Population bulk segregant mapping uncovers resistance mutations and the mode of action of a chitin synthesis inhibitor in arthropods

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Because of its importance to the arthropod exoskeleton, chitin biogenesis is an attractive target for pest control. This point is demonstrated by the economically important benzoylurea compounds that are in wide use as highly specific agents to control insect populations. Nevertheless, the target sites of compounds that inhibit chitin biogenesis have remained elusive, likely preventing the full exploitation of the underlying mode of action in pest management. Here, we show that the acaricide etoxazole inhibits chitin biogenesis in *Tetranychus urticae* (the two-spotted spider mite), an economically important pest. We then developed a population-level bulk segregant mapping method, based on high-throughput genome sequencing, to identify a locus for monogenic, recessive resistance to etoxazole in a field-collected population. As supported by additional genetic studies, including sequencing across multiple resistant strains and genetic complementation tests, we associated a nonsynonymous mutation in the major *T. urticae* chitin synthase (*CHS1*) with resistance. The change is in a C-terminal transmembrane domain of CHS1 in a highly conserved region that may serve a noncatalytic but essential function. Our finding of a target-site resistance mutation in *CHS1* shows that at least one highly specific chitin biosynthesis inhibitor acts directly to inhibit chitin synthase. Our work also raises the possibility that other chitin biogenesis inhibitors, such as the benzoylurea compounds, may also act by inhibition of chitin synthases. More generally, our genetic mapping approach should be powerful for high-resolution mapping of simple traits (resistance or otherwise) in arthropods.

insecticide | cuticle | toxicology | development | diflubenzuron

Chitin is among the most abundant natural polymers, and in arthropods it serves as the skeletal polysaccharide in the cuticle and the midgut peritrophic matrix. Chitin consists of GlcNAc residues linked by β-(1–4) glycosidic bonds, and the pathway leading to the formation of chitin in arthropods can be divided into three steps. These steps include sugar transformations leading to the amino sugar GlcNAc, activation to UDP-GlcNAc, and polymerization by a membrane integral and processive type II glycosyltransferase called chitin synthase (1, 2). Because vascular plants and vertebrates use alternative polymers for support, such as cellulose, lignin, or collagen, the chitin biosynthesis pathway is an attractive target for the development of pesticides to selectively control arthropods in agriculture and human health. In fact, peptidyl nucleoside antibiotics, such as polyoxins and nikkomycins that are derived from *Streptomyces* spp., are known substrate analogs at the catalytic site of insect and fungal chitin synthases. Their utility has been limited, however, by their low cuticular permeability, hydrolytic instability in the gut, and, to some extent, toxicity in vertebrates (2).

As opposed to substrate analogs, several widely used and economically important pesticides (or pesticide classes) disrupt chitin biosynthesis in arthropods, but with modes of action that are unknown (1, 2). These pesticides include the economically important benzoylureas, of which diflubenzuron is the best known example. Benzoylureas are potent insecticides that inhibit chitin biosynthesis in insects, but not in fungi, and have been developed into a large and successful class of insecticides with high selectivity (3–5). Several lines of evidence using cell-free systems have revealed that benzoylureas do not inhibit the catalytic step of chitin synthesis, or the sugar transformations in the biochemical pathways leading to GlcNAc (1, 2). Likewise, the mode of action is also unknown for etoxazole, a commercialized oxazoline that is a potent acaricide and that is now used worldwide (6). In the lepidopteran *Spodoptera frugiperda,* etoxazole affected chitin content in vivo and inhibited the incorporation of C^{14}-GlcNAc into isolated integument pieces in vitro, an effect similar to the benzoylurea diflubenzuron. Based on the similarity of action between benzoylureas and etoxazole, it has been suggested that these compounds share a similar mode of action to inhibit chitin biosynthesis (7). For the long-studied benzoylureas, which were discovered in the 1970s, many theories of the mode of action have been proposed (1), most of which claim a mechanism independent of a direct interaction with chitin synthase itself.

In establishing the molecular action and resistance mechanisms of these and other pesticides, a limiting factor has been the lack of genetic systems, and accompanying genomic resources, for efficient identification of resistance mutations that suggest molecular mechanisms (8). In this study, we investigated the action and resistance mechanisms of etoxazole in field-collected strains of the two-spotted spider mite, *Tetranychus urticae*, a globally important polyphagous pest (9). *T. urticae* is especially well suited for characterizing resistance mechanisms—resistance arises easily (9), crosses are straightforward, and the small genome size (90 Mb) facilitates genomic analyses (10). We developed and used a bulk segregant analysis (BSA) mapping method (11) that we adapted to fully exploit the power of large and genetically diverse mite populations. This method allowed genetic mapping of resistance to a small genomic region and, as supported

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by complementary approaches, the identification of a resistance mutation that defines the molecular target of etoxazole.

