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

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ABSTRACT

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. Phylogenic 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 37.9% (*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. *Ace*1 and *Ace*2) were silenced in *S. litura* confirms its insecticidal effectiveness, which provided a molecular basis in biological pest control approach.

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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 that 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. *Ace*1 and *Ace*2) (Kim and Lee, 2013). *Ace*1 refers to AP-*Ace* gene as it is an additional gene which is found in insects other than the Cyclorrhapha flies, whereas *Ace*2 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 *Ace*1 and *Ace*2 have been reported in 27 insect species, for instance, *Myzus persicae*, *Plutella xylostella*, *Rhopalosiphum padi* and *Sitobion avenae*, *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; Zhang 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 *Tribolium castaneum* plays an essential non-cholinergic role in female

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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 $25 \pm 1\,°C$, a photoperiod of 14L: 10D and a relative humidity of 60–70%. The adult males and females were allowed to emerge in transparent cages and were fed a diluted honey solution (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 databanks 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.or), 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: TcaAce (CAA27169, *Torpedo californica*); TcAce1 (HQ260968, *Tribolium castaneum* Ace1); TcAce2 (HQ260969, *Tribolium castaneum* Ace2); BmAce1(EU262633, *Bombyx mandarina* Ace1); BmAce2 (EU262632, *Bombyx mandarina* Ace2); SaAce1 (AY819704, *Sitobion avenae* Ace1); SaAce2 (AY707319, *Sitobion avenae* Ace2); RpAce1 (AY667435, *Rhopalosiphum padi* Ace1); RpAce2 (AY707318, *Rhopalosiphum padi* Ace2); DmAce (X05893, *Drosophila melanogaster* Ace); AgAce1 (XM_321792, *Anopheles gambiae* Ace1); AgAce2 (BN000067, *Anopheles gambiae* Ace2); LdeAce1 (FJ647186, *Liposcelis decolor* Ace1); LdeAce2 (FJ647187, *Liposcelis decolor* Ace2); OvAce1 (FJ228227, *Orchesella villosa* Ace1); OvAce2 (FJ228228, *Orchesella villosa* Ace2); LeAce1 (EU854149, *Liposcelis entomophila* Ace1); LeAce2 (EU854150, *Liposcelis entomophila* Ace2); BgAce1 (DQ288249, *Blattella germanica* Ace1); BgAce2 (DQ288847, *Blattella germanica* Ace2); BtAce1 (EF675188, *Bemisia tabaci* Ace1); BtAce2 (EF675190, *Bemisia tabaci* Ace2); CqAce1 (XM_001847396, *Culex quinquefasciatus* Ace1); CqAce2 (XM_001842175, *Culex quinquefasciatus* Ace2); BmoAce1 (NP_001037380 *Bombyx mori* Ace1); BmoAce2 (NP_001108113 *Bombyx mori* Ace2); ApAce1 (XM_001948618, *Acyrthosiphon pisum* Ace1); ApAce2 (XM_001948953, *Acyrthosiphon pisum* Ace2); NvAce1 (XM_001600408, *Nasonia vitripennis* Ace1); NvAce2 (XM_001605518, *Nasonia vitripennis* Ace2); PhAce1 (AB266605, *Pediculus humanus corporis* Ace1); PhAce2 (AB266606, *Pediculus humanus corporis* Ace2); CpAce1 (DQ267977, *Cydia pomonella* Ace1); CpAce2 (DQ267976, *Cydia pomonella* Ace2); HaAce1 (DQ001323, *Helicoverpa assulta* Ace1); HaAce2 (AY817736, *Helicoverpa assulta* Ace2); AaAce1 (AB218421, *Aedes albopictus* Ace1); AaAce2 (AB218420, *Aedes albopictus* Ace2); AgoAce1 (AF502081, *Aphis gossypii* Ace1); AgoAce2 (AF502082, *Aphis gossypii* Ace2); CtAce1 (AB122151, *Culex tritaeniorhynchus* Ace1); CtAce2 (AB122152, *Culex tritaeniorhynchus* Ace2); SgAce1 (AF321574, *Schizaphis graminum* Ace1); MpAce1 (AF287291, *Myzus persicae* Ace1); MpAce2 (AY147797, *Myzus persicae* Ace2); CpiAce1 (AJ489456, *Culex pipiens* Ace1); CpiAce2 (AM159193, *Culex pipiens* Ace2); MdAce (AY134873, *Musca domestica* Ace); PxAce1 (AY970293, *Plutella xylostella* Ace1); PxAce2 (AY061975, *Plutella xylostella* Ace2); CsAce1 (EF453724, *Chilo suppressalis* Ace1); CsAce2 (EF470245, *Chilo suppressalis* Ace2); CmAce2 (FN538987, *Cnaphalocrocis medinalis* Ace2); PhcAce1 (AB266614, *Pediculus humanus capitis* Ace1); PhcAce2 (AB266615, *Pediculus humanus capitis* Ace2); TuAce (AY188448, *Tetranychus urticae* Ace); AaeAce1 (EF209048, *Aedes aegypti* Ace1); LmAce1 (EU231603, *Locusta migratoria manilensis* Ace1); LbAce1 (FJ647185, *Liposcelis bostrychophila* Ace1); LbAce2 (EF362950, *Liposcelis bostrychophila* Ace2); NlAce (FM866396, *Nilaparvata lugens* Ace); CcAce (EU130781, *Ceratitis captitata* Ace); AmAce (AB181702, *Apis mellifera* Ace); BdAce2 (AY155500, *Bactrocera dorsalis* Ace2); HiAce (AY466160, *Haematobia irritans* Ace); CppAce1 (AY762905, *Culex pipiens pallens* Ace1); HarAce2 (AF369793, *Helicoverpa armigera* Ace2); BoAce (AF452052; *Bactrocera oleae* Ace); LcAce (U88631, *Lucilia cuprina* Ace); NcAce (AF145235, *Nephotettix cincticeps* Ace); LdAce2 (L41180, *Leptinotarsa decemlineata* Ace2); SlAce1 (KY130418, *Spodoptera litura* Ace1); SlAce2 (KY130419, *Spodoptera litura* 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-pupae, 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 TRIzol 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 DNase1 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 iQ2 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 $2^{-\triangle\triangle CT}$ 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

