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# Maize toxin degrades peritrophic matrix proteins and stimulates compensatory transcriptome responses in fall armyworm midgut

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ABSTRACT

Understanding the molecular mechanisms underlying insect compensatory responses to plant defenses could lead to improved plant resistance to herbivores. The Mp708 inbred line of maize produces the maize insect resistant 1-cysteine protease (Mir1-CP) toxin. Reduced feeding and growth of fall armyworm larvae fed on Mp708 was previously linked to impairment of nutrient utilization and degradation of the midgut (MG) peritrophic matrix (PM) by Mir1-CP. Here we examine the biochemical and transcriptional responses of fall armyworm larvae to Mir1-CP. Insect Intestinal Mucin (IIM) was severely depleted from pure PMs treated *in vitro* with recombinant Mir1-CP. Larvae fed on Mp708 midwhorls excrete frass largely depleted of IIM. Cracks, fissures and increased porosity previously observed in the PM of larvae fed on Mp708 midwhorls could ensue when Mir1-CP degrades the IIM that cross-links chitin fibrils in the PM. Both targeted and global transcriptome analyses were performed to determine how complete dissolution of the structure and function of the PM is prevented, enabling larvae to continue growing in the presence of Mir1-CP. The MGs from fall armyworm fed on Mp708 upregulate expression of genes encoding proteins involved in PM production as an apparent compensation to replace the disrupted PM structure and restore appropriate counter-current MG gradients. Also, several families of digestive enzymes (endopeptidases, aminopeptidases, lipases, amylase) were more highly expressed in MGs from larvae fed on Mp708 than MGs from larvae fed on diets lacking Mir1-CP (artificial diet, midwhorls from Tx601 or B73 maize). Impaired growth of larvae fed on Mp708 probably results from metabolic costs associated with higher production of PM constituents and digestive enzymes in a compensatory attempt to maintain MG function.



## 1. Introduction

Fall armyworm, *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae) is a devastating pest throughout much of the western hemisphere. This insect thrives on a wide range of plants including the important crops, maize (*Zea mays* L.) and rice (*Oryza sativa* L.). Unfortunately, methods for controlling this herbivore are limited. Although chemical insecticides can be an effective control, they are

*Abbreviations:* ARH, arylphorin; BLAST, basic local alignment search tool; BLASTn, Nucleotide–nucleotide BLAST; BLASTp, Protein–protein BLAST; BLASTx, Nucleotide 6-frame translation-protein BLAST; tBLASTx, Nucleotide 6-frame translation-nucleotide 6-frame translation BLAST; CAD, cadherin; CP, carboxypeptidases; CDA, chitin deacytlase; CTR, chymotrypsin; CHTG, Chymotrypsinogen; CYP, cytochrome P450; $C_T$, cycle threshold; cDNA, copy deoxyribonucleic acid; DAVID, Database for Annotation, Visualization and Integrated Discovery; FBgn, FlyBase gene number; IIM, insect intestinal mucin; JA, jasmonic acid; 2-D LC-MS/MS, two dimensional liquid chromatography tandem mass spectrometry; LYSO, lysozyme; MA plots, $\log_2$-ratio of two expression levels/intensities versus the mean $\log_2$-expression of the same two expression levels/intensities; Mir1-CP, maize insect resistant 1 cysteine protease; MG, midgut; NAC, no amplification control; NTC, no template control; PM, peritrophic matrix; PRTS, 30 k protease; *Q*-values, adjusted probability-values found using an optimized false discovery rate approach; qRT-PCR, quantitative reverse-transcription polymerase chain reaction; QTLs, quantitative trait loci; RNA-seq, high throughput sequencing of RNA after conversion to cDNA; TMM, trimmed mean of M values; TYRP, trypsin.

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expensive and not environmentally friendly, and the fall armyworm has developed resistance to many of these (Yu, 1992; Yu et al., 2003; Virla et al., 2008). Transgenic maize cultivars that express a Bt-toxin are less effective against fall armyworm than other lepidopteran herbivores (Luttrell et al., 1999; Buntin et al., 2000) and field resistance to this technology was recently observed in Puerto Rico (Storer et al., 2010). Furthermore, there are concerns about public acceptance and the high cost of transgenic crops that often precludes their use by farmers in developing countries. To extend the repertoire of potential control strategies for this herbivore, we are studying the mechanism of native host plant resistance in the maize inbred line Mp708 developed by traditional plant breeding (Williams et al., 1990). This resistance thwarts feeding by fall armyworm and numerous other lepidopteran pests (Davis et al., 1988). When fall armyworm larvae feed on whorl tissue from Mp708, their growth is retarded by approximately 50% compared to susceptible maize inbreds, and the time required to reach pupation increased by several days (Chang et al., 2000). Analysis of nutritional indices from larvae fed on Mp708 indicated that their nutrient utilization was severely impaired (Chang et al., 2000).

