**Annual Progress Report / Final Report**

**Title:** Development of Genomic Resources for Management of Verticillium wilt of Potato

**Personnel:**

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| **Name** | **Title** | **Affiliation** | **Email** | **Phone** |
| David Wheeler | Assistant Professor | Washington State University | david.wheeler@wsu.edu | 509-335-3733 |
| Jeremiah Dung | Associate Professor | Oregon State University | jeremiah.dung@oregonstate.edu | 541-475-7107 |

**Reporting period**: Year Initiated: 2020; Terminating Year: 2021.

**Summary of accomplishments:**

The goal of this project was to generate genomic resources for potato, brown mustard, and *Verticillium dahliae*. Previously, the authors detected a diversity of differentially expressed genes (DEGs) in potato, mint, brown mustard and *V. dahliae* with RNA-sequencing (RNA-seq) technology*.* Some of these genes, however, could be artifacts and not responsible for any biological differences during infection. Thus, the objective of this project was to validate a subset of these DEGs in potato, brown mustard, and *V. dahliae* during infection. Potato and brown mustard plants were grown in a greenhouse. Plants were inoculated with strain 653 of *V. dahliae* that is virulent towards potato, strain 111 which virulent towards mint, or mock-inoculated with sterilized water. All plants were harvested 10 days after inoculation. RNA was isolated thereafter. DEGs that were first detected with RNA-seq were validated with a quantitative real time-PCR (qRT-PCR) method. DEGs were selected for validation if they exhibited the highest fold change values from the RNA-seq study or were assigned putative roles in pathogenicity, virulence, or defense. A total of 26 DEGs were validated, including 12, 10, and 4 from potato, brown mustard, and *V. dahliae*, respectively. The direction of DEGs were similar between both RNA-seq and qRT-PCR data sets. Similarly, the correlation coefficient (*r*) between fold changes obtained from RNA-seq and qRT-PCR methods were 0.91, 0.97, and 0.85 forpotato, brown mustard, and *V. dahliae,* respectively. For potato, defense response genes like *PR04, PRR1*, *WRK40*, *TIF5A* and several with unknown biological functions were validated. For *V. dahliae*, genes with putative virulence functions like peptidase, hydrolase, and oxidoreductase were validated. This study confirms the transcriptional changes in potato, brown mustard, and *V. dahliae* during infection previously observed with RNA-seq technology. Results from this study have (i) provided genomic resources for disease resistance breeding in potato, (ii) increased our understanding of non-host resistance, and (iii) helped identify host and pathogen genes that are required for symptomatic and asymptomatic interactions. Thus, the most significant accomplish of this research is that the results should help breeders and pathologists develop potatoes that are resistant to *V. dahliae*.

**Activities or experiments conducted:**

**Sample collection:**

Potato cv. ‘Russet Burbank’, and brown mustard cv. ‘ISCI 99’, were planted in 3.5” pots filled with Turface® (Profile Products LLC, Buffalo Grove, IL) in the greenhouse. Plants were fertigated with 20-10-20 NPK. After emergence, plants were drenched with two strains of *V. dahliae*, 653 (virulent towards potato) and 111 (virulent towards mint) (**Fig. 1A-D**) (Dung et al. 2013). Both strains can colonize brown mustard but do not incite symptoms (Wheeler and Johnson, 2016). After inoculation, ten replicates of each treatment were arranged in a randomized complete block design. Ten days after inoculation, plants were destructively sampled, and flash-frozen in liquid nitrogen. The collected plant samples were stored at -80°C for ten days and lyophilized for 48 hours.

