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Microarray Analysis of Arabidopsis Wrky33 Mutants in Response to the Necrotrophic Fungus Botrytis Cinerea

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MICROARRAY ANALYSIS OF ARABIDOPSIS *WRKY33* MUTANTS IN RESPONSE TO THE NECROTROPHIC FUNGUS *BOTRYTIS CINEREA*

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Under the Supervision of Dr. Synan AbuQamar

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Declaration of Original Work

I, Shamma Saeed Salem Al Shamsi, the undersigned, a graduate student at the United Arab Emirates University (UAEU), and the author of this thesis entitled "*Microarray Analysis of Arabidopsis* WRKY33 *Mutants in Response to the Necrotrophic Fungus Botrytis cinerea*", hereby, solemnly declare that this thesis is my own original research work that has been done and prepared by me under the supervision of Dr. Synan AbuQamar, in the College of Science at UAEU. This work has not previously been presented or published, or formed the basis for the award of any academic degree, diploma or a similar title at this or any other university. Any materials borrowed from other sources (whether published or unpublished) and relied upon or included in my thesis have been properly cited and acknowledged in accordance with appropriate academic conventions. I further declare that there is no potential conflict of interest with respect to the research, data collection, authorship, presentation and/or publication of this thesis.

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Abstract

Plants are exposed to many environmental stresses that affect their growth and development. These stresses include biotic stresses (via organisms) and abiotic stresses (via environment). Plants respond to these stresses by transcriptional reprogramming and different signaling pathways. *Arabidopsis thaliana* has shown great sensitivity to the biotic stress *Botrytis cinerea*. The *WRKY33* gene plays an important role in plant defense mechanism against this pathogen. The overall goal is to identify common regulated genes of *wrky33* mutant and 35S:*WRKY33* overexpressing transgenic lines in response to *B. cinerea*; ultimately to improve plant stress tolerance in Arabidopsis. The specific aims are to: (1) identify Arabidopsis regulated genes in response to *B. cinerea* infection; (2) determine common up- and down-regulated genes in response to *B. cinerea* infection in Arabidopsis *wrky33* mutant and 35S:*WRKY33* overexpression lines; and (3) characterize the function of the common genes in response to *B. cinerea*. To achieve these objectives, a microarray-based analysis of Arabidopsis wild-type, *wrky33* mutant and 35S:*WRKY33* overexpression line in response to *B. cinerea* will be studied for their regulation to disease and stress responses. The results of this study will help identifying the up- and down-regulated defense genes to these stresses and improving crop tolerance to these stresses. In addition, Arabidopsis expression profiling of defense regulated genes in response to these stresses will help understanding how the plants respond to stress in field conditions. Overall, this can help introducing defense genes that make the crops more resistance to different stresses, particularly *B. cinerea*.

Keywords: *Arabidopsis, Botrytis cinerea*, defense response, microarray, WRKY33 transcription factor.

Title and Abstract (in Arabic)

استخدام تقنية المايكروأري في تحليل استجابة نبتة (Arabidopsis) المعدلة وراثيا للإصابة بالفطر المميت (*Botrytis cinerea*)

الملخص

تتعرض النباتات بشكل عام للكثير من الضغوطات البيئية التي تؤثر على نموها و تطورها. فقد تنشأ هذه الضغوطات إما بفعل كائنات حية أخرى (biotic stresses) أو بسبب عوامل غير حية مثل درجة الحرارة، الملوحة، و الجفاف (abiotic stresses). عادةً تستجيب النباتات لمثل هذه الضغوطات عن طريق اعادة برمجة نظام النسخ لحمضها النووي و أيضا من خلال ارسال مسارات مختلفة من الاشارات الكيميائية حتى تتمكن خلايا النبتة من التواصل فيما بينها بشكل فَعَّال. لقد لوحظ بأن لدى نبات الأرابيدوبسيس تاليانا (*Arabidopsis thaliana*) حساسية شديدة لفطر العفن الرمادي المميت (*Botrytis cinerea*). كما لاحظ العلماء مدى فاعلية جين (*WRKY33*) في آليات الدفاع ضد مسببات الأمراض لدى النباتات.

إن الغاية الأساسية من هذه الأطروحة هي التعرف على الجينات التي تنظم استجابة نبات الأرابيدوبسيس (*Arabidopsis*) لمقاومة الفطر السابق ذكره. بحيث سيتم دراسة ذلك على ثلاث نباتات من الأرابيدوبسيس اثنان منها معدلة وراثية إما بحذف جين (*WRKY33*) أو بزيادة انتاجه (*35S:WRKY33*) أما النبات الثالث فهو غير معدل وراثيا. كما تتلخص تلك الغاية من خلال الأهداف التالية: (1) تحديد الجينات المُنظمة لإستجابة نبات الأرابيدوبسيس (*Arabidopsis*) لهجوم فطر العفن الأسود(*B. cinerea*)؛ (2) التعرف على الجينات التي ستُثَبَّط و الجينات التي سيزيد نشاطها في النباتات المعدلة وراثيا بعد إصابتها بالفطر؛ و (3) توصيف وظيفة الجينات المشتركة في الاستجابة ضد فطرالعفن الأسود. و للوصول لهذه الأهداف تم استخدام بعض التقنيات و منها تقنية المايكروأري. من المتوقع بأن نتائج هذه الدراسة ستساعد العلماء على تحديد مدى نشاط الجينات الدفاعية في عملية تحمل الضغوطات المختلفة. مما سيوفر فهماً عميقاً حول كيفية استجابة النباتات و المحاصيل الزراعية بشكل خاص للآفات المختلفة، بالتحديد فطر العفن الأسود.

مفاهيم البحث الرئيسية: نبات الأرابيدوبسيس تاليانا، العفن الرمادي، تقنية المايكروأري، استجابة الدفاع، عامل النسخ WRKY33.

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Special thanks go to my family and friends for helping me along the way. I am sure they suspected it was endless.

Dedication

To my beloved parents and family

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List of Abbreviations

ABA	Abscisic acid
CWDEs	Cell wall degrading enzymes
ET	Ethylene
EXLA2	Expansin-like A2
GAs	Gibberellins
HUB1	Histone mono-ubiquitination1
JA	Jasmonate
MAMPs	Microbe-associated molecular patterns
MAPK	Mitogen Activated Protein Kinase
OPDA	12-oxophytodeniec acid
PP	Phytoprostanes
PRRs	Pattern recognition receptors
PG	Poly-galacturonase
PGIPs	Poly-galacturonase-inhibiting proteins
R	Resistance
ROIs	Reactive oxygen intermediates
SAR	Systemic acquired resistance
SA	Salicylic acid
PPA_1	Phytoprostane A_1
PPI	protein-protein interaction
GO	Gene ontology
PCR	Polymerase chain reaction
qRT-PCR	Quantitative real-time polymerase chain reaction
dpi	Days post infection

hpi	Hours post inoculation
BiNGO	Biological networks gene ontology
REF	*Rubber elongation factor*
BDGs	*B. cinerea*-down regulated genes
BUGs	*B. cinerea*-up regulated genes
*PBDG*s	PPA_1/*B. cinerea*-down regulated genes
*PBUG*s	PPA_1/*B. cinerea*-up regulated genes
OBDGs	OPDA/*B. cinerea*-down regulated genes
OBUGs	OPDA/*B. cinerea*-up regulated genes

Chapter 1: Introduction

1.1 The importance of Arabidopsis

Arabidopsis thaliana is a member of the family Brassicaceae which is small in size and has small leaves and a main stem which can reach in the adult stage up to 30–40 cm terminating on an inflorescence (Soledade et al. 2010). This plant has lately shown significant importance in research, due to its short life cycle, small sequenced genome, large number of mutant lines and genomic resources, and possibility to be genetically transformed. Together, these have enabled Arabidopsis to be considered as a model plant (Conn et al. 2013).

1.2 *Botrytis cinerea*

Botrytis cinerea is among the top ten fungal pathogens that causes plant diseases and negatively affects the agribusiness section for a wide range of crops (Dean et al. 2012). This pathogen usually affects many types of crops such as: grapes, forming a mold that is grayish in color and powdery in texture known as the gray mold (Snowdon 1990). Moreover, *B. cinerea* usually infects the weak damaged parts in a plant, and then spreads to the whole plant. *B. cinerea* uses multiple strategies to penetrate and attack its host tissues by physical or chemical means (Isaac 1992).

1.3 Host invasion by of *B. cinerea*

During the pathogenesis, *B. cinerea* induces host cell death by producing toxins, cell wall degrading enzymes (CWDEs) and reactive oxygen intermediates (ROIs) (Prins et al. 2000; Muckenschnabel et al. 2002; Wolpert et al. 2002). Although cell death and accumulation of ROIs are associated with plant resistance to biotrophic

pathogens (Lamb and Dixon 1997), the ROIs can also increase plant susceptibility to necrotrophs (Govrin and Levine 2000). In addition, the plant polygalacturonase-inhibiting proteins (PGIPs) counteract polygalacturonase (PG) which are important host colonizing factors for some fungal pathogens (Ferrari et al. 2003). Although the cell wall and cuticle protect plants against pathogen penetration or infection, Arabidopsis mutants defective in components of the cell wall and cuticle were resistant to *B. cinerea* (Kurdyukov et al. 2006; Chassot et al. 2007; AbuQamar et al. 2013). In fact, the cell wall and cuticle are primary barriers against pathogen attacks that may decrease or enhance plant resistance to pathogens. For instance, a loss-of-function of the *HISTONE MONOUBIQUITINATION1* (*HUB1*) gene, encoding an E3 ligase required for histone H2B ubiquitination, reduces the cell wall thickness and increases the susceptibility to *B. cinerea* and *Alternaria brassicicola* (Dhawan et al. 2009).

1.4 Plant-pathogen interaction

Plant responses to necrotrophic fungi are complex and multigenic. They often depend on plant species, pathogens and their virulence and signaling pathways being involved (Wolpert et al. 2002; Glazebrook 2005). A mixture of effectors and microbe-associated molecular patterns (MAMPs) play important roles in the determination of plant-pathogen interactions. High-throughput technologies such as microarray are now commonly used to study the molecular mechanisms that control plant responses to environmental stresses, hormonal signals and pathogens.

1.5 Plant recognition of the pathogen

Similarly, to animals, plants recognize elicitors derived from pathogens to activate innate immune defense responses (Montesano et al. 2009). In contrast to race-specific elicitors or resistance (R) genes described for biotroph-plant interactions, plants recognize a pathogen -regardless of its lifestyle- via MAMP general elicitors (Jones and Dangl 2006; Boller and Felix 2009). Chitins and glucans are fungal MAMPs that plants can recognize by pattern recognition receptors (PRRs). The Arabidopsis FLS2 and EFR receptor kinases independently recognize the bacterial flagellin (flg22) and elongation factor Tu (elf18) epitopes, respectively, as MAMPs (Gomez-Gomez and Boller 2000; Kunze et al. 2004; Zipfel et al. 2006). Recognition of *B. cinerea* MAMPs activates plant innate immunity system through mitogen activated protein kinase (MAPK)-based signaling cascades (Ren et al. 2008; Pitzschke et al. 2009), suggesting that the MAMP signaling mediates a conserved MAPK pathways and confers resistance to both bacterial and fungal pathogens. In Arabidopsis, systemic acquired resistance (SAR) can also be initiated upon MAMP recognition to induce defense responses (Mishina and Zeier 2007).

1.6 Plant defense mechanisms

Plant hormones also play crucial roles in triggering defense responses to pathogens. For example, signaling pathways involving salicylic acid (SA), ethylene (ET), jasmonate (JA), abscisic acid (ABA), auxin and gibberellins (GAs) may act independently, synergistically or antagonistically to confer to plants the resistance to diseases (Glazebrook 2005; AbuQamar et al. 2006; Berrocal-Lobo and Molina 2008; Koornneef and Pieterse 2008; Llorente et al. 2008; Laluk et al. 2011a, b).

