Cloning, expression, and functional analysis of two acetylcholinesterase genes in *Spodoptera litura* (Lepidoptera: Noctuidae)

Abdalla M.A. Salim, Muhammad Shakeel, Jinyun Ji, Tinghao Kang, Yashu Zhang, Ehsan Ali, Zhao Xiao, Yanhui Lu, Hu Wan, Jianhong Li

A B S T R A C T

Two acetylcholinesterase genes (SlAce1 and SlAce2) were cloned from *Spodoptera litura*, which is an important pest that causes widespread economic damage to vegetables and ornamental plants. We analyzed their expression patterns and compared their biological functions by using RNA interference. Our results showed that SlAce1 and SlAce2 cDNA contains 2085 bp and 1917 bp nucleotides and encoding proteins of 694 and 638 amino acid residues, respectively. Phylogenetic analysis indicated that the lineage of SlAce genes and SlAce1 was completely different from SlAce2. Although both genes were expressed in all developmental stages and majorly in the brain. The expression levels of the both genes were suppressed by inserting their related dsRNA in the 6th instar larvae, which led to 47.3% (SlAce1) and 37.3% (SlAce2) mortality. Interestingly, the suppression of the SlAce2 transcripts also led to significant reductions in the fecundity, hatching, and offspring in the parental generation of *S. litura*. It is concluded that SlAce2 is responsible for the hydrolysis of acetylcholine and also plays role in female breeding, embryo progress, and the development of progeny. Considerable larval mortality was observed after both AChE genes (i.e. Ace1 and Ace2) were silenced in *S. litura* confirms its insecticidal effectiveness, which provided a molecular basis in biological pest control approach.

© 2017 Elsevier Inc. All rights reserved.

1. Introduction

Acetylcholinesterase (AChE, EC 3.1.1.7) is a key enzyme that terminates neurotransmission by the rapid hydrolysis of the neurotransmitter acetylcholine at cholinergic synapses in all animals (Soreq and Seidman, 2001). Several studies concentrated on AChE in arthropods because it is the target of organophosphate and carbamate insecticides. In addition, the mutations in the AChE reduce target sensitivity and thus conferring pesticide resistance (Fournier and Mutero, 1994; Weill et al., 2002; Weill et al., 2003). The first insect AChE gene (Ace) sequenced was from *Drosophila melanogaster* in 1986, and it has been described as an Ace-orthologous (AO-Ace) gene (Hall and Spierer, 1986), whereas, the first Ace-paralogous (AP-Ace) gene was sequenced from *Schizaphis graminum*. Surprisingly, *D. melanogaster* has only one Ace gene as confirmed by its genome sequence (Myers et al., 2000), whereas most other insect species have more than one AChE gene (i.e. Ace1 and Ace2) (Kim and Lee, 2013). Ace1 refers to AP-Ace gene as it is an additional gene which is found in insects other than the Cyclorrhapha flies, whereas Ace2 refers to AO-Ace gene in relation to the *D. melanogaster* Ace gene as it is common to all species of insect (Kono and Tomita, 2006). AChE-encoding cDNAs of arthropods have been sequenced from at least 43 insect species (Lu et al., 2012a). Among these, both Ace1 and Ace2 have been reported in 27 insect species, for instance, *Myzus persicae*, *Plutella xylostella*, *Rhopalosiphum padi* and *Sitobion avenue*, *Bombyx mori*, *Bemisia tabaci*, *Chilo suppressalis* and *Nilaparvata lugens* (Nabershima et al., 2003; Baek et al., 2005; Chen and Han, 2006; Seino et al., 2007; Alon et al., 2008; Jiang et al., 2009; Li et al., 2012). The presence of two Ace genes in insect species has led to research into their function, especially regarding their connection to insect susceptibility to pesticides (Revuelta et al., 2009; Lu et al., 2012b; Xiao et al., 2015) and have become targets for the development of new insecticides (Pang, 2006; Lang et al., 2010; Pang et al., 2012).

