Next-generation molecular diagnostics (TaqMan qPCR and ddPCR) for monitoring insecticide resistance in *Bemisia tabaci*

Short running title: ddPCR diagnostics for *Bemisia tabaci* pooled samples

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ABSTRACT

BACKGROUND: Insecticide resistance has developed in several populations of the whitefly *Bemisia tabaci* worldwide and threatens to compromise the efficacy of chemical control. The molecular mechanisms underpinning resistance have been characterised and markers associated with the trait have been identified, allowing the development of diagnostics for individual insects.

RESULTS: TaqMan and Droplet Digital PCR (ddPCR) assays were developed and validated, in individual and pooled whitefly samples, respectively, for the following target-site mutations: the acetylcholinesterase (*ace1*) F331W mutation conferring organophosphate-, the voltage-gated sodium channel (*vgsc*) mutations L925I and T929V conferring pyrethroid- and the acetyl-CoA carboxylase (*acc*) A2083V mutation conferring ketoenol- resistance. The ddPCR's Limit of Detection (LoD) was <0.2% (i.e., detection of 1 heterozygote whitefly in a pool of 249 wild type individuals). The assays were applied in 11 of *B. tabaci* field populations from four locations in Crete, Greece. The F331W mutation was detected to be fixed or close to fixation in 8/11 *B. tabaci* populations, and at lower frequency in the remaining ones. The pyrethroid resistance mutations were detected in very high frequencies. The A2083V spiromesifen resistance mutation was detected in 8/11 populations (frequencies=6.16%-89.56%). Spiromesifen phenotypic resistance monitoring showed that the populations tested had variable levels of resistance, ranging from full susceptibility to high resistance. A strong spiromesifen resistance phenotype-genotype (A2083V) correlation (r_s = -0.839, p = 0.002) was observed confirming the ddPCR diagnostic value.

CONCLUSION: The ddPCR diagnostics developed in this study are a valuable tool to support evidence based rational use of insecticides and resistance management strategies.

Keywords: ddPCR; TaqMan diagnostics; agricultural pests; resistance monitoring; target site resistance

1. INTRODUCTION

The silverleaf whitefly, *Bemisia tabaci* Gennadius (Homoptera: Aleyrodidae), is a major pest of economic importance worldwide. It feeds on over 700 plant species[1-3] and damages crop plants by sucking phloem sap and transmitting plant viruses[4, 5].

The application of chemical insecticides is the primary means of control of *B. tabaci*. Several insecticides, belonging to different modes of action (MoA), such as modulators of insect nicotinic acetylcholine receptors (nAChRs) (neonicotinoids), sodium channel modulators (pyrethroids), acetylcholinesterase (AChE) inhibitors (organophosphates - OPs) and, more recently, lipid biosynthesis inhibitors (ketoenols) have been used for the control of *B tabaci* populations worldwide. The intensive use of insecticides against *B. tabaci* has led to the development of resistance to most of the insecticides employed for its control, with over 688 examples of insecticide resistance to more than 67 active components recorded for this species[6, 7].

Target site mutations represent a major resistance mechanism that can provide highly relevant information for resistance monitoring. Target site mutations are well characterized in *B. tabaci* and have been shown to confer strong resistance phenotypes providing clear predictive value for resistance. For example, the Acetyl-coenzyme A carboxylase (*ACC*) mutation A2083V, associated with resistance to ketoenols (known inhibitors of ACC, a key enzyme for lipid biosynthesis)[8] has been functionally demonstrated to confer potent resistance to commercially available ketoenols, i.e., a resistant ratio (RR) for spiromesifen of >890, a RR for spirotetramat of >874 and a RR for spirodiclofen of >3600[8]. Additional validated target-site mutations in *B. tabaci* have been described in genes encoding the acetylcholinesterase enzyme (*Ace-1:* F331W), associated with resistance to acetylcholinesterase inhibitors (organophosphates (OPs) and carbamates) (MoA: AChE inhibitors)[9] and the voltage-gated sodium channel (*VGSC*/L925I, T929V), associated with resistance to pyrethroids (MoA: Sodium channel modulators)[10, 11]. Metabolic resistance has also been reported in *B. tabaci*. Most notably, the overexpression of the

cytochrome P450 monooxygenase CYP6CM1, leads to increased detoxification of neonicotinoids and additionally confers cross resistance to other chemical classes of insecticide (e.g., pymentrozine, pyriproxyfen). A field applicable immunostrip kit, which detects the protein levels of CYP6CM1, has been developed as a rapid resistance monitoring tool to support *B. tabaci* oriented insecticide resistance management (IRM) strategies[12].

Insecticide resistance monitoring is an important prerequisite for the application of evidence-based IRM strategies, in order to sustain the effectiveness of chemical control applications. Bioassays have been traditionally used for this purpose, but their application comes with limitations, such as the inability to pinpoint specific resistance mechanisms, the maintenance of live pest populations and low sensitivity [13, 14].

Molecular diagnostics that are built on robust markers based on scientific evidence of the underlying molecular mechanisms mutations can complement bioassays and can prove a powerful tool in modern IRM. Target site mutations are currently frequently assayed via classical diagnostic methods, such as Sanger sequencing, allele specific PCR (PASA) and PCR-RFLP[11, 13-15]. Such assays are typically used to genotype individual pest insects. However, the use of diagnostics to test pooled samples can significantly increase practicality and cost-effectiveness of monitoring efforts, as we have previously shown for vector monitoring[16, 17]. Recently, we used the latest generation of PCR, i.e., droplet digital PCR (ddPCR), to develop and apply highly sensitive diagnostics for the accurate detection and monitoring of emerging low-frequency insecticide resistance mutations in pooled samples of *T. urticae*[18]. DdPCR allows the accurate detection of very small amounts of target DNA within a large background of non-target DNA[19, 20], and thus is an ideal method for assaying target site mutations even in large pooled samples[13, 16, 18].

Here, we developed, validated and applied ddPCR diagnostics for detecting insecticide resistance mutations in bulk pooled samples of several *B. tabaci* populations from Crete, Greece. We then

validated the diagnostic value of the molecular assays using insecticide bioassays for the compound spiromesifen and the *ACC* mutation A2083V.

2. MATERIALS AND METHODS

2.1 *B. tabaci* field populations and control samples

Eleven field *B.tabaci* populations were collected from four distinct locations of Crete, Greece, from 2017 to 2020 and utilized in our analyses (Suppl. Table S1). The biotype status was assessed according to Tsagkarakou et al, 2007[21] and it was found that all populations belonged to the *B. tabaci* MED species, mitochondrial type Q1. Bt1 was collected from cucumbers in Ierapetra (insecticide application history not documented). Bt2 was collected from infested eggplants in Ierapetra, with an application history of sulfoxaflor, spirotetramat, acetamiprid and pymetrozine treatments. Bt3 was also collected from eggplants in Ierapetra with an application history of spiromesifen, spirotetramat, flonicamid, pymetrozine and flupyradifurone. Bt4 was collected from cucumbers in Tympaki, with an application history of spirotetramat, sulfoxaflor and flonicamid. Bt5 was collected form eggplants in Tympaki where only flonicamid has been applied to control the infestation. Bt6 was collected from melons in Tympaki, with an application history of sulfoxaflor, spirotetramat and thiamethoxam. Bt7 was also collected from melons in Tympaki, with an application history of spirotetramat, deltamethrin, thiamethoxan and pymetrozine. Bt8 was collected from cucumbers in Ierapetra, with an unknwon application history. Bt9 was collected from peppers in Ierapetra with an application history of the ketoenols spirotetramat and spiromesifen. Bt10 was collected from roses in Rethymnon with an application history of sulfoxaflor. Bt11 was collected from eggplants in Ierapetra, with an application history of acetamiprid, flupyradifurone, pyriproxifen, spirotetramat and spiromesifen (Suppl. Table S1). Application history refers to the last season prior to whitefly collection. All populations were

reared on cotton plants in standard insectary conditions (23–26 °C, 14:10 light:dark photoperiod and 70% relative humidity) for 1-5 generations before they were used.

