



Molecular Entomology

Trehalose promotes the response of CYP genes in *Tribolium castaneum* (Coleoptera: Tenebrionidae) to high-CO₂ stress

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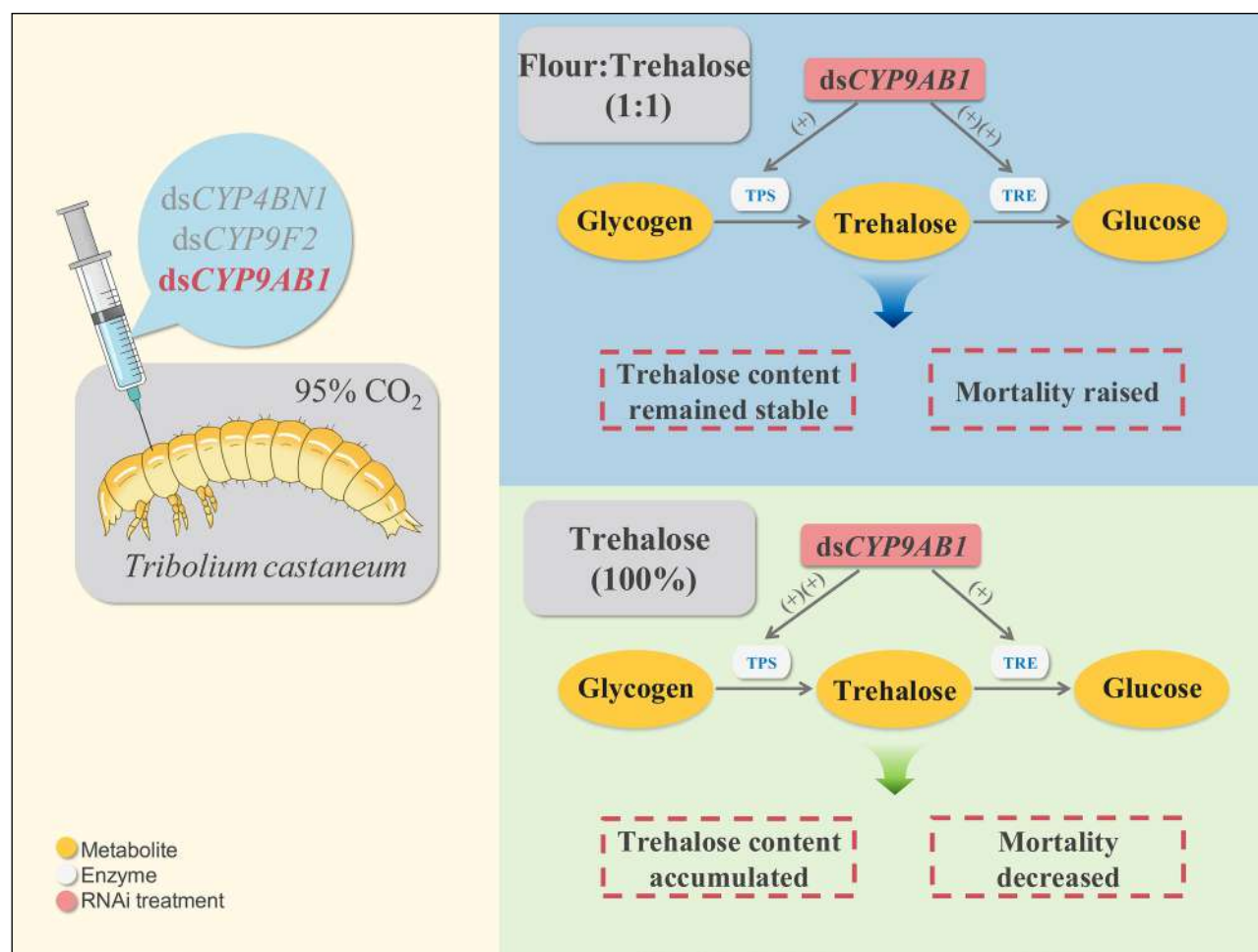
Cytochrome P450 monooxygenases (CYP450) and trehalose play a significant detoxification role under high CO₂ stress. Notably, CYP450 significantly affects trehalose metabolism of *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae), a devastating stored pest. To explore whether trehalose enhances CYP gene responses to CO₂ stress, investigations were conducted on the 95% CO₂ tolerance in 8th-instar *T. castaneum* larvae, whose specific CYP genes—*TcCYP4BN1*, *TcCYP9F2*, and *TcCYP9AB1*—were silenced, across different trehalose dietary regimes (50% flour + 50% trehalose or 100% trehalose). The 95% CO₂ tolerance response was systematically evaluated through multi-dimensional analysis of gene expression levels, carbohydrate contents, and enzyme activities. Results indicated that compared with the 50% flour + 50% trehalose feeding regimen, trehalose-only diet groups exhibited downregulation of trehalose metabolism-related genes, with the notable exception of the ds*CYP9F2* experimental group. As to carbohydrate contents, glucose content increased significantly on 100% trehalose diet by inhibiting the expression of *TcCYP9AB1*, but it decreased in the other 2 groups, a pattern that also held true for glycogen. Together, these results demonstrate that trehalose does enhance the response of CYP genes to CO₂ stress, and that *TcCYP9AB1* is more responsible for modulating trehalose metabolism. Future research could investigate the molecular mechanisms underlying these regulatory processes and their practical applications, potentially enhancing biocontrol techniques and advancing pest management solutions.

Keywords: trehalose, cytochrome P450 monooxygenase, CO₂ stress, stored pest, RNA interference

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Graphical abstract



Introduction

Food grain is an important material basis for human survival and an important strategic material for the national economy and livelihood of some (Cui et al. 2023). However, during the food storage stage, the activity of pests has given rise to great losses on grain storage (Ingegno and Tavella 2022). Among these destructive insects, *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae), a prevalent stored pest, causes substantial economic losses in diverse storage environments (Manandhar et al. 2018, Natonek-Wiśniewska et al. 2022). To control these pests, modified atmosphere storage technology, utilizing carbon dioxide or nitrogen to alter the gas composition, is gaining popularity as a safe and cost-effective solution relative to chemical fumigants (Paul et al. 2020). High N₂ is believed to reduce O₂ levels (Huang et al. 2024a), while CO₂ (10 to 20%) can be directly toxic or inhibitory to insects (Badre et al. 2005). In *Rhyzopertha dominica* (Fabricius) (Coleoptera: Bostrichidae), higher mortality rates have been observed with the rising CO₂ gas pressure (Sadeghi et al. 2021). Also, in *Plodia interpunctella* (Hübner) (Lepidoptera: Pyralidae), more significant decrease in survival rates has been noted after CO₂ was added to low O₂ (Huang et al. 2024b). Despite remarkable strides in modified atmosphere storage technology, the development of resistance to high CO₂ levels in pests has presented

considerable challenges to widespread adoption and practical application (Wu et al. 2022). Therefore, investigating the molecular mechanisms behind CO₂-enriched modified storage technology could further influence and improve the field of grain preservation.

Trehalose is known for its role as a biochemical protector in promoting organismal survival under adverse environmental stress (Watanabe 2006, Ribeiro et al. 2024). As a non-reducing disaccharide widely distributed in nature, trehalose can be synthesized by trehalose-6-phosphate synthase (TPS) and degraded by trehalase (TRE) (Kaur et al. 2024). TPS is broadly categorized into 2 subfamilies: class I and class II (Wang et al. 2019, Zou et al. 2024), while TRE is categorized into 2 forms based on its cellular distribution: the soluble form, Trehalase 1 (TRE1), and the membrane-bound form, Trehalase 2 (TRE2) (Tellis et al. 2023, Zhang et al. 2024). As early as 2003, trehalose has been reported to enhance hypoxia adaptability mainly by reducing protein aggregation in cells (Chen et al. 2003), and it has currently found to help insects cope with various abiotic and biotic stresses (Tao et al. 2023). Due to these properties, its protective effects under toxic stress are of great interest (Singh et al. 2011, Bao et al. 2021), and its metabolic pathways are being considered as targets for pest control (Tang et al. 2016, Pan et al. 2020, Wu et al. 2022).