Even though genetic studies in Arabidopsis and tomato implicate that SA-mediated responses and SAR are associated with resistance to biotrophic pathogens (Govrin and Levine 2002), JA and ET are key regulators of plant responses to necrotrophic pathogens such as *B. cinerea* (Thomma et al. 1999; Diaz et al. 2002; Glazebrook, 2005; AbuQamar et al. 2006; 2008). Recently, the cyclopentenone, 12-oxo-phytodeniec acid (OPDA) and phytoprostanes (PP), have been reported to accumulate upon infection by various pathogens (Thoma et al. 2003; Block et al. 2005; Mueller et al. 2008; AbuQamar et al. 2013; Sham et al. 2014, 2015). OPDA, a JA precursor, is produced enzymatically from α-linolenic acid and forms JA and/or its conjugates by OPR3 followed by three steps of ß- oxidation (Mueller 1997). Phytoprostanes (PP), on the other hand, are produced nonenzymatically from α-linolenic acid via a free radical-catalyzed pathway. Mutations in *OPR3* and *expansin-like A2* (*EXLA2*) genes can modulate gene expression through cyclopenteone/COI1 independently from JA under biotic stress (Ribot et al. 2008; AbuQamar et al. 2013). Yet, little is known about the role of electrophilic oxylipins OPDA or PPA_1 in plant response to *B. cinerea*.

Nonetheless, gene expression profiling has been established in response to necrotophic pathogens in many plant species such as Arabidopsis and tomato (Schenk et al. 2003; AbuQamar et al. 2006; Hernandez -Blanco et al. 2007; Windram et al. 2012; Smith et al. 2014; Sham et al. 2014, 2015). Previously, *wrky33-1* and *wrky33-2* were identified as Arabidopsis mutants with increased susceptibility to *B. cinerea* and other necrotophic pathogens (Zheng et al. 2006). Ectopic overexpressing lines of WRKY33 showed enhanced resistance to *B. cinerea* compared with the wild-type. Here, we aimed at identifying transcriptional responses mediated by WRKY33 at early stages of *B. cinerea* infection using microarray-based analysis to examine the

expression profiling in Arabidopsis *WRKY33* transgenic plants. The expression levels of 2714 genes were altered at least twofold: 1660 genes were *B. cinerea*-upregulated genes (*BUG*s) and 1054 genes were *B. cinerea*-downregulated genes (*BDG*s), representing, respectively, about 7.3% and 4.6% of the overall *B. cinerea*-responsive genes. About 4821 genes of the *B. cinerea*-regulated genes were dependent on the presence of *WRKY33*, and 3356 of them were dependent on its absence. We also determined functional classes, and predicted networks of proteins and motifs related to defense responses and/or non-defense pathways regulated by *B. cinerea* infection. Plant response to *B. cinerea* can be regulated by electrophilic oxylipins, opening the door for opportunities to establish network models of defense signaling pathways during *B. cinerea*-Arabidopsis interactions.

Chapter 2: Materials and Methods

2.1 Plant growth, disease assay and fungal growth

Arabidopsis wild-type, *wrky33-1* mutant and 35S:*WRKY33* overexpression transgenic plants (Zheng et al. 2006) generated from Arabidopsis Col-0 background were used in this study. For disease assays, photos and qRT-PCR analysis, detached leaves (five-week old plants grown in soil) were drop-inoculated with 3 µL of *B. cinerea* spore suspension containing 3×10^5 spores mL^{-1}. For percentage of decayed plants experiment, whole plants (five-week-old) grown in soil were spray-inoculated with *B. cinerea* spore suspension containing 3×10^5 spores mL^{-1}, using a Preval sprayer (Valve Corp., Yonkers, NY, USA). The spore suspension was prepared under the hood; to prevent the risk of contamination as follows: *B. cinerea* strain *BO5-10* was grown on 2 × V8 agar (36% V8 juice, 0.2% CaCO3, 2% Bacto-agar) and then mycelium-containing agar was transferred to fresh 2 × V8 agar and incubated at 20-25°C. Fungal spores (conidia) were then collected from 10-day-old *B. cinerea* cultures and used in the infection assays (AbuQamar et al. 2006).

After inoculation, detached leaves/plants were transferred into a growth chamber and kept under a sealed transparent cover to maintain high humidity at a fluorescent light intensity of 150 µE m^{-2} sec^{-1}; 8 h light/16 h dark and 21 ± 2 °C temperature. Responses to *B. cinerea* infection were assayed at 0 and 24 hours post inoculation (hpi), or otherwise stated. Plants were then visually and regularly examined at 1 and 3 days post infection (dpi) and *B. cinerea*-decayed (rotten) plants were obtained at 2, 4 and 6 dpi.

2.2 RNA extraction and expression analysis of *B. cinerea*-inoculated plants

RNA was isolated from the whole plant tissue. Plant tissue (10g) was homogenized with a Brinkmann Polytron in equal volumes of grinding buffer (50 mM Tris HCl, pH 8.0/4% sodium *p*-aminosalicylic acid/1% sodium naphthalene 1,5-disulfonic acid) and water-saturated phenol. After separating the phases by centrifugation, the aqueous phase was extracted again with phenol/ $CHCl_3$, and then with $CHCl_3$ alone. The aqueous phase was then made 0.5 M LiCl/1 mM EDTA/0.1% Na_2DodSO_4. Up to 0.5 g of oligo (dT) cellulose was added and shaken for 10 min. The slurry was poured into a small glass column and poly $(A)^+$ RNA was collected (Lagrimini et al. 1987). Moreover, real time quantitative-PCR (qRT-PCR) expression analyses were completed using gene-specific primers, with *Arabidopsis thaliana*-specific *Actin2* (*AtActin2*; *At3G18780*) as an endogenous reference for normalization. A minimum of three technical replicates was used for each sample with a minimum of two biological replicates for qRT-PCR (AbuQamar et al. 2013). *B. cinerea* growth in inoculated plants was evaluated by qRT-PCR analysis based on the levels of *B. cinerea ActinA* DNA at 1 and 3 dpi (Benito et al. 1998; van Wees et al. 2003). The relative amplifications of *B. cinerea*-specific *ActinA* (*BcActinA*) to that of the Arabidopsis Actin2 was determined (Bluhm and Woloshuk 2005). Gene expression levels were analyzed with qRT-PCR using gene-specific primers (Table 1) at 0 and 24 hpi with *B. cinerea*. Expression levels of genes were measured in triplicates. To confirm that the efficiencies of the target and reference reactions were equal, reactions were performed using the primers for each gene with serialized dilutions of cDNA as template. After confirming that the efficiencies of the primers were suitable, expression levels were calculated by the comparative cycle threshold method with *AtActin2* (Bluhm and Woloshuk 2005).

Table 1: List of primers (Sequence 5' to 3') used in this study

Description	Left primer sequence	Right primer sequence
AtActin2	GTCGTACAACCGGTATTGTGCTG	CCTCTCTCTGTAAGGATCTTCATGAG
BcActinA	ACTCATATGTTGGAGATGAAGCGCA	AATGTTACCATACAAATCCTTACGGA
At1g60730	AATATGGAATCAGGTATGCAGAGGG	GGCAACATCTACTCGCATTAAACTA
BAP1	CCCAACGAATGATTTCATGGGAAGG	TGACGATCCCACACTTATCACCAAA
GER5	TGGAACTGTCTATCTTTCGAATGCTC	ACCCTGTAGTAGCTCCAAGATTCTT
At5g25930	GAGAAGGAGTTTATTGCTGAAGTTG	AGCTTTGAATCTTCCCTTGAGATAC
CSLE1	CTGGCCTCTGTATAAAGGTATGTTG	GGTACAGGCAGATAAAGCTAAAACA
At4g24160	GGTCATTTTGTGTTCATAGACAACC	GAGTTGTTGATCATGAGAGGAGTCT
At3g44190	GACTTCTCCAGCGGTTAATATCAC	CATTGTGACCAGTAGCAATAACAAG
TolB-related	CAAAGTATCTAAATCCGACGGTTC	GTGTAGATTACATGACGCTCTGTTG
HSP70	GACCAAGCTATTGAATGGTTAGATG	ATAATAGGGTTGCAAAGAGACTCG
HSF4	GCTAGTTGATGATCATAGCACAGAC	GAAGATCTTTAGCAAACTCTGCTGT
UGT87A2	GGAGAGAGGAGATCAAGGAAGTAGT	GACTGATTTCACTAAGGTCACAAGC
CYP89A9	GAAGAGATCAGAGAAGAGGATTTGG	ATGGTAAGACAAGTAATGACCAGGA
CSLD5/SOS6	ATTCAGAGTGCGTTCTGAGCTATAC	TTTAGCATCTCGTCTTCTTCACTCT
CAX7	CCTGGTTGTTTGTTCTGTTCTACTT	TAGGAGACAGCTTCAAAACCTTAGA
NIT4	CATCAAATCTTCCCTGAGATTGAC	CGGGAGTATCGTAGAAGACTGTAGA
TI1	CTATCGTTTCCATCTTCGTTGTCT	GTATTCTTTCAAGCACTCGTTACCT
At4g20860	CTACTCCGACAAGAGAACAATTCC	GGGAATCTAGTGTATGAGTGGTGAG
CYP71B6	TACTCGTCCAAAGCTATCTATCACC	AGTTTCCGCACATCTCTGTAGTAAT
CAD1	ATGAGTTCTTCAGAGAGTGTGGAAA	TAACAGACCTGCGAGTGATAGTATG
MRP4	GATCAGACCAATGTCGATATCCTTA	CGTACTGGCAGGTAACTATGAAAAT
ANAC053	GACGAAGAACTCGTTCGTTACTATC	GGCTCAGATTTGTATACATCGGTAA
CYP72A8	GATACTACTTGGTCGAACCGTAGAG	GATGAACCATAAGAACAGGAATCAC
At1g13990	CAGGCTAGACCCAAAACTAAATTC	GGTCACAGTATCTAACAGCCTCATT
At5g03490	TGTTATTGTTGCCGGGAACTAAATC	AAGTCAAGTAGAGGAAGTAAGTGGC
RD2	TCTTCTTCGTCTCTCTGTGTGTGTA	GCATACAATACATTCATTCCTGAGC
At1g72900	TCAGGGTAACTACTTTGAAAGCCA	AGCAGAACCTTTTGCTTCTTGAGA
At4g30490	TACTACTAGGGTCACCGTCTCAGAT	CAGATATCACCAGTCATGAGTTCAC
Peptidase C15	TTCAGAGAATCCTACTGAGAAGATAGC	CAAGAACACTACAGCTACCAAGACA

2.3 Sample preparation, microarray hybridization and data analysis

Five-week-old whole plants were spray-inoculated with *B. cinerea* spore suspension containing 3×10^5 spores mL^{-1} in inoculation buffer using a Preval sprayer. Control plants (mock) were sprayed with 1% Sabouraud maltose broth buffer, and then kept in the same condition as the *B. cinerea*-inoculated plants, as described above.

RNA samples used for array hybridizations were prepared from tissues infected with *B. cinerea* with each sample containing the entire aboveground part of the inoculated plant and collected at 0 and 24 hpi. Three technical replicates of RNAs were pooled for each genotype per each time point for labeling and hybridization from three independent biological replicates. RNA quality was checked by running an aliquot of 2-µg RNA solution on agarose gel. Sixty micrograms of the total RNAs was purified using Qiagen RNAeasy Mini Kit (Valencia, CA, USA) and used for the subsequent experiments. Following that, cDNA synthesis, samples labeling, array hybridization, scanning, and data processing were conducted (Zhu and Wang 2000).