In addition to their roles in cholinergic synapses, non-cholinergic roles for cholinesterases (i.e., cell adhesion, neurite growth, and apoptosis) have been reported in vertebrates (Park et al., 2004; Zang and Greenberg, 2012). Suppression of Ace genes in *Helicoverpa armigera* was recently found to influence larval growth and development (Kumar et al., 2009). In *P. xylostella*, *PxAce1* is more critical than *PxAce2* for the non-cholinergic functions in controlling larval development and motor capacity (He et al., 2012). Furthermore, *TcAce2* in *Triboletum castaneum* plays an essential non-cholinergic role in female
breeding, embryo expansion, and the development of descendants (Lu et al., 2012b).

The AChE genes (RpAce1 and RpAce2, as well as SaAce1 and SaAce2) have also been highlighted in R. padi and correspondingly in S. avenae, R. padi and S. avenae, devastating aphid pests of wheat (Triticum spp.), sorghum and other cereal grain crops worldwide (Chen and Han, 2006). Their AChE activity has been described as a combination of the two types of AChE genes (Lu and Gao, 2009; Lu et al., 2013a). Despite recent inquiries into the relevant contributions of Ace1 and Ace2 to the catalytic action of AChE in R. padi and S. avenae, their roles in pesticide endurance and non-cholinergic functions remain poorly understood.

This paper investigates two Ace genes of the cutworm S. litura, focusing on the cloning, phylogenies, and determination of the transcript level of SlAce1 and SlAce2 in different developmental stages and tissues. RNA interference (RNAi) of SlAce1 and SlAce2 was used to assess their biological functions in the cutworm and to obtain insight into better strategies for insect pest control.

2. Material and methods

2.1. Insects

The insects were reared on an artificial diet and temperature at 25 ± 1 °C, a photoperiod of 14L: 10D and a relative humidity of 60–70%. The adult males and females were allowed to emerge in transparent cages breeding, embryo expansion, and the development of descendants (Lu et al., 2013b; Wan et al., 2014).

