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Two NADH-cytochrome b_5 reductases from the beet armyworm, *Spodoptera exigua*: Gene identification, cloning and expression profiles

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ABSTRACT

NADH-cytochrome-b_5 reductase (CbR) was a flavoprotein and multi-functional redox enzyme, purportedly shuttled electron to the substrate-complex for various physiological reaction, such as the P450s metabolism reaction, desaturation and elongation of fatty acyl-CoA etc., and was potentially used in food industry, biosensor and diagnostic areas. In this work, two full-length cDNAs of SeCbR genes, with the different N- terminal nucleotide sequence, were isolated and partially characterized. The cloned SeCbR1 (SeCbR-like3) and SeCbR2 (SeCbR-like2) had complete open reading frame, were predicted to encode 324 and 311 amino acids respectively, and shared high identities with CbRs of several other species. For disclosing more information of SeCbR1 and SeCbR2, both genes were analyzed by bio-information software and detected with RT-qPCR method. Both genes had the trans-membrane segment at the N- terminus. And the phylogenetic tree result showed that both genes are belong to the CbR family and had closest relationship to the CbR of Helicoverpa armigera. The RT-qPCR results signified that SeCbR1 and SeCbR2 expressed in most developmental stages of S. exigua except egg, as well as in tissues of cuticle, fatbody and midgut. It is anticipated that our initial finding of SeCbR1 and SeCbR2 genes could generate the basement for further studies of them at the molecular level. (words 204; limitation 250)

Key words: clone, mRNA expression level, NADH-cytochrome b_5 reductase (CbR), *Spodoptera exigua* (4 words)

INTRODUCTION

NADH-cytochrome-b5 reductase (EC.1.6.6.2, CbR), also known as flavoprotein, is a multi-functional redox enzyme, which is responsible to shuttle electron to other enzymes and could affect the progress of the P450s metabolism reaction [1, 2], desaturation and elongation of fatty acyl-CoA [3], biosynthesis of cholesterol [4], hydroxylamine reduction [5] , plant correct pollen and seed maturation [6] and so on. CbR have been isolated from the fungus, plant, insect and mammal [7-14] and deeper studies have been conducted on the fungus (*Mortierella alpina 1S-4*), plant [cotton (*Gossypium hirsutum* L.), bean (*Phaseolus vulgaris* L.) and tung (*Vernicia fordii*)] and mammal (human, known as methemoglobin reductase, and rat). However only a little information is currently available for the insect's CbR except in *Ceratitis capitata*, *Helicoverpa armigera* and *Musca domestica* [15-18].

The beet armyworm, *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae) as one polyphagous insect, has led to severe damage to various crops, such as corn, vegetables and other economic crops for a long time [19-21], due to the failure of chemical control. Therefore, research about its resistance to insecticide should be done to reduce the loss of farmer. As well-known, P450 enzyme is the most detoxification enzyme of insect resistance, and cytochrome

b_5 system, including CbR and cytochrome b_5 as electron transfer, could affect the activity of P450 detoxification [2, 22]. In current study, the full-length cDNAs of *SeCbR* were firstly cloned and characterized from *S. exigua* and the mRNA expression level of *Se*CbR1 and SeCbR2 in tissue-distribution and developmental stages were examined by reverse transcription polymerase chain reaction (RT-qPCR) analysis. These investigations would provide a foundation for further study on the function of *Se*CbR and the interactions of *Se*CbR with other components of P450s in *S. exigua*, and promote the study of CbR in insects.

EXPERIMENTAL SECTION

Insect cultures

The *S. exigua* were reared on artificial diets in an air-conditioned room at 27±1°C, 75–80% relative humidity, with a 16:8 (Light: Dark) photoperiod and without exposure to any insecticides.

Total RNA isolation and cDNA synthesis

Total RNA from the whole worm of different developmental stages (1^{st} to 5^{th} instars larvae, pupae and adult) and various tissues (cuticle, fatbody and midgut) of 5^{th} instars larvae of *S. exigua* were individually extracted by the SV Total RNA Isolation System (Promega, Madison, WI, USA) on three repetitive preparations, and were quantified by Nano Drop (Nano Drop Technologies, San Diego, CA, USA).

