Parasitology

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The alcohol dehydrogenase with a broad range of substrate specificity regulates vitality and reproduction of the plant-parasitic nematode *Bursaphelenchus xylophilus*

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Research Article

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Abstract

Pine wilt disease, which is caused by the pine wood nematode (PWN), *Bursaphelenchus xylophilus*, has caused huge damage to pine forests around the world. In this study, we analysed the PWN transcriptome to investigate the expression of genes related to the associated bacterial species *Pseudomonas fluorescens* and found that the gene *adh*-1 encoding alcohol dehydrogenase (ADH) was upregulated. The open reading frame of *adh*-1, which encoded a protein of 352 amino acid residues, was cloned from *B. xylophilus*. Recombinant ADH with a relative molecular weight of 39 kDa, was present mainly in inclusion bodies and was overexpressed in *Escherichia coli* BL21 (DE3) and purified after refolding. The biochemical assay revealed that recombinant ADH could catalyse the dehydrogen reaction of eight tested alcohols including ethanol in the presence of NAD^+. Quantitative real-time RT-PCR analysis indicated that ethanol upregulated *adh*-1 expression in PWN. Results of RNA interference and inhibition of ADH treatment indicated that downregulating expression of *adh*-1 or inhibition of ADH could reduce ethanol tolerance and the vitality and reproduction ability of *B. xylophilus*, suggesting that *adh*-1 is involved in pathogenicity of PWN.

Introduction

The pine wood nematode (PWN), *Bursaphelenchus xylophilus*, is the causal agent of pine wilt disease (PWD), which causes major economic and ecological losses by damaging pine trees worldwide (Mamiya, 1983; Mota *et al*., 1999; Braasch *et al*., 2001). The PWN is transmitted to pine trees by pine sawyer beetles such as *Monochamus alternatus* that feed on pine branches. Once PWN infects susceptible pine trees, early nematode stages feed on the epithelial cells around the resin ducts followed by feeding on fungi growing in dead trees (Hasegawa and Miwa, 2008). The PWN population in an infected pine tree is very low at the early infection stage (Kuroda, 1991), but it quickly increases when the pine is approaching death, and air embolisms form in xylem tissue, which stops water movement and ultimately results in pine wilt (Kuroda and Ito, 1992).

Ikeda and Oda (1980) reported that PWN-infected pine produced a large amount of volatile compounds such as ethanol after oleoresin leakage stopped; ethanol was later shown to have a stimulating effect on oviposition of PWN (Shuto and Watanabe, 1988). Oku (1988) reported that low concentrations of dihydroconiferyl alcohol produced by PWN-infected pines stimulated multiplication of PWN. In the nematode *Caenorhabditis briggsae*, low concentrations of ethanol significantly increased population growth (Lu *et al*., 1978). These studies hint at a probable relationship between alcohols produced by PWN-infected pines and PWN reproduction, but the molecular mechanism of the influence of alcohols on PWN is still unclear.

Alcohol dehydrogenases (ADH, EC 1.1.1.1) are a group of dehydrogenase enzymes distributed in many organisms with the reduction of nicotinamide adenine dinucleotide (NAD^+) (Reid and Fewson, 1994). ADH belongs to the medium chain dehydrogenase/reductase protein family (MDR) that contains the zinc-dependent ADH-Zn and related proteins (Persson *et al*., 2008). As an important part of MDR, the ADH system, which catalyses the interconversion between ethanol and acetaldehyde, plays an important role in sugar metabolism including ethanol production and assimilation and cofactor balancing (Lin *et al*., 2010). In *Caenorhabditis elegans*, the reaction rate for alcohol oxidation catalysed by ADH is more rapid with higher molecular weight alcohols as substrates than with ethanol (Williamson *et al*., 1991).

Although it is controversial that bacteria associated with PWN are the casual pathogens of PWD, previous studies reported that the disease was induced by a synergistic effect of axenic PWN and pathogenic bacteria, and that pathogenic bacteria carried by PWN or axenic PWN alone could not induce PWD (Zhao *et al*., 2003; Nascimento *et al*., 2015; Proença *et al*., 2017). *Pseudomonas fluorescens* GcM5-1A, one of the pathogenic bacteria species associated with

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PWN, produces an antioxidant 1-cys peroxiredoxin and probably protects PWN from H_2O_2 damage in host pines (Liu *et al.*, 2015). In the present study, we used transcriptome analysis to investigate the effect of *P. fluorescens* GcM5-1A on gene expression of PWN. The gene *adh*-1 of aseptic PWN encoding ADH was found to be upregulated by re-association with *P. fluorescens* GcM5-1A isolated from wild PWN (unpublished data). We hypothesized that *adh*-1 might have participated in regulating the interaction between nematodes and pathogenic bacteria. To further elucidate the roles of ADH in PWN, we cloned *adh*-1, characterized the recombinant ADH, and investigated the effects of expression levels of *adh*-1 on PWN vitality and reproduction.

Materials and methods

PWN and associated bacteria

PWN was isolated from wilted Japanese black pines in Nanjing, China and cultured on *Botrytis cinerea* (Guo *et al.*, 2017). *Pseudomonas fluorescens* GcM5-1A (CCTCC No: M204065) was originally separated from wild PWN and maintained in Luria–Bertani (LB) medium (Liu *et al.*, 2015). Aseptic PWNs were obtained according to a method described previously (Han *et al.*, 2003; Zhao *et al.*, 2011). To prepare PWN associated with *P. fluorescens* GcM5-1A, about 5000 aseptic PWNs in 500 μL sterilized water were mixed with equal volume of *P. fluorescens* GcM5-1A culture (1.0×10^6 cfu mL^{-1}). The nematodes were cultured at 25 °C for 12 h, followed by inoculating them to *B. cinerea* growing on potato dextrose agar (PDA) medium. *Psuedomonas fluorescens* GcM5-1A carried by PWN was confirmed by re-isolating bacteria on LB medium.

Extraction of mRNA from PWN

Total RNA of PWN was extracted using Trizol reagent (Invitrogen, Waltham, MA, USA) and treated with DNase I (Cwbio, Beijing, China). Extracted RNA was measured using ultraviolet absorbance at A260/280 (TU-1810, Persee, China) and detected using electrophoresis on a 1.5% denatured agarose gel.

