Molecular mechanisms of *Tetranychus urticae* chemical adaptation in hop fields

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The two-spotted spider mite, *Tetranychus urticae* Koch is a major pest that feeds on >1,100 plant species. Many perennial crops including hop (*Humulus lupulus*) are routinely plagued by *T. urticae* infestations. Hop is a specialty crop in Pacific Northwest states, where 99% of all U.S. hops are produced. To suppress *T. urticae*, growers often apply various acaricides. Unfortunately *T. urticae* has been documented to quickly develop resistance to these acaricides which directly cause control failures. Here, we investigated resistance ratios and distribution of multiple resistance-associated mutations in field collected *T. urticae* samples compared with a susceptible population. Our research revealed that a mutation in the cytochrome b gene (G126S) in 35% tested *T. urticae* populations and a mutation in the voltage-gated sodium channel gene (F1538I) in 66.7% populations may contribute resistance to bifenazate and bifenthrin, respectively. No mutations were detected in Glutamate-gated chloride channel subunits tested, suggesting target site insensitivity may not be important in our hop *T. urticae* resistance to abamectin. However, P450-mediated detoxification was observed and is a putative mechanism for abamectin resistance. Molecular mechanisms of *T. urticae* chemical adaptation in hopfields is imperative new information that will help growers develop effective and sustainable management strategies.

As a flavoring and stability ingredient in beer, hop (*Humulus lupulus*) is an economically important crop in the Pacific Northwest (PNW) of the United States. The U.S. hop industry is concentrated in the three PNW states, Washington, Oregon, and Idaho, which represent over 99% of the nation's and 30% of the world's hop acreage in 2013. The preliminary production of the U.S. hops crop was valued at $249 million in 2013\(^1\). Hop is a dioeciously perennial specialty crop that is planted in female monoculture\(^3\). Hops bloom in the PNW initiated by long days, and un-pollinated flowers develop into cones that ripen between mid-August to mid-September. The commercial products from hops are resin and hop oil extracted from the lupulin gland of the hop cone\(^4\). In the State of Washington hops are only grown commercially in the Yakima Valley including three distinct growing areas: the Moxee Valley, the Yakama Indian Reservation, and the Lower Yakima Valley. Each of these areas are within a 50-mile radius (80 kilometers) of each other in shrub-steppe habitats characterized by low annual winter precipitation and hot dry summers. Climate, experienced growers, established infrastructure, and modern drip irrigation techniques enhance optimal hop production in the Yakima Valley and make it among the most productive hop growing regions in the world\(^5\). In 2013, Washington growers produced 79% of the U.S. hops crop\(^1\).

Integrated pest management strategies have been developed to optimize production of high-quality hops\(^6\). To date, several plant pathogens and arthropods have been reported as pests of hops in the PNW\(^8\). Among these, *Tetranychus urticae* is the most serious and prevalent arthropod pest in warmer...
94 unique insecticide/acaricide active ingredients in 468 documented cases worldwide. This species has been found to be resistant to agroecosystem and offers a refuge to escape pesticide exposure. Indeed, the two-spotted spider mite has populations. Studies reported that T. urticae genes that are correlated with abamectin resistance in etoxazole, hexythiazox, and clofentezine in extremely short life span with relatively high fecundity, and arrhenotokous reproduction. As its name implies, T. urticae is able to produce webs from silk glands located at each palp. The webbings made by T. urticae may work as a shelter to avoid pesticide exposure and protect it from other life-threatening conditions including wind, rain, and natural enemies. Moreover, T. urticae undergoes diapause in soil, tree bark, ground cover and dried leaves when decreasing temperature, photoperiod, and decline in the quality of food supply occur due to plant senescence. Diapause facilitates T. urticae adaptation to the agroecosystem and offers a refuge to escape pesticide exposure. Indeed, the two-spotted spider mite has been reported to be the world’s most resistant arthropod; this species has been found to be resistant to 94 unique insecticide/acaricide active ingredients in 468 documented cases worldwide.

The mechanisms of pesticide resistance exhibited by arthropods typically evolve along several trajectories, including behavioral avoidance, decreased cuticular penetration, enhanced sequestration or metabolic detoxification, and target site insensitivity. Among these, target site insensitivity to acaricides in T. urticae have been investigated extensively. For example, several mutations in the acetylcholinesterase (AChE) gene have been documented in organophosphate resistant T. urticae strains from Europe and Korea. Two mutations were identified in the Glutamate-gated chloride channel (GluCl) genes that are correlated with abamectin resistance in T. urticae populations. Studies reported that resistance to bifenthrin commonly used for T. urticae control was tightly linked to multiple mutations at the Quinol oxidation (Qo) site of mitochondrial cytochrome b (cytb) gene. Pyrethroid resistance in T. urticae has been associated with several amino acid substitutions in the voltage-gated sodium channel (VGSC) gene. Additionally, recent studies revealed that a mutation on the chitin synthase gene may contribute to resistance to etoxazole, heptylthiazox, and clofentezine in T. urticae.

