Dear editor,

Thank you for giving us this opportunity to revise our manuscript "Characterization of a Mn-SOD from the desert beetle *Microdera punctipennis* and its increased resistance to cold stress in *E. coli* cells". We have cautiously read and carefully considered the reviewer's comments, and revised our MS in detail according to these comments. The revised parts are given in blue font. The question responses are as follows. We appreciate the reviewer's valuable comments, which inspired us to improve and think over more about our research.

Kind regards!

General comments

Reviewer: Aarushi Sharma

1. Methods section needs to be more detailed. Specifically, details on statistical analysis in methods only mention Tukey's post-hoc test alone. It does not mention one-way ANOVA as its primary statistical test run by authors for identifying significant changes in their treatment data. Moreover, some of the experiments were analyzed by paired Student's t-test which needs to be mentioned in the methods.

Response: We have detailed the methods for data analysis in the Method section as "One-way analysis of variance and Tukey's multiple comparison test were used for data analysis in gene expression, SOD activity as well as $O_2^{\bullet-}$ contents in *E. coli* cells. Paired *t*-test was employed for data analysis in the diameter of inhibition zone, relative conductivity and MDA contents in *E. coli* cells.

2. There is some detail on how relative electrical conductivity assay was performed and results analyzed in the supplementary file. However, authors should explain the complete procedure in the methods section.

Response: We have detailed how relative electrical conductivity and MDA content assay were performed in the Methods section.

3. Though the overexpression data in *E. coli* is well put together and supports the hypothesis strongly, it would be great if authors could in near future show protection from cold stress by Mn-SOD via knock-down or knock-out models for Desert Beetle.

Response: Thanks for this essential point. We will construct the Mn-SOD knock-down model in the desert beetle in near future to check the function of Mn-SOD under cold stress in the beetle.

Commented [SA(1]: Please add that as a weakness and future direction in your discussion.

Reviewer: Yinnian Feng

1. As the authors mentioned in the intro part (line 77), the average temperature of the soil the beetle would confront in Jan are -12 ℃ to -5 ℃. However, the experimental temperature in this paper for beetle individuals was performed at 4 ℃. I am more concerned

that the temperature difference will affect some of the data shown in the paper. I am also curious about the reasons that the authors wouldn't perform these experiments in -5 ℃. Maybe the authors should add some sentences in the experiment part to address this. I did find out that the *E-coil* experiment was performed at -4 ℃. I think this temperature is much closer to the real temperature the enzyme will suffer.

Response: Thanks for this question. The reason we treat the insect at 4 ℃ instead of -5 ℃ is that in our previous work (*Hou et al., 2010*) we found that 4 ℃ is the low temperature at which the insect has already responded to cold stress. In the desert during spring and autumn when the insect is still active the air temperature often fluctuates sharply ranging from about 25 ℃ to 0 ℃, which may cause more serious cold injure to insect than to the diapause one, thus we perform the experiment at 4 ℃. For *E. coli*, because 4 ℃ is not serious harmful to its survival, we performed the experiment at -4 ℃ so as to discriminate the results between the control and the cold treatment groups. We have supplemented as follows: "As 4 °C is the low temperature at which the insect has begun to respond to cold stress (*Hou et al., 2010*), the beetle individuals were exposed....."

2. The mRNA experiment shown in Fig. 4 is interesting but confused. In A, it shows that the hindgut and fat body rank the first and second tissues that have more *MpmMn-SOD* expression. However, in B it only shows the expression of the overall mRNA in the beetle. In my opinion, B needs to show the different expressions in all the tissue listed in A, at least show the data in hindgut and fat body would be better. Also, the data is compared to the head at 0h, which would give you the highest fold-change as the head has the lowest amount of MpmMn-SOD. But why you don't compare it to the other 4 tissues listed in A?

Response：Thanks for these comments. The tissue specific expression results showed that hindgut and fat body were the main location for *MpmMn-SOD*. These results are in consistent with the function of hindgut and fat body. Hindgut plays important roles in detoxification and elimination of toxins. And fat body is one of the prime sites for antioxidant enzymes. The reason we tested the overall mRNA instead of each tissue mRNA at 4° C is that we focused on examining whether the expression of *MpmMn-SOD* responses to low temperature stimulation, since hindgut and fat body are the main sites for detoxification and antioxidation, it is less possible that under cold conditions the expression of the tissue distribution would change. But we agree that it is better to show the data in hindgut and fat body.

For data processing, we apologize for the unclear writing in figure 4 legend. In figure 4A, the expression level of *MpmMn-SOD* gene in different tissues were normalized to head. In figure 4B, the expression level at different time periods was normalized to 0 h, not 0 h and head. We have rewritten the legend as "..... **(A)** The expression profile of *MpmMn-SOD* gene in different tissues. Values are represented as fold change compared to the

Commented [SA(2]: The authors have perhaps not understood the reviewer's comments here. In figure 4B, it is still unclear what the control at 0h is. In the correctly performed experiment, it should be mRNA level of the same tissue at 0h. Secondly, the labeling on top of bars (c, b, cd etc.) have not been explained in the legend. Please rectify these issues.

control (head); **(B)** Temporal expression of *MpmMn-SOD* gene under 4 °C cold stress. Values are represented as fold change compared to the control (0 h)."

3. Again, in figure 4B, at 11h the mRNA level seems to go back, and it is very interesting. It looks like another round of cold-defense is going on? I recommend the authors could briefly explain this in results and discussion.

Response: The cold expression profile of *MpmMn-SOD* presented a stress-responsive pattern, that is, the expression levels fluctuates during the whole stress period. These fluctuations indicate that cells can adjust the amount of the enzyme timely and economically, because stress-responsive expression is at the cost of the inhibition of other genes expression. It is unsafe for cells to overexpress the stress-responsive gene all the time. However, with the prolong of the cold stress, ROS increases again, and cells need to produce more *MpmMn-SOD* to deal with the excessive ROS (*Xikeranmu et al., 2019*), so it looks like another round of cold-defense is going on.

We have supplemented a brief explanation in the results part as "The cold expression profile presented a stress-responsive pattern. The large fluctuation of *MpmMn-SOD* expression during the cold treatment indicated that cells adjusted the level of the enzyme timely and finely.

In Discussion part, we supplemented a discussion as "It is noticeable that the cold expression profile of *MpmMn-SOD* under 4 °C presented as a stress-responsive type, which is characterized with great fluctuations during the cold treatment period, the first large peak appeared at 1.5 h, and the second large peak appeared at 11 h which was 67.3-fold of the control. It looks like another round of cold defense is going on. These results may be interpreted as that the cells cold timely adjust the amount of the enzyme to keep a relative intracellular balance, because stress-responsive expression is at the cost of the inhibition of other genes expression. On the other hand, with the prolong of cold stress, ROS increases again, and cells need to produce more *MpmMn-SOD* to deal with the excessive ROS. Our previous work (*Xikeranmu et al., 2019*) showed that there is a rapid increase of $O_2^{\bullet-}$ contents in the beetle after an exposure at 4 °C for 10 h, which is consistent with the appearance of the second large expression peak in this work."

4. The SDS-PAGE experiment in Fig.5 A, there is a clear and strong up-regulation band in lane 2, located in ~20 KDa, what is this? It seems that the band disappeared in the lane 4. If lane 2 and lane 4 are both induced samples and the expression of pET32a-mMn-SOD wouldn't affect the ~20KDa protein, should it be also on the lane 4, same position? Can you explain this?

Commented [SA(3]: Not a correct word to use here.

Commented [SA(4]: prolonging

Deleted: the

Deleted: increased

Response: In Fig.5 A lane 2 the clear and strong up-regulation band located in ~20 kDa is the fusion protein Trx-His, the tag protein on the vector plasmid BL21(pET32a), which is 18.5kDa. The construction of the recombinant plasmids pET-32a (MpmMn-SOD) is illustrated below, which produces fusion protein Trx-His-MpmMn-SOD. The molecular weight of this fusion protein is about 41 kDa (18.5kDa + 23.69 kDa, the predicted molecular weight of MpmMn-SOD), and appeared in lane 4. Because *MpmMn-SOD* gene was fused with *trx-his* tag on the vector, the induced expressed protein would be a big fusion protein Trx-His-MpmMn-SOD, and Trx-His tag protein cannot be expressed alone in lane 4, so there is no 20 kDa band in lane 4.

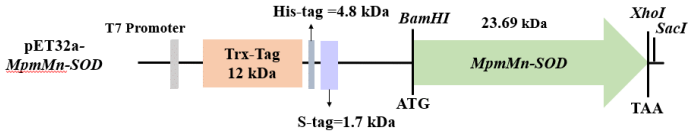


Diagram of the recombinant plasmids pET-32a (MpmMn-SOD)

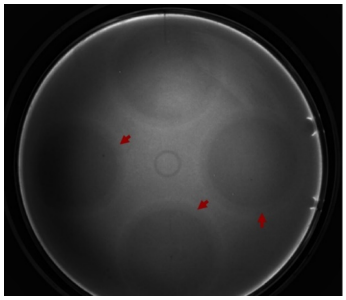
5. Since the data in Fig.4 showed the experiment till 11 h (11 h shows a growing again and is interesting), it would be better that the same time data points (8 h, 9 h, 11 h?) are shown and compared in Fig.6. Then you can compare the activity of the enzyme both in beetle and *E.coil*.

Response: As -4 °C is harmful to bacteria survival, with the time prolonging part of the cells died, so the experiment was not extended longer than 6 h. We appreciate this suggestion and would carefully design the next comparing experiment. To avoid the influence of cells die to experimental results, we may treat the *E. coli* cells at 4 °C for 0.5~11 h, then we can compare the activity of the enzyme both in beetle and *E. coli*.

6. The Oxford-cup experiment in Fig. 7 is also interesting. Although the control showed a bigger circle compared to the MpmMn-SOD one at same H_2O_2 concentration, the circles in the control experiments are very dim and absent of a clear, sharp circle boundary. Why this happened? Can you explain?

Response: It is true, the inhibition circle in the control group is dim and absent of a clear, sharp circle boundary. We have repeated this experiment several times, and all got the same results. By amplifying the picture, we found that there is a dim circle outside the inhibition area (see arrows in the picture). That's why the boundary looks unclear. The reason for the occurrence of this dim circle may due to the weak resistance of the control bacteria to H_2O_2, they might be very sensitive to H_2O_2, even trace peroxide around the cup would kill some cells, thus a tiny circle formed.

Commented [SA(5]: How were the 4 concentrations chosen? Please explain that as well.



