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Arginine kinase in the cladoceran *Daphnia magna*: cDNA sequencing and expression is associated with resistance to toxic *Microcystis*

Kai Lyu[a], Lu Zhang[a], Xuexia Zhu[a], Guilian Cui[a], Alan E. Wilson[b], Zhou Yang[a,*]

[a] *Jiangsu Key Laboratory for Biodiversity and Biotechnology, School of Biological Sciences, Nanjing Normal University, 1 Wenyuan Road, Nanjing 210023, China*

[b] *School of Fisheries, Aquaculture, and Aquatic Sciences, Auburn University, Auburn, AL 36849, USA*

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ABSTRACT

Nutrient loading derived from anthropogenic activities into lakes have increased the frequency, severity and duration of toxic cyanobacterial blooms around the world. Although herbivorous zooplankton are generally considered to be unable to control toxic cyanobacteria, populations of some zooplankton, including *Daphnia*, have been shown to locally adapt to toxic cyanobacteria and suppress cyanobacterial bloom formation. However, little is known about the physiology of zooplankton behind this phenomenon. One possible explanation is that some zooplankton may induce more tolerance by elevating energy production, thereby adding more energy allocation to detoxification expenditure. It is assumed that arginine kinase (AK) serves as a core in temporal and spatial adenosine triphosphate (ATP) buffering in cells with high fluctuating energy requirements. To test this hypothesis, we studied the energetic response of a single *Daphnia magna* clone exposed to a toxic strain of *Microcystis aeruginosa*, PCC7806. Arginine kinase of *D. magna* (Dm-AK) was successfully cloned. An ATP-gua PtransN domain which was described as a guanidine substrate specificity domain and an ATP-gua Ptrans domain which was responsible for binding ATP were both identified in the Dm-AK. Phylogenetic analysis of AKs in a range of arthropod taxa suggested that Dm-AK was as dissimilar to other crustaceans as it was to insects. Dm-AK transcript level and ATP content in the presence of *M. aeruginosa* were significantly lower than those in the control diet containing only the nutritious chlorophyte, *Scenedesmus obliquus*, whereas the two parameters in the neonates whose mothers had been previously exposed to *M. aeruginosa* were significantly higher than those of mothers fed with pure *S. obliquus*. These findings suggest that Dm-AK might play an essential role in the coupling of energy production and utilization and the tolerance of *D. magna* to toxic cyanobacteria.

1. Introduction

Concomitant with the eutrophication of lakes, nuisance cyanobacterial blooms have become increasingly frequent and widespread for several decades (Chislock et al., 2013a; Merel et al., 2013). In addition to worsening the quality of water resources for human use, high cyanobacterial abundances may also have considerable negative impacts on herbivorous zooplankton, including the important generalist zooplankton genus, *Daphnia* (Reynolds, 1994; Wilson et al., 2006; Yang et al., 2011, 2012).

Cyanobacteria have several characteristics that make them insufficient as a food source for zooplankton. For example, grazing-resistant forms, such as large filaments and colonies, may mechanically interfere with feeding (De Bernardi et al., 1990; Yang et al., 2006), while the low content of essential fatty acids in cyanobacteria can suppress growth rates of zooplankton (Von Elert and Wolffrom, 2001). Moreover, some cyanobacterial genera can produce toxins (e.g. microcystins; MCs) (Catherine et al., 2013) that can inhibit zooplankton by reducing filtering rates or causing rapid death (DeMott et al., 1991; Lürling and Beekman, 2006; Xiang et al., 2010). Despite disagreement on the causal factors, cyanobacteria in the diet typically reduced zooplankton growth and reproduction (Lürling, 2003; Martin-Creuzburg et al., 2005; Wilson et al., 2006; Lyu et al., 2013a,b,c; Hochmuth and De Schamphelaere, 2014), which can lead to zooplankton population declines that can reduce energy transfer efficiency in aquatic food-webs (Miner et al., 2012; Ger et al., 2014).

To withstand toxic cyanobacteria, some *Daphnia* species have been shown to adapt to toxic cyanobacteria (Gustafsson et al., 2005; Guo and Xie, 2006; Wilson and Hay, 2007; Chislock et al., 2013b; Jiang et al., 2013). For example, Hairston et al. (1999) first showed the evolution of resistance in *Daphnia galeata* to

* Corresponding author. Tel.: +86 25 85891671.
E-mail address: yangzhou@njnu.edu.cn (Z. Yang).

