Characterization of the Cyclin-Dependent Kinase 6 Gene in *Apis cerana cerana* in Response to Multiple Environmental Stresses

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Cyclin-dependent kinases (CDKs) are serine/threonine kinases that play critical roles in the cell cycle regulation. Herein, we describe the identification of a CDK gene from *Apis cerana cerana*, named *AccCDK6*. The full-length cDNA is 1,778 bp long, including an ORF of 1,380 bp that encodes a polypeptide of 459 amino acid residues. Multiple sequence alignment analysis showed that the predicted AccCDK6 sequence shares a high similarity with CDK6 genes of other species, and this protein may share an evolutionary predecessor with *Drosophila* CDK4. The expression patterns of the gene were also analysed, and the transcript was detected throughout the larval, pupal, and adult developmental stages. Furthermore, the expression level of the mRNA of the gene in adult workers was influenced by H_2O_2, ultraviolet (UV) light, temperature (42 °C), $HgCl_2$, and pyriproxyfen. These results indicate that *AccCDK6* responds to multiple environmental stresses and may also participate in intracellular reactions of reactive oxygen species (ROS) and development processes in honey-bees.

Key words: Cyclin-Dependent Kinase 6, *Apis cerana cerana*, Cloning, Semi-Quantitative RT-PCR

Introduction

An individual cell may experience one of five fates during its lifetime: proliferation, differentiation, quiescence, senescence, or apoptosis. A proliferating cell progresses through the cell cycle, which is divided into four main phases, namely the G1 phase (or Gap 1), the S phase, the G2 phase, and the M phase. Once a cell is finishing the last cell cycle, it enters the G_1 phase, and processes that determine the cell fate occur during this period (Blomen and Boonstra, 2007). Besides, the length of the G_1 phase plays important roles in differentiation of some types of cells (Singh and Dalton, 2009; Salomoni and Calegari, 2010; Orford and Scadden, 2008). To ensure that the cell cycle can go through smoothly, multiple proteins are working co-operatively. Cyclins and the cyclin-dependent kinases (CDKs) are the primary regulatory proteins (Blomen and Boonstra, 2007). CDKs can bind to and be activated by cyclins to form cyclin-CDK complexes, and participate in cell cycle regulation. To date, 11 CDKs have been identified, with CDK4 and CDK6 playing particularly important roles. CDK4 and CDK6 have a high identity and similar functions, both of them are cyclin D-dependent kinases and can only bind to cyclin D (Malumbres and Barbacid, 2005; Matsushime *et al*., 1992; Meyerson and Harlow, 1994; Bird, 2003). However, they are not considered to be the only two cyclin D-dependent kinases. In mammalians, there are three types of cyclin D, *i. e.* cyclin D1, D2, and D3, and the latter two can also bind to and activate CDK2 (Ewen *et al*., 1993), but only the cyclin D-CDK4/6 complex is thought to be the unique G_1-specific cyclin-CDK complex that plays an important role during the early G_1 phase of the cell cycle. The cyclin D-CDK4/6 complex can phosphorylate the retinoblastoma (Rb) protein (pRB) and pRB-related "pocket proteins" (Sherr and Robert, 1999, 2004) and act as a tumour suppressor by inhibiting E2F transcription factors, whose activities are necessary for entry of the S phase (Blomen and Boonstra, 2007).

Many researchers have focused on the important regulatory functions of the cyclin D-CDK4/6 complex. As early as 1999, Cheng *et al.* have demonstrated that a severe reduction in CDK4

and CDK6 activities can be well tolerated and has no overt effects on the cell cycle. Later, mutant mice, including a CDK6-knockout, a CDK4/CDK6 double-mutant ($CDK4^{-/-}CDK6^{-/-}$), and a triple-mutant of all three D-type cyclins (cyclin $D1^{-/-}D2^{-/-}D3^{-/-}$), were generated for in-depth studies. The CDK6-knockout mice developed relatively normally, while $CDK4^{-/-}CDK6^{-/-}$ and cyclin $D1^{-/-}D2^{-/-}D3^{-/-}$ individuals were all dead before the end of gestation (Malumbres *et al*., 2004; Kozar *et al*., 2004).

CDK6 and CDK4 have long been regarded as functionally homologous kinases, but many differences were also found between them, such as timing activity, localization, tumour selectivity, and possibly even their substrates (Grossel and Hinds, 2006). In the past decades, many independent studies have indicated a novel role for CDK6 in differentiation. In multiple types of cells, such as murine erythroid leukemia (MEL) cells and the monocytic cell line RAW264.7, the down-regulation of CDK6 protein levels can trigger the entry of differentiation, whereas the overexpression of CDK6 leads to inhibition or even failure of differentiation (Matushansky *et al*., 2000, 2003; Ericson *et al*., 2003; Ogasawara *et al*., 2004a, b). These functions are apparently not shared with CDK4 (Grossel and Hinds, 2006).

