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RNA interference of glutamate-gated chloride channel decreases abamectin susceptibility in *Bemisia tabaci*

Peiling Wei[a,b,1], Wunan Che[c,1], Jinda Wang[d], Da Xiao[a], Ran Wang[a,*], Chen Luo[a,*]

[a] Institute of Plant and Environment Protection, Beijing Academy of Agriculture and Forestry Sciences, Beijing 100097, China
[b] Department of Entomology, China Agricultural University, Beijing, 100193, China
[c] Department of Pesticide Sciences, Shenyang Agricultural University, Shenyang 110866, China
[d] National Engineering Research Center of Sugarcane, Fujian Agricultural and Forestry University, Fuzhou 350002, China

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ABSTRACT

The *Bemisia tabaci* (Gennadius) cryptic species complex comprises very destructive insect pests of agricultural crops worldwide and has been found to be resistant to various insecticides in China. Abamectin is one of the most widely used insecticides for insect pest control and the glutamate-gated chloride channel (GluCl) in insects was presumed to be the main target site of abamectin. In this study, a 1353 bp full-length cDNA encoding GluCl (named BtGluCl, GenBank ID: MF673854) was cloned and characterized from *B. tabaci*. BtGluCl encodes 450 amino acids, which shares 71–81% identity with other insect GluCl isoforms. Spatial and temporal expression revealed BtGluCl was highly expressed in the 4th nymphal instar and adult head, and the least expressed in the 1st nymphal instar and adult leg. Dietary ingestion of dsBtGluCl significantly reduced the mRNA level of BtGluCl in the treated adults by 62.9% and greatly decreased abamectin-induced mortality. Thus, our results could be conducive to further understanding the mechanisms of resistance to abamectin in arthropods.

1. Introduction

Avermectins are a class of macrocyclic lactones with nematocidal, acaracidal and insecticidal activity, produced by a soil actinomycete, *Streptomyces avermitilis*, and highly effective when used against targeted species [1]. Abamectin (avermectin B1) is one of the most widely used avermectins for pest control in agriculture [1], and ivermectin (the semisynthetic 22, 23-dihydroderivative of avermectin B1) is used against a wide range of nematode and arthropod parasites [2]. Emamectin is derived from abamectin via a five-step synthesis and has a much higher potency than abamectin against lepidopteran insects [2,3]. Avermectins interact with various ligand-gated ion chloride channels, such as glutamate-gated chloride channels (GluCls), histamine-gated chloride channels and γ-aminobutyric acid-gated chloride channels [4–7].

In the nervous system of invertebrates, GluCls play important roles in mediating inhibitory synaptic transmission as one of critical neurotransmitter receptors [8,9]. GluCls composed of five subunits, and each subunit contains an extracellular N-terminal domain that establishes the glutamate-binding site and four transmembrane α-helices that form a channel domain [9,10]. Presently, GluCls have only been described in invertebrates with different numbers of orthologous GluCl genes [9]. There is only one GluCl gene found in insects such as *Drosophila melanogaster*, *Apis mellifera* and *Tribolium castaneum* [9,11,12], whereas six orthologous GluCl genes have been identified in the nematode *Caenorhabditis elegans* and the mite *Tetranychus urticae* [13,14]. Glutamate-gated chloride channels have been identified as the primary target of avermectins in arthropods. In spider mites, GluCls have been proved as the target of avermectins for many years [15–17]. In the studies of *Plutella xylostella*, deletion and mutation have been identified in the GluCls which is associated with resistance to abamectin [7,18]. Furthermore, studies have reported that the α subunit is important for the action of glutamate and avermectins in insects, but, in *C. elegans*, both α and β subunits are required for the action [19,20].

The whitefly, *Bemisia tabaci* (Gennadius) is a worldwide agricultural pest that spread over tropical, subtropical, and low-latitude temperate regions globally and causes serious damage to > 600 plants by sucking the phloem sap and acting as a vector of viruses [21]. The most common and efficient way to control whitefly infestations is by chemical treatments, but then frequent and extended applications of insecticides have caused insect pests to develop insecticidal resistance [22–26] and fortunately, abamectin is still useful and popular for controlling whitefly [23,25]. However, because of a knowledge gap in the underlying mechanisms of insecticide resistance, and there have

* Corresponding authors at: Institute of Plant and Environment Protection, Beijing Academy of Agriculture and Forestry Sciences, Beijing 100097, China.
E-mail addresses: rwang1105@126.com (R. Wang), luochen1010@126.com (C. Luo).
[1] These authors contributed equally to this work.