In addition to trehalose-mediated protection, the cytochrome P450 monooxygenase (CYP450) gene families, among the largest in nature, also contribute to environmental stress tolerance across animals, plants, and microorganisms (Baldwin et al. 2021, Shelomi 2022, Chakraborty et al. 2023). In *Apis cerana cerana* (Fabricius) (Hymenoptera: Apidae) and *T. castaneum*, specific CYP genes are reportedly involved in pesticide resistance and detoxification (Zhang et al. 2021, Tan et al. 2023). Furthermore, CYP genes are also responsively expressed and participate in resistance mechanisms under physical stressors such as hypoxia and ultraviolet radiation (Sang et al. 2012, Zhu et al. 2016). These findings suggest that understanding the underlying mechanisms is crucial for discovery of novel control targets and strategies, with some reports highlighting the importance of CYP450 in *T. castaneum*'s response to extreme environments (Yao et al. 2019, Wang et al. 2020, Chen et al. 2022). However, the same function in insects lacks enough research (Pender and Horvitz 2018), signaling a need for more scientific investigations.

In the current study, experiments were conducted on the 95% CO₂ tolerance of 8th-instar *T. castaneum* larvae where we silenced *TcCYP4BN1* (GenBank: NM_001130521 (National Center for Biotechnology Information 2024a), NCBI Nucleotide Database), *TcCYP9F2* (GenBank: NM_001134234 (National Center for Biotechnology Information 2024b), NCBI Nucleotide Database), and *TcCYP9AB1* (GenBank: XM_967453 (National Center for Biotechnology Information 2024c), NCBI Nucleotide Database) genes, which may be correlated with the trehalose metabolism pathway, across different trehalose regimes (50% flour + 50% trehalose or 100% trehalose). We sought to investigate whether trehalose enhances the response of CYP genes to CO₂ atmosphere stress, offering new solutions to the problem of pest resistance in grain storage under controlled atmosphere conditions.

Materials and Methods

Insect Cultures

The insects *T. castaneum* were sustained under laboratory conditions with the whole wheat flour as the primary nutrient source. The rearing environment was precisely controlled, with

temperature regulated at 29 ± 1 °C, humidity maintained at 65 ± 5% RH, and a 0L : 24 D photoperiod implemented throughout the cultivation period.

RNA Extraction and cDNA Synthesis

Total RNA extraction from *T. castaneum* larval specimens was conducted using the Trizol reagent kit (Invitrogen, Carlsbad, California, United States). The extracted RNA quality verification involved dual assessment procedures: 1 µl RNA for purity/concentration determination using the NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, United States), and 2 µl RNA for structural integrity confirmation via 1% agarose gel electrophoresis (Kim et al. 2024). Any residual RNA was maintained at -80 °C for future analysis. The first-strand cDNA synthesis was achieved using the PrimeScript RT Reagent Kit With gDNA Eraser (TaKaRa, Kyoto, Japan) and stored at -20 °C (Tang et al. 2016).

Cloning of *TcCYP4BN1*, *TcCYP9F2*, and *TcCYP9AB1* Genes

Partial ORF sequences of *TcCYP4BN1*, *TcCYP9F2*, and *TcCYP9AB1* were amplified from the synthesized cDNA using the Ex Taq kit (TaKaRa, Kyoto, Japan) (Osanai et al. 2006). Specific primers for amplification (Table 1) were designed with Primer 6.0 software (Premier Biosoft International, Palo Alto, California, United States) (Long et al. 2024). After confirming these amplified target fragments by electrophoresis, the corresponding gel bands were excised and purified with the DNA Gel Extraction Kit (US Everbright, Jiangsu, China). Quality and concentration of the purified DNA were assessed using a NanoDrop 2000 spectrophotometer prior to -20 °C storage (Kim et al. 2024). To obtain the connecting solution, 3 µl of the purified DNA were mixed with 3.5 µl Solution I and 0.5 µl pMD18-T Vector Cloning Kit (Takara, Kyoto, Japan) in PCR tubes, followed by brief centrifugation and incubation at 16 °C for 30 min (Zhang et al. 2020). Plasmid transformation was performed using DH5α competent cells, after which distinct circular colonies from the Petri dish were selected, dissolved in 30 µl of sterile water, and directly utilized as templates for colony PCR to verify cloning

Table 1. Primer sequences used for dsRNA synthesis and qRT-PCR detection of genes associated with trehalose metabolism in *T. castaneum*

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')	Function of Primers
<i>dsCYP4BN1</i>	GCATCAACGAGAAGTCCACA	AGTCAGCAAACAGTCCCTAA	dsRNA synthesis
<i>dsCYP4BN1-T7</i>	T7-GCATCAACGAGAAGTCCACA	T7-AGTCAGCAAACAGTCCCTAA	
<i>dsCYP9AB1</i>	GTTCTATCTGATGAGCAAAGC	CGATGACGTACTCCTTCG	qRT-PCR detection
<i>dsCYP9AB1-T7</i>	T7-GTTCTATCTGATGAGCAAAGC	T7-CGATGACGTACTCCTTCG	
<i>dsCYP9F2</i>	CCTACAAATACTGGACCGA	CAGGAAGTACCCCAACAA	
<i>dsCYP9F2-T7</i>	T7-CCTACAAATACTGGACCGA	T7-CAGGAAGTACCCCAACAA	
<i>TcCYP4BN1</i>	GGCAGGCCTCTAACTCAAGA	CGTAACTGCGGGACTTTTGT	
<i>TcCYP9AB1</i>	GGGCGTCACGATACCAGATA	TCCTCCCAGATGAAGTGCTG	
<i>TcCYP9F2</i>	ACCGGCTACCAAGAATCCAA	GTGACCTTTCCGTTGCAGTT	
<i>TcTRE1-1</i>	AACGACTCGCAATGGCTGG	CGGAGGCGTAGTGGAATAGAG	
<i>TcTRE1-2</i>	GTGCCCCAATGGGTTTATCG	CAACCACAACACTTCCTTCG	
<i>TcTRE1-3</i>	CCTCTCATTTCGTACAAAGCG	AAGCGTTTGATTCTTTGCG	
<i>TcTRE1-4</i>	ACGGTGCCCCGCTACTACTA	GTGTAGGTGGTCCCGTTCTTG	
<i>TcTRE2</i>	CTCAGCCTGGCCCTTAGTTG	GGAGTCCTCGTAGATGCGTT	
<i>TcTPS1</i>	CGATTCTGACTACAACGGCTGC	GTGGTGTAGCATTGCCAGTGC	
<i>TcTPS2</i>	ACCTTGCCATCATCCCTCC	GCCCAACATTGCTTCACA	
<i>TcRPL13a</i>	ACCATATGACCGCAGGAAAC	GGTGAATGGAGCCACTTGTT	

T7: GGATCCTAATACGACTCACTATAGG (5'-3').

success. After PCR amplification, 1% agarose gel electrophoresis was used to detect the correctness of the target fragment of the colony amplified using PCR. Positive clones were cultured overnight in LB-Amp medium (37°C, 250rpm). Then, 500 µl of the clones were submitted to Zhejiang Sunya Biotech Co., Ltd (Zhejiang, China) for sequencing. Following the same method, the Green fluorescent protein gene (GFP) was cloned by using the pMD18-T plasmid with the GFP sequence as the template.

Synthesis and Microinjection of dsRNA

The primers (Table 1) designed using Primer 6.0 software with a T7 promoter were used to perform cross-PCR reaction on the correctly sequenced plasmids, after which the double-stranded RNA (dsRNA) of the target genes were synthesized via the T7 RiboMax Express RNAi System kit (Promega, Madison, Wisconsin, United States) (Xu et al. 2020, Long et al. 2024). The total RNA integrity and concentration were verified through agarose gel electrophoresis and spectrophotometry separately. The dsGFP was synthesized for the control group using the same method. Synthesized products were finally stored at -80°C.

Tribolium castaneum larvae at the eighth instar were immobilized on ice and microinjected using a Transferman 4r microinjection (Eppendorf, Hamburg, Germany) with 100 nL dsRNA (2,000 ng/µl) targeting *TcCYP4BN1*, *TcCYP9F2*, or *TcCYP9AB1* through the abdominal intersegmental membrane (second-third segments) (Guan et al. 2024). The same amount of dsGFP was injected into the control group. Each dsRNA injection group had about 800 larvae in total.