Many studies have addressed the role of the cyclin D-CDK4/6 complex in the duration of the G_1 phase and in cellular differentiation throughout embryo development, and the complexity of a system involving three D-type cyclins and two G_1-specific cyclin D-dependent kinases has provided much matter for research. Previous works and the completed genome sequence showed that single genes for cyclin D and its G_1-specific kinase partner, designated *DmCDK4* (GenBank accession number AAM68505), are present in *Drosophila melanogaster* (Sauer *et al*., 1996; Adams *et al*., 2000). Accordingly, research on insect G_1-specific cyclin D-dependent kinases mainly focused on DmCDK4. Unlike the cyclin D-CDK4/6 complex in mammalian cells, *Drosophlia* CDK4 and cyclin D do not seem to be absolutely essential. Researchers found that the cyclin D-CDK4/6 complex can promote the accumulation of cell mass, rather than regulating the G_1/S phase directly. Furthermore, a lack of CDK4 was not lethal in flies: progress into and through cell cycle was observed, though with reduced growth level of cells and organs (Meyer *et al*., 2000; Datar *et al*., 2000). In contrast, ectopic expression of cyclin D/CDK4 led to larger cells and nuclear area in *Drosophila* fat body (Baltzer *et al*., 2009), and an increased mitochondrial quantity and mitochondrial cyclooxygenase (COX) activity (Frei *et al*., 2005). However, in *Caenorhabditis elegans*, *CDK4* has been demonstrated to be essential (Park and Krause, 1999). In addition, the completion of the genome sequence of *Apis mellifera* indicated that this organism expresses only a single cyclin D-dependent kinase, *AmCDK6* (GenBank accession number XP_391955). However, few publications were found regarding the differences, or potential identity, between *DmCDK4* and *AmCDK6*.

Apis cerana cerana is a species of honey-bee which uniquely exists in China and plays a critical role in the ecological balance and diversity of species. With an imminent threat of extinction, molecular biology research on *A. cerana cerana* should be conducted to provide a theoretical basis for protection. In addition, environmental challenges, such as increased ultraviolet radiation intensity, and heavy metal and pesticide pollution, are becoming increasingly serious and the damages to beneficial insects are becoming more prominent. Indeed, honey-bees are among the most severely affected animals. Researchers have demonstrated that, in plants, environmental stresses can affect the cell cycle by influencing the expression of CDK inhibitors via the abscisic acid (ABA) or jasmonic acid (JA) signaling pathways (Inzé, 2005). Conversely, such research focused on insects is relatively nonexistent. Therefore, the influences of environmental stresses on cell cycle regulation genes in honey-bee should be studied. Here, a cyclin-dependent kinase gene of *A. cerana cerana*, *AccCDK6*, was identified and the structure and expression patterns were also analysed. In this study, it was found that the newly characterized gene is highly similar to *Apis mellifera CDK6*. Semi-quantitative reverse-transcription-polymerase chain reaction (RT-PCR) indicated that the *AccCDK6* gene is expressed throughout the larval, pupal, and adult developmental stages. Furthermore, the expression level of the gene can be influenced by H_2O_2, ultraviolet (UV) light, temperature (42 °C), $HgCl_2$, and pyriproxyfen during the adult stage. These results suggest that *AccCDK6* participates in both environmental defences and development in honey-bees.

Material and Methods

Insect materials and treatments

All colonies of *A. cerana cerana* used in the experiments were maintained in our laboratories. Pupae white (PW) and adult workers were chosen for cloning of cDNA and genomic sequence, and 1-day-old workers were used for expression analysis. The workers were kept in an incubator and fed with sugar, pollen, and water. All colonies were divided into 6 groups, with 40 individuals in each group. Larvae, pupae, and adult workers were divided by ages as group 1. Group 2 was housed in an incubator at 42 °C. Groups 3 to 6 were subjected to the following environmental treatments, respectively: 2 mM H_2O_2, ultraviolet (UV) light (30 mJ/cm^2), $HgCl_2$ (3 μg/l) or 20 mg/l pyriproxyfen. The untreated individuals in each group were used as controls. All samples were flash-frozen in liquid nitrogen and stored at –80 °C until use.

RNA isolation, reverse-transcription, and DNA preparation

Total RNA was isolated from UV-treated adult workers and PW using Trizol reagent (TransGen, Beijing, China), incubated with RNase-free DNase I (Fermentas, Vilnius, Lithuania) for 30 min to degrade genomic DNA, and then stored at –80 °C until use. The first-strand cDNA was synthesized from 5 μg of total RNA using the EasyScript First-Strand cDNA Synthesis SuperMix (TransGen) according to the manufacturer's protocols. The DNA was extracted from adult workers using the cetyl trimethyl ammonium bromide (CTAB) method. The first-strand cDNA and genomic DNA were both kept at –20 °C.

Cloning and sequencing of internal fragment

A pair of primers, pM1 and pM2, was designed based on the nucleotide sequences of the CDK6 and CDK4 genes of other insects to obtain an internal, conserved fragment. PW cDNA was used as template for PCR, which was carried out according to the following program: 94 °C for 10 min, 35 cycles at 94 °C for 50 s, 50 °C for 50 s, 72 °C for 1 min, and then 72 °C for 10 min.

