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Original article

Molecular cloning, characterisation and expression of methionine sulfoxide reductase A gene from *Apis cerana cerana*

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Abstract – Methionine sulfoxide reductases (Msrs) catalyse the reduction of methionine sulfoxide to methionine and play key roles in protein repair and reactive oxygen species scavenging. Here, an *MsrA* gene-designated *AccMsrA* was isolated from *Apis cerana cerana* for the first time. The full-length cDNA of *AccMsrA* is 1,540 bp, and it encodes a protein with 217 amino acids. Sequence alignment analysis showed that AccMsrA shares high similarity with other insect MsrAs. Analysis of the 5′-flanking region of *AccMsrA* revealed a group of transcription factor-binding sites that are associated with the regulation of development and responses to environmental stresses. Quantitative real-time PCR showed high expression levels of *AccMsrA* mRNA in prepupa and in the head of adult workers. Furthermore, the expression of *AccMsrA* was upregulated by multiple oxidative stresses, including ultraviolet light (30 mJ/cm^2), heat (42°C) and H_2O_2 (2 mM). These results indicate that *AccMsrA* might fulfill an important role in the regulation of insect development and in their responses to various environmental stresses. This report is the first description of the characteristics of the *AccMsrA* gene in the Chinese honeybee, and it establishes a primary foundation for further study.

methionine sulfoxide reductase A / *Apis cerana cerana* / cDNA / genomic DNA / quantitative real-time PCR

1. INTRODUCTION

Organisms generate a large amount of reactive oxygen species (ROS) when they are challenged with external environmental stresses. ROS generated by stimulation lowers the cellular antioxidant capacity, alters protein function and damages proteins (Zhang et al. 2010; Brunell et al. 2010; Brot and Weissbach 1991; Moskovitz 2005). Methionine is one of the most oxidation-sensitive amino acids. Methionine can be easily oxidised to methionine sulfoxide (MetO) by ROS, and the resulting MetO can be reduced back to methionine by antioxidant enzymes. Methionine sulfoxide-S-reductase (MsrA) is one such antioxidant enzyme that can act not only as a repair enzyme, but also as an indirect scavenger of ROS by reducing the amount of MetO (Weissbach et al. 2002).

The first MsrA enzyme was identified in *Escherichia coli* and was shown to convert MetO to Met (Brot et al. 1981). Subsequent studies have clearly elucidated the importance of MsrAs in protecting cells against oxidative

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damage. For instance, deletion or silencing of the *MsrA* gene in yeast or animal cells renders the cells more sensitive to oxidative stress (Moskovitz et al. 1997; Zhou et al. 2009). In human epidermal melanocytes, a small interfering RNA (siRNA) specific for *MsrA* was used to suppress its expression, and this decreased *MsrA* expression led to an increased sensitivity to H_2O_2 oxidative stress, resulting in more cell death (Zhou et al. 2009). In vivo, mice lacking MsrA accumulate high levels of oxidised protein under 100% oxygen treatment and exhibit sensitivity to oxidative stress (Moskovitz et al. 2001). Moreover, the overexpression of the *MsrA* gene in animal cells improves resistance to oxidative stresses (Zhang et al. 2010; Yermolaieva et al. 2004). In PC-12 cells, the overexpression of bovine *MsrA* protected neuronal cells from hypoxia/reoxygenation injury, causing diminished ROS accumulation and facilitating cell survival under hypoxia treatment (Yermolaieva et al. 2004). Furthermore, fruit flies overexpressing the *MsrA* gene show significant resistance to paraquat-induced oxidative stress and a markedly extended lifespan (Ruan et al. 2002).

Previous studies of MsrAs have mainly focused on antioxidant defence and modulation of the aging process and little information about the role of MsrA in bees has been available until now. Honeybees are pollinating insects that play an important role in maintaining ecological balance and increasing crop yields. When collecting pollen, honeybees encounter diverse environmental stresses, such as sunlight (ultraviolet (UV) radiation) and high temperatures that cause the formation of ROS (Kielbassa et al. 1997; Heise et al. 2003), which inevitably results in oxidative damage. In this study, we isolated *AccMsrA*, an *MsrA* gene from *Apis cerana cerana* encoding a putative methionine sulfoxide-S-reductase, and we identified a series of transcription factor-binding sites related to tissue development and stress responses. *AccMsrA* mRNA was detected in different developmental stages and tissues by quantitative real-time PCR (qRT-PCR), and a high level of expression was observed in fifth instar larva and in the head of adult workers. Furthermore, the accumulation of *AccMsrA* mRNA was induced by UV light, heat and H_2O_2. Together, these results indicate that *AccMsrA* might play an important role in the regulation of insect development and responses to various environmental stresses.

2. MATERIALS AND METHODS

2.1. Animals and treatments

Chinese honeybees (*A. cerana cerana*) maintained at Shandong Agricultural University, China, were used in the study. The colonies were fed in incubators and were kept at a constant temperature (33°C) and humidity (80%). The entire body of second (L2) larval instars, fourth (L4) larval instars, prepupal (PP) phase pupa, earlier pupal phase (Pw) pupa, pink (Pp) phase pupa and dark (Pd) phase pupa were taken from the hive, and the adult workers (1-day-old) were collected at the entrance of the hive when returning to the colony after foraging (Bitondi et al. 2006). Larvae, pupae and 1-day-old adults and the head, thorax, abdomen and midgut which were dissected from the adults were frozen in liquid nitrogen and stored at −80°C. The 1-day-old adults were divided into four groups (n= 10–15). Group 1 was treated with 30 mJ/cm^2 of UV light. Group 2 was subjected to 42°C heat. Group 3 was treated with 2 mM H_2O_2. The control bees in group 4 were fed a pollen and sucrose solution exclusively. Three bees were harvested at the appropriate times in each condition and stored at −80°C until use.

2.2. RNA and DNA extraction

Total RNA was extracted with TRIZOL reagent (Invitrogen, USA) according to the manufacturer's instructions. RNase-free DNase-I (Promega, USA) was applied to eliminate potential genomic DNA contamination. First-strand cDNA was synthesised with 2 μg of total RNA by reverse transcriptase (TransGen biotechnology, Beijing) at 42°C for 50 min using the oligo-d$(T)_{18}$-adaptor primer. Genomic DNA was isolated from larvae using the EasyPure Genomic DNA Extraction Kit (TransGen biotechnology, Beijing) according to the manufacturer's protocol.

Table I. Primers used in this study.

