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Study of Defense Genes Expression Induced by Leaf Herbivory in Roots of Insect-resistant Maize

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ABSTRACT

There is a long-term coevolutionary relationship between plants and their insect herbivores, which results in plants mounting defenses against the insects. The study of plant herbivore defense is a popular field that allows researchers to investigate the mechanisms of plant defense. The motivation of this study is to obtain information, which can be used in the long-term to design herbivore control for agricultural crops.

By examining the plant defense responses in leaf and root, I hypothesize that aboveground herbivory induces defense gene transcript levels in both the aboveground and belowground parts of the insect-resistant maize inbred line Mp708. To prove this hypothesis, I examined changes in different defense gene expression in response to caterpillar feeding in maize whorl and root tissues. The results indicate that leaf herbivory does induce defense gene expression in insect-resistant maize leaves and roots.

Previous research has shown that a cysteine protease called Mir1-CP accumulates in the whorls of insect-resistant maize Mp708. This research investigates *mir1* gene transcript levels and Mir1-CP accumulation in the leaves and roots after feeding by the fall armyworm (*Spodoptera frugiperda*) larvae, in V7 growth stage of maize. Experiment observations suggest that there is aboveground and belowground communication of maize.

Defense gene expression during the different growth stages of maize was also investigated in this study. At different development stages maize may exhibit various levels of insect resistance and defense. This could result in variations of defense gene transcript levels in response to herbivory.

This research reveals that aboveground herbivory increases defense genes transcript levels both in aboveground and belowground parts of insect-resistant maize inbred line Mp708. And different growth stages exhibit different expression patterns of these genes in Mp708.

Table of Contents

LIST OF TABLES viii
LIST OF FIGURES ix
ACKNOWLEDGEMENTS xi
1. Background 1
1.1 Insect-Resistance Mechanisms in Plants 1
1.1.1 Constitutive Defense 2
1.1.2 Inducible Defense 2
1.1.2.1 JA Signaling Pathway 3
1.2 Insect-Resistant Maize Inbred Lines 6
1.2.1 Defense Genes in Mp708 7
1.2.1.1 Maize Insect Resistance Genes (*Mir*) 7
1.2.1.2 Lipoxygenase Gene (*Lox*) 8
1.2.1.3 Chitinase Gene (*Chi*) 9
1.2.1.4 Maize Protease Inhibitor Gene (*MPI*) 10
1.2.1.5 Pathogenesis-Related Protein Gene (*PR*) 10
1.2.1.6 Ribosome-Inactivating Protein Gene (*RIP*) 11
1.2.1.7 Rubisco Small Subunit Gene (*SSU*) 12
1.3 Aboveground and Belowground Communication 12
1.3.1 Evidences in Mp708 Maize Inbred Line 14
1.4 Maize Development Stages 15
1.5 Fall Armyworm (*Spodoptera frugiperda*) (J. E. Smith) 16
1.5 Objectives and Hypotheses 19
1.5.1 Objectives 19
1.5.2 Hypotheses 20
2. Materials and Methods 21
2.1 Plant and Insect Materials 21

2.2 Insect Infestation 22
2.3 RNA and cDNA Preparation 22
2.3.1 RNA Extraction 22
2.3.2 Reverse Transcription-PCR 23
2.4 Conventional PCR for Gene Amplification 23
2.4.1 Primer Design 23
2.4.2 Conventional PCR Analysis 26
2.4.3 Cloning 26
2.4.4 Sequence Analysis 27
2.5 Gene Expression Analysis 27
2.5.1 Quantitative Real Time-PCR 27
2.5.2 Statistical Analysis 28
2.6 Protein Extraction and Accumulation Analysis 28
2.6.1 Protein Extration 28
2.6.2 Protein Accumulation 29
3. Results 31
3.1 Result of Insect Infestation 31
3.2 Amplification of Defense Genes 32
3.2.1 Amplification of *Mir1* 32
3.3 Quantification of Defense Genes Expression in V7 Stage Mp708 Plant in Response of Foliar Herbivory 33
3.3.1 *Mir1* Gene Expression in V7 Stage Maize 34
3.3.2 *Mir2* and *Mir3* Genes Expression in V7 Stage Maize 35
3.3.3 *Lox3* and *Lox6* Genes Expression in V7 Stage Maize 35
3.3.4 *MPI, RIP2, Chi1, PR10* Genes Expression in V7 Stage Maize 36
3.3.5 *SSU1* Gene Expression in V7 Stage Maize 36
3.4 Quantification of Defense Genes Expression in Different Maize Growth Stages 37
3.4.1 *Mir1* Gene Expression in Different Maize Growth Stages 37

3.4.2 *Mir2* and *Mir3* Genes Expression in Different Maize Growth Stages 37
3.4.3 *Lox3* and *Lox6* Genes Expression in Different Maize Growth Stages 38
3.4.4 *RIP2* Gene Expression in Different Maize Growth Stages 39
3.4.5 *MPI* Gene Expression in Different Maize Growth Stages 39
3.4.6 *PR10* Gene Expression in Different Maize Growth Stages.................................. 39
3.4.5 *Chi1* Gene Expression in Different Maize Growth Stages.................................... 40
3.4.7 *SSU1* Gene Expression in Different Maize Growth Stages.................................. 40
3.5 Mir1-CP Protein Accumulation 40
4. Discussion 78
4.1 Pros and Cons of Experiment Designs 78
4.2 Results Discussion... 79
4.2.1 Model of Aboveground and Belowground Communication 79
4.2.2 *Mir2, Mir3, MPI, Lox3, Lox6, RIP, Chi1, PR10* Genes Expression in the V7 Growth Stage 81
4.2.3 *Mir* Genes Expression in Plant Development... 82
4.2.4 Other Genes Expression Variation in Plant Development 83
4.3 Summary 84
References 86
Appendix 106

LIST OF TABLES

Table 2.1.: Oligonucleotides of primers designed for targeted genes *mir1, mir2, mir3, lox3, lox6, RIP2, MPI, Chi1, PR10, SSU*..25

LIST OF FIGURES

Figure 1.1 A simplified diagram of the steps that occur in response to caterpillar feeding and that lead to the production of plant proteins involved in direct defenses…………………..………....5

Figure 1.2 Damage caused by fall armyworm in corn. ……………………………..….……18

Figure 3.1 Pictures of damaged aboveground and undamaged root tip tissues………..………42

Figure 3.2 Conventional PCR analysis …………………………………...………...……..43

Figure 3.3 Specificity of Real-Time PCR for amplification of *Zea mays* gene *mir1*, as determined by melting curve analysis..……………………………………………………..45

Figure 3.4 Real-Time PCR standard curves from 10-fold serial dilutions of most the concentrated samples of the *Zea mays mir1*……………………………………………………46

Figure 3.5 Histogram of *mir1* gene expression in leaf and root tissues from fall armyworm fed and non-fed V7 growth stage 08 Mp7plant……………………..……………….……...……47

Figure 3.6 Histogram of *mir2* gene expression in leaf and root tissues from fall armyworm fed and non-fed V7 growth stage Mp708 plant…………………………………………......……48

Figure 3.7 Histogram of *mir3* gene expression in leaf and root tissues from fall armyworm fed and non-fed V7 growth stage Mp708 plant………………………......................................49

Figure 3.8 Histogram of *lox3* gene expression in leaf and root tissues from fall armyworm fed and non-fed V7 growth stage Mp708 plant…………………………………….……..........50

Figure 3.9 Histogram of *lox6* gene expression in leaf and root tissues from fall armyworm fed and non-fed V7 growth stage Mp708 plant………………………………....….….…..…..51

Figure 3.10 Histogram of *RIP2* gene expression in leaf and root tissues from fall armyworm fed and non-fed V7 growth stage Mp708 plant………………………………..............................52

Figure 3.11 Histogram of *MPI* gene expression in leaf and root tissues from fall armyworm fed and non-fed V7 growth stage Mp708 plant………..…………………..............................53

Figure 3.12 Histogram of *PR10* gene expression in leaf and root tissues from fall armyworm fed and non-fed V7 growth stage Mp708 plant…………………………..…………………..……54

Figure 3.13 Histogram of *Chi1* gene expression in leaf and root tissues from fall armyworm fed and non-fed V7 growth stage Mp708 plant. …………….……………………………………...55

Figure 3.14 Histogram of *SSU1* gene expression in leaf and root tissues from fall armyworm fed and non-fed V7 growth stage Mp708 plant. ………………….…………….........................56

Figure 3.15 *mir1* gene expression in leaf and root tissues from fall armyworm fed and non-fed Mp708 during plant development (From V1 growth stage to V11 growth stage)……………..…....57

Figure 3.16 *mir2* gene expression in leaf and root tissues from fall armyworm fed and non-fed Mp708 during plant development (From V1 growth stage to V11 growth stage)……………… 59

Figure 3.17 *mir3* gene expression in leaf and root tissues from fall armyworm fed and non-fed Mp708 during plant development (From V1 growth stage to V11 growth stage)……….……..61

Figure 3.18 *lox3* gene expression in leaf and root tissues from fall armyworm fed and non-fed Mp708 during plant development (From V1 growth stage to V11 growth stage)…….................63

Figure 3.19 *lox6* gene expression in leaf and root tissues from fall armyworm fed and non-fed Mp708 during plant development (From V1 growth stage to V11 growth stage) …….……….65

Figure 3.20 *RIP2* gene expression in leaf and root tissues from fall armyworm fed and non-fed Mp708 during plant development (From V1 growth stage to V11 growth stage)……………….67

Figure 3.21 *MPI* gene expression in leaf and root tissues from fall armyworm fed and non-fed Mp708 during plant development (From V1 growth stage to V11 growth stage)… ....…69

Figure 3.22 *PR10* gene expression in leaf and root tissues from fall armyworm fed and non-fed Mp708 during plant development (From V1 growth stage to V11 growth stage)……………….71

Figure 3.23 *Chi1* gene expression in leaf and root tissues from fall armyworm fed and non-fed Mp708 during plant development (From V1 growth stage to V11 growth stage)……………….73

Figure 3.24 *SSU1* gene expression in leaf and root tissues from fall armyworm fed and non-fed Mp708 during plant development (From V1 growth stage to V11 growth stage)……………….75

Figure 3.25 Immunoblot analysis of Mir1-CP protein expression in leaf and root tissues from V7 stage Mp708 plant.……………………..……………..……………………………….……………77

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1. Background

This chapter provides the background and literature review for this thesis. Mechanisms of insect-resistance in plants, the insect-resistant inbred line of maize and insect pests used in this research are illustrated. The defense genes involved in JA signaling pathway examined for gene expression are also introduced in this chapter. The possibility of aboveground and belowground interactions in the maize plant leads to the formulation of my hypothesis.

Section 1.1 introduces two types of insect-resistance mechanisms in plants: constitutive and inducible defenses. The JA signaling pathway, as an important mechanism in inducible defense, is described in details in this section. Section 1.2 provides information about the insect-resistant maize inbred line Mp708, which is the model plant for my research. Defense genes including *mir1, mir2, mir3, lox3, lox6, MPI, RIP2, Chi1, PR10*, and *SSU1* are introduced in Section 1.2. Section 1.3 provides the literature review about aboveground and belowground communication in maize and other plants. Section 1.4 defines the maize developmental stages. Section 1.5 describes damage caused by the maize herbivore—fall armyworm (FAW) larvae, as well as FAW's life cycle. At last, this research's objectives and hypotheses are discussed in section 1.6.

1.1 Insect-Resistance Mechanisms in Plants

This study investigates mechanism of the interaction between insects and crop plants to determine strategies for a cost-effective, environmentally safe way to control herbivory, which will be beneficial for agriculture and many other fields. This is important since hunger is becoming more and more severe in the world and agricultural crops experience losses of up to forty-five percent due to pests, diseases and competitions with weeds (Charles et al., 2010). Insects cause nearly sixteen percent of maize crop losses (Schultz, 2009).

Plant defense against herbivorous insect includes a range of regulation and adaptations, which improves the plant's survival by reducing the negative impact of the insects. Plants use several strategies to defend against damage caused by herbivores. For example, many plants produce secondary metabolites, which influence normal behavior, growth, reproduction or survival of herbivores (Via et al. 1995). These chemical defenses usually act as toxins to herbivores, or reduce plant's digestibility (Karban and Myers, 1989). Other defensive strategies used by plants include escaping from or avoiding herbivores, for example, by growing in a location where plants are not easily found or accessed by insects, or by changing seasonal growth patterns (Karban and Baldwin, 1997). Some plants use a particular strategy to encourage the natural enemies of herbivores. This strategy, in turn, provides protection against herbivores. Each type of defense can be either constitutive or inducible (Dangl, 2001).

1.1.1 Constitutive Defense

Resistance or tolerance of plants to insect herbivores and pathogens is mediated via constitutive or induced defense mechanisms (Purrington, 2000). Constitutive defenses are those that are present in the plant without herbivory. Structural constitutive defenses include cuticle, trichomes, spines, and bark. Chemical constitutive defenses include secondary metabolites such as isoprenes, phenolic compounds, and alkaloids (Wink, 1988).

1.1.2 Inducible Defense

My research focuses on inducible defenses in response to herbivory. Inducible defenses play a major role in resistance to insects. The effects of inducible defense on insects can include increase of toxicity, delay of larval development, or increase of attack by insect parasitoids (Agrawal, 1998). Inducible defenses are a result of attack by chewing insects. This response may

be caused by Jasmonic acid (JA) which induces a number of proteins that provide improved defense to later insect attack (Mauricio et al., 1997). JA biosynthesis pathway is a well known herbivory defense mechanism pathway in plant kingdom.

Many insects cause damage to host plants by either chewing foliar tissues or root tissues. The wounding induces responses that include the production of protease inhibitors and other antifeedants such as alkaloids and phenolic compounds (Baldwin and Preston, 1999). Additionally, in response to herbivore damage to aboveground foliar tissues, the whole plant releases a complex array of volatiles which attract parasitic insects to feed on, or to deposit eggs into the larvae of the herbivorous insect, which would cause the death of the insect larvae (Farmer, 2001). With regards to the interactions between insect herbivores and monocotyledonous plants, it is well known that, maize releases a mixture of volatiles containing several terpenes. The released terpenes, including linalool, therefore attract parasitic wasps to attack the larvae (Turlings and Tumlinson, 1992; Alborn et al., 1997). In this research, another interaction between herbivore and monocotyledonous plant (*Zea mays*) has been investigated.

1.1.2.1 JA Signaling Pathway

One of well known induced defense mechanisms is JA signaling pathway. As signals that are released in plant when plant is attacked by pathogens or insects, jasmonic acid (JA) activates an entire defense gene expression program that includes the synthesis of a series of different plant defense proteins as an early component (Maleck and Dietrich, 1999). JA and its derivatives can modulate aspects of production of viable pollen, root growth, fruit ripening, and plant resistance to insects and pathogens (McConn et al., 1997). It has been shown that JA, salicylic acid (SA) in

JA biosynthesis pathways and ethylene signaling pathways are integrated in the regulation of stress response and plant development (Stotz et al., 2002).

It has been found that there are at least 41 genes responding to JA which are called JA responsive genes (Loughrin et al., 1995; Rapusas et al., 1996; Gouinguene et al., 2001). It has been proposed that JA activates expression of some genes encoding antifungal proteins (Shinshi et al., 1995; Wu and Bradford, 2003) and cell wall proteins (Heil, 1999; Heil and Baldwin, 2002). Figure 1.1 illustrates a simplified diagram of the steps that occur in response to insect wounding and lead to the production of plant proteins involved in direct defense in the insect-resistant maize inbred line Mp708. After an herbivore signal is received by plant, linolenic acid is converted to JA through a series of reactions. One of key enzymes catalyzing some of the reactions is lipoxygenase (LOX) encoded by *lox* genes. JA triggers the activation of downstream genes: *mir1* and its homolog genes, *MPI, RIP, Chi1* and *PR10* (Ankala et al., 2009; Shivaji et al., 2010; Pechan et al., 1999; Heitz et al., 1997; Laudert and Weiler, 1998; Mussig et al., 2000; Ishiguro et al., 2001; Seo et al., 2001; Sembdner and Parthier, 1993; Gols et al., 1999; Reinbothe et al., 1999).

JA influences defense gene expression at various levels, including gene activation and transcription, post-transcriptional RNA processing and transcript stability, translation, and post-translational steps including protein modifications and protein degradation (Sembdner and Parthier, 1993).

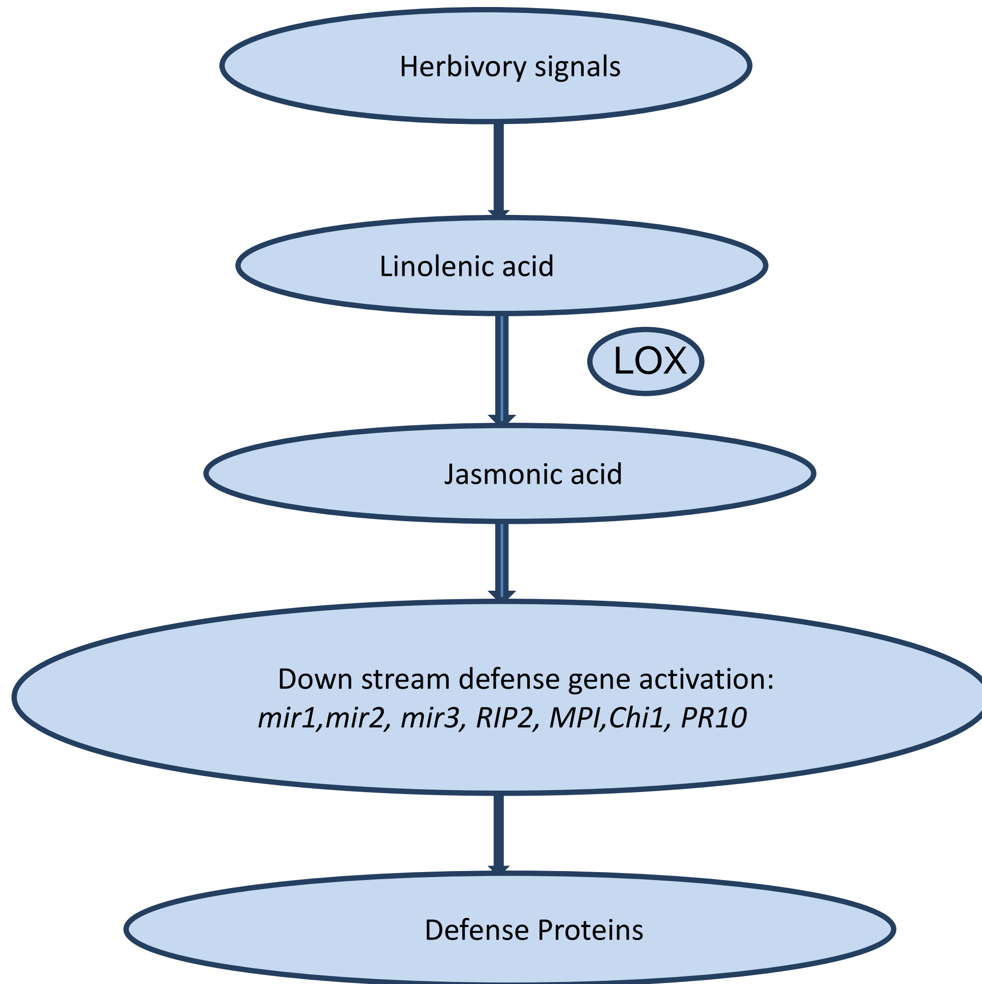


Figure1.1 Diagram of JA defense signaling pathway that occur in response to caterpillar feeding in miaze. Transcript levels for the genes in the boxes were measured in this study.

1.2 Insect-Resistant Maize Inbred Lines

In this research, Mp708 inbred line was chosen as the studied host plant because of its high resistance to insect feeding. The insect-resistant maize inbred line Mp708 (Williams and Davis, 1984; Williams and Davis, 1985; Williams et al., 1987) was developed using Antiguan germplasm obtained from CIMMYT (Internal Maize and Wheat Improvement Center) and designated MBR (Multiple Borer Resistant) lines. Mp708 shows resistance against feeding by fall armyworm (*Spodoptera frugiperda*) and other *Lepidopteran* species such as Europe corn borer (*Ostrinia nubilalis*), sugarcane borer (*Diatraea saccharalis*), corn earworn (*Helicoverpa zea*), tobacco budworm (*Heliothis virescens*), Asian corn borer (*Ostrinia frunicalis*), spotted stem borer (*Chilo partellus*), and African maize stem borer (*Busseda fusca)* (Williams and Buckley, 1990; Williams et al., 1989).

It has been reported that larvae reared on Mp708 leaf material weight less and take longer to pupate than those reared the same whorl and leaf part of susceptible maize inbred line (Tx601) (Williams et al., 1989). Larvae fed on yellow-green whorl tissues from resistant plants have lower growth rates and lower efficiency of conversion of both ingested and digested food into body substance than those fed on yellow-green whorl tissues of susceptible maize or artificial diets (Chang et al., 2000; Pechan et al., 2002).

High hemicellulose content, low protein content, and leaf toughness might be related with reduced larval growth (Williams et al., 1998). Although Mp708 inbred resistance mechanism is not fully uncovered, there is an analysis which suggests that the resistance of Mp708 inbred line is a quantitative genetic trait (Brooks et al., 2007).

1.2.1 Defense Genes in Mp708

In this research, several genes expression in response to herbivory is examined. Many genes are involved in insect resistance in Mp708. This study analyzes genes upstream and downstream in the JA pathway, including *mir1, mir2, mir3, lox3, lox6, MPI, RIP2, Chi1, PR10*. This research examine *SSU* gene, which encodes the small subunit of the CO_2 fixing enzyme rubisco, which is not related with JA pathway. *SSU* gene is used as a control for the experiments.

1.2.1.1 Maize Insect Resistance Genes (*Mir*)

Previous research has identified a novel insect defense mechanism in maize (*Zea mays*) genotypes that have genetic resistance to feeding by FAW (Pechan et al., 2000). For example, Mp708 exhibits less foliar damage than the susceptible line (Tx601); caterpillars that feed on the resistant plants are significantly smaller than caterpillars that feed on the susceptible lines (Williams et al., 1989).

