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Molecules and Cells

Identification of *cdc25* Gene in Pinewood Nematode, *Bursaphelenchus xylophilus*, and Its Function in Reproduction

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The *cdc25* gene, which is highly conserved in many eukaryotes, encodes a phosphatase that plays essential roles in cell cycle regulation. We identified a *cdc25* ortholog in the pinewood nematode, *Bursaphelenchus xylophilus*. The *B. xylophilus* ortholog (*Bx-cdc25*) was found to be highly similar to *Caenorhabditis elegans cdc-25.2* in sequence as well as in gene structure, both having long intron 1. The *Bx-cdc25* gene was determined to be composed of seven exons and six introns in a 2,580 bp region, and was shown to encode 360 amino acids of a protein containing a highly-conserved phosphatase domain. *Bx-cdc25* mRNA was hardly detectable throughout the juvenile stages but was highly expressed in eggs and in both female and male adults. Functional conservation during germline development between *C. elegans cdc25* and *Bx-cdc25* was revealed by *Bx-cdc25* RNA interference in *C. elegans*.

INTRODUCTION

CDC25 phosphatase is an essential cell cycle regulator in eukaryotes that dephosphorylates and activates cyclin-dependent kinases to promote cell cycles. It was first identified in *Schizosaccharomyces pombe* (Russell and Nurse, 1986). The *cdc25* gene family is evolutionarily conserved from yeast to human. *Drosophila* has two *cdc25* genes, *string* and *twine* (Edgar and O'Farrell, 1989). *Caenorhabditis elegans* has four genes: *cdc-25.1, -25.2, -25.3*, and *-25.4* (Ashcroft et al., 1998). Mammals have three *cdc25* genes: *Cdc25A*, *Cdc25B*, and *Cdc25C* (Sadhu et al., 1990). The two *Drosophila* orthologs of *cdc25* have distinct roles in cell cycles. STRING is required for mitosis and TWINE is required for meiosis (Alphey et al., 1992; Courtot et al., 1992; Lehman et al., 1999; Reed, 1995). Similarly, while mammalian CDC25A, CDC25B, and CDC25C are all required for cell proliferation, their expression patterns and functions seem to be distinct: *Cdc25C* is abundant in germ cells but *Cdc25B* is abundant in somatic cells (Sadhu et al., 1990; Wu and Wolgemuth, 1995). In *C. elegans*, CDC-25.1 functions predominantly in mitotic proliferation of the germ cells (Ashcroft and Golden, 2002; Ashcroft et al., 1999; Kim et al., 2009a). CDC-25.2, CDC-25.3, and CDC-25.4 appear to have distinct and essential functions in other types of cell cycle controls (our unpublished results). Collectively, the data concerning *cdc25* orthologs in multicellular organisms suggests that *cdc25* family members are key factors regulating various cell cycles during development.

Pine-wilt disease caused by pinewood nematode (PWN), *Bursaphelenchus xylophilus,* has become problematic because of its fast spread throughout East Asia, North America, and Europe. Developing anti-nematodal drugs against PWN is urgently required. To eradicate PWN, selecting more specific and potent targets for anti-nematodal drugs is essential. Recently, expressed sequence tag (EST) sequences of *B. xylophilus* have been analyzed and deposited to GenBank (Kikuchi et al., 2007), which has made identifying potential molecular targets for the anti-nematodal drugs much easier.

It was reported that embryogenesis and life cycle of *B. xylophilus* are very similar to those of *C. elegans* (Hasegawa et al., 2004). Repression or depletion of CDC25 activity causes embryonic lethality and sterility in *C. elegans* (Ashcroft and Golden, 2002; Ashcroft et al., 1999; Kim et al., 2009a), suggesting that CDC25 is a potential target for developing potent anti-nematodal drugs against PWN. This idea is further supported by our previous results showing that CDC25 inhibitors and flavones can repress overexpressed CDC25 activity (Kim et al., 2009b), thereby causing high embryonic lethality and sterility (Lee et al., 2008).

In this study, we screened for *cdc25* orthologs in PWN and isolated *Bx-cdc25,* which appears to exist as a single copy gene in the genome. We determined that the gene encodes 360 amino acids of a protein containing a highly conserved catalytic phosphatase domain, and shows functional conservation between *C. elegans* and *B. xylophilus* in reproduction.

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MATERIALS AND METHODS

Isolation of *Bx-cdc25* gene

To isolate *cdc25* gene in *B. xylophilus,* an EST database developed at Chungbuk National University was searched using sequence homology with the four *cdc25* family members in *C. elegans*: *cdc-25.1* (GenBank accession number: AF039038), *cdc-25.2* (AF039048), *cdc-25.3* (Z11115), and *cdc-25.4* (Z48795).

