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High resolution genetic mapping uncovers chitin synthase-1 as the target-site of the structurally diverse mite growth inhibitors clofentezine, hexythiazox and etoxazole in *Tetranychus urticae*

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Abstract

The acaricides clofentezine, hexythiazox and etoxazole are commonly referred to as ‘mite growth inhibitors’, and clofentezine and hexythiazox have been used successfully for the integrated control of plant mite pests for decades. Although they are still important today, their mode of action has remained elusive. Recently, a mutation in *chitin synthase 1* (*CHS1*) was linked to etoxazole resistance. In this study, we identified and investigated a *T. urticae* strain (HexR) harboring recessive, monogenic resistance to each of hexythiazox, clofentezine, and etoxazole. To elucidate if there is a common genetic basis for the observed cross-resistance, we adapted a previously developed bulk segregant analysis method to map with high resolution a single, shared resistance locus for all three compounds. This finding indicates that the underlying molecular basis for resistance to all three compounds is identical. This locus is centered on the *CHS1* gene, and as supported by additional genetic and biochemical studies, a non-synonymous variant (I1017F) in *CHS1* associates with resistance to each of the tested acaricides in HexR. Our findings thus demonstrate a shared molecular mode of action for the chemically diverse mite growth inhibitors clofentezine, hexythiazox and etoxazole as inhibitors of an essential, non-catalytic

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activity of CHS1. Given the previously documented cross-resistance between clofentezine, hexythiazox and the benzyolphenylurea compounds flufenoxuron and cycloxuron, CHS1 should be also considered as a potential target-site of insecticidal BPUs.

1. Introduction

Phytophagous mites of the genus *Tetranychus* and *Panonychus* are serious pests on plants worldwide (Jeppson et al., 1975; Zhang, 2003). Among these, the two-spotted spider mite, *Tetranychus urticae*, is of particular importance, as this extreme generalist species has been documented on more than 1000 plant species including numerous crops of economic significance (Jeppson et al., 1975; Migeon and Dorkeld, 2013). Although biological control of *T. urticae* has been successfully implemented in many greenhouses and protected crops (Gerson and Weintraub, 2012; Perdakis et al., 2008; Sabelis, 1981), the species is primarily controlled by acaricides in open field crops (Dekeyser, 2005; Marcic, 2012; Van Leeuwen et al., 2010; Zhang, 2003). However, spider mites rapidly develop resistance to diverse acaricides (Dermauw et al., 2012; Van Leeuwen et al., 2010), a major factor threatening the efficient control of spider mites in agriculture.

It is therefore crucial to maintain the efficacy of the available acaricide portfolio by developing and implementing efficient resistance management strategies. In this respect, understanding the mode of action of acaricides – and in particular identifying their molecular targets – is of particular importance (Van Leeuwen et al., 2012b). Knowledge of target-site resistance alleles may allow for screening of field populations with high-throughput molecular diagnostic tools, facilitating the implementation of resistance management strategies based on resistance gene allele frequencies in a geographical or plant host manner. Further, the elucidation of acaricide modes of action allows the grouping of compounds into classes to avoid selection pressure on the same molecular target and hence delay resistance development (Nauen et al., 2012). A clear example on how molecular information about target-sites can directly influence resistance management practices has recently been documented for the acaricides bifenazate and acequinocyl. When bifenazate was launched, the mode of action was not fully understood but reported to be neurotoxic (Dekeyser, 2005). In greenhouses in the Netherlands, bifenazate was consequently used in rotation with acequinocyl, a known complex III inhibitor. However, a case of maternally inherited bifenazate resistance pointed towards a resistance gene in the mitochondria (Van Leeuwen et al., 2006). It was subsequently shown that mutations in the cytochrome b subunit of complex III underlie bifenazate resistance (Van Leeuwen et al., 2008), and that these mutations cause cross-resistance between bifenazate and acequinocyl (Van Nieuwenhuyse et al., 2009). As a consequence, bifenazate and acequinocyl should no longer be alternated as they both select for the same target-site mechanism.

This example is illustrative of the fact that the mode of action of acaricides is often less well understood as compared to the mode of action of insecticides. Today, few insecticides are on the market for which the molecular mode of action is unknown (Krämer et al., 2011). In contrast, for a number of frequently used acaricides, including dicofol, fenbutatin oxide and propargite, the molecular target site has not been determined. One class of valuable acaricides for which the modes of action are poorly documented consists of the compounds

clofentezine, diflovidazin and hexythiazox that have been generically grouped as ‘mite growth inhibitors’ (Fig. 1). A thorough investigation is particularly relevant for clofentezine (a tetrazine acaricide, Fig. 1a) and hexythiazox (a thiazolidinone compound, Fig. 1b), as both acaricides have been widely used for more than 30 years, and are still valuable tools for mite control. Their popularity is mainly due to an excellent ecotoxicological profile, as they are safe for beneficial insects and predatory mites, and because they provide long residual control (Aveyard et al., 1986; Bretschneider and Nauen, 2008; Yamada et al., 1987). Both compounds further share a broad-spectrum activity against several plant-feeding mite species, including *Tetranychus* spp and *Panonychus* spp, and an excellent efficacy on eggs and/or larvae and nymphs (but not adults). Clofentezine is mainly used as a potent contact ovicide (Aveyard et al., 1986; Neal et al., 1986), and is thought to act by interfering with cell growth and cell differentiation during the final phases of embryonic and early larval development (Bretschneider and Nauen, 2008). Diflovidazin (also known as flufenazine, Fig. 1c) has similar properties as clofentezine, but the introduction of fluorine atoms in the *ortho* position of the phenyl ring resulted in improved translocation properties (Pap et al., 1996). Hexythiazox was launched in 1985, soon after clofentezine (Yamada et al., 1987). It quickly became, and continues to be, widely used in integrated pest management programs throughout the world. In comparison with clofentezine, it has a more pronounced activity on larvae, proto-, and deutonymphs. More recently, in 1998 the oxazoline compound etoxazole was also introduced as an acaricide (Fig. 1d) (Ishida et al., 1994). Like hexythiazox, it has excellent activity on eggs, larvae and nymphs but lacks activity on adults (Bretschneider and Nauen, 2008; Dekeyser, 2005; Suzuki et al., 2002). Etoxazole was described as about 100-fold more toxic in comparison to hexythiazox, but with altered selectivity. It has a less favorable ecotoxicological profile (Kim and Yoo, 2002) and also exhibits activity on juvenile stages of aphids (Bretschneider and Nauen, 2008; Ishida et al., 1994).