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been low level of resistance to abamectin in *B. tabaci* in the field [27,28], we investigated the relationship between BtGluCl and the susceptibility to abamectin in *B. tabaci*. In this study, we isolated a full-length GluCl cDNA (named as BtGluCl) from *B. tabaci* and used dietary ingestion of double-stranded RNA (dsRNA) to knock down target gene in whitefly. Then, we studied the influence of BtGluCl-dsRNA on susceptibility of *B. tabaci* adults to abamectin. In this work, we explored that silencing of BtGluCl using RNAi technique decreases the susceptibility of *B. tabaci* to abamectin, and it could be contributed to further work on the mechanisms of resistance in arthropods.

2. Materials and methods

2.1. Insects

B. tabaci lab-MED strain originally obtained from the Institute of Vegetables and Flowers in the Chinese Academy of Agricultural Sciences, and established in the laboratory at the Institute of Plant and Environment Protection, Beijing Academy of Agriculture and Forestry Sciences, China. The insects were reared on cotton plants (*Gossypium hirsutum* L. var. 'Shiyuan 321') grown in a glasshouse under a 16 h: 8 h, light: dark photoperiod at 25–28 °C and 60–80% humidity. Adult whiteflies less than five days old were used for bioassays and RNA extractions.

2.2. Insecticide and bioassay

A formulated insecticide containing abamectin at a concentration of 18 g/L EC (Jiangsu Fengyuan Biochemical Co., Ltd., China), was used for the bioassays. We used the leaf-dipping bioassay method with a total of eight different abamectin concentrations (0.8 mg/L, 0.4 mg/L, 0.2 mg/L, 0.1 mg/L, 0.05 mg/L, 0.025 mg/L, 0.0125 mg/L, 0.00625 mg/L) [29]. Leaf discs (22 mm diameter) from cotton plants were dipped in one of the eight concentrations of insecticide solution or in distilled water (control) for 20 s. After the discs dried, each was placed at the bottom of a flat-bottom, 78-mm-long glass tube containing agar (2 mL of 15 g/L) with the adaxial surface facing down. Adults of *B. tabaci* were transferred into these tubes by inverting the tubes above the leaves of cotton plants used to rear whiteflies in the glasshouse. This allowed the mixed adults to fly into the tube. After 20–30 adults had flown into a tube, the tube was sealed with a cotton plug. The tubes were maintained in a growth chamber at 27 ± 1 °C with 60 ± 10% RH and a 16:8 h (L: D) photoperiod. Whitefly mortality was recorded after 48 h and immobile adults were scored as dead. In the bioassay, four replicate tubes represented each working concentration of insecticide and the LC_{50} value was calculated with a Probit statistical model and PoloPlus software [30].

2.3. Cloning and sequencing of full-length BtGluCl cDNA

Total RNA was extracted from six different developmental stages, including eggs, 1st to 4th instar nymphs and adults. In addition, tissue-specific expression profiles were tested from diverse body parts (head, thorax, abdomen, leg and wing) of adults with a Trizol kit (Invitrogen, CA, USA) according to the manufacturer's instructions. The first-strand cDNA was synthesized using Prime Script™ 1st Strand cDNA Synthesis Kit with gDNA Eraser (Perfect Real Time) (TaKaRa, Dalian, China) from 1 μg of total RNA. The synthesized cDNA was then used for reverse transcription-polymerase chain reaction (RT-PCR) and quantitative RT-PCR (qRT-PCR). Based on our previous transcriptome data [31], the Open Reading Frame (ORF) of the GluCl gene was predicted by ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). Subsequently, primers (Supporting Information 1) were designed to amplify complete ORFs of the GluCl gene and confirm the reliability of the assembled sequence. The fragments after sequencing were assembled and aligned with DNAMAN (DNAMAN 5.2.2, Lynnon BioSoft). ExPASy Proteomics Server (http://cn.expasy.org/tools/pi_tool.html) was used to compute the isoelectric point and molecular weight of deduced protein sequences. The signal peptide was predicted using the SignalP 4.1 server (http://www.cbs.dtu.dk/services/SignalP/). The matured GluCl protein sequences from *B. tabaci* and other insect pest species (see Supporting Information 2) were aligned using ClustalX 1.83. A phylogenetic tree was constructed in MEGA5.1 using the neighbor-joining method with a bootstrap value of 1000. Transmembrane domains were predicted using TMHMM 2.0 (http://www.cbs.dtu.dk/services/TMHMM-2.0/). The regions of putative motifs were predicted by ExPASy ScanProsite (http://prosite.expasy.org/scanprosite/) or alignment to other GluCls of invertebrates.

