The response to cabbage looper infestation in Arabidopsis is altered by lowering levels of Zat18 a Q-type C2H2 zinc finger protein*

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ABSTRACT

Q-type C2H2 zinc finger proteins (ZFPs) play a role in plant stress response. Overexpression of individual ZFPs enhances tolerance to abiotic stresses such as drought or salt. Overexpression of ZFP, Zat18 enhances tolerance to drought, whereas plants with lower amounts are less resilient. The effect of Zat18 loss on gene expression has not been characterized. Overexpression of an ortholog of Zat18 in potato, StZFP2 enhances tolerance to infestation by Manduca sexta. This work focuses on two Zat18 mutant lines, a knockout and knockdown. The two lines differ in response to infestation. Typically, a chewing insect induces the jasmonic acid (JA) pathway. While infestation induces JA pathway genes, Zat18 mutant lines downregulate abscisic acid signaling and induce ethylene signaling genes ERF1 and EBF2 as well as salicylic acid response gene NPR3. Knockdown Zat18, cause far more changes in the infestation response compared with Col-0 or the Zat18 knockout line.

Introduction

Enhancing the plant’s ability to withstand or fight off insect pests has been a persistent goal of plant breeders. As there is increasing public concern towards spraying crops with toxic chemicals that can damage the environment, affect the health of farm workers or consumers has also made improving plant resistance within the plant an important aim worldwide. A better understanding of how plant’s balance growth and protection from insect, pathogen or abiotic stresses through pivotal signaling genes may ultimately allow breeders important tools for new tolerant plant varieties.

The plants response to chewing insects results in an increase in the hormones jasmonic acid (JA) and abscisic acid (ABA) (Vos et al. 2019), while attack from necrotrophic pathogens induces JA and ethylene (Eth) (Vos et al. 2019; Yang et al. 2019). Biosynthesis of JA and ABA occurs upon infestation as well as a complex regulatory shift from growth and development to defense (Huot et al. 2014). Several recent reviews are available for more detail on the induction and control of the defense pathway against insects (Erb and Reymond 2019; Yang et al. 2019). For example, JAZ genes are rapidly induced and act to ultimately control the defense response. However, experiments in Arabidopsis deleting multiple JAZ genes demonstrate that the loss of five genes was required to alter the defense response (Guo et al. 2018).

Plant defense from insects, necrotrophic and biotrophic pathogens is induced upon attack and results in changes in the specific regulatory proteins from growth to defense, and whose proteins induce defenses based on levels of signaling molecules regulating the pathway (Huot et al. 2014). For example, when chewing insects attack, JA and ABA are induced which ultimately results in the induction of defense genes such as VSP1 or VSP2 which have been used in Arabidopsis as markers for the JA/ABA defense pathway (Lorenzo et al. 2004). Alternatively, JA and Eth are induced upon attack by necrotrophic pathogens and the common downstream marker for the pathway in Arabidopsis is PDF1.2 (Lorenzo et al. 2003). The hormone salicylic acid (SA) is induced upon attack by biotrophic pathogens (Backer et al. 2019) results in the induction of defense proteins such as PR1 (pathogen gene response gene 1).

