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Identification and functional characterization of a novel acetyl-CoA carboxylase mutation associated with ketoenol resistance in *Bemisia tabaci*

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ABSTRACT

Insecticides of the tetronic/tetramic acid family (cyclic ketoenols) are widely used to control sucking pests such as whiteflies, aphids and mites. They act as inhibitors of acetyl-CoA carboxylase (ACC), a key enzyme for lipid biosynthesis across taxa. While it is well documented that plant ACCs targeted by herbicides have developed resistance associated with mutations at the carboxyltransferase (CT) domain, resistance to ketoenols in invertebrate pests has been previously associated either with metabolic resistance or with non-validated candidate mutations in different ACC domains. A recent study revealed high levels of spiromesifen and spirotetramat resistance in Spanish field populations of the whitefly *Bemisia tabaci* that was not thought to be associated with metabolic resistance. We confirm the presence of high resistance levels (up to > 640-fold) against ketoenol insecticides in both Spanish and Australian *B. tabaci* strains of the MED and MEAM1 species, respectively. RNAseq analysis revealed the presence of an ACC variant bearing a mutation that results in an amino acid substitution, A2083V, in a highly conserved region of the CT domain. F1 progeny resulting from reciprocal crosses between susceptible and resistant lines are almost fully resistant, suggesting an autosomal dominant mode of inheritance. In order to functionally investigate the contribution of this mutation and other candidate mutations previously reported in resistance phenotypes, we used CRISPR/Cas9 to generate genome modified *Drosophila* lines. Toxicity bioassays using multiple transgenic fly lines confirmed that A2083V causes high levels of resistance to commercial ketoenols. We therefore developed a pyrosequencing-based diagnostic assay to map the spread of the resistance alleles in field-collected samples from Spain. Our screening confirmed the presence of target-site resistance in numerous field-populations collected in Sevilla, Murcia and Almeria. This emphasizes the importance of implementing appropriate resistance management strategies to prevent or slow the spread of resistance through global whitefly populations.

1. Introduction

The control of many of the world's most damaging insect pests continues to rely primarily on the application of chemical insecticides. However, many insect species exhibit an exceptional ability to develop resistance to insecticides, with the cotton whitefly *Bemisia tabaci* Gennadius (Hemiptera: Aleyrodidae) one of the best examples of this. *B. tabaci* is one of the world's most destructive sucking pests causing

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damage to a wide range of food and commodity crops (Byrne and Bellows, 1991; De Barro et al., 2011). It's ability to rapidly develop resistance to control agents is enhanced by its short life-cycle, high fecundity and arrhenotokous parthenogenesis of haploid males. This is further exacerbated by high insecticide spraying frequency, with compounds often applied at reduced recommended rates against isolated populations established in greenhouses, thus increasing selection pressure (Nauen and Denholm, 2005; Horowitz et al., 2020).

The development of resistance against conventional insecticides has stressed the need for chemicals with novel modes of action to facilitate the development and deployment of Insecticide Resistance Management (IRM) strategies (Grávalos et al., 2015). However, new product development is hampered by the limited availability of molecular targets and an increasingly demanding regulatory environment (Sparks and Nauen, 2015). Although the insecticide market is currently dominated by compounds targeting nerve and muscle function, new chemistries addressing other modes of action have been introduced in recent years. Among these, are the family of tetronic/tetramic acid derivatives or cyclic ketoenols, assigned to group 23 of the IRAC mode of action classification. This group currently contains three commercially available cyclic ketoenol compounds, i.e. spirodiclofen, spiromesifen and spirotetramat (Sparks and Nauen, 2015). A fourth ketoenol compound, spiropidion will be introduced to the market in the near future (Muehlebach et al., 2020). These compounds are lipid biosynthesis inhibitors targeting acetyl-CoA carboxylase (ACC, EC 6.4.1.2), an enzyme known to catalyze the rate limiting step in fatty acid biosynthesis (Nauen, 2005; Lümmen et al., 2014). The first commercial ketoenol, spirodiclofen, was successfully launched for the control of herbivorous mites and psyllids, while the later introduced spiromesifen and spirotetramat are effective against whiteflies, thrips, aphids and other sucking pests (Bretschneider et al., 2003; Nauen et al., 2005; Brück et al., 2009; Bretschneider et al., 2012; Guillén et al., 2014). Following uptake by plants, ketoenols are hydrolyzed to the active enol form, enabling translocation in the xylem as well as in the phloem of crop plants (Brück et al., 2009). It is the enol form that is active against ACC (Bretschneider et al., 2012; Lümmen et al., 2014). Ketoenol insecticides show a favourable ecotoxicological profile, being compatible with managed bee pollinators and biological control agents (Bielza et al., 2005, 2009).

ACC catalyzes the carboxylation of acetyl-CoA to malonyl-CoA, which is the first committed and rate-limiting step in fatty acid biosynthesis. The reaction is biotin-dependent and proceeds in two steps (biotin carboxylation and then transfer of the carboxyl group to the acyl-CoA acceptor; Lümmen et al., 2014). In prokaryotes, each step is performed by distinct protein subunits, while in eukaryotes large multidomain ACC enzymes are responsible for the whole catalytic reaction. Mammalian genomes contain two genes encoding ACC isoforms ACC1 and ACC2, with different localization - ACC1 is found in the endoplasmic reticulum of lipogenic tissues while ACC2 is associated with mitochondria (Tong, 2005). In arthropods, multidomain ACCs are encoded by a single gene (Parvy et al., 2012; Demaeght et al., 2013), with the encoded biotin carboxylase (BC) domain catalyzing the first step of the reaction, with the second step mediated by the encoded carboxyltransferase (CT) domain (Tong, 2013; Tong, 2017).

The insecticide spirotetramat-enol binds to the CT domain of insect and mite ACC as shown by in vitro Michaelis Menten kinetic experiments (Lümmen et al., 2014). Specific mutations in the CT domains of plant ACCs are known to interfere with the binding of herbicidal inhibitors (Jang et al., 2013; Kaundun, 2014). However, generating the corresponding mutations in spider mite ACC (L1736I/A, and A1739V/S) did not affect the inhibition of spider mite ACC by spirotetramat-enol, possibly indicating different binding modes of the ketoenols and the herbicidal ACC inhibitors (Lümmen et al., 2014). Interestingly, a mutation (E645K) found between the BC and BCC domains in *Trialeurodes vaporariorum* has been associated with spiromesifen resistance (Karatolos et al., 2012). Similarly, a single non-synonymous substitution (A1079T) outside the CT domain is reported in spirodiclofen-resistant populations of the spider mite *Tetranychus urticae* (Khajehali, 2010; Wybouw et al., 2019), however metabolic resistance mediated by overexpressed carboxylesterases has been described to be of major importance in spider mites (Bajda et al., 2015; Wei et al., 2020). Finally, *ACC* overexpression and a combination of *ACC* overexpression and 14 non-synonymous mutations in the BC and CT domain were associated with low to moderate levels of spirotetramat resistance in nymphs of a laboratory-selected *Aphis gossypii* strain (Wei et al., 2016; Pan et al., 2017). The same authors also speculated that *CYP380C6*, an overexpressed cytochrome P450-monooxygenase, is possibly involved in spirotetramat resistance in *A. gossypii* (Pan et al., 2018).