Previous research results suggest that there are biochemical differences between callus of the resistant and susceptible inbreds. A 33-KD cysteine protease was found in Mp708 callus, which was named as Mir1-CP. The negative correlation between larval weight and Mir1-CP concentration suggests that Mir1-CP protein is involved in retarding larval growth (Jiang et al., 1995). Mir1-CP inhibits caterpillar growth by as much as 80% (Pechan et al., 2000). When caterpillars ingest Mir1-CP, this cysteine protease permeabilizes and attacks caterpillars' peritrophic matrix (PM). Caterpillar's PM is a structure that surrounds the food bolus and protects the intestinal microvilli from toxins and mechanical damage (Pechan et al., 2002). It is also reported that Mir1-CP accumulation is developmentally regulated and it is most abundant in the yellow-green mid-whorl region of resistant plant genotypes (Pechan et al., 2000). These

studies indicate Mir1-CP affects the digestive system of the insect herbivore, and the up-regulation of Mir1-CP in response to feeding by caterpillar larvae is involved in the insect defense mechanism.

Ankala et al., 2009 demonstrated that JA biosynthesis inhibitors reduce Mir1-CP accumulation in maize leaves in response to larval damage. Blocking of the JA signaling pathway reduced *mir1* transcript levels and increased insect predation (Ankala et al., 2009). Their work provided evidence that mir1 is downstream gene of JA pathway.

Mir1, mir2 and *mir3* genes were obtained by screening a callus cDNA library from the maize inbred Mp708 with oligonucleotides derived from the N-terminal amino acid sequence of the 33 kD protease. Then three *mir* genes encoding putative cysteine proteases were identified (Pehcan et al., 1999). The amino acid signal sequences of *mir1, mir2* and *mir3* genes suggest that the presumed cysteine proteases encoded by those genes are secreted. The predicted molecular mass of the mature proteins with the signal and propeptide sequences removed were 25.4, 35.5 and 37.2 kD for *mir1*, *mir2*, and *mir3*, respectively (Pechan et al., 1999). There was 45–55% similarity among *mir* cNDAs at the nucleotide sequence levels, which suggest that *mir2* and *mir3* encoded proteins may have similar function as Mir1-CP, and they are may be involved in the insect defense of Mp708 maize (Pehcan et al., 2000).

1.2.1.2 Lipoxygenase Gene (*Lox*)

Plant lipoxygenases (LOX) catalyze the oxidation of polyunsaturated fatty acids, creating fatty-acid hydroperoxides (oxylipins) (Gao et al., 2009). Accumulating evidence suggests diverse physiological functions of oxylipins in plants, such as growth, development, resistance to insects or pathogens, and tolerance to salt, drought, and cold (Howe and Schilmiller, 2002).

Lipoxygenase (LOX) mediates an essential step in JA biosynthesis by catalyzing oxygenation of fatty acids to their hydroperoxy derivatives (Lee SH et al., 2005). Plant LOX regulates programmed cell death (Cacas et al., 2005; Rustérucci et al., 1999), and it plays a role in defense responses to pathogens, also in plant developmental processes. In maize, 9-lipoxygenase (*lox3)* gene has functions of controlling development, root-specific expression of defense genes, and resistance of root-knot nematodes (Feussner and Wasternack; 2002). Another *lox* gene, Lipoxygenase6 (*lox6)* is regulated by phytohormones and pathogen infection (Gao et al., 2008). *Lox* genes are required for JA synthesis, which defines this class of genes as major herbivory defense genes (Gao et al., 2009). Shiwaji et al., 2010 found that transcript levels of *lox1* and *lox3* were higher in Mp708 than in Tx601 after insect feeding (Shiwaji et al., 2010). This finding suggested lox important role in Mp708 insect resistance.

1.2.1.3 Chitinase Gene (*Chi*)

It is known that chitinases play an important role in plant defense against pathogens (Fan et al., 2007). It has been proposed that, plants which constitutively express high levels of chitinases have enhanced resistance to pathogens (Broglie et al., 1991). Chitinases are enzymes that catalyze the hydrolysis of ß-1,4-N-acetylglucosamine linkages present in chitin (Benhamou et al., 1993). Chitin is one of the most important biopolymers in nature. It is mainly produced by fungi, arthropods and nematodes. In insects, it functions as scaffold material, supporting the cuticles of the epidermis and trachea as well as the peritrophic matrices lining the gut epithelium. Insect growth and morphogenesis are strictly dependent on the capability to remodel chitin-containing structures (Merzendorfer and Zimoch., 2003). Chitinases break down insect chitin and chitinases therefore inhibit insect growth (Schlumbaum et al., 1986). In Mp708, transcript level of Chitinase gene increased after 24 h of insect feeding compared to control plant (Shiwaji et al.,

2010), and this result suggested that high chitinase expression in Mp708 could contribute to the insect resistance of this maize inbred (Pechan et al. 2002).

1.2.1.4 Maize Protease Inhibitor Gene (*MPI*)

MPI gene encodes a maize protease inhibitor protein. The mRNA of *MPI* accumulates in response to mechanical wounding (Bode et al., 1992). In mechanically damaged leaves of maize, *MPI* gene expression is induced, and *MPI* mRNA accumulation is affected by the degree of damage inflicted on the foliar tissues of maize plant (San Segundo et al., 2005). It is known that mechanical wounding results in a increasing level of *MPI* transcripts (Koiwa et al., 1997). When larvae are fed on maize leaves, MPI protein accumulates in tissues adjacent to the wounding site. Besides, the level of MPI accumulation is higher in leaves chewed by larvae than in leaves that have been damaged mechanically (Solomon et al., 1999). MPI also can inhibit the two types of insect digestive protease, elastase and chymotrypsin (Strobl et al., 1998) suggesting its significant role in the host-herbivory defense system. MPI accumulates by as early as 4 hr after mechanical wounding, insect feeding, or pathogen infection in maize plant (Cordero et al. 1994). In Mp708 inbred line, it has been found that fall armyworm feeding resulted in increased transcript levels of *MPI* gene. (Shiwaji et al., 2010)

1.2.1.5 Pathogenesis-Related Protein Gene (*PR*)

Pathogenesis-related proteins (PR) also play a role in the plant defense mechanism. Previous studies suggested that PR proteins could participate in plant resistance in incompatible interactions (Constabel and Brisson, 1992; Matton et al., 1989). PR proteins are a ubiquitous class of intracellular defense proteins (Somssich et at., 1988). The well-characterized *PR* genes induced by pathogen invasion provide excellent models to study the transcriptional regulation of

defense genes. *PR* genes are subdivided into 11 classes based on sequence homology (Van Loon et al., 1994). The *PR10* gene family is subdivided into two groups and encodes small, acidic intracellular proteins of 15 to 18 kD (Osmark et al., 1998). Studies revealed that *PR10* has a homology to a ribonuclease (RNase) isolated from phosphate-starved ginseng cells, which supports the assumption that PR10s may possess such activity (Moiseyev et al., 1994; Swoboda et al., 1996). PR10 protein has been associated with ribonuclease activity from different plant species (Liu and Ekramoddoullah, 2006). *PR10* gene is activated following biotic stress due to pathogens in maize plant (Elliot et al., 2003).

1.2.1.6 Ribosome-Inactivating Protein Gene (*RIP*)

Ribosome-inactivating proteins (RIP) are plant enzymes that are potent inactivators of the eukaryotic protein synthesis system (Stirpe et al., 1992). RIP acts as N-glycosidase which inactivates eukaryotic ribosomes by enzymatically cleaving a specific adenine residue in the 28s ribosomal RNA. This irreversible modification makes the ribosome unable to bind elongation-factor-2, therefore blocks the normal translation (Walsh et al., 1991; Bass et al., 1992). RIP is proposed to protect plants against insect herbivory because it inactivates ribosomes from phylogenetically distant species, including pathogens and insects (Mundy, 1994). Kim et al., 1994 constructed transgenic rice with a maize *RIP* gene highly expressed. Kim et al., 1994's research resulted in fungal disease-resistant transgenic rice (Kim et al., 1994). Their results suggest *RIP* genes' critical role in insect-resistant maize plants. In bioassays, purified maize RIP1 deterred insect feeding (Dowd et al., 1998). *RIP2* was identified in maize by Bass et al. 1995 and reported to be strongly induced in the developing maize kernel during water deficits (Zinselmeier et al., 2002). Given these roles for *RIP2*, the transcriptional changes for *RIP2* may have an effect on herbivore defense.

1.2.1.7 Rubisco Small Subunit Gene (*SSU*)

The enzyme ribulose 1,5 bisphosphate carboxylase (Rubisco) is located in the chloroplast where it acts as the primary carboxylating enzyme in photosynthesis (Cavalier-Smith, 1993). The enzyme is composed of two types of protein subunit: eight large subunits (50-58kD), which are encoded in chloroplast DNA and synthesized in the chloroplast (Pawlowski et al., 1994), and eight small subunits (12-18kD), which are encoded in the nucleus, synthesized in the cytoplasm and transported into the chloroplast. The genes encoding for the small subunit named *SSU* are expressed only in light, and only in green cells (Wray et al., 1995).

It has been established that insect infection leads to major changes in the primary metabolism of the plant, including synthesis of Rubisco (Kombrink and Hahlbrock, 1990; Somssich and Hahlbrock, 1998). Insect infection might cause changes in gene expression of *SSU*, however, it should not be affected in the roots by aboveground herbivory. Therefore, *SSU* was selected in this research as control gene.

1.3 Aboveground and Belowground Communication

Insect herbivores can attack plants in both aboveground and belowground parts. The wounding signals from the herbivore attack could boost the local and system defense response. It has been found that signals are exchanged between roots and leaves upon herbivore attack. Leaf-root communication is supposed to take place through the internal vascular network between phloem and xylem bundles in the plant (Orians, 2005) or through the external route of volatile signaling. Erb et al ., 2008 proposed that signaling communication between aboveground leaf tissues and belowground root tissues would involve root-to-leaf transport via xylem vessels,

bidirectional translocation via the phloem, exchange between the vascular tissues or nonvascular cell-to-cell signaling (Erb et al ., 2008).

Plants show resistance against herbivory by a complex mechanism. The spatial and temporal dynamics of aboveground and belowground herbivores, plant pathogens, and plant responses, can differ in space and time. Combining both aboveground and belowground compartments in studies of insect-resistance throughout the life cycle of plants will improve our understanding of ecology and evolution in the plant kingdom (Atkins and Smith, 2007), which leads to the main goal of this research.

Soler et al., 2005 proposed that infestation by root herbivores induces aboveground resistance against chewing herbivory on leaf tissues, which boosts the systemic production of defensive metabolites (Soler et al., 2005). Previous research suggests that root herbivory modulates leaf defenses, thereby altering leaf herbivore performance (Bardgett and Wardle, 2003). Supportive evidence reveals that belowground attack by root herbivory triggers a local and systemic increase in abscisic acid (ABA) accumulation, and ABA-inducible gene expression level in the leaves, causing desiccation of the leaves (Christmann et al., 2005). Root treatments increase leaf concentrations of terpenoids in cotton and maize (Bezemer et al., 2003); phenolics in *Brassica nigra* (van Dam et al., 2005), pyrrolizidine alkaloids in *Senecio jacobea* (Hol et al., 2004); glucosinolates in *Brassica spp.* (van Dam et al., 2004), and protease inhibitors in *Nicotiana attenuata* (van Dam et al., 2001). Various studies on interactions between aboveground and belowground responses in plant have found an increase in basic levels of aboveground defenses following belowground herbivory. For example, in response to rootworm infestation, maize shows statistically significant levels of induction in defense to leaf herbivory (Gange et al.,

1989). Fungus pathogen causes significantly smaller lesions in root infested plant (Erb et al., 2008).

In the reverse direction, leaf herbivory may also have a positive or negative effect on the root defense response to herbivory (Frost et al., 2008). For example, leaf herbivory or aboveground wounding with regurgitate treatment can induce nicotine and protease inhibitors in root tissues of *N. attenuate* (Baldwin et at., 1994), glucosinolates in root tissues of *Brassica campestris* (Ludwig-Müller et at., 1997). Shoot herbivory also has negative effects on root insect defense response in some plant genotypes with respect to defense genes, proteins, and hormones (Christmann et al., 2007).

1.3.1 Evidences in Mp708 Maize Inbred Line

For the insect-resistant inbred line Mp708, the basal level of shoot defense response to caterpillar is induced by leaf herbivory (Shivaji et al., 2010). López et al., 2007 demonstrated that, Mir1-CP was present in xylem and phloem of Mp708 following the aboveground herbivory, which suggested that it could be transported throughout the plant. The primary sites of Mir1-CP synthesis in the whorl were the vascular parenchyma and bundle sheath cells (López et al., 2007). After foliar feeding, the amount of Mir1-CP in the root xylem increased and it appeared to move from xylem parenchyma into the root metaxylem elements (López et al., 2007). The accumulation of Mir1-CP in maize vascular elements suggests Mir1-CP may move through these tissues to defend against insect herbivores. (López et al., 2007).

This study aimed at investigating the interaction between the aboveground and belowground parts in insect-resistant maize inbred line Mp708, by feeding FAW in aboveground leaf tissues.

Both leaf and root tissues were examined in this study to determine whether leaf herbivory has effects on the root herbivore defense.

1.4 Maize Development Stages

Maize (*Zea mays*) development stages are definitions developed by maize researchers for standardization of growth. Standardization of definition of growth stages allows researchers to compare the phenology of maize under different environmental conditions and experimental treatments (Beauchamp and Lathwell, 1967; Ritchie et al., 1993).

Maize growth stages are divided into two broad categories: vegetative stages (V) and reproductive stages (R). The vegetative growth stages start with corn emergence (VE) and finish with tasseling (VT). Stages between VE and VT are designated numerically as V1, V2 …through V(n) (Nielsen, 2007a). The (n) represents stage with top leaf fully expanded before the VT stage. From green house observation, in Mp708 inbred line, the reproductive phase starts after 10 to 12 leaves are fully expanded, depending on environment and nutrition. A leaf is considered fully expanded if the leaf collar (discolored line between the leaf blade and leaf sheath) is visible. The reproductive (R) stages are designated with R1 through R6. These stages start with silking (R1) and finish with physiological maturity. Following R1, stage R2 refers to blister, R3 refers to milk, R4 refers to dough, R5 refers to dent, and R6 refers to physiological maturity (Ritchie et al., 1993; Nielsen, 2007b).

Additionally, growth stages can be grouped into four major periods which are: seedling growth (stages VE and V1), vegetative growth (stages V2, V3,…Vn), flowering and fertilization (stages VT, R0, and R1), grain filling and maturity (stages R2 and R6) (Manrique et al., 1991).

Maize plants develop 20 to 21 total leaves, silk about 65 days after emergence, and mature around 125 days after emergence. The specific time interval, however, can vary among hybrids, environments, planting date, location and other conditions. The length of time between each growth stage, therefore, is dependent upon these circumstances. Not all plants in a field or in a green house reach a particular growth stage at the same time. Usually it is assumed that the crop reaches a specific stage when at least 50% of the plant shows the corresponding features (Cooper and Law, 1978).

1.5 Fall Armyworm (*Spodoptera frugiperda*) (J. E. Smith)

Fall armyworm (FAW) is chosen as model insect in this research because fall armyworm is one of the more difficult insect pests to control in maize. While fall armyworm can damage corn plants in nearly all stages of development, it will concentrate on later plantings that have not yet silked (Hardy et al., 1985). Fall armyworm can only be effectively controlled while the larvae are small. Early detection and proper timing of an insecticide application is critical (Schultz, 2009).

FAW moths disperse long distances annually. As a regular and serious pest, its range tends to be mostly in the southeastern states (Hardy et al., 1985). For heavy populations of one or more larvae per plant, FAW are very difficult to control (Hardy et al., 1985).

FAW preferred host plants include corn, sorghum, alfalfa, bean, peanut, potato, turnip, spinach, tomato, cabbage, cotton, tobacco and most of plants in the grass family (Sparks, 1979). In maize plants, FAW larvae of the early instar cause damage by consuming foliage, young larvae initially consume leaf tissues from one side. By the second or third instar, larvae begin to eat the yellow-green part of the whorl. Serious caterpillar infections usually cause extensive defoliation (Marenco et al., 1992). Larvae burrow into the growing point bud destroying the

growth potential of plants. FAW can feed on tassels of maize. FAW caterpillar also feeds on kernels by burrowing through the husk the ear. Figure 1.2 shows how FAW larvae cause damage to different parts of the maize plant.

The time required for completing the life cycle of FAW varies depending on environment. The life cycle of FAW is completed in about 30 days during the summer, 60 days in the spring and autumn, and 80 to 90 days in the winter. The number of generations occurring in areas also varies depending on the environment. The female usually deposits eggs on a layer of foliage. Eggs usually hatch in two to three days during the summer season (Vickery, 1929). During other seasons when temperature is lower, the egg stage of FAW expands to a longer period.

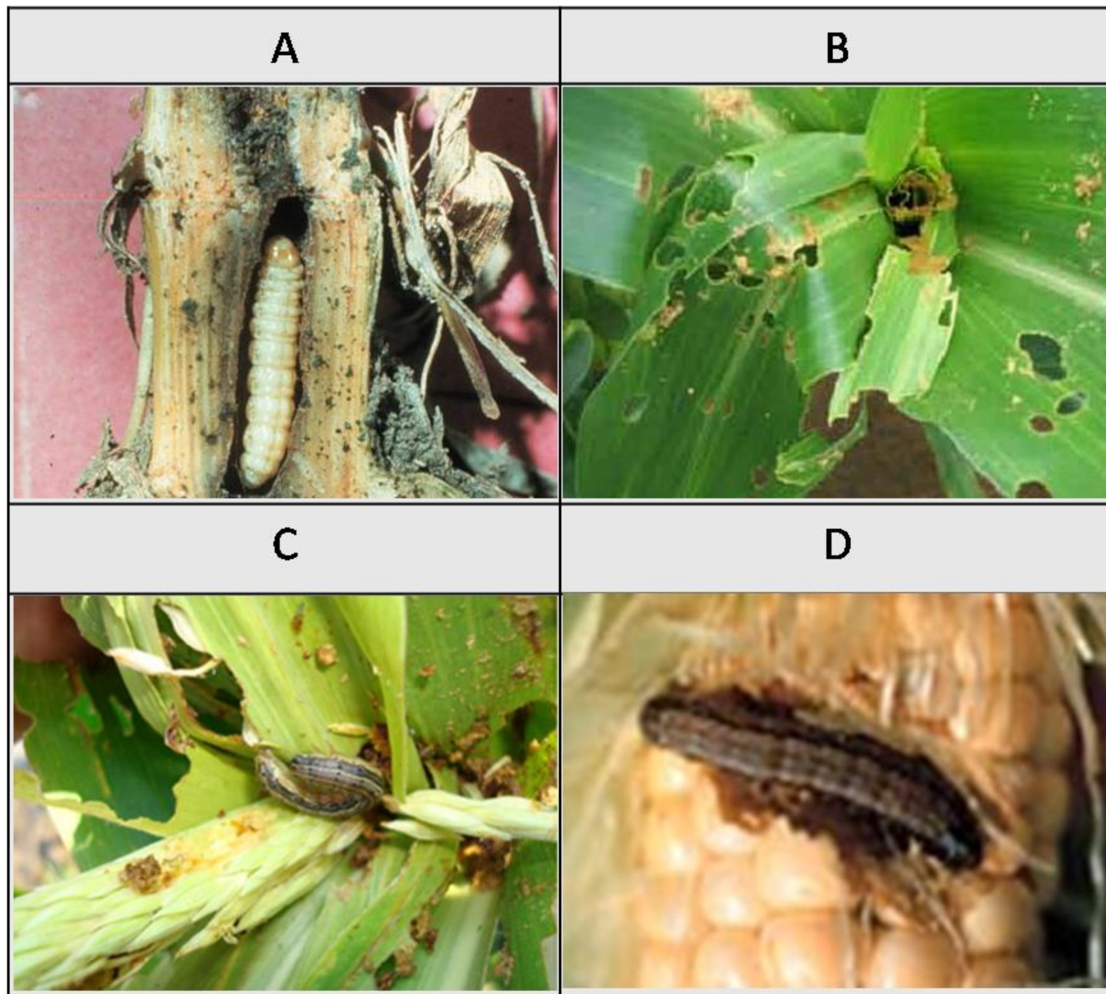


Figure 1.2 Damage caused by fall armyworm in maize. (A) corn whorl damage (http://ipmworld.umn.edu/chapters/maize), (B) corn leaf damage (http://ipmworld.umn.edu/chapters/maize), (C) corn tassel damage (geneticmaize.com/sweet-sweet-corn/), (D) corn cob damage (geneticmaize.com/sweet-sweet-corn/).

1.5 Objectives and Hypotheses

1.5.1 Objectives

This study aims at investigating communication of aboveground and belowground responses to leaf herbivory in maize plant. It is known that the cysteine protease Mir1-CP accumulates in the whorls of insect-resistant maize Mp708 in response to caterpillar larvae feeding the whorls (Pechan et al., 1999). Mir-CP is also found in the xylem and phloem of Mp708 in response to aboveground herbivory by FAW (López et al., 2007). It has been reported that Mir1-CP accumulates in the metaxylem elements of the root in response to foliar feeding by FAW (López et al., 2007). Furthermore, no Mir1-CP accumulates in the whorl when roots are removed prior to FAW feeding (López et al., 2007).

In this research, one of the research purposes is to examine defense gene expression in the whorls and roots of Mp708 maize plants that were subjected to foliar herbivory by FAW.

Defense gene expression patterns in different growth stages of maize have not been studied in Mp708. Different development stages may exhibit different levels of a defense response. Testing gene expression levels variation in response to larvae feeding at different growth stages will provide a better understanding of the whole scale of plant insect defense mechanisms.

In this study, I examined: 1) defense genes induction by herbivory in aboveground and belowground parts of insect-resistant maize inbred line Mp708; 2) variation pattern of defense gene expression during Mp708 development stages; 3) defense proteins accumulation in aboveground and belowground parts of Mp708.

