

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

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36 **Abstract**

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

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62

63 **Keywords**

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

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68 **1. Introduction**

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

79 Diamide insecticides act as conformation sensitive activators of the insect ryanodine
80 receptor (RyR), a large (homo)tetrameric calcium-channel located in the sarco- and
81 endoplasmic reticulum in neuromuscular tissues (Ebbinghaus-Kintscher et al., 2006; Cordova
82 et al., 2006; Lümmen et al., 2007; Sattelle et al., 2008). The endogenous activation of RyRs is
83 mediated by calcium influx, driven by voltage-gated calcium channels upon depolarization of
84 the cell membrane (Lümmen 2013). The symptomology of poisoning after diamide
85 application involves muscle contraction, paralysis and eventually death (Tohnishi et al.,
86 2005; Cordova et al., 2006). In contrast to mammals which possess three *RyR* genes (Rossi
87 and Sorrentino 2002), insects encode a single *RyR* gene with an open reading frame (ORF) of
88 >15000 nucleotides translated into a RyR protomer with a molecular weight of more than
89 5000 kDa, as first described for *Drosophila melanogaster* (Takeshima et al., 1994). RyRs were
90 shown to be composed of six helical transmembrane spanning domains at the C-terminal
91 end containing the calcium ion-conducting pore, and a large N-terminal cytosolic domain
92 (Lümmen 2013). Recently Yan et al., (2015) published a rabbit RyR1 structure determined by
93 single-particle electron cryomicroscopy which resolved a large portion of the
94 homotetrameric channel protein (Yan et al., 2015).

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

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

103 Diamide insecticides are used globally straight and in mixtures for foliar, drench and seed
104 treatment applications in a broad range of agricultural and horticultural cropping systems.
105 The popularity and widespread uptake of diamides has increased selection pressure for the
106 evolution of resistance, particularly in the case of lepidopteran pests (Teixeira and Andaloro
107 2013). The global status of diamide resistance issues in lepidopteran pests was recently
108 reviewed by Nauen and Steinbach (2016). High levels of resistance compromising the
109 efficacy of diamides at recommended field rates were reported in very few species. The first
110 serious cases of resistance were described for diamondback moth strains collected in the
111 Philippines and Thailand (Trocza et al., 2012), followed by China (Wang and Wu 2012; Wang
112 et al., 2013; Gong et al., 2014), Brazil (Ribeiro et al., 2014), Taiwan, India, USA, Japan, Korea
113 and Vietnam (Steinbach et al., 2015). The underlying basis of resistance in a number of field-
114 collected diamondback moth strains appears to be largely due to target-site mutations in the
115 transmembrane domain of the RyR (Trocza et al., 2012; Guo et al., 2014; Steinbach et al.,
116 2015). In particular the G4946E mutation - located at the interface between helix S4 and the
117 S4-S5 linker - was functionally linked to high levels of diamide resistance (Steinbach et al.,
118 2015; Trocza et al., 2015). The functional significance of other mutation sites such as
119 I4790M and Q4594L, present in recently collected Chinese *P. xylostella* populations, is less
120 clear (Guo et al., 2014). Nevertheless, homology modeling of the *Plutella* RyR based on the
121 structure of rabbit RyR1 (Yan et al., 2015), revealed that the I4790M mutation in helix S2 is
122 in quite close proximity to G4946E, suggesting a potential functional role in diamide binding
123 (Steinbach et al., 2015). Apart from diamondback moth, high levels of diamide cross-
124 resistance under applied conditions were only described in two other lepidopteran pest
125 species, the smaller tea tortrix, *Adoxophyes honmai* (Uchiyama and Ozawa 2014) and the
126 tomato the leafminer, *Tuta absoluta* (Meyrick) (Roditakis et al., 2015, Silva et al., 2016).