3′ and 5′ RACE experiments

Based on the nucleotide sequence of the internal, conserved fragment, sequence-specific primers were designed for 3′ RACE (rapid amplification of cDNA ends) and 5′ RACE experiments. The sequences of both the 3′ and 5′ ends of the CDK gene were obtained by nested PCR. To amplify the 5′ end of the CDK gene, the first-strand cDNA was purified by the Wizard DNA Clean-up System (Promega, Madison, USA), and then terminal deoxynucleotide transferase (TaKaRa, Dalian, China) was used for homopolymeric tailing to the 5′ end with dCTP. The first-time PCR was carried out, using the 5′ sequence-specific primer CDK5y and the adapter primer AAP, as follows: 94 °C for 10 min, 35 cycles at 94 °C for 50 s, 48 °C for 50 s, 72 °C for 1 min, and then 72 °C for 10 min. To increase the specificity, the inner primers, CDK5n-2 and AUAP, were used to amplify the 5′ end sequence following the same program with nested PCR.

The primers CDK3y and CDK3n-1 were used as the outer and inner primers paired with the adapter primers B26 and B25, respectively, for the 3′ RACE experiments. The PCR program was the same as that used in the 5′ RACE experiments, except that the annealing temperature was 48 °C.

Cloning of full-length cDNA and genomic sequence

The fragments obtained in the RACE experiments were analysed using the software DNAMAN version 5.2.2 (Lynnon Biosoft Company, Quebec, Canada), and a downstream primer, pM3, was designed based on the result. The primers pM1 and pM3 were used to amplify the full-length cDNA sequence of the CDK gene from first-strand cDNA of PW, following the program: 94 °C for 10 min, 35 cycles at 94 °C for 50 s, 47 °C for 50 s, 72 °C for 1.5 min, and then 72 °C for 10 min. This pair of primers was also used to obtain the genomic sequence from the genomic DNA of adult workers, with an elongation time of 2 min. All primers used for cloning (Table I) were synthesized by the Sangon Biotechnology Company (Shanghai, China). All of the PCR products were isolated by 1% agarose gel electrophoresis, purified, and then cloned into the pMD18-T vector (TaKaRa). The recombination plasmid was transformed into competent cells of *Escherichia coli* strain DH5α; positive clones were screened by PCR and sequenced by Sangon Biotechnology Company.

Bioinformation and phylogenetic analysis of the CDK gene

The open reading frame (ORF) searching and translation, together with molecular weight and isoelectric points (pI) prediction, were conducted by the software DNAMAN version 5.2.2. The nucleotide or amino acid sequences of similar enzymes were retrieved from the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/, NCBI, Bethesda, USA). Multiple sequence alignment among these enzymes was performed using DNAMAN version 5.2.2. Bioinformatics tools available at the NCBI server (http://blast.ncbi.nlm.nih.gov/Blast.cgi) were used to detect the functional domains of these proteins. Based on the alignment of different CDK amino acid sequences, phylogenetic analysis was conducted using the neighbour-joining (NJ) method in MEGA 4.1.

Semi-quantitative RT-PCR analysis

Semi-quantitative RT-PCR experiments were carried out to investigate the expression patterns of *AccCDK6* at the mRNA level. A pair of specific primers (RT1 and RT2, Fig. 1 and Table I) was designed to amplify a 374-bp fragment using an initial predenaturation at 94 °C for 5 min, followed by 28 cycles at 94 °C for 30 s, 53 °C for 30 s, and 72 °C for 30 s, and a final additional extension at 72 °C for 5 min. The sequence corresponding to primer RT1 is separated by the first intron in the genomic sequence, as shown in Fig. 1. The house-keeping gene, *A. mellifera β-actin* (GenBank accession number XM623378), was also amplified using primers R and F as inner standard (Yu *et al.*, 2011) under the same program described above to estimate equal amounts of RNA among the samples. To increase the integrity, three parallel experiments were performed. The stained PCR bands were normalized against that of *β-actin* using the Quantity-One™ and the ratios of *AccCDK6* to *β-actin* were calculated using the Quantity-One™ image analysis software implemented in VersaDoc 4000 (Bio-Rad, Hercules, CA, USA), respectively.

Table I. Primers used in the experiments.

Abbreviation	Sequence (5′ to 3′)
pM1	ATGGCCGGGACATCTCGA
pM2	GCATCCAACAGACCAAATATCAACAG
cdk5y	TTGGATACCGTAACATCAGTCTGC
cdk5n-2	TGCTCAAGTCATGTTCCTTCTCG
cdk3y	GAACACGGGACAAGTCGTAGC
cdk3n-1	GAGGTTGACTTCTGTGGTCGTG
pM3	TTGGATACCGTAACATCAGTCTGC
RT1	CCGCACATTGTAAGATTATTGGAC
RT2	CTAGGAGGACTTCTGGAGCC
R	GTTTTCCCATCTATCGTCGG
F	TTTTCTCCATATCATCCCAG
AAP	GGCCACGCGTCGACTAGTAC $(G)_{14}$
AUAP	GGCCACGCGTCGACTAGTAC
B26	GACTCGAGTCGACATCGA $(T)_{18}$
B25	GACTCGAGTCGACATCGAT

