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Full Length Research Paper

Comparative analysis of two acetylcholinesterase genes of *Bombyx mandarina* and *Bombyx mori*

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Acetylcholinesterase (AChE), which contains two subfamilies, *ace*1 and *ace*2 in insects, was identified to be the target of organophosphorous and carbamate insecticides. Resistance to insecticides is apparently different between *Bombyx mori* and *Bombyx mandarina.* To compare the sequences and tissue expressions of the two *ace*s between the two species, cDNAs encoding two *ace* genes were cloned and designated as *Bmm-ace*1 and *Bmm-ace*2 from the larvae of the *B. mandarina*. The amino acid sequence of *Bmm-ace*1 shared 99.71% homology with its homolog, *Bm-ace*1, in *B. mori*, with two mutations (G664S and S307P) and the amino acid sequence of *Bmm-ace*2 shared 99.37% homology with *Bm-ace*2, in *Bombyx mori*, with four mutations (M18I, N233S, I310V and G621S). Analysis of tissue expression showed that *ace*1 genes of the two species were highly expressed only in brain tissues and fat bodies, while *ace*2 genes were expressed in all tissues tested; the expression level of *Bmm-ace*1 and *Bm-ace*1 in brain tissue was almost the same, while the expression level of *Bmm-ace*2 was 4.17 fold as high as that of *Bmm-ace*2 in brain tissue. The results indicated that *ace* gene mutations and the difference in the expression level of *ace*2 were speculated to be the molecular basis for the difference in sensitivity to organophosphate insecticides between *B. mori* and *B. mandarina.* This was the first experimental report in which the *ace*2 gene was closely related to insecticide resistance in silkworm.

Key words: *Bombyx mori*, *Bombyx mandarina*, acetylcholinesterase gene, expression, insect, resistance.

INTRODUCTION

Acetylcholinesterase (AchE[2] EC 3.1.1.7) encoded by acetylcholinesterase gene (*ace*) can terminate neurotransmission in the postsynaptic membrane by hydrolysis of the neurotransmitter, acetylcholine (ACh) (Fournier and Mutero, 1994). Organophosphate insecticide is currently one of the major insecticides used in farming, and AChE was one of the principal targets of these insecticides. Organophosphate and carbamate insecticides inhibited AChE, resulting in the accumulation of ACh in the postsynaptic membrane, and then excess ACh caused desensitization of AChR, inducing the confusion of ACh signaling (Voss and Matsumura, 1964).

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Abbreviations: Ach, Acetylcholine; **AchE,** acetylcholinesterase; **AChR,** acetylcholine receptor; **RT-PCR,** reverse transcription-polymerase chain reaction.

Insect AChE gene was firstly cloned from *Drosophila melanogaster* (Hall and Spierer, 1986). Alterations in the structure of AChE were the main reasons for its insensitivity to organophosphate insecticides. According to researches into resistant strains of *D. melanogaster*, there were five mutations in AChE including F115S, I119V, I119T, G303A and F368Y, each of which could cause AChE insensitivity to organophosphate insecticides, thus increasing mutants' resistance (Mutero et al., 1994). Moreover, based on researches into resistant strains of *Bactrocera oleae* (Vontas et al., 2002), *Anopheles gambiae* (Weill et al., 2003) and *Aedes aegypti* (Ffrench-Constant et al., 1998), mutations in AChE, namely, G488S, G119S and G105S were found, respectively.

Lepidopteran insects are classified as one of the major pests. However, there has been no reliable evidence concerning the relationship between mutations in AChE and insecticide resistance in lepidopteran insects other than the mutation found in the *ace*1 of *Plutella xylostella* (Baek et al., 2005).

The domesticated silkworm, *B. mori*, which has been used as a source of silk, had lost some characteristics because of long-term breeding in artificial conditions. The wild silkworm, *B. mandarina*, is very similar to *B. mori* in morphological and physiological characteristics (Astaurov et al., 1959; Xia et al., 2004; Yoshitake, 1968). From the close relationship between the two species, it was generally believed that *B. mandarina* was the original type of the domesticated silkworm *B. mori* (Banno et al., 2004). Due to long-term natural selection, there was a difference in resistance to insecticides between the two species (Shen et al., 2003). *Bombyx mori* had a weak resistance to insecticide, and its production was reduced by more than 30% annually because of insecticide poisoning. On the other hand, being one of the major pests in mulberry fields, *B. mandarina* showed increasing resistance to insecticides owing to its wide use. Recently, the cDNAs of two acetylcholinesterase genes in *B. mori* were cloned and analyzed (Seino et al., 2007; Shang et al., 2007). There are presently no reports regarding the *ace* genes of *B. mandarina*. In order to explore the mechanisms under-lying the difference in resistance to organophosphate insecticide between *B. mori* and *B. mandarina* in this study, full-length cDNAs of *Bmm-ace*1 and *Bmm-ace*2 *of B. mandarina* were cloned and then compared with their homologs, *Bm-ace*1 and *Bm-ace*2*,* respectively. Furthermore, expression of the genes in different tissues of *B. mori* and *B. mandarina* was also studied.

