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Identification of the chitinase genes from the diamondback moth, *Plutella xylostella*

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Abstract

Chitinases have an indispensable function in chitin metabolism and are well characterized in numerous insect species. Although the diamondback moth (DBM) *Plutella xylostella*, which has a high reproductive potential, short generation time, and characteristic adaptation to adverse environments, has become one of the most serious pests of cruciferous plants worldwide, the information on the chitinases of the moth is presently limited. In the present study, using degenerated polymerase chain reaction (PCR) and rapid amplification of cDNA ends-PCR strategies, four chitinase genes of *P. xylostella* were cloned, and an exhaustive search was conducted for chitinase-like sequences from the *P. xylostella* genome and transcriptomic database. Based on the domain analysis of the deduced amino acid sequences and the phylogenetic analysis of the catalytic domain sequences, we identified 15 chitinase genes from *P. xylostella*. Two of the gut-specific chitinases did not cluster with any of the known phylogenetic groups of chitinases and might be in a new group of the chitinase family. Moreover, in our study, group VIII chitinase was not identified. The structures, classifications and expression patterns of the chitinases of *P. xylostella* were further delineated, and with this information, further investigations on the functions of chitinase genes in DBM could be facilitated.

Keywords: chitinase, phylogenesis, *Plutella xylostella*

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Introduction

Chitin is a characteristic constituent of the cuticles of insects and other arthropods (Kramer *et al*., 1995; Merzendorfer, 2006). In addition to cuticles, chitin is also found in the peritrophic matrix, tracheae, and at muscle attachment points (Kramer & Koga, 1986; Hegedus *et al*., 2009). The chitin-containing tissues must be remodeled during insect growth and development, and the chitinases (EC 3.2.1.14) are the enzymes responsible for the degradation of the linear polysaccharides in chitin (Kramer & Muthukrishnan, 2005). Insect chitinases belong to glycosyl hydrolase family 18 (GH18) and are responsible for the endo-degradation of chitin; thus, chitinases are a target in pest management (Merzendorfer & Zimoch, 2003). Insect chitinases and chitinase-like genes were recently identified from the completed genome sequences in *Drosophila melanogaster, Tribolium castaneum* and *Anopheles gambiae* (Zhu *et al*., 2004, 2008*c*; Zhang *et al*., 2011*a*). Moreover, the deduced proteins of these genes have been classified into eight groups based on the phylogenetic

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analysis of catalytic domain (CD) sequences (Kramer & Muthukrishnan, 1997; Arakane & Muthukrishnan, 2010).

Although several chitinase genes from lepidopteran species have been characterized, including those of *Manduca sexta* (Kramer *et al.*, 1993), *Bombyx mori* (Kim *et al.*, 1998; Mikitani *et al.*, 2000; Abdel-Banat & Koga, 2001), *Hyphantria cunea* (Kim *et al.*, 1998), *Spodoptera litura* (Shinoda *et al.*, 2001), *Choristoneura fumiferana* (Zheng *et al.*, 2002), *Helicoverpa armigera* (Ahmad *et al.*, 2003), *Lacanobia oleracea* (Fitches *et al.*, 2004), *Spodoptera frugiperda* (Bolognesi *et al.*, 2005) and *Ostrinia nubilalis* (Khajuria *et al.*, 2010), only one chitinase gene or cDNA was identified in most of these species. Furthermore, most of the identified lepidopteran chitinases are group I chitinases, which are the enzymatically well characterized chitinases that are isolated from molting fluid or integument; there are some exceptions, such as a few bacterial-type chitinase genes (*Cht-h*) (Daimon *et al.*, 2003, 2005), a gut-specific group IV chitinase gene in *O. nubilalis* (Khajuria *et al.*, 2010) and certain chitinase-like genes (Imaginal disk growth factor, *IDGF*) in group V (Zhang *et al.*, 2006; Wang *et al.*, 2009). However, as whole-genome sequences are continually reported, remarkable advances have occurred in the understanding of the entire family of lepidopteran chitinases. Based on the screening of the genome of *B. mori*, lepidopteran species have multiple genes that encode chitinase proteins (Nakabachi *et al.*, 2010; Pan *et al.*, 2012). In addition to the eight groups that were described previously, two new groups of chitinases (groups IX and X) were created in a genome-wide analysis of the tobacco hornworm *M. sexta* (Tetreau *et al.*, 2015).

In this study, we characterized the entire chitinase gene family from *Plutella xylostella*, which is a major pest of several agricultural crops worldwide. We first cloned four chitinase genes using degenerated polymerase chain reaction (PCR) and rapid amplification of cDNA ends (RACE-PCR) strategies before the complete genome sequence and the transcriptomic database of *P. xylostella* were released (Jouraku *et al.*, 2013; You *et al.*, 2013). BLAST searches of these databases identified an additional 11 genes that encoded chitinase proteins. The characteristics of the chitinase genes in the diamondback moth *P. xylostella*, 15 in total, were determined in this study, including the domain structures, expression patterns, and phylogenetic relationships among the chitinase genes from different orders, particularly Lepidoptera.

Materials and methods

Insect culture

The *P. xylostella* were maintained in a growth chamber under a 16:8 h light:dark photoperiod at 25 ± 2°C and 60–70% relative humidity (RH). The larvae were reared on 10–15-day-old cabbage (*Brassica rapa* L.), and the adults were fed honey as a dietary supplement.

Total RNA extraction and synthesis of first-strand cDNA

The total RNA was extracted from the larvae of all instars of *P. xylostella* using a TRIzol total RNA isolation kit (Invitrogen) according to the manufacturer's instructions. One microgram of total RNA was used as a template for first-strand cDNA synthesis using the ImProm-II™ Reverse Transcription system (Promega) with an Oligo-$(dT)_{20}$ primer. The reverse transcription was performed at 42°C for 60 min and was terminated at 72°C for 10 min.

Degenerate PCR amplification of putative chitinase gene fragments

The partial chitinase fragments were amplified with a pair of degenerate primers. The forward and reverse primers that corresponded to two conserved regions of the Family 18 insect chitinases were synthesized according to the amino acid sequences DLDWEYP and WAIDMDDF, respectively. The primers and the conditions for PCR are shown in tables S1 and S2, respectively. The PCR products were separated on 1% agarose gel, and a product mixture of approximately 700 bp was excised from the gel. After purification using a DNA gel extraction spin column (Bioman), the 700 bp fragments were subcloned into the T and A cloning vectors (RBC Bioscience). The positive clones were selected, and the plasmids were prepared for DNA sequencing. The sequencing results showed that five different chitinase fragments were amplified by degenerated PCR after a BLAST search of the NCBI database.