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

Primer name	Primer sequences	Product size (bp)
PCR for cDNA sequences		
SlAce1-F	ATGCGCGTGGTGTTGGCAGCGCTGACGG	2085
SlAce1-R	TTACACGATATACTTGAACAT	
SlAce2-F	ATGATCAGCAACACGAAGATTGTGTTC	1917
SlAce2-R	TTACAACGCGACGGTTGTGGC	
Quantitative real-time PCR		
SlAce1-(Q)F	GGAAGAGTAAGAGGCATCA	163
SlAce1-(Q)R	GTGGCAGTGTCGTAGTAT	
SlAce2-(Q)F	CTAGGACCGTTAAGATTC	234
SlAce2-(Q)R	CTCCGTAAGAGGTTTATC	
RPL10-F	GACTTGGGTAAGAAGAAG	189
RPL10-R	GATGACATGGAATGGATG	
GAPDH-F4	GGGTATTCTTGACTACAC	184
GAPDH-R2	CTGGATGTACTTGATGAG	
double-stranded RNA synthesis[a]		
ds*SlAce1*-F	ggatcctaatacgactcactataggTTGGCAGACAGGCTATT	371
ds*SlAce1*-R	ggatcctaatacgactcactataggTTCTCCATTCGGGTTTAT	
ds*SlAce2*-F	ggatcctaatacgactcactataggACACTGGCATTTTGGGATT	392
ds*SlAce2*-R	ggatcctaatacgactcactataggATCCGCCAACACTTCCGC	

[a] Sequences underlines indicate T7 RNA Polymerase promoter.