Results

Etoxazole Inhibits Chitin Synthesis in *T. urticae*. Etoxazole causes lethality in mite embryos and developing stages at the time of eclosion or ecdysis. Although etoxazole is not toxic to adults, fertility of treated females was strongly affected (Fig. S1*A*), and eggs deposited by treated females develop normally to the red-eye stage, but larvae fail to hatch (Fig. S1 *B* and *C*). These specific symptoms are nearly identical to those observed with benzoylureas in insects (12). To establish the effect of etoxazole on chitin deposition in mites, we treated synchronized female deutonymphs with etoxazole and collected the teleiochrysalis stage just before ecdysis. As revealed by calcofluor white (CFW) staining, levels of chitin in the cuticle were drastically decreased by treatment with etoxazole (Fig. 1), confirming the inhibitory effect of etoxazole on chitin biosynthesis observed in insects where etoxazole is much less potent.

Genetic Basis of Etoxazole Resistance. To determine the molecular action of etoxazole, we first characterized an etoxazole-resistant *T. urticae* strain from Japan (strain EtoxR), where etoxazole has been in use for more than a decade, and field resistance has been reported (13). In comparison with the etoxazole-susceptible London strain, EtoxR had a resistance ratio of 48,000-fold (Fig. 2*A* and Table 1). Mortality in F1 progeny of reciprocal crosses between EtoxR and London revealed that resistance was recessive and not maternally inherited (Fig. 2*A*); as shown by mortality in haploid F2 males obtained from F1 virgin females of reciprocal crosses, etoxazole resistance segregates as a single resistance locus ($\chi = 6.07$, df = 9, $P < 0.05$; Fig. 2*A* and Table 1).

Identification of the Etoxazole Resistance Locus. To identify the resistance locus in EtoxR, we sequenced, with Illumina methods (14), the EtoxR strain to ~21-fold genomic coverage. Alignment of EtoxR reads to the 90-Mb London genome sequence (10) revealed that the EtoxR strain is highly polymorphic, with 766,572 SNPs relative to London (minor allele frequency >10%; Fig. S2 *A* and *B*). As assessed across the genome for EtoxR, no extended regions of fixed sequences were apparent (Fig. S2*C*). This observation suggested that etoxazole resistance arose from a variant that persisted in the population long enough for recombination to break down hitchhiking with distantly linked variants (i.e., we found no evidence of a selective sweep fixing a tens to hundreds of kilobases region of the genome).

We therefore developed a BSA approach to localize the resistance locus to a small genomic region. We crossed ~700 EtoxR males with ~650 London females, and we propagated the resulting progeny to the F4 generation. This cross was done under high density and with a large population size (~50,000 mites at the F4 generation) to facilitate maximal intercrossing and recombination between the genomes of the susceptible and resistant strains to break apart haplotypes near the resistance mutation. We then treated a subset of the progeny of F4/F5 mites (the selected population) with a dose of etoxazole lethal for the London strain and kept the remaining F4 mites as the control (unselected) population. We next sequenced with Illumina methods genomic DNA from the selected and unselected populations. By aligning Illumina reads to the London reference genome sequence, we determined allele frequencies for each sample at 182,361 SNPs that were fixed (or nearly so) in the parental EtoxR strain relative to the reference London genome sequence (*SI Text*). Given the monogenic, recessive

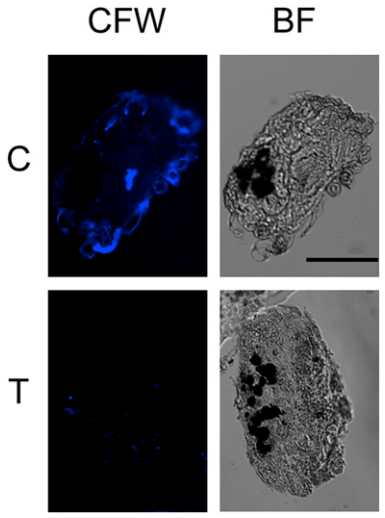

Fig. 1. CFW staining of cryosections from *T. urticae*. Cryosections from control (C) or etoxazole-treated mites (T) were stained with 0.01% (wt/vol) CFW to visualize chitin deposition in the cuticle. Fluorescence was recorded using identical settings for exposure time and grayscale profiles. The representative image shows an inhibitory effect of etoxazole on chitin formation. BF, bright field. (Scale bar, 100 μm.)