Affymetrix microarrays (Arabidopsis Genome ATH1 array) used in this study were containing 22,810 total probe sets representing approximately 25,000 genes. These samples are wild-type, *wrky33* and 35S*:WRKY33* plants inoculated with mock (control: Wt-0; *wrky33-0* and *35S:WRKY33-0*) or *B. cinerea* (Wt-24; *wrky33-24* and *35S:WRKY33-24*). Data were analyzed using R software (https://www.r-project.org/) with Affy and MAS5 packages for data analysis and normalization; Affy package for quantifying signal intensity and MAS5 for the detection calls of each probe ID displayed as Present 'P', Absent 'A' and Marginal 'M'. Genes with expressions labeled as 'A' or 'M' across all the samples were removed from the analysis. Log^{2-} transformed expression level data were used to generate scatter plots to detect the effect of *B. cinerea* infection on plant gene expression. Comparisons of three independent replicates for each set of experiments were performed. At each time point, the overall gene expression difference between mock-inoculated (control) and pathogen-inoculated samples of *wrky33* mutant or *35S:WRKY33* overexpression and wild-type were determined by pairwise comparison. Normalized fold change for each gene was calculated by dividing its expression level in *B. cinerea*-treated samples over

its expression level in the control (mock-treated samples). A twofold difference was set as the threshold for considering a gene as to be *BUGs* or *BDGs*. *BUGs* or *BDGs* were considered to be WRKY33-dependent if their average expression levels following *B. cinerea* inoculation in the mutant (*wrky33*) or the overexpressing line (35S:*WRKY33*) vs. wild plant, were twofold induced or repressed. Microarrays data for seedlings treated with OPDA and PPA_1 (Taki et al. 2005; Mueller et al. 2008) were used in the analysis.

2.4 Bioinformatics analyses of Arabidopsis potential protein-protein interactions

Arabidopsis protein-protein interaction (PPI) dataset obtained from The Arabidopsis Information Database (TAIR; www.arabidopsis.org) was used for bioinformatics analyses (motif analysis, conserved domain, functional domain, biological processes, molecular functions, gene ontology).

Mfinder/MDraw tools: The tool, MDraw (http://www.weizmann.ac.il/mcb/UriAlon/), was used to provide a universal visualization tool for directed biological networks and to visualize the network motifs embedded in them by using 1000 randomized network; *P*-value <0.01; Z-score >1; and frequency >4, to describe a network motif (Milo et al. 2002). MDraw relies on MFinder (Kashtan et al. 2004), a software tool that detects network motifs. Motifs found in the input network were available including statistical measures. Accordingly, the patterns with significant measures are the ones that describe the network motifs. The *P* -value measure (ranging between 0-1) indicates the number of random networks in which a motif Gp (a subgraph identified in the input network) occurred more often than in the input network, divided by the total number of random networks. The

smaller the *P* -value, the more significant the predicted motif. The motif id is unique for each motif present which has a universal shape.

CentiScaPe: Cytoscape (http://www.cytoscape.org/) and CentiScaPe plugin (http://chianti.ucsd.edu/cyto_web/plugins/index.php) were used to arrange nodes according to their priority/importance in the network (Scardoni et al., 2009). The number of connections from a single node was obtained by the degree of the node. The motifs obtained by MFinder were used to sort out the desired set of genes from the whole set of Arabidopsis protein interactions data. Data were then used to build a new network based on the motif id assigned by the MFinder. The new network was analyzed to predict genes in the network according to the degree of the gene.

Network motifs: Network motifs are illustrative sub-graphs in a specific network or among various networks. Each of sub-graph, defined by a particular pattern of interactions between vertices, may reflect a framework in which particular functions are fulfilled. Motifs are a useful concept to predict structural design principles of complex networks (Masoudi-Nejad et al. 2012). Each type of network seems to display its own characteristic motifs (*e.g.* ecological networks have different motifs than gene regulation networks). Network motifs in both directed and undirected networks were analyzed (Masoudi-Nejad et al. 2012). In directed networks, the edges or connections between nodes have a specific direction (*e.g.* transcriptional regulatory network) whereas in indirected networks, the edges between nodes can be in both directions (*e.g.* PPI network).

Gene Ontology Analysis: Gene Ontology (GO) of the whole set of genes or proteins was determined by the Cytoscape 2.6 plugin BINGO 2.3 (Maere et al. 2005). The annotation of the genes was set for the biological process and the molecular functions to determine the specific function and process in which each gene/protein is involved.

Overrepresented GO ”biological process” categories were identified using a hypergeometric test with a significance threshold of 0.05 after Benjamini and Hochberg FDR correction (Benjamini and Hochberg 1995) with the whole annotated genome as the reference set.

2.5 Statistical analysis

All experiments were repeated at least three times with similar results. Results were expressed as means ± standard deviation (SD) of the number of experiments. Data of *B. cinerea* growth in inoculated plants represent the mean ± SD from a minimum of 16 plants. Analysis of variance and Duncan’s multiple-range test were performed to determine the statistical significance (SAS Institute 1999). Mean values followed by an asterisk are significantly different from the corresponding control ($P < 0.05$).

Chapter 3: Results

3.1 *B. cinerea* infection in *WRKY33* transgenic plants

Arabidopsis *WRKY33* gene and its role in resistance to *B. cinerea* was previously identified (Zheng et al. 2006). Although no visible symptoms were observed when detached leaves were drop-inoculated with *B. cinerea* spores at one-day post-inoculation (dpi), lesions spread more rapidly in the *wrky33* mutant than those in wild-type or 35S:*WRKY33* transgenic plants at 3 dpi (Figure 1a), which agrees with previous observations (Zheng et al. 2006). The disease expansion by 5 dpi, resulting in clear necrotic and chlorotic lesions in the mutant leaves; whereas disease lesions remained restricted in 35S:*WRKY33* plants at 5 dpi. In wild-type plants, lesions expanded until 5 dpi, with chlorosis surrounding them.

B. cinerea infections were confirmed in all Arabidopsis genotypes by qRT-PCR using *B. cinerea ActinA* gene as a target amplicon. In the *wrky33* mutants, disease symptoms appeared more quickly than in wild-type plants. As expected, at 1 and 3 dpi, loss-of-function mutants accumulated a significantly higher amount of fungal DNA than in the wild-type (Figure 1b). Under favorable growth conditions, infection with *B. cinerea* continued to spread out and infest the *wrky33* mutant, while in the wild-type the infection was slower at all-time points tested, resulting in 83% completely rotten mutant plants compared with 40% of the wild-type when inoculated at 6 dpi (Figure 1c). When we tested the outcome of overexpression of 35S:*WRKY33* in transgenic plants infected with *B. cinerea*, it was found that the infection was effective at one dpi and the symptoms were less severe than in the wild-type at 3 dpi (Figure 1b-c). Moreover, most the overexpression lines survived at the same period of infection

(Figure 1c), indicating that the constitutive overexpression of *WRKY33* gene enhanced resistance to *B. cinerea*.

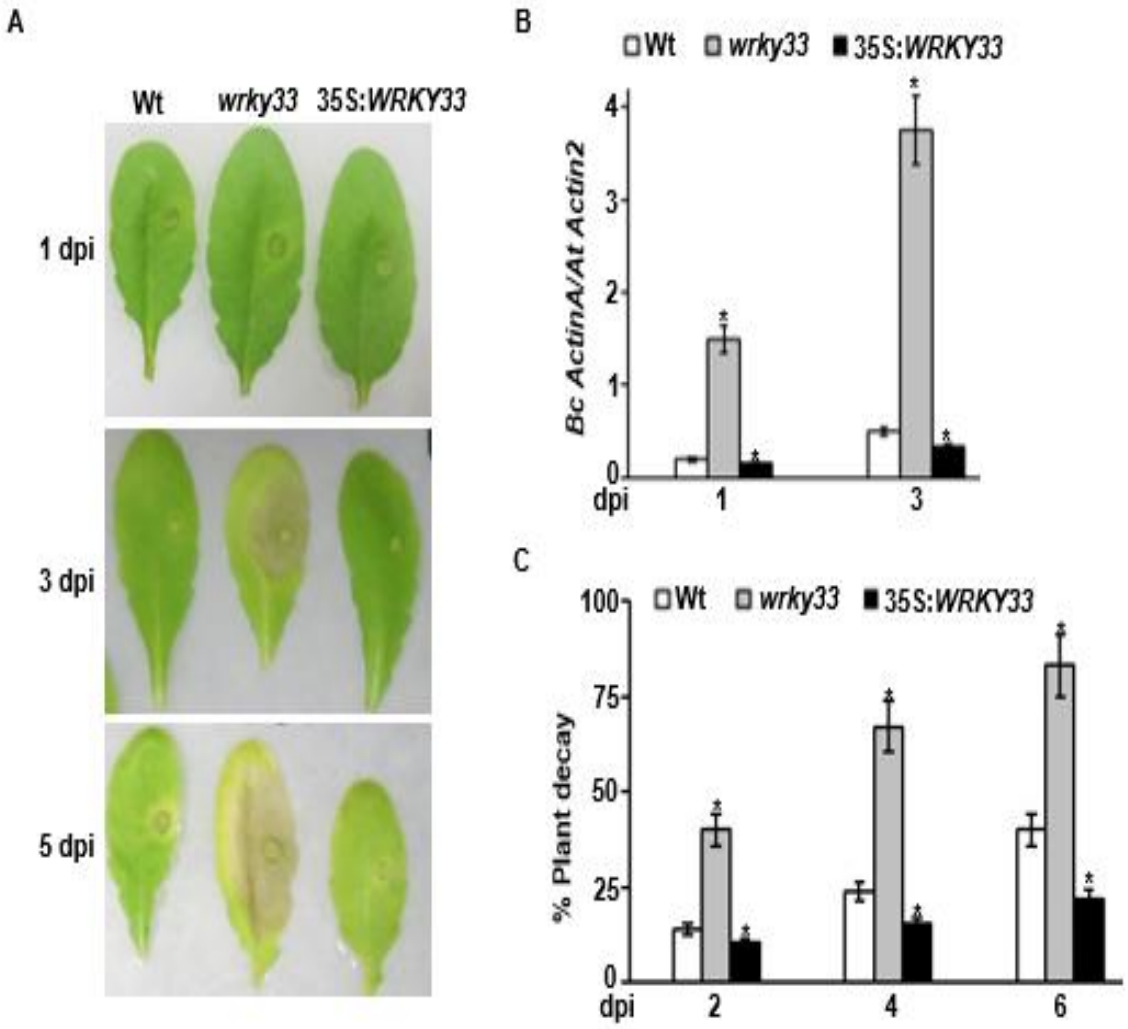

Figure 1: Disease progress of mutant and overexpression plants to *B. cinerea.*

Disease symptoms (a) and fungal growth (b) in leaves after drop-inoculation; and percentage of decayed plants (c) after spray-inoculation with *B. cinerea*. In (b) qRT-PCR amplification of *B. cinerea ActinA* relative to Arabidopsis *Actin2* gene. In (c) plants were considered decayed when they were completely rotten due to *B. cinerea* infection. Data represent the mean ± SE from a minimum of 30 plants. Analysis of variance and Duncan's Multiple Range Test were performed to determine the statistical significance of the differences between the mean values using SAS software (SAS Institute 1999). Mean values followed by an asterisk is significantly different from wild-type at the tested time (P=0.05). Experiments were performed as described in Methods and repeated at least three times with similar results. *Bc ActinA*, *B. cinerea ActinA* gene; *At Actin2*, *Arabidopsis thaliana Actin2* gene; dpi, days post inoculation.

3.2 Identification of *BUG*s and *BDG*s

The development of disease symptoms in Arabidopsis wild-type, *wrky33* mutants and ectopic overexpression plants were analyzed (Figure 1). A comparison of the gene expression levels between transgenic plants was done using Arabidopsis whole-genome Affymetrix gene chip (ATH1) representing approximately 25,000 genes to identify regulated genes by *B. cinerea* infection. Many *BUG*s and *BDG*s were identified. Differentially regulated genes have been identified by comparing the expression profiles of *B. cinerea*-inoculated and non-inoculated tissues (Figure 2a) at 0 and 24 hours post-inoculation (hpi) in three Arabidopsis genotypes: wild-type, *wrky33-1* mutant and 35S:*WRKY33* overexpression transgenic plants.

