nac055	Col-0	Bu et al. 2008
gh3.2	Col-0/ KanR	González-Lamothe et al. 2012
gh3.2	Ler	González-Lamothe et al. 2012
gh3.3-1	Col-0	Gutierrez et al. 2012
gh3.3-2	Col-0	Gutierrez et al. 2012
gh3.2 gh3.3-1	Col-0	this study
wrky33 gh3.2 gh3.3-1	Col-0	this study
cyp707a1-1	Col-0	Okamoto et al. 2006
cyp707a2-1	Col-0	Okamoto et al. 2006
cyp707a3-1	Col-0	Okamoto et al. 2006
nac061	Col-0	this study
wrky33 nac061	Col-0	this study
rbohd	Col-0	Torres et al., 2002
wrky70	Col-0	GK_752F08
wrky40	Col-0	Shen et al., 2007
wrky46	Col-0	GABI_ 038C07

The *Arabidopsis* mutant *gh3.2* was kindly provided by Dr. Kamal Bouarab; *gh3.3* was kindly provided by Dr. Catherine Bellini; *erf5*, *erf6* and *erf5erf6* were kindly provided by Dr. Gary Stacey; *dde2 ein2 pad4* were kindly provided by Dr. Kenichi Tsuda; *sid2*, *nced3*, *nced5*, and *nced3 nced5* were kindly provided by Dr. Annie Marion-Poll; *nac019*, *nac055*, and *nac019 nac055* were kindly provided by Dr. Chuanyou Li; *cyp707a01*, *cyp707a02*, and *cyp707a03* were kindly provided by Dr. Yong Xiang; *aba2* and *aba3* were kindly provided by Dr. Akira Mine.

Table5.2. *Arabidopsis* transgenic lines

Name	Background	Construct	Reference/Source
WRKY33HA	Col-0 *wrky33*	*pWRKY33:WRKY33-HA*	Birkenbihl et al. 2012
OE-WRKY33HA	Col-0 *wrky33*	*35S:WRKY33-HA*	Birkenbihl et al. 2012
OE-NCED3	Col-0	*35S:NCED3*	Fan et al. 2009
OE-NCED5	Col-0	*35S:NCED5*	Fan et al. 2009

The *Arabidopsis* transgenic plants *OE-NCED3* and *OE-NCED5* were kindly provided by Dr. Jun Fan.

5.1.2. Pathogens

All pathogens employed in this study are depicted in Table 5.3. In addition, source or reference is stated.

5.1.3. Oligonucleotides

Primers used in this study were purchased from Sigma-Aldrich or Invitrogen and resuspended in H_2O to a concentration of 100µM. Working solutions were further diluted to 10µM. Primers used for fungal growth biomass are summarized in Table 5.4, for qPCR in Table 5.5, for ChIP-PCR or LinDA-PCR in Table 5.6, and for genotyping in Table 5.7, respectively.

Table 5.3. pathogens used in this study

Strain	Description	Reference/Source
2100	Isolated from leaf of *Vicia faba* (United Kingdom)	CECT (Spanish Type-Culture Collection)
BMM	Isolated from *Pelargonium zonale*	Zimmerli *et al.* 2001
B05.10	Obtained after benomyl treatment of a *Vitis* isolate (Germany)	Büttner *et al.* 1998; Quidde *et al.* 1998
B05.10: Δ*bcaba1*	B05.10, Δ *bcaba1::hygR* (T7, T10)	Siewers et al. 2004
B05.10: Δ*bcaba3*	B05.10, Δ *bcaba3::hygR* (T14, T22)	Siewers et al. 2006

Table 5.4. Oligonucleotides used for fungal growth biomass

Gene	Locus	Forward primer (5' - 3')	Reverse primer (5' - 3')	Purpose
BC-CutA		AGCCTTATGTCCCTTCCCTTG	GAAGAGAAATGGAAAATGGTGAG	fungi biomass
ATSK11	AT5G26751	CTTATCGGATTTCTCTATGTTTGGC	GAGCTCCTGTTTATTTAACTTGTACATACC	fungi biomass

Table 5.5. Oligonucleotides used for qPCR

Gene	Locus	Forward primer (5' - 3')	Reverse primer (5' - 3')	Purpose
CYP71A12	AT2G30750	TTGTAGGCCGATTTGACTGGA	ACGGAAGATGGAAATGCAATG	qPCR
CYP71A13	AT2G30770	ATTCGGATCAGGGAGAAGGATA	CGATACCAATGGCTTCAGTTAGAT	qPCR
PAD3	AT3G26830	GGTTTCTCGACAGTTCCGTTGA	TTCAACAATGCCATCTCAACAAGTA	qPCR
WRKY38	AT5G22570	CATAACTTGAAAGCGGTCCAC	AAATGAACTCCCCACACGAA	qPCR
WRKY50	AT5G26170	GATCTTGTGTCTGCGGTTTC	CAGAAGCAGTGGCTGTAGCA	qPCR
WRKY53	AT4G23810	CCAGAGTCAAACCAGCCATTA	CGTATCAGGGAACGAGAAAAC	qPCR
WRKY41	AT4G11070	CCGTCGGATTTCACTGGA	GCCTGTGTTAATCTCAGCCG	qPCR
WRKY48	AT5G49520	TCAACATCACCAGCCCTACA	CATATCATAACCAAAGCCGGG	qPCR
WRKY55	AT2G40740	CTTCCGGGTCACATACCGT	TGAAATCCATGTTGGTTCCG	qPCR
NAC061	AT3G44350	CAGTATCTGTCGAATCTACA	TTGGTTTCCTCCTGAATGTG	qPCR
NAC090	AT5G22380	GCTGAGACATCGTTCCGTG	CAGTCCCACATCGGTTCTG	qPCR
GH3.2	AT4G37390	TGGAGCAGCAGAAGCATCAT	TGTCGCCAACTCTGTAACGG	qPCR
GH3.3	AT2G23170	ACCTATGCTGGGCTTAACCGT	AGAGCGATGCGTTCTCAACC	qPCR
AKT1	AT2G26650	ATAGCCGGGAAGCTGGTA	TGGAACCCAATTCTAGCAACT	qPCR
NCED3	AT3G14440	GGAGAAGGAGGAGAGGAAGA	CGACCTGCTTCGCCAAATCAT	qPCR
NCED5	AT1G30100	CGAGGAGAGTTGGGAATCGG	TGGTTTAACATATCCGCCGAA	qPCR
CYP707A3	AT5G45340	CAGATGGTCAATCGTAGGGC	TTTCGTTCCAAGGCAATAGG	qPCR
WAKL7	AT1G16090	CAGTGGCTACAGAGGCAATCC	TCACAATCATACCCTTTCCCA	qPCR
NPR3	AT5G45110	CGACATCCTCGACGATTTCC	CATGTTGTGTTGTGCAGGTCA	qPCR
AAO1	AT5G20960	AACGGTTGACACAATCCCAAG	GCAGTGAACAGAAGCCGCTAA	qPCR
ACS2	AT1G01480	AGTTTCCGACGACTTTACGAG	GAAGAGGTGAGTGTGGTGACA	qPCR
JRG21	AT3G55970	AGGGCAATGTTCCTATCGAGC	GACATTTGCTACGTGGGCCT	qPCR
ERF5	AT5G47230	TGGAGAGACGTTTCCGTTTGT	CGGTCAACTGGGAATAACCA	qPCR
ERF1	AT3G23240	TCGGCGATTCTCAATTTTTCG	CCGTCTCATCGAGTGTTTCCT	qPCR
ACS6	AT4G11280	AAACCGAACTATGGCGTGTG	TCATGGCAATGGAACGAAC	qPCR
LOX4	AT1G72520	CTCCGTCATCACCACCATC	AAAATACTTTTGAGGATCTTCGATA	qPCR
WRKY33	AT2G38470	CTCGTGGTAGCGGTTACGCC	CCTTTGCTCTAGAGAATCCACC	qPCR