Specific comments:

1. The term BL21 in the abstract is very confused to me. I don't think that people would know it is an *E-coil* if they are not cell biologists. Maybe add it after "An E. coli system" in line 24, such as "An *E. coli* system (BL21) was applied ...."

Response: Done.

2. Line 45, "reactive oxygen species" should be "ROS" as you mentioned the acronym before.

Response: We changed “reactive oxygen species" to "ROS" in line 45.

3. Line 53, "Superoxide dismutases" should be "Superoxide dismutases (SODs)", as you used SOD below.

Response: We changed “Superoxide dismutases " to " Superoxide dismutases (SODs)" in line 53.

4. Line 62, "MnSOD" should be "Mn-SOD".

Response: "MnSOD" was changed to "Mn-SOD"

5. Line 144, "MpmMnSOD" should be "MpmMn-SOD".

Response: "MpmMnSOD" was changed to "MpmMn-SOD".

6. Sentence from Lines 301 to 302, please rewrite it.

Response: Sentence from Lines 301 to 302 were rewritten as " Antioxidant activity assay was performed to evaluate whether overexpression of mMpMn-SOD could enhance the tolerance of the transformed bacteria to oxidative stress. "

7. Lines 403 and 411, two "further"s seem weird. Could change the second further to some other words, such as "last but not least"?

Response: We have changed the word "further" to "finally" in line 411.

8. Line 542, the reference is wrong. The doi is same as the reference in line 538. Also, I couldn't find the reference.

Response: The reference in line 542 was corrected. The doi was rewritten as "https:// doi: 10.13441/j.cnki.cykx.2018.02.002. "

9. In SI material all the "datas" should be "data". "Data" is used as a plural noun in technical English, when the singular is datum.

Response: Done.

10. Figure 5 labels A and B in figure are missing.

Response: Labels A and B were added in figure 5.

Reviewer 3

1. The paper requires corrections of a few errors in the text e.g. line 382 where the time required to reach 125-fold is missing.

Response: We are sorry for the mistake. We've added it.

2. The description and the y-axis title differ in figure 6B. Is it $O_2\bullet^-$ content or $O_2\bullet^-$ content reduction?

Response: We are sorry for making these mistake. In the revised version we have corrected them.

3. I would encourage the authors to include a section with commonly used acronyms.

Response: We have added the commonly used acronyms in the MS in the end of the Methods section.

4. Do insects have any other antioxidant enzymes? I believe it would enhance the knowledge of the reader if you were to mention in the introduction, others that might participate in the same process.

Response: Yes, same as with other organisms, insect has several antioxidant enzyme systems beyond SODs. We have added the information in the introduction as: "The dominant ROS, superoxide anion ($O_2\bullet^-$), is converted to hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD), then transformed to water via catalase (CAT) or glutathione peroxidase (GPx) (*Schafer and Buettner, 2001*)".

5. Figure 2: Can you please add to your description, what the colors cyan, pink and black represent?

Response: Fig.2 was created with DNAMAN 6.0 (http://www.lynnon.com). In this software different colors represent different sequence homology levels. We have added the following information in the description: The amino acid homology up to 100% is in black, 75%~99% is in pink, and 50%~74% in cyan.

6. What was the purpose of the analysis behind figure 3? Is there anything you can infer based on the classification about the behavior of the protein? Can any similarities be drawn with the enzyme version expressed by *T. molitor*? While you mention it in a few lines (231-235) in the results section, a bigger discussion about the different clades of enzymes in the introduction or results would help.

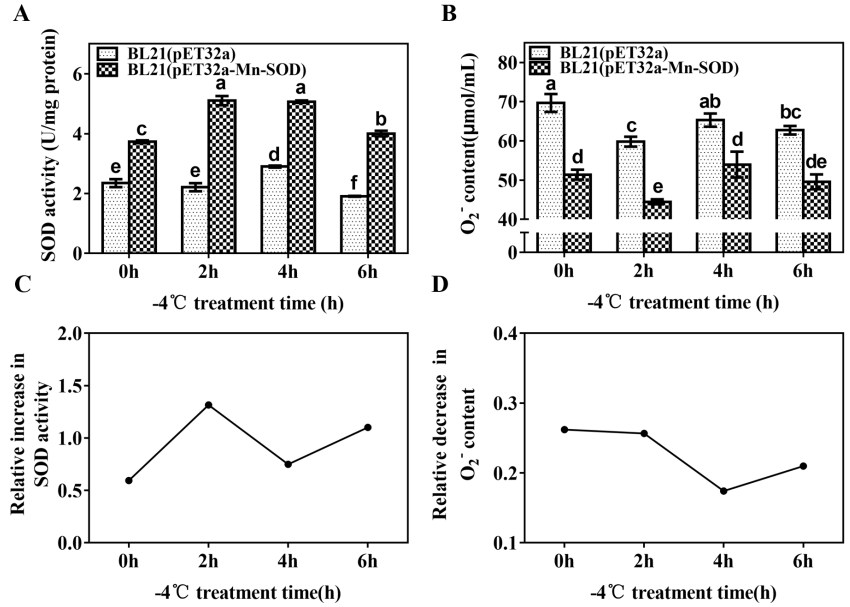
Response: Figure 3 is a phylogenetic analysis of SODs in insects. After we have *in silicon* characterized the Mn-SOD gene in *M.punctipennis* by BLAST at GenBank, a phylogenetic analysis may further help to classify the type of this SOD gene and to show its relationship with SODs from other insects. Fig.3 shows the phylogenetic tree has two separate clusters

from the same root, Mn-SOD and Cu/Zn-SOD, which is in accordance with their distinct metal cofactor requirements. We may infer that Mn-SOD and Cu/Zn-SOD originate from a same ancestor, and Mn-SOD may have evolved longer than Cu/Zn-SOD as its branch length is longer than that of the Cu/Zn-SOD. Within Cu/Zn-SOD clade, ecCu/Zn-SOD and icCu/Zn-SOD were classified as two subgroups. ecCu/Zn-SOD subgroup diverged earlier than icCu/Zn-SOD. The latter sub-family may evolve by gene replication. MpmMn-SOD was clustered with Mn-SODs, and was closed to *Tenebrio molitor* Mn-SOD, although their amino acid sequence identity(similarity) is only 35.27%. These two insects are belonging to Tenebrionidae (Coleoptera), the close relationship of their Mn-SOD sequences may roughly reflect their taxonomic relationships.

We have supplemented results description and discussion in the Results and Discussion sections respectively.

7. Figure 6: Why does the SOD activity increase not correlate with a drop in $O_2^{\bullet -}$ levels? What do you hypothesize as the reason for increase in $O_2^{\bullet -}$ levels at 6 h?

Response: Firstly, we apologize for the inappropriate data process, which caused Fig.6 an incorrect data represents. In Fig.6 the SOD increment was obtained simply by subtracting the SOD activity in BL21(pET-32a-MpmMn-SOD) from that in the control bacteria BL21(pET-32a), this is an absolute difference, so does for $O_2^{\bullet -}$ reduction. As in each sample, especially at different time point, the levels of SOD activity of BL21(pET-32a-MpmMn-SOD) are different from each other, it is inappropriate to compare each sample



directly by using the absolute difference. Now we corrected this by using a relative difference, which is obtained by dividing the absolute difference by the value of BL21(pET-32a-MpmMn-SOD). The relative value can measure the difference of each sample at a same baseline. The corrected relative increase of SOD activity and relative decrease of O_2•- contents are given in Fig8.C and Fig.8D respectively in the revised version. To let the results be clearer to read Fig.6 was modified and supplied with the measured values of SOD activity (Fig.8A) and O_2•$^-$ contents (Fig.8B), respectively (because OD595 readings were supplemented as in Fig6 in the revised version, the original Fig.6 was remarked as Fig.7, and so on for the following figures); The corresponding relative differences were given in Fig. 8C and Fig.8D, respectively.

From Fig.8C and Fig.8D we can see that the two curves had similar changing trends under cold conditions. The relative increase of SOD activity at 2 h and 6 h of the cold treatment was 2.3 and 2 times of those of the control(p<0.05) (Fig.8C) respectively. Correspondingly, the relative decrease of O_2•- contents at 2 h and 6 h of the cold treatment (Fig.8D) was higher than that at 4 h. This means under cold conditions the more increase in SOD activity, the more decrease in O_2•- contents. The low levels of those values at 4 h of cold treatment may due to a self-regulation of the cells to keep a relative subcellular balance in SOD gene expression. However, we noticed that at 0 h, the relative decrease of O_2•- contents was roughly at the same level as at 2 h under cold conditions. This result may be explained as the transformed bacteria BL21(pET32a-Mn-SOD) had higher SOD activity than the control and it functioned well at room temperature.

We have revised the description about these results as follows: We detected the changes of the SOD activity and O_2•$^-$ contents in the MpmMn-SOD transformed bacteria BL21(pET32a-Mn-SOD) after -4 °C treatment. The results showed that compared to BL21(pET32a), in all the samples BL21(pET32a-Mn-SOD) had significant high SOD activity (Fig. 8A). During the cold treatment SOD activity of BL21(pET32a-Mn-SOD) was significantly higher than the control at 0 h without cold treatment (Fig. 8A), suggesting that cold stress significantly stimulated SOD activity of BL21(pET32a-Mn-SOD). While for BL21(pET32a) only at 4 h of the cold treatment SOD activity significantly increased (Fig. 8B). Taken together, these results suggested that the over-expressed MpmMn-SOD in bacteria not only increased SOD activity overall, but also enhanced the response of SOD activity of the cells to cold stress. Accordingly, the O_2•$^-$ contents in BL21(pET32a-Mn-SOD) were significantly lower than the control bacteria overall, suggesting that the over-expressed MpmMn-SOD effectively scavenged O_2•$^-$ contents in cells (Fig. 8B). Pearson's correlation analysis showed that in BL21(pET32a-Mn-SOD) under -4 °Ctreatment, the SOD activity and O_2•$^-$ contents were negatively correlated with a correlation coefficient of -0.995 (p<0.05).

Commented [SA(6]: Whatever the authors are trying to convey here is not clear at all.

Commented [SA(7]: Relative to 0h. Please describe each of those letters above bar graphs in legend.

Commented [SA(8]: Figure 8A, shouldn't it be? 8B indicates superoxide level changes.

Deleted: significantly

The relative increase in SOD activity at 2 h and 6 h of the cold treatment was 2.3 and 2.0 times of those of the control (Fig.8C). Correspondingly, the changes of the relative decrease in $O_2\bullet^-$ contents under cold conditions (Fig.8D) was roughly the same as the changes of the relative increase in SOD activity. That means under cold conditions the more increase in SOD activity, the more decrease in $O_2\bullet^-$ contents.

