Key Genes in the Jaz Signaling Pathway Are Up-regulated Faster and More Abundantly in Caterpillar-resistant Maize

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Abstract

Jasmonic acid (JA) and its derivatives, collectively known as jasmonates (JAs), are important signaling hormones for plant responses against chewing herbivores. In JA signaling networks, jasmonate ZIM-domain (JAZ) proteins are transcriptional repressors that regulate JA-modulated downstream herbivore defenses. JAZ repressors are widely presented in land plants, however, there is only limited information about the regulation/function of JAZ proteins in maize. In this study, we performed a comprehensive expression analysis of ZmJAZ genes with other selected genes in the jasmonate pathway in response to feeding by fall armyworm (*Spodoptera frugiperda*, FAW), mechanical wounding, and exogenous hormone treatments in two maize genotypes differing in FAW resistance. Results showed that transcript levels of JAZ genes and several key genes in JA-signaling and biosynthesis pathways were rapidly and abundantly expressed in both genotypes in response to these various treatments. However, there were key differences between the two genotypes in the expression of *ZmJAZ1* and *ZmCOI1a*, these two genes were expressed significantly rapidly and abundantly in the resistant line which was tightly regulated by endogenous JA level upon feeding. For instance, transcript levels of *ZmJAZ1* increase dramatically within 30 min of FAW-fed Mp708 but not Tx601, correlating with the JA accumulation. The results also demonstrated that wounding or JA treatment alone was not as effective as FAW feeding; this suggests that insect-derived factors are required for optimal defense responses.

Introduction

During their long history of co-evolution with insects, plants have developed various defense strategies to fight against herbivorous pests. Specific hormones are produced by plants and used as signals to activate defense-related genes thus resulting in specific defense responses (Pieterse et al. 2009). In particular, jasmonic acid (JA) and its derivatives play a dominant role in regulating gene expression responding to mechanical wounding and herbivore attack (Howe et al. 2018; Wasternack and Hause 2013; Zhang et al. 2017). Salicylic acid (SA) (Vlot et al. 2009) and ethylene (ET) (Van Loon et al. 2006) are documented as major plant defense-related hormones as well. In general, SA is pivotal in resistance against piercing and/or sucking insects and biotrophic pathogens, whereas JA and ET are associated with defense against chewing herbivores and necrotrophic pathogens (Glazebrook 2005; Trujillo and Shirasu 2010). In addition to biotic/abiotic stresses, JAs are also involved in other aspects of plant growth including seed development, root growth, tendril coiling, flowering, and senescence (Goossens et al. 2016; Shyu and Brutnell 2015; Wasternack and Song 2016).

The molecular basis of JA biosynthesis and mechanism of JA-regulated defense signaling networks have been extensively reviewed in Arabidopsis (Goossens et al. 2016; Wasternack and Song 2016; Zhang et al. 2017). The synthesis of JA is initiated by releasing α-linolenic acid (α-LA) from chloroplast membrane lipids. In the chloroplast, α-LA is converted to 12-oxo-phytodienonic acid (OPDA) in the sequential reactions catalyzed by lipoxygenase (LOX), allene oxide synthase (AOS), and allene oxide cyclase (AOC). OPDA is then transported to the peroxisome, where this precursor is processed to yield active *cis*-JA, which is easily epimerized to more stable *trans*-JA, through the serial reactions catalyzed by

OPDA reductase (OPR3), OPC-8:0 CoA ligase (OPLC1), and three rounds of β-oxidation. Newly synthesized JA can be further modified to several biological active derivatives by conjugating enzymes upon transport to the cytoplasm, including MeJA that is formed by JA methyltransferase. Among many metabolic conversions of JA derivatives, jasmonoyl-isoleucine ((+)-7-iso-JA-L-Ile, abbreviated as JA-Ile) catalyzed by JASMONATE RESISTANT 1(JAR1) is critical for plant direct defense against herbivore feeding (Erb et al. 2012; Fonseca et al. 2009).

JA perception and signaling require JA-Ile and the help of the ubiquitin-proteasome system for defense gene activation. The current simplified model for JA perception and signaling in responses to stresses is briefly described here. In the resting stage, JASMONATE ZIM DOMAIN (JAZ) (Chini et al. 2007; Thines et al. 2007) proteins repress JA-responsive gene expression by binding to positive transcriptional factors (TFs), such as MYC2 that initiate expression of early JA-responsive genes (Liu et al. 2019). In response to herbivory or wounding damage, JA-Ile accumulates in injured tissues within minutes (Fonseca et al. 2009). This bioactive JA-Ile signal is then perceived by CORONATINE INSENSITIVE1 (COI1), the F-box protein of the SCF^{COI1} E3 ubiquitin-ligase (SKP1/Cullin/F-box protein complex) (Devoto et al. 2002; Xie et al. 1998). The binding of the hormone to COI1 recruits the JAZ proteins, tags them with ubiquitin, and mediates their targeted degradation by the 26S proteasome (Chini et al. 2007; Katsir et al. 2008; Thines et al. 2007). This targeted degradation of JAZ repressors liberates the TFs (i.e., MYC2), thus activating transcription of JA-responsive genes. Activation of gene expression is seen approximately after 1 hr and later followed by metabolic changes (Furstenberg-Hagg et al. 2013). JAZ gene expression is also MYC2 dependent, but it is under negative feedback control. With the accumulation of newly-translated JAZ proteins, repression of JA biosynthesis and the associated pathways is regained (Chini et al. 2007; Chung et al. 2009; Farmer 2007; Pauwels and Goossens 2011; Thines et al. 2007). Other plant hormones, such as abscisic acid (ABA) (Ton et al. 2009), auxins (Kazan and Manners 2009), and gibberellin (GAs) (Navarro et al. 2008) are also reported to function in the regulation of plant defense signaling networks. Crosstalk between these plant hormones is common (Kazan and Manners 2012; Qi et al. 2014; Song et al. 2014), and MYC2 has been reported to mediate signaling crosstalks between JA and other plant hormones, including ABA, ET, GA, and SA (Goossens et al. 2016; Han 2017; Kazan and Manners 2013).

Key components in the JAZ signaling pathway have been well studied in Arabidopsis. Among the 13 AtJAZ genes, some have shown induced expression under insect feeding and wounding (Chung et al. 2008; Howe and Jander 2008; Thireault et al. 2015) with possible functional redundancy (Pauwels and Goossens 2011). In addition to Arabidopsis, JAZ proteins are commonly present in other land plants (Garrido-Bigotes et al. 2019). Evidence shows that the JAZ repressor model is not only critical in regulating biotic/abiotic stress responses, but also extends to aspects of plant growth and reproduction (Howe et al. 2018). JAZ proteins have been largely investigated in many dicots including Arabidopsis (Chini et al. 2007; Thines et al. 2007; Yan et al. 2007), apple (An et al. 2017; Li et al. 2014), cotton (Li et al. 2017; Zhao et al. 2016), grape (Zhang et al. 2012), rubber (Chao et al. 2019; Pirrello et al. 2014; Tian et al. 2010), tomato (Chini et al. 2017; Ishiga et al. 2013), and tobacco (Oh et al. 2013; Oh et al. 2012; Shoji et al. 2008), but far less is known about JAZ function in monocots, except for rice (Hakata et al. 2017;

Taniguchi et al. 2014; Tian et al. 2019; Toda et al. 2013; Wu et al. 2015). Recently, JAZ genes have been found in several other monocot species (Huang et al. 2016; Liu et al. 2017; Sen et al. 2016), such as maize (Han and Luthe 2021; Zhang et al. 2015b; Zhou et al. 2015), Brachypodium (Zhang et al. 2015a), wheat (Wang et al. 2017), and sorghum (Bai et al. 2011). In our previous study, 16 JAZ candidates were identified from the maize genome based on conserved ZIM and Jas domains, and three homologous pairs of ZmJAZs (*ZmJAZ1a/ 1b*; *ZmJAZ2a/ 2b*; *ZmJAZ3-1a/ 3-1b*; Table 1) were cloned and preliminarily characterized for two maize inbreds (Mp708 and Tx601) (Han and Luthe 2021). However, functional characterization of the ZmJAZ genes has not been reported.