Research to understand the Mp708 mechanism slowing larval growth revealed that resistance is a multigene trait regulated by several quantitative trait loci (QTLs) (Brooks et al., 2005, 2007). Among these traits, Mp708 has at least two unique characteristics: 1] the plants are "genetically primed" to withstand larval feeding due to constitutively elevated levels of jasmonic acid (JA) and herbivore defense gene transcripts (Shivaji et al., 2010); and 2] there is an exceedingly rapid accumulation of the maize insect resistant 1-cysteine protease (Mir1-CP) in the whorl within 1 h of larval attack (Pechan et al., 2000). This protease is a highly potent insecticidal protein that attacks the fall armyworm peritrophic matrix (PM) (Pechan et al., 2002; Mohan et al., 2006), a semipermeable protective structure that lines the larval midgut (MG) and surrounds the food bolus preventing food particles from directly contacting epithelial cells and MG-microvilli (Wang and Granados, 2001). The proteolytic activity of Mir1-CP permeabilizes the PM (Mohan et al., 2006), which ultimately impairs the insect's ability to utilize nutrients from its diet (Chang et al., 2000). Although it is known that Mir1-CP attacks PM proteins, we do not know if all PM proteins are equally vulnerable to proteolysis by Mir1-CP, or if specific PM proteins are targeted. For example, it has been shown that the PM structural protein, Insect Intestinal Mucin (IIM), is degraded by the metalloprotease, enhancin, produced by the *Trichoplusia ni* granulovirus during its infection of *T. ni* (Hübner) larvae (Lepidoptera: Noctuidae) (Wang and Granados, 1997a).

With exception to the order Hemiptera, all other representatives of the class Insecta contain a PM during all or most of their life stages. The PM facilitates the digestive processes in the MG, maintains its structural integrity and protects the insect from infection by microorganisms and parasites (Hegedus et al., 2009). It is mainly composed of chitin, glycosaminoglycans and proteins (Wang and Granados, 2001; Hegedus et al., 2009; Toprak et al., 2010a). The proteins can be divided into nonstructural and structural proteins collectively called peritrophins. Nonstructural proteins in the PM include chitin-modifying enzymes (e.g., chitin deacetylase, chitinase) and several families of digestive enzymes (e.g., lipases, serine proteases, exopeptidases, endopeptidases) (Toprak et al., 2010a). The composition of the PM, however, depends on the insect species. For example, the major proteins found in the PM of *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) were chitin deactylase-like and mucin-like (Campbell et al., 2008). In the fall armyworm, anterior MG columnar cells secrete a peritrophin (Bolognesi et al., 2001). Peritrophins are referred to as IIMs when they contain both chitin binding and mucin domains (Hegedus et al., 2009; Toprak et al., 2010a) with disagreement in the number of mucin domains required to constitute an IIM.

Besides its role in digestion, the PM plays an important role in detoxification of plant toxins consumed by the insect (Hegedus et al., 2009; Hakim et al., 2010; Toprak et al., 2010a). Thus, disrupting the PM, or making it more permeable, induces behavioral and physiological changes in caterpillars, such as cessation of feeding, reduced growth and increased susceptibility to toxins and pathogens (Wang and Granados, 2001; Guo et al., 2007; Hegedus et al., 2009). Although knowledge of the defensive mechanisms deployed by maize in response to feeding by fall armyworm and other caterpillars is increasing (Lopez et al., 2007; Mohan et al., 2008; Shivaji et al., 2010; Gill et al., 2011; Smith et al., 2012), little is known about responses to maize toxins by fall armyworm larvae. In particular, we sought to illuminate changes in gene expression that occur in the larval MG tissue during the larval counter defense response. A better understanding of PM constituents that protect the MG from damage will provide insights into potential new targets for pest control because plant defenses often target PM structural components such as chitin and proteins. Hence, we performed biochemical experiments to determine the PM protein targets of Mir1-CP and conducted both targeted and global transcriptome analysis of MGs from fall armyworm larvae that fed on either resistant or susceptible maize genotypes.

## 2. Materials and methods

### 2.1. Plant and insect material

Seed for the maize (*Z. mays* L.) inbreds, Mp708, Tx601 and B73, were supplied by the U.S. Department of Agriculture (USDA), Agriculture Research Service (ARS), Corn Host Plant Resistance Research Unit at Mississippi State University (Mississippi State, MS). In the experiments reported, Mp708 developed by Williams and colleagues (Scott et al., 1982; Williams and Davis, 1982,1984; Williams et al., 1990; Williams and Davis, 2000,2002) was used as the insect resistant inbred line. This inbred was selected from a cross between Tx601 and the resistant inbred Mp704 (Williams et al., 1990). The B73 and Tx601 inbred lines are susceptible to aboveground damage by several lepidopteran pests while the Mp708 inbred is resistant to these insects and accumulates Mir1-CP in response to caterpillar feeding (Williams et al., 1990; Pechan et al., 2000; Lopez et al., 2007). The whorls of Tx601 and B73 do not accumulate Mir1-CP (Pechan et al., 2002) and both are highly susceptible to fall armyworm herbivory. Seeds (one to two per pot) were planted in Hagerstown loam in 2.8 l or 5 l pots. Plants were grown under conditions simulating Pennsylvania summer conditions in a greenhouse. Plants were harvested at approximately the V8 stage (Ritchie et al., 1992).

The USDA-ARS Laboratory at Mississippi State University also supplied fall armyworm eggs that hatched into larvae used in experiments measuring IIM degradation, MG transcripts by quantitative reverse-transcription polymerase chain reaction (qRT-PCR) and RNA-seq. Larvae used in the PM and qRT-PCR experiments were reared on a casein-based artificial diet until the second instar when they were placed in 30 ml clear plastic diet cups lined on the bottom with 1% agar and containing excised yellow-green midwhorl foliage. Old foliage and frass were removed every other day and replaced with fresh foliage. Larvae were reared in an environmental chamber at 27 °C, 14:10 (light:dark) photoperiod, and 70% relative humidity. In the qRT-PCR experiment, MGs were dissected from larvae in the penultimate instar.

Beginning as neonates, larvae used in the RNA-seq experiment were reared on yellow-green midwhorl foliage as described above. Midguts were dissected from larvae 2 d after molting to the last