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Genetic characterization of multiple insecticide resistances in *Cydia pomonella* (L.) using RNAseq

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With 2 figures and 1 table

Abstract: The codling moth, *Cydia pomonella*, is a major pest of apple and pear orchards. However, with intensive use of pesticide, an increasing number of resistances has been reported. Detoxification is one of the main mechanisms involved in resistance in Lepidoptera, and there is still much to discover about the molecular mechanisms underlying it. This study was conducted to decipher the molecular mechanisms involved in the insecticide resistance of *C. pomonella* and to identify genetic markers associated with resistance upon exposure to several pesticides. To that aim, an experimental evolution was carried out from a wild population resistant to several active substances. We generated three lines by combining genetic crosses and experimental selection using chlorantraniliprole, deltamethrin and spinosad. High throughput RNA-sequencing (RNA-seq) was performed on the three lines to verify whether similar mechanisms were involved in pesticide resistance across lines and to assess the role of detoxification and other mechanisms in resistance. In total, we found 592 mutations and 157 differentially expressed genes. Of the candidate genes identified as involved in resistance, a very limited number were shared between lines. We found in total 20 genes belonging to already known candidate genes, with 2 of them known to be involved in *C. pomonella* resistance (*CYP4C1* and *CYP6B2*) and identified several genes that have never been linked with resistance before (mainly associated with odorant perception and stress).

Keywords: Codling moth, insecticide resistance, RNA sequencing, polymorphism, detoxification enzymes, metabolic resistance, transcriptomics, Tortricidae

1 Introduction

Resistance to pesticides is a textbook example of rapid environmental change driven by anthropogenic activity (Palumbi 2001). Indeed, a number of different species evolved resistance resulting from the massive application of pesticides (Georghiou 1990). Understanding the ins and outs of the adaptation to pesticides is a key objective, both from a theoretical and applied point of view, particularly concerning integrated pest management.

The underlying mechanisms involved in insecticide resistance are diverse. They can be sorted into two major classes: mechanisms linked to a modification of the molecular target site of the pesticide (or TSR) and mechanisms that reduce the effective amount of pesticide available to be fixed at the target site (NTSR) (Li et al. 2007). TSR is commonly achieved by mutations in the open reading frame of the target itself, whereas NTSR can be caused by a variety of mechanisms. Behavioural resistance, reduced penetration or metabolic

mechanisms such as efflux, sequestration or detoxification (with enhanced activity or overexpression of detoxification enzymes) can all be involved in NTSR (R4P Network, 2016). When the target of the insecticide is identified, TSR can be easily genotyped and monitored (Boaventura et al. 2020). By contrast, NTSR involves a large number of genes and potentially multiple pathways (Amezian et al. 2021). Knowledge of the genetic bases of NTSR mechanisms is incomplete, especially for non-model species. Identifying the exact genes involved in NTSR and consequently developing molecular tools to monitor NTSR is therefore a challenge (Amezian et al. 2021).

Among the most studied NTSR mechanisms are the detoxification pathways involving enzymes such as cytochromes P450 (P450), glutathione S-transferases (GST), carboxylesterases (CbE), UDP-glucuronosyltransferases (UGT) or transmembrane proteins such as ABC-transporters (Li et al. 2007; Gott et al. 2017). These different proteins belong to pathways that can be activated differently (e.g.

independently, in parallel or synergistically) depending on the pest species and the insecticide involved (Amezian et al. 2021).

The massive use of various chemical insecticides in the last decades has led pests to combine resistance to several active substances (Georghiou 1990). Two different genetic determinisms can lead to resistance to several active substances: (i) multiple resistance, which is the result of an accumulation of several independent resistance alleles, each of them conferring resistance to one active substance, and (ii) cross-resistance, which is the result of one genetic event, where the resistance to several active substances is mediated by a single allele only (R4P Network 2016). Because they are less specific, NTSR, including detoxification pathways, might be involved in cross-resistance. From a management perspective, it is important to distinguish multiple resistance from cross-resistance in resistant pest populations.

