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MAIZE DEFENSE RESPONSES TO CATERPILLARS AT THE MOLECULAR, PHENOTYPIC AND ECOLOGICAL SCALES

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ABSTRACT

Maize (*Zea mays ssp.* mays), one of the most important crops worldwide, has to face attack from numerous insects during its life cycle in the field. The major pests of maize include caterpillars, beetles, aphids, and thrips. The aim of the dissertation was to study maize defense responses to caterpillars at the molecular, phenotypic and ecological scales. More specifically, maize defense responses to fall armyworm (FAW, *Spodoptera frugiperda*) and black cutworm (BCW, *Agrotis ipsilon*) in the Order of Lepidoptera were investigated in various experimental settings. Chapter 2 assessed maize defense responses to FAW in the nested association mapping (NAM) founder lines, and illustrated possible resistance mechanisms. Insect bioassays indicated that the constitutive defense of the 25 founder lines fall into a continuum. High levels of *rip2* and *mpi* transcript expression and RIP2 protein accumulation in resistant genotypes correlated with their high constitutive defenses to FAW. There was a correlation between *rip2* expression levels and RIP2 accumulation upon FAW herbivory in the diverse maize inbred lines. Chapter 3 focused on the soil-mediated cover crop effect on corn defense response to BCW, and linked the molecular aspects of corn defense responses to agroecosystem and farming management practices. BCW grew better when corn plants obtained adequate amount of N from soil provided by cover crops, so cover crops benefited both corn plants and BCW at the phenotypic level. Secondly, cover crops increased the expression of several corn defense genes to deter BCW herbivory at the molecular level. Therefore, the relationship of cover crops and corn defense responses to BCW is not a simple linear relationship. The Appendix Chapter investigated an important herbivore defense gene maize insect resistance 1 (*mir1*) expression in 23 NAM founder lines, and it was found that maize natural sequence polymorphism affects the expression of *mir1*. 23 maize NAM founder lines including B73 did not express *mir1* in their whorls before or after FAW infestation and the presence of the 4789 bp region including a CACTA transposable element in *mir1* promoter of these lines is likely one of the regulatory sequences controlling *mir1* expression in diverse maize lines whorls. The second regulatory sequence controlling *mir1* expression is an inverted-repeat transposable element (MITE) in Mp708 *mir1* intron three. This dissertation primarily tackled the question of how maize plant protects and defends itself against caterpillars within systems of various scales. It has added to the basic scientific knowledge of maize and

insect interactions, and provided a fresh view of key components not only as separate research objects, but also from a holistic perspective to understand the hidden mechanisms.

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Chapter 1

Introduction

Maize (*Zea mays* ssp. *mays*) is one of the most important cereal crop species in human agriculture. Between 2016 and 2017, the global corn production is predicted to be 40,861 million bushels, of which the U.S. will produce 37% and China will produce 21% of the total (World Markets and Trade USDA 2017). In 2016, U.S. growers planted 94.0 million acres and harvested 86.7 million acres of corn, of which the total production was 15.1 billion bushels and resulted in a crop value equaling 51.5 billion dollars (World of Corn 2017 http://www.worldofcorn.com/#/). U.S. corn usage in 2016 included animal feed, biofuel ethanol, food industry (corn syrup, sweetener, starch, cereal, and beverage alcohol) and exportation to other countries (ERS Feed Outlook USDA 2017; ProExporter Network 2017).

With the constant changes in the global climate, growth of maize is challenged by various environmental stresses including abiotic and biotic stresses. One of the biotic stresses is due to numerous insect pests that attack maize in the field. They feed on different tissues and organs of maize and have preferences for specific developmental stages. The aboveground insects include armyworms, corn borers, corn earworm and corn leaf aphids that belong to various feeding guilds, and they attack leaves, stalks, ears and flowers (Thomas 1993a). The belowground insects include corn rootworm, wireworm, black cutworm and corn root aphid, and they feed on roots and stems (Thomas 1993b). A large amount of maize yield is lost due to insect damage and this causes significant economic losses for the growers every year (O'Day et al. 1998; Rice et al. 1999).

To fight against insect assaults, plants produce a vast majority of repellent, antinutritive and toxic chemicals to deter herbivory. Such chemicals and plant physical barriers, such as trichomes and thorns, constitute plant direct defense (Howe and Jander 2008). Plants also have indirect defenses that use plant-derived volatiles to attract natural enemies of the herbivore (Kessler and Baldwin 2002). Plant defenses against herbivores can also be classified into constitutive and induced defenses. The former consists of the presence of certain defensive traits regardless of herbivore attack (Howe and Jander 2008). For example, a fall armyworm (*Spodoptera*

frugiperda) resistant maize line constitutively produces a toxic cysteine proteinase in low amount in the whorl region of leaf prior to herbivory (Pechan et al. 2000). Induced defenses are only triggered after herbivore challenge to generate a plethora of defensive chemicals in damaged and undamaged tissues to thwart herbivorous insects (Bostock 2005).

The plant hormone jasmonic acid (JA) and its derivatives play a central role in regulating plant responses to insect herbivory. JA synthesis begins in the chloroplast of the plant cell. Released from plant cell membrane by an acyl hydrolase or a phospholipase, linolenic acid is converted to 12-oxo-phytodienoic acid (OPDA) by lipoxygenase (LOX), allene oxide synthase (AOS) and allene oxide cyclase (AOC). Then OPDA is transported to peroxisome and reduced to 3-oxo-2-(2'(Z)-pentenyl)-cyclopentane-1-octanoic (OPC-8:0) by OPDA reductase 3 (OPR3). OPC-8:0 CoA ligase (OPLC1) catalyzes transfer of the acetyl group from acetyl-CoA to OPC-8:0 to form OPC-8:CoA. OPC-8:CoA goes through three cycles of β-oxidation, resulting in (+)-7-iso-JA (Engelberth et al.). JA can be converted to numerous biologically active forms such as methyl-JA (MeJA) and jasmonoyl-L-isoleucine (JA-Ile), as well as inactive derivatives as a way of regulating JA levels and signaling processes in the plant (Schaller et al. 2004; Howe and Jander 2008; Schaller and Stintzi 2009).

In plants, JA is a master regulator of their defense responses to insects. Caterpillar herbivory triggers JA biosynthesis and signaling to allow the plant to switch on downstream JA-inducible defense genes, generating defense proteins and secondary metabolites to thwart caterpillar herbivory (Howe and Jander 2008; Wasternack and Hause 2013). A general model of this induction pathway for maize is well illustrated in Shivaji et al. (2010).

The innate host plant resistance to insects in maize can be attributed to some of its biochemical properties (McMullen et al. 2009; Meihls et al. 2012). There are secondary metabolites functioning to defend maize against insect herbivores. These include DIMBOA (2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one), which is the major maize benzoxazinoid that acts as defense metabolite (McMullen et al. 2009; Meihls et al. 2013). It was identified in maize seedlings and demonstrated to be the chemical component in resistance to European corn borer (ECB) (Klun et al. 1967; Scriber et al. 1975). It deters ECB feeding and reduces larvae growth (Houseman et al. 1992). The biosynthesis pathway has ten *bx* genes controlling the enzymatic reactions and conversions of DIMBOA (Gierl and Frey 2001; Jonczyk et al. 2008; Butrón et al. 2010). Another

group of defense metabolites are the phenolic acids, especially chlorogenic acid and maysin (Szalma et al. 2005). These compounds were originally isolated from the silks of a maize landrace showing resistance to corn earworm (CEW) (Waiss et al. 1979; Byrne et al. 1996). They have antinutritive effects on CEW growth and development (Wiseman et al. 1992).

There are several proteins contributing to maize resistance to insects. One of these is maize protease inhibitor (MPI). MPI can effectively inhibit elastase and chymotrypsin activities from the cotton leafworm (*Spodoptera littoralis*) midgut extract (Tamayo et al. 2000). Ribosome-inactivating protein 2 (RIP2) is a protein synthesis inhibitor that has site-specific RNA N-glycosidase activity to arrest translation (Bass et al. 2004). Synthesized as an inactive proenzyme in maize, RIP2 is induced by caterpillar feeding, and it resists digestion in caterpillar gut and is eliminated in the frass (Chuang et al. 2014). Its toxicity makes it one of the defensive proteins that guard maize against insects. A unique cysteine protease, maize insect resistance 1 (Mir1-CP), was identified in the germplasm developed in Mississippi. Mir1-CP targets the caterpillar peritrophic matrix (PM) (Pechan et al. 2002), resulting in this germplasm showing resistance to several lepidopteran insect pests and insects from other feeding guilds (Davis et al. 1988; Gill et al. 2011; Louis et al. 2015).

During herbivory, maize plant is able to emit green leafy volatiles (GLV) to attract arthropod predators of insect pests (Turlings 1992; Engelberth et al. 2004; Farag et al. 2005), which is known as an indirect defense. The volatile mixture is composed of indoles and a large number of terpenes and sesquiterpenes (Ton et al. 2007; Unsicker et al. 2009). For example, maize terpene synthase 23 (TPS23) synthesizes the sesquiterpene, (*E*)-β-caryophyllene as an airborne signal to attract natural enemies of caterpillars and rootworms (Köllner et al. 2008; Capra et al. 2015).

The above innate host plant resistance to insects primarily focuses on the inherent traits of the maize plant, but altering the external environmental inputs can also influence plant defense responses to insects. It is known that exogenous nitrogen (N) supply influences plant resistance to insect herbivores (Mattson 1980; Zangerl and Bazzaz 1992; Awmack and Leather 2002). In the native tobacco plant (*Nicotiana attenuata*), low nitrogen supply decreased the insect elicitor-induced phytohormones, jasmonic acid and salicylic acid. It also reduced nicotine and trypsin protease inhibitor, which are N-rich defense compounds, but increased C-containing defense chemicals (chlorogenic acid and rutin). However, the emission of volatile organic compounds

(VOCs) was not affected by low or high nitrogen supply. Low nitrogen supply also reduced the expression of herbivore-inducible genes at the transcriptional level (Lou and Baldwin 2004). The above results partially support the carbon-nutrient balance theory in that when a plant has an excess N supply, it is likely to produce more N-containing defense metabolites, and when under N limitation, a plant is likely to produce more C-containing defense metabolites (Mattson 1980). In another study, nitrogen deficiency affected soybean plant growth, and thus brought a reduction in the growth of fall armyworm and its parasitoid. But nitrogen deficiency did not alter the range of herbivore-induced VOCs in soybean, leading to no change in parasitoid behavior response (Winter and Rostás 2010).

The broad objective of this dissertation was to study maize defense responses to caterpillars at the molecular, phenotypic and ecological scales. In current U.S. agriculture, genetically engineered (GE) maize crop is widely adopted to control various insect pests, but GE maize has its own shortcomings and exerts unfavorable influences on agricultural ecosystems and triggers fierce controversies in the public eyes (Wolfenbarger and Phifer 2000; National Research Council 2010; Nicolia et al. 2014). On the other hand, it is essential and valuable to investigate the natural resistance mechanisms of plants to insects and the environmental factors involved. From the perspective of basic science, this investigation illuminates the interaction of plants and insects, how plants defend themselves, how insects counteract plant defenses, and how the interaction forms and evolves over time in the context of evolutionary biology. From the perspective of applied science, the results of this investigation can be used in designing and engineering new insect resistant crops, improving current GE crops, and designing and implementing novel agricultural ecosystems. As for maize, it has the highest phenotypic and genetic diversity among the crop species, which has created new resources and directions for studying maize defense responses to insects. Newly identified resistance mechanisms can be incorporated into various maize breeding programs as novel avenues for integrated pest management. Further, U.S. growers will benefit from the study of maize defense responses to insects in reducing the cost of purchasing GE corn seeds. At the ecological and agricultural levels, the new knowledge will demystify the underlying on-going processes of how cover crop, cash crop, soil, insect pests, and related environmental factors interact and provide positive influences on agroecosystems.

In Chapter 2, the aim was to use maize genetic diversity resources to study how maize inbred lines with divergent genetic backgrounds respond to fall armyworm, and which molecules contributed to the divergence in the defense responses. The fall armyworm is a major caterpillar pest on maize across southeastern U.S. and South America. Currently, there are few studies on how the maize Nested-Association Mapping (NAM) founder lines respond to caterpillar herbivory; hence, this study focuses on assessing maize resistance to fall armyworm in the founder lines and illustrating possible resistance mechanisms.

In the Appendix Chapter, the aim was to study how maize natural allelic variation affects *mir1* expression in the NAM founder lines. Mir1-CP and *mir1* were identified from insect resistant maize inbred lines that originated from Mississippi. However, since maize has the highest genetic diversity among crop species, it is not known how maize natural allelic variation affects *mir1* expression upon fall armyworm infestation in the founder lines and what are the potential regulatory sequences controlling *mir1* expression.

In Chapter 3, the aim was to evaluate how cover crop utilization in organic farming systems influences maize defense responses to black cutworm. Black cutworm (*Agrotis ipsilon*) is a problem for organic farming systems using cover crops as the main avenue in managing soil fertility. Black cutworm adults oviposit in nearby cover crops in early spring and young larvae establish on these plant materials and migrate to emerging corn seedlings for feeding. Cover crop effects on soil may affect plant defense responses to insects in the following cash crops. We performed both field and greenhouse experiments for two years to examine whether cover crops affect maize resistance to black cutworm and whether the resistance relates to the expression of herbivore defense genes in maize.

This dissertation contains the results and implications of several novel research projects with a wide scope. Chapter 2 studied the defense responses of diverse maize inbred lines to fall armyworm both phenotypically and molecularly. The Appendix Chapter took a genetic and molecular approach to pinpoint the causative regions controlling the expression of an important maize herbivore defense gene. Chapter 3 extended the scope to an ecological scale in the pioneering research to reveal molecular mechanisms of soil-mediated cover crop effects on maize defense responses to black cutworm in actual farming systems.

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Chapter 2

Maize NAM Founder Lines Exhibit Diverse Responses to Caterpillar Herbivory

Introduction

Maize (*Zea mays* ssp. *mays*) is one of the most important crops in worldwide agriculture. Because of its rich nutritional value and high grain yield, maize has become excellent food source and shelter for a broad variety of insect species, and therefore it has to face attack from numerous insects during its life cycle in the field (O'Day et al. 1998). The fall armyworm, *Spodoptera frugiperda* (J. E. Smith) is a major caterpillar pest to corn across southeastern United States and South America (William et al. 1978; Wiseman and Davis 1979; Davis 1980). Its damage costs growers millions of dollars each year. In the southern U.S., fall armyworm (FAW) larvae feed inside the developing whorl of knee-high to tasseling (V8 to VT) corn. The adult moths can migrate long distances and cause heavy larval infestations that can completely defoliate a cornfield overnight.

Transgenic maize hybrids producing the *Bacillus thuringiensis* (Bt) insecticidal protein have effectively controlled several caterpillar pests, but FAW has been reported to start evolving resistance to transgenic maize in Brazil, Puerto Rico, and the southeastern U.S., thus making transgenic maize less powerful for FAW control (Huang et al. 2014; Omoto et al. 2014; Bernardi et al. 2015). On the other hand, a series of maize inbred lines highly resistant to FAW and southwestern corn borer (SWCB) were selected through a breeding program (Scott et al. 1982; Williams and Davis 1982). One of the resistant maize inbred lines, Mp708, shows resistance to several lepidopteran pest species (Davis et al. 1988). Mp708 has been intensively studied to illustrate the mechanism of its natural resistance (Jiang et al. 1995; Pechan et al. 2000; Pechan et al. 2002; Shivaji et al. 2010). However, other than the resistant maize inbred lines developed in Mississippi, there have been few reports on exploiting other sources of maize natural resistance to FAW that include the identification of resistant inbreds and analysis of resistance mechanisms. Therefore, it has become important and crucial to start searching and exploiting other sources of maize natural resistance to FAW.

The maize genome contains higher molecular diversity than other plant species, resulting in tremendous phenotypic diversity (Tenaillon et al. 2001; Wright et al. 2005). The genetic difference between two maize inbred lines is as much as the difference between human and chimpanzee (Chimpanzee Sequencing and Analysis Consortium 2005). The high genetic diversity of maize and the availability of a large set of germplasm makes it possible to search for the inbred lines with natural resistance to FAW. One outstanding diversity panel is the maize nested association mapping (NAM) population. (Liu et al. 2003; Yu et al. 2008; McMullen et al. 2009). It includes 25 diverse founder lines, a common parent B73, and 200 recombinant inbred lines for each founder line and B73 cross. Its purpose is to encompass a large amount of maize genetic diversity for dissecting the genetic architecture of complex traits and pinpointing functional valuable alleles controlling important agronomic traits. A broad range of research on the maize NAM population has emerged recently, including the study of the genetic components of plant height, flowering time, kernel composition, and resistance to northern leaf blight and aphids (Buckler et al. 2009; Poland et al. 2011; Tian et al. 2011; Cook et al. 2012; Meihls et al. 2013; Peiffer et al. 2013; Peiffer et al. 2014). Linkage mapping and genome wide association mapping (GWAS) both identified significant quantitative trait loci (QTL) and multiple candidate genes related to these important traits. However, there were no studies on how NAM founder lines respond to caterpillar herbivory; thus, this study focuses on assessing maize resistance to FAW in the founder lines and illustrating possible resistance mechanisms.

Plant direct defense against insects include a vast majority of repellent, anti-nutritive and toxic chemicals, and physical barriers like trichomes and thorns (Howe and Jander 2008). Constitutive and induced defenses are two forms of plant direct defenses. Constitutive defenses are always present regardless of herbivore attack, but induced defenses are only triggered after herbivore challenge to generate a large amount of defensive chemicals in damaged and undamaged tissues to thwart herbivorous insects (Bostock 2005).

Ribosome-inactivating protein 2 (RIP2) is a protein synthesis inhibitor that has site-specific RNA N-glycosidase activity to arrest translation (Bass et al. 2004). “It blocks translational elongation by depurinating residues on the large ribosomal RNA component” (Endo et al. 1987; Endo and Tsurugi 1987; Nielsen et al. 2001). Synthesized as an inactive proenzyme in maize plant, RIP2 protein is induced by caterpillar feeding, can function and resist digestion in caterpillar gut, and is eliminated in the frass (Chuang et al. 2014). Its toxicity makes it one of the defensive proteins

that guard maize against insects. Protease inhibitors also are in the arsenal that plants use to thwart herbivore and pathogen attacks (Koiwa et al. 1997). Herbivores, including insects, have various proteases in their digestive system that digest and degrade proteins for nutrient assimilation. Plant protease inhibitors bind to these proteases and decrease their activity, thus preventing herbivores from efficiently attaining nutrients. Protease inhibitors and their role in herbivore defense have been well-studied in tomato and potato (Green and Ryan 1972; Koiwa et al. 1997). Maize also has protease inhibitors and the mRNA for maize proteinase inhibitor (MPI) accumulates upon fungal infection in germinating maize embryos (Cordero et al. 1994). Wounding, abscisic acid, methyl jasmonate, and caterpillar feeding also can induce *mpi* mRNA accumulation. *mpi* mRNA accumulation exhibits a systemic pattern in that upon wounding, both local and systemic *mpi* induction were detected (Cordero et al. 1994). MPI protein can effectively inhibit elastase and chymotrypsin activities from the cotton leafworm (*Spodoptera littoralis*) midgut extract (Tamayo et al. 2000).

Here, the constitutive and induced defenses of eight selected maize inbred lines were investigated from the phenotypic to molecular level to determine how these genotypes respond to FAW herbivory. The first hypothesis tested was that the 25 founder lines show a continuum of constitutive defenses to FAW. The second hypothesis tested was that the eight selected genotypes show divergence in constitutive and induced defenses. The last hypothesis tested was that the selected eight genotypes show divergent patterns in *rip2* and *mpi* mRNA and RIP2 protein accumulation prior to and after FAW herbivory.

Materials and Methods

Plant materials and FAW rearing

Maize (*Zea mays*. ssp. *mays*) NAM founder line seeds were obtained from the North Central Regional Plant Introduction Station (NCRPIS). Mp708 seeds were obtained from Dr. William Paul Williams (USDA-ARS Corn Host Plant Resistance Research Laboratory, Mississippi State University, USA). B73 seeds were produced at the Pennsylvania State University research farm at Rock Springs, PA.

To achieve a high germination rate, seeds of all inbred lines were placed into Ziploc plastic containers with four layers of paper towel covering the top and bottom of the seeds. Ziploc containers were wrapped with aluminum foil to maintain a dark environment for germination.

Tiny holes were punched in aluminum foil to ensure that there was sufficient air for germination. Paper towels were damped to maintain moisture and the seeds were put into a 25 °C incubator. It usually took 4 to 5 days for the radicle and cotyledon to emerge and then seedlings were transferred into the potting mix (PRO-MIX BX mycorrhizae, PRO-MIX, USA). After the cotyledon and the first two true leaves emerged, the seedlings were transferred into pots containing topsoil (Hagerstown silt loam).

For the first group of seven bioassays, two maize plants were planted in one 18L black plastic pot with topsoil. Osmocote Plus (The Scotts Company, Marysville, OH) was used as fertilizer. When the plants reached V8 to V10 stage (Ritchie et al. 1986), they were used in bioassays. For all the other experiments, a single maize plant was planted in a 4 L pot with topsoil and fertilized as above, and when plants reached V8 to V10 stage, they were used for experiments.

Plants were grown in the Plant Science Department greenhouse at The Pennsylvania State University (University Park, PA, USA). The temperature was from 20 to 28 °C. The photoperiod was 16 hr light:8 hr dark. Photosynthetically active radiation ranged from 500 to 1200 µmol $m^{-2}s^{-1}$. Maize plants were watered with tap water as needed. Because the growth rate of the maize inbreds differed, they were planted in a staggered manner so they reached the same growth stage for experiments. For example, slow growing lines were planted early and fast growing lines were planted late.

FAW eggs were obtained from the USDA-ARS Corn Host Plant Resistance Research Laboratory, Mississippi State University, USA. Eggs were hatched in a 27 °C incubator. After hatching, neonates were reared on an artificial diet (Peiffer and Felton 2005) in the same incubator with a 14 hr light:10 hr dark.

For bioassays, larvae were reared on artificial diet for four days before the experiment. When larvae reached 3.5 to 5.5 mg, they were used in bioassays. For infestation experiments, individual larvae were reared on artificial diet until the fourth or fifth instar.

FAW bioassay

The first set of seven bioassays was done over one year because it was impossible to grow all the NAM founder lines and conduct bioassays at the same time. The 25 NAM founder lines were divided into seven groups based on their growth rate. In the summer of 2012, all founder lines

were grown at The Pennsylvania State University research farm at Rock Springs, PA to observe the phenology. In each bioassay, B73 and Mp708 served as controls because previous results showed that Mp708 is a FAW resistant line and B73 is a FAW susceptible line.

In each bioassay, three or four founder lines were tested. Five or six maize plants of each genotype were cut from the whorl region. The inner most three layers of whorl leaves (above the newest leaf collar) were used in bioassay because this is the preferred FAW feeding site (Pechan et al. 2000). The tip, base, and midrib of the leaves were discarded. The leaves were cut into 1-1.5 cm pieces and mixed together from five or six plants.

To maintain moisture for FAW larvae during the bioassay, 1% agar (Difco, BD USA) was premade and 3 ml of the agar solution was added to each 1 oz diet cup and solidified. A predetermined excess amount of maize leaves was put into each cup to provide sufficient leaf material for 4 days of feeding.

Thirty FAW larvae were tested for each founder line. At the start of the bioassay, FAW larvae were selected in a weight range from 3.5 to 5.5 mg and they were placed in the diet cups. The bioassays were conducted in a 27 °C incubator with a14 hr light:10 hr dark. After 4 days, FAW larvae were removed and final weights recorded.

The data entered for statistical analysis is Relative Growth Rate (RGR). It was calculated as {(Weight final-Weight initial)/((Weight final+ Weight initial)/2)}/ 4 days (Mohan et al. 2008). To combine the results from seven bioassays, all RGR values were divided by the average RGR of B73 and it was called B73 normalized RGR (nRGR).

The bioassay with selected six NAM lines, B73, and Mp708 was conducted in the same way.

Infested leaf bioassay

The purpose of the infested leaf bioassay was to use the growth inhibition of FAW to indicate the amount of induced defense for each genotype after infestation. Five starved FAW larvae were placed in the whorl region of each plant, and 14 to 15 maize plants were infested for each genotype. After 24 hr, leaf tissue near the feeding sites was collected (up to 1.5 cm surrounding the feeding site), cleaned by paper towel, cut into 1-1.5 cm pieces, and mixed for each genotype. The remainder of the bioassay was conducted as described above.

On the same day, another bioassay using 14 to 15 uninfested maize plants of each genotype was initiated. The purpose was to make sure the infested and control plants were cultivated at the same time, so other than the infestation treatment, there was no difference between the infested and control plants. The initial and final weights (4 days feeding on leaves) of FAW were recorded. The percentage of growth inhibition was calculated as {(Average of Weight control - Weight infested)/Average of Weight control}*100% for individual larva fed on each genotype.

FAW infestation experiment

FAW were reared to the fourth or fifth instar and starved overnight. Maize plants ready to be infested were moved into another greenhouse. Five starved FAW larvae were placed in the whorl region of each maize plant, and four maize plants were infested for each genotype. The infestation period was 24 hr. Then leaves were cut from the whorl region and 0.1 g of tissue adjacent to the feeding site (approximately 5 mm surrounding the feeding site) was collected, immediately frozen in liquid N_2 and stored in -80 °C freezer. Leaf samples from control plants were collected at 0 hr time point.

RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR)

The leaf tissues were homogenized using the Geno/Grinder 2000 (SPEX Certiprep, USA). Total RNA was extracted from leaf tissues using the TRIzol reagent and protocol (Life Technologies, USA). The RNA pellet was dissolved in diethylpyrocarbonate (DEPC)-treated water. After extraction, RNA was subjected to DNase digestion to remove residue genomic DNA. The reaction contained 26 µl RNA, 3 µl DNase buffer and 1 µl DNase (New England BioLabs, USA). It was incubated at 37 °C for 30 min and then DNase was inactivated at 65 °C for 10 min. Nanodrop 2000 (Thermo-Fisher Scientific, USA) was used to measure RNA concentration and its purity. For reverse transcription PCR, 1 µg total RNA was added into a 20 µl reaction with oligo-dT 20 primers following the manufacturer's protocol. The reverse transcription PCR program was 37 °C for 2 hr and 85 °C for 5 sec. The kit was the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA).

qRT-PCR was performed in an ABI 7500 Fast Real Time PCR system. Primer Express software was used to design gene-specific primers (Life Technologies, USA). Individual cDNAs were diluted 10 times before running qRT-PCR. The qRT-PCR reaction (10 µl) contained 1 µl diluted cDNA, 1 µl H_2O, 1 µl forward primer (1 µm), 1 µl reverse primer (1 µm), and 5 µl SYBR Green

Master Mix (Roche Applied Science, USA). The program was as follows: step 1, 50 °C for 2 min and 95 °C for 10 min; step 2, 95 °C for 15 sec and 60 °C for 1 min, 40 cycles; step 3, 72 °C for 10 min; step 4, dissociation stage Since the eight selected inbred lines belong to distinct maize genetic/breeding groups, the use of a single control gene for calculating the relative quantification of target gene expression by 2-ΔΔCT method was not a good option (data not shown). The criteria for an adequate control gene in qRT-PCR is that its cycle threshold (Ct) values need to be constant and least variable among treatments, tissues, and developmental stages (Huggett et al. 2005; Kozera and Rapacz 2013). Four candidate control genes (*ubiquitin*, *actin*, *gapdh* and *apt1*) were tested in the eight maize inbred lines prior to and after FAW infestation. It was found that the choice of two control genes and the use of the geometric mean of *gapdh* and *apt1* according to Vandesompele et al. (2002) for calculation by 2-ΔΔCT method resulted in the least variation of control genes and provided an accurate quantification of target gene expression. Therefore, *gapdh* and *apt1* were chosen as control genes. Two endogenous genes *gapdh* and *apt1* were used to calculate the relative quantification of target gene expression by 2-ΔΔCT method (Vandesompele et al. 2002; Lin et al. 2014). The list of primers is shown in **Supplementary Table 2.2**. The original data are shown in **Supplementary Table 2.3**.

Data Analyses

All the statistical analyses were performed in Minitab 17 Statistical Software (Minitab Inc., USA).

B73 normalized relative growth rate (nRGR) of FAW larvae fed on 25 NAM founder lines, Mo17, and Mp708 was analyzed as a one-way ANOVA using genotype as the independent variable ($F=7.47$, $P<0.001$). A Fisher pairwise comparison was performed to annotate the statistical significant difference.

The relationship of FAW larvae nRGRs fed on NAM founder lines and the phylogenetic relationship was analyzed as a one-way ANOVA using genetic group as the independent variable ($F=7.08$, $P<0.001$). Minitab 17 optimized the λ value for data transformation and transformed the data automatically. A Fisher pairwise comparison was performed to annotate the statistical significant difference of means.

RGR of FAW larvae fed on six NAM founder lines, B73, and Mp708 was analyzed as a one-way ANOVA using genotype as the independent variable ($F=92.03$, $P<0.001$). A Fisher pairwise comparison was performed to annotate the statistical significant difference.

FAW growth inhibition was analyzed as a one-way ANOVA using genotype as the independent variable ($F=23.26$, $P<0.001$). A Fisher pairwise comparison was performed to annotate the statistical significant difference.

Target gene relative quantification was analyzed as a two-way ANOVA using genotype and infestation as the independent variables. Minitab 17 optimized the λ value for data transformation and transformed the data automatically. *rip2* relative quantification statistics was shown as: Genotype $F=83.84$, $P<0.001$; Infestation $F=324.63$, $P<0.001$; Genotype*Infestation $F=18.56$, $P<0.001$. *mpi* relative quantification statistics was shown as: Genotype $F=4.75$, $P<0.001$; Infestation $F=995.68$, $P<0.001$; Genotype*Infestation $F=15.12$, $P<0.001$. Since there was interaction between genotype and infestation for *rip2* and *mpi* relative quantification, a Fisher pairwise comparison was performed to annotate the statistically significant differences**.**

Immunoblot analysis of RIP2 protein

Leaf tissues were homogenized using the Geno/Grinder 2000 (SPEX Certiprep, USA). Then the proteins were extracted in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (Laemmli, 1970) with commercial protease inhibitor cocktail added (Sigma-Aldrich, USA). Protein concentrations were determined by a Non-interfering Protein assay kit (G-Biosciences, USA).

Then 30 µg of total protein from each sample was loaded in each gel lane and separated by SDS-PAGE. Protein gels were blotted onto nitrocellulose membranes using the Panther Semi-Dry Electroblotter (Thermo Scientific, USA). Dr. Rebecca Boston (North Carolina State University, Raleigh, NC, USA) kindly provided the polyclonal antibody to RIP protein. Immunoblots were conducted using 1:10,000 diluted anti-RIP antibody and 1:10,000 diluted anti-rabbit secondary antibody conjugated with horseradish peroxidase (Thermo Fisher Scientific, USA) (Chuang et al. 2014). Target protein RIP2 was detected by chemiluminescence (West Femto Maximum Sensitivity Substrate, Thermo Scientific, USA). Finally, the nitrocellulose membranes were reversibly stained with Ponceau S to determine if there was equal protein loading (Salinovich and Montelaro 1986).

Results

Bioassays

Laboratory insect feeding bioassays were used to evaluate the constitutive defenses of NAM founder lines to FAW. The principle of bioassay is that when caterpillars feed on susceptible plants, they grow bigger and faster than caterpillars that feed on resistant plants. By measuring the weight of caterpillars, the susceptibility or resistance of plants can be determined (Chang et al. 2000; Williams and Buckley 2008). Based on previous field phenology observations, NAM founder lines were divided into seven groups according to their growth rate. Mp708 is a FAW resistant inbred line (Williams et al. 1990; Pechan et al. 2000) and B73 is a FAW susceptible inbred line, so they served as positive and negative controls in each bioassay set.

