**Title:** Endophytic and pathogenic strains of *Verticillium dahliae* elicit defense responses in phylogenetically distant hosts

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**Abstract**

The fungus *Verticillium dahliae* is an endophyte and pathogen of hundreds of plant species.The goal of this research was to characterize host and *V. dahliae* genes during symptomatic and asymptomatic infections. To accomplish this goal, we tested the null hypotheses that differences in gene expression do not exist (i) within hosts during infection with different *V. dahliae* isolates that vary in aggressiveness*,* (ii) between *V. dahliae* isolates within a host, and (iii) between *V. dahliae* isolates across symptomatic and asymptomatic hosts. Potato, peppermint, and brown mustard plants were inoculated with two isolates of *V*. *dahliae* that vary in aggressiveness. Whole plants were harvested 10 days post inoculation. Dual RNA-sequencing (RNA-seq) was completed. In total 2,214, 1588, 2,079 and 41 differentially expressed genes (DEGs) were detected from potato, peppermint, brown mustard, and *V. dahliae*, respectively*.* Of these, 12, 4, 10, and 4 genes were validated for potato, peppermint, brown mustard, and *V. dahliae* with reverse transcription quantitative PCR (RT-qPCR). For both symptomatic hosts, potato and mint, at least twice as many DEGs were detected from plants inoculated with the most aggressive isolate of *V. dahliae* compared to plants inoculated with the less aggressive isolate. Of the characterized DEGs, genes associated with both pattern-triggered immunity (PTI) and effector-triggered immunity (ETI) were detected from all hosts, regardless of symptom expression. For *V. dahliae*, only two DEGs were detected between isolates within a host: one from potato and one from peppermint. Of these genes, one was uncharacterized and one was associated with oxioreductase activity. Lastly, a total of 15 *V. dahliae* DEGs were detected between asymptomatic mustards plants and either symptomatic potato or peppermint plants. While some of these DEGs were uncharacterized, others were involved in melanin biosynthesis, catalytic, peptidase, oxioreductase, and hydrolase activity. This research has documented the similarities and differences in host and fungal gene expression during infection with isolates of *V. dahliae* that vary in aggressiveness.

**Introduction**

Members of the fungal genus *Verticillium* infect hundreds of plant species (Berlanger and Powelson, 2000). Isolates of the most economically destructive species of *Verticillium*, *V. dahliae* Kleb., (Pegg and Brady, 2002) can behave as both pathogens and endophytes on different hosts (Malcolm et al 2015; Wheeler et al. 2018). For example, some hosts, like brown mustard (*Brassica juncea* L.), are colonized but do not express detectable symptoms (Wheeler and Johnson, 2016). Other hosts, like potato (*Solanum tuberosum* L.) and peppermint (*Mentha* x *piperita* L.) are susceptible, express acute symptoms, and respond to infection with reductions in biomass (Dung et al. 2010; Johnson and Dung, 2010). Although the pathogen is generally considered to have a wide host range, intraspecific differences in aggressiveness has been documented amongst *V. dahliae* isolates, including those from potato and peppermint (Douhan and Johnson 2001; Johnson and Dung, 2010).

Successful management of these symptomatic reactions to *V. dahliae* could potentially be improved with more information about the genetic dynamics that influence symptomology. As of now, management of Verticillium wilts is difficult because *V. dahliae* produces survival structures called microsclerotia that can survive for 14 years (Wilhelm 1955), can symptomatically or asymptomatically infect and reproduce on a wide range of crops and weeds (Berlanger and Powelson, 2000), is sometimes (Tsror et al. 2005) but not always sensitive to soil-fumigants (Woodward et al. 2011). Additionally, resistance to the fungus is not always present in cultivars that possess other desirable traits (Johnson and Dung, 2010). Solutions to some of these management obstacles could conceivably be revealed by information about the genes that dictate biological processes in both *V. dahliae* and its hosts.

The authors are not the first to make this observation. Reports of transcriptional differences among isolates of *V. dahliae* and its hosts are abound. For example, differentially expressed genes (DEG) of *V. dahliae* isolates were detected by Duressa *et al*. 2013, Jin *et al*. 2019, and Jiménez-Ruiz *et al*. 2019 under different conditions. Similarly, DEGs from various hosts infected with *V. dahliae* have been reported by Guo *et al.* 2017, Sun *et al.* 2017, Tan *et al.* 2015. Finally, at least one study documented the transcriptomes of both *V. dahliae* and one of its hosts, *Arabidopsis*, during infection (Scholz *et al.* 2018). Thus, gene expression profiles *V. dahliae* and its symptomatic hosts have received considerable attention.

In contrast, the authors are not aware of any studies that have documented the transcriptomes of multiple *V. dahliae* isolates and asymptomatic or symptomatic hosts during infection. The goal of this research is to fill this gap. To accomplish this goal, the asymptomatic host, brown mustard, and symptomatic hosts, including potato and peppermint, were separately inoculated with one of two isolates of *V. dahliae* that varied in aggressiveness on potato and peppermint. Dual RNA-seq was subsequently completed. DEGs were detected and validated. The results documented here demonstrate that transcriptomes of hosts and isolates are mostly similar despite overt differences in symptomology and aggressiveness.

**Materials and Methods**

To test the hypotheses stated above, a dual RNA-seq trial was completed and validated with real-time quantitative reverse transcription PCR (RT-qPCR). Each trial is described below.

**RNA seq trial**

The treatment structure of the dual RNA seq trial was a 3 x 3 completely crossed factorial design. The first factor, host, consisted of three levels: Russet Burbank potato, Black Mitchum peppermint, and brown mustard ISCI 99. Similarly, the second factor, fungus, consisted of three levels: *Verticillium dahliae* isolate 653 (highly aggressive towards potato and weakly aggressive towards peppermint), *V. dahliae* isolate 111 (highly aggressive towards peppermint and mildly aggressive towards potato), and a water control. Neither isolate elicits visible symptoms on brown mustard. Each treatment was replicated 10 times and arranged in a randomized complete block design in a greenhouse.

