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Molecular characterization and tissue expression analysis of five genes for chitinase in the red palm weevil, *Rhynchophorus ferrugineus* (Coleoptera: Curculionidae)

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Insect chitinases are hydrolytic enzymes that cleave chitin of the cuticle and peritrophic membrane during molting. Multiple genes encode insect chitinases, which are characterized as having diverse chemical and enzymatic properties depending on the time and the site of expression. This work was done to isolate and characterize chitinase genes from the red palm weevil (RPW), *Rhynchophorus ferrugineus* (Oliver), a cryptic pest of many palm trees. The isolated five genes were phylogenetically clustered into five different groups (I, II, III, VI, and VII) of the glycoside hydrolase family 18 (GH18). Domain structure analysis revealed that *RfCht1* (group I), *RfCht3* (group VI), and *RfCht5* (group VII) each retained a single catalytic domain of the GH18, whereas *RfCht2* (group III)) and *RfCht4* (group II) possessed two and five GH18 catalytic domains, respectively. *RfCht1*, *RfCht2*, and *RfCht3* each retained a single chitin-binding domain (CBD) and *Rf*Cht4 retained five CBDs, but *RfCht5* lacked CBD. Developmental and tissue expression profiles showed high levels of transcripts of the five genes in the newly hatched first instar larvae. *RfCht1* and *RfCht2* transcripts were expressed constitutively almost with high levels in young and mature eggs, in all tested larval instars, pre-pupae, pharate pupae, and adults; whereas *RfCht3* and *RfCht5* transcripts were expressed as low levels in the early instars larvae few hours before molting. In late developmental stages and mature eggs, *RfCht3*, *RfCht4*, and *RfCht5* were expressed as low levels mainly in the cuticle. This study presents the first report on chitinase genes in the RPW and suggests that these genes have additional roles in the weevil development, which require further elucidation.

Key words: Chitinase, conserved motifs, cuticle, domain structure, expression profile, *Rhynchophorus ferrugineus*

INTRODUCTION

Insect body contains rigid and insoluble chitin, as a component of the exoskeleton, trachea, and the peritrophic membrane (PM) that surrounds the food in the midgut. Chitin in these organs provides protection to

insects against environmental and mechanical injuries but it limits the growth and development of insects. Therefore, the cuticles and PM are degraded periodically and reshuffled to allow growth and development (Merzendorfer and Zimoch, 2003; Muthukrishnan et al., 2016). Insect chitinases play crucial roles to degrade chitin in the old cuticles and PM during the larval molting and pupation and act defensively to prevent bacteria and fungi from penetrating the PM. They belong to family 18 glycosyl hydrolases (GH18). The GH18 genes of chitinases have potential use for pest management as biopesticides (Kramer and Muthukrishnan, 1997). Insect chitinase genes have been suggested as targets for gene silencing via RNA interference (RNAi) (Al-Ayedh et al., 2016; Cao et al., 2017; Su et al., 2016; Zhu et al., 2008a) and have also been proposed as appropriate candidates in host-mediated silencing of pest genes (HMSPG) for control of diseases and insect pests of date palm (Niblett and Bailey, 2012).

Chitinases have been isolated and characterized in many insects including *Anopheles gambiae*, *Bombyx mori*, *Chilo suppressalis*, *Drosophila melanogaster*, *Manduca sexta*, *Mythimna separata*, *Nilaparvata lugens*, *Ostrinia nubilalis*, *Tribolium castaneum*, and economically important other species (Shen and Jacobs-Lorena, 1997; Zhu et al., 2008b; Khajuria et al., 2010; Zhang et al., 2011a; Huang et al., 2012; Pan et al., 2012; Tetreau et al., 2015; Xi et al., 2015; Su et al., 2016; Cao et al., 2017). Functional analyses of particular chitinases revealed that insect chitinases belong to a large family of enzymes with diverse domain architecture, expression patterns, tissue specificity, and function. They have been grouped into, at least, eleven groups based on domain arrangement and/or tissue specificity of expression and phylogenetic analyses as well as functional analyses (Nakabachi et al., 2010; Tetreau et al., 2015). Groups I and II chitinases are found in molting fluid (Koga et al., 1992; Qu et al., 2014). Groups III and VIII members have a membrane-spanning domain and are involved in regulating abdominal contraction and wing expansion. Group V members are mainly imaginal disc growth factor genes that are necessary for adult eclosion (Zhu et al., 2008a). It has been reported that in *N. lugens* at least four groups of chitinases (groups I, II, III, and V) are involved in nymph-nymph molt (Xi et al., 2015). The presence of several functional chitinases with distinct domain configuration suggests that they have other functions besides the digestion of chitin in the old cuticle. These could include providing primers for elongation of chitin and processing of mature chitin chains for a higher level of organization (Muthukrishnan et al., 2016).

The red palm weevil (RPW), *Rhynchophorus ferrugineus* (Olivier) (Coleoptera: Curculionidae), is a noxious internal feeder attacking many palm species including coconut, *Cocos nucifera*, date palm, *Phoenix dactylifera,* Canary Islands palm, *Phoenix canariensis*, and African oil palm, *Elaeis guineensis* (Dembilio et al., 2012; Fiaboe et al., 2012; Hoddle et al., 2013). The entire larval life cycle of the weevil is concealed within the palm trunk, which makes its early infestations difficult to detect (Faleiro et al., 2012; Hoddle et al., 2013; Llácer et al., 2010; Murphy and Briscoe, 1999). Food and Agriculture Organization of the United Nations (FAO) has classified RPW as category-1 pest on date palm in the Middle East (Al-Dosary et al., 2016).

To date, the large-scale dataset on the RPW transcriptome analyses though provided substantial information on the weevil's development that could have important practical applications (Wang et al., 2013); however, no information exists about particular genes, especially those functions relating to processes of ecdysis and metamorphosis of the developmental stages of the RPW. Thus, we isolated, amplified, cloned, and sequenced five genes for chitinase were isolated from the RPW to gain insight into their structural and functional domains architecture and also to study the mRNA expression patterns of these genes in different developmental stages of the RPW.

