Ryanodine receptor point mutations confer diamide insecticide resistance in tomato leafminer, *Tuta absoluta* (Lepidoptera: Gelechiidae)

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Abstract

Insect ryanodine receptors (RyR) are the molecular target-site for the recently introduced diamide insecticides. Diamides are particularly active on Lepidoptera pests, including tomato leafminer, *Tuta absoluta* (Lepidoptera: Gelechiidae). High levels of diamide resistance were recently described in some European populations of *T. absoluta*, however, the mechanisms of resistance remained unknown. In this study the molecular basis of diamide resistance was investigated in a diamide resistant strain from Italy (IT-GELA-SD4), and additional resistant field populations collected in Greece, Spain and Brazil. The genetics of resistance was investigated by reciprocally crossing strain IT-GELA-SD4 with a susceptible strain and revealed an autosomal incompletely recessive mode of inheritance. To investigate the possible role of target-site mutations as known from diamondback moth (*Plutella xylostella*), we sequenced respective domains of the RyR gene of *T. absoluta.* Genotyping of individuals of IT-GELA-SD4 and field-collected strains showing different levels of diamide resistance revealed the presence of G4903E and I4746M RyR target-site mutations. These amino acid substitutions correspond to those recently described for diamide resistant diamondback moth, i.e. G4946E and I4790M. We also detected two novel mutations, G4903V and I4746T, in some of the resistant *T. absoluta* strains. Radioligand binding studies with thoracic membrane preparations of the IT-GELA-SD4 strain provided functional evidence that these mutations alter the affinity of the RyR to diamides. In combination with previous work on *P. xylostella* our study highlights the importance of position G4903 (G4946 in *P. xylostella*) of the insect RyR in defining sensitivity to diamides. The discovery of diamide resistance mutations in *T. absoluta* populations of diverse geographic origin has serious implications for the efficacy of diamides under applied conditions. The implementation of appropriate resistance management strategies is strongly advised to delay the further spread of resistance.

Keywords

Tuta absoluta, diamide resistance, flubendiamide, chlorantraniliprole, ryanodine receptor, target-site mutation

1. Introduction

The chemical class of diamide insecticides is a relative recent introduction to the market for the control of herbivorous crop pests, particularly lepidopteran larvae (Nauen, 2006; Jeanguenat, 2013). The phthalic acid diamide flubendiamide (Tohnishi et al., 2005; Hirooka et al., 2007) was introduced in 2006, followed by the anthranilic diamides chlorantraniliprole and cyantraniliprole (Lahm et al., 2007, 2009). The global turn-over of the whole chemical class was >$1.2 billion in 2013, representing approx. 8% of the insecticide market (Sparks and Nauen, 2015). Both flubendiamide and chlorantraniliprole are highly active against a broad range of lepidopteran pests at low application rates, show low acute mammalian toxicity, a favorable environmental profile, and can be used in integrated pest management programmes (Tohnishi et al., 2005; Lahm et al., 2007).

Diamide insecticides act as conformation sensitive activators of the insect ryanodine receptor (RyR), a large (homo)tetrameric calcium-channel located in the sarco- and endoplasmic reticulum in neuromuscular tissues (Ebbinghaus-Kintscher et al., 2006; Cordova et al., 2006; Lümmen et al., 2007; Sattelle et al., 2008). The endogenous activation of RyRs is mediated by calcium influx, driven by voltage-gated calcium channels upon depolarization of the cell membrane (Lümmen 2013). The symptomology of poisoning after diamide application involves muscle contraction, paralysis and eventually death (Tohnishi et al., 2005; Cordova et al., 2006). In contrast to mammals which possess three *RyR* genes (Rossi and Sorrentino 2002), insects encode a single *RyR* gene with an open reading frame (ORF) of >15000 nucleotides translated into a RyR protomer with a molecular weight of more than 5000 kDa, as first described for *Drosophila melanogaster* (Takeshima et al., 1994). RyRs were shown to be composed of six helical transmembrane spanning domains at the C-terminal end containing the calcium ion-conducting pore, and a large N-terminal cytosolic domain (Lümmen 2013). Recently Yan et al., (2015) published a rabbit RyR1 structure determined by single-particle electron cryomicroscopy which resolved a large portion of the homotetrameric channel protein (Yan et al., 2015).

Biochemical studies with a series of *Bombyx mori* RyR deletion mutants suggested that the diamide binding site is likely to be located in the C-terminal transmembrane spanning domain (Kato et al., 2009). This was further supported by the identification of target-site mutations, G4946E and I4790M, in the RyR transmembrane domain of diamide resistant strains of diamondback moth *Plutella xylostella* (Troczka et al., 2012; Guo et al., 2014), and functionally confirmed by radioligand binding studies (Steinbach et al., 2015), and

fluorescence based reporter assays using Sf9 cells stably expressing a modified (G4946E) diamondback moth RyR (Troczka et al., 2015).

Diamide insecticides are used globally straight and in mixtures for foliar, drench and seed treatment applications in a broad range of agricultural and horticultural cropping systems. The popularity and widespread uptake of diamides has increased selection pressure for the evolution of resistance, particularly in the case of lepidopteran pests (Teixeira and Andaloro 2013). The global status of diamide resistance issues in lepidopteran pests was recently reviewed by Nauen and Steinbach (2016). High levels of resistance compromising the efficacy of diamides at recommended field rates were reported in very few species. The first serious cases of resistance were described for diamondback moth strains collected in the Philippines and Thailand (Troczka et al., 2012), followed by China (Wang and Wu 2012; Wang et al., 2013; Gong et al., 2014), Brazil (Ribeiro et al., 2014), Taiwan, India, USA, Japan, Korea and Vietnam (Steinbach et al., 2015). The underlying basis of resistance in a number of field-collected diamondback moth strains appears to be largely due to target-site mutations in the transmembrane domain of the RyR (Troczka et al., 2012; Guo et al., 2014; Steinbach et al., 2015). In particular the G4946E mutation - located at the interface between helix S4 and the S4-S5 linker - was functionally linked to high levels of diamide resistance (Steinbach et al., 2015; Troczka et al., 2015). The functional significance of other mutation sites such as I4790M and Q4594L, present in recently collected Chinese *P. xylostella* populations, is less clear (Guo et al., 2014). Nevertheless, homology modeling of the *Plutella* RyR based on the structure of rabbit RyR1 (Yan et al., 2015), revealed that the I4790M mutation in helix S2 is in quite close proximity to G4946E, suggesting a potential functional role in diamide binding (Steinbach et al., 2015). Apart from diamondback moth, high levels of diamide cross-resistance under applied conditions were only described in two other lepidopteran pest species, the smaller tea tortrix, *Adoxophyes honmai* (Uchiyama and Ozawa 2014) and the tomato the leafminer, *Tuta absoluta* (Meyrick) (Roditakis et al., 2015, Silva et al., 2016).