MATERIALS AND METHODS

Insects

The larvae of *B. mori* (Dazao strain) and *B. mandarina* (Suzhou strain), maintained in our laboratory, were reared on mulberry leaves under a 12 h light/dark photoperiods. 20 g of fresh mulberry leaves were soaked in a solution containing 2.5 µg/mL phoxim for 1 min. After drying in air, the leaves were used to rear the larvae of *B. mori* and *B. mandarina* (10 larvae with 5 males and 5 females in each group).

Chemicals

T_4 DNA Ligase plasmid extraction Kit and gel extraction Kit were the products of Shanghai Shenergy Biocolor Bioscience and Technology Company. DNA molecular weight Marker, restriction enzymes, reaction buffers and other routine chemical reagents were all purchased from TAKARA Biotechonolgy (Dalian) Co., Ltd. Primers were synthesized by Shanghai Sangon Biological Techonology and Services Co., Ltd. The reagent, phoxim was purchased from Sigma-Aldrich Company.

Extraction of total RNA and RT-PCR

The larvae of *B. mori* and *Bombyx mandarina* on the third day of the 5th instar were dissected, and their respective hemolymph, brain tissue, midgut, fat bodies and silk gland selected. Total RNAs was extracted from these tissues, respectively, by using TRIzol according to the manufacturer's instructions (TAKARA Biotechonolgy (Dalian) Co., Ltd), and then stored at -70°C. RT-PCR was carried out by using M-MLV RTase cDNA Synthesis Kit according to the manufacturer's instructions (TAKARA Biotechonolgy (Dalian) Co., Ltd). Primers, Type P1 (5'-TTG TGG GTG TAG GTG CCA GCG ACG GTA T-3') and Type M1 (5'- ACT TAT ATG GTG TAT TTG AAC AGT GCT GTG CCT GTA-3'), were designed according to sequences 26 bp upstream and 2029 bp downstream of ATG of *ace*1 of *B. mori* (GenBank Accession No. DQ186605), respec-tively, and PCR cycling conditions were as follows: 94°C for 3 min; 35 cycles of 98°C for 20 s, 68°C for 3 min 30 s; and a final extension at 72°C for 10 min. Primers, *ace*2 P2 (5'- GAA TCA CAA TGA TCA ACT ACG GCA AGA TT-3') and *ace*2 M2 (5'- TAC AAA GCA ATA GTG ATT GCC AAA GTG GTG-3'), were designed according to sequences 8 bp upstream and 1869 bp downstream of *ace*2 of *B. mori* (Accession No. DQ115792), respectively, and PCR cycling conditions were as follows: 94°C for 3 min; 35 cycles of 94°C for 35 s, 63°C for 40 s, 72°C for 150 s; and a final extension at 72°C for 10 min. Using the cDNA of the brain tissue of *B. mandarina* as template, RT-PCR was carried out.

The RACE for full length cDNA cloning

According to the sequencing result of *Bmm-ace*1*,* the following primers for 5' RACE were designed at the 5' of *Bmm-ace*1: Ache1-RT: 5'- (P) TCG CTC GTG ATT AG -3'; AChE1-S1: 5'- ACC AAG ACT CGA AGA CCA CG -3'; AChE1-A1: 5'- CGT GGT GCC TCG CCC GGT GC -3'; AChE1-S2: 5'- GAG AAC CAA GTA TGA GGA GAG -3'; AChe1-A2: 5'- GCT CGT GCG GAC CGG CAA GG -3'. According to the sequencing result of *Bmm-ace*2*,* the following primers for 5' RACE were designed at the 5' of *Bmm-ace*2: AChe2-RT: 5'- (P) TTC GCA AAC GGG AT -3'; AChe2-S1: 5'- CGG TCT CAT CAA AGG ATA CGC -3'; AChe2A1: 5'- GTA GTT TGT GTT GTA GAT GTT GTG G -3'; AChe2-S2: 5'- ACT GTA ATG GGA CGC GAG GT -3'; AChe2-A2: 5'- CAA AAG TAC CGG ATA TGA GC -3'. 5'-RACE was performed according to the instructions of TAKARA 5'-Full RACE Core Set.

The 3' anchored oligo-dT primer used in the synthesis of the first-strand cDNA in 3' RACE was 3′Race-dT (5'- ACG CTA CAC GAC TCA CTA ATG GGC T_{12} N -3'), and anchored primer was 3'Race-M (5'- ACG CTA CAC GAC TCA CTA ATG GGC TT -3'). For *Bmm-ace*1, the gene specific primer in the first round was None3 race1 (5'- CAG CAA ACG CTT AAT GAG ATA TTG GGC -3') and the nested specific primer in the second round was None3 race2 (5'- TAA TGG CTG CTA CCA ATA AAC CAG AGC -3'). For *Bmm-ace*2, the gene specific primer in the first round was the 3' race 1 (5'- TCT GGG GAG AAT GGA TGG GTG T -3') and the nested specific primer was the 3' race 2 (5'- CCT CCC TGT AAC CCT TCT CAC CAC -3').