RNA sequencing analysis

Approximately 5 μg total RNAs extracted from aseptic PWNs and PWNs carrying *P. fluorescens* GcM5-1A, respectively, were used to construct RNA sequencing libraries with the TruseqTM RNA sample prep Kit from Illumina. The aseptic PWNs and PWNs carrying *P. fluorescens* GcM5-1A RNA samples were indexed with the adapters and sequenced on an Illumina HiSeq 4000. Sequence analysis and functional annotation of the differentially expressed genes (DEGs) were performed according to the method described previously (Liu *et al.*, 2017).

Gene cloning and construction of expressing plasmid

According to the sequence of *adh*-1 revealed by transcriptome analysis, the forward primer p1 and reverse primer p1 (Table 1) were used to amplify *adh*-1 by polymerase chain reaction (PCR) using cDNA reverse-transcribed from mRNA of PWN. The PCR program was as follows: pre-denaturing at 94 °C for 10 min, followed by denaturing at 94 °C for 50 s, annealing at 56 °C for 45 s and extension at 72 °C for 55 s. The resulting PCR product was digested by *Nde* I and *Xho* I, ligated into pET-15b (Novagen, Gibbstown, NJ, USA), and sequenced.

The ORF of *adh*-1 from PWN was analysed using the program ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The structural features of *adh*-1 were analysed using the PWN genome from WormBase ParaSite (http://parasite.wormbase.org/index.html). The computed parameters of ADH were obtained from ProtParam (http://web.expasy.org/protparam/). Bioinformatics websites were used to analyse the characteristics of predicted ADH protein including signal peptides (http://www.cbs.dtu.dk/services/SignalP-4.0/) and transmembrane helices (http://www.cbs.dtu.dk/services/TMHMM/).

Table 1. PCR primers used in this study

Name of primer	Sequence (5′→3′)
Forward primer p1	AAACATATGGCTACCTTGGAGCCCATTC
Reverse primer p1	ACTCGAGTTACTTCCACAAGTCCAAAAC
Forward primer p2	CTGCTGAGCGTGAAATCGT
Reverse primer p2	GTTGTAGGTGGTCTCGTGGA
Forward primer p3	CGTGCTGGTGTTAAGTGGAT
Reverse primer p3	CTTTGAGGGCTTTGTAGGC
RNAi-BxADH-T7R	GATCACTAATACGACTCACTATAGGGACCTTC ACGTATGGCTGGGAGATTC
RNAi-BxADH-F	TTGTTGGAAGGATCCATCGCGATGG
RNAi-BxADH-R	ACCTTCACGTATGGCTGGGAGATTC
RNAi-BxADH-T7F	GATCACTAATACGACTCACTATAGGGTTGTT GGAAGGATCCATCGCGATGG
RNAi-GFP-T7R	GATCACTAATACGACTCACTATAGGGCAAAG ATGACGGGAACTAC
RNAi-GFP-F	GATAATGGTCTGCTAGTTG
RNAi-GFP-R	CAAAGATGACGGGAACTAC
RNAi-BxADH-T7F	GATCACTAATACGACTCACTATAGGGGATAA TGGTCTGCTAGTTG

The T7 promoter sequences were underlined.

Expression and purification of the recombinant ADH

Escherichia coli BL21 (DE3) (TaKaRa, Dalian, China) was transformed with the expression vector pET-15b-Adh to construct engineered bacteria. Expression of the recombinant ADH and collection of inclusion bodies were conducted according to a method described previously (Liu *et al.*, 2015).

Inclusion bodies were dissolved in 10 mL lysis buffer (8 M urea, 0.1 mM phenylmethanesulfonyl fluoride, 20 mM Tris-Cl, 500 mM NaCl, 5 mM imidazole, pH 8.0), and the insoluble materials were removed by centrifugation at 10 000 ***g*** for 30 min under 4 °C. The resulting supernatant was diluted with refolding buffer (20 mM Tris-Cl, 500 mM NaCl, 5 mM imidazole, pH 8.0) to gradually decrease concentrations of urea from 8 to 1 M at 4 °C. Refolded recombinant ADH was purified using Ni-NTA affinity chromatography (Liu *et al.*, 2015). The purified protein was analysed by SDS-PAGE with a 12% separating gel, and protein concentrations were quantified using Coomassie Brilliant Blue G-250 with bovine serum albumin as the standard (Bradford, 1976).

Characterization of the recombinant ADH

ADH activity of different substrates was assayed as described previously (Cho and Jeffries, 1998) with some modifications. The reaction mixture contained 100 mM Tris-HCl buffer (pH 8.3), 5.0 mM NAD^+, 50 μL recombinant ADH, 100 mM substrate and 0.05 mM $ZnSO_4$, in a total volume of 1.0 mL. One unit of enzyme activity was expressed as the amount of ADH to increase 1.0 μM of NADH per minute at 25 °C.

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In the assay to investigate the effects of temperatures and pH values on ADH activity, the reaction was performed using 1-propanol as substrate. To determine the influence of temperatures on the activities of the ADH activity, reactions were carried out at 10–80 °C according to the method described above. To measure the effect of pH values on ADH activity, 100 mM sodium acetate-acetic (pH 4.0–6.0), Tris-HCl buffer (pH 7.0–8.0) and glycine-sodium hydroxide buffer (pH 9.0–10.0) were used and the reaction was carried out at 25 °C.

Expression analysis of adh-1 by quantitative real-time RT-PCR

To investigate the effect of ethanol on *adh*-1 expression, about 20 000 aseptic PWN were, respectively, treated in 0.1, 0.5 and 1.0% ethanol for 24 h at 25 °C. PWN incubated in sterilized water under the same conditions were used as a control. To study the effect of *P. fluorescens* GcM5-1A on *adh*-1 expression, approximately 20 000 aseptic PWN and PWN carrying *P. fluorescens* GcM5-1A were inoculated on PDA plates growing *B. cinerea* and incubated for 7 days at 25 °C.

Gene expression of the *adh*-1 transcript in PWN was measured using qRT-PCR with forward primer p3 and reverse primer p3 (Table 1) (Xu *et al*., 2015). The PWN actin gene, a constitutively expressed gene, was selected as the internal control using forward primer p2 and reverse primer p2 (Table 1). The data were analysed using the $2^{-\triangle\triangle Ct}$ method. There were three biological replicates for this experiment.