The codling moth, *Cydia pomonella*, is a major pest of apple and pear orchards present on all continents (Blommers 1994). Insecticides used to control this pest range from neurotoxics and compounds provoking muscle paralysis (pyrethroids, organophosphates, spinosyns, diamides) to growth regulators (insect growth regulators: juvenile hormones or ecdysone antagonists). Following the intensive use of these products, chemical control failures started to be reported in the 1980s (Reyes et al. 2009). In *C. pomonella*, several TSRs have been documented: such as *kdr* or *MACE*, conferring resistance to pyrethroids and organophosphates, respectively (Brun-Barale et al. 2005, Cassanelli et al. 2006).

Increased activity of different detoxification enzymes, such as mono-oxygenase cytochromes P450, glutathione S-transferases (GST) and esterases (CbE) have also been documented in resistant *C. pomonella* populations (Ju et al. 2021). Using quantitative PCR, studies have investigated variation in expression associated with resistance phenotypes of a handful of targeted genes coding for detoxification enzymes. Regarding P450 genes, overexpression of *CYP6B2*, *CYP4C1* and *CYP4D2* (Dai et al. 2022) or *CYP9A61* (Ju et al. 2021) conferred resistance to pyrethroids and organophosphates. For GST genes, overexpression of *CpGSTd1* or overexpression of *CpGSTd3*, *CpGSTd4* and *CpGSTe3* conferred resistance to pyrethroids (Ju et al. 2021). Last, regarding CbE genes, overexpression of *CpCE-1* conferred resistance to organophosphates and carbamates (Ju et al. 2021). Even though these studies identified candidate genes involved in NTSR mechanisms, they offer only a partial view of the genetic bases involved as they use classical molecular methods, allowing to observe only a few genes involved in one or two insecticide resistances. In addition to that, knowledge about polymorphisms affecting NTSR mechanisms in *C. pomonella* is scarce as opposed to other *Lepidoptera* (Boaventura et al. 2020; He et al. 2012). Indeed, in the codling moth, only Wan et al. (2019) found that the overexpression of *CYP6B2* was associated with 3 SNP in its promoter region.

In this context, the present study aimed at combining experimental evolution and RNA-seq to gain knowledge on gene expression and polymorphism variation associated with resistance to several active substances in *C. pomonella*, without *a priori* on the genes involved. We used a wild population characterized as resistant to several active substances to establish three lines selected with three different insecticide active substances: deltamethrin, a pyrethroid for which gene expression and polymorphism variation involved in NTSR was already documented in *C. pomonella* and other insect species (Li et al. 2007; Ju et al. 2021); chlorantraniliprole, for which a P450 gene was found to be involved in resistance in *Plutella xylostella* (Mallott et al. 2019), where mutations in its promoter region enhance its expression level, and spinosad, where the involvement of different detoxification enzymes has been shown in resistant populations of several insect species (Sparks et al. 2012), but their genetic basis has not yet been studied.

Based on the experimental design of Cattell et al. (2020), we combined genetic crosses with a susceptible strain and gradients of selection to identify variations specifically associated with resistance to each insecticide. This allowed us to test whether common NTSR mechanisms could be at work in resistance to several insecticides (cross-resistance vs multiple resistance). In the case of cross-resistance, we hypothesized that the variation in gene expression and the polymorphism associated with resistance would be shared between the lines. On the contrary, in the case of multiple resistance, our experimental design would allow to segregate specific differential gene expression and polymorphism associated with resistance to each insecticide.

2 Materials and methods

2.1 Population sampling and rearing

During an official insecticide resistance monitoring in France in 2016, a population sampled in a commercial apple orchard in Cavaillon (PACA, FR) was identified as resistant to *Cydia pomonella* *Granulovrius*-M, thiacloprid, deltamethrin, chlorantraniliprole and phosmet (Table S1, Supplementary Information). Since this population showed signs of resistance to the main modes of action used to control *C. pomonella*, diapausing larvae from this orchard were sampled during autumn 2018 for the purpose of this study.