2.2. Cloning of cDNA fragments of SlAce1 and SlAce2 and phylogenetic analysis

The clone containing the SlAce1 or SlAce2 cDNA of interest was isolated from an expressed sequence tag (EST) collection generated from a cDNA library of whole bodies of S. litura (Wan et al., 2014). Plasmid Mini Kit (Omega, Norcross, GA, USA) was used to extract the Plasmid DNA and the DNA sequences were determined by a company (TSING KE Biological Technology, China). The sequences were compared using the DNASIS and BLAST databases of NCBI (http://www.ncbi.nlm.nih.gov/BLAST). MacVector (ver. 6.5, Oxford Molecular Ltd.) was used to align SlAce amino acid sequences. The tertiary structure was predicted using SWISS-MODEL (http://swissmodel.expasy.org), phylogenetic analysis was performed using Molecular Evolutionary Genetics Analysis (MEGA 6.0). For construction the phylogenetic tree to evolutionary relationships among Torpedo californica, Tetranychus urticae and 46 insect species constructed the maximum likelihood method. GenBank accession numbers of sequences utilized: Sequences used: TcaAce1); ApAce2 (XM_001605518, Nasonia vitripennis Ace1); NvAce2 (XM_001600408, Nasonia vitripennis Ace1); PhAce1 (AB266605, Pediculus humanus corporis Ace1); PhAce2 (AB266606, Pediculus humanus corporis Ace2); CpaAce1 (DQ267977, Cydia pomonella Ace1); CpaAce2 (DQ267976, Cydia pomonella Ace2); HaAce1 (DQ001323, Helicoverpa assulta Ace1); HaAce2 (AY187736, Helicoverpa assulta Ace2); AeAce1 (AB128421, Aedes albopictus Ace1); AeAce2 (AB128420, Aedes albopictus Ace2); AgoAce1 (AF502081,Apis gossypii Ace1); AgoAce2 (AF502082, Aphis gossypii Ace2); CtAce1 (AB121251, Culex tritaeniorhynchus Ace1); CtAce2 (AB121252, Culex tritaeniorhynchus Ace2); SgAce1 (AF321574, Schizaphis graminis Ace1); MpcAce1 (AF287291, Myzus persicae Ace1); MpcAce2 (AY147797, Myzus persicae Ace2); CpiAce1 (AY498546, Culex pipiens Ace1); CpiAce2 (AM159193, Culex pipiens Ace2); MdAce (AY134873, Musca domestica Ace1); PxAce1 (AY970293, Plutella xylostella Ace1); PxAce2 (AY061975, Plutella xylostella Ace2); CsAce1 (EF453724, Chilo suppressalis Ace1); CsAce2 (EF407254, Chilo suppressalis Ace2); CmAce1 (FN538987, Cnaphalocrocis medinalis Ace1); PhAce1 (AB266614, Pediculus humanus capitis Ace1); PhAce2 (AB266615, Pediculus humanus capitis Ace2); TuAce (AY188448, Tetanychus urticae Ace1); AaeAce1 (EF209048, Aedes aegypti Ace1); LmAce1 (EU231603, Locusta migratoria mandissi Ace1); LbAce (FJ647185, Liposcelis bostrychophila Ace1); LbAce2 (EF362950, Liposcelis bostrychophila Ace2); NiAce (FM863696, Nilaparvata lugens Ace1); CcAce (EU130781, Ceratitis capitata Ace1); AmAce (AB181702,Apis mellifera Ace1); BdAce2 (AY155500, Bactrocera dorsalis Ace2); HiAce (AY466160, Haematobia irritans Ace1); CpcAce1 (AY762905, Culex pipiens pellens Ace1); HarAce2 (AF369793, Helicoverpa armigera Ace1); BoAce (AF452052, Bactrocera oleae Ace1); LcAce (U88651, Lucilia cuprina Ace1); NaAce (AF145235, Nephrotettix cinereus Ace); LdAce2 (L41180, Leptinotarsa decemlineata Ace2); SlAce1 (KY130418, Spodoptera littoralis Ace1); SlAce2 (KY130419, Spodoptera littoralis Ace2).

2.3. SlAce genes expression pattern in different developmental stages and tissues

Total RNA was extracted from different stages: 400 one day old eggs, 100 first instar larvae, 50 second instar larvae, 30 third instar larvae, 5 fourth, fifth, and sixth instar larvae, 5 pre-pupa, and 5 first-day (male and female), 5 sixth-day (male and female), and 5 twelfth-day (male and female) pupae; and 5 one-day-old adults (male and female) and 5 seven-day-old (female and male) adults for each biological replicate. For tissue expression, same-size individual healthy larvae at the 6th instar were selected, and the brain, midgut, fat body, epidermal tissue and hemolymph were examined. RNA extraction was performed using the Rtrlzol reagent (Invitrogen) based on the manufacturer's protocol. AUV1800 spectrophotometer (Shimadzu, Kyoto, Japan) was used to determine the purity of the RNA samples. Finally, after DNaseI treatment, reverse transcriptase system was used to synthesize cDNA according to the manufacturer's protocol.

The SsoFast™ EvaGreen® Supermix (Bio-Rad, Hercules, CA, USA) was used on Bio-Rad iQ Optical (Bio-Rad) to perform qRT-PCR. The reaction process was done for 30 s at 95 °C, followed by 40 cycles at 95 °C for 5 s and 60 °C for 10 s. Finally, a dissociation analysis from 55 to 95 °C was carried out in order to make sure the purity of amplified product (Shakeel et al., 2015). The relative expression was calculated using the 2−ΔΔCT method (Livak and Schmittgen, 2001), and we selected ribosomal protein L10 (RPL10) as a reference gene for normalization in tissues and GAPDH for normalization in developmental stages. Both (RPL10 and GAPDH) reference genes have already been validated in a previous report by (Lu et al., 2013b). The primers used for qRT-PCR are provided in Table 1.

