Comparative Analyses of Cu-Zn Superoxide Dismutase (SOD1) and Thioredoxin Reductase (TrxR) at the mRNA Level between *Apis mellifera* L. and *Apis cerana* F. (Hymenoptera: Apidae) Under Stress Conditions

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**Abstract**

This study compared stress-induced expression of Cu-Zn superoxide dismutase (SOD1) and thioredoxin reductase (TrxR) genes in the European honeybee *Apis mellifera* L. and Asian honeybee *Apis cerana* F. Expression of both SOD1 and TrxR rapidly increased up to 5 h after exposure to cold (4°C) or heat (37°C) treatment and then gradually decreased, with a stronger effect induced by cold stress in *A. mellifera* compared with *A. cerana*. Injection of stress-inducing substances (methyl viologen, [MV] and H2O2) also increased SOD1 and TrxR expression in both *A. mellifera* and *A. cerana*, and this effect was more pronounced with MV than H2O2. Additionally, we heterologously expressed the *A. mellifera* and *A. cerana* SOD1 and TrxR proteins in an *Escherichia coli* expression system, and detection by SDS-PAGE, confirmed by Western blotting using anti-His tag antibodies, revealed bands at 16 and 60 kDa, respectively. Our results show that the expression patterns of SOD1 and TrxR differ between *A. mellifera* and *A. cerana* under conditions of low or high temperature as well as oxidative stress.

**Key words**: *Apis mellifera*, *Apis cerana*, Cu-Zn superoxide dismutase, thioredoxin reductase, stress

Oxidative stress generates reactive oxygen species (ROS), such as superoxide radicals, hydrogen peroxide, and hydroxyl radicals, resulting in damage to proteins, lipids, and DNA (Mount 1996, Dalton et al. 1999). Superoxide dismutase (SOD), which reduces such damage by responding to oxidative stress, converts superoxide into H2O2, which is then converted into H2O by catalase, glutathione peroxidase and reductase, or thioredoxin (Trx) peroxidase and reductase (Fridovich 1995). SOD typically generates hydroxyl (OH) groups in association with a metal cofactor. One of the SOD enzymes, Cu-Zn SOD (SOD1), is a key superoxide-scavenging enzyme found primarily in the cytoplasm and carries out a protective function against oxygen toxicity in all aerobic organisms (Crapo et al. 1992). Another SOD, Mn-SOD, is present in mitochondria, the major sites of superoxide production (Crapo et al. 1992). Vertebrates inevitably produce ROS during respiration (Gazl et al. 2006), and researchers have investigated SOD1 gene expression in insects, such as *Drosophila*, reporting that the enzyme also plays a significant role in eliminating ROS in insects. For example, Phillips et al. (1989) found that SOD1-deactivated insects had shortened lifespans due to their inability to remove ROS.

In fact, many studies have examined the effects of ROS removal on life expectancy, ultimately highlighting the importance of SOD1 (Orr and Sohal 1994, Sohal et al. 1995, Parkes et al. 1998, Sun and Tower 1999). Studies on the formation of ROS and the importance of SOD1 to ROS removal in *Bombus ignitus* have also been reported in Choi et al. (2006a). Thioredoxin reductase (TrxR), a selenoprotein and flavoprotein, contains a NADPH-binding site and disulfide bonds that are related to both oxidative and reductive reactions (Mustacich and Powis 2000). TrxRs catalyze the NADPH-dependent reduction of the redox protein Trx (Matsu and Powis 2000), and the reduced form of Trx is an excellent catalyst in interactions with factors that react to various types of oxidative stress. In addition, TrxRs are involved in cell growth, transformation, and oxidation prevention and are responsible for many other functions, such as the reduction of endogenous and exogenous compounds (Mustacich and Powis 2000). The function of Trx becomes even more important considering that glutathione reductase, the disulfide bonds of which are involved in extensive redox reactions (Bauer et al. 2003), is not expressed in such insects as *Drosophila*. The sequences of the SOD1 and TrxR genes of the...
European honeybee *Apis mellifera* (Lee et al. 2010) and the Asian honeybee *Apis cerana*, with the former being larger and brighter yellow in color, have been previously reported in Lee et al. (2010).

