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Five hepatopancreatic and one epidermal chitinases from a pandalid shrimp (*Pandalopsis japonica*): Cloning and effects of eyestalk ablation on gene expression

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ABSTRACT

Six cDNAs encoding chitinase proteins in *Pandalopsis japonica* were isolated by using polymerase chain reaction (PCR) cloning methods and bioinformatic analysis of expressed sequence tags (ESTs). The cDNAs, designated Pj-Cht1, 2, 3A, 3B, 3C, and 4, encoded proteins ranging from 388 to 607 amino acid residues in length (43.61–67.62 kDa) and displayed a common structural organization: an N-terminal catalytic domain, a Thr/Pro-rich linker region, and either 0 (Pj-Cht2, 3A), 1 (Pj-Cht1, 3B, and 3C), or 2 (Pj-Cht4) C-terminal chitin-binding domain(s) (CBD). Pj-Cht1 and 2 lacked the 5′ end of the open reading frame (ORF); the other Pj-Chts contained the complete ORF. All known decapod crustacean chitinases were segregated into at least four groups based on phylogenetic analysis and domain organization. Group 1 chitinases, represented by Pj-Cht1, were most closely related to insect group I chitinases and may function in the digestion of the peritrophic membrane. Group 2 chitinases including Pj-Cht2 show different domain organizations and pI value from other chitinases and appear to function in degradation of the old exoskeleton during the premolt period. Group 3 chitinases, represented by Pj-Cht3A, 3B, and 3C, may function in digestion of chitin-containing food and defense against pathogens. Group 4 chitinases, represented by Pj-Cht4, have two CBDs and their functions are unknown. Five Pj-Chts (Pj-Cht1, 3A, 3B, 3C, and 4) are expressed in the hepatopancreas and intestine, whereas Pj-Cht2 is expressed in epidermis and SG/XO complex suggesting crustacean chitinases can be classified into two groups (hepatopancreatic and epidermal) based on the expression profile. Eyestalk ablation (ESA) down-regulated the hepatopancreatic chitinase expression (Pj-Cht1, 3A, and 3C); Pj-Cht3B expression was not significantly affected by ESA. By contrast, mRNA levels of Pj-Cht2 were significantly upregulated in 7 days post-ESA. Pj-Cht4 mRNA levels were too low for measurement with quantitative polymerase chain reaction. ESA had no significant effect on chitinase expression in the intestine. These data indicate that Pj-Cht1, 3A, 3B, 3C, and 4 are hepatopancreatic chitinases that may function in the digestion of ingested chitin and the modification of peritrophic membrane in the intestine. By contrast, epidermal chitinase, Pj-Cht2 may play a role in chitin metabolism during molt cycle as shown in other crustacean group 2 chitinases.

1. Introduction

Chitin is a linear polymer of β-1, 4-linked N-acetyl-D-glucosamine that forms the major structural constituent of the exoskeleton and the intestinal peritrophic membrane (PM) in arthropods (Kramer and Koga, 1986; Cabib, 1987; Kramer and Muthukrishnan, 1997; Lehane, 1997). The articulated exoskeleton provides protection, mechanical support, and an attachment framework for the musculature (Cisne, 1974; Lehane, 1997). The PM protects the intestinal lining from abrasion by food particles. Turnover of chitinous structures, controlled by several chitinolytic enzymes, is essential for arthropod growth and development (Zou and Bonvillain, 2004; Merzendorfer, 2006). During the premolt period the epidermis secretes chitinases that degrade the inner layers of the old exoskeleton while synthesizing a new exoskeleton (Zou and Bonvillain, 2004). The digestive tract secretes chitinases that degrade ingested chitin, modify the PM, and defend against viral pathogens (Watanabe et al., 1998; Pan et al., 2005; Zhang et al., 2010). Chitinase (1,4-β-poly-N-acetylglucosaminidase, EC 3.2.1.14) is an endoglycosidase that cleaves internal linkages in chitin polymers, producing oligomeric fragments and chitobiose. These, in turn, are hydrolyzed to N-acetyl-β-glucosamine by N-acetyl-β-glucosaminidase and chitobiosidase (Tronsmo and Harman, 1993; Zou and Bonvillain, 2004).

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Due to their importance in growth and development, many genes encoding chitinolytic proteins have been isolated and characterized in insect species (Zhu et al., 2008a). Genome-wide analysis has identified more than a dozen new genes encoding chitinolytic enzymes. Sixteen genes encoding chitinases or chitinase-like proteins occur in *Tribolium castaneum* and *Drosophila melanogaster* and 20 genes occur in *Anopheles gambiae* (Zhu et al., 2008a; Zhang et al., 2011). Functional studies suggest that the chitinases have specific roles in insect growth and development (Zhu et al., 2008b).

In contrast to insect species, no genome-wide analysis has been carried out in crustacean species and cDNAs encoding chitinase and chitinase-like proteins have been characterized in relatively few species. Most studies of crustacean chitinases were from Dendrobranchiata, most of which are commercially important species. cDNAs encoding three different chitinases (to avoid confusion we renamed the MjCht1, Cht2, and Cht3) were isolated from kuruma prawn, *Marsupenaeus japonicus* (Watanabe et al., 1996; Watanabe and Kono, 1997; Watanabe et al., 1998). In addition to *M. japonicus*, a full length chitinase cDNA (PmChi-1) was isolated from tiger shrimp, *Penaeus monodon*, which was differentially expressed in the hepatopancreas during the molt cycle (Tan et al., 2000). More recently, three chitinases (PmChi1, Chi2 and Chi3) were identified from *P. monodon* (Proespraiwong et al., 2010). PmChi-1 and PmChi1 appear to be encoded by the same gene; PmChi1 has a 46-amino acid extension at the C-terminus (Proespraiwong et al., 2010). It might be possible that differences in the PmChi-1 and PmChi1 sequences were due to variation in shrimp stocks (Proespraiwong et al., 2010). Most recently, at least six chitinase cDNAs (LvCht1 to Cht6) were identified from *Litopenaeus vannamei* (Huang et al., 2010), suggesting that more chitinase-like genes are present in decapod crustacean genomes.

Compared with those of insect species, the biochemical properties of crustacean chitinases are not well established. A recombinant *P. monodon* chitinase1 protein from the bacterial expression system (rPmChi1) has maximal activity at pH 5 (Proespraiwong et al., 2010). Chitobiase activity in *L. vannamei* was also measured using the chitin analog, *p*-Nitrophenyl-N-acetyl-β-D-glucosaminide (pNP-β-D-GlcNAc). Its optimum activity occurs at pH 5.2 suggesting that the optimal pH for crustacean chitinolytic enzymes is acidic (Xie et al., 2004).

A major production site for the chitinase genes is the hepatopancreas. For example, cDNAs encoding chitinases from *L. vannamei* (LvCht1, LvCht3) are expressed in the hepatopancreas (Huang et al., 2010). Similar results were also found for *M. japonicus* chitinases MjCht1and MjCht3 (Watanabe et al., 1996, 1998). By contrast, several chitinases are expressed in other tissues. MjCht2 from *M. japonicus* is expressed in the tail fan and blade at the onset of molting (Watanabe and Kono, 1997). LvCht2 from *L. vannamei* is expressed at low levels in cuticular tissues (Huang et al., 2010). These results suggest that chitinases expressed in integument may be involved in molting.

In this study, we identified six distinct cDNA sequences encoding chitinases from *Pandalopsis japonica* (designated Pj-Cht1, 2, 3A, 3B, 3C and 4). Structural and phylogenetic analysis classified the Pj-Chts and other decapod chitinases into four distinct groups. Tissue expression profiles of each Pj-Cht were determined using end-point polymerase chain reaction (PCR), which supported our classification based on structural characteristics. Quantitative PCR (qPCR) was used to determine the effects of eyestalk ablation (ESA) on the expression of each Pj-Cht in the intestine and hepatopancreas.

2. Materials and methods

2.1. Animals and experimental treatment

Intermolt adult shrimp, *P. japonica* were purchased from a local seafood market and kept in seawater tanks at 6 °C. After 3 days of acclimation, eyestalks were removed and the wound was cauterized to minimize loss of hemolymph. Intact animals maintained under the same conditions served as controls. Tissues from intact, 3 days post-ESA, and 7 days post-ESA animals were frozen in liquid N_2 and stored at −70 °C until RNA extraction.

