Journal: BMC genomics or BMC Plant Biology

Comprehensive Transcriptomic analysis reveals turnip mosaic virus infection and its aphid vector *Myzus persicae* cause large changes in gene regulatory networks and co-transcription of alternative spliced mRNAs in *Arabidopsis thaliana*.

Venura Herath[1], Clare L. Casteel[2,] Jeanmarie Verchot[3]

[1]Department of Agricultural Biology, Faculty of Agriculture, University of Peradeniya, Sri Lanka. 20400

[2]Department of Plant Pathology and Plant-Microbe Biology, Cornell University, Ithaca, NY, USA.

[3]Department of Plant Pathology & Microbiology, Texas A&M University, College Station, TX 77845

Co-Corresponding author: Jeanmarie Verchot (jeanmarie.verchot@ag.tamu.edu)

Keywords: potyvirus, aphids, hormone signaling, transcriptomics

Abstract

Background: Virus infection and aphid herbivory induce robust changes in gene expression relating to environmental stress. This study employed high-throughput transcriptomic and alternative splicing analysis to understand the separate and combined impacts on host gene expression in *Arabidopsis thaliana* by *Myzus persicae* (green peach aphid), and turnip mosaic virus (TuMV).

Results: By investigating changes in transcript abundance, the data shows that aphids feeding on virus infected plants intensify the number of differentially expressed stress responsive genes compared to challenge by individual stressors. This study presents evidence that the combination of virus-vector-host interactions induces significant changes in hormone and secondary metabolite biosynthesis, as well as downstream factors involved in feedback loops within hormone signaling pathways. This study also shows that gene expression is regulated through alternative pre-mRNA splicing and the use of alternative transcription start and termination sites.

Conclusions: These combined data suggest that complex genetic changes occur as plants adapt to the combined challenges posed by aphids and the viruses they vector. This study also provides new datasets that could be used in the future to dissect the genetic mechanisms mediating tripartite interactions and inform future breeding programs.

Background

Many animal and plant infecting viruses have been known to elicit spatiotemporal changes in host gene expression in a manner that impairs the translation of cellular mRNA and host defenses to favor virus gene expression, known as host gene shut-off [1]. More than two decades ago, researchers showed that transient downregulation of host gene expression accompanies pea seed-borne mosaic virus (PSbMV; a potyvirus) replication in embryonic and cotyledon tissues [2] and the simultaneous increase in HSP70 and polyubiquitin expression [3]. In the past several years, researchers have shown that key viral proteins directly influence plant gene regulatory networks and, in some cases facilitate aphid infestation and transmission by modulating plant transcription factors, protein turnover pathways, and defense signaling pathways [4–6].

The potyvirus genera provides a robust number of virus species known to manipulate host gene expression. Virus-encoded HC-Pro, VPg, NIa-Pro, NIb (RNA-dependent RNA polymerase), and 6K2 proteins directly influence host gene expression to create an environment favoring infection and herbivory [7–9]. HC-Pro for example, which is well known for binding virions to aphid stylets for vector transmission, also binds to the RAV2 transcription factor to induce host stress and defense-related gene expression including factors that interfere with antiviral silencing [10]. In addition, HC-Pro and RAV2 combine to influence the expression of genes involved in responses to wounding, JA, cold, and heat stress [10]. The VPg-NIa-Pro blocks host translation by sequestering in the nucleus and nucleolus with the eukaryotic translation initiation factor 4E (eIF4E), ribosomal protein S6 kinase, and the poly(A) binding protein (PABP)[9, 11–13]. The potyviral NIa-Pro, increases aphid performance on infected *Arabidopsis thaliana* plants by inhibiting ethylene dependent plant defenses [14]. The NIb protein is known to recruit many cellular proteins including RNA helicase-like proteins AtRH8 and AtRH9, and NbEXPA1 to viral replication complexes in the cytoplasm, while in the nucleus it interacts with SUMO-conjugating enzyme 1 (SCE1) to block antiviral NPR1 activities [7, 9]. Mutations disrupting the nuclear translocation of NIb abolish virus infection suggesting that NIb has some undiscovered abilities to influence host gene expression [7]. The TuMV 6K2 protein while embedded in the ER triggers the unfolded protein response (UPR) by activating the bZIP60, bZIP28 and bZIP17 transcription factors and increasing the expression of chaperones needed for proper protein folding [15, 16]. The bZIP60 and bZIP28 combined influence virus titers in systemically infected plants.

While the vast majority of transcriptomic studies involving virus and insect challenges to plants have focused on changes in mRNA abundance [17, 18], new studies involving sugarcane mosaic virus (SCMV), potato virus Y (PVY), and bean common mosaic virus (BCMV) are noting

the accumulation of different mRNA isoforms that are regulated through alternative splicing of pre-mRNAs [19–23]. For example, JAZ proteins represent a group of transcriptional repressor proteins known to repress JA responses. AS of the JAZ10 pre-mRNA generates 3 variants encoding proteins with different C-terminal domains which also differ in their sensitivity to rising jasmonic acid-isoleucine (JA-Ile) concentrations (JA)-induced degradation[24]. The maize phytoene synthase 1 (ZmPSY1) gene encodes a key enzyme in carotenoid biosynthesis that is required for normal chloroplast function and is a pro-viral host factor influencing the pathogenesis of SCMV. Two splicing variants of ZmPSY1 known as T001 and T003 only differ in the length of the 3' UTR which is a region that controls its own mRNA translation activity. Research indicates that SCMV alters the splicing pattern to favor accumulation of the T001 transcript which plays a more significant role in promoting SCMV titer and disease while preserving chloroplast functions [22].

Despite the frequent co-occurrence of aphids and viruses in natural environments and synergistic interactions, the molecular mechanisms mediating these two agents together have received limited attention (But see: Safari et al., 2019; Gadhave et al., 2020; Pan et al., 2021; Ray and Casteel, 2022). Furthermore, only a few studies have investigated the role of AS in the regulation of plant-insect interactions. For example, a study of *Zea mays* (maize) under normal conditions and after aphid herbivory found minimal overlap between genes regulated by both expression and AS in response to aphids [29]. Similarly, a transcriptomic study of *Manduca sexta* caterpillar feeding on *Nicotiana attenuata* leaves generated mostly distinct sets of differentially spliced genes and differentially expressed genes in the leaves and roots [30]. To address some of these gaps in knowledge, our study aims to expand the understanding of plant transcriptional response to stress using *Arabidopsis thaliana*, *Myzus persicae* (green peach aphid), and TuMV as a model system. We reanalyzed our published RNA-seq experiments in new ways to generate novel hypotheses concerning the function of plant transcriptional responses to aphids, TuMV, and the dual challenge of TuMV and aphids. Furthermore, we identified isoforms in the RNAseq data, enabling the identification of unique and previously unexplored transcriptional responses. The research outcomes hold substantial importance in enhancing our understanding of plant defense mechanisms against the combined threats of aphids and viruses.

Methods

Plant Material and Aphid/Virus Treatments

Recently, we characterized the transcriptome of *Arabidopsis thaliana* plants with and without TuMV infection and aphid-infestation [31]. Wild-type *A. thaliana* ecotype Columbia-0 plants were obtained from the Arabidopsis Biological Resource Center and grown under controlled conditions of 25/20 °C day/night with a photoperiod of 14/10 h day/night, a relative humidity of 50%, and a light intensity of 200 mmol m−2 s−1. After three weeks of growth, half of the plants were rub-inoculated with TuMV:GFP, which was propagated from the infectious clone pCAMBIA:TuMV-GFP as in [32]. One week after infection, fully infected leaves were identified by fluorescence under UV light, and 15 adult apterous aphids were caged on one leaf per plant for six uninfected and six infected plants. A corresponding set of six infected and six uninfected plants received cages with no aphids as controls for aphid feeding. Caged leaves were developmentally matched, and infected infection was verified before caging based on GFP visualization (Supplementary Figure 1A). After 48 hours of aphid placement, cages and aphids were removed, and leaves were pooled for every two plants resulting in replicates for each treatment.