Expression analysis of the genes in tissues

The following primers for *Actin*-3 were designed: *Actin*-3 P1 (5'-AAC ACC CCG TCC TGC TCA CTG -3') and *Actin*-3 P2 (5'- GGG CGA GAC GTG TGA TTT CCT -3'). In accordance with the conserved sequences of *ace*1 and *ace*2 of *B. mori* and *B mandarina* and two genomic sequences of *Bombyx mori* (DQ186606 and DQ115793), the following primers across introns were designed: 814 ace P1 (5'- CCG ACG GAT ATT TGA ACC A -3'), 814 ace P2 (5'- GTG TAG TAA TGA GGC GAA GAC C -3'), 814 ace1P1 (5'-ATG GTC GGA GAC TAT CAT TTC ACT -3') and 814 ace1 P2 (5'-GCG GCT CTG GTT TAT TGG T -3'). The cDNAs of hemolymph, brain tissue, midgut, fat body and silk gland of *B. mori* and *B. mandarina* were used as templates and normalized by *Actin*-3 gene and then the expression of *ace*1 and *ace*2 was studied in the tissues of *B. mori* and *B. mandarina* PCR cycling conditions for

for *Actin*-3 were as follows: 94°C for 2 min; 22 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 30 s; and a final extension at 72°C for 10 min. PCR cycling conditions for *ace*1 were as follows: 94°C for 2 min; 28 cycles of 94°C for 30 sec, 52.9°C for 30 s, 72°C for 30 s; and a final extension at 72°C for 10 min. PCR cycling conditions for *ace*2 were as follows: 94°C for 2 min; 28 cycles of 94°C for 30 s, 51.1°C for 30 s, 72°C for 30 s; and a final extension at 72°C for 10 min.

Sequence analysis

Sequences were analyzed by using online software (http://www.ncbi.nlm.nih.gov/blast, http://bio-soft.net/sms/and http://npsa-pbil.ibcp.fr), and a phylogenic tree was constructed by using MEGA 4.0. The amino acid sequences were used for alignment available in the GenBank: *B. mandarina ace*2 (EF166089), *B. mori ace*2 (EU328262), *Helicoverpa armigera ace*2 (AY142325), *Cydia pomonella ace*2 (DQ267976), *Plutella xylostella ace*2 (AY061975), *Helicoverpa assulta ace*2 (AY817736), *Nephotettix cincticeps ace*1 (AY256851), *Plutella xylostella ace*1 (AY970293), *Helicoverpa armigera ace*1 (DQ064790), *B. mandarina ace*1 (EF190220), *B. mori ace*1 (EU328261), *Cydia pomonella ace*1 (DQ267977), *Blattella germanica ace*1 (DQ288249), *Chilo suppressalis ace*1 (EF453724) and *Helicoverpa assulta ace*1 (DQ001323).

RESULTS

Cloning of full-length *Bmm-ace*1 and *Bmm-ace*2 cDNAs

With the total RNA of brain tissue of *B. mandarina* as template, two fragments with lengths of 2078 and 1917 bp were amplified by RT-PCR, respectively. Two fragments of 336 and 333 bp for the 5' UTR of the genes *Bmm-ace*1 and *Bmm-ace*2 containing the overlapping sequence were amplified in 5' RACE. In the same way, the fragments with 144 bp *ace*1 and 225 bp *ace*2 containing the overlapping sequence were obtained in 3'RACE. The full-length fragments of the genes of *Bmm-ace*1 and *Bmm-ace*2 were obtained, as shown in Figures 1 and 2. The *Bmm-ace*1 gene contains a 2052 bp ORF, 231 and 140 bp of 5' and 3'UTR, respectively, while the *Bmm-ace*2 gene contains a 1917 bp ORF, 333 and 225 bp of 5' and 3'UTR.

Comparison of amino acid sequences encoded by *Bmm-ace*1 and *Bm-ace*1 (EU328261) showed that there were 681 identical amino acid residues out of 683, with 2 mutations including S307P and G664S. By comparing *Bmm-ace*2 and *Bm-ace*2 (EU328262) amino acid sequences, the four mutations including M18I, N233S, I310V and G621S634 were found in the amino acid residues.

Gene expression in tissues

By RT-PCR assay, the results showed that both *Bm-ace*1 and *Bmm-ace*1 were highly expressed in the brain and fat body. However, the former was with minor expression while the latter without detectable expressions in the midgut. Both *Bm-ace*2 and *Bmm-ace*2 were expressed in the 5 tissues tested with a high expression in the brain and fat body; the expression level of *ace*1 in the brain was the same between *B. mori* and *B. mandarina*, while the expression level of *ace*2 in the brain of *B. mandarina* was 4.17 folds as high as that of *B. mori* (Figure 3).

The expression of the genes after stimulation of the reagent, phoxim

After stimulation by the reagent, phoxim in fat bodies, expressions of both *ace*1 and *ace*2 decreased apparently (Figures 4 and 5). The expression of *ace*1 and *ace*2 of *B. mandarina* decreased by 82.67 and 30.56 %, while that of *Bombyx mori* by 81.11 and 84.50 %, respec-tively. In hemolymph, the expression of *Bmm-ace*2 increased by 1.8 folds. The expression of *Bm-ace*1 in-creased by 1.28 folds in the midgut. No changes in expression of both genes were observed in other tissues of both insects. The results indicated that *Bmm-ace*2 might be more effective in insecticide resistance.

