Genetic basis of acaricide resistance

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

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General discussion

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Pesticide resistance continues to be a major concern for crop protection (McCaffery and Nauen 2006; Sparks and Nauen 2015; Van Leeuwen et al. 2020). Increasingly more studies and economic investments have been devoted to understanding the evolutionary origins and mechanisms of pesticide resistance as well as developing resistance management (RM) strategies and efficient pesticides (Clark and Yamaguchi 2001; Heckel 2012; Hawkins et al. 2019; Sparks 2013; Sparks and Nauen 2015; Van Leeuwen et al. 2010; Van Leeuwen and Dermauw 2016). The spider mite *Tetranychus urticae* is considered as one of the most resistant arthropod pests based on the number of compounds to which resistance has been reported (Whalon et al. 2008; Van Leeuwen et al. 2010). Pesticide resistance mechanisms can be categorized in two groups: 1) toxicodynamic changes, such as a reduction in the sensitivity or availability of the target-site of the pesticide due to point mutation(s), gene knockout or gene amplification; and 2) toxicokinetic changes that reduce the amount of pesticide that reaches the target-site through changes in exposure, penetration, transportation, metabolism or excretion (Feyereisen et al. 2015; Li et al. 2007; Van Leeuwen and Dermauw 2016). In this thesis, I explored the risk of resistance to novel acaricides and identified new toxicodynamic and toxicokinetic changes that confer resistance in *T. urticae*. Here, I discuss my results in light of existing knowledge on pesticide resistance and RM strategies.

Novel mutations confer toxicodynamic resistance to Q_0 inhibitor acaricides

Genetic variation conferring target-site resistance is either present in pest populations due to natural polymorphism or arises as *de novo* mutations under pesticide pressure (ffrench-Constant 2017; ffrench-Constant 2013; Hawkins et al. 2019). A high number of mutations have been associated with acaricide resistance in *T. urticae* [reviewed by Van Leeuwen and Dermauw (2016)], including the mitochondrial cytochrome b (*cytb*) mutations G126S, I136T, S141T, A133T, D161G, P262T, I256V and N321S, which alone or in different combinations confer strong to medium levels of resistance to the Q_0 inhibitors bifenazate and acequinocyl (Van Leeuwen and Dermauw 2016; Kim et al. 2019). In chapter 2 of this thesis, I identified two novel mutations in the highly conserved region of the Q_0 site of cytb of *T. urticae* strains that exhibited resistance to Q_0 inhibitors, the single mutation G132A and the combined mutations G126S and A133T, previously reported in *Panonychus citri*. Using reciprocal crosses and

backcrosses, I provided several lines of evidence for the causal role of the novel mutations in resistance. Resistance mutations have been suggested to be employed as molecular markers to predict resistance in RM. The strength and reliability of such molecular markers have recently been discussed by Van Leeuwen et al. (2020) (Van Leeuwen et al. 2020). Strength of the resistance phenotype attributable to the target-site mutation is a major factor affecting the reliability of a target-site mutation as a molecular marker to predict resistance in RM. In *T. urticae*, it is feasible to determine the phenotypic strength of mitochondrial mutations by reciprocal crosses that identifies the level of maternal inheritance of resistance and repeated back-crossing of resistant females to susceptible males that excludes the possible influence of nuclear genome in resistance(Riga et al. 2017; Bajda et al. 2017). In addition, the reliability of a target-site mutation as a molecular marker in RM depends on the repertoire of the different resistance mechanisms that pest populations across the globe have evolved. In *T. urticae*, the worldwide frequency of a whole panel of different target-site mutations has been investigated, revealing that identical mutations are often present across continents which make them reliable candidates as molecular markers (Van Leeuwen et al. 2020). In this study, reciprocal crosses and repeated backcrossing indicated that G132A is completely responsible for resistance to bifenazate and the dual G126S+A133T substitutions are largely linked to resistance to acequinocyl. It therefore seems that the two novel mutations can be used as reliable molecular marker to predict resistance to Q_0 inhibitor acaricides in RM of *T. urticae*.

