Title: **Identification and characterization of striking multiple-insecticide resistance in a *Tetranychus urticae* field population from Greece**

Running title: Wide spectrum resistant *Tetranychus urticae* due to accumulation of multiple mechanisms

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Abstract

BACKGROUND: *Tetranychus urticae* is a notorious crop pest with world-wide distribution that has developed resistance to a wide range of acaricides. Here, we investigated the resistance levels of a *T. urticae* population collected from an ornamental greenhouse in Peloponnese, Greece, and analyzed its resistance mechanisms at the molecular level.

RESULTS: Toxicological assays showed resistance levels against compounds with different mode of action, with resistance ratios scaling at: 89-fold for abamectin, >1000-fold for clofentezine, >5000-fold etoxazole, 27-fold for fenpyroximate and pyridaben, 20- and 36-fold for spirodiclofen and spirotetramat, respectively and 116- and >500-fold for cyenopyrafen and cyflumetofen, respectively.

Bioassays with synergists indicated the involvement of detoxification enzymes in resistance to abamectin but not to cyflumetofen and spirodiclofen. RNAseq analysis showed significant over-expression of several genes encoding detoxification enzymes such as cytochrome P450 monooxygenases and UDP-glycosyltransferases, which have been previously associated with acaricide resistance. Known target-site resistance mutations were identified in *acetyl-choline esterase, chitin synthase 1* and *NDUFS7/psst*, but also discovered putative novel resistance mutations in targets such as the *glutamate-gated chloride channel subunit 3*. Interestingly, target site resistance mutations against pyrethroids or bifenazate were not identified possibly indicating a recent reduced selection pressure in Greece, as well as a possible opportunity to rotate these chemistries.

CONCLUSION: We identified and characterized a striking case of multiple acaricide resistance in a field population of *T. urticae*. Exceptionally strong resistance phenotypes, with accumulation of multiple resistance mutations and over-expression of P450s and other detoxification genes in the same field population is reported.

Keywords: Two-spotted spider mite, transcriptomics, mutations, acaricide, multi-resistance

1 INTRODUCTION

The two spotted spider mite *Tetranychus urticae* is one of the most notorious and destructive pests around the globe. As a polyphagous pest, it infests over 1000 plant species including many food crops, but also many ornamental plants.[1] Its control is mainly based on the use of acaricides. Organophosphates (OPs), pyrethroids and macrocyclic lactones, such as abamectin, act on the nervous system of spider mites.[2, 3] OPs were among the first insecticides used for the control of *T. urticae* but failure due to resistance appeared in the early 1950s.[4] Pyrethroids act on voltage gated sodium channel of insects and spider mites, but display low toxicity in mammals.[4] Abamectin has strong acaricidal, insecticidal and nematicidal activity and is produced by the fermentation of *Streptomyces avermitils.*[5] Etoxazole, which has low potency against insects,[6] and tetronic acid derivatives, such as spirodiclofen and spirotetramat, act on growth and development of spider mites although on different targets.[7,8] The mitochondrial electron transport inhibitors (METI) act on the mitochondrial respiratory pathway. Fenpyroximate and pyridaben act on complex I at the ubiquinone site of NADH oxidoreductase (METI-Is),[9] while bifenazate act on Q_o site of cytochrome b complex III (METI-III).[2] The more recently developed METIs cyflumetofen and cyenopyrafen act on complex II of the mitochondrial respiratory chain (METI-IIs).[10] Their main use is against spider mites, although some active ingredients are effective against insect pest species, and to some extend are selective towards insect predators.[11]

However, due to the frequent application of such compounds and biological features of *T. urticae* (high reproduction rate, arrhenotokous parthenogenesis, short life cycle), this species develops resistance very rapidly. *T. urticae* has developed resistance to more than 90 different compounds, and is considered one of the top resistant species (https://www.pesticideresistance.org/).[12] Still, to date, the cases of strong and multiple-

insecticide resistance in field populations are very limited, [13-17] and most of the reports refer to laboratory strains after selection.[18-21]

Several studies have reported *T. urticae* strains with high levels of resistance against certain active ingredients,[22-25] including some cases of cross- or multiple- resistance.[18,26-28] Several mechanisms have been associated to these phenotypes, with the most studied ones being target-site mutations,[9,27,29-32] as well as over-expression of detoxification enzymes.[33-39]

Target-site mutations associated with acaricide resistance in *T. urticae* have been discovered in genes encoding (a) acetylcholinesterase (*AChE*), which is associated with resistance to organophosphates and carbamates,[32] (b) voltage-gated sodium channel (*vgsc*), that confers resistance to pyrethroids,[16,27,29] (c) glutamate-gated chloride channels (*GluCl1/3*), which are linked with resistance to abamectin,[30,31] (d) cytochrome b (*cytb*), which is associated with bifenazate and acequinocyl resistance,[26,40] (e) chitin synthase 1 (*chs1*), which has been linked with high levels of resistance to the mite growth inhibitors (MGIs) etoxazole, clofentezine and hexythiazox[22,41], (f) PSST subunit (*psst/NDUFS7*) of the mitochondrial respiratory complex I, which has been associated with resistance to pyridaben, tebufenpyrad and fenpyroximate[9,42] and (g) in succinate dehydrogenase subunits B and C (*SdhB* and *SdhC*, respectively) conferring resistance to several complex II inhibitors.[43]

Although the presence of certain point mutations in populations might be sufficient to cause field failure of acaricides,[44] there are several cases where detoxification enzymes are involved in acaricide resistance. In particular, it has been previously shown that the P450 CYP392A16 was over-expressed in an abamectin resistant strain and capable of metabolizing abamectin.[45] Furthermore, recombinant UDP-glycosyltransferases (UGTs) derived from resistant *T. urticae* strains and/or strains that have been adapted to different host plants, were able to glycosylate a range of acaricides including abamectin.[37,46] ATP-binding cassette (ABC) transporters have been found to be overexpressed in multi-resistant strains [34] and the genes Pgp1 and 2, members

of the ABCB subfamily, are potentially involved in resistance to abamectin.[39] Additionally, CYP392A11 metabolizes the METI acaricides fenpyroximate and cyenopyrafen,[36] while the glutathione S-transferase GSTd05 metabolizes cyflumetofen.[47] Furthermore, a P450 from *T. cinnabarinus*, CYP389C16, was found to be over-expressed in moderate resistant strains and is capable of metabolizing the de-esterified metabolite of cyflumetofen,[48] while down-regulation of esterases has been proposed to play a role in resistance to cyflumetofen.[49] Finally, resistance to keto-enols, such as spirodiclofen, has been linked to the metabolism of the compound by CYP392E10,[33] much likely in combination with sequestration of spirodiclofen by an esterase (CCE04).[50]

Here, we investigated the resistance levels of a *T. urticae* population collected from a gypsum greenhouse in Trizina, Peloponnese, Greece, with history of heavy acaricide application and analyzed the resistance mechanisms at the molecular level.

2 MATERIALS AND METHODS

2.1 *T. urticae* strains

The susceptible strains, London and BC2018-14, and the field population Trizina were used in this study. Hereafter, we will refer to these strains as S-1, S-2 and Trz, respectively. S-1 is a green color-morph strain, was originally collected from the Vineland region (Ontario, Canada), and was used for sequencing the complete *T. urticae* genome.[51] The S-2 strain is a red color-morph strain, collected in 2018 from citrus in the region of Valencia (Spain, coordinates 39°13'50.88"N 0°22'42.62"W), with an acaricide application history of clofentezine, hexythiazox and etoxazole. Trz is a red color- morph population and was collected in 2018 from a gypsum greenhouse in Trizina (Peloponnese, Greece). Primarily, the acaricide application history included the use of abamectin, bifenazate and clofentezine active ingredients in the last 2-3 years. All strains were reared on 3-week old potted kidney bean

plants (*Phaseolus vulgaris L.*) at 25 ± 1 °C, 16:8 light:dark photoperiod and maintained without exposure to insecticides.

