Microarray analysis of Arabidopsis WRKY33 mutants in response to the necrotrophic fungus Botrytis cinerea

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Abstract

The WRKY33 transcription factor was reported for resistance to the necrotrophic fungus Botrytis cinerea. Using microarray-based analysis, we compared Arabidopsis WRKY33 overexpressing lines and wrky33 mutant that showed altered susceptibility to B. cinerea with their corresponding wild-type plants. In the wild-type, about 1660 genes (7% of the transcriptome) were induced and 1054 genes (5% of the transcriptome) were repressed at least twofold at early stages of inoculation with B. cinerea, confirming previous data of the contribution of these genes in B. cinerea resistance. In Arabidopsis wild-type plant infected with B. cinerea, the expressions of the differentially expressed genes encoding for proteins and metabolites involved in pathogen defense and non-defense responses, seem to be dependent on a functional WRKY33 gene. The expression profile of 12-oxo-phytodienoic acid- and phytoprostane A₁-treated Arabidopsis plants in response to B. cinerea revealed that cyclopentenones can also modulate WRKY33 regulation upon inoculation with B. cinerea. These results support the role of electrophilic oxylipins in mediating plant responses to B. cinerea infection through the TGA transcription factor. Future directions toward the identification of the molecular components in cyclopentenone signaling will elucidate the novel oxylipin signal transduction pathways in plant defense.

Introduction

Plant responses to necrotrophic fungi are complex and multigenic traits. They often depend on plant species, pathogens and their virulence and signaling pathways being involved [1, 2]. A number of effectors and microbe-associated molecular patterns (MAMPs) play important roles in determining plant-pathogen interactions. High-throughput transcriptomic approaches such as microarray are now commonly used to study the molecular mechanisms that control plant responses to environmental stresses, hormonal signals and pathogens. 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 [3]. During the pathogenesis, B.
cinerea induces host cell death by producing toxins, cell wall degrading enzymes and reactive oxygen intermediates (ROIs) [1, 4, 5]. Although cell death and accumulation of ROIs are associated with plant resistance to biotrophic pathogens [6], the ROIs can also increase plant susceptibility to necrotrophs [7]. In addition, the plant polygalacturonase-inhibiting proteins counteract polygalacturonase which are important host colonizing factors for some fungal pathogens [8]. 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 [9–11]. 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 [12].

Similarly to animals, plants recognize elicitors derived from pathogens to activate innate immune defense responses [13]. In contrast to race-specific elicitors or resistance genes described for biotroph-plant interactions, plants recognize a pathogen -regardless of its lifestyle- via MAMP that serve as general elicitors [14, 15]. Chitins and glucans are fungal MAMPs that plants can recognize by pattern recognition receptors. The Arabidopsis receptor kinases, FLS2 (flagellin sensing 2) and EFR (elongation factor Tu receptor), independently recognize the bacterial flagellin (flg22) and elongation factor Tu (elf18) epitopes, respectively, as MAMPs [16–18]. Recognition of B. cinerea MAMPs activates plant innate immunity system through mitogen activated protein kinase (MAPK)-based signaling cascades [19, 20], 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 [21]. Plant hormones also play crucial roles in triggering defense responses to pathogens. For example, signaling pathways involving salicylic acid (SA), ethylene (ET), jasmonate (JA), ABA, auxin and gibberellins may act independently, synergistically or antagonistically to confer the plants resistance against diseases [2, 22–27].

Even though genetic studies in Arabidopsis and tomato implicate that SA-mediated responses and SAR are associated with resistance to biotrophic pathogens [7], JA and ET are key regulators of plant responses to necrotrophic pathogens such as B. cinerea [2, 22, 28–30]. Recently, the cyclopentenone, 12-oxo-phytodienic acid (OPDA) and phytoprostanes, have been reported to accumulate upon infection by various pathogens [11, 31–35]. OPDA, a JA precursor, is produced enzymatically from α-linolenic acid and forms JA and/or its conjugates by OPDA reductase (OPR3) followed by three steps of β-oxidation [36]. Phytoprostanes, 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 cyclopentene/coronatine insensitive 1 (COI1) independently from JA under biotic stress [11, 37]. Yet, little is known about the role of electrophilic oxylipins OPDA or phytoprostane A1 (PPA1) in plant response to B. cinerea.