ICS1	AT1G74710	CATTGATCTATGCGGGGACAG	TGGACAAAAGCTCGTACCTGAG	qPCR
NPR1	AT1G64280	AGGCACTTGACTCGGATGATATTG	CTTCACATTGCAATATGCAACAGC	qPCR
NPR4	AT4G19660	TCGTATCCCGAGAAAGGCAC	AGCTGGTGATGAAGAAGAAAGACAA	qPCR
TRXH5	AT1G45145	TGAATTGCAAGCTGTTGCTC	GCAGAAGCTACAAGACCACC	qPCR
FMO1	AT1G19250	CGTCCAAAGCAGCTCGAAC	CGTGGAAATGCAATGACGTTT	qPCR
PR2	AT3G57260	TTCAAACCCGTACGACACTG	TCTGGCGTCGTGAGGAGGAA	qPCR
EDS1	AT3G48090	AAGCATGATCCGCACTCG	CGAAGACACAGGGCCGTA	qPCR
PAD4	AT3G52430	GGTTCTGTTCGTCTGATGTTT	GTTCCTCGGTGTTTTGAGTT	qPCR
NIMIN-1	AT1G02450	TTCAAACCCGTACGACACTG	TCTGGCGTCGTGAGGAGGAA	qPCR
PR1	AT2G14610	TTCTTCCCTCGAAAGCTCAA	AAGGCCCACCAGAGTGTATG	qPCR
NAC019	AT1G52890	GGAGGAAGTCGAGAGCAGTCA	CAAACCCACCAACTTGCCC	qPCR
NAC055	AT3G15500	CGCAGCAACAAACTGAGGG	CCCGAGTACCCAAATCCGTT	qPCR
MPK11	AT1G01560	CGATGAAGCCTTGTGCCA	CCTTGATGTTCTCTTCCGTCA	qPCR
BIR1	AT5G48380	GGGTAACCTCGTGGAATGG	CTTCAAACATGGTTGGCCTC	qPCR
CDPK1	AT1G18890	GGATGTCGATGGAAATGGG	CGCTTAGAACACTGGCGTCT	qPCR
CRK36	AT4G04490	GACAAGGTGGGTTTGGATCTG	GTCCTGAGCCTCCAGCTAATC	qPCR
ORA59	AT1G06160	AGGCAGCCTCGCAGTACTCAA	CTCTTCAAGGCTATCACCGGA	qPCR
PDF1.2	AT5G44420	TGCATGATCCATGTTTGGCTC	ACGCACCGGCAATGGTGGAA	qPCR
ABI1	AT4G26080	AAGGGAAAGATCCTGCGGC	TCCGAGGCTTCAAATCAACC	qPCR
AMT1	AT5G05730	AGTGACCCGCAAGACGAA	TCACAAATGCAGATTCAGCC	qPCR
AOC3	AT3G25780	CTGAAAAGAGCGGTGACAGAT	CACCAGTGACAGCGAGGAAC	qPCR
JAR1	AT2G46370	CCGTATGTGCCAAAGCTGAGAC	GGTTTCTCCTCTCCTTCCCCT	qPCR
JRG21	AT3G55970	AGGGCAATGTTCCTATCGAGC	GACATTTGCTACGTGGGCCT	qPCR
THI2.1	AT1G72260	TGCCCAGGCTCTTCATTG	AGAGGCGTGGCTTTAGGA	qPCR
VSP2	AT5G24770	ACCGTTGGAAGTTGTGGAAG	CCAAATCAGCCCATTGATCT	qPCR
LOX2	AT3G45140	TACTTTCCCAACCGACCAAC	CCTGTTTCTGCGATGGGTAT	qPCR
MYC2	AT1G32640	GCGGTTTTATCTCCGAATGA	GCGATGAAGGTAAACG	qPCR (from Dr. Akira Mine)
DDE2	AT5G42650	ATGTGTTGTGGTCGAATGGAC	AACGAATCATATCGCCGGA	qPCR
AOC1	AT3G25760	CGCATTCAGCTTTAGGTTTGG	CGTTCTTCTCAGGGACGTGT	qPCR

HSFA4A	AT4G18880	GAATGATGGCTTCTGGCAGC	TTGAACTTCCCGTTGCTCG	qPCR
EXP	AT4G26410	GAGCTGAAGTGGCTTCCATGAC	GGTCCGACATACCCATGATCC	qPCR

Table 5.6. Oligonucleotides used for ChIP-qPCR

Gene	Locus	Forward primer (5' - 3')	Reverse primer (5' - 3')	Purpose	
CYP71A12	AT2G30750	CAAGTTTGACTGACCCATCT	GATTTAGAAGTTTGAATAACCC	ChIP-qPCR	
CYP71A13	AT2G30770	GGTCGCATTCAACAGCTAAG	GGAAGTTTGACTTACCCATTCA	ChIP-qPCR	LinDA-qPCR
PAD3	AT3G26830	AGGAGGTGTGCAATATGGAC	TAGGTTGCGCTGACCAAACA	ChIP-qPCR	LinDA-qPCR
WRKY38	AT5G22570	CAAAAGTATATATTTGACTAAAGCTGG	TTAAACCAAGTAGTTGGAACGTAG	ChIP-qPCR	
WRKY50	AT5G26170	GAATGGTACATAACAAGTCCTC	TGTGTTTCCAAGCAATAGAGACAT	ChIP-qPCR	
WRKY53	AT4G23810	AGTTAGGCTATTTAATGCGTATATCT	CATTGCTTTCAATCCCTTTGATAT	ChIP-qPCR	
WRKY53	AT4G23810	GAGAGTGACGCCATTATAAAAATTA	CAAAAAGAAAATCAATATTCAAAAGGAC	ChIP-qPCR	
WRKY53	AT4G23810	CCTTTGACCTTATACTCTTTCACTA	TTGACCAAATGACCAAACCATAAAT	ChIP-qPCR	
WRKY41	AT4G11070	GGTTAAACTAAAATAAACCCAAAGTTG	TGAAAATTTGACCAAGTGAGCAAAC	ChIP-qPCR	
WRKY48	AT5G49520	GCTTGGTTGAATAACTGATGGT	ATGATTGACCACAGGATCATAG	ChIP-qPCR	LinDA-qPCR
WRKY55	AT2G40740	GTAATGTTGAAATTTGAAAGAAATAAATAATC	TCTAACTAATGATAACCCATTGACC	ChIP-qPCR	
NAC061	AT3G44350	AAAGCACTCACTGTCTCACGTAT	GGCCCATCTTTGTGATAATTTC	ChIP-qPCR	LinDA-qPCR
NAC090	AT5G22380	AACTGGCAAGCCAAATTAAAAGAT	CGGATATTGGTAAAGACAAGGA	ChIP-qPCR	
GH3.2	AT4G37390	GCCATGCCTTCTTTGGAT	TTTGCCTGAGATTTCCTTGA	ChIP-qPCR	LinDA-qPCR
AKT1	AT2G26650	AAATGTTGGTTTCCACGTTT	GGAGCTTTAGTCGTCAAGTAGTT	ChIP-qPCR	LinDA-qPCR
NCED3	AT3G14440	CCATGCTTTAGTGACGTTTACTTG	TTACTTCCGATAAACAGCTTCAATC	ChIP-qPCR	
NCED3	AT3G14440	TTGTCGGGTTGGTGTCCTC	AAATACAGTTGCCGGTCAAAGA	ChIP-qPCR	
NCED5	AT1G30100	CCGGTAAATATTGTGAACCTTT	CCAAGTAACTGTCACCAATCAC	ChIP-qPCR	LinDA-qPCR
CYP707A3	AT5G45340	TTTGTCGATCTCTGACCGATTT	TCACTTTAACGAAGCGCAAAC	ChIP-qPCR	LinDA-qPCR
CYP707A3	AT5G45340	TCACAGGCAGAAAGTCAATTT	CCTATATATGTGCTTAGAACTTAATCAGA	ChIP-qPCR	LinDA-qPCR
WAKL7	AT1G16090	AGTTGACAAAGTAAGAGGCAGGA	ACAGTTGCCTACTTGGACCAC		LinDA-qPCR
RING1A	AT5G44280	AAATTCAACCATTCACCGTTT	ACAATTCAAACCGGCAGC		LinDA-qPCR