RNA interference of adh-1 in PWN

RNA interference (RNAi) was used to identify additional functions of *adh*-1. The DNA templates (248 bp) for synthesizing double-stranded RNA (dsRNA) corresponding to *adh*-1 were amplified by PCR using pET-15b-Adh as a template with two pairs of primers: RNAi-BxADH-F/RNAi-BxADH-T7R and RNAi-BxADH-T7F/RNAi-BxADH-R (Table 1). The DNA templates (248 bp) of non-endogenous gene *gfp* (the gene coding green fluorescent protein) were amplified by PCR using pET-15b-gfp as a template with two pairs of primers: RNAi-GFP-T7R/RNAi-GFP-F and RNAi-GFP-R/RNAi-BxADH-T7F (Table 1). The dsRNA was prepared using the MEGscript RNAi Kit (Invitrogen, Vilnius, Lithuania) and an RNAi soaking method was used according to a previous study (Xu *et al*., 2015). Around 3000 aseptic nematodes were soaked in 50 μL *adh*-1 dsRNA solutions (1.0 $\mu g\ \mu L^{-1}$) and then incubated for 72 h at 20 °C. Aseptic nematodes soaked in 50 μL sterilized water and *gfp* dsRNA solutions (1.0 $\mu g\ \mu L^{-1}$) were used as double negative controls. qRT-PCR was performed to verify mRNA abundance of *adh*-1 after RNAi.

Influence of RNAi on ADH activity and alcohol tolerance of PWN

To investigate the influence of PWN after RNAi on ADH activity, about 8000 nematodes soaked in dsRNA solutions and sterilized water were collected by centrifugation at 3000 rpm for 5 min and then washed three times using sterilized water. The collected nematodes were suspended in 100 μL saline, and then ground using a grinding rod at 4 °C. The extract of PWN was centrifuged at 12 000 ***g*** for 30 min at 4 °C, and the supernatant was collected. ADH activity was assayed as described above, and the reaction was performed using ethanol as a substrate.

To further investigate the tolerance of nematodes to alcohol after RNAi, about 200 previously dsRNA-soaked PWNs were treated with 0.1, 0.5, 1.0, 2.0 and 3.0% ethanol for 24 h at 25 °C. PWNs soaked in sterilized water under the same conditions were used as a control. The survival rates of PWN were counted using a stereo microscope (SZX16, Olympus, Japan).

Influence of RNAi on vitality and reproduction of PWN

In order to understand the relationship between *adh*-1 expression and vitality of PWN, about 50 nematodes from each experimental group and control group were observed under a stereo microscope. Head thrashes per minute were used to evaluate the vitality of nematodes (Xu *et al*., 2015).

To investigate the effect of RNAi of *adh*-1 on the reproduction of PWN, 15 pairs of female and male PWNs soaked in *adh*-1 dsRNA solutions, sterilized water (control) and *gfp* dsRNA solutions (control) were picked and transferred onto a PDA plate with *B. cinerea*. After cultivation at 25 °C for 8 days, PWNs were isolated using the Baermann funnel method and counted under a stereo microscope.

Influence of fomepizole on ADH activity of PWN

The ADH inhibitor, fomepizole (Oka, China), can be used to inhibit ADH activity (Battistella, 2002). To further explore the possible biological functions of ADH in nematodes, about 3000 aseptic nematodes soaked in 0.02 mM fomepizole solution were incubated at 160 rpm for 24 h at 25 °C. PWNs soaked in sterilized distilled water were used as the control. ADH activity for ethanol with or without 0.02 mM fomepizole was assayed as described above.

The number of head thrashes per minute was used to evaluate the vitality of PWN. To study the effect of fomepizole on PWN reproduction, 15 pairs of female and male nematodes soaked in 0.02 mM fomepizole solutions were picked and transferred onto PDA plates growing *B. cinerea* at 25 °C for 9 days; nematodes treated with distilled water were used as a control. The feeding of PWN on *B. cinerea* was observed, and then PWNs were collected from PDA plates growing *B. cinerea* using the Baermann funnel method and the nematodes were counted under a stereo microscope.

Data analysis

All of the experiments were performed three times. Data of repeated experiments were represented as means ± standard deviation (S.D.). Differences between control and experimental samples were analysed using SPSS 17.0 software (SPSS, Chicago, IL, USA). The statistical significance of the control and experimental samples was performed using Student's *t*-tests. A P value <0.05 was defined as statistically significant.

Results

Differentially expressed genes analysis of aseptic PWN and aseptic PWN carrying P. fluorescens GcM5-1A

Pseudomonas fluorescens GcM5-1A could significantly affect the gene expression of aseptic PWNs. Transcriptome analysis indicated that 178 DEGs were obtained in PWNs carrying *P. fluorescens* GcM5-1A compared with aseptic PWNs, which included 47 upregulated genes and 35 downregulated genes with at least a 2-fold change at the expression level. A total of 68 DEGs were annotated using NCBI database, and the top significantly 20 up and downregulated genes from 68 annotated DEGs based on the fold change were described in Table 2. The gene *adh*-1 encoding ADH was one of the genes in PWN which was upregulated by the association of *P. fluorescens* GcM5-1A.

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Table 2. Top 20 up and downregulated genes in PWNs carrying *P. fluorescens* GcM5-1A based on the fold change

