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Key Words:	avermectin, ivermectin, arthropoda, GluCl, Rdl, two-electrode voltage-clamp electrophysiology

Untangling a Gordian knot: the role of a GluCl3 I321T mutation in abamectin resistance in *Tetranychus urticae*

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Abstract

BACKGROUND

The cys-loop ligand-gated ion channels, including the glutamate-gated chloride channel (GluCl) and GABA-gated chloride channel (Rdl) are important targets for drugs and pesticides. The macrocyclic lactone abamectin primarily targets GluCl and is commonly used to control the spider mite *Tetranychus urticae*, an economically important crop pest. However, abamectin resistance has been reported for multiple *T. urticae* populations worldwide, and in several cases was associated with the mutations G314D in *GluCl1* and G326E in *GluCl3*. Recently, an additional I321T mutation in *GluCl3* was identified in several abamectin resistant *T. urticae* field populations. Here, we aim to functionally validate this mutation and determine its phenotypic strength.

RESULTS

The *GluCl3* I321T mutation was introgressed into a *T. urticae* susceptible background by marker-assisted backcrossing, revealing contrasting results in phenotypic strength, ranging from almost none to 50-fold. Next, we used CRISPR-Cas9 to introduce I321T, G314D and G326E in the orthologous *Drosophila GluCl*. Genome modified flies expressing GluCl I321T were threefold less susceptible to abamectin, while CRISPRed G314D and G326E flies were lethal. Last, functional analysis in *Xenopus* oocytes revealed that the I321T mutation might reduce GluCl3 sensitivity to abamectin, but also that all three *T. urticae* Rdls are affected by abamectin at high concentrations.

CONCLUSION

Three different techniques were used to characterize the role of I321T in GluCl3 in abamectin resistance and, combining all results, our analysis suggests that the I321T mutation has a complex role in abamectin resistance. Given the reported subtle effect, additional synergistic factors in resistance warrant more investigation.

Keywords: avermectin, ivermectin, arthropoda, GluCl, Rdl, two-electrode voltage-clamp electrophysiology

1 INTRODUCTION

Macrocyclic lactones such as avermectins and milbemycins are natural fermentation products of *Streptomyces* bacteria. They exhibit strong insecticidal, nematicidal, and acaricidal activity with low toxicity in mammals and have been widely used in pest control and as anthelmintics in animal and human health for several decades.[1–5] Macrocyclic lactones are neurotoxins known to act on cys-loop ligand-gated ion channels (LGICs). These channels contain five homologous subunits, where each subunit consists of a large N-terminal extracellular domain, four hydrophobic alpha-helical transmembrane segments (TMs), an intracellular loop between TM3 and TM4 and a short extracellular C terminus to form a central ion channel lining (Fig. 1a).[6–9] In invertebrates, the glutamate-gated chloride channel (GluCl), GABA-gated chloride channel (GABACl, also known as Resistance to dieldrin (Rdl)), the histamine-gated chloride channel (HisCl) and the pH-sensitive chloride channel (pHCl) belong, amongst others, to the cys-loop LGIC family.[2,10–14] In *Caenorhabditis elegans* six GluCl and one Rdl gene have been identified,[7,8,15,16] while most insects, except Lepidoptera and aphids, only have a single GluCl and Rdl gene. Genome sequencing of the spider mite *Tetranychus urticae* and the honeybee mite *Varroa destructor*, revealed the presence of five GluCl and three Rdl genes in *T. urticae,* while three Rdl genes have been found in *V. destructor*.[14,17–21] The GluCl has an important role in the nervous system, including modulation of locomotion, regulation of feeding and mediation of sensory inputs and is the primary target of macrocyclic lactones, while Rdl is considered as a secondary target of these compounds.[15,22–26] Fuse et al., for example, showed that the EC_{50} of ivermectin, an avermectin, was more than 150-fold larger for *Musca domestica* Rdl than for GluCl.[26] Of peculiar note, cys-loop channels, sensitive to ivermectin, consisting of both GluCl and Rdl subunits have previously been reported.[12,27,28]

The spider mite *T. urticae* is a highly polyphagous pest and develops resistance very rapidly.[29,30] A frequently used compound to control *T. urticae* is abamectin, a mixture of avermectins containing a minimum of 80% avermectin B_{1a} and maximum 20% avermectin B_{1b}. Due to its extensive application for decades, increasing field abamectin resistance in *T. urticae* has been reported.[31–36] Backcrossing experiments and F_2 screen showed that two mutations, G314D in *GluCl1* and G326E in *GluCl3*, were associated with abamectin resistance in *T. urticae*.[14,37] Based on the crystal structure of the *C. elegans* GluClα-subunit and its binding-mode to ivermectin, a residue in the

third transmembrane region (TM3), equivalent to the position of the above two mutations, was likely involved in the formation of the allosteric site and located closest to ivermectin.[7] In addition, functional validation using two electrode voltage-clamp electrophysiology and *Xenopus* oocytes also revealed that a G326E substitution in *T. urticae* GluCl3 completely abolished the agonistic activity of abamectin.[38] Further, an identical substitution at an equivalent position in *Plutella xylostella* GluCl reduced the sensitivity to abamectin by about 500-fold,[39] while a G329D substitution (corresponding to G314D in *T. urticae* GluCl1) in *Haemonchus contortus* GluCl, abolished binding with the macrocyclic lactone milbemycin A_4.[40]

Recently, a target-site (GluCl) screening of 32 European field *T. urticae* strains, revealed the presence of a potential new target-site mutation, I321T in *GluCl3*, in four abamectin resistant strains.[36] This mutation was also found in another abamectin resistant strain from Peloponnese, Greece in a recently published paper of Papapostolou et al. 2020.[41] This mutation is adjacent to A309V, a previously reported abamectin resistance associated mutation in *P. xylostella*.[39] In addition, two other mutations V327G (adjacent to the important G326 residue) and L329F were also found in *GluCl3* of a strain exhibiting an around 500-fold resistance ratio (RR). L329 corresponds to M345 of *C. elegans* GluClα, a residue predicted to be involved in ivermectin binding;[7] while two substitutions at a position equivalent to L329F (L315F and L319F in GluCl of *M. domestica* and *Bombyx mori*, respectively) also showed reduced sensitivity to ivermectin.[42,43] However, none of these recently identified mutations (I321T, V327G and L329F) have been functionally validated in *T. urticae*. Characterizing the properties of these mutations could help in understanding the macrocyclic lactone resistance mechanisms in *T. urticae* and, in the long term, might aid in designing effective pest management strategies. In this study, the main aim is to characterize the I321T mutation in *GluCl3*. First, we performed electrophysiological analyses to clarify the effect of abamectin on GluCl3 wild-type, I321T GluCl3 and Rdls in *T. urticae*. Next, marker-assisted back-crossing was used to introduce the mutation into a *T. urticae* line with a susceptible genetic background, and the relative contribution of the mutation to abamectin resistance was assessed. Finally, CRISPR-Cas9 technology was used to introduce the I321T mutation in the *Drosophila* GluCl homologue, and the effect of I321T on abamectin resistance was compared against the nos.Cas9 stock and G314D and G326E flies.

2 MATERIALS AND METHODS

2.1 Mite strains

The abamectin susceptible strain SR6 was collected from tomato plants in Italy in 2017. This strain was inbred by six rounds of mother-son mating as previously described in Bryon et al. 2017 and screened for the presence of mutations in *GluCl1*/*2*/*3*/*4*/*5* TM3 and *Rdl1*/*2*/*3* TM2/TM3 regions (see 2.2).[44] The abamectin resistant strain IT6 has been previously described and of all five GluCl genes only *GluCl3* carried an I321T mutation,[36] while the TM2/TM3 region of *Rdl1*/*2*/*3* was screened in this study (see 2.2.) The abamectin resistant strain TR2 (corresponding to sample number 2 in İnak et al. 2019) has been previously described and lacks the previously documented abamectin resistance mutations, G314D in *GluCl1* and G326E in *GluCl3*.[45] The TM3 region of *GluCl2*/*4*/*5*, I321T mutation in *GluCl3* and TM2/TM3 region *Rdl1*/*2*/*3* of TR2 were screened in this study (see 2.2). In our previous study, the strains IT1, IT5 and ES1 were screened for the presence of mutations in TM3 of *GluCl1*/*2*/*3*/*4*/*5* [36] while the TM2/TM3 regions of *Rdl1*/*2*/*3* of these strains were screened in this study. Furthermore, all GluCl and Rdl genes from MR-VL from Belgium[46], were also screened in this study. Detailed information of all strains can be found in Table S1. All *T. urticae* strains were maintained on non-sprayed kidney bean leaves (*Phaseolus vulgaris* L. cv. “Prelude”) under laboratory conditions (25±1°C, 60% relative humidity, and 16:8 hr light: dark photoperiod).

2.2 Survey of genotypes in GluCl TM3 and Rdl TM2/TM3 regions

Genomic DNA (gDNA) of strains IT1, IT5, IT6, ES1, MR-VL and SR6 was extracted from approximately 200 *T. urticae* female adults as described earlier by Van Leeuwen et al. (2008). gDNA of the TR2 strain was extracted from approximately 100 adult female mites with the Qiagen DNeasy Blood & Tissue Kit following the manufacturer’s instructions. Quality and quantity of DNA were checked via a spectrophotometer (Thermo Scientific NanoDrop 2000 or a DeNovix DS-11 (DeNovix, Willmington, DE, USA)). PCR amplification of the TM3 regions from five GluCl genes and TM2/TM3 fragments from three Rdl genes was performed as described by Dermauw et al..[14] Primers used for PCR are listed in Table S3. PCR-products were purified using E.Z.N.A. Cycle Pure Kit (Omega Bio-Tek) and were sequenced (LGC genomics, Germany). All sequenced data was analysed using BioEdit version 7.0.5.2..[47]

2.3 Toxicity bioassays

Toxicity bioassays were carried out with commercially formulated abamectin (Vertimec 18 g a.i./L EC or Agrimec 18 g a.i./L EC). At least five concentrations (causing between 20 and 80% mortality) were tested in four replicates and blank controls were sprayed with deionised water only. For each replicate, about 20-30 young adult female mites were transferred to 9-cm^2 bean leaf disks and sprayed with serial pesticide dilutions at 1 bar pressure. For IT6 and congenic lines (2.4), a Cornelis spray tower was used for spraying leaf disks and 0.8 mL of pesticide dilution was applied to obtain a 1.5 ± 0.05 mg aqueous acaricide deposit cm^{-2}.[45,46,48] For TR2 and congenic lines (2.4), a Potter spray tower (Burkard Scientific Ltd, Uxbridge, UK) was used for spraying and 2 mL of acaricide dilution was applied to obtain a 1.95 ± 0.05 mg aqueous acaricide deposit cm^{-2}. LC_{50} and LC_{90} values and their 95% confidence intervals (CIs) were determined with PoloPlus (LeOra Software, Berkeley, CA, USA, 2006).[49] Resistance ratios (RRs) were considered significantly different when their 95% CI did not include the value 1.[50]

2.4 Backcrossing experiments

2.4.1 Establishment of congenic lines

To assess the resistance levels associated with the I321T mutation, we used a marker assisted backcrossing approach to produce near-isogenic sister lines as previously outlined in Riga *et al.* 2017.[51] Briefly, a haploid male of a resistant strain (IT6= BC1 or TR2= BC2) was crossed with a virgin female of the susceptible strain SR6, in three and four replicates respectively. The resulting heterozygous virgin females of each replicate cross were backcrossed to susceptible males of SR6 and heterozygote genotypes (I321/T321) were identified by PCR and sequencing as described in section 2.4.2. This process was repeated for seven generations. In the last generation, backcrossed heterozygous virgin females were crossed with their first-born sons, representing either the I321 or T321 genotype respectively, and resulting in congenic homozygous lines (with either the I321/I321 or T321/T321 genotype).

