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Regulatory functions of trehalose-6-phosphate synthase in the chitin biosynthesis pathway in *Tribolium castaneum* (Coleoptera: Tenebrionidae) revealed by RNA interference

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Abstract

RNA interference (RNAi) is a very effective technique for studying gene function and may be an efficient method for controlling pests. Trehalose-6-phosphate synthase (*TPS*), which plays a key role in the synthesis of trehalose and insect development, was cloned in *Tribolium castaneum* (Herbst) (*TcTPS*) and the putative functions were studied using RNAi via the injection of double-stranded RNA (dsRNA) corresponding to conserved TPS and trehalose-6-phosphate phosphatase domains. Expression analyses show that *TcTPS* is expressed higher in the fat body, while quantitative real-time polymerase chain reaction results show that the expression of four trehalase isoforms was significantly suppressed by dsTPS injection. Additionally, the expression of six chitin synthesis-related genes, such as hexokinase 2 and glutamine-fructose-6-phosphate aminotransferase, was suppressed at 48 and 72 h post-dsTPS-1 and dsTPS-2 RNA injection, which were two dsTPS fragments that had been designed for two different locations in TcTPS open reading frame, and that trehalose content and trehalase 1 activity decreased significantly at 72 h post-dsRNA injection. Furthermore, *T. castaneum* injected with dsTPS-1 and dsTPS-2 RNA displayed significantly lower levels of chitin and could not complete the molting process from larvae to pupae, revealing abnormal molting phenotypes. These results demonstrate that silencing *TPS* gene leads to molting deformities and high mortality rates via regulation of gene expression in the chitin biosynthetic pathway, and may be a promising approach for pest control in the future.

Keywords: *Tribolium castaneum*, trehalose-6-phosphate synthase, RNA interference, chitin biosynthesis pathway, pest control

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Introduction

Trehalose, a non-reducing disaccharide formed by two glucose molecules linked by a 1α–1α bond, is widely present in bacteria, fungi, plants, and invertebrates (Avonce *et al.*, 2006) and can be induced under various conditions of stress (Li *et al.*,

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2009; Thorat *et al.*, 2012; Sánchez-Fresneda *et al.*, 2013). Trehalose is also referred to as 'blood sugar' because of its important functions in insect growth and development (Wyatt, 1967; Tang *et al.*, 2012). The molecular characterization of trehalose biosynthetic pathways and corresponding genes in various organisms has revealed at least five biosynthetic pathways for trehalose (Elbein *et al.*, 2003; Avonce *et al.*, 2006), which includes TPS/TPP (trehalose-6-phosphate synthase/trehalose-6-phosphate phosphatase), TreY/TreZ (maltooligosyl trehalose synthase/maltooligosyl trehalose trehalohydrolase), TreP (trehalose phosphorylase), and TreT (trehalose glycosyltransferring synthase) pathways (Avonce *et al.*, 2006). Insect trehalose is mainly synthesized by TPS/TPP (Tang *et al.*, 2010). It has also been reported that insects have two distinct types of trehalase – soluble trehalase (trehalase 1 or TRE1) and membrane-bound trehalase (trehalase 2 or TRE2) – which function was degraded trehalose to glucose (Chen *et al.*, 2010*a*; Tang *et al.*, 2012; 2016; Zhao *et al.*, 2016).

Since the first insect *TPS* gene was discovered and cloned in the fruit fly *Drosophila melanogaster* in 2002 (Chen *et al.*, 2002), many *TPS* homologs have been cloned and reported from a variety of insects, including the cotton bollworm (*Helicoverpa armigera*) (Xu *et al.*, 2009), the brown planthopper (*Nilaparvata lugens*, Stål) (Chen *et al.*, 2010*b*; Yang *et al.*, 2017), the beet armyworm (*Spodoptera exigua* Hübner) (Tang *et al.*, 2010), the locust (*Catantops pinguis* Stål) (Tang *et al.*, 2011), the German cockroach (*Blattella germanica* Linnaeus) (Chen & Zhang, 2015), the Chinese citrus fly (*Bactrocera minax* Enderlein) (Xiong *et al.*, 2016), and the Colorado potato beetle (*Leptinotarsa decemlineata* Say) (Shi *et al.*, 2016). Moreover, two *TPS* genes in *B. germanica* (*BgTPS*1: KR050213 and *BgTPS*2: KR050214) and in *N. lugens* (*NlTPS1*: GQ397450 and *NlTPS2*: KU556826) have been cloned (Chen & Zhang, 2015; Yang *et al.*, 2017). Furthermore, some cases of singular *TPP* genes in insects have been found in GenBank [e.g., the Formosan subterranean termite (*Coptotermes formosanus* Oshima; JN565075), the fruit fly *D. melanogaster* (NM_135269), and the diamondback moth (*Plutella xylostella* Linnaeus; XM_011559193)]; however, it is shorter than TPS, contains one TPP domain, and is highly similar to the TPS of each respective species, with only a few differences in protein sequence at the *N*-terminus.

The chitin biosynthesis pathway is vital to insect growth and development, and includes trehalases (*TRE*), hexokinases (*HK*), glucose-6-phosphate isomerase (*G6PI*), glutamine-fructose-6-phosphate aminotransferase (*GFAT*), and chitin synthase (*CHS*) (Tang *et al.*, 2010; Chen *et al.*, 2010*b*). It has been reported that *TPS* is also vital to insect growth and development. For example, introducing mutations into the *TPS* gene of *D. melanogaster* larvae has been shown to be fatal (Chen *et al.*, 2002). Abnormalities in the molting process have been observed in *S. exigua*, *N. lugens*, *L. decemlineata*, and *B. minax* as a result of *TPS* inhibition (Tang *et al.*, 2010; Chen *et al.*, 2010*b*; Shi *et al.*, 2016; Xiong *et al.*, 2016; Yang *et al.*, 2017). As TPS is known to be vital to insect growth and development, the present study investigated the putative functions of TPS in *Tribolium castaneum* using RNAi via the injection of double-stranded RNA (dsRNA) corresponding to conserved TPS and TPP structure. In this study, we aimed to (a) synthesize dsRNA fragments from the conserved domains of TPS-1 and TPS-2 from the TPS sequence of *T. castaneum* (*TcTPS*: XM_970683, fig. 1), (b) analyze the functions of the *TPS* gene using RNAi, and (c) assess the viability of this technique for use as a potential method of pest control for *T. castaneum*, especially via the chitin synthesis pathway.

RNA interference (RNAi) is widely used to study gene function by suppressing gene expression via the injection of dsRNA (Fire *et al.*, 1998). RNAi technology also has been widely used to investigate the functions of essential genes in insects (Tomoyasu & Denell, 2004; Chen *et al.*, 2008; Gong *et al.*, 2012; Wang *et al.*, 2017; Zhang *et al.*, 2013; Asokan *et al.*, 2013; Qi *et al.*, 2015), and has been particularly employed to silence vital genes (Belles, 2010; Liu *et al.*, 2010; Wang *et al.*, 2012). Similar studies using RNAi have resulted in diverse malformed phenotypes in insects and show varying efficiencies in the insect tissues investigated (Zhu *et al.*, 2008; Belles, 2010; Scott *et al.*, 2013; Xi *et al.*, 2015). The red flour beetle (*T. castaneum*, Herbst 1797; Coleoptera) is a holometabolous insect with embryonic, larval, pupal, and adult stages, and is considered to be a serious pest of stored grains (Adarkwah *et al.*, 2010). Several studies have revealed that RNAi in *T. castaneum* is efficient in maintaining the silencing effect for a longer period and inducing a higher rate of deformations (Chen *et al.*, 2010*a*; Xi *et al.*, 2015), even in the postembryonic stages (Tomoyasu & Denell, 2004; Arakane *et al.*, 2005; Minakuchi *et al.*, 2008, 2015). Previous studies have also shown that *T. castaneum* is an effective and reliable insect for conducting RNAi research (Noh *et al.*, 2012; Tang *et al.*, 2016).

