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A network approach to investigating the role of the *Sorghum bicolor* transcription factor SbWRKY86 in promoting plant resistance to aphids

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The thesis of Armando Vazquez is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

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ABSTRACT OF THE THESIS

A network approach to investigating the role of the *Sorghum bicolor* transcription factor SbWRKY86 in promoting plant resistance to aphids

by

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Plants defend themselves against pathogens and pests by mounting complex responses involving many defensive genes, proteins and metabolites. These immune responses are often activated by transcription factors that regulate expression of genes required for immunity. *Melanaphis sacchari* (Sugar cane aphid, SCA) has become a major

pest to *Sorghum bicolor* (sorghum), posing a threat to this livestock feeding and biofuel producing plant. Recently, a sorghum transcription factor, *SbWRKY86*, was identified through a genome-wide association study as being associated with SCA resistance and was subsequently shown to confer aphid resistance when ectopically expressed in both *Nicotiana benthamiana* and *Arabidopsis thaliana*. However, how *SbWRKY86* promotes aphid resistance was not understood. Here, we investigated the resistance phenotype further through transcriptomic profiling of transgenic *Arabidopsis thaliana* lines heterologously expressing *SbWRKY86* as a fusion with yellow fluorescent protein and under the control of the Cauliflower Mosaic Virus (CaMV) 35S constitutive promoter (35S*::SbWRKY86-YFP* lines). Genes that were differentially expressed in 35S*::SbWRKY86-YFP* lines were extracted and used to probe a predicted protein-protein interaction (PPI) network. The integrated analysis identified potential network components associated with *SbWRKY86* function. Specifically, a network of genes related to carbon/nitrogen metabolism, production of the cell wall polymer callose, and heat shock proteins were upregulated in these lines. Visualization of callose deposits in the leaves of 35S*::SbWRKY86-YFP* plants demonstrated increased frequency as compared to wild type in response to the immunostimulatory microbe-associated molecular pattern flg22. These results suggest that *SbWRKY86* may in part mediate resistance to *M. sacchari* through regulation of callose production.

1. Introduction

Plants face a variety of threats to their survival, ranging from biotic stresses such as pathogens and pests to abiotic stresses such as extreme temperatures and drought. These challenges cause damage, leading to crop losses each year and contributing to food shortages and decreased food security (Savary *et al.*, 2019). One biotic stress that has contributed to the damage of multiple crop species is the invasion and feeding by the insect Aphidoidea (Aphid). Aphids cause direct physical damage through the destruction of vascular tissue and photosynthetic cells (Al-Mousawi *et al.*, 1983), and the introduction of harmful toxins into the plants (Fouche *et al.*, 1984). Aphids can also transmit plant pathogens (Gray *et al.*, 1991), and siphon important plant nutrients directly from the phloem sap (Schobert *et al.* 1989). These aphid-related stresses result in stunting of plant growth, reduction of crop production (Burd *et al.*, 1992), and increasing the plant's susceptibility to abiotic related stresses such as drought (Riedell *et al.*, 1989). However, plants are not helpless, and have evolved an arsenal of defenses to combat against feeding insects like aphids. To protect themselves from biotic stresses, plant immune systems incorporate physical barriers against aphid feeding (Luna *et al.*, 2011). Additionally, nonprotein amino acids like L-canavanine disrupt herbivore protein function and specialized metabolites such as tannins have been shown to provide defense against herbivores through direct toxicity (Rosenthal *et al.*, 2001, Luna *et al.*, 2011, Bernays *et al.*, 1980). Plants also produce a variety of defensive proteins such as proteinase inhibitors, that help combat against some feeding herbivores and their digestive enzymes (Brown *et al.*, 1984). Understanding the dynamic immune system is a prerequisite to developing sustainable solutions to combat pathogens and pest, such as using genetic tools to modify

plants and increase their resistance to these attackers (Oliva *et al.,* 2019) or even identifying metabolites that can increase resistance (Bleeker *et al.,* 2012).

Sorghum (*Sorghum bicolor*) is grown widely throughout the southeastern U.S. and primarily used as a biofuel feedstock in the production of ethanol and as livestock feed (Dahlberg *et al.,* 2019). The goal of reducing carbon emissions has led to a rise in the usage, production, and research into biofuels such as ethanol from multiple crop plants. Among these, sorghum has emerged as a dominant biofuel candidate for growth in the U.S. due to its ability to grow on marginal lands and in drought conditions that are unsuitable for food crop production (Fargioni *et al.,* 2008). Sorghum is also widely used in livestock feed within the U.S., costing less to produce than corn (Ronda *et al.,* 2018). Given these factors, sorghum is an important grain for food stability and energy diversification within the U.S. (Ronda et al., 2018). Recently sugarcane aphid, *Melanaphis sacchari* (Zehntner) (Hemiptera: Aphididae), emerged as a pest of sorghum in North America (Brewer et al., 2016). First reported in the U.S. in 2013, *M. sacchari* caused devastating losses in the Southeast and persists as an economically important pest (Bowling *et al.,* 2016). By 2015 *M. sacchari* was present in 17 states which together are responsible for 98% of the total domestic sorghum crop production (Bowling *et al.,* 2016). Sugarcane aphids damage sorghum plants through direct feeding, siphoning away important nutrients and leading to the severe health decline of the plant (Bowling *et al.,* 2016). Sugarcane aphids also damage sorghum plants through the buildup of honeydew that they excrete onto the foliar surface, which leads to proliferation of sooty mold that grows on the honeydew (Narayana *et al.,* 1975). The sooty mold decreases the foliar surface area exposed to sunlight, reducing the

plant's photosynthetic capacity and potentiating damage (Bowling *et al.,* 2016). Overall, damage caused by sugarcane aphid can lead to losses of up to 50% in grain yields (Bowling *et al.,* 2016).

As sugarcane aphid is now distributed throughout the U.S. regions where sorghum is cultivated and remains a damaging pest, there is a growing need to investigate potential sources of natural aphid resistance in sorghum. One method of investigation is by probing aphid resistance across a population of genetically diverse sorghum lines through Genome-Wide Association Studies (GWAS) to identify genetic loci associated with aphid resistance. This method has been used previously within sorghum to identify loci associated with resistance to pathogens such as *Colletotrichum sublineola* (Prom *et al.,* 2019). GWAS are useful to identify an association between genetic variation, often single nucleotide polymorphisms (SNPs), and phenotypes (Prom *et al.,* 2019). After genotyping a population of many hundreds of individual cultivars of a given crop species, this population is then evaluated for phenotypes of interest, such as resistance to a specific disease, pest, or pathogen to identify associations between SNP markers and phenotypes. Statistical analyses of phenotypic data in combination with SNP positions can reveal SNPs with significant statistical association with a specific phenotype like pest resistance and pinpoint specific chromosomal loci (Prom *et al.,* 2019).

2. Introduction to Preliminary Results

2.1 Association Mapping for SCA resistance in sorghum

Previously in the Huffaker lab a GWAS study was conducted to identify SNPs associated with sugarcane aphid resistance in sorghum (SCA; Fig. 1). 696 sorghum

lines from two large sorghum association panels, the Sorghum Association Panel (SAP) and the Sorghum Bioenergy Association Panel (BAP), were used for mapping (Fig. 1). A unified set of SNPs for both sorghum association panels was assembled by Drs. Geoffrey Morris & Zhenbin Hu, Colorado State University. SCA resistance in a field setting was assessed for all lines in Tifton, Georgia in 2017 by Dr. Xinzhi Ni, USDA-ARS. Total SCA damage (1-9 ranking system) was used as a mapping trait (Sharma *et al.*, 2012) and TASSEL 5.0 association software was used with both GLM and unified MLM models to analyze data (Bradbury *et al.*, 2007). Out of 528,052 SNPs genotyped from all the lines, 99,189 were polymorphic in at least 85% percent of the population. Results of the GWAS revealed a single locus on chromosome 9 associated with SCA resistance in sorghum (Fig. 1a). Using a Bonferroni adjusted p-value threshold of $p < .05$, the associated locus interval was found to contain approximately 40 genes coding for proteins including an F-box protein, a ubiquitin hydrolase, a WRKY transcription factor, and a PP2C (Fig. 1b, 1d). The most highly associated SNPs were found within the coding region of the WRKY transcription factor, SbWRKY86 (Sobic.009G238200; Fig. 1c). This study identified a single unique mapping interval on chromosome 9 associated with SCA resistance. To identify possible candidate genes associated with SCA resistance in sorghum located within this newly identified locus on chromosome 9, further transcriptional analysis experiments were performed.

2.2 Identifying aphid-induced genes with the SCA Resistance locus

Transcriptional analysis through RNA-seq allows for the simultaneous quantification of relative gene expression for all detectable transcripts in a sample (Kurkurba *et al.,* 2015). In this case, RNA-seq was performed on sorghum plants infested with SCA; differentially expressed genes within the chromosome 9 locus were examined, with the gene encoding *SbWRKY86* found to have the greatest increase in expression of all genes in the loci 48 h after SCA infestation (Fig. 2a). In a previous study it was reported that *SbWRKY86* was expressed exclusively in the resistant sorghum genotype exposed to aphid herbivory (Kiana and *Szczenpaniec et al.,* 2018). Expression of genes in this locus was compared with expression data for maize genes that are syntenic orthologs (Fig. 2a). RNA-seq data from maize infested with *Rhopalosiphum maidis* (corn leaf aphid) revealed expression of only one orthologous gene, GRMZM2G063880, to be strongly induced by aphids (Fig. 2c). Intriguingly, GRMZM2G063880 is the syntenic ortholog of SbWRKY86 (Fig. 2d). Expression patterns uncovered through these transcriptional studies provide evidence to prioritize *SbWRKY86* as the candidate gene involved in SCA resistant in sorghum.