NPR3	AT5G45110	TCCTTGTCCAAAGTTTCTTCC	TCAACCAAACCCAGAACCA		LinDA-qPCR
AAO1	AT5G20960	TCTTACCTCCTCGGATTCAAT	ACTTGTATTTCTTCTAGTCGTCCAC		LinDA-qPCR
ACS2	AT1G01480	TGCAGACTACAACATACCAAATG	CCAAGGCTATTTCTAAGCAACTT		LinDA-qPCR
JRG21	AT3G55970	CAACGACTAAGACGCTCTCACA	GCAAAGAATGTAATCATAGACGTGC		LinDA-qPCR
ERF5	AT5G47230	AAACATGAGATATTGACCGGC	TCGACTTGTTCAGACAGAATCAC		LinDA-qPCR
ERF1	AT3G23240	AGGATTGTCTTTAAGCATGTGC	CGCTCCTCAATACTCATGGA		LinDA-qPCR
ACS6	AT4G11280	TTCTTTCATGGAACTTTCGTTG	GATGTGTTGGGAAGTGAGATTG	ChIP-qPCR	
LOX4	AT1G72520	ACGTTGAACATTAAAGTGTCCG	GCCTTGAGAAAGAGAGAGCA	ChIP-qPCR	
WRKY33	AT2G38470	AAGCATTGAGCCGCCCCTCA	ATGAAGAAGAGTAGTTTCTGAG	ChIP-qPCR	
ORA59	AT1G06160	TGTGGACACCAAATGATAAAGAG	CAAGATTAAGAAGTTGAATTGGCTG	ChIP-qPCR	
NUDT6	AT2G04450	GAAGAGATTGCAGGAGATGG	TTGAGGAGGTTGGCGTGATC	refrerence	

Table 5.7. Oligonucleotides used for genotyping

Gene	Locus	Forward primer (5' - 3')	Reverse primer (5' - 3')	Purpose
GH3.2	AT4G37390	TACGTAACCACCGGAACTTTG	AGAGCGGATGATTGTTGATTG	genotyping
GH3.3	AT2G23170	TTTTAACGTATTAATCTTGGCACG	GGGAACAACAACATGATCCCT	genotyping
NCED3	AT3G14440	ACAGAGGCTCTCCTCCGTAAC	GTCAGCCACGAGAAGCTACAC	genotyping
NCED5	AT1G30100	TAACACCAAACCCAACCAAAC	TGACTCAACCCAAACCATCTC	genotyping
WRKY33	AT2G38470	CTCCTTCTCTTGTCTCTCCTTCC	TTGTGATTAAAGCTCCTGTGGTT	genotyping
SALKLBB1.3		ATTTTGCCGATTTCGGAAC		genotyping
GK-LB8409		ATATTGACCATCATACTCATTGC		genotyping

5.1.4. Antibodies and enzymes

Antibodies

Listed below are primary and secondary antibodies used for immunoblot detection.

Table5. 8. Primary antibodies

Antibody	Source	Dilution	Reference	Purpose
α -HA	Rabbit (polycl.)	1:5000 in 5% milk	Sigma	ChIP
α -HA	Rat (monocle.)	1:5000 in 5% milk	Roche	Western

Table 5.9. List of secondary antibodies used in this study

Antibody	Source	Dilution	Reference	Purpose
α-rat IgG	HRP[a]	1:10.000 in 5% milk	Sigma	Western
α-mouse IgG	HRP[a]	1:5000 in 5% milk	ECL (GE ealthcare)	Western

[a.] horseradish peroxidase

Enzymes

Taq-Polymerase used for standard PCR-reactions was ordered from Ampliqon (Odense, Denmark) and used according to the manufacturer's instructions.

5.1.5. Chemicals

Laboratory grade chemicals and reagents were purchased from Invitrogen (Karlsruhe, Germany), Merck (Darmstadt, Germany), Roth (Karlsruhe, Germany) and Sigma-Aldrich (München, Germany) unless stated otherwise.

Pierce ProteinA agarose for ChIP-experiments and Pierce Western substrate were purchased from Thermo Fisher Scientific (Rockford, USA).

5.1.6. Media

Sterilized media was used for growing bacteria, fungi or *Arabidopsis* plants *in vitro*. For sterilization, media was autoclaved for 20 min at 121°C and cooled down prior adding heat instable antibiotics or other supplements. Heat instable compounds were filter-sterilized before use. Agar for agar plate preparation was purchased from Becton (Franklin Lakes, USA) and MS medium was ordered from Duchefa (Haarlem, Netherlands) or Sigma.

Escherichia coli medium	Luria-Bertani (LB) broth or agar plates
Agrobacterium tumefaciens medium	YEB broth or agar plates
Arabidopsis thaliana medium	½ Murashige-Skoog (MS) medium including vitamins and 0.5% sucrose
Botrytis cinerea medium	PD broth or agar plates

5.1.7. Buffers and solutions

General buffers and solutions are displayed in the following listing. Buffers and solutions not displayed here are described in the corresponding methods. All buffers and solutions were prepared using Milli-Q water. Buffers and solutions for molecular biological experiments were autoclaved or filter-sterilised.