1.5.2 Hypotheses

This study tested the following hypotheses: 1) aboveground herbivory induces defense gene expression both in aboveground and belowground parts of insect-resistant maize inbred line Mp708; 2) Different growth stages exhibit different expression levels of defense gene expression in Mp708 plants.

2. Materials and Methods

This research uses Mp708 and FAW as plant and insect materials. After feeding by FAW for 4 hours, plant tissues were collected. RNA was extracted and reverse transcription PCR was conducted for cDNA synthesis. Conventional PCR, gene cloning and sequencing were carried out for amplification of defense genes and check of designed primers. Defense gene transcript levels were determined by quantitative Real-Time PCR. Defense proteins accumulation was examined by immunoblot analysis.

In this chapter, experimental materials and methods are described. Section 2.1 provides information on plant and insect larvae growing condition. Section 2.2 describes how larval infestation was done. Section 2.3 and section 2.4 discusses processes used for RNA extraction from tissues and cDNA synthesis. Section 2.5 describes methods for design of primer pairs and Table 2.1 provides nucleotide sequences of each primer pair and expected sequence length. Section 2.6, 2.7 and 2.8 are integrated to address PCR, cloning, sequencing processes which are conducted for primer specificity check. Then Section 2.9 and 2.10 illustrates quantitative Real Time-PCR and data analysis of qRT-PCR for results test. At last, section 2.10 discusses the immunoblot experiments used for determining protein accumulation.

2.1 Plant and Insect Materials

The insect-resistant maize (*Zea mays*) inbred line Mp708 (Williams et al., 1990) was used in this study. Plants were grown and maintained in greenhouse under 100W Super Spectrum Lights. Fall armyworm (*Spodoptera frugiperda*) insect larvae eggs were obtained from Corn Host Research Unit Insect-Rearing laboratory of the USDA-Agricultural Research Service in

Starkville, MS. Larvae were reared in a growth chamber at 27 ^{o}C on a wheat-germ based artificial diet (Davis, 1989) to fourth instar.

2.2 Insect Infestation

In order to test the effect of FAW herbivory of plant defenses, FAW larvae were applied to plants growing at different vegetative stages (V1-V11). The experiment had three plant replicates each growth stage equivalent to fifty-two caterpillar fed plants. The same amount of control plants without caterpillar feeding were grown at same time. Fourth instar FAW larvae (five per plant) were placed in the whorl of different growth stages maize plants for feeding. After 4 hours feeding, larvae were damaging the whorls, leaves and tassels. Then larvae were removed. The plants were left growing for 24 hours. Approximately ten 100 mg leaf tissue samples immediately adjacent to the wounding sites caused by insect feeding were harvested. 2cm long root tip tissues of same caterpillar fed plants were collected and cleaned. Around ten 100 mg root tip tissues were collected per plant. Control plants at the same growth stage were left completely untouched. For the control plants leaf and root tissues, same amount of tissues were collected with the same location as samples collected from larvae fed plants. Then tissue samples were immediately placed in liquid nitrogen, and were stored at –80 ℃ freezer for protein and gene expression analyses.

2.3 RNA and cDNA Preparation

2.3.1 RNA Extraction

Three leaf samples and three root samples from each plant were homogenized in liquid nitrogen. The total RNA was extracted from maize leaf and root tissues using the RNeasy Plant

Mini Kit (Qiagen, Valencia, CA) in accordance with the manufacturer's recommendations. Purified RNA was stored at -80 ℃ in water until cDNA synthesis. The integrity and size distribution of the purified RNA was checked by denaturing agrose gel electrophoresis at 100V for 1h, followed by 2% ethidium bromide staining. The concentration of obtained RNA was measured by GeneQuant Pro (Amersham Bioscience, Golden Valley, MN).

2.3.2 Reverse Transcription-PCR

The cDNA from leaf and root tissues was synthesized using Applied Biosystem's High Capacity cDNA Reverse Transcription Kit, with Oligo(DT)$_{20}$ (50 μM) from Integrated DNA Technologies (IDT) as primer. 1 μg extracted RNA was pipetted into each reaction tube; d_2H_2O was added to total volume 15.2 μl. 4.8 μl RT master mix, i.e., 2 μl 10×RT buffer, 0.8 μl 25×dNTP mix, 1 μl Oligo(DT)$_{20}$ and 1 μl MultiScribe Reserve Transcriptase, was pipetted into each reaction tube on ice. All the tubes were incubated at room temperature for 10 minutes. Reverse transcription-PCR experiment was conducted for each tube. The condition of the reverse transcription-PCR experiment was: 37 ℃ 120mins, 85 ℃ 5secs. The samples were stored at 4 ℃ for 12 hours. The cDNA synthesis reaction products were stored at -20 ℃.

2.4 Conventional PCR for Gene Amplification

2.4.1 Primer Design

Zea mays nucleotide sequences were obtained from the GenBank database. A BLAST (Basic Local Alignment Search Tool) against the GenBank EST database was used to find the maize nucleotide corresponding to target genes to be found. Primers were designed with IDT SciTools OligoAnalyzer 3.1 and were synthesized by Integrated DNA Technologies Company

(http://www.idtdna.com/) (see Table 2.1). Primer concentrations giving the lowest threshold cycle (Ct) value were selected for further analysis. These primers resulted in the PCR amplification of an approximately 100bp ampification fragments.

Table 2.1 Oligonucleotides of primers designed for targeted genes *mir1, mir2, mir3, lox3, lox6, RIP2, MPI, Chi1, PR10, SSU1*. Each expected amplification fragment length is shown.

Gene name	Orientation	Sequence	Length (bp)
mir1	Forward	5'-AACGCGATCGCGACGGGTAAC-3'	123
	Reverse	5' - GTTGCCGATGACGAACCGGAAC -3'	
mir2	Forward	5'-ATCAACAAGATCGTGACAGGCAGCCT-3'	128
	Reverse	5'-TTCTTGATCATGAACACGAAAGCATTGTCC-3'	
mir3	Forward	5'- CAACAATAGCAGCTGTGGAAGGCATCAA-3'	110
	Reverse	5'- ATTGCACCCCTGATTGTATGAAGTGTCA-3'	
lox3	Forward	5'- AAAATACCAGCCCTCGAGGACCTG-3'	134
	Reverse	5'-ATCCAACCTGTCTTGTCCTCTTTGATGATC-3'	
lox6	Forward	5'-TGTACGACTACGCGCTGTACAACGA -3'	98
	Reverse	5'- GGATCTGTTTTGGCGGCTGGTCG-3'	
RIP2	Forward	5'-CGAAATCTTCCCCGTGGAGGACAC -3'	111
	Reverse	5'-ACGGGCTGGACGATGCCTTTA -3'	
MPI	Forward	5'- GACGCCAAGAAGGTGATCCTCA -3'	98
	Reverse	5'- GACACGGTTAGGGCGATAATCCG-3'	
Chi1	Forward	5'- TTCAACTTCATCCTCGCCTTCGC -3'	107
	Reverse	5'- AGGTTCCCCGTGTCCCAGAA -3'	
PR10	Forward	5'- TTCAAAAACTTCCCCGGTCACACAATG-3'	109
	Reverse	5'- CGTCCAGGAACTCGAGCCTCT-3'	
SSU1	Forward	5'- TTCAGCAAGGTCGGCTTCGTGTAA-3'	137
	Reverse	5'- GCCTCCTGCAGCTCCTTGTA-3'	

2.4.2 Conventional PCR Analysis

The newly synthesized cDNA was used for PCR. Each reaction tube contained 1μl of cDNA, 2μl of defense genes specific forward and reverse primer pairs (1:10 diluted), 4 μl dNTP (2.5 mm), 0.5μl Taq DNA polymerase, 5μl 10X Ex Taq reaction buffer and water was added to a final volume of 50 μl. The thermo cycling conditions of the PCR experiment were: 1) initial denaturation at 95 ℃ for 2 min; 2) 30 amplification cycles, each cycle consisting of denaturation at 95 ℃ for 30 sec, annealing at 60 ℃ for 1 min, extension at 72 ℃ for 1 min; and 3) post-extension at 60 ℃ for 1 min. The PCR products were stored at -20 ℃.

2.4.3 Cloning

PCR products were analyzed on a 2% agarose/ethidium bromide gel run in 1× tris-borate-EDTA buffer (Tris Base (10.78 g/L), Boric Acid (5.50 g/L), EDTA, Disodium, Dihydrate (0.74 g/L)). These cDNA fragments were recovered by excision from the gel, purified by QIAquick Gel Extraction Kit (Qiagen, Valencia, CA). Then the fragments were cloned using Promega pGEM-T Easy Vector System (Promega, Medison, WI).

Ligation with T4 DNA ligase (1μ pGEM-T Easy vector, 5μl 2X ligase buffer, 1μl T4 DNA ligase, 3μl insert cDNA) was stored at 4 ℃ for 12 hours. Then transformation with Top10 competent cells was carried out. Recombinants, which were white colonies, were picked when they were plated onto Luria-Bertani broth plates (10 mg of X-Gal per liter, 10 mg of IPTG per liter, 10 mg of ampicillin per liter). X-Gal is 5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside. IPTG is isopropyl-b-D-thiogalactopyranoside. White colonies were grown overnight in liquid Luria Bertani broth medium. For sequencing, plasmids were isolated and purified from each clone culture using FastPlasmid Mini Kit (5Prime, Gaithersburg, MD).

2.4.4 Sequence Analysis

The nucleotide sequence was determined in DNA sequencing facility of Huck Institute for the Life Science at Pennsylvania State University, University Park, PA. Homology search within the databases was done using the BLAST program of the DNA databank of NCBI http://www.ncbi.nlm.nih.gov/Genbank/). Alignments of amino acid sequences were compared against GenBank, using the BLAST program to confirm identity.

2.5 Gene Expression Analysis

2.5.1 Quantitative Real Time-PCR

In order to test gene expression, quantitative real time-PCR (qRT-PCR) was conducted by using ABI's 7500 Fast Real-Time PCR system. The qRT-PCR products binding the fluorescent DNA dye SYBR green (FastStart Universal SYBR Green Master (Rox), from Roche Diagnostics) were detected. Gene specific primer pairs were diluted to 100nM. cDNA from leaf and root tissues were ten times diluted. ABI's MicroAmp Fast Optional 96-well Reaction Plate and Base, as well as ABI's Optical Adhesive Cover were used. Template cDNA was amplified in a total reaction volume of 10 µl for each well (2 µl cDNA, 1 µl water, 1 µl forward primer, 1 µl reverse primer and 5 µl SYBR master mix).

Serial dilutions of cDNA were amplified to construct the standard curve. The Ct values, defined as the number of cycles needed to reach a defined relative fluorescence level which were calculated by the 7500 Fast System SDS Software (Applied Biosystems, USA), were plotted as a linear function of the log of the DNA concentration. A melting curve was plotted at the end of each run, to verify the specificity of the amplification product. The reaction efficiency for

standard and sample curves was measured as described in the ABI's 7500 Fast Real-Time PCR manual. The products from the real-time PCR were electrophoresed in a 2% agarose gel and visualized by ethidium bromide staining to determine if they were the correct size.

The housekeeping gene *actin* from maize plant was used as a constitutively expressed endogenous control. An equal fluorescence yield during qRT-PCR per initial *actin* transcript was assumed. Non-template control was used to check for primer-dimer. Calculations analyses were performed in the ABI 7500 Fast System SDS software.

Each cDNA sample was amplified 3 replicas (wells) to minimize the loading error; therefore the standard Ct error was below 0.3. All reactions were heated to 95 ℃ for 10 min, followed by 40 cycles of 95 ℃ for 15 sec and 60 ℃ for 1 min, and 1 cycle of 65 ℃ for 3 min.

2.5.2 Statistical Analysis

Fisher's and Tukey's separation of means test was applied to analyze relative expression values by SAS statistical program. By mean separation test, each mean of gene expression values was compared to the other means. Then the significance of difference was obtained with each pair of comparison.

2.6 Protein Extraction and Accumulation Analysis

2.6.1 Protein Extration

Frozen leaf and root tissue samples (200 mg) from V7 growth stage Mp708 maize plant were ground to a powder in liquid N_2 using a mortar and pestle. Just as the liquid N_2 evaporated, 0.4 ml of 2X sodium dodecyl sulfate (SDS) sample buffer (60 mM pH 6.8 TrisHCL, 3% SDS, 10%

glycerol, 0.05 % BFB, 10% ßME (freshly added)) was added and the tissues were further ground. After thawing, the homogenates were heated at 100 ℃ for 10 min and then were briefly centrifuged at 12,000 × g to pellet the cellular debris. The resulting supernatant was stored at –20 ℃ until analyzed.

One-dimensional SDS-polyacrylamide gel electrophoresis (PAGE) (Shapiro et al., 1967) was performed, with 20 µg of protein loaded per lane with synthesized Mir1-CP as control. Constant voltage of 50 V was applied until the tracking dye reached the bottom of the gel. For immunoblot, a duplicate SDS-PAGE gel was run and stained with Coomassie brilliant blue to confirm that equal amounts of protein were loaded in each lane.

2.6.2 Protein Accumulation

Following SDS-PAGE, immunoblot of proteins was performed. After tracking dye reached the bottom of the SDS-PAGE gel, the proteins were transferred from gel to polyvinylidene difluoride (PVDF) membrane with constant voltage of 12V applied for 45 min. The membrane and blot paper were soaked in gel running buffer (Tris base 0.3%, Glycine 1.44%, SDS 0.1%). Immunolabeling of blots was performed. The membrane was soaked in 30 ml 5% non-fat diet milk 30 min for blot. After washing by 1X TTBS buffer (10X TTBS buffer with 9% NaCl 90 g, 0.186% Tris base, 1.34% Tris HCl, 1% Tween-20), the membrane was treated with 15 ml first antibody (1.5 µl for monoclonal antibody against Mir1-CP, 2.5% non-fat milk with 1X TTBS buffer) at 4 ℃ over night. After washing by 1X TTBS buffer, the membrane with treated with 15 ml secondary antibody (1.5 µl goat anti-rabbit horseradish peroxidase-conjugated serum with 1X TTBS) at room temperature for 1 hour. Then the membrane was treated with SuperSignal West

Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific, Rockford, IL). And signals indicating Mir1-CP protein were detected at membrane.

3. Results

Experimental results are presented and interpreted in this chapter. The result of FAW larvae infestation on Mp708 is illustrated in section 3.1. The amplification products of defense genes by conventional PCR are shown in section 3.2. The quantification of defense gene expression in V7 stage Mp708 induced by foliar herbivory is exhibited in Section 3.3. Those defense genes include *mir1, mir2, mir3, MPI, lox3, lox6, RIP2, Chi1, PR10* and *SSU1*. Observations of defense gene expression induction in different maize growth stages are discussed in section 3.5. Mir-CP accumulation in Mp708 aboveground and belowground parts is addressed in section 3.6 .

3.1 Result of Insect Infestation

In the field, this resistant line (Mp708) exhibits less foliar damage than the susceptible line (Tx601) (Jiang et al., 1995). Caterpillars that feed on the resistant plants are significantly smaller than those that feed on the susceptible lines (Tx601) (Jiang et al., 1995). To determine if the defense genes (*mir1, mir2, mir3, MPI, lox3, lox6, RIP2, Chi1, PR10,* and *SSU1*) expression levels are induced by larvae feeding on the insect-resistant inbred Mp708, the following experiments were conducted. Fourth instar larvae were placed in the whorl of Mp708 plants for 4 hours. The larvae were removed from plants, then after 24 hours, and the above ground damaged tissues and below ground tissues were collected (Figure3.1). In above ground tissues, the green-yellow mid-whorl tissues were damaged seriously. FAW larvae also fed on green leaves, making holes on leaves. FAW does not feed on root tissues, which kept the belowground part undamaged. In this experiment, aboveground damaged yellow-green whorls, damaged adjacent green leaf tissues and belowground root tip tissues was examined for defense gene

expression. To illustrate the changes of target gene expression over maize development, tissues were taken at different growth stages of maize, from V1 stage through V11 stage.

3.2 Amplification of Defense Genes

To determine whether the defense genes were transcripted in the FAW fed plants, conventional reverse transcription-PCR experiments were conducted.

RNA was extracted from leaf and root tissues from FAW fed plants and non-fed control plants. The samples were collected from Mp708 maize plants which were at V1 (1 collared leaf is visible) to the V11 (11 collared leaf is visible) growth stages. cDNA was synthesized from RNA isolated from maize tissues. Primers were designed for amplifying a conserved region with approximate 100 base pairs of the interested genes (refer to Appendix for each gene expected amplification sequence).

3.2.1 Amplification of *Mir1*

The cDNA fragments were verified by the agarose gel electrophoresis of the PCR amplification products. A single band of the expected 123bp for *mir1* was observed (Figure 3.2). Agarose gel pictures indicated approximately 123bp length cDNA fragments in both aboveground and belowground, in different growth stages (V1-V9).

This cDNA fragments were recovered by excision from the gel, purified and cloned using pGEM-T Easy vector system. The plasmids sequencing results demonstrated that the fragment was cDNA *mir1*.

These results reveal that *mir1* gene transcripts in both the damaged aboveground and undamaged belowground of insect-resistant inbred line. Bands with different intensity suggest different transcript levels, which mean this gene's expression level varies between aboveground and belowground. The results also show *mir1* gene is expressed in different growth stages (from V1 growth stages toV9 stages), which indicates this gene is expressed throughout vegetative stages. The variation of transcript levels among different growth stages, indicates *mir1* is expressed differently during maize development (Figure3.2).

3.3 Quantification of Defense Genes Expression in V7 Stage Mp708 Plant in Response of Foliar Herbivory

After verifying that the designed primers specifically amplified target genes, experiments were taken to optimize the quantification of *mir1, mir2, mir3, MPI, lox3, lox6, RIP, Chi1, PR10*, *SSU1* and *Actin*, by quantitative Real Time-PCR (qRT-PCR). Melting curve analysis was done for each gene to determine if the primer pairs were specifically amplifying the desired gene. Each amplification product has a sequence-specific melting temperature, and therefore each unique DNA species can be identified based on its peak fluorescence at a specific temperature. The annealing temperature of each qRT-PCR cycle was adjusted systematically until the melting curve analysis revealed a single fluorescent peak at the expected temperature. Melting curve profiles of qRT-PCR products indicated the amplification of a single PCR amplification. Figure 3.3 shows the melting curve analysis for *mir1* specific PCR products. The presence of a single peak at the expected temperature indicates that the primers are gene specific. This type of analysis was done for all genes tested.

To assess the range of PCR linearity, standard curves were constructed on cDNA of defense genes. Cycle threshold (Ct) values from serial dilutions of genomic DNA were plotted versus the logarithm of the DNA concentration. Figure 3.4 shows PCR standard curve for *mir1* using 10 fold serial dilutions. The correlation larger than 0.98 and slope of -3.0 indicates that the designed primers for this specific gene can be used for qRT-PCR experiment to detect gene expression level.

The transcripts level of the defense genes *mir1, mir2, mir3, MPI, lox3, lox6, RIP2, Chi1, PR10, SSU1* was quantified by quantitative RT-PCR. Regression coefficients (R2) ranged from 0.98 to 1.00, and slopes were approximately -3.0.

3.3.1 *Mir1* Gene Expression in V7 Stage Maize

cDNA was amplified and quantified by qRT-PCR to examine the expression of defense gene transcripts. The expression of genes was normalized by representing the results as a ratio of target gene expression to *actin* expression.

The level of *mir1* transcripts in the V7 stage FAW fed Mp708 leaf tissues is shown in Figure3.5. When compared to non-fed control Mp708 maize plant leaf tissues, the level of *mir1* transcripts increased slightly (same character shows no significant different in transcript level). In the root tissues from the same plant, the level of *mir1* transcripts was approximately 10 times higher in the FAW fed Mp708 when compared to control plant root tissues. In V7 Mp708 control plant, *mir1* transcript levels in root tissues were lower than that in leaf tissues. After attacked by FAW larvae in leaf, gene transcript levels increased dramatically in root tissues of V7 stage maize.

These results indicated that for Mp708 plants fed by FAW larvae which were growing at V7 stage, the *mir1* gene transcript level increased significantly in belowground root tissues, and there is no significant increase of gene expression level in aboveground tissues.

3.3.2 *Mir2* and *Mir3* Genes Expression in V7 Stage Maize

The same experiment procedures were conducted with *mir2* and *mir3* genes. The level of *mir2* transcripts in the FAW fed V7 stage Mp708 leaf tissues was examined (Figure3 .6). *Mir2* gene showed significant induced expression in fed plants. While in non-fed plants, *mir2* transcript levels in root tissues and leaf tissues were barely detectable. After feeding by FAW, gene expression increased dramatically in leaf and root tissues to more than 1000 fold when compared to the non-fed plant at the same (V7) growth stage. The result indicated that after plant fed by FAW larvae, *mir2* transcript levels increased significantly in belowground and aboveground leaf tissues of V7 growth stage Mp708.

Similar results were obtained for *mir3* gene expression data analysis, which is a homolog to *mir2*. There was relative low expression in aboveground and belowground of insect-resistant maize control plant. Gene expression level increased to nearly100 folds in leaves and 5 folds in roots of FAW-fed V7 plants (Figure3 .7).

3.3.3 *Lox3* and *Lox6* Genes Expression in V7 Stage Maize

The expression level of *lox3* in Mp708 at the V7 stage in response to the FAW foliar feeding was examined by qRT-PCR. Statistical analysis revealed this defense gene's transcript level increased significantly in Mp708 leaves and roots in response to FAW feeding. The relative

expression levels increased in both leaf and root tissues (Figure 3.8). However, the extent of induction was not as great as that of the *mir2* and *mir3* transcripts.

Figure3.9 showed that there was no significant increase in *lox6* transcripts in the whorl in response to FAW feeding. However, this gene was significantly up-regulated by caterpillar feeding in the root in response to FAW feeding in the leaf of V7 Mp708.

