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Genetic basis of acaricide resistance

Identification and characterization of the risk and mechanisms of resistance to bifenthrin, acequinocyl, and the novel acaricide pyflubumide in Tetranychus urticae

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Identification and characterization of new mutations in mitochondrial cytochrome b that confer resistance to bifenthrin and acequinocyl in the spider mite *Tetranychus urticae*

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Abstract

BACKGROUND: In spider mites, mutations in the mitochondrial cytochrome b Q₀ pocket have been reported to confer resistance to the Q₀ inhibitors bifenthrin and acequinocyl. In this study we surveyed populations of the two-spotted spider mite *Tetranychus urticae* for mutations in cytochrome b, linked newly discovered mutations with resistance and assessed potential pleiotropic fitness costs.

RESULTS: We identified two novel mutations in the Q₀ site: G132A (equivalent to G143A in fungi resistant to strobilurins) and G126S + A133T (previously reported to cause bifenthrin and acequinocyl resistance in *Panonychus citri*). Two *T. urticae* strains carrying G132A were highly resistant to bifenthrin but not acequinocyl, whereas a strain with G126S+A133T displayed high levels of acequinocyl resistance, but only moderate levels of bifenthrin resistance. Bifenthrin and acequinocyl resistance were inherited maternally, providing strong evidence for the involvement of these mutations in the resistance phenotype. Near isogenic lines carrying G132A revealed several fitness penalties in *T. urticae*; a lower net reproductive rate (R₀), intrinsic rate of increase (r_m) and finite rate of increase (LM); a higher doubling time (DT); and a more male-biased sex ratio.

CONCLUSIONS: Several lines of evidence were provided to support the causal role of newly discovered cytochrome b mutations in bifenthrin and acequinocyl resistance. Because of the fitness costs associated with the G132A mutation, resistant *T. urticae* populations might be less competitive in a bifenthrin-free environment, offering opportunities for resistance management.

INTRODUCTION

The spider mite *Tetranychus urticae* Koch (Arthropoda: Acari: Tetranychidae) is an important cosmopolitan pest damaging many agricultural crops. Frequent acaricide applications are needed to control this species, which have led inevitably to the development of resistance. This species is considered one of the most pesticide-resistant arthropods based on the number of active ingredients to which resistance has been reported.^{1,2} Pesticide resistance evolves via two main mechanisms: (i) toxicodynamic changes, such as a reduction in the sensitivity or availability of the target-site due to point mutation(s), gene knockout or amplification; and (ii) toxicokinetic changes that reduce the amount of pesticide that reaches the target-site through changes in exposure, penetration, transportation, metabolism and excretion.^{3,4}

Resistance mechanisms are often costly; for example, point mutations in essential target genes can convey pleiotropic effects and affect other phenotypic traits in addition to pesticide resistance.⁵⁻⁷ Reproduction, dispersal, generation time and longevity have been reported to be negatively affected by target-site resistance mutations.⁸⁻¹² Also, for the spider mite *T. urticae*, fitness costs have been reported after marker-assisted backcrossing, but not for all resistance mutations.¹¹

Although environmentally friendly methods such as biological control have increased in importance, especially in greenhouse crops,^{13,14} spider mites such as *T. urticae* are still mainly controlled using acaricide applications.¹⁵ The hydrazine carbazate acaricide bifenthrin is one of the most recently developed and frequently used acaricides with excellent selectivity to all life stages of *Tetranychus* spp. and *Panonychus* spp.^{16,17} Bifenthrin was first classified as a neurotoxin,¹⁸ but later studies revealed a mitochondrial mode of action via inhibition of electron transport.^{12,19} Bifenthrin resistance has been shown to inherit maternally and high levels of resistance are linked tightly with mutation(s) at highly conserved regions (the cd1-helix and ef-helix) of the cytochrome b Q₀ site of mitochondrial complex III (bc1 complex, ubiquinone: cytochrome c oxidoreductase enzyme complex).

Mitochondrial complex III is an essential enzyme complex in the electron transport chain that plays a critical role in the biochemical generation of adenosine triphosphate (ATP) via oxidative phosphorylation. The catalytic core of this enzyme complex is composed of three subunits in eukaryotes: cytochrome b, Rieske iron-sulphur protein (ISP) and cytochrome c1 proteins.

Cytochrome b is encoded by the mitochondrial genome, whereas the other subunits are encoded by the nuclear genome. Electrons are transported from low-potential ubiquinol to a higher potential cytochrome c via the Q-cycle pathway.^{20,21} This pathway requires two separate quinone-binding sites: the quinol oxidation site (Q₀ site) and the quinone reduction site (Q_i site). These two sites are located on opposite sides of the membrane and are linked by a transmembrane electron-transfer pathway. Pesticides that inhibit the normal functioning of Q₀ sites have been developed from different chemical classes including, in addition to the carbazate bifenthrin, the 2-hydroxynaphthoquinones (HONQs) and the b-methoxyacrylates (MOAs) with the strobilurins as a commercially successful family of potent fungicides.²²⁻²⁴ Acequinocyl is the only commercialized acaricide of the naphthoquinone analogue group²⁵ and is commonly used against all stages of *T. urticae* and other spider mite species.¹⁸ Cross-resistance between bifenthrin and acequinocyl associated with cytochrome b mutations has been reported from *T. urticae* and *Panonychus citri*

populations.^{19,24} The strobilurin fungicides were originally isolated from the mycelium of the basidiomycete *Strobilurus tenacellus* strain No. 2160226 and are currently considered one of the most important classes of agricultural fungicides.^{27,28} The first field resistance to strobilurin fungicides was reported in wheat powdery mildew populations in northern Germany in 1998.²⁹ Later studies revealed that resistance to this group of fungicides in plant pathogenic fungi is most often due to point mutation(s) in the Q₀ region of mitochondrial cytochrome b.^{30–32}

In this study, we discovered a G132A mutation in cytochrome b of *T. urticae*, equivalent to G143A in fungi, which has been reported as the most frequent mutation associated with strobilurin resistance.^{30,33–36} During a survey investigating the frequency of G132A in *T. urticae* field strains, we also uncovered for the first time the combination of G126S+A133T in *T. urticae*, reported previously in the spider mite *P. citri*.²⁴ We provide strong evidence of the causal role of these resistance mutations by revealing maternal inheritance and determine the strength of the phenotype by introgression of the mitochondrial haplotype in a susceptible genomic background. Lastly, we used the generated isogenic lines to assess potential fitness costs associated with G132A in *T. urticae*.