Materials and methods

Insect rearing

The experimental beetle populations of *T. castaneum* were raised in whole-wheat flour with 5% yeast powder in the dark at 30 °C (Minakuchi *et al.*, 2008; 2015). Developmental stages were synchronized by providing fresh coarse wheat bran to replace old coarse wheat bran daily, while the old coarse wheat bran mixture (containing *T. castaneum* eggs) was cultured for future experiments.

RNA extraction, cDNA synthesis, and cloning of TPS

Total RNA was extracted from the whole body of *T. castaneum* larvae or adults using TRIzol reagent (Life Technolodies, Carlsbad, California, USA) following the manufacturer's instructions. The RNA concentration was determined by measuring the absorbance at 260 nm using a spectrophotometer (NanoDrop™ 2000, Thermo Scientific, Waltham, MA, USA). Purified RNA was stored at −80 °C for future experiments. First-strand cDNA synthesis was performed using the PrimeScript® RT reagent Kit with gDNA Eraser (TaKaRa Bio Inc., Dalian, China) according to the manufacturer's protocol.

Primers were designed using a cDNA template according to *TcTPS* (XM_970683). A primer pair – TcTPS-F and TcTPS-R – was designed to amplify the open reading frame (ORF) of *TcTPS* (table 1). The reaction mixture included 10× polymerase chain reaction (PCR) buffer with 0.1 mM dNTPs, 0.2 µM of each primer, and 0.5 U HiFi Taq DNA polymerase (TransGen Biotech, Beijing, China), for a total of 25 µl. PCRs were performed according to the following conditions: 10 min at 94 °C; followed by 30 cycles of 30 s at 94 °C, 30 s at 55 °C, and 160 s at 72 °C and a final extension at 72 °C for 10 min. Products were analyzed by agarose gel electrophoresis. Bands corresponding to the expected size (approximately 2500 bp) were excised and purified using a DNA gel extraction kit (Omega Bio-Tek, Inc., Norcross, Georgia, USA). Purified DNA was ligated into the pMD18-T vector (TaKaRa Bio Inc., Dalian, China) and sequenced.

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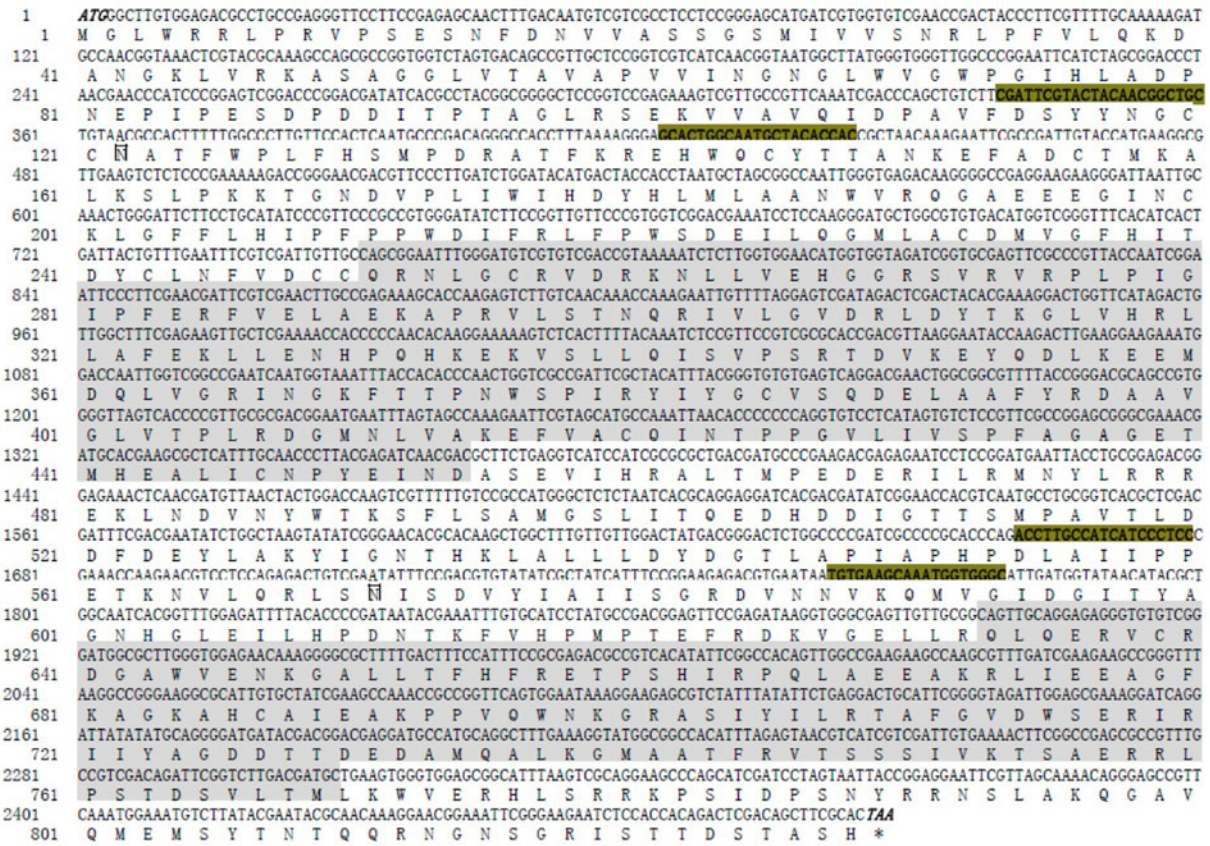

Fig. 1. Nucleotide and deduced amino acid sequences of *Tribolium castaneum* trehalose-6-phosphate synthase (*TPS*). Both initiation and termination codons are indicated by bold typeface and italic. Two conserved domains used for dsRNA synthesis of *TPS* (751–1362 bp) and trehalose-6-phosphate phosphatase (*TPP*) (1897–2308 bp) are shaded in gray. qRT–PCR primers sequences used to detect *TPS-1* and *TPS-2* are indicated by bold typeface and gold-colored shading. The two glycosylation sites are boxed.