2.4. dsRNA synthesis and application

dsRNA targeting SlAce1 or SlAce2 was prepared utilizing the T7 RiboMAX™ Express RNAi System (Promega) based on the
manufacturer’s protocol. Forward and reverse primers containing a T7 RNA Polymerase promoter were designed to amplify 371 bp and 392 bp for SlAce1 and SlAce2, respectively (Table 1). pGEM-T Easy Vector (Promega) plasmids carrying the gene fragments were used as the template to generate corresponding dsRNA, which was then suspended in DEPC-treated water. The same procedure as mentioned above (Section 2.3) was followed to assess dsRNA purity and quantity. The concentration of dsRNA was adjusted to 1 μg μL−1, and the sample was stored at −20 °C until use. GFP (dsGFP) dsRNA used as a control was produced as described above. Newly molted Sixth instar larvae were injected with 5 μL of SlAce1 and SlAce2 dsRNA, with three replications, for a minimum of 30 insects in each control or treatment. Samples were collected at 24 h, 48 h, and 72 h. The controls were treated with dsGFP. The insects injected were reared under standard conditions for monitoring insect (larval) mortality. The remaining transcript levels were further evaluated using qPCR. To determine the effects of dsSlAce2 on insect growth and development, a previously described method (Lu et al., 2012a, b) was followed. Insects were evaluated by treating several 6th-instar larvae with dsSlAce2 or dsGFP as the control. The treated larvae developed into pupae and adults. The males and females were separated and coupled for crossbreeding as follows: (I) adult males and females developed from larvae injected with dsGFP; (II) adult males and females developed from larvae injected with dsSlAce2; (III) an adult male developed from a larva injected with dsSlAce2 paired with an adult female developed from a larva injected with dsGFP; (IV) an adult male developed from a larva injected with buffer paired with an adult female developed from a larva injected with dsSlAce2. The treatments included eight male and female couplings, with three replications each. The number of egg masses oviposited by each female was observed daily and accumulated until the female died, whereas egg parturition was evaluated five days after the eggs accumulated.

Table 1
PCR primers used to amplify cDNA sequences of SlAce genes, dsRNA and qPCR.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequences</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR for cDNA sequences</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SlAce1-F</td>
<td>ATGCCGGTGGTTGGCAGCCTGACGG</td>
<td>2085</td>
</tr>
<tr>
<td>SlAce1-R</td>
<td>TTAACGATAATACTTGAACCATC</td>
<td>1917</td>
</tr>
<tr>
<td>SlAce2-F</td>
<td>ATGATCAGCAACACGAAGATTGTTC</td>
<td></td>
</tr>
<tr>
<td>SlAce2-R</td>
<td>TTACAACGCGACGGTTGGC</td>
<td></td>
</tr>
<tr>
<td>Quantitative real-time PCR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SlAce1- (Q)F</td>
<td>GGAAGAACTAGGAGCACTCA</td>
<td>163</td>
</tr>
<tr>
<td>SlAce1- (Q)R</td>
<td>GTGCCGTCCTCCTCAATAT</td>
<td>234</td>
</tr>
<tr>
<td>SlAce2- (Q)F</td>
<td>CTGGCGAGATTGTTAT</td>
<td></td>
</tr>
<tr>
<td>SlAce2- (Q)R</td>
<td>ATCCGCCAACACTTCCGC</td>
<td></td>
</tr>
<tr>
<td>RPL10-F</td>
<td>CACTGGAGGAAAGAGGAT</td>
<td>189</td>
</tr>
<tr>
<td>RPL10-R</td>
<td>CATCAGAATGGCAGTG</td>
<td></td>
</tr>
<tr>
<td>GAPDH-F4</td>
<td>GGTATCTTCTGACTACAC</td>
<td>184</td>
</tr>
<tr>
<td>GAPDH-R2</td>
<td>CTGATCTTACGTGAG</td>
<td></td>
</tr>
<tr>
<td>double-stranded RNA synthesisa</td>
<td></td>
<td></td>
</tr>
<tr>
<td>dsSlAce1-F</td>
<td>ggtatcctgagaagcagttggcagacctgctatt</td>
<td>371</td>
</tr>
<tr>
<td>dsSlAce1-R</td>
<td>ggtatcctgagaagcagttggcagacctgctatt</td>
<td>392</td>
</tr>
<tr>
<td>dsSlAce2-F</td>
<td>ggtatcctgagaagcagttggcagacctgctatt</td>
<td></td>
</tr>
<tr>
<td>dsSlAce2-R</td>
<td>ggtatcctgagaagcagttggcagacctgctatt</td>
<td></td>
</tr>
</tbody>
</table>