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1    ACT CTG TGT CGG TGA TCA CTA CTG TTC GCT CCG CAG ATT CCT TTT   45
46   CGT GTT CTT CCT TGC ATC AAA ATG GTT GAC GCC GCC GTT GCC GAG   90
                                 M  V  D  A  A  V  A  E     8
91   AAA TTG GAA GCT GGA TTC CAG AAG CTC CAG GAA GCC ACC AAC TGC  135
9     K  L  E  A  G  F  Q  K  L  Q  E  A  T  N  C     23
136  AAG TCT CTG TTG AAG AAG CAC CTC ACT CGC GAG ATC TTC GAC AAG  180
24    K  S  L  L  K  K  H  L  T  R  E  I  F  D  K     38
181  ATC AAG GAT CTC AAG ACC TCC TTC GGA TCC ACC CTT CTC GAT GTC  225
39    I  K  D  L  K  T  S  F  G  S  T  L  L  D  V     53
226  ATC CAA TCT GGT GTT GAG AAC TTG GAC TCT GGA TTC GGT GTG TAC  270
54    I  Q  S  G  V  E  N  L  D  S  G  F  G  V  Y     68
271  GCC CCC GAT GCC GAA GCT TAC AGC GTT TTC AAC GAC CTC TTC GAA  315
69    A  P  D  A  E  A  Y  S  V  F  N  D  L  F  E     83
316  CCC ATG ATC TGC GAT TAC CAC ACC GGA TTC AAG CCC GGA GAT GCT  360
84    P  M  I  C  D  Y  H  T  G  F  K  P  G  D  A     98
361  CAC CCA CCC AGG GAC TTT GGT GAT CTC GAG ACT TTC GGC AAC TTG  405
99    H  P  P  R  D  F  G  D  L  E  T  F  G  N  L    113
406  GAC CCC GAG GGC GCC TTC ATC GTC TCC ACC CGC GTC CGT TGC GGC  450
114   D  P  E  G  A  F  I  V  S  T  R  V  R  C  G    128
451  CGA TCC TTG GCC GGC TAT GCC TTC AAC CCT TGC TTG ACT GAG GCC  495
129   R  S  L  A  G  Y  A  F  N  P  C  L  T  E  A    143
496  AAC TAC AAG GAG ATG GAA GAG AAA GTC GTC GCC AGC TTG TCC TCC  540
144   N  Y  K  E  M  E  E  K  V  V  A  S  L  S  S    158
541  TTG GAA GGC GAA CTC AAG GGA ACT TAC TAC CCA TTG ACT GGC ATG  585
159   L  E  G  E  L  K  G  T  Y  Y  P  L  T  G  M    173
586  ACC AAG GAA GTC CAG ACC CAG CTC ATC CAG GAT CGT TTC CTC TTC  630
174   T  K  E  V  Q  T  Q  L  I  Q  D  R  F  L  F    188
631  AAG GAG GGA GAT CGC TTC CTT CAG GCT GCC AAC GCC TGC CGC TAC  675
189   K  E  G  D  R  F  L  Q  A  A  N  A  C  R  Y    203
676  TGG CCC ACC GGA CGT GGC ATC TAC CAC AAC GAC GCC AAG ACC TTC  720
204   W  P  T  G  R  G  I  Y  H  N  D  A  K  T  F    218
721  TTG GTT TGG TGC AAC GAG GAA GAT CAC TTG CGC ATC ATC TCC ATG  765
219   L  V  W  C  N  E  E  D  H  L  R  I  I  S  M    233
766  CAG AAA GGT GGT GAC TTG AAG GCC GTC TAT GCC CGT CTC GTT AAC  810
234   Q  K  G  G  D  L  K  A  V  Y  A  R  L  V  N    248
811  GCC ATC AAC GAA ATC GAG AAG AGG ATT CCC TTC TCT CAC CAC GAT  855
249   A  I  N  E  I  E  K  R  I  P  F  S  H  H  D    263
856  AAA TAC GGT TTC TTG ACC TTC TGC CCA ACC AAC TTG GGC ACC ACC  900
264   K  Y  G  F  L  T  F  C  P  T  N  L  G  T  T    278
901  ATC CGC GCT TCC GTC CAC ATT GCG CTG CCC AAA TTG GCT GCT GAT  945
279   I  R  A  S  V  H  I  A  L  P  K  L  A  A  D    293
946  CTT GCC AAG CTC GAA GAG GCC GCC GGA AAG TTC AAC CTC CAG GTC  990
294   L  A  K  L  E  E  A  A  G  K  F  N  L  Q  V    308
991  CGT GGA ACT GCT GGT GAA CAC ACC GAA GCC GAA GGT GGT GTG TAC 1035
309   R  G  T  A  G  E  H  T  E  A  E  G  G  V  Y    323
1036 GAC ATC TCC AAC AAA CGC CGC ATG GGT CTG ACT GAA TAC CAG GCC 1080
324   D  I  S  N  K  R  R  M  G  L  T  E  Y  Q  A    338
1081 GTC AAG GAG ATG TAC GAT GGT CTC CAG GAG CTC ATC CGC ATG GAG 1125
339   V  K  E  M  Y  D  G  L  Q  E  L  I  R  M  E    353
1126 AAA GAG GCT GCT TAA ATC TCT TCC TTT CTC TCT GTC TCT CCC ACA 1170
354   K  E  A  A  *
1171 GCC CTT CTT AAT AAT AAT AAT ATA TTA TTA CAC CAT TAG CCA TGT 1215
1216 GTT AAG ATC TGC TTA AAA AGC GTA TTT GAA CTG ATT ATT GTA GAT 1260
1261 GTA TTG GGC CTA ATT TGC GAC ATC CAT TGG AAC CGT TTT CTT TCT 1305
1306 CCT TCT TCT TCT CTA TTA AAG TTG TTG TTG TTG TTG TAC AAC 1350
1351 GGT GTG AGA TAC GGT TTT AAC ACA CCG CCA CGT CAT CGA GTT TTG 1395
1396 AAA GTC CTT TGT ATC CGA AGA ATT CTT CTG TGT CCA TTG TGT GTA 1440
1441 CAT CCA CGA CGG AAC GGG TTG GAA GGC TCT GCA GGA TCC ACT GCT 1485
1486 CGA GAT TTG CAC TCG ATA GTC GAA ATA GCG CTC GAT TCT ACT CTT 1530
1531 CAT TGT AAC GAA TAT TGT TTT TTT GAT ATT TGT TAC AAT ACC AGA 1575
1576 GTG CGT TAT TAA TGG CAC ATA TCG AAT TTG GAA GAG AAA GAG ACT 1620
1621 GCA AAG AGA TGT AAA ACA TGG AGA AAG CCC ACG TAT ATC AGT GAT 1665
1666 TTG TTC AAA AAA ACC GCA AAA AAA AAA AAG AAT ACA CAG AAA CCA 1710
1711 TCC CTA AAA AAA AAA AAA AAA AAA AAA AAA AAA               1743
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Fig. 1. Nucleotide and deduced amino acid sequences of *D. magna* arginine kinase (Dm-AK). The nucleotide sequence is numbered from 5′ end, and the single letter amino acid code is shown below the corresponding codon. The start codon (ATG) and the end codon (TAA) highlighted in fluorescent green color. Potential ATP: guanido phosphotransferases active site highlighted in pink color. Four protein kinase C phosphorylation sites are highlighted in yellow color. One N-myristoylation site is highlighted in gray color. Six casein kinase II phosphorylation sites are highlighted in turquoise color. The termination code is marked with an asterisk. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