In order to design the most effective and sustainable T. urticae management strategy, our long-term goals include revealing the mechanisms underlying the chemical adaptation of T. urticae in the field. We initially calculated the baseline concentration response curves of T. urticae population susceptible to three acaricides: abamectin, bifenthrin, and bifenthrin. We chose these three compounds because they are currently the most commonly used acaricides for T. urticae control in hopyards according to the spray records we investigated (Fig. 1). Recently, field control failures with these acaricides have been observed in the Yakima Valley of Washington State. We collected 31 T. urticae field populations from hopyards in the Yakima Valley during summer 2013 (Fig. 2) and evaluated the acaricide resistance levels in most of these populations compared with a susceptible strain. We also investigated the distribution pattern of resistance-associated target site mutations in these field collected T. urticae populations. Finally, the relative expressions of several detoxification-related P450 genes in field T. urticae populations were compared with that of the susceptible population.

**Results**

**Baseline toxicity of the lab susceptible population.** To establish baseline levels of susceptibility and discriminating concentrations for three acaricides, their toxicity was first evaluated in the susceptible T. urticae population. All acaricides tested caused 100% mortality of spider mites at concentrations equivalent to the field rates of 25 mg a.i./L (abamectin), 899 mg a.i./L (bifenazate) and 120 mg a.i./L (bifenthrin). Probit analysis showed that the dose responses of susceptible T. urticae to these three acaricides are significantly lower than field rates (Table 1).
Acaricide resistance levels in field populations. The toxicities of abamectin and bifenazate were assessed for *T. urticae* populations collected from 13 and 12 hopyards, respectively (Tables 2 and 3). In the bioassays with abamectin, the LC$_{50}$s ranged from 1.36 to 26.05 mg a.i./L and the resistant ratios (RRs) compared with the susceptible strain varied from 5.96 to 114.25 (Table 2). Low resistance levels (RR $<10$) were observed in 10.5% of the surveyed populations, 10.5% had high resistance (RR $>100$), and the majority of the surveyed populations (79%) exhibited moderate resistance (RR $=10–100$) to abamectin (Fig. 3A). The RR of the *T. urticae* population in the organic hopyard (Granger 2) compared with the susceptible population was 11.23, which is the 3rd lowest resistance among surveyed populations and the highest level of mortality (100%) at the field rate. Samples collected from the Granger 4 hopyard showed the lowest resistance ratio (RR $=5.96$) compared with the susceptible population. There were three 1st year (baby) hopyards (Prosser 3, 4, and 5) surveyed in 2013. The RRs of samples collected from these baby hopyards ranged from 21.80 to 114.25, exhibiting a moderate to high degree of resistance (Table 2). There were multiple collections from certain hopyards (Prosser 2, 3 and 4) during the course of summer 2013. Specifically, six collections were taken from the Prosser 2 hopyard starting from mid-June till just prior to harvest in late August during which abamectin was applied twice. The RR increased 6-fold from the middle of July to mid-August (Table 2). The RRs in samples collected from Prosser 3 and 4 increased 1.7-fold and 2.3-fold in four and five weeks, respectively. The highest resistance level to abamectin was recorded at the Prosser 4 (RR $=114.25$) (Table 2).

In the bioassays with bifenazate, the LC$_{50}$s ranged from 3.93 to 78.97 mg a.i./L and the RRs varied from 4.79 to 96.30 (Table 3). Populations exhibiting low resistance levels (RR $<10$) accounted for 37.5% of the populations surveyed, and 62.5% of the populations exhibited moderate resistance (RR $=10–100$) to bifenazate (Fig. 3B). The lowest RR to bifenazate, 4.79, was recorded from the samples collected from the organic hopyard (Granger 2). The RRs of samples collected from the 1st year hopyards showed low to moderate level of resistance (Table 2). The highest RR to bifenazate was observed in the sample collected from Granger 3 (RR $=96.30$) (Table 3). Due to the limited number of collected *T. urticae* individuals in four populations, only the discriminating dose of bifenazate was evaluated (Table 3).

Table 1. Baseline toxicity of acaricides in susceptible *T. urticae*.

| Acaricide | Field rate (mg a.i./L) | N | LC$_{50}$ (mg a.i./L) | 95% CI | Slope ± SEM | Bioassay method | X$_{df}$ | df |
|-----------|----------------------|---|----------------------|-------|-------------|-----------------|--------|----|
| Abamectin | 23                   | 4100 | 0.228 | 0.12–0.33 | 1.87 ± 0.07 | Leaf disc | 36.02 | 3  |
| Bifenazate| 899                  | 2195 | 0.820 | 0.79–0.85 | 5.69 ± 0.46 | Leaf disc | 0.12  | 1  |
| Bifenthrin| 120                  | 2300 | 17.970 | 8.42–44.60 | 1.73 ± 0.08 | Sticky tape | 73.55 | 3  |

Evaluation of target site mutations. The occurrence of 16 mutations in four target genes, *GluCl1* and *GluCl3* (target of abamectin; Fig. S1), *cytb* (target of bifenazate; Fig. S2), and *VGSC* (target of bifenthrin; Fig. S3), was examined in *T. urticae* field populations by direct sequencing of PCR products. By visual examination of sequencing chromatographs at the mutation sites, we could identify samples that contained wild-type, resistant, or both alleles. The combination of mutations in field *T. urticae* populations collected from PNW hopyards exhibited a unique pattern (Table 4). Only two mutations, G126S
### Table 2. Toxicity to abamectin of *T. urticae* populations collected in 2013. % Mortality stands for the % mortality at field rate of abamectin, which is 22.5 mg a.i./L. RR represents Resistance Ratio = LC50 of field population/ LC50 of susceptible population. These populations were reared on lima bean plants for 1 month in the lab prior to bioassay due to the limited spider mite number. Organic hopyard. Molecular data shown in Table 4. *No data available.