2.2 Insecticides and chemicals

Ten formulated acaricides/insecticides of different MoA were used for toxicity assays: abamectin (Vertimec 18EC), clofentezine (Apollo 50SC), spirodiclofen (Envidor 240SC), bifenazate (FloramiteSC), etoxazole (Baroque 11SC), cyflumetofen (Nealta 20SC), pyridaben (Nexter 20EC), spirotetramat (Movento 150OD), cyenopyrafen (Starmite 30SC) and fenpyroximate (Kendo 5.34SC). Furthermore, the following inhibitors were used in synergism assays: piperonyl butoxide (PBO; 95%), a P450 inhibitor, S,S,S-tributylphosphorothioate (DEF; 98%), an esterase inhibitor, and diethyl maleate (DEM; 95%), a GST inhibitor. PBO and DEM were purchased from Sigma-Aldrich Corporation, whereas DEF was purchased from Chem Service Ink.

2.3 Toxicity and synergism assays

Adulticidal bioassays were conducted with 20 adult female mites of 2-3 days old which were transferred on the upper side of 9 cm^2 square-cut kidney bean leaf discs on wet cotton wool.[20] The plates were sprayed with 1 ml of spray fluid at 1 bar pressure with a Potter Spray Tower (Burkard Scientific, UK) to obtain a homogenous spray film. To assess the toxic effects of spirodiclofen, spirotetramat and etoxazole, larvicidal bioassays were performed as previously described,[19] while ovicidal bioassays were performed for clofentezine.[20] Approximately 20 females were allowed to lay eggs for 5 hours on a leaf disc. After spray treatment, leaf discs were placed at 25 ± 0.5 °C, 60% RH and 16:8 h (light:dark) photoperiod. Three replicates of at least five serial dilutions of each acaricide and a control of deionized water were tested. Mortality was assessed after 24 h for bifenazate and cyflumetofen and 48 h for all other adulticide acaricides. Adult mites were considered alive if they could walk twice the distance of their body size after being prodded with a hair brush.[23] Mites treated with MGIs and keto-

enols, were considered as unaffected, if they displayed the same developmental stage as water treated control at the time of scoring. LC_{50} values, slopes, RRs and 95% confidence intervals (CIs) were calculated by probit analysis (POLO, LeOra Software, Berkeley, USA).[52] Resistance ratio was considered significant if the 95% CI do not include the value of 1.[52] In case 5000 mg l^{-1} did not cause 50% mortality, no further attempts were made to determine LC_{50}s.

Toxicity assays in presence of synergists were conducted as previously described.[19] Synergists (PBO, DEF or DEM) were dissolved in N, N-dimethylformamide and emulsifier W (3:1, w:w) and then diluted with deionized water to reach the final concentration. For the Trz population, all synergists (PBO, DEF and DEM) were applied at 1000 mg/L for all insecticides, except in the case of the larvicide assay with spirodiclofen (300 mg/L). For both susceptible strains, DEM and PBO were applied at a concentration of 1000 and 300 mg/L, respectively, while DEF was used at 300 mg/L for adulticide assays and at 100 mg/L for the larvicide assays with spirodiclofen. The concentration of synergists applied in each strain was the highest concentration causing up to 5% mortality to the mites. Larvae or adults were pretreated with the synergist and the acaricide of interest was applied on larvae or adults 4- and 24-hours post-synergist treatment, respectively. Mortality was scored as mentioned above. Synergism ratios (SR) were determined by PoloPC (LeOra Software, Berkeley, USA)[52] and considered significant if the 95% CI of the synergist ratio did not include the value 1.

2.4 RNA isolation, library construction, sequencing, cDNA synthesis and gDNA extraction

Four biological replicates of 200 1-3 days old adult females from the *T. urticae* strains S-1, S-2 and Trz population, respectively, were used for total RNA extraction, using the RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. The extracted RNA was treated with

Turbo DNase (Ambion), in order to remove any traces of genomic DNA. The purity and concentration of RNA were estimated using a Nanodrop spectrophotometer. RNA samples were sent to Macrogen (Korea) for mRNA library construction with the Illumina Truseq stranded mRNA sample preparation kit and sequenced in the Illumina Novaseq platform yielding 100 bp PE reads. One μg of RNA was used for cDNA synthesis using the reverse transcriptase kit from Minotech (Heraklion, Greece), according to the manufacturer's instructions. gDNA extractions were performed in pools of 50 adult female mites following the CTAB method as previously described.[53]

2.5 RNAseq data analysis

After assessing sample quality with FASTQC, RNAseq reads from the resistant Trz population and the susceptible S-1 and S-2 strains were mapped on the reference *T. urticae* str. London genome using hisat2.[54] Next, the RNAseq reads were counted at the gene level using featureCounts to estimate gene expression (data are accessible in the GEO database under the accession number GSE155156) and a differential expression analysis between the multi-resistant and the susceptible strain was performed using EdgeR. Functional enrichment analyses on the differentially expressed gene sets were done using g:Profiler.[55] To detect non-synonymous polymorphisms in target genes, we used samtools-1.10 to generate an mpileup file with the default parameters and identified high-quality SNPs using VarScan 2.4.4.[56] Finally, the variant call data was loaded into the Integrative Genomics Viewer v2.6.3 [57] and the identified SNPs in target genes were manually inspected.

2.6 Detection of single nucleotide polymorphisms (SNPs)

gDNA and/or cDNA was used as template in PCR reactions in order to screen for known or novel mutations. Primers used for the amplification of target-site gene regions are listed on Table S1. PCR reactions were performed with KAPA Taq PCR Kit (Kapa-Biosystems) in 50 μL containing 1 μL of dNTPs (10mM each), 5 μL of 10X Buffer, 2 μL of each primer (10μM),

0.3 μL (1.5units) Taq DNA polymerase and 2 μL template. PCR was performed under the temperature cycling conditions of: 5 min at 95 °C, 40 cycles of 30 sec at 95 °C, 30-45 sec at 52-57 °C (depending on the primer set for the gene of interest), 30 sec at 72 °C, followed by final extension of 4 min at 72 °C. PCR products were purified by using the Nucelospin Extract 2.0 kit (Macherey Nagel, Düren, Germany), according to manufacturer's instructions. Nucleotide sequences of purified PCR products were determined for both strands at CeMIA sequencing facility (CEMIA, SA., Greece). Obtained sequences were analysed with BioEdit v 7.0.1.[58] The presence/absence of target site mutations was based on visual examination of sequencing chromatographs and RNAseq analysis as described in 2.5 section.

3 RESULTS

3.1 Characterization of resistance levels of the field population

Toxicity assays of the Trz field population showed moderate to high resistance to most compounds used in this study, in comparison to the susceptible S-1 and S-2 strains, indicating a multiple-resistant profile (Table 1). Specifically, Trz population displayed high insensitivity to MGIs, as well as, extremely high resistance levels (>500-fold) to the METI-II acaricide, cyflumetofen. Resistance to abamectin was high (~90-fold), while lower resistance levels (from 13- to >40-fold) were observed for the remaining of the compounds tested (Table 1). Furthermore, the toxicity assay data indicated potential cross-resistance between acaricides with the same mode of action, more specifically for MGIs (etoxazole and clofentezine), METI-I acaricides (pyridaben and fenpyroximate), METI-II acaricides (cyenopyrafen and cyflumetofen) and the different keto-enol compounds (spirodiclofen and spirotetramat).

3.2 Synergistic toxicity bioassays

To investigate the potential metabolic mechanisms, the effects of synergists to cyflumetofen, abamectin and spirodiclofen were determined against S-1, S-2 strains and Trz population (Table 2). Resistance to cyflumetofen of the Trz population is not affected by the application

of any synergist. PBO, DEM and DEF synergized the toxicity of abamectin in Trz by 7.0-, 1.9- and 18.91-fold, respectively. Remarkably, synergists synergized abamectin toxicity in the S-2 but not in the S-1 strain. All synergists enhanced spirodiclofen toxicity in the Trz population. However, synergist ratios were low and both PBO and DEF synergized spirodiclofen toxicity in the S-2 strain at similar levels. Interestingly, DEF reduced the toxicity of cyflumetofen to both susceptible strains, S-1 and S-2, while a similar effect was observed for DEM and PBO in the S-2 strain.

3.3 Transcriptomic analysis

RNA sequencing (RNAseq) was performed in order to compare gene expression profiles between the resistant Trz population and the susceptible S-1 and S-2 strains. Four replicates were sequenced for each strain yielding a total of 265.9 million reads for Trz, 286.4 million reads for S-1, and 246.1 million reads for S-2. A Principal Components Analysis (PCA) showed that replicates of these populations were clearly separated from each other (Figure S1), thus ensuring the validity of the subsequent comparative analyses.