In the Discussion section we have revised as follows: Within our expectations, the enzyme activity during the cold treatment period was significantly higher than the control bacteria, and cold stress could stimulate SOD activity of the bacteria cells. Correspondingly, the $O_2\bullet^-$ contents were significantly lower than the control bacteria during the cold treatment period, indicating that the overexpression of MpmMn-SOD in *E. coli* cells enhanced cells ability to scavenge ROS thus to reduce oxidative damage under cold conditions. The changing trends of the relative increase of SOD activity and the relative decrease of $O_2\bullet^-$ contents under cold stress was consistent, implying the more increase in SOD activity, the more decrease in $O_2\bullet^-$ contents. The low levels of these values at 4 h of cold treatment may due to a self-regulation of the cells to keep a relative subcellular balance in SOD gene expression. However, we noticed that at 0 h, the relative decrease of $O_2\bullet^-$ contents were roughly at the same level as at 2 h under cold conditions. This result may be explained as BL21(pET32a-Mn-SOD) had higher SOD activity than the control and it functioned well at room temperature.

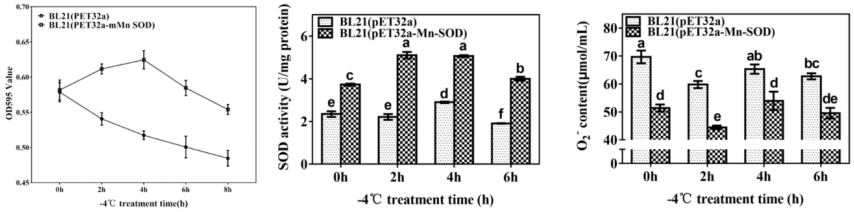
8. Does the over-expressed enzyme in BL21 bacteria localize to the mitochondria and if it does not, would that affect its effective activity? Further, I encourage the authors to have the manuscript proofread.

Response: Thanks for this question. Since *E. coli* BL21 belongs to prokaryote, it has no organelles such as mitochondria. Therefore, the over-expressed enzyme in BL21 bacteria does not localize to the mitochondria but to the intracellular. It is possible the enzyme's activity would, to some extent, be affected in *E. coli* system, however, the results in this study showed that the over-expressed enzyme could effectively scavenge $O_2\bullet^-$ and protect cells against cold stress. We have supplemented the figure of the bacteria survival at -4 °C **(**figure 6) to show this effectiveness. The manuscript has been proofread.

Commented [SA(9]: Sentence seems incomplete here.

9. In the experiments leading to Figure 6, it is mentioned that OD 595 readings were taken at the end of the assay. Would you mind sharing this as well? I would like to see how viability is affected by prolonged treatment to low temperatures and how this might affect the data.

Response: OD595 readings are presented below (left figure). To make the content of this manuscript more coherent, OD595 readings were supplemented in Fig.6 in the revised version. The survival of the experimental bacteria BL21(pET32a-Mn-SOD) is significantly better than the control bacteria BL21(pET32a) after the cold treatment, suggesting that MpmMn-SOD significantly enhanced the cold resistance of the transformed bacteria. With the prolonging of the cold stress, OD595 values began to decrease after a peak at 4h till at 8 h lower than the control at 0 h. These changes were consistent with the changes of SOD activity in BL21(pET32a-mMn-SOD) during the cold stress (middle figure). The $O_2^{\bullet-}$ contents were negatively correlated to SOD activity.



Deleted: and integrity

Characterization of a Mn-SOD from the desert beetle *Microdera punctipennis* and its increased resistance to cold stress in *E. coli* cells

Zilajiguli Xikeranmu, Ji Ma, Xiaoning Liu*

Xinjiang Key Laboratory of Biological Resources and Genetic Engineering, College of Life Science and Technology, Xinjiang University, 666 Shengli Road, Urumqi 830046, China

Corresponding Author:
Xiaoning Liu
Xinjiang Key Laboratory of Biological Resources and Genetic Engineering, College of Life Science and Technology, Xinjiang University, Urumqi 830046, China
Email address: liuxn0103@sina.com (Xiaoning. Liu)

Abstract

Insects have developed a complex network of enzymatic antioxidant systems for handling reactive oxygen species (ROS) generated during stress. Superoxide dismutases (SODs) play a determinant role in balancing ROS in insect. However, studies devoted to SODs functions in insects under cold stress are limited. In the present study, we attempted to identify and characterize a mitochondrial manganese SOD (mMn-SOD) from the desert beetle *Micordera punctipennis* (denoted as MpmMn-SOD) and explore its protective effects on bacteria cells under cold stress. MpmMn-SOD is composed of 202 amino acids with conserved domains required for metal ions binding and enzyme activity. qRT-PCR experiments revealed that the expression of *MpmMn-SOD* was ubiquitous but tissue-specific and was induced by cold stress. An *E. coli* system (BL21) was applied to study the function of MpmMn-SOD. The MpmMn-SOD gene was cloned into the prokaryotic expression vector pET-32a to generate a recombinant plasmid pET-32a (*MpmMn-SOD*). After transformation of the plasmid into *E. coli* BL21, the fusion proteins Trx-His-MpmMn-SOD was overexpressed and identified by SDS-PAGE and Western blotting. The transformed bacteria BL21 (pET32a-mMn-SOD) showed enhanced cold resistance compared to the control bacteria BL21 (pET32a). Its SOD activity under -4 °C had a significant negative correlation (r =-0.995) with superoxide anion $O_2^{\bullet-}$ contents. Antioxidant activity assay showed that the death zones of BL21 (pET32a-mMn-SOD) were smaller in diameter than the control bacteria. Accordingly, the transformed bacteria had lower electric conductivity and malondialdehyde (MDA) contents than the control bacteria under cold stress. Taken together, our results showed that cold stress stimulated the expression

Deleted: was

of *MpmMn-SOD* in *M. punctipennis*. The *E. coli* cells that overexpress MpmMn-SOD could increase their resistance to cold stress by scavenging ROS, and mitigate potential cell damage caused by ROS under cold conditions.

Introduction

Oxygen is essential for most life forms. The full reduction of oxygen to H_2O by cytochrome oxidase is a key step in the mechanism of aerobic ATP formation (*Hermes-Lima et al., 2001*). However, the partial reduction of oxygen leads to the formation of various reactive oxygen species (ROS). Superoxide anion radical ($O_2\bullet^-$) is usually the first ROS to be generated. The equilibrium between the production and the scavenging of ROS may be perturbed by various biotic and abiotic stress factors such as salinity, UV radiation, drought, heavy metals and temperature extremes (*Sarvajeet Singh & Narendra, 2010*). Insects are constantly subjected to changes in environmental temperature. Low temperature is a major environmental constraint that impacts the geographic distribution and seasonal activity patterns of insects (*Denlinger DL, 2010*). Cold stress may result in oxidative stress with the accumulation of ROS (*Gharari et al., 2014; Jithesh et al., 2006*).

Unbalanced high levels of ROS in living organisms under stress can cause potential damage to biological macromolecules (*Gutierrez et al., 2010*). To defend against the oxidative injury of ROS, cells are equipped with myriad antioxidant enzymes to scavenge and detoxify the accumulated oxyradicals (*Arenas-Ríos et al., 2007; Park SY, 2004; Vaughan, 1997*). Enhanced antioxidants could provide this same action to support winter survival by cold-hardy insects (*Denlinger DL, 2010*). The dominant ROS, superoxide anion ($O_2\bullet^-$), is converted to hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD), then transformed to water via catalase (CAT) or glutathione peroxidase (GPx) (*Felton and Summers, 1995*; *Schafer and Buettner, 2001*).

SODs are the main antioxidant enzyme families in organisms. They are considered as the first defense line against oxidative stresses due to their function of converting $O_2\bullet^-$ to H_2O_2 and H_2O (*Ackerman & Brinkley, 1966; McCord & Fridovich, 1988*). SOD is unique in that its activity determines the concentration of O_2 and H_2O_2, the two Haber-Weiss reaction substrates, and it is therefore, central in the defense mechanism (*Bowler, 1992*). SODs are classified into three distinct groups in eukaryotes: intracellular copper/zinc SOD (icCuZn-SOD), extracellular copper/zinc SOD (ecCuZn-SOD, or EC-SOD) and manganese SOD (Mn-SOD) (*Zelko et al., 2002*).

Mn-SOD has received much attention because mitochondria is the main source of ROS (*Kailasam et al., 2011; Li et al., 2011*). Two types of Mn-SOD are known in eukaryotes:

Deleted: ,
Deleted: t
Deleted: by

mitochondrial Mn-SOD (mMn-SOD) that has a mitochondrial transit peptide for translocation and cytosolic Mn-SOD (cMn-SOD) without the peptide (*Lin et al., 2010*). Temperature stress was reported as one of the key mediators of ROS generation (*Harari et al., 1989; Rauen et al., 1999*). The mitochondrial electron-transport chain is responsible for a significant proportion of intracellular superoxide radical production (*Møller, 2010*). Low temperature can fall down the rate of enzymatic reactions, leading to a decrease in demand for ATP and accumulation of electrons in certain points of the respiratory chain. This situation promotes a sudden increase in the production of several ROS which relieve the burden of excess reducing potential. Cold stress is therefore associated with an increased intracellular oxidative stress, and an increase in antioxidants activity appears to be one of features of cold-adaptations (*Chattopadhyay, 2002*). Previous studies have shown that the expression of *Mn-SOD* gene is induced in response to cold stress in several insect species (*Kim et al., 2010; Gao XL et al., 2013; Gao XM et al., 2013; Jia et al., 2014*). Beetle *Micordera punctipennis* (Coleoptera: Tenebrionidae) is an endemic species in Gurbantunggut Desert in Xinjiang, China (*Hou et al., 2010*). The adult is cold hardy, the average temperatures of the soil surface and soil-in-5cm in January were -12 °C and -5 °C, respectively (*Huang et al., 2005*). In the low temperature transcriptome of *M. punctipennis*, GO (Gene ontology) term analysis showed that Mn-SOD is one of the eight significantly up-regulated genes that are related to abiotic stress response (*Tusong et al., 2016*). It is possible that Mn-SOD might be present in the mitochondrial matrix, near the primary source of superoxide, as occurs in other species and may respond to oxidative stress caused by cold stress. However, the function and characteristics of this protein in *M. punctipennis* are currently unclear. In this study we aim at (1) isolating and characterizing a mitochondrial Mn-SOD gene (*MpmMn-SOD*) from *M. punctipennis*; (2) investigate *MpmMn-SOD* distribution patterns in different tissues and temporal expression profiles at mRNA level after being challenged by low temperature in order to explore one of the possible mechanisms of the insect response to cold stress; (3) analyzing the antioxidant activity of the recombinant MpmMn-SOD and the $O_2^{\bullet-}$ contents under cold stress by over-expressing this protein in bacteria.; (4) examining the protective effects of MpmMn-SOD on the bacteria cells carrying *MpmMn-SOD* gene under cold stress. The results will help to primarily study the possible function of MpmMn-SOD in the desert beetle under cold conditions.

