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Overexpression of the UDP-glycosyltransferase UGT34A23 confers resistance to the diamide insecticide chlorantraniliprole in the tomato leafminer, *Tuta absoluta*

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A B S T R A C T

The tomato leafminer, *Tuta absoluta*, is an invasive crop pest that has evolved resistance to many of the insecticides used for its control. To facilitate the investigation of the underpinning mechanisms of resistance in this species we generated a contiguous genome assembly using long-read sequencing data. We leveraged this genomic resource to investigate the genetic basis of resistance to the diamide insecticide chlorantraniliprole in Spanish strains of *T. absoluta* that exhibit high levels of resistance to this insecticide. Transcriptomic analyses revealed that, in these strains, resistance is not associated with previously reported target-site mutations in the diamide target-site, the ryanodine receptor, but rather is associated with the marked overexpression (20- to >100-fold) of a gene encoding a UDP-glycosyltransferase (UGT). Functional expression of this UGT, *UGT34A23*, via ectopic expression in *Drosophila melanogaster* demonstrated that it confers strong and significant resistance *in vivo*. The genomic resources generated in this study provide a powerful resource for further research on *T. absoluta*. Our findings on the mechanisms underpinning resistance to chlorantraniliprole will inform the development of sustainable management strategies for this important pest.

1. Introduction

The tomato leafminer, *T. absoluta*, is an economically important insect pest of tomato crops worldwide. This invasive species spread from South America to Spain in 2006, and subsequently radiated throughout Europe, Africa and the Middle East (Biondi et al., 2018). In some tomato producing regions, *T. absoluta* has resulted in the destruction of whole crops, in some cases inflating market prices by up to 400% (Toesland, 2016). The control of *T. absoluta* has relied heavily on the use of chemical insecticides leading to the evolution of resistance to many of the insecticides used for control (Guedes et al., 2019).

The toxicodynamic process of synthetic insecticides often involves interactions with target receptors in the insect nervous system. Their effects are most frequently overcome through the evolution of one or both of two mechanisms, target-site resistance and metabolic resistance. In the case of diamide insecticides, which act on the insect ryanodine receptor (RyR), both mechanisms have been implicated in resistant insects (Guedes et al., 2019; Richardson et al., 2020). In the case of

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T. absoluta, target-site resistance mutations in the RyR gene (at positions G4946 and I4790) have been identified that maintain receptor function whilst altering its structure/electrochemical profile sufficiently to minimise the toxicological effects of diamide insecticides (Douris et al., 2017; Grant et al., 2019; Guedes et al., 2019; Roditakis et al., 2017). This mechanism has been demonstrated for *T. absoluta* populations from Italy, Greece, Spain and Brazil (Roditakis et al., 2017). Diamide target-site mutations have also been identified at low frequencies in UK populations showing moderate (9.5-fold) tolerance to the diamide chlorantraniliprole (Grant et al., 2019). Furthermore, these mutations were rapidly selected to fixation in the laboratory resulting in a strain with potent (>5000-fold) resistance (Grant et al., 2019). A second strain from North Yorkshire also showed tolerance relative to a reference susceptible strain (TA1), however, selection failed to elevate resistance in this population (Grant et al., 2019). In this case, analysis of the RyR gene sequence of this strain before and after selection identified no known target-site mutations. Furthermore, an absence of target-site resistance was also observed in European populations with tolerance to the compound (Grant et al., 2019).

The identification of diamide resistant *T. absoluta* strains that do not carry known target-site mutations suggests that other mechanisms may underpin their resistance, such as enhanced metabolic detoxification. Metabolic resistance results from alterations in metabolic systems that enhance detoxification or sequestration of toxins, reducing their cellular concentrations and thus minimising interactions with the target-site (Li et al., 2007). The detoxification proteins most commonly involved in metabolic insecticide resistance fall into one of five major families: Cytochrome P450s (P450s), esterases (ESTs), uridine 5′-diphosphoglycosyltransferase (UGTs), glutathione S-transferases (GSTs) and ATP-binding cassette transporters (ABC transporters) (Li et al., 2007; Perry et al., 2011). These can function independently, but most often in a three phase, step-wise process typically comprising modification, conjugation, and excretion, and metabolic resistance is often associated with the increased expression of one or more genes encoding these enzymes (Li et al., 2007; Perry et al., 2011).

Previous studies of *T. absoluta* have implicated metabolic detoxification in resistance to pesticides in the absence of target site resistance (Barati et al., 2018; Campos et al., 2015). For example, fluorescence and absorbance assays were used to show elevated P450 and EST activity in spinosad resistant populations from Chile (Reyes et al., 2012), elevated levels of CYPs and GSTs in pyrethroid resistant populations from Brazil (Silva et al., 2015), and enhanced activity of CYPs and ESTs in organophosphate resistant Iranian populations (Barati et al., 2018). While, to date, metabolic mechanisms of resistance have not been directly linked to diamide resistance in *T. absoluta,* synergist assays on a diamide resistant strain collected in Italy showed that S,S,S-tributyl phosphorotrithioate (DEF) and to a lesser, extent piperonyl butoxide (PBO) and diethyl maleate (DEM) can partially suppress chlorantraniliprole resistance, suggesting esterases, and potentially P450s and GSTs may play a role in resistance (*Roditakis* et al., *2017*).

In other insect pests detoxification enzymes have been directly linked to diamide resistance. These include flavin monooxygenases in *Plutella xylostella*, and P450s and UGTs in *P. xylostella*, *Chilo suppressalis, and Aphis gossypii* (*Li* et al., *2018*; *Mallott* et al., *2019*; *Xu* et al., *2019*; *Zeng* et al., *2021*; *Zhao* et al., *2019*). Finally, ABC transporters have also been implicated in diamide resistance in *C. suppressalis*, although a causal role has not been demonstrated using functional approaches (*Meng* et al., *2020*).

Chlorantraniliprole remains an important pesticide in the control of *T. absoluta* in many countries. Losing the efficacy of this pesticide through the evolution of resistance would be a significant economic blow to the tomato industry as there are limited compounds that can replace it in current integrated pest management (IPM) strategies (Biondi et al., 2018; Grant et al., 2019; Guedes et al., 2019). It is therefore vital that the efficacy of this compound against *T. absoluta* populations is monitored and the mechanisms through which resistance evolves characterised. However, in relation to the latter, investigation of metabolic resistance in *T. absoluta* is currently hampered by the quality of genomic resources available for this species. Previous work has sequenced and assembled a draft genome for this species (Tabuloc et al., 2019). However, the use of short-read sequencing and the high heterozygosity of *T. absoluta* resulted in a highly fragmented assembly with a contig N50 of just 26 Kb and scaffold N50 of 113 Kb (Tabuloc et al., 2019). This lack of contiguity can impact the quality of global gene expression analysis, which is often the starting point for the investigation of metabolic resistance. Here we addressed this issue by using long single-molecule sequencing in combination with short-read sequencing to generate a new more contiguous draft genome of *T. absoluta*. We then leveraged this resource in combination with biological, transcriptomic and functional approaches to investigate the role of metabolic mechanisms in chlorantraniliprole resistant strains of *T. absoluta* that are not impacted by target-site resistance.