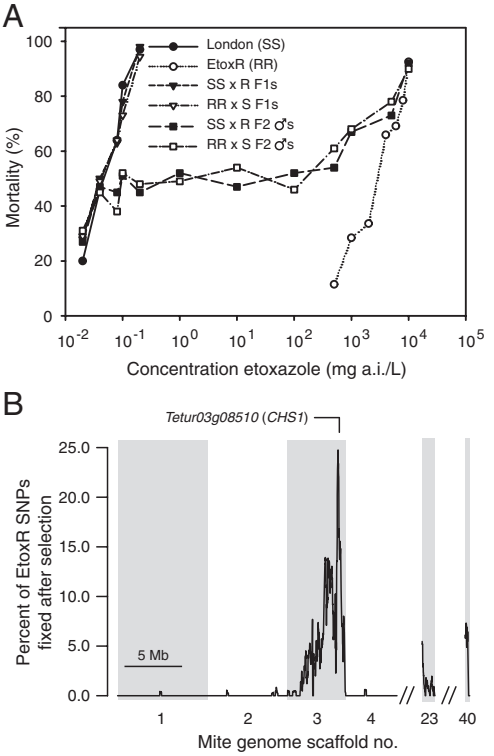

Fig. 2. Genetics and mapping of etoxazole resistance in *T. urticae*. (*A*) Concentration response relationship of etoxazole toxicity on London, EtoxR, and crosses. High resistance is inherited recessively and is not maternal [compare reciprocal F1s (triangles) with parental strains (circles)]. A pronounced mortality plateau at 50% for haploid males that are the progeny of unfertilized F1 females (squares) demonstrates control by a single major factor. (*B*) Fixation of EtoxR SNPs in the selected population, as assessed with a sliding window of 150 kb (windows were sequentially offset by 10 kb; the percent of fixed EtoxR SNPs in a widow following selection is plotted; see Figs. S2 and S3 for details). Scaffolds 1, 2, and 4 are representative of the genome average (no evidence of selection); only scaffolds 3, 23, and 40 show marked evidence of shifts in allele frequencies in response to etoxazole selection (Fig. S3). The position of *CHS1* is indicated at top.

Table 1. Toxicological parameters of etoxazole

Strain	LC_{50}	95% CI	*n*	Slope (SE)	χ^2 (df)	RR	D
London	0.056	0.046–0.065	855	3.1 (0.30)	27.1 (18)	1	
EtoxR	2,700	2,320–3,175	806	1.8 (0.13)	22.6 (19)	48,000	
Strain005R	21,000	13,057–60,578	753	1.3 (0.21)	19.8 (15)	375,000	
London × EtoxR	0.046	0.039–0.053	1,039	2.1 (0.18)	22.5 (14)	0.82	−1.0
EtoxR × London	0.046	0.041–0.051	1,354	2.0 (0.12)	14.9 (14)	0.82	−1.0
London × Strain005R	1.8	1.4–2.3	2,098	0.79 (0.04)	32.3 (24)	32	−0.37
Strain005R × London	3.2	2–6.3	1,346	0.62 (0.05)	19.4 (14)	57	−0.46
Strain005R × EtoxR	3,800	3,416–4,386	828	2.4 (0.15)	18.4 (13)	67,000	
EtoxR × Strain005R	3,600	3,179–4,139	660	3.3 (0.21)	20.4 (13)	64,000	

Concentration-mortality data of etoxazole on larvae of strains and their crosses. CI, confidence interval; D, degree of dominance; *n*, no. of mites; RR, resistance ratio.

nature of resistance, in the selected population, the genomic region harboring the resistance allele would be expected to exhibit a shift in allele frequencies for SNPs specific to the EtoxR strain (i.e., the causal region/variant should be fixed, whereas the rest of the genome should segregate for alleles from both parental strains; for the unselected population, all loci should segregate). Compared with the unselected population, a sliding window analysis of the 44 largest scaffolds in the *T. urticae* genome assembly (which contain 94.6% of the 90-Mb genome) revealed a pronounced fixation of EtoxR-specific SNPs specific to the selected population on the distal end of scaffold 3 (Fig. 2*B*, Fig. S3, and *SI Text*). Only two other scaffolds (23 and 40) showed appreciable signatures of selection, but to a far lesser extent (the *T. urticae* scaffolds are unordered, and whether scaffolds 23 and 40 are adjacent to scaffold 3 in the genome is unknown). Our population-level BSA mapping thus resolved the resistance locus to a small region on scaffold 3 at ∼4.5 Mb.

