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Identification of a laccase gene involved in shell periostracal tanning of the clam *Meretrix petechialis*

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ABSTRACT: Tanning is a complex extracellular process that is a mechanism for stabilizing proteinaceous extracellular structures. Phenoloxidases play important roles in cross-linking during tanning, and laccase (EC 1.10.3.2) is a member of the phenoloxidase enzyme class. In this study, we identified a laccase gene (*MpLac*) from the clam *Meretrix petechialis* and found that *MpLac* might be involved in shell periostracal tanning of clams. Using whole-mount *in situ* hybridization, we found that *MpLac* mRNA in the larval clam was mainly expressed in the mantle edge. In the adult clam, our quantitative real-time PCR results showed that the mantle was also a tissue with a high *MpLac* expression level; in addition, by combining the results of fluorescence *in situ* hybridization, H&E staining and transmission electron microscopy, we found that the inner epithelium of the outer fold of the mantle edge, which is involved in periostracum formation, was the exact region in which *MpLac* mRNA was expressed. Furthermore, knocking down the expression of *MpLac* by RNA interference (RNAi) bleached the new shell periostracum. All of our results suggest the involvement of *MpLac* in shell periostracal tanning of *M. petechialis*.

KEY WORDS: *Meretrix petechialis* · Laccase · Shell periostracum · Tanning

1. INTRODUCTION

Mollusks are generally covered with an exoskeleton (shell) consisting of calcium carbonate and an organic matrix (periostracum) (Lowenstam & Weiner 1989, Marin & Luquet 2004). The periostracum is mainly a quinone-tanned protein layer (Degens et al. 1967) and is among the mechanically strongest but chemically most inert structures in the animal kingdom (Waite 1983). The periostracum, which is located outside the shell surface, protects the calcified shell from corrosion and is also involved in shell calcification (Taylor & Kennedy 1969). Periostracum formation originates in the periostracal groove (PG) of the mantle edge, and the proximal epithelium is involved in periostracum thickening (Bevelander & Nakahara 1967, Kniprath 1972). First, the soluble precursor of the periostracum (i.e. periostracin) is secreted (Waite et al. 1979). Then, via a tanning process, periostracins are cross-linked and become rigid, forming the insoluble periostracum (Saleuddin & Petit 1983).

Tanning (i.e. sclerotization and pigmentation) is a complex extracellular process first proposed >70 yr ago as a mechanism for stabilizing the proteinaceous extracellular structures found throughout the animal kingdom (Pryor 1940). Phenoloxidases play important roles in the cross-linking process during tanning (Andersen 2005, 2010, Checa 2000). In animals, there are 2 main types of phenoloxidases: tyrosinase

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(EC.1.10.3.1) and laccase (EC.1.10.3.2). For laccase, 2 isoforms (laccase1 and laccase2) have been found in insects (Dittmer et al. 2004, Arakane et al. 2005). Laccase2 was reported to be indispensable for tanning of the cuticle (exoskeleton) in the insect *Tribolium castaneum* (Arakane et al. 2005). The involvement of laccase2 in cuticle tanning has also been found in other insect species (Andersen 2005, Niu et al. 2008, Elias-Neto et al. 2013). In mollusks, laccase has been identified as an immune factor (Luna-Acosta et al. 2010, 2011, Le Bris et al. 2013, 2014), but little is known about its role in periostracum formation.

The Asiatic hard clam *Meretrix petechialis* is mainly distributed in the coastal areas of South and Southeast Asia and is an important commercial marine bivalve species (Tang et al. 2006). *M. petechialis* has a typical periostracum outside the calcified shell (Yue et al. 2015); thus, this species is an excellent model for investigation of shell periostracal tanning. In the present study, we identified a laccase-like gene from *M. petechialis* (*MpLac*) and investigated whether laccase is also involved in the tanning of the clam periostracum. We detected the expression patterns of *MpLac* in both larval and adult clams, and gained insights into the function of *MpLac* in shell periostracal tanning. Furthermore, in an RNA interference (RNAi) experiment, we observed changes in the shell periostracum when *MpLac* expression was knocked down. Our study will be helpful for understanding the molecular mechanisms underlying periostracum formation in clams.

2. MATERIALS AND METHODS

2.1. Full-length cDNA cloning and sequence analysis

After a basic local alignment search tool (BLAST) analysis of the expressed sequence tag (EST) sequences from the cDNA library of *Meretrix petechialis* that we had previously constructed (Yue et al. 2015), one EST sequence (~400 bp) showed the highest similarity to the laccase gene. The full-length laccase cDNA of *M. petechialis* (*MpLac*) was then obtained by using the rapid amplification of cDNA ends (RACE). The 3′ and 5′ RACE reactions were performed following the instructions of the SMART RACE cDNA amplification kit (Clontech). The PCR products were gel-purified and then individually cloned into the pMD19-T simple vector (TaKaRa). The recombinant plasmid was transformed into competent *Escherichia coli* cells, which were then cultured on Luria-Bertani (LB) plates at 37°C overnight. Positive clones with the recombinant plasmid were identified by PCR. The recombinant plasmid was extracted from the mass culture of positive clones and then sequenced by the Sanger method. The entire assembled cDNA sequence was further verified by PCR amplification and sequencing.

The amino acid sequence of *MpLac* was deduced from the cDNA sequence. The signal peptide was predicted by both neural networks and hidden Markov models on the Signal IP 3.0 Server (Bendtsen et al. 2004). The isoelectric points (pI) and molecular weight of the deduced protein were determined using the 'Compute pI/MW' tool on the ExPASY Server (www.expasy.org/tools) (Gasteiger et al. 2005).

Phylogenetic analysis was done with amino acid sequences by the neighbour-joining (NJ) method with the program Mega 3.0. Besides *MpLac*, other laccases from different species (i.e. oyster *Crassostrea gigas*, freshwater snail *Biomphalaria glabrata*, amphioxus *Branchiostoma belcheri*, sponge *Amphimedon queenslandica*, silkworm *Bombyx mori*, moth *Manduca sexta*, green rice leafhopper *Nephotettix cincticeps*, beetle *Tribolium castaneum*) were subjected to the phylogenetic tree construction. The laccase from sponge *A. queenslandica* was set as an outgroup.