Seeds of brown mustard, potato plantlets, and cuttings of peppermint with 2-4 nodes were planted in Turface® (Profile Products LLC, Buffalo Grove, IL) to enable easy harvesting. Plants were fertigated with 100 ppm of 20-10-20 NPK (Peters Professional, Summerville, SC) daily until all plants emerged from the Turface. Upon emergence of all plants, the Turface was either drenched with 100ml of 1 x 106 *V. dahliae* conidia/ml or sterile distilled water. After inoculation, plants were grown under a 15 hour/day photoperiod for 10 days.

Whole plants were harvested 10 days post inoculation (dpi). Harvested plants were subsequently flash frozen in liquid nitrogen, stored at -80°C for 1 week, and lyophilized. Once dried, whole plants were grounded with a mortar and pestle. RNA was extracted with a modified version of Kumar *et al.* 2007 (**Supplementary document 1**). Estimates of RNA quality and quantity were determined by fragment analysis (Advanced Analytical Technologies Inc., Ankeny, IA) at Washington State University’s Center for Reproductive Biology, Molecular Biology and Genomics Core.

Samples of 3 biological replicates from each treatment were sent to Novogene corporations (Beijing, China) for library preparation and sequencing. In short, after RNA qualification, mRNA was enriched with oligo (dT) beads, cDNA was synthesized and purified with end-repairs and the addition of poly (A) tails and ligation of adapters. Fragments were amplified and sequenced on an Illumina NovaSeq 6000 platform (Illumina, San Diego, CA).

For potato and *V. dahliae,* RNA fragments were mapped to each respective reference genome (potato: PRJNA63145; Xu *et al.* 2011; *V. dahliae*:PRJNA225532; Klosterman et al. 2011). For peppermint and brown mustard, RNA fragments were first filtered to remove reads with adapter contamination, reads comprised of 10% or more uncertain nucleotides, or reads comprised of 50% or more of low-quality nucleotides. After filtering, clean reads were assembled with Trinity (Grabherr *et al.* 2011). Redundant contigs were identified and removed with hierarchical clustering in Corset (Davidson and Oshlack, 2014). Unigenes were then selected as the longest transcript for each cluster.

Gene expression levels were determined and DEGs were identified with the DESeq2 package version 1.26.0 (Love *et al.* 2014) in R version 3.6.2 (R Core Team (2019)). Reads with fewer than 1 count across samples were removed to expedite analysis. Contrasts between hosts within an isolate of *V. dahliae* and between isolates within a host were calculated after log fold change shrinkage (LFC) with the lfcShrink() function in R. *P-*values were adjusted to *q*-values = 0.001 to control for the False-Discovery Rate (FDR).

Gene expression levels for potato and *V. dahliae* were determined with the expected fragments per kilobase of transcript per million fragments mapped (FPKM) (Trapnell *et al.* 2010). For peppermint and brown mustard, the abundance of reads were estimated with RNA-seq by Expectation-Maximization (RSEM) with Bowtie 2 (Langmead *et al.* 2012). FPKM was then used to calculate gene expression levels. DEGs were identified with the DESeq2 package version 1.26.0 (Love *et al.* 2014) in R version 3.6.2 (R Core Team (2019)).

Functional annotation of unigenes was completed with seven databases: NR, NT, Pfam, KOG/COG, Swiss-Prot, KEGG, and GO. Alignment of unigenes to protein databases was accomplished with several software packages. Alignment of unigenes in NR, Swiss-Prot, and KOG was completed with Diamond 0.8.22 (Buchfink *et al.* 2015) with evalue thresholds of 1 × 10−5 for NR and Swiss-Prot and 1 × 10−3 for KOG. Alignment of unigenes in NT was completed with NCBI’s Blast 2.2.28+ (Altschul *et al.* 1990) with an e-value threshold of 1 × 10−5. Alignment of unigenes in Pfam was completed in HMMER (Eddy 2011) with an e-value threshold of 1 × 10−2. Alignment of unigenes in GO was completed with results from Pfam and NR in Blast2GO v2.5 (Götz et al., 2008) with an e-value of 1 × 10−6. Finally, alignment of unigenes in KEGG was completed with the KAAS-KEGG Automatic Annotation Server with an e-value of 1 × 10−10 (Moriya *et al.* 2007).

GO enrichment analysis of significant DEGs was performed using Blast2GO v4.0 for each host. Statistically significant GO terms were identified based on Fisher’s exact test, and Benjamini-Hochberg FDR corrected *P*-values less than 0.05.

**Validation trial**

 A subset of the DEGs identified in the RNA-seq experiment described above were validated with an independent experiment. Plants were grown, inoculated, and RNA was isolated, quantified and qualified as described above. cDNA was synthesized from 1 µg of total RNA in 20µl reaction volume using qScriptTM cDNA SuperMIX (QuantaBio) following the manufacturer’s instructions. The cDNA was diluted to 1:5 in DNAase and RNAase free sterile water for brown mustard, potato, and peppermint gene validation. For V. dahliae gene validation, cDNA was diluted to 1:3. All cDNA was stored at -20°C prior to validation.

A total of 28 DEGs were selected for validation from RNA-seq results. DEGs were selected for each host if they exhibited the highest fold change values or represented putative pathogenicity or virulence-related genes. Primers were designed for each gene using NCBI Primer-BLAST. Primers with 19-23 bp size, 40-60% GC content, amplicon size of 65-180 bp with no self-annealing and primer dimer formation were used. Primer sequences and amplicon lengths are presented in **Table 1**.