A recent resistance monitoring study of *B. tabaci* populations collected in Spain has identified strains with high levels of resistance to spiromesifen and spirotetramat (Bielza et al., 2019). Interestingly, bioassays of these strains using inhibitors of common detoxification enzymes in combination with these insecticides failed to restore susceptibility, strongly suggesting that enhanced detoxification does not critically contribute to resistance to ketoenols in *B. tabaci* (Bielza et al., 2019). Thus, resistance is conferred by either a target-site mechanism or a metabolic mechanism that is not inhibited by the specific synergists used. To further investigate the molecular mechanisms of resistance to insecticidal ACC inhibitors in these strains in this study, we performed an RNAseq transcriptomic analysis to screen for known or novel ACC target-site mutations. We also conducted reciprocal crossings to explore the genetics of resistance. Furthermore, we engineered, by CRISPR/Cas9 genome editing, *Drosophila melanogaster* lines (see Douris et al., 2020; this issue for a comprehensive review of this approach) bearing candidate target site resistance mutations present in the *ACC* gene. These were used in toxicity bioassays to validate the role of the mutations in conferring resistance to different commercial ketoenols while avoiding confounding genetic effects. Lastly, we developed a diagnostic tool to in order to investigate the spread of the resistance allele in field populations found in major Spanish vegetable production areas.

2. Materials and methods

2.1. Chemicals

Spirodiclofen (PubChem CID 177863), spiromesifen (PubChem CID 9907412) and spirotetramat (PubChem CID 9969573) (Fig. 1) were of technical grade and provided in-house (Bayer AG, Monheim, Germany). Technical grade spiropidion (PubChem CID 58537978; Muehlebach et al., 2020) was synthesized in-house. For *Drosophila* feeding bioassays we used commercial formulations of spiromesifen, spirodiclofen and spirotetramat, i.e. Oberon 240 SC, Envidor 240 SC and Movento 150 OD (all from Bayer). Emulsifier W (EW; CAS No. 104376–72-9) used as a detergent was obtained from Lanxess (Leverkusen, Germany). All other chemicals and solvents used were of analytical grade and obtained from Sigma Aldrich (St. Louis, MO) unless otherwise stated.

2.2. Insect strains

The *B. tabaci* strains used in this study and their respective properties are shown in Table 1. All strains were maintained on cotton plants (*Gossypium hirsutum*) under greenhouse conditions at 25 ± 1 °C, 60% relative humidity and a photoperiod of 16 h light/8 h dark (L16:D8). All strains collected in Australia were maintained at conditions as described elsewhere (Hopkinson and Pumpa, 2019). *B. tabaci* biotypes were determined as described by Shatters et al. (2009).

Strain SUD-S is an insecticide susceptible reference strain, whereas strains UNAI and ESP07 are known to be resistant against neonicotinoid insecticides (Nauen et al., 2015). Strain R-SPI3 originates from strain ARO1 (Bielza et al., 2019) and was maintained under selection pressure on cotton plants treated with 200 mg L^{-1} spiromesifen.

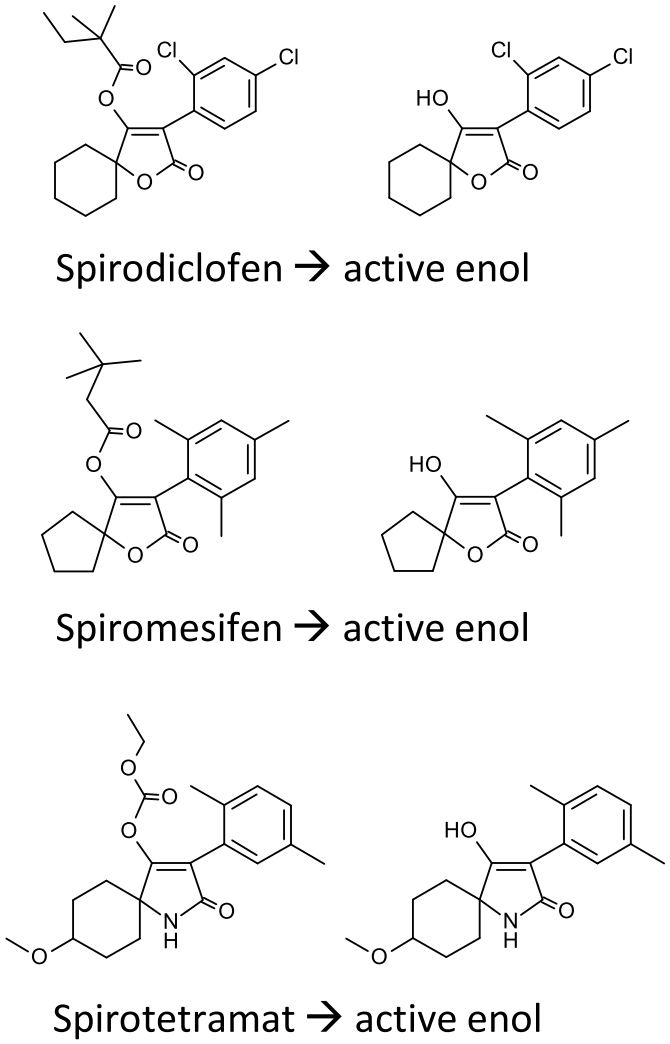

Fig. 1. Chemical structures of commercial tetronic- and tetramic acid derivatives and their activated enols known to inhibit acetyl-CoA carboxylase (ACC).

Table 1
Origin and characteristics of different strains of *Bemisia tabaci* used in this study.

Strain	Species	Country	Region	Crop	Year
SUD-S	–	Sudan	Lab (reference)	Unknown	1978
UNAI	MEAM1	Brazil	Unai	Bean	2009
ESP07	MED	Spain	Almeria	Pepper	2007
ARO1	MED	Spain	Murcia	Pepper	2014
R-SPI3	MED	Spain	ARO1 selected: 200 ppm spiromesifen continuously		
SU07–1	MEAM1	Australia	Canberra	Poinsettia	1995
Bowen-11	MEAM1	Australia	Queensland	Tomato	2011
Bowen-16	MEAM1	Australia	Queensland	Tomato	2016
Ayr	MEAM1	Australia	Queensland	Cantaloupe	2016
AY16-1R	MEAM1	Australia	Ayr selected: 1000 ppm spirotetramat continuously		

The Australian *B. tabaci* strains were maintained and bioassayed in the Department of Agriculture and Fisheries lab (Queensland, Australia). Strain SU07–1 is a laboratory reference strain collected in Australia in 1995 and has had no exposure to insecticides since the time of collection (Hopkinson and Pumpa, 2019). The Ayr and Bowen strains were collected in 2016 from cantaloupe in Queensland at 19.4840S 147.1331E and tomato at 20.0004S 148.1110E, respectively. Founding populations of around 200 insects were collected for each strain. The Bowen population collected in 2011 was from tomatoes. Strain AY16-1R was kept under constant selection pressure with 1000 mg L^{-1} spirotetramat.

The *Drosophila* strain used for CRISPR/Cas9 genome modification was strain y1 M{nos-Cas9.P}ZH-2A w* (Port et al., 2014), where Cas9 is expressed in the germ line under the control of *nanos* promoter; this strain is referred to below as nos.Cas9 (strain #54591 in Bloomington *Drosophila* stock center). The background strain *yellow white* (*yw*) and the 2nd chromosome balancer line *yw*; *CyO / Sco* are part of the IMBB/FORTH facility fly collection (kindly provided by Professor Christos Delidakis, IMBB and University of Crete). All flies were kept at 25 °C, 60–70% relative humidity and 12:12 h photoperiod on a standard fly diet.

2.3. B. tabaci toxicity bioassays

All Australian whitefly strains were tested in a leaf-dip bioassay as recently described (Hopkinson and Pumpa, 2019). All other strains were tested according to Nauen et al. (2008) with slight modifications. Briefly, bioassays were conducted on 3-week-old cotton plants. One leaf from cotton plants having two true leaves was removed and the remaining one was infested with adult whiteflies (approx. 150 insects per leaf) for 18–24 h. Afterwards the whiteflies were removed, and the plants kept at 22 °C, 60% relative humidity and a photoperiod of L16:D8 for 10–12 days to allow for 2nd (early 3rd) instar development. Infested leaves were treated with serial dilutions of ketoenol insecticides using a purpose-built spraying device. Ketoenols were applied as technical material of the highest purity available. Stock solutions of the technical material were prepared by dissolving 75 mg a.i. in 1.2 mL dimethylformamide incl. 12.5% emulsifier W, 150 µL 100 mg L^{-1} RME EW 500 (rape methyl ester) and 150 µL aqueous ammonia sulfate (AS) solution (1000 mg L^{-1}). Dilutions were prepared using aqueous RME EW 500 and AS (1 g L^{-1} each). Each concentration was tested thrice in three replicates and controls were treated with aqueous RME EW 500 and AS (1 g L^{-1} each). Mortality was scored based on eclosed adults and empty pupae, respectively 14 days after treatment. After correction for control mortality (Abbot, 1925) data were analysed by probit analysis using Polo PC (LeOra Software, CA).