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

135 develop resistance to multiple classes of insecticide (Siqueira et al., 2000, 2001; Silva et al.,
136 2011; Haddi et al., 2012; Silva et al., 2016). Baseline susceptibility studies showed diamide
137 insecticides are highly effective against *T. absoluta* (Roditakis et al., 2013; Campos et al.,
138 2015). However, recently, *T. absoluta* collected from Italian greenhouse tomatoes exhibited
139 resistance to diamides of more than 1000-fold when compared to a susceptible reference
140 strain (Roditakis et al., 2015). Despite the reliance on diamides for control of *T. absoluta* in
141 many countries, the molecular mechanisms conferring diamide resistance in *T. absoluta* and
142 implications for resistance management remain unknown. In the present study we
143 investigated the molecular basis of diamide resistance in *T. absoluta* populations collected in
144 both Europe as well as South America and describe target-site resistance to this insecticide
145 class in *T. absoluta* for the first time.

146

147 **2. Materials and methods**

148 *2.1 Insect strains*

149 The *T. absoluta* field strains used in this study were collected from infested tomato (*Solanum*
150 *lycopersicum* L.) crops in Italy, Greece, Spain and Brazil between 2014 and 2015. A number
151 of tomato leaves infested with *T. absoluta* larvae were collected in large plastic bags and
152 transferred to the respective laboratory for experimentation. Details for each collected
153 strain are provided in Table 1. At least 300 individuals were collected in each location and
154 the larvae were allowed to develop on 3-6 week old potted tomato plants. All populations
155 were maintained in insect cages at 26 ± 1 °C, 65 % RH and 16 h light : 8 h dark photoperiod.
156 The susceptible reference strains (GR-Lab, ES-Sus and BCS-TA-S) were maintained for several
157 years under laboratory conditions without any exposure to insecticides. Strain IT-GELA-SD4
158 was obtained after four sequential selection cycles of strain IT-GELA-14-1 with the
159 insecticide chlorantraniliprole using foliar applied doses of 100 mg L⁻¹ (first selection) and
160 300 mg L⁻¹ (subsequent selections). At least 1000 2nd instar larvae were used for each
161 selection cycle.

162

163 *2.2 Chemicals and insecticides*

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

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

169

170 *2.3 Insect bioassays*

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

185 Lethal concentration values for 50 % mortality (LC₅₀) and 95 % confidence limits (CL 95 %)
186 were obtained by probit analysis using the software packages PriProbit 3.4 (Sakuma, 1998)
187 or Polo Plus (LeOra software, USA). LC₅₀-values were considered significantly different when
188 their 95 % confidence limits did not overlap. Percentage mortality values generated in
189 bioassays was corrected using Abbott's formula (Abbott, 1925).

190

191 *2.4 Synergist bioassays*

192 Synergist bioassays were performed following IRAC method 022 as described above with
193 strain IT-GELA-SD4 (selected with chlorantraniliprole). Two hours prior to bioassay, larvae
194 were exposed to sublethal doses (≤ LC₀₅) of different synergists via contact on fresh dried
195 residues in coated glass vials (30 ml volume). Synergists used were piperonyl butoxide (PBO,
196 Sigma, UK), S,S,S tributyl phosphorotrithioate (DEF, Sigma, UK) and diethyl maleate (DEM,
197 Sigma, UK), known to inhibit cytochrome P450 monooxygenases, esterases and glutathione
198 S-transferases, respectively. For vial coating 300 µl of acetonic solutions of PBO (0.1 g L⁻¹),
199 DEF (0.1 g L⁻¹) or DEM (0.3 g L⁻¹) were added into each vial. Afterwards vials were placed

200 horizontally on rotating metal rods for 1 h. After rotation the vials were allowed to dry for
201 another hour before adding the larvae. The synergistic ratio was calculated by dividing the
202 calculated LC₅₀-value of synergist-exposed larvae by the LC₅₀-value of larvae not exposed to
203 synergists.