RNA Extraction, Library Preparation, and Sequencing

The SV Total RNA Isolation Kit (Promega, Madison, WI, USA) was used to extract total RNA from pooled plant samples and treat it with DNAse. RNA purity was assessed using a Nanodrop 8000 (Thermo Scientific, Whatham, MS, USA). The A260/A280 ratios of samples ranged between 1.9–2.2. RNA integrity was assessed using Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) and all samples had an RNA integrity number (RIN number) >7.0. mRNA purification, fragmentation, cDNA synthesis, second-strand synthesis, adapter ligation, cDNA and library purification were performed as previously described (Bera et al., 2022). Sequencing was performed using an Illumina HiSeq 2000 instrument at the Genomics Resources Core Facility (GRCF) at Weill Cornell Medical Center (New York City, NY, USA). The reads from this experiment are available from the NCBI SRA (PRJNA60524) (Supplementary Figure 1B).

Transcriptomic Analysis

Read quality was assessed using FastQC (v 0.11.9) and then the reference-guided mapping was carried out using the *A. thaliana* genome assembly (TAIR10) in the Arabidopsis Information Resource database (https://www.arabidopsis.org/index.jsp, last accessed 15.03.2022) using HISAT2 (v2.2.1) [33]. The workflow is illustrated in Supplementary Figure 1B. The SAM files were converted to BAM files and indexed using SAMtools (v1.10) [34, 35]. Assembly alignment quality was assessed using FASTQC (v. 0.11.9). Transcripts assembly and abundance were determined using StringTie (v2.1.0) [36] and using the annotations obtained from Thalemine (v5.1.0-

20221003). Raw sequence counts were calculated using HT-Seq (v2.0.1) (Putri et al., 2022). Overall, between 10.5 and 15.7 million raw reads were mapped to the reference Arabidopsis genome, and among these, between 4.6 and 13.9 million clean reads were detected across treatments using HISAT2 software (Table S1). The total alignment rate including unique and multi-mapped reads with the reference genome ranged between 63 and 98%.

Differential expression analysis was carried out using EdgeR (v.3.43.7) (Robinson et al., 2009) in R-Studio Desktop (v. 2023.06.0 build 421) or RStudio server hosted in Cornell University and the Texas A&M University high-performance computing portal running R (v. 4.2.1) framework. Differentially regulated genes with ≤−1 or ≥1 log2-fold difference with a false discovery rate (FDR) of ≤0.05 at each time point were further analyzed. Volcano plots were generated using EnhancedVolcano (v. 1.19.0) [37].

Gene Annotation and GO Enrichment Analysis

Gene descriptions and gene annotations were retrieved using the Gene Annotation search tool (https://www.arabidopsis.org/tools/bulk/go/index.jsp; last accessed on 04.06.2023) on curated gene descriptions and annotations from TAIR [38]. GO enrichment analysis was conducted using ShinyGO v0.76 with the following settings; FDR cutoff 0.05, a minimum pathway size 2, and pathway database GO: Biological Process. Visualization of GO enrichment plots were carried out using the built-in tool available in the ShinyGO [39].

Assignments of gene families were carried out using a locally executed uniport database. Amino acid sequences of the genes were extracted using TBTools (v.1_098722) and local blast database of UNIPROT (release-2022_01) was generated using BLAST+ executables (v.2.3.10+) [40]. A BLASTp search of differentially expressed genes (DEGs) was carried out against the UNIPROT database and based on the resulting family names, were assigned using the UNIPROT ID mapping tool (https://www.uniprot.org/id-mapping).

Abscisic Acid Levels

Tissue was collected, lyophilized, and ground to a fine powder, and 50 mg was weighed out for each sample. ABA was extracted from tissue using an iso-propanol:H_20: hydrochloric acid extraction buffer (2:1:0.005) spiked with 1000 ng/µL of deuterated standard of ABA ((+)-ABA-d6; Cayman Chemical, MI, USA). Extracts were analyzed using a Dionex UHPLC system (Thermo Scientific, Whatham, MA, USA) with a Kinetix C18 column of particle size 1.7 µm, length 150 X 2.1 mm, 100 Å (Phenomenex, USA), and an Orbitrap-Q Exactive mass spectrometer (Thermo

Scientific, Whatham, MA, USA). ABA and the standard were separated, identified, and the peak area determined as in [41] . ABA concentrations were quantified by comparing the peak area of the endogenous ABA with 1000 ng of spiked deuterated ABA-d6 and standardized to sample dry mass.

Ab initio promoter analysis

The 1000 bps upstream promoter regions of the differently expressed genes were extracted using from the Arabidopsis thaliana Genome Annotation Official Release (Starting Version: Araport11; Update date: September 2022) hosted in the The Arabidopsis Information Resource (TAIR) database (https://www.arabidopsis.org/index.jsp). A total of non-redundant 741 promoter cis-elements belonging to taxa group plantae was retrieved from JASPAR database ver. JASPAR_2022_9_NR. Promoter enrichment was carried out using Ciiider software using the site count and coverage p values of 0.05. The frequencies of cis-element binding sites were visualized using the heatmap tool of TBTools ver.2_008. Identification of ABA-related cis-element was carried out manually by conducting a comprehensive literature analysis.

Isoform Analysis

A Kallisto (v.0.46.2) transcriptome index was created using the working model transcripts that include both high-confidence and working gene models of the Arabidopsis Col-0 Assembly (TAIR10). Transcript abundance and estimates were also calculated using Kallisto (v.0.46.2) [42]. Transcript isoform analysis was carried out using IsoformSwitchAnalyzeR (v.2.1.2) [43](Supplementary Figure 1C). Transcript expression values were imported from Kallisto into IsoformSwitchAnalyzeR using the importRdata function [44]. The isoform switch test was carried out using DEXSeq implemented in IsoformSwitchAnalyzeR [45]. Then, predictions of premature termination codons (PTC) and thereby NMD-sensitivity were carried out [45]. The coding potentials of the transcripts were analyzed using CPC2 [46]. Domain architectures of the resulting proteins were identified using the Pfam database [47]. The presence of the signal peptides was inquired using SignalP (v.5.0) [48] and protein disorder was assessed using IUPred2A [49]. Predictions of the consequences of isoforms were conducted and visualized using IsoformSwitchAnalyzeR (v.2.1.2) [45]. See supplemental Figure 1 for an overview of workflow.

RESULTS

TuMV and aphids regulate greater numbers of transcripts together than alone

We reported Arabidopsis transcriptome responses to *M. persicae* aphid-infestation and TuMV infection to investigate the impacts of these challengers on plant protein turnover pathways [6](See Supp. Fig. 1 for the experimental plan). In revisiting these data, we expanded our efforts to obtain a comprehensive view of the plant transcriptomic responses to the individual and combined treatment of aphid feeding and TuMV infection. Consistent with our previous analysis of differentially expressed genes (DEGs;(Bera et al., 2022)) the log_2-fold changes were higher both in the positive and negative direction when aphids were feeding on TuMV-infected plants than on healthy (Mock) plants treated with aphid or in TuMV infected plants not treated with aphids (Fig. 1)**.** The combination of Aphids + TuMV produced 847 DEGs (339 up; 508 down; Table 1) while aphid-infested plants produced 45 DEGs (9 down, 36 up, Table 1) and TuMV-infected plants generated 177 DEGs (87 up; 90 down, Table 1). A total of 2419 DEGs (1135 up, 1284 down) were exclusive to the Aphid+TuMV treatment and not identified in the mock treated plants (Table 1).

The coordinated genetic responses to aphids feeding on TuMV infected plants did not appear to be additive based on the numbers DEGs obtained following the individual aphid or TuMV challenge treatments. The log_2-fold changes were also higher in the positive and negative direction when comparing Aphid + TuMV responsive genes to aphids alone or TuMV alone (Fig. 2A and 2B). A subset of 45 DEGs that were responsive to aphid treatment were oppositely influenced by the combination of Aphids +TuMV treatment (Fig. 2C). Another set of 50 DEGs induced by TuMV treatment showed a different pattern of dysregulation by the combination of Aphids + TuMV treatment (Fig. 2D). Such complex effects on gene expression points to compounding molecular interactions [28].

Aphids feeding on TuMV infected plants intensify the pattern of stress responsive genes compared to the individual challengers.