Nonetheless, gene expression profiling has been established in response to necrotrophic pathogens in many plant species such as Arabidopsis and tomato [22, 34, 35, 38–42]. Previously, wrky33-1 and wrky33–2 were identified as Arabidopsis mutants with increased susceptibility to B. cinerea and other necrotrophic pathogens [43]. 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. We also determined functional classes 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.

Materials and methods

Plant growth, disease assay and fungal growth

Arabidopsis wild-type, wrky33-1 mutant and 35S:WRKY33 overexpression transgenic plants [43] in Col-0 background were used in this study. Seeds of the wrky33 mutant and 35S:WRKY33 overexpressing transgenic lines were kindly provided by Dr. Tesfaye Mengiste and Dr. Zhixiang Chen, Purdue University (West Lafayette, IN, USA). 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 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 as previously described [22].

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} s^{-1}; 8 h light/16 h dark and 21 ± 2°C temperature. Responses to B. cinerea infection were assayed at 0 and 24 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.

RNA extraction and expression analysis

RNA extraction and real time quantitative-PCR (qRT-PCR) expression analyses were performed as described previously [11, 44]. 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 [45, 46]. The relative amplifications of B. cinerea-specific ActinA (BcActinA) to that of the Arabidopsis thaliana-specific Actin2 (AtActin2; At3G18780) was determined [47]. Gene expression levels were analyzed with qRT-PCR using gene-specific primers (S1 Table) at 0 and 24 hours post inoculation (hpi) with B. cinerea. The AtActin2 was used as an endogenous reference for normalization. Expression levels were calculated by the comparative cycle threshold method, and normalization to the control was performed as previously described [47].

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 with three whole plants each. 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 (Qiagen, Valencia, CA, USA)
and used for the subsequent experiments. cDNA synthesis, samples labeling, array hybridization, scanning, and data processing were conducted as previously described [48].

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 (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. Log₂-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 by its expression level in the control (mock-treated samples). A twofold difference at $P \leq 0.05$ was set as the threshold for considering a gene as to be B. cinerea induced genes (BIGs) or B. cinerea repressed genes (BRGs). The cutoffs of the fold change and $P$-value were chosen to filter false positives and to compare our data analyses with those in the microarray literatures. BIGs or BRGs 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.

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 20 plants. Analysis of variance and Duncan’s multiple-range test were performed to determine the statistical significance [49]. Mean values followed by an asterisk are significantly different from the corresponding control ($P<0.05$).

Results

B. cinerea infection in WRKY33 transgenic plants

The role of Arabidopsis WRKY33 gene in resistance to B. cinerea were previously reported [43]. 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 (Fig 1A), which is in agreement with previous observations [43]. We also noticed that the disease expanded by day 5 of the fungal inoculation, 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 (Fig 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 (Fig 1C). When we tested the outcome of overexpression of 35S:WRKY33 in transgenic plants infected with B. cinerea, we found that the infection was effective at one dpi and the symptoms were less severe than in the wild-type at 3 dpi (Fig 1B and 1C). Moreover, the majority of the overexpression lines survived at the same period of infection (Fig 1C), indicating that the constitutive overexpression of WRKY33 gene enhanced resistance to B. cinerea.

Differentially expressed Arabidopsis genes during B. cinerea infection

WRKY33 is highly induced upon B. cinerea infection [43]. The development of disease symptoms in Arabidopsis wild-type, wrky33 mutants and ectopic overexpression plants were analyzed (Fig 1). We compared the gene expression levels in these transgenic lines using Arabidopsis whole-genome Affymetrix gene chip (ATH1) representing approximately 25,000 genes to identify regulated genes by B. cinerea infection. Many BIGs and BRGs were identified. Differentially expressed genes (DEGs) have been identified by comparing the expression profiles of B. cinerea-inoculated and non-inoculated tissues (Fig 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 \cite{22, 40}. Fold expression changes have been calculated by dividing the normalized gene expression level of \textit{B. cinerea}-infected sample by their corresponding controls (no infection). In wild-type plants, we found 1660 BIGs and 1054 BRGs at 24 hpi compared with 0 hpi in wild-type. Gene identifications for 1660 BIGs and 1054 BRGs 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 \cite{50}.