The selected time point (24 hpi) was used to compare differences in gene expression because most changes in gene expression occur between 18-30 hpi (AbuQamar et al., 2006, Windram et al., 2012). Fold expression changes have been calculated by dividing the normalized gene expression level of *B. cinerea*-infected sample by their corresponding controls (no infection). In wild-type plants, we found approximately 1660 *BUG*s and 1054 *BDG*s at 24 hpi (Table S1; https://drive.google.com/file/d/0Bx4SCX1UvbirTmZBTEdlVVpSMEU/view?usp=sharing). Based on their functional similarities, *BUG*s and *BDG*s were classified into distinct groups that suggest potential functional components of signaling pathways and cellular activities associated with Arabidopsis resistance to *B. cinerea* (Figure 3).

Among the regulated genes by *B. cinerea*, we found number of genes encoding known regulatory, developmental and structural proteins that have previously been reported (AbuQamar et al. 2006; Sham et al. 2014, 2015). Most *BUG*s and *BDG*s encode functional proteins involved in plant responses to stress stimuli, signal transduction pathways, transport and energy pathways, metabolic and biological

processes (Figure 3). The fraction of genes involved in kinase activities was more prominent among the *BUG*s compared with the *BDG*s. A certain number of *BUG*s and *BDG*s were without known functions. Notably, there were significant differences in the number of genes that were upregulated in different cytoplasmic components and in the cell wall (Figure 3). Most of the *BDG*s encode enzymes (i.e. hydrolyases, transferases), transporters and receptors that are highly involved in cellular activities and localized in the plastids, membranes and cell wall. Altogether, the expression levels of *BUG*s and *BDG*s in various subcellular locations is consistent with the role of extracellular and intracellular components in plant response to *B. cinerea* infection.

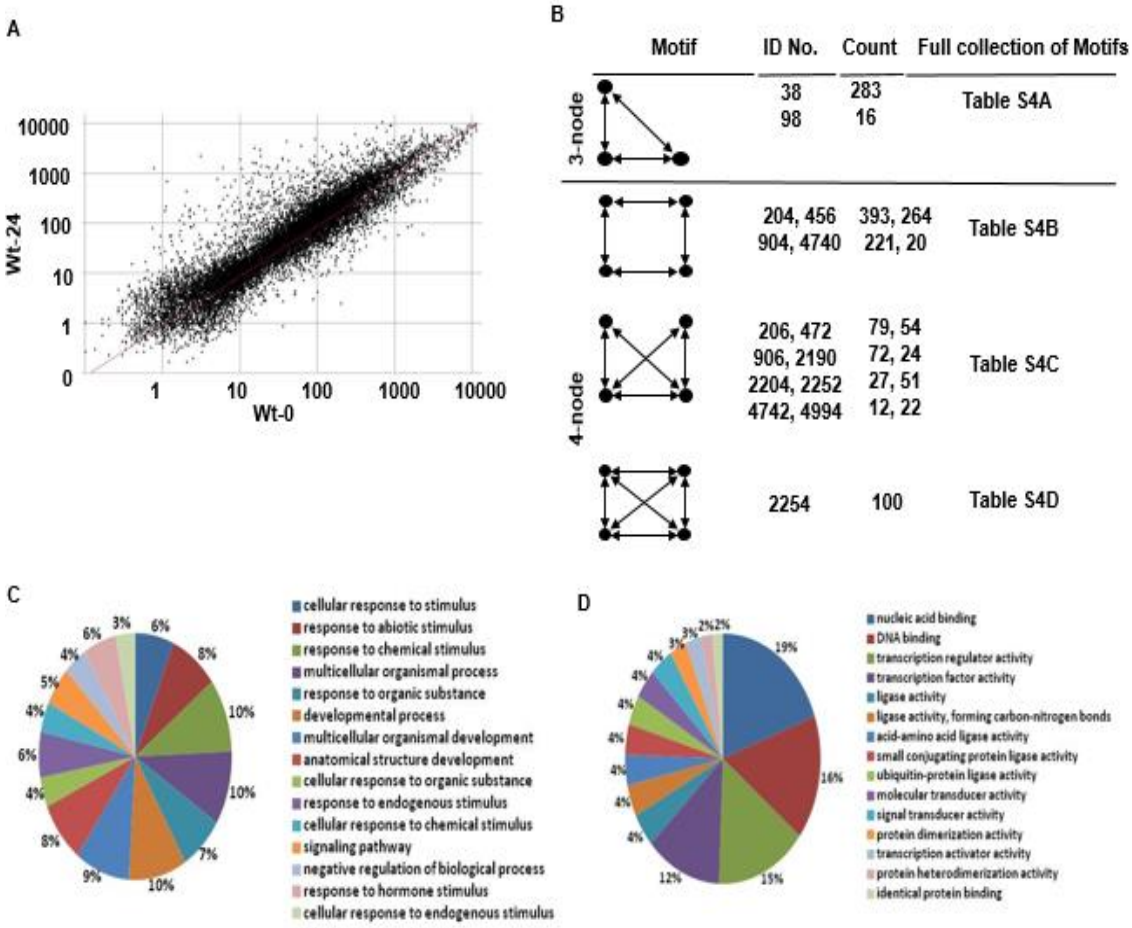

Figure 2: Scatter-plot comparisons of differentially expressed genes and motif patterns in the *B. cinerea*-Arabidopsis interaction network.

Normalized expression value for each probe set in wild-type plants infected with *B. cinerea* at 24 hpi (Wt-24) is plotted on the Y-axis; the value in wild-type plants sampled before *B. cinerea* treatment (0 hpi; WT-0) is plotted on the X-axis (a); examples of significant 3-node motif and 4-node motifs (b); and GO Terms relating to biological process (c); or molecular function (d) category overrepresented in clusters of genes differentially expressed after *B. cinerea* infection of Arabidopsis plants. In (b), the number of instances (counts) of each motif corresponding to the ID No. is indicated. Top fifteen GO IDs according to their corrected *P*-values. The full collection of motifs and their individual subgraphs are in Table S2; (https://drive.google.com/file/d/0Bx4SCX1UvbirTmZBTEdlVVpSMEU/view?usp=sharing) and Fig. 4;, respectively. Criteria used in this analysis were: *P*-value <0.01; Mfactor> 1.10; and uniqueness ≥ 4 were used to define significant patterns. Each of the motifs was given a unique pattern or motif ID as previously described (Taylor et al. 2007). In (c-d), pie chart shows percentage of *BUG*s and *BDG*s in the various functional classes according to the Cytoscape plugin BiNGO as described (Maere et al. 2005). Corrected *P*-values less than 5×10^{-5} were considered statistically significant. Gene identifications for 2714 genes were entered for this analysis. All GO terms are available in Table S3; https://drive.google.com/file/d/0Bx4SCX1UvbirTmZBTEdlVVpSMEU/view?usp=sharing.

Figure 3: Functional classes of *BUGs* (a) and *BDGs* (b) at 24 hpi compared with 0 hpi in wild-type.

Gene identifications for 1660 *BUG*s and 1054 *BDG*s were entered for this analysis. Error bars are SD. GO categories that a significantly over or underrepresented at $p < 0.05$ are in black text. Normalized frequency of genes to the number of genes on the microarray chip was determined as described (Provart and Zhu 2003).

3.3 Network motif-based predictions of Arabidopsis-*B. cinerea* interaction

B. cinerea-responsive genes were identified based on the transcriptome profiling following infection in Arabidopsis. Differentially expressed genes were analyzed for the presence of network motifs (Milo et al. 2002). The whole set of Arabidopsis protein-protein interaction (PPI) data was analyzed using Cytoscape tool. The aim was to determine the biological processes and molecular functions of the genes/proteins involved in the motifs and the centrality measure of these genes, to predict the importance of the genes in a desired network. The Biological Networks Gene Ontology (BiNGO) tool was used to determine the gene ontology (GO) of the genes present in the motifs and ranked according to the importance of the gene obtained using the Cytoscape results (Maere et al. 2005).

We aimed at identifying networks that are used to describe complex systems and to display motifs and patterns within the network, rather than random networks. *BUG*s and *BDG*s were analyzed for the presence of network motifs using the MFinder (Kashtan et al. 2004) in order to identify candidate regulatory genes. Each of the motifs belonging to a universal shape in the network was given a unique pattern or motif ID (Taylor et al. 2007). Based on the motif ID assigned, a new network predicted genes in the Arabidopsis-*B. cinerea* interaction according to the degree of a node in a network which corresponds to the highest number of connections or edges the node to other nodes in the interaction network. The obtained MDraw results showed patterns of 3-node and 4-node motifs in the network (Figure 2b). The simplest network patterns containing information about the genetic-interaction modes are 3-node motifs. ID# 38 and ID# 98 were identified as patterns of the 3-node motifs, counting for 283 and 16 instances, respectively. The analyses also determined 3 sets of patterns of the 4-node motifs that were frequently found, with a total of 13 possible patterns being significant.

Examples of such diagrams illustrating the shape of motifs (and their ID numbers) and the full collection of motifs are given in (Figure 4 and Table S2; https://drive.google.com/file/d/0Bx4SCX1UvbirTmZBTEdlVVpSMEU/view?usp=sharing) respectively. The motifs differ from each other in the processes they are involved in, suggesting different roles of these motifs in functional and metabolic pathways present in genetic interaction networks.

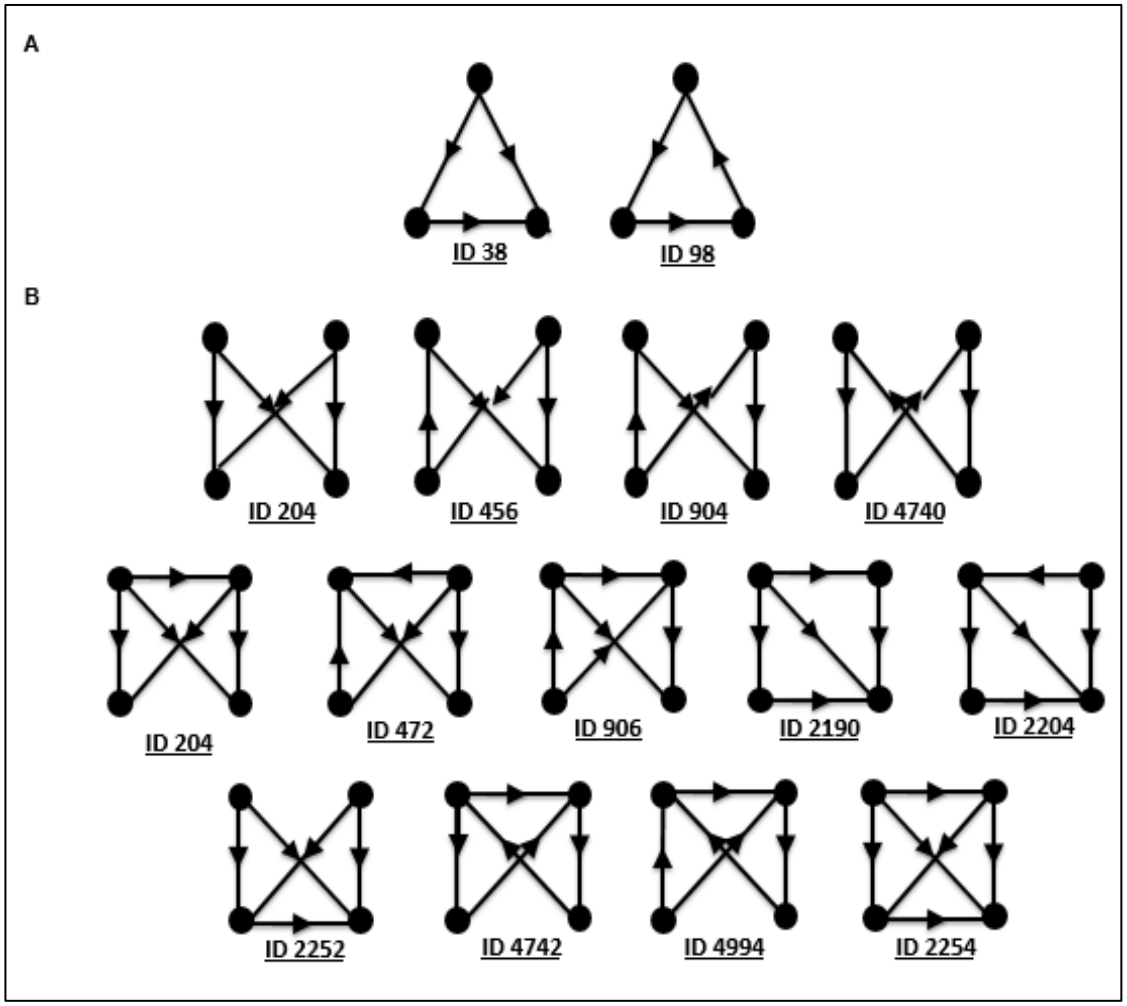

Figure 4: Top fifteen (a) 3 node- and (b) 4 node-subgraphs and their motif IDs.