Table 1
Bioassay of the abamectin on *B. tabaci* MED.

Insecticide	Number[a]	Slope ± SE	LC_{50} (mg/L)	LC_{50} (95% FL) (mg/L)[b]	X^2 (df)
Abamectin	990	1.16 ± 0.08	0.04	0.03–0.05	4.20 (6)

[a] Number of adults tested.
[b] Concentration of insecticide killing 50% of adults and its 95% fiducial limits.

2.4. qRT-PCR analysis of BtGluCl expression profiles

The relative transcription levels of BtGluCl in different developmental stages (eggs, 1st to 4th instar nymph, adults) and various body parts from the adults (head, thorax, abdomen, leg and wing) were examined using qRT-PCR method. The qRT-PCR was performed using gene-specific primers (Supporting Information 1) and SYBR Premix EX Taq™ (Takara, Japan) in three biological replicates per developmental stage or body part sample on an ABI 7500 system (Applied Biosystems, CA, USA). Reactions, 20 μL in total, contained 2.0 μL cDNA (200 ng/μL), 10 μL SYBR Premix Ex TaqTM (Takara, Japan), 1 μL forward primer (10 μM), 1 μL reverse primer (10 μM). 0.4 μL Rox Reference Dye II and 5.6 μL nuclease free water. Thermal cycling conditions were: 95 °C for 30s, 40 cycles of 95 °C for 5 s, 62 °C for 34 s. After the cycling protocol, a melting curve analysis from 60 °C to 95 °C was applied to all reactions to verify a single PCR product. The *β-actin* and *EF-1α* of *B. tabaci* were used as housekeeping genes to correct for sample-to-sample variation. The results were conducted in terms of the $2^{-\triangle\triangle Ct}$ method [32]. All statistical analyses on the expression of BtGluCl in different growth stages and adult body parts were analyzed by one way ANOVA followed by Tukey' HSD for multiple comparisons using the software of SPSS [33].

2.5. dsRNA synthesis and RNAi

RNA interference (RNAi) was carried out to evaluate the role of BtGluCl in *B. tabaci* and the double stranded RNA (dsRNA) were synthesized using a T7 RiboMAX Express RNAi kit (Promega, Madison, WI, USA). Relevant information on the primers used for dsRNA synthesis is shown in Supporting Information 1. The dsRNA concentration was measured using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). RNAi was conducted by directly feeding dsRNA to *B. tabaci* adults in a feeding chamber as described in past studies [24,34]. Adult whiteflies continuously ingested nuclear-free water (CK), enhanced green fluorescent protein dsRNA (dsegfp) and GluCl dsRNA (dsGluCl) with diets for two days, respectively. Both concentrations of dsGluCl and dsegfp are 0.5 μg/μL, and the diet solution contains 5% yeast extract and 30% sucrose (Weight/volum). Each RNAi experiment was conducted with six replicates in each treatment or the control. About 40 newly emerged adults (mixed sexes) were added into each replicate, which were placed in a growth chamber at 27 ± 1 °C with 60 ± 10% RH and a 16:8 h (L: D) photoperiod. Among the six replicates, three of them were used to monitor the