***CHS* Variant Associated with Etoxazole Resistance.** In analyzing scaffold 3, we found that one of two chitin synthase genes in the *T. urticae* genome, *tetur03g08510*, is coincident with the resistance locus; *tetur03g08510* is among the 20 genes (±43 kb) closest to the peak of the BSA sliding window analysis (Fig. 2*B*).

Although chitin synthase is an obvious prima facie candidate to be the molecular target of etoxazole (Fig. 1), its coincidence with the resistance locus was nonetheless surprising. The symptoms of poisoning of etoxazole and benzoylureas are identical (Fig. S1), and though the mode of action had already been suggested to be the same (7), benzoylureas have never been shown to directly inhibit the catalytic activity of a chitin synthase. To further evaluate *tetur03g08510* as a potential target, we therefore performed several additional studies. First, because etoxazole causes lethality in developing mites (embryos, larvae, and nymphs, with adults being insensitive), we asked if *tetur03g08510* is the major chitin synthase expressed during development. As assessed with stage-specific RNA sequencing (RNA-seq) profiling data from an earlier study (10), and as validated in the current study by quantitative RT-PCR, *tetur03g08510* is the predominant chitin synthase expressed in the embryo and developmental stages (a second chitin synthase, *tetur08g00170* is expressed weakly during *T. urticae* development, and at higher levels in adults; Table S1 and Fig. S4). The latter findings suggest that *tetur03g08510* is a member of class A genes encoding chitin synthase 1 (CHS1), which are expressed in the epidermis and tracheal system for cuticle formation during development (2).

Second, we determined by PCR and Sanger sequencing the *tetur03g08510* (hereafter called *CHS1*) coding sequence from additional strains, three of which have similar levels of etoxazole resistance but are unrelated (Fig. 3*A* and *SI Text*). Compared with two susceptible strains, and as assessed across all resistant strains, a single nonsynonymous variant, an isoleucine (I)-to-phenylalanine (F) change at position 1017 (I1017F), was uniquely associated with etoxazole resistance (Fig. 3*B*). Intriguingly, Strain005 initially exhibited only partial etoxazole resistance, and segregated for the I1017F variant (allele frequencies 0.45/0.55; Fig. 3 *A* and *B* and *SI Text*). Selection of Strain005 with etoxazole produced a uniformly resistant population (Strain005R; Fig. 3 *A* and *B* and Table 1), and in a single generation, the I1017F variant became fixed. Similar to EtoxR, etoxazole resistance in the selected population of Strain005 was not maternally inherited, was intermediately recessive, and was caused by a single major factor (χ = 24.3, df = 15, $P < 0.05$; Fig. S5 and Table 1). In a reciprocal cross of Strain005R with EtoxR, F1 mites were etoxazole resistant. Thus, the resistance factors in the two strains fail to genetically complement each other (i.e., restore the WT phenotype of etoxazole susceptibility). This result is only expected if the same gene underlies resistance in both strains (Fig. 3*C*).

Finally, the resistant strains are from disparate geographic locations (e.g., Belgium and Greece in Europe, and Japan in Asia; Fig. 3). Though only an A–T transversion at position 1 in codon 1017 can cause an I-to-F change, the I1017F variant was present on two different haplotype groups (i.e., compare strains EtoxR and Strain005 with strains MRVL and TuSB9). Although some evidence for recombination or gene conversion is apparent, the observed pattern is indicative of recurrent mutation and selection, a phenomenon expected only in the presence of a strong selective agent (e.g., a highly potent pesticide; *Discussion*). Furthermore, patterns for an adjacent synonymous SNP in the Strain005 population indicate that the origin for the I1017F transversion in Strain005 (Fig. S6) may be independent of that for EtoxR, even though Strain005 and EtoxR share a globally similar *CHS1* haplotype (Fig. 3*B*).

Collectively, our data implicate a single CHS1 amino acid change as conferring target site resistance to etoxazole.