Virus can stimulate numerous physiological and defense responses through the induction of SA, JA, and gene silencing machinery, and therefore we hypothesize that TuMV infection also primes adaptive machineries for insect compatible interactions by pre-inducing genes. For hypothesis development, we first performed Gene Ontology (GO) enrichment analysis to identify the biological and molecular processes over-represented among the DEG datasets. Tables S2 through S9 were manually sorted into categories based on GO terms associated with the DEGs. The highest priority was to group genes associated with external and endogenous stimuli, abiotic or biotic stimuli. Since most genes had multiple GO terms, we reported additional GO terms for "response to hormone" or "response abiotic stimuli" in adjacent columns.

Dot plots were used to visually compare 42 enriched GO terms relating to host defenses, regulation of defenses, responses to various pathogens, insects, and symbionts across treatments (Fig. 3). Forty GO terms were among the upregulated gene sets following Aphid + TuMV challenge while only 4 or 11 of these GO terms were identified among the upregulated gene sets following treatment with only aphids or TuMV, respectively (Fig. 3A), indicating that the combined challenges amplified the array of cellular defense genes that were activated. A subset of 6 terms that were among the downregulated in response to aphid challenge (Aphid vs. Mock) as well as the upregulated genes when aphids challenged virus infected plants (Fig. 3A) suggesting that there is fine-tuning of gene expression based on the individual or combined challenges. Such fine tuning of gene expression often occurs at the promoter or transcriptional level.

Virus-plant, and aphid-plant interactions, whether compatible or incompatible, are linked to changes in abiotic stress tolerance as well as rapidly changing redox homeostasis [50]. Tables S2 through S5 clearly show an abundance of DEGs that are directly or indirectly associated with oxygen metabolism. Many potyviruses, including TuMV, were reported to enhance the activity of antioxidant enzymes, lipid peroxidation, protein oxidation, H_2O_2 accumulation, and the loss of chlorophyll levels [51, 52]. We also examined 25 enriched GO terms associated with abiotic stress and oxidative stress or metabolism (Fig. 3B and C). Following TuMV+ Aphid treatment, 9 GO biological process terms were upregulated and 17 GO terms were downregulated (Fig. 3B and C). Treatment with aphids alone or TuMV alone failed to produce any upregulated GO terms in this category once again suggesting that the combination of challengers produced unique genetic responses. Aphid only treatment and TuMV only treatment each showed 5 enriched GO terms were downregulated and were categorically different from each other (Fig. 3B and C). As before, the genetic responses to aphids feeding on virus infected plants were the opposite of aphids feeding on healthy plants.

2.3 Regulatory changes associated with aphids and TuMV infection point to key metabolic and hormone regulated molecular networks

The expanded list of DEGs in the Aphid + TuMV treatment compared to aphids alone or TuMV alone includes genes encoding catalytic and biosynthetic enzymes across the plant metabolic network. Enzymes are up and downregulated to activate or deactivate branch metabolic pathways to support adaptive responses to environmental challenges [53]. Metabolic pathways controlling polyphenol and phytohormone synthesis are often controlled by positive and negative feedback loops between the metabolites and their enzymatic genes [54, 54–56]. Therefore, GO

analysis of DEGs was performed to identify key secondary metabolites and phytohoromones that influence cellular adaptive responses.

Thirty-three GO terms for secondary metabolic and biosynthetic processes were examined revealing various glucosinolate (GSL), phenylpropanoids, flavonoids, and anthocyanin pathway genes and regulators **(**Tables S2 through S9, Fig. 4A**).** For TuMV+Aphid challenged plants, KEGG enrichment revealed 37 genes linked to GSL biosynthesis from tryptophan, which coincided with the upregulated GO terms including the biosynthesis and catabolism of phenol containing compounds, sulphur compounds, GSLs, and indole containing compounds **(**Table S10**).** Notably, there are 37 unique GSL biosynthesis genes when comparing aphids feeding on TuMV infected plants to TuMV infected plants alone, indicating a specific influence of aphids on the GSL pathway. Aphids feeding on healthy plants downregulate 5 KEGG genes linked to indole-GSL activation and indole -3-acetate (IAA) inactivation according to the GO terms **(**Table S10**).** Given that indolic-GSLs are sulfur and nitrogen containing compounds involved in plant defenses against herbivores and pathogens, associated with auxins and phytoalexins, these data combined with data from Figures 2, 3, and Table S10 support the hypothesis that a greater number of DEGs are involved in GSL biosynthesis or its regulation in response to these combined challengers [57]. Importantly, since the differential expression of GSL and indolic-GSL biosynthetic genes are regulated by transcription factors and plant hormones including ABA, JA, SA, and ET these data suggest a significant role for plant hormones in regulating the responses to the combined challengers [57].

For TuMV+Aphid challenged plants, AraCyc identified 49 total pathway genes involved in flavonoid biosynthesis and anthocyanidin modifications while KEGG identified 34 terms for flavonoid and brassinosteroid biosynthesis. TuMV alone induced 29 flavonoid biosynthesis associated genes, while aphids alone induced genes were involved in flavonoid biosynthesis and anthyocyandin modification (Table S10). Considering that plant phytohormones such as auxin, JA, GA, and transcription factors mediate controls on the expression and repression of flavonoid and anthocyanin synthesis while transcriptional regulators influence their gene expression (Naik 2022) the combined alterations in GSL, flavonoid, anthocyanidin, and brassinosteroid biosynthetic pathways suggests a major role for hormone signaling in transcriptional reprogramming of plant cells.

TuMV infection is well known for altering transcriptional programing through interactions with the cellular unfolded protein response machinery [15, 16, 58] however little is known about how aphids influence cellular reprogramming in the same level **(**Fig. 4B). Heat maps were generated using 12 GO terms surrounding protein folding and maturation. As expected TuMV infected plants showed a 10-fold enrichment of genes involved in chaperone-mediated protein

folding [15, 16, 58–60]and no terms were among the downregulated genes (Fig. 4B). Aphids feeding on TuMV infected plants led to significant enrichment of 11 GO terms and none were downregulated. KEGG analysis identified 92 genes involved in ribosome biogenesis that are uniquely influenced by aphids feeding on TuMV infected plants rather than healthy plants. Protein maturation and quality control appear to be critical during compatible interactions involving TuMV alone or Aphids+TuMV treatment (Fig. 4B).

2.4 TuMV and aphids combined influence ABA-dependent host transcriptional responses to aphid infestation.

The role of SA and JA signaling in regulating virus defenses has received more attention than the role of ABA. There are a few examples where ABA functions to mediate plant-virus interactions partially through effects in the RNA silencing pathway [61–64]. TuMV is reported to alter ABA homeostasis, differentially influence the expression of ABA-responsive genes, and increase host sensitivity to water stress [65]. Here, ABA concentrations were elevated 2.5-fold in aphid infested Arabidopsis plants and 5-fold elevated when aphids were feeding on TuMV infected plants (Fig. 5A). When manually curating genes in the Supplementary Tables, there were wider number of genes that were identified by Thalemine and TAIR to be engaged in hormone responses, especially ABA, than determined via GO analysis (Tables S2 through S9).

To understand the influence of ABA on transcriptional reprogramming in response to aphids and TuMV infection, we selected 1,000 bp upstream of the predicted transcription start site for the 25 genes that were upregulated when comparing Aphids + TuMV to TuMV infected plants (Table 1) and the 1135 genes upregulated by Aphids + TuMV when compared to aphids alone (Suppl. Figure 2, Fig. 5B). The 25 genes that are uniquely responsive to Aphids + TuMV include receptor-like proteins, protein kinases, and other enzymes linked to biotic and abiotic stress. Multiple types of TFs are involved in ABA responses including the ABA-insensitive (ABI) factors, Group A basic region/leucine zipper (bZIP) factors which bind to the ABA responsive (AREB/ABF), cis regulatory elements (CREs) in promoters NAC domain factors, WRKY factors, MYB factors, C2H2-type zinc finger (ZFPs), basic helix-loop-helix (bHLH) factors [66–72]. Therefore, we examined a wide range of potential TF CREs to best discover crucial ABA-related factors. Our analysis produced 48 TF CREs linked to hormone signaling. The most striking outcome was that the ABA-response ATHB-6 binding site was represented between 20 and 80 copies per gene. The WRKY48 binding site, which is not ABA-responsive per se, was the second highest representation, between 10 and 25 copies (Fig. 5B). Interestingly, WRKY48 was initially reported to be a transcriptional activator that represses plant basal defenses including PR gene

expression leading to enhanced growth of *Pseudomonas syringae* (DC3000) [73]. While ATHB and WRKY factors represent families of regulators involved in biotic and abiotic stress and ABA signaling [69], this is the first report suggesting ATHB-6 as well as WRKY48 may be essential for interactions involving host tolerance to aphids and plant virus infection. The binding sites for two additional ABA responsive factors, AHL12 and DOF1.6, occurred an average of ≥10 copies per gene (Fig. 5B). Further studies will be needed to determine if these factors interact in a meaningful way to influence gene expression.