Genes	Description	Fold change	*q*-value	Genes	Description	Fold change	*q*-value
vap-1↑	Venom allergen-like protein 1	5.15	0.02	LOC106670128↓	Fatty-acid amide hydrolase 2-A-like	−5.42	0.02
grl-9↑	GRound-Like (grd related)	4.46	0.02	SRAE2000138100↓	Rac GTPase-activating protein 1	−4.97	0.02
cyp-33C9↑	Cytochrome P450-33C9	4.44	0.02	SRAE1000223500↓	Chitin binding domain protein	−4.92	0.02
LOC103491921↑	Cucumis melo CWF19-like	4.05	0.02	*nhr*-28↓	Nuclear hormone receptor	−4.64	0.02
LOC111268644↑	Phytanoyl-CoA dioxygenase	3.88	0.02	LOC108375683↓	Serine/threonine-protein kinase tousled-like 2	−4.56	0.02
Phyh↑	Protein PHYH	3.88	0.02	*cre-hsp*-70↓	CRE-HSP-70 protein	−4.37	0.02
cp↑	Cysteine proteinase	3.78	0.02	LOC108552010↓	Polyubiquitin-A isoform X3	−4.07	0.02
Bm104380↑	Transthyretin-like family protein	3.75	0.02	LOC105338232↓	Acetyl-CoA carboxylase	−3.91	0.02
dhrs-4↑	Dehydrogenase/ reductase SDR family member 4	3.68	0.02	*sir*-2.1↓	NAD-dependent protein deacetylase sir-2.1	−3.87	0.02
lys-2↑	Protein LYS-2	3.67	0.04	SRAE1000112300↓	Transcription factor IIIB 90 kDa subunit	−3.64	0.02
LOC100631470↑	Pyruvate dehydrogenase E1 component subunit beta	3.33	0.02	*vig*-1↓	VIG (Drosophila Vasa Intronic Gene) orthologue	−3.43	0.02
NECAME15558↑	Transthyretin-like family protein	3.29	0.02	LOC111682366↓	Enhancer of rudimentary homologue	−3.40	0.02
gsts-1↑	Glutathione *S*-transferase-1	3.25	0.02	*sft*-4↓	Protein SFT-4	−3.27	0.04
cyp-42A1↑	Cytochrome P450-42A1	3.16	0.04	LOC107450227↓	Serine/threonine-protein kinase PLK1-like	−3.24	0.04
LOAG07633↑	Oxidoreductase	3.09	0.02	SRAE0000031300↓	Elongation factor 1-beta	−3.11	0.02
Gpx↑	Glutathione peroxidases	3.07	0.02	*ima*-3↓	Importin subunit α-3	−3.02	0.04
ap↑	Aspartic protease	3.02	0.02	*mog*-5↓	ATP-dependent RNA helicase mog-5	−2.99	0.02
ttr-46↑	Transthyretin-like protein 46	3.01	0.02	LOAG05446↓	Elongation factor 2	−2.90	0.02
adh-1↑	Alcohol dehydrogenase-1	2.65	0.02	PCSK2↓	Neuroendocrine convertase 2	−2.73	0.02
hsp-21↑	Small HSP21-like protein	2.58	0.02	LOC102531939↓	40S ribosomal protein SA-like	−2.71	0.02

Up arrows were defined as upregulated genes. Down arrows were defined as downregulated genes. The significant DEGs listed had *q*-values $\leqslant 0.05$

Cloning and sequencing of the gene encoding ADH from PWN

The coding sequence of *adh*-1 was amplified by PCR using cDNA reverse-transcribed from mRNA of PWN. Sequence analysis showed that the PCR product contained a 1059 bp open reading frame (ORF) which encoded a protein of 352 amino acids with a relative molecular mass of 39 kDa (GenBank accession no: KY670592) (Fig. 1A). Protein BLAST showed that the deduced amino acid sequence of *adh*-1 exhibited a relatively high level of identity with the ADH proteins of *C. elegans* (GenBank accession no: NP_505991) and *C. briggsae* (GenBank accession no: XM_002636504), with identities of 55 and 54%, respectively (Fig. 1B). The ADH of PWN also shared 11% identity with the protein from *Drosophila melanogaster* (GenBank accession no: NM_001032095). The theoretical pI of ADH from PWN was 8.65. Results from SignalP 4.1 and TMHMM server 2.0 showed that the deduced amino acid sequences of *adh*-1 did not contain a signal peptide sequence or transmembrane regions. Alignment of the ORF with the genomic sequences of PWN revealed that PWN *adh*-1 of PWN contained three exons of 291, 189 and 579 bp and two introns of 92 and 206 bp. Sequences of the introns corresponded to the 'GT-AG rule' for *cis*-splicing (Fig. 1C).

Expression and purification of the recombinant ADH

To study the characteristics of ADH of PWN, the ORF of *adh*-1 was cloned into vector pET-15b to construct pET-15b-Adh. The recombinant ADH was overexpressed in *E. coli* BL21 (DE3) harbouring pET-15b-Adh by IPTG induction at 28 °C. SDS-PAGE analysis revealed that the relative molecular mass of recombinant

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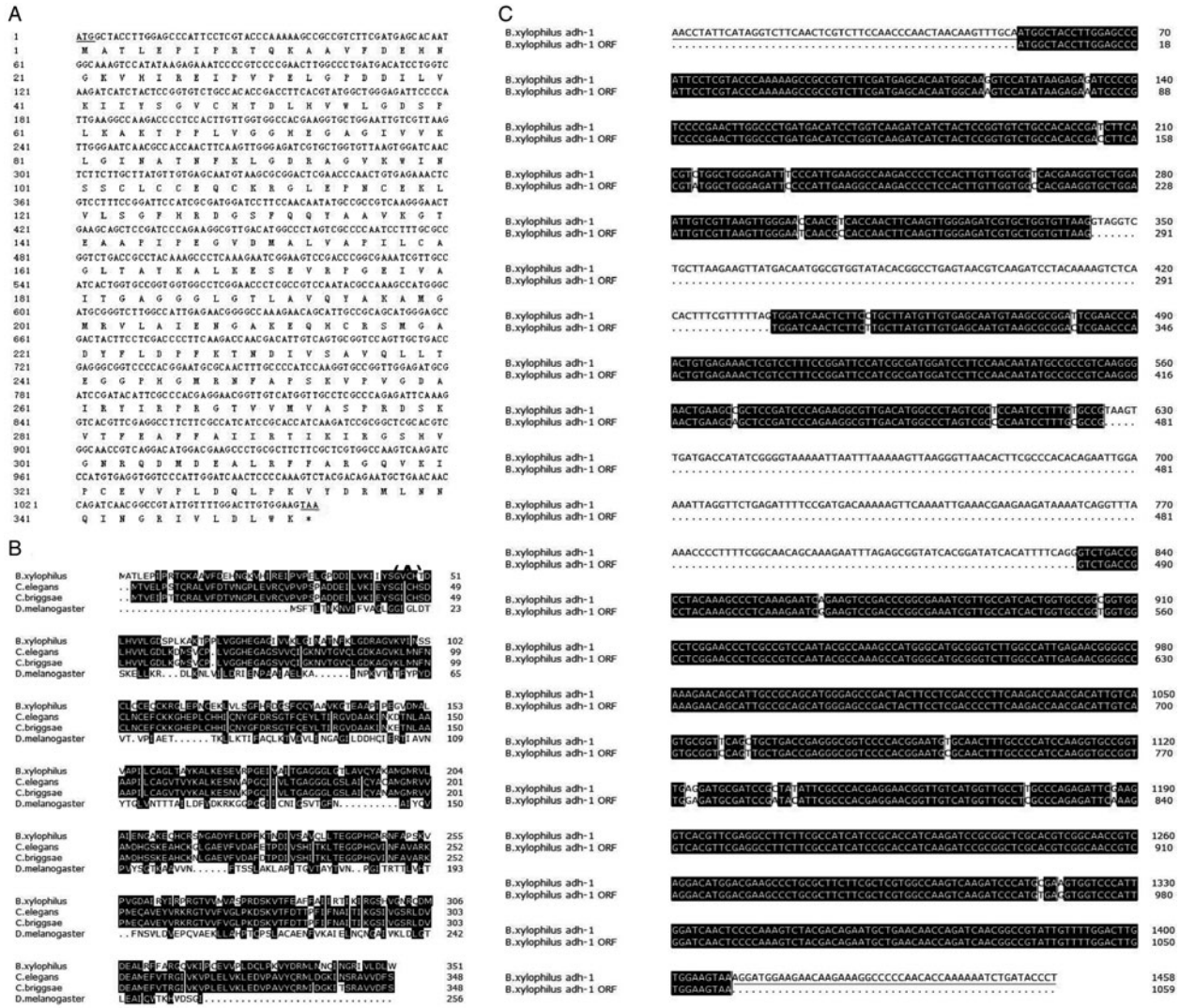