MATERIALS AND METHODS

Chemicals and mite strains

Commercial formulations of the mitochondrial complex III electron transport inhibitors bifenthrin (Floramite[®] 240 g L⁻¹ SC) and acequinocyl (Cantack[®] 150 g L⁻¹ SC) were purchased from Intergrow (Aalter, Belgium). All other chemicals were analytical grade and purchased from Sigma-Aldrich (Zwijndrecht, Netherlands), unless stated otherwise. The JP-R strain³⁷ and the laboratory susceptible Wasatch strain³⁸ have been described previously. In addition, 23 field strains were collected from different geographical areas across Europe between 2016 and 2019 for resistance mutation screening (Table 1). All mites were reared on kidney bean plants, *Phaseolus vulgaris* L. cv. ‘Speedy’ or ‘Prelude’, at 25±1 °C, 60% relative humidity and a 16:8 h light/dark photoperiod.

Survey of cytochrome b variants

DNA extraction and polymerase chain reaction (PCR) amplification of cytochrome b were performed as described by Van Leeuwen *et al.*¹² Briefly, ~200 adult females were collected and homogenized in 800 μL sodium dodecyl sulfate (SDS) buffer (200 mmol L^{-1} Tris-HCl, 400 mmol L^{-1} NaCl, 10 mmol L^{-1} EDTA, 2% SDS at pH 8.3) followed by phenol-chloroform extraction. For single mite DNA extraction, a single adult female was homogenized by hand in 20 μL mixture of STE buffer (100 mmol L^{-1} NaCl, 10 mmol L^{-1} Tris-HCl, 1 mmol L^{-1} EDTA, pH 8.0) and proteinase K (10 mg mL^{-1} , 2 mL) in a 1.5 mL Eppendorf tube. The mixture was then incubated at 37 $^{\circ}\text{C}$ for 30 min followed by 95 $^{\circ}\text{C}$ for 5 min.¹² PCR was performed using the Expand Long Range PCR kit (Roche, Zwijndrecht, the Netherlands) and the primers Cytbdia2F (5'-TTAAGAACTCCTAAAACCTTTTCGTTTC) and Cytbdia2R (5'-GAAACAAAAATTATTATTCCC-CAAC). PCR products were purified with a Cycle-Pure Kit (E.Z.N.A.TM) and sequenced with the original PCR primers and four internal sequencing primers (cytbWTF, 5'-CGGAATAATTTTACAAATAAATCATGTC; cytbWTR, 5'-TGGTACAGATCGTAGAATTGCG; PEWYF1, 5'-AAAGGCTCATCTAACCAAATAGG; PEWYR2, 5'-AATGAAATTTCTGTAAAAGGGTATTC).¹² Sequence data were analysed using BioEdit software.³⁹ Sequences have been submitted to the NCBI repository (Table 1).

Generation of isofemale and introgressed lines

Isofemale lines were established from the FS1 and FS8 strains and were labelled as iso-FS1 and iso-FS8, respectively. Approximately 500 mated female mites were transferred to detached bean leaves on wet cotton wool in Petri dishes and sprayed with 200 mg L^{-1} bifentazate. Five Petri dishes were prepared per strain. After 72 h, ten alive females were selected at random from the sprayed arenas and transferred to 9 cm^2 bean leaf discs individually. Mites were allowed to lay eggs for 3–4 days. DNA of each single female was extracted as described above. Progeny of a single female with the G132A (iso-FS1) and G126S+A133T (iso-FS8) mutations were used to create the isofemale lines. Introgressed lines were established using the backcrossing methods described by Bajda *et al.*^{40,41} Briefly, JP-R and iso-FS8 virgin females were crossed with susceptible Wasatch males. A virgin F1 female was backcrossed to

Wasatch males, and the backcrossing was repeated seven times. After backcrossing, mites were transferred to full bean plants and were allowed to expand their population size for toxicity and fitness costs experiments. Introgressed lines that carry G132A and G126S+A133T are labelled JP-R-BC (1-3) and iso-FS8-BC (1-3), respectively.

Table 1. The cytochrome b Q₀ genotypes of the surveyed *T. urticae* strains.

| Strain | Host | Origin | Q ₀ genotype | Access. Nr. |
|---------|-------------|--------------------|-------------------------|-------------|
| JP-R | Rose | Japan ^a | G132A | MN029033 |
| Wasatch | Tomato | United States | - | MN276073 |
| FS1 | Rose | the Netherlands | G132A | MN029034 |
| FS2 | Potted rose | the Netherlands | - | MN029035 |
| FS3 | Cucumber | the Netherlands | G126S | MN029048 |
| FS4 | Gerbera | the Netherlands | - | MN276066 |
| FS5 | Rose | the Netherlands | G126S | MN276067 |
| FS6 | Rose | the Netherlands | G126S | MN276068 |
| FS7 | Cucumber | United Kingdom | G126S | MN029041 |
| FS8 | Strawberry | United Kingdom | G126S/A133T | MN029042 |
| FS9 | Rose | United Kingdom | G126S | MN029043 |
| FS10 | Cucumber | United Kingdom | - | MN029044 |
| FS11 | Strawberry | United Kingdom | P262T | MN276069 |
| FS12 | Cucumber | Belgium | - | MN029036 |
| FS13 | Raspberry | Germany | - | MN029037 |
| FS14 | Hop | Germany | - | MN029038 |
| FS15 | Hop | Germany | - | MN029039 |
| FS16 | Hop | Germany | - | MN029040 |
| FS17 | Carnation | Italy | - | MN276070 |
| FS18 | Rose | Italy | - | MN276071 |
| FS19 | Citrus | Italy | - | MN276072 |
| FS20 | Strawberry | Spain | - | MN029045 |
| FS21 | Cucumber | Spain | - | MN029046 |
| FS22 | Rose | Romania | - | MN029047 |

All strains were field collected in Europe, except JP-R and Wasatch, which were laboratory strains. The substitutions in the conserved regions of the cytochrome b Q₀ pocket (the cd1-helix and ef-helix) are described using the GSS genotype as reference (EU556751.1).