Molecular biology work:

DNA extraction buffer	Tris-HCl, pH 7.5	200 mM
	NaCl	250 mM
	EDTA	25 mM
	SDS	0.5%
DNA gel loading dye (6x)	Sucrose	4 g
	EDTA (0.5 M)	2 ml
	Bromphenol blue	25 mg
	H_2O to 10 ml	

TE buffer	Tris-HCl pH 8.0	10 mM
	EDTA	1 mM

SDS-PAGE:

Laemmli buffer (2x)	Tris-HCl pH 6.8	0.125 M
	SDS	4%
	Glycerol	20% (v/v)
	Bromphenol blue	0.02%
	Dithiothreitol (DTT)	0.2 M

SDS running buffer (10x)	Tris	30.3 g
	Glycine	144 g
	SDS	10 g
	H_2O to 1000 ml	

Western blotting:

Transfer buffer (10x)	Tris	30.3 g
	Glycine	144 g
	H_2O to 1000 mL	
	Before using dilute 100mL 10x buffer with 700ml H_2O and add 200mL methanol.	

Ponceau S staining — Ponceau S working solution was prepared by dilution of ATX Ponceau S concentrate (Sigma-Aldrich) 1:5 in H_2O.

PBS(T) buffer (10x)

NaCl	80 g
KCl	2 g
Na_2HPO_4	14.4 g
KH_2PO_4	2.4 g
Ajust pH to 7.4 (HCl), H_2O to 1000 Ml	

For 1 x PBST working solution, dilute 100mL 10x buffer, and then add Tween 20.

Tween 20	0.05%

Pathogen infection

Vogelbuffer 1L

Sucrose	15 g
Na-citrate	3 g
K_2HPO_4	5 g
$MgSO_47H_2O$	0.2 g
$CaCl_22H_2O$	0.1 g
NH_4NO_3	2 g

5.2. Methods

5.2.1. Maintenance and cultivation of Arabidopsis plants

Arabidopsis thaliana seeds were sowed out on 42 mm Jiffy-7 pots soaked in four liters of water containing 0.1% Wuxal fertilizer (Manna, Germany), to avoid unspecified pathogen infections derived from garden soil. Plants were grown for 4-5 weeks under short-day conditions (10 hours light at 23°C/14 hours darkness at 20°C and 60% humidity) in closed cabinets (Schneijder chamber) until use. For pathogen infection plants were transferred to designated growth-chambers for the respective pathogens.

5.2.2. Crossing Arabidopsis plants

Individual flowers with immature stamina were emasculated with fine tweezers. Stigmas were pollinated by tapping three to four donor stamens from different flowers onto them. Mature siliques containing F1 seeds were harvested and allowed to dry. F1 seeds were grown and allowed to self-pollinate. Produced F2 seeds were sown and used for genotyping.

5.2.3. Pathogen infections and quantification of fungal growth by qPCR

B. cinerea strain 2100 and BMM was cultivated on potato dextrose plates at 22℃ for 10 days. *B. cinerea* strain B05.10 and mutants (*BcABA1*, *BcABA3*) were cultivated on potato dextrose plates with light and dark alternatively (12h/12h) for 10-14 days. Spores were collected,

washed, and frozen at -80C in 0.8% NaCl at a concentration of 10^7 spores mL^{-1}. For inoculation of *Arabidopsis* plants the spores were diluted in Vogel buffer prepared as previously described. For droplet inoculations, 2 µL of $2.5*10^5$ spores mL^{-1} was applied to single leaves of 4-week-old intact plants. The leaves were detached from the plants only after completion of the experiment and only for documentation purposes. The same spore concentration was used for spray inoculations of entire 4-week-old intact plants. For mock treatment, Vogel buffer alone was used. Plants were kept prior to and during the infection under sealed hoods under high humidity. The infection was carried out on the bench at room temperature or plants were put in the climate chamber under a strict light (12h/12h, light/dark) and temperature (22℃/20℃) regime.

Quantification of fungal biomass relative to plant biomass by qPCR was basically performed as previously described (Gachon and Saindrenan, 2004). Leaves of the indicated Arabidopsis lines were inoculated with two 2-µL droplets of *B. cinerea* spores and DNA extracted 3 days later from whole leaves of similar fresh weight. The relative amounts of *B. cinerea* and Arabidopsis DNA were determined by qPCR employing specific primers for Cutinase A and SKII, respectively.

5.2.4. DNA isolation

For genotyping, 10 mg plant leaf material was collected into 96-well Collection Microtubes (Qiagen, Hilden, Germany) containing ~20 1.0 mm Zirconia Beads (BioSpec). Samples were frozen in liquid nitrogen and homogenized using a TissueLyser (Qiagen, Hilden, Germany) for 2x 30 sec at 30 strokes/sec. 400 µL DNA extraction buffer was added to the homogenized samples and shaked again in the TissueLyser for 10 sec at 30 strokes/sec. The following DNA extraction was performed according to Kotchoni et al (Kotchoni and Gachomo, 2009). 2 µl of extracted DNA was used for subsequent PCR analysis.

5.2.5. RNA Isolation and cDNA synthesis

Total RNA was extracted from 100 mg plant leaf material of 4-5 week old *Arabidopsis* plants. Frozen samples were homogenized with ~20 1.0 mm Zirconia Beads (BioSpec) in a Mini- BeadBeater-6 (BioSpec) for 2x 30 seconds or using a TissueLyser (Qiagen, Hilden, Germany) for 30 sec at 30 strokes/sec. RNA was isolated using the TRI Reagent (Ambion) following the manufacturer's protocol. Concentration and quality was determined

using a NanoDrop photometer (PeqLab). RNA with a 260/280 and 160/230 ratio of ~2.0 was used for cDNA synthesis.

5 µg total RNA was used for cDNA synthesis with oligodT-primers employing the 'SuperScript II first-strand synthesis system for reverse-transcription PCR' (Invitrogen) following the manufacturer's protocol. cDNA was solved in 50 µl water and subsequently used for quantitative real-time PCR.

5.2.6. Polymerase chain reaction (PCR)

Standard PCR was performed using the Ampliqon (Odense, Denmark) *Taq* polymerase. The standard PCR reaction mix (Table 5.10) and thermal profile (Table 5.11) is depicted below.

Table 5.10. Stadard PCR reaction mix

Reagent	Amount
DNA template	10-50 ng
PCR amplification buffer	1/10 of reaction volume
dNTP mix (dATP, dGTP, dCTP, dTTP)	0,2 mM each
forward primer	0,5 µM
reverse primer	0,5 µM
Taq DNA polymerase	2,5-5 units
sterile H_2O	Variable

Table 5.11. Standard PCR thermal profile

Step	Temp.	Time	
Initial denaturation	95°C	3 min.	
Denaturation	95°C	30 sec.	35x
Annealing	55°C	30 sec.	
Extension	72°C	20-120 sec	
Final extension	72°C	5 min.	

5.2.7. qRT-PCR analysis of Arabidopsis gene expression

Total RNA was isolated from leaves at 8, 14, 24, and 48 hpi as described above and reverse transcribed with oligo(dT) primer to produce cDNA as mentioned above. cDNA corresponding to 2.5ng of total RNA was subjected to qPCR with gene-specific primers using the SYBR Green reagent (Bio-Rad). The qPCRs were performed on the iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad) with three biological replicates. 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 analysis. 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. The standard qPCR-program is depicted in Table 5.12. Results were analysed using the BioRad iQ5 software and Microsoft Office Excel.