At the time of launch, etoxazole was reported to lack cross-resistance when applied to hexythiazox and/or clofentezine resistant spider mites (Ishida et al., 1994). Nevertheless, subsequent studies have demonstrated cross-resistance between etoxazole and both clofentezine and hexythiazox. Strong cross-resistance between clofentezine and hexythiazox was reported in *T. urticae* and *P. ulmi* strains from Australia (Herron et al., 1993; Thwaite, 1991), as well as in multiple European *P. ulmi* strains (Grosscurt et al., 1994). More recently, cross-resistance was also detected between etoxazole and hexythiazox in *T. urticae*, and genetic linkage between hexythiazox and etoxazole resistance was tested with genetic crosses (Asahara et al., 2008). These experiments revealed that etoxazole resistance was caused by a single recessive locus, while hexythiazox resistance was under control of more than one major factor. Notably, one genetic factor for hexythiazox resistance was linked to the etoxazole resistance locus, raising the possibility of a shared resistance mechanism (Asahara et al., 2008).

Despite toxicological and genetic studies, the nature of cross-resistance to hexythiazox, clofentezine and etoxazole –either through alterations in a common target-site, or alternatively by a common detoxification pathway – has remained unknown. For etoxazole, the recessive nature of resistance in some *T. urticae* populations (Uesugi et al., 2002; Van Leeuwen et al., 2012a), coupled with the recently released draft genome sequence for the

species, enabled positional cloning of a monogenic resistance locus. By developing a population-level bulk segregant analysis (BSA) method based on high-throughput genome sequencing, we localized the etoxazole resistance factor to a very small region on scaffold 3 of the *T. urticae* genome assembly. As supported by additional genetic and biochemical studies, a single non-synonymous mutation (I1017F) in the *chitin synthase 1* gene (*CHS1*) was uncovered as the causal mutation (Van Leeuwen et al., 2012a).

In this case, cloning of the resistance allele led to the identification of *CHS1* as the molecular target, a finding supported by the demonstration of reduced chitin deposition in susceptible animals treated with etoxazole (Nauen and Smagghe, 2006; Van Leeuwen et al., 2012a). In this study, we adapted a related BSA method (Van Leeuwen et al., 2012a) for high-resolution genetic mapping to test if high-level resistance to the mite growth inhibitors clofentezine and hexythiazox shares the same genetic basis, and hence suggests the same molecular target, as observed for etoxazole. To do this, we isolated a field strain of *T. urticae* with very high levels of resistance to all three compounds, established that resistance was recessive and monogenic in each case, and mapped resistance for all three acaricides by independent selections of a derived population that segregated for resistance to each compound. Our genetic data strongly supports a shared underlying molecular basis for resistance to all three compounds via the impairment of an essential, non-catalytic activity of *CHS1*.

2. Material and methods

2.1 Chemicals

Commercial formulations of etoxazole (Borneo, 120 g l⁻¹ SC), hexythiazox (Nissorun, 250 g l⁻¹ SC) and clofentezine (Apollo, 500 g l⁻¹ SC) were purchased from FytoVanhulle, Belgium.

2.2 Mite husbandry and strains

The susceptible laboratory strain (London) originates from the Vineland region in Ontario (Canada), and was used as the reference strain in the *T. urticae* genome project (Grbic et al., 2011). The strain is susceptible to most currently used acaricides (Khajehali et al., 2011). The hexythiazox resistant strain (HexR) originates from a field strain, collected in Ghent, Belgium. The strain was maintained on potted bean plants sprayed with 500 mg l⁻¹ hexythiazox. Strains EtoxR, MR-VL, TuSb9 and Strain005R were previously described as resistant to etoxazole (Van Leeuwen et al., 2012a) and have been maintained under a constant selection pressure of 1000 mg l⁻¹ etoxazole. These strains of *T. urticae* have been maintained on 3-week-old potted kidney bean plants (*Phaseolus vulgaris* L., cv. Prelude) in a climatically controlled room or incubator at 26 (± 0.5) °C, 60% relative humidity, and 16:8 light:dark photoperiod (hereafter standard incubation conditions).

Dutch and North American strains used for haplotype screening were collected in the Netherlands from commercial rose growers in 2009 (Khajehali et al., 2011) and from within 10 km of the University of Utah, Salt Lake City, Utah, USA in 2012 or 2013 (Table S1).