Rapid amplification of cDNA ends

The SMART™ RACE cDNA Amplification kit (Clontech) was used to clone the full length of the chitinase cDNAs. The 3′ RACE and 5′ RACE cDNAs were synthesized from the total RNA isolated as previously described using SMARTScribe™ Reverse Transcriptase (Clontech), according to the manufacturer's instructions. Three forward and three reverse gene-specific primers were designed based on the known cDNA sequences amplified by the degenerate PCR. The amplification of the cDNA to end was achieved using the Universal Primer A Mix (UPM) supplied in the kit and pairing with one forward gene-specific primer in the 3′ RACE PCR reaction and with one reverse gene-specific primer in the 5′ RACE PCR reaction. The primers, conditions of the PCR, and amplified range are shown in tables S1 and S2. The PCR products were recovered and were cloned into the RBC vector as previously described. The positive clones were selected for insert sequencing.

Identification of chitinase-like genes from P. xylostella *genome and transcriptomic databases*

The genome of *P. xylostella* (GenBank accession AHIO00000000) (http://www.ncbi.nlm.nih.gov/assembly/GCF_000330985.1) was screened for genes that encoded chitinase-like proteins by using BLAST searches. The BLASTP searches were performed at the website of the NCBI (http://www.ncbi.nlm.nih.gov/) using the amino acid sequences of the chitinase-like proteins of *D. melanogaster*, *T. castaneum* and *A. gambiae* obtained from the NCBI and of *M. sexta* from the Manduca base (http://agripestbase.org/manduca) as queries. KONAGAbase (http://dbm.dna.affrc.go.jp/px/), a DBM comprehensive transcriptomic and draft genomic sequences database, was also searched using the identical BLASTP method.

Default parameters were used and then the candidate chitinase-like genes were confirmed by searching the BLASTX algorithm against the nonredundant (nr) NCBI nucleotide database.

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Domain architecture and phylogenetic analysis

The domain structures of the putative chitinase genes were analyzed using the SMART online program (http://smart.embl-heidelberg.de/), and the signal peptides were predicted through the SignalP 4.0 program (http://www.cbs.dtu.dk/services/SignalP). The transmembrane regions were analyzed using TMHMM Server version 2.0 (http://www.cbs.dtu.dk/services/TMHMM). The CD search at the NCBI (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) was used to confirm the predicted domains. The phylogenetic tree was generated based on the amino acid sequences of the CD. First, the amino acid sequences were aligned using ClustalX version 2.0 (Larkin *et al.*, 2007), and the phylogenetic trees were inferred with the neighbor-joining (NJ) method (p-distance model, uniform rates and complete deletion of gaps or missing data). The NJ tree was constructed using the program MEGA version 6.0 (Tamura *et al.*, 2013). The bootstrap probability for each node was calculated by generating 5000 bootstrap replicates. The letters A–E denote multiple CDs from the N- to the C-terminuses in the same protein.

Reverse transcription PCR (RT-PCR) analysis

To investigate the stage-specific expressions of the chitinase genes, the total RNA was isolated from each of eight developmental stages, including the egg, first to fourth instar larvae, prepupa, pupa and adult, using the TRIzol Total RNA Isolation kit (Invitrogen). The total RNA was also isolated from fourth instar larval tissue samples, including the midgut, hemolymph, Malpighian tube and carcass (whole larva after the gut was removed), to study the tissue-specific expression. The cDNA synthesis was previously described, and the information for the primer sequences is shown in table S3. The PCR products were resolved on 1.5% agarose gel and were visualized by staining with ethidium bromide. The β-actin gene from *P. xylostella* was used as the loading reference for RT-PCR analyses. The RT-PCR was repeated a minimum of three times for each gene at each developmental stage and for each tissue type of the fourth instar larva.

Results

Identification and classification of chitinase genes in P. xylostella

Four RACE-PCR cloned chitinase genes from *P. xylostella* were denoted as *PxCht1*, *PxCht2*, *PxCht3* and *PxCht3-like*. The complete cDNA sequences and the corresponding amino acid sequences were submitted to the GenBank database with the nucleotide accession numbers of FJ613480, JQ417265, JQ417267 and JQ417266, respectively. The phylogenetic analysis of the four chitinase proteins (accession: ACU42267, AFI55112, AFI55114 and AFI55113) with proteins from *A. gambiae* (*AgCht*), *Aedes aegypti* (*AaCht*), *T. castaneum* (*TcCht*) and *D. melanogaster* (*DmCht*) was generated using the method proposed by Zhang *et al.* (2011*a*). In fig. 1, *PxCht1* and *PxCht2* are grouped into groups I and III, respectively, with bootstrap values greater than 90%. However, *PxCht3* and *PxCht3-like* were tentatively placed in the group IV with low bootstrap values.

The BLAST searches of the *P. xylostella* genome (assembly DBM_FJ_V1.1) and transcriptomic databases (KONAGAbase) identified 15 genes that encoded chitinase proteins: thirteen *Cht* genes, one *IDGF* gene and one *Cht-h* gene respectively. In fig. 2, the molecular phylogenetic analysis of the 15 *P. xylostella* chitinases (table 1) with other four lepidopteran chitinases is shown. The 13 *Danaus plexippus*, 13 *Papilio xuthus*, 11 *Bombyx mori* and 11 *M. sexta* chitinase proteins obtained from the NCBI, NCBI, both NCBI and silkDB (http://silkworm.genomics.org.cn/) and the Manduca base, respectively (table S5), were included in the analysis. The chitinase genes detected in *P. xylostella* were named with a number that corresponded to the phylogenetic group in which they were grouped: *PxCht* (chitinases, all groups except group V) or *PxIDGF* (chitinase-like proteins, the Imaginal Disk Growth Factors, group V). The four RACE-PCR cloned *Cht* genes that were described previously were found again in the screening of the *P. xylostella* genome. Thus, hereafter in this study, *PxCht1*, *PxCht2*, *PxCht3* and *PxCht3-like* were renamed *PxCht5*, *PxCht7*, *PxCht25-1* and *PxCht25-2*, respectively. The detailed information is listed in table 1 for the genes that encoded the chitinase proteins searched from *P. xylostella*.

The chitinase genes from five lepidopterans were clustered into 12 groups (fig. 2): 11 of the groups (I−X and h) were previously reported in *M. sexta* (Tetreau *et al.*, 2015) and one new group was created. The 15 *P. xylostella* chitinases were clustered into 10 of these 11 groups, not including group VIII, and two chitinases might be in a new group of the chitinase family. Eight of the 11 groups in *P. xylostella* (groups I-III, V, VI, IX, X and h) contained a single chitinase gene: *PxCht5* in group I, *PxCht10* in group II, *PxCht7* in group III, *PxIDGF* in group V, *PxCht6* in group VI, *PxCht1* in group IX, *PxCht3* in group X and *PxCht-h* in group h. The other three groups (IV, VII and the new group) contained 2, 3 and 2 chitinase genes, respectively: *PxCht8-1* and *PxCht8-2* in group IV, *PxCht2-1*, *PxCht2-2* and *PxCht2-3* in group VII, and *PxCht25-1* and *PxCht25-1* in the new group. Moreover, both *PxCht2-3* and *PxCht6* were predicted to have two transcript variants, and the letters *a* and *b* denote each of the isoforms.