^a Selected by cyenopyrafen, a complex II inhibitor, under laboratory conditions

Toxicity bioassays

To determine bifentazate and acequinocyl toxicity, dose–response bioassays were conducted with female adult mites, as described by Van Leeuwen *et al.*⁴² Briefly, we tested a minimum of five concentrations in four replicates. For each replicate, ^{20–35} adult females were transferred to 9 cm² bean leaf discs on wet cotton wool. Arenas were sprayed with 1 mL of acaricide solution or deionized water (as control) at 1 bar pressure in a Potter spray tower resulting in 2 mg aqueous deposit per cm². Mortality was recorded after 24 h. The 50% lethal concentration (LC₅₀) values and their 95% confidence limits were calculated from probit regressions using (POLO-Plus; LeOra Software, Berkeley, CA, USA, 2006).

Reciprocal crosses

To elucidate the mode of inheritance of bifentazate and acequinocyl resistance, reciprocal crosses were set up between the JP-R (G132A), iso-FS1(G132A) and iso-FS8 (G126S+A133T) resistant lines and the susceptible Wasatch strain. To create hybrid F1 females, ~80 teleiochrysalid females and 100 adult males were placed on detached bean leaves on wet cotton wool and allowed to mate. After 2 days, females were collected and transferred daily to a fresh 9 cm² bean leaf disc and allowed to lay eggs. F1 adult females were used for toxicity bioassays. The degree of dominance (*D*) was calculated using the Stone⁴³ formula:

$$D = (2X_2 - X_1 - X_3) / (X_1 - X_3),$$

where $X_1 = \log_{10} LC_{50}$ of the resistant strain, $X_3 = \log_{10} LC_{50}$ of the susceptible strain and $X_2 = \log_{10} LC_{50}$ of the F1 females obtained from the reciprocal cross.

Fitness cost of G132A

To explore potential fitness costs associated with the G132A mutation, demographic experiments were conducted with the three independent JP-R backcrossed lines in comparison with the parental Wasatch line as control.

2.6.1 Developmental time, immature stage survivorship, and sex ratio

For each introgressed line and the Wasatch control, 100 females were collected randomly from stock cultures and transferred to a detached bean leaf on wet cotton wool in three replicates. Females were allowed to lay eggs for 4–5 h and the numbers of eggs were recorded. After 8 days, the development of the offspring was followed every 12 h, and the eclosion time and sex of the adults were recorded.

2.6.2 Oviposition and adult longevity

From the three introgressed lines and the Wasatch control, 40 female teleiochrysalids were placed individually with an adult male on a 2 cm² leaf disc (in total 4 × 40 = 160 leaf discs for each mite couple). Every 12 h, all disc arenas were checked for female oviposition and death. Every 24 h, each mite couple was transferred to a fresh leaf disc until the female died. Pre-oviposition, oviposition and post-oviposition periods were determined as the time between adult female emergence and the first egg, the time between the first and last day of oviposition, and the time between the day when no eggs were deposited and death of the female, respectively.

Statistical analysis

Statistical analysis was conducted within the R framework (R Core Team, version 3.1.2, R Foundation for Statistical Computing, Vienna, Austria) for all data. Normality of variances was tested using a Shapiro–Wilk test. A generalized linear model with a negative binomial error distribution was used to analyse the data for female longevity, pre-oviposition period, oviposition period, post-oviposition period and the number of eggs. Sex ratio data were analysed using a generalized linear model with a binomial error distribution. A general linear model was used to analyse immature stage survivorship (ISS) data that were normally distributed. Differences between the introgressed lines were determined using Tukey’s HSD test at a 95% confidence level. Life table analysis was performed based on the lifetable R script.⁴⁴ The intrinsic rate of increase (r_m) was calculated with the equation

$$\sum_{x=x_0}^{\Omega_g} e^{-r_m l_x} m_x = 1$$

where l_x is the proportion of females surviving to age x and m_x is the mean number of female progeny per adult female at age x . The net reproductive rate or mean number of daughters produced per female was calculated from

$$R_0 = \sum_{x=x_0}^{\Omega_g} l_x m_x$$

and the mean generation time from

$$T = \frac{\ln(R_0)}{r_m}$$

The finite rate of increase and doubling time were inferred from the equations: $LM = e^{r_m}$ and $DT = \frac{\ln 2}{r_m}$. Variance for the life table parameters was estimated using the jackknife resampling method.⁴⁵ Because the jackknife method is an asymptotic procedure that is sensitive to a highly skewed distribution,⁴⁶ the symmetry of our data set was measured with the function skewness from package moments prior to the final analysis.⁴⁷ Subsequently, mean jackknife values and their standard errors (SE) were calculated for the five LT parameters.⁴⁸ Mean jackknife values for lines carrying mutations were then compared to Wasatch using Dunnett's test (adjusted P -value <0.05).