Table 5.12. qPCR thermal conditions

Stage	Temperature (°C)	Duration (sec)	No. of cycles
Initial denaturation	95	180	1
Denaturation	95	10	
Annealing	58	30	40
Elongation	72	30	
Denaturation	95	60	1
Renaturation	55	60	1
Melting Curve	55 to 95 Increase set point temperature after cycle 2 by 0,5 °C	10	81

5.2.8. Denaturing SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Polyacrylamide gels for SDS-PAGE were prepared by pouring first the resolving gel followed by the stacking gel containing a comb to produce slots for sample loading. Composition of gels is listed in Table 5.13.

Table 5.13. Composition of polyacrylamide gels (10ml)

	10% resolving	12% resolving	4% stacking
H_2O	3.8 mL	3.3 mL	3.1 mL
30% Acrylamide/Bis solution 29:1 (BioRad)	3.4 mL	4.0 mL	0.67 mL
Tris-HCl pH 8.8	2.5 mL	2.5 mL	-
Tris-HCl pH 6.8	-	-	1.25 mL
10% SDS	100 µL	100 µL	50 µL
10% ammonium persulfate (APS)	100 µL	100 µL	50 µL
TEMED (BioRad)	10 µL	10 µL	5 µL

For comparable starting amounts, about 100mg leaf tissue was harvested to 1.5mL Eppendorf tubes each with ~20 1.0 mm Zirconia Beads (BioSpec), then frozen in liquid nitrogen and homogenized using a TissueLyser (Qiagen, Hilden, Germany) for 2x 30 sec at 30 strokes/sec. Equal amounts of 2x Laemmli sample buffer was added to each tube, samples were boiled for 5-10min in a heating block and used directly for western blot analysis. Gels were placed into electrophoresis tanks submerged in 1x SDS running buffer. A prestained protein ladder (Spectra Multicolor Broad Range Protein Ladder, Thermo Scientific) was loaded alongside with the denatured protein samples and samples were separated at 80-120V.

5.2.9. Western blot and immunodetection of proteins

Proteins separated by SDS-PAGE were electro-blotted onto PVDF transfer membranes (Thermo Scientific) in electrophoresis tanks submerged in 1x transfer buffer for 120 min at 150mA. Equal protein transfer was monitored by staining membranes with Ponceau S (Sigma-Aldrich). Destained membranes were blocked for 1h in 5% milk in PBST before incubation in 5% milk in PBST containing primary antibody overnight. The appropriate horseradish peroxidase (HRP)-conjugated secondary antibody was applied and proteins were detected using Enhanced Chemiluminiscence Reagent (ECL; Pierce Thermo Scientific) either by exposure on a photographic film (BioMax light film, Kodak) or by using the ChemiDoc MP imaging system (BioRad).

5.2.10. Chromatin preparation and immunoprecipitation

4-week-old wild-type plants or plants expressing *WRKY33-HA* from the native *WRKY33* promoter (*pWRKY33:WRKY33-HA*) were spray inoculated or mock treated for 14 hours. ChIP

assays were performed following the protocol Gendrel et al. (2005) with some modifications. Each 2g 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 30s each with 30s of cooling on ice in between. The sheared chromatin (25µg) was diluted 10-fold in ChIP dilution buffer 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; Sigma) 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 ug of RNAase (Roche) and 2µg 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, pH8.0. One microliter was used for qPCR assays.

5.2.11. ChIP-seq assay

ChIP DNA was recovered using a QIA quick PCR Purification kit (Qiagen) and subjected to a modified linear DNA amplification (LinDA) protocol described recently (Shankaranarayanan et al., 2011). The major modification included two rounds of '*in vitro* transcription' by T7 RNA polymerase. The resulting LinDA DNA was used to generate sequencing libraries bearing barcodes using a NEBNext ChIP-Seq Library Pre Reagent Set for Illumina kit (New England Biolabs). The barcoded libraries were pooled and sequenced by Illumina HiSeq2500. The ChIP sequencing was performed in the Max Planck Genome Centre Cologne and resulted ~10 million 100bp single-end reads per sample.

5.2.12. ChIP-seq data analysis

Before mapping, remaining LinDA adapters and low quality sequences were removed from the sequencing data using a two-step procedure. In this procedure, first Bpm and T7-Bpm sites were trimmed from the 5' end using cutadapt (version 1.2.1) (Martin, 2011) with options –e 0.2, -n 2 and –m 36 (otherwise default settings were used), and subsequently poly-A and poly-T tails and low quality ends were trimmed and reads with overall low quality or with less than 36 bases remaining after trimming were removed using PRINSEQ lite (version 0.20.2)

(Schmieder and Edwards, 2011) with options –trim_qual_right/left 20, trim_tail_right/left 3 –min_len 36, -min_qual_mean 25. After this pre-processing steps, the remaining high quality reads were mapped to the *Arabidopsis thaliana* reference genome TAIR10 (http://www.arabidopsis.org) using Bowtie (version 0.12.7) (Langmead et al., 2009) with options –best –m 1 to extract only uniquely mapped reads and allowing two mismatches in the first mapping steps (default settings).

To identify genomic DNA regions enriched in sequencing reads in the ChIP sample compared to input control as well as in inoculated compared to mock-treated samples ('peak regions'), the peak calling algorithm of the QuEST program (version 2.4) (Valouev et al., 2008) was applied using the transcription factor mode (option "2"), with permissive parameter settings for the peak calling (option "3"). Each of the two biological replicates was first analyzed separately and additionally, to obtain more exact peak locations for the consistent peaks, the mapped reads of the two replicates were pooled and peaks were also called for the pooled samples. To annotate the peak location with respect to annotated gene features in TAIR10 the annotatePeaks.pl function from the Homer suite (Heinz et al., 2010) was used with default settings. To extract consistent peaks between the replicates, a custom R (http://www.r-project.org) script was used that identified overlapping peak regions between the replicates. Two peak regions were counted as overlapping, if they overlapped by at least 50% of the smaller region and a peak region was counted as consistent, if it was found to be overlapping between the two individual replicates as well as the pooled sample.

To search for conserved binding motifs in the consistent WRKY33 binding regions, for each consistent peak the 500bp sequence surrounding the peak maximum was extracted and submitted to the online version of MEME-ChIP (Machanick and Bailey, 2011). MEME-Chip was run with default settings, but a custom background model derived from the Arabidopsis genome was provided and "Any number of repetitions" of a motif was allowed. For visualization, prominent motifs identified within MEME-ChIP by either MEME or DREME were chosen. To extract the number/percentage of peak regions that contain a certain motif, the online version of FIMO (Grant et al., 2011) was run with the peak sequences and the motif of interest (MEME/DREME output) as input and a p-value threshold of 0.001. Note: These analysis were performed by Dr. Barbara Kracher.