3.3.4 *MPI, RIP2, Chi1, PR10* Genes Expression in V7 Stage Maize

The expression of other defense genes *RIP2, MPI, PR10, Chi1* in response to foliar feeding by FAW at V7 stage of Mp708 plant were examined. The qRT-PCR experiments indicated that transcript levels for *RIP2, MPI, Chi1, PR10* increased in both the leaf and root tissues, following FAW herbivory (Figure 3.10, Figure3.11 Figure3.12, and Figure3.13). Statistical analysis indicated significant difference in the expression of the defense genes in the roots of FAW fed and unfed plants. In V7 growth stage of Mp708, all of these transcript levels were relatively high in the roots of FAW fed plants compared to the controls.

3.3.5 *SSU1* Gene Expression in V7 Stage Maize

The expression level of *SSU1* in Mp708 in response to the FAW foliar feeding was examined by qRT-PCR. Statistical analysis revealed this defense gene transcript level increased significantly in Mp708 leaves in response to FAW feeding. *SSU1* gene expression levels in roots were not different between control and fed plants. It should be noted that the relative amount of *SSU1* transcripts was much lower than that of the defense genes analyzed (Figure3.14).

3.4 Quantification of Defense Genes Expression in Different Maize Growth Stages

The experiments determined how the expression of key herbivore defense genes changes throughout plant development. The results of qRT-PCR show that as the maize plant mature there is variation in the level of defense gene expression. It was observed that defense genes transcript levels increased in both the leaf and root in response to herbivory. This is especially true for root tips where expression in foliar fed plants was often 100 times greater in leaves of control plants.

3.4.1 *Mir1* Gene Expression in Different Maize Growth Stages

Figure 3.15 indicates changes in *mir1* gene expression level induced by FAW herbivory. In the whorl tissue adjacent to the feeding site, relative *mir1* transcript levels were the highest at the V5 stage. However the fold-induction was highest at the V1 stage. In root tissues, gene expression in the caterpillar fed plants reached the highest at the V11 growth stage. The level of *mir1* transcripts induced belowground was higher than the level aboveground. The various growth stages showed different levels of *mir1* gene expression both above and belowground. In the whorl, expression of mir1 appeared to be higher in response to FAW feeding in V1 to V3 stages. With the exception of the V11 stage, the expression of *mir1* in the roots in response to herbivory also was higher in the V1 to V3 stages. The highest expression in the root at the V11 growth stage corresponds to the time just before the reproductive stage.

3.4.2 *Mir2* and *Mir3* Genes Expression in Different Maize Growth Stages

The expression pattern for *mir2* and *mir3* during development was quite different from that of *mir1* (Figure 3.16, Figure3.17). In general the induced expression of *mir2* in the whorl was

higher than its induced expression in root. The maximum expression level for *mir2* was in the whorl at the V7 stage and the fold-induction (20 fold) also was the highest at the time. *Mir2* expression in the leaf in response to herbivory was greater from the V5 to the V11 stages. In contrast, the expression *mir2* in the roots in response to foliar herbivory was highest at the seedling stage, V1. The induced expression of *mir3* also was higher in the whorl than in the roots and it occurred at the V9 stage. The fold-induction (10 fold) was highest at the V9 stage as was the case for *mir2*. *Mir3* expression in the roots in response to foliar herbivory was the highest at the V1 stage. However the constitutive expression of *mir3* also was high at this stage. In fact, aside from the V1 stage, there was little to no induced expression of *mir2* or *mir3* in the roots during development.

3.4.3 *Lox3* and *Lox6* Genes Expression in Different Maize Growth Stages

There also were different patterns in leaves and roots for *lox3* and *lox6* expression (Figure 3.18, Figure3.19). The highest induced level of *lox3* expression occurred during the V5 stage in the roots. Stages V1, V3, V9 and V11 had the highest induced expression in the whorl, but they were lower than that of the V5 stage in the root. The greatest fold-induction was 4 in the root at the V5 stage.

For *lox6*, the induced expression in the whorl at the V9 and V11 stages was higher than in the root, but there was little to no induction of *lox6* at the earlier developmental stages. The greatest induced expression in the root was at V5, as was the case for *lox3*. Except for V5, the level of *lox6* induction in the roots in response to foliar feeding was not pronounced.

3.4.4 *RIP2* Gene Expression in Different Maize Growth Stages

Analysis of the defense gene *RIP2* showed different in induction patterns during development for the leaves and roots (Figure 3.20). In response to the FAW feeding the levels of *RIP2* transcripts increased the most at the V3 through V7 stages in the whorl. The pattern in the roots was different from that of the whorl in that the highest expression was at V7. However, the fold induction at the V7 stage was 6 fold in the leaves and approximately 8 fold in the roots. There was no or little induction of *RIP2* at the V1, V9 and V11 stage in the leaves and the V1 and V3 stages in the roots.

Defense gene expression induction indicated different pattern for leaves and roots separately for *RIP2* gene (Figure3.20). As maize developing and growing, defense gene expression changed significantly. In response to the FAW insect feeding, the average genes transcript levels increased significantly compared to the average gene transcript levels in non-fed maize plants.

3.4.5 *MPI* Gene Expression in Different Maize Growth Stages

In response to the FAW insect feeding, *MPI* transcripts levels increased significantly in the leaves at the V3 and V7 stage, whereas the expression increased significantly at the V5 and V7 stages in the roots (Figure3.21). There were different developmental patterns of induction for *MPI* in leave and roots.

3.4.6 *PR10* Gene Expression in Different Maize Growth Stages

There also was a different pattern of transcript induction for the *PR10* defense gene during development (Figure3.22). In response to herbivory, the average genes transcript levels in FAW fed plants was induced to the highest level in V5 and V9 growth stages in leaf tissues. In root

tissues, gene expression in insects attacked plant reached to the highest levels at V3 and V7 growth stages.

3.4.5 *Chi1* Gene Expression in Different Maize Growth Stages

For *Chi1,* the induction of transcripts in response to foliar FAW feeding was the highest at in both the whorl and roots (Figure 3.23). Induction in the roots in response to foliar herbivory was higher in the roots at the V5 and V9 stages than in the whorl.

3.4.7 *SSU1* Gene Expression in Different Maize Growth Stages

As expected, *SSU1* expression levels were very low in the roots and there were no large changes in transcript abundance in response to herbivore feeding in the whorl (Figure3.24). Surprisingly expression levels in the leaf increased in response to herbivory at all stages except the V1 and V9 stages.

3.5 Mir1-CP Protein Accumulation

Accumulation of transcripts does not always correlate with protein accumulation. Therefore an immunoblot experiment was conducted to determine if Mir1-CP protein accumulated in the root tips of V7 plants in response to foliar feeding by the FAW.

The results of this experiment indicated that the whorl of larval fed plants had much abundance of Mir1-CP protein than the control whorl (Figure 3. 25), although some Mir1-CP protein was present in the unwounded whorl and this has been observed previously (Pechan et al., 2000). Mir1-CP protein was detected in the root tips of foliar fed plants, but level was much lower than in the whorl. No Mir1-CP protein was detected in control roots.

The qRT-PCR and immonoblot results suggested that there was a certain type of aboveground to belowground signaling that associated for the accumulation of Mir1-CP protein in the root tips. The mechanism of this communication remains unknown.

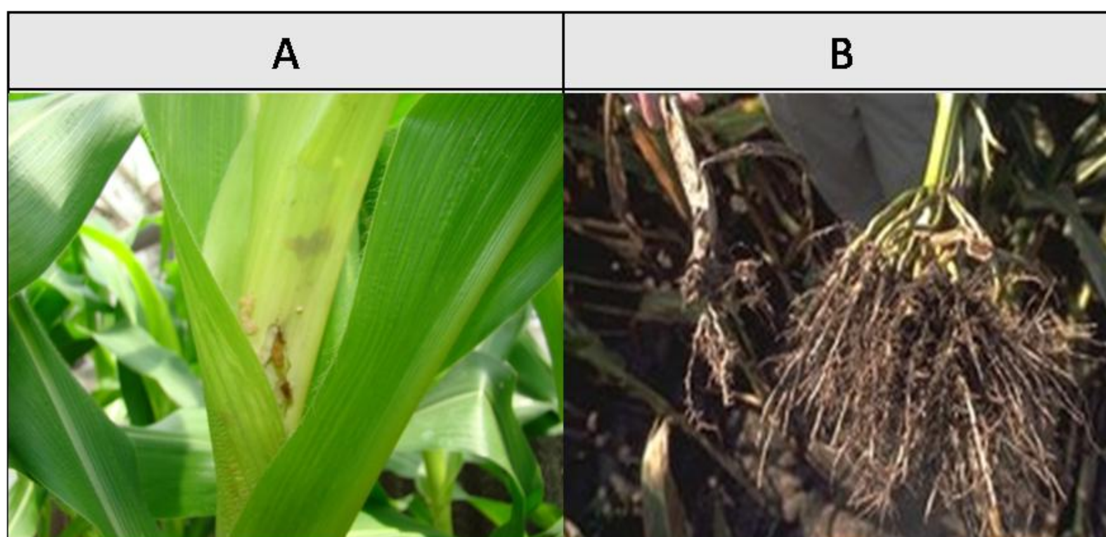


Figure 3.1 Pictures of damaged Mp708 leaf after 4 hours feeding by FAW (A) and undamaged roots from the same plant (B).

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

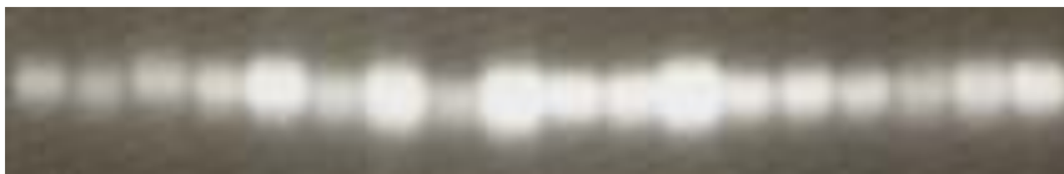


Figure 3.2 Conventional qualitative PCR analysis. The primers amplified the 123bp product from FAW fed and Non-fed Mp708 different growth stages maize plant.

Lane 1 ---*mir1* gene in V1 growth stage leaf tissues of FAW fed Mp708 plant;

Lane 2 ---*mir1* gene in V1 growth stage leaf tissues of Mp708 control plant;

Lane 3 ---*mir1* gene in V1 growth stage root tip tissues of FAW fed Mp708 plant;

Lane 4 ---*mir1* gene in V3 growth stage root tip tissues of Mp708 control plant;

Lane 5 ---*mir1* gene in V3 growth stage leaf tissues of FAW fed Mp708 plant;

Lane 6 ---*mir1* gene in V3 growth stage leaf tissues of Mp708 control plant;

Lane 7 ---*mir1* gene in V3 growth stage root tip tissues of FAW fed Mp708 plant;

Lane 8 ---*mir1* gene in V3 growth stage root tip tissues of Mp708 control plant;

Lane 9 ---*mir1* gene in V5 growth stage leaf tissues of FAW fed Mp708 plant;

Lane 10 ---*mir1* gene in V5 growth stage leaf tissues of Mp708 control plant;

Lane 11 ---*mir1* gene in V5 growth stage root tip tissues of FAW fed Mp708 plant;

Lane 12 ---*mir1* gene in V5 growth stage root tip tissues of Mp708 control plant;

Lane 13 ---*mir1* gene in V7 growth stage leaf tissues of FAW fed Mp708 plant;

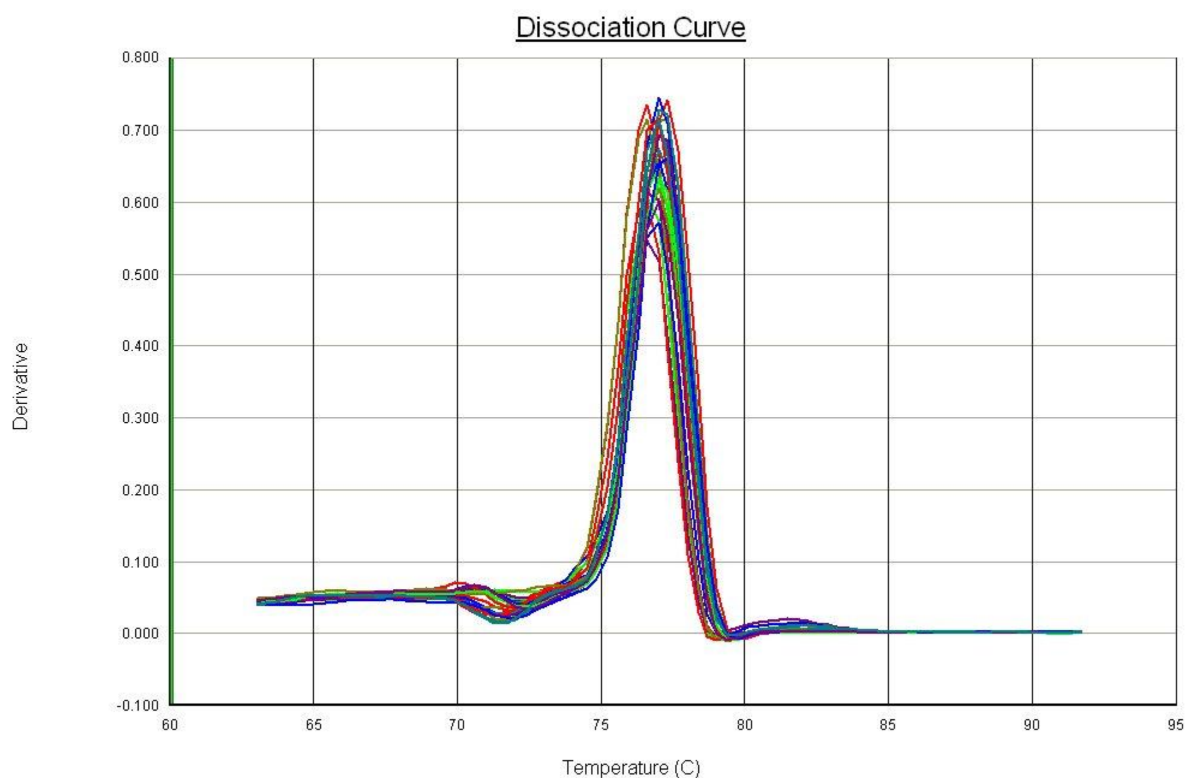
Lane 14 ---*mir1* gene in V7 growth stage leaf tissues of Mp708 control plant;

Lane 15 ---*mir1* gene in V7 growth stage root tip tissues of FAW fed Mp708 plant;

Lane 16 ---*mir1* gene in V7 growth stage root tip tissues of Mp708 control plant;

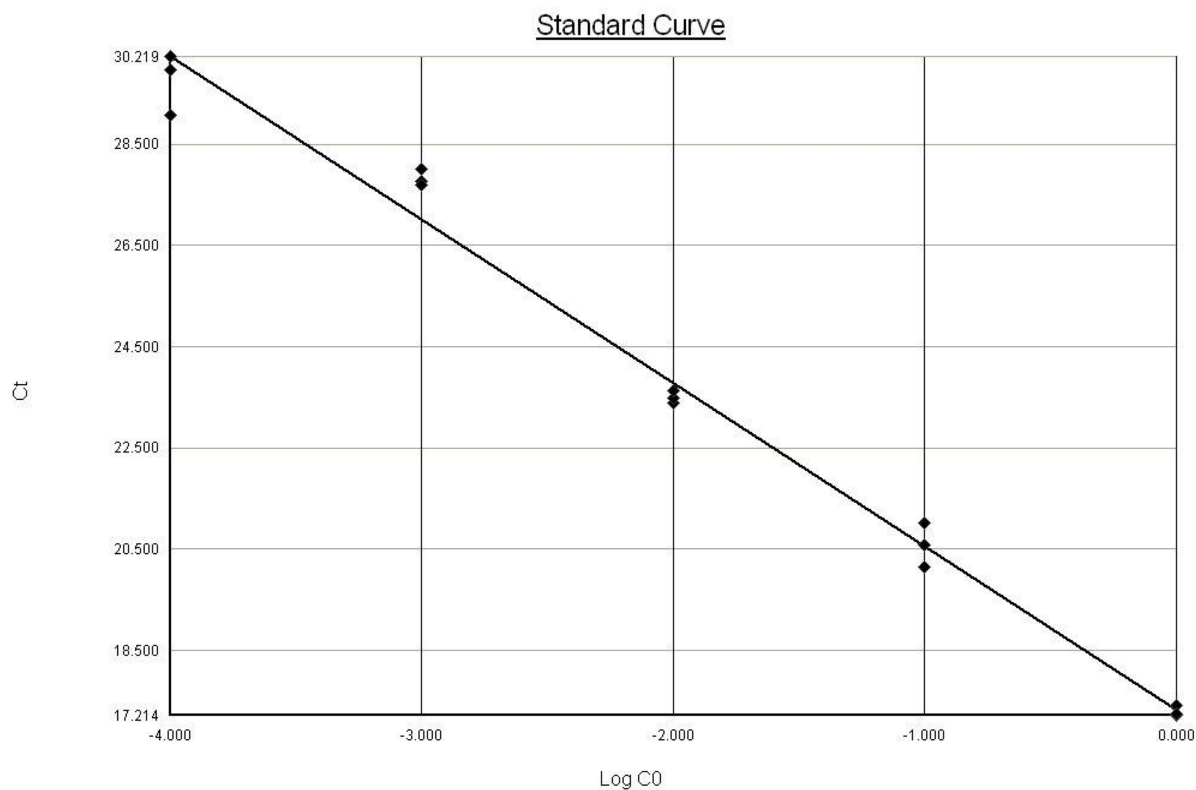
Lane 17 ---*mir1* gene in V9 growth stage leaf tissues of FAW fed Mp708 plant;

Lane 18 ---*mir1* gene in V9 growth stage leaf tissues of Mp708 control plant.



Detector=Mir1-CP, TM=60.1 °C
Well(s): D1=F12
Document: Juliaplate9mir1(06-29-09) (ddCt plate)

Figure 3.3 Specificity of quantitative Real-Time PCR for amplification of *mir1*, as determined by melting curve analysis. Melting peaks were determined by plotting the continuous negative derivative of fluorescence emitted by each sample as PCR products were slowly melted.



Detector : Mir1-CP, Slope: -3.044979, Intercept: 17.61965, R2: 0.988184
Document: juliaplate25standardmir1(Standard Curve)

Figure 3.4 Quantitative Real-Time PCR standard curves from 10 fold serial dilutions of for *mir1* cDNA. Log concentration of each standard was plotted against the cycle number where the fluorescence was greater than background.

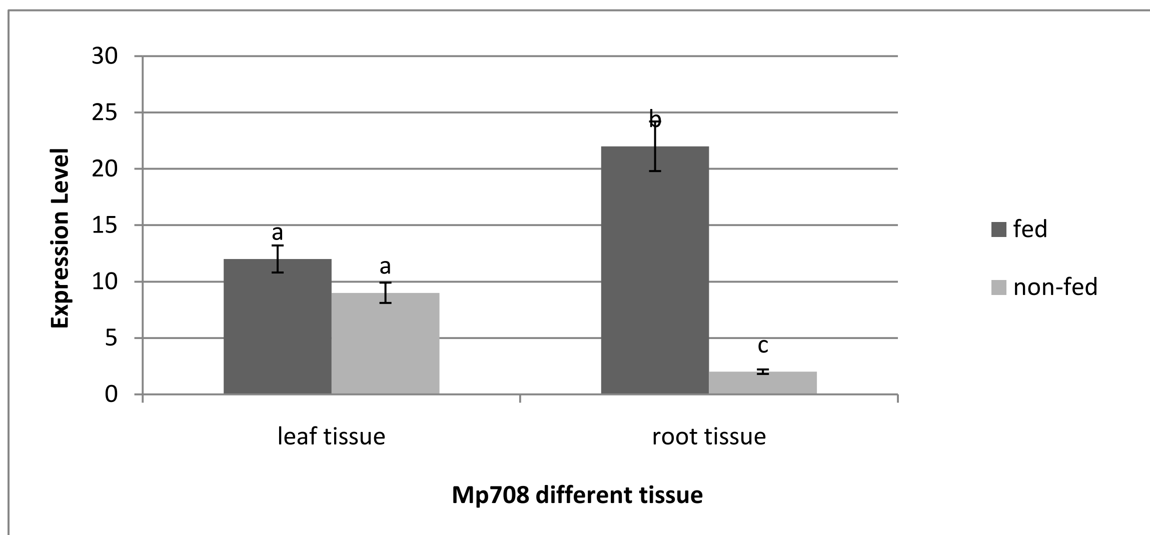


Figure 3.5 Histogram of *mir1* gene expression in leaf and root tissues from FAW fed and non-fed V7 growth stage Mp708. Bars with the same letter indicates that there is no statistical difference in gene expression values, and different letters indicates significant differences ($p<0.05$). There was no significant increase of *mir1* gene transcript levels in Mp708 leaf tissues, while there was a significant increase in transcript levels in Mp708 root tissues in response to FAW feeding.

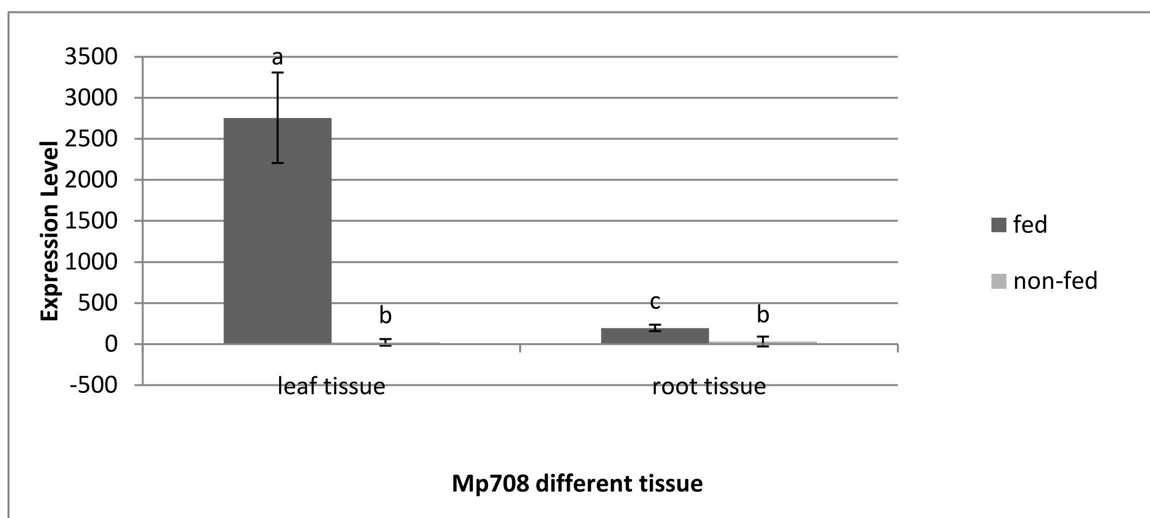


Figure 3.6 Histogram of *mir2* gene expression in leaf and root tissues from FAW fed and non-fed V7 growth stage Mp708. Bars with the same letter indicates that there is no statistical difference in gene expression values, and different letters indicates significant differences ($p<0.05$). There was a significant increase of *mir2* gene transcript level in Mp708 leaf and root tissues.