G132A, a strong case of convergent evolution across kingdoms

Convergent evolution is the process where two reproductively isolated populations/lineages evolve similar traits independently. This process can be the result of adaptation to similar environments or ecological niches. The extent of convergent molecular evolution is still largely unknown. Convergent evolution across mite and insect species has already been observed (Nyoni et al. 2011; Eleftherianos et al. 2008; Rinkevich et al. 2013; Douris et al. 2016). For example, the M918T mutation in the sodium channel gene, which is associated with strong resistance to pyrethroid insecticides in the peach-potato aphid (*Myzus persicae*), has also been reported from pyrethroid resistance in the tomato red spider mite (*Tetranychus evansi*) (Nyoni et al. 2011; Eleftherianos et al. 2008; Rinkevich et al. 2013). Another example is the BPU resistance-conferring mutation I1042M in the chitin synthase 1 (*CHS1*) gene of *Plutella xylostella* which is at same position as the I1017F mutation which confers resistance to etoxazole, clofentezine and hexythiazox in spider mites (Demaeght et al.

2014). In addition, it has been reported that both *T. urticae* and its phytoseiid predator *Kampimodromus aberrans* have evolved resistance to acetylcholine esterase (AchE) inhibitors by the single point mutation G119S (Cassanelli et al. 2015). Here, this has been beneficial for integrated *T. urticae* management programs using both resistant predatory mites and AchE inhibitors (Cassanelli et al. 2015).

In chapter 2 of my thesis, I uncovered an additional strong case of convergent evolution across the animal and fungal kingdoms. The novel mutation G132A which confers resistance to Q_0 inhibitor acaricides in *T. urticae* is equivalent to the G143A mutation in pathogenic fungi where it confers resistance to Q_0 inhibitor strobilurin fungicides (Lümmen 2007; Fernández-Ortuño et al. 2008; Fisher and Meunier 2008; Gisi et al. 2002). The observed convergent evolution confirmed that the both groups of acaricides and fungicides have the same target site.

Pleiotropic effects associated with resistance mutations

Pleiotropy is the phenomenon whereby one gene controls multiple phenotypic traits (Paaby and Rockman 2013). Fisher's geometrical model of adaptation predicts that resistance by *de novo* target-site mutations can be costly for species in a pesticide-free environment (Fisher 1999).

In chapter 2, I observed several fitness costs associated with the G132A mutation in the highly conserved region of the Q_0 site of *cytb* of *T. urticae*. Resistant mites showed a lower net reproductive rate (R0), intrinsic rate of increase (rm) and finite rate of increase (LM), a higher doubling time (DT) and a more male-biased sex ratio. There are a number of studies reporting fitness costs linked to point mutations in target genes of pesticide-resistant arthropods (Kliot and Ghanim 2012; Bajda et al. 2018). However, the majority of these studies have a common weakness in their experimental design in that resistant and susceptible populations have different genetic backgrounds (Kliot and Ghanim 2012; Ffrench-Constant 2017). These genetic differences are not necessarily coupled with the target-site mutation and corresponding resistance phenotype, but may result in different fitness (Raymond et al. 2011; Anopheles gambiae 1000 Genomes Consortium 2017; Varzandeh et al. 1954). For mutations encoded by mitochondrial genomes, it is possible to establish near-isogenic resistant and susceptible lines with a similar nuclear genome by backcrossing resistant females to susceptible males for several generations. Comparison of the near-isogenic resistant and susceptible lines allow for maximizing the reliability of linking changes in fitness

components with the target-site mutation under study (Bajda et al. 2017; Riga et al. 2017; Bajda et al. 2018; Brito et al. 2013). In chapter 2 of this thesis, I followed the same approach to explore fitness consequences of the G132A mutation in resistant mites.

As for RM, understanding the fitness consequences associated with the resistance can be useful to estimate the establishment, spread and persistence of resistance. In light of our findings, it seems that in an acaricide-free environment the resistant genotypes with G132A mutation might be less competitive and grow slower than susceptible genotypes. In addition, the more male-biased sex ratio in resistant populations could further reduce the frequency of transmission of the mutation since the mutation is encoded in the mitochondrial genome. Together, it appears that management of the G132A resistance might be easier than that of mutations without fitness costs, such as G126S+S141F and P262T (Bajda et al. 2018).