Genomic DNA isolation, polymerase chain reaction (PCR) amplification, and sequencing

Total genomic DNA was extracted from *B. xylophilus* cultured at 25°C on potato dextrose agar (PDA) medium with the fungus *Botrytis cinerea* as previously described (Hasegawa et al., 2004; Lee et al., 2008). The extracted DNA was then amplified by PCR using the following primers: 5′-GAA CAC TTG GGA GGA TTC GG-3′ (forward primer, starting with G of the first ATG in exon 1), and 5′-GAT CTG ATG ATG GAC ACC CT-3′ (reverse primer, located at the end of exon 7). PCR was performed for 35 cycles with an annealing temperature at 59°C. The amplified genomic DNA was sequenced by automated DNA sequencing in both directions using the forward primers 5′-GAA CAC TTG GGA GGA TTC GG-3′ and 5′-CGC GAC CAA CTA CCC TCA TC-3′ and the reverse primers 5′-CCA ATC CCA AAC CCA TTT GC-3′, 5′-TCG GTA CAT TTG CAC TCA AC-3′, and 5′-GAT CTG ATG ATG GAC ACC T-3′. The genomic sequence of *Bx-cdc25* has been deposited to GenBank; the accession numbers are GQ245975 for the gene and ACV52012 for the protein.

Genomic Southern hybridization

Genomic DNA was purified from the mixed-stage *B. xylophilus* worms. The worms were recovered from culture plates and washed more than twice with distilled water to remove any remaining *B. cinerea*. Collected worms were lysed for 16 h at 50°C in SNET lysis buffer [20 mM Tris-Cl (pH 8.0), 5 mM EDTA, 400 mM NaCl, and 1% sodium dodecyl sulfate (SDS)] with proteinase K (0.7 mg/ml) and RNAse A (20 μg/ml), prior to isolation of total genomic DNA by standard phenol/chloroform extraction and ethanol precipitation. *B. xylophilus* genomic DNA was digested with three different pairs of restriction enzymes: *Sal*I-*Spe*I, *Pst*I-*Spe*I, and *Dra*I-*Spe*I. The digested genomic DNA was separated by electrophoresis, transferred to a Hybond-N^+ nylon membrane (Amersham Biosciences) by capillary action, and fixed with an ultraviolet cross-linker (1200 × 100 μJ/cm^2). Randomly-primed DIG-labeled DNA probe (Roche Applied Science) was generated from a 563 bp DNA fragment corresponding to the highly conserved phosphatase domain in *Bx-cdc25* gene (Fig. 1E). Hybridization with the probe was carried out either at 37°C (moderate stringency) or at 42°C (high stringency) using DIG-Easy Hyb (Roche Applied Science), and the blot was washed at room temperature in 2× SSC (pH 7; 30 mM sodium citrate, 0.3 M NaCl) with 0.1% SDS, followed by washing either at 42°C (moderate stringency) or at 60°C (high stringency) in 0.5× SSC (pH 7; 7.5 mM sodium citrate, 75 mM NaCl) with 0.1% SDS. Hybridized bands were visualized according to the manufacturer's instructions (Roche Applied Science) with CSPD as a substrate for alkaline phosphatase, and band intensities on the X-ray film exposed to chemiluminescence were quantified using Quantity-One® software (Bio-Rad).