Tissue-specific expression of TcTPS using quantitative real-time PCR (qRT–PCR)

Tissue-specific expression of the TPS conserved domains sequences was analyzed via qRT–PCR using gene-specific primers for qTcTPS-1 and qTcTPS-2 (table 1). The epidermis (Ep), midgut (Mg), Malpighian tubules (Mt), and fat body (Fb) from larvae of last-instar larvae were dissected in a saline solution (0.75% NaCl) and stored at −80 °C for future analyses. Total RNA was isolated from these tissues and reverse transcribed into cDNA as previously described. Expression was detected using qRT–PCR with the Bio-Rad CFX96™ system (Bio-Rad Laboratories, Redmond, Washington, USA) and SsoFast™ Eva Green Supermix (Bio-Rad Laboratories) according to the manufacturer's protocol. Each reaction was performed in a final volume of 20 µl, containing 1 µl cDNA sample (or standard), 1 µl (10 µmol μl^{-1}) each primer, 7 µl RNase-free and DNase-free water, and 10 µl SsoFast™ Eva Green Supermix. The thermocycler profile was as follows: 3 min of initial denaturation at 95 °C; 40 cycles of denaturation at 95 °C for 5 s, annealing at 55–62.5 °C for 20 s; and melting curve performed at 65–95 °C (according to the instructions for SsoFast™ Eva Green Supermix). Standard curves were obtained using ten-fold serial dilutions of pooled total RNA. In addition primers specific to *T. castaneum* β-*actin* (*Tc*β-*actin*) were used as a control in the qRT–PCR analyses (table 1).

dsRNA synthesis from the conserved domains of TPS-1 and TPS-2 and subsequent injection

The two conserved domains (TPS-1 and TPS-2) from *TcTPS* were used as target sequences in dsRNA production (fig. 1). The lengths of the resulting TcTPS-1 and TcTPS-2 dsRNAs were 612 and 412 bp, respectively, corresponding to sites 751–1361 (251–454 aa) and 1897–2308 (633–770 aa), respectively. Two pairs of primers, with the T7 RNA promoter sequence flanking the 5′-ends, were designed according to these regions and synthesized using the T7 RiboMAX™ Express RNAi system (Promega Corporation, Madison, Wisconsin, USA) (table 1). Templates for *in vitro* transcription were prepared via PCR using gene-specific primers.

The NT-88-V3 series (Nikon Corporation, Tokyo, Japan) micromanipulator system was used for injection. The dorsal side of the first abdominal segment of *T. castaneum* was selected as the injection point, and 0.5–0.7 µg of dsRNA (3 µg μl^{-1}), targeting *TcTPS* sequences and green fluorescent protein (*GFP*) (control) were injected into last-instar larvae (Noh *et al.*, 2012; Tang *et al.*, 2016).

Insect selection, phenotype observations, and sample collection

Following injection of last-instar insects, 30–50 larvae were pooled as one group for injection as a replicate, and each

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Table 1. *Tribolium castaneum*-specific primers used in this study and GenBank accession no. for each sequence.

PCR fragment	Primer name	Nucleotide sequences-F (5′–3′)	Nucleotide sequences-R (5′–3′)	(GenBank)
cDNA cloning	TcTPS	ATGGGCTTGTGGAGACGCCTGCC	TTAGTGCGAAGCTGTCGAGTCTGTGG	XM_970683
dsRNA synthesis	dsTcTPS-1	GGATCCTAATACGACTCACTATAGGCAGCGGAA TTTGGGATGTCG	GGATCCTAATACGACTCACTATAGGGTCGTTGATCT CGTAAGGGTTGC	XM_970683
	dsTcTPS-2	GGATCCTAATACGACTCACTATAGGCAGTTGCAG GAGAGGGTGTGTC	GGATCCTAATACGACTCACTATAGGCATCGTC AAGACCGAATCTG	XM_970683
	dsGFP	GGATCCTAATACGACTCACTATAGGAAGGGCGA GGAGCTGTTCACCG	GGATCCTAATACGACTCACTATAGGCAGCAGG ACCATGTGATCGCGC	
qRT–PCR	qTcTPS-1	CGATTCGTACTACAACGGCTGC	GTGGTGTAGCATTGCCAGTGC	XM_970683
	qTcTPS-2	ACCTTGCCATCATCCCTCC	GCCCACCATTTGCTTCACA	XM_970683
	qTcHK1	CGCACCGAATGCCAGAATC	GACCCACCCGACATCGATT	XM_961317
	qTcHK2	CGAATCGGCCTAATAGTTGGC	GACGGAGCCCTCGATTTCAT	XM_965552
	qTcCHS1	CGTATAGCCGCCGACTTGAA	CCTATGACGAGAGCACCCAAGA	NM_001039402
	qTcCHS2	GAGTTGTGGCAATGTTCTCGC	GTGGTGTGGCCCTTGGTT	AY295879
	qTcG6PI	GTGATGCCGGAGGTGAAT	CACGTCGGTGATGGGCTT	NM_001145486
	qTcGFAT	GGAACTGGACATGGACCGTA	GAACGGTGAGGATGCGAGTT	XM_961890
	qTcPFK	GAGCAAGGACATGGAAGGGAA	CCAACCCAACCAGCCACTT	XM_961686
	qTcTRE1-1	CGACCTGAAATTAGCCCAGAAG	GGCTCCCCCACTCTTTC	XM_968798
	qTcTRE1-2	GTGCCCAATGGGTTTATCG	CAACCACAACACTTCCTTCG	XM 968883
	qTcTRE1-3	GGGACCGTGGGTTAAACCAAC	CTGTGGCCGCTGAACTGAA	XM_968859
	qTcTRE1-4	ACGATATCGAGCTCAGAGTCC	TACGTCTCTGTGGTGTTCGC	XM_968826
	qTcTRE2-F	GGTGCGCTCCAACTACAAAG	GCTCCGCAGTTCCGTGTAG	EFA11183
	Tcβ-actin	AGGGCGTCATGGTCGGTAT	TTCTACAACGAGCTCCGCG	NM_001172373

injection treatment for dsTPS-1, dsTPS-2, and dsGFP was performed in nine replicates. Abnormal insects were observed and counted every 12 h after RNAi treatment (for a total of 72 h). Individuals displaying phenotypic abnormalities were collected at 48 and 72 h post-injection to detect gene expression via qRT–PCR, while some abnormal insects were also collected for the determination of trehalose concentration, chitin content, and trehalase activity.

Determination of chitin content

The abnormal larvae of *T. castaneum*, 48 h post-injection, were used for the determination of chitin content. These insects were first dried in an oven at 50 °C for 4 days, milled to a powder, and weighed. The chitin extraction from *T. castaneum* was performed as follows: briefly, the powder was treated with 50 ml of 4 M HCl solution at 75 °C for 2 h to remove minerals and catechols. Subsequently, the sample was filtered, and the residue was rinsed thoroughly with distilled water. To deproteinize the sample, the filtrate was treated with 50 ml of 4 M NaOH solution for 20 h at 150 °C. The mixture was filtered and washed repeatedly with distilled water. Finally, the sample was placed in an oven at 60 °C for 24 h to be dried. Thereafter, the dry weight of chitin contents was calculated (Kaya *et al.*, 2013; 2015; Tang *et al.*, 2017). Additionally isolation chitin samples were led to air-dry in shade at room temperature for 10 days. Then dried samples were weighted and determined (Kaya *et al.*, 2015).