Biological processes and molecular functions of the motifs within proteins/genes were also analyzed (Scardoni et al. 2009; Smoot et al. 2011). To enrich our experimental data with network topological parameters and to

integrate the molecular interaction networks with gene expression profiles, the Cytoscape plugin, CentiScaPe was used (Masoudi-Nejad et al. 2012). Cytoscape plugin computes several network centrality parameters and analyzes existing relationships between experimental data provided by the user and node centrality values computed by the plugin. CentiScaPe was used to identify network nodes relevant from both experimental and topological pointviews, thus allowing easy node categorization and experimental prioritization. Accordingly, GO was determined and the gene was overrepresented according to its biological activities and molecular functions in plants using Cytoscape plugin BiNGO (Maere et al. 2005). *B. cinerea* infection induced regulatory and structural genes known to function in different pathways (AbuQamar et al. 2006; Sham et al. 2014, 2015). *B. cinerea*-regulated genes encode proteins that closely function in plant responses to different stimuli and stresses, including abiotic stress, general and chemical stimuli, and other related responses to developmental processes (Figure 2c; Table S3; https://drive.google.com/file/d/0Bx4SCX1UvbirTmZBTEdlVVpSMEU/view?usp=sharing). Analysis of the molecular function of the identified GO terms illustrated that DNA binding, transcription factors and transcription regulators activities were heavily involved in defense response against *B. cinerea* (Figure 2d; Table S3; https://drive.google.com/file/d/0Bx4SCX1UvbirTmZBTEdlVVpSMEU/view?usp=sharing). Results on molecular function from this research confirm the data of previous studies that defense response against *B. cinerea* identify

transcription factor families during the infection of this pathogen (Windram et al. 2012; Segarra et al. 2013).

3.4 *BUG*s and *BDG*s are dependent on Arabidopsis *WRKY33*

The basal expression level of the early *B. cinerea*-regulated genes selected from wild-type samples altered in the transgenic plants was determined. In the absence of the pathogen, the expression of 171 genes were constitutively expressed between the wild-type and *wrky33*; of which 148 (86.6%) genes were at least twofold higher in *wrky33* than in wild-type samples (Figure 5a; Table S4; https://drive.google.com/file/d/0Bx4SCX1UvbirTmZBTEdlVVpSMEU/view?usp=sharing). By contrast, 23 (13.4%) genes were at least twofold lower in *wrky33*. Comparing the expression profiles from non-infected plants revealed that 332 genes were constitutively and differentially expressed between the wild-type and 35S:*WRKY33* lines, 251 (75.6%) of them were upregulated and 81 (24.4%) were downregulated (Figure 5a; Table S4; https://drive.google.com/file/d/0Bx4SCX1UvbirTmZBTEdlVVpSMEU/view?usp=sharing). This indicates that the basal expression level of several genes is dependent on *WRKY33*, which seems to be required for gene regulation before and during *B. cinerea* infection.

The normalized transcriptional levels of all potentially *B. cinerea*-regulated genes in *wrky33* and 35S:*WRKY33* background lines were compared at 24 hpi (Figure 5b, c). Upon *B. cinerea* infection, expression levels of 1660 *BUG*s and 1054 *BDG*s in *wrky33* mutant and *WRKY33* overexpression lines were compared with the wild-type. The goal is to determine whether the expression levels of *BUG*s or *BDG*s are potentially dependent on *WRKY33* or not. The expressions of 4821 genes were

altered more than twofold in *wrky33* mutants; 921 upregulated and 3900 downregulated, corresponding to 4% and 17% of the whole transcriptome, respectively (Figure 5a; Table S5; https://drive.google.com/file/d/0Bx4SCX1UvbirTmZBTEdlVVpSMEU/view?usp=sharing, with a common set of 789 up- and 847 downregulated genes showing similar changes upon infection in both *wrky33* and wild-type plants (Figure 5d; Table S5; https://drive.google.com/file/d/0Bx4SCX1UvbirTmZBTEdlVVpSMEU/view?usp=sharing). When the *WRKY33* overexpression transgenic plants were infected with *B. cinerea*, the transcript levels increased in 1099 genes (4.8% of the transcriptome), but decreased in 2257 of the genes (9.9% of the transcriptome) (Figure 5a). We also figured out about 924 up- and 914 downregulated genes in the overexpression line were commonly changed in the wild-type plants (Figure 5e; Table S6; https://drive.google.com/file/d/0Bx4SCX1UvbirTmZBTEdlVVpSMEU/view?usp=sharing). Expression levels of 869 and 207 genes were up- and down-regulated, respectively, in the wild-type; whereas the differential expression of 3183 (132 up- and 3051 downregulated) genes was triggered by the loss-of-*WRKY33* function. Similarly, the expression was induced in 732 up of the genes but reduced in 139 genes in the wild-type; thus, more than 1500 (174 up- and 1339 downregulated) genes were altered in the gain-of-*WRKY33* function (Figure 5e). In addition, all reciprocal combinations of common differentially expressed genes between wild-type and *wrky33* plants as well as wild-type and 35S:*WRKY33* overexpression plants infected with *B. cinereal* were determined (Figure 5d, E; Table S7; https://drive.google.com/file/d/0Bx4SCX1UvbirTmZBTEdlVVpSMEU/view?usp=sharing). Regardless of the transcript level differences between the wild type, *wrky33* mutant and *35S:WRKY33* overexpressing line, several genes associated with JA

pathway (*AOC3*, *OPR1*, *PDF1.2* and *JAZ1*) and ET pathway (*ERF1*, *ERF 15*, *ORA59*, *ACS6* and *ERF-1*) were upregulated at 24 hpi with *B. cinerea* in both transgenic lines (Tables S5 and S6; https://drive.google.com/file/d/0Bx4SCX1UvbirTmZBTEdlVVpSMEU/view?usp=sharing). Similarly, the expression of SA pathway-associated genes, *SID2*, *EDS5* and *PR1*, was induced upon the fungal attack in *wrky33* and *35S:WRKY33* genotypes. This confirms previous published datasets comparing expression levels of hormone signaling pathways in wild type- and *wrky33*-infected plants (Birkenbihl et al. 2012).

In addition, camalexin biosynthetic genes, C*YP71A13* and *PAD3*, were also upregulated in both *WRKY33* mutant and overexpressing transgenic lines infected with *B. cinerea*. The transcript level of genes encoding proteins that are involved in the regulation of cellular redox homeostasis, such as *GRX48*, *CKX4*, *RBOHD* and *TRX-H5*, increased in *wrky33* mutants after *B. cinerea* attack. The latter genes were also induced at 24 hpi with the same pathogen. Together, the collected data suggest a regulatory role of *WRKY33* in mediating gene expression which corresponds to disease responses in its mutant and overexpressing lines.

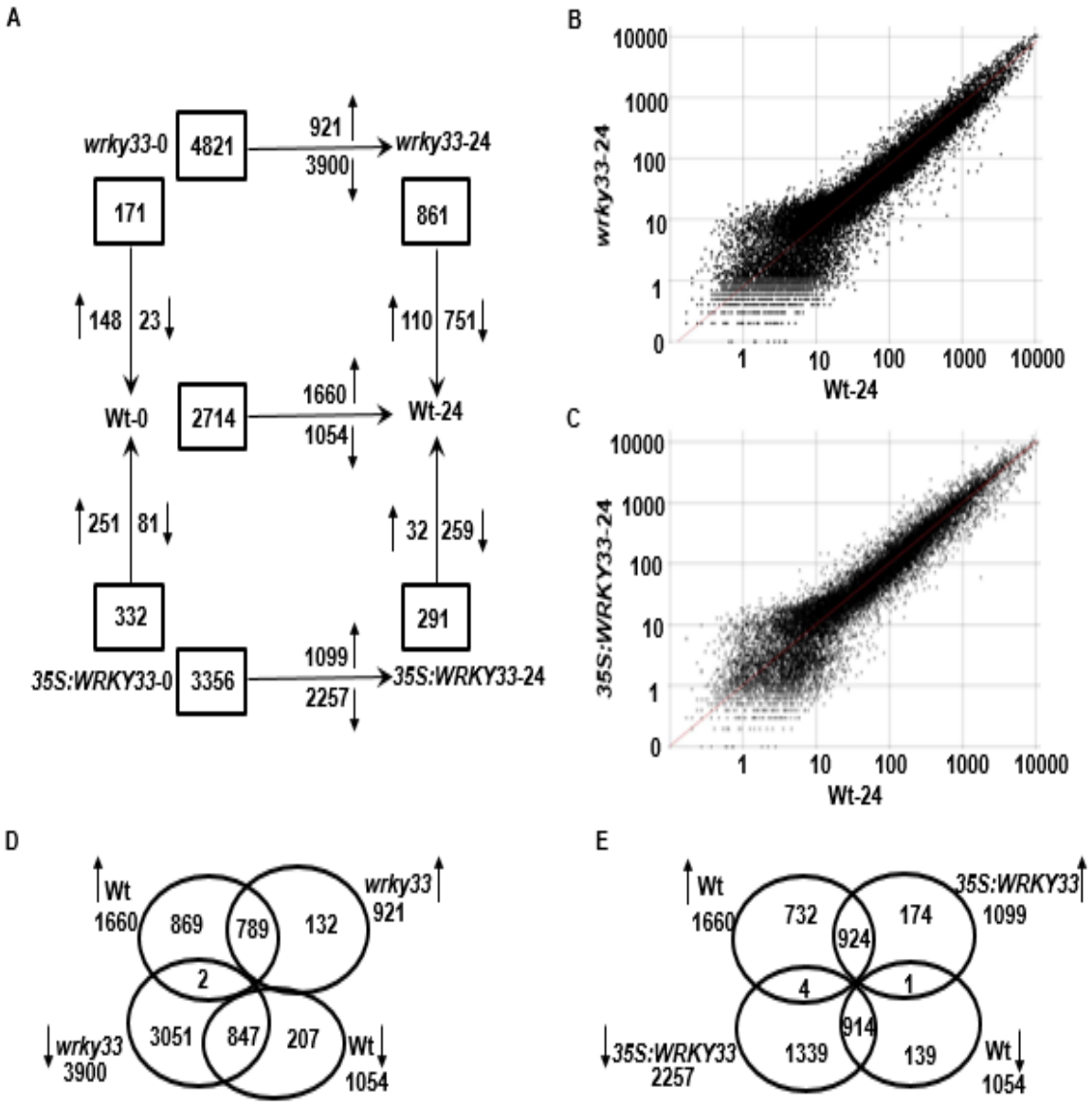

Figure 5: Transcriptional reprogramming and scatter-plot comparisons of differentially expressed gene in *WRKY33* transgenic plants.

The numbers of differentially expressed genes (≥ 2-fold at $P \leq 0.05$) between wild-type, *wrky33* and 35S:*WRKY33* at 0 or 24 hpi of inoculation with *B. cinerea* (a); normalized expression value for each probe set in wild-type plants infected with *B. cinerea* at 24 hpi is plotted on the Y-axis; the value in *B. cinerea*-treated (b) *wrky33* mutant and (c) 35S:*WRKY33* plants infected with *B. cinerea* at 24 hpi is plotted on the X-axis; and Venn diagram showing the overlapping numbers of *BUG*s and *BDG*s in wild-type and (d) *wrky33*; or (e) 35S:*WRKY33* plants at 24 hpi with *B. cinerea*. In (c), boxes represent total number, and arrows represent the number of *BUG*s and *BDG*s between the treatments and the genotypes tested. Wt, wild-type; *wrky33*, *wrky33* mutant; 35S:*WRKY33*, 35S:*WRKY33* overexpression transgenic line; hpi, hours post inoculation.