2.2. Quantitative real-time PCR

Using quantitative real-time PCR (qRT-PCR) with the specific primers lac-RT-F and lac-RT-R (Table 1), the relative mRNA expression of *MpLac* was compared among different clam tissues, including the adductor muscle, foot, hepatopancreas, haemolymph, mantle and gill. The different tissues of 5 clams were dissected separately and immediately stored at −80°C for subsequent RNA extraction. In addition, qRT-PCR was also used to detect changes in the *MpLac* expression level in the mantle during the RNAi experiment. The samples used in the qRT-PCR are described in detail in Section 2.6.

Total RNA was extracted separately from each sample, and cDNA was synthesized as the qRT-PCR template. qRT-PCR was performed using a QuantiNova SYBR Green PCR Kit (Qiagen) with an ABI 7500 Fast Real-Time Thermal Cycler (ABI). The PCR parameters were 95°C for 30 s, followed by 40 cycles of 95°C for 10 s and 60°C for 30 s. Each 10 µl volume of the PCR reaction mix contained 20 ng of the template, 0.2 µM of each primer and 5 µl of the QuantiNova SYBR Green PCR Master Mix. Each reaction

was performed in triplicate. β-actin was set as an internal reference to normalize expression levels among samples (Yue et al. 2011). The $2^{-\Delta\Delta CT}$ method was used to analyze the relative mRNA expression level of *MpLac* among samples (Livak & Schmittgen 2001). Data were examined for homogeneity of variances by *F*-test and then analyzed by *t*-test using SPSS v.13.0 software. A difference was considered statistically significant if $p < 0.05$.

2.3. Whole-mount *in situ* hybridization

Clam larvae at 3 different developmental stages, including the D-veliger early stage, D-veliger late stage and postlarval stage, were separately collected and immediately fixed in 4% paraformaldehyde. To detect the locations of *MpLac* mRNA in the whole clam during development, whole-mount *in situ* hybridization (WMISH) was applied to the clam larvae. The detailed WMISH method is described in Yue et al. (2013). Briefly, a cDNA fragment of *MpLac* (~600 bp) was amplified using the specific primers lac-ISH-F and lac-RT-R (Table 1) and then cloned into the pGEM-T-easy vector (Promega). A digoxigenin-labelled RNA probe with the *MpLac* antisense or sense sequence was synthesized following the general protocol of the DIG RNA labelling kit (Roche). The larvae were prehybridized in the hybridization buffer for 4 h at 65°C. Then, the larvae were hybridized in the hybridization buffer with the antisense probe or the sense probe (negative control) for 12–14 h at 59°C, followed by sequential washing. Blocking and antibody (i.e. alkaline phosphatase conjugated anti-digoxigenin antibody) incubation were subsequently performed. Finally, the larvae were stained with the colourimetric AP substrate (NBT/BCIP stock solution). The specific blue-violet-coloured precipitate indicated the location of *MpLac* mRNA. Images were captured with a digital camera mounted on a microscope (Nikon H600L).

2.4. Fluorescence *in situ* hybridization

The mantle edge zone was dissected from the adult clam and immediately fixed in 4% paraformaldehyde. Routine paraffin section preparation was applied to the mantle edge tissue. Sections with a 6 µm thickness were cut and then transferred onto poly-L-lysine-coated slides (Boster). To detect the mRNA expression of *MpLac* in the shell-forming tissue (i.e. mantle), these sections were analyzed with fluorescence *in situ* hybridization (FISH) targeted to *MpLac* mRNA. The *MpLac* RNA probes used for FISH were the same as those used for WMISH. The FISH method was modified from that described in Yue et al. (2013). Briefly, the dewaxed sections on the slides were prehybridized in the hybridization buffer for 4 h at 65°C and then hybridized in the hybridization buffer with 1 ng μl^{-1} of the antisense or sense probe (negative control) for 12–14 h at 59°C. After stringent washing and blocking, slides were incubated with anti-digoxigenin-fluorescein and Fab fragments (Roche) in blocking buffer for 2 h at 37°C. Evans blue dye was used to contrast dye the non-antigen locations of the mantle edge. Unbound antibodies were removed by washing in 1× PBS. Finally, slides were mounted with ProLong Diamond Antifade Mountant (Invitrogen) and a coverslip and photographed with an LSM710 confocal laser scanning microscope (Zeiss).

2.5. Hematoxylin and eosin staining and transmission electron microscopy

To further determine the locations of *MpLac* mRNA in the mantle edge, hematoxylin and eosin (H&E) staining and transmission electron microscopy (TEM) were performed. The microstructure of the mantle edge was revealed by H&E staining, while the ultrastructure of the mantle edge was revealed by TEM. The detailed method of H&E staining followed the descriptions in the 'H&E staining protocol' on the website IHCWORLD (www.ihcworld.com/_protocols/special_stains/h&e_ellis.htm) (Martoja & Martojapierson 1967). The TEM procedure followed the descriptions in Yue et al. (2010). To achieve accurate localization, semi-thin sections stained with 1% toluidine blue solution were prepared before cutting the ultrathin sections.