The normalized relative growth rate (nRGR) of FAW larvae fed on different genotypes showed a range from 1.0021 (larvae fed on M37W) to 0.9042 (larvae fed on Mp708), which indicates that the constitutive defense of 25 founder lines fall into a continuum (**Figure 2.1**). Typically, the average weight of FAW larvae reared on Mp708 was approximately 50 to 60% of those reared on B73. The results indicated that the reference maize inbred B73 was one of the most susceptible genotypes to FAW. Previous studies about the genetic structure of the 25 founder lines showed that they are clustered into six distinct genetic groups (Liu et al. 2003). These groups are sweetcorn, popcorn, tropical or semitropical (TS), Stiff Stalk (SS), non-Stiff Stalk (NSS), and mixed origin group. After analyzing the relationship of FAW growth and the genetic groups, it was found that the constitutive defense of the founder lines was not independent of their phylogenetic relationship (**Figure 2.2**). Lines in the TS group had the highest constitutive defense indicated by low pooled FAW nRGR. Lines in the NSS and mixed groups had a medium level of constitutive defense. Lines in the popcorn, sweet corn, and SS groups had the lowest constitutive defense shown by high pooled FAW nRGR.

After the initial assessment of constitutive defense, six founder lines, B73, and Mp708 were selected for further study to investigate how differently they respond to FAW herbivory and what contributes to the divergence of responses. There were two reasons for selecting M37W, IL14H, Hp301, Ms71, NC350, and CML333. First, it was based on their level of constitutive defense to FAW within the 25 founder lines. CML333 and NC350 are resistant, while the other inbred lines

are susceptible. Second, it was based on the genetic structure. One representative inbred line was selected from the corresponding genetic group (**Figure 2.1**).

A second bioassay divided the eight genotypes into five susceptible and three resistant lines in regard to constitutive defense (**Figure 2.3** [A]). The result confirmed the position of each inbred line in the continuum of constitutive defense to FAW. It is interesting that CML333 is the most resistant inbred line. Mp708, serving as a control for the bioassays and also a known FAW resistant genotype, was less resistant than CML333 in this bioassay. NC350 can be considered as moderately resistant, since it behaved better than the other five genotypes, but not as well as CML333 or Mp708. M37W, IL14H, B73, Ms71 and Hp301 are similarly susceptible to FAW.

To investigate how induced defenses differ among the eight inbred lines, an infested leaf bioassay was conducted. In the experiment, the RGR of FAW larvae reared on infested and control leaf tissues were compared, and then growth inhibition was calculated and used to indicate the level of induced defenses of the eight inbred lines (**Figure 2.3** [B]). FAW fed on infested Mp708 plants showed the highest reduction in growth, which was inhibited by approximately 55%. FAW fed on infested Hp301 and Ms71 plants showed 42% growth inhibition. FAW fed on infested M37W, CML333, and B73 plants fell into the same statistical group and ranked third in their growth inhibition, approximately 30%. FAW fed on infested IL14H and NC350 were least inhibited in their growth, which was less than 10%. These results showed that the resistant control Mp708 was able to mount the highest induced defense after FAW infestation. CML333 can mount a medium level of defense induction, while NC350, which has relatively high constitutive defenses, had a low level of induced defense after FAW herbivory. As for the susceptible genotypes, Hp301 and Ms71 both can mount a high level of induced defense. M37W and B73 are medium in this regard. IL14H had the lowest level of induced defense. Based on the bioassay results indicating the constitutive and induced defenses, the above eight genotypes have been divided in two ways (**Figure 2.3** [C]). The separation of resistant and susceptible genotypes is in terms of their constitutive defense levels, and the induced defense is categorized as highly, moderately, and minimally inducible genotypes.

Ribosome-inactivating protein 2 (*rip2*) gene expression

To examine the possible molecular mechanisms for constitutive and induced resistance level, the abundance of mRNA encoding RIP2 was determined in the eight genotypes. Except NC350 and

IL14H, *rip2* expression levels were significantly higher for the other six genotypes after FAW infestation than in unchallenged plants. This indicates that FAW infestation successfully triggered the accumulation of *rip2* mRNA (**Figure 2.4** [A]). On the other hand, CML333, Mp708, NC350 and Hp301 had higher constitutive levels of *rip2* mRNA, while M37W, Ms71 and B73 had the lowest constitutive levels. This correlated with the high constitutive defense levels of CML333, Mp708, and NC350 that were resistant to FAW. The constitutive *rip2* mRNA level for the genotype IL14H was close to zero. When it comes to the induced level of *rip2* mRNA, M37W, and Ms71 showed the highest induction after FAW infestation. This supported the results showing that Ms71 and M37W were able to mount higher induced defenses compared to CML333, B73, NC350, and IL14H (**Figure 2.3** [B]). B73, CML333, Mp708, and Hp301 showed a medium level of *rip2* mRNA induction. It is interesting to note that FAW infestation did not induce *rip2* mRNA accumulation in NC350 or in IL14H. NC350 and IL14H *rip2* mRNA accumulation and their low level of induced resistance shown in **Figure 2.3** [B] indicate that these two genotypes can barely elevate their defenses after FAW infestation.

RIP2 protein accumulation

In addition to measuring *rip2* transcript abundance, the accumulation of RIP2 after FAW infestation was determined by immunoblot analysis (**Figure 2.4** [B]). For uninfested control plants, a very low amount of RIP2 was present in NC350, CML333, and Mp708, but this band was not detectable in M37W, B73, IL14H, Hp301, or Ms71. This result correlated well with *rip2* expression analysis showing that CML333, Mp708, and NC350 had higher constitutive levels of *rip2* mRNA, while M37W, Ms71, and B73 had lower levels. The presence of RIP2 in NC350, CML333, and Mp708 control plants could partially contribute to their higher constitutive defense to FAW.

Following infestation, all genotypes except IL14H had increased levels of RIP2. For the susceptible genotypes, M37W and Ms71 appeared to have the highest RIP2 accumulation. B73 had medium RIP2 accumulation, while Hp301 had the lowest RIP2 protein accumulation. This result correlated well with *rip2* mRNA accumulation in these four genotypes after FAW infestation. For the three resistant genotypes, RIP2 appeared to be greater in NC350, CML333, and Mp708 after infestation even though its mRNA accumulation was not as high in these resistant genotypes.

The most interesting result was from IL14H where FAW feeding did not enhance the accumulation of RIP2 and it was not detected in infested plants. This was in good agreement with IL14H *rip2* expression since the values were close to zero in both control and infested IL14H plants.

Maize proteinase inhibitor (*mpi*) gene expression

For all the inbred lines, *mpi* gene expression was significantly higher after FAW infestation which means that infestation triggered the accumulation of *mpi* mRNA (**Figure 2.5**). Hp301, Mp708, CML333, and NC350 had higher constitutive level of *mpi* mRNA than the other four genotypes, and this helped to explain the higher constitutive defense of CML333, NC350, and Mp708. After FAW infestation, IL14H showed the highest induction of *mpi* gene expression. Ms71, M37W, and Hp301 did not show statistically significant difference in the induction of *mpi* gene expression, but the trend was clear in that Ms71> M37W > Hp301. This also could contribute to their higher induced defense levels. Mp708 showed a medium level of *mpi* mRNA accumulation after FAW herbivory. CML333, B73, and NC350 had the smallest induction in *mpi* expression after FAW infestation. In the case of NC350, its small induction in *mpi* expression correlated well with its low induced defense.

Discussion

Measuring the constitutive defense as a quantitative trait

Of all the important agronomic traits, maize resistance or defense response to insects is one of the hardest to study since both the maize plant and the insects are living organisms with complex genomes. Maize resistance to insect pests is a quantitative trait as demonstrated in the literature. The design of the maize NAM population combined the advantages of linkage analysis and association mapping with both high statistical power and resolution (Liu et al. 2003; Yu et al. 2008); therefore, it has successfully pinpointed loci responsible for complex traits down to single-gene levels and identified numerous candidate genes (Zhang et al. 2015; Xiao et al. 2017; Yang and Holland 2017; Zhang et al. 2017). The current study has shown that the constitutive defense of 25 NAM founder lines to FAW fall into a continuum in both laboratory and greenhouse settings. The future direction is to employ the whole NAM population or several recombinant inbred line (RIL) families in field studies to perform linkage mapping and association mapping for finding the genes underlying maize host-plant resistance to caterpillar

pests. The genotyping data for NAM population is available (Bukowski et al. 2015) and a good field phenotyping method is to infest the maize plants with FAW neonates and visually evaluate the damage. The newly discovered genes can be combined with genetic engineering for developing novel stacked insect resistance traits in maize or used in traditional breeding for breeding naturally insect resistant maize lines.

The NAM founder lines have 13 TS lines, six NSS lines, four mixed origin lines, one SS line, two sweet corn lines, and one popcorn line. The numbers of inbred lines per genetic group are not identical. B73 (SS), IL14H (sweet corn), P39 (sweet corn), and Hp301 (popcorn) had low constitutive defenses to FAW (**Figure 2.1**), and led to the corresponding genetic groups with low constitutive defense. Lines in TS, NSS, and mixed origin groups showed variation in constitutive defenses with both susceptible and resistant genotypes, and after pooling the data, these three genetic groups had higher constitutive defenses especially the TS group.

Constitutive and induced defenses

In this study, constitutive and induced defenses were investigated separately. Mp708 had both high constitutive and induced defenses. This genotype resulted from a breeding program targeting FAW and SWCB resistance. One factor contributing to resistance in Mp708 is the maize insect resistance1-cysteine protease (Mir1-CP) (Pechan et al. 2000; Mohan et al. 2008). Its mRNA and protein are present in a low amount in the whorl region of control plants prior to FAW infestation (Shivaji et al. 2010). Upon FAW infestation, *mir1* mRNA levels remain unchanged or down regulated during the first 24h, but the protein accumulates rapidly adjacent to the feeding site in the whorl region (Pechan et al. 2000;Shivaji et al. 2010). The other seven genotypes tested in this study do not accumulate *mir1* transcripts (data not shown). So, the other two resistant line CML333 and NC350 must rely on alternative resistance mechanisms.

CML333 had the highest constitutive defense, but its induced defense was at a medium level. NC350 had a medium constitutive defense and minimal induced defense. It is possible that physical properties such as leaf toughness are major contributors to their constitutive defenses that cannot be dramatically altered upon FAW herbivory during the 24h infestation period. Therefore, the induced defenses due to shifts in plant metabolism were not high. Unlike Mp708, CML333 and NC350 did not come from any specific breeding program for insect resistance, but originated from a tropical or semitropical environment. In that way, their high and medium

constitutive defenses were probably a result of their artificial selection in a high insect pressure environment.

The genotypes M37W, B73, IL14H, Ms71, and Hp301 had low constitutive defenses. However, Hp301 and Ms71 were able to mount highly induced defenses. That means upon FAW herbivory, Hp301 and Ms71 rapidly and efficiently altered their metabolism and produced a large amount of defensive metabolites and proteins to fight back. M37W and B73 had medium levels of induced defenses. IL14H had both low constitutive and induced defenses, and the absence of *rip2* mRNA and RIP2 before and after FAW infestation correlated well with that.

Here, the results have shown that a maize genotype with high constitutive resistance does not necessarily have highly induced defense. A maize genotype with low constitutive resistance can mount highly induced defense. It seems that maize plants make trade-offs to manage constitutive and induced defenses to FAW. For maize breeding programs, targeting both high constitutive and induced defenses to insect herbivory should be considered and evaluated. In the field, both constitutive and induced defenses are functioning together to protect maize plants against insect herbivory, and maize must fine-tune its metabolism to achieve these goals.

***rip2* transcript and RIP2 protein accumulation**

In control plants, Mp708, NC350, and CML333 had higher level of *rip2* mRNA and RIP2 than M37W, Ms71, and B73. These results partially explained their high constitutive defense to FAW. Although Hp301 had higher level of *rip2* mRNA in control plants than M37W, Ms71 and B73, RIP2 protein was absent prior to FAW infestation. This result correlated with the lower constitutive defense of Hp301. It is possible that RIP2 did not accumulate in Hp301 controls due to some type of post-transcriptional regulation, such as impaired translation of *rip2* mRNA, or post-translational proteolytic degradation of RIP2.

After FAW infestation, the fold-induction of *rip2* mRNA in M37W and Ms71 (approximately 75- and 50-fold, respectively) was greater than that in B73 and Hp301 (approximately 30- and 6-fold, respectively). RIP2 accumulation was also higher in these genotypes than B73 and Hp301 after FAW infestation. Therefore, in these susceptible genotypes, *rip2* mRNA and RIP2 protein accumulation appeared to be correlated. For the resistant genotypes CML333, Mp708, and NC350, the fold-induction of *rip2* mRNA accumulation was lower (approximately 10-, 6- and 5-fold, respectively). However, there was abundant accumulation of RIP2 in these genotypes. This

is probably due to synthesis and accumulation of RIP2 in control plants that increases during infestation. Alternatively, there could be different alleles of *rip2* genes in susceptible and resistant genotypes, and differences in gene sequence could result in changes in protein accumulation. This is supported by the different molecular masses of RIP2 from the inbreds included in the study. Another study also showed that the size of RIP2 differed among several other maize inbred lines (Chuang et al. 2014).

The most interesting result was from IL14H. In this genotype, *rip2* mRNA levels were barely detectable in control and infested plants. Furthermore, RIP2 protein did not accumulate in infested plants. It was previously reported that RIP2 accumulated after FAW herbivory in 13 different maize genotypes and two teosintes, so it appears as a common maize defensive protein (Chuang et al. 2014). For IL14H, it could be that *rip2* is not present in its genome or that a transposable element disrupts its expression.

Gene expression analysis showed that CML333, Mp708, and NC350 had higher constitutive levels of *rip2* and *mpi* transcripts than the other genotypes. Since *rip2* and *mpi* are both downstream JA-inducible defense genes in the maize direct defense to caterpillar herbivory pathway, it is likely that the three resistant genotypes have constitutively elevated jasmonic acid (JA) levels that turn on downstream defense responses even before caterpillar feeding. Thus, the possibility of elevated JA levels contributed to the overall high constitutive defense in these three resistant genotypes. This scenario has been demonstrated in Mp708, which showed higher JA and cis-12-oxo-phytodienoic acid (OPDA) levels together with higher expression of JA biosynthesis and downstream defense genes compared to its susceptible parent (Shivaji et al. 2010).

At the phenotypic level, this study has shown that the eight selected maize inbred lines contain different constitutive and induced defenses to FAW. At the molecular level, *rip2* mRNA and RIP2 abundance prior to and after FAW infestation in the above genotypes also display different patterns. This study is an initial step to investigate the molecular mechanisms of resistance to caterpillar pests in the NAM founder lines and much remains unknown about them.

Future perspectives

Since the maize inbred lines with high constitutive and induced defenses to FAW were identified here and since constitutive and induced defenses are traits that can be phenotyped and quantified

accurately and efficiently, a future direction will be to employ linkage analysis and genome wide association study (GWAS) to identify significant quantitative trait locus (QTL) and potential candidate genes involved in maize constitutive and induced defenses to caterpillar pests.

Another direction will be to perform proteomic analysis to identify potential maize defense proteins that are up or down regulated in its defense response to caterpillar pests. In this way, we can determine if defense genes identified by GWAS and proteins identified by proteomic analysis converge and lead to the identification of new resistance factors in maize.

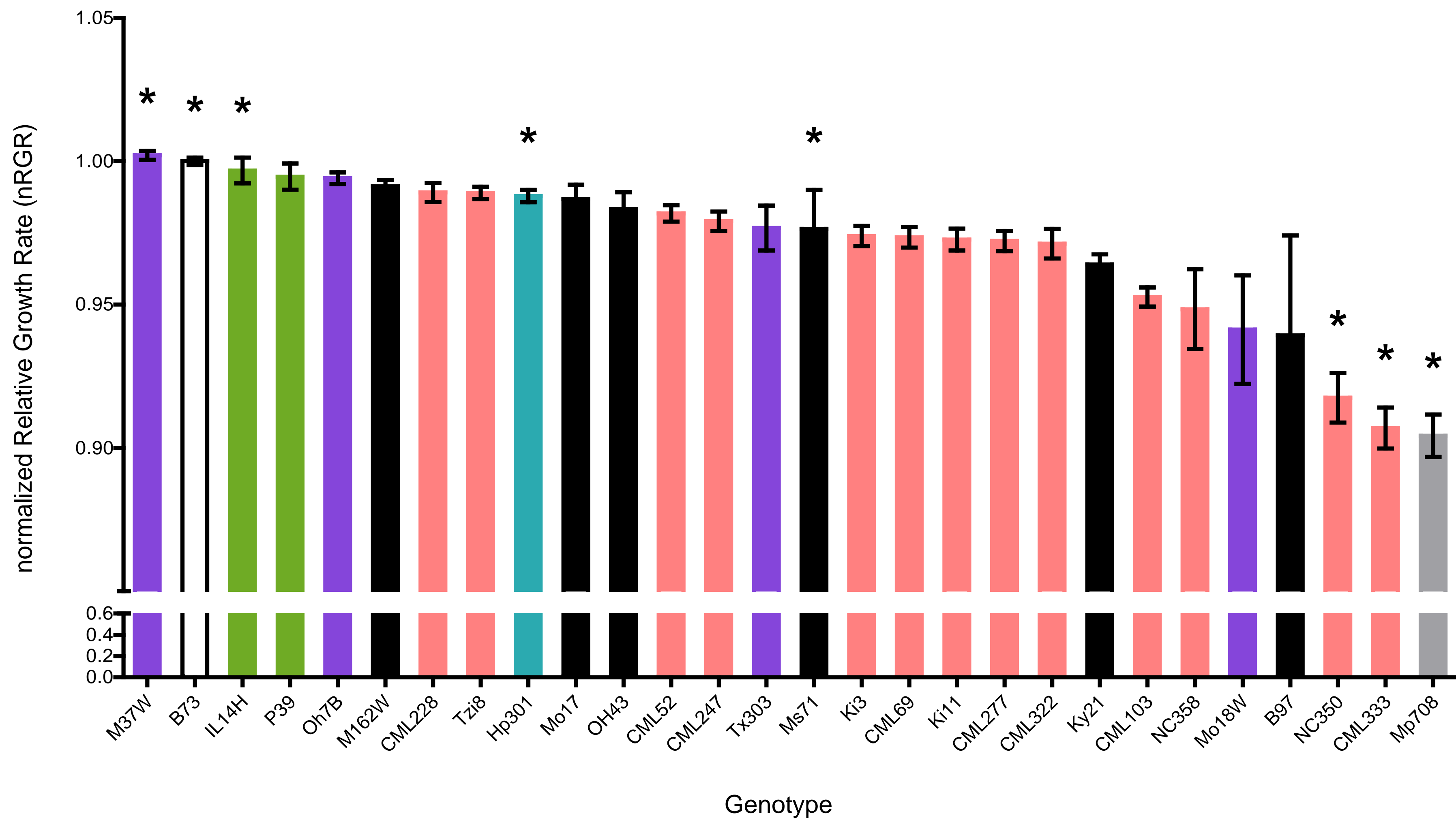
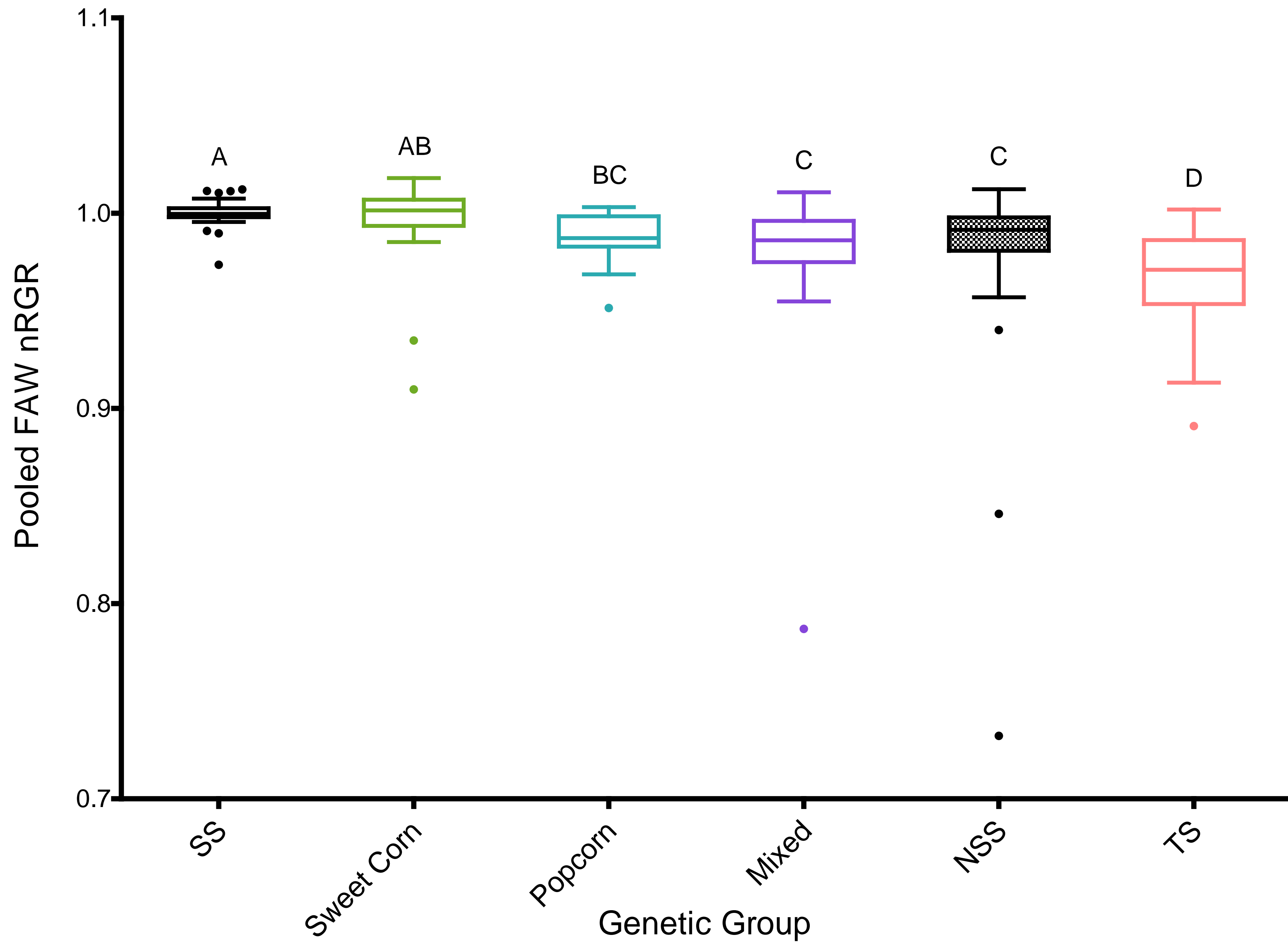

normalized Relative Growth Rate (nRGR)
1.05
1.00
0.95
0.90
0.6
0.4
0.2
0.0
M37W
B73
IL14H
P39
Oh7B
M162W
CML228
Tzi8
Hp301
Mo17
OH43
CML52
CML247
Tx303
Ms71
Ki3
CML69
Ki11
CML277
CML322
Ky21
CML103
NC358
Mo18W
B97
NC350
CML333
Mp708
Genotype

Figure 2.1. B73, Mo17, Mp708, and 25 NAM founder lines have different levels of constitutive defense to FAW. The constitutive defense is indicated by normalized relative growth rate (nRGR) of FAW. Leaves from five to six maize plants per genotype were cut into 1-1.5cm pieces and mixed. Thirty FAW larvae fed on each genotype for 4 days and initial and final weights were recorded. Relative growth rate (RGR) was calculated as {(final weight – initial weight)/[(final weight + initial weight)/2]}/days. All RGR values were divided by B73 average RGR for normalization to perform statistical analysis. The statistics are shown in **Supplementary Table 2.1**. Error bar indicates the standard error of the mean (SEM).

Each genotype can be placed in a specific genetic group as described by Liu et al. 2003. The groups included sweetcorn (green), popcorn (turquoise), tropical or semitropical (TS) (peach), Stiff Stalk (SS) (white), non-Stiff Stalk (NSS) (black), and mixed origin group (purple). Mp708 is shown in gray. The bars with asterisks indicate the eight selected inbred lines for following experiments.

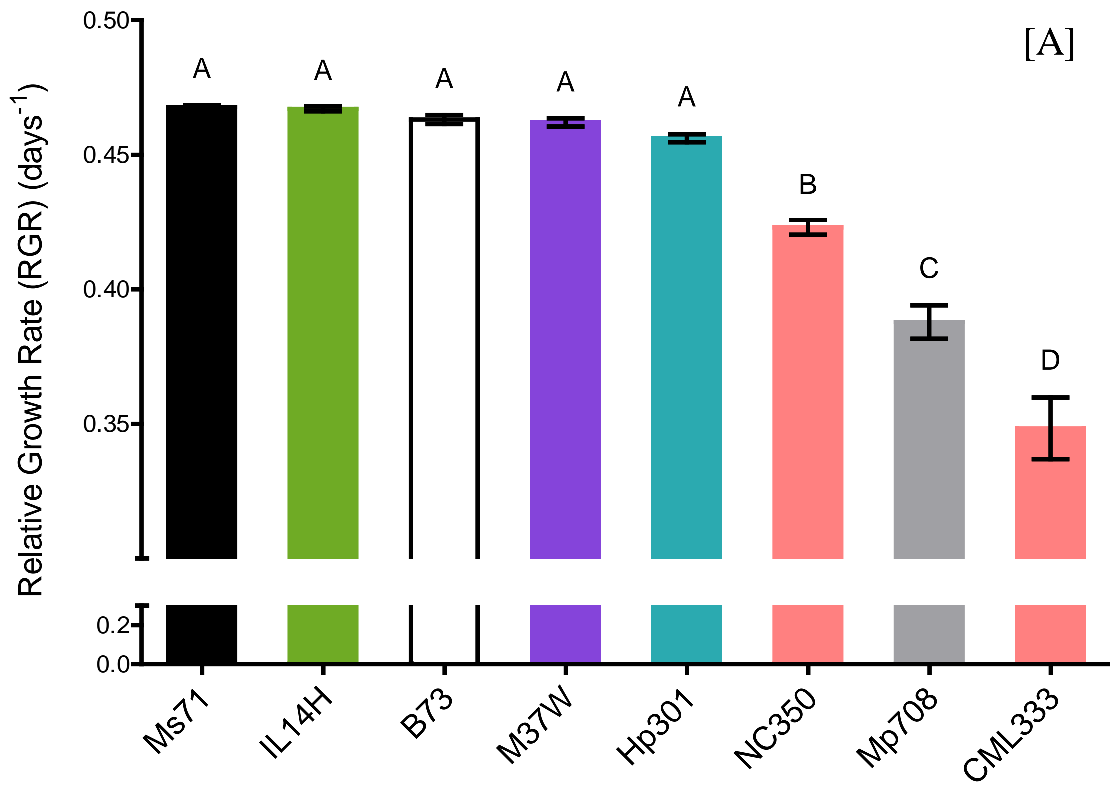


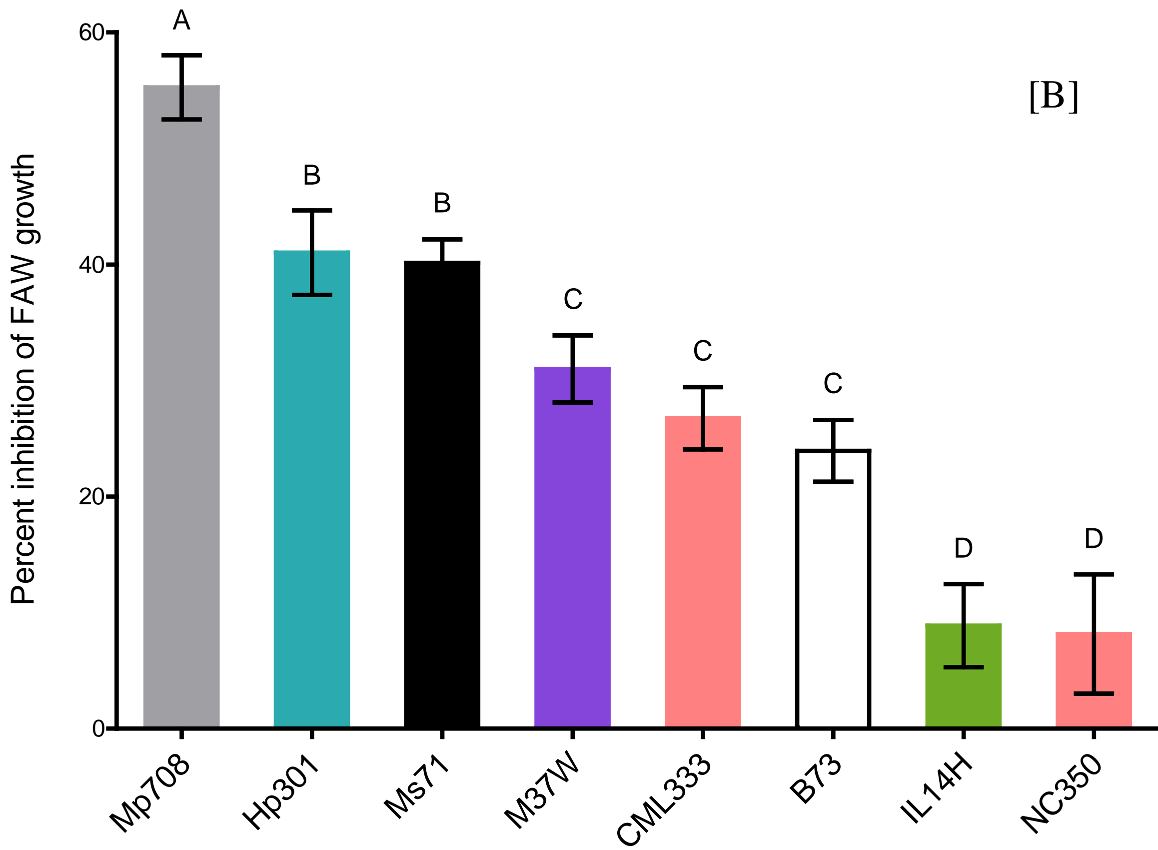
1.1
1.0
0.9
0.8
0.7
Pooled FAW nRGR
A
AB
BC
C
C
D
SS
Sweet Corn
Popcorn
Mixed
NSS
TS
Genetic Group

Figure 2.2. The constitutive defense of the NAM founder lines to FAW is not independent of their phylogenetic relationship.

The genetic groups are according to Liu's assignment (Liu et al. 2003), including sweetcorn (green), popcorn (turquoise), tropical or semitropical (TS) (peach), Stiff Stalk (SS) (black), non-Stiff Stalk (NSS) (black and dots), and mixed origin group (purple). The box plot whisker is plotted by Tukey method and sample size varies between 27 and 30 FAW larvae per genetic group.

For each genetic group, the consecutive FAW nRGRs per inbred line were added and averaged by the number of inbred lines, and resulted in the consecutive input values (pooled FAW nRGR) for single genetic group. For example, the first FAW nRGRs from M162W, Mo17, OH43, Ms71, Ky21, and Mo18W were added, averaged by six (six inbred lines in NSS group), and resulted in the first pooled FAW nRGR for NSS group. After the calculation, each genetic group had 27 to 30 pooled FAW nRGRs as biological replicates. The median is shown for each genetic group, but letters indicate significant differences of means determined by Fisher least significant difference (LSD) test ($P<0.001$).


