Maize Endochitinase Expression in Response to Fall Armyworm Herbivory

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Abstract

A large percentage of crop loss is due to insect damage yearly, especially caterpillar damage. Plant chitinases are considered excellent candidates to combat these insects since they can catalyze chitin degradation in peritrophic matrix (PM), an important protective structure in caterpillar midgut. Compared to chemical insecticides, chitinases could improve host plant resistance and be both economically and environmentally advantageous. The focus of this research was to find chitinase candidates that could improve plant resistance by effectively limiting caterpillar damage. Five classes of endochitinase (I-V) genes were characterized in the maize genome, and we further isolated and cloned four chitinase genes (chitinase A, chitinase B, chitinase I, and PRm3) present in two maize (*Zea mays* L.) inbred lines Mp708 and Tx601, with different levels of resistance to caterpillar pests. Further, we investigated the role of these maize chitinases in response to fall armyworm (*Spodoptera frugiperda*, FAW) attacks. Results from gene expression and enzyme assays from maize leaves indicated that both chitinase transcript abundance and enzymatic activity increased in response to FAW feeding and mechanical wounding. Furthermore, chitinase retained activity inside the caterpillar's midgut since specific activity was detected in both the food bolus and frass. When examined under scanning electron microscopy, PMs from Tx601-fed caterpillars showed structural damage when compared to diet controls. Analysis of chitinase transcript abundance after caterpillar feeding and proteomic analysis of maize leaf trichomes in the two inbreds suggested that the chitinase PRm3 in Tx601 has potential insecticidal properties.

Introduction

Herbivorous insects are one major cause of crop losses worldwide. Maize (*Zea mays* L.), as one of the most important crops, suffers great damage from a wide range of insect pests especially in tropical regions (McMullen et al. 2009), and fall armyworm (*Spodoptera frugiperda*, FAW) is one of them. To control this devastating pest, current pest control methods largely rely on chemical insecticides or genetically modified crops (GMOs) (Aranda et al. 1996; Bohorova et al. 2001; Bokonon-Ganta et al. 2003; Huang 2020). However, the major problem associated with the above methods, in addition to environmental health safety risks and public acceptance of GMOs, is that FAW has quickly developed resistance over the years (Bernardi et al. 2015; Flagel et al. 2018; Gutierrez-Moreno et al. 2019; Horikoshi et al. 2016; Storer et al. 2010; Storer et al. 2012; Virla et al. 2008; Zhu et al. 2015). Sustainable strategies for insect control are urgently needed since they are target-specific and environmentally friendly. One approach is enhancing resistance by developing more pest-resistant plants (Corrado et al. 2008). For this purpose, more attention should be given to the identification and characterization of endogenous genes that can increase maize resistance against herbivore pests.

Chitinases are enzymes that catalyze the degradation of chitin. Chitin, a linear polymer of β-1,4-N-acetyl-D-glucosamine (GlcNAc) residues, is one of the most abundant polysaccharides in nature and a structural component of many organisms. Because chitin is often localized in specific parts of organisms such as fungal cell walls and the exoskeleton and gut linings of insects, it is an excellent target for pest control management (Kramer and Muthukrishnan 1997). Chitinases are produced by a large number of organisms regardless if they contain chitin or not, including bacteria, fungi, plants, invertebrates, and vertebrates (Tronsmo and Harman 1993). Chitinases from different organisms have different biological functions (Table S1). In bacteria, chitinases provide nutrients by degrading chitin; in fungi, chitinases are used for cell growth and division; in insects, chitinases are used when they molt their exoskeleton and when they form the peritrophic matrix (PM). Unlike others, there is no natural substrate for chitinase in plants, however, plant chitinolytic enzymes play an important role in defense against pathogens and herbivorous pests (Flach et al. 1992; Gooday 1999; Hamid et al. 2013; Kramer and Muthukrishnan 1997; Lawrence and Novak 2006; Sharma et al. 2011). Reports of plants using chitinases to fight against fungal pathogens are well documented, some have direct antifungal activity because fungal chitin is a key structural component for their hyphal walls (Graham and Sticklen 1994; Peethambaran et al. 2010; van Loon et al. 2006), and some indirectly affect defenses by releasing oligomers from fungal chitin that act as elicitors for pathogen-responsive defense pathway (Grover 2012). In short, chitinases are involved in many plant growth and development processes and are induced under various biotic/abiotic stresses (Grover 2012).

Classifications of chitinases are based on protein sequences, domain compositions, and catalytic properties. Glycoside hydrolases (GH) families 18 and 19 are regarded as endochitinases (EC 3.2.1.14) which cleave the chitin chain randomly (Henrissat 1999; Kesari et al. 2015; Shoresh and Harman 2008). While GH family 20, which cleaves GlcNAc residues from the non-reducing end of the chitin molecule, are considered as exochitinases (β-hexosaminidase, EC 3.2.1.52) (Shoresh and Harman 2008). GH19 chitinases, with an α-helix rich lysozyme-like catalytic domain (Oyeleye and Normi 2018), carry out the hydrolysis of the β-1,4-glycosidic linkage utilizing an inverting mechanism; while GH18 chitinases, which contain triosephosphate isomerase (TIM) barrel $(\beta/\alpha)_8$ domains in their catalytic regions(Hamid et al. 2013; Oyeleye and Normi 2018), cut the chitin polymer through a retaining mechanism (Iseli et al. 1996). Chitinases can be further subdivided into five major classes (class I, II, III, IV, and V) taking into account their structural and functional characteristics (Beintema 1994; Hamel et al. 1997). Members from class I, II, and IV contain globular domains (Hamid et al. 2013), which are of plant origin and belong to GH19 family. Whereas, members from class III and V belong to GH18 family. Class III chitinases are typical of plant and fungal origin, while class V chitinases are typical of bacterial origin. It is worth noticing that GH18 and 19 chitinases share no sequence similarity and differ in catalytic mechanisms, thus resulting in different activity and substrate specificities. GH19 chitinases have been well documented to have more antifungal properties than GH18 chitinases (Oyeleye and Normi 2018). There is limited information regarding the role of plant chitinases in response to insect pests. In one case, transgenic tomato expressing poplar chitinase *WIN6*, a class 19 chitinase, retarded the development of the Colorado potato beetle (Lawrence and Novak 2006). However, several studies have shown that chitinases from GH18 family can enhance the plant resistance against herbivory pests by targeting the degradation of insect peritrophic matrix (PM) (Corrado et al. 2008; Kramer and Muthukrishnan 1997). The PM is a semipermeable lining in the intestine of many invertebrates that is primarily composed of chitin and glycoproteins (Hegedus et al. 2009). PMs are normally produced during feeding and physically protect the midgut epithelium against food abrasion, digestive enzymes, toxins, and microorganisms (Hegedus et al. 2009; Lehane 1997; Mason et al. 2019). Since the insect PM is continuously exposed to ingested plant materials, compounds having the potential to damage PM would impair normal digestive processes and biological functions (Hegedus et al. 2009; Zhu-Salzman et al. 2008).

In maize, four plant endochitinase genes have been found previously: chitinase A (Chaudet et al. 2014; Huynh et al. 1992; Peethambaran et al. 2010; Ray et al. 2016), chitinase B (Huynh et al. 1992), chitinase I (Hawkins et al. 2015; Peethambaran et al. 2010; Wu et al. 1994), and chitinase PRm3 (Didierjean et al. 1996; Peethambaran et al. 2010). Chitinase A and chitinase B are from class IV chitinases, and chitinase I is an acidic member of the class I chitinase, which all belong to GH19. However, PRm3 is a member of class III chitinases and belongs to GH18. Previous reports showed that expression of *ChiA*, *ChiI*, and *PRm3* was induced in response to abiotic or biotic stresses (Didierjean et al. 1996; Pechanova et al. 2011; Peethambaran et al. 2010; Tiffin 2004). Proteomic analysis of frass collected from FAW larvae that fed on different maize inbreds (Mp708, Tx601, and B73) indicated that maize chitinase peptides were present in the frass (Chuang et al. 2014a; Ray et al. 2016). This is noteworthy because it has been shown that proteins involved in plant defense against herbivores are not completely degraded in the insect gut and can be detected in the frass whereas non-defensive proteins such as Rubisco are completely digested (Chen et al. 2007; Chen et al. 2005). Plants that accumulate high levels of chitinases in response to insect feeding could interrupt nutrient uptake in the insect by disrupting the PM and thereby enhance plant resistance to the herbivore. However, there is little research definitively documenting that plant chitinases have a detrimental effect on lepidopteran larvae or their PMs.

The overall objective of our study was to determine if maize chitinases play a role against caterpillar herbivores in plant defense. This study focused on the endochitinases that cleave polymers internally and hence may be instrumental in damaging the insect PM. Four maize endochitinases detected from a previous proteomic study (Chuang et al. 2014a; Ray et al. 2016) were cloned and sequenced from two maize inbred lines-Mp708 and Tx601 that differ in native host plant resistance to FAW. These included chitinase A (ChiA), chitinase B (ChiB), chitinase I (ChiI), and PRm3. The first goal was to determine if chitinase expression and activity were induced in maize leaves in response to herbivore feeding and mechanical wounding and if maize chitinase could survive digestion and retain activity inside the FAW midguts. The second goal was to determine if the PM of plant-fed FAW showed signs of damage during this process. While both maize inbreds tested in this study mount inducible defenses against FAW feeding, such as rapid accumulation of transcripts for defensive proteins (Shivaji et al. 2010), Mp708 mounts a more rapid and robust response than Tx601. So the third goal was to determine if chitinase expression/activity profile was differently regulated in the two inbreds responding to herbivore feeding or mechanical wounding.

