Journal of Asia-Pacific Entomology 19 (2016) 341–345

Contents lists available at ScienceDirect

Journal of Asia-Pacific Entomology

journal homepage: www.elsevier.com/locate/jape

Effects of carboxylesterase gene silence on wheat aphid *Sitobion avenae* (Fabricius)

Yifan Zhang [a,1], Fei Deng [a,1], Yongliang Fan [b], Zhangwu Zhao [a,*]

[a] Department of Entomology, China Agricultural University, Beijing 100193, China
[b] State Key Laboratory of Crop Stress Biology for Arid Areas, College of Plant Protection, Northwest A&F University, Yangling, Shaanxi 712100, China

ARTICLE INFO

Article history:
Received 5 November 2015
Revised 21 February 2016
Accepted 23 March 2016
Available online 31 March 2016

Keywords:
Carboxylesterase
RNAi
Sitobion avenae
Wheat aphid

ABSTRACT

Multifunctional carboxylesterase (CarE) has been found in all animals, plants and microbes, and belongs to a superfamily enzyme of serine hydrolase involved in detoxification, allelochemical tolerance and some specific hormone or pheromone metabolism. Insects usually utilize carboxylesterases to detoxify xenobiotics, and positively correlated with insect resistance to some insecticides. Despite the importance of CarEs in insects, carboxylesterases and their functions in wheat aphid *Sitobion avenae* (Fabricius) have not been clear. In this study, a sequence that encodes a carboxylesterase protein from *S. avenae* (*SaCarE*) was sequenced and cloned. After aligning the encoded amino acid sequence of the *SaCarE* gene with other known *CarEs* of insects, we found that the *CarE* gene was highly conserved in insects. The *SaCarE* mRNA levels at different developmental stages of *S. avenae* were gradually increased from the first instar of nymphs to adult stage. RNAi was employed to further explore its functions, in which oral ingestion of *SaCarE* double-stranded RNA from the third instar nymph significantly knocked down *SaCarE* expression, and significantly decreased ecdysis index in *S. avenae*. These results indicate that *SaCarE* is functional in *S. avenae* and could serve one of the potential target genes for management of *S. avenae*.

Introduction

Carboxylesterase (CarE) widely exists in animals, plants and microorganisms (Satoh and Hosokawa, 1998; Marshall et al., 2003; Miyazaki et al., 2003; Furihata et al., 2004). It is one group of important enzymes involved in insect detoxification, and plays an important role in insect resistance mechanism. When insects are exposed to exogenous harmful materials such as medicine, pesticide, herbicide, phenols, alkaloids and plant secondary metabolites, the activity of carboxylesterase increases and the adaptability of insects is enhanced. Studies have found that many insects resistance to organophosphorus insecticide agents were associated with excessive expression of a carboxylesterase gene (Vaughan and Hemingway, 1995; Karunaratne et al., 1999). CarE activity reflects the resistance of the peach aphid *Myzus persicae* to organophosphate, carbamate and pyrethroid pesticides (Bizzaro et al., 2005). In addition, carboxylesterase is also involved in the degradation of insect pheromones and hormones. Durand found that the carboxylesterase in antennae of *Spodoptera littoralis* could metabolize smell matters of plant leaf (Durand et al., 2010). Other studies found that antennae rich in esterase are associated with the degradation of the ester type of sex pheromone in *Apis mellifera* (Kamikouchi et al., 2004) and in two pest moths, *Spodoptera littoralis* and *Sesamia nonagrioides* (Maïbèche-Coisne et al., 2004). Reports indicate that carboxylesterase plays an important role in the formation and development of the neural regulation, in which carboxylesterase participated in neural development adjustment in bees (Biswas et al., 2010). The actions of CarE to exogenous and endogenous compounds are through degrading sulphur ester keys, amide keys and ester bond hydrolyze (Miyazaki et al., 2003).