MATERIALS AND METHODS

RPW rearing and tissue collection

RPW was reared in the laboratory and the insectary facilities of the Date Palm Research Center of Excellence, King Faisal University. For egg laying, male and female adults were fed on sugarcane kept in TATAY storage boxes (51 cm × 38 cm × 26 cm) made of polypropylene and bisphenol A (BPA) free (www.tatay.com) with perforated lids as described elsewhere (El-Shafie et al., 2013). Eggs were removed with a brush and placed into Petri dishes that contained cotton and moist filter paper and incubated at 28°C until the eggs hatch. First instar larvae were collected daily and reared on pineapples and date palm trunk. Samples of different developmental stages were collected periodically for integument and tissue collection. Larvae were dissected by cutting off their heads using a standard stainless steel entomology dissection set. The integument was cut longitudinally to separate the adipose tissues and the guts. The dissected tissues were immediately frozen in liquid nitrogen. Eggs, elytra, forewings, and the adult's body were directly frozen in liquid nitrogen. All samples were stored at -80°C for the subsequent experiments.

BLAST® search and sequence alignment

The online Basic Local Alignment Search Tool (BLAST) was used to search for potential chitinase genes sequences in the RPW Transcriptome Shotgun Assembly (TSA) (Wang et al., 2013). *T. castaneum* and *B. mori* chitinase gene sequences available in the

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Table 1. Primer sequences for amplification and expression profiling of the RPW chitinase genes (*RfChts*).

Primer name	Sequence (5′→ 3′)	Target Gene	Purpose
RfCht1-1	GGACACTCGGTTGTGGTGCTTTTGGTGG	*RfCht1*	Full length cDNA cloning and qPCR
RfCht1-5c	TTCGGAAACAAAATTGATTTATTTTCGT		Full length cDNA cloning
RfCht1-2	CCTGGGATATTCTCATCATTGATCCTGA		RT-PCR
RfCht1-13c	CAGCCCAGTTTCCACGCAAATCGTACGT		RT-PCR
RfCht2-1	GCCAAGGACGTCGACTGGGCTGTCGTGG	*RfCht2*	Full length cDNA cloning
RfCht2-4c	GCGATGTTAATATATTTTTATTATAAGA		Full length cDNA cloning
RfCht2-7	CATCCGACAACGCAGTGAGCGCCTCCAG		qPCR
RfCht2-5c	TGCATTTGATGGAGAGTGTACGGTATATTTTG		qPCR
RfCht2-9c	GTCGGGACCTACTGGAACAGCAGCTGAG		qPCR
RfCht3-1	GAATTAAGTCTTCGTACCGTACGCTTAG	*RfCht3*	Full length cDNA cloning
RfCht3-3c	CGGTGAGGATGGGGTTGTTGTAACCACT		Full length cDNA cloning and qPCR
RfCht3-3	GGATAACTGCAGCTACTAGCAAATCTAG		qPCR
RfCht3-6c	CGTTGATGGTCGTTGACGCCTGATATTG		qPCR
RfCht4-1	CGTTACTTGGCGACATCGGCATCGACGG	*RfCht4*	Full length cDNA cloning
RfCht4-6c	AGACGTTATCGGTGTATTCTTTTTGACA		Full length cDNA cloning
RfCht4-2	GGTGGAACTCGCCAGGATGCTCCCCATC		qPCR
RfCht4-2c	CGCCCAGTTGGTTACGTAGCACACCACC		qPCR
RfCht4-12	GCGTATTCGCATGGGCTGCAGATCTGGA		qPCR
RfCht4-14c	GCTGATCCGTCGCTGACCAATCTAGAGT		qPCR
RfCht5-1	TACCAGTTAGAACGGTCGAGCTTCGACC	*RfCht5*	Full length cDNA cloning
RfCht5-2c	AAATCCCATAAGGAACAATATAGTATAAAATA		Full length cDNA cloning and qPCR
RfCht5-2	CCCCTGACAAGCACCGACGTCCTCAGCG		qPCR
RfCht5-1c	CGCTGAGGACGTCGGTGCTTGTCAGGGG		qPCR
RfCht5-3	CATAGGCGGTTGGAACGAGGGTTCCACC		qPCR

GenBank (https://www.ncbi.nlm.nih.gov/genbank/) were used to search for similar sequences in the RPW TSA dataset. The identified RPW TSA sequences were pools of unannotated sequences with gaps in sequenced contigs. Multiple sequence alignment was done using MEGA7 (Kumar et al., 2016) and BioEdit (http://www.mbio.ncsu.edu/BioEdit/BioEdit.html) softwares in order to locate the four highly conserved signatures in the amino acids of all known insect chitinases (Zhang et al., 2011b). Only RPW TSA contigs with the conserved four regions were used to synthesize the primers (Table 1) used for partial amplification of chitinase genes.

RNA isolation and first strand cDNA synthesis

Frozen RPW tissues were ground into fine powder in liquid nitrogen using mortar and pestle. Total RNA was isolated using RNeasy Plus Universal Mini Kit (QIAGEN) according to the manufacturer's protocol. Elongase™ enzyme mix was obtained from Invitrogen® and the recombinant Taq DNA polymerase was purchased from Fermentas®. Reverse transcription of RNA to synthesize first strand cDNAs for *RfChts* was done using a random hexamer primer and RevertAid RT Kit obtained from Thermo Fisher Scientific according to the manufacturer's protocol. Double-stranded cDNA was amplified using the first strand cDNA as template and a gene-specific primer (Table 1). Primers used to amplify the full-length cDNAs and to study the expression patterns of the RPW chitinase genes, and for sequencing are shown in Table 1. The thermocycler used for cDNA amplification was Veriti® Thermal Cycler (96 well) supplied by Applied Biosystems™. Amplified PCR products were electrophoresed on 0.7% agarose D1 (Pronadisa) gel, stained with ethidium bromide, visualized using INGENIUS Syngene Bio Imaging System, and documented using GeneSnap software from Syngene. Then, the cDNAs were purified either from the excised gel using QIAquick® Gel extraction kit (Qiagen) or directly from the PCR products using the DNA Pure Kit (Geneaid®) following the manufacturers' protocols. The recovered cDNAs were used for the subsequent PCR amplification, cloning, or direct sequencing.