Materials And Methods

Plant Cultivation and Sample Collection. Seeds for maize inbreds, Mp708 and Tx601, and the hybrid Mp704 x Mp708 were obtained from W. P. Williams (USDA-ARS Corn Host Plant Resistance Research Unit) at Mississippi State University (Mississippi State, MS). These inbred lines show differential resistance to FAW feeding. Resistant inbred Mp708 was developed for resistance to fall armyworm and other leaf-feeding caterpillars using traditional breeding strategies (Williams et al. 1990). Tx601, which was used as one of the breeding parents for developing Mp708, is relatively susceptible to FAW feeding when compared to Mp708. Mp708 was selected from a cross between Tx601 and the other resistant inbred Mp704 (Williams et al. 1990). The Mp704 x Mp708 hybrid also is resistant. Seeds were sown in 18 L pots filled with topsoil (Hagerstown Loam). Plants were grown in the Plant Science greenhouse at The Pennsylvania State University (University Park, PA), the average temperature was maintained at 28°C under a 16 hr photoperiod. Plants were harvested around the V8 to V9 (Ritchie et al. 1984) leaf stage. Following FAW infestations (see below) tissue adjacent to the feeding sites was collected, immediately frozen in liquid nitrogen, and stored at -80°C. Samples from undamaged control plants were collected at the same time. For wounding experiments, plants were wounded with a specially designed wounding tool (Bosak 2011).

Insect and Sample Collection. FAW eggs were also obtained from USDA-ARS Corn Host Plant Resistance Research Laboratory at Mississippi State University. After they hatched, larvae were reared on an artificial diet (Peiffer and Felton 2005) at 27°C with a 16 hr photoperiod. Larvae used in the experiments measuring chitinase expression and activity were reared until the fifth-instar, then three to five larvae were starved for 1 hr and carefully placed in the maize whorls. After 24 hr of infestation, larvae were retrieved, immobilized on ice, and dissected. The food bolus and PM in the midguts were removed, frozen in liquid nitrogen, and stored at -80°C. Frass collection was performed as described by Chuang et al (2014a). Larvae used for scanning electron microscopy (SEM) experiments were reared on the artificial diet until the early second-instar, then starved for 1 hr and three larvae per plant were transferred to the whorl region of Tx601. Three days after transfer to plants, larvae were collected, weighed, immobilized on ice, dissected and processed for the microscopic procedures. Relative growth rate (RGR) was calculated using the formula RGR=(W2-W1)/((W2 + W1)/2xdays) g g-1 d-1 (Hoffmann and Poorter 2002; Mohan et al. 2008).

cDNA Cloning and Sequencing. Four chitinase genes (chitinase A, chitinase B, chitinase I, and PRm3) were cloned from cDNA of two maize inbreds Mp708 and Tx601. Genes of interest were amplified with *Taq* polymerase (New England Biolabs, Beverly, MA) using gene-specific primers. Products from PCR amplification, approximately 1 kb in size, were gel-purified and ligated into the pGEM®-T Vectors (cat. No. A1360, Promega). After transformation into competent *Escherichia Coli* TOP10 cells (Invitrogen), white colonies were picked, and followed by sequencing using vector-specific primers T7 (GTAATACGACTCACTATAGGG) and SP6 (GCTATTTAGGTGACACTATAG) on an Applied Biosystems 3100 DNA sequencer. The DNA sequences of the chitinase genes were then analyzed using the SeqMan from DNASTAR. All primers used in this study are listed in Table S6.

Chitinase Sequences from Maize Genome Database. To identify candidate chitinase (EC 3.2.1.14) genes in maize (B73), maizeGDB (Schaeffer et al. 2011), maize Gramene database (Monaco et al. 2014), Pfam (Finn et al. 2014), and Cazy (Lombard et al. 2014) databases were used to search homologous sequences containing conserved catalytic domains (GH18 and GH19). After manually eliminating the redundant sequences, the retrieved sequences were confirmed by InterPro (Hunter et al. 2012). A literature search was also performed in Pubmed. Nucleotide sequences of maize chitinase genes were obtained from the maizeGDB (Table S2), other chitinase genes were obtained from GenBank. Tandem duplication of chitinase genes was defined as adjacent homologous genes on the same chromosome, but with one or no intervening gene in maize chromosomes (Zhang et al. 2012).

Classification of Chitinases. Deduced amino acid sequences were used for the classification of maize chitinases based on their structural features (Hamel et al. 1997). As described in Hamel et al (1997), Class I chitinases encoded the signal peptide (SP), chitin-binding domain (CBD), a catalytic domain, and a carboxy-terminal extension (CTE). Some class I chitinases lacked the CTE. Class II chitinases lacked the coding sequences for both CBD and CTE. Class IV

chitinases contained the CBD but CTE was absent, they also shared several deletions in the coding sequences of catalytic domains comparing with class I catalytic domains. Class III and V chitinases encoded an SP and a catalytic domain, lacking the CBD, and shared no similarity with other chitinase classes.

Phylogenetic Tree. Phylogenetic analysis was conducted using MEGA version 6 (Hall 2013; Tamura et al. 2013). The multiple maize chitinase amino acid sequences were aligned by MUSCLE. The resulting alignment was used for phylogenetic analysis. The best substitution model (WAG + G) was selected for Maximum Likelihood (ML) inference. ML method was then used for phylogenetic tree construction, with 1000 bootstrap replicates.

RNA Extraction and cDNA Synthesis. Total RNA from leaf samples was extracted using TRIzol Reagent (Invitrogen) and re-suspended in DEPC (diethylpyrocarbonate) water. The RNA was then treated with DNase (Progema Corp., Madison, WI) following the standard protocol. Total RNA was quantified by absorbance at 260nm, and an aliquot of 1 µg of total RNA was reverse transcribed with ABI high capacity cDNA reverse transcription kit (Foster City, CA), 2.5 µM oligo-dT20 was used in the standard reaction.

Quantitative RT-PCR (qRT-PCR). Maize chitinase gene expression was measured using quantitative RT-PCR (qRT-PCR). Primer Express software (version 3, ABI) was used to design primers for qRT-PCR, using SYBR as a detection dye. All primers were tested in absolute quantification and confirmed that slopes were approximately − 3.3 and R^2 values were close to 1. qRT-PCR reactions, containing 10 µl of the reaction mix, were performed in an Applied Biosystems (ABI) 7500 Fast Real-Time PCR machine. All reactions were run in triplicates under the default conditions: 50°C for 2 min, 95°C for 10 min; 95°C for 15 s, and 40 cycles of 60°C for 1 min; 72°C for 10 min. At the end of each run, a dissociation curve was conducted using the manufacturer's default setting. Relative gene expression levels were analyzed by ABI 7500 Fast SDS Software (version 1.4), normalized using actin as the reference gene, and the fold-changes relative to the control sample were determined. All primers used in this study are listed in Table S7.

Protein Extraction and Chitinase assay. Mp708 and Tx601 plants were either mechanically wounded or infested with fifth-instar FAW larvae for 24 hr. FAW larvae that fed on maize leaves were collected and the food bolus was dissected from the midguts. Frass from FAW larvae was also collected. The samples were frozen in liquid nitrogen and ground to fine powder using Geno/Grinder 2000 (SPEX Certi-Prep, Metuchen, NJ). Then they were homogenized in extraction buffer (Bekesiova et al. 2008) and centrifuged at 13,000 rpm at 4°C for 20 min. The supernatants were transferred to 1.5 ml microcentrifuge tubes and the protein concentrations were measured using the modified Bradford method (Bradford 1976).

Chitinase activity from leaves, food bolus, and frass of insects was measured using chitin-azure (cat. No. C3020, Sigma) as a substrate. The reaction was carried out in 2 ml microcentrifuge tubes containing 1 mg of chitin-azure, 950 µl of 0.2 M sodium phosphate buffer (pH 7.2), and 50 µl of the enzyme extracts. The mixture was incubated at 37°C for 24 hr, and the samples were centrifuged for 10 min at 10,000 rpm. The absorbance of the supernatants was measured at 570 nm using a sample processed identically without enzyme as blank. One unit of chitinase activity was defined as the amount of enzyme that results in an increase of 0.01 in absorbance. Chitinase specific activity was calculated as units per milligram of total protein (U/mg).

Scanning Electron Microscopy (SEM). Damage to PMs from FAW larvae fed on Tx601 plants was examined by scanning electron microscopy (SEM). The midguts were dissected from cold anesthetized larvae. The caterpillar was pinned, cut longitudinally from the dorsal side; the midgut was gently teased open with fine forceps to expose the PM and food bolus, and the PMs containing food bolus were isolated. The samples were fixed in primary fixative (1.5 % formaldehyde and 2.5 % glutaraldehyde in 0.1 M phosphate buffer, pH 7.4) overnight at 4°C, then washed in the same buffer and postfixed in 1 % osmium tetroxide for 1 hr at room temperature. Following serial steps of ethanol dehydration, samples were critical point dried in a Baltec CPD-030 Critical Point Dryer (Techno Trade, Manchester, NH), mounted on aluminum stubs with double-sided carbon tape, and sputter-coated with gold-palladium in a Baltec SCD-050 Sputter Coater (Techno Trade, Manchester, NH). Detailed experimental steps for SEM were described in Plymale et al. (2008). The samples were then examined with JSM 5400 (JEOL, Peabody, MA) at an accelerating voltage of 20kV, and digital images were recorded using image archiving software (IMIX-PC v.10, Princeton, NJ). For SEM, four to six PMs per treatment were thoroughly examined at several magnifications. The same method for SEM was used for trichome number counting and length measurements (see below).