I1017F Is Located near the Sequence Motif WGTR and Does Not Abolish the CFW Resistance Phenotype of Yeast *chs3*Δ Cells. The I1017F mutation is located within the last transmembrane helix of the predicted C-terminal 5 transmembrane segment (5TMS) cluster originally reported for insect chitin synthases. This region has been suggested to be involved in pore formation and chitin extrusion (Fig. 4) (2, 15). The 5TMS cluster is followed by a conserved WGTR motif and a coiled-coil region, which could mediate oligomerization. Chitin synthase 3 (CHS3) from *Saccharomyces cerevisiae* has a homologous TMS cluster built of three transmembrane helices, which correspond to the last three helices of the 5TMS cluster found in insects and mites (although the predicted coiled-coil region is absent). Remarkably, many amino acids within the last transmembrane helix are either identical or similar between insect/mite CHS1 and yeast CHS3 (Fig. 4), and the isoleucine at the 1017 resistance position is conserved across all insects we analyzed (Fig. 4). Resistant mites with the I1017F

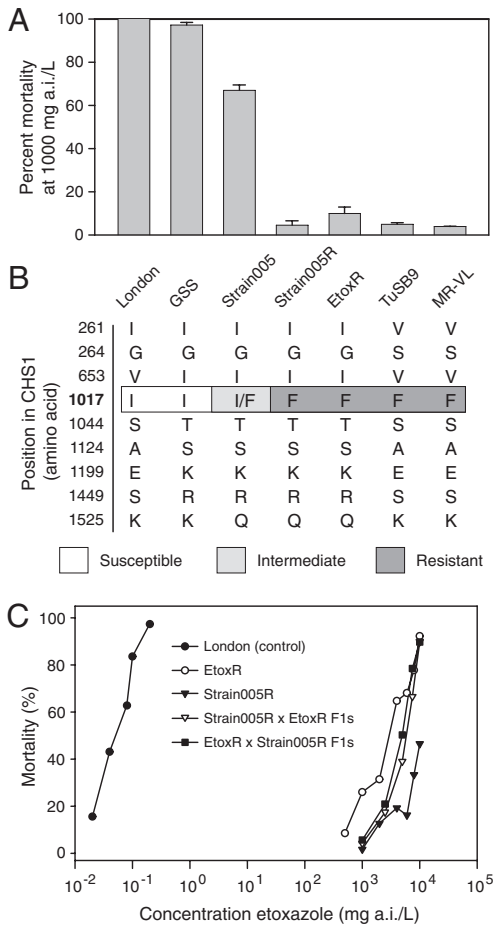

Fig. 3. A nonsynonymous SNP in *CHS1* is associated with etoxazole resistance. (*A*) Etoxazole resistance in strains of diverse geographical origin as documented by mortality at a discriminating dose. Error bars represent SE. (*B*) A single I-to-F variant is associated with etoxazole resistance, and becomes fixed in a segregating population (Strain005) after a single selection (Strain005R). Strains from different regions display different haplotypes. Only nonsingleton nonsynonymous variants are displayed (see Fig. S3 for additional information). (*C*) Concentration response relationship of etoxazole toxicity in London, EtoxR, Strain005R, and crosses (see legend). Because Strain005R × EtoxR progeny are not susceptible, recessive resistance in the two strains (Fig. 2*A* and Fig. S5) must share the same genetic basis. Strain origins are Belgium (MR-VL), Canada (London), Holland (Strain005/005R), Japan (EtoxR), Greece (TuSB9), and Germany (GSS).