2.3 Bioassays

To assess the toxic effects of etoxazole and hexythiazox, larval bioassays were performed as previously described (Van Pottelberge et al., 2009). Clofentezine acts primarily as an ovicide, and bioassays were performed on eggs instead of larvae. Briefly, 20–30 adult female mites were allowed to deposit eggs for 6 h on 9 cm² square bean leaf discs, which were placed on wet cotton wool under standard incubation conditions as described above. Larvae (for etoxazole and hexythiazox) and 2-day old eggs (for clofentezine) were sprayed with 0.8 ml spray fluid at 1 bar pressure (1.5 mg aqueous deposit per cm²). Serially diluted concentrations of etoxazole, hexythiazox and clofentezine were tested in 3–4 replicates, along with a water-sprayed control. Mortality was assessed after 4 days; mortality of the water-sprayed controls never exceeded 10%. Dose–response relationships (lethal concentrations and their 95% confidence limits) were analyzed by Probit analysis (POLO; LeORa Software). Resistance ratios were calculated by dividing LC₅₀ values by that of the London (susceptible) strain.

2.4 Establishing the mode of inheritance

Crossing experiments were performed as previously described (Van Leeuwen et al., 2012a; Van Leeuwen et al., 2004). Larvae of the F1 hybrid progeny were examined for etoxazole, hexythiazox and clofentezine susceptibility. The degree of dominance was calculated using the respective LC₅₀ values in F1s, according to the formula of Stone (Stone, 1968). To test whether resistance was inherited as a single major factor, unfertilized F1 females (after removing males at the deutonymph stage from the population) were allowed to deposit eggs, resulting in haploid male progeny. The observed response was compared with the expected response under monogenic inheritance with a χ^2 goodness-of-fit analysis. The expected response was calculated with the formula $C = 0.5 w (\text{parent 1}) + 0.5 w (\text{parent 2})$, where C is the expected mortality and w is the observed mortality of the parents at a certain concentration (Georgiou, 1969).

2.5 Bulk segregant analysis

2.5.1 Sample preparation for bulk segregant analysis—One male of the hexythiazox resistant strain (HexR) was placed on a square bean leaf disc on wet cotton wool. Every 3 days, 2–3 virgin females of the London susceptible strain were placed with the resistant male for fertilization. In total, 15 females were collected and allowed to deposit eggs on a detached bean leaf disc. The resulting ~200 F1 females were collected and allowed to grow in bulk on potted bean plants under standard incubation conditions. At the F2, the population of mixed stages was split into 4 subpopulations (started from ~1000 mites each): the first population was allowed to grow without selection pressure, the second population was selected with 1000 mg l⁻¹ etoxazole, the third population was selected with 1000 mg l⁻¹ hexythiazox and the fourth population was selected with 1000 mg l⁻¹ clofentezine. Selection was performed on sprayed bean plants. After another 3 generations, ~500 plant-selected F5 fertilized female mites from each of the split populations were transferred to detached bean leaves on wet cotton wool in petri dishes, and allowed to lay eggs. After hatching, the larvae from populations previously selected were sprayed with 1000 mg l⁻¹ etoxazole, hexythiazox or clofentezine, to assure no susceptible genotypes were

present. Approximately 5000 F6 females from each of the four populations were finally collected and DNA was extracted as previously described (Van Leeuwen et al., 2012a). Quality and quantity of the gDNA samples were assessed using NanoDrop ND-1000 spectrophotometer (Nanodrop Technologies) and Quant-iT dsDNA Assay Kit (Invitrogen), respectively.

2.5.2 DNA sequencing, read mapping, SNP analysis—DNA extracted from the parental strains (London and HexR) and the selected and non-selected progeny were used to construct 6 genomic DNA libraries. Barcoded DNA sequencing libraries were constructed according to standard Illumina protocols for each of the six sample pools (Bentley et al., 2008). Samples were then multiplexed and paired-end sequenced with 100bp reads on a single Illumina Hi-Seq lane by Fasteris SA (Genève, Switzerland).

Paired-end DNA sequence reads for each sample were aligned to the *T. urticae* genome (London strain; Grbic et al., 2011) with bowtie2 (Langmead and Salzberg, 2012) using the following parameters: --very-sensitive, -X 1000 (maximum fragment insert length), and -I 200 (minimum fragment insert length). A 92.9% average alignment rate was observed, after which resulting BAM files were processed with SAMtools (Li et al., 2009) to remove PCR duplicates that are a source of noise in assessing allele frequencies at segregating sites. After processing the BAM files, we performed joint genotype calling using the Genome Analysis Tool Kit's (GATK v2.5–2, McKenna et al., 2010) Unified Genotyper (DePristo et al., 2011; Van der Auwera et al., 2013) with default settings. Subsequently, we used custom python scripts to retrieve the following information from the variant call format (VCF) file generated by Unified Genotyper for each sample: (1) the consensus genotype call (GT field), (2) the allele frequency (parsed from the AD field), and (3) the coverage of the variants (DP field). No call variants were treated as missing data.

2.5.3 Bulk segregant analysis of selected mites—To identify the region for monogenic recessive resistance to the three acaricides, we implemented two BSA mapping approaches. First, between each of the three selected populations and the unselected population, we assessed changes in allele frequency at 1.3 million SNPs identified in HexR relative to the London reference genome sequence (a sliding window analysis was used and applied to 44 scaffolds in the *T. urticae* assembly of size >150 kb that cumulatively comprise 94.6% of the assembled genome). As a complementary approach, we also performed a sliding window analysis to identify the HexR genomic locus we expected to be fixed after selection by each acaricide. For this analysis, we used a set of 121,430 SNPs that we determined to be fixed (or nearly fixed) between HexR and the sensitive London strain that represent parent-of-origin informative markers. The SNPs were selected according to the following criteria: (1) we required the SNPs to be within 5% allele frequency of being fixed for alternate alleles between the two parental lines and (2) that they remained segregating in the unselected mites (>5% minor allele frequency), which acted to remove spurious regions that became fixed in the generation of all lines prior to selection (i.e. due to drift or segregation distortion).