Results

Cloning of full-length cDNA of the CDK gene

Using the reverse-transcription products of total RNA as a template, a fragment of 1,082 bp was obtained by PCR with a pair of sequence-specific primers, pM1 and pM2, and then a 495-bp fragment was obtained in the 3′ RACE experiment. The 5′ RACE experiment was performed using the purified and tailed cDNA template, and a fragment of 430 bp was isolated. These three fragments were analysed using DNAMAN version 5.2.2. According to the result, primer pM3 was designed to confirm the sequence, together with pM1. Using this pair of primers, a 1,778-bp full-length cDNA sequence was obtained (GenBank accession number FJ_966898) and named *Apis cerana cerana CDK6* (*AccCDK6*). It includes an ORF of 1,380 bp, a 300-bp 5′ UTR (untranslated region), and a 98-bp 3′ UTR (Fig. 1). The entire ORF encodes a polypeptide of 459 amino acid residues (Fig. 1), with a predicted molecular mass of 52.9 kDa and an isoelectric point of 5.50.

Isolation of genomic sequence of the CDK gene

Primers pM1 and pM3 were also used to determine the genomic sequence of the CDK gene from adult worker DNA. The length of the genomic sequence of the CDK gene was 2,009 bp. Alignment of the genomic and cDNA sequence, respectively, showed that the ORF is divided into four exons by three introns, and no intron was found in the untranslated regions. These three introns were 62 bp, 87 bp, and 85 bp, respectively, in length and located at nucleotides 960–1,020, 1,363–1,448, and 1,608–1,691 of the genomic sequence, respectively.

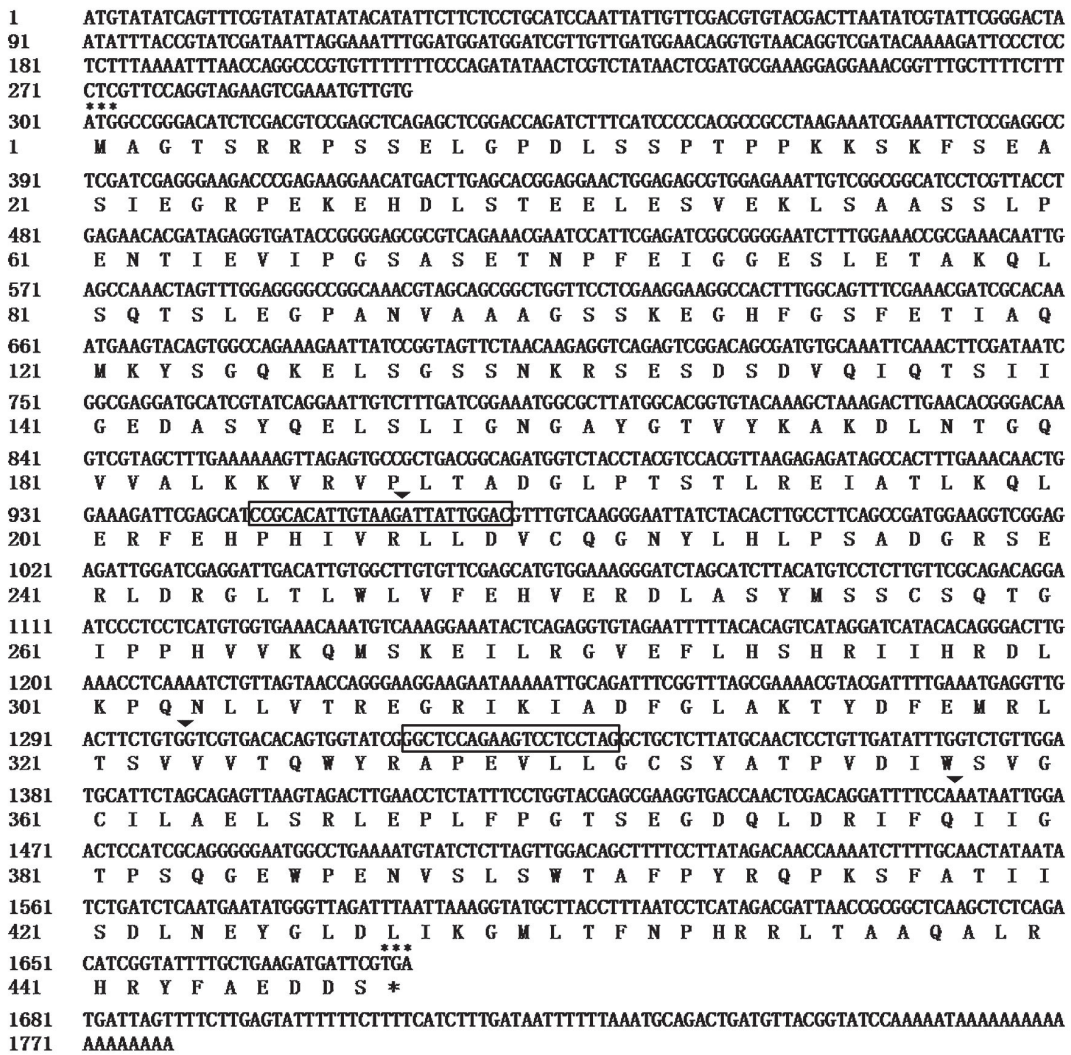

Fig. 1. Nucleotide sequence and deduced amino acid sequence of *AccCDK6* gene. The full-length cDNA is 1,778 bp (GenBank accession number FJ_966898) including an ORF that encodes a polypeptide of 459 amino acid residues. The start and stop codons are marked by asterisks (*) above the letters, and the black triangles indicate the position of the three introns. The sequences of the primers used in the semi-quantitative RT-PCR amplifying the *AccCDK6* fragment are framed; one of them is separated by the first intron in the genomic sequence.