2.4. Reciprocal crossing experiments

Leaves with nymphs close to emergence (red eye nymphs) were removed from the two populations selected for reciprocal crossing experiments, i.e. strains SU07–1 (susceptible) and AY16-1R (resistant and kept under selection pressure with 1000 mg L^{-1} spirotetramat). To obtain unmated individuals for the crosses, we separated nymphs prior to emergence. Sex of pupae was determined by size (Horowitz et al., 2003), with male pupa smaller than female. With the aid of a stereo microscope, male and female nymphs were segregated to obtain the following, male SU07–1, female SU07–1, male AY16-1R, and female AY16-1R. We then placed these sections of leaf into a cage (66 × 36 × 30 cm) containing a clean cotton plant and created the following parental populations AY16-1R ♀ × ♂ SU07–1 and SU07–1 ♀ × ♂ AY16-1R. Bioassays to test for ketoenol efficacy were done as described above with some modifications. Briefly, four strains were assayed, the susceptible (SU07–1), spirotetramat resistant (AY16-1R) and the hybrid progeny of the crosses (AY16-1R ♀ × ♂ SU07–1 and SU07–1 ♀ × ♂ AY16-1R). In these assays we used technical spirotetramat (0.1 g) dissolved in 10 mL acetone, then added to water with the adjuvant Agral (0.01% Agral). The concentration range tested was 1 to 1000 mg L^{-1} in ½ log increments, and a treated control (0.01% Agral solution) was included. Each concentration including control was tested in 5 replicates. Approximately 30 insects per experimental unit were tested and the range across all 4 bioassays was 15–36, i.e. 172–216 insects tested per replicate. The degree of dominance (D_{LC}) was calculated according to Bourguet et al. (1996):

$$D_{LC} = \frac{\log(LC_{50}RS)\text{-}\log(LC_{50}SS)}{\log(LC_{50}RR)\text{-}\log(LC_{50}SS)}$$

2.5. RNAseq and transcript annotation

Total RNA was extracted from whitefly adults of *B. tabaci* strains SUD−S, UNAI, ESP07A, ARO1 and R-SPI3 (5 mg each; and four biological replicates per strain) with TRIzol® reagent (Invitrogen, CA, USA) and followed by RNA purification with the RNeasy® Plus Universal Mini Kit (QIAGEN, Germany) according to the manufacturer's instruction including a genomic DNA eliminator column step. Library preparation was done with 3 µg of total RNA using KAPA Stranded mRNA-Seq Kit Illumina Platform (KAPA) and NimbleGen SeqCap Adapter Kit A and B (Roche) according to the manufacturer instructions. The RNA was quantified by spectrophotometry (NanoQuant Infinite 200Pro, Tecan, Switzerland). RNA sequencing of prepared *B. tabaci* libraries was conducted with the Illumina NextSeq 500/550 High output v2 sequencing reagent Kit (150 cycles) according to the manufacturer's instructions. The libraries were sequenced on an Illumina NextSeq 500 machine, and custom-made transcriptomes were created by assembling reads of each sample with Trinity 2014–04-13p1 (Grabherr et al., 2011). Assembled full-length *ACC* variants were mapped to scaffold 707 of the *B. tabaci* genome (Chen et al., 2016) spanning the *ACC* gene to check for non-synonymous mutations, particularly in the CT domain. The resulting transcripts were annotated with the RefSeq assembly ASM185493v1 and UniProt, and the longest ORFs of apparent *ACC* genes were translated to yield the protein sequences. The obtained protein sequences were aligned with Clustal Omega v1.2.3 (Sievers and Higgins, 2014). All assembled full-length *ACC* genes were submitted to NCBI and deposited in GenBank.

2.6. ACC expression analysis by RT-qPCR

Total RNA was extracted as described above and 2 µg RNA was used for cDNA synthesis using the iScript gDNA Clear cDNA Synthesis Kit according to the manufacturer's instructions. PCR reactions (20 µL) contained 5 µL of cDNA (25 ng), 10 µL of SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, USA), and 1 µM of each primer (Table S1). Samples were run on a CFX384 Real Time System (Bio-Rad) using the following temperature cycling conditions: 30 s at 95 °C followed by 35 cycles of 95 °C for 15 s, 60 °C for 15 s and 60 °C for 15 s. A final melt-curve step was included post-PCR (ramping from 65 to 95 °C by 0.5 °C every 5 s) to confirm the absence of any non-specific amplification. The amplification efficiency for each primer pair was assessed using a serial dilution of 25 ng to 0.04 ng of cDNA. Efficiency was over 99% for each primer pair used. Each qPCR experiment consisted of five independent biological replicates and three technical replicates. *ACC* expression data were analysed according to the ΔΔCT method (Pfaffl, 2001) using qbase + v3.1 (Biogazelle, Belgium). The following reference genes were used for normalization: *HSP90*, *PPIA*, and *EF-1α* (Li et al., 2013; Table S1).

2.7. Pyrosequencing diagnostic to genotype ACC alleles

Genomic DNA was extracted from individual whiteflies (n = 15–20) by disruption in 200 µL nuclease-free water using a 3-mm tungsten bead (Qiagen) in a bead mill (Retsch MM300) set on 25 Hz for two bursts of 30 s. The tube was then incubated for 6 min at 65 °C and subjected to another milling step for 15 s at 20 Hz. Subsequently the extract was transferred to a PCR plate and incubated for 5 min at 98 °C. For A2083V genotyping, a short fragment of 120 bp was amplified by PCR using 7 µL of sample homogenate as template. The PCR protocol comprised 25 µL reaction mixture containing 12.5 µL 1× Jump start Taq ReadyMix (Sigma), 0.125 µL each 0.5 µM forward and reverse primers (Table S1) and 5.25 µL nuclease free water. Cycling conditions were 3 min at 95 °C, followed by 45 cycles of 30 s at 95 °C, 45 s at 52 °C and 45 s at 72 °C, and a final elongation step of 5 min at 72 °C. For genotyping A2151V, a short 103 bp fragment was amplified following the same protocol as described above (for primer pairs see Table S1). The cycling conditions were 2 min at 94 °C, followed by 45 cycles of 30 s at 94 °C, 45 s at 55 °C and 45 s at 72 °C, and a final elongation step of 5 min at 72 °C. The pyrosequencing reactions were carried out according to the manufacturer's instructions using the PSQ 96 Gold Reagent Kit (Qiagen), and the sequencing primer ACC-A2083Vseq and ACC-A2151Vseq, respectively for genotyping (Table S1). The resulting pyrograms were analysed using PyroMark Q96 ID Software 2.5 (Qiagen).

2.8. Amplification and sequencing of ACC target regions in Drosophila

DNA from nos.Cas9 *Drosophila* adults was extracted with DNAzol (MRC) following the manufacturer instructions. Four primer pairs (ACC1F/ACC1R, ACC2F/ACC2R, ACC3F/ACC3R, ACC4F/ACC4R, Table S2) were designed based on the *ACC* gene sequence in order to amplify and sequence overlapping fragments that correspond to a 4254 bp sequenced genomic region of strain nos.Cas9 (2R: 7977099:7981352, numbering according to BDGP6 genome assembly, GenBank Acc. No.: AE013599.5) that contains the positions E793 (equivalent to *T. vaporariorum* E645), A1293 (equivalent to *T. urticae* A1079) and A2225 (equivalent to A2083 in *B. tabaci*). The amplification reactions were performed using KapaTaq DNA Polymerase (Kapa Biosystems). The conditions used were 95 °C for 2 min for initial denaturation followed by 30–35 cycles of denaturation at 95 °C for 30 s, annealing at 56 °C–58 °C for 30 s, extension at 72 °C for 90 s and a final extension step for 2 min. The PCR products were purified with a PCR clean-up kit (Macherey-Nagel, Germany) according to the manufacturer's instructions. Sequencing of the products was performed in both directions at Macrogen (Netherlands).