Distribution of carboxylesterase in insects is different. The larval carboxylesterase are mainly distributed in intestine, fat body, body wall, malpighian tube and other organs, while carboxylesterase of adult worms are mainly distributed in the legs, body wall and head. The expression level of CarE also varies in different insects as well as in different developmental stages of insects. Nowadays, carboxylesterase genes in many insects have been cloned, such as *M. persicae*, *Aphis gossypii*, *Rhopalosiphum padi*, *Spodoptera frugiperda*, *Spodoptera exigua*, *Helicoverpa armigera* (Blackman et al., 1996; Cao et al., 2008). However, it has not been reported in wheat aphid *Sitobion avenae* (Fabricius), and its function is still unclear. In order to

* Corresponding author at: Department of Entomology, College of Agriculture and Biotechnology, China Agricultural University, Beijing 100193, China. Tel./fax: +86 10 62734225.
E-mail address: zhaozw@cau.edu.cn (Z. Zhao).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.aspen.2016.03.011
1226-8615/

investigate the function of carboxylesterase in *S. avenae*, we used the RNAi to knock down the gene expression and observed its effects on growth and development of the wheat aphid.

Materials and methods

Insects

The colony of wheat aphid, *S. avenae* (Fabricius), was a laboratory strain kindly provided by Dr. Dunlun Song, China Agricultural University. Aphids were reared on wheat seedlings at 22 ± 1 °C under a 16:8 h light/dark photoperiod with new wheat seedlings provided once a week.

Cloning of CarE

Total RNA was extracted from the whole body of adult aphids using the TRIzol Reagent (Tiangen, Beijing, China). Extracted RNA concentration and purity was determined by spectrophotometry-NanoDrop 2000 (Thermo Scientific, Pittsburgh, PA). The synthesis of cDNA was performed with Fast Quant RT Kit (Tiangen, Beijing, China) according to the manufacturer's instructions. CarE coding sequence was amplified from *S. avenae* cDNA by PCR using 2×Taq PCR MasterMix Kit (Aidlab Biotechnologies, Beijing, China) with degenerate primers (Table 1). The PCR was performed at 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s, finished with an extension step at 72 °C for 10 min. PCR products were run on 1.5% agarose gel and stained with ethidium bromide. PCR products were purified using Universal DNA Purification Kit (Tiangen, Beijing, China). The purified fragments were cloned into PMD19-T Simple vector and transformed into *Escherichia coli* DH5α cells.

Sequence analysis

The CarE sequences of *S. avenae* were blasted at NCBI website (http://www.ncbi.nlm.nih.gov/) to ensure that no more than a 20-bp homology existed between the CarE sequences and any other genes in *S. avenae*. The CarE sequence was translated into protein with online software (http://insilico.ehu.es/translate/) to establish the degree of similarity and conservation among different CarE orthologs.

Preparation of dsRNA

The dsRNAs were synthesized *in vitro* with T7 RiboMAX™ Express RNAi System (Promega, USA). A CarE coding fragment of 312 bp was selected as RNAi target sequence. PCR primers with T7 promoter sequences were used to prepare double-stranded RNA (Table 1). The primers for green fluorescent protein gene (GFP), which served as a control in artificial feeding, are also shown in Table 1. The PCR of the target fragments was performed at 94 °C for 3 min, followed by 38 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 40 s, finishing with an extension step at 72 °C for 10 min. PCR products were purified using Universal DNA Purification Kit (Tiangen, Beijing, China). The dsRNA was synthesized by following the instructions of T7 RiboMAX™ Express RNAi System Kit, and was further purified and quantified by spectrophotometer at 260 nm.

CarE expression analysis at different instars of nymphs and adults

To study temporal expression of *CarE* during the different developmental stages, we explored different instars of larvae and adults. The temporal expression of *CarE* in *S. avenae* was estimated by qRT-PCR using RealMasterMix (SYBR Green) on StepOne™ Real-time PCR System (USA). The primers for qRT-PCR were designed and shown in Table 1. The ribosomal protein L7 (RpL7) gene showed a stable expression in different developmental stages (Wang et al., 2012) and was chosen as the control. Cycling for each reaction was carried out in a final volume of 20 μl containing 1 μl of the cDNA sample, 0.6 μl (10 pmol/μl) of each primer, 10 μl 2× SuperReal PreMix Plus, 2 μl 50× ROX Reference Dye and 5.8 μl ddH_2O.

