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Cloning arginine kinase gene and its RNAi in *Bursaphelenchus xylophilus* causing pine wilt disease

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Abstract Since *Bursaphelenchus xylophilus* causes serious losses in pine forestry, new ways of controlling this nematode damage are urgently needed. Arginine kinase (AK) is a phosphotransferase, which plays a critical role in cellular energy metabolism in invertebrates. It is only present in invertebrates and may be a suitable chemotherapeutic target in the control of this pest. RNA interference (RNAi) technology has been developed in biological science in recent decades as a powerful tool to silence the target gene function in the post-translational status. In this study, one AK gene, *Bx*AK1 (GeneBank accession No. EU853862) was firstly cloned, and then its functions were identified by RNAi technology. Results show that the full-length cDNA of the *Bx*AK1 gene contains 1206 base pairs and an 1086 bp open reading frame encoding 361 amino acids. The length of the *Bx*AK1 genomic coding region contains 2430 bp consisting of four introns of 421, 117, 475, and 268 bp respectively, and five exons of 57, 207, 309, 360, and 215 bp respectively. A dsRNA targeting *Bx*AK1 was constructed and tested for its RNAi effect on *B. xylophilus* by soaking bioassays. RNAi not only significantly increased the mortality of *B. xylophilus*, but also greatly reduced its fecundity and fertility. These results suggest that RNAi targeting *Bx*AK1 may be an effective approach for controlling nematode pests.

Keywords *Bursaphenlenchus xylophilus* · Arginine kinase · Gene cloning · RNA interference (RNAi)

Introduction

The nematode *B. xylophilus*, the causal agent of Pine Wilt Disease (PWD), is carried from tree to tree by the cerambycid beetle *Monochamus* spp. (Ryss et al. 2005). PWD constitutes one of the most serious worldwide conifer diseases affecting the native species of *Pinus* spp. from Far East forest lands (Japan, China, Korea and Taiwan) (Yano 1913; Cheng et al. 1983; Tzean and Jan 1985a, b; Yi et al. 1989; Shin and Han 2006), and more recently from Europe (Portugal and Spain) (Mota et al. 1999; Fonseca et al. 2010; 2012; Abelleira et al. 2011; Robertson et al. 2011). This disease causes significant economic and environmental

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damage to the affected countries, with huge annual losses of timber (Mamiya 2004). In order to eradicate pine wilt disease, insecticides such as sodium N-methyldithiocarbamate, methyl bromide, and fenitrothion have been applied in the past by fumigation or aerial spray (Dwinell 1997; Kwon et al. 2005; Sung et al. 2005). In addition, avermectins, such as emamectin benzoate and abamectin, which target the glutamate-gated chloride channels of nematodes, have been injected into pinewood trunks (Takai et al. 2001, 2004; Yates et al. 2003). With the rise in environmental concerns about the negative effects of sustained nematicide use, a novel and robust method to control this pest is urgently needed.

Following the discovery of gene silencing with dsRNA (Fire et al. 1998), RNAi was developed as an effective tool for gene function study and genetic manipulation in plants and animals (Aravin et al. 2001; Wesley et al. 2001; Sugimoto 2004). Moreover, the application of RNAi in plants to control plant parasitic nematodes may become an important part of an integrated pest management through the development of stable transgenic plants that encode nematode-specific dsRNAs (Huang et al. 2006; Steeves et al. 2006; Yadav et al. 2006; Fairbairn et al. 2007; Sindhu et al. 2009). The efficacy of gene silencing has previously been demonstrated in *Globodera pallida* (Urwin et al. 2002; Kimber et al. 2007), *Meloidogyne incognita* (Bakhetia et al. 2005; Rosso et al. 2005; Shingles et al. 2007) and *B. xylophilus* (Park et al. 2008) using long dsRNA silencing triggers. In *B. xylophilus*, several essential genes of myosin heavy chain, tropomyosin, heat shock protein 70, and cytochrome C have been silenced (Park et al. 2008). Since mammals may have orthologs of these essential genes, their RNAi may have an adverse effect on mammals.