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 ds*SlAce2* 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 ds*SlAce2* 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 ds*SlAce2*; (III) an adult male developed from a larva injected with ds*SlAce2* 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 ds*SlAce2*. 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.
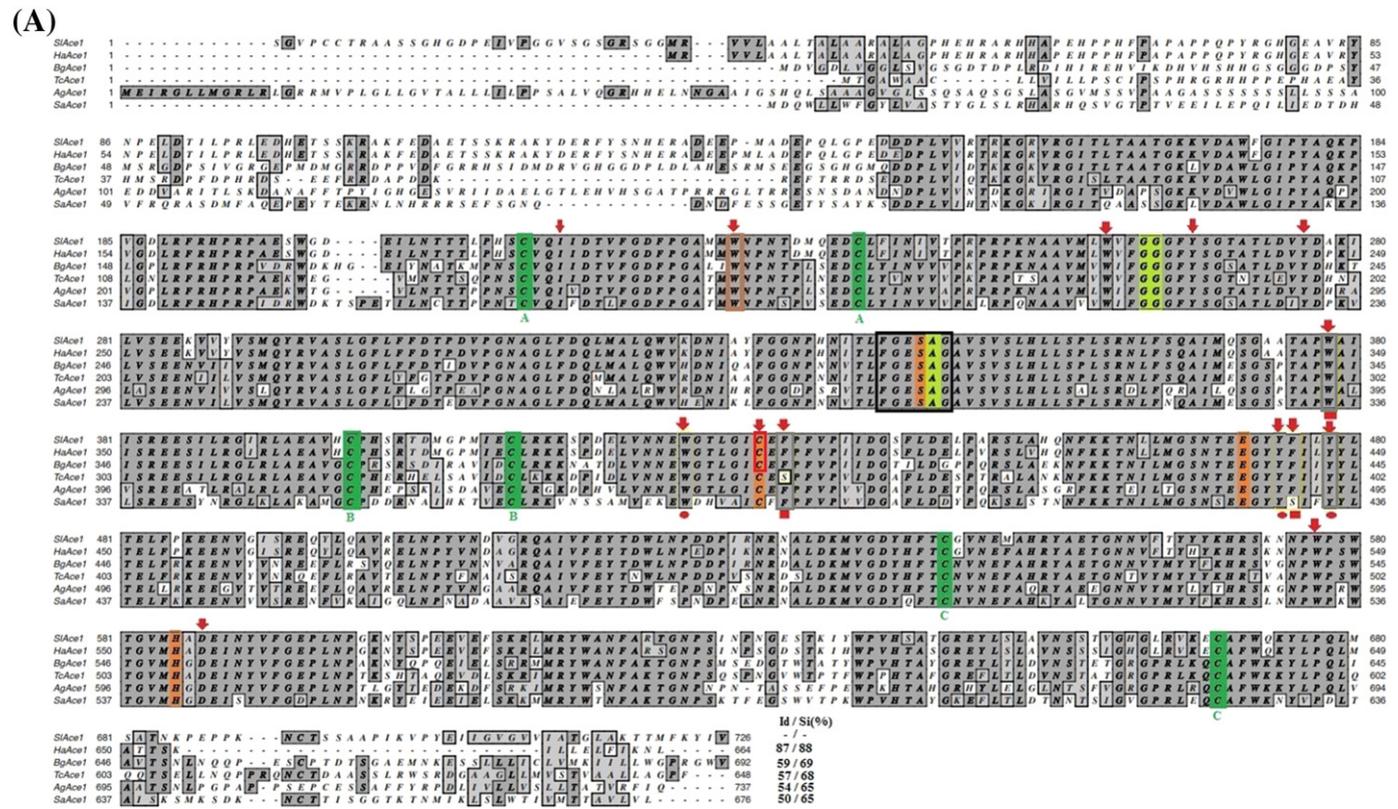
Fig. 1. Gene cloning of *SlAce1* and *SlAce2*: (A) Multiple comparisons of amino acid sequences of *SlAce1* with other insects were performed. The catalytic triads are shown in orange; the oxianion hole is shown in lime. Acyl pockets are identified by red cycles; peripheral anionic subsites are identified by red squires. The choline-binding sites are shown in brown box, the characteristic FGESAG motif are indicated in black box, and the positional conserved cysteine residues in AChE1s are identified in red box. The positions of aromatic residues lining the active site gorge with red arrows and cysteine residues forming intramolecular disulfide bonds are in green with the letters A, B, and C. GenBank accession numbers of sequences used: HaAce1 (DQ001323, *Helicoverpa assulta* Ace1); BgAce1 (DQ288249, *Blattella germanica* Ace1); TcAce1 (HQ260968, *Tribolium castaneum* Ace1); AgAce1 (XM_321792, *Anopheles gambiae* Ace1); SaAce1 (AY819704, *Sitobion avenae* Ace1); SlAce1 (KY130418, *Spodoptera litura* Ace1). (B) Alignment of the amino acid sequence of SlAce2 with known insects is shown. HaAce2 (AY817736, *Helicoverpa assulta* Ace2); BgAce2 (DQ288847, *Blattella germanica* Ace2); TcAce2 (HQ260969, *Tribolium castaneum* Ace2); AgAce2 (BN000067, *Anopheles gambiae* Ace2); SaAce2 (AY707319, *Sitobion avenae* Ace2); SlAce1 (KY130419, *Spodoptera litura* Ace2).