Experimental Setup

Following microinjection with dsCYP4BN1, dsCYP9F2, dsCYP9AB1, or dsGFP, larvae from each injection group were randomly divided into 2 equal subgroups (about 400 larvae per subgroup). These subgroups were then fed 2 distinct trehalose dietary regimens: 1 subgroup received 50% flour + 50% trehalose, and the other received 100% trehalose. This experimental setup created a total of 8 final treatment groups (4 dsRNA types × 2 diets). After 48 h, we evaluated mortality rates from each treatment group. Surviving larvae (minimum 260 per group) were collected and stored at -80°C for a subsequent analysis of gene expression levels, carbohydrate contents, and enzyme activities.

Mortality Analysis

Larvae that died within 3 h of microinjection were identified as mechanical injury death and excluded from subsequent mortality analysis. Mortality rates were evaluated after 48 h of microinjection in each group maintained under 95% CO₂ with either 50% flour + 50% trehalose or 100% trehalose diets. For each treatment group, a minimum of 370 larvae were used for mortality calculation, with 4 biological replicates per treatment group. Larvae were recorded death if they were immobile after being touched with a brush.

Quantitative Real-Time Polymerase Chain Reaction

Tribolium castaneum larvae that survived in the 48-h mortality assessment were collected from the 8 previously described treatment groups. Each treatment group combined 1 of 4 dsRNA microinjections (dsCYP4BN1, dsCYP9F2, dsCYP9AB1, or dsGFP) with 1 of 2 trehalose dietary regimens (50% flour +

50% trehalose or 100% trehalose) under a 95% CO₂ atmosphere. Three biological replicates (10 larvae each) were analyzed per group. For each biological replicate, 3 technical replicates were performed to ensure experimental reliability. Utilizing Primer 6.0 software, quantitative real-time polymerase chain reaction (qRT-PCR) primers (Table 1) were designed based on coding sequences of trehalose pathway genes in *T. castaneum*, with *Ribosomal Protein L13a* (*RPL13a*) serving as endogenous reference (Long et al. 2024). Gene expression quantification employed TB Green Premix Ex Taq II (Tli RNaseH Plus) (TaKaRa, Kyoto, Japan) on a Bio-Rad CFX96 system. qRT-PCR reaction system: 5 µl of an SYBR Green master mix, 0.4 µl (10 pmol) of forward/reverse primers, 1 µl of cDNA, 2.8 µl of RNase Free ddH₂O. qRT-PCR reaction procedure: pre-denaturation at 95°C for 30 s, denaturation at 95°C for 5 s, extension at 60°C for 20 s (39 cycles). The melt curve analysis was conducted in a range of 65 to 95°C. Relative expression was analyzed using 2^{-ΔΔCT} methodology (Livak and Schmittgen 2001).

Determination of Carbohydrate Contents and Trehalase Activity

Samples for determining carbohydrate contents and trehalase activity were collected from the larvae that survived in the 48-h mortality assessment in the 8 previously described treatment groups. Each treatment group combined 1 of 4 dsRNA microinjections (dsCYP4BN1, dsCYP9F2, dsCYP9AB1, or dsGFP) with 1 of 2 trehalose dietary regimens (50% flour + 50% trehalose or 100% trehalose) under a 95% CO₂ atmosphere. Four biological replicates (15 larvae each) were analyzed per group. Following homogenization in 1,000 µl PBS (pH 7.0), samples underwent 30-min sonication and primary centrifugation (1,000×g, 20 min, 4°C). The resultant supernatant was divided into 2 aliquots: 350 µl for simultaneous quantification of glycogen, trehalose, and total protein, while the remaining 350 µl was ultracentrifuged (20,800×g, 60 min). After ultracentrifugation, 300 µl of ultra-supernatant and resuspended pellet (300 µl PBS) were respectively analyzed for glucose concentration, trehalase activity, and protein content. Trehalase activity assays incorporated 60 µl sample (supernatant/pellet suspension), 75 µl 40 mM trehalose (Sigma-Aldrich, Saint Louis, Missouri, United States), and 165 µl PBS, incubated at 37°C for 60 min prior to heat inactivation (100°C, 5 min). Reaction termination with 260 µl 12 N H₂SO₄ preceded trehalase activity measurement via Glucose (Go) Assay Kit (Sigma-Aldrich, Saint Louis, Missouri, United States) with absorbance value measured at 540 nm using a microplate reader (Thermo Fisher Scientific, Waltham, Massachusetts, United States). Protein determinations utilized the BCA Protein Assay Kit (Beyotime, Shanghai, China), while trehalose measurements followed anthrone methodologies (Zhang et al. 2017, Yu et al. 2020, Ge et al. 2021).

Determination of CYP450 Activity

Cytochrome P450 monooxygenase activity was determined using the Insect Cytochrome P450 Enzyme-Linked Immunosorbent Assay (ELISA) Kit (COIBO BIO, Shanghai, China). Samples for determining CYP450 activity were collected from the larvae that survived in the 48-h mortality assessment in the 8 previously described treatment groups. Each treatment group combined 1 of 4 dsRNA microinjections (dsCYP4BN1, dsCYP9F2, dsCYP9AB1, or dsGFP) with 1 of 2 trehalose

dietary regimens (50% flour + 50% trehalose or 100% trehalose) under a 95% CO₂ atmosphere. For each group, 4 biological replicates (30 larvae each) were homogenized in 30 µl PBS and flash-frozen. Prior to analysis, samples were thawed (2 to 8 °C), mixed with 270 µl PBS, and centrifuged (2,500 rpm, 20 min) to collect the supernatant. Standard solutions (50 µl per well) and diluted test samples (40 µl diluent + 10 µl sample) were loaded onto the ELISA plate. After adding 100 µl enzyme conjugate (excluding blank wells), the plate was incubated at 37 °C for 60 min. Wells were washed 5 times with 20× diluted buffer (30 s interval per wash), followed by sequential addition of 50 µl substrates A and B for 15 min of dark incubation at 37 °C. Reactions were terminated with 50 µl stop solution (blue-to-yellow color transition), and absorbance was measured at 450 nm within 15 min using a microplate reader.

Statistical Analysis

All data were organized with Microsoft Excel (Microsoft Corporation, Redmond, Washington, United States) and analyzed with IBM SPSS Statistics 23.0 (IBM Corporation, Armonk, New York, United States). Results are presented as mean ± standard error (SE) and visualized in bar graphs with GraphPad Prism 9.0.0 (GraphPad Software, Boston, Massachusetts, United States). For gene expression data, student's independent samples *t*-test was used to compare differences between the dsCYP treatment group and the dsGFP control group in RNA interference efficiency and expression levels of trehalose metabolic pathway genes (ie *TRE1-1*, *TRE1-2*, *TRE1-3*, *TRE1-4*, *TRE2*, *TPS1*, and *TPS2*). For data of mortality, carbohydrate contents (ie glucose, trehalose, and glycogen), and enzyme activities (ie soluble trehalase, membrane-bound trehalase, and CYP450), Shapiro-Wilk test and Levene's test were used first to test normality and homoscedasticity respectively. Where data (ie mortality on 100% trehalose diet, carbohydrate contents, and enzyme activities) were normal and homoscedastic, one-way analysis of variance (ANOVA) was then used to determine the effect of CYP genes knockdown. When the one-way ANOVA results were significant ($P < 0.05$), means were separated by Tukey Honestly Significant Difference (HSD) post-hoc test at $\alpha = 0.05$. However, since mortality data on 50% flour + 50% trehalose diet were non-normal and heteroscedastic, Welch's ANOVA was used to test if mortality differed between groups: dsGFP, dsCYP4BN1, dsCYP9F2, and dsCYP9AB1 (Delacre et al. 2018). When the mortality differences were significant ($P < 0.05$), Games-Howell post-hoc test at $\alpha = 0.05$ was used to examine pairwise differences between means (Zhang et al. 2022, Agbangba et al. 2024). As to gene expression analysis (qRT-PCR), 3 biological replicates per treatment group were used, while 4 biological replicates per treatment group were applied for measurements of mortality rates, carbohydrate contents, and enzyme activities.