Q-type C2H2 Zinc finger proteins (ZFPs) containing two zinc fingers are often induced during environmental stress (Kielbowicz-Matuk 2012; Xie et al. 2019). Q-type refers to the zinc finger domains containing an invariant QALGGH motif within their two zinc fingers. The zinc fingers bind to promoter motifs within genes regulated by the specific ZFP. These genes were first discovered in petunia by Takatsuji et al. (1992). Arabidopsis has 18 Q-type ZFPs with two zinc fingers, acting as transcription factors (Ciftci-Yilmaz and Mittler 2008). Another important domain in these Q-type ZFPs is the EAR domain named for ethylene-responsive element-binding factor (ERF)-associated amphiphilic repression (Sakamoto et al. 2004). As a negative regulator of transcription, the EAR motif is an active repressor of genes bound to the zinc fingers (Kagale and Rozwadowski 2011). Overexpression has been associated with increases in tolerance to stress (Xie et al. 2019). Recently, overexpression of the ZFP, Zat18 enhanced tolerance to drought stress (Yin et al. 2017). The Zat18 mutant lines (from T-DNA insertions at the Salk institute) were also less tolerant than wildtype Arabidopsis (Yin et al. 2017). The authors also studied the difference between wildtype and the over-expressor line under control and drought stress conditions using RNA-Seq, with an eye to identify a suite of genes that might enhance the plant’s ability to tolerate water loss.
Recently, we have shown that an ortholog of Zat18, StZFP2 overexpressed (OE) in potato results in lower larval weight in *Manduca sexta* when feeding on OE-StZFP2 (Lawrence and Novak 2018), thus providing a slightly enhanced tolerance to this insect pest. The effect of Zat18 mutant lines with different amounts of expression was subjected to infestation by the chewing insect *T. ni* to determine how the loss of Zat18 affects gene expression. The opportunity to test the Salk T-DNA Zat18 mutant lines versus the wildtype Col-0 upon infestation was performed and RNA-Seq was used to identify the genes differentially expressed in wildtype and mutant lines during the early stages of attack.

**Materials and methods**

**Plants**

ArabiPots (9 × 9 × 125 cm, Lehle Seeds) in a 3 × 6 pot flat configuration (18 pot capacity) were filled with a 75/25 mix of Premier ProMix Germinating Mix PGX (Griffin) and Emerald profile field and fairway calcined clay granules and placed in an irrigation tray filled with water until the soil medium was thoroughly moist. Seeds for Zat18 Salk T-DNA promoter insertion lines 132289C and 027144C, known in this work as 289 or 144, respectively, were purchased at the Arabidopsis Biological Resource Center. Both the mutant lines and wild type Col-0 seeds (Lehle Seeds) were sown on the surface of each pot and watered in with a hose fitted with a misting nozzle. After removing any excess water in the irrigation tray, flats were covered with plastic wrap and humidity dome and placed at 4°C for 4 days to stratify germination and break any dormancy. Flats were transferred to a Conviron growth chamber with a 22°C, 12 h, 150 µmol/m²/s light and 18°C, 12 h dark photoperiod. Plastic wrap was removed after germination. Plants were thinned after the second set of true leaves appeared and domes were removed one week later. Plants were sub-irrigated with 10-15-10 liquid fertilizer only when the soil became dry.

**Insects and infestation**

*Trichoplusia ni*, cabbage looper (CL) eggs were acquired from Benzon Research and reared on a diet until they reached 5th instar (King and Hartley 1985). Six-week-old plants were used for infestation. For control time zero, leaves from three plants of each variety were excised and immediately frozen in liquid nitrogen. For the 1 and 4 h time points, six plants of each variety were excised and immediately frozen in liquid nitrogen using a mortar and pestle. RNA was isolated using TRIzol according to manufacturer’s instructions. Two micrograms of RNA was used to make cDNA with Superscript VILO IV mastermix (Thermo Fisher Scientific). qRT-PCR was performed with 25 ng cDNA, Taqman Gene Expression Assays PR1, VSP2, PDF1.2, ACTIN, ZAT18 (Table 1) and Taqman Universal Master Mix II, with UNG, using the 7500 Real Time PCR System (Thermo Fisher Scientific). The Comparative Ct method was used to calculate transcript abundance for each gene (Schmittgen and Livak 2008). The results are graphed using the ΔΔCt values as ‘mean transcript levels’ with ACTIN as an endogenous control.