2.6. RNAi

RNAi was performed to detect the shell colour change when *MpLac* expression was knocked down. The cDNA fragment of *MpLac* was amplified using the T7 promoter-linked primer pair (lac-T7-F and lac-T7-R) (Table 1) and used as the PCR template to synthesize the *MpLac* double-stranded RNA (dsLac). The fragment of a pEGFP vector plasmid (Clontech) was amplified by EGFP-T7-F and EGFP-T7-R primers (Table 1) and used as the PCR template to synthesize the EGFP double-stranded RNA (dsEGFP). dsLac and dsEGFP were synthesized and purified

Table 1. Primers used in this study

Primer	Sequence (5′–3′)
lac-RT-F	CTA CCT GCT GGC GGT TAT GTC
lac-RT-R	CTC CTC CTC CGT GAA AGT CCT
lac-ISH-F	ATT TGC GAT GAA TCT AAG TGT GC
lac-T7-F	GCG TAA TAC GAC TCA CTA TAG GGA ACC AAT TAA CTA ACA CGC CTT TG
lac-T7-R	GCG TAA TAC GAC TCA CTA TAG GGG CTT ATC CCA TTC ATG TGA CCC
EGFP-T7-F	GCG TAA TAC GAC TCA CTA TAG GGA GCC ATA CCA CAT TTG TAG AGG
EGFP-T7-R	GCG TAA TAC GAC TCA CTA TAG GGC GCT TTC TTC CCT TCC TTT

with a TranscriptAid T7 High Yield Transcription Kit (Thermo) according to the manufacturer's instructions. The quantity and integrity of dsLac/dsEGFP were determined using a Nanodrop ND1000 spectrophotometer (Thermo) and agarose gel electrophoresis, respectively.

Clams with maroon shell colour (strain WM) (Yue et al. 2015) were acclimated in the laboratory for a week and then randomly divided into 3 groups, i.e. the dsLac-injected group, dsEGFP-injected group (negative control) and PBS-injected group (blank control). Based on different groups, 20 µl of dsLac solution (1.5 µg μl^{-1} in PBS), dsEGFP (1.5 µg μl^{-1} in PBS) or PBS was injected into the mantle tissue of each clam. qRT-PCR was used to test the efficiency of the *MpLac* knockdown by detecting the change in the relative mRNA expression of *MpLac* during RNAi. Five clam mantles were collected from each group at 0, 24 and 48 h post-injection (hpi) for RNA extraction. Then the cDNA was synthesized and used as the qRT-PCR template. In order to monitor the changes in shell colour, the remaining 5 clams in each group were cultivated with injections of dsLac, dsEGFP or PBS once per week for 3 wk. All clams were photographed using a camera (SONY) before the 1st injection and after the 3rd injection.

3. RESULTS

3.1. Characterization of the whole *MpLac* cDNA sequence

As Fig. 1 shows, the complete cDNA sequence of *MpLac* with a poly-(A) tail is 2405 bp, including a 2130-bp open reading frame (ORF) encoding a polypeptide with 709 amino acid residues. Signal P prediction indicated that the 27 N-terminal amino acids formed a signal peptide. The predicted mature protein was thus composed of 682 amino acids, with a theoretical mass of 76.35 kDa and a pI of 5.67. The cDNA and the deduced amino acid sequence have been deposited in GenBank under accession number MG845688. BLASTX searches against the NCBI database revealed that the deduced protein of *MpLac* shared the highest identity with a *Crassostrea gigas* laccase sequence.

The alignment result used for the phylogenetic tree construction is shown in Fig. S1 in the Supplement at www.int-res.com/articles/suppl/b028p055_supp.pdf. The phylogenetic tree (Fig. 2) showed that *MpLac* has the closest relationship with the laccase of the freshwater snail *Biomphalaria glabrata* and the oyster *C. gigas* (XP_019920445.1, XP_019920446.1, EKC25244.1, EKC25250.1) but was distantly related to the laccases (1 and 2) of insect species. This result indicates that *MpLac* is systematically closer to the laccases from other mollusks than the insect laccases. Like insects, mollusk species may have multiple laccase isoforms, e.g. oyster *C. gigas*.

3.2. Tissue distribution of *MpLac* mRNA in both larval and adult clams

The distribution of *MpLac* mRNA in whole clam larvae at different developmental stages was detected by WMISH. At the early stage of D-veliger larvae (24 h post-fertilization [hpf]), the time when the prodissoconch began to form, *MpLac* mRNA was detected along the mantle edge and occupied a large zone (Fig. 3a). With larval development, the mantle-edge zone with *MpLac* mRNA was reduced at the late D-veliger larvae stage (48 hpf) (Fig. 3b). At the postlarval stage (10 d post-fertilization [dpf]), *MpLac* mRNA mainly appeared in the mantle edge and near the gill (Fig. 3c).

The tissue distribution of *MpLac* mRNA in adult clams was analysed by qRT-PCR. Results showed that *MpLac* mRNA was mainly expressed in the mantle and gill tissues and was expressed in other tissues at a significantly lower level ($p < 0.05$) (Fig. 4).