Arginine kinase (ATP: L-arginine phosphotransferase, EC 2.7.3.3, AK) plays a critical role in cellular energy homeostasis in invertebrates by catalyzing a reversible transfer of phosphate from MgATP to arginine, yielding phosphoarginine as follows: MgATP+Arginine <=> Phosphoarginine+MgADP (Newsholme et al. 1978). AK only exists in the tissues of invertebrates (e.g., epithelial cells, muscle fibers, and neurons) (Lang et al. 1980; Chamberlin 1997; Kucharski and Maleszka 1998), and functions as a central regulator of temporal and spatial ATP buffers directly associated with muscle contraction, ATP regeneration and energy transport in cellular energy metabolism (Ellington 2001). Since AK is absent in vertebrates, and the biosynthetic pathway of phosphoarginine is totally different from those in mammalian tissues, AK may be a useful chemotherapeutic target in invertebrate pest control (Pereira et al. 2000; Brown and Grossman 2004; Wu et al. 2007). Genes encoding proteins with essential functions are supposed to be the best RNAi targets for increasing morbidity and mortality in nematodes. Since AK regulates the cellular energy reserve in invertebrates, it is proposed that RNAi be used to silence the AK gene in order to induce high mortality in nematodes. Most of the previous studies on AK focused on elucidating its catalytic mechanism and identifying its substrate analog inhibitors. Although arginine kinase genes from many organisms, including nematodes such as *Caenorhabditis elegans*, *Toxocara canis* and *Heterodera glycines,* have been cloned (Dumas and Camonis 1993; Suzuki and Furukohri 1994; Strong and Ellington 1995; Suzuki and Furukohri 1997; Kucharski and Maleszka 1998; Suzuki et al. 1999; Pereira et al. 2000; Astrofsky et al. 2002; Suzuki et al. 2002; Compaan and Ellington 2003; Matthews et al. 2003; Perovic-Ottstadt et al. 2005; Uda et al. 2006; Wickramasinghe et al. 2007; Liu et al. 2009), only the AK gene cloned from *Phyllotreta striolata* has been used to develop RNAi in insect pest control (Zhao et al. 2008).

In order to find new approaches to prevent the spreading of pine wilt disease using RNAi technology, one arginine kinase gene from *B. xylophilus* was cloned, and RNAi experiments were conducted. An increase in the mortality rate and a reduction of the fecundity rate of nematodes were observed in the soaking bioassay, which suggests that the targeting of AK by RNAi may be an effective and efficient approach for controlling *B. xylophilus*.

Materials and methods

Growth of *B. xylophilus*

B. xylophilus isolates were collected from chips of infested pine wood of *Pinus massoniana* in Guangdong province in China using the Baermann funnel method, and identified using a microscope. Isolates of *B. xylophilus* were reared on a lawn of *Pestalotiopsis* sp. cultured on potato dextrose agar (PDA) plates at 25 °C in the dark (Wang et al. 2011).

Bacterial strain and plasmid

Escherichia coli JM109 was used for cloning and propagation of the plasmids, and was maintained on Luria-Bertani medium. Plasmid pMD-18T (TaKaRa, Japan) and pGEM-T Easy Vector (Promega, USA) were used for cDNA cloning. Plasmid DNA was isolated from *E. coli* cells using the Plasmid DNA Extracting Kit (Sangon Company, China), following the protocols recommended by the manufacturer.

RNA extraction and cDNA synthesis

B. xylophilus worms were washed off the PDA plates (plus *Pestalotiopsis* sp.) with distilled water and allowed to settle in a microtube. The supernatant was removed and the worms were washed three additional times in distilled water, after which the pellet (approximately 100 μl) was dissolved in 1 ml TRizol reagent (Invitrogen, USA), and the lysate was extracted, firstly with chloroform and then with isopropanol. Total RNA was treated with DNase, and was measured by ultraviolet absorbance at $A_{260/280}$ (Eppendorff AG 22331, Germany). Total RNA was treated with DNase, and was measured by ultraviolet absorbance at $A_{260/280}$ (Eppendorff AG 22331, Germany). The yield of RNA was calculated as follows: $A_{260} \times 40 \times$ dilution factor $= \mu g$ RNA ml^{-1}. The first-strand cDNA was synthesized using 5 μg of total RNA, Oligo (dT) primer, Random primer and M-MLV ReverseTranscriptase according to the manufacturer's instructions from the M- MLV RTase cDNA Synthesis Kit (Takara, Japan).

Full-length cDNA clone of arginine kinase gene and sequence analysis

For cloning the full-length cDNA, a pair of primers, the oligo (dT) adaptor and a 'universal' primer (Table 1) for RT-PCR was designed according to Suzuki and Furukohri (1994). The 3′ end of the cDNA sequence was obtained by PCR amplification. The reaction solution consisted of 10 μl of the template cDNA, 0.5 μl of the oligo (dT) adaptor (20 μM), 0.5 μl the 'universal' primer (20 μM), 5 μl 10×Ex Taq Buffer, 0.25 μl TaKaRa Ex Taq (5 $U\mu l^{-1}$), and 33.75 μl ddH_2O. The amplification profile was as follows: 1 cycle at 95 °C for 1 min, and 35 cycles at 94 °C for 40 s, 60 °C for 30 s, and 72 °C for 3 min. After a final extension of 10 min at 72 °C, the samples were stored at 4 °C. A SMART RACE cDNA amplification kit (Clontech, USA) was used for the 5′ end of the cDNA sequence PCR amplification. In brief, the forward primer UPM supplied by the kit and the gene-specific reverse primer for *B. xylophilus* arginine kinase AK5R1 were used for the first round of PCR.

