Sequencing and Expression Characterization of Antifreeze Protein Maxi-Like in Apis cerana cerana

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Abstract

Antifreeze proteins (AFPs) are biological cryoprotectants with unique properties that play a crucial role in regulating the molecular mechanisms governing cold resistance in insects. To identify and characterize Apis cerana cerana AFP (AcerAFP), we cloned the full-length cDNA of AcerAFP and examined its expression patterns in A. cerana cerana. A nucleotide alignment analysis showed that the entire coding region of AcerAFP is 1095 bp and encodes a polypeptide of 365 amino acids. The amino acid sequence of this protein exhibits 63–96% homology with AFP homologs from other hymenopterans. α-helices form the main secondary and tertiary structures of AcerAFP, which is similar to the molecular structure of fish AFP type-I. The expression profiles of AcerAFP revealed that expression was tissue, sex, and developmentally specific. In response to cold stress, the mRNA and protein expression of AcerAFP were both induced by low temperatures, and were also related to the concentrations of several cryoprotectants, including glucose, glycerin, glutamic acid, cysteine, histidine, alanine, and methionine. In addition, we found that the knockdown of AcerAFP by RNA interference remarkably increased the total freezing temperature of hemolymph in A. cerana cerana, where levels of AcerAFP mRNA were correlated with the expression of most antifreeze-related proteins. Taken together, these results suggest that AcerAFP plays an essential role as a biological cryoprotectant in honeybees, and is in turn regulated by small cryoprotectants and antifreeze-related proteins.

Key words: antifreeze protein, Apis cerana cerana, cold stress, expression patterns, RNA interference

Introduction

Insects living at high latitudes or altitudes are exposed to fluctuating thermal environments. For survival, the majority of freeze-tolerant insects (which can survive freezing) have to hide in local shelters to escape the low temperatures and must bear some of the brunt of low temperature exposure (Doucet et al. 2009). To counter these adverse conditions, insects have evolved a suite of physiological and molecular adaptations, which mainly include the synthesis of low-molecular-weight compounds and cryoprotective proteins (Bale 2002). These small compounds primarily consist of hydrocarbons, amino acids, and polylols, which are involved in energy metabolism and accumulation of cryoprotectants (Holden and Storey 1994). The antifreeze proteins (AFPs), antifreeze glycoproteins, heat shock proteins (HSPs), serine/threonine kinases (STKs), ice-nucleating proteins, membrane protectants, and other similar but less well-characterized proteins serve as prominent cryoprotective agents with unique characteristics and special properties (Bang et al. 2013, King and MacRae 2015). Among these proteins, AFPs have gained much attention because they are known to protect organisms from freezing by lowering the freezing temperature ($T_f$) (Wen et al. 2016).

AFPs have been identified in marine organisms, including fishes, and also in insects, microalgae, bacteria, and fungi (Doucet et al. 2009). AFPs were first discovered in fishes in the Arctic and Antarctic regions, and have been classified into four types (types I, II, III, and IV). The four types of AFPs are fundamentally different in terms of their primary sequences and three-dimensional (3D) structures, but show corresponding properties in binding ice and lowering the freezing points (Davies et al. 2002, Venketesh and Dayananda 2008). In contrast to fish AFPs, insect AFPs possess very high thermal hysteresis (TH) activity, where some isoforms exhibit a specific activity up to 100 greater than that of fish AFPs (Liou et al. 1999). The TH, a measure of antifreeze activity, refers to the difference between the melting and freezing points of a
solution. At present, insect AFP research is focused on the coleopteran species *Tenebrio molitor* and *Dendroides canadensis*, and on the lepidopteran species *Chloristoneura fumiferana*, where insect AFPS have been shown to exhibit similarities in the heterogeneity of structure, patterns of amino acid repeats, and α-helix structures (Li et al. 1998, Graether et al. 2000, Liou et al. 2000). The special characteristics and structures of AFPS are responsible for their specific orientations and functions. Owing to the distinctive overwintering methods of different insects, the relationships between the presence and expression levels of AFPS and photoperiods, environmental temperatures, development stages, and the sexes of insects have been investigated (Horwath et al. 1996, Graham et al. 2000). The results of such multifaceted analyses indicate that insect AFPS contribute significantly to protecting insects against low temperatures in a variety of ways (Qin et al. 2006).

Honeybees, one of the most common eusocial insects, are extremely sensitive to fluctuations in climate. Low temperatures not only delay larval and pupal development but also significantly influence the physiological activities of the adults. In the winter, when the ambient temperature is very low, the overwintering workers and the queen are clustered inside the hive, living on the honey reserves that were accumulated before the onset of winter. Workers maintain a temperature of about 20°C inside the colony by contracting their flight muscles, where the energy for heat production is supplied by the stored honey (Winston 1992). Individual honeybees are poikilothermic and maintain their body temperatures at or near a constant by burning calories during flight (Coelho 1991). When body temperatures decrease below 10°C, honeybees fall into cold-induced state of torpor (i.e., chill-coma) from which they cannot voluntarily recover without external warming (Heinrich 1980). As such, honeybees should be classified as freeze-avoiding insects (i.e., they die if frozen).

