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Research paper

Comparative RNA-sequencing profiling reveals novel Delta-class glutathione S-transferases relative genes expression patterns in *Tribolium castaneum*

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ABSTRACT

Glutathione S-transferases (GSTs) are a large group of enzymes having both detoxification roles conferring insecticide resistance and specialist metabolic functions. *Tribolium castaneum GST Delta 1* (*TcGSTd1*) has been found playing crucial role in insecticide resistance and biological processes in insect species. However, the regulatory system of *TcGSTd1* has still rarely been known. Comparing the transcriptome profile of RNAi treated larvae (ds-*TcGSTd1*) and control larvae of *T. canstaneum* by using RNA-sequencing, we obtained 14,284,085 sequence reads aligned with 13,275 genes. And 512 differentially expressed genes (DEGs) were identified from ds-*TcGSTd1* treated group. *Est/CCE*, *CYP*, *MRPs* were significantly down-regulated in ds-*TcGSTd1* group when compared with control group, which illustrated that they cooperated with *TcGSTd1* to reduce the activity of cellular metabolism system. While, *SNO* was up-regulated in ds-*TcGSTd1* insects suggested it may also involve in detoxifying alkaloid of insect metabolism system. These results established that *TcGSTd1* not only acts as a vital gene for phase II cellular detoxification but also participates in phase 0, I, and III cellular detoxification by cooperating with *CSPs*, *OBPs*, *CYP9*, *ESTB1*, *CCE6*, *MRPs* and other detoxification genes. Knockdown of *TcGSTd1* also suppressed several genes encoding antioxidant enzymes, e.g. *CuZnSOD*, *Duox*, *Prx*, *HPX*, *CPO*, and *MCORP*. Suggested that they may modulate the function of *TcGSTd1* on lifespan, immune, development and reproduction. All these results shed the new insights into the regulatory mechanism of *TcGSTd1* involved in insect physiology and could further facilitate the research of suitable and sustainable managements for the pest control.

1. Introduction

Glutathione S-transferases (GSTs, EC 2.5.1.18) are a diverse family of enzymes involved in the detoxification of endogenous or exogenous toxic compounds in both prokaryotic and eukaryotic cells. They mainly act by conjugating the thiol group from the tripeptide glutathione (GSH) to electrophilic centers of nonpolar compounds (Hayes et al., 2005). Then substrates can render more water soluble by targeting to specific GSH, thus easier to excrete from cells (Ketterman et al., 2011). GSTs possessed a wide range of substrates specificities as endogenous substrates including reactive DNA bases, reactive unsaturated hydroperoxides, organic hydroperoxides and epoxides which were generated by oxidative stress (Cnubben et al., 2001; Yan et al., 2013). The absence of Se-dependent glutathione peroxidases (GPOXs) in insects raise the underlying putative peroxidase function of GSTs in antioxidant defense, with the basic function of reducing organic hydroperoxides within lipoproteins and membranes (Fournier et al., 1992; Parkes et al., 1993; Duan et al., 2013). GSTs also participate in many non-catalytic functions such as the intracellular biosynthesis of hormones, transport fatty acids, hematin and involve in stress signal processing (Lumjuan et al., 2007; Ketterman et al., 2011; Wongtrakul et al., 2014).

In insects, GSTs can be divided into two major groups: microsomal- and cytosolic-GSTs. Microsomal-GSTs were bounded in membranes which were evolutionarily and structurally distinct from cytosolic-GSTs. However, several studies indicated that cytosolic-GSTs were the common GSTs in insect species, and they can further divide into six major classes (Delta, Epsilon, Sigma, Theta, Omega, and Zeta). Among them, Sigma, Omega, Zeta, and Theta are broadly existed across Metazoa, whereas Delta and Epsilon are specific to insects (Friedman, 2011). In last decades, the researches of insect GSTs has been focused on the conferring insecticide resistance and protecting against cellular damage which caused by oxidative stress (Zou et al., 2000). Increased GSTs activity and/or elevated the expression of GSTs have been

Abbreviations: GST, glutathione S-transferase; DEG, differential expressed genes; RNAi, RNA interference; GSH, glutathione; RPKM, reads per kilobases per million reads; GO, gene ontology; KEGG, Kyoto Encyclopedia of Genes and Genome; FDR, false discovery rate; qRT-PCR, quantitative real-time polymerase chain reaction; rps3, ribosomal protein S3.