The WRKY is a large plant-specific family of transcription factors that have been shown to regulate multiple responses to abiotic and biotic stresses across plant species (*Wang et al.,* 2018; Gao *et al.,* 2020). For example, in Arabidopsis, *AtWRKY33* is upregulated in response to plant infection with the fungal pathogen *Botrytis Cinerea* (Botrytis) and mediates increased resistance to the fungal pathogens Botrytis and *Alternaria Brassicicola* (Alternaria) using *AtWRYK33* overexpression Arabidopsis lines, while *AtWRYK33* mutant lines showed decrease resistance to both pathogens.

(Zheng *et al.*, 2006). In contrast, *AtWRKY38* and *AtWRKY62* are negative regulators of defense; overexpression of these genes in Arabidopsis reduced basal plant defenses against the bacterial pathogen *Pseudomonas syringae* strain DC3000 (Pst DC3000) (Kim *et al.*, 2008). WRKY transcription factors have been shown to act through both the Jasmonic Acid (JA) and the Salicylic Acid (SA) pathways (Li *et al.*, 2006), two major signaling pathways involved in plant responses to both biotic and abiotic stresses (Feys *et al.*, 1994; Palva *et al.*, 1994). The widespread role of WRKY family transcription factors in plant defense supports a role for *SbWRKY86* in contributing to aphid resistance.

2.3 Using heterologous expression systems to probe the role of *SbWRKY86* in SCA resistance

To examine a potential role for SbWRKY86 in aphid resistance, the gene was cloned from BTx623, the genome-sequenced sorghum line, and heterologously expressed in *Nicotiana benthamiana* with the constitutive cauliflower mosaic virus 35S (CaMV-35S) promoter. Expression of 35S*::SbWRKY86-YFP* in *N. benthamiana* through agrobacterium-mediated transient transformation was used to test the role of *SbWRKY86* in resistance to *Myzus persicae* (Green Peach Aphid, GPA) proliferation. *N. benthamiana* plants transiently expressing 35S*::SbWRKY86-YFP* that were infested with a single adult GPA for 7 days, had a reduced GPA numbers compared to aphids grown on plants carrying an empty vector (Fig. 3a). Expression of SbWRKY86 was visualized by western blotting, showing that the protein was expressed for the complete duration of the GPA bioassay (Fig. 3b). *SbWRKY86* was also heterologously expressed

in Arabidopsis plants, a tractable heterologous expression system for probing the function of genes under different experimental conditions (Wang *et al.,* 2020, Yesilirmak *et al.,* 2009), following Agrobacterium-mediated stable transformation of 35S*::SbWRKY86-YFP*. Three independent transgenic Arabidopsis lines, 10, 13, and 14, constitutively expressing 35S*::SbWRKY86-YFP* were generated. Expression of SbWRKY86 was visualized by western blotting demonstrating successful heterologous expression in all independent transgenic lines (Fig. 3c). Transgenic plants expressing SbWRKY86 were more resistant to aphids following a 4-day infestation with a single adult GPA for each plant, showing a significant reduction in aphid proliferation in the transgenic lines compared to wild-type plants (Fig. 3d). Both results further support that *SbWRKY86* confers enhanced aphid resistance, however the mechanisms underlying this resistance are not yet known.

3. Materials

3.1 Plant Materials and Growth Conditions

Arabidopsis thaliana seeds were sterilized and were stratified by incubation for 48 hrs at 4°C and then germinated in plates containing half-strength Murashige and Skoog (MS) media (Murashige and Skoog 1962; PhytoTechnology Laboratories). The pH was adjusted to 5.8 using KOH with 0.8% phytagel (PhytoTechnology Laboratories). Arabidopsis were kept in plates or transferred to BM2 soil. *Nicotiana benthamiana* seeds were germinated in BM2 soil supplemented with 20-20-20 General purpose fertilizer (Jack's Professional, https://www.jrpeters.com/). Arabidopsis and *N. benthamiana* plants were cultivated in a room at 22 ± 2 °C, 16 h light, 150 µmol m –2 s

–1 /8 h dark, or 12 h light, 150 µmol m –2 s –1 /12 h dark. A colony of *Melanaphis sacchari* originated from Kettleman City, California was obtained from Dr. Spencer Walse (USDA-ARS, Parlier CA) and grown on 3- to 6-week-old sorghum plants. The tobacco-adapted red lineage of *Myzus persicae* (Ramsey *et al.*, 2014) was obtained from Dr. Georg Jander (Boyce Thompson Research Institute) and grown on 4- to 6-week-old *A. thaliana* ecotype Columbia-0 (Col-0) plants.

To generate constructs, the coding region of *SbWRKY86* (Sobic.009G238200) from the Sorghum bicolor line BTx623 was amplified from cDNA via PCR and cloned into the pENTR/D-TOPO vector (Invitrogen). Gateway cloning using the pGWB441 destination vector was used to create overexpression cassettes driven by the Cauliflower Mosaic Virus (CaMV) 35S promoter and producing a C-terminal yellow fluorescent protein (YFP) fusion (Nakagawa *et al.*, 2007). Constructs were confirmed by Sanger DNA sequencing and transformed into *Agrobacterium tumefaciens* strain GV3101 (pMP90). Arabidopsis Col-0 plants were transformed with *A. tumefaciens* carrying the 35S*::SbWRKY86-YFP* expression construct by the floral-dip method (Clough and Bent 1998), and successful transformants were selected on half strength MS media supplemented with 100 µg mL -1 kanamycin. All experiments used plants from homozygous T_2 and T_3 generations, confirmed expressing SbWRKY86-YFP protein through western blotting. For transient expression of SbWRKY86-YFP in *Nicotiana benthamiana*, leaves were infiltrated with *A. tumefaciens* carrying the vectors as described (Sparkes et al. 2006). Experiments using these plants were

performed 24 h post-agroinfiltration and SbWRKY86-YFP protein expression was confirmed through western blotting.

3.2 Preparation and Processing of Arabidopsis RNA-seq data

For the study of altered basal gene expression in the Arabidopsis lines heterologously expressing 35S::*WRKY86-YFP*, plants were grown for 25 days. Two insertional lines, 35S::*SbWRKY86-YFP* lines 13 and 14, were used for comparison to Col-0 wild type as a control. Biological replicates consisted of individual leaves from four separate plants pooled together. Wild-type Col-0 plants were used as controls. Total RNA was isolated with the Spectrum Plant Total RNA kit (Sigma) and DNAse-treated using the Turbo DNA-free kit (Ambion). Preparation of RNA-seq data was performed by Novogene Corporation (Novogene, UC Davis, USA). RNA quality and quantity was analyzed using the Agilent Bioanalyzer 2100 (Agilent technologies, Santa Clara, CA, US) and a cDNA library was constructed and sequenced using an Illumina HiSeq platform PE150 to generate paired end reads that were used for analysis. The raw RNA-seq reads were deposited to the NCBI Sequence Read Archive (SRA) and are available under the BioProject accession number PRJNA786556. Bioinformatics analysis of Arabidopsis RNA-seq data was performed by Novogene Corporation. Raw reads were filtered for adaptor contamination, to eliminate reads with more than 10% uncertainty and more than 50% low quality nucleotides, followed by alignment using HISAT2 (v2.0.5, -dta – phred 33) (Kim *et al.,* 2015). Featurecounts (v1.5.0-p3, default settings) was used to generate read counts from aligned files (Liao et al. 2014).

Different gene expression analysis was performed using DESeq2 (v1.20.0) and the P values were adjusted using the Benjamini and Hochberg's method (Love *et al.,* 2014).

3.3 Network of analysis of differentially expressed genes in transgenic 35S*::SbWRKY86-YFP* Arabidopsis lines

Differentially expressed genes were defined as >2 fold-change upregulated or downregulated genes and adjusted P value < 0.05. A list of 87 differentially expressed genes, that overlapped between both transgenic lines was generated and ran through STRING-db (Szklarczyk *et al.,* 2019). The STRING Protein–Protein Interaction Networks Functional Enrichment Analysis program was used to infer a protein interaction network for the protein products of this gene list. This network was imported into Cytoscape for further annotation, analysis and visualization (Shannon *et al.,* 2003). The log2(fold-change) values of 35S*::SbWRKY86-YFP* lines 13 were used as a gradient for the interior color of the node. R metap package was used to combine p-values through Fisher's method (Dewey *et al.,* 2020). The negative $\log_{10}$ of these combined p-values of the genes was used a gradient for the color of node border. Node size was assigned by degree of connectivity. Nodes with no degree of connectivity, 41 total, within the network were excluded. Potential hub genes with a high degree of connectivity were identified within the network using cytoHubba plugin in Cytoscape (Chin *et al.,* 2014). After filtering and annotating the nodes, the network was investigated for genes associated with plant biotic/abiotic defense (Fig. 4).

3.4 Quantification of callose deposition in Arabidopsis plants

For the callose leaf deposition assays 10-12 day old seedlings of wild-type Col-0 and transgenic 35S*::SbWRKY86-YFP* lines 13 and 14 Arabidopsis plants were used.