The Chinese honeybee (*Apis cerana cerana*) is an important endemic species in China that can be used as an effective model organism for studying cold resistance in honeybees (Xu et al. 2017). Most of the current studies on insect AFPS are focused on solitary species of freeze-tolerant insects, while reports regarding social Hymenoptera are scant. In the present study, we cloned the full-length cDNA of the *A. cerana cerana* AFP Maxi-like gene (henceforth *AcerAFP*) and analyzed the structure and functions of the corresponding protein using bioinformatics. The expression of *AcerAFP* was determined by real-time polymerase chain reaction (PCR) and western blot analysis. Moreover, RNAi was used to validate the function of *AcerAFP* and the correlation of AFP with antifreeze-related proteins. Through these in-depth studies, we have attempted to augment the existing level of knowledge regarding the sequence and function of honeybee AFPS and to provide a theoretical basis for understanding the mechanisms of the evolution of cold resistance in eusocial Hymenoptera.

**Materials and Methods**

**Insects and Treatments**

*A. cerana cerana* were collected from the colony maintained by the apiculture laboratory at Shaxi Agriculture University, Shaxi, China. Newly emerged adult worker bees (*n* = 300) were sampled 1, 5, 10, 15, 20, 25, and 30 d after emergence, and labeled with paint. These bees represented seven different age groups—adu1, adu5, adu10, adu15, adu20, adu25, and adu30, respectively. For tissue-specific expression analyses, the antennae, head, thorax, abdomen, legs, and wings of newly emerged bees (*n* = 100) were dissected on ice, and the tissues and whole body were frozen immediately in liquid nitrogen, and stored at −80°C.

Adult bees (post-emergence age 20 d) were equally divided into two groups (*n* = 60/group), which were maintained at constant temperatures of 0 and 10°C, and 50% relative humidity, in incubators for 0, 2, 4, 6, and 8 hr. Postemergence 10-d-old drones were collected in the same manner which was used to collect the workers. All bee specimens were immediately frozen in liquid nitrogen at the indicated time points and stored at −80°C.

**Measurement of TH Activity**

Two samples of hemolymph collected from 50 Chinese honeybees before and after RNA interference and purification of the heterologous expression of AFSs were selected to measure TH activity (THA). Bovine serum albumin (BSA) solution was used as a control. THA was determined using a differential scanning calorimeter (DSC Q20) (DSC Q20, TA Instruments, New Castle, DE, USA). After sampling, the temperature of samples was lowered from room temperature to −30°C, and then warmed to 15°C. Subsequently, samples were continuously cooled to −30°C and then warmed to −1°C (holding temperature, *T_h*), held at *T_h* for 3 min, and lowered to −30°C again. The rate of temperature variation was 1°C/min. The *T_h* was the temperature at which samples crystallized after the holding temperature. The THA = *T_h* − *T_f*. The total freezing temperature was the temperature at which the samples froze completely.

**Cloning *AcerAFP* cDNA**

The extraction of total RNA and the isolation of the full-length cDNA sequence of *AcerAFP* were performed following previously described methods (Zhao et al. 2014). The primers used in this study are listed in Supp Table 1 (online only). Total RNA was isolated from 10 worker bees using TRZol reagent (Invitrogen, Carlsbad, CA). Conditions for PCR amplifications were as follows: 4 min at 94°C; followed by 38 cycles at 94°C for 30 s, 58°C for 1 min, 72°C for 1 min 30 s, and a final extension at 72°C for 8 min. Purified PCR products of the correct sizes were ligated into the pGM-T vector (Tiangen Biotech, Beijing, China), and transformed into DH5α-competent cells. Positive colonies were identified by PCR and sequenced by the Huada Gene Research Center (Beijing, China).

**Bioinformatic and Phylogenetic Analyses**

ProtParam (http://web.expasy.org/protparam/) was used to predict the physicochemical properties of *AcerAFP*. Putative signal peptides and functions of predicted were performed using the SignalP 3.0 Server (Bendtsen et al. 2004). The PredictProtein web-resource (https://www.predictprotein.org/) was used to predict secondary structures. The Swiss model homology-modeling server (http://swissmodel.expasy.org/interactive) was queried to locate 3D structure models of *AcerAFP*. Phylogenetic and molecular evolutionary analyses were performed with Molecular Evolutionary Genetics Analysis (MEGA version 4.1), software using the neighbor-joining method. Homology searches were conducted on the NCBI platform (www.ncbi.nlm.nih.gov). The promoter was predicted using the BDGP web resource (http://www.fruitfly.org/seq_tools/promoter.html). The online tool PROMO (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoInoti.cgi?dirDB=TF_8.3) was used to predict putative cis-acting elements.