Results

Detection of Silencing Efficiency and Mortality after Feeding Trehalose under 95% CO₂

Tribolium castaneum larvae received dsCYP injections and were fed with diets of either 50% flour + 50% trehalose or 100% trehalose, to explore the effects of CYP450 and trehalose on their resistance to 95% CO₂ stress. Changes in expression of target genes were then monitored. The results indicated that trehalose

feeding still maintains the potency of RNA interference under 95% CO₂. Specifically, the expression of *TcCYP4BN1*, same as that of *TcCYP9AB1*, significantly decreased when trehalose was added (*TcCYP4BN1*: $t_{50\% \text{ flour} + 50\% \text{ trehalose}} = 8.249$, $df=4$, $P=0.001$; $t_{\text{trehalose}} = 6.334$, $df=4$, $P=0.003$; *TcCYP9AB1*: $t_{50\% \text{ flour} + 50\% \text{ trehalose}} = 6.978$, $df=4$, $P=0.002$; $t_{\text{trehalose}} = 4.123$, $df=4$, $P=0.015$; Fig. 1A and C). Also, expression levels of *TcCYP9F2* notably fell far below those in the control groups ($t_{50\% \text{ flour} + 50\% \text{ trehalose}} = 9.061$, $df=4$, $P < 0.001$; $t_{\text{trehalose}} = 25.148$, $df=4$, $P < 0.001$; Fig. 1B).

After 48 h, mortality of *T. castaneum* was observed and analyzed. Results indicated that under a 95% CO₂ atmosphere, mortality rates fluctuated depending on RNAi and trehalose diet. On a mixed diet of 50% flour + 50% trehalose, the mortality increased in all dsCYP treatment groups ($F=21.280$, $df=3$, 6.078, $P=0.001$). On 100% trehalose diet, dsCYP4BN1 and dsCYP9F2 slightly increased the mortality, while dsCYP9AB1 decreased mortality compared with the control ($F=8.272$, $df=3$, 12, $P=0.003$; Fig. 2).

Effect on Carbohydrate Contents under 95% CO₂ after Trehalose Feeding and dsRNA

Under 95% CO₂ conditions, carbohydrate contents were assessed in *T. castaneum* subjected to a combination of trehalose dietary regimes (50% flour + 50% trehalose or 100% trehalose) and dsCYP treatments after 48 h. The results indicated that carbohydrate contents changed little (Fig. 3). As to glucose content, it was generally reduced in the dsCYP groups on 50% flour + 50% trehalose diet ($F=3.922$, $df=3$, 12, $P=0.037$), and significantly increased in the dsCYP9AB1 group on 100% trehalose diet ($F=14.285$, $df=3$, 12, $P < 0.001$; Fig. 3A). Glycogen content increased in the dsCYP9AB1 group but decreased in the other 2 groups on 50% flour + 50% trehalose diet ($F=5.070$, $df=3$, 12, $P=0.017$). On 100% trehalose diet, compared with the dsGFP, both the dsCYP4BN1 and dsCYP9F2 treatment groups exhibited reduced glycogen content, while the dsCYP9AB1 demonstrated an increased glycogen content ($F=2.798$, $df=3$, 12, $P=0.085$; Fig. 3C). Trehalose content showed no significant differences among groups on a mixed diet ($F=0.490$, $df=3$, 12, $P=0.696$). However, on 100% trehalose diet, dsCYP groups had notably higher trehalose levels than the control ($F=5.016$, $df=3$, 12, $P=0.018$). Overall, the trehalose content from 50% flour + 50% trehalose diet was slightly higher than that from 100% trehalose diet in *T. castaneum* (Fig. 3B).

Effect on Trehalase Activity under 95% CO₂ after Trehalose Feeding and dsRNA

After 48 h of co-treatments with trehalose dietary regimes (50% flour + 50% trehalose or 100% trehalose) and dsCYP under 95% CO₂, the trehalase activity was quantified. Soluble trehalase activity slightly decreased in both the dsCYP4BN1 and dsCYP9AB1 treatment groups on 50% flour + 50% trehalose diet, or diverged on 100% trehalose diet compared with the controls, while dsCYP9F2 maintained equivalent activity on 50% flour + 50% trehalose diet but increased on 100% trehalose diet, though no significant changes were observed in either diet condition ($F_{50\% \text{ flour} + 50\% \text{ trehalose}} = 0.968$, $df=3$, 12, $P=0.440$; $F_{\text{trehalose}} = 2.406$, $df=3$, 12, $P=0.118$; Fig. 4A). Membrane-bound trehalase activity remained stable on 50% flour + 50% trehalose diet ($F=1.495$, $df=3$, 12, $P=0.266$) but dropped significantly by 22.4% with the dsCYP9AB1 treatment on 100% trehalose diet ($F=3.556$, $df=3$, 12, $P=0.048$; Fig. 4B).

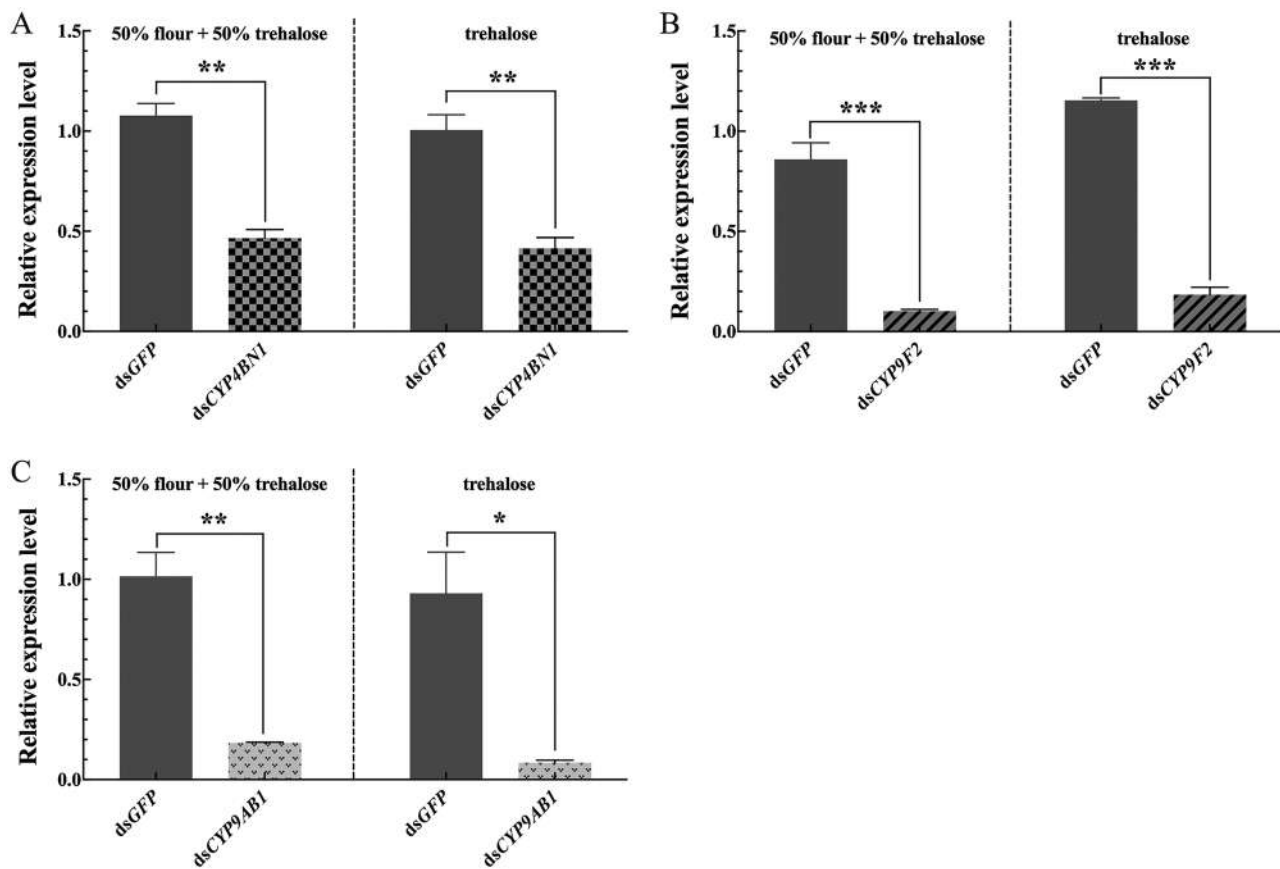


Fig. 1. The dsRNA interference efficiency in *T. castaneum* under 95% CO₂ stress after 48h of trehalose dietary regimes (50% flour + 50% trehalose or 100% trehalose) and dsCYP treatments. A) dsCYP4BN1 interference efficiency; B) dsCYP9F2 interference efficiency; C) dsCYP9AB1 interference efficiency. Each group included 3 biological replicates with 10 larvae each. Each biological replicate included 3 technical replicates. Values are presented as mean $\pm$ SE. *** P <0.001, ** P <0.01, * P <0.05 (independent samples t -test).