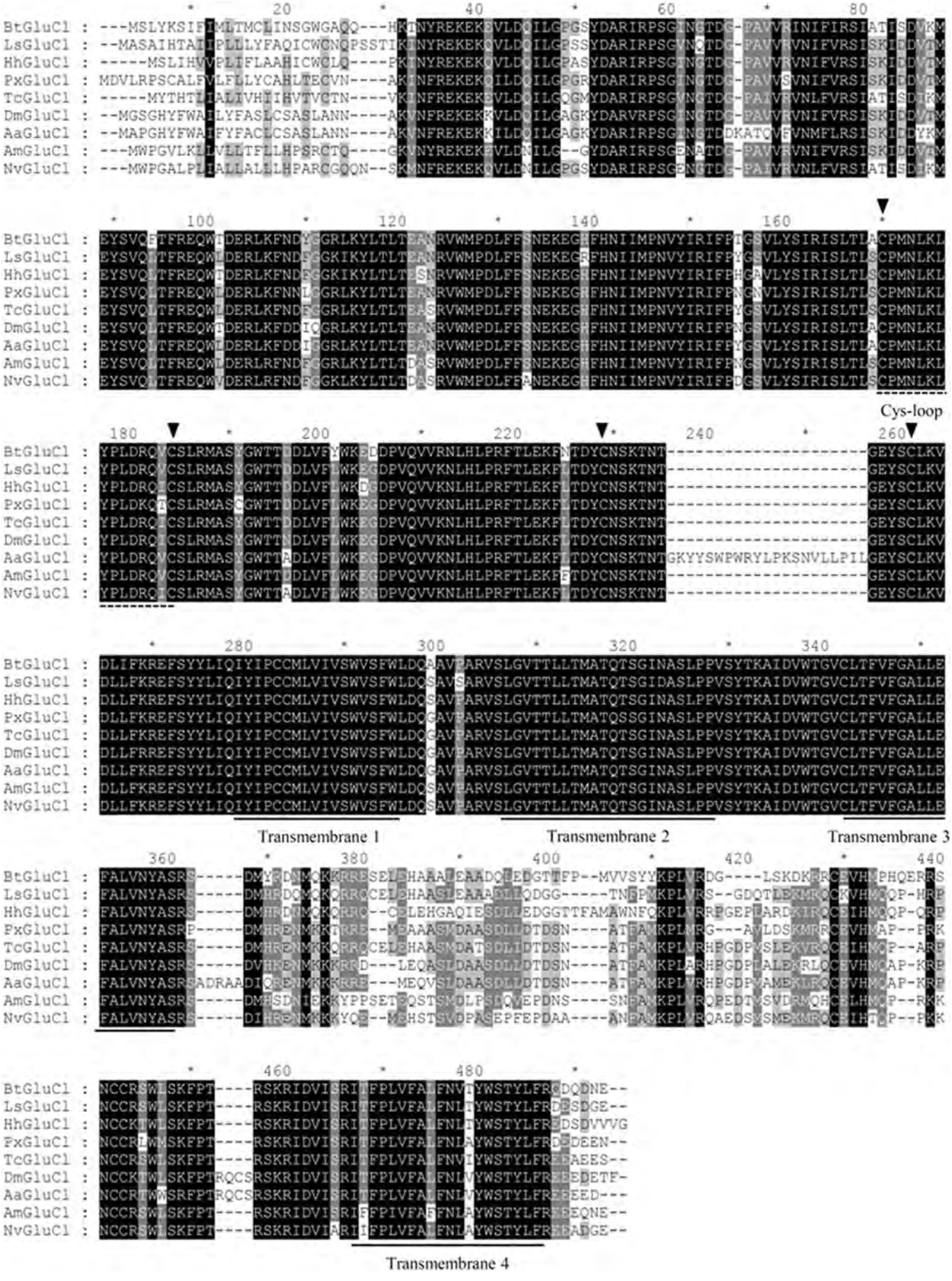

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Fig. 1. Alignment of the insect GluCls. Four transmembrane domains (TM1-4) and four sites of cysteine residues are highlighted respectively by black lines and black triangles. The Cys-loop, the neurotransmitter-gated ion-channels signature, is underlined by a dotted line. The corresponding GenBank IDs are listed in Supporting Information 4.

Table 2
Comparison of GluCl sequences from different organisms.