| Population | Date     | % Mortality | N   | LC50 (mg a.i./L) | 95% CI        | Slope ± SEM | RR  | X2 | df |
|------------|----------|-------------|-----|-----------------|---------------|-------------|-----|----|----|
| *Granger 1* | 16 Jul   | 80.0        | 180 | 8.24            | 6.22–10.73    | 1.84 ± 0.26 | 36.14 | 1.64 | 2  |
| *Granger 1* | 20 Aug   | 92.5        | 238 | 7.47            | 5.77–8.89     | 3.63 ± 0.60 | 32.76 | 3.79 | 4  |
| *Granger 2* | 16 Jul   | 100.0       | 180 | 2.56            | 1.82–3.36     | 2.20 ± 0.29 | 11.23 | 1.16 | 2  |
| *Granger 3* | 25 Jul   | 74.0        | 178 | 9.72            | 7.48–12.79    | 2.07 ± 0.33 | 42.63 | 1.24 | 2  |
| *Granger 4* | 25 Jul   | 92.5        | 240 | 1.36            | 0.15–3.91     | 0.97 ± 0.13 | 5.96  | 11.89 | 4  |
| *Granger 5* | 25 Jul   | 95.0        | 200 | 8.80            | 5.59–11.72    | 4.18 ± 0.66 | 38.60 | 3.27 | 3  |
| *Mabton 1*  | 15 Jul   | 90.0        | 199 | 4.24            | 1.82–3.36     | 2.20 ± 0.29 | 11.23 | 1.16 | 2  |
| *Moxee 1*   | 18 Jul   | 50.0        | 160 | 13.36           | 10.95–16.50   | 3.33 ± 0.63 | 58.60 | 0.59 | 3  |
| *Prosser 1* | 16 Jun   | 93.5        | 276 | 3.08            | 0.33–9.52     | 0.94 ± 0.12 | 13.51 | 14.59 | 4  |
| *Prosser 2* | 16 Jun   | 95.0        | 200 | 1.94            | 1.03–2.94     | 1.36 ± 0.21 | 8.51  | 1.94 | 3  |
| *Prosser 4* | 14 Jul   | 87.5        | 200 | 2.85            | 1.76–4.04     | 1.48 ± 0.21 | 12.50 | 1.29 | 3  |
| *Prosser 2* | 28 Jul   | 89.0        | 198 | 7.12            | 2.12–15.36    | 2.01 ± 0.25 | 31.23 | 9.15 | 3  |
| *Prosser 2* | 10 Aug   | 73.0        | 198 | 16.65           | 6.86–19.41    | 2.00 ± 0.27 | 51.10 | 4.13 | 3  |
| *Prosser 2* | 19 Aug   | 75.0        | 237 | –               | –             | 1.69 ± 0.23 | –     | 31.43 | 4  |
| *Prosser 2* | 22 Aug   | 70.0        | 219 | –               | –             | 3.34 ± 0.78 | –     | 9.40  | 3  |
| *Prosser 3* | 14 Jul   | 85.0        | 220 | 4.97            | 1.28–9.97     | 1.68 ± 0.21 | 21.80 | 7.47 | 3  |
| *Prosser 3* | 19 Aug   | 75.0        | 220 | 8.60            | 5.04–15.69    | 1.36 ± 0.18 | 37.72 | 3.62 | 4  |
| *Prosser 4* | 17 Jul   | 62.5        | 200 | 11.37           | 6.01–35.94    | 1.04 ± 0.26 | 49.87 | 1.03 | 3  |
| *Prosser 4* | 03 Sep   | 47.5        | 240 | 26.05           | 16.24–62.98   | 1.28 ± 0.26 | 114.25 | 0.33 | 3  |
| *Prosser 4* | 08 Sep   | 54.0        | 139 | –               | –             | 1.54 ± 0.44 | –     | 5.39  | 2  |
| *Prosser 5* | 24 Jul   | 92.5        | 218 | 8.47            | 5.98–10.50    | 2.98 ± 0.69 | 37.15 | 1.61 | 3  |

### Table 3. Toxicity to bifenazate of *T. urticae* populations collected in 2013. % Mortality stands for the % mortality at ¼ the field rate of bifenazate, which is 224 mg a.i./L. RR represents Resistance Ratio = LC50 of field population/ LC50 of susceptible population. Organic hopyard. Molecular data shown in Table 4. *No data available.

