**Final report for Northwest Potato Research Consortium**

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

**PERSONNEL:**

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**REPORTING PERIOD**: Year Initiated: 2020; Terminating Year: 2021.

**ACCOMPLISHMENTS:**

The goal of this project is to provide genomic resources of potato, brown mustard, and *Verticillium dahliae* for scientists and stakeholders. More specifically, this project aims to validate the differentially expressed genes (DEGs) in potato, brown mustard and *Verticillium dahliae* in response to two strains of *V. dahlia*e. The quantitative Real Time-PCR (qRT-PCR) method was used to validate the expression changes of the DEGs. The DEGs with the highest fold change values in previous RNA-Seq study or with putative role in pathogenicity or virulence or defense were selected for this validation project. A total of 26 DEGs comparison from RNA-seq results were validated, including 12, 10, and 4 for *Solanum tuberosum* (potato), *Brassica junceae* (brown mustard) and *V. dahliae*, respectively. Gene expression changes in qRT-PCR were in the same direction (up or down-regulated) as RNA-Seq results and confirmed to be consistent with RNA-Seq study. The correlation coefficient (R) between fold changes obtained from RNA seq and qPCR methods were 0.91, 0.85, and 0.96 for *S. tuberosum, V. dahliae and B. juncea*, respectively. In hosts, defense response genes like *PR04, PRR1*, *WRK40*, *TIF5A* and few with unknown biological functions were validated and in pathogen, virulence-related genes like peptidase, hydrolase, and oxidoreductase were validated. This study confirms the transcriptional changes in potato, brown mustard and *V. dahliae*. The findings from this study will contribute towards disease resistance breeding in potato and provides the basis for host-pathogen interaction studies.

**PROCEDURE:**

**Sample collection:**

The 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 as recommended. After emergence, plants were drenched with two strains of *Verticillium dahliae*, Vd-653 and Vd-111. Vd-653 is highly aggressive and Vd-111 is less aggressive against potato (Dung et al. 2013). Both strains can colonize brown mustard but do not show symptoms (Wheeler and Johnson 2016). 100 ml of inoculum at a concentration of 1 x 106 conidia/ml was drenched in each treated plants and same quantity of sterile water was drenched in control plants (Fig. 1A-D). The greenhouse experiment was conducted in a randomized complete block design with a 2×3 factorial arrangement with ten replications. Ten days after *V. dahliae* 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 72-2000 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 S. tuberosum and B. juncea gene validation and 1:3 for V. dahliae gene validation and stored at -20°C.

**Gene selection and primer design**

A total of 32 DEGs comparisons from RNA-seq results were selected for validation. The gene expression change was analyzed utilizing the commonly used method, quantitative Real Time-PCR (qRT-PCR) (Taylor et al. 2010). For potato and brown mustard genes, three pairwise comparisons were made: Vd-653 vs control, Vd-111 vs control and Vd-111 vs Vd-653. Likewise, different treatment combinations of the potato and mustards were also compared for the *V. dahliae*. The differentially expressed genes (DEGs) with the highest fold change values or putative pathogenicity or virulence-related genes were selected for each host. 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). The primer sequence and amplicon length 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 experiments were 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. No template control was included in each reaction as a negative control. Primer efficiency and Ct values were calculated using LinRegPCR software (Ruijter et al. 2009). The Ct value for each biological replicate was calculated by taking the average Ct values of three technical replicates. The Log2 fold change value was derived using the delta-delta Ct method for each comparison (Livak and Schmittgen 2001). House-keeping genes, Solanum tuberosum elongation factor 1-α (EF1α), Brassica juncea actin gene (ACT-2) and Verticillium dahliae elongation factor 1-α (EF1α) were used for normalization of the DEGs for potato, brown mustard, and *V. dahliae*, respectively. The fold change values obtained from qRT-PCR were compared with fold changes obtained from RNA sequencing to check whether the gene expression level changes are in the same direction or not (i.e. upregulation and downregulation). The correlation coefficient was calculated between fold change obtained from these two methods for each host.