Previous research has shown that JA levels are elevated under the challenge of herbivore feeding or wounding in maize plants (Huffaker et al. 2013; Lyons et al. 2013). JA has been shown to regulate the expression of the insecticidal protease Mir1 (maize insect resistance1) in the caterpillar-resistant maize inbred line Mp708 (Ankala et al. 2009; Shivaji et al. 2010). Mp708 originated from Antiguan germplasm and was developed using traditional plant breeding methods to improve resistance against whorl-feeding lepidopteran herbivores including fall armyworm (FAW) and the southwestern corn borer (SCB) (Williams et al. 1990). Mp708 inbreds show superior herbivore resistance against leaf-feeding caterpillars, as well as corn leaf aphids (Louis et al. 2015), and western corn rootworm (Castano-Duque et al. 2017). Quantitative trait loci (QTL) mapping has shown that its resistance to FAW and SCB is due to several insect-resistant QTLs (Brooks et al. 2007; Brooks et al. 2005). Results from Shivaji *et al.* (2010) also indicated that Mp708 had constitutively higher JA levels than Tx601, the susceptible inbred line that was used as a breeding parent for the development of Mp708 (Williams et al. 1990). Consequently, Mp708 appears to be genetically primed to respond to herbivory with a rapid defense response.

Since JA accumulation is induced by herbivory and promotes resistance in maize (Ankala et al. 2009; Shivaji et al. 2010), we propose that JAZ genes play a similar role in maize as in Arabidopsis. In addition, we were interested to know if there are differences in JAZ gene expression between Mp708 and Tx601 that might account for the higher constitutive JA levels and more rapid deployment of defenses. One possible reason could be differences in the JAZ repressor proteins, however, we found no major sequence differences in the selected ZmJAZ genes (Han and Luthe 2021). Consequently, we hypothesized that differences in JAZ gene expression might contribute to the difference in endogenous JA levels. To test this, we performed a comprehensive gene expression analysis of JAZ and JA-related genes in response to treatments that included insect feeding, mechanical wounding, and phytohormone treatments. In addition to JAZ genes, the expression of several primary responses and JA-biosynthesis genes were also tested including COI1, MYC2, LOX1, AOS, and OPR2. JA levels also were measured in both maize inbred lines following selective feeding periods, and temporal and genotype-specific accumulation of JA was observed. Investigation of the temporal induction pattern for JAZ and JA-related genes in response to various conditions in different genotypes revealed a few key differences between the two genotypes that could extend our understanding of insect resistance in maize and provide avenues for further studies.

Materials And Methods

Plant Materials and Insect Rearing. Seeds for the maize inbreds, insect-resistant Mp708, insect-susciptableTx601, and B73 were obtained from W. P. Williams (USDA-ARS Corn Host Plant Resistance Research Unit) at Mississippi State University (Mississippi State, MS, U.S.A.). Three seeds were sown in each 18 L pot filled with topsoil (Hagerstown Loam). All plants were grown in the Plant Science greenhouse at The Pennsylvania State University (University Park, PA, U.S.A.), maintained at the average temperature of 28°C under a 16 hr photoperiod and water as needed. Plants at the V8 to V9 (Ritchie et al. 1984) leaf stage were used for the various treatments (see below), including FAW infestation, wounding and hormone treatments, and JA measurements.

FAW eggs were also obtained from USDA-ARS Corn Host Plant Resistance Research Laboratory at Mississippi State University. Larvae were reared on an artificial diet (Peiffer and Felton 2005) at 27°C with a 16-hr photoperiod until the fifth-instar. For transcript abundance measurements, three to five larvae were starved for 1 hr and carefully placed in the maize whorls. Following FAW infestations, tissue adjacent to the feeding sites was collected at 0, 10 min, and 30 min, 1 hr, 6 hr, and 12 hr after feeding, immediately frozen in liquid nitrogen and stored at -80°C. Experiments were repeated three times with 3-4 plants for each time point and genotype. For wounding experiments, plants were wounded with a specially designed wounding tool (Bosak 2011).

Hormone Applications. To determine the effect of the exogenous hormone on JA-related gene expression, methyl-jasmonate (MeJA) was tested. MeJA (Bedoukian Research Inc., Danbury, CT) was diluted with ethanol to make 10% (v/v) MeJA stock (stored at - 20°C), and a fresh working solution of 0.01% (v/v) was prepared with sterile water before use. Plants at the V8-V9 stage were sprayed with 0.01% MeJA and quickly covered with the sealed plexiglass chamber.

To test the effect of MeJA along with wounding and ethylene (ET), leaves were wounded first, then sprayed with MeJA, ET, or MeJA and ET with proper controls (unwounded, wounded, and wounded and buffer solution only). ET solution was prepared as 3 mM 2-chloroethyl phosphonic acid (Ethephon, Sigma-Aldrich, St. Louis, MO). For controls, plants were sprayed with double distilled water containing the same ethanol concentration as in MeJA working solution. Leaf tissues were then collected after 1 hr and 6 hr and immediately frozen in liquid nitrogen. Experiments were repeated twice with 3 plants for each time point.

Analysis of Jasmonates after FAW Feeding. Following FAW infestations, approximately 100 mg of leaf tissue adjacent to the feeding site was collected at each time point and quickly frozen in liquid nitrogen. Six plants were sampled for each time point and genotype. Jasmonates (JA) were extracted using the vapor phase extraction method described previously by Schmelz et al. (2004). In summary, samples were transferred to pre-weighed tubes containing Zirmil beads (0.9-1.1mm diameter, Saint-Gobain, Le Pontet, FRA), 400 ul of propanol/H_2O/HCl (2:1:0.002), and the internal standard consisting dihydro jasmonic acid and homogenized. Dichloromethane (1 ml) was added to each sample, followed by another homogenization step. The organic phase was transferred and methylated by adding 3 ul of 2 M trimethylsilyl diazomethane in hexane and 200 ul of ether/methanol (9:1). After incubation, the contents

were evaporated under the stream of nitrogen and the volatile was trapped on 30 mg of Super Q adsorbent (80/100 mesh, Altech Associates Inc., State College, PA). The trapped volatiles was then eluted with 150 ul of dichloromethane and analyzed by chemical ionization- gas chromatograph-mass spectrometer (GC-MS, Agilent) fitted with an HP-1MS column (Agilent, 30m x 0.25 mm ID x 0.25 μm film thickness). A more detailed method was described by Bosak (2011). Endogenous JA was identified and quantified by comparing it with the internal standards. The resulting amounts of JA were divided by the fresh weight of each sample.

Identification of Putative Maize COI1 and MYC2 Genes. To identify the putative homologous sequences of COI1 and MYC2 in maize, BLAST search in maize genome database (http://www.maizesequence.org) (Monaco et al. 2014) was carried out using the previously identified sequence as a query. The retrieved sequences were manually confirmed by Pfam (Finn et al. 2014). Synteny information was obtained from Plant Genome Duplication Database (http://chibba.agtec.uga.edu/duplication/#Zea_mays) (Lee et al. 2012), and orthologous sequences were checked from the Rice Orthologous database (http://rice.plantbiology.msu.edu/annotation_pseudo_pog.shtml) (Ouyang et al. 2007). The homologous genes for each species were listed in Supplemental Table 1 (COI1) and Table 2 (MYC2).

Sequence alignments and phylogenetic analysis of maize COI1 and MYC2 genes

Phylogenetic analysis was conduct using MEGA version 6 (Hall 2013; Tamura et al. 2013). The multiple amino acid sequences of COI1 and MYC2 from maize, rice, and Arabidopsis were aligned by the ClustalW, respectively. Each resulting alignment was used for further analysis, and a phylogenetic tree was constructed using the Neighbor-Joining method, with 1000 bootstrap replicates.

RNA Extraction and cDNA Synthesis. Approximately 100 mg of frozen leaf samples were homogenized using the Genogrinder 2000 (SPEX Certi Prep, Metuchen, NJ). Total RNA was extracted using TRIzol Reagent (Invitrogen) and re-suspended in DEPC (diethylpyrocarbonate)-treated water. The RNA was then treated with DNase (Progema Corp., Madison, WI, U.S.A.) to remove contamination from residual genomic DNA. Total RNA was quantified with NanoDrop Spectrophotometer ND-1000 (Thermo Fisher Scientific Inc., Waltham, MA). The cDNA was reverse transcribed from 1 μg of total RNA with ABI high capacity cDNA reverse transcription kit (Foster City, CA, U.S.A.), and 2.5 μM oligo-dT_{20} was used in the standard reaction.