**Stranded RNA-Seq**

Purified RNA was submitted to Georgia Genomics and Bioinformatics Core Facility (Athens, GA) for transcriptome analysis by next-generation sequencing. Total RNA was quality controlled by Bioanalyzer or Fragment analyzer to determine RNA concentration and integrity. Sample concentration was normalized in 25 µL of nuclease-free H₂O prior to library preparation. Libraries were prepared using KAPA’s Stranded mRNA-seq kit (#KK4821) with halved reaction volumes. During library preparation, mRNA was selected using oligo-dT beads, the RNA was fragmented, and cDNA generated using random hexamer priming. The number of cycles for library PCR was determined based on kit recommendations of the amount of total RNA used during library preparation. Libraries were quality tested using qubit or plate reader, depending on the number of samples, and fragment analyzer was also used to determine library concentration and the size distribution of the library, respectively. Pooling was done using qPCR concentrations. The final pool was examined by Qubit, Fragment Analyzer, and qPCR for amount and integrity.

**Sequencing**

RNA libraries were prepared for sequencing on Illumina instruments using Illumina’s Dilute and Denature protocol. Pooled libraries were diluted to 2nM, then denatured using NaOH. The denatured library was further diluted to 2.2 pM, and PhiX was added for a 1% library volume. RNA pools were typically run on the NextSeq 550 instruments.

**RNA-seq analysis**

Paired-end reads were pruned for adaptors and quality using BBduk (version 37.66) from the BBTools software suite (https://iigi.doe.gov/data-and-tools/bbtools/bbt-tools-user-guide/bbmap-guide/). The Arabidopsis genome, GCF_000001735.4_TAIR10.1_genomic.fna and corresponding GFF file were downloaded from the NCBI database (https://www.ncbi.nlm.nih.gov). Alignments of paired-end reads to the Arabidopsis genome were performed using HISAT2 version 2.0.5 using default values and 40 threads (Kim et al. 2019). The resulting sam files from HISAT2 were converted to bam files using the samtools (http://samtools.sourceforge.net/). Raw counts of reads mapped to

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**Table 1. Taqman Assays used in this work.**

| Locus   | Gene | Tagman Assay ID |
|---------|------|----------------|
| A2G214610 | PR1  | At02170748_s1  |
| A2G24770 | VSP2 | At02304127_g1  |
| A5G44420 | PDF1.2 | At02315604_g1 |
| A5G99810 | ACTIN | At02335714_g1 |
| A7G35600 | ZAT18 | At02188341_s1  |
genes were extracted from the bam files with the program featureCounts and subread package (http://subread.sourceforge.net/), and a summary table of raw counts was created using Perl and awk scripts. The DESeq2 program from the R statistical software suite uses raw counts as input and performs normalization by calculating a size factor using geometric mean and median (Love et al. 2014). After size factors were calculated to normalize the data, the estimate of dispersion was determined. DESeq2 then uses a negative binomial GLM fitting and Wald statistic in the determination of differentially expressed transcripts. The adjusted value (p-adj) was calculated using the Benjamin–Hochberg correction, where the p−adjusted value < = 0.05 was chosen as the value used in determining statistically significant deferentially expressed genes. Genes with fold change more than 2 or less than –2 and a false discovery rate genome was obtained from The Arabidopsis Information Resource (TAIR) https://www.arabidopsis.org/ by inputting the IDs from the GFF file.

**Statistical analysis of qRT-PCR**

Two-way ANOVA was conducted on the ΔΔCt values (Khan-Malek and Wang 2011) using SAS Proc MIXED (SAS v9.4 2016) to accurately model among-genes and between-treatments. Pairwise means comparisons were conducted using the SLICE option of the LSMEANS statement to compare among times for each gene and treatment and to compare treatments for each gene at each time, using the Sidak method to adjust p-values to protect against obtaining false positive comparisons. Letters indicating significant differences among means were generated using the pdmix800 SAS macro (Saxton 1998).

**Genome ontology and Kegg mapping**

A classification tool was used to identify differences between genotypes and induced or repressed genes upon infestation. The internet tool http://bar.utoronto.ca/ntools/cgi-bin/ntools_classification_supervisor.cgi was used to determine the biological process of genes in each genotype and time of infestation. Only genes with adjusted p-values less than 0.05 were selected and the upregulated genes were >2.0 log2 fold change and downregulated genes were <-2 log2 fold change. One and 4 h up- and downregulated genes were separately plotted because the combination of both was too complex to easily identify differences. Kegg mapping was also used (https://www.genome.jp/kegg/mapper.html).