**RESULTS/DISCUSSION:**

This study validated the differentially expressed genes from previous RNA-seq results utilizing qRT-PCR. A total of 26 out of 32 comparisons of differential gene expressions (DEGs), including 12 for potato, 10 for brown mustard and 4 for *V. dahliae* were validated. These comparisons include expression changes of 9, 5, and 4 genes for potato, brown mustard, and *V. dahliae*, respectively. All the validated comparisons had gene expression changes in the same direction (up or down-regulation) as RNA-seq result (Figure 2). The correlation coefficient of gene expression changes (log2fold change) between qRT-PCR and RNA seq were 0.91, 0.85, and 0.96 for potato, brown mustard, and *V. dahliae* respectively (Figure 3). Since RNA-Seq and qRT-PCR use different normalization process for data analysis, a correlation was performed over other statistical analysis to compare fold change between these two methods. To be more specific, read counts in RNA-Seq data were normalized using DESeq2 size factor (Love et al. 2014); and Ct values in qRT-PCR were normalized using house-keeping genes of the respective host.

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). Different sensitivity of two methods, use of different template RNA for quantification and inconsistent expression of transcripts in some treatments could be potential reasons for such differences in results (Wang et al.2016). For Brassica 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 no gene expression in those treatments throughout all biological replications in RNA-Seq count data.

Validated genes include those with functional role in defense response in hosts and pathogenicity in *V. dahliae*. The Pathogenicity related (PR) like genes *PR04\_SOLLC*, *PRR1\_TOBAC* which have a role in plant defense (Vleeshouwers et al. 2000) were significantly upregulated when challenged with Vd-653 isolate compared to control and Vd-111 isolate in potato. Similarly, transcription factor, *WRK40\_ARATH*, which play an important role in the signal transduction in plant upon pathogen recognition was also differentially expressed in potato (Eulgem and Somssich 2007). Jasmonic acid (JA) regulation gene, *TIF5A* was upregulated in Vd-111 compared to Vd-653 infected potato plants. JA regulates the host defense against necrotrophic pathogen like *V. dahliae* (Schlolz et al. 2018). Similarly, oxidoreductase and *lipoxygenase* (*LOX12*) gene was also confirmed to be upregulated in response to Vd-111 infection. In addition, genes involved in oxidoreductase, DNA binding, catalytic, and transferase activities were validated using the qRT-PCR method in potato. In *V. dahliae*, genes such as peptidase, hydrolase, oxidoreductase, and catalytic activity with putative role in virulence were also validated. The heptaketide hydrolase, *AYGI*, differentially expressed in this study was 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, DNA binding transcription factor, RNA binding were validated.

Previously, RNA-Seq study also identified a good number of differentially expressed genes in potato, brown mustard and *V. dahliae* with no known biological function and their role in defense or pathogenicity. The qRT-PCR study confirmed those few uncharacterized genes as well. The further investigation and functional analysis of these genes would help to decipher the host-pathogen interaction and could be used as novel targets for Verticillium wilt management.

This qRT-PCR study confirms the transcriptional changes in potato, brown mustard and *V. dahliae* in response to *V. dahliae* infection. The study provides the bigger picture of changes at the molecular level upon *V. dahliae* infection and could be used as a basis to solve complex puzzle of host and *V. dahliae* interaction. Also, validated defense response genes in the host could be used as an important genomic resource in disease resistance breeding program.