2.4.2 Single mite DNA extraction and genotyping

DNA from individual adult females was extracted using the Qiagen DNeasy Blood & Tissue Kit following the manufacturer instructions, or by homogenization in 20 µL STE buffer (100 mM NaCl, 10 mM Tris- HCl and 1 mM EDTA) with 1 mg ml^{-1} proteinase K (Sigma-Aldrich, Overijse, Belgium) and incubated for 30 min at 37 °C and 5 min at 95 °C.[51] The primers used to amplify the TM3 of GluCl3 were as follows: 5'-3'

CCGGGTCAGTCTTGGTGTTA and 3'- 5' CACCACCAAGAACCTGTTGA.[14] Then standard PCR was conducted with single mite DNA. PCR products were purified and sequenced (LGC genomics, Germany and Macrogen Inc., Seoul, Korea). All sequenced data was analysed using BioEdit version 7.0.5.2.[47]

2.4.3 Toxicity bioassays of congenic lines

Toxicity bioassays with abamectin were carried out as described in 2.3.

2.5 CRISPR-Cas9 genome editing *in Drosophila*

2.5.1 *Drosophila* stocks

Genome modification of *Drosophila* was performed in the strain y1 M{nos-Cas9.P} ZH-2A w*,[52] in which Cas9 is expressed under the control of the *nos* promoting element (strain #54591 at the Bloomington *Drosophila* stock center, hereafter referred to as nos.Cas9). We also used a balancer stock for the third chromosome (*yw*; TM3 Sb e / TM6B Tb Hu e), hereafter referred to as TM3/TM6B. Flies were cultured under laboratory conditions (25 °C, 60-70% relative humidity, and 12:12 hr light: dark photoperiod) and fed on a standard fly diet.

2.5.2 *Drosophila* DNA purification and amplification

DNA was purified from *Drosophila* by DNAzol® (MRC) according to manufacturer instructions. PCR amplification with relevant primer pairs (Table S3) was performed with Taq DNA Polymerase (MINOtech BIOTECHNOLOGY) and the conditions used were 94 °C for 2 min, followed by 35 cycles of denaturation at 94°C for 45 sec, annealing at 57 °C (generic primers) or 51 °C (specific primers) for 30 sec, and extension at 72 °C for 30 sec followed by a final extension step at 72 °C for 5 min.

2.5.3 Genomic engineering strategy

An *ad hoc* CRISPR-Cas9 genomic engineering strategy was designed in order to generate the mutations of interest (GluCl1 G314D, GluCl3 G326E and GluCl3 I321T, *T. urticae* numbering) at the *GluCl* gene (CG7535) of *D. melanogaster* (Fig. S1).

In order to identify possible variations from the published genome sequence, two sets of primers pairs were used (GluExFw/GluExRv and GluInFw/GluInRv, Table S3) and a nos.Cas9 DNA template to amplify and sequence a 1771 bp DNA fragment corresponding to the genomic region 3R: 19,763,419-19,765,189 (BDGP6.28 genome assembly) encompassing exons 8 and 9 of GluCl (Fig. S1). Potential CRISPR targets in the region of interest were identified using the online tool Optimal Target Finder[53]

(http://tools.flycrispr.molbio.wisc.edu/targetFinder/) and a target with no predicted off-target hits was selected.

A relevant sgRNA expressing plasmid was generated using vector pU6-BbsI chiRNA[54] by sub cloning a double stranded DNA oligo (generated by annealing sense and antisense sgRNA oligos (Table S3). To facilitate Homologous Directed Repair (HDR), three donor plasmids were synthesized *de novo* (donorI321T, donorG314D and donor G326E (Genscript, NJ, USA)) containing two ~800 bp homology arms flanking the CRISPR target. The donor sequences correspond to the genomic region 3R: 19,763,457-19,765,164 and contain the non-synonymous substitution that generates the relevant mutations as well as certain synonymous mutations incorporated for diagnostic purposes or to avoid cleavage of the donor plasmid by the CRISPR/Cas9 machinery. Plasmid donorG314D contained the non-synonymous GGC→GAC substitution (leading to the G314D mutation), as well as synonymous mutations to abolish an existing ClaI restriction site and introduce a new MluI restriction site (Fig. S2a). Similarly, plasmid donorG326E contained at the same site the non-synonymous GGC→GAG substitutions (leading to the G326E mutation), as well as synonymous mutations to abolish an existing XhoI restriction site and introduce a new AccI restriction site (Fig. S2b), while plasmid donorI321T contained the non-synonymous ATC→ACC substitution (leading to the I321T mutation and abolishment of ClaI) as well as certain synonymous mutations to introduce MluI and inhibit recognition and cleavage of the plasmid by CRISPR/Cas9 (Fig. S2c). Note that the G→D or G→E mutations occur at the same position (G312 in *Drosophila* GluCl) for donorG314D and donorG326E, respectively.

2.5.4 Generation and screening of genome modified flies

Preblastoderm nos.Cas9 embryos were injected with a plasmid mixture containing 75 ng/ µL of sgRNA plasmid and 100 ng/ µL of donor plasmid in injection buffer (2 mM Sodium phosphate pH 6.8-7.8, 100 mM KCl). Injected G_0 adults were back-crossed to nos. Cas9 and G_1 progeny was initially screened *en masse* to identify crosses that had produced G_1 flies that underwent HDR events (Fig. S3a). Screening was performed by DNA extraction from batches of ~30 individuals per vial, digesting ~2 µg of total DNA with *Cla*I (for G314D and I321T) or XhoI (for G326E) in order to preferentially digest wild-type alleles but not the genome modified alleles that contain the donor sequence where the corresponding site is absent, and using ~30 ng of the digested DNA as

template for amplification. Screening was performed either with a "generic" primer pair yielding a 489 bp product that was further digested with *Mlu*I (for G314D and I321T) or AccI (for G326E) that were introduced into the genome modified allele, or in the case of I321T with a "specific" primer combination (Dmel_GluCl_generic_F / Dmel_GluCl_specific_R, Table S3) that generates a diagnostic fragment (429 bp) in the presence of the modified allele.

In case the presence of genome modified alleles was indicated in the pool, individual G_1 flies from the same original cross were outcrossed with nos.Cas9 flies and after generating progeny (G_2), they were screened individually to identify crosses bearing genome modified alleles. Several lines originating from positive G_1 flies were established, and individual G_2 flies (expected to be heterozygous for the mutant allele at a 50% ratio) were balanced against a TM3 / TM6B balancer strain and then individually screened (Fig. S3b) to select crosses generating G_3 progeny containing the genome modified allele against the balancer chromosome. Then, single balanced G_3 flies were back-crossed with the same balancer fly stock and following a final molecular screening, adults with *Stubble* phenotype from two positive lines (heterozygous for the mutation) were collected and crossed among themselves to generate two homozygous strains bearing the I321T mutation. The established lines were sequence verified (Fig. S3c). Sequencing reactions were performed at CeMIA sequencing facility (CEMIA, S.A., Greece).

2.5.5 Bioassays

Abamectin was used for toxicity bioassays as a commercial insecticide formulation (Vertimec 18 g a.i./L EC). Bioassays were performed as previously described.[55] Briefly, 2nd instar larvae were collected and transferred in batches of 20 into new vials containing fly food supplemented with different insecticide concentrations. Total development time was monitored for a period of 10-15 days for each applied insecticide concentration, and pupation efficiency was used as a proxy for survival. Five to six insecticide concentrations that cause 5-95% mortality (when applicable) were tested in triplicates, together with relevant negative (no insecticide) controls for I321T and the wild-type (nos.Cas9) strains.

2.5.6 Statistical analyses

Observed mortality for each insecticide concentration was corrected for control (no insecticide) mortality using Abbott's formula.[56] The LC_{50} values and 95% confidence

intervals (CIs) were calculated via probit analysis using PoloPlus (LeOra Software, Berkeley, California), resistance ratios (RRs) were considered significantly different when the 95% CI of the RRs did not include the value 1.[50]

2.6 Two-electrode voltage-clamp electrophysiology

2.6.1 Vector construction and cRNA synthesis

The TuGluCl3 I321T plasmid was generated by GenScript (Piscataway, New Jersey, USA) using site-directed mutagenesis and the TuGluCl3 WT plasmid, previously described in Mermans et al 2017, as template. The TuRdl# constructs were *in silico* generated in an identical way as TuGluCl3 WT constructs[38] except that the *T. urticae* GluCl3 coding sequence was replaced by either *T. urticae* Rdl1 (GenBank acc. AB567686.1, TuRdl1), Rdl2 (*tetur36g00580*, accessible at ORCAE[57] ((https://bioinformatics.psb.ugent.be/orcae/overview/Tetur)), TuRdl2) and Rdl3 (*tetur36g00590* accessible at ORCAE, TuRdl3) (also see File S1). All *T. urticae* Rdl coding sequences were codon optimized for *Xenopus* expression using the OptimumGene™-Codon Optimization software of GenScript. Next, TuRdl# constructs were synthesized *de novo* and ligated into a pUC57 plasmid by Genscript. After transformation into DH5α competent *Escherichia coli* (Invitrogen, Carlsbad, CA, USA) GluCl and Rdl plasmids were purified using the E.Z.N.A.® Plasmid DNA Mini Kit I (Qiagen, Germantown, MD, USA). The plasmids were linearized with NotI-HF restriction endonuclease (New England BioLabs Inc., Ipswich, MA, USA) followed by extraction using the phenol and chloroform method with ethanol precipitation [58]. Capped mRNAs were generated using the mMESSAGEmMACHINE T7 transcription kit (Thermo Fischer Scientific, Waltham, MA, USA) according to manufacturer instructions. Synthesized cRNAs were purified with a RNeasy MinElute Cleanup Kit (Qiagen, Germantown, MD, USA), dissolved in nuclease-free water and stored at -80 °C until use.

2.6.2 Oocyte injection and two-electrode voltage clamp electrophysiology

Defolliculated, stage V–VI *X. laevis* oocytes were acquired form Ecocyte Bioscience (Castrop-Rauxel, Germany) in Tris-buffered Barth's solution (Ecocyte Bioscience). Oocytes were microinjected using a Nanoject III Programmable Nanoliter Injector (Drummond Scientific Co., Broomali, PA, USA) with 75 nL of cRNA solution (25–50 ng/µL) and incubated at 18 °C in sterile Barth's solution. Recordings were made 1–4 days post-cRNA injection. Two-electrode voltage-clamp (TEVC) measurements were

conducted with the fully automated Roboocyte2 (Multi Channel Systems MCS GmbH, Reutlingen, Germany); oocytes were held in a standard 96-well microtitre plate and impaled with two glass microelectrodes filled with 0.1 M KCl 1.5 M potassium acetate solution. Oocyte membrane potentials were fixed at -60mV throughout the experiment.