Table 1 List of primers used in this study

Primer	Sequences
oligo(dT)	5′-GGATCCGAATTCCCCGGGT-3′
universal	5′-GT(ACGT)TGG(AG)T(ACGT)AA(TC)GA(AG)GA(AG)GA(TC)CA-3′
UPM	5′-CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT-3′
AK5R1	5′-GGATGACGGGCGAATGGCTGCTT-3′
NUP	5′-AAGCAGTGGTATCAACGCAGAGT-3′
AK5R2	5′-TTGGCCGCAATGATCTTGAGTCCCT-3′
F30	5′-GGGTGTTTGGCTCGTTCGG-3′
R1189	5′-GAAATGTCGTAGATTCCCTCCGCCG-3′
F139	5′-GTTGGACTTTATGCTCCGGATG-3′
R737	5′-CGGGCGAATGGCTGCT-3′
*Bx*AK1F	5′- TCGGAGACGGTTGTGTGAAGATG-3′
*Bx*AK1T7R	5′-TAATACGACTCACTATAGGGCAAGCACGGGTTGAATGGGT-3′
*Bx*AK1R	5′- CAAGCACGGGTTGAATGGGT-3′
*Bx*AK1T7F	5′-TAATACGACTCACTATAGGGTCGGAGACGGTTGTGTGAAGATG-3′
*Bxactin*F1	5′- TGCCGCTCTTGTTGTGG-3′
*Bxactin*R1	5′- GGAAAGGACGGCTTGGAT-3′

The second round of PCR was performed using a nested forward primer NUP and the second gene-specific reverse primer (Table 1). The amplification was conducted in a Peltier Thermal Cycler Chromo 4 PTC 200 (Bio-Rad, USA). The resulting 3′ and 5′ RACE PCR amplification products were gel-purified and cloned into pMD 18 T-Vector, and sequenced at SHENGGONG Biotechnology in Shanghai. The full-length cDNA of *B. xylophilus* arginine kinase was obtained through sequence assembly by searching for the sequence overlap region. Alignment of the full-length cDNA with other homologous sequences was conducted with DNAMAN software (Lynnon Biosoft, Canada) and through GenBank searches (http://www.ncbi.nih.gov/).

Genomic cloning of the arginine kinase gene from *B. xylophilus*

The genomic DNA was extracted from *B. xylophilus* using a conventional phenol–chloroform method (Smits et al. 1991). PCR was performed with 1 μg genomic DNA using two non-redundant primers (F30/R1189 and F139/R737) (Table 1) designed based on the cDNA sequence, respectively. The DNA was amplified for 35 cycles, denaturized for 30 s at 94 °C, annealed for 30 s at 55 °C, and extended for 1 min at 72 °C, using *Ex Taq* HS DNA polymerase (Takara, Japan). The amplicon was separated by electrophoresis on 1 % agarose gel and purified with an agarose gel DNA Purification Kit (Takara, Japan), then sequenced after being cloned into pGEM-T Easy Vector (Promega, USA).

Phylogenetic studies

Alignments and ClustalX analyses were performed using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) with amino acid sequences of arginine kinase genes available on NCBI databases, the accession numbers of which are listed in the legend of Fig. 2. A phylogenetic tree was constructed by the neighbour-joining method (Saitou and Nei 1987), using MEGA (Molecular Evolutionary Genetics Analysis, USA).

RNAi preparation

A fragment (495 bp) of the ORF of *Bx*Ak1 was cloned into the vector pMD18-T (Takara, Japan), and the constructed vector was further confirmed by sequencing. Based on the fragment, two primer pairs of *Bx*AK1F/*Bx*AK1T7R and *Bx*AK1R/*Bx*AK1T7F (Table 1) were designed to amplify the sense and anti-sense single stranded RNAs product. The sense and anti-sense RNA were transcribed using the T7 transcription kit (TOYOBO, Japan) according to the manufacturer's instructions. Sense and anti-sense transcripts were annealed for 30 min at 37 °C and analyzed by agarose gel electrophoresis. The dsRNA was purified with 1/10 amount of KAc (3 mol^{-1}) and three amounts of 95 % ethylalcohol overnight, washed by 75 % ethylalcohol three times, and finally stored at -80 °C for later use.

Measurement of phenotypes after soaking

The RNAi soaking method was basically performed according to Urwin et al. (2002). Freshly cultured *B. xylophilus* nematodes (a mixture of adults and juveniles, approximately 3,000 individuals) were soaked in 50 μl of 2 μg μl^{-1} dsRNA solution. Nematode suspensions were shaken lightly (200 rpm) in a rotary incubator for 4 h at 20 °C. Nematodes soaked in DDW with non-dsRNA were taken as a control. After soaking, the samples of each treatment were thoroughly washed several times in DDW and allowed to recover at 20 °C in distilled water. Their morphology, locomotion and mortality were observed and recorded at 4, 8, 12, 24, 36, 48, and 60 h after soaking, respectively. Juveniles/adults were scored as being dead if they did not move after being poked with a platinum wire. All assays were performed in triplicate.