Bioinformation and phylogenetic analysis of the CDK gene

Multiple alignments were conducted and a phylogenetic tree was constructed to examine the relationship between the predicted AccCDK6 protein and CDK4/6 proteins of other species. The deduced amino acid sequence of AccCDK6 has 99% identity with AmCDK6 (XP_391955) and 67% identity with NvCDK4 (*Nasonia vitripennis* CDK4, NP_001154936). AccCDK6 also shares high similarities with other CDK4/6 proteins except for amino acid residues 1 to 117, within which BLAST analysis showed little known functional domains. As shown in Fig. 2, the predicted AccCDK6 amino acid sequence shares highly conservative properties in some functional domains with both CDK6 and CDK4 proteins of insects and mammals, such as activity sites, ATP binding sites, substrate binding position, activity loop, and CDK/cyclin interface. These function-

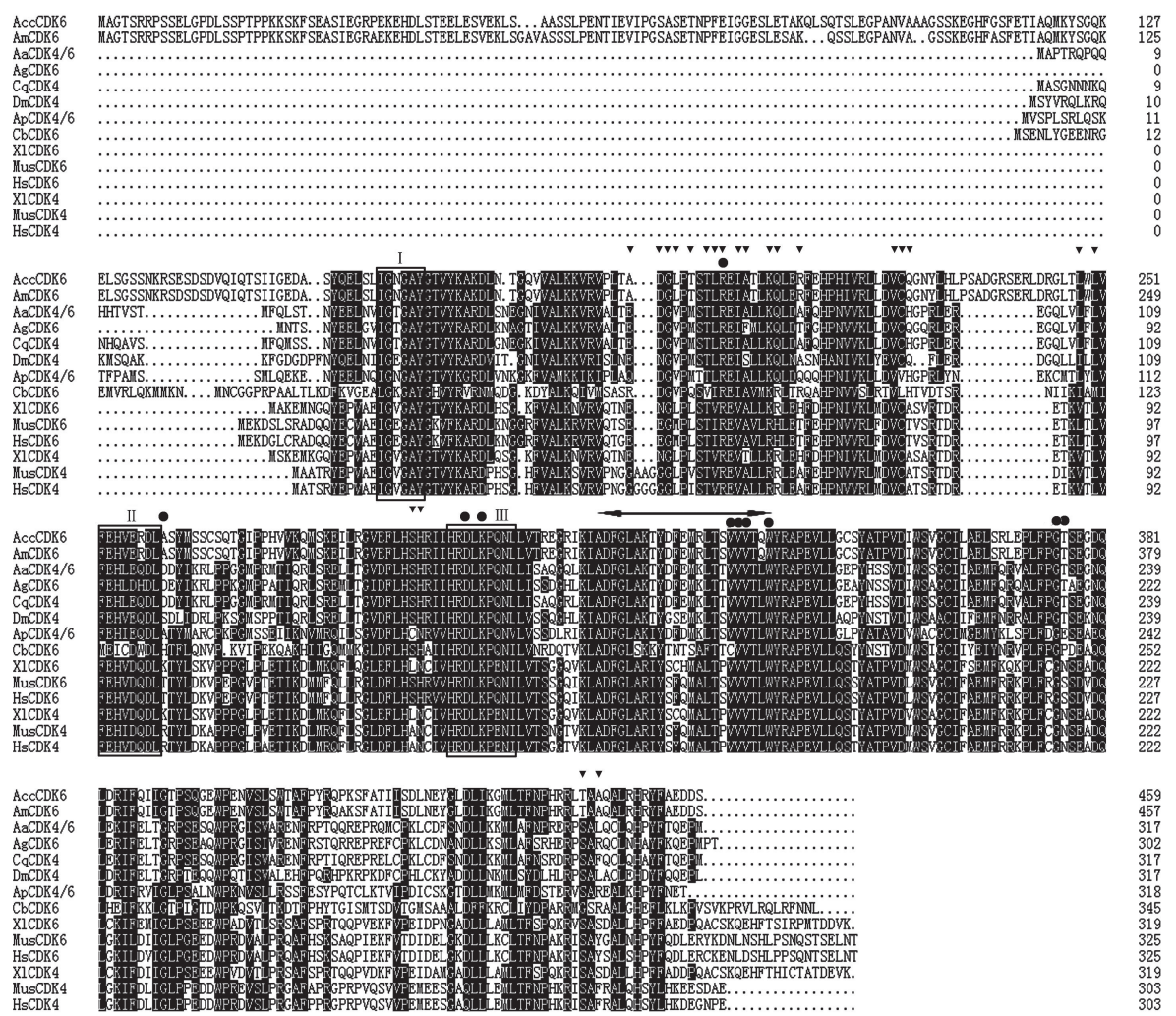

Fig. 2. Multiple amino acid alignment of vertebrate and invertebrate CDKs conducted by the software DNAMAN version 5.2.2. Active sites are boxed and numbered from I to III, while substrate-binding positions, CDK/cyclin interface, and activity loop are indicated by dots, triangles, and ←→, respectively. The first and second active sites also function as ATP binding sites. The following nucleotide and amino acid sequences retrieved from NCBI are shown in the alignment: AccCDK6 (*Apis cerana cerana*, FJ_966898); CbCDK6 (*Caenorhabditis briggsae*, AF520618); ApCDK4/6 (*Acyrthosiphon pisum*, XP_001947810); AaCDK4/6 (*Aedes aegypti*, XP_001659194); AgCDK6 (*Anopheles gambiae*, XP_315841); AmCDK6 (*Apis mellifera*, XP_391955); CqCDK4 (*Culex quinquefasciatus*, XP_001866128); DmCDK4 (*Drosophila melanogaster*, AAM68505); HsCDK4 (*Homo sapiens*, CAG47043); HsCDK6 (*Homo sapiens*, NP_001250); MusCDK4 (*Mus musculus*, AAH52694); MusCDK6 (*Mus musculus*, AAD43504); XlCDK4 (*Xenopus laevis*, CAA61666); XlCDK6 (*Xenopus laevis*, NP_001088955).