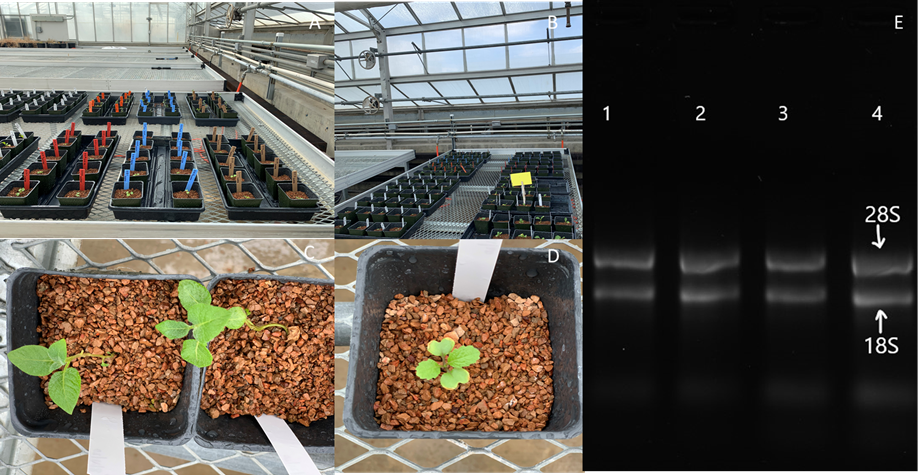


Figure 1. A, B) Greenhouse experiment, C) Potato plantlets, D) Mustard plants, and E) Total RNA integrity test on 1% agarose gel. Lane 1,2,3 and 4 indicates RNA samples with two 28S and 18S ribosomal RNA.

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

|  |  |  |  |
| --- | --- | --- | --- |
| Gene name | Forward sequence (5’ to 3’) | Reverse sequence (5’ to 3’) | Amplicon size (bp) |
| St\_*PR04\_SOLLC* | GCCGTGCAATTGTGGGTGTC | CGCACACTTTTCCACTAGCAC | 76 |
| St\_*ABAH1\_ARATH* | CCACTTCCTCCTGGTACTTTAGG | AACTTGTTTAGCTGCCTCTGG | 177 |
| St\_PGSC0003DMG400024310 | GAGAAGGAAGATTGGTGGGACA | CTACCCATCCCTCCTCCACA | 105 |
| St\_*LOX12\_SOLTU* | ATTAGCTCTGTTCAAGGTGATCC | TCTCCAAGTAGGCTGGATTGC | 70 |
| St\_*PRR1\_TOBAC* | TGTCTTTTGCCCTTGAAGGCT | GACAACGTCTCACCAGCTCT | 115 |
| St\_*CHSB\_SOLTU* | GAGCTCAAGGAGAAATTTAAGCG | ACAACAACTATGTCTTGCCTTGC | 149 |
| St\_*EDL3\_ARATH* | AATGGTCGGATCGGAGGAGA | TCGGATTACACCCGCAACAG | 70 |
| St\_*WRK40\_ARATH* | AGACAACCCATCTCCAAGAGC | TCGATTGGTCTTCCACGCTT | 95 |
| St\_*TIF5A\_ARATH* | ATGTCCGAGCCTTCATCACC | GGAGCAACTAGTGATGGTATGGT | 130 |
| St\_ *EF1α* | ATTGGAAACGGATATGCTCCA | TCCTTACCTGAACGCCTGTCA | 101 |
| Vd\_*AOX\_PODAS* | GCTGCGTGGAAGTTTGTGC | TTCTTGTCAACCTGCTGCTCA | 83 |
| Vd\_*YDDQ\_BACSU* | AAGATTGTGCTCGTCGGGTA | TCTCAGCCAGAGCAACCTTC | 163 |
| Vd\_mRNA\_1341 | GCTGTCCGCATCTGACTTGT | GGTGACGTTGAACTTTGCCA | 97 |
| Vd\_*AYG1\_ASPFU* | GATTCGGCTGACCCAGACAG | ACCTTGCCCATATCGAACCG | 89 |
| Vd\_*EF1α* | CTTCCAACGTCATCACCT | CTCAGAGCGAACTCATACT |  |
| Bj\_Cluster-15354.86688 | ATTCACACTGCTCCACGCTA | GGCTGAAGGGTGAGAATGGG | 78 |
| Bj\_*NUP1\_ARATH* | CCATCCTTGCTTGGATTGCC | ATGCAGGAGGCTAAGGTTGG | 110 |
| Bj\_*PDRP2\_ARATH* | TATAAAGCAGGCAGCGAAGC | GAGAGCACTCCCCAACGAT | 105 |
| Bj\_*SCL1\_ARATH* | AACTGCTGAAAAGGATGACAAGT | TGCTCTTGCTGCTTTCCGTT | 84 |
| Bj\_Cluster-15354.44072 | TGCGTTCCTCAGAACCAGAG | AGCTTCTTCTCCACTGCTGAC | 106 |
| Bj\_Cluster-15354.125147 | GCGAGAGCAAGACACAAAAGG | TGTTTGCTGAGGGGAGTTCG | 77 |
| Bj\_*ACT-2* | TGGGTTTGCTGGTGACGAT | TGCCTAGGACGACCAACAATACT | 290 |



**Figure 2:** Validation of relative expression changes of selected DEGs of *S. tuberosum*, *V. dahliae*, and *B. juncea* with the qRT-PCR method. The fold change was calculated using method for qRT-PCR and DESeq2 for RNA-Seq. The x-axis represents the name of the genes, and the y-axis represents Log2 fold change value for the given comparison (shown in legend).