RT-qPCR was performed in a QuantStudioTM Real-Time PCR System (Applied Biosystems) using SYBRTM Select Master Mix (2X) (Applied Biosystems) in 10µl reaction volumes. The reaction mixture consisted of 5µl SYBR Select Master Mix(2X), 0.5µl of each forward and reverse primers (10µM), 1µl diluted cDNA and 3µl sterile water. The cycling conditions for RT-qPCR were 95°C for 10 minutes, followed by 40 cycles of 95°C for 15s and 60°C for 1 min. Melting curve analysis was performed from 60°C to 95°C with fluorescence reading acquired at 0.5°C increments per cycle to confirm the presence of a single amplified product. Three biological and technical replications were used for all DEGs. Negative controls consisted of samples without cDNA. Primer efficiency and Cycle threshold (Ct) values were calculated using LinRegPCR program (Ruijter *et al*. 2009). Primer efficiencies of DEGs ranged between 72 to 97%. The log2 fold change value was derived using the delta-delta Ct method for each comparison (Livak and Schmittgen 2001). Normalization of the DEGs for potato was completed with the house-keeping gene elongation factor 1-α (EF1α), and with the actin gene (ACT*)* for brown mustard, peppermint, and V. *dahliae* (**Table 1**). To confirm the direction of fold change values RT-qPCR data were compared with fold changes obtained from RNA-sequencing. Correlations between fold change estimates from these methods were calculated for each host.

**Results**

**RNA seq trial**

Summary statistics for reference-based transcriptome assemblies for potato and *V. dahliae* are presented in **Table 2**. For potato, an average of 68% of total reads mapped back to the reference genome across all samples. For *V. dahliae,* an average of 0.1% of total reads mapped back to the reference genome across all samples.

Summary statistics for *de novo-based* transcriptome assemblies for peppermint and brown mustard are presented in **Table 3**. For both peppermint and brown mustard, an average of 98% of raw reads were retained after read cleaning. Further, for both peppermint and brown mustard, 99% of total transcripts were identified as unique unigenes.

The number of DEGs varied across hosts and *V. dahliae* isolates. Tables of DEGs are provided as supplementary files (**Supplementary Tables 2-5**).

The total number of detected DEGs varied among treatments (**Figure 1**). For brown mustard, a total of 2,079 DEGs were detected (**Figure 1Aa, Supplementary Table 2**). Of these, approximately 60% were upregulated (**Supplementary Table 2**). Approximately 40% of all DEGs were identified from the contrast between plants infected with *V. dahliae* isolate 653 (aggressive towards potato) and the non-inoculated control. Conversely, only about 23% of total DEGs were identified from the contrast between plants infected with *V. dahliae* isolate 111 and the non-inoculated control. In comparison, about 1% DEGs were identified from the contrast between plants infected with different isolates of *V. dahliae.* Shared DEGs were not detected in any of the contrasts for brown mustard (**Figure 1Aa**).

For potato, a total of 2,214 DEGs were detected (**Figure 1Ab, Supplementary Table 3**). Of these, approximately 47% were upregulated (**Supplementary Table 3**). As above, approximately, 47% of all DEGs were identified from the contrast between plants infected with *V. dahliae* isolate 653 and the non-inoculated control. Further, about 19% of the DEGs were identified from the contrast between plants infected with *V. dahliae* isolate 111 and the non-inoculated control. Like mustard, about 1% of DEGs were identified from the contrast between plants infected with different isolates of *V. dahliae.* Finally, 0.1% of DEGs were shared among all of the contrasts for potato (**Figure 1Ab**).

For mint, a total of 1,588 DEGs were detected (**Figure 1A, Supplementary Table 4**). Of these, approximately 30% were upregulated (**Supplementary Table 4**). Approximately 83% of all DEGs were identified from the contrast between plants infected with *V. dahliae* isolate 111 and the non-inoculated control. Conversely, only about 1% of these DEGs were identified from the contrast between plants infected with *V. dahliae* isolate 653 and the non-inoculated control. Still fewer DEGs, about 0.5%, were identified from the contrast between plants infected with different isolates of *V. dahliae.* Lastly, 0.25% of DEGs were shared among all of the contrasts for brown mint (**Figure 1Ac**).

For *V. dahliae,* a total of 62 DEGs were detected (**Figure 1B, Supplementary Table 5**). Of these DEGs, 40% were upregulated (**Supplementary Table 5**). Of the 62 total DEGs, only two DEGs were detected from comparisons between isolates within a host (**Supplementary Table 5**). For the *V. dahliae* DEGs detected between hosts, 27 and 44% were detected between the asymptomatic host, brown mustard, and either symptomatic host. The remaining *V. dahliae* DEGs were detected between symptomatic hosts.

Like the differences in DEGs detected among hosts, gene expression patterns of *V. dahliae* varied across hosts and *V. dahliae* isolates (**Figure 2**). In general, patterns in gene expression were patchy within each host and between *V. dahliae* isolates*.* For example, brown mustard genes expressed in response to infection with *V. dahliae* isolates varied from each other as much as each did from plants that were not inoculated (**Figure 2a**). Similarly, for potato, overt differences in gene expression between non-inoculated plants and those inoculated with either isolate of *V. dahliae* represented only a subset of the total genes (e.g. *LOX12*) (**Figure 2b**). However, exceptions to this observation were noted. For example, potato plants inoculated with either isolate of *V. dahliae* expressed lower levels of several genes (top rows of **Figure 2b**) compared to non-inoculated plants. Likewise for peppermint, some plants inoculated with either of the *V. dahliae* isolates exhibited gene expression patterns more similar to non-inoculated plants than inoculated plants. Finally, for *V. dahliae*, the differences in gene expression between isolates are largely eclipsed in magnitude by the differences observed between hosts. In other words, the strong vertical patterns in **Figure 2d** demarcate differences between hosts, not isolates.

