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Research

Molecular Characterization and Expression Analysis of Two Acetylcholinesterase Genes From the Small White Butterfly *Pieris rapae* (Lepidoptera: Pieridae)

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Abstract

Acetylcholinesterases (AChEs) are essential for the hydrolysis of the neurotransmitter acetylcholine and play crucial roles in the termination of neurotransmission. AChEs are encoded by the *ace* genes. However, the *ace* genes from the small white butterfly, *Pieris rapae* (L.) (Lepidoptera: Pieridae), remained uncharacterized. In this study, two *ace*s (*Prace1* and *Prace2*) were identified from *P. rapae*. *Prace1* encoded a PrAChE1 protein consisting of 694 amino acid residues, and *Prace2* encoded the 638-amino-acid PrAChE2. The two identified PrAChEs both had features typical of AChEs, including the catalytic triad, choline-binding sites, an oxyanion hole, an acyl pocket, a peripheral anionic subsite, an FGESAG motif and 14 conserved aromatic amino acids. Phylogenetic analysis showed that *Prace1* and *Prace2* were clustered into two distinct groups: *ace1* and *ace2*, respectively. The two *Prace*s were distributed on different genomic scaffolds: *Prace1* on scaffold 156 and *Prace2* on scaffold 430. Additionally, *Prace1* consisted of three exons and two introns, whereas *Prace2* consisted of six exons and five introns. One amino acid mutation (Gly324Ala) in PrAChE1 and two (Ser291Gly and Ser431Phe) in PrAChE2 were consistent with mutations in other insect AChEs that are associated with insecticide insensitivity. Both *Prace1* and *Prace2* were highly expressed at the fifth-instar larval stage and in the larval head, and the transcriptional levels of *Prace1* were significantly higher than those of *Prace2* in all of the tested life stages and tissues. This is the first report characterizing two *ace* genes in *P. rapae*. The results pave the way for functional study of these genes.

Key words: *Pieris rapae*, AChE, *ace* gene, expression profile, target site insensitivity

Acetylcholinesterase (AChE; EC 3.1.1.7) is a key enzyme required for the hydrolysis of the neurotransmitter acetylcholine (ACh) and plays an essential role in the control of synaptic transmission in all animals (Kim and Lee 2018). AChE is encoded by the *ace* gene (Kim and Lee 2018). In the class Insecta, two types of *ace* genes (*ace1* and *ace2*) have been identified in numerous insect species from various orders, including mosquitoes (Weill et al. 2002, 2004), moths (Shang et al. 2007, Hui et al. 2011), butterflies (Li et al. 2015), aphids (Li and Han 2002, Nabeshima et al. 2003), planthoppers (Kwon et al. 2012, Li et al. 2012), honeybees (Kim et al. 2012b), beetles (Revuelta et al. 2011, Wang et al. 2017a), cockroaches (Kim et al. 2006, 2010), and bugs (Hwang et al. 2014, Liu et al. 2017a). However, in the fruit fly *Drosophila melanogaster* Meigen (Diptera: Drosophilidae), the house fly *Musca domestica* L. (Diptera: Muscidae) , and other fly species in the Cyclorrhapha suborder of Diptera, only a single *ace2* gene has been identified (Weill et al. 2002, Huchard et al. 2006). The absence of *ace1* in these species is probably due to gene loss (Huchard et al. 2006).

Many studies have demonstrated that the two types of *ace* genes have different expression profiles in most insect species. In *Helicoverpa assulta* (Guenée) (Lepidoptera: Noctuidae) (Lee et al. 2006), *Blattella germanica* L. (Blattodea: Blattellidae) (Kim et al. 2006), and *Nilaparvata lugens* (Stål) (Hemiptera: Delphacidae) (Li et al. 2012), expression levels of the *ace1* gene were much higher than those of *ace2* in all tested body parts. In these insect species, the *ace1* gene is expected to encode a main catalytic enzyme. Conversely, in the silk moth *Bombyx mori* (L.) (Lepidoptera: Bombycidae), *ace2* is expressed at a significantly higher level than *ace1*, and AChE2 is thought to be the main catalytic enzyme (Chen et al. 2009). Kim and Lee (2013) examined the AChE activities in 100 insect species and found that AChE1 was the main catalytic enzyme in 67 species, whereas AChE2 acts as the main catalytic enzyme in the remaining 33 species. Recently, the RNA interference (RNAi) technique was used to study the physiological functions of the *ace* genes in vivo. In *B. germanica* and *Tribolium castaneum* (Herbst) (Coleoptera:

Tenebrionidae), knockdown of *ace1* significantly increased the sensitivity of individuals to AChE insecticides (Revuelta et al. 2009, Lu et al. 2012b), while silencing of *ace2* delayed insect development and reduced egg laying and hatching (Lu et al. 2012b). Furthermore, in *B. mori* and *Plutella xylostella* (L.) (Lepidoptera: Plutellidae), silencing both *ace1* and *ace2* genes impacted larval motor ability and development, and even resulted in death (He et al. 2012, Ye et al. 2017). These results showed that the *ace1* and/or *ace2* genes have non-neuronal functions.