Tuta absoluta is a multivoltine, invasive pest that originated from Latin America and has recently spread into Europe, Africa and the Middle East, threatening tomato production in both open field and greenhouse crops (Desneux et al., 2010). Recent investigations have shown that *T. absoluta,* which first invaded the Mediterranean Basin in 2006, most likely originates from Chile (Guillemaud et al., 2015). Given its invasive nature and destructive potential it has quickly gained status as a global pest of key concern (Desneux et al., 2011). *Tuta absoluta* control relies heavily on insecticide treatments (Roditakis et al., 2013), however, reports of control failure have clearly illustrated the potential of this pest to

develop resistance to multiple classes of insecticide (Siqueira et al., 2000, 2001; Silva et al., 2011; Haddi et al., 2012; Silva et al., 2016). Baseline susceptibility studies showed diamide insecticides are highly effective against *T. absoluta* (Roditakis et al., 2013; Campos et al, 2015). However, recently, *T. absoluta* collected from Italian greenhouse tomatoes exhibited resistance to diamides of more than 1000-fold when compared to a susceptible reference strain (Roditakis et al., 2015). Despite the reliance on diamides for control of *T. absoluta* in many countries, the molecular mechanisms conferring diamide resistance in *T. absoluta* and implications for resistance management remain unknown. In the present study we investigated the molecular basis of diamide resistance in *T. absoluta* populations collected in both Europe as well as South America and describe target-site resistance to this insecticide class in *T. absoluta* for the first time.

2. Materials and methods

2.1 Insect strains

The *T. absoluta* field strains used in this study were collected from infested tomato (*Solanum lycopersicum* L.) crops in Italy, Greece, Spain and Brazil between 2014 and 2015. A number of tomato leaves infested with *T. absoluta* larvae were collected in large plastic bags and transferred to the respective laboratory for experimentation. Details for each collected strain are provided in Table 1. At least 300 individuals were collected in each location and the larvae were allowed to develop on 3-6 week old potted tomato plants. All populations were maintained in insect cages at 26 ± 1 °C, 65 % RH and 16 h light : 8 h dark photoperiod. The susceptible reference strains (GR-Lab, ES-Sus and BCS-TA-S) were maintained for several years under laboratory conditions without any exposure to insecticides. Strain IT-GELA-SD4 was obtained after four sequential selection cycles of strain IT-GELA-14-1 with the insecticide chlorantraniliprole using foliar applied doses of 100 mg L^{-1} (first selection) and 300 mg L^{-1} (subsequent selections). At least 1000 2^{nd} instar larvae were used for each selection cycle.

2.2 Chemicals and insecticides

All chemicals and organic solvents used were of analytical grade. For all bioassays and insecticide treatments commercial formulations of the diamide insecticides chlorantraniliprole (Altacor® 35WG, DuPont, France) and flubendiamide (Belt® 24WG, Bayer

CropScience AG, Germany) were used. For Brazilian strains another commercial formulation of chlorantraniliprole was used (Premio® 200SC, DuPont, Brazil).

2.3 Insect bioassays

Leaf dip bioassays with the European populations were principally conducted according to IRAC method 022 (www.irac-online.org) with slight modifications described elsewhere (Roditakis et al., 2013). Briefly, either tomato leaflets cut in square pieces, or entire leaves were immersed in serial insecticide concentrations containing Triton X-100 (0.2 g L^{-1}) as a non-ionic wetting agent. Treated leaves were allowed to dry for 1-2 hours at room temperature and subsequently placed adaxially on moist tissue paper in a multi-well repli-dish. A single 2nd instar larva was placed in each well, subsequently all wells were sealed with transparent ventilated adhesive lids. Bioassays with the Brazilian field strains were conducted with a slightly different method according to Campos et al., (2015). All bioassays were incubated in growth chambers at 25 ± 0.5 °C and 65 ± 5 % relative humidity. Larval mortality was assessed after 3 days of exposure. Mortality evaluations were performed with the aid of a light source and magnifying glass. Larvae were carefully removed from tomato leaf galleries and considered dead if they were unable to move the length of their bodies after gentle prodding with a camel-hair brush.

Lethal concentration values for 50 % mortality (LC_{50}) and 95 % confidence limits (CL 95 %) were obtained by probit analysis using the software packages PriProbit 3.4 (Sakuma, 1998) or Polo Plus (LeOra software, USA). LC_{50}-values were considered significantly different when their 95 % confidence limits did not overlap. Percentage mortality values generated in bioassays was corrected using Abbott's formula (Abbott, 1925).

2.4 Synergist bioassays

Synergist bioassays were performed following IRAC method 022 as described above with strain IT-GELA-SD4 (selected with chlorantraniliprole). Two hours prior to bioassay, larvae were exposed to sublethal doses ($\leq LC_{05}$) of different synergists via contact on fresh dried residues in coated glass vials (30 ml volume). Synergists used were piperonyl butoxide (PBO, Sigma, UK), S,S,S tributyl phosphorotrithioate (DEF, Sigma, UK) and diethyl maleate (DEM, Sigma, UK), known to inhibit cytochrome P450 monooxygenases, esterases and glutathione S-transferases, respectively. For vial coating 300 µl of acetonic solutions of PBO (0.1 g L^{-1}), DEF (0.1 g L^{-1}) or DEM (0.3 g L^{-1}) were added into each vial. Afterwards vials were placed

horizontally on rotating metal rods for 1 h. After rotation the vials were allowed to dry for another hour before adding the larvae. The synergistic ratio was calculated by dividing the calculated LC_{50}-value of synergist-exposed larvae by the LC_{50}-value of larvae not exposed to synergists.

2.5 Genetics of diamide resistance

Tuta absoluta larvae of strains GR-Lab and IT-GELA-SD4 were allowed to develop and pupate under controlled conditions (see above) in small plastic rearing cages. Pupae were subsequently collected and sexes separated based on external morphology (Solomon, 1962). Subsequently they were placed individually in transparent glass tubes where the emergence of adults was checked daily. The sex of adult moths was checked again for confirmation of the initial classification of pupae (Coelho and França, 1987). Subsequently 100 virgin females of strain IT-GELA-SD4 were crossed with 100 males of the susceptible strain GR-Lab and *vice versa*. Second instar larvae of the F1 generation of reciprocal crosses were subsequently bioassayed with chlorantraniliprole to determine LC_{50}-values as described above. The degree of dominance was calculated using the formula $D= (2X_2-X_1-X_3)/(X_1-X_3)$ (Stone, 1968). The values X_1, X_2 and X_3 are the $\log(LC_{50})$ of the resistant strain IT-GELA-SD4, the F1 generation and the susceptible strain GR-Lab, respectively. The F1 generation of the reciprocal crosses was subsequently back-crossed with the parental IT-GELA-SD4 strain to check for monogenic resistance. Expected mortality of the backcross generation (F2) under a monogenic model was calculated using the following formula: expected % mortality F2 at [c] = 0.5 x (% mortality of F1 at [c] + % mortality of strain IT-GELA-SD4 at [c]), where [c] refers to the respective concentration of chlorantraniliprole.

2.6 Biochemical assays

All enzymatic assays were repeated at least three times. Detoxification enzyme activities were determined in strains GR-Lab (susceptible), IT-GELA-14-1 (field-collected) and IT-GELA-SD4 (selected). Glutathione S-transferase (GST), esterase (EST) and cytochrome P450 monooxygenase (P450) activities were determined using a SpectraMax M2e microplate reader (Molecular Devices, Berkshire, UK). For GST and EST activities, groups of five 3rd instar larvae were homogenized on ice in 100 µl of 0.1 M sodium phosphate buffer, pH 7.2 and centrifuged for 5 min at 4 °C and 5,000 g. The supernatants were used as enzyme source. Total protein content of the enzyme solution was determined by the Bradford method using bovine serum albumin as the standard (Bradford, 1976).