Cloning of full-length cDNA of SeCbR

The total RNA (2μg) was reverse transcribed to the first-stranded cDNA with *EasyScript* Two-Step RT-PCR SuperMix (TransGen Biotech, Beijing, China) and SMARTer™ RACE cDNA Amplification Kit (Clontech Laboratories, Inc. Dalian, China) following the specification. The degenerate primers (DCbR1 and DCbR2, Table 1) were designed according to the conserved partial amino acid residues (DCbR1: GLPIGQHI and DCbR2: MIAGGTGIAPM) from other known species referring early report [16]. The partial *Se*CbR gene was cloned following the procedure: 94 °C, 5 min; 35 cycles PCR (94 °C, 30 s; 58 °C, 30 s; 72 °C, 30s); and 72 °C, 10 min. The PCR products were purified using Wizard® SV Gel and PCR Clean-Up System (Promega), inserted into to pGEM-T vector, and transformed into *Escherichia coli* XL10 cell (Vazyme Biotech Co., Ltd, Nanjing, China). The transformants were screened with LB-*ampicillin* (60 μg/ mL) agar plates. The positive clones were verified and sequenced by Invitrogen (Life Technologies, Beijing, China). Subsequently, two specific primers for 5′-RACE (TCbR2) and 3′-RACE (TCbR1) were designed on basic of the partial *Se*CbR nucleotide sequence and the 5′ and 3′ parts of the corresponding cDNA were obtained with RACE technique. Finally, the full-length ORF of *SeCbR*s were cloned by specific primers.

Table 1 Primers used in this study

Primer name	Primer sequence (5′→3′)	Function
DCbR1	GGNYTNCCNRTYGGNCARCAYAT	RACE Degenerate primers
DCbR2	CATNGGNGYRATNCCNGTTCCNCCNGCRATCAT	
TCbR2	AGTGTTGAGACATCTTACCACCGTCAG	5′RACE
UPM	CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT **&** CTAATACGACTCACTATAGGGC	Provided by kit
TCbR1	ATGTGCACCCTAAATTCCCTGACGG	3′RACE
qCbR1	GGCGGCTGTGGTGGATTTTT	RT-qPCR analysis
qCbR2	TGCCAAGTCCACAACATGTTCTAG	
qCbR3	GCTTGTACTTCTTTCTTGGCTGGTT	
β-actin 1	TCCTCCGTCTGGACTTGGC	House-keeper gene
β-actin 2	CCTTGATGTCACGCACGATTT	

Sequence analysis

The full-length cDNAs of *Se*CbR1 and *Se*CbR2 were assembled and the homology of deduced amino acid sequences among various species CbR were analyzed by DNAMAN software package (Version 6.0, Lynnon Biosoft, Canada) in terms of the sequence database from National Center for Biotechnology Information (NCBI) respectively. The theoretical isoelectric point (pI) and molecular weight (Mw) of putative *Se*CbR1 and *Se*CbR2 were calculated using the Compute pI/Mw (http://www.expasy.ch/tools/pi_tool.html). The signal peptide, trans-membrane (TM) segment and functional domain of the these putative protein were predicted by the Simple Modular Architecture Research Tool (SMART, http://smart.embl-heidelberg.de/) [23]. The hydrophobicity scales were predicted using Hphob/ Kyte & Doolittle with ProtScale (http://web.expasy.org/protscale/) [24]. The phylogenetic tree was

constructed on MEGA 6.0, using the default settings with the maximum likelihood method [25].

RT-qPCR analysis

After 2μg RNase-treated total RNA was reverse transcribed to cDNA, the RT-qPCR assay was performed in final volume of 20 μL including 10 μL 2×Ultra SYBR Mixture (with ROX) solution (CWBiotech, Beijing, China), 4 μL each of forward and reverse primer (1 μM) (qCbR1 and qCbR3 for *SeCbR1*, and qCbR2 and qCbR3 for *SeCbR2*), 0.5 μL cDNA template and 1.5 μL RNase-free water. The reaction was started at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1min. The RT-qPCR was repeated three times for each gene and the dissociation curve analysis of amplified products was proceeded at PCR reaction end to verify the specifity and unique of the PCR product. The cycle threshold (Ct) values of *SeCbR1*, *SeCbR2* and β-actin (as keeping gene) were determined, respectively. At last, the the RT-qPCR data were analyzed on the SDS 1.4 software plate (Applied Biosystems, CA, USA) by comparative Ct method [26]. The statistical analysis herein were conducted using one-way ANOVA and the results were represented as mean ± standard error (SE). The $p<0.05$ was considered statistically significant differences.