[A]
Relative Growth Rate (RGR) (days-1)
0.50
0.45
0.40
0.35
0.2
0.0
A
A
A
A
A
B
C
D
Ms71
IL14H
B73
M37W
Hp301
NC350
Mp708
CML333


[B]
Percent inhibition of FAW growth
60
40
20
0
A
B
B
C
C
C
D
D
Mp708
Hp301
Ms71
M37W
CML333
B73
IL14H
NC350

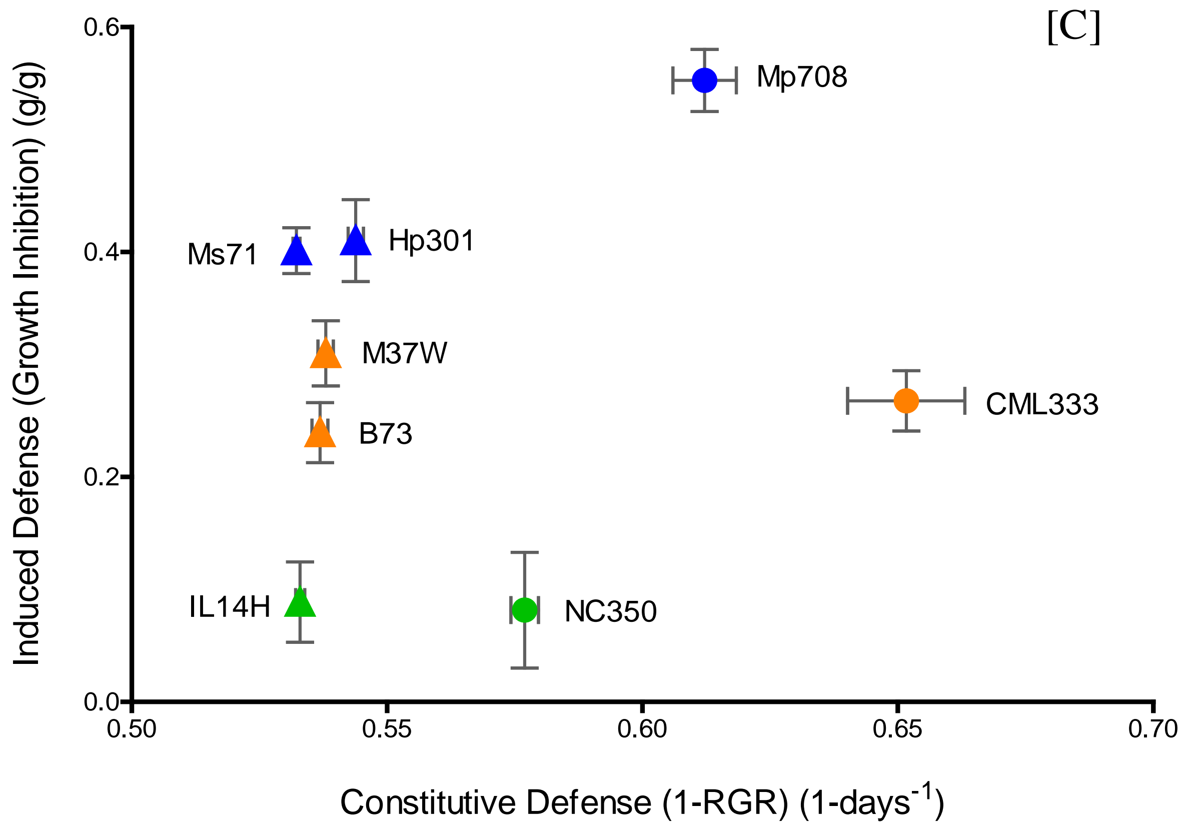


Figure 2.3. The eight selected maize inbred lines differ in the constitutive and induced defenses to FAW. Colored bars in [A] and [B] represent the genotypes selected from each genetic group in accordance with **Figure 2.1**.

[A] Validation of the constitutive defense of the eight inbred lines shown by the relative growth rate of FAW. Leaves from five to six maize plants per genotype were cut into 1-1.5cm pieces and mixed. Thirty FAW larvae fed on each genotype for 4 days and initial and final weights were recorded. Relative growth rate (RGR) was calculated as {(final weight – initial weight)/[(final weight + initial weight)/2]}/days. Letters indicate significant differences determined by Fisher LSD test (P<0.001). Error bar indicates SEM.

[B] The eight inbred lines have different levels of induced defense to FAW. The induced defense is indicated by percent inhibition of FAW growth from the infested-leaf bioassay. The percent inhibition of FAW growth was calculated as {(Average of Weight control - Weight

infested)/Average of Weight control} *100 for each larva. 14 to 15 maize plants per genotype were infested with five 5th instar FAW for 24 hr, and then leaf tissues around the feeding sites were collected and mixed. On the same day, leaves from 14 to 15 control maize plants per genotype were collected and mixed. 30 FAW larvae fed on each genotype/treatment for 4 days. Because FAW fed on infested and control plants started with the same initial weights, the final weights of FAW fed on infested and control plants were used to calculate the growth inhibition. Letters indicate significant differences determined by Fisher LSD test ($P<0.001$). Error bar indicates SEM.

[C] The eight inbred lines exhibit a divergent pattern in the constitutive and induced defenses. Constitutive defense is indicated by 1 – FAW relative growth rate, and induced defense is indicated by FAW growth inhibition. The circles are resistant genotypes (Mp708, CML333, and NC350). The triangles are susceptible genotypes. Blue indicates highly inducible genotypes (Mp708, Hp301, and Ms71). Orange indicates medium inducible genotypes (CML333, M37W, and B73). Green indicates minimally inducible genotypes (IL14H and NC350). Error bars (both directions) indicate SEM.

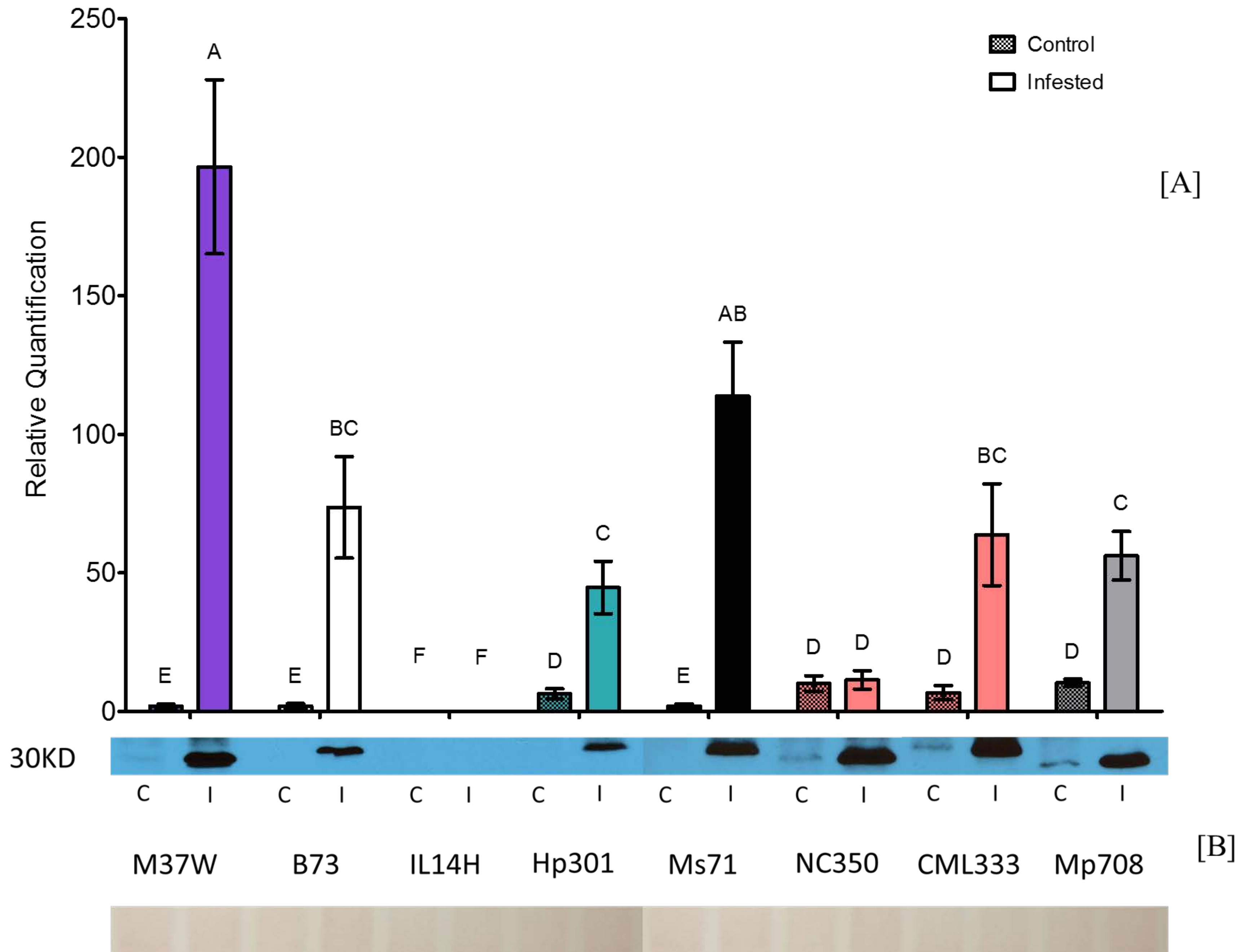

250
200
150
100
50
0
Relative Quantification
Control
Infested
[A]
A
BC
F
F
C
D
E
E
E
AB
D
D
D
BC
D
C
30KD
C
I
M37W
B73
IL14H
Hp301
Ms71
NC350
CML333
Mp708
[B]

Figure 2.4. Ribosome-inactivating protein 2 (*rip2*) transcript and protein accumulation in response to FAW infestation are correlated. Colored bars in [A] represent the genotypes selected from each genetic group in accordance with **Figure 2.1**.

[A] Gene expression analysis of *rip2* mRNA in the eight inbred lines prior to and after FAW infestation. Individual maize plant per genotype was infested with five 5th instar FAW larvae for 24 hr. Uninfested plants were used as the control. Total RNA was isolated from the leaf tissues around the feeding sites. Relative expression was determined by qRT-PCR in each biological replicate and normalized to the expression of two endogenous genes (n=3 to 4, error bar indicates SEM). Letters indicate significant differences determined by Fisher LSD test ($P<0.001$).

[B] Immunoblot analysis of RIP2 protein in the eight inbred lines prior to and after FAW infestation. Individual maize plant per genotype was infested with five 5th instar FAW larvae for 24 hr. Leaf tissues around the feeding sites were collected. Leaf samples from control plants were collected at 0 hr time point. Each lane represents the combination of four biological replicates. Equal amounts of proteins (30 µg) were loaded in each lane. C: control plant; I: infested plant. Maize leaf Rubisco large unit stained by Ponceau S indicates equal amounts of proteins loading per lane.

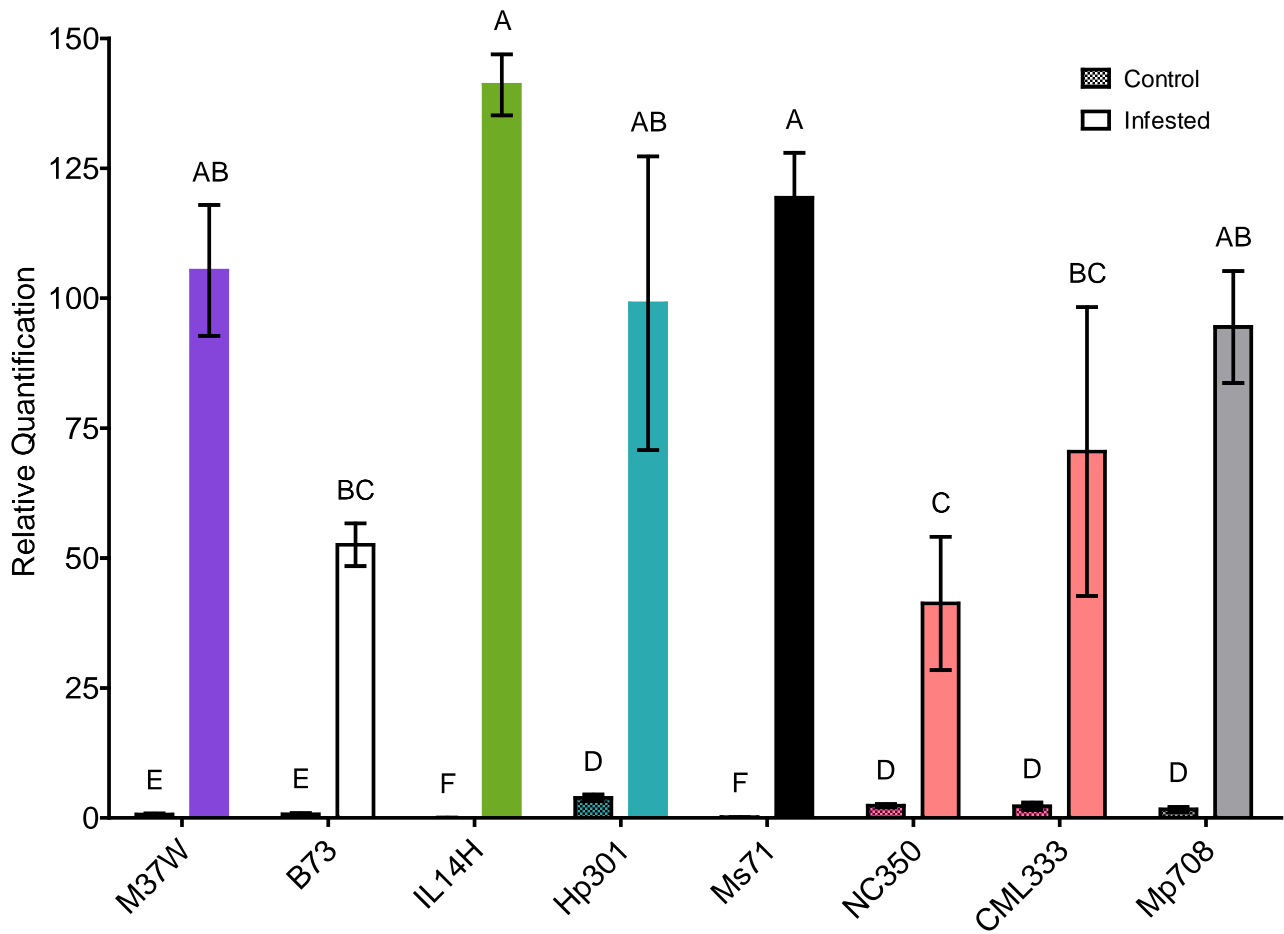


Figure 2.5. Gene expression analysis of maize proteinase inhibitor (*mpi*) mRNA in the eight inbred lines prior to and after FAW infestation. Colored bars represent the genotypes selected from each genetic group in accordance with **Figure 2.1**. Individual maize plant per genotype was infested with five 5th instar FAW larvae for 24 hr. Uninfested plants were used as the control. Total RNA was isolated from the leaf tissues around the feeding sites. Relative expression was determined by qRT-PCR in each biological replicate and normalized to the expression of two endogenous genes (n = 3 to 4, error bar indicates SEM). Letters indicate significant differences determined by Fisher LSD test (P<0.001).

Supplementary Data

Supplementary Table 2.1. Normalized relative growth rate (nRGR) of FAW fed on B73, Mo17, Mp708, and 25 NAM founder lines for 4 days. Genetic group and mean nRGR for each genotype are shown. Letters indicate significant differences determined by Fisher LSD test (P<0.001). Ranking equals the statistical result.

Genotype	Genetic Group	Mean	Ranking
M37W	Mixed	1.0021	A
B73	SS	1.0000	AB
IL14H	Sweet corn	0.9968	ABC
P39	Sweet corn	0.9947	ABC
Oh7B	Mixed	0.9941	ABC
M162W	NSS	0.9913	ABC
CML228	TS	0.9891	ABCD
Tzi8	TS	0.9890	ABCD
Hp301	Popcorn	0.9879	ABCD
Mo17	NSS	0.9868	ABCD
OH43	NSS	0.9833	ABCD
CML52	TS	0.9818	ABCD
CML247	TS	0.9791	ABCDE
Tx303	Mixed	0.9767	ABCDE
Ms71	NSS	0.9765	ABCDE
Ki3	TS	0.9739	BCDEF
CML69	TS	0.9735	BCDEF
Ki11	TS	0.9727	CDEF
CML277	TS	0.9722	CDEF
CML322	TS	0.9713	CDEF
Ky21	NSS	0.9640	DEFG
CML103	TS	0.9526	EFG
NC358	TS	0.9484	FG
Mo18W	Mixed	0.9413	GH
B97	NSS	0.9393	GH
NC350	TS	0.9175	HI
CML333	TS	0.9070	I
Mp708	NA	0.9042	I

Supplementary Table 2.2. List of gene specific primers for quantitative real-time PCR in maize.

Gene	Forward	Reverse	NCBI Accession Number
Adenine phospohoribosyltransferase 1 (*apt1*)	AGGCGTTCCGTGACACCATC	CTGGCAACTTCTTCGGCTTCC	BT085274.2
Glyceraldehyde-3-phosphate dehydrogenase (*gapdh*)	GCTGCCAAGGCTGTTGGTAAAGTT	AGGTCAACAACCGAGACATCCACA	XM_008679567.1
Maize proteinase inhibitor (*mpi*)	GCGGATTATCGCCCTAACC	CGTCTGGGCGACGATGTC	X78988.2
Ribosome-inactivating protein 2 (*rip2*)	GAGATCCCCGACATGAAGGA	CTGCGCTGCTGCGTTTT	L26305.1

Supplementary Table 2.3. qRT-PCR original data for *mpi* and *rip2* relative quantification.

The square root (*apt1*gapdh*) was calculated from the original cycle threshold (Ct) values of *apt1* and *gapdh*.

Genotype	Infestation	Square root (*apt1*gapdh*)	*mpi* relative quantification	*rip2* relative quantification
B73	Control	21.71324	0.999998	0.937169
B73	Control	20.70986	1.115443	1
B73	Control	22.91384	0.499862	4.518946
B73	Control	21.39603	0.116209	1.228733
B73	Infested	23.34488	46.80673	65.93753
B73	Infested	22.79694	60.53489	67.18555
B73	Infested	22.25585	50.33916	36.9882
B73	Infested	24.05434	132.3392	124.4551
Mp708	Control	23.86156	3.051083	9.036134
Mp708	Control	22.35121	1.091997	13.69287
Mp708	Control	23.33768	0.916014	10.94975
Mp708	Control	21.81087	1.465743	7.417926
Mp708	Infested	24.14873	123.1686	52.66874
Mp708	Infested	23.32798	80.26737	37.56002
Mp708	Infested	23.43223	75.68787	79.91388
Mp708	Infested	23.77598	98.615	54.42205
CML333	Control	19.68438	0.67884	1.5741
CML333	Control	22.1437	3.456858	5.763101
CML333	Control	22.94292	3.431112	14.04574
CML333	Control	20.9562	1.386996	5.390232
CML333	Infested	22.58852	23.04336	31.57396
CML333	Infested	23.87068	145.557	411.5215
CML333	Infested	22.5662	34.48653	64.50527
CML333	Infested	23.34217	78.89861	95.22787
M37W	Control	21.6463	0.383702	3.141479
M37W	Control	21.4647	1.177279	3.0332
M37W	Control	20.11318	0.286567	1.017006
M37W	Control	20.86553	0.774473	0.504061
M37W	Infested	23.48921	83.86325	197.451
M37W	Infested	23.89744	141.7492	144.967
M37W	Infested	24.47418	96.86768	284.7057
M37W	Infested	23.35849	98.85561	158.9727
Hp301	Control	23.28414	4.160921	8.641531
Hp301	Control	22.93776	2.704791	3.839453
Hp301	Control	22.59807	4.786036	10.22632

Hp301	Control	21.50529	0.291598	2.534033
Hp301	Infested	23.61577	34.28568	31.2529
Hp301	Infested	23.40438	79.84546	36.51766
Hp301	Infested	24.49333	113.3064	72.67914
Hp301	Infested	24.31429	168.5521	38.1189
IL14H	Control	22.80498	0.055159	0.008669
IL14H	Control	21.44712	0.009105	0.002464
IL14H	Control	22.74726	0.051226	0.013152
IL14H	Control	22.3427	0.009476	0.004002
IL14H	Infested	24.15866	129.3756	0.083841
IL14H	Infested	22.96596	68.48499	0.006105
IL14H	Infested	24.07193	148.1268	0.021643
IL14H	Infested	24.20448	145.6374	0.059401
NC350	Control	22.09284	2.45649	9.279084
NC350	Control	20.93943	1.621343	5.546483
NC350	Control	21.88821	3.105807	15.20372
NC350	Control	21.25401	2.278006	25.77956
NC350	Infested	20.76225	26.33676	12.54804
NC350	Infested	19.81814	14.51933	5.071058
NC350	Infested	22.73755	52.94082	16.43492
NC350	Infested	22.88505	71.29891	73.67954
Ms71	Control	21.54508	0.206018	1.236471
Ms71	Control	20.33126	0.030522	0.562296
Ms71	Control	21.61284	0.226189	3.991645
Ms71	Control	20.68225	0.038903	1.739166
Ms71	Infested	24.324	128.9581	130.9745
Ms71	Infested	23.71996	102.0915	74.85896
Ms71	Infested	23.8914	126.9537	135.4431

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Chapter 3

Soil-Mediated Cover Crop Effect on Corn Defense Responses to Black Cutworm

Introduction

Since agriculture entered the industrialized era, it became largely dependent on external inputs like synthesized fertilizers, herbicides and pesticides. Humans successfully produced more food, fiber and agricultural products with synthesized chemicals, but these chemicals have a negative impact on the environment and do not benefit the sustainability of ecosystems (Altieri 1998; Horrigan et al. 2002). On the contrary, organic agriculture is gaining popularity worldwide (Gliessman 1990; Willer et al. 2014a; Willer et al. 2014b; Balogun et al. 2016). "Organic agriculture is an ecological production management system that promotes and enhances biodiversity, biological cycles and soil biological activity. It is based on minimal use of off-farm inputs and on management practices that restore, maintain and enhance ecological harmony" (Gold 2014). It incorporates farming practices according to naturally occurring biological processes and biodiversity rather than external inputs with adverse effects (LEISA 2006a, 2006b). "Organic practices include enhancing predatory beneficial insects and micro-organisms, rotating crops, and using cover crops, biologic pesticides, herbicides and minimum tillage" (IFOAM 2002). Organic agriculture can be economically more competitive than conventional agriculture. Higher market prices and premiums are the main drives for organic products profitability even with less crop yield and higher labor cost (Pimentel et al. 2005; Seufert et al. 2012; Crowder and Reganold 2015). Organic products deliver healthy and nutritious food (Brandt and Mølgaard 2001; Brandt et al. 2011; Barański et al. 2014; Lairon and Huber 2014). With its potential to meet multiple sustainability goals, organic agriculture is likely to continue to expand globally.

Cover crops are a major component of soil fertility management practice for organic farming (O.F.R.F. 1998). Cover crops include plants such as small grains, legumes, Brassicas, and others that are planted between cash crop seasons to keep a living cover on the landscape (PFI http://practicalfarmers.org/member-priorities/cover-crops/). The implementation of cover crops

has a long-standing history in agricultural practices (Salon 2009). Cover crops have their main influences on soil nutrient cycling (Blanco-Canqui et al. 2015). The influences include supplying nutrients to the following cash crops, nutrient retention and reduction in leaching, and adding soil organic matter (Kuo and Sainju 1998; Blanco-Canqui et al. 2012; Licht et al. 2016). Cover crops also play a role in weed suppression (Teasdale et al. 1991; Creamer et al. 1996; Bryant et al. 2013) and insect pest regulation (Bottenberg et al. 1999; Crowder et al. 2010) in organic cropping systems. With the combined functions, cover crops can promote the cash crop yield during harvest season (Decker et al. 1994; Kuo and Jellum 2002; Tonitto et al. 2006; Quemada et al. 2013). Therefore, using cover crops will achieve the goal of sustainable agriculture not only in providing profitability to farmers compared to other agricultural management practices, but also in providing key services to current agricultural ecosystems.

Black cutworm (BCW), *Agrotis ipsilon* (Hufnagel), is one of the most widely distributed species of the cutworm family. The range of its distribution includes six of the seven continents except Antarctica (Harris et al. 1962; Rings et al. 1974). Adult female moths lay eggs singly, or in masses, on grasses, weeds, and plant debris. Larvae go through six or seven instars to reach pupation. Black cutworm feeds on various plants and is a notorious pest to both agricultural important crops and noneconomic plant species. It can cause damage to seedling stage of field corn, cotton, tobacco, wheat, vegetables, and turf grasses (Rings et al. 1975). Injury consists of cutting young seedlings; chewing into stems, roots, bulbs, and tubers; killing turf and golf course grasses. A previous plant cover of weeds, grasses, and plant debris will attract adult moths to oviposit, thus worsening the damage to following crops (Williamson and Shetlar 1995).

Black cutworm is an early season pest for corn plant throughout the Corn Belt in May and early June (Jarvis et al. 1981; Showers et al. 1985). When the larvae are small (3rd instar or younger), they do not have any cutting capability, therefore the damage is shown as leaf feeding damage, mainly small pinholes within the whorl (Archer and Musick 1977). As the larvae develop from 4th to 7th instars, they acquire their cutting ability. The corn seedlings can be cut just above or below the soil surface, and large larvae will drag a cut plant into the burrow and feed on it later (Archer and Musick 1977; Showers et al. 1983). Corn seedlings are vulnerable to BCW from the coleoptile through the four-leaf growth stage (Sherrod et al. 1979; Mulder and Showers 1983).

Black cutworm can especially be a problem for organic farming systems using cover crops as the main avenue in managing soil fertility. The reason is that prior to corn planting in early spring, BCW adults are able to lay eggs in nearby cover crops. The newly hatched larvae can feed and establish on cover crop plant materials or weeds, and they migrate to emerging corn seedlings for feeding. Therefore, BCW is an issue with cover crop farming systems (Waldron and Woodsen 2014).

Even though recent literature suggests that cover crops help reduce the presence of insect pests while attracting more natural enemies (Tillman et al. 2004; Prasifka et al. 2006; Schmidt et al. 2007; Koch et al. 2012), it is not always the case. The relationships of cover crop and insect pest and parasitoid and predator communities varies depending on the type of cover crop, whether it is single species or mixture, the following cash crop, the type of insect pest, as well as the type of parasitoid and predatory invertebrates. In a study performed in Minnesota, the addition of a rye cover crop in soybean production system successfully suppressed soybean aphid *Aphis glycine* at five out of six experimental sites because of increased plant diversity (Koch et al. 2015). However, in a study performed in Iowa, the presence of rye cover crop in cornfield was positively related to increased abundance of true armyworm *Pseudaletia unipuncta* (Haworth) and greater insect injury to corn; however, common stalk borer *Papaipema nebris* (Guenee) population was not affected by the presence of rye cover crop (Dunbar et al. 2016). In an annual bioenergy cropping system where maize stover was used for ethanol production, cereal rye/Austrian winter pea cover crop mixture showed neutral effects on predatory arthropod community and natural biological control services (insect egg prey removal rate) in Michigan and Wisconsin (Fox et al. 2016).

The above studies investigated the function of cover crop in insect pest regulation at a broad ecological scale. The experiments consisted of various cropping systems in multiple locations and years, as well as large-scale insect sampling. But if we take a closer look, a missing key point in these studies is the hidden mechanism that affects the arthropod community. The typical progression in cover crop use is that the cover crop is terminated and turned into plant debris before cash crop is planted. Thus, the cover crop exerts its influence on soil during its growing period (different ecosystem services), and then cash crop is planted and the growth and physiology of the cash crop is affected by the soil. Consequently, plant nutrients, secondary metabolites, and volatile compounds that could be involved in cash crop resistance or

susceptibility to herbivores vary. Therefore, the responses of insect pests and natural enemy population vary. What is unknown is how cover crops exert their influence on cash crop growth and physiology via soil-mediated effects, what changed in cash crop both physiologically and molecularly, and if these changes affect cash crop defense responses to insect pests.

The legacy effects of cover crop on soil quality may affect plant defense responses to insect pests in cash crops. Our study is about 1] how cover crop diversity affects the ability of the corn plant to mount a defense response against insect pests and 2] the mechanism of soil-mediated cover crop effect on cash crop plant resistance to insect pest at the molecular level. We performed both field and greenhouse experiments to examine whether cover crops affect corn resistance to BCW and whether the resistance relates to the expression of herbivore defense genes in corn. We hypothesize that corn grown in soil from the three and six species cover crop mixture will retard BCW growth and have more robust defense responses compared to the corn grown in fallow soil. Evaluation of the molecular defense response to plant-feeding insects will provide the initial information needed to illustrate a more complete picture of mechanisms contributing to changes in overall arthropod pest resistance that occur when plants are grown under organic management.

Materials and Methods

Experimental design

The following experiments belonged to a three-year research project investigating multiple functions of cover crop cocktails for organic cropping systems (Kaye et al. 2011). The three-year project included a field experiment from 2012-2015 at the Russell E. Larson Agricultural Research Center near Rock Springs, PA. A detailed description of precipitation, temperature and soil types for the research site can be found in Murrell et al. (2016).

The research project used a randomized complete block design with four replications in which 12 cover crop diversity treatments were embedded in a rotation of corn (*Zea mays* subsp. *mays.*), soybean (*Glycine max*), and winter wheat (*Triticum aestivum*). The main plot (24 × 348 m) was divided into 12 subplots (24 × 29 m) in a split-plot design and the cover crop diversity treatments were planted in these subplots in a randomized order (Murrell et al. 2016; Supplemental Figure 1). A detailed description of what the cover crop diversity treatments were, the seeding rate of cover crop monoculture and mixtures, how they were planted, and the management and cultivation of the research site, can be found in Murrell et al. (2016).

The cover crop treatments we studied for corn defense to BCW were a nitrogen management three species mixture (3SppN), six species mixture (6Spp), and no cover crop (fallow soil). Because corn is a high-nitrogen (N) demanding crop species (Vanotti and Bundy 1994), this cash crop was preceded by the 3SppN cover crop mixture to increase N inputs. The 3SppN mixture included Austrian winter pea (*Pisum sativum*), common medium red clover (*Trifolium pratense*), and cereal rye (*Secale cereale*) that will maximize N fixation in the fall and spring, and N uptake by the rye will reduce N losses and stimulate N fixation (Kaye et al. 2011). Rye mixed with legume cover crops can improve the synchrony of cover crop N mineralization with corn N demand (Stute and Posner 1995). The 6Spp cover crop mixture included six cover crop species and they were forage radish (*Raphanus sativus*), winter canola (*Brassica napus*), and oats (*Avena sativa* L.) in addition to those in the 3sppN mixture. The aim was to assess whether high diversity (6Spp vs. 3Spp) was necessary or redundant in relation to corn defense against BCW. The fallow soil treatment provided the baseline information regarding whether cover crop treatments exhibit a real effect on corn defense against BCW.

Another experimental factor was manure application to corn subplots. For manure application, subplots were fertilized with dairy bedded-pack manure incorporated in spring prior to corn planting (43 Mg ha^{-1}in 2014, and 47 Mg ha^{-1}in 2015) (Kaye et al. 2011). Manure exclusion subplots were not fertilized. The purpose was to evaluate whether manure affects corn defense against BCW.

2014 and 2015 summer growing corn for insect bioassay

MC4050 conventional corn seed (King's Agriseeds, PA, USA) were soaked in tap water overnight to ensure the success of germination. For each cover crop treatment, 30 seeds (ten seeds per row and three rows) were planted in manure application and manure exclusion subplots. Row 10/11/12 were used in manure application subplots and row 4/5/6 were used in manure exclusion subplots. Ten feet buffering zone was avoided to eliminate the edge effect.

In 2014, the planting was finished on June 16th, two weeks after the cash crop corn planting. The seeds were placed between the cash crop corn plants and the depth was 3 cm. The plants were harvested on July 22nd to conduct the insect bioassays.

In 2015, the planting was finished on June 5th and it was one week after the cash crop corn planting. The seeds were placed between the cash crop corn plants and the depth was 5 cm. The plants were harvested on July 7th to start the insect bioassays.