RESULTS

Cytochrome b genotypes of JP-R and the field strains

During a cross-resistant screen of the Japanese JP-R strain selected for cyenopyrafen resistance,³⁷ we found strong bifentazate resistance, and therefore sequenced the complete cytochrome b gene. Aligning the cytochrome b sequence of JP-R against those of the susceptible strains Wasatch and GSS revealed a novel amino acid substitution (G132A) (Table 1 and Fig. 1). To explore the spread of this mutation in Europe, several field-collected strains were screened (Table 1). We found four mutations in the conserved cd1- and ef-helices of the Q₀ pocket of cytochrome b of mitochondrial complex III (G126S, G132A, A133T and P262T). The novel G132A uncovered in JP-R was also identified in FS1, a strain from the Netherlands. In addition, a novel mutation combination (G126S+A133T) was identified in strain FS8 from the UK. This combination of mutations has been reported previously from *P.*

citri, but not *T. urticae*²⁴ (Table 1 and Fig. 1). Lastly, the well characterized P262T was found in a population from strawberry in the UK. Additional substitutions were also found in non-conserved regions. The G126S mutation was found by itself in five strains collected from the Netherlands and the UK (Table 1 and Fig. 1), but whether the mutation alone confers resistance remains to be investigated.

Resistance to bifentazate and acequinocyl

The results of the toxicity tests are listed in Table 2. The JP-R and FS1 strains that carry the G132A mutation were resistant to bifentazate ($LC_{50} > 150 \text{ mgL}^{-1}$), but not to acequinocyl. By contrast, the FS8 strain with the G126S+A133T haplotype showed high levels of acequinocyl resistance ($LC_{50} > 600 \text{ mgL}^{-1}$), and only very moderate resistance to bifentazate (Table 2). Levels of resistance between parental and introgressed lines were comparable across all independent replicates for G132A (Table 2). For the G126S+A133T haplotype, resistance ratios for acequinocyl were twofold lower after introgression, but LC_{50} values remained very high (Table 2).

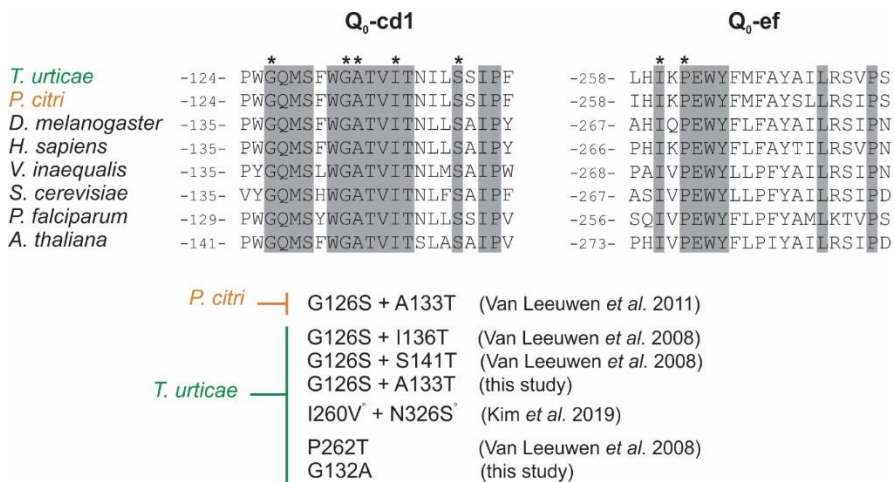


Figure 1. Target-site mutations in the cd1- and ef-helices of mitochondrial cytochrome b in spider mites that confer Q₀I resistance. An amino acid alignment is shown of the cytochrome b cd1- and ef-helices of the spider mites *Tetranychus urticae* and *Panonychus citri*, the fruit fly *Drosophila melanogaster*, human *Homo sapiens*, the fungi *Venturia inaequalis* and *Saccharomyces cerevisiae*, the protozoan *Plasmodium falciparum* and the plant *Arabidopsis thaliana*. Fully conserved residues are shaded in grey. Asterisks indicate the locations of point mutations that are linked to Q₀I resistance in spider mites. The validated substitutions in the Q₀ pocket that cause bifentazate and acequinocyl resistance in spider mites are outlined below the alignment. ^o, mutations were originally reported as I256V and N321S.

Table 2. Bifenazate and acequinocyl resistance in *T. urticae* strains with novel Q₀ mutations.

| Strain | Q ₀ genotype | Bifenazate | | | Acequinocyl | | |
|------------|-------------------------|----------------------------------|-------------|-------|---------------------------------|-------------|--------|
| | | LC ₅₀ (95%CI) (mg/ L) | Slope ± SE | RR | LC ₅₀ (95%CI) (mg/L) | Slope ±SE | RR |
| Wasatch | Wild-type | 6.93 (6.31 - 7.51) | 4.88 ± 0.49 | - | 10.71 (10.23 - 11.15) | - | - |
| JP-R | G132A | 221.29 (192.80 - 250.93) | 2.49 ± 0.19 | 31.93 | 39.86 (34.37 - 45.00) | 3.36 ± 0.40 | 3.72 |
| JP-R-BC1 | | 164.13 (144.41 -185.17) | 3.15 ± 0.27 | 23.68 | 23.19 (20.45 - 25.64) | 4.68 ± 0.48 | 2.17 |
| JP-R-BC2 | | 153.84 (136.16 - 173.02) | 3.14 ± 0.25 | 22.2 | 23.86 (21.01 - 26.24) | 5.31 ± 0.59 | 2.23 |
| JP-R-BC3 | | 180.13 (148.97 -211.24) | 3.17 ± 0.34 | 26 | 18.09 (16.13 - 19.85) | 4.92 ± 0.49 | 1.69 |
| FS1 | | 126.8 (113.5 - 141.28) | 3.26 ± 0.25 | 18.3 | - | - | - |
| iso-FS1 | | 261.35 (229.37 - 295.09) | 3.12 ± 0.27 | 37.71 | 37.97 (34.04 - 41.81) | 3.59 ± 0.29 | 3.55 |
| FS8 | | 51.42 (46.02 - 56.12) | 4.87 ± 0.51 | 7.42 | - | - | - |
| iso-FS8 | G126S+A133T | 79.22 (72.20 - 85.72) | 5.47 ± 0.46 | 11.43 | 1340.51 (1053.38 - 1636.39) | 1.67 ± 0.16 | 125.16 |
| isoFS8-BC1 | | 42.32 (38.25 - 50.00) | 5.10 ± 0.49 | 6.11 | 699.291 (584.34 - 824.97) | 2.62 ± 0.21 | 65.29 |
| isoFS8-BC2 | | 45.62 (39.98 - 52.08) | 4.32 ± 0.40 | 6.58 | 617.56 (494.55 -751.54) | 2.92 ± 0.25 | 57.66 |
| isoFS8-BC3 | | 49.00 (42.47 - 57.00) | 4.64 ± 0.42 | 7.1 | 820.47 (695.79- 956.49) | 2.43 ± 0.2 | 76.6 |