The amplification efficiency of the gene was estimated by primer efficiency test. The PCR program consisted of an initial denaturation at 95 °C for 15 min followed by 40 cycles of denaturation at 94 °C for 10 s, annealing at 57 °C for 20 s and elongation at 72 °C for 30 s. An actin fragment was amplified as an internal control. Relative expression of the CarE gene in different stages was conducted according to threshold cycle (Ct) value based on the $2^{-\Delta\Delta CT}$ method. Each experiment was independently repeated three times and three technical replicates were performed in each of these three biological repetitions. All values were the means of three individual measurements ± SE. All the data are presented as the relative mRNA expression.

Feeding effects of dsCarE

The diet used for rearing *S. avenae* was a meridic artificial diet (see Deng and Zhao, 2014). The nymphs of the third instar were fed on three different dsCarE-containing diets (5, 10 and 20 ng/μl), and mortality was respectively recorded every day. A total of 15 individuals were transferred into a vial using a soft writing brush and the vial was sealed with stretched Parafilm. The diet containing dsCarE (2 μl dsCarE were added to every 100 μl artificial diets, which were changed every two days) was fed to the nymphs until the fourth day for expression analysis of CarE. From the background experiments above, 20 ng/μl concentration of dsCarE was selected to investigate the effects on the aphids, and dsGFP of the same concentration was also mixed into artificial diet to serve as control. The isometric DEPC H_2O was mixed into artificial diet to serve as another control.

Gene silencing analysis

The expression of CarE mRNA after dsRNA feeding was investigated by qRT-PCR as described above. Total RNA was isolated and cDNA was synthesized from day 1 through day 6 after feeding. cDNA for qRT-PCR

Table 1
CarE primers used for cDNA fragment cloning, qRT-PCR analysis and dsRNA synthesis.

Application of primers	Primers sequence (5′–3′)	PCR product size (bp)
cDNA sequencing of CarE	F: TACCCTACGCTCAACCAC R: GAACAGACGCTGATCCTG	489
dsCarE	F: TAATACGACTCACTATAGGGATGGTCTGGAGTTTT R: TAATACGACTCACTATAGGGATTACGATTCAACACTA	312
dsGFP	F: TAATACGACTCACTATAGGCACAAGTTCAGCGTGTCCG R: TAATACGACTCACTATAGGGTTCACCTTGATGCCGTTC	439
qRT-PCR analysis of CarE	F: TCCTGGAAACGTGGGCTTGAA R: AACAGACGCTGATCCTGCACTTT	127
qRT-PCR analysis of RpL7	F: CCGAAAAGCTGTCATAATGAAGACC R: GGTGAAACCTTGTCTACTGTTACATCTTG	231

was produced from 1 μg total RNA. The procedure of qRT-PCR was same as the aforementioned procedures.

Growth and development analysis

The growth and development of aphids were observed every day. Then, ecdysis index was calculated by molting numbers of aphids when the aphids were fed for 6 days ($I_m = \sum$(molting number/live insects number of the day before)). The ecdysis index was statistically analyzed in each experiment. The experiment was repeated three times. Statistical analysis of data was performed by one-way ANOVA in SPASS.

Results

Sequencing and dsRNA synthesis

A 460 bp *CarE* gene sequence from *S. avenae* was obtained by PCR, and the sequence shows 98.26% identity with the counterpart of *A. gossypii* in GenBank (accession number EU783916.1) (Fig. 1). The phylogenetic tree of deduced amino acid sequences showed that its amino acid sequence is also closest to *A. gossypii* (Fig. 2).

CarE expression analysis at different instars of nymphs and adults

The expression of *CarE* gene was steadily increasing from the 1st instar to the adult stage, and reached the highest in adulthood. The expression levels of the 4th instar and adult were significantly higher than that on other instars (Fig. 3).