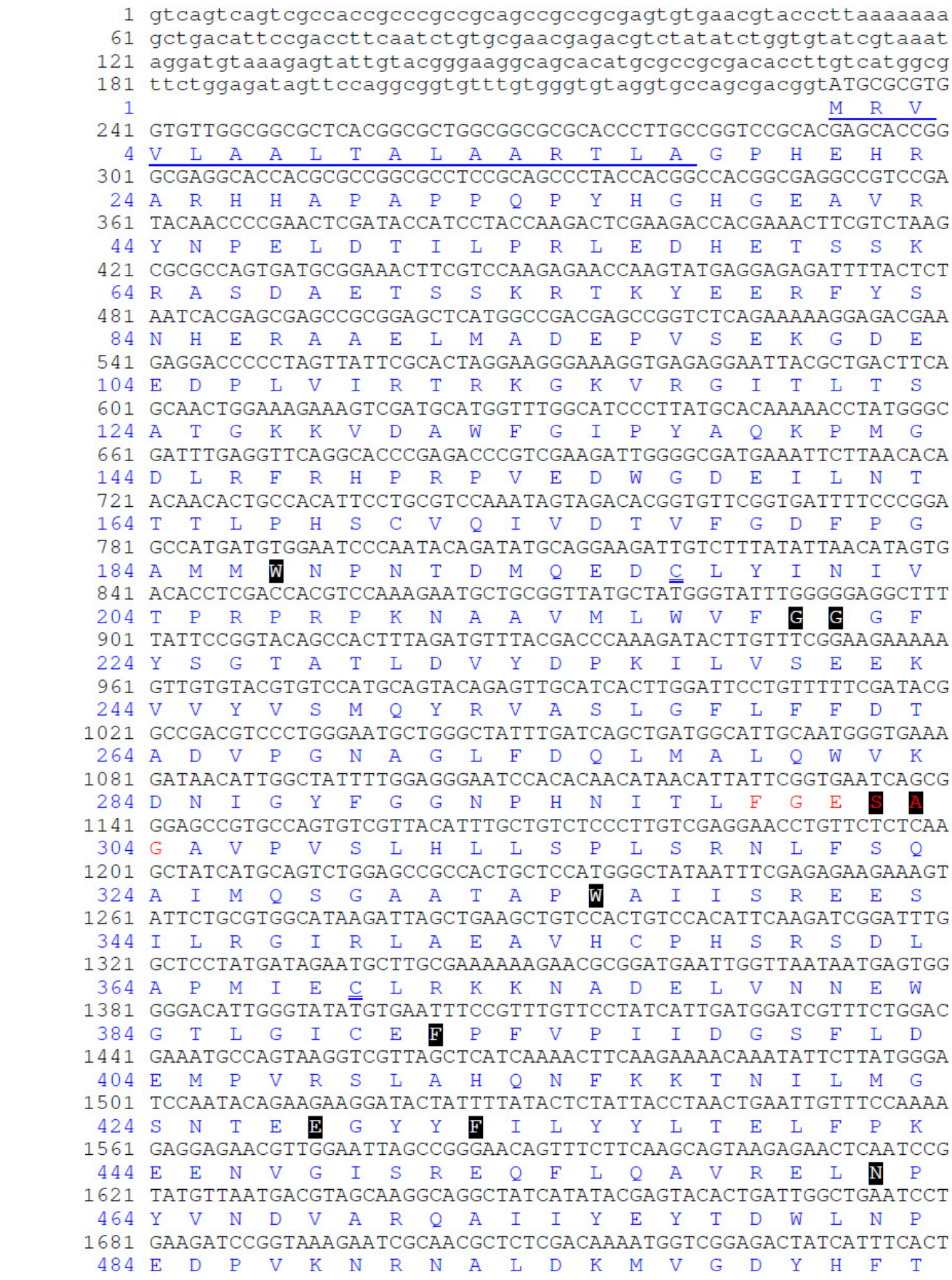
Phylogenetic analysis

Phylogenetic analysis showed that *Bmm-ace*1 and *Bmm-ace*2 had the closest genetic relationship with *Bm-ace*1 and *Bm-ace*2, respectively, with an amino acid identity of 99.71 and 99.37%, respectively (Figure 6). *Bmm-ace*1 and *Bmm-ace*2 showed only 30.82% identity between each other. Compared with *ace*1, *ace*2 was more evolutionally conserved. These results indicated that *ace*2 might be closely related to insecticide resistance.

DISCUSSION

B. mandarina was long regarded as a pest in mulberry fields. However, with the development of research into functional genomics of *B. mori* in recent years, importance had been attached to *B. mandarina* in that genes of *B. mandarina* were used to compare their homologs in *B. mori*. In this study, we firstly obtained full-length sequences of two *ace* genes from *B. mandarina* by using RACE, according to the homology genes in other lepidopteran insects (Seino et al., 2007; Hall and Spierer, 1986; Gao and Zhu, 2002).