**Results**

Two Salk T-DNA insertion lines 132289C (289) and 027144C (144) purchased from ABRC (Arabidopsis Biological Resource Center) map to the putative promoter of the Zat18 gene (AT3G53600) (Figure 1(A)). The expression of the Zat18 mutants upon infestation by the chewing insect pest, C. Trichoplusia ni (Hübner) was compared to the wild-type Col-0 (Figure 1(B)). Upon infestation Col-0 was induced dramatically at 1 h. The Zat18 mutant line 289 retains a small amount of Zat18 expression and will be referred to as the knockdown line. Zat18 mutant line 144 is barely detectable using qRT-PCR and will be referred to as the knockout line for Zat18. Classic marker genes were used to compare the response to infestation by chewing insects in Arabidopsis wildtype Col-0 and the Zat18 knockout and knockdown lines. Taqman assays used for qRT-PCR were purchased (Table 1) for Zat18 and markers for genes downstream of the following defense pathways VSP2 for jasmonic acid/abscisic acid (JA/ABA), PR1 for SA, and PDF1.2 associated with jasmonic acid/ethylene response (JA/Eth).

VSP2 is significantly induced by infestation in all genotypes, and its expression is not influenced by the Zat18 mutants (Figure 1(C)). This suggests that the loss of Zat18 is not affecting the JA pathway. PDF1.2 upon infestation in Col-0 and line 144 is not reduced by infestation, while PDF1.2 is significantly induced in the knockdown Zat18 mutant 289 by 4 h of infestation. Clearly, the lower level of Zat18 but not the knockout line is causing this change. PDF1.2 is downstream of the JA/Eth pathway. PR1 is a downstream gene in the SA pathway. PR1 is repressed in the knockdown line 289 at every time point in comparison with Col-0. While the expression of mutant line 144 is not significantly changed over time, the knockdown mutant 289 is significantly repressed by infestation at 4 h. There is a significant decrease in the expression of PR1 in Col-0 and the mutant lines at 4 h of infestation. However, the knockdown line 289 represses PR1 at all time points and by 4 h reduces its response to infestation. PDF1.2 is induced by infestation in the knockdown line, while PR1 is repressed by infestation in the knockdown line. However, PR1 in both Col-0 and knockout of Zat18 are not induced or repressed by infestation. Consequently, the presence of a low level of Zat18 causes a change in the response to SA and JA/Eth markers, but the knockout line does not. While the JA pathway marker gene, VSP2 is induced upon infestation in all three lines, clearly, a smaller amount of Zat18 alters the response to infestation. RNA-Seq was performed to identify infestation responsive genes in the wildtype Arabidopsis line Col-0 and the Zat18 knockout line 144 and knockdown line 289. Comparisons between qRT-PCR and RNA-Seq results for Zat18 and three classic marker genes verify the reliability of the RNA-Seq results (Figure 1(B-C)).

To get a global picture of the difference between the genes induced and repressed by infestation in wildtype and Zat18 mutant lines, the classification supervisor at Bar Toronto was used to identify gene ontology (GO) terms. GO terms were identified, plotted for upregulated and downregulated genes at 1 and 4 h of infestation in Col-0, 289 and 144 (Figure 2). Although there are additional GO terms for Biological Process, only those that were significantly different in at least one comparison (such as Col-0 4 h versus Col-0 0 h) were plotted. For example, although signal transduction genes in Col-0 at 4 h were present, since the value was not significant the comparison was not plotted. Signal transduction, response to stress and abiotic and biotic stress have the highest values at both 1 and 4 h of infestation (Figure 2). However, signal transduction at 4 h in Col-0 had decreased to 0.92 with a p value of 0.065 while at 1 h the normalized frequency was 2.5. The effect of loss of Zat18 either slowed the expression of genes involved in signal transduction and the stress response or upregulated a different set of genes later in infestation. The knockout line 144 line maintained a higher GO value at 4 h of infestation for signal transduction and general stress response in comparison to Col-0 and the knockdown line 289. Thousand and thirty-three genes were upregulated at 4 h in Col-0 (Figure 2).
Since signal transduction is a large category of upregulated genes upon infestation and affected by Zat18 mutations, it was more closely examined using KEGG mapper (https://www.genome.jp/kegg/mapper.html). Focusing on hormone signal transduction, the RNA-Seq data was sorted for comparison of 0 h to either 1 h or 4 h of infestation in Col-0 and Zat18 lines 289 and 144. KEGG mapper not only identified genes involved in hormone signal transduction but also compared genotype differences at each time point and lowercase letters compare time point differences for each genotype.