Isofemale lines were created from field strains with novel Q₀ mutations and are specified by an 'iso' prefix. Strains with a 'BC' suffix were created by repeated back-crossing. Only adult females were used in the bioassays.

Mode of inheritance of bifentazate and acequinocyl resistance

Reciprocal crosses revealed complete maternal inheritance of bifentazate resistance in the G132A lines (Table 3 and Fig. 2), linking the mutation to the phenotype. The limited bifentazate resistance observed in iso-FS8 with the G126S+A133T haplotype also showed complete maternal inheritance. There was a very strong maternal effect in the inheritance pattern of acequinocyl resistance in the reciprocal cross of iso-FS8 × Wasatch. By contrast, the very low resistance to acequinocyl in G132A lines did not inherit maternally (Table 3 and Fig. 2), indicating that G132A does not confer acequinocyl resistance. The LC50 values and dominance levels for all reciprocal crosses are specified in Table 3.

Table 3. Mode of inheritance of Q₀I resistance in *T. urticae* strains with novel Q₀ mutations.

| Q ₀ genotype | Cross (♀ x ♂) | Bifenazate | | | | Acequinocyl | | |
|-------------------------|-------------------|------------|---------------------------|-------|-------------------------------------|-------------------|-------|--|
| | | F1 (mg/L) | LC ₅₀ (95% CI) | D | F1 LC ₅₀ (95% CI) (mg/L) | D | | |
| G132A | JP-R × Wasatch | 300.04 | (257.39 - 347.51) | 1.14 | 24.39 | (21.94 - 26.85) | 0.25 | |
| | Wasatch × JP-R | 7.59 | (7.08 - 8.05) | -0.95 | 19.64 | (17.88 - 21.38) | -0.08 | |
| | iso-FS1 × Wasatch | 167.04 | (147.87 - 187.38) | 0.75 | 36.71 | (31.99 - 40.97) | 0.95 | |
| | Wasatch × iso-FS1 | 7.45 | (6.69 - 8.08) | -0.96 | 24.73 | (21.62 - 27.4) | 0.33 | |
| G126S + A133T | iso-FS8 × Wasatch | 61.84 | (58.81 - 64.87) | 0.80 | 555.74 | (439.98 - 676.06) | 0.64 | |
| | Wasatch × iso-FS8 | 5.85 | (5.43 - 6.25) | -1.14 | 28.72 | (25.55 - 32.43) | -0.59 | |

D is the degree of dominance. Only adult females were used in the bioassays.