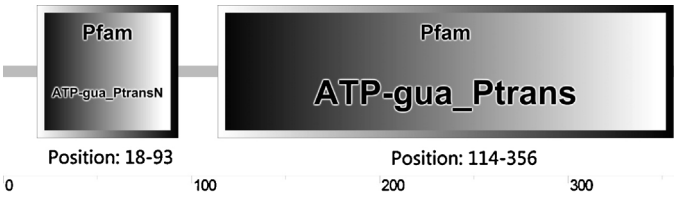

Fig. 2. The architecture of deduced Dm-AK amino acid (356 amino acids). An ATP-gua PtransN and an ATP-gua Ptrans domain were at the position 18–93 and 114–356, respectively. Protein domains were predicted by SMART program.

cyanobacteria by contrasting the performance of *Daphnia* clones hatched from contrasting periods in the eutrophication history of Lake Constance to diets lacking or containing toxic *Microcystis aeruginosa*. Since then, related studies have shown that adaptations in *Daphnia* to toxic cyanobacteria occur across lakes that vary in productivity and abundance of cyanobacteria (Sarnelle and Wilson, 2005) and that the effects of *Daphnia* resistance on algal abundance can be as important as the presence or absence of *Daphnia* (Sarnelle and Wilson, 2005; Chislock et al., 2013b). Even short-term exposure to *M. aeruginosa* can quickly induce resistance to cyanobacteria in *Daphnia* (Gustafsson and Hansson, 2004) or cause feeding selectivity to reduce the ingestion of cyanobacteria (Tillmanns et al., 2011). One possible explanation for these observations is that tolerant zooplankton may elevate energy production, thereby adding more energy allocation to detoxification expenditure (Bossuyt and Janssen, 2004; Ortiz-Rodríguez and Wiegand, 2010; Bergman Filho et al., 2011). Recent results on the gene expression of key enzymes of carbohydrate and protein metabolism showed disruptions on the energy producing pathways of *D. magna* exposed to *M. aeruginosa*. These findings suggest that important functions of energy status to maintain metabolic homeostasis may compete with energy deficiency and toxicity due to feeding on cyanobacteria (Schwarzenberger et al., 2009; Asselman et al., 2012).

In invertebrates, arginine kinase (ATP: arginine N-phosphotransferase, EC 2.7.3.3; AK) which is analogous to the creatine kinase (CK) reaction in vertebrates, serves as a core in temporal and spatial ATP buffering in cells with high, fluctuating energy requirements (muscle, nerves, etc.) (Uda et al., 2006). AK catalyzes the reversible substrate-level phosphorylation of arginine by MgATP to form phosphoarginine and MgADP, thereby regenerating ATP during bursts of cellular activity (Zhou et al., 1998; Alonso et al., 2001). A growing literature has revealed an evolutionary relationship between invertebrate AK and vertebrate CK and their analogous metabolic roles (Bogdan, 2001). AK has been found to synthesize phosphagen in one direction when ATP supply is abundant, and in the opposite direction to mediate the rapid breakdown of phosphagen during acute stress response. In recent studies, it was elucidated that the expression of AK correlated closely with salinity change in blue crab, *Callinectes sapidus* (Kinsey and Lee, 2003), acclimation to cadmium in crab, *Eriocheir sinensis* (Silvestre et al., 2006), the exposure to lead in yabby, *Cherax destructor* (Morris et al., 2005), and hypoxic stress in the kuruma prawn *Marsupenaeus japonicas* (Abe et al., 2007). Therefore, AK is believed to play a crucial role in environmental stress responses in terms of regulating energy production and utilization in crustaceans.

However, little is known about AK characterization and functions of *Daphnia* which have been shown to locally adapt to