Insect AChEs are molecular targets of organophosphate and carbamate insecticides (Casida and Durkin 2013). These insecticides bind to the serine residue located on the active center of AChEs, inhibiting the ACh hydrolyzing activity (Casida and Durkin 2013). In those insect species that have both *ace1* and *ace2* genes, point mutations in *ace1* often cause amino acid substitutions in AChE1, and this is the main mechanism for resistance to organophosphate and carbamate insecticides (Lee et al. 2015). For example, an A216S substitution in AChE1 increases chlorpyrifos resistance in *Apolygus lucorum* (Meyer-Dür) (Wu et al. 2015), and a G119S mutation in several mosquito species leads to insecticide insensitivity (Weill et al. 2004). Besides these single mutations, multiple mutations in *ace1* have also been shown to increase insecticide resistance. For instance, three mutations (G119S, F331C, and I332L) in AChE1 confer resistance to chlorpyrifos in *N. lugens* (Zhang et al. 2017), and four substitutions (G119A, F/Y330S, F331H, and H332L) in the same insect species are associated with reduced sensitivity to carbofuran (Kwon et al. 2012).

The small white butterfly, *Pieris rapae* (L.) (Lepidoptera: Pieridae), is a serious insect pest of cultivated crucifers (Shen et al. 2016). In different geographic regions of China, various organophosphate and carbamate insecticides have been widely used to control this species (Wang et al. 2017b). However, excessive spraying of insecticide leads to resistance; several insecticides, including dichlorvos and malathion, have become ineffective in the control of *P. rapae*, even at relatively high doses (Li et al. 1991, Peng et al. 1996). Point mutations in the *ace* genes may be associated with insecticide insensitivity in this species. However, to date, little information has been published on the *ace* genes in *P. rapae*. In this study, we identified two *ace* genes (*Prace1* and *Prace2*) by searching a previously released *P. rapae* transcriptome (Qi et al. 2016). The phylogenies, gene structures and expression patterns of the *Prace* genes, as well as some amino acid substitutions in the PrAChE proteins, were analyzed. The results pave the way for a better understanding of the physiological functions of the two genes, which may be useful in combating insecticide resistance.

Materials and Methods

Insect Rearing and Sample Collection

Male and female adults of *P. rapae* were captured from an experimental cabbage (*Brassica pekinensis*) field in Anhui Agricultural University, Hefei, Anhui, China in May 2017. The butterflies were transported to the laboratory and kept in mating cages (75 × 75 × 75 cm; each cage had 10 males and 10 females). They were provided daily a 10% (v/v) honey solution. After mating, the females laid eggs on cabbage leaves, which were placed into the cages beforehand. The hatched first-instar larvae were reared with cabbage leaves until pupation. The pupae were kept separately in glass tubes for emergence. The rearing conditions were 25 ± 1°C, 65% RH, and a 16:8 (L:D) h photoperiod (Liu et al. 2017a).

P. rapae were collected at different developmental stages, including second- to fifth-instar larvae, pupae, and adult males and females. At each stage, 30 individuals were sampled. We also dissected different tissues from 40 fourth-instar larvae. These tissues included the head, ventral nerve cord, midgut, and integument. All of the samples were frozen immediately with liquid nitrogen and stored at −80°C prior to use.

RNA Isolation and cDNA Synthesis

Total RNA was isolated using RNAiso Plus reagent (Takara, Dalian, China) and treated with RNase-free DNase I (Takara) to reduce DNA contamination. The quality and concentration of RNA samples were determined by agarose gel electrophoresis and a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE). The first-strand cDNA was synthesized using a ReverTra Ace qPCR RT Master Mix with gDNA Remover (Toyobo, Osaka, Japan). Each cDNA sample was diluted to 20 ng/µl with nuclease-free water.

Homology Search

A larval transcriptome dataset of *P. rapae* has been released (Qi et al. 2016). This dataset was searched to identify *ace* genes. The protein sequences of *ace1* and *ace2* from *B. mori* were used as queries (Shang et al. 2007). The search was performed using the Basic Local Alignment Search Tool (BLAST) program (Altschul et al. 1997). The cutoff e-value was set as 1×10^{-5}. The identified *P. rapae ace* genes were further validated by searching against the National Center for Biotechnology Information (NCBI)'s non-redundant database using the BLASTX online program (http://blast.ncbi.nlm.nih.gov/ blast.cgi).

Molecular Cloning

The complete open reading frames (ORFs) of *Prace1* and *Prace2* were cloned from larval cDNA using KOD FX DNA polymerase (Toyobo, Osaka, Japan). The gene-specific primers used are listed in Supp Table S1 (online only). PCR amplification was done using the following parameters: 94°C for 2 min, followed by 35 cycles at 98°C for 30 s, 52°C for 30 s and 68°C for 2 min, and one additional cycle at 68°C for 10 min. The PCR products were separated by agarose gel electrophoresis, purified from the gel, and sequenced from both 5′- and 3′-directions (Sangon Biotech., Shanghai, China).

Bioinformatic Analyses

The theoretical molecular weight and isoelectric point of the identified proteins were calculated using the ExPASy tool (http://web.expasy.org/compute_pi/). A signal peptide cleavage site was predicted using SignalP (http://www.cbs.dtu.dk/services/SignalP/). Functional motifs and key catalytic residues were predicted using NCBI's CD-search program (https://www.ncbi.nlm.nih.gov/structure/cdd/wrpsb.cgi). The genomic DNA sequence of *P. rapae* and other model insect species [*B. mori*, *Acyrthosiphon pisum* (Harris) (Hemiptera: Aphididae), *T. castaneum* and *D. melanogaster*] were downloaded from NCBI's genome database (https://www.ncbi.nlm.nih.gov/genome/), and the exon–intron structures were determined by aligning cDNA sequences of the genes with genomic DNA sequences using the Splign program (https://www.ncbi.nlm.nih.gov/sutils/splign/splign.cgi). The protein sequences of different insect *ace*s were aligned using Clustal Omega (http://www.ebi.ac.uk/tools/msa/clustalo/). The alignments were imported into MEGA7.0 software, and a phylogenetic tree was generated using the neighbor-joining method with 1,000 bootstrap replications (Tamura et al. 2013). The *ace* gene from *Torpedo californica* Ayres was used as an outgroup. The GenBank accession numbers of insect *ace*s used are listed in Supp Table S2 (online only).