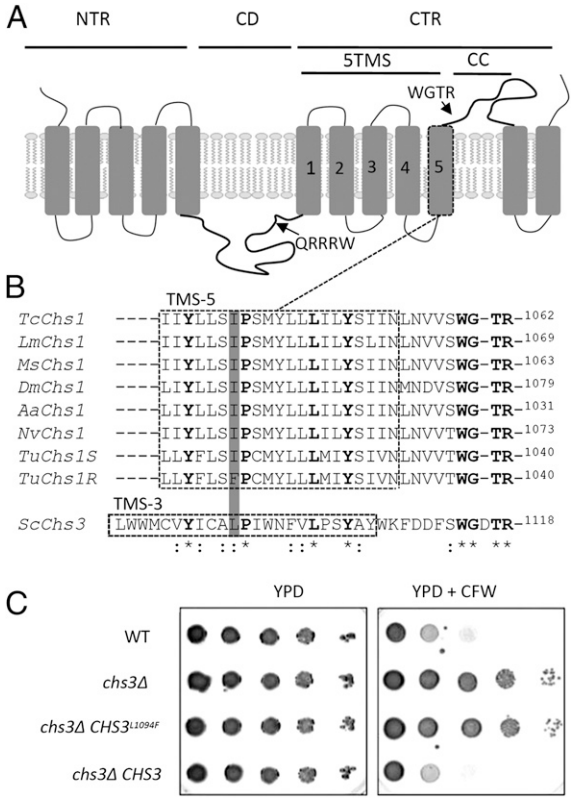

Fig. 4. Location of the mutation conferring etoxazole resistance, and evaluation of its effect on chitin biosynthesis in yeast. (*A*) Schematic representation of domain architecture of CHS1 from *T. urticae*. NTR, N-terminal region; CD, catalytic domain; CTR, C-terminal region; 5TMS, cluster of five transmembrane segments; and CC, coiled-coil motif. Rectangular boxes represent transmembrane domains. Arrows point to signature sequences QRRRW (catalytic domain) and WGTR (N-terminal region). (*B*) Aligned amino acid sequences of helix 5 in the 5TMS clusters of CHS1 of representative insect species, *T. urticae*, and the corresponding helix 3 of *S. cerevisiae*. Universally conserved residues are marked in bold. The position of the I1017F substitution in etoxazole-resistant mites is indicated in gray. S, etoxazole susceptible; R, etoxazole resistant; Tc, *Tribolium castaneum*; Lm, *Locusta migratoria*; Ms, *Manduca sexta*; Dm, *D. melanogaster*; Aa, *Aedes aegypti*; Nv, *Nasonia vitripennis*; Tu, *T. urticae*; Sc, *S. cerevisiae*. (*C*) Complementation of the CFW resistance phenotype in *chs3*Δ strains. *chs3*Δ cells were transformed with pRS415 plasmids containing the ORFs for *CHS3* or its mutant version $CHS3^{L1094F}$. WT, *chs3*Δ, *chs3*Δ*CHS3*, and *chs3*Δ$CHS3^{L1094F}$ cells were grown overnight in liquid YPD medium and spotted after dilution with water onto YPD plates with or without 50 μg/mL CFW. In contrast to *chs3*Δ *CHS3,* the CFW phenotype in *chs3*Δ $CHS3^{L1094F}$ cells is not restored, indicating that $CHS3^{L1094F}$ is nonfunctional.

variant are viable, although more subtle effects on fitness are a possibility. We therefore tested the effect of the analogous mutation in yeast, the only established system for mutational analysis of CHS enzyme complexes, and where sensitive assays of function are available.

To do this, we generated a mutated version of the enzyme mimicking the I-to-F exchange at the corresponding amino acid position of CHS3, which is a leucine residue (Fig. 4), and evaluated chitin formation in vivo by complementation of the CFW resistance phenotype of *chs3*Δ yeast cells lacking a functional version of CHS3. CFW is a fluorescent dye that binds to nascent chitin chains and inhibits fungal growth. Because *chs3*Δ cells lack much of the cell wall chitin, and hence CFW binding capacity, they are thus resistant to CFW. Hence, resistance correlates with reduction in chitin levels and can be used to evaluate chitin formation in vivo (16). In contrast to WT cells, which were highly sensitive to CFW, *chs3*Δ cells exhibited significant resistance to CFW. CFW sensitivity could be restored in *chs3*Δ cells when the cells were transformed with a pRS415 plasmid expressing WT *CHS3*. However, when *chs3*Δ cells were transformed with a pRS415 plasmid carrying the L1094F mutation in *CHS3*, the cells did not become sensitive to CFW, indicating that $CHS3^{L1094F}$ encodes a protein with markedly reduced function, even though the full-length protein was expressed at levels similar to WT in yeast cells (Fig. S7).

Discussion

In our study, we show that the potent acaricide etoxazole inhibits chitin synthesis in spider mites, and that resistance in field strains is monogenic and recessive, with exceptionally high resistance ratios (a typical feature of target site resistance). Multiple lines of evidence suggest that etoxazole inhibits chitin biogenesis by a direct effect on chitin synthase. First, by population BSA mapping, we localized the etoxazole resistance locus to a small genomic interval coincident with the chitin synthase (*CHS1*) that is expressed in the stages most susceptible to etoxazole. Second, we identified a single nonsynonymous change (I1017F) in CHS1

that was invariantly associated with resistance across multiple unrelated strains. Additionally, the transversion producing the I1017F change was globally distributed in the northern hemisphere, and present on highly distinct haplotypes. We believe this pattern is most consistent with recurrent mutation and selection, although a role for the rapid spread and introgression of the I1017F variant remains possible (i.e., from standing genetic variation). Regardless, these patterns are difficult to explain in the absence of strong selection, a feature of pesticide use. In fact, the phenomenon of recurrent mutation has been observed in other instances of target site resistance (17).