2.2. Isolation of an initial chitinase-like cDNA by the differential-display reverse transcription-PCR (DDRT-PCR)

A partial chitinase cDNA sequence, designated Pj-Cht1, was initially isolated from DDRT-PCR using cDNA synthesized from hepatopancreas from intact and ESA animals. Total RNA was purified from the different tissues using Trizol reagent according to the manufacturer's instructions (Invitrogen, USA), quantified by absorbance at 260 nm (Nanodrop Technologies, Inc., USA), and stored at −70 °C. DDRT-PCR was carried out using the Gene Fishing™ DEG Premix Kit (Seegene Inc., Korea) according to the manufacturer's instructions. The differently amplified PCR products between the control and ESA groups were isolated from gel slices using Gel Extraction Kit (Geneall Inc., Korea), ligated into TA plasmid vector with the pGEM-T Easy Cloning Kit (Promega, USA), and transformed into One Shot Top 10 *E. coli* strain (Invitrogen, cDNAs were sequenced with an automated DNA sequencer (ABI Biosystem, USA). The nucleotide sequence similarities were examined by BLAST software (http://www.ncbi.nlm.nih.gov/BLAST/).

5′ Rapid Amplification of cDNA Ends (RACE) was used to obtain the remainder of the open reading frame (ORF) and the 5′ untranslated region (UTR). The DNA Walking SpeedUp™ Kit (Seegene, Korea) was used according to the manufacturer's instructions. All primers used in the experiment were designed using IDTSciTools program (http://www.idtdna.com/SciTools/SciTools.aspx) and were synthesized by Bioneer Company, Korea (Table 1). For 5′ RACE three consecutive nested PCR reactions with sequence specific reverse primers (Table 1) and the manufacturer's universal forward primers were carried out. Reaction mixtures (20 μL) contained 1 μL cDNA (100 ng), 2 μL 5 μM universal primers, 1 μL 10 μM reverse specific primers (Table 1), 0.1 μL rTaq polymerase (Takara Bio Inc., Japan), 1.33 μL dNTP (2.5 mM each), and 2 μL buffer (10×). The first PCR was done in two steps: a first step of one cycle at 94 °C for 5 min, 42 °C for 1 min, and 72 °C for 2 min, in which only the universal primer annealed to the complementary sequence of the cDNA template; a second step of 30 cycles at 94 °C for 30 s, 65 °C for 40 s, and 72 °C for 100 s. The second and third PCRs were the same as the second step of the first PCR. 5′ RACE of other two Pj-Chts (3A and 3C) were performed as described above.

2.3. 3′ RACE PCR

The 3′ ends of the Pj-Cht2, 3A, 3C were obtained by 3′ RACE. cDNA was synthesized in a reaction (10 μL) containing 2 μg total RNA, 0.5 μL DNase1, 1 μL 10x DNase buffer. Total reaction was kept 37 °C for 30 min followed by 70 °C for 10 min. After adding 1 μL 20 μM oligo-dT primer (Table 1) and 4 μL dNTPs (2.5 mM each) the reaction was terminated by heating at 70 °C for 5 min and was chilled on ice for 2 min. 5x First-strand buffer (4 μL), 2 μL dithiothreitol (0.1 M), and 1 μL RNase out (Invitrogen, USA) were added and the reaction was incubated at 37 °C for 50 min. Enzyme inactivation was done for 70 °C for 15 min. cDNA was quantified and stored at −20 °C. PCR was carried out using sequence-specific forward primers and linker primers (Table 1). The reaction (30 μL) contained 1 μL cDNA (100 ng), 1 μL primers (10 μM), 0.2 μL Ex Taq Polymerase (Takara), 2 μL dNTPs (2.5 mM each) and 3 μL 10x buffer (Takara). PCR conditions were 1 min at 94 °C; 30 cycles at 94 °C for 1 min, 50 °C for 30 s, and 72 °C for 1 min; and post extension for 5 min at 72 °C. PCR products were purified, cloned, and sequenced as described above.

Table 1
Primers used to clone cDNAs encoding *P. japonica* chitinases.

Primer name	Sequence(5′-3′)	Description
Pj-Cht1 R1	GATGTGGGTACAGAGGTCTGCAG	5′ Race reverse specific primer
Pj-Cht1 R2	GAAGATTCTCGACGTCGTAGATTGCTTC	5′ Race reverse specific primer
Pj-Cht1 R3	CGACGTCGTAGATTGCTTCTTCTGGC	5′ Race reverse specific primer
Pj-Cht1 FC	AATTGAAGACCACCCTAGCTGTTG	ORF confirm forward primer
Pj-Cht1 RC1	ATGAACAGGGCGTTTCACCATGTC	ORF confirm reverse primer
Pj-Cht1 RC2	CTACTTCAGTGACACGTGTACCAT	ORF confirm reverse primer
Pj-Cht2 DEG F1	TGYTAYTWYACNAAYTGGGCN	1st degenerate forward primer
Pj-Cht2 DEG F2	TGGGCNTGGTAYMGNCMN	2nd degenerate forward primer
Pj-Cht2 DEG R1	RTCNARRTCNARNGCCCADAT	1st degenerate reverse primer
Pj-Cht2 DEG R2	NGCCCADATCATNCCNCC	2nd degenerate reverse primer
Pj-Cht2 F1	AGAACAGAATCGGACCCTACGCCTACTCA	3′ Race 1st specific forward primer
Pj-Cht2 F2	AAGAAGTCTCAATACATCCGGGAT	3′ Race 2nd specific forward primer
Pj-Cht2 PCF1	AGTACAGCCAGAGGATATCGAC	Sequence confirm forward primer
Pj-Cht2 PCF2	GACCCCCACCCATCTGTACCCA	Sequence confirm forward primer
Pj-Cht2 PCR1	TCATTGATGATTGATAAAGATCATATGC	Sequence confirm reverse primer
Pj-Cht2 PCR2	CACCCATACTATAAATTGAAATGGTTG	Sequence confirm reverse primer
Pj-Cht3A R1	GTCATTATAAGGATCGAACACC	5′ Race reverse specific primer
Pj-Cht3A R2	CCGGAATTCTTCAGTCCAGCA	5′ Race reverse specific primer
Pj-Cht3A R3	GTTGGGTCGATGTCCTCTACTTC	5′ Race reverse specific primer
Pj-Cht3A 3F	CATTTGACGACGTGGTTCATC	3′ RACE forward primer
Pj-Cht3A FC	CATTTGACGACGTGGTTCATC	ORF confirm forward primer
Pj-Cht3A RC	GGCTAATGGGTTGACGAGGAT	ORF confirm forward primer
PjCht3B FC	GTTCATCATGAAGTTGTTACTCCT	ORF confirm forward primer
Pj-Cht3B RC	CTTGCGTCTTCTACTATGCTGGG	ORF confirm reverse primer
Pj-Cht3C R1	CCAAGTCCAGCAAATGCATAGATGAG	5′ Race reverse specific primer
Pj-Cht3C R2	CCCAAGAGCTGAAGTAGCATACCATTG	5′ Race reverse specific primer
Pj-Cht3C R3	CTCAGCGAGGGAGAGGTAGAC	5′ Race reverse specific primer
Pj-Cht3C-F1	CCTTCATCGACTCGTCAATTG	3′ Race 1st specific forward primer
Pj-Cht3C F2	GAAGGCTCATGACTTCGATGG	3′ Race 2nd specific forward primer
Pj-Cht3C FC1	TTGCCAACAGCCCATCTTGGC	ORF confirm forward primer
Pj-Cht3C FC2	AACAGCCCATCTTGGCGTGGTT	ORF confirm forward primer
Pj-Cht3C RC	GCTGTGCTAAGTCAGTTCTCAGC	ORF confirm reverse primer
Pj-Cht4 F1	CAACATTCTCAGGATGAGGTTCTTG	ORF confirm forward primer
Pj-Cht4 F2	CAGGATGAGGTTCTTGTGTCTC	ORF confirm forward primer
Pj-Cht4 R1	GCAAACAACAAAAGTAGAGGCCTAC	ORF confirm reverse primer
Pj-Cht4 R2	CTATAAACTTTTCATATCTCTTTTCCAT	ORF confirm reverse primer
M13F (−40)	CAGGAAACAGCTATGAC	Vector FWD primer for DNA sequencing
M13R (−20)	GTAAAACGACGGCCAG	Vector RVS primer for DNA sequencing
DEG-3Race	CTGTGAATGCTGCGACTACG	3′ Race 1st reverse primer
ACP3	TGAATGCTGCGACTACGA	3′ Race 2nd reverse primer
Oligo-dT	TTTTTTTTTTTTTTT	Primer for RT reaction for 3 RACE
DEG ACP 86	GTCTACCAGGCATTCGCTTCATIIIIIAGGCGCTCTC	Specific FWD primer for DDRT-PCR
DEG ACP2	CTG TGA ATG CTG CGA CTA CGA TII III T(15)	Specific RVS primer for DDRT-PCR