Materials & Methods

Insect treatments, total RNA extraction and cDNA synthesis

The beetles were collected from Wujiaqv (N 44° 29′, E 87° 31′, 410 m), which is about 100 km northeast of the geological center of Asia. The samples were returned to the laboratory

and kept in large plastic beakers containing dry sands at 30 ± 0.5 °C, 16:8 h (light: dark) photoperiod, relative humidity (RH) of 30 ± 6%. Adults were fed with wheat bran and fresh cabbage leaves.

Beetles were dissected in cold 1×PBS (phosphate belanced solution) to isolate different tissues, such as head, midgut, hindgut (containing Malpighian tubule), fat body and carcass (whole body after head, gut and fat body were removed).

As 4°C is the low temperature at which the insect beginsto respond to cold stress (*Hou et al., 2010*), the beetle individuals were exposed at 4 °C for different time periods (0.5 h, 1 h, 1.5 h, 2 h, 3 h, 5 h, 7 h, 9 h and 11 h, respectively, three replicates per treatment group). The individuals at room temperature (about 25 °C) without any cold treatment were used as control. After the cold treatment, beetles were immediately frozen in liquid nitrogen for RNA extraction. Total RNA extraction was performed by using Trizol reagent (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. RNA concentration was quantified by using a Nano-Drop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, USA). The cDNA was synthesized from 1.0 μg total RNA based on Reverse Transcriptase M-MLV (Takara, China) according to the manufacturer's instructions.

Deleted: has begun

Cloning of the full-length *MpmMn-SOD* cDNA

MpmMn-SOD fragment (transcriptomic ID c41919) which had the up-regulated expression at 4 °C was selected from the transcriptomic data of *M. punctipennis* (*Tusong et al., 2017*). The lacked 3'-sequence were obtained by SMARTer™ RACE cDNA Amplification Kit (Clontech, Beijing, China). Primers used in this experiment were detailed in Table. The PCR program was 95 °C for 5 min followed by 30 cycles of 94 °C for 30 s, 63 °C for 30 s, 72 °C for 1 min and a final extension at 72 °C for 10 min. For verification, PCR products were purified, and cloned into pMD18-T vector (Trans GenBiotech, Beijing, China), and then were transformed into competent *E. coli* cells (DH5a) for Sanger sequencing by Quintarabio, Urumqi, China.

The deduced amino acid domains in MpmMn-SOD were analyzed using the BLAST search program (http://blast.ncbi.nlm.nih.gov/ Blast.cgi). The physicochemical properties were predicted by using AntPASy's ProtParam Online Tool. Multiple sequence alignments among insect species in different orders were created with DNAMAN 6.0 software (http://www.lynnon.com). The signal peptide cleavage site was examined with SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP/) program. TargetP 1.1 (http://www.cbs.dtu.dk/services/TargetP/) was used to predict presence of a putative mitochondrial targeting sequence (MTS). Phylogenetic analysis was performed by IQTREE 1.6.2. The phylogenetic tree was constructed based on predicted amino acid sequences using the Maximum Likelihood (ML)

method with 5000 replicates bootstrap. Mn-SOD sequences in different insect species were downloaded from the database in NCBI website.

mRNA level of *MpmMn-SOD* detected by Fluorescent real-time quantitative PCR (RT-qPCR)

The expression of *MpmMn-SOD* transcript was assayed on a 7500 Real Time PCR System (Applied Biosystems, USA) using SYBR Green Mix to determine the expression profiles of *MpmMn-SOD* gene in *M. punctipennis* at 4 °C for 0.5~11 h as described above. Translation elongation factor (*EF-α*) was used as a reference gene to normalize the target gene expression levels among samples (*Xikeranmu et al., 2019*). Primers for RT-qPCR are detailed in Table. The qPCR amplification conditions were 94 °C for 2 min, followed by 40 cycles at 94 °C for 30 s and 62 °C for 30 s. The relative expression of the target gene was calculated using the comparative $2^{-\Delta\Delta CT}$ method. The change of the gene expression levels at 4 °C was normalized to the gene in the control at 25 °C.

The expression of *MpmMn-SOD* mRNA in head, midgut, hindgut, fat body and carcass were separately detected by RT-qPCR. The expression level of *MpmMn-SOD* in different tissues was normalized to that of the head which had the lowest expression level. The value at each time point was given as mean ± *S.E.* (*n*=3).

Prokaryotic expression and Western blot analysis of the fusion protein Trx-His- MpmMn-SOD

To obtain the recombinant MpmMn-SOD protein and examine whether it possesses antioxidant activity, DNAMAN was used to design primers containing *Bam*HI and *Xho*I restriction sites (Table) to amplify the coding sequence (CDS) of *MpmMn-SOD* gene. The amplified fragments were digested with the endonucleases, and subcloned into a pET-32a (+) expression vector that was digested with the same enzymes. The constructed plasmid denoted as pET-32a (MpmMn-SOD) was transformed into competent cells of *E. coli* BL21 (DE3). The parent vector pET-32a without inserts gene was transformed into BL21 (DE3), and used as a control. The two transformed bacteria were induced with 0.5 mM isopropyl β-D-thiogalactoside (IPTG) at 25 °C for 10 h to overexpress fusion proteins Trx-His-MpmMn-SOD (41 kDa) and Trx-His (the tag protein on the vector, 18.5 kDa) respectively in *E. coli*. Luria broth (LB) was used for bacterial culture medium. Expression efficiency of different transformants was assessed by analysis of the target protein band in dodecyl sulfate, sodium salt (SDS)-Polyacrylamide gel electrophoresis (SDS-PAGE). The correct expression of the proteins was further confirmed by Western blotting with anti-His antibody (Zsbiotech, Beijing, China).

Table

Measurement of SOD activity and $O_2\bullet^-$ content in the MpmMn-SOD overexpressed bacteria at -4 ℃

As 4 °C is not enough to influence bacteria survival within short time, we treated the MpmMn-SOD overexpressed bacteria under -4 °C. BL21(pET-32a) were set as the control. Cultures of the two bacteria were induced to produce proteins Trx-His-MpmMn-SOD and Trx-His separately by addition of IPTG described above, and 5 mL cultures of the bacteria were exposed to -4 °C for 0 h, 2 h, 4 h and 6 h, respectively. At the end of the cold treatments, the bacteria in each group were recovered at 37 °C for 1 h, and OD595 was determined for making survival curve. The control was -4 °C for 0 h no cold treatment. Each treatment had three replicates.

Then, the cells were harvested by centrifugation (12000 rmp,10 minutes, at 4 °C). The collected cells were sonicated in an ice bath after suspension in PBS. The supernatants were collected as crude enzyme liquids and were quantified using the BCA Protein Assay Kit (Thermo Scientific Pierce, IL, USA). $O_2\bullet^-$ content was measured according to the hydroxylamine oxidation method described by Wang et al (*Wang & Luo, 1990*). The SOD activity was detected using the CuZn/Mn-SOD Assay Kit (Jiancheng, Nanjing, China) following the manufacturer's protocol with minor modification (*Meng et al., 2013*).

The increase of SOD activity was calculated by subtracting the SOD activity of BL21(pET-32a-MpmMn-SOD) from that of the control BL21(pET-32a), then dividing the difference by the SOD activity of BL21(pET-32a-MpmMn-SOD). So, did for calculating the decrease of $O_2\bullet^-$ content.

Antioxidant activity assay by the Oxford Cup method

To test whether MpmMn-SOD has antioxidant activity, the tolerance to hydroperoxide of the *E. coli* cells overexpressing Trx-His-MpmMn-SOD was determined by the Oxford Cup method (*Liu, 2018*). The bacteria BL21(pET-32a-MpmMn-SOD) and BL21(pET-32a) were grown overnight at 37 °C in LB (Luria-Bertani) broth containing ampicillin (Amp^+) (50 mg/L), and then diluted 1: 100 in LB medium. The diluted cells were further incubated at 37 °C until a final optical density of 0.4~0.6 at 595 nm. These cells were induced with 0.3 mM IPTG at 25°C for 10 h. Then, 100 μL BL21(pET-32a-MpmMn-SOD) and BL21(pET-32a) were, respectively, added to fresh LB (Amp^+) solid medium in plates. After the medium is solidified, five Oxford cups were placed on the plate, then 100 μL of different concentrations (100, 75, 50, 25 and 0 mmol/L) of H_2O_2 were added to the top of the oxford cup. The plate was incubated overnight at 37 °C. Three replicates per treatment group. BL21(pET-32a) cells were used as the control. The agent diffused into the surrounding area through the Oxford Cup to form a

decreasing concentration gradient. Observe the zone of inhibition formed around the cup and record the diameter of the zone. The inhibition zones were measured as described by Burmeister et al. (*Burmeister et al., 2008*).

Measurement of the Relative electrical conductivity (REC) and Malondialdehyde (MDA) content of bacteria BL21(pET-32a-MpmMn-SOD) under -4 ℃

The influence of low temperature on cell membrane permeability was determined by measuring the relative electrical conductivity (REC) in bacteria BL21(pET-32a-MpmMn-SOD) and BL21(pET-32a), respectively. BL21(pET-32a) was used as the control. After the recombinant protein was expressed by IPTG induction, 5 mL of the bacteria were centrifuged at 6000 rpm for 10 min to collect cells. The cells were washed with 5% dextrose solution until the bacterial solution's REC (denoted as L1) was comparable to that of 5% glucose solution. 5 mL of the isotonic bacterial solutions was stored at −4 °C for 0 h, 2 h, 4 h and 6 h, respectively, following which REC was measured for each time point (denoted as L2). After boiling for 5 min, the REC (denoted as L0) of the treated bacteria solution was measured again. The final relative conductivity was calculated as: REC (%) =100× (L2 -L1)/L0.

For MDA determination, 5 mL of the cultures were exposed at −4 ℃ for 0 h, 2 h, 4 h and 6 h, respectively after IPTG induction. Then, 400 μL MDA extract solution (MDA Assay Kit, Solarbio, Beijing, China) were added to the bacteria cells (about two million) to lyse the cells. The mixture was centrifuged at 8000 rpm for 10 min at 4 °C. The supernatant was collected and set on ice bath. The MDA content was determined by using MDA Assay Kit (Solarbio, Beijing, China) according to the manufacturer's protocol.