The plants were treated with water or 1µM flg22 for 24 h. The leaves were then placed in 98% (v/v) ethanol for 48 h to clear the chlorophyll and once the leaves become transparent, they were placed in 70% (v/v) ethanol. After washing three times using distilled water, leaves were stained for 3 to 4 h in 150 nM K 2 HPO 4 (pH 9.5) containing 0.01% (v/v) Aniline Blue (Sigma-Aldrich). The leaves were mounted on slides using 50% (v/v) glycerol and callose deposits were visualized by microscopy using an ultraviolet (UV) filter in Nikon Eclipse microscope. Callose spots were counted per mm 2 of leaf tissue on the adaxial side of each clip-caged leaf segment using ImageJ (Schindelin *et al.,* 2012). Trainable Weka Segmentation (TWS) plugin for ImageJ was used to analyze leaf images (Arganda-Carreras *et al.,* 2017). Leaf images were scaled to mm per pixel and areas were calculated for each leaf image. TWS was used to calculate the total number of callose particle counts on the leaf itself. Callose deposits per millimeter squared were produced for each leaf as described in (Mason *et al.,* 2020). For each transgenic line, leaves were treated with flg22 or with DI water as a control, and two trials were run with 4 leaves for each line per trial. One-way ANOVA and an unpaired t-test were performed using GraphPad Prism version 8.0.1 for Windows, GraphPad Software, San Diego, California USA, www.graphpad.com, to compare control and treated leaves of the 35S*::SbWRKY86-YFP* 13 and 14 lines vs the Columbia 0 (Col-0) wild type control and treated leaves with a total of n=4 for each treatment condition. Callose deposits per millimeter squared was used in this comparison. GraphPad Prism v 8.0.1 was also used to generate bar graphs and show statistical significance for the callose quantification data.

4. Results

4.1 Transcriptional profiling of 35S*::SbWRKY86-YFP* expressing Arabidopsis lines to identify mechanisms contributing to conferred resistance to aphid attack.

The primary function of WRKY transcription factors in mediating stress responses across different plant species is through transcriptional upregulation of genes involved in stress mitigation. To further investigate how heterologous expression of SbWRKY86 might enhance aphid resistance, we generated transgenic 35S*::SbWRKY86-YFP* lines in Arabidopsis thaliana. We performed RNA-Seq transcriptional profiling of basal gene expression patterns in two independent 35S*::SbWRKY86-YFP* transgenic lines, Sb-13 and Sb-14, in comparison to control Col-0 wild-type plants (Fig. 4). In total, 171 and 334 differentially expressed genes (DEGs) were significantly upregulated and 178 and 48 were significantly downregulated in Sb13 an Sb14, respectively, compared to Col-0. DEGs were identified in both transgenic lines compared to WT plants as defined by a fold change in expression of two or more. A total of 87 genes were differentially expressed in both Arabidopsis transgenic lines compared to WT, with 23 significantly downregulated and 64 significantly upregulated.

The list of 87 overlapping DEGs were used to more precisely characterize the transcriptional response of Arabidopsis plants heterologously expressing 35S*::SbWRKY86-YFP* and to identify associated immune-related processes. First, the list of 87 DEGs was analyzed using String-db (Szklarczyk *et al.,* 2019) to compose an initial protein-protein interaction (PPI) network which was further annotated and analyzed in Cytoscape (Fig. 4). The PPI evidence of associated from String-db was

used to further narrow down the gene lists, leaving the network with 46 genes total (Fig. 4). These genes were prioritized based on their interconnections within the PPI network to further explore interactions between the gene protein products.

Identification of hub genes and potentially important genes in PPI networks within Cytoscape have been identified through the usage of degree of connectivity in previous studies (Chen *et al.,* 2019). This allowed for the prediction of key patterns and hub genes within the Arabidopsis 35S*::SbWRKY86-YFP* induced PPI network by using the degree of connectivity of each protein and their expression within the network. Through this method of network analysis, multiple patterns and possible important genes within the network were identified with putative roles in plant immune responses against aphids. These genes include Heat Shock Proteins (HSPs), genes related to Carbon/Nitrogen response, genes related to the deposition of the cell wall polymer callose, and genes generally related to immunity (Fig. 4). Specifically, we identified Heat Shock Proteins (HSP81-3), (HSP70-4), (HSP70-3), that were upregulated and present in the PPI-Network. Genes involved in Carbon/Nitrogen plant responses such as Protein Exordium-like1 (PHI-1), ARABIDOPSIS TOXICOS EN LEVADURA 31 (ATL31, CARBON/NITROGEN INSENSITIVE 1 (CNI1)), and Protein QQS (QQS) were also upregulated (Fig. 4) (Schröder *et al.,* 2011, Li *et al.,* 2015, Engelsdorf *et al.,* 2013). Additionally, the degree of connectivity for ATL31 was among the highest in the network. Along with ATL31, other genes tied to callose production and the formation of cell wall appositions, a major form of defense for multiple plant species

against aphid feeding (Hao *et al.*, 2008, Rubiato *et al.*, 2022), such as SYNTAXIN OF PLANTS 122 (SYP122) were upregulated (Fig. 4).

4.2 Quantification of *SbWRKY86*-mediated effects on callose deposition

Callose deposition is an important plant defense response against aphids, used to block phloem-feeding insects from access to sieve elements (Will *et al.*, 2006). We hypothesized that one mechanism by which *SbWRKY86* could enhance plant resistance to aphids was through upregulation of genes involved in callose deposition. To test this hypothesis, callose deposition was quantified in leaves of Arabidopsis 35S*::SbWRKY86-YFP* lines 13 and 14 compared to wild type. Callose deposition was triggered using a bacterial-derived pathogen-associated molecular pattern (PAMP) flg22 and was quantified as previously described as callose deposit per mm^2 of leaf tissue on the adaxial side of each leaf (Mason *et al.*, 2020). An image of a flg22-treated leaf was used to establish a classifier for identifying callose depositions using ImageJ and TWS. This initial classifier was generated by creating two classes of items, callose deposits to be quantified on the leaf image and the rest of the leaf structures that were not callose. These items were manually annotated within TWS and used to train the classifier. If the classifier was insufficient in differentiating between callose deposits and leaf structures, then the classifier was further trained by increasing the number of items annotated in the initial leaf image. TWS allows for the visualization of what the classifier algorithm is denoting as a callose deposit in the image, thus allowing for quality control checking of the classifier. Once the classifier was deemed sufficient to quantify callose deposits in the leaf image accurately, it classifier was used

to automatically quantify callose depositions amongst all other leaf images. Differences in leaf size were accounted for in TWS by cropping the leaf images to only contain the leaf surface area. This area was then used to normalize each leaf for callose deposits in the measured area. Increased callose deposition was observed in the *SbWRKY86*-expressing lines compared to wild type, indicating a role in callose regulation for *SbWRKY86* (Fig. 5a,b).

5. Discussion

5.1 GWAS identification of *SbWRYK86*

Through GWAS, a unique mapping interval on chromosome 9 associated with SCA resistance was identified (Figure 1). This study identifies a new possible locus in Sorghum associated with resistance to this aphid pest. Previously, GWAS has been used to identify genes associated with Arabidopsis resistance to green peach aphid, *Myzus persicae*. This led to the identification of the *WRKY22* gene being responsible for Arabidopsis susceptibility to green peach aphid (Kloth *et al.,* 2016). GWAS has also been previously used in maize to identify multiple O-methyl transferase genes related to resistance to corn leaf aphid, *Rhopalosiphum maidis* (Meihls *et al.,* 2013). Within this sorghum study, GWAS was used to identify the mapping interval on chromosome 9, and then further transcriptional analysis was performed on genes within the mapping interval in the presence of SCA infestation (Fig. 1,2). *SbWRKY86* was the gene with the greatest increase in expression 48h post infestation (Fig. 2). Expression of *SbWRKY86* was significantly higher in SCA-resistant sorghum lines as compared to susceptible lines. Together this endogenous expression data indicated that

SbWRKY86 was potentially the candidate gene contributing to sorghum aphid resistance.

5.2 Investigation of a role for *SbWRKY86* in contributing to aphid resistance

To further investigate *SbWRKY86* contributions to aphid resistance, heterologous expression experiments were performed in both Arabidopsis and *N. benthamiana*. Within both plants species, heterologous expression of 35S*::SbWRKY86-YFP* resulted in an increased resistance to aphids, supporting the role of *SbWRKYK86* in SCA resistance in sorghum (Fig. 3). As a family, the WRKY transcription factors have been well documented to be involved in defense responses against diverse attacking organisms. Members of the WRKY transcription factor family have been shown to be responsible for resistance to fungal pathogens, bacterial pathogens, and aphids (Zheng *et al.,* 2006; Kim *et al.,* 2008; Kloth *et al.,* 2016). Silencing of the *WRKY53* gene in wheat (*Triticum aestivum* L.) led to increased aphid reproduction on silenced plants (Eck *et al.,* 2010). Overexpression of a WRKY transcription factor (*CmWRKY48*) in *Chrysanthemum morifolium* (chrysanthemum) lines led to a reduction of aphid reproduction (Li *et al.,* 2015). The reduced aphid numbers observed in our study is comparable to the CmWRKY48 and provides additional evidence that members of the WRKY TFs can mediate aphid resistance within multiple plant species. Considering the role that WRKY transcription factors play in regulating the expression of defense genes, and our results showing that *SbWRKY86* can confer resistance against aphids in Arabidopsis, *N. benthamiana*, (Fig. 3a, 3d, 6a) the evidence suggests that *SbWRKY86*-mediated aphid resistance is conserved across diverse plant species. These results

provide supporting evidence that modulating expression of the *SbWRKY86* transcription factor could be an effective strategy for increasing crop resistance to insects like aphids.