2.6.3 Actions of L-glutamic acid and abamectin on wild type and I321T GluCl3

Test solutions of L-glutamic acid monosodium salt monohydrate (CAS number 6106-04-3; Sigma–Aldrich) and abamectin (CAS number 71751-41-2, more than 80% avermectin B1a and less than 20% avermectin B1b; Sigma–Aldrich) were freshly prepared from stock solutions made with dimethyl sulfoxide (DMSO) and diluted with Normal Frog Ringer (NFR) solution (Ecocyte Bioscience). DMSO concentrations for test solutions did not exceed 1%. Dose-response curves to the natural agonist and compounds were obtained by sequential applications for 30 s of increasing concentrations with a 90s recorded wash-out (NFR) between applications for the channel to recover from desensitization. Abamectin application was proceeded by L-glutamic acid (at EC_{50} concentration) to normalize the response and to validate GluCl expression. An extra two min non-recorded wash-out was maintained between abamectin applications to further allow the current to return to baseline as desensitization from abamectin is slow. Next, abamectin was co-applied with L-glutamic acid for 30 sec to both wild type and I321T injected oocytes to test abamectin potentiation of glutamate-induced currents. Responses to L-glutamic acid applications were normalized (I% = (I/ Imax) × 100) and graphed as means ± SEM (standard error of the mean) using at least 6 oocytes. TEVC recordings were analyzed using the Roboocyte 2+ V. 1.4.3. software (Multi Channel Systems MCS GmbH), EC_{50}, pEC_{50} values and Hill coefficients were calculated by fitting a four-parameter logistic curve (Hill equation) on response data using SigmaPlot software 13.0 (Systat Software, San Jose, CA, USA).

2.6.4 Actions of γ-aminobutyric acid and abamectin on wild type *T. urticae* Rdls

The activity of γ-aminobutyric acid (GABA; CAS number 56-12-2; Sigma–Aldrich) and abamectin on three *T. urticae* Rdls (Rdl1, Rdl2 or Rdl3) channels was examined as described above, with a prolonged abamectin application of 3 min. To assess the antagonistic effect of abamectin, oocytes were tested based on Rufener *et al*. 2017.[59] Oocytes were first exposed to GABA (EC_5, EC_{50} or EC_{90} concentration) 4 times for 30 s every 1.5 min at the beginning of the experiment to test for Rdl expression and to

stabilize the response. Subsequently, oocytes were pre-exposed for 75 s to abamectin (1 nM-10 μM) followed by 30 s of co-applied GABA (EC_{50}) and abamectin. Both compounds were washed out (non-recorded) for 3.5 min before increasing to the next concentration.

2.7 Figure editing

CorelDRAW Home & Student ×7, SigmaPlot 13.0 and GraphPad Prism 5 software were used to process and edit the figures.

3 RESULTS

3.1 Genotyping of GluCl TM3 and Rdl TM2/TM3 regions in *T. urticae* field isolates

During a target-site (GluCl) screening of 32 European field *T. urticae* strains, we previously detected an I321T (ATT->ACT) mutation in GluCl3 in four abamectin resistant strains from Spain and Italy (Fig. S4 and Table S2).[36] To further explore the occurrence of this mutation in Europe, a number of additional strains were tested which revealed the occurrence of the mutation in a abamectin resistant field strain from Turkey (TR2) and a multi-acaricide resistant strain from Belgium, MR-VL[46] (Fig. S4 and Table S2). The mutation I321T was fixed in MR-VL but was segregating in TR2. PCR and sequencing confirmed the absence of TM3 mutations in all five GluCl genes in the susceptible strain SR6. As Rdl is considered as a potential secondary target of macrocyclic lactones,[12,27,28] the TM2/TM3 fragments of three Rdl genes were also screened for all seven strains in this study but no mutations were identified in any of the tested strains.

3.2 Abamectin resistance levels

The results of the toxicity tests are listed in Table S2. The strains IT1, IT5, IT6 and TR2 with only the I321T mutation in TM3 region of GluCl3, were all resistant to abamectin with LC_{50}s over 10 mg a.i./L and resistance ratios (RRs) ranging from 46.63- to 104.0-fold compared to the susceptible strain SR6. The ES1 strain with three segregating mutations I321T/V327G/L329F in GluCl3 showed the highest levels of abamectin resistance (78.59 mg a.i. /L) with RRs reaching 327.5 -fold. Another strain MR-VL (with the I321T mutation in TM3 region of GluCl3) exhibited low levels of abamectin resistance (1.79 mg a.i. /L) with only 7.46-fold RR.

3.3 Backcrossing experiments

3.3.1 Establishment of congenic lines

Two backcross experiments (BC1 and BC2) were carried out between a parental resistant strain, fixed for the I321T GluCl3 mutation, and a susceptible strain, lacking the mutation (BC1: ♀ SR6 x ♂ TR2 and BC2: ♀ SR6 x ♂ IT6). After seven rounds of backcrossing and the final cross between heterozygous backcrossed females and their sons, two lines that were homozygous for the I321T mutation (TRA_R1, TRA_R2; T321/T321 genotype), and two congenic control lines, lacking the mutation (TRA_C1, TRA_C2; I321/I321 genotype), were obtained for BC1. For the BC2 experiment, three lines with the T321/T321 genotype (ITA_R1, ITA_R2, ITA_R3) and three susceptible control lines with the I321/I321 genotype (ITA_C1, ITA_C2, ITA_C3) were obtained.

3.3.2 Toxicity assays of congenic lines

Before the start of the backcross experiments, abamectin was tested on all parental strains (SR6, TR2 and IT6). The abamectin RR of TR2 and IT6 was 82 and 105 -fold respectively (Table 1). In BC1, the lines with the T321/T321 genotype showed resistance to abamectin with RRs up to 50-fold (Table 1) while for BC2, RRs of the lines with the T321/T321 genotype were less than two-fold and not significantly different from 1 (Table 1).

3.4 CRISPR-Cas9 genome editing in *Drosophila*

3.4.1 Introduction of I321T in *Drosophila* GluCl

Several genome-edited fly lines bearing an I321T (*T. urticae* GluCl3 numbering) mutation in the GluCl gene were established. We confirmed the presence of HDR-derived alleles in five out of eighteen different G_0 lines that gave G_1 progeny (Fig. S3). Two phenotypically homozygous lines were sequence verified, bearing the correct mutation (Fig. S3c) at the *Drosophila* GluCl gene. One of these was used for downstream toxicity bioassays. Feeding toxicity bioassays with *Drosophila* larvae against abamectin were carried out using nos.Cas9 flies as controls. *Drosophila* larvae were in continuous contact with the insecticide containing food. Since the fly larvae are not readily visible in the food, we considered pupation efficiency as a measurable proxy of eventual survival. The results (Table 2) indicate that flies bearing the I321T mutation are more resistant to abamectin than the control (nos.Cas9) flies carrying the wild-type *Drosophila* GluCl allelic combination. As shown in Table 2, LC_{50} value for nos.Cas9 is

0.018 mg/L (95% CI: 0.014 - 0.020), while for I321T flies the corresponding value is 0.048 mg/L (95% CI: 0.039 - 0.055), which translates to a resistance ratio (RR) of 2.7 folds. It must be noted, however, that I321T flies apparently exhibited slower development when exposed to abamectin, even at the lowest concentration (c. 15 days from egg to adult at 25°C compared to 10-11 days for the nos.Cas9 background strain)

3.4.2 Introduction of G314D or G326E in *Drosophila* GluCl

Several genome-edited fly lines bearing the G314D of G326E mutation (*T. urticae* GluCl1 and GluCl3 numbering, respectively) in the GluCl gene were generated. The presence of HDR-derived alleles in ten out of thirty-nine different G_0 lines that gave G_1 progeny for G314D and seven out of seventeen G_0 lines for G326E was confirmed. Several independent lines were established and balanced, but at the final crossing step virtually no phenotypically homozygous flies could be identified, indicating an associated lethality in both genome-modified alleles. A very small number of "escaper" flies without the balancer-associated marker *Stubble* was eventually observed after several generations of inter-crosses between heterozygotes derived from the same or different G_0 lines (three flies in total among several thousands of screened progeny). These flies were all male, much smaller than their heterozygous siblings and when crosses were attempted, all three were sterile and only survived for a few days. In order to further investigate the G314D/G326E associated lethality, a complementation experiment was performed by crossing heterozygous G314D/TM3 or G326E/TM3 flies with strain #25726 from Bloomington *Drosophila* stock centre (genotype w[1118]; Df(3R)BSC636/TM6C, cu[1] Sb[1]) which carries a chromosomal deletion at chromosome 3R at a region that includes the GluClα gene of *Drosophila*. These crosses never produced any progeny not bearing the parental balancer chromosomes, indicating that indeed the lethality is linked to the deleted region that contains GluClα. Taken together, these observations suggest it is highly likely that both G314D and G326E are essentially lethal, i.e. have severe impact on channel function and viability.

3.5 Two-electrode voltage-clamp electrophysiology

3.5.1 Responses of *T. urticae* GluCl3 WT and GluCl3 I321T to L-glutamic acid and abamectin

Two homomeric GluCl channels, consisting either solely of GluCl3 wild type (WT) subunits or GluCl3 I321T subunits, were expressed in *Xenopus* oocytes and the

responsiveness of these channels was examined for L-glutamic acid and abamectin. As previously described in Mermans et al. 2017, GluCl3 WT channels generate robust responses to L-glutamic acid with rapid inward currents and fast desensitization.[38] In contrast, only few of the GluCl3 I321T injected oocytes showed clear responses to L-glutamic acid, with generally smaller amplitudes of currents but with the same response profile as GluCl3 WT indicating that only in a fraction of the oocytes functional GluCl3 I321T receptors were formed (Fig. 2a). L-glutamic acid dose-response curves resulted in EC_{50}s of 476.4 µM (pEC_{50} = 3.32 ± 0.01) and 311.3 µM (pEC_{50}=3.51 ± 0.02) and Hill coefficients of 2.34 ± 0.14 and 2.42 ± 0.31 for GluCl3 WT and GluCl3 I321T respectively (Fig. 2d, Table 3).

Next, to investigate whether both channels were activated by abamectin, injected oocytes were first tested for expression through application of EC_{50} L-glutamic acid. When clear inward currents were observed, the oocytes were subjected to an increasing concentration of abamectin (1 nM - 100 µM) with an extensive wash of 3.5 min in between every concentration. Abamectin elicited inward currents through GluCl3 WT channels with a slow onset and a very slow desensitization in comparison to L-glutamic acid responses. The same was observed for oocytes expressing GluCl3 I321T, but again with a significantly (around 10-fold) smaller amplitude of currents compared to GluCl3 WT (Fig. 2b). While GluCl3 WT channels showed a clear dose dependent response, GluCl3 I321T channels seemed more insensitive to the increase of concentrations of abamectin (Fig. 2c). Therefore, an unambiguous abamectin EC_{50} could only be calculated for GluCl3 WT (EC_{50} =447 nM (pEC_{50} = 6.32 ± 0.12)). No responses to L-glutamic acid and abamectin were observed in any oocyte injected with water alone (data not shown).