**RNA Extraction and cDNA synthesis:**

Total RNA was extracted from all collected samples following the method described by Kumar et al. 2007 with some modification. To avoid genomic DNA contamination, total RNA was digested with Ambion DNAse I (RNase-free) (Invitrogen). Quantification of total RNA was completed with a QUBIT fluorometer (Life Technologies), and integrity was assessed by gel electrophoresis. The quantities of total RNA in the samples ranged from 72 to 2000 ng/µl. RNA samples free from genomic DNA and with two clear bands representing 28S and 18S ribosomal RNA were used for the cDNA synthesis and qRT-PCR validation (**Fig. 1E**). 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 potato and mustard gene validation and 1:3 for V. dahliae gene validation. cDNA was stored at -20°C prior to validation.

**Gene selection and primer design:**

A total of 36 DEGs comparisons from our RNA-seq results were selected for validation. For potato and brown mustard genes, three pairwise comparisons were completed: 653 vs control, 111 vs control, and 111 vs 653. Likewise, all possible treatment combinations of the potato and mustards were used for the *V. dahliae* genes. DEGs were selected for each host if they exhibited the highest fold change values (the largest differences in gene expression) or represented putative pathogenicity or virulence-related genes. Primers spanning exon-exon junction (if applicable) were designed for each gene using NCBI Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast). Primer sequences and amplicon lengths are presented in **Table 1**. Primers with 19-23 bp size, 40-60% GC content, amplicon size of 70-180 bp with no self-annealing and primer dimer formation were used.

**Quantitative real-time PCR:**

qRT-PCR was performed in a QuantStudioTM Real-Time PCR System (Applied Biosystems) using SYBRTM Select Master Mix (2X) (Applied Biosystems) in 10µl reaction volume. 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 qRT-PCR 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 genes. Negative controls consisted of samples without cDNA. Primer efficiency and Cycle threshold (Ct) values were calculated using LinRegPCR software (Ruijter et al. 2009). The Ct value for each biological replicate was calculated by averaging Ct values from three technical replicates. 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 and V. *dahliae* (**Table 1**). To confirm the direction of fold change values qRT-PCR data were compared with fold changes obtained from RNA-sequencing. Correlations between fold change estimates from these methods were calculated for each host.