Trichome Number Counting and Length Measurement. Three to four V8-V9 stage Mp708 and Tx601 plant leaves were used for trichome measurements. A paper punch was used to cut two leaf disks the yellow-green portion of each leaf, a total of six to eight leaf disks from each inbred were collected, treated, and observed under SEM. Each leaf disk was measured twice at two different places. Then trichome density was calculated as the total number of trichomes per mm^2. Trichome length was measured using Sigma Scan Pro 5.0 software (Systat Software, Inc, San Jose, CA).

Trichome Isolation. Mp704 x Mp708 and Tx601 plants were grown in Baccto potting soil (Michigan Peat Company) at Mississippi State University. Plants at the V8 stage were used for trichome isolation. To remove the trichomes, all leaves that contained trichomes were first removed from the plant and rinsed with distilled water to minimize contamination in the samples. Then leaves were cut into small pieces and dipped into liquid nitrogen. Using a small paintbrush dipped in phosphate buffered saline (pH 7.4) containing 0.005% Triton X-100, 0.1% protease inhibitor cocktail (Sigma), and 0.5% polyvinylpyrrolidone, the trichomes were gently removed onto a Petri dish. The trichomes were collected in the dish and transferred into a microcentrifuge tube. The samples were weighed and stored at -80°C for further analysis.

Proteomic Analysis of Trichomes. The trichomes were first ground into a fine powder in liquid nitrogen using a mortar and pestle and the proteins were extracted using the phenol extraction method (Hurkman and Tanaka 1986; Pechanova et al. 2008). Proteins were quantified using the RC DC Protein Assay (BioRad) with bovine serum albumin as a standard. The resulting proteins were digested with sequencing-grade modified trypsin (Promega) (Donaldson et al. 2009).

Three technical replicates of each genotype were analyzed by (1-D) LC/MS/MS using the ProteomeX Workstation (ThermoFinnigan) as described in Pechanova et al. (2010). The raw mass spectrometry data were subjected to database searching with SEQUEST (Bioworks 3.1, ThermoElectron) to identify the proteins based on the tryptically digested peptides in the sample. The ProtIDer maize database was used (AgBase; http://agbase.msstate.edu). The

ProtIDer maize database was created by the translation of assembled (TCs) and singleton ESTs from the TIGR maize gene index. For each sequence, the protein in the UniProt Knowledge Base that gave the highest match using blastx (e-value of less than e-10) was used as the template for translation. The resulting database was supplemented with the non-redundant rice protein dataset from NCBI and also with mitochondrial proteins from Arabidopsis and chloroplast proteins from rice and maize. The SEQUEST report was then subjected to filtering using the PepSort program. Only proteins with tryptic peptides with a delta correlation (Cn) of greater than 0.08 and a correlation (Xcorr) greater than 1.9, 2.2, and 3.75 for charged states of + 1, +2, and + 3, respectively were kept. After using the PepSort program, the list of proteins was manually inspected, and only those proteins with at least one unique peptide were kept.

Statistical Analysis. For qRT-PCR data, values for relative expression were were Box-Cox transformed (LaLonde 2005; Osborne 2010) to increase homogeneity. A general linear model (PROC GLM) was used to assess the chitinase gene expression changes. Least significant difference (LSD) test was used to determine significant differences (α = 0.05) among treatments. The same method was used for chitinase activity data analysis, FAW growth analysis, and trichome analysis. All analyses were performed using SAS software version 9.3 (SAS Institute, Cary, NC).

Results

Cloning and Classification of Chitinases Genes from Maize Inbreds Mp708 and Tx601. To investigate the aforementioned maize chitinases from the inbred lines used in our lab, four cDNAs encoding maize chitinases A (GRMZM2G051943), chitinase B (GRMZM2G005633), chitinase I (GRMZM2G14561), and PRm3 (GRMZM2G453805) were cloned and sequenced from Mp708 and Tx601. Classifications and properties of these chitinase genes are shown in Table 1, such as chromosome location and amino acid numbers. The deduced amino acid sequences of cloned chitinases were then aligned with the corresponding sequences from the maize inbred B73 (Schaeffer et al. 2011; Schnable et al. 2009) and the conserved domains were pointed out in the alignments (Supplemental Fig. 1), such as signal peptide (SP), chitin-binding domain (CBD), and the catalytic domain. Although the maize inbreds tested in this study had different levels of resistance, chitinase sequences were almost identical: they shared an apparent 98%-100% sequence identity, at both nucleotide and amino acid levels (Supplemental Fig. 1). When comparing all cloned maize sequences, chitinases from the same family had a higher sequence identity. Class I chitinase (ChiI, GH19) shared > 58% nucleotide sequence identity and > 38% peptide sequence identity with class IV chitinases (ChiA and ChiB, GH19), however, class III chitinase (PRm3, GH18) only had > 40% and > 11% nucleotide and peptide sequence identity with class IV chitinases, respectively.

Table 1
Classification of endochitinases in maize

Name[a]	Family	Class	Bin No.[b]	Protein (aa)			Exon	Intron	SP[c]	CBD[d]	CATALYTIC[e]	Organ[f]	Stage[g]	QTLs[h]
				Mp708	Tx601	B73								
Chitinase I	19	I	6.01	321	321	261	1	0	y	cd06921	cd00325	l, h	S, R	
Chitinase A	19	IV	2.04	277	277	280	2	1	y	cd00035	cd00325	s	S	
Chitinase B	19	IV	10.04	282	282	281	2	1	y	cd00035	cd00325	s	S	FAW/SWCB
PRm3	18	III	3.08	294	295	295	1	0	y	n	cd02877	l	S	SWCB
[a] Nomenclature of chitinase genes from reference literature in PubMed														
[b] Chromosome number and bin location from maizeGDB														
[c] Signal peptide sequences predicted from SignalP: y, yes														
[d] Conserved chitin binding domain sequences predicted from GenBank: n, none														
[e] Conserved catalytic domain sequences predicted from GenBank														
[f] Organs with highest expression from maizeGBD: h, husk; l, leaf; s, seed														
[g] Developmental stage with highest expression from maizeGDB: S, seedling; R, reproductive														
[h] Possible insect resistance traits from two QTLs mapping studies (Brooks et al., 2007)														

The lengths of the predicted mature proteins from the cloned maize chitinases were 277 amino acids corresponding to a molecular mass of approximately 29 kDa for ChiA, 282 amino acids corresponding to a mass of 29 kDa for ChiB, 321 amino acids with a molecular mass of 33 kDa for ChiI, and 294/295 (Mp708/Tx601) amino acid with a molecular mass of 30 kDa for PRm3. ChiA and ChiB appeared to be the product of a recent duplication event (Tiffin 2004), and they shared > 92% nucleotide sequence identity and > 85% peptide sequence identity. Based on the predicted chromosomal bin number (Schaeffer et al. 2011) for each cloned gene, it was possible to determine if the chitinase gene mapped in regions associated with known QTL for resistance to FAW and

southwestern corn borer (SWCB) (Brooks et al. 2007; Brooks et al. 2005). For example, chitinase B was located in the SWCB and FAW QTL on chromosome 10, bin 0.04, PRm3 was in the SWCB QTL on chromosome 3, bin 0.08, and chitinase I was near two QTL (SWCB and FAW QTL) on chromosome 6, bin 0.01.

Genomewide Identification and Classification of Putative Maize Chitinase Genes. In addition to the chitinases listed above, we were also interested in identifying other potential chitinase genes in maize *in silico*. Homologous chitinase sequences were found in the maize genome through the extended searches described in Materials and Methods. After manual elimination of redundant and unspecific sequences, thirty-three sequences (Table S2) were identified as possible candidate endochitinase genes: eighteen from GH18 and fifteen from GH19 chitinase family. The candidate genes went through a literature search, and the descriptions used in this study followed those used in previous papers. Many annotated sequences from search results shared high percentages of homology in the catalytic region, however, they belonged to other families of chitinolytic enzymes (i.e. lysozyme, EC 3.2.1.17; endo-β-N-acetylglucosaminidase, EC 3.2.1.96) rather than endochitinases (EC 3.2.1.14) specifically, thus these sequences were excluded from our study. For this reason, although our result was in agreement with the previous study (Hawkins et al. 2015; Shoresh and Harman 2008), GRMZM2G312226 (chitinase 8-like), GRMZM2G412577 (chitinase 10-like), and GRMZM2G400497 (chitinase 10-like) were excluded here. We found several chitinase genes tandemly arranged or on the opposite strand of DNA in a nearly identical position, and they shared a high percentage of sequence similarity. Five such gene clusters were found in the maize genome: chitinase I cluster GRMZM2G145461 and GRMZM2G145518; endochitinase A cluster GRMZM2G051943, GRMZM2G051921, GRMZM2G052175 and GRMZM2G122708; Hevamine-A cluster GRMZM2G837822, GRMZM2G430936, GRMZM2G430942, and GRMZM2G023650; Xylanase inhibitor protein cluster GRMZM2G328171 and GRMZM2G162359; and chitinase 2 cluster GRMZM2G162505 and GRMZM2G057093. It was suggested this could be due to recent duplication events in maize genomes (Hawkins et al. 2015). Transposable elements (TE) were also identified to be widely present within or flanking these similar sequences when blasted against the Maize Transposable Elements Database (Wessler et al. 2009). According to the maize transcriptional analysis conducted by Sekhon et al. (2011), the highest chitinase expression levels were found in seeds, silks, or leaves. There was no clear correlation between sequence similarity and gene expression pattern. Likewise, seven loci were found in regions of FAW QTL and nine loci were found in regions of SWCB QTL (Table S2).