Sequence alignment and phylogenetic analyses

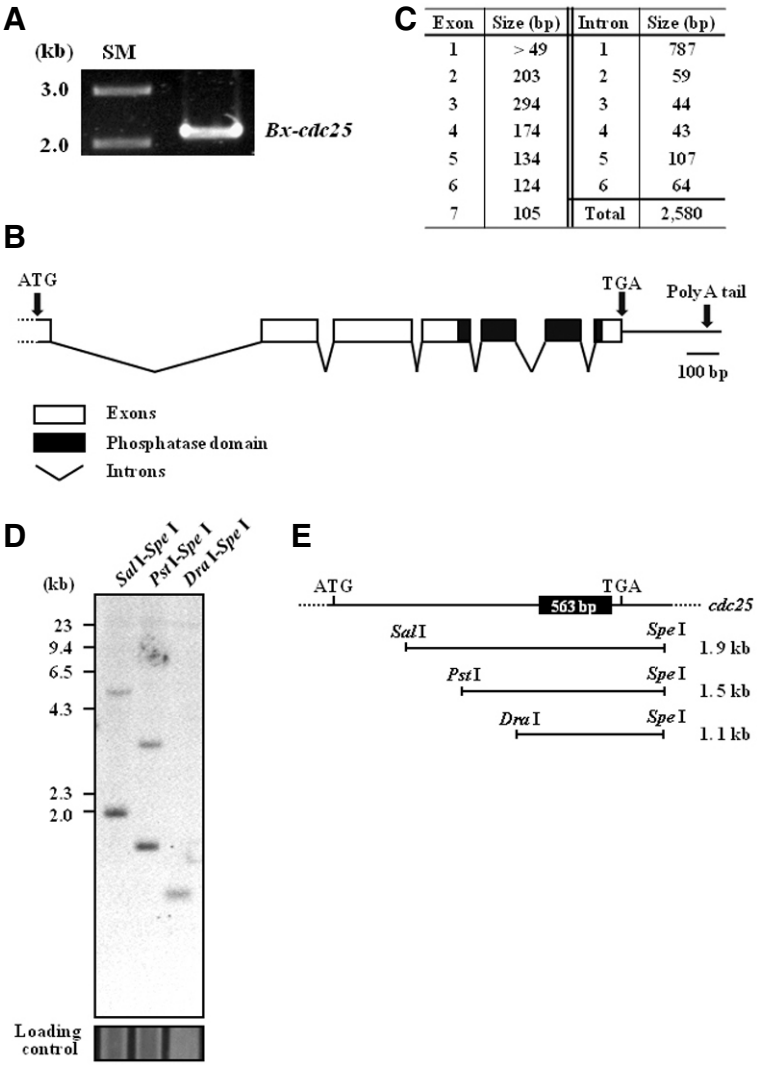
The putative phosphatase domains identified in the deduced CDC25 amino acid sequences from the *cdc25* cDNA sequences were used for comparison. ClastalW version 2 was used for sequence alignment (http://www.ebi.ac.uk/Tools/clustalw2). GenBank accession numbers of CDC25 proteins used for analyses were as follows: *Homo sapiens* CDC25A, AAA58415; *Homo sapiens* CDC25B, AAA58416; *Homo sapiens* CDC25C, AAA35666; *Sus scrofa* (pig) CDC25C, CAA55123; *Mus musculus* (mouse) CDC25A, AAA85580; *Mus musculus* CDC25B, AAB22026; *Mus musculus* CDC25C, AAA74912; *Drosophila melanogaster* (fruitfly) TWINE, AAA28413; *Drosophila melanogaster* STRING, AAA28916; *Caenorhabditis elegans* (nematode) CDC25.1, AAK29711; *Caenorhabditis elegans* CDC-25.2, AAB94237; *Caenorhabditis elegans* CDC-25.3, CAA77456; *Caenorhabditis elegans* CDC-25.4, CAA88725; *Caenorhabditis briggsae* (nematode) CDC-25.1, CAP31584; *Caenorhabditis briggsae* CDC-25.2, CAP26876; *Caenorhabditis briggsae* CDC-25.3, CAP28061; *Caenorhabditis briggsae* CDC-25.4, CAP22347; *Schizosaccharomyces pombe* (yeast) CDC25, AAA35294; *Arabidopsis thaliana* CDC25, AAO39886. Phylogenetic analysis was performed using MacVector version 10.0.2 (Accelrys).

Reverse transcriptase-PCR (RT-PCR)

The mRNA level of *Bx-cdc25* at each developmental stage including egg stage, three juvenile stages, and male and female adult stages was analyzed by RT-PCR. Total RNA was extracted from *B. xylophilus* at each developmental stage using TRIzol Reagent (Invitrogen). Total cDNA was synthesized using reverse transcriptase (Invitrogen), and *Bx-cdc25* cDNA was amplified with the same primers used for *Bx-cdc25* genomic DNA amplification. Except for the egg stage, the cDNA was further amplified by semi-nested PCR using another reverse primer (5′-TCG GTA CAT TTG CAC TCA AC-3′). *actin* cDNA was amplified as an internal control (GenBank accession number, EU100952), using primers 5′-GAG GCC CAG TCC AAG AGA-3′ (Forward) and 5′-TGT TGG AAG GTG GAC AGG-3′ (Reverse).

RNA interference (RNAi) analysis

Soaking RNAi was performed as previously described (Maeda et al., 2001; Tabara et al., 1998). DNA template was synthesized from T3-3483 clone containing partial *Bx-cdc25* cDNA sequence using T7 primer (5′-GTA ATA CGA CTC ACT ATA GGG C-3′) and CMo422 primer (5′-GCG TAA TAC GAC TCA CTA TAG GGA ACA AAA GCT GGA GCT-3′) by PCR. PCR was performed for 35 cycles of denaturing at 94°C for 1 min, annealing at 55°C for 30 s, and extension at 72°C for 2.5 min. The amplified template DNA was purified with a gel extraction kit (Qiagen), and used for synthesizing dsRNA in Transcription Buffer (Stratagene) with a mixture of NTPs (2 mM of each; TaKaRa Bio), 10 mM dithiothreitol (DTT), and 100 U of T7 RNA polymerase (Stratagene) at 37°C for 2 h. Then, synthesized double-strand (ds) RNA was purified by DNase I digestion (10 U; TaKaRa Bio.), phenol-chloroform-isoamylalcohol/chloroform extraction, and isopropanol precipitation. The dsRNA of *Ce-cdc-25.1* (GenBank accession No.: AF039038) was also synthesized as a control. Twenty synchronized L4-stage *C. elegans* worms were put into soaking buffer containing synthesized dsRNA, and soaked for 24 h at 20°C. Then, the soaked worms were recovered to NGM agar plates containing *Escherichia coli* OP50. Among the 20 soaked worms, eight healthy worms were selected and individually transferred to fresh plates every 24 h for 7 days, and phenotypes of the progeny including brood size, embryonic lethality, and percent larval development were measured. Alternatively, more than 20 L1 worms were soaked under the same conditions, recovered, and their sterility/fertility was judged when they grew into adult worms. Each experiment was repeated more than three times.