**Heterologous Expression and Purification of AFP**

The *AcerAFP* sequence was subcloned into the pEASY-Blunt vector for expression in *Escherichia coli* BL21b cells (Novagen, San Diego, CA). The primers for the pMAL-CX-6His recombinant vectors are listed in Supp Table 1 (online only). The expression of AFP was induced by isopropyl thiogalactoside (0.5 mmol/liter) (Sangon, Shanghai, China) at 11°C for 8 hr. The heterologous expression of *AcerAFP* was examined via western blot analyses using anti-6×His tag antibodies. The *AcerAFP* in the supernatant was collected and purified using an anti-6×His tag antibody. The purified *AcerAFP* was dissolved
in phosphate-buffered saline (pH 7.4, 2.0 mg/mL) and its concentration was determined using the Bradford method (Yu et al. 2016).

Expression Analysis of Temporal-Spatial Development and Responses to Low Temperature Using Real-Time PCR and Western Blot Analyses

The real time quantitative reverse transcriptase PCR (qRT-PCR) analysis was performed following previously described methods using the SYBR Premix Ex Taq kit (Takara, Dalian, China) (Zhao et al. 2014). The A. cerana cerana β-actin gene (HM640276.1) was used as an internal control. Transcript levels were evaluated in three independent biological replicates with three technical repeats for each primer pair. The relative quantification of gene expression was analyzed using the comparative 2−ΔΔCt method (Livak and Schmittgen 2001). The western blot analysis was performed following previously described methods (Zhao et al. 2015). For western blot analysis, a peptide corresponding to amino acids 18–31 of AcerAFP was synthesized in the Huada Protein Research Center (Beijing, China). New Zealand white rabbits were immunized with the synthesized peptide coupled to keyhole limpet hemocyanin to prepare the antiserum. The anti-AcerAFP serum was used as the primary antibody at a dilution of 1:1,000 (v/v). The BCA Protein Assay Kit (Boster, Wuhan, China) was used to detect the proteins. Peroxidase-conjugated goat anti-rabbit IgG (Boster) was used as the secondary antibody at a dilution of 1:3,000 (v/v). Subsequently, a super ECL chemiluminescence plus (Boster) was used for visualization after membranes were washed. The relative intensities of the aforementioned proteins were analyzed using Image Lab Software (Bio-Rad Laboratories, PA). The AcerAFP content was normalized to β-actin level in each lane.

RNA Interference

The dsRNAs were synthesized using the T7RiboMAX Express RNAi System (Promega, Madison, WI, USA) following the manufacturer’s protocol. Primer sequences (RIafp/RIgfp) are listed in Supp Table 1 (online only). The newly emerged adult worker bees were divided at random into three groups (n = 50/group) and were used in RNAi experiments. Each individual in the two treatment groups was fed with 10 μL (10 ng) of either dsAcerAFP or ds green fluorescent protein (dsGFP) and were designated as the dsAcerAFP group and the dsGFP group, respectively. The third honeybee group was left untreated and was designated as the control check (CK) group. Subsequently, ‘normal’ individuals (levels of physiological activity similar to those of the control group) were sampled for 5 d. This experiment was repeated in triplicate.

The Correlation Analyses Between AcerAFP Expression Levels and Small Cryoprotectants Concentrations

The concentrations of small cryoprotectants were listed in Supp Table 3 (online only). The Pearson correlation indices, which calculated by SPSS statistics 17.0, were used to evaluate the correlations between AcerAFP expression levels and small cryoprotectants.

Gene Expression Profiles of Cold-Related Proteins Before and After RNAi

To explore the expression profiles of cold-related protein genes before and after RNAi, we performed qRT-PCR on several cold stress-related different expression genes (9 HSPs, 7 STKs, and 12 zinc finger proteins [ZFPs]). The mRNA of genes expression of these cold-related proteins was detected in our previous experiments (Xu et al. 2017). Primers used for qRT-PCR analyses on the gene expression of cold-related protein genes are listed in Supp Table 1 (online only). The tested samples were collected from the dsAcerAFP and dsGFP groups after 3 d of treatment. The methods of analyses were the same as in the Expression Analysis of Temporal-Spatial Development and Responses to Low Temperature Using Real-Time PCR and Western Blot Analyses.