For GST activity, the homogenate (10-20 µg of total protein) was mixed with 200 µL of substrate solution (10 mM reduced glutathione, 3 mM 1-chloro-2,4-dinitrobenzene (CDNB) in 100m M sodium phosphate buffer, pH 6.5). The activity was measured kinetically at 340 nm for 5 min and expressed as µmol mg protein^{-1} min^{-1} using the extinction coefficient of the resulting 2,4-dinitrophenylglutathione conjugate at 340nm (9.6 mM^{-1} cm^{-1}).

EST activities were determined using 1- and 2-naphthyl acetate (NA) as substrate. Homogenate (2-5 µg of total protein) was placed in a microplate well, and 200 µl naphthyl acetate working solution was added (0.3 mM 1-NA or 2-NA in sodium phosphate buffer 0.2 M, pH 7.2). After 30 min incubation, 50 µl of 6.4 mM Fast Blue B salt (Sigma Aldrich) diluted in 35 mM sodium phosphate buffer pH 7, containing 35 g L^{-1} SDS were added to each well. The formation of the 1- or 2-naphthol Fast Blue dye complex was measured at 570 nm and converted to specific activity expressed as 1- or 2-naphthol mg protein-1 min-1, using a standard curve of 1- or 2-naphthol. Standard curves were established to determine the quantity of formed final product.

P450 activity was determined by the O-deethylation of 7-ethoxycoumarin (7-EC) using a protocol adapted for *in vivo* analysis in microplates (Reyes et al., 2012). Briefly twenty 3rd instar larvae were dissected individually placed in the wells of black microplates. Each well contained 0.4 mM 7-EC in 0.1 ml of 50 mM HEPES buffer, pH 7.0. Ten wells without dissected insects were used as controls. After 4 h incubation at 30 °C, the enzymatic reaction was stopped by adding 0.1 ml 0.1 mM glycine buffer (pH 10.4):ethanol (v/v). Umbelliferone fluorescence was measured at 465 nm while exciting at 390 nm. A standard curve was established using umbelliferone and cytochrome P450 activity was expressed as pg product insect^{-1} min^{-1}.

2.7 Radioligand binding studies

Tuta absoluta adults of strains BCS-TA-S and IT-GELA-SD4 were flash-frozen in liquid nitrogen, stored at -80°C and collected over many generations to get the appropriate biomass necessary for conducting radioligand binding assays. Thoracic endoplasmic/sarcoplasmic reticulum membranes were prepared as described earlier (Steinbach et al., 2015). Radioligand binding assays were performed using 50 µg membrane protein per assay. Enhancement of ryanodine binding was measured using 4.0 nM [^{3}H]ryanodine as a function of increased diamide insecticide concentration as described elsewhere (Steinbach et al., 2015). Equilibrium binding parameters were calculated using the software package GraphPad Prism 5 (GraphPad Inc.).

2.8 Identification and sequence verification of the T. absoluta *RyR*

The *T. absoluta* RyR was manually curated from an unpublished transcriptome of this species, generated by Illumina sequencing of different life stages of a diamide susceptible strain, followed by *de novo* assembly using Trinity (Grabherr et al., 2011). To verify the transcriptome sequence 14 primer pairs were designed (Table S1), spanning the entire ORF. RNA was extracted from pools of 10-20 larvae using the ISOLATE II RNA Mini Kit (Bioline) and quantified using a NanoDrop® 1000 (ThermoScientific, USA). 5µg was used for cDNA synthesis using SuperScript III RT (ThermoScientific, USA) and random hexamers (Promega, USA). PCR reactions (25µl total volume) contained Dreamtaq mastermix (ThermoScientific, USA) 1µl of cDNA and 10 pmol of each primer pair. Cycling conditions were 95^{o}C for 2min followed by 35 cycles of 95^{o}C for 20s, 50^{o}C for 20s and 72^{o}C for 2 min, with a final extension step of 72^{o}C for 5min. PCR products were visualised on a 1% TAE agarose gel electrophoresis and purified via QIAquick PCR purification kit (Qiagen, Germany). All PCR products were sequenced using the Sanger method by Eurofins (Germany). Sequencing results were analysed using Geneious software (Biomatters, New Zeland). The final curated sequence was deposited in NCBI under GenBank accession no. KX519762.

2.9 Pyrosequencing of PCR amplified RyR cDNA of T. absoluta *for genotyping*

Individuals of different strains of *T. absoluta* were genotyped for the recently described diamondback moth RyR mutations G4946E (Troczka *et al.,* 2012; Steinbach et al., 2015) and I4790M (Guo *et al.,* 2014). Genomic DNA was extracted from individual larvae using the DNAdvance Tissue Kit (Agencourt) according to the supplier's recommended protocol. Primer pairs were designed based on the obtained RyR full-length cDNA sequence (GenBank no. KX519762). A short gene fragment of 228 bp and 190 bp for G4946E and I4790M genotyping, respectively was amplified by PCR from 50 ng aliquots of *T. absoluta* gDNA using the primer pairs Ta_I4790-F, Ta_I4790-R-btn and Ta_G4946-F-btn, Ta_G4946-R, respectively (Table S2). The pyrosequencing protocol for the detection of I4790M (position 4746 in *Tuta* RyR) comprised 40 PCR cycles with 0.5 µM forward and biotinylated reverse primer in 30 µl reaction mixtures containing 1 × Taq enzyme reaction mix (JumpStart™ Taq ReadyMix™, Sigma Aldrich) and cycling conditions of 95 °C for 3 min, followed by 40 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min, and a final elongation step at 72 °C for 5 min. The same protocol was used for genotyping G4946E (position 4903 in *Tuta* RyR) with a few modifications: the forward primer was biotinylated rather than the reverse primer and the temperature for primer annealing was set to 53 °C for 30 s. The single strand DNA required

for pyrosequencing was prepared as described in Troczka *et al.,* (2012). The pyrosequencing reactions were carried out according to the manufacturer's instructions using the PSQ 96 Gold Reagent Kit (Qiagen), and the sequencing primer Ta_I4790-Seq-F (I4790M) or Ta_G4946-Seq-R (G4946E) for genotyping. The pyrograms, indicating the genotype, were analyzed using the SNP Software (Qiagen).

2.10 Sanger sequencing

Sanger sequencing was performed in order to partially sequence the RyR domain of Brazilian strains BR-GML1 and BR-PSQ that failed to produce results in G4946E-pyrosequencing diagnostics. Degenerate primers, Ta_SangerSeq-F and Ta_SangerSeq-R (Table S2), were designed based on a multiple nucleotide alignment of *T. absoluta* (GenBank no. KX519762), *P. xylostella* (JN801028), *B. mori* (XM_004924859) and *C. suppressalis* (JX082287). A 285 bp fragment of *T. absoluta* RyR was amplified from gDNA by PCR using Q5® High-Fidelity DNA Polymerase 2x Master Mix according to the manufacturer's instruction (New England BioLabs Inc., USA). The cycling conditions were 98 °C for 30 s, followed by 35 cycles of 98 °C for 10 s, 50 °C for 30 s and 72 °C for 30 s, and a final elongation step at 72 °C for 2 min. The purified PCR products were sequenced by Eurofins Genomics (Ebersberg, Germany).