Subsequently, abamectin potentiation of L-glutamic induced currents was examined in both GluCl3 channels by perfusion with 1 mM of L-glutamic acid followed by co-application of 1 mM L-glutamic acid and 100 nM abamectin. The amplitude of currents induced by the L-glutamic acid was increased by the perfusion of abamectin in wild-type GluCl3. In contrast, no clear potentiation of glutamate-induced currents was observed in oocytes expressing GluCl3 I321T (Fig. 2c). GluCl3 G326E was tested again in this study and, as in our previous study, GluCl3 G326E was not activated by abamectin even when tested at high concentrations nor could abamectin potentiate the L-glutamic acid response when co-applied (Fig. 2b&c).[38]

3.5.2 Responses of *T. urticae* RdIs to GABA and abamectin

All three channels showed robust responses to the natural agonist GABA, generating rapid inward currents with a slow desensitization as long as the agonist was applied, followed by a very rapid desensitization once wash-out with NFR was started. The averaged dose-response curves for GABA were characterized by an EC_{50} of 15.45 µM (pEC_{50}= 4.8 ± 0.007), 68.6 µM (pEC_{50}=4.16 ± 0.034) and 139.8 µM (pEC_{50}=3.85 ± 0.035) for Rdl1, Rdl2 and Rdl3 respectively (Fig. 3c and Table 4). To assess the agonistic effect of abamectin, the compound was first applied alone to Rdl1, Rdl2 or Rdl3 expressed in oocytes for 3min, but the channels were not activated by abamectin (Fig. 3a). Although, when tested at high concentrations (10-100 µM), abamectin elicited very small inward currents which are negligible in amplitude compared to the GABA response (Fig. S6). To test antagonistic properties of abamectin, a cumulative exposure was used where the oocytes were pre-incubated with abamectin for 75 s followed by co-application of abamectin and GABA to ensure the maximum inhibitory effect. Perfusion of abamectin produced a dose-dependent inhibition of the GABA response for Rdl1, Rdl2 and Rdl3 regardless when EC_5, EC_{50} or EC_{90} of GABA was applied (Fig. 3b-d, Fig. S5 and Table 4).

4 DISCUSSION

Abamectin has been used extensively for spider mite control, and over the years various levels of abamectin resistance have been detected in *T. urticae* populations worldwide.[31,33,36,45,60] The molecular mechanism underlying abamectin resistance in *T. urticae* have been investigated in a number of strains and both enhanced metabolic detoxification as well as target-site resistance mutations were shown to be at play. In particular, gene-expression analysis and functional characterization have pointed towards the involvement of cytochrome P450s (P450s), glutathione-S-transferases (GSTs) and uridine diphosphate (UDP) - glycosyltransferases (UGTs).[36,61,62] In addition, two mutations, G314D in *GluCl1* and G326E in *GluCl3*, were shown to be genetically linked to abamectin resistance and both G314D and G326E have been functionally validated by two-electrode voltage-clamp electrophysiology in *Xenopus*.[14,37–40] Intriguingly, in contrast to the electrophysiological validation, introgression of mutations in GluCls in a susceptible background have revealed only a relatively weak resistance phenotype. It is therefore clear that the interplay between different receptors, their mutations and potential synergistic actions of additional

mechanisms of resistance, is far from completely understood, and in part also the objective of this study.

Recently, abamectin and milbemectin (cross)-resistance mechanisms have been studied in an exceptionally wide collection of strains sampled across Europe. Aside from confirming the presence of both increased detoxification and previously described target-site mutations, this study also uncovered the presence of a potential new target-site mutation, I321T in GluCl3. Surprisingly, this mutation was only found in populations consisting of the red color morph of *T. urticae*.[36] We further confirm in this study the presence of this mutation in two additional red *T. urticae* strains, TR2 and MR-VL from Turkey and Belgium, respectively[45,46] (Table S2) and recently, it was also detected in a Greek multi-resistant red spider mite population.[41] Based on receptor sequences of green and red color morphs (Fig. S4), phylogenetic constraints do not seem to explain why this mutation only occurs in red morphs, and given the geographical origin of the strains under investigation, a single origin and spread of the mutation does not seem likely, although it is impossible to exclude.

The potential relevance of I321T in abamectin resistance can first be inferred from its location in TM3, which is at the periphery of the predicted glutamate binding site and was previously reported to be involved in ivermectin binding, based on the GluClα crystal structure of *C. elegans*.[7] In addition, an A309V mutation in *P. xylostella* GluCl, adjacent to I321 in *T. urticae* GluCl3, was also strongly associated with abamectin resistance (Fig. 1a&b).[39,63]

We used the *Xenopus* system to investigate the role of the I321T mutation in the interaction of abamectin with GluCl. Compared to GluCl3 WT, functional GluCl3 I321T was only successfully expressed in a limited number of oocytes, and L-glutamic acid induced currents had a significantly lower amplitude in these channels. The lack of GluCl3 I321T expression could either be due to a reduced translation of the exogenous cRNA in *Xenopus* oocytes or it is possible that only few of the formed receptors are functional i.e. that binding of ligands and ivermectin is compromised or that the ion channel is unable to open to its normal extend. Either way, the GluCl3 I321T mutation seems to influence GluCl3 I321T expression as both GluCl3 WT and G326E showed consistent expression in *Xenopus* oocytes when established with the same protocol.[38]

Nevertheless, L-glutamic acid dose-response curves could be calculated for both GluCl3 WT and GluCl3 I321T channels, and the L-glutamic acid EC_{50} for both

channels differed by 1.5 fold (Table 3) only, which is in line with previous studies that examined the effect of GluCl resistance mutations using *Xenopus*.[38,39] For both GluCl3 WT and GluCl3 I321T abamectin induced currents could be observed, but, as with L-glutamic acid induced currents, the amplitude of response was significantly lower for GluCl3 I321T. Further, no clear potentiation of glutamate-induced currents was observed after co-application of 1 mM L-glutamic acid and 100 nM abamectin in GluCl3 I321T expressing oocytes while such increase was observed for GluCl3 WT. This subtle effect of the I321T mutation on abamectin response is in contrast to the G314D and G326E mutation, which completely abolished the agonist activity of abamectin and milbemycin A_4, another macrocyclic lactone (Fig. 2b).[38] But in line with the study of Wang et al., the A309V mutation in *P. xylostella* GluCl (corresponding to A320 and located next to I321 in *T. urticae* GluCl3) only resulted in 4.8 fold reduced sensitivity, while the G315E mutation (corresponding to G326E in *T. urticae* GluCl3) reduced the sensitivity to abamectin by 493-fold.[39] This difference in effect might be explained by the interaction of these residues with macrocyclic lactones. Based on the crystal structure of *C. elegans* GluClα and its predicted binding to ivermectin, A320 and I321 are not predicted to interact with ivermectin while G326E is predicted to have van der Waals interactions with ivermectin and is located closest to ivermectin (Fig. 1b).[7] However, Wang et al. 2017, predicted that *P. xylostella* GluCl A309V mutants might allosterically offset the functional effect of abamectin upon binding,[39] and our findings suggest that this prediction also holds for the GluCl3 I321T mutation, with I321T having an effect on abamectin action, much likely by reducing channel sensitivity.

To further corroborate the possible likelihood of this mutation to be involved in abamectin resistance, the relative phenotypic contribution of the I321T mutation was first characterized by marker-assisted backcrossing. Two independent backcrossing experiments (BC1 and BC2) originating from two genetically different strains from distinct geographical origin were performed. Each experiment resulted in congenic lines, either homozygous for the mutant (T321/T321) or wildtype (I321/I321) GluCl3 allele. For BC1, resistance ratios reached over 50-fold (Table 1) compared to the wildtype lines, but in contrast, for BC2 only low levels of abamectin resistance were observed in the mutant lines. The result of BC2 is similar to Riga et al. 2017, where only low levels of resistance were observed when either G314D in GluCl1 or G326E in GluCl3 was introgressed, indicating that a mutation in GluCl1 and GluCl3 alone was insufficient to convey resistance.[51]

Marker-assisted back-crossing is expected to uncouple multiple resistance factors and reduces the proportion of donor genome with every cycle. However, if resistance genes are closely located together on the chromosome, chances to capture recombination events between adjacent genes are rare. Hence, to explain the variance between the mutant congenic lines of BC1 and BC2, one could argue that in BC1 the uncoupling of resistance genes might not have succeeded because of linkage. The fact that the mutation alone does not convey high resistance levels was also clear from the laboratory reference resistant strain MR-VL. Although this strain was fixed for the mutation, it is not highly resistant to abamectin (Table 1). Since this strain has not been selected with abamectin for more than ten years, additional factors that might contribute to resistance were probably lost, mimicking the effect of introgression. What these extra resistance factors might be is hard to resolve and could be any regulatory element of a detoxification enzyme or factors resulting in compositional changes of GluCl channels (i.e. overexpression/ underexpression of certain GluCl genes).

Indeed, the differences between BC1 and BC2 and low resistance levels of MR-VL point towards a lack of understanding of the role of mutations in resistance that was already previously clear from introgression work with other mutations in GluCl1 and GluCl3. These mutations also provide a weak phenotype *in vivo*, despite the complete lack of agonistic activity in electrophysiological studies.[40] The complexity of the channel confirmation might be one of the factors involved. For example, a GluCl channel could consist of five GluCl3 encoded subunits, each carrying the I321T mutation (homomeric GluCl channel), or could be composed of GluCl subunits with or without mutation (heteromeric GluCl channel with subunits encoded by different genes) (Fig. 1c); the latter channel being likely more sensitive to abamectin. Alternatively, less-sensitive abamectin receptors could be formed through combination of GluCl and non-GluCl subunits.[12] In Xue et al. 2020, it was shown that two cys-loop ligand gated ion channel subunit genes (*tetur02g11020* and *tetur02g11170*), were overexpressed in abamectin resistant strains. The *Drosophila* ortholog (CG12344) of these genes has only been poorly characterized but encode subunits of a cys-loop LGIC predicted to be most related to vertebrate glycine receptors (http://flybase.org/reports/FBgn0033558.html). Interestingly, vertebrate glycine receptors are 100-fold less sensitive to ivermectin compared to invertebrate GluCls as they lack the TM3 glycine residue at a position corresponding to G314/G326 in *T. urticae* GluCl1/GluCl3[14,64,65] and hence heteromeric cys-loop channels consisting of *GluCl* and *tetur02g11020/tetur02g11170* encoded

subunits might be less sensitive to abamectin. Alternatively, less-sensitive abamectin receptors might also consist of a mix of GluCl and Rdl subunits (Fig. 1c). Such channels have been reported before,[12] and homomeric Rdl channels were reported to be more than 150-fold less sensitive to ivermectin compared to GluCl channels.[26]

Therefore, to infer whether abamectin also acts on *T. urticae* Rdl channels, three homomeric *T. urticae* Rdl channels,[14] consisting either of *T. urticae Rdl1*, *Rdl2* or *Rdl3* encoded subunits) were expressed using *Xenopus* oocytes. Abamectin alone did not invoke Rdl currents but had an antagonistic effect on all three *T. urticae* Rdl channels (Fig. 3 and Table 4). These observations are in line with Xu et al. 2017, in which *Tetranychus* Rdl channels were not activated by abamectin or ivermectin alone. However, a follow-up study of the same group showed that abamectin has an agonistic action on *Tetranychus* Rdl2.[66,67] On the other hand, for insect and nematode Rdls, the action of ivermectin was shown to be dependent on application conditions[26,68] and our results are in in agreement with previous electrophysiology studies showing that application of ivermectin alone does not have any effect on the receptor[69,70] and reports showing that ivermectin is an antagonist of GABA induced Rdl currents at a GABA concentration higher than the EC_{50}.[26,68,69] This suggests that indeed, to survive high concentration of abamectin, a detoxification component or additional factor is needed to protect against additional Rdl interactions.