(Analyzed by GeneDoc, http://www.nrbsc.org/gfx/genedoc/ebinet.htm) The species and their corresponding GenBank IDs are: *Bemisia tabaci* (BtGluCl), this study; *Laodelphax striatellus* (LsGluCl), AEE39458.1; *Halyomorpha halys* (HhGluCl), XP_014282874.1; *Plutella xylostella* (PxGluCl), ACT09139.1; *Tribolium castaneum* (TcGluCl), NP_001107775.1; *Drosophila melanogaster* (DmGluCl), ABG57261.1; *Aedes aegypti* (AaGluCl), XP_021704264.1; *Apis mellifera* (AmGluCl), NP_001071277.1; *Nasonia vitripennis* (NvGluCl), NP_001171232.1.

	B. tabaci	L. striatellus	H. halys	P. xylostella	T. castaneum	D. melanogaster	A. aegypti	A. mellifera	N. vitripennis
B. tabaci	*	81%	81%	77.%	80%	77%	71%	76%	76%
L. striatellus		*	86%	81%	83%	81%	75%	79%	80%
H. halys			*	79%	83%	81%	75%	78%	78%
P. xylostella				*	83%	81%	77%	78%	79%
T. castaneum					*	83%	78%	81%	85%
D. melanogaster						*	86%	79%	79%
A. aegypti							*	72%	75%
A. mellifera								*	85%
N. vitripennis									*

* Means no comparison.

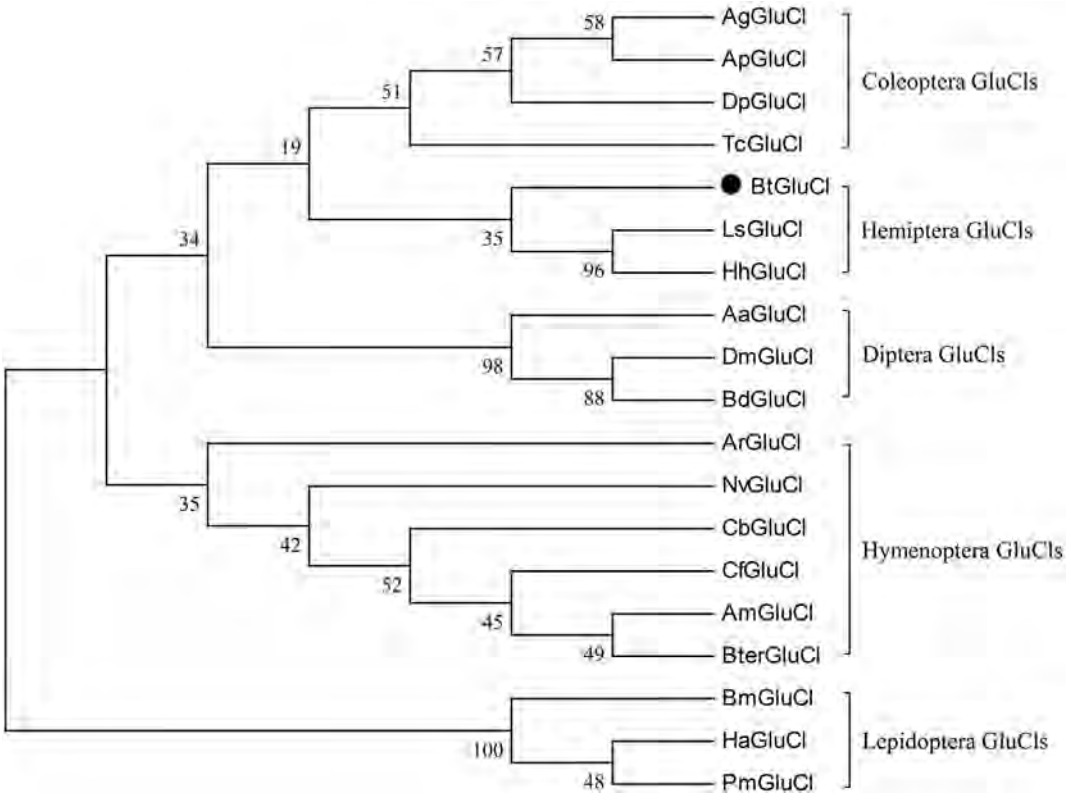

Fig. 2. The phylogenetic tree of deduced BtGluCl compared to other known GluCls of different species. The corresponding GenBank IDs are listed in Supporting information 2.

change of the BtGluCl transcript level, and the other three replicates for each treatment and the control were exposed to LC_{50} of abamectin using the bioassay method as described above. Mortality of whiteflies was recorded after 48 h. All statistical analyses on the expression of BtGluCl and mortality were analyzed by one way ANOVA followed by Tukey' HSD for multiple comparisons using the software of SPSS [33].