The BSA approach with high-throughput sequencing that we adapted to mite populations is conceptually similar to that reported in several recent studies with plants and yeast (18, 19). The approach allowed us to resolve, with no prior expectation, a resistance locus to a small genomic region in an arthropod that lacks traditional genetic resources but for which a complete genome sequence is now available. In applying BSA to map resistance in *T. urticae*, as well as for follow-up studies, two life-history characteristics were particularly helpful. First, spider mites can be easily reared in extremely large populations over many generations. This feature allows for dense recombination following bulk crosses, a prerequisite for high-resolution mapping that is the key to elucidate the molecular nature of resistance. A second point is that, along with large population sizes, haplodiploid sex determination may facilitate the evolution and spread of resistance (e.g., recessive resistance is immediately uncovered in haploid males), which may have contributed to our finding of multiple haplotypes that conferred resistance—an observation that ultimately allowed us to identify a single associated DNA lesion. Nevertheless, though *T. urticae* is especially amenable for the genetic approaches we used, the BSA method should be applicable to studies in other arthropods where genomic resources are or will become available. Moreover, it should be of value to study nonresistance traits also (18), at least where the underlying genetic architecture is comparatively simple.

To date, the absence of heterologous expression systems that recapitulate the full chitin synthase function in arthropods (catalysis and translocation) have hampered studies to understand chitin synthases, as well as their mode of inhibition. Nevertheless, genetic studies have supported a role for the C-terminal region, including the SWG(D)TR motif and the ensuing region near the location of the etoxazole resistance mutation. In *Drosophila melanogaster*, four embryonic lethal mutations affect the C-terminal region of *krotzkopfverkehrt* (*kkv*), which encodes CHS1 (20–22). In particular, the kkv^{JH9} mutation is caused by a G1077D exchange in the WGTR motif of the extracellular domain. Moreover, the C-terminal regions of each of the CHS1, CHS2, and CHS3 proteins in yeast are required for chitin synthesis (23, 24). When we mimicked the analogous I-to-F change in yeast CHS3 by site-directed mutagenesis (Fig. 4), the resulting protein no longer had WT function. Whereas previous mutational analysis in yeast has focused on the C-terminal nonmembrane integral regions, our result suggests that the last transmembrane helix preceding the S/WGDTR motif is also crucial for CHS activity in yeast. This experimental finding was in contrast to the observation that the I1017F change in mite CHS1 does not abolish all function. It does, however, implicate the respective transmembrane region as important for a key aspect of chitin synthase function. These findings may reflect differences between the distantly related yeast CHS3, as well as CHS1s of insects, compared with the mite CHS1. Whether such differences underlie variation in relative susceptibilities to etoxazole will require further study [e.g., yeast is not susceptible to etoxazole (Fig. S8), and insects less so than mites].

It was previously suggested that the transmembrane helices of the C-terminal part of chitin synthases are involved in pore formation, which is required for the translocation of nascent chitin polymers across the membrane (2, 15). Therefore, binding of etoxazole to the 5TMS cluster could block chitin translocation across the membrane; alternatively, it could impair oligomerization of CHS units, a crucial step in the formation of a functional CHS complex (25) similar to cellulose synthesizing rosette complexes (2, 25, 26), or the proper function of the adjacent coiled-coil motif. The replacement of the isoleucine residue by the aromatic phenylalanine in the 5TMS helix of the CHS1 might prevent etoxazole from interacting with the transmembrane helices to affect chitin synthesis. Intriguingly, the 1017 residue is immediately adjacent to a universally conserved proline, and several studies have demonstrated that transmembrane proline residues may be involved in formation of molecular hinges important for integral membrane protein function (27).

Finally, although etoxazole has low potency in insects, identical symptoms of poisoning are observed following exposure to both benzoylureas and etoxazole, and thus the same mode of action has been postulated (7). Though the sulfonylurea receptor (SUR) has been suggested as the direct target of benzoylureas (28, 29), with inhibition affecting chitin biosynthesis indirectly by altering vesicle trafficking, its role in chitin synthesis inhibition is controversial (30, 31). In the case of etoxazole, our identification of a monogenic resistance locus on scaffold 3 rules out the SUR receptor as the molecular target for this compound (SUR is on scaffold 11; *SI Text*). Our findings suggest that CHSs should be reexamined as the target of the economically important benzoylureas, and potentially other chitin biosynthesis inhibitors, keeping in mind that inhibition may result from the disruption of a function distinct from the enzymatic addition of GlcNAc monomers to the growing chitin chain by arthropod CHS enzyme complexes (i.e., oligomerization or polymer translocation that may require helix–helix interactions). In support of this view, benzoylurea derivatives have been reported that are able to adopt α-helical conformations and occupy binding sites for α-helices involved in protein–protein interactions (32).