Patterns in DEGs were further investigated with volcano plots (**Figure 3A**). For the DEGs from host plants, the largest consistent differences in the magnitude of gene expression were observed in asymptomatic brown mustard plants. However, the single largest change in relative expression was detected in peppermint plants infected with *V. dahliae* isolate 111 compared to inoculated plants. Further, the most statistically significant changes associated with the smallest *q*-values occurred in potato and peppermint plants inoculated with *V. dahliae* isolate 111 compared to inoculated plants (**Figure 3A**).

For DEGs from *V. dahliae* isolates, the largest differences in the magnitude of gene expression were observed in *V. dahliae* isolate 111 recovered from peppermint and compared to mustard or potato (**Figure 3B**). Small but statistically significant differences in gene expression were detected within potato and peppermint. Moreover, the most statistically significant changes associated with the smallest *q*-values occurred in all comparisons between hosts and within a *V. dahliae* isolate (**Figure 3B**).

Significant gene ontology (GO) terms from three main GO categories: biological process, molecular function, and cellular component were identified for DEGs using the GO database in Blast2GO. A total of 322, 235, and 156 significantly enriched GO terms were identified for potato, brown mustard, and peppermint, respectively. The largest number of GO terms belonged to the biological process (GO:0008150) followed by molecular function (GO:0003674) and cellular component (GO: 0005575) **(Supplementary Table 6). However,**no significantly enriched GO terms were identified for Verticillium dahliae. The top 15 GO terms for each host are presented in **Figure 4**. Five GO terms within the metabolic process sub-group and three within the catalytic activity sub-group were common in all three hosts. In potato, a higher number of significantly (adjusted *P* < 0.05) enriched GO terms were related to stimulus response (GO:0050896) compared to brown mustard and peppermint **(Supplementary Table 6).** The GO terms related to stimulus response in potato included response to stress (GO:0006950), response to biotic stimulus (GO:0009607), response to fungus (GO:0009620), immune response (GO:0006955), defense response (GO:0006952), response to reactive oxygen species (GO:0000302), chitin catabolic process (GO:0006032), and chitin metabolic process (GO:0006030). However, in brown mustard, two GO terms belonging to the same category were detected namely, response to stress (GO:0006950) and response to oxygen-containing compounds (GO:1901700). In peppermint, only one GO term, response to stimulus (GO:0050896), was significantly enriched.

A separate GO enrichment analysis was performed to elucidate biological differences in each host during infection with the *V.* *dahliae* isolates that varied in aggressiveness. Different types of significantly enriched GO terms were observed with different frequencies for each host and comparison (**Supplementary Table 7**). The brown mustard, endophyte of both isolates, had nearly equal number of significantly enriched GO terms in both comparisons (111 vs. control and 653 vs. control) **(Table 4)**. Interestingly, more GO terms were detected from potato DEGs within the isolate 653 (aggressive towards potato) vs. control contrast compared to the isolate 111 (less aggressive towards potato) vs. control contrast **(Table 4)**. A similar pattern was observed in peppermint as well. For peppermint, DEGs observed in 111 (aggressive towards peppermint) vs. control had 98 significantly enriched GO terms, whereas DEGs from 653 (less aggressive towards peppermint) vs. control had no significantly enriched GO terms **(Table 4)**. In addition, GO analysis for DEGs from 653 vs. 111 in potato identified two GO terms; defense response and response to biotic stimulus, whereas no significant GO terms were detected in brown mustard and peppermint for the same comparison **(Table 4)**.

**Validation trial**

A subset of the DEGs identified with RNA-seq data above were validated with RT-qPCR. A total of 30 of 43 DEG comparisons, including 10 from brown mustard, 12 from potato, 4 from peppermint, and 4 from *V. dahliae*, were validated. These comparisons include expression changes of 5, 9, 3 and 4 genes for brown mustard, potato, peppermint, and *V. dahliae*, respectively. The DEGs with similar direction (up or down-regulation) of fold changes in both RT-qPCR and RNA-seq data are presented in **Figure 5**. The correlation coefficient (*r*) of gene expression changes (log2fold change) between RT-qPCR and RNA seq were 0.97, 0.91, and 0.86, and 0.85 forbrown mustard, potato, peppermint, and *V. dahliae,* respectively.

Generally, the magnitude of gene expression change was lower in RT-qPCR compared to RNA-seq (**Figure 5**). Differences in the magnitude of fold changes likely arose from the different normalization methods used for RNA-seq and RT-qPCR (Love *et al.* 2014). A total of 13 comparisons of DEGs did not exhibit the same expression patterns as those from the RNA seq data. More specifically, a total of 8, 2, 2, and 1 DEGs with different expression patterns were from brown mustard, potato, peppermint and *V. dahliae*, respectively.

For the hosts, the biological function of the DEGs varied depending on the isolate of *V. dahliae* with which they were inoculated. For example, Pathogenicity-related (PR) genes like *PR04* and *PRR1* (Vleeshouwers et al. 2000) were significantly upregulated (adjusted *P* value < 0.05) in potato plants when challenged with the aggressive 653 isolate compared to the control and less aggressive 111 isolate. The transcription factor *WRK40*, which plays an important role in plant signal transduction upon pathogen recognition (Eulgem and Somssich 2007), was also differentially expressed in potato infected with isolate 111 compared to the non-inoculated control.