Quantitative RT-PCR (qRT-PCR). Maize gene expression was measured using quantitative RT-PCR (qRT-PCR) carried out in an Applied Biosystems (ABI) 7500 Fast Real-Time PCR machine using SYBR as detection dye. All gene-specific primers for qRT-PCR were designed with Primer Express software (version 3, ABI), listed in Supplemental Table 3. All primers were confirmed in absolute quantification, with a slope of approximately -3.3 and R^2 values close to 1. Each reaction contained with a 10 μl reaction mix and was run in triplicate under the default conditions: 50°C for 2 min, 95°C for 10 min; 95°C for 15 sec, and 40 cycles of 60°C for 1 min; 72°C for 10 min. At the end of each run, a dissociation curve was conducted using the manufacturer's default setting. Actin was used as the reference gene to normalize the variance

among samples. The relative gene expression level was analyzed by ABI 7500 Fast SDS Software (version 1.4), and the fold-changes relative to the control sample were calculated.

Since the high sequence similarity for each JAZ homologous pairs (Supplemental Table 4), no gene-specific primers could be generated to distinguish specific maize JAZ genes (*ZmJAZ1a/1b*, *ZmJAZ2a/2b*, *ZmJAZ3-1a/3-1b*) for qRT-PCR. Therefore, the primer used a sequence conserved in Zm*JAZ1*, Zm*JAZ2,* and Zm*JAZ3*, respectively, and thus detected both of the JAZ homologs. For this reason, the relationship of the homeologous pair in maize is unclear. They could act independently or redundantly, synergistically or competitively.

Statistical Analysis. All qRT-PCR data were analyzed using SAS software version 9.3 (SAS Institute, Cary, NC). Relative expression values were Box-Cox transformed (LaLonde 2005; Osborne 2010) to increase homogeneity. A general linear model (PROC GLM) was then used to assess the gene expression fold-changes. For multiple samples, the least significant difference (LSD) test was used to determine significant differences (α=0.05) among treatments.

Results

JAZ Transcript Levels were Temporally Induced in Maize by FAW Infestation. To explore the potential role of ZmJAZ in the JA-regulated defense response, we first investigated the expression profile of JAZ genes in response to FAW feeding. To establish the timing of the feeding response, gene expression was monitored up to 12 hr after FAW infestation in both Mp708 and Tx601. The early time points (10 and 30 min) were selected because as a primary defense regulator, JAZ genes are known for their rapid induction following mechanical wounding and herbivore feeding in Arabidopsis (Chung et al. 2008; Koo et al. 2009). Figure 1a shows that all JAZ transcripts accumulated in leaves in response to FAW feeding, but their individual expression patterns were different. Overall, *ZmJAZ1* transcripts accumulated to a much greater level than *ZmJAZ2* or *ZmJAZ3* (see y-axis scales in Fig. 1a). In Mp708, *ZmJAZ1* transcripts started to accumulate as early as 30 min after infestation. After a slight dip at 1 hr, *ZmJAZ1* transcript levels increased again at 6 hr and remained high at 12 hr. Although *ZmJAZ1* expression was upregulated in Tx601 upon feeding, there was no peak expression at 30 min and there was no dramatic change throughout the remainder of the time course. Expression of *ZmJAZ1* was significantly higher in Mp708 than Tx601 at 30 min and 6 and 12 hr. Unlike *ZmJAZ1, ZmJAZ2* transcripts did not have the early 30 min peak, but gradually increased to peak at 1 hr in Mp708 and 6 hr in Tx601. However, there were no significant differences in *ZmJAZ2* expression between the two genotypes and its overall expression levels were the lowest of the three JAZ genes tested. *ZmJAZ3* transcripts had similar temporal profiling as *ZmJAZ2* and peak induction was seen at 1 hr for both Mp708 and Tx601, except the expression was significantly higher at 30 min in Mp708. We also tested the JAZ gene expression in another caterpillar susceptible maize inbred line B73 in response to FAW feeding (Supplemental Fig. 1a-c). Similar results were observed as in Tx601, except the expression levels continued to increase at 12 hr. Taken together, these results confirmed that FAW feeding could induce the expression of *ZmJAZ1-3*, and expression of *ZmJAZ1* was regulated differently in Mp708 than in Tx601.

Faw Feeding can Increase Endogenous JA Levels in Maize Leaves. To test the effect of feeding on JA accumulation, time courses of JA production from leaves of Mp708 and Tx601 in response to FAW infestation were measured, and the amounts of active (*cis*-JA), inactive (*trans*-JA), and total JA were determined (Fig. 2). In both inbreds, *cis*-JA (Fig. 2a) levels rapidly accumulated within 10 min of FAW feeding, and Mp708 had a significantly greater induction level than Tx601; *cis*-JA levels increased to approximately 648 and 374 ng/g fresh weight in Mp708 and Tx601, respectively. *Cis*-JA levels kept increasing until a peak was reached at 1 hr, and they dropped significantly at 6 hr. Following herbivory, although *cis*-JA levels were higher in Mp708 than Tx601, they were only statistically different after 10 min of FAW infestation. *Trans*-JA levels were significantly higher in Mp708 at the resting stage but tended to be higher in Tx601 after feeding (Fig. 2b). Interestingly, the ratio of *cis*/*trans*-JA varied in maize inbreds as well. In undamaged control plant, ~50% of the JA from Mp708 leaves was in *trans*-conformation, but within 10 min after insect damage, ~80% of the JA was found in the *cis*-conformation, compared with ~40% and ~60% from Tx601 leaves, respectively. When combined, no dramatic differences in total JA content were seen between the two genotypes, except constitutively (0 min) and 10 min after feeding, where total JA levels were significantly higher in Mp708 (Fig. 2c). In the un-fed control, the constitutive levels of *cis*- and *trans*-JA in undamaged Mp708 leaves (controls) were approximately 132 and 124 ng/g fresh weight, respectively. As expected, these values were 2 to 4-fold higher than those in Tx601, which were approximately 54 and 31 ng/g fresh weight, respectively. Our results supported the finding of Shivaji *et al.* (2010) reporting significantly higher concentrations of both *cis*- and *trans*-JA in undamaged Mp708 leaves. In combination with the qRT-PCR data, the results show *ZmJAZ* expression levels correlate with JA accumulation at the feeding sites. In addition, the ability of Mp708 to rapidly accumulate *cis*-JA as soon as 10 min after infestation may help to account for its increased resistance because it can modulate defenses more quickly.

JA Biosynthesis Genes were Upregulated in Maize by FAW Infestation. As stated previously, elevated JA levels were observed between 10 and 30 min near the caterpillar feeding sites, and remained high at least for an hour. Next, we compared changes in the transcript levels of key genes potentially involved in the JA biosynthesis pathway. *ZmLOX1* (AF271894), *ZmAOS* (AY488135), and *ZmOPR2* (AY921639) were selected because they are the closest homologs in maize compared with the JA biosynthesis pathway in rice and Arabidopsis (Ankala et al. 2013; Zhang et al. 2005). As shown in Figure 3a, *ZmLOX1*, *ZmAOS,* and *ZmOPR2* were up-regulated by herbivore feeding. For *ZmLOX1*, known for its up-regulation by wounding and MeJA (Kim et al. 2003), there was a gradual increase in transcript levels up to 12 hr and there were no significant differences in expression between Mp708 and Tx601. *ZmAOS* is considered a rate-limiting step in JA biosynthesis (Wasternack 2007), and its transcripts increased throughout the feeding periods, and only were significantly higher in Mp708 than Tx601 at 30 min (Fig. 3a). Previous groups have reported that constitutively higher levels of OPDA and JA were observed in Mp708 than Tx601 (Shivaji et al. 2010; Varsani et al. 2019), surprisingly, *ZmOPR2* expression tended to be higher in Tx601 than in Mp708 at all times tested (Fig. 3 and 5). Although *ZmOPR2* expression responded to feeding, it might not be a major contributor to JA biosynthesis upon FAW feeding in Mp708 (Borrego and Kolomiets 2016; Pingault et al. 2021; Zhang et al. 2005) and it was not responsive to MeJA treatment

(Fig. 3b). Again, gene expression was also measured in B73 (Supplemental Fig. 1g-i) and the trends were similar to those in Tx601. Our results agreed with previous reports showing that genes in JA-biosynthesis pathways were under positive feedback regulation upon herbivore feeding (Wasternack and Song 2016).