5.2.13. RNA-seq assay

Total RNA was extracted from mock treated (14hpi) and *B. cinerea* infected (14hpi) 4-week-old plants (Col-0 and *wrky33*) with using RNeasy Plant Mini (Qiagen) according to the manufacturer's instructions, and mRNA sequencing libraries were constructed with barcodes using the TrueSeq RNA Sample Preparation Kit (Illumina). The barcoded libraries were pooled together and sequenced by Illumina HiSeq2500. Three biological replicates were processed and RNA sequencing was performed by the Max Planck Genome Centre Cologne resulting in 25-45 million 100 bp single end reads per sample. Total reads were mapped to the *Arabidopsis* genome (TAIR10) using TopHat (version 2.0.10) (Kim et al., 2013a) with settings –a 10 –g 10 and splice sites provided based on TAIR10 gene annotations.

5.2.14. Statistical Analysis of RNA-seq

The mapped RNA-seq reads were transformed into a count per gene using the function coverageBed of the bedTools suite (Quinlan and Hall, 2010). Genes with less than 50 reads in all samples together were discarded, and subsequently the count data of the remaining genes were log-transformed and normalized by the function voom from the R package limma (version 3.10.3) (Smyth et al., 2005) to yield log2 counts per million. To analyze differential gene expression between genotypes (WT, *wrky33*) and treatments (mock treated, *B. cinerea* infected), we fitted a linear model with the explanatory variable "genotype_treatment" (i.e. including both genotype and treatment) using the function lmFit (R package limma). Subsequently, we performed moderated t-tests over the four contrasts of interest. Two contrasts compare *B. cinerea* infected vs mock treated samples within each genotype and the other two contrasts compare *wrky33* vs. WT Col-0 plants within each treatment. In all cases, the resulting P values were adjusted for false discoveries due to multiple hypothesis testing via the Benjamini–Hochberg procedure. For each contrast, we extracted a set of significantly differentially expressed genes between the tested conditions (adj. p value < 0.05, log2FC≥1). Note: These were performed by Dr. Barbara Kracher.

5.2.15. Gene Ontology analysis

The Bingo 2.44 plug-in implemented in Cytoscape v2.81 was used to determine and visualize the GO enrichment according to the GOslim categorization. A hypergeometric distribution statistical testing method was applied to determinate the enriched genes and the Benjamini and Hochberg FDR correction was performed in order to limit the number of false positives. The FDR was set up to 0.05 for the ChIP-seq and expression data, respectively.

5.2.16. Phytohormone measurements and quantification

Sample processing, data acquisition, instrumental setup, and calculations were performed as described (Ziegler et al., 2014). Instrument specific parameters for the detection of SA are shown in Table 5.14. (3,4,5,6-D_4)-SA was obtained from Campro Scientific (Veenendal, The Netherlands) and used as internal standard for SA quantification (1.5 ng per sample). Note: These experiments were performed by Dr. Jörg Ziegler (IPB Halle, Germany).

Table 5.14. parameters for the detection of SA.

MS parameters for MRM-transition of salicylic acids (SA)

Hormone	MRM transitions	Declustering potential (DP), V	Entrance potential (EP), V	Cell entrance potential (CEP), V	Collision potential (CE), V	Cell exit potential (CEX), V
SA	**137→93**	-25	-5.5	-14	-22	0
	137→65	-25	-5.5	-14	-44	0
SA-D_4	**141→97**	-25	-5.5	-14	-22	0

Quantifier and qualifier transitions are indicated in bold and italics, respectively

Abbreviations

-	fused to (in the context of gene/protein fusion constructs)
%	percent
°C	degrees Celsius
3'	downstream region (of a gene or sequence)
35S	*35S* promoter from Cauliflower mosaic virus
5'	upstream region (of a gene or sequence)
ABA	abscisic acid
AP2/ERF	APETALA2/ETHYLENE RESPONSE FACTOR
Avr	avirulence
B.c.	*Botrytis cinerea*
bp	base pair
CC	coiled-coil
cDNA	copied DNA
ChIP	chromatin immunoprecipitation
ChIP-seq	ChIP sequencing
CNL	CC-NLR
CPKs	calcium-dependent protein kinases
CWDEs	cell wall-degrading enzymes
d	day
ddH_2O	deionised distilled water
dH_2O	deionised water
DNA	desoxyribonucleic acid
DNase	desoxyribonuclease
dNTP	desoxynucleosidetriphosphate
EDS1	Enhanced disease susceptibility 1
EDTA	ethylenediaminetetraacetic acid
EP	exopolysaccharide
ET	ethylene
ETI	effector-triggered immunity
EtOH	ethanol
g	gram
G. orontii	Golovinomyces orontii
h	hour
hpi	hours post infection
HR	hypersensitive response
HRP	horseradish peroxidase
ICS1	ISOCHORISMATE SYNTHASE 1
Ile	isoleucine
IP	immunoprecipitation
JA/JAs	jasmonic acid/jasmonates
JA-Ile	JA-isoleucine
JAR1	JASMONATE RESISTANT 1

kb	kilobasepair
kDa	kilodalton
L	liter
LOX2	LIPOXYGENASE 2
LRR	leucine-rich repeats
m	milli
M	molar (mol/L)
MAMP	microbe-associated molecular pattern
MAPK	mitogen activated protein kinase
min	minute
mM	millimolar
mo	mock
mRNA	messenger RNA
MW	molecular weight
NB	nucleotide binding
NB-LRR	nucleotide-binding-lucine-rich repeat
ng	nanogram
NLR	NB-LRR receptor
nm	nanometer
NPR1	Nonexpressor of PR genes1
N-terminal	amino-terminal
OPDA	12-oxo-phytodienoic acid
ORA59	OCTADECANOID-RESPONSIVE ARABIDOPSIS59
ox	overexpressor
p value	probability value
P.	*Pseudomonas*
PAA	polyacrylamide
PAD3	PHYTOALEXIN DEFICIENT 3
PAD4	*Phytoalexin deficient 4*
PAGE	polyacrylamide gel-electrophoresis
PBS	phosphat buffered saline
PCD	programmed cell death
PCR	polymerase chain reaction
PDF1.2	PLANT DEFENSIN 1.2
pH	negative decimal logarithm of the H^+ concentration
PR	*Pathogenesis related*
PRR	pattern recognition receptor
Pst	*Pseudomonas syringae* pv. *tomato*
PTI	pattern-triggered immunity
pv.	pathovar
qRT-PCR	quantitative real-time PCR
R	*Resistance*
RLK	receptor-like kinase
RNA	ribonucleic acid

Abbreviations

RNA-seq	RNA sequencing
ROS	reactive oxygen species
rpm	rounds per minute
RPS	*Resistance to P. syringae*
RT	room temperature
RT-PCR	reverse transcription-polymerase chain reaction
SA	salicylic acid
SD	standard deviation
SDS	sodium dodecyl sulphate
sec	second
SID2	SALICYLIC ACID INDUCTION DEFICIENT 2
SNC1	*Suppressor of npr1, constitutive 1*
Taq	Thermophilus aquaticus
TEMED	N,N,N',N'-Tetramethylethylenediamine
TF	transcription factor
TIR	Toll/Interleukin-1 receptor like
TNL	TIR-NLR
Tris	Tris-(hydroxymethyl)-aminomethane
U	unit
V	Volt
VSP2	VEGETATIVE STORAGE PROTEIN 2
w33	*wrky33*
WRKY	WRKY transcription factor
WT	wild-type
µ	micro

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Supplementary tables

Table S1. List of WRKY33 regulated target genes involved in cell death.