BCW rearing

BCW eggs were purchased from Benzon Research (Benzon Research, Carlisle, PA). Eggs were hatched in at 27 °C in an incubator. After hatching, neonates were reared on an artificial diet (Peiffer and Felton 2005) in the same incubator with 14 hr light:10 hr dark.

For insect bioassays, larvae were reared on artificial diet for four days before the experiment. When larvae reached 3.5 to 5.5 mg, they were used in insect bioassays. For greenhouse infestation experiments, larvae were reared on artificial diet until the 3rd or 4th instar.

Insect bioassay

The BCW non-choice feeding bioassay was set up over three days. On the first day, 15 to 20 corn plants were collected from field treatments by cutting them at the base of the stem. Plants were taken back to the laboratory and stored at 4 °C overnight. On the second day, two to three layers of leaves in the whorl region were cut into 1 to 1.5 cm pieces and the midribs were discarded. Leaf segments were placed in 1 oz plastic cups to provide sufficient materials for four days of feeding. The leaves were stored at 4 °C cold room overnight. On the third day, 20 BCW larvae were selected per combination of treatments for bioassay and total 480 larvae were used. Their initial weight range was 5 to 7 mg and this criterion reduced the size variation among BCW larvae. The bioassays were conducted in a 27°C incubator with a 14 hr light:10 hr dark. After four days, BCW larvae final weights were recorded. The data entered for statistical analysis is Relative Growth Rate (RGR). It was calculated as {(weight final-weight initial)/ ((weight final+ weight initial)/2)}/ 4 days (Mohan et al. 2008).

2014 and 2015 summer growing corn for greenhouse infestation

Cover crops were terminated in spring by flail mowing followed by incorporation with a moldboard plow (Murrell et al. 2016; Table 1). Soil from the research site was collected after the cover crop was incorporated into the soil. For each cover crop treatment, 18.9 liter (5 gallons) of soil was collected from manure application and manure exclusion subplots. Ten soil cores evenly distributed per row in five separated rows of the middle area in each subplot were taken using a

bulb planter with avoiding buffering areas (ten feet away from the edges of the subplots). The sampling depth was 10 cm (4 inches) from soil surface. A total of 24 soil buckets were filled and they were transported back to campus and stored at room temperature in the basement of the Agricultural Sciences and Industries Building (ASI) at The Pennsylvania State University before further processing. Storage at room temperature was to mimic similar field temperatures at the end of May. The buckets had lids loosely capped on top so that there was some airflow into the buckets. After five days, soil was hand mixed. The soil was poured into plastic bins. Small rocks were taken out and plant residues were chopped when necessary. For each bucket, 50 soil cores were homogenized by hand, and after mixing, the soil was poured back into the original bucket and stored at room temperature in the ASI basement before planting corn.

Corn planting for the greenhouse infestation experiment was on the same day as the cash crop corn was planted in the field to ensure the collected soil was stored for the same length of time as the soil in the field. The soil was poured into 15 cm circumference (6-inch) standard plastic pots and one corn seed was planted in each pot. The MC4050 conventional corn seeds were soaked in tap water overnight to ensure the success of germination. The soil samples were collected on May 20th 2014 and the greenhouse planting was finished on June 2nd, 2014. The soil samples were collected on May 22nd, 2015 and the greenhouse planting was finished on May 29th, 2015. The difference of greenhouse planting between 2014 and 2015 was that one seed was planted per pot in 2014, but it did not always ensure the success of germination. So, two MC4050 seeds were planted per pot in 2015, and after a week, one seedling was removed and each pot had one corn seedling. Corn plants reaching V4 (Ritchie et al. 1986) stages were used for infestation.

MC4050 corn grew in the Plant Science Department greenhouse at The Pennsylvania State University for four weeks (University Park, PA, USA). The temperature was from 22 to 30 °C and it was the ambient natural photoperiod for greenhouse environment. Photosynthetically active radiation ranged from 500 to 1200 µmol $m^{-2}s^{-1}$. Corn plants were watered with tap water as needed. Weeds in the pots were taken out by hand as needed. All the replicates were grown in the same greenhouse.

Greenhouse infestation

In 2014, BCW were reared to the 4th instar and starved for 24 hr before infestation to ensure that they would feed on corn. A single corn plant was infested with two BCW larvae for 24 hr, and

four plants were infested for each combination of treatments. After infestation, 0.1 g leaf tissue adjacent to the feeding site (approximately 5 mm surrounding the feeding site) was collected, immediately frozen in liquid N_2, and stored in -80 °C freezer. Leaf tissues from the control plants were collected at 0 hr. In 2015, BCW were reared to the 3rd instar and starved for 24 hr before infestation to make sure they would feed on corn. Each corn plant was infested with four BCW larvae for 48 hr, and four plants were infested for each combination of treatments. The rest was the same as 2014.

RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR)

The leaf tissues were homogenized using the Geno/Grinder 2000 (SPEX Certiprep, USA). Total RNA was extracted from leaf tissues using the TRIzol reagent and protocol (Life Technologies, USA). The RNA pellet was dissolved in diethylpyrocarbonate (DEPC)-treated water. After extraction, RNA was subjected to DNase digestion to remove residue genomic DNA. The reaction contained 26 µl RNA, 3 µl DNase buffer and 1 µl DNase (New England BioLabs, USA). It was incubated at 37 °C for 30 min and then DNase was inactivated at 65 °C for 10 min. Nanodrop 2000 (Thermo-Fisher Scientific, USA) was used to measure RNA concentration and its purity. For reverse transcription PCR, 1 µg total RNA was added into a 20 µl reaction with oligo-dT 20 primers following the manufacturer's protocol. The reverse transcription PCR program was 37 °C for 2 hr and 85 °C for 5 sec. The kit was the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA).

qRT-PCR was performed in an ABI 7500 Fast Real Time PCR system. Primer Express software was used to design gene-specific primers (Life Technologies, USA). Individual cDNAs were diluted 10 times before running qRT-PCR. The qRT-PCR reaction (10 µl system) contained 1 µl diluted cDNA, 1 µl H_2O, 1 µl forward primer (1 µm), 1 µl reverse primer (1 µm), and 5 µl SYBR Green Master Mix (Roche Applied Science, USA). The program was as follows: step 1, 50 °C for 2 min and 95 °C for 10 min; step 2, 95 °C for 15 sec and 60 °C for 1 min, 40 cycles; step 3, 72 °C for 10 min; step 4, dissociation stage. Endogenous gene *actin* was used in 2014 and two endogenous genes *gapdh* and *apt1* were used in 2015 to calculate the relative quantification of target gene expression by 2-ΔΔCT method (Vandesompele et al. 2002; Lin et al. 2014). The list of primers is shown in **Supplementary Table 3.1**.

Statistical analyses

All the statistical analyses were performed in SAS 9.4 Software (SAS 9.4, USA).

The RGRs of BCW in 2014 and 2015 were analyzed in response to cover crop treatment, manure treatment, and cover crop*manure interaction, by mixed models, and the experimental block was assigned as a random effect (PROC GLIMMIX, SAS 9.4). Pairwise comparisons with a Tukey adjustment were performed following significant effects.

Messenger RNA (mRNA) relative quantification of target genes in 2014 and 2015 were analyzed in response to cover crop, manure and infestation treatments, and four interaction terms, by mixed models, and the experimental block was assigned as a random effect (PROC GLIMMIX, SAS 9.4). Pairwise comparisons with a Fisher least significant difference (LSD) test were performed following significant effects.

Results

The reason for growing corn plants in field for insect bioassay was that the field provides a realistic growing condition truly reflecting what is happening in the agroecosystem. Thus, the results will deliver more biological and ecological meanings for organic farming systems. The second reason is that conducting insect bioassay requires many corn plants. This was a more feasible alternative than growing corn in soil brought to the greenhouse from the field. Therefore, plants were grown in the field and collected for the insect feeding bioassays.

Since the greenhouse infestation experiments were to artificially infest the corn plants with BCW and examine defense gene expression, it was infeasible and uncontrollable to grow corn in field for artificial infestations. Therefore, we collected soil after cover crop for growing corn plants in the greenhouse.

2014 and 2015 BCW bioassays

Statistical analysis showed that cover crop and manure treatments had significant effects on BCW growth in 2014 (**Table 3.1**). BCW fed on corn grown in 3SppN soil and in manure application subplots had higher RGRs (**Figure 3.1**). Also, the interaction of cover crop and manure treatments had a significant effect on BCW growth, indicating that the cover crop effect was influenced by the manure.

In manure exclusion subplots, the RGR of BCW fed on corn grown in 3SppN soil (3SppN corn) was the highest (**Figure 3.1**). The RGR of BCW fed on corn grown in fallow and 6Spp soils

(fallow and 6Spp corn) were lower, but similar. In manure application subplots, the RGR of BCW fed on fallow corn was the highest, but not statistically different from the RGR of BCW fed on 3SppN. The RGR of BCW fed on 6Spp corn was the lowest. Secondly, the RGRs of BCW fed on 6Spp and fallow corn were higher than those from manure exclusion. However, manure application did not create a difference on the RGR of BCW fed on 3SppN corn compared with manure exclusion.

Statistical analysis showed that cover crop had a significant effect on BCW growth in 2015, but manure did not (**Table 3.1**). BCW fed on fallow and 6SppN corns had higher RGRs (**Figure 3.2**). Also, the interaction of cover crop and manure treatments had a significant effect on BCW growth, indicating that the cover crop effect was influenced by the manure.

In manure exclusion subplots, the RGRs of BCW fed on 6Spp and fallow corn were higher than that on 3SppN corn (**Figure 3.2**). In manure application subplots, there were no significant differences of BCW growth among the cover crop treatments. Secondly, manure application resulted in lower RGRs for BCW fed on 6Spp and fallow corn than manure exclusion, but the RGR of BCW fed on 3SppN corn higher than manure exclusion.

Effects of BCW on corn defense gene expression in 2014

Caterpillar herbivory triggers jasmonic acid (JA) biosynthesis and signaling pathways in corn, and therefore the corn plant turns on downstream JA-inducible defense genes and generates defense proteins and secondary metabolites to thwart caterpillar herbivory (Howe and Jander 2008; Wasternack and Hause 2013). A general model of this induction pathway is well illustrated in Shivaji et al. (2010). The primary interest of this study is to determine if cover crop and manure treatments have effects on corn defense gene expression. To examine the possible molecular mechanisms linking the cover crop with corn resistance to BCW, the abundances of mRNAs encoding *aos*, *rip2*, *mpi* and *tps23* were determined in a greenhouse infestation experiment.

Allene oxide synthase (AOS) "catalyzes the first step in the biosynthesis of jasmonic acid from lipoxygenase-derived hydroperoxides of free fatty acids" and is a crucial regulatory step in octadecanoid biosynthesis (Laudert and Weiler 1998; Sivasankar et al. 2000). Ribosome-inactivating protein 2 (RIP2) is induced by caterpillar feeding. Since RIP2 resists digestion in caterpillar gut and is toxic, it is one of the defensive proteins that protects maize against

herbivory (Chuang 2012; Chuang et al. 2014). Maize proteinase inhibitor (MPI) inactivates proteolytic enzymes in insect gut to function as a defensive protein (Green and Ryan 1972; Koiwa et al. 1997). Wounding, abscisic acid, methyl jasmonate and caterpillar feeding induce *mpi* mRNA accumulation (Cordero et al. 1994; Tamayo et al. 2000). During herbivory, corn leaves are able to emit green leafy volatiles (GLV) to attract arthropod predators of insect pests (Turlings 1992; Engelberth et al. 2004; Farag et al. 2005). Maize terpene synthase 23 (TPS23) synthesizes the sesquiterpene, (*E*)-β-caryophyllene, as an airborne signal to attract natural enemies of caterpillar and rootworms (Köllner et al. 2008; Capra et al. 2015). The expression of the genes described above appears to be regulated at the transcriptional level.

Infestation showed significant effects on gene expression levels of target genes in 2014 and 2015 (**Table 3.2** and **3.3**). When corn plants were infested with BCW larvae, target genes mRNA accumulated at much higher levels than that in control plants, indicating that the BCW infestation in the greenhouse were successful in both years.

Statistical analysis showed that cover crop treatment had a significant effect on *aos* expression in 2014 (**Table 3.2**). 3SppN corn accumulated more *aos* mRNA compared to 6Spp and fallow corn, whereas 6Spp and fallow corns accumulated the same level of *aos* mRNA (**Figure 3.3**). Secondly, the manure treatment did not have a significant effect on *aos* expression and is not shown. The statistics showed that neither cover crop nor manure had a significant effect on *rip2* expression (**Table 3.2**).

Effects of BCW on corn defense gene expression in 2015

In 2015, the abundances of mRNA encoding *mpi* and *tps23* in addition to *aos* and *rip2* were determined in the greenhouse infestation experiment. The difference between the infestation experiments in 2014 and 2015 was that 48 hr infestation period was chosen in 2015 instead of 24 hr in 2014. The reason was that the feeding damage was not equal for all combinations of treatments at 24 hr in 2015.

The statistics showed that cover crop did not have a significant effect, but manure had a marginal effect on *aos* expression (**Table 3.3**). Corn plants in manure exclusion subplots accumulated more *aos* mRNA than corn plants in manure application subplots even though the difference was not statistically significant.

Statistical analysis showed that neither cover crop nor manure had a significant effect on *rip2* expression. However, the interaction between cover crop and manure treatments was statistically significant (**Table 3.3**). With manure application, 6Spp corn accumulated more *rip2* mRNA than fallow corn. 3SppN corn accumulated *rip2* mRNA at an intermediate level between 6Spp and fallow corn (**Figure 3.4**). With manure exclusion, plants grown in the three soil types accumulated similar levels of *rip2* mRNA. Secondly, manure application did not generate a difference in *rip2* mRNA levels in corn plants grown in 3SppN and fallow soils compared with manure exclusion. However, manure application in 6Spp soil enabled the corn plants to accumulate higher *rip2* mRNA than manure exclusion in 6Spp soil. Statistical analysis showed that neither cover crop nor manure had a significant effect on *mpi* expression (**Table 3.3**).

Statistical analysis showed that neither cover crop nor manure had a significant effect on *tps23* expression. However, the interaction between cover crop and manure treatments was statistically significant (**Table 3.3**). With manure application, 6Spp corn plants accumulated more *tps23* mRNA than fallow corn. 3SppN corn accumulated *tps23* mRNA at an intermediate level between 6Spp and fallow corns (**Figure 3.5**). With manure exclusion, the three soil types resulted in plants that accumulated similar levels of *tps23* mRNA. Secondly, corn plants grown in manure application and manure exclusion subplots did not have a difference in *tps23* mRNA accumulation for three levels of cover crop treatments.

Discussion

2014 and 2015 BCW bioassays

In manure exclusion subplots, BCW fed on 3SppN corn had higher RGR than BCW fed on fallow and 6Spp corn. Since corn is a high N demanding species, 3SppN soil provided more nitrogen (**Supplementary Figure 3.1**), resulting in corn with higher nitrogen content in leaves and other tissues. Therefore, BCW fed on 3SppN corn could have assimilated more nitrogen in various forms and gained more weight. In manure application subplots, BCW fed on fallow and 3SppN corns had higher growth than BCW fed on 6Spp corn. This most likely correlated with higher soil N in 3SppN and fallow soils than in 6Spp soil. Secondly, the effect of manure addition on BCW growth was dependent on cover crop. In 6Spp and fallow soils, manure increased BCW growth, but in 3sppN soil, manure did not create a difference in BCW growth. Again, this could be due to elevated soil N in 6Spp and fallow soils from manure application that

led to elevated N content in 6Spp and fallow corn. The increased N in the plant could have a positive effect on BCW growth. However, 3SppN corn absorbed adequate amount of N from 3SppN soil provided by the legume cover crops so that the extra N from manure made little difference in 3SppN corn N content. So, manure did not create a difference in BCW growth for 3SppN soil.

In 2015, BCW growth was opposite of the trend in 2014. It was mainly because of the environmental effects. Since cover crop diversity treatments were embedded in a rotation of corn, soybean, and winter wheat, the corn plants were grown in a different set of subplots in 2015 compared to in 2014. Also, the corn plants grew four weeks in field during 2015 instead of five weeks during 2014.

2014 gene expression analysis

In the JA biosynthetic pathway, AOS converts 13-hydroperoxy octadecadienoic acid (13-HPOT) into allene oxide (Laudert and Weiler 1998; Sivasankar et al. 2000). 3SppN corn accumulated more *aos* mRNA than 6Spp and fallow corn, which suggests that the JA biosynthetic pathway was more likely upregulated in 3SppN corn. Thus, 3SppN corn had the potential to produce more JA upon BCW herbivory, and could be more resistant to BCW at the molecular level. 6Spp and fallow corns accumulated similar levels of *aos* mRNA, which means that the additional three cover crop species in 6Spp soil may act antagonistically/counteractively to pea, clover and rye (3SppN) in term of enhancing corn resistance to BCW. The soil nitrogen (N) content showed that 3SppN soil had more N than 6Spp and fallow soils in both manure application and exclusion subplots (**Supplementary Figure 3.1**). Higher N level in 3SppN soil would give the corn plants more resources to make defense molecules, therefore 3SppN corn accumulated more *aos* mRNA after 24 hr of BCW feeding.

It has been known that *rip2* mRNA abundance is a good prediction of RIP2 protein levels in various maize inbred lines (Chuang 2012; Chuang et al. 2014). In 2014, cover crop and manure treatments had neutral effects on *rip2* expression. Therefore, cover crop and manure treatments were not likely to alter the accumulation of RIP2 protein in corn plants upon BCW herbivory. If we look at how insect herbivory triggers plant defense responses, including production of JA, subsequent JA signaling, and induction of downstream defense genes (Howe and Jander 2008; Wasternack and Hause 2013), 3SppN corn accumulated more *aos* mRNA, but the effect may not

have been relayed to the final steps of defense gene expression, so there was no difference in *rip2* mRNA accumulation among these three levels of cover crop treatments. BCW is a slow feeder compared to fall armyworm or corn earworm. BCW hide in soil surface during the day and feed on young corn plants during night, whereas other caterpillars constantly feed (Clement and McCartney 1982; Reese 1986). In 24 hr feeding period, even though 3SppN corn accumulated more *aos* mRNA, it is possible that the signal was not delivered to downstream defense gene expression because of BCW sporadic feeding.

2015 gene expression analysis

Because BCW did not create enough damage in all treatments during the 24 hr feeding period in 2014, the infestation period was changed to 48 hr in 2015 to give BCW more time to feed.

For *rip2*, cover crop and manure had no effects on it*s* expression, but the interaction between cover crop and manure had a significant effect. With manure exclusion, three levels of cover crop treatments resulted in corn plants accumulating similar levels of *rip2* mRNA, but with manure applied, 6Spp corn accumulated more *rip2* mRNA than fallow corn. Even though 3SppN and 6Spp cover crop were likely to provide more nutrients for following corn cash crop, the corn plant could have been limited in the amount of resources needed to balance growth and defense. Thus, it cannot allocate more nitrogen or other resources to produce defense proteins, which ultimately led to similar levels of *rip2* mRNA in manure exclusion treatment. On the other hand, manure provided more resource to corn plants and the restriction of allocating resources between growth and defense was turned off. Therefore, 6Spp corn was able to accumulate more *rip2* mRNA in response to BCW infestation.

For *tps23*, cover crop and manure had no effects on its expression, but the interaction between cover crop and manure had a significant effect. With manure application, 6Spp corn accumulated more *tps23* mRNA than fallow corn, while with manure exclusion, there was no difference in t*ps23* mRNA levels among cover crop treatments. TPS23 is one of the herbivore-induced terpene synthases in maize and produces sesquiterpenes as a volatile signal to attract natural enemies of lepidopteran herbivores (Köllner et al. 2013). Similar to *rip2* mRNA accumulation, manure has provided the corn plants with enough resources to turn on the production of volatile organic compounds (VOCs) as a method of indirect defense.

It is known that different insect herbivory triggers *de novo* JA biosynthesis within 30 minutes (JA level reaches a peak at 30 minutes), but after a few hours, JA level returns to normal (Howe and Jander 2008; Koo and Howe 2009). On the contrary, downstream defense genes and proteins take hours to respond to insect attack and are persistent in the feeding sites for days (Kant et al. 2004; Reymond et al. 2004; Chen et al. 2005; Chuang et al. 2014). The corn plant uses JA as a signal for insect herbivory, therefore generating the signal is a fast response; however, as defense proteins and metabolites are weapons to deter herbivory, the response is slower and longer.

The discrepancy between 2014 and 2015 gene expression results could also be due to a longer feeding period in 2015 (24 hr vs. 48 hr). Within 24 hr in 2014, 3SppN corn accumulated more *aos* mRNA, but the difference was not detected in *rip2* mRNA. A possible scenario is that 24 hr BCW feeding was long enough for cover crop to make a difference in JA biosynthesis, but not long enough to affect downstream defense gene expression. Within 48 hr in 2015, cover crop did not affect *aos* mRNA level, but affected *rip2* and *tps23* mRNA levels. It was likely that JA level passed the peak and went down to normal at the 48 hr time point, but it was long enough for the effect to be shown at defense gene expression. Thus, 6SppN corn accumulated more *rip2* and *tps23* mRNA than fallow corn. Current research on BCW is rare. Since young BCW larvae show a slow and sporadic feeding pattern on corn, how fast corn plant responds to such feeding pattern is unknown; this requires further investigation to find the best time points for sampling.

One ecosystem function of the cover crop is to provide nutrients to the following cash crop. The combination of legume with cereal and Brassica cover crop species aids in converting N into a form that can be biologically absorbed and prevents N from leaching to minimize its loss. It is not known how much N is contributed by cover crop residue to subsequent cash crop and whether the amount of N is in excess or limitation. A model-data fusion approach was used to build a model based on multiple datasets to tackle the challenge of predicting how cover crop residue decomposition contributed to N availability to subsequent corn crop in term of yield. The model suggested that the effectiveness of N provisioning to the corn crop favors a low cover crop biomass C/N ratio and a high cover crop biomass N ratio. Therefore, it will be convenient to predict whether cover crops are able to provide sufficient or limited N to corn plant (White et al. 2016).

Insect bioassays demonstrated that cover crops might have increased BCW growth by elevating soil N levels. The addition of manure did not always increase BCW growth. Secondly, cover crops increased the expression of several defense genes expression levels when corn plants were challenged by BCW. BCW grew better when corn plants obtained adequate amount of N from soil provided by cover crops, so the presence of cover crops benefited both corn plants and BCW at the phenotypic level. However, corn defense gene expression was elevated because of cover crops, so the presence of cover crops benefited corn plant, not BCW, at the molecular level. Therefore, the relationship of cover crops and corn resistance to BCW is not a simple linear relationship.

When we think about what happens in reality in a farmer's field, what suggestions should we give to the farmers after reaching these conclusions? The main purpose for a farmer to implement cover crops is to help the soil nutrient cycling process (Kaye et al. 2011). These conclusions suggested that in regard to BCW control, there is a possibility that cover crops may benefit BCW growth because BCW is able to obtain enough nutrients from healthy corn plants. But at the same time, cover crops also benefit the corn plants by improving their molecular herbivore defense responses. So, a farmer can keep the warning in mind, but not to worry so much.

This is the initial step in the investigation of the molecular mechanisms of soil-mediated cover crop effects on corn defense responses to BCW. The whole system has four levels of players in interactions: cover crop - soil - corn plant - BCW. It has opened the gate for illuminating the mechanisms contributing to changes in overall arthropod pest resistance that occur when crops are grown under organic management.

Table 3.1. Mixed model analyses of black cutworm (BCW) relative growth rate (RGR) by cover crop and manure treatments in 2014 and 2015.

Effect	2014			2015		
	df	F	P	dF	F	P
Cover Crop	2	22.72	<.0001	2	25.02	<.0001
Manure	1	16.82	<.0001	1	1.16	0.2820
Cover Crop*Manure	2	10.69	<.0001	2	20.14	<.0001

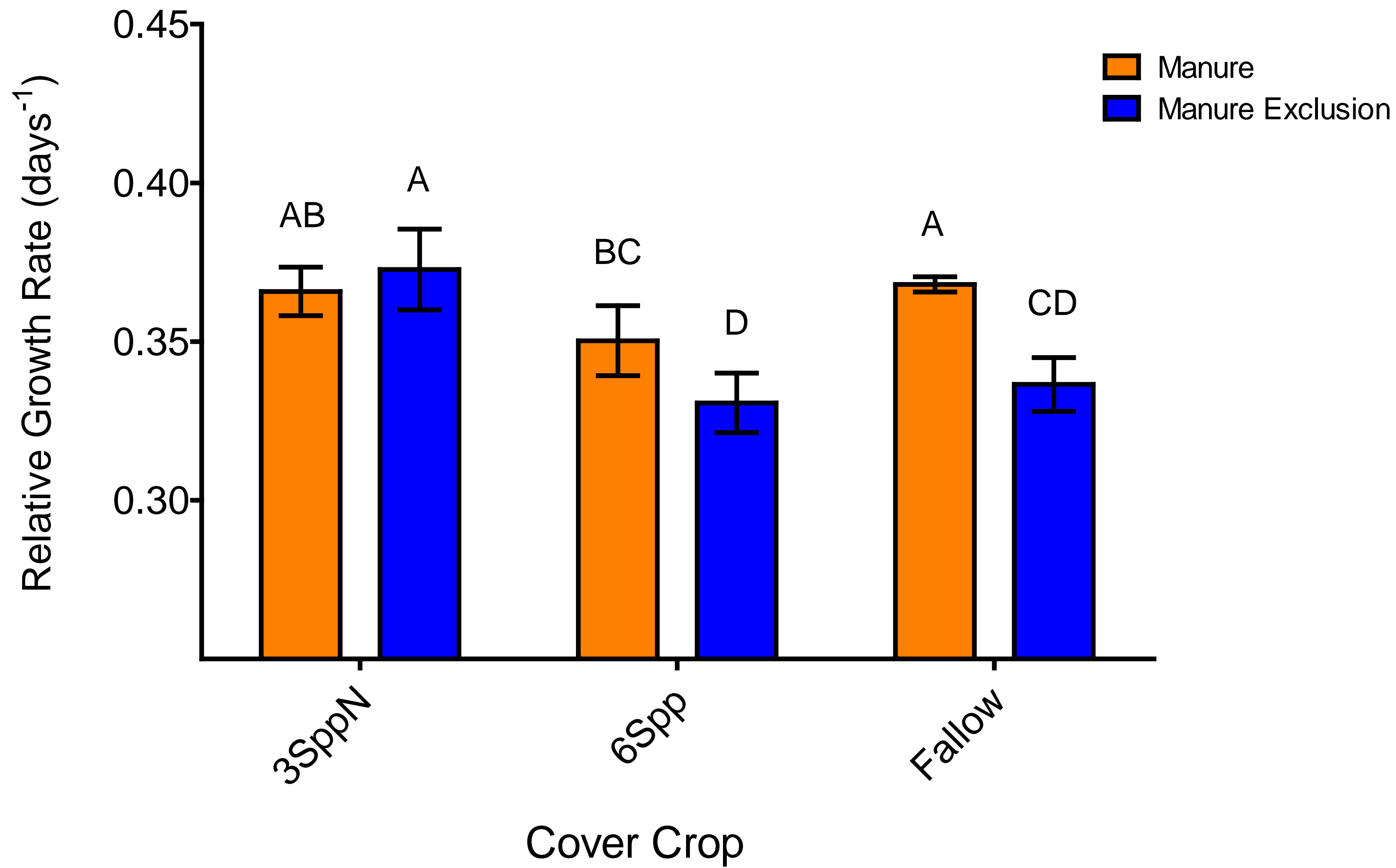
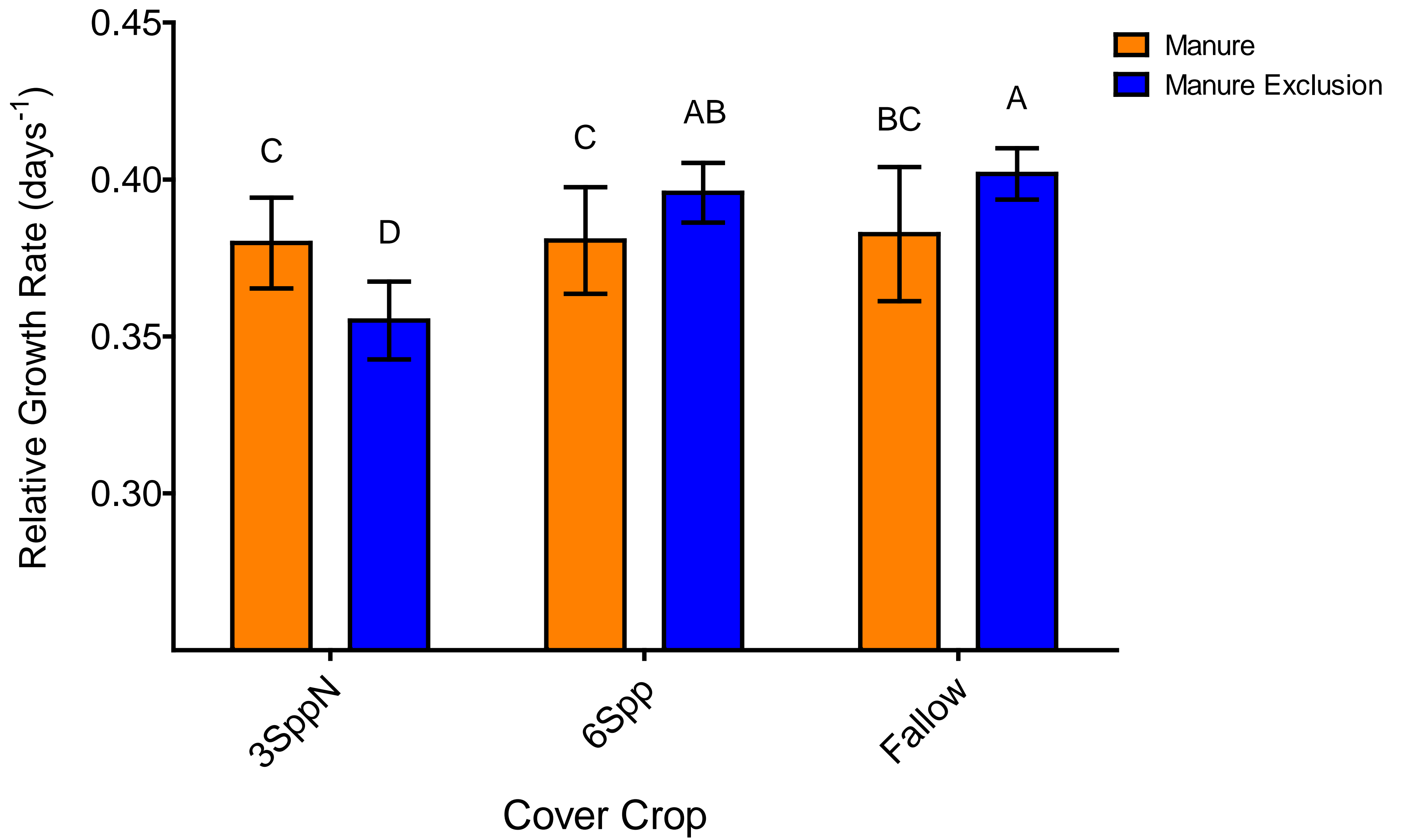

0.45
0.40
0.35
0.30
Relative Growth Rate (days^{-1})
Manure
Manure Exclusion
AB
A
BC
D
A
CD
3SppN
6Spp
Fallow
Cover Crop

Figure 3.1. Relative growth rate (RGR) of BCW fed on corn grown in manure application and exclusion subplots for 4 days in 2014. Leaves from 15 to 20 corn plants of each combination of treatments were cut into 1 - 1.5 cm pieces and mixed together. 20 BCW larvae fed on these leaves 4 days and initial and final weights recorded. RGR was calculated as {(final weight – initial weight)/[(final weight + initial weight)/2]}/4 days. Orange bars indicate manure application subplots; blue bars indicate manure exclusion subplots.