204

205 *2.5 Genetics of diamide resistance*

206 *Tuta absoluta* larvae of strains GR-Lab and IT-GELA-SD4 were allowed to develop and pupate
207 under controlled conditions (see above) in small plastic rearing cages. Pupae were
208 subsequently collected and sexes separated based on external morphology (Solomon, 1962).
209 Subsequently they were placed individually in transparent glass tubes where the emergence
210 of adults was checked daily. The sex of adult moths was checked again for confirmation of
211 the initial classification of pupae (Coelho and França, 1987). Subsequently 100 virgin females
212 of strain IT-GELA-SD4 were crossed with 100 males of the susceptible strain GR-Lab and *vice*
213 *versa*. Second instar larvae of the F1 generation of reciprocal crosses were subsequently
214 bioassayed with chlorantraniliprole to determine LC₅₀-values as described above. The degree
215 of dominance was calculated using the formula $D = (2X_2 - X_1 - X_3) / (X_1 - X_3)$ (Stone, 1968). The
216 values X₁, X₂ and X₃ are the log(LC₅₀) of the resistant strain IT-GELA-SD4, the F1 generation
217 and the susceptible strain GR-Lab, respectively. The F1 generation of the reciprocal crosses
218 was subsequently back-crossed with the parental IT-GELA-SD4 strain to check for monogenic
219 resistance. Expected mortality of the backcross generation (F2) under a monogenic model
220 was calculated using the following formula: expected % mortality F2 at [c] = 0.5 x (%
221 mortality of F1 at [c] + % mortality of strain IT-GELA-SD4 at [c]), where [c] refers to the
222 respective concentration of chlorantraniliprole.

223

224 *2.6 Biochemical assays*

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

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

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

248 P450 activity was determined by the O-deethylation of 7-ethoxycoumarin (7-EC) using a
249 protocol adapted for *in vivo* analysis in microplates (Reyes et al., 2012). Briefly twenty 3rd
250 instar larvae were dissected individually placed in the wells of black microplates. Each well
251 contained 0.4 mM 7-EC in 0.1 ml of 50 mM HEPES buffer, pH 7.0. Ten wells without
252 dissected insects were used as controls. After 4 h incubation at 30 $^{\circ}\text{C}$, the enzymatic reaction
253 was stopped by adding 0.1 ml 0.1 mM glycine buffer (pH 10.4):ethanol (v/v). Umbelliferone
254 fluorescence was measured at 465 nm while exciting at 390 nm. A standard curve was
255 established using umbelliferone and cytochrome P450 activity was expressed as pg product
256 $\text{insect}^{-1} \text{ min}^{-1}$.

257

258 2.7 Radioligand binding studies

259 *Tuta absoluta* adults of strains BCS-TA-S and IT-GELA-SD4 were flash-frozen in liquid
260 nitrogen, stored at -80°C and collected over many generations to get the appropriate
261 biomass necessary for conducting radioligand binding assays. Thoracic
262 endoplasmic/sarcoplasmic reticulum membranes were prepared as described earlier
263 (Steinbach et al., 2015). Radioligand binding assays were performed using 50 μg membrane
264 protein per assay. Enhancement of ryanodine binding was measured using 4.0 nM
265 [^3H]ryanodine as a function of increased diamide insecticide concentration as described
266 elsewhere (Steinbach et al., 2015). Equilibrium binding parameters were calculated using the
267 software package GraphPad Prism 5 (GraphPad Inc.).

268 *2.8 Identification and sequence verification of the T. absoluta RyR*

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

284

285 *2.9 Pyrosequencing of PCR amplified RyR cDNA of T. absoluta for genotyping*

286 Individuals of different strains of *T. absoluta* were genotyped for the recently described
287 diamondback moth RyR mutations G4946E (Trocza et al., 2012; Steinbach et al., 2015) and
288 I4790M (Guo et al., 2014). Genomic DNA was extracted from individual larvae using the
289 DNAdvance Tissue Kit (Agencourt) according to the supplier's recommended protocol.
290 Primer pairs were designed based on the obtained RyR full-length cDNA sequence (GenBank
291 no. KX519762). A short gene fragment of 228 bp and 190 bp for G4946E and I4790M
292 genotyping, respectively was amplified by PCR from 50 ng aliquots of *T. absoluta* gDNA using
293 the primer pairs Ta_I4790-F, Ta_I4790-R-btn and Ta_G4946-F-btn, Ta_G4946-R, respectively
294 (Table S2). The pyrosequencing protocol for the detection of I4790M (position 4746 in *Tuta*
295 RyR) comprised 40 PCR cycles with 0.5 µM forward and biotinylated reverse primer in 30 µl
296 reaction mixtures containing 1 × Taq enzyme reaction mix (JumpStart™ Taq ReadyMix™,
297 Sigma Aldrich) and cycling conditions of 95 °C for 3 min, followed by 40 cycles of 95 °C for
298 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
299 same protocol was used for genotyping G4946E (position 4903 in *Tuta* RyR) with a few
300 modifications: the forward primer was biotinylated rather than the reverse primer and the
301 temperature for primer annealing was set to 53 °C for 30 s. The single strand DNA required