Quantitative Real-Time PCR

Quantitative real-time PCR (qPCR) was performed to investigate the transcription profiles of the *Prace* genes. Each 20 µl reaction mixture contained 10 µl SYBR Green Realtime PCR Master Mix (Toyobo, Osaka, Japan), 1 µl (20 ng) cDNA template, 0.4 µl (0.2 µM) sense primer, 0.4 µl (0.2 µM) anti-sense primer, and 8.2 µl nuclease-free water. Primers (Supp Table S1 [online only]) used for qPCR were designed using the BatchPrimer3 program (https://probes.pw.usda.gov/batchprimer3/), and two housekeeping genes (*β-actin* and *18S rRNA*) were used as references to normalize target gene expression (Liu et al. 2017b).

Reactions were run on a CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA). The thermal cycling conditions were: one cycle of 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C for 25 s. A heat-dissociation protocol was included in the thermal cycle to confirm that only one single target gene was amplified by each primer pair. Also, a no-template control and a no-transcriptase control were both included in the assay to detect potential contamination. The experiment was biologically repeated three times. The gene expression level was quantified using a modified Pfaffl method (Liu et al. 2015). The relative mRNA levels of *Prace1* and *Prace2* in different samples were calculated by a double

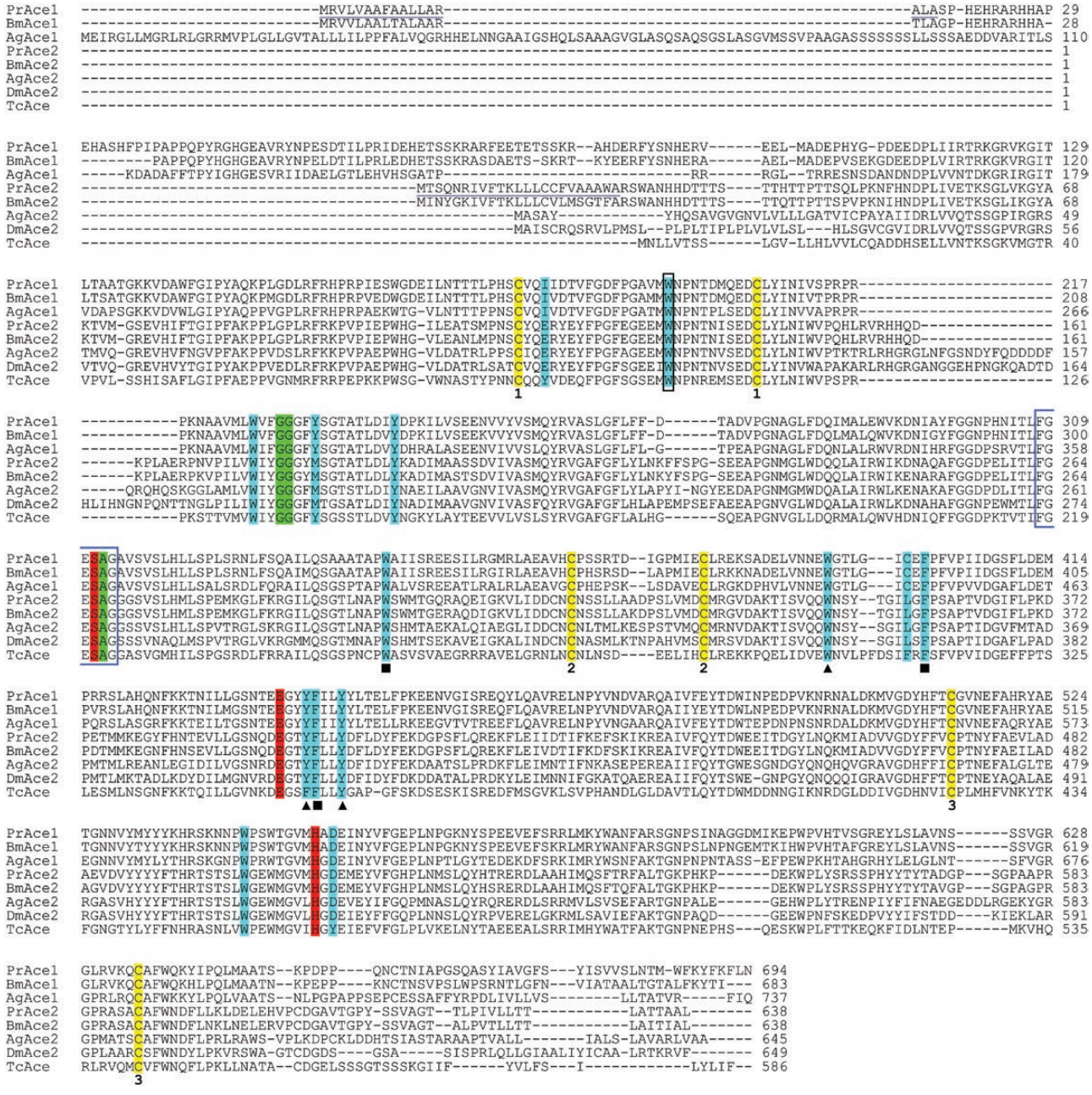

Fig. 1. Multiple sequence alignment of amino acid sequences of *ace* genes from *P. rapae* (Pr), *B. mori* (Bm), *Anopheles gambiae* Giles (Ag), *D. melanogaster* (Dm), and *T. californica* (Tc). Signal peptides are underlined; the catalytic triads are shown in red; the oxyanion hole is shown in green. Acyl pockets are identified by solid triangles; peripheral anionic subsites are identified by rectangles. The choline-binding site is shown in a black box, and the FGESAG motif is indicated by a blue box. Fourteen aromatic residues lining the catalytic gorge are highlighted in cyan, and cysteine residues forming intramolecular disulfide bonds are in yellow with the numbers 1, 2, and 3. GenBank accession numbers of these sequences are listed in Supp Table S2 (online only).

normalization method described elsewhere (Cui et al. 2012, Wang et al. 2016b, Yu et al. 2017). The lowest expression level was used as a calibrator (set as one-fold).

Statistics

Data were analyzed using Data Processing System (DPS) software v9.5 (Tang and Zhang 2013). The differences among multiple samples were compared by one-way analysis of variance (ANOVA) with Tukey's post hoc test. The differences between two samples were compared by two-tailed Student's *t*-test. The level of significance was set at $P < 0.05$.

Results

Identification and Characterization of Two *Prace* Genes

By searching the *P. rapae* transcriptome dataset, the *Prace1* and *Prace2* genes (GenBank accession numbers: MH105065 and KY021892, respectively) were successfully identified from *P. rapae*. They were further verified by PCR amplification and DNA sequencing (data not shown). *Prace1* contained a complete ORF consisting of 2,085 bp nucleotides, encoding a PrAChE1 protein consisting of 694 amino acid residues. *Prace2* had a complete ORF of 1917 bp, encoding a 638-amino-acid PrAChE2 protein (Fig. 1). The theoretical isoelectric points of PrAChE1 and PrAChE2 were 6.1 and 5.3, respectively, and the calculated molecular weights were 78.4 and 71.7 kDa, respectively.