Manure
Manure Exclusion
Relative Growth Rate (days^{-1})
0.45
0.40
0.35
0.30
C
D
C
AB
BC
A
3SppN
6Spp
Fallow
Cover Crop

Figure 3.2. Relative growth rate (RGR) of BCW fed on corn grown in manure application and exclusion subplots for 4 days in 2015. Leaves from 15 to 20 corn plants of each combination of treatments were cut into 1 - 1.5 cm pieces and mixed together. 20 BCW larvae fed on these leaves for 4 days with initial and final weights recorded. RGR was calculated as {(final weight – initial weight)/[(final weight + initial weight)/2]}/4 days. Orange bars indicate manure application subplots; blue bars indicate manure exclusion subplots.

Table 3.2. Mixed model analyses of allene oxide synthase (*aos*) and ribosome-inactivating protein 2 (*rip2*) mRNA relative quantification (RQ) by cover crop, manure and infestation treatments in 2014.

Effect	*aos*			*rip2*		
	df	F	P	df	F	P
Cover Crop	2	3.64	0.0283	2	1.84	0.1449
Manure	1	0.22	0.6492	1	0.01	0.9314
Infestation	1	140.94	<.0001	1	334.89	<.0001
Cover Crop*Manure	2	0.19	0.8216	2	0.12	0.8860
Cover Crop*Infestation	2	0.39	0.6784	2	0.69	0.5062
Manure*Infestation	1	0.07	0.7959	1	0.22	0.6545
Cover Crop*Manure*Infestation	2	0.38	0.6709	2	0.44	0.6358

Table 3.3. Mixed model analyses of allene oxide synthase (*aos*), ribosome-inactivating protein 2 (*rip2*), maize protease inhibitor (*mpi*) and terpene synthase 23 (*tps23*) mRNA relative quantification (RQ) by cover crop, manure and infestation treatments in 2015.

Effect	*aos*			*rip2*			*mpi*			*tps23*		
	df	F	P	df	F	P	df	F	P	df	F	P
Cover Crop	2	2.01	0.1373	2	0.37	0.6899	2	0.19	0.8268	2	0.29	0.7490
Manure	1	3.84	0.0518	1	0.87	0.3527	1	0.01	0.9312	1	0.14	0.7098
Infestation	1	70.24	<.0001	1	194.86	<.0001	1	548.45	<.0001	1	271.10	<.0001
Cover Crop*Manure	2	1.41	0.2475	2	4.15	0.0174	2	1.56	0.2129	2	3.70	0.0269
Cover Crop*Infestation	2	2.57	0.0799	2	0.16	0.8547	2	0.13	0.8804	2	0.00	0.9996
Manure*Infestation	1	0.96	0.3298	1	0.74	0.3894	1	1.11	0.2932	1	0.01	0.9157
Cover Crop*Manure*Infestation	2	1.17	0.3121	2	0.28	0.7556	2	0.00	0.9962	2	0.93	0.3960

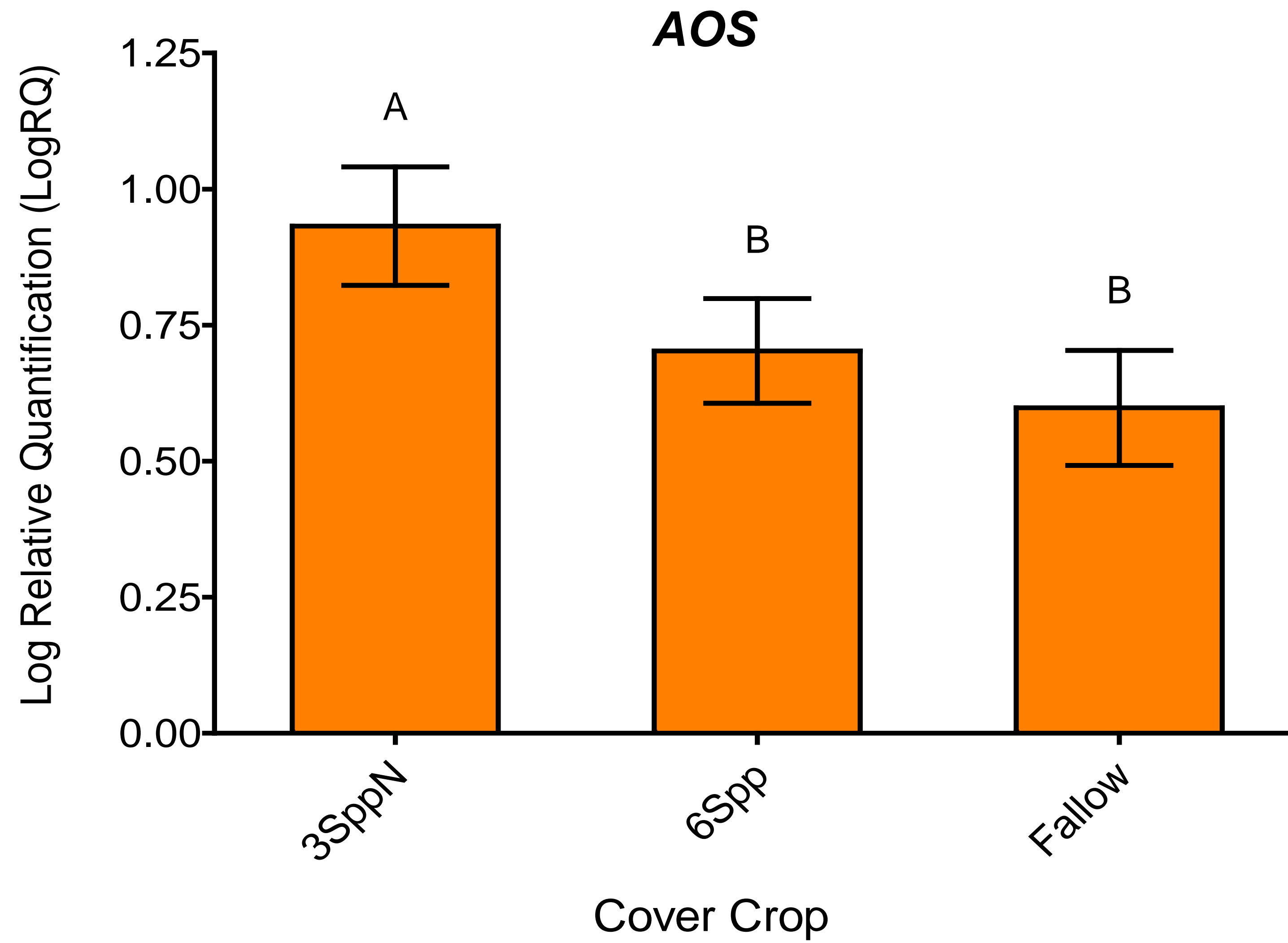

AOS
Log Relative Quantification (LogRQ)
1.25
1.00
0.75
0.50
0.25
0.00
A
B
B
3SppN
6Spp
Fallow
Cover Crop

Figure 3.3. 2014 gene expression analysis of allene oxide synthase (*aos)* mRNA levels in corn grown in soils from cover crop treatments. Each corn plant was infested with two fourth instar BCW larvae for 24 hr**.** Uninfested plants were used as controls. Total RNA was isolated from the leaf tissues around the feeding sites. Relative expression was determined by qRT-PCR in each biological replicate and normalized to the expression of endogenous gene *actin* (n=50 to 54, error bar indicates SEM). Letters indicate significant differences determined by Fisher LSD test (P=0.0283).

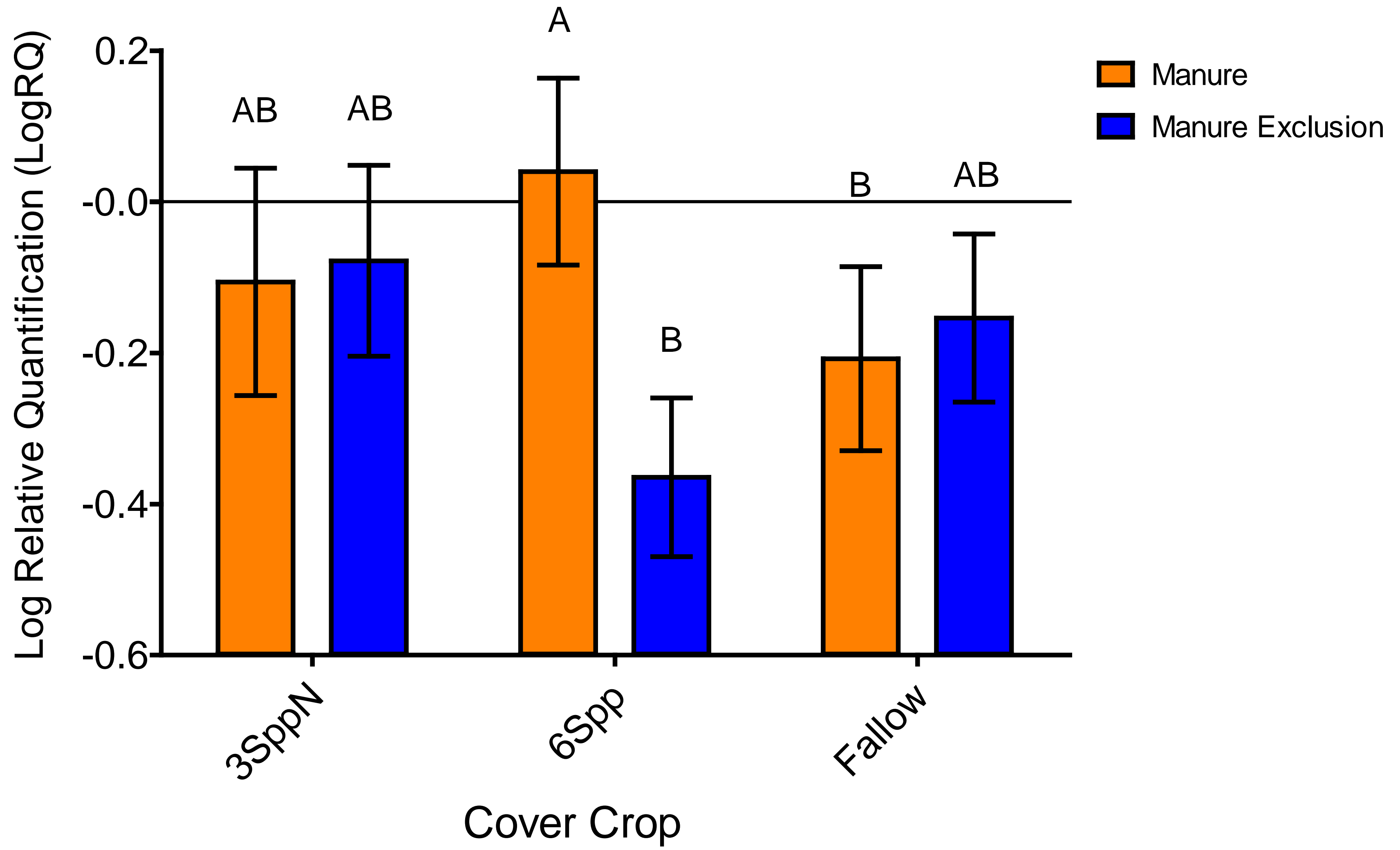
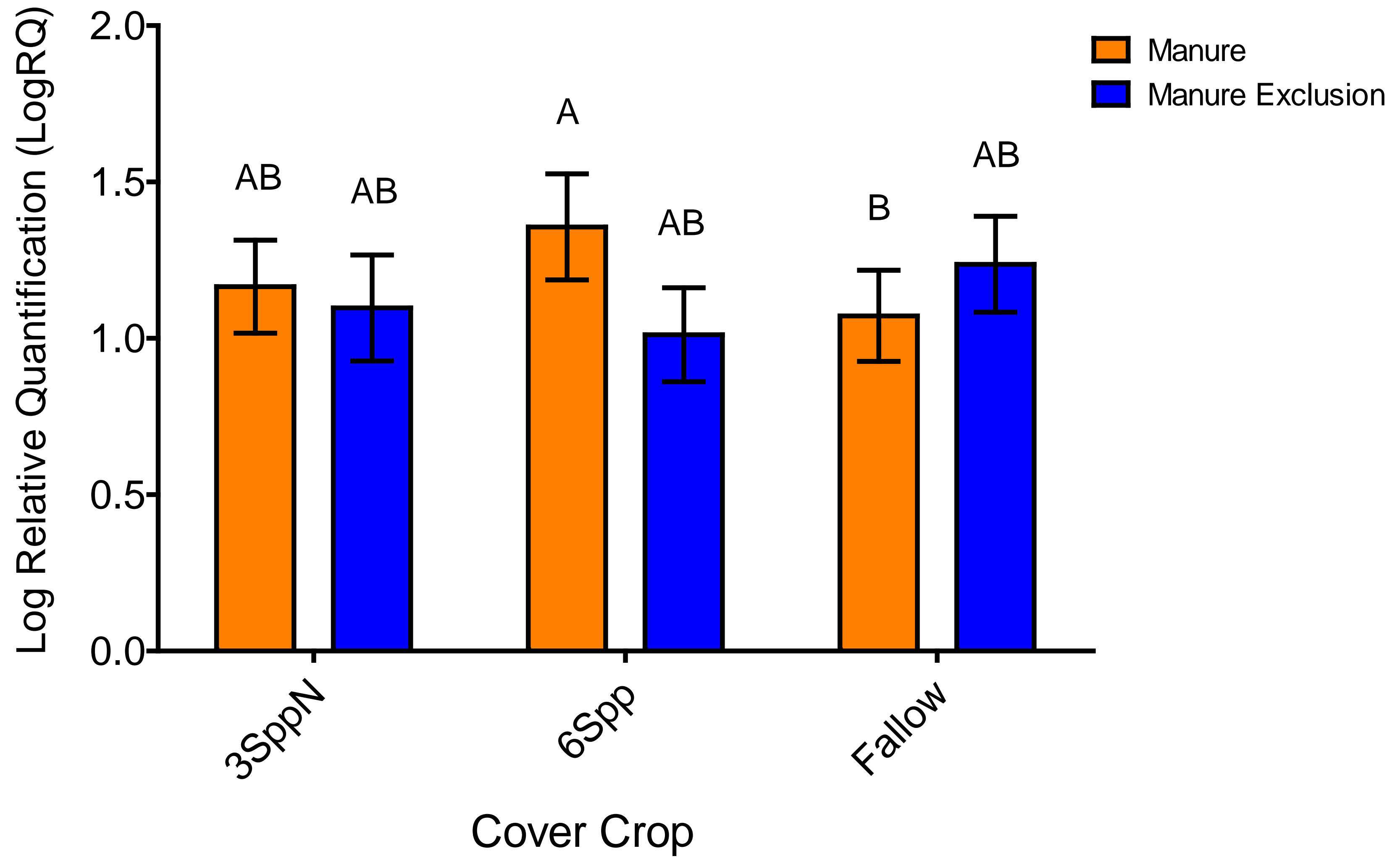

RIP2
Log Relative Quantification (LogRQ)
0.2
-0.0
-0.2
-0.4
-0.6
AB
AB
A
B
B
AB
Manure
Manure Exclusion
3SppN
6Spp
Fallow
Cover Crop

Figure 3.4. 2015 gene expression analysis of *ribosome-inactivating protein 2 (rip2)* mRNA levels in corn grown in soils from combinations of treatments. Each corn plant was infested with four third instar BCW larvae for 48 hr. Uninfested plants were used as controls. Total RNA was isolated from the leaf tissues around the feeding sites. Relative expression was determined by qRT-PCR in each biological replicate and normalized to the expression of two endogenous genes *ubiquitin* and *apt1* (n=28 to 32, error bar indicates SEM). Letters indicate significant differences determined by Fisher LSD test (P=0.0174).



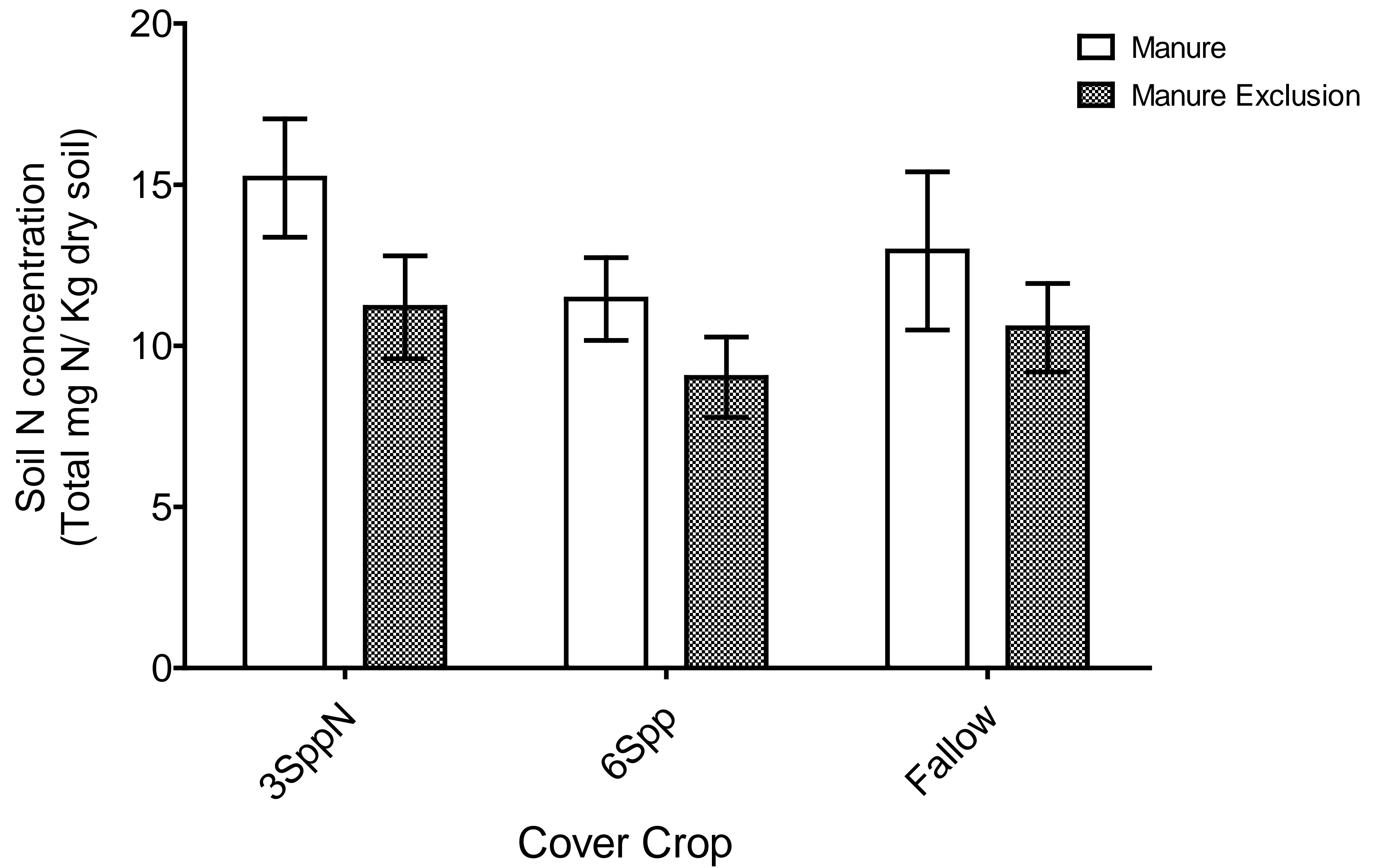
TPS23
Log Relative Quantification (LogRQ)
2.0
1.5
1.0
0.5
0.0
Manure
Manure Exclusion
AB
AB
A
AB
B
AB
3SppN
6Spp
Fallow
Cover Crop

Figure 3.5. 2015 gene expression analysis of *terpene synthase 23* (*tps23*) mRNA levels in corn grown in soils from combinations of treatments. Each corn plant was infested with four third instar BCW larvae for 48 hr. Uninfested plants were used as controls. Total RNA was isolated from the leaf tissues around the feeding sites. Relative expression was determined by qRT-PCR in each biological replicate and normalized to the expression of two endogenous genes *ubiquitin* and *apt1* (n=28 to 32, error bar indicates SEM). Letters indicate significant differences determined by Fisher LSD test (P=0.0269).

Supplementary Data

Supplementary Table 3.1. List of gene specific primers for quantitative real-time PCR in maize.

Gene	Forward	Reverse	NCBI Accession Number
Actin	GGAGCTCGAGAATGCCAAGAGCAG	GACCTCAGGGCATCTGAACCTCTC	U60511.1
Adenine phospohoribosyltransferase 1 (*apt1*)	AGGCGTTCCGTGACACCATC	CTGGCAACTTCTTCGGCTTCC	BT085274.2
Allene oxide synthase (*aos*)	CAAACCGACGAATTTGAGCAA	GGAGGCTCGCAACAAGTTG	NM_001111774
Glyceraldehyde-3-phosphate dehydrogenase (*gapdh*)	GCTGCCAAGGCTGTTGGTAAAGTT	AGGTCAACAACCGAGACATCCACA	XM_008679567.1
Maize proteinase inhibitor (*mpi*)	GCGGATTATCGCCCTAACC	CGTCTGGGCGACGATGTC	X78988.2
Ribosome-inactivating protein 2 (*rip2*)	GAGATCCCCGACATGAAGGA	CTGCGCTGCTGCGTTTT	L26305.1
Terpene synthase 23 (*tps23*)	TCACCCATGAGTGCCTCAGA	GTTGACCGCCCTCTCTAGAAGA	EU259634.1


Soil N concentration
(Total mg N/ Kg dry soil)
20
15
10
5
0
Manure
Manure Exclusion
3SppN
6Spp
Fallow
Cover Crop

Supplementary Figure 3.1. Soil nitrogen content of the corresponding subplots where corn plants were grown for BCW non-choice feeding bioassay during 2014. (Adopted from Cover Crop Cocktail project 1.0)

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Chapter 4

Conclusion and Future Directions

As one of the New World Crops, maize is native to Mexico and was domesticated from the wild grass teosinte, especially *Zea mays* ssp. *Parviglumis*, by primitive Mexican people less than 10,000 year ago (Doebley et al. 1990). The domestication created a handful of maize landraces and spread them through the South America continent (Freitas et al. 2003; Vigouroux et al. 2008; Jaenicke-Després and Smith 2010). After Christopher Columbus discovered the new continents in the Americas, maize was distributed to the rest of the world in the 15th and 16th centuries (Mintz 1998; Jones et al. 2011). Current elite maize lines were created and improved by modern breeding in the past century and have served as one of the foundations for global agriculture.

During its artificial selection, maize and numerous insects have established a long history of interaction and coevolution. Numerous insects shifted to maize as their food source with maize distribution in post-Columbian eras (Nault 1990) and become the pests of maize. The aim of the dissertation was to study maize defense responses to caterpillars at the molecular, phenotypic and ecological scales. More specifically, maize defense responses to fall armyworm (FAW, *Spodoptera frugiperda*) and black cutworm (BCW, *Agrotis ipsilon*) in the Order of Lepidoptera were investigated in various experimental settings. Overall, the findings of the dissertation have significant implications for both the basic and applied research areas of maize and insect interaction. The findings and significance are summarized and highlighted for individual chapters in the following paragraphs.

In Chapter 2, maize NAM founder lines were tested with FAW to determine their defense responses to the Lepidopteran pest. FAW bioassays showed that the constitutive defense of 25 founder lines fall into a continuum ranging from the most susceptible to the most resistant genotypes and that the constitutive defenses of the founder lines was not independent of their phylogenetic relationship. Secondly, the subset of eight inbred lines used in this study exhibited a divergent pattern in the constitutive and induced defenses. Lastly, high levels of *rip2* and *mpi* transcript expression and RIP2 protein accumulation in resistant genotypes correlated with their high constitutive defenses to FAW. Furthermore, there was a correlation between *rip2* expression

levels and RIP2 accumulation upon FAW herbivory in the diverse maize inbred lines. The findings can be used to employ the whole NAM population or several RIL families in leveraging the genetic groups in field studies to perform linkage and/or association mapping to identify genes underlying maize host-plant resistance to caterpillar pests. The resistant genotypes and newly discovered genes can be combined with genetic engineering for developing novel stacked insect resistance traits in maize or used in traditional breeding for breeding naturally insect resistant maize lines.

In Chapter 3, the soil-mediated cover crop effect on corn defense response to BCW was the focus, and it linked the molecular aspects of corn defense responses to agroecosystem and farming management practices. Insect bioassays showed that in 2014, BCW fed on 3SppN corn had the highest growth because 3SppN cover crops elevated soil nitrogen level. BCW grew better when corn plants obtained adequate amount of N from soil provided by cover crops, so cover crops benefited both corn plants and BCW at the phenotypic level. Secondly, cover crops increased the expression of several corn defense genes to deter BCW herbivory at the molecular level. Therefore, the relationship of cover crops and corn defense responses to BCW is not a simple linear relationship. This is the initial step investigating the molecular mechanisms of soil-mediated cover crop effects on corn defense responses to BCW within the agricultural system containing four levels of players in interactions: cover crop - soil - corn plant - BCW.

In the Appendix Chapter, an important herbivore defense gene *mir1* expression was investigated in 23 NAM founder lines, and it was found that maize natural sequence polymorphism affects the expression of *mir1*. qRT-PCR and RT-PCR results suggested that *mir1* is not expressed in the 23 NAM founder lines whorls before or after FAW infestation. Also, these founder lines contain a 4789 bp regulatory sequence in *mir1* promoters. Therefore, it is speculated that the 4789 bp is one of the causative regulatory sequences controlling *mir1* expression in diverse maize line whorls. A 796 bp MITE in Mp708 *mir1* intron three is likely the second regulatory sequence controlling *mir1* expression. With the above results, it will be of interest to further study what evolution or selection *mir1* has gone through since it performs such an important function in maize insect resistance. Furthermore, the findings can be incorporated with genetic engineering to turn on *mir1* expression in previous non-expressing maize lines as a method of increasing maize natural defense levels to insect herbivores.

In conclusion, this dissertation covers a broad range of most popular and ground-breaking research projects with bioinformatics, laboratory bench work, greenhouse experiments, and on-site field trials, tackling the question of how maize plant protects and defends itself against caterpillars within systems of various scales. It has added to the basic scientific knowledge of maize and insect interactions, and provided a fresh view of key components not only as separate research objects, but also from a holistic perspective to understand the mechanisms. As for potential applications, the results can be used in designing and engineering new naturally insect resistant maize, improving current GE maize, and designing and implementing novel agricultural ecosystems to achieve the goal of insect pest management. In the long run, U.S. growers will benefit from having more choices for corn varieties other than GE maize, and being more informative in making wise decisions for insect pest management. As such, the environment will be more balanced and sustainable.

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Appendix Chapter

Natural allelic variation affects maize insect resistance 1 (*mir1*) expression in the NAM founder lines

Introduction

Insect pests are a problem for the southern United States and Central to South American maize production because of the geographic locations and warm climates (CIMMYT 1987). Maize geneticists and entomologists (USDA-ARS Corn Host Plant Resistance Research Laboratory, Mississippi State University) established a breeding program to develop insect resistant maize lines for the southern United States (William et al. 1978; Davis 1980; Scott et al. 1982; Williams and Davis 1982; Williams et al. 1990). The original resistant landrace used in the breeding program came from Antigua in Central America. Thereafter, a series of insect resistant maize inbred lines showing resistance to fall armyworm (FAW) and southwestern corn borer (SWCB) were developed and released.

When researchers were looking for the resistance mechanism, they identified a 33-KD protein by comparing two-dimensional protein gel electrophoretic profiles of callus from maize resistant (Mp708 and Mp704) and susceptible (Tx601 and Ab24E) lines (Jiang et al. 1995). This protein is a cysteine protease and named maize insect resistance 1-cysteine protease (Mir1-CP). Mir1-CP targets the caterpillar peritrophic matrix (PM) (Pechan et al. 2002). The PM is composed of a network of chitin fibers, glycoproteins, and proteoglycans, and it lines insect midgut. The PM is active in insect digestion and nutrient absorption and protects midgut from chemical and mechanical damage. Scanning electron microscopy showed that the PM of FAW reared on Mp708, or transgenic maize callus over expressing Mir1-CP, was severely damaged with holes and cracks, while the PM of FAW reared on Tx601, or non-transformed callus, maintained its integrity during the experiment.

The *mir1* cDNA encoding Mir1-CP was isolated from a callus cDNA library of Mp708 (Pechan et al. 1999). The deduced amino acid sequence analysis showed that Mir1-CP is produced as a pre-proprotein. The “pre” signal sequence directs the protein to its targeted localization. Then the “pro” sequence is removed to activate the enzyme. In a Mp708 × A619 F2 mapping population,

researchers identified key QTL regions on maize chromosome 1, 5, 6, 7 and 9 conferring resistance to FAW and SWCB, and *mir1* was mapped to chromosome 6 at bin 6.02 (Brooks et al. 2005; Brooks et al. 2007).

Mp708 has shown resistance to several lepidopteran insect pests and insects from other feeding guilds, and this resistance is related to the presence Mir1-CP in the whorl and roots (Davis et al. 1988; Gill et al. 2011; Louis et al. 2015). As such, a powerful maize defense protein, Mir1-CP, and Mp708 provide a new opportunity for incorporating this natural resistance into maize lines for applied agricultural production.

However, up to now the missing part is the identity of the genetic element(s) that control(s) *mir1* expression in Mp708. Is the *mir1* gene present or missing in other maize inbred lines? Are these lines able to express *mir1* upon insect herbivory? If they are, which of them are? If there are not, why do some lines not express *mir1*? What evolution or selection has *mir1* gone through since it performs such an important function in maize insect resistance?

The maize genome contains higher molecular diversity than other crop species, resulting in tremendous phenotypic diversity (Tenaillon et al. 2001; Wright et al. 2005). The high genetic diversity of maize and the availability of a large set of germplasm makes it possible to study how *mir1* gene allelic variation affects its expression among the diverse inbred lines upon insect herbivory. One outstanding diversity panel is the maize nested association mapping (NAM) population (Liu et al. 2003; Yu et al. 2008; McMullen et al. 2009). It includes 25 diverse founder lines, a common parent B73, and 200 recombinant inbred lines for the cross between each founder line and B73. The use of the NAM population is to encompass a large amount of maize genetic diversity for dissecting the genetic architecture of complex traits and pinpointing functional valuable alleles controlling important agronomic traits. A broad range of research on the maize NAM population has emerged recently, including the study of the genetic components of plant height, flowering time, leaf architecture, kernel composition, and resistance to northern leaf blight and aphids (Buckler et al. 2009; Poland et al. 2011; Tian et al. 2011; Cook et al. 2012; Meihls et al. 2013; Peiffer et al. 2014). Linkage mapping and genome wide association mapping (GWAS) both identified significant quantitative trait loci (QTL) and multiple candidate genes related to these important traits.

Here, we tested 23 NAM founder lines for *mir1* expression and confirmed *mir1* expression by amplifying its coding DNA sequence. *mir1* gene and promoter sequences were compared in inbred lines Mp708 and B73. Since the NAM founder lines originated with diverse genetic backgrounds, the first hypothesis was that some of the founder lines could express *mir1* in the whorl region upon FAW herbivory. The second hypothesis was that the regulatory sequence controlling *mir1* expression is not a single region, but multiple regions.

Materials and Methods

Plant materials and FAW rearing

Maize (*Zea mays*. ssp. *mays*) NAM founder line seeds were obtained from the North Central Regional Plant Introduction Station (NCRPIS). Mp708 seeds were obtained from Dr. Paul Williams (USDA-ARS Corn Host Plant Resistance Research Laboratory, Mississippi State University, USA). B73 seeds were produced at the Pennsylvania State University research farm at Rock Springs, PA.