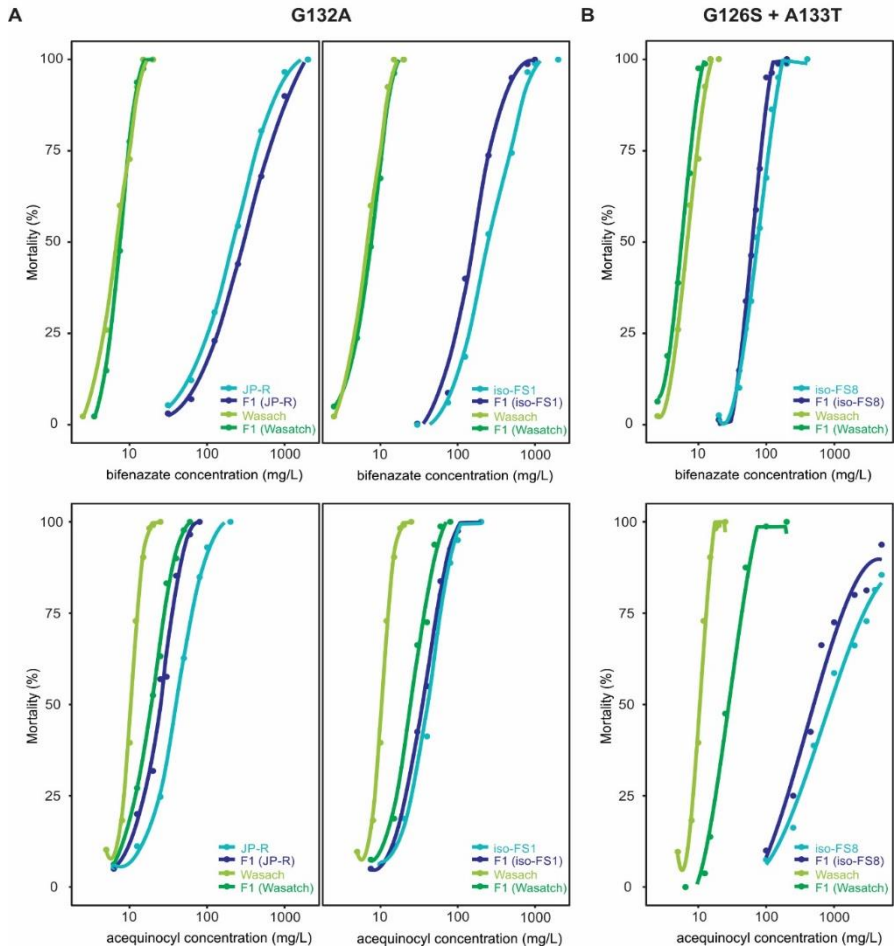


Figure 2. Bifenazate and acequinoyl dose–response toxicity data of susceptible reference and resistant strains carrying new Q₀I resistant mutations and their reciprocal crosses revealing the mode of inheritance. (A) Dose–response curves show that the JP-R and iso-FS1 strains that carry G132A were resistant to bifenazate, but susceptible to acequinoyl. Wasatch was susceptible to both acaricides. Reciprocal crosses showed that bifenazate resistance is maternally inherited. The mother for each cross is given in parentheses. (B) Dose–response curves show that the iso-FS8 strain carrying G126S+A133T showed high levels of acequinoyl resistance, and moderate resistance to bifenazate. Wasatch was susceptible to both acaricides. Reciprocal crosses showed that both bifenazate and acequinoyl resistance is maternally inherited. The mother for each cross is given in parentheses.

Fitness costs

Adult males and females of Wasatch emerged earlier than the introgressed lines JP-R-BC (1–3) (female: $df = 3$, $F = 11.12$, $P < 0.001$ and male: $df = 3$, $F = 7.29$, $P < 0.001$) (Fig. S1 and Fig. 3). Significant differences were observed between the three introgressed resistant lines JP-R-BC and the bifentazate susceptible strain Wasatch in terms of ISS ($F = 4.13$; $df = 3$, $P = 0.015$), sex ratio ($\chi^2 = 9.30$; $df = 3$; $P = 0.023$), longevity ($\chi^2 = 17.76$; $df = 3$; $P < 0.001$), oviposition period ($\chi^2 = 17.62$; $df = 3$; $P < 0.001$), total number of eggs laid per female ($\chi^2 = 12.61$; $df = 3$; $P = 0.005$) and post-oviposition ($\chi^2 = 7.97$; $df = 3$; $p = 0.46$), but not pre-oviposition period ($\chi^2 = 0.12$; $df = 3$; $P = 0.989$) (Fig. 3).

3.4.1 Fertility life table parameters

All life table parameters, net reproductive rate (R_0), the intrinsic rate of increase (rm), the finite rate of increase (LM), mean generation time (T) and the doubling time (DT) of the three introgressed lines carrying resistance mutations JP-R-BC (1–3) and Wasatch, are summarized in Table 4. All three introgressed lines of JP-R showed significantly smaller values for R_0 , rm and LM and significantly longer DT compared with their congenic line, Wasatch (Table 4 and Fig. 4). No significant difference was found in T .

Table 4. The effect of G132A on fertility life table parameters in *T. urticae*.

| Q ₀ genotype | Line | N | R ₀ ± SE | T ± SE | DT ± SE | rm ± SE | LM ± SE |
|-------------------------|----------|----|---------------------|---------------|--------------|---------------|---------------|
| Wild-type | Wasatch | 38 | 28.96 ± 2.58a | 17.83 ± 0.24a | 3.66 ± 0.08a | 0.19 ± 0.004a | 1.21 ± 0.005a |
| | JP-R-BC1 | 38 | 12.88 ± 0.96b | 17.58 ± 0.17a | 4.76 ± 0.12b | 0.14 ± 0.004b | 1.16 ± 0.004b |
| G132A | JP-R-BC2 | 39 | 19.61 ± 1.55b | 17.72 ± 0.22a | 4.12 ± 0.07b | 0.17 ± 0.003b | 1.18 ± 0.004b |
| | JP-R-BC3 | 39 | 13.71 ± 1.10b | 17.38 ± 0.17a | 4.59 ± 0.12b | 0.15 ± 0.004b | 1.16 ± 0.005b |

Net reproductive rate (R_0), the intrinsic rate of increase (rm), the finite rate of increase (LM), mean generation time (T) and the doubling time (DT) of three near-isogenic lines of *T. urticae* (JP-R-BC1-3) and Wasatch were calculated. Means with different letters (a,b) within a column were significantly different at $\alpha = 0.05$. N, number of females.