The differential expression (DE) analysis identified 1460 differentially expressed genes (DEGs) ($|\log_2 FC| > 1.5$ and FDR < 0.05), 594 of which were over-expressed in the Trz population compared to S-1, whereas the remaining 866 genes were under-expressed (Figure 1A, Table S2). The comparison between Trz and the other susceptible strain S-2, showed that there are 662 over-expressed genes (Figure 1B, Table S3), whereas 305 were under-expressed. The commonly over-expressed genes in Trz vs both strains are 180 (Table S4, Figure 2), whereas 113 are commonly under-expressed (Table S5, Figure 2).

Functional enrichment analysis was performed in order to find enriched Gene Ontology (GO) terms in the over-expressed genes that could potentially be associated with the observed resistant phenotype. This analysis revealed a statistically significant (p-value < 0.05) enrichment for GO terms associated with cytochrome P450 (CYP) and other detoxification

enzymes activities (Table 3, Figure S2). Enrichment of GO terms such as iron ion binding (GO:0005506), heme binding (GO:0020037), tetrapyrrole binding (GO:0046906), monooxygenase activity (GO:0004497), and transferase activity (GO:0016757, GO:0016758) suggests that metabolic detoxification processes might be at least partly responsible for the multi-resistance phenotype of this population. Importantly, these GO terms were also enriched in the over-expressed genes found in the Trz vs S-2 comparison (Figure S2B), as well as in the 180 genes that are over-expressed in both comparisons (Figure S2C). These results support the hypothesis that detoxification genes may play an important role in the resistant phenotype of the Trz population.

3.4 Differential expression of detoxification genes

Twenty-nine detoxification genes were commonly differentially expressed in Trz population compared to both susceptible strains. More specifically, 8 P450s, 7 CCEs, 6 UGTs and 4 ABC transporters were over-expressed in the Trz population (Table 4). CYP392A15, CYP392D5p, CYP392E8, CYP392E2, and CYP392D10p were among the most highly over-expressed P450s. Seven CCE genes were over-expressed in the resistant population, including CCEincTu11, TuCCE35 and TuCCE01 that have already been identified over-expressed in other resistant strains.[35, 59] Furthermore, UGT69 (tetur22g00510), UGT55 (tetur15g00340) and UGT79p (tetur139g00010) were the most over-expressed UGTs in our dataset which is in line with previous studies.[35, 37] Among the over-expressed ABC transporters in Trz population, the TuABCC-02 (tetur01g10390) and pABC-23 (tetur19g01730) are the most highly over-expressed and is in line with a previous study.[35]

In contrast, 5 detoxification genes were under-expressed in Trz population when compared to both susceptible strains. These include a cytochrome P450 (CYP392A12, tetur03g00830), two GSTs (tetur05g05250 and tetur396g00010), an ABC transporter (tetur02g13710) and a UGT (tetur07g06450).

3.5 Detection of target-site mutations

RNAseq analysis was used to identify previously documented target-site mutations and to screen for novel ones (Table 5). In specific cases, PCR and sequencing was also performed (Table 5). The I1017F mutation on *chs1*, which is associated with high resistance levels to MGIs,[22, 41] was fixed in the Trz population (Table 5, Figure S3), but also present at low frequency in the S-1 and S-2 populations, with the S-2 population having a documented application history of MGIs (see Materials and methods). Additionally, two other point mutations, I261V and G264S, were identified in *chs1* of both Trz population and S-2 strain, which have been previously identified in other resistant strains, but their link with resistance to MGIs has not been established[41] (Table 5). The H92R was identified in PSST subunit, although not fixed and an additional mutation was uncovered (Table 5). Sequence analysis of the four genes that encode succinate dehydrogenase (SDH, complex II), the target of cyflumetofen, revealed a fixed putative novel mutation (Q32R) in the *SdhD* gene in Trz (Table 5, Figure S3), but not in S-1 or S-2. The recently reported abamectin resistance mutation I321T[46] was also identified in the *GluCl3* gene (Table 5, Figure 3), but none of the previously characterized abamectin resistance mutations G314D and G326E in *GluCl1* and *GluCl3*, respectively were identified.[30, 31]

Furthermore, two non-synonymous mutations (resulting in T1158N and T2315L) were identified in the Trz population but were located outside the carboxyl transferase (CT) domain of the *ACCase* gene. T1158N was also fixed in the S-2 strain (Table 5). Through RNAseq we also investigated the presence of previously characterised point mutations in the *AchE* gene of the Trz population. G119S was not identified, but the frequency of the previously documented F331W or F331Y resistance mutation summed up to 100% .[32, 60] Notably, the F331W mutation was also found at 41.9 % and 100% frequency in S-1 and S-2 populations, while the F331Y mutation was not present in either of the susceptible populations. Finally, the Trz population

does not bear any of the previously reported resistance mutations in the *vgsc* [16, 27] or cytochrome b [26, 40, 61] that have been associated with resistance to pyrethroids and bifenazate, respectively.

4 DISCUSSION

We identified and characterized a striking case of multiple acaricide resistance in a field population of *T. urticae* from ornamental plants in Peloponnese, Greece. We report strong resistance phenotypes and accumulation of multiple target-site resistance mutations in conjunction with the over-expression of several detoxification genes. This is not only interesting from a scientific point of view, given that only few studies have documented such multi – resistance [13, 14, 17, 62] , but especially worrying in terms of the remaining options for pest management. The intensity and spectrum of the documented resistance, with LC_{50}s exceeding the recommended field dose for almost all registered chemicals, is of great concern. Toxicity assays revealed extremely high resistance levels to METI-II acaricides and MGIs and high to moderate levels to the remaining tested acaricides. Extremely high resistance levels to the METI-II acaricide cyflumetofen (RR>500; Table 1) were observed in the Trz population, without further selection in the laboratory. Such high resistance levels to cyflumetofen have, to our knowledge, only been reported in a Japanese field strain after further selection in the laboratory, attaining LC_{50}s of higher than 10000 mg/L and cyflumetofen RR exceeding 5000-fold.[43] Cyflumetofen, together with cyenopyrafen belong to the same IRAC group (group 25) and although cyenopyrafen is not registered yet in Greece, cross-resistance was observed in our study, similar to a previous report.[18] Additionally, cross-resistance between METI-I and METI-II acaricides have also been reported,[59, 63] and was also observed in the present study.

Cyflumetofen acts as a complex II inhibitor in the mitochondrial electron transport chain.[10] A number of frequently used fungicides, the boscalid, fluopyram and penthiopyrad,[64] have a similar mode of action as succinate dehydrogenase inhibitors (SDHI), and fungicide resistance has been associated with mutations in *SdhB*, C and D, which form the ubiquinone binding site.[65]

Recently, the point mutations I260T/V and S56L, located in conserved regions in *SdhB* and C, respectively were identified in *T. urticae* strains and associated with high resistance levels to three acaricidal SDHIs.[43] However, none of the aforementioned substitutions were detected in our field population. The identified point mutation (Q32R) in the Trz population is located at the N-terminus of the *SdhD* gene, which lacks conservation. However, the potential role of Q32R mutation in cyflumetofen resistance remains to be investigated functionally, particularly given the limited observed synergism with classical detoxification enzyme inhibitors. Interestingly, the synergist DEF substantially reduced cyflumetofen toxicity in susceptible strains, which is in line with other studies[18,49], and most likely caused by inhibition of hydrolytic activation of cyflumetofen. Additionally, the RNAseq analysis indicated that the known cyflumetofen metaboliser GSTd05[47] was over-expressed in the Trz population compared to the S-2 strain, but not the S-1. Furthermore, other GSTs, such as GSTd10, are largely over-expressed, and should be functionally characterized.

Moderate resistance levels to METI-I acaricides were recorded in the Trz population, approximately 27-folds RR to both fenpyroximate and pyridaben. However, the LC_{50} values were >800 a.i. mg/L, substantially exceeding the 100-200 a.i. mg/L recommended field dose, possibly indicating control failure. Moderate to high RRs to METI-I acaricides have been reported previously.[13, 28, 66] A point mutation in *psst/NDUFS7* of Complex I has previously been associated with resistance to METI-Is in both *Tetranychus* and *Panonychus* species[9, 67, 68] and this mutation was also identified in the Trz population, although not fixed. In addition, an A55T substitution was also uncovered, but this mutation was not in a conserved region, nor near the binding site of METI-Is. As the main target-site mutation was not fixed, additional mechanism might contribute to METI-I resistance, as indicated previously.[9,36] Specifically, functionally expressed CYP392A11 could metabolise fenpyroximate and the METI-II

acaricide cyenopyrafen *in vitro*,[36] and this P450 is over-expressed in Trz population compared to S-2 strain, but not when compared to both strains.