5.3 Transcriptomic analysis of transgenic Arabidopsis lines expressing *SbWRKY86* identify potential mechanisms for regulation of aphid resistance

Within the network of genes that were differentially expressed in 35S*::SbWRKY86-YFP* compared to wild type, multiple heat shock proteins, (HSP81-3), (HSP70-4), (HSP70-3), were upregulated (Fig. 4). HSPs are known to play a major role in Arabidopsis' response to abiotic stresses such as light stress and heat stress. HSPs are also potentially involved in some capacity for plant defense, as they are induced in the presence of viral infection in plants such as *Nicotiana benthamiana* (Chen *et al.,* 2008, Scarpeci *et al.,* 2008). Some HSPs, such as *HSP90* have been shown to be essential in pathways related to *Macrosiphum euphorbiae* (potato aphids) resistance within tomato (*Solanum lycopersicum*; Bhattarai *et al.,* 2007). It has also been shown that the hsp20-like gene *SLI1* is responsible for reducing *Myzus persicae* (green peach aphid) feeding efficiency in Arabidopsis through restriction of phloem sap ingestion (Kloth *et al.,* 2017). It is known that aphid infestation of plants causes oxidative stress; *Brachycorynella asparagi* (Mordv.) aphid infestation of *Asparagus officinalis* L. results in increased hydrogen peroxide levels and the generation of Superoxide Anion Radicals (Borowiak-Sobkowiak *et al.,* 2016). This increased oxidative stress eventually leads to plant growth inhibition, yellowing, reduced crop output, and eventual death of the plant (Borowiak-Sobkowiak *et al.,* 2016). Previously, heat shock proteins (HSPs)

have been shown to reduce and protect against the effects of oxidative stress in plants (Downs *et al.,* 1999). It is possible that *SbWRKY86*-mediated upregulation of heat shock proteins can protect against oxidative stress caused by aphid infestation. Protection against oxidative stress by HSPs could contribute to this SCA resistant phenotype.

Within the PPI network, multiple Carbon/Nitrogen Metabolism related genes were seen upregulated such as Protein EXORDIUM-like 1 (PHI-1), ARABIDOPSIS TOXICOS EN LEVADURA 31 (ATL31), and Protein QQS (QQS) (Fig. 4). PHI-1 has previously been seen to play a role in carbon allocation and was induced by carbon starvation (Schröder *et al.,* 2011). QQS regulates carbon and nitrogen metabolism in Arabidopsis and other plant species (Li *et al.,* 2015). Previously (ATL31) and other members from the ATL gene family were observed as upregulated in response to Microbe-associated molecular patterns (MAMPs) such as flg-22, suggesting a possible role in pathogen response and plant defense in Arabidopsis (Maekawa *et al.,* 2012). ATL31 also contributes to regulation of carbon/nitrogen metabolism (Sato *et al.,* 2009). Carbon/Nitrogen metabolism critically affects defense responses in plants; increased carbon levels can lead to increased defense responses and viral resistance in tobacco plants (Herbers *et al.,* 2000). Carbon availability in the form of sugars and metabolism is important for plant defense, as reduced carbon availability has been shown to cause reduced callose response, reduced defense responses, and reduced Hypersensitive cell death, leading to decreased plant resistance to pathogens (Essmann *et al.,* 2008). Nitrogen metabolism and availability in the form of amino acids has also

been shown to affect plant resistance; increased nitrogen availability resulted in enhanced resistance of potato to *Alternaria solani*, but increased susceptibility to *Phytophthora infestans* (Mittelstraß et al., 2006). As aphids feed directly from the phloem of the plant, they siphon away valuable resources like sugars and amino acids, thus altering the C/N ratio and availability in the plant (Schobert *et al.*, 1989). *SbWRKY86* regulation of multiple carbon/nitrogen metabolism related genes could affect the plant's allocation of sugars and amino acids, potentially preventing the aphids from accessing these nutrients needed as a food source and thereby negatively impacting the aphid's ability to reproduce on these plants. *SbWRKY86*-mediated changes in carbon/nitrogen metabolism could also affect activation of defenses to improving plant outcome during aphid infestation. The availability of carbon in the form of sugars is important for other defense related mechanisms such as the formation of callose as physical barriers to aphid penetration which require these carbon-based compounds for creation.

5.4 Expression of *SbWRKY86* associated with significant increases in callose deposition

In addition to regulating carbon/nitrogen metabolism, ATL31 has also been shown to increase callose response within Arabidopsis in response to flg22 (Maekawa *et al.,* 2012). Based on our transcriptomic analysis and the role of callose deposition in resistance to aphids we hypothesized that callose deposition could be upregulated by *SbWRKY86* expression. Two independent Arabidopsis transgenic lines expressing 35S*::SbWRKY86-YFP,* were tested for flg22-induced callose deposition compared to

WT Col-0 plants. The results showed that flg22-induced callose deposition were significantly higher in 35S*::SbWRKY86-YFP* lines compared to WT plants (Fig. 5). Callose deposition was also evaluated in sorghum lines susceptible (PI597950) and resistant (PI656043) to SCA after three days of SCA infestation. The resistant sorghum line had statistically significant increases in callose deposits as compared to the susceptible line (Fig. 6a). This increased callose response could be tied to a *SbWRKY86*-mediated effects on expression of a sorghum ortholog of ATL31, as overexpression of ATL31 in Arabidopsis also increased callose levels (Maekawa *et al.,* 2012). ATL31 and it's known interactor SYP122 mediate the formation of papillae in response to fungal pathogen infection (Maekawa *et al.,* 2012). Papillae, also known as cell wall appositions, are cell wall structures formed by plants in response to pathogens to physically prevent cell wall penetration. Papillae are predominantly composed of callose, and contain phenolics, reactive oxygen species (ROS) and cell wall structural proteins (Bayles *et al.,* 1990). The formation of papillae facilitated by ATL31 and SYP122 could also function as feeding deterrent against aphids. Callose plays a vital role in plant's defense against invading pests by providing a physical barrier to impede feeding from herbivores such as aphids (Varsani *et al.,* 2019). Aphids feed on plants through their stylet which penetrates the plant's sieve tube to feed on the sugars and nutrients contained in the phloem (Will *et al.,* 2015). Callose deposition can block sieve elements, preventing further aphid feeding (Will *et al.,* 2006). This can lead to a reduced survival rate for these aphids that are unable to obtain the nutrients necessary for reproduction and survival. Previously, increased callose deposition has been

associated with aphid resistance in both soybean and maize (Yao *et al.,* 2020; Varsani *et al.,* 2019). Together this suggests that increased callose deposition mediated by *SbWRKY86* may be a contributing factor to conferred aphid resistance.

6. Future directions in *SbWRKY86*'s role in aphid resistance

The data obtained in this study provides evidence supporting a role for *SbWRKY86* in plant resistance to aphid attack and elucidates several potential mechanisms by which this may occur. The GWAS and transcriptomic results highlighted *SbWRKY86* as a likely candidate contributor to aphid resistance. Additionally, the results provide other potential genes that could be involved in response to aphids. Through the usage of the FarmCPU GWAS method, the identification of additional significant SNPS located near Sobic.001G055100 (F-box domain containing protein), Sobic.004G287000 (SEC14 ctyosolic factor family protein), and Sobic.010G156100 (GDSL-like Lipase/Acylhydrolase superfamily protein). The data is the first of its kind exploring SCA resistance in sorghum and can pave the way for future experiments investigating sorghum aphid resistance. Additional support for the role of *SbRYK86* as the gene contributing to SCA resistance in sorghum was provided by the heterologous expression experiments demonstrating aphid resistance in both Arabidopsis and *N. benthamiana*. However, additional experiments in sorghum using *SbWRKY86* knock-out lines and stable overexpression lines will be necessary to provide more conclusive evidence for the role of *SbWRKY86* in aphid resistance. Transcriptomic and PPI-network analyses of transgenic 35S*::SbWRKY86-YFP* showed an upregulation of multiple genes that could have a potential role in resistance to aphids. Additional work

using method such as T-DNA or CRISPR-Cas9 knockouts, miRNA or virus-induced gene silencing, or transgenic expression of gene candidates with follow up aphid trials could provide some insight into how important these genes are for aphid resistance in different plant models. In this study we showed that transgenic expression of 35S*::SbWRKY86-YFP* in Arabidopsis increased both the expression of genes regulating callose deposition, and levels of callose deposition, which can contribute to aphid resistance.

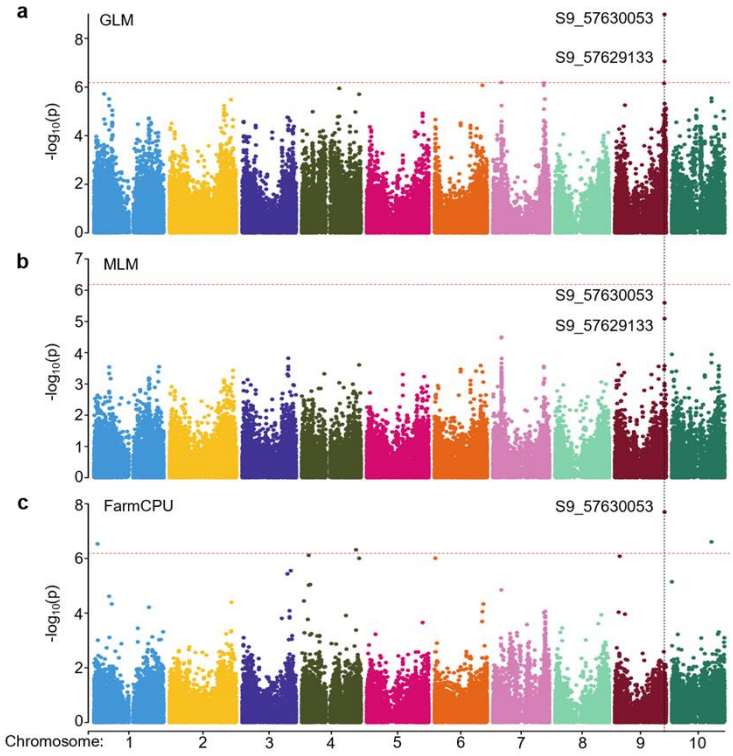
7. Conclusions

This study furthers the investigation of *SbWRKY86*, a gene candidate that was identified through GWAS mapping by measuring SCA resistance across two sorghum mapping populations. The data suggests that *SbWRKY86* contributes to SCA resistance in sorghum. Additionally, the data uncovers several mechanisms by which SCA resistance may be conferred and brings up the possibility of the transcriptional regulation of genes involved in heat shock responses, Carbon/Nitrogen metabolism and callose response as potential candidates for regulation of the SCA resistance phenotype. The identification of these potential mechanisms through the PPI network provides a good basis of investigation to explore further. *SbWRKY86* was shown to increase callose response within Arabidopsis and preliminary data indicates this may occur in sorghum as well. Further investigation of *SbWRKY86*'s effect and potential action mechanisms can provide new insight into aphid resistance within sorghum and can lead to the discovery a multifaceted defense response responsible for this

resistance. This can also provide greater insight into the defense mechanisms employed by sorghum to defend against herbivores.