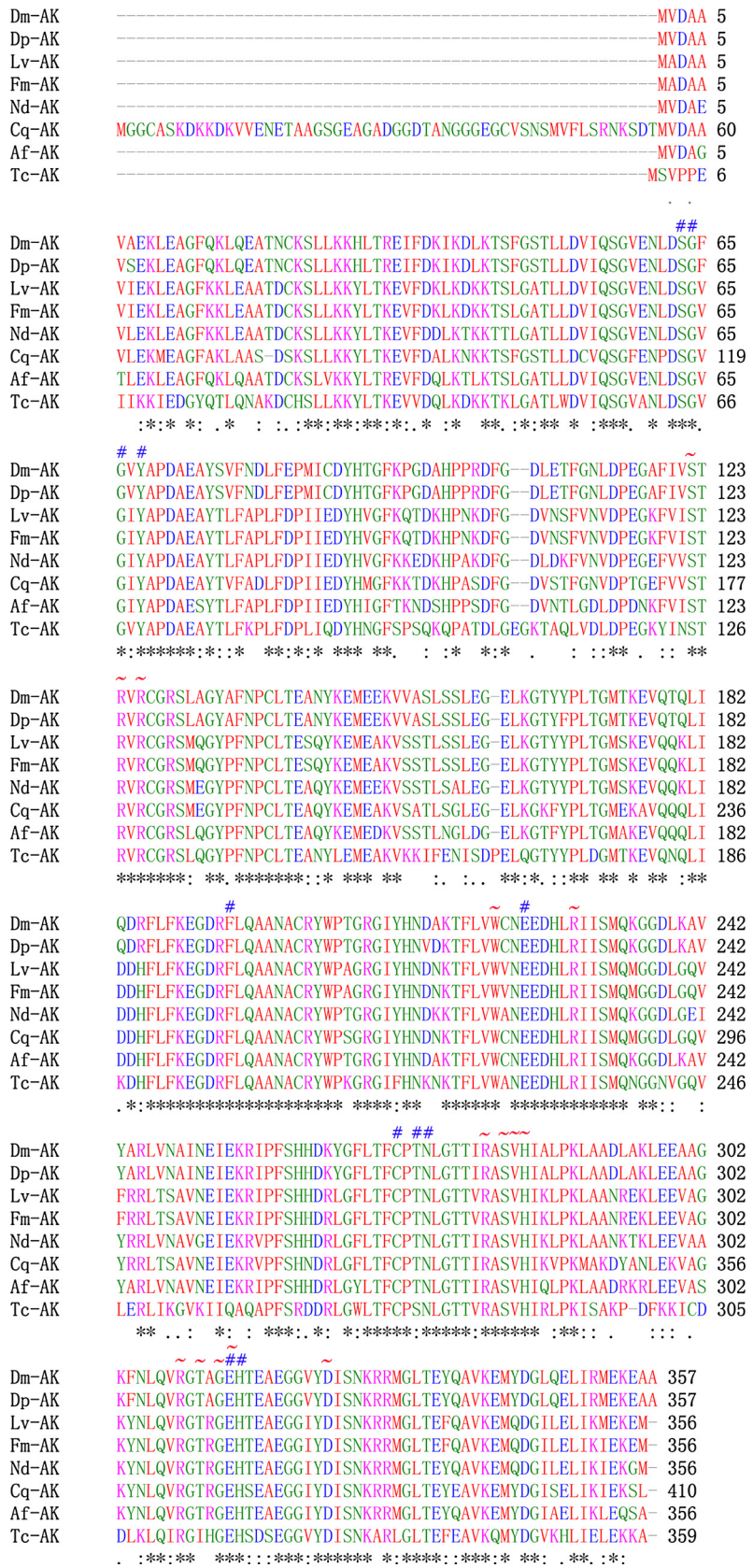

Fig. 3. Multiple alignment of AK amino acid sequences from Dm-AK, Dp-AK, Lv-AK, Fm-AK, Nd-AK, Cq-AK, Af-AK and Tc-AK. Identical (*) and similar (. or:) amino acid residues are indicated. Gaps (-) were introduced to maximize the alignment. The 11 residues interacted with arginine was remarked with '#'. The 14 residues interacted with ADP were remarked with '~'.

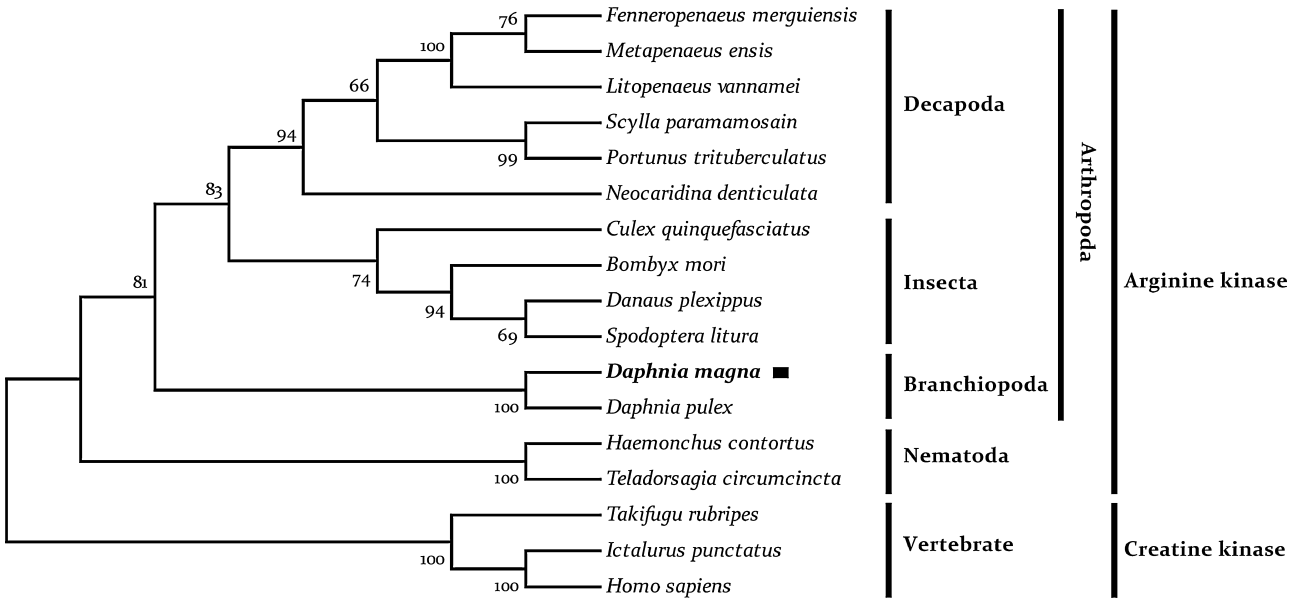

Fig. 4. Phylogenetic analysis of Dm-AK with other invertebrate arginine kinases (AKs) and vertebrate creatine kinases (CKs). Neighbor-Joining tree was produced with the Mega5 software. 1000 bootstraps carried out to check the repeatability of the result.

toxic cyanobacteria. Given that the energy status of an organism at any given time could affect its capacity to cope with stress, we tested the hypothesis that upregulated AK gene expression and ATP content may promote rapid adaptation of resistance to toxic *M. aeruginosa* in *D. magna*. Elucidation of the energy dynamics involved with *M. aeruginosa* tolerance might allow us to better understand earlier documented local adaptation in *Daphnia* to cyanobacteria and subsequently allow a mechanistic understanding of the phenotypic plasticity processes in *Daphnia* populations. To prepare for this bioassay, a full-length cDNA of *D. magna* (Dm-AK) was identified by sequencing and structural characterization.

2. Materials and methods

2.1. Daphnia and algae culture conditions

The *D. magna* clone used in the experiment was established from a single parthenogenetic female obtained from the State Key Laboratory of Pollution Control and Resource Reuse, Nanjing University (Nanjing, China); this isoclonal population is maintained under laboratory conditions in 3 L beakers filled with M4 medium and fed with *Scenedesmus obliquus* (1.5 mg C L^{-1}) at 25 °C under fluorescent light at 40 μmol photons m^{-2} s^{-1} with a light–dark period of 12:12 h (Yang et al., 2012). Medium was gently aerated with filtered air for 24 h before use and animals were transferred to new medium twice a week.

The microcystin-producing cyanobacteria, *M. aeruginosa* (PCC7806), and the chlorophyte, *S. obliquus* (FACHB-416; nontoxic food source), were obtained from the Freshwater Algae Culture Collection of the Institute of Hydrobiology at the Chinese Academy of Sciences. The *M. aeruginosa* strain, which grows as single or paired cells, was chosen for this study to avoid any mechanical interference issues that might influence *Daphnia* fitness. Both algal cultures were maintained in BG-11 medium (Stanier et al., 1971) under the same conditions as *D. magna*. Diet algal densities were determined using a blood-cell counting chamber under an inverted Nikon microscope at 400× magnification and converted to total organic carbon (TOC) using a previously developed calibration curve between cell densities and TOC (Supplementary Data (SD) Fig. S1).