Compared with their respective homologs, *Bm-ace*1 and *Bm-ace*2, the genes of *Bmm-ace*1 and *Bmm-ace*2 cloned in this study had some corresponding amino acid mutations. Two mutations (G664S and S307P) occurred in the *ace*1 gene, and four mutations (M18I, N233S, I310V and G621S) occurred in the *ace*2 gene. Specifically, two mutations (G-to-S and I-to-V) of the *ace*2 gene might be closely related to organophosphate insecticide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121 aggatgtaaagagtattgtacgggaaggcagcacatgcgccgcgacaccttgtcatggcg
181 ttctggagatagttccaggcggtgtttgtgggtgtaggtgccagcgacggtATGCGCGTG
1 M R V
241 GTGTTGGCGGCGCTCACGGCGCTGGCGGCGCGCACCCTTGCCGGTCCGCACGAGCACCGG
4 V L A A L T A L A A R T L A G P H E H R
301 GCGAGGCACCACGCGCCGGCGCCTCCGCAGCCCTACCACGGCCACGGCGAGGCCGTCCGA
24 A R H H A P A P P Q P Y H G H G E A V R
361 TACAACCCCGAACTCGATACCATCCTACCAAGACTCGAAGACCACGAAACTTCGTCTAAG
44 Y N P E L D T I L P R L E D H E T S S K
421 CGCGCCAGTGATGCGGAAACTTCGTCCAAGAGAACCAAGTATGAGGAGAGATTTTACTCT
64 R A S D A E T S S K R T K Y E E R F Y S
481 AATCACGAGCGAGCCGCGGAGCTCATGGCCGACGAGCCGGTCTCAGAAAAAGGAGACGAA
84 N H E R A A E L M A D E P V S E K G D E
541 GAGGACCCCCTAGTTATTCGCACTAGGAAGGGAAAGGTGAGAGGAATTACGCTGACTTCA
104 E D P L V I R T R K G K V R G I T L T S
601 GCAACTGGAAAGAAAGTCGATGCATGGTTTGGCATCCCTTATGCACAAAAACCTATGGGC
124 A T G K K V D A W F G I P Y A Q K P M G
661 GATTTGAGGTTCAGGCACCCGAGACCCGTCGAAGATTGGGGCGATGAAATTCTTAACACA
144 D L R F R H P R P V E D W G D E I L N T
721 ACAACACTGCCACATTCCTGCGTCCAAATAGTAGACACGGTGTTCGGTGATTTTCCCGGA
164 T T L P H S C V Q I V D T V F G D F P G
781 GCCATGATGTGGAATCCCAATACAGATATGCAGGAAGATTGTCTTTATATTAACATAGTG
184 A M M W N P N T D M Q E D C L Y I N I V
841 ACACCTCGACCACGTCCAAAGAATGCTGCGGTTATGCTATGGGTATTTGGGGGAGGCTTT
204 T P R P R P K N A A V M L W V F G G G F
901 TATTCCGGTACAGCCACTTTAGATGTTTACGACCCAAAGATACTTGTTTCGGAAGAAAAA
224 Y S G T A T L D V Y D P K I L V S E E K
961 GTTGTGTACGTGTCCATGCAGTACAGAGTTGCATCACTTGGATTCCTGTTTTTCGATACG
244 V V Y V S M Q Y R V A S L G F L F F D T
1021 GCCGACGTCCCTGGGAATGCTGGGCTATTTGATCAGCTGATGGCATTGCAATGGGTGAAA
264 A D V P G N A G L F D Q L M A L Q W V K
1081 GATAACATTGGCTATTTTGGAGGGAATCCACACAACATAACATTATTCGGTGAATCAGCG
284 D N I G Y F G G N P H N I T L F G E S A
1141 GGAGCCGTGCCAGTGTCGTTACATTTGCTGTCTCCCTTGTCGAGGAACCTGTTCTCTCAA
304 G A V P V S L H L L S P L S R N L F S Q
1201 GCTATCATGCAGTCTGGAGCCGCCACTGCTCCATGGGCTATAATTTCGAGAGAAGAAAGT
324 A I M Q S G A A T A P W A I I S R E E S
1261 ATTCTGCGTGGCATAAGATTAGCTGAAGCTGTCCACTGTCCACATTCAAGATCGGATTTG
344 I L R G I R L A E A V H C P H S R S D L
1321 GCTCCTATGATAGAATGCTTGCGAAAAAAGAACGCGGATGAATTGGTTAATAATGAGTGG
364 A P M I E C L R K K N A D E L V N N E W
1381 GGGACATTGGGTATATGTGAATTTCCGTTTGTTCCTATCATTGATGGATCGTTTCTGGAC
384 G T L G I C E F P F V P I I D G S F L D
1441 GAAATGCCAGTAAGGTCGTTAGCTCATCAAAACTTCAAGAAAACAAATATTCTTATGGGA
404 E M P V R S L A H Q N F K K T N I L M G
1501 TCCAATACAGAAGAAGGATACTATTTTATACTCTATTACCTAACTGAATTGTTTCCAAAA
424 S N T E E G Y Y F I L Y Y L T E L F P K
1561 GAGGAGAACGTTGGAATTAGCCGGGAACAGTTTCTTCAAGCAGTAAGAGAACTCAATCCG
444 E E N V G I S R E Q F L Q A V R E L N P
1621 TATGTTAATGACGTAGCAAGGCAGGCTATCATATACGAGTACACTGATTGGCTGAATCCT
464 Y V N D V A R Q A I I Y E Y T D W L N P
1681 GAAGATCCGGTAAAGAATCGCAACGCTCTCGACAAAATGGTCGGAGACTATCATTTCACT
484 E D P V K N R N A L D K M V G D Y H F T

Figure 1. Continued.

Figure 1. The full-length *ace*1 cDNA from *B. mandarina* and the deduced amino acid sequence.The residues of precursor are numbered at the right from the first methionine. N-terminal underlined amino acids indicate putative signal peptides; C-terminal underlined amino acids indicate hydrophobic amino acid tails; the blackened residues indicate characteristic amino acids including the following motifs: catalytic triad, acel pocket, anionic subsite, and oxianion holes; the double-underlined Cys residues indicate the interfaces for intra- and intermolecular disulfide bonds; the shaded amino acids indicate putative ω-sites; the stop codon was indicated with asterisk.

resistance as mentioned in the introduction.