Phylogenetic analysis using deduced protein sequences of all putative chitinases from B73 and cloned sequences from Mp708 and Tx601 are presented in Fig. 1. All five classes of endochitinases were present in maize genome based on domain features, and each GH family comprised evolutionary-conserved individual classes as predicted previously, since chitinases from the same classes tended to cluster together and had similar splicing patterns. For instance, although there were a few exceptions, normally class I and III had one to two exons, class IV and class V had two to three exons, and class II typically had three exons, as shown in Table S2. Our results also showed a large evolutionary distance between GH18 and GH19 families, which suggested a subsequent diversification from an ancient origin. Besides, the distribution of chitinase classes was different for these two: GH18 family consisted mostly of class III chitinases, only two (GRMZM2G141456 and GRMZM2G090441) belonged to class V; while GH19 family had a relatively equal gene number from class I, II, and IV.

Chitinase Gene Expression and Enzymatic Activity were Induced by Mechanical Wounding and Insect Herbivory. Maize chitinase genes were reported to be expressed in many organs such as leaves, husks, roots, seeds, silks and tassels, and during different development stages including seedling, vegetative and reproductive stages (Table S2). However, far less is known about their expression in response to herbivory (Ray et al. 2016; White et al. 1997). In this study, we examined the expression of chitinase genes (*ChiA, ChiB, ChiI*, and *PRm3*) in Mp708 and Tx601 in response to mechanical wounding and FAW feeding. In general, qRT-PCR results from Fig. 2 showed that transcript abundance for all four chitinase genes increased dramatically in response to wounding and feeding in both maize inbreds, compared to undamaged controls. FAW infestation resulted in a slightly greater transcript accumulation than mechanical wounding, although the differences were not statistically significant, except for *ChiI*. Since FAW feeding process combines wounding with saliva deposition (Chuang et al. 2014a), our data suggested that saliva deposited was needed for maximum induction of *ChiI* gene expression (Fig. 2c).

When the gene expression level was compared between two inbreds, differences were observed even in controls. It appeared that Mp708 and Tx601 had different chitinase expression profiles. In Mp708, *ChiA* and *ChiI* had significantly higher basal expression levels than Tx601 (Fig. 2a and 2c). Plus, there was more *ChiB* gene expression in Mp708 than in Tx601(Fig. 2b) under both treatments, whereas *PRm3* transcripts accumulated to a significantly higher abundance in Tx601 (Fig. 2d). Interestingly, it was noted that the fold-change of *ChiB* and *ChiI* transcript induction were generally much greater (about 10-fold) than those of *ChiA* and *PRm3* (see y-axis scales in Fig. 2).

Because chitinase genes were differentially expressed in response to FAW infestation and wounding, we also measured chitinase activity in maize leaves under the same experimental conditions (Table S3). The data were reported as total chitinase specific activity because the enzymatic activity assay used here cannot discriminate among different types of chitinases. Unexpectedly, our results showed that activity significantly increased only in Tx601 in response to FAW feeding when comparing with undamaged control. Unlike the relatively large increase in chitinase transcript abundance in Mp708, it appears that there was only a marginal increase in total chitinase activity in FAW-fed Mp708 leaves, and it was not statistically greater than the control.

Chitinase Activity was Detected in Plant-fed FAW Midguts and Frass. Although we found both chitinase gene expression and activity were up-regulated in maize leaves upon insect damage, it remains debatable if these enzymes would retain activity inside the caterpillar since pH is typically alkaline in the midgut and plant chitinases have been shown to have little or no activity at high pH (Dow 1992; Pegg and Young 1982; Zhe-fu et al. 1992). Previously, Chen et al (2007; 2005) reported that several plant defense enzymes could retain enzymatic activity in the midgut. Another indirect clue supporting maize chitinases might maintain enzyme activity was the presence of chitinase peptides in FAW frass (Chuang et al. 2014a; Ray et al. 2016), this finding suggested chitinases could withstand insect's digestive process. To test this hypothesis, contents from food bolus and frass were collected from FAW larvae reared on Mp708, Tx601, or artificial diet, and chitinase specific activity was tested using chitin-azure method. Results in Fig. 3 clearly showed chitinase activity was present in food bolus and frass from plant-fed FAW larvae, and its value was dramatically higher compared to the enzymatic activity in both FAW-fed and un-fed maize leaves. This is probably due to the degradation of plant proteins as leaf materials move through FAW digestive system and thus resulting in higher specific activity. As expected, plant-fed larvae had significantly higher chitinase activity both in food bolus and frass compared to diet-fed caterpillars. The

basal activity detected in diet samples was possibly due to endochitinase in wheat germ that was used to make insect diet (Molano et al. 1979). When comparing between two genotypes, Tx601-fed FAW larvae again had higher chitinase specific activity in the food bolus than Mp708-fed ones, however, the value was not significantly different. However, specific activity in frass from Tx601-fed larvae was significantly higher than Mp708-fed larvae even though Tx601 is less resistant than Mp708. It is unlikely the chitinase activity in the food bolus was insect-derived because no activity was detected in the PM after removing all the plant debris (data not shown).

Tx601-fed FAW Larvae Shown Growth Retardation and PM Damage under SEM. In response to herbivore feeding, defense enzymes that degrade PM proteins and/or disrupt the chitin network could cause direct deleterious effects on the structural integrity of the PM (Corrado et al. 2008; Gongora et al. 2001; Gopalakrishnan et al. 1995; Huber et al. 1991; Martinez et al. 2012; Pechan et al. 2002). Since chitinase activity was detected in the FAW midgut, we further explored the effect of maize chitinases on the PM by examining PM structure using scanning electron microscopy (SEM) after FAW larvae fed on Tx601 plants or artificial diet for three days (Fig. 4). Tx601 was selected because it had higher chitinase activity in frass and food bolus than Mp708 and does not express the protease, Mir1-CP (Pechan et al. 2000), whose proteolytic activity could mask the effects of chitinases. Since past studies have shown that Mp708-fed FAW larvae had damaged PMs that were attributed to the accumulation of Mir1-CP, a cysteine protease that attacks the PM scaffold protein insect intestinal mucin (IIM) (Fescemyer et al. 2013; Mohan et al. 2006; Pechan et al. 2002). In this study, the ectoperitrophic layer (closest to the midgut microvilli) of the PMs was first examined at lower magnifications to visualize their typical morphology, then they were examined at higher magnifications to observe damaged areas. All PMs showed signs of mechanical cracks or tears that occurred during the preparation procedures (data not shown), which could be easily distinguished since the edges of the damaged areas were clean and showed no signs of degradation (Pechan et al. 2002). PMs from diet-fed larvae were used as control.

At lower magnification, SEMs of control PMs showed a wrinkled, unbroken surface, without any apparent disruptions (Fig. 4a). Even at higher magnification, PM surfaces looked continuous with no signs of damage (Fig. 4b). On the contrary, PMs dissected from Tx601-fed caterpillars showed more severe damage than control. They had noticeable roughened surfaces, as well as perforations and abrasions on the epithelial side (Fig. 4c). Areas of discontinuity were also found scattered along the examined surface. Damage varied from mild to severe depending on the regions of the PM examined. The scattered flakes of PM, one example of severe damage, were further examined at higher magnification and additional damage was visualized including holes, cracks, structural discontinuities, and abrasions (Fig. 4d). The discontinuous areas exhibited multiple layers of membranes with loosely aggregated spherical particles, shown in Fig. 4e and 4f, which could be disintegrated protein or lipid components of the PM formed during the degeneration process (Adang and Spence 1981).

Both diet- and Tx601-fed FAW larvae had fully filled PMs, therefore gut contents were readily observed in the food bolus. Plant materials from Tx601-fed PMs were of various sizes and shapes and most of the leaves were cut into roughly rectangular or square shapes approximately 200 x 500 µm in size (data not shown). Certain leaf structures, such as trichomes were still visible on the leaf fragments. In many cases, the prickle hair class of trichomes (Freeling and Lane 1994) were observed penetrating PMs (Fig. 4g and 4h), which had an average length of approximately 44 µm. The perforation could have occurred because PM was weakened by the induced plant chitinases, thus allowing the trichomes to pierce the PM more easily. Larvae weights were also measured before and after the feeding and relative growth rates were calculated for each treatment. Results from Table 2 showed that Tx601-fed larvae weight 67% less compared to diet-fed ones, and had a significantly slower growth rate as well. Although the mortality rate of FAW larva feeding on Tx601 plants was not as high as Mp708 (Chang et al. 2000), it did significantly delay the caterpillar growth when compared to diet-fed ones (Chuang et al. 2014b). Altogether our results showed that the induction of chitinases by insect feeding could contribute to the plant's resistance to herbivory since chitinases from maize leaves remained active in the insect midgut, which allowed them to continuously degrade chitin in the PM layers.

Table 2

FAW weight and RGR[a] after feeding on diet[b] or Tx601[c] plants for three days

	FAW		
	Initial/g	Final/g	RGR[c]
Diet[a]	0.0165 ± 0.0045	0.0855 ± 0.0229*	0.45*
Tx601[b]	0.0138 ± 0.0027	0.0276 ± 0.0064	0.22
[a] RGR=(W2-W1)/((W2 + W1)/2xdays) g g-1 d-1			
[b] twenty 2nd -3rd instar FAW caterpillar to start with, 17–20 caterpillars retrieved later, most of them were around 4th -5th instar			
[c] twenty 2nd -3rd instar FAW caterpillars to start with, 10–15 caterpillars retrieved later, most of them were around 3rd -4th instar			
* The data represented are mean values with error bars (+ SE, n = 20). Asterisks indicate significant differences (P < 0.05)			

Since trichomes were easily visible on leaf fragments in the food bolus, we also examined the structure and density of trichomes on maize leaves from the yellow-green portion of the whorl, which was the primary FAW feeding site (Pechan et al. 2000). Three types of trichomes, macrohair, prickle hair, and bicellular microhair, were present on maize leaf (Freeling and Lane 1994; Poethig 1990). SEM analysis of trichomes from Mp708 and Tx601 was shown in Fig. 5 and representatives of each trichome class were labeled. The appearance of trichomes differed between the two inbreds. Macrohairs on Tx601 leaves were almost twice as long as those on Mp708 (Table 3). Likewise, the Tx601 prickle hairs and bicellular mircohairs also were significantly longer than those in Mp708. Measurements of trichome density indicated that the macrohairs were dramatically denser on Tx601 while the bicellular microhairs were denser in Mp708.