Function analysis of CarE

To find a suitable dose of dsCarE, three different concentrations of dsCarE were fed starting from the third instar (L3) nymph, and the survival rate was observed every day. The relative expression of *CarE* mRNA on the fourth day after ingestion was measured by qRT-PCR. Results

```
1       ------------------------------------------------------------
2       ATGGAAGTCGTCATTGAACAAGGTGCTCTAAAAGGACTTAAAAAAAAAAACGCTATTGTCA 60


1       ----------------------------------------------------TAAATAAATTA 11
2       AACAAACCTTACGTCAGTTTTCTAGGCATACCCTACGCTCAACCACCCGTTAACGACTTA 120
                                                           *:** .*.***

1       AG-------GCTCCTGTCAA-CATCCCGGATGGTCTGGAGTTTTAAATGCTGTTTCAGAA 63
2       AGATTCAAGGCTCCTGTCAAACATCCCGGATGGTCTGGAGTTTTAAATGCTGTTTCAGAA 180
        **       ********** ****************************************

1       AGAGACAAATGCACGCAGTACGTTTTTATGACGAATCACATCGTTGGAAGTGAAGATTGC 123
2       AGAGACAAATGCACGCAGTACGTTTTTATGACGAATCACATCGTTGGAAGTGAAGATTGC 240
        ************************************************************

1       TTGTACCTAAATATATCGGTGCCACAG---AACGAATTGAATGGAAAACTTGCTGTTATG 180
2       TTGTACCTAAATATATCGGTGCCACAGCAGAACGAATTGAATGGAAAACTTGCTGTTATG 300
        ***************************   ****************************

1       ATATTCATACATGGAGGTGCCTTTAACTATGGCAGTGGGTCAATGAATGAATATTCTCCC 240
2       ATATTCATACATGGAGGTGCCTTTAACTATGGCAGTGGGTCAATGAATGAATATTCTCCC 360
        ************************************************************

1       GATTATTTTATCGACGAAAACGTGATTGTCGTCACAATAAATTATCGTCTAAACGCCCTA 300
2       GATTATTTTATCGACGAAAACGTGATTGTCGTCACAATAAATTATCGTCTAAACGCCCTA 420
        ************************************************************

1       GGATTTCTAAACTTGGATATTGACGAGTGTCCTGGAAACGTGGGCTTGAAAGATCAACTA 360
2       GGATTTCTAAACTTGGATATTGACGAGTGTCCTGGAAACGTGGGCTTGAAAGATCAACTA 480
        ************************************************************

1       TTTGCAATCAAATGGGTTAAAGCGAATATAGCTGCATTTGGGGGTGATGTAAACAATATC 420
2       TTTGCAATCAAATGGGTTAAAGCGAATATAGCTGCATTTGGGGGTGATGTAAACAATATC 540
        ************************************************************

1       ACCATATTCGGTGAAAGTGCAGGATCAGCGTCTGTTCATC---------------------- 460
2       ACCATATTCGGTGAAAGTGCAGGATCAGCGTCTGTTCATTATCACACAATATCACCACAA 600
        ****************************************
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Fig. 1. Sequence alignment of *CarEs* in *S. avenae* and *A. gossypii*. 1 and 2 represent *CarE* gene sequences of *S. avenae* and *A. gossypii*, respectively.

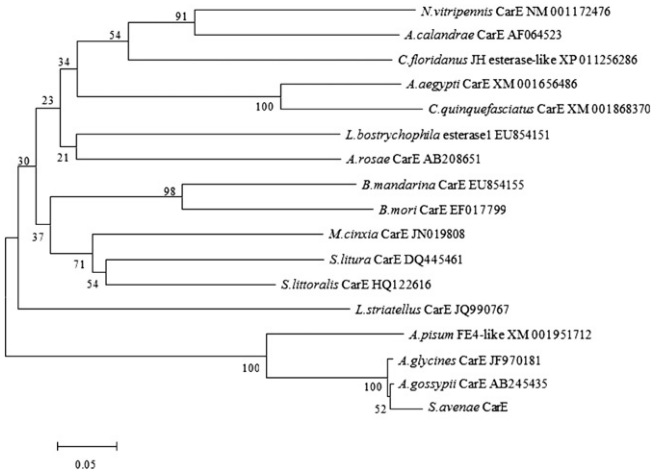

