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Mutations on M3 helix of *Plutella xylostella* glutamate-gated chloride channel confer unequal resistance to abamectin by two different mechanisms

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1 **Abstract**

2 Abamectin is one of the most widely used avermectins for agricultural pests control, but the
3 emergence of resistance around the world is proving a major threat to its sustained application.
4 Abamectin acts by directly activating glutamate-gated chloride channels (GluCl_s) and
5 modulating other Cys-loop ion channels. To date, three mutations occurring in the
6 transmembrane domain of arthropod GluCl_s are associated with target-site resistance to
7 abamectin: A309V in *Plutella xylostella* GluCl (PxGluCl), G323D in *Tetranychus urticae*
8 GluCl1 (TuGluCl1) and G326E in TuGluCl3. To compare the effects of these mutations in a
9 single system, A309V/I/G and G315E (corresponding to G323 in TuGluCl1 and G326 in
10 TuGluCl3) substitutions were introduced individually into the PxGluCl channel. Functional
11 analysis using *Xenopus* oocytes showed that the A309V and G315E mutations reduced the
12 sensitivity to abamectin by 4.8- and 493-fold, respectively. In contrast, the substitutions
13 A309I/G show no significant effects on the response to abamectin. Interestingly, the A309I
14 substitution increased the channel sensitivity to glutamate by one order of magnitude
15 (~12-fold). Analysis of PxGluCl homology models indicates that the G315E mutation
16 interferes with abamectin binding through a steric hindrance mechanism. In contrast, the
17 structural consequences of the A309 mutations are not so clear and an allosteric modification
18 of the binding site is the most likely mechanism. Overall the results show that both A309V
19 and G315E mutations may contribute to target-site resistance to abamectin and may be
20 important for the future prediction and monitoring of abamectin resistance in *P. xylostella* and
21 other arthropod pests.

22

23 **Keywords:** Abamectin, Glutamate-gated chloride channel, Target-site resistance, Molecular
24 modelling

25

1 **1. Introduction**

2 Avermectins are naturally occurring 16-membered macrocyclic lactones produced by
3 fermentation of the soil-dwelling microorganism *Streptomyces avermitilis*. These compounds
4 have potent activity against many kinds of endoparasitic nematodes and ectoparasitic
5 arthropods (Campbell, 2012). Since their first isolation in the 1970s, they have been applied
6 globally for crop protection and animal health purposes. Within this family, abamectin
7 (avermectin B₁) is one of the most widely used compounds for agricultural pest management
8 (Clark et al., 1995) and ivermectin (a synthetic derivative of avermectin B₁, IVE) is active at
9 extremely low dosage against a range of nematode and arthropod parasites (Campbell et al.,
10 1983). Avermectins are classified as neurotoxins, which act by directly activating or
11 potentiating glutamate-gated chloride channels (GluCl_s) (and to a lesser extent γ -amino
12 butyric acid (GABA)-gated chloride channels). GluCl_s contribute extensively to invertebrate
13 nervous system function, including modulating locomotion, regulating feeding and mediating
14 sensory inputs (Wolstenholme, 2012). As a result, modification of channel function by
15 avermectins action can result in paralysis and death (Bloomquist, 1996, 2003).

16 GluCl_s belong to the Cys-loop superfamily of ligand-gated ion channels that assemble as
17 pentamers to form a central ion-conducting pore; these GluCl receptors are present only in
18 invertebrates (Jones and Sattelle, 2008; Wolstenholme, 2012). GluCl_s were first described in
19 electrophysiological studies on locust muscle, when a biphasic change in its membrane
20 potential was recorded and glutamate-enhanced chloride permeability resulted in the
21 hyperpolarizing phase (Cull-Candy, 1976). The first cloned genes encoding GluCl subunits
22 were glucl- α and glucl- β (now referred to *glc-1* and *glc-2*, respectively) from the nematode
23 *Caenorhabditis elegans*. When expressed in *Xenopus* oocytes, GluCl_s composed of glucl- α
24 subunits responded to IVE but not to L-glutamate whereas homomeric glucl- β channels were
25 unresponsive to IVE but were activated by L-glutamate (Cully et al., 1994; Daeffler et al.,
26 2014). The co-expression of both α and β subunits produced channels that responded to both
27 glutamate and IVE independently and so more closely resembled the naturally-occurring
28 receptor (Cully et al., 1994; Vassilatis et al., 1997; Slimko et al., 2002; Degani-Katzav et al.,
29 2016). The *C. elegans* GluCl gene family has since been expanded to six members (Jones and

1 Sattelle, 2008). Genomic analysis has shown that a single GluCl gene, with variant transcripts
2 produced by mRNA splicing and extensive editing, is present in several insects, including
3 *Drosophila melanogaster*, *Apis mellifera*, *Nasonia vitripennis*, and *Tribolium castaneum*
4 (Semenov and Pak, 1999; Jones and Sattelle, 2006, 2007; Jones et al., 2010; Knipple and
5 Soderlund, 2010). Multiple GluCl genes have been identified in other organisms: two in
6 *Aplysia californica*, six in *Tetranychus urticae* and one in *Lepeophtheirus salmonis* (Tribble et
7 al., 2007; Kehoe et al., 2009; Dermauw et al., 2012).

8 Several experiments based on functional analysis of IVE-resistant GluCl channels in
9 nematodes predicted that IVE acts by insertion between transmembrane domain of GluCl
10 (Dent et al., 2000; Njue et al., 2004). Recently, our knowledge of the structural and functional
11 properties of the GluCl has been greatly advanced by the determination of the
12 three-dimensional crystallographic structure of *C. elegans* GluCl α channel (CeGluCl α), which
13 presented the first high-resolution view of a eukaryotic Cys-loop receptor (Hibbs and Gouaux,
14 2011). This X-ray structure revealed that GluCl α are arranged with five-fold symmetry and the
15 transmembrane M2 helices form the pore lumen. The pore-lining residues of the M2 helices
16 therefore determine the ion selectivity and conductance properties of the channel (Hibbs and
17 Gouaux, 2011). This structure was co-crystallized with IVE, which binds in the
18 transmembrane domain at subunit interfaces, lying between M3 and M1 helices of two
19 adjacent subunits and making binding contacts with M2 and the M2-M3 loop. The
20 endogenous ligand glutamate binds in the extracellular domain of the receptor in the classical
21 neurotransmitter position, which is also located at the interface of adjacent subunits.