3. Results

3.1 Leaf-dip bioassays and stability of resistance

Three diamide susceptible laboratory reference strains, five field-collected strains from Italy, Greece, Spain and Brazil, and one chlorantraniliprole-selected strain originating from Italy (IT-GELA-SD4) were included in this study. The obtained LC_{50}-values for chlorantraniliprole in the susceptible reference strains GR-Lab, ES-Sus and BCS-TA-S differed less than 2-fold (between 0.18 and 0.31 mg L^{-1}; Table 2). Resistance ratios for chlorantraniliprole in field collected strains varied between 8 (strain ES-MUR-14, Spain) and >3000 (strain BR-PSQ, Brazil). Strain IT-GELA-SD4 selected with chlorantraniliprole for four generations exhibited a resistance ratio 4-fold higher than the parental strain IT-GELA-14-1 (Table 2). Strain IT-GELA-SD4 was mass reared and chosen for more detailed genetic and molecular studies on diamide resistance in *T. absoluta*. Whereas parental strain IT-GELA-14-1 lost some of its resistance over time, we observed a stable diamide resistance of selected strain IT-GELA-SD4 over a period of 10 months (data not shown).

335 *3.2 Genetics of resistance of strain IT-GELA-SD4*

336 Results of the bioassays with chlorantraniliprole against strains GR-Lab (S) and IT-GELA-SD4
337 (R), as well as their reciprocal crosses are presented in Table 3. Although the LC_{50}-values
338 obtained in bioassays with the respective F1 generation of reciprocal crosses were 3-fold
339 different, such marginal differentiations could be more likely caused by bioassay variably
340 factors rather than sex linkage. Therefore, negligible maternal effect was observed
341 suggesting that diamide resistance in *T. absoluta* is autosomFLal inherited. The calculated
342 degree of dominance (D) was -0.63 and -0.29 for (S)♀ x (R)♂ and (S)♂ x (R)♀, respectively,
343 suggesting an incompletely recessive mode of inheritance. Female F1 hybrids of (S)♀ x (R)♂
344 crosses were backcrossed with males of IT-GELA-SD4 and tested for monogenic resistance.
345 However the obtained experimental dose-response curve for chlorantraniliprole did not
346 clearly plateau at 50% mortality and was significantly different from theoretical
347 considerations assuming monogenic inheritance (X^2 = 28.3, df = 8, P < 0.05), possibly
348 indicating that diamide resistance in strain IT-GELA-SD4 is not controlled by a single trait (Fig.
349 1).
350
351 *3.3 Diamide cross-resistance, effect of synergists and activity levels of detoxification enzymes*
352 *in strain IT-GELA-SD4*

353 Strain IT-GELA-SD4 showed strong cross-resistance to the phthalic acid diamide
354 flubendiamide (RR >2500-fold), demonstrating the presence of a resistance mechanism
355 affecting both anthranilic and phthalic acid diamides (Table 4). In order to investigate the
356 possible involvement of a metabolic mechanism of resistance we tested the effect of
357 synergists on the efficacy of both chlorantraniliprole and flubendiamide against strain IT-
358 GELA-SD4. Glutathione depletion by DEM and inhibition of cytochrome P450
359 monooxygenases by PBO prior to diamide exposure resulted in synergistic ratios less than or
360 equal to 2-fold, suggestive of a rather limited impact of these detoxification mechanisms in
361 strain IT-GELA-SD4. In bioassays with the esterase inhibitor DEF combined with
362 chlorantraniliprole we observed a synergistic ratio of 11-fold compared to
363 chlorantraniliprole alone, suggesting the possible presence of an esterase-mediated
364 mechanism of resistance for this particular insecticide only (Table 4), since comparable
365 synergistic action with DEF was not observed when flubendiamide was tested. However, the
366 chlorantraniliprole resistance ratio in strain IT-GELA-SD4 was still >100-fold in DEF
367 synergised bioassays when compared to the susceptible reference strain GR-Lab, clearly
368 indicating the presence of additional mechanisms of resistance. The activity levels of

detoxification enzyme families measured with artificial model substrates were similar in larvae of strains GR-Lab, IT-GELA-14-1 and IT-GELA-SD4 (Table 5), with no significant increase in activity observed in GSTs, CEs, or P450s in strain IT-GELA_SD4 compared with the GR-Lab susceptible strain.

3.4 Radioligand binding studies

Comparative binding studies using thoracic membrane preparations of strains BCS-TA-S and IT-GELA-SD4 were carried out with the diamide insecticide flubendiamide, known to act, like chlorantraniliprole, as a positive allosteric activator of [^{3}H]ryanodine binding to lepidopteran RyRs (Steinbach et al., 2015). Strain IT-GELA-SD4 shows high levels of resistance ($\geq$1000-fold) against both flubendiamide and chlorantraniliprole when compared to the susceptible reference strains in this study (Table 4). Radioligand binding studies using thoracic endo-/sarcoplasmic membranes of adults of strain BCS-TA-S and IT-GELA-SD4 revealed a relative increase of [^{3}H]ryanodine binding as a function of flubendiamide concentration at an EC_{50} of 3.03 ± 1.45 nM and >1000 nM, respectively (Fig. 2). This equates to RyR target-site insensitivity of >300-fold to flubendiamide in membrane preparations of strain IT-GELA-SD4 when compared to membranes isolated from the diamide susceptible strain BCS-TA-S.

3.5 Cloning, sequencing and characterisation of the T. absoluta RyR

A single contig of 16,431 bp encoding the *T. absoluta* RyR was identified in our unpublished transcriptome of this species based on BLAST annotation of the whole transcriptome against the non-redundant protein database of NCBI. This transcript included the full length coding sequence of the RyR gene of 15,363 bp encoding 5121 amino acids and shows high levels of sequence similarity with the RyR mRNA of other Lepidoptera (Table 6). The complete coding sequence of this contig was independently sequence verified by PCR and the final curated sequence was deposited in NCBI under GenBank accession no. KX519762. The predicted protomer has a molecular mass of 578.965 kDa and shares features common to other characterized insect RyRs, including a large extracellular N-terminal region followed by a highly conserved transmembrane region comprising 6 predicted transmembrane S1-S6 (Fig. 3) toward the COOH terminus of the sequence with the probable pore-forming domain located between domains S5 and S6. Several putative resistance hot-spots have been described in the RyR of resistant *P. xylostella* (G4946E, E1338D, Q4594L and I4790M) two of which (G4946E and I4790M) have been most strongly implicated in resistance as detailed in the introduction. The RyR sequence of the diamide susceptible *T. absoluta* strain sequenced

had the 'susceptible' amino acid at position 4903 (corresponding to G4946 in *P. xylostella*), 4746 (I4790 in *P. xylostella*), 4540 (Q4594 in *P. xylostella*) but an aspartic acid (D) at position 1339 (E1338 in *P. xylostella*), suggesting the E1338D substitution first reported in *P. xylostella* is not a *bona fide* resistance mutation.