Gene	Description	score_ChIP	log2FC Bc KO-WT
AT5G05730	AMT1	22,94	-1,60
AT2G30770	CYP71A13	12,74	-3,17
AT3G26830	CYP71B15	56,29	-1,61
AT4G23810	ATWRKY53	49,21	1,45
AT5G22570	ATWRKY38	13,52	4,50
AT2G38470	ATWRKY33	30,45	-3,32
AT5G60900	RLK1	28,11	1,17
AT2G32680	AtRLP23	54,73	1,06
AT4G35600	CST	17,28	1,64
AT4G23170	CRK9	18,13	1,33
AT3G09830	protein kinase	39,86	1,16
AT5G48380	BIR1	29,67	1,88
AT4G26070	ATMEK1	26,55	1,36
AT1G01560	ATMPK11	14,73	2,61
AT5G01540	LECRKA4.1	24,64	1,07
AT4G04490	CRK36	22,37	2,01
AT1G18890	ATCDPK1	13,03	1,27
AT3G45290	ATMLO3	22,02	1,41
AT1G11310	ATMLO2	24,36	1,09
AT2G39200	ATMLO12	40,71	2,06
AT5G61210	ATSNAP33	39,65	1,39
AT1G19250	FMO1	11,40	1,75
AT3G22160	JAV1	22,09	1,16
AT3G57260	AtPR2	20,18	3,78
AT4G14400	ACD6	20,11	1,04
AT1G29690	CAD1	20,96	1,02
AT1G07000	ATEXO70B2	17,84	1,32
AT3G11840	PUB24	24,07	1,11
AT1G76970	Target of Myb1	16,21	1,63
AT3G49350	RabGAP/TBC domain	11,75	1,20
AT4G14365	XBAT34	53,67	1,70
AT3G01830	CML40	17,98	2,09
AT2G30550	DALL3	14,30	1,44
AT5G45110	ATNPR3	11,61	1,78
AT5G50200	ATNRT3.1	14,59	1,87
AT5G40780	LHT1	22,52	1,38
AT4G39670	glycolipid transfer	18,06	1,26
AT5G44070	ARA8	27,54	1,20
AT5G24530	DMR6	16,85	2,73

AT1G08450	AtCRT3	41,77	1,94
AT4G34150	calcium-dependent	13,10	1,16
AT4G12470	AZI1	33,92	2,13

Table S2. List of WRKY33 regulated targets representing kinase activity.

Gene	Description	score_ChIP	log2FC Bc KO-WT
AT5G48380	BIR1	29,67	1,88
AT4G08850	LRR-RLP	17,63	1,30
AT2G25440	RLP20	16,99	1,11
AT2G32680	RLP23	54,73	1,06
AT3G11080	RLP35	16,14	2,53
AT3G28890	RLP43	13,88	2,22
AT1G47890	RLP7	11,05	1,55
AT1G51850	LRR-PK	15,51	2,91
AT1G51790	LRR-PK	13,10	2,22
AT3G14840	LRR-PK	28,68	1,26
AT4G11480	CRK32	15,58	1,76
AT4G23170	EP1	18,13	1,33
AT4G23220	CRK14	72,22	1,17
AT1G70520	CRK2	14,66	1,08
AT4G23320	CRK24	28,25	1,54
AT4G04490	CRK36	22,37	2,01
AT4G23150	CRK7	14,37	1,86
AT2G32800	LECRK-S.2	24,78	1,48
AT4G04960	LECRK-VII.1	40,93	-1,75
AT5G01540	LECRK-VI.3	24,64	1,07
AT5G01550	LECRK-VI.2	26,55	2,57
AT4G27300	G-type	19,12	-1,00
AT1G61460	G-type	12,39	1,15
AT1G61420	G-type	24,14	1,12
AT1G11330	G-type	17,06	1,25
AT5G63650	SNRK2.5	16,00	-1,42
AT5G47850	CCR4	46,02	2,34
AT4G35600	CONNEXIN 32	17,28	1,64
AT3G09830	CCR-like	39,86	1,16
AT2G17220	KIN3	18,83	1,02
AT5G42440	AT5G42440	25,14	1,34
AT5G38210	AT5G38210	24,92	1,06
AT1G67000	AT1G67000	16,36	1,12
AT5G47070	AT5G47070	18,48	1,00
AT2G47060	PTI1-4	23,08	1,13
AT1G65790	RK1	13,17	1,70
AT5G60900	RLK1	28,11	1,17
AT1G01560	MPK11	14,73	2,61
AT4G26070	MEK1	26,55	1,36
AT1G18890	CDPK1	13,03	1,27

AT1G21270	WAK2	35,90	1,01

Table S3. List of WRKY33 target genes encoding transcription factors.

Gene	Description	score_ChIP	log_2FC Bc KO-WT
AT1G25560	EDF1	24,29	-0,42
AT2G41710	AP2-like ERF	12,46	-0,18
AT4G37750	ANT	24,36	-0,05
AT5G07580	ERF/AP2	11,97	-0,98
AT1G04370	ATERF14	17,63	-0,71
AT4G17500	ATERF-1	50,34	0,01
AT3G23240	ATERF1	35,69	-0,13
AT5G47230	ATERF-5	21,67	-1,35
AT5G53290	CRF3	28,46	0,36
AT5G25190	ESE3	16,85	-0,97
AT1G71520	ERF020	16,21	0,22
AT1G33760	ERF022	19,47	-0,95
AT1G64380	ERF061	12,53	1,14
AT1G06160	ORA59	21,31	-2,45
AT3G23220	ESE1	12,60	0,15
AT5G43410	ERF096	14,94	-1,14
AT4G34410	RRTF1	18,27	1,16
AT2G33710	ERF112	29,24	-0,37
AT5G07310	ERF115	31,44	n.d.
AT1G68550	CRF10	17,35	-0,75
AT1G78080	RAP2.4	21,24	0,44
AT3G27785	MYB118	12,46	n.d.
AT3G23250	ATMYB15	24,71	1,28
AT1G66230	AtMYB20	22,37	-0,44
AT1G74650	ATMYB31	19,12	-0,74
AT5G06100	ATMYB33	12,89	0,03
AT4G12350	AtMYB42	19,61	-0,56
AT5G16600	AtMYB43	11,05	0,29
AT1G18570	AtMYB51	37,46	-0,50
AT1G68320	AtMYB62	32,85	0,09
AT5G11050	MYB64	23,86	n.d.
AT5G65790	ATMYB68	17,28	-2,64
AT2G23290	AtMYB70	13,45	-0,84
AT4G37260	ATMYB73	17,21	0,62
AT4G05100	AtMYB74	14,94	0,85
AT4G22680	AtMYB85	24,99	-0,69
AT5G62470	ATMYB96	13,31	0,34
AT5G05090	MYB	17,91	1,39
AT3G10760	MYB	25,28	-0,21
AT1G25550	MYB like	15,44	0,62