2.9. Strategy for genome editing

An ad hoc CRISPR-Cas9 strategy was implemented in order to generate *Drosophila* strains bearing each mutation of interest in the *ACC* gene. Based on the genomic sequence obtained for strain nos.Cas9 (see Section 2.8), several CRISPR targets in the corresponding regions were identified using the Optimal Target Finder online tool (http://tools.flycrispr.molbio.wisc.edu/targetFinder, Gratz et al., 2014). CRISPR targets which had minimal predicted off-target effects were selected.

In order to obtain the modification equivalent to E645K we used two sgRNA targets flanking the target sequence, namely LsgRNA645 and RsgRNA645 (Fig. S1). Similarly, to obtain A1079T we used LsgRNA1079 and RsgRNA1079, while a single target sgRNA2083 was used to obtain A2083V (Fig. S1). In order to generate the respective sgRNAs targeting those sequences, five different RNA expressing plasmids were generated (LsgRNA645/RsgRNA645, LsgRNA1079/RsgRNA1079 and sgRNA2083, respectively) based on the vector pU6-*Bbs*I chiRNA (Gratz et al., 2013) following digestion with BbsI and ligation of five relevant double stranded oligos, which were generated by annealing the corresponding single stranded sense/antisense oligos (Table S2). Following ligation and transformation, single colonies for each construct were picked and checked for the correct insert by performing colony PCR using T7 universal primer and the reverse primer for each dsDNA. The sequence of each sgRNA expressing plasmid was verified by sequencing (Macrogen, Amsterdam).

Three different donor plasmids, DonorE645K, DonorA1079T and DonorA2083V were synthesized de novo (Genscript) to facilitate Homologous Directed Repair (HDR) for the generation of strains E645K, A1079T and A2083V, respectively (newly synthesized sequences were subcloned in pUC57 vector *Eco*RV site). Each plasmid contains two ~900 bp homology arms flanking the target region between the respective sgRNA targets. The target regions were specifically designed in order to contain the desired mutations along with certain additional

synonymous mutations (Fig. S1) serving either as molecular markers (to facilitate molecular screening of CRISPR events), or to prevent unwanted CRISPR digestion of the donor itself.

For the generation of the E645K strain, the modifications introduced were synonymous changes in the target sequences identified by Cas9 endonuclease, the generation of an *Afl*II restriction site that specifically cuts mutant allele DNA and the removal of an *Xho*I restriction site, which would only cut non-modified (wild-type) allele DNA (Fig. S1). Similarly, for the generation of the A1079T strain, we generated synonymous modifications in the PAM and target sequences which introduced a specific *Pst*I site to the modified allele and removed a *Stu*I site that would cut only wild type allele DNA (Fig. S1). Finally, for the generation of the A2083V strain, we introduced synonymous modifications in the PAM and target sequences, generating a *Sac*I site specific for the modified allele, and deleting a *Nla*IV site that cuts only non-modified (wild-type) allele DNA (Fig. S1).

Furthermore, the introduction of specific mutations into the donor plasmids used for the generation of the genome modified alleles, enabled us to design specific diagnostic primer pairs in order to rapidly and efficiently screen for mutants. Thus, primer pair SpecFor645/SpecRev645 amplifying a diagnostic 1037 bp fragment was used for E645K screening, while primers ACC3F/SpecRev1079 generating a specific 690 bp fragment were used for A1079T screening, and primer pair GenFor2083/SpecRev2083 (Table S2) generating a diagnostic 224 bp fragment were used for screening A2083V.

2.10. Molecular screening and establishment of genome modified lines

Injection of nos.Cas9 pre-blastoderm embryos was performed at the IMBB/FORTH facility with injection mixes containing 75 ng μL^{-1} of each sgRNA plasmid vector and 100 ng μL^{-1} of donor template. Hatched larvae were transferred into standard fly artificial diet and after 9–13 days G_0 surviving adults were collected and individually backcrossed with nos.Cas9 flies. In order to screen for CRISPR events, G_1 generation progeny from each cross were pooled into batches of ~30 and genomic DNA extraction was performed *en masse* in order to be screened. Initially, 2 μg of pooled gDNA were digested with enzymes that cut only the wild type alleles but not potential mutant alleles in each DNA pool (XhoI, StuI and NlaIV for E645K, A1079T and A2083V respectively). We then performed PCR amplification with relevant generic and/or specific primer combinations (see Section 2.9) in order to generate relevant diagnostic fragments specific to genome modified alleles. PCR was performed with Kapa Taq polymerase as previously described using ~60 ng of digested template DNA mix. Crosses that proved positive for genome modified alleles were further explored in order to identify individual flies bearing mutant alleles and establish homozygous lines using a previously described crossing scheme (Douris et al., 2017). DNA was extracted from several homozygous or heterozygous (for E645K) adults, amplified by using relevant primer pairs and the amplification fragments containing the region of interest were sequence verified (Macrogen, Amsterdam, The Netherlands) for the presence of the desired mutations (Fig. 2).

2.11. Toxicity bioassays

Feeding bioassays were performed as described previously (Douris et al., 2016; Ffrench-Constant and Bass, 2017) to investigate the response to different ketoenols in *Drosophila* larvae. More specifically, 2nd instar larvae were collected and transferred in batches of 20 into new vials containing fly food supplemented with different insecticide concentrations. Larval development, molting, pupal eclosion and adult survival were monitored for a period of 10–12 days. Five to ten concentrations of each ketoenol that cause 5–95% mortality (when applicable) were tested in triplicate, together with relevant negative (no insecticide) controls. The same concentrations were tested both in genome modified (mutant) and wild-type (nos.Cas9) strains.

2.12. Data analysis

Dose dependent mortality curves for *Drosophila* as well as *B. tabaci* were constructed from dose-response data, and LC_{50} values, 95% confidence limits and slopes were calculated using PoloPlus (LeOra Software, Berkeley, California). The relative potency ratio among responses was calculated. Responses were considered significantly different when the 95% confidence interval of relative potency ratio did not include the value 1. Mortality was corrected for control mortality using Abbott's formula (Abbot, 1925). Sequence alignments and exon mapping was done using Geneious software v. 10.2.3 (Biomatters Ltd., New Zealand). Gene expression data were analysed by one-way ANOVA with post hoc Benjamini and Hochberg correction for significant differences between strains.

3. Results

3.1. B. tabaci nymph bioassays with ketoenols

Field-collected MED strain ARO1 showed a 4-fold and 77-fold resistance to spirotetramat and spiromesifen, respectively. After laboratory selection with 200 mg L^{-1} of spiromesifen, the resulting strain R-SPI3 exhibited significantly increased levels of resistance of 93-fold and > 640-fold against the commercial ketoenol insecticides spirotetramat and spiromesifen, respectively (Table 2). An additional bioassay was conducted with spiropidion, a novel not yet commercialized ketoenol of a different tetramic acid chemotype with a spiro *N*-methoxy piperidine ring substituent (Muehlebach et al., 2020). However, nymph bioassays revealed resistance ratios of 29-fold and > 750-fold against spiropidion in strains ARO1 and R-SPI3, indicating high levels of spiropidion cross-resistance selected by spirotetramat treatment in strain R-SPI3 (Table 2). A Spanish MED type strain ESP07 collected in 2007 was fully susceptible to all tested ketoenols, likewise a Brazilian MEAM1 strain, UNAI, when compared to the susceptible reference strain SUD-S (Table 2).