To score offsprings survival, 10 *B. xylophilus* females with eggs were soaked for 4 h in 30 μl soaking buffer containing 2 μg μl^{-1} dsRNA, while 10 *B. xylophilus* soaked in DDW with non-dsRNA were taken as a control. The nematodes were transferred to a PDA plate with *Pestalotiopsis* sp. with 30 males of *B. xylophilus*. Living nematodes were counted three times in 4 and 8 days. All assays were performed in triplicate.

Statistical analysis

The data shows the mean±standard deviation (SD) of three independent experiments. The statistical significance

was determined using a Student's *t* test. A P value <0.05 was considered significant.

Semi-quantitative RT-PCR analyses for *Bx*Ak1 expression of the RNAi soaking *B. xylophilus*

RNA was extracted from dsRNA treated and control mixed stages of *B. xylophilus* using an RNeasy mini kit (Qiagen, Germany), according to the manufacturer's instructions. Residual genomic DNA was removed by using 20 U RNase-free DNase I (Qiagen, Germany). cDNA was synthesized using a Multi Scribe RT High-capacity cDNA archive kit (Applied Biosystems, USA) with random primers according to the manufacturer's instructions. Arginine kinase cDNA was detected by PCR using gene-specific primers *Bx*AK1F and *Bx*AK1R (Table 1), in which 2 μg *B. xylophilus* cDNA was used as a template in a 60 μl reaction. The PCR protocol and PCR reaction conditions used in this experiment were optimized to determine the comparative level of gene expression. To reach the plateau phase of PCR amplification, different numbers of amplification cycles (19, 21, 23, 25, 27, 29, 31, 33, 35, and 37 cycles) were used. Every two cycles, 10 μl aliquots of the reaction were removed from the PCR samples until 37 cycles were completed. PCR products were analyzed on 1 % agarose gels stained with ethidium bromide. The *actin* gene of *B. xylophilus* (EU100952)of 409 bp was amplified as an endogenous control using the primers *Bxactin*F1 and *Bxactin*R1 (Table 1). All assays were performed in triplicate.

Results

Full length *Bx*AK1 cDNA cloning and its sequence

RACE strategy was used to obtain the full-length cDNA sequence of the *Bx*AK1 gene. Subsequently, a 1206 bp cDNA was cloned from the *B. xylophilus*, including an open reading frame (ORF) of 1086 bp (GenBank accession no EU853862). The ORF began with an ATG initiation codon at nucleotide 35 and terminated with a TAA at nucleotide 1119 (Fig. 1). Further analysis suggests that the *B. xylophilus* *Bx*AK1 protein is composed of 361 amino acids with a molecular mass of approximately 40.3 kDa. The amino acid sequence prediction was deduced from NCBI.

Genomic cloning of an arginine kinase gene from *B. xylophilus*

The length of the *Bx*AK1 genomic coding region was 2430 bp. The exon/intron boundaries were determined by aligning the genomic sequence with the corresponding cDNA sequences. The BxAK1 genomic DNA contained four introns (421, 117, 475, and 268 bp) and five extrons (57, 207, 309, 360, and 215 bp) (supplement Fig. 1), and their splice site sequences followed the conserved 'GT-AG' rule of cis-splicing in the free-living nematode *C. elegans* (Mount 1982). This is the first report on the genome sequence of the arginine kinase gene in *B. xylophilus*.

Sequence comparison

3,935 amino acid sequences of arginine kinase were searched against the NCBI database and it was found that, to date, only invertebrates own arginine kinase. This indicates that arginine kinases are specifically conserved in invertebrates. Then, 52 arginine kinases were selected from six categories, namely, Nematoda, Arthropoda, Mollusca, Cnidaria, Annelida, and Echinodermata, for a sequence comparison. Apart from *Bx*AK1, no other arginine kinase of *B. xylophilus* was retrieved, although the *B. xylophilus* genome sequence has recently been published (Kikuchi et al. 2011). These results show that the arginine kinase genes of *B. xylophilus*, *C. elegans*, *H. glycines*, and *Toxocara canis* were present in the same branch of the phylogenetic tree and *Bx*AK1 had a higher similarity to the AK of *C. elegans* than to the AK of *H. glycines* (Fig. 2).

RNAi by soaking *B. xylophilus* in dsRNA

After soaking for 4 h in dsRNA solution, all of the *B. xylophilus* became twisted, and circled with trembling before death (Fig. 3). *B. xylophilus* mortality due to gene silencing was recorded every 4 h after the four-hour soaking and then every 12 h until 60 h after soaking. As shown in Fig. 4, the *Bx*AK1 knockdown obviously resulted in a high mortality of nematodes compared with the control group ($P<0.05$), at each point of observation (Fig. 4).