The Trz population showed extremely high resistance levels to MGIs (RR>1000-fold), such as etoxazole and clofentezine which is in line with previous studies.[22, 41] The I1017F mutation in *chs1*, previously linked with high levels of resistance to MGIs (etoxazole, clofentezine and hexythiazox),[22, 41] was fixed in the Trz population. As introgression by marker-assisted back-crossing has revealed the very strong phenotype associated with this mutation, it probably fully explains the observed etoxazole and clofentezine resistance levels[44] (Table 1). Interestingly, fitness cost experiments indicated that the near-isogenic lines bearing I1017F have slower developmental and mean generation time than their susceptible counterparts, while mixed cage experiments showed that resistant homozygotes dropped below 10%. Thus, adopting strategies such as refugia of susceptible mites and rotation of MGIs in distant time intervals might be useful insecticide resistance management strategy, especially since it is recessive.[69]

Resistance to abamectin is also high in the Trz population, although higher RRs have been documented in field populations from other countries.[13, 17, 24, 45, 46] Although we did not identify any of the previously associated mutations (G314D in *GluCl1* and G326E in *GluCl3* [30, 31]), a putative novel resistance mutation (I321T) in *GluCl3* (Table 5) was identified, which has also been very recently detected in other European *T. urticae* populations in association with abamectin resistance.[46] Interestingly, a mutation at a neighbouring position (A309V) has been implicated in abamectin resistance in *Plutella xylostella* (Figure 3).[70, 71] Also, I321 is adjacent to one of the *Caenorhabditis elegans* GluClα TM3 residues that, based on the crystal structure, was predicted to be involved in ivermectin binding.[72] Whether and to what extent the I321T mutation contributes to abamectin resistance remains to be investigated functionally using previously established methodologies. [44, 71, 73, 74] Synergism toxicity assays revealed that PBO synergizes the toxic effect of abamectin in Trz population indicating the involvement of P450

enzymes in the resistant phenotype, whereas DEM and DEF do not particularly enhance abamectin toxicity when taking into account both susceptible strains. The P450 CYP392A16, which has been functionally expressed and is able to metabolize abamectin,[45] was slightly over-expressed in the Trz population compared to the S-1 strain, but not compared to S-2, indicating the possible involvement of alternative resistance mechanism(s).

Twenty-fold resistance to spirodiclofen and cross-resistance to spirotetramat (RR: 36 fold) was detected in the Trz population. Although, the obtained resistance ratio to spirodiclofen is moderate, compared to other studies,[13] it is significantly higher than cases previously reported in Greece, during a large monitoring survey.[62] In addition, it is likely to have an operational impact, since the LC_{50} value is >100 mg a.i./L, exceeding the 40 mg a.i./L field dose. Spirodiclofen targets *ACCase* and at present, only one mutation (A1079T) has been identified in the *ACCase* gene of a spirodiclofen-resistant strain of *T. urticae*.[75] This mutation is located outside of the CT domain and genome editing in the model organism *Drosophila melanogaster* did not support a role in resistance to keto-enols.[76] Recently, a novel point mutation (A2083V) was identified in the CT domain of *ACCase* in *Bemisia tabaci* and genome editing in *D. melanogaster* confirmed high resistance levels to keto-enols.[76] Here, we did not identify any non-synonymous mutations in the CT domain, while two mutations, T1158N and T2315L, with the latter not being fixed, were identified in less conserved regions. By employing the CRISPR technology [73, 77] or multiple backcrossing, the role of these mutations in keto-enol resistance could be validated. Synergism assays indicated that DEM only slightly synergized spirodiclofen toxicity in the Trz population, but overall synergists did not particularly enhance its toxicity. CCE04 and CYP392E10, that have been implicated in resistance to spirodiclofen,[33, 50] were not found in either comparison (Tables S1-2), which is in line with synergism assays.

Only limited decreased susceptibility to bifenazate was observed, although it is included in the application history for the control of the Trz population. Similarly to our study, moderate

404 resistance to bifenazate was observed in field collected strains from other countries.[15, 66]
405 Resistance to bifenazate has been linked with point mutations in cytb, and only recently, a new
406 mutation was uncovered from resistant field strains in the Netherlands.[26, 40, 44] Surprisingly, we
407 did not detect any of the previous documented mutations,[15, 40] possibly reflecting rational use
408 and application of the specific compound.
409 Trz population does not bear any of the previously identified mutations in *vgsc*,[16, 27, 29]
410 indicating the limited use of pyrethroids for the control of *T. urticae* populations recently in
411 Greece, but also a potential to possibly rotate old pyrethroid chemistries for *T. urticae* control,
412 if similar patterns are observed in a wider sampling from the region. Finally, several
413 segregating point mutations were identified in the *AchE* gene. Interestingly, the G119S was not
414 present, while the frequency of F331W and F331Y summed up to 100% frequency in the Trz
415 population,[32] possibly reflecting the past use of organophosphates for *T. urticae* control in
416 Greece and the low fitness cost of these resistant mutations.
417 The RNAseq analysis indicated the consistent over-expression of several additional genes in
418 Trz population when compared against both susceptible strains, that might be associated with
419 resistance phenotypes against specific or multiple active ingredients. For example, several
420 cytochrome P450s, such as CYP392D10p CYP392E8, CYP392A15 and CYP392D5p, some
421 of which have been also associated with acaricide resistance in other studies.[35, 45, 46] However,
422 functional expression remains to establish a functional link between the over-expressed genes
423 and the observed/recorded phenotype.

424 **5 CONCLUSIONS**

425 We report on a multi-resistant *T. urticae* field population collected from Trizina, Peloponnese,
426 Greece. This population showed extremely high resistance levels to a range of compounds such
427 as abamectin, etoxazole, clofentezine, cyflumetofen, fenpyroximate and spirodiclofen.
428 RNAseq analysis indicated that many detoxification genes (P450s and UGTs) were over-

expressed, but also previously documented target site resistance mutations were present. A number of interesting SNPs were also uncovered in *SdhD, GluCl3* and *ACCase* genes that await functional validation. Such resistance cases compromise effective acaricide resistance management and their early detection is of outmost importance for evidence-based management. Interestingly, target site mutations against pyrethroids or bifenazate were not detected, thus possible rotation of these compounds might be considered for the control of *T. urticae* populations with similar phenotype.

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SUPPORTING INFORMATION

Supporting information might be found in the online version

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Tables

Table 1: Toxicity assays of ten acaricides/insecticides to *Tetranychus urticae* strain S-1 and Trz population with probit regression parameters and resistance ratios

Active ingredient	Strain	Slope ± s.e.	LC_{50} (95% CI) †	χ^2 (df)	RR (95% CI)
Abamectin	S-1	5.75±0.79	0.19 (0.17-0.21)	11.560 (13)	1
	Trz	4.77±0.60	17.13 (15.16-19.23)	6.87 (10)	89.91 (76.11-106.20)*
Clofentezine	S-1	2.36±0.16	6.34 (5.14-7.76)	34.75 (15)	1
	Trz		>10000		>1666*
Etoxazole	S-1	5.92±0.81	1.91 (1.66-2.12)	14.18 (13)	1
	Trz		>10000		>5235*
Spirodiclofen	S-1	13.48 ±1.56	6.45 (5.80-7.02)	45.67 (12)	1
	Trz	2.74±0.20	131.30 (103.67-160.34)	58.14 (18)	20.36 (18.07-22.94)*
Spirotetramat	S-1	5.32±0.68	1.17 (0.88-1.40)	27.47 (10)	1
	Trz	1.09±0.06	42.25 (22.41-70.23)	71.11 (12)	36.20 (28.72-45.64)*
Pyridaben	S-1	2.32±0.56	29.07 (14.25-39.00)	18.32 (15)	1
	Trz	1.46±0.18	813.11 (578.57-1134.88)	8.16 (10)	27.97 (17.62- 44.40)*
Fenpyroximate	S-1	1.558±0.16	40.75 (28.17-55.78)	10.53 (16)	1
	Trz	1.28±0.19	1105.42 (794.65-1449.62)	2.81 (13)	27.13 (17.36-42.39)*
Cyenopyrafen	S-1	2.40±0.38	3.89 (2.68-4.91)	6.48 (10)	1
	Trz	3.22±0.43	453.74 (376.38-526.93)	10.18 (12)	116.66 (83.87- 162.29)*
Cyflumetofen	S-1	8.26±0.99	19.99 (18.58-21.35)	11.37 (13)	1
	Trz		>10000		>500.35*
Bifenazate	S-1	5.98±0.63	2.32 (1.99-2.86)	44.38 (16)	1
	Trz	3.63±0.37	30.62 (26.66-35.18)	9.93 (16)	13.18 (11.15- 15.58)*

†: Lethal concentration expressed in mg L^{-1}

*: Treatment effect was considered significant if 95% CI RR do not contain the value 1.