Structure of chitinase genes from P. xylostella

Fig. 3 shows the domain architecture of the deduced amino acid sequences from the *P. xylostella* chitinase genes. Most of the predicted amino acid sequences contained single copy of putative CD (GH18 domain: pfam00704) (see: http://www.ncbi.nlm.nih.gov/cdd, for more information on the conserved domains), whereas *PxCht7* had two copies of this domain and *PxCht10* had five copies. One or more chitin-binding domains (CBD) (CBM-14: pfam01607) were detected in *PxCht3*, *PxCht5*, *PxCht6*, *PxCht7*, *PxCht8-1*, *PxCht8-2* and *PxCht10*. Additionally, *PxCht-h* contained a polycystic kidney disease 1 domain (PKD1: smart00089).

Fig. 4 shows the alignment with the putative CD of the 15 chitinase proteins from *P. xylostella*. Most sequences contained four highly conserved regions (CR I-CR IV), a characteristic feature of all insect chitinases (Kramer & Muthukrishnan, 1997; De la Vega *et al.*, 1998). The signature sequences were KXXXXXGGW, FDGXDLDWEYP, MXYDXXG and GXXXWXXDXD in which X was a non-specified amino acid. In *PxCht1* and *PxCht3*, all four regions were poorly conserved and devoid of the residue E, which is the putative proton donor in the catalytic mechanism in the CR II (Watanabe *et al.*, 1993; Lu *et al.*, 2002). The CD range label in boldface indicates the presence of E residue in CR II (table 1). Moreover, genome assembly gaps were found in several *PxCht* sequences (denoted with asterisks in table 1).

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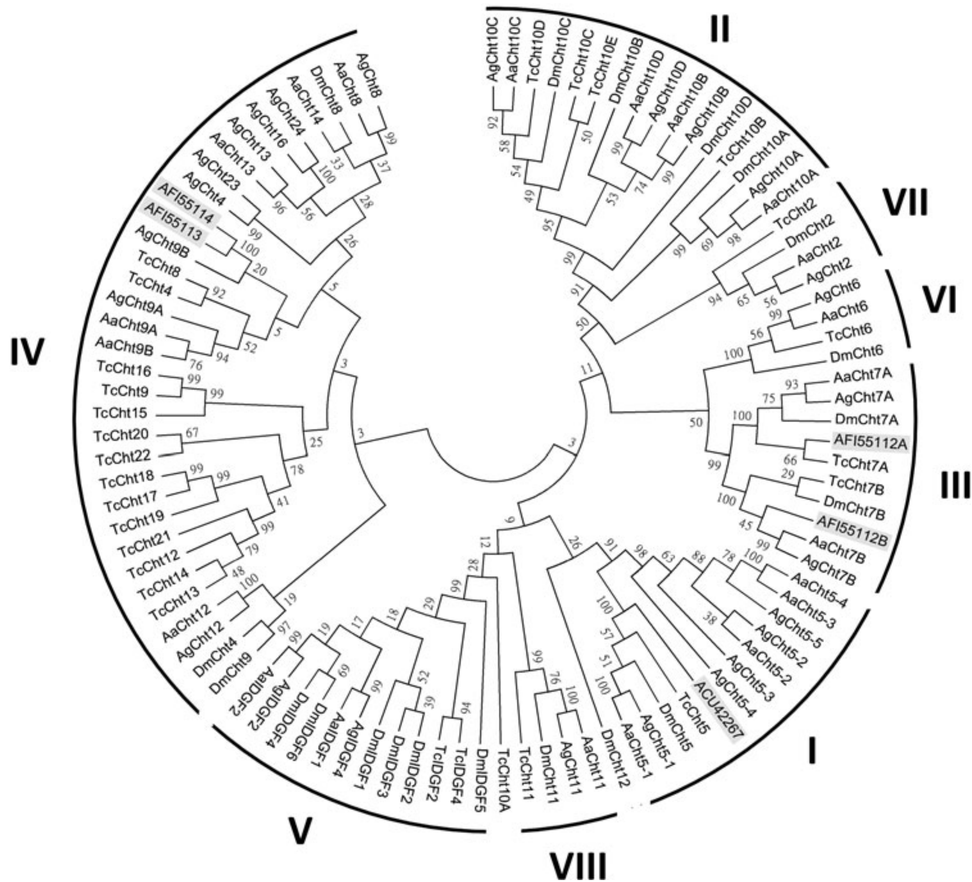

Fig. 1. Phylogenetic analysis of chitinase proteins based on catalytic domain sequences. The RACE-PCR cloned chitinase (accession: ACU42267, AFI55112, AFI55114 and AFI55113) and chitinases listed in table S4 were used. Phylogenetic trees of insect chitinases were generated with the MEGA6 software after alignment using ClustalX2 software. Bootstrap values were obtained with the neighbor-joining method using 5000 replications. The letters A–E denote multiple CDs from N- to C-terminuses in the same protein.

Expression of PxCht *genes in different developmental stages and tissues*

The stage- and tissue-specific expression patterns of the *PxCht* genes were analyzed with RT-PCR. Fig. 5 shows that *PxCht1*, *PxCht2-1*, *PxCht2-2*, *PxCht2-3*, *PxCht5*, *PxCht7*, *PxCht8-1*, *PxCht8-2* and *PxIDGF* were expressed at all developmental stages, *PxCht6* and *PxCht-h* were expressed in most stages except for the adult stage, and *PxCht10* was not detected in pupae and adults. *PxCht3* was detected only at the prepupal and pupal stages, and *PxCht25-1* and *PxCht25-2* were larval stage specific. Based on the tissue-specific expression patterns, *PxCht5*, *PxCht7* and *PxIDGF* were expressed in all the tissues that were examined, *PxCht1*, *PxCht6*, *PxCht10* and *PxCht-h* were expressed in most of the tissues, *PxCht1*, *PxCht10* and *PxCht-h* were not detected in the hemolymph, and *PxCht6* was not expressed in the gut tissue. Additionally, *PxCht8-1*, *PxCht8-2*, *PxCht25-1* and *PxCht25-2* were gut-specific, and *PxCht2-1*, *PxCht2-2* and *PxCht2-3* were carcass-specific. Furthermore, the expression of *PxCht3* was not found in all the tissues dissected from fourth instar larvae.