22 The diamondback moth, *Plutella xylostella*, is one of the few insect species that has
23 evolved field resistance to all primary classes of insecticides (Furlong et al., 2013). As such it
24 represents a valuable agricultural pest model to study the genetics and mechanisms of
25 resistance development (Furlong et al., 2013). Gene mutation-mediated resistance
26 mechanisms have been widely studied in *P. xylostella*. For example, mutations have been
27 identified in occurring in genes encoding acetylcholinesterase, sodium channel, nicotinic
28 acetylcholine receptor, GluCl, ryanodine receptor and ABC transporter C2. In addition, such
29 mutations have been implicated in resistance to organophosphate, pyrethroid, spinosad,

1 abamectin, chlorantraniliprle, Bt Cry1Ac toxin, indoxacarb and metaflumizone insecticides,
2 respectively (Schuler et al., 1998; Lee et al., 2007; Baxter et al., 2010, 2011; Troczka et al.,
3 2012; Guo et al., 2014; Wang et al., 2016a, 2016b, 2016c). Abamectin has been used for *P.*
4 *xylostella* control since the 1980s and at least 72 cases from four countries have been reported
5 of field resistance to this chemical (APRD, 2017). A detailed study of the molecular
6 mechanisms of abamectin resistance is important for designing strategies aimed at
7 circumventing resistance it. In our previous work we identified a point mutation (A309V) in
8 the transmembrane domain of *P. xylostella* glutamate-gated chloride channel receptor,
9 PxGluCl, which has been evaluated to contribute ~10-fold increase to abamectin resistance in
10 the resistant Roth-Abm strain (Wang et al., 2016b). We believe that characterizing the
11 pharmacological properties of this mutation that evolved in PxGluCl could help in exploring
12 the resistance mechanisms in this and other species and might have relevance in the
13 development of new macrocyclic lactones insecticides.

14 In addition to A309V, only three other mutations in GluCl_s have been reported in
15 association with resistance to avermectins (IVE & abamectin) in other arthropods (Fig. 1).
16 The P299S mutation of the *D. melanogaster* DmGluCl channel is associated with a 3-fold
17 resistance to IVE in this insect (Kane et al., 2000). The TuGluCl1 G323D and TuGluCl3
18 G326E mutations are associated with 18- and 2000-fold resistance to abamectin in *T. urticae*,
19 respectively (Kown et al., 2010; Dermauw et al., 2012). Of these, only the P299S mutation
20 has been characterized electrophysiologically and a 14-fold reduction in IVE sensitivity was
21 found when this substitution was introduced into DmGluCl (Kane et al., 2000). In the current
22 study, we expressed A309V or G315E (equivalent to G323 in TuGluCl-1 and G326 in
23 TuGluCl-3) mutants of PxGluCl in *Xenopus laevis* oocytes and compared their functional
24 properties with wild-type receptors. In addition, further mutations of A309 to glycine or
25 isoleucine were generated to investigate how substitutions at this position on the M3 helix
26 could affect the functional effects of glutamate or abamectin. Homology models were
27 generated to provide structural insight into the molecular mechanisms that underlie the
28 abamectin resistance of these mutations.

29 **2. Materials and methods**

1 2.1. Chemicals and reagents

2 L-Glutamic acid ($\geq 99\%$) and abamectin ($B_{1a} = 91.5\%$), used for two-electrode
3 voltage-clamp recording, were purchased from Sigma-Aldrich (St. Louis, MO, USA) and
4 Veyong bio-chemical (Shijiazhuang, Hebei, China), respectively. All the restricted enzymes
5 used in this study, including *EcoRI*, *XbaI*, *SmaI*, *NotI* and the klenow fragment were
6 purchased from Thermo Fisher Scientific (Waltham, MA, USA).

7 2.2. Site-directed mutagenesis and cRNA synthesis

8 Total RNA of 4th-instar larvae from *P. xylostella* susceptible Roth strain was extracted by
9 using the SV Total RNA Isolation System Kit (Promega, Madison, WI, USA) according to the
10 manufacturer's protocol. First-strand cDNA was synthesized from 1 μ g total RNA using an
11 oligo(dT)₁₅ primer and M-MLV reverse transcriptase (Promega, Madison, WI, USA). The
12 full-length cDNA of wild-type PxGluCl (GenBank accession no. JX014231.1) was amplified
13 by PrimeSTAR HS DNA polymerase (TaKaRa, Japan) and PCR (35 cycles of 98 °C for 10 s,
14 53 °C for 15s, and 72 °C for 90 s) with the forward primer PxGluCl_*EcoRI*_F and the
15 corresponding reverse primer PxGluCl_*XbaI*_R (Table 1). Site-directed mutagenesis was
16 performed by fusion PCR with wild-type PxGluCl cDNA as the template. All the cDNA
17 constructs were ligated into the pEASY-Blunt Cloning vector (Beijing TransGen Biotech Co.
18 Ltd, Beijing, China) and the isolated plasmid DNAs were examined by nucleotide sequencing
19 (Life Technology, Shanghai, China). Five full-length *P. xylostella* GluCl cDNA (wild-type,
20 A309V, A309I, A309G and G315E) were subcloned into the pGH19 vector, a modified
21 version of plasmid pGEMHE, which contains 5'- and 3'-untranslated *X. laevis beta-globin*
22 gene regions, and the presence of the mutations/substitutes were reconfirmed by sequencing.
23 Plasmids were linearized with *NotI*, and capped RNAs were carried out using the T7
24 mMessage mMachine Kit (Ambion, Life Technologies, Paisley, UK) according to the
25 manufacturer's instruction. Synthesized cRNAs were recovered by precipitation with
26 isopropanol, dissolved in nuclease-free water at a final concentration of 500 ng/ μ L and stored
27 at -80 °C until use.