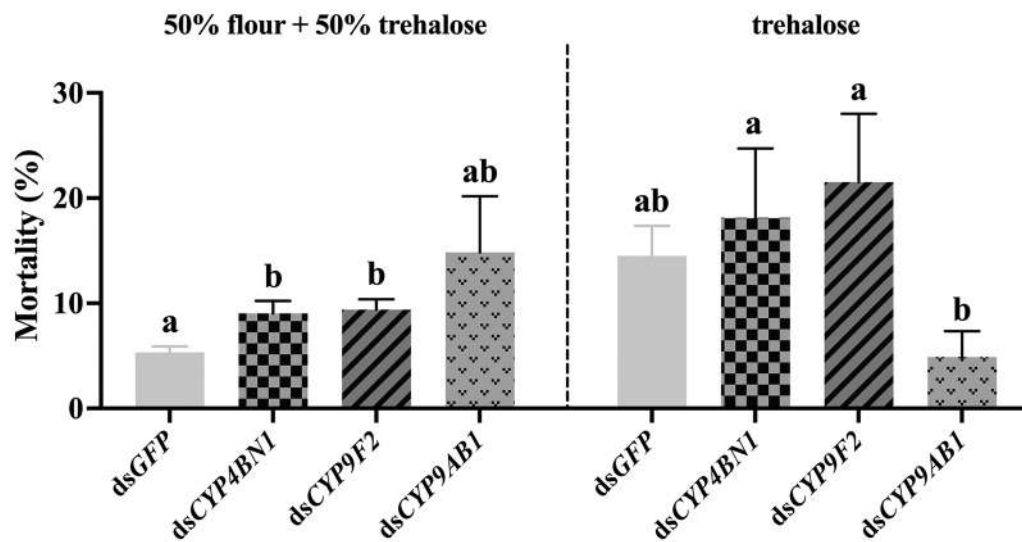


Fig. 2. Mortality in *T. castaneum* under 95% CO₂ stress after 48h of trehalose dietary regimes (50% flour + 50% trehalose or 100% trehalose) and dsCYP treatments (dsGFP, dsCYP4BN1, dsCYP9F2, or dsCYP9AB1). Each group included 4 biological replicates. Values are presented as mean $\pm$ SE. Different letters indicate significant differences between groups. The 50% flour + 50% trehalose dietary groups were analyzed using Welch's ANOVA followed by Games-Howell post-hoc test at α =0.05 (letters ordered from smallest to largest mean), while the 100% trehalose dietary groups were compared by one-way ANOVA followed by Tukey HSD post-hoc test at α =0.05 (letters ordered from largest to smallest mean).

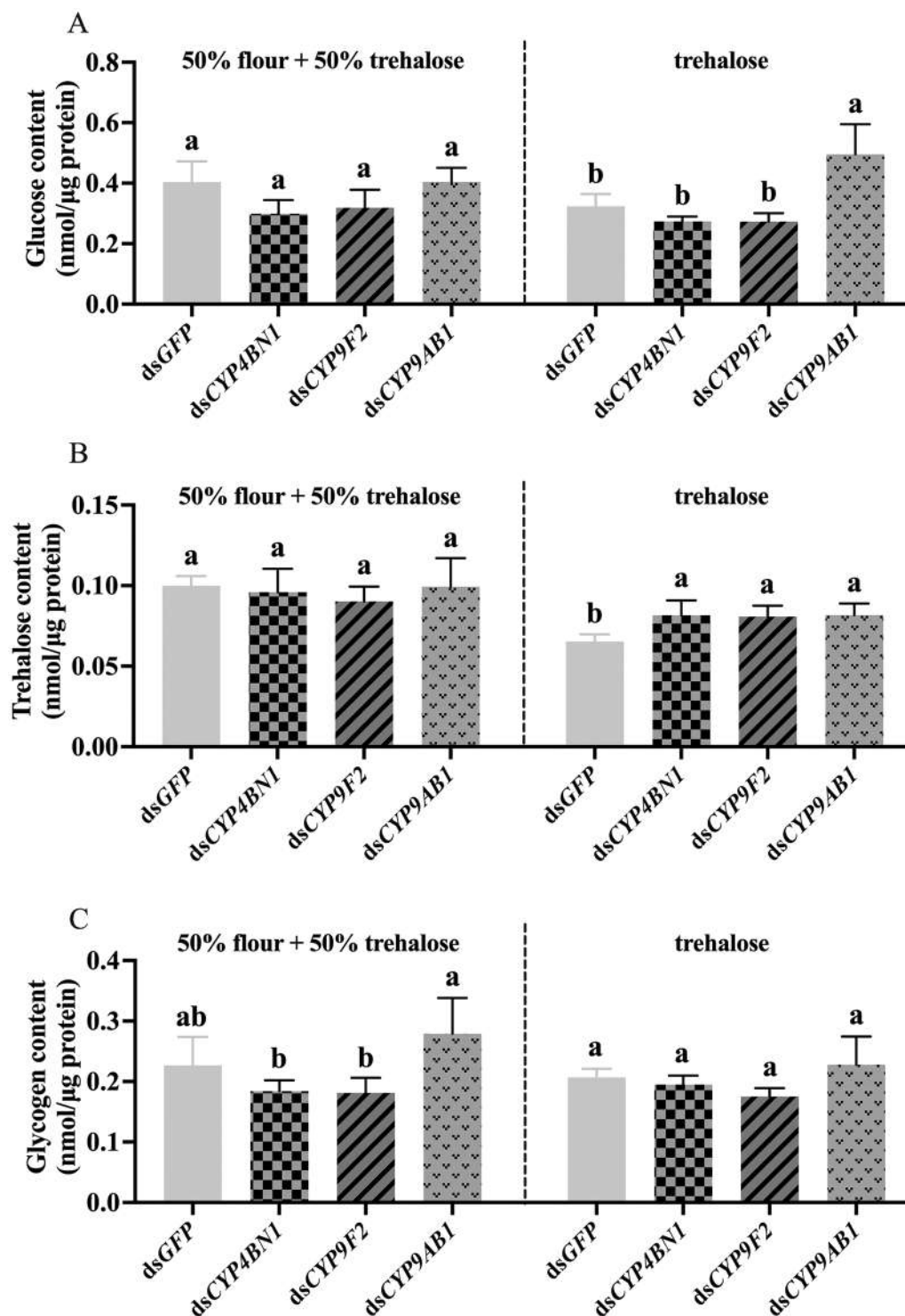


Fig. 3. The effects of trehalose dietary regimes (50% flour + 50% trehalose or 100% trehalose) and CYP gene silencing (dsGFP, dsCYP4BN1, dsCYP9F2, or dsCYP9AB1) on glucose A), trehalose B), and glycogen C) content in *T. castaneum* under 95% CO₂ stress after 48 h. Each group included 4 biological replicates with 15 larvae each. Values are presented as mean ± SE. Different letters indicate significant differences between groups (Tukey HSD post-hoc test at α = 0.05).

Effect on CYP450 Activity under 95% CO₂ after Trehalose Feeding and dsRNA

Following a 48-h exposure to trehalose dietary regimes (50% flour + 50% trehalose or 100% trehalose) and dsCYP treatments under 95% CO₂ conditions, the CYP450 activity was assessed. On 50% flour +

50% trehalose diet, the dsCYP9AB1 group had notably higher CYP450 activity than the control, while the other 2 groups showed a slight increase ($F=12.345$, $df=3, 12$, $P<0.001$). On 100% trehalose diet, the CYP450 activity showed little difference among the 4 groups ($F=0.772$, $df=3, 12$, $P=0.532$; Fig. 5).