3.6 Genotyping for RyR target-site mutations by pyrosequencing

Individuals of a number of strains included in this study were analyzed for the presence and frequency of mutations at positions G4903 and I4746, previously associated with RyR target-site resistance against diamides in *P. xylostella* at the corresponding positions (Steinbach et al., 2015; Guo et al., 2014). A partial amino acid sequence of the *T. absoluta* RyR C-terminal transmembrane domain indicating potential mutation sites (based on diamide resistant *P. xylostella* RyR) is shown in Fig. 3. The pyrosequencing assay using genomic DNA of *T. absoluta* worked well for all strains and both mutation sites (Figs. S1 and S2), except for G4903 of the two Brazilian field strains BR-GML1 and BR-PSQ, due to their polymorphism in the nucleotide sequence of the *RyR* regions selected for primer design as revealed by Sanger sequencing (Fig. S3). However Sanger sequencing of partial *RyR* sequences of both Brazilian field strains highly resistant to diamides revealed the presence of the G4903E mutation (Fig. 4) at a frequency of 100%, albeit only a relatively small number of samples was analysed (Table 7). Furthermore, pyrosequencing of DNA samples of these strains confirmed the presence of the mutations I4746M, and a previously un-reported amino acid substitution, I4746T, at low frequency (Table 7). The diamide susceptible strains BCS-TA-S, GR-Lab and ES-Sus were all wildtype homozygous at positions G4903 and I4746 (Table 7). The laboratory selected strain IT-GELA-SD4 was found to contain a mixture of individuals of different genotypes none of which had the wildtype G4903 amino acid residue, but rather were homozygous for G4903E, or G4903V, a novel substitution not yet reported at this position. In addition the IT-GELA-SD4 strain also carried the I4746M mutation at high frequency (80 % of individuals tested were homozygous for this mutation). Strain GR-IER-15-2 which exhibited 55-fold resistance to chlorantraniliprole exhibited 10 % and 20 % of sequenced individuals homozygous for G4903E and G4903V, respectively. Most individuals were wildtype I4746, though we detected a low frequency of I4746M heterozygotes. Strain ES-MUR-14 collected in Spain in 2014, which showed very low resistance to chlorantraniliprole (8-fold), was exclusively wild-type at position 4903 but carried a low frequency (33%) of individuals heterozygous for I4746M.

4. Discussion

Diamide insecticides have been introduced for European tomato leafminer control only very recently and the first field failures were reported in greenhouse tomatoes in Italy in 2014 after repeated applications of chlorantraniliprole (Roditakis et al., 2015). The resistance cases in Italy are no longer restricted to greenhouse tomatoes in Sicily, but have now also been reported in other regions in the south, e.g. Puglia (Stefan Herrmann, Bayer CropScience, personal communication). Diamide resistance ratios of more than 1000-fold highlight the severity of the problem in Italy, triggered by repeatedly treating consecutive generations of this pest and failure to implement appropriate resistance management strategies as recently proposed (Teixeira and Andaloro, 2013). *Tuta absoluta* was first reported in Europe (Spain) in 2006, but after only 5 years had spread to most tomato growing regions in the Mediterranean Basin, underlining its exceptional invasive potential (Desneux et al., 2011). Aggressive control measures comprising almost weekly insecticide applications targeting consecutive generations of the pest, known to complete 10-12 generations per year under greenhouse conditions, facilitated resistance development to diamides in a relatively short time ($\leq$ 4 years), similar to observations made in repeatedly treated diamondback moth populations in cabbage fields in different geographic locations (Troczka et al., 2012; Wang and Wu, 2012; Gong et al., 2014; Steinbach et al., 2015).

In this study we also investigated field-collected *T. absoluta* populations showing low to high levels of diamide resistance sampled outside of Italy, specifically Greece, Spain and Brazil. High levels of diamide resistance were also found in Brazilian populations, whereas moderate and low levels of resistance were detected in populations collected in Greece and Spain in 2015 and 2014, respectively. We went on to select one of the Italian strains collected in 2014 (IT-GELA-14-1) for additional generations with chlorantraniliprole and investigated the molecular basis of resistance in detail. Our study was informed by recent work conducted on diamide resistant diamondback moth populations, which revealed amino acid substitutions in the *Plutella* RyR transmembrane domain that confer target-site resistance (Troczka et al., 2012, 2015; Guo et al., 2014; Gong et al., 2014; Steinbach et al., 2015). We isolated the full-length *T. absoluta RyR* gene and focused on the C-terminal domain spanning approx. 450 amino acids and containing six highly conserved transmembrane segments previously shown to play a major role in diamide insecticide binding (Kato et al., 2009). It was recently shown that the G4946E mutation in the diamondback moth RyR has strong functional implications for both the direct binding of

tritiated diamide insecticides and their ability to allosterically enhance [^{3}H]ryanodine binding in isolated thoracic microsomal membrane preparations (Steinbach et al., 2015). This was confirmed in a second study measuring calcium transients in diamide treated Sf9 insect cells stably expressing *Plutella* RyR constructs carrying the G4946E mutation when compared to cells expressing wildtype receptors (Troczka et al., 2015).

Here we have discovered the corresponding mutation, G4903E, in the RyR of the diamide selected *T. absoluta* strain IT-GELA-SD4. Furthermore, we also identified an alternative amino acid substitution at the same position, G4903V, that has not been previously described. Both precisely correspond to the G4946E mutation site functionally proven to alter diamide binding in the RyR´s of diamide resistance *Plutella*. As previously demonstrated for *P. xylostella* we confirmed the strong impact of mutations at this site using radioligand binding studies on thoracic membrane preparations of tomato leafminer adults of strain IT-GELA-SD4 in comparison to a susceptible reference strain (BCS-TA-S). This binding assay is considered to be a reliable and sensitive indicator of diamide action on insect RyR´s (Lümmen et al., 2007; Qi et al., 2014). Flubendiamide allosterically increased [^{3}H]ryanodine binding in RyR preparations of the susceptible strain BCS-TA-S at low nanomolar concentrations (EC_{50}-value: 3 nM), i.e. in the range of EC_{50}-values recently reported for other lepidopteran membrane preparations. The EC_{50}-value for [^{3}H]ryanodine binding stimulation by flubendiamide in strain IT-GELA-SD4 was at least 300-fold higher, thus confirming insensitivity and the relevance of the RyR G-to-E (or -V) target-site mutation in a species other than diamondback moth.