2.4. Expressional analysis of chitinase mRNAs

The tissue expression of Pj-Cht1, 2, 3A, 3B, 3C and 4 was examined using end-point RT-PCR and qPCR. Total RNA was isolated and quantified as described above. cDNA was synthesized from the total RNA of gill, epidermis, gonad, hepatopancreas, deep abdominal flexor and extensor muscles, heart, thoracic and abdominal ganglia, brain, sinus gland, and intestine. RNA preparations were treated with DNaseI (Promega, USA) to remove genomic DNA. cDNA was synthesized using random hexamer as primers for the reverse transcription. Reactions (20 μL) contained 1 μL cDNA (100 ng), 1 μL 4 μM sequence specific primers (Table 2), 0.2 μL Ex Taq polymerase (Takara), 2 μL dNTP (2.5 mM each), and 2 μL 10x buffer. PCR conditions were 3 min at 94 °C, followed by 30 cycles at 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s, and final extension at 72 °C min for 5 min. PCR products were separated by 1.5% agarose gel electrophoresis and stained with ethidium bromide. The sequence-specific primers for each target gene were designed using the Primer Quest tool (http://www.idtdna.com) and 18SrRNA primers served as a positive control.

qPCR was carried out using the DNA Engine Chromo4 Real-Time Detector (Bio-Rad, USA) to measure expression levels of Pj-Cht1, Pj-Cht2, Pj-Cht3A, Pj-Cht3B, Pj-Cht3C and Pj-Cht4 between intact and ESA animals. Five samples of each group were analyzed individually. SYBR Green premix Ex TaqTM (Takara) was used with 100 ng cDNA as template. Real-time PCR was carried out under the same conditions as used for end-point RT-PCR described above, except 40 cycles were performed. Standard curves were constructed to quantify copy numbers and confirm primer efficiency as described in (Kim et al., 2008). The copy number from each developmental stage was normalized by the copy number of 18S rRNA in the same sample.

2.5. Bioinformatic analysis of six Pj-Chts

Deduced amino acids sequences were obtained using an ORF finder program (http://ncbi.nlm.nih.gov/gorf/gorf.html). Amino acid sequence alignment was performed using ClustalW2 program, which is offered by the European Bioinformatics Institute (http://www.ebi.ac.uk./Tools/msa/clustalw2/), and presented using the GeneDoc program (http://www.nrbsc.org/gfx/genedoc/index.html). Theoretical isoelectric points (pI) and molecular masses were calculated with the Compute pI/Mw program (http://expasy.org/tools/pi_tool.html). Signal peptide and putative proteolytic cleavage sites were predicted by the SignalP 3.0 Server (http://www.cbs.dtu.dk/services/SignalP/). Phylogenetic diagram was prepared by the minimum evolution method by Molecular Evolutionary Genetics Analysis (MEGA4) and bootstrapping replications were 1000 (Tamura et al., 2007). Since chitinase proteins vary in domain composition and numbers of amino acid residues, the tree was constructed using the catalytic domain. Chitinases with multiple catalytic domains were excluded from the analysis. Putative chitinase-like imaginal disc growth factor (TcIDGF4) from *T. castaneum* was used as an outgroup member. Different

Table 2
Primers used for quantitative PCR.

Primer name	Sequence (5′-3′)	Description
Pj-Cht1 RTFD	CAAAGCCGACACTCACTCCATGTT	Pj-Cht1 FWD primer for qPCR
Pj-Cht1 RTRD	TCTCGTGGCTGGAGGGATTCTTGA	Pj-Cht1 RVS primer for qPCR
Pj-Cht2 RTFD	ACCCTACGCCTACTCAGGCAATCAAT	Pj-Cht2 FWD primer for qPCR
Pj-Cht2 RTRD	TTGTGGTGGAGATGGAACAGGCTT	Pj-Cht2 RVS primer for qPCR
Pj-Cht3A RTFD	TTCCTGAAGGCCATCAACAGGATT	Pj-Cht3A RVS primer for qPCR
Pj-Cht3A RTRV	CCATCCCTCAACAACAACGAGTTTGC	Pj-Cht3A RVS primer for qPCR
Pj-Cht3B RTFD	TGCAGATGACGACGATTGTACCCA	Pj-Cht3B FWD primer for qPCR
Pj-Cht3B RTRV	ACCCTTGCGTCTTCTACTATGCTG	Pj-Cht3B RVS primer for qPCR
Pj-Cht3C RTFD	CCAGATCCACTCCAGAGCCCACTA	Pj-Cht3C FWD primer for qPCR
Pj-Cht3C RTRV	TACAGCGCAGACAGAGTCTTTCCA	Pj-Cht3C RVS primer for qPCR
Pj-Cht4 RTFD	TCCTTATGCCGTGTTCACCTGGAA	Pj-Cht4 FWD primer for qPCR
Pj-Cht4 RTRV	ATGTGTCCACGTGGGAACTGTGAT	Pj-Cht4 RVS primer for qPCR
18S rRNA-F	ATGAGAGTGCTCAAAGCAGGCTACTC	Forward primer for 18S rDNA expression
18S rRNA-R	GGCGAATCGCTAGTCAGCATCGTT	Reverse primer for 18S rDNA expression

types of domains were analyzed using SMART modular architectural analysis programs (http://smart.embl-heidelberg.de/) (Schultz et al., 1998).

2.6. Statistical analysis

Statistical analysis of Pj-Chts was evaluated by comparing the mean values using Sigma plot program. The results were considered significant at $P<0.05$.

3. Results

3.1. Cloning of six Pj-Chts

A partial cDNA encoding Pj-Cht1 was identified as one of the major hepatopancreatic genes affected by eyestalk ablation. A 948 bp 3′ RACE product was amplified by the DEG ACP 86 primers (Table 1). This sequence exhibited 60% amino acid sequence similarity with the chitinase 1 precursor from the mud crab, *Scylla serrata*. Since the DDRT-PCR system is based on the amplification of the 3′end 5′ RACE was used to obtain the remainder of the open reading frame (ORF) and 5′ untranslated region (UTR). Unfortunately, we failed to obtain the 5′end of the ORF and 5′UTR sequence. Partial cDNA sequence of Pj-Cht2 was isolated from the epidermis using degenerate primers designed by comparison of crustacean group 2 chitinases (Table 1). Although we failed to obtain the 5′ coding region and UTR, additional 3′ sequence was obtained by 3′ RACE. The Pj-Cht2 sequence (1433 bp) showed 71% sequence identity and 83% similarity to the *S. serrata* chitinase 2. Three additional chitinase cDNAs, designated Pj-Cht3A, 3B, and 3C, were isolated from RACE products while trying to obtain the full length Pj-Cht1. A 5′ RACE product, designated Pj-Cht3A, encoded the 5′-UTR and N-terminal region that differed from Pj-Cht1. Subsequent 3′ RACE using a sequence-specific primer to the Pj-Cht3A (Table 1) obtained the 3′ sequence of Pj-Cht3A, as well as a second sequence, designated Pj-Cht3B, that differed from Pj-Cht3A in the ORF and 3′UTR. A partial sequence (245 bp) of a third chitinase, designated Pj-Cht3C, was isolated from 5′ RACE while trying to obtain the upstream region of Pj-Cht3A (Table 1). The complete cDNA sequence of Pj-Cht3C was obtained with 5′ and 3′ RACE, which produced 61 bp and 1277 bp products, respectively. The complete ORFs of Pj-Cht3A, 3B, and 3C were verified by PCR and sequencing (Table 1).

Finally, a sixth cDNA sequence, designated Pj-Cht4 (2492 bp), was originally identified from a neuronal tissue EST database, which was constructed from a neuronal cDNA library of *P. japonica*. The complete ORF (1824 bp) was confirmed by PCR using two gene-specific forward and reverse primers (Table 1).

3.2. Nomenclature and classification of six Pj-Chts from P. japonica

The properties of the six cDNAs encoding *P. japonica* chitinases are summarized in Table 3. Phylogenetic analysis of the six Pj-Chts and other arthropod chitinase and chitinase-like proteins revealed that crustacean chitinases can be classified into at least four groups (Fig. 1). In order to avoid confusion with the classification of insect chitinases, Arabic numbers were used for the crustacean groups. Members of Group 1 included chitinases from four penaeid shrimps (MjCht1, LvCht1, Fcchi-1 and PmChi1), one from a brachyuran species (SsCht1), and Pj-Cht1 from this study. Group 1 chitinases were more closely related to insect group I chitinases than to any other crustacean chitinases (Fig. 1). Pj-Cht2 was clustered with LvCht2, MjCht2, and Ss2Cht2, forming Group 2. Group 3 chitinases consisted of Pj-Cht3A, 3B, and 3C, LvCht3, and MjCht3. Multiple copies of Group 3 chitinases were found only in *P. japonica*. Group 4 consisted of Pj-Cht4 and LvCht4. In addition, two chitinase sequences, LvCht5 and LvCht-6, were identified in *L. vannamei* (Huang et al., 2010) that did not cluster with chitinases in crustacean chitinase groups 1–4. As no orthologs of LvCht5 and LvCht6 have been identified in other crustaceans, they could not classify definitively into one or two additional groups. Interestingly, LvCht5 is most closely related with an insect Group VII chitinase (DmCHT2) from *D. melanogaster* (Fig. 1).