Individually extracted and sequenced *B. tabaci* DNAs available from a previous study by Tsagkarakou et al[15] were used as controls, for the following mutations: *Ace1* F331W and *Vgsc* L925I and T929V. For the *Acc* A2083V assay, we sequenced individual whitefly samples (N =30 in total, Suppl. Table S3) in the framework of the current study (section 2.4) from resistant and susceptible populations collected in Greece and Spain, which were employed as control samples.

2.2 Toxicity assays

Spiromesifen (Oberon 240SC), was applied on nymphs as described in Ilias et al [22]. Briefly, sixty mature whiteflies, of mixed sex, were placed in ventilated boxes with leaves and allowed to oviposit for 24 hours. The whiteflies were subsequently removed, and the eggs observed on a daily basis until they developed into 2nd instar nymphs. At least three leaves were dipped in the insecticide dilution for 5 s, and mortality was calculated after 10-12 days. Nymphs that developed into pupae or adults (emerged) were considered alive [22]. The recommended field dose (RD) of spiromesifen was applied (150 mgL^{-1}). The percentage mortality for each insecticide was corrected using Schneider-Orelli's formula [23], i.e., Corrected % mortality = [(mortality % in treatment- mortality % in control) / 100 - mortality % in control)] x 100.

2.3 Extraction of gDNA

Extraction of gDNA was performed on individuals and pools of adult whiteflies, following the CTAB method as previously described.[24] The concentrations of extracted gDNAs were determined with the Qubit™ dsDNA BR Assay (Invitrogen, Carlsbad, CA) on a Qubit fluorometer 2.0 (Invitrogen, Carlsbad, CA). The mean ± SE double stranded DNA (dsDNA) concentration was 3.01 ± 0.69 ng/μL. The gDNA samples were stored at -20ºC until analysis.

2.4 Genotyping of *ACC* A2083V control samples by sequencing

Individual whiteflies from resistant and susceptible populations of Greece and Spain (N =30 in total, Suppl. Table S3) were used as control samples for the *Acc* A2083V assay. Two primers were designed (F: 5'-AAATTCGGTGCCTACATCGT-3', R: 5'-CTGCTTTCGGGATCGGCGTA-3') based on the mRNA sequence of the *B. tabaci* ACCase gene, and used to amplify a gene fragment of 158 bp encompassing the A2083V mutation site. PCR reactions (50 µl) contained 2.0 µl genomic DNA, 0.25 mM dNTPs, 0.25 µM of each primer, 5 µl of 10x Buffer and 1 U Kapa Taq DNA polymerase (Kapa Biosystems). PCRs were performed under the following conditions: one step of initial denaturation at 95 °C for 3min; 35 cycles at 95 °C for 30 s, 56 °C for 30 s and 72 °C for 20 s; and one final extension step at 72 °C for 2 min. PCR fragments were purified using the Nucleospin Gel and PCR Clean-Up purification kit (Macherey – Nagel) according to the manufacturer's instructions and sequenced at CeMIA SA (Larissa, Greece) with the same primers that were used for PCR. The Sequencing data were analyzed using BioEdit 7.0.5.3 software.

2.5 TaqMan qPCR assays

A set of four TaqMan assays were designed *de novo* and optimized for the purposes of this study (Suppl. Table S4). They can be applied on individual whiteflies for genotyping target site mutations associated with resistance to different pesticide MoAs; specifically, for acetylcholinesterase inhibitors (organophosphates/carbamates) (F331W, *Ace1*), sodium channel modulators (pyrethroids) (L925I and T929V, *Vgsc*), and lipid biosynthesis inhibitors targeting *Acc* (Cyclic ketoenols) (A2083V, *Acc*). Wild type (wt) probes were labelled with HEX and mutant (mut) probes were labelled with FAM fluorescent dyes. All probes had a 3′non-fluorescent quencher and a minor groove binder (MGB) at the 3' end. Probe concentrations were optimized (Suppl. Table S4) using probe matrices for each assay, and qPCR reactions were performed as previously described[18]. Sample calling was performed using the Allelic Discrimination module of the Bio-Rad CFX Maestro 2.3 software (version 5.3.022.1030)

2.6 Droplet Digital PCR (ddPCR) assays

ddPCR was performed using the QX200 Droplet Digital PCR System (Bio-Rad, Hercules, CA), and was optimized for each assay using synthetic double stranded DNA sequences (gBlocks™ gene fragments, IDT) of known wild type and mutant sequence concentration and copy numbers as previously described[18] . The optimized conditions for the four assays of the present study are given in Suppl. Table S5.

Reactions (final volume of 20 μL) included ddPCR Supermix for probes at 1×, 5 U restriction enzyme EcoRI-HF® (New England Biolabs), 5 ng of dsDNA, and primers and probes specific to each assay (Suppl Table S5) with droplets prepared and cycled as previously described[18]. The thermal cycling protocol comprised: 95 ^{o}C for 10 min, and 50 cycles of 94 ^{o}C for 30 s, 54 or 60 ^{o}C for 1 min (Suppl. Table S5), and 98^{o}C for 10 min. Endpoint fluorescence was measured, raw data were processed and percentage mutant allelic frequency (%MAF) was calculated as previously described[18].

2.8 Statistical Analyses

The limit of detection (LoD) for each ddPCR assay was defined as the lowest MAF that could be reliably detected with accuracy calculated according to Lavebratt et al[25]. Correlation analysis between A2083V MAFs and spiromesifen mortality rates was performed using the Spearman's correlation coefficient (r_s). Statistical analyses were performed using the SPSS Statistics software package version 17.0.

3. RESULTS

3.1 Development of TaqMan qPCR assays for detecting *B. tabaci* target site mutations in individual insect specimens

TaqMan qPCR assays were developed and optimized in terms of primer and probe concentrations (Suppl Table S4), using gBlocks™ gene fragments, for the following mutations: F331W (*Ace1*), L925I, T929V (*Vgsc*) and A2083V (*Acc*). The optimized TaqMan assays were applied in individual samples for validation purposes. Specifically, DNA samples isolated from previously used *B. tabaci* populations of known Sanger-sequenced genotypes for the mutations F331W, L925I and T929V and DNA samples sequenced in the framework of this study for the A2083V mutation were analysed. The validation showed that there was complete concordance between the genotypes scored by Sanger sequencing and TaqMan assays (Suppl. Table S3), covering all possible genotypes (wild type, mutant and heterozygous). Representative examples for all four mutations, i.e., F331W, L925I, T929V, and A2083V are presented in Figure 1 (parts A-D, respectively). In the HEX versus FAM fluorescence plots of Figure 1, an increase in HEX fluorescence indicates a wild type sample, an increase of FAM fluorescence indicates a mutant sample and an intermediate increase in both HEX and FAM fluorescence indicates a heterozygote whitefly sample. The calling of samples was made automatically using the qPCR machine's software.