All results within this thesis have been included in published material as it appears in Poosapati S, Poretsky E, Dressano K, Ruiz M, Vazquez A, Sandoval E, Estrada-Cardenas A, Duggal S, Lim JH, Morris G, Szczepaniec A, Walse SS, Ni X, Schmelz EA, Huffaker A. A sorghum genome-wide association study (GWAS) identifies a WRKY transcription factor as a candidate gene underlying sugarcane aphid (Melanaphis sacchari) resistance. Planta. 2022 Jan 12;255(2):37. The thesis author was a co-author of this paper.

Figures



d

Method	Chr	Position	-log10(P)	Gene ID	Description
GLM	9	57630053	8.99	Sobic.009G238200	WRKY family transcription factor
GLM	9	57629133	7.06	Sobic.009G238200	WRKY family transcription factor
GLM	7	9746662	6.19	Sobic.007G082300	BTB/POZ/MATH-domains containing protein
MLM	9	57630053	5.60	Sobic.009G238200	WRKY family transcription factor
MLM	9	57629133	5.09	Sobic.009G238200	WRKY family transcription factor
FarmCPU	9	57630053	7.70	Sobic.009G238200	WRKY family transcription factor
FarmCPU	10	45992029	6.61	Sobic.010G156100	GDSL-like Lipase/Acylhydrolase superfamily
FarmCPU	1	4120345	6.53	Sobic.001G055100	F-box domain containing protein
FarmCPU	4	62932125	6.32	Sobic.004G287000	SEC14 cytosolic factor family protein

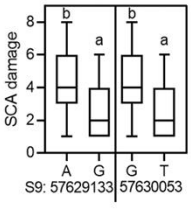


Figure 1. GWAS reveals a single locus on chromosome 9 associated with SCA resistance in sorghum. GWAS performed to identify loci associated with sugarcane aphid (SCA) damage ratings in sorghum. (a-c) Series of Manhattan plots displaying significance of association between single-nucleotide polymorphisms (SNPs) and ordered quantile normalized transformation of scored SCA damage ratings in both sorghum association panels, SAP and BAP. (a-c) The x-axis represents chromosomal locations, and the y-axis represents the -log10 (P values) for genotypic associations obtained between three different algorithm models. The horizontal line represents the threshold of significance for Bonferroni adjusted genome-wide significance ($P < 0.05$). The vertical line represents the top significant SNPs associated with SCA on chromosome 9, which were all present in Sobic.009G238200, a gene encoding SbWRKY86. (d) Table of most statistically significant SNPs associated with the SCA damage rating and nearest corresponding genes detected for each model. (e) Box-plots showing the distribution of the raw sugarcane aphid (SCA) damage score for variants of the two SNPs (S9_57629133, S9_57630053) significantly associated with SCA damage. Different letters (a,b) represent significant differences ($P < 0.05$) as determined by a pairwise Student's t test.

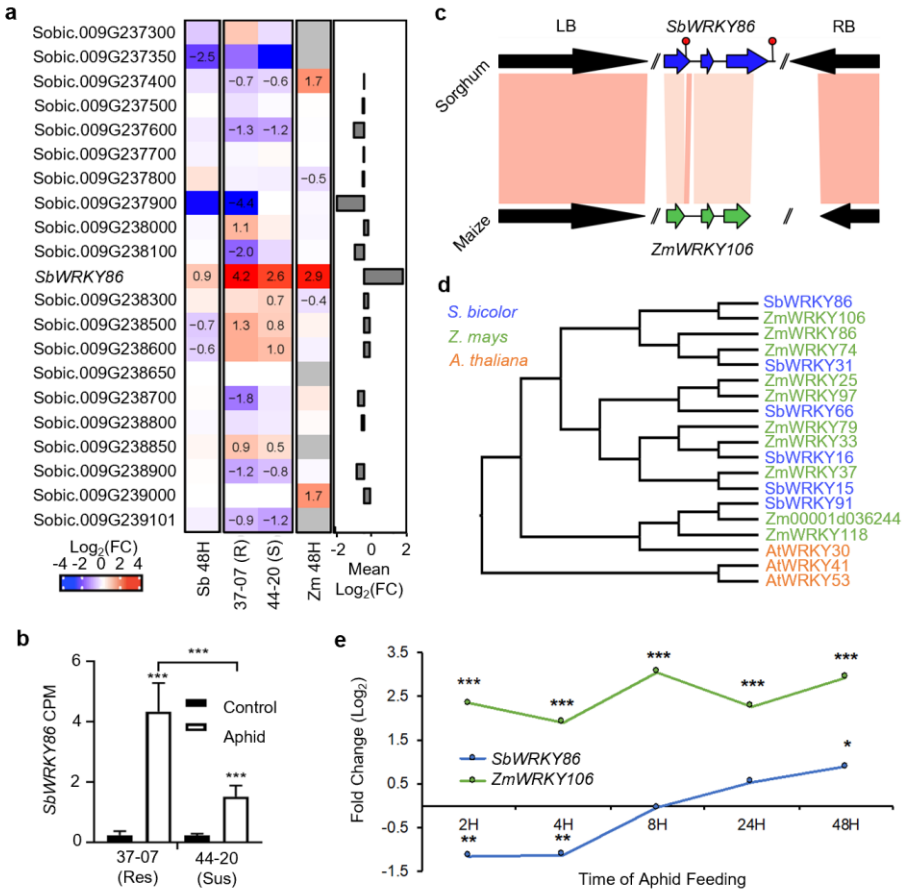


Figure 2. SbWRKY86 found to have the greatest increase in expression of all genes in the chromosome 9 loci 48 h after SCA infestation in Sorghum. (a) Heatmap of RNA-seq gene expression data with cell values represented as Log2 (Fold Change, FC) as compared to controls. (a) Sb 48H column represents data obtained in this study for gene expression in sorghum line BTx623, 48 h post-infestation with SCA as compared to un-infested control plants. The center two columns represent sorghum expression data obtained 24 h post-SCA infestation in an SCA-resistant line, 37-07 (R) versus an SCA-susceptible line, 44-20 (S) (Kiani and Szczepaniec et al. 2018). Column labeled Zm 48H represents expression data from maize for syntenic orthologous genes contained in the corresponding region of the maize genome; expression values are fold change 48 h post-infestation with corn leaf aphid as compared to un-infested leaves (Tzin et al 2015). (b) RNA-seq expression of SbWRKY86, expressed counts per million reads mapped (CPM), 24 h post-SCA infestation versus un-infested controls in the SCA-resistant line, 37-0 (Res) and SCA-susceptible line, 44-20 (Sus) (Kiani and Szczepaniec et al. 2018). (c) Comparison of sorghum SbWRKY86 with locus the corresponding maize syntenic orthologue ZmWRYK106, including the neighboring genes upstream (LB- left border) and downstream (RB- right border) for reference. (d) Phylogenetic Tree of clade containing SbWRKY86 obtained from the complete tree of predicted WRKY proteins based on PlantTFDB annotation of the sorghum (S. bicolor), maize (Z. mays) and Arabidopsis (A. thaliana) proteomes. (e) Comparison of time-course RNA-seq data from sorghum line BTx623 and maize line B73 (Tzin et al 2015) showing the Log2 (Fold Change, FC) in expression for SbWRKY86 and ZmWRKY106 genes post-aphid infestation versus un-infested control plants. Adjusted P-values were calculated for all experiments using the ExacTest edgeR Function (*, **, *** for $P < 0.05$, <0.01, <0.001).