Table 3

Maize leaf trichome[a] length and density for Mp708 and Tx601

	Length (micron)			Density (No./mm^2)		
	mh	ph	bm	mh	ph	bm
Mp708	455.83 ± 14.35	32.06 ± 0.49	38.3 ± 0.3	3.37 ± 0.2	59.04 ± 3.32	35.67 ± 1.58 *
Tx601	823.5 ± 29.17 *	44.06 ± 0.76 *	48.24 ± 0.44 *	4.56 ± 0.16 *	56.49 ± 3.54	30.02 ± 1.5

[a] Abbreviation: mh, macrohair; ph, prickle hair; bm, bicelluar microhair (Freeling and Lane 1994)

* The data represented are mean values with error bars (+ SE, n = 6 or 8). Letters indicate significant differences by least significant difference test ($P < 0.05$).

Supplementary Materials

Supplemental Table 1 List of Endochitinases

Supplemental Table 2 List of maize endochitinase genes

Supplemental Table 3 Specific activity of maize endochitinase from various tissues measured using chitin-azure[a]

Supplemental Table 4 Proteins Identified in Mp704 x Mp708 Trichomes

Supplemental Table 5 Parameters of proteins identified in Tx601 trichomes

Supplemental Table 6 Primers used in chitinase gene cloning

Supplemental Table 7 Primers used in qRT-PCR

Supplemental Fig. 1 Alignment of endochitinase amino acid sequences from three different maize inbreds. The deduced amino acid sequences of chitinase A, chitinase B, chitinase I, and chitinase PRm3 were aligned from maize inbreds B73, Mp708, and Tx601. Numbers correspond to the amino acid position. Black backgrounds indicate identical or similar amino acids. The dashes denote spaces required for optimal alignment. The signal peptide, chitin-binding domain, and catalytic domain are labeled. The conserved catalytic residues were indicated by open black boxes and labeled with stars. The sources for plant chitinases were listed in Table 1. Sequences were aligned using Vector NTI 11 program from Invitrogen

Maize PRm3 Sequences were Similar to other GH18 Family Chitinases with Reported Anti-herbivory Activity. PRm3, a GH18 chitinase, was found in maize leaves and its expression was dramatically induced more robustly in FAW-fed Tx601 plants than in Mp708, although gene sequences were almost identical between the two genotypes. Furthermore, PRm3 was also detected from Tx601 trichomes in the proteomic analysis conducted using leaf trichomes from resistant hybrid Mp704 x Mp708 and Tx601 (see Tables S4 and S5, supplemental data). Results showed eighty-five proteins were identified in the resistant hybrid Mp704 x Mp708 and approximately 20 of these were putative defense proteins. While many of these proteins were peroxidases, one chitinase was found that belongs to rice GH family 18. Meanwhile, sixty-one proteins were identified in Tx601 trichomes and approximately 12 were putative defense proteins. Again, peroxidases were the most abundant defense proteins identified, and the GH18 chitinase PRm3 was detected in Tx601 trichomes.

One common feature of chitinases currently known to damage the PM is that they belong to GH18 family (Corrado et al. 2008; Gongora et al. 2001; Gopalakrishnan et al. 1995; Harrison et al. 2008; Martinez et al. 2012; Regev et al. 1996). Because of its potential insecticidal activity, we conducted a sequence comparison using deduced amino acid sequenced from maize PRm3 and selected GH18 chitinases reported to have anti-herbivory activity, including chitinases from *S. marcescens* (Regev et al. 1996), *S. albidoflavus* (Martinez et al. 2012), *M. sexta* (Gopalakrishnan et al. 1995), *S. frugiperda* (Bolognesi et al. 2005), AcMNPV (Corrado et al. 2008), and SfMNPV (Harrison et al. 2008). Results from protein alignments presented high sequence similarity at the active sites, and two sets of conserved catalytic residues were found (Fig. 6). In the first conserved region (amino acids 99 to 106), five residues in the motif (K-X-X-X-S-I-X-G-G) were almost identical to the compared sequences. In the second conserved region (amino acids 146 to 153), motif (D-G-X-D-X-D-X-E) from all compared chitinases were identical and sequence is believed to be crucial for the catalytic activity (Oyeleye and Normi 2018; Robertus and Monzingo 1999). The phylogenetic relationship among these enzymes is shown in Fig. 6b. In general, maize chitinases were more closely related to insect chitinases than viral or bacterial chitinases, except for *S. albidoflavus* chiA.

Discussion

Chitinases are present in a wide range of prokaryotic and eukaryotic organisms, including maize. In this study, we identified 33 candidate endochitinase (EC 3.2.1.14) genes from the maize genome, consisting of various members from GH18 and GH19 family. Most maize endochitinases had the predicted molecular mass of approximately 30 kDa and a wide range of pI values from 4–10. The expression of these genes was detected in multiple organs and development stages (Table S1 and S2). Several tandem repeats of chitinase gene clusters were found on chromosomes 1, 2, 3, 6, and 7. Several candidates mapped within or near herbivore-resistance QTL for leaf-feeding insects SWCB and FAW (Brooks et al. 2007) and were found on every maize chromosome except chromosome 9 (Table 2). The occurrence of these chitinase genes in or near the known QTL for caterpillar resistance suggests that they could play a role in plant defenses. Plant chitinases have very diverse domain structures, catalytic characteristics, substrate specificities, and chitin degradation products. Consequently, these differences give plants the flexibility to adapt to a wide spectrum of stresses (Broadway et al. 1998; Kramer and Muthukrishnan 1997; Oyeleye and Normi 2018). For instance, when comparing the catalytic domains, class I and class IV chitinases were structurally similar except for several short deletions, therefore class IV chitinases were a bit smaller due to these deletions. A study suggested that the shorter catalytic domain evolved in class IV chitinases could accommodate shorter substrates (Chaudet et al. 2014). The role of chitin binding domain (CBD) in chitinase activity is not clearly

understood, however, this domain might contribute to substrates binding at active sites and hydrolysis (Li et al. 2005). Furthermore, chitinases from two GH families (18 and 19) shared little to no sequence similarity to each other, and are evolutionary divergent as supported by phylogenetic analysis (Fig. 1). Variations in the topology of active sites for chitinases from a particular GH family (Oyeleye and Normi 2018) suggests that different chitinases may have specific functions in maize defense responses.

Since peptides from chitinase A, B, I, and PRm3 were identified from maize-fed FAW frass (Chuang et al. 2014a; Ray et al. 2016), we further cloned and compared the coding sequences of these genes in maize inbreds Mp708 and Tx601 (Table 1). Results from sequence comparisons showed that nucleotide sequences from the two inbreds were almost identical except for a few polymorphisms. ChiA, ChiB, and ChiI were more closely related to each other than PRm3 (Supplemental Fig. 1). Interestingly, when compared to the maize ancestor teosinte (*Zea mays* ssp *parviglumis*), both nucleotide and amino acid sequences of GH19 chitinases ChiA, ChiB, and ChiI were 97%-100% identical to the corresponding sequences from teosinte, which indicates they were quite conserved during the domestication of maize. On the other hand, none of the teosinte subspecies exhibited sequence identities to the GH18 chitinase PRm3. Sequence similarities shared with PRm3 were rather seen from other monocot crops such as sorghum (~ 90%) and rice (~ 80%).

Despite the extensive reviews of plant chitinases and their antifungal activity, few have been studied for the role in insect resistance (Lawrence and Novak 2006; Ray et al. 2016). Chitinases have been reported to disrupt the PM structure and consequently may be one of the defense protein plants use to increase their insect resistance (Herrera-Estrella and Chet 1999; Tellam et al. 1999; White et al. 1997). Since maize ChiA, ChiB, ChiI, and PRm3 were highly expressed in leaf, silk, and husk tissues, they may serve as a defense for the developing ear against FAW and fungal pathogens (Table 1). In our study, we investigated the gene expressions of the aforementioned chitinases in two maize inbreds in response to herbivore feeding and mechanical wounding. Surprisingly, chitinase gene expression was differently regulated in Mp708 and Tx601 despite the sequence identity between the two. Although transcript levels all significantly increased in maize leaves when challenged by FAW infestation, *ChiA*, *ChiB*, and *ChiI* (all GH19, known for antifungal activity) were more abundant in Mp708-fed leaves, while *PRm3* (GH18) was dramatically higher in Tx601 (Fig. 2). Similarly, under wounding treatments, *ChiB* expression was significantly greater in Mp708, and *PRm3* was constantly higher in Tx601. It was not surprising to find that wounding alone could significantly induce the chitinase expression, as a previous study (Ray et al. 2016) showed that wounding in maize leaves suppressed the growth of fungal pathogen *Cochliobolus heterostrophus*. One explanation was maize plants might prime themselves to inhibit opportunistic fungal pathogen since wounding caused by herbivore feeding could provide an opening for them (Clarke et al. 1998). In addition, chitin oligomers from the degraded PM could also serve as an elicitor to activate the pathogen defense pathway and further prevent diseases from entering the wounded area (Cohen-Kupiec and Chet 1998; Eckardt 2008; Ray et al. 2016). It is unlikely that the differences in gene expression between Mp708 and Tx601 were due to polymorphisms in coding sequences of the respective genes, but could be due to variations in the promoter regions or signals that interacting with them. For instance, Mp708 plants have higher constitutive JA levels than Tx601 and thus are primed and ready for insect attack (Luthe et al. 2011; Shivaji et al. 2010). This priming could explain why Mp708 has higher basal chitinase expression for *ChiA* and *ChiI* than Tx601, except *PRm3*. In a recent study, exogenously applied ChiA reduced the growth of Tx601-fed FAW larvae by 80% (Mason et al. 2019). In another study, Pr4 and ChiA found in B73-fed FAW frass suppressed pathogen growth by inducing pathogen defense pathways, however, they also attenuated insect-induced responses in maize, and exogenously applying ChiA had no impact on caterpillar growth (Ray et al. 2016). Taken together, it seems that chitinase gene regulation most likely depends on the genetic background of the maize plant.