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

307

308 *2.10 Sanger sequencing*

309 Sanger sequencing was performed in order to partially sequence the RyR domain of Brazilian
310 strains BR-GML1 and BR-PSQ that failed to produce results in G4946E-pyrosequencing
311 diagnostics. Degenerate primers, Ta_SangerSeq-F and Ta_SangerSeq-R (Table S2), were
312 designed based on a multiple nucleotide alignment of *T. absoluta* (GenBank no. KX519762),
313 *P. xylostella* (JN801028), *B. mori* (XM_004924859) and *C. suppressalis* (JX082287). A 285 bp
314 fragment of *T. absoluta* RyR was amplified from gDNA by PCR using Q5® High-Fidelity DNA
315 Polymerase 2x Master Mix according to the manufacturer's instruction (New England
316 BioLabs Inc., USA). The cycling conditions were 98 °C for 30 s, followed by 35 cycles of 98 °C
317 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
318 purified PCR products were sequenced by Eurofins Genomics (Ebersberg, Germany).

319

320 **3. Results**

321 *3.1 Leaf-dip bioassays and stability of resistance*

322 Three diamide susceptible laboratory reference strains, five field-collected strains from Italy,
323 Greece, Spain and Brazil, and one chlorantraniliprole-selected strain originating from Italy
324 (IT-GELA-SD4) were included in this study. The obtained LC₅₀-values for chlorantraniliprole in
325 the susceptible reference strains GR-Lab, ES-Sus and BCS-TA-S differed less than 2-fold
326 (between 0.18 and 0.31 mg L⁻¹; Table 2). Resistance ratios for chlorantraniliprole in field
327 collected strains varied between 8 (strain ES-MUR-14, Spain) and >3000 (strain BR-PSQ,
328 Brazil). Strain IT-GELA-SD4 selected with chlorantraniliprole for four generations exhibited a
329 resistance ratio 4-fold higher than the parental strain IT-GELA-14-1 (Table 2). Strain IT-GELA-
330 SD4 was mass reared and chosen for more detailed genetic and molecular studies on
331 diamide resistance in *T. absoluta*. Whereas parental strain IT-GELA-14-1 lost some of its
332 resistance over time, we observed a stable diamide resistance of selected strain IT-GELA-SD4
333 over a period of 10 months (data not shown).

334

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 LC₅₀-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 ($\chi^2 = 28.3$, $df = 8$, $P < 0.05$), 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

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

373

374 *3.4 Radioligand binding studies*

375 Comparative binding studies using thoracic membrane preparations of strains BCS-TA-S and
376 IT-GELA-SD4 were carried out with the diamide insecticide flubendiamide, known to act, like
377 chlorantraniliprole, as a positive allosteric activator of [³H]ryanodine binding to lepidopteran
378 RyRs (Steinbach et al., 2015). Strain IT-GELA-SD4 shows high levels of resistance (≥ 1000 -fold)
379 against both flubendiamide and chlorantraniliprole when compared to the susceptible
380 reference strains in this study (Table 4). Radioligand binding studies using thoracic endo-
381 /sarcoplasmic membranes of adults of strain BCS-TA-S and IT-GELA-SD4 revealed a relative
382 increase of [³H]ryanodine binding as a function of flubendiamide concentration at an EC₅₀ of
383 3.03 ± 1.45 nM and >1000 nM, respectively (Fig. 2). This equates to RyR target-site
384 insensitivity of >300 -fold to flubendiamide in membrane preparations of strain IT-GELA-SD4
385 when compared to membranes isolated from the diamide susceptible strain BCS-TA-S.

386

387 *3.5 Cloning, sequencing and characterisation of the *T. absoluta* RyR*

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

403 had the 'susceptible' amino acid at position 4903 (corresponding to G4946 in *P. xylostella*),
404 4746 (I4790 in *P. xylostella*), 4540 (Q4594 in *P. xylostella*) but an aspartic acid (D) at position
405 1339 (E1338 in *P. xylostella*), suggesting the E1338D substitution first reported in *P.*
406 *xylostella* is not a *bona fide* resistance mutation.