As there was no conclusive outcome from the backcrossing experiments, we next used the CRISPR-Cas9 technology to introduce the I321T mutation in the GluCl homologue of *D. melanogaster*. In the past, this technology was shown to be a very useful tool to elucidate the role of a single amino acid substitution in resistance against insecticides.[71] Two phenotypically homozygous fly lines bearing an I321T mutation in *GluCl* were generated. Toxicity bioassays with one of these lines revealed moderate abamectin resistance levels in the *GluCl* I321T line compared to the control. In addition, we also observed a fitness-cost (although we did not quantify) in the *GluCl* I321T line, with developmental time being much slower in I321T flies exposed to abamectin compared to control flies. To better interpret the effects of the *GluCl* I321T mutation in *Drosophila*, we also tried to introduce the previously documented abamectin resistance mutations, G314D in *GluCl1* and G326E in *GluCl3*, in *Drosophila* GluCl. Unfortunately, no phenotypically homozygous flies could be identified at the final crossing step, and crosses between heterozygous G314D/TM3 or G326E/TM3 flies and a fly strain that had a chromosomal deletion in the GluClα region, yielded no viable progeny; strongly

suggesting these mutations cause lethality. A lethal phenotype for flies with CRISPRed resistance mutation(s) has been reported before; see for example Bajda et al. 2017, Douris et al 2017 or Douris et al, 2020.[55,71,72] Hence, *Drosophila* is not always a suitable species for reverse genetics with resistance mutations that were detected in other species. In the case of *T. urticae* and abamectin resistance, this is further complicated by the fact that there is only one *GluCl* gene in *Drosophila* while there are at least five in *T. urticae*.[14,17]

Hence, ideally, one would introduce the GluCl mutations in *T. urticae.* As it was recently shown that CRISPR-Cas9 can be used to create gene knockouts in *T. urticae*,[73] this type of gene-editing might soon be a feasible genetic tool for this species. Nevertheless, the lethality of the G314D/G326E mutations in *Drosophila* does imply that these residues have an important role in channel functioning, and is in line with a previously documented fitness cost of G314D and G326E in *T. urticae*.[74]

5 CONCLUSION

To conclude, the role of the GluCl3 I321T mutation in abamectin resistance of *T. urticae* was examined by *in vitro* functional expression and electrophysiology, backcrossing experiments and CRISPR-Cas9 gene editing in *Drosophila*. Based on backcross experiments, the I321T mutation alone did not always result in high abamectin resistance levels, a finding also reported for other mutations in GluCl1 and GluCl3. Genome editing in *Drosophila* confirmed the role of GluCl3 I321T in resistance, but again with low resistance levels. Functional analysis using *Xenopus* oocytes showed that the I321T mutation could reduce GluCl sensitivity to abamectin, but whether this reduction is actually due to the effect of the I321T mutation or resulting from decreased expression remains unclear. We confirm the antagonistic effect of abamectin on all three Rdls, suggesting that abamectin also acts on Rdls at high doses in *T. urticae*. We therefore propose that the I321T GluCl3 mutation plays a role in abamectin resistance, but only in combination with synergistic additional factors that deserve more investigation.[75]

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7 SUPORTING INFORMATION

Supporting information might be found in the online version of this article.

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9 TABLES

Table 1. Toxicity of abamectin to adult females of backcrossed lines and their parental strains

	Strain	Genotype	Slope(±SE)	LC_{50}(95% CI) (mg a.i./L)	RRs[a,b] (95%CI)
♀SR6 x♂TR2 (BC1)					
Backcrossed lines	TRA_R1	321T	2.85 (±0.28)	6.00 (5.36-6.80)	44.14 (37.38-52.12)*
	TRA_R2	321T	3.17 (±0.31)	8.10 (7.08-9.04)	59.51 (50.31-70.39)*
	TRA_R3	321T	3.67 (±0.31)	5.85 (5.32-6.38)	42.70 (37.08-49.78)*
	TRA_C1	I321	2.51 (±0.22)	0.15 (0.14-0.18)	1.13 (0.95-1.34)
	TRA_C2	I321	2.71 (±0.22)	0.13 (0.12-0.15)	0.98 (0.83-1.16)
Parental strains	SR6_BC1	I321	2.84 (±0.23)	0.14 (0.12-0.15)	1.00
	TR2	321T	2.52 (±0.25)	11.19 (8.57-14.50)	82.26 (69.24-97.72)*
♀SR6 x♂IT6 (BC2)					
Backcrossed lines	ITA_R1	321T	4.08 (±0.30)	0.42 (0.38-0.47)	1.80 (1.57-2.06)*
	ITA_R2	321T	4.63 (±0.44)	0.33 (0.30-0.36)	1.39 (1.21-1.59)*
	ITA_R3	321T	4.71 (±0.42)	0.34 (0.30-0.37)	1.42 (1.24-1.64)*
	ITA_C1	I321	3.36 (±0.30)	0.22 (0.19-0.26)	0.94 (0.81-1.10)
	ITA_C2	I321	4.82 (±0.41)	0.18 (0.15-0.21)	0.75 (0.66-0.87)*
	ITA_C3	I321	4.03 (±0.31)	0.26 (0.23-0.31)	1.11 (0.96-1.28)
Parental strains	SR6_BC2	I321	3.31 (±0.25)	0.24 (0.21-0.26)	1.00
	IT6	321T	3.33 (±0.22)	24.96 (22.76-27.20)	105.5 (92.02-120.9)*

[a]The resistance ratio (RR) compared to the susceptible strain SR6

[b]An asterisk (*) indicates the RR was considered significantly different from 1 based on non-overlap of 95% CI (PoloPlus LeOra Software).[50]

Table 2. Log-dose probit-mortality data for abamectin tested against larvae of *Drosophila* genome modified strain I321T vs wild-type control (nos.Cas9). LC_{50} values are given in mg a.i./L. The resistance ratio (RR) is calculated by dividing the LC_{50} of strain I321T by the LC_{50} of the wild type strain nos.Cas9.

Compound	Strain	Slope ± SE	LC_{50} (95% CI) (mg a.i./L)	RRs[a,b] (95% CI)
abamectin	nos.Cas9	7.32 (±1.34)	0.018 (0.014-0.020)	1
	I321T	3.70 (±0.40)	0.048 (0.039-0.055)	2.66 (2.30-3.06)*

[a]The resistance ratio (RR) compared to the wild type strain nos.Cas9.
[b]An asterisk (*) indicates the RR was considered significantly different from 1 based on non-overlap of 95% CI (PoloPlus LeOra Software).[50]

Table 3. L-glutamic acid and abamectin responses of wild- and mutant GluCls expressed in *Xenopus* oocytes. Data represent the mean of 8 oocytes ± SEM.

	Wild-type			GluCl3 I321T		
	EC_{50} (μM)	pEC_{50}	n_H†	EC_{50} (μM)	pEC_{50}	n_H†
L-glutamic acid	476.4	3.32 ± 0.01	2.34 ± 0.14	311.3	3.51 ± 0.02	2.42 ± 0.31
abamectin	0.447	6.32 ± 0.12	0.97 ± 0.23	-	-	-

†n_H: Hill coefficient

Table 4. GABA and abamectin responses of three wild-type *T. urticae* Rdls expressed in *Xenopus* oocytes. Data are the mean of 6-8 oocytes ± SEM

	GABA				Abamectin			
	n†	EC_{50} (μM)	pEC_{50}	n_H‡	n†	IC_{50} (μM)	pIC_{50}	n_H‡
Rdl1	8	15.5	4.81 ± 0.007	5.61 ± 0.56	8	0.102[a]	6.98 ± 0.34	-0.47 ± 0.24
					6	0.058[b]	7.23 ± 0.17	-0.45 ± 0.11
					6	0.179[c]	6.74 ± 1.98	-0.21 ± 0.51
Rdl2	6	68.6	4.16 ± 0.034	1.56 ± 0.16	6	0.226	6.64 ± 0.21	-1.07 ± 0.43
Rdl3	6	139.8	3.85 ± 0.035	3.36 ± 0.65	6	0.502	6.29 ± 0.35	-0.49 ± 0.18

†number of oocytes

‡n_H: Hill coefficient

[a] Co-application of GABA (EC_{50}) and abamectin

[b] Co-application of GABA (EC_5) and abamectin

[c] Co-application of GABA (EC_{90}) and abamectin

10 FIGURES LEGENDS

Figure 1. Identification of an I321T mutation in GluCl3 of abamectin resistant *T. urticae* strains. a) Individual GluCl channel subunit, consisting of four transmembrane (TM) domains; the I321T mutation (indicated with a red star) found in abamectin resistant *T. urticae* is located in TM3; b) Alignment of TM3 of *T. urticae GluCl1* and *GluCl3* with GluCl of *Drosophila melanogaster*, *Plutella xylostella* and *Caenorhabditis elegans*. The I321T mutation is indicated with a red star, while the A309V mutation in *P. xylostella GluCl* and G314D / G326E in *T. urticae GluCl1* / *GluCl3*, which were previously associated with abamectin resistance,[14,37,38,63] are indicated with a square and triangle, respectively. *C. elegans GluCl*α residues that were previously shown to be involved in ivermectin binding are underlined.[7] An 80% threshold was used for identity (black background) and similarity shading (grey background). Abbreviation of species names: Tu, *T. urticae*; Ce, *C. elegans*; Dm, *D. melanogaster*; Px, *P. xylostella*; c) GluCl channels consist of five GluCl subunits, which in *T. urticae* might be encoded by the same gene (e.g. *GluCl3* carrying the I321T mutation) or can be encoded by different genes (e.g. *GluCl3* I321T and *GluCl1*) or, as was shown for *D. melanogaster*,[12] ivermectin receptors might be formed that consist of both GluCl and Rdl subunits.

Figure 2. L-glutamic acid and abamectin activation of GluCl3 WT and GluCl3 I321T expressed in *Xenopus* oocytes. a): Examples of electrical current responses. The period of L-glutamic acid application time is indicated by the bar above the trace as well as the concentrations applied (mM); b): Examples of electrical current responses for abamectin. The period of application is indicated by the bar above the trace as well as the concentrations applied; c): Abamectin potentiation of L-glutamic induced currents; perfusion of GluCl3 WT, GluCl3 I321T and GluCl3 G326E injected oocytes with 1 mM of L-glutamic acid followed by co-application of 1 mM L-glutamic acid and 100 nM abamectin; d) L-Glutamic acid dose-response curves for the activation of wild-type and I321T GluCl3; e): Abamectin dose-response curves for the activation of wild-type and I321T GluCl3; Error bars indicate SEM (n=8).

Figure 3. Antagonistic activity of abamectin on the Rdl1, Rdl2 and Rdl3 GABA receptors expressed in *Xenopus* oocytes. a): A current trace when abamectin was

administered to Rdl1; b): Current traces from a cumulative exposure to increasing dosage of abamectin obtained for a *Xenopus oocyte* expressing Rdl1. The bars indicate the time period of co-application of GABA (15 μM) and increasing concentrations of abamectin (1 nM - 10 μM); c): GABA dose-response curves for the activation of Rdl1, Rdl2 or Rdl3; d): Inhibition dose-response curve measured for abamectin obtained from oocytes expressing Rdl1, Rdl2 or Rdl3. Error bars indicate SEM (n=6-8).