2. Materials and methods

2.1. Insect strains

The TA1 strain of *T. absoluta* was collected in Murcia, Spain from field-grown tomatoes in 2010. The Sus and Mur strains were collected in Murcia, Spain from tomatoes in commercial glasshouses in 2011 and 2014 respectively. The Ssus and Sres strains were collected from tomatoes in commercial glasshouses in Cartagena, Spain in 2011 and Los Palacios y Villafranca, Spain in 2017 respectively. Approximately 50 larvae were collected and used to initiate each strain. Insects were maintained in bug dorm cages (MegaView science co.) in controlled environment rooms at 25 °C, 60% R.H., 16:8 light-dark cycle and fed *ad libitum* on tomato plants (var. Money Maker).

2.2. Sequencing and assembly of a reference T. absoluta genome

Prior to sequencing, the TA1 strain of *T. absoluta* was inbred to maximise homozygosity and facilitate subsequent genome assembly. Pupae from a single pair mating of the TA1 strain were collected, sexed, and paired in isolated chambers containing a single tomato leaf (var. Money Maker). All pupae were collected from the most fecund pair and sexed. The sibling offspring were then paired off again in isolated chambers to breed. This process was repeated over 6 generations until further matings were inhibited by inbreeding depression. The culture was then snap frozen in liquid nitrogen and stored at −80 °C prior to DNA extraction.

We employed a hybrid long and short-read approach to sequence the genome of the inbred *T. absoluta* strain using PacBio single-molecule real-time (SMRT) sequencing and Illumina paired-end sequencing respectively. For the generation of Illumina libraries, genomic DNA was extracted from a single L4 larvae using the E.Z.N.A Insect DNA kit (Omega Bio-Tek) following the manufacturer's protocol. This provided sufficient DNA for the preparation of a single PCR-free paired-end library that was sequenced on an Illumina HiSeq 2500 using a 250 bp read metric. For the generation of PacBio libraries, high molecular weight DNA was extracted from a pool of 30 L4 larvae using the Genomic-tip kit (Qiagen) according to the manufacturer's instructions, and used to generate long-read PacBio libraries which were sequenced using five PacBio Sequel SMRT cells. DNA quantity and quality was assessed by spectrophotometry using a NanoDrop (Thermo Scientific), Qubit assay (ThermoFisher) and gel electrophoresis.

To improve annotation of genes in the genome assembly, and to investigate the molecular basis of chlorantraniliprole resistance, the replicated transcriptomes of the Sus, Mur, Ssus and Sres strains were sequenced. For this, RNA was extracted from 4 replicate pools of 10 whole larvae (L2-L3 stage) of each strain using the Isolate RNA Mini Kit (Bioline) according to the manufacturer's instructions. Samples were taken from the same generation used for insecticide bioassays (see

below). The quantity and quality of RNA was checked as described above. RNA was used as a template for the generation of barcoded libraries (TrueSeq RNA library preparation, Illumina) and RNA samples sequenced on an Illumina HiSeq2500 flowcell using a 100 bp paired-end read metric. Note that a transcriptome of the TA1 strain generated from eggs, larvae, pupae and adults has been generated previously (Berger et al., 2016).

PacBio long-reads were assembled using Canu v2.0 (Koren et al., 2017) (using default parameters), and then polished with both long- and short-reads using Racon -v1.3.171 (Vaser et al., 2017) and Pilon -v1.2272 (Walker et al., 2014) respectively. The hybrid assembler DBG2OLC v20180222 (Ye et al., 2016) was also used to assemble both short- and long-reads together (using default parameters). The Canu and DBG2OLC assemblies were then merged using QuickMerge v0.3 (Chakraborty et al., 2016), and redundancy in the merged assembly removed (de-duplicated) using Redundans (Pryszcz and Gabaldon, 2016). The completeness and contiguity of the assembly was checked using KATv2.4.2 (Mapleson et al., 2017) and BUSCO tools v4.1.2 (Simão et al., 2015).

Prior to gene prediction the assembly was soft masked for repetitive elements with RepeatMasker -v4.0.780 using repeat libraries generated by RepeatModeler -v2.0.2 (Smit and Hubley, n.d.). Protein coding genes were predicted using GeneMark-ES –v4.3.881 and AUGUSTUS –v3.3.082 implemented in the BRAKER -v2.1.283 pipeline using the RNA-seq datasets detailed above as evidence (Brůna et al., 2021). RNA-seq data was mapped to the repeat masked genome using HISAT2 v2.0.584 (Pertea et al., 2016) followed by sorting and indexing with SAMtools -v1.385 (Li et al., 2009). BRAKER2 was run with UTR training and prediction enabled with the parameters –softmasking—gff3—UTR = on. Following gene prediction, genes that contained in frame stop codons were removed using the BRAKER2 script getAnnoFastaFromJoingenes.py and the completeness of each gene set was checked by BUSCO analysis (Simão et al., 2015) using the longest transcript of each gene as the representative transcript. Functional annotation of the *de novo* predicted gene models was performed based on homology searches against the NCBI nr and Interpro databases using BLAST2GO –v5.2.5 (Conesa et al., 2005).

The genome assembly and sequence data generated in this study has been deposited with the NCBI Short Read archive in BioProject PRJNA836608.

2.3. Insecticide assays

Leaf-dip bioassays were completed according to test method 22 guidelines produced by the Insecticide Resistance Action Committee (IRAC, n.d.) which subjects pooled replicates of larvae to a range of pesticide concentrations. The pesticide was diluted in Triton X-100 (0.2 g L−1) to make a range of concentrations expected to induce 0–100% mortality in *T. absoluta*. Five replicates of fresh tomato leaves (var. Money Maker) were immersed in each of the varying concentrations of pesticide solutions and allowed to dry for ~2 h. Once dried, each individual leaf was placed in a Petri dish on a moist sheet of filter paper. 250 L2-L3 larvae were collected from the relevant culture and 10 larvae were placed on each leaf. The larvae were left in CT rooms at 25 °C, 60% R.H., 16:8 light-dark cycle for 72 h. After 72 h mortality was scored. Probit analysis and the calculation of LC_{50} values were performed using GenStat 15th Edition (VSN International). Resistance ratios (RR) were calculated by dividing the LC_{50} value of the target population by the LC_{50} of the susceptible strain.