RESULTS AND DISCUSSION

Cloning and sequence analysis of SeCbR

Following on the RT-PCR and RACE manipulation, four cDNA fragments, including 1200 bp (3′ RACE), 450bp (degenerate PCR), and 700 and 450 bp (5′ RACE) were obtained (Fig. 1). Subsequently, two full-length cDNAs of *SeCbR* were obtained after assembling the PCR fragments. The nucleotide sequence analysis revealed that the complete cDNA of *SeCbR1* and *SeCbR2* contained a 252 bp and 39 bp 5′ untranslated region (5′ UTR) and a 544 bp and 545 bp 3′ UTR with a poly-A tail, respectively. The ORFs length of *SeCbR1* and *SeCbR2* was 972 bp and 933 bp, and encoded 323 and 310 amino acids with the predicted molecular mass of 36.5KDa (pI, 7.15) and 35.2 KDa (pI, 8.57), respectively. The *SeCbR1* and *SeCbR2* were deposited in the GenBank database with accession numbers HQ852050.1 (*SeCbR1*) and JX569756 (*SeCbR2*), respectively.

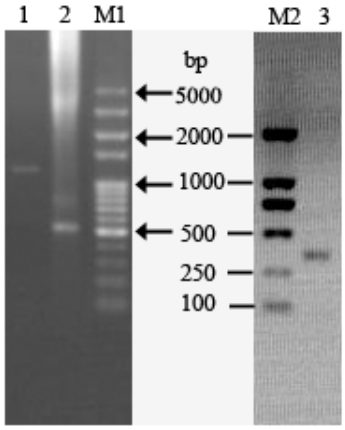

Fig. 1 The PCR products of *SeCbR1* and *SeCbR2* by 3′ and 5′ RACE amplification detected on 1% agarose gel following electrophoresis and visualized with ethidium bromide (10 μg/lane). Lane M1, 100bp Plus DNA marker; M2, D2000 DNA marker; Lane 1 and 2, products of 3′ RACE and 5′ RACE; Lane 3, PCR product from the degenerate oligonucleotide primer, respectively

The putative *Se*CbR1 and *Se*CbR2 protein possess the characteristic features of CbR protein family, including the FAD- and NADH-binding domains (Fig. S1).