2.2. Dm-AK cDNA sequencing

We collected four 5-day-old *D. magna* and homogenized them using a pestle prior to extracting total RNA using the Trizol technique following manufacturer instructions (Takara, Japan). In order to remove any traces of genomic DNA, the RNA was treated with DNase I (Invitrogen, USA). The integrity of the RNA was verified with a 2100 bioanalyzer (Agilent). cDNA was synthesized from 5 μg total RNA by reverse transcription using a M-MLV RTase cDNA Synthesis Kit (Takara, Japan). Then, we used AK1103-F and AK1103-R (Table S1) as the PCR primers to amplify Dm-AK coding sequences. The primer set was designed against the conserved regions of the corresponding genes in *E. sinensis*, *Hydrochara obtusata*, and *Conchoecetes artificiosus*. PCR program and vector ligation was extensively described in SD.

2.3. Rapid amplification of cDNA ends (RACE)

The Dm-AK partial cDNA sequence was extended using 5′ and 3′ RACE (SMARTer^{TM} RACE cDNA Amplification Kit, Clontech). The 3′ RACE PCR reaction was carried out in a total volume of 50 μL containing 2.5 μL (800 ng μL^{-1}) of the first-strand cDNA reaction as a template, 5 μL of 10× Advantage 2 PCR buffer, 1 μL of 10 mM dNTPs, 5 μL (10 mM) gene-specific primer (3′-AK-1 and 3′-AK-2; SD Table S1), 1 mL of Universal Primer A Mix (UPM; Clontech), 34.5 μL of sterile deionized water, and 1 U 50× Advantage 2 polymerase mix (Clontech). For the 5′ RACE, UPM was used as forward primers in PCR reactions in conjunction with the reverse gene-specific primers (5′-AK-1, 5′-AK-2 and 5′-AK-3; SD Table S1). All RACE PCR was performed based on manufacturer instructions. After ligating into the vector, the samples were sequenced at Springen (Nanjing), China.

2.4. Characterization of AK

The cDNA sequence and deduced amino acid sequence of AK were analyzed using the BLAST algorithm (http://www.ncbi.nlm.nih.gov/blast). Translation and protein analyses were performed using ExPASy tools (http://us.expasy.org/tools/). The ClustalW Multiple Alignment program (http://www.ebi.ac.uk/clustalw/) was used to create the multiple sequence alignment. The predicted

molecular weight was calculated using an online tool (http://expasy.org/cgi-bin/pi_tool). To establish the tertiary structure of mature Dm-AK, a 1.6 Å crystal structure of *Litopenaeus vannamei* AK monomer from PDB database (ID, 4bg4.2) was selected as the template by Swiss-Model (http://swissmodel.expasy.org/). The structural models were visualized with DeepView and RasMol programs. An unrooted phylogenetic tree was constructed based on the amino acid sequences alignment by the neighbor-joining (NJ) algorithm embedded in MEGA 5.0 program (http://www.megasoftware.net/). The reliability of the branching was tested by bootstrap resampling (1000 pseudoreplicates).

2.5. Exposure protocol

To investigate energetic response of *D. magna* F_0 (mother generation) when exposed to toxic *M. aeruginosa*, 60 neonates less than 12 h old from an isoclonal population were collected, randomly divided into two groups, and transferred into three replicate 500 mL beakers (10 neonates per each beaker), respectively. One group, hereafter called the S-group, was daily fed only *S. obliquus* with a total food concentration of 1.5 mg C L^{-1} for three weeks. The other group, hereafter called the M-group, was daily fed a mixture of *S. obliquus* and an increasing amount of toxic *M. aeruginosa*, giving the same total carbon concentration as in S-group. During the first week of exposure, M-group was fed 10% *M. aeruginosa* with increasing relative carbon concentrations of *M. aeruginosa* in the second (20%) and third (40%) weeks. After three weeks, we collected F_1 (offspring; <12 h of old) released by F_0 mothers from each experimental group (i.e. S-group and M-group) to investigate the energetic response of F_1 when exposed to toxic *M. aeruginosa*. In addition, all living F_0 were collected and frozen in −80 °C for subsequent analysis. All offspring (F_1) produced from both experimental groups were fed mixture of 50% *S. obliquus* + 50% *M. aeruginosa* (50% So + 50% Ma; total 1.5 mg C L^{-1}) daily for 14 days. Likewise, a total of 60 neonates less than 12 h of old from both the S-group and M-group were transferred to three replicate 500 mL beakers (10 neonates per each beaker; called SM-group and MM-group), respectively. After 14 days, we collected all live offspring and their mothers as described above for analysis of Dm-AK gene expression and ATP content. The schematic diagram of the exposure protocol is shown in SD Fig. S2. For all groups (i.e. S-group, M-group, SM-group and MM-group), animals were placed under the same conditions used for *Daphnia* culture maintenance (Section 2.1); diets were renewed daily.

2.6. Dm-AK gene expression by quantitative PCR (Q-PCR)

Total RNA of the exposed individuals was isolated as mentioned in Section 2.2, and 5 μg of total RNA was reverse-transcribed into cDNA with a PrimeScript RT Reagent Kit (Takara, Japan). For quantification of the Dm-AK expression, a pair of gene specific primers (AK-Q-F and AK-Q-R; SD Table S1) was used, and the primers actin-F and actin-R (SD Table S1) were used to amplify actin as an internal control. The amplification efficiencies of two primers are between 95% and 105%, examined by using the *Ct* slope method. The thermal conditions were: one cycle of 95 °C for 10 s, followed by 40 cycles of 95 °C for 5 s, 60 °C for 10 s and 72 °C for 20 s. Dissociation curve analysis of amplification products was performed at the end of the reaction to confirm that a specific single PCR product was amplified and detected. Gene expression was calculated by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

2.7. ATP content

ATP content was determined using a bioluminescence ATP assay kit (Beyotime, Nanjing). The individuals were disrupted in 200 μL of lysis buffer and centrifuged at 12,000 × *g* to collect the cell supernatant. An aliquot (100 μL) of an ATP detection working solution was added to each well of a black 96-well culture plate, and the plate was then incubated for 3 min at room temperature. Then, 40-μL samples of the cell lysate were added to the wells, and the luminescence was measured by Mithras LB 940 Multimode Microplate Reader (Berthold, Germany). For the internal calibration, 10 μL of ATP standard was added to the sample and light emission ($RLU_{internal\ standard}$) was measured again. The amount of the ATP in each well was calculated according to the following equation:

$$\text{ATP, mmol} = ((RLU_{sample} - RLU_{background})/RLU_{ATP\ standard}) \times \text{ATP standard, mmol.}$$

The RFU values as well as ATP concentrations in the samples were both expressed as percentages of the controls.