Risk of resistance to the novel complex II inhibitor pyflubumide

A RM strategy for *T. urticae* is development of novel acaricides with new modes of action which limits cross-resistance to other commercially available compounds (Nauen et al. 2012; Van Leeuwen et al. 2015). Pyflubumide is a recently developed acaricide with excellent activity against phytophagous mites of the genera *Tetranychus* and *Panonychus* (Nakano et al. 2015). Pyflubumide, together with cyenopyrafen and cyflumetofen, are the first commercially developed acaricides that act as complex II inhibitors in the mitochondrial electron transport chain (Van Leeuwen et al. 2015; Furuya et al. 2017). In chapter 3, I uncovered decreased susceptibility to pyflubumide in a cyenopyrafen-selected Japanese strain, JPR (Sugimoto and Osakabe 2014; Khalighi et al. 2014). I further selected for pyflubumide resistance in JPR through two different laboratory selection regimes, and obtained two highly resistant strains, JPR-R1 and JPR-R2. Pyflubumide resistance evolved very quickly under this selection regime, which indicates a high risk of development of resistance to pyflubumide in agricultural areas that have been exposed to cyenopyrafen; other complex II inhibitors probably confer a similar risk.

Molecular mechanisms of resistance to pyflubumide

Structurally, pyflubumide is similar to complex II inhibitor carboxamide fungicides (Furuya et al. 2017). Resistance to this group of fungicides has been reported to be conferred by point mutations in the complex II subunits of many fungal plant pathogens (Avenot and Michailides 2010; Sierotzki and Scalliet 2013; Oliver 2014). I did not find any target-site resistance mutation candidates in the pyflubumide-resistant strains by sequencing all complex II subunits which is in line with a previous study that did not find target-site resistance in the cyenopyrafen-resistant strain JPR (Khalighi et al. 2016). However, a recent study (Sugimoto et al. 2020) reported target-site mutations in two subunits of mitochondrial complex II that confer resistance to complex II inhibitors, including pyflubumide and cyenopyrafen. Their microsatellite linkage map which was followed by comparison of partial amino acid sequences of subunits gathered evidence that in subunit succinate dehydrogenase b (SdhB), the I260T mutation is linked to cyflumetofen resistance, whereas the I260V mutation causes pyflubumide resistance. In addition, the S56L mutation in subunit succinate dehydrogenase C (SdhC) was reported to confer resistance to cyenopyrafen (Sugimoto et al. 2020).

Based on transcriptomic comparisons, Sugimoto et al. (2020) also indicated that three cytochrome P450 (CYP) genes and six carboxyl/choline esterase (CCE) genes are involved in resistance to pyflubumide (Sugimoto et al. 2020). Our synergism experiments of chapter 3 strongly suggested that CYPs are involved in pyflubumide detoxification. Follow-up genetic and molecular investigations were performed to test this hypothesis and are discussed below.

Transcriptomic approaches have increasingly been used to determine the genetic basis of resistance in pest species such as *T. urticae* (Van Leeuwen and Dermauw 2016). In chapters 3 and 4 of my thesis, transcriptome studies were performed to identify the molecular mechanism of resistance to pyflubumide in *T. urticae*.

Gene-expression microarrays and RNA sequencing (RNAseq) are two powerful and widespread high-throughput transcriptomic technologies which allow to measure genome-wide gene expression on large numbers of individuals in populations simultaneously (Alvarez et al. 2015). RNA-seq was developed with some advantages over microarray technology. Compared with array technology, RNA-seq is not dependent on existing genome data (Rao et al. 2019; Wang et al. 2010; Iyer et al. 2015; Li et al. 2006; Yan et al. 2015; Wang et al. 2009; Wilhelm and Landry 2009), it can quantify expression across a larger dynamic range (Wang, K. et al. 2010;

Wilhelm and Landry 2009; Zhao et al. 2014), and it can detect a higher percentage of differentially expressed genes, particularly genes with low expression levels (Zhao et al. 2014; Wang et al. 2014; Li, Junqin et al. 2016).