3.3. Structural characteristics of six Pj-Chts

In order to compare the structural organization of the six Pj-Chts, multiple amino acid alignments were carried out (Fig. 2). Except for Pj-Cht1 and 2, the complete ORFs were identified in the other four

Table 3
Properties of cDNAs encoding *P. japonica* chitinases.

Name	Accession number	Total length (bp)	ORF (bp)	5′UTR (bp)	3′UTR (bp)	Amino acid residues	Mass (kDa)	pI
Pj-Cht1	JF694836	1687	–	–	245	479	54.22	4.52
Pj-Cht2	JN982965	1433	–	–	167	421	47.25	6.23
Pj-Cht3A	JF694837	1479	1167	22	290	388	43.61	4.67
Pj-Cht3B	JF694838	1672	1452	7	213	483	53.06	4.40
Pj-Cht3C	JF694839	1736	1455	61	220	484	53.88	4.59
Pj-Cht4	JF694840	2492	1824	13	655	607	67.62	4.77

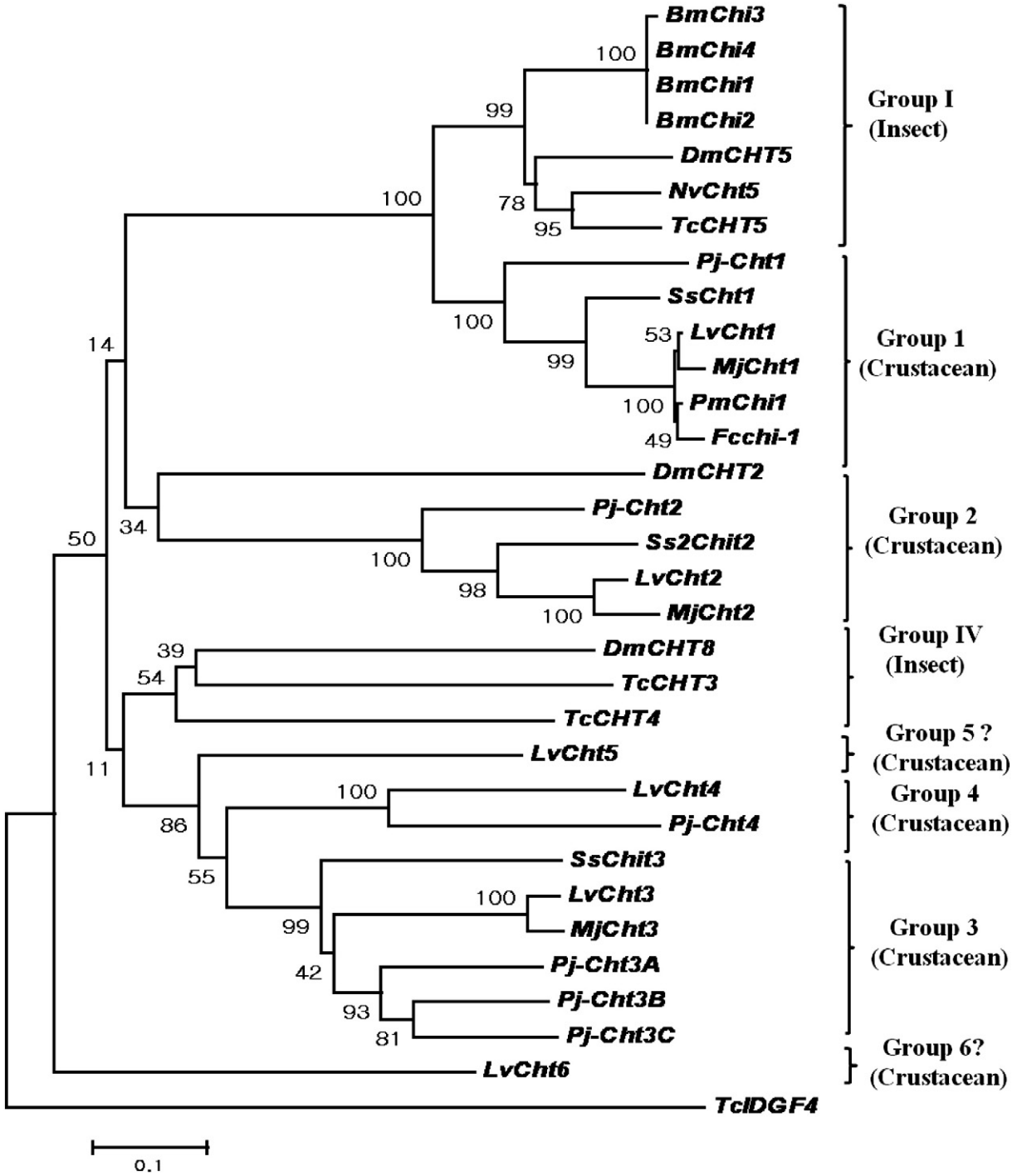

Fig. 1. Phylogenetic tree of crustacean and insect chitinases. The analysis was restricted to the catalytic domain using Molecular Evolutionary Genetics Analysis (MEGA4) software (see Materials and methods). The diagram was generated by minimum evolution method and bootstrapping test was performed with 1000 replicates. The scale bar represents 0.1 amino acid substitution per site. The decapod chitinases cluster into at least 4 groups (1 to 4); the insect chitinases cluster into Groups I and IV. Species and GenBank accession numbers: *P. japonica*, Pj-Cht1 (JF694836), Pj-Cht2 (JN982965) Pj-Cht3A (JF694837), Pj-Cht3B (JF694838), Pj-Cht3C (JF694839) and Pj-Cht4 (JF694840); *L. vannamei*, LvCht1 (EU883591), LvCht2 (FJ888479), LvCht3 (EU381118), LvCht4 (FJ888480), LvCht5 (FJ888481), and LvCht6 (GQ916594); *P. monodon*, PmChi1 (AAD40313); *M. japonicus*, Mj-Cht1 (BAA12287), Mj-Cht2 (D89751), and Mj-Cht3 (BAA22854); *F. chinensis*, Fcchi-1 (ABB85237); *S. serrata*, SsCht1 (ACG60512), Ss2Cht2 (ACZ53950), and SsCht3 (EU402970); *B. mori*, BmChi1 (NP001166831), BmChi2 (NP001037480); BmChi3 (NP001166832), and BmChi4 (NP001166833); *T. castaneum*, TcCHT3 (AAW67570), TcCHT4 (ABL73927), TcCHT5 (AAV74190), and TcIDGF4 (imaginal disc growth factor; DQ659254); *D. melanogaster*, DmCHT2 (NP477298); DmCHT5 (NP650314), and DmCHT8 (NP611542); and *N. vitripennis*, Nv-Cht5 (NP001128139).

Pj-Cht cDNAs. The Pj-Chts encoded proteins ranging from 388 to 607 amino acid residues, with estimated masses between 43.61 and 67.62 kDa (Table 3). Except for Pj-Chit1 and 2, which are incomplete cDNA sequences, Pj-Chts had a common structural organization: an N-terminal signal sequence, a large catalytic domain, a Thr/Pro-rich linker region, and a C-terminal chitin-binding domain (CBD). The Pj-Chts differed primarily in the number of CBDs and the length of the Thr/Pro-rich linker region. Pj-Cht3A lacked a CBD; Pj-Cht1, 3B, and 3C contained 1 CBD; and Pj-Cht4 had 2 CBDs. In addition, Pj-Cht4 had a large expansion of the Thr/Pro-rich linker region (Fig. 2). Except for Pj-Cht2, which is close to neutral (6.23), other five Pj-Chts exhibited acidic isoelectric points (Table 3). Putative signal