3.2 Development of ddPCR assays for the hypersensitive monitoring of *B. tabaci* target site mutations in pooled samples

DdPCR assays were optimised in terms of optimal separation of droplet populations by testing different annealing temperatures and dsDNA template quantities. Final optimised reaction conditions are provided in Suppl. Table S5.

Following assay optimisation, the mutant gBlock™ synthetic double stranded DNA sequences were diluted in a stable background of wild-type DNA to produce control samples with different MAFs (100.0%, 10.0%, 1.0%, 0.2%, 0.1%, and 0.0%) and to calculate the limit of detection (LoD) for each assay. This was defined as the lowest MAF that can reliably be detected and is distinguishable from the wild type background. i.e., 0.1% for A2083V and L925I assays, which

corresponds to the reliable detection of 1 heterozygote whitefly in pool of 499 wild type individuals, and 0.2% for F331W and T929V representing 1 heterozygote in a background of 249 wild type individuals (Figure 2 and Suppl. Table S5). Next, mutant and wild type allele reaction copies (MUTcp and WTcp) were used to calculate the percentage mutant allelic frequency using the following formula: (%MAF) = (MUT cp/ (MUTcp + WTcp)) × 100%. The frequencies of A2083V, F331W, L925I and T929V target site mutations defined by ddPCR in eleven *B. tabaci* populations are presented in Table 1.

The sequencing of individual control samples that were destined to be used for the validation of the A2083V assays revealed the presence of two different mutant alleles, namely mutation 1: GTA and mutation 2: GTC (Suppl. Figure S1). Notably, the GTA allele was found only in individuals originating from Greece and the GTC allele was detected only in individuals originating from Spain as shown in Lueke et al [8] and data published in this paper (Suppl. Fig S1). We took into consideration the existence of the two mutant alleles during A2083V assay design and thus we opted to include a "degenerate" mutant probe that binds to and detects both alleles. When genotyping the study's *B. tabaci* populations from Crete we noticed the existence of two distinct droplet populations in the mutant channel and hypothesized that both GTA and GTC alleles are present. To test that hypothesis, we assayed, with ddPCR, individual control samples that were verified by sequencing to bear either the GTA (Suppl Figure S2, part A) or the GTC (Suppl Figure S2, part B) mutation. We then superimposed the two control sample types (GTA and GTC) using the ddPCR software (QuantaSoft™ Analysis Pro, v1.0596) and verified that the two droplet populations can indeed be readily distinguished based on the FAM amplitude fluorescence (Figure 2, Part C). By taking advantage of this information, we were able to delineate that the field *B. tabaci* populations belonged to one of the following groups: bearing only the GTA mutation (example in Suppl. Figure S2, part D), which was the majority of cases (Table 1) bearing only the GTC mutation (Suppl. Figure S2, part E), and bearing both mutations (Suppl. Figure S2, part F).

3.3 Application of ddPCR assays in pooled samples of *B. tabaci* field populations

The acetylcholinesterase inhibitor (OPs/carbamate) resistance mutation F331W was detected in all eleven populations. It was found to be fixed (MAF = 100.0%) in Bt3, Bt5, Bt6 and Bt8 and close to fixation in Bt9 (99.13%), Bt4 (95.89%), Bt7 (96.55%) and Bt2 (93.41%). Bt11 showed a 61.74% MAF, whereas a more moderate presence of the mutation was recorded in Bt10 (47.91%) and Bt1 (47.52%).

Both mutations that are implicated in sodium channel modulator (pyrethroid) resistance (L925I and T929V) were detected in all populations. More precisely, populations Bt1, Bt11 and Bt2 showed L925I MAFs of 97.36%, 96.0% and 91.39% respectively, coupled with T929V MAFs of 1.79%, 2.35% and 8.87% respectively. Populations Bt5, Bt3, Bt6, Bt7 and Bt8 presented with MAFs in the range of 70.26% to 81.03% for L925I, whereas T929V ranged from 19.13% to 25.21%. In population Bt9 a MAF of 59.48% was recorded for L925I (35.12% for T929V) and population Bt4 showed a MAF of 37.27% for L925I, which was coupled with a frequency of 60.62% for T929V. Finally, in Bt10, a moderate presence of these mutations (17.51% for L925I and 42.11% for T929V) was documented.

The A2083V mutations, associated with spiromesifen resistance was detected in 8 out of 11 populations. The highest frequency was recorded for Bt3 (89.56%), followed by Bt8 (78.15%) and Bt9 (72.28%). Bt4 and Bt2 presented an A2083V MAF of 60.67% and 40.37%, respectively, whereas lower frequencies were recorded for Bt11 (20.0%), Bt6 (7.18%) and Bt7 (6.16%). Bt1, Bt5 and Bt10 did not carry these mutations.

3.4 Phenotypic characterization of populations for spiromesifen resistance and correlation with the frequency of A2083V

Eleven *B. tabaci* field populations were collected from various cites of Crete. Detailed information about the collection location, host plants, and history of application for these populations is

provided in Table S1. The standard toxicity bioassay was applied to evaluate phenotypic resistance for spiromesifen (complete results are presented in Suppl. Table S2), where only the RD dosage (150 mgL^{-1}) was applied. Populations Bt2 (% mortality = 22.0), Bt3 (% mortality = 10.0), Bt11 (%mortality = 10.3), Bt4 (% mortality = 9.5), Bt8 (% mortality = 4.2), and Bt9 (% mortality = 7.2) showed very high insensitivity when the RD was applied. Population Bt7 displayed moderate resistance (% mortality = 48.0). Among the populations that showed low insensitivity to spiromesifen were Bt1 (% mortality = 94.4) and Bt5 ((% mortality = 81.0), whereas Bt6 showed full susceptibility (% mortality = 100.0).

In order to perform a phenotype-genotype correlation analysis we used the spiromesifen toxicity data (Suppl. Table S2) and the relevant molecular data for the A2083V mutation (Table 1) that has been linked to spiromesifen resistance[8]. This analysis revealed a strong negative correlation (r_s = -0.839, p = 0.002) between A2083V MAFs and spiromesifen mortality at RD (Figure 3) for the *B. tabaci* populations tested in our study.