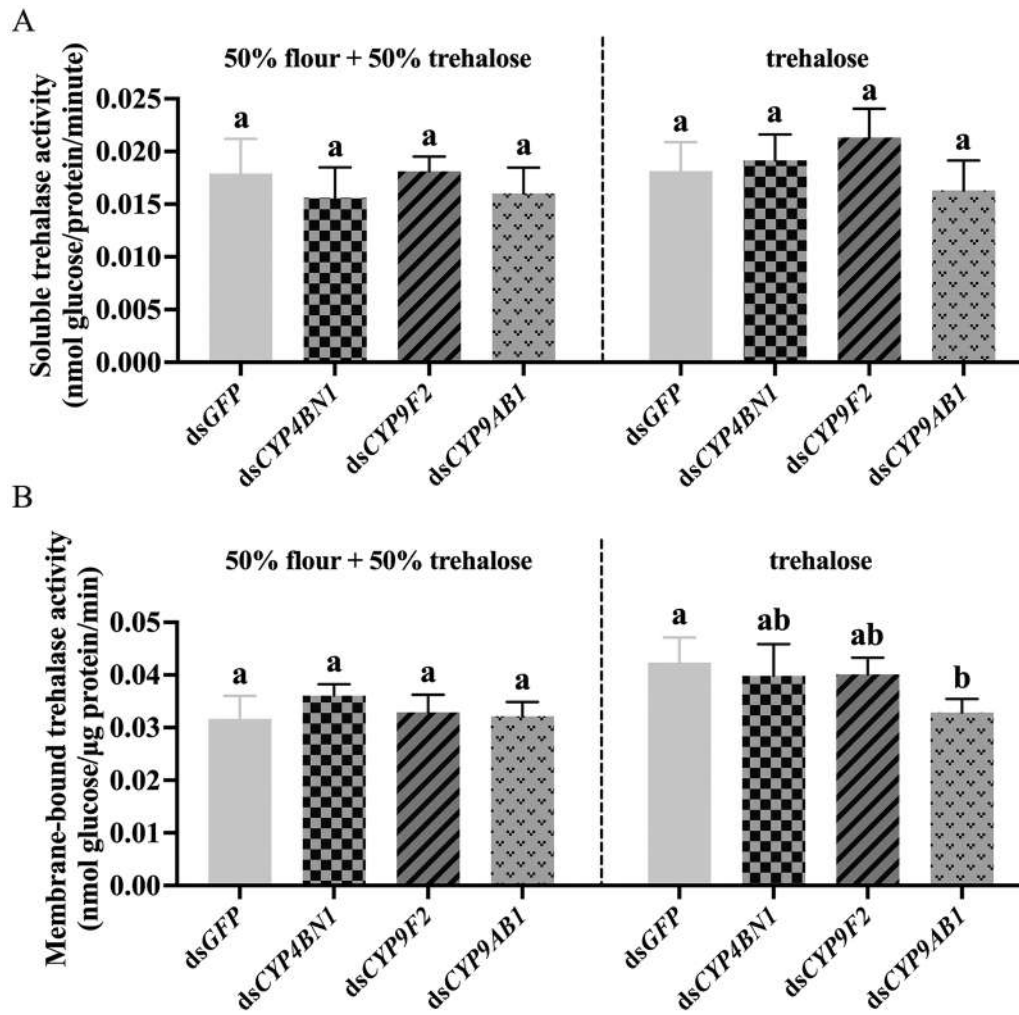


Fig. 4. The effects of trehalose dietary regimes (50% flour + 50% trehalose or 100% trehalose) and CYP gene silencing (dsGFP, dsCYP4BN1, dsCYP9F2, or dsCYP9AB1) on soluble A) and membrane-bound B) trehalase activity in *T. castaneum* under 95% CO₂ stress after 48 h. Each group included 4 biological replicates with 15 larvae each. Values are presented as mean ± SE. Different letters indicate significant differences between groups (Tukey HSD post-hoc test at $\alpha=0.05$).

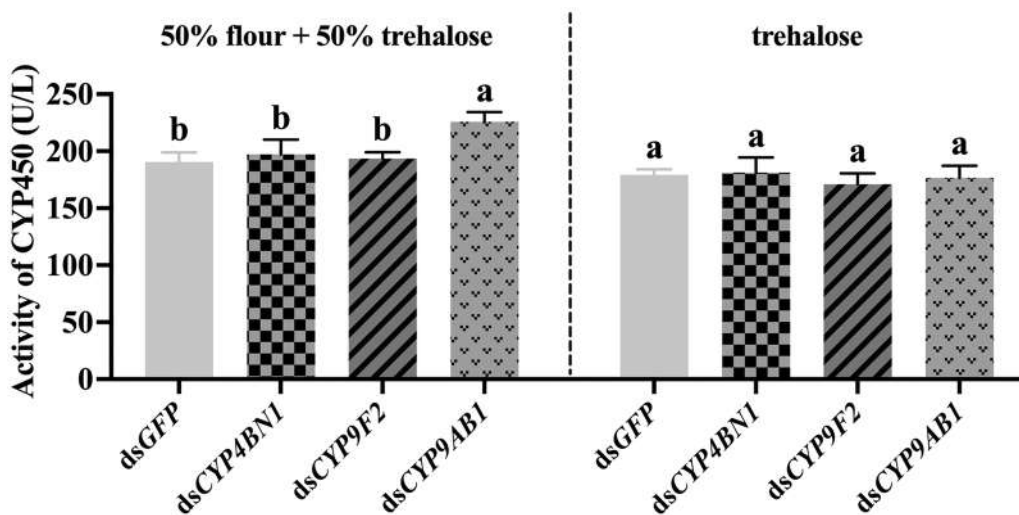


Fig. 5. The effects of trehalose dietary regimes (50% flour + 50% trehalose or 100% trehalose) and CYP gene silencing (dsGFP, dsCYP4BN1, dsCYP9F2, or dsCYP9AB1) on CYP450 activity in *T. castaneum* under 95% CO₂ stress after 48 h. Each group included 4 biological replicates with 30 larvae each. Values are presented as mean ± SE. Different letters indicate significant differences between groups (Tukey HSD post-hoc test at $\alpha=0.05$).

Effect on Trehalose Metabolism Pathway under 95% CO₂ after Trehalose Feeding and dsRNA

To investigate dsCYP impact on trehalose metabolism under 95% CO₂ and trehalose dietary regimes (50% flour + 50% trehalose or 100% trehalose), key gene expressions in the trehalose metabolic pathway were assessed. On 50% flour + 50% trehalose diet, the dsCYP4BN1 treatment significantly down-regulated *TcTRE1-1*, *TcTRE2*, *TcTPS1*, and *TcTPS2* (*TcTRE1-1*: $t=7.085$, $df=4$, $P=0.002$; *TcTRE2*: $t=5.168$, $df=4$, $P=0.007$; *TcTPS1*: $t=3.878$, $df=4$, $P=0.018$; *TcTPS2*: $t=4.270$, $df=4$, $P=0.013$; Fig. 6A), while dsCYP9F2 decreased *TcTRE1-1*, *TcTRE1-3*, and *TcTPS1* (*TcTRE1-1*: $t=11.609$, $df=4$, $P<0.001$; *TcTRE1-3*: $t=2.810$, $df=4$, $P=0.048$; *TcTPS1*: $t=4.003$, $df=4$, $P=0.016$; Fig. 6B). In contrast, dsCYP9AB1 up-regulated *TcTRE2*, *TcTRE1-2*, *TcTRE1-3*, and *TcTPS2* (*TcTRE2*: $t=-4.533$, $df=4$, $P=0.011$; *TcTRE1-2*: $t=-3.561$, $df=4$, $P=0.024$; *TcTRE1-3*: $t=-4.280$, $df=4$, $P=0.013$; *TcTPS2*: $t=-4.081$, $df=4$, $P=0.015$; Fig. 6C).

