An Epsilon Class Glutathione S-Transferase Gene Contributes to the Phytochemical Susceptibility of *Tribolium castaneum* (Coleoptera: Tenebrionidae)[1]

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Abstract Evidence is accumulating that insect epsilon class glutathione S-transferases (GSTs) play an important role in the resistance to xenobiotics such as insecticides. A *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae) epsilon class GST gene, *TcGSTe17*, was previously found to be significantly induced following exposure to the plant derivative eucalyptol. However, whether *TcGSTe17* is involved in resistance to eucalyptol remains unclear. In this study, *TcGSTe17* was cloned from the *T. castaneum*. Development stage expression profiling revealed that *TcGSTe17* was expressed at all development stages of *T. castaneum* but was highly expressed in late-stage larvae. Subsequent expression profiling of tissues showed that *TcGSTe17* was highly expressed in the gut of larvae and the fat body of adults, and also revealed gender-specific expression patterns. In addition, the transcripts of *TcGSTe17* were significantly increased following exposure to eucalyptol, and RNAi increased the susceptibility of the beetles to eucalyptol. Collectively, these results suggest that *TcGSTe17* contributes to the susceptibility of *T. castaneum* to eucalyptol. These findings provide new data for the prevention of pest resistance.

Key Words epsilon class glutathione S-transferases, *Tribolium castaneum*, RNAi, phytochemical susceptibility

Glutathione-S-transferases (GSTs; EC 2.5.1.18) are a kind of superfamily of enzymes that are widely distributed in animals, plants, and microorganisms (Sheehan et al. 2001). GSTs are related to many physiological processes of organisms, including synthesis of endogenous hormones, metabolism of exogenous substances, and anti-oxidative stress (Enayati et al. 2005). In the metabolism of exogenous substances, GSTs are a class of phase II detoxification enzymes that can metabolize secondary products from phase I (Pavlidi et al. 2018). According to their subcellular location, GSTs can be classified into mitochondrial, microsomal, and cytosolic GSTs, of which only two classes (microsomal and cytosolic GSTs) have been reported in insects (Ketterman et al. 2011). Cytosolic GSTs of insects can be further classified into six

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groups: delta, epsilon, sigma, omega, zeta, and theta, of which only delta and epsilon are unique to insects (Ranson et al. 2002).

The epsilon class of GSTs, which is one of the largest subfamilies of insect GSTs, are mainly implicated in the resistance to xenobiotics such as insecticides (Hu et al. 2022). With the development and accessibility of sequencing, numerous GST genes, including those from the epsilon class, have been identified and characterized from insect genomes (Han et al. 2016, Hu et al. 2022, You et al. 2015), thereby facilitating the study of insect GSTs. For instance, evidence is accumulating that the epsilon class GSTs are associated with insecticide resistance in insects including *Aedes aegypti* (L.) (Lumjuan et al. 2005, 2011; Morou et al. 2010), *Bombyx mori* (Yamamoto et al. 2013, Zhou et al. 2015), *Anopheles gambiae* Giles (Ding et al. 2005, Muleya et al. 2008, Wang et al. 2008), *Spodoptera litura* F. (Deng et al. 2009, Hirowatari et al. 2018, Li et al. 2021, Xu et al. 2015), *Bactrocera dorsalis* (Hendel) (Lu et al. 2016, 2020), *Locusta migratoria* (L.) (Zhang et al. 2022), *Cydia pomonella* (L.) (Hu et al. 2020a), *Musca domestica* L. (Nakamura et al. 2013), and *Leptinotarsa decemlineata* (Say) (Han et al. 2016). Additionally, in the above insects, all epsilon class GSTs could be induced or overexpressed after exposure to insecticides, and reduction of the relevant epsilon class GSTs led to the enhanced susceptibility of insects to insecticides. In contrast, there is less research on insect-specific epsilon class GSTs involved in resistance to plant derivatives.