407

408 3.6 Genotyping for RyR target-site mutations by pyrosequencing

409 Individuals of a number of strains included in this study were analyzed for the presence and
410 frequency of mutations at positions G4903 and I4746, previously associated with RyR target-
411 site resistance against diamides in *P. xylostella* at the corresponding positions (Steinbach et
412 al., 2015; Guo et al., 2014). A partial amino acid sequence of the *T. absoluta* RyR C-terminal
413 transmembrane domain indicating potential mutation sites (based on diamide resistant *P.*
414 *xylostella* RyR) is shown in Fig. 3. The pyrosequencing assay using genomic DNA of *T.*
415 *absoluta* worked well for all strains and both mutation sites (Figs. S1 and S2), except for
416 G4903 of the two Brazilian field strains BR-GML1 and BR-PSQ, due to their polymorphism in
417 the nucleotide sequence of the *RyR* regions selected for primer design as revealed by Sanger
418 sequencing (Fig. S3). However Sanger sequencing of partial *RyR* sequences of both Brazilian
419 field strains highly resistant to diamides revealed the presence of the G4903E mutation (Fig.
420 4) at a frequency of 100%, albeit only a relatively small number of samples was analysed
421 (Table 7). Furthermore, pyrosequencing of DNA samples of these strains confirmed the
422 presence of the mutations I4746M, and a previously un-reported amino acid substitution,
423 I4746T, at low frequency (Table 7). The diamide susceptible strains BCS-TA-S, GR-Lab and ES-
424 Sus were all wildtype homozygous at positions G4903 and I4746 (Table 7). The laboratory
425 selected strain IT-GELA-SD4 was found to contain a mixture of individuals of different
426 genotypes none of which had the wildtype G4903 amino acid residue, but rather were
427 homozygous for G4903E, or G4903V, a novel substitution not yet reported at this position. In
428 addition the IT-GELA-SD4 strain also carried the I4746M mutation at high frequency (80 % of
429 individuals tested were homozygous for this mutation). Strain GR-IER-15-2 which exhibited
430 55-fold resistance to chlorantraniliprole exhibited 10 % and 20 % of sequenced individuals
431 homozygous for G4903E and G4903V, respectively. Most individuals were wildtype I4746,
432 though we detected a low frequency of I4746M heterozygotes. Strain ES-MUR-14 collected
433 in Spain in 2014, which showed very low resistance to chlorantraniliprole (8-fold), was
434 exclusively wild-type at position 4903 but carried a low frequency (33%) of individuals
435 heterozygous for I4746M.

436

437

438 **4. Discussion**

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

456 In this study we also investigated field-collected *T. absoluta* populations showing low to high
457 levels of diamide resistance sampled outside of Italy, specifically Greece, Spain and Brazil.
458 High levels of diamide resistance were also found in Brazilian populations, whereas
459 moderate and low levels of resistance were detected in populations collected in Greece and
460 Spain in 2015 and 2014, respectively. We went on to select one of the Italian strains
461 collected in 2014 (IT-GELA-14-1) for additional generations with chlorantraniliprole and
462 investigated the molecular basis of resistance in detail. Our study was informed by recent
463 work conducted on diamide resistant diamondback moth populations, which revealed amino
464 acid substitutions in the *Plutella* RyR transmembrane domain that confer target-site
465 resistance (Troczka et al., 2012, 2015; Guo et al., 2014; Gong et al., 2014; Steinbach et al.,
466 2015). We isolated the full-length *T. absoluta* RyR gene and focused on the C-terminal
467 domain spanning approx. 450 amino acids and containing six highly conserved
468 transmembrane segments previously shown to play a major role in diamide insecticide
469 binding (Kato et al., 2009). It was recently shown that the G4946E mutation in the
470 diamondback moth RyR has strong functional implications for both the direct binding of