On 100% trehalose diet, the dsCYP4BN1 treatment sharply reduced most genes, except for *TcTRE1-3* (*TcTRE1-1*: $t=4.013$, $df=4$, $P=0.016$; *TcTRE1-2*: $t=5.018$, $df=4$, $P=0.007$; *TcTRE1-3*: $t=1.548$, $df=4$, $P=0.196$; *TcTRE1-4*: $t=6.760$, $df=4$, $P=0.002$; *TcTRE2*: $t=4.753$, $df=4$, $P=0.009$; *TcTPS1*: $t=4.159$, $df=4$, $P=0.014$; *TcTPS2*: $t=12.827$, $df=4$, $P<0.001$; Fig. 7A). dsCYP9F2 notably increased *TcTRE1-2* and decreased *TcTRE1-4*, with minor changes in other genes (*TcTRE1-1*: $t=0.689$, $df=4$, $P=0.529$; *TcTRE1-2*: $t=-3.637$, $df=4$, $P=0.022$; *TcTRE1-3*: $t=-2.220$, $df=4$, $P=0.091$; *TcTRE1-4*: $t=3.990$, $df=4$, $P=0.016$; *TcTRE2*: $t=-0.175$, $df=4$, $P=0.869$; *TcTPS1*: $t=-0.822$, $df=4$, $P=0.457$; *TcTPS2*: $t=-2.696$, $df=4$, $P=0.054$; Fig. 7B). In the dsCYP9AB1 treatment, key genes *TcTRE2*, *TcTPS1*, and *TcTPS2* saw significant increases, with *TcTPS2* experiencing the most pronounced upregulation (*TcTRE1-1*: $t=-0.638$, $df=4$, $P=0.558$; *TcTRE1-2*: $t=-0.998$, $df=4$, $P=0.375$; *TcTRE1-3*: $t=-0.182$, $df=4$, $P=0.865$; *TcTRE1-4*: $t=-1.822$, $df=4$, $P=0.143$; *TcTRE2*: $t=-2.869$, $df=4$, $P=0.046$; *TcTPS1*: $t=-4.402$, $df=4$, $P=0.012$; *TcTPS2*: $t=-5.211$, $df=4$, $P=0.006$; Fig. 7C).

Discussion

Cytochrome P450 monooxygenases are a class of important detoxification enzymes, crucial for the physiological functions of insects, while trehalose serves as a blood sugar regulator and a versatile compound that protects cells against various stress factors (Elbein et al. 2003, Kuczyńska-Wisnik et al. 2024, Wang et al. 2024). In this study, we used qRT-PCR to analyse the expressions of *TcCYP4BN1*, *TcCYP9F2*, and *TcCYP9AB1* genes in individuals after dsRNA injection and trehalose feeding. It was found that dsRNA still maintained its inhibitory efficacy despite the presence of exogenous trehalose, as evidenced by the downregulation of CYP gene expressions (Fig. 1).

To delve deeper into the interplay between trehalose supplementation and CYP450 in *T. castaneum* under 95% CO₂, we calculated the mortality of dsCYP4BN1-, dsCYP9F2-, and dsCYP9AB1-treated groups. The results revealed that on 100% trehalose diet, dsCYP treatments still increased mortality of *T. castaneum* larvae compared with those on a mixed diet. However, under dsCYP9AB1 treatment the mortality increased on a mixed diet, but decreased surprisingly on 100% trehalose diet compared with the controls (Fig. 2). Distinct from dsCYP9AB1, dsCYP4BN1 and dsCYP9F2 treatments showed lower

mortality on a mixed diet and higher mortality on 100% trehalose diet, which was more similar to that of dsGFP (Fig. 2). This divergence suggests that *TcCYP9AB1* may play a unique role in trehalose metabolism during high-CO₂ stress. The elevated mortality in dsCYP9AB1-treated larvae on a mixed diet implies that *TcCYP9AB1* may regulate trehalose metabolism under physiological conditions, with its silencing compromising larval adaptation to high-CO₂ stress. Strikingly, the reduced mortality observed in the 100% trehalose group upon *TcCYP9AB1* knockdown may point to the role of accumulated trehalose as a protector, which is known to provide energy and protection against various stress (Tellis et al. 2023). Moreover, in recent studies, a strong association has been found between CYP genes and trehalose metabolism. For example, *TcCYP314A1* has been identified as effective for trehalose metabolism and synthesis of insect 20E in *T. castaneum* (Zhou et al. 2022). Also, when *TcCYP6K1* was knocked down via RNAi, both an increased larval mortality and trehalose levels were observed in *T. castaneum* under 75% CO₂ stress (Guan et al. 2024), further emphasizing the correlation between CYP genes and trehalose metabolism. Thereby, a critical role for *TcCYP9AB1* in trehalose metabolism, with a tighter link relative to the other 2 target genes, is the most likely model to account for our results.

Previously empirical evidence implies in several species, including *Acyrtosiphon pisum* (Harris) (Hemiptera: Aphididae) and *Drosophila melanogaster* (Diptera: Drosophilidae), that carbohydrate levels generally shift when insects are fed diets with varying sugar amounts as a metabolic regulatory mechanism to maintain energy homeostasis (Wang et al. 2021, Strilbytska et al. 2022). However, we observed no extremely significant biochemical changes, neither in the levels of glucose and glycogen nor in the activity of trehalase and CYP450, when *T. castaneum* was treated with trehalose feeding and RNAi (Figs 3–5). A potential explanation may be that stored product insects can regulate their physiological metabolic processes through their well-developed regulatory mechanisms to cope with environmental stress (Harrison et al. 2006). In *Callosobruchus chinensis* (Hope) (Coleoptera: Bruchidae), the magnitude of metabolic alteration under hypoxic stress showed significant fluctuations relative to controls (Cui et al. 2019), further suggesting that insects may maintain homeostasis through self-regulatory mechanisms to adapt to environmental stress. Upon closer analysis of the results, we posit that the *TcCYP9AB1* gene exhibits a stronger association with trehalose metabolism compared with the other 2 target genes, as evidenced by the significant increase in glucose content on 100% trehalose diet in the dsCYP9AB1 group, whereas the other 2 treatment groups showed decreases (Fig. 3A). Similar trends were also observed in glycogen content (Fig. 3C).

Studies have elucidated the contributions of carbohydrate metabolism to biological processes, and its critical role as a link between protein, lipid, nucleic acid, and secondary biomass metabolisms (Pan et al. 2020). Moreover, the potential regulatory mechanism underlying insect resistance to high CO₂ stress, when studied, can be fostered to facilitate the growth of sustainable storage-pest management solutions (Guan et al. 2024). Therefore, we measured the expression levels of *TcCYP4BN1*, *TcCYP9F2*, *TcCYP9AB1*, and genes related to the trehalose metabolic pathway via qRT-PCR, to further explore the intricate relationship between trehalose and CYP genes under stress conditions. Experiments evaluating the expression of related