1 2.3. *Functional expressions of PxGluCl variants in X. laevis oocytes*

2 Mature healthy *X. laevis* oocytes (stage V–VI) were treated with collagenase (type IA; 2
3 mg/ml; Sigma, USA) in calcium-free Barth's solution (88 mM NaCl, 2.4 mM NaHCO₃, 15
4 mM Tris-HCl, 1 mM KCl and 0.82 mM MgCl₂) for about 25 min at room temperature, rinsed
5 three times, and manually defolliculated before injection with cRNA. Oocytes were injected
6 with 5-15 ng cRNA in a volume of 32.2 nL into the cytoplasm by using a Drummond variable
7 volume microinjector. After injection, calcium-containing Barth's solution (0.77 mM) with
8 antibiotics (100 units/mL penicillin, 100 µg/mL streptomycin, 4 µg/mL kanamycin and 50
9 µg/mL tetracycline) was used for oocytes' incubation at 18 °C. Experiments were carried out
10 at room temperature between 3 and 5 days after injection.

11 2.4. *Two-electrode voltage clamp electrophysiology*

12 Two-electrode voltage clamp was used for recording whole-cell currents from the
13 injected *Xenopus* oocytes. The oocytes were held in a recording bath and continuously
14 perfused with a Ringer's solution (115 mM NaCl, 2.5 mM KCl, 1.8 mM CaCl₂ and 10 mM
15 Hepes, pH 7.3). The glass capillary electrodes were filled with 3M KCl and have a resistance
16 of 0.5-1.5 MΩ. Glutamate and abamectin induced inward currents were recorded with an
17 OC-725C oocyte clamp (Warner Instruments, Hamden, CT, USA) at a holding potential of -60
18 mV. Data acquisition and analysis were carried out using iWorx 408 data acquisition system
19 and LabScribe software (iWorx Systems, Inc. Dover, NH, USA). Glutamate was dissolved in
20 Ringer's solution and applied to the oocytes for 3 s. After the glutamate perfusion, the oocytes
21 were washed by Ringer's solution for 2 min to assess their reproducibility. Abamectin was
22 first dissolved in DMSO (0.1 mM) and then diluted with Ringer's solution. The abamectin
23 solution containing ≤ 0.1% DMSO was applied to oocytes for 15 s, and the oocytes were then
24 washed for 3.5 min. Because of the effects of abamectin was irreversible, activated currents
25 were normalized to the saturating glutamate-induced current in the same oocyte.
26 Dose-response data were analyzed by nonlinear regression analysis using GraphPad Prism 5.0
27 (GraphPad Software Inc., San Diego, CA, USA). Data were obtained for at least four oocytes
28 from at least two frogs, and the values are given as means ± SEM. Statistical analysis was
29 determined by a two-tail Student's *t*-test.

1 2.5. Homology modeling of PxGluCl mutants

2 A homology model of the open-state *P. xylostella* glutamate-gated chloride channel
3 (GenBank accession no. JX014231.1) was generated as described (Wang et al., 2016b). A
4 docking prediction of avermectin B1a with the open-state *P. xylostella* open-state model was
5 also previously generated (Wang et al., 2016b). In this study, mutations were introduced into
6 the channel model using Swiss-PdbViewer software (Guex et al., 1999) followed by 30 steps
7 of steepest descent then 50 steps of conjugate gradient energy minimization. Figures were
8 produced using PyMOL (DeLano Scientific, San Carlos, CA, USA).

9 2.6. Genomic DNA extraction and mutations screening of PxGluCl in field populations

10 To determine whether the A309V and G315E mutations of PxGluCl have evolved in field
11 population of *P. xylostella*, PCR amplification was carried out with genomic DNA isolated
12 from individual larvae using the AxyPrep™Multisource Genomic DNA Miniprep Kit
13 (Axygen Biosciences, Union, CA, USA) according to the manufacturer's protocol. A genomic
14 DNA fragment of PxGluCl, covering the M3 region, was amplified as described previously
15 (Wang et al., 2016b). 20-40 samples from seventeen *P. xylostella* populations collected in
16 China during 2013-2015 were successful examined by direct nucleotide sequencing using a
17 gene-specific primer (Wang et al., 2016b). The genotypes of 309 and 315 positions of the
18 PxGluCl were identified according to sequence chromatograms.

19 3. Results

20 3.1. Response of wild-type and mutant channels to glutamate

21 We cloned the full-length cDNA sequence of wild-type PxGluCl (the most common
22 isoform 3c9a) from the susceptible Roth strain. The natural-occurring mutations
23 PxGluCl-A309V (found in *P. xylostella* by Wang et al., 2016b) and PxGluCl-G315E
24 (equivalent to G326E mutation found in *T. urticae* by Dermauw et al., 2012) were then
25 introduced and two other mutants (PxGluCl-A309I, PxGluCl-A309G) were generated by
26 site-directed mutagenesis (Table 1 and Fig. 1). After expression in *Xenopus* oocytes,
27 application of glutamate to these five channels induced inward currents in a dose-dependent
28 fashion (Fig. 2). The dose-response curves gave EC₅₀s of 19.93 ± 3.26 μM, 13.24 ± 1.05 μM,

1 $1.67 \pm 0.20\mu\text{M}$, $34.60 \pm 2.83\mu\text{M}$ and $10.35 \pm 3.67\mu\text{M}$ for wild-type, A309V, A309I, A309G
2 and G315E channels, respectively, and Hill coefficients of 1.75 ± 0.18 , 0.98 ± 0.06 , $2.47 \pm$
3 0.31 , 0.70 ± 0.05 and 1.03 ± 0.09 for wild-type, A309V, A309I, A309G and G315E channels,
4 respectively (Table 2). The A309V and G315E increased the potency of glutamate by 1.5- and
5 1.9-fold, respectively, without significant differences observed in EC_{50} s when compared with
6 the wild-type channel.

7 Interestingly, the A309I mutant exhibited an approximately 12-fold increase in glutamate
8 sensitivity (Fig. 2B), and the difference of EC_{50} s and Hill coefficients between A309I and
9 wild-type channel were significant ($p < 0.05$). In contrast, the A309G mutant was less
10 sensitive to glutamate, with a 1.7-fold reduction compared with wild-type. The Hill
11 coefficient for the A309I and A309G mutants was 2.5 and 0.7, respectively, which suggests
12 that the number of occupiable glutamate-binding sites in these receptors changed compared to
13 wild-type.