a Sequences underlines indicate T7 RNA Polymerase promoter.
2.5. SlAce knockdown determination using qRT-PCR

Total RNA was extracted from the 6th-instar larvae in each treatment and control at 24 h, 48 h and 72 h using the TRIzol reagent (Invitrogen). First-strand cDNA was synthesized as described above. qRT-PCR was used to detect the level of gene silencing compared with controls (dsGFP).

2.6. Statistical analysis

All trials were carried out in triplicate, and the data were assayed using an analysis of variance (ANOVA) followed by Dancans multiple range test using the SPSS statistical software package (version 11.5; SPSS, Inc.). Mean separation was done according to a least significant differences (LSD) test at \( P < 0.05 \), with alpha = 0.05.

3. Results

3.1. Sequence analysis of Spodoptera litura AChE genes

In the present study, two types of Ace cDNAs that included the opening reading frame (ORF) were identified from a S. litura cDNA library. These two cDNAs were named SlAce1 and SlAce2. The nucleotide sequences of SlAce1 and SlAce2 have been deposited in GenBank under accession numbers KY130418 and KY130419, respectively. The ORF of cDNA fragments encoding SlAce1 is 2085 bp and encodes a protein consisting of 694 amino acids. SlAce2 has an ORF of 1917 bp that encodes...
a putative protein consisting of 638 amino acids. Multiple sequence alignment of deduced amino acid sequences AChE1, AChE2, and AChEs from other insect species exhibited that both S. litura AChE1 and AChE2 contain specific characteristics of functional AChE, having a catalytic triad, an oxanion hole, an acyl pocket, sites of choline-binding, and a peripheral anionic subsite (Fig. 1A, B). Both SlAChEs not only possessed conserved motifs, but also have other characteristic features, including “FGESAG” motif and the conserved 14 aromatic amino acids in lining of catalytic gorge. Six cysteine residues involved in the formation of the intramolecular disulfide bonds and a positional conserved cysteine residue were also observed in both of the two SIaChEs (Fig. 1A, B).

SlAce1 and SlAce2 sequences were confirmed according to sequence similarities with other known insect Ace genes. The deduced amino acid sequence of SlAce1 showed the highest homology to Helicoverpa assulta (87%) and high homologies to ace1-type genes from Blattella germanica (69%), Tribolium castaneum (68%), Anopheles gambiae (65%) and Sitobion avenae (65%) (Fig. 1A). Deduced amino acid sequences of SlAce2 exhibited high homologies to ace2-type genes from Helicoverpa assulta (97%), Blattella germanica (78%), Tribolium castaneum (77%), Sitobion avenae (67%) and Anopheles gambiae (49%) (Fig. 1B). The tertiary structures of SlAce1 and SlAce2 were predicted using the SWISS-MODEL program. The human butryryccholinesterase (hBChE) (Protein Data Bank ID: 5HQ3.1.A (Goldenzweig et al., 2016)) and D. melanogaster (PDB ID: 1DX4.1.A (Harel et al., 2000)) were used as templates for SIaChE and SlACE2, respectively. In SlAce1 (Fig. 2A), Cys432 is exposed to solvent and nearby to covalent bonding at the opening of the active-site gorge. The resulting 3D structures revealed in the active-site gorge that appear to be generated by the amino acid residues of SlAChE including Trp137, Trp505, Asp515, Met515 and Gly133 (Fig. 2B).