4. DISCUSSION

We report the development of a suite of target site molecular diagnostics for *B. tabaci* insecticide resistance against three different MoAs (lipid biosynthesis inhibitors, AChE inhibitors and sodium channel modulators) [6, 7]. The panel includes four TaqMan-chemistry based assays (detecting F331W, L925I, T929V and A2083V) that we firstly developed for use in conventional qPCR machines for genotyping of individuals (Figure 1, Suppl. Table S3). Aiming to provide a solution for the screening of large pools of whitefly samples we subsequently adapted these assays for ddPCR (Figure 2, Table 1). The use of pooled samples can increase the practicality of resistance monitoring by significantly reducing time and resources[17] as well as provide very high sensitivity, when the assays are performed via ddPCR[16, 18]. Interestingly, a previous attempt has been made to

assees pooled whitefly samples for L925I and T929V mutations, using amplicon sequencing, which is a higly reliable and robust method[26]. However, amplicon sequencing is also considerably less sensitive than ddPCR, as it can reach an LoD of 5.0% at best, and requires a multistep protocol that also includes bioinformatic analyses to obtain results[27, 28]. We report here ddPCR LoDs ranging from 0.1% (detection of 1 heterozygote in a pool of 499 wild type individuals) to 0.2% (1 heterozygote in 249 wild type individuals) (Figure 2). Thus, ddPCR diagnostics can detect incipient resistance, a key objective in insecticide resistance management (IRM). Concerning cost effectiveness, the cost for a standard 2-plex reaction for pools of 100 insects is 0.07 € per insect. This is considerably lower compared to individual genotyping (e.g., for TaqMan genotyping a cost of ~1.0 € /per insect was previously reported[29]) and can decrease even more if even larger pools are used.

The ddPCR assay panel was implemented in a pilot study using pooled *B. tabaci* field populations (Table 1). Molecular monitoring for OP/carbamate resistance through F331W mutation in *Ace1* showed that 8 out of 11 *B. tabaci* populations (Bt2-Bt9) were fixed (100.0%) or close to fixation (93.41-99.13%) for this mutation. Interestingly, there were also populations (Bt1, Bt10, Bt11) that exhibited lower F331W MAFs (47.52-61.74%). Previous studies performed in Crete[15] had shown that F331W mutation was fixed in all *B. tabaci* populations, due to a high selection pressure from repeated OP and carbamate treatments. More recentlly, OP/carbamate applications have been significantly restricted and this may explain the reversal of F331W fixation that was observed in our study, given also the high fitness cost of this mutation[15].

Molecular monitoring for pyrethroid resistance (L925I and T929V in the *vgsc*) revealed the high frequency of either the L925I or T929V mutations in 10 out of 11 populations. Only one population, Bt10, presented moderate MAFs (Table 1). The high presence of L925I in a population was always accompanied by a lower presence of T929V (majority of cases), or *vice versa*. This can be explained by the fact that the L925I and T929V mutations are mutually exclusive. An

earlier study conducted in *B. tabaci* populations from Mediterranean countries[30] also documented a very high frequency of these mutations. The maintenance of high L925I or T929V frequencies in the Cretan populations could be explained by the continuance application of pyrethroids and the lack of any major fitness costs of these mutations as previously suggested[15].

The resistance screening for spiromesifen, performed both by the A2083V molecular assay (Table 1) and toxicity bioassay (Suppl. Table S2) shows that resistance to ketoenols is present in *B. tabaci* field populations in Crete. More precisely, populations Bt2, Bt3, Bt4, Bt8, Bt9, and Bt11 showed low mortality (4.2-22.0%) rates at the RD and this is in line with the MAFs detected for A2083V (20.0% - 89.56%). Populations Bt6, Bt1 and Bt5 that showed high response or even full susceptibility to spiromesifen (81.0-100% mortality) presented with zero or very low A2083V MAFs (0.0-7.18%). The Bt7 population that presented with moderate resistance (mortality = 48.0%) also showed very low frequency of the A2083V mutation (6.16%). While spiromesifen resistance has also been recorded in *B. tabaci* populations in Spain[8, 31] Brazil[32] and China[33], it has not been described in prior studies (2006-2008) in Greece.

A strong phenotype-genotype correlation between the spiromesifen mortality and the mutation frequency of A2083V was revealed in the populations tested in our study (Figure 3). This is in accordance with studies that have functionally validated the role of A2083V in resistance to spiromesifen and other ketoenols[8] and associated strong phenotypic resistance to ketoenols with high A2083V mutational frequency in *B. tabaci* populations from Spain[31]. The fact that metabolic mechanisms do not seem to significantly contribute to the resistance phenotype[31] enhances the operational relevance of the A2083V as a single marker for spiromesifen resistance. While our data demonstrate the diagnostic value of the A2083V ddPCR assays; larger field studies will help to assess the precise predictive strength of this mutation. We have previously revealed strong correlation between etoxazole resistance and the I1017F mutation[18] and tebufenpyrad / fenpyroximate resistance and the frequency of the H92R mutation[34]. Such studies demonstrate the

considerable potential of these mutations to be used as molecular diagnostics for insecticide resistance.

Interestingly, our pilot screening of A2083V mutations in *B. tabaci* field populations from Crete revealed the presence of the GTC mutant allele which was previously detected only in populations from Spain[8] (and data published in this paper, Suppl. Fig S1). Our methodology offers new opportunities to effectively monitor the geographical distribution of the A2083V mutation as a resistance marker. This type of multiplexing capability is a unique property of ddPCR, which in our case was made possible by using a single degenerate mutant probe that allows the differential detection of both GTA and GTC mutant alleles, and together with the wild type allele allows for triplex allele detection in a single reaction (Suppl Figure S2) using only two optical channels. Degenerate bases are equimolar mixtures of two or more different bases at a given position within the sequence that result to a final product which is a blend of two or more different sequences made simultaneously during one synthesis. The competition and different binding of the probes to the template produces differential fluorescent patterns resulting in distinct droplet clusters[19] (Suppl Figure S2). This opportunity that ddPCR provides can be exploited even further via higher order multiplexing using different assay concentrations to detect up to 16 different targets in a single reaction as shown recently[35]. This could further increase the throughput and lower the cost of resistance monitoring significantly.

Robust molecular markers can prove of great value for IRM. Such cases are not exclusive to *B. tabaci* but are also in place for other major agricultural pests, like *T. urticae* (e.g., I1017F[18] and H92R[34]). However, in cases where such evidence is lacking, association between resistance and molecular markers across geographical regions and functional validation of the genotype-phenotype relationship should be established. This would help to evaluate the spatiotemporal diagnostic value of the marker, in order for its application to provide robust predictive value.

5. CONCLUSION

We report here the development of molecular diagnostics for *B. tabaci* that can be applied to individual or pooled samples of insects and prove a valuable tool for next-gen hypersensitive insecticide resistance monitoring, to support evidence based IRM.

ACKNOWLEDGMENTS

This work was supported by the European Union's Horizon 2020 research and innovation program (Grant: 773902-SuperPests). The research has also been co-financed by the following sources: (a) Greek national funds through the Public Investments Project (PIP) of General Secretariat for Research & Technology (GSRT), under the Emblematic Action "Agro4Crete : Research in the Agri-Food Sector of Crete", which is part of Subproject 2, "Pilot application of new standards of agricultural production" of the project sector of agri-food" (project code 2018 ΣΕ01300000), (b) The project "Smart Diagnostic tools and database to support precision plant protection in horticultural crops in Crete' 'SmartPP' funded by the Crete Operational Program 2014-2020 and co-funded by the European Regional Development Fund (ERDF), under the Priority Axis "Enhancing the Competitiveness, Innovation, and Entrepreneurship of Crete", Action 1.b.1: Demonstration - Experimental Development Projects, Promoting Research and Innovation in RIS3Crete, and (c) by Greece and the European Union (European Social Fund—ESF) through the Operational Programme "Human Resources Development, Education and Lifelong Learning" in the context of the project "Strengthening Human Resources Research Potential via Doctorate Research" (MIS-5000432), implemented by the State Scholarships Foundation (IKY) (M.S.).