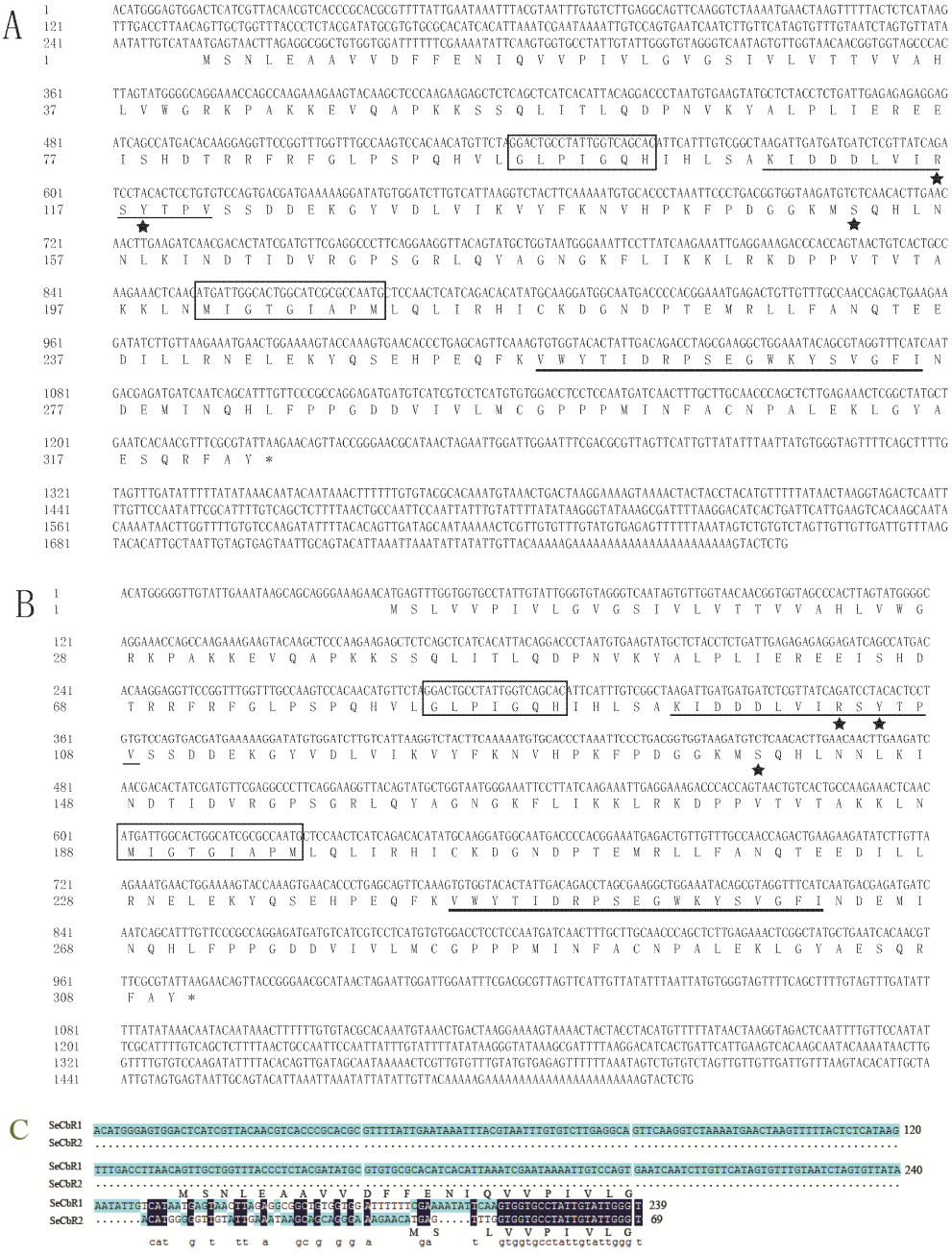

Fig. S1 Analysis of nucleotide sequence of *Se*CbR1 (A) and *Se*CbR2 (B). The related amino acid sequences according to the degenerate primers was boxed in A and B. The difference between *Se*CbR1 and *Se*CbR2 was shown in C. The single underline was the FAD binding domain and the bold underline was the NADH binding domain in A and B, respectively. The pentagon in A and B was for the flavin binding domain. The stop codon is in asterisk

No signal peptide was found in the secondary structures of these two proteins following the SMART searching, however, a typical N-terminal TM domain was found in *Se*CbR1 and *Se*CbR2 protein (Fig. S2).

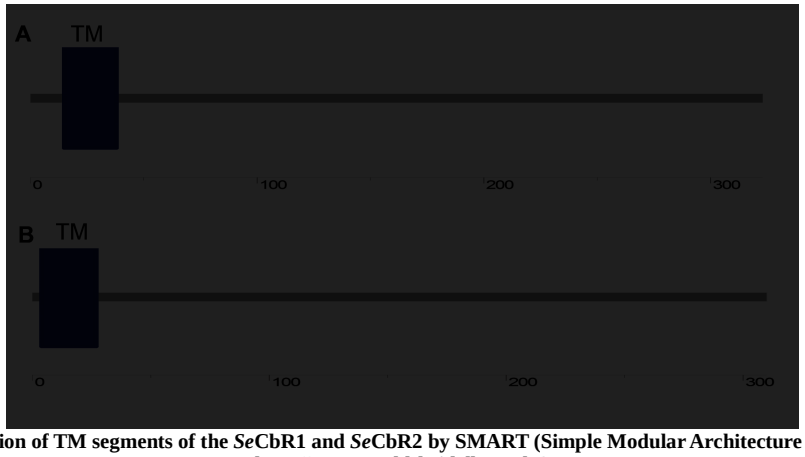

Fig. S2 Prediction of TM segments of the *Se*CbR1 and *Se*CbR2 by SMART (Simple Modular Architecture Research Tool, http://smart.embl-heidelberg.de/)