Results

THA in the Chinese Honeybee

The TH activities of hemolymph and BSA were detected using DSC (Fig. 1). Our results show that a heat-flow peak appeared in the hemolymph of the Chinese honeybee when the temperature of samples decreased from −1°C (T_h) to −30°C, while the temperature of the peak (T_f) was −1.53°C. In contrast, the control BSA solution exhibited no heat-flow peak in the cooling process. This indicates that the hemolymph of the Chinese honeybee had a THA of 0.53°C, whereas the control BSA solution exhibited no THA, suggesting the presence of AFPs in the hemolymph of Chinese honeybees. The total freezing temperature is the temperature at which the samples froze completely in the process of decreasing from T_h to −30°C. The total

![Fig. 1. TH values of Chinese honeybee hemolymph and BSA. (A) Result obtained from the DSC scan of Chinese honeybee hemolymph. T_h, the hold temperature; T_f, the total freezing temperature. (1) Result obtained from the DSC scan of the BSA solution.](image-url)
freezing temperature of hemolymph of Chinese honeybee and BSA were no −9.46 and −9.23°C, respectively.

**Cloning and Sequence Analysis of AcerAFP**

The full-length *AcerAFP* gene sequence (GenBank accession number: KX458243) is 1,200 bp in length and contains a 42-bp 5' untranslated region (UTR) and a 63-bp 3'-UTR. Sequence analyses show that the open reading frame is 1,095 bp long, and encodes a 365-amino acid protein with an estimated isoelectric point of 6.09. A hydrophobic signal peptide with 16 amino acid residues was identified (Fig. 2A). The prediction of secondary protein structures revealed that 96.43% of the amino acids formed helices, and 3.57% formed loops (Fig. 2B). The tertiary structure of AcerAFP confirms that α-helices constitute the main structure.
of the protein, whereas random coils constitute only a minor portion of its structure (Fig. 2C).

An alignment of AcerAFP with homologous proteins from other hymenopteran insects using NCBI BLASTp (Fig. 2D) indicates that more than 59% of the protein sequences of AcerAFP were comparable to AFP protein sequences from other insects. AcerAFP was highly similar to AmelAFP (96%) from the European honeybee Apis mellifera, while it was less similar to the corresponding AFP from the more distantly related parasitic wood wasp Orestus abietinus (63%). We found that AcerAFP amino acid numbering 87–269 were relatively well conserved. The phylogenetic tree constructed based on this finding directly reflects the relationship between the selected insects (Fig. 2E). The tree had two main groups: the first group (including A. cerana cerana) consisted of the superfamily Apoidea, whereas the other group consisted of the three hymenopteran families Formicidae, Tenthredinidae, and Orussidae.

Genomic Structure and Putative cis-Acting Elements in 5′-UTR of AcerAFP

The sequence of 5′-UTR of AcerAFP (results of FASTA in Suppl. Table 2 online only) was obtained from the NCBI database (GenBank ID: NW_016019342.1 [369089... 392989]) (Fig. 3). The promoter prediction for AcerAFP shows that the transcription start site is 560 nucleotides upstream from the translation start site. The promoter region (ATATTATATATATAAATGAACGAG AATTCTTTCAATATTTGAATAAAAT) extend from nucleotides −44 to +6. The results of the prediction analyses revealed that some sequences involved in embryo or tissue development, including Cf2-II (n = 9), Dfd (n = 1), BR-C (n = 13), Nit2 (n = 8), and CdxA (n > 50) listed in Suppl. Table 4 (online only). Several important transcription factors associated with environmental stress and immune responses, such as heat shock factors (n = 14), activating protein-1 (n = 4), nuclear factor kappa B (n = 1), and zinc-finger transcription factors (n = 4) were also identified in 5′-UTR of AcerAFP.

Heterologous Expression of AFP

AcerAFP was heterologously expressed in E. coli (Fig. 4A). Dodecyl sulfate, sodium salt-polyacrylamide gel electrophoresis (SDS–PAGE) analysis revealed that the recombinant protein was soluble, and had a molecular mass of ~125 kDa (results of western blot in Supp Figure 1 [online only]), which is consistent with the predicted molecular mass of 60 kDa (containing MBP-6xHis tags of ~64 kDa). These results of DSC in Fig. 4B indicate that the AcerAFP solution had a THA of 0.52°C, and exhibited a deeper total freezing temperature (~14.52°C) than the hemolymph in the Chinese honeybee. These results also indicate that the purified AcerAFP obtained in the present study belongs to the AFP family.

Expression Patterns in Temporal-Spatial Development of Honeybee

To determine the temporal and spatial expression patterns of AcerAFP mRNA, real-time PCR was performed using cDNAs prepared from various developmental stages and tissues (Fig. 5). The results of temporal expression-pattern analysis showed that AcerAFP mRNA levels continued to drop from 1- to 15-d-old adults and then remained constant from 20- to 30-d-old adults. The highest and lowest levels of AcerAFP were detected in 1- and 15-d-old adults, respectively (Fig. 5A). We also found that the highest level of AcerAFP mRNA occurred in the legs, followed by the thorax and wings. The lowest level of expression occurred in the abdomen (Fig. 5B). AcerAFP mRNA expression levels varied among sexes and were higher in workers than in drones (Fig. 5C).