14 *3.2. Response of wild-type and mutant channels to abamectin*

15 Unlike the glutamate response, application of abamectin induced slow-to-activate and
16 irreversible currents (Fig. 3A), and it was observed in all channels in a dose-dependent
17 manner (Fig. 3B). The current did return to original baseline even after washing for several
18 minutes with Ringer's solution. The EC_{50} s of abamectin calculated from the dose-response
19 relationships of wild-type, A309V, A309I, A309G and G315E channels were $0.16 \pm 0.05\mu\text{M}$,
20 $0.78 \pm 0.02 \mu\text{M}$, $0.25 \pm 0.01\mu\text{M}$, $0.15 \pm 0.02 \mu\text{M}$ and $80.32 \pm 23.67 \mu\text{M}$, respectively (Table
21 2). The results showed that the curve for abamectin was right-shifted in A309V (Fig. 3B), and
22 the EC_{50} reduced 5.0-fold but significantly ($p = 0.0284$), when compared with that of the
23 wild-type. However, both of the A309I and A309G mutated channels had similar EC_{50} values
24 ($p > 0.05$) to that wild-type channel (Table 2 and Fig. 3B).

25 In contrast, the G315E channel showed greatly reduced sensitivity to abamectin, yielding
26 a 493-fold right-shift of EC_{50} compared with that of wild-type (Table 2 and Fig. 3B), and the
27 observed reduction of EC_{50} between G315E and wild-type channel were significantly different
28 ($p = 0.0173$). Especially, the response of G315E channel to the highest concentration of

1 abamectin used in this study (0.3 mM), reached only $63 \pm 4.1\%$ of the response to saturating
2 glutamate-activated current (0.1 mM). It is therefore predicted that the G315E mutation in
3 PxGluCl may abolish abamectin potency.

4 *3.3. Molecular models of PxGluCl mutants*

5 A model of PxGluCl in the open state and in complex with abamectin was previously
6 generated (Wang et al., 2016b) using the crystal structure of CeGluCl α in complex with IVE
7 (Hibbs and Gouaux, 2011) as the homology template. As found previously with the A309V
8 mutation, there were no steric clashes encountered when A309 was substituted with either
9 glycine or isoleucine (Fig. 4). Furthermore, even introduction of the larger isoleucine
10 side-chain did not result in novel binding contacts forming with abamectin. This is due to the
11 register of the M3 helix whereby the 309 side chain is orientated away from the insecticide
12 binding site (Fig. 4).

13 G315 is also located on M3 but, in contrast to A309, the side chain of this residue is
14 orientated towards the ligand binding domain (Fig. 5). This glycine is predicted to form van
15 der Waals interactions with abamectin, which is also found with IVE and the conserved M3
16 glycine in the open-state CeGluCl α structure (Hibbs and Gouaux, 2011). The effect of the
17 G315E mutation was to produce a major steric clash with the benzofuran moiety of abamectin
18 (Fig. 5).

19 *3.4. Mutation screening in field populations*

20 As has been described in Wang et al. (2016b), we have established a rapid detection
21 method for the mutation occurred in M3 by using the direct-sequencing chromatograph. The
22 A309V mutation was first identified in Roth-Abm, which was obtained from a field
23 population TH with high-level of abamectin resistance (Pu et al., 2010), indicating that
24 A309V was present in the field. Both TH13 and TH16 were also collected in 2013 and 2016
25 from Tonghai County, Yunnan Province, China. Genotype detection has revealed that the
26 TH16 population consisted of 16 homozygous wild-type (Ala at position 309) and 4
27 heterozygous (Ala/Val) individuals, and the mutant frequency reached to 10% (Table 3). The
28 TH13 population collected 2yrs ago had also been detected carrying 6.25% mutant alleles. By

1 contrast, the A309V mutation was not detected in five populations from Southeast China
2 which had evolved high levels of resistance to abamectin (49- to 253-fold, unpublished data).
3 However, 2 of 8 populations (KS14 and KS15) from Eastern China have been shown to carry
4 3.33% and 1.67% mutant alleles, respectively, although both of them had low levels of
5 resistance to abamectin. In general, heterozygous and homozygous mutant-type individuals
6 with the A309V mutation have been detected in field populations collected from China, but
7 the reported mutations (G323D and G326E in TuGluCl) equivalent to G315 position of
8 PxGluCl have not been screened in the 17 field populations.

9 **4. Discussion**

10 In this study we cloned and functionally expressed PxGluCl from an
11 abamectin-susceptible strain of *P. xylostella*. The functional effects of mutations relevant to
12 abamectin resistance were also characterized. In addition, populations of abamectin-resistant
13 *P. xylostella* were genotyped to detect the occurrence of mutations at the A309 or G315
14 positions in the field. We previously demonstrated that the A309V mutation of PxGluCl was
15 strongly associated with a 10-fold increase in abamectin resistance in *P. xylostella* Roth-Abm
16 strain (Wang et al., 2016b). Here we show by expressing the wild-type or A309V channels in
17 oocytes that the mutant exhibits a modest but significant (~5-fold) reduction of abamectin
18 sensitivity relative to the wild-type channel. This finding strongly suggests that target-site
19 resistance plays a role in abamectin resistance of *P. xylostella* pest populations in the field.

20 We hypothesized that A309V may confer resistance by allosterically modifying the
21 abamectin binding site (Wang et al., 2016b). To investigate this further, A309G and A309I
22 mutants were generated, corresponding to residues with side chains that are either smaller or
23 bigger than wild-type or A309V. The A309G mutation showed equivalent abamectin
24 sensitivity compared with wild-type whereas the A309I channel showed a 1.5-fold decrease in
25 abamectin sensitivity, which contrasts with the 5-fold resistance exhibited by A309V.
26 Modelling of the receptor shows, as with A309V (Wang et al., 2016b), the A309G and even
27 the larger A309I side chain can be accommodated without sterically perturbing the structure
28 (Fig. 4). In addition, substitutions at the 309 position are still too far from the abamectin
29 binding site to interact directly with the ligand, further supporting the hypothesis of an

1 allosteric mechanism underlying resistance.