al domains are marked by different symbols in Fig. 2. The phylogenetic tree (Fig. 3) reveals that AccCDK6 is highly homologous to insect CDK6 s or CDK4 s. However, the similarity between AccCDK6 and mammalian CDK6 is less profound than the similarity between mammalian CDK6 and CDK4 of the same species.

Developmental expression pattern of AccCDK6

CDK6 or CDK4 is universally needed by mitotic cells. Here, to investigate the expression patterns between different developmental stages, semi-quantitative RT-PCR was performed. The mRNA level of *AccCDK6* was high during the

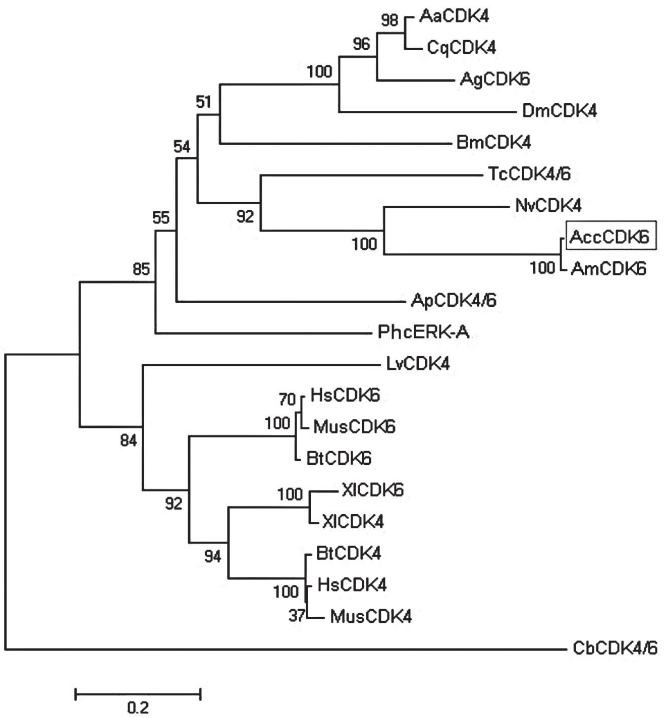

Fig. 3. Phylogenetic tree of CDK4/6 proteins from different species established by MEGA 4.1 software. The amino acid sequences of *AccCDK6* and *CbCDK4/6* were deduced by the software DNAMAN version 5.2.2. AccCDK6 is boxed. Sequences of following proteins were analysed in the tree: AccCDK6, CbCDK4/6, ApCDK4/6, AaCDK4, AgCDK6, AmCDK6, BmCDK4 (*Bombyx mori*, NP_001162052), BtCDK4 (*Bos taurus*, AAI09859), BtCDK6 (*Bos taurus*, DAA30804), CqCDK4, DmCDK4, HsCDK4, HsCDK6, LvCDK4 (*Lytechinus variegates*, AAK95393), MusCDK4, MusCDK6, NvCDK4 (*Nasonia vitripennis*, NP_001154936), TcCDK4/6 (*Tribolium castaneum*, XP_975093), PhcERK-A (*Pediculus humanus corporis*, EEB10788), XlCDK4, XlCDK6. The information on not defined sequences is provided in Fig. 2.

pupal state, while the quantities in larval and adult stages were both lower than that in the pupal. The expression levels in larval and adult stages were nearly equal, as shown in Fig. 4A. This result indicates that *AccCDK6* may not only participate, but also play an important role in the transition from larval to adult state.