Tribolium castaneum (Herbst) (Coleoptera: Tenebrionidae) is a worldwide grain storage pest, causing billions of dollars of damage annually. It has been reported that *T. castaneum* has developed resistance to 33 active ingredients, ranking in the top 20 arthropod resistance lists (Kalsi and Palli 2017). Therefore, it is vital to find plant-derived natural products with insecticidal activity. Eucalyptol is an effective component of secondary metabolites in many plants, including *Senecio cannabifolius* Less. (Wu et al. 2006, Yang et al. 2021), *Cinnamum cassia* Ness ex Blume, and *Laurus nobilis* L. (Wang et al. 2019). In addition, various studies have reported that eucalyptol has insecticidal and repellent effects on insects including *A. aegypti*, *M. domestica*, and *Chrysomya megacephala* (F.) (Klocke et al. 1987, Sukontason et al. 2004). In our previous study, eucalyptol could cause a time-dependent increase in *T. castaneum* larval mortality, and investigation of the molecular mechanism of action of eucalyptol on *T. castaneum* indicated that an epsilon class GST gene (*TcGSTe17*) was significantly upregulated after exposure to eucalyptol (Gao et al. 2023). However, whether *TcGSTe17* is involved in susceptibility to eucalyptol has yet to be elucidated. Therefore, the purpose of this study was to further investigate the causal role of *TcGSTe17* in susceptibility to eucalyptol. Findings from the study will provide new data for the generation and prevention of insect resistance.

Materials and Methods

Insect rearing. The Georgia-1 (GA-1) strain of *T. castaneum* used in this study was maintained at Nanjing Normal University for 13 years and originated from Kansas State University (Manhattan, KS). The insects were raised in jars containing wheat flour and Brewer's yeast (19:1) maintained at 30°C and a relative humidity (RH) of 40% with a 14:10 h light:dark cycle (Gao et al. 2020, Xiong et al. 2019a).

Gene cloning and bioinformatics analysis. The open reading frame (ORF) cDNA sequence of *TcGSTe17* was augmented by RT-PCR (primers listed in Table 1).

Table 1. Primers used for this research.

Gene	Sequence (5'-3')	Primer length (bp)	Utility
TcGSTe17-FF	ATGCGCCCCAAGTTGTAC	18	Clone
TcGSTe17-FR	TTAACACCGGTTTAGAATCC	20	
RPS3-F	TCAAATTGATCGGAGGTTTG	20	qRT-PCR
RPS3-R	GTCCCACGGCAACATAATCT	20	
TcGSTe17-F	GAAGGAGGTTTCTTGCGACA	20	qRT-PCR
TcGSTe17-R	CTTCGTATGGTATCGAGTTCAGA	23	
dsVER-F	TAATACGACTCACTATAGGG GTCTTGGTGGACCAAG	35	RNAi
dsVER-R	TAATACGACTCACTATAGGG CCGCCATTTCGTGATC	34	
dsGSTe17-F	TAATACGACTCACTATAGGG ACAATTCTCCATCCCTACGCTG	42	RNAi
dsGSTe17-R	TAATACGACTCACTATAGGG CAAACCGCAATCGTTCGTCTAT	42	

The underline of dsRNA primers is T7 promoter sequence.

The recovery-purification of the PCR product was done by using a FastPure Gel DNA Extraction Mini Kit (Vazyme, Nanjing, China), and the purified product was inserted into the PET28a (+) vector (Novagen, Darmstadt, Germany). The recombinant plasmid was transformed into *Escherichia coli* Trans1T1 (TransGene, Beijing, China) and sequenced by Traditional Sanger sequencing (General Biol, Nanjing, China). In addition, the sequence of *TcGSTe17* (accession no. TC003347) was downloaded from the iBeetle-Base (https://ibeetle-base.uni-goettingen.de/). Prediction in the ORF sequence, deduced proteins, molecular weight, and pI were performed using the ExPASy online server (https://www.expasy.org). Signalp-5.0 (http://www.cbs.dtu.dk/services/SignalP/) was used for predicting the location of a signal peptide.

Sample collection, RNA extraction, and cDNA synthesis. The different stages and tissues of *T. castaneum* were sampled as previously described (Xie et al. 2020). Briefly, approximately 50 mg of 1-d-old and 3-d-old embryos were sampled, whereas, three individuals were sampled for each of 1-d-old larvae, 18-d-old larvae, 1-d-old pupae, 5-d-old pupae, 1-d-old adults, and 10-d-old adults. The gut, fat body, integument, and hemolymph of the larvae were sampled from approximately 100 18-d-old larvae, and the gut, integument, ovary, fat body, testis, and head were sampled from approximately 100 10-d-old adults. Three biological replicates were conducted for each sample. The total RNA of each sample was extracted according to the manufacturer's instructions of the RNA Extraction Reagent kit (Vazyme). Subsequently, 1 μg of purified total RNA was used for cDNA synthesis according to the instructions of HiScript® III RT SuperMix (Vazyme).