3. Results

3.1. Toxicity of abamectin to B. tabaci

The toxicitiy of the abamectin to *B. tabaci* was determined by using the leaf-dipping method. According to the bioassay results, 0.04 mg/L of abamectin was used as LC_{50} to treat *B. tabaci* (Table 1).

3.2. Analysis of BtGluCl amino acid sequence and alternative splicing

The ORF of BtGluCl was a 1353 bp contiguous sequence and length of the deduced protein sequence contained 450 amino acids. The predicted protein BtGluCl, which has a molecular weight of 52.26 kDa and an isoelectric point of 8.62, was submitted to the National Center for Biotechnology Information (NCBI) with GenBank ID: MF673854. Analyses of the amino acid sequence of BtGluCl showed that there are four typical transmembrane (TM) domains in the C-terminal region and these domains shared high identities with GluCls of other insects (Fig. 1). There are four extracellular cysteine residues (C1–C4) and a neurotransmitter-gated ion-channels signature (Cys-loop), CPMNLKLYPLDRQVC, which is located between C1 and C2 (Fig. 1). After sub-cloning and sequencing of multiple BtGluCl cDNA fragments,

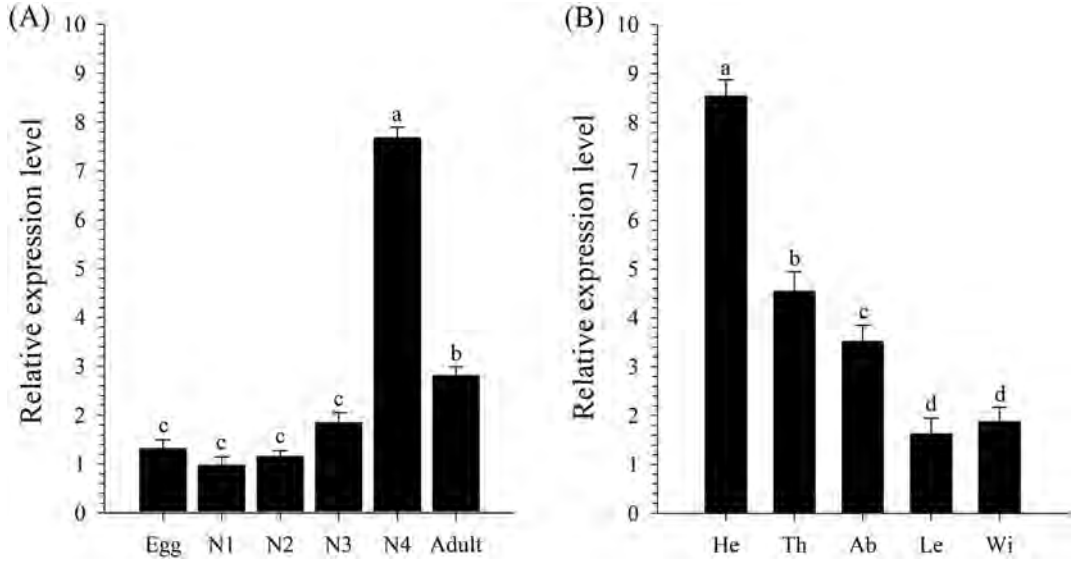

Fig. 3. Spatial and temporal expression levels of BtGluCl mRNA. (A) Expression levels of BtGluCl mRNA in six development stages: egg, 1st, 2nd 3rd, 4th instar nymphal (N1-N4) and adult. (B) BtGluCl mRNA in body parts of adults. The He, Th, Ab, Le and Wi represent the head, thorax, abdomen, led and wing, respectively. Different lowercase letters (a, b and c) indicate significant differences ($p < 0.05$) based on one way ANOVA followed by Tukey's HSD for multiple comparisons. Means with standard error bars are represented from three replicates.