Primer	Primer sequence (5′→3′)	Description
For cloning the full-length cDNA		
MP1	GGAATGGGATGTTTCTGG	Conserved fragment primer, forward
MP2	CGAAGTCGATATTTCTGATG	Conserved fragment primer, reverse
3P1	CACGTGAACAAGAGCAATACAAAC	3′-RACE forward primer, primary
3P2	GTTTTATCTAGCAGAAGATTATCATC	3′-RACE forward primer, nested
B26	GACTCTAGACGACATCGA$(T)_{18}$	Universal primer, primary
B25	GACTCTAGACGACATCGA	Universal primer, nested
5P1	CAAGTTCTAATTACACCGGG	5′-RACE reverse primer, primary
5P2	CGCCAAATAAACAATCACCGGC	5′-RACE reverse primer, nested
AAP	GGCCACGCGTCGACTAGTAC$(G)_{16}$	Abridged anchor primer
AUAP	GGCCACGCGTCGACTAGTAC	Abridged universal amplification primer
FP1	CTTTCAGTCACCAACGAAATATC	Full-length cDNA primer, forward
FP2	GCTCAAGACTTATTTGCGAGAG	Full-length cDNA primer, reverse
For cloning the genomic and 5′-flanking region		
G1	CTTTCAGTCACCAACGAAATATC	Genomic sequence primer, forward
G2	CGCCAAATAAACAATCACC	Genomic sequence primer, reverse
G3	GGTGATTGTTTATTTGGCG	Genomic sequence primer, forward
G4	TTTGTATTGCTCTTGTTCACG	Genomic sequence primer, reverse
G5	CGTGAACAAGAGCAATACAAA	Genomic sequence primer, forward
G6	GCTCAAGACTTATTTGCGAGAG	Genomic sequence primer, reverse
PP1	TGTTTTTTGATTACTTCGCA	I-PCR forward primer, outer
PP2	CCAGAAACATCCCATTCC	I-PCR reverse primer, outer
PP3	CTGCGTTATTAAATACGCACGC	I-PCR forward primer, inner
PP4	GATATTTCGTTGGTGACTGAAAG	I-PCR reverse primer, inner
Primers used for Quantitative real-time PCR and protein expression assays		
QP1	GGTGATTGTTTATTTGGCG	Real-time PCR primer, forward
QP2	TTTGTATTGCTCTTGTTCACG	Real-time PCR primer, reverse
β-action-u	GTTTTCCCATCTATCGTCGG	Standard control primer, forward
β-action-d	TTTTCTCCATATCATCCCAG	Standard control primer, reverse
Yup	AGTACTCTTTCAGTCACCAACGAAATATC	Protein expression primer, forward
Ydown	GGTACCGCTCAAGACTTATTTGCGAGAG	Protein expression primer, reverse

2.3. Synthesis of the *AccMsrA* cDNA

To isolate the internal conserved cDNA fragment of *AccMsrA*, primers MP1 and MP2 were designed and synthesised (Shanghai Sangon Biotechnological Company, China) based on amino acid and nucleotide sequences that are conserved among *Aedes aegypti*, *Culex quinquefasciatus* and *Apis mellifera*, which were obtained from NCBI GenBank. Then PCR was performed. Based on the sequence of the cloned internal fragment, the 5′ and 3′ ends of the mRNA were cloned by rapid amplification of cDNA ends (RACE) methodology using gene-specific primers. In 3′ RACE-PCR, the primers 3P1 and B26 were utilised in the primary PCR, and the nested PCR was performed with the primers 3P2 and B25 using the primary PCR products as the template. In 5′ RACE-PCR, the

Table II. PCR amplification conditions.

Primer pair	Amplification conditions
MP1, MP2	5 min at 94°C, 40 s at 94°C, 40 s at 49°C, 40 s at 72°C for 35 cycles, 5 min at 72°C
3P1, B26	5 min at 94°C, 40 s at 94°C, 40 s at 47°C, 50 s at 72°C for 31 cycles, 5 min, at 72°C
3P2, B25	5 min at 94°C, 30 s at 94°C, 30 s at 48°C, 50 s at 72°C for 35 cycles, 5 min at 72°C
5P1, AAP	5 min at 94°C, 40 s at 94°C, 40 s at 49°C, 40 s at 72°C for 31 cycles, 5 min at 72°C
5P2, AUAP	5 min at 94°C, 30 s at 94°C, 30 s at 49°C, 30 s at 72°C for 35 cycles, 5 min at 72°C
FP1, FP2	5 min at 94°C, 60 s at 94°C, 60 s at 47°C, 50 s at 72°C for 35 cycles, 5 min at 72°C
G1, G2	5 min at 94°C, 30 s at 94°C, 30 s at 51°C, 60 s at 72°C for 35 cycles, 5 min at 72°C
G3, G4	5 min at 94°C, 60 s at 94°C, 60 s at 47°C, 60 s at 72°C for 35 cycles, 5 min at 72°C
G5, G6	5 min at 94°C, 60 s at 94°C, 60 s at 47°C, 60 s at 72°C for 35 cycles, 5 min at 72°C
PP1, PP2	10 min at 94°C, 50 s at 94°C, 75 s at 50°C, 2 min at 72°C for 30 cycles, 5 min at 72°C
PP3, PP4	10 min at 94°C, 50 s at 94°C, 75 s at 51°C, 2 min at 72°C for 30 cycles, 5 min at 72°C
β-u, β-d	5 min at 94°C, 30 s at 94°C, 15 s at 50°C, 30 s at 72°C for 35 cycles, 5 min at 72°C
QP1, QP2	5 min at 94°C, 40 s at 94°C, 15 s at 50°C, 40 s at 72°C for 35 cycles, 5 min at 72°C
Yup, Ydown	5 min at 94°C, 60 s at 94°C, 60 s at 47°C, 50 s at 72°C for 35 cycles, 5 min at 72°C

first-strand cDNA was purified with DNA Clean-Up System (Promega, USA) and then polyadenylated at its 5′ end with dGTP by terminal deoxynucleotidyl transferase (TaKaRa, Japan) according to the manufacturer's instructions. PCR was initially performed with the primers 5P1 and AUAP. Subsequently, the primary PCR products were used as the template and the primers 5P2 and AAP were chosen to amplify the related sequence. All PCR products were cloned into the pMD18-T vector (TaKaRa, Japan) and then sequenced. All primers used in the present study are shown in Table I, and all of the PCR amplification conditions are shown in Table II.