Discussion

The first insect chitinase gene was cloned from *M. sexta* (Kramer *et al.*, 1993). In the early stages of research, the cDNA cloned from several insects indicated that only a single chitinase gene was found in each species. However, in later studies, large and diverse groups of the chitinase genes were found. The phylogenetic analysis based on the protein sequences of the CDs first assigned these chitinase proteins into five separate groups (I–V) (Zhu *et al.*, 2004, 2008*c*), and then the family of chitinase proteins was expanded to eight groups (I–VIII) (Zhang *et al.*, 2011*a*). Although *B. mori* was the first species from which multiple chitinase genes were identified (Kim *et al.*, 1998; Abdel-Banat & Koga, 2001; Daimon *et al.*, 2003), the progression of investigation on the entire family of lepidopteran chitinase genes is much slower than that of dipteran and coleopteran species. A better understanding of the lepidopteran chitinase genes was achieved as genome sequences became available (Pan *et al.*, 2012; Tetreau *et al.*, 2015). Currently, the chitinase family of genes is classified into 11 groups (groups I–X and h, a lepidopteran-specific chitinase) (Tetreau *et al.*, 2015).

In this study, we successfully identified 15 individual chitinase genes from *P. xylostella*, which is a recalcitrant pest of several agricultural crops. Based on the phylogenetic and structural analyses, these chitinase genes were named in accordance with the nomenclature reported in recent studies (Zhang *et al.*, 2011*a*; Tetreau *et al.*, 2015). *PxCht5*, *PxCht7*, *PxCht25-1* and *PxCht25-2* were first cloned by RACE-PCR

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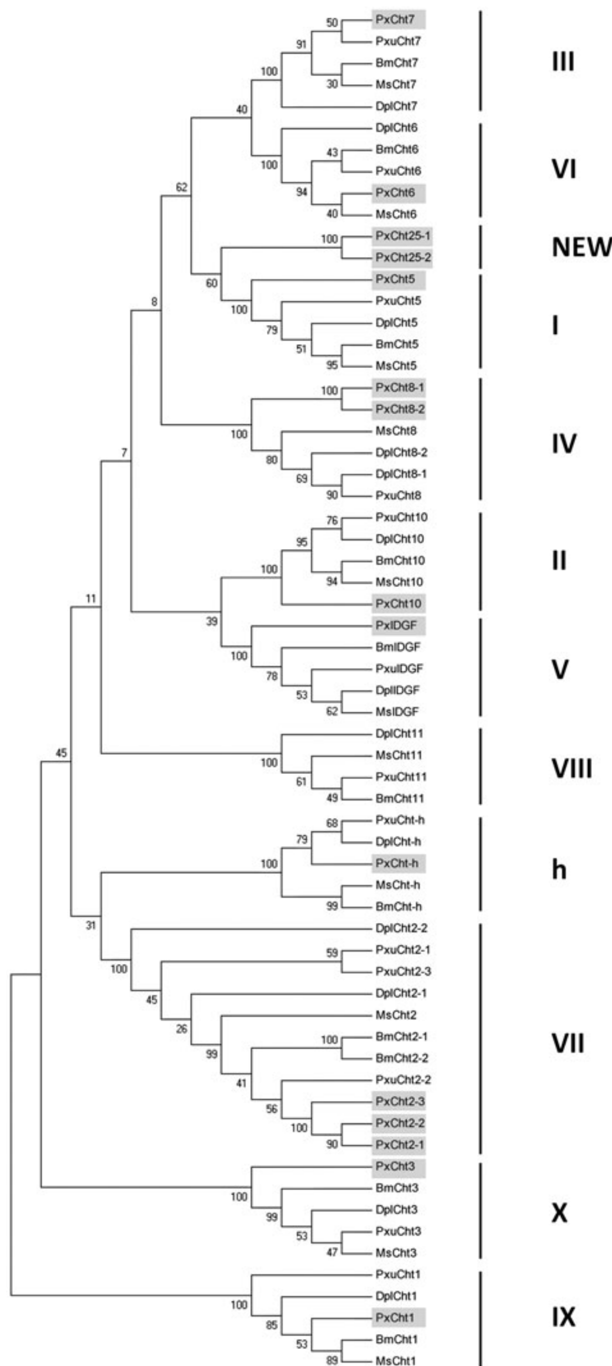

Fig. 2. Phylogenetic analysis of lepidopteran chitinase proteins based on catalytic domain sequences. The chitinase proteins listed in table 1 and table S5 were used. Phylogenetic trees of lepidopteran chitinases were generated with the MEGA6 software after alignment of first catalytic domain using ClustalX2 software. Bootstrap values were obtained with the neighbor-joining method using 5000 replications.

and were also detected in the later genome searching method later. Our cloned cDNA added 153 and 27 transcript bases to patch the genome assembly gaps in *PxCht5* and *PxCht7*, respectively. According to the results of the domain and phylogenetic analyses (figs 1, 2 and 3), *PxCht5* was classified in group I and was typically multi-domain, including a signal peptide, a CD, an S/T-rich linker that was heavily glycosylated, and a CBD with six consensus cysteines (belonging to chitin-binding module 14). Based on the results of RNA interference to silence the *TcCht5* gene, the group I chitinase might only be required for pupal-adult molting in *T. castaneum* (Zhu *et al.*, 2008*a*). However, the results were different in *S. exigua* and suggested that the group I chitinase had an important role during the larval–pupal and pupal–adult stages, and down-regulated expression caused abnormal and lethal effects (Zhang *et al.*, 2012). The current results suggested that *PxCht5* might be involved in multiple functions associated with chitin turnover because the transcripts were detected in all stages and tissues, similar to other lepidopteran group I chitinases (fig. 5).

PxCht7 contained 2 CDs and 1 CBD, which was a characteristic of group III chitinases (fig. 3). The *Cht7* proteins typically possess a transmembrane region at the N-terminus and are predicted to be membrane-anchored proteins. Additionally, a signal peptide existed in front of the transmembrane segment of the *Cht7* proteins in *D. melanogaster* and *A. gambiae* but not in *T. castaneum* (Zhu *et al.*, 2008*c*) and *M. sexta* (Tetreau *et al.*, 2015). In the current study, *PxCht7* had only a signal peptide and lacked a transmembrane region. The lack of a transmembrane region in *PxCht7* suggested that the protein was not membrane-anchored, similar to the other putative group III chitinases. For example, a chitinase from the hard tick *Haemaphysalis longicornis* (You *et al.*, 2003) is the only biochemically well characterized group III chitinase that contains only a signal peptide in front of 2 CDs. The chitinase of hard ticks is located between the old and the new cuticle in molting nymphs, suggesting a role in molting. Another example is *Cht4* from a pea aphid (*Acyrthosiphon pisum*) (Nakabachi *et al.*, 2010), whose expression level is dominant in embryos, indicating that this chitinase was essential for embryonic development in aphids. In *T. castaneum*, *Cht7* functions in tissue differentiation rather than in molting because when the *TcCht7* was silenced, there was defective abdominal contraction, elytral expansion and hindwing folding (Zhu *et al.*, 2008*a*). *PxCht7* was expressed in all stages and tissues (fig. 5); however, the function of group III chitinases in Lepidoptera remains unclear.