| Population | Date     | % Mortality | N   | LC50 (mg a.i./L) | 95% CI        | Slope ± SEM | RR  | X2 | df |
|------------|----------|-------------|-----|-----------------|---------------|-------------|-----|----|----|
| Granger 1  | 30 Aug   | 82          | 157 | 47.86           | 11.39–138.08  | 1.72 ± 0.22 | 58.37 | 3.07 | 2  |
| *Granger 2* | 16 Jul   | 100         | 120 | 3.93            | 0.34–7.11     | 1.89 ± 0.64 | 4.79  | 0.02 | 1  |
| *Granger 3* | 20 Aug   | 76          | 197 | 78.97           | 55.99–107.50  | 1.71 ± 0.19 | 96.30 | 2.49 | 3  |
| *Granger 5* | 25 Jul   | 93          | 160 | 4.88            | 0.89–10.76    | 0.99 ± 0.21 | 5.95  | 1.87 | 2  |
| *Mabton 1* | 27 Jun   | 96          | 60  | –               | –             | –           | –    | –   | –  |
| *Mabton 2* | 27 Jun   | 96          | 60  | –               | –             | –           | –    | –   | –  |
| *Moxee 1*  | 18 Jul   | 160         | 90  | 18.88           | 9.71–30.14    | 1.38 ± 0.22 | 23.02 | 1.95 | 2  |
| *Prosser 1* | 14 Jul   | 100         | 60  | –               | –             | –           | –    | –   | –  |
| *Prosser 2* | 28 Jul   | 85          | 160 | –               | –             | 1.44 ± 0.21 | –     | 3.23 | 2  |
| *Prosser 3* | 29 Jul   | 88          | 160 | 25.49           | 3.87–66.98    | 1.50 ± 0.21 | 31.09 | 2.51 | 2  |
| *Prosser 4* | 08 Sep   | 90          | 160 | 6.87            | 1.36–13.66    | 1.58 ± 0.42 | 8.38  | 0.72 | 2  |
| *Prosser 5* | 24 Jul   | 95          | 157 | 9.31            | 3.91–15.69    | 1.29 ± 0.29 | 31.35 | 0.95 | 2  |
and F1538I, in cytb and domain III of VGSC, respectively, were identified (Table 4). There were no mutations observed in GluCl1, GluCl3, and other region of cytb and VGSC.

No mutations observed in Glutamate-gated chloride channel genes. Inhibitory Glutamate-gated chloride channels (GluCls), members of the cys-loop ligand-gated ion channel (cysLGIC) superfamily, are extrajunctional or postsynaptic receptors found in muscle or neural ganglion of most protostome phyla including Chelicerates such as T. urticae. The genome of T. urticae contains six orthologous GluCl genes. Previous studies revealed that two mutations in two different GluCl channel subunits, GluCl1 and GluCl3, were related to abamectin resistance in T. urticae. Thus we designed primers to sequence the fragments containing these two mutations (Fig. S1) from susceptible and all hop field populations of T. urticae. Surprisingly, there were no mutations identified from the samples tested (Table 4), suggesting target site insensitivity-mediated resistance is not the mechanism leading to the abamectin resistance that we observed in T. urticae field populations.

Identification of mutations in the cytb gene. Recent studies suggested that bifenazate resistance was closely correlated with mutation(s) in the mitochondrial cytb. A combination of at least two cd1 helix mutations in the Qo pocket (G126S and I136T or G126S and S141F) and one mutation in the ef helix of Qo pocket (P262T) were linked with a high level of bifenazate resistance in T. urticae. We sequenced an 828 bp fragment of the T. urticae cytb gene, which included the G126, I136, S141, D161 and P262 sites (Fig. S2) that have been demonstrated to confer bifenazate resistance in T. urticae. One amino acid substitution, G126S, was detected in T. urticae field populations. 35% of field samples contained only the resistant allele, 20% contained both alleles (G/S) and 15% only the susceptible allele (G) (Table 4; Fig. 4A). Since the G126S mutation alone only causes low to moderate bifenazate resistance, this result is consistent with the bifenazate resistance phenotype observed (Table 3).

Identification of mutations in the voltage-gated sodium channel gene. The voltage-gated sodium channel (VGSC) is an integral transmembrane protein that is responsible for the rapidly rising phase of action potentials on the neuronal membranes. Due to its essential role in electrical signaling, VGSC is the target of several neurotoxins, including pyrethroids and DDT. Many amino acid substitutions associated with pyrethroid resistance in arthropods are located in transmembrane segments 4–6 of domain II (IIS4-IIS6) including M918 (super kdr), L925, T929, L932, V1010, L1014 (kdr), and L1024. One mutation within the intracellular inter linker connecting domains II and III (A1215D) and one mutation in domain III (F1538I) were detected in a highly bifenthrin resistant T. urticae strain from Greece. Thus we amplified three fragments of the VGSC from the domain II, II-III inter linker, and domain III regions (Fig. S3). We identified only one amino acid substitution, F1538I. It was observed in 16 out of 24 field samples tested (66.7%), 12 of which contained both alleles (F/I) and 4 of which were only contained the isoleucine substitution (I) (Table 4; Fig. 4B).