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associated with resistance to a wide range of insecticides including organophosphates, organochlorines, and pyrethroids (Reidy et al., 1990; Huang et al., 1998; Ranson et al., 2002; Lumjuan et al., 2011). Basically, GSTs show directly detoxify organophosphates and organochlorines through conjugate with those electrophilic compounds with GSH, and making the resultant productions more water soluble and excretable than the non-GSH conjugated substrates (Udomsinprasert et al., 2005). But the evidence that GSTs can directly metabolize pyrethroids was insufficient. Besides, GSTs can detoxify lipid peroxidation products induced by insecticides, which also demonstrated in coffering resistance (Kostaropoulos et al., 2001; Vontas et al., 2001).

Delta is the largest cytosolic-GSTs subgroups in *Drosophila melanogaster* (11), *Anopheles gambiae* (17), *Acyrthosiphon pisum* (16), *Aedes aegypti* (8), *Nasonia vitripennis* (4), *and Bombyx mori* (5), while there are only 3 members in *Tribolium castaneum* (Shi et al., 2012; Schama et al., 2016). *Drosophila GSTd1* (CG10045) have DDT dehydrochloinase activity suggested that *GSTd* play an important role in DDT metabolism. And a number of Delta GSTs in *D. melanogaster* have GSH peroxidase activity against cumene hydroperoxide or catalyze the lipid peroxidation productions, which indicated that metabolism of products of lipid peroxidation is a biochemical pathway with detoxification as well as regulatory functions (Tang and Tu, 1994; Sawicki et al., 2003). Two another Delta GST genes, *AgGST1-5* and *AgGST1-6*, were isolated from a DDT resistant strain of *A. gambiae* and their proteins expressed in *Escherichia coli* exhibited very high level of activity to DCNB which indicated that they were involved in insecticide resistance (Ranson et al., 1997). While, a Delta GST has been cloned from a pyrethroid resistant strain of *Nilaparvata lugens* and the recombinant protein has high peroxidase activity, which proposed its critical function for preventing oxidative damage (Vontas et al., 2002). However, not all Delta GSTs show the similar functions and there were several differences in subfamilies of Delta GSTs. In *Culex pipiens*, CpGSTd1 exhibited peroxidase activity and metabolize DDT, but CpGSTd2 appeared as no activity. And, both of them do not appear to play major roles in permethrin resistance in mosquitoes (Samra et al., 2012). In addition, it is hard to detect the expression of some Delta GSTs in the detoxification organ (fat boby), which suggested that Delta GSTs have variety functions in *Bombyx mori* (Yu et al., 2008) or *Tenebrio molitor* (Liu et al., 2015). Thereby, the physiological functions of Delta GSTs are varied from one insect to another, and some of these still poorly understood.

The red flour beetle, *T. castaneum* (Coleoptera, Tenebrionidae) is a worldwide notorious agricultural pest of stored grain and cereal products. Our previous study has identified 3 Delta GSTs of *T. castaneum* (Shi et al., 2012). The purification proteins exhibited a high activity toward to CDNB and knockdown any one of them in *T. castaneum* caused increasing susceptibility to insecticides which suggested they play an important role in insecticide resistance. Intriguingly, loss of *TcGSTd1* caused approximately 100% mortality which has never been reported in any insects before (data not shown). And it is still unknown how *TcGSTd1* performs its functions in the red flour beetle. Therefore, in order to clarify the specific function and especial information in regulatory system of *TcGSTd1* gene. RNA-sequencing technology and RNAi were merged to investigate the potential functions of *TcGSTd1*, and our study can shed new lights in signaling modulated systems of relative genes in insect species.