2.4. Differential gene expression analysis

The replicated RNAseq data generated for the Sus, Ssus, Mur and Sres strains was used for differential gene expression analysis. RNAseq reads were aligned to the genome assembly created in this study using HISAT2 v2.1.0 (Pertea et al., 2016), and gene expression estimated using the htseq-count tool implemented in the HTSeq package (Anders et al., 2015). EdgeR v3.9 (Robinson et al., 2010) was used to identify significantly differentially-expressed genes in pairwise comparisons of chlorantraniliprole susceptible and resistant strains using a corrected p-value threshold of $p < 0.05$ and a fold change >2. Lists of differentially expressed genes were compared using Jvenn (Bardou et al., 2014).

2.5. Genotyping of the ryanodine receptor gene for resistance-associated mutations

To investigate the role of target-site mutation in the resistance of the Sus, Ssus, Mur and Sres strains to chlorantraniliprole the RNAseq data generated for each strain was mapped to the gene encoding the RyR, the chlorantraniliprole target-site, using Geneious mapper with default parameters (Geneious v10.2.6, Biomatters Ltd.) Alignments were manually checked for the presence of mutations and indels.

2.6. Transgenic expression of candidate genes in Drosophila melanogaster

The candidate resistance gene, *UGT34A23,* identified from transcriptome profiling (see results below) was synthesised (Twist Bioscience) and cloned into the pUASTattB plasmid (GenBank: EF362409.1). This construct was transformed into the *D. melanogaster* germline carrying an attP docking site on chromosome 2 (attP40) and the phiC31 integrase gene under the control of the vasa regulatory region on the X chromosome (y w M (eGFP, vas-int, dmRFP)ZH-2A: P [CaryP]attP40). Transgenic strains were balanced and the expression of the transgene confirmed using quantitative PCR (qPCR). For this, RNA was extracted from four biological replicates of five mixed sex adults of the transgenic fly strain and the progeny of crosses of this strain with the Act5C-GAL4 strain (see below) using the Isolate II RNA Mini Kit (Bioline). cDNA was synthesised from 1 μg of RNA per replicate using the Maxima H Minus First Strand cDNA Synthesis Kit (ThermoFisher) using both random hexamer and oligo (dT) primers. QPCR analysis was performed using the primers detailed in Table S6, with the efficiency of PCR for each primer pair assessed using a serial dilution of 100 ng–0.01 ng of cDNA. PCR reactions (20 μl) contained 10 ng of cDNA, 10 μl of SYBR Green JumpStart Taq Readymix (Sigma), and 0.25 μM of each primer. Each qPCR experiment consisted of three technical replicates and four biological replicates. Reactions were run on a Real-Time PCR System (BioRad CFX96) using temperature cycling conditions of: 3 min at 95 °C followed by 40 cycles of 95 °C for 30s, 57 °C for 20s and 72 °C for 25s. A melt curve step was included (ranging from 72 °C to 95 °C by 1 °C every 5s) to confirm the absence of non-specific amplification. Data were analysed using the ΔΔCT method (Livak and Schmittgen, 2001) from the geometric mean of two *D. melanogaster* housekeeping genes (RPL32 and SDHA, Table S6).

To conduct insecticide bioassays virgin females of the Act5C-GAL4 strain ("y[1] w[*]; P(Act5C-GAL4-w)E1/CyO,""1; 2″) (Bloomington Stock Center) were crossed with UAS- *UGT34A23* males to allow for mating and oviposition. Bioassays using a total of 5 concentrations (1 mg L-1, 10 mg L-1, 100 mg L-1, 1000 mg L-1 and 10,000 mg L-1) made from technical grade chlorantraniliprole (Sigma) were performed to assess the susceptibility of transgenic adult female flies to this compound. A total of 100 μl of each insecticide concentration was overlaid on 1.5% agar (1% sucrose, 0.5% acetic acid) in standard fly vials and allowed to dry at room temperature overnight. 20 adult females (2–7 days post-eclosion) were added to each vial and mortality assessed after 24 h. Five replicates were conducted for each concentration. A *D. melanogaster* strain of the same genetic background to the transgenic strain but lacking a transgene (progeny of crosses of the Act5C-GAL4 and UAS-no transgene strains) was included in bioassays as a reference control. Control mortality was assessed using vials containing agar but lacking insecticide. LC_{50} values and 95% fiducial limits were calculated with 2 parameter log-logistic models using the R package 'drc' in RStudio. Significance testing between LC_{50} values was conducted using the

EDcomp function in 'drc'.

2.7. Curation and phylogeny of T. absoluta UGT genes

T. absoluta UGT genes were mined from the genome assembly generated in this study based on the BLAST annotations obtained during gene annotation. To confirm the recovered genes are expressed, RNAseq data was aligned to each candidate UGT gene in the genome using Geneious v10.2.6. Exons for each UGT were extracted and concatenated and coding sequences were translated into their protein sequence. According to their amino acid sequence identity (aaID), the *T. absoluta* UGTs were grouped with other lepidopteran UGTs using CD-HIT (Li and Godzik, 2006) at 60% and 45% aaID as cut-off values. Families were defined as sharing 45% or more aaID and subfamilies defined at 60% aaID or greater. For phylogenetic analysis, UGT protein sequences from *T. absoluta* were compared with those of *Spodoptera exigua* (n = 32), *Plutella xylostella* (n = 21) and *Chilo suppressalis* (n = 25). Protein sequences were aligned using MUSCLE (Edgar, 2004) and a Maximum Likelihood phylogeny performed in Geneious with 5000 bootstrap replications (version 10.2.6). Amino acid substitution was modelled using the LG replacement matrix (Le and Gascuel, 2008). *P. xylostella* Fringe (GenBank accession: MK530647.1), was used as an outgroup.