Cytochrome P450-mediated metabolic detoxification. Besides target site insensitivity, cytochrome P450-mediated detoxification had been shown to be one of the most important mechanisms in acaricide resistance of T. urticae. The genome of T. urticae contains 86 P450 genes. We examined the relative expression of three P450s, CYP385C4, CYP389A1, and CYP392D8, belonging to the CYP3, CYP4, and CYP2 clans, respectively. We chose these three P450s because they have been shown to exhibit more than two-fold up regulation after switching host plants and their expression patterns have been linked to acaricide resistance in T. urticae. The expressions of these three P450s in five field populations
from five major locations were compared with their expressions in the susceptible population. As shown in Fig. 5, CYP385C4 had significantly higher expression in all five field populations. However, this increase in expression was not large (less than two-fold). CYP389A1 only showed significantly higher

| Population | Date     | GluCl1 (G323D) | GluCl3 (G326E) | Cytb | VGSC II | VGSC II–III | VGSC III (F1538I) |
|------------|----------|----------------|----------------|------|---------|-------------|-------------------|
| "Susceptible" | 10 June | G              | G              | No   | No      | A           | F                 |
| Grandview  | 23 Sep   | G              | G              | –    | No      | A           | I                 |
| "Granger 1" | 16 Jul   | G              | G              | No   | No      | A           | F/I               |
| "Granger 2" | 16 Jul   | G              | G              | No   | No      | A           | F                |
| "Granger 3" | 25 Jul   | G              | G              | No   | No      | A           | F                |
| "Granger 3" | 20 Aug   | G              | G              | –    | No      | A           | F/I               |
| "Granger 4" | 25 Jul   | G              | G              | G126G/S | No      | A           | F/I               |
| "Granger 5" | 25 Jul   | G              | G              | No   | No      | A           | F/I               |
| Mabton 1   | 15 Jul   | G              | G              | No   | No      | A           | F/I               |
| "Mabton 1" | 16 Jul   | G              | G              | No   | No      | A           | F/I               |
| "Mabton 2" | 15 Jul   | G              | –              | No   | –      | –           | –                 |
| Mabton 3   | 02 Jul   | G              | G              | No   | No      | A           | F/I               |
| "Moxee 1"  | 18 Jul   | G              | G              | G126S | No      | A           | F                |
| "Moxee 2"  | 29 Aug   | G              | G              | G126S | No      | A           | F/I               |
| "Prosser 1"| 14 Jul   | G              | G              | G126G/S | No      | A           | F/I               |
| "Prosser 2"| 28 Jul   | G              | G              | No   | No      | A           | F                |
| "Prosser 2"| 14 Jul   | G              | G              | No   | No      | A           | F                |
| "Prosser 3"| 14 Jul   | G              | G              | No   | No      | A           | F                |
| "Prosser 3"| 19 Aug   | G              | G              | –    | No      | A           | F                |
| "Prosser 4"| 17 Jul   | G              | G              | –    | No      | –           | I                 |
| "Prosser 4"| 03 Sep   | G              | G              | –    | No      | A           | F/I               |
| "Prosser 4"| 08 Sep   | G              | G              | G126G/S | No      | A           | I                 |
| "Prosser 5"| 24 Jul   | G              | G              | G126G/S | No      | A           | F/I               |
| "Prosser 5"| 21 Aug   | G              | G              | G126S | No      | A           | I                 |

Table 4. Target site mutations in the susceptible and field Tetranychus urticae populations for GluCl1, GluCl3, Cytb, and VGSC. Samples were collected for both DNA and RNA extraction. Organic field. Spider mite samples were reared on lima bean plants after collection and sampled for DNA extraction one month later. Bioassay data shown in Table 1–3. No: stands for no mutation identified. -No data available.

Figure 4. Pie charts showing proportions of resistance associated allele for G126S on cyt b (A) and F1538I on VGSC (B). The colors green, blue, and orange stand for the susceptible allele, double alleles, and resistant allele, respectively.
expression in the Prosser 2 population. The expression of CYP392D8 was strikingly higher in all five field populations, exhibiting levels 5 to 40-fold higher than the susceptible strain. It indicates that CYP392D8 may play an important role in acaricide resistance of T. urticae populations in hopyards.

Discussion

Due to a very short residual effectiveness, abamectin has become the predominant acaricide applied to control T. urticae outbreaks in August as the hops near harvest. Annually, approximately 98% of the hop acreage in Washington is treated with abamectin at least once and 80% is treated at least three times. The widespread use of abamectin on hops raises the distinct possibility of control failure as a result of resistance. From sampling the same hop yard over multiple time points in the same season, we found increasing levels of abamectin resistance, suggesting selection pressure from abamectin applications was driving increasing resistance. For instance, multiple collections in the Prosser 2 hopyard showed that the RR to abamectin increased 6-fold from the middle of July to mid-August (Table 2). The highest LC50s to abamectin were recorded in the Moxee 1 and Prosser 4 T. urticae populations (Table 2). However, Moxee 1 had only two acaricide applications during 20138. The possibility for the reported highest abamectin resistance ratio in Moxee 1 could be the entire application history of abamectin in this field that remains unknown. There may have been high abamectin selective pressure over multiple overwintering populations in this field prior to 2013. The high level of abamectin resistance in the Prosser 4 population was also unexpected because this sample was collected from the 1st year baby hopyard8. Prior to planting hops, the crop planted in Prosser 4 was Concord grapes. A recent study reported infestation of T. urticae in grape yards and high abamectin resistance in 45% T. urticae populations from these grape yards in Brazil40. Nevertheless, our previous investigation suggested that T. urticae is not a pest of grape yards in Washington State41 and thus we do not expect the fields have been extensively sprayed with abamectin.