For determining the adverse effects of dsRNA on *B. xylophilus*, adult nematodes were grouped and soaked in a solution of dsRNA. Because the life cycle of *B.*

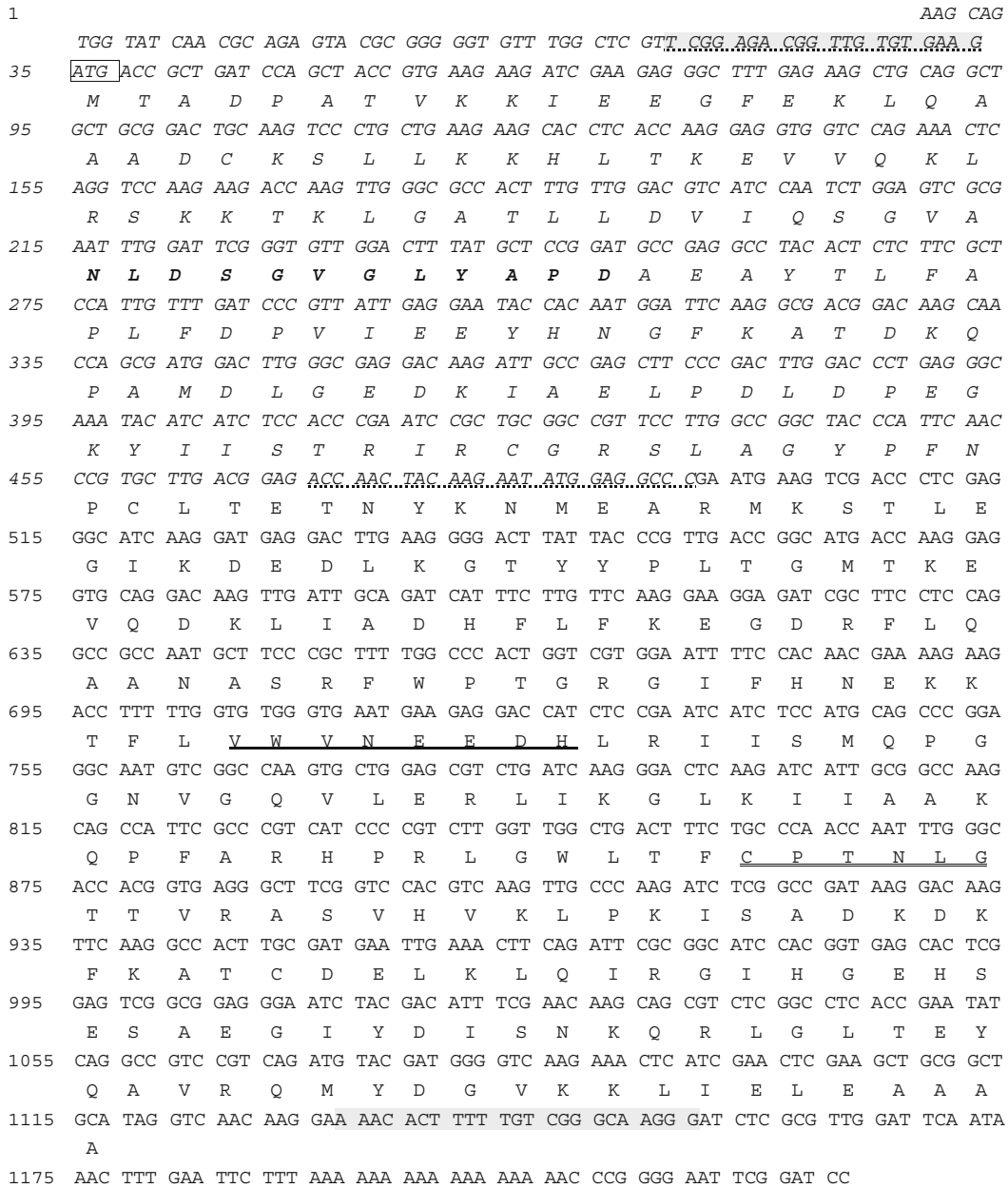

Fig. 1 *Bx*AK1cDNA sequence and its deduced amino acid sequence. The highly conserved motif, a reactive Cysteinyl residue as well as substrate binding site (GS) known for the Arginine kinase, are indicated. The highly conserved motif is marked by the underline; a reactive Cysteinyl residue is marked by double underlines; and the substrate binding site(GS) is in bode italics. Primers from the kit are in italics, primers used for *BxAK1* cloning are marked in dark gray; and primers used for RNAi are marked by dots

xylophilus is normally 4–5 days, days 4 and 8 after soaking were chosen as points of observation. A decrease in the nematode vitality and fertility was observed according to the number of juveniles born by soaked females. Ten female adults treated with RNAi produced about 22.3 % and 68.3 % fewer juveniles than those produced by the control after 4 and 8 days, respectively ($P<0.05$) (Fig. 5).