Fig. 1. Open reading frame, amino acid sequence and gene structures of *adh*-1 from *B. xylophilus*. (A) Nucleotide and deduced amino acid sequence of *adh*-1 from *B. xylophilus*. The initial codon is marked by underlining and the stop codon is indicated by an asterisk. (B) Alignment of the amino acid sequences of ADH from *B. xylophilus*, *C. elegans*, *C. briggsae* and *D. melanogaster*. Letters in black boxes indicate conserved amino acid residues. (C) Structural features of *adh*-1 from *B. xylophilus*. 5′/3′ UTRs are marked by underlining. Letters in black boxes and open boxes indicate exons and introns, respectively.

protein with a His-tag at its N-terminus was approximately 39 kDa (Fig. 2A), which conformed to the predicted molecular size.

The recombinant ADH appeared mainly in inclusion bodies which could be refolded by stepwise dilution with refolding buffer (Fig. 2A). The re-natured recombinant ADH with a His-tag was further purified using Ni^{2+} affinity chromatography, and the homogeneity of ADH was verified by SDS-PAGE (Fig. 2A).

Characteristics of the recombinant ADH

The refolded recombinant ADH was used to measure dehydrogenase activity using NAD^+ as a cofactor, and methanol, ethanol, 1-propanol, 1-butanol, isoamylol, sorbitol, D-mannitol and D-pinitol as substrates. The results showed that the recombinant ADH of PWN could catalyse the dehydrogen reaction of the eight tested substrates, and the activity with 1-propanol was higher than with the other substrates (Fig. 2B).

Temperature and pH values had obvious influences on the activity of the recombinant ADH. The activity of ADH towards 1-propanol as a substrate increased with increasing temperature under 30 °C, and decreased quickly when reaction temperature reached 40 °C. The optimal temperature for the recombinant ADH of PWN was 30 °C (Fig. 2C). The recombinant ADH showed relatively good catalytic activity to 1-propanol over a broad pH scale from 7.0 to 9.0, and the optimal pH was 8.0 (Fig. 2D).

Induced expression of adh-1 by ethanol and P. fluorescens *GcM5-1A*

To investigate the effect of ethanol on *adh*-1 gene expression in PWN, qRT-PCR was performed. The result revealed that ethanol induced the expression of the *adh*-1 gene. Compared with PWN in sterilized water, the *adh*-1 expression level was upregulated 1.35-, 1.76- and 2.07-fold when PWN were treated with 0.1, 0.5 and 1.0% ethanol, respectively (Fig. 3A). This result suggested that the *adh*-1 gene had a close relationship with ethanol metabolism in PWN.

To analyse the roles of *adh*-1 in association with *P. fluorescens* GcM5-1A to PWN, *adh*-1 gene expression of PWN was evaluated with qRT-PCR. Results showed that the expression level of *adh*-1 of PWN increased dramatically when aseptic PWN were subjected to inoculation with *P. fluorescens* GcM5-1A, and *adh*-1 gene expression level was upregulated 2.65-fold compared with aseptic nematodes (Fig. 3B). This result indicated that ADH of