2.6 Cryosectioning and calcofluor white staining

Samples for cryo-sectioning were prepared by synchronizing subcultures of the susceptible London strain and the hexythiazox/clofentezine-resistant strain HexR on detached bean leaves under standard incubation conditions. After mites molted into deutonymphs, the plates were immediately sprayed with solutions containing either 100 mg l⁻¹ hexythiazox, 100 mg l⁻¹ clofentezine or with a water control. All deutonymphs on treated and control plates entered the teleiochrysalis stage, and were collected just before ecdysis and fixed in 1.5 mL of 4% (wt/vol) formaldehyde in PBS, pH 7.6, supplemented with 0.05% (wt/vol) Triton-X. Cryoprotection, tissue embedding, serial cryosectioning and calcofluor white (CFW) staining were performed as described previously (Van Leeuwen et al., 2012a). CFW emission was viewed with an Olympus IX70 fluorescence microscope using the DAPI filter set (Olympus). Identical settings for exposure time and grayscale profiles were used for all specimens. The grayscale intensities of CFW fluorescence across cuticles were analyzed with the MetaMorph software package (Version 7.6.2.0) (MDS Analytical Technologies, Sunnyvale, CA) using the Linescan tool. Values are given as mean ± SEM (n=20).

2.7 Detection of the I1017F substitution in geographically diverse strains

With DNA from strains from The Netherlands (Khajehali et al., 2011), PCR amplification was performed with primers 5'-CTTCACCGTCTGCCGTATTT-3' (forward) and 5'-CTTTTCGTCGTTTGGTTTGG-3' (reverse) to detect the I1017F SNP previously implicated as the etoxazole resistance mutation (Van Leeuwen et al., 2012a). PCR reactions were performed in 50 µl containing 10x PCR-buffer, 2 mM MgCl₂, 0.2 µM of each primer, 0.2 dNTP mix, 2 µl template and 1 U Taq DNA polymerase (Invitrogen) under the following conditions: an initial denaturation step of 2 min at 94 °C, 35 cycles of 30 s at 94 °C, 30 s at 55 °C, 45 s at 72°C and a final extension of 3 min at 72°C. PCRs were purified using E.Z.N.A. Cycle-Pure Kit (Omega Biotek). In a similar manner, genotyping for the I1017F SNP was performed in four strains from the state of Utah, USA (Table S1). For these strains, PCR was performed with primer sequences AGATCCTTTACGTCTGGGGC (forward) and CAATTGGGACTCGTTTCTTTTCA (reverse) in 50 µl containing 5 µl 10x PCR-buffer, 2 mM MgCl₂, 0.2 µM of each primer, 0.2 µM dNTP mix, 1 µl template and 1.25 U Taq DNA polymerase (New England Biolabs, Ipswich, MA). PCR reaction conditions were as follows: initial denaturation of 95 °C for 2 min followed by 35 cycles at 95 °C for 30 s, 51 °C for 1 min and 68 °C for 1 min followed by a final extension 68 °C for 5 min. For genotyping at the I1017F SNP, purified PCR amplicons were Sanger sequenced at LGC Genomics or at the University of Utah Core Facility and variants were determined by inspection of sequencing chromatographs.

3. Results

3.1 Toxicology and genetic basis of etoxazole, hexythiazox and clofentezine resistance

To characterize the genetic basis of cross-resistance among clofentezine, hexythiazox and etoxazole, we isolated a *T. urticae* strain, HexR, which is resistant to all three compounds. Toxicity data of each compound for the HexR strain, as well as for the London reference strain from which the genome was sequenced (Grbic et al., 2011), are presented in Table 1. London was extremely sensitive to etoxazole, consistent with our earlier studies (Khajehali

et al., 2011; Van Leeuwen et al., 2012a), and was also highly susceptible to both hexythiazox and clofentezine (LC_{50} values ranged from 0.036 to 1.9 mg l⁻¹ active ingredient for the three compounds; Table 1). Strikingly, the LC_{50} values for HexR exceeded 5000 mg l⁻¹ for each compound, resulting in high resistance ratios (RRs) (Table 1).

In another strain (EtoxR), we previously demonstrated that etoxazole resistance with a high RR was recessive and monogenic (Van Leeuwen et al., 2012a). To establish the inheritance patterns of resistance to etoxazole, hexythiazox and clofentezine in strain HexR, we crossed HexR to the susceptible London strain. Mortality in F1 progeny revealed that resistance to all three compounds is (incompletely) recessive with D-values of -0.72, -0.87 and -0.98 for etoxazole, hexythiazox and clofentezine respectively (Table 1). In addition, resistance to all three compounds segregates as a single factor, as evidenced by the ~50% plateau of mortality in haploid F2 males across a large range of discriminating acaricide doses (etoxazole: $\chi^2=23$, df=14, $P<0.05$; hexythiazox: $\chi^2=19$, df=13, $P<0.05$; clofentezine: $\chi^2=18$, df=14, $P<0.05$; Fig. 2).

