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De novo sequencing and transcriptome analysis of *Stichopus horrens* to reveal genes related to biosynthesis of triterpenoids

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ABSTRACT

As the widespread species in the tropical Indo-Pacific area, the sea cucumber *Stichopus horrens* is characterized by the presence of appreciable amounts of triterpene glycosides. However, genes involved in biosynthesis of the triterpenoids in sea cucumber have rarely been researched. Here, we aim to explore the triterpenoid biosynthesis of *S. horrens* collected in the south sea of China. In this study, the *de novo* sequencing of *S. horrens* was carried out by Illumina HiSeq 2000. A total of 99,854,932 high quality sequence reads were obtained. These raw data were assembled into 168,151 contigs, and 78,024 unigenes were left after redundance removing. The transcriptomic data clearly showed that triterpenoid biosynthesis was *via* the MVA pathway in *S. horrens*. The content of total triterpene glycosides was examined by method of orcinol reaction, and 0.116 ± 0.012 mg/g and 0.016 ± 0.003 mg/g in intestine and body wall, respectively. qRTPCR showed that both oxidosqualene cyclases OSC1 and OSC2 are highly expressed in intestine, suggesting their commitment to triterpenoid biosynthesis. We also reported some potential genes encoding Cytochrome P450 and glycosylations enzymes, which might be involved in the late step of biosynthesis of triterpenoid glycosides. Overall, the present study provided the basic data for future molecular studies on biosynthesis of triterpene glycosides in sea cucumber.

1. Introduction

Sea cucumbers, belonging to the class *Holothuroidea* of *Echinodermata* phylum, are characterized by the presence of appreciable amounts of triterpene glycosides (Silchenko and Kalinovsky, 2012). Although widely distributed in higher plants, these compounds are also found in asteroids (Mackie and Turner, 1970) and sponges (Colorado-Rios et al., 2013; Colorado et al., 2013; Kubanek et al., 2002; Petrichtcheva et al., 2002) in the marine environment. The triterpene glycosides are supposed as the main important secondary metabolites accounting for the poisonous features of sea cucumber and function in defense against predators and pathogens (Kim and Himaya, 2012; Osbourn et al., 2011). Studies have demonstrated that sea cucumber triterpene glycosides have many beneficial bioactive properties including antitumor, antifungal, and immune-modulating effects (Menchinskaya et al., 2013; Prokofieva et al., 2003). To date, over 100 sea cucumber triterpene glycosides had been reported (Caulier et al., 2011; Dang et al., 2007), and the number increases as new triterpene glycosides with novel structures have been successively identified.

The majority of known sea cucumber glycosides possess 18(20)-lactone in aglycones (so-called holostane aglycones), and the aglycones preferably have a 7(8)- or 9(11)-double bond and a sugar chain of monosaccharide units (usually include D-xylose, D-glucose, D-quinovose, D-3-O-methylxylose and 3-O-methyl-D-glycose) (Bahrami et al., 2014). *De novo* biosynthesis of triterpene glycosides in sea cucumber was confirmed by radiolabeling experiments (Elyakov et al., 1975). The radiolabeled acetate and mevalonate could be used as the precursors of 2,3-oxidosqualene, which was usually transformed into lanosterol and parkeol (lanosta-9(11), 24-dien-3b-ol) after cyclization (Cordeiro and Djerassi, 1990; Cordeiro et al., 1988). Although whether lanosterol and parkeol are incorporated into glycosides are somewhat controversial (Silchenko and Kalinovsky, 2012), these two compounds are important intermediates for synthesis of aglycones containing 18(20)-lactone. Genes involved in formation of triterpene backbone in sea cucumber are scarcely reported, however, biosynthesis of triterpenes are demonstrated in other organism and biosynthetic enzymes are well functionally characterized (Abe, 2007). The biosynthetic pathway for the backbone of triterpenoids from sea cucumber could probably be proposed by combining examining structures of triterpene glycosides, tracing intermediates and *de novo* transcriptomic sequencing.

Stichopus horrens is an important tropical commercial species, and successfully cultivated in China (Hu et al., 2013). This sea cucumber has been reported to contain triterpene glycosides with the typical 18(20)-lactone in aglycones (Cuong et al., 2017), providing a wonderful

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model for glycosides synthesis in sea cucumbers. In order to elucidate the genes involved in biosynthetic pathway of triterpenes in sea cucumber, we sequenced *S. horrens* transcriptome, trying to predict potential genes most likely involved in biosynthesis of triterpene backbone. Cytochrome P450 (CYP450) and glycosyl transferases (GTs) involved in the late steps of glycoside biosynthesis were also analyzed.

2. Materials and methods

2.1. Animals

The sea cucumber *S. horrens* with average wet weight of 127.2 ± 3.4 g was collected from the coast of Dongying, Hainan, China in winter 2016. The temperature and salinity were 24.8 °C and 32 ppt respectively.