3. Results

3.1. A long-read genome assembly of T. absoluta

Kmer analysis using Illumina reads derived from sequencing the TA1 strain of *T. absoluta* revealed two coverage peaks at around 18X and 36X, and estimated a heterozygosity rate of 1.45% and a genome size of 478 Mb (Fig. S1). The latter is consistent with a previous estimate obtained from kmer analysis of 492 Mb (Tabuloc et al., 2019) but below the estimated diploid genome size obtained by flow cytometry of 1129 Mb (Gandhi Gracy et al., 2019). Long and short-read data was assembled into 3111 scaffolds with an N50 of 1124.7 Kb resulting in a final assembly of 819 Mb. The completeness of the gene space in the assembled genome was assessed using the Benchmarking Universal Single-Copy Orthologues (BUSCO) pipeline (Simão et al., 2015), with 93.3% of the Arthropoda test gene set found to be present as complete single copies (Table 1, Fig. S1). Thus, the new *T. absoluta* assembly represents a good quality contiguous assembly and a significant improvement on the existing short read assembly of this species which has a contig N50 of 26.36 Kb (Tabuloc et al., 2019) (Table S1). Structural genome annotation using a workflow incorporating RNAseq data predicted a total of 40,252 protein-coding genes (Table 1) in the assembly. Of these, 34,736 were successfully assigned functional annotations based on BLAST searches against the non-redundant protein database of NCBI and the InterPro database (Fig. S1).

Table 1
Summary statistics of the *T. absoluta* genome assembly. The BUSCO score presented is the percentage of the Arthropoda test gene set (n = 1013) found to be present as complete single copies.

Assembly statistic	Value
Number of total contigs	7407
Number of total scaffolds	3111
Contig N50	117,487 bp
Contig L50	1157
Longest contig	3,129,931 bp
Scaffold N50	1,124,664 bp
Scaffold L50	167
Longest scaffold	20,421,755 bp
Total assembled size	818,551,225 bp
GC content	35.45
% Complete BUSCOS	93.3
Total gene content	40,252
Total gene annotated	34,736

3.2. Identification of chlorantraniliprole resistance in strains of T. absoluta from Spain

The sensitivity of four strains of *T. absoluta*, collected from commercial glasshouses in Spain (Ssus, Sres, Sus and Mur), to chlorantraniliprole was compared by full dose–response insecticide bioassays (Table 2). The Ssus and Sus strains both exhibited high sensitivity to this compound with LC_{50} values of 0.088 and 0.15 mg L^{-1} respectively. The Mur strain exhibited moderate levels of resistance to chlorantraniliprole with an LC_{50} value of 1.6 mg L^{-1}, 18.2-fold higher than that of the most susceptible strain Ssus. Finally, the Sres strain exhibited extremely high levels of resistance (44,614-fold) with an LC_{50} of 39,261.6 mg L^{-1}.

3.3. Chlorantraniliprole resistance does not result from mutation of the diamide target-site

To investigate the molecular basis of resistance in the Mur and Sres strains we performed replicated messenger RNA sequencing (RNAseq) of each strain and the two susceptible strains Ssus and Sus. The data obtained was first leveraged to investigate the role of target-site mutation in resistance. For this, RNAseq data was mapped to the gene encoding the RyR, and examined for potential target-site alterations associated with resistance (Fig. 1). No nonsynonymous mutations across the whole RyR gene coding sequence were identified in the Sus and Mur strains, including at sites previously implicated in resistance (Roditakis et al., 2017). In contrast, some RNAseq reads of the Sres strain carried mutations at the sites of two previously described resistance mutations encoding the G4946V and I4790L substitutions (*P. xylostella* numbering) (Grant et al., 2019; Roditakis et al., 2017). However, based on the number of wildtype and mutant reads mapping at these sites, the frequency of both mutations in the Sres strain was found to be very low (frequency of 0.04 and 0.06 respectively), with more than 99% of the reads mapping to these sites representing wildtype (i.e. chlorantraniliprole susceptible) sequences (Fig. 1). Intriguingly, the chlorantraniliprole susceptible strain Ssus also carried a point mutation at one of these positions resulting in the G4946L replacement, again at very low frequency (0.04%) (Fig. 1). The extremely low frequency of these mutations in the Sres and Ssus strains would not be expected to confer any measurable resistance phenotype. Thus, these findings provide clear evidence that the resistance of the Sres and Mur strains to chlorantraniliprole is not a result of mutation of the target-site of this insecticide.

3.4. Chlorantraniliprole resistance is associated with marked overexpression of a UDP-glycosyltransferase

To explore the role of metabolic mechanisms in chlorantraniliprole resistance, we used the RNAseq data to conduct differential gene expression analyses, first comparing each of the two resistant *T. absoluta* strains with each of the two susceptible strains (Fig. 2, Dataset S1). A total of 228 to 4128 genes were found to be significantly upregulated and 290 to 3868 downregulated in the four pairwise comparisons (Fig. 2, Dataset S1). Cross referencing of genes consistently differentially

Table 2
Sensitivity of field-collected strains of *T. absoluta* from Spain to chlorantraniliprole. Data shows LC_{50} values (& 95% CIs) for clones derived from full dose response bioassays. Resistance ratios (RR) shown are relative to the Ssus strain. Nc = not calculable due to insufficient mortality at the highest dose tested.

Strain	LC_{50} (mg L^{1})	CI 95%	RR
Sus	0.15	0.12–0.20	1.7
Mur	1.577	1.073–2.676	18.2
Ssus	0.088	0.053–0.140	1
Sres	3926	816 - nc	44613.6