2.6 ABA independent changes influencing TuMV and aphid related plant drought tolerance.

Given that ABA is a primary phytohormone that regulates plant responses to drought, we would expect that the heightened ABA levels would partially explain the improved drought tolerance seen in TuMV infected plants. GO analysis presented in Fig 3 shows clusters of genes associated with the term "response to water deprivation" as downregulated by aphid + TuMV treatment, although genes associated with the GO term "drought recovery" seem unaffected. The Arabidopsis 9-*cis*-epoxycarotenoid dioxygenase-3 (AtNCED3) is required for ABA biosynthesis and its transcripts, along with ENO2 were shown to be drastically induced in drought-treated plants [74]. AtNCED-3 and other ABA biosynthetic genes influence stomatal closure (Fig. 6A)[75]. DREB/CBF proteins are AP2 transcription factors that also influence MED16/SFR6 (AT4G04290; upregulated in Table S6) expression [76]. RDR29A expression is controlled by MED16 and linked to drought stress (Fig. 6A).

Table S7 lists genes that are downregulated in response to aphids feeding on TuMV infected plants and includes AtNCED3 (AT3G14440) and seven other genes linked to the regulation of stomata closure: AT2G40820, AT3G10660, AT5G37500, AT2G04570, AT4G17970, AT3G01500, AT5G65590. SnRK2 kinases are also ABA inducible and directly phosphorylate the ABA-responsive element-Binding Factor (ABF)-type transcript factors [71, 77]. In Table S3 and S7 the SnRK2.5 (AT5G63650) are downregulated by TuMV infection and Aphids+TuMV treatment. These data suggest that the ABA dependent transcriptional regulation of these factors may be suppressed rather than induced. We performed RT-qPCR to detect expression of NCED3, ENO2, and RDR29A using RNA from healthy and TuMV-infected Arabidopsis plants which were infested with aphids (Fig. 6B). All three genes were suppressed by TuMV infection or aphids feeding on TuMV infected plants compared to mock controls. ENO2 was induced, but not NCED3 or RDR29A, by aphids feeding on healthy plants (Fig. 6B). A heat map was assembled to examine 29 genes that are regulated by MED16 in an ABA-dependent manner and the result show that only 4 genes were suppressed by Aphids alone, another set of 8 genes were

suppressed by TuMV alone, and 22 were either unaffected or suppressed by the aphids + TuMV treatment (Fig. 6C). Interestingly, fibrillarin (At4G25630), which is a nucleolar protein that interacts with viral proteins in the nucleus or nucleolus to disable anti-viral responses and promote infection. For potyviruses, the VPg interacts with fibrillarin in the nucleus and researchers have speculated that the interaction affects host transcription and pre-mRNA processing for host-gene shut off [78].

The Tables S3, S6, and S7 identify *DREB1A, DREB2C, DREB4, DREB26, RAP2.4,* and *TG* (At4G25480, AT1G21910, AT1G78080, AT2G40340, AT1G36060, AT5G52020) among the differentially regulated genes suggesting fine tuning among these transcription factors to regulate the physiological conditions of challenged plants. Heat maps comparing the expression of 14 AP2 proteins that are classified as DREB/CBF family proteins and linked to biotic stress responses indicate that the combination of Aphids +TuMV suppressed expression of most of these genes when compared to mock plants or plants treated with aphids alone. TuMV alone, aphids alone had little to no effect on their expression (Fig. 6D).

2.6 Regulation of transcript isoform accumulation

ABA is also known to induce RNA alternative splicing (AS) in response to environmental challenges and growth [79]. New high throughput sequencing approaches have revealed that plant and animal infecting viruses modulate RNA alternative splicing (AS). There are few examples describing AS influences immune responses in virus-host interactions [80–82]. In the case of sugarcane mosaic virus (SCMV) the AS maize phytoene synthase 1 (*ZmPSY1*) ensures persistent virus infection while decreasing chloroplast damage [83]. Others suggest that viruses may stimulate AS to highjack host factors from the normal cellular functions to aid in virus replication and accumulation.

Table 2 shows that there are 21 and 30 genes with differentially used isoforms as the result of aphid infestation alone or TuMV infection alone, respectively. The combination of Aphid +TuMV saw an increase of number of genes to 36 and when we compare these datasets there are 22 novel genes resulting from Aphid+TuMV compared to aphid alone and 20 genes when compared to TuMV alone. Given the hypothesis that AS can expand the complexity of the cellular proteome during biotic stress, these data show very few genes show alternate isoform usage in comparison to the overall number of DEGs. However, these data provide the first evidence that aphids along with TuMV can influence pre-mRNA splicing carried out by the spliceosome. There were 29 isoform switches that were exclusively influenced by Aphids + TuMV when compared to

aphids alone and 21 exclusive isoform switches when compared to TuMV alone (Table 2). Interestingly RNA-dependent RNA polymerase 6 (RdR6) which is critical in gene silencing defenses to virus infection, was among the genes with isoform changes. There were many other genes involved in pathogen defense or abiotic stress. There were many TFs or mRNA binding factors affecting RNA metabolism including translation and pre-mRNA splicing. Several factors were associated with chloroplast metabolism (Table S11 and S12). Interestingly the U2AF65A (AT4G36690) which is an important subunit for U2 small nuclear ribonucleoproteins (snRNP) was identified in Supplementary Table 12. ABA is also known to regulate *U2AF65A* splicing which in turn influences ABA-mediated flowering and drought stress. While protein functional domains there were only 2 factors, based on GO terms, for which alternate isoforms resulted in a change in protein subcellular targeting. At3G2360 is an mRNA binding factor for which one isoform associates with the Golgi and vacuole, and the other isoform is extracellular. The other factor is AT1G23750 which is involved in nucleic acid binding and chlorophyll biosynthesis and one isoform is nuclear while the alternative isoform is cytoplasmic (Table S11 and 12). One explanation is that virus infection or aphids might highjack these factors for compatible interactions although further investigations are needed to study these interactions.

The dominant mode for alternative transcription events in TuMV infected plants and aphids +TUMV infected plants was the production of isoforms via alternative transcription start (ATSS) and termination (ATTS) sites. There was also a positive shift in the number of intron retention (IR) events when we compared aphids +TuMV to aphids alone (Fig. 7A, B). More often intron retention resulted in a gain in coding sequences. For the most part, aberrant RNAs are degraded through nonsense mediated decay (NMD). For the most part, NMD sensitive isoforms were slightly increased by TuMV infection, but not altered by aphids feeding on TuMV infected plants when compared to aphids alone (Fig. 7C). There was little evidence linking ABA signaling to AS pathways in aphid and TuMV response based on the identified genes or the pattern of splicing events which commonly include intron retention, and alternative 5' or 3' splicing sites.