Statistical analysis

One-way analysis of variance and Tukey's multiple comparison test were conducted for data analysis for gene expression, SOD activity measurement, $O_2^{\bullet-}$ content determination. Paired *t*-test was employed for analyzing data from experiments measuring diameter of inhibition zone, relative conductivity and MDA content. Spearman's correlation analysis was used for correlation analysis of SOD activity and $O_2^{\bullet-}$ content. Data were shown as mean ± *S.E.*

Commonly used acronyms

thioredoxin–histone(Trx-His), intracellular copper/zinc superoxide dismutase(icCu-Zn/SOD), extracellular copper/zinc superoxide dismutase(ecCuZn-SOD or EC-SOD), cytosolic manganese superoxide dismutase(cMn-SOD),isopropyl β-D-thiogalactoside (IPTG), mitochondrial targeting sequence (MTS), Gene ontology(GO), relative humidity(RH), phosphate belanced solution(PBS), dodecyl sulfate, sodium salt (SDS)-Polyacrylamide gel electrophoresis(SDS-PAGE), Luria-Bertani(LB), Relative electrical conductivity(REC).

Deleted: Set

Deleted: then

Deleted: measure the

Deleted: respectively

Deleted: is

Deleted: after IPTG induction

Deleted: in

Deleted: as well as

Deleted: data analysis

Deleted: in

Results

Identification and characterization of the MpmMn-SOD sequence

We obtained the *MpmMn-SOD* sequence from the transcriptomic data of *M. punctipennis*, and then confirmed this sequence by cDNA cloning. The full-length cDNA was 1359 bp including an ORF of 609 bp, a 3'-UTR of 750 bp with a poly (A) tail and a single polyadenylation signal (AATAAA). The open reading frame (ORF) was 609 bp encoding a protein of 202 amino acids (GenBank accession no. MK676072.1), no signal peptide was predicted. It was predicted as a Mn-SOD sequence containing one N-glycosylation site (NGTL) (circled in Fig.1). The calculated molecular mass was 22 kDa, and the estimated pI was 6.54. Analyses using the online tool TargetP revealed a putative N-terminal mitochondrial targeting sequence (MTS) consisting of 12 amino acids (underlined in Fig.1), indicating this protein may exist in mitochondria. We designated this sequence MpmMn-SOD.

Fig 1

Fig 1. Nucleotide and deduced amino acid sequences of a Mn-SOD from the beetle *M. punctipennis*. The letters in box indicate the start codon (ATG) and the stop codon (TAA). The putative mitochondrial targeting sequence (MTS) is underlined in black. The putative polyadenylation signal (AATAAA) and poly A are underlined in blue. The potential N-glycosylation site is shown in pink (NGTL). The Mn-SOD signature motif (DIWEHAYY) is highlighted in cyan. The four highly conserved amino acids (His26, His76, Asp162, and His166) critical for Mn-binding are circled in red.

Comparison of the predicted amino acids of MpmMn-SOD with Mn-SODs from different insect species indicated the high conservation of four manganese binding sites (His26, His76, Asp162 and His166), and one signature of Mn-SOD from 162 to 169 (DV/IWEHAYY) was also conserved across these insect species (Fig. 2). MpmMn-SOD was most like the yellow meal worm Mn-SOD, the two sequences both had a shortened N-terminal sequence compared to Mn-SODs from insects in other taxonomic order. However, the identity of these two sequences was only 35.27%, suggesting that MpmMn-SOD was a novel Mn-SOD in insects.

Fig 2

Fig 2. Multiple alignments of the deduced amino acid sequences of the Mn-SODs from the beetle *M. punctipennis* and other known insect species. The Mn-SOD signature DIWEHAYY is boxed in red (labeled Signature). Mn-binding sites are indicated with triangles. The amino acid homology up to 100% is shown in black, 75% ~99% is in pink and 50% ~74% in cyan. *M. punctipennis*: *Microdera punctipennis; T. molitor: Tenebrio molitor; A. mellifera*: *Apis mellifera; B.tabaci: Bemisia tabaci; H.armigera: Helicoverpa armigera; T. castaneum: Tribolium*

castaneum.

To further analyze MpmMn-SOD sequence with SOD sequences in other insects at evolutionary perspective, phylogenetic analysis was conducted. The results revealed two separate clusters, Mn-SOD and Cu/Zn-SOD, in the phylogenetic tree with strong bootstrap (100%) support, in accordance with their distinct metal cofactor requirements (Fig. 3). MpmMn-SOD was clustered with Mn-SODs. Within Cu/Zn-SOD clade, ecCu/Zn-SOD and icCu/Zn-SOD were classified as two subgroups with strong bootstrap support (98%), and ecCu/Zn-SOD subgroup was the basic form. MpmMnSOD was closed to the Mn-SOD from the yellow meal worm *Tenebrio molitor*.

Fig 3

Fig 3. Phylogenetic analysis of SOD sequences from *M. punctipennis* and other insect species based on predicted amino acid sequences. *A.cerana cerana: Apis cerana cerana; A. glabripennis: Anoplophora glabripennis; A. planipennis: Agrilus planipennis; A.rosae: Athalia rosae; A. tumida: Aethina tumida; B.mori: Bombyx mori; B.tabaci: Bemisia tabaci; C.formosanus: Coptotermes formosanus; C. lapponica: Chrysomela lapponica. D. helophoroides: Dastarcus helophoroides; D.melanogaster: Drosophila melanogaster; D.plexippus: Danaus plexippus; G.morsitans morsitans: Glossina morsitans morsitans; H.saltator: Harpegnathos saltator; M. punctipennis: Microdera punctipennis; O.biroi: Ooceraea biroi; P. coochleariae: Phaedon cochleariae; T. castaneum: Tribolium castaneum; T. molitor: Tenebrio molitor; Z. nevadensis: Zootermopsis nevadensis.*

Expression of *MpmMn-SOD* gene in different tissues

To examine the tissue distribution profile of *MpmMn-SOD* expression, the mRNA levels from head, midgut, hindgut, fat body and carcass were measured by using RT-qPCR. The results showed that *MpmMn-SOD* expressed in all the tissues we have checked, but the expression levels varied greatly among the tissues. The highest was in hindgut followed by fat body, midgut and carcass; the lowest was in head (Fig. 4A). Compared to that of the head, the expression level in hindgut, fat body, midgut and carcass was 57-fold, 17-fold, 5.3-fold and 3.5-fold of that of the head, respectively ($F_{(4,10)} = 111.645$, $P < 0.01$), suggesting a tissue specific expression pattern.

Fig 4

Fig 4. Relative mRNA levels of *MpmMn-SOD* gene detected by RT-qPCR. (A) The expression profile of *MpmMn-SOD* gene in different tissues. Values are represented as fold change compared to that of the head; **(B)** Temporal expression of *MpmMn-SOD* gene under 4 °C cold stress. Values are represented as fold change compared to the control (0 h). Different letters above each column indicate statistical significance. $P<0.05$ (lower-case letters), $P<0.01$(capital letters).

Temporal expression of *MpmMn-SOD* in *M. punctpennis* at 4 °C

To determine the effect of cold stress on the expression of *MpmMn-SOD*, the beetles were exposed to 4 °C for different time periods, as 4 °C is the low temperature at which the insect has begun to respond to cold stress. The results showed that the mRNA level of *MpmMn-SOD* was significantly increased after the cold exposure compared with the control (25 °C), and this simulative effect was very significant ($F_{(9,18)} = 80.07, P < 0.001$) (Fig. 4B). It was approximately 9.9-fold, 22.5-fold and 125-fold of the control after cold exposure for 0.5 h,1 h and 1.5 h ($P <$ 0.01) respectively. The second large expression peak appeared at 11 h, which was about 67.3-fold of the control. From 2 h to 9 h the level slightly fluctuated with a small peak of 16-fold of the control at 3 h. This cold expression profile presented a stress-responsive pattern. The large fluctuation of *MpmMn-SOD* expression during the cold treatment indicated that cells could adjust the level of the enzyme timely and finely.

Prokaryotic expression and Western blot analysis of the fusion protein Trx-His- MpmMn-SOD

To study the enzyme activity of MpmMn-SOD, we inserted MpmMn-SOD into pET32a expression vector and transformed *E. coli* BL21 with this recombinant plasmid. pET32a alone was also transformed into *E. coli* BL21 as the control. The fusion protein Trx-His-MpmMn-SOD and the tag protein Trx-His were separately over-expressed in the two transformants through IPTG induction. The expression of these two proteins were analyzed on SDS-PAGE (Fig. 5A). A clear thick band of about 41 kDa appeared in lane 4 after IPTG induction, it matched the calculated size of the molecular mass of Trx-His-MpmMn-SOD; and a clear thick band of about18.5 kDa appeared in lane 2 after IPTG induction, it matched the calculated size of the molecular mass of Trx-His. The two proteins were absent in the un-induced samples. We further confirmed the fusion protein by Western blotting using anti-His antibody (Fig. 5B). The result indicated that the fusion protein Trx-His-MpmMn-SOD was correctly expressed in *E. coli*.

Fig 5

Fig 5. Analysis of the fusion protein Trx-His-mMpMn-SOD overexpressed in BL21 cells. **(A)** SDS-PAGE analysis of the whole cell lysate. **(B)** Western blot analysis. M: protein marker; lane1: non-induced BL21(pET32a); lane2: induced BL21(pET32a); lane3: un-induced BL21(pET32a-mMn-SOD); lane4: induced BL21 (pET32a-mMn-SOD).

The tolerance of the MpmMn-SOD overexpressed BL21 (pET32a-mMn-SOD) to oxidative stress

Antioxidant activity assay was performed to evaluate whether the overexpress mMpMn-SOD could enhance the tolerance of BL21 (pET32a-mMn-SOD) to oxidative stress. The results showed that the death zones around the discs on the BL21 (pET32a-mMn-SOD) plate were smaller in diameter than the control BL21 (pET32a) (Fig. 6A). The diameters of the inhibit zones on agar plates were significantly smaller than the control bacteria (Fig.6B), demonstrating that the mMn-SOD-overexpressing *E. coli* was more tolerant to H_2O_2-mediated oxidative damage than the control BL21 (pET32a).