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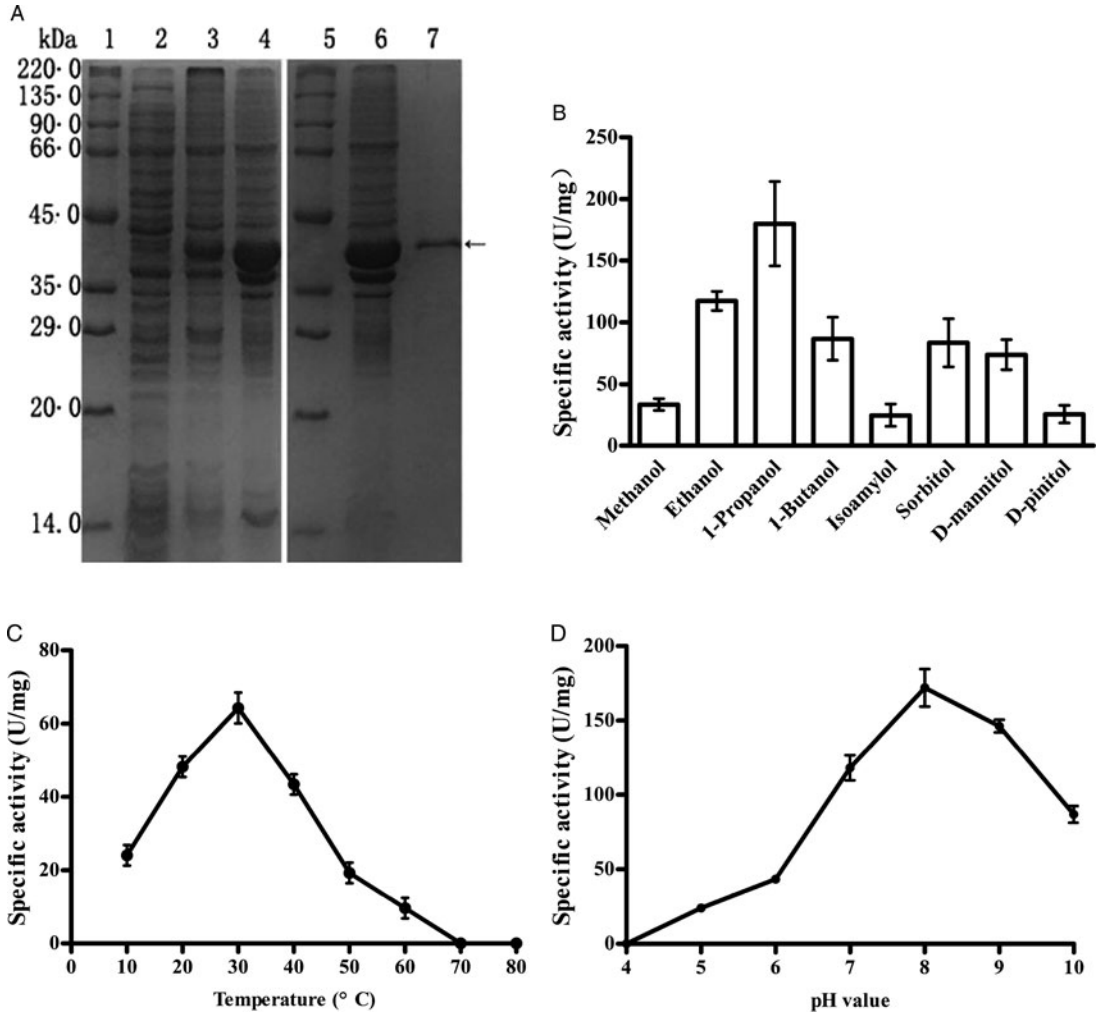

Fig. 2. Purification and characterization of the recombinant ADH. (A) SDS-PAGE analysis of the expression and purification of recombinant ADH. 1,5, Standard proteins; 2, total proteins of *E. coli* BL21 (DE3); 3, total proteins of *E. coli* BL21 (DE3) harbouring pET-15b-Adh; 4, inclusion bodies; 6, washed inclusion bodies; 7, purified recombinant ADH. (B) Relative activities of recombinant ADH on eight substrates. (C, D) Effects of temperatures and pH values on the activity of recombinant ADH.

PWN might participate in regulating the interaction between PWN and its associated bacteria.

Regulation of ADH activity and tolerance to ethanol of PWN using RNAi

The effect of RNAi on *adh*-1 expression level was evaluated by qRT-PCR, and the 248 bp dsRNA sequences that contained conservative regions of *adh*-1 were used as interfering RNA. The expression of *adh*-1 of PWN soaked in *adh*-1 dsRNA solutions decreased significantly compared with PWN soaked in sterilized water (Fig. 3C). When the mRNA level of *adh*-1 in the control was 1.00, the level in PWN treated with RNAi was only 0.06. This result showed that the expression of *adh*-1 in PWN could be strongly inhibited by RNAi. The results of qRT-PCR also showed that the *gfp* dsRNA has no significant effects on the expression level of *adh*-1 of PWN compared with sterile water.

Compared with PWNs soaked in sterilized water, the specific activity of ADH in RNAi nematodes decreased significantly, to only 32% of that in control (Fig. 3D). This result indicated that ADH activity in RNAi nematodes was reduced.

The RNAi PWNs were treated with ethanol of different concentrations, and their tolerances to ethanol were investigated and compared with that of PWNs in sterilized water. The assay result revealed that mortalities of PWNs increased with increasing ethanol concentrations. The survival rate of RNAi PWNs significantly decreased compared with that of PWNs treated with distilled water (Fig. 3E), which indicated that *adh*-1 might be involved in the tolerance of PWN to ethanol.

Influence of vitality and reproduction ability of PWN by RNAi

To further analyse the influence of *adh*-1 gene on the behaviour of PWN, the RNAi PWNs were observed under a stereo microscope and their head thrashes were counted. Compared with PWNs in sterilized water and *gfp* dsRNA solutions, the RNAi nematodes moved much slower. The head thrashes of RNAi PWNs and those in sterilized water and *gfp* dsRNA solutions were 22, 51 and 50 thrashes min^{-1}, respectively (Fig. 3F), which indicated that the vitality of PWNs was significantly reduced by downregulation of *adh*-1 expression. In addition, the results showed that there was no significant effect of non-endogenous genes (*gfp*) dsRNA on the vitality of PWN (Fig. 3F).

To investigate the effect of RNAi on PWN population growth, nematodes were inoculated on *B. cinerea* and cultured, and subsequently re-isolated and counted. The results showed that the population of RNAi PWN was significantly reduced compared with the nematodes in sterilized water and *gfp* dsRNA solutions, with populations of 6348, 17 450 and 16 981, respectively (Fig. 3G). Furthermore, there was no obvious difference for effects