Exon	Size (bp)	Intron	Size (bp)
1	> 49	1	787
2	203	2	59
3	294	3	44
4	174	4	43
5	134	5	107
6	124	6	64
7	105	Total	2,580

Fig. 1. Genomic DNA structure of *Bx-cdc25* and Southern blotting analysis. (A) PCR-amplified 2.2 kb *Bx-cdc25* genomic DNA fragment. (B) Genomic DNA structure of the *Bx-cdc25* gene. White boxes indicate exons and the black region corresponds to the conserved phosphatase domain of CDC25 super family. The start codon ATG, stop codon TGA, and poly A tail site are indicated by arrows. (C) The *Bx-cdc25* 2,580 bp genomic coding sequence contains seven exons and six introns. (D) Southern blot analysis. Twenty micrograms of *B. xylophilus* genomic DNA was double-digested with pairs of enzymes indicated at the top of the panel and separated on a 0.9% agarose gel. The blot was hybridized with a DNA probe generated by random priming from a 563 bp DNA fragment where the conserved phosphatase domain is located, which is indicated by a black box (E). Predicted sizes of the hybridizing DNA fragments after double digestions are depicted (E).

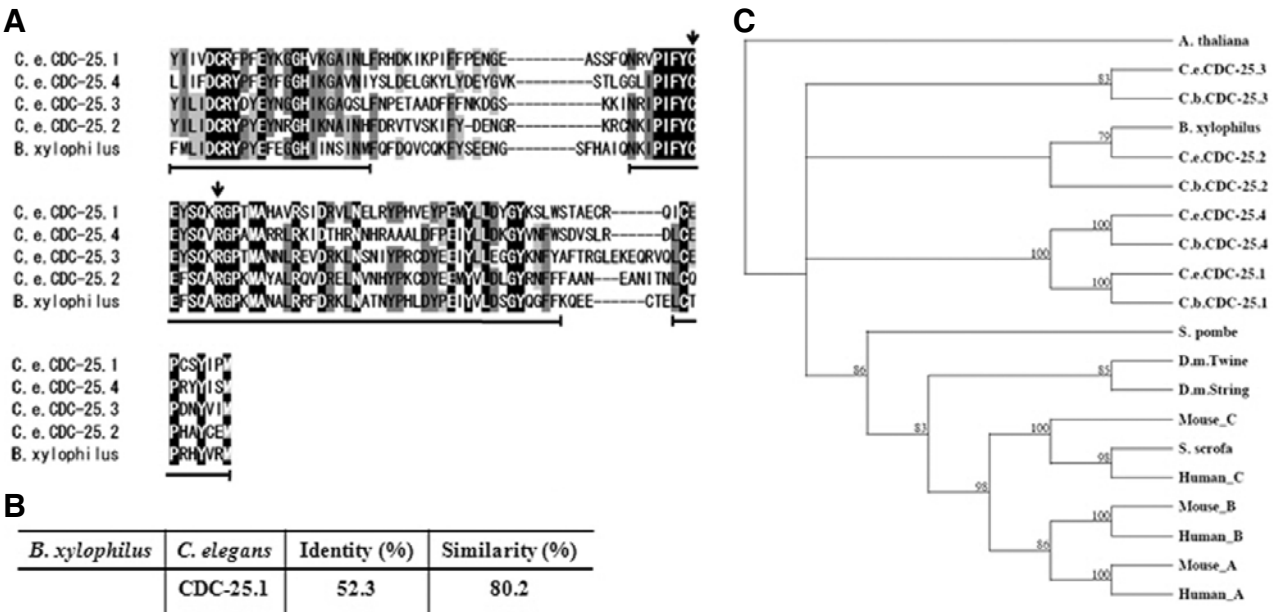
Brood size was defined as the total number of hatched and non-hatched embryos produced by a mother worm. Embryonic-lethality was defined as the percentage of non-hatched embryos among the total laid embryos. Percent larval development was defined as the percentage of worms that reached adulthood among the total hatched larvae. A worm was judged to be sterile if it did not produce any fertilized embryos.