However, Bradenburg and Kennedy42 reported that wind dispersal was a key factor causing the infestations of T. urticae from corn fields to surrounding crops. Thus, the resistant T. urticae populations we detected may have been transported from adjacent crops to the 1st year hopyard through wind dispersal.

GluCls together with gamma-aminobutyric acid (GABA)-gated channels and histamine-gated chloride channels (HisCls) are known targets of the macrocyclic lactones, the avermectins (including abamectin) and ivermectins26,33,43,44. The point mutation G323D in GluCl1 was tightly linked to a moderate abamectin resistance (17.9-fold) in the AbaR strain26. Two point mutations, G323D and G326E, in GluCl3, respectively, were identified in a > 2,000-fold abamectin resistant strain21. However, there was no mutation on GluCl subunits detected in any hop samples (Table 4), suggesting target site insensitivity is not likely the mechanism involved in resistance to abamectin in T. urticae field populations. Our results

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**Figure 5.** Relative expression of CYP385C4, CYP389A1 and CYP392D8 in field T. urticae populations compared with that of the susceptible strain. The mRNA levels were quantified by qRT-PCR and normalized with reference genes Actin and RP49. The data shown are mean ± SEM (n = 3). Statistical significance of the gene expression between two samples was calculated using Student’s t test (two-tailed distribution). *p-value < 0.05, **p-value < 0.01.
are comparable with a study by Khajehali et al.⁴⁵ which found no GluCl mutations in 15 *T. urticae* strains collected from rose greenhouses in the Netherlands, although 10 of those strains displayed abamectin resistance. Many recent studies also suggested that abamectin target site mutations are not especially common in *T. urticae* populations worldwide. For example, the G326E was detected in only seven out of 51 *T. urticae* populations sampled from 27 countries and five continents⁴⁶. The G323D mutation was only found in two Greek samples in the same survey⁴⁶. In another study with 25 Korean *T. urticae* populations, only one field-collected *T. urticae* sample contains G323D mutation⁴⁷.

Previous synergism tests and transcriptomic data indicated that additional mechanisms such as enhanced metabolic detoxification by cytochrome P450s may be implicated in the abamectin resistance phenotype²⁷,⁴⁸,⁴⁹. A genome microarray analysis revealed several cytochrome P450 genes were up-regulated in an abamectin resistant strain⁵⁰. Further evidence confirmed the function of one of these P450s, CYP392A16, in metabolizing abamectin⁵⁰. Unfortunately, this study was published after the completion of our study, and we did not have enough sample material remaining to test for expression of this gene. However, of the three P450s we did examine in our study, one Clan 2 P450, CYP392D8, showed constitutive over-expression in all five field collected samples compared to the susceptible population, indicating its potential function in abamectin resistance (Fig. 5).

Bifenazate is a hydrazine carbazate acaricide that was discovered in 1990 by Uniroyal Chemical and first registered in the state of Washington in 2002⁵¹. Because of the quick knockdown and long residual effects on many economically important phytophagous mite species and low toxicity on predatory mites and beneficial insects, bifenazate is widely used as a selective acaricide to control *T. urticae* in hopyards. Our bioassay data demonstrated that the majority of field *T. urticae* populations (62.5%) in hopyards exhibit moderate levels of resistance to bifenazate (Fig. 3B). Our target site mutation screening revealed that a mutation G126S on *cytb* gene occurs in 35% of *T. urticae* populations (Fig. 4A). It should be noted that G126S (GGA to AGA) is the same mutation as described in previous studies²²,²⁷,²⁸. G126S is the most common substitution on *cytb* gene of *T. urticae* that was identified in several bifenazate resistant populations²²,²⁷,²⁸,⁴⁵,⁴⁶. Previous studies showed that mutations on the G137 site in hopyards are comparable with a study by Khajehali & et al.⁴⁵ which found no GluCl mutations in 15 *T. urticae* strains collected from rose greenhouses in the Netherlands, although 10 of those strains displayed abamectin resistance. Many recent studies also suggested that abamectin target site mutations are not especially common in *T. urticae* populations worldwide. For example, the G326E was detected in only seven out of 51 *T. urticae* populations sampled from 27 countries and five continents⁴⁶. The G323D mutation was only found in two Greek samples in the same survey⁴⁶. In another study with 25 Korean *T. urticae* populations, only one field-collected *T. urticae* sample contains G323D mutation⁴⁷.

In summary, *T. urticae* populations in hopyards exhibit a low to moderate level of acaricide resistance. The mechanisms of acaricide resistance in *T. urticae* are likely mediated by a number of different pathways: not only target site insensitivities but also enhanced metabolic detoxification. It is a common phenomenon that multiple genes or mechanisms confer resistance simultaneously to a certain pesticide²⁸,²⁰,⁶⁰–⁶⁴. Therefore, we plan a genome-wide investigation to identify a more complete set of candidate resistance genes from *T. urticae* populations of hopyards. Our data also suggests that acaricide spray history, neighboring plants, and time of the season are important factors in correctly diagnosing acaricide resistance in *T. urticae*. Developing a baseline effective dose for commonly used acaricides and screening local *T. urticae* populations with resistance-associated molecular markers would be a proactive approach toward *T. urticae* resistance management. Our study reveals a unique phenotypic and genotypic pattern underpinning the chemical adaptation of *T. urticae* in hop fields which will be of assistance in developing diagnostic tools for integrated *T. urticae* management.