The hydrophobic index results, from ProtScale predication, evoked that the *N*-terminal domain of *Se*CbR1 and *Se*CbR2 were observed to be highly hydrophobic (Fig. S3), suggesting that these protein might be the membrane-bound protein, which may anchor to the membrane through the *N*-terminus. Both predicted results from SMART and ProtScale could authenticate each other.

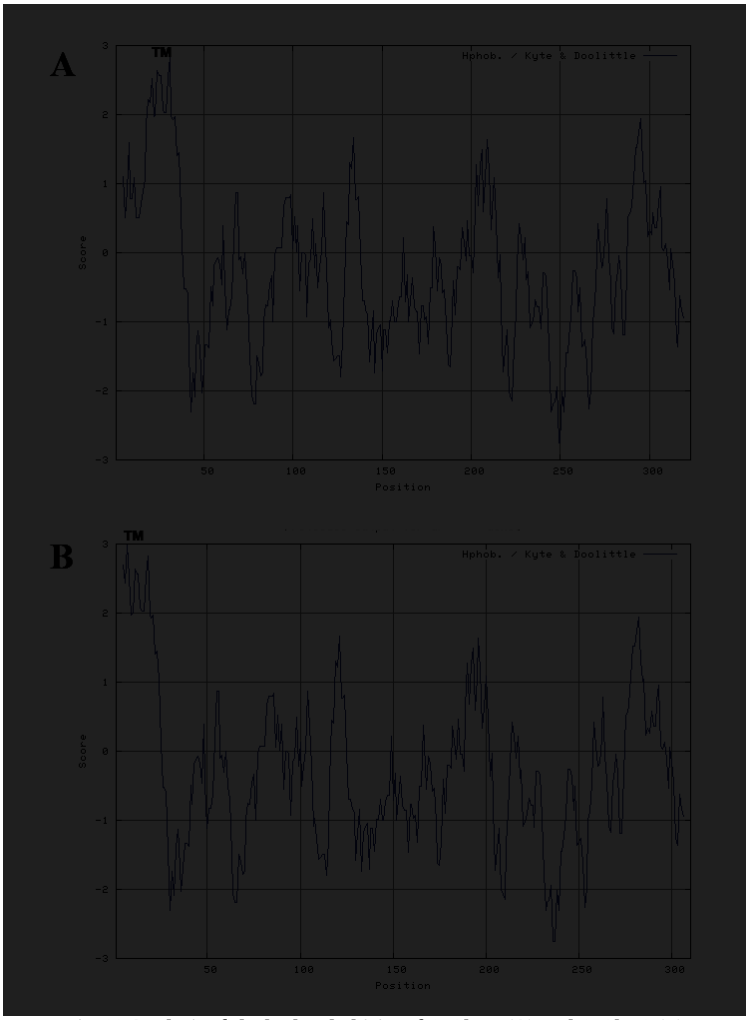

Fig. S3 Analysis of the hydrophobicity of *Se*CbR1 (A) and *Se*CbR2 (B)
Note. TM indicated the trans-membrane.

The amino acid sequences of *SeCbR1* and *SeCbR2* were aligned with those of *CbR* in other organism by introducing gaps to achieve maximum homology (Fig. 2). The results showed that the CbR of thale cress (*Arabidopsis thaliana*, AED92466.1), yeast (*Saccharomyces cerevisiae*, CAA86908.1), nematode (*Caenorhabditis remanei*, XP_003112622.1), fruit fly (*Drosophila melanogaster*, AHN58060.1), cotton bollworm (*Helicoverpa armigera*, ADO08221.1 & ADY89570), beet armyworm (*Spodoptera exigua*, ADX95747.1 & AGL61416.1), zebrafish (*Danio rerio*, AAQ97765.1), rat (*Rattus norvegicus*, EDM09729.1) and human (*Homo sapiens*, AAP97209.1) genes shared 32.41%, 29.39%, 53.54%, 54.41%, 83.69%, 81.85%, 54.77%, 50.77% and 51.08% identities with *SeCbR1* and possessed 33.76%, 29.86%, 55.13%, 55.59%, 81.23%, 84.44%, 56.09%, 52.25% and 52.85% identities with *SeCbR2*, respectively.