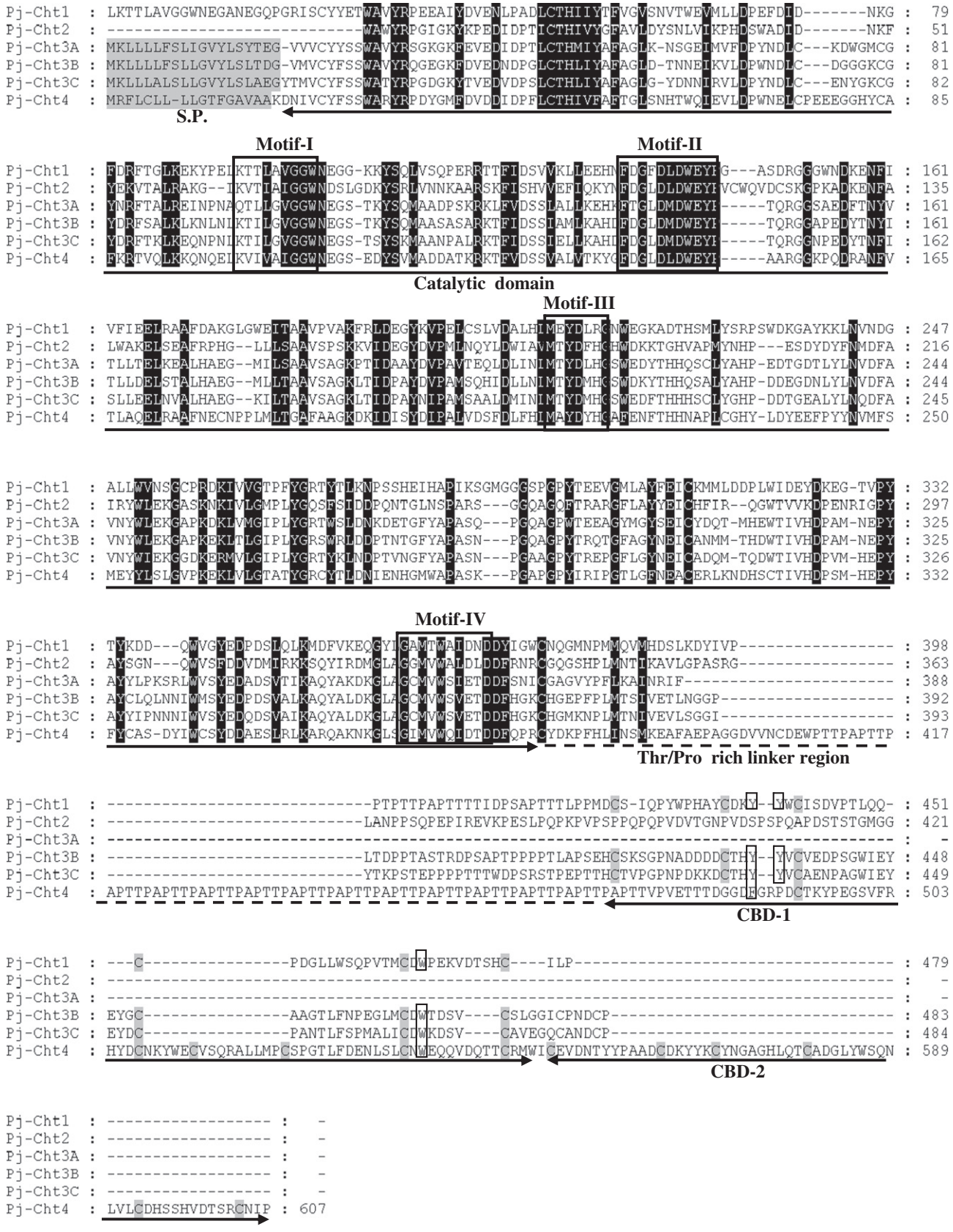

Fig. 2. Alignment of deduced amino acid sequences of *P. japonica* chitinase cDNAs. Alignments of the CBD were modified manually to match cysteine spacing, which is conserved for substrate binding. The proteins had the same structural organization: N-terminal signal peptide (shaded), catalytic domain with 4 conserved motifs (I, II, III, and IV), a Thr/Pro-rich linker region, and 0 (Pj-Cht3A, Pj-Cht2), 1 (Pj-Cht1, 3B, and 3C), or 2 (Pj-Cht4) C-terminal chitin-binding domain(s) (CBD). Pj-Cht1, 2 sequence lacked the 5′end of the ORF that contained the signal peptide. Aromatic residues and 6 cysteines in the CBD are highlighted.

peptide sequences were identified in all Pj-Chts, except for Pj-Cht1 and 2, which lacked the 5′ end of the ORF. Pj-Cht 3A and 3B shared a common cleavage site, which was located between Gly19 and Val20. The Pj-Cht3C cleavage site was located between Gly19 and Tyr20. The Pj-Cht4 cleavage site was located between Ala17 and Lys18. These results indicate that Pj-Cht3A, 3B, 3C, and Cht4 are secretory proteins.

Analysis of amino acid sequence identity confirmed the phylogenetic analysis. Pj-Cht3B and Pj-Cht3C were most closely related to each other (72%), whereas the lowest amino acid sequence identity was found between Pj-Cht2 and Pj-Cht4 (35%). Pj-Cht1 showed 66% amino acid sequence identity to the chitinase 1 from *S. serrata*, (SsChit1; GenBank accession number ACG60512). Pj-Cht3A and Pj-Cht3C showed 63% and 62% identities, respectively, to *L. vannamei* Cht3 (LvCht3, ABY70643), whereas Pj-Cht3B showed 60% identity to the chitinase 3 from *S. serrata* (SsChit3, ABY85409). Pj-Cht4 showed 62% identity with the chitinase 4 from *L. vannamei* (LvCht4, ACR23314). Pj-Cht2 exhibited 72% amino acid identity with chitinase2 from *S. serrata* (Ss2Chit2, ACZ53950) and *P. monodon* (PmChi2, ADG22164).

The catalytic domain of the Pj-Chts contained the four highly conserved motifs, designated I, II, III, and IV, that are also found in insect chitinases (Fig. 3A).The sequences of Motifs I (KXXXXXGGW) and II (FDGXDLDWEYP) were highly conserved in the Pj-Chts, except for Pj-Cht3A, in which Lys (K) was replaced by Gln (Q) in Motif I and Asp (D) was replaced by Thr (T) in Motif II. Motifs III (MXYDXXG) and IV (GXXXWXXDXD) were also well conserved in all Pj-Chts. CBDs of the Pj-Chts were aligned with CBDs in other crustacean chitinases (Fig. 3B). Pj-Cht3A and Pj-Cht2 lacked a CBD and therefore were not included. Both CBDs in Pj-Cht4 were included in the alignment. The chitinase CBDs belong to the carbohydrate binding module 14 (CBM-14) family (http:www.cazy.org/fam/CBM14.html). Aromatic residues (Trp, Tyr) that are critical for substrate binding were conserved, except for the second CBD in Group 4 chitinases. Six cysteine residues, which form three disulfide bridges to stabilize protein structure (Akagi et al., 2006; Huang et al., 2010), were also well conserved within the CBD.

3.4. Expression analysis of the six Pj-Chts

Tissue distribution of six Pj-Cht transcripts were determined by end-point RT-PCR and subsequent agarose-gel electrophoresis of PCR products (Fig. 4). In order to exclude the individual variation, total 16 cDNAs for each tissue were tested. Except for Pj-Cht2, the hepatopancreas and intestine were the major sites for expression of all other Pj-Chts (Pj-Cht1, 3A, 3B, 3C, and 4). A lesser amount of PCR product of Pj-Cht3C was amplified from sinus gland/X-organ complex