RESULTS

Isolation of *cdc25* gene in *B. xylophilus*

BLAST search of the PWN EST database using the *C. elegans cdc25* (*Ce-cdc25*) genomic sequence as a query sequence gave two positive *B. xylophilus* cDNA clones: T3-3483 and T3-5872. These clones contained partially overlapping nucleotide sequences that contained nearly full length *B. xylophilus cdc25* (*Bx-cdc25*) cDNA ranging from the first ATG to the poly A tail when they were combined. To determine *Bx-cdc25* gene structure, the genomic DNA sequence of *cdc25* was determined by sequencing the PCR-amplified genomic DNA fragment (Fig. 1A). The 2,580 bp fragment was predicted to encode a 360 amino acid protein containing a conserved phosphatase domain in the C-terminal half (Fig. 1B). Seven exons and six introns were evident including the very long intron 1 (787 bp) (Figs. 1B and 1C). The 5′ end of exon 1 has remained undetermined. The putative exon 1 containing the predicted first ATG is presented as an open box (Fig. 1B). The 3′untranslated region (UTR) after the stop codon and before the poly A attachment site was 329 bp. The 5′UTR remains to be determined. When the GenBank database (http://www.ncbi.nlm.nih.gov) was BLAST searched with the *Bx-cdc25* genomic sequence, two partially matching sequences, CJ980250 and CJ985384, were found. They had been deposited in the database in a recent study (Kikuchi et al., 2007). Both sequences were approximately 500 bp and were identical to the partial nucleotide sequence of *Bx-cdc25* presently isolated.

Southern blot analysis of *B. xylophilus* genomic DNA was performed to examine copy numbers of the *Bx-cdc25* gene. The blot was hybridized with a 563 bp DNA probe generated from the phosphatase domain spanning the end of exon 4 to the beginning of exon 7 of the *Bx-cdc25* gene (Figs. 1B, 1D, and 1E). The genomic DNA that was double-digested with *Sal*I-*Spe*I, *Pst*I-*Spe*I, and *Dra*I-*Spe*I was analyzed (Figs. 1D and 1E); the expected sizes of hybridizing bands were 1.9 kb, 1.5 kb, and 1.1 kb, respectively (Fig. 1E), if there was only one copy for the *Bx-cdc25* gene. We found two hybridizing bands for *Sal*I-*Spe*I and *Pst*I-*Spe*I digests; a band of the expected size (lower band) and a larger band of an unexpected size (upper



B

B. xylophilus	*C. elegans*	Identity (%)	Similarity (%)
CDC25	CDC-25.1	52.3	80.2
	CDC-25.2	68.6	79.1
	CDC-25.3	60.5	76.7
	CDC-25.4	53.5	69.8

Fig. 2. Amino acid sequence alignment of the phosphatase domains among Bx-CDC25 and four Ce-CDC25 proteins. (A) The phosphatase domains of four *C. elegans* CDC25 proteins and *B. xylophilus* CDC25 protein are aligned, and three conserved motifs within the domain are indicated with horizontal bars: the CH2-A motif, the CX_5R active site motif, and the CH2-B motif. The invariant cysteine (C) and arginine (R) residues in the CX_5R motif are marked with arrows. Letters in black boxes, grey boxes, and light grey boxes indicate invariant amino acid residues, highly conserved amino acid residues, and moderately conserved amino acid residues, respectively, among the CDC25 proteins. Gaps (-) were generated for the best alignment. (B) Identities and similarities between amino acid sequences of the phosphatase domains of *B. xylophilus* CDC25 protein and each of four *C. elegans* CDC25 proteins. (C) Phylogenetic analyses of twenty CDC25 family members. Phylogenetic tree was constructed based on the phosphatase domain sequences using MacVector software (Version 10.0.2; Accelrys). The twenty CDC25 proteins used for analysis comprised three from *Homo sapiens* (Human_A, Human_B, Human_C), one from *Sus scrofa* (S. scrofa), three from *Mus musculus* (Mouse_A, Mouse_B, Mouse_C), two from *Drosophila melanogaster* (D.m.Twine, D.m.String), four from *Caenorhabditis elegans* (C.e.CDC-25.1, C.e.CDC-25.2, C.e.CDC-25.3, C.e.CDC-25.4), four from *Caenorhabditis briggsae* (C.b.CDC-25.1, C.b.CDC-25.2, C.b.CDC-25.3, C.b.CDC-25.4), one from *Schizosaccharomyces pombe* (*S. pombe*), one from *Arabidopsis thaliana* (*A. thaliana*), and *B. xylophilus* CDC25 (*B. xylophilus*). *S. pombe* and *A. thaliana* proteins are used as outgroups of the tree. Bootstrap analysis was accomplished using 1,000 replicates to appraise the reliability of different phylogenetic groups, and numbers above the branches are the percentage of 1,000 replicates. GenBank accession numbers of the CDC25 proteins used for the analysis are listed in "Materials and Methods."