3.2 Bulk segregant analysis of clofentezine, hexythiazox and etoxazole resistance

Previously, we applied BSA methods on large populations of mites to identify a *CHS1* allele underlying recessive, monogenic resistance to etoxazole in strain EtoxR (Van Leeuwen et al., 2012a). To identify the locus (or loci) conferring resistance to each of the three compounds in HexR, for which resistance is inherited in a similar fashion, we applied related BSA methods to populations derived from crossing a single HexR male to susceptible London females. The resulting F2 intercross progeny were then divided into four replicate experimental populations: one was left untreated while the other three were subjected to treatment with discriminating doses of etoxazole, hexythiazox, and clofentezine (see Methods). From the F6 generation, bulked mites were collected from all populations and DNA was prepared. These samples, along with DNA of the parental strains (HexR and London), were used to produce between 32- to 64-fold genome coverage per sample with 2 x 100 bp paired-end Illumina sequencing reads.

For strain HexR, which is not inbred, we identified 1.3 million single nucleotide polymorphisms (SNPs) in alignments of Illumina reads to the ~90 Mb draft genome sequence from strain London (see Methods). Of these, 121,431 were identified as biallelic SNPs that were fixed in HexR as compared to the London parent (hereafter, 'HexR-specific SNPs'). Genome-wide, the density of all SNPs in HexR, as well as for HexR-specific SNPs, was comparatively even (Fig. S1A,B). An exception was a sharp peak in the density of HexR-specific SNPs on scaffold 3 at about 4.5 Mb (Fig. S1B). As the HexR strain has been maintained with ongoing selection with hexythiazox (see Methods), one possibility is that the region of this peak harbors the resistance allele for hexythiazox; that is, the high frequency of SNPs fixed in HexR in this region reflects the selective sweep of hexythiazox on a resistance allele.

To test this, and to genetically resolve the resistance loci for all three compounds, we compared changes in allele frequencies for all segregating SNPs between the unselected and each of the three selected populations in a sliding window analysis. Individual comparisons between the unselected population and the etoxazole, hexythiazox, and clofentezine selected

populations revealed striking, yet essentially identical changes in allele frequencies on a distal region of scaffold 3. The peak regions are coincident with *CHS1* previously implicated in etoxazole resistance (Fig. 3A), as well as to the region enriched for the fixed HexR-specific SNPs in the parental HexR strain (Fig. S1B). Notable, though less marked, changes were also observed for scaffolds 23 and 40, further supporting the conclusion of Van Leeuwen et al. that scaffolds 3, 23, and 40 are adjacent in the genome (in the current draft assembly, numbering is based solely on scaffold length) (Van Leeuwen et al., 2012a).

In addition to the expectation that allele frequencies should deviate between the unselected and selected populations (Fig. 3), a second expectation is that the haplotype for the recessive resistance locus (or loci) contributed by the HexR haploid male parent should be fixed following selection in the derived experimental populations. Because of drift (or potentially segregation distortion) during the production of the selected populations, fixation or partial fixation of regions contributed by the HexR parent could spuriously occur that would be independent of selection by the compounds; these regions could potentially confound a genome-wide scan to detect acaricide-induced fixation of alleles from the resistant parent. Nevertheless, despite minor peaks on several scaffolds, one and only one sharp peak of nearly complete fixation of HexR-specific SNPs was identified after selection with each compound (Fig. 3B,C). Each peak was coincident on scaffold 3 with the maximal peaks observed for allele frequency divergence between the unselected and the three selected populations as inferred with all SNPs (compare Fig. 3B to Fig. 3A). As evaluated with several sliding window sizes (Table S2), the midpoints of the window with the highest fixation index clustered closely to *CHS1* (typically either several kb to tens of kb upstream or downstream of *CHS1*). For instance, with a window size of 150 kb that was used previously for BSA mapping in *T. urticae* (Van Leeuwen et al., 2012a), the midpoints of the maximally fixed windows following selection with clofentezine, hexythiazox and etoxazole were within an ~14 kb region centered on *CHS1* (Fig. 3C, D; Table S2).

3.3 The effect of hexythiazox and clofentezine on chitin deposition and embryo development

As revealed by BSA mapping, a single locus underlies recessive, monogenic resistance to clofentezine, hexythiazox and etoxazole in strain HexR. Strikingly, the locus is coincident with *CHS1*, the proposed molecular target for etoxazole as revealed by BSA in strain EtoxR (Van Leeuwen et al., 2012a). If hexythiazox and clofentezine also interfere with *CHS1*, a possibility raised by our mapping data and the known cross-resistance among the compounds, reduced chitin deposition is expected in susceptible mites treated with the respective compounds. Further, such a reduction should be absent in resistant mites. To test this, we treated synchronized cultures of susceptible (London) and resistant (HexR) female deutonymphs with hexythiazox and clofentezine and performed cryo-sectioning and CFW staining as previously described (Van Leeuwen et al., 2012a). CFW is a fluorescent dye that binds to nascent chitin chains, and thus CFW incorporation into cuticle reflects chitin deposition (Meissner et al., 2010). The level of CFW staining was significantly reduced in cuticles of susceptible deutonymphs treated with hexythiazox (Fig. 4A; $P < 0.001$, student's t-test), a finding identical to that observed previously for etoxazole in an analogous experiment (Van Leeuwen et al., 2012a). Deutonymphs of resistant mites treated with

hexythiazox showed strong CFW signals in cuticles that were not significantly different from the untreated control of sensitive mites (Fig. 4A; $P=0.126$, student's t-test).

As opposed to that observed for hexythiazox and etoxazole, treatment of susceptible deutonymphs with clofentezine did not result in significantly reduced chitin levels (Fig. 4B). These results are consistent with the well-documented specificity of clofentezine, which acts mainly as an ovicide (Aveyard et al., 1986; Bretschneider and Nauen, 2008; Bryan et al., 1981). Due to technical reasons, we were unable to assess chitin deposition in embryos by CFW staining of isolated cuticle sections. However, we did assess the developmental effects of hexythiazox and clofentezine on embryos. Both hexythiazox and clofentezine treated eggs developed to the red-eye stage, but failed to hatch (Fig. S2). These symptoms are identical to those previously observed with etoxazole treatment (Van Leeuwen et al., 2012a; Fig. S2). Collectively, these results support a shared mode of action for the three compounds at the one developmental stage (embryos) for which all are effective.