Detection of gene expression. The expression of each gene (Table 1) was determined by quantitative real-time polymerase chain reaction (qRT-PCR). According to the instructions of ChamQ SYBR qPCR Master Mix (Vazyme), qRT-PCR was conducted on an ABI Q6 instrument (Applied Biosystems, Foster City, CA) using the following conditions: 95°C for 10 min; 40 cycles of 95°C for 15 s, and 60°C for 60 s; followed by 95°C for 15 s, 60°C for 60 s, and 95°C for 15 s. The qRT-PCR system included 3.5 μl of RNase-free water, 0.25 μl of each primer, 5 μl of ChamQ SYBR qPCR Master Mix, and 1 μl of cDNA. The $2^{-\Delta\Delta CT}$ method was used to calculate gene expression from the data (Begum et al. 2009, Livak and Schmittgen 2001).

Dip bioassays of eucalyptol. Eucalyptol (99%, CAS: 470-82-6) was obtained from the Aladdin Company (Shanghai, China). The median lethal concentration (LC_{50}) (43.294 mg/ml) of eucalyptol to *T. castaneum* was prepared by dissolving eucalyptol in acetone (Gao et al. 2023). Subsequently, dip bioassays of eucalyptol were used to treat beetles (Lu et al. 2012). Briefly, 60 12-d-old larvae were divided into two groups, and one group was treated with 100 μL of LC_{50} eucalyptol for 1 min, and the other group was treated with the same volume of acetone for 1 min. Each treatment was performed in three independent biological replicates. Subsequently, the treated insect life stages were dried on filter paper for 2 min and transferred to glass vials containing wheat flour. Mortality was measured at 12, 24, 36, 48, 60, and 72 h after exposure. The larvae were considered dead if they do not respond when touched with a brush. Meanwhile, the surviving larvae were sampled at 12, 24, 36, 48, 60, and 72 h after exposure. The total RNA of each sample was extracted and reverse transcribed into cDNA for qRT-PCR.

dsRNA synthesis, injection, and eucalyptol bioassay after RNAi. According to the instructions of the TranscriptAid T7 High Yield Transcription Kit (Fermentas, Vilnius, Lithuania), the dsRNA of genes (Table 1) was synthesized and 200 nl was then injected into the body of each larva by microinjector. Simultaneously, each larva was injected with an equal volume of IB (Injection Buffer) or dsVer (*T. castaneum* vermillion, accession no. AY052390) as a control. The interference efficiency of the dsRNA was detected on the third day. For the eucalyptol bioassay following RNAi of *TcGSTe17*, the dsRNA-treated larvae of *T. castaneum* were treated with eucalyptol by drip method (Zhang et al. 2022). Briefly, 90 12-d-old larvae were divided into three groups, and these groups were injected with IB, dsVer, or dsGSTe17, respectively. Three biological replicates were administered for each injection treatment. The surviving larvae were treated with eucalyptol on the third day after injection, and mortality was recorded at 72 h after exposure.

Results and Discussion

cDNA cloning, sequence analysis, and molecular docking analysis. The cDNA of *TcGSTe17* was further cloned and analyzed to characterize *TcGSTe17* (accession no. TC003347). Sequence analysis revealed that full-length *TcGSTe17* was 1263 bp, which contained an ORF of 597 nucleotides encoding 198 amino acids (Fig. 1). The predicted molecular mass and isoelectric point of *TcGSTe17* were 22.5 kDa and 6.61, respectively. In most organisms, the molecular mass of cytosolic GSTs ranges from 21 to 29 kDa (Blanchette et al. 2007, Mannervik et al. 1988), indicating that *TcGSTe17* is in accordance with the characteristic of GSTs. In addition, the predicted *TcGSTe17* protein contained GST_N_3 and GST_C, and no signal peptide