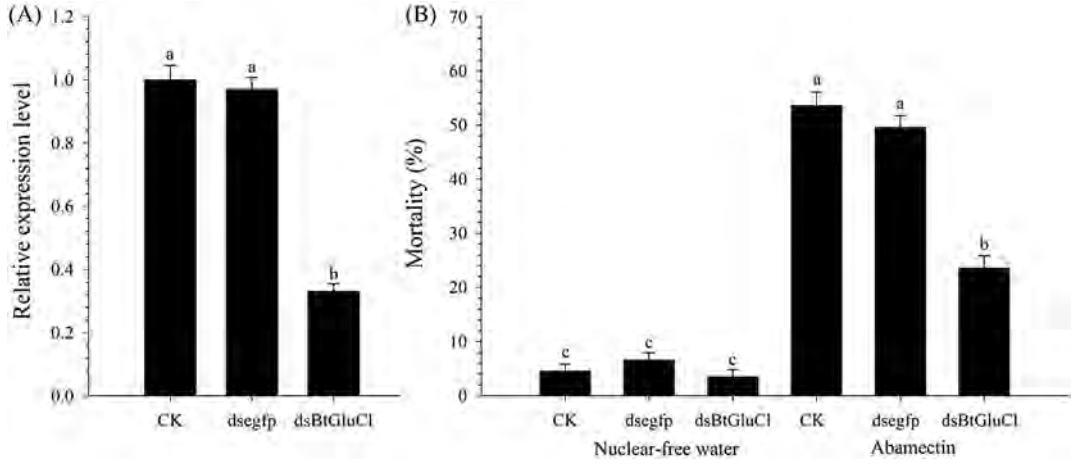

Fig. 4. Effects of dietary introduction of dsBtGluCl on the relative BtGluCl transcript level (A) and mortality of adult whiteflies (B). Newly-emerged adults were continuously exposed to dsRNA for two days. Mortality was evaluated two days after abamectin or nuclear-free water treatments. Different lowercase letters (a, b and c) indicate significant differences ($p < 0.05$) based on one way ANOVA followed by Tukey's HSD for multiple comparisons. Means with standard error bars are represented from three replicates.

two alternative splicing sites were detected in the predicted GluCl domain between amino acid residues 20–24 and 376–379, and then three variants were found: BtGlu1, BtGlu2, BtGlu3 and BtGlu5; BtGlu4, BtGlu6 and BtGlu7; BtGlu8, BtGlu9 and BtGlu10 (Supporting information 3), and these three variants showed similar frequency from the results of sequencing.

3.3. Comparison of phylogenetic relationships of GluCls with other insects

As shown in Table 2, the alignment of amino acid sequences demonstrated that BtGluCl shared the greatest identity with *Laodelphax striatellus* GluCl (81%) and *Halyomorpha halys* (81%). The shared identities of BtGluCl and the GluCl of *T. castaneum*, *D. melanogaster*, *Plutella xylostella*, *A. mellifera*, *Nasonia vitripennis* and *Aedes aegypti* were 80%, 77%, 77%, 76%, 76% and 71%, respectively. To investigate the evolutionary relationships among GluCls, a phylogenetic tree was constructed using Clustal X and MEGA 5.1 and is displayed in Fig. 2. GluCls from different orders of insects are clearly clustered into groups of diptera, lepidoptera, coleoptera, hymenoptera and hemiptera.

3.4. Spatial and temporal expression of BtGluCl

The mRNA levels of BtGluCl were measured in eggs, 1st through 4th instar nymphs and adults. As shown in Fig. 3A, the relative expression of BtGluCl in the 4th instar nymphal stage was significantly higher than all other stages. Relative expression levels of BtGluCl in the 1st, 3rd, 4th instar nymphal and adult stages were, respectively, 1.3-, 1.2-, 1.9-, 7.7- and 2.8- fold less than the 2nd instar nymphal stage. Of the BtGluCl expression levels that were determined from the adult body parts, mRNA was most abundantly expressed in the head by 1.9-, 2.4-, 5.3-, and 4.6-fold higher levels than compared to the thorax, abdomen, leg and wing, respectively (Fig. 3B).