2.4. Amplification of the genomic sequence of *AccMsrA* and its 5′-flanking region

To clone the genomic DNA of *AccMsrA*, four gene-specific primers were designed based on the sequence of the coding region of *AccMsrA*. Genomic DNA from *A. cerana cerana* was used as the template, and the primers G1/G2, G3/G4 and G5/G6 were utilised to identify three genomic DNA fragments. The PCR products were purified and cloned into the vector pMD18-T (TaKaRa, Japan) and then sequenced. To amplify the 5'-flanking region of *AccMsrA*, inverse polymerase chain reaction (IPCR) was used. Total genomic DNA from *A. cerana cerana* was extracted, completely digested with the restriction endonuclease *Eco*RI and then self-ligated to form plasmids using T4 DNA ligase (TaKaRa, Dalian, China). Based on the *AccMsrA* genomic sequence, the specific primers PP1/PP2 and PP3/PP4 were designed and used in the IPCR to clone the 5′-flanking region as described by Liu (Liu et al. 2009). The PCR products were purified and cloned into pMD18-T and then sequenced. Additionally, the MatInspector database (www.cbrc.jp/research/db/TFSEARCH.html) was used to search for transcription factor-binding sites in the 5′-flanking region.

2.5. Sequence analysis and phylogenetic construction

The deduced amino acid sequence of AccMsrA was analysed and predicted using DNAman version 5.2.2 (Lynnon Biosoft Company). A multiple sequence alignment of AccMsrA with three other insects obtained from the NCBI database was also performed with DNAman version 5.2.2. A phylogenetic tree was generated with 11 MsrAs from invertebrates and plants

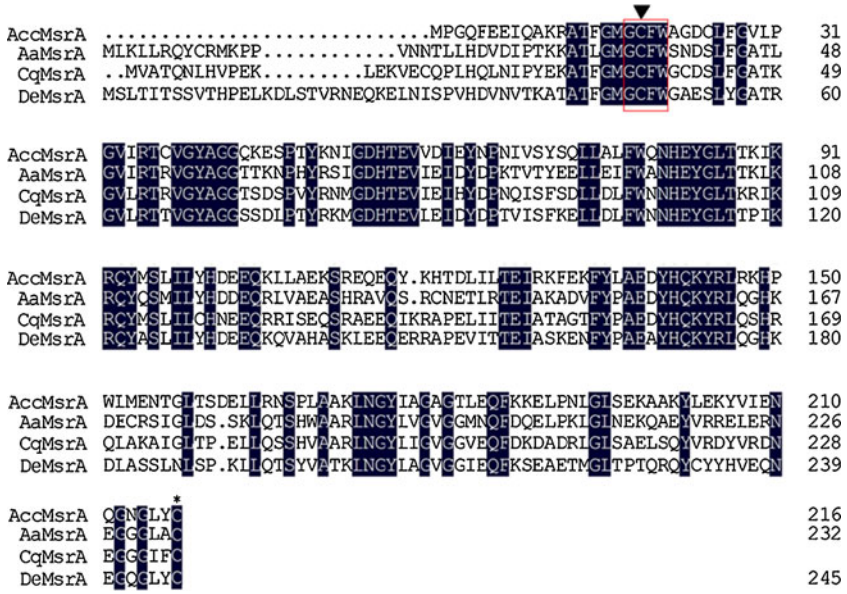

Figure 1. Alignment of the deduced AccMsrA protein sequence with other known insect MsrAs. The amino acid sequences used in the study are from *Aedes aegypti* (AaMsrA, GenBank accession no. XP001649797), *Culex quinquefasciatus* (CqMsrA, GenBank accession no. XP001868737), *Drosophila erecta* (DeMsrA, GenBank accession no. XP001973088) and *Apis cerana cerana* (AccMsrA, GenBank accession no. HQ219724). Identical amino acid residues in this alignment are shaded in *black*; gaps are introduced for optimal alignment and maximum similarity among all compared sequences. The short sequence motif characteristic of MsrAs is marked by a *box*, and conserved MsrA Cys sites are marked by a *black down-pointing triangle* and *asterisk*.

obtained from the NCBI database by MEGA version 4.

2.6. Expression analysis of *AccMsrA* by real-time quantitative PCR

Specific primer pairs (QP1/QP2) were designed according to the cDNA of *AccMsrA* for use in qRT-PCR reactions. The housekeeping gene *β-actin,* which is equally expressed in organisms, was used as a control. Reactions were performed using the SYBR® PrimeScript™ RT-PCR Kit (TaKaRa Code: DRR063A) on a CFX96TM Real-time System (Bio-Rad) under the following thermal cycling profile: 95°C for 30 s, followed by 40 cycles of amplification (95°C for 10 s, 50°C for 15 s and 72°C for 15 s) and a melt cycle from 65°C to 95°C. The Ct of each sample was used to calculate the ΔC_T values. The $-\Delta\Delta C_T$ method was used to calculate the different expression levels of *AccMsrA*. The different transcript levels of all samples and significant differences among samples were verified by Statistical Analysis System (SAS) version 9.1.

3. RESULTS

3.1. Cloning and sequence analysis of *AccMsrA*

Based on the conserved region of insect *MsrA* genes, primers MP1 and MP2 were designed to amplify the middle region of *AccMsrA*. A fragment of about 600 bp was obtained, and then RACE-PCR was performed according to the internal sequence. The deduced full-length cDNA of *AccMsrA* (GenBank accession no. HQ219724) is 1,540 bp, and it contains a 136-bp 5′ untrans-