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1       ccttactgtagcgttaaaagtcgtcgtaacgtttaacgagttcataatatttggtaataaaatgtttatgtggtgacttaaacc
85      ATGGAAAGAACAACAATGGGCGCATATATTGGCCAATTGTATTTGATAACGATAATTGCCATCGTACCAGAAGCTATTTCTGACGGCCAC
        M E R T T M G A Y I G Q L Y L I T I I A I V P E A I S D G H  30
175     ACCAATCCAGGAACATGTGATATGGGCGCTTCAAAGTGTGACCTTTACCTTGATGTGAGCCTCTGGCTACCAATGCGACGTAACTATGGA
        T N P G T C D M G A S K C D L Y L D V S L W L P M R R N Y G  60
265     GAGAAAGTTTACATAAATGACACGGATGGTCGTTTATATGTACACGGTGATGAGTCGTACAATCAGATAGACCCAGATGATGTTGTCCTA
        E K V Y I N D T D G R L Y V H G D E S Y N Q I D P D D V V L  90
355     GCAGATGGATCGCCACGGGAACGAACACTGACACTTTTTAACAAGACAATGCCTGGCCCAACGTTAATTGTCTACGTTAATCAAGAGGTT
        A D G S P R E R T L T L F N K T M P G P T L I V Y V N Q E V 120
445     AAAGTTCACGTGAGGAATCAAATGTTAAGTGATGGAGTCACGGTCCATTTTCATGGTATTGAAATGCGAGGCACACCCTGGATGGACGGA
        K V H V R N Q M L S D G V T V H F H G I E M R G T P W M D G 150
535     GCAGCGTTTGTCACCCAATGTCCGATTTTACCTGGACAAACGTTTACGTATAAGTTCACACCTAAGCGTAAGGGGACTTATTTTTATCAT
        A A F V T Q C P I L P G Q T F T Y K F T P K R K G T Y F Y H 180
625     TCTCATACCGGCGCACAGATGAGTATGGGCTTGGTTGGGGCATTTATTGTCAAAGAGAGAAAATTAGATGAACTCGAGGAACATGTGATG
        S H T G A Q M S M G L V G A F I V K E R K L D E L E E H V M 210
715     GTACTACAAGACTACAATAATGCACAGTCGTCCGATGAGTTATTTGCAAGTACAGGTTTACTAGGATTTATAAGTACAGAAGGTGAACCA
        V L Q D Y N N A Q S S D E L F A S T G L L G F I S T E G E P 240
805     TTTGCCTCAGAATTAAGAACTGACGGTTCGTACTCTGCTTCGATAAAAATAACATCGTCACTAATAAATGGCAAAGGCAGACTGTTCGAT
        F A S E L R T D G S Y S A S I K I T S S L I N G K G R L F D 270
895     CCTCAGGGAAACCAATTAACTAACACGCCTTTGACTGTATTTCCTGTGAAGCAAGGCCAAGCTTATAGGTTCAGGGTAATCGGCGGGGCA
        P Q G N Q L T N T P L T V F P V K Q G Q A Y R F R V I G G A 300
985     TATTCCTTGCAACACAAAGTTTCTATAGACAATCATAAACTTAAACTAGTTGCAGCCGATGGGAACGACATTGATCCAATAATTGCCGAT
        Y S L Q H K V S I D N H K L K L V A A D G N D I D P I I A D 330
1075    TCTTTTATGATACATTCTGGAGAAAGATTTGATTTTATCATTGATGCTAATCAAACAGTTGATAATTATTGGATTAGAGCGGAAACATTG
        S F M I H S G E R F D F I I D A N Q T V D N Y W I R A E T L 360
1165    CAGAAATATACTAACTATACAGGATTTGCAATCTTGCGATATGAGGGAGCTGAGAGTATTGATCCAACAAGTTCGGTTCAACAATGCTCA
        Q K Y T N Y T G F A I L R Y E G A E S I D P T S S V Q Q C S 390
1255    ATGGAAAACATGTGTACAGTTGTCAACTGTCCTTTTGAAACGTATCCCAACTGGACCTGCATAACAACAGATCAAATTCACTCAACTACG
        M E N M C T V V N C P F E T Y P N W T C I T T D Q I H S T T 420
1345    CCAGCTCCAACCCATAACTCTGGGAGTTTCAAAGAATTTTTCGTTAGCGTTGGATTTGCTCAGGCAGAGAACAATGTCTTTATGGGTCAC
        P A P T H N S G S F K E F F V S V G F A Q A E N N V F M G H 450
1435    ATGAATGGGATAAGCCTTAAGTTACCAAGTGTTTCCGCATTAACGCAACCAAAAGAGGTGACTGATATTTGCGATGAATCTAAGTGTGCG
        M N G I S L K L P S V S A L T Q P K E V T D I C D E S K C A 480
1525    GCGGGTAAATTTTGCAGATGTTATCGTTCTCTTGAAGTGAATTTCGGAGATGTCGTACAGATAAACTTCTTCAGTGCAGGAAAATATAAC
        A G K F C R C Y R S L E V N F G D V V Q I N F F S A G K Y N 510
1615    CTTGCCAGCCACCCTATACACATACATGGATACTCGTTTCACGTTTTAAAAGTTGGTTATCGGCAGGAAAATTCCAGCGTTGTAAATGCT
        L A S H P I H I H G Y S F H V L K V G Y R Q E N S S V V N A 540
1705    CAATTCCTTAATCAATATATCAGTTGTCCAAACGAATTTTGTTACCGTAATTTGGGATGGGCTAATCAAAGCTGGAAAGGAGGAAACATT
        Q F L N Q Y I S C P N E F C Y R N L G W A N Q S W K G G N I 570
1795    CCCGGGATTGATTTAGATCGGGCACCGCGTAAGGATACAGTTACCCTACCTGCTGGCGGTTATGTCGTTGTAAGATTTGTTGCAGACAAT
        P G I D L D R A P R K D T V T L P A G G Y V V V R F V A D N 600
1885    CCAGGTCTCTGGTACATCCATTGTCACCAAGAATACCATGCTCAAAAGGGTTTAGGATTACTACTTAATGATTCATTCTCAGCAATCCCT
        P G L W Y I H C H Q E Y H A Q K G L G L L L N D S F S A I P 630
1975    GCCCCACCCGAGGGATTTCCTGAATGTAGAAGCTTTCCGCCTCCAGACTGGCTGACTTCAGACAAACGGGAATCATTGCCCAAAGACGAT
        A P P E G F P E C R S F P P P D W L T S D K R E S L P K D D 660
2065    GAAAATACAACAGTTGCACGTACAGACGGAAGGACTTTCACGGAGGAGGAGTTCTGGGGAATGTTTGGAGCGCTTCTGTTCGTGATTTTA
        N T T V A R T D G R T F T E E E F W G M F G A L L F V I L 690
2155    TTACAGTTCATCATTTCTGTGTGGTGCTTAAGAAAAACCAAATCTTCCGTATCGTTCTAGaacagacaaaagaagaaaaaagtacacttt
        L Q F I I S V W C L R K T K S S V S F *
2245    tggattaaaataacttagtatttttagttctcatttcctctgtatgtattcacaaactgctgtatttataaaaaaactgtaactcgcttt
2335    ataagatttttatgtaacctcgttgagatgaatacaattaaagacaaaaaaaaaaaaaaaaaaaaaaaa                    2405
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Fig. 1. Nucleotide sequence (upper) and deduced amino acid sequence (single letter code, below) of the full-length *MpLac* cDNA. Nucleotide and amino acid numbers are shown on the left and right, respectively. Underlined amino acids are the predicted signal peptide. *: stop codon

3.3. *MpLac* mRNA localization in the mantle edge of adult clams

Based on the results of H&E staining (Fig. 5a), the mantle edge of *M. petechialis* was divided into 3 folds: the inner fold (IF), middle fold (MF) and outer fold (OF). The PG was between the MF and OF; the periostracum originated from the PG and adjoined the inner epithelium of the OF; the periostracum was thin near the PG and intensified gradually with distance from the PG. These results imply that the inner epithelium of the OF plays an important role in periostracum formation. TEM results also showed that large amounts of electron-opaque granules were secreted from the inner epithelium of the OF to the electron-dense periostracum (Fig. 5b), further implying that the inner epithelium of the OF is involved in periostracal secretion.