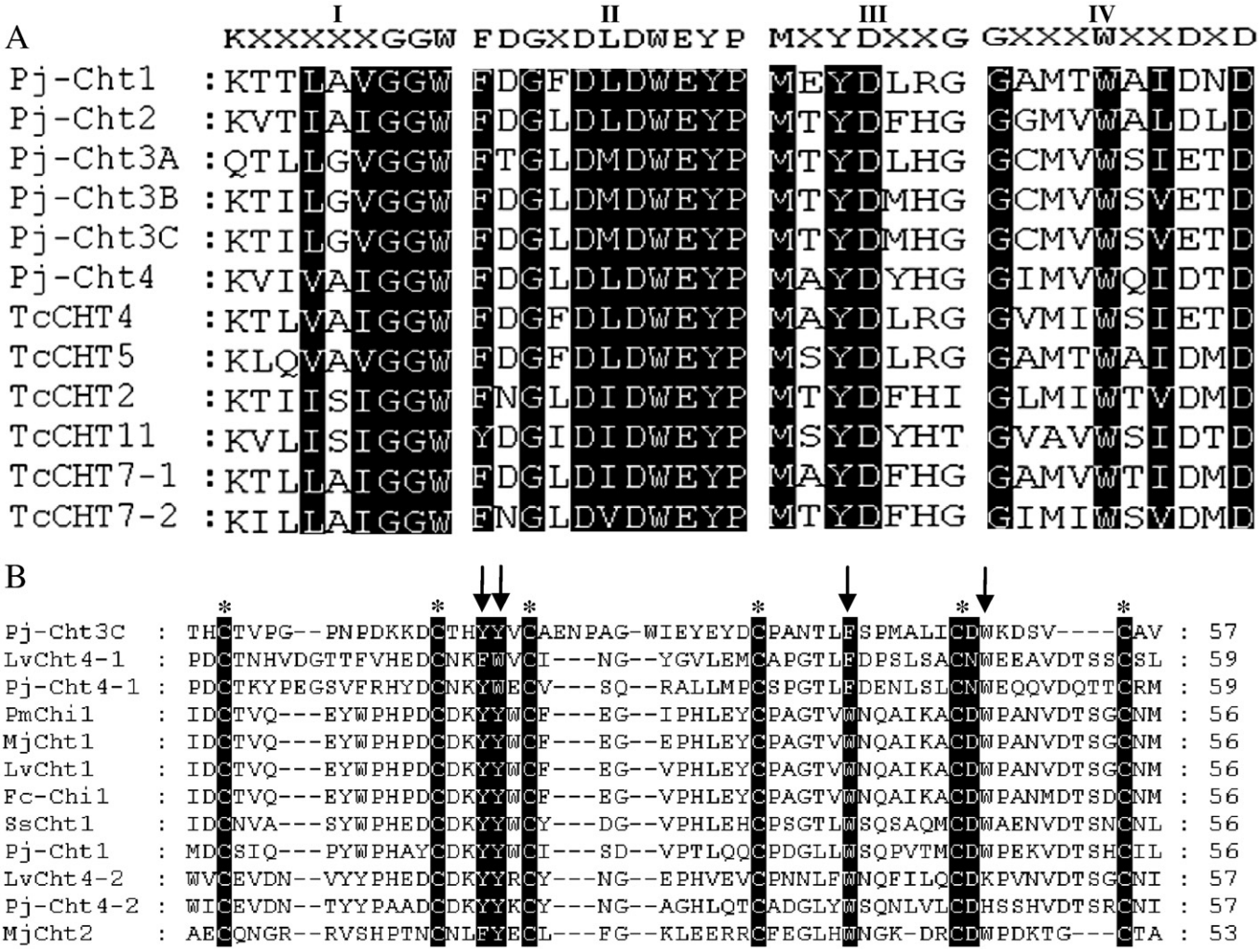

Fig. 3. Alignment of the catalytic and chitin-binding domains in crustacean and insect chitinase and chitinase-like proteins. (A) Comparison of the amino acid sequences of motifs I, II, III, and IV in the catalytic domain. (B) Comparison of the amino acid sequences of the CBD. Conserved cysteines involved in intramolecular disulfide bridges are indicated by asterisks (*). Conserved aromatic residues are indicated by arrows. The species and GenBank accession numbers are the same as in the legend to Fig. 1.

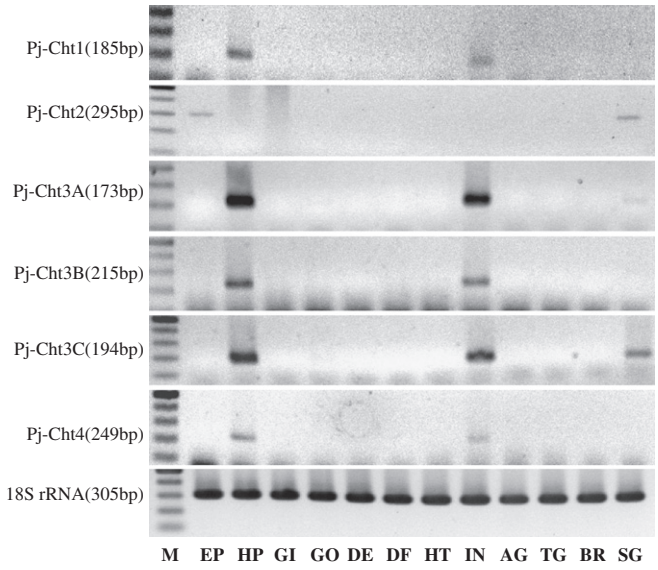

Fig. 4. Tissue expression of *P. japonica* chitinases. End-point RT-PCR products of Pj-Cht1, 2, 3A, 3B, 3C and 4 were separated on a 1.5% agarose gel and stained with ethidium bromide (inverted image). Amplification of an 18S rRNA product was used as a positive control. Abbreviations: AG, abdominal ganglion; BR, brain; DE, deep abdominal extensor muscle; DF, deep abdominal flexor muscle; EP, epidermis; GI, gill; GO, gonad, HP, hepatopancreas; HT, heart; IN, intestine; M, molecular size marker; SG, sinus gland; and TG, thoracic ganglion.

(SG/XO) cDNA. Contrary to other Pj-Chts, Pj-Cht2 transcript was detected in epidermis and SG/XO and no expression was identified in either hepatopancreas or intestine (Fig. 4).

qPCR was used to determine the effects of molt induction by ESA on the expression of Pj-Chts. ESA is widely used to induce molting in decapods (Brown and Cunningham, 1939; Kyer, 1942; Warner and Stevenson, 1972; Meade and Watts, 2001; Okumura and Aida, 2001). Effects of ESA were different between hepatopancreatic chitinase transcripts (Pj-Cht1, 3s) and epidermal chitinase transcript (Pj-Cht2). mRNA levels of Pj-Cht1, Cht3A, Cht3B, and Cht3C in the hepatopancreas were decreased by ESA (Fig. 5). Pj-Cht1, 3A, and 3C mRNA levels decreased 2.6-fold, 3.6-fold, and 3.4-fold, respectively, by 3 days post-ESA. Pj-Cht3B mRNA level showed a similar change as Pj-Cht3A and 3C, but the difference between the intact and 3 days post-ESA means was not significantly different. The expression of Pj-Cht1, 3A, 3B, and 3C appeared to recover partially by 7 days post-ESA. Expression of Pj-Cht4 was below the detection limit for qPCR. By contrast, expression of Pj-Cht2 was induced by ESA in epidermis (Fig. 6). Its expression level increased 3.6 fold by 7 days post-ESA. ESA had no significant effect on the expression of the six Pj-Chts in the intestine (data not shown). Expression was much lower in the intestine than in the hepatopancreas by factors between 7.2×10^{-7}-fold and 0.4-fold.

4. Discussion

Chitinases and chitinase-like proteins from *P. japonica* and other decapod crustaceans were classified based on structural organization, tissue expression patterns, and sequence similarity. There are at least 4 groups, and the six Pj-Cht cDNAs fall into all 4 groups (Fig. 1). It is likely that the number of groups will increase as more chitinase and chitinase-like genes are characterized. Six cDNAs have been characterized in *L. vannamei*, two of which (LvCht5 and Cht6) do not cluster with the chitinases in Groups 1–4 (Fig. 1). Three chitinase cDNAs have been identified in *P. monodon*, four in *S. serrata*, and three in *M. japonicus*. We do not exclude the possibility of additional chitinase genes in decapod crustaceans, given that between 16 and 20 chitinase genes have been identified in insect genomes. A recent genome-wide study identified 17 chitinase genes in *Daphnia pulex* that may have resulted from intra-chromosomal tandem duplication (McTaggart et al., 2009).

The catalytic domains of arthropod chitinases contain four conserved motifs. Among them, motif II is essential for catalytic activity, but the precise functions of the three other motifs are yet to be established (Arakane and Muthukrishnan, 2010). Motif II of all six Pj-Chts was highly similar to chitinases in other arthropod species, indicating that the Pj-Cht proteins are catalytically active. However, amino acid substitutions occurred in Pj-Cht3A; Lys (K) was replaced by Gln (Q) in Motif I and Asp (D) was replaced by Thr (T) in Motif II (Fig. 3A). In *Manduca sexta*, chitinases in which Trp (W) is replaced by glycine are inactive, but retain chitin-binding activity (Huang et al., 2000). Hydrolysis of the glycosidic bonds in chitin uses a general acid/base mechanism (Sinnott, 1990; McCarter and Stephen Withers, 1994; Davies and Henrissat, 1995). Among the four acidic residues within Motif II (FDGXDLDWEYP), the two amino acids at positions #7 and #9 participate directly in catalysis, in which the Glu (E) acts as proton donor and Asp acts as a nucleophile (Perrakis et al., 1994; Terwisscha van Scheltinga et al., 1994). The two acidic residues are conserved in the six Pj-Chts (Fig. 3A). The replacement of Asp at position #2 with Thr in Pj-Cht3A may not have a significant effect on catalytic activity.

The CBDs of invertebrate chitinases are about 65 amino acids in length (Shen and Jacobs-Lorena, 1999). The putative function of the CBD is to localize the enzyme on chitin filaments, which increases the efficiency of chitin degradation (Linder and Teeri, 1997). Aromatic residues and disulfide bonds in the CBD are essential for saccharide binding (Shen and Jacobs-Lorena, 1999). Although the precise mechanism is not yet established, the aromatic residues of the chitin- or cellulose-binding proteins are involved in interacting with polysaccharide. The chitinase in the PM of *A. gambiae* binds chitin, but not cellulose (Shen and Jacobs-Lorena, 1998). The spacing of the aromatic residues and/or other residues may also be important for determining binding specificity (Shen and Jacobs-Lorena, 1999). Aromatic residues within all the Pj-Chit CBDs were well conserved (Fig. 3B). One exception was found in the second CBD of crustacean group 4 chitinases, in which Trp was replaced by either Lys or His, suggesting the second CBD may possess different binding specificity than the first CBD. The crustacean CBDs have six cysteines that form intramolecular disulfide bridges. The CBD is absent in Pj-Cht3A, suggesting that the protein interacts with chitin at only the catalytic domain. Both native chitinases and mutant without CBD had activity but its activity is about three times lower than normal enzyme suggesting CBD facilitates substrate binding (Iseli et al., 1993).