In order to measure changes in expression of the target mRNA induced by RNAi, after RNA was

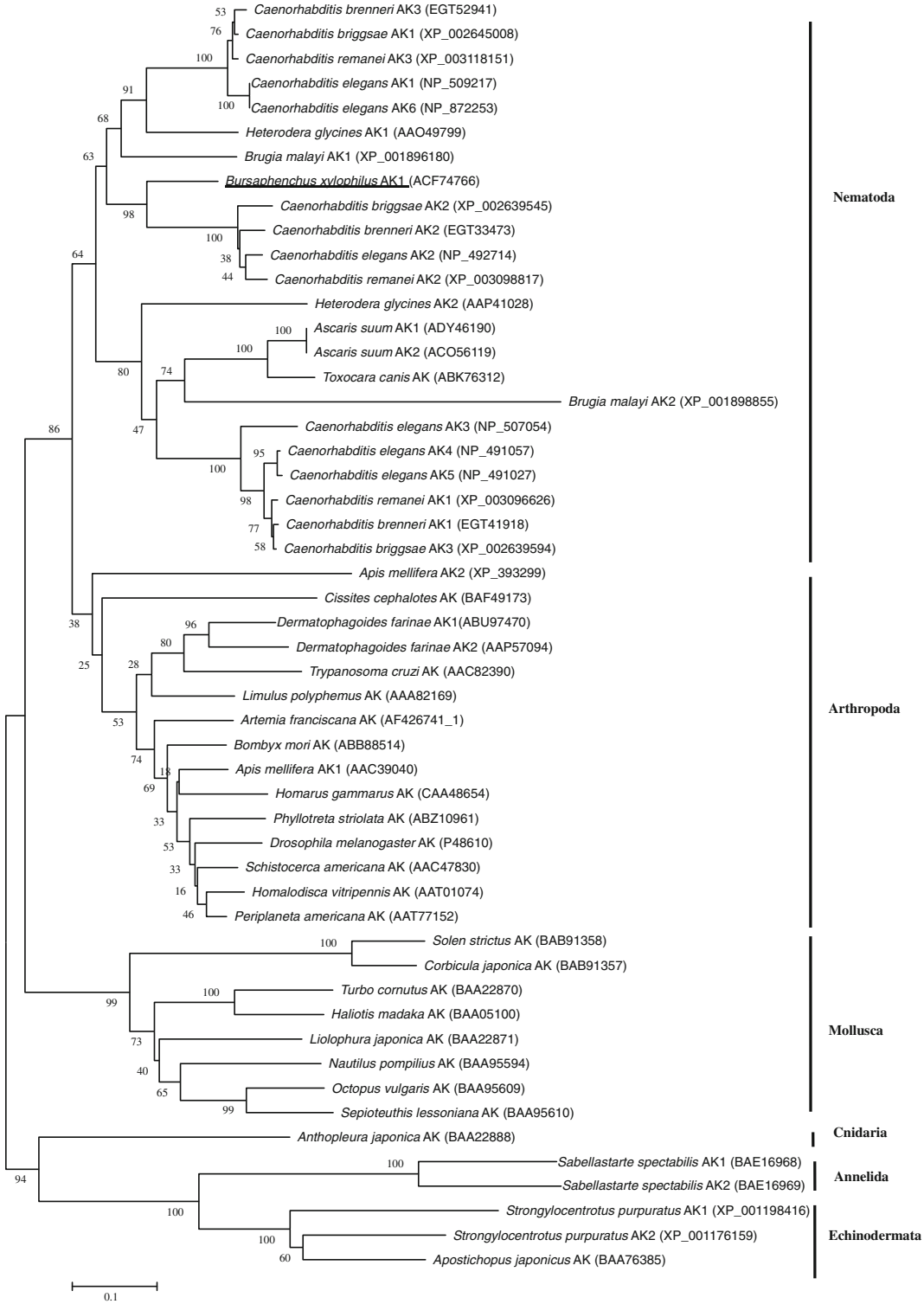

Fig. 2 Phylogram constructed on the basis of amino acid sequences depicting the evolutionary relationships among arginine kinases of different species *(Tree Dom Viewer,* http://www.bioinformatics.nl/tools/treedom/*)*, including 52 various invertebrate members (*Bursaphenchus xylophilus* AK1, highlighted by the underline). Accession number of the sequences in brackets. Distances on the *X*-axis correspond to the grade of sequence homology; distances on the *Y*-axis are arbitrary