**Results and discussion:**

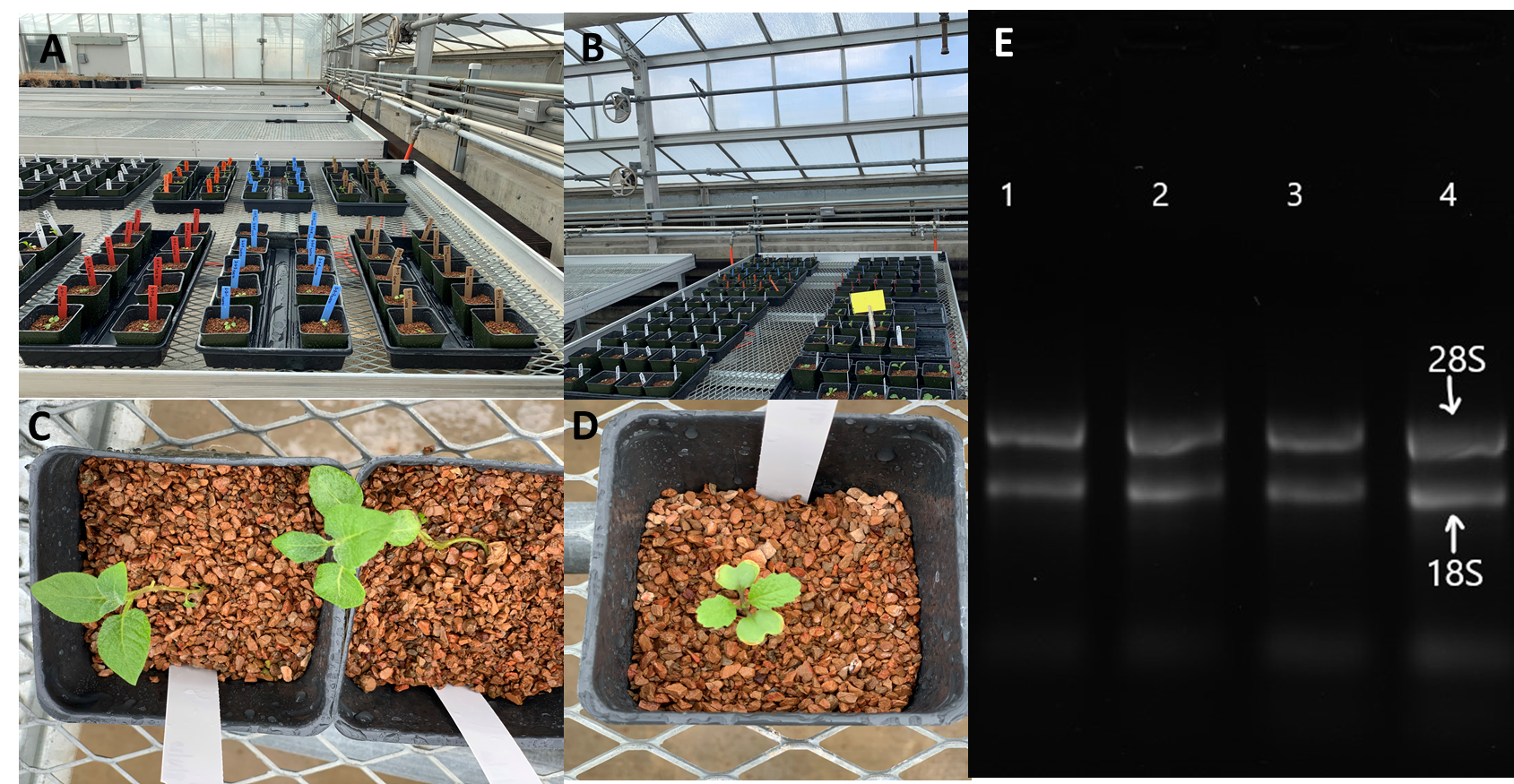
This study validated the differentially expressed genes from previous RNA-seq results utilizing qRT-PCR. A total of 26 out of 36 comparisons of DEGs, including 12 for potato, 10 for brown mustard and 4 for *V. dahliae* were validated. These comparisons include expression changes of nine, five, and four genes for potato, brown mustard, and *V. dahliae*, respectively. The direction (up or down-regulation) of fold changes were the same in both qRT-PCR and RNA-seq data (**Figure 2**). The correlation coefficient of gene expression changes (log2 fold change) between qRT-PCR and RNA seq were 0.91, 0.85, and 0.97 for potato, brown mustard, and *V. dahliae* respectively (**Figure 3**).

Two genes of potato, *PRS2\_SOLTU* and *IER1\_SOLLC,* and four genes of brown mustard, *UVB31\_ARATH*, *LUC7L3*, Cluster-15354.91015, and Cluster-15354.125147, did not show a similar expression pattern as RNA-seq (data not shown). Potential sources for such differences in results likely include differences in the sensitivity of the two methods, environmental differences, the use of a different template RNA for quantification, and inconsistent expression of transcripts in some treatments (Wang et al. 2016). For brown mustard genes, *UVB31\_ARATH* and Cluster-15354.125147, there was either no amplification or very poor amplification in certain treatments. This inconsistency in amplification can be explained by the absence of gene expression in those treatments throughout all biological replications in RNA-seq count data. Additionally, the reference genome against which differentially expressed genes were mapped was different from the species used in these experiments (*B. napus* vs. *B. juncea*).