band) for each digest (Fig. 1D). This result suggested either the presence of two copies of the *Bx-cdc25* gene or partial digestion with the restriction enzymes. To distinguish these possibilities, the same blot was re-hybridized and washed at higher temperatures. If the unexpected upper bands were generated by partial digestion, the hybridization intensity would remain at similar levels as that of the lower bands at higher temperatures. However, if the upper bands were derived from the second gene of *Bx-cdc25,* the hybridization intensity would be significantly reduced at higher temperatures compared to that of the lower bands, because the second gene should not contain 100% matching sequence with the first gene that was used to make the hybridization probe. We found that the hybridization intensities of the upper bands were not significantly reduced compared to those of the lower bands after high temperature hybridization and washing (data not shown). In addition, the genomic DNA double-digested with *Dra*I-*Spe*I gave only one hybridizing band even after moderate temperature hybridization and washing (Fig. 1D), implying that *Bx-cdc25* exists most likely as a single-copy gene in the *B. xylophilus* genome, and that the hybridizing bands of unexpected sizes were likely generated by partial digestions.

Phylogenetic analyses of Bx-CDC25

The alignment of phosphatase domains of Bx-CDC25 and four Ce-CDC25 proteins revealed high sequence identities and similarities (Figs. 2A and 2B). Three motifs within the phosphatase domain were evident in the Bx-CDC25 protein as previously described for other CDC25 proteins (Fauman and Saper, 1996). The regions comprised a CH2-A motif, a region of 21 amino acids (IIDCRYPYEYxGGHIxGAxNL) containing an invariant aspartic acid residue (D); the active site motif (CX_5R), which has been proposed to form a phosphate binding loop with invariant cysteine (C) and arginine (R) residues; and the CH2-B motif (CxPxxYxxM) that is absolutely conserved among all the CDC25 family members (Ashcroft et al., 1998; Fauman and Saper, 1996). All four Ce-CDC25 proteins showed more than 50% identity to Bx-CDC25 protein (Fig. 2B). Among them, CDC-25.2 showed the highest identity (68.6%). To investigate further the phylogenetic relationship among CDC25 proteins of multicellular organisms, the phosphatase domain sequences of 20 different CDC25 proteins

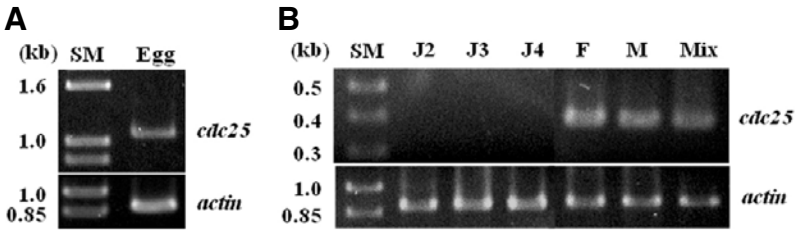


Fig. 3. *Bx-cdc25* mRNA expression during the development. (A) mRNA expression of *Bx-cdc25* in the egg monitored by RT-PCR. The RT-PCR product size of *Bx-cdc25* was 1,078 bp. (B) During the juvenile stages (J2-J4) *Bx-cdc25* mRNA expression was not detectable, but the female (F) and male (M) adults and the mixed-stage worms (Mix) evidently gave the mRNA expression. The RT-PCR product size of *Bx-cdc25* in this experiment was 404 bp. RT-PCR products were separated by 2% agarose gel electrophoresis with DNA size markers (SM) indicated on the left of each panel. Expression of actin mRNA in each sample was also monitored by RT-PCR as an internal loading control.

including Bx-CDC25 were compared (Fig. 2C, Supplementary Fig. S1). A high degree of conservation was observed within their phosphatase domains, suggesting functional conservation as well. This analysis also demonstrated that Ce-CDC-25.2 was the closest protein to Bx-CDC25 among the 19 family members examined, with the second closest one being Cb-CDC-25.2 (Fig. 2C).

Expression pattern of *Bx-cdc25* mRNA

To examine the mRNA expression pattern of *Bx-cdc25* during development, RT-PCR was performed using total RNA extracted from eggs (Fig. 3A), juvenile-stage worms (J2-J4), and the female (F) and male (M) adult worms (Fig. 3B). *B. xylophilus* molts from J1 to J2 inside the egg and hatches at J2 stage (Hasegawa et al., 2004); progress through J2, J3, and J4 juvenile stages; then become adults (Oh et al., 2009). We found that *Bx-cdc25* mRNA was abundantly expressed in the eggs and both female and male adult worms, but not in juvenile worms (Figs. 3A and 3B). Total RNA from the mixed-stage population (Mix) also gave a strong signal of *Bx-cdc25* mRNA (Fig. 3B).