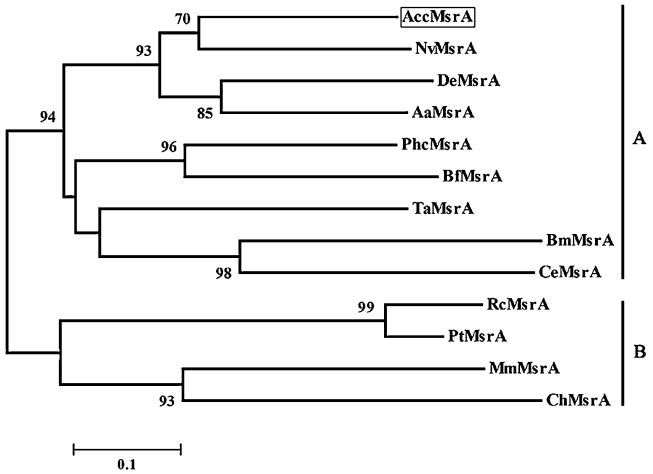

Figure 2. Phylogenetic analysis of MsrAs from different species. Amino acid sequences of MsrAs were obtained from GenBank as follows: *Nasonia vitripennis* (NvMsrA, GenBank accession no. XP001603389), *Drosophila erecta* (DeMsrA, GenBank accession no. XP001973088), *Aedes aegypti* (AaMsrA, GenBank accession no. XP001649797), *Pediculus humanus corporis* (PhcMsrA, GenBank accession no. XP002426843), *Branchiostoma floridae* (BfMsrA, GenBank accession no. XP002594093), *Trichoplax adhaerens* (TaMsrA, GenBank accession no. XP002113880), *Brugia malayi* (BmMsrA, GenBank accession no. XP001893631), *Caenorhabditis elegans* (CeMsrA, GenBank accession no. NP495540), *Ricinus communis* (RcMsrA, GenBank accession no. XP002530748), *Populus trichocarpa* (PtMsrA, GenBank accession no. XP002310304), *Methanococcus maripaludis* (MmMsrA, GenBank accession no. YP001097623), *Cytophaga hutchinsonii* (ChMsrA, GenBank accession no. YP678260) and *Apis cerana cerana* (AccMsrA, GenBank accession no. HQ219724).

lated region (UTR) and a 753-bp 3′ UTR. The cDNA harbours a 651-bp open reading frame that encodes a protein of 217 amino acids. The AccMsrA protein has a theoretical molecular mass of 25.0 kDa and a predicted isoelectric point of 6.78. Compared with MsrA sequences from other insects, *AccMsrA* exhibits higher A+T content (72.6%), which is consistent with the genomic characteristics of *A. mellifera* (Honeybee Genome Sequencing Consortium 2006).

3.2. Characterisation and phylogenetic analysis of *AccMsrA*

A multiple sequence alignment showed that AccMsrA shares 49.6%, 45.8% and 51.3% identity with the MsrAs from *A. aegypti*, *Drosophila erecta* and *C. quinquefasciatus*, respectively (Figure 1). The highly conserved motif GCFW, a fingerprint of MsrAs, was found in the N-terminus. A cysteine residue was identified in the conserved GCFW region that is involved in the catalytic mechanism. Moreover, the conserved Cys site in the MsrA amino acid sequences of insects was observed in C-terminus end, and it is distinct from previous studies.

To investigate the evolutionary relationships among AccMsrA and other MsrA proteins, a phylogenetic tree was generated based on the MsrA amino acid sequence alignment. The phylogenetic tree displays two distinct clusters: A, metazoans and B, plants and bacteria (Figure 2). AccMsrA and *Nasonia vitripennis* (NvMsrA) are in the same clade, belonging to Hymenoptera, and they are close to the Diptera-originated *D. erecta* MsrA (DeMsrA) and *A. aegypti* MsrA (AaMsrA). Thus, the function of AccMsrA might be similar to those of NvMsrA, DeMsrA and AaMsrA.

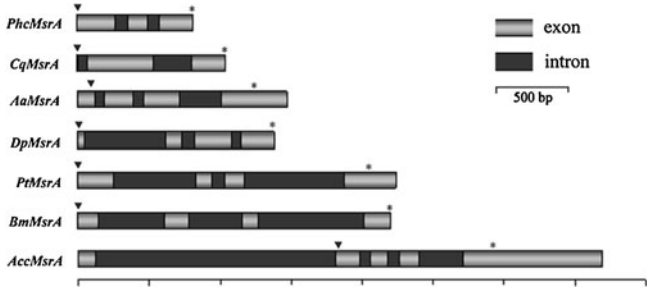

Figure 3. Schematic representation of the genomic organisation. Lengths of the exons and introns of the genomic DNA of *Pediculus humanus corporis* (*PhcMsrA*, GenBank accession no. 8229465), *Culex quinquefasciatus* (*CqMsrA*, GenBank accession no. 6052472), *Aedes aegypti (AaMsrA*, GenBank accession no. 5565445), *Drosophila persimilis* (*DpMsrA*, GenBank accession no. 6597072), *Populus trichocarpa* (*PtMsrA*, GenBank accession no. 7495540), *Bombyx mori* (*BmMsrA*, GenBank accession no. 732898) and *Apis cerana cerana* (*AccMsrA*, GenBank accession no. HQ219725) are indicated according to the scale below. The exons are highlighted with *white bars*, and the introns are indicated with *grey bars*. The initiation codon (ATG) is marked by a *black down-pointing triangle*. The stop codon (TAA) is marked by *asterisk*.

3.3. Genomic structure analysis of *AccMsrA*

To further elucidate the genomic structure of *AccMsrA*, three fragments of about 1,851, 521 and 623 bp were obtained using the specific genomic primers G1+G2, G3+G4 and G5+G6, respectively. By assembling the cDNA fragments and intron sequences, we obtained the *AccMsrA* genomic sequence, which spans a region of 3,690 bp (GenBank accession no. HQ219725) that contains five exons and four introns (Figure 3). All introns of *AccMsrA* had the expected characteristics typical of introns, such as high A+T content (Table III) and signals flanked by the 5′ splice donor GT and the 3′ splice donor AG. Furthermore, the exon–intron structure is fairly conserved among MsrAs from different species. Notably, the first intron of *AccMsrA* is located in the 5′ UTR; however, no intron was detected in the UTR of *MsrA* genes from other species. This result implies that the features of introns are specific to different species in the process of evolution.

3.4. Identification of putative *cis*-acting elements in the 5′-flanking region of *AccMsrA*

To obtain more information on *AccMsrA* gene expression and regulation, a 1,400 bp 5′-flanking

Table III. Exon and intron sizes of the AccMsrA gene and AT content

Exon			Intron		
Number	Size (bp)	AT content (%)	Number	Size (bp)	AT content (%)
1	1–127 (127)	77.95	1	128–1,804 (1,677)	84.14
2	1,805–1,971 (167)	60.48	2	1,972–2,051 (80)	82.5
3	2,052–2,172 (121)	66.94	3	2,173–2,250 (78)	84.62
4	2,251–2,387 (137)	27.01	4	2,388–2,702 (315)	10.48
5	2,703–3,690 (988)	25.40			