**Figure 1.** (A) Map of tDNA insertions in Zat18 mutants 289 and 144. Zat18 transcript is in green. qRT-PCR experiment was analyzed with 3-way Anova. Capital letters compare genotype differences at each time point and lowercase letters compare time point differences for each genotype. (B) Expression of Zat18 upon infestation in Col-0 (green) 289 (yellow) and 144 (blue). Zat18 is induced by infestation within 1 h in Col-0, reduced in knockdown mutant 289 and lost in knockout 144. (C) VSP2 is induced in all phenotypes. No difference in the expression of PDF1.2 or PR1 in Col-0 or knockout mutant 144. PDF1.2 is significantly reduced in the knockdown Zat18 mutant 289 at 0 and 1 h of infestation and significantly induced by infestation at 4 h. PR1 is repressed only in the knockdown line 289 at all timepoints.
transduction, but also placed them onto a pathway map. The infestation response of Col-0 was monitored and then compared to the Zat18 mutant lines. While genes involved in the JA pathway were induced upon infestation, there were no differences in expression between Col-0 and the ZAt18 line 289 and 144 (Figure 3). Seven JAZ genes were enhanced in expression upon infestation in comparison to time 0 h. JAZ1, JAZ3, JAZ5, JAZ6, JAZ9, JAZ10 and TIFY10B were all induced (Figure 3). JAR1 was induced by infestation in all three lines, but the p adj value was 0.07, so was not
considered significantly induced by infestation (Figure 3). Another sign that the Zat18 loss does not affect the JA pathway is by comparing genes involved in JA biosynthesis. Again, Kegg mapper was used to identify genes upregulated by infestation in the synthesis of JA (Table 3). Eight genes were induced in Col-0 at 1 h of infestation: LOX3, LOX4, AOS, AOC1, AOC2, AOC3, OPR3 and OPCL1. By 4 h two additional genes were upregulated, LOX2 and another OPR gene, AT1G09400. ZAT18 knockout line 144 induced the same genes except for AT1G09400. ZAT18 knockdown line 289 induced the same JA biosynthesis genes as 144, with the addition of OPR1 and an OPCL1-like gene AT1G20480. This supports the conclusion that the JA pathway is not drastically altered by the lack of Zat18.

In this work two ABA receptor genes PYL5 and PYL6 were induced in Col-0 and both Zat18 mutant lines (Table 2). By 4 h of infestation ABF1, a transcription factor that binds to downstream ABA response genes was induced in all three lines (Table 2). ERF2 was also induced by infestation in all three lines. The gene RGL3 is induced by infestation (Table 2). RGL3 was induced by infestation. On the other hand, RGL2 another DELLA protein in the GA pathway was repressed by infestation (Table 2).

Alteration of the level of Zat18 affects the response to infestation for several hormone pathways.

Zat18 loss differentially affected ABA signaling. For example, the ABA receptor PYL4 was induced by infestation in 289 (Table 4). PP2C’s, negative regulators of ABA signal transduction, bind to ABA receptors. One of these, ABI5 was induced by Col-0 and 144 at 4 h of infestation, while ABI1 was induced in 289. Several additional ABI-like negative regulators were repressed in 289 at 1 h, such as HA1, HA2, and ABI2. HAB1 was repressed at 1 and 4 h in 289 (Table 4). The transcription factor ABF2 was repressed at 1 and 4 h in 289 only and ABF3 was also repressed at 4 h in 289. In the absence of ABA PP2C repressors bind to SnRK2.3, which was induced in all genotypes at 4 h, with 144 and 289 also induced at 1 h of infestation. SnRK2.8 on the other hand was repressed at 4 h of infestation in both 144 and 289, but not Col-0.