Fig 6

Fig 6. Antioxidant activity assay on LB agar plates containing *E. coli* cells overexpressing MpmMn-SOD. Oxford cups containing different concentrations of H_2O_2 were used to generate oxidative stress to the cells. (A) Inhibited zones of the bacteria on agar plates. Numbers 1~4 on each disc represents different H_2O_2 concentrations from 100 mM to 25 mM. mMn-SOD: *E. coli* cells BL21(pET32a-mMn-SOD); Control: *E. coli* cells BL21(pET32a). **(B)** Quantitative diameters of the inhibited zones in histograms. Values are compared to the control bacteria between the same group. The data are the mean ± *S.E.* of three replicate

Survival of the MpmMn-SOD overexpressed BL21(pET32a-mMn-SOD) under cold stress at -4 °C

As *E. coli* is not significantly harmed by exposure to 4 °C for a limited time, we exposed the MpmMn-SOD overexpressed bacteria BL21(pET32a-mMn-SOD) to –4 °C to test the protective function of MpmMn-SOD for the bacteria under cold stress. The survival curve of BL21(pET32a-mMn-SOD) was a convex type, while it was a rough negative line for the control bacteria (Fig.7), indicating the cold resistance of BL21(pET32a-mMn-SOD) was significantly increased compared to the control bacteria BL21(pET32a). The OD595 of BL21(pET32a-mMn-SOD) was 0.63 at 4 h of the cold treatment, which was significantly higher than the control bacteria of 0.52 under the same conditions.

Fig. 7

Fig.7. Survival curve of the MpmMn-SOD overexpressed BL21 (pET32a-mMn-SOD) at -

386 **4 ℃**

387 We detected the changes of the SOD activity and $O_2\bullet^-$ content in the MpmMn-SOD
388 overexpressed bacteria after -4 °C treatment. The results showed that the cold stress
389 significantly stimulated SOD activity of BL21(pET32a-Mn-SOD) compared to BL21(pET32a)
390 (Fig. 8A), suggesting the over-expressed MpmMn-SOD in bacteria not only increased SOD
391 activity overall, but also enhanced the response of SOD activity of the cells to cold stress.
392 Accordingly, the $O_2\bullet^-$ content in BL21(pET32a-Mn-SOD) was significantly lower than the
393 control bacteria, suggesting that the over-expressed MpmMn-SOD effectively scavenged $O_2\bullet^-$
394 in cells (Fig. 8B). Pearson's correlation analysis showed that the SOD activity and $O_2\bullet^-$ content
395 of BL21(pET32a-Mn-SOD) under -4 °Ctreatment were strong negatively correlated, and the
396 correlation coefficient was -0.995 (p<0.05). The relative increase of SOD activity at 2 h and 6
397 h of the cold treatment was 2.3 and 2 times of those of the control (Fig.8C). Correspondingly,
398 at 2 h and 6 h time points the relative decrease of $O_2\bullet^-$ content (Fig.8D) was high. The two
399 indexes had similar changing trends, suggesting that the more increase in SOD activity, the
400 more decrease in $O_2\bullet^-$ content under -4 °C temperature.
401

Fig. 8

402 **Fig. 8. SOD activity and $O_2\bullet^-$ content in bacteria BL21 in response to -4 °C cold stress.** **(A)**
403 SOD activity in bacteria BL21 (pET32a) and (pET32a-mMn-SOD). **(B)** $O_2\bullet^-$ content in bacteria
404 BL21 (pET32a) and (pET32a-mMn-SOD). **(C)** Relative increase in SOD activity. **(D)**
405 Relative decrease in $O_2\bullet^-$ content. Different letters over each column indicate statistical
406 significance, $P < 0.05$.

407 **Relative electrical conductivity (REC) and Malondialdehyde (MDA) content of the**
408 **MpmMn-SOD overexpressed BL21 (pET32a-mMn-SOD) under cold stress at -4 ℃**

409 The excessive accumulation of ROS under cold stress may cause lipid peroxidation which
410 leads to damage of cell membranes. Therefore, electrolyte leakage and MDA level in the BL21
411 (pET32a-mMn-SOD) and the control BL21 (pET32a) under -4 °C cold stress were determined.
412 After cold stress, the two groups of bacteria showed increased REC and MDA content (Fig.9),
413 indicating that -4 °C caused cells damage to both groups. However, the increasing trends of the
414 two indexes in BL21(pET32a-mMn-SOD) were slower than those of the control BL21(pET32a)
415 during the cold treatment. At 4 h and 6 h of the cold treatment, both REC and MDA content in
416 the control group were significantly higher than the experimental group (Fig. 9A, 9B),
417 indicating that the cell membrane injury in BL21(pET32a-mMn-SOD) caused by the cold stress
418 was less serious than that in the control bacteria. These results suggested that the overexpressed
419 MpmMn-SOD conferred cold tolerance to cells via increasing their ability for ROS-scavenging

thus reducing membrane damage.

Fig 9

Fig 9. Protective effect of MpmMn-SOD on bacteria BL21(pET32a-mMn-SOD) in response to -4 °C cold stress. (A) Relative conductivity of BL21(pET32a-mMn-SOD) and BL21(pET32a) under -4 °C. **(B)** MDA content of BL21 (pET32a-mMn-SOD) and BL21 (pET32a) under -4 °C. Paired t-test was conducted to analyze the difference between BL21 (pET32a-mMn-SOD) and BL21 (pET32a) at each time treatment group. The symbol * indicates statistical significance $P < 0.05$. Values are expressed as means ±*S.E.* (n=3).

Discussion

When insects suffer from environmental stresses such as extreme temperatures, reactive oxygen species (ROS) are spawned (*Gao XM et al., 2013*). Metalloenzyme SOD is the most effective intracellular enzymatic antioxidant which is ubiquitous in all aerobic organisms and in all subcellular compartments prone to ROS mediated oxidative stress. It removes highly toxic $O_2^{\bullet-}$ and hence prevents the risk of hydroxyl radical OH• generation via the metal catalyzed Haber-Weiss-type reaction (*Fridovich, 1978*). Mn-SOD is considered as a general stress responsive factor whose expression might be influenced by a variety of intracellular and environmental cues including cold stress at transcriptional and/or translational levels (*Cho et al., 2006; Zelko et al., 2002*). Only a little is known to date about oxidative stress induced by cold and functional characterization of SOD in cold-hardy insects. In the present study, a *mMn-SOD* gene, *MpmMn-SOD*, from the desert beetle *Microdera punctipennis* was cloned, characterized and the cold protective effect of MpmMn-SOD protein was investigated for the first time.

Sequence analysis showed that *MpmMn-SOD* encodes four metal-binding residues (His26, His76, Asp162, and His166) and a highly conserved Mn-SOD amino acid motif DI/VWEHAYY, suggesting that these sites were essential to the structure and function of Mn-SODs. The identification of the signature sequence and the conserved metal-binding residues suggested that MpmMn-SOD possessed the essential properties of Mn-SOD family. Many mitochondrial proteins are synthesized as precursors containing MTS. The finding of MTS in MpmMn-SOD sequence suggested that MpmMn-SOD was of precursor type being transported into the mitochondria (*Yamamoto et al., 2005c*).

A BLASTP search at GenBank revealed that MpmMn-SOD sequence was most close to amino acid sequence of Mn-SOD from the yellow meal worm *T. molitor* with identity of 35.27%, indicating that MpmMn-SOD was more diverged from the other Mn-SODs in insects.

Phylogenetic analysis confirmed this relationship. These two insects are belonging to the family of Tenebrionidae (Coleoptera), their Mn-SOD sequences both were short at the N-terminal. The close relationship of their Mn-SOD sequences may roughly reflect their taxonomic relationships. The phylogenetic tree revealed that Mn-SOD and Cu/Zn-SOD may originate from a same ancestor, and Cu/Zn-SOD may have evolved longer than Cu/Zn-SOD. Besides, Cu/Zn-SOD clade was subdivided into ecCu/Zn-SOD and icCu/Zn-SOD, and ecCu/Zn-SOD subgroup showed more divergency than icCu/Zn-SOD, these two protein sub-families may evolve by gene replication.

Previous study suggested that *Mn-SOD* in insect is widely distributed in a variety of cells and tissues (*Zelko et al., 2002*). We found that *MpmMn-SOD* also distributes in all the tested tissues, but the expression levels varied greatly among tissues, and the highest was in hindgut, followed by in fat body. Thus, *MpmMn-SOD* may mainly function in hindgut and fat body. The hindgut includes Malpighian tubule which plays an important role in detoxification and elimination of toxins (*Beyenbach et al., 2010*). And fat body is one of the prime sites for antioxidant enzymes (*Kwang Sik et al., 2005; Yamamoto et al., 2005a; Yamamoto et al., 2005b; Yamamoto et al., 2005d*). Our result is similar to those on *Glossina morsitans* (*Munks et al., 2010*) and *Agrilus planipennis* (*Rajarapu et al., 2011*), they both have significant SOD mRNA levels in fat body and hindgut. The great up-regulation of *MpmMn-SOD* in hindgut and fat body, in turn, indicated that these two tissues were important sites for resisting oxidative attack.

Mn-SOD has been considered a stress-responsive factor and its expression at the transcriptional and translational levels might be influenced by a variety of intracellular and environmental factors, including cold stress (*Fukuhara et al., 2002*). The *Mn-SOD* mRNA of the fall webworm *Hyphantria cunea* (*Kim et al., 2010*) and the bee *Apis cerana* (*Jia et al., 2014*) were highly increased at 4 °C. Similar result is also reported in oriental fruit fly *Bactrocera dorsalis* exposed to 0 °C (*Gao et al., 2013*). Our previous work found that *MpmMn-SOD* was one of the eight significantly up-regulated genes related to abiotic stress response in the transcriptomic data of the cold treated insects (*Tusong et al., 2017*). Here, we confirmed the expression profiling of *MpmMn-SOD* at 4 °C for different time lengths to detect the responsive pattern of the gene to cold stress. We found that *MpMn-SOD* mRNA levels were very sensitively modulated by 4 °C cold stress. Within 0.5 h of 4 °C treatment, its expression increased to 9.9-fold of the control, and reached to 125-fold of the control at 1.5 h, strongly indicating that cold stress stimulates the expression of *MpmMn-SOD*. Our previous study on the *MpCu/Zn-SODs* showed that 4 °C stimulate the expression of *MpecCu/Zn-SOD* but not *MpicCu/Zn-SOD* (*Xikeranmu et al., 2019*). Compared with *MpecCu/Zn-SOD*, the *MpmMn-SOD* mRNA level was much higher than that of *MpecCu/Zn-SOD* which was 6.8-fold of the control 4 °C for 0.5

h, implying that *MpmMn-SOD* may play major role under cold stress. This was in consistent with the location of MpmMn-*SOD* in mitochondria, where the electron-transport chain is responsible for a significant proportion of intracellular superoxide radical production *(Møller, 2010)*. The rapid increase of the *MpmMn-SOD* levels under cold acclimation may reflect the adaptation of *M. punctipennis* to Guerbantonggut desert which is characterized with rapid and large temperature fluctuation. Similar result was found in the polychaete *Perinereis nuntia* treated with Cd (50 μg/L), in which *Mn-SOD* had a greater susceptibility than *Cu/Zn-SOD* (*Won, 2014*). It is noticeable that the cold expression profile of *MpmMn-SOD* under 4°C presented as a stress-responsive type, which is characterized with drastic fluctuation during the cold treatment period, the first and second large peaks appeared at 1.5h and 11 h of the cold treatment, which were 125-fold and 67.3-fold of the control respectively. The appearance of the second large peak looks like another round of cold defense is going on. These results may be interpreted as the cells timely adjusting the level of the enzyme to surrounding temperature to keep a relative intracellular balance, because stress-responsive expression is at the cost of the inhibition of other genes expression. On the other hand, with the prolong of the cold stress, ROS increased again, and cells need to produce more MpmMn-SOD to deal with the excessive ROS. Our previous work (*Xikeranmu et al., 2019*) showed that there was a rapid increase of $O_2^{\bullet-}$ content in the beetle after an exposure at 4 °C for 10 h, which is consistent with this result in this work.