The amino acid identity between *Prace1* and *Prace2* was low (40% identity; Table 1), but the genes showed high identities with their respective orthologs from other insect species: *Prace1* showed the highest amino acid identity (90%) with its ortholog from the butterfly species *Melitaea cinxia* (L.) (Lepidoptera: Nymphalidae), and 82–87% identities with *ace1* genes from several moth species including *B. mori*, *P. xylostella*, *H. armigera*, and *Cydia pomonella* (L.) (Lepidoptera: Tortricidae); *Prace2* showed the highest amino acid identity (93%) with *M. cinxia ace2*, and 91–94% identities with *ace2* genes from the aforementioned moth species (Table 1).

The identified PrAChE1 and PrAChE2 proteins both have a predicted signal peptide at their N-terminus (Fig. 1), suggesting that the two proteins could be secreted into the extracellular fluid. Multiple sequence alignment showed that PrAChE1 and PrAChE2 both have the conserved motifs of AChEs, including the catalytic triad, choline-binding sites, an oxyanion hole, an acyl pocket, and a peripheral anionic subsite (Fig. 1). Other typical features, such as the characteristic FGESAG motif and the 14 conserved aromatic amino acids in the lining of catalytic gorge, were also present in both PrAChEs (Fig. 1). Moreover, both proteins have six positional conserved cysteine residues, which are essential for the formation of the intramolecular disulfide bonds (Fig. 1).

Phylogenetic Analysis

A neighbor-joining tree was generated to investigate the phylogenetic relationships of insect *ace* genes (Fig. 2). This showed that insect *ace* genes are segregated into two distinct groups: *ace1* and *ace2*. *Prace1* and *Prace2* fell into the *ace1* and *ace2* groups, respectively (Fig. 2). In the *ace1* group, *Prace1* and the *M. cinxia ace1* were clustered into one branch; in the *ace2* group, *Prace2* was clustered into a branch together with its orthologs from *Papilio polytes* L. (Lepidoptera: Papilionidae), *P. xuthus* L. (Lepidoptera: Papilionidae), and *P. machaon* L. (Lepidoptera: Papilionidae) (Fig. 2).

Exon–Intron Structures of *ace* Genes From *P. rapae* and Other Insect Species

The genomic locations and exon–intron structures of two *Prace* genes were analyzed by aligning their cDNA sequences with genomic sequences (Fig. 3). The results showed that the two *Prace*s were located on different genomic scaffolds. *Prace1* was located on scaffold 156, while *Prace2* was on scaffold 430. The lengths of genomic DNA sequences for *Prace1* and *Prace2* were 2,348 bp and 15,083 bp, respectively (Fig. 3). *Prace1* consisted of three exons and two introns, whereas *Prace2* had six exons and five introns (Fig. 3). In addition, the donor and acceptor sites of all of the introns in both genes obeyed the classical GT–AG rule (data not shown).