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

476 Here we have discovered the corresponding mutation, G4903E, in the RyR of the diamide
477 selected *T. absoluta* strain IT-GELA-SD4. Furthermore, we also identified an alternative
478 amino acid substitution at the same position, G4903V, that has not been previously
479 described. Both precisely correspond to the G4946E mutation site functionally proven to
480 alter diamide binding in the RyR's of diamide resistance *Plutella*. As previously demonstrated
481 for *P. xylostella* we confirmed the strong impact of mutations at this site using radioligand
482 binding studies on thoracic membrane preparations of tomato leafminer adults of strain IT-
483 GELA-SD4 in comparison to a susceptible reference strain (BCS-TA-S). This binding assay is
484 considered to be a reliable and sensitive indicator of diamide action on insect RyR's
485 (Lümmen et al., 2007; Qi et al., 2014). Flubendiamide allosterically increased [³H]ryanodine
486 binding in RyR preparations of the susceptible strain BCS-TA-S at low nanomolar
487 concentrations (EC₅₀-value: 3 nM), i.e. in the range of EC₅₀-values recently reported for other
488 lepidopteran membrane preparations. The EC₅₀-value for [³H]ryanodine binding stimulation
489 by flubendiamide in strain IT-GELA-SD4 was at least 300-fold higher, thus confirming
490 insensitivity and the relevance of the RyR G-to-E (or -V) target-site mutation in a species
491 other than diamondback moth.

492 The IT-GELA-SD4 strain also carries an additional mutation, I4746M, at very high frequency.
493 This corresponds to the I4790M mutation recently described in the RyR of diamide resistant
494 populations of diamondback moth from China (Guo et al., 2014). In our study this mutation
495 was present in combination with G4946E so the impact of I4790M on diamide binding and
496 its contribution to resistance remains unclear. Nevertheless a recent study using homology
497 modelling of the *Plutella* RyR (Steinbach et al., 2015), based on a recently published
498 vertebrate RyR1 structure determined by single-particle electron cryomicroscopy (Yan et al.,
499 2015), has suggested that both sites may contribute to diamide binding. The model suggests
500 that the G4946E mutation is located at the interface between helix S4 and the S4-S5 linker,
501 thought to have a critical role in RyR gating by impacting the movement of pore-associated
502 helices (Ramachandran et al., 2013). The second mutation I4790M was shown to be located
503 in helix S2 in close proximity to G4946E. Whereas G4946 is highly conserved in all insect
504 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 Andoloro, 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 Andoloro,
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.

558

559

560 **Acknowledgements**

561 The work of Hellenic Agricultural Organisation - 'Demeter' was partially supported by an
562 ARIMnet2 StomP grand to A.T and E.R. The Universidad Politécnica de Cartagena group
563 would like to thank for partial financial support the Ministerio de Economía y Competitividad
564 of Spain and FEDER (AGL2011-25164). Lidia García-Vidal holds a grant from the MECD
565 (FPU13/01528). The *Tuta absoluta* strain from Gela, Sicily was collected under the frame a
566 resistance monitoring program established among the Hellenic Agricultural Organisation -
567 'Demeter' and DuPont De Nemurs (data published in 2015). Finally, the Hellenic Agricultural
568 Organisation - 'Demeter' would like to thank Fytochem S.A., Neo Mirtos, Ierapetra for
569 supplies of plant material.

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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

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

803 ^a Resistance ratio (RR) calculated is based on strain GR-Lab
 804 ^b Resistance ratio (RR) calculated is based on strain ES-Sus
 805 ^c Resistance ratio (RR) calculated is based on strain BCS-TA-S
 806 ^d Confidence limits 95%

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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

881 **Table 4**
 882 Log-dose probit-mortality data for chlorantraniliprole (CPR) and flubendiamide (FLB) tested
 883 in combination with synergists against 2nd instar larvae of *Tuta absoluta*
 884

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

885 ^a Confidence limits 95%

886 ^b Synergistic ratio (LC₅₀ without synergist divided by LC₅₀ + synergist)

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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 \pm 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 \pm 10	265 \pm 13	0.258 \pm 0.058	16.0 \pm 2.65
IT-GELA-14-1	166 \pm 26	251 \pm 6.7	0.198 \pm 0.036	20.1 \pm 2.45
IT-GELA-SD4	141 \pm 4.1	249 \pm 2.9	0.217 \pm 0.022	20.9 \pm 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 μ mol 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)