PxCht25-1 and *PxCht25-2* were cloned by using the same primers because their open reading frame shared a high level of sequence similarity, and the major difference between the two mRNA sequences was found in the 3'UTR. Table 2 shows the percentage of protein sequence identity. Similar to multiple chitinases from Dipteran and *T. castaneum* that could not be clustered in other groups, *PxCht25-1* and *PxCht25-2* were tentatively placed in the most divergent group IV (fig. 1). In the phylogenetic relationship among Lepidopteran chitinases, they fell in group I with NJ tree (fig. 2) but not with Maximum likelihood tree (fig. S1). Based on the sequence similarity and the phylogenetic analysis, we proposed that *PxCht25-1* and *PxCht25-2* were paralogous genes that could be in a new group of the chitinase family because they could not form monophyletic groups with any known chitinase consistently. Both *PxCht25-1* and *PxCht25-2* proteins possessed a signal peptide and a CD (fig. 3) and showed similar patterns of expression, exclusively expressed in the larval stages and gut tissue, whereas *PxCht25-2* was highly expressed in the first and second larval instars (fig. 5). The domain architectures and expression patterns of *PxCht25-1* and *PxCht25-2* were

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Table 1. Information on genes encoding chitinase and chitinase-like proteins searched from *P. xylostella* genome and transcriptomic databases. Genome assembly gap found in scaffold is denoted with an asterisk, the presence of glutamic acid residues in the CD is shown in boldface, and NS represents not shown in the prediction.

Accession	Gene name	Gene locus (scaffold)	Length (aa)	SP	CD	CBD	PI	Mw
XP_011547843	PxCht1	LOC105380051 (13)	392	1–21	79–384	NS	8.80	44832.77
XP_011554399	PxCht2-l	LOC105385677 (104)	498	NS	**15–362**	NS	5.96	56452.84
XP_011549318	PxCht2-2	LOC105381318 (59)	517	1–21	**34–381**	NS	5.62	58465.06
XP_011553720	PxCht2-3a	LOC105385097 (93)	517	1–21	**34–381**	NS	5.71	58472.14
XP_011553722	PxCht2-3b		517	1–21	**34–381**	NS	5.74	58521.20
XP_011561741	PxCht3	LOC105391876 (329)	2146	NS	2–242	317–381 393–453 2078–2138	5.79	235937.30
ACU42267 XP_011558385	PxCht5	LOC105389028* (287)	558	1–19	**22–374**	501–558	5.01	62029.17
XP_011560386	PxCht6a	LOC105390728* (264)	2445	1–35	**38–412**	512–572,	5.11	271933.56
XP_011560387	PxCht6b		1694	1–35	**38–412**	512–572 1635–1693	8.31	186405.71
AFI55112 XP_011553989	PxCht7	LOC105385326* (87)	988	1–26	**96–441** **530–870**	921–979	6.05	111483.08
XP_011552347	PxCht8-l	LOC105383909 (119)	567	1–21	**24–373**	510–567	4.54	61766.47
XP_011562207	PxCht8-2	LOC105392303* (350)	567	1–21	**24–376**	510–567	4.60	61640.33
XP_011550146	PxCht10	OC105382027 (44)	3425	1–25	145–487 1190–1532 **2141–2488** **2576–2921** 3087–3396	522–577 1673–1726 1791–1845 1874–1927 1964–2016 2031–2084 3017–3068	5.78	382619.83
AFI55114 XP_011549424	PxCht25-l	LOC105381408 (199)	386	1–19	**21–362**	NS	4.63	41539.17
AFI55113 XP_011551844	PxCht25-2	LOC105383493 (57)	386	1–19	**21–362**	NS	4.67	41601.19
BAF36822 PXUG_V1_002709	PxIDGF		433	1–17	24–412	NS	7.69	47942.28
PXPG_V2_004242	PxCht-h		558	1–21	**155–541**	NS	5.68	61236.47
XP_011551549	PxENGase	LOC105383219 (173)	897	NS	98–157	NS	5.04	99792.63

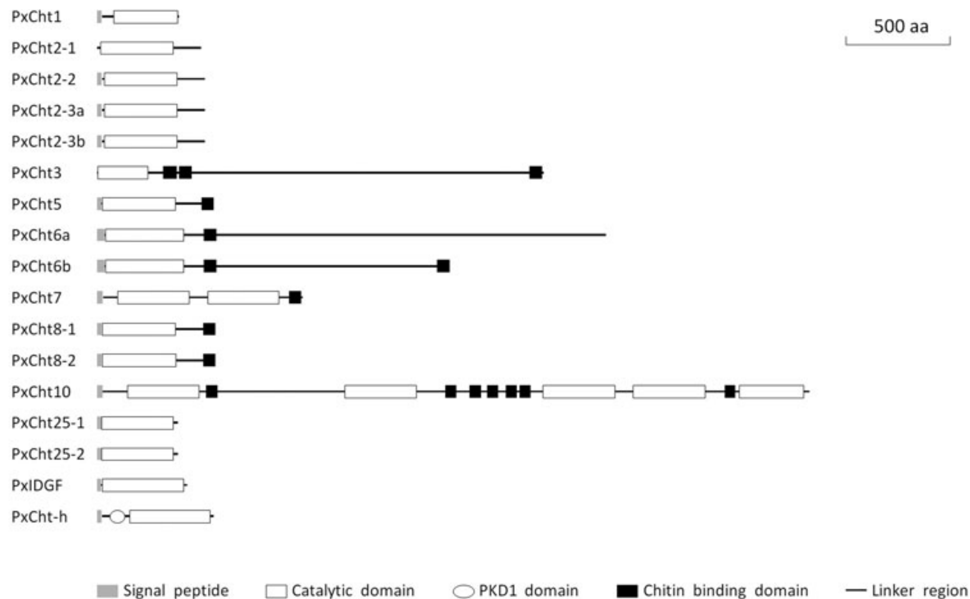

Fig. 3. Domain architecture of the chitinase proteins of *Plutella xylostella*. The domain structure was analyzed with the SMART tool (http://smart.embl-heidelberg.de/). The accession numbers of all the proteins used are listed in table 1.