In addition to gene expression, we also found chitinase specific activity was induced in maize by both wounding and feeding treatments (Table S3). Next, we determined if chitinases were active in the insect midgut and if the midgut lining showed signs of damage after ingesting maize leaves. Results from activity analysis showed that specific activity from both the FAW food bolus and frass increased significantly after 24 hr of feeding in both inbred lines when compared to diet-fed control (Fig. 3). This large increase in specific activity inside FAW midgut suggested that maize chitinases were stable and active when ingested and processed in the midgut. This finding is significant, since it has always been questioned if plant chitinases retain activity in the alkaline lepidopteran midgut (Broadway et al. 1998). One possible explanation for the retention of chitinase activity is the micro-environment created inside the midgut that may provide suitable conditions for enzyme activity. In our study, SEM images (Fig. 4) clearly shown that partially digested leaves were inside the midgut and hence, they could sustain a favorable pH range for the chitinases. However, which chitinase or combinations of chitinases contributed to the activity could not be determined due to the limitation of the enzymatic assay method used. Since both chitinase transcript levels and enzyme activities were induced after feeding, it suggests that a combination of chitinases could play a role in maize defense against herbivory.

Chitinase activity was also differently regulated in each maize genotype, in addition to gene expression. Unlike Mp708, significantly greater enzyme activity was detected in Tx601 compared to control in response to feeding. Also, samples collected from the food bolus and frass of Tx601-fed caterpillars had higher chitinase specific activity than Mp708-fed ones (Fig. 3). There are several possible causes for this increased specific activity in Tx601 despite the greater transcripts increase in Mp708. Mp708 expresses a potent defense protein, Mir1-CP (Pechan et al. 2002), which can dramatically damage the PM by degrading the integral PM protein, insect intestinal mucin (IIM) (Fescemyer et al. 2013). Enhancin, a baculovirus metalloprotease, has been also shown to attack this important PM structural protein (Wang and Granados 1997). As a result, high level of Mir1-CP presenting in the food bolus of Mp708-fed larvae may have inactivated or degraded the chitinases induced by FAW infestation. A previous study also shows that the digestion process is impaired in Mp708-fed FAW larvae (Chang et al. 2000), so caterpillars fed on Tx601 could have digested more protein in the food bolus resulting in lower overall protein content and higher specific activity. Last but not least, it is possible PRm3, which was more abundant in Tx601, is either has more potent enzymatic activity or is more resistant to degradation in the midgut than the other chitinases induced during infestation (Chen et al. 2007; Koga et al. 1999).

Previous literature shows certain GH18 chitinases that are widely distributed in both prokaryotes and eukaryotes have antiherbivore activity. For example, a chitinase from *Serratia marcescens* was shown to perforate *Spodoptera* larval midgut PM (Regev et al. 1996). *Streptomyces albidoflavus* chitinases were found to enhance resistance both to cabbage looper (Gongora et al. 2001) and coffee berry borer (Martinez et al. 2012). *Manduca sexta* chitinase had been found to increase the mortality of tobacco budworm when expressed in a recombinant baculovirus (Gopalakrishnan et al. 1995). Moreover, chitinases from the virus, *Autographa californica* nuclear polyhedrosis virus (AcMNPV), and *Spodoptera frugiperda* nucleopolyhedrovirus (SfMNPV) were found to enhance

mortality of Lepidoptera larvae (Corrado et al. 2008; Harrison et al. 2008). As a member of class III chitinase, maize PRm3 had ~ 80% sequence identity at the conserved catalytic domains (K-X-X-X-S-I-X-G-G motif and catalytic motif DXDXE, Fig. 6) to other GH18 chitinases with anti-herbivory activity (Corrado et al. 2008; Gongora et al. 2001; Gopalakrishnan et al. 1995; Harrison et al. 2008; Martinez et al. 2012; Regev et al. 1996). This conservation of sequence among chitinases having insecticidal activity and PRm3 suggests that it also may be involved herbivore resistance. It will be interesting to perform additional experiments with purified PRm3 chitinase to see if it has direct deleterious effects on caterpillar growth and PMs.

Morphological observations also suggest that the PRm3 enzyme is a good candidate for insect defense in Tx601. When the damaged PM dissected from larvae reared on Tx601 were examined, it appeared that the hydrolytic enzymes from the ingested plant material had weakened the PMs since these PMs were not only more fragile than the diet-fed ones during the sample processing, but showed obvious signs of physical damage compared to those reared on artificial diets. Detailed structural abnormalities were observed including membrane flakes, multiple holes, spheroid-shaped particles embedded in membrane layers, and trichome piercing (Fig. 3). The spheroid-shaped particles on the PM surface have been previously seen, but without known function (Adang and Spence 1981; Brandt et al. 1978; Pechan et al. 2002). Trichome structure was clearly visible under SEM, and we also observed the phenomenon of trichomes perforating the PM. This was typically observed near the areas where degenerated PM patches were seen (Fig. 4). This could be explained by the increased PRm3 chitinase levels present in Tx601 leaves causing PM degradation. It was also shown that Tx601 trichomes contained PRm3 that could directly deliver this enzyme to the midgut after leaves were consumed.

Trichomes are hair-like structures developed from the aerial epidermis on the leaves of many plant species. They can be glandular or non-glandular, unicellular, or multicellular and vary in size, number, shape, and chemical composition (Johnson 1975; Serna and Martin 2006; Tian et al. 2012; Werker 2000; Yoshida et al. 2009). Previous studies show that trichomes not only act as a physical barrier that interferes with insect herbivore movement and feeding, they also poison, trap or repel insect herbivores by releasing toxic chemicals (Elle and Hare 2000; Holeski et al. 2010; Levin 1973; Peiffer et al. 2009; Tian et al. 2012; Wagner 1991). In this study, our data also suggested that trichomes may be related to insect defense by facilitating PM penetration and resulting in growth delay of FAW. For example, growth retardation was seen in FAW reared directly on Tx601 leaves (this study) or those exogenously treated with ChiA or bacteria on Tx601 leaves (Mason et al. 2019). However, in another inbred line B73, directly applying ChiA did not slow the caterpillar growth (Ray et al. 2016). One reason for the differences could be due to the maize trichome structure and composition. Since Tx601 has significantly longer and denser trichomes than Mp708 and B73, it can damage PM by physical penetration and further altering the caterpillar gut microbiome, which in turn reduces larvae performance (Mason et al. 2019; Williams et al. 2000). Overall, our results strongly indicated that the maize chitinases could disrupt the PM and have the potential to significantly disrupt insect digestion or nutrient intake, thus increase the susceptibility to abrasion or pathogens (Aranda et al. 1996; Gongora et al. 2001; Hegedus et al. 2009).

Conclusions

Chitinases are reported to adversely affect insect pests, together with other natural products like toxins, lectins, proteases, and α-amylase inhibitors (Broadway et al. 1998; Kramer and Muthukrishnan 1997; Oyeleye and Normi 2018). In this study, we found distinctive groups of chitinases in maize with different molecular structures and catalytic mechanisms in support of previous studies, and here we focused on exploring the role of endochitinases in maize insect defense. Variations were observed in chitinases gene and activity regulation in response to insect feeding and mechanical wounding from different GH families, and some chitinases may be more effective in defense than others. When comparing maize inbreds with different levels of herbivore resistance, we found those chitinase sequence identities were extremely high. Nevertheless, each inbred line had a specific induction profile for chitinases suggesting that different strategies may be deployed for herbivore defense. The physical/physiological effects of plant chitinases allow us to evaluate its impact as a target-specific natural resistance factor, and among all four chitinases tested, PRm3 could be a better candidate for a potential pest control agent.