In the spring of 2019, 1500 emerging adults from the Cavaillon population were allowed to reproduce in the laboratory. After a week of mating, eggs were collected and stored until larvae hatching. First instar larvae were then individually placed in hemolysis tubes filled with artificial rearing diet (Guennelon et al. 1981). Emerging adults were transferred into plastic containers (5L) at a 1:1 male:female ratio for mating. The population was maintained under laboratory conditions ($23 \pm 2^\circ\text{C}$, RH $65 \pm 5\%$ and 16:8 L:D h) without any exposure to insecticide for 5 generations

to reach a satisfactory growth rate and a sufficient number of individuals to perform the experimental evolution (Table S2, Supplementary Information).

2.2 Experimental evolution with three insecticides

The following insecticides were used (commercial formulations): deltamethrin (Decis®, Decis Protech Bayer Sas [15 g.L⁻¹]), chlorantraniliprole (Coragen®, Coragen Cheminova Agro France SAS [200 g.L⁻¹]) and spinosad (Success 4®, Success 4 Corteva Agriscience France S.A.S. [480 g.L⁻¹]). These insecticides were chosen based on several criteria: the Cavaillon population's susceptibility to these active substances, their use in the field and the frequency of resistances in France. Deltamethrin has been used for decades in field and is still being used, despite resistances being reported repeatedly for over 25 years, including TSR and increased activity of detoxification enzymes (Reyes et al. 2009). Chlorantraniliprole is currently used as one of the main control substances in conventional agriculture (Bosch et al. 2018) and only emerging resistances, associated with P450 increased activity, have been observed in the field (Bosch et al. 2018). Spinosad is one of the main control substances used in organic orchards, there is no knowledge of gene expression or polymorphism variation associated with resistance to this active substance as its frequency in the field is rare (Siegwart, pers. comm.). These formulations were diluted in osmosed water to obtain different concentrations of the insecticide applied on artificial diet during the experimental evolution (Table S3, Supplementary Information).

From generation F6, we created 3 insecticide-selected lines, with 3 replicates per selected line and one line without insecticide selection pressure (*i.e.* 10 lines in total). Each selected line has been fed with diet amended with one insecticide. To this aim, 15 µl of the insecticide solution were added on the top of the artificial rearing diet in each tube, then dried before depositing one neonatal larva. Mild and strong selection pressures were applied at generations F6 and F7, respectively (Fig. 1). These smooth selection pressures were chosen to increase the frequency of resistance-conferring alleles while minimizing the probability of eliminating low-effect alleles in our population (Roush & Daly 1990). The concentrations of the different insecticide solutions applied for these selection pressures were calculated from tube bioassays performed on individuals of the previous generation (Appendix S1, Supplementary Information). If the selection pressure was unsuccessful, another generation was put under selection. At generation F7, if the mortality induced by the selection pressure corresponded to that expected, surviving adults were crossed with a susceptible strain to segregate the alleles involved in the resistance. This crossing was conducted in order to break genetic linkages and to mitigate false positives coming from potential hitchhiking effects. We also wanted to segregate the associated alleles according to our different lines in case of a multiple resistance,

since our lines came from one initial population rather than a composite initial population. Then, each line was allowed to reproduce freely during one generation to avoid applying selection pressure on 100% heterozygotes, and thus be able to maintain recessive alleles conferring resistance. Similarly, 129 individuals from generation F8 of the control line were crossed with 132 individuals from the susceptible strain, to form a hybrid control line. Finally, at generation F9 (F10 for the deltamethrin lines), each line, except the hybrid control line, was randomly divided into 3 sub-lines (27 sub-lines in total) and exposed to a gradient of concentrations of the insecticide used for its selection: no exposure, medium dose (MD) and high dose (HD) (Fig. 1).

The effectiveness of the selection was assessed after each round of selection by calculating the corrected mortality using Abbott's formula. For each F9-10 sub-line, individuals surviving the insecticide treatment were collected at emergence and stored at -80°C until RNA extractions. Microplate ingestion bioassays were conducted to get an indication of the susceptibility of the initial population to the three insecticides and of the F9-10 sub-lines (Appendix S2, Supplementary Information).