To achieve a high germination rate, seeds of all inbred lines were placed into Ziploc plastic containers with four layers of paper towel covering the top and bottom of the seeds. Ziploc containers were wrapped with aluminum foil to maintain a dark environment for germination. Tiny holes were punched in aluminum foil to ensure there was sufficient air for germination. Paper towels were damped to maintain moisture and the seeds were put into a 25 °C incubator. It usually took four or five days for the radicle and cotyledon to emerge and then the seedlings were transferred into the potting mix (PRO-MIX BX mycorrhizae, PRO-MIX, USA). After the cotyledon and the first two true leaves emerged, the seedlings were transferred into pots containing topsoil (Hagerstown silt loam).

A single maize plant was planted in a 4 L pot with topsoil and Osmocote Plus (The Scotts Company, Marysville, OH) was used as fertilizer. When plants reached V8 to V10 stage (Ritchie et al. 1986), they were used for FAW infestation.

Plants were grown in the Plant Science Department greenhouse at The Pennsylvania State University (University Park, PA, USA). The temperature was from 20 to 28 °C. The photoperiod was 16 hr light:8 hr dark. Photosynthetically active radiation ranged from 500 to 1200 µmol $m^{-2}s^{-1}$. Maize plants were watered with tap water as needed. Because the growth rate of the maize

inbreds differed, they were planted in a staggered manner so they reached the same growth stage for experiments. For example, slow growing lines were planted early and fast growing lines were planted late.

FAW eggs were obtained from the USDA-ARS Corn Host Plant Resistance Research Laboratory, Mississippi State University, USA. Eggs were hatched in a 27 °C incubator. After hatching, neonates were reared on an artificial diet (Peiffer and Felton, 2005) in the same incubator with a 14 hr light:10 hr dark. For infestation experiments, individual larvae were reared on artificial diet until the 4th or 5th instar.

FAW infestation experiment

FAW were reared to the 4th or 5th instar and starved overnight. Maize plants ready to be infested were moved into another greenhouse. Five starved FAW larvae were placed in the whorl region of each maize plant and four maize plants were infested for each genotype. The infestation period was 24 hr. Then leaves were cut from the whorl region and 0.1 g of tissue adjacent to the feeding site (approximately 5 mm surrounding the feeding site) was collected, immediately frozen in liquid N_2 and stored in -80 °C freezer. Leaf samples from control plants were collected at 0 hr time point.

Mp708 and OH43 root tips were collected from control and infested plants. The roots were washed at the root wash facility at the Plant Science Department (University Park, PA, USA). When maize plants reached V8 to V10 stage, primary and secondary roots withered and lateral and crown roots proliferated. Therefore, 1 to 1.5 cm long root tips from several lateral and crown roots were collected, immediately frozen in liquid N_2 and stored in -80 °C freezer.

RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR)

The leaf and root tissues were homogenized using the Geno/Grinder 2000 (SPEX Certiprep, USA). Total RNA was extracted from leaf and root tissues using the TRIzol reagent and protocol (Life Technologies, USA). The RNA pellet was dissolved in diethylpyrocarbonate (DEPC)-treated water. After extraction, RNA was subjected to DNase digestion to remove residue genomic DNA. The reaction contained 26 µl RNA, 3 µl DNase buffer and 1 µl DNase (New England BioLabs, USA). It was incubated at 37 °C for 30 min and then DNase was inactivated at 65 °C for 10 min. The Nanodrop 2000 (Thermo-Fisher Scientific, USA) was used to measure

RNA concentration and its purity. For reverse transcription PCR (RT-PCR), 1 µg total RNA was added into a 20 µl reaction with oligo-dT 20 primers following the manufacturer's protocol. The reverse transcription PCR program was 37 °C for 2 hr and 85 °C for 5 sec. The kit was the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA).

qRT-PCR was performed in an ABI 7500 Fast Real Time PCR system. Primer Express software was used to design gene-specific primers (Life Technologies, USA). Individual cDNAs were diluted 10 times before running qRT-PCR. The qRT-PCR reaction (10 µl system) contained 1 µl diluted cDNA, 1 µl H_2O, 1 µl forward primer (1 µm), 1 µl reverse primer (1 µm), and 5 µl SYBR Green Master Mix (Roche Applied Science, USA). The program was as follows: step 1, 50 °C for 2 min and 95 °C for 10 min; step 2, 95 °C for 15 sec and 60 °C for 1 min, 40 cycles; step 3, 72 °C for 10 min; step 4, dissociation stage. Endogenous gene *actin* was used to calculate the relative quantification of *mir1* gene expression by 2-ΔΔCT method (Vandesompele et al., 2002; Lin et al., 2014). The list of primers is shown in **Supplementary Table A.1**. The original data are shown in **Supplementary Table A.2**.

Data Analyses

mir1 gene relative quantification was analyzed as a two-way ANOVA using genotype and infestation as the independent variables in Minitab 17 Statistical Software (Minitab Inc., USA). Minitab 17 optimized the λ value for data transformation and transformed the data automatically. *mir1* relative quantification statistics was shown as: Genotype $F=17.04$, $P<0.001$; Infestation $F=115.74$, $P<0.001$; Genotype*Infestation $F=3.90$, $P<0.001$. Since there was interaction between genotype and infestation for *mir1* relative quantification, a Fisher pairwise comparison was performed to determine statistically significant differences**.**

Reverse transcription polymerase chain reaction (RT-PCR)

The *mir1* full length mRNA in Mp708 was retrieved from NCBI database and used for designing RT-PCR primers. The primer sequences were as follows: *mir1* CDS forward ATG CGC CCA ACA CGC TCC GCT GTG T (5′ to 3′); m*ir1* CDS reverse CTA AGC AAG AAC CAT CTT GAT CAC A (5′ to 3′).

Template cDNA was the undiluted cDNA product. The 20 µl RT-PCR reaction system consisted of 12.4 µl H_2O, 4 µl 5X Phusion HF buffer, 0.4 µl 10 mM dNTPs, 1 µl forward primer (10 µm),

1 µl reverse primer (10 µm), 1 µl template cDNA, and 0.2 µl Phusion DNA polymerase (Phusion® High-Fidelity DNA Polymerase, New England BioLabs, USA). The RT-PCR cycling condition was as follows: initial denaturation 98 °C for 30 sec; 40 cycles with 98 °C for 10 sec, 60 °C for 30 sec and 72°C for 45 sec; final extension 72 °C for 10 min; hold at 4 °C.

Sequence alignment and query into maize HapMap V3.2.1

Different sequence alignments and how single nucleotide polymorphisms (SNPs) were retrieved from maize HapMap V3.2.1 (imputed) are clearly illustrated in the **Results** session.

Results

***mir1* gene expression in 23 maize NAM founder lines**

MaizeGDB and related databases have provided various sequence information about maize NAM founder lines (Harper et al. 2016). With *mir1* expression data, it is possible to relate the result of mRNA level with sequence data to look for the potential causative genetic regions controlling *mir1* expression.

Exogenous ethylene and methyl jasmonate application induced *mir1* mRNA accumulation after 24 hr treatments (Ankala et al. 2009). Third instar FAW infestation for 24 hr in Mp708 whorl region increased *mir1* mRNA level by 1.25-fold compared to control plants. Another paper reported that *mir1* mRNA level decreased by 1.7-fold after 24 hr of FAW infestation compared to Mp708 control plants (Shivaji et al. 2010). These results suggest that there is variability in *mir1* transcript accumulation in response to FAW feeding.

At the protein level, immunoblot analysis showed that Mir1-CP rapidly accumulated within 1 hr of FAW infestation in Mp708 whorls (Pechan et al. 2000). Mir1-CP also was more abundant in roots than in the whorls of Mp708 unifested plants (Lopez et al. 2007). However, within 24 hr of FAW infestation, Mir1-CP substantially accumulated in the whorl. Therefore, it was hypothesized that Mir1-CP was recruited and moved within the vascular system from root to leaf when Mp708 encountered FAW herbivory in the aboveground tissues. Further evidence was provided by an excised root experiment, which showed that root removal decreased Mir1-CP accumulation in the whorl in response to FAW herbivory compared to intact Mp708 plants.

The rapid accumulation of Mir1-CP and its highly increased abundance together with little or no change in *mir1* mRNA levels in Mp708 whorls upon FAW herbivory suggested that there are differences in the regulation of *mir1* mRNA and Mir1-CP accumulation.

The qRT-PCR results showed that nine founder lines did not accumulate *mir1* mRNA near the feeding sites in the whorls prior to and after FAW infestation. These NAM lines were B73, B97 CML103, CML228, Ki11, M162W, M37W, Ms71, and NC358. These data were not shown because qRT-PCR could not detect any *mir1* fragment and there was no cycle threshold (Ct) value for *mir1*, and therefore the relative quantification of *mir1* could not be calculated. Notably, FAW infestation was not able to induce *mir1* mRNA accumulation in B73 whorl region.

In the other14 founder lines, *mir1* mRNA levels were low in control plants compared to the Mp708 control (**Figure A.1**). FAW infestation significantly induced *mir1* mRNA accumulation in Ky21 whorls. FAW Infestation induced *mir1* mRNA accumulation in OH7B and OH43 whorls, but the levels were lower than in Mp708 and Ky21. Nevertheless, the induction levels were higher than in the other 11 founder lines. It was considered that these 11 founder lines could not express *mir1* in the whorl upon FAW infestation, since *mir1* relative quantification values were less than 0.4. Because *mir1* transcript levels increased in Ky21 and OH43, they were selected for further study.

Further evidence from amplification of *mir1* coding DNA sequence

Currently, the NAM founder lines are being re-sequenced and aligned with B73 reference genome (Bukowski et al. 2015). It is not known how maize genetic diversity influences the NAM founder line transcriptomes in response to insect herbivory. Since qRT-PCR amplifies a small fragment (<150 bp) of cDNA, it is not known how valid the *mir1* expression data were considering there is little information on the transcriptomes of NAM founder lines. Therefore, amplification of *mir*1 coding DNA sequence (CDS) was performed to validate the results from gene expression measured by qRT-PCR.

Previous studies investigated *mir1* transcript changes in Mp708 whorls during FAW herbivory, or treatments with phytohormones and their corresponding inhibitors (Ankala et al. 2009; Shivaji et al. 2010). Mir1-CP is more abundant in Mp708 root than leaf prior to FAW infestation (Lopez et al. 2007), so it is speculated that *mir1* transcript is more abundant in uninfested Mp708 roots than in leaf. However, it is unknown how *mir1* transcript changes in Mp708 roots in response to

FAW herbivory from the aboveground tissues. Therefore, Mp708 root tips were used as positive controls and the first RT-PCR was to test the positive controls. The results showed that *mir1* CDS was amplified in the root tips of control Mp708 plants, but not in those from infested plants. *mir1* CDS was also amplified in Mp708 control and infested whorls, but the amount was much lower than in root tips (**Figure A.2** [A]).

Since *mir1* qRT-PCR expression results showed that *mir1* mRNA accumulated in Ky21, OH7B, and OH43 infested whorls, the second RT-PCR tested leaves and roots from these inbred lines. The results showed that *mir1* CDS was not amplified in the whorl of Ky21 infested plants. Nor was it amplified in the whorl or root tips of control OH43 plants (**Figure A.2** [B] lane 1 to 4). *mir1* CDS was amplified in OH43 infested plant root tips, but not in the whorl region (**Figure A.2** [B] lane 5 and 6).

Gene expression analysis showed that Ky21 accumulated a large amount of *mir1* mRNA after FAW infestation. However, *mir1* CDS was absent in the whorls of Ky21 infested plants. Considering the genetic diversity within the founder lines, qRT-PCR probably amplified some other gene mRNA instead of *mir1*. In the whorls of OH43 infested plants, *mir1* CDS was absent. Since in Mp708 whorl region, *mir1* CDS abundance was very low and gene expression showed that Mp708 had a higher *mir1* mRNA level than OH43, it was likely that RT-PCR could not amplify *mir1* CDS due to its extremely low abundance in OH43 infested plant whorl region. RT-PCR suggested that *mir1* mRNA did not accumulate in OH43 infested plant whorls, but did in OH43 infested plant roots.

Comparison of *mir1* gene sequences in maize inbred lines Mp708 and B73

Dr. Yang Han in Dr. Luthe's lab cloned and sequenced *mir1* gene and promoter from Mp708 (Han unpublished data). Using the *mir1* gene sequence as an input to search against B73 genome assembly RefGen_V3, it was found that *mir1* gene is present in B73 genome (MaizeGDB). Its Gramene ID is GRMZM2G150276 and alternative name is ZEAMMB73_043905. (**Figure A.3** [A]).

Next, BLAST global alignment (Needleman-Wunsch Global Align Nucleotide Sequences) was used to compare *mir1* gene sequences in Mp708 and B73. The result showed that the percent identity of the two sequences is 67% (1857/2758) and gap is 862/2758 (31%). The gap is due to a 792 base pair (bp) insertion in Mp708 *mir1* gene that is absent in B73 (**Figure A.3** [B]). *mir1*

full length mRNA was sequenced in Mp708, so it was retrieved from the NCBI database; the NCBI Reference Sequence ID is NM_001112101.1. By aligning *mir1* mRNA, Mp708 *mir1* gene, and B73 *mir1* gene, the location of the 792 bp region is clear. The three sequences were compared by **mu**ltiple **s**equence **c**omparison by **l**og-**e**xpectation (MUSCLE http://www.ebi.ac.uk/Tools/msa/muscle/). The result showed that the 792 bp region lies within the third intron of the Mp708 *mir1* gene (**Figure A.3** [C]) and it was visually identified as a CACTA transposable element (TE) (**Figure A.3** [D]). The left and right terminal inverted repeats (TIRs) are 10 bp long and reverse complementary to each other. The left and right TIRs are characterized by 3 bp CTA target site duplication (TSD). Therefore, the sequence characteristics suggest that it is a CACTA type TE (Li et al. 2009; Yang et al. 2013). The regulatory sequence is only 796 bp with TIRs and TSD included. These characteristics also suggest that it is likely a miniature inverted-repeat transposable element (MITE). MITEs are usually a few hundred bp long and found in introns (Jiang and Wessler 2001; Feschotte et al. 2002). However, B73 only has a right 3 bp CTA TSD compared to Mp708. The MITE in Mp708 *mir1* gene intron three is likely one of the regulatory sequences controlling *mir1* expression.

Comparison of *mir1* promoter sequences in maize inbred lines Mp708 and B73

Using the *mir1* promoter sequence from Mp708 to blast B73 assembly RefGen_V3, the B73 *mir1* promoter was identified. The two promoter sequences were aligned by MUSLE. The result showed that the major difference between Mp708 and B73 *mir1* promoters is a 4789 bp region in the middle of the B73 promoter (**Figure A.4**; **Supplementary Figure A.1**). NCBI nucleotide blast showed that the identity is 1791/1955 (92%) and the gap is 81/1955 (4%) in the first part of Mp708 and B73 *mir1* promoters, while in the last part of Mp708 and B73 *mir1* promoters, the identity is 359/402 (89%) and the gap is 16/402 (3%).

From the 4789 bp region in B73 *mir1* promoter, a CACTA TE was visually identified (**Figure A.4**; **Supplementary Figure A.1**). The left and right TIRs are 13 bp long and reverse complementary to each other. The left and right TIRs are characterized by 3 bp ACT TSDs. The length of the TE is 3787 bp. Therefore, the sequence characteristics suggest that it is a CACTA type TE (Li et al. 2009; Yang et al. 2013). However, the TE is absent in Mp708. The TE in B73 *mir1* promoter is likely another regulatory sequence controlling its expression.

mir1 promoter genotypic data in 23 maize NAM founder lines

As of now, 1210 teosinte, landrace and maize lines have had their whole genomes sequenced, and the data were used to generate maize HapMap V3. Because of the tremendous genetic diversity in the *Zea mays* species ranging from domestication to modern breeding, the B73 reference genome only captured a small fraction of *Zea mays* haplotypes. The maize HapMap V3 provides genotypic data on polymorphism sites including most of the *Zea mays* haplotypes (Bukowski et al. 2015).

The *mir1* promoter position on B73 RefGen_V3 is chromosome 6 from 89074184 to 89081327, and the 4789bp region is from 89074576 to 89079364. Using the 4789 bp in B73 to query HapMap V3.2.1 (imputed) for 23 founder lines, 245 SNPs were retrieved. If the region is not present in the maize NAM founder lines, the query should not return any SNPs. The 4789 bp region contains 245 SNPs and it was found that the SNPs are present in the 23 founder lines. This suggested that similar to B73, the region is present in all of the tested 23 founder lines (**Table A.1**). Specific SNPs vary among the founder lines. The tested 23 founder lines have the 4789 bp region in the *mir1* promoter, and therefore, they are B73-like instead of Mp708-like for this region.

Discussion

The qRT-PCR results showed that nine founder lines did not accumulate *mir1* mRNA near the feeding sites in the whorls prior to and after FAW infestation. These NAM lines are B73, B97 CML103, CML228, Ki11, M162W, M37W, Ms71, and NC358. For the other eleven founder lines, *mir1* relative quantification values were less than 0.4, much lower than Mp708, and therefore it was considered that these eleven founder lines could not express *mir1* in the whorl prior to and after FAW infestation. These lines are CML247, CML277, CML333, CML52, CML69, Hp301, IL14H, Ki3, Mo17, Mo18W, and NC350 (**Figure A.1**). So, twenty maize NAM founder lines cannot express *mir1* before or after FAW herbivory in the whorls.

mir1 expression was also queried with the online maize NAM qTeller database (http://qteller.com/NAM/) and the results showed that *mir1* RNA-seq expression is extremely low or close to zero in the shoot tissues of the above twenty founder lines without FAW infestation except B97, CML277, and Ki11 (**Supplementary Figure A.2**). The database did not specify which part of shoot tissues and what developmental stages of the founder lines were used for RNA-seq, but the results have lent strong support to the *mir1* expression qRT-PCR data using

the whorls of the founder lines at V8 to V10 growth stages. The discrepancy of B97, CML277 together with Ki11 *mir1* qRT-PCR and RNA-seq expression is likely due to the differences of the two techniques, shoot sampling locations, and/or plant developmental stages.

RT-PCR suggested that *mir1* mRNA did not accumulate in OH43 infested whorls, but in OH43 infested roots. This implies that the regulation of *mir1* expression in maize whorl and root tissues in response to FAW herbivory is in a tissue-specific manner. Even though qRT-PCR showed that Ky21 and OH43 expressed *mir1* after FAW infestation, RT-PCR showed that *mir1* CDS was not amplified in Ky21 and OH43 infested whorl regions. Therefore, it is concluded that *mir1* is not expressed in the 23 NAM founder lines whorls before or after FAW infestation.

Maize inbred line B73 has the *mir1* gene in its genome and the major difference between Mp708 and B73 *mir1* genes is that Mp708 has a 796 bp miniature inverted-repeat transposable element (MITE) in *mir1* intron three. This MITE is likely one of the regulatory sequences controlling *mir1* expression. MITEs are closely located with genes in maize and rice genomes (Wessler et al. 2001). They are speculated to regulate nearby gene expression via MITEs-generated small RNAs (Kuang et al. 2009). A large number of MITEs are transcribed in the rice cultivar Nipponbare (*Oryza sativa* ssp. *japonica*) and it was shown that genes physically close to MITEs have lower expression levels compared to genes far away from MITEs in Nipponbare (Lu et al. 2011).

Since this MITE is not present in the reference genome B73 RefGen_V3, it is not assembled or annotated in B73. Maize HapMap V3.2.1 could not give any information on this transposable element in the NAM founder lines, so it is unknown whether NAM founder lines have this MITE or not. This MITE could be an insertion in Mp708 *mir1* gene or a deletion in B73 *mir1* gene depending on its presence in the other founder lines. Without knowing the information on this MITE from other founder lines, the two possibilities of its origin cannot be assessed.

The major difference between Mp708 and B73 *mir1* promoters is that a 4789 bp region lies in the middle of the B73 *mir1* promoter. Also, the region contains a 3787 bp CACTA transposable element in B73. This 4789 bp region including the transposable element is assembled and annotated in the reference genome B73 RefGen_V3, so maize HapMap V3.2.1 has given genotypic information for the region in the NAM founder lines. It is found that the 4789 bp region is present in the tested 23 founder lines (**Table A.1**). In other words, these founder lines are identified as B73-like because they have the 4789 bp region in their *mir1* promoters.

The 23 founder lines contain the 4789 bp region in *mir1* promoters and *mir1* is not expressed in these founder lines whorls before or after FAW infestation. Therefore, it is likely one of the regulatory sequences controlling *mir1* expression in diverse maize line whorls. Also, the 4789 bp sequence including the transposable element is a deletion in Mp708 since 23 founder lines have it and do not express *mir1* in their whorls.

In conclusion, 23 maize NAM founder lines including B73 do not express *mir1* in their whorls before or after FAW infestation, and the presence of the 4789 bp region including a CACTA transposable element in *mir1* promoter of these lines is likely one of the regulatory sequences controlling *mir1* expression in diverse maize lines whorls. The second regulatory sequence controlling *mir1* expression is a MITE in Mp708 *mir1* intron three. Its presence in other NAM founder lines cannot be determined with currently available genotypic data. This study took a genetic and molecular approach to pinpoint the causative genetic elements controlling the expression of an important maize herbivore defense gene. Even though the findings are not conclusive, it has tested 23 maize NAM founder lines and identified two potential regulatory sequences. With the advance of next-generation sequencing and the genomes of diverse maize germplasms assembled and annotated, the mystery of *mir1* gene regulation will likely be solved in the near future. It will be of interest to further study what evolution or selection *mir1* has gone through since it performs such an important function in maize insect resistance. Furthermore, the findings can be incorporated with genetic engineering to turn on *mir1* expression in previous non-expression maize lines as a method of increasing maize natural defense levels to insect herbivores.

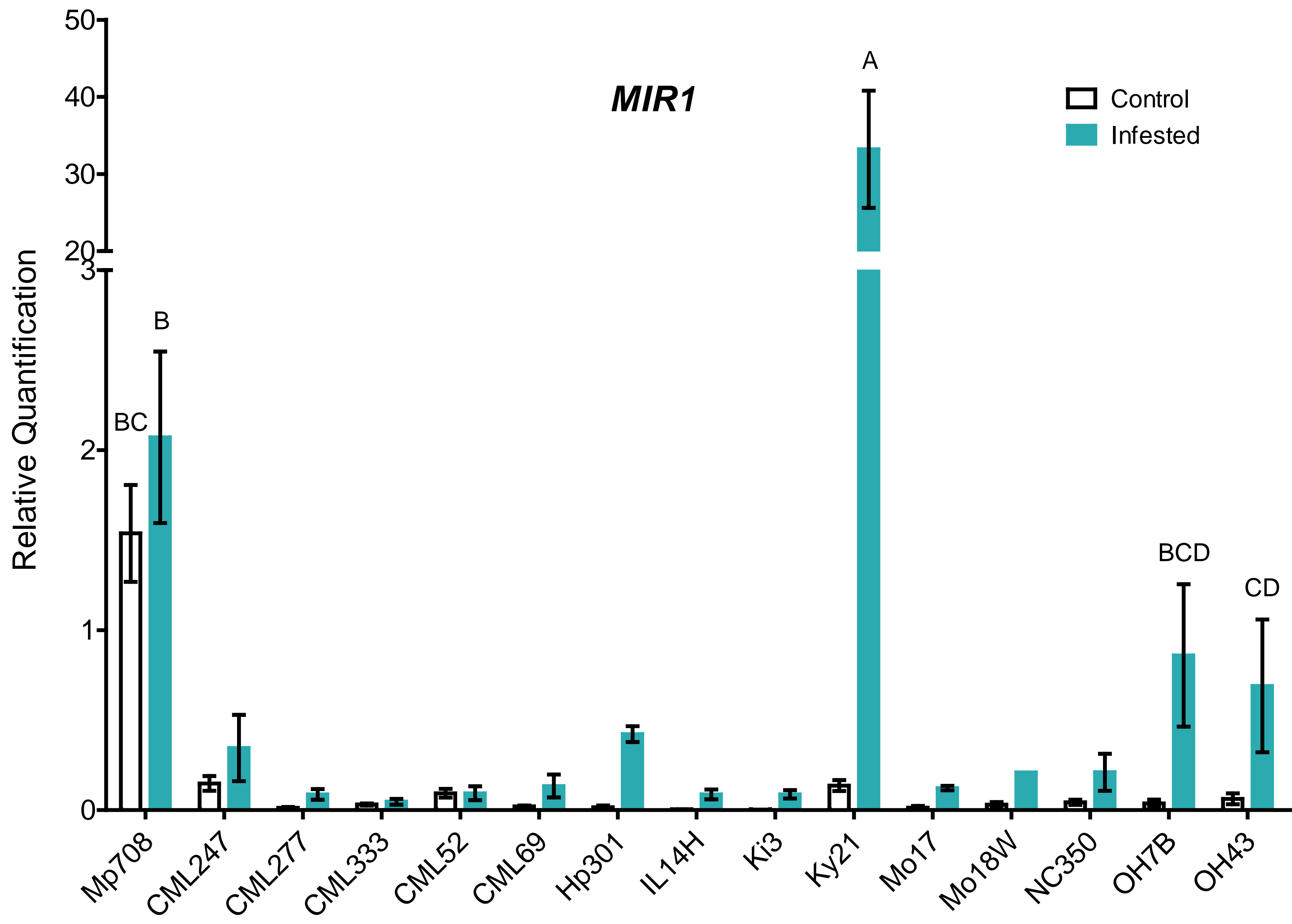

MIR1
Control
Infested
Relative Quantification
50
40
30
20
3
2
1
0
A
B
BC
BCD
CD
Mp708
CML247
CML277
CML333
CML52
CML69
Hp301
IL14H
Ki3
Ky21
Mo17
Mo18W
NC350
OH7B
OH43

Figure A.1. Maize insect resistance 1 (*mir1*) is not expressed in the aboveground tissues of the majority of NAM founder lines.

mir1 gene expression was analyzed in the whorls of 14 NAM founder lines prior to and after FAW infestation. Individual maize plant of each genotype was infested with five 5th instar FAW larvae for 24 hr. Uninfested plants were used as controls. Total RNA was isolated from the leaf tissues around the feeding sites in the whorls. Relative expression was determined by qRT-PCR in each biological replicate and normalized to the expression of *actin* (n=3 to 4, error bar indicates SEM). Letters indicate significant differences determined by Fisher least significant difference (LSD) test ($P<0.001$).

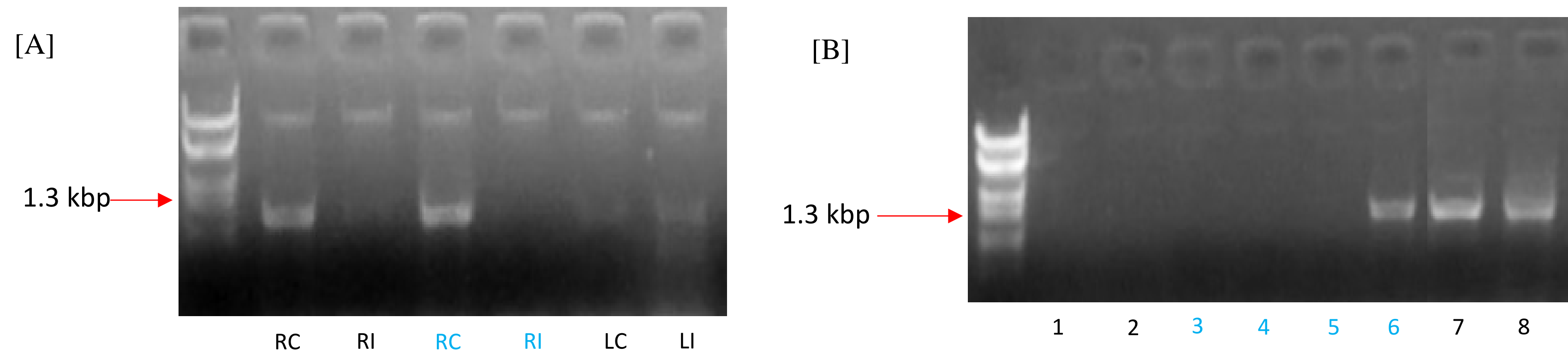
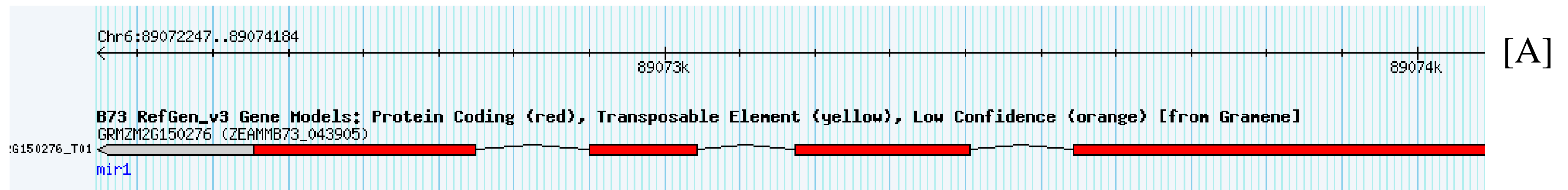
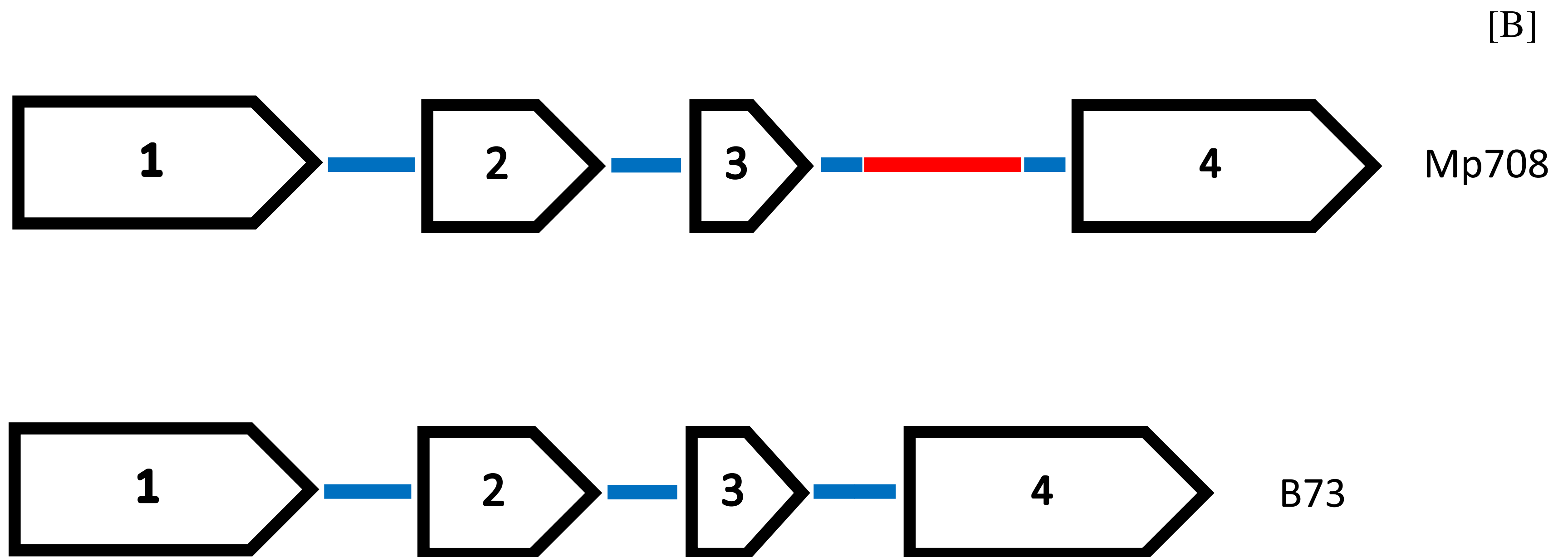


Figure A.2. RT-PCR amplification of *mir1* 1.2 kbp coding DNA sequence (CDS).