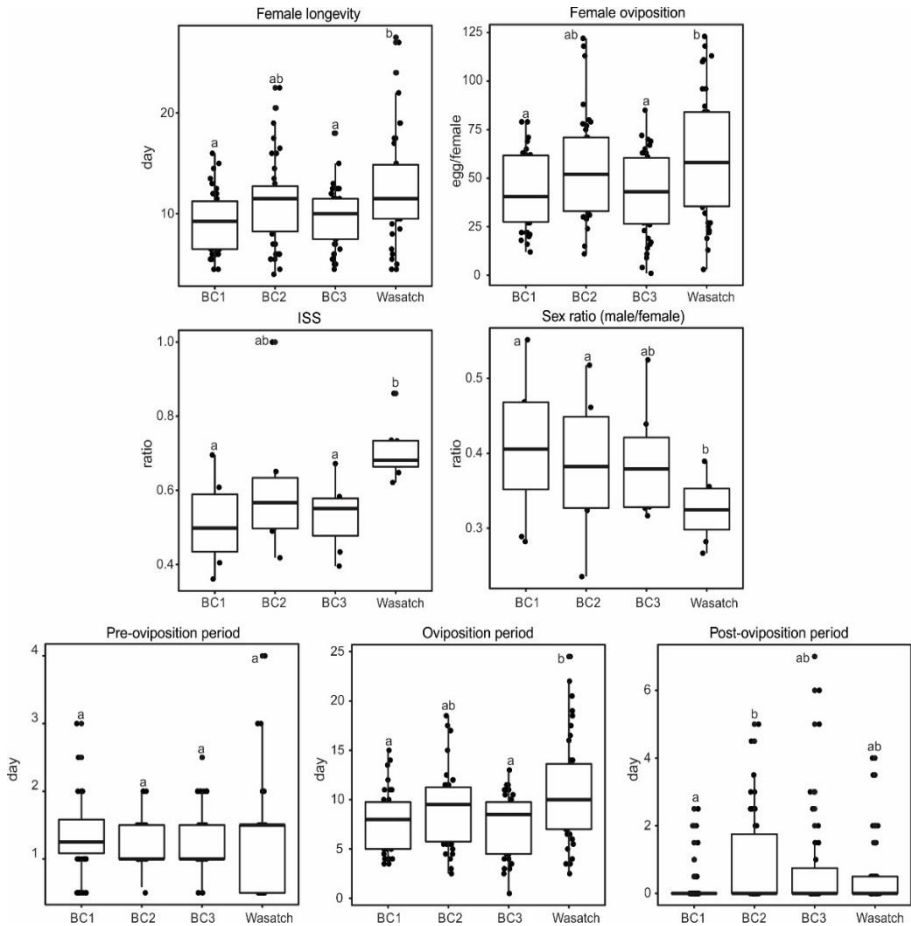


Figure 3. The effect of G132A on single-generation life-history traits in *Tetranychus urticae*. Three introgressed lines carrying the G132A substitution were compared with Wasatch in terms of female longevity, female oviposition, immature stage survivorship (ISS), sex ratio (proportion of males), pre-oviposition period, oviposition period and post-oviposition period. Letters (a, b) indicate significant differences at $\alpha = 0.05$. The bottom and top of the boxplots depict the first and third quartiles. The central line shows the median, and the whiskers extend to the most extreme data points which are no more than 1.5 times the interquartile range from the box.

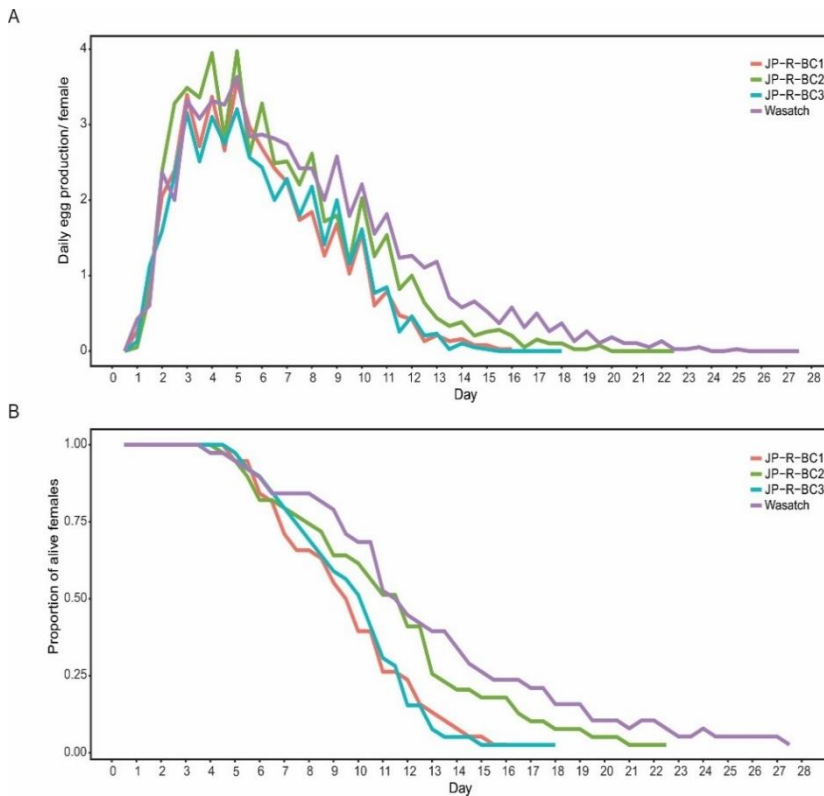


Figure 4. The effect of G132A on female longevity and oviposition in *Tetranychus urticae*. (A) Daily egg production per female. (B) Proportion of alive females over the course of the experiment.

DISCUSSION

Because of its excellent efficacy and safety toward biological control agents such as predatory mites,^{15,16,50} bifentazate has been frequently used worldwide. Soon after its introduction in the European Union (EU), resistance was reported in *T. urticae* populations from greenhouse roses in the Netherlands.¹² Surprisingly, bifentazate resistance inherits maternally and investigation of resistance mechanisms led to the discovery of a mitochondrial mode of action,¹² instead of the previously proposed interaction with GABA-gated chloride channels.^{51,52} Mitochondrial genome sequencing revealed mutations at conserved sites in the mitochondrial cytochrome b gene, suggesting that bifentazate acts as a Q_0 inhibitor.^{12,19,53} In spider mites, reciprocal genetic crosses between populations can be conducted easily, and should thus be the standard in validating the role of specific mutations in cytochrome b in Q_0 I resistance.

Because cytochrome b is encoded by the mitochondrial genome, maternal inheritance is uniquely associated with these resistance-conferring mutations. In addition, for a number of cytochrome b mutations, repeated backcrossing to a susceptible line has confirmed the very potent resistant phenotype in bifenazate resistance.⁴⁰ Over the years, a number of mutations conferring bifenazate and acequinocyl resistance have been validated by revealing a maternal inheritance, both in *T. urticae* as *P. citri* populations (Fig. 1). Although a number of other mutations has been reported, formal evidence of their contribution to bifenazate resistance is lacking.⁵⁴ The same is true for G126S, which was reported initially in combination with other cd1-helix mutations, but the mutation alone has never been validated to confer a resistant phenotype, despite a recent report.⁵⁴ This is in contrast with mutations in (or close to) the ef-helix, where P262T and I256V alone confer bifenazate and acequinocyl resistance respectively (Fig. 1).^{12,49}