Fig 2. Scatter-plot comparison and functional classification of DEGs in the \textit{B. cinerea}-Arabidopsis interaction network. (A) Normalized expression value for each probe set in wild-type plants infected with \textit{B. cinerea} at 24 hpi (WT-24) is plotted on the Y-axis; the value in wild-type plants sampled before \textit{B. cinerea} treatment (0 hpi; WT-0) is plotted on the X-axis. Functional classes of (B) BIGs; and (C) BRGs at 24 hpi compared with 0 hpi in wild-type. Gene identifications for 1660 BIGs and 1054 BRGs 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 \cite{50}.

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we found a number of genes encoding known regulatory, developmental and structural proteins that have previously been reported [22, 34, 35]. Most BIGs and BRGs encode functional proteins involved in plant responses to stress stimuli, signal transduction pathways, transport and energy pathways, metabolic and biological processes (Fig 2B and 2C). The fraction of genes involved in kinase activities was more prominent among the BIGs compared with the BRGs. A certain number of BIGs and BRGs 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 (Fig 2B). Most of the BRGs 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 BIGs and BRGs in various subcellular locations is consistent with the role of extracellular and intracellular components in plant response to B. cinerea infection.

**DEGs are dependent on Arabidopsis WRKY33**

We determined the basal expression level of the early regulated genes selected from wild-type samples altered in the transgenic plants. In the absence of the pathogen, the expression of 171 genes were differentially 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 (Fig 3A; S3 Table). By contrast, the expression of 23 (13.4%) genes were at least twofold lower in wrky33. Comparing the expression profiles from non-infected plants revealed that 332 genes were differentially expressed between the wild-type and 35S:WRKY33 lines, 251 (75.6%) of them were induced and 81 (24.4%) were repressed (Fig 3A; S3 Table). This indicates that the basal expression level of several genes is dependent on WRKY33.

The normalized transcriptional levels of all potentially DEGs in wrky33 and 35S:WRKY33 background lines were compared at 24 hpi (Fig 3B and 3C). Upon B. cinerea infection, expression levels of 1660 BIGs and 1054 BRGs in wrky33 mutant and WRKY33 overexpression lines were compared with the wild-type. The goal is to determine whether the expression levels of BIGs or BRGs are potentially dependent on WRKY33 or not. We found that the expressions of 4821 genes were altered more than twofold in wrky33 mutants; 921 induced and 3900 repressed, corresponding to 4% and 17% of the whole transcriptome, respectively (Fig 3A; S4 Table), with a common set of 789 up- and 847 repressed genes showing similar changes upon infection in both wrky33 and wild-type plants (Fig 3D; S4 Table). The B. cinerea-inducible or -repressed gene was considered to be dependent on WRKY33 if the average expression level following B. cinerea inoculation in wrky33 mutant line was 2-fold repressed or induced, respectively, than the expression level in the wild-type plant. About 45% (751/1660) of the B. cinerea-induced genes in wild-type plants were also repressed in the wrky33 mutant inoculated by the same pathogen (Fig 3A). On the other hand, the expression level of a set of genes (110/1054) representing 10.4% of the whole Arabidopsis genome was greatly reduced in wild-type plants but increased in the wrky33 mutant following B. cinerea inoculation. This alteration in the expression levels of DEGs between the wild-type and wrky33 mutant suggests a potential involvement of DEGs in the WRKY33-dependent response to B. cinerea. 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) (Fig 3A). We also figured out 924 up- and 914 repressed genes in the overexpression line were commonly changed in the wild-type plants (Fig 3E; S5 Table). 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 repressed) 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 down-regulated) genes were altered in the gain-of-WRKY33 function (Fig 3E). We also determined all reciprocal combinations of common DEGs between wild-type, wrky33 and 35S:WRKY33 at 0 or 24 hpi of inoculation with B. cinerea. 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. Venn diagram showing the overlapping numbers of BIGs and BRGs in wild-type and (D) wrky33; or (E) 35S:WRKY33 plants at 24 hpi with B. cinerea. In (A, D and E), boxes represent total number, and arrows represent the number of BIGs and BRGs between the treatments and the genotypes tested. Wt, wild-type; wrky33, wrky33mutant; 35S:WRKY33, 35S:WRKY33 overexpression transgenic line; hpi, hours post inoculation.