Validated genes include those with functional role in defense response in hosts and pathogenicity in *V. dahliae*. In potato, the Pathogenicity-related (PR) like genes *PR04\_SOLLC* and *PRR1\_TOBAC* were significantly upregulated (adjusted *P* value < 0.05) when challenged with the 653 isolate compared to the control and 111 isolate in potato; these genes have been shown to play a role in plant defense (Vleeshouwers et al. 2000). Similarly, the transcription factor *WRK40\_ARATH*, which plays an important role in plant signal transduction upon pathogen recognition, was also differentially expressed in potato (Eulgem and Somssich 2007). Ajasmonic acid (JA) regulation gene, *TIF5A*, was upregulated in potato plants inoculated with strain 111 compared to strain 653. JA regulates the host defense against hemibiotrophic pathogens like *V. dahliae* (Scholz et al. 2018). Similarly, oxidoreductase and *lipoxygenase* (*LOX12*) genes were also confirmed to be upregulated in response to infection by strain 111. In addition, genes involved in oxidoreductase, DNA binding, catalytic, and transferase activities were validated in potato using qRT-PCR. For *V. dahliae*, genes with putative roles in virulence such as peptidase, hydrolase, oxidoreductase, and catalytic activity were also validated. The heptaketide hydrolase, *AYGI*, was differentially expressed in this study and was previously shown to play a role in melanin biosynthesis (Fujii et al. 2004). *AYGI* was also differentially expressed in *V. dahliae* in vitro studies(Duressa et al. 2013). In brown mustard, DEGs involved in nuclear mRNA export, ATP binding, kinase activity, DNA binding transcription factor, and RNA binding were validated.

Previously, our RNA-seq study identified at least 7, 9, and 7 DEGs in potato, brown mustard and *V. dahliae* respectively with no known biological function. The qRT-PCR study confirmed those few uncharacterized genes as well. Further characterization and functional analysis of these novel transcripts will provide a better understanding of the mechanisms by which this pathogen infects symptomatic and asymptomatic hosts. In the future, these novel targets could be utilized as important genomic resources to manage Verticillium wilt of potato.

This qRT-PCR study confirms the transcriptional changes in potato, brown mustard and *V. dahliae* during infection. The study describes changes in symptomatic vs asymptomatic hosts at the molecular level upon *V. dahliae* infection by strains differing in aggressiveness on three important crops grown in Pacific Northwest potato production systems. Further, our observations could be used to help solve complex puzzles between host and *V. dahliae* interactions. Also, the validated defense response genes could be used as important genomic resources in disease resistance breeding programs. Finally, the suppression of *V. dahliae* pathogenicity genes (identified in this RNA-seq study) with host-induced gene silencing could yield fruitful results, as it has for the papaya industry that was once paralyzed by papaya ringspot virus.



**Figure 1:** A, B) Greenhouse experiment, C) Potato plantlets, D) Mustard plants, and E) Total RNA integrity test on 1% agarose gel. Lane 1,2,3 and 4 indicates RNA samples with two 28S and 18S ribosomal RNA.

**Table 1:** List of primer sequence and functions of differentially expressed genes (DEGs) used for the qRT-PCR validation