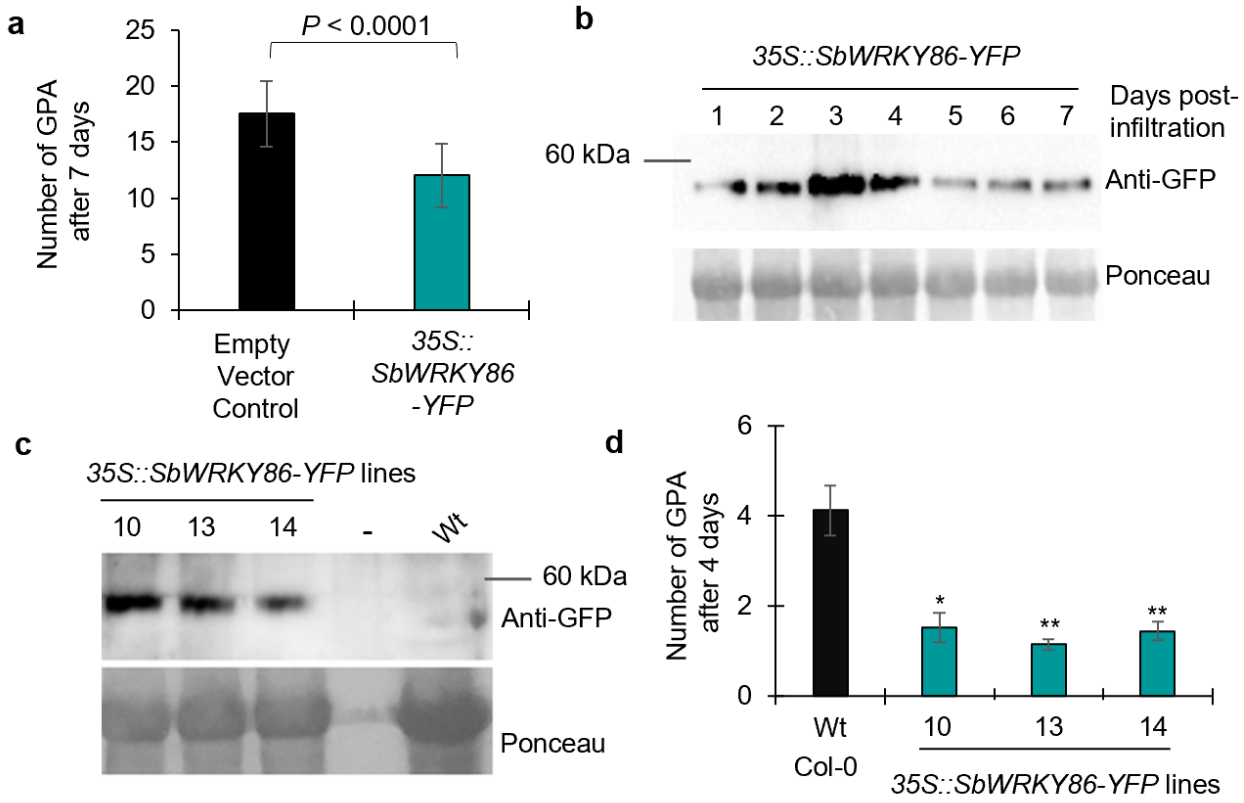


Figure 3. Heterologous expression of SbWRKY86 confers increased resistance to green peach aphid (GPA, Myzus Persicae) in both Nicotiana benthamiana and Arabidopsis thaliana. (a) Mean (N=15, ± SEM) GPA 7 d after N. benthamiana leaves expressing 35S::SbWRKY86-YFP were infested with a single wingless GPA adult confined by a clip-cage. N. benthamiana leaves expressing an empty vector used as control. (b) Expression of SbWRKY86-YFP protein in agroinfiltrated *benthamiana* leaves were confirmed for the GPA bioassay, anti-GFP antibody and Ponceau staining used to confirm equal protein loading. (c) Expression of SbWRKY86-YFP protein in independent lines (10,13,14) of Arabidopsis thaliana expressing 35S::SbWRKY86-YFP detected using anti-GFP antibody and Ponceau staining used to confirm equal protein loading. (d) Number of aphids 4 d post-infestation with a single alate GPA adult for each of the three independent A. thaliana lines expressing SbWRKY86-YFP as compared to wildtype (Wt) Col-0 control plants. (a,d) Error bars represent SEM, n=15 and n =25 for experiments shown in a and d respectively. Single asterisk (*) indicating a significant difference of P < .0002 and asterisks (**) indicating P < .0001 as determined by a Student t-tests (two-tailed distribution, unpaired) within each graph.

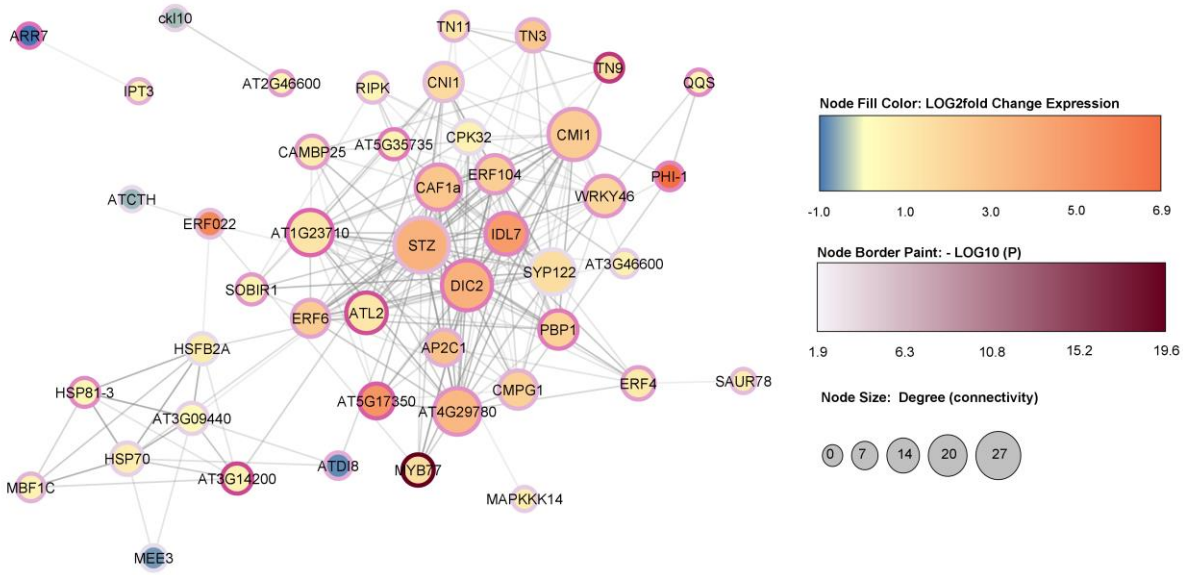


Figure 4. Network analysis of transcriptional changes in SbWRKY86-YFP A.thaliana lines 13,14. This protein-protein interaction network was created using STRING analysis of predicted proteins encoded by DEGs through RNA-seq transcriptional analysis of basal expression in A. thaliana 35S::SbWRKY86-YFP expressing lines 13 and 14 compared to wild type Col-0 plants. Interior node color represents the magnitude of log2 fold-change in expression between Col-0 and SbWRKY86-expressing lines. Color gradient of the node color represents the -log10 (P-value) for statistical significance in differential expression, using a combined P-value from both line's expression data using Fisher's method. The degree of connectivity of each node is represented by the node size. Strength of correlations are represented by the edge width, with a wider edge corresponding to a higher correlation. A list of Arabidopsis gene IDs and annotations associated with the network are detailed Table (1). A list of Log2FC gene expression values for Arabidopsis genes within the network are detailed in Table (1).

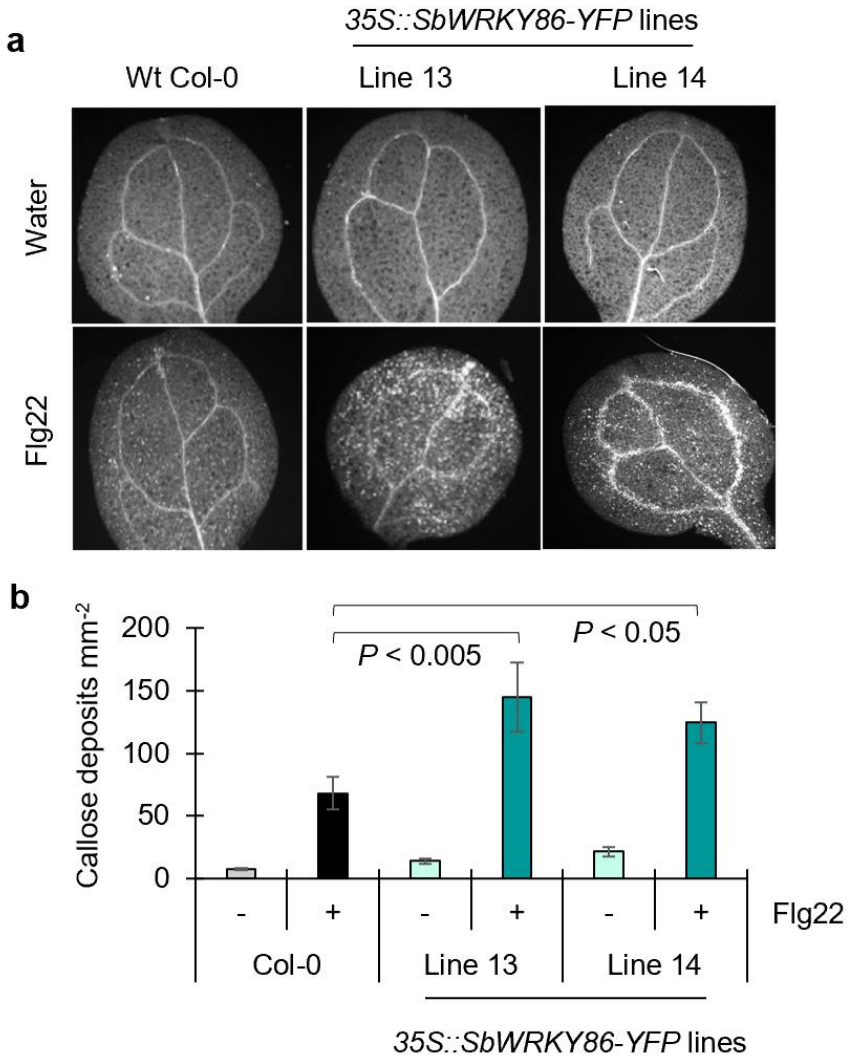


Figure 5. Increased Callose depositions in A. thaliana lines heterologously expressing SbWRKY86-YFP compared to Col-0 (Wt). (a) Callose deposits in leaves of wildtype (Wt) Col-0 and two independent lines of A. thaliana expressing 35S::SbWRKY86-YFP 13 and 14, before and after 24 h after treatment with a 1 µM solution of flg22 or water. (b) Quantification of callose deposits per mm2 of leaf tissue with (+) and without (-) pretreatment of 1µM flg22 were calculated using Trainable Weka Segmentation (TWS) plugin for ImageJ. (N=8; ± SEM), with significant differences between flg22-treated samples determined by pairwise Student's t-test.