In general, AS of transcripts is a feature of plant adaptation to environmental stress. To test this hypothesis, we examined the AS events of four genes that were found to be uniquely regulated by Aphids + TuMV when compared to TuMV or aphids from Tables S11 and S12: PUX2, AGL31, Sec14-family protein, and an unknown factor in relation to a wide range of environmental stressors as reported in public databases (Fig. 8). Interestingly, the levels of PUX2 isoform 1 decreases while isoform 2 increases in the presence of Aphid +TuMV when compared to aphids alone suggesting that TuMV infection preconditioned the cells for further abilities to

respond to aphid infestation. Across 15 treatments involving abiotic stress or hormones, only SA treatment showed a rise in isoform 2 and decrease in isoform 1. All other treatments, including ABA retained higher isoform 1 than 2 levels (Fig 8). For AGL31 isoform 1 and 3 are not statistically significant while isoform 5 is downregulated by the combined aphid +TuMV treatment. Across the same 15 treatments in Fig. 8, the isoforms 3 and 5 are widely altered due to environment or hormone challenges. Sec14A isoforms 1 increases and isoform 3 decreases due to Aphid+TuMV, and across the 15 treatments. In the case of the uncharacterized AT1G23750, isoform 1 is decreased and isoform 2 is increased. The opposite is true for abiotic stress treatment (Fig. 8). These data suggest that the complex gene regulatory networks affecting the accumulation of transcript isomers underly Aphid+TuMV infection overlap genetic responses to other environmental challenges. These data point to fine tuning of gene expression involving pre-mRNA processing alongside differential gene expression.

DISCUSSION

This transcriptome study was undertaken to understand the landscape of cellular reprogramming that occurs when aphids are feeding on TuMV-infected or healthy plants. Remarkably fewer genes were influenced by aphids alone and TuMV alone compared to the combined challengers. It is arguable that since the plants are already virus infected that genetic modifications by the combined challengers are important to create an environment that favors acquisition feeding, enable vector borne transmission, or promotes aphid reproduction. Regarding plant response to aphids, there has been very little information available concerning the potential signaling hubs that integrate sensing functions relating to the virus and its vector.

The cumulative assessment of genes that are exclusively affected by aphids alone or Aphids +TuMV was examined. Aphids feeding on mock Arabidopsis plants specifically downregulate suberin biosynthetic processes, regulators of defense responses or responses to external stimuli such as wounding or insects, and phenylpropanoid biosynthetic processes. At the same time, there is exclusive upregulation of sulfate assimilates, certain pathogen defense responses, regulation of flavonoid, anthocyanin, and pigment biosynthetic processes, and inositol phosphate dephosphorylation. Genes that are exclusively downregulated by aphids feeding on TuMV infected plants encode factors engaged in cold responses, photosynthetic processes, NADH and NAH(P)H dehydrogenase complex assembly, lipid and fatty acid biosynthesis, and circadian rhythms.

Genes that are exclusively affected by TuMV alone or Aphids +TuMV also presented GO terms that were exclusively influenced by TuMV or the combination of challengers. Among the

upregulated were genes involved in systemic acquired resistance, SA metabolism, fungal defenses, immune responses, and leaf senescence. Genes that are exclusively downregulated by Aphids +TuMV that are not influenced by TuMV alone include genes involved in responses to JA, ABA, water deprivation, and UV-B, as well as anthocyanin and flavonoid biosynthetic processes.

Around 50% of the DEGs that were altered in response to aphids, TuMV, or the combination of both challenges were related to abiotic and biotic stress responses (Tables S2-S8), plant development, hormone signaling, and innate immunity. Herbivore-associated molecular patterns (HAMPS), pathogen-associated effector triggered immunity (ETI) and pattern triggered immunity (PTI) involve common phytohormone signaling pathways led by salicylic acid (SA) and jasmonic acid (JA). In this study, 29 genes associated with SA and JA-dependent defense pathways were upregulated when aphids were feeding on TuMV infected plants, but not on healthy plants or in plants that were only infected with TuMV. The list includes phenylalanine ammonia lyase 1 (PAL1; At2G37040) and SARD1 (At1G73805) which are essential for SA biosynthesis. A greater number of genes involved in JA metabolism were observed including several lipoxygenases, jasmonate induce oxygenase, and jasmonate-ZIM (JAZ) domain proteins [84, 85]. DEGs associated with oxidative stress and ROS-scavenging include cytochrome P450 monooxygenases (CYPs) and 2-oxoglutarate-dependent oxygenases (2ODO), superoxide dismutase, catalase, and peroxidases. These families participate in a broad range of cellular metabolic processes and are important for plant-environment interaction [86, 87].

Receptor like proteins (RLP) play significant roles in plant immunity, response to environmental challenges, as well as in plant growth and development [88, 89]. Comparing aphids feeding on TuMV infected plants to feeding on healthy plants, the upregulated genes (Table S6) included 13 LRR proteins, 32 RLPs and receptor like kinases (RLKs), 13 serine/threonine kinases, and 25 other kinases. Among the downregulated gene are 11 LRR proteins, and 44 receptor kinases and other kinases (Suppl Table 7). RLPs are typically located at the cell surface with an extracellular leucine rich repeat domain (LRR), as well as receptor like kinases (RLK), and intracellular kinases (KIN). For example, RLP38 (At3G23120) which is related to Clavata 2 (CLV2) was upregulated by TuMV and by aphids in the presence of TuMV, but not by aphids alone (Supplementary Table 2 and 6). Other Clavata signaling pathway genes are downregulated by aphids feeding on TuMV infected plants, but not by aphids alone include CLV1 (AT1G75820), CLV3 (AT1G70895), CLE9 (AT1G26600), CLE10 (AT1G69320), CLE17 (AT1G70895), CLE21 (AT5G64800), CLE26, (AT1G69970), CLE42 (AT2G34925), BAM2 (AT3G49670) (Supplementary Table 7). CLAVATA -type receptors are known for their role in apical meristem

development [90], and more recently are shown to be crucial in plant response to abiotic stress, plant-microbe interactions, and parasitic nematodes which also can that secrete CLE-like effectors into plants [91] . Aphids potentially deliver effector molecules through their saliva into plants, and it is worth speculating that may deposit CLE peptides which could impact host gene expression or feedback loops regulating clavata signaling [91]. Not many LRR proteins that recognize aphid signals have been identified and as plant viruses are obligate pathogens it is unclear if virus infection increases or decreases expression of defense related RLPs and RLKs that would otherwise respond to aphid feeding.

Abscisic acid (ABA) is also important for diverse plant-pathogen interactions, abiotic stress, as well as plant growth and development. MED16 is a component of ABA-transcriptional regulation bridging the ABF transcription factors with RNA polymerase II. MED16 plays a crucial role in defense response against aphids and TuMV infection and we recently showed that its nuclear localization if negatively impacted by NIa-Pro (Ray et al., 2023). In this study we identified a number of genes that are regulated by ABA and MED16 that are downregulated when aphids infest TuMV infected plants compared to healthy plants. While dysregulation could be explained by disarming MED16 the robust downregulation points to the possibility of an unknown suppressor that competes with MED16 at the same promoters. These data support a model in which MED16 is a potential signaling hub for precise genetic changes in response to TuMV and aphids. However, the results of RT-qPCR indicated that NCED3 and ENO2 are downregulated by TMV and TuMV+aphid treatment suggesting that the increase in drought protection does not involve these two factors. Because we identified Visual cues such as leaf yellowing are known to attract insects to plants, and therefore the identification of genes regulating chlorophyll content and leaf chlorosis among the differentially regulated genes was not surprising.

It has been recently reported that ABA treatment and other abiotic stress treatments of Arabidopsis leads to dramatic changes in mRNA alternative splicing events [79]. Putative alternative splicing events have been recently reported in studies involving other plant viruses such as bamboo mosaic virus, bean common mosaic virus, and panicum mosaic virus. [21, 63, 93]. When we compared the outcomes of aphids feeding on TuMV versus healthy plants, there was a steep increase in the use of alternative transcription start sites and termination sites. Surprisingly the combination of aphid feeding on TuMV infected plants showed more intron retention in transcripts than in TuMV infected plants, or aphid infestation of healthy plants. Most of the genes with shifting isoform usage encoded, regulatory factors in the nucleus, chloroplast, mitochondria, or peroxisomes. While the mechanism for virus or vector induced AS in plants is not known, it is possible that expression of Med factors which regulate RNA pol II, downregulation

of fibrillarin which regulates mRNA splicing, and perhaps the recruitment of different splicing factors in the splicing complex could explain this phenomenon. Until now most AS splicing events are reported from transcriptome studies and the functional significance of the AS events regulated by plant virus infection or aphids is not yet known, but these events likely contribute to plant adaptation to challenges.