968 **Table 6**
 969 Comparison of insect ryanodine receptor protein sequences (given in % identity)
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	<i>Tuta absoluta</i>	<i>Cnaphalocrocis medinalis</i>	<i>Helicoverpa armigera</i>	<i>Plutella xylostella</i>	<i>Tribolium castaneum</i>	<i>Drosophila melanogaster</i>	<i>Acyrtosiphon pisum</i>
<i>T. absoluta</i>	---						
<i>C. medinalis</i>	93.1	---					
<i>H. armigera</i>	92.7	94.3	---				
<i>P. xylostella</i>	90.7	92.2	92.2	---			
<i>T. castaneum</i>	81.8	82.6	82.0	81.0	---		
<i>D. melanogaster</i>	77.9	78.6	78.3	77.5	77.7	---	
<i>A. pisum</i>	77.3	77.9	77.2	76.5	79.1	74.5	---

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1016 **Table 7**

1017 Genotyping by pyrosequencing of individuals of *Tuta absoluta* (n = 8-12) for the presence of
 1018 RyR mutations in the C-terminal transmembrane domain at amino acid positions G4903 and
 1019 I4746 corresponding to G4946 and I4790 in diamondback moth. All three diamide
 1020 susceptible strains (BCS-TA-S, GR-Lab and ES-Sus) were homozygous wildtype (SS) at the
 1021 respective positions.

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Strain	G4903			V4903		E4903		I4746			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

1023 ^a Frequency estimated by Sanger sequencing (pooled n ≥ 4)

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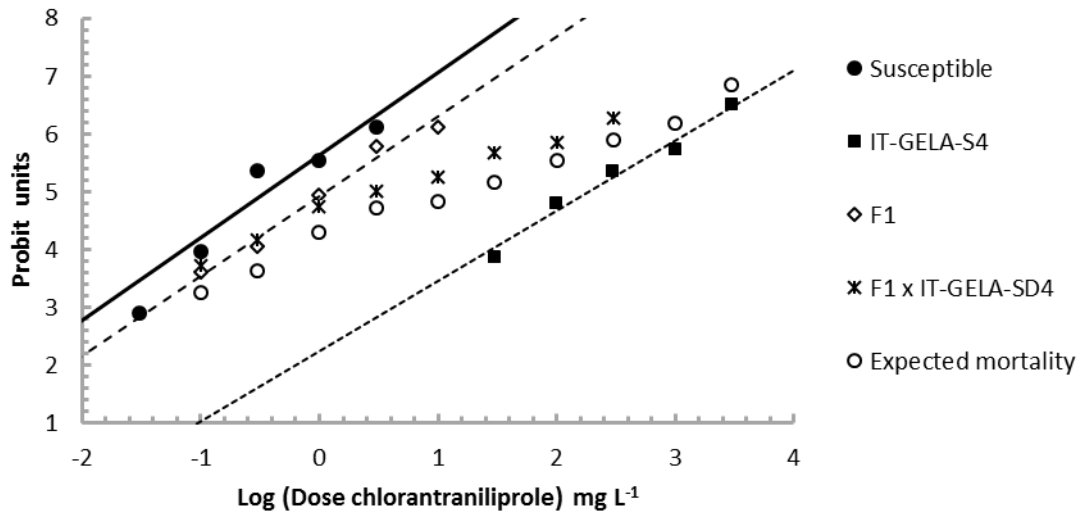
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1054 **Fig. 1. Log-dose probit-mortality lines for chlorantraniliprole tested against 2nd**
 1055 **instar larvae of *Tuta absoluta*.** Probit lines for strains GR-LAB (susceptible), IT-GELA-
 1056 SD4, F1-a hybrids (GR-LAB♀ x IT-GELA-SD4♂), F1-a♀ x IT-GELA-SD4♂ backcross and
 1057 its calculated theoretical backcross based on monogenic resistance. Data obtained
 1058 for F1 hybrids resulting from GR-LAB♂ x IT-GELA-SD4♀ crosses are not shown as
 1059 they were not significantly different.