719 **Table 2.** Toxicity of cyflumetofen, abamectin and spirodiclofen with and without synergists to

720 susceptible strains (S-1 and S-2) and resistant (Trz) population of *Tetranychus urticae*

Compound	Strain	LC_{50} † (95% CI)	Slope ± SE	χ^2 (dF)	SR (95% CI)
Cyflumetofen	Trz	> 10000			-
	Trz + PBO	>5000			
	Trz + DEM	>5000			
	Trz + DEF	>5000			
	S-1	19.99 (18.58-21.35)	8.26±0.99	11.36 (13)	-
	S-1 +PBO	19.43 (10.44-23.90)	4.12±1.11	20.67 (14)	1.03 (0.84-1.26)
	S-1 +DEM	29.34 (25.12-32.77)	7.35±1.61	15.91 (17)	0.68 (0.53-0.78) *
	S-1 +DEF	921.24 (813.78-1012.78)	6.10±0.82	7.43 (16)	0.022 (0.019-0.025) *
	S-2	13.98 (12.78-15.14)	6.92±0.97	6.75 (12)	-
	S-2 + PBO	108.67 (42.92-144.92)	5.55±1.50	4.15 (10)	0.13 (0.08-0.19) *
	S-2 + DEM	139.70 (101.80-177.00)	2.89±0.43	4.30 (11)	0.10 (0.08-0.13) *
	S-2 + DEF	779.54 (721.32-825.97)	12.90±2.50	6.46 (11)	0.018 (0.016-0.020)*
Abamectin	Trz	17.13 (15.16-19.23)	4.77±0.60	6.87 (10)	-
	Trz + PBO	2.41 (1.77-3.03)	2.50±0.32	14.75 (15)	7.11 (5.34-9.47)*
	Trz + DEM	9.03 (6.78-10.75)	4.08±0.74	7.58 (19)	1.90 (1.49-2.42)*
	Trz + DEF	0.91 (0.72-1.05)	3.77±0.64	7.04 (19)	18.91 (15.28-23.41)*
	S-1	0.19 (0.17-0.21)	5.75±0.79	11.60 (13)	-
	S-1 +PBO	0.19 (0.17-0.21)	5.94±0.53	62.55 (21)	0.98 (0.86-1.12)
	S-1 +DEM	0.19 (0.18-0.21)	5.31±0.54	13.30 (18)	0.98 (0.84-1.13)
	S-1 +DEF	0.20 (0.19-0.22)	6.69±0.61	21.23 (18)	0.93 (0.82-1.07)
	S-2	0.76 (0.60-0.83)	13.38±3.23	13.64 (11)	-
	S-2 + PBO	0.20 (0.16-0.24)	2.94±0.34	9.89 (16)	3.78 (3.05-4.68)*
	S-2 + DEM	0.36 (0.26-0.43)	5.66 ± 1.3	10.82 (19)	2.10 (1.66-2.65)*
	S-2 + DEF	0.04 (0.04-0.05)	6.69±1.33	7.27 (19)	17.29 (14.53-20.57)*
Spirodiclofen	Trz	131.31 (103.67-160.34)	2.74±0.20	58.14 (18)	-
	Trz + PBO	43.93 (34.67-52.87)	2.39±0.21	15.01 (15)	2.99 (2.41-3.71)*
	Trz + DEM	91.63 (71.20-113.64)	1.61±0.12	15.00 (16)	1.43 (1.10-1.86)*
	Trz + DEF	96.17 (70.61-123.28)	2.50±0.21	36.80 (12)	1.36 (1.14-1.63)*
	S-1	6.45 (5.80-7.02)	13.48±1.56	45.67 (12)	-
	S-1 +PBO	5.84 (5.23-6.46)	3.21±0.23	13.37 (12)	1.11 (1.00-1.22)
	S-1 +DEM	9.38 (7.57-10.7)	5.30±0.70	13.32 (10)	0.70 (0.60-0.78) *
	S-1 +DEF	7.73 (5.71-9.14)	4.28±0.63	11.67 (10)	0.834 (0.70-1.00)
	S-2	15.68 (14.22-17.05)	5.29±0.51	18.07 (25)	-
	S-2 + PBO	5.62 (2.37-7.96)	2.88±0.36	56.66 (13)	2.79 (2.23-3.48)*
	S-2 + DEM	14.06 (9.67-16.97)	3.31±0.46	35.08 (17)	1.11 (0.93-1.34)
	S-2 + DEF	9.27 (7.66-10.99)	2.70±0.24	19.50 (15)	1.69 (1.42-23.01) *

721 † Lethal concentration expressed in mg L^{-1}

722 * Treatment effect was considered significant if the 95% CI SR does not contain the value 1.

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Table 3. List of the enriched GO terms in the over-expressed genes

Source[a]	Term Name	Term ID	Adjusted p-value
GO:MF	iron ion binding	GO:0005506	2.69e-09
GO:MF	heme binding	GO:0020037	7.87e-09
GO:MF	tetrapyrrole binding	GO:0046906	7.87e-09
GO:MF	oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen	GO:0016705	2.65e-06
GO:MF	monooxygenase activity	GO:0004497	8.98e-06
GO:MF	cofactor binding	GO:0048037	6.14e-05
GO:MF	oxidoreductase activity	GO:0016491	9.58e-05
GO:MF	transferase activity, transferring glycosyl groups	GO:0016757	1.20e-04
GO:MF	transferase activity, transferring hexosyl groups	GO:0016758	6.98e-04
GO:MF	transition metal ion binding	GO:0046914	3.05e-03
GO:BP	oxidation-reduction process	GO:0055114	3.86e-05
GO:BP	non-motile cilium assembly	GO:1905515	4.62e-04
GO:CC	peroxisome	GO:0005777	1.06e-04
GO:CC	microbody	GO:0042579	1.06e-04
GO:CC	BBSome	GO:0034464	1.68e-04
KEGG	Linoleic acid metabolism	KEGG:00591	4.42e-05
KEGG	Retinol metabolism	KEGG:00830	3.65e-04

[a] Abbreviations used: MF: Molecular Function, BP: Biological Process, CC: Cell Component.

Table 4. Differentially expressed detoxification genes in Trz population compared to S-1 and S-2 strains

Gene ID	log_2FC in S-1	log_2FC in S-2	Description
tetur03g00010	11.85	1.52	probable pseudogene
tetur03g05110	6.15	4.28	CYP392D10p probable pseudogene
tetur03g09941	4.2	3.61	CYP392A15
tetur03g05020	3.7	6.43	CYP392D5p probable pseudogene
tetur27g00350	3.6	3.79	CYP392E8
tetur06g02400	3.03	1.64	CYP392E2
tetur03g05040	2.59	1.82	fragment
tetur03g05060	2.14	2.35	probable pseudogene
tetur27g00330	2.07	3.55	CYP392E6
tetur602g00010	1.99	1.56	CYP385B1-p pseudogene
tetur16g03790	1.76	1.54	CYP392A10
tetur29g00990	8.78	1.99	CCEincTu18
tetur29g00950	7.66	1.63	CCEincTu11
tetur309g00020	3.3	2.46	TuCCE71
tetur17g00300	3.17	1.86	TuCCE45
tetur01g08680	2.92	2.75	TuCCE01
tetur24g02580	2.65	3.55	CCEincTu10
tetur11g05770	2.55	2.42	TuCCE35
tetur22g00510	4.28	7.01	teturUGT69
tetur139g00010	3.59	7.39	teturUGT79p
tetur15g00340	3.42	3.99	teturUGT55
tetur22g00360	2.94	2.82	teturUGT63
tetur05g00090	2	3.05	teturUGT23
tetur02g09830	1.5	2.32	teturUGT10
tetur01g10390	3.18	2.75	TuABCC-02
tetur19g01710	2.46	1.66	pABC-22
tetur19g01730	1.88	1.67	pABC-23
tetur07g04290	1.82	1.81	TuABCC-21