We also compared the exon–intron structures of *Prace*s with *ace* genes from other model insect species including *B. mori*, *A. pisum*, *T. castaneum*, and *D. melanogaster* (Fig. 3). The results showed that *Prace1* shared the same exon numbers (three exons) with its ortholog from *B. mori*. The *A. pisum ace1* and *T. castaneum ace1* had five and two exons, respectively (Fig. 3). *Ace2* genes in *P. rapae*, *B. mori*, and *T. castaneum* shared the same exon numbers (six exons), whereas the *A. pisum ace2* and *D. melanogaster ace2* had eight and nine exons, respectively (Fig. 3).

Amino Acid Variations in AChEs in *P. rapae* and Other Insect Species

In order to explore key amino acid variations potentially associated with insecticide insensitivity, we compared the amino acid sequences of the AChEs of *P. rapae* with those of other insect species in which

Table 1. Percent identities of amino acid residues between the *ace* genes from different lepidopteran species, including *P. rapae* (Pr-prefix), *M. cinxia* (Mc), *B. mori* (Bm), *P. xylostella* (Px), *H. armigera* (Ha), and *C. pomonella* (Cp)

	Prace1	*Mcace1*	*Bmace1*	*Pxace1*	*Haace1*	*Cpace1*	*Prace2*	*Mcace2*	*Bmace2*	*Pxace2*	*Haace2*	*Cpace2*
Prace1	–											
Mcace1	90	–										
Bmace1	85	89	–									
Pxace1	82	85	85	–								
Haace1	86	88	89	85	–							
Cpace1	87	87	88	86	86	–						
Prace2	40	38	37	37	37	37	–					
Mcace2	39	37	36	36	36	37	93	–				
Bmace2	40	37	36	36	36	36	92	92	–			
Pxace2	40	37	37	36	36	37	92	93	92	–		
Haace2	38	37	36	36	36	37	94	95	95	94	–	
Cpace2	39	37	37	36	36	36	91	93	92	93	94	–

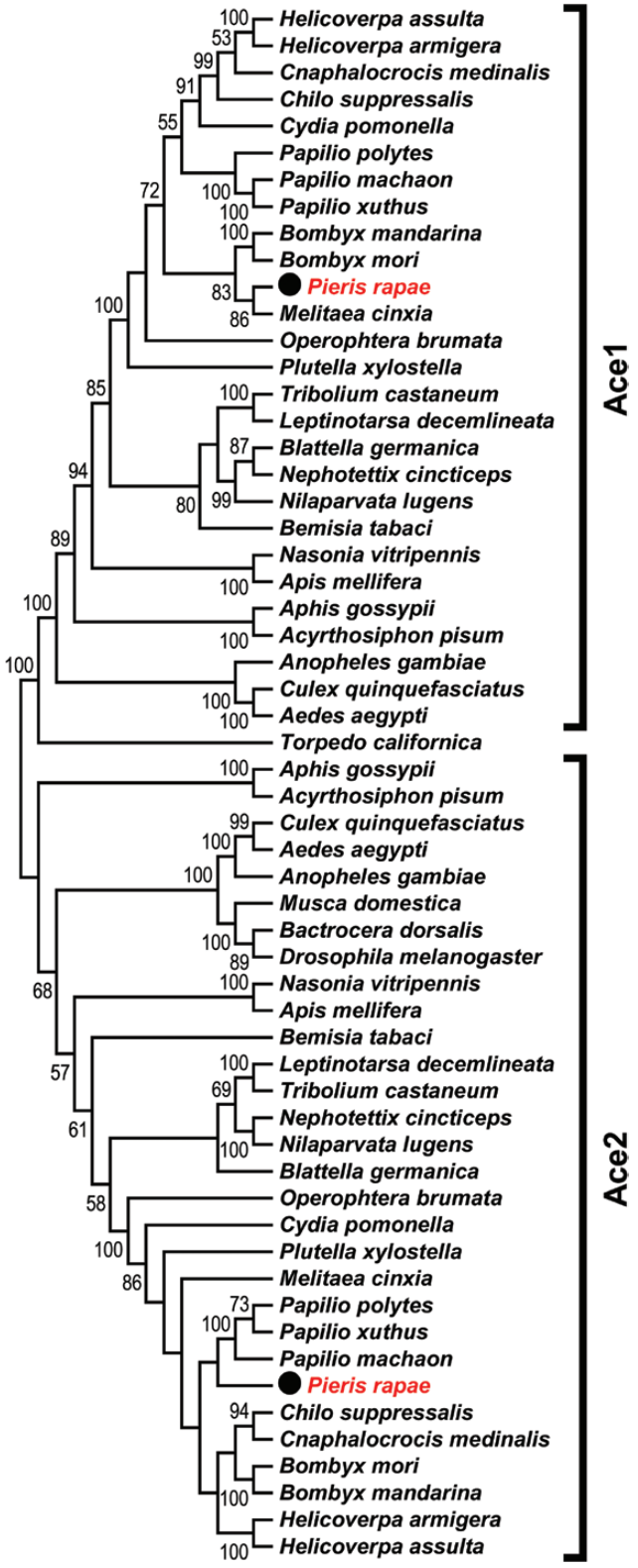

Fig. 2. Phylogenetic relationships of insect *ace* genes. Bootstrap values of >50% are shown at each node. The *ace* gene from *T. californica* was used as the outgroup. The two *P. rapae ace* genes are marked by solid circles. GenBank accession numbers of the sequences used are listed in Supp Table S2 (online only).