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Fig 3. Transcriptional reprogramming and scatter-plot comparisons of DEGs in WRKY33 transgenic plants. (A) The numbers of DEGs (≥ 2-fold at P ≤ 0.05) between wild-type, wrky33 and 35S:WRKY33 at 0 or 24 hpi of inoculation with B. cinerea. 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. Venn diagram showing the overlapping numbers of BIGs and BRGs in wild-type and (D) wrky33; or (E) 35S:WRKY33 plants at 24 hpi with B. cinerea. In (A, D and E), boxes represent total number, and arrows represent the number of BIGs and BRGs between the treatments and the genotypes tested. Wt, wild-type; wrky33, wrky33mutant; 35S:WRKY33, 35S:WRKY33 overexpression transgenic line; hpi, hours post inoculation.

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35S:WRKY33 genotypes. This confirms previous published datasets comparing expression levels of hormone signaling pathways in wild type- and wrky33-infected plants [51]. In addition, camalexin biosynthetic genes, cytochrome P450 (CYP71A13) and phytoalexin deficient 3 (PAD3) were also induced 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 glutaredoxin (GRX48), cytokinin oxidase/dehydrogenase (CKX4), NADPH/respiratory burst oxidase protein D (RBOHD) and thioredoxin-H5 (TRX-H5), increased in wrky33 mutants after B. cinerea attack. The latter genes were also induced at 24 hpi with the same pathogen. Together, our data suggest a regulatory role of WRKY33 in mediating gene expression which corresponds to disease responses in its mutant and overexpressing lines.

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 [52, 53]. The two groups of B. cinerea-responsive genes (BIGs and BRGs; S2 Table) were analyzed with OPDA- or PPA_1-regulated genes to determine possible correlations between the four groups [33, 54]. It has been reported that WRKY33 regulates the expression of many genes encoding components associated with hormonal signaling pathways during B. cinerea infection [51, 55]. To determine whether WRKY33 regulates non-enzymatic targets in the Arabidopsis genome following infection with B. cinerea, the expression of BIGs and BRGs in the WRKY33 mutant and overexpressing transgenic line with that of OPDA and PPA_1 regulators were thus compared. Although none of the OPDA-downregulated genes [54] were repressed by B. cinerea infection (Fig 4), a group of genes that were 2-fold induced by OPDA treatment [54] and B. cinerea infection, thus termed as OBIGs, were demonstrated (Table 1; S7 Table). Of the OPDA-upregulated genes identified [54], 24.3% (17/70) were also induced by B. cinerea infection in wild-type plants (Fig 4). The OBIGs encode a subset of proteins including kinases, Aldo/keto reductase, FAD-linked oxidoreductase, ABA-responsive and other related proteins. Seven of the 17 (41%) OBIGs were dependent on WRKY33 (Fig 4). Targets of the OBIGs, DREB2A (At5g05410) and B-box zinc-
finger (At2g47890) proteins, are involved in pathogen attack signaling and abiotic stress signaling [56, 57] were altered in both WRKY33 mutant and overexpression backgrounds (Table 1). The Arabidopsis oxidative stress-related gene, GPX6 (At4g11600) encoding glutathione peroxidase protein [58], was the only gene that was induced by both OPDA and B. cinerea in wrky33 mutant background (Table 1). On the other hand, the OBIG-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. Together, WRKY33 transcription factor was found to have a potential role in OPDA-mediated regulation of gene expression.

In addition, DEGs upon B. cinerea infection were also compared with PPA1-regulated genes [33]. Two distinct groups were identified: genes that were induced by both PPA1 and B. cinerea (termed as PBIGs) and genes that were repressed by both PPA1 and B. cinerea (termed as PBRGs) (Table 2; S7 Table). In Arabidopsis wild-type plants, 25.5% (19/47) and 50.0% (17/34) of induced or repressed genes by PPA1 were also induced or repressed by B. cinerea, respectively (Fig 4).