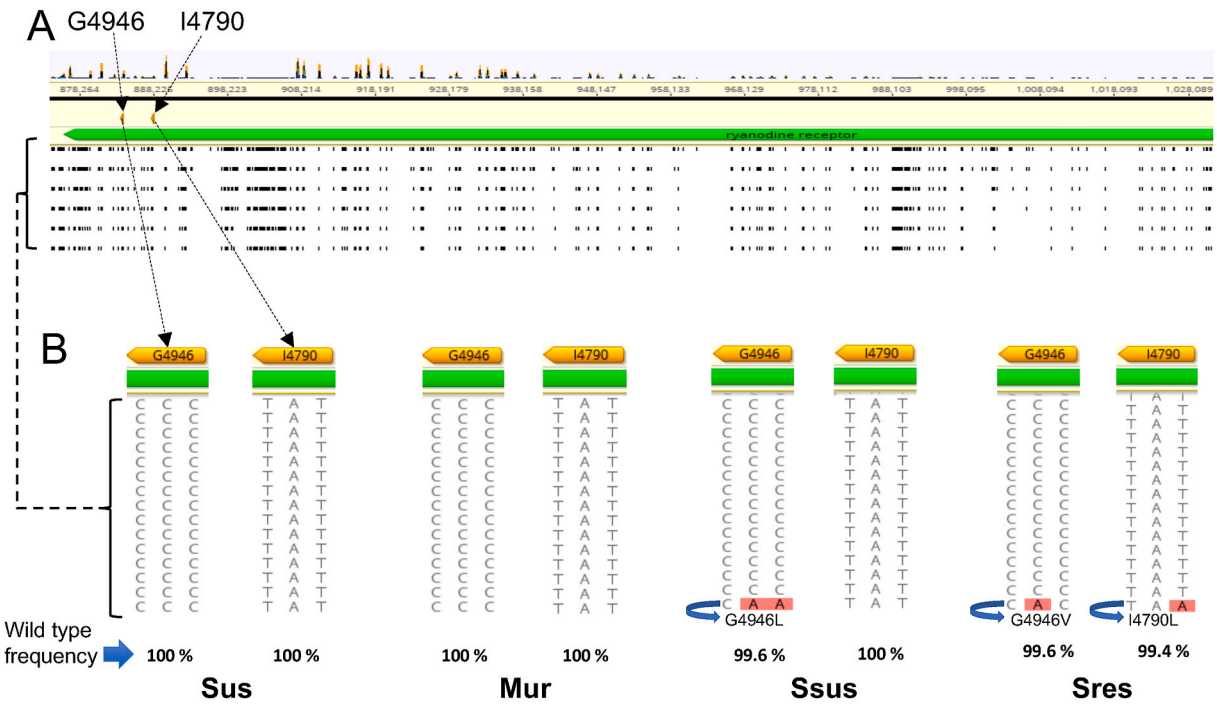

Fig. 1. Investigation of the role of mutations in the ryanodine receptor (RyR) in resistance to chlorantraniliprole in *T. absoluta* strains from Spain. The (A) RNAseq reads (black dashes) mapped to the RyR gene (green bar) with previously reported resistance mutation sites (G4946 and I4790) highlighted. For clarity read coverage is shown up to 6X and true coverage is displayed by the plot at the top of figure. (B) Close up subset of mapped transcripts for all four strains at the known resistance mutation sites showing the frequency of reads encoding the wild type (i.e. insecticide susceptible) residue. The impact of mutations present at low frequency in the Ssus and Sres strains on the encoded amino acid sequence are indicated.

expressed in all comparisons of resistant and susceptible strains resulted in a list of 20 upregulated genes and 24 downregulated genes (Fig. 2, Table S2). Interrogation of the final gene list for genes encoding detoxification genes most commonly implicated in insecticide resistance identified just one P450 (gene ID g33632, annotated as cytochrome P450 9e2-like), and one UDP-glycosyltransferase (gene ID g995, officially named as *UGT34A23*) as overexpressed in both resistant strains compared to both susceptible strains. However, in the case of the *CYP9E2-like* P450 gene, the level of overexpression in comparisons of the Mur strain (which exhibited a phenotype of modest resistance to chlorantraniliprole) with the two susceptible strains was higher (7.1-fold compared to Ssus and 7.2-fold compared to Sus) than comparisons of the Sres strain (which exhibited extremely high levels of resistance to chlorantraniliprole) with the susceptible strains (4.4-fold compared to Sus and 4.2-fold compared to Ssus) (Fig. 3). Thus, the profile of expression of *CYP9E2-like* in the two resistant strains is not consistent with their resistance phenotypes, suggesting that overexpression of this P450 gene is not the molecular explanation for chlorantraniliprole resistance. In contrast, the pattern of expression of *UGT34A23* in the two resistant strains was more consistent with the resistance profile of these strains. Specifically, this gene was highly upregulated in the Sres strain (149.1-fold up regulated in the Sres vs Sus comparison and 116.9-fold up regulated in the Sres vs Ssus comparison) (Fig. 3). Furthermore, while *UGT34A23* was also overexpressed in the Mur strain compared to both susceptible strains, the level of overexpression was notably lower (22.6-fold compared to Ssus and 20.6-fold compared to Sus), correlating with the lower level of resistance observed in this strain (Fig. 3). This gene was therefore prioritised for further characterisation.

Further mapping of RNAseq data to the locus of the genome where *UGT34A23* is located confirmed that a full length UGT was expressed, identifying a 1566 bp open reading frame encoding a protein of 521 amino acids (Fig. S2). The sequence obtained exhibits high levels of homology to fully characterised UGTs from a range of lepidopterans, with phylogenetic analysis placing the gene in the UGT34 family which is known to be conserved in Lepidoptera (Fig. 4). We also leveraged the new reference genome to examine the relationship of *UGT34A23* to the wider UGT superfamily of enzymes in *T. absoluta* by manually curating a total of 27 full length UGT genes from the genome (Table S3). Phylogenetic analysis distributed the *T. absoluta* UGT genes throughout 11 subfamilies containing genes from all comparison species (Fig. 4).

3.5. *UGT34A23 confers resistance to chlorantraniliprole in vivo*

To functionally validate the role of *UGT34A23* in chlorantraniliprole resistance, a transgenic strain of *D. melanogaster* was created carrying this gene. Quantitative PCR confirmed that when this strain was crossed with the Act5C-GAL4 driver strain (see methods), the transgene was highly overexpressed in the resulting progeny (Table S4). In insecticide bioassays the progeny of these crosses exhibited striking and significant (10.8-fold, $p < 0.01$) resistance to chlorantraniliprole compared with control flies of the same genetic background but without a transgene (progeny of crosses of the Act5C-GAL4 and UAS-no transgene strains) (Fig. 5, Table S5), demonstrating that expression of *UGT34A23* is sufficient to confer a resistant phenotype to this compound *in vivo*.

4. Discussion

The genome sequence of *T. absoluta* reported here represents a significantly more complete and contiguous assembly than the previously reported short-read only assembly of this species. In 2019 Tabuloc et al. (2019) released a draft genome to facilitate the successful creation of a diagnostic for accurately identifying *T. absoluta* species from closely related and morphologically similar species. Our sequencing approach differed from that previously employed through the additional use of PacBio long-read technology, generating reads that span the low complexity and repetitive regions of the genome, creating more accurate *de novo* assemblies (Pollard et al., 2018). Our approach resulted in more assembled content (819 versus 677 Mb) and much greater contiguity,