We thank Prof. Sofia Agelaki and the Laboratory of Translational Oncology, School of Medicine, University of Crete for kindly providing us access to the ddPCR platform.

CONFLICT OF INTEREST DECLARATION

The authors declare that there is no conflict of interest.

TABLES

Table 1 Mutant allele frequencies measured by ddPCR in pools of *B. tabaci* field populations (N = 50 individuals per pool).

Pesticides	AChE inhibitors (OPs, Carbamates)	Sodium channel modulators (Pyrethroids)		Lipid biosynthesis inhibitors targeting ACC (Tetronic & tetramic acid derivatives / ketoenols)
Mutation Gene	**F331W** *Ace1*	**L925I** *Vgsc*	**T929V**	**A2083V** *Acc*
Population	**%MAF**	**%MAF**		**%MAF**
Bt1	47.52	97.36	1.79	0.00
Bt2	93.41	91.39	8.87	40.37[a]
Bt3	100.00	73.46	21.85	89.56 [a]
Bt4	95.89	37.27	60.62	60.67[b]
Bt5	100.00	81.03	19.13	0.00
Bt6	100.00	75.92	23.34	7.18
Bt7	96.55	70.26	24.79	6.16[c]
Bt8	100.00	72.64	25.21	78.15
Bt9	99.13	59.48	35.12	72.28
Bt10	47.91	17.51	42.11	0.00
Bt11	61.74	96.00	2.35	20.00

AChE: Acetylcholinesterase; OPs: Organophosphates; ACC: acetyl-CoA carboxylase; MAF: Mutant Allelic Frequency.

[a] Mutation 1: GTA; [b]Both mutation 1: GTA and mutation 2: GTC; [c]Mutation 2: GTC

FIGURE LEGENDS

Figure 1 TaqMan assays for the detection of the F331W (part A), L925I (part B), T929V (part C) and A2083V (part D) mutations in individual whitefly samples. In the scatter HEX versus FAM fluorescence plots each square represents a wild type, each triangle a heterozygote and each circle a mutant sample. Graphs and sample calling were generated made using the Allelic Discrimination module of the Bio-Rad CFX Maestro 2.3 software.

Figure 2 ddPCR assay for the detection of the F331W (part A), L925I (part B), T929V (part C), and A2083V (part D) mutations in pooled whitefly samples and quantification of their mutant allelic frequencies (MAFs). MAF samples were prepared by mixing gBlock™ wild type (WT) and mutant (MUT) sequences of known copy numbers resulting in the following frequencies: 10.0%, 1.0%, 0.2% and 0.1%, in order to find the LoD for each assay. Ctrl: Control samples. NTC: No Template Control; WT: Wild type gBlock™ control (0.0% MAF); MUT: Mutant gBlock™ control (100% MAF).

Figure 3 Correlation between the results generated by spiromesifen toxicity assays and A2083V ddPCR molecular diagnostics. MAF: mutant allelic frequency; RD: recommended dose. r_s: Spearman's correlation coefficient.

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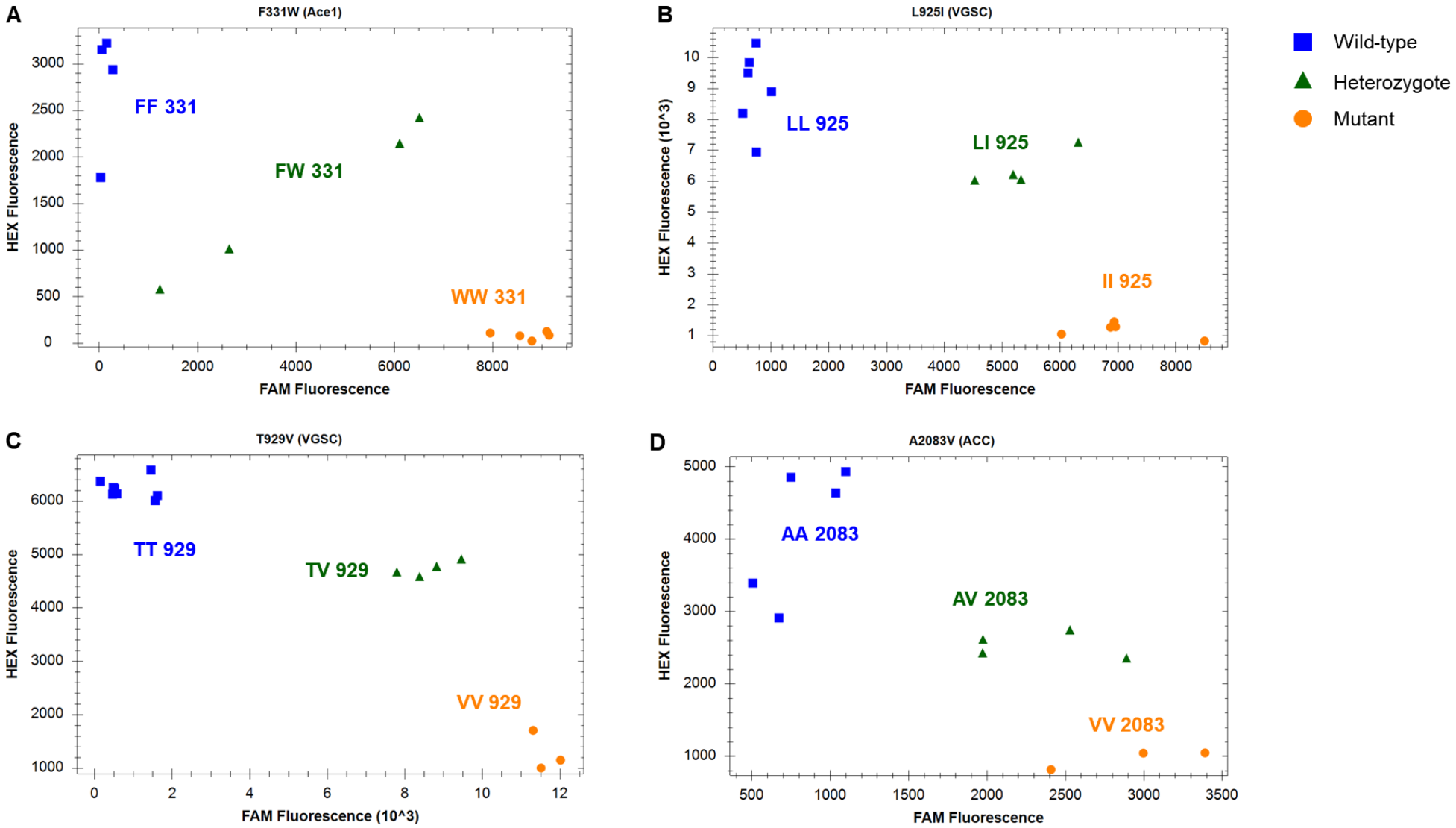