Expression of AcerAFP in Response to Low Temperatures

The relative expression of AcerAFP mRNA and protein at different low temperatures and durations of exposure were determined by qRT-PCR and western blot analyses (Fig. 6). AcerAFP mRNA expression patterns varied significantly with varying low-temperature treatments. When exposed to 10°C, levels of AcerAFP mRNA continued to increase for 4 hr, when the highest expression levels were detected, after which the levels decreased and were maintained initial levels. When exposed to 0°C, AcerAFP mRNA expression levels increased initially and then decreased, where the highest and lowest expression levels occurred at 4 and 8 hr, respectively. By comparing the expression levels of AcerAFP mRNA at different temperatures for the same durations of exposure, we observed that expression levels at 10°C were higher than that at 0°C. Unsurprisingly, there were significant differences in AcerAFP protein expression levels at various low temperatures. At 10°C, AcerAFP levels decreased initially (2 hr), and then increased from 4 to 6 hr, followed by a drop to a lower level at 8 hr. Thus, the highest and lowest expression levels occurred at 6 and 2 hr, respectively. At 0°C, trends in expression-level changes in AcerAFP protein fluctuated, where they steadily decreased from 0 to 4 hr, and after a little recovery at 6 hr, decreased further to a lower level. The highest and lowest expression levels occurred at 4 and 6 hr, respectively. In general, the expression level of AcerAFP was higher at 10°C than at 0°C.

The determination of the concentration of different cryoprotectants under different temperature treatments (Supp Table 3 [online only]) revealed that the concentrations of glucose, glycogen, and amino acids varied significantly with different temperature treatments. It is believed that the metabolisms of these materials are involved in developing cold resistance in honeybees. Pearson correlation analysis was used to evaluate the interactions between the expression of AcerAFP and the concentrations of cryoprotectants (Table 1). The results of correlation analyses show that there is a strong relationship between AcerAFP mRNA expression levels and the concentrations of glucose, glycogen, and glutamic acid. The concentrations of the amino acids cysteine, histidine, alanine, and methionine were also found to be related to the expression of the AcerAFP protein.

RNAi of AcerAFP in Honeybees

The RNAi, mediated by dsRNA, was used to explore the function of AcerAFP. The result of gene silencing is shown in Fig. 7A and B. AcerAFP mRNA levels in the CK group decreased at first and then increased before finally decreasing. Constant levels of AcerAFP mRNA were maintained at two development stages: from 1 to 2 d and from 3 to 5 d. The expression levels of AcerAFP mRNA in the dsGFP group were lower at 1 d and were maintained at a constant level from 2 to 4 d. There were significant differences between the mRNA levels of the dsGFP group and the CK group at 1 and 2 d. These differences may be reduced in situations of stress by feeding dsRNA. In comparison to the CK and dsGFP groups, AcerAFP levels in the dsAcerAFP group decreased from 1 to 3 d, then recovered on day 4 and exhibited no difference from the levels expressed in the CK and dsGFP groups on day 5. A comparison of the inhibitory effect of RNAi from 1 to 5 d revealed that RNAi mediated by dsAcerAFP on 3 d was the most effective, where the gene-silencing efficiency was 77.82%. The result of western blot analyses showed that expression levels declined to 43.65% after feeding with dsAcerAFP. Thus, the samples from the dsAcerAFP and dsGFP groups after 3-day treatment were used for further analyses.
The TH activities of the hemolymph of the Chinese honeybee after a 3-d RNAi treatment were detected using DSC (Fig. 7C). The results showed that the THA of the hemolymph had risen from 0.53 to 0.56°C and that the total freezing temperature was reduced from −9.46 to −6.77°C before and after RNAi treatments, respectively.

In addition, we detected the expression levels of 28 candidate genes involved in cold hardiness (Xu et al. 2017)—belonging to three protein families (9 HSPs, 12 ZFPs, and 7 STKs)—in the dsAcerAFP and dsGFP groups (Fig. 7D–F). The results show that the expressions of 19 genes changed significantly, 9 of which were upregulated and 10 of which were downregulated. Two upregulated genes (HSC70-3 and HSF2BP) and three downregulated genes (HSC70-4, HSP60, and HSP90) belonged to the HSP family. Three upregulated genes (ZBED1, ZFP25, and ZIP19) and seven downregulated genes (ZFP36, ZFP431, ZFP708, ZFPN, ZFPR, ZMPN13, and ZIP13) belonged to the ZFP family. Finally, we only detected four upregulated genes (CG31145, STKA2, mig15, and PLK1) from the STKs.