Gene cloning and sequencing

The PCR-amplified cDNAs were cloned into pGEM®-T Easy vector (Promega, Madison, WI, USA) according to the manufacturer's protocol. Ligation, cloning, and transformation processes were carried out according to the standard protocols (Sambrook et al., 1989). The manipulated plasmids were transformed into *Escherichia coli* strain DH5α. Plasmids maintained by the bacterium were isolated using Wizard® Plus SV Minipreps DNA Purification System (Promega, Madison, WI, USA) according to the supplier's instructions. Due to the big sizes of some cDNA clones, multiple

Table 2. Properties of the identified red palm weevil, *Rhynchophorus ferrugineus*, chitinases (*RfChts*).

Gene name	Group	cDNA length (bp)	Coding region (bp)	5′-untranslated region	3′-untranslated region	Amino acids	Isoelectric point	Molecular weight (kDa)	GenBank accession #
RfCht1	I	1,828	103~1,722	102	106	540	5.43	60.70	KX954127
RfCht2	III	3,638	68~3,031	67	607	988	6.64	112.49	KY576032
RfCht3	VI	4,124	169~4,083	168	41	1305	5.33	145.53	KY576033
RfCht4	II	9,058	209~8,746	208	312	2846	6.53	319.97	KY576034
RfCht5	VII	1,595	61~1,404	60	191	448	6.73	50.74	KY576035

sequencing rounds were carried out to clarify dubious and long uncovered reads. Sequencing was done at Macrogen service facilities (Seoul, South Korea).

Domain structure and phylogenetic analyses

Multiple sequence alignment of the deduced amino acids of *RfChts* and the molecular phylogenetic analyses were performed using the software MEGA7 (Kumar et al., 2016). Chitinase domain structure analysis was done using InterPro: protein sequence analysis and classification database (https://www.ebi.ac.uk/interpro/).

Expression profiles of *RfChts* genes in different developmental stages and tissues

The expression profiles of the five *RfCht* genes at different stages of development including eggs, larvae, pre-pupae, pupae, adults and appendages were tested. Eggs were collected 12- and 24-h after laying and tissues of the middle-aged larvae were collected at 0- to 96-h pre-molting. Reverse transcription PCR was done in a 25-µl reaction mixture containing 1 µl template first strand cDNA, 10 pmol/µl each primer, 12.5 µl Master Mix (Biomatik Corporation, Canada), and nuclease-free water. The thermocycler program for RT-PCR was as follows: initial denaturation cycle at 94°C for 3 min followed by 30 cycles at 94°C for 25 s, 60°C for 25 s, and 72°C for 2 min, and a final extension at 72°C for 10 min. PCR products were analyzed on 1% agarose gel. Experiments were replicated at least three times using independent total RNA preparations. RPW's ribosomal protein S3 (*RfRpS3*) was used as an internal reference gene for RT-PCR analysis.

RESULTS

Sequence analysis of cDNAs for the genes of chitinase

Five chitinase cDNA amplicons from *R. ferrugineus* were sequenced (*RfCht1*, *RhCht2*, *RfCht3*, *RfCht4*, and *RfCht5*) and the sequences were deposited in the GenBank database and the accession numbers are shown in Table 2. The *RfCht1* full sequence is 1,828 bp long. It consists of 1620 bp open reading frame (ORF), 102 bp 3′-untranslated region, and 106 bp 5′-untranslated region (Table 2). The translated region consists of 540 amino acids (Figure 1). The first 20 amino acids constitute a putative signal peptide, as predicted by the online SignalP 4.1 Server (Petersen et al., 2011) that targets the protein to the extracellular space or sorts it into plasma membrane to face in both cases carbohydrates of the extracellular matrix (Kawamura et al., 1999; Royer et al., 2002; Arakane et al., 2003).

Analysis of the putative consensus signature domains of the isolated cDNA revealed that the protein consists of a single catalytic domain of the glycoside hydrolase family 18 (GH18). The catalytic domain covers the deduced amino acids span from 18 to 383. The amino acids from 270 to 346 form a chitinase insertion domain (CID). The C-terminus amino acids from 484 to 539 constitute a chitin-binding domain (CBD), which belongs to family 14 of carbohydrate-binding modules (CBM14). *RfCht2* is 3,638 bp long covering an ORF of 2,964 bp that encodes a putative protein of 988 amino acids including a putative signal peptide, two GH18 catalytic domains in which two CIDs are embedded, and has a single C-terminal CBD. *RfCht3* is 4,124 bp long. It consists of 3,915 bp ORF that encodes a putative protein of 1,305 amino acids including a putative signal peptide, a GH18 catalytic domain in which a CID is embedded, and has a single C-terminal CBD. On the other hand, *RfCht4* is the largest gene for chitinase from the RPW found in this study. The gene is 9,058 bp long with an ORF of 8,538 bp that encodes a putative protein of 2,846 amino acids. The identified sequence of *RfCht4* lacks a signal peptide but it contains five GH18 catalytic domains in which five CIDs are embedded, and has five CBDs. One CBD located between the first and the second catalytic domains, three CBDs located between the second and the third catalytic domains, and one CBD located between the fourth and the fifth catalytic domains (Figure 1 and Table 2). In comparison, *RfCht5* is the smallest gene among the *RfChts* identified in this study. *RfCht5* consists of 1,595 bp with an ORF of 1,344 bp that encodes a putative protein of 448 amino acids (Table 2). It contains a predictable signal peptide, a single GH18 catalytic domain but lacks