3.5 Regulation of cyclopentenone-induced genes during *B. cinerea* infection

The cyclopentenone oxilipins, OPDA and PPA_1 are formed via enzymatic and nonenzymatic free radical-catalyzed pathways, respectively (Mueller and Berger 2009; Schaller and Stintzi 2009). The two groups of *B. cinerea*-responsive genes (*BUGs* and *BDGs*; Table S1; https://drive.google.com/file/d/0Bx4SCX1UvbirTmZBTEdlVVpSMEU/view?usp=sharing) were analyzed with OPDA- or PPA_1-regulated genes to determine possible correlations between the four groups (Taki et al. 2005; Mueller et al. 2008). It has been reported that WRKY33 regulates the expression of many genes encoding components associated with hormonal signaling pathways during *B. cinerea* infection (Birkenbihl et al. 2012; Liu et al. 2015). To determine whether WRKY33 regulates non-enzymatic targets in the Arabidopsis genome following infection with *B. cinerea*, the expression of *BUG*s and *BDG*s in the *WRKY33* mutant and overexpressing transgenic line with that of OPDA and PPA_1 regulators were thus compared. A group of genes that were 2-fold upregulated by OPDA treatment (Taki et al. 2005) and *B. cinerea* infection, thus termed as OPDA/*B. cinerea*-upregulated genes (*OBUG*s), were demonstrated (Table 2; Table 3). The *OBUG*s encode a subset of proteins including kinases, Aldo/keto reductase, FAD-linked oxidoreductase, ABA-responsive and other related proteins. Seven of the 17 (41%) *OBUG*s were dependent on *WRKY33* (Table 2). Targets of the *OBUGs*, *DREB2A* (*At5g05410*) and B-box zinc-finger (*At2g47890*) proteins, are involved in pathogen attack signaling and abiotic stress signaling (Chen et al. 2010; Bhosale et al. 2013) were altered in both *WRKY33* mutant and overexpression backgrounds. The Arabidopsis oxidative stress-related gene, *GPX6* (*At4g11600*) encoding glutathione peroxidase protein (Mhamdi et al. 2010), was the only gene that was upregulated by both OPDA and *B. cinerea* in *wrky33* mutant background (Table

2). On the other hand, the *OBUG*-induced genes, *mildew resistance locus O6* (*MLO6*), *zinc-finger* (*RHL41*), *Fe superoxide dismutase* (*FDS1*) and *rubber elongation factor* (*REF*), were regulated by 35S:*WRKY33* only. On the other hand, the transcript level of the *OBDG* which encodes a receptor-like kinase (*At4g67360*) was regulated by the presence of *WRKY33* gene. Together, WRKY33 transcription factor was found to have a potential role in OPDA-mediated regulation of gene expression.

Table 2: Regulation of genes by OPDA treatment and *B. cinerea* infection

Description	Gene Locus	Fold induction [a]		Expression requires [d]
		OPDA [b]	*B. cinerea* [c]	
***OBUG*s**				
DRE-binding protein 2A (DREB2A)	*At5g05410*	4.4	3.9	*w33*, 35S:*W33*
B-box zinc-finger	*At2g47890*	3.1		*w33*, 35S:*W33*
Glutathione peroxidase 6 (GPX6)	*At4g11600*	3.2	2.3	*w33*
Mildew resistance locus O6 (MLO6)	*At1g61560*	3.9	9.1	35S:*W33*
Zinc-finger Zat12 (RHL41)	*At5g59820*	3.5	14.1	35S:*W33*
Iron superoxide dismutase 1 (FSD1)	*At4g25100*	2.5	-3.3	35S:*W33*
Rubber elongation factor protein (REF)	*At1g67360*	2.0	4.0	35S:*W33*
***OBDG*s**				
Ser/Thr receptor-like kinase (RLK3)	*At4g23190*	-3.3	15.0	35S:*W33*

[a] Fold induction = normalized OPDA treatment or *B. cinerea* inoculation/normalized no OPDA treatment or *B. cinerea* inoculation. Data set on at least twofold induction or halffold repression after treatment/inoculation.
[b] OPDA-upregulated genes data were obtained from Taki et al. (2005) at 3 hpt.
[c] *B. cinerea*- upregulated genes data were obtained from this study at 24 hpi.
[d] Gene regulation is dependent on *WRKY33* (Tables S5 and S6; https://drive.google.com/file/d/0Bx4SCX1UvbirTmZBTEdlVVpSMEU/view?usp=sharing).
*OBUG*s, OPDA-*B. cinerea* upregulated genes; *w33, wrky33*; 35S:*W33*, 35S:*WRKY33*.

In addition, *B. cinerea*-regulated candidate genes were also compared with PPA_1-regulated genes (Mueller et al. 2008). Two distinct groups were identified: PPA_1/*B. cinerea*-upregulated genes (*PBUG*s) and PPA_1/*B. cinerea*-downregulated genes (*PBDG*s) (Tables 3 and 4). About 22.2% (12/54) and 14.7% (5/34) of upregulated or downregulated genes by PPA_1 were also upregulated or downregulated by *B. cinerea*, respectively. *PBUG*s appear to fall in a gene category related to

detoxification or stress responses such as the cytochrome P450, UDP-glucoronosyl transferases, transporters, heat shock factors/proteins, and TolB-related proteins. By contrast, *PBDG*s encode proteins involved in cell growth, cell wall biosynthesis or cell cycle such as hydroxyproline-rich glycoproteins, expansin B3, cyclin-dependent kinase (CDK), pectinase and cellulose synthase. Two of the *PBUG*s genes were dependent on *WRKY33* (Table 4). The *TolB-related* (*At4g01870*) and *mildew resistance locus O12* (*MLO12*; A*t2g39290*) responsive genes which were previously expressed in response to fungal infections (Pan et al., 2013; Sham et al. 2015), were also upregulated by *B. cinerea* in wild-type plants; thus regulated by the absence or presence of *WRKY33* (Table 4). It is worth mentioning that WRKY proteins specifically bind to a DNA motif (TTGACT/C; also termed the W-box) (Rushton et al., 2010). MLO12 contains W-box motif in its promoter. *AGP17*- and *At3g02120*-downregulation to both of PPA_1 treatment and with *B. cinerea* infection was dependent on 35S:*WRKY33* only. Although, we figured out that 6 *PBDG*s were differentially expressed in *wrky33* mutant only; 3 others were dependent for their downregulation to both the mutant and the overexpressing line of *WRKY33* (Table 4). The data indicate that *WRKY33* transcriptionally regulates genes commonly involved in plant response to PPA_1 and *B. cinerea*, suggesting that *WRKY33* may play a role in the non-enzymatic pathway that is responsible for the synthesis of PPA_1 oxylipin that is involved in plant stress responses.

Table 3: Regulation of genes by OPDA or PPA1 treatment and *B. cinerea* infection

Description	Gene Locus	Fold induction [a]		
		OPDA [b]	PPA_1 [c]	*B. cinerea* [d]
***OBUG*s**				
Receptor-related protein kinase like	*At5g25930*	7.1		9.5
12-Oxo-phytodienoate reductase (OPR2)	*At1g76690*	6.2		5.1
Aldo/keto reductase family	*At1g60730*	4.6		7.4
FAD/NAD (P)-binding oxidoreductase family	*At3g44190*	4.3		2.0
Hydrolase, α/β fold family	*At4g24160*	4.1		2.4
Auxin-responsive family	*At5g35735*	3.4		2.4
Kunitz family trypsin/protease inhibitor	*At1g17860*	3.4		2.1
ABA-responsive GEM-related 5 (GER5)	*At5g13200*	3.2		2.7
Cellulose synthase like E1 (CSLE1)	*At1g55850*	3.1		5.4
BON Associated Protein (BAP1)	*At3g61190*	2.5		5.8
***PBUG*s**				
UDP-glucoronosyl transferase 73B3 (UGT73B3)	*At4g34131*		105.4	2.7
Class I small heat shock (HSP17.6)	*At2g29500*		57.8	2.2
Heat shock factor (HSF4)	*At4g36990*		12.3	10.2
ABC transporter	*At3g47780*		9.6	2.4
Multidrug-resistant ABC transporter (MDR4)	*At2g47000*		8.7	19.0
Heat shock protein 70 (HSP70)	*At3g12580*		5.4	7.4
Glycosyl hydrolase family 81	*At5g15870*		3.7	2.4
UDP-glucoronosyl transferase 87A2 (UGT87A2)	*At2g30140*		3.7	10.2
12-Oxo-phytodienoate reductase 1 (OPR1)	*At1g76680*		3.3	5.1
Cytochrome P450 (CYP89A9)	*At3g03470*		3.1	2.5
***PBDG*s**				
Cyclin-dependent kinase regulator (CYCA1;1)	*At1g44110*		-4.4	-4.3
CYCLIN-dependent Kinase B2;1 (CDKB2;1)	*At1g76540*		-3.1	-2.8
SNAP receptor (syntaxin 111; SYP111)	*At1g08560*		-4.0	-2.3
Cellulose synthase 5 (CESA5)	*At5g09870*		-5.3	-3.1
Expansin B3 (EXPB3)	*At4g28250*		-4.9	-3.0
Pectin lyase-like superfamily protein	*At3g06770*		-4.1	

[a] Fold induction = normalized OPDA or PPA_1 treatment or *B. cinerea* inoculation/normalized no OPDA or PPA_1 treatment or *B. cinerea* inoculation. Data set on at least twofold induction or repression after treatment/inoculation.
[b] OPDA-upregulated genes data were obtained from Taki et al. (2005) at 3 hpt.
[c] PPA_1-upregulated genes data were obtained from Mueller et al. (2008) at 4 hpt.
[d] *B. cinerea*- upregulated genes data were obtained from this study at 24 hpi.
[d] Gene regulation is dependent on *WRKY33* (Tables S5 and S6; https://drive.google.com/file/d/0Bx4SCX1UvbirTmZBTEdlVVpSMEU/view?usp=sharing).
*OBUG*s, OPDA-*B. cinerea* upregulated genes; *PBUG*s, PPA_1-*B. cinerea* upregulated genes; *PBDG*s, PPA_1-*B. cinerea* downregulated genes.

Table 4: Regulation of genes by PPA1 treatment and *B. cinerea* infection

Description	Gene Locus	Fold induction [a]		Expression requires [d]
		PPA_1 [b]	*B. cinerea* [c]	
***PBUG*s**				
TOLB protein-related	*At4g01870*	20.1	4.5	*w33*, 35S:*W33*
Mildew resistance locus O12 (MLO12) [e]	*At2g39200*	7.1	2.3	*w33*, 35S:*W33*
***PBDG*s**				
Arabinogalactan protein 17 (AGP17)	*At2g23130*	-5.2		35S:*W33*
Hyp-rich glycoprotein family protein	*At3g02120*	-4.6		35S:*W33*
Cellulose synthase-like 5 (CSLD5), Salt Overly Sensitive 6 (SOS6)	*At1g02730*	-3.7		*w33*, 35S:*W33*
Auxin Inducible 2-11 (AUX2-11)	*At5g43700*	-3.8		*w33*, 35S:*W33*
Actin-11 (ACT11)	*At3g12110*	-3.6		*w33*, 35S:*W33*
ASCICLIN-like arabinogalactan 18 precursor (FLA18)	*At3g11700*	-5.1		*w33*
Pectin lyase-like superfamily protein	*At3g62110*	-4.5		*w33*
Cellulose synthase 6 (CESA6)/Isoxaben resistant 2 (IXR2)	*At5g64740*	-3.1		*w33*
CYCLIN D3 (CYCD3)	*At4g34160*	-3.5		*w33*
Short hypocotyl 2 transcription factor (SHY2)	*At1g04240*	-3.4		*w33*
Auxin-induced 13 (IAA13)	*At2g33310*	-3.2		*w33*

[a] Fold induction = normalized PPA_1 treatment or *B. cinerea* inoculation/normalized no PPA_1 treatment or *B. cinerea* inoculation. Data set on at least twofold induction after treatment/inoculation.
[b] PPA_1-upregulated genes data were obtained from Mueller et al. (2008) at 4 hpt.
[c] *B. cinerea*-upregulated genes data were obtained from this study at 24 hpi.
[d] Gene regulation is dependent on *WRKY33* (Tables S5 and S6; https://drive.google.com/file/d/0Bx4SCX1UvbirTmZBTEdlVVpSMEU/view?usp=sharing).
*PBUG*s, PPA_1-*B. cinerea* upregulated genes; *PBDG*s, PPA_1-*B. cinerea* downregulated genes; *w33, wrky33*; 35S:*W33,* 35S:*WRKY33*.
[e] Presence of WRKY33 DNA binding motif (Liu et al. 2015).