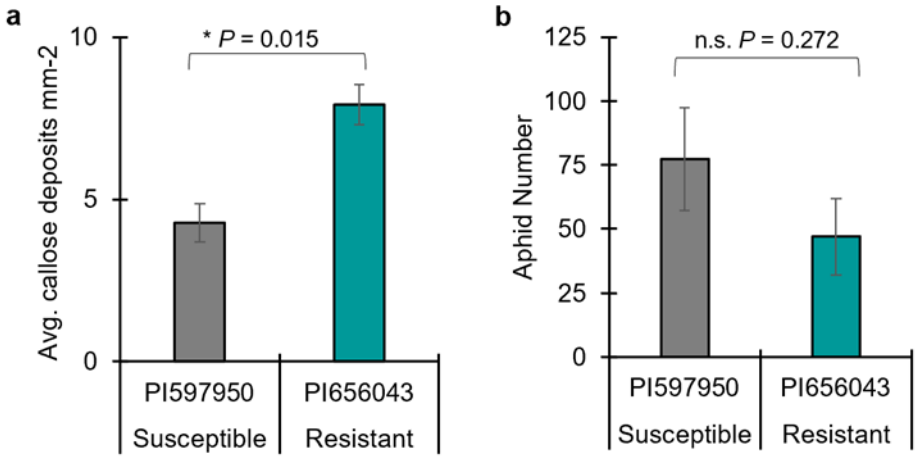


Figure 6. Increased Callose deposition in leaves of SCA resistant Sorghum Line vs susceptible line post SCA infestation. (a) Quantification of the average callose deposits in leaves of the SCA-susceptible line PI 597950 versus the SCA-resistant line PI656043 3 days post-infestation with 10 wingless adult SCA. (b) Count of SCA 3 days post-infestation on the resistant vs susceptible plants used for callose assays. (N=4, SEM) Statistical test used to compare genotypes is pairwise Student's t-test.

Table 1. List of DEGs in both A.Thaliana lines Sb13 and Sb14 heterologously expressing SbWRKY86-YFP, and their respective Log2FC values, gene description, and significance values

Gene ID	Gene Name	Log2FC Sb13	Sb13 P value (adjusted)	Log2FC Sb14	Sb14 P value (adjusted)	Gene Description
AT3G50060	MYB77	1.65	0.041909087	2.70	4.91E-34	Transcription factor MYB77 [Source:UniProtKB/Swiss-Prot;Acc:Q9SN12]
AT3G19030	-	1.96	0.008726283	2.07	1.98E-16	AT3g19030/K13E13_15 [Source:UniProtKB/TrEMBL;Acc:Q9LJ63]
AT1G72920	TIR-NBS9|TN9	1.59	0.025434292	2.13	1.79E-15	Similar to part of disease resistance protein [Source:UniProtKB/TrEMBL;Acc:Q9SSN4]
AT3G16720	ATL2|TL2	1.18	0.004279116	1.91	1.35E-12	RING-H2 finger protein ATL2 [Source:UniProtKB/Swiss-Prot;Acc:Q8L9T5]
AT3G14200	-	0.62	1.44E-05	0.68	3.71E-14	Chaperone DnaJ-domain superfamily protein [Source:UniProtKB/TrEMBL;Acc:Q9LJG5]
AT1G23710	-	1.30	0.005536365	1.98	9.05E-11	At1g23710 [Source:UniProtKB/TrEMBL;Acc:Q9ZUC4]
AT5G17350	-	0.98	6.99E-05	6.11	1.73E-09	Uncharacterized protein At5g17350 [Source:UniProtKB/TrEMBL;Acc:Q9LF49]
AT4G24570	DIC2|UCP4	3.73	0.004279116	4.93	4.12E-09	DIC2 [Source:UniProtKB/TrEMBL;Acc:A0A178URN9]
AT4G27657	-	2.05	0.002000733	2.21	4.16E-09	At4g27657 [Source:UniProtKB/TrEMBL;Acc:Q8LF18]
AT5G35735	-	0.61	0.003577502	0.95	5.86E-09	Cytochrome b561 and DOMON domain-containing protein At5g35735 [Source:UniProtKB/Swiss-Prot;Acc:Q9FKH6]
AT3G44260	CAF1-9|ATCAF1A|CAF1A	2.70	0.047643701	3.36	1.35E-08	Probable CCR4-associated factor 1 homolog 9 [Source:UniProtKB/Swiss-Prot;Acc:Q9LXM2]
AT4G29780	-	3.36	0.020788343	4.45	5.33E-08	Nuclease [Source:UniProtKB/TrEMBL;Acc:Q84J48]
AT5G61600	ERF104	2.36	0.042593606	3.10	9.18E-08	Ethylene-responsive transcription factor ERF104 [Source:UniProtKB/Swiss-Prot;Acc:Q9FKG1]
AT4G27280	KRP1|CMI1	2.40	0.042262335	3.17	1.27E-07	Calcium-binding protein KRP1 [Source:UniProtKB/Swiss-Prot;Acc:O81831]
AT3G10930	IDA-LIKE7|IDL7	4.72	0.00098801	5.80	1.48E-07	Uncharacterized protein At3g10930 [Source:UniProtKB/TrEMBL;Acc:Q84TK4]
AT2G41010	CAMBP25|ATCAMBP25	0.96	0.042262335	1.33	1.55E-07	Calmodulin-binding protein 25 [Source:UniProtKB/Swiss-Prot;Acc:O80683]
AT3G15210	ERF4|ATERF-4|ATERF4|RAP2.5	1.06	0.035055874	1.71	3.16E-07	Ethylene-responsive transcription factor 4 [Source:UniProtKB/Swiss-Prot;Acc:O80340]
AT2G46600	KIC	0.52	0.016553256	0.92	4.39E-07	Calcium-binding protein KIC [Source:UniProtKB/Swiss-Prot;Acc:Q9ZPX9]
AT2G31880	SOBIR1|EVERSHED|EVR	0.68	0.008641071	0.97	4.77E-07	Leucine-rich repeat receptor-like serine/threonine/tyrosine-protein kinase SOBIR1 [Source:UniProtKB/Swiss-
AT3G30720	QQS|DEG6	0.52	0.00070464	0.66	4.77E-07	Protein QQS [Source:UniProtKB/Swiss-Prot;Acc:Q3E7K4]
AT5G16200	-	1.91	0.015921742	2.38	6.55E-07	50S ribosomal protein-like protein [Source:UniProtKB/TrEMBL;Acc:Q9LF08]
AT3G16510	-	1.13	0.034601308	1.57	6.71E-07	At3g16510 [Source:UniProtKB/TrEMBL;Acc:Q9LK74]
AT5G54490	PBP1	2.17	3.19E-05	2.54	7.06E-07	PBP1 [Source:UniProtKB/TrEMBL;Acc:A0A178URF6]
AT4G17490	ERF6|ATERF6|ERF-6-6|ERF6	2.46	0.038115717	3.55	9.00E-07	Ethylene-responsive transcription factor 6 [Source:UniProtKB/Swiss-Prot;Acc:Q8VZ91]
AT1G35140	EXL1|HUP46|PHI-1	6.95	0.000116057	9.92	1.05E-06	Protein EXORDIUM-like 1 [Source:UniProtKB/Swiss-Prot;Acc:Q9C6E4]
AT2G46400	WRKY46|ATWRKY46	1.94	0.001546423	2.25	1.65E-06	Probable WRKY transcription factor 46 [Source:UniProtKB/Swiss-Prot;Acc:Q9SKD9]
AT5G43500	ARP 9	1.10	1.42E-19	0.69	6.01E-06	Actin-related protein 9 [Source:UniProtKB/Swiss-Prot;Acc:Q9LSW2]
AT2G30020	AP2C1	2.96	0.034716625	3.67	6.42E-06	PP2C-type phosphatase AP2C1 [Source:UniProtKB/TrEMBL;Acc:F6LPR5]
AT1G72940	TIR-NBS11|TN11	1.29	0.039402459	1.81	1.31E-05	At1g72940/F3N23_14 [Source:UniProtKB/TrEMBL;Acc:Q9SSN2]
AT3G24500	MBF1C|ATMBF1C	0.67	0.023666886	0.99	1.90E-05	MBF1C [Source:UniProtKB/TrEMBL;Acc:A0A178VDH9]
AT5G56010	HSP90-3|HSP81.3|HSP81-3|ATHSP90-3|ATHSP90.3	0.48	1.80E-05	0.48	1.98E-05	Heat shock protein 90-3 [Source:UniProtKB/Swiss-Prot;Acc:P51818]
AT1G27730	ZAT10|STZ	3.67	0.028099908	4.34	2.53E-05	Zinc finger protein ZAT10 [Source:UniProtKB/Swiss-Prot;Acc:Q96289]
AT1G49780	PUB26	0.85	0.00098801	0.95	2.53E-05	U-box domain-containing protein 26 [Source:UniProtKB/Swiss-Prot;Acc:Q9FXA4]
AT1G66090	TIR-NBS3|TN3	2.67	0.008520412	3.47	2.73E-05	Disease resistance protein (TIR-NBS class) [Source:UniProtKB/TrEMBL;Acc:Q9C515]
AT2G05940	RIPK|PBL14	0.51	0.046103053	0.62	3.63E-05	Serine/threonine-protein kinase RIPK [Source:UniProtKB/Swiss-Prot;Acc:Q9ZUF4]
AT1G66160	PUB20|ATCMPG1|CMPG1	2.23	0.00296368	3.00	9.75E-05	U-box domain-containing protein 20 [Source:UniProtKB/Swiss-Prot;Acc:Q9C8D1]
AT5G10550	GTE2	0.38	0.012249247	0.57	0.000121055	Transcription factor GTE2 [Source:UniProtKB/Swiss-Prot;Acc:Q9LXA7]
AT5G08760	STMP8	0.72	0.000971711	0.68	0.000126774	unknown protein; FUNCTIONS IN: molecular_function unknown; INVOLVED IN: biological_process unknown;
AT1G33760	ERF022	5.65	0.007586913	7.64	0.000313597	Ethylene-responsive transcription factor ERF022 [Source:UniProtKB/Swiss-Prot;Acc:Q9LQ28]
AT2G30040	MAPKKK14	0.87	0.037653304	1.33	0.00172509	Mitogen-activated protein kinase kinase kinase 14 [Source:UniProtKB/TrEMBL;Acc:O64741]
AT3G54830	-	0.64	1.35E-05	0.49	0.00172509	-
AT5G27420	ATL31|CNI1	1.81	0.000687359	2.31	0.003469131	E3 ubiquitin-protein ligase ATL31 [Source:UniProtKB/Swiss-Prot;Acc:Q8LGA5]
AT1G62422	-	0.73	0.032643609	0.87	0.003674183	F2401.15 [Source:UniProtKB/TrEMBL;Acc:Q9MAV1]
AT1G78080	RAP2-4|ATWIND1|WIND1	0.42	0.009051859	0.45	0.005344971	Ethylene-responsive transcription factor RAP2-4 [Source:UniProtKB/Swiss-Prot;Acc:Q8H1E4]
AT1G21550	CML44	1.53	0.01712685	1.81	0.006029273	Probable calcium-binding protein CML44 [Source:UniProtKB/Swiss-Prot;Acc:Q9LPK5]