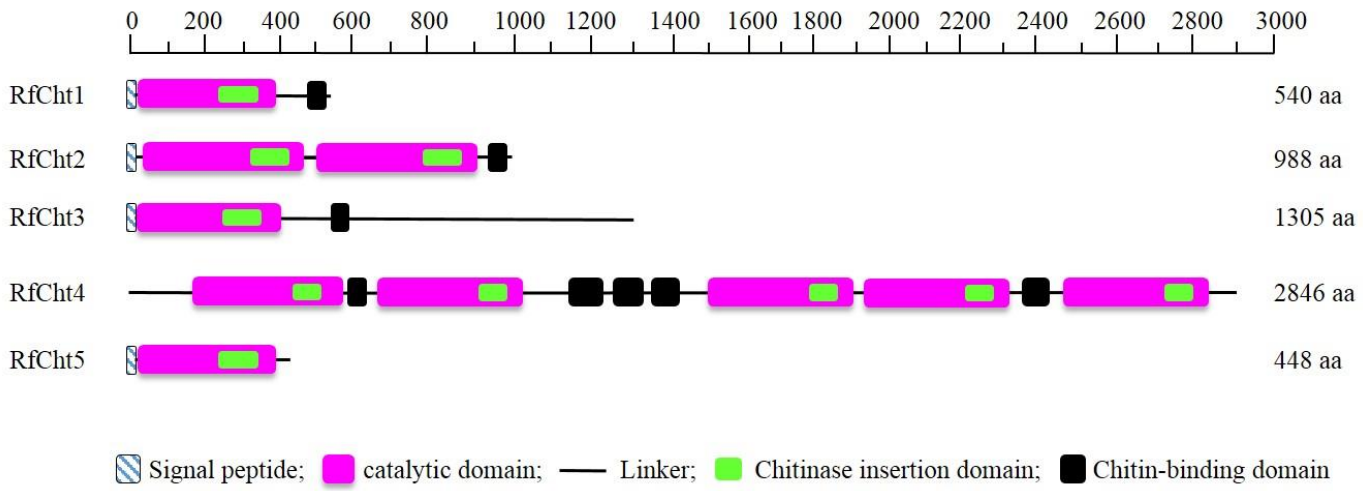

Figure 1. Domain organization of putative chitinases from RPW. The signal peptide was predicted by SignalP 4.1 server. The other domains were analyzed by the program InterPro. All investigated *RfChts* except *RfCht5* possess single or multiple chitin-binding domains (CBD) resembling the carbohydrate-binding module (CBM-14) found in peritrophins.

CBD (Figure 1). The five *RfChts* have many serine/ threonine (S/T) residues in the sequences between the catalytic domains and the CBDs. Similar to other insect chitinases, all CBDs of *RfChts* retain six conserved cysteines (C) that probably form three disulfide bridges that increase the affinity of chitinase for the insoluble substrate during chitin hydrolysis.

GenBank database homology searches and the sequence alignments revealed that the deduced amino acids of the five *RfChts* share common consensus signature sequences that identify them as GH18 chitinases (Figure 2) and these are well-characterized from many insects belonging to different orders and species (Zhu et al., 2008b; Su et al., 2016). All the five *RfChts* retain the signature sequences in the conserved region I (KxxxxxGGW) and in the conserved region IV (GxxxWxxDxDD) of GH18 chitinases. It is notable that the conserved region II (FDGxDLDWEYP) is common in all identified *RfChts* except *RfCht5* where the residue tyrosine (Y) was replaced by phenylalanine (F). Moreover, in the *RfCht4* the conserved glutamate residue (E) in the second catalytic domain was replaced by asparagine (N) (Figure 2).

Phylogenetic analysis of deduced protein sequences of five *RfCht* genes

A phylogenetic tree was constructed by using deduced amino acids of *RfChts* and sequences of chitinase and/or chitinase-like proteins from 21 insect species belonging to seven orders (Figure 3). The tree was generated using maximum likelihood method. Phylogenetically, *RfCht1* belongs to group I chitinases and is closely related to a hypothetical protein from the mountain pine beetle, *Dendroctonus ponderosae* (Keeling et al., 2013) and to chitinase 5 (*TcCht5*) of the red flour beetle, *T. castaneum* (Zhang et al., 2011a). *RfCht2*, *RfCht3*, *RfCht4,* and *RfCht5* phylogenetically belong to the groups III, VI, II, and VII, respectively (Figure 3).

Expression profiling of *RfChts* in the tissues and developmental stages

Expression profiles of *RfChts* primary transcripts in eggs, larvae, adults, and the appendages were determined by RT-PCR. Transcripts of *RfCht1* and *RfCht2* were expressed in the larval cuticles 96-h pre-molting and the expression continues to the onset of molting (Figure 4). *RfCht2* seems more steadily expressed 14-h pre-molting; however, 96- to 24-h pre-molting, fewer transcripts were expressed relative to the transcripts found 14- to 0-h pre-molting. The transcripts of both chitinases were found in the adipose tissues and the gut of larvae, as well as in the guts of both males and females, the elytra, and the hindwings (Figure 4). The transcripts of *RfCht3* were uniformly detectable 14- to 0-h pre-molting but in less quantity relative to *RfCht1* and *RfCht2* transcripts. *RfCht3* was not detected in cuticular tissues 96- to 24-h pre-molting and it was not found in adult weevils or their appendages. *RfCht4* transcripts were detected only in cuticles that were collected 96-, 8- and 4-h pre-molting. It seems there is no steady expression titer for *RfCht4* in the cuticle. Transcripts of *RfCht4* were not detected in adults or their appendages. Faint transcripts of *RfCht5* were detected in larval cuticular tissues 48- to 0-h pre-molting but were not found in other tissues and developmental stages.