Furthermore, a phylogenetic tree was constructed to analyze the lineage of SlAce genes with AChEs from other species in two major clusters: AChE1 and AChE2 (Fig. 3). The phylogenetic analysis showed that both SlAce genes are grouped into two major clusters (AChE1 and AChE2). It is worth noting that, per the present nomenclature, the reported PhAce1, MpAce1, AgAce1, AgoAce1 and CtAce1 in the Ace1 group should be named CtAce1, MpAce1, AgAce1, AgoAce1 and CtAce1, respectively; whereas the reported CtAce2, MpAce2, AgAce2, AgoAce2 and PhAce2 in the Ace2 group should be named CtAce2, MpAce2, AgAce2, AgoAce2 and PhAce2, respectively (Fig. 3).

3.2. SlAce genes expression profiles in S. litura

As shown in Fig. 4A and B, both SlAce genes were expressed during different developmental stages in S. litura, including the egg, 1st to 6th instars, pupae and adults on various days. The highest expression level of SlAce1 was observed in 6th-instar larvae and the lowest during the first six days of the female pupal stage. Additionally, the expression level in 1st-day female adults was significantly higher than in male adults, whereas the level in 7th-day female adults was lower than in adult males (Fig. 4A). The highest level of SlAce2 expression was noted in male pupae on the first day, whereas the lowest expression was observed during the 12th-day male pupae. The expression level of SlAce2 of the 1st day of male pupae was significantly greater than in female pupae, and there were no significant differences between 6th and 12th-day male and female pupae. Moreover, no significant difference in the transcript level of SlAce2 was detected on the first day of the female and male adult stage. In contrast, expression during the first seven days in female adults was significantly lower than in male adults (Fig. 4B). The expression levels of SlAce1 and SlAce2 genes were also relatively high on the first day of the egg stage. Furthermore, qRT-PCR was used to measure the expression level of the two Ace genes in S. litura 6th-instar larvae, as indicated in Fig. 4C (SlAce1) and Fig. 4D (SlAce2). The results showed different levels of expression for these two genes in all measured tissues. For SlAce1, expression was the highest in the brain, followed by the midgut, the hemolymph, the fat body and the epidermis. In contrast, SlAce2 showed the highest expression in the brain, followed by the epidermis, the midgut, the hemolymph and the fat body.
3.3. Gene silencing through larval injection of dsSlAce

When 6th-instar larvae were injected with 5 μL of dsSlAce1 or dsSlAce2, suppression of these genes gradually increased until reaching the highest level of silencing on day 3. The level of gene suppression at different intervals (SlAce1 and SlAce2 on day 1, SlAce1 and SlAce2 on day 2, and SlAce1 and SlAce2 on day 3) remained at 26.8% and 45.45%, 18.3% and 37.2%, and 5.9% and 27.1%, respectively, compared with the control treatment (Fig. 5A and B).

![Gene silencing via the insertion of dsSlAce into larvae. Transcript levels were expressed as percentages compared with corresponding controls. The relative transcript levels of SlAce1 and SlAce2 in the dsRNA-injected larvae of common cutworm were determined using RT-qPCR.](image)
3.4. Injection with dsRNA delays pupation and emergence

To determine the effects of dsSlAce1 and dsSlAce2 on pupation and emergence, 6th instar larvae were injected with 5 μl dsRNA against SlAce1 or SlAce2, which considerably postponed pupation and emergence of S. litura. Although larvae treated with buffer (control) showed 100% pupation at 3 days after injection (Fig. 6A), larvae treated with dsRNA (dsAce1 or dsAce2) exhibited pupation rates of only 86% and 73.2%, respectively. Regarding eclosion, 100% was obtained within 14 days after 6th instar larvae were injected dsGFP (Fig. 6B). In contrast, the larvae treated with dsRNA showed only 89.2% and 77.7% eclosion for dsSlAce1 and dsSlAce2, respectively. Larval mortality was evaluated in 24-h intervals after dsRNA injection, the results indicated a mortality rate of 47.3% and 37.9% for SlAce1 and SlAce2, respectively; in comparison, only 7% mortality was observed when larvae were injected with dsGFP (Fig. 6C).