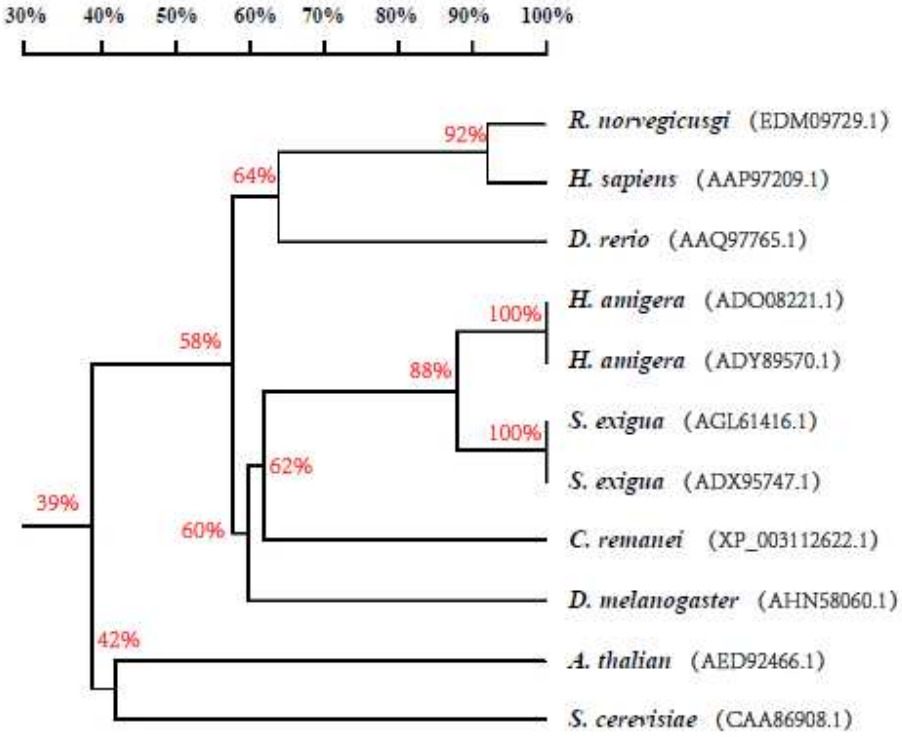

Fig. 2 Maximum likelihood homology tree obtained from multiple sequence of several species. The organisms' names and GenBank numbers were listed below: *A. thalian* (AED92466.1), *C. remanei* (XP_003112622.1), *D. melanogaster* (AHN58060.1), *D. rerio* (AAQ97765.1), *H. amigera* (ADO08221.1 and ADY89570.1), *H. sapiens* (AAP97209.1), *R. norvegicusgi* (EDM09729.1), *S. exigua* (AGL61416.1 and ADX95747.1) and *S. cerevisiae* (CAA86908.1)

Phylogenetic analysis of the SeCbR

Phylogenetic analysis and multiple sequence alignments of *Se*CbR deduced amino acid sequences with other *CbR* proteins from NCBI substantiated that *SeCbR1* and *SeCbR2* are the new members of the *CbR* family. To summarize, four clans (I, II, III and IV) were separated. It was noteworthy that the sequences of *HaCbR* was the closest to that of *SeCbR* (Fig. 3),

From the cluster group, there may be at least four types of CbRs in the organisms. However, the *HaCbR1*, *HaCbR2*, *SeCbR1* and *SeCbR2* did not belong to separated cluster.