OPDA may function independently from JA (Mueller et al. 2008; Böttcher and Pollmann, 2009). Many genes containing a TGA motif (TGACG) in the 500 bp upstream of their promoters may contain binding sites for TGA transcription factors (Lam et al. 1989). The analysis was set on upregulated genes by PPA_1 and OPDA treatments (Mueller et al. 2008) and *B. cinerea* infection. Of the 48 upregulated genes by the two cyclopentenone oxylipins (Mueller et al., 2008), 29 (60.4%) were *B. cinerea*-induced, of which 18 (62.1%) were dependent on the presence of TGA2/5/6

(Tables 5 and 6). Five of the *OBUG/PBUG*s were dependent on *WRKY33*. Upon infection with the plant pathogen *B. cinerea*, some induced genes were responsive to treatments with PPA_1 and OPDA. These genes could be regulated by a common pathway in which *WRKY33* may act through TGA transcription factors. For example, *WRKY75* and *cytochrome P450* (*CYP72A15*) expression was increased after 24 hpi with *B. cinerea*; thus, this change was impaired by TGA or WRKY33 transcription factors (Table 5). Both WRKY75 (Table 5) and PAD3 (Table 6) contain W-box motif in their loci. On the other hand, other regulators which do not contain a TGA motif, such as *At3g21700* (*SGP2*), *At5g17860* (*CAX7*) or *At2g43510* (*TI1*), were transcriptionally dependent on WRKY33 after infection. This suggests a regulation of some *OBUG/PBUG*s by WRKY33 upon infection with *B. cinereal.*

Table 5: Upregulated genes by PPA1 and OPDA treatments and *B. cinerea* inoculation dependent on TGA2/5/6 and WRKY33

Array Element	Gene Locus	Description	Fold Induction			Expression requires [c]	TGACG presence
			PPA_1 [a]	OPDA[a]	*Bc* [b]		
***OBUG/PBUG*s**							
245976_at	*At5g13080*	WRKY75 transcription factor (WRKY75) [d]	10.4	4.4	41	*w33*, 35S:*W33*	+
258094_at	*At3g14690*	Cytochrome P450 (CYP72A15)	11.1	4.0	1.3	*w33*, 35S:*W33*	+
257951_at	*At3g21700*	GTP binding (SGP2)	2.7	2.3	5.3	*w33*, 35S:*W33*	-
250054_at	*At5g17860*	Calcium exchanger 7 (CAX7)	2.3	3.9	20.4	35S:*W33*	
260551_at	*At2g43510*	Trypsin inhibitor protein (TI1)	2.3	7.3	7.1	*w33*	-

[a] Normalized fold induction of genes by PPA_1 and OPDA (75 µM) of at least twofold in Arabidopsis wild-type plants relative to controls but no induction in *tga2/5/6*. PPA_1- and OPDA-induced genes data were obtained from Mueller et al. (2008) at 4 hpt.
[b] Normalized fold induction of genes by *B. cinerea* of at least twofold in Arabidopsis wild-type plants relative to controls (Table S1; https://drive.google.com/file/d/0Bx4SCX1UvbirTmZBTEdlVVpSMEU/view?usp=sharing). *B. cinerea*-induced genes data were obtained from this study at 24 hpi.
[c] Gene upregulation is dependent on *WRKY33* (Tables S5 and S6; https://drive.google.com/file/d/0Bx4SCX1UvbirTmZBTEdlVVpSMEU/view?usp=sharing).
PPA_1, phytoprostane-A_1; OPDA, 12-oxo-phytodienoic acid; *Bc*, *B. cinerea*; *w33*, *wrky33*; 35S:*W33*, 35S:*WRKY33*.
[d] Presence of WRKY33 DNA binding motif (Liu et al. 2015)

Table 6: Upregulated gens by PPA_1 and OPDA treatments and *B. cinerea* inoculation

Gene Locus	Description	Fold Induction			TGACG presence
		PPA_1 [a]	OPDA[a]	*Bc* [c]	
OBUG/PBUGs					
At4g20860	FAD-binding Berberine family	25.7	6.6	7.6	+
At1g15520	ABC transporter (PDR12)	24.5	18.7	82.5	+
At2g47730	Glutathione *S*-transferase PHI 8 (GST6)	22.6	5.1	2.1	+
At1g33590	Disease resistance LRR protein-related	2.3	2.5	2.4	+
At3g26830	Phytoalexin deficient 3 (PAD3) [c]	9.6	7.9	103.5	-
At5g22300	Nitrilase 4 (NIT4)	9.3	6.6	15.6	+
At3g14620	Cytochrome P450 family (CYP72A8)	8.2	2.7	5.5	-
At3g10500	NAC domain containing protein 53 (ANAC053)	4.7	2.1	6.7	+
At5g03490	UDP-glucoronosyl transferase family protein	3.7	2.5	4.1	+
At1g72680	Cinnamyl alcohol dehydrogenase (CAD1)	3.3	2	3.2	+
At1g72900	Disease resistance protein (TIR-NBS class)	3.3	3.7	8.0	+
At3g01970	WRKY45 transcription factor (WRKY45)	3.2	4.1	39.3	-
At1g13990	Expressed protein	3	3	3.0	+
At5g19440	Alcohol dehydrogenase	2.9	2.4	2.3	-
At2g21620	Responsive to dessication 2 (RD2)	2.7	2.1	4.2	+
At1g23440	Pyrrolidone-carboxylate peptidase family protein	2.5	2.1	4.0	+
At4g30490	AFG1-like ATPase family protein	2.2	2.2	2.9	-
At5g65300	Expressed protein	2.2	2.5	4.4	+
At2g47800	Multidrug resistance-associated protein4 (MRP4)	2.1	2.7	5.6	+
At2g24180	Cytochrome P450 family protein	2.1	2	8.4	-
At3g14690	Cytochrome P450 (CYP72A15)	11.1	4.0	1.3	+
At3g21700	GTP binding (SGP2)	2.7	2.3	5.3	-
At5g17860	Calcium exchanger 7 (CAX7)	2.3	3.9	20.4	
At2g43510	Trypsin inhibitor protein (TI1)	2.3	7.3	7.1	-

[a] Normalized fold induction of genes by PPA_1 and OPDA (75 µM) of at least twofold in Arabidopsis wild-type plants relative to controls but no induction in *tga2/5/6*. PPA_1- and OPDA-induced genes data were obtained from Mueller et al. (2008) at 4 hpt.

[b] Normalized fold induction of genes by *B. cinerea* of at least twofold in Arabidopsis wild-type plants relative to controls (Table S1; https://drive.google.com/file/d/0Bx4SCX1UvbirTmZBTEdlVVpSMEU/view?usp=sharing). *B. cinerea*-induced genes data were obtained from this study at 24 hpi.

[c] Presence of WRKY33 DNA binding motif (TTGACT/C; W-box).

PPA_1, phytoprostane-A_1; OPDA, 12-oxo-phytodienoic acid; *Bc*, *B. cinerea*

3.6 Validation of *OBUGs* and/or *PBUGs* dependent on *WRKY33* to *B. cinerea* infection

The results for *OBUG*s or *PBUG*s obtained from microarray data were confirmed by qRT-PCR analysis that revealed that some of the OPDA- or/and PPA_1-regulated genes were specifically regulated by *B. cinerea* (Figure 6). Similar to the observed microarray analysis, all tested *OBUG*s were induced by *B. cinerea* infection in wild-type plants only. However, the transcript levels of these genes change when the *WRKY33* gene was either absent or overexpressed (Figure 6a). For example, the *OBUG*s (*At5g05410*, *At3g14890* and *At4g11600*) were repressed in *wrky33* mutants (Figure 6a). Except of *At4g11600* that showed comparable expression levels with the wild type, the other two genes had lower transcript levels in the *WRKY33* overexpression lines. Even though the stress-responsive genes, *At4g01870* and *At2g39200*, were the only genes that were induced by the three genotypes by *B. cinerea*, their expression was altered in *WRKY33* loss- and gain-of-function plants (Figure 6a). In addition, gene expression of *PBDG* results obtained by qRT-PCR were similar to those by microarray. The induction of *At3g02120* transcript was not altered by the *WRKY33* loss-of-function; the other *PBDG*s showed a significant increase in the transcript levels in *wrky33* mutant when treated with the same pathogen (Figure 6a). Similarly, there was a significant induction in the 35S:*WRKY33* overexpression transgenic lines, suggesting that these genes play a role in *B. cinerea* defense.

Next, the array results for TGA dependent-*OBUG*/*PBUG*-inducible genes (Table 5) were verified upon infection with *B. cinerea* in all *WRKY33* backgrounds by qRT-PCR. Similar patterns of gene expression were observed in both qRT-PCR and microarray analyses (Figure 6b). The expression profiles of *OBUG*/*PBUG*s were dependent on the TGA transcription factor in *B. cinerea*-stressed plants (Table 5). We

also found a regulation of *B. cinerea*-induced *WRKY33* in plant defense system, affecting the cyclopentenone pathway TGA-dependent. Our results showed that *At5g13080, At3g14690* and *At3g21700* were induced by *B. cinerea* in wild-type; thus, this induction was significantly altered in the other *WRKY33* genotypes. Similar to the microarray analysis, *At5g17860* and *At2g43510* induction was dependent on the absence and presence of WRKY 33, respectively (Figure 6b). Together, this suggests that there might be a gene regulation programing by OPDA and PPA_1 that can be induced by *B. cinerea* through WRKY33.