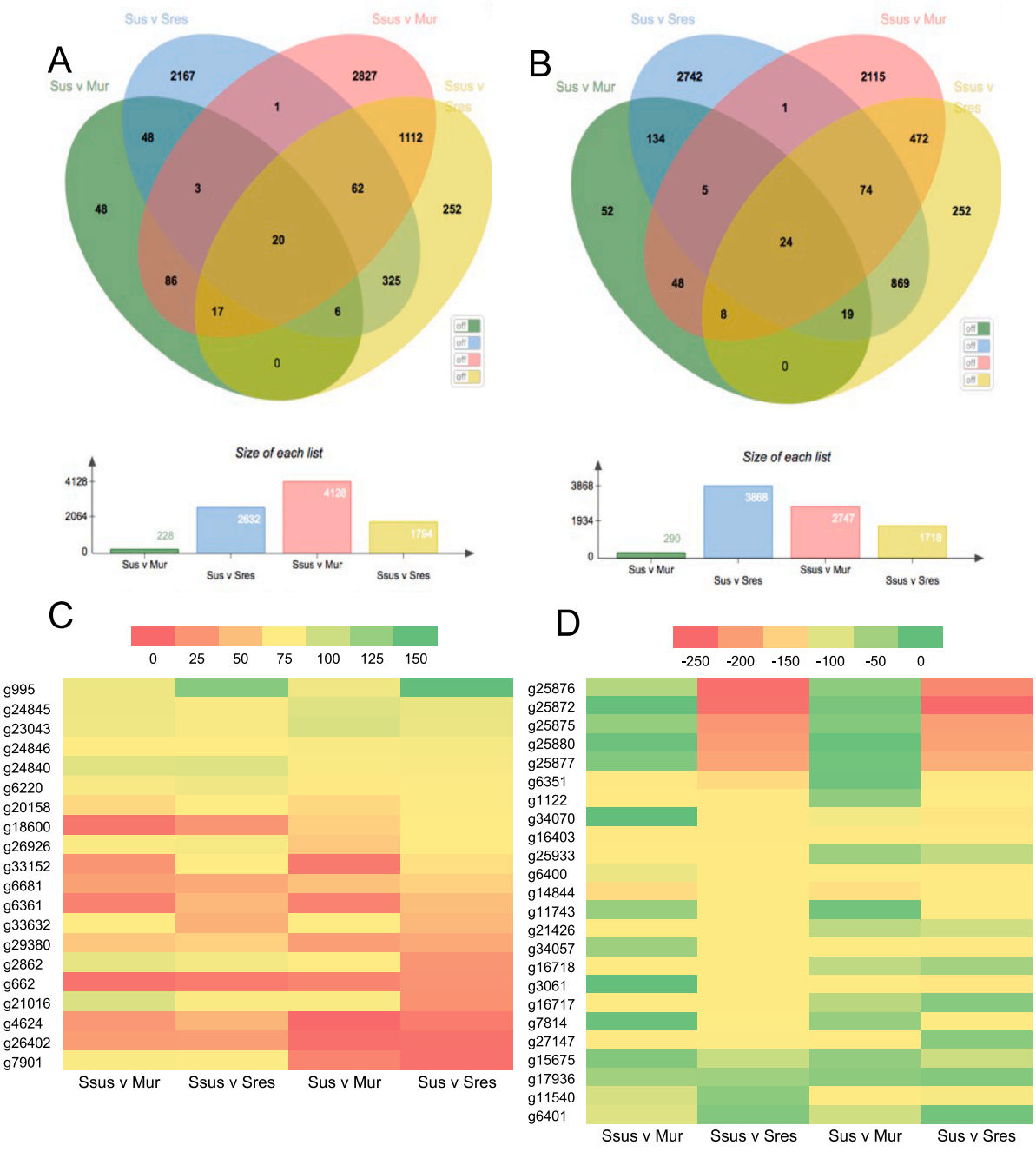

Fig. 2. Identification of differentially expressed genes in in comparisons of chlorantraniliprole susceptible and resistant strains of *T. absoluta*. Panels A and B display Venn diagrams showing genes commonly up (A) and down (B) regulated in comparisons of susceptible and resistant strains. The bar charts below each Venn indicate total number of genes differentially expressed in each RNAseq comparison. Panels C and D display expression heat maps of fold change of each comparison for the subset of genes that were consistently up (C) and down (D) regulated across all pairwise comparisons.

with an order of magnitude higher scaffold N50 (1124.66 versus 97.12 Kb) and lower L50 (167 versus 1786) (Tabuloc et al., 2019). Improved sequence contiguity increases the completeness of genes and genomic elements in the assembly and provides a greater understanding of how loci are ordered and oriented, thereby enabling, and/or improving the quality of, a range of downstream genomic and transcriptomic analyses (Grau et al., 2018). It is worth noting, however, that the size of our assembly (819 Mb) is larger than the size estimates of around 500 Mb obtained from kmer analysis in this and previous studies (Tabuloc et al., 2019), but below the estimated diploid genome size obtained by flow cytometry of 1129 Mb (Gandhi Gracy et al., 2019). Sequence-based methods have been previously shown to underestimate genome size (Pflug et al., 2020), however, the difference in size of our genome assembly with the genome size estimate derived from flow cytometry, may indicate that some genomic content, likely repetitive in nature, is not represented in our assembly. Nevertheless, BUSCO analysis revealed good representation of gene content in our assembly. Thus, the new genome assembly of *T. absoluta* represents a valuable resource for future research on this important agricultural pest. In the current study we leveraged this resource to advance understanding of the evolution of insecticide resistance in this important global insect pest, uncovering a novel mechanism underpinning resistance to a diamide insecticide widely used for control.

We provide several lines of evidence that metabolic detoxification is

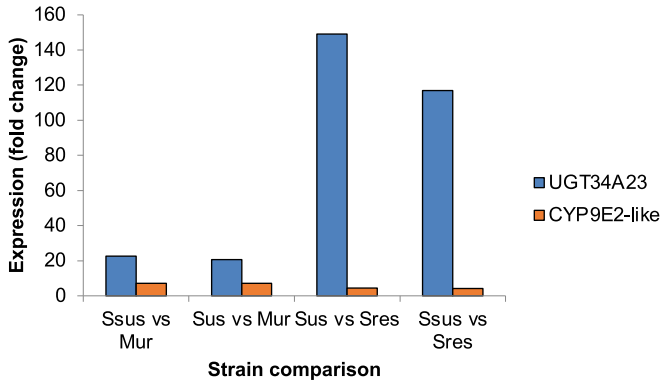

Fig. 3. Expression of *UGT34A23* and *CYP9E2-like* in comparisons of chlorantraniliprole susceptible and resistant strains of *T. absoluta*. Expression is shown as fold-change between each resistant and susceptible strain and is derived from RNAseq analysis.

a key determinant of the resistance of *T. absoluta* to the pesticide chlorantraniliprole through the increased expression of *UGT34A23*. Firstly, we show that two strains of *T. absoluta* exhibiting moderate and extremely high levels of resistance to chlorantraniliprole do not carry target-site resistance mutations in the RyR at appreciable frequencies. This suggests that the resistance of these strains does not result from modification of the diamide target-site. Secondly, differential gene expression analyses provided a short list of just 20 genes that were consistently and significantly overexpressed in both chlorantraniliprole resistant strains compared to both susceptible strains. However, of these genes, there was just one strong candidate resistance gene, *UGT34A23*, based on the pattern of expression in the resistant strains. Specifically, the level of overexpression of this gene in the resistant strains correlated with their sensitivity to chlorantraniliprole, with *UGT34A23* > 100-fold overexpressed in comparisons of the highly resistant Sres strain with both susceptible strains, and >20-fold overexpressed in the moderately resistant Mur strain. Finally, the marked (~11-fold) tolerance of transgenic flies expressing *UGT34A23,* compared to flies of the same genetic background but lacking the transgene, demonstrates that overexpression of this gene is sufficient to confer resistance *in vivo*. Together these