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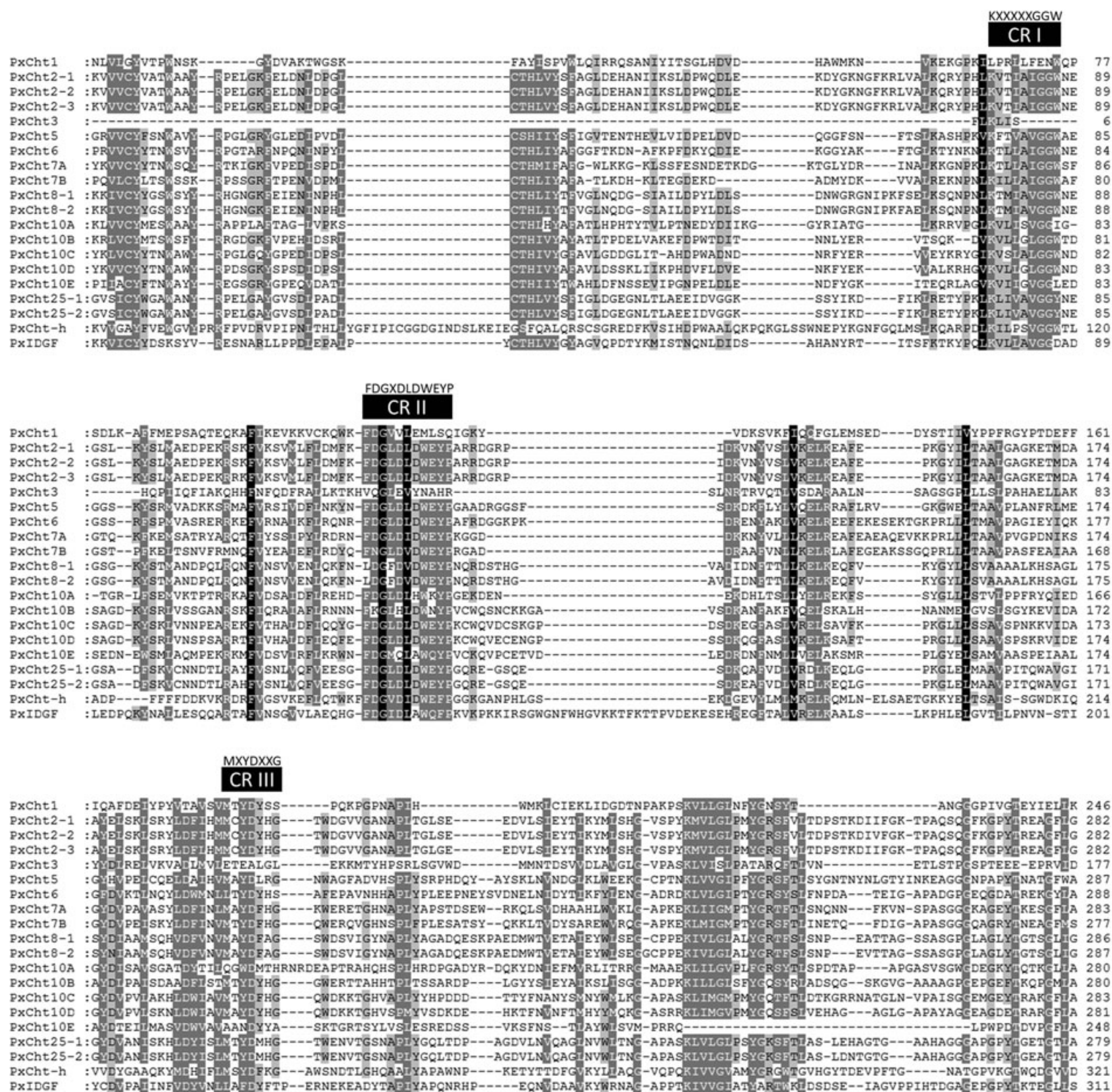

Fig. 4. Conserved regions in catalytic domains of chitinase proteins in *Plutella xylostella*. Multiple alignments of the CD sequences were generated with the GeneDoc tool. Four conserved regions (CR I-IV) are labeled. Their amino acid sequences are shown above the boxes. The accession numbers of all the proteins used are listed in table 1.

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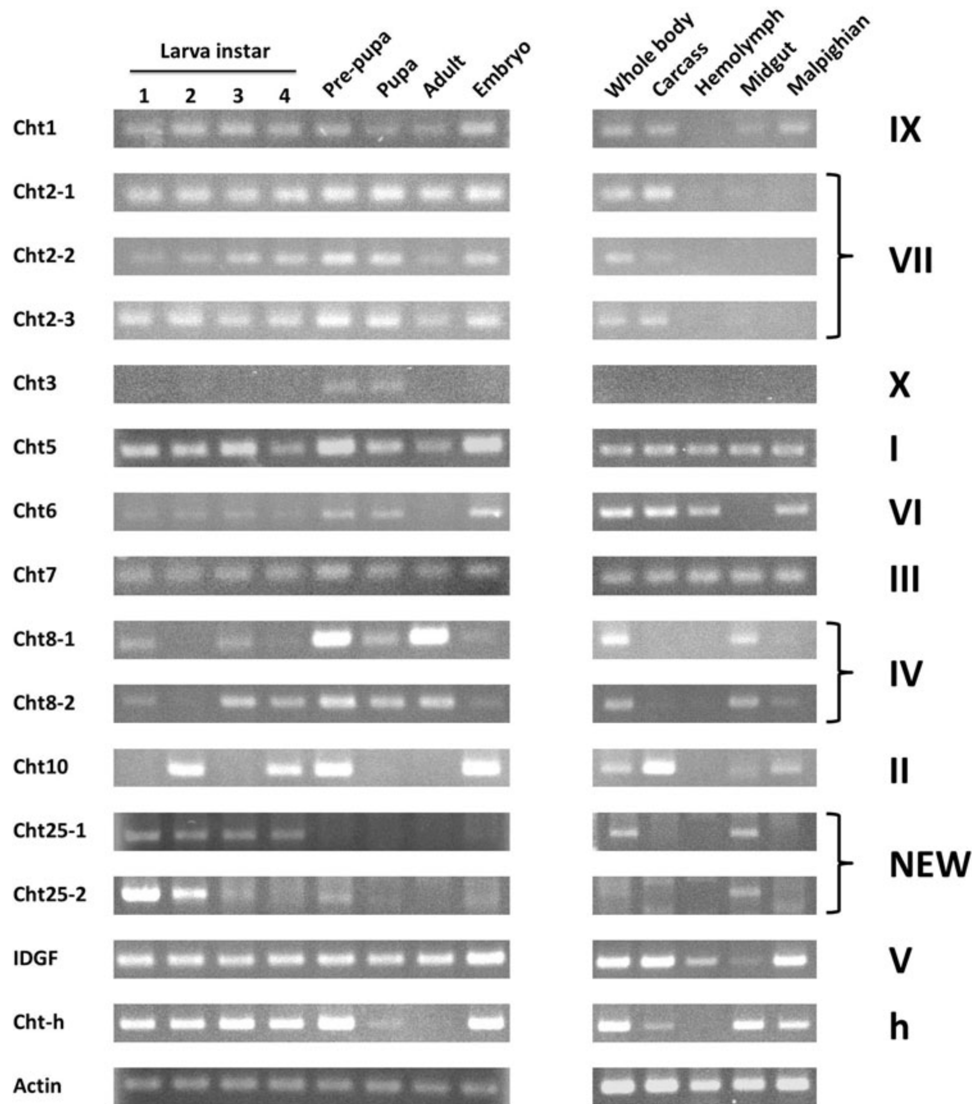