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Host | Gene name | Putative molecular/ biological function | Forward sequence (5’ to 3’) | Reverse sequence (5’ to 3’) | Amplicon size (bp) |
| Potato | *PR04\_SOLLC* | Defense response against pathogens | GCCGTGCAATTGTGGGTGTC | CGCACACTTTTCCACTAGCAC | 76 |
| Potato | *ABAH1\_ARATH* | Oxidoreductase activity | CCACTTCCTCCTGGTACTTTAGG | AACTTGTTTAGCTGCCTCTGG | 177 |
| Potato | PGSC0003DMG400024310 | Heterocyclic compound binding/ cation binding | GAGAAGGAAGATTGGTGGGACA | CTACCCATCCCTCCTCCACA | 105 |
| Potato | *LOX12\_SOLTU* | Oxidoreductase/ dioxygenase activity | ATTAGCTCTGTTCAAGGTGATCC | TCTCCAAGTAGGCTGGATTGC | 70 |
| Potato | *PRR1\_TOBAC* | Defense response against pathogen | TGTCTTTTGCCCTTGAAGGCT | GACAACGTCTCACCAGCTCT | 115 |
| Potato | *CHSB\_SOLTU* | Transferase activity | GAGCTCAAGGAGAAATTTAAGCG | ACAACAACTATGTCTTGCCTTGC | 149 |
| Potato | *EDL3\_ARATH* | Stress response | AATGGTCGGATCGGAGGAGA | TCGGATTACACCCGCAACAG | 70 |
| Potato | *WRK40\_ARATH* | Signal transduction/ Transcriptional reprogramming | AGACAACCCATCTCCAAGAGC | TCGATTGGTCTTCCACGCTT | 95 |
| Potato | *TIF5A\_ARATH* | Jasmonic acid (JA) regulation | ATGTCCGAGCCTTCATCACC | GGAGCAACTAGTGATGGTATGGT | 130 |
| Potato | *EF1α* | Housekeeping gene | ATTGGAAACGGATATGCTCCA | TCCTTACCTGAACGCCTGTCA | 101 |
| *V. dahliae* | *AOX\_PODAS* | Oxidoreductase activity | GCTGCGTGGAAGTTTGTGC | TTCTTGTCAACCTGCTGCTCA | 83 |
| *V. dahliae* | *YDDQ\_BACSU* | Hydrolase activity | AAGATTGTGCTCGTCGGGTA | TCTCAGCCAGAGCAACCTTC | 163 |
| *V. dahliae* | mRNA\_1341 | nk1 | GCTGTCCGCATCTGACTTGT | GGTGACGTTGAACTTTGCCA | 97 |
| *V. dahliae* | *AYG1\_ASPFU* | Melanin biosynthesis | GATTCGGCTGACCCAGACAG | ACCTTGCCCATATCGAACCG | 89 |
| *V. dahliae* | *ACT* | Housekeeping gene | GGCTTCCTCAAGGTCGGCTATG | GCTGCATGTCATCCCACTTCTTC |  |
| Brown mustard | Cluster-15354.86688 | nk | ATTCACACTGCTCCACGCTA | GGCTGAAGGGTGAGAATGGG | 78 |
| Brown mustard | *NUP1\_ARATH* | Nucleus or nuclear membrane organization | CCATCCTTGCTTGGATTGCC | ATGCAGGAGGCTAAGGTTGG | 110 |
| Brown mustard | *PDRP2\_ARATH* | ATP binding/ Phosphotransferase activity | TATAAAGCAGGCAGCGAAGC | GAGAGCACTCCCCAACGAT | 105 |
| Brown mustard | *SCL1\_ARATH* | Transcription regulation | AACTGCTGAAAAGGATGACAAGT | TGCTCTTGCTGCTTTCCGTT | 84 |
| Brown mustard | Cluster-15354.44072 | RNA binding | TGCGTTCCTCAGAACCAGAG | AGCTTCTTCTCCACTGCTGAC | 106 |
| Brown mustard | *ACT-2* | Housekeeping gene | TGGGTTTGCTGGTGACGAT | TGCCTAGGACGACCAACAATACT | 290 |

1nk: Gene with not known biological or molecular function

**Figure 2:** Validation of relative expression changes for selected DEGs of potato, brown mustard, and *V. dahliae* with qRT-PCR data. The fold change was calculated using method for qRT-PCR and 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).

**Figure 3:** Scatter plots showing the linear relationship between qRT-PCR and RNA-seq gene expression changes in potato, *V. dahliae*, and brown mustard. R-value represents the correlation coefficient for the respective host.

**Publications:**

A peer-reviewed publication and a *Potato Progress* article are in preparation. We will submit the peer-reviewed paper in 2021 and the *Potato Progress* report shortly thereafter.

**Presentations and reports:**

A brief summary of some of this work was presented at the Western Washington Potato Workshop and the Columbia Basin Soil Health Working Group Meeting in 2020.

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