Additionally, a jasmonic acid (JA) regulation gene, *TIF5A*, that regulates defense responses against hemibiotrophic pathogens like *V. dahliae* (Scholz *et al*. 2018) was upregulated in potato plants inoculated with the less aggressive isolate 111 compared to the more aggressive isolate 653. Similarly, oxidoreductase and *lipoxygenase* (*LOX12*) genes were upregulated in response to infection by isolate 111 compared to isolate 653. However, two defense response genes of potato, *PRS2* and *IER1* showed the opposite expression in the RT-qPCR data compared to the data from RNAseq. Potential sources for these differences likely include differences in the sensitivity of the two methods, environmental differences, and the use of a different cDNA for quantification (Wang et al. 2016).

For brown mustard, DEGs involved in nuclear mRNA export, ATP binding, kinase activity, DNA binding transcription factor, and RNA binding were validated with RT-qPCR. For example, *NUP1* involved in mRNA transport was differentially upregulated by 3 and 3.5 folds in brown mustard plants inoculated with isolates 653 and 111 respectively compared to non-inoculated control. Similarly, *PDRP2* involved in ATP binding, and transferase activity was downregulated in brown mustard plants infected with 653 and 111 compared to non-inoculated control. *SCL1*, a transcription regulator, was also differentially downregulated in 111 inoculated plants compared to plants inoculated with 653 and non-inoculated plants.

Several DEGs were identified in peppermint following inoculation with *V*. *dahliae*. Most up-regulated genes were homologous with *Arabidopsis* *thaliana* genes that are potentially involved in pathogen recognition, such as FB30 (putative F-box protein) and EGL1 (transcription activator), and responses to stress, such as IFRH (isoflavone reductase) and PER45 (peroxidase). Peppermint genes that were downregulated tended to be involved in photosynthesis, including homologues of CB21 (chlorophyll a-b binding protein) and PNSB3 (photosynthetic NDH subunit). Genes associated with photorespiration (RBS2), cell morphogenesis (ARP3), and ion transport (CNGC5) were also down-regulated. For *V. dahliae*, genes with putative roles in virulence such as peptidase, hydrolase, oxidoreductase, and catalytic activity were differentially expressed. AOX is involved in oxidoreductase activity was differentially upregulated in isolate 111 compared to isolate 653 upon infection in potato. The heptaketide hydrolase, *AYG1*, which plays a role in melanin biosynthesis (Fujii *et al*. 2004) was differentially expressed in isolate 111 during infection of brown mustard compared to potato. In addition, differential expression of *AYG1* was also observed in both isolates during infection of both potato and peppermint. Similarly, another melanogenesis associated gene, scytalone dehydratase (*SCYD*) (Kubo *et al*. 1996), was also downregulated in isolate 111 during infection of potato compared to peppermint. In addition, putative virulence factor, pectate lyase, *PLYF* was downregulated in isolate 111 during infection of brown mustard compared to peppermint.

**Discussion**

Verticillium wilts are problematic diseases for many plant species in temperate regions around the world (Pegg and Brady, 2002). Management of Verticillium wilts is difficult, in part, because of the variation in symptom expression and aggressiveness across hosts and isolates of *Verticillium dahliae*, respectively*.* The short-term goal of this research was to document the transcriptomes of hosts and isolates along the spectrums of symptom expression and aggressiveness, respectively. Long-term, this research should provide resources for other scientists to study and develop management strategies for Verticillium wilts.

To accomplish these goals, transcriptomes were characterized (i) within potato, brown mustard, and peppermint plants in response to infection by different *V. dahliae* isolates, (ii) between *V. dahliae* isolates within each host, and (iii) between *V. dahliae* isolates across symptomatic and asymptomatic hosts.

When brown mustard, potato, and peppermint were challenged with *V. dahliae,* transcriptional responses to the fungus were similar for some hosts and different for others. For example, at least one gene associated with anthocynanin biosynthesis, *ELG1* (Zhou *et al.* 2022)*,* was upregulated exclusively in peppermint plants, a species that often expresses anthocyanescence symptoms when infected with *V. dahliae.* Additionally and despite differences in symptom expression between the symptomatic hosts, potato and peppermint, and the asymptomatic hosts, brown mustard, all were enriched for metabolic processes, catalytic activity (eg. oxido-reductase, hydrolase, lyase, transferase) and stimulus response. These transcriptional similarities, despite overt differences in symptomology, are consistent with a recent report from the Arabidopsisand *Fusarium oxysporum* system (Guo et al. 2021) where pathogenic and endophytic isolates of *F. oxysporum* elicited similar responses from Arabidopsis. Moreover, this observation supports the hypothesis that differences in symptomology are not always categorically reflected in host transcriptomes.

Potato, peppermint, and brown mustard also produced DEGs associated with pattern-triggered immunity (PTI) defense responses when challenged with at least one isolate. For example, plasma membrane (PM)-localized pattern recognition receptors (PRRs) were detected from potato and brown mustard plants inoculated with both isolates (**Supplementary tables 2 and 3**). Similarly, genes from the lysin motif receptor-like kinase, *LYK5,* were upregulated in peppermint plants inoculated with isolate 111 compared to plants that were not inoculated (**Supplementary table 4**). These genes help detect microbe-associated molecular patterns (MAMPs) or damage-associated molecular patterns (DAMPs) from microbes and induce subsequent defense responses or activate downstream signaling pathways (Bigeard et al. 2015; Huang et al. 2020; Jones and Dangl, 2006). Thus, both isolates elicit MAMPs responses in potato and brown mustard and isolate 111 elicits MAMPs responses in peppermint. Although differentially regulated PRRs were not detected in peppermint plants when challenged with isolate 653, differential regulation of receptor like cytoplasmic kinases (RLCKs) (eg. *PBL15*, *CRK2*) and mitogen-activated protein kinase 9 (*MPK9*) were observed. RLCKs and MPK are part of PTI signaling components that are activated by PRRs for downstream signaling (Yuan et al. 2021). Although expected for the symptomatic hosts, it was not obvious, *a priori*, that PRR related genes would be differentially expressed in asymptomatic brown mustard plants. Fortunately, these results too were corroborated in the Arabidopsis and *F. oxysporum* system by (Guo et al. 2021).