**Figure 3:** Scatter plots showing the linear relationship between qRT-PCR and RNA-Seq gene expression changes in *S. tuberosum*, *V. dahliae*, and *B. juncea*. R-value represents the correlation coefficient for the respective host.

***Publications*:**

Manuscript in preparation

***Presentations and Reports:***

None to date.

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**REFERENCES:**

1. Dung, J.K., Peever, T.L. and Johnson, D.A. 2013. *Verticillium dahliae* populations from mint and potato are genetically divergent with predominant haplotypes. Phytopathology. 103:445-459.
2. Duressa, D., Anchieta, A., Chen, D., Klimes, A., Garcia-Pedrajas, M.D., Dobinson, K.F. and Klosterman, S.J. 2013. RNA-seq analyses of gene expression in the microsclerotia of *Verticillium dahliae*. BMC genomics. 14:607.
3. Eulgem, T. and Somssich, I.E. 2007. Networks of WRKY transcription factors in defense signaling. Current Opinion in Plant Biology. 10:366-371.
4. Fujii, I., Yasuoka, Y., Tsai, H.F., Chang, Y.C., Kwon-Chung, K.J. and Ebizuka, Y. 2004. Hydrolytic polyketide shortening by ayg1p, a novel enzyme involved in fungal melanin biosynthesis. Journal of Biological Chemistry. *279*:.44613-44620.
5. Kumar, GNM, Iyer S, Knowles NR. 2007. Extraction of RNA from Fresh, Frozen, and Lyophilized Tuber and Root Tissues. Journal of Agricultural and Food Chemistry. 55:1674-1678. doi: 10.1021/jf062941m
6. Livak, K.J. and Schmittgen, T.D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2− ΔΔCT method. Methods. 25:402-408.
7. Love, M.I., Huber, W. and Anders, S. 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome biology. 15:550.
8. Ruijter, J.M., Ramakers, C., Hoogaars, W.M.H., Karlen, Y., Bakker, O., Van den Hoff, M.J.B. and Moorman, A.F.M. 2009. Amplification efficiency: linking baseline and bias in the analysis of quantitative PCR data. Nucleic acids research. 37: 45. doi:10.1093/nar/gkp045
9. Scholz, S.S., Schmidt-Heck, W., Guthke, R., Furch, A.C., Reichelt, M., Gershenzon, J. and Oelmüller, R. 2018. *Verticillium dahliae*-Arabidopsis interaction causes changes in gene expression profiles and jasmonate levels on different time scales. Frontiers in Microbiology. 9:217.
10. Taylor, S., Wakem, M., Dijkman, G., Alsarraj, M. and Nguyen, M., 2010. A practical approach to RT-qPCR publishing data that conform to the MIQE guidelines. Methods. 50:S1-S5.
11. Vleeshouwers, V.G., Van Dooijeweert, W., Govers, F., Kamoun, S. and Colon, L.T. 2000. Does basal PR gene expression in Solanum species contribute to non-specific resistance to Phytophthora infestans?. Physiological and Molecular Plant Pathology. 57:35-42.
12. Wang, L., Wang, Y., Cao, H., Hao, X., Zeng, J., Yang, Y. and Wang, X. 2016. Transcriptome analysis of an anthracnose-resistant tea plant cultivar reveals genes associated with resistance to Colletotrichum camelliae. PLoS One. 11:e0148535.
13. Wheeler, D.L. and Johnson, D.A. 2016. *Verticillium dahliae* infects, alters plant biomass, and produces inoculum on rotation crops. Phytopathology. 106:602-613.