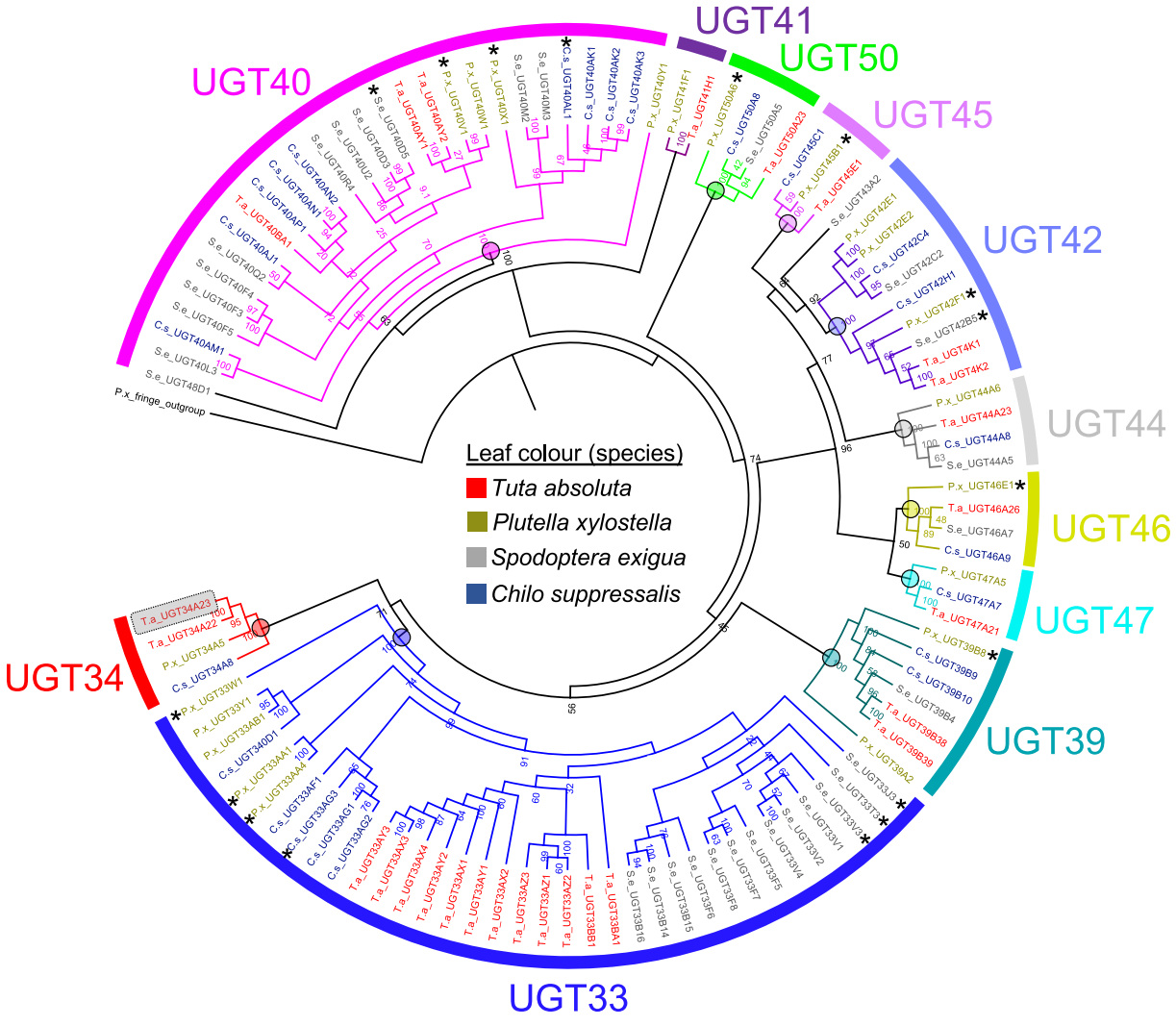

Fig. 4. Phylogenetic relationship of the UDP-glycosyltransferases (UGTs) of *Tuta absoluta* and three other lepidopteran insect species. Protein sequences of 27 *T. absoluta* (T.a) UGTs, 32 *Spodoptera exigua* (S.e) UGTs, 21 *Plutella xylostella* (P.x) UGTs and 24 *Chilo suppressalis* (C.s) UGTs were used to estimate a Maximum Likelihood phylogeny in Geneious (version 10.2.6). Amino acid substitution was modelled using the LG replacement matrix (Le and Gascuel, 2008). *P. xylostella* Fringe (GenBank accession: MK530647.1) was used as an outgroup. Nodes are marked with bootstrap support values, based on 5000 replicates. Branches are coloured by UGT family with the basal nodes of each marked with a circle of the same colour. The leaf colours represent the four species: red for *T. absoluta*, gold for *P. xylostella*, grey for *S. exigua* and blue for *C. suppressalis*. T.a_UGT34A23 implicated in this study in resistance to chlorantraniliprole is marked with a grey rectangle; other UGT genes implicated in resistance to chlorantraniliprole in previous studies are highlighted with black stars.

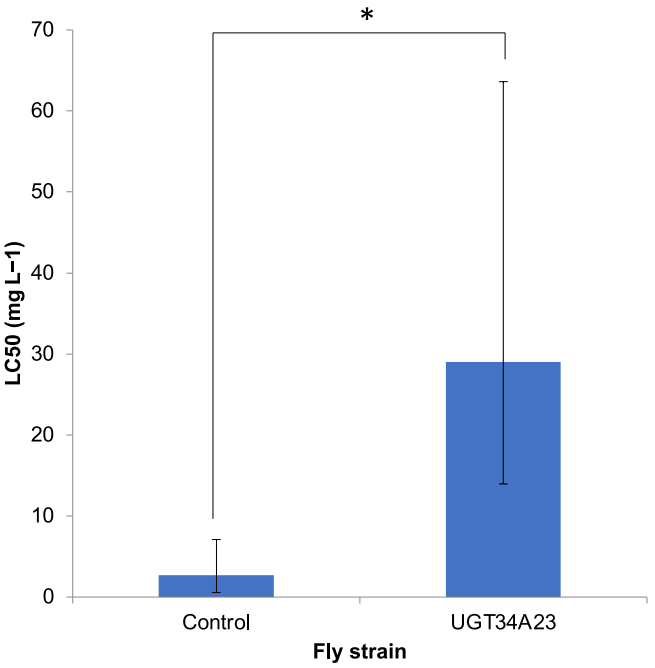

Fig. 5. UGT2C1-like confers resistance to chlorantraniliprole *in vivo*. Bar charts display the sensitivity of a transgenic strain of *Drosophila melanogaster* expressing *UGT2C1-like* to chlorantraniliprole. Data shows LC_{50} values (& 95% FIs) derived from full dose response insecticide bioassays. Star indicates a significant difference ($p < 0.001$) in the LC_{50} values obtained relative to those of a control *D. melanogaster* strain of the same genetic background but lacking the transgene (progeny of crosses of the Act5C-GAL4 and UAS-no transgene strains).