AChEs have mutations involved in insecticide insensitivity (Table 2). The result showed that one amino acid variation in PrAChE1 was at the same position as a Gly324Ala mutation in *P. xylostella*, and two amino acid variations in PrAChE2 corresponded to a Ser291Gly mutation in *Leptinotarsa decemlineata* Say (Coleoptera: Chrysomelidae) and a Ser431Phe mutation in *Aphis gossypii* Glover (Hemiptera: Aphididae) (Table 2).

Expression Profiles of the *Prace* Genes

Relative expression levels of *Prace1* and *Prace2* in various larval tissues were determined by qPCR. The mRNA transcripts of both genes were detected in all of the tested samples, and the expression patterns of the two genes were quite similar: high levels of transcription were observed in the head, whereas relatively low levels of mRNA transcripts were found in the ventral nerve cord, midgut, and integument (Fig. 4A and B). In addition, transcriptional levels of *Prace1* were significantly higher than those of *Prace2* in all of the tested tissues (Fig. 4C).

Expression profiles of the two *Prace* genes at different developmental stages were also determined. The two genes had a similar transcription pattern: relatively low mRNA levels in the second-instar larval stage, which increased gradually from the third- to the fifth-instar larval stage, and reached their peak levels at the fifth-instar larval stage (Fig. 4D and E). Both genes were then downregulated during the pupal stage, and upregulated again at the adult stage (Fig. 4D and E). *Prace1* had significantly higher mRNA levels than *Prace2* in all tested developmental stages (Fig. 4F).

Discussion

AChE in insects is extremely important not only because it is a key enzyme in synaptic transmission, but also because it is the target of organophosphate and carbamate insecticides (Kim and Lee 2018). Point mutations in insect *ace* genes usually result in insecticide resistance (Lee et al. 2015). Therefore, these genes have attracted much attention in insecticide toxicological studies (Alizadeh et al. 2014, Miao et al. 2016, Zhou et al. 2016). Recently, it was shown that knockdown of *ace1* genes led to increased sensitivity of insects to AChE insecticides (Revuelta et al. 2009, 2011, Lu et al. 2012b), and that silencing *ace1* and/or *ace2* impacted insect development (He et al. 2012, Lu et al. 2012b, Salim et al. 2017). This suggests that these genes could be utilized for developing RNAi-based pest control strategies (Kim et al. 2015). Prior to this study, *ace* genes had been isolated from a great number of insect species (Kim and Lee 2018), but little was known about *ace* genes in *P. rapae*. Here, we report the identification and characterization of *Prace1* and *Prace2* in this insect species. Having obtained the *Prace* sequences will allow future study of the physiological functions of these two genes.

Phylogenetic analysis showed that insect *ace genes* were divided into two distinct groups: *ace1* and *ace2*. This phylogeny is consistent with those that have been published previously (Wang et al. 2016a, Salim et al. 2017). In this tree, *Prace1* and *Prace2* were clustered into the *ace1* and *ace2* groups, respectively, with strong bootstrap support. The significant divergence between *Prace1* and *Prace2* implied that the two genes may have distinct functions. Furthermore, *Prace1* and *Prace2* were located on different genomic scaffolds. In other insect species, *ace genes* are also distributed on distinct chromosome scaffolds (Weill et al. 2002, Seino et al. 2007, Lu et al. 2012a). Additionally, *Prace1* was shown to have three exons, and *Prace2* was shown to have six. Definition of the exon–intron structures provides useful information for evolutionary study of insect *ace* genes.

In many insect species, point mutations in AChEs affect sensitivity to organophosphate and carbamate insecticides (Lee et al. 2015). For the purpose to find potential point mutation sites that may be associated with insecticide resistances in two PrAChEs, we compared the amino acid variations of the PrAChEs with AChEs from