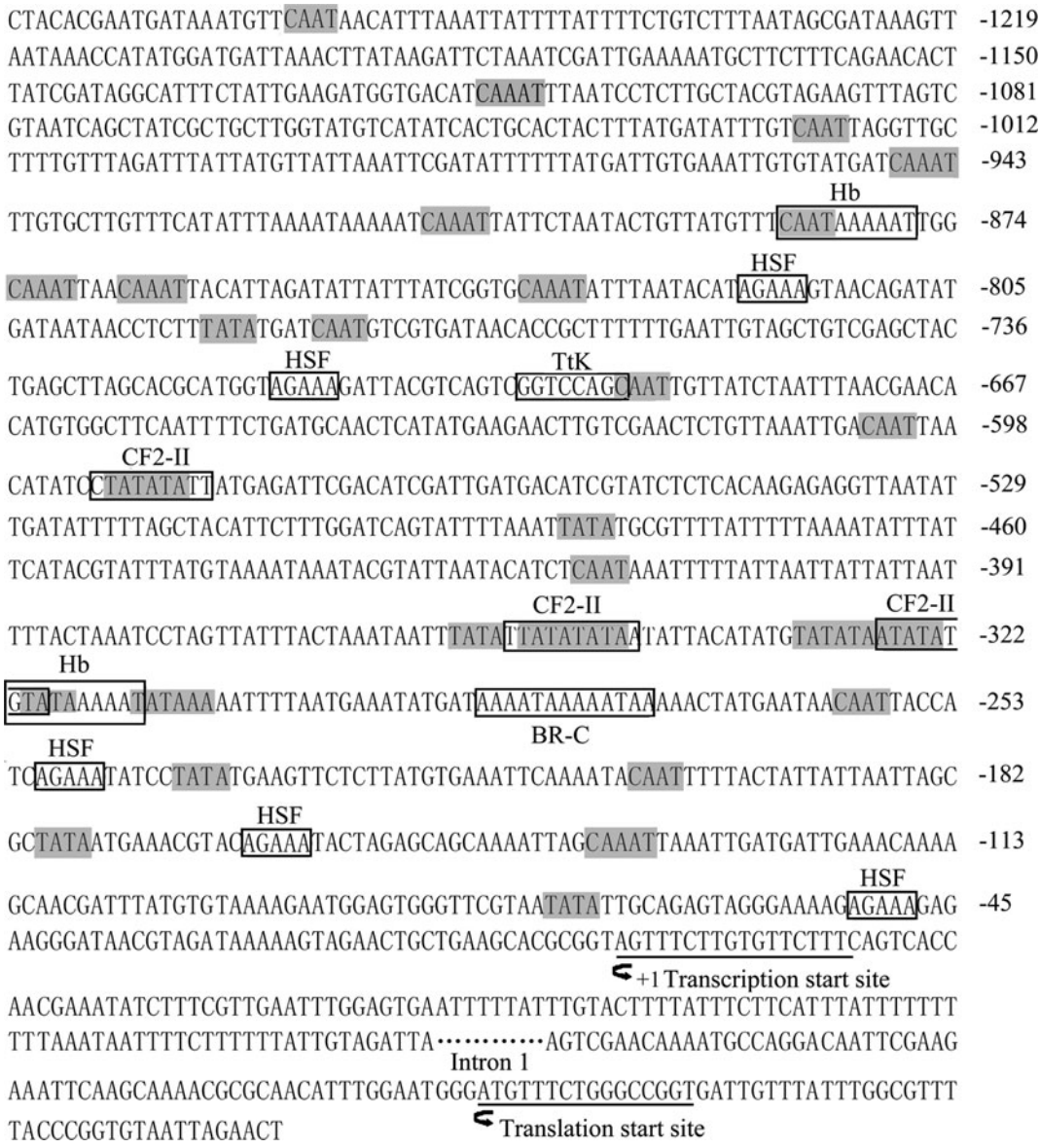

Figure 4. Nucleotide sequence of the 5′-flanking region of *AccMsrA*. The transcription start site and the translation start site are marked with *arrowheads*. The putative transcription factors are indicated, their binding sites are *boxed*, and the putative core promoter consensus sequences are highlighted in *grey*.

region of *AccMsrA* was generated from genomic DNA with the primers PP3 and PP4. Using a comparison with the *AccMsrA* cDNA sequence, the putative transcription start site was defined as +1 at 1,364 bp upstream from the ATG translation start site (Figure 4). Then several transcription factor-binding sites in the 5′-flanking region of *AccMsrA* were predicted by the web software program MatInspector (www.cbrc.jp/research/db/TFSEARCH.html). Of these sites, five heat shock factor (HSF), which are involved in heat stress responsiveness, were identified (Fernandes

Table IV. Putative transcription factor-binding sites in the 5′-flanking region of AccMsrA: positions and possible functions.

Transcription factor	Putative-binding site and position(bp)	Function
HSF	AGAAA: -52, -165, -249,-715, -819	Heat shock factors (Fernandes et al. 1994)
Ttk	GGTCCAGC: -697	Regulation of developmental genes (Read and Manley 1992)
CF2-II	CTATATATT: -590,TTATA-TATA: -353,ATATATGTA: -326	Developmentally regulated (Hsu et al. 1992)
Hb	GTATAAAAAT: -320,CAA-TAAAAAT: -885	Function in the segmentation of early embryo (Stanojević et al. 1989)
BR-C	AAAATAAAAATAA: -286	Controls a tissue-specific response to the steroid hormone ecdysone at the onset of metamorphosis (Kalm et al. 1994)

et al. 1994). In addition, a series of binding sites related to development were found including: (a) tramtrack (Ttk), which is related to the regulation of developmental genes (Read and Manley 1992); (b) cell factor 2-II (CF2-II), which is related to developmental regulation (Hsu et al. 1992); (c) hunchback (Hb), which functions in early embryo segmentation (Stanojević et al. 1989); and (d) Broad-Complex (BR-C), which directly controls a tissue-specific response to the steroid hormone ecdysone at the onset of metamorphosis (Kalm et al. 1994). The predicted transcription factor-binding sites and their positions are shown in Figure 4 and Table IV, respectively. Together, these results indicate that *AccMsrA* might be involved in responses to external factors and might also have an effect on development from the early embryo to adulthood.