Determination of trehalase activities, trehalose, and glucose concentration

The activity of trehalase was determined as described previously with some modifications (Tatun *et al.*, 2008; Yang *et al.*, 2017). Every 10–15 individuals were homogenized with phosphate buffer (pH 7.0), and the homogenate was centrifuged at 1000*g* for 20 min at 4 °C. Supernatant (350 µl) was obtained and centrifuged at 20,800*g* for 60 min at 4 °C. The remaining supernatant was used for the determination of protein concentration, glycogen, and trehalose. The supernatant collected after high-speed centrifugation was used for the determination of trehalase 1 (soluble trehalase) activity and protein content, and the sediment [suspended by phosphate buffer (pH 7.0)] was used for the determination of trehalase 2 (membrane-bound trehalase) activity and protein content. Samples (both supernatant and resuspended pellet) (60 µl) were incubated at 37 °C in the presence of 165 µl phosphate buffer and 75 µl 40 mM trehalose (Sigma-Aldrich Co., St. Louis, Missouri, USA) in a final volume of 300 µl. After 1 h, samples were centrifuged at 12,000*g* for 10 min at 4 °C, and trehalase activity was determined in 10 µl supernatant using the glucose (Go) Assay Kit (Sigma-Aldrich). The concentration of protein was determined using the BCA protein Assay Kit (Pierce Biotechnology, Rockford, Illinois, USA) according to the manufacturer's protocol.

For glucose measurement, the larvae were homogenized in 0.2 and 0.3 ml of PBS, respectively, after which the glucose levels were measured with GO reagent (Sigma-Aldrich). The trehalose content was determined in 10 µl samples obtained as described above using the anthrone-sulfuric acid method. Another 10 µl of sample, obtained as described above, were incubated at 37 °C for 4 h in the presence of starch glycosidase enzymes, and the glucose content subsequently determined

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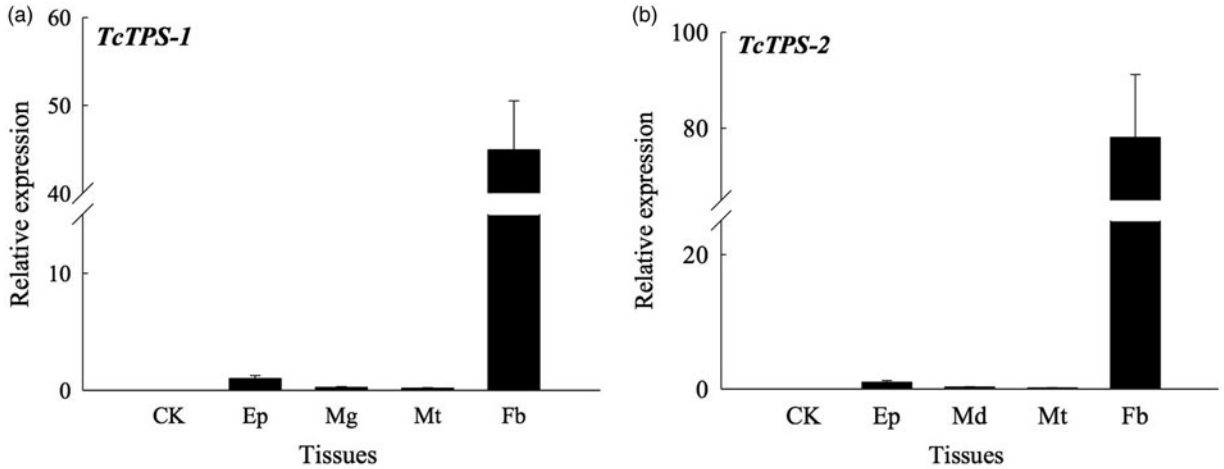

Fig. 2. qRT–PCR analyses of *TcTPS* in tissues of last-instar larvae of *Tribolium castaneum*. Two fragments of TPS-1 and TPS-2 have been used to detect the TPS gene expression in four different tissues of *T. castaneum*. Total RNA was extracted from control group (no any tissues cDNA added, CK), epidermis (Ep), midgut (Md), Malpighian tubules (Mt), and fat body (Fb) tissues and the expression of TPS gene was obtained by qRT–PCR, using *Tc*β-*actin* as the internal control. Primers TPS-1 and TPS-2 correspond to amplicons only TPS-1 domain and only TPS-2 domain, respectively. Values are means ± SEs from three independent measurements.

using the glucose (Go) Assay Kit (Sigma-Aldrich) according to the manufacturer's instructions.

Quantification of mRNA expression

The effects of RNAi on the expression of several genes in the chitin biosynthesis pathway were analyzed using qRT–PCR. Total RNA (1 µg) from each sample was reverse transcribed according to the methods as previously described. All primers were designed to determine the expression of the corresponding homologous gene sequences from the chitin synthesis pathway (table 1). The quantification of mRNA expression detection as previously described. In addition, primers specific to *Tc*β-*actin* were used as a control in the qRT–PCR analyses (table 1).

Statistical analyses

The mRNA expression level in the dsGFP-injected groups was designated as controls. All the data obtained in this study are expressed as the mean ± standard error (SE) of 3–6 replicates, and were determined by one-way analysis of variance and Tukey–Kramer HSD (honestly significant difference) test using JMP software (version 7.0.1 for Macintosh; SAS Institute Inc., Cary, North Carolina, USA) (Tang *et al.*, 2014*b*). Asterisks (*) indicate significant differences in mRNA expression between the dsGFP group and dsTcTPS-injected groups measured at the same time ($P < 0.05$), while double asterisks (**) indicate highly significant differences ($P < 0.01$).

Results

Analysis of full-length TcTPS ORF and tissue-specific expression

TcTPS is 2487 bp in length and contains an ORF of 828 aa, and includes conserved TPS and TPP structures (fig. 1). Our results indicate that TPS is composed of approximately 500 aa (from 27 to 497 aa), while TPP contains 242 aa (from 533 to 774 aa). The theoretical TPS protein has an isoelectric point of 6.58 and a molecular weight of 93.07 kDa, has two N-glycosylation sites, and possesses no signal peptides or transmembrane structures (fig. 1). The tissue-specific expression of *TcTPS* was determined in the midgut, fat body, epidermis, and Malpighian tubules, through qRT–PCR. Our results show that, of the four tissues studied, *TcTPS* expression was detected in the fat body at a very higher level (fig. 2).

Efficiency and specificity of RNAi targeting of TcTPS and expression-induced changes in trehalose metabolism

Significant decreases in *TPS-1* and *TPS-2* expression were observed at 48 and 72 h post-dsTPS-1 ($P < 0.01$) or dsTPS-2 injection ($P < 0.05$), respectively (fig. 3a, b). Our results also indicate that while *TPS-1* expression was significantly higher 72 h post-dsTPS-2 RNA injection, *TPS-2* expression in the dsTPS-1-injected group was higher than in the dsTPS-2 group at 48 h. Furthermore, four soluble trehalase isoforms in *T. castaneum*, *TcTRE1-1*, *TcTRE1-2*, *TcTRE1-3*, *TcTRE1-4*, and a membrane-bound trehalase of *TcTRE2* genes' expressional analysis as detected by qRT–PCR, the results showed that their expression decreased significantly at 48 and 72 h, with the exception of *TRE1-1* at 72 h in dsTPS-1-injected individuals (fig. 3c, e–g). However, the expression of *TcTRE1-2* was increased significantly at 48 h post-dsTPS-1 and dsTPS-2 injection ($P < 0.05$) (fig. 3d).