Previous study has shown that there were splicing pattern differences between *Bmm-ace1* and *Bm-ace*1 (Li et al., 2008). However, the results of tissue expression demonstrated that the expression level of *Bmm-ace*1 was the same as that of *Bm-ace*1 in brain tissues, indicating that different splicing patterns had no impact on the expression level of *ace*1 gene.

The results of tissue expression also showed that while *Bm-ace*1 was expressed in the midgut tissue, *Bmm-ace*1 was not expressed in the same tissue. Furthermore, organophosphate insecticide is a stomach poison that can inhibit AChE in the midgut. Accordingly, it was speculated that AChE produced by the expression of *Bm-ace*1 in the midgut tissue caused the sensitivity of *B. mori* to insecticides, and that the failure of *B. mandarina* to produce AChE in the midgut resulted in its insensitivity to insecticides. However, this speculation should be verified by comparing the differences in organophosphate insecticide metabolism in the midgut between *B. mori* and *B. mandarina*.

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   1 gactcagtctacactgcttcgttggtggagacgtaactcgcgagtcgcgctctccgccct
  61 cagtactttatagtgtacaaatcgcgagtgaacgtgtgaggcttcattcgaacttaatgt
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   1                                   M  I  N  Y  G  K  I  V  F
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  10 T  K  L  L  L  C  V  L  I  S  G  T  F  A  R  S  W  A  N  H
 421 CATGATACCACAACATCTACAACACAAACTACACCAACGACAAGTCCGGTACCAAAAAAT
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  70 T  V  M  G  R  E  V  H  I  F  T  G  I  P  F  A  K  P  P  L
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  90 G  P  L  R  F  R  K  P  V  P  I  E  P  W  H  G  V  L  E  A
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 230 A  P  G  S  M  G  L  W  D  Q  Q  L  A  I  R  W  I  K  E  N
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 270 G  S  V  S  L  H  M  L  S  P  E  M  K  G  L  F  K  R  G  I
1201 TTGCAATCAGGAACGTTGAATGCACCTTGGAGTTGGATGACTGGAGAAAGAGCTCAAGAT
 290 L  Q  S  G  T  L  N  A  P  W  S  W  M  T  G  E  R  A  Q  D
1261 GTTGGAAAAGTATTAATTGATGACTGTAACTGCAACAGTAGTCTTTTAGCCAAAGATCCT
 310 V  G  K  V  L  I  D  D  C  N  C  N  S  S  L  L  A  K  D  P
1321 AGTCTCGTAATGGATTGCATGCGCGGAGTTGACGCTAAAACGATTTCTGTTCAGCAATGG
 330 S  L  V  M  D  C  M  R  G  V  D  A  K  T  I  S  V  Q  Q  W
1381 AATTCTTATACTGGAATTTTGGGTTTTCCGTCCGCACCTACGGTTGATGGTATTTTTTTG
 350 N  S  Y  T  G  I  L  G  F  P  S  A  P  T  V  D  G  I  F  L
1441 CCAAAAGATCCTGATACCATGATGAAGGAAGGAAATTTCCATAATAGTGAAGTGCTACTT
 370 P  K  D  P  D  T  M  M  K  E  G  N  F  H  N  S  E  V  L  L
1501 GGCAGTAACCAAGACGAAGGGACATATTTTTTGCTGTACGACTTCCTGGATTATTTCGAA
 390 G  S  N  Q  D  E  G  T  Y  F  L  L  Y  D  F  L  D  Y  F  E
1561 AAGGATGGGCCTAGTTTTCTTCAGAGGGAGAAATTTCTCGAAATCGTTGACACTATTTTC
 410 K  D  G  P  S  F  L  Q  R  E  K  F  L  E  I  V  D  T  I  F
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Figure 2. Continued.