Transcripts of the five chitinases were further

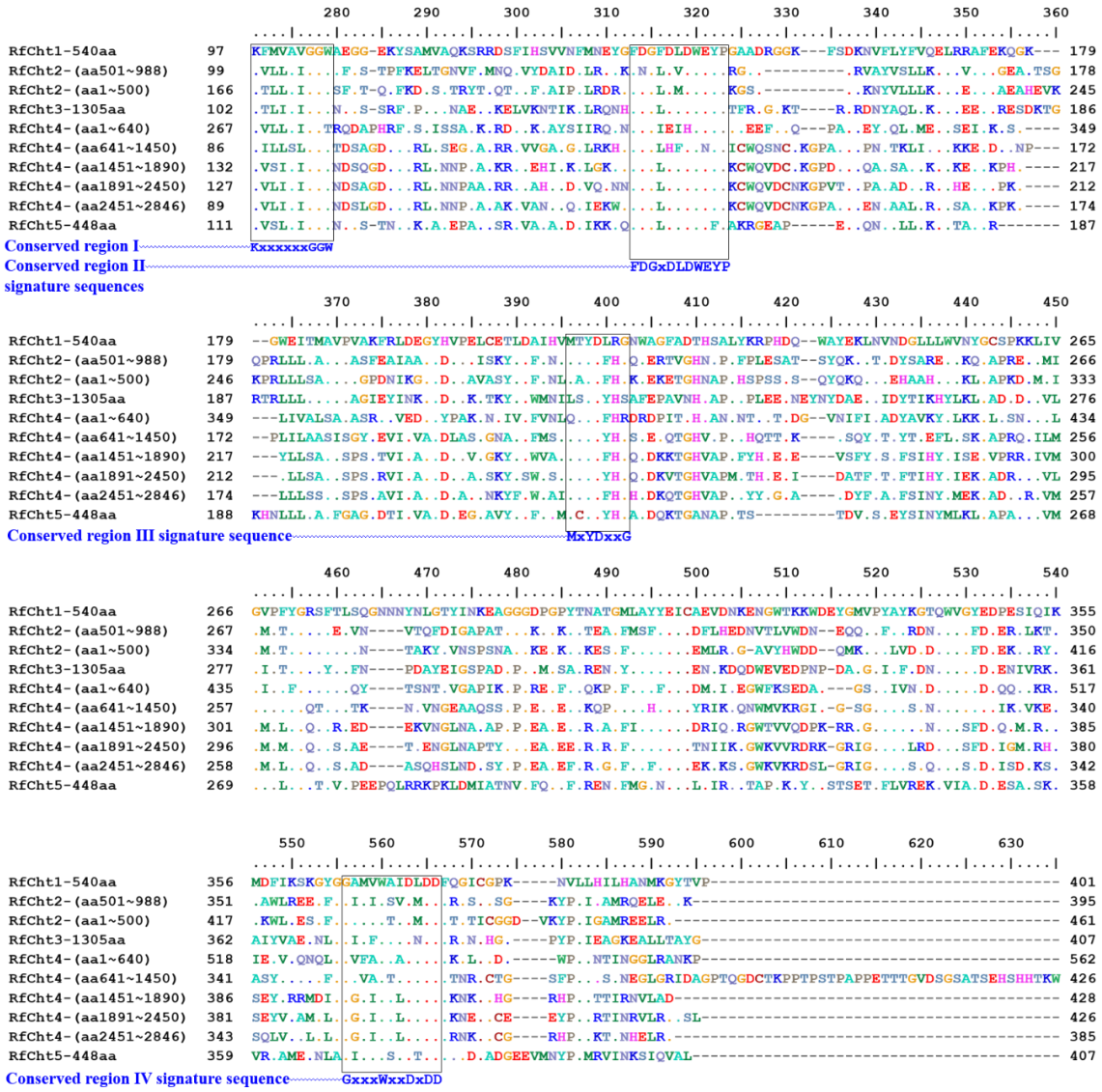

Figure 2. Multiple sequence alignment of the deduced amino acid for *RfChts*. MEGA7 (Kumar et al., 2016) and BioEdit softwares performed the multiple sequence alignment. Dots indicate identical amino acid sequences. Conserved regions I, II, III, and IV in insect chitinases were boxed. The signature sequences for each conserved region were also depicted.

investigated in the cuticles and internal organs of the larvae inhabiting the cocoon, pre-pupae, and pupae (Figure 5). *RfCht1* and *RfCht2* were found in all tested stages and tissues including adult wings, 12- and 24-h old eggs. *RfCht3*, *RCht4*, and *RfCht5* transcripts were very low relative to those for *RfCht1* and *RfCht2*. It is clear that the transcripts of *RfCht3*, *RCht4,* and *RfCht5* were steadily expressed but in low quantities in the last instar larvae within the cocoon, pre-pupae, and pupae. Transcripts of all the five *RfCht* genes were expressed in 24-h old eggs though more transcripts of *RfCht1* were expressed followed by *RfCht2*, *RfCht3*, *RfCht5*, and *RfCht4*. *RfCht2* transcripts were prominent in 12-h old eggs and hardly detectable for the other chitinases (Figure 5). Apparently, the transcripts of *RfCht2* were higher followed by the transcripts of *RfCht4*, *RfCht5*, *RfCht1*, and *RfCht3* in first instar larvae just emerged from the eggs (Figure 6).

DISCUSSION

Chitinases are indispensable enzymes involved in chitin metabolism leading to molting and eclosion of insects and other arthropods. The matrix polymer chitin has been designed to provide the cuticle with flexibility in response