Materials and Methods

Bioassays. To assess the toxic effects of etoxazole, a larval bioassay was developed based on previously published assays for ovi/larvicides (33). Briefly, 20–30 adult female mites were allowed to lay eggs for 6 h on 9-cm^2 square bean leaf discs, which were placed on wet cotton under standard incubation conditions (for mite husbandry and strain details, see *SI Text*). After hatching (typically 3–4 d), larvae were sprayed with 0.8 mL spray fluid at 1 bar pressure (1.5 mg aqueous deposit per cm^{-2}). Serial diluted concentrations of etoxazole (trade name Borneo, 11% suspension concentrate) were tested in 3–4 replicates, along with a water control. Mortality was assessed after 4 d; control mortality 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 LC_{50} values by that of the London (susceptible) strain.

Establishing the Mode of Inheritance. Reciprocal crossing experiments were undertaken as previously described (34). F1 hybrid progeny of reciprocal crosses were examined for etoxazole susceptibility in the larval stage as described above. Based on calculated LC_{50} values, the degree of dominance was determined (35). A subset of the F1 progeny was allowed to develop into unfertilized females (by removing males from the population at the deutonymphal stage). These females lay haploid eggs, and the resulting F2 male larvae (etoxazole is equally toxic to both sexes) were used in toxicity experiments to test the hypothesis of monogenic resistance. For monogenic-recessive inheritance of resistance in reciprocal crosses (Fig. 2*A* and Fig. S5), a plateau is expected at 50% mortality across a range of discriminating doses (36). We also compared the observed response with the expected response under the hypothesis of monogenic inheritance. The expected response was calculated with the formula C = 0.5 W (parent 1) + 0.5 w (parent 2) (36), where *C* is the expected mortality and *w* the observed mortality of the parental types at a given concentration. A χ^2 goodness-of-fit analysis was used to examine how well the observed response fit the expected response.

Sample Preparation for Bulk Segregant Analysis. For crossing, ~650 females in the teleiochrysalis stage of the London strain were placed along with 700

males of the EtoxR strain on a detached bean leaf. After 3 d, the fertilized females were collected and allowed to grow in bulk on potted bean plants using standard incubation conditions. After the fourth generation, the population was split and placed on either treated plants (1,000 mg/L etoxazole, selected) or plants sprayed with water (unselected). Selected and unselected F5 females were further allowed to lay eggs on detached bean leaves on cotton that were sprayed as previously described with 1,000 mg active ingredient per liter etoxazole and water, respectively. This procedure ensured that all susceptible genotypes were wiped out in the selected population, which was confirmed in a toxicity experiment. Finally, ~2,500 females (F6 generation) were collected from each treatment. Mites were homogenized in extraction buffer [2% (wt/vol) SDS, 200 mM Tris·HCl, 400 mM NaCl, and 10 mM EDTA] and incubated at 60 °C for 30 min with rotation. DNA was prepared by chloroform–phenol extraction (37) and isopropanol precipitation, and stored in 75% (vol/vol) ethanol.

Genome Sequencing, SNP Detection, and BSA. DNA prepared from the parental strains, London (susceptible) and EtoxR (resistant), as well as the unselected and selected F6 females (see above) was used to construct four genomic DNA libraries that were sequenced on an Illumina Genome Analyzer II (GAII) using standard Illumina protocols (14). A single GAII lane was used to produce 6–28 million 80- to 82-bp single-end reads per library. The resulting reads were aligned to the *T. urticae* genome sequence for SNP discovery and genotyping, and the resulting genotypic data were used for BSA (*SI Text*).

Complementation Assays in Yeast. To test the effect of a L1094F substitution in yeast CHS3, we performed complementation assays monitoring CFW resistance in yeast cells that were deficient in the *CHS3* gene (16). For this purpose, a *chs3*Δ yeast strain (in BY4741; *MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) was transformed by electroporation with pRS415 plasmid carrying either the ORF of WT *CHS3* (accession no. NM_001178371.1, NCBI RefSeq database) or mutated $CHS3^{L1094F}$, which was generated by site-directed mutagenesis and verified by nucleotide sequencing of the complete ORF (see *SI Text* for details). Yeast cells of various genotypes (WT, *chs3*Δ, *chs3*Δ*CHS3*, and *chs3*Δ$CHS3^{L1094F}$) were cultured overnight and plated in dilution series to assay CFW sensitivity (see *SI Text* for culture details and Western blot confirmation of expression).

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