The IT-GELA-SD4 strain also carries an additional mutation, I4746M, at very high frequency. This corresponds to the I4790M mutation recently described in the RyR of diamide resistant populations of diamondback moth from China (Guo et al., 2014). In our study this mutation was present in combination with G4946E so the impact of I4790M on diamide binding and its contribution to resistance remains unclear. Nevertheless a recent study using homology modelling of the *Plutella* RyR (Steinbach et al., 2015), based on a recently published vertebrate RyR1 structure determined by single-particle electron cryomicroscopy (Yan et al., 2015), has suggested that both sites may contribute to diamide binding. The model suggests that the G4946E mutation is located at the interface between helix S4 and the S4-S5 linker, thought to have a critical role in RyR gating by impacting the movement of pore-associated helices (Ramachandran et al., 2013). The second mutation I4790M was shown to be located in helix S2 in close proximity to G4946E. Whereas G4946 is highly conserved in all insect species, methionine 4790 - described as a RyR mutation site in diamide resistant *P. xylostella*

505 - is wildtype in the RyR´s of several other insect species that are highly sensitive to
506 chlorantraniliprole, a fact prompting Steinbach et al., (2015) to speculate that this site
507 determines binding specificity and differences in selectivity profiles between anthranilic and
508 phthalic diamides rather than field-relevant resistance (reviewed in Nauen and Steinbach,
509 2016). Diamide resistance in *T. absoluta* strain IT-GELA-SD4 is autosomally inherited, and as
510 an incompletely recessive trait (heterozygotes display a near susceptible phenotype), a
511 finding corresponding to the inheritance of diamide resistance mediated by target-site
512 mutations in diamondback moth (Guo et al., 2014; Steinbach et al., 2015).

513 To facilitate the genotyping of target-site resistance in *T. absoluta* field samples (e.g.
514 conserved and shipped in alcohol) we developed a pyrosequencing method based on
515 genomic DNA recently successfully used to monitor the geographic spread of RyR target-site
516 resistance in diamondback moth (Steinbach et al., 2015). While pyrosequencing individuals
517 for the presence of I4746M in both Brazilian strains included in this study, we detected
518 another novel mutation, I4746T, carried in the homozygous form or as combined I4746T/M
519 heterozygotes. However, both Brazilian strains also expressed the G4903E RyR target-site
520 mutation as revealed by Sanger sequencing of the respective domain in the *RyR* gene.
521 Genotyping by pyrosequencing of the slightly resistant (8-fold) Spanish field strain ES-MUR-
522 14 failed to detect G4903E (or 4903V) either in the heterozygous or homozygous form,
523 whereas a Greek field strain collected in 2015 and exhibiting 55-fold resistance to
524 chlorantraniliprole carried G4903V and G4903E in the homozygous form at low frequencies
525 of 20 % and 10 %, respectively. Interestingly both these strains also carried the I4746M
526 mutation at similar low frequency and in the heterozygous form. Given this finding it is likely
527 that the greater level of diamide resistance in the Greek field strain GR-IER-15-2 is conferred
528 by the low frequency of the G4903V and G4903E mutations in this strain. In the case of the
529 Spanish strain we either did not pyrosequence enough individuals to detect rather low
530 frequencies of G4903E/V genotypes, or another, less potent, mechanism of resistance might
531 explain resistance. A possible candidate mechanism is enhanced detoxification by
532 cytochrome P450s, as recently implicated in Brazilian *T. absoluta* strains exhibiting variation
533 in monooxygenase activity correlated with their response to the diamides chlorantraniliprole
534 and cyantraniliprole (Campos et al., 2015). However, most if not all studies on lepidopteran
535 pests so far published, failed to clearly demonstrate strong evidence for metabolic
536 mechanisms of diamide resistance powerful enough to cause field failure at recommended
537 rates (Nauen and Steinbach, 2016). This is largely supported by our results showing a lack of
538 elevated levels of detoxification enzymes in the selected IT-GELA-SD4 strain when compared

539 to a susceptible reference strain. However, in contrast to this finding, we observed notable
540 synergism of chlorantraniliprole by DEF in strain IT-GELA-SD4 suggesting that esterases may
541 play some role in enhancing chlorantraniliprole toxicity.

542

543 Tomato leafminer is the second lepidopteran pest species, after diamondback moth, to
544 develop extremely high levels of diamide resistance mediated by confirmed RyR target-site
545 mutations that confer sufficient levels of resistance to compromise efficacy under field
546 conditions at recommended application rates. Diamides were only introduced to the market
547 10 years ago, but due to their high efficacy at low rates they quickly gained widespread
548 adoption reflected in global sales and application frequency in certain agri- and horticultural
549 settings (Teixeira and Andaloro, 2013; Sparks and Nauen, 2015). Unfortunately, it is likely
550 that additional crop pest species are at high risk of developing target-site resistance to this
551 insecticide class unless appropriate insecticide resistance management (IRM) strategies,
552 particularly those based on mode of action rotation, are adhered to (Teixeira and Andaloro,
553 2013; Sparks and Nauen, 2015; Nauen and Steinbach, 2016). In this regard the
554 implementation of IRM programmes to protect new modes of action such as RyR
555 modulators from rapid resistance development needs to be an essential cornerstone of
556 modern crop protection in order to prolong the life of the limited arsenal of actives currently
557 available for control and guarantee sustainable crop yields.

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Table 1

Information on field and laboratory strains of *Tuta absoluta* used in the present study. All strains were collected and/or maintained on tomato plants. Strain IT-GELA-SD4 was obtained from strain IT-GELA-14-1 after 4 selection cycles with chlorantraniliprole (see M&M).

	Strain	Country	Location	Year
Reference lab	GR-Lab	Greece	Peloponnese	2010
	ES-Sus	Spain	Murcia, Aguilas	2011
	BCS-TA-S	Brazil	Paulinia, SP	2005
Field-collected	GR-IER-15-2	Greece	Ierapetra, Kalogeri	2015
	IT-GELA-14-1	Italy	Sicily, Gela	2014
	ES-MUR-14	Spain	Murcia, Lorca	2014
	BR-GML1	Brazil	Gameleira, BA	2014
	BR-PSQ	Brazil	Pesqueira, PE	2014
Selected	IT-GELA-SD4	Italy	Sicily , Gela	2014

Table 2

Log-dose probit-mortality data for chlorantraniliprole against 2nd instar larvae of different strains of *Tuta absoluta* in foliar bioassays (96h)

	Strain	N	LC_{50} [mg L^{-1}]	CL 95%[d]	Slope	RR
Reference	GR-Lab	192	0.31	0.22-0.45	1.6	
	ES-Sus	300	0.18	0.14-0.23	2.3	
	BCS-TA-S	210	0.21	0.15-0.29	1.4	
Field	GR-IER-15-2	145	17	8.7-42		55[a]
	IT-GELA-14-1	191	56	14-120	1.0	181[a]
	ES-MUR-14	210	1.5	1.1-2.1	1.6	8[b]
	BR-GML1	296	92	60-130	1.2	438[c]
	BR-PSQ	292	650	420-920	1.2	3095[c]
Selected	IT-GELA-SD4	127	230	110-430	1.2	742[a]

[a] Resistance ratio (RR) calculated is based on strain GR-Lab

[b] Resistance ratio (RR) calculated is based on strain ES-Sus

[c] Resistance ratio (RR) calculated is based on strain BCS-TA-S

[d] Confidence limits 95%

Table 3

Log-dose probit-mortality data for chlorantraniliprole tested against reciprocal crosses of 2nd instar larvae of diamide susceptible (S) and resistant (R) *Tuta absoluta* strains GR-Lab and IT-GELA-SD4, respectively.