In addition, trehalose was quantified at 48 and 72 h post-dsTPS-1 and dsTPS-2 RNA injection. These results show that the trehalose content decreased, most notably at 72 h compared with the dsGFP RNA injection group (fig. 4a). Although increases in glucose levels were observed, the differences were not statistically significant (fig. 4b). These results indicate that the trehalose synthesis pathway was effectively inhibited. In addition, the results demonstrate that the activities of both soluble trehalase (trehalase 1 or TRE1) and membrane-bound trehalase (trehalase 2 or TRE2) were decreased post-dsTPS-1 and dsTPS-2 RNA injections (fig. 4c, d). The activity of

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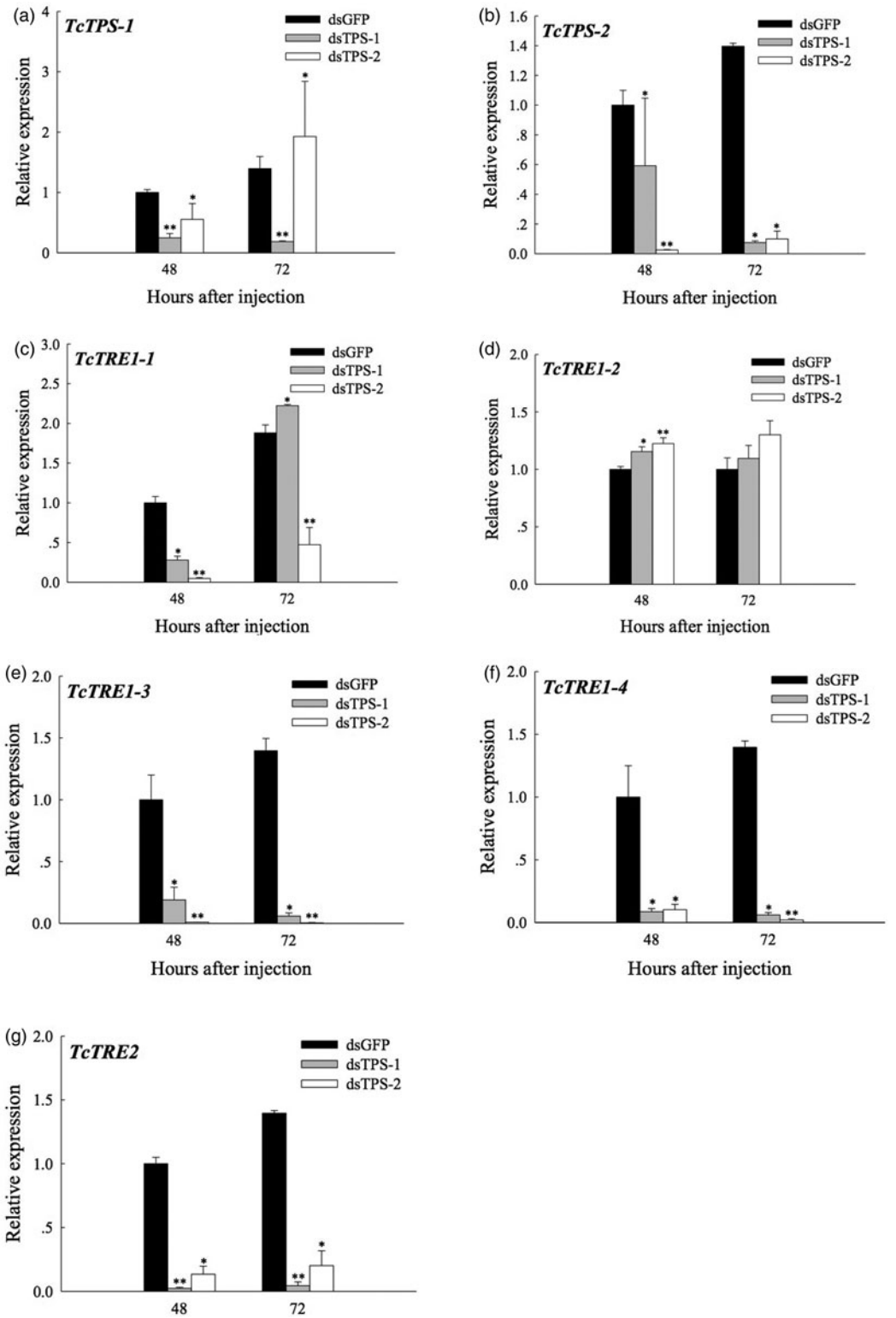

Fig. 3. Effects of dsTPS-1 and dsTPS-2 RNAi injection on genes involved in trehalose metabolism in last-instar larvae after 48 and 72 h. This experiment included dsGFP, dsTPS-1, and dsTPS-2 treatment groups. Approximately 30–50 larvae were pooled as one group for injection as a replicate. The mRNA levels of two *TcTPS-1*, *TcTPS-2*, and five trehalases (*TRE1-1*, *TRE1-2*, *TRE1-3*, *TRE1-4*, and *TRE2*), relative to *Tc*β*-actin* expression, were measured via qRT–PCR. (a–g) indicate *TcTPS-1*, *TcTPS-2*, *TcTRE1-1*, *TcTRE1-2*, *TcTRE1-3*, *TcTRE1-4*, and *TcTRE2*, respectively. Each point represents the mean ± standard error from dsGFP group and dsTcTPS-injected groups. Asterisks (*) indicate significant differences between dsGFP injection group and dsTPS-1 or dsTPS1-2 injection group (Tukey's method, $*P < 0.05$, $**P < 0.01$). TPP, trehalose-6-phosphate phosphatase; TPS, trehalose-6-phosphate synthase.

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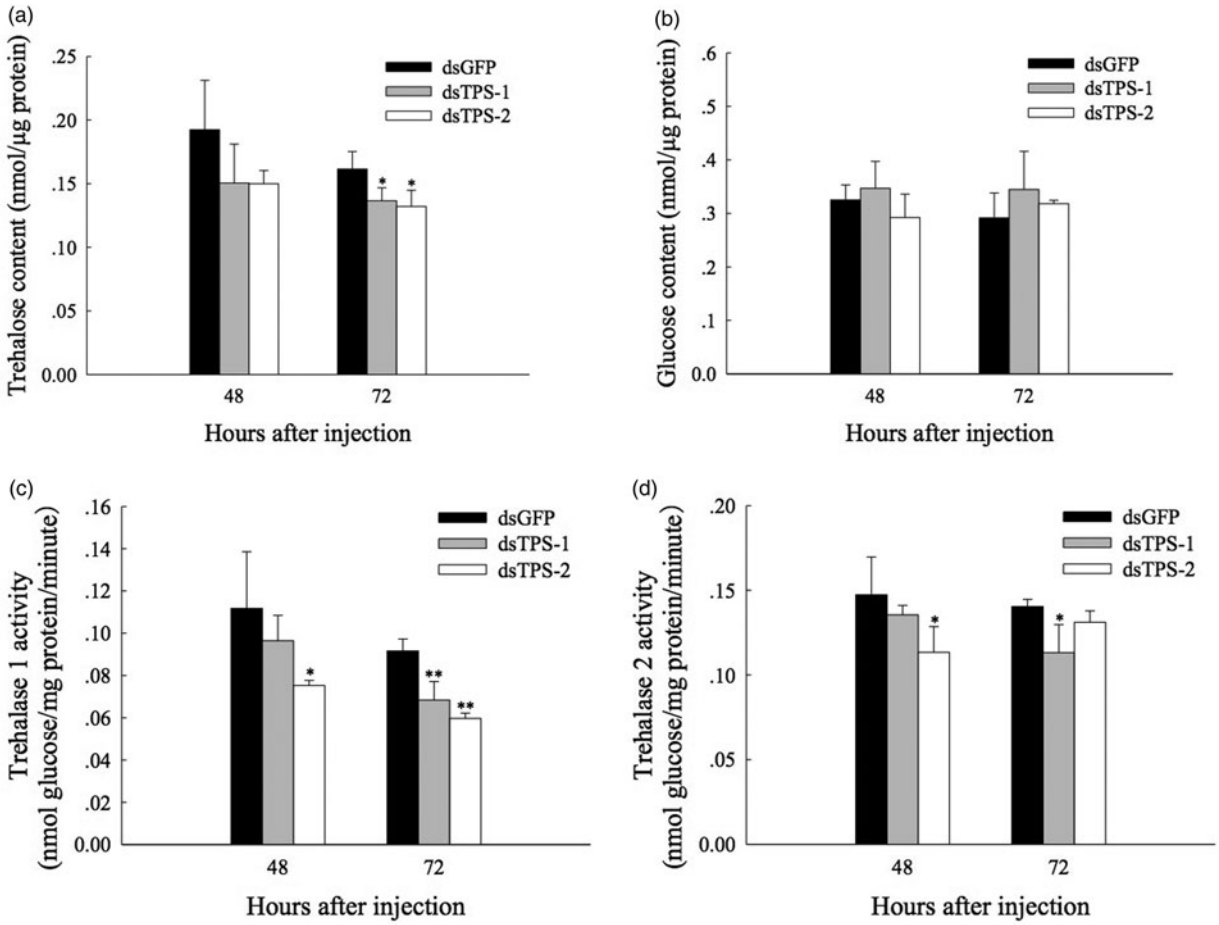