Deleted: cold timely

The anti-oxidative activity of MpmMn-SOD was examined by investigating the involvement of MpmMn-SOD in anti-oxidative stress by agar plate diffusion assay. The bacteria that overexpressed MpmMn-SOD had significant smaller diameters of the inhibit zones on agar plates than the control bacteria, demonstrating that MpmMn-SOD can significantly enhance cells tolerance to H_2O_2-mediated oxidative stress. Jia et al (*Jia et al. 2014*) observed similar results with ours, which shows the diameters of the death zones between the *Apis cerana* mMn-SOD-overexpressing bacteria and the control bacteria are obviously different under oxidative stressors. Our results showed that MpmMn-SOD indeed is an antioxidant enzyme that protect cells from oxidative damage.

Overexpression of MpmMn-SOD in BL21(pET32a-mMn-SOD) showed significant protective effect for the bacteria under cold stress, the survival curve of BL21(pET32a-mMn-SOD) at -4 °C was a convex type, while it was almost a negative line for the control bacteria, suggesting the cold resistance of BL21(pET32a-mMn-SOD) was significantly increased compared to the control bacteria BL21(pET32a). Further, we determined the SOD activity and the $O_2^{\bullet-}$ content of the transformed bacteria under -4 °C conditions. Within our expectations, the enzyme activity during the cold treatment period was significantly higher than the control

bacteria, and cold stress could stimulate SOD activity of the bacteria cells. Correspondingly, the O_2•- content was significantly lower than the control during the cold treatment period, indicating that the overexpression of MpmMn-SOD in *E. coli* cells enhanced cells ability to scavenge ROS thus to reduce oxidative damage under cold conditions. The changing trends of the relative increase of SOD activity and the relative decrease of O_2•- content under cold stress was consistent, implying the more increase in SOD activity, the more decrease in O_2•- content. The low levels of these two indexes at 4 h of cold treatment may due to a self-regulation of the cells to keep a relative subcellular balance in SOD gene expression. However, we noticed that at 0h, the relative decrease of O_2•- content was roughly at the same level as at 2 h under cold conditions. This result may be explained as BL21(pET32a-Mn-SOD) had higher SOD activity than the control and it functioned well at room temperature.

Finally, we investigated the protective effect of MpmMn-SOD to BL21 (pET32a-mMn-SOD) under -4 °C cold conditions. ROS accumulation can lead to membrane peroxidation and thus destroy cell structure and function (*Mittler et al., 2004*). Thus, we measured relative electrolyte leakage and MDA level in the bacteria cells. Within prediction, the plasma membrane leakage and MDA contents in BL21 (pET32a-mMn-SOD) and BL21 (pET32a) both increased under -4 °C cold stress, but the upward trend of the conductivity and MDA levels in BL21 (pET32a-mMn-SOD) were significantly lower than the control bacteria (Fig.9). These results suggested that the damage degree to cell membrane under cold stress to the transgenic bacteria was significantly less than in control bacteria. Therefore, the high activity of MpmMn-SOD in the transformed bacteria should play its role in eliminating ROS, and thus preventing the membrane lipids from peroxidation. The present results agree with the work of Kwon et al (*Kwon et al., 2010*), who suggest that overexpression of SOD induced tolerance to membrane damage.

Conclusions

In conclusion, the identified and characterized mitochondrial manganese superoxide dismutase gene (*MpmMn-SOD*) from the desert beetle *Microdera punctipennis* was tissue-specific, and cold inducible. It had anti-oxidative activity. The MpmMn-SOD overexpressed bacteria treated at -4 °C showed increased cold resistance. Analysis of the SOD activity and O_2•- content in the MpmMn-SOD overexpressed bacteria treated at -4 °C revealed a significant negative correlation, implying that MpmMn-SOD cold act as a defense mechanism to mitigate cell damage caused by ROS under cold conditions. Accordingly, the MpmMn-SOD overexpressed bacteria could decrease the plasma membrane damage caused by lipid peroxidation and kept better plasma membrane integrity under cold stress. Our findings provide

basic data for further study the function, antioxidant mechanism and physiological responses of *Mn-SOD* gene in model species exposed to temperature changes. Obviously, additional studies based on our data are needed to gain further insights into the complex role of *Mn-SOD* gene in insect of cold tolerance.

Acknowledgements

We would like to acknowledge our fellow scholars, Fengjuan Zhang and Maimaitiaili abudunasier for help collecting and identifying the insects used in this research.

Funding

The research was funded by the National Natural Science Foundation of China (No. 31360527), and Tianshan Cedar Project in 2017(2017xs20).

Competing Interests

The authors declare that they have no competing interests.

Author Contributions

Zilajiguli Xikeranmu, Xiaoning Liu and Ji Ma conceived and designed the study. Zilajiguli Xikeranmu conducted all the experiments involved in this study. Zilajiguli Xikeranmu wrote the paper with contributions from Xiaoning Liu and Ji Ma. All authors read and approved the final version of the manuscript.

Data Availability

All data, models, and code generated or used during the study appear in the submitted article.

References

Ackerman NB, Brinkley FB. 1966. Oxygen tensions in normal and ischemic tissues during hyperbaric therapy. Studies in rabbits. *Jama the Journal of the American Medical Association* **198:**1280-1283 https://doi: 10.1001/jama.1966.03110250094027.

Arenas-Ríos E, León-Galván MA, Mercado PE, López-Wilchis R, Cervantes DLMI, Rosado A. 2007. Superoxide dismutase, catalase, and glutathione peroxidase in the testis of the Mexican big-eared bat (*Corynorhinus mexicanus*) during its annual reproductive cycle. *Comparative Biochemistry & Physiology Part A Molecular & Integrative Physiology* **148:**150-158 https:// doi:10.1016/j.cbpa.2007.04.003.

Beyenbach K, Skaer H, and Dow J. 2010. The Developmental, Molecular, and Transport Biology of Malpighian Tubules. *Annual Review of Entomology* **55:**351-374 https://doi: 10.1146/annurev-ento-112408-085512.

Bowler C. 1992. Superoxide dismutase and stress tolerance. *Annurevplant Physiolplant Molbiol* **43:**83-116 https://doi: 10.1146/annurev.arplant.43.1.83.

Burmeister C, Lã¼Ersen K, Heinick A, Hussein A, Domagalski M, Walter RD, and Liebau E. 2008. Oxidative stress in *Caenorhabditis elegans*: protective effects of the

Omega class glutathione transferase (GSTO-1). *Faseb Journal* **22:**343-354 https:// doi: 10.1096/fj.06-7426com.

Chattopadhyay MK. 2002. Low temperature and oxidative stress. *Current Science* **83:**109.

Cho YS, Choi BN, Kim KH, Kim SK, Dong SK, Bang IC, Nam YK. 2006. Differential expression of Cu/Zn superoxide dismutase mRNA during exposures to heavy metals in rockbream (*Oplegnathus fasciatus*). *Aquaculture* **253:**667-679 https:// doi:10.1016/j.aquaculture.2005.05.047.

Denlinger DL. 2010. Low temperature biology of insects. *Cambridge University Press*. https://doi.org/10.1017/CBO9780511675997.

Felton GW, Summers CB. 1995. Antioxidant systems in insects. *Archives of Insect Biochemistry and Physiology.* **29(2):**187-197 https://doi. 10.1002/arch.940290208.

Fridovich I. 1978. The biology of oxygen radicals. *Science* 201:875-880 https:// doi:10.1126/science.210504.

Fukuhara R, Tezuka T, Kageyama T. 2002. Structure, molecular evolution, and gene expression of primate superoxide dismutases. *Gene* **296:**99-109 https:// doi:10.1016/s0378-1119(02)00837-5.

Gao XL, Li JM, Wang YL, Jiu M, Yan GH, Liu SS, Wang XW. 2013. Cloning, expression and characterization of mitochondrial manganese superoxide dismutase from the Whitefly, *Bemisia tabaci*. *International Journal of Molecular Sciences* **14:**871-887 https:// doi:10.3390/ijms14010871.

Gao XM, Jia FX, Shen GM, Jiang HQ, Dou W, Wang JJ. 2013. Involvement of superoxide dismutase in oxidative stress in the oriental fruit fly, *Bactrocera dorsalis*: molecular cloning and expression profiles. *Pest Management Science* **69:**1315-1325 https://doi: 10.1002/ps.3503

Gharari Z, Nejad RAK, Shekasteband R, Najafi F, Nabiuni M, Irian S. 2014. The role of *Mn-SOD* and *Fe-SOD* genes in the response to low temperature in chs mutants of *Arabidopsis*. *Doga Turkish Journal of Botany* **38:**80-88 https:// doi:10.3390/ijms14010871.

Harari PM, Fuller DJM, Gerner EW. 1989. Heat shock stimulates polyamine oxidation by two distinct mechanisms in mammalian cell cultures. *International Journal of Radiation Oncology Biology Physics* **16:**451-457 https:// doi:10.1016/0360-3016(89)90341-6.

Hermes-Lima M, Storey JM, Storey KB. 2001. Chapter 20 Antioxidant defenses and animal adaptation to oxygen availability during environmental stress. *Cell & Molecular Response to Stress* **2:**263-287 https://doi.org/10.1016/S1568-1254(01)80022-X.