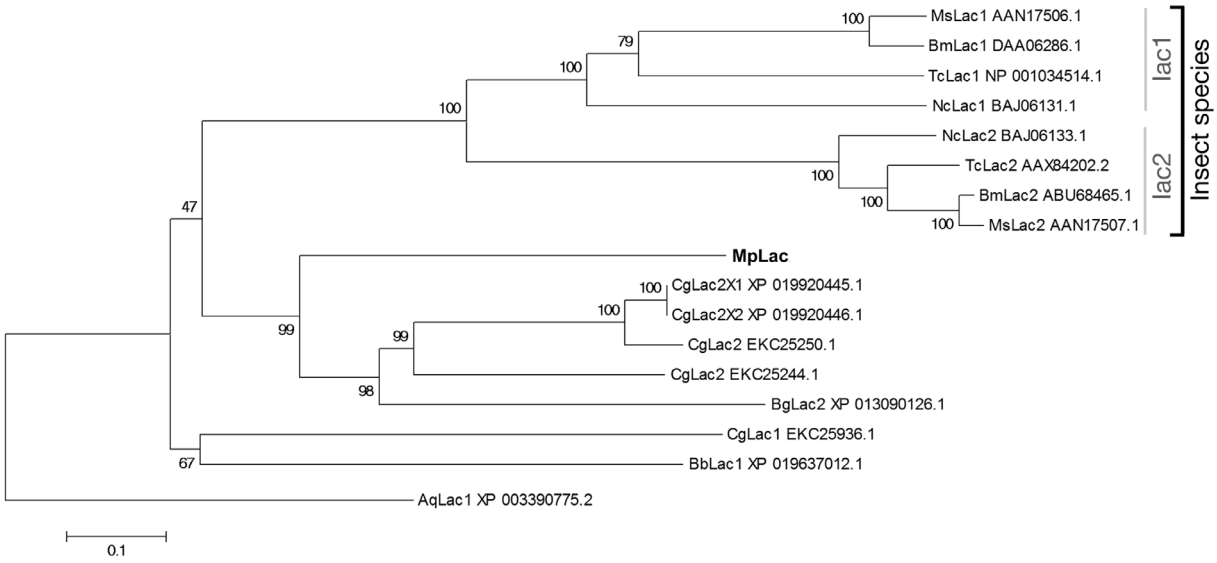

Fig. 2. Phylogenetic tree depicting the relationship between *MpLac* (bold) and laccases of other species. The sequences applied to the phylogenetic tree construction were as follows: *Crassostrea gigas* (Cg), laccase-2 isoform X1, laccase-2 isoform X2, laccase-2, laccase-2, laccase-1; *Biomphalaria glabrata* (Bg), laccase-2; *Branchiostoma belcheri* (Bb), laccase-1; *Amphimedon queenslandica* (Aq), laccase-1; *Bombyx mori* (Bm), laccase-1, laccase-2; *Manduca sexta* (Ms), laccase-1, laccase-2; *Nephotettix cincticeps* (Nc), laccase-1, laccase-2; *Tribolium castaneum* (Tc), laccase-1, and laccase-2. The laccase from sponge *A. queenslandica* was set as an outgroup. The tree was constructed using the neighbor joining method; bootstrap confidence was calculated from 1000 replications. The scale bar represents 0.1 amino acid substitutions per site