Identification of COI1 and MYC2 Homologs in the Maize Genome. COI1, an F-box protein, serves as a receptor for JA signaling by binding directly to bioactive JA-Ile and is critical for all JA-mediated responses (Yan et al. 2009). In Arabidopsis, expression of primary responsive genes (i.e. JAZs) is induced in a COI1-dependent manner by wounding or feeding (Chung et al. 2008). There is only one AtCOI1 gene in the Arabidopsis genome (Devoto et al. 2002; Xie et al. 1998), but three closely related homologs are present and expressed in the rice genome (Hu et al. 2006; Lee et al. 2013; Ye et al. 2012). Two out of three COI1 genes-OsCOI1a and OsCOI1b-could be the result of a duplication event, due to high sequence identity. Arabidopsis point mutation *coi1-1* does not respond to JA-Ile, while either *OsCOI1a* or *OsCOI1b* can complement this mutation and restore JA signal transduction (Lee et al. 2013; Lee et al. 2015). After a BLAST search using homologous rice sequences, a total of five putative ZmCOI genes were identified in the maize genome, and these homologs were named based on synteny information (Supplemental Table 1). However, in this study one sequence (GRMZM2G035314) was manually deleted due to lack of critical F-box sequence. ZmCOI1b-1 and ZmCOI1b-2 were possibly the products of a recent duplication event in maize because they have located in the maize syntenic region and shared a 93% amino acid sequence identity.

Phylogenetic analysis was further performed using the deduced amino acid sequences from maize, rice, and Arabidopsis (Supplemental Fig. 2b). Based on the sequence alignment, these plant COI1 homologs shared high sequence conservation at the amino acid level and the characteristic features of F-Box motif and leucine-rich repeats (LRR) were observed in all COI1s (Xie et al. 1998). The essential amino acid residues required for COI1-interaction were conserved across all COI1s (Lee et al. 2013), as marked in Supplemental Figure 2a. It showed that the COI1 sequences from rice were more closely related to maize rather than Arabidopsis (Supplemental Fig. 2b). For example, for each syntenic group (COI1 and 2), the ZmCOI1 shared an approximate 84% amino acid sequence identity with OsCOI1, but only approximately 56% identity with AtCOI1. Results from an *in silico* study showed that, among four ZmCOI1 genes, only ZmCOI1a had a predicated nuclear localization. For these reasons, ZmCOI1a was chosen for expression analyses.

MYC2 is from the family of the basic helix-loop-helix (bHLH) transcription factors (TF), which is a direct target for JAZ proteins during JA-induced gene expression (Cheng et al. 2011). In Arabidopsis, AtMYC2 is well characterized as the master regulator in JA repression and point of crosstalk with other signaling pathways (Kazan and Manners 2013; Wang et al. 2015). In addition to the active nuclear localization signal, MYC2 TF contains three important domains: an N-terminal transcriptional activation domain (TAD) and adjacent JAZ interaction domain (JID) which required for JAZ protein binding, and a C-terminal conserved plant bHLH domain (Cheng et al. 2011; Fernandez-Calvo et al. 2011). Earlier research shows that one maize putative TF from the bHLH family named MYC7E shares high sequence similarity with Arabidopsis MYC2 (Abe et al. 2003; de Pater et al. 1997; Loulergue et al. 1998), and it was induced

upon wounding and JA-Ile treatment in maize leave (Engelberth et al. 2012; Fu et al. 2020). A rice homologous sequence (bHLH137) from the bHLH family was also detected and tested (Toda et al. 2013; Zhu et al. 2005). These MYC2 homologs all show high sequence similarity and have the same gene structure as AtMYC2 with only one exon, unlike other rice homologs (bHLH148) that have three exons (Li et al. 2006; Seo et al. 2011). This finding was confirmed in another study by Wang et al. (2015). In this study, we found an additional homologous sequence (GRMZM2G001930) using the previously identified MYC7E sequence, and paralogous information was obtained from the maize genome database (http://www.maizesequence.org) (Monaco et al. 2014). Supplemental Table 2 listed the candidate maize MYC2 homologous, and both genes (ZmMYC2a and 2b) had predicated nuclear localization. The two ZmMYC2 homologs shared over 90% sequence identity at amino acid level, and over 80% and 50% identity to OsMYC2 and AtMYC2, respectively. The phylogenetic data (Supplemental Fig. 3) showed high conservation for ZmCOI1 and ZmMYC2 genes at the nucleotide level and suggested the potential role of ZmMYC2 genes in JA-related plant responses and therefore, they were selected for expression analyses.

Gene Expression of ZmCOI1 and ZmMYC2 was Responsive to FAW Infestation in Maize Leaves. Since there is limited expression data about *ZmCOI1* and *ZmMYC2*, and no report of feeding data in maize to date, to better understand the regulation of these signaling pathway genes, we measured their expression levels in response to caterpillar feeding. Transcript levels of *ZmCOI1a* (GRMZM2G125411), *ZmMYC2a* (GRMZM2G001930), and *ZmMYC2b* (GRMZM2G049229) were tested. These genes were chosen since they were the most closely related maize homologs to those in rice and Arabidopsis (Lee et al. 2013; Loulergue et al. 1998) and had a predicted nuclear localizationResults from feeding-induced expression (Fig. 4a) revealed that *ZmCOI1a* transcripts were up-regulated as soon as 10 min (2.9-fold change) after FAW infestation in Mp708 compared to the undamaged control. After this transient peak, the transcript levels decreased dramatically and then increased slightly in Mp708 at 6 hr and 12 hr. But other than 10 mins, there were no significant differences in *ZmCOI1a* transcript levels between Mp708 and Tx601 during the remainder of the time course. *ZmMYC2* expression also was induced by FAW feeding and similarly, transcripts of *ZmMYC2a* and *ZmMYC2b* both accumulated within 10 min of feeding, and gradually increased over time until they began to diminish at 12 hr. Similar trends but with weaker induction levels were observed in Tx601, and there were no significant differences in the expression of these two genes between Tx601 and Mp708. The expression levels of *ZmCOI1a* and *ZmMYC2* were analyzed in B73 as well (Supplemental Fig. 1d-f) and were generally similar to those of Tx601. In summary, the data show that *ZmCOI1a* and *ZmMYC2a/b* exhibited an inducible expression pattern in response to herbivore feeding at very early stage (10 min), which indicated their potential involvement in JA-related defense responses in maize.

Effects of JA, Wounding, and Ethylene (ET) Treatments on JA-Related Gene Expression in Maize. To further investigate if exogenous hormone application could induce JA-related defense gene expression, plants were first sprayed with MeJA, and leaf samples were collected from both inbreds at the same time intervals used in the feeding experiments. MeJA was used since it can penetrate the cell membranes easily, and also can be transmitted by airborne diffusion due to its volatility (Farmer and Ryan 1990). After MeJA treatments, qRT-PCR data shown an inducible expression pattern of all selected genes tested

in this study. These results are shown in panel B of Figures 1, 3, and 4. In general, gene expression responded more slowly in MeJA-treated Mp708 and Tx601 compared with FAW-fed plants. For example, JAZ transcript level increased dramatically at 6 hr after MeJA treatment and remained relatively constant at 12 hr (Fig. 1b). This is in contrast to FAW feeding where JAZ transcript accumulation occurred as early as 30 min. The only time point showing significant differences between Mp708 and Tx601 was 12 hr after MeJA treatment when the accumulation of all three *ZmJAZ* remained higher in Mp708 than Tx601. Similar results were seen for JA biosynthesis gene (*ZmLOX1*) and JA signaling (*ZmCOI1a* and *ZmMYC2a/b*) genes; transcript levels of these marker genes were induced by MeJA, but induction time and intensity differed when compared with FAW feeding (Fig. 3b and Fig. 4b). However, in Tx601 MeJA-induced *ZmAOS* expression was similar to feeding-induced levels, whereas in Mp708 *ZmAOS* transcript levels appeared to have a typical MeJA-induced pattern: a delayed peak of induction but greater fold-change at later time points. Transcript levels of *ZmOPR2* were also differently regulated as previously mentioned (Louis et al. 2015; Zhang et al. 2005) and it was not responsive to MeJA treatment in Mp708, while there was only a weak expression in Tx601 (see y-axis scales in Fig. 3a and 3b).