Fig. 4. Trehalose and glucose levels and trehalase 1 (soluble trehalase) and trehalase 2 (membrane-bound trehalase) activities were detected at 48 and 72 h after dsGFP, dsTPS-1, and dsTPS-2 dsRNA injection. Approximately 30–50 larvae were pooled as one group for injection as a replicate. (a–d) indicate trehalose levels, glucose levels, trehalase 1 activity, and trehalase 2 activity, respectively. All *Tribolium castaneum* larvae were divided into six groups and injected with dsGFP, dsTPS-1, and dsTPS-2. Insects were collected and used to detect trehalose and glucose levels, and trehalase isoenzyme activity after 48 h. Each group had three to five replicates.

trehalase 1 was 0.0916 in the dsGFP RNAi treatment group, and was significantly higher at 72 h than both post-dsTPS-1 RNA (0.0684) and dsTPS-2 RNA injection (0.0597) ($P < 0.01$) (fig. 4c). In addition, trehalase 2 activity was significantly decreased 48 h post-dsTPS-2 RNA injection and 72 h post-dsTPS-1 RNA injection (fig. 4d).

Effects of RNAi on phenotype, development, mortality rates, and chitin

The results showed that high mortality and presented various abnormal phenotypes were found in insect when *TPS* gene expression knockdown by RNAi (fig. 5a). These results indicate that a portion of *T. castaneum* larvae is not able to complete the molting process. In addition, chitin content was observed to decrease significantly, where the chitin content was 10% in the control (dsGFP RNAi) treatment, and was 8% and 7% in TPS-1 and TPS-2 dsRNA-injected groups at 48 h, respectively (fig. 5b). The abnormality and mortality rates were 26% and 38%, respectively, 72 h post-injection with dsTPS-1 and 31% and 28% at 72 h post-injection, respectively, post-injection with dsTPS-2 (fig. 5c, d). The abnormality and mortality rates of insects injected with dsGFP were 0% and 4%, respectively, at 72 h, and were significantly lower ($P < 0.05$) than those in the dsTPS-1 and dsTPS-2 treatment groups. Several abnormal molting/deformed phenotypes were observed post-dsTPS-1 and dsTPS-2 injection during development (from larvae to pupae).

Effects of TcTPS RNAi on the gene expression of genes involved in the chitin biosynthesis pathway

Real-time fluorescence qRT–PCR was used to measure the effect of RNAi targeting of *TPS-1* and *TPS-2* on the mRNA expression of genes related to chitin biosynthesis. The results showed that the expression of the six genes tested (*HK2*, *G6PI*, *GFAT*, *PFK*, and *CHS2*) were significantly or extremely significantly downregulated from 48 to 72 h ($P < 0.05$ or < 0.01, respectively) (fig. 6). The expression of *CHS1* significantly decreased at 48 h post-dsTPS-1 injection, and then significantly increased at 72 h, post-injection with dsTPS-2 in comparison to the dsGFP-injected group (fig. 6f).

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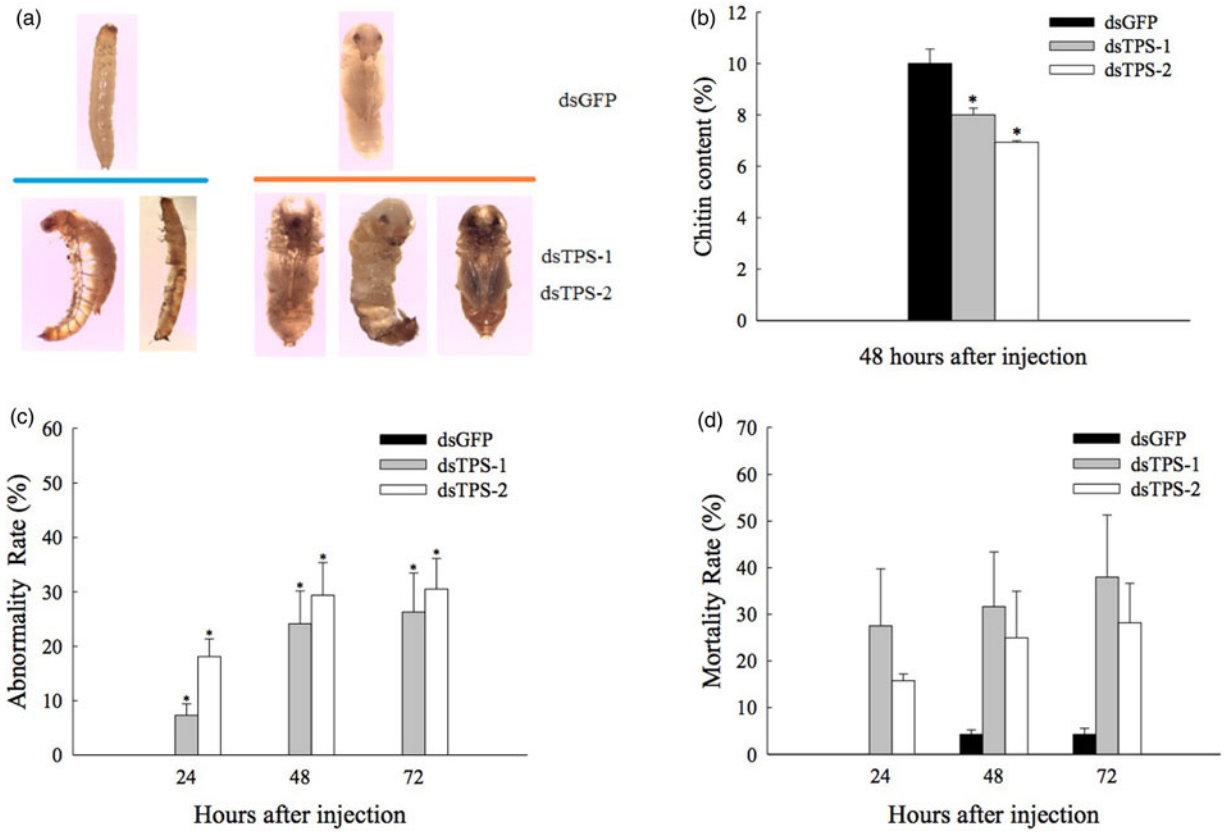