findings provide unequivocal evidence of the role of *UGT34A23* in chlorantraniliprole resistance.

UGTs are phase II metabolic enzymes that catalyse the conjugation of xenobiotics to form water-soluble inactive compounds that can be readily excreted and have been increasingly implicated in resistance to insecticides in a range of insect pest species (Li et al., 2017; Ma et al., 2021; Pym et al., 2022; Tian et al., 2019; Yiou et al., 2018; Zeng et al., 2021). This includes to diamide insecticides, with studies on *P. xylostella* implicating *UGT2B17* in resistance to chlorantraniliprole (Li et al., 2017), work on *C. suppressalis* linking the upregulation of the UGT genes *UGT40AL1* and *UGT33AG3* to tolerance to chlorantraniliprole (Zhao et al., 2019), and a recent study on *Aphis gossypii* demonstrating that UGT341A4, UGT344B4 and UGT344M2 can confer resistance to cyantraniliprole (Zeng et al., 2021). Together with the results of our study these findings clearly demonstrate that upregulation of UGTs is a convergent mechanism of resistance to chlorantraniliprole across multiple insect species.

Analysis of the *T. absoluta* genome identified 27 full length genes encoding UGTs and phylogenetic analysis revealed they were distributed among 11 families with the majority of the UGTs falling into the lepidopteran specific family UGT33 (Ahn et al., 2012) as was also observed in other Lepidoptera, such as *P. xylostella* (X. Li et al., 2018) and *S. exigua* (Hu et al., 2019). The phylogenetic analysis also showed that UGTs identified as being differentially expressed in response to pesticide resistance in *P. xylostella* (X. Li et al., 2018) and *S. exigua* (Hu et al., 2019) had a closely related UGT in *T. absoluta* (Fig. 4). This finding suggests that *T. absoluta* UGTs may also have the direct potential, through upregulation, or the evolutionary potential, through adaptation of substrate specific sites, to facilitate detoxification to a broad range of pesticides.

The results of our study have significant applied implications in relation to the sustainable control of *T. absoluta*. The chlorantraniliprole resistant populations of *T. absoluta* investigated in this study were all from Spain. In future it will be important to establish if the metabolic mechanism of diamide resistance identified in this study is restricted to populations in certain regions of Spain or is more widespread, as reported for target-site resistance to this insecticide class in *T. absoluta* (Guedes et al., 2019; Roditakis et al., 2017). In this regard, Spain is a major exporter of tomatoes, for example, in 2018 Spain exported 112,025 metric tonnes of tomatoes to the UK (Blazquez, 2021). Thus, there is a risk of *T. absoluta* carrying this mechanism spreading to, and compromising susceptible populations in, other countries via these trade routes. These results are also important in relation to pest management strategies as they demonstrate that resistance to chlorantraniliprole can develop in *T. absoluta* in the absence of target-site resistance. Molecular-based resistance assays to identify mutations in insecticide target-sites have been proposed as alternatives to traditional bioassays to determine the resistance status of pest populations and inform pesticide application (Troczka et al., 2012). However, the presence of metabolic resistance could lead to false predictions from data derived from such assays. To detect the metabolic resistance described in this study, attempts to assess field resistance through molecular assays would have to be complemented by phenotyping by bioassays. The latter could also include the use of inhibitors of UGTs and/or other enzyme systems (i.e. synergist assays) to provide evidence of potential metabolic resistance in the population. Such assays may also help facilitate selection of appropriate synergist assays for the inhibition of UGT activity. In this regard the findings presented in this study could inform the development of pesticide formulations through the incorporation of synergists. Specifically, the knowledge that UGTs confer diamide resistance provides motivation to test compounds known to inhibit UGTs for use in such formulations. Although, such a strategy does not impact resistance mediated by target-site resistance, this practice has been used in the past with PBO added to pyrethroid based pesticides to maintain their efficacy in the face of metabolic detoxification by P450s (Gleave et al., 2021). PBO has also been shown to reduce the production of CHP-glucose conjugates, the products of UDP-glucosyltransferase detoxification reactions in *M. domestica* exposed to pyraclofos (Lee et al., 2006). The potential of UGT inhibition has been recently demonstrated in an imidacloprid resistant population of *Diaphorina citri,* where UGTs were found to be highly overexpressed. Application of two synergists, 5-nitrouracil and sulfinpyrazone resulted in a significant increase of pesticide toxicity (5.89- and 8.15-fold, respectively) (Tian et al., 2019). Furthermore, in human drug research a wide range of compounds have been identified that inhibit a broad range of UGT activity including atazanavir and erlotinib for 1A1, Hecogenin for 1A4, Niflumic acid for 1A9, Fluconazole for 2B7 and *S*-Nicotine for 2B10 (Oda et al., 2015). The fact that *UGT34A23* is readily expressed in *D. melanogaster* as a functional enzyme means the transgenic line created in this study could be used as a screening tool for the assessment of novel synergists, aiding the enhancement of contemporary pesticide products and extending the effective lifespan of active ingredients. In some countries such as the UK, the importance of maintaining diamide pesticides through such processes is heightened due to the loss of efficacy of other pesticides used in current IPM strategies such as spinosad (Grant et al., 2019).

In summary, we present a new more complete and contiguous reference genome for *T. absoluta*. We identify potent diamide resistance in strains of *T. absoluta* from Spain in the absence of appreciable target-site resistance, and link this to the dramatic overexpression of a single UGT gene. The outputs of this study provide: i) a powerful genomic resource to facilitate future research on *T. absoluta*, ii) new insight into the role of UGTs in insecticide resistance, and, iii) knowledge and tools that can be utilised to develop strategies for the sustainable control of a highly damaging globally distributed crop pest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ibmb.2023.103983.

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