Similarly, evidence of effector-triggered immunity (ETI) was also detected in all host plants challenged with select isolates. Genes from the receptor-like cytoplasmic kinase genes were detected from peppermint plants challenged with either isolate (**Supplementary table 4**). These types of genes interact with effectors from pathogens and help regulate ETI (Guy et al. 2013). Similarly, transcripts of a pathogenesis-related R gene (*PRR1*) were recovered from both potato and brown mustard plants when challenged with both isolates or isolate 111, respectively. The evidence of PTI and ETI in all three hosts was further supported by the expression and differential regulation of putative pathogenicity related genes (eg. *AYG1*, *PLYF*, *SCYD*) and effectors (eg. isochorismatase) of *V. dahliae* in all three hosts (Kubo et al. 1996; Fuji et al. 2004; Liu et al. 2014; Yang et al. 2018) (**Supplementary table 5**)*.* Detection of these defense-related genes in asymptomatic hosts, like brown mustard, was corroborated in the maize and *Fusarium virguliforme* by Baetsen-Young *et al* 2021. In this system, the researchers attributed phenotypic differences between hosts to differential temporal activation of defense responses between asymptomatic maize plants and symptomatic soybean plants (Baetsen-Young *et al* 2021). To determine if temporal differences in defense responses predict phenotypes in the *V. dahliae* system, additional time course experiments are needed.

Relatively few differences in gene expression were detected within *V. dahliae.* Most *V. dahliae* DEGs (n=25), for example, were detected between brown mustard and peppermint plants inoculated with isolate 111 while only two DEGs were detected between brown mustard and potato plants inoculated with isolate 653. Thus *V. dahliae* appears to produce more similar gene expression profiles between brown mustard and potato than between brown mustard and peppermint. This is consistent with the inference that populations of *V. dahliae* from brown mustard co-occur with those from potato (Wheeler et al. 2018) and, if we assume genetic similarity translates to transcriptome similarity, may therefore produce transcriptomes in each host.

Only two DEGs were detected between the two isolates of *V. dahliae* within the hosts. The DEG detected between isolates during infection of potato was characterized while the DEG detected between isolates during infection of peppermint was not characterized. Within potato, transcripts of alternative oxidase (*AOX*) were upregulated when inoculated with isolate 111 compared to isolate 653. Since fungal AOX proteins are involved in pathogenesis, fungicide resistance, stress signaling, cellular development, and mycotoxin production (Tian *et al.* 2020), *AOX* genes may warrant further investigation to development better management strategies for Verticillium wilt of potato. Within peppermint, transcripts of an uncharacterized gene were downregulated when inoculated with isolated 653 compared to isolate 111. Within brown mustard, no DEGs were detected between isolates of *V. dahliae.* Although a null result, the absence of DEGs between isolates during infection of asymptomatic brown mustard raises interesting questions about the biology of endophytic vs pathogenic relationships. For example, do all isolates produce the same transcriptomes during infection of asymptomatic hosts, or is there yet-to-be-sampled variation? Given that only one DEG was detected between isolates during infection of either symptomatic host, perhaps some isolates produce similar transcriptomes in response to most hosts unless provided sufficient time to develop transcriptomes specific for certain hosts, like potato and peppermint. More research is needed to address these questions since transcriptomics of endophytic and pathogenic fungi during infection of different asymptomatic and symptomatic hosts appears to be a neglected topic.

In summary, the transcriptomes of all hosts were relatively similar despite differences in symptom expression. DEGs were detected across all hosts but not across all isolates of *V. dahliae.* Evidence of PTI and ETI were detected among symptomatic potato, and peppermint and asymptomatic brown mustard plants inoculated with *V. dahliae* isolates. Finally, only a few DEGs were detected across isolates of *V. dahliae* within each host. Collectively, these results support the hypothesis that transcriptomes of hosts and *V. dahliae* isolates are more similar than different. Additional research is needed to identify sources of differences between hosts with and without symptoms and between pathogenic and endophytic isolates.

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**Tables**

**Table 1.** List of primer sequence and putative functions of differentially expressed genes (DEGs) used for the RT-qPCR validation