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1621 AAGGACTTTTCTAAAATTAAGAGAGAAGCCATTGTGTTCCAGTATACAGATTGGGAAGAG
 430 K  D  F  S  K  I  K  R  E  A  I  V  F  Q  Y  T  D  W  E  E
1681 ATCACCGACGGATATTTGAACCAGAAGATGATAGCTGATGTCGTAGGCGACTACTTCTTC
 450 I  T  D  G  Y  L  N  Q  K  M  I  A  D  V  V  G  D  Y  F  F
1741 GTATGCCCCACTAACTACTTCGCCGAAATACTTGCCGACGCCGGTGTCGATGTTTACTAT
 470 V  C  P  T  N  Y  F  A  E  I  L  A  D  A  G  V  D  V  Y  Y
1801 TACTATTTTACTCATCGTACCAGCACAAGTCTCTGGGGAGAATGGATGGGTGTGATGCAT
 490 Y  Y  F  T  H  R  T  S  T  S  L  W  G  E  W  M  G  V  M  H
1861 GGTGACGAAATGGAATATGTTTTTGGACATCCCTTGAACATGTCCCTTCAGTACCATTCC
 510 G  D  E  M  E  Y  V  F  G  H  P  L  N  M  S  L  Q  Y  H  S
1921 CGGGAGCGTGATTTAGCAGCACACATTATGCAGTCTTTCACACAGTTTGCTCTTACCGGA
 530 R  E  R  D  L  A  A  H  I  M  Q  S  F  T  Q  F  A  L  T  G
1981 AAACCTCACAAACCTGACGAGAAGTGGCCTCTGTACTCCCGGTCTTCGCCTCATTACTAC
 550 K  P  H  K  P  D  E  K  W  P  L  Y  S  R  S  S  P  H  Y  Y
2041 ACATACACGGCAGTGGGTCCAAGCGGTCCAGCCGGACCCCGCGGCCCGCGTGCCTCCGCT
 570 T  Y  T  A  V  G  P  S  G  P  A  G  P  R  G  P  R  A  S  A
2101 TGCGCTTTCTGGAACGATTTCTTGAACAAACTTAACGAGTTGGAGCGTGTACCGTGTGAC
 590 C  A  F  W  N  D  F  L  N  K  L  N  E  L  E  R  V  P  C  D
2161 GGCGCCGTGACCGGTCCTTACAGCAGTGTCGCCAGCACTGCCCTCCCTGTAACCCTTCTC
 610 G  A  V  T  G  P  Y  S  S  V  A  S  T  A  L  P  V  T  L  L
2221 ACCACTTTGGCAATCACTATTGCTTTGTAAattttaaattaaaaataatgtagtcttgtc
 630 T  T  L  A  I  T  I  A  L  *
2281 cgcgcttcgaagtgaaaaggactattaaagtgaataataacggctgtatgtgtgtagacg