2.2. RNA isolation and sequencing

Briefly, the body wall and intestine of sea cucumbers were sampled and the total RNA was isolated using the Qiagen RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer's technical instructions. To remove genomic DNA, the total RNA was treated with RNase-free DNase I contained in this kit for 15 min at room temperature. The purified RNA was analyzed on a Nanodrop spectrophotometer (Nanodrop 2000, Thermo Scientific) followed by agarose electrophoresis to determine the quantity. Equal amounts of mRNA from body wall and intestine were pooled and subject to cDNA library construction and RNA sequencing. Sequencing was carried out as follows: Poly (A) mRNA was purified from the total RNA using the oligo (dT) magnetic beads, and fragmented into small pieces with fragmentation buffer. The 200–700 bp fragments were used to synthesize the first-strand and double-strand cDNA with random hexamer-primers, after which end repair, dA tailing, adaptor ligation and sequential DNA fragment enrichment were performed. The concentration of the cDNA library was further checked with a Bioanalyzer 2100 (Agilent Technologies, USA) and sequenced using Illumina HiSeq 2000(Illumina Inc., San Diego CA, USA).

2.3. Sequence assembling and analysis

Raw reads produced from sequencing machines cleaned by removing the adaptor sequences and any ambiguous or low quality bases. *De novo* transcriptomic assembly was carried out with the short-read assembly soft package Trinity (r20131110) under default settings.

Blastx analysis of unigenes longer than 200 bp was conducted against NCBI non-redundant protein database (NR) (www.ncbi.nlm.nih.gov) and SwissProt database with a cutoff E-value of 0.00001, or PFAM database (http://pfam.sanger.ac.uk/) COG database (www.ncbi.nlm.nih.gov/COG/) and KEGG database (http://www.genome.jp/kegg/) with a cutoff E-value of 0.001. The Best Hit of the highest sequence similarity from all Blast results was parsed for a homology-based functional annotation to the giving unigenes. Based on the annotation information from NR and PFAM, GO annotation was also performed using Blast2GO soft (http://www.balst2go.org/version 2.5), and the classification of GO functions was conducted using R software.

2.4. Quantitative study of saponins and gene expression

The procedures of saponins extraction and purification were adapted from Campagnuolo et al. (2001) and Dyck et al. (2009), and quantitative measurement by orcinol reaction method was adapted from Dyck et al. (2010) as follows. In brief, body wall or intestine was sampled and extracted with ethanol/water (v/v = 70/30) and evaporated. Then the dry extract was diluted in 90% methanol and partitioned against n-hexane, CCl_4 and CCl_3. The hydromethanolic was further purified on column filled with Amberlite XAD-4 and partitioned against iso-butanol to obtain the purified saponins. Finally, the crude saponins was mixed with aqueous orcinol solution (1.6%) and sulfuric acid solution (60%), and incubated at 80 °C for chromogenic reaction. The mixture's absorbance was measured at 540 nm. A 10, 5, 2.5 1, 0.5 and 0.25 mg/mL solution of D-xylose was used to make a standard curve. Saponins extraction and measures are independent triplicate.

Table 1
Primers used in qRTPCR.

Primer	Sequence (5′ → 3′)
QC1	Forward: GCCTTGGAAGTCTGCTTGGAT Reverse: GCTTTGGACTCTGGACCATCT
QC2	Forward: ACTTTCGACAATACAATAAGGG Reverse: AAGGGACATTTCTCGTTCATC
β-Actin	Forward: GACCTACCACCTATTCTTCACC Reverse: TTCACATCAACCGACTATCCC

Total RNA from body wall and intestine of four individuals was extracted with the method described above, and cDNA was synthesized by EvoScript Universal cDNA Master kit (Roche). Quantitative real-time PCR (qRTPCR) was conducted in a StepOnePlus Real-Time PCR System (ABI) using the FastStart Universal SYBR Green Master (ROX) Kit (Roche). Primers are showed in Table 1. Each assay was performed with β-actin as the internal control, and body wall served as the reference tissue to normalized target gene expression level. Measures were from three independent individuals, each in two biological replicates. A $2^{-\Delta\Delta Ct}$ method was adapted from Schmittgen and Livak (2008) to calculated the gene relative expression.

2.5. Statistical analyses

Mean ± standard deviation (S.D.) was calculated for each of the results. Saponin content and gene expression level were analyzed by one-way ANOVA in SPSS 19.0. Differences were set as statistical significance at $p < .05$.

3. Results

3.1. Generation and assembly of de novo transcriptomic sequencing data

To obtain an overview of the *S. horrens* gene expression profile, cDNA samples from body wall and intestine were pooled and sequenced on an Illumina HiSeq2000 machine. A total of 99,854,932 clean sequence reads were obtained after trimmed (Table 2). These raw data were assembled into 168,151 isotigs. All isotigs were subjected to CD-hit program to remove redundant sequences under default settings, leaving 78,024 unigenes. The mean unigenes size was 1600 bp with lengths ranging from 201 bp to 36,149 bp and N50 of 2727 bp (Table 2, Fig. 1).

3.2. Functional annotation of unigenes

Unigenes were performed blastx alignment against public databases

Table 2
Overview of sequencing and *de novo* assembly.

Item	Value
Number of raw reads	104,592,100
No. of clean reads	99,854,932
Average GC content (%)	41.52
Number of unigenes	78,024
Total length of unigenes (bp)	124,838,545
Range of unigenes lengths	201–36,149
Mean length of unigenes (bp)	1600
N50 of unigenes (bp)	2727

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