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Host | Gene name | Putative biological/ molecular function | Forward sequence (5’ to 3’) | Reverse sequence (5’ to 3’) | Amplicon size (bp) |
| brown mustard | Cluster-15354.86688 | Unknown | ATTCACACTGCTCCACGCTA | GGCTGAAGGGTGAGAATGGG | 78 |
| brown mustard | *NUP1* | Nucleus or nuclear membrane organization | CCATCCTTGCTTGGATTGCC | ATGCAGGAGGCTAAGGTTGG | 110 |
| brown mustard | *PDRP2* | ATP binding/ Phospho- transferase activity | TATAAAGCAGGCAGCGAAGC | GAGAGCACTCCCCAACGAT | 105 |
| brown mustard | *SCL1* | Transcription regulation | AACTGCTGAAAAGGATGACAAGT | TGCTCTTGCTGCTTTCCGTT | 84 |
| brown mustard | Cluster-15354.44072 | RNA binding | TGCGTTCCTCAGAACCAGAG | AGCTTCTTCTCCACTGCTGAC | 106 |
| brown mustard | *ACT-2* | Housekeeping gene | TGGGTTTGCTGGTGACGAT | TGCCTAGGACGACCAACAATACT | 290 |
| potato | *PR04* | Plant defense | GCCGTGCAATTGTGGGTGTC | CGCACACTTTTCCACTAGCAC | 76 |
| potato | *ABAH1* | Stress response | CCACTTCCTCCTGGTACTTTAGG | AACTTGTTTAGCTGCCTCTGG | 177 |
| potato | PGSC0003DMG400024310 | Heterocyclic compound binding/ cation binding | GAGAAGGAAGATTGGTGGGACA | CTACCCATCCCTCCTCCACA | 105 |
| potato | *LOX12* | Oxidoreductase/ dioxygenase activity | ATTAGCTCTGTTCAAGGTGATCC | TCTCCAAGTAGGCTGGATTGC | 70 |
| potato | *PRR1* | Plant defense | TGTCTTTTGCCCTTGAAGGCT | GACAACGTCTCACCAGCTCT | 115 |
| potato | *CHSB* | Transferase activity | GAGCTCAAGGAGAAATTTAAGCG | ACAACAACTATGTCTTGCCTTGC | 149 |
| potato | *EDL3* | Stress response | AATGGTCGGATCGGAGGAGA | TCGGATTACACCCGCAACAG | 70 |
| potato | *WRK40* | Transcription regulation | AGACAACCCATCTCCAAGAGC | TCGATTGGTCTTCCACGCTT | 95 |
| potato | *TIF5A* | Defense response | ATGTCCGAGCCTTCATCACC | GGAGCAACTAGTGATGGTATGGT | 130 |
| potato | *EF1α* | Housekeeping | ATTGGAAACGGATATGCTCCA | TCCTTACCTGAACGCCTGTCA | 101 |
| peppermint | *CNGC5* | Probable cyclic nucleotide-gated ion channel | TAATCTGCGTCGGCAGTGAA | AGCGCGAGGGAGCTAGTGA | 65 |
| peppermint | *EGL1* | Transcription activator, involved in trichome and root cell formation, anthocyanin biosynthesis | ACCCTTGGCCGACATTGA | GCATTATCCCCTGAAGATCTCACT | 83 |
| peppermint | *PMTK* | Methyltransferase activity | CATTTCGCCAAAGAGCTCAAT | CCGATGCAGCATGGTACAAC | 94 |
| *V. dahliae* | *AOX* | Oxidoreductase activity | GCTGCGTGGAAGTTTGTGC | TTCTTGTCAACCTGCTGCTCA | 83 |
| *V. dahliae* | *YDDQ* | Hydrolase activity | AAGATTGTGCTCGTCGGGTA | TCTCAGCCAGAGCAACCTTC | 163 |
| *V. dahliae* | mRNA\_1341 | Unknown | GCTGTCCGCATCTGACTTGT | GGTGACGTTGAACTTTGCCA | 97 |
| *V. dahliae* | *AYG1* | Melanin biosynthesis | GATTCGGCTGACCCAGACAG | ACCTTGCCCATATCGAACCG | 89 |
| *V. dahliae* | *ACT* | Housekeeping | GGCTTCCTCAAGGTCGGCTATG | GCTGCATGTCATCCCACTTCTTC | NA |

**Table 2.** Summary statistics for reference-based transcriptome assembly of *Solanum tuberosum* and *Verticillium dahliae*. Included are the total number of reads generated, reads that map to the reference genomes, reads that map to multiple locations, reads that map to only one location, and reads that did not map to the respective reference genome.

|  |  |  |
| --- | --- | --- |
| **Source** | ***Solanum tuberosum*** | ***Verticillium dahliae*** |
| Total reads | 65,720,887 ± 16,583,820 | 77,301,461 ± 6,069,398 |
| Mapped reads | 44,601,321 ± 11,149,227 | 103,065 ± 66,259 |
| Multiple mapped reads | 1,764,566 ± 428,612 | 439 ± 327 |
| Uniquely mapped reads | 42,836,754 ± 10,728,311 | 102,625 ± 65,971 |
| Unmapped reads | 21,119,565 ± 5,447,805 | 77,198,396 ± 6,074,812 |

**Table 3.** Summary statistics for *de novo* transcriptome assembly of *Mentha x piperita* and *Brassica juncea*. Included are the sequencing read counts, the number of reads recovered after filtering, the total number of clean nucleotides, the percentage of bases with correct nucleotide recognition greater than 99.9%, the total number of transcripts and unigenes, and the mean lengths of transcripts and unigenes.

|  |  |  |
| --- | --- | --- |
| **Source** | ***Mentha x piperita*** | ***Brassica juncea*** |
| Raw reads | 69,008,844 ± 17,431,082 | 65,190,911 ± 21,342,502 |
| Clean reads | 67,599,600 ± 17,071,506 | 63,754,238 ± 20,863,378 |
| Clean nucleotides (G) | 10 ± 2 | 10 ± 3 |
| Q30 (%) | 96 ± 0.1 | 95 ± 2 |
| Number of transcripts | 266,580 | 223,003 |
| Mean length of transcripts (nt) | 937 | 790 |
| Number of unigenes | 266,009 | 222,364 |
| Mean length of unigenes (nt) | 684 | 792 |

**Table 4:** Number of significantly enriched GO terms in brown mustard, potato, and peppermint for differentially expressed genes (DEGs) in different comparisons. DEGs observed in each host for comparisons: control vs. *Verticillium dahliae* isolate 111, control vs. *Verticillium dahliae* isolate 653, and *Verticillium dahliae* isolates 653 vs. 111 were used to identify numbers of significant GO terms.

|  |  |  |  |
| --- | --- | --- | --- |
| Comparison | Number of GO terms | | |
| brown mustard | Potato | Peppermint |
| 111 vs control | 89 | 25 | 152 |
| 653 vs control | 103 | 98 | 0 |
| 653 vs 111 | 0 | 2 | 0 |
| Total | 192 | 125 | 152 |

**Figures**

**Figure 1.** Comparisons of differentially expressed genes (DEGs) between (**A**): brown mustard (a), potato (b), and peppermint (c) infected with *Verticillium dahliae* isolate 653 and non-inoculated control (pink), *V. dahliae* isolate 111 and non-inoculated control (blue), and between *V. dahliae* isolates 653 and 111 (orange) and (**B**): *V. dahliae* isolates recovered from each host. Numbers within each venn diagram represent the shared number of DEGs between comparisons.