[A] Amplification of *mir1* CDS in Mp708 whorl and root tips prior to and after FAW infestation. RC: root control (two biological replicates indicated by black and blue RC); RI: root infested (two biological replicates indicated by black and blue RI); LC: leaf control; LI: leaf infested.

[B] Amplification of *mir1* CDS in Ky21 and OH43 whorls and root tips prior to and after FAW infestation. Lane 1 and 2: Ky21 LI (two biological replicates); Lane 3: OH43 LC; Lane 4: OH43 LI; Lane 5: OH43 RC; Lane 6: OH43 RI; Lane 7 and 8 Mp708 RC (two biological replicates). LC: leaf control; LI: leaf infested; RC: root control; RI: root infested.


Chr6:89072247..89074184
89073k
89074k
B73 RefGen_v3 Gene Models: Protein Coding (red), Transposable Element (yellow), Low Confidence (orange) [from Gramene]
GRMZM2G150276 (ZEAMMB73_043905)
G150276_T01
mir1
[A]


[B]
1
2
3
4
Mp708
1
2
3
4
B73

[C]

```
CLUSTAL multiple sequence alignment by MUSCLE (3.8)


B73mir1gene         AAAATGGCAGCTGCGGAGCTGCCATCTTTGCACGAAGACACACACACACAGCAGCTGAGC
Mp708mir1mRNA       ---------------------------------------------------------AGC
Mp708mir1gene       AAAATGGCAGCTGCGAAGCTGCCATCTTTGCACGAAGACACACACACACAGCAGC--AGC
                                                                             ***

B73mir1gene         AG----CAGCAA------CACGCTCCCATCATCTCTCGCCATGCGCCCAACACGCTCCCC
Mp708mir1mRNA       AGTTCACAGTTAAGTCTTCTCATTCCCATCATCTCTCGCCATGCGCCCAACACGCTCCGC
Mp708mir1gene       AGTTCACAGTTAAGTCTTCTCATTCCCATCATCTCTCGCCATGCGCCCAACACGCTCCGC
                    **    ***  *      * *  *********************************** *

B73mir1gene         TGTGCCGGCTACGGCGCTGCTCCTGCTTGCCGTGGCACTGGCACTGGCCGCCACGGCGGC
Mp708mir1mRNA       TGTGTCGGCTACGGCGCTGCTCCTGCTTGCCGTGGCACTGGCACTGGCCGCCACGGCGGC
Mp708mir1gene       TGTGTCGGCTACGGCGCTGCTCCTGCTTGCCGTGGCACTGGCACTGGCCGCCACGGCGGC
                    **** *******************************************************

B73mir1gene         GGCCCGCCACTTCTACACCACCACCACCACCACCCGCGTCCCGGCGCCAGCGGAGCGGGC
Mp708mir1mRNA       GGCCCGCCACTCCTA---CACCACCACCACCACCCGCGTCCCGGCGCCAGCGGAGCGGGC
Mp708mir1gene       GGCCCGCCACTCCTA---CACCACCACCACCACCCGCGTCCCGGCGCCAGCGGAGCGGGC
                    *********** ***   ******************************************

B73mir1gene         GGACGAGGAGGTAAGGCGCATGTACGAGGCGTGGAAGTCGAAGCACGGGCGCGGCGGCAG
Mp708mir1mRNA       GGACGAGGAGGTGCGGCGCATGTACGAGGCGTGGAAGTCGAAGCACGGGCGCGGCGGCAG
Mp708mir1gene       GGACGAGGAGGTGCGGCGCATGTACGAGGCGTGGAAGTCGAAGCACGGGCGCGGCGGCAG
                    ************  **********************************************

B73mir1gene         CAGCAACGACGACTGCGACATGGCGCCCGGCGATGATGAGC---AGGAGGAGGACCGCCG
Mp708mir1mRNA       CAGCAACGACGACTGCGACATGGCGCCCGGCGATGATGAGCAGGAGGAGGAGGACCGCCG
Mp708mir1gene       CAGCAACGACGACTGCGACATGGCGCCCGGCGATGATGAGCAGGAGGAGGAGGACCGCCG
                    *****************************************   ****************

B73mir1gene         GCTGCGGCTGGAGGTGTTCCGCGACAACCTTCGGTACATCGACAAGCACAACGCGGAGGC
Mp708mir1mRNA       GCTGCGGCTGGAGGTGTTCCGCGACAACCTTCGGTACATCGACGCGCACAACGCGGAGGC
Mp708mir1gene       GCTGCGGCTGGAGGTGTTCCGCGACAACCTTCGGTACATCGACGCGCACAACGCGGAGGC
                    *******************************************  ***************

B73mir1gene         GGACGCTGGGCTCCACACCTTCCGCCTCGGCCTCACCCCCTTCGCCGACCTCACCCTGGA
Mp708mir1mRNA       GGACGCTGGGCTCCACACCTTCCGCCTCGGCCTCACCCCCTTCGCCGACCTCACCCTGGA
Mp708mir1gene       GGACGCTGGGCTCCACACCTTCCGCCTCGGCCTCACCCCCTTCGCCGACCTCACCCTGGA
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Start Codon

Exon 1

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                  ************************************************************

B73mir1gene       CGAGTACCGCGGCCGCGTCCTCGGATTCCGCGCCCGCGCCCGCCGCAGCGGCGCCCGCTA
Mp708mir1mRNA     GGAGTACCGTGGCCGCGTCCTCGGCTTTCGCGCCCGCGGCCGCCGCAGCGGCGCCCGCTA
Mp708mir1gene     GGAGTACCGTGGCCGCGTCCTCGGCTTTCGCGCCCGCGGCCGCCGCAGCGGCGCCCGCTA
                   ******** ************** ** ********** *********************

B73mir1gene       CGGCCACGGCCACGGCTACCGCGCCCGTCCCCGCGGCGGCGACCTCCTCCCCGACGCCAT
Mp708mir1mRNA     CGGCTCCGGCTACAGCG------------TCCGCGGCGGCGA---CCTCCCCGACGCCAT
Mp708mir1gene     CGGCTCCGGCTACAGCG------------TCCGCGGCGGCGA---CCTCCCCGACGCCAT
                  ****  **** ** **              ************   ***************

B73mir1gene       CGACTGGCGCCAGCTTGGCGCCGTCACCGAGGTCAAGGACCAGCAACAGTGCGGTCCGTA
Mp708mir1mRNA     CGACTGGCGCCAGCTTGGCGCCGTCACCGAGGTCAAGGACCAGCAACA------------
Mp708mir1gene     CGACTGGCGCCAGCTTGGCGCCGTCACCGAGGTCAAGGACCAGCAACA----GGTCCGTA
                  ************************************************

B73mir1gene       GCATATATACTCCGACCCATAGCGCGCCGTACTCTTACATA-TGTGTGCGTGC-TCTCCG    Intron 1
Mp708mir1mRNA     ------------------------------------------------GTGC--------
Mp708mir1gene     GC----ATACTCCGACCCATCGCGCGCCGTACTCGTACATATTGTGTGCGTGCTTCTCCG

B73mir1gene       GCGAGAAGCTTGTTGC-GGCTACTT-----ATCTGATGACGATCTCTGTGGGGGGC---A
Mp708mir1mRNA     ------------------------------------------------------------
Mp708mir1gene     GCGAG-AGCTTGTTGCTGGCTATTTATCTGATCTGATGACGATCTCCGTTGGGGACTGGG

B73mir1gene       TGCAATGCATGCATGCTTGCA----GGTGGGTGCTGGGCGTTCTCGGCGGTGGCGGCCAT
Mp708mir1mRNA     -------------------------GGTGGGTGCTGGGCGTTCTCGGCGGTGGCGGCCAT
Mp708mir1gene     GGGCATGCATGCATGCTTGCAGTGCGGTGGGTGCTGGGCGTTCTCGGCGGTGGCGGCCAT
                                           ***********************************

B73mir1gene       CGAGGGGATCAACGCGATCGCGACGGGTAACCTGGTGTCGCTGTCGGAGCAGGAGATCAT
Mp708mir1mRNA     CGAGGGGGTGAACGCGATCGCGACGGGTAACCTGGTGTCGCTGTCGGAGCAGGAGATCAT
Mp708mir1gene     CGAGGGGGTGAACGCGATCGCGACGGGTAACCTGGTGTCGCTGTCGGAGCAGGAGATCAT
                  ******* * **************************************************

B73mir1gene       CGACTGCGACGCCCAGGACAGCGGCTGCGACGGCGGGCAGATGGAGAACGCGTTCCGGTT
Mp708mir1mRNA     CGACTGCGACGCCCAGGACAGCGGCTGCGACGGCGGGCAGATGGAGAACGCGTTCCGGTT    Exon 2
Mp708mir1gene     CGACTGCGACGCCCAGGACAGCGGCTGCGACGGCGGGCAGATGGAGAACGCGTTCCGGTT
                  ************************************************************
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B73mir1gene        CGTCATCGGCAACGGCGGGATCGACACCGAGGCCGACTACCCCTTCATCGGAACCGACGG
Mp708mir1mRNA      CGTCATCGGCAACGGCGGGATCGACACCGAGGCCGACTACCCCTTCATCGGAACCGACGG
Mp708mir1gene      CGTCATCGGCAACGGCGGGATCGACACCGAGGCCGACTACCCCTTCATCGGAACCGACGG
                   ************************************************************

B73mir1gene        CACTTGTGACGCCAGCAAGGTCGGTATGGGTGCTGGCGTGCTGCTGCTTTTGCTTCGTCG
Mp708mir1mRNA      CACTTGTGACGCCAGCA-------------------------------------------
Mp708mir1gene      CACTTGTGACGCCAGCAAGGTCGGTATGGGTGCTGGCGTGCTGCTGCTTTTGCTTCGTCG
                   *****************

B73mir1gene        ATCGATGGATGGATGATGCGGTAGTACTGCTGCTAATATAACGGAGAGATCGATATGACT    Intron 2
Mp708mir1mRNA      ------------------------------------------------------------
Mp708mir1gene      ATCGATGGATGGATGATGCG---GTACTGCTGCTAATATAACGGAGAGATCGATATGACT

B73mir1gene        GTGTGTGTACGTGTTCAATTCAATGCAGGAGAACAACGAGAAGGTCGCCACCATAGATGG
Mp708mir1mRNA      --------------------------AGGAGAAGAACGAGAAGGTCGCCACCATAGATGG
Mp708mir1gene      GTGTGTGTACGTGTTCAATTCAATGCAGGAGAAGAACGAGAAGGTCGCCACCATAGATGG
                                             ******* *************************

B73mir1gene        GTTGGTGGAGGTGGCGAGCAACAACGAGACGGCGCTGCAGGAGGCGGTGGCGATCCAGCC    Exon 3
Mp708mir1mRNA      GTTGGTGGAGGTGGCGAGCAACAACGAGACGGCGCTGCAGGAGGCGGTGGCGATCCAGCC
Mp708mir1gene      GTTGGTGGAGGTGGCGAGCAACAACGAGACGGCGCTGCAGGAGGCGGTGGCGATCCAGCC
                   ************************************************************

B73mir1gene        CGTCAGTGTCGCCATCGACGCAAGCGGGCGTGCGTTCCAGCACTACAGTTCGGTAA----
Mp708mir1mRNA      CGTCAGTGTCGCCATCGACGCAAGCGGGCGTGCGTTCCAGCACTACAGTTC---------
Mp708mir1gene      CGTCAGTGTCGCCATCGACGCAAGCGGGCGTGCGTTCCAGCACTACAGTTCGGTAAGCTA
                   ***************************************************

B73mir1gene        ------------------------------------------------------------
Mp708mir1mRNA      ------------------------------------------------------------
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B73mir1gene        ------------------------------------------------------------
Mp708mir1mRNA      ------------------------------------------------------------
Mp708mir1gene      TAACCGCCAGTGCTAGGGGCCAGTAGAAATCATCATTTGTACAGGCGGGTAACTGAGGAC
```

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B73mir1gene         ------------------------------------------------------------
Mp708mir1mRNA       ------------------------------------------------------------
Mp708mir1gene       CGCCAGTGCAAATCGTTTCCAGGAAACATAAAACATATTTTAAAAATAGTTAAAAAATTT

B73mir1gene         ------------------------------------------------------------
Mp708mir1mRNA       ------------------------------------------------------------
Mp708mir1gene       ATTTTTATTAGGCCCACCCCACCCGCCAGTCCCGCCAAGTCGCAAGTCGCGGCATTTTTC

B73mir1gene         ------------------------------------------------------------
Mp708mir1mRNA       ------------------------------------------------------------
Mp708mir1gene       GCGCGCTACGCGGTTGCTAGTATTCGAACCGGCGACCTCACCCTCGCGCGTACCCTCCCC

B73mir1gene         ------------------------------------------------------------
Mp708mir1mRNA       ------------------------------------------------------------
Mp708mir1gene       TACCACTCCGTCTATGACATGTCTTGTGTCTAGTTTGTAGTTGTTTTCTCCACATATTAC

B73mir1gene         ------------------------------------------------------------
Mp708mir1mRNA       ------------------------------------------------------------      Intron 3
Mp708mir1gene       AACCATTTGAGTGTAAATTGCTTATTTGAGACCCTAAACGAATTCAAAAAAACAAGTTGT

B73mir1gene         ------------------------------------------------------------
Mp708mir1mRNA       ------------------------------------------------------------
Mp708mir1gene       CAACTACAAAGATAAATAACTTTTGAAGTTCTACAACTTTTATTTTGACACTTTTTTCAT

B73mir1gene         ------------------------------------------------------------
Mp708mir1mRNA       ------------------------------------------------------------
Mp708mir1gene       CCGAGGTAGTTTGCAAAATTTGAATTTTAAATTTGACATACTTAGATTCAATTTTTGAGA

B73mir1gene         ------------------------------------------------------------
Mp708mir1mRNA       ------------------------------------------------------------
Mp708mir1gene       ACCGAAATGAGTTCAAATAAAAAAGTTGTCAACTACAAAGTTTCATAACTTTTAGAGATC

B73mir1gene         ------------------------------------------------------------
```

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Mp708mir1mRNA      ------------------------------------------------------------
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B73mir1gene        ------------------------------------------------------------
Mp708mir1mRNA      ------------------------------------------------------------
Mp708mir1gene      GGATAAAAATGATTTCTAGTGGCGGTTCCTTAAGAAAACCGCCACTAGAAATAGCACAGT

B73mir1gene        ------------------------------------------------------------
Mp708mir1mRNA      ------------------------------------------------------------
Mp708mir1gene      CGGTGGTGAAGCGAAACCGCCTGTAAAAATATATCGTCCCCGCAGGCTTTGAGCCTTTTT

B73mir1gene        --------GCTAGCTAGCTAGCTAAGCATGCATGAGAATGAGATCCATCAGACGACGACG
Mp708mir1mRNA      ------------------------------------------------------------
Mp708mir1gene      GTACTAGTGCTAGCTAGCTAGCTAAGCATGCATGAGAATGAGATCCATCAGACGACGACG

B73mir1gene        ACGACGACCGTCACAGATTTAGGAAGAACTAGCTAGCTAGGTTCTGTGCGTGTCTGTCTG
Mp708mir1mRNA      ------------------------------------------------------------
Mp708mir1gene      ACGACGACCGTCACAGATTTAGGAAGAACTAGCTAGCTAGGTTCTGTGCGTGTCTGTCTG

B73mir1gene        TGTGATGAACTGTTGTAGTATGCACTATAATGCAGGGCATCTTCAACGGGCCATGCGGGA
Mp708mir1mRNA      ----------------------------------GGGCATCTTCAACGGGCCATGCGGGA
Mp708mir1gene      TGTGATGAACTGTTGTAGTATGCACTATAATGCAGGGCATCTTCAACGGGCCATGCGGGA
                                                     **************************

B73mir1gene        CGAGCCTGGACCACGGCGTCACGGCGGTGGGCTACGGCAGCGAGAGCGGCAAGGACTACT
Mp708mir1mRNA      CGAGCCTGGACCACGGCGTCACGGCGGTGGGCTACGGCAGCGAGAGCGGCAAGGACTACT
Mp708mir1gene      CGAGCCTGGACCACGGCGTCACGGCGGTGGGCTACGGCAGCGAGAGCGGCAAGGACTACT
                   ************************************************************

B73mir1gene        GGATCGTGAAGAACTCGTGGAGCGCCAGCTGGGGCGAGGCCGGCTACATCCGCATGAGGC
Mp708mir1mRNA      GGATCGTGAAGAACTCGTGGAGCGCCAGCTGGGGCGAGGCCGGCTACATCCGCATGAGGC
Mp708mir1gene      GGATCGTGAAGAACTCGTGGAGCGCCAGCTGGGGCGAGGCCGGCTACATCCGCATGAGGC
                   ************************************************************

B73mir1gene        GCAACGTGCCCCGGCCCACGGGCAAGTGCGGCATCGCCATGGACGCGTCCTACCCTGTGA      Exon 4
Mp708mir1mRNA      GCAACGTGCCCCGGCCCACGGGCAAGTGCGGCATCGCCATGGACGCGTCCTACCCTGTGA
```

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Mp708mir1gene      GCAACGTGCCCCGGCCCACGGGCAAGTGCGGCATCGCCATGGACGCGTCCTACCCTGTGA
                   ************************************************************

B73mir1gene        AGGACACCTACCACGACCCCGGCACCGGCACCGGCACCGCCACGGCTACGGCAGCTGCCA
Mp708mir1mRNA      AGGACACCTACCA---------CCCCGGCACCGGCACCGCCACGGCTAGGGCAGCTGCCA
Mp708mir1gene      AGGACACCTACCA---------CCCCGGCACCGGCACCGCCACGGCTAGGGCAGCTGCCA
                   *************         * ************************ ***********

B73mir1gene        TGGATGTGATCAAGATGGTTCTTGCTTAGGAGGGAGCGAGCGGAGCAGGCAGCAGAGAGC     Stop Codon
Mp708mir1mRNA      TGGATGTGATCAAGATGGTTCTTGCTTAGGAGGGAGCGAGCGGAGCAGGCAGCAGAGAGC
Mp708mir1gene      TGGATGTGATCAAGATGGTTCTTGCTTAGGAGGGAGCGAGCGGAGCAGGCAGCAGAGAGC
                   ************************************************************

B73mir1gene        CGATGGTCTTGTCGTGTTGAACTTTACATATGGTAGCTAGGTACCACTGGGGATAATTAA
Mp708mir1mRNA      CGAGGGTCTTGTCGTGTTGAACTTTACATAGGGTAGCTAGGTACCACTGGGGATAATTAA
Mp708mir1gene      CGAGGGTCTTGTCGTGTTGAACTTTACATAGGGTAGCTAGGTACCACTGGGGATAATTAA
                   *** ************************** *****************************

B73mir1gene        GTTAATCCGTTATGGTGTGGCAAGTTAATTAATATGTGCGTATCTCTTTTGATGAGTGCC
Mp708mir1mRNA      GTTAATCCGTTATGGTGTGGCAAGTTAATTAATATGTGCGTATCTCTTTTGATGAGTGGC
Mp708mir1gene      GTTAATCCGTTATGGTGTGGCAAGTTAATTAATATGTGCGTATCTCTTTTGATGAGTGGC
                   ********************************************************** *

B73mir1gene        ATATATGATGTAATGAAGTTACATAAACTCAAATAAAGTCGTAATCGTAATGGTAAA---
Mp708mir1mRNA      ATATATGATGTAATGAAGTTACATAAACTCAAATAAAGTCGTAATCGTAATGGTAAAAAA
Mp708mir1gene      ATATATGATGTAATGAAGTTACATAAACTCAAATAAAGTCGTAATCGTAATGGTAAAG--
                   *********************************************************