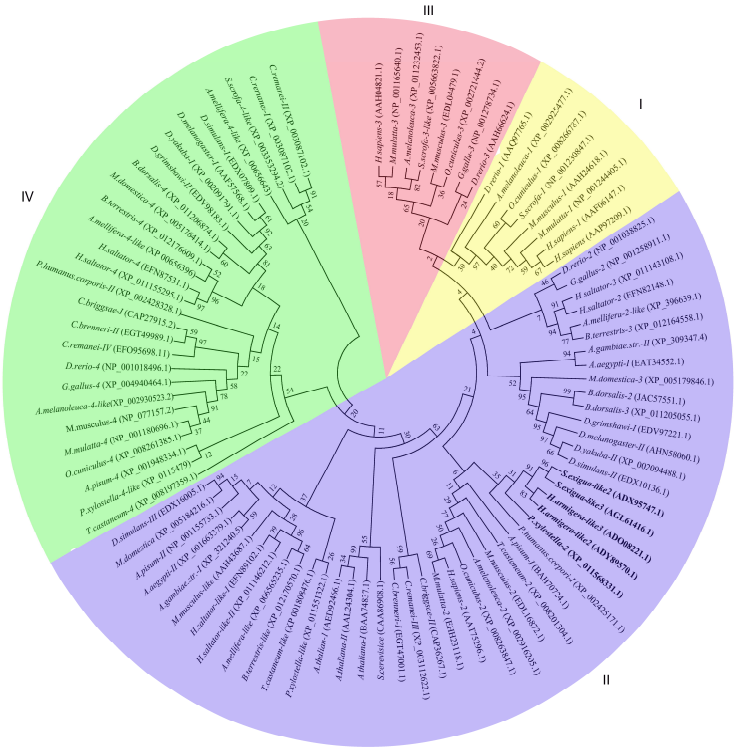

Fig. 3 The maximum likelihood phylogenetic tree of *SeCbR1* and *SeCbR2* with those of other organisms. The deduced amino acid sequences were aligned by Clustal Walgorithm. The *SeCbR1* and *SeCbR2* are underlined. The regular roman letters, I, II, III and IV, indicated the CbRs, that did not be defined the group of CbR by the submitter in the NCBI database. The bold roman letters represented the cluster of CbR and the yellow, blue, pink, and green indicated the I, II, III and IV cluster of CbR, respectively

Expression level of SeCbR1* and *SeCbR2 mRNA in S. exigua

RT-qPCR results showed that *SeCbR1* and *SeCbR2* mRNA were differently expressed in most life stages except egg (Fig. 4 A), and all test tissues (Fig. 4 B). The expression levels of *SeCbR1* and *SeCbR2* mRNA were varied through the different developmental stages, with the highest level occurring in the 4th and 5th instar larvae, respectively, and the lowest expression level found in 2nd instar larvae. There were 6.40- and 5.49- fold higher of *SeCbR1* and *SeCbR2* mRNA expression level in highest level comparing to those in the lowest level, respectively.

The tissue-dependent expression results clearly showed that the midgut is the highest expression tissue of *SeCbR1* and *SeCbR2* mRNA in test tissues. There were 7.75- and 5.19-fold higher of *SeCbR1* and *SeCbR2* mRNA expression level in midgut than that in cuticle, respectively.