3.4 A widely distributed CHS1 variant is associated with clofentezine, hexythiazox and etoxazole resistance

In earlier work, we identified the amino acid variant I1017F as causal for monogenic, recessive resistance to etoxazole as it was the only change in CHS1 shared among unrelated *T. urticae* strains with high resistance ratios (Van Leeuwen et al., 2012a). Strain HexR was not included in our earlier study, but it also harbors the I1017F variant as assessed from aligned Illumina reads (Fig. 5). The striking similarities in genetic mapping for resistance to etoxazole, hexythiazox and clofentezine, as well as the presence of I1017F in HexR, raise the possibility that the same molecular change underlies target-site resistance to all three acaricides. To assess this possibility, we determined the toxicity of clofentezine and hexythiazox for four previously reported etoxazole resistant strains for which only the I1017F variant is universally shared at CHS1 (Fig. 5). These strains, EtoxR, TuSb9, Strain 005R and MR-VL, originate from continental Europe or Japan (Van Leeuwen et al., 2012a). Each of these etoxazole resistant strains is also highly resistant to both hexythiazox and clofentezine with LC_{50} values exceeding 5000 mg l⁻¹ (Table S3).

To further understand the prevalence and geographical distribution of the I1017R variant, we surveyed by PCR and Sanger sequencing 17 additional strains collected from Europe and North America. First, we examined a collection of 13 *T. urticae* strains that were sampled from roses in commercial rose-growing areas across the Netherlands, and that were analyzed earlier by Khajehali et al. (2011). Using DNA samples from these strains previously used for the detection of resistance mutations, we found that one strain segregated for the I1017F substitution (Table S1), and that it was fixed in another strain. Second, in four strains collected from the western United States, the SNP was present (and fixed) in only a single strain. In combination with our earlier work (Van Leeuwen et al., 2012a), these data show that the I1017F variant is broadly distributed, at least in the northern hemisphere, but of moderate prevalence.

4. Discussion

The compounds clofentezine, hexythiazox and etoxazole are commonly referred to as ‘mite growth inhibitors’, and grouped together in group 10 of the IRAC (Insecticide Resistance Action Committee) mode of action classification scheme (Nauen et al., 2012). Hexythiazox and clofentezine have been widely used for decades, while today etoxazole is becoming more important as an acaricide. Based on data from 2010, hexythiazox and etoxazole are among the top 10 selling acaricides worldwide (Agrobase, <http://www.agrobase-logigram.com>). However, the mode of action of hexythiazox and clofentezine have remained unknown.

In this study, we identified a *T. urticae* strain (HexR) harboring recessive, monogenic resistance to each of hexythiazox, clofentezine, and etoxazole. As assessed by BSA methods applied to a cross with HexR, a single shared resistance factor for all three compounds was located on scaffold 3, and as revealed with parent of origin markers, the midpoints of fixation for the HexR specific locus underlying resistances clustered within a few tens of kb. This level of mapping resolution is remarkable, and additional studies are needed to determine if our results are representative of other genomic locations. It should be noted, however, that classic studies with pigmentation loci in *Tetranychus pacificus* have suggested that *Tetranychus* mites have extraordinarily high levels of recombination genome-wide (Helle and Vanzon, 1970) (as with traditional genetic mapping, recombination frequency is the major determinant of mapping resolution in BSA approaches). In contrast to our earlier work to localize etoxazole resistance by BSA in strain EtoxR (Van Leeuwen et al., 2012a), for the current study we used a single male from strain HexR to found all experimental populations. As male mites are haploid, they contribute a single haplotype for the entire genome. This reduced the genetic complexity in our cross, and facilitated the use of strain-of-origin markers in the BSA approaches we employed. Our findings thus establish the use of single males to found experimental populations as an attractive option for BSA studies in mites. This is of particular relevance to potential genetic studies of polygenic traits, examples of which include many instances of metabolic pesticide resistance (Li et al., 2007; Van Leeuwen et al., 2010), and presumably host plant adaptation (Dermauw et al., 2013; Gould, 1979; Jaquiéry et al., 2012; Sezer and Butlin, 1998; Via, 1990), where locus and allelic heterogeneity can confound the detection of quantitative trait loci.

In a recent study, we proposed that the target site of etoxazole is CHS1, and that a single substitution in a transmembrane region confers resistance (Van Leeuwen et al., 2012a). Our current BSA findings with strain HexR identify the same genomic region that was previously documented for target-site resistance to etoxazole in strain EtoxR. In EtoxR, HexR and other strains highly resistant to all three compounds, only a single nonsynonymous variant in CHS1 is shared. This I1017F variant affects an α -helix in a conserved transmembrane domain that is proposed to be part of the enzyme’s chitin translocation pore (Merzendorfer, 2013). Therefore, we propose that etoxazole, clofentezine and hexythiazox all bind to the pore region and impair chitin translocation. This mechanism would prevent chitin deposition in the cuticle, which is observed in nymphal stages after administration of etoxazole and hexythiazox, but not of the ovicide clofentezine, which may be metabolically inactivated more efficiently in larvae and nymphs. Large differences