Because of the market for bee products, such as honey, propolis, and royal jelly, honeybees constitute an economically important insect. Furthermore, the market value of foods pollinated by honeybees exceeds U.S. $212 billion worldwide (Gallai et al. 2009). However, due to a phenomenon called Colony Collapse Disorder (CCD), >30% of worker bees have disappeared in the United States since 2006, and >25% have disappeared globally (van Engelsdorp et al. 2007). Although many studies have been performed to clarify the causes of CCD around the world, the disorder is not fully understood. Some of the various hypotheses regarding honeybee CCD include climate change and virus infection (UDSA 2012; Goulson et al. 2015). In particular, drastic climate change due to global warming could increase external stress on honeybees, and environmental stresses are thought to be associated with increases in disease susceptibility, which is attributable to evolutionary trade-offs between the energy demands required to address stress versus pathogens. For instance, temperature stress affects the expression of immune response genes in the alfalfa leafcutting bee *Megascolia rotundata* (Xu and James 2012). Chalkbrood occurs in other species of bees, most notably honeybees (*A. mellifera*), and larvae are more susceptible to the disease when maintained at a temperature slightly below the normal nest temperature (Vojvodic et al. 2011). Two honeybee species are reared in Korea, *A. cerana* and *A. mellifera*, and differences in their response to temperature is an interesting topic because the former produces less honey and has a high rate of swarming and absconding; there is also a lack of established beekeeping methods for *A. cerana*. Nonetheless, the honey produced by *A. cerana* sells at a higher price in Korea than *A. mellifera* honey due to its rarity. Therefore, the objectives of this study were to investigate the gene expression of the antioxidant enzymes SOD1 and TrxR in response to various stresses in *A. mellifera* and *A. cerana* and to characterize the SOD1 and TrxR proteins using an *Escherichia coli* expression system.

**Materials and Methods**

**Insects**

*A. mellifera* and *A. cerana* worker bees were obtained from a farm located in Cheungwon, Chungbuk Province, Republic of Korea (November 2011). The insects were acclimated at 25–27°C with a photoperiod of 16:8 (L:D) and 40–60% relative humidity for 3 d prior to utilization.

**Stress Conditions**

Approximately 10 d after adult emergence, *A. mellifera* and *A. cerana* worker bees were exposed to 4 or 37°C, and samples of five bees were collected after 1, 3, 5, 7, and 9 hr. The control group was maintained at 27°C. In addition, ~10 d after adult emergence, *A. mellifera* and *A. cerana* worker bees were injected with methyl viologen (MV) and H₂O₂ using a micro-applicator (Burkard Scientific, Middlesex, UK). MV was dissolved in phosphate-buffered saline (PBS), and 2 μl (10 mM) was injected into each individual (*n = 5*); 2 μl of H₂O₂ (10%) was injected into each individual. Samples of five bees were collected after 1, 3, 5, 7, and 9 hr. Control group was injected with 2 μl of PBS buffer. This test was performed with five replicates.