(B)
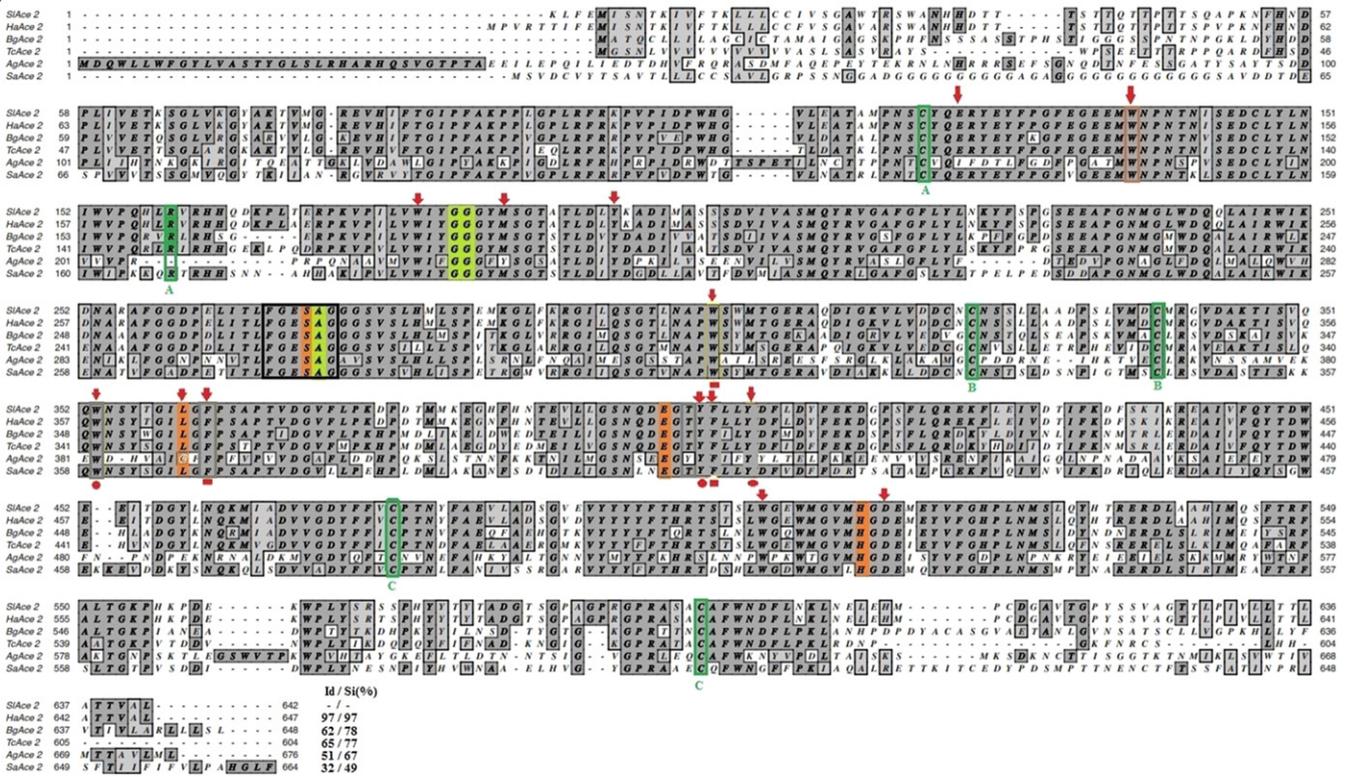
Fig. 1 (*continued*).

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) **(B)**
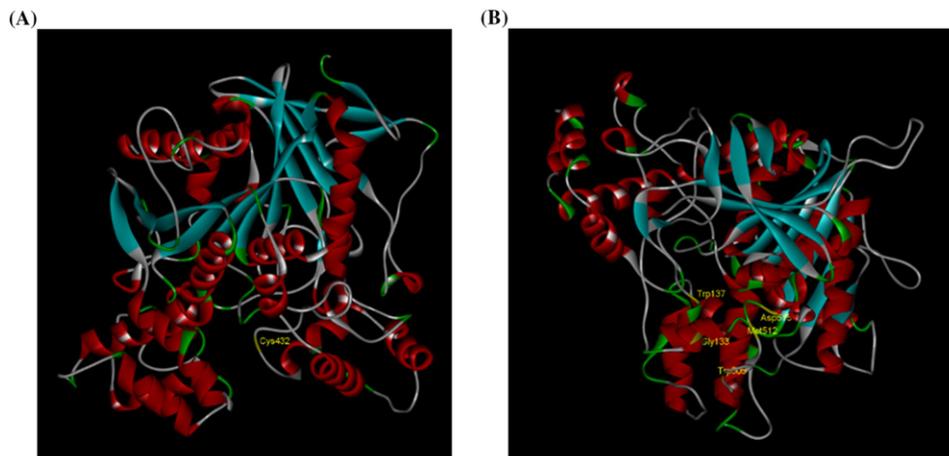
Fig. 2. The predicted tertiary structure of SlAce1 and SlAce2. (A) Overlay of *SlAce1* and a human butyrylcholinesterase from a perspective looking down onto the active site. (B) Overlay of SlAce2 and *D. melanogaster* acetylcholinesterase from a perspective looking down onto the active site.

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 oxyanion hole, an acyl pocket, sites of choline-binding, and a peripheral anionic subsite (Fig. 1A, B). Both SlAChEs not only possess 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 SlAChEs (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 butyrylcholinesterase (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 SlAce1 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 SlAce2 including Trp137, Trp505, Asp515, Met515 and Gly133 (Fig. 2B).