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Methods

Mite samples. The susceptible acaricide naïve *T. urticae* strain (SS) was originally collected from weeds in Montana in 1995 and reared under laboratory conditions without exposure to any pesticides. This population was reared on 2-week-old lima bean plants (*Phaseolus lunatus* L.) at 28 ± 2 °C, 70 ± 5 RH and a photoperiod of 16:8 (L:D) h in an isolated walk-in growth chamber at the Irrigated Agricultural Research and Extension Center (IAREC) in Prosser, WA. Bean plants were grown from seeds (Buckeye Seed Supply, Canton, OH) with medium grade vermiculite (Therm-o-rock West Inc.) soaking in water in the greenhouse. New, healthy, lima bean plants were provided for *T. urticae* and plants were replaced every seven days. To prevent mite migration, the colonies were maintained in 27-L plastic tubs filled with soapy water Huffaker moats.

Thirty-one field *T. urticae* populations were collected from commercial hopyards located within the Yakima Valley of Washington State from June to September in 2013. There were five major locations: one sample was collected in Grandview, WA (46°15′13″N 119°54′36″W), eight in Granger, WA (46°20′40″N 120°11′29″W), four in Matbon, WA (46°12′42″N 119°59′47″W), two in Moxee, WA (46°33′23″N 120°23′14″W), and 16 in Prosser, WA (46°12′25″N 119°45′56″W) (Fig. 2; Tables 2–4). The samples collected from the same location at different times were treated as different populations. Mite-infested hop leaves were stored in a plastic bag and transported to the lab in a cooling box within a few hours of collection. Spider mites were identified under a dissection scope according to morphological characteristics. Approximately 50–100 adults were stored in 95% ethanol for genomic DNA extraction. About 300 adult *T. urticae* from each of five major locations listed in Table 4 were also stored in RNAlater® (Sigma-Aldrich, Saint Louis, MO) for RNA extraction. Remaining mites were used for *T. urticae* extraction. About 300 adult mites were used for bioassay analysis of LC50 values was based on non-overlapping 95% CI. Resistance ratios (RRs) were calculated because pyrethroids are shown to have repellent effects on mites. In this method, ten female adult spider mites were placed dorsal side down on a strip of double-sided sticky Scotch® tape (3 cm × 1.2 cm) stuck on a glass slide (7.5 cm × 2.5 cm). The commercially formulated bifenthrin was Bifenture® EC, a pyrethroid provided by United Phosphorus (25.1% a.i. Bifenthrin). These bifenthrin solutions were serially diluted in distilled water for 4–7 concentrations ranging from 0.1–67 mg a.i/L and 899 mg a.i/L, respectively. The field rate solutions were prepared in the lab using commercial formulated acaricides and distilled water. These solutions were serially diluted in distilled water for 4–7 concentrations ranging from 0.1–67 mg a.i./L and 0.44–889 mg a.i./L for Epi-mek® and Acramite®, respectively.

Bioassays and data analysis. Leaf disc bioassays were used to estimate the LC50 (lethal concentration required to kill 50% of the individuals in a population) of abamectin and bifenazate for lab susceptible and field spider mite populations. The method followed that of Knight *et al.*. Briefly, ten female adult spider mites were placed on the back of a bean leaf disc (2 cm diameter) with a fine brush. Two leaf discs were arranged on water-saturated cotton (4 cm × 4 cm) in a single petri dish (9 cm diameter, 1.5 cm height; Alkali Scientific, Pompano Beach, FL). The water-saturated cotton was pushed up against the perimeter of the leaf disc to prevent mites from walking off the disc. Two commercially formulated acaricides for leaf disc bioassay are Epi-mek® 0.15 EC (2% a.i. Abametacin, Syngenta Crop Protection) and Acramite® 50WS (50% a.i. Bifenazate, Chemtura Agro Solutions). The recommended field concentrations for these two acaricides are 23 mg a.i./L and 899 mg a.i./L, respectively. The field rate solutions were prepared in the lab using commercial formulated acaricides and distilled water. These solutions were serially diluted in distilled water for 4–7 concentrations ranging from 0.1–67 mg a.i./L and 0.44–889 mg a.i./L for Epi-mek® and Acramite®, respectively.

The sticky tape method was used to estimate the LC50 to bifenthrin for the lab susceptible strain because pyrethroids are shown to have repellent effects on mites. In this method, ten female adult spider mites were placed dorsal side down on a strip of double-sided sticky Scotch® tape (3 cm × 1.2 cm) stuck on a glass slide (7.5 cm × 2.5 cm). The commercially formulated bifenthrin was Bifenture® EC, a pyrethroid provided by United Phosphorus (25.1% a.i. Bifenthrin). These bifenthrin solutions were serially diluted in distilled water for 4–7 concentrations ranging from 6–120 mg a.i./L.