In this thesis, I used both gene-expression microarrays (chapter 3) and RNA-seq techniques (chapter 4) to identify candidate genes underlying pyflubumide resistance. In Khalighi et al. (2016), cyenopyrafen resistance was reported to be strongly linked to the overexpression of CYPs, specifically *CYP392A11* and *CYP392A12*; they generated genome-wide gene expression data and incorporated a meta-analysis of previously obtained gene expression data (microarray) in their studies. The two CYPs were suggested as molecular diagnostic markers for monitoring cyenopyrafen resistance in the field (Khalighi et al. 2016). In chapter 3, I investigated the constitutive and induced transcription patterns in susceptible and resistant mites and observed overexpression of five CYP and four CCE genes in the JPR-R strains. However, gene-expression patterns showed to be complex and failed to clearly identify CYP candidate(s) that might be involved in resistance. To some extent, the GE microarray methodology can explain the lack of transparency in our analysis (Hughes et al. 2001). GE microarray techniques estimate transcript abundance through hybridization of cyanine-labelled mRNA to an array of complementary probes with low-specificity for intraspecies variation. Here, a GE microarray analyzer measures fluorescent intensity which mirrors the gene expression level (Templin et al. 2002). Genetic variations such as copy number variations (CNVs) and polymorphisms in the coding regions can confound hybridization of target DNA or RNA to the probes and lead to a lower clarity. In addition, it has been shown that polymorphisms in the probe–target sequences can affect probe-hybridization affinities due to mismatches between a microarray probe and its target sequence. As a result, it can lead to a reduced signal intensity and appearance of false-positive results (Benovoy et al. 2008).

As discussed below, I used a combination of high-resolution BSA genetic mapping and RNA-seq as a more efficient approach to characterize pyflubumide resistance (chapter 4).

Genetic mapping by bulked segregant analysis in spider mites

In species with genetic and genomic resources such as *Drosophila melanogaster*, advanced genetic designs such as genome-wide association studies (GWAS) have

been developed to discover the association between phenotype and genotype (Hales et al. 2015; Groen and Whiteman 2016). For many species, however, GWAS is not applicable due to the lack of comprehensive genetic resources. In a number of arthropod species, traditional linkage mapping studies were successful to identify loci for trait variation using intensive genotyping of single individuals in segregating populations (Uesugi et al. 2002; Zhan et al. 2009; Smith et al. 2015; Linnen et al. 2018; Sugimoto et al. 2020). Recently, microsatellite linkage map technique was used for genetic mapping of a highly resistance *T. urticae* strains to complex II inhibitor acaricides. This study successfully identified target-site mutations associated with the high levels of resistance. Despite all recent technological advances, genotyping hundreds of individuals in this method remains time-consuming and expensive (Bailey and Keifer 1943; Polilov 2015; Kurlovs et al. 2019). To circumvent these obstacles, a growing number of studies have employed the bulked segregant analysis (BSA) method. This cross-based method is becoming an important tool for the rapid mapping of both monogenic and polygenic traits (Kurlovs et al. 2019). In *T. urticae*, BSA approaches have successfully been used to identify monogenic loci conferring resistance to mite growth inhibitors (Van Leeuwen et al. 2012; Demaeght et al. 2014), and polygenic loci underlying resistance to METI-I acaricides (Snoeck et al. 2019) and the lipid synthesis inhibitor spirodiclofen (Wybouw et al. 2019). This approach has also been successfully adapted to characterize the genetic basis of easy-to-score phenotypes such as carotenoid-based pigmentation (Bryon et al. 2017; Wybouw et al. 2019).

In chapter 4, I first applied classic genetic crosses which revealed an incomplete recessive polygenic mode of inheritance of pyflubumide resistance. I further subjected a segregating population (parental strains JPR-R1 and Wasatch, which are resistant and sensitive, respectively) to multiple rounds of selection by pyflubumide, and used high-quality SNP loci for BSA genetic mapping to identify loci responding to selection for this acaricide. Our findings from classic genetic crosses, indicated that resistance to pyflubumide is polygenic. It was confirmed with the genetic mapping that identified three different JPR-R1 allele frequency peaks [two peaks on chromosome 1 (QTL-1 and QTL-2), and one peak on chromosome 2 (QTL-3)] in the pyflubumide-selected populations, compared to their paired controls. The main genes of interest in QTL-1, -2, and -3 were *CYP392A16*, *CYP392E8*, and cytochrome P450 reductase (CPR), respectively. Transcription of *CYP392A16* and *CYP392E8* were both higher in pyflubumide-selected populations, compared to the susceptible controls. However, DNA read coverage indicated that the segregating populations and the two parental strains were not copy number variable. Therefore, the higher transcription levels of the *CYP* genes was not due to copy number variation. In

contrast, JPR-R1 and selected lines harbored three *CPR* copies, compared to a single copy in the susceptible parental and control lines. This ratio was reflected in the RNA read data, such that pyflubumide-selected population displayed ~3.66 up-regulation of *CPR* transcription compared with control populations.