Declarations:

Declarations

Competing interests

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

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Authors' contributions

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

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Figures

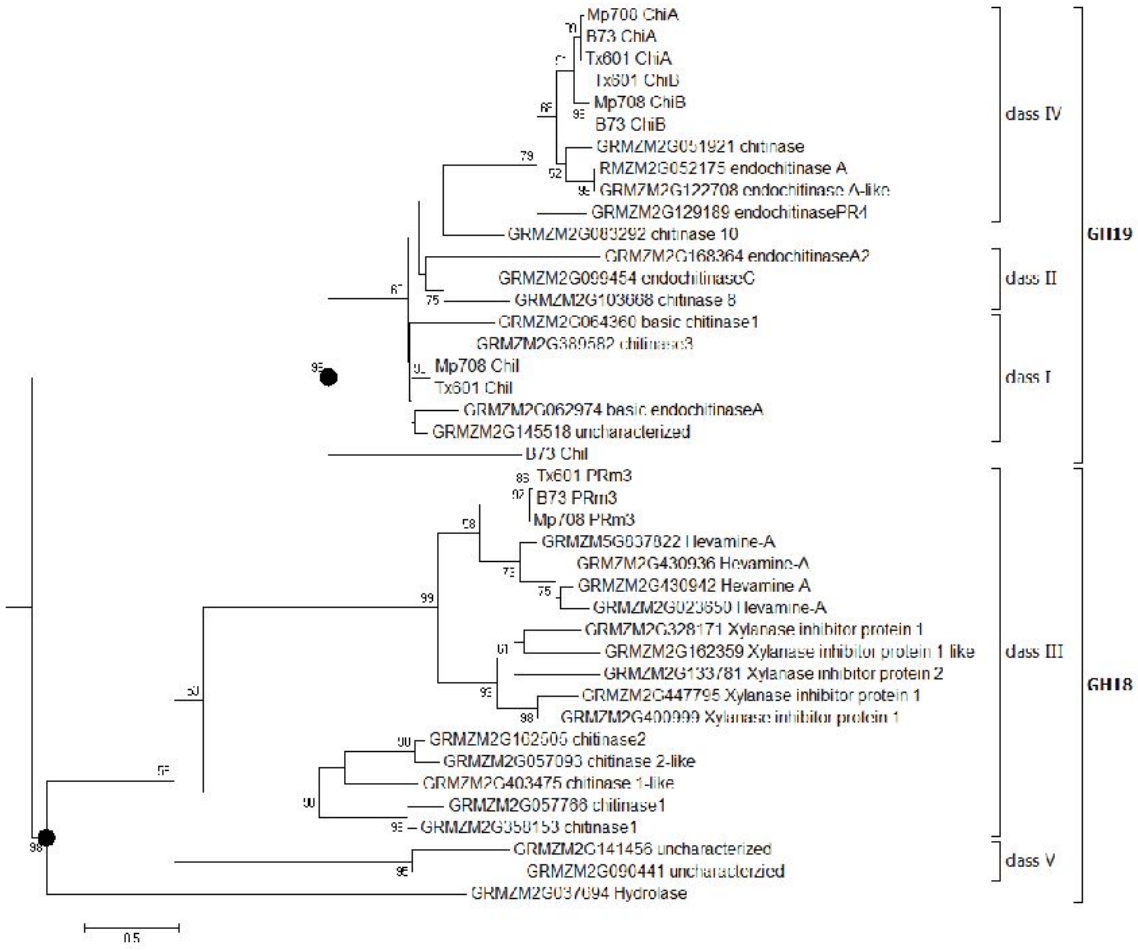

Figure 1

Phylogenetic tree of maize chitinases. The tree was constructed using the amino acid sequences by Maximum Likelihood methods with MEGA version 6, the numbers on the branch indicate bootstrap values from 1000 replicates. Filled circles indicate the sequence partition between family 19 and family 18 chitinases. Members belonging to the same class were within each bracket. Sources of the amino acid sequences are listed in Table S2

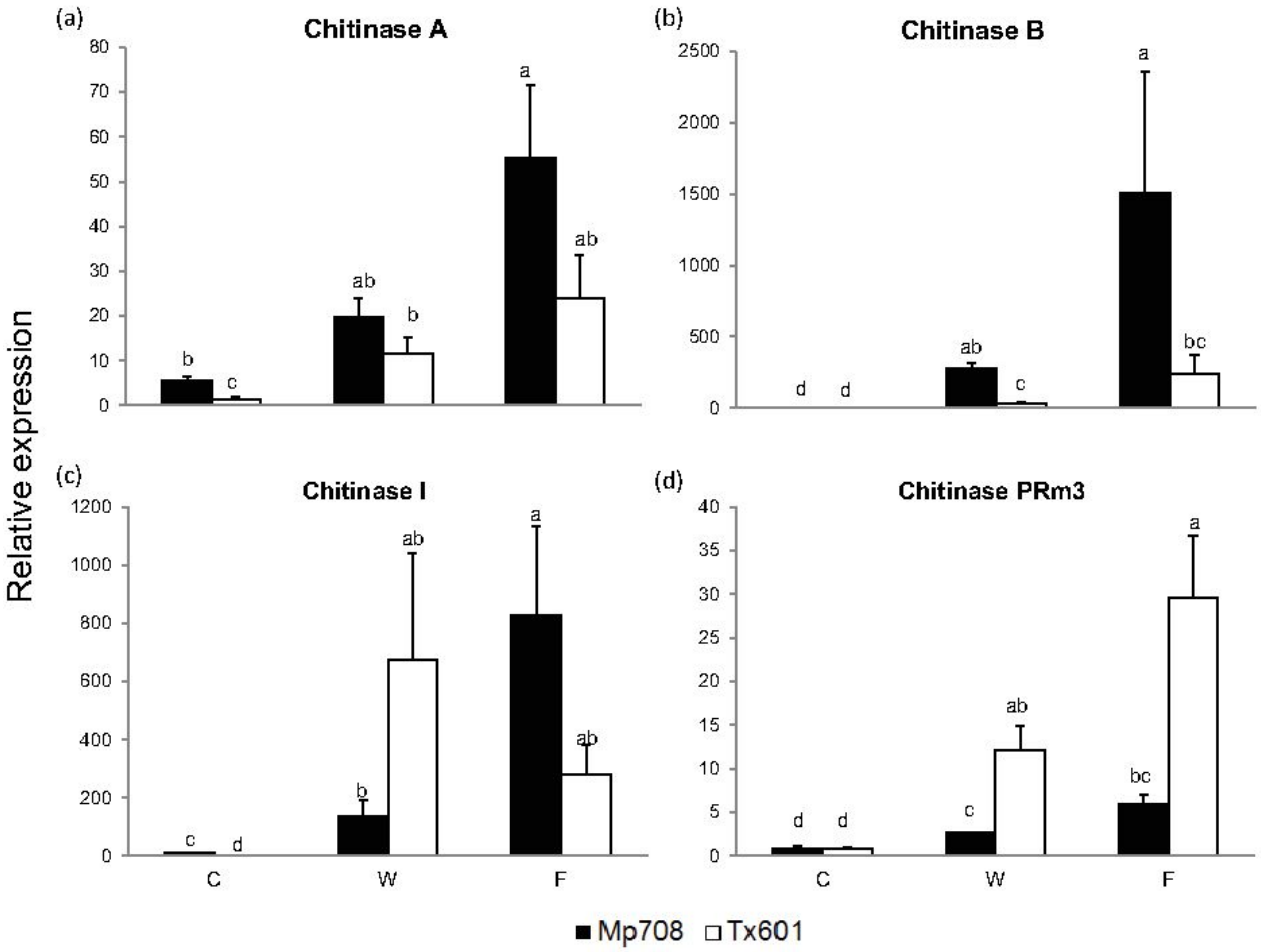

Figure 2

Relative expression of chitinase transcripts in maize leaves in response to various treatments. Quantitative RT-PCR was performed for (a) chitinase A, (b) chitinase B, (c) chitinase I, and (d) chitinase PRm3. Whorls from V8 stage Mp708 and Tx601 were mechanically wounded (W), or fed (F) by fall armyworm larvae for 24 hr. Undamaged plants were used as control (C). Total RNA was isolated from control whorls and the damaged areas on the leaves. Relative transcript abundance was calculated using actin as the reference gene. The data represented were mean values with error bars (+SE, n=9). Letters indicate significant differences by the least significant difference test ($P<0.05$)

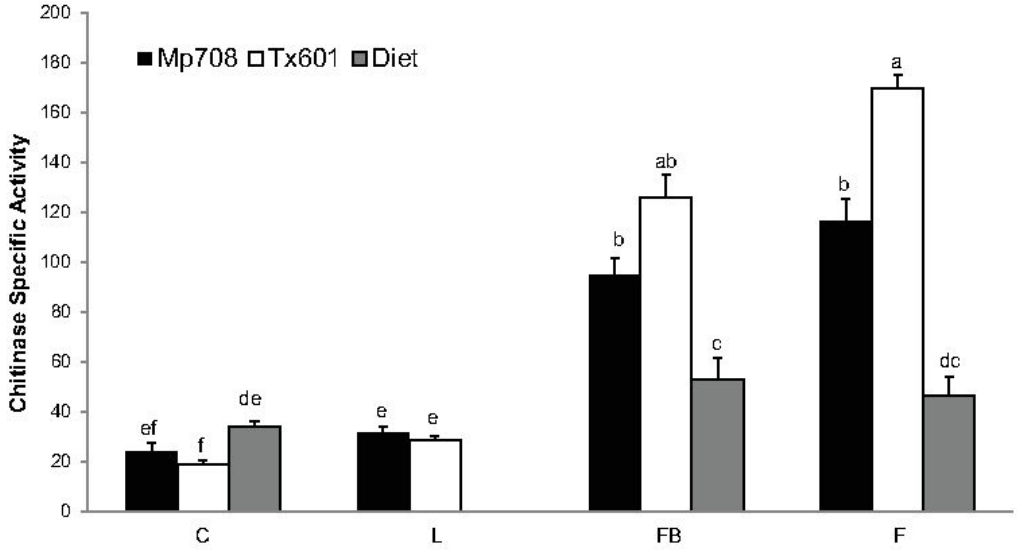

Figure 3

Chitinase specific activity in maize leaves and fall armyworm food bolus and frass after feeding for 24 hr on Mp708 or Tx601. V8 maize whorls from Mp708 and Tx601 were infested with FAW larvae for 24 hr (L). Undamaged plants were used as control (C). FAW larvae that fed on these plants were collected and food bolus (FB) was dissected from the midguts. Frass (F) from FAW larvae was also collected. Chitinase activity was measured in the collected materials using chitin-azure as a substrate as described in Materials and Methods. Food bolus and frass from larvae fed on artificial diet were used as a reference control. The data represented are mean values with error bars (+SE, n=3 or 6). Letters indicated significant differences by the least significant difference test ($P<0.05$)