2.3 RNA sequencing

For each sub-line (generation F9-10) and the hybrid control line, the thoraxes of five individuals kept at -80°C were pooled and used for the RNA extraction (28 pools in total), using RNeasy Plus Mini Kit (Qiagen, Hilden, GE). Thoraxes were grounded using a sterile 3.15 mm diameter steel bead on a 1600 MiniG tissue homogenizer (Spex SamplePrep, NJ USA) at 1500 rpm for 15 seconds, in 350 µl of RLT buffer. RNA extraction was carried out according to the manufacturer's instructions. Total RNA quality and quantity were assessed with a Nanodrop ND-1000 (ThermoScientific, MT USA), and integrity was verified by 1% agarose gel electrophoresis. Library preparation, validation and sequencing via DNA nanoball were performed by BGI (Hong Kong). All sequence data has been deposited and archived in GenBank under BioProject PRJNA943149.

2.4 Data analysis

2.4.1 RNAseq data

Quality control of the obtained sequences was assessed using FastQC (Babraham Bioinformatics). Sequences were filtered based on their length, pairing and quality with TrimGalore (Babraham Bioinformatics) using the following parameters: stringency: 3, minimum length: 25 bp, trimming of Ns from either side of the reads (--stringency 3 --length 25 --trim-n).

Mapping on the *C. pomonella* reference genome (Wan et al. 2019) was performed with the STAR algorithm (Dobin et al. 2013) with the standard parameters. Transcript discovery was performed using StringTie software (John Hopkins University), using the following parameters: minimum length allowed for the predicted transcripts: 120 bp