This is the first time that the CPR copy number difference has become putatively associated with resistance in arthropod pests. In *T. urticae CYP392A16* and *CYP392E8* have already been reported to be involved in the detoxification of the pesticide abamectin (Riga et al. 2014). However, two previous studies with *T. urticae* demonstrated a mutation (D384Y) in CPR potentially involved in resistance to spirodiclofen (Wybouw et al. 2019) and METI-I acaricides (Snoeck et al. 2019). Recently, using a microsatellite linkage map, two QTLs were identified to be associated with pyflubumide resistance in *T. urticae* (Sugimoto et al. 2020). One QTL centers on *tetur01g15710*, the gene that codes for SdhB, the molecular target of pyflubumide (Furuya et al. 2015). In addition, genotyping a panel of *T. urticae* strains revealed a strict association between the non-synonymous substitution I260V of SdhB and pyflubumide resistance. The second QTL of Sugimoto et al. 2020 included three cytochrome P450 genes (*CYP392A8*, *CYP392B1*, and *CYP392B2*) and six CCE genes associated with pyflubumide resistance. Our investigations did not reveal any traces of I260V or other candidate target-site resistance mutations in SdhB, nor were any of our three QTLs centered on SdhB. The three *CYP* genes of the second QTL of this study also were not differentially transcribed between the pyflubumide-selected and control populations, and they were not included in any of the three QTLs of our study.

Below, I discuss a number of factors that are expected to impact the resolution of mapping. These factors include the reproductive mode, crossing method, and number of screened progenies. Because of the haplodiploid reproduction of spider mites [diploid females develop from fertilized eggs and haploid males from unfertilized ones], a cross between a resistant male and a susceptible female introduces a single resistant genotype in female offspring, which improves the resolution of BSA genetic mapping (Demaeght et al. 2014). In addition, female mites can mate with their sons which is used as a fast method compared with sibling mating to establish highly homozygous inbred lines to increase mapping resolution in crossing experiments (Bryon et al. 2017; Van Petegem et al. 2018). I also used a single male set-up that incorporated susceptible inbred lines to increase mapping resolution. To increase the number of recombination break-points, a high number of generations and large population size is needed before phenotyping starts. It has been shown that BSA can detect many small-effect loci with high resolution if mapping is performed on the progeny of crossed populations with more than 10^5 individuals (Ehrenreich et al.

2010). A high population can easily be achieved in *T. urticae* due its high reproductive capacity, allowing extensive recombination. In addition, it is also feasible to establish a large number of replicates from the initial bulked population to mitigate the effect of drift under acaricide selection pressure. In addition, recombination occurs independently in each replicate which can further facilitate the investigation for polygenic traits. This would allow application of a permutation-based test that uses information from replicates to detect whether the peaks are due to selection or to genetic drift. This method has already successfully been performed in other *T. urticae* resistance studies (Wybouw et al. 2019; Snoeck et al. 2019).

Conclusion

This thesis is another proof of the great ability of *T. urticae* to develop resistances to acaricides. Cross-resistance to the compounds with similar modes of action limits the options to develop novel acaricides. I, however, identified fitness penalties associated with resistance mutation, which can make its management relatively easy. Resistance genes can be used as molecular markers to monitor resistance in the field. Previous studies on similar compounds were helpful in the identification of resistance genes to Q_0 inhibitor acaricides. However, it was not straightforward to uncover the genetic basis of pyflubumide resistance. To this end, I took several experimental steps including screening for target-site mutations, transcriptomic analyses and high-resolution BSA genetic mapping. Collectively, I uncovered a strong contribution of CYP metabolism to pyflubumide resistance in *T. urticae*. The methodology of this thesis can be used in future investigations to determine the mechanism of resistance to acaricides in *T. urticae*.

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