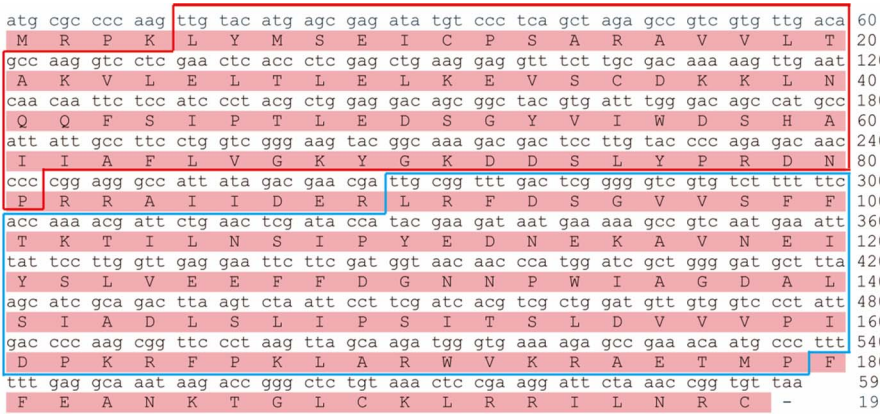

Fig. 1. Nucleotide and deduced amino acid sequences of *TcGSTe17* from *T. castaneum*. The red frame represents the N-terminus of *TcGSTe17*, and the blue frame represents the C-terminus.

was predicted at the N-terminus of *TcGSTe17* (Fig. 1). This finding may be related to the fact that *TcGSTe17* is a cytosolic GST (Hu et al. 2022).

Expression profile of *TcGSTe17*. The spatio-temporal expression of *TcGSTe17* was detected to further analyze the function of *TcGSTe17*. Development stage expression profiling indicated that the transcripts of *TcGSTe17* were expressed at all development stages of *T. castaneum* and were highly expressed in late-stage larvae (Fig. 2), which may be related to the resistance to xenobiotics during insect development. The period when insects consume most is the larval stage, and some studies have shown that continued development and increased food intake of larvae determine the increase of GST expression (You et al. 2015). This could explain why TcGSTe17 was highly expressed in the larvae stage. Similar results have been reported in several epsilon class GST genes from other insects, including *C. pomonella* (Hu et al. 2020a, 2022), *Plutella xylostella* (L.) (You et al. 2015), *Bombyx mori* L. (Yu et al. 2008), *Nilaparvata lugens* (Stal) (Sun et al. 2013), *Chironomus tentans* F. (Li et al. 2009), and *Pieris rapae* (L.) (Liu et al. 2017).

The expression profiling of *T. castaneum* tissues indicated that *TcGSTe17* was highly expressed in the gut of larvae (Fig. 3a) and the fat body of adults (Fig. 3b). The gut and fat body are important metabolic tissues of insects and are usually associated with the detoxification of xenobiotics (Hu et al. 2020a, b). The high expression of *TcGSTe17* in these tissues implies that this gene also might be associated with the detoxification of xenobiotics. *SoGSTe12*, involved in the tolerance of phosphine, is highly expressed in the gut of *Sitophilus oryzae* (L.) (Hu et al. 2018), which is consistent with our results. In addition, *TcGSTe17* is highly expressed in the gonads including ovary and testis (Fig. 3b). Similarly, some epsilon class GST genes showed gender-specific expression patterns in *C. pomonella* (Hu et al. 2020a, 2022) and *B. dorsalis* (Lu et al. 2016), indicating that this is a common phenomenon, and the detoxification capacity between males and females probably differs.

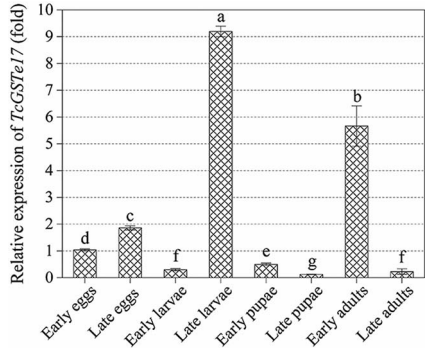

Fig. 2. The expression of *TcGSTe17* in different developmental stages of *T. castaneum*. The gene *rps3* was used as a housekeeping gene for the normalization of data. The error bars indicate the standard errors for three independent biological replicates. One-way analysis of variance (ANOVA) in combination with a least significant difference (LSD) test was performed for the significance analysis of all developmental stages. Different letters at the top of the bars indicate significant differences under $P < 0.05$.