PBIGs appear to fall in a gene category related to detoxification or stress responses such as the cytochrome P450, UDP-glucuronosyl transferases, transporters, heat shock factors/proteins, and TolB-related proteins. By contrast, PBRGs 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 PBIGs (16.7%) and 11 of the PBRGs (64.7%) genes were dependent on WRKY33, respectively (Fig 4). The TolB-related (At4g01870) and mildew resistance locus O12 (MLO12; At2g39290) responsive genes which were previously expressed in response to fungal infections [35, 59], were also induced by B. cinerea in wild-type plants; thus regulated by the absence or presence of WRKY33 (Table 2). It is worth mentioning that WRKY proteins specifically bind to a DNA motif (TTGACT/C; also termed the W-box) [60], where 80% of the identified WRKY33 binding regions contained the W-box motif [55]. MLO12 contains W-box motif in its promoter. AGP17- and At3g02120 repressed to both of PPA1 treatment and with B. cinerea infection was dependent on 35S:WRKY33 only (Table 2). Although, we figured out that 6 PBRGs were differentially expressed in wrky33 mutant only; 3 others were dependent for their suppression to both the mutant and the overexpressing line of WRKY33 (Table 2). Our data

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^aFold induction = normalized OPDA treatment or B. cinerea inoculation/normalized no OPDA treatment or B. cinerea inoculation. Data set on at least twofold induction or repression after treatment/inoculation.

^bOPDA-upregulated genes data were obtained from [54] at 3 hpt.

^cB. cinerea-induced genes data were obtained from this study at 24 hpi.

^dGene regulation is dependent on WRKY33 (S4 and S5 Tables).

OBIGs, OPDA-B. cinerea-induced genes; w33, wrky33; 35S:W33, 35S:WRKY33.

Table 1. Regulation of genes by OPDA treatment and B. cinerea infection.
indicate that WRKY33 transcriptionally regulates genes commonly involved in plant response to PPA1 and *B. cinerea*, suggesting that WRKY33 may play a role in the non-enzymatic pathway that is responsible for the synthesis of PPA1 oxylipin involved in plant stress responses.

Previous studies have reported that OPDA and PPA1 may function through TGA transcription factors, independently from JA [33, 61, 62]. Many genes (53% of the whole genome) containing a TGA motif (TGACG) in the 500 bp upstream of their promoters may contain binding sites for TGA transcription factors [63]. It has been reported that 60% of the PPA1- and 30% of the OPDA-inducible are dependent on the TGA transcription factors TGA2/5/6 [33]. Microarray analysis revealed that electrophilic oxylipins are involved in mediating responses to *B. cinerea* infection and abiotic stress through TGA transcription factors [34, 35].

We set our analysis on induced genes by PPA1 and OPDA treatments [33] and *B. cinerea* infection. Of the 52 induced genes by the two cyclopentenone oxylipins [33], 26 (50.0%) were *B. cinerea*-induced and 21 (40.4%) were dependent on the presence of TGA2/5/6 (Fig 4). Five of the identified OBIG/PBIGs (19.2%) were dependent on WRKY33. Upon infection with the plant pathogen *B. cinerea*, some induced genes were responsive to treatments with PPA1 and OPDA. These genes could be regulated by a common pathway in which WRKY33 may act through TGA transcription factors. Of the five OBIG/PBIGs that were dependent of WRKY33, two were in a TGA-dependent manner, representing 40% of the OBIG/PBIGs (Fig 4). 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 3). Both WRKY75 (Table 3) and PAD3 (S8 Table) contain W-box motif in their loci.

Table 2. Regulation of genes by PPA1 treatment and *B. cinerea* infection.