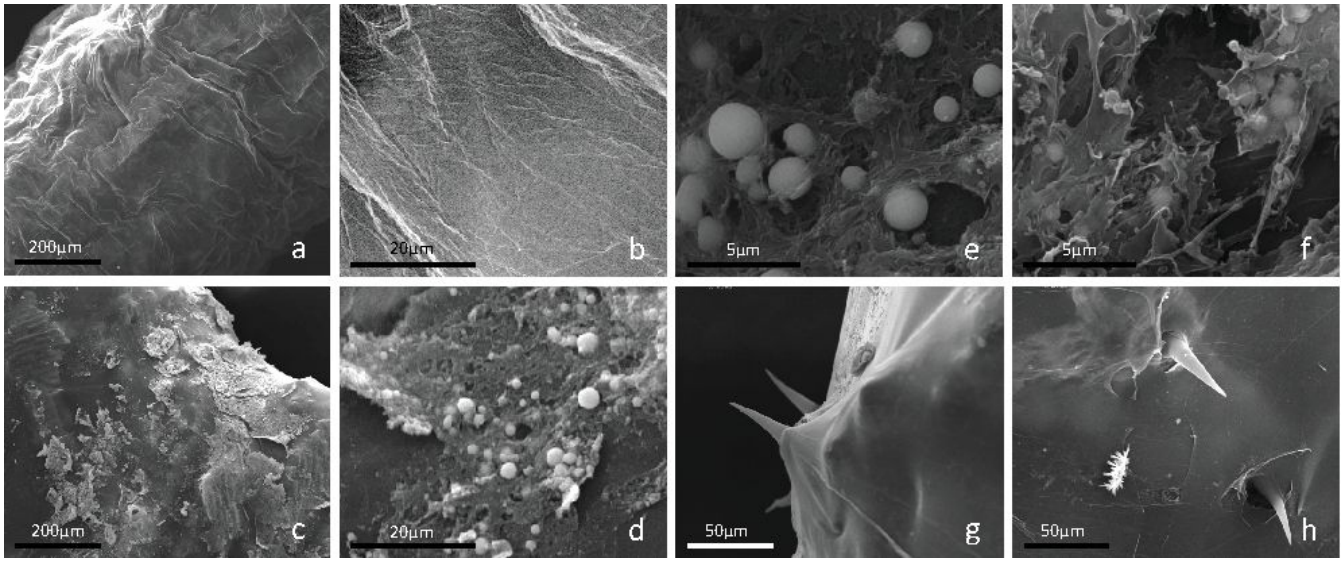

Figure 4

Scanning electron micrographs showing the peritrophic matrix (PM) of fall armyworm caterpillars. (a) and (b) PM from FAW larvae reared on artificial diet (magnification X100, and X500 respectively). (c) and (d) PM from FAW larvae reared on Tx601 plant showing the damaged area (magnification X100, and X500 respectively). (e) and (f) Enlargement of PM flakes from FAW larvae reared on Tx601 showing perforations and spherical particles (magnification X5000). (g) and (h) showing trichomes penetrating the peritrophic matrix (PM) of fall armyworm larvae reared on Tx601 (magnification X100). The scale was indicated on each micrograph

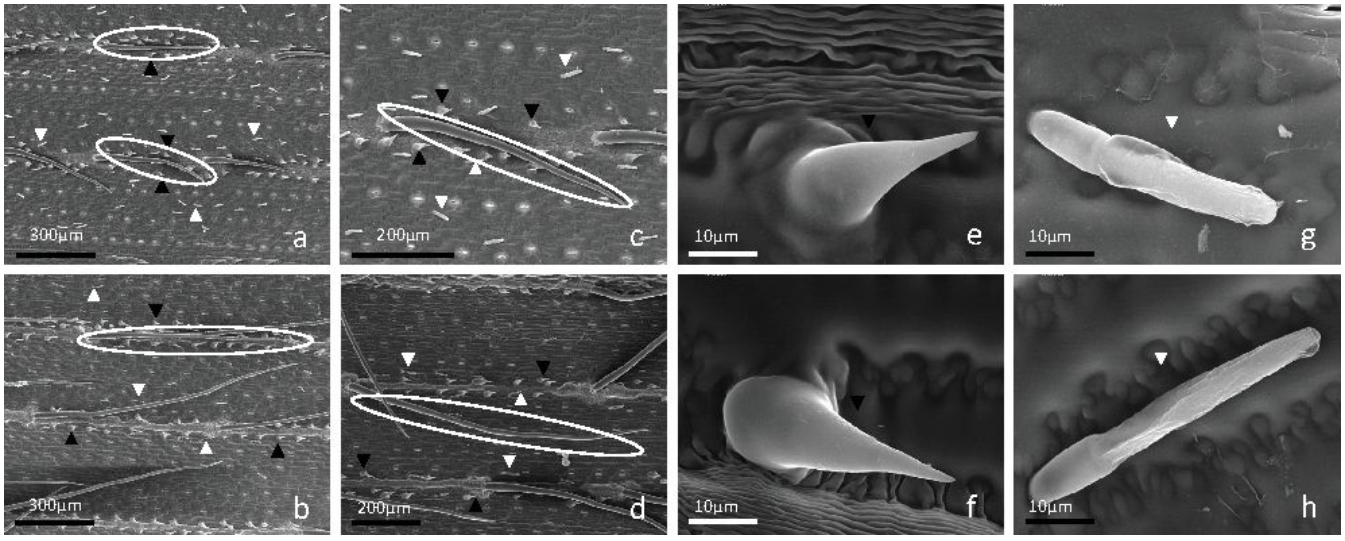

Figure 5

Scanning electron micrographs of trichomes from maize inbreds Mp708 and Tx601. Three types of maize trichomes were observed: macrohair (circled), prickle hair (black arrows), and bicellular microhair (white arrows). Trichome morphology on the leaf surface of Mp708 (a) and Tx601 (b) is shown at 75X magnification. Enlargement of trichome macrohair (circled) from Mp708 (c) and Tx601 (d) is shown at 150X and100X magnification, respectively. Enlargement of trichome prickle hair (black arrows) and bicellular microhair (white arrows) from Mp708 (e, g) and Tx601 (f, h), are shown at 2000X magnification respectively. The scale was indicated on each micrograph

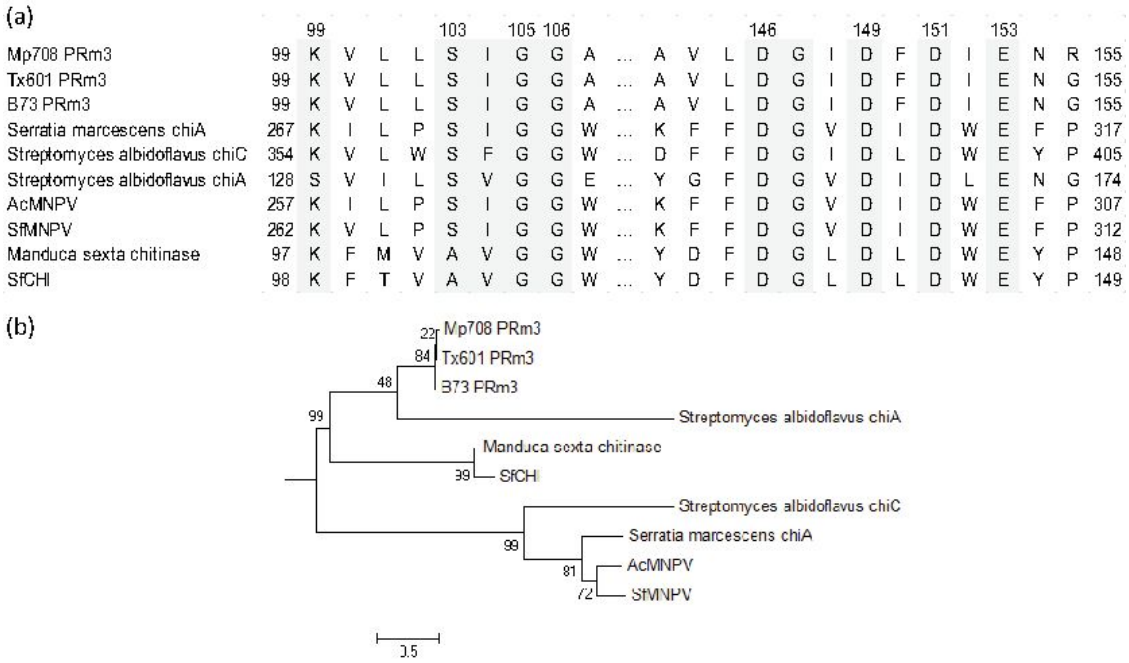

Figure 6

Phylogenetic analysis of selected family 18 endochitinases. (a) Comparison of the conserved region of the amino acid sequences. Numbers correspond to the amino acid position. Gray backgrounds indicate identical or similar amino acids. (b) Phylogenetic tree of chitinases. The tree was constructed using the amino acid sequences by Maximum Likelihood methods with MEGA 6, the numbers on the branch indicated bootstrap values from 1000 replicates. Sequences listed here were deduced sequence of chitinase from maize inbred B73, Mp708, and Tx601 (this work), Serratia marcescens (Regev et al., 1996), Streptomyces albidoflavus (Martinez et al., 2012), Autographa californica nuclear polyhedrosis virus (AcMNPV) (Corrado et al., 2008), Spodoptera frugiperda nucleopolyhedrovirus (SfMNPV) (Harrison et al., 2008), Manduca sexta (Gopalakrishnan et al., 1995), and Spodoptera frugiperda (Bolognesi et al., 2005). Sequences were analyzed using MEGA 6

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementalFig1.pdf
- SupplementalTable17.xlsx