The linker region of arthropod chitinases has high proportions of threonines, prolines and few serine residues (Arakane et al., 2003). This region is heavily O-glycosylated and hydrophilic, which contributes to an extended conformation and may confer conformational and energetic stability to the protein. Although there is no general rule of residues for O-glycosylation, prolines are often located at the −1 and +3 positions from the O-glycosylation site on serine or threonine residues. Moreover, the abundance of both prolines and serines or threonines also affects glycosylation frequency (Chen et al., 2007). The preference of threonine instead of serine in Pj-Chts may have functional significance, but further study is needed (Fig. 2).

All five hepatopancreatic chitinases have acidic isoelectric points (Table 3). The hepatopancreas-specific chitinase from *F. chinensis* (*Fcchi-3*; pI = 4.73) may be involved in the digestion of chitin-containing food (Zhang et al., 2010). A recombinant *P. monodon* chitinase 1 protein (rPmChi1) from a bacterial expression system has maximal activity at pH 5 (Proespraiwong et al., 2010). As shown in crustacean chitinases, most insect chitinase-like genes also have acidic pIs. In *T. castaneum*, for example, the pIs range between 4.3 and 7.0 (Zhu et al., 2008a). Most are acidic (pH ~ 4.5), but three chitinases (TcCHT4, TcCH7, and TcCHT10) are between pH 6.0 and 7.0. Interestingly, TcCH7 and TcCHT10 are two key enzymes, in which knockdown of the two transcripts results in abnormality in molting (Zhu et al.,

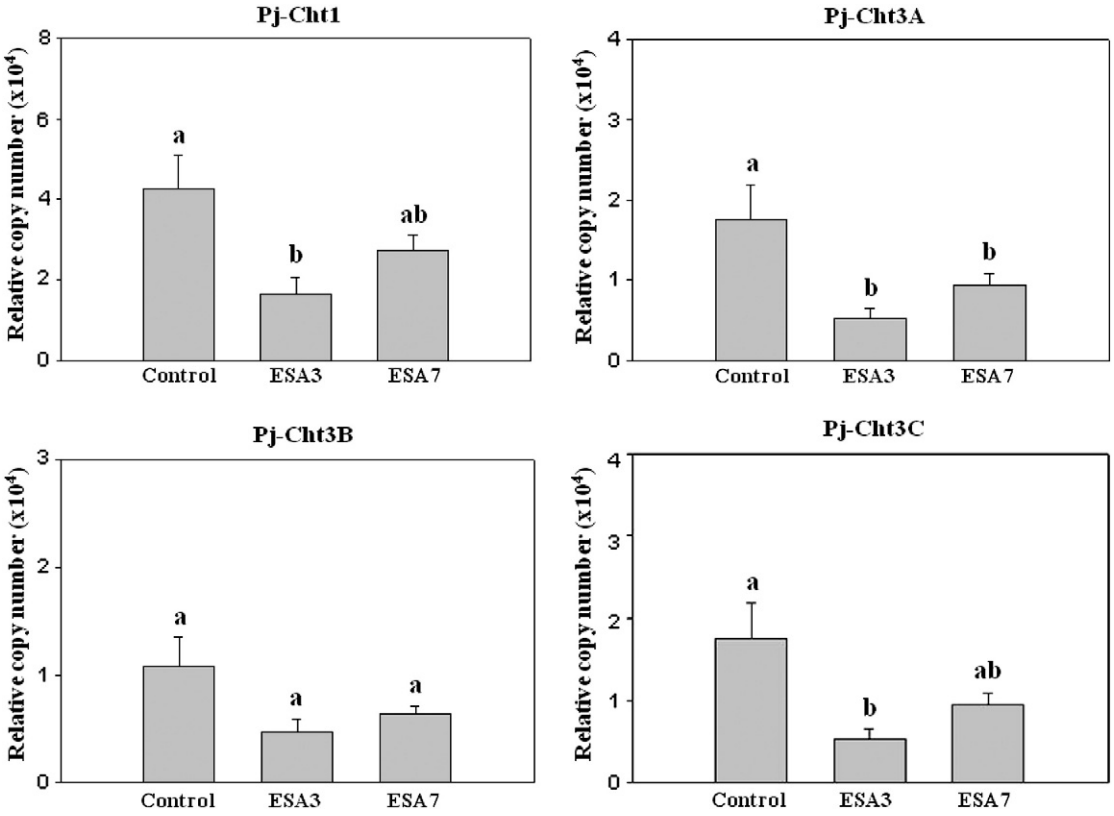

Fig. 5. Effect of eyestalk ablation (ESA) on expression of *P. japonica* chitinases in hepatopancreas. mRNA levels of Pj-Cht1, 3A, 3B, and 3C were quantified in hepatopancreas by qPCR and data were expressed as relative copy number (see Materials and methods). The Pj-Cht4 mRNA level was below the detection limit for qPCR. Means that share the same letter are not significantly different ($P > 0.05$).

2008b). Pj-Cht2 had an estimated pI similar to that of TcCHT10 and TcCHT7 (Table 3), which suggests that a neutral pI may be characteristic of chitinases involved in chitin metabolism during the molting cycle. Until now, no functional study has been carried out on crustacean group 2 chitinases, and the optimum pH of the catalytic activity may distinguish hepatopancreatic chitinases from epidermal chitinases.

Crustacean group 1 chitinases were most closely related to insect group I chitinases (Fig. 1). Insect group I chitinases appear to be involved in stage-specific molting processes. In a dsRNA knockdown experiment of group I chitinase, TcCHT5, lethal phenotypes were only observed at pharate adults in which the animals failed to fully shed their old cuticles (Zhu et al., 2008b). Hepatopancreatic expression of PmChi-1from *P. monodon* varies with molt stage with the highest expression at premolt (Stage D_2), suggesting it functions in the digestion of cuticle prior to ecdysis (Tan et al., 2000). Expression of two different FcChi and FcChi-1 transcripts in whole juvenile shrimp during the molting cycle found considerable fluctuations in different molt stages. During the premolt period, the lowest expression of both transcripts occurred in stages D_0 and D_2, whereas the highest expression occurred in stages D_1 and D_3 (Priya et al., 2009). In addition, two hepatopancreatic chitinases including groups 1 and 3 chitinases, are down-regulated by increased ecdysteroid in crayfish, *Cherax quadricarinatus* (Shechter et al., 2007). In our present study, we also found that expression levels of Pj-Cht1 and 3 were down-regulated by ESA. It has been proposed that crustacean group 1 chitinases have a role in the digestion of peritrophic membrane before molting.

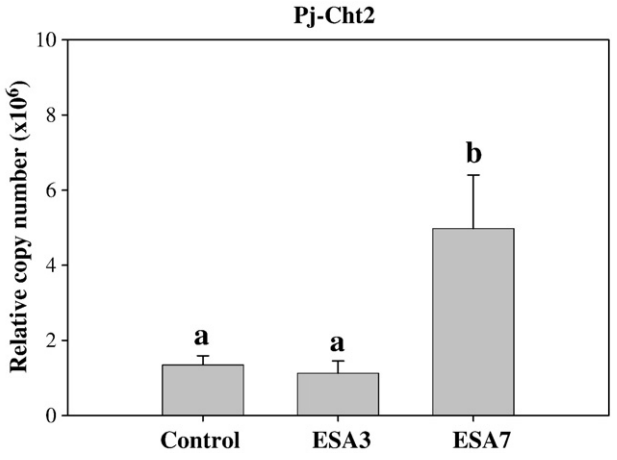

Fig. 6. Effect of eyestalk ablation (ESA) on expression of *P. japonica* chitinases in epidermis. mRNA levels of Pj-Cht2 were quantified in epidermis by qPCR and data were expressed as relative copy number (see Materials and methods). Means that share the same letter are not significantly different ($P > 0.05$).