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<tr>
<th>Description</th>
<th>Gene locus</th>
<th>Fold inductiona</th>
<th>Expression requiresd</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PBIGs</strong></td>
<td></td>
<td>PPA1b</td>
<td><em>B. cinerea</em>c</td>
</tr>
<tr>
<td>TOLB protein-related</td>
<td>At4g01870</td>
<td>20.1</td>
<td>4.5</td>
</tr>
<tr>
<td>Mildew resistance locus O12 (MLO12)d</td>
<td>At2g39200</td>
<td>9.6</td>
<td>2.3</td>
</tr>
<tr>
<td><strong>PBRGs</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arabinogalactan protein 17 (AGP17)</td>
<td>At2g23130</td>
<td>-5.2</td>
<td>-1.8</td>
</tr>
<tr>
<td>Hyp-rich glycoprotein family protein</td>
<td>At3g02120</td>
<td>-4.6</td>
<td>-1.1</td>
</tr>
<tr>
<td>Cellulose synthase-like 5 (CSDL5), Salt Overly Sensitive 6 (SOS6)</td>
<td>At1g02730</td>
<td>-3.7</td>
<td>-1.1</td>
</tr>
<tr>
<td>Auxin Inducible 2–11 (AUX2-11)</td>
<td>At5g43700</td>
<td>-3.8</td>
<td>-2.1</td>
</tr>
<tr>
<td>Actin-11 (ACT11)</td>
<td>At3g12110</td>
<td>-3.6</td>
<td>-1.8</td>
</tr>
<tr>
<td>ASCiCLIN-like arabinogalactan 18 precursor (FLA18)</td>
<td>At3g11700</td>
<td>-5.1</td>
<td>-1.6</td>
</tr>
<tr>
<td>Pectin lyase-like superfamily protein</td>
<td>At3g62110</td>
<td>-4.5</td>
<td>-1.3</td>
</tr>
<tr>
<td>Cellulose synthase 6 (CESA6)/Isoxaben resistant 2 (IXR2)</td>
<td>At5g64740</td>
<td>-3.1</td>
<td>-1.1</td>
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<td>CYCLIN D3 (CYCD3)</td>
<td>At4g34160</td>
<td>-3.5</td>
<td>-1.4</td>
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<tr>
<td>Short hypocotyl 2 transcription factor (SHY2)</td>
<td>At1g04240</td>
<td>-3.4</td>
<td>-1.8</td>
</tr>
<tr>
<td>Auxin-induced 13 (IAA13)</td>
<td>At2g33310</td>
<td>-3.2</td>
<td>-1.7</td>
</tr>
</tbody>
</table>

afold induction = normalized PPA1 treatment or *B. cinerea* inoculation/normalized no PPA1 treatment or *B. cinerea* inoculation. Data set on at least twofold induction or repressed after treatment/inoculation.

bPPA1-upregulated genes data were obtained from [33] at 4 hpt.

c*B. cinerea*-repressed genes data were obtained from this study at 24 hpi.

dGene regulation is dependent on WRKY33 (S4 and S5 Tables).

PBIGs, PPA1-*B. cinerea* induced genes; PBRGs, PPA1-*B. cinerea* repressed genes; w33, wrky33, 35S:W33, 35S:WRKY33.

ePresence of WRKY33 DNA binding motif [55].

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Table 3. Upregulated genes by PPA, and OPDA treatments and *B. cinerea* inoculation dependent on TGA2/5/6 and WRKY33.

<table>
<thead>
<tr>
<th>Array element</th>
<th>Gene locus</th>
<th>Description</th>
<th>Fold induction</th>
<th>Expression requires&lt;sup&gt;a&lt;/sup&gt;</th>
<th>TGACG presence&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>PPA&lt;sup&gt;a&lt;/sup&gt;</td>
<td>OPDA&lt;sup&gt;a&lt;/sup&gt;</td>
<td><em>Bc</em>&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>OBIG/PBIGs</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>245976</td>
<td>At5g13080</td>
<td>WRKY75 transcription factor (WRKY75)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>10.4</td>
<td>4.4</td>
<td>41</td>
</tr>
<tr>
<td>258094</td>
<td>At3g14690</td>
<td>Cytochrome P450 (CYP72A15)</td>
<td>11.1</td>
<td>4.0</td>
<td>1.3</td>
</tr>
<tr>
<td>257951</td>
<td>At3g21700</td>
<td>GTP binding (SGP2)</td>
<td>2.7</td>
<td>2.3</td>
<td>5.3</td>
</tr>
<tr>
<td>250054</td>
<td>At5g17860</td>
<td>Calcium exchanger 7 (CAX7)</td>
<td>2.3</td>
<td>3.9</td>
<td>20.4</td>
</tr>
<tr>
<td>260551</td>
<td>At2g43510</td>
<td>Trypsin inhibitor protein (T11)</td>
<td>2.3</td>
<td>7.3</td>
<td>7.1</td>
</tr>
</tbody>
</table>

<sup>a</sup>Normalized fold induction of genes by PPA<sub>a</sub> and OPDA (75 μM) of at least twofold in Arabidopsis wild-type plants relative to controls but no induction in tga2/5/6. PPA<sub>a</sub>- and OPDA-induced genes data were obtained from [33] at 4 hpt.