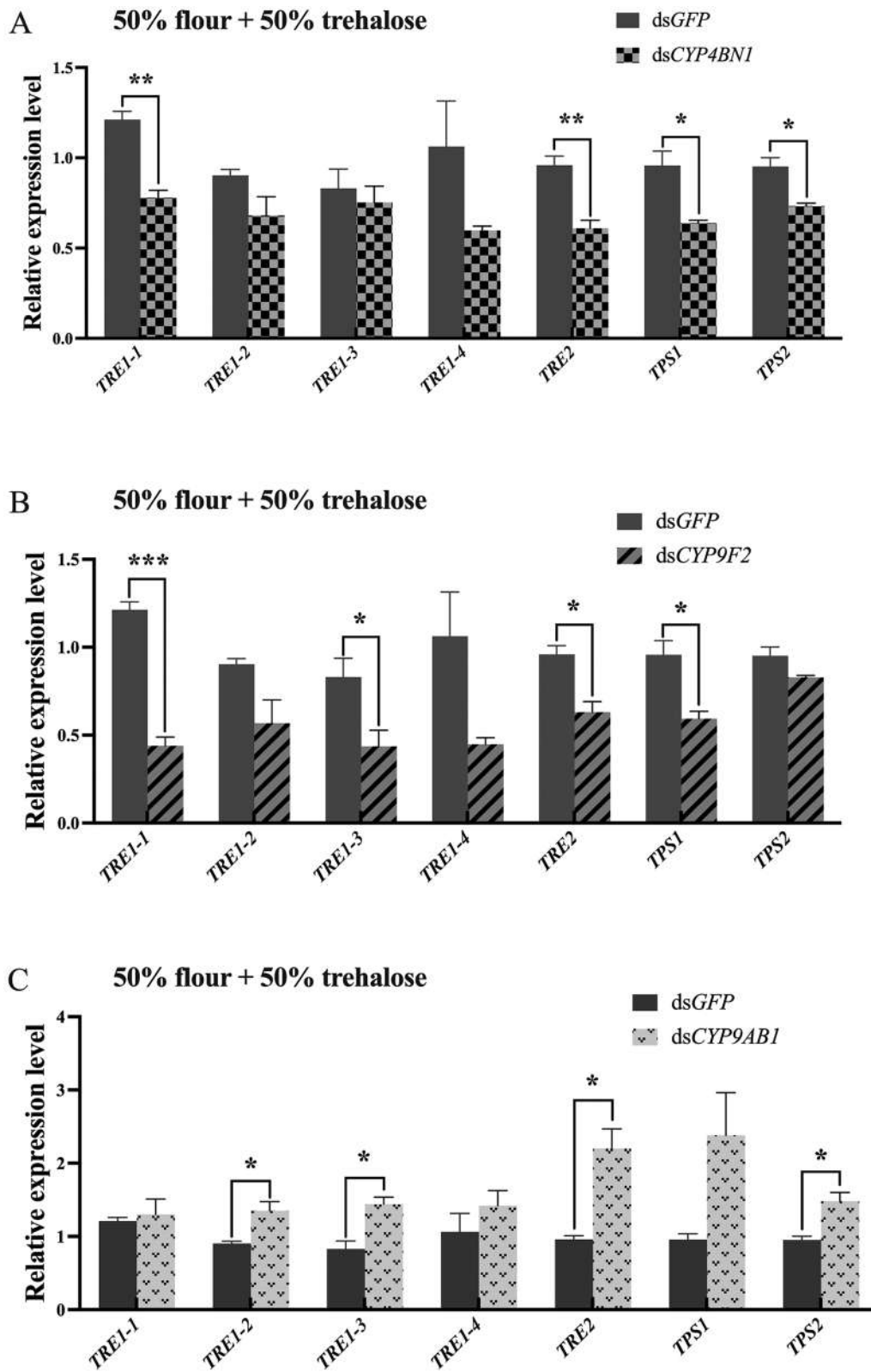


Fig. 6. On 50% flour + 50% trehalose diet, effects of dsCYP treatments on trehalose metabolic gene expression in *T. castaneum* under 95% CO₂ stress. A to C) display the impact of *TcCYP4BN1* A), *TcCYP9F2* B), and *TcCYP9AB1* C) RNA interference on the expression of trehalose metabolic pathway genes (*TRE1-1*, *TRE1-2*, *TRE1-3*, *TRE1-4*, *TRE2*, *TPS1*, and *TPS2*) in larvae. TRE, trehalase; TPS, trehalose-6-phosphate synthase. Each group included 3 biological replicates with 10 larvae each. Each biological replicate included 3 technical replicates. Values are presented as mean $\pm$ SE. *** P < 0.001, ** P < 0.01, * P < 0.05 (independent samples *t*-test).

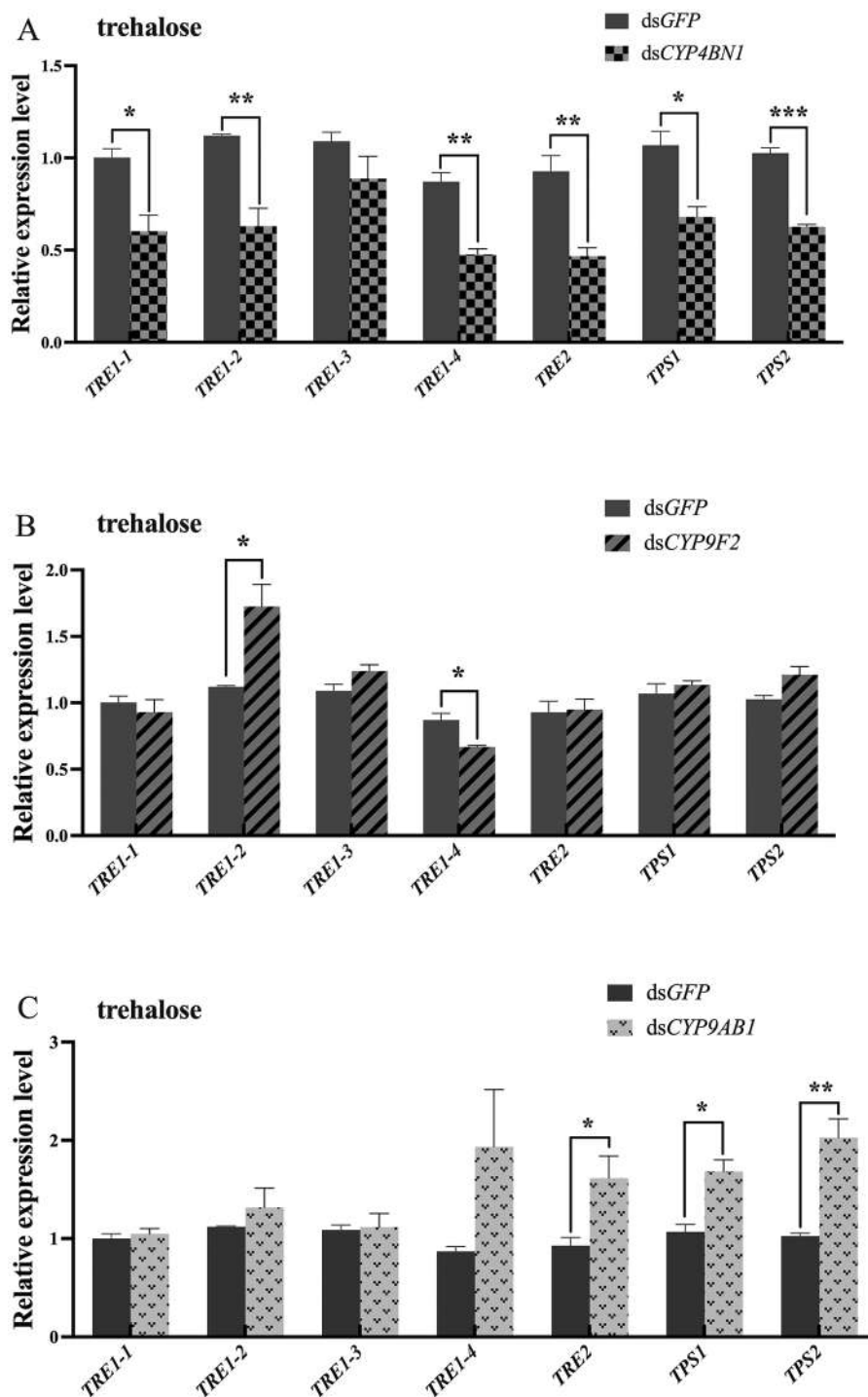


Fig. 7. On 100% trehalose diet, effects of dsCYP treatments on trehalose metabolic gene expression in *T. castaneum* under 95% CO₂ stress. A to C) display the impact of *TcCYP4BN1* A), *TcCYP9F2* B), and *TcCYP9AB1* C) RNA interference on the expression of trehalose metabolic pathway genes (*TRE1-1*, *TRE1-2*, *TRE1-3*, *TRE1-4*, *TRE2*, *TPS1*, and *TPS2*) in larvae. TRE, trehalase; TPS, trehalose-6-phosphate synthase. Each group included 3 biological replicates with 10 larvae each. Each biological replicate included 3 technical replicates. Values are presented as mean $\pm$ SE. *** P <0.001, ** P <0.01, * P <0.05 (independent samples t -test).

genes in all groups allowed us to confirm the possibilities that trehalose can enhance the response of CYP genes to CO₂ atmosphere stress, and that trehalose intake may alleviate the upregulation of key genes involved in trehalose metabolism caused by dsRNA, as evidenced by a decrease in the expression of genes associated with trehalose metabolism pathway in groups

exclusively fed trehalose, with the exception of the dsCYP9F2, compared with groups fed 50% flour + 50% trehalose (Figs 6 and 7). Consistent with our conjectures, trehalose and *TcCYP9E2* have a synergistic effect in *T. castaneum* coping with high CO₂ stress (Zhou et al. 2023). Also, trehalose and *TcCYP6K1* can improve the ability of *T. castaneum* to

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