Crustacean group 2 chitinases may correspond to the insect group II chitinases and may be involved in molting. These chitinases are expressed in integument and are up-regulated during premolt (Watanabe and Kono, 1997). Pj-Cht2 is also expressed in epidermis and up-regulated by ESA (Figs. 4 and 6). Although the expression patterns of crustacean group 2 chitinases are similar to insect group II chitinases, a major difference between the two groups is the number of catalytic domains. Group II chitinases have multiple catalytic domains and CBDs and are involved in molting and metamorphosis in insect species, such as *T. castaneum*. For example, TcCHT10 has five catalytic and CB domains (Fig. 7). It is involved in molting throughout development, including embryo, hatch, larvae, pupae, and metamorphosis. Group III chitinase, such as TcCHT7, lacks a signal peptide sequence and contains two catalytic domains and one CBD and it plays a role in regulating abdominal contraction and wing expansion (Zhu et al., 2008b). Unlike insect groups II and III chitinases, all known crustacean chitinases have only a single catalytic domain (Fig. 7). Crustacean group 2 chitinases have a unique domain organization

in which the CBD is located at N-terminus and the catalytic domain is located at the C-terminus. As only one complete cDNA of crustacean group 2 chitinases has been characterized (Mj-Cht2), it may be too early to generalize. In addition, Mj-Cht2 has an unusually long 5′UTR (~1.4 kb) and shows several unidentified sequences (Watanabe and Kono, 1997), which may explain why group 2 chitinases have not been obtained from other decapods. We do not exclude the possibility that crustacean group 2 chitinases contain multiple catalytic domains and CBDs. We confirmed that there is no CBD at the C-terminus of Pj-Cht2 but failed to obtain additional catalytic domains or CBDs at the N-terminus. More sequence information is needed in a variety of decapod species to establish the domain organization of this group.

Group 3 chitinases, such as Pj-Cht3A, 3B, and 3C, may have function in digestion of chitin-containing food. There were no detectable abnormalities in the *T. castaneum*, which were treated with dsRNAs of insect group IV (TcCHT2,-6,-8,-14,-16) and group V (TcIDGF2) chitinases, which show similar domain organization to crustacean group 3 chitinases, suggesting that they are not involved in molting process (Zhu et al., 2008b). Among the crustacean group 3 chitinases, Pj-Cht3A has no CBD and its domain organization is similar to that of the chitinase IV group in insects. This is the first description of a chitinase lacking a CBD in crustaceans. Pj-Cht3 appears to be a distinct gene product and not the product of alternative splicing, as its sequence is different from other two Pj-Cht3 cDNAs. In addition to chitin hydrolysis in the hepatopancreas, crustacean group 3 chitinases may function in defense from pathogens. The expression of Fcchi-3 in hepatopancreas is down-regulated by challenge to white spot syndrome virus (WSSV) (Zhang et al., 2010). Although it is not yet clear if chitinases are involved directly in defense system, Pan et al. (2005) showed that three chitinase genes are upregulated in the hepatopancreas of WSSV-resistant *M. japonica*.

Crustacean group 4 chitinases, such as Pj-Cht4, contain two CBDs at the C terminus, which is unique to crustaceans; no such organization has been found in insect chitinases. An ortholog was also identified in *L. vannamei* (Huang et al., 2010). The functional significance of two CBDs is unknown. Although Pj-Cht4 shows a representative hepatopancreatic pI value (4.77), its level of expression was too low to determine the effects of ESA by qPCR.

In present study, five Pj-Chts (Pj-Chit1, 3A, 3B, 3C, 4) were expressed predominantly in the hepatopancreas. Since the highest chitinolytic

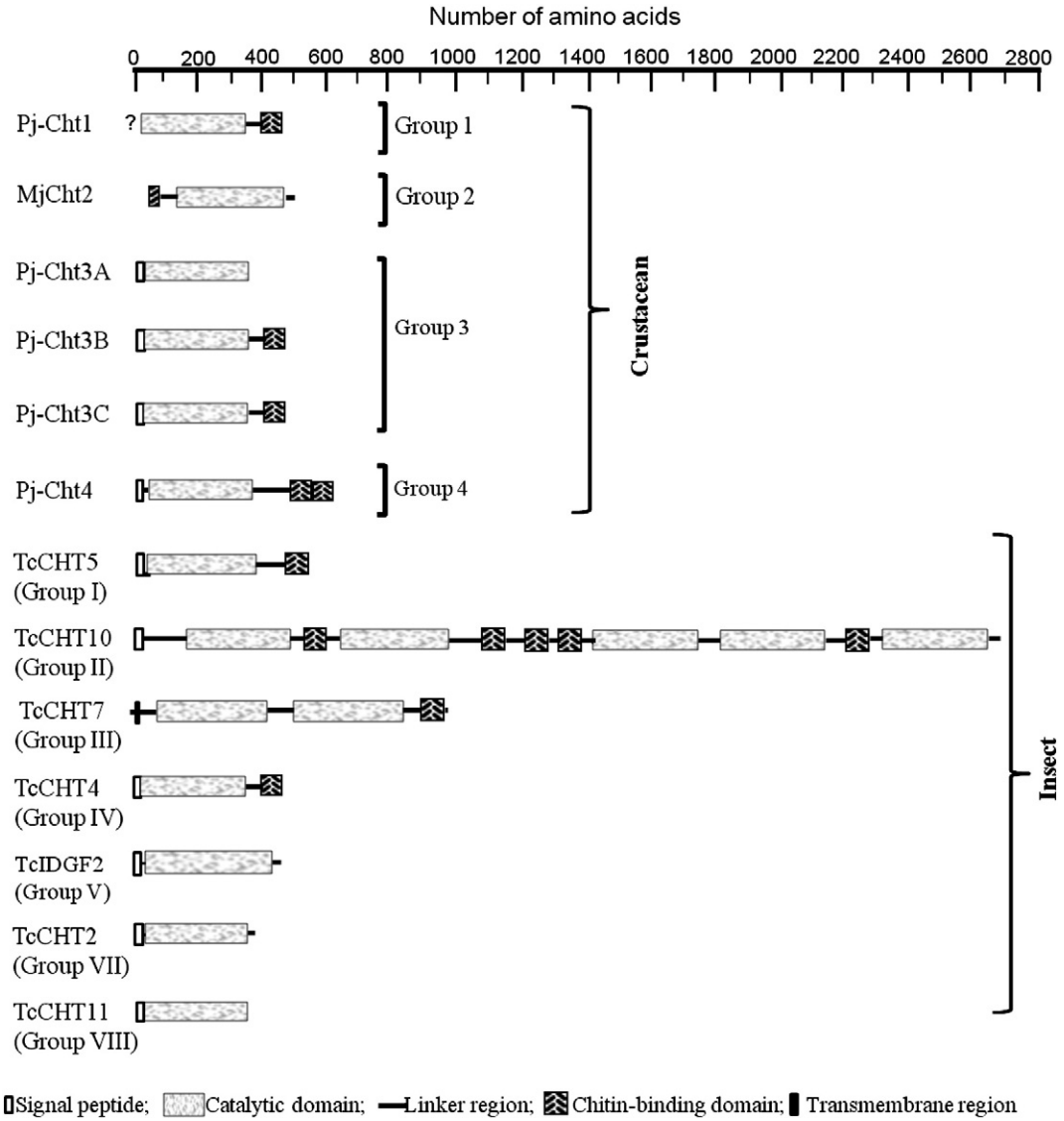

Fig. 7. Domain organization of chitinase and chitinase-like proteins representing crustacean and insect groups. SMART software was used to identify the functional domains (see Materials and methods). Crustacean groups 1 to 4 are represented by Pj-Cht1, Mj-Cht2, Pj-Cht3A, B, C, and Pj-Cht4, respectively. Insect groups I to VIII are represented by *T. castaneum* genes (Insect group VI was excluded) (Arakane and Muthukrishnan, 2010). All have N-terminal signal peptides, except insect group III, which has an N-terminal transmembrane domain. The CBD, if present, is located at the C-terminus of crustacean groups 1, 3, and 4 chitinases. The CBD in group 2 chitinases is located at the N-terminal region.

activity occurs in the epidermis and not the hepatopancreas during premolt, the hepatopancreatic chitinases may not be directly involved in the degradation of the old exoskeleton. Only the crustacean group 2 chitinases including Pj-Cht2 are expressed in the epidermal tissues, such as gill and tail fan (Watanabe and Kono, 1997; Huang et al., 2010). Crustacean growth requires periodic ecdyses, in which the old exoskeleton is replaced by a larger exoskeleton that expands to make room for tissue growth (Skinner, 1985). ESA decreases four chitinase mRNA levels (Fig. 5). By contrast, ecdysteroids generally have a stimulatory effect on mRNA and protein synthesis (Gorell and Gilbert, 1969; Skinner, 1985). However, our finding is similar to other results showing the down-regulation of chitinase-like proteins by elevated ecdysteroid titers (Shechter et al., 2007). The down-regulation of chitinase expression may be in response to reduced feeding prior to molting (Shechter et al., 2007).

In summary, we isolated six distinct cDNAs encoding chitinase homologs expressed in the hepatopancreas and epidermis that cluster with Groups 1, 2, 3, and 4 chitinases from other decapod crustacean species. Comparative structural and expression analysis indicated that the hepatopancreatic chitinases represented by Pj-Chit1, 3s, and 4 are acidic and may function in the modification of the PM and the digestion of chitin ingested in food. Group 2 chitinases including Pj-Cht2 show neutral pI value and may function in the degradation of the old exoskeleton during premolt, but further study is needed. Arthropods express a diversity of chitinases and chitinase-like proteins, which indicates a complexity of chitin metabolism at all life history stages.

Acknowledgments

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