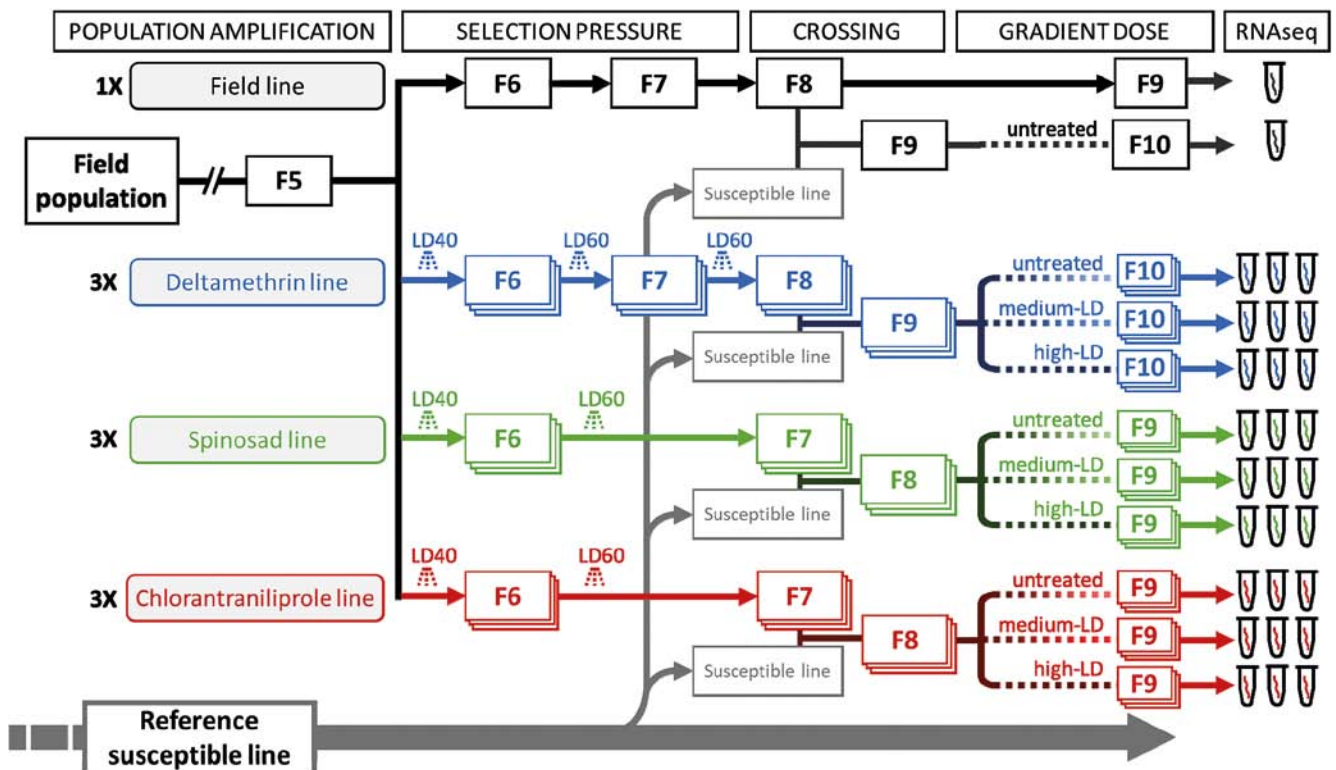


Fig. 1. Experimental evolution design. Colours indicate insecticide lines and dashed lines indicate sub-lines exposed to gradient of insecticide doses with corresponding lethal doses (LD) indicated.

(-m 120 --rf). Read count was performed using the Salmon algorithm (Patro et al. 2017), using the default parameters, on two datasets: one consisting of all the transcripts found and another consisting of our local resistance candidate genes database (Appendix S3, Supplementary Information). Where available, the gene name and annotation from the reference genome were retrieved under the name cpoXXXXX (as defined in the reference genome). If not, we used the naming convention MSTRG.XXXXX. The annotation of the remaining genes was done by performing a BLAST against the nr database on the NCBI website using the standard parameters (a query coverage percentage and identity of at least 80% and an e-value of less than 0.05).

2.4.2 Differential expression

In order to identify the genes activated or repressed by insecticide ingestion, the differential expression levels were assessed by comparing the read counts across the whole genome, for each selected line independently, between F9-10 control and MD sub-lines, and between F9-10 control and HD sub-lines (*i.e.* 6 independent analyses in total). Differential expression was estimated using the ‘edgeR’ package with a glm method (quasi-negative binomial model) (Robinson et al. 2010). The control of the false discovery rate for multiple independent test statistics was performed

following the Benjamini and Hockberg sequential procedure (Benjamini & Hochberg 1995). Genes showing a fold change greater than 3 (in either direction) and a significant corrected P-value ($\alpha < 0.05$) were considered differentially expressed.

2.4.3 SNP Filtering and research of variants

Following the method recommended by Faucon et al. (2017), we first filtered data based on quality and coverage: variants were called against the whole *C. pomonella* genome using the VarScan software, version 2.4.2 (Koboldt et al. 2012), with the following parameters: locus coverage > 20 in all conditions, ignore loci with average base quality < 20, ignore reads with mapping quality ≤ 20 and ignore variants with less than 5% supporting reads. Then, for each selected line separately, we applied the following pipeline to filter polymorphisms linked with selection pressure according to their frequency.

$$[F_{\text{SNP}}(\text{hybrid control line}) < F_{\text{SNP}}(\text{F9-10 no exposure})] \text{ in at least } 2 \text{ lines}$$

and

$$[F_{\text{SNP}}(\text{F9-10 medium LD}) < 15 \text{ AND } F_{\text{SNP}}(\text{F9-10 high LD}) < 15] \text{ in } 2 \text{ lines}$$

and

$$[F_{\text{SNP}}(\text{F9-10 medium LD}) < 15 \text{ OR } F_{\text{SNP}}(\text{F9-10 high LD}) < 15] \text{ in the } 3^{\text{rd}} \text{ lines}$$

The frequency of alleles positively associated with resistance was expected to increase from the hybrid control line to the unexposed F9-10 individuals and to increase from the F9-10 unexposed individuals to the F9-10 exposed to a medium and a high LD. The frequency of alleles negatively associated with resistance was expected to behave reciprocally. To take into account the possible effect of the low number of individuals pooled randomly in each sample on the outcome of the variation of the frequency in a single sample, the possibility for one of the three lines not to follow the expected evolution of allele frequencies was allowed. Then, to determine the effect of polymorphisms in our transcriptomic data, coding effects of SNPs were predicted using SnpEff 5.0c (Cingolani et al. 2012). Variants were categorized as synonymous, non-synonymous, upstream, downstream and according to their localization. Finally, we performed a BLAST of this list against our candidate gene database of genes associated with resistance.