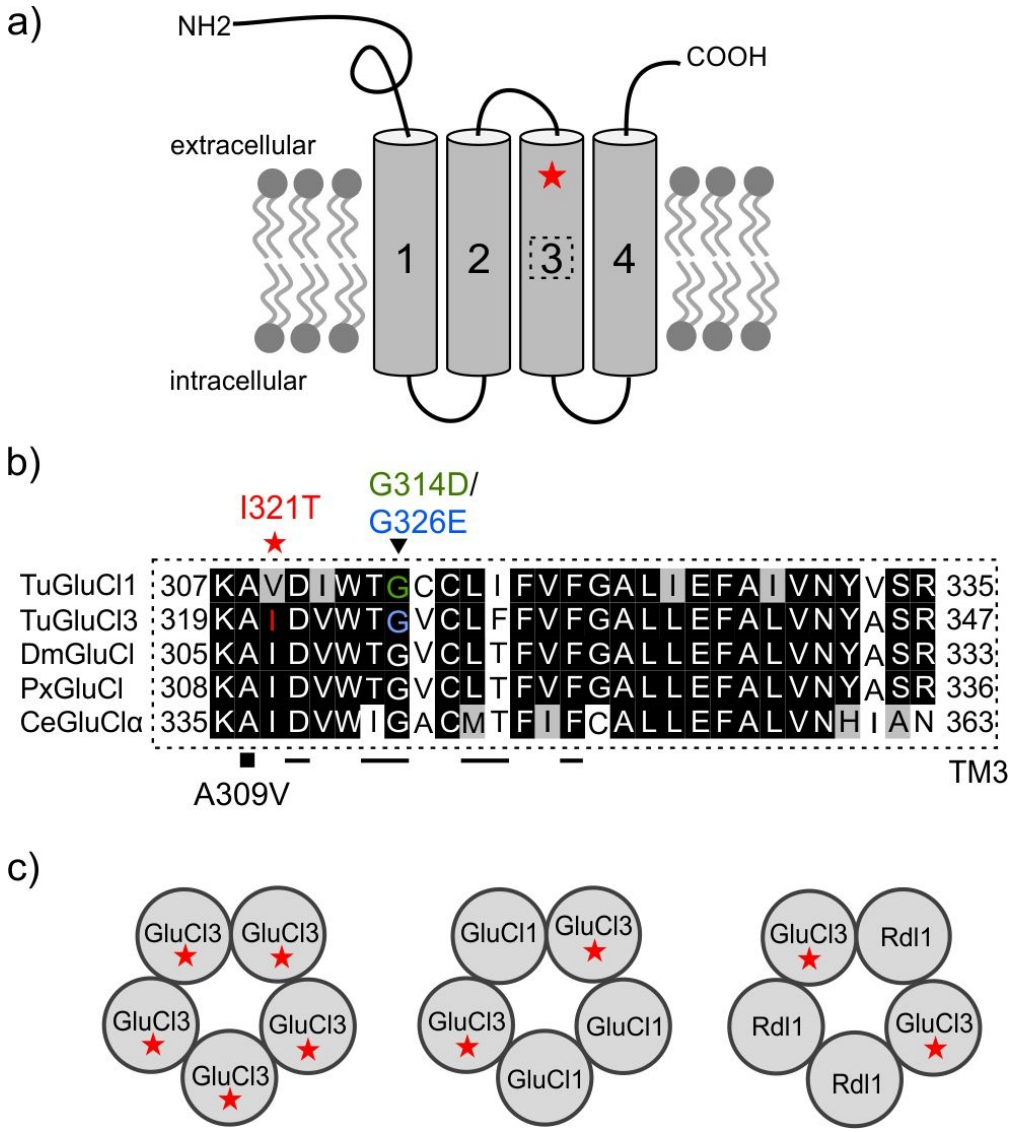

Figure 1. Identification of an I321T mutation in GluCl3 of abamectin resistant *T. urticae* strains. a) Individual GluCl channel subunit, consisting of four transmembrane (TM) domains; the I321T mutation (indicated with a red star) found in abamectin resistant *T. urticae* is located in TM3; b) Alignment of TM3 of *T. urticae GluCl1* and *GluCl3* with GluCl of *Drosophila melanogaster*, *Plutella xylostella* and *Caenorhabditis elegans*. The I321T mutation is indicated with a red star, while the A309V mutation in *P. xylostella* GluCl and G314D / G326E in *T. urticae GluCl1* / *GluCl3*, which were previously associated with abamectin resistance,[14,37,38,61] are indicated with a square and triangle, respectively. *C. elegans GluCla* residues that were previously shown to be involved in ivermectin binding are underlined.[7] An 80% threshold was used for identity (black background) and similarity shading (grey background). Abbreviation of species names: Tu, *T. urticae*; Ce, *C. elegans*; Dm, *D. melanogaster*; Px, *P. xylostella*; c) GluCl channels consist of five GluCl subunits, which in *T. urticae* might be encoded by the same gene (e.g. *GluCl3* carrying the I321T mutation) or can be encoded by different genes (e.g. *GluCl3* I321T and *GluCl1*) or, as was shown for *D. melanogaster*,[12] ivermectin receptors might be formed that consist of both GluCl and Rdl subunits.

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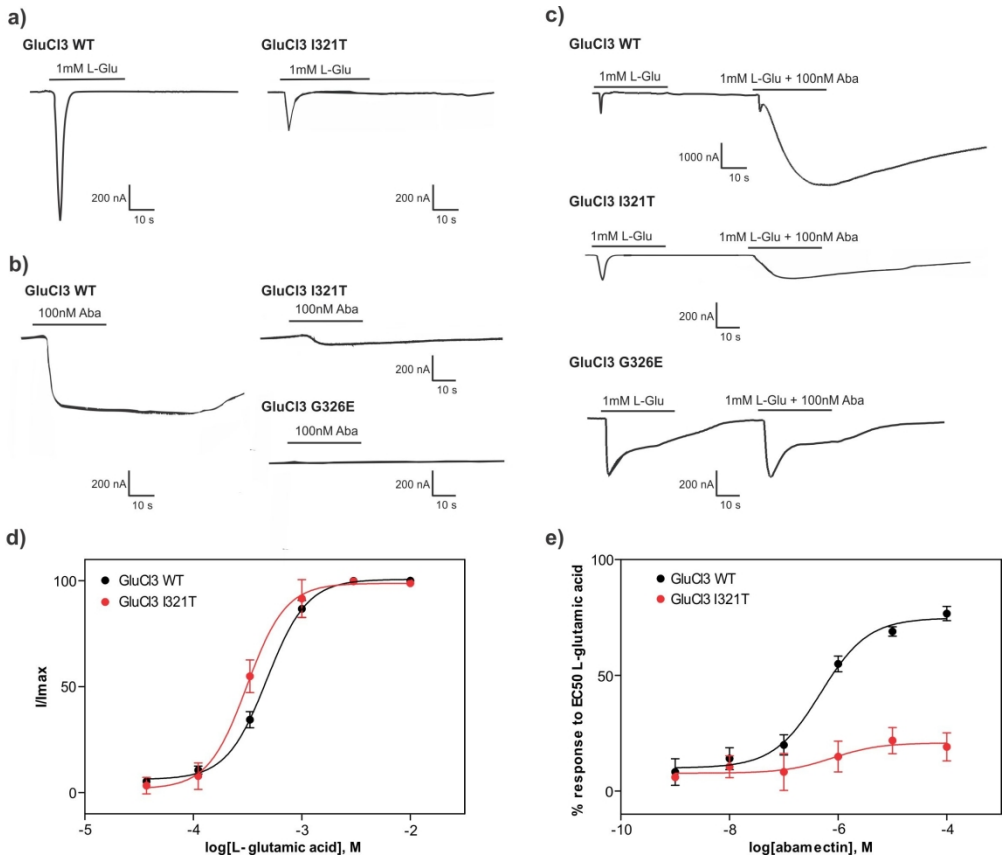

Figure 2. L-glutamic acid and abamectin activation of GluCl3 WT and GluCl3 I321T expressed in *Xenopus* oocytes. a): Examples of electrical current responses. The period of L-glutamic acid application time is indicated by the bar above the trace as well as the concentrations applied (mM); b): Examples of electrical current responses for abamectin. The period of application is indicated by the bar above the trace as well as the concentrations applied; c): Abamectin potentiation of L-glutamic induced currents; perfusion of GluCl3 WT, GluCl3 I321T and GluCl3 G326E injected oocytes with 1 mM of L-glutamic acid followed by co-application of 1 mM L-glutamic acid and 100 nM abamectin; d) L-Glutamic acid dose-response curves for the activation of wild-type and I321T GluCl3; e): Abamectin dose-response curves for the activation of wild-type and I321T GluCl3; Error bars indicate SEM (n=8).

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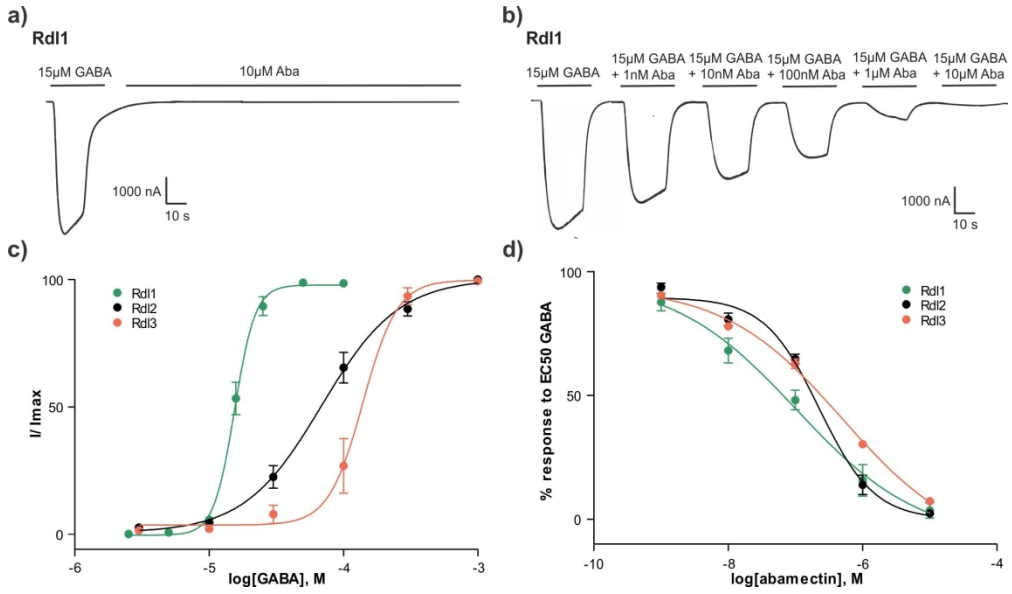

Figure 3. Antagonistic activity of abamectin on the Rdl1, Rdl2 and Rdl3 GABA receptors expressed in *Xenopus* oocytes. a): A current trace when abamectin was administered to Rdl1; b): Current traces from a cumulative exposure to increasing dosage of abamectin obtained for a Xenopus oocyte expressing Rdl1. The bars indicate the time period of co-application of GABA (15 µM) and increasing concentrations of abamectin (1 nM - 10 µM); c): GABA dose-response curves for the activation of Rdl1, Rdl2 or Rdl3; d): Inhibition dose-response curve measured for abamectin obtained from oocytes expressing Rdl1, Rdl2 or Rdl3. Error bars indicate SEM (n=6-8).