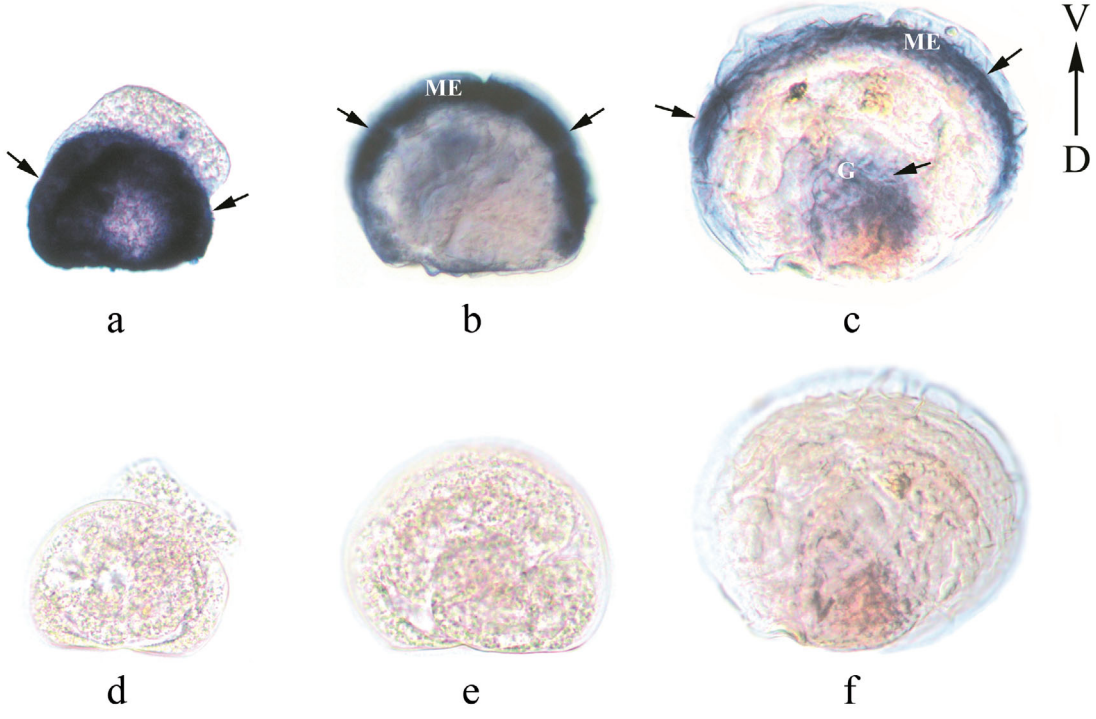

Fig. 3. *MpLac* mRNA expression at different larval stages of *Meretrix petechialis* revealed by whole-mount *in situ* hybridization, showing larvae hybridized with (a–c) the antisense probe and (d–f) the sense probe (negative control). Lateral view of the (a,d) early D-veliger larva; (b,e) late D-veliger larva; and (c,f) postlarvae. Arrowheads: positive staining (blue-violet colour) of *MpLac* mRNA. ME: mantle edge; G: gill; V: venter; D: dorsum

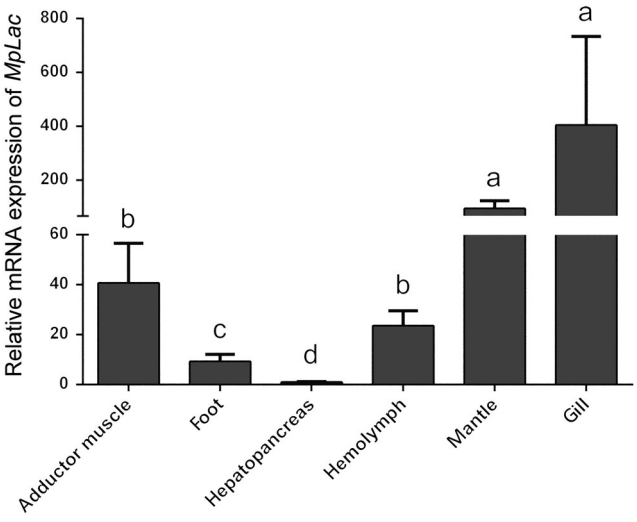

Fig. 4. Relative mRNA expression of *MpLac* in various tissues of adult clams. Error bars: SD. Columns with different letters are significantly different from each other ($p < 0.05$)

Since the periostracum formed in the mantle edge of the clam, we detected *MpLac* expression in the mantle edge by FISH to identify the function of *MpLac* in periostracum formation. FISH results showed that the *MpLac* mRNA was located in the mantle-edge epithelium and was mainly limited to the inner epithelium of the OF, which was adjacent to the periostracum (Fig. 6a).

3.4. Change in periostracum colour after knocking down *MpLac* expression

To test whether the shell colour (i.e. the periostracum colour) of clams is related to *MpLac* expression level, an RNAi experiment was performed. The qRT-PCR results showed that at 24 hpi, *MpLac* expression level was significantly decreased in the dsLac-injected group compared to the PBS-injected group (blank control) and dsEGFP-injected group (negative control) ($p < 0.05$) (Fig. 7). At 48 hpi, *MpLac* expression level was significantly lower in the dsLac-injected group than the dsEGFP-injected group ($p < 0.05$). Compared to the PBS-injected group, the mean of *MpLac* expression level was 25% lower in the dsLac-injected group. The non-significant difference in *MpLac* expression level between the dsLac-injected and PBS-injected group was because there was a large standard deviation root in the huge expression difference among the PBS-injected clams (Fig. 7). Thus, we think the knockdown may be still effective at 48 hpi. Changes in the shell colour were checked for the clams in all 3 groups. Our results showed that in the dsLac-injected clams, the colour of the new shell was bleached (Figs. 8 & S2). No such changes were detected in the dsEGFP- and PBS-injected clams (Fig. 8).

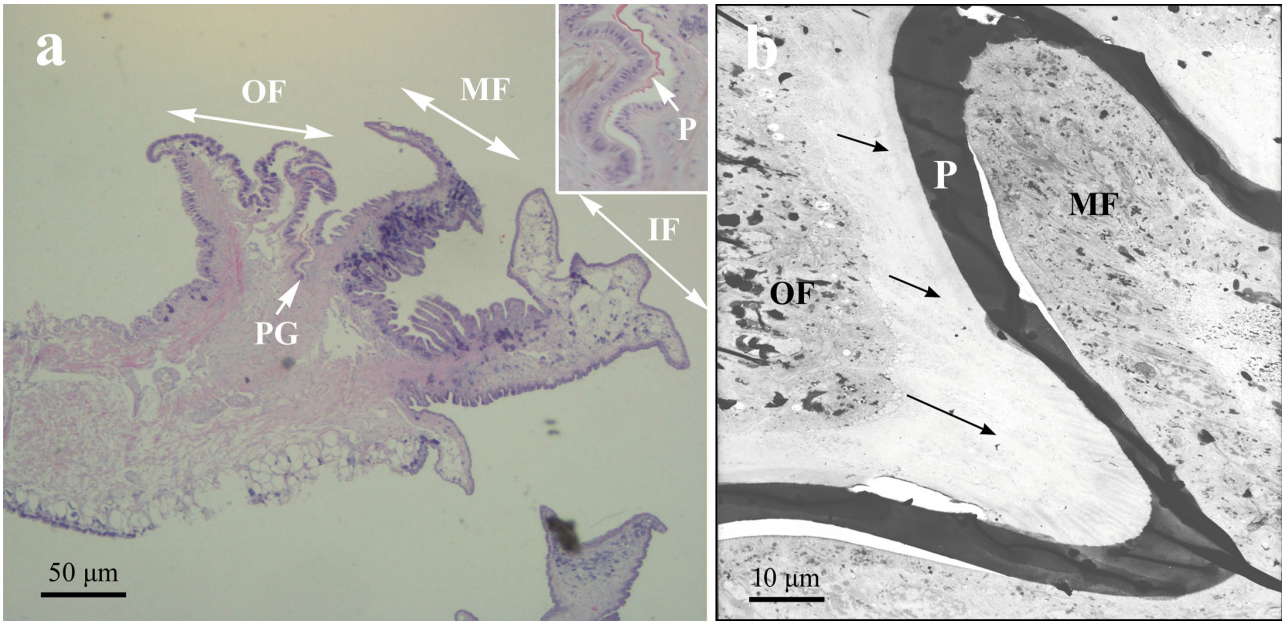

Fig. 5. (a) Microstructure of the mantle edge of the adult clam revealed by H&E staining and (b) ultrastructure revealed by transmission electron microscopy. The magnified image of the periostracal groove (PG) region is shown at the top-right corner of image (a). OF: outer fold; MF: middle fold; IF: inner fold; P: periostracum. Black arrows: massive granules being secreted from the inner epithelium of the OF to the P