Fig. 5. Expression of *P. xylostella* chitinase genes in different developmental stages and different tissues as evaluated by RT-PCR. RNA samples were isolated from eight developmental stages and five tissues in the fourth instar larvae. The primers used for the RT-PCR are shown in table S3. The actin (*PxActin*) gene was used as the reference gene.

similar to one *O. nubilalis* chitinase, which was the first identified group IV chitinase in Lepidopteran (Khajuria *et al.*, 2010). The larval and gut-specific chitinases are important in regulating the chitin content of the peritrophic matrix (PM) and are essential for larval growth and development; *PxCht25-1* and *PxCht25-2* were likely to have a similar function.

Sequencing gaps were found in *PxCht8-2*, which lead to incorrect genome assembly. A genomic PCR was performed to obtain the missing sequences. The complete *PxCht8-2* genome sequence was reassembled and the deduced protein sequence length was updated from 370 to 567 bp (fig. S2). Most group IV chitinases are characterized by the absence of a CBD and an S/T-rich region and are expressed only in the gut or the fat body (Yan *et al.*, 2002; Arakane & Muthukrishnan, 2010). *PxCht8-1* and *PxCht8-2* were orthologous to *Cht8s* (fig. 2), members of group IV chitinases that contain a CBD (fig. 3) (Zhu *et al.*, 2008*c*; Tetreau *et al.*, 2015). Additionally, *PxCht8-1* and *PxCht8-2* had similar patterns of expression and were expressed exclusively in gut tissue and throughout all stages of development (fig. 5). No developmental defects were observed when the expression of *TcCht8* was down-regulated by RNAi (Zhu *et al.*, 2008*a*). Further experiments are required to determine whether the *Cht8s* have a role in the digestion of chitin-containing material or in the immunity against pathogens containing chitin.

Similar to the new group and group IV chitinases, group VII in *P. xylostella* consisted of multiple genes (*PxCht2-1*, *PxCht2-2* and *PxCht2-3*). Multiple group VII chitinase genes were also observed in *D. plexippus*, *B. mori* and *P. xuthus* (table S5). Multiple group I chitinases have been identified in three mosquito species, including five in *A. gambiae*, four in *Ae. aegypti* and three in *C. quinquefasciatus* (Zhang *et al.*, 2011*b*). A

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Table 2. Protein sequence identity profiles of *PxCht2s* (A), *PxCht8s* (B) and *PxCht25s* (C). *PxChts* belong to the same group and were aligned and statistically analyzed using the GeneDoc tool. Statistical reports show the calculations for exact matches, Juxtaposition greater than zero and aligned with gaps from top to bottom; absolute values are on the left and percent values are on the right.

A	PxCht2-1	PxCht2-2	PxCht2-3a	PxCht2-3b
PxCht2-1	498	94%	94%	94%
	0	95%	94%	94%
	0	3%	3%	3%
PxCht2-2	490	517	98%	99%
	493	0	99%	99%
	19	0	0%	0%
PxCht2-3a	488	511	517	99%
	491	515	0	99%
	19	0	0	0%
PxCht2-3b	489	512	514	517
	491	515	515	0
	19	0	0	0

B	PxCht8-1	PxCht8-2
PxCht8-1	567	95%
	0	96%
	0	1%
PxCht8-2	543	567
	552	0
	6	0

C	PxCht25-1	PxCht25-2
PxCht25-1	386	98%
	0	99%
	0	0%
PxCht25-2	379	386
	384	0
	0	0

gene cluster consisting of multiple *Cht5* genes may have resulted from gene tandem duplications. A similar phenomenon of gene duplication for two *LmCht5* genes in *Locusta migratoria* suggested that the gene duplication of *Cht5* might be not unique to the mosquito lineage (Li *et al.*, 2015). However, the gene duplication of group I chitinase was not observed in *P. xylostella*, but was observed in the new group and groups IV and VII. The locations of *PxCht25s*, *PxCht8s* and *PxCht2s* were on the different scaffold (table 1). The duplication of these chitinases in the new group and groups IV and VII have not been previously reported and the mechanisms are required to further determine. The similar patterns of expression among *PxCht2s* suggested that they might be involved in the chitin turnover associated with molting because the transcripts were detected primarily in the carcass (fig. 5).

The generation of multiple isoforms from a single group I chitinase gene through alternative splicing was first reported in *B. mori* (Abdel-Banat & Koga, 2002). Both *PxCht2-3* and *PxCht6* were predicted to have two transcript variants, and *PxCht6* is a member of the group VI chitinases (fig. 2). The alternative splicing event in *PxCht6* led to the generation of two isoforms that differed in protein length and structure, and *PxCht6b* had an additional CBD at the C-terminal region (fig. 3). A similar phenomenon was also found for *MsCht6* during the search for the transcripts in Manduca Base (ftp://ftp.bioinformatics.ksu.edu/pub/Manduca/OGS2/OGS2_20140407_ transcripts.fa). In *PxCht2-3*, the alternative splicing event led to equal lengths and only three differences in their deduced amino acid sequences (table 2).