Strain	N	LC_{50} [mg L^{-1}]	CL 95%[a]	Slope	D[b]
GR-Lab (S)	192	0.36	0.23-0.54	1.4	
IT-GELA-SD4 (R)	189	185	111-288	1.2	
F1: (S)♀ x (R)♂	145	1.1	0.67-1.8	1.4	-0.64
F1: (S)♂ x (R)♀	208	3.3	1.9-5.1	1.4	-0.29

[a] Confidence limits 95%

[b] Degree of dominance

Table 4

Log-dose probit-mortality data for chlorantraniliprole (CPR) and flubendiamide (FLB) tested in combination with synergists against 2nd instar larvae of *Tuta absoluta*

Strain	N	LC_{50} [mg L^{-1}]	CL 95%[a]	Slope	RR	SR[b]
CPR						
GR-Lab	187	0.18	0.13-0.30	1.4		
IT-GELA-SD4	92	244	29-410	2.5	1356	
IT-GELA-SD4 +DEF	168	23	14-35	1.7	128	11
IT-GELA-SD4 +PBO	100	161	82-430	1.4	894	2
IT-GELA-SD4 +DEM	95	169	80-1000	1.0	939	1
FLB						
GR-Lab	186	0.79	0.31-1.5	1.1		
IT-GELA-SD4	160	2100	1300-4300	1.4	2658	
IT-GELA-SD4 +DEF	100	1255	496 - 16035	0.87	1589	2
IT-GELA-SD4 +PBO	116	1284	626 - 3520	0.78	1625	2
IT-GELA-SD4 +DEM	112	1700	550-5700	1.3	2152	1

[a] Confidence limits 95%

[b] Synergistic ratio (LC_{50} without synergist divided by LC_{50} + synergist)

Table 5

Detoxification enzyme activity measured with model substrates for esterases (EST), glutathione S-transferases (GST) and microsomal monooxygenases (P450) in different strains of *Tuta absoluta.* Results are mean values ± SD (n=3) with no significant differences between strains.

Strain	EST 1-NA[a]	EST 2-NA[b]	GST CDNB[c]	P450 7-EC[d]
GR-Lab	122 ± 10	265 ± 13	0.258 ± 0.058	16.0 ± 2.65
IT-GELA-14-1	166 ± 26	251 ± 6.7	0.198 ± 0.036	20.1 ± 2.45
IT-GELA-SD4	141 ± 4.1	249 ± 2.9	0.217 ± 0.022	20.9 ± 2.11

[a] nmol 1-naphthol/min x mg protein (1-NA = 1-naphthylacetate)

[b] nmol 2-naphthol/min x mg protein (2-NA = 2-naphthylacetate)

[c] µmol 2,4-dinitrophenyl-S-glutathione/min x mg protein (CDNB = 1-Chloro-2,4-dinitrobenzene)

[d] pg 7-OH-coumarin/min x larva (7-EC = 7-Ethoxycoumarin)

Table 6

Comparison of insect ryanodine receptor protein sequences (given in % identity)

	Tuta absoluta	*Cnaphalocrocis medinalis*	*Helicoverpa armigera*	*Plutella xylostella*	*Tribolium castaneum*	*Drosophila melanogaster*	*Acyrthosiphon pisum*
T. absoluta	---						
C. medinalis	93.1	---					
H. armigera	92.7	94.3	---				
P. xylostella	90.7	92.2	92.2	---			
T. castaneum	81.8	82.6	82.0	81.0	---		
D. melanogaster	77.9	78.6	78.3	77.5	77.7	---	
A. pisum	77.3	77.9	77.2	76.5	79.1	74.5	---

Table 7

Genotyping by pyrosequencing of individuals of *Tuta absoluta* (n = 8-12) for the presence of RyR mutations in the C-terminal transmembrane domain at amino acid positions G4903 and I4746 corresponding to G4946 and I4790 in diamondback moth. All three diamide susceptible strains (BCS-TA-S, GR-Lab and ES-Sus) were homozygous wildtype (SS) at the respective positions.

Strain	G4903	V4903		E4903		I4746	M4746		T4746		M/T 4746
(Genotype)	(SS) %	(SR) %	(RR) %	(SR) %	(RR) %	(SS) %	(SR) %	(RR) %	(SR) %	(RR) %	(RR) %
BCS-TA-S	100	0	0	0	0	100	0	0	0	0	0
GR-Lab	100	0	0	0	0	100	0	0	0	0	0
IT-GELA-SD4	0	0	33	0	67	4	16	80	0	0	0
GR-IER-15-2	70	0	20	0	10	76	24	0	0	0	0
ES-Sus	100	0	0	0	0	100	0	0	0	0	0
ES-MUR-14	100	0	0	0	0	67	33	0	0	0	0
BR-GML1	0	0	0	0	100[a]	67	0	0	0	33	0
BR-PSQ	0	0	0	0	100[a]	75	0	0	0	12.5	12.5

[a] Frequency estimated by Sanger sequencing (pooled n $\geq$ 4)

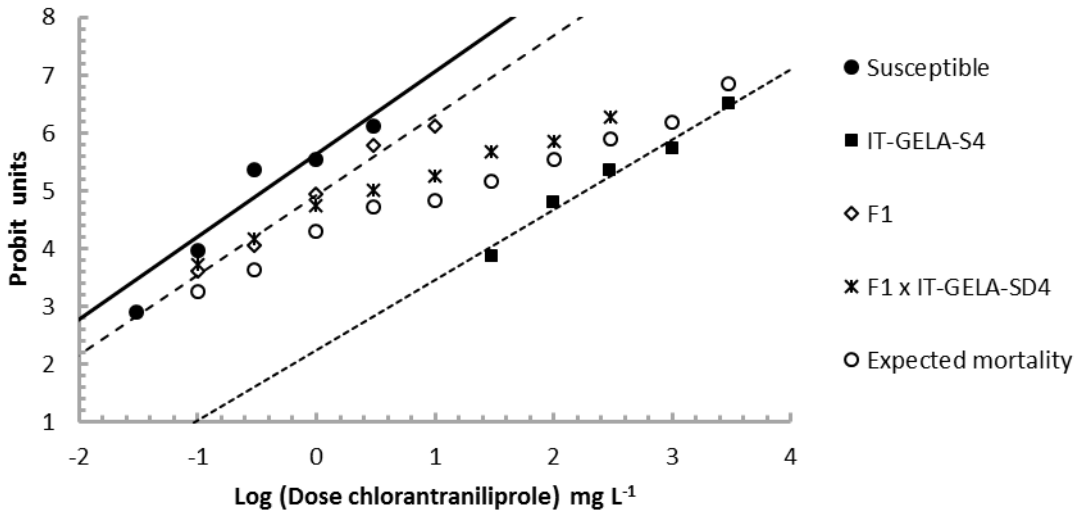

Fig. 1. Log-dose probit-mortality lines for chlorantraniliprole tested against 2nd instar larvae of *Tuta absoluta*. Probit lines for strains GR-LAB (susceptible), IT-GELA-SD4, F1-a hybrids (GR-LAB♀ x IT-GELA-SD4♂), F1-a♀ x IT-GELA-SD4♂ backcross and its calculated theoretical backcross based on monogenic resistance. Data obtained for F1 hybrids resulting from GR-LAB♂ x IT-GELA-SD4♀ crosses are not shown as they were not significantly different.