A second set of bioassays (leaf-dip instead of spraying) was conducted with Australian MEAM1-type strains Bowen-11, Bowen-16 and AY16-1R (lab selected with 1000 mg L^{-1} spirotetramat) in comparison to the susceptible reference strain SU07–1 (Table 3). Strain Bowen-11 collected in 2011 from Queensland tomatoes showed no resistance, whereas strain Bowen-16 collected five years later from tomatoes in the same region showed an LC_{50}-value for spirotetramat of > 1000 mg L^{-1} equating to a resistance ratio of > 165-fold. Similarly, strain AY16-R1, originating from a field population (Ayr) collected from cantaloupe in Queensland in 2016, maintained under constant spirotetramat selection pressure, showed a resistance ratio of > 165-fold (Table 3).

3.2. RNAseq, ACC assembly and variant calling in different B. tabaci strains

Illumina RNAseq analysis was conducted with five strains (SUD−S, UNAI, ESP07, ARO1 and R-SPI3) with > 22 Mio raw reads generated per strain. A complete overview of the statistics of the *de-novo* RNAseq assemblies is given in Table S3. An earlier study strongly suggested that enhanced detoxification did not critically contribute to the observed ketoenol resistance in strains ARO1 and R-SPI3 but rather implicated a mechanism based on target-site resistance (Bielza et al., 2019). Therefore, we concentrated our approach on *ACC* variant analysis for all individually analysed strains and biological replicates. The obtained full-length ACC consensus sequences for strains SUD−S, UNAI, ESP-07 and R-SPI-3 (ARO1 selected) contain 2342 amino acids, encompassing catalytic domains BC (PF02785.15; AA 499–605) and CT (PF01039.18; AA 1658–2210). Sequences are shown in Fig. S2 and were submitted to GenBank; the respective accession numbers are MN567040, MN567041, MN567038 and MN567039, respectively (Fig. S2). We identified only two non-synonymous mutations in the fully assembled

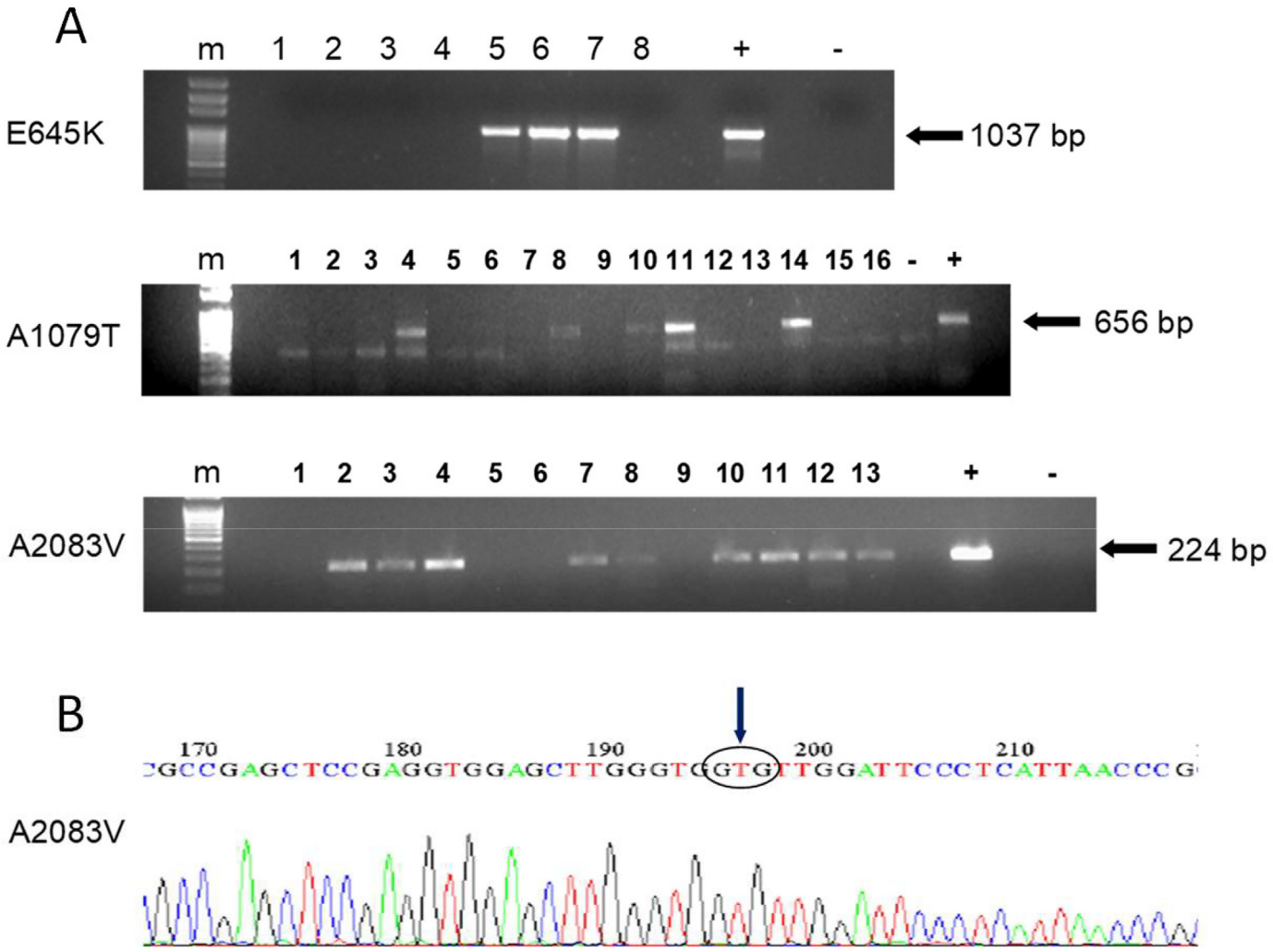

Fig. 2. Screening for genome modified flies. (A) (top): Screening of individual E645K flies (1–8, G3 crosses to yw, CyO / Sco) by DNA amplification with primer pair SpecFor645/ SpecRev645 to generate an expected product size of 1037 bp. (middle): Screening of individual A1079T flies (1–14, G2 crosses to nos.Cas9) by DNA amplification with primer pair ACC3F/ SpecRev1079 with an expected product size of 656 bp. (bottom): Screening of individual A2083V flies (1–13, G3 crosses to yw, CyO / Sco) by DNA amplification with primer pair GenFor2001/SpecRev2001 with an expected product size of 224 bp +: positive control (donor plasmid). -: negative control (nos.Cas9 DNA template). m: molecular weight marker. (B): Sequencing of a homozygous A2083V individual (line 14.3). The arrow points at the nucleotides encoding Val (GTG, encircled) instead of Ala (GCC) at position 2225 of the Drosophila ACC amino acid sequence (equivalent to A2083 in *B. tabaci* and A2001 in *Saccharomyces cerevisiae*).

Table 2
Log-dose probit-mortality data for three ketoenol insecticides (a.i.) against 2nd/ 3rd instar nymphs of different strains of *Bemisia tabaci* after foliar application. The resistance ratio (RR) is calculated by dividing the LC_{50} of respective strain by the LC_{50} of strain SUD−S.