A
F331W (Ace1)
HEX Fluorescence
FAM Fluorescence
FF 331
FW 331
WW 331
B
L925I (VGSC)
HEX Fluorescence (10^3)
FAM Fluorescence
LL 925
LI 925
II 925
C
T929V (VGSC)
HEX Fluorescence
FAM Fluorescence (10^3)
TT 929
TV 929
VV 929
D
A2083V (ACC)
HEX Fluorescence
FAM Fluorescence
AA 2083
AV 2083
VV 2083
Wild-type
Heterozygote
Mutant

Figure 1

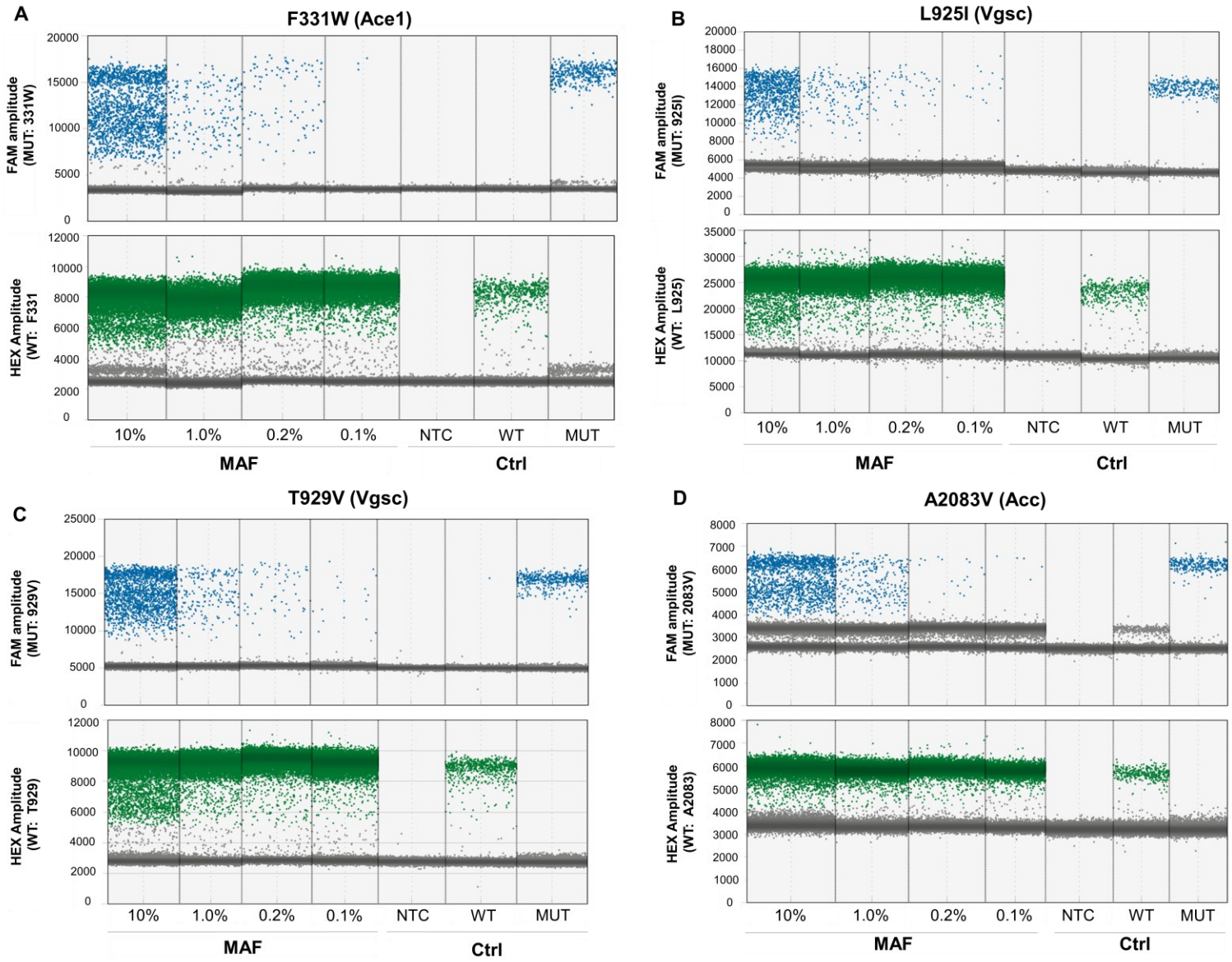

A
F331W (Ace1)
FAM amplitude (MUT: 331W)
HEX Amplitude (WT: F331
B
L925I (Vgsc)
FAM amplitude (MUT: 925I)
HEX Amplitude (WT: L925)
C
T929V (Vgsc)
FAM amplitude (MUT: 929V)
HEX Amplitude (WT: T929)
D
A2083V (Acc)
FAM amplitude (MUT: 2083V)
HEX Amplitude (WT: A2083)
10%
1.0%
0.2%
0.1%
NTC
WT
MUT
MAF
Ctrl