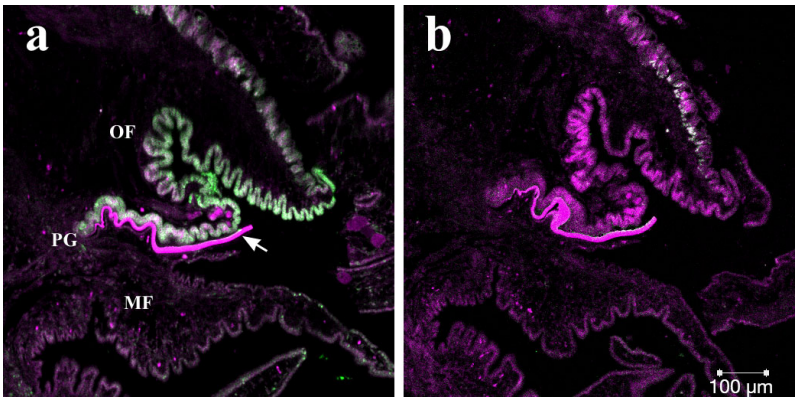

Fig. 6. *MpLac* mRNA localization in the mantle edge of the adult clam revealed by fluorescence *in situ* hybridization, showing slides hybridized with (a) the antisense probe and (b) the sense probe (negative control). Green fluorescence signals indicate *MpLac* mRNA. OF: outer fold; MF: middle fold; PG: periostracal groove. White arrow indicates the periostracum

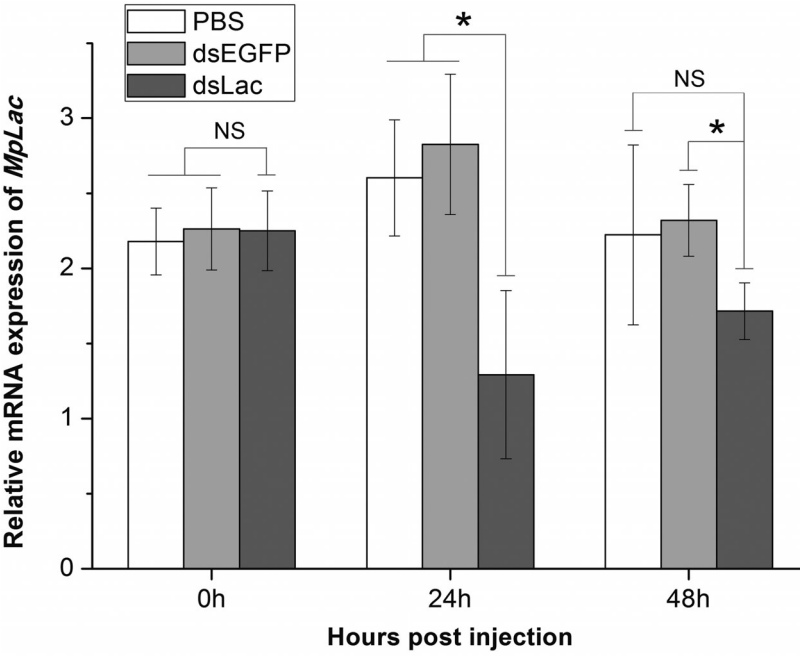

Fig. 7. Relative mRNA expression of *MpLac* in the mantle of clams injected with dsLac/dsEGFP/PBS (see Section 2.6). Error bars: SD. (*) represent a significant difference ($p < 0.05$) between the dsLac-injected group and any of the control groups (dsEGFP/PBS-injected groups). NS: no significant difference

4. DISCUSSION

Laccases comprise a group of multi-copper enzymes. In many insects, laccase has been reported to be involved in the tanning of larval, pupal and adult cuticles (Andersen 2005, Arakane et al. 2005, Niu et al. 2008, Elias-Neto et al. 2013). In this study, we identified a laccase gene from the clam *Meretrix petechialis* and investigated whether laccase was also involved in the tanning of the clam periostracum.

M. petechialis has a typical periostracum outside the calcified shell; the colour of this periostracum determines the colour of the shell (Yue et al. 2015). Periostracum synthesis starts at the early stages of development, and the periostracum grows continuously over the entire life of the bivalve. The larval shell of the bivalve undergoes 3 stages during ontogenesis, namely prodissoconch-I, prodissoconch-II and dissoconch, all of which contain the periostracum structure (Marin & Luquet 2004). The prodissoconch-I and prodissoconch-II form in the course of D-veliger larval development, and the dissoconch appears after metamorphosis of the veliger larva to the juvenile larva (Che et al. 2001). In this study, with the WMISH method, *MpLac* expression was detected in 3 developmental stages of clam larvae, i.e. the early stage of D-veliger larvae, the late stage of D-veliger larvae and the postlarval stage, which separately secreted the prodissoconch-I, prodissoconch-II and dissoconch shells. The WMISH results (Fig. 3) showed that *MpLac* mRNA was detected in all 3 larval stages and mainly appeared along the mantle edge. The zone of the mantle edge with *MpLac* mRNA is larger in the early D-veliger larvae than in the late D-veliger larvae and postlarvae; this may be because the shell field spreads with the fast lateral extension of the periostracum during the growth of the prodissoconch-I shell (Marin & Luquet 2004) or due to the undifferentiation of the mantle edge at the early D-veliger larval stage (Arandaburgos et al. 2014). Shell growth in bivalves is related to the activities of mantle epithelial cells (Jolly et al. 2004). The periostracum, as a part of the mollusk shell, is secreted by the mantle (Addadi & Weiner 1985). Our results, that *MpLac* transcripts were mainly expressed in the mantle edge of the larval clam, suggest that *MpLac* is likely involved in the formation of the larval clam periostracum.