Hou F, Ma J, Liu X, Wang Y, Liu XN, Zhang FC. 2010. Seasonal changes in antifreeze protein gene transcription and water content of beetle *Microdera punctipennis* (Coleoptera, Tenebrionidae) from Gurbantonggut desert in Central Asia. *Cryo Letters* **31:**359-370 https://doi:10.1016/S1568-1254(01)80022-X.

Huang RX, Hong-Ying HU, Wei WU, Fan ZT, Suo FY. 2005. Formation and Evolution of Desert Insects in Xinjiang and Its Adjacent Regions. *Arid Land Geography*. **28(1) :** 38-44 https://doi:10.13826/j.cnki.cn65-1103/x.2005.01.008

Jaramillo-Gutierrez G, Molina-Cruz A, Kumar S, and Barillas-Mury C. 2010. The Anopheles gambiae oxidation resistance 1 (*OXR1*) gene regulates expression of enzymes that detoxify reactive oxygen species. *Plos One* **5:**e11168 https://

638 doi:10.1371/journal.pone.0011168 https://doi: 10.1007/s00299-006-0127-4
639 **Jia H, Sun R, Shi W, Yan Y, Li H, Guo X, Xu B. 2014.** Characterization of a mitochondrial
640 manganese superoxide dismutase gene from *Apis cerana cerana* and its role in oxidative
641 stress. *Journal of Insect Physiology* **60:**68-79 https://doi:10.1007/s12562-011-0334-y
642 **Jithesh MN, Prashanth SR, Sivaprakash KR,Parida A. 2006.** Monitoring expression
643 profiles of antioxidant genes to salinity, iron, oxidative, light and hyperosmotic stresses
644 in the highly salt tolerant grey mangrove, *Avicennia marina* (Forsk.) Vierh. by mRNA
645 analysis. *Plant Cell Reports* **25:**865-876 https://doi: 10.1007/s00299-006-0127-4.
646 **Kailasam M, Kaneko G, Oo AKS, Ozaki Y, Thirunavukkarasu AR, Watabe S. 2011.**
647 Effects of calorie restriction on the expression of manganese superoxide dismutase and
648 catalase under oxidative stress conditions in the rotifer *Brachionus plicatilis*. *Fisheries*
649 *Science* **77:**403-409 https://doi:10.1007/s12562-011-0334-y.
650 **Kim YI, Kim HJ, Kwon YM, Kang YJ, Lee IH, Jin BR, Han YS, Cheon HM, Ha NG, Seo**
651 **SJ. 2010.** Modulation of MnSOD protein in response to different experimental
652 stimulation in *Hyphantria cunea*. *Comparative Biochemistry & Physiology Part B*
653 *Biochemistry & Molecular Biology* **157:**343-350 https://
654 doi:10.1016/j.cbpb.2010.08.003.
655 **Kwang Sik L, Seong Ryul K, Nam Sook P, Iksoo K, Pil Dong K, Bong Hee S, Kwang Ho**
656 **C, Seok Woo K, Yeon Ho J,Mong LS. 2005.** Characterization of a silkworm
657 thioredoxin peroxidase that is induced by external temperature stimulus and viral
658 infection. *Insect Biochemistry and Molecular Biology* **35:**73-84 https://
659 doi:10.1016/j.ibmb.2004.09.008.
660 **Kwon SY, Jeong YJ, Lee HS, Kim JS, Cho KY, Allen RD, Kwak SS. 2010.** Enhanced
661 tolerances of transgenic tobacco plants expressing both superoxide dismutase and
662 ascorbate peroxidase in chloroplasts against methyl viologen-mediated oxidative stress.
663 *Plant Cell & Environment* **25:**873-882 https:// doi:10.1046/j.1365-3040.2002.00870.x.
664 **Li C, He J, Su X, Li T. 2011.** A manganese superoxide dismutase in blood clam *Tegillarca*
665 *granosa* : Molecular cloning, tissue distribution and expression analysis. *Comparative*
666 *Biochemistry & Physiology Part B Biochemistry & Molecular Biology* **159:**64-70
667 https://doi:10.1016/j.cbpb.2011.02.003
668 **Lin YC, Lee FF, Wu CL, Chen JC. 2010.** Molecular cloning and characterization of a
669 cytosolic manganese superoxide dismutase (cytMnSOD) and mitochondrial manganese
670 superoxide dismutase (mtMnSOD) from the kuruma shrimp *Marsupenaeus japonicus*.
671 *Fish & Shellfish Immunology* **28:**143-150 https://doi:10.1016/j.fsi.2009.10.012.
672 **Liu YC, Su H, Wang PC, Xu YL, Wei TC, Wang HB, Xu YS. 2018.** Cloning，Fusion
673 Expression and Enzyme Activity Analysis of Extracellular Copper Zinc Superoxide
674 Dismutase in Mulberry Pyralid，*Glyphodes pyloalis*. *Science of Sericulture* **44(2):**
675 0188-0195 https:// doi: 10.13441/j.cnki.cykx.2018.02.002.
676 **McCord JM, Fridovich I. 1988.** Superoxide dismutase: The first twenty years (1968–1988).
677 *Free Radical Biology Medicine* **5:**363-369 https:// doi:10.1016/0891-5849(88)90109-8.
678 **Meng Q, Jing C, Xu C, Huang Y, Wang Y, Wang T, Zhai X, Wei G, Wen W. 2013.** The
679 characterization, expression and activity analysis of superoxide dismutases (SODs)
680 from *Procambarus clarkii*. *Aquaculture* **406:**131-140 https://
681 doi:10.1016/j.aquaculture.2013.05.008.

Mittler R, Vanderauwera S, Gollery M, Breusegem FV. 2004. Reactive oxygen gene network of plants. *Trends in Plant Science* **9:**490-498 https:// doi: 10.1016/j.tplants.2004.08.009.

Møller IM. 2010. ROS signaling—specificity is required. *Trends Plant Science* **15(7):**370-374 https:// doi:10.1016/j.tplants.2010.04.008.

Munks RJ, Sant'Anna MR, Grail W, Gibson W, Igglesden T, Yoshiyama M, Lehane SM, Lehane MJ. 2010. Antioxidant gene expression in the blood-feeding fly *Glossina morsitans morsitans*. *Insect Molecular Biology* **14:**483-491 https:// doi:10.1111/j.1365-2583.2005.00579.x

Park SY KY, Yang DJ, Yoo MA. 2004. Transcriptional regulation of the *Drosophila* catalase gene by the DRE/DREF system. *Nucleic Acids Research* **32(4):**1318-1324. https:// doi:10.1093/nar/gkh302.

Rauen U, Polzar B, Stephan H, Mannherz HG, Groot HD. 1999. Cold-induced apoptosis in cultured hepatocytes and liver endothelial cells: mediation by reactive oxygen species. *Faseb Journal* **13:**155-168 https:// doi:10.1096/fasebj.13.1.155.

Sarvajeet Singh G,Narendra T. 2010. Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. *Plant Physiology Biochemistry* **48:**909-930 https:// doi: 10.1016/j.plaphy.2010.08.016.

Schafer FQ, Buettner GR. 2001. Redox environment of the cells as viewed through the redox state of the glutathione disulfide/glutathione couple. *Free Radical Biology and Medicine* **30:**1191-1212 https:// doi: 10.1016/S0891-5849(01)00480-4.

Swapna Priya R, Praveen M, Herms DA, Pierluigi B, Omprakash M. 2011. Antioxidant genes of the emerald ash borer (*Agrilus planipennis*): gene characterization and expression profiles. *Journal of Insect Physiology* **57:**819-824 https:// doi: 10.1016/j.jinsphys.2011.03.017.

Tusong K, Guo XX, Meng S, Liu XN, Ma J. 2017. Comparative analysis of the transcriptome of the overwintering desert beetle *Microdera punctipennis*. *Cryobiology* **78:**80-89 https:// doi:10.1016/j.cryobiol.2017.06.009.

Tusong K, Liu XY, Liu XN, Ma J. 2016. Transcriptomic analysis of the desert beetle *Microdera punctipennis*(Coleoptera: Tenebrionidae) in response to short-term cold stress. *Acta Entomologica Sinica*. **59(6):**581-591 https://doi:10.16380/j.kcxb.2016.06.001.

Vaughan M. 1997. Oxidative Modification of Macromolecules Minireview Series. *Journal of Biological Chemistry* **272:**18513-18513 https:// doi:10.1074/jbc.272.30.18513.

Wang AG, Luo GH. 1990. Quantitative Relation between the Reaction of Hydroxylamine and Superoxide Anion Radicals in Plants. *Plant Physiology Communications*. **6:**55-57. https:// doi:10.13592 /j.cnki.ppj.1990.06.031.

Won EJ, Ra K, Kim KT, Lee JS, Lee YM. 2014. Three novel superoxide dismutase genes identified in the marine polychaete *Perinereis nuntia* and their differential responses to single and combined metal exposures. *Ecotoxicology and Environmental Safety* **107:**36-45 https:// doi:10.1016/j.ecoenv.2014.03.026.

Xikeranmu Z, Abdunasir M, Ma J, Tusong K, and Liu XN. 2019. Characterization of two copper/zinc superoxide dismutases (Cu/Zn-SODs) from the desert beetle *Microdera punctipennis* and their activities in protecting *E. coli* cells against cold. *Cryobiology* **87:**

15-27. https:// doi:10.1016/j.cryobiol.2019.03.006.
Yamamoto K, Banno Y, Fujii H, Miake F, Kashige N, Aso Y. 2005a. Catalase from the silkworm, *Bombyx mori* : Gene sequence, distribution, and overexpression. *Insect Biochemistry & Molecular Biology* **35:**277-283 https:// doi:10.1016/j.ibmb.2005.01.001.
Yamamoto K, Zhang P, Banno Y, Fujii H, Miake F, Kashige N, Aso Y. 2005b. Superoxide Dismutase from the Silkworm,*Bombyx mori*:Sequence,Distribution,and Overexpression. *Journal of the Agricultural Chemical Society of Japan* **69:**507-514 https:// doi: 10.1271/bbb.69.507.
Yamamoto K, Zhang P, He N, Wang Y, Aso Y, Banno Y,Fujii H. 2005c. Molecular and biochemical characterization of manganese-containing superoxide dismutase from the silkworm, *Bombyx mori*. *Comparative Biochemistry & Physiology Part B Biochemistry & Molecular Biology* **142:**403-409 https:// doi:10.1016/j.cbpb.2005.09.002.
Yamamoto K, Zhang P, Miake F, Kashige N, Aso Y, Banno Y, Fujii H. 2005d. Cloning, expression and characterization of theta-class glutathione S -transferase from the silkworm, *Bombyx mori*. *Comparative Biochemistry & Physiology Part B Biochemistry*