2341 ttgatattagaattatttcttaatttagtaacattagagacattgcatatcgaaaagggt
2401 atagaaatttggtaggatctgaagaagaaattggacaatgtaatggaatgttggtgtaag
2461 tgaaaaaaaaaaaaa
```

Figure 2. The full-length *ace2* cDNA from *B. mandarina* and the deduced amino acid sequence. The details are identical to Figure 1.

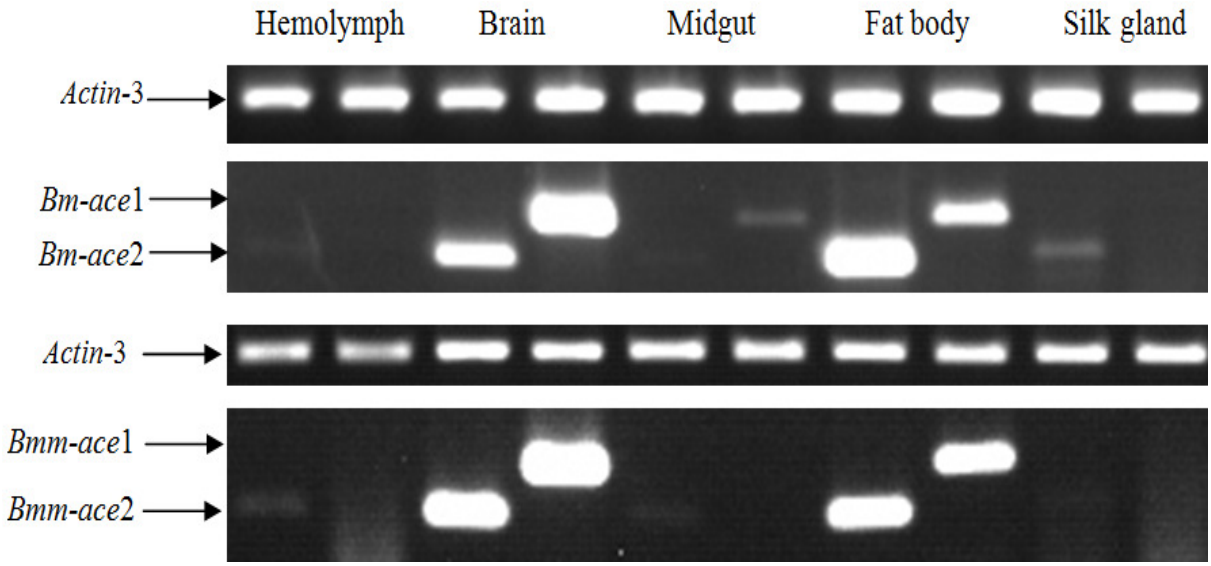

Figure 3. Expression of the *ace* genes in silkworm tissues by RT-PCR assay. Both the genes of *Bm-ace*1 and *Bmm-ace*1 were only highly expressed in the brain and fat body. The genes of *Bm-ace*2 and *Bmm-ace*2 were expressed in the five tissues tested with high expression in the brain and fat body. The expression level of *ace*1 in the brain was the same between *B. mori* and *B. mandarina* and the expression level of *ace*2 in the brain of *B. mandarina* was 4.17 folds as high as that of *B. mori* .

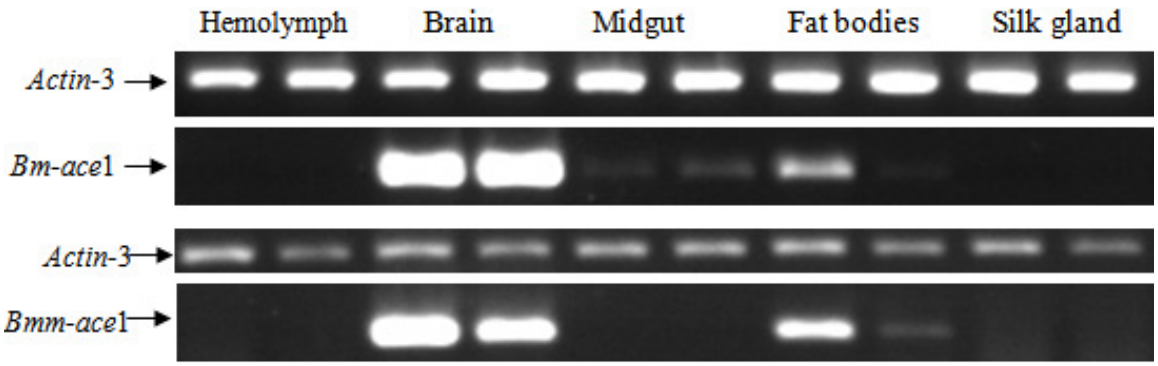

Figure 4. Expression analysis of the genes *Bmm-ace*1 and *Bm-ace*1 in larva tissues induced by the reagent phoxim. The lanes 1, 3, 5, 7 and 9 indicate no induction while the lanes 2, 4, 6, 8 and 10 indicate induced blood, brain, midgut, fat body and silk gland, respectively.

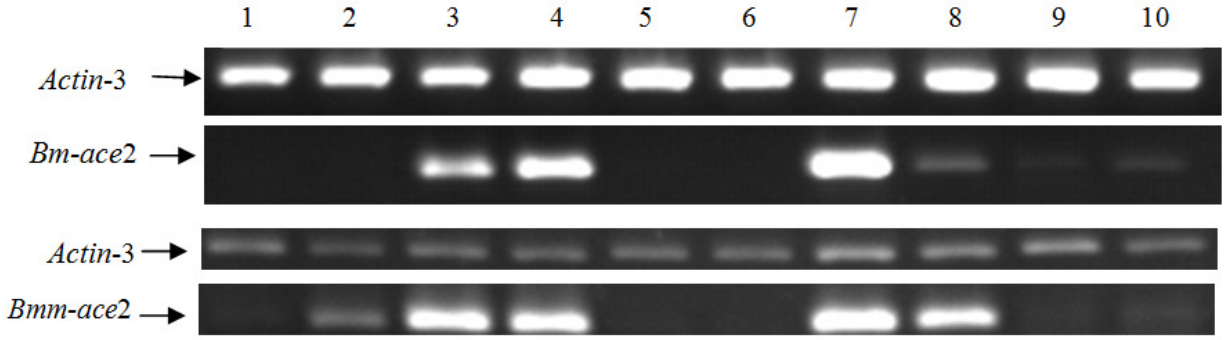

Figure 5. Expression analysis of the genes *Bmm-ace*2 and *Bm-ace*2 in larva tissues induced by the reagent phoxim. The details are identical to Figure 4.

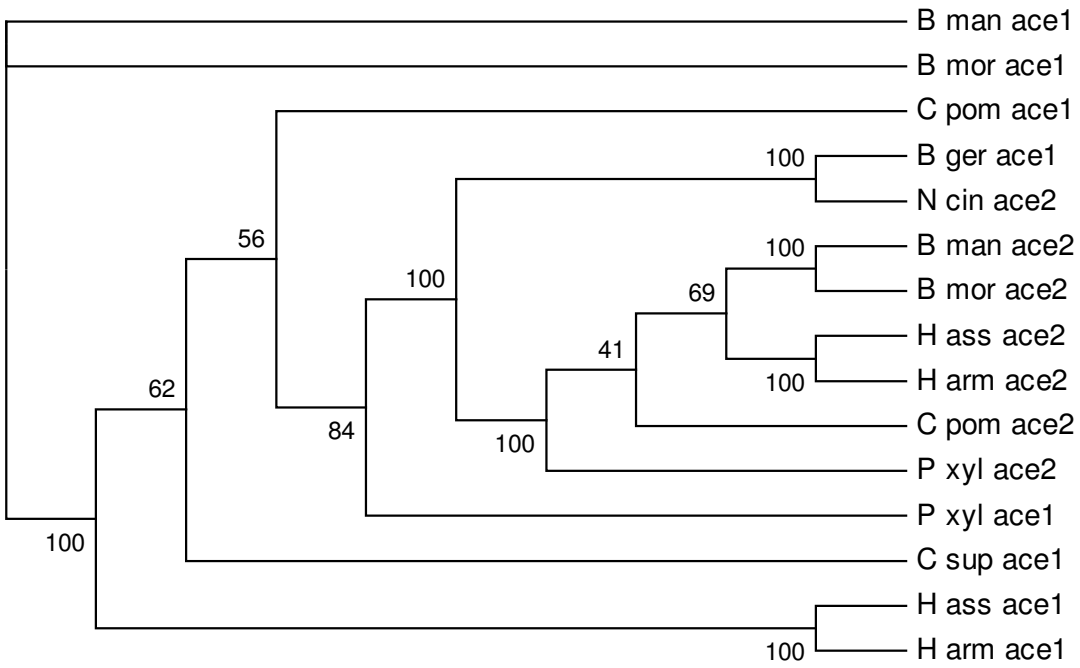

Figure 6. Unrooted phylogenic tree of the insect *ace* genes constructed by the neighbor-joining method.

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