Since MeJA treatment alone could not effectively mimic the same induction response as FAW infestation, we then examined the effect of mechanical wounding combined with MeJA application. The expression level of JA-related genes was examined first in mechanically wounded Mp708 and Tx601 plants without applying exogenous MeJA. Analysis 30 min after wounding showed no differences in expression relative to the controls (data not shown), so two additional time points (1 hr and 6 hr) were then tested. Since only subtle changes were seen at 1 hr post wounding (data not shown), qRT-PCR results for the two maize genotypes at 6 hr post wounding were presented in Figure 5. The results indicated that selected gene expression levels for JA signaling and biosynthesis pathways increased at 6 hr after wounding similar to feeding for 6 hr, except for *ZmJAZ1* and *ZmMYC2a/b*. For these genes, wounding alone would not effectively induce transcript accumulation to the same level as feeding, and our results differ from the previous founding in Arabidopsis (Fu et al. 2020). When the treatments of wounding and JA application were combined, the transcript levels of all tested genes dramatically increased at 6 hr compared to wounding only (Fig. 5). However, the induction of *ZmCOI1a* transcripts was largely due to wounding instead of MeJA, since there was no difference at expression level between wounding with MeJA and wounding with buffer treatments (Fig. 5d).

In addition to JA, ethylene (ET) is also associated with defense against pests and pathogens (Glazebrook 2005; Trujillo and Shirasu 2010). JA and ET have been reported to have either synergistic (Ankala et al. 2009; Harfouche et al. 2006) or antagonizing (Rojo et al. 1999; Tian et al. 2014) roles in plant response regulations, and ET-related genes were also regulated by MYC2 in Arabidopsis. Louis group (2015) found Mp708 plants use ET instead of JA to regulate *mir1* expression in response to corn leaf aphid. However, the regulatory mechanisms for crosstalk between JA and ET in herbivore resistance remain inconclusive (Onkokesung et al. 2010). Since EAR (ethylene-response factor amphiphilic repression) motif was found in maize JAZ genes (Han and Luthe 2021), considering the possible involvement of JAZ proteins in hormone crosstalk, we also examined the effect of exogenous ET on gene expression. Mechanically wounded maize leaves were treated with either Ethephon, an ethylene generating compound (Ankala et al.

2009), or a combination of MeJA and Ethephon, and samples were harvested and analyzed at 6 hr (Fig. 5) after treatments. In the ET and wounding combined treatments, expression levels of tested genes were either equivalent to (*ZmJAZ2*, *ZmMYC2*, *ZmLOX1*, *ZmAOS*, and *ZmOPR2*) or less than (*ZmJAZ1*) the undamaged controls, and only two genes-*ZmJAZ3* and *ZmCOI1a*-were positively induced. When JA, ET, and wounding treatments were combined the results differed, JA not only rescued the transcription activation that was reduced by ET, in some cases (e.g. *ZmCOI1*, *ZmLOX1*, and *ZmAOS*), ET appeared to enhance the JA response. The data suggested that ET alone was not as effective as JA in enhancing defense gene expression in maize, but it did enhance the JA regulated defense responses, which is unlike the antagonistic role of ET in Arabidopsis (Wasternack and Song 2016) and tomato (Tian et al. 2014). The effects of mechanical wounding and hormone treatment were different between Mp708 and Tx601. In general, gene expression levels were significantly higher for wounding alone or wounding with hormone treatments in Mp708 than Tx601, except for *ZmOPR2* (Fig. 5i). Collectively, the results indicate that JA is critical for the up-regulation of maize defense responses, and ET could facilitate the induction of the JA-related gene expression.

Discussion

Since the first discovery of JA almost four decades ago, a large body of evidence suggests that the JA pathway is universally present throughout the plant kingdom and plays a central role in the regulation of stress responses and physiological development in plants (Furstenberg-Hagg et al. 2013; Howe and Jander 2008; Howe et al. 2018; Wang et al. 2015; Wasternack and Hause 2013). In the current JA signaling model using Arabidopsis, three modules must be present: (1) JAZ protein, (2) SCF^{COI1} co-repressor complex, and (3) TF (i.e. MYC2) interact with JAZs (Sheard et al. 2010; Wasternack 2014). Orthologs of JAZ, COI1, and MYC2 have been identified in maize, and comparison between different plants reveals striking functional conservations in signaling pathways during evolution (Fu et al. 2020; Giri et al. 2017; Huang et al. 2014; Lee et al. 2013). Numerous studies have shown that most of the plant JAZ genes are rapidly induced by either JA-treatment (Chini et al. 2007; Ge et al. 2015; Hakata et al. 2017; Li et al. 2014; Thines et al. 2007; Zhang et al. 2012), wounding (Chao et al. 2019; Oh et al. 2012; Pirrello et al. 2014; Shoji et al. 2008; Tian et al. 2010; Yan et al. 2007; Ye et al. 2009) or herbivore feeding (Chung et al. 2008; Oh et al. 2012) in both dicots and monocots, and some are also differently regulated in response to abiotic and biotic stresses (An et al. 2017; Chini et al. 2017; Huang et al. 2016; Liu et al. 2017; Pirrello et al. 2014; Saha et al. 2016; Sun et al. 2017; Wu et al. 2015). However, no study has investigated JAZ/JA induction patterns in response to caterpillar infestation using maize genotypes that differ in herbivore resistance. Besides, another goal of our study was to determine if there were differences in the expression of JA pathway genes between Mp708 and Tx601 that could contribute to the higher constitutive levels and earlier accumulation of JA in Mp708.

Although the role of JA in dicots in conjunction with insect defense has been well studied, there is limited information about its role in maize herbivore defense. In this study, when we measured and compared the JA levels between two inbred lines in response to FAW infestation, Mp708 and Tx601 had different

temporal induction levels; in general, (*cis*-) JA levels in insect-resistant inbred Mp708 rose earlier and stayed higher (Fig. 2). Specifically, in addition to the (two-fold) higher resting *cis*-JA level in Mp708, a significant accumulation was also observed 10 min post-feeding, which was equivalent to the peak induction level in Tx601 at 1 hr. The peak JA accumulation occurred 1 hr after feeding in both inbreds, and then rapidly decreased at 6 hr due to JAZ negative feedback regulation: the abundant presence of newly synthesized JAZ proteins can facilitate a timely termination of the signaling pathway and also depletion of JA during this process (Howe et al. 2018). Engelberth et al. (2007) measured the JA levels in mechanically wounded maize seedlings and observed a similar trend with JA accumulation starting at 10 min after wounding and peaking at about 1 hr. In plants, the newly synthesized JA is in the *cis*-formation, and later turns to more stable *trans*-formation rapidly (Schaller et al. 1998; Vick and Zimmerman 1986). *Cis*-JA has been believed to be crucial for biological function (Fonseca et al. 2009; Holbrook et al. 1997), although other studies show that *trans*-JA also has biological activities in the plant as well (Koda et al. 1992). This abundance of *cis*-JA in Mp708 at early stages suggested it was critical for the defense activation in maize which might lead to different insect resistance.

Transcript abundance was also examined in response to feeding, wounding, MeJA, and ET treatments, and qRT-PCR results showed temporal accumulation of transcripts for key JA-signaling (i.e. JAZ, COI1, and MYC2) and biosynthesis (i.e. LOX, AOS, and OPR) genes under various conditions. Interestingly, we notice that the rapid burst of *cis*-JA levels in FAW-damaged maize leaves during the first 60 min after feeding showed a strong correlation with the initial accumulation of JAZ and JA-related gene transcripts, suggesting JA played an important role in the activation and regulation of JAZ gene expression in maize. For instance, the (*cis*-) JA levels in both Mp708 and Tx601 at the damage site dramatically increased within 10 min (Fig. 2), and this rapid burst of JA led to the up-regulation of gene expression especially in Mp708, including *ZmCOI1a* and *ZmMYC2a/b* at 10 min (Fig. 4), and *ZmJAZ* at 30 min (Fig. 1). Various downstream JA-related genes including the JA biosynthesis genes *ZmLOX1*, *ZmAOS*, and *ZmOPR2* were subsequently induced, ranging from 30 min to 6 hr post-feeding (Fig. 3). Consequently, the rapid resynthesis of JAZ repressor could provide a mechanism to attenuate the duration of JA activation, since JA is antagonistically linked between plant defenses, growth, and reproduction (Havko et al. 2016; Koo et al. 2009). In conclusion, FAW infestation increased *ZmJAZ* expression in a JA-dependent manner and jasmonate-mediated transcriptional responses are tightly integrated with the accumulation of JA and JAZ transcripts.