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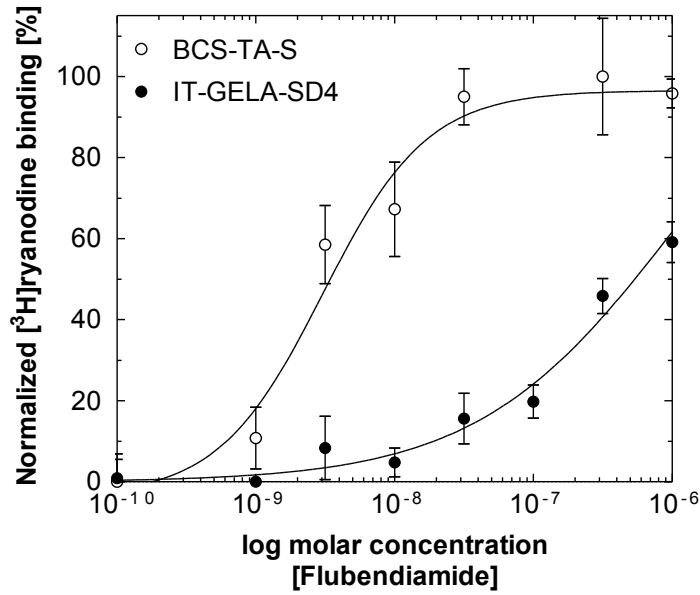
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1076 **Fig. 2. Radioligand binding assays using thoracic microsomal membrane**
 1077 **preparations of diamide susceptible (BCS-TA-S) and resistant (IT-GELA-SD4) *Tuta***
 1078 ***absoluta* strains.** Relative increase of [³H]-ryanodine binding as a function of diamide
 1079 insecticide concentration reveal functional implications of the detected G4903E/V
 1080 and I4746M RyR mutation in strain IT-GELA-SD4 for diamide binding. Data are mean
 1081 values ± SD (n=4).

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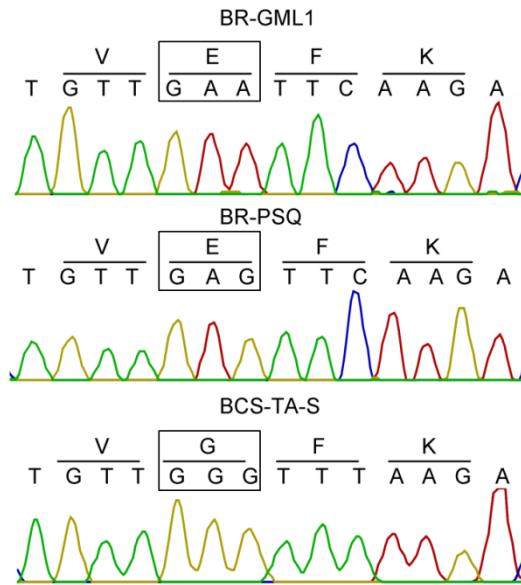
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1097 **Fig. 3. Partial amino acid sequence of the C-terminal transmembrane domain of**
 1098 **the RyR from *Tuta absoluta*.** The positions of the transmembrane-spanning domains
 1099 S1 to S6 are designated with red bars and based on those recently predicted for the
 1100 diamondback moth RyR (Steinbach et al., 2015). The highly conserved pore helix and
 1101 the luminal loop are designated with grey and orange bars, respectively. The S4-S5
 1102 linker region (marked in magenta) is close to the amino acid residue G4903, which
 1103 corresponds to the mutation site recently shown to confer diamide resistance in
 1104 diamondback moth (G4946E). Two further mutation sites, I4790M and Q4594L
 1105 recently linked to diamide resistance in diamondback moth correspond to amino
 1106 acid residues I4746 and Q4540, respectively. (For interpretation of the references to
 1107 color in this figure legend, the reader is referred to the web version of this article.)

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1112 **Fig. 4. Sanger sequencing revealed mutations in the *RyR* gene in Brazilian *Tuta***
 1113 ***absoluta* field strains.** The lower nucleotide sequence obtained from a diamide
 1114 susceptible laboratory strain (BCS-TA-S) shows the reference sequence GGG (boxed)
 1115 coding for a glycine at position 4903 in the *T. absoluta* *RyR*. The upper
 1116 chromatograms and corresponding sequences clearly show the presence of different
 1117 single nucleotide polymorphisms (boxed) in diamide-resistant Brazilian field strains
 1118 BR-GML1 (GAA) and BR-PSQ (GAG), resulting in the amino acid substitution G4903E,
 1119 which corresponds to the mutation site recently shown to confer diamide target-site
 1120 resistance in diamondback moth (G4946E).

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