Furthermore, a phylogenic 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 PhCAce1, MpAce1, AgAce1, AgoAce1 and CtAce1 in the Ace2 group should change to PhAce2, AgAce2, AgoAce2, MpAce2 and CtAce2, respectively, whereas the reported CtAce2, MpAce2, AgAce2, AgoAce2 and PhcAce2 in the Ace1 group should be named CtAce1, MpAce1, AgAce1, AgoAce1 and PhcAce1, 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.
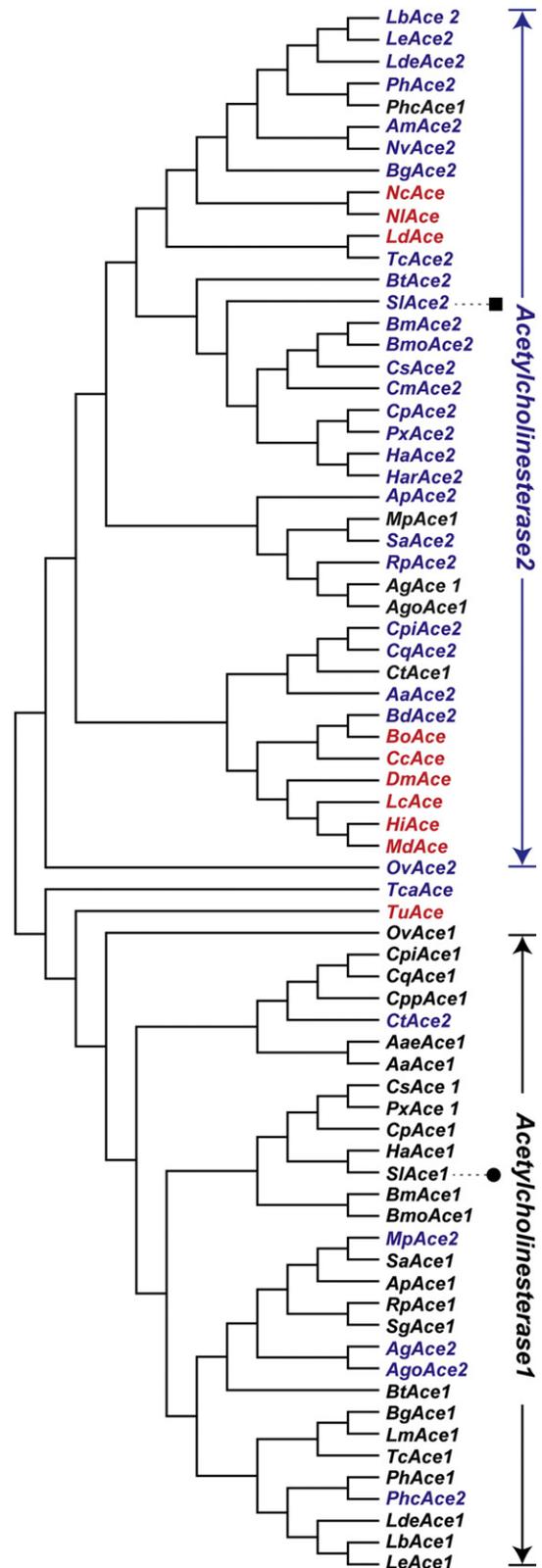
Fig. 3. Phylogenetic analysis of *Spodoptera litura* and other known insect AChE sequences. Rooted phylogenetic tree of deduced *Ace* amino acid sequences from *Torpedo californica* and 46 insects were constructed using a maximum likelihood method. The names of the insects in this figure are given as the first letter of the genus followed by the first one or two letters of the specific name.
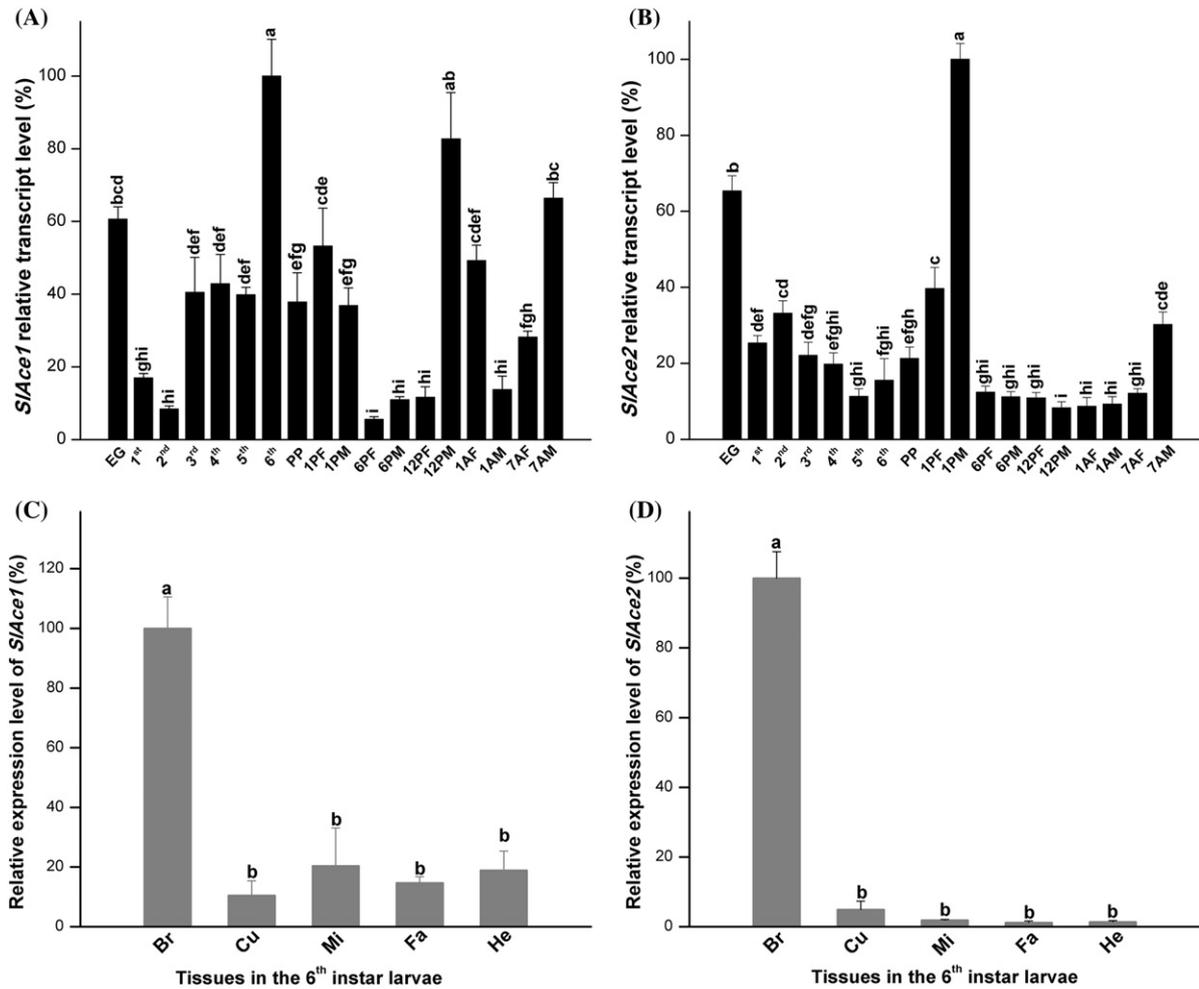
Fig. 4. Expression profile of the *SlAce1* (A) and *SlAce2* (B) genes in different developmental stages in *S. litura*. EG (1-d eggs)1st (first instar larvae), 2nd (second instar larvae), 3rd (third instar larvae), 4th (fourth instar larvae), 5th (fifth instar larvae), 6th (sixth instar larvae), PP (prepupae),1PF (1-d female pupae), 1PM (1-d male pupae), 6PF (6-d female pupae), 6PM (6-d male pupae), 12PF (12-d female pupae), 12PM (12-d male pupae), 1AF (1-d female adults), 1AM (1-d male adults), 7AF (7-d female adults) and 7AM, 7-dmale adults. The expression of the *SlAce1* (C) *SlAce2* (D) genes in different tissues of 6th instar larvae. Br (Brain), Cu (Cuticle), Mi (Midgut), Fa (Fat body), He (Hemolymph). The results are presented as the mean and standard errors of three biological replicates. Different litters on the bars of the histogram indicate significant differences based ANOVA followed by Dancans multiple range test ($P < 0.05$).