Fig. 3. The nucleotide sequence and putative transcription factor binding sites of the 5′-flanking regions of AcerAFP. AP1, activating protein-1; HSF, heat shock factors; NFKB, nuclear factor kappa B; ZIC, zinc finger transcription factor.
Fig. 4. Dodecyl sulfate, sodium salt-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the expression of AcerAFP and the THA of purified AcerAFP. (A) M: molecular marker (kDa); Lane 1, non-induced expression; Lanes 2, induced overexpression of pMAL-AcerAFP-6×His in cell; Lanes 3, The purity protein after purification with 6×His tag antibody. (B) Result obtained from the DSC scan of the purified AcerAFP. $T_h$, the hold temperature; $T_f$, the total freezing temperature.

Fig. 5. Expression profile of AcerAFP as determined by qPCRs. (A) The relative expression of AcerAFP at different developmental stages. (B) The relative expression of AcerAFP in different tissues. AB, abdomen; AN, antennae; HE, head; LE, legs; TO, thorax; WB, whole body; WI, wings. The different letters above the columns indicate significant differences ($P < 0.05$) according to Duncan’s multiple range tests. (C) The relative expression of AcerAFP in different sexes. $P < 0.01$ indicates highly significant difference between the different sexes.

Fig. 6. Expression profiles of AcerAFP as determined by qPCR (A) and western blot analysis under low temperature stress (B). The data represent the means ± SEM of three independent experiments. The different letters above the columns indicate significant differences ($P < 0.05$) according to Duncan’s multiple range tests.
Fig. 7. Effects of AcerAFP RNAi in adults. (A) The mRNA levels of AcerAFP are shown in RNAi tests. (B) The expression level of AcerAFP in the honeybees with RNAi treatment on the third day. *P* < 0.01 indicate highly significant difference between the different treatments. (C) Result obtained from the DSC scan of the hemolymph was collected from 50 Chinese honeybees after RNA interference. *T*<sub>h</sub>, the hold temperature; *T*<sub>tf</sub>, the total freezing temperature. (D) The mRNA expression level of HSPs in dsAcerAFP group and dsGFP group. HSC70-3, heat shock 70 kDa protein cognate 3, 107997049; HSC70-4, heat shock 70 kDa protein cognate 4-like, 107996313; HSP10, heat shock protein 10 kDa, 108000341; HSP60, heat shock protein 60 kDa, 108000480; HSP90, heat shock protein 90, 408928; sHSP22.6, small hsp protein 22.6 gene, KF150018.1; sHSP24.2a, small heat shock protein 24.2a gene, KF150019.1; HSF2BP, heat shock factor 2-binding protein-like, 107996546; HSF5, heat shock factor protein 5-like, 102674894. (E) The mRNA expression levels of ZFPs in dsAcerAFP group and dsGFP group. ZMPN13, zinc metalloproteinase nas-13-like, 107994936; ZFP25, zinc finger protein 25-like, 107998072; ZIP13, zinc transporter ZIP13 homolog, 410710; ZBED1, zinc finger BED domain-containing protein 1-like, 108004414; ZFP36, zinc finger protein 36, C3H1 type-like 3, 410758; ZIP9, zinc transporter ZIP9, 107995642; ZFP708, zinc finger protein 708-like, 107997300; ZFPR, zinc finger protein rotund-like, 107998742; ZFP431, zinc finger protein 431-like, 108001041; ZFPN, zinc finger protein Noc-like, 108002202; ZKSCN5, zinc finger protein with KRAB and SCAN domains 5-like, 107993647; ZFPS82, zinc finger protein 582, 108002579. (F) The mRNA expression levels of serine/threonine-protein kinases in dsAcerAFP group and dsGFP group. CG31145a, extracellular serine/threonine protein CG31145, 107998746; STKA2, serine/threonine-protein kinase AurA-2-like, 108004128; PAK1, serine/threonine-protein kinase PAK mbi, 108003675; MKX1, MAP kinase-interacting serine/threonine-protein kinase 1-like, 108000389; STYK, serine/threonine-tyrosine-interacting protein kinase-like, 107993674; mig15, serine/threonine-protein kinase mig-15, 108003038; PLK1, serine/threonine-protein kinase PLK1-like, 107999607. **There were significant difference among the expression levels of antifreeze-related protein genes in different treatment groups.