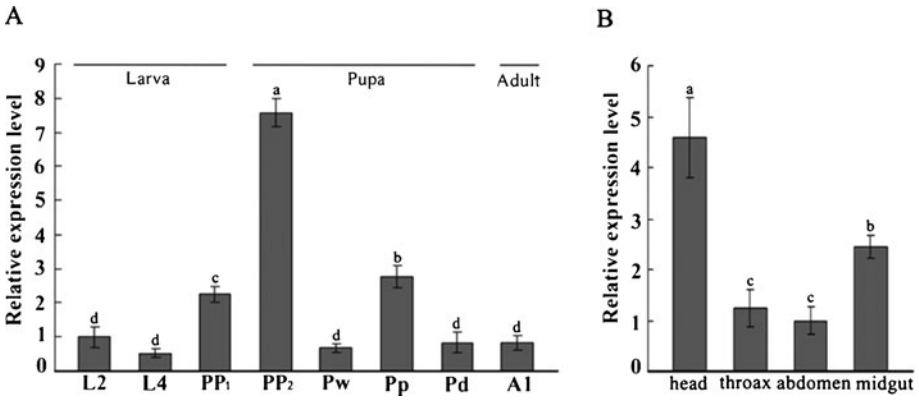

Figure 5. *AccMsrA* mRNA expression in different developmental phases (**a**) and tissues (**b**) determined by qRT-PCR in *A. cerana cerana*. **a** Expression of *AccMsrA* in the entire body of second (L2) larval instars, fourth (L4) larval instars, prepupal (PP) phase pupa, earlier pupal phase (Pw) pupa, pink (Pp) phase pupa and dark (Pd) phase pupa and adult workers (1-day-old). **b** Distribution of *AccMsrA* in brain, abdomen, thorax and midgut. *Vertical bars* represent the mean±standard error of mean (S.E.M.) (n=3). *Different letters above the bars* indicate significant difference as determined using SAS software analysis (P<0.05).

3.5. Expression pattern of *AccMsrA* in different developmental stages and tissues

To determine the expression of *AccMsrA* in different developmental stages, qRT-PCR was carried out using RNA extracted from larvae, pupae and 1-day old adults. As shown in Figure 5a, the expression of *AccMsrA* was detected at all developmental stages. It is interesting to observe that there was a significant high expression ($P<0.05$) of the levels of *AccMsrA* mRNA in the PP2 stage. Moreover, high expression levels were observed in the PP1 and Pp stages.

A tissue-specific expression analysis of *AccMsrA* was also performed using RNA extracted from the head, thorax, abdomen and midgut of adult *A. cerana cerana* (Figure 5b). The expression levels in the head and midgut were 2.5-fold and 1.2-fold greater than those in the abdomen.

3.6. Expression profile of *AccMsrA* under diverse environmental stresses

MsrAs play an important role in the oxidative stress response. Previous studies reported that the expression of MsrA could be regulated by environmental stressors such as UV light, heat

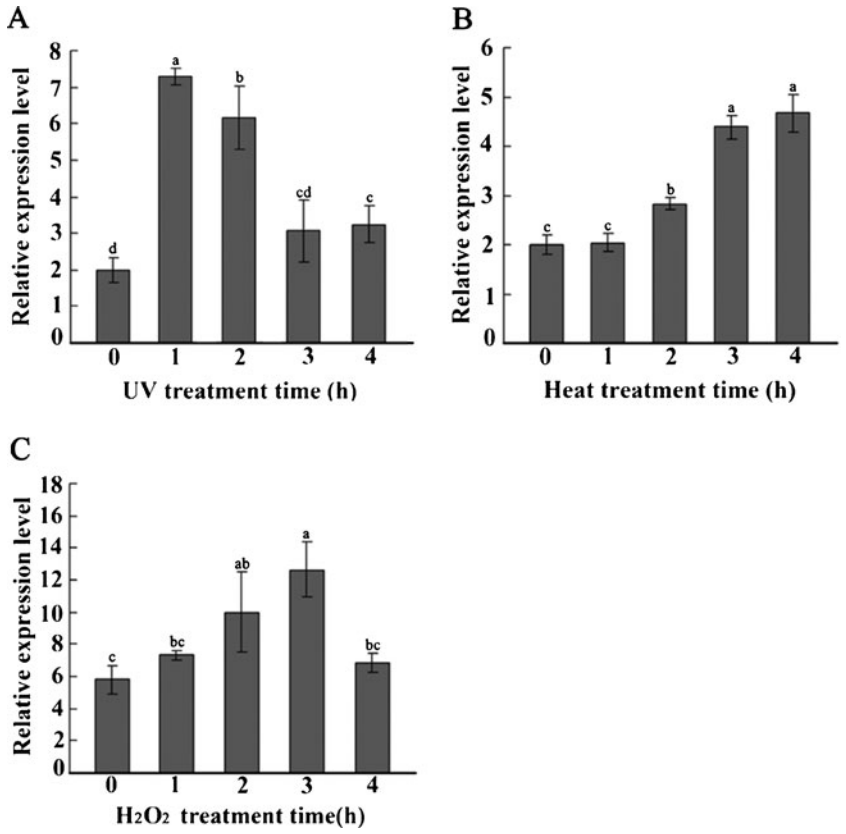

Figure 6. *AccMsrA* mRNA expression under different environmental stresses in *A. cerana cerana.* **a** *AccMsrA* mRNA expression in *A. cerana cerana* after UV light treatment, as measured by qRT-PCR. **b** *AccMsrA* mRNA expression in *A. cerana cerana* after heat treatment, as measured by qRT-PCR. **c** *AccMsrA* mRNA expression in *A. cerana cerana* after H_2O_2 treatment, as measured by qRT-PCR. *Vertical bars* represent the mean±S.E.M. ($n=3$). *Different letters above the bars* indicate significant difference as determined using SAS software analysis ($P<0.05$).

and H_2O_2 (Ogawa et al. 2006; Sreekumar et al. 2005). To investigate whether *AccMsrA* is involved in the response to these environmental stresses, qRT-PCR was performed. UV light induced the expression of *AccMsrA* rapidly; the expression level of *AccMsrA* peaked at 1 h and then declined (Figure 6a). When bees were exposed to heat treatment, the expression of *AccMsrA* showed no significant change between 0 and 1 h, and then it increased gradually after 1 h and remained high through 3 h (Figure 6b). When treated with H_2O_2, the *AccMsrA* expression pattern was similar to that under UV light treatment. However, the highest expression level was observed 3 h after H_2O_2 treatment (Figure 6c). Thus, these results demonstrate *AccMsrA* might participate in anti-oxidant systems under environmental stresses in bees.