Fig. 2. The phylogenetic relationships of insect carboxylesterases. The tree was constructed by the neighbor-joining method using MEGA software. Bootstrap analyses of 5,000 replications are shown. The sequences were obtained from GenBank. Notes: CarE: carboxylesterase; JH: juvenile hormone.

revealed that the 20 ng/μl dose dsCarE was effective (Fig. 4). When the aphids were continuously fed with dsCarE, *CarE* expression was significantly reduced compared to control group ($P < 0.05$) on the sixth day (Fig. 5). Moreover, a significant differential in the rate of development was observed in this experiment. The development of the aphids fed dsCarE was gradually slowed down. After 6 days of dsCarE ingestion, the ecdysis index of each experimental group was calculated. The ecdysis index of the group fed dsCarE was lower than that of the controls. In the treatment group, the ecdysis index was 1.34 ± 0.05, while it was 1.73 ± 0.07 in the dsGFP group and 1.75 ± 0.10 in the DEPC H_2O group (Fig. 6), indicating that CarE plays an important role in growth and development of *S. avenae*. However, the survival rate had no significant differences between the treatment group and the control group (Supplementary Fig. 1).

Discussion

In this study, a fragment of cDNA sequence encoding a carboxylesterase gene was cloned from the Homopterous insect *S. avenae*. The successful cloning of *SaCarE* provides a basis for cloning of the corresponding full-length carboxylesterase cDNA and further

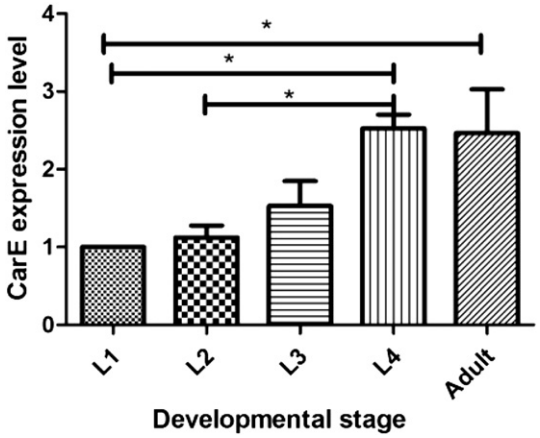

Fig. 3. The *CarE* expression levels in different developmental stages of *S. avenae*. *, indicates $p < 0.05$ between treatment and control analyzed by one-way ANOVA. Error bars are standard errors of three independent biological replicates.

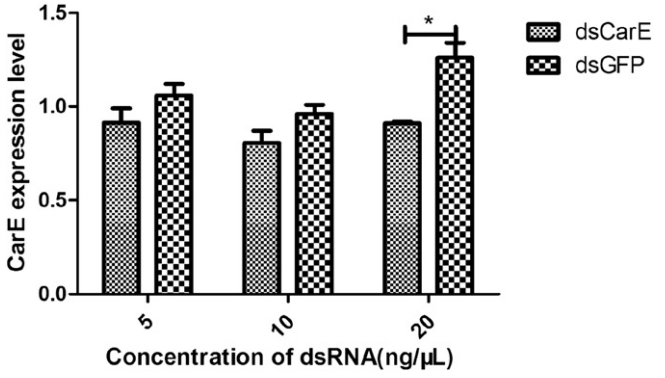

Fig. 4. Relative expression of *CarE* treated by different concentration of dsCarE. *, indicates $p < 0.05$ between treatments and controls analyzed by one-way ANOVA. Error bars are standard errors of three independent biological replicates.

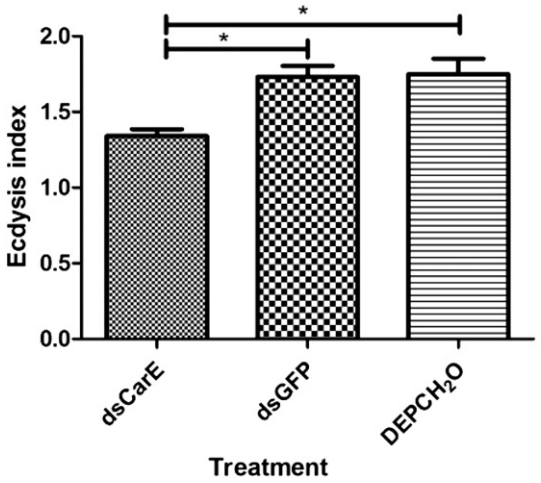

Fig. 6. The ecdysis index of *S. avenae*. *, indicates $p < 0.05$ between treatments and controls analyzed by one-way ANOVA. Error bars are standard errors of three independent biological replicates.

research on its properties and biological functions. To detect the expression pattern of *SaCarE*, qRT-PCR was used to determine the relative expression level of the *SaCarE* in various developmental stages. *SaCarE* transcript was detected at all life stages, and the highest expression was shown in the adult stage. This is in agreement with the finding of carboxylesterase E4 (Xu et al., 2014) of *S. avenae*. Carboxylesterase E4 has been proved to detoxify the phoxim insecticide.