Figure 2

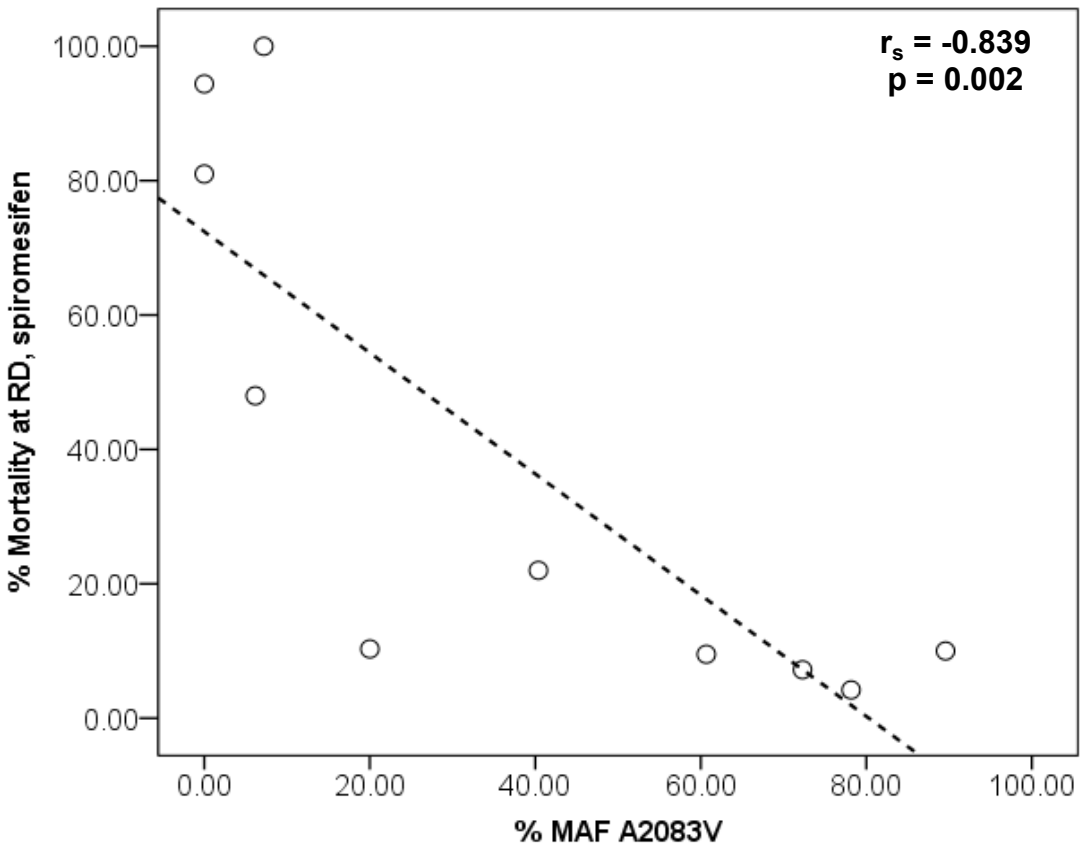

r_s = -0.839
p = 0.002
% Mortality at RD, spiromesifen
100.00
80.00
60.00
40.00
20.00
0.00
0.00
20.00
40.00
60.00
80.00
100.00
% MAF A2083V

Figure 3

SUPPLEMENTAL INFORMATION

Supplementary Table S1 Characteristics of the *B.tabaci* field populations collected from Crete

Population	Locality	Host Plant	Pesticide Application History	Collection Date
Bt1	Ierapetra, Lasithi Prefecture	Cucumber	N/A	25 May 2017
Bt2	Ierapetra, Lasithi Prefecture	Eggplant	Sufloxaflor, spirotetramat, acetamiprid, pymetozine	1 June 2018
Bt3	Ierapetra, Lasithi Prefecture	Eggplant	Spiromesifen, spirotetramat, flonicamid, pymetrozine, flupyradifurone	10 May 2019
Bt4	Tympaki, Heraklion Prefecture	Cucumber	Spirotetramat, sufloxaflor, flonicamid	30 June 2020
Bt5	Tympaki, Heraklion Prefecture	Eggplant	Flonicamid	4 October 2018
Bt6	Tympaki, Heraklion Prefecture	Melon	Sufloxaflor, spirotetramat, thiamethoxam	20 June 2018
Bt7	Tympaki, Heraklion Prefecture	Melon	Spirotetramat, delamethrin, thiamethoxam, pymetrozine	7 June 2019
Bt8	Ierapetra, Lasithi Prefecture	Cucumber	N/A	24 June 2020
Bt9	Ierapetra, Lasithi Prefecture	Pepper	Spirotetramat, spiromesifen	24 June 2020
Bt10	Rethymnon Prefecture	Rose	Sufloxaflor	6 October 2020
Bt11	Ierapetra, Lasithi Prefecture	Eggplant	Acetamiprid, flupyradifurone, pyriproxifen, spirotetramat, spiromesifen	9 March 2020

N/A: Not available

Supplementary Table S2 Toxicity assays of five pesticides for *Bemisia tabaci* field populations

Pesticide (RD)	**Spiromesifen** (150 mgL^{-1})
Population	**% Mortality**
Bt1	94.4
Bt2	22.0
Bt3	10.0
Bt4	9.50
Bt5	81.0
Bt6	100
Bt7	48.0
Bt8	4.20
Bt9	7.20
Bt10	Nd
Bt11	10.3

RD: Recommended Dose. Nd: not determined.

Supplementary Table S3 Results of the validation of the TaqMan assays.

Assay (mutation)	Sample Category (N analyzed)	Expected Genotype [based on Sequencing]	Observed Genotype [based on TaqMan qPCR assay]	Agreement
Ace1 (F331W) *	Wild type (N = 4)	FF	FF	100%
	Heterozygous (N= 4)	FW	FW	
	Mutant (N = 5)	WW	WW	
Vgsc-A (L925I) *	Wild type (N = 6)	LL	LL	100%
	Heterozygous (N = 4)	LI	LI	
	Mutant (N = 5)	II	II	
Vgsc-B (T929V) *	Wild type (N = 9)	TT	TT	100%
	Heterozygous (N = 4)	TV	TV	
	Mutant (N = 3)	VV	VV	
Acc (A2083V) **	Wild type (N = 12)	AA	AA	100%
	Heterozygous (N = 8)	AV	AV	
	Mutant (N = 10)	VV	VV	

* Control samples available from Tsagkarakou et al (doi:10.1016/j.pestbp.2009.03.002).

**Control samples sequenced as part of this study (Suppl Figure S1).

Supplementary Table S4 List of primers and probes used in the study.

Assay (mutation)	Oligo name	Sequence 5'→3' (including dyes)	Final concentration*
Ace1 (F331W)	331F	ATCCTCATGGGGAGCAACAC	800 nM
	331R	GACACCGCCTGGATGAACTG	800 nM
	331Pwt	HEX-ACATGATGAAGTAGTTSC-MGB	300 nM
	331Pmut	FAM-ACATGATCCAGTAGTTSC-MGB	300 nM
Vgsc-A (L925I)	925/929F	TTCAATCATGGGCCGAACAG	800 nM
	925/929R	AGTTGCATTCCCATCACAGCA	800 nM
	925Pwt	HEX-AATTTCCTAAGGCCCCA-MGB	300 nM
	925Pmut	FAM-AATTTCCTATGGCCCCA-MGB	300 nM
Vgsc-B (T929V)	925/929F	TTCAATCATGGGCCGAACAG	800 nM
	925/929R	AGTTGCATTCCCATCACAGCA	800 nM
	929Pwt	HEX-ACAAAAGTCAAATTTCC-MGB	500 nM
	929Pmut	FAM-CAAAAACCAAATTTCC-MGB	300 nM
Acc (A2083V)	2083F	TACATCGTGGACGAGTTGCG	800 nM
	2083R	TTTCGGGATCGGCGTACAT	800 nM
	2083Pwt	HEX-CAACGACKGCCCARG-MGB	300 nM
	2083Pmut	FAM-ACGACKACCCARGCTC-MGB	100 nM