Fig. 5. Phenotypes, abnormality, mortality rates, and chitin content in response to injection with dsTPS-1 and dsTPS-2 RNA fragments in *Tribolium castaneum*. Approximately 30–50 larvae were pooled as one group for injection as a replicate. (a) Abnormal phenotypes (larvae–pupae). (b) Chitin content changes at 48 h. (c) Abnormality rates. (d) Mortality rates 24–72 h after dsGFP, dsTPS-1, and dsTPS-2 RNA injection. Each point represents the mean ± standard error from dsGFP group and dsTcTPS-injected groups. Asterisks (*) indicate significant differences between dsGFP injection group and dsTPS-1 or dsTPS1-2 injection group (Tukey's method, $*P < 0.05$, $**P < 0.01$). GFP, green fluorescent protein; TPS, trehalose-6-phosphate synthase.

Discussion

RNAi is an effective and important tool used to study gene function, as well as used to systematically downregulate genes of interest and analyze theirs in a biological process (Yilmazel *et al*., 2014). However, it is critical that the siRNA specifically targets the expression of the gene of interest but has no off-target effects (OTEs) on other genes (Seinen *et al*., 2011), and some reports have shown that off-targeting can widely occur during RNAi (Nunes *et al*., 2013), especially in *Drosophila* and mammalian cells (Kulkarni *et al*., 2006; Ma *et al*., 2006; Moffat *et al*., 2007; Seinen *et al*., 2010; 2011). In the previous reports, one method was described which enables the identification of potential off-targets at a genome-wide level wobble mismatches (Seinen *et al*., 2010). The dsRNAs predictions about OTEs based on sequence similarity: there is a high prevalence of predicted off-targets when this method was used to investigate the occurrence of six genes targets of dsRNA sequences (Seinen *et al*., 2011). Therefore, it is important to ensure that the dsRNA length and corresponding siRNA sequence do not exert OTEs (Moffat *et al*., 2007; Koch & Kogel, 2014), and it can minimize off-targeting effects by use of highly specific sequences in siRNA and controlling their expression through specific and inducible promoters (Kola *et al*., 2015). In our study, only one TPS gene was found in *T. castaneum*, and TPS-1 and TPS-2 dsRNA sequences have been blast with the genomic sequence in order to reduce OTEs (fig. 1). Importantly, it is known that human and other mammal species did not synthesize trehalose because it lacks *TPS* and *TPP* genes, which can be used as a potential pest control target genes.

To date, multiple *TPS* genes have been cloned in *N. lugens* and *B. germanica* (Chen & Zhang, 2015; Yang *et al*., 2017), while instances of singular *TPP* genes can also be found in the NCBI database. In addition, similarly to insects, four *TPS* isoforms, varying at the 5′ end, have been cloned in the blue crab (*Callinectes sapidus* Rathbun); however, an analysis of their structures revealed that *TPS-mus1* contains TPS and TPP conserved structures, and *TPS-mus-1b* and *TPS-mus-1c* contain only one TPS structure (Shi & Chung, 2014). In our study, in the four tissues studied here (epidermis, midgut, fat body, and Malpighian tubule), *TcTPS* was found to be expressed very higher in the fat body (fig. 2), similar to *B. minax TPS*, which exhibited the highest levels of expression in the fat body (Xiong *et al*., 2016). However, in *S. exigua*, *TPS* was shown to be expressed not only in the fat body and ovary (Tang *et al*., 2010), but also in the midgut, hemolymph, and leg muscles (Cui & Xia, 2009; Chen *et al*., 2010*b*).

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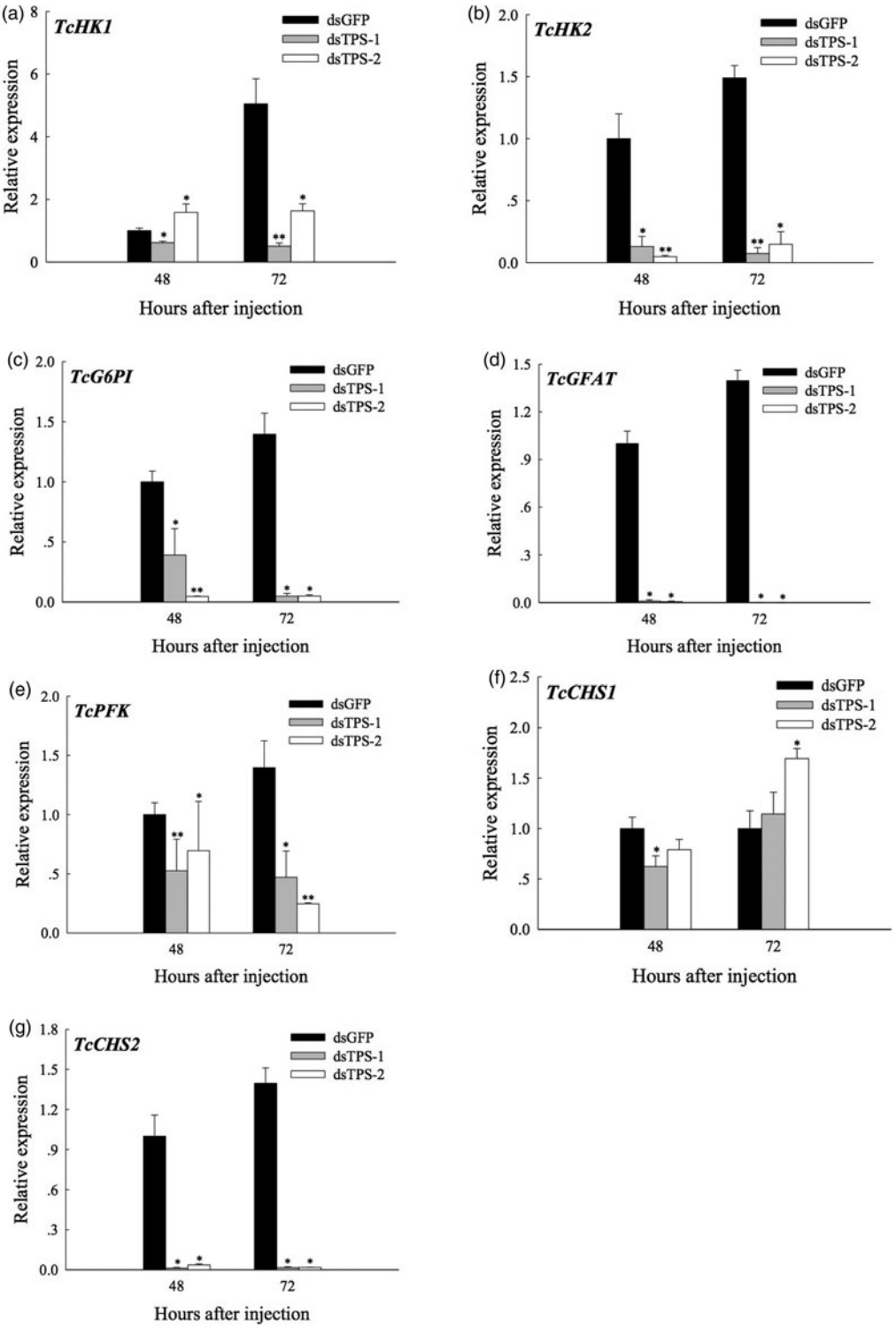