Expression analysis of AccCDK6 under environmental stresses

Studies in mammalians have indicated that, during the later stages of development and in adults, D-type cyclin-CDK4/6 complexes appear to play essential roles as master integrators of the various mitogenic and antimitogenic signals conveyed by the extracellular environment (Bockstaele *et al.*, 2006). Therefore, the responses of *AccCDK6* in adult workers to various kinds of environmental stresses were examined. As shown in Fig. 4B, the level of *AccCDK6* mRNA was enhanced by H_2O_2, UV light, and temperature and reached the highest level at 1 h, 3 h, and 3 h of exposure, respectively. Additionally, $HgCl_2$ and pyriproxyfen were used to examine influences of a heavy metal and a pesticide on *AccCDK6* expression. As shown in Fig. 4C, the expression level of *AccCDK6* increased and reached the highest level at about 24 h after induction by $HgCl_2$ (3 μg/l), and showed a slight increase at about 0.5 h when treated with 20 mg/l pyriproxyfen. The response times for these stresses are different, so it can be inferred that the effects on *AccCDK6* of these stimulants are not direct but may rather be mediated by other proteins or signaling pathways. These results indicate that *AccCDK6* might participate in the responses of organisms or cells to damage caused by disadvantageous environmental factors.

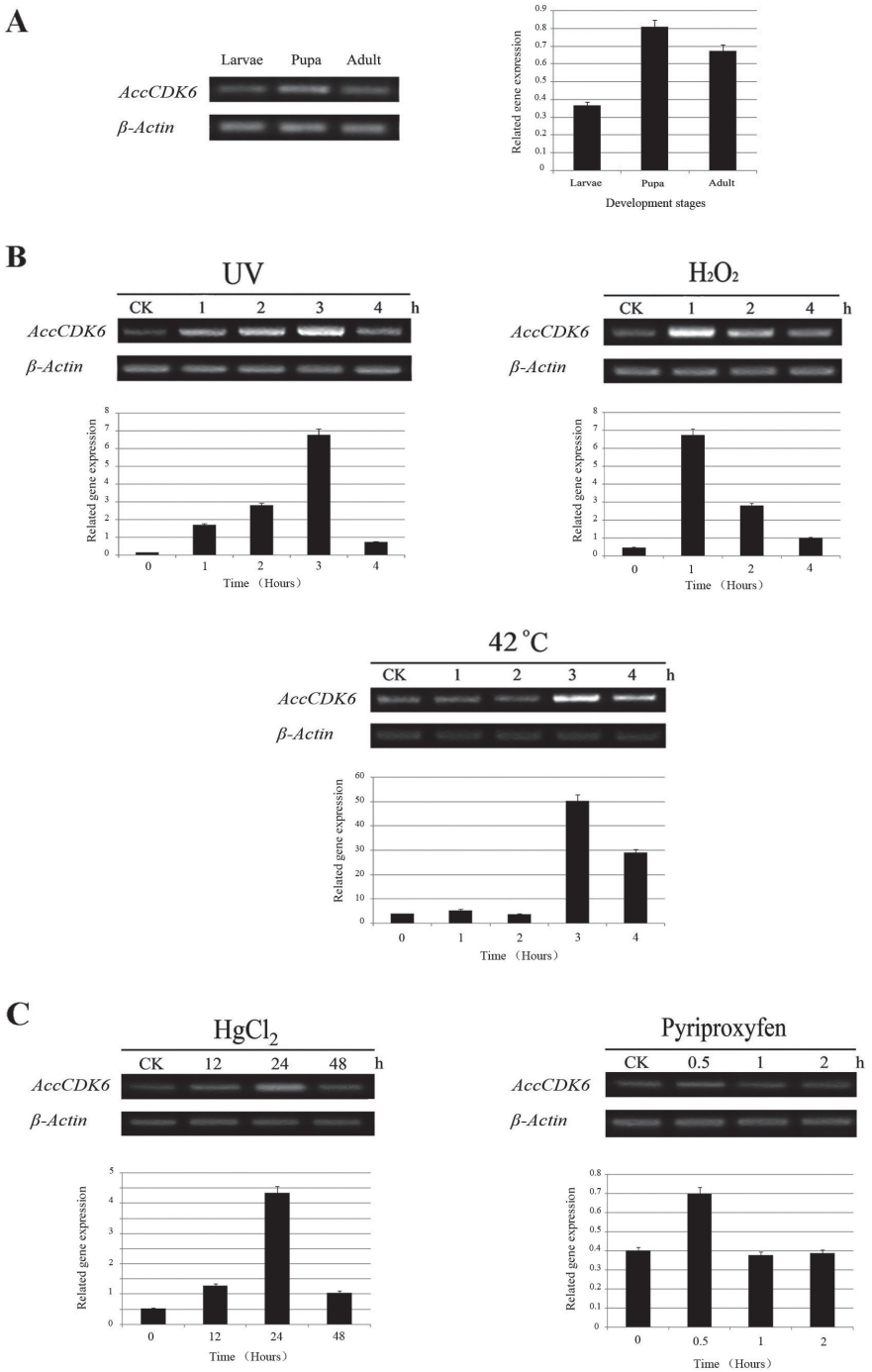

Fig. 4. Expression analysis of *AccCDK6* on mRNA level. (A) Expression pattern in developmental stages. (B) Expression patterns under environmental stresses: UV light, temperature (42 °C), and H_2O_2. (C) Expression patterns in the presence of the pollutants $HgCl_2$ and pyriproxyfen. Expression patterns of *AccCDK6* were studied in different developmental stages, under different environmental and pollution stresses. The *β-actin* gene was amplified as internal standard under the same program to estimate equal amounts of RNA among different samples. To increase integrity, three parallel experiments were carried out.