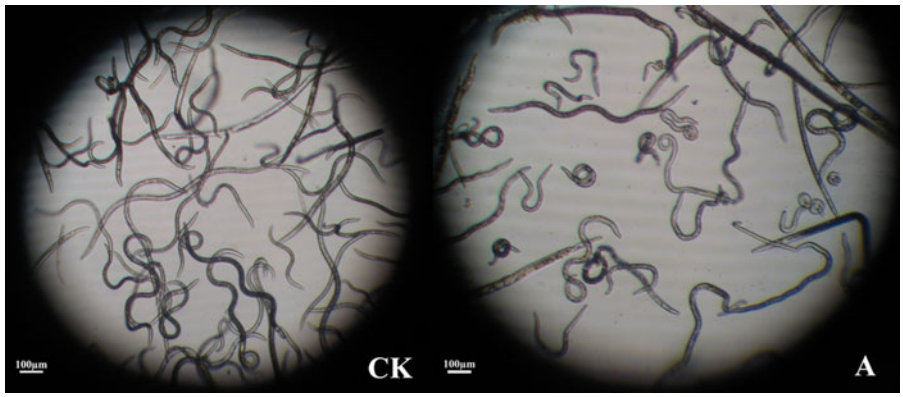

Fig. 3 Morphology and locomotation of *B. xylophilus* after soaking in 2 μgμL^{-1} dsRNA of *Bx*AK1 for 4 h. CK, control; A, treatment

isolated from the nematodes which had been soaked in dsRNA solutions of the target genes for 4 h, mRNA levels of the target *Bx*Ak1 gene and the actin gene as a control, were detected by RT-PCR respectively. The intensity of the amplified cDNA fragments separated on agarose gels after the 29 cycles of PCR reactions clearly showed that the target mRNA expression had disappeared (Fig. 6).

Discussion

This study represents the first report of cloning and RNAi analysis of the AK gene from *B. xylophilus*, a causative agent of pine wilt disease. The sequence analysis of *Bx*AK1 revealed an open reading frame showing 80 % nucleotide identity to the *C. elegans* AK2. In addition, the highly conserved motif CPTNLGT (positions 275–281) around a reactive cysteinyl residue (position 275) is the signature pattern for the arginine kinase family (Fig. 1). The gene containing a well-conserved Cys 275, which plays an important role in substrate binding, is directly involved in AK catalysis reaction (Gattis et al. 2004); and two highly conserved amino acids, Asp 63 and Arg 197, which are responsible for the synergy in the substrate binding of AK (Zhou et al. 1998). Arginine kinase plays a critical role in cellular energy homeostasis in invertebrates by catalyzing a reversible transfer of phosphate from Mg-ATP to arginine, yielding phosphoarginine (Newsholme et al. 1978). Our study showed that adverse effects on survival, fecundity and fertility by RNAi of the AK genes observed in *B. xylophilus* was similar to that in *Phyllotreta striolata* (Zhao et al. 2008), suggesting that the *Bx*Ak1 gene of *B. xylophilus* is an orthologue of AK from invertebrates.

Although dsRNA targeting AK can adversely affect these invertebrates, causing a reduction in their survival, fecundity and fertility both in *B. xylophilus* and *P. striolata*, their target mRNA expression reduction degrees were different. Surprisingly, the target mRNA expression of *Bx*AK1 in *B. xylophilus* had almost

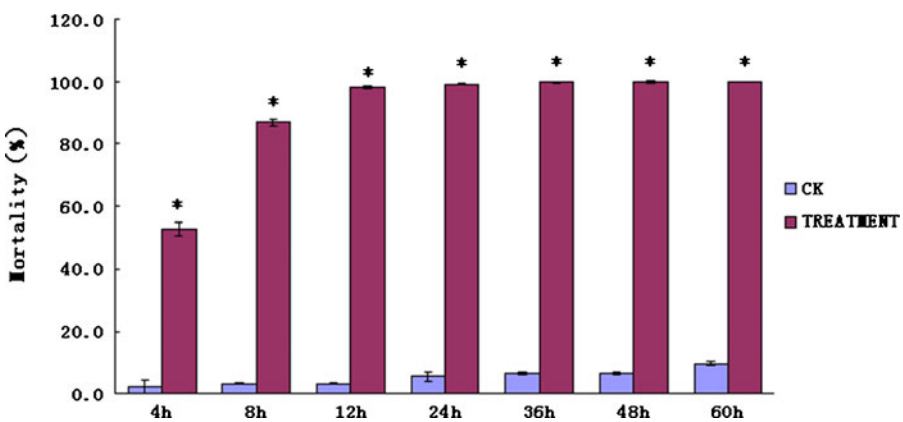

Fig. 4 Mortality of *Bursaphenchus xylophilus* caused by BxAK1 RNAi at different observation point. (* $P<0.05$, *t* test)