<sup>b</sup>Normalized fold induction of genes by *B. cinerea* of at least twofold in Arabidopsis wild-type plants relative to controls (S2 Table). *B. cinerea*-induced genes data were obtained from this study at 24 hpi.

<sup>c</sup>Gene upregulation is dependent on WRKY33 (S6 and S7 Tables). PPA<sub>a</sub>, phytoprostane-Α<sub>1</sub>; OPDA, 12-oxo-phytodienoic acid; *Bc*, *B. cinerea*; w33, wrky33; 35S:W33, 35S:WRKY33.

<sup>d</sup>Presence of WRKY33 DNA binding motif [55].

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On the other hand, other regulators which do not contain a TGA motif, such as *At3g21700* (SGP2), *At5g17860* (CAX7) or *At2g43510* (T11), were transcriptionally dependent on WRKY33 after infection. This suggests a regulation of some OBIG/PBIGs by WRKY33 upon infection with *B. cinerea*.

Validation of OBIGs and/or PBIGs dependent on WRKY33 to *B. cinerea* infection

The results for OBIGs or PBIGs obtained from microarray data were confirmed by qRT-PCR analysis that revealed that some of the OPDA- or and PPA<sub>a</sub>-regulated genes were specifically regulated by *B. cinerea* (Fig 5). Similar to the observed microarray analysis, all tested OBIGs 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 (Fig 5A). For example, the OBIGs (*At5g05410*, *At3g14890* and *At4g11600*) were repressed in wrky33 mutants (Fig 5A). 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 (Fig 5A). In addition, gene expression of PBRG 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 PBRGs showed a significant increase in the transcript levels in wrky33 mutant when treated with the same pathogen (Fig 5A). 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, we verified the array results for TGA dependent-OBIG/PBIG-inducible genes (Table 3) 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 (Fig 5B). The expression profiles of OBIG/PBIGs were dependent on the TGA transcription factor in *B. cinerea*-stressed plants (Table 3). 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 (Fig 5B). Together, this suggests that there might be a gene regulation programing by OPDA and PPA1 that can be induced by B. cinerea through WRKY33.

Fig 5. Expression of OBIGs/PBIGs in response to B. cinerea. Relative expression levels obtained through qRT-PCR for (A) OBIGs, PBIGs or PBRGs; and (B) OBIGs/PBIGs 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 ≥ 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.

doi:10.1371/journal.pone.0172343.g005
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 induced and repressed genes during B. cinerea pathogenesis; (ii) compare and link the DEGs after B. cinerea infection in presence of WRKY33 gene; and (iii) determine possible correlations of OPDA- and/or PPA1-regulated genes in response to B. cinerea in presence of TGA2/5/6 and WRKY33 as stress-associated 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 [45] 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 [27, 43, 64], 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 [22, 34, 35, 40, 51, 65, 66].