Table 1. Continued

AT3G46600	SCL30	0.73	0.010817799	1.09	0.006029273	Scarecrow-like protein 30 [Source:UniProtKB/Swiss-Prot;Acc:Q9SNB8]
AT4G25620	-	0.95	0.018437086	0.99	0.006725183	At4g25620 [Source:UniProtKB/TrEMBL;Acc:Q8GYX5]
AT3G09440	HSP70-3	0.33	0.012249247	0.39	0.006871758	Heat shock protein 70 (Hsp 70) family protein [Source:UniProtKB/TrEMBL;Acc:A0A178VI76]
AT5G46490	-	0.64	1.97E-05	0.51	0.00770196	Disease resistance protein (TIR-NBS-LRR class) family [Source:UniProtKB/TrEMBL;Acc:Q9FHF6]
AT5G65010	ASN2	0.41	0.000656323	0.34	0.00964433	asparagine synthetase 2 [Source:TAIR;Acc:AT5G65010]
AT3G12580	MED37C|ATHSP70|HSC70-4|HSP70	0.86	0.007970748	0.87	0.010897131	Probable mediator of RNA polymerase II transcription subunit 37c [Source:UniProtKB/Swiss-Prot;Acc:Q9LHA8]
AT1G72430	SAUR78	0.81	0.000176083	0.64	0.011656754	Auxin-responsive protein SAUR78 [Source:UniProtKB/Swiss-Prot;Acc:Q9C9E1]
AT5G62020	HSFB2A|AT-HSFB2A	0.98	0.046191385	0.97	0.01477171	HSFB2A [Source:UniProtKB/TrEMBL;Acc:A0A384KDZ5]
AT3G63110	IPT3|ATIPT3	0.58	2.40E-05	0.46	0.014924908	IPT3 [Source:UniProtKB/TrEMBL;Acc:A0A178V8J2]
AT3G50800	-	3.01	0.007730045	3.96	0.016421153	Uncharacterized protein At3g50800 [Source:UniProtKB/TrEMBL;Acc:Q9SVL8]
AT3G52400	SYP122|ATSYP122	1.63	0.035129778	2.06	0.018258273	Syntaxin-122 [Source:UniProtKB/Swiss-Prot;Acc:Q9SVC2]
AT5G03380	HIPP06|ATHMP43	0.69	0.000651135	0.52	0.022553876	Heavy metal-associated isoprenylated plant protein 6 [Source:UniProtKB/Swiss-Prot;Acc:Q9LZF1]
AT3G21150	BBX32|ATBBX32|EIP6	1.67	0.031365617	2.03	0.023458279	B-box zinc finger protein 32 [Source:UniProtKB/Swiss-Prot;Acc:Q9LJB7]
AT1G33600	-	0.60	2.18E-05	0.40	0.026842183	Leucine-rich repeat (LRR) family protein [Source:UniProtKB/TrEMBL;Acc:Q9FW48]
AT3G57530	CPK32	0.66	0.022042245	1.31	0.027472896	Calcium-dependent protein kinase 32 [Source:UniProtKB/Swiss-Prot;Acc:Q6NLQ6]
AT1G01120	KCS1	0.40	0.045131831	0.39	0.038779875	3-ketoacyl-CoA synthase [Source:UniProtKB/TrEMBL;Acc:A0A178W124]
AT4G32060	MICU	0.30	0.012818338	0.35	0.040536673	Calcium uptake protein, mitochondrial [Source:UniProtKB/Swiss-Prot;Acc:Q9SZ45]
AT1G75750	GASA1	0.43	0.016016368	0.45	0.04569351	GASA1 [Source:UniProtKB/TrEMBL;Acc:A0A178W4S5]
AT4G02410	LECRK43|ATLPK1|LPK1|LECRK-IV.3	0.52	0.006673464	0.61	0.049975425	L-type lectin-domain containing receptor kinase IV.3 [Source:UniProtKB/Swiss-Prot;Acc:O81292]
AT4G19240	-	-7.64	2.03E-08	-7.63	7.95E-09	Uncharacterized protein AT4g19240 [Source:UniProtKB/TrEMBL;Acc:O49681]
AT1G19050	ARR7	-0.97	7.45E-06	-1.01	6.76E-07	At1g19050 [Source:UniProtKB/TrEMBL;Acc:Q2HIJ6]
AT3G62820	-	-0.76	7.45E-06	-0.59	0.004915022	Plant invertase/pectin methylesterase inhibitor superfamily protein [Source:UniProtKB/TrEMBL;Acc:Q9LZI3]
AT4G13577	-	-1.12	7.45E-06	-1.32	5.90E-07	-
AT3G55500	EXP16|EXPA16|ATEXP16	-1.83	7.87E-06	-0.93	0.040356219	Expansin-A16 [Source:UniProtKB/Swiss-Prot;Acc:Q9M2S9]
AT5G66400	RAB18|ATDI8	-0.81	3.13E-05	-0.69	0.004850226	RAB18 [Source:UniProtKB/TrEMBL;Acc:A0A178UN24]
AT2G42190	-	-0.43	0.000101177	-0.39	0.001218002	Expressed protein [Source:UniProtKB/TrEMBL;Acc:O48526]
AT3G53980	-	-2.13	0.001140638	-2.06	0.001233263	At3g53980 [Source:UniProtKB/TrEMBL;Acc:Q9M329]
AT1G64390	ATGH9C2|CH9C2	-0.40	0.001427431	-0.44	0.013214753	Endoglucanase 6 [Source:UniProtKB/Swiss-Prot;Acc:Q42059]
AT1G69040	ACR4	-0.49	0.00224545	-0.57	1.04E-05	ACT domain repeat 4 [Source:UniProtKB/TrEMBL;Acc:F4I0I7]
AT1G10470	-	-0.30	0.002729651	-0.25	0.04171285	Two-component response regulator ARR4 [Source:UniProtKB/Swiss-Prot;Acc:O82798]
AT1G70420	-	-0.45	0.005771502	-0.52	0.000540353	At1g70420/F17O7_4 [Source:UniProtKB/TrEMBL;Acc:O64594]
AT5G18460	-	-0.50	0.007724588	-0.60	0.001417475	At5g18460 [Source:UniProtKB/TrEMBL;Acc:Q8LFH3]
AT5G45950	-	-0.30	0.007821572	-0.38	0.008137597	GDSL esterase/lipase At5g45950 [Source:UniProtKB/Swiss-Prot;Acc:Q9FJ41]
AT5G24470	APRR5|PRR5	-0.59	0.008799914	-0.71	0.000348272	Two-component response regulator-like APRR5 [Source:UniProtKB/Swiss-Prot;Acc:Q6LA42]
AT2G25900	ATCTH|TZF1	-0.47	0.010797128	-0.43	0.04171285	Zinc finger CCCH domain-containing protein 23 [Source:UniProtKB/Swiss-Prot;Acc:O82307]
AT3G23340	-	-0.38	0.022142088	-0.48	0.003720732	Casein kinase 1-like protein 10 [Source:UniProtKB/Swiss-Prot;Acc:Q9LW62]
AT5G20150	ATSPX1|SPX1	-0.43	0.025375716	-0.56	0.002334948	SPX domain-containing protein 1 [Source:UniProtKB/Swiss-Prot;Acc:Q8LBH4]
AT5G02890	-	-0.58	0.02732768	-0.65	0.005968321	At5g02890 [Source:UniProtKB/TrEMBL;Acc:Q9LYZ6]
AT3G45160	-	-0.43	0.034574613	-0.65	0.032216431	At3g45160 [Source:UniProtKB/TrEMBL;Acc:Q9M1U3]
AT5G15490	UGD3	-0.36	0.034601308	-0.33	0.027455149	UDP-glucose 6-dehydrogenase [Source:UniProtKB/TrEMBL;Acc:A0A178UJW9]
AT2G05380	-	-0.57	0.042262335	-0.72	0.001717837	glycine-rich protein 3 short isoform [Source:TAIR;Acc:AT2G05380]
AT2G21650	ATRL2|MEE3|RSM1	-0.70	0.043860629	-0.69	0.022009625	RSM1 [Source:UniProtKB/TrEMBL;Acc:A0A178VZA8]

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