The effects of eucalyptol on the expression profile of *TcGSTe17*. qRT-PCR was performed to investigate the effect of eucalyptol on the transcripts of *TcGSTe17*. In comparison to the control, the transcripts of *TcGSTe17* were significantly upregulated following exposure to eucalyptol (Fig. 4), suggesting that *TcGSTe17* could be

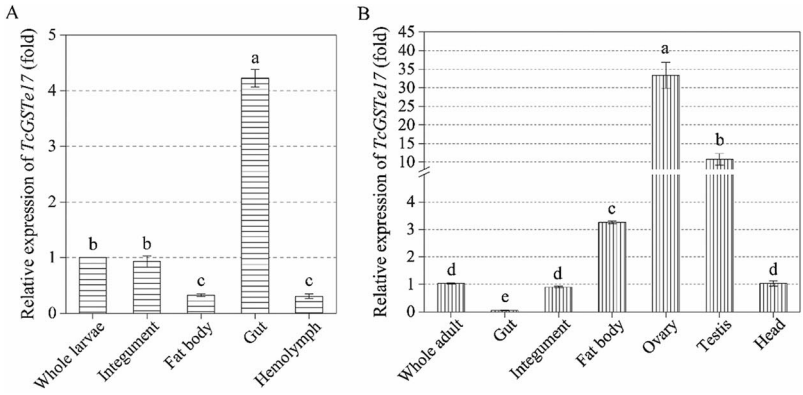

Fig. 3. The expression of *TcGSTe17* in different tissues of *T. castaneum*. The gene *rps3* was used as a housekeeping gene for the normalization of data. The error bars indicate the standard errors for three independent biological replicates. One-way analysis of variance (ANOVA) in combination with a least significant difference (LSD) test was performed for the significance analysis of all tissues. Different letters at the top of the bars indicate significant differences under $P < 0.05$.

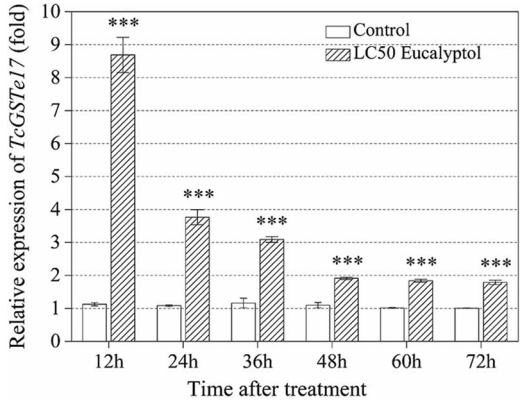

Fig. 4. The expression of *TcGSTe17* in *T. castaneum* after exposure to eucalyptol. The 12-day-old *T. castaneum* larvae were treated with acetone (control) or eucalyptol. Then, the expression of *TcGSTe17* was detected at 12, 24, 36, 48, 60, and 72 h by qRT-PCR. The gene *rps3* was used as a housekeeping gene for the normalization of data. The error bars indicate the standard errors for three independent biological replicates. Student's *t* tests were performed for the significance analysis. The asterisks at the top of bars indicate significant differences between control and treatment (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

significantly induced by eucalyptol. Insect GSTs are instrumental in the tolerance of xenobiotics such as insecticides and phytochemicals. For example, *LdGSTe2a* and *LdGSTe2b* were significantly overexpressed in *L. decemlineata* under the stress of three different insecticides (Han et al. 2016). Similarly, the mRNA levels of *PrGSTe1*, *PrGSTe2*, and *PrGSTe3* in *P. rapae* were upregulated following exposure to different insecticides (Liu et al. 2017). The mRNA levels of *TcGSTe17* were increased in the samples at 12 and 24 h after eucalyptol exposure and then decreased at 36, 48, 60, and 72 h (Fig. 4). This is a normal phenomenon of the stress response of insects. In general, when insects are attacked by xenobiotics, the expression level of detoxification enzyme genes will increase in response to the stress of foreign substances, but over time, these detoxification enzyme genes slowly return to normal levels of expression (Xiong et al. 2019b). This could explain the tendency of relative mRNA expression of *TcGSTe17* after eucalyptol exposure. Consistent with the trend in our results, the transcripts of *CpGSTe1* in *C. pomonella* first increased and then decreased with extended exposure time (Hu et al. 2022).