### Table 1. The results of correlation analyses between AcerAFP expression levels and concentrations of cryoprotectants.

| Classification | Sugar | Polyols | Amino acids |
|----------------|-------|---------|-------------|
| **Cryoprotectant** | mRNA level | Protein level | mRNA level | Protein level | mRNA level | Protein level |
| **Glucose** | -0.956<sup>a</sup> | 0.447 | -0.952<sup>a</sup> | -0.410 | -0.461 | -0.798<sup*a</sup> | 0.275 | -0.561 | -4.26 |
| **Glycerin** | -0.970<sup>a</sup> | -0.577 | 0.07 | -0.148 | -0.970<sup>a</sup> | -0.468 | -0.600 |
| **Aspartic** | -0.461 | -0.372 | -0.725<sup>b</sup> | -0.597 | -0.613 | -0.578 | -0.546 |
| **Glutamic** | -0.372 | -0.372 | -0.725<sup>b</sup> | -0.597 | -0.613 | -0.578 | -0.546 |
| **Cysteine** | 0.275 | -0.561 | -0.644 | -0.644 | -0.652 |
| **Serine** | -0.561 | -0.644 | -0.644 | -0.652 |
| **Glycine** | -4.26 | -0.600 | -0.644 | -0.652 |

<sup>a</sup>Significant correlations between the expression of AcerAFP and the concentrations of cryoprotectants at 0.01 level.

<sup>b</sup>Significant correlations between the expression of AcerAFP and the concentrations of cryoprotectants at 0.05 level.
Discussion

Insects have a wide distribution across the various cold climatic zones on earth. Insect AFPs show diversity and variability between varied species, and can even exhibit significant differences within the same species. Insect AFP cDNAs generally encode a group of small proteins (7–21 kDa) rich in Cys or Thr residues, which together comprise nearly 40% of the constituent amino acids. These small AFPs are also composed of varying numbers of tandem 12–15 residue repeats. A model structure, obtained using X-ray crystallographic analysis, indicated that the insect AFPs fold as a β-helix (Doucet et al. 2009). In the present study, we found that AcerAFP encodes a 365-amino acid protein with a molecular weight of ~60 kDa that is rich in Ala and has some (n = 11) repeats of four residues, AAXA, in the constituent amino acid arrangement. The secondary and tertiary structures of the protein were mainly composed of α-helices. From the perspective of protein structure, AcerAFP did not resemble other insect AFPs, and was more similar to the fish type-I AFP. In sculpins and winter flounders, type-I AFP is rich in Ala, and its structure is composed entirely of α-helices (Low et al. 1998; Graether and Sykes 2004). The comparability of the protein sequences of AcerAFP with the protein sequences of other hymenopteran insects was 63–96%. These results indicate that the AFPs are highly divergent, likely because of the differences in the behaviors of individual species.

There are several factors that have been shown to affect the expressions of AFPs, such as environment temperature, humidity, and the length of photoperiod (Graham et al. 2000). Other than these external environmental factors, many autoregulatory factors are also crucial in affecting the presence of AFP transcripts (Qin and Walker 2006). For example, in C. fumiferana, AFP transcripts are most abundant in the second instar of the overwintering larvae, and are localized mainly in the fore and midguts of the larval body (Qin et al. 2006). In T. molitor, the presence of AFPs has been correlated with the stages of development (Graham et al. 2000). In the present study, the expression levels of AcerAFP mRNA in the newly emerged adult workers continued to drop until they were 15-d-old adults, and then remained relatively stable (from 15- to 30-d-old adults). This is likely because the newly emerged honeybee is too weak to resist cold stress, where elevated levels of AcerAFP help to increase survival to adulthood. Furthermore, before honeybees become old enough to engage in foraging activities (~15 d), cold hardness is well established, and AcerAFP mRNA expression is maintained at a lower level. AcerAFP mRNA was found to be expressed in all body tissues of honeybees, where particularly higher expression levels were detected in the locomotive organs (legs, thorax, and wings), possibly because locomotive organs have been shown to be sensitive to changes in environmental temperatures (Tosi et al. 2016).

Many species of insects in colder climates survive low temperatures by increasing AFP levels in their bodies. A study on T. molitor showed that exposing small larvae to 4°C for 4 wk increased AFP concentrations in the hemolymph by more than 20-fold (Graham et al. 2000). In the present study, as the expression of AcerAFP was affected by both decreased temperature and the duration of exposure, it is likely that AFP confers cold-resistance in these insects. The significantly higher expression of AcerAFP at 10°C than at 0°C could be the result of metabolic torpor at 0°C. The tendency of AcerAFP expression to increase at the early stages (0–4 hr) indicates that honeybees increase concentrations of AFP and improve their cold hardiness soon after exposure to low temperatures. The lower expression levels of AcerAFP after 4 hr suggest that the accumulation of AcerAFP might be detrimental to the health of honeybees (Kawahara et al. 2009). There was little difference between observed in western blot analyses, which is possibly a result of the differential expression levels of the different genes. In addition, we noted differences between the levels of mRNA and protein expression. This could be a result of the delayed effect of protein expression, existing mechanisms of post-translational control.