**Quantitative Real-Time PCR**

*A. mellifera* and *A. cerana* individuals were frozen in liquid nitrogen prior to whole-body homogenization in lysis buffer using a porcelain mortar and pestle. Total RNA was extracted using Easy-spin Total RNA Extraction Kit (iNtRON, Seoul, Republic of Korea) according to the manufacturer’s instructions. The extracted RNA was quantified and utilized for qRT-PCR. cDNA was then synthesized using Maxime RT PreMix (iNtRON, Seoul, Republic of Korea) following the manufacturer’s instructions. The extracted RNA was quantified and utilized for qRT-PCR. cDNA was then synthesized using Maxime RT PreMix (iNtRON, Seoul, Republic of Korea) following the manufacturer’s instructions. The extracted RNA was quantified and utilized for qRT-PCR. cDNA was then synthesized using Maxime RT PreMix (iNtRON, Seoul, Republic of Korea) following the manufacturer’s instructions. Primers for SOD1 and TrxR were designed based on the consensus sequence of previously reported genes (Lee et al. 2010) (Table 1). The housekeeping gene β-actin was used for normalization. qRT-PCR analyses were performed using 10 μl SYBR Premix Ex Taq (2×), forward primer (5 pmol/μl), reverse primer (5 pmol/μl), and 2 μl synthesized cDNA with a Rotor-Gene Q cycler (QIAGEN, Hilden, Germany). After incubation at 95°C for 5 min, a total of 40 cycles of PCR were conducted with the following conditions: 10 s at 95°C, 10 s at 55°C, and 15 s at 72°C. Calculations were performed using the Ct values obtained at the end of the PCR reaction with the Delta-Delta Ct method (Livak and Schmittgen 2001). Based on the equation ΔΔCt = (Ct target – Ct reference)treatment – AVERAGE (Ct target – Ct reference)controls, the expression level of pre-treatment samples (controls) was set to a value of 1; the results of post-treatment samples represents the fold change relative to the control sample, and the positive or negative nature of the ΔΔCt values indicates up- or down-regulation.

### Table 1. Primers used for qRT-PCR

| Target gene (GenBank No.) | Primer name | Primer sequence (5’→3’) | Product size (mer) |
|--------------------------|------------|-------------------------|------------------|
| SOD1* (NM001178027.1)   | SOD-F1     | TCCGTGAAGGTCACCGGTCAAGT | 104              |
|                          | SOD-R1     | GCACCAGACATTGTAACACCATGG |                |
| TrxR (GU188975.1/AY329357.1) | TrxR-R1 | CCTGTTGCTATACATGCGGTCG | 141              |
|                          | TrxR-R2    | TGGTGTGGCTATACATGCGGTCG |                |
| β-actin (AB072495.1/AB023025.1) | β-actin-F | TGCCAACACTGTCCGTTCTCG | 140              |
|                          | β-actin-R  | AGAATTGACCCACCAATCCA  |                |

*A A common sequence between *A. mellifera* and *A. cerana*.

### Table 2. Primers used for amplification and cloning

| Target gene | Primer name | Primer sequence (5’→3’)* | Size (mer) |
|-------------|------------|--------------------------|-----------|
| SOD1        | SOD-F2     | AAAGGATCCATGACTAAAGCAGTGGTGCG | 28        |
|             | SOD-R2     | TTTAGCTTCATTTTTGAATTCCAAATGCC | 33        |
| TrxR        | TrxR-F2    | AAAGGATCCATGCAACACAATTTTGCG | 28        |
|             | TrxR-R2    | TTTAGCTTCATGCAACACAATTTTGCG | 29        |

*Restriction sites are underlined.
**E. coli Expression System**

1) Construction of recombinant plasmid: To clone the target genes into the *E. coli* expression vector, a primer set was designed for insertion between *BamH* I and *Hind* III sites (Table 2). The PCR program consisted of an initial denaturation at 95°C for 5 min followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 62°C for 30 s, and extension at 72°C for 45 s, with a final extension at 72°C for 5 min. After the PCR products were confirmed on a 1% agarose gel, TA-cloning was performed with the pGEM-T vector system (Promega, Madison, WI) following the manufacturer’s instructions. Positive clones were first screened by agarose gel, TA-cloning was performed with the pGEM-T vector system (Promega, Madison, WI) following the manufacturer’s instructions. DNA sequencing was performed by Macrogen Inc. (Seoul, Republic of Korea). The constructs (pT-AmSOD1, pT-AmTrxR, pT-AcSOD1, and pT-AcTrxR) were digested with *BamH* I and *Hind* III, and the fragments were cloned into the expression vector pET-21 a (+) (Clontech, Mountain View, USA) for recombinant expression. The plasmids were transformed into TOP10 competent cells, which were grown on LB plates with ampicillin. The positive colonies were cultured in LB medium with ampicillin, and the recombinant plasmids (pET-AmSOD1, pET-AmTrxR, pET-AcSOD1, and pET-AcTrxR) were isolated. 2) Expression of recombinant protein: Recombinant plasmids were transformed into BL21 competent cells, the gene expression host, with selection on LB plates with ampicillin. Each selected positive colony was first cultured in LB liquid medium with ampicillin to an of OD600 0.6, the exponential growth phase. Next, 1.5 ml of the cultured cells from the primary culture was grown in a secondary culture in LB liquid medium with ampicillin to an OD600 of 1.0. Isopropyl-β-D-thiogalactopyranoside (IPTG, 1 mM) was then added to induce the expression of SOD1 and TrxR proteins. The cells were collected by centrifugation at 10,000 × g for 10 min and washed with PBS. The cells were sonicated in lysis buffer (50 mM Tris-HCl, 5% glycerol, 50 mM NaCl, pH 7.0) and centrifuged at 13,000 rpm for 5 min. The supernatants were subjected to 10% SDS-PAGE and Western blot analysis.