3.5. Effect of dsBtGluCl on BtGluCl expression and abamectin tolerance

To further investigate the function of BtGluCl, dsBtGluCl was synthesized and then fed to *B. tabaci* adults of the Lab-MED strain. After two days of feeding on dsBtGluCl, BtGluCl transcript level was suppressed by 62.9% of the CK level, and dsegfp ingestion did not alter

BtGluCl expression level compared to the CK level (Fig. 4A). In addition, after the abamectin-treatment the ingestion of dsBtGluCl by adults resulted in a significantly lower mortality compared to the control (Fig. 4B). Therefore, ingestion of dsBtGluCl greatly decreased abamectin-induced mortality in *B. tabaci*.

4. Discussion

In this study, we characterized a *GluCl* gene expressed in *B. tabaci*. Orthologous GluCls have been cloned in hemipteran *L. striatellus*; lepidopteran *Papilio machaon*, *P. xuthus* and *P. xylostella*; dipteran *D. melanogaster*, *Musca domestica* and *Anopheles gambiae*; and hymenopteran *Ooceraea biroi* and *A. mellifera* [35–42]. All insect GluCls include four cysteine residues within the N terminal region and the four proposed transmembrane regions. An amino acid sequence alignment showed that BtGluCl shares 71–81% sequence identities with other insect homologues. Moreover, the phylogenetic result shows that BtGluCl is distantly related to other insect GluCls. These results indicate that BtGluCl could be orthologous with other insect GluCls genes and these GluCls of insect might be evolved from a common ancestral gene.

Temporal and spatial expression analysis showed that BtGluCl is expressed in all the development stages (egg, first through fourth instar nymphs and adult) and adult body parts (list them too) that we examined. Expression levels in fourth instars and adults were significantly higher than other stages. In addition, we found BtGluCl is most abundant in the head of the adult. The same result was confirmed in the adults of *L. striatellus* [43] and *A. gambiae* [40]. In *M. domestica*, all variant transcripts of MdGluCl were abundant in the adult head compared with the pupal head, the larval head, and the whole embryo [44]. In *L. striatellus*, LsGluCl was expressed at all developmental stages and highly expressed in the adult stage compared with immature developmental stages [43]. Similarly, in the carmine spider mite, TcGluCl5 showed the highest expression in the adult stage. However, of four other TcGluCls in the spider mite, the highest transcription levels were detected in the larval stage [17]. Above evidences indicate that transcript profiles of GluCl are spatially similar and, on the contrary, the temporal transcript profiles of GluCl varies in different insects.

Alternative splicing of pre-mRNA transcripts is a prevalent feature of gene processing and is critical for protein diversity. To date, splicing variants have been observed in several insects. *D. melanogaster*, *A. mellifera*, *N. vitripennis* and *P. xylostella* have two alternative variants [7,9,38,45], whereas *T. castaneum*, *M. domestica* and *Bombyx mori* have three alternative variants [11,44,46]. Recently, Meyers et al. was the first to identify two alternative GluCl mRNA forms with different splice sites in *A. gambiae* [40]. Alternatively spliced GluCl genes have now been found in *L. striatellus* as well [43]. In the present study, we identified alternative splicing of GluCl mRNAs in *B. tabaci* for the first time.

RNAi technology has been widely used to identify or validate target genes for insecticides [47]. As potential targets for insecticides, a change of expressed levels of these genes had been proven to mediate resistance in many pests [48–51]. We found that dietary ingestion of dsBtGluCl significantly reduced the mRNA level of BtGluCl in the treated adults by 62.9%, and greatly decreased abamectin-induced mortality in *B. tabaci* suggesting that BtGluCl gene encoded a functional GluCl that mediates abamectin toxicity to *B. tabaci*. Besides, RNAi and toxicity test indicated a similar relationship of GluCls and the toxic effects of abamectin in the carmine spider mite [17]. Another study also documented that RNAi suppression of the glutamate receptor gene caused a decrease of susceptibility to abamectin in *P. xylostella* [51]. All of these studies showed that the dsRNA GluCls could reduce the expression of target genes and supplied direct evidence to indicate that GluCls are the molecular targets for avermectins in different pest by RNAi. In this study, we tested the role of BtGluCl in *B. tabaci* in response to abamectin. Furthermore, our conclusions also provide a baseline to understand the evolution and function of BtGluCl.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pestbp.2017.12.004.

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