Insecticide	Strain	LC_{50} [mg L^{-1}]	CL95%	Slope ± SE	RR
Spiromesifen	SUD-S	1.56	1.09–2.21	2.19 ± 0.18	
	UNAI	2.57	2.10–3.13	2.02 ± 0.16	1.6
	ESP07	2.40	2.05–2.81	3.31 ± 0.33	1.5
	ARO1	120	45.7–1010	0.93 ± 0.10	77
	R-SPI3	> 1000	–	–	> 640
Spirotetramat	SUD-S	2.72	2.24–3.29	2.20 ± 0.19	
	UNAI	1.84	1.58–2.16	3.38 ± 0.38	< 1
	ESP07	3.00	1.67–5.77	3.51 ± 0.33	1.1
	ARO1	10.1	6.29–15.9	1.22 ± 0.083	3.7
	R-SPI3	254	219–298	3.48 ± 0.39	93
Spiropidion	SUD-D	1.33	1.11–1.59	2.56 ± 0.23	
	UNAI	3.12	2.58–3.75	2.30 ± 0.20	2.3
	ESP07	1.90	1.44–2.51	1.87 ± 0.14	1.4
	ARO1	38.4	32.1–46.0	2.52 ± 0.23	29
	R-SPI3	> 1000	–	–	> 750

Table 3
Log-dose probit-mortality data for formulated spirotetramat (Movento 240SC) against 2nd/3rd instar nymphs of Australian MEAM1 strains of *Bemisia tabaci* after foliar application. Strain AY16-1R was kept under constant selection pressure using 1 g L^{-1} spirotetramat (Movento 240SC).

Insecticide	Strain	Species	LC_{50} [mg L^{-1}]	CL95%	RR
Spirotetramat	SU07–1	MEAM1	6.05	4.85–7.36	
	Bowen-11	MEAM1	3.59	2.43–4.56	1
	Bowen-16	MEAM1	> 1000	–	> 165
	AY16-1R	MEAM1	> 1000	–	> 165

ACC gene of the highly resistant strain R-SPI3 mapping to exon 28 of the *ACC* gene located on scaffold 707 of the recently assembled *B. tabaci* genome (Chen et al., 2016; Fig. S3). The resulting amino acid substitutions were A2083V and A2151V, whereby A2083 is located in a highly conserved region and A2151 in a much less conserved region among different insect and spider mite species (Fig. 3). Next, we genotyped 15–20 individual whiteflies per strain by pyrosequencing analyses for the presence of the detected mutations in the respective CT domain (Table 4). Strains SUD−S, UNAI and ESP07 were homozygous

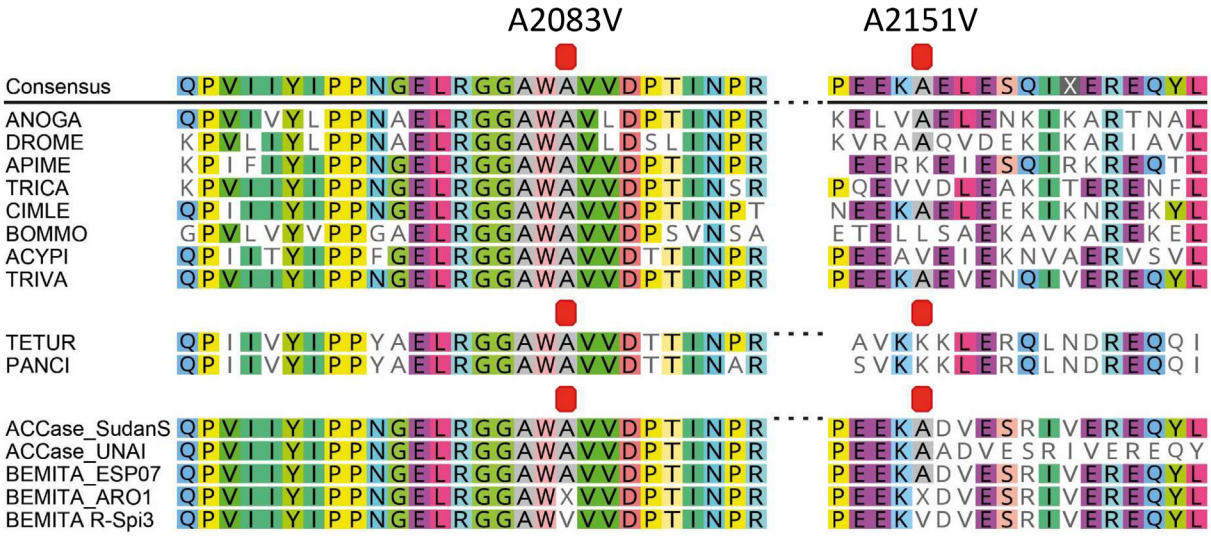

Fig. 3. Amino acid alignment of a partial sequence of the carboxyl transferase domain of ACC of different organisms. The position of the ACC A2083V and A2151V mutation sites detected in ketoenol resistant *Bemisia tabaci* strain R-SPI3 are marked by a red dot. GenBank accession numbers for the different sequences are given in the table next to the alignment. GenBank accession numbers of full length ACC genes of strains SUD−S, UNAI, ESP07 and R-SPI3 are MN567040, MN567041, MN567038 and MN567039, respectively. Abrreviations: ANOGA *Anopheles gambiae* (XP_001688518), DROME *Drosophila melanogaster* (NP_610342), APIME *Apis mellifera* (XP_006565238), TRICA *Tribolium castaneum* (XP_008194740), CIMLE *Cimex lectularius* (XP_014259658.1), BOMMO *Bombyx mori* (XP_012549774), ACYPI Acyrtosiphon pisum (XP_003245354), TRIVA *Trialeurodes vaporariorum* (Karatolos et al., 2012), TETUR Tetranychus urticae (AFQ61042), and PANCI Panonychus citri (AID68367.1). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 4
Genotyping by pyrosequencing of individual female adults of *Bemisia tabaci* for the presence and frequency of ACC A➔V mutations in the carboxyltransferase domain at amino acid positions A2083 and A2151. All three ketoenol susceptible strains (SUD−S, UNAI and ESP07) were A2083 homozygous wildtype. ARO1(S) represent survivors of 2nd/3rd instar nymphs of strain ARO1 sprayed with a high dose of 1000 mg L^{-1} spiromesifen. Bowen-16 and AY16-R1 are strains from Australia that are highly resistant to ketoenols.

Strain	ACC 2083			ACC 2151		
(Genotype, %)	A/A	A/V	V/V	A/A	A/V	V/V
SUD-S	100	0	0	100	0	0
UNAI	100	0	0	100	0	0
ESP07	100	0	0	40	20	40
ARO1	67	29	4	23	48	29
ARO1(S)	0	80	20	0	47	53
R-SPI3	0	0	100	0	0	100
Bowen-16	0	27	73	100	0	0
AY16-1R	0	14	86	100	0	0

for A2083, whereas R-SPI3 was expectedly homozygous V2083. Strain ESP07 – fully susceptible to ketoenols – consists of different A2151/V2151 genotypes, i.e. 23% homozygous for A2151 29% homozygous for V2151 (29%) and 48% heterozygous for A2151/V2151, suggesting that A2151V is unlikely to be causal for ketoenol resistance. Strain ARO1 were of mixed A/V2083 genotypes and when treated with spiromesifen at 1000 mg L^{-1} the survivors (ARO1(S)) were either heterozygous A/V2083 or homozygous V2083 (Table 4). Individuals of strain R-SPI3 were all homozygous for V2083 and V2151, whereas highly resistant individuals of the Australian strains Bowen-16 and AY16-R1 were mostly homozygous for V2083 homozygous (73% and 86% homozygous respectively) and fully homozygous for A2151, supporting a causal role for A2083V in ketoenol resistance.

3.3. ACC expression level

ACC expression level in adults of strains SUD−S, UNAI, ESP07, ARO1 and R-SPI3 was normalized to EF-1α and HSP90 reference genes and relative to the susceptible strain (SUD−S). No significant differences in expression was observed between strains ($P > .05$) (Fig. 4).