Fig. 1. Transcript levels of SOD1 and TrxR were determined in *A. mellifera* (A, B) and *A. cerana* (C, D) after exposure to thermal stress. Workers were incubated at 4°C (low) or 37°C (high) for 9 hr. Controls were kept at 27°C. Total RNA was extracted from whole *A. mellifera* and *A. cerana* bodies and used for qRT-PCR. The values are presented as the mean ± SD. The significance of the difference compared with the control value is indicated by *(P < 0.01)*.
Statistical Analysis
The gene expression levels of SOD1 and TrxR were compared by one-way analysis of variance. The means were separated by Tukey's test when significance at $P < 0.01$ and 0.05 was found (SAS Institute 2003). For all treatments, each assay was replicated five times with five independent tissue preparations.

Results and Discussion

SOD1 and TrxR Expression after Temperature Treatments
To identify the effect of the external temperature on SOD1 and TrxR expression, *A. mellifera* and *A. cerana* adults were exposed to low (4°C) or high (37°C) temperature conditions for 1, 3, 5, 7, and 9 hr; the controls were maintained at 27°C. As determined by qRT-PCR, expression of both SOD1 and TrxR rapidly increased in *A. mellifera* up to 5 hr after the treatment and then gradually decreased (Fig. 1A and B). The results in *A. cerana* showed a similar trend (Fig. 1C and D), with these genes in both species having a relatively higher induction after exposure to low temperature (4°C) than high temperature (37°C). Additionally, the relatively higher expression level in *A. mellifera* compared with *A. cerana* is consistent with the fact that *A. cerana* appeared to be less stressed than *A. mellifera*. Based on visual observation, *A. cerana* was more active than *A. mellifera* at a low temperature, demonstrating that *A. cerana* is more tolerant to cold than *A. mellifera*. Sakagami (1959) also reported that *A. cerana* is more tolerant and less aggressive than *A. mellifera* and commented on the superiority of *A. mellifera* in terms of their larger colony size, strong fighting capacity. Choi et al. (2006a) reported that SOD1 expression in bumblebees increased up to 3 hr after treatment at 4°C and then gradually decreased, but that treatment for 2 hr at 37°C decreased SOD1 expression; they also found the highest level of expression of a Trx-like protein at ~4 hr after the thermal stress treatment, which decreased thereafter (Choi et al. 2006b). Similarly, in a study by Kim et al. (2005), the greatest increase in peroxiredoxin expression in *Gryllotalpa orientalis* occurred at ~3 hr after thermal stress treatment (4 and 37°C). Lee et al. (2005) reported that the highest expression of thioredoxin