Bx-cdc25 RNAi in *C. elegans*

To assess the functions of *Bx-cdc25,* we performed RNAi analysis. RNAi technology was first developed in *C. elegans* to knock-down gene function (Fire et al., 1991; 1998). RNAi in *B. xylophilus* was first attempted using dsRNA of myosin heavy chain (*Bx-myo-3*), tropomyosin (*Bx-tmy-1*), heat shock protein 70 (*Bx-hsp-1*), and cytochrome C (*Bx-cyt-2.1*) by three methods: soaking, electroporation, and microinjection (Park et al., 2008). Depending on the developmental stages, efficiency of the three RNAi methods differed. Soaking RNAi to the adult worms was moderately efficient (Park et al., 2008). Therefore, soaking RNAi of *Bx-cdc25* to *B. xylophilus* adult worms was performed. Embryonic lethality and survival rate were measured after RNAi, but its effect was negligible (data not shown).

Because of the high sequence similarity in the phosphatase domains between *Bx-cdc25* and *Ce-cdc25*, we assumed a functional conservation between the two genes. Therefore, we attempted soaking RNAi of *Bx-cdc25* in *C. elegans* to assess the function of *Bx-cdc25*. We measured brood size, embryonic lethality, percent larval development, and sterility after the RNAi (Fig. 4). As previously reported with RNAi of *Ce-cdc-25.1* (Ashcroft et al., 1999), reduced brood size (35.9% of control), high embryonic lethality (94.2%), and high sterility (90.3%) were observed in *C. elegans* worms treated with *Ce-cdc-25.1* dsRNA. Treatment of *C. elegans* worms with *Bx-cdc25* dsRNA also caused reduced brood size (49.9% of control), embryonic lethality (12.3%), and sterility (14.6%), but the percent larval development was not affected (Fig. 4). These results suggest that *Bx-cdc25* likely possesses similar biological functions as *Ce-cdc25* during the germline development of *B. xylophilus.*

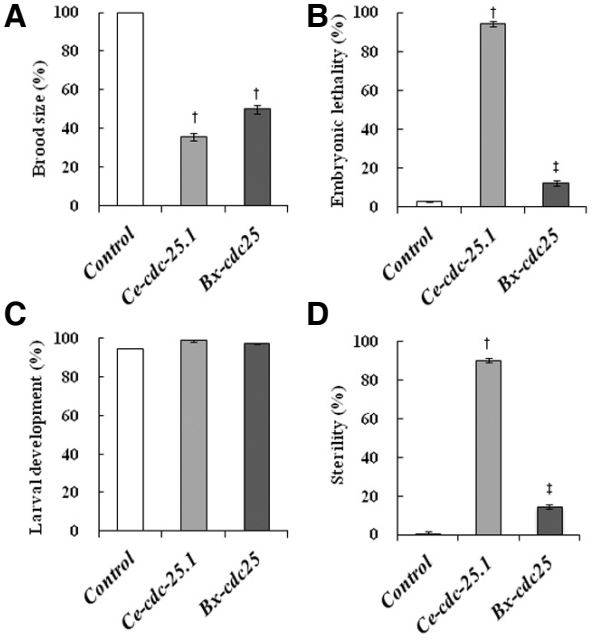


Fig. 4. *Bx-cdc25* RNA interference in *C. elegans*. Brood size (A), embryonic lethality (B), larval development (C), and sterility (D) were measured after performing soaking RNAi of *Bx-cdc25* in *C. elegans*. For (A), (B), and (C), L4-stage mother worms were treated with soaking RNAi, and their progeny were scored (total numbers of treated mothers ranged from 22 to 29). For (D), L1-stage worms were treated with soaking RNAi, and observed when they grew into adults (total numbers of treated L1 worms ranged from 50 to 156). *Ce-cdc-25.1* RNAi was also performed as a positive control. Control indicates mock RNAi using buffer solution without dsRNA, which served as a negative control. Each RNAi experiment was performed more than three times, and the result is presented as the average ± S.D. The *p* values for mean differences were calculated using KESS (stat.snu.ac.kr/time). $^{\dagger}p < 0.001$, $^{\ddagger}p < 0.05$, otherwise, $p > 0.05$ against the control.