2 Intriguingly, A309 substitutions also affected glutamate sensitivity. A 1.7-fold reduction
3 of glutamate sensitivity was detected with A309G, a 1.5-fold increase in sensitivity with
4 A309V and surprisingly the A309I had increased glutamate sensitivity by one order of
5 magnitude (12-fold). The 309 position is located on the top of the M3 helix and so distal from
6 the glutamate binding site (see Fig. 6A in Wang et al., 2016b). Nevertheless, it is contiguous
7 with the M2-M3 linker, which is critical for transmitting neurotransmitter-induced
8 conformational changes in the extracellular domain to the transmembrane domain to gate the
9 pore (Althoff et al., 2014). Degani-Katzav et al. (2016) identified a mutation in the
10 IVE-binding site of M1 (α L279W; equivalent to $\alpha(-)$ L218 of CeGluCl crystal structure) that
11 reduced the EC₅₀ of glutamate by about 25-fold, when compared with the *C. elegans* wild
12 type receptor. Similarly, Daeffler et al. (2014) introduced several mutant amino acids of
13 varying size and polarity in to the M2 helix of *C. elegans* GluCl β receptor, four of these
14 mutations led to 9- to 44-fold increase in channel sensitivity to glutamate, respectively. In the
15 current study, it appears that larger residues at the top of M3 of PxGluCl facilitate channel
16 opening, although the model reveals that the replacement of the alanine side chain with the
17 larger isoleucine side chain didn't produce any steric clashes. Further studies will be required
18 to determine why the A309I mutant is more sensitive to glutamate and to explain why the Hill
19 coefficient has increased from 1.8 to 2.5, which suggests the presence of an additional
20 glutamate binding site. Yet the effect of the A309I mutation clearly demonstrates the
21 influence of this 309 position on GluCl pharmacology.

22 In the case of the A309V receptor, it is possible that this mutation does not allosterically
23 modify the binding site for abamectin, thus inhibiting its binding. Instead this mutation may
24 allosterically counteract the functional effect of the insecticide upon binding. We attempted to
25 investigate this by quantifying binding affinity using radioligand binding studies of
26 [³H]-avermectin to membranes of the PxGluCl β s expressed in *Drosophila* S2 cell lines.
27 Unfortunately these studies were not successful, due to extremely high levels of nonspecific
28 binding of [³H]-avermectin to membranes (data not shown). This is almost certainly due to the
29 very lipophilic nature of the radioligand. Similar problems have been reported in binding

1 studies with pyrethroids on insect sodium channels (Rossignol, 1988; Pauron et al., 1989;
2 Dong and Scott, 1994).

3 The current work also represents the first effort to characterize the pharmacological effect
4 of naturally occurring G315E mutation. This mutation is tightly linked to abamectin
5 resistance in the MAR-AB strain of *T. urticae*, with a resistance level of more than 2000-fold
6 (Dermauw et al., 2012). The equivalent G323D mutation is associated with a 18-fold
7 resistance to abamectin in an isogenic-resistant strain (Kwon et al., 2010). Our
8 electrophysiology findings show that introducing the G315E mutation into PxGluCl results in
9 493-fold resistance to abamectin. Glycine is entirely conserved in M3 helix of GluCls and the
10 role of substitutions at this “M3-Gly” position in conferring resistance to avermectin
11 compounds has been well characterized (Lynagh and Lynch, 2012). Human glycine receptor
12 $\alpha 1$ receptor (GlyR $\alpha 1$) has an alanine at the equivalent position and showed that
13 IVE-sensitivity (50-fold) can be conferred on an IVE-insensitive receptor by introducing a
14 M3-Gly at this position. Furthermore, the reciprocal introduction in the *Haemonchus*
15 *contortus* $\alpha 3$ BGluCl, G329A (the human GlyR $\alpha 1$ equivalent position) shifts the EC₅₀ of IVE
16 activation from 39 nM up to 1200 μ M. This indicates that M3-Gly is essential for high IVE
17 sensitivity in both glycine- and glutamate- gated Cys-loop receptors (Lynagh and Lynch,
18 2010). The proposed resistance mechanism for M3-Gly mutations (Lynagh and Lynch, 2012)
19 is that a larger side chain at this position sterically obstructs entry of an avermectin ligand to
20 the narrow gap between the M3 helix of principal subunit and M1 of the complementary
21 subunit and thus limits access to the insecticide binding site.

22 Recently, resistance to abamectin began to emerge and develop in field populations of
23 various arthropod pests in worldwide, including twenty-one species from Lepidoperan, Acari,
24 Blattodea, Coleoptera, Diptera, Hemiptera, Mesostigmata and Thysanoptera (APRD, 2017).
25 Without doubt, identification of abamectin-resistant mutations will be very valuable for
26 elucidating the molecular mechanisms and monitoring the development of resistance to
27 abamectin. In this work, in total 503 larvae sampled from 17 field *P. xylostella* populations
28 were analyzed for the estimation of the frequency of the A309V and G315D/E mutations,
29 previously associated with abamectin resistance in arthropods. The A309V mutation was

1 detected in four populations with lower frequencies. Two populations collected from Tonghai
2 county in 2013 and 2016 (TH13, TH16), where abamectin was mainly employed in the
3 control of *P. xylostella* and control failure always reported by local farmers, showed 6.25%
4 and 10% mutant allele frequencies. In contrast, G315D/E has not been detected in any tested
5 populations from China. Resistance development of field populations is complex, and
6 resistant phenotype might be associated with metabolic detoxification of abamectin and other
7 potential mechanisms. Although insecticide resistance development may or may not result in
8 control failure, it has given rise to the loss of many selective compounds that could otherwise
9 have formed part of insecticide resistance management (IRM) and, more broadly, integrated
10 pest management programs for *P. xylostella*. In view of the A309V mutation detected in *P.*
11 *xylostella* field populations, and the G314D and G326E mutations have been reported in *T.*
12 *urticae*, originating from rose greenhouses with varied frequencies (from 5% to 100%) (Ilias
13 et al., 2016), molecular diagnostic assays for early identification and monitoring of
14 low-frequency A309V and G315D/E alleles of PxGluCl in a globally collected samples is
15 required in IRM strategies of *P. xylostella*.