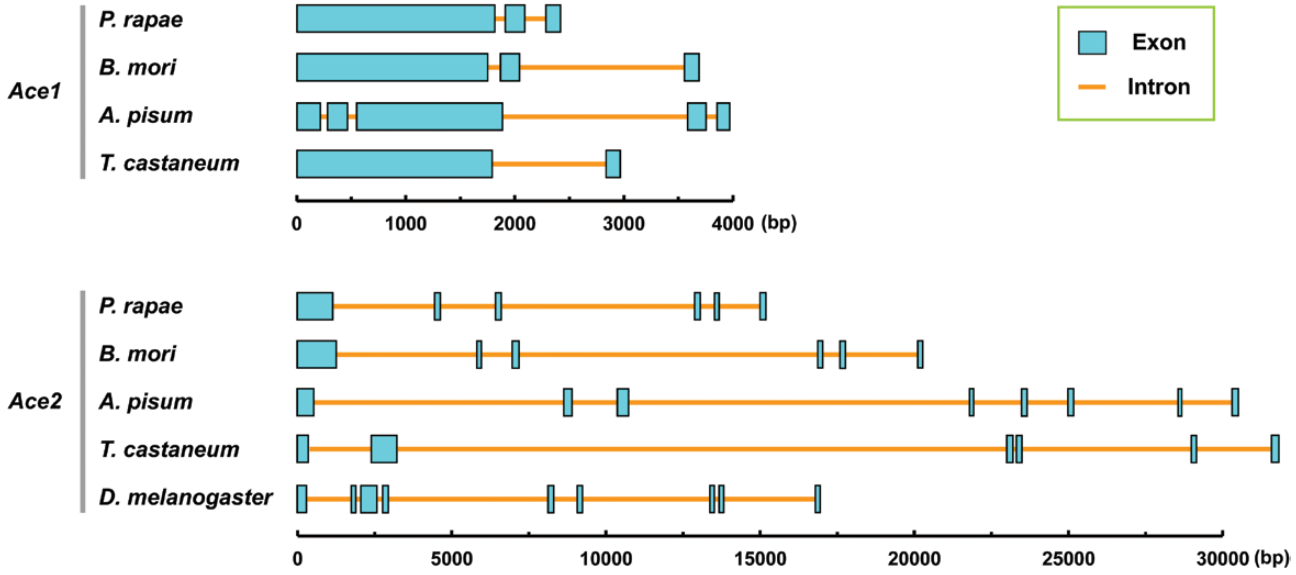

Fig. 3. Schematic diagram of the exon–intron structures of *ace* genes in different insect species including *P. rapae*, *B. mori*, *A. pisum*, *T. castaneum*, and *D. melanogaster*.

Table 2. Amino acid variations in *P. rapae* AChEs compared with mutations in AChEs from other insect species that are potentially associated with insecticide insensitivity

AChE	Insect species	Mutation	Reference	*P. rapae*[a]
1	*Myzus persicae* (Sulzer)	S431F	Nabeshima et al. (2003	Y
	A. gossypii Glover	S431F	Benting and Nauen (2004)	Y
	An. gambiae Giles	G119S	Weill et al. (2004)	G
	Culex pipiens L. (Diptera: Culicidae)	G119S	Weill et al. (2004)	G
	C. pomonella (L.)	F399V	Cassanelli et al. (2006)	F
	Bemisia tabaci (Gennadius) (Hemiptera: Aleyrodidae)	F392W	Alon et al. (2008)	F
	N. lugens (Stål)	G119A	Kwon et al. (2012)	G
		G119S	Zhang et al. (2017)	G
		F/Y330S	Kwon et al. (2012)	Y
		F331C	Zhang et al. (2017)	F
		F331H	Kwon et al. (2012)	F
		I332L	Kwon et al. (2012) and Zhang et al. (2017)	I
	P. xylostella (L.)	A298S	Kim et al. (2011, 2012a)	A
		G324A	Kim et al. (2011, 2012a)	A
	Chilo suppressalis (Walker) (Lepidoptera: Crambidae)	A314S	Jiang et al. (2009)	A
	A. lucorum (Meyer-Dür)	A216S	Wu et al. (2015)	A
2	*D. melanogaster* Meigen	F115S	Mutero et al. (1994)	F
	M. domestica L.	G262V	Walsh et al. (2001)	A
	L. decemlineata Say	S291G	Zhu et al. (1996)	G
	A. gossypii Glover	F139L	Li and Han (2004)	F
		A302S	Toda et al. (2004)	A
		S431F	Toda et al. (2004)	F

[a]These amino acid residues are from the wide type *P. rapae* AChEs.

other insect species. In PrAChE1, only one amino acid residue variation corresponds to a mutated residue (Gly324Ala) in *P. xylostella*. This Gly324Ala mutation has been shown to be important in the development of resistance to 2,2-dichlorovinyl dimethyl phosphate in *P. xylostella* (Kim et al. 2012a). In PrAChE2, two amino acid variations were found: one corresponded to the Ser291Gly mutation in *L. decemlineata*, and the other corresponded to the Ser431Phe mutation in *A. gossypii*. The Ser291Gly mutation in *L. decemlineata* may change the structure of the α-helix of AChE and may be associated with increased resistance to azinphos-methyl (Zhu et al. 1996). The Ser431Phe mutation in *A. gossypii* is 1 of 14 aromatic residues lining the active site gorge and may play a role in pirimicarb insensitivity (Toda et al. 2004). However, since the *P. rapae* individuals used in this study were from a laboratory colony in which sensitivity to insecticides has not yet been evaluated, it is not possible to assess the relationship between frequencies of mutations and levels of insecticide resistance. Further biochemical analysis is needed to test whether these amino acid variations influence insecticide sensitivity.

In insects possessing two *ace* genes, it is hypothesized that the central nervous system-distributed *ace*s is responsible for neuronal function, while *ace*s expressed in other tissues may have non-neuronal roles, such as xenobiotic defense (Kim and Lee 2018).