Infestation of 289 also resulted in induction of the SA pathway transcription factor NPR3 and ethylene pathway gene EBF2 (Table 4). The transcription factor ERF1 was induced in 144. Negative regulators of the GA pathway GID1A were induced by 144 and 289 with GID1B also induced in 289 (Table 4). Clearly, lower levels of Zat18 affect the expression of these GA negative regulators. ABA Eth and GA pathways are clearly altered by lower levels of Zat18, while the JA pathway was not affected during infestation.

The auxin pathway is also affected by the loss of Zat18 expression upon infestation. The expression of auxin

Figure 3. Ten genes involved in JA signaling were responsive to infestation. JAZ3, JAZ5, JAZ6, JAZ9 and JAZ12 were induced to a higher level in the knockout line 144 at 1 h of infestation. Col-0 was repressed by infestation at 1 and 4 h. JAR1, JAZ1, TIFY10B and JAZ10 were induced by infestation but not clearly affected by loss of Zat18.
pathway gene DFL2 was induced at 1 h in 289 but only at 4 h in either Col-0 or 144 (Table 5). Once again, the intermediate level of 289 had a greater effect on auxin gene expression than complete loss of Zat18. IAA19 was induced in 144 at 1 h and 289 at 1 and 4 h while it was not induced in Col-0 upon infestation. IAA30 was induced in both Zat18 mutant lines but not in Col-0. On the other hand, GH3.1 was induced in Col-0 and 144, but not in 289 at 1 h. WES1 was induced in the Zat18 mutant lines but not in Col-0. Several auxin pathway genes were also repressed during infestation. For example, AXR3 was repressed in both Col-0 and Zat18 mutant lines but not in 289 at 1 and 4 h while it was not induced in Col-0 and 144 at 1 h. WES1 was induced in both Zat18 mutant lines but not in Col-0. Several auxin pathway genes were also repressed during infestation. For example, AXR3 was repressed in Col-0 and 144 while IAA12 was repressed in the Zat18 mutant lines and not Col-0. Finally, GH3 was repressed in Col-0 and 144 while IAA12 was repressed in both Col-0 and Zat18 mutant lines.

Table 4. Bold genes are induced by infestation italic genes are repressed.

| Pathway | line | 1h | 4h |
|---------|------|----|----|
| ABA     | Col-0 | SnRK2.3 | SnRK2.3 |
|         | 144   | SnRK2.3 | SnRK2.3 |
|         | 289   | SnRK2.3 | SnRK2.3 |
| ABA     | Col-0 | AB1     | AB1     |
|         | 144   | AB1     | AB1     |
|         | 289   | AB1     | AB1     |
| ABA     | 144   | AB1     | AB1     |
|         | 289   | SnRK2.8 | SnRK2.8 |
|         | 289   | HAB1    | HAB1    |
|         | 289   | HAB1    | HAB1    |
|         | 289   | AB2     | AB2     |
|         | 289   | AB2     | AB2     |
|         | 289   | AB3     | AB3     |
|         | 289   | AB3     | AB3     |
|         | 289   | EBF1    | EBF1    |
|         | 289   | EBF1    | EBF1    |
|         | 289   | EBF2    | EBF2    |
|         | 289   | EBF2    | EBF2    |

Table 5. Bold genes are induced by infestation italic genes are repressed.