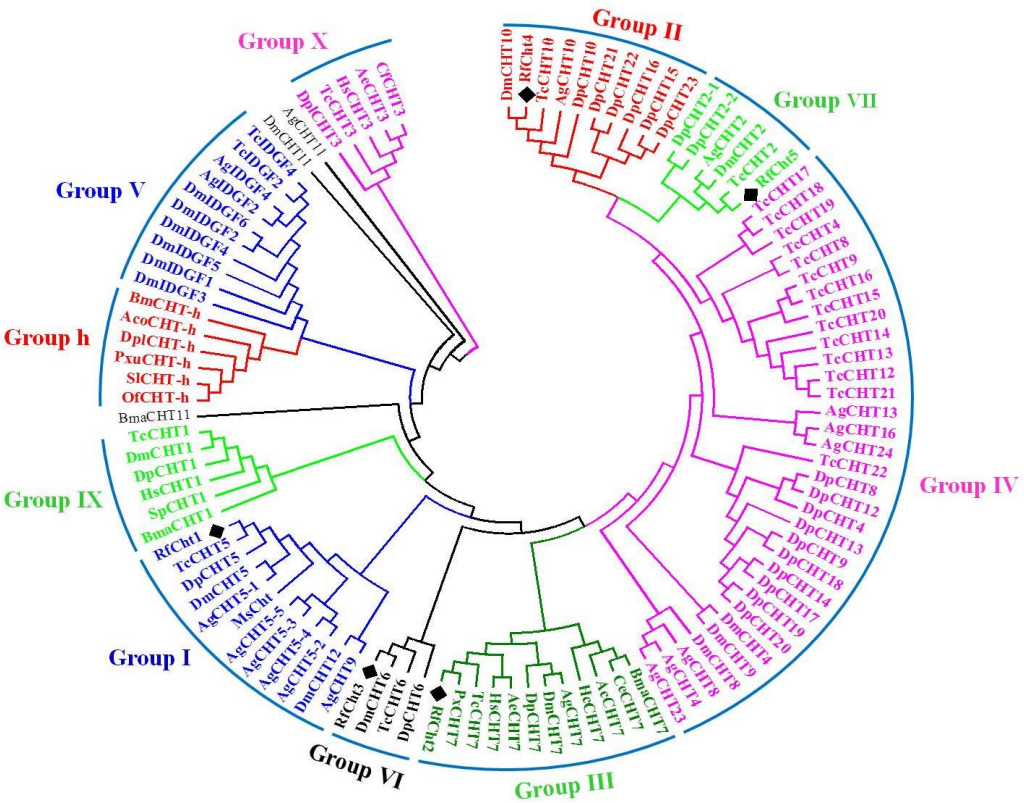

Figure 3. Molecular phylogenetic tree of *RfChts*. The tree was inferred by using the Maximum Likelihood method based on the JTT matrix-based model implemented in MEGA7. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 107 amino acid sequences downloaded from the GenBank. The sequences represent chitinases and chitinase-like proteins from 21 species of arthropods belonging to seven orders. *Acromyrmex echinatior* (Ae) – Hymenoptera; *Agrius convolvuli* (Aco) – Lepidoptera; *Ancylostoma ceylanicum* (Ac) – Nematoda; *Anopheles gambiae* (Ag) – Diptera; *Bombyx mori* (Bm) – Lepidoptera; *Brugia malayi* (Bma) – Nematoda; *Caenorhabditis elegans* (Ce) – Nematoda; *Camponotus floridanus* (Cf) – Hymenoptera; *Danaus plexippus* (Dpl) – Lepidoptera; *Daphnia pulex* (Dp) – Crustacea; *Drosophila melanogaster* (Dm) – Diptera; *Haemonchus contortus* (Hc) – Nematoda; *Harpegnathos saltator* (Hs) – Hymenoptera; *Manduca sexta* (Ms) – Lepidoptera; *Ostrinia furnacalis* (Of) – Lepidoptera; *Papilio xuthus* (Pxu) – Leidoptera; *Plutella xylostella* (Px) – Lepidoptera; *Rhynchophorus ferrugineus* (Rf) – Coleoptera; *Spodoptera litura* (Sl) – Lepidoptera; *Strongylocentrotus purpuratus* (Sp) – Echinodermata; *Tribolium castaneum* (Tc) – Coleoptera.

to the need to adapt to extensively diverse environmental stresses (Muthukrishnan et al., 2016). Insect chitinases have been characterized as potential targets for pest management either via directly targeting them (Su et al., 2016; Cao et al., 2017) or via manipulating their inhibitors as biopesticides (Arakane and Muthukrishnan, 2010; Hirose et al., 2010). *R. ferrugineus* lives and develops inside the palm trunk, where there are various biomass-degrading microbes that do not harm the weevil. In this context, it has been reported that polar surface cuticular extracts from adults and larvae inhibited the growth of Gram-positive bacteria and the entomopathogenic fungi *Beauveria bassiana* (Mazza et al., 2011).

Here, cDNAs have been isolated and synthesized for the chitinase genes from *R. ferrugineus* larvae that had not yet undergone apolysis. During this time the molting fluid fills the space between the digested old cuticle and the newly synthesized cuticle (Kramer et al., 1985). The identified chitinase genes shared the common consensus signature sequences found in all investigated insect chitinases, namely the conserved regions I, II, III, and IV that classify them into GH18. The largest gene for chitinase isolated from the RPW is *RfCht4* and the smallest one is *RfCht5*. The five *RfChts* identified in this study have divergent numbers of catalytic domains, CBDs, and CIDs that are believed to interact with oligosaccharides during catalysis (Li and Greene, 2010). The CID facilitates orienting and binding to longer chitin substrates when inserted into the triose phosphate isomerase (TIM) barrel, a conserved protein fold consisting of eight α-helices and eight parallel β-strands that alternate along the peptide backbone (Li and Greene,