16 In conclusion, here we functionally characterized the potency of abamectin and the
17 endogenous neurotransmitter glutamate on the activity of wild-type and mutant PxGluCls.
18 Two naturally occurring mutations in the M3 helix of arthropod GluCls that are associated
19 with abamectin resistance reduced the channel sensitivity to this compound. We suggest that
20 two different mechanisms are involved in abamectin target-site resistance, with the G315
21 mutation directly sterically hindering abamectin binding while the A309V mutation modifies
22 the allosteric binding site. Fitness analysis is required to define the physiological function
23 and/or negative effects of these mutations on insects. Our study provides direct evidences that
24 A309V and G315E mutations on M3 helix can result in abamectin resistance. We also suggest
25 that resistant alleles should be monitored continuously, which will provide useful information
26 for investigating the evolution and spread of well-characterized resistance genes.

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3 Council (BBSRC) in collaboration with Syngenta (Grant BB/M503083/1).

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1 **Table 1**

2 Sequences of primers used in this study.

Primer	Use	Sequence (5' to 3')	
PxGluCl_EcoRI_F	Full-length cDNA amplification and fusion PCR	CGGAATTCGGTTTGCTGAGTTGGAGAATGGACG	3 4 5
PxGluCl_XbaI_R	Full-length cDNA amplification and fusion PCR	GCTCTAGATGCCAGTGGAACGATGCTGATGC	6
Px_A309V_F	Amplify the downstream fragment of A to V mutation	GTATCATAACAAAAGGTCATAGACGTATGGACTGG	7
Px_A309V_R	Amplify the upstream fragment of A to V mutation	CCAGTCCATACGTCTATGACCTTTGTGTATGATAC	8
Px_A309I_F	Amplify the downstream fragment of A to I mutation	GTATCATAACAAAAGATCATAGACGTATGGACTGG	9
Px_A309I_R	Amplify the upstream fragment of A to I mutation	CCAGTCCATACGTCTATGATCTTTGTGTATGATAC	10 11
Px_A309G_F	Amplify the downstream fragment of A to G mutation	GTATCATAACAAAAGGGCATAGACGTATGGACTGG	12
Px_A309G_R	Amplify the upstream fragment of A to G mutation	CCAGTCCATACGTCTATGCCCTTTGTGTATGATAC	13
Px_G315E_F	Amplify the downstream fragment of G to E mutation	TAGACGTATGGACTGAGGTTTGTAAACATTCG	14
Px_G315E_R	Amplify the upstream fragment of G to E mutation	CGAATGTTAAACAAACCTCAGTCCATACGTCTA	15

16

1 **Table 2**

2 EC₅₀ values and Hill coefficients of L-glutamate- and abamectin-induced responses of
 3 PxGluCl_s expressed in *Xenopus laevis* oocytes. Data were obtained by nonlinear regression
 4 analyses and indicated as means and 95% confidence limits (n = 5 ~ 6).

5

Variants	L-glutamate (μM)					Abamectin (μM)				
	n	EC ₅₀	95% CL	n _H	95%CL	n	EC ₅₀	95% CL	n _H	95%CL
wild-type	6	19.93	17.43-22.79	1.8	1.4-2.1	5	0.163	0.118-0.226	1.4	0.8-1.9
A309V	6	13.24	11.73-14.94	1.0	0.9-1.1	5	0.780	0.648-0.940	1.0	0.8-1.2
A309I	6	1.673	1.514-1.847	2.5	2.0-2.9	5	0.250	0.155-0.404	1.1	0.6-1.6
A309G	6	34.60	27.47-43.60	0.7	0.6-0.8	5	0.153	0.132-0.177	1.8	1.4-2.2
G315E	6	10.35	8.689-12.33	1.0	0.9-1.2	5	80.320	53.87-119.7	0.5	0.4-0.6

1 **Table 3**

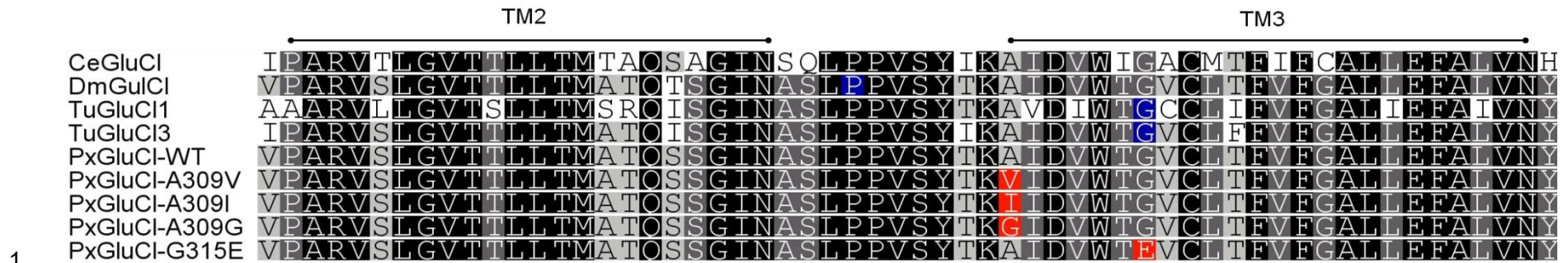
2 Levels of resistance to abamectin and the A309V mutation frequencies in field *P. xylostella*
 3 populations from China.

4

Populations	RF ^a	N ^b	A309V			Mutation frequency (%)
			Ala/Ala	Ala/Val	Val/Val	
Pops. from Southwest China						
TH13	345	40	35	5	0	6.25
MD13	491	36	36	0	0	0
SH13	383	36	36	0	0	0
TH16	92	20	16	4	0	10
Pops. from Southeast China						
GZ14	49	30	30	0	0	0
ZC14	151	30	30	0	0	0
SZ14	253	30	30	0	0	0
HZ14	162	30	30	0	0	0
HK15	86	22	22	0	0	0
Pops. from Eastern China						
NJ14	4.7	30	30	0	0	0
HF14	12	30	30	0	0	0
JN14	24	30	30	0	0	0
KS14	3.1	30	29	0	1	3.33
NJ15	14	30	30	0	0	0
HF15	3.3	30	30	0	0	0
JN15	2	30	30	0	0	0
KS15	12	30	29	1	0	1.67