2.8. Statistical analyses

All biochemical and molecular data were expressed as mean ± 1 standard error (SE). Significant differences were evaluated by one-way analysis of variance (ANOVA) followed by the Duncan multiple range test ($\alpha = 0.05$). All tests were run with the SigmaPlot 11.0 software package.

3. Results

3.1. Dm-AK cDNA sequence

By 5′ and 3′ RACE PCR, a 1745-bp full-length cDNA sequence (Fig. 1) which contained a 1074 open reading frame (ORF) encoding 357 amino acids (AA) was obtained. The full-length cDNA contained a 5′ untranslated region of 66 nucleotides, a 3′ untranslated region of 604 nucleotides; an atypical polyadenylation consensus signal (^{1696}AATACA^{1701}) at the 555 bp followed by the stop codon and a poly (A) tail. The ORF is capable of encoding a polypeptide of 356 AA with an estimated molecular mass of 39.9 kDa and a predicted isoelectric point (pI) of 5.56. Dm-AK cDNA sequence was deposited in the Genbank (accession numbers: KJ944636).

3.2. Dm-AK motifs

The ExPASy motif analysis of Dm-AK AA sequence (Fig. 1) contained a potential ATP: guanido phosphotransferases active site (^{271}CPTNLGT^{277}), four protein kinase C phosphorylation sites (^{122}STR^{124}, ^{206}TGR^{208}, ^{278}TIR^{280}, ^{326}SNK^{328}), six casein kinase (^{49}TLLD^{52}, ^{56}SGVE^{59}, ^{157}SSLE^{160}, ^{260}SHHD^{263}, ^{311}TAGE^{314}, ^{316}TEAE^{319}) and one N-myristoylation site (^{209}GIYHN^{214}). A long ADP binding site was in the AA sequence between 122 and 324. Another long arginine binding site was in the AA sequence between 63 and 315. Additionally, prediction of protein domains by SMART program revealed an ATP-gua PtansN and an ATP-gua Ptrans domain at the positions 18–93 and 114–356, respectively (Fig. 2).

The Dm-AK AA identity and similarity percentages were calculated using ClustalW program (Fig. 3) and revealed that it shared high similarity with other AKs, such as 99% with *Daphnia pulex*, 80% with *Artemia franciscana*, 79% with *Neocaridina denticulate*, 78% with *L. vannamei*, and 78% with *Fenneropenaeus merguiensis*. The conserved AA residues such as 11 residues (S63, G64, G66, Y68, F194, E225, C271, T273, N274, E314, H315, the symbol # shown in Fig. 3) interacted with arginine and 14 residues (S122, R124, R126, W221, R229, R280, S282, V283, H284, R309,T311, G313, E314, D324, the symbol ~ shown in Fig. 3) interacted with ADP were well identified in Dm-AK.

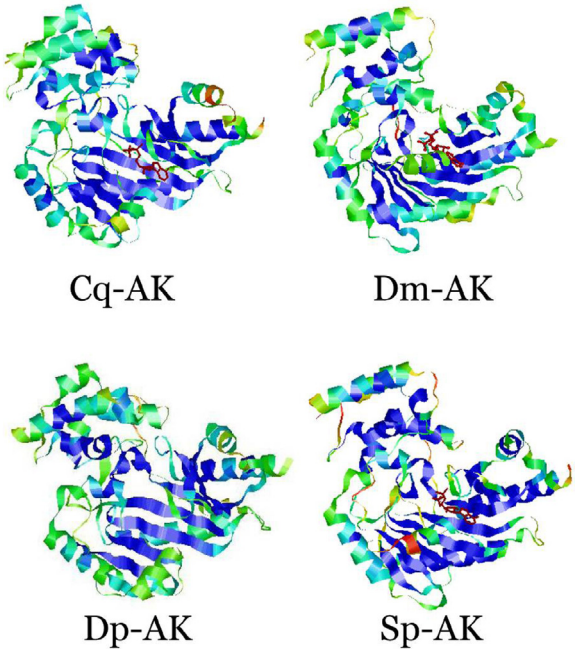

Fig. 5. The predicted spatial structures of Cq-Ak, Dm-AK, Dp-AK and Sp-AK predicted by SWISS-MODEL program.

3.3. Phylogenetic analysis of Dm-AK

A phylogenetic tree for the sequences of selected vertebrate's CKs and invertebrate's AKs was constructed by the NJ method and maximum parsimony method (Fig. 4). The NJ tree showed that invertebrate AKs could be separated from vertebrate CKs. Among invertebrate AKs, they were further divided into two distinct clades of arthropod AKs and nematode AKs. The arthropod AK clade contained three clusters. Cluster 1 included decapod AKs from *F. merguiensis* AK to *N. denticulate* AK. Cluster 2 included insect AKs, such as *Spodoptera litura* AK and *Culex quinquefasciatus* AK. Cluster 3 contained the Branchiopod AK (i.e. Dm-AK and Dp-AK). The vertebrates, such as *Takifugu rubripes*, *Ictalurus punctatus* and *Homo sapiens* formed creatine kinases (CKs) group.

3.4. Dm-AK tertiary structure

The SWISS-MODEL prediction algorithm established the potential tertiary structure of the AKs from *C. quinquefasciatus*, *D. magna*, and *Scylla paramamosain* based on the template of *L. vannamei* AK (Fig. 5). Results showed that *C. quinquefasciatus*, *D. magna*, *D. pulex*, and *S. paramamosain* shared different identities with *L. vannamei*: 83.15%, 77.18%, 76.62% and 93.54%, indicating that the three-dimensional structure of Dm-AK was more similar with Dp-AK than the others.