B73mir1gene        ------------
Mp708mir1mRNA      AAAAAAAAAAAA
Mp708mir1gene      ------------
```

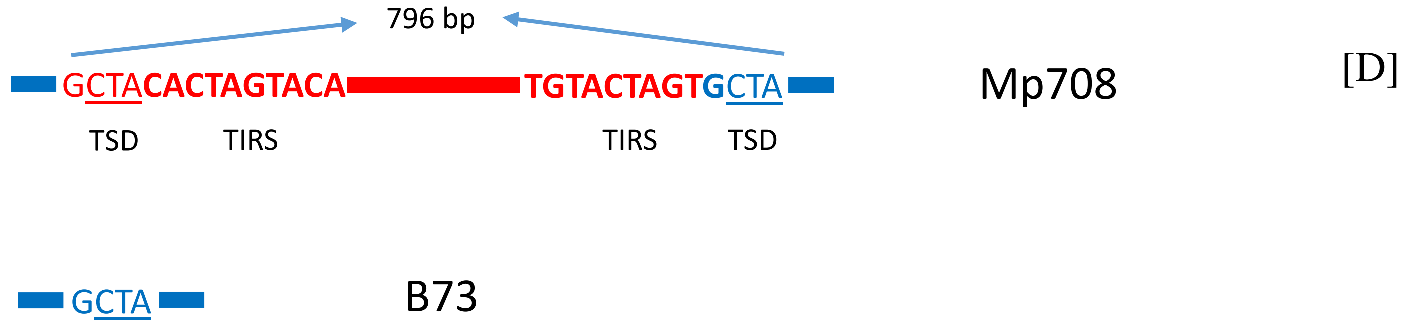


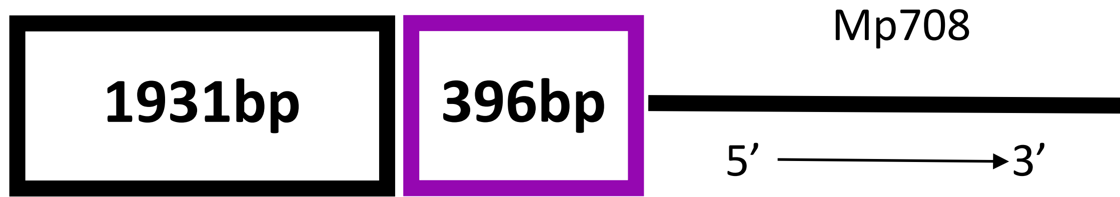
Figure A.3. *mir1* gene is present in maize inbred line B73.

[A] *mir1* gene is shown on B73 genome assembly RefGen_V3 (MaizeGDB).

[B] Schematic representation of *mir1* gene structures in maize inbred lines Mp708 and B73. *mir1* has four exons and three introns in both inbred lines. Arrow pentagons represent exons and lines represent introns. The red line represents a 792 bp region in Mp708 *mir1* gene intron three.

[C] B73 *mir1* gene, Mp708 *mir1* mRNA and gene were compared and aligned by MUSCLE. Nucleotide matches are indicated by asterisks. Start and stop codons together with exons and introns are indicated by different colors. The left and right TIRs and TSDs of the transposable element are underlined in Mp708 *mir1* intron three.

[D] The 792 bp region is a CACTA type transposable element in Mp708 *mir1* intron three. In Mp708, the left and right terminal inverted repeats (TIRs) are CACTAGTACA and TGTACTAGT. The left and right target site duplication (TSDs) are CTA. B73 *mir1* only has a right TSD.


1931bp
396bp
Mp708
5′
3′

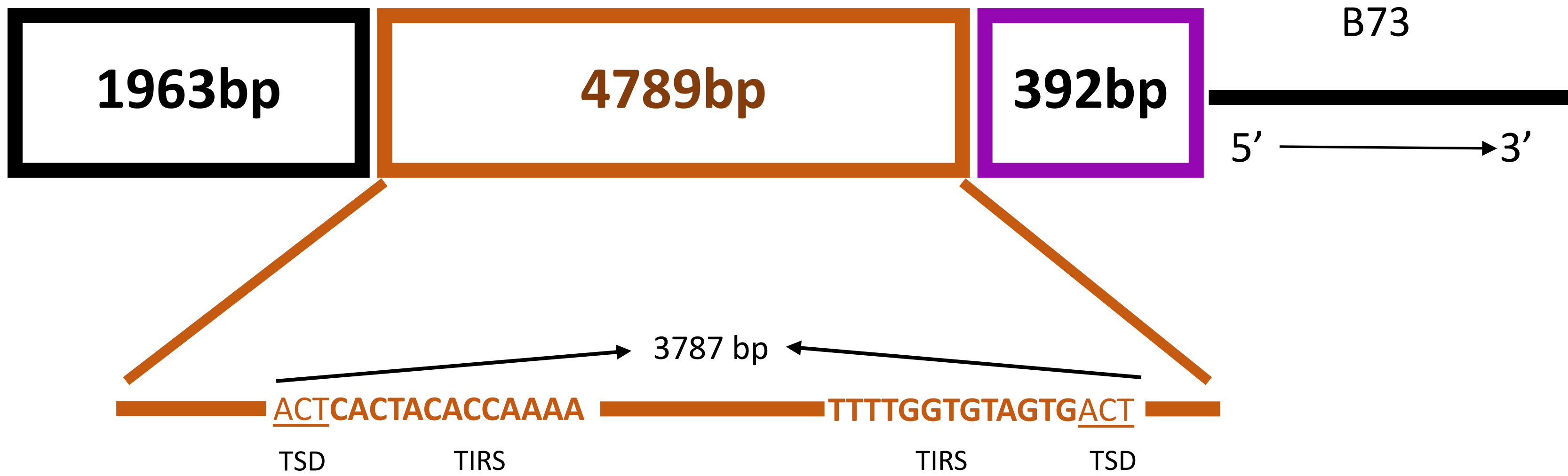

1963bp
4789bp
392bp
B73
5′
3′
3787 bp
ACTCACTACACCAAAA
TTTTGGTGTAGTGACT
TSD
TIRS
TIRS
TSD

Figure A.4. Schematic representation and sequence alignment of *mir1* promoters in Mp708 and B73.

Schematic representation of *mir1* promoter structures in maize inbred lines Mp708 and B73. Rectangles represent promoter and lines represent gene. The black rectangle is the first part of the promoter; the brown orange rectangle is a 4789 bp region in B73 *mir1* promoter; the purple rectangle is the last part of the promoter. A 3787 bp CACTA transposable element in present in B73 *mir1* promoter (highlighted under the brown orange rectangle). The left and right TIRs are in capital letters. The left and right TSDs are ACT.

Table A.1. The 23 tested NAM founder lines are identified as B73-like because they have the 4789 bp region in their *mir1* promoters. The original output of SNPs from maize HapMap V3.2.1 (imputed) is not shown.

Genotypes	***mir1* promoter B73-like**	***mir1* promoter Mp708-like**
Mp708		×
B73	×	
B97	×	
CML103	×	
CML228	×	
CML247	×	
CML277	×	
CML333	×	
CML52	×	
CML69	×	
Hp301	×	
IL14H	×	
Ki11	×	
Ki3	×	
Ky21	×	
M162W	×	
M37W	×	
Mo17	×	
Mo18W	×	
Ms71	×	
NC350	×	
NC358	×	
OH7B	×	
OH43	×	

Supplementary Data

Supplementary Table A.1. List of gene specific primers for quantitative real-time PCR in maize.

Gene	Forward	Reverse	NCBI Accession Number
Actin	GGAGCTCGAGAATGCCAAGAGCAG	GACCTCAGGGCATCTGAACCTCTC	U60511.1
Maize insect resistance 1 (*mir1*)	GAGGGTCTTGTCGTGTTGAACTT	GCCACACCATAACGGATTAACTT	NM_001112101.1

Supplementary Table A.2. qRT-PCR original data for *mir1* relative quantification.

Genotype	Infestation	*mir1* relative quantification
Mp708	Control	1.785
Mp708	Control	1.828
Mp708	Control	1
Mp708	Infested	1.376
Mp708	Infested	1.855
Mp708	Infested	2.983
CML247	Control	0.23
CML247	Control	0.115
CML247	Control	0.1
CML247	Infested	0.895
CML247	Infested	0.207
CML247	Infested	0.136
CML247	Infested	0.143
CML277	Control	0.005
CML277	Control	0.012
CML277	Control	0.006
CML277	Control	0.027
CML277	Infested	0.051
CML277	Infested	0.117
CML277	Infested	0.026
CML277	Infested	0.154
CML333	Control	0.019
CML333	Control	0.036
CML333	Control	0.038
CML333	Infested	0.052
CML333	Infested	0.088
CML333	Infested	0.016
CML333	Infested	0.03
CML52	Control	0.037
CML52	Control	0.087
CML52	Control	0.156
CML52	Control	0.094
CML52	Infested	0.132
CML52	Infested	0.055
CML69	Control	0.013
CML69	Control	0.017
CML69	Control	0.02
CML69	Control	0.034

CML69	Infested	0.05
CML69	Infested	0.258
CML69	Infested	0.094
Hp301	Control	0.007
Hp301	Control	0.025
Hp301	Infested	0.479
Hp301	Infested	0.335
Hp301	Infested	0.453
IL14H	Control	0.006
IL14H	Control	0.004
IL14H	Control	0.001
IL14H	Infested	0.016
IL14H	Infested	0.133
IL14H	Infested	0.076
IL14H	Infested	0.124
Ki3	Control	0.003
Ki3	Control	0.002
Ki3	Infested	0.127
Ki3	Infested	0.116
Ki3	Infested	0.025
Ki3	Infested	0.083
Ky21	Control	0.201
Ky21	Control	0.085
Ky21	Control	0.086
Ky21	Control	0.174
Ky21	Infested	29.647
Ky21	Infested	18.260
Ky21	Infested	30.558
Ky21	Infested	54.410
Mo17	Control	0.004
Mo17	Control	0.023
Mo17	Infested	0.13
Mo17	Infested	0.141
Mo17	Infested	0.086
Mo17	Infested	0.132
Mo18W	Control	0.016
Mo18W	Control	0.044
Mo18W	Infested	0.210
NC350	Control	0.035
NC350	Control	0.071
NC350	Control	0.025
NC350	Infested	0.103
NC350	Infested	0.113

NC350	Infested	0.416
OH7B	Control	0.016
OH7B	Control	0.059
OH7B	Infested	1.488
OH7B	Infested	0.963
OH7B	Infested	0.129
OH43	Control	0.014
OH43	Control	0.012
OH43	Control	0.135
OH43	Control	0.09
OH43	Infested	0.239
OH43	Infested	0.382
OH43	Infested	1.793
OH43	Infested	0.347

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CLUSTAL multiple sequence alignment by MUSCLE (3.8)


Mp708mir1promoter      CTCTCAGACCAAATCCATGTGTGTTGAGTGAGAATTGAGATGGGCA-GGATTACTCCAAA
B73mir1promoter        CTCTCAGACCAAATCCATGTGTGTTGAGTGAGAATTGAGATGGGCAGGGATTACTCCAAA
                       ********************************************** *************

Mp708mir1promoter      TCCCTTTGTTTTGGACGGGGACTTGGTTTATTTTCATCAACAATTCCAGCCCGCCCTGAT
B73mir1promoter        TTCCTTTGTTTTGGACGGGGACTTGGTTTATTTTCACCAACAATTCCAGCCCGCCTTGAT
                       * ********************************** ****************** ****

Mp708mir1promoter      TGCCTGCCTTAGAAATGACCGTGGTTGTACTGTTGGAGACTTGGTTTGTTTTAGTGGCCC
B73mir1promoter        TGCCTGCCTTAGAAATGACCGTGGTTGTACTGTTGGAGACTTGGTTTGTTTTACTGGCCC
                       ***************************************************** ******

Mp708mir1promoter      TGGCGTGAAACACAGACT--GGCCCTGTACAAGAAAATATTAAAAAACTATAGGTTTTCT
B73mir1promoter        TGGCGTGAAACACAGACTGAGACCCTGTACAAGGAAATA-TAAAAAACTATAGGTTTTCT
                       ******************  * *********** ***** ********************

Mp708mir1promoter      TGTTGTGTTATCTCGCCGATGTTAAAATTAGACTGTGTATCAAATAGCAGATCATAGCCT
B73mir1promoter        TG-TGTGTTATCTCGTCGATTTCAAAACTAGACTGTGTAGCAAATAGCAGATCATAGCCT
                       ** ************ **** * **** *********** ********************

Mp708mir1promoter      AACACACGATCACACATGTACGAGTTGAGGGCTGAGGCTCTAGAATTCTAGGGACTCTGT
B73mir1promoter        AACACACAATCACACATGTACGAGTTG--------GGCCCTAGAATTCTAGGGACTCTGT
                       ******* *******************        *** *********************

Mp708mir1promoter      ACGGTTGGCCCATTTACGCTGGGTCATGTAGGGGCCTAGCTCTAAGGCCGTTTTCAGTAG
B73mir1promoter        GCAGTTGGCCCATTTACGTTGGATCATGTAGGGGCCTAGCTGTAAGGCCGTTTTCAACAG
                        * *************** *** ****************** **************  **

Mp708mir1promoter      AGGCCCAGTATAAATGATA-----------------------------------------
B73mir1promoter        AGGCCCAATATAAATGATAAAAAAAAGTTCATATCCGTACCTTGGCCCAGGCCGTTTTCAA
                       ******* ***********

Mp708mir1promoter      ---------------------AAAAAAAGTTCATATCCGTAGCTTGGCCCGTCAATCAAG
B73mir1promoter        CAGAGGCCCAATATAAATGATAAAAAAAGTTCATATCCGTACCTTGGCCCATCAATCATG
                                            ******************** ******** ******* *

Mp708mir1promoter      TCGTGGAAAAGAAGTGTACTTTACTTTTAATAATGCATGCTCTGTCAAGATGTCTGCTTG
B73mir1promoter        TCGTGGAAAAGAAGTGTACTTTACTTTTAATAATGCATGCTCTGTCAAAATGTCCGGTTG
                       ************************************************ ***** * ***

Mp708mir1promoter      GC----------------TTTACTATAAAATTCTACGGTGGTTTGTTTCTATGCCGAGGC
B73mir1promoter        GCTTTAATTCAAAACGGTTTTACTGTAAAATTCTAGAGTAGTTTGTCTCTGTATTACAAT
                       **                ****** **********  ** ****** *** *

Mp708mir1promoter      TTCAGTCCGAATAGTTGGCTTTAATCCAAAACGAATAACTTGTTGTATCTTTTTGTCTAT
B73mir1promoter        T----GTCGAATAACTGACTTTAATCCGAAACGAACAAC----TGTATCTTTTTGTCTAT
                       *      ******  ** ********* ******* ***    *****************

Mp708mir1promoter      ATACTAATTCTAAGATATATTGGAGCAGATGCCACGACAGTTTCATAAATATTGTCTGCA
B73mir1promoter        ATACTAATTCTAAGATATATTCGAGCAGATGCCACGACAGTTTCATAAATATTGTCTGCA
                       ********************* **************************************

Mp708mir1promoter      AGGCACAAACCGTCTAGATAACCACACAACAAATTGTCTACTCTGTTGCTGCAGTACATA
B73mir1promoter        AGGCACAAACCGTATAGATAACCACACAACAAATTGTCTACTCTGTTGCTGCAGTACATA
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                           ************* **********************************************

Mp708mir1promoter          AATATGTTTTTGGTTGTAAAGAAACTAGGCTACATCACGATTCACACCACGTGCTGACGA
B73mir1promoter            AATATGTTTTTGGTTGTAAAGAAACTAGGCTACATCACGATTCACACCACGTGCTGACGA
                           ************************************************************

Mp708mir1promoter          CGTGAAGCATCACCCGATTTTCACGGCGGGGCACCGGGCACGGCCTCCAGCACCAGCCGC
B73mir1promoter            CGTGAAGCATCACCAGATTTTCACGGCGGGGCACCGGGCACGGCCTCCAGCACCAGCCGC
                           ************** *********************************************

Mp708mir1promoter          CACGGCGACGCAAACGTCAAGAGCTGCGTGCCCAAACGAGGGACAGGTCAGATGGGCGAG
B73mir1promoter            CACGGCGACGCAAACGTCAAGAGCTGCGTGCCCAAACGAGGGACAGGTCAGATGGGCGAG
                           ************************************************************

Mp708mir1promoter          GAAAAGCGCCCTGTTTCCTGCCCGGAGCGCCATGATGGCTCCTCGTGGCCTTTTCGTCGC
B73mir1promoter            GAAAAGCGCCCTGTTTCTTGCCCGGAGCGCCATGATGGCCCCTCGTGGCCTTTTCGTCGC
                           ***************** ********************* ********************

Mp708mir1promoter          TCGTTATTTTTAATCCCTCGACAAGATGCTTTTCTGCACGTTTGCGCATTAACACACACA
B73mir1promoter            TCGTTATTTTTAATCCCTCGACAAGATGCTTTTCTGCACGTTTGCGCATTAACACACACA
                           ************************************************************

Mp708mir1promoter          CACACACAC----AGAGAGAGAGAGAGAGAGCTCGTAGAATGGCACGCACACGCTCCCTG
B73mir1promoter            CACACACACAGAGAGAGAGAGAGAGAGAGAGCTCGTAGAATGGCACGCACACGCTCCCTG
                           *********    ***********************************************

Mp708mir1promoter          TGTGCTGTGCTCCACAGTCGTCGCCGTCGTCTGGGCCCGCCGGCCGGCGGCGACCGGATC
B73mir1promoter            TGTGCTGTGCTCCACAGTCGTCGCCGTCGTCTGGGCCCGCCGGCCGGCGGCGACCGGATC
                           ************************************************************

Mp708mir1promoter          GCCACCGCGAGTACACGGTCTCGCCGCACGGCGCACAAGGTATGGGGGGCGTGGGAGTGC
B73mir1promoter            GCCACCGCGAGTACACGGTCTCGCCGCACGGCGCACAAGGTATGGGGGGCGTGGGAGTGC
                           ************************************************************

Mp708mir1promoter          GCGGTGCCAGTCAGGGTCTCGCGTTCGCCATTAGTGGTGCCGTTTAGAATATGGCAAAGG
B73mir1promoter            GCGGTGCCAGTCAGGGTCTCGCGTTCGCCATTAGTGGTGCCGTTTAGAATATGGCAAAGG
                           ************************************************************

Mp708mir1promoter          ATTATGCGTGCCCAACTTGTGGTGGACGTGTAGCTAGCCACGCGCGCCTTCTTCGTCACT
B73mir1promoter            ATTATGCGTGCCCAACTTGTGGTGGACGTGTAGCTAGCCACGCGCGCCTTCTTCGTCACT
                           ************************************************************

Mp708mir1promoter          CGTCACCGCGCGCTAAACGACCGGCTGGTGTAACCTTACCACGGCCACGTCGAGGGATCG
B73mir1promoter            CGTCACCGCGCGCTAAACGACCGGCTGGTGTAACCTTACCACGGCCACGTCGAGGGATCG
                           ************************************************************

Mp708mir1promoter          TCGACGTCCAAATCCGAGTACAAAAGCAAAGCACCCATCATTAACTAGCGCCGCCTACAC
B73mir1promoter            T---CGTCCAAATCCGAGTACAAAAGCAAAGCACCCATCATTAACTAGCGCCGCCTACGC
                           *   ****************************************************** *

Mp708mir1promoter          ATCGTAGGATAAGCACGCTGATGGTGATGGTATAGTACGTACGTAGTAGTACATC-TTTT
B73mir1promoter            ATCGTAGGATAAGCACGCTGATGGTGATG--CTAGTACGTACGTAGTAGTTCATCTTTTT
                           *****************************   ****************** **** ****

Mp708mir1promoter          TTTTAATTGACTCTGGTGGTGATCGCAGTAGTGCAGACGACGGAGTGCTCCAGCAACGGG
B73mir1promoter            TTTTAATTGACTCTGGTGGTGATTGC---AGTGCAGACGACGGAGTGC------------
                           *********************** **   *******************
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Mp708mir1promoter      ACGCTGATGAACAGGGAGATAGTATAGCTCGAGCTCCCTAATATAATCCACACGCTTGCA
B73mir1promoter        -----------CAGGGCGATAGTATAGCTCGAGCTCCCTAATATAATCCACACGCTTGCA
                                  ***** *******************************************

Mp708mir1promoter      CTGGTCTCGAGAATGAGA-AATCTATTCACCAGTTTGTGTTGTGCAGACTCATGCAGTAA
B73mir1promoter        CTGGTCTCGAGAATGAGATAGTCTATTCACCAGTTTGTGTTATGCAGACTCATGCAGTA-
                       ****************** * ******************** *****************

Mp708mir1promoter      TATCTAGAGACCGAGTGTGTCGTCATCAAGGATTACCAAACCCCTTTTGGGTAAAAAAAA
B73mir1promoter        -ATCTAGAGACCGAGTGTGTCATCATCAAGGATTACCAAACCCTTATCGGTCAAAAAAAA
                        ******************** ********************* * * **  ********

Mp708mir1promoter      AACCCGCTACACCTACCGGCAACGGTTTACCGCCTGTAAACCAATGAAAACCGATAAAAC
B73mir1promoter        AACCCGCT--ACCCACCGGTAACGGTCTAGCGCCAGTAAACTGATG----CCGGTAAAAT
                       ********  *** ***** ****** ** **** ******  ***    *** ***** 

Mp708mir1promoter      CGACCGG-TTTGACCAAACCAAATTTAAACAATTTTAAAAC-GTTTTTTTTTGAAAAACT
B73mir1promoter        CGGCCGGTTTTGGCTAAACCAAATTTAAACAAATTCAAAACGGTTTTTCGTTGAAAAATT
                       ** **** **** * ***************** ** *****  ******  ******** *

Mp708mir1promoter      GGCAGTAAACCGTTCAATACCATCGATAAACCGTCT------------------------
B73mir1promoter        GTCGGTGAACAGCTCAATACCGCCGATAAACCGTCCATTAAACCGTTTTTTTGTTTTACA
                       * * ** *** * ********  ************

Mp708mir1promoter      ------------------------------------------------------------
B73mir1promoter        TAAAAAATGAAAAAAGGTGACATGATTCACTTGTAATTTGCTTAAATTCACGTTGCATAG

Mp708mir1promoter      ------------------------------------------------------------
B73mir1promoter        TAAATACTAGTTGATCCGTATAACTTATGTTCACCAAAATGACATACAACATACATACGC

Mp708mir1promoter      ------------------------------------------------------------
B73mir1promoter        AACCACAAACTTCAATTCTCATGAAACAAACAATCATCAGATTTCATAGATACTCAAATA

Mp708mir1promoter      ------------------------------------------------------------
B73mir1promoter        CAAGTTCTTTTAGAGTTCTCCCACGACACATAGGTCGACATAATTCACAATACCCACGTA

Mp708mir1promoter      ------------------------------------------------------------
B73mir1promoter        TTCAGCTCGAGTCATACTACTCAGTCATATGGGAAGGATCCTGATACGTGCCCATTCTGT

Mp708mir1promoter      ------------------------------------------------------------
B73mir1promoter        CTATGTGCGATATGTTCCTTCAATCAAGTACCACCCCTTCCTCTCTTCGTTTCTCTAAAG

Mp708mir1promoter      ------------------------------------------------------------
B73mir1promoter        TACTTACACTCGAAACATGTGTCAACCTTCTCTTCGTGCTCTCAGATTAGGTCGAACATG

Mp708mir1promoter      ------------------------------------------------------------
B73mir1promoter        TCTCATATAAATAAGAACAATACATAATATCATGTAACGAGAGTAGAATAAAAAACATAT
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Mp708mir1promoter        ------------------------------------------------------------
B73mir1promoter          TACTTGTAACAAATGACATATTAACATGAAACAATAAAACATCGAAACATATTTTCATTC

Mp708mir1promoter        ------------------------------------------------------------
B73mir1promoter          TTTCCTCTATGCCCGAGATGCTCCTTCAACCAGATATCACCCCTCCGCGCGCTCTTTGTT

Mp708mir1promoter        ------------------------------------------------------------
B73mir1promoter          CCTTTACAATACTTACACTTAAAACCTGCACCAACCTTCTCTTCGCACTGACAGACTAAA

Mp708mir1promoter        ------------------------------------------------------------
B73mir1promoter          TCCAACATGTGTCCTACAAATGTGACAAAAATAATGCAAGTATGCAACACATTAGTAGCA

Mp708mir1promoter        ------------------------------------------------------------
B73mir1promoter          AATAATATTACCAGCTACATATATAACTAAATATTAACGTATATGTATGACTAAACATTA

Mp708mir1promoter        ------------------------------------------------------------
B73mir1promoter          ACAATACTAACGTATATAACTCACTACACCAAAAACATACACTTCCTACGGTTTTTTAAC

Mp708mir1promoter        ------------------------------------------------------------
B73mir1promoter          TTCCTACAGCTTTTATAACAAACCGTAGGAAATAAATAACTTCCGAGAGGCAACTAGTAG

Mp708mir1promoter        ------------------------------------------------------------
B73mir1promoter          CTCTAGGAAATAAACATAACTTCCTACGGTATTTTATAAAAGCTGTAGGAAGTTAACTTT

Mp708mir1promoter        ------------------------------------------------------------
B73mir1promoter          CACACGCTGATCCATGTGTGTGGTCAAGCGAGCCGCTAACTTCCTACGGCCGACTCTAGG

Mp708mir1promoter        ------------------------------------------------------------
B73mir1promoter          AAGTTACCATGGGCAGCTAACTTCCTACGACCTCCTCTAGGAAGTTAGCTTTCAGTTGCT

Mp708mir1promoter        ------------------------------------------------------------
B73mir1promoter          GATCTACGGATGCGGTAAAACGAGCCGCTAACTTCTTACGACCGCCTCTAGGAAGTTAGC

Mp708mir1promoter        ------------------------------------------------------------
B73mir1promoter          TTTCATCTTTTGACCAGTCAAACGAAAAGCTAACTTTCTACGGCCGCCTCTAGGAAGTTC

Mp708mir1promoter        ------------------------------------------------------------
B73mir1promoter          GCTGCCCATGCTAACTTCCTACGGCCTCCTCTAGGAAGTTAGCTTTCAGCTTTTGACCAG

Mp708mir1promoter        ------------------------------------------------------------
B73mir1promoter          TCAAACAAAAAGGTAACTTCCTACGGCCTGCTCTAGGAAGTTAATTAACTTCCTAGAATA

Mp708mir1promoter        ------------------------------------------------------------
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B73mir1promoter        CATGTACAATCTCTAGGAAGTTACGTAACTTCCTACAGCTTTACTCTAGGAAGTTATATT

Mp708mir1promoter      ------------------------------------------------------------
B73mir1promoter        TTTTATCTTAACCCGCAAATCAACCTTATCTTCTCTCACTTTCCTTTCTCTCCCGTTTCT

Mp708mir1promoter      ------------------------------------------------------------
B73mir1promoter        TCCACCTACCATAGAGCGTGCCCAAGGCTCCATCCGCCGGAGCGCGCCCCCGGCCTAG

Mp708mir1promoter      ------------------------------------------------------------
B73mir1promoter        CCCGCCGCGCCCGCCGGAGCTCCCGCCCCCGACCGCGCCCGCCCTCGGCCCCGGCCGCCG

Mp708mir1promoter      ------------------------------------------------------------
B73mir1promoter        CGCCCACCGGAGCGTGCGCCCCCGGCCCCGCCCGCCACGCCCGCCGGAGCGCCCACCCTC

Mp708mir1promoter      ------------------------------------------------------------
B73mir1promoter        GGCCCCGCCCGCCGCGCCCCTAACATCACAAAAGACATAATTTCTGCTAGAAATAATCAT

Mp708mir1promoter      ------------------------------------------------------------
B73mir1promoter        GTTGGTATTATTTTTGCTCCCACCTATGGTTTATTTTGCCCAAGTCTATTTAATCTGCTT

Mp708mir1promoter      ------------------------------------------------------------
B73mir1promoter        ATTGTCATCACGAATATTTATGTGCTGAGTCTGTGTACTGGCTTTGAGCATCTGGCAATT

Mp708mir1promoter      ------------------------------------------------------------
B73mir1promoter        TGCGGTCTCTAGGCAACTTCCATGTGACTTTACTTGCTTGTATTTAGGAGGATCGCCCAA

Mp708mir1promoter      ------------------------------------------------------------
B73mir1promoter        CAAGGTCAGAGCCAACACTACCTTTTAGTACTAGTTGTGACCAAAGTAACCGTGCTTTTA

Mp708mir1promoter      ------------------------------------------------------------
B73mir1promoter        TCTTCCTTTATTTTTGTTTTTTGTCCAACCCTTTACAACAATATTTTAGTAGTTGTAAAC

Mp708mir1promoter      ------------------------------------------------------------
B73mir1promoter        AATAATTTTGCAGTGCTCGTCTCTTAGGTGTAACCGTTGTCAATCAACTGTTTCCCAGCT

Mp708mir1promoter      ------------------------------------------------------------
B73mir1promoter        GACTAGTCAATAATTTGTCTAATTCCCTCGACTTCATACCAGGCCGAACAATAACCCAAC

Mp708mir1promoter      ------------------------------------------------------------
B73mir1promoter        TGTTCTTCAATTTGGCAGCCTGTTGTATCCTGTTTGTCTTGTTTTGTTCCTTGGATAGAT

Mp708mir1promoter      ------------------------------------------------------------
B73mir1promoter        TATCGGTCTAGTGTTCTGAATTCTTCATTGCTTTGCCATTGGAACGCAGAATTAATTCAT
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Mp708mir1promoter      ------------------------------------------------------------
B73mir1promoter        CTAGTTGAGTCCCATGCATGTGTCTGGTTGCTACAATCGGAAATGCATAGGCCCTCAAAG

Mp708mir1promoter      ------------------------------------------------------------
B73mir1promoter        TCCAATAACAAACGTGTAGTGGGCTGGTAATGATGTTTTTTTCATTGATCCCGAATTCTC

Mp708mir1promoter      ------------------------------------------------------------
B73mir1promoter        GTCAAATAGTGAAAATCAGCTTAATTATGAGGCAGGAGATTGCTTACTGTATTCTAGGGA

Mp708mir1promoter      ------------------------------------------------------------
B73mir1promoter        TCACTGTCTTGCGTTCTGAATCTTGTGTGTGCTACACACAGCAGGCTCTGGGTCAACTTT

Mp708mir1promoter      ------------------------------------------------------------
B73mir1promoter        TGACCTAGATTAATACCAACATAATTTCCAATATATCCCCTAACATCTATTTCCTTATCT

Mp708mir1promoter      ------------------------------------------------------------
B73mir1promoter        TTCAGGTATGCCATCACAAAAGCTTTTGTTATAGCTTTTGTGATGACTTTCTTCTCAGTG

Mp708mir1promoter      ------------------------------------------------------------
B73mir1promoter        TTTGACGTTCCTGTCTTCTGGCCTATACTCCTCTGCTACTGGATTGTTCTCTTTGTCCTT

Mp708mir1promoter      ------------------------------------------------------------
B73mir1promoter        ACAATGAAGCGCCAGATTATACATATGATCAAATACAAATATGTGCCTTTCAGTATCGGG

Mp708mir1promoter      ------------------------------------------------------------
B73mir1promoter        AAGCAGGTCAGTACCTACCACTAAACTCATTGTTTTTTGTTTATTCACTGAATTGTCATG

Mp708mir1promoter      ------------------------------------------------------------
B73mir1promoter        CGTTTGACGCCAACTGCTGTGGGTATCATGGGAGTTGATTGTCTAAGTAACACTCGGACC

Mp708mir1promoter      ------------------------------------------------------------
B73mir1promoter        AAAGTTATGAAGTTGTCTTGTCAATCCTAGTCGTCATGGGACAGACTACGTAACTGCTGT

Mp708mir1promoter      ------------------------------------------------------------
B73mir1promoter        GGGTATCAATTGTTTGAATATTAATTATGAGCATTAAATATAAATTTAATTAGGTTTAAT

Mp708mir1promoter      ------------------------------------------------------------
B73mir1promoter        AGATCCATCTCATCTTTTAGTCTTCATATGTATAATTAGTATTATAATTAATTTATATTT

Mp708mir1promoter      ------------------------------------------------------------
B73mir1promoter        AATACCCAAAATTATCATCCAAGTATTCGATGTGACAGAGACTAAATTTTAGTTGGGTGA
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Mp708mir1promoter      ------------------------------------------------------------
B73mir1promoter        AACCAAACAACCCTATATATATCAGAACTATTATACTAGATGTTCTAGAGTACAAATCAA

Mp708mir1promoter      ------------------------------------------------------------
B73mir1promoter        GAATGAAGATAACTCTAGGGTGGTGGTTTCATGTAGGTTGCAAATTGGGCTGAAAAACAT

Mp708mir1promoter      ------------------------------------------------------------
B73mir1promoter        GAACCTGAGTGAAGCCATCCTAGATGCAACCCGTGTATCAGCTGCCCAGAAATTCCATTC

Mp708mir1promoter      ------------------------------------------------------------
B73mir1promoter        TCCTGATTGTGTGACTAATCGTTATTAGTTGATGACTAATCGGATGACCATAAAACTAAT

Mp708mir1promoter      ------------------------------------------------------------
B73mir1promoter        TCCATTCTCCTTCTGTTTGTCTGAATAATCGTGATTAGTTGATGACTAATTGGATGACTA

Mp708mir1promoter      ------------------------------------------------------------
B73mir1promoter        GACAACTAATAGGCCTAATTGAGTCGTATTCCCTATTTGGGTCCAGGATGCCAACTAGGA

Mp708mir1promoter      ------------------------------------------------------------
B73mir1promoter        CAACTATTCACAATTAATCAGATGACCTAATAACACTGTCATAGACACTATTTTTCTCTT

Mp708mir1promoter      ------------------------------------------------------------
B73mir1promoter        CCAGTTTATTAACGTTTGCTTTTATTAAACGCCATTAGATGTGGTCCATGGATCAATTTT

Mp708mir1promoter      ------------------------------------------------------------
B73mir1promoter        ACCCGTATTTTGTGACGTTAAAGAAATGCTTGTGTAGTTGACACATAATATTATGTATTT

Mp708mir1promoter      ------------------------------------------------------------
B73mir1promoter        TCTGAAGTGCCTATTGTTTCATTTCTCATGATTTTCCCCAATTGTGATGCCAGAAATATG

Mp708mir1promoter      ------------------------------------------------------------
B73mir1promoter        GTGGGAAGAAGAGTGTCGTGAGCACCAGTTCCTCAAAAGACTGATTTCGAACCTGCCCCA

Mp708mir1promoter      ------------------------------------------------------------
B73mir1promoter        GTAGGAACTTGCCAGGGGAACAAGATACGGTGGATGATAATGTTTGATGTGCTCAATACC

Mp708mir1promoter      ------------------------------------------------------------
B73mir1promoter        AAGTGTAATACTTCATTCATATCTAGGAGGTAGACGGATTTTCGCTCCTGTTTCTGACTC

Mp708mir1promoter      ------------------------------------------------------------
B73mir1promoter        TGGGCTCACTGTTTAATTCCATGTTGTGCTTAGGTTGCTTCCTCCTATGCTTGTATAACG
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Mp708mir1promoter      ------------------------------------------------------------
B73mir1promoter        AGTTTGCGTCTCGAATAAATTATTTCGTTTTAGTTACTACGTATCTGGGCCCTAAAACAA

Mp708mir1promoter      ------------------------------------------------------------
B73mir1promoter        TAAGCTCTATGCTCTCAGCAAGTTTGGTATGAGATATTGTCTCTTATTAGTATTGGATGA

Mp708mir1promoter      ------------------------------------------------------------
B73mir1promoter        GATGTGTCTTATTATGATTATGGATAAGACTTTTGTGATGTAATTGATGAAACTATGGAT

Mp708mir1promoter      ------------------------------------------------------------
B73mir1promoter        TTAAATATTGTTATGTGATTGTGTGAGAGATGTTTTATGTGAAAATATATTGTGCTATAT

Mp708mir1promoter      ------------------------------------------------------------
B73mir1promoter        AGCTGTTGATATATTTGTTATGTTGTGTAATGTGGTGTAAAATAAAAACAAACAACATTT

Mp708mir1promoter      ------------------------------------------------------------
B73mir1promoter        GTAATGCTGGTCAAATTAACTTCCTAGAGGCCAATTGGGTCGTAGGAAGTTAGCTAGGGG

Mp708mir1promoter      ------------------------------------------------------------
B73mir1promoter        CTATAGTTAGTCGTAGGAAGTTAGCCAGCTAACTTCCTAGGGGTACAGTCAGCCGTAGGA

Mp708mir1promoter      ------------------------------------------------------------
B73mir1promoter        AGTTAATCAGCTCACGGCGCTGACGGCGTGAAGCTACTACCTTCCGAGAACCTAGTAACT

Mp708mir1promoter      ------------------------------------------------------------
B73mir1promoter        TCCGAGAGGCTTCTTTGGCTGTAGGAAGTTAGCTAACTTCCTAGAGGGCTCTAGGAAGTT

Mp708mir1promoter      ------------------------------------------------------------
B73mir1promoter        AAGCTAACTTCCGACAAAAAATTTCCGAGAGCCAAACTTCCGACGGGATGGCTTAACTTC

Mp708mir1promoter      ------------------------------------------------------------
B73mir1promoter        CGAGAGTTCAGCTAACTTCCTAGAGTTTAGGTCTAACTTTCTAGGGTTTAGGCTCTAGGA

Mp708mir1promoter      ------------------------------------------------------------
B73mir1promoter        AGTTCACTATTTTGGTGTAGTGACTACACATTAACAACATTAATATATAACTAGATATTA

Mp708mir1promoter      ------------------------------------------------------------
B73mir1promoter        ACAAGACGTCATCACATAAAAAAAATATGAAACCCTAAAACCAAAAAACCAAATTTAGAG

Mp708mir1promoter      ------------------------------------------------------------
B73mir1promoter        ACTAAAATTACTAGTAGCGGGTCCATACGTTTTCCTATTTTTTAATCGACTAAACTAAAT

Mp708mir1promoter      -------------------------ATTACTCACTAGAACATGTAGTTTTTTAATGTCAG
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B73mir1promoter        TAAATTTAGATATCATGTATATCAGATTACTCACTAGAACATGTAGTATTTTAATGTCCG
                                                ********************** ********** *

Mp708mir1promoter      ACGG-TTAGCCCTTATTTCCGACGGTTTTCGCCGTCCAAAAATTTTATATTTTACAGTTG
B73mir1promoter        ACAGTTTAGGCCTTATTTTCGACGACTTTGGCTAT-CGGAAATTTTATATTTCAGTGTTG
                       ** * **** ******** *****  *** **  * *  ************* *  ****

Mp708mir1promoter      CGCTGCATTCCAGTCCTAAAAGCACGATTTCAGATTTTGGAAATCACTTTTATTCTCTAG
B73mir1promoter        TGTTGCATTCCAATCCTAAGAGCACGATTTCAGATTTTGGAAATCACTTTTATTCTCTAG
                        * ********* ****** ****************************************

Mp708mir1promoter      CTTCATCATACCAAGGGTTAGCAACTGCTGCTTGTATTGCTTGCAGATGTGACATCGCCT
B73mir1promoter        CTTCATCATACCAA-GGTTAGCAACTGCTGCTTGTATTGCTTGCAGACGTGACATCGCCT
                       ************** ******************************** ************

Mp708mir1promoter      CAATCTCCAGTGCAACTTCAACAGCA-CCATTTGGTCTCTAACACCAAAACCCAAATCAA
B73mir1promoter        CAATCTCCAGTGCAACTTCAACAGCATCCATTTGGTCTGTAACAGCAAAACCCAAATCAA
                       ************************** *********** ***** ***************

Mp708mir1promoter      CGTCTTAGCGTGCGTGCGTGCATGCATGCGGGCAGCCCTCTGTGTAAGACATGCATGCGT
B73mir1promoter        CGTCTC----CGCATGCATGCATGCATGCGGGCAGCCCTCTGTTTAAGA----CGTGCGT
                       *****      ** *** ************************* *****    * *****

Mp708mir1promoter      CAAGGTCCTCAAGGATATATAAGATGGTGGGTCCG----GTTTGTTCGTTCTCGTTGCCC
B73mir1promoter        CAAGGTCCTCAAGGATATATAAGATGGTGGGTCCGGTTCGTTCGTTCGTTCTCGTTGCCT
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Mp708mir1promoter      GGCGCGGCTAT
B73mir1promoter        GGCGCGGCTAT
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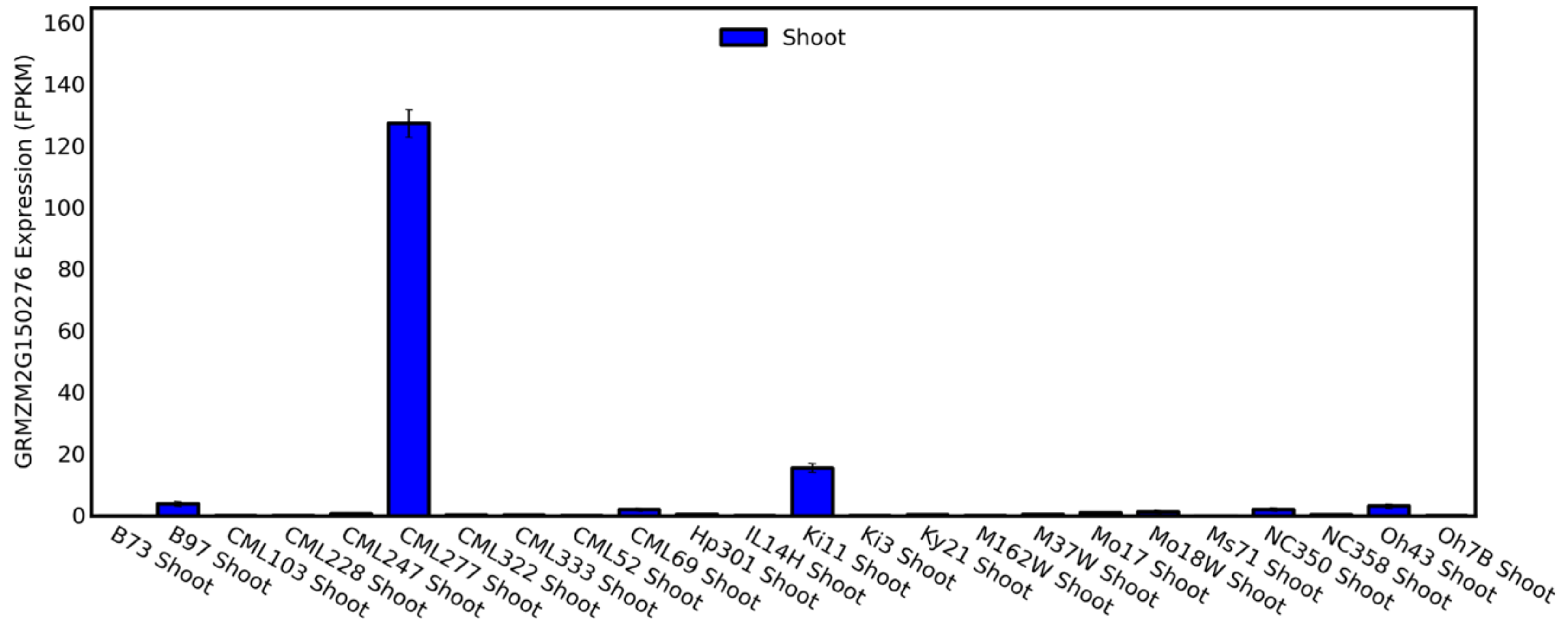
Supplementary Figure A.1. Mp708 and B73 *mir1* promoter sequences were compared and aligned by MUSCLE. Nucleotide matches are indicated by asterisks. The 4789 bp genetic region of B73 *mir1* promoter is in red. The left and right TIRs and TSDs of the transposable element are underlined in B73 *mir1* promoter.



Supplementary Figure A.2. qTeller *mir1* RNA-seq expression in shoot tissues of 23 maize NAM founder lines (This figure is adopted from http://qteller.com/NAM/tmp/GRMZM2G150276.png).

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