3.3. Gene silencing through larvae injection of dsSlAce

When 6th-instar larvae were injected with 5 μL of ds*SlAce1* or ds*SlAce2*, 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).
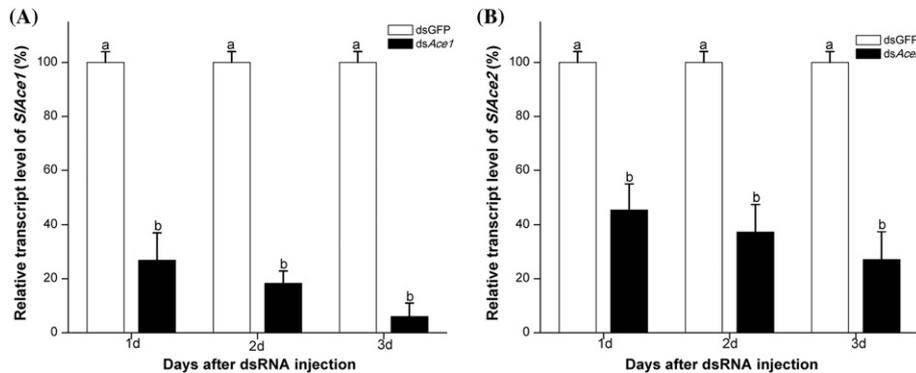
Fig. 5. Gene silencing via the insertion of ds*SlAce* in to larvae. Transcript levels were expressed as percentages compared with corresponding controls. The relative transcript levels of *SlAce1* and *SlAce2* in the dsRNA-injected larvae of common cutworm were determined using RT-qPCR. The outcomes are displayed as the means and standard errors of three biological replicates. The letters on the bars of the histogram indicate significant differences.