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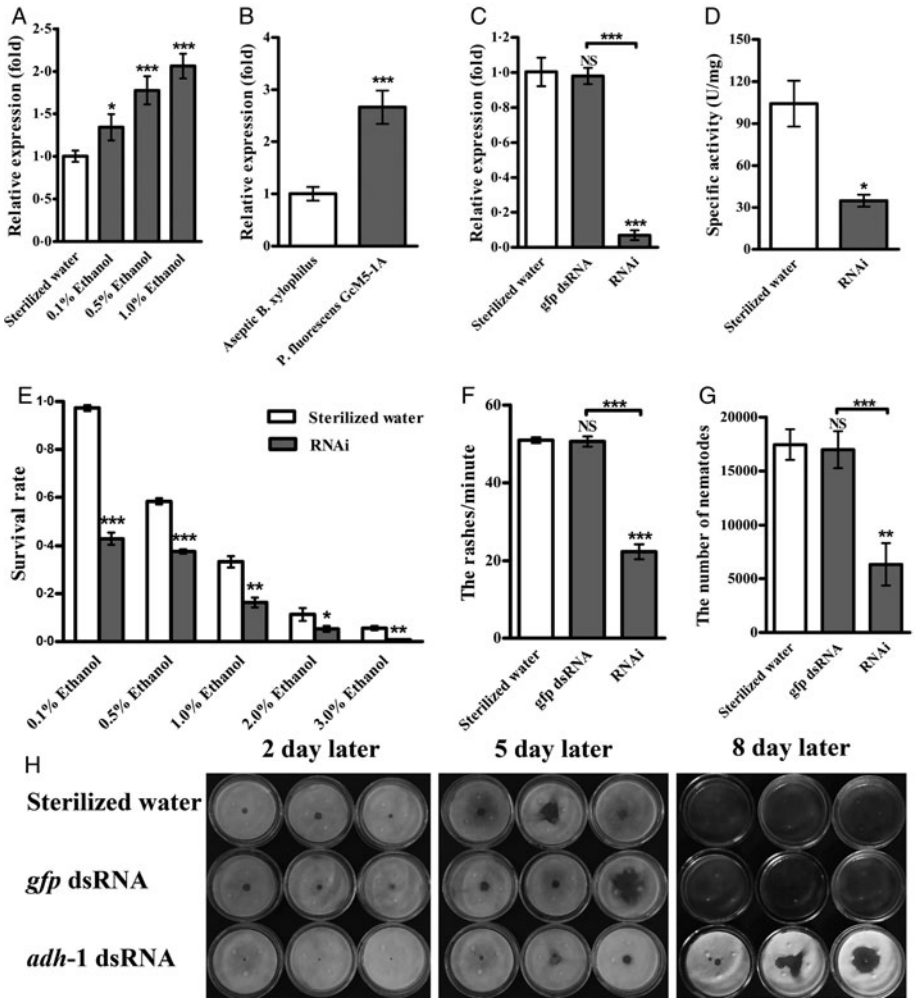

Fig. 3. Expression and function analysis of *adh*-1 of *B. xylophilus*. (A, B) Expression of *adh*-1 regulated by ethanol and associated *P. fluorescens* GcM5-1A. (C, D) RNAi analysis by qRT-PCR and measurement of ADH activity of RNAi *B. xylophilus*. (E, F, G) Tolerance to ethanol, vitality and reproduction ability of *B. xylophilus* with *adh*-1 downregulated by RNAi. (H) The feeding of *B. cinerea* by RNAi-treated PWNs. *Bursaphelenchus xylophilus* soaked in sterilized water and *gfp* dsRNA solutions were used as double negative controls. Asterisks on top of the bars indicate statistically significant differences (*$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$), and NS shows no significant difference.

of *gfp* dsRNA and sterile water on the reproduction of PWN (Fig. 3G). The feeding rate of *B. cinerea* for RNAi-treated PWN was obviously slower than that of PWN in sterilized water and *gfp* dsRNA solutions. Almost all of the hyphae on the plates had been consumed by PWNs in the double negative control groups at 8 days, while only a small portion of the hyphae was consumed by RNAi-treated PWNs (Fig. 4H). Compared with PWN in sterilized water, there was no significant difference for effects of *gfp* dsRNA on the feeding of PWN (Fig. 4H).

Influence of fomepizole on PWN

To further verify the functions of ADH in PWN, the ADH inhibitor fomepizole was used to assess ADH activity *in vivo* and thereby the influences on vitality and reproduction of PWN. The ADH activity of PWN pretreated with fomepizole solution was only 31% of that of PWN in control, which indicated that fomepizole effectively inhibited ADH of PWN *in vivo* (Fig. 4A).

Fomepizole-treated PWN moved more slowly than control nematodes. The number of head thrashes per minute for fomepizole-treated PWN was 16 compared with 46 for that in the control (Fig. 4B), which showed that the ADH inhibitor fomepizole significantly reduced the vitality of PWN.

To investigate the influence of fomepizole on PWN population growth, PWNs treated with fomepizole and distilled water were inoculated on *B. cinerea* and cultured for 9 days. The population of fomepizole-treated PWN was significantly reduced compared with that of PWN treated with distilled water, to only 16% of that in the control group (Fig. 4C). The feeding rate of *B. cinerea* for fomepizole-treated PWN was obviously slower than that of PWN in the control group. Almost all of the hyphae on the plates were consumed by PWN in the control, while only a small portion of the hyphae was consumed by fomepizole-treated PWN (Fig. 4D).

Discussion

PWD is a complex and destructive disease to most pine species and virulence of the PWN has a close relationship to the spread of PWD. PWN isolates from different resources show different abilities to damage pines (Mota *et al.*, 2006), probably due to genome-wide variations (Palomaresrius *et al.*, 2015). Many pathogenesis-related genes of PWN, such as the pectate lyase gene (Qiu *et al.*, 2016), dumpy genes (Wang *et al.*, 2016) and cytochrome P450 genes (Xu *et al.*, 2015), were verified to participate in cell wall degradation of many pine species, and to growth and development processes of PWN. Numerous novel detoxification proteins genes, such as the glutathione *S*-transferases gene (Espada *et al.*, 2016*b*) and effector genes (Espada *et al.*, 2016*a*), are important for mediating interactions of PWN with its host. In addition, some studies suggested that the bacteria associated with PWN also probably due to pathogenicity (Zhao *et al.*, 2011), and bacteria benefited PWN by supplying nutrition or protecting PWN from oxidation stress (Vicente *et al.*, 2013; Zhao *et al.*, 2014). However, the exact molecular mechanism of PWN pathogenicity has not been fully elucidated.