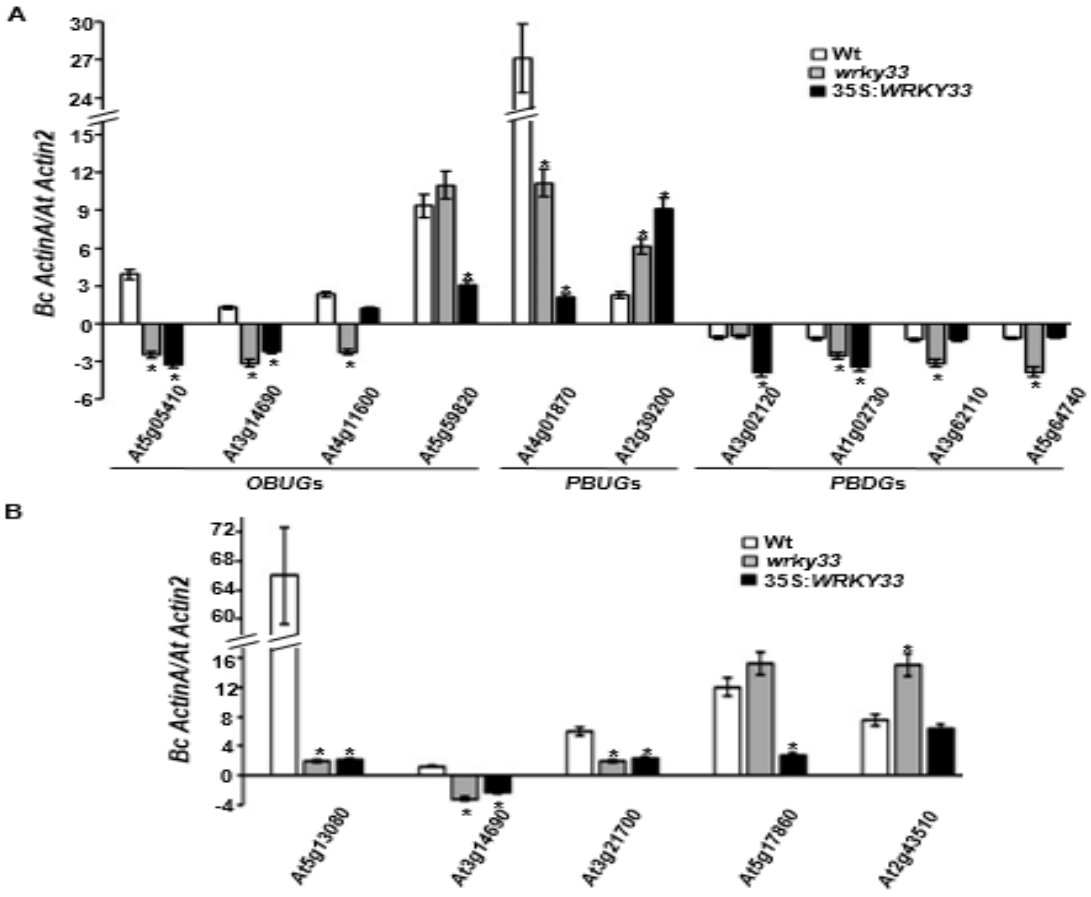

Figure 6: Expression of O*BUGs*/*PBUGs* in response to *B. cinerea.*

Relative expression levels obtained through qRT-PCR for *OBUG*s; *PBUGs*/*PBDG*s (a); and *OBUG*s/*PBUG*s (b) after infection with *B. cinerea* at 24 hpi. Expression of *B. cinerea*-inducible genes was quantitated relative to control conditions (no infection), and corrected for expression of the control gene (*AtActin2*). Error bars for qRT-PCR values are the standard deviations ($n \geq 3$). Mean values followed by an asterisk is significantly different from wild-type at the tested time (P=0.05). Experiments were repeated at least three times with similar results.hpi, hours post inoculation; *At Actin2, Arabidopsis thaliana Actin2* gene.

Chapter 4: Discussion

A global gene expression profiling using Affymetrix microarrays was performed in Arabidopsis *wrky33* mutant and 35S:*WRKY33* overexpressing transgenic plants during infection with the necrotrophic fungus *B. cinerea*. Our aim was to (i) identify upregulated and downregulated genes during *B. cinerea* pathogenesis; (ii) compare and link the *B. cinerea*-regulated genes in presence of *WRKY33* gene; and (iii) determine possible correlations of OPDA- and/or PPA_1-regulated genes in response to *B. cinerea* in presence of *TGA2/5/6* and *WRKY33* as target genes. We first assayed *wrky33* mutants with *B. cinerea* treatments and then assessed the susceptibility/resistance to the pathogen by quantifying the *B. cinerea ActinA* expression qRT-PCR (Benito et al. 1998) and by comparing the percentage of decayed plants in *wrky33* mutants, overexpression transgenic lines and wild-type plants. The *B. cinerea* hyphal growth and the number of rotten plants were much lower in the ectopic overexpression transgenic lines (35S:*WRKY33*) than in wild-type plants, suggesting an enhanced resistance to *B. cinerea* in these transgenic lines. This finding appears in agreement with previously tested visual observations, measurements of lesion diameter and fungal biomass (Zheng et al. 2006; Luo et al. 2010; Laluk et al. 2011b), suggesting that the Arabidopsis *WRKY33* gene is required for resistance to *B. cinerea*. Earlier studies of Arabidopsis defense mechanisms against *B. cinerea* have identified a certain number of defense-related genes or regulatory proteins using transcriptome and proteome analyses (AbuQamar et al. 2006; Mulema et al. 2011; Birkenbihl et al. 2012; Mulema and Denby 2012; Windram et al. 2012; Sham et al. 2014, 2015).

While the biological processes underlying plant responses to necrotrophs are still not fully understood, changes in Arabidopsis gene expression profiling and regulated genes were determined using microarray-based analysis after inoculation with *B. cinerea*. Necrosis, chlorosis, tissue maceration and plant decay are common symptoms of fungal infection in Arabidopsis (Fig. 1; AbuQamar et al. 2006). The time point was set at 24 hpi because it has proven that this short period allows to identify genes potentially involved in the early production of toxin and host specificity (AbuQamar et al. 2006; Muelema et al. 2011; Windram et al. 2012). We also combined high-throughput microarray technology and computational network to unravel the complex Arabidopsis-*B. cinerea* interaction. Most of the *BUG*s encode proteins that were responsive to biotic, abiotic and chemical stimuli, and signal transduction at 24 hpi. On the other hand, the major categories of the *BDG*s include genes encoding proteins belong to electron transport, responses to environmental cues, photosynthesis and other metabolic processes. This confirms that *B. cinerea*-upregulated proteins fall in the categories of response to chemical stimuli and plant hormone signal transduction; whereas downregulated proteins are involved in the photosynthesis, chlorophyll metabolism and carbon utilization categories (AbuQamar et al. 2006; Mulema et al. 2011; Sham et al. 2014, 2015). *B. cinerea*-upregulated proteins include kinases, transferases and other enzymes that are commonly induced upon pathogen infections to activate signal transduction pathways and metabolic reactions. Extracellular proteins or those localized within plastids, including chloroplasts, were downregulated as a defense response by the pathogen attack (Mulema and Denby 2012). Out of the 1660 of *BUG*s, 789 and 924 genes that were dependent on the presence and absence of *WRKY33,* respectively. On the other hand, a lesser number of genes were constitutively regulated by *WRKY33* encoding transcription factors

required for resistance to pathogens (Zheng et al. 2006). The target genes of the transcription factor WRKY33 are involved in the crosstalk between SA and JA/ET signaling and camalexin biosynthesis pathways (Birkenbihl et al. 2012). Our microarray analysis demonstrated similar results with other studies. For example, genes that are either considered as JA-responsive or involved in biosynthesis of JA were differentially expressed at 24 hpi in *wrky33* mutant and/or *35S:WRKY33* overexpressing lines compared with wild-type (Table S5; https://drive.google.com/file/d/0Bx4SCX1UvbirTmZBTEdlVVpSMEU/view?usp=sharing; Birkenbihl et al. 2012). Similarly, genes involved in JA/ET-mediated signaling, SA signaling, camalexin biosynthesis, and redox homeostasis were differentially regulated by WRKY33 in Arabidopsis plants inoculated with *B. cinerea*. At early stages of the infection with *B. cinerea*, *WRKY33*-impaired mutants contain high levels of SA; then, at later stages of infection, a downregulation of JA-associated responses occurs, which in turn, activates ZIM-domain genes and consequently represses JA signaling pathways (Birkenbihl et al. 2012). An early transcriptional response mediated by WRKY33 in Arabidopsis towards this necrotrophic fungus suggests that *WRKY33* altered expression will affect *B. cinerea*-regulated genes. Moreover, the elevated levels of ABA in *wrky33* mutant accompanied with the repression of *NCED3/NCED5* –involved in ABA biosynthesis– suggest a negative regulation of ABA signaling by WRKY33 in resistance against *B. cinerea* (Liu et al. 2015). Altogether, WRKY33 is associated with the regulation of hormonal signaling pathways upon *B. cinerea* attack. However, this does not rule out the possibility that WRKY33 may also play a role in the regulation of non-hormone targets in cyclopentenone signaling during defense responses to *B. cinerea*.

The OPDA is an active and immediate precursor of JA (Schaller and Stintzi 2009) and plays an independent role in mediating resistance to pathogens and pests (Böttcher and Pollmann 2009). The PPA_1 is a cyclopentenone isoprostane produced by the action of reactive oxygen species (ROS) from α-linolenic acid in plants (Thoma et al. 2003; Mueller and Berger 2009). In Arabidopsis, upon *B. cinerea* infection, ROS and a set of enzymes are induced, which in turn, undergo the nonenzymatic and enzymatic pathways, respectively. These events will lead to the accumulation and activation of cyclopentenones, phytoprostanes (*i.e.* PPA_1) and OPDA. PPA_1 enhances the expression of detoxification enzymes whereas OPDA induces a number of genes through COI1-dependent pathways. In addition, OPDA may function independently from COI1 (Mueller et al. 2008; Ribot et al. 2008; AbuQamar et al. 2013; Sham et al. 2014, 2015). PPA_1 also increases the phytoalexin biosynthesis rates, induces the expression of ABA- and auxin-responsive genes and genes involved in primary and secondary metabolism processes. The transcriptional profiles of many OPDA- and PPA_1-regulated genes during *B. cinerea* infection confirm previous results and show some overlap between genes upregulated by cyclopenetenone oxylipins and pathogens. For example, Arabidopsis plants treated with *P. syringae* accumulate nonenzymatically-formed hydroxyl fatty acids and PPs (Grun et al. 2007). OPDA, PPA_1 and other PPs accumulate after infection with necrotrophic pathogens independent of JA (Thoma et al. 2003; AbuQamar et al. 2013; Stotz et al. 2013). The induced expression of *WRKY33* and the increased susceptibility of its mutant upon infection with *B. cinerea* (Fig. 1; Zheng et al. 2006; Birkenbihl et al. 2012) suggest a key regulatory role of *WRKY33* gene in plant defense against *B. cinerea* invasion. In addition, COI1 which is required for JA signaling and resistance to *B. cinerea*, represses the basal expression of *WRKY33*. Previous studies have reported that OPDA

and PPA_1 may function through TGA transcription factors, independently from COI1 (Mueller et al. 2008) or through COI1 but independently of JA (Stotz et al. 2013). A large number of previously identified PPA_1/OPDA-responsive genes that are dependent on TGA2/5/6 (Mueller et al. 2008; Taki et al. 2008; Stotz et al. 2013) were also upregulated by *B. cinerea* (Table 5). About 91% of these regulated genes were also dependent of the presence/absence of WRKY33 transcription factor confirming previous regulation of these genes in response to *B. cinerea* (Ferrari et al. 2007). We speculate that this regulation is not only TGA-dependent but also WRKY33-dependent. Upon *B. cinerea* infection, the MAP kinases MPK3 and MPK6, directly phosphorylate WRKY33 *in vivo*, which in turn binds directly to PAD3 promoter, and subsequently this activates the expression of PAD3, the camalexin biosynthetic gene (Mao et al. 2011). We also found not only PAD3 contain W-box DNA-binding motif (Rushton et al. 2010) within their loci, but also MLO12 and WRKY75 that can be specifically bind to WRKY33 transcription factor (Tables 2 and 3), suggesting that these targets could potentially bind directly to WRKY33. This is in match with Liu et al. (2015) that WRKY33 regulates target genes involved in cell death (MLO12 and PAD3) or encoding transcription factors (WRKY75).

Chapter 5: Conclusion

Our goal was to identify common regulated genes of *wrky33* mutant and 35S:*WRKY33* overexpressing transgenic lines in response to *B. cinerea*; ultimately to improve plant stress tolerance in Arabidopsis. In this study, we identified a number of potential defense-related genes that coordinate regulatory pathways through *WRKY33* in mediating resistance to *B. cinerea*. Furthermore, there is a possibility to improve crop resistance by introducing the *WRKY33* gene into other crops (*i.e.* Solanacea family) generating resistant/tolerant modified crops to *B. cinerea*. The comparison of the expression profiling of the *WRKY33* gene in response to the *B. cinerea* may help biotechnologist in developing transgenic crops which can be resistant to that pathogen. It is recommended that future research must be done to investigate specific molecular functions of common regulated genes of WRKY33 transgenic lines post *B. cinerea* infection. In addition, further studies are needed to elucidate in detail the function and mechanism of cyclopentenone metabolism during *B. cinerea* and other necrotrophic pathogens infections.

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