3.5. Dm-AK mRNA expression and ATP content after digesting *Microcystis* in the F_0 and F_1 generations

Dm-AK transcript level in the presence of *M. aeruginosa* was 0.618 ± 0.046, about 30% lower ($P < 0.05$) than that of the 100% *S. obliquus* (Fig. 6A). Similarly, ATP content was significantly decreased since *D. magna* F_0 fed with a mixture of *M. aeruginosa* and *S. obliquus* ($P < 0.05$).

For offspring of S-group and M-group *D. magna* that were cultured in a mixture of 50% So + 50% Ma, Dm-AK transcript level in the MM-group was (5.457 ± 0.481) significantly higher than that of SM-group (0.995 ± 0.005) ($P < 0.05$; Fig. 6B). Also, ATP content in the MM-group was significantly elevated compared to the SM-group ($P < 0.05$).

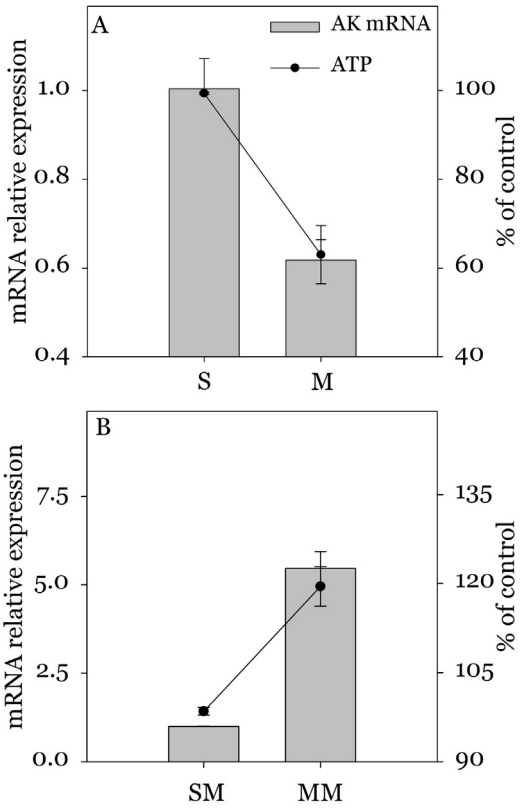

Fig. 6. Changes of Dm-AK mRNA expression and ATP content in F_0 (A): S-group (S) and M-group (M) and F_1 (B): SM-group (SM) and MM-group (MM). All values represent the mean ± SE ($n = 3$).

4. Discussion

Arginine kinase (AK) plays an important role in cellular energy metabolism in invertebrates (Alonso et al., 2001), which can catalyze the phosphoarginine and ADP to L-arginine and ATP on substrate level when energy is needed (Tanaka et al., 2007; Trautmann, 2009). Several AKs were cloned or identified from other crustaceans (Arockiaraj et al., 2011; Shen et al., 2011; Chen et al., 2013; Xie et al., 2014). In this study, an AK gene was cloned from *D. magna* with an ORF of 1074 bp encoding for 357 AA. The deduced AA sequence of Dm-AK shared high similarity with AKs of other arthropods (Fig. 3).

Dm-AK AA contained an ATP PtransN domain from 18 to 93 which was described as a guaninine substrate specificity domain (GS) and an ATP-gua Ptrans from 114 to 356 which was responsible for ATP binding (Ellington, 2001). The conserved amino acid residues in all AKs, including the 11 amino acid residues (S63, G64, G66, Y68, F194, E225, C271, T273, N274, E314, H315) that interacted with arginine and 14 arg residues (S122, R124, R126, W221, R229, R280, S282, V283, H284, R309, T311, G313, E314, D324) that interacted with ADP, were all identified in CfAK (Tada and Suzuki, 2010). D62 and R197 residues are suggested to play a key role in stabilizing the substrate-bound structures of AK by forming an ion pair (Suzuki et al., 2000) and are conserved in most AK sequences, including the unusual *Stichopus* AK, which evolved secondarily from CK (Suzuki et al., 1999). Tyr at position 89 is also a key residue in typical invertebrate AKs and is strictly conserved. This residue is not directly involved in substrate binding but it is located close to the site that binds with the substrate arginine (Zhou et al., 1998). It has been

found that site-directed mutagenesis of this residue significantly and specifically affects guanidine substrate (Edmiston et al., 2001; Tanaka and Suzuki, 2004). Furthermore, Dm-AK AA sequence had a myristoylation site (^{209}GIYHN^{214}), predicted by ExPASy motif analysis. The myristoylation signal group mediates the binding of the protein to the cell membrane and is characterized by the following sequence: (N-terminal G-[not E, D, R, K, H, P, F, Y and W]-X-X-[any of S, T, A, G, C, H, and N]-[not P]). In the case of sea urchin flagellar CK which has a myristoylated N-terminus, the enzyme is localized in the flagellar cell membrane (Quest et al., 1992). Likewise, Dm-AK might target the cell membrane.

The phylogenetic analysis indicated that all AKs were clustered together as one subgroup and all CKs as another group (Fig. 4), consistent with the notion of a common origin for these two phosphagen kinases with different substrate specificity (Ellington, 2001). Related to Dp-AK, Dm-AK was positioned within the branchiopod branch of the arthropod cluster, but it was not the sister group of AK of decapod crustaceans which was sister group of Insecta (Labbe and Little, 2009). This result was in line with the paraphyly hypothesis of Crustacea related to Insecta (Cook et al., 2005; Legg et al., 2013), which also has been confirmed by our previous study (Lyu et al., 2014). The tertiary structure of Dm-AK was more similar to Dp-AK than to Cq-AK and Sp-AK, which was consistent with the AK phylogeny (Fig. 4). We speculated that the AK function of *D. magna* was the most close to the congeneric *D. pulex*. All these findings supported a number of previous studies that provided evidence for an excellent conservation of both primary, secondary and tertiary structures amongst phosphagen kinases (Ellington, 2001; Yao et al., 2005).