188x109mm (300 x 300 DPI)

File S1 - Sequences of *T. urticae* GluCl and Rdl constructs that were ligated into a pUC57 vector (FASTA format).

>TuGluCl3

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>TuRdl1

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Color legend:
T7 promotor
Xenopus 5'UTR
GCCACC: KOZAK sequence
CDS
TuGluCl3 I321T: site-directed mutagenesis ATC ->ACC
Xenopus 3'UTR
SP6

Untangling a Gordian knot: the role of a GluCl3 I321T mutation in abamectin resistance in *Tetranychus urticae*

Wenxin Xue, Catherine Mermans, Kyriaki-Maria Papapostolou, Mantha Lamprousi, Iason-Konstantinos Christou, Emre Inak, Vassilis Douris, John Vontas, Wannes Dermauw, Thomas Van Leeuwen

Supplementary Figures

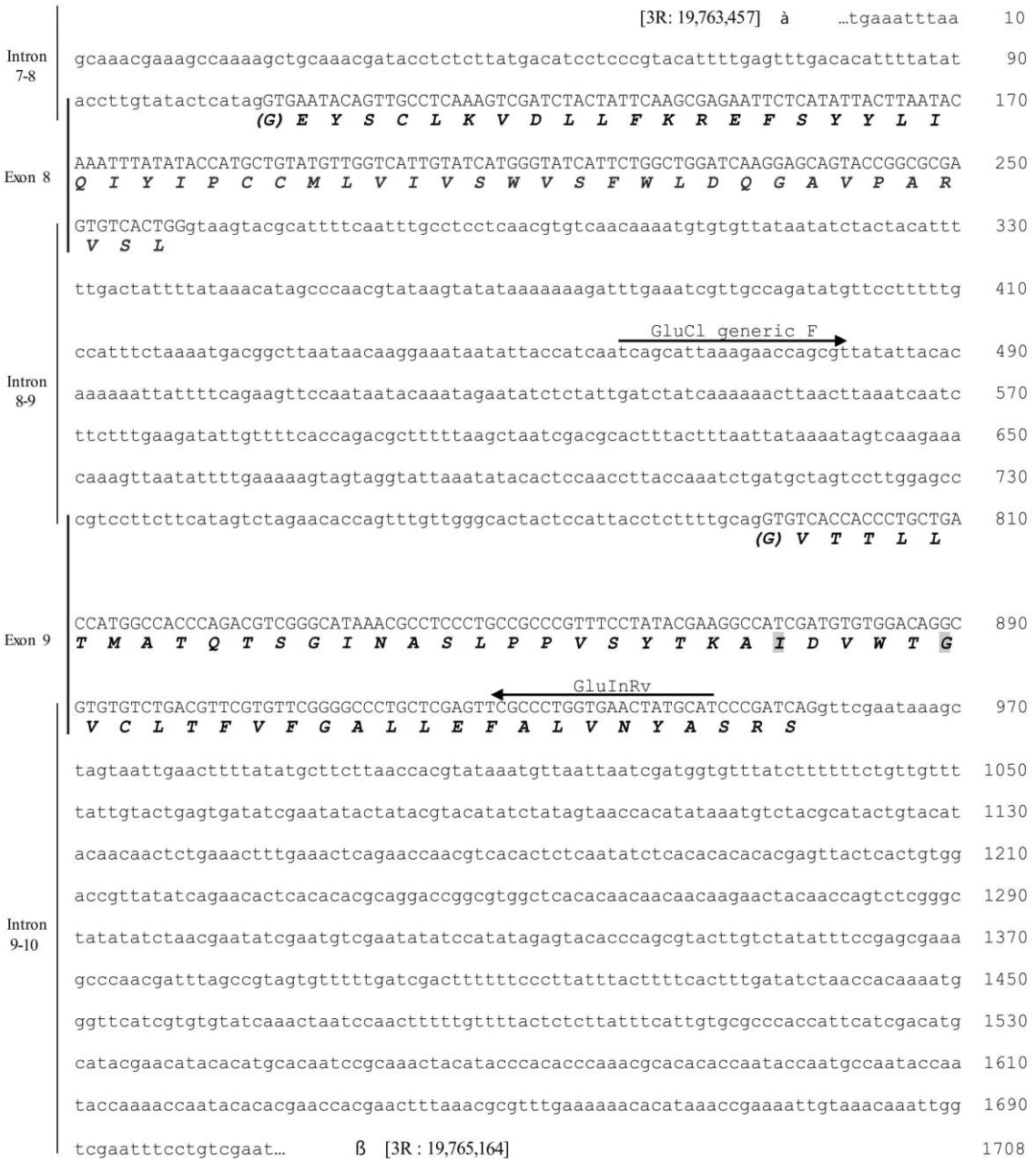

Figure S1. *Drosophila* GluCl target region. Nucleotide and deduced amino acid sequence of a 1708 bp fragment (region 3R: 19,763,457-19,765,164 of the BDGP6.22 genome assembly) of the *D. melanogaster* GluCl used for homology-directed repair, encompassing exon 9 that contains the target aminoacid positions I307 (equivalent to I321 in Tu_GluCl3) and G312 (equivalent to either G314 or G326 in Tu_GluCl1 and Tu_GluCl3 respectively), shown in light gray shading. Intron sequences are shown in lowercase letters. Horizontal arrows indicate the positions of primers GluCl_generic_F / GluInRV (Table S3) used for amplification and sequencing of the genome modified alleles.

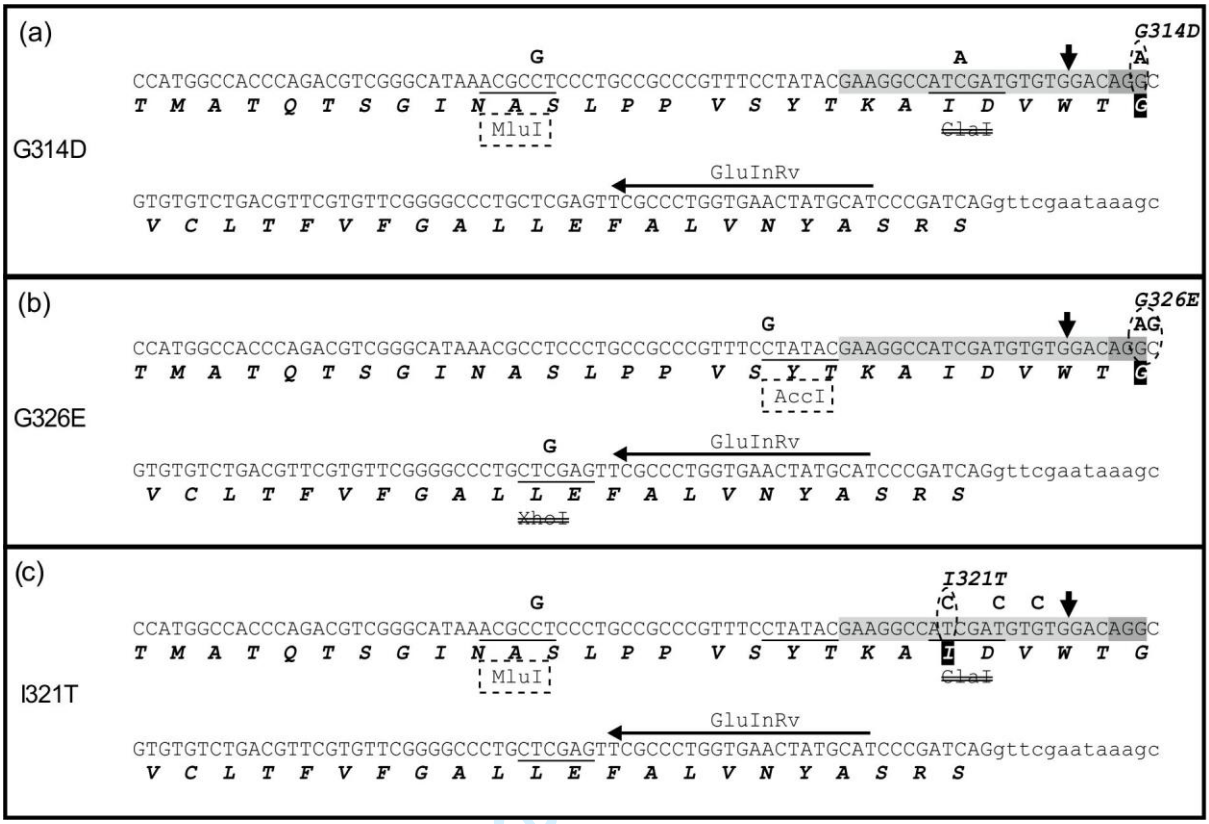

Figure S2. CRISPR/Cas9 strategy for generation of genome modified flies. (a): strategy for G314D mutation, (b): strategy for G326E mutation, (c): strategy for I321T mutation. All panels represent the nucleotide and deduced amino acid sequence of a 160 bp fragment of the *D. melanogaster GluCl* containing part of exon 9 (uppercase) that bears the target positions, and adjacent intron (lowercase). Light gray area indicates the CRISPR/Cas9 target selected (sgRNA), while the dark gray area indicates the corresponding PAM (-*NGG*) triplet. The vertical arrow denotes the break point for CRISPR/Cas9-induced double stranded break. The ovals mark the non-synonymous differences between target (wild-type) and donor (genome modified) sequences used to generate each mutation. Synonymous mutations incorporated for diagnostic purposes, as well as to avoid cleavage of the donor plasmid by the CRISPR/Cas9 machinery, are shown above the nucleotide sequence. Restriction sites abolished because of the genome modification are shown with strikethrough letters and the corresponding sequence is underlined. Restriction sites introduced because of the genome modification are shown with a dashed box and the corresponding sequence is also underlined. The horizontal arrow indicates the position of primer GluInRV (Table S3) used for sequencing of the genome modified alleles.