Discussion

CDK6 and CDK4 are highly homologous proline-dependent serine/threonine kinases that belong to the CDK enzyme family and act as regulators of cell cycle progression (Sherr and Robert, 2004; Ortega *et al*., 2002). Until now, many studies have focused on the functions of the mammalian G_1-specific cyclin D-dependent kinases, CDK4 and CDK6, and have provided multiple significant results. However, attention on the insect G_1-specific cyclin D-dependent kinases had mostly concentrated on *Drosophila* CDK4, with limited conclusions. In this study, for the first time cloning and analysis of the expression pattern of the cyclin-dependent kinase 6 gene in *A. cerana cerana* were undertaken. Multiple alignments of CDK4s and CDK6s of various species revealed that the predicted AccCDK6 amino acid sequence contains a unique fragment of 117 amino acid residues, and in AmCDK6 a highly similar fragment of 115 amino acid residues was also found. Our BLAST analysis showed that this region is exclusive to the honey-bee; information regarding the functions of this region is scarce thus far. Except for this polypeptide fragment, AccCDK6 showed a very high amino acid identity with both the CDK6 and CDK4 proteins of other species, especially in conserved function domains, as shown in Fig. 2. The polygenetic relationships showed that the deduced amino acid sequence of AccCDK6 shared high similarity with AmCDK6, and also with CDK4 or CDK6 of other insects, but AccCDK6 was less homologous to CDK4s of vertebrata. In insects, only one of the two genes exists in a given species, *i. e.* either CDK4 or CDK6. In the vertebrate *Xenopus laevis*, CDK6 was even more homologous to *X. laevis* CDK4 than to CDK6 of other species. According to the results of our multiple alignments and phylogenetic relationship analysis, we conclude that the ancestor of CDK4 and CDK6 derived from an ancestral signal protein. This protein evolved into two different proteins with many similar functions during the evolution of vertebrates, while during the evolution of insects, a signal gene remained CDK4 in *Drosophila* or CDK6 in the honey-bee. Thus, whether CDK4 and CDK6 are both present in a genome may be regarded as a taxonomic trait to a certain degree.

Previous research in mammals has demonstrated that CDK6 can regulate the activities of transcription factors that control the expression of differentiation-specific genes (Doonan and Kitsios, 2009). In the life of the honey-bee, the pupal stage is a period during which both growth and developmental events occur, for example, enlargement of the individual, formation of the head, thorax, abdomen, and legs, as well as the change of the colour of the body surface. The developmental stage-specific expression analysis revealed that the expression level of *AccCDK6* was higher during the pupal stage than that during the larval and adult stage, respectively. It is possible that this increase in the transcript level of *AccCDK6* was to meet the requirements of cell division and differentiation in the growth and developmental process. Otherwise, a rise in transcript levels may lead to an increased quantity of mitochondria and of mitochondrial COX activity (Frei *et al*., 2005), providing sufficient energy to ensure growth and developmental processes.

Recently, evidence has increased that the catalytic activity of CDK2 plays a critical role in the DNA damage response (Yata and Esashi, 2009). Another investigation uncovered that a minimum level of CDK activity is sufficient to promote DNA repair (Cerqueira *et al*., 2009). Meanwhile, molecular analysis showed that, in tissues lacking CDK4 and CDK6, interactions between cyclin D and CDK2 were enhanced (Malumbres *et al*., 2004). Therefore, CDK2 and CDK6 may share some similar functions. In our studies it was found that the expression of *AccCDK6* can be increased by UV light treatment suggesting that *CDK6* may also participate in the response to DNA damage caused by UV light. Honey-bees are able to regulate their nest temperature within a narrow range between 32 and 36 °C. When the temperature exceeds 36.5 °C, the time necessary for an egg to develop into an adult shortens, though the reason for this change remains unclear. The expression pattern of *CDK6* at 42 °C provided us with a possible explanation. A higher temperature can lead to increased levels of CDK6 mRNA, which may enhance the expression of related differentiation-specific genes, thus accelerating the developmental processes.

It has been demonstrated that environmental stresses such as H_2O_2, heavy metals, and heat, can enhance the endogenous reactive oxygen species (ROS) formation (Narendra *et al*., 2007). In addition, pesticides can suppress embryogenesis and adult formation, and can block the synthesis of

chitin of insects. All of these four environmental stresses can increase the expression level of *AccCDK6* to a certain degree. Our results suggest that *AccCDK6* participates in ROS-mediated reactions and may also be associated with the stability of the cellular membranes in honey-bee. In the future, cloning and analysis of the *AccCDK6* promoter, more expression analyses under different treatments, and transgenic experiments will be conducted to gain a better understanding of the functions of AccCDK6, with a particular emphasis on the cell cycle regulation and holometabolous development progress in honey-bees. This will provide us with more data on insect CDK4/6 functions.

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