The group II chitinase (*PxCht10*) was large chitinase that had multiple CDs and CBDs (fig. 3), whose number and location showed conserved arrangements in the same order. The arrangement of CD and CBD units in lepidopterans is Ⓒ-[D]-Ⓒ-[D]-[D]-[D]-[D]-[D]-Ⓒ-Ⓒ-[D]-Ⓒ in which Ⓒ represents a CD and [D] represents a CBD, according to three group II chitinases from *B. mori* (Pan et al., 2012), *M. sexta* (Tetreau *et al.*, 2015) and *Danaus plexippus* (GeneBank: EHJ65741). Dipterans have 4 CDs and 4 CBDs (Zhu *et al.*, 2008*c*; Zhang *et al.*, 2011*a*), with the arrangement Ⓒ-[D]-[D]-[D]-Ⓒ-Ⓒ-[D]-Ⓒ, and Coleopterans have 5 CDs and 5 CBDs (Royer *et al.*, 2002; Arakane & Muthukrishnan, 2010), with the arrangement Ⓒ-[D]-Ⓒ-[D]-[D]-[D]-Ⓒ-Ⓒ-[D]-Ⓒ. A comparison of the CRIIs in each of the CDs from the three lepidopteran group II chitinases showed that the glutamate residue was uniformly detected in the third and the fourth CRIIs. *TcCht10* was expressed in all stages and played a vital role in the embryo hatch, larval molt, pupation and adult metamorphosis (Zhu *et al.*, 2008*a*); *PxCht10* might be not involved in pupation and adult metamorphosis because the transcripts were not detected in the pupal and adult stages (fig. 5). However, further investigations are needed to verify this assumption.

PxCht1 was in group IX (fig. 2) and was previously identified as Stabilin-1 interacting chitinase-like protein (SI-CLP). The SI-CLP proteins are secreted by lysosomes to interact with the transmembrane receptor stabilin-1 and are involved in protein sorting during endocytosis (Kzhyshkowska *et al.*, 2006). Similar to *MsCht1* (Tetreau et al., 2015), *PxCht1* had a different set of conserved residues in CRII (fig. 4). In group I to IV chitinases, the consensus sequence for CRIIs consisted of FDG(L/F)DLDWE(Y/F)P, whereas the CRII sequence in *PxCht1* was FDGVVLEMLSQ, which was devoid of the residue E, only had the first aspartic acid and appeared to lack the other acidic groups shown to influence catalytic activity (Lu *et al.*, 2002; Zhang *et al.*, 2002). *PxCht3* was in group X (fig. 2), which contained one CD followed by two very closely spaced tandem CBDs and a very long C-terminal stretch ending with a third CBD (fig. 3). *PxCht3* appeared to be missing the CRI and have suffered an alteration of the typical CRII to VQGLE (fig. 4), which was similar to*MsCht3* (Tetreau *et al.*, 2015). Notably, different patterns of expression were found in group IX and X chitinases between *P. xylostella* and *M. sexta* (fig. 5) (Tetreau *et al.*, 2015). *MsCht1* was expressed exclusively in adult testes and ovaries, whereas *PxCht1* was expressed in all stages, and *MsCht3* was expressed in the fourth instar larvae, whereas *PxCht3* was expressed exclusively in the pupae.

One *PxIDGF* sequence was reported previously in the NCBI (accession AB282642) and was also found in KONAGAbase (PXUG_V1_002709). All IDGFs were in group V chitinases (Zhu *et al.*, 2008*c*). The crystalline structure and the homology modeling suggest that all chitinase-like proteins have a (βα)8 triose-phosphate isomerase barrel structure (Varela *et al.*, 2002), and the group V chitinase-like proteins have an extra loop between the β−4 strand and the α−4 helix (Zhu *et al.*, 2004, 2008*c*). The growth-promoting function was demonstrated on *Drosophila* IDGFs (Kawamura *et al.*,

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1999). The IDGF proteins carry carbohydrate-binding activity but lack enzymatic activity (Zhu *et al.*, 2008*b*) and most likely act as chitolectins and bind to cell surface receptors or glycoproteins. Furthermore, in the current study, *PxIDGF* was expressed at all developmental stages and in all tissues (fig. 5), which was consistent with previous findings in *B. mori* (Pan *et al.*, 2010) and *M. sexta* (Tetreau *et al.*, 2015).

PxCht-h was identified in KONAGAbase (PXPG_V2_004242). *Cht-h* is a chitinase-like gene specific to lepidopteran insects (Zhang *et al.*, 2011*a*). Because the deduced amino acid sequences showed extensive structural similarities with bacterial and baculoviral chitinases, *Cht-h* might be derived from a bacterial or baculoviral chitinase gene through horizontal gene transfer. Additionally, the *Cht-h* proteins exhibit exo-chitinase activities that act in concert with endo-chitinases. In an immunohistochemical study, the *B. mori Cht-h* was localized in the chitin-containing tissues during the molting stages (Daimon *et al.*, 2005). Moreover, a lethal effect was observed at the pupal and adult stages after injection with *Cht-h* dsRNA into *S. exigua* larvae indicating that *SeCht-h* played an important role in the larval-pupal and pupal-adult stages (Zhang *et al.*, 2012). However, *PxCht-h* might be not involved with the pupal-adult stage because of low and no expression in the pupal and adult stages, respectively (fig. 5).

An endoβ-*N*-acetylglucosaminidase (ENGase) (EC 3.2.1.96), which belong to the glycosyl hydrolase family 85 (GH85), was also detected in our genome-wide search (table 1). The ENGases are included in the GH18 chitinase-like superfamily because they are phylogenetically closely related to the GH18 chitinases (Funkhouser & Aronson, 2007). Moreover, both *PxIDGF* and *PxCht-h* were only detected in KONAGAbase but not in the genome search, and group VIII (*Cht11*) was not identified using either search approach. Tetreau *et al.* (2015) described the evolution of the 11 groups of chitinases in 14 species from seven different phylogenetic groups and suggested that group VIII chitinases were found in all insect species and even in Nematoda (Tetreau *et al.*, 2015). Therefore, the identification of *PxCht11* is expected upon release of a new version of the DBM genome.

The insect chitinase families most likely evolved from a common ancestor, which was evident because the chitinases shared a high degree of conservation of amino acid sequences and domain organization in each group. Lepidopteran insects have a similar assortment of genes that encode chitinase and chitinase-like proteins but differ in the total number compared with the other insect orders. In conclusion, we identified 15 chitinase genes in *P. xylostella*, and we showed the phylogenetic relationships with other lepidopteran chitinases. Although we did not identify a group VIII chitinase, one new phylogenetic group of chitinases was found in addition to the 11 groups described previously. The different patterns of expression suggest that the DBMs carry numerous chitinase proteins to efficiently degrade the different types of chitin (α, β and γ forms) and the modified forms (deacetylated chitin) that occur in different extracellular structures and developmental stages (Merzendorfer & Zimoch, 2003). Additional experiments are required to determine which of these chitinases are enzymatically active and to characterize their functions in the chitin metabolism of insects. In our laboratory, the expressions of *PxCht5*, *PxCht7* and *PxCht25* genes were performed in *Escherichia coli*, yeast and a baculovirus system for enzymatic characterization (data not shown). A better understanding of the chitinases in Lepidoptera might help to develop novel chitin-targeted strategies for pest control.

Supplementary Material

The supplementary material for this article can be found at http://dx.doi.org/10.1017/S0007485316000511.

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