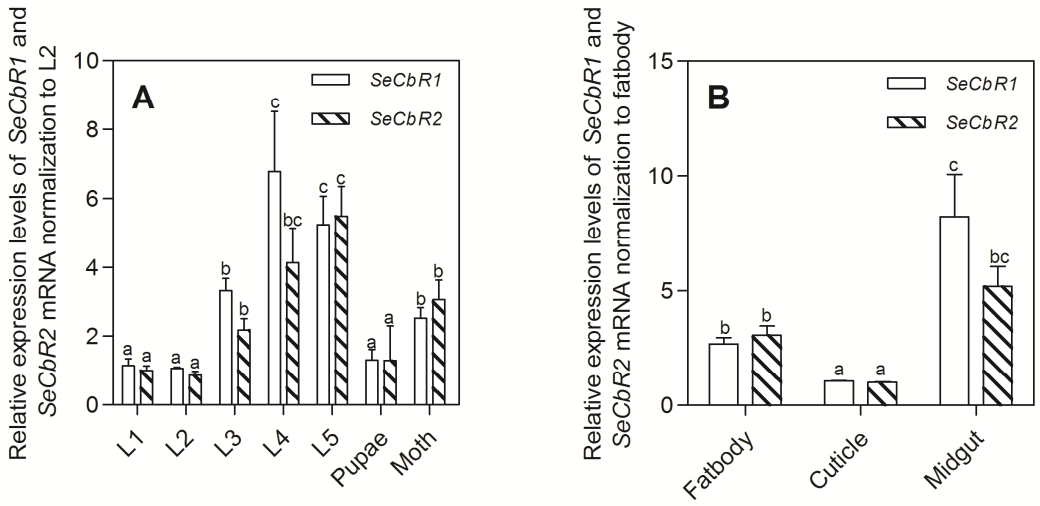

Fig. 4 Spatial expression levels of *SeCbR1* and *SeCbR2* mRNA in different developmental stages (A) and tissues of 5th instar larvae (B). L1, L2, L3, L4 and L5 represented the 1st, 2nd, 3rd, 4th and 5th instar larvae of *S. exigua*, respectively. Different lowercase letters (a, b and c) indicated significant difference ($p<0.05$) based on Duncan's new multiple range tests (DMRT). The error bars represent the standard errors (SE) of three replicates

DISCUSSION

The CbR is an archaic multi-functional redox enzyme, which is paid high attention due to its function in human disease of recessive congenital methemoglobinemia (RCM). And its activity always was used to diagnosis of RCM. Except that, the CbR could transfer the electron to the cytochrome b_5 and be involved in important metabolic process, such as cytochrome P450 metabolism reaction [27], cholesterol biosysthesis and other reactives in insects [15, 28, 29]. Syed et al. (2011) pointed that CbR responsible to shuttle electron to the substrate complex for P450s as an electron-transfer intermediate provider [2].

In current study, two SeCbR genes were isolated and the results exhibited that *SeCbR1* and *SeCbR2* have the same nucleotide sequences in *C*-terminus, but different in *N*-terminus, and interestingly similar phenomenon was discovered in human, insect, plant and yeast as well [10, 30, 31]. The length of these proteins with 311 and 324 amino acid were consistent to the CbR gene feature of less 350 amino acids, and other typical features, such FAD-binding domain, NADH-binding domain, and β-sheet structure between FAD- and NADH-binding domain, were found as well. The putative of *Se*CbR was structurally related to those of other species indicating that they may posse similar functions with the known CbRs. Via prediction of SMART program, the TM segment were founded in the *N*-terminus of *Se*CbR1 and *Se*CbR2, respectively. Therefore, two CbRs here may be located on the cytosolic side of the endoplasmic reticulum with its *N*-terminus. The position of hydrophobicity of SeCbR *N*-terminus was agreement with the TM position.

According to the polygenetic tree, four clans of CbR (I, II, III and IV) were generated. Interestingly, CbR1 and CbR3 only found in mammal, however, CbR2 and CbR4 existed in mammal, insect, plant and nematode. Combining with the homology result, the SeCbR was closest relationship with the CbR of *H. armigera*, which belong to the Noctuidae family as well as *S. exgua*. And the diamondback moth (*P. xylostella*) belonging to the Plutellidae family taken the second place to SeCbR. However, the *Ha*CbR1, *Ha*CbR2, *Se*CbR1 and *Se*CbR2 did not belong to separated groups that may be due to the alternative splicing or other reasons. Hence, more research should be done to clarify the CbR clusters.

The expression level of *SeCbR1* and *SeCbR2* mRNA were changed with the growth of *S. exigua*, the highest level was found in the 4th and 5th larval instars, respectively and the lowest level in pupae. Meanwhile, the *SeCbR1* and *SeCbR2* mRNA transcripts in different tissues were different, and the maximum level was observed in the midgut. In general, the expression levels of certain protein in tissues is related or affect its function. Such tissue distribution pattern of CbR was similar with that of not only *Ha*CbR but also the P450 in *H. armiger* [16, 32]. That phenomenon

was agreement with fact that its involvement in the function of P450s.

CONCLUSION

In brief, the current study provides the primary information on molecular about nucleotide and amino acid sequence, putative structure and expression pattern of CbR in *S. exigua*. However, for discovering specific pharmaceutical function of *Se*CbR, further studies and more work still should be undertaken.

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