Fig. 6. Effects of dsTPS-1 and dsTPS-2 RNAi on the expression of chitin synthesis-related genes in last-instar larvae of *Tribolium castaneum*. Approximately 30–50 larvae were pooled as one group for injection as a replicate. *TcHK1 and TcHK2*: hexokinases, *TcG6PI*: glucose phosphate isomerase, *TcGFAT*: glutamine-fructose-6-phosphate aminotransferase, *TcCHS1* and *TcCHS2*: chitin synthase, *TcPFK*: phosphofructokinase. The mRNA expression levels were measured via qRT–PCR. Each point represents the mean ± standard error from the dsGFP group and dsTcTPS-injected groups. Asterisks (*) indicate significant differences between dsGFP injection group and dsTPS-1 or dsTPS1-2 injection group (Tukey's method, $*P < 0.05$, $**P < 0.01$). TPS, trehalose-6-phosphate synthase.

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In the present study, insect can't complete the molting process because of the inhibition of *TPS* genes by RNAi (fig. 5a), this result is in agreement with *N. lugens* and *S. exigua TPS* genes' study (Tang *et al*., 2010; Yang *et al*., 2017), but the mortality rate is different. It has been reported that the inhibition of *TPS* expression in *N. lugens* resulted in mortality rates of up to 60% in dsTPS1 RNA groups (2.4 times higher than dsGFP controls), and 25% when subjects were fed the corresponding dsRNA for 10 days (Chen *et al*., 2010*b*). The inhibition of *TPS* expression via dsTPS injection resulted in mortality rates of over 65% in *S. exigua*, while the mortality of dsGFP-injected individuals was only 10% (Tang *et al*., 2010). The mortality rates in the present study reached 37.98% and 28.22% when *T. castaneum* was injected with dsTPS-1 and dsTPS-2 RNA fragments, respectively (fig. 5d). In recent studies, mortality rates of up to 28 and 55% were observed in *B. minax* and *L. decemlineata*, respectively, when *TPS* gene expression was knocked down by RNAi (Shi *et al*., 2016; Xiong *et al*., 2016). The enzyme trehalase catalyzes the degradation pathway of trehalose, and observations regarding the function of decrease in TRE via dsTPS-1 or dsTPS-2 RNAi show that mortality rates are similar or even higher than those observed in insect studies where *TRE* expression is inhibited by RNAi in *S. exigua* (Chen *et al*., 2010*a*) and in the small brown planthopper *Laodelphax striatellus* Fallen (Zhang *et al*., 2012).

In addition, similar abnormal phenotypes, such as molting deformity, have been observed as a result of the knockdown of *TRE* and *TPS* genes (fig. 5a; Tang *et al*., 2016; Zhao *et al*., 2016; Yang *et al*., 2017). This indicates that TPS and TRE play a key role, not only in trehalose synthesis, but also in chitin synthesis. Our results show that the content of trehalose decreased significantly at 72 h, while glucose increased slightly at 72 h post-injection with dsTPS-1 and dsTPS-2 RNA (fig. 4a, b). These findings are similar to those observed in *B. minax*, where the knockdown of the *TPS* gene resulted in a significant decrease in trehalose and a significant increase in glucose (Xiong *et al*., 2016). Similarly, the trehalose content in the hemolymph, fat body, epidermis, and brain/corpora cardiaca/corpora allata complex of *L. decemlineata* were significantly decreased when the *TPS* gene was inhibited by way of RNAi (Shi *et al*., 2016). However, in *N. lugens*, trehalose content increased in response to dsNlTPS1 and dsNlTPS2 knockdown, and the activities of both the soluble and membrane-bound TREs decreased, where TRE2 activity was extremely significantly decreased at 48 h (Yang *et al*., 2017). In our study, the activities of TRE1 and TRE2 also decreased (fig. 4c, d), where TRE1 decreased significantly 72 h post-dsTPS-1 and dsTPS-2 RNA injection. At the same time, the trehalase gene expressions were regulated and two trehalase activities changed after *TcTPS* RNAi. These results indicated that TPS effectively regulates chitin biosynthesis or degradation by the way of TRE. This hypothesis has been confirmed via knockdown of the *TPS* gene by RNAi in *N. lugens*, where a portion of genes involved in the chitin biosynthesis pathway and nearly all of the chitinase genes were observed to have decreased expression (Yang *et al*., 2017).

TPS has been shown to play an important regulatory role in insect growth and development, especially in the chitin synthesis pathway (Tang *et al*., 2010; Chen *et al*., 2010*b*). Inhibition of the expression of the four *TRE* isoforms by injection with dsTPS-1 or dsTPS-2 RNA (with one exception) (fig. 3) indicates that knockdown of *TPS* results in altered expression of *TRE1* and *TRE2*. In our previous study, we demonstrated that chitin content decreased significantly at 48 h when a trehalase inhibitor validamycin had been injected into *N. lugens* (Tang *et al*., 2017). Likewise, the chitin content also decreased significantly at 48 h in response to dsTPS-1 and dsTPS-2 RNA injection in to *T. castaneum* larvae in the present study (fig. 5b). Similarly, chitin content decreased significantly at day 8 when the *TPS* gene was knocked down by RNAi in *L. decemlineata* (Shi *et al*., 2016).

It is well known that the chitin biosynthesis pathway is a well-established pathway to target for pest management and control. The discovery and characterization of increasing numbers of important regulatory genes in the chitin biosynthesis pathway will promote the further development of pest management science. Our study shows significant decreases in the expression of chitin content and genes related to chitin synthesis (figs. 5b and 6), although the mRNA expression of *CHS1* was not suppressed in response to *TPS* knockdown (fig. 6f). In the present study, while we observed phenotypes expressing molting deformities in response to *TPS* inhibition, similar phenotypes were observed in response to the inhibition of genes related to chitin synthesis (Arakane *et al*., 2005, 2008). Therefore, imbalances in chitin metabolism could result in the molting difficulties observed in *T. castaneum* in the present study, as the expression of chitinase was not measured. Above all, *TPS* is a suitable potential pest control gene because it has not been shown to exist in humans and other mammals (Tang *et al*., 2014*a*).

In conclusion, we provide insights regarding the role of *TPS* in regulating the chitin biosynthesis pathway, and help to elucidate the molecular mechanisms underlying the gene regulation of chitin synthesis by *TPS* during the molting process. In future studies, we aim to screen the genes related to the trehalose synthesis pathway via TPS/TPP and provide evidence for their role in the regulation of trehalose synthesis genes in chitin metabolism. In the meantime, the application of inhibitors to prevent trehalose synthesis points toward a promising future in formulating strategies for insect pest control.

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