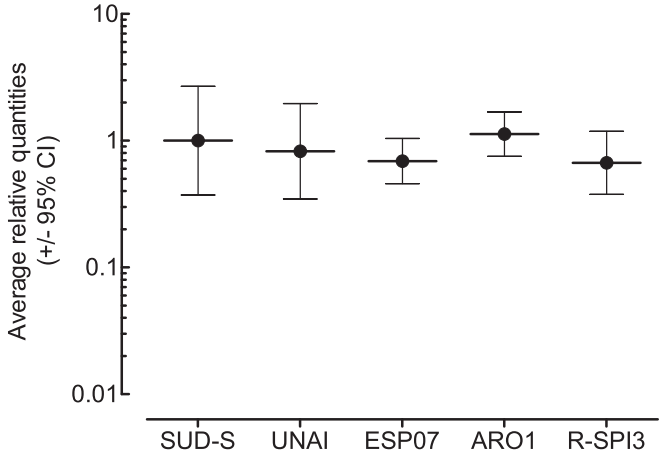

Fig. 4. Expression level (log scale) of ACC in adults of different strains of *Bemisia tabaci*. The expression level was normalized to the EF-1α and HSP90 reference genes and is shown relative to the susceptible strain (SUD−S). Data were analysed by one-way ANOVA with post hoc Benjamini and Hochberg correction and revealed no significant differences between strains ($P > .05$).

3.4. Genetics of ketoenol resistance

Reciprocal crosses of Australian strains SU07–1 (fully susceptible) and AY16-R1 (highly resistant) revealed autosomal inheritance, but slight differences in LC_{50}-values between F1 progeny were observed. The estimated degree of dominance (D_{LC}) in F1 ♀SU07–1 × ♂AY16-1R and F1 ♀AY16-1R × ♂SU07–1 heterozygotes were < 0.93 and 1.0, suggesting an almost completely dominant mode of inheritance, i.e. heterozygotes express a resistant phenotype (Table 5).

Table 5
Log-dose probit-mortality data for spirotetramat (a.i.) tested against 2nd/3rd instar nymphs (mixed sex) of resistant (AY16-1R), susceptible (SU07–1) and reciprocal F1 crosses of *Bemisia tabaci*.

Strain	LC_{50} (mg L^{-1})	CL95%[a]	Slope ± SE	RR[b]	D_{LC}[c]
SU07–1	2.67	2.01-3.49	2.45 ± 0.33		
AY16-1R	> 1000	–	–	> 375	
F1 ♀SU07–1 x ♂AY16-1R	647	287–2310	0.45 ± 0.062	242	< 0.93
F1 ♀AY16-1R x ♂SU07–1	> 1000	–	–	> 375	1.0

[a] Confidence limits, 95%.
[b] Resistance ratio (LC_{50} of AY16-1R or F1 strain divided by LC_{50} of SU07–1).
[c] Degree of dominance (according to Bourguet et al., 1996).

3.5. CRISPR/Cas9 in Drosophila

In order to investigate the contribution of different mutations to the resistant phenotypes, we generated genome modified fly lines bearing the equivalent of the A2083V mutation identified in resistant *B. tabaci*. For comparison we also generated the mutation A1079T previously reported in *T. urticae* ACC, as well as the E645K mutation previously identified in *T. vaporariorum*. We identified the orthologous *ACC* gene in *Drosophila* and in order to generate the relevant mutations (Fig. S1) we injected nos.Cas9 embryos with the appropriate gRNAs/donor plasmid mixes and screened progeny for genome modified alleles. For the E645K mutation, there were indications for the presence of HDR-derived alleles within the sample in five out of forty-six different lines that gave G_1 progeny, for the A1079T mutation four out of thirty-one lines, and for the A2083V sixteen out of thirty-three lines. G_1 individuals from each of these positive original (G_0) lines were crossed to balancer flies and screened to identify positive heterozygotes (Fig. 2). Several independent lines were established and at least one became readily homozygous after balancing for the A1079T and A2083V substitutions, however, none of the five independent lines yielded homozygous progeny for the E645K mutation. Sequence verification of the relevant genomic region showed all lines to be genome-modified as expected, bearing the correct mutation and other inserted markers at the target region of the *ACC* gene. Homozygous, sequence verified lines carrying A1079T or A2083V alleles were selected for further insecticide toxicity bioassays.

3.6. The Drosophila line bearing T. urticae mutation A1079T does not exhibit resistance to spirodiclofen

Toxicity assays with *Drosophila* larvae against spirodiclofen were carried out for two different strains bearing the A1079T genome modification using wild-type nos.Cas9 flies as controls. *Drosophila* larvae were in continuous contact with the insecticide-containing food. Since the fly larvae are not readily visible in the food, we considered pupation efficiency as a measurable proxy of eventual survival. The results showed that the fly strain bearing the A1079T mutation exhibits no differences in sensitivity to spirodiclofen relative to the control (nos.Cas9) flies carrying the wild-type *Drosophila* allele - LC_{50} for nos.Cas9 of 3.83 mg L^{-1} [95% CL 2.41, 7.31] versus 3.36 mg L^{-1} [95% CL 2.97, 3.67] for A1079T flies. It must also be noted that A1079T flies exhibit slower development (c. 15 days from egg to adult at 25 °C compared to 10–11 days for the nos.Cas9 background strain – data not shown).

3.7. Drosophila lines bearing the A2083V mutation of B. tabaci exhibit high levels of resistance to spirodiclofen, spiromesifen and spirotetramat

Toxicity assays with *Drosophila* larvae against all three ketoenols were carried out for three different strains bearing a homozygous A2083V genome modification that resembles the allele identified in resistant *B. tabaci* populations, using wild-type nos.Cas9 flies as the control. The LC_{50} values (with their corresponding 95% confidence intervals) and the associated resistance ratios against all three commercially available ketoenol compounds are shown in Table 6. The resistance of the A2083V strain vs nos.Cas9 flies against spirodiclofen and spiromesifen was found to be remarkably high (with resistant larvae manage to pupate at insecticide concentrations as high as 5000 μg mL^{-1}) generating resistance ratios of > 3600 against spiromesifen and > 890 against spirodiclofen. Similarly, a very high (yet measurable) resistance ratio is recorded against spirotetramat (RR: 874). Notably, this is despite the fact that A2083V flies also exhibited slower development (14–16 days from egg to adult) as well as somewhat increased overall mortality compared to nos.Cas9 strain of the same genetic background. Furthermore, while nos.Cas9 larvae do not survive ketoenol toxicity bioassays (for all three compounds tested), A2083V flies are able to complete pupation and emerge as adults; however, more than half of the emerging adults die during emergence while the remainder die in early adulthood, indicating that the resistance phenotype does not fully extend to adults, neither comes without a strong fitness cost, at least in *Drosophila*.

Table 6
Log-dose probit-mortality data for different commercial ketoenol insecticides tested against Drosophila larvae of genome-modified strain A2083V and non-modified wild-type control (nos.Cas9). LC_{50}-values are given in mg L^{-1}. The resistance ratio (RR) is calculated by dividing the LC_{50} of strain A2083V by the LC_{50} of strain nos.Cas9.

Compound	Strain	LC_{50} (CL95%)	Slope ± SE	RR
Spiromesifen	nos.Cas9	1.38 (1.22–1.53)	5.32 ± 0.548	1
	A2083V	> 5000	n/a	> 3616
Spirodiclofen	nos.Cas9	5.62 (5.35–5.87)	8.83 ± 0.842	1
	A2083V	> 5000	n/a	> 890
Spirotetramat	nos.Cas9	1.01 (0.908–1.14)	5.69 ± 0.953	1
	A2083V	882 (378–9770)	0.972 ± 0.351	874

3.8. Genotyping of Spanish field-collected B. tabaci strains by pyrosequencing

We developed and validated a pyrosequencing method (Fig. S4) based on genomic DNA for high-throughput ACC resistance allele detection allowing us to test field-collected samples preserved in alcohol. In order to determine the frequency of ACC target-site mutations in *B. tabaci* populations in Spain we collected 30 field strains in greenhouses in Almeria, Murcia and Sevilla in Ffrench-Constant and Bass, 2017. Genotyping by pyrosequencing of 15 individuals per sample revealed the presence of the resistance allele, i.e. V2083 homozygotes or A/V2083 heterozygotes at high frequency in populations from several places (Fig. 5).