In this study, we report for the first time a single mutation in the cd1-helix, G132A, that confers resistance to bifenazate. The mutation was uncovered after a cross-resistance screen of JP-R,37 a strain of Japanese origin, and was subsequently also detected in a Dutch field strain, FS1. Both lines harbouring the G132A mutation and backcrossed lines displayed similar LC₅₀ values, and RR and resistance inheritance was perfectly maternal. This strongly validates the role of the G132A mutation in bifenazate resistance. However, the mutation did not confer acequinocyl cross-resistance. Bifenazate resistance levels of 30-fold with LC₅₀ values of 150–200mgL⁻¹ are very significant in the light of field rate (e.g. Bifenazate at 96mg a.i. L⁻¹ in the EU) and could cause field failure, but nevertheless are much lower than those previously reported in the cd1 helix (LC₅₀ >10 000).^{12,19} This suggests that a combination of mutations is needed to attain these very high resistance levels. Interestingly, this mutation is the main resistance factor in pathogenic fungi resistant to strobilurins, which are classified as MOAs and Q₀I inhibitor fungicides,^{22,23,55,56} providing a strong example of convergent evolution across kingdoms. Screening of field-collected European *T. urticae* populations also led to the discovery of another novel combination of mutations: G126S+A133T. This Q₀ pocket haplotype is associated with high levels of acequinocyl and bifenazate resistance in *P. citri*.²⁴ In our study, the combination of G126S and A133T in *T. urticae* conferred only moderate levels of resistance to bifenazate but high resistance to acequinocyl. It is surprising that this combination of substitutions confers such different levels of bifenazate resistance in *P. citri* and *T. urticae*, particularly because the resistant phenotype inherited maternally in both species, and additional (nuclear) factors in resistance can thus be ruled out. For G132A, it is clear that bifenazate must be the most relevant selective force in *T. urticae* field populations, as it does not confer

acequinocyl resistance. The opposite is likely true for G126S+A133T, as the effect seems to be much more pronounced on acequinocyl toxicity, and hence it is tempting to speculate that frequent acequinocyl use lies at the basis of resistance development.

After repeated backcrossing to the susceptible Wasatch strain, we obtained congenic lines harbouring the mitochondrial haplotype of JP-R (G132A) and the nuclear background of Wasatch. As this uncouples the mitochondrial resistance mutations from confounding genomic factors, it is not only a validation of the phenotypic strength, but also a powerful approach to assess fitness costs. Our analyses of the G132A congenic lines revealed a lower R_0 , r_m and LM, and a higher DT compared with Wasatch. It therefore seems that in an acaricide-free environment the resistant genotypes might be less competitive and will grow slower than susceptible genotypes. In addition, we found that the resistant genotype is more male biased, which could further reduce the frequency of G132A transmission. Our findings could be important for the management of G132A-conferred resistance in the field. It appears that the management of G132A resistance might be easier than that of the mutations without fitness costs, such as G126S+S141F and P262T.¹¹

There are several reports on the fitness of resistant fungal species that carry G143A (G132A in spider mites). Some species, such as *Plasmopara viticola*^{57,58} and *Magnaporthe oryzae*⁵⁹ show lower fitness. For example, conidia production of the field G143A azoxystrobin-resistant mutant of *M. oryzae* is lower than that of the susceptible wild-types.⁵⁹ Other studies failed to find fitness costs in resistant species such as *Blumeria graminis*,⁶⁰ *Alternaria alternata*,⁶¹ *Botrytis cinerea*⁶² and *Colletotrichum acutatum*.⁶³ These fitness studies, however, did not provide direct evidence for the association of fitness consequences with the G143A mutation. To evaluate the role of G143A in fungicide resistance and its impact on the fitness of fungi, the mutation was introduced into the cytochrome b of the yeast species *Saccharomyces cerevisiae* as a model system.⁶⁴ Although confirming involvement of the mutation in resistance, they showed that the mutation has a slightly deleterious effect on the bc1 function of the site mimic of some, but not all, pathogenic fungi species. The authors therefore argued that a small variation in the Q₀ site can affect the impact of the G143A mutation on bc1 activity, and can differentially affect the fitness between species. In light of this, it is not surprising that different spider mite mutations can confer different levels of fitness penalties.

CONCLUSION

In conclusion, new cytochrome b mutations were uncovered, and several lines of evidence support the causal role of these mutations in bifentazate or acequinocyl resistance. Patterns of maternal inheritance and introgression experiments identified G132A as tightly linked with high levels of bifentazate resistance. In *T. urticae*, G126S+A133T conferred very high acequinocyl resistance, with only limited levels of bifentazate cross-resistance. Investigation into the fitness costs revealed that strains harbouring G132A might be more easily managed.

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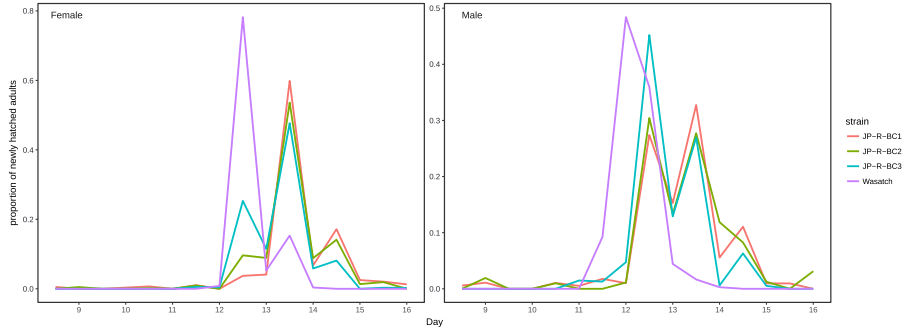
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Supporting information



Supplemental Figure 1. The effect of G132A on male and female development time in *T. urticae*. Emergence of the adult mites was scored in intervals of 12 h.