3.5. dsAce2 injection affects egg laying, egg hatching and progeny development

Because dsSlAce2 treatment of 6th instar larvae impacted insect endurance in the same age group, we also investigated (1) whether dsSlAce2 in 6th instar larvae influences reproduction in mature females, (2) whether the impact of RNAi is inherited by the next generation, and (3) which sex is responsible for such inheritance (Fig. 7A). We observed remarkable decreases in egg laying (Fig. 7B), egg hatching (Fig. 7C), and larval body weight in the next age group (Fig. 7D) when female larvae were injected with dsSlAce2. The numbers of pupae and adults among progeny were reduced when female larvae were injected with dsSlAce2 compared to dsGFP (Fig. 7E).

4. Discussion

Acetylcholinesterase is reported to play a canonical biological role in the termination of impulse transmissions at cholinergic synapses by rapidly hydrolyzing its substrate acetylcholine to acetate and choline in animals (Soreq and Seidman, 2001). In addition to the cholinergic roles in cholinergic synapses, the non-cholinergic roles for cholinesterase (i.e., cell adhesion, neurite growth, and apoptosis) have also been reported in vertebrates (Park et al., 2004; Zhang and Greenberg, 2012) and invertebrates (Kumar et al., 2009; He et al., 2012; Lu et al., 2012b). The first insect AChE orthologous gene (Ace2 or AO-Ace) and a paralogous gene (Ace1 or AP-Ace) were reported in D. melanogaster in 1986 (Hall and Spierer, 1986) and in Schizaphis graminum in 2002, respectively (Gao et al., 2002). Since, related cDNAs (encoding both Ace1 and Ace2) have been sequenced in at least 27 insect species (Lu et al., 2012a). To our knowledge, this is the first study on the two Ace genes in common cutworm species. The SlAce1 cDNA consists of 2,085bp and encodes 694 amino acid residues, whereas the SlAce2 cDNA contains 1,917bp and encodes 638 amino acid residues. The SlAce genes are recognized by their similarities and identities with other known insect Aces genes and are substantially more similar to lepidopteran Aces than any other Ace genes (Fig. 1A, B). Based on the deduced amino acid sequences, the SlAce1 and SlAce2 encoded by the two genes contain characteristic amino acids, including the catalytic triad, anionic choline-binding site, an oxyanion hole, acyl pocket, peripheral anionic subsite, the characteristic “FGESAG” motif surrounding the active serine residue, and the conserved 14 aromatic amino acids in the lining of catalytic gorge (Wang et al., 2016b). Using a reported simulation-refined model of D. melanogaster (Harel et al., 2000), and the same multiple molecular dynamics simulation method to model and refine SlAce1, we obtained models of SlAce1 which is in complex with acetylcholine. The SlAce1 model has an active site that is almost identical to those of A. gambiae and S. graminum APAChEs, and it has Cys432 at the opening of the active-site gorge just like the insect-specific C286 of A. gambiae and C289 of S. graminum that are susceptible to sulfhydryl agents (Pang, 2006, 2007) (Fig. 2A). Nevertheless, the AChE1-specific Cys288 residue was proposed to be accessible to sulfhydryl agents for modification (Pang et al., 2012). Furthermore, the phylogenetic tree analysis revealed
that the genes from *S. litura* are similar to those from *Helicoverpa assulta* (HaAce1) and *Bombyx mandarina* (BmAce1) whereas Ace2 was more closely associated with the proteins of *Bombyx mandarina* (BmAce2) and *Bemisia tabaci* (BtAce2). Overall, these data indicate that SlAce1 and SlAce2 are members of the AChE family.