4. Discussion

We have investigated and characterized levels of ketoenol resistance in different cryptic species and strains of *B. tabaci*, including a MED strain (R-SPI3) exhibiting strong resistance to spirotetramat (93-fold) and spiromesifen (> 640-fold). An earlier study indicated that enhanced detoxification was not a major component of resistance to ketoenols in *B. tabaci*, suggesting ACC target-site resistance may be the underpinning mechanism (Bielza et al., 2019). A comparison of the sequences between the fully assembled *ACC* gene obtained by RNAseq revealed two non-synonymous mutations that were found only in the resistant strains: an alanine to valine substitution at position 2083 (equivalent to A2083V) is located in a highly conserved region of the ACC gene in exon 28 and an alanine to valine substitution at position

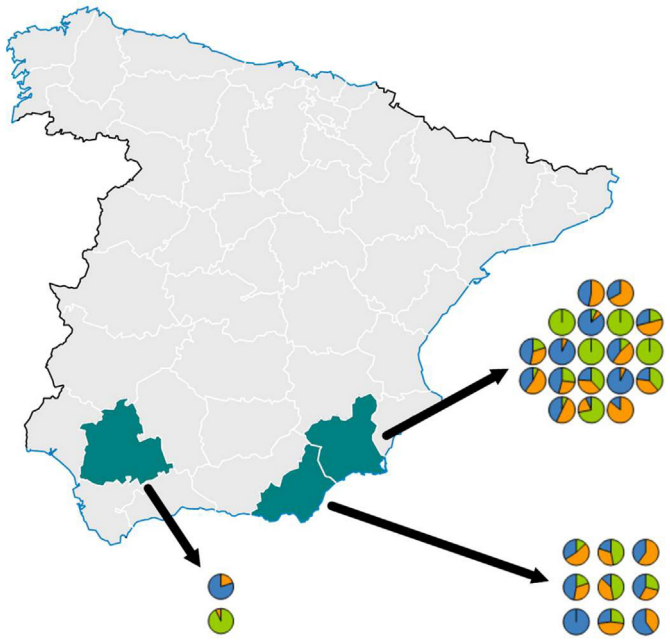

Fig. 5. Genotyping by pyrosequencing for the presence of the ACC target-site mutation A2083V in adults of 30 *Bemisia tabaci* populations collected from different regions in Spain in 2017. Pie charts display the proportion of genotypes assigned to RR resistant homozygotes (blue), SR heterozygotes (orange) and SS susceptible homozygotes (green). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2151 (A2151V) is located in a much less conserved region (Fig. 3).

Genome edited *Drosophila* flies bearing the A2083V mutation in their orthologous ACC gene provided compelling evidence for the causal role of this mutation in the resistance phenotype. Several independent homozygous lines were established for the A2083V mutation. These flies exhibited slower development and a clear reduction in fitness compared to nos.Cas9 control flies of the same genetic background but without the mutation. Toxicity assays with the transgenic lines revealed that the mutation confers potent resistance to spirodiclofen, spiromesifen and spirotetramat i.e. resistance ratios of > 3600- and > 890- and 874-fold, respectively relative to the control line. Consistent with this, a combination of forward genetics and genome editing approaches using the model nematode *C. elegans* showed that the same mutation (homologous mutation A1847V in *C. elegans*) in the CT domain of pod-2 (ACC) confers spirotetramat resistance (Guest et al., 2020, this issue). Interestingly, spirotetramat is less affected when compared to the other tested ketoenol insecticides in both whiteflies and transgenic Drosophila. Further docking studies and ACC homology modelling may provide structural insights into whether this is due to differences in binding to the CT domain target-site.

The ACC mutation and the associated striking ketoenol resistance phenotype has been observed locally (Spain) (Bielza et al., 2019), but it seems that it has also high potential to spread to other places such as Australia. Indeed, both phenotypic and genotyping data suggest that the mutation evolved independently in two different geographies and cryptic species (MEAM1 and MED), probably reflecting high selection pressure from the use of ketoenols for control.

Experiments to shed light on the genetics of ketoenol resistance were carried out and the observed differences in LC_{50} values between the reciprocal crosses is most likely due to the reproduction system with diploid females and haploid males, because nymphs of mixed sex were tested. As males result from unfertilized eggs one would expect a certain proportion of S males along with RS females from the F1 ♀SU07–1 (SS) x ♂AY16-1R (R) cross, thus explaining why this cross is more sensitive to spirotetramat than the other (eggs of unfertilized ♀AY16-1R (RR) would develop into ♂R). The autosomal dominant mode of inheritance also has major implications for IRM, as it facilitates the rapid spread of resistance. In this context it would be important to know whether the observed fitness costs in *Drosophila* are also present in whiteflies. Although the Drosophila approach is very powerful in validating the causal link between mutations and resistance phenotypes (Douris et al., 2020, this issue), fitness costs of resistance mutations are probably better assessed in the pest where they are uncovered, for example via near isogenic lines (NILs) as previously outlined (ffrench Constant and Bass Ffrench-Constant and Bass, 2017; Bajda et al., 2018).

Previous studies had reported mutations that correlated with resistance in *T. vaporariorum* (E645K) and the spider mite *T. urticae* (A1079T), but both mutations are located outside the ACC CT domain that interacts with ketoenols (Lümmen et al., 2014). In addition, a number of mutations in *A. gossypii* were reported, of which none seemed relevant based on their position (Fig. S5) (Pan et al., 2017). Based on our experiments with *Drosophila*, there is no evidence of a causal role of A1079T in resistance, while for E645K no homozygotes could be generated precluding functional analyses and suggesting that introduction of the relevant point mutations in the *Drosophila* orthologue may be constrained by sequence context. The A1079T mutation was uncovered via an unbiased genomic mapping approach in a spirodiclofen resistant line, that pointed toward 3 QTLs, including the location of ACC (Wybouw et al., 2019). QTLs are fairly large genomic regions and although other relevant genes were not reported in the *ACC* QTL, it still remains possible that resistance is caused by other genes, or even by non-coding genetic changes. However, given the similar location of the whitefly and plant mutations providing resistance to ACC inhibitors, it now seems less likely that the lack of phenotype is due to limitations of *Drosophila* as a system. As CRISPR-Cas9 genome editing is now possible in the spider mite *T. urticae* (Dermauw et al., 2020), it might be feasible in the near future to test these hypotheses by introducing this mutation in a spider mite susceptible line.

Similarly, despite the lack of homozygous E645K *Drosophila* lines that would provide definitive evidence regarding the potential role of this mutation in ketoenol resistance, the fact that it is located outside the CT domain argues for caution. Nevertheless, as this mutation in *T. vaporariorum* alters a protein kinase C site, and the effect of this substitution is hence potentially sequence and species specific in allosteric modulation of ACC, genome editing in *Drosophila* might not be a suitable surrogate for functional characterization. Importantly however, a more recent study failed to associate the frequency of the E645K mutation in *T. vaporariorum* with spiromesifen resistance in field populations from southern Greece (Kapantaidaki et al., 2018). Thus, it is plausible that E645K is not a bona fide ketoenol resistance mutation in contrast to early reports (Karatolos et al., 2012). The possible presence of the A2083V in *T. vaporariorum* field populations which have recently developed higher levels of ketoenol resistance remains to be investigated.

In conclusion while the possibility of alternative/additional mutation(s), or additional mechanism(s) associated or contributing to the ketoenol resistance phenotype in whiteflies or other target species should not be discounted our work clearly illustrates the important role of A2083V in conferring resistance to this insecticide group. Given this the pyrosequencing genotyping method that was developed and validated in this study, based on genomic DNA for high-throughput *ACC* resistance allele detection, provides a powerful tool to test field-collected samples preserved in alcohol, in order to guide evidence-based IRM.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pestbp.2020.104583.

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