In this paper, we reported an *adh*-1 gene encoding ADH from PWN and a method for preparation of the recombinant ADH. A

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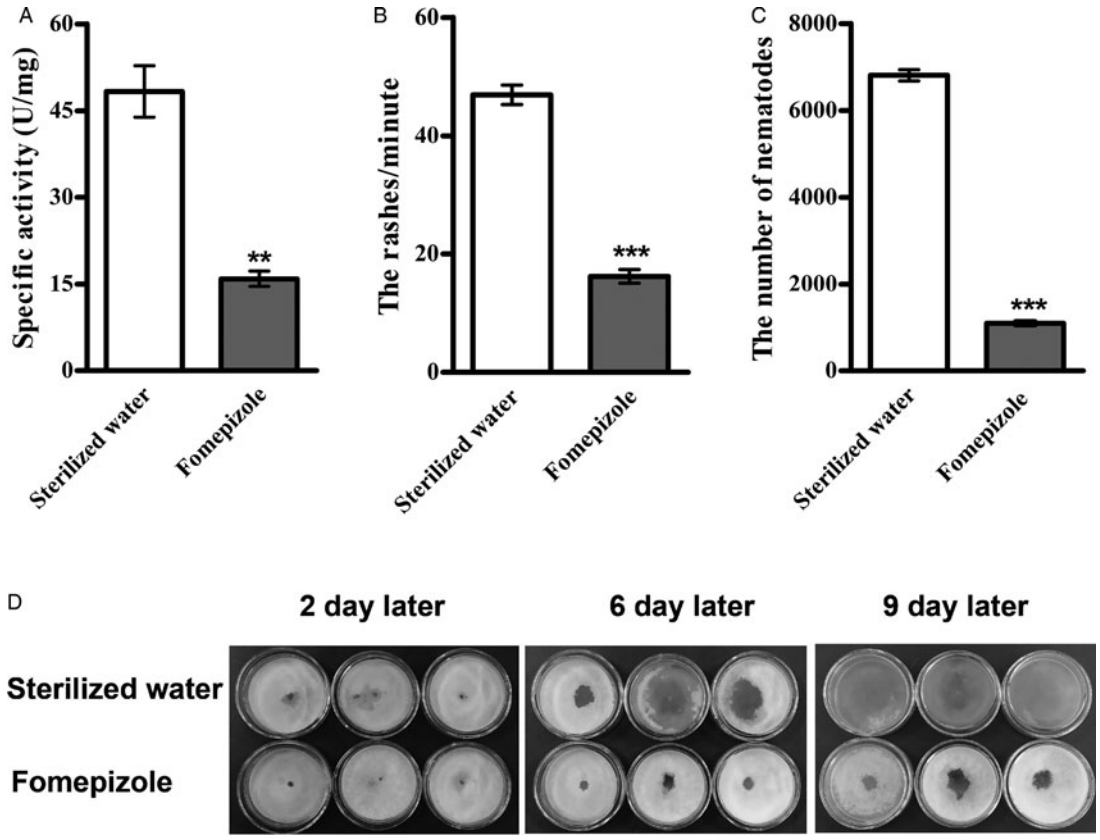

Fig. 4. Influence of fomepizole on *B. xylophilus*. (A, B, C) ADH activity, vitality and reproduction ability of *B. xylophilus* treated with fomepizole. (D) The feeding of *B. cinerea* by *B. xylophilus* treated with fomepizole. *B. xylophilus* soaked in sterilized water were used as a control. Asterisks on top of the bars indicate statistically significant differences (*$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$).

biochemical assay indicated that the recombinant ADH had a broad range of substrate specificity, which could catalyse the oxidation of eight substrates including ethanol. Interestingly, the recombinant ADH displayed obvious activity with D-pinitol, a chemical component in pine trees such as *Pinus taeda* (Pullman and Buchanan, 2008). D-pinitol was reported to be selected first as a carbohydrate resource for growth by *Leptographium procerum* associated with red turpentine beetle. D-pinitol benefited development of the beetle larvae by avoiding competition for glucose, and this adaption was regulated by bacteria carried by this insect (Zhou *et al.*, 2016). D-pinitol was also reported to be an oviposition stimulant for the common grass yellow butterfly, *Eurema mandarina* (Mukae *et al.*, 2016). Whether D-pinitol could be utilized by PWN through ADH to stimulate oviposition is worth further study.

PWN infection causes pines to release a large amount of ethanol and other volatile compounds, which was especially obvious on day 10 when oleoresin leakage stopped (Ikeda and Oda, 1980). Similarly, the population of PWN starts to increase explosively 2 weeks after infection (Kiyohara and Tokushige, 1971), which means that there might be a close relationship between the alcohols in volatile compounds produced by nematode-infected pines and PWN reproduction. There have been some reports showing that alcohols are related to nematode reproduction (Lu *et al.*, 1978; Oku, 1988; Shuto and Watanabe, 1988). However, the mechanism of how alcohols promote reproduction of PWN is not clear. The results of RNAi of *adh*-1 and fomepizole tests in this study indicated that ADH encoded by *adh*-1 was associated with PWN reproduction. Alcohols might participate in regulating reproduction of PWN through ADH. In addition, our RNAi results indicated that *adh*-1 was involved in tolerance of PWN to ethanol, which might help us understand how PWN deals with alcohols in pine hosts.

Ethanol has been reported to have obvious influences on the behaviour of *C. elegans*. Ethanol could dose-dependently cause an acute depression of the nematode crawling on an agar surface, but the nematode could partially recover its locomotion speed despite the continued presence of the drug (Davies *et al.*, 2015). Starvation induces collective behaviour in L1 larvae of *C. elegans*. This aggregation requires a small amount of ethanol or acetate in the environment and requires functional ADH encoded by gene *adh*-1 (Artyukhin *et al.*, 2015). In this study, we found that the expression of *adh*-1 could be enhanced by ethanol treatment and inhibited by RNAi of *adh*-1. The vitality of PWN was significantly reduced after interference of *adh*-1 expression or if ADH was inhibited by fomepizole. This result means that *adh*-1 might be a key gene regulating the vitality behaviour of PWN. Furthermore, *adh*-1 in aseptic PWN was upregulated by re-association with *P. fluorescens* GcM5-1A, a bacterial strain carried by wild PWN. Previous studies showed that *P. fluorescens* GcM5-1A increased egg production, developmental rate, body length and diameter of PWN (Zhao *et al.*, 2007). Other bacteria associated with PWN were also reported to be related to PWN virulence, and PWN isolates with different virulence possessed different bacteria and diverse carbon metabolism (Wu *et al.*, 2013). Therefore, we hypothesize that *adh*-1 of PWN probably plays some roles in the interaction of PWN with its associated bacteria, and even with PWN virulence. However, the mechanism needs to be further investigated.

Data

Nucleotide sequence data reported in this paper is available in GenBank (Accession no: KY670592).

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Conflict of interest. None.

Ethical standards. Not applicable.

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