DISCUSSION

We identified *Bx-cdc25* gene and examined its phylogenetic relationship to other CDC25 family proteins. We further uncovered potential roles of *Bx-cdc25* during development. The phosphatase domain of Bx-CDC25 protein showed high sequence similarity to those of *C. elegans* CDC-25 proteins, implying that they are functionally conserved as well. Especially, Ce-CDC-25.2 protein showed the highest scores of identity for both amino acid sequence (68.6%) and nucleotide sequence (65.1%) to Bx-CDC25, which was even higher than those be-

tween Ce-CDC-25.2 and the other Ce-CDC25 proteins. Ce-CDC-25.2 was also shown to be the closest ortholog to Bx-CDC25 among the 19 CDC25 family members examined in phylogenetic analysis. In addition, both *Bx-cdc25* and *Ce-cdc-25.2* genes contain a long intron 1; *Bx-cdc25* intron 1 is 787 bp and *Ce-cdc-25.2* intron 1 is about 5 kb, while the other introns are relatively short for both genes. In general, the typical size of *C. elegans* intron is around 47 bp (Fedorov et al., 2003). If intron 1 has a functional activity, it is possibly conserved between the two species. In *C. elegans*, some intron 1, which is relatively longer than the other introns, often serves as a regulatory site for gene expression. For example, the first intron of *snap-25* (about 7.8 kb) has four distinct regulatory elements that are required for its specific expression in different neuronal cells (Hwang and Lee, 2003). Functional significance of *Bx-cdc25* intron 1 during the development remains to be elucidated.

Bx-cdc25 is probably present as a single-copy gene in *B. xylophilus* genome as predicted by Southern blot analysis. In addition, RT-PCR performed with primers for the conserved phosphatase domain produced a single band, and BLAST search for *cdc25* gene in a *B. xylophilus* EST database gave two overlapping clones with identical sequences. Many organisms possess multiple *cdc25* genes. *C. elegans* has four *cdc25* genes, *cdc-25.1, -25.2, -25.3*, and *-25.4* (Ashcroft et al., 1998); so does *C. briggsae* (www.wormbase.org). However, *S. pombe* and *Arabidopsis thaliana* have one *cdc25* gene (Landrieu et al., 2004; Russell and Nurse, 1986). In *C. elegans,* four *cdc25* genes appear to have distinct expression patterns during the development (our unpublished results), suggesting that function of each member is also different. Although *B. xylophilus* has female and male sexes it appears to have a single copy of *cdc25*, suggesting that cell cycle regulation mechanism is probably common in both sexes, between mitosis and meiosis, and among different tissues in *B. xylophilus* compared to *C. elegans*. Alternatively, other cell cycle regulators such as cyclin-dependent kinases and cyclins may contribute to confer specificity on each type of cell cycle regulation in *B. xylophilus*. Recently, Kikuchi's group deposited 6487 *B. xylophilus* genes to GenBank (Kikuchi et al., 2007), which are good resources to further identify other cell cycle regulators and to examine their functions at the molecular level. If more cell cycle regulators are uncovered, we may be able to understand how various cell cycles are regulated by a single copy of *cdc25* gene in *B. xylophilus*.

In this study, we performed RNAi of *Bx-cdc25* gene in *C. elegans*. The results suggest that, like *Ce-cdc-25.1*, *Bx-cdc25* functions in germline development. Depletion of *Ce-cdc-25.1* expression by RNAi causes embryonic lethality in *C. elegans* (Ashcroft et al., 1999), and its loss-of-function mutants are sterile due to under-proliferation of germ cells (Ashcroft and Golden, 2002; Kim et al., 2009a). RNAi of *Bx-cdc25* in *C. elegans* also caused the reduced brood size, embryonic lethality, and sterility, although the levels of effects were lower than those of *Ce-cdc-25.1* RNAi. On the other hand, RNAi of *Ce-cdc-25.1* or *Bx-cdc25* did not inhibit larval development, suggesting that they have little impact on postembryonic somatic development. In *C. elegans*, *cdc-25.1* mRNA level is very low at the L1 stage, and it is gradually increased (although still remaining low) throughout the larval development (Kim et al., 2009a). However, the mRNA level is drastically increased when worms reach the adult stage, indicating that *cdc-25.1* mRNA levels correlate with the number of germ cells (Kim et al., 2009a). Similarly, *Bx-cdc25* mRNA expression was hardly detectable throughout the juvenile stages of *B. xylophilus,* but a significant level of the mRNA was detected at the adult stage and in the eggs, suggesting that *Bx-cdc25* mRNA levels may also be correlated with germ cell proliferation and embryogenesis, like *Ce-cdc-25.1* mRNA. Since *Bx-cdc25* is likely a single-copy gene, and its expression levels are very low in *B. xylophilus* juveniles, it is uncertain how the cell cycle is regulated during the juvenile stages. Although there are four *cdc25* genes in *C. elegans*, their mRNA levels are very low during the early larval stages for all four genes (our unpublished results), implying that little CDC25 activity is required for larval development. A similar situation may exist for *B. xylophilus.*

B. xylophilus causes pine-wilt disease, which is a serious problem for pinewoods in many countries. Developing nematocides against *B. xylophilus* is urgently required. Our study supports the idea that *Bx-cdc25* is a potential target.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org)

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