The gene expression of insects shows different patterns in different developmental stages and tissues, such as *BmAce2* gene expression of silkworm (*Bombyx mori*) was significantly higher in males than females during the mature stage (Chen et al., 2009). In contrast, Ace1 expression was greater than Ace2 in insects such as *P. xylostella* (Baek et al., 2005),
H. assulta (Lee et al., 2006) and Tribolium castaneum (Lu et al., 2012b). In C. pipiens, two ace genes displayed the same description pattern at different developmental stages (Huchard et al., 2006). Our results indicated that the expression of SlAce1 was significantly greater in the 6th instar (100-fold), whereas it was its lowest in the first six days of the female pupae (5.6-fold). Moreover, expression rates of the 1st day female adults (49.2-fold) were considerably higher than for mature males (13.8-fold), whereas the expression rates of the 7th day female adults (28.2-fold) were less than those of the mature males (66.4-fold) (Fig. 4A). SlAce2 was more prominent in the 1st day male pupae (100-fold) than the female pupae (39.7-fold), and there were no significant differences between the 6-12 (4.1/4.2-fold) and 12-10 (8.7/8.3-fold) male and female pupae, respectively. Additionally, on the first day of female and male adults (8.7/9.3-fold), no significant impact on the transcript levels of SlAce2 was detected. In contrast, the first seven days of mature females (12.1-fold) had significantly lower transcript levels than the mature males (30.2-fold), as shown in Fig. 4B. In the German cockroach, both Ace genes were described in dissimilar tissues, and the relative transcript level of Bgace1 was approximately 10-fold greater than that of Bgace2 in both of the studied tissues (Kim et al., 2006). In this study, we found that the expression of Ace genes in the brain of the common cutworm was substantially higher than that for other tissues (Fig. 4C, D).

In the present study, we also employed the RNAi technique to systematically investigate the functions of Ace1 and Ace2 in the common cutworm S. litura. Ace1 is the major AChE responsible for the physiological hydrolysis of ACh at the synapses in C. pipiens (Bourguet et al., 1996), P. xylostella (He et al., 2012), and C. suppressalis (Hui, 2011). Thus far, studies have suggested that the Ace gene encodes major AChE and that this gene is likely connected with OP and CB pes-ticide tolerance (Weill et al., 2003; Baek et al., 2005; Labbé et al., 2007; Allot and Weill, 2008; Djogbenou et al., 2008; Wang et al., 2016a). The present results appear to be consistent with these studies. Notably, both dsRNA-Ace1 and dsRNA-Ace2 drastically suppressed their respective transcript levels from day 1 to day 3 and reached their highest silencing at day 3 post-injection (Fig. 5 A and B). After the 6-day larvae were treated with dsSlAce1 or dsSlAce2, we observed 47.3% and 37.9% mortality within three days. In comparison, the insertion of dsGFP (control) did not lead to any significant mortality (Fig. 6C). These results clearly indicated that both SlAce1 and SlAce2 are vital for insect survivability because the silencing of either AChE genes would drastically suppress SlAce1 and SlAce2 in both of the studied tissues (Fig. 4C, D).

In summary, the present study is the first to investigate the functional differences in the AChE genes (SlAce1 and SlAce2) in S. litura. Based on the results, we can conclude that Ace1 has cholinergic function because the suppression of its transcript by RNAi impacts the common cutworm’s life span and could lead to death and Ace2 has an additional non-cholinergic as its suppression caused significant reduction in female breeding, embryo progress, and the development of progeny.

Acknowledgments

This research was supported by the National Natural Science Foundation of China (No. 31201544) and the National Key Technology R&D Program of China (No. 2012BAD27802).

References


Lu, Y., Park, Y., Gao, X., Zhang, X., Yao, J., Pang, Y.P., Jiang, H., Zhu, K.Y., 2012b. Cholinergic function because the silencing of either AChE genes would drastically suppress SlAce1 and SlAce2 in both of the studied tissues (Fig. 4C, D).