762 **Table 5.** Overview of target-site mutations found in the Trz population

Gene[A]	Mutation[d]	Frequency in Trz	Frequency in S-1	Frequency in S-2
tetur03g08510 (*chs1*)[a]	I261V	100%	0%	100%
	G264S	100%	0%	100%
	I1017F[e]	100%	13.26±11.14%	27.32±13.63%
tetur08g03210 (*SdhA*)[a]	I50V[e]	28.84±3.81%	0%	100%
	V143I[e]	100%	45%	100%
	I245V[e]	100%	45%	100%
	T498S[e]	60.5±2.59%	42.3±3%	100%
tetur30g00210 (*SdhC*)[a]	T107S	25%	0%	0-2%
tetur20g00790 (*SdhD*)[a]	Q32R[e]	100%	0%	0%
tetur10g03090 (*GluCl3*)[a]	I321T[e]	100%	0%	0%
tetur19g00850 (*AchE*)[b]	V7I	0%	31.09±12.32%	0%
	G119S	0%	56.35±7.71%	0%
	A201S	24.59±4.40%	0%	100%
	T280A	26%	0%	100%
	F331Y	78.25±4.51%	0%	0%
	F331W	21.75±4.51%	41.9±3.30%	100%
tetur21g02170(*ACCase*)[a]	T1158N	100%	0%	100%
	T2315L	58.42±2.17%	0%	0%
tetur07g05240 (*PSST*)[c,a]	A55T	41.85±0.96%	0%	0%
	H92R (H110R)	50.19±3.52%	22.08±1.61%	0%

763 [A] the position of the identified point-mutations is according to a) *Tetranychus urticae* numbering, b) *Torpedo*
764 *californica* mature sequence numbering, c) *Yarrowia pilolytica*
765 [d] Mutation frequency was estimated using VarScan
766 [e] these mutations were first identified using the RNAseq data, but have also been verified by sequencing of the
767 respective PCR products.
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Figure legends

Figure 1: Overview of the differentially expressed genes ($log_2|FC| > 1.5$ and FDR < 0.05) with an emphasis on over-expressed detoxification enzymes. A. Comparison between Trz population and S-1 strain. B. Comparison between Trz population and S-2 strain.

Figure 2: Summary of the genes that are differentially expressed between Trz population and susceptible strains, S-1 and S-2. The Venn diagram represents the numbers of over- and under-expressed genes in Trz population compared to S-1 and S-2 strains. The overlapping part represents the differentially expressed genes in Trz population compared to both susceptible strains. Over- and under-expressed genes are depicted by upward and downward arrows, respectively.

Figure 3: Alignment of the transmembrane 3 region of *Tetranychus urticae* GluCls and *Plutella xylostella* GluCl. An 80% threshold was set to show identity (black shading). The amino acid position that corresponds to the substitution identified in *P. xylostella* is depicted with a diamond. The filled circle corresponds to the position of the novel substitution found in Trz population and the substitution is indicated in red font. All *T. urticae* GluCl sequences are obtained from ORCAE database, except the sequence of Trz that was included for reasons of comparison. Species abbreviations: Tu, *Tetranychus urticae*; Px, *Plutella xylostella.*

Figure S1: Principal components analysis of the gene expression levels between the multi-resistant Trz population and the susceptible strains S-1 and S-2. The samples of each strain are clustered together and are also clearly separated from each other, thus demonstrating that the samples are suitable for any downstream comparative analyses.

Figure S2: Over-represented GO terms in the genes that are over-expressed in the resistant Trz population. (A) Comparison with the S-1 strain. (B) Comparison with the S-2 strain. (C) Comparison with both susceptible strains (S-1 and S-2)

Figure S3: Chromatographs from sequencing of *GluCl3*, *SdhD* and *chs1* genes. A. Sequencing of *GluCl3* from the two susceptible (S-1 and S-2) strains and Trz population indicate the SNP at nucleotide level. B. Sequencing of *SdhD* gene from the two susceptible (S-1 and S-2) strains and Trz population indicate the SNP at nucleotide level. C. Sequencing of *chs1* gene from the S-1 strain and Trz population indicate the SNP at nucleotide level.