4. DISCUSSION

In the present study, we report the cloning and characterisation of the *AccMsrA* gene from *A. cerana cerana* for the first time. Multiple sequence alignment indicated that the deduced amino acid sequence of AccMsrA shares high similarity with MsrAs from other insects. The Cys in the GCFW fingerprint region of the N-terminus and three other Cys sites, located at positions 25, 37 and 216, were observed in the AccMsrA sequence. Previous studies have showed that the catalytic mechanism of MsrAs involves three cysteine residues. The first Cys site, CysA, belongs to the fingerprint GCFW of N-terminus and attacks the sulfoxide group of S-MetO to release the reduced methionine, while the second and third Cys sites, CysB and CysC, facilitate responsiveness to thioredoxin as the reductant (Weissbach et al. 2002, 2005). MsrAs from bovine, human and *Caenorhabditis elegans* have similar structures (Kauffmann et al. 2005; Lee et al. 2005). Interestingly, a Cys site in the MsrA amino acid sequences of the insects was observed in the C-terminus end, in contrast to other studies. This suggests that MsrAs in insects potentially possess different functions. However, further study would be required for a comprehensive analysis. Phylogenetic analysis showed that AccMsrA belongs to the Hymenoptera insect. In addition, it is interesting to observe that the first intron is located in the 5′ UTR, suggesting that the *AccMsrA* gene has evolved differently from other species. However, further studies should be performed.

Previous studies have shown that MsrAs scavenge ROS and protect proteins by reducing the sulfoxide group of S-MetO (Levine et al. 1999). The expression level of MsrA genes is influenced by ROS (Picot et al. 2005; SreekuMar et al. 2005). Stresses such as UV light, heat and H_2O_2 always increase ROS (Heise et al. 2003). The expression of *MsrA* is upregulated by UVA and H_2O_2 in human keratinocytes (Ogawa et al. 2006). In our multiple stresses study, the transcript levels of *AccMsrA* in honeybees were upregulated by exposure to UV light (UV, 30 mJ/cm2), heat (42°C) and H_2O_2 (2 mM), with UV light having the most profound influence. This suggests that *AccMsrA* may be involved in insects' response to environmental stresses and that it may play a central role in ROS reduction. In addition, binding sites for putative stress response transcription factors, such as the HSFs, were identified in the 5′-flanking region of *AccMsrA*.

MsrA is considered the major component of the honeybee antioxidant system (Corona and Robinson 2006). Corona et al. (2005) have indicated that mRNA from the *MsrA* gene is expressed widely in the head, thorax and abdomen of adult workers and that expression levels tend to increase with age. Here, qRT-PCR has demonstrated that the *AccMsrA* gene was highly expressed in the head and midgut. However, transcription factor-binding sites for BR-C, which functions in tissue-specific responses, were observed in the promoter sequence of *AccMsrA*. This indicates that *AccMsrA* may be involved in the clearance of ROS in the head and midgut. High expression profiles of *AccMsrA* were observed in the PP2 and Pp developmental stages, which have histodifferentiation. We hypothesise that the significant change of *AccMsrA* expression was regulated by

hormones such as ecdysone. Previous studies have demonstrated that steroid hormones such as ecdysone are systemic signalling molecules that temporally coordinate the juvenile-adult transition in insects and control many aspects of development including moulting and metamorphosis (McBrayer et al. 2007; Rewitz et al. 2009; Gilbert et al. 2002). Moreover, the *MsrA* gene in *Drosophila* Kc cells was induced by ecdysone independently of ROS treatment (Roesijadi et al. 2007). Additionally, transcription factor-binding sites related to development such as Ttk, CF2-II and Hb were observed in the 5′-flanking region. This suggests that *AccMsrA* gene participates in the developmental regulation the growth and differentiation of bees.

The identification of the MsrA gene from *A. cerana cerana* represents a critical step in understanding the various roles played by the MsrA gene in response to environmental stresses and in the regulation of the growth and development of bees. However, further elucidation of the mechanisms involved is necessary in the honeybees.

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Clonage moléculaire, caractérisation et expression du gène codant pour la réductase A du sulfoxyde de méthionine chez *Apis cerana cerana*.

Réductase A du sulfoxide de méthionine / *Apis cerana cerana* / ADNc / ADN génomique / PCR quantitative en temps réel

Klonierung, molekulare Charakterisierung und Expression des Methioninsulfoxidreduktase-A-Gens von *Apis cerana cerana.*

Methioninsulfoxidreduktase-A / *Apis cerana cerana* / cDNA / genomische DNA / quantitative real-time PCR

REFERENCES

Bitondi, M.M.G., Nascimento, A.M., Cunha, A.D., Guidugli, K.R., Nunes, F.M.F., Simões, Z.L.P. (2006) Characterization and expression of the Hex 110 gene encoding a glutamine-rich hexamerin in the honey bee, *Apis mellifera*. Arch Insect Biochem **63**, 57–72

Brot, N., Weissbach, H. (1991) Biochemistry of methionine sulfoxide residues in proteins. Biofactors **3**, 91–96

Brot, N., Weissbach, L., Werth, J., Weissbach, H. (1981) Enzymatic reduction of protein-bound methionine sulfoxide. Proc Natl Acad Sci U S A **78**, 2155–2158

Brunell, D., Weissbach, H., Hodder, P., Brot, N. (2010) A high-throughput screening compatible assay for activators and inhibitors of methionine sulfoxide reductase A. Assay Drug Dev Technol **8**, 615–620

Corona, M., Robinson, G.E. (2006) Genes of the antioxidant system of the honey bee: annotation and phylogeny. Insect Mol Biol **15**, 687–701

Corona, M., Hughes, K.A., Weaver, D.B., Robinson, G.E. (2005) Gene expression patterns associated with queen honey bee longevity. Mech. Ageing Dev **126**, 1230–1238

Fernandes, M., Xiao, H., Lis, J.T. (1994) Fine structure analyses of the *Drosophila* and *Saccharomyces* heat shock factor-heat shock element interactions. Nucleic Acids Res **22**, 167–173

Gilbert, L.I., Rybczynski, R., Warren, J.T. (2002) Control and biochemical nature of the ecdysteroidogenic pathway. Annu. Rev. Entomol. **47**, 883–916

Heise, K., Puntarulo, S., Portner, H.O., Abele, D. (2003) Production of reactive oxygen species by isolated mitochondria of the Antarctic bivalve *Laternula elliptica* (King and Broderip) under heat stress. Comp. Biochem. Physiol. C. **134**, 79–90

Honeybee Genome Sequencing Consortium (2006) Insights into social insects from the genome of the honeybee *Apis mellifera*. Nature. **443**, 931–949

Hsu, T., Gogos, J.A., Kirsh, S.A., Kafatos, F.C. (1992) Multiple zinc finger forms resulting from developmentally regulated alternative splicing of a transcription factor gene. Science. **257**, 1946–1950