![Fig. 2. Transcript levels of SOD1 and TrxR were determined in *A. mellifera* (A, B) and *A. cerana* (C, D) after in vivo injection of MV and H2O2. Workers were injected with 10 mM MV (3 µl) or 10 mM H2O2 (3 µl). Controls were injected with PBS buffer (3 µl). Total RNA was extracted from whole *A. mellifera* and *A. cerana* bodies and used for qRT-PCR. The values are presented as the mean ± SD. The significance of the difference compared with the control value is indicated by *($P < 0.01$).](Journal of Insect Science, 2016, Vol. 16, No. 1)
peroxidase (TPx) in Bombyx mori occurred at ~5 hr after thermal stress treatment rather than at 3 hr. These results suggest that SOD1 and TPx are sensitive to thermal stress, with expression tending to increase with changes in temperature. Indeed, the timing of gene expression in response to environmental stimuli is very important for combating such stress, and based on the above results, gene expression can be achieved in 3 and 5 hr. Temperature stress is a key mediator in ROS formation (Lindquist 1986; Rauen et al. 1999). In previous studies, insect antioxidant enzyme genes, such as G. orientalis SOD1 (Kim et al. 2005b), B. mori TPx (Lee et al. 2005), and G. orientalis Prx (Kim et al. 2005a), were up-regulated by both cold and heat stresses. In this study, the induction of honeybee SOD1 by temperature stress, a mediator of ROS, suggests that SOD1 may play an important role as an antioxidant enzyme by reducing the high level of intracellular superoxide radical induced by extracellular stimuli such as low or high temperature.

SOD1 and TrxR Expression After MV or H2O2 Injection

When MV was injected, the expression of both SOD1 and TrxR in A. mellifera increased up to the maximum levels at 3 hr and then decreased (Fig. 2A and B). The expression of both SOD1 and TrxR displayed a similar pattern in A. cerana, with higher expression than in the control group (Fig. 2C and D). MV, commonly known as paraquat (PQ), is an oxidative stress inducer and despite the fact that its ingestion can cause death by multi-organ failure (Bus et al. 1974), is one of the most widely used herbicides. When injected with H2O2, however, the expression of SOD1 and TrxR in A. mellifera and A. cerana did not change compared with the control. Choi et al. (2006a,b) reported that a Trx-like protein in the fat body of B. ignitus workers was up-regulated by H2O2 treatment. In addition, the TPx gene was found to be dramatically increased in the fat body of B. mori (Lee et al. 2005). In our study, expression of both SOD1 and TrxR after H2O2 injection was found in whole-body A. mellifera and A. cerana samples. Therefore, additional studies are needed.

Characterization of SOD1 and TrxR Expression Using an E. coli Expression System

To characterize the SOD1 and TrxR proteins, each gene from A. mellifera and A. cerana was cloned into the E. coli expression vector pET-21a (+). We previously reported that the A. mellifera SOD1 and TrxR genes are 532 bp and 1,674 bp long, respectively (Lee et al. 2010); the A. cerana SOD1 and TrxR genes are 474 and 1,620 bp long, respectively. The A. mellifera SOD1 and TrxR proteins consist of 152 and 491 amino acids, respectively, and those of A. cerana 152 and 494 amino acid, respectively. Although the two species are genetically similar, the nucleotide and amino acid sequences of their genes differ. The cloning of each gene was confirmed via restriction enzyme digestion and sequencing, and the plasmids containing each recombinant protein were termed pET-AmSOD, pET-AcSOD, pET-AmTrxR, and pET-AcTrxR (Fig. 3A). Recombinant SOD1 and TrxR expression was analyzed by SDS-PAGE (Fig. 3B), which revealed bands of ~16 and 60 kDa, respectively. In addition, each recombinant protein was confirmed by Western blot analysis using an anti-His monoclonal antibody (Fig. 3C). This is the first report describing the expression of SOD1 and TrxR proteins from A. mellifera and A. cerana. Lee et al. reported the identification of a silkworm TPx after thermal stress (4 and 37°C), and TPx proteins of ~25 kDa have been expressed in Sf9 insect cells. Although there are slight differences in the expression of SOD1 and TrxR genes under different stress conditions, these proteins may play significant roles as antioxidant enzymes by reducing high levels of intracellular superoxide radical induced by thermal stress or the injection of stress inducers.

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