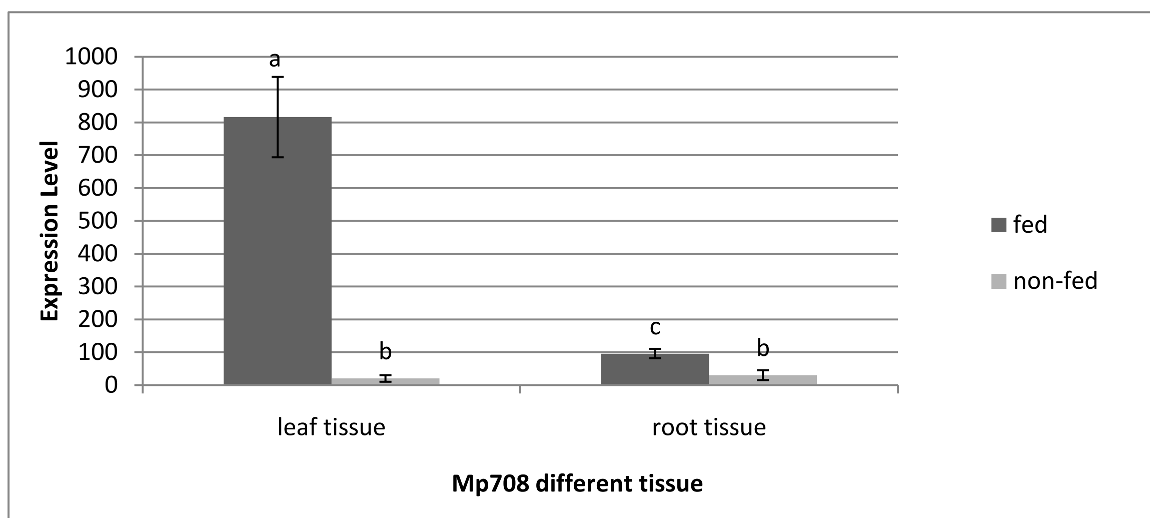


Figure 3.7 Histogram of *mir3* gene expression in leaf and root tissues from FAW fed and non-fed V7 growth stage Mp708. Bars with the same letter indicates that there is no statistical difference in gene expression values, and different letters indicates significant differences ($p<0.05$). There was a significant increase of *mir3* gene transcript level in Mp708 leaf and root tissues.

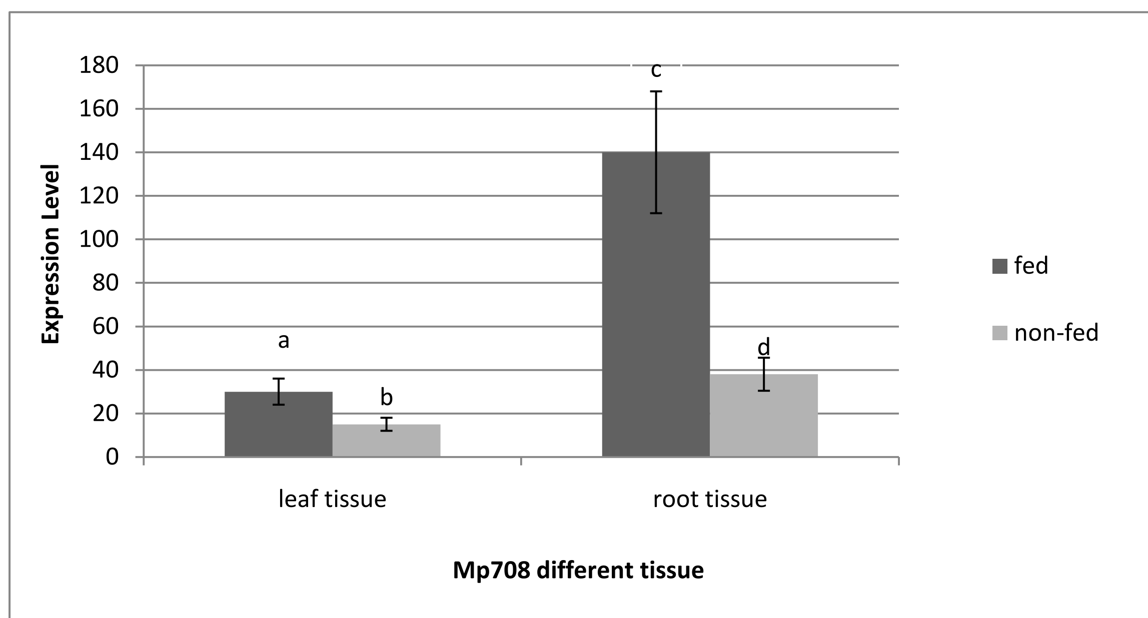


Figure 3.8 Histogram of *lox3* gene expression in leaf and root tissues from FAW fed and non-fed V7 growth stage Mp708. Bars with the same letter indicates that there is no statistical difference in gene expression values, and different letters indicates significant differences ($p<0.05$). There was a significant increase of *lox3* gene transcript level in Mp708 leaf and root tissues.

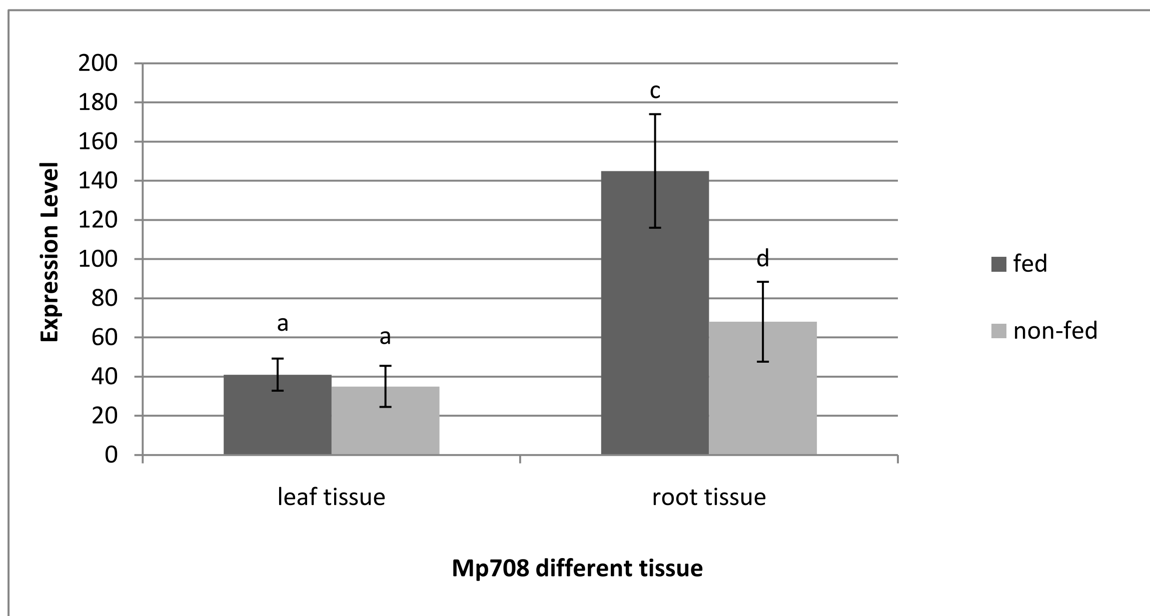


Figure 3.9 Histogram of *lox6* gene expression in leaf and root tissues from FAW fed and non-fed V7 growth stage Mp708. Bars with the same letter indicates that there is no statistical difference in gene expression values, and different letters indicates significant differences ($p<0.05$). There was a significant increase of *lox6* gene transcript level in Mp708 leaf and root tissues.

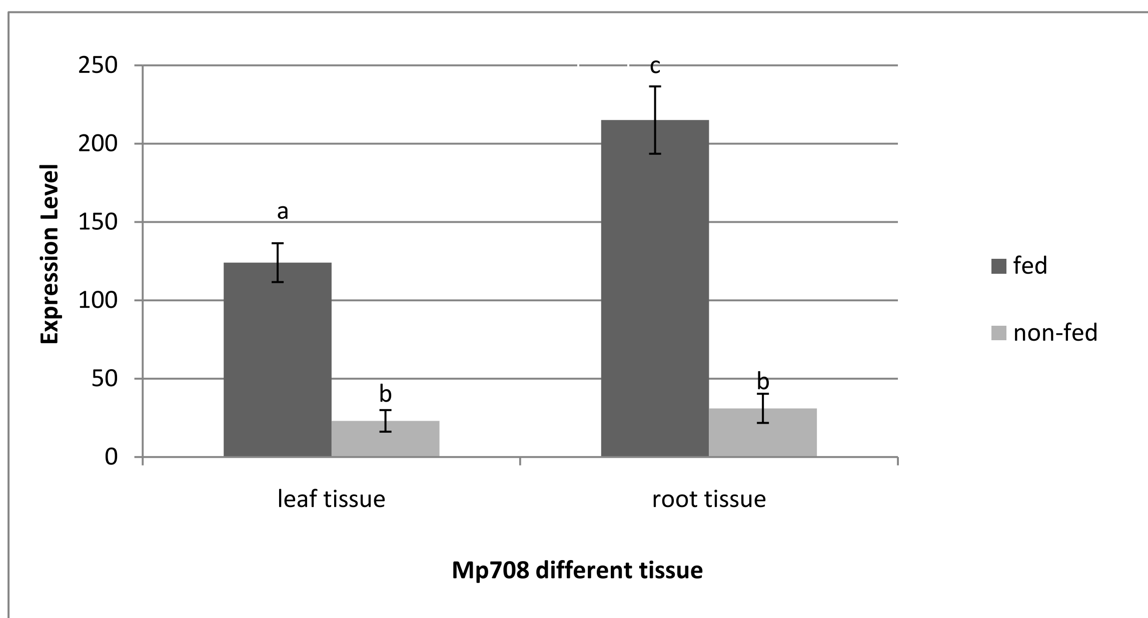


Figure 3.10 Histogram of *RIP2* gene expression in leaf and root tissues from FAW fed and non-fed V7 growth stage Mp708. Bars with the same letter indicates that there is no statistical difference in gene expression values, and different letters indicates significant differences ($p<0.05$). There was a significant increase of *RIP2* gene transcript level in Mp708 leaf and root tissues.

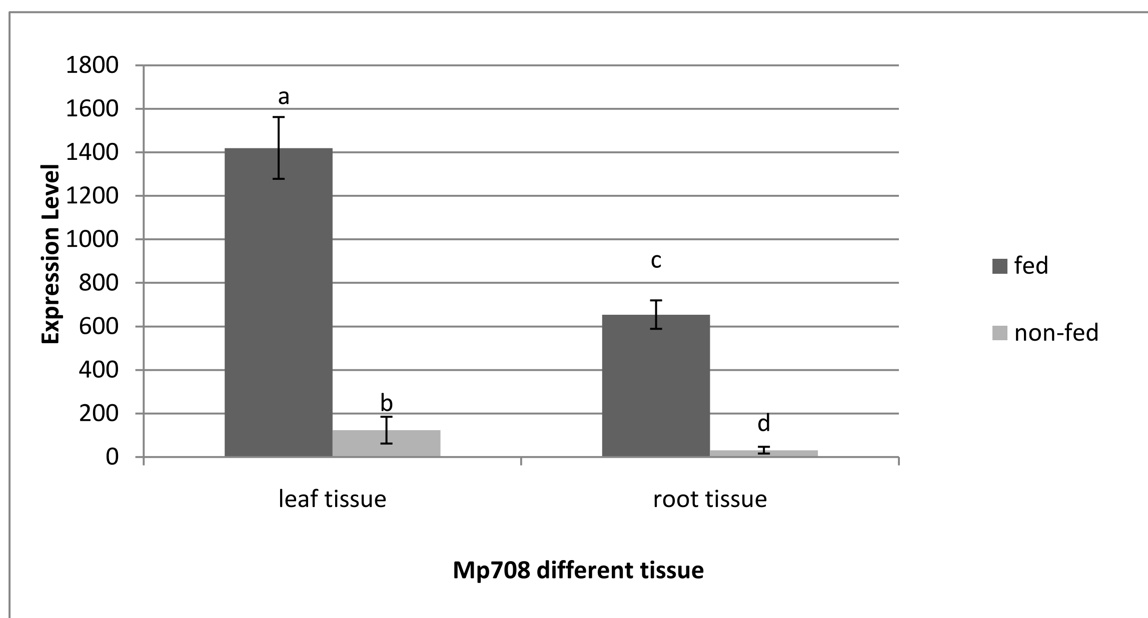


Figure 3.11 Histogram of *MPI* gene expression in leaf and root tissues from FAW fed and non-fed V7 growth stage Mp708. Bars with the same letter indicates that there is no statistical difference in gene expression values, and different letters indicates significant differences ($p<0.05$). There was a significant increase of *MPI* gene transcript level in Mp708 leaf and root tissues.

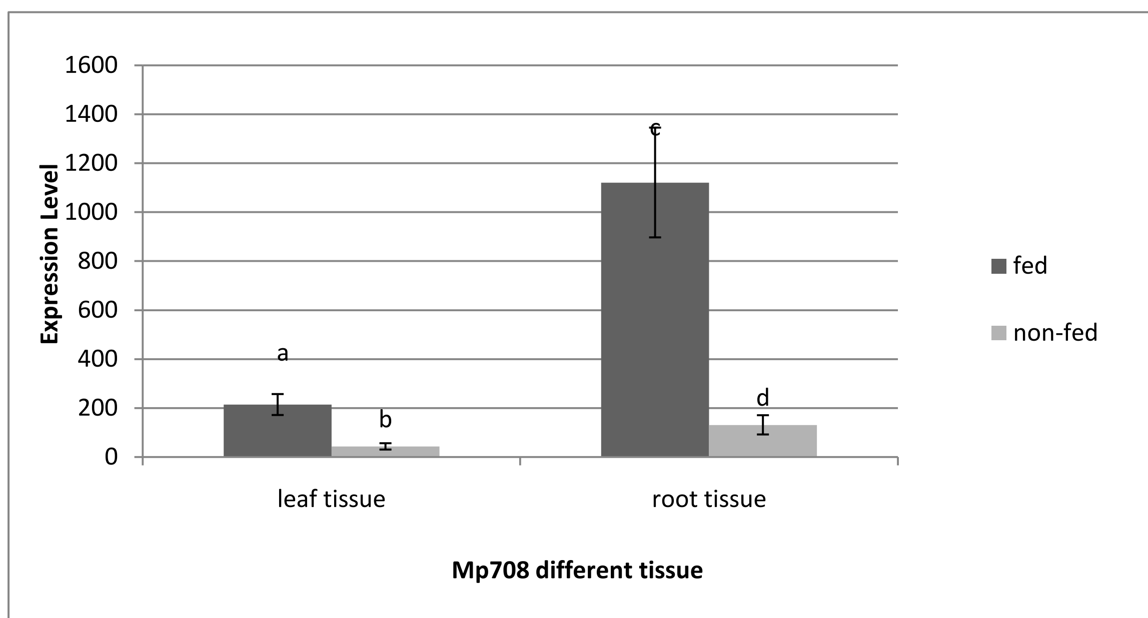


Figure 3.12 Histogram of *PR10* gene expression in leaf and root tissues from FAW fed and non-fed V7 growth stage Mp708. Bars with the same letter indicates that there is no statistical difference in gene expression values, and different letters indicates significant differences ($p<0.05$). There was a significant increase of *PR10* gene transcript level in Mp708 leaf and root tissues.

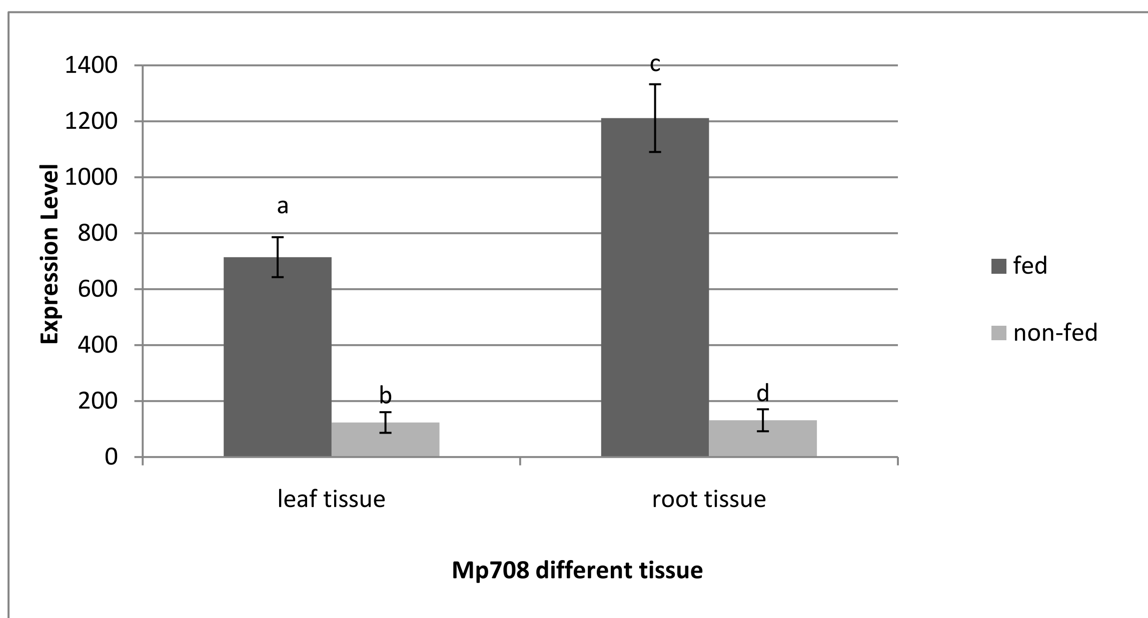


Figure 3.13 12 Histogram of *Chi1* gene expression in leaf and root tissues from FAW fed and non-fed V7 growth stage Mp708. Bars with the same letter indicates that there is no statistical difference in gene expression values, and different letters indicates significant differences ($p<0.05$). There was a significant increase of *Chi1* gene transcript level in Mp708 leaf and root tissues.

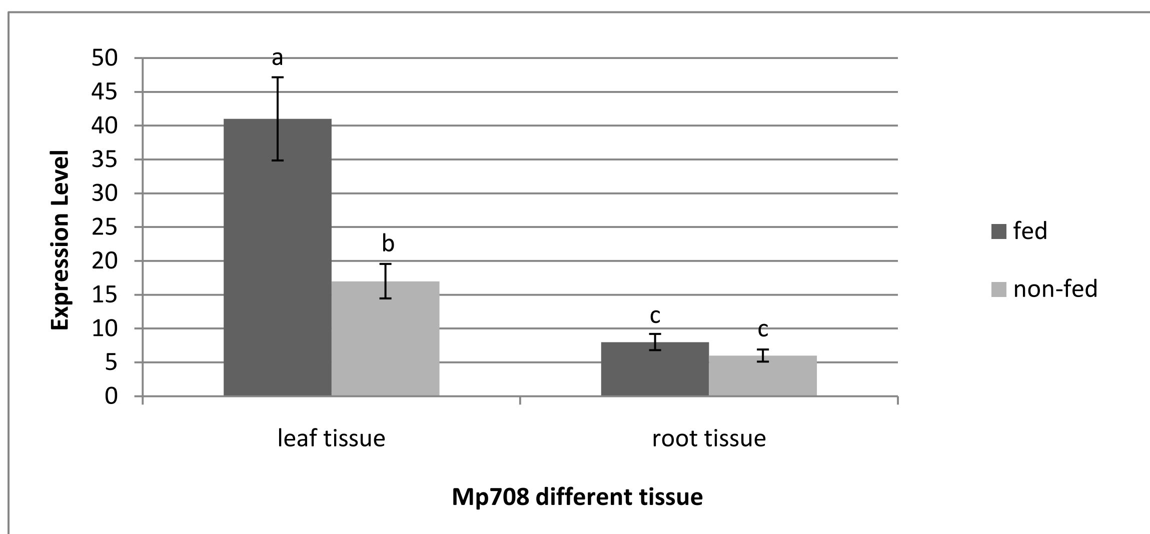
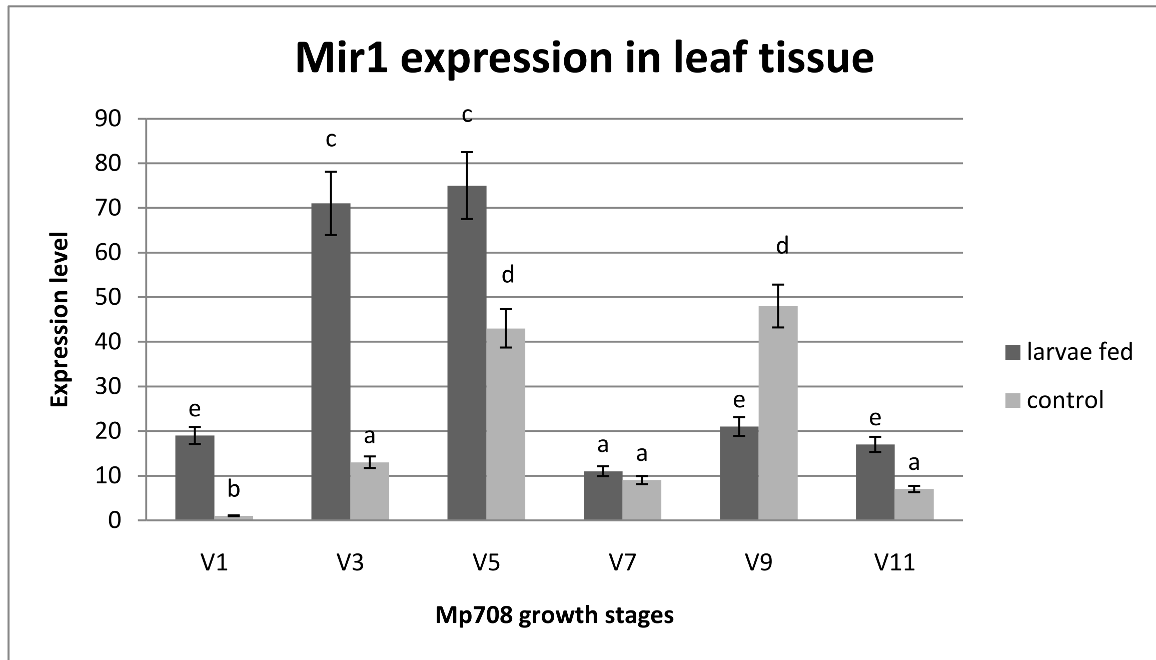


Figure 3.14 Histogram of *SSU1* gene expression in leaf and root tissues from FAW fed and non-fed V7 growth stage Mp708. Bars with the same letter indicates that there is no statistical difference in gene expression values, and different letters indicates significant differences ($p<0.05$). There was a significant increase of *SSU1* gene transcript level in Mp708 leaf tissues. There was no significant increase of *SSU1* gene transcript level in Mp708 leaf tissues.