Figures

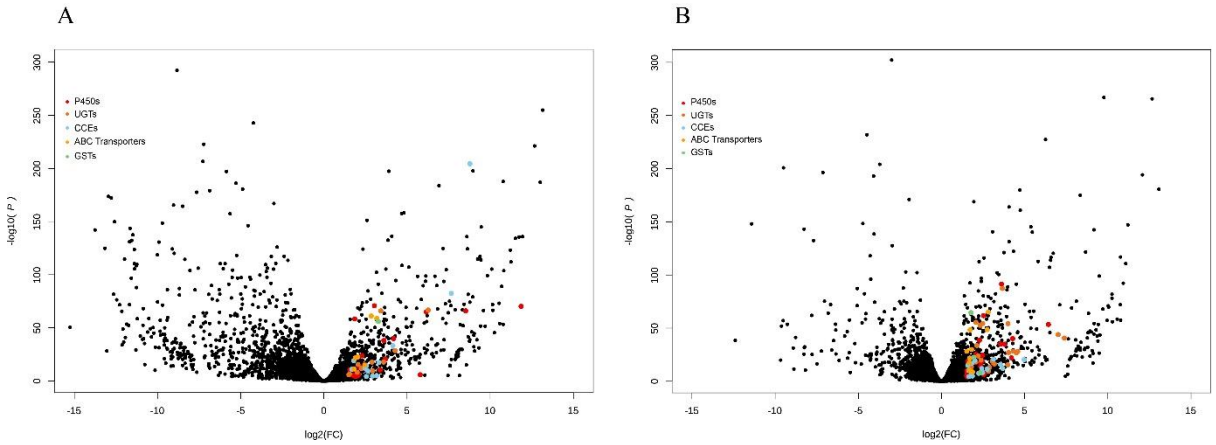


Figure 1

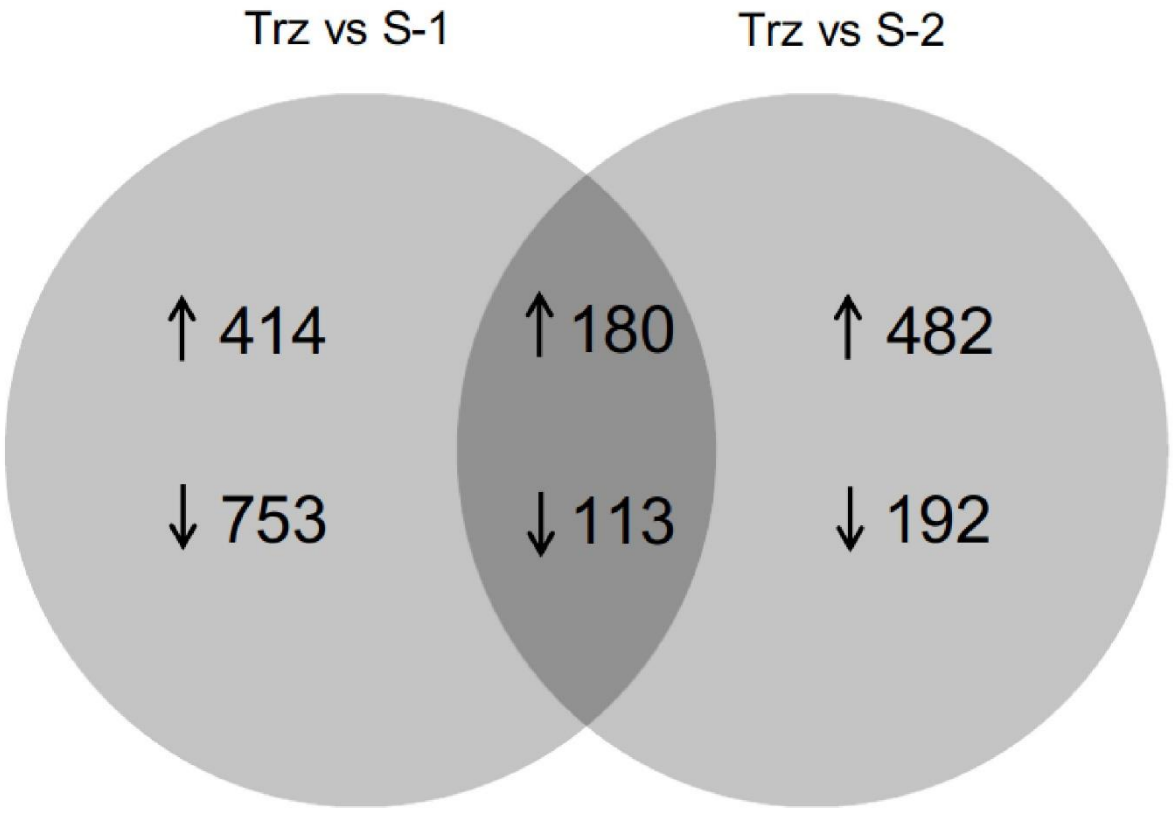


Figure 2

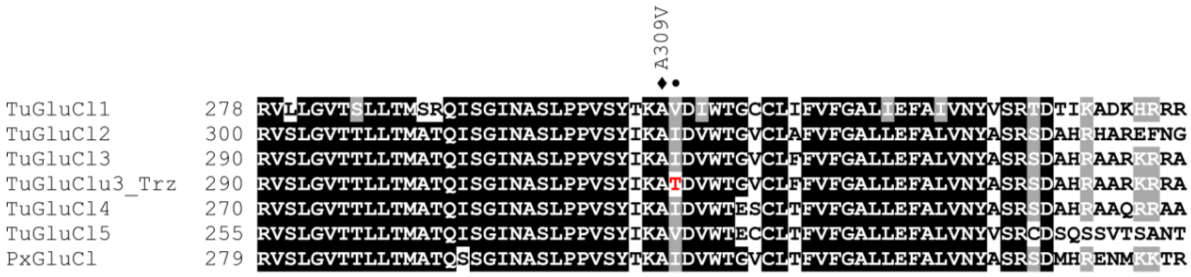
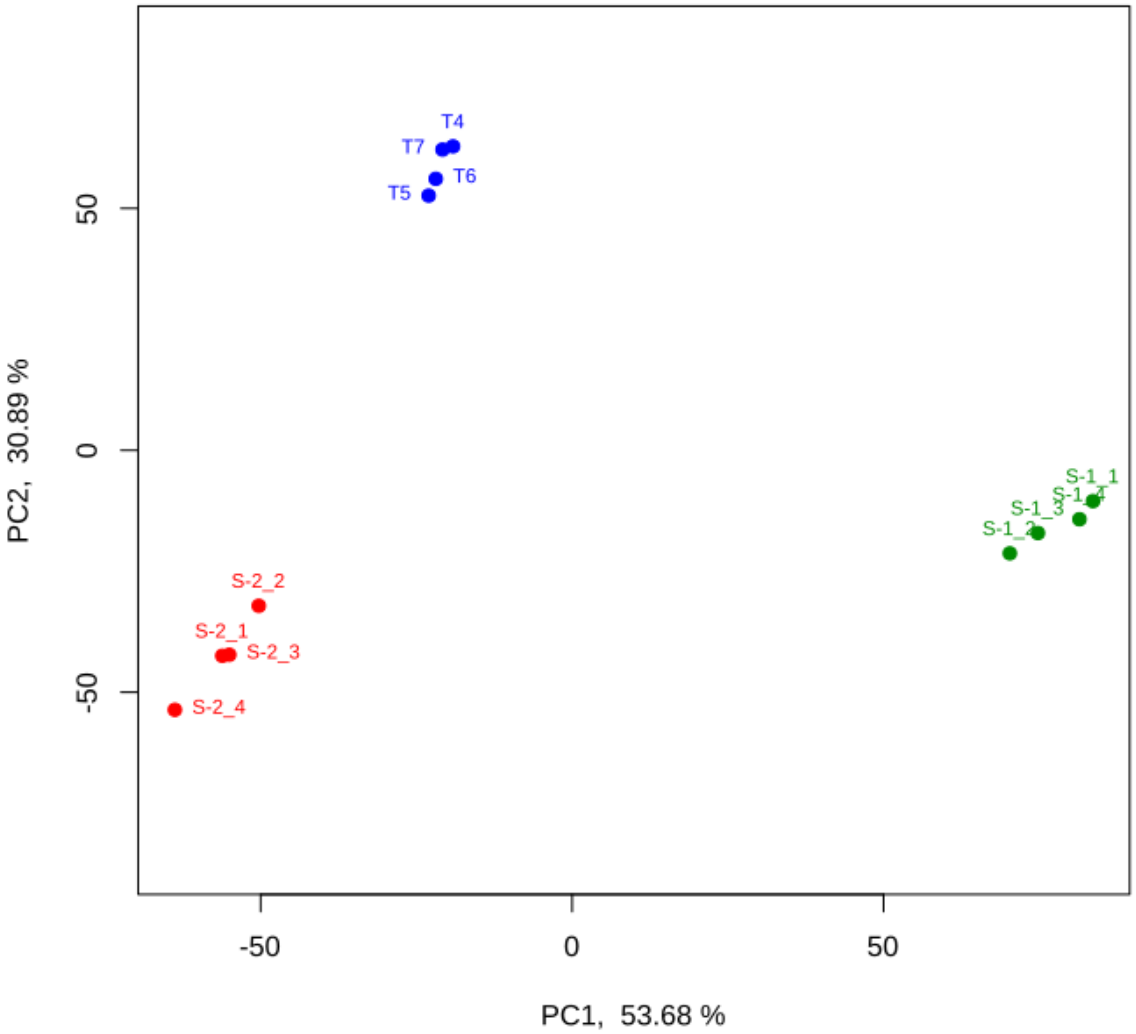


Figure 3

Table S1. Primers used in this study

Gene	TeturID	Primer	Sequence (5'-3')	PCR product size	Template	Reference
GluCl1	*tetur02g04080*	Turt GluCl1_F	TTGGATTGACCCTAACTCAGCA	261bp	gDNA	27
		Turt GluCl1_R	TTGCACCAACAATTCCTTGA			
GluCl3	*tetur10g03090*	Turt GluCl3_F	CCGGGTCAGTCTTGGTGTTA	253bp	gDNA	
		Turt GluCl3_R	CACCACCAAGAACCTGTTGA			
SdhA	*tetur08g03210*	08g03210_F1	TGCGATTGATTCAAAAACTCA	760bp	gDNA	
		08g03210_R1	AGGCTCGACCATATCCTCCT			
		08g03210_F2	AGCTCATCGATGTTGCTGTG	760bp	gDNA	
		08g03210_R2	AGCAGCGTAAAGACCAGGAA			
		08g03210_F3	GAGCCTATTCCCGTTCTTCC	780bp	gDNA	
		08g03210_R3	CGGGAGGAACTGTTTGACAT			
SdhB	*tetur01g15710*	sdhB_F	AGTTGCTTTCCTTGGCTTCA	950bp	gDNA	21
		sdhB_R	ACCAGTTACTTGGGGGCTTT			
SdhC	*tetur30g00210*	sdhC_F	AAATCATGTTATTTCCACGTTTGA	800bp	gDNA	
		sdhC_R	GCAATTGGTTACGGGTAGTTTAGTAT			
SdhD	*tetur20g00790*	sdhD_F	CCATGAACCGAGTTTTGTCA	800bp	gDNA	
		sdhD_R	CGATGACTTTTCCGTAATTCCT			
chs1	*tetur03g08510*	CHS1_F	CTTCACCGTCTGCCGTATTT	541bp	gDNA	18, 37
		CHS1_R	CTTTTCGTCGTTTGGTTTGG			
ACCase	*tetur10g00850*	ACC_A_int1	AAAGGCGCTGGCTTTATTTT	695/796bp	cDNA/gDNA	this study
		ACC_A_FW	ATATTTGGTCCCCGTGAAGA			
		ACC_A_RV	CGTCATTTCTGTGCATAGCTT	694/760bp	cDNA/gDNA	
		ACC_A_int2	GCCTTTACCGATAATGGAACC			
		ACC_B_int2	TTGATAAAACGCCTTGAGCA	719bp	gDNA	
		ACC_B_RV	TGCCATGGAATGATACCTCTAA			
		ACC_B_RV_bis	CCGCATAGAACCATTTTTCC	1509bp (with ACC_B_FW)	gDNA	
		ACC_B_FW	AGCCAGAAGGTTCAGTCGAA	797bp	gDNA	