between detoxifying enzyme activity in the egg versus other developmental stages have been previously documented for *T. urticae* (Demaeght et al., 2013). Furthermore, differences in the reactivity of clofentezine (and its derivative diflovidazin) compared to hexythiazox and etoxazole are suggested by calculating Fukui functions (Fig. S3). These functions indicate spatial positions where electrophilic attack leads to favorable changes in electron density (Beck, 2005). As visualized in Fig. S3, the maxima of the Fukui functions of hexythiazox and etoxazole are spread across various structural features of the molecules, whereas in clofentezine and diflovidazin reactivity is very much centered to the hetero aromatic 1,2,4,5-tetrazine system. This strongly suggests that clofentezine and diflovidazin are more prone to oxidative attack. Secondly, due to the bulky *ortho*-substituents, the resulting twist of clofentezine and diflovidazin leads to a pronounced exposure of these reactive features and a very good accessibility by nucleophilic reaction-partners. In conclusion, the performed calculations support that clofentezine and diflovidazin are more prone to oxidative attack than hexythiazox and etoxazole. However, the undeniable influence of quaternary structure of oxidative detoxification enzymes such as cytochrome P450s needs to be considered, and is not taken into account by such calculations. Nonetheless, empiric data on several insecticides revealed that such pronounced differences are typically reflected by their respective metabolic stabilities (Jeschke et al., 2013).

The proposed resistance mutation changes a central Ile of the α -helix into an aromatic Phe and may block binding of the inhibitors without substantially changing the overall channel width of the transition pore as etoxazole and hexythiazox resistant strains deposit chitin at similar levels in their cuticle as evidenced by CFW staining (see Fig. 4 and (Van Leeuwen et al., 2012a)). Other chitin synthesis inhibitors used as insecticides may also function through transition pore inhibition, such as the benzoylphenyl urea (BPU) compound, diflubenzuron. In line with this assumption, BPU compounds are proposed to act on a post- catalytic step, which was deduced from the observation that they only inhibit chitin synthesis in cell-intact systems but not in cell-disrupted systems (Merzendorfer, 2006). The behavior of the chitin synthesis inhibitors suggests that chitin synthesis, translocation and fibrillogenesis are coupled processes in cell-intact systems and uncoupled processes in cell-disrupted systems, where inhibition of the translocation pore would not interfere with the polymerization reaction. Among the BPUs, some compounds such as flucycloxuron and flufenoxuron have specific acaricidal activity. In support of a shared resistance mechanism between mite growth inhibitors and BPUs that potentially act through modifications of a shared target-site is the work of Yamamoto et al. (1995c), where it was shown that a hexythiazox-selected resistant strain of *P. citri* was cross-resistant to clofentezine (>1600 fold), flufenoxuron and flucycloxuron. Although more than 20 active ingredients were tested in their study, cross-resistance with other compounds was not detected and was specific to the mite growth inhibitors and BPUs (Yamamoto et al., 1995c). Given that the inheritance of hexythiazox resistance in this *P. citri* strain was monogenic and recessive (Yamamoto et al., 1995a), as observed in *T. urticae* in this study, we hypothesize that a similar target-site mutation, rather than a common detoxification pathway, underlies cross-resistance. Cross-resistance between hexythiazox, clofentezine and the BPU flucycloxuron, has also been reported in the field for *P. ulmi* (Grosscurt et al., 1994). Together, this suggests that a similar mutation to that reported here might confer cross-resistance between hexythiazox, clofentezine and various

BPU compounds across different mite species, and may imply a shared mode action with benzoylphenyl urea compounds. Notably, hexythiazox, diflovidazin, etoxazole, and flucycloxuron share a halogenated ring structure, which might act as a structural determinant for binding specificity. As suggested by earlier studies, we note that BPU compounds may have additional target sites, such as sulfonyleurea receptors (Matsumura, 2009) or other members of the ABC transporter family (Meyer et al., 2013), although recent evidence reviewed in Dermauw and Van Leeuwen (2014) does not support this hypothesis.

As revealed by Van Leeuwen et al. (2012a) and by the current study, the I1017F resistance mutation is broadly distributed across Eurasia and is also present in North America. The association between the I1017F variant and high-level resistance to clofentezine and hexythiazox, which have been widely used for decades, may explain the broad geographical distribution of the I1017F variant. In fact, the presence of the I1017F variant on diverse *CHS1* haplotypes strongly suggests that it has arisen independently at least twice (Van Leeuwen et al., 2012; Fig. 5 and Supplemental Table 1). Nevertheless, in field populations the resistance mutation is far from fixed, even in strains from regions where acaricides are widely used for mite control (Supplemental Table 1). This may suggest that the mutation, which is in one of the most highly conserved regions of *CHS1*, has a negative effect on fitness in the field. In support of this, a fitness cost linked to hexythiazox resistance (with cross-resistance to clofentezine and flufenoxuron) was previously documented in *P. citri* (Yamamoto et al., 1995b, 1996). As mentioned above, inheritance patterns of resistance in this species mirrors that seen in our etoxazole, clofentezine and hexythiazox resistant lines of *T. urticae* (Van Leeuwen et al., 2012a; this study), and might suggest that a similar fitness cost is linked to the *CHS1* variant. Alternatively, resistance to hexythiazox is known to be polygenic in some *T. urticae* strains (Asahara et al., 2008), which is indicative of metabolic resistance. If metabolic resistance to hexythiazox and clofentezine is widespread, positive selective pressure on the I1017F target-site mutation may be modest. Collectively, these factors, in isolation or in combination, may explain why hexythiazox and clofentezine have not become obsolete for mite control, as well as why etoxazole could be successfully launched for use worldwide. However, historic selection by older pesticide classes for a target-site mutation in *CHS1* that also confers cross-resistance to etoxazole raises the possibility that the efficacy of this newer and highly potent acaricide may be comparatively short-lived. In this context, and to minimize the likelihood of further evolution of resistance, the sequential use of hexythiazox, clofentezine, and etoxazole to control mite populations should be avoided when implementing resistance management strategies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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HIGHLIGHTS