A



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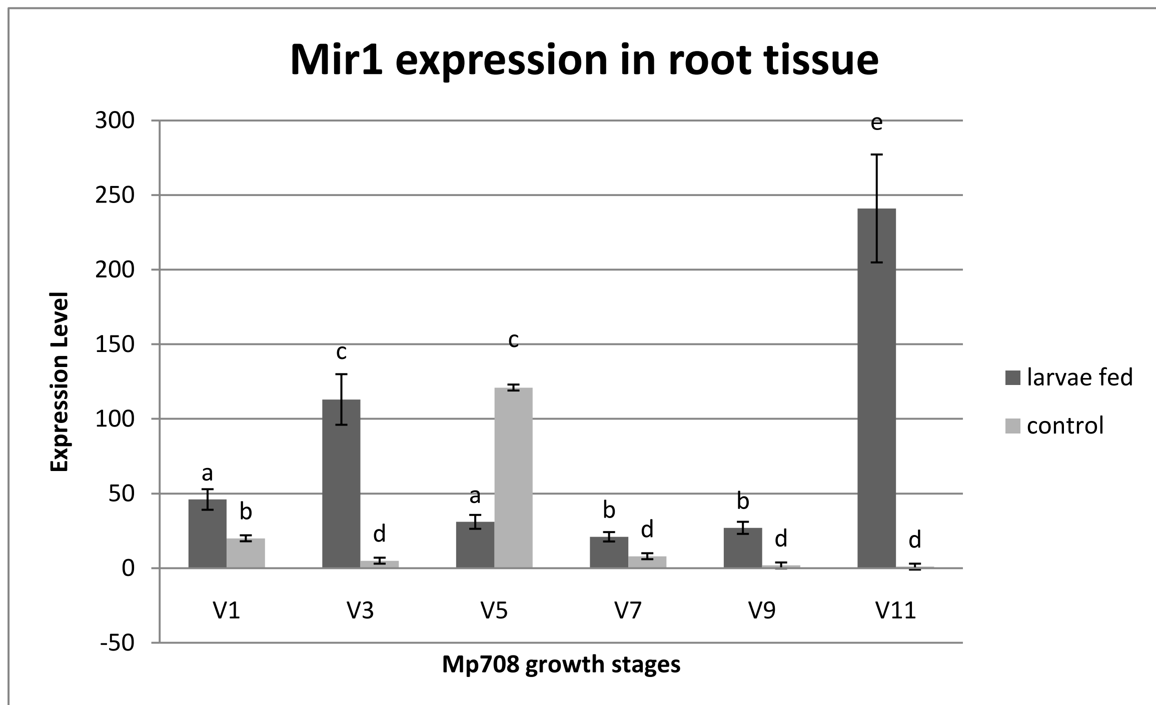
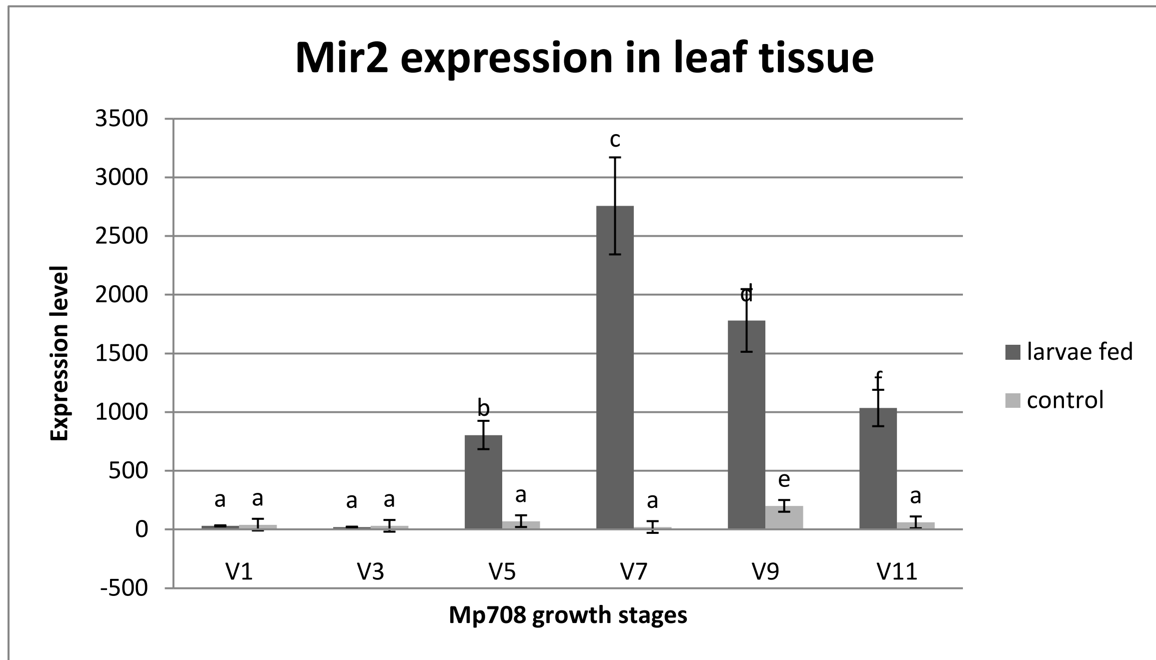


Figure 3.15 (**A**) *mir1* gene expression in leaf tissue from caterpillar fed and non-fed Mp708 maize during development (From V1 growth stages to V11 growth stages). (**B**) *mir1* gene expression in root tissue from caterpillar fed and non-fed Mp708 maize plant during plant development (From V1 growth stages to V11 growth stages). Bars with same letters indicated that there were no statistical differences in gene expression values and different letters indicate statistical difference ($p<0.05$).

A



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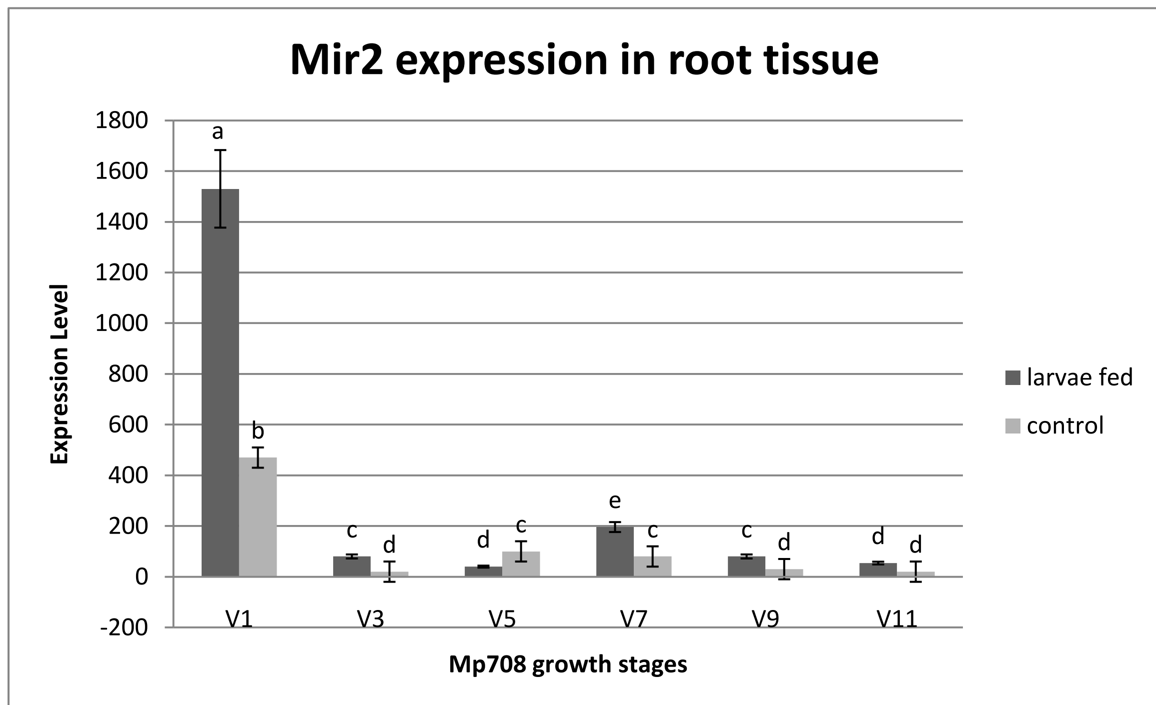
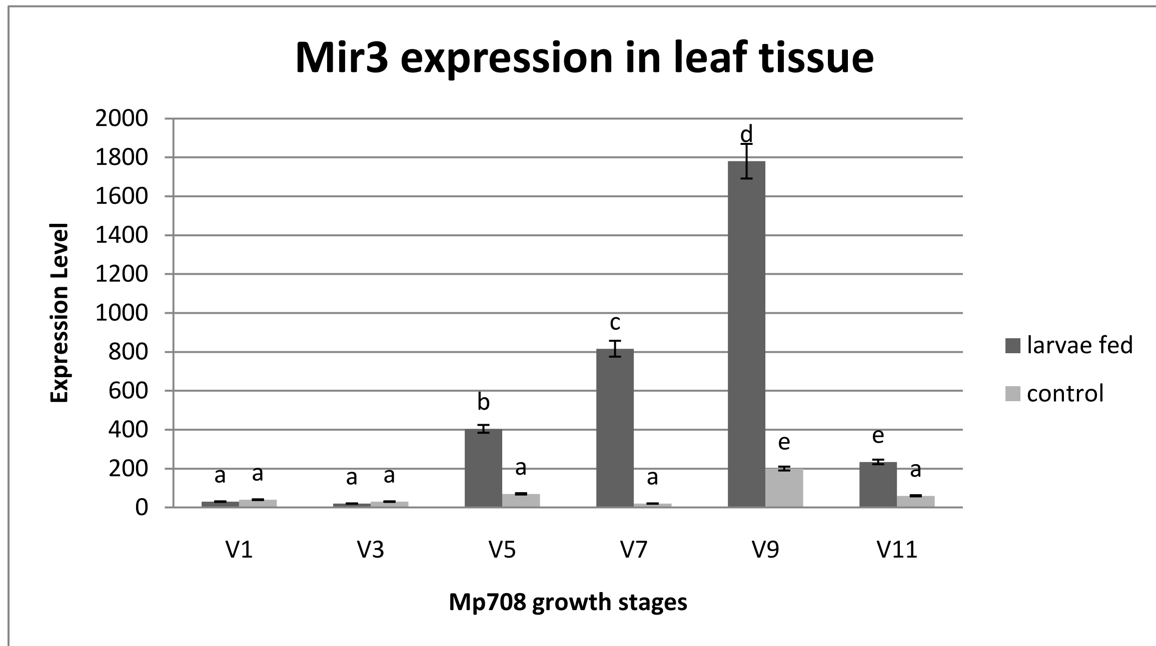


Figure 3.16 (**A**) *mir2* gene expression in leaf tissue from caterpillar fed and non-fed Mp708 maize during development (From V1 growth stages to V11 growth stages). (**B**) *mir2* gene expression in root tissue from caterpillar fed and non-fed Mp708 maize plant during plant development (From V1 growth stages to V11 growth stages). Bars with same letters indicated that there were no statistical differences in gene expression values and different letters indicate statistical difference ($p<0.05$).

A



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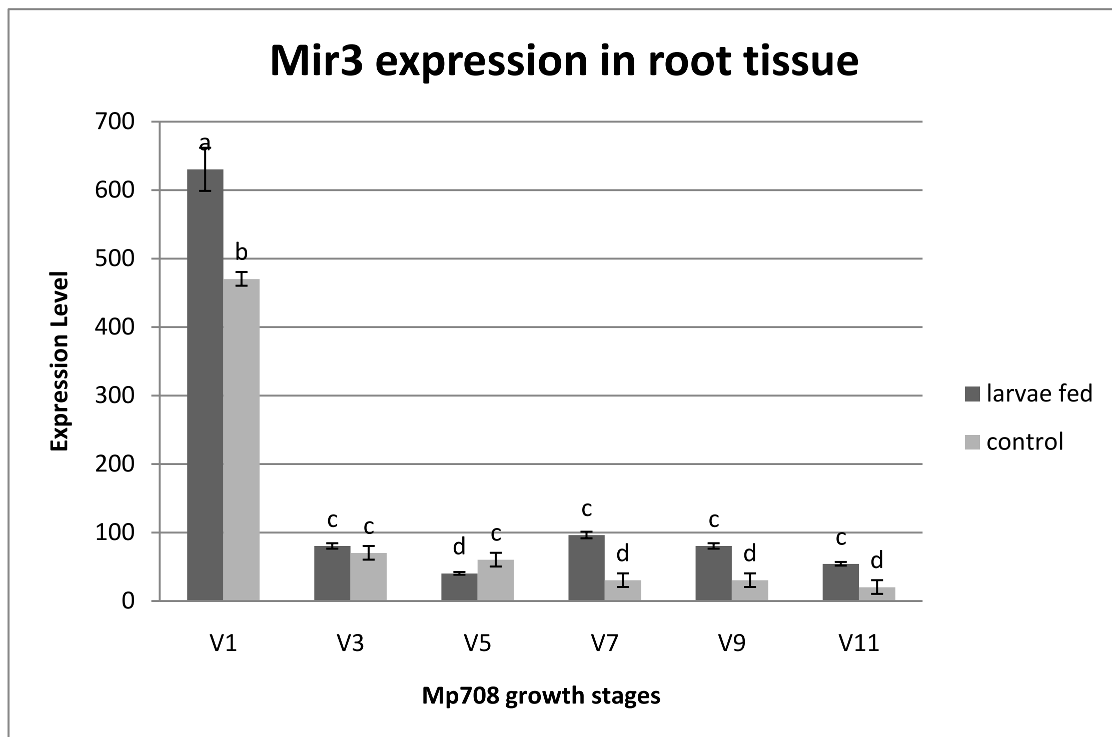
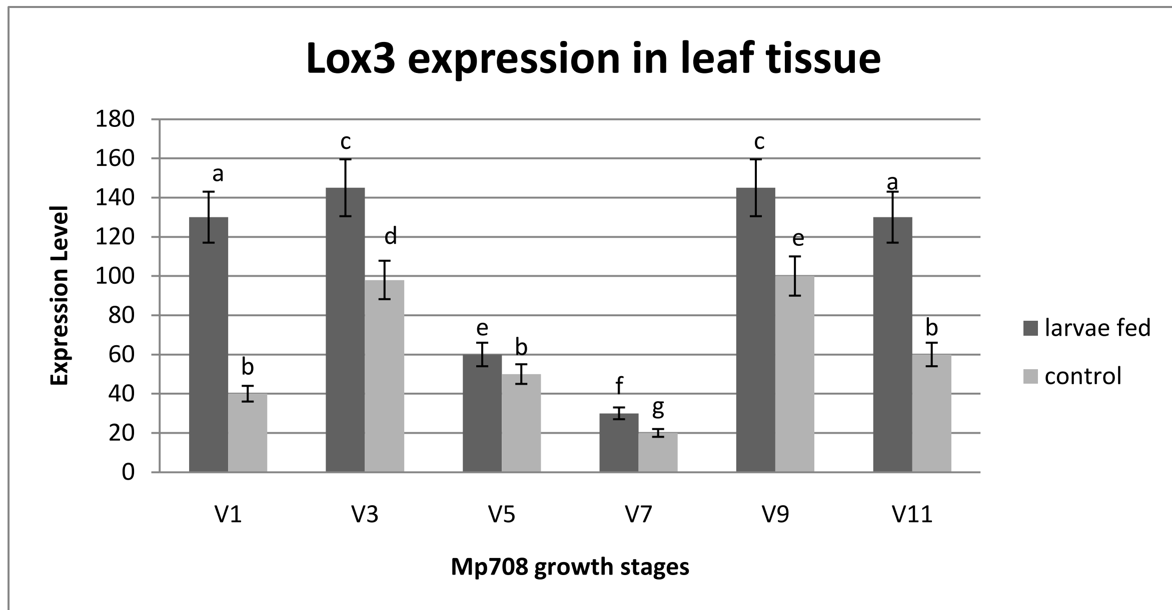


Figure 3.17 (**A**) *mir3* gene expression in leaf tissue from caterpillar fed and non-fed Mp708 maize during development (From V1 growth stages to V11 growth stages). (**B**) *mir3* gene expression in root tissue from caterpillar fed and non-fed Mp708 maize plant during plant development (From V1 growth stages to V11 growth stages). Bars with same letters indicated that there were no statistical differences in gene expression values and different letters indicate statistical difference ($p<0.05$).

A



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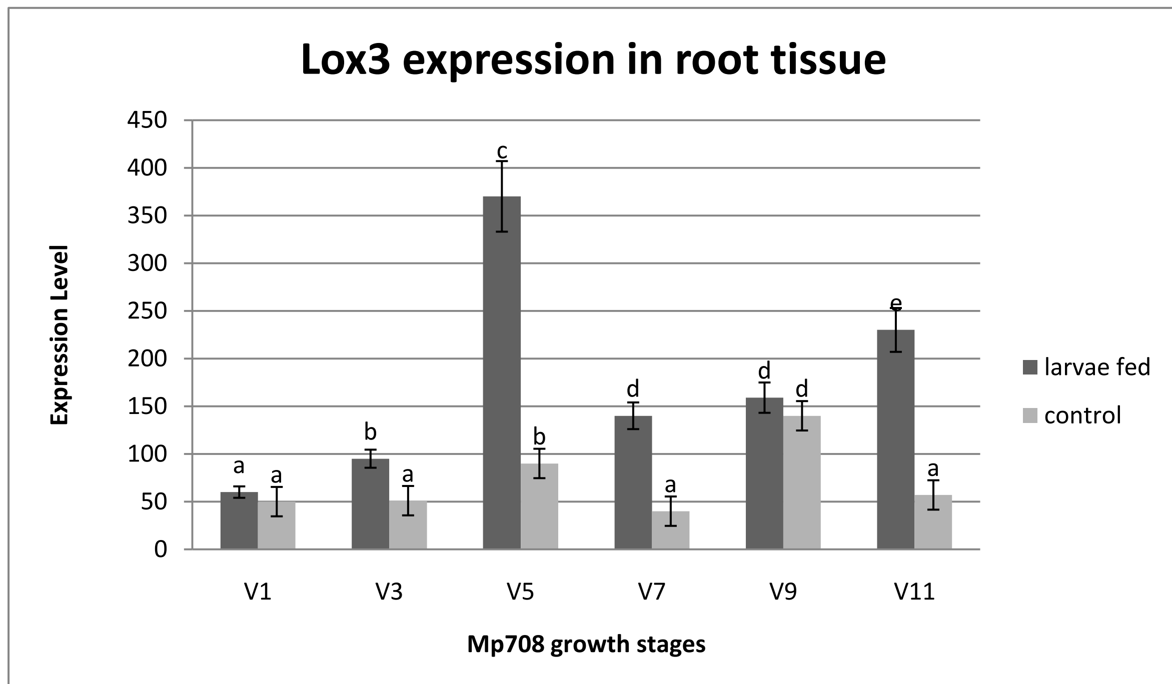
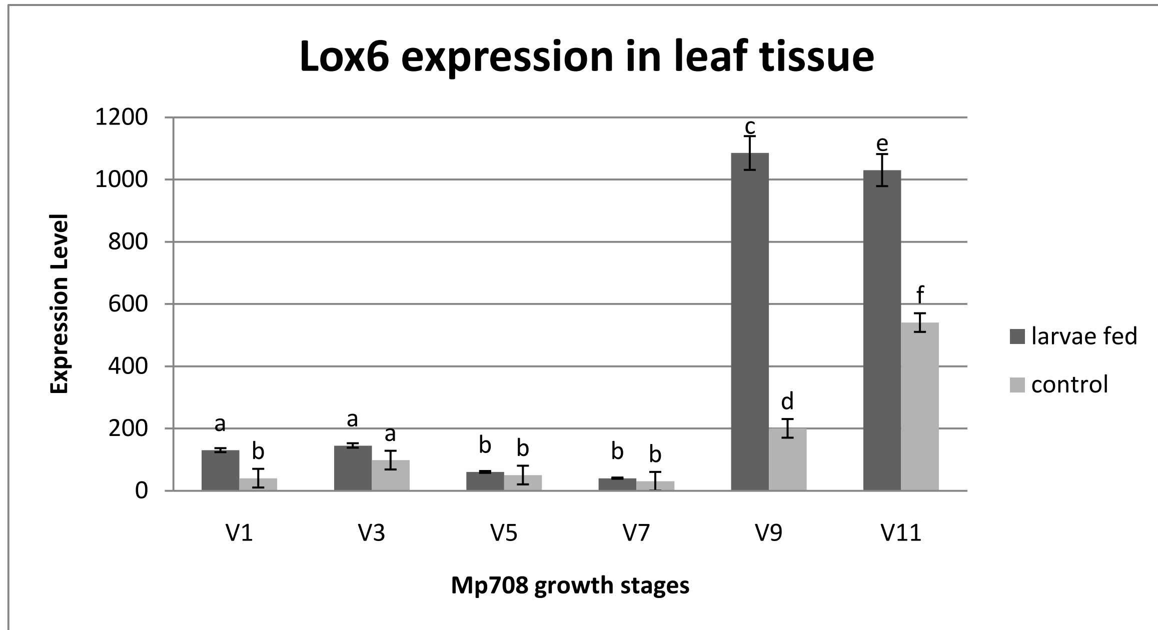


Figure 3.18 (**A**) *lox3* gene expression in leaf tissue from caterpillar fed and non-fed Mp708 maize during development (From V1 growth stages to V11 growth stages). (**B**) *lox3* gene expression in root tissue from caterpillar fed and non-fed Mp708 maize plant during plant development (From V1 growth stages to V11 growth stages). Bars with same letters indicated that there were no statistical differences in gene expression values and different letters indicate statistical difference ($p<0.05$).