As stated previously, genes in JAZ signaling were differentially expressed and regulated in response to multiple stimuli. Besides hormones, insect cues were also involved in modulating maize defense against herbivores. Expression analysis from this study showed that feeding, among all the treatments, always triggered a quicker up-regulation of JAZ gene and JA-signaling genes (COI1 and MYC2) in maize (Fig. 1, 4, 5). For example, it appeared that FAW feeding initiated a more rapid induction of *ZmJAZ1* in Mp708 than Tx601, however, this did not occur when plants were wounded or treated with MeJA (Fig. 1). MeJA-treatment induced expression in a delayed but more intense fashion while wounding alone could induce only moderate responses. Similar results were also observed in rice in response to wounding and MeJA treatments (Hakata et al. 2017). On the other hand, in Arabidopsis, most JAZ transcript levels rapidly

increased responding to either mechanical wounding or jasmonate treatment (Howe et al. 2018). This strongly suggests that insect-derived factors are needed in maize to enhance the plant defense response (Chuang et al. 2014; Fescemyer et al. 2013). Some of these factors include continuous physical damage as opposed to one-time wounding (Mithofer et al. 2005), oral secretions (Chuang et al. 2014), and feces deposition (Ray et al. 2015). Therefore, HAMPs and DAMPs (herbivore- and damage-associated

molecular patterns) must be involved in maize defense pathways (Erb et al. 2012).

Crosstalk between plant hormones is common, in Arabidopsis, GA and JA can crosstalk by interchanging their repressor components during trichome formation (Qi et al. 2014), and JA and ET show antagonism during development (Zhang et al. 2014). In tomato, they also found that ET/ethephon treatment significantly decreased JA levels (Tian et al. 2014). Interestingly, JA-ET crosstalk is present in maize plants as well. Based on the results from wounding combined with hormone treatments, our observations indicated that when applied alone JA but not ET is required for increased transcript abundance after wounding, however, when JA and ET were combined there was enhanced defense gene induction in some cases (e.g. *ZmCOI1*, *ZmLOX1*, and *ZmAOS*) (Fig. 5). Ankala et al. (2009) previously demonstrated that ET working downstream of JA signaling regulated both *mir1* transcript and protein accumulation in Mp708 upon FAW infestation. Later Louis et al. (2015) determined that ET regulated the induction of *mir1* in Mp708 leaves in response to feeding by corn leaf aphids. However, this induction was independent of JA or SA. Unlike the antagonistic effect of JA-ET in Arabidopsis and tomato, maize may have a different regulatory mechanism for JA and ET crosstalk, and further investigation is needed (Fu et al. 2020).

Since our data agreed with the current model of JA regulation in Arabidopsis, we propose a similar JAZ repressor model for core JA signaling in maize (Supplemental Fig. 4). So how do the Mp708 and Tx601 maize plants use the same signaling network differently to render contrasting levels of insect resistance? One explanation points to the gene sequences. Although there were some polymorphisms between the two maize inbreds at both nucleotide level and amino acid level, ZmJAZ genes from Mp708 and Tx601 share > 99% sequence identity (Supplemental Table 4). Other possible explanations include the variability in promoter sequences, post-transcriptional modification, or protein stability. For example, alternative splicing patterns for ZmJAZ transcripts have been identified (Han and Luthe 2021) which might alter protein stability or binding efficiency. Last but not least, our study revealed that two key genes *ZmJAZ1* and *ZmCOI1a* also were differentially regulated between Mp708 and Tx601. To be specific, the transient peak of *ZmCOI1a* accumulation in FAW-fed Mp708 at 10 min was absent in FAW-fed Tx601, similarly, the transient peak of *ZmJAZ1* at 30 min was also missing in Tx601 (Fig. 1 and 4). In conclusion, our results suggest in maize resistance line Mp708, a timely transcriptional reconfiguration of JAZ signaling regulated by COI1 and JA may play a role in its resistance. Mp708 leaves had a higher JA level during the early stages of insect feeding, which in turn could enhance gene activation in the JAZ pathway since it is dose-dependent (Chini et al. 2016). Once defense signaling was activated, transcript levels for JAZ and other early signaling genes were maintained at the relatively greater abundance and for longer time periods in Mp708 (i.e. *ZmJAZ1*, *ZmAOS*, *ZmMYC2*), thanks to the transient early expression of Mp708 *ZmCOIa,* which could bind JAZ proteins in the presence of JA-Ile to stabilize the downstream gene

expression. So, this longer and more abundant induction of *ZmJAZ1* in Mp708 did not attenuate or switch off signaling, consequently, more *de novo* JA was synthesized due to the positive regulation of JA biosynthesis. This could explain why endogenous JA levels remained higher in Mp708 once the system was back to the resting stage. Our study provides more insight into the temporal pattern of maize signaling events before and after FAW infestation and demonstrates that in Mp708, JAZ and other selected genes are involved in the defense response, thus providing better crop protection and growth/defense balance. Still, the underlining mechanisms that lead to this robustness for defense activation need further investigation.

Although ZmJAZ genes were expanded through gene duplication (Han and Luthe 2021), the response of individual ZmJAZ gene to the input signal and physiology function was different. In this study, *ZmJAZ1* (from group1) and *ZmJAZ3* (from group 3) were induced more robustly and rapidly upon feeding, whereas *ZmJAZ2* (from group 2) and *ZmJAZ3* had a greater induction in response to wounding. Mp708 *ZmJAZ1* always had the highest induction levels in response to FAW feeding and MeJA treatment, which suggested its involvement in defense. Our results also indicated *ZmJAZ* genes from different groups may have diverse functions, other than direct involvement in insect resistance (Vernoud et al. 2009). Interestingly, when summarizing past JAZ reviews, JAZ genes from group 1 tended to show increased induction in response to wounding, pests, pathogens, or hormone treatments (see Supplemental Table 5). In addition to the functional similarity, they seemed to share high amino acid sequence identity at conserved TIFY and Jas domains and have similar exon-intron structures dependent on the phylogenetic distance (Supplemental Fig. 5). However, domain variances between groups were common as well. For example, in maize, CMID domain (cryptic MYC-interaction domain) was commonly found in genes from groups 1 and 3, but not in group 2. In Arabidopsis, functional CMIDs have been identified in AtJAZ1 and AtJAZ10, which were suggested to have a high binding affinity to MYC transcription factors, but the exact function was still inconclusive (Howe et al. 2018). JAZ genes from group 2 had a different motif, EAR motif. In Arabidopsis, some JAZ proteins also have the EAR motif (AtJAZ5-8 and AtJAZ13) (Kagale et al. 2010; Thireault et al. 2015) which helps recruitment of the general co-repressor TOPLESS (TPL) or other TFs to repress jasmonate responses (Chini et al. 2016). These differences in JAZ gene structures may provide regulation diversity to various elicitors and different hormone regulation pathways. Since ZmJAZ family contained more groups than those tested in this study, our result highlights the need for additional work to reveal the JAZs' role in maize defense.

Conclusion And Future Work

In this study, we wanted to determine if there were differences in the induction of JA responsive genes involved in defense signaling in two maize genotypes that differ in herbivore resistance. Mp708 has demonstrated resistance to not only FAW but also corn rootworm (Castano-Duque et al. 2017) and corn leaf aphid (Louis et al. 2015). Previous work has demonstrated that Mp708 has elevated constitutive levels of JA and OPDA (Shivaji et al. 2010) that allow it to be genetically primed in response to herbivory. The cause of this early robust response remains elusive. A previous study shows that there are no obvious allelic differences in the coding sequences of the JAZ genes in the two genotypes. This study

indicated that transcript levels of *ZmJAZ1* increase significantly within 30 min of FAW infestation in Mp708 and not Tx601. Differences in the temporal expression of the JA-associated gene and the level of induction suggest that Mp708 has a more rapid and robust defense response. Since there are multiple isoforms for each key protein/enzyme in the JA-related defense pathway, additional genetic evidence is needed to conclusively determine its role in maize plants.

Declarations

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Declarations:

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Competing interests

The authors have no conflicts of interest to declare that are relevant to the content of this article

Availability of data and materials

All data generated or analyzed during this study are included in this published article and its supplementary information files.