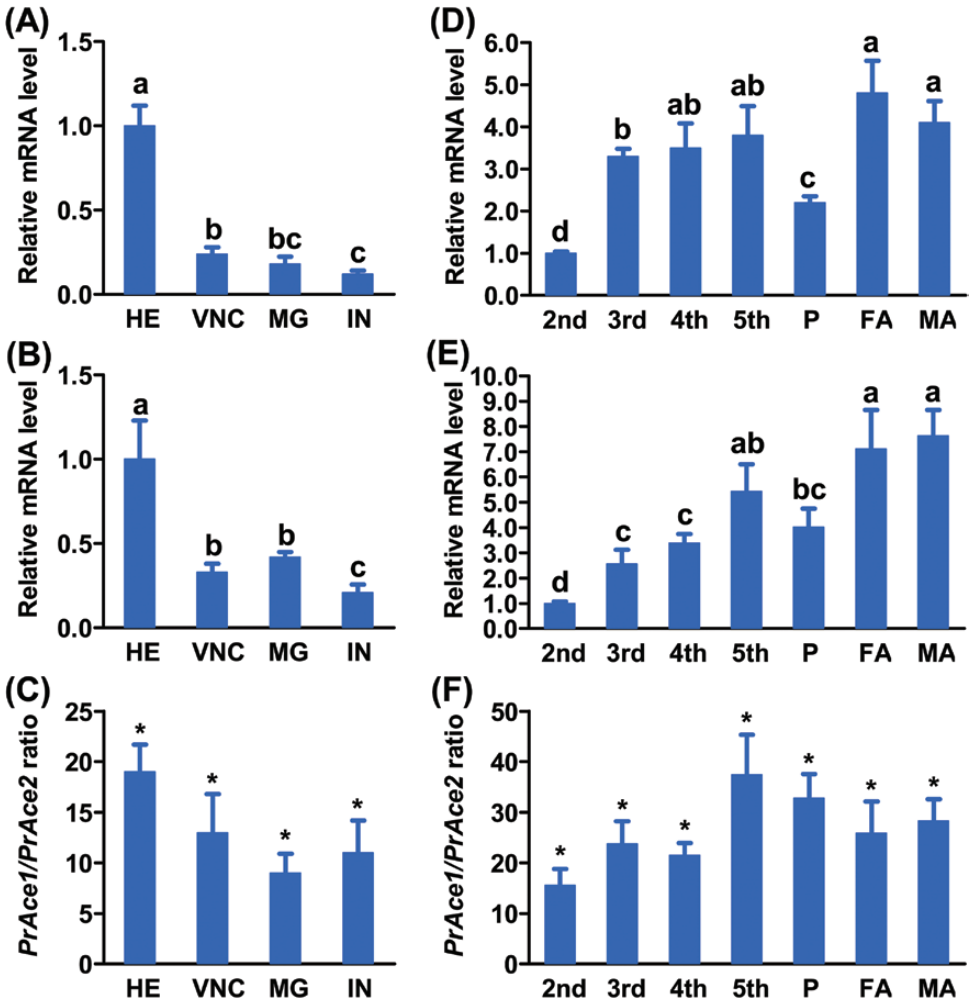

Fig. 4. Expression profiles of *Prace1* and *Prace2*. Left panel: relative mRNA levels of (A) *Prace1* and (B) *Prace2* in various larval tissues. HE: head; VNC: ventral nerve cord; MG: midgut; IN: integument. (C)The ratios between *Prace1* and *Prace2* mRNA levels in these tissues. Right panel: relative mRNA levels of (D) *Prace1* and (E) *Prace2* at different developmental stages. Second to fifth: second- to fifth-instar larvae; P: pupae; FA: female adults; MA: male adults. (F) The ratios between *Prace1* and *Prace2* mRNA levels in these stages. The lowest expression level was used as a calibrator (set as one-fold). The error bars represent the standard error of the mean of three biological repeats. Different lowercase letters indicate significant differences among multiple samples (one-way ANOVA with Tukey's post hoc test; $P < 0.05$); asterisks represent significant differences between *Prace1* and *Prace2* mRNA abundances in a sample (two-tailed Student's *t*-test; $P < 0.05$).

Therefore, determination of the expression patterns of two *Prace* genes is conducive to predict their physiological functions. By using qPCR, the transcription profiles of *Prace1* and *Prace2* were investigated. Both genes were highly expressed at the fifth-instar larval stage and in the larval head. This pattern is similar to *ace gene* expression in *H. assulta* (Lee et al. 2006), *T. castaneum* (Lu et al. 2012a), *Cnaphalocrocis medinalis* (Guenée) (Lepidoptera: Crambidae) (Wang et al. 2016a), and *Spodoptera litura* (Fabricius) (Lepidoptera: Noctuidae) (Salim et al. 2017). However, in other insects, *ace genes* have different expression profiles. For instance, in *N. lugens*, the highest levels of *ace1* and *ace2* were found at the first-instar nymph and egg stages, respectively (Li et al. 2012); and in *B. mori*, *ace1* was highly expressed in muscle and *ace2* was ubiquitously expressed in various tissues (Ye et al. 2017).

This study also compared the expression levels of the two *Prace* genes and found that transcript levels of *Prace1* were significantly higher than *Prace2* in various tissues and at different developmental stages. This is consistent with previous reports of *ace* genes in the majority of insect species (Kim et al. 2006, Lee et al. 2006, Revuelta et al. 2011, Wang et al. 2016a). However, in *B. mori*, *ace2* has a different transcription profile and is expressed more abundantly than *ace1* (Chen et al. 2009, Ye et al. 2017). Although the relative transcription level of a gene does not necessarily fully reflect its physiological importance, the significantly higher expression level of *Prace1* does imply that this gene may play an important role in neurotransmission and/or other biological processes. However, functional studies are needed to demonstrate the precise functions of the two *Prace* genes in *P. rapae*.

In conclusion, this study examines sequence characteristics, phylogenetic relationships, exon–intron structures, genomic locations and expression patterns of two *ace* genes in *P. rapae*. We found that the transcription levels of *Prace1* were significantly higher than those of *Prace2* in all of the tested samples, implied that *Prace1* may play an important role in physiological process. In addition, amino acid mutations were discovered in both PrAChE1 and PrAChE2 and may be associated with insecticide insensitivity. Further investigation is needed to elucidate the function of the two genes.

Supplementary Data

Supplementary data are available at *Journal of Insect Science* online.

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Conflict of Interest

The authors declare that they have no conflicts of interest.

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