Diagram

Description automatically generated

**Diagram, venn diagram

Description automatically generated**

**Figure 2.** Heatmaps for the top 20 differentially expressed genes (DEG) for (a) brown mustard, (b) potato, (c) peppermint, and (d) *Verticillium dahliae*.DEGs are clustered with *k-*means by rows. Columns represent treatments. DEGs without recognizable gene names were not homologous to genes from online repositories. Each cell represents a biological replicate. The color of each cell reflects the relative changes in gene expression illustrated in the legends.



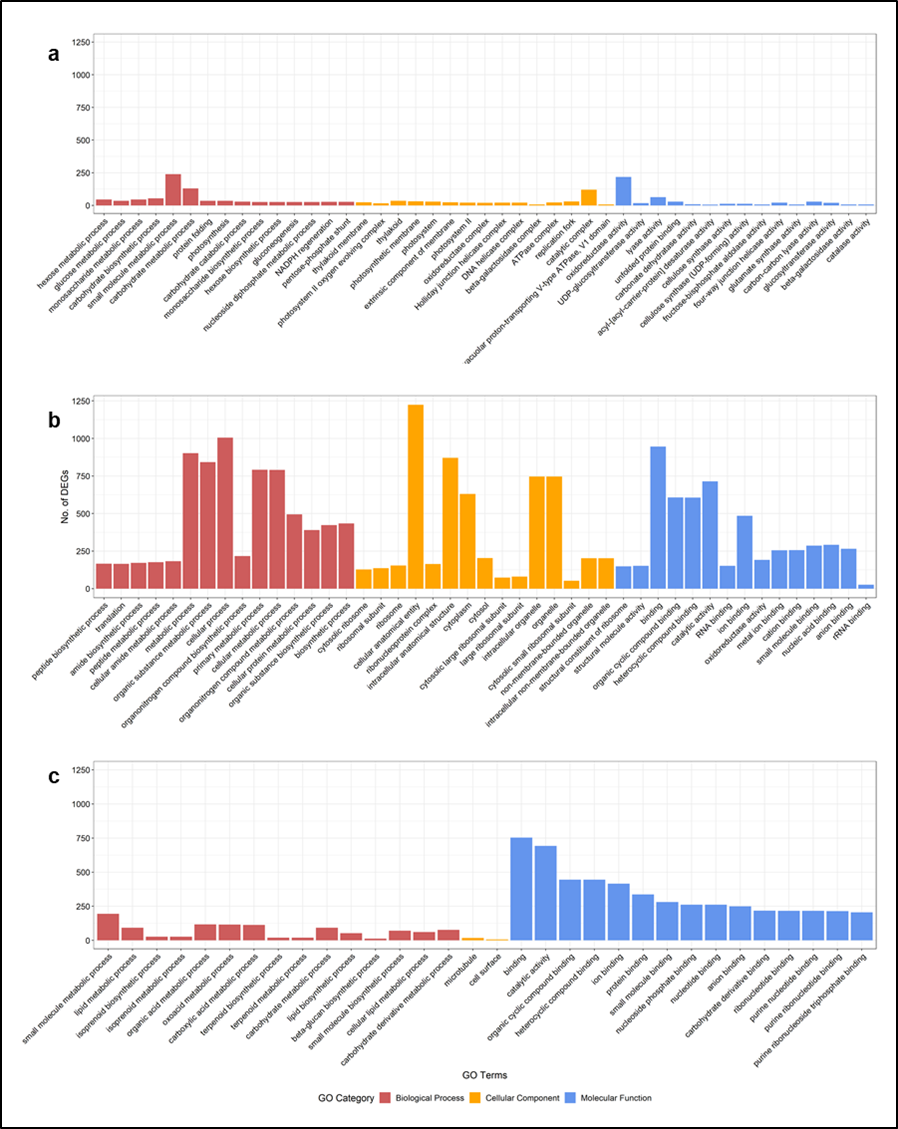
**Figure 3.** Volcano plots for (**A**) brown mustard (a), potato (b), and peppermint (c) and (**B**) *Verticillium dahliae.*  Log-transformed *P*-values are expressed as a function of the fold-change between comparisons of interest. Panel **A** illustrates comparisons between *V. dahliae* isolate 653 vs. non-inoculated control, *V. dahliae* isolate 111 vs. non-inoculated control, and between both *V. dahliae* isolates. Panel **B** illustrates comparisons between *V. dahliae* isolates within a host and between hosts within an isolate. Black dots represent genes with relatively small fold-changes and large, non-significant, *P*-values. Yellow dots represent genes with relatively large fold-changes but large, non-significant, *P*-values. Grey dots represent genes with relatively small fold-changes but small *P*-values. Red dots represent genes with relatively large fold-changes and small *P*-values.

**Diagram

Description automatically generatedChart, radar chart

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**Figure 4**. Top 15 GO terms of DEGs detected in brown mustard (a), potato (b), peppermint (c). The number of DEGs is expressed as a function of GO terms. GO categories are represented by colors; biological process (brick red), cellular component (orange), and molecular function (blue).



**Figure 5.** Validation of relative expression changes for selected DEGs of brown mustard, potato, peppermint, and *V. dahliae* with RT-qPCR data. The fold change for three biological replicates was calculated using the method for RT-qPCR and average fold change was derived using DESeq2 for RNA-seq. The Log2 fold change value (y-axis) for each comparison (shown in legend) is expressed a function of each gene (x-axis).



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