Code Availability

Not applicable

Authors' contributions

Yang Han and Dawn Luthe contributed to the study conception and design. Material preparation, data collection, and analysis were performed by Yang Han. The first draft of the manuscript was written by Yang Han and Dawn Luthe commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Ethics approval

Not applicable

Consent to participate

Not applicable

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Not applicable

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Figures

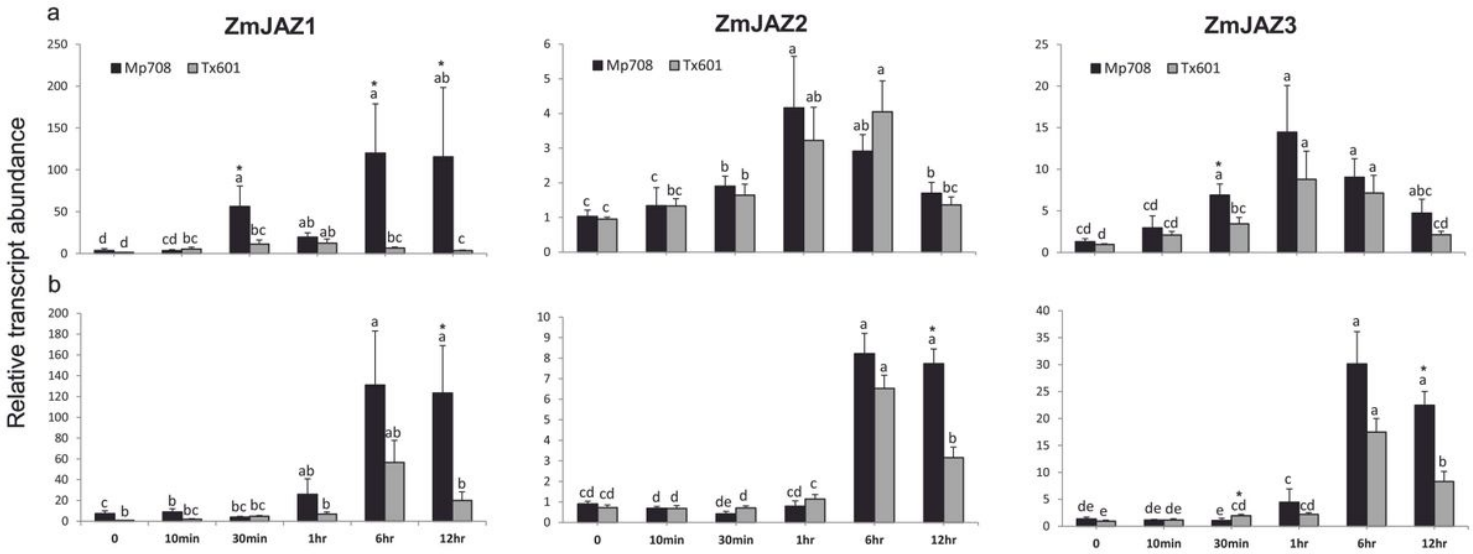

Figure 1

Expression of JAZ genes in response to FAW feeding and exogenous MeJA in maize leaves. V-8 stage plants from maize inbred lines Mp708 and Tx601 were (a) infested with FAW larvae, or (b) sprayed with 0.01% MeJA. Leaf tissues from the feeding sites or whorls were harvested for RNA extraction at the indicated time points. Undamaged plants (0) were used as control. Gene expression of ZmJAZ1-3 was monitored using quantitative RT-PCR. Relative transcript abundance was calculated using actin as the reference gene. The data represented were mean values with error bars (+SE, n=9 for feeding, n=6 for MeJA). Different letters indicate significant differences by the least significant difference test ($P<0.05$). Asterisks indicate significant differences compared between Mp708 and Tx601 plants ($P<0.05$)

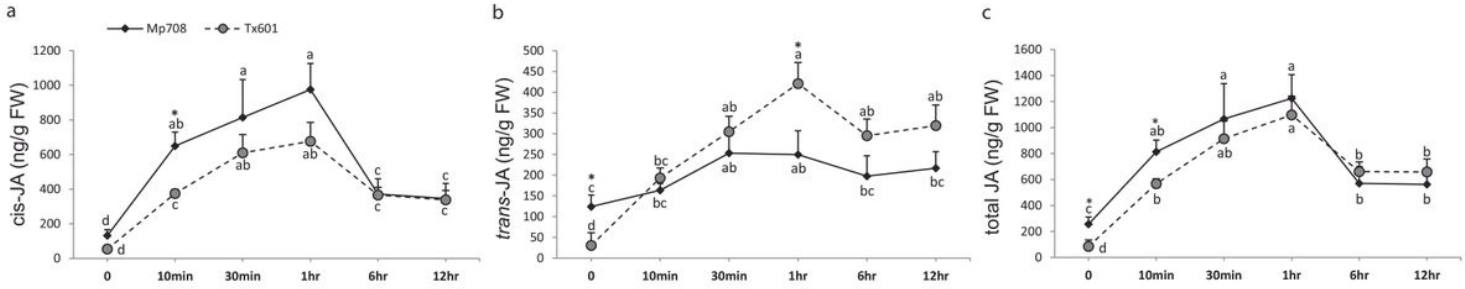

Figure 2

Rapid accumulation of JAs in response to FAW feeding in maize leaves. Time course of (a) cis-JA, (b) trans-JA, and (c) total JA accumulation from maize inbred line Mp708 and Tx601 in response to FAW feeding were measured. Larvae were allowed to feed on V-8 stage maize plants of Mp708 and Tx601, leaf tissues surrounding the feeding sites and unwounded control plants (0) were harvested for JA extractions at the indicated time points. JA (measured as cis- and trans-JA) levels were determined by CI-GC/MS described in “Materials and Methods”. The data represented were mean values with error bars (+SE, n=6). Letters indicate significant differences by the least significant difference test (P<0.05). Asterisks indicate significant differences compared between Mp708 and Tx601 plants (P<0.05). FM, fresh mass

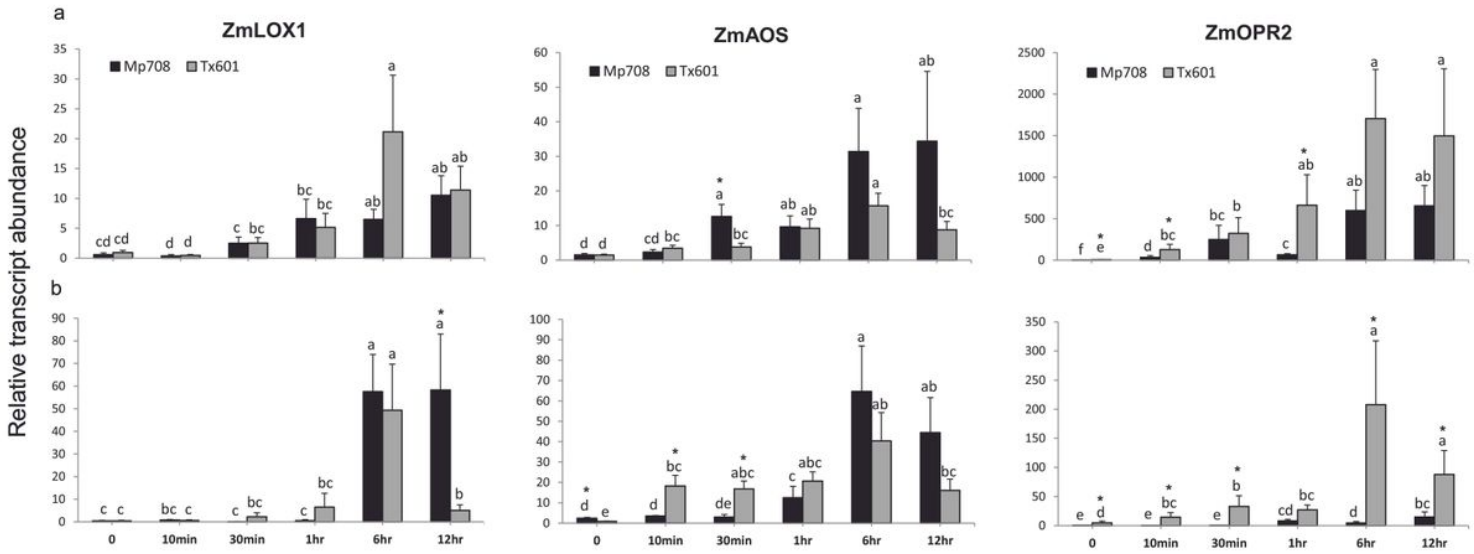

Figure 3

Expression of JA biosynthesis genes in response to FAW feeding and exogenous MeJA in maize leaves. V-8 stage plants from maize inbred lines Mp708 and Tx601 were (a) infested with FAW larvae, or (b) sprayed with 0.01% MeJA. Leaf tissues from the feeding sites or whorls were harvested for RNA extraction at the indicated time points. Undamaged plants (0) were used as control. Quantitative RT-PCR was performed for ZmLOX1, ZmAOS, and ZmOPR2. Relative transcript abundance was calculated using actin as the reference gene. The data represented were mean values with error bars (+SE, n=9 for feeding, n=6 for MeJA). Different letters indicate significant differences by the least significant difference test (P<0.05). Asterisks indicate significant differences compared between Mp708 and Tx601 plants (P<0.05)