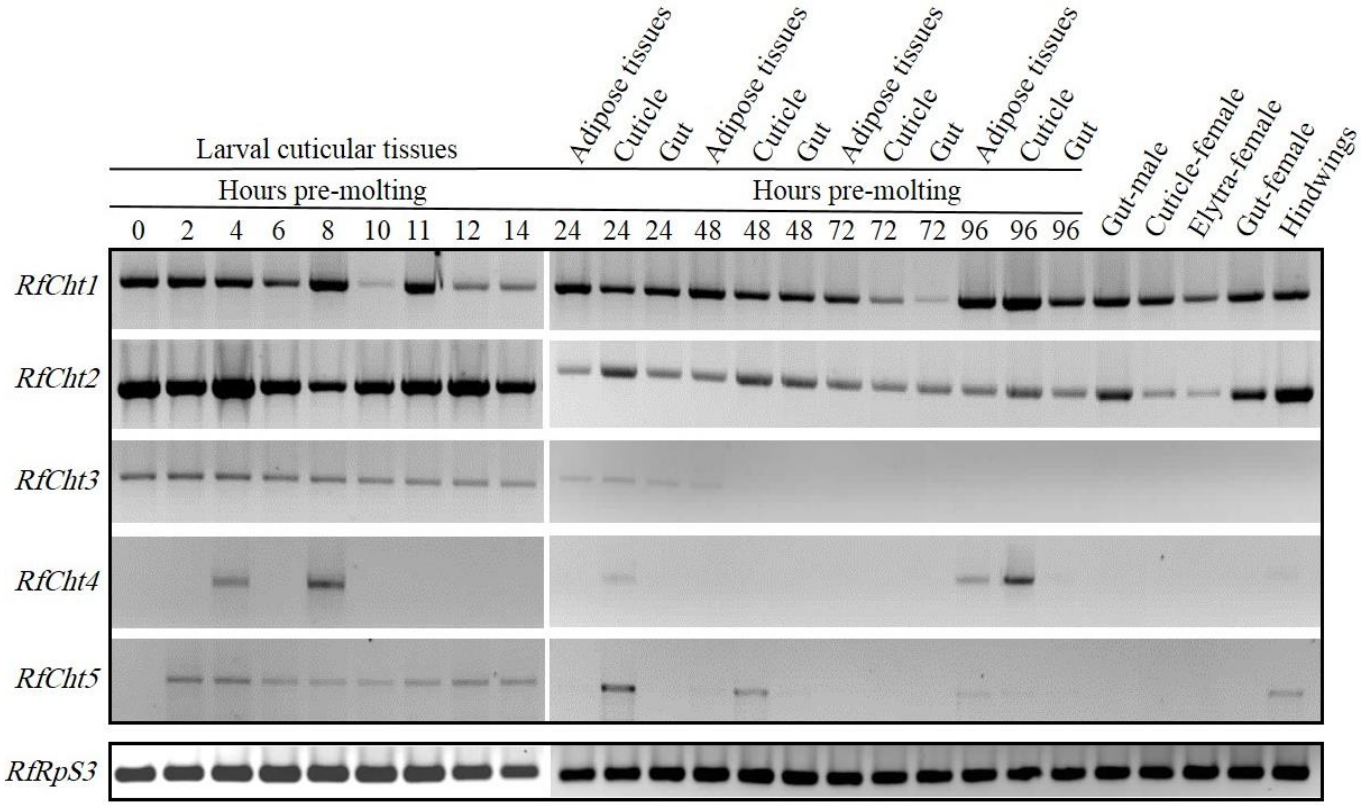

Figure 4. *RfChts* expression profiles in different tissues of middle-aged larvae and adults. The expression patterns of five *RfCht* genes were evaluated 96- to 0-hours pre-molting in larval adipose tissues, cuticles, and guts using RT-PCR. Likewise, the expression patterns were investigated in the adult's cuticle, gut, elytra, and hindwings. *RfRpS3* was used as reference gene for the RT-PCR.

2010). Moreover, with the exception of *RfCht5* that has no CBD, the CBDs of *RfChts* have linker sequences with six conserved cysteine residues. It has been reported that the common spacing between the conserved cysteines in the CBDs of GH18 chitinases appears to be as follows: $^{1}Cx_{11\text{-}24}$-$^{2}Cx_{5\text{-}6}$-$^{3}Cx_{9\text{-}19}$-$^{4}Cx_{10\text{-}17}$-$^{5}Cx_{4\text{-}14}$-^{6}C, where x is any other amino acid (Arakane and Muthukrishnan, 2010; Su et al., 2016). However, the six cysteine residues in *RfCht1* gene were arranged as follows: $^{1}Cx_{9}$-$^{2}Cx_{5}$-$^{3}Cx_{9}$-$^{4}Cx_{12}$-$^{5}Cx_{10}$-^{6}C. It is obvious that the spacing between the first and the second conserved cysteines in *RfCht1* CBD is only nine amino acids, that is, the spacing is shorter in *RfCht1* compared to the chitinase genes isolated from the RPW and from other insects. However, the spacing between the remaining conserved cysteines in the five *RfChts* investigated here is within the range commonly found in other insect chitinases. These linker regions can be heavily glycosylated and are believed to increase the stability of chitinases when present in a protease-rich environment such as the gut or the molting fluid (Abdel-Banat and Koga, 2002; Arakane et al., 2003; Arakane and Muthukrishnan, 2010).

Notable observations were found in the conserved region II (FDGxDLDWEYP) where the conserved tyrosine (Y) was replaced by phenylalanine (F) in *RfCht5* and the conserved glutamate residue (E) in the second catalytic domain of *RfCht4* was replaced by asparagine (N). These replacements were also observed in *CpCht10* from *Culex pipiens* and *PhcCht10* from *Pediculus humanus corporis* (Arakane and Muthukrishnan, 2010). Generally, glutamate residue (E) is the most critical in the conserved motif II and is believed to be the proton donor required for cleavage of the glycosidic bond as evidenced by the replacement of the residue (E) by a glutamine (Q) or with an aspartic acid (D) which resulted in complete loss of enzyme activity (Lu et al., 2002). Structurally, the identified *RfChts* typically resemble other known chitinases that identified from other insect species, especially in their physical and multi-domain architectures (Pan et al., 2012; Huang et al., 2012).

Insect chitinases and chitinase-like proteins are categorized into distinct groups according to phylogenetic kinships. Group I chitinases are secreted proteins that are the most abundant enzymes in molting fluid and/or integument, and represent the prototype enzyme of GH18, with a single copy each of the catalytic domain and chitin-binding domain (CBD) connected by linker polypeptide rich with S/T residues (Arakane and