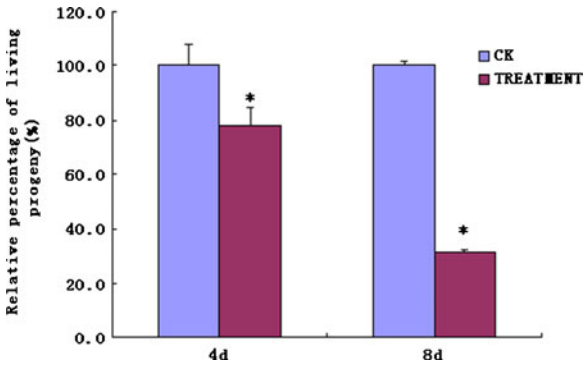

Fig. 5 The average percentage of *B. xylophilus* offsprings from adult females after soaking in dsRNA or CK on Day 4 and Day 8 after inoculation, respectively. (* $P<0.05$, *t* test)

disappeared, but soaking living females deposited their offsprings 4 days after being cultivated in a medium of *Pestalotiopsis* sp., which may be because of the existence of another AK gene in *B. xylophilus.* Furthermore, *C. elegans* has six homogeneous genes coding AK (NP_491057, NP_509217, NP_872253, NP_491027, NP_492714, NP_507054) and *H. glycines* has two AK homogeneous genes (AAO49799, AAP41028), so more than one AK gene could exist in *B. xylophilus*. Homogeneous AK genes were searched against the genomic sequence of *B. xylophilus* (Kikuchi et al. 2011) and *Meloidogyne incognita* (Abad et al. 2008), but no valuable information was found. Therefore, more experimental work needs to be done to clone more homogeneous AK genes in *B. xylophilus*. The AK gene was named *Bx*AK1, since it is the first AK gene cloned from *B. xylophilus*.

There was a time-lag between the soaking and dying of *B. xylophilus* after their recovery from the distilled water. The requirement for RNAi amplification and

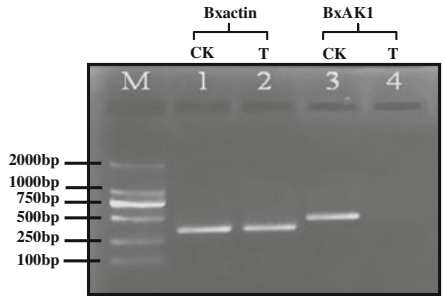

Fig. 6 RT-PCR analysis of *BxAK*1 transcript levels in *B. xylophilus* after treatment with dsRNA. In *B. xylophilus* a 409 bp fragment of the *Bx*actin gene was amplified as endogenous control and the amplicon from the arginine kinase transcript was 495 bp in length. *Bx*Ak1—Arginine kinase Ak1 of *B. xylophilus*

systemic spread could partly account for this phenomenon. Minute amounts of dsRNA ingested into *C. elegans* induced the expression of four distinct RdRps, triggering a systemic silencing driven by core RNAi genes, such as RNA–dependent RNA polymerase (RdRP) and RNA channel transporter SID (Fire et al. 1998; Winston et al. 2002; Siomi and Siomi 2009). According to Abad et al. (2008), four RdRPs in *C. elegans* all have at least one homologue in *M. incognita* based on a sequence comparison. The *B. xylophilus* genome (Kikuchi et al. 2011) encodes more predicted orthologues of *C. elegans* RNAi pathway effectors (37 of a potential 78) than found in *M. incognita* (Korf 2004) and *M. hapla* (Lukashin and Borodovsky 1998). RNA-dependent RNA polymerases (RdRps) are expanded relative to *C. elegans*, with four ego-1-, two rrf-1-, and three rrf-3-like orthologues. The fact that many genes of myosin, heavy chain, tropomyosin, heat shock protein 70, cytochrome C, and β-1,4-endoglucanases gene had also been silenced successfully in *B. xylophilus* (Park et al. 2008; Cheng et al. 2010) may indicate that it is possible to develop *Bx*AK1 RNAi technology to control *B. xylophilus*.

More than twenty successful applications of RNAi have been reported in plant parasitic nematodes, including cyst and root knot nematodes (Urwin et al. 2002; Rosso et al. 2005). Targeting essential genes for nematode development or parasitism can be used to develop new control methods against damaging nematode species. Plants that express dsRNA or hairpin RNA in the nematode feeding cells provide a new option for the creation of resistant varieties. RNAi-based resistant plants are being developed against viruses and insects using hairpin RNA directed to the essential genes of these pathogens (Baum et al. 2007; Shimizu et al. 2009). However, despite several reports of successful plant-mediated RNAi, no plant species engineered to control plant parasitic nematodes in this way has appeared on the market yet.

An important issue arising from this study is whether or not other nematodes and insect species will be affected by the application of a dsRNA targeting *Bx*AK1 gene. Because the AK is highly conserved in nematodes and insects (Fig. 2), dsRNA targeting *Bx*AK1 has the potential to be used as a general biocontrol agent for pests. This study has demonstrated that dsRNA targeting *Bx*AK1 was effective for killing *B. xylophilus*. It may kill non-target organisms such as silkworms (*Bombyx mori*) and honeybees

(*Apis mellifera*). Therefore, further experiments are necessary to determine the effectiveness of dsRNA targeting *BX*AK1 in controlling other nematodes and insects.

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