Leaf discs or glass slides were treated topically with 2 ml of acaricide solutions with a Potter spray tower (Burkard Manufacturing, Richmansworth, Herts, UK). The tower was calibrated to deliver 1.1 kg/cm² which allowed 2.0 ± 0.1 mg/cm² spray fluid. Each bioassay consisted of 4–7 acaricide concentrations with 4–6 replicates for each concentration. The spider mites exposed to distilled water in the Potter spray tower were used as the non-treated control. The treated leaf discs or glass slides were maintained at 25 ± 2 °C and a photoperiod of 16:8 (L:D) h after the initiation of the bioassay. Mortality was evaluated after 24 h. Mortality was assessed by gently touching each individual spider mite with a fine camel hair paint brush under a dissecting stereomicroscope. The individuals with no response were counted as dead. The few moribund individuals that were not able to maintain balance and show uncoordinated twitching were also recorded as dead. The slope, intercept, and LC50 (corrected against the untreated control) were evaluated with Abbott's formula calculated by log-dose probit analysis (POLO Probit 2014). The statistical analysis of LC50 values was based on non-overlapping 95% CI. Resistance ratios (RRs) were calculated through dividing LC50 values of field samples by the LC50 value of the lab susceptible population.

Resistance-associated amino acid substitution screening. Genomic DNA was extracted using a DNeasy Blood & Tissue kit (QIAGEN) from 10 adult mites for each population. The DNA was stored at −20 °C till use. The genomic DNA was used as a template for PCR performed in a Peltier-Effect thermal cycler (MJ Research, Inc., Canada). Primers for PCR amplification of regions with resistance-associated point mutations are listed in Table S1. PCR was performed using Phusion High-Fidelity DNA Polymerase (Thermo Scientific, Pittsburgh, PA) under the following cycling parameters: 95 °C for 3 min 50 s, 35 cycles of 94 °C for 35 s, 55 °C for 35 s, and 72 °C for 3 min, with final extension for 10 min at 72 °C. PCR products were purified using DNA Clean & Concentrator (Zymo Research, Irvine, CA) following the
manufacturer’s protocol. The purified DNA from each individual was directly sequenced using primers described above (Table S1) for PCR amplification. Each individual PCR product was sequenced using ABI BigDye Terminator Version 3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA) and ABI 3730 at the Center for Reproductive Biology Molecular Biology and Genomics Core facility at Washington State University. The obtained sequences were analyzed with BioEdit 7.0.1 software (Ibis Biosciences, Carlsbad, CA). The occurrence of mutations was evaluated according to the inspection of sequencing chromatographs, as containing one or both alleles. Each sample was sequenced three times with independently prepared genomic DNAs.

RNA extraction, cDNA synthesis and qRT-PCR. Total RNA from 100 spider mites per population was extracted using TRIZOL reagent (Invitrogen) following manufacturer’s protocol. The quality of total RNA was checked by gel electrophoresis and spectrometry analyses. The total RNA was treated with DNase I (Ambion Inc., Austin, TX) to remove contaminating DNA. DNase I treated total RNA was used as a template for cDNA syntheses by M-MLV reverse transcriptase (Promega, Madison, WI). qRT-PCR was performed using a CFX96™ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA). Each qRT-PCR reaction (10 μl final volume) contained 5 μl iQ™ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA), 1.0 μl of cDNA, 3.6 μl ddH₂O, and 0.4 μl forward and reverse gene specific primers (Table S4, stock 10 μM). An initial incubation of 95°C for 3 min, followed by 40 cycles of 95°C for 10 s, 55°C for 60 s was used. The qRT-PCR for each sample was conducted with two technique replicates and three biological replicates. The no-template control and internal controls were included in each plate. Actin and rp49 were used as reference genes for internal controls. Relative expression levels for target genes, in relation to two reference genes, actin and rp49 were calculated by the 2^(-ΔΔCt) method. Both the PCR efficiency and R² (correlation coefficient) value were taken into consideration in estimating relative quantities. PCR efficiency between 95% and 105% and R² value > 0.99 for each gene were considered as qualified for further analysis.

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**Acknowledgements**
We are grateful to Tora Brooks, Bianca Mendoza, and Christina Nguyen (Washington State University) for their technical assistance. Special thanks go to Timothy W. Moural and Mark Lavine (Washington State University) for their help on English editing. This work was supported by a USDA/NIFA grant (D.B.W and L.C.L), Hop Research Council, WSU Proposal Development Stimulus Program, and WSU Emerging Research Issues Program.

**Author Contributions**
D.B.W., L.C.L., T.G.P. and F.Z. designed the experiments. T.G.P. collected all field spider mite samples and performed bioassays. F.Z., J.B. and M.M. performed the molecular experiments and analyzed the data. F.Z., T.G.P. and J.B. prepared all figures and tables. F.Z., T.G.P., J.B., M.M., L.C.L. and D.B.W. contributed reagents/materials/analysis tools. F.Z. wrote the manuscript. T.G.P., D.B.W. and L.C.L. revised the manuscript. All authors reviewed the manuscript.

**Additional Information**
Supplementary information accompanies this paper at http://www.nature.com/srep

**Competing financial interests:** The authors declare no competing financial interests.

**How to cite this article:** Piraneo, T. G. *et al.* Molecular mechanisms of *Tetranychus urticae* chemical adaptation in hop fields. *Sci. Rep.* **5**, 17090; doi: 10.1038/srep17090 (2015).

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