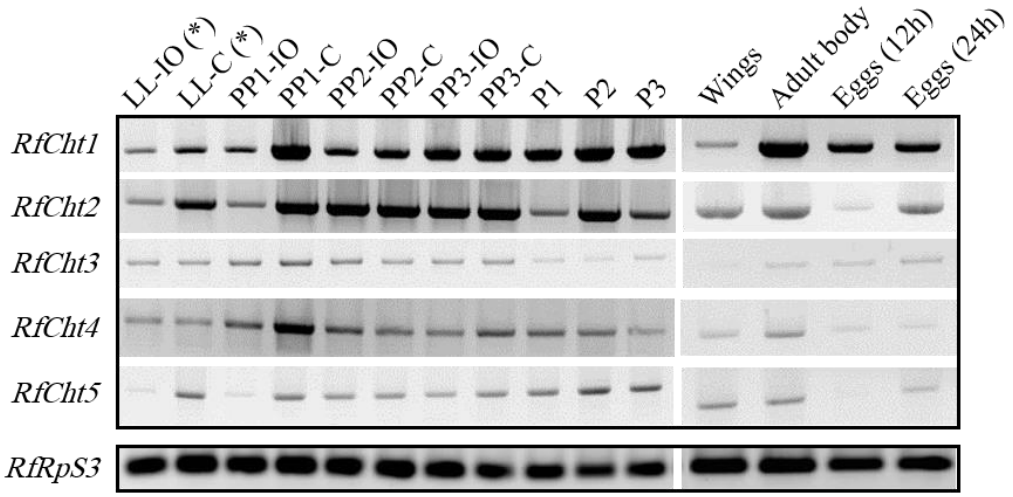

Figure 5. *RfChts* expression profiles in eggs, tissues of last instar larvae, pre-pupae, pupae, and adult weevil. The expression profiles were tested in cuticular (C) and internal organ (IO) tissues from the last instar larvae (LL) that have spun the cocoon (*) and inhabited inside, from three pre-pupae (PP), and from three pupae in addition to 12-hour and 24-hour old eggs. The expression was also evaluated in the adult's body as well as the wings. *RfRpS3* was used as reference gene for the RT-PCR.

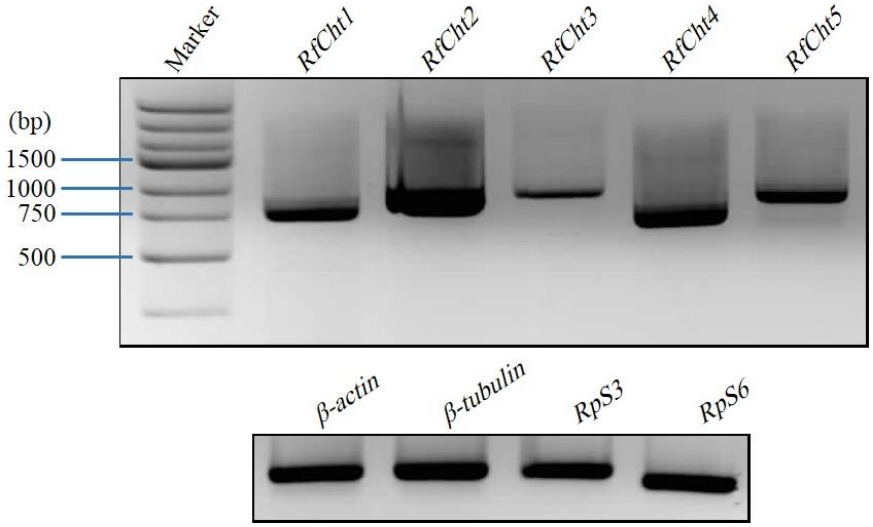

Figure 6. Evaluation of the primary transcripts for *RfChts* in the first instar larvae. The RT-PCR analysis was done to evaluate the expression of the five *RfChts* in tissues of the first instar larvae that instantly hatched from eggs. The housekeeping genes *Rfβ-actin*, *Rfβ-tubulin*, *RfRpS3*, and *RfRpS6* were used as reference genes for the RT-PCR.

Muthukrishnan, 2010; Khajuria et al., 2010; Tetreau et al., 2015). Previous studies reported that group II chitinases are larger-sized secreted proteins with multiple catalytic domains and CBDs (Zhu et al., 2008c; Tetreau et al., 2015). Group III chitinases contain two catalytic domains and are predicted as membrane-anchored proteins. Group IV chitinases are the most divergent. They usually lack a CBD and/or an S/T-rich linker domain and are predicted to be secreted proteins found in the gut or fat body. Group V proteins include the putative chitinase-like imaginal disc growth factors (IDGFs). In *T. castaneum* and *D. melanogaster* genomes, multiple genes were found to encode groups IV and group V chitinase-like proteins. In contrast, groups I, II, and III are each

represented by only a single gene in each species (Zhu et al., 2008c; Tetreau et al., 2015). Group VI chitinases resemble group I chitinases; however, the C-terminal S/T-rich linker extends the molecular mass of proteins in this group. Group VII chitinases structurally resemble group IV but phylogenetically are placed as an outlier of group II (Merzendorfer, 2013). The group has an N-terminal signal peptide and a GH18 catalytic domain, but it lacks a CBD.

The expression patterns of *RfChts* are similar to the expression patterns of a chitinase-like gene cluster (*AgCht5*) from the African malaria mosquito, *A. gambiae* (Diptera: Culicidae), where the five genes in the cluster showed different expression patterns at different developmental stages (Zhang et al., 2011a). It has been reported that expression of insect chitinases is time controlled and released only during molting. Chitinase transcripts are not detectable in cuticular tissue until after the cessation of feeding in each larval instar and appear immediately prior to pupation before dropping to undetectable levels 1 to 2 days into the molt cycle (Muthukrishnan et al., 2016). In contrast, our study showed that transcripts of *RfChts* were detectable in all stages at earlier times before the larvae had commenced the molting. Similar expression patterns were reported in *N. lugens* chitinase-like gene family (Xi et al., 2015). This suggests that at least some RPW chitinases may have been enrolled in functions other than molting. Due to the concealed living behavior of the weevil within the trunk of the host plant, a place where many species of microbes survive and propagate, it is probable that some of the chitinases might be involved in the immune defense to protect the weevil from microbial infection.

Molecular characterization and phylogenetic studies of five *R. ferrugineus* chitinases revealed RPW conserved the functional domain consensus commonly found in all identified chitinases from insects belonging to different orders. However, the expression pattern of some chitinases in the tissues of *R. ferrugineus* and developmental stages is unique. Expression of RPW chitinases varies from being in short time and in specific patterns usually found in other insects to an unusual constitutive expression throughout the larval developmental stages. This might be related to the living habitat of this insect pest, where an array of microbial inhabitants exists in the same habitation. Further studies are necessary to understand the specific roles of the constitutively expressed chitinases in *R. ferrugineus*.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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