There is a great deal of research showing that small cryoprotectants and large molecular-weight proteins related to cold tolerance work together to improve the adaptability of insects to cold environments (Doucet et al. 2009). Unlike the cold-related proteins, changes in the concentrations of small cryoprotectants can directly reflect an organism’s ability to resist cold. Research on Drosophila melanogaster shows that the reserves of glycerogen, triacylglycerols, and proline might be important in coping with cold (Chen and Walker 1994). The larvae of the cold-hardy gall fly (Eurosta solidaginis) exhibit high rates of glycerin, sorbitol, glucose, and trehalose biosynthesis (Holden and Storey 1994). Studies on other overwintering insects have shown that glycoproteins and various amino acids are also related to the cold-stress response (Huang et al. 1990, Chen et al. 2005). On the basis of the aforementioned findings and components of fuel for over-wintering, we propose that the synthesis of glucose, glycerin, and various amino acids likely plays a key role in improving the adaptability of Chinese honeybees to cold (Chen and Walker 1993, Rochefort et al. 2011). This hypothesis was proven to be likely in the earlier stages of our study (Suppl Table 3 [online only]). Previous studies have shown that AFPs and some cryoprotectants are able to influence each other, and that the combination of cryoprotectants and AFPs also provide a novel approach to cold protection (Wen et al. 2016). In our study, the expression levels of AcerAFP were related to the concentrations of glucose, glycerin, glutamic acid, cysteine, histidine, alanine, and methionine, which corroborate the findings of Wen et al. (2016). Thus, AcerAFP may improve energy metabolism to reduce injuries caused by low temperatures.

RNA interference biotechnology has already proven its usefulness in functional genomic research on insects, and will soon prove to be very promising in medicine to control cancers and viral diseases (Huvenne and Smagghe 2010). In early studies on insects, most of experiments were conducted by directly injecting dsRNA into the organism (Hossain et al. 2008). Some studies showed the ability of organisms to autonomously take up dsRNA through food and assimilate it in the gut, thereby enabling efficient insect control (Baum et al. 2007). In this study, we inhibited AcerAFP mRNA by feeding dsRNA to A. cerana cerana. The results showed that the effect of dsGFP was less obvious, where there were no off-target effects, as there is no GFP target in honeybees (Elias-Neto et al. 2010). In contrast to the CK and dsGFP groups, mRNA and protein expression of the AcerAFP in the dsAcerAFP group decreased substantially (77.82 and 43.65%, respectively). These results, similar to previous findings (Zhang et al. 2014), showed that RNA interference by feeding dsRNA was a feasible and effective method in Chinese honeybees. In comparing the results of DSC before and after RNAi, we found that the silencing of AcerAFP considerably increased the total freezing temperature; however, there was no significant change in the THA of hemolymph in A. cerana cerana. A probable cause of this unexpected result is that there were undetected changes in the level(s) of other antifreeze-related protein(s). More research will be needed to further explore this possibility. Combined with the results obtained from the DSC scan of the purified AcerAFP, we speculate that AcerAFP increases cold resistance by lowering the total freezing temperature of the hemolymph in these honeybees.

In addition to small cryoprotectants, cold-related proteins play crucial roles in imparting cold resistance via their own unique mechanisms.
properties and structures (Molle and Kremer 2010, Bahar et al. 2013). HSPs are a super family of chaperone proteins that are rapidly biosynthesized in response to various environmental stressors through translation, the folding of newly synthesized proteins, and the degradation of unstable and misfolded proteins (Garrido et al. 2012, Sun et al. 2016). HSFBP primarily modulates HSF activation, and also appears to be the most insensitive to temperature changes of the HSPs (Fu et al. 2006). The ZFP family is large and widely distributed in plants, animals, and microorganisms (Miller et al. 1985). Zinc fingers can bind to DNA, RNA, and DNA-RNA hybrids, and are also involved in protein-protein interactions, regulating the expression of the target genes of transcriptional and translational processes (Pavlevich and Pabo 1991, Neely et al. 1999, White et al. 1985). Zinc fingers can bind to DNA, RNA, and DNA-RNA hybrids, and are also involved in protein-protein interactions, regulating the expression of the target genes of transcriptional and translational processes (Pavlevich and Pabo 1991, Neely et al. 1999, White et al. 1985).

In our study, some HSPs, ZFPs, and STKs were significantly altered in response to the RNA interference of AFP in Chinese honeybees, suggesting that the expression of AcerAFP is involved in the expression and activity of these cold-related genes. Furthermore, there may be a link between putative transcription factor binding sites in the 5’-UTR of AcerAFP, similar to the results of genomic structures.

Supplementary Data
Supplementary data are available at Journal of Insect Science online.

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