To explore the function of *SaCarE*, we conducted RNAi knockdown by continuous feeding of dsCarE to reduce the carboxylesterase expression starting from the third instar nymph. A reduction of *SaCarE* mRNA and a decrease of insect growth and development were observed in the dsCarE diet feeding assay. This indicates that wild-type carboxylesterase in *S. avenae* is important for maintaining normal growth besides of the function on resistance of heterogeneous substances.

In recent years, several efficient methods to deliver dsRNA into insects have been developed to knock down a specific gene expression. Besides, microinjection is the most direct method to knock down the expression of target genes. Previous studies have demonstrated that feeding-based RNAi methods can specifically lead to gene silencing in several insects. Because injection may cause mechanical injury, lead to a high mortality rate and is not an effective means to treat large numbers of insects for pest control. We used a feeding-based RNAi to knock down the expression of the *SaCarE* gene in this study. After 6 days of dsCarE ingestion, the expression of *CarE* showed a low level compared with the control group. With an increase of the dsCarE levels, the effect of gene silencing is more obvious suggesting that a sufficient dose of dsRNA is required to induce a systemic and sufficient knockdown of *CarE*. This result indicated that the dsCarE was effective in aphids RNAi with the significant reduction of *SaCarE* expression.

In conclusion, this study demonstrates that the *SaCarE* gene is a feasible candidate for RNAi targeting of genes in *S. avenae*. Hence, targeting of *SaCarE* may offer a potential approach for the development of the RNAi-based transgenic plant for pest control.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.aspen.2016.03.011.

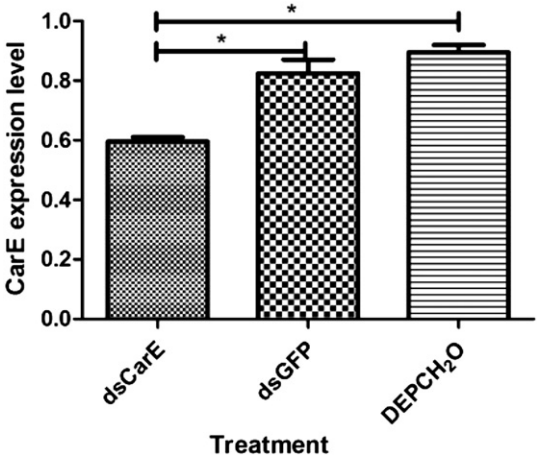

Fig. 5. Inhibition of *CarE* expression by dsCarE. *, indicates $p < 0.05$ between treatments and controls analyzed by two-way ANOVA. Error bars are standard errors of three independent biological replicates.

Acknowledgments

We thank Dr. Julian Chen (Institute of Plant Protection, Chinese Academy of Agricultural Sciences) for supplying us with *S. avenae* artificial food. This work was supported by the Public Welfare Project of the Ministry of Agriculture of the People's Republic of China (Grant No. 201103022) to Z. Zhao.

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