Kalm, L.V., Crossgrove, K., Seggern, D.V., Guild, G.M., Beckendorf, S.K. (1994) The Broad-Complex directly controls a tissue-specific response to the steroid hormone ecdysone at the onset of *Drosophila* metamorphosis. EMBO J. **13**, 3505–3516

Kauffmann, B., Aubry, A., Favier, F. (2005) The three-dimensional structures of peptide methionine sulfoxide reductases: current knowledge and open questions. Biochim. Biophys. Acta. **1703**, 249–260

Kielbassa, C., Roza, L., Epe, B. (1997) Wavelength dependence of oxidative DNA damage induced by UV and visible light. Carcinogenesis **18**, 811–816

Lee, B.C., Lee, Y.K., Lee, H., Stadtman, E.R., Lee, K.H., Chung, N. (2005) Cloning and characterization of antioxidant enzyme methionine sulfoxide-S-reductase from *Caenorhabditis elegans*. Arch. Biochem. Biophys. **434**, 275–281

Levine, R.L., Berlett, B.S., Moskovitz, J., Mosoni, L., Stadtman, E.R. (1999) Methionine residues may protect proteins from critical oxidative damage. Mech. Ageing Dev. **107**, 323–332

Liu, Y., Gao, Q., Wu, B., Ai, T., Guo, X. (2009) NgRDR1, an RNA-dependent RNA polymerase isolated from *Nicotiana glutinosa*, was involved in biotic and abiotic stresses. Plant Physiol. Biochem. **47**, 359–68

McBrayer, Z., Ono, H., Shimell, M., Parvy, J.P., Beckstead, R.B., Warren, J.T., Thummel, C.S., Dauphin-Villemant, C., Gilbert, L.I., O'Connor, M. B. (2007) Prothoracicotropic hormone regulates developmental timing and body size in *Drosophila*. Dev. Cell. **13**, 857–871

Moskovitz, J. (2005) Methionine sulfoxide reductases: ubiquitous enzymes involved in antioxidant defense, protein regulation, and prevention of aging-associated diseases. Biochim. Biophys. Acta. **1703**, 213–219

Moskovitz, J., Berlett, B.S., Poston, J.M., Stadtman, E. R. (1997) The yeast peptide-methionine sulfoxide reductase functions as an antioxidant in vivo. Proc. Natl. Acad. Sci. U S A **94**, 9585–9589

Moskovitz, J., Bar-Noy, S., Williams, W.M., Requena, J., Berlett, B.S., Stadtman, E.R. (2001) Methionine sulfoxide reductase (MsrA) is a regulator of antioxidant defense and lifespan in mammals. Proc Natl Acad Sci U S A **6**, 12920–12925

Ogawa, F., Sander, C.S., Hansel, A., Oehrl, W., Kasperczyk, H., Elsner, P., Shimizu, K., Heinemann, S.H., Thiele, J.J. (2006) The repair enzyme peptide methionine-S-sulfoxide reductase is expressed in human epidermis and upregulated by UVA radiation. J. Invest. Dermatol. **126**, 1128–1134

Picot, C.R., Petropoulos, I., Perichon, M., Moreau, M., Nizard, C., Friguet, B. (2005) Overexpression of MsrA protects WI-38 SV40 human fibroblasts against H_2O_2-mediated oxidative stress. Free Radic. Biol. Med. **39**, 1332–1341

Read, D., Manley, J.L. (1992) Alternatively spliced transcripts of the *Drosophila* tramtrack gene encode zinc finger proteins with distinct DNA binding specificities. EMBO J. **11**, 1035–1044

Rewitz, K.F., Larsen, M.R., Lobner-Olesen, A., Rybczynski, R., O'Connor, M.B., Gilbert, L.I. (2009) A phosphoproteomics approach to elucidate neuropeptide signal transduction controlling insect metamorphosis. Insect Biochem. Mol. Biol. **39**, 475–483

Roesijadi, G., Rezvankhah, S., Binninger, D.M., Weissbach, H. (2007) Ecdysone induction of MsrA protects against oxidative stress in *Drosophila*. Biochem. Biophys. Res. Commun. **354**, 511–516

Ruan, H., Tang, X.D., Chen, M.L., Joiner, M.L., Sun, G., Brot, N., Weissbach, H., Heinemann, S.H., Iverson, L., Wu, C.F., Hoshi, T. (2002) High-quality life extension by the enzyme peptide methionine sulfoxide reductase. Proc. Natl. Acad. Sci. U. S. A. **99**, 2748–2753

Sreekumar, P.G., Kannan, R., Yaung, J., Spee, C.K., Ryan, S.J., Hinton, D.R. (2005) Protection from oxidative stress by methionine sulfoxide reductases in RPE cells. Biochem. Biophys. Res. Commun. **334**, 245–53

Stanojević, D., Hoey, T., Levine, M. (1989) Sequence-specific DNA-binding activities of the gap proteins encoded by hunchback and Krüppel in *Drosophila*. Nature **341**, 331–335

Weissbach, H., Etienne, F., Hoshi, T., Heinemann, S.H., Lowther, W.T., Matthews, B., St John, G., Nathan, C., Brot, N. (2002) Peptide methionine sulfoxide reductase: structure, mechanism of action, and biological function. Arch. Biochem. Biophys **397**, 172–178

Weissbach, H., Resnick, L., Brot, N. (2005) Methionine sulfoxide reductases: history and cellular role in protecting against oxidative damage. Biochim. Biophys. Acta. **1703**, 203–212

Yermolaieva, O., Xu, R., Schinstock, C., Brot, N., Weissbach, H., Heinemann, S.H., Hoshi, T. (2004) Methionine sulfoxide reductase A protects neuronal cells against brief hypoxia/reoxygenation. Proc. Natl. Acad. Sci. U. S. A. **101**, 1159–1164

Zhang, C., Jia, P., Jia, Y., Weissbach, H., Webster, K., Huang, X., Lemanski, S.L., Achary, M., Lemanski, L.F. (2010) Methionine sulfoxide reductase A (MsrA) protects cultured mouse embryonic stem cells from H_2O_2-mediated oxidative stress. J. Cell Biochem. **111**, 94–103

Zhou, Z., Li, C.Y., Li, K., Wang, T., Zhang, B., Gao, T.W. (2009) Decreased methionine sulphoxide reductase A expression renders melanocytes more sensitive to oxidative stress: a possible cause for melanocyte loss in vitiligo. Br. J. Dermatol. **161**, 504–509