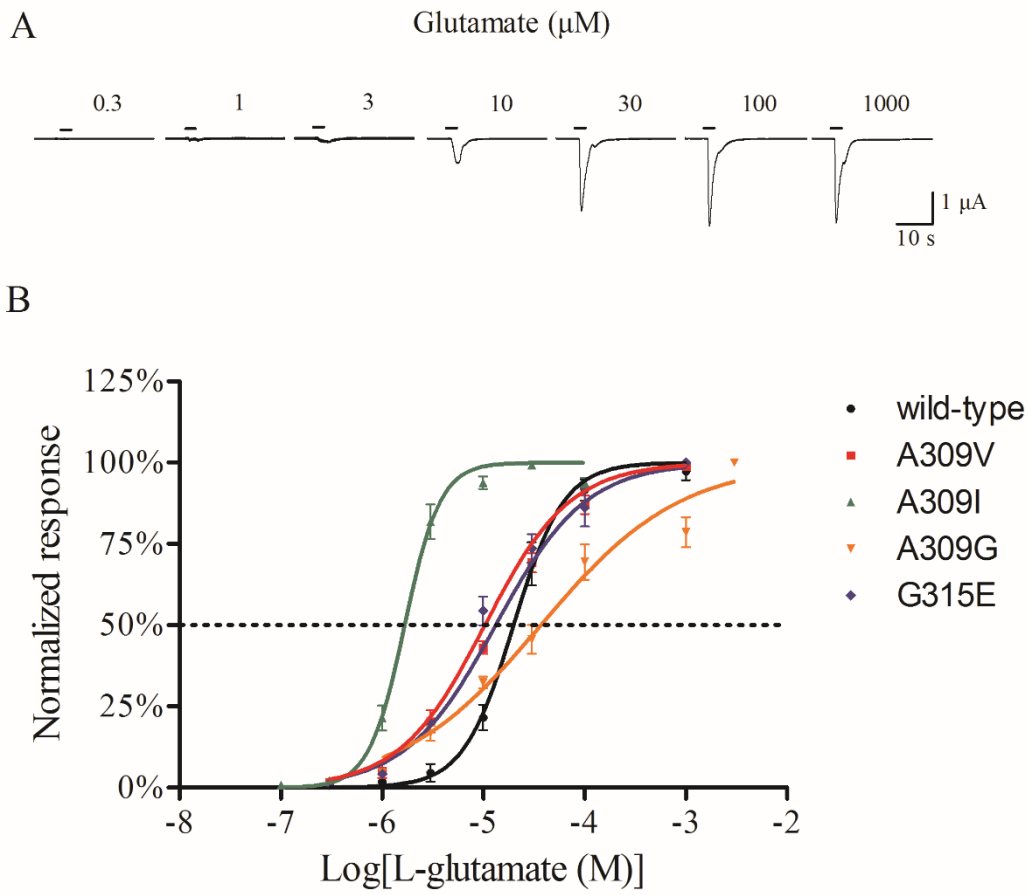
^a RF (resistance factor) = LC₅₀ (resistant strain) ÷ LC₅₀ (Roth). Unpublished data.

^b Number of insects genotyped. A, alanine (Ala); V, valine (Val).



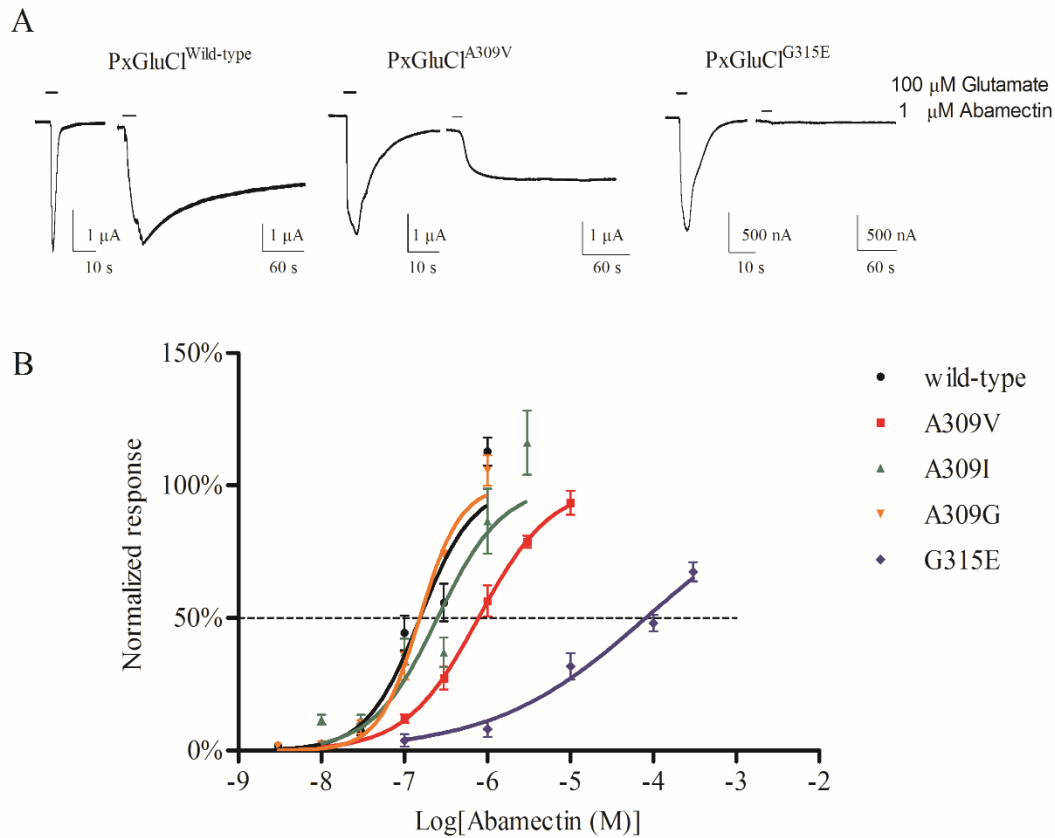
1
2

3 **Fig. 1.** Alignment of the amino acid sequences in transmembrane domain 2 and 3 of glutamate-gated chloride channel genes of *C. elegans*
 4 (CeGluCl, GenBank accession code AAA50785.1), *D. melanogaster* (DmGluCl, GenBank accession code U58776.1), *T. urticae* (TuGluCl1,
 5 TuGluCl3, GenBank accession code XM_015938823.1 and NP_001310061.1) and *P. xylostella* (PxGluCl-WT, PxGluCl-A309V, PxGluCl-A309I,
 6 PxGluCl-A309G, PxGluCl-G315E). Fully conserved residues are shaded in black, while lower similar positions are shaded in grey. The P229S,
 7 G314D and G326E mutations in DmGluCl, TuGluCl1 and TuGluCl3, associated with ivermectin/abamectin resistance, are indicated in blue
 8 (Kane et al., 2000; Kwon et al., 2010; Dermauw et al., 2012). The A309V mutation in PxGluCl associated with abamectin resistance (Wang et al.,
 9 2016b) and the introduced A309I/G and G315E substitutes in this study are indicated in red.