To summarize, large numbers of genes that are differentially regulated exemplify crosstalk between cellular immunity, cell survival, adaptation, and responses to environmental stress. These include factors that are essential for cell survival and some that are either hijacked by virus infection or have dual functions in defense or adaptation. Alternative splicing of pre-mRNAs serves to add or remove protein domains and this appears to be an essential component of the arms race between viruses, herbivores, and their hosts**.**

Acknowledgments

The authors thank Maria Persuad-Fernandez and Tyseen Murad for help with plant and aphid colony care and experiments. We also thank REU scholars Geysira Vela and Lindsay Vaughan for assistance with building Supplementary Tables.

Ethics Declarations

Ethics approval and consent to participate: Not applicable.

Consent for publication: All authors consent to the publication of this article

Availability of data and material: We have no research data declarations to make.

Funding: This research was supported by a National Science Foundation award to CLC (1723926) and to Verchot (1759034) and an REU award led by Dr Wonbo Shim (202367037-40307).

Competing interests: The authors have no competing interests as defined by BMC, or other interests that might be perceived to influence the results and/or discussion reported in this paper.

References

1. Chase AJ, Semler BL. Viral subversion of host functions for picornavirus translation and RNA replication. 2012.

2. Aranda M, Maule A. MINIREVIEW Virus-Induced Host Gene Shutoff in Animals and Plants. 1998.

3. Aranda MA, Escaler M, Wang D, Maule AJ. Induction of HSP70 and polyubiquitin expression associated with plant virus replication. Proceedings of the National Academy of Sciences. 1996;93:15289–93.

4. Casteel CL, De Alwis M, Bak A, Dong H, Whitham SA, Jander G. Disruption of Ethylene Responses by Turnip mosaic virus Mediates Suppression of Plant Defense against the Green Peach Aphid Vector. Plant Physiol. 2015;169:209–18.

5. Patton MF, Bak A, Sayre JM, Heck ML, Casteel CL. A polerovirus, Potato leafroll virus, alters plant–vector interactions using three viral proteins. Plant Cell Environ. 2020;43:387–99.

6. Bera S, Arena GD, Ray S, Flannigan S, Casteel CL. The Potyviral Protein 6K1 Reduces Plant Proteases Activity during Turnip mosaic virus Infection. Viruses. 2022;14:1–16.

7. Shen W, Shi Y, Dai Z, Wang A. The RNA-dependent RNA polymerase NIb of potyviruses plays multifunctional, contrasting roles during viral infection. Viruses. 2020;12.

8. Gaucherand L, Gaglia MM. Annual Review of Virology. 2022. https://doi.org/10.1146/annurev-virology-100120.

9. Yang X, Li Y, Wang A. Research Advances in Potyviruses: From the Laboratory Bench to the Field. Annu Rev Phytopathol. 2021;59:1–29.

10. Endres MW, Gregory BD, Gao Z, Foreman AW, Mlotshwa S, Ge X, et al. Two plant viral suppressors of silencing require the ethylene-inducible host transcription factor RAV2 to block RNA silencing. PLoS Pathog. 2010;6.

11. Eskelin K, Hafrén A, Rantalainen KI, Mäkinen K. Potyviral VPg Enhances Viral RNA Translation and Inhibits Reporter mRNA Translation In Planta . J Virol. 2011;85:9210–21.

12. Hafrén A, Lõhmus A, Mäkinen K. Formation of Potato Virus A-Induced RNA Granules and Viral Translation Are Interrelated Processes Required for Optimal Virus Accumulation. PLoS Pathog. 2015;11:1–28.

13. Beauchemin C, Laliberté J-F. The Poly(A) Binding Protein Is Internalized in Virus-Induced Vesicles or Redistributed to the Nucleolus during Turnip Mosaic Virus Infection. J Virol. 2007;81:10905–13.

14. Casteel CL, De Alwis M, Bak A, Dong H, Whitham SA, Jander G. Disruption of Ethylene Responses by Turnip mosaic virus Mediates Suppression of Plant Defense against the Green Peach Aphid Vector . Plant Physiol. 2015;169:209–18.

15. Gayral M, Arias Gaguancela O, Vasquez E, Herath V, Flores FJ, Dickman MB, et al. Multiple ER-to-nucleus stress signaling pathways are activated during plantago asiatica mosaic virus and turnip mosaic virus infection in Arabidopsis thaliana. The Plant Journal. 2020;103:1233–45.

16. Gaguancela O, Zúñiga LP, Arias AV, Halterman D, Flores FJ, Johansen IE, et al. The IRE1/bZIP60 pathway and Bax inhibitor 1 suppress systemic accumulation of potyviruses and potexviruses in Arabidopsis and Nicotiana benthamiana plants. Molecular Plant-Microbe Interactions. 2016;29:750–66.

17. Voelckel C, Jander G. Annual Plant Reviews, Insect-Plant Interactions. John Wiley & Sons; 2014.

18. Åhman I, Kim S-Y, Zhu L-H. Plant Genes Benefitting Aphids—Potential for Exploitation in Resistance Breeding. Frontiers in Plant Science. 2019;10.

19. Herath V, Verchot J. Transcriptional regulatory networks associate with early stages of potato virus X infection of Solanum tuberosum. Int J Mol Sci. 2021;22:2837.

20. Glushkevich A, Spechenkova N, Fesenko I, Knyazev A, Samarskaya V, Kalinina NO, et al. Transcriptomic Reprogramming, Alternative Splicing and RNA Methylation in Potato (Solanum tuberosum L.) Plants in Response to Potato Virus Y Infection. Plants. 2022;11.

21. Martin K, Singh J, Hill JH, Whitham SA, Cannon SB. Dynamic transcriptome profiling of Bean Common Mosaic Virus (BCMV) infection in Common Bean (Phaseolus vulgaris L.). BMC Genomics. 2016;17.

22. Du K, Jiang T, Chen H, Murphy AM, Carr JP, Du Z, et al. Viral perturbation of alternative splicing of a host transcript benefits infection. Plant Physiol. 2020;184:1514–31.

23. Su CF, Das D, Muhammad Aslam M, Xie JQ, Li XY, Chen MX. Eukaryotic splicing machinery in the plant–virus battleground. Wiley Interdiscip Rev RNA. 2023; February:1–23.

24. Zhang F, Ke J, Zhang L, Chen R, Sugimoto K, Howe GA, et al. Structural insights into alternative splicing-mediated desensitization of jasmonate signaling. Proc Natl Acad Sci U S A. 2017;114:1720–5.

25. Gadhave KR, Gautam S, Rasmussen DA, Srinivasan R. Aphid Transmission of Potyvirus: The Largest Plant-Infecting RNA Virus Genus. Viruses. 2020;12.

26. Pan L-L, Miao H, Wang Q, Walling LL, Liu S-S. Virus-induced phytohormone dynamics and their effects on plant–insect interactions. New Phytologist. 2021;230:1305–20.

27. Safari M, Ferrari MJ, Roossinck MJ. Manipulation of Aphid Behavior by a Persistent Plant Virus. J Virol. 2019;93:1–10.

28. Ray S, Casteel CL. Effector-mediated plant–virus–vector interactions. Plant Cell. 2022;34:1514–31.

29. Song J, Liu H, Zhuang H, Zhao C, Xu Y, Wu S, et al. Transcriptomics and alternative splicing analyses reveal large differences between maize lines B73 and Mo17 in response to aphid Rhopalosiphum padi infestation. Front Plant Sci. 2017;8.

30. Ling Z, Zhou W, Baldwin IT, Xu S. Insect herbivory elicits genome-wide alternative splicing responses in Nicotiana attenuata. Plant Journal. 2015;84:228–43.

31. Bera S, Arena GD, Ray S, Flannigan S, Casteel CL. The potyviral protein 6K1 reduces plant protease activity during Turnip mosaic virus infection. bioRxiv. 2021;:2021.09.05.459032.

32. Casteel CL, Yang C, Nanduri AC, De Jong HN, Whitham SA, Jander G. The NIa-Pro protein of Turnip mosaic virus improves growth and reproduction of the aphid vector, Myzus persicae (green peach aphid). The Plant Journal. 2014;77:653–63.

33. Kim D, Langmead B, Salzberg SL. HISAT: A fast spliced aligner with low memory requirements. Nat Methods. 2015. https://doi.org/10.1038/nmeth.3317.