3.4. Injection with dsRNA delays pupation and emergence

To determine the effects of ds*SlAce1* and ds*SlAce2* 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 (ds*Ace1* or ds*Ace2*) 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 ds*SlAce1* and ds*SlAce2*, 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 ds*SlAce2* treatment of 6th-instar larvae impacted insect endurance in the same age group, we also investigated (1) whether ds*SlAce2* 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 ds*SlAce2*. The numbers of pupae and adults among progeny were reduced when female larvae were injected with ds*SlAce2* 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 cholinebinding 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
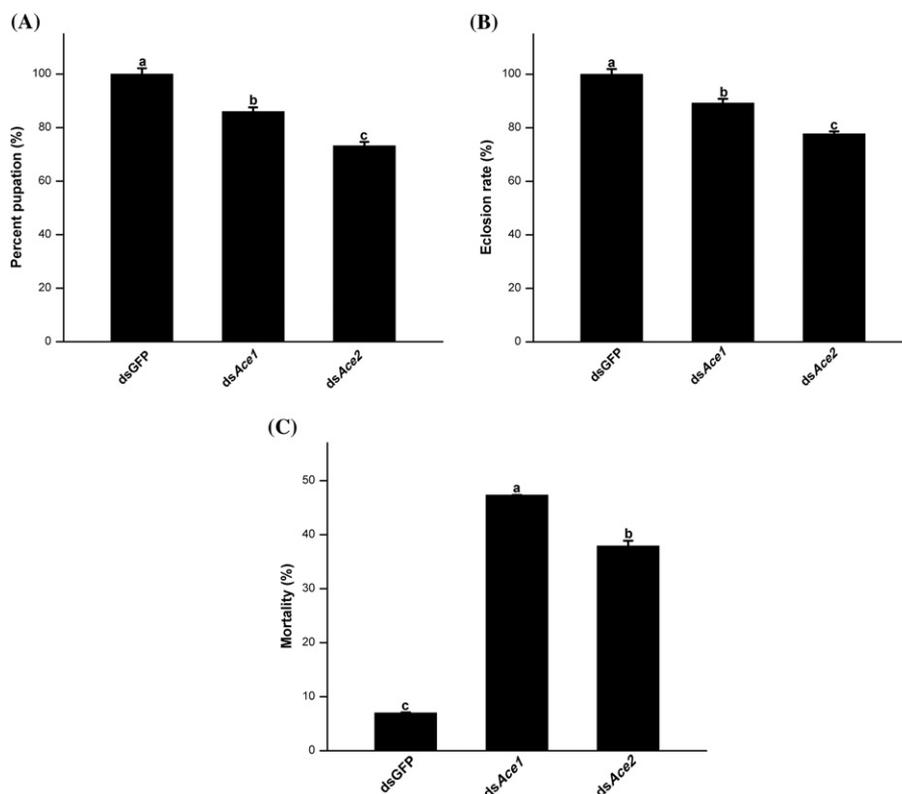
Fig. 6. The influence of gene suppression mediated by RNAi on pupation (A), eclosion (B) and mortality (C) after 6th instar larvae of *S. litura* were inserted with dsGFP (control), ds*SlAce1*, or ds*SlAce2* (treatments). The outcome is presented as the means and standard errors of three replicates (each replicate was conducted with at least 30 insects).
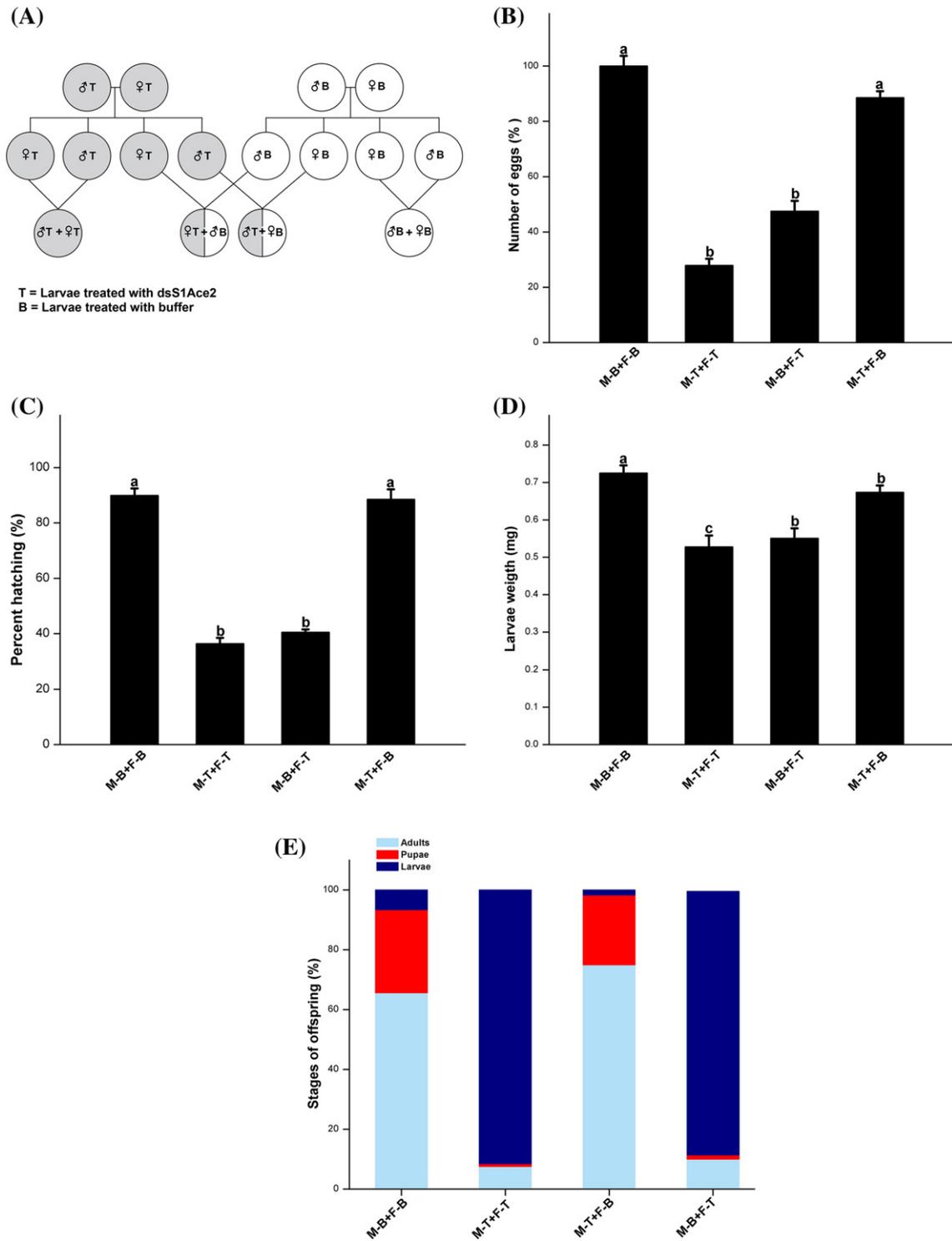
Fig. 7. The influence of generations of RNAi for ds*SlAce2* after 6-day larvae of *S. litura* were injected with ds*SlAce2* (A). ♂**T** × ♀**T**: a male injected with ds*SlAce2* mating with a female injected with ds*SlAce2*; ♂**B** × ♀**T**: a male injected with dsGFP mating with a female injected with ds*SlAce2*; ♂**T** × ♀**B**: a male injected with ds*SlAce2* mating with a female injected with dsGFP; ♂**B** × ♀**B**: a male injected with dsGFP mating with a female injected with dsGFP. An aliquot of 5 μL of ds*SlAce2* was used in all treatments. The experiment was implemented in three replicates (each with eight male and female