A



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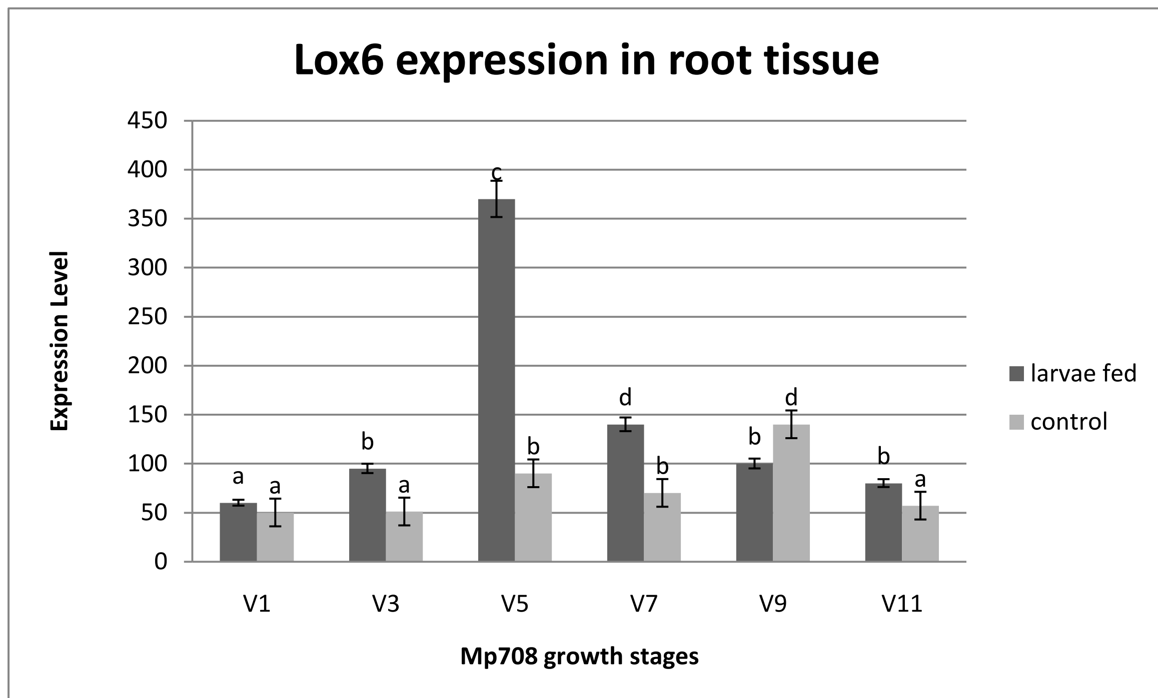
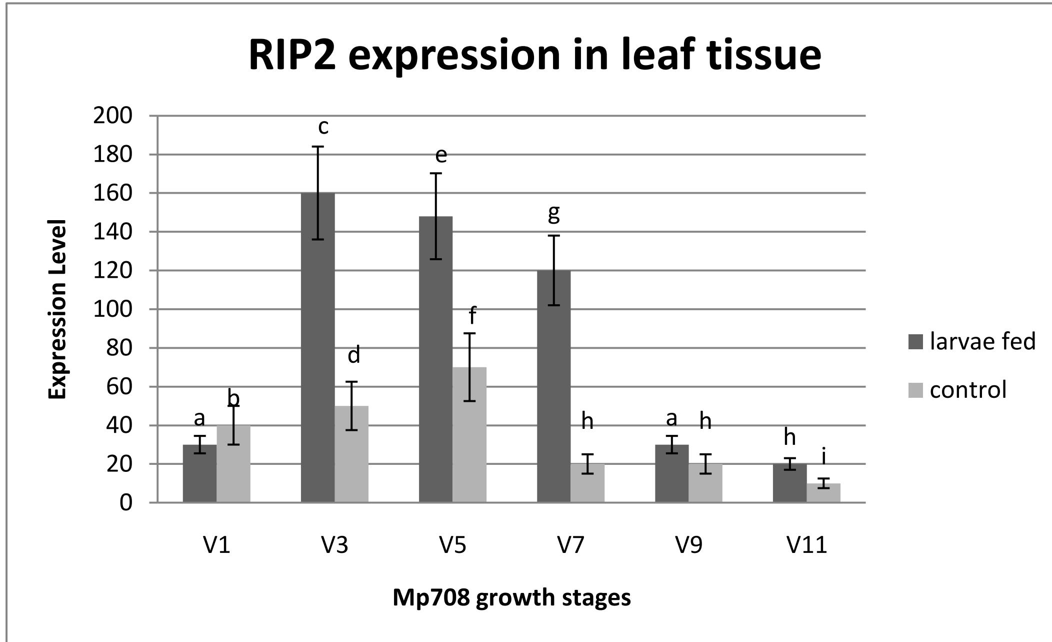


Figure 3.19 (**A**) *lox6* gene expression in leaf tissue from caterpillar fed and non-fed Mp708 maize during development (From V1 growth stages to V11 growth stages). (**B**) *lox6* gene expression in root tissue from caterpillar fed and non-fed Mp708 maize plant during plant development (From V1 growth stages to V11 growth stages). Bars with same letters indicated that there were no statistical differences in gene expression values and different letters indicate statistical difference ($p<0.05$).

A



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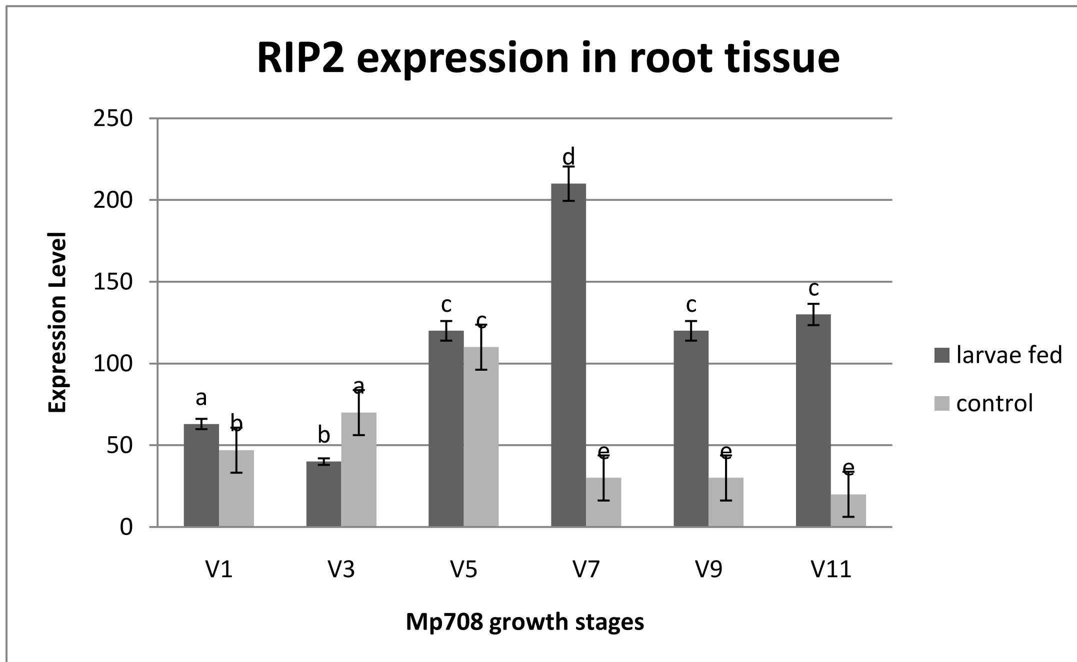
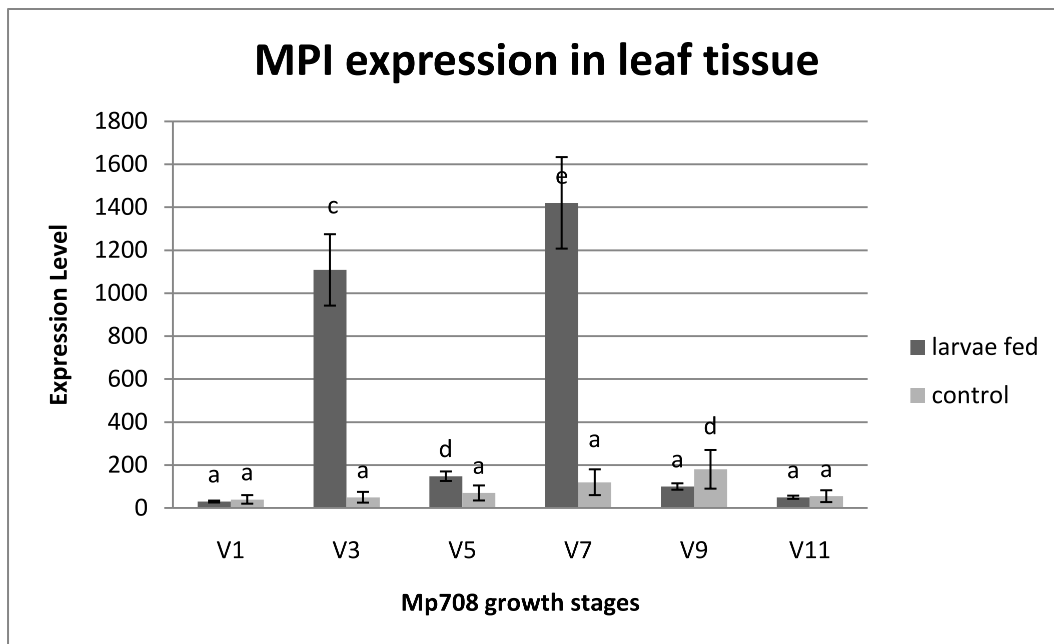


Figure 3.20 (**A**) *RIP2* gene expression in leaf tissue from caterpillar fed and non-fed Mp708 maize during development (From V1 growth stages to V11 growth stages). (**B**) *RIP2* gene expression in root tissue from caterpillar fed and non-fed Mp708 maize plant during plant development (From V1 growth stages to V11 growth stages). Bars with same letters indicated that there were no statistical differences in gene expression values and different letters indicate statistical difference ($p<0.05$).

A



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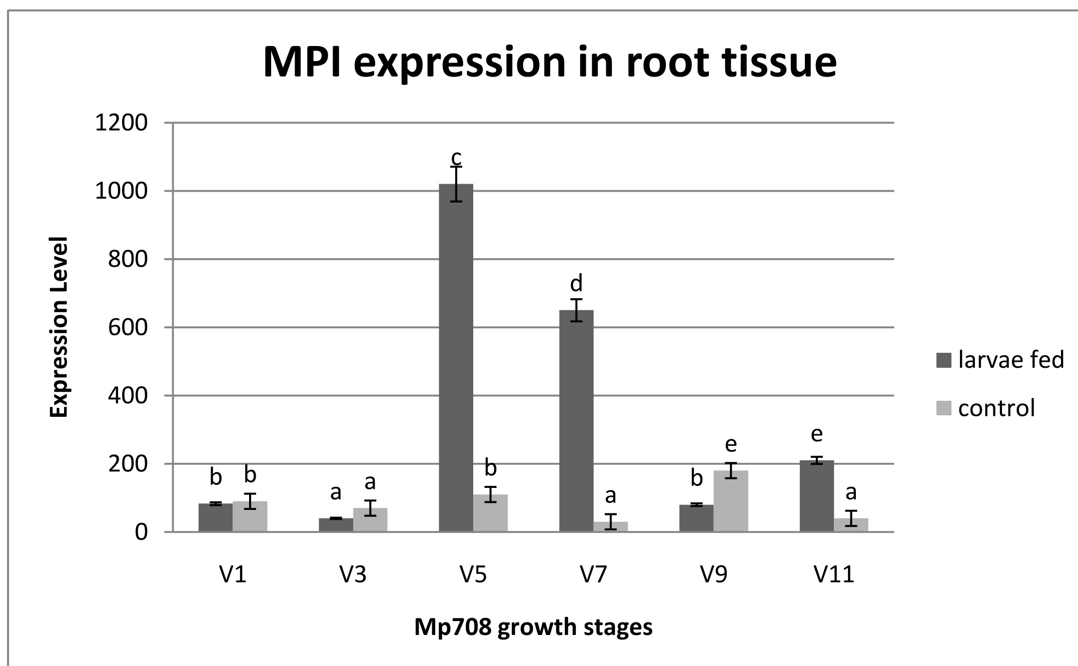
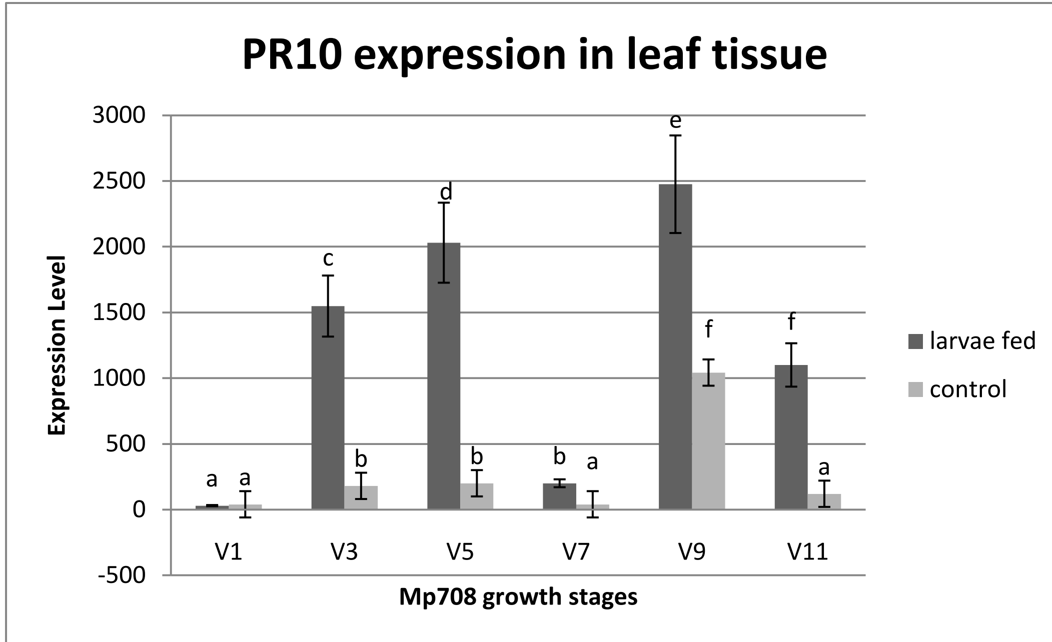


Figure 3.21 (**A**) *MPI* gene expression in leaf tissue from caterpillar fed and non-fed Mp708 maize during development (From V1 growth stages to V11 growth stages). (**B**) *MPI* gene expression in root tissue from caterpillar fed and non-fed Mp708 maize plant during plant development (From V1 growth stages to V11 growth stages). Bars with same letters indicated that there were no statistical differences in gene expression values and different letters indicate statistical difference ($p<0.05$).

A



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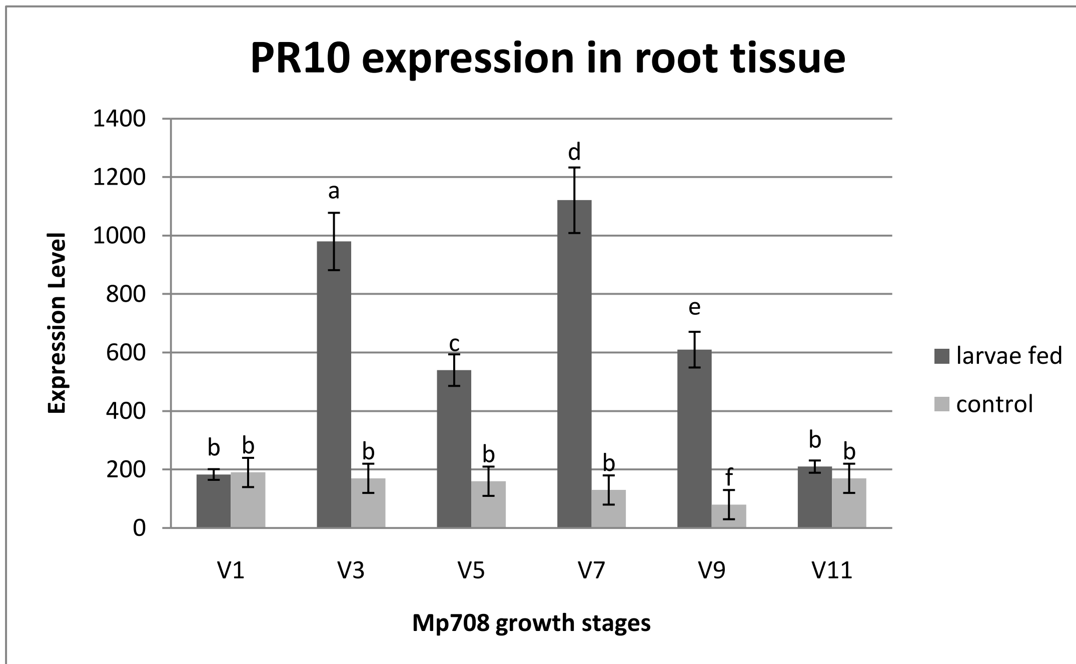
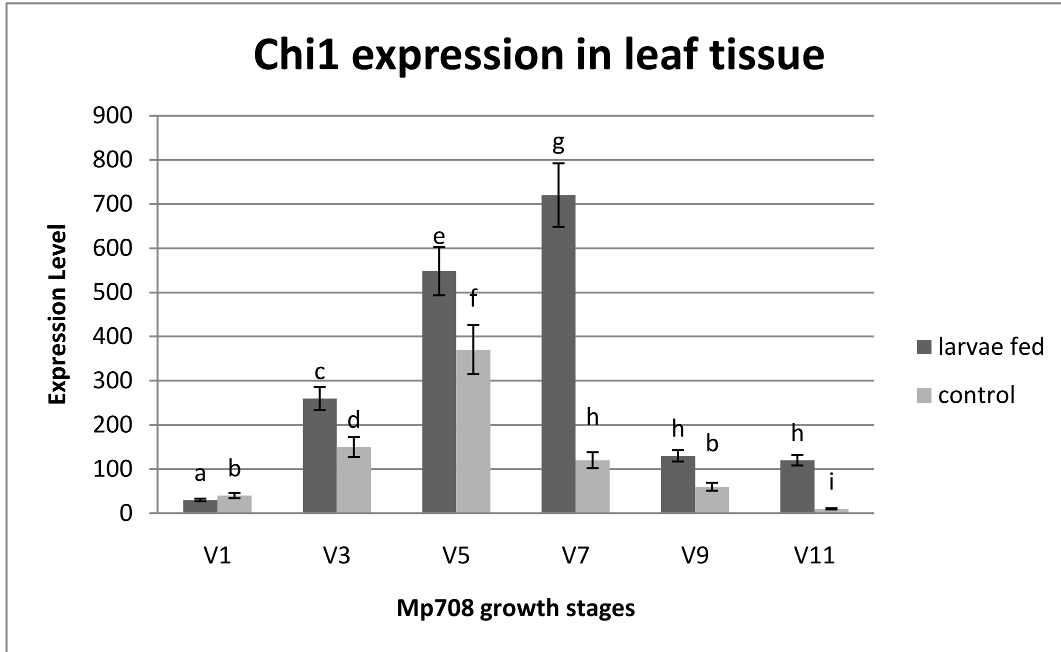


Figure 3.22 (**A**) *PR10* gene expression in leaf tissue from caterpillar fed and non-fed Mp708 maize during development (From V1 growth stages to V11 growth stages). (**B**) *PR10* gene expression in root tissue from caterpillar fed and non-fed Mp708 maize plant during plant development (From V1 growth stages to V11 growth stages). Bars with same letters indicated that there were no statistical differences in gene expression values and different letters indicate statistical difference ($p<0.05$).