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) [22]. We set up the time point 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 [22, 40, 65]. We also used high-throughput microarray technology to unravel the complex Arabidopsis-B. cinerea interaction. In Arabidopsis wild-type plants, the expression levels of 2714 genes was altered at least twofold or more compared to non-infected plants with 1660 genes being up-regulated and 1054 genes being repressed, representing 7.3% and 4.6% of the overall Arabidopsis transcriptome, respectively. Most of the BIGs 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 BRGs include genes encoding proteins belong to electron transport, responses to environmental cues, photosynthesis and other metabolic processes. This confirms that the 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 in response to this necrotrophic fungal pathogen [22, 34, 35, 65]. The 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 [66]. Out of the 1660 of BIGs, 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 [43]. The target genes of the transcription factor WRKY33 are involved in the crosstalk between SA and JA/ET signaling and camalexin biosynthesis pathways [51, 67]. 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 (S6 Table) [51, 68]. 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 [51, 69]. An early transcriptional response mediated by WRKY33 in Arabidopsis towards this necrotrophic fungus suggests that WRKY33 altered expression will affect gene regulation upon infection with B. cinerea. 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 [55]. 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 [54] and plays an independent role in mediating resistance to pathogens and pests [61]. The PPA1 is a cyclopentenone isoprostane produced by the action of reactive oxygen species (ROS) from α-linolenic acid in plants [31, 54]. 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. PPA1) and OPDA. PPA1 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 [11, 33–35, 37]. PPA1 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 PPA1-regulated genes during B. cinerea infection confirm previous results and show some overlap between genes upregulated by cyclopentenone oxylipins and pathogens. For example, Arabidopsis plants treated with P. syringae accumulate nonenzymatically-formed hydroxyl fatty acids and PPs [70]. OPDA, PPA1 and other phytoprostanes accumulate after infection with necrotrophic pathogens independent of JA [11, 31, 62]. The induced expression of WRKY33 and the increased susceptibility of its mutant upon infection with B. cinerea (Fig 1) [43, 51] 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 PPA1 may function through TGA transcription factors, independently from COI1 [33] or through COI1 but independently of JA [62]. A large number of previously identified PPA1/OPDA-responsive genes that are dependent on TGA2/5/6 [33, 54, 62] were also induced by B. cinerea (Table 3; S8 Table). 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 [71]. 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 [72]. Liu and colleagues [55] have reported that several WRKY33-regulated proteins, including MLO12, are involved in cell death. In addition to PAD3, we found MLO12 and WRKY75 (Tables 2 and 3) [55] contain the W-box DNA-binding motif in their promoter [60], suggesting that WRKY75 is binding to (and thus presumably regulating) WRKY33. Thus, the regulation between WRKY33 and its downstream targets in response to B. cinerea is underway.
In this study, we identified a number of potential defense-related genes that coordinate regulatory pathways through WRKY33 in mediating resistance to B. cinerea. Further investigations are needed to elucidate in detail the function and mechanism of cyclopentenone metabolism during B. cinerea and other necrotrophic pathogens infections.

Supporting information
S1 Table. List of qRT-PCR primers (sequence 5’ to 3’) used in this study. (PDF)
S2 Table. Expression levels and fold (A) induction in all BIGs or (B) repression in all BRGs, selected from wild-type samples. (XLSX)
S3 Table. List of probe sets/array elements and locus identifiers corresponding to genes that are (A) inducible or (B) repressible basal expression in wrky33 mutant; or (C) inducible or (D) repressible basal expression in 35S:WRKK33 overexpression transgenic lines. (XLSX)
S4 Table. List of probe sets/array elements and locus identifiers corresponding to genes that are (A) induced or (B) repressed in wrky33 mutant; and commonly (C) induced or (D) repressed in both wild-type and wrky33 mutant in response to B. cinerea inoculation. (XLSX)
S5 Table. List of probe sets/array elements and locus identifiers corresponding to genes that are (A) induced or (B) repressed in 35S:WRKY33 overexpression; and commonly (C) induced or (D) repressed in both wild-type and 35S:WRKY33 overexpression in response to B. cinerea inoculation. (XLSX)
S6 Table. List of probe sets/array elements and locus identifiers corresponding to genes that are commonly (A) induced or (B) repressed in wild-type, wrky33 mutant and 35S:WRKY33 overexpression in response to B. cinerea inoculation. (XLSX)
S7 Table. Regulation of genes by OPDA or PPA1 treatment and B. cinerea infection. (PDF)
S8 Table. Induced genes by PPA1 and OPDA treatments and B. cinerea inoculation. (PDF)

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Formal analysis: SAQ.
Funding acquisition: SAQ.
Investigation: SAQ.
Methodology: AS SA SAQ.
Project administration: SAQ.
Resources: RI SAQ.
Software: AS SAS.
Supervision: SAQ.
Validation: AS SAS.
Visualization: AS SAS SAQ.
Writing – original draft: SAQ.
Writing – review & editing: AS KM SAS SA RI SAQ.

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