AT4G38620	ATMYB4	18,34	0,38
AT1G14350	AtMYB124	28,11	0,70
AT1G14600	MYB like	19,61	-0,49
AT5G24110	ATWRKY30	29,88	0,01
AT2G04880	ATWRKY1	16,78	0,26
AT4G01250	AtWRKY22	23,51	0,47
AT2G40740	ATWRKY55	12,53	1,88
AT4G31550	ATWRKY11	22,87	0,50
AT2G23320	AtWRKY15	33,14	0,16
AT4G23550	ATWRKY29	18,98	0,38
AT2G38470	ATWRKY33	30,45	-3,32
AT5G22570	ATWRKY38	13,52	4,50
AT4G11070	AtWRKY41	35,61	2,72
AT4G04450	AtWRKY42	11,68	0,09
AT5G49520	ATWRKY48	19,26	1,38
AT5G26170	ATWRKY50	23,65	3,56
AT4G23810	ATWRKY53	49,21	1,45
AT1G29280	ATWRKY65	26,62	-0,04
AT1G80590	ATWRKY66	14,51	n.d.
AT1G29860	ATWRKY71	17,63	0,49
AT5G13080	ATWRKY75	30,02	-0,38
AT3G15510	ANAC056	18,55	-0,93
AT1G61110	ANAC25	35,83	n.d.
AT5G13180	ANAC083	22,09	-0,20
AT2G43000	ANAC042	15,15	-0,51
AT3G15500	ANAC055	24,71	-0,96
AT5G22380	NAC090	12,82	3,49
AT3G44350	NAC061	21,81	3,89
AT3G12910	NAC	17,63	-0,12
AT1G35560	TCP23	25,49	-0,50
AT3G15030	MEE35	35,83	-0,28
AT5G23280	TCP7	13,31	-0,89
AT2G45680	TCP9	17,42	-0,19
AT1G61660	bHLH112	19,61	0,97
AT2G43140	bHLH129	21,60	1,21
AT2G40200	bHLH51	18,83	-0,23
AT4G29100	bHLH68	11,97	-0,28
AT5G56960	bHLH041	36,96	0,59
AT2G31730	bHLH	21,81	0,23
AT3G20770	AtEIN3	44,11	0,36
AT4G38900	bZIP like	20,04	0,26
AT1G42990	ATBZIP60	50,63	0,47
AT5G28770	AtbZIP63	31,01	-1,53
AT1G78600	BBX22	16,00	-0,62
AT3G19580	AZF2	16,64	1,38

AT5G66730	ENY	12,53	0,27
AT3G60580	C2H2-like	19,75	0,42
AT2G01940	ATIDD15	17,13	-1,20
AT5G60470	C2H2 and C2HC	12,82	0,64
AT5G04340	C2H2	15,22	0,04
AT2G40140	ATSZF2	19,90	0,76
AT3G55980	ATSZF1	42,77	0,16
AT4G29190	AtC3H49	18,48	-0,99
AT5G46910	C5HC2 type	17,13	0,45
AT1G30810	JMJ18	18,62	0,52
AT1G51700	ADOF1	42,06	0,80
AT4G24060	DOF4.6	16,78	0,50
AT5G02460	DOF5.1	11,97	-0,15
AT4G27310	BBX28	13,24	-0,61
AT4G39070	BBX20	13,24	0,75
AT2G41310	ARR8	28,18	-0,12
AT5G24470	APRR5	12,25	0,30
AT1G21450	SCL1	25,77	0,61
AT5G59450	SCL11	26,98	-0,23
AT1G07530	ATGRAS2	13,03	-0,36
AT3G46600	SCL30	16,43	0,31
AT5G48150	PAT1	26,55	-0,02
AT4G17230	SCL13	33,49	0,35
AT1G07520	GRAS	13,38	0,27
AT3G06740	GATA15	15,65	-0,13
AT4G17570	GATA26	17,63	0,29
AT5G66320	GATA5	24,99	-0,06
AT3G54810	BME3	16,92	-0,04
AT5G39760	AtHB23	20,67	-0,14
AT4G00730	AHDP	13,45	-0,24
AT3G61890	ATHB-12	13,24	0,08
AT4G37790	HAT22	14,23	-0,13
AT3G60390	HAT3	15,79	0,77
AT5G44180	RLT2	11,12	0,31
AT3G18010	WOX1	32,50	-0,44
AT2G43500	RWP-RK	23,22	-0,09
AT1G76350	RWP-RK	16,71	0,01
AT2G36960	TKI1	29,81	0,29
AT5G46760	MYC3	16,78	-0,31
AT2G22770	NAI1	12,53	0,64
AT2G27100	SE	17,21	-0,01
AT4G18880	AT-HSFA4A	78,59	0,76
AT1G06040	BBX24	15,44	-0,34
AT3G14020	NF-YA6	16,14	-0,60
AT1G55110	AtIDD7	10,13	-0,17

AT4G23800	3xHMG-box2	16,78	1,25
AT1G80420	ATXRCC1	45,88	-0,28
AT2G22630	AGL17	13,03	1,69

Note: Transcription factors highlight in red color indicate WRKY33-regulated transcription factors.

Acknowledgments

This work has been carried out at the Department of Plant Microbe Interactions, Max-Planck-Institute for Plant Breeding Research with the PhD fellowship from International Max Planck Research Schools (IMPRS).

I extremely appreciate my supervisor, Dr. Imre E. Somssich, for his excellent guidance, patience, precise and consistent encouragement, support and promotion my PhD research over the years. He is always available for discussing science and solving my problems in the research. He is always patient to answer my strange and even stupid questions. Most importantly, he gives me a lot of freedom to try any possible ideas, but also pinpoints the problem and brings me back to the right direction. Special thanks also for his patient and conscientious language correcting on my thesis and manuscript.

I would like to express my deep gratitude to Prof. Dr. Paul Schulze-Lefert, thanks him for providing so excellent place and environment for my PhD research, and thanks him for continuously supporting my study in the department.

I also want to thank my thesis committee members, Prof. Dr. Paul Schulze-Lefert, Prof. Dr. Ulf-Ingo Flügge and Prof. Dr. Ute Höcker, for their kindly reviewing my thesis and scientific discussion.

Great thanks are given to Dr. Barbara Kracher in Dept. of Plant-Microbe Interactions, MPIPZ, for her great helps and excellent works in bioinformatics; and to Dr. Jörg Ziegler in IPB Halle, Germany, for his great helps and excellent works in hormone measurements.

I sincerely acknowledge to Dr. Rainer P. Birkenbihl, for his invaluable helps and teaching on my experiments. Without his patient and experienced guidance, finishing this work would take more time. Rainer, thank you so much!

I wish to thank Ms. Elke Logemann, she is experienced in so many experiments, and many thanks for her warm and selfless helps during my working in the past three years.

I also wish to thank my colleagues Ms. Lydia Bollenbach, Dr. Moritz Schön, Dr. Armin Töller and Dr. Mario Roccaro, thanks for their helps during my works.

I also want to thank Dr. Julia Schumacher and Prof. Dr. Paul Tudzynski from University of Münster, Münster, for their kindly providing *Botrytis cinerea* strain B05.10 and the associated mutants. Thanks Dr. Floriane L'Haridon from University of Fribourg, Switzerland, kindly providing *B. cinerea* strains BMM and B05.10.

Many thanks to people who kindly share their *Arabidopsis* mutants. Thanks for all their previous works.

Finally, I appreciate my parents and my family for their unconditional love and support !

Erklärung

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Köln, 15th November, 2014 _____

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