A



B

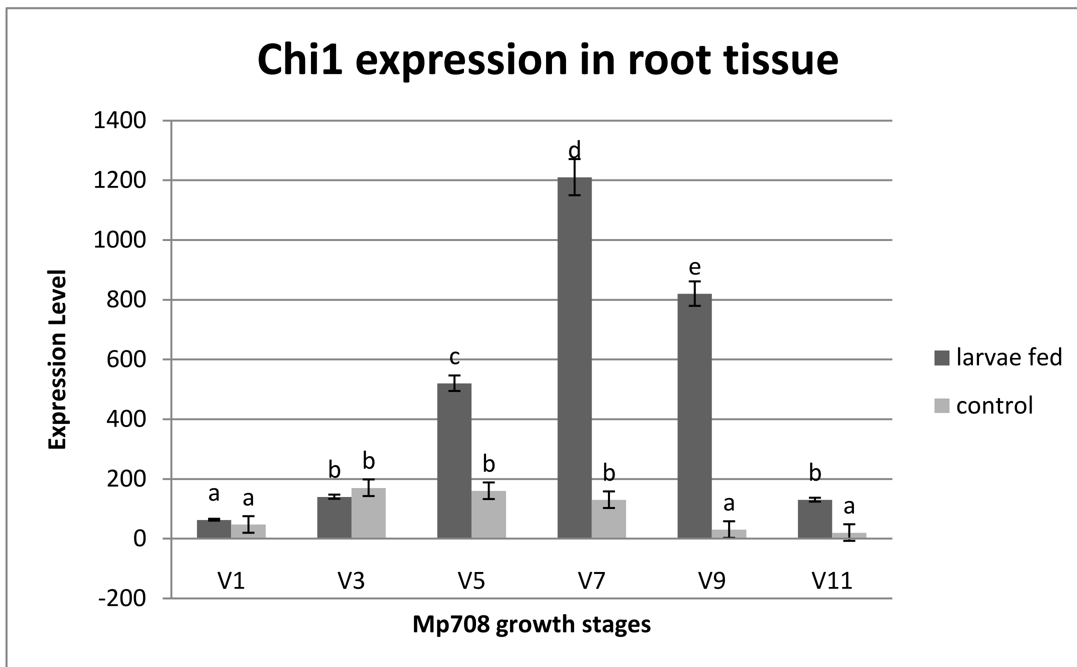
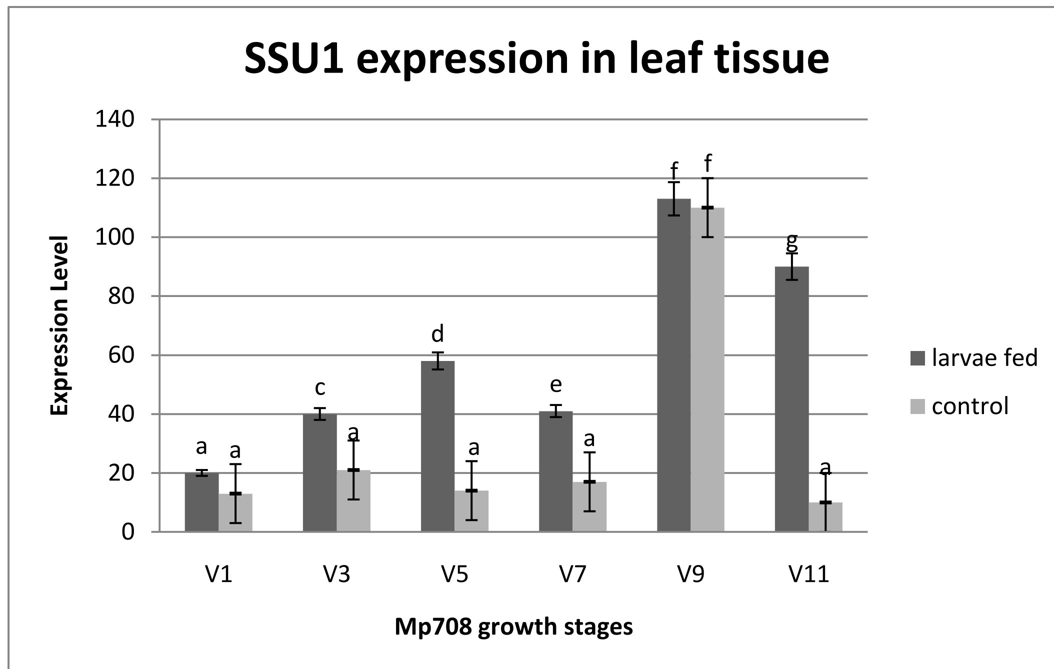


Figure 3.23 (**A**) *Chi1* gene expression in leaf tissue from caterpillar fed and non-fed Mp708 maize during development (From V1 growth stages to V11 growth stages). (**B**) *Chi1* gene expression in root tissue from caterpillar fed and non-fed Mp708 maize plant during plant development (From V1 growth stages to V11 growth stages). Bars with same letters indicated that there were no statistical differences in gene expression values and different letters indicate statistical difference ($p<0.05$).

A



B

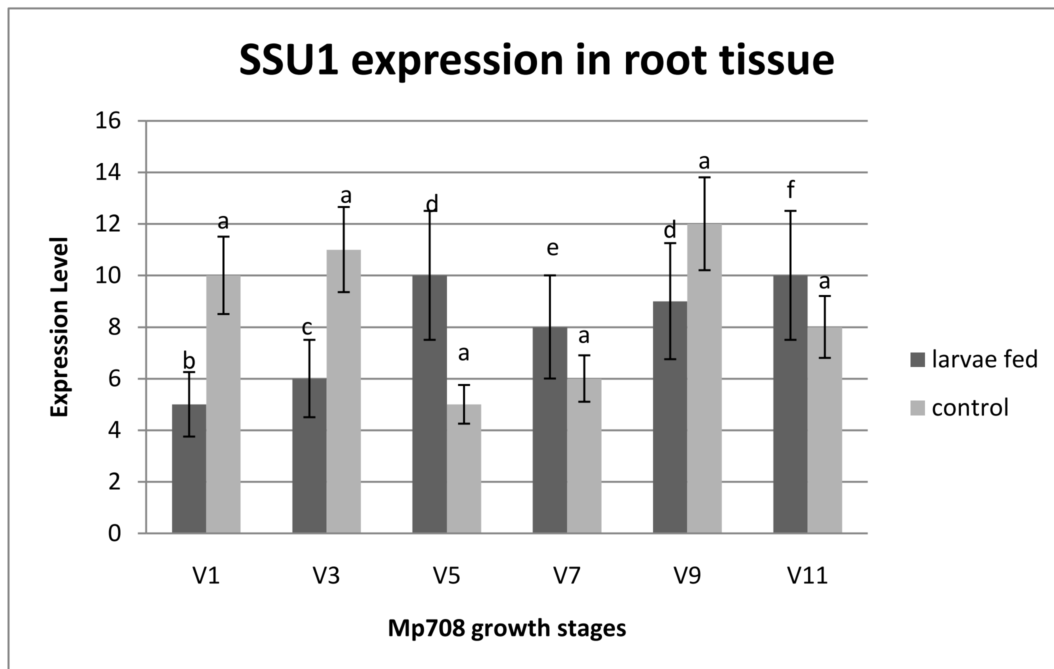


Figure 3.24 (**A**) *SSU1* gene expression in leaf tissue from caterpillar fed and non-fed Mp708 maize during development (From V1 growth stages to V11 growth stages). (**B**) *SSU1* gene expression in root tissue from caterpillar fed and non-fed Mp708 maize plant during plant development (From V1 growth stages to V11 growth stages). Bars with same letters indicated that there were no statistical differences in gene expression values and different letters indicate statistical difference ($p<0.05$).

Figure 3.25 Immunoblot analysis of Mir1-CP protein expression in leaf and root tissues from V7 stage Mp708 plant. The arrow indicates Mir1-CP protein in FAW fed V7 stage plant whorl (lane 1); Mir1-CP protein in non-fed V7 plant whorl (lane 2); Mir1-CP protein root tips from fed plant at V7 stage (lane 3); Mir1-CP protein in root tips from control plant at V7 stage (lane 4).

4. Discussion

In this thesis, the question of whether above ground herbivory induces defense gene expression in aboveground and belowground tissue of the maize inbred line Mp708 is addressed. The findings suggest that aboveground feeding results in belowground accumulation of defense gene transcripts and the defense protein Mir-CP.

4.1 Pros and Cons of Experiment Designs

The plant used for this project was insect-resistant maize inbred line Mp708. This inbred line was selected by traditional breeding and it shows remarkably strong insect defense responses compared to the insect susceptible inbred line Tx601 (Williams and Davis, 1984; Williams and Davis, 1985). This characteristic makes it an ideal plant for studying mechanism of plant herbivore defense.

The project was conducted using FAW larvae as herbivore. FAW is found in the tropics and the US (Johnson, 1987). It disperses long distances annually during the summer months making it a widespread pest that is of great harm to crops. FAW larvae feed extensively on whorl leaf tissue, which results in crop losses. The above characteristics of FAW make it an important pest for insect control research (Williams et al., 1987; Pechan et al., 2000). Besides FAW, there are other maize herbivores, such as European corn borer (*Ostrinia nubilalis*), corn earworn (*Helicoverpa zea*), Asian corn borer (*Ostrinia frunicalis*), spotted stem borer (*Chilo partellus*), and African maize stem borer (*Busseda fusca)* (Williams and Buckley, 1990; Williams et al., 1989). Although studying on other insects may provide more information on maize insect defenses, this work used FAW because it is a major insect of maize and a general *Lepidopteran* pest.

Previous genetic analyses of Mp708 plants indicated that resistance to FAW was regulated by several defense genes (Williams et al., 1989; Khairallah et al., 1998). In this study, RT- PCR was executed to quantify gene expression. The qRT-PCR provides precise and accurate quantification of gene transcript the relative abundance. This technique has become one of the most widely used methods of gene quantification because of the following advantages: 1) large dynamic range 2) tremendous sensitivity 3) high sequence-specificity (Wong et al., 2005). One of the limitations of qRT-PCR is the unavoidable experimental variation. In most cases qRT-PCR provides relative quantification instead of absolute data such as transcript copy number (Herman et al., 2008).

In order to examine defense proteins accumulation in different tissues of plant, immunoblot analysis was conducted. It provides direct visual evidence of protein abundance. This technique is a quick and inexpensive method for comparison the abundance of a target protein. Although the relative abundance of proteins within a tissue are better determined using immunohistochemistry (Bakalova et al., 2005), immunoblot was chosen for practical reasons.

4.2 Results Discussion

4.2.1 Model of Aboveground and Belowground Communication

The qRT-PCR results indicated that, in V7 growth stage, the *mir1* gene transcript levels increased significantly in root tissues of larval fed plants, however in leaf tissues the difference was not neat. When compared to the non-fed control, Mp708 root tips, the quantitative value of *mir1* transcripts was approximately 10 times higher ($P < 0.05$) in the root tips from larval fed Mp708 maize plant. In the V7 growth stage, after leaf herbivory, the increase of *mir1* gene transcript levels in leaf tissues was not as significant as gene transcript levels in root tissues. The

link between increased *mir1* expression in the root and leaf herbivory provided supportive evidence that *mir1* expression is induced in belowground following aboveground larvae feeding.

The immunoblot experiment results indicated that Mir1-CP accumulation in Mp708 plants whorl at V7 growth stage when plants were attacked by FAW feeding. Mir1-CP was detected in the roots of these plants, there was much less in the roots compared to the leaf tissues. In control plants, no detectable amount of Mir1-CP was found. These observations indicated that after larvae feeding, most Mir1-CP accumulated in the leaf tissues of V7 plants in response to FAW herbivory. We can conclude that Mir1-CP accumulation aboveground was induced by foliar herbivory and the protein accumulated to a lowest extent in the roots.

Since Mir1-CP is toxic to *lepidopteran* larval digestive system (Mohan et al., 2006), its accumulation in aboveground tissue increases plant resistance to leaf herbivory. López et al., 2007 found Mir1-CP in the phloem and the metaxylem of the leaf and root, respectively. It has been established that herbivory signals are exchanged between roots and leaf upon herbivore attack (López et al., 2007). Leaf-root communication is proposed to take place through the internal vascular network between phloem and xylem bundles in the plant (Orians, 2005) or the external route of volatile signaling. Combined with this evidence, I propose that, Mir1-CP is synthesized in root, and then Mir1-CP move into the vascular tissues and is possibly transported to aboveground tissues attacked by herbivores.

Mir1-CP presence and abundance in the metaxylem elements suggests that it might be transported in the xylem sap to the aboveground leaf tissues from belowground root tissues (López et al., 2007). A study reported that some xylem sap proteins are actively secreted into the xylem stream and they may travel to the leaf, where they function in cell wall repair, and insect

defense (Sakuta and Satoh, 2000). This research suggests that certain signals triggered by herbivory could travel from the leaves to the roots and result in the accumulation of Mir1-CP in the root. Then, Mir1-CP might travel back to the leaf.

4.2.2 *Mir2, Mir3, MPI, Lox3, Lox6, RIP, Chi1, PR10* Genes Expression in the V7 Growth Stage

The herbivory induction of *mir2* transcripts in leaf tissues of V7 growth stage Mp708 was examined in this thesis. Compared to non-fed control Mp708 leaf tissue, gene transcript levels increased dramatically in larval damaged leaf tissues. Compared to root tissues from control plants, *mir2* transcript levels was approximately 10 times higher in the root tips from larvae fed Mp708 maize plant. The result indicated that V7 stage Mp708 plants, FAW feeding increased *mir2* transcripts level significantly both belowground and aboveground. Similar results were obtained for *mir3* gene expression data analysis, which is a homology of *mir2*. In control plants, these genes were expressed in aboveground and belowground parts. Genes expression level increased thousands of times after plants are fed by FAW larvae.

In this research, expression levels for the defense genes *MPI, lox3, lox6, RIP, Chi1, PR10* were examined in Mp708 at the V7 stage following herbivory. The qRT-PCR experiments indicated that the transcript levels of these defense genes increased significantly in Mp708 not only in aboveground tissues but also in belowground tissues in response to insect feeding. These results indicated these genes functioned in plant-insect interactions to defend the plant aboveground and belowground.

Insect herbivory is known to promote an increase in JA accumulation and ethylene emission (Argandona et al., 2001). One of the known mechanisms is that the insect attack in aboveground

sends signals that activate the JA biosynthesis pathway. This process indicates that the transcript levels of genes regulating the JA biosynthesis pathway also should change in abundance (Wu and Bradford, 2003). One of the primary effects of JA accumulation and ethylene emission is to alter the expression of various defense genes (Jones et al., 1995). Previous research illustrated that in some plants, insect feeding signals increased transcript levels of defense genes, including glucanase, peroxidase chalcone synthase, ripening-related genes, ethylene biosynthesis genes (Botella et al., 1996; Ecker and Davis, 1987; Cervantes et al., 1994). This study suggested that in Mp708, insect feeding on leaf tissues induces transcript levels of these JA-related defense genes, including: *mir1*, *mir2*, *mir3*, *MPI, lox3, lox6, RIP2, Chi1, PR10,* in aboveground and belowground plant tissues.

4.2.3 *Mir* Genes Expression in Plant Development

This study also investigated that as maize grows, whether defense gene expression changes throughout maize's lifespan. In response to the FAW insect feeding, the average gene transcript levels increased significantly compared to the average genes transcript levels in non-fed maize plant. In some particular growth stages, transcripts level for *mir1*, *mir2, mir3* and other genes increased up to 1000 times in both leaf and root tissues of FAW larvae fed plants compared to control plants. Different growth stages exhibited different levels of gene induction. Defense genes transcript levels were much higher at some particular vegetative stages, suggesting that expression of these genes could be developmentally regulated.

For *mir1*, the greatest of induction occurred at the V5 stage in the leaf, but it was at the V11 stage in the roots. The average gene transcript levels up-regulated by foliar herbivory

aboveground was higher than that belowground. Different growth stages had different gene induction pattern in aboveground and belowground part of maize plant.

For *mir2* and *mir3*, the expression pattern during development differed from that of *mir1*. The average expression level for FAW damaged foliage leaf tissues was much higher than transcript level of root tissues. *Mir2* and *mir3* expression in response to larvae feeding reached to the highest levels in V7 and V9 plant leaf tissues. In root, gene expression showed highest peaks at the V1 young seedling stage. Transcript induction in aboveground was much higher than the level in belowground. In root tissues, *mir2* and *mir3* gene expression was constantly lower in the maize plant attacked by FAW.

4.2.4 Other Genes Expression Variation in Plant Development

Defense gene expression resulted in different patterns for leaves and roots for *lox3, lox6, RIP2, Chi1, MPI, PR10, SSU1*.

Lox3 and *lox6* expression in response to larvae feeding reached to the highest level in V9 leaf tissues. In roots, gene expression was highest at V3 young seedling stage. *Lox* expression increased during germination and the active vegetative growth stages (Feussner and Wasternack, 2002), and expression of *lox* was induced to high levels during seed development and middle of vegetative stages in maize (Santino et al., 2005).

SSU1 gene was only expressed in the leaf and it is known to be induced by insect infection. *SSU1*expression increases after FAW leaf herbivory. No significant expression of *SSU1* in root tissues of larval fed and control plants provided a good control for other genes which were expressed in roots.

For all defense genes, different growth stage exhibited different level of gene induction in the aboveground and belowground of insect-resistant inbred maize plant. On average, the insect feeding regulated defense gene expression to a higher level in both leaf and root tissues at some particular growth stages of maize.

As plants develop from seeds to seedlings, juveniles and mature stages, their developments affects resistance to herbivore damage. Ecological and evolutionary theories about interactions between plants and herbivores are largely based on observations and experiments conducted at a single growing stage (Bonhomme et al., 1984). Owing to resource allocation and architectural constraints in plants, and the influence of herbivore foraging behavior, resistance to herbivores is likely to change during plant development (Boege et al., 2005), which is probably why there were different levels of defense gene induction in whorls and roots during maize development. All defense mechanisms are likely to vary with plant development progress, given the differences in physiological constraints and in the selective pressure of herbivores during plant development. Plant development changes the defense to biotic or abiotic stress including insect foliar herbivory (Weiner et al., 2004). With increasing plant age, the carbon–nutrient balance, storage capacity, and shoot: root ratio also increases, whereas growth rates and metabolic activity decrease (Weiner et al., 2004). The functional priorities of growth, resistance, and reproduction also change as plants develop.

4.3 Summary

Plants respond to herbivory in a complex environment. The spatial and temporal dynamics of aboveground and belowground herbivores, plant pathogens, and plant responses, can differ in space and time. The variations depend on different temperature, humidity, nutrition, and .etc.

Combination of both aboveground and belowground compartments in studies of insect-resistant throughout the life cycle of plants will improve our understanding of ecology and evolution in the plant world. Interpretation of gene expression level changes in response to larvae feeding at different growth stages of maize help us understand whole scale of plant insect defense mechanisms.

In summary, whether aboveground herbivory up-regulates the abundance defense related genes transcripts in aboveground and belowground tissues of Mp708 was examined, from corresponding result, the model of aboveground and belowground communication has been proposed. Also, the expression pattern of defense genes and their transcript levels changes in maize development stages have been investigated. The mechanism and reasons underlying these phenomena has been interpreted in this report. This study is important towards investigating mechanism of insect and crop plant interactions that may help to find out a cost-effective, environmentally safe solution for controlling herbivore damage in crops.

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Appendix

Expected Nucleotide Sequence Amplification of Defense Genes

Mir1 amplification cDNA nucleotide sequence (123bp):

AACGCGATCGCGACGGGTAACCTGGTGTCGCTGTCGGAGCAGGAGATCATCG
ACTGCGACGCCCAGGACAGCGGCTGCGACGGCGGGCAGATGGAGAACGCGTTCCGG
TTCGTCATCGGCAAC

Mir2 amplification cDNA nucleotide sequence (128bp):

ATCAACAAGATCGTGACAGGCAGCCTCATCTCGCTGTCGGAGCAGGAGCTTA
TCGACTGCGATAAGTTCCAGGACCAGGGCTGCGATGGCGGCTTAATGGACAATGCTT
TCGTGTTCATGATCAAGAA

Mir3 amplification cDNA nucleotide sequence (110bp):

CAACAATAGCAGCTGTGGAAGGCATCAACCAGATTGTTACAGGTGACTTGAT
CTCCTTGTCTGAGCAAGAGCTTGTCGACTGTGACACTTCATACAATCAGGGGTGCAA
T

Lox3 amplification cDNA nucleotide sequence (134bp):

AAAATACCAGCCCTCGAGGACCTGCGGAAGCAGTTCCCACTCGAGCTCGTCA
AGGATGTCCTCCCGGTCGGCGGCGACTACCTCCTCAAGCTCCCCATGCCGCAGATCA
TCAAAGAGGACAAGACAGGTTGGAT

Lox6 amplification cDNA nucleotide sequence (98bp):

TGTACGACTACGCGCTGTACAACGACCTGGGGAACCCAGACCTGCGCCAGGA
CCTGGCGCGCCCCGTGCTGGGAGGATCCCAGGAGTACCCGTACCCTCGGCGTACCA
AGACCGGCCGACCAGCCGCCAAAACAGATC

RIP2 amplification cDNA nucleotide sequence (111bp):

CGAAATCTTCCCCGTGGAGGACACGGCCTACCCTTACAGCGCCTTCATCACCTCC GTCCGGAAAGACGTGATCAAATACTGCACCAACCATACAGGCATCGTCCAGCCCGT

MPI amplification cDNA nucleotide sequence (98bp):

GACGCCAAGAAGGTGATCCTCAAGGACAAGCCGGACGCCGACATCGTGGTG CTGCCCGTCGGCTCCGTGGTGACCGCGGATTATCGCCCTAACCGTGT

Chi1 amplification cDNA nucleotide sequence (107bp):

TTCAACTTCATCCTCGCCTTCGCCATGGACTACACGCCGGTGAACCAGCATCC CACGCCGGCGCCCACCAACGGCGTGTTCAGCCCGTTCTGGGACACGGGGAACCT

PR10 amplification cDNA nucleotide sequence (109bp):

TTCAAAAACTTCCCCGGTCACACAATGCAAACTCGTAACATGTCACTGCTCCT CCTGCGTGTGTGCAGCCATGCCGTTCGGCTTCGTGAAGGAGAGGCTCGAGTTCCTG

SSU1 amplification cDNA nucleotide sequence (137bp):

TTCAGCAAGGTCGGCTTCGTGTACCGCGAGAACTCCACCTCCCCGTGCTACTACG ACGGCCGCTACTGGACCATGTGGAAGCTGCCCATGTTCGGCTGCAA