| Pathway | line | 1h | 4h |
|---------|------|----|----|
| ABA     | Col-0 | DFL2 | DFL2 |
|         | 144   | DFL2 | DFL2 |
|         | 289   | DFL2 | DFL2 |
| ABA     | Col-0 | IAA19 | IAA19 |
|         | 144   | IAA19 | IAA19 |
|         | 289   | IAA19 | IAA19 |
| ABA     | 144   | IAA30 | IAA30 |
|         | 289   | IAA30 | IAA30 |
| ABA     | 144   | GH3.1 | GH3.1 |
|         | 289   | GH3.1 | GH3.1 |
| ABA     | 144   | DH4    | DH4    |
|         | 289   | IA6    | IA6    |
| ABA     | 144   | WES1   | WES1   |
|         | 289   | WES1   | WES1   |
| ABA     | 144   | BRU6   | BRU6   |
|         | 289   | BRU6   | BRU6   |
| ABA     | 144   | AXR3   | AXR3   |
|         | 289   | AXR3   | AXR3   |
| ABA     | 144   | IAA12  | IAA12  |
|         | 289   | IAA12  | IAA12  |
| ABA     | 144   | AT4G13360 (GH3) | AT4G13360 (GH3) |

Discussion

Upon infestation by chewing insects, plants respond by increasing levels of JA and ABA (Erb and Reynold 2019; Vos et al. 2019). JAZ genes downregulate the JA response so that long-term expression of defense genes does not occur. While the plant would be well defended against pest insects, its growth is curtailed and stunted. JAZ genes while unique are somewhat redundant to one another, thus requiring mutation in five JAZ genes before a loss of function line has a measurable difference in response (Guo et al. 2018). RGL3 is a DELLA protein that negatively regulates growth and enhances JA defense (Wild et al. 2012). The expression of negative regulators GID1A and GID1B inhibit the expression of the JA pathway, responsible to growth and development (Huot et al. 2014). This interplay between GA and JA expression in this case enhances the plant response to defense. This may result in an enhanced defense response upon infestation. This interplay between GA and JA expression in this case enhances the plant response to defense.

While this work does not demonstrate a specific gain or loss in the JA response to infestation upon loss of Zat18 expression, it does show an interplay between levels of Zat18 expression and hormonal pathways upon infestation in Arabidopsis. Clearly levels of Zat18 alter the hormonal response to infestation and result in changes in the expression of standard defense genes such as PDF1.2. Over-expression of Zat18 results in enhanced tolerance to drought, and the loss of Zat18 also diminishes the plants recovery from drought (Yin et al. 2017). In our hands, the response of Zat18 mutant lines to infestation did not result in a lower JA response, but affected several other pathways, such as ABA, Eth, GA and auxin suggesting that this C2H2 transcription factor has a more indirect effect on plant gene expression. Perhaps it acts as an intermediary between pathways with net effects on ABA, Eth, GA and Auxin during infestation. Alternatively, insect infestation results in a complex response in the plant, with the physical damage brought on by the insect (DAMPs), the water loss that ensues upon chewing insect damage and any specific microbial (MAMP) or herbivore (HAMP) affects the plant’s response. Given that there was no net change in the JA pathway, but there were several differences between the response to infestation in ABA, Eth, GA and auxin pathways demonstrates the interrelationship between hormonal pathways during chewing insect defense. While it has been demonstrated that alteration in Zat18 expression affects the response to drought (Yin et al. 2017), this work demonstrates its
involvement and the interplay between hormone pathways upon infestation. Another important aspect to this work is the intermediate level of Zat18 expression in the 289 knockdown line seems to have a greater effect on the ABA pathway, causing the inhibition of expression of ABF2, ABF3, HAB1, HAI1, HAI2, ABI2 and ABI3. The intermediate level of Zat18 also results in the induction of the SA pathway gene NPR3 and the induction of the GA pathway repressors GID1A and GID1B.

Conclusion
This work enhances our perception of the role Zat18 plays in response to insect feeding. It also clearly shows that while knockout lines are crucial to understanding the role a gene plays in the plant response, knockdown lines reveal unexpected allelic differences that result from altering expression levels of a C2H2 ZFPs. Arabidopsis has remarkable tools available for researchers, such as the T-DNA insertion lines from the Salk Institute used in this work. Further, the Zat18 overexpression line produced by Yin et al. (2017) could clarify whether enhanced levels of Zat18 improve defense against insect pests.

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