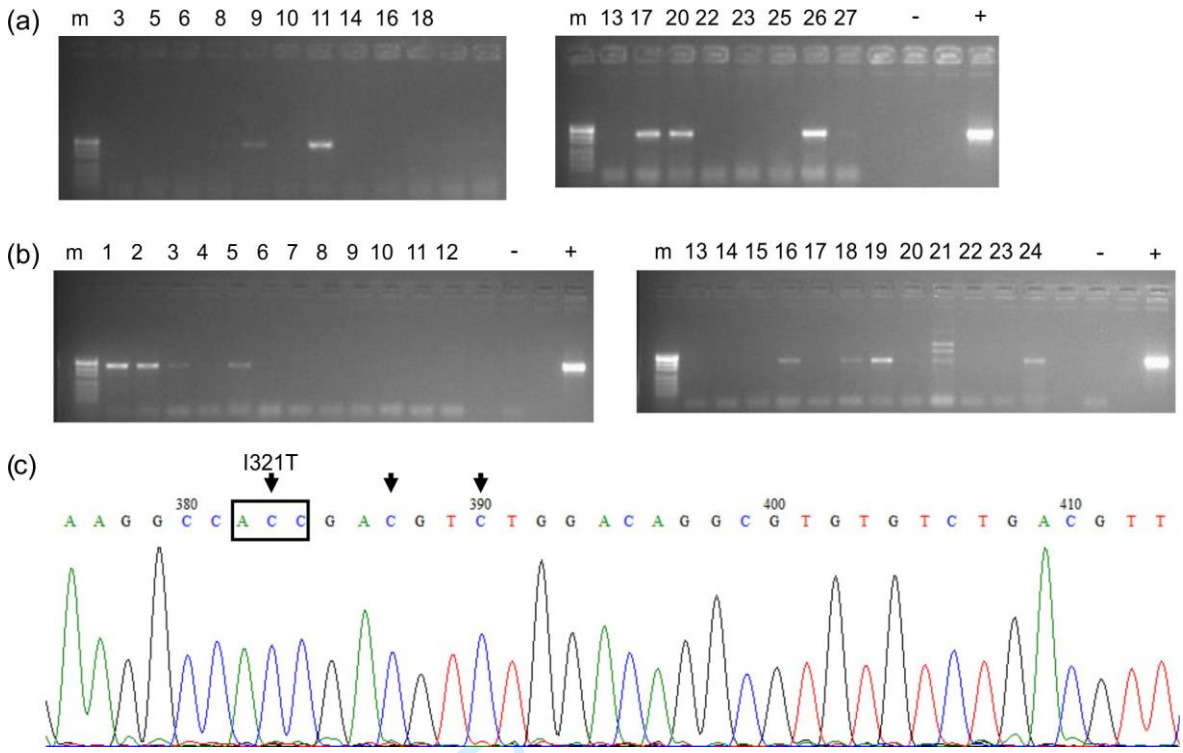

Figure S3. Screening for genome-modified I321T flies. (a): PCR screening following digestion with ClaI of template DNA from pools of G_1 flies derived from different G_0 (injected) individuals using a specific primer combination (GluCl_generic_F / GluCl_specific_R, Table S3) that provides a diagnostic 429 bp band for the I321T mutation. (b): Screening of individual balanced G_2 flies for I321T alleles using the specific primer combination (GluCl_generic_F / GluCl_specific_R, Table S3); several positive crosses are visible. m: MW marker -: nos.Cas9 DNA (negative control), +: donor plasmid template (positive control). (c): Sequencing of the relevant GluCl region in homozygous genome modified flies. Vertical arrows indicate modified nucleotides. The boxed ACC triplet encodes I321T.

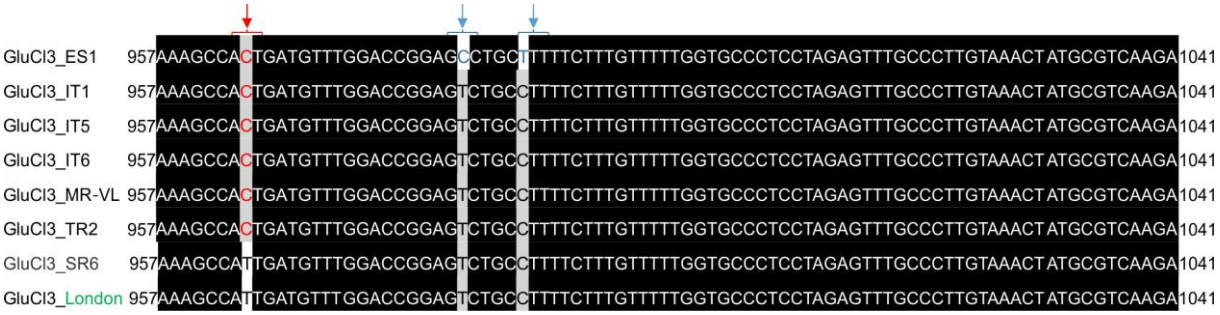

Figure S4. Nucleotide alignment of the TM3 region of *GluCl3* of red morph *T. urticae* strains investigated in this study and a green morph *T. urticae* strain (London). The mutation I321T (ATT-> ACT) characterized in this study in six *T. urticae* strains (IT1, IT5 IT6, ES1, MR-VL and TR2) is indicated with a red arrow and a red font. The V327G (GTC-> GCC) and L329F (CTT-> TTT) mutation found in ES1 in our previous study is indicated with a blue arrow and blue font.[36] The London (green font) abamectin susceptible strain originated from a wild-collected *T. urticae* population from the Vineland region (Ontario, Canada) and its TM3 *GluCl3* sequence was downloaded from the ORCAE website (http://bioinformatics.psb.ugent.be/webtools/bogas/overview/Tetur) with accession number *tetur10g03090*).[14,73] An 90% threshold was used for identity (black background) and similarity shading (grey background).

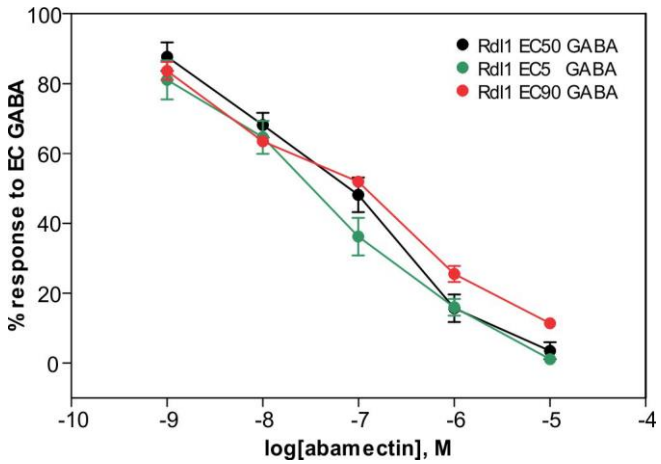

Figure S5. Dose-response regression curve measured for abamectin obtained from oocytes expressing Rdl1 and tested with the EC_5, EC_{50} or EC_{90} of GABA. Error bars indicate SEM (n=8).

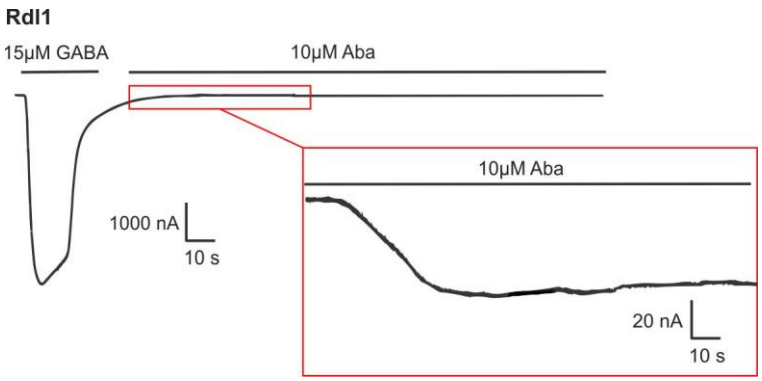

Figure S6. Detail of a current trace when abamectin was administered to Rdl1 expressing oocytes.

Untangling a Gordian knot: the role of a GluCl3 I321T mutation in abamectin resistance in *Tetranychus urticae*

Wenxin Xue, Catherine Mermans, Kyriaki-Maria Papapostolou, Mantha Lamprousi, Iason-Konstantinos Christou, Emre Inak, Vassilis Douris, John Vontas, Wannes Dermauw, Thomas Van Leeuwen

Supplementary Tables

Table S1. *T. urticae* populations used in this study

Strain	DOC[a]	Origin	Host plant	Reference
IT1	06/2017	Granieri, Italy	Carnation	Xue et al. 2020
IT5	05/2018	Granieri, Italy	Carnation	
IT6	05/2018	Vittoria, Italy	Gerbera	
ES1	05/2017	Sevilla, Spain	Strawberry	
MR-VL	2005	Gent, Belgium	poplar cuttings, beans or ornamentals	Van Leeuwen et al., 2005 (fenbutatin oxide selection line)
TR2	11/2017	Mersin, Turkey	Zucchini	İnak et al., 2019
SR6	2017	Italy	Tomato	This Study

[a]Date of collection

Table S2. *GluCl1/2/3/4/5* TM3 and *Rdl1/2/3* TM2/TM3 region genotypes and abamectin resistance in the surveyed *T. urticae* populations

Strain	GluCls	Rdls	Abamectin		
			Slope(±SE)	LC50s (95% CI)/ mg L^{-1}	RRs[a,b] (95%CI)
IT1	I321T in *GluCl3*	-	3.31 (±0.32)	14.64 (12.08-17.12)	61.00 (50.33-71.33)*
IT5	I321T in *GluCl3*	-	4.28 (±0.13)	14.26 (12.45-16.15)	59.42 (51.88-67.29)*
IT6	I321T in *GluCl3*	-	3.33 (±0.22)	24.96 (22.76-27.20)	104.0 (94.8-113.3)*
ES1	I321T/V327G/L329F in *GluCl3*	-	2.53 (±0.18)	78.59 (67.63-90.84)	327.5 (281.8-378.5)*
MR-VL	I321T in *GluCl3*	-	3.78 (±0.28)	1.79 (1.66-2.12)	7.46 (6.92-8.83)*
TR2	I321T in *GluCl3*	-	2.52 (±0.25)	11.19 (8.57-14.50)	46.63 (35.71-55.77)*
SR6	-	-	3.31 (±0.25)	0.24 (0.21-0.26)	1.00

[a]Resistance Ratios compared to the susceptible strain SR6
[b]An asterisk (*) indicates the RR was considered significantly different from 1 based on non-overlap of 95% CI (PoloPlus LeOra Software).[50]

Table S3. Primers used in this study

Primer Name	Sequence (5' - 3')	Use
Tu_GluCl1_dia_F	TTGGATTGACCCTAACTCAGCA	survey of TM3 region of GluCls (Dermauw et al. 2012)
Tu_GluCl1_dia_R	TTGCACCAACAATTCCTTGA	
Tu_GluCl2_dia_F	TCATCGTCTCTTGGGTCTCC	
Tu_GluCl2_dia_R	CCCATCGTCGTTGATACCTT	
Tu_GluCl3_dia_F	CCGGGTCAGTCTTGGTGTTA	
Tu_GluCl3_dia_R	CACCACCAAGAACCTGTTGA	
Tu_GluCl4_dia_F	TATTCCAGCCCGAGTTTCAC	
Tu_GluCl4_dia_R	AATCGGAGGTTGACTTGGTG	
Tu_GluCl5_dia_F	ATGTTGGTCATCGTTTCGTG	
Tu_GluCl5_dia_R	AATCGGGATTGAATTTGCTG	
Tu_Rdl1_TM3F	TGCGAAATTCAGTTGACTCG	survey of TM2/TM3 region of Rdls
Tu_Rdl1_TM3R	CCAATTGTTGGAAGCGATTT	
Tu_Rdl2_TM3F	CCTGCAAGCCTTATAGTGGTG	
Tu_Rdl2_TM3R	CACTTTGTTCGGCTACAGCA	
Tu_Rdl3_TM3F	TCCCTGCCAGCCTTATAGTG	
Tu_Rdl3_TM3R	ATGGCAATCCGTTTACCAAG	
sgRNA sense	CTTCGAAGGCCATCGATGTGTGGAC	sgRNA
sgRNA antisense	AAACGTCCACACATCGATGGCCTTC	
GluExFw	TGGATGGCATTTCTCTGTACCT	Genomic region amplification / sequencing
GluExRv	GCAAGGAACCGAACAAATCGT	
GluInFw	ACCTAACCCCTTTTGCAGGT	Genomic region sequencing
GluInRv	ATGCATAGTTCACCAGGGCG	
GluCl_generic_F	TCAGCATTAAAGAACCAGCGT	Sequencing, allele screening
GluCl_specific_R	CACGCCTGTCCAGACGTCGG	