3 Results

3.1 Experimental evolution

For the first generation of selection (F6), corrected mortality (CM) ranged from 7.83 to 29.9%, thus consistent with the mild selection pressure targeted in our experimental design (Fig. S1, Supplementary Information). For the second generation of selection (F7), CMs of the spinosad and chlorantraniliprole selected lines were 72.4% and 73.8%, respectively, thus the strong selection was considered effective. By contrast, selection on the deltamethrin-selected lines led to a 12.5% corrected mortality, which was much lower than intended. A third round of selection was thus applied to the deltamethrin-selected lines (F8, Fig. 1) and led to a corrected mortality of 62.9%. Mortalities in sub-lines of the F9-10 generations were for chlorantraniliprole, spinosad and deltamethrin 52.9%, 43.2% and 48.2% for medium dose and 78.3%, 70.6% and 93.5% for high dose, respectively (Fig. S1, Supplementary Information). Bioassays performed on the initial generation (P0) confirmed 2017 results for deltamethrin, with a significant difference in mortality compared to the susceptible reference (LD50 P-value < 0.001) and a resistance ratio (RR50) of 13.07. Conversely, the 2017 resistance to chlorantraniliprole was not confirmed (LD50 P-value = 0.1807, RR50 = 1.06). Last, for spinosad that was not tested in 2017, we found a weak signal of resistance (LD50 P-value < 0.039, RR = 1.66). As expected, following the crossing with the susceptible reference strain, F9-10 RR50 decreased and the LD50 were not significantly different among control, medium LD and high LD sub-lines. However, we observed that RR increased with exposition to pesticides in F9-10 sub-lines (Table S4, Supplementary Information).

3.2 Sequencing metrics and parameters

The DNB sequencing allowed us to obtain an average of 46×10^6 150-bp reads per sample with a mean coverage of 45X. (see details per sub-line in Table S5, Supplementary Information). Filtering them according to read pairing, sequencing quality and mapping accuracy allowed for over 80% of the total reads to be successfully mapped on the *C. pomonella* genome.

3.3 Differentially expressed genes

Overall, we identified 80 differentially expressed genes between the control and medium LD sub-lines, and 77 differentially expressed genes between the control and high LD sub-lines ($|\log_2FC| > 1.5$ and FDR < 0.05) (Fig. S2, Supplementary Information). We found no differentially expressed genes in common between deltamethrin, spinosad and chlorantraniliprole selected lines.

More specifically, for the deltamethrin-selected lines, 3 genes were overexpressed and 3 underexpressed between the control and medium LD sub-lines. All of them were uncharacterized or hypothetical proteins. Between the control and high LD sub-lines, 8 genes were overexpressed and 7 underexpressed. Among them, 12 were identified, including a glutathione S-transferase 1 involved in detoxification mechanisms (cpo154510) which was overexpressed.

For the chlorantraniliprole-selected lines, 24 genes were overexpressed and 18 underexpressed between the control and medium LD sub-lines. Among them, 12 were identified, such as a cuticle protein (MSTRG.06627), an endochitinase (cpo145340), a pupal cuticle protein (cpo162620), which play a part in cuticle synthesis and composition and were all overexpressed. Between the control and high LD sub-lines, 15 genes were overexpressed and 10 underexpressed. Among them, two genes involved in detoxification mechanisms were identified: CYP4C1 (cpo142370) and a farnesoate epoxidase (cpo111450) which were underexpressed and overexpressed, respectively. Several genes involved in stress or immune response were also overexpressed between the sub-lines (Fig. 2).

Between the control and medium LD spinosad sub-lines, 24 and 8 genes were overexpressed and underexpressed, respectively. Among them, 11 were identified, such as a CYP6B5 (MSTRG.09738) and a probable CYP303A1 (cpo122510) involved in detoxification, over and underexpressed, respectively. Between the control and high LD sub-lines, 22 genes were overexpressed and 15 underexpressed, including CYP6B2 (cpo040630) which was overexpressed: Overall, we also found genes involved in olfactory and pheromone systems overexpressed.

Interestingly, we found genes involved in immunity over and underexpressed for the three insecticide-selected lines and genes involved in pheromone perception in chlorantraniliprole-selected and spinosad-selected lines (Fig. 2).