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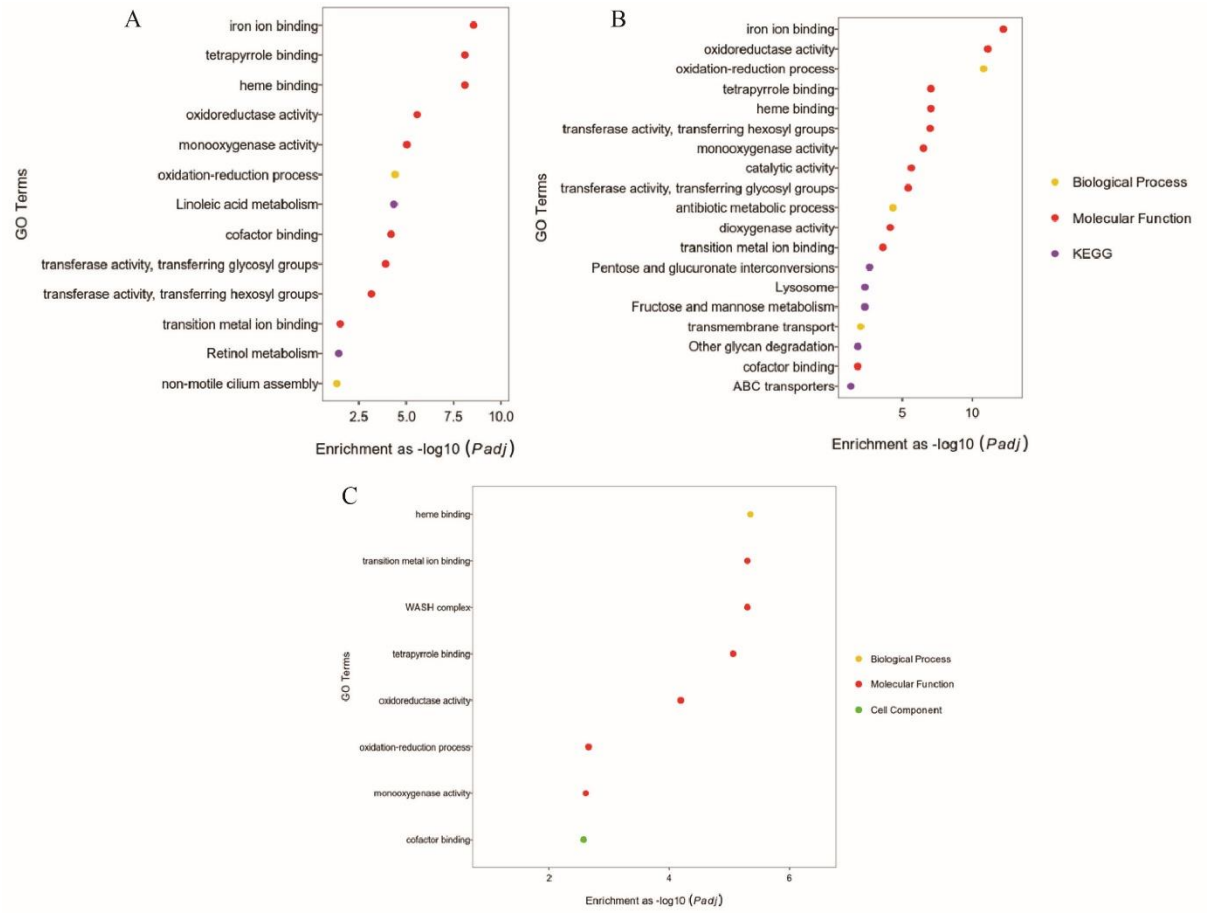
849 **Figure S1**


A
iron ion binding
tetrapyrrole binding
heme binding
oxidoreductase activity
monooxygenase activity
oxidation-reduction process
Linoleic acid metabolism
cofactor binding
transferase activity, transferring glycosyl groups
transferase activity, transferring hexosyl groups
transition metal ion binding
Retinol metabolism
non-motile cilium assembly
GO Terms
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Enrichment as -log10 (Padj)
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oxidoreductase activity
oxidation-reduction process
tetrapyrrole binding
heme binding
transferase activity, transferring hexosyl groups
monooxygenase activity
catalytic activity
transferase activity, transferring glycosyl groups
antibiotic metabolic process
dioxygenase activity
transition metal ion binding
Pentose and glucuronate interconversions
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tetrapyrrole binding
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oxidation-reduction process
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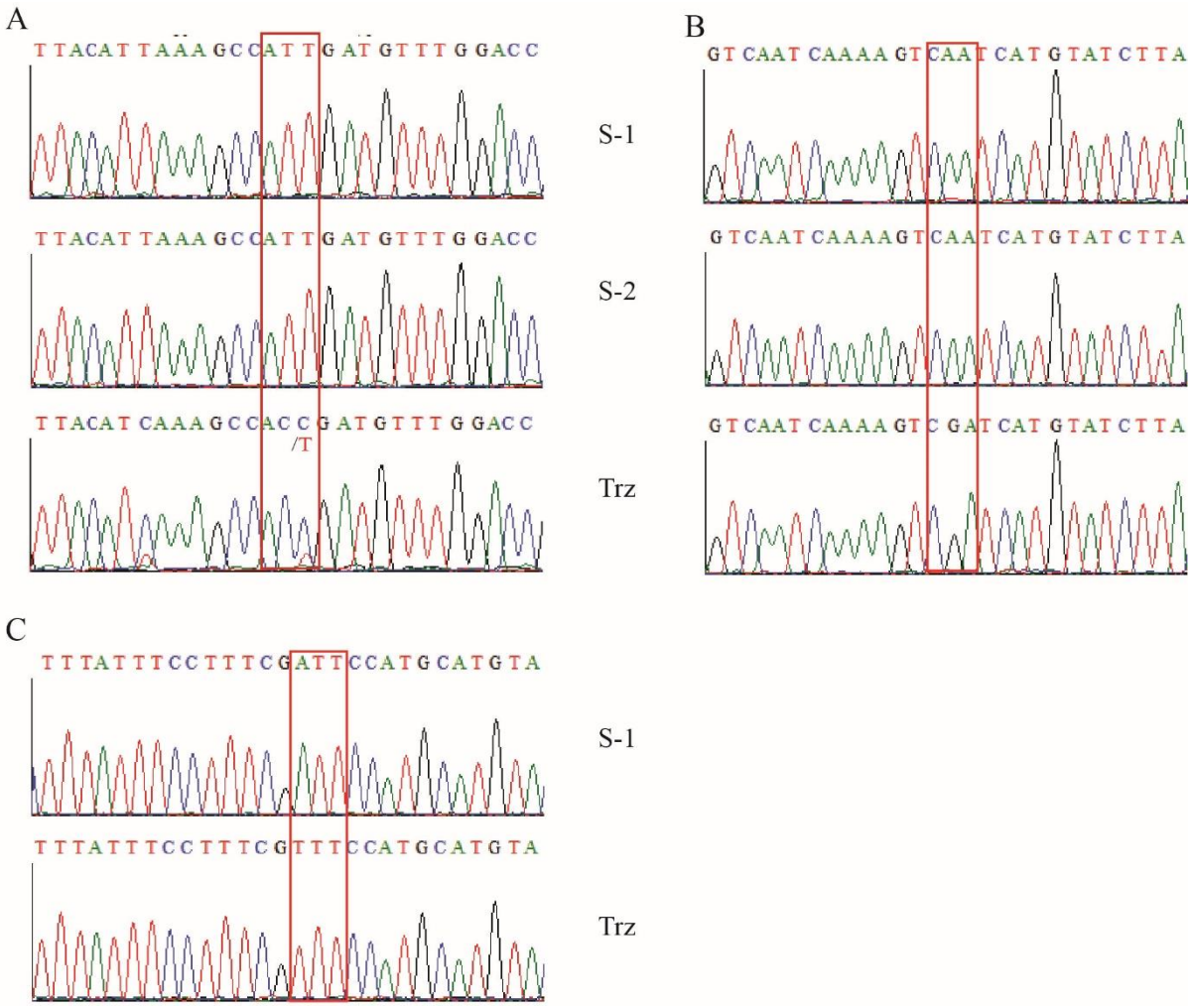
Biological Process
Molecular Function
Cell Component

Figure S2



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853 **Figure S3**

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