- We identified a *T. urticae* strain harboring recessive, monogenic resistance to each of hexythiazox, clofentezine, and etoxazole
- BSA mapping identifies the same genomic region associated with resistance to all three compounds.
- The use of single males to found experimental populations enhances mapping resolution
- Chitin staining in hexythiazox treated mites supports that a I1017F variant of chitin synthase-1 confers resistance to a broad class of mite growth inhibitors

Fig. 1. Chemical structures of the mite growth inhibitors used in this study
Clofentezine (a), hexythiazox (b), diflovidazin (c) and etoxazole (d).

Fig. 2. Genetics of clofentezine, hexythiazox and etoxazole resistance

Concentration response relationship of clofentezine (A), hexythiazox (B) and etoxazole toxicity (C) on London, HexR and cross progeny. Resistance to all three compounds is (incompletely) recessive as evidenced by high mortality in the F1 generation (triangles). The mortality plateau at 50% within the range of discriminating pesticide doses for F2 haploid males (squares) demonstrates that resistance to all three compounds segregates as a single locus.

Fig. 3. Bulk segregant genetic mapping of a shared resistance locus for etoxazole, hexythiazox, and clofentezine

(A) Sliding window analysis examining the deviation in allele frequency between each of the various selected, and the unselected, F6 intercross progeny for all SNP positions in 150kb windows across the *T. urticae* genome (with 10kb offset). Genomic data derived from pooled mites treated with either etoxazole, hexythiazox or clofentezine are marked in red, black or blue respectively. Scaffolds are presented in order of length and are marked by alternating grey rectangles. The position of chitin synthase (*CHS1*) is highlighted with an arrow. (B) Fixation of HexR-specific SNPs across the pesticide treated progeny. The percent of parent-of-origin informative SNPs segregating in the unselected population that became fixed for resistant parent alleles in the selected lines is plotted in 150kb windows across the genomic scaffolds (plotting parameters are same as in A). (C) Peak of differentiation centers on *CHS1*. A large proportion HexR-specific alleles become fixed in the selected progeny on *T. urticae* scaffold 3 and centers nearby *CHS1*. (D) Distance of the sliding window peak to previously identified *CHS1* I1017F variant associated with etoxazole resistance. The

distance from the midpoint of the most differentiated genomic window to *CHS1*, and specifically to the I1017F SNP (also see Table S2), is shown for each of the selected lines. Individual genes are demarcated with boxes (exons) connected by lines segments (introns) with vertical position indicating gene strand.

Fig. 4. Cuticular chitin in cryosections from different *T. urticae* strains

Cryosections were prepared from sensitive control strain treated with water (C), hexythiazox- and clofentezine resistant strain HexR (R), and hexythiazox- and clofentezine-treated sensitive strain London (S). The specimens were stained with 0.01% (w/v) CFW to visualize chitin deposition in the cuticle. Fluorescence was recorded using identical settings for exposure time and grayscale profiles. *Above*, the representative images show the effects of hexythiazox (left) and clofentezine (right) on chitin deposition in the cuticle. Scale bar, 100 μ m. *below*, the densitometric analysis of CFW fluorescence in mite cuticles is shown. Significant differences in fluorescence intensities compared with sensitive control strains; $p < 0.001$ (Student's t-test).

Fig. 5. Location of the I-to-F mutation associated with etoxazole, hexythiazox and clofentezine resistance

(A) Schematic of CHS1 domains from *T. urticae*. The I1017F mutation is located in the last transmembrane helix. LB, lipid bilayer; 5TMS, cluster of five transmembrane segments; and CC, coiled-coil motif. Rectangular boxes represent trans-membrane domains (Van Leeuwen et al., 2012a). (B) The single I-to-F mutation is universally shared at CHS1 in geographically diverse strains conferring resistance to etoxazole, hexythiazox and clofentezine (see also Supplemental Table 3; Van Leeuwen et al. (2012a)).

Table 1

Toxicological parameters of etoxazole, hexythiazox and clofentezine.

Compound	Strain	LC ₅₀ (95%CI) (mg l ⁻¹)	LC ₉₉ (95%CI) (mg l ⁻¹)	Slope (±SE)	χ ² (df)	RR	D
Etoxazole	London	0.036 (0.032 – 0.041)	0.18 (0.13 – 0.27)	3.4 (±0.23)	30 (13)		
	HexR	>5000				>138888	
	London x HexR	0.19 (0.15 – 0.24)	2.9 (2.1 – 4.4)	2 (±0.12)	16 (11)		-0.72
Hexythiazox	London	1.9 (1.6 – 2.1)	15 (11 – 21)	2.6 (±0.14)	22 (14)		
	HexR	>5000				>2632	
	London x HexR	3.2 (2.7 – 3.7)	29 (23 – 41)	2.4 (±0.15)	22 (15)		-0.87
Clofentezine	London	1.2 (1.1 – 1.4)	5.9 (4.6 – 8.1)	3.4 (±0.31)	6.9 (6)		
	HexR	>5000				>4166	
	London x HexR	1.3 (1.2 – 1.5)	14 (11 – 22)	2.3 (±0.16)	5.8 (7)		-0.98

CI, confidence interval. RR, resistance ratio. D, dominance.