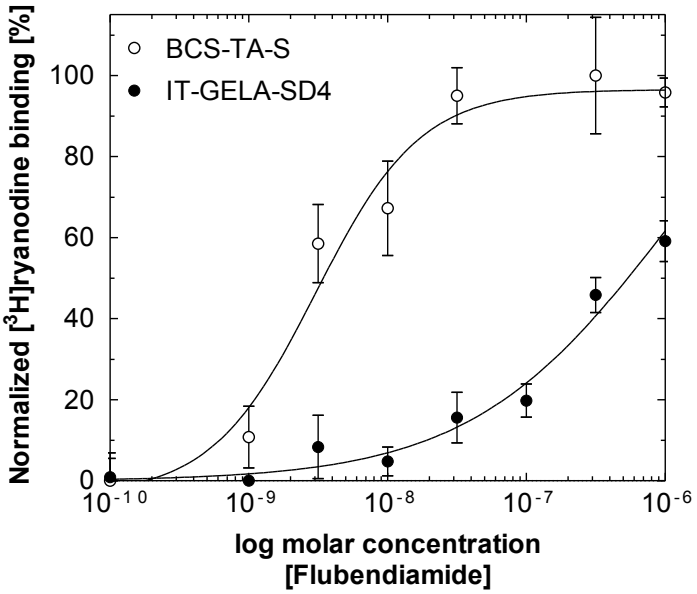

Fig. 2. Radioligand binding assays using thoracic microsomal membrane preparations of diamide susceptible (BCS-TA-S) and resistant (IT-GELA-SD4) *Tuta absoluta* strains. Relative increase of [^{3}H]-ryanodine binding as a function of diamide insecticide concentration reveal functional implications of the detected G4903E/V and I4746M RyR mutation in strain IT-GELA-SD4 for diamide binding. Data are mean values ± SD (n=4).

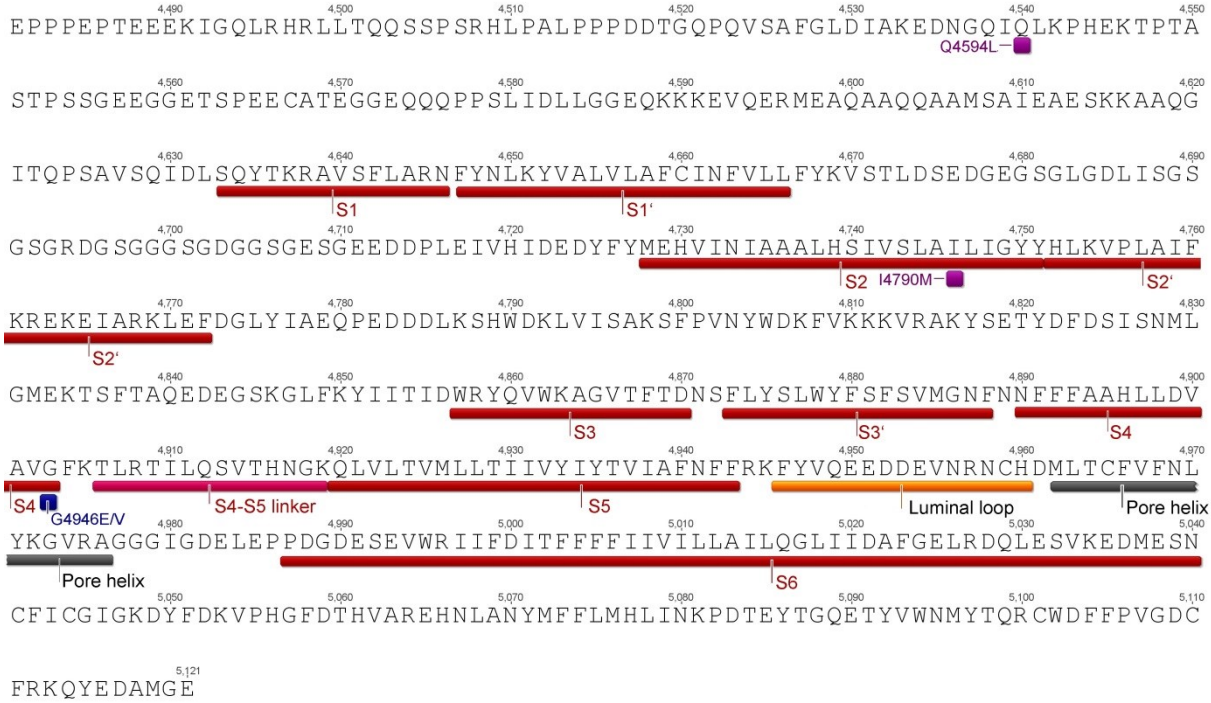

Fig. 3. Partial amino acid sequence of the C-terminal transmembrane domain of the RyR from *Tuta absoluta*. The positions of the transmembrane-spanning domains S1 to S6 are designated with red bars and based on those recently predicted for the diamondback moth RyR (Steinbach et al., 2015). The highly conserved pore helix and the luminal loop are designated with grey and orange bars, respectively. The S4-S5 linker region (marked in magenta) is close to the amino acid residue G4903, which corresponds to the mutation site recently shown to confer diamide resistance in diamondback moth (G4946E). Two further mutation sites, I4790M and Q4594L recently linked to diamide resistance in diamondback moth correspond to amino acid residues I4746 and Q4540, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

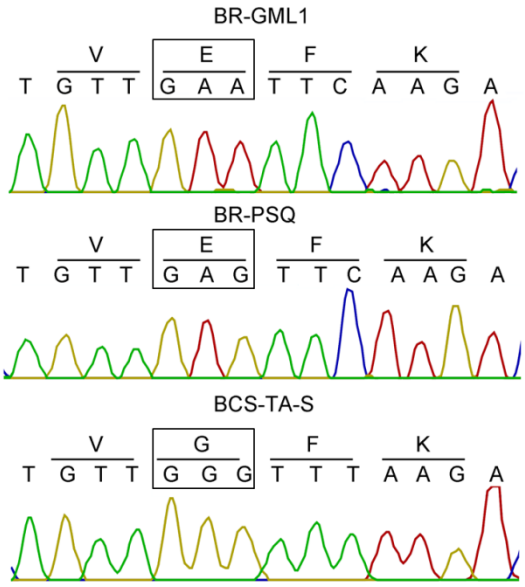

Fig. 4. Sanger sequencing revealed mutations in the *RyR* gene in Brazilian *Tuta absoluta* field strains. The lower nucleotide sequence obtained from a diamide susceptible laboratory strain (BCS-TA-S) shows the reference sequence GGG (boxed) coding for a glycine at position 4903 in the *T. absoluta* RyR. The upper chromatograms and corresponding sequences clearly show the presence of different single nucleotide polymorphisms (boxed) in diamide-resistant Brazilian field strains BR-GML1 (GAA) and BR-PSQ (GAG), resulting in the amino acid substitution G4903E, which corresponds to the mutation site recently shown to confer diamide target-site resistance in diamondback moth (G4946E).