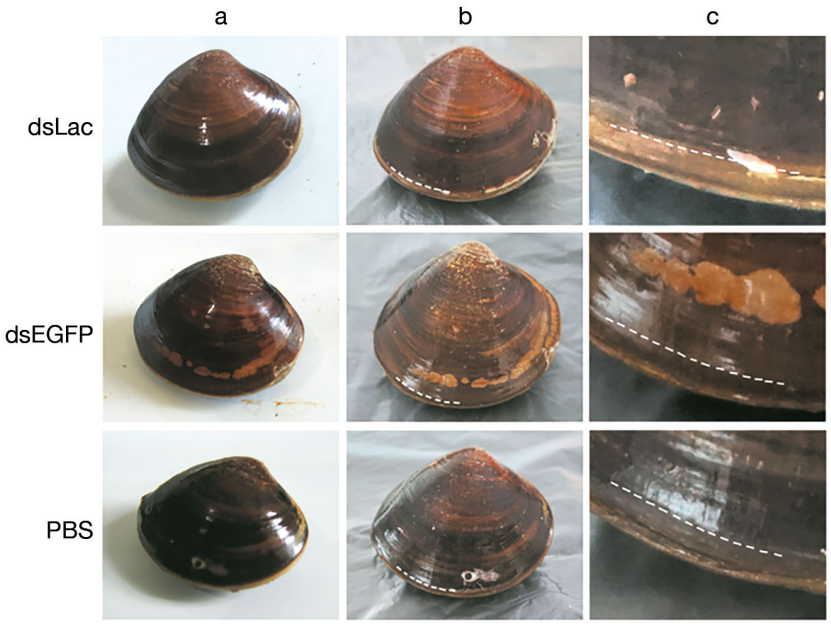

Fig. 8. Changes in periostracum colour among clams injected with dsLac/dsEGFP/PBS (see Section 2.6). (a) Clam images before the first injection; (b) clam images after the last injection; (c) magnified shell-margin images of the clams after the last injection. Dashed line: boundary between the old and new shells

In the adult clam, similar to in the larval clam, the mantle was also a tissue with a high expression level of *MpLac* (Fig. 4), further implying the involvement of *MpLac* in periostracum formation. Results of H&E staining and TEM (Fig. 5) showed that the periostracum of the adult clam originated in the PG of the mantle edge, and the inner epithelium of the OF was involved in periostracum thickening. Our results were consistent with the results in other bivalve species (Bevelander & Nakahara 1967, Kniprath 1972). Notably, the FISH results (Fig. 6) showed that *MpLac* transcripts were not only located in the epithelium of the mantle edge but were also mainly limited to the region of the inner epithelium of the OF. These results imply that *MpLac* is involved in the periostracum formation of the adult clam. The expression locations of *MpLac* in both larval and adult clams suggest a close relationship between *MpLac* and the periostracum.

Additionally, an RNAi experiment was performed to verify the role *MpLac* plays in periostracum tanning of the clam *M. petechialis*. RNAi is a widely used method to silence gene expression in mollusks (Suzuki et al. 2009). For instance, RNAi was used to suppress the expressions of some shell-related genes in the pearl oyster, and the influence of RNAi on shell formation was observed (Fang et al. 2011, Jiao et al. 2012); RNAi was also used to identify the downstream genes of the signalling pathway in scallops and pearl oysters (Wang et al. 2011, Oyanedel et al. 2016, Zhang et al. 2017). A previous study showed that RNAi of laccase-2 in the insect *Tribolium castaneum* made the cuticle bleach; thus, laccase is the phenoloxidase gene required for beetle cuticle tanning (Arakane et al. 2005). In this study, an RNAi experiment was performed to detect the change in shell colour (i.e. the periostracum colour) of clams after the expression of *MpLac* was knocked down. Strain WM, developed by our lab and displaying an overall maroon shell-colour (Yue et al. 2015), was used in the RNAi experiment. During the RNAi experiment, we found that the new shell colour of the clam was bleached after *MpLac* expression was knocked down (Figs. 8 & S2). Even though the *MpLac* expression began to increase again at 48 hpi, an obvious colour bleaching was observed in the dsLac-injected clams, which may be due to the additive effect of repeated RNAi. The results of RNAi further suggested that *MpLac* was a phenoloxidase gene required for the periostracal tanning of clams.

The complete cDNA sequence of *MpLac* we obtained was 2405 bp, including a 2130-bp ORF encoding a polypeptide with 709 amino acid residues (Fig. 1). The results of our phylogenetic analysis (Fig. 2) showed that *MpLac* is systematically closer to the laccases from other mollusks than the insect laccases. Asano et al. (2014) also indicated that mollusk laccases, which were devoid of the cysteine-rich domain, clustered together and were isolated from the cluster of insect laccases, implying that mollusk laccases represent a new sub-family of laccases. Our study identified a laccase gene from the clam *M. petechialis* and demonstrates the relationship between the laccase (*MpLac*) and shell periostracal tanning for the first time.

In conclusion, a laccase-like gene (*MpLac*) was identified in the clam *M. petechialis*, and the *MpLac* transcripts were mainly expressed in the mantle edge of both larval and adult clams. Furthermore, *MpLac* transcripts were mainly limited to the inner epithelium region of the OF of the mantle edge, which is responsible for periostracum thickening. In addition, knocking down *MpLac* expression bleached the new shell periostracum. All of our

results indicate the involvement of *MpLac* in the shell periostracal tanning of *M. petechialis*. This study will be helpful for understanding the molecular mechanisms underlying periostracum formation in clams.

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