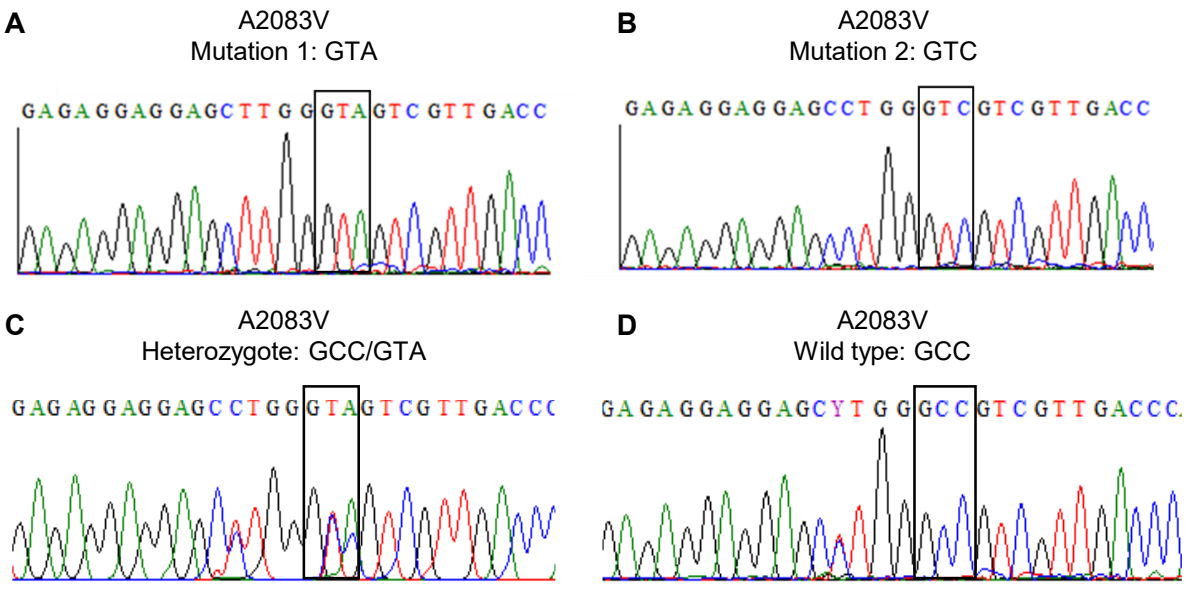
*Final concentration referrers to TaqMan qPCR assays. F: Forward; R: Reverse; P: Probe; Wt: Wild-type; Mut: mutant; MGB: Minor Groove Binder.

Supplementary Table S5 Optimized ddPCR reaction conditions and analytical properties per assay

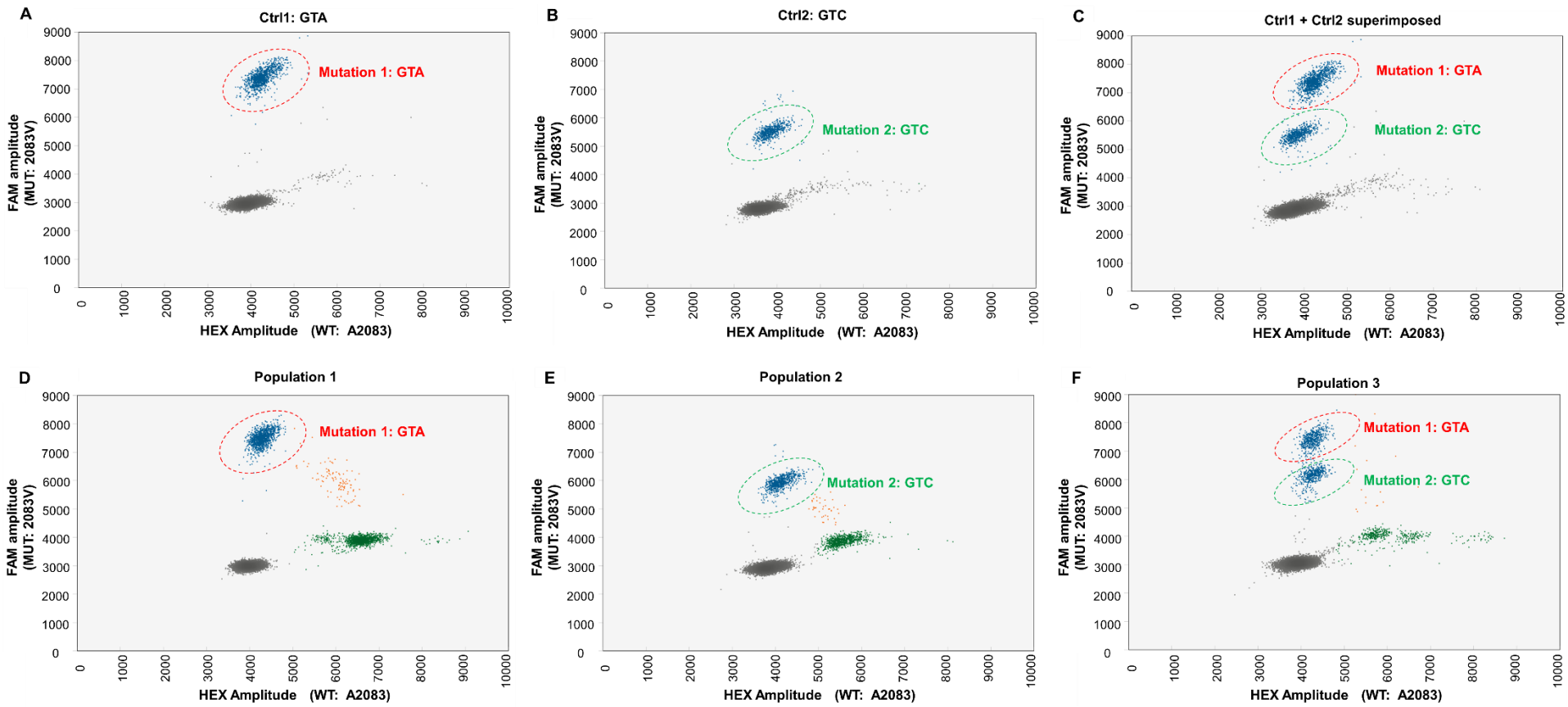
Assay (mutation)	Oligo*	Concentration	dsDNA quantity	Annealing Temperature	N of cycles	LoD for MAF	Accuracy for MAF
Ace1 (F331W)	F, R Pwt Pmut	1200 nM 600 nM 600 nM	5 ng	60ºC	50	0.20%	0.61%
Vgsc-A (L925I)	F, R Pwt Pmut	1200 nM 600 nM 600 nM	5 ng	54ºC	50	0.10%	0.76%
Vgsc-B (T929V)	F, R Pwt Pmut	1200 nM 600 nM 500 nM	5 ng	60ºC	50	0.20%	1.14%
Acc (A2083V)	F, R Pwt Pmut	1200 nM 600 nM 400 nM	5 ng	54ºC	50	0.10%	1.37%

*Sequence same as in Suppl. Table S3

F: Forward primer; R: Reverse primer; Pwt: Wild type probe; Pmut: Mutant probe from Suppl Table S4; LoD: Limit of Detection; MAF: Mutant allelic Frequency.



Suppl Figure S1 Sequencing chromatographs of a region of the *ACC* gene derived from individual *B. tabaci* insects. The A2083V mutations are indicated in boxes. Mutation 1: GTA was detected in individual samples from Greece (N=5) (Part B) and Mutation 2: GTC was detected in individual samples from Spain (N=5) (Part C). In parts C and D, representative heterozygote (N=8) and wild type samples (N=12), respectively, are also presented from Greece.



Suppl Figure S2 Differential detection of the two mutant A2083V alleles (GTA and GTC) in the same optical channel (FAM) though ddPCR. Individual control samples bearing the GTA (Part A) or GTC (Part B) codons are presented individually and superimposed (Part C), showing distinguishable droplet clusters. Field populations bearing either the GTA, GTC codons or both codons are presented in Part D, Part E and Part F, respectively.