pairs). The influence of generations of RNAi for ds*SlAce2* on female egg laying (B), egg hatching (C), larval body weight (D) and distribution of stages of the next generation (E) after 6th instar larvae of *S. litura* were injected with ds*SlAce2*. An aliquot of 5 μL ds*SlAce2* was inserted into all medications. The results are shown as the means and standard errors of three replicates (each was performed with eight pairs of the female and male). The letters above the standard error bars indicate considerable dissimilarities.

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-day (12.4/11.2-fold) and 12-day (10.8/8.3-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*. AChE1 is the major AChE responsible for the physiological hydrolysis of AChE at the synapses in *C. pipiens* (Bourguet et al., 1996), *P. xylostella* (He et al., 2012), and *C. suppressalis* (Hui et al., 2011). Thus far, studies have suggested that the *Ace1* gene encodes major AChE and that this gene is likely connected with OP and CB pesticide tolerance (Weill et al., 2003; Baek et al., 2005; Labbé et al., 2007; Alout 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 ds*SlAce1* or ds*SlAce2*, 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 lead to death. Notably, the current study also showed that the suppression of the *Ace2* transcript led to significant reductions in the common cutworm related to fecundity, hatching and offspring in the parental generation (Fig. 7B, C and E). This outcome suggested that *Ace2* of *S. litura* has non-cholinergic functions.

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 *Ace*2 has an additional non-cholinergic as its suppression caused significant reduction in female breeding, embryo progress, and the development of progeny.

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