34. Etherington GJ, Ramirez-Gonzalez RH, MacLean D. Bio-samtools 2: A package for analysis and visualization of sequence and alignment data with SAMtools in Ruby. Bioinformatics. 2015. https://doi.org/10.1093/bioinformatics/btv178.

35. Danecek P, Bonfield JK, Liddle J, Marshall J, Ohan V, Pollard MO, et al. Twelve years of SAMtools and BCFtools. Gigascience. 2021;10:1–4.

36. Pertea M, Pertea GM, Antonescu CM, Chang TC, Mendell JT, Salzberg SL. StringTie enables improved reconstruction of a transcriptome from RNA-seq reads. Nat Biotechnol. 2015;33:290–5.

37. Blighe, Rana, Lewis. EnhancedVolcano: Publication-ready volcano plots with enhanced colouring and labeling. https://github.com/kevinblighe/EnhancedVolcano. 2018.

38. Berardini TZ, Reiser L, Li D, Mezheritsky Y, Muller R, Strait E, et al. The arabidopsis information resource: Making and mining the “gold standard” annotated reference plant genome. Genesis. 2015. https://doi.org/10.1002/dvg.22877.

39. Xijin Ge S, Jung D, Yao R. ShinyGO: a graphical gene-set enrichment tool for animals and plants. Bioinformatics. 2020;36:2628–9.

40. Chen C, Chen H, Zhang Y, Thomas HR, Frank MH, He Y, et al. TBtools - an integrative toolkit developed for interactive analyses of big biological data. Mol Plant. 2020. https://doi.org/10.1016/j.molp.2020.06.009.

41. Bera S, Blundell R, Liang D, Crowder DW, Casteel CL. The Oxylipin Signaling Pathway Is Required for Increased Aphid Attraction and Retention on Virus-Infected Plants. J Chem Ecol. 2020. https://doi.org/10.1007/s10886-020-01157-7.

42. Bray NL, Pimentel H, Melsted P, Pachter L. Near-optimal probabilistic RNA-seq quantification. Nat Biotechnol. 2016. https://doi.org/10.1038/nbt.3519.

43. Vitting-Seerup K, Sandelin A, Berger B. IsoformSwitchAnalyzeR: Analysis of changes in genome-wide patterns of alternative splicing and its functional consequences. Bioinformatics. 2019;35:4469–71.

44. Soneson C, Love MI, Robinson MD. Differential analyses for RNA-seq: transcript-level estimates improve gene-level inferences. F1000Res. 2015;4:1521.

45. Vitting-Seerup K, Sandelin A. The landscape of isoform switches in human cancers. Molecular Cancer Research. 2017;15:1206–20.

46. Kang YJ, Yang DC, Kong L, Hou M, Meng YQ, Wei L, et al. CPC2: A fast and accurate coding potential calculator based on sequence intrinsic features. Nucleic Acids Res. 2017;45:W12–6.

47. El-Gebali S, Mistry J, Bateman A, Eddy SR, Luciani A, Potter SC, et al. The Pfam protein families database in 2019. Nucleic Acids Res. 2019;47:D427–32.

48. Almagro Armenteros JJ, Tsirigos KD, Sønderby CK, Petersen TN, Winther O, Brunak S, et al. SignalP 5.0 improves signal peptide predictions using deep neural networks. Nat Biotechnol. 2019;37:420–3.

49. Mészáros B, Erdös G, Dosztányi Z. IUPred2A: Context-dependent prediction of protein disorder as a function of redox state and protein binding. Nucleic Acids Res. 2018;46:W329–37.

50. Song Y, Feng L, Alyafei MAM, Jaleel A, Ren M. Function of chloroplasts in plant stress responses. International Journal of Molecular Sciences. 2021;22.

51. Wei T, Zhang C, Hou X, Sanfaçon H, Wang A. The SNARE Protein Syp71 Is Essential for Turnip Mosaic Virus Infection by Mediating Fusion of Virus-Induced Vesicles with Chloroplasts. PLoS Pathog. 2013;9.

52. Hernández JA, Gullner G, Clemente-Moreno MJ, Künstler A, Juhász C, Díaz-Vivancos P, et al. Oxidative stress and antioxidative responses in plant–virus interactions. Physiol Mol Plant Pathol. 2016;94:134–48.

53. Carthew RW. Gene Regulation and Cellular Metabolism: An Essential Partnership. Trends in Genetics. 2021;37:389–400.

54. Chaves M, Oyarzún DA. Dynamics of complex feedback architectures in metabolic pathways. Automatica. 2019;99:323–32.

55. Sharma A, Shahzad B, Rehman A, Bhardwaj R, Landi M, Zheng B. Response of phenylpropanoid pathway and the role of polyphenols in plants under abiotic stress. Molecules. 2019;24.

56. Liu W, Feng Y, Yu S, Fan Z, Li X, Li J, et al. The flavonoid biosynthesis network in plants. International Journal of Molecular Sciences. 2021;22.

57. Harun S, Abdullah-Zawawi M-R, Goh H-H, Mohamed-Hussein Z-A. A Comprehensive Gene Inventory for Glucosinolate Biosynthetic Pathway in Arabidopsis thaliana. J Agric Food Chem. 2020;68:7281–97.

58. Zhang L, Chen H, Brandizzi F, Verchot J, Wang A. The UPR Branch IRE1-bZIP60 in Plants Plays an Essential Role in Viral Infection and Is Complementary to the Only UPR Pathway in Yeast. PLoS Genet. 2015;11:e1005164.

59. Williams B, Verchot J, Dickman MB. When supply does not meet demand-ER stress and plant programmed cell death. Front Plant Sci. 2014;5 JUN:1–9.

60. Herath V, Gayral M, Miller RK, Verchot J. BIP and the unfolded protein response are important for potyvirus and potexvirus infection. Plant Signal Behav. 2020;15:1807723.

61. Alazem M, Lin NS. Interplay between ABA signaling and RNA silencing in plant viral resistance. Curr Opin Virol. 2020;42:1–7.

62. Alazem M, Kim KH, Lin NS. Effects of abscisic acid and salicylic acid on gene expression in the antiviral RNA silencing pathway in arabidopsis. Int J Mol Sci. 2019;20.

63. Alazem M, Lin KY, Lin NS. The abscisic acid pathway has multifaceted effects on the accumulation of Bamboo mosaic virus. Molecular Plant-Microbe Interactions. 2014;27:177–89.

64. Chiu YH, Hung YL, Wang HP, Wei WL, Shang QW, Pham TH, et al. Investigation of P1/HC-pro-mediated ABA/calcium signaling responses via gene silencing through high- and low-throughput RNA-seq approaches. Viruses. 2021;13.

65. Manacorda CA, Gudesblat G, Sutka M, Alemano S, Peluso F, Oricchio P, et al. TuMV triggers stomatal closure but reduces drought tolerance in Arabidopsis. Plant Cell Environ. 2021;44:1399–416.

66. Podzimska-Sroka D, O'Shea C, Gregersen PL, Skriver K. NAC transcription factors in senescence: from molecular structure to function in crops. Plants. 2015;4:412–48.

67. Kim D, Jeon SJ, Yanders S, Park S, Kim HS, Kim S. MYB3 plays an important role in lignin and anthocyanin biosynthesis under salt stress condition in Arabidopsis. Plant Cell Rep. 2022;41:1549–60.

68. Wang T, Tohge T, Ivakov A, Mueller-Roeber B, Fernie AR, Mutwil M, et al. Salt-related MYB1 coordinates abscisic acid biosynthesis and signaling during salt stress in arabidopsis. Plant Physiol. 2015;169:1027–41.

69. Rushton DL, Tripathi P, Rabara RC, Lin J, Ringler P, Boken AK, et al. WRKY transcription factors: Key components in abscisic acid signalling. Plant Biotechnology Journal. 2012;10:2–11.

70. Tang W, Luo C. Overexpression of Zinc Finger Transcription Factor ZAT6 Enhances Salt Tolerance. Open Life Sci. 2018;13:431–45.

71. Gao S, Gao J, Zhu X, Song Y, Li Z, Ren G, et al. ABF2, ABF3, and ABF4 Promote ABA-Mediated Chlorophyll Degradation and Leaf Senescence by Transcriptional Activation of Chlorophyll Catabolic Genes and Senescence-Associated Genes in Arabidopsis. Mol Plant. 2016;9:1272–85.