2. Material and methods

2.1. Insect strains

In this study, *T. castaneum* Georgia-1 (GA-1) strain was used as experimental animal. Insects were reared in whole wheat flour containing 5% brewer's yeast at constant temperature (30 °C) and relative humidity (55%) under standard conditions (Reidy et al., 1990).

2.2. Double-strand RNA synthesis and injection

For double-strand RNA (dsRNA) synthesis, primers containing *TcGSTd1*-specific sequences (sense primer: 5′-TATTAAGATCAACCCGCAAC-3′, antisense primer: 5′-TCGACCATCTGCTTAAAGAT-3′) and the T7 polymerase promoter (TAATACGACTCACTATAGGG) at the 5′-end of both the sense primer and *anti*-sense primer were designed to amplify dsDNA of *TcGSTd1*. And *TcGSTd1* dsRNA synthesis and RNAi were performed as described previously (Li et al., 2014b). Larvae injected with *TcGSTd1* dsRNA were denoted as ds-*TcGSTd1* group, and larvae injected with an equal volume of buffer were denoted as control group.

2.3. RNA preparation and Illumina sequencing

Control and RNAi of *TcGSTd1* beetle samples were collected and used for RNA-sequencing. Total RNA was isolated separately from of control and ds-*TcGSTd1* insects at the fifth day after injection by using the RNAiso™Plus Trizol reagent (TaKaRa), which according to the manufacturer's protocol. The quality and quantity of total RNA samples were assessed by using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA), and experimental samples were normalized to the same concentration. The integrity of RNA was detected by using a 1% agarose gel and Agilent 2100 Bioanalyzer. Poly (A) mRNA was isolated by using oligo(dT) beads and was disrupted into short fragments (approximately 200 bp). The fragments were purified with a QIAquick PCR Extraction Kit (Qiagen, Germany), followed by end repairing and adaptor ligation. Experimental cDNA syntheses were performed by using Illumina Tru-Seq™ RNA sample preparation kit according to the manufacturer's protocol. Single-end RNA-sequencing libraries of control and ds-*TcGSTd1* samples were prepared and then sequenced on the Illumina HiSeq™ 2000 platform. And the raw data of RNA-sequencing were deposited to the NCBI Sequence Read Archive (SRA) database (http://trace.ncbi.nlm.nih.gov/Traces/sra_sub/sub.cgi?login=pda), and the accession numbers were SRR3087513 (control) and SRR2087948 (ds-*TcGSTd1*).

2.4. Quality control and functional annotation

Sequencing-received raw image data were transformed by base calling into sequence data. The raw reads generated from the sequencing machines were cleaned by discarding the adaptor and low-quality sequences. The clean reads then assembled and mapped to the reference genome of *T. castaneum* (http://www.beetlebase.org/) conducting with SOAPaligner/soap2 using the default parameters (Li et al., 2009). Failed mapped reads were progressively trimmed off 1 base at a time from the 3′-end and mapped to the genome again until a match was found (unless the read had been trimmed by <27 bases). The data obtained were analyzed for the following functional annotation of all detected expressed genes.

Firstly, ERANGE software (version 4.0) (http://woldlab.caltech.edu/gitweb/) was applied to calculate the gene locus expression level by assigning reads to their sites of origin and counting them. The expression level of a gene from the RNA-sequencing was normalized by RPKM method (Wang et al., 2009), a normalized measure of read density that allows transcriptional levels to be compared both within and between samples. The cutoff value for determining the gene transcriptional profiling of each gene was determined based on a 95% confidence interval for all the RPKM values. Then, all detected beetle genes were assigned to different function categories by the software Blast2GO (version 2.3.5) (http://www.blast2go.org/) (Conesa et al., 2005). And the GO classification was carried out using WEGO (Web Gene Ontology Annotation Plot, http://wego.genomics.org.cn/cgibin/wego/index.pl) to classify these GO terms by performing Fisher's exact test with an FDR correction to obtain an adjusted *p*-value between certain test gene groups and the whole annotation genome (Ye et al.,