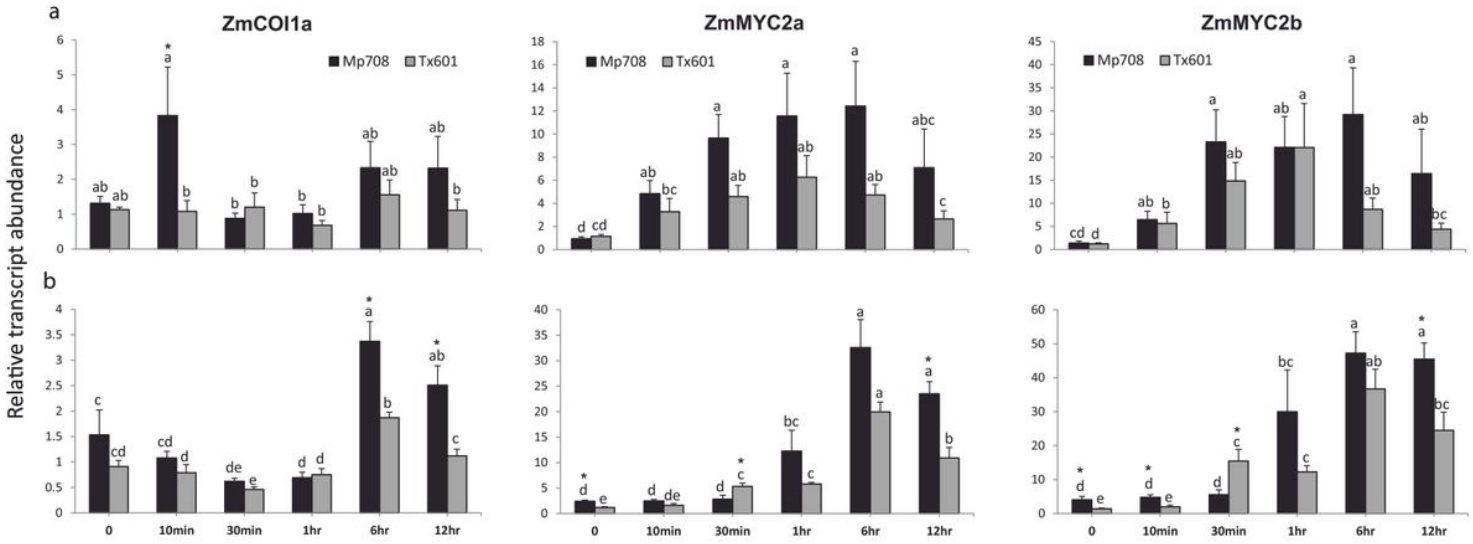

Figure 4

Expression of COI1 and MYC2 genes in response to FAW feeding and exogenous MeJA in maize leaves. V-8 stage plants from maize inbred lines Mp708 and Tx601 were (a) infested with FAW larvae, or (b) sprayed with 0.01% MeJA. Leaf tissues from the feeding sites or whorls and undamaged plants (0) were harvested for RNA extraction at the indicated time points. Quantitative RT-PCR was performed for ZmCOI1a, ZmMYC2a, and ZmMYC2b. Relative transcript abundance was calculated using actin as the reference gene. The data represented were mean values with error bars (+SE, n=9 for feeding, n=6 for MeJA). Different letters indicate significant differences by the least significant difference test ($P<0.05$). Asterisks indicate significant differences compared between Mp708 and Tx601 plants ($P<0.05$)

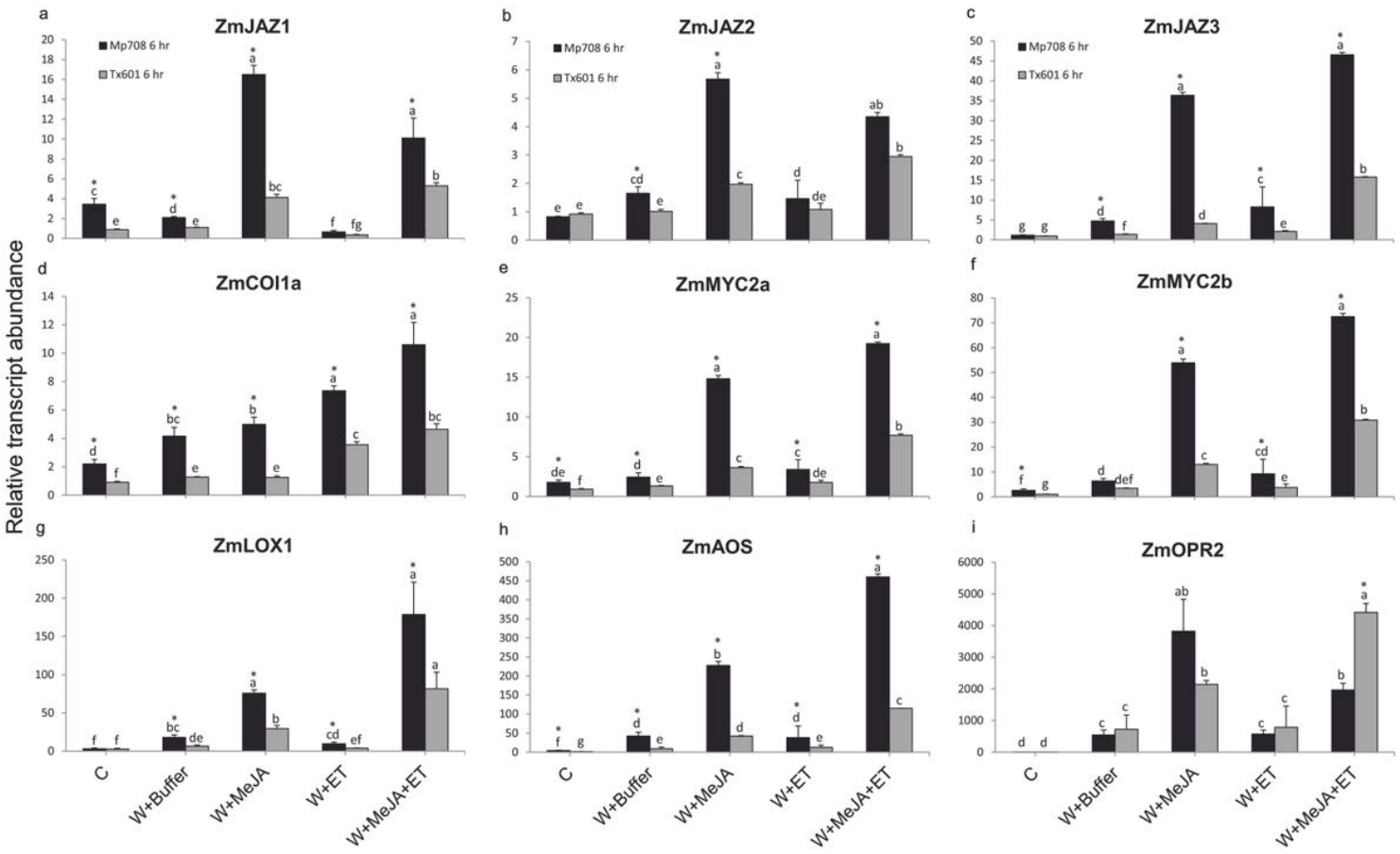

Figure 5

Effects of wounding, exogenous MeJA, and ET on the expression of JA signaling and biosynthesis genes in maize leaves. V8 plant from two maize inbreds (Mp708 and Tx601) were mechanically damaged with the wounding tool (W) and sprayed with solvent buffer (W+Buffer) or 0.01% MeJA (W+MeJA) or 3mM ET (W+ET) or MeJA and ET (W+MeJA+ET) for 6 hr. Undamaged plants were used as control (C). Total RNA was isolated from control whorls and the damaged areas on the leaves. Quantitative RT-PCR was performed for ZmJAZ1-3 (a-c), ZmCOI1a, ZmMYC2a/b (d-f), and ZmLOX1, ZmAOS, ZmOPR2 (g-i). Relative transcript abundance was calculated using actin as the reference gene. The data represented were mean values with error bars (+SE, n=3). Different letters indicate significant differences by the least significant difference test (P<0.05). Asterisks indicate significant differences compared between Mp708 and Tx601 plants (P<0.05)

Supplementary Files

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