The substrate-level phosphorylation activity of AK is indispensable for the physiological process of energy production and utilization, and it plays an essential role in the whole life of invertebrate animals, especially when they are challenged by environmental stressors. To investigate chronic effects of *M. aeruginosa* on energy production in *D. magna*, *D. magna* individuals were daily fed with the presence of *M. aeruginosa* for 21 days. Significant inhibition was detected in the Dm-AK expression and ATP content for individuals ingesting toxic *M. aeruginosa* (Fig. 6A), suggesting that *M. aeruginosa* may impair the ability of *D. magna* to produce sufficient energy through hampered AK expression. Cyanobacteria, including *Microcystis*, are well-known to be of low food quality for zooplankton due to its toxicity (Lampert, 1981), mechanical interference (Shao et al., 2014), or nutritional deficiencies (Müller-Navarra, 1995). The inhibited AK expression could not be due to mechanical interference given that the *M. aeruginosa* strain used in this study grew as single cells.

Hence, in the current study, MCs and nutritive deficiencies apparently cause energetic insufficiency of *D. magna*. On the one hand, as one of most common and potent toxins, MCs often inhibit protein phosphorylation and induce oxidative stress (Wiegand and Pflugmacher, 2005), which could subsequently result in disorder of cellular homeostasis and energy production. Ortiz-Rodríguez and Wiegand (2010) found that enzymatic response of lactate dehydrogenase (LDH) was negatively affected by exposure to MCs; this enzyme is involved in the production of energy, being particularly important when a considerable amount of additional energy is urgent. Also, recent results on the gene expression of key enzymes of carbohydrate and protein metabolism showed disturbances on the energy producing pathways of *D. magna* exposed to *M. aeruginosa* (Schwarzenberger et al., 2009). On the other hand, the MCs detoxification process requires significant energy, which may require additional resources. Moreover, Von Elert and Wolffrom (2001) demonstrated that *Microcystis* which lacked an important compound (e.g. lipid substance) making it a poor food for *Daphnia*. Hence, the low ATP content of *Daphnia* fed a diet containing toxic *M. aeruginosa* relative to conspecifics fed *Scenedesmus* (Fig. 6A) was likely due to that *Microcystis* could not transfer sufficient resource to *Daphnia*.

Previous studies have shown that rapid adaptations in *Daphnia* can promote resistance to toxic cyanobacteria, and that these adaptations can be transferred to future generations (Gustafsson et al., 2005; Sarnelle and Wilson, 2005; Guo and Xie, 2006; Jiang et al., 2013). For example, Gustafsson and Hansson (2004) reported that the survivorship, growth, and reproduction of *D. magna* were enhanced if the animals had experienced previous exposure to toxic *Microcystis*. Also, smaller cladocerans, such as *Moina* and *Ceriodaphnia*, may develop stronger tolerance to cyanobacteria after previous exposure compared with larger *Daphnia* species (Guo and Xie, 2006). Jiang et al. (2013) demonstrated that rapid adaptation caused not only an increase in fitness of 20% in the presence of cyanobacteria, but also a fitness reduction of 25% in the absence of cyanobacteria, in the form of reduced survivorship and reproductive output. In the present study, we found that the Dm-AK expression and ATP content in neonates whose mothers were fed with a mixture of 50% So + 50% Ma were significantly higher than offspring whose mother fed with pure *S. obliquus* (Fig. 6B). These data implied that increasing mRNA expression could help to produce more ATP (also shown in Fig. 6B) which could lead to enhanced resistance to toxic cyanobacteria. It has been well-accepted that *Daphnia* in a poor-food environment produce small broods of large neonates (Garbutt et al., 2013), whereas daphnids in a good-food environment produce large broods of small neonates (Enserink et al., 1990; Gliwicz and Guisande, 1992; Coors et al., 2004). The large neonates contain a higher amount of lipids (Enserink et al., 1993) and are assumed to be of higher quality than small neonates. Consequently, large *Daphnia* may increase their energy allocation (due to higher amount of lipids) to self-maintenance and may then increase their stress resistance. Our experimental data (Fig. 6B) suggest that *Daphnia* elevated their energy production corresponding to *Microcystis* exposure if mothers had been exposed to *Microcystis*. A candidate gene study of *D. magna* identified that three genes involved in glycolysis and the tricarboxylic acid cycle were up-regulated 8–10 folds in individuals exposed to toxic *Microcystis* relative to those exposed to non-toxic strains (Schwarzenberger et al., 2009). Moreover, Schwarzenberger et al. (2014) recently reported that the increases of permeases gene expression were detected in the tolerant *Daphnia* clone, leading to higher production of transporter protein, which subsequently results in a stronger excretion of microcystins from the cells. Given that excreting microcystins process is energy-dependent (Campos and Vasconcelos, 2010; Bieczynski et al., 2014), it is reasonable that elevated Dm-AK expression and ATP content may promote microcystins excretion. Hence, our results together with results of Schwarzenberger et al. (2009, 2014) could partly explain why subsequent offspring had higher fitness when the previous generation had been exposed to toxic *Microcystis*. Further studies on the gene-regulated pathways involving maternal effects in *Daphnia* are necessary, in light of the publication of the *Daphnia* genome (Colbourne et al., 2011) which brings *Daphnia* research into a new era where we face the challenging task of connecting genome structure, function and expression with variation in ecologically important traits.

5. Conclusion

We cloned arginine kinase gene of *D. magna* and showed that it possesses all the features commonly found in invertebrates, indicating Dm-AK belonged to the conserved ATP: arginine N-phosphotransferase family. Dm-AK might be related to cyanobacteria resistance, as its expression was enhanced after exposure to toxic *Microcystis* in *Daphnia* whose mothers were previously

exposed to toxic cyanobacteria. Although the *Daphnia -Microcystis* system is well-characterized in terms of physiology and ecology, the genes and defense mechanisms that regulate this 'arms race' are poorly resolved. Our data are an important step toward understanding the molecular basis of phenotypic plasticity and coevolution in cyanobacteria–zooplankton interactions.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.aquatox.2014.12.023.

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