Functional analysis of *TcGSTe17* by RNAi. To further investigate whether *TcGSTe17* is involved in resistance to eucalyptol in *T. castaneum*, 12-d-old larvae were injected with dsGSTe17 and then exposed to eucalyptol. The interference efficiency of the dsRNA was detected on the third day. Compared with the control, the transcripts of *TcGSTe17* were significantly decreased (Fig. 5a). Subsequently, the surviving larvae were treated with eucalyptol to assess the tolerance of dsGSTe17-treated larvae to eucalyptol. In comparison to the control (IB- or dsVer-treated larvae), the mortality of dsGSTe17-treated larvae was markedly increased after exposure to

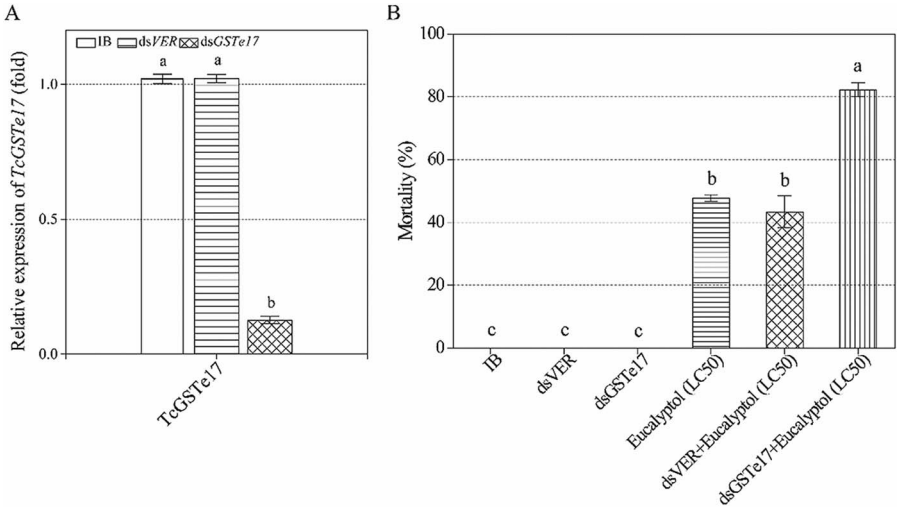

Fig. 5. The effect of RNAi treatment on the expression of *TcGSTe17* (a) and the susceptibility of *T. castaneum* larvae to eucalyptol (b). The gene *rps3* was employed as a housekeeping gene for the normalization of data. The error bars indicate the standard errors for three independent biological replicates. One-way analysis of variance (ANOVA) in combination with a least significant difference (LSD) test was performed for the significance analysis. Different letters at the top of the bars indicate significant differences under $P < 0.05$.

eucalyptol (Fig. 5b), indicating that *TcGSTe17* contributes to the susceptibility of *T. castaneum* to eucalyptol. Similarly, RNAi of epsilon class *SlGSTe12* in *S. litura* (Li et al. 2021), *BdGSTe2*, *BdGSTe3*, *BdGSTe4*, and *BdGSTe8* in *B. dorsalis* (Lu et al. 2016, 2020), *LmGSTe4* in *L. migratoria* (Zhang et al. 2022), and *AaGSTe2* and *AaGSTe7* in *A. aegypti* (Lumjuan et al. 2011) also significantly increased the mortality from insecticides, indicating that epsilon class GSTs are involved in insecticide susceptibility. As a plant-derived compound, eucalyptol is not an insecticide but has significant activity against insects (Klocke et al. 1987, Sukontason et al. 2004). Similar results were found in our previous study (Gao et al. 2023), which suggests that eucalyptol is a potential active ingredient for controlling pests. Our study also demonstrates that an epsilon class GST (*TcGSTe17*) is associated with phytochemical tolerance, which could further validate the contribution of epsilon class GSTs in phytochemical resistance and provide strong support in the development of new strategies to control pests.

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