72. Hwang K, Susila H, Nasim Z, Jung JY, Ahn JH. Arabidopsis ABF3 and ABF4 Transcription Factors Act with the NF-YC Complex to Regulate SOC1 Expression and Mediate Drought-Accelerated Flowering. Mol Plant. 2019;12:489–505.

73. Xing DH, Lai ZB, Zheng ZY, Vinod KM, Fan BF, Chen ZX. Stress- and pathogen-induced Arabidopsis WRKY48 is a transcriptional activator that represses plant basal defense. Mol Plant. 2008;1:459–70.

74. Prakash V, Nihranz CT, Casteel CL. The Potyviral Protein 6K2 from Turnip Mosaic Virus Increases Plant Resilience to Drought. Molecular Plant-Microbe Interactions®. 2023;36:189–97.

75. Sussmilch FC, Brodribb TJ, McAdam SAM. Up-regulation of NCED3 and ABA biosynthesis occur within minutes of a decrease in leaf turgor but AHK1 is not required. J Exp Bot. 2017;68:2913–8.

76. Boyce JM, Knight H, Deyholos M, Openshaw MR, Galbraith DW, Warren G, et al. The sfr6 mutant of Arabidopsis is defective in transcriptional activation via CBF/DREB1 and DREB2 and shows sensitivity to osmotic stress. Plant Journal. 2003;34:395–406.

77. Wang RS, Pandey S, Li S, Gookin TE, Zhao Z, Albert R, et al. Common and unique elements of the ABA-regulated transcriptome of Arabidopsis guard cells. BMC Genomics. 2011;12.

78. Decle-Carrasco S, Rodríguez-Zapata LC, Castano E. Plant viral proteins and fibrillarin: the link to complete the infective cycle. Molecular Biology Reports. 2021;48:4677–86.

79. Yang X, Jia Z, Pu Q, Tian Y, Zhu F, Liu Y. ABA Mediates Plant Development and Abiotic Stress via Alternative Splicing. Int J Mol Sci. 2022;23.

80. Boudreault S, Roy P, Lemay G, Bisaillon M. Viral modulation of cellular RNA alternative splicing: A new key player in virus–host interactions? Wiley Interdiscip Rev RNA. 2019;10:1–20.

81. Mandadi KK, Scholthof KBG. Genome-wide analysis of alternative splicing landscapes modulated during plant-virus interactions in brachypodium distachyon. Plant Cell. 2015;27:71–85.

82. Zheng Y, Wang Y, Ding B, Fei Z. Comprehensive Transcriptome Analyses Reveal that Potato Spindle Tuber Viroid Triggers Genome-Wide Changes in Alternative Splicing, Inducible trans-Acting Activity of Phased Secondary Small Interfering RNAs, and Immune Responses. J Virol. 2017;91:1–18.

83. Du K, Jiang T, Chen H, Murphy AM, Carr JP, Du Z, et al. Viral perturbation of alternative splicing of a host transcript benefits infection. Plant Physiol. 2020;184:1514–31.

84. Sun Y, Detchemendy TW, Pajerowska-Mukhtar KM, Mukhtar MS. NPR1 in JazzSet with Pathogen Effectors. Trends Plant Sci. 2018;23:469–72.

85. Tungadi T, Watt LG, Groen SC, Murphy AM, Du Z, Pate AE, et al. Infection of Arabidopsis by cucumber mosaic virus triggers jasmonate-dependent resistance to aphids that relies partly on the pattern-triggered immunity factor BAK1. Mol Plant Pathol. 2021;22:1082–91.

86. Hagel JM, Facchini PJ. Expanding the roles for 2-oxoglutarate-dependent oxygenases in plant metabolism. Nat Prod Rep. 2018;35:721–34.

87. Zeng WY, Tan YR, Long SF, Sun ZD, Lai ZG, Yang SZ, et al. Methylome and transcriptome analyses of soybean response to bean pyralid larvae. BMC Genomics. 2021;22.

88. Mang H, Feng B, Hu Z, Boisson-Dernier A, Franck CM, Meng X, et al. Differential regulation of two-tiered plant immunity and sexual reproduction by ANXUR receptor-like kinases. Plant Cell. 2017. https://doi.org/10.1105/tpc.17.00464.

89. Kourelis J, Kamoun S. RefPlantNLR: a comprehensive collection of experimentally validated plant NLRs. 2020;:1–14.

90. Hirakawa Y. CLAVATA3, a plant peptide controlling stem cell fate in the meristem. Peptides. 2021;142.

91. Bashyal S, Gautam CK, Müller LM. CLAVATA signaling in plant–environment interactions. Plant Physiol. 2023. https://doi.org/10.1093/plphys/kiad591.

92. Ray S, Murad T, Arena G, Arshad K, Arendsee Z, Herath V, et al. Viral protease NIa-Pro increases Mediator Subunit16 cleavage and plant susceptibility to turnip mosaic virus and its aphid vector Myzus persicae. BioRxiv. 2023. https://doi.org/10.1101/2023.10.24.563845.

93. Pyle JD, Mandadi KK, Scholthof KBG. Panicum mosaic virus and its satellites acquire RNA modifications associated with host-mediated antiviral degradation. mBio. 2019;10.

Figure Legends.

Fig. 1. Differentially expressed number of genes (DEGs). Volcano plots show the significantly upregulated genes in blue and downregulated genes in red (p<0.05) when comparing Arabidopsis plants infected with TuMV and healthy (mock) plants, plants infested with aphids and mock plants, and aphids feeding on TuMV infected plants compared with mock plants.

Fig. 2. Aphids feeding on healthy versus TuMV infected plants produce different gene expression patterns. **A**, **B** Volcano plots show numbers of DEGs following treatment of Arabidopsis plants with aphids and TuMV versus aphids alone or TuMV alone. **C, D** Heatmaps showing the relative expression of candidate target genes, that were responsive to the combination of Aphids +TuMV treatment and oppositely influenced by aphid (n=45) or TuMV (n=50) treatment alone. Rows represent genes and columns represent treatments. Clusters of genes were labeled depending upon their particular expression patterns.

Fig. 3. Dot plots from gene ontology analysis representing the fold enrichment of DEGs associated with environmental responses. Rows represent GO terms and columns indicate treatments with aphids, TuMV, or aphids+TuMV. Blue dots represent downregulated and red dots represent upregulated genes. **A** Analysis of enriched GO terms relating to plant-microbe interactions. **B** Enriched GO terms relating to abiotic stress. **C** Enriched GO terms relating to oxidative stress.

Fig. 4. Dot plots from gene ontology analysis representing the fold enrichment of DEGs**. A** Rows represent biosynthetic and metabolic pathways. **B** Rows represent GO terms for protein maturation processes.

Fig. 5. ABA levels and genes with ABA responsive elements that are DEGs in this study. **A** Graph shows the concentration of ABA in mock, aphid treatment, TuMV infection, and aphids feeding on TuMV infected plants. **B** Genes with ABA responsive cis regulatory elements (CREs) in the promoters. The number of each category of ABA response and other CREs are identified in each column.

Fig. 6. Analysis of ABA-dependent and independent drought responsive genes. **A** Pathway overview of hallmark genes controlling drought responses via MED16 and independent of MED16. **B** RT-qPCR analysis of NCED3, ENO2, and RDR29A expression. Heatmaps showing **C** ABA dependent changes and **D** DREB dependent gene expression that are specific to aphids alone, TuMV alone or Aphids + TuMV treatment.

Fig. 7. Isoform enrichment analysis. **A** Illustration of classes of isoform changes that can occur during transcription and pre-mRNA processing. **B**. Number of significant isoforms according to the alternative transcription event seen when comparing TuMV infected and mock plants, or aphids+TuMV and aphid infested plants. **C**. Consequence of the alternative isoforms based on the categories presented across the top of each grid. The Y axis shows number of isoforms influenced and the X axis indicates the gain or loss according to the category.

Fig. 8. Isoform enrichment of four genes. Colored bars represent isoforms indicated in the legends for each graph. X axis indicates the environmental or hormonal challenge affecting isoform accumulation. Y axis indicates transcripts per million.