1

2 **Fig. 2.** Glutamate responses of wild- and mutant-type homomeric PxGluCl1s expressed in
 3 *Xenopus* oocytes. (A) Traces of electrical responses induced by L-glutamate from the
 4 wild-type ion channels. (B) Normalized dose-response curves for glutamate induced currents
 5 for oocytes expressing wild-type, A309V, A309I, A309G and G315E mutant channels. The
 6 data are means \pm SEM (n = 6).



1

2 **Fig. 3.** Abamectin responses of wild- and mutant-type homomeric PxGluCl_s expressed in

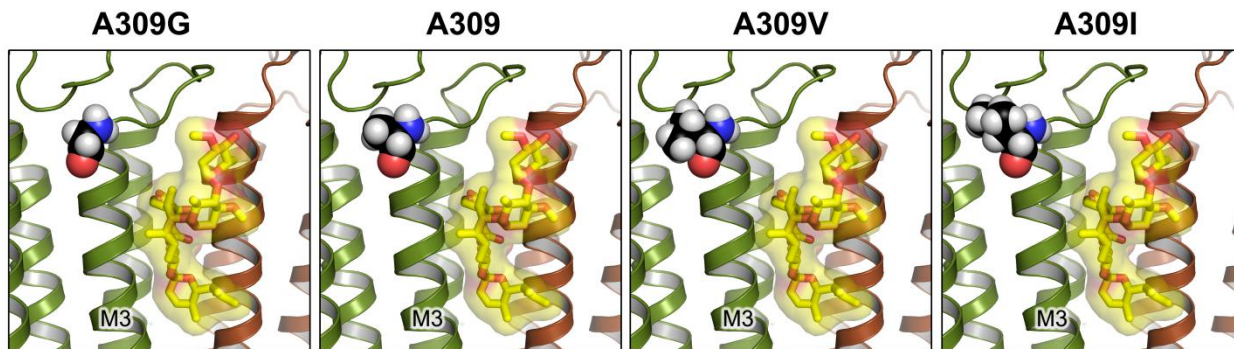
3 *Xenopus* oocytes. (A) Examples of electrical responses induced by saturated glutamate (100

4 μ M) and abamectin (1 μ M) in the wild-type, A309V and G315E mutant ion channels,

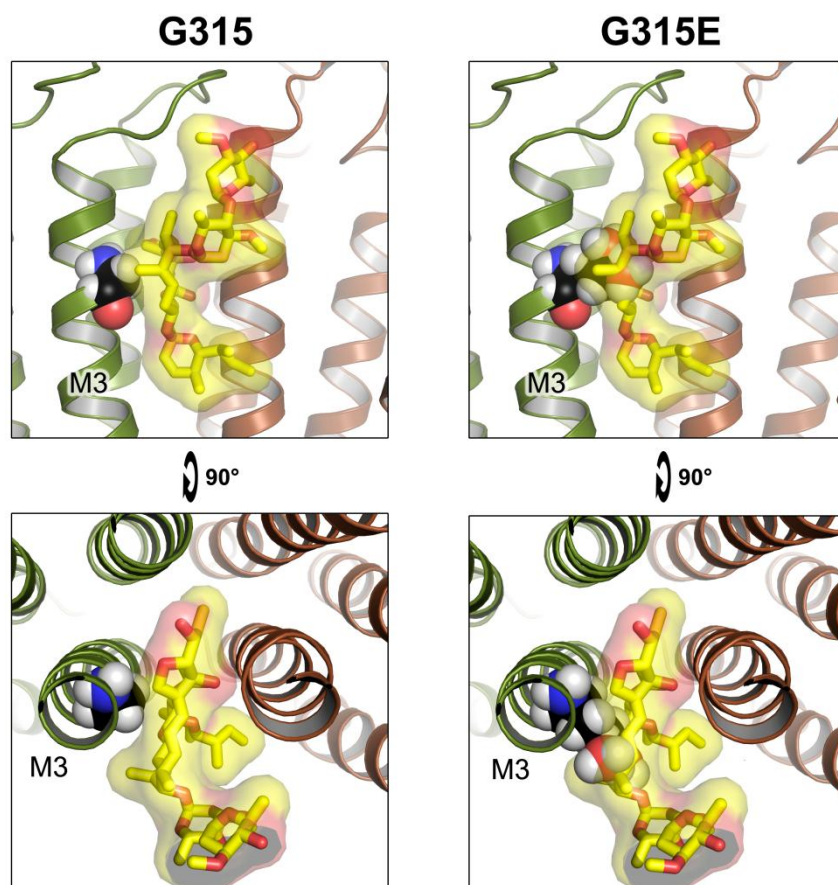
5 respectively. (B) Normalized dose-response curves for abamectin induced currents for oocytes

6 expressing wild-type, A309V, A309I, A309G and G315E mutant channels. The data are means

7 \pm SEM (n = 5).



1
2 **Fig. 4.** Models of wild-type PxGluCl and A309 mutations. Side view of the transmembrane
3 domain with channel monomers shown as cartoon. The 309 position is shown in black
4 spacefill and abamectin is shown as yellow sticks with a transparent surface.



1
 2 **Fig. 5.** Models of wild-type PxGluCl and the G315E mutation. Side view and extracellular
 3 view of the abamectin binding site with channel monomers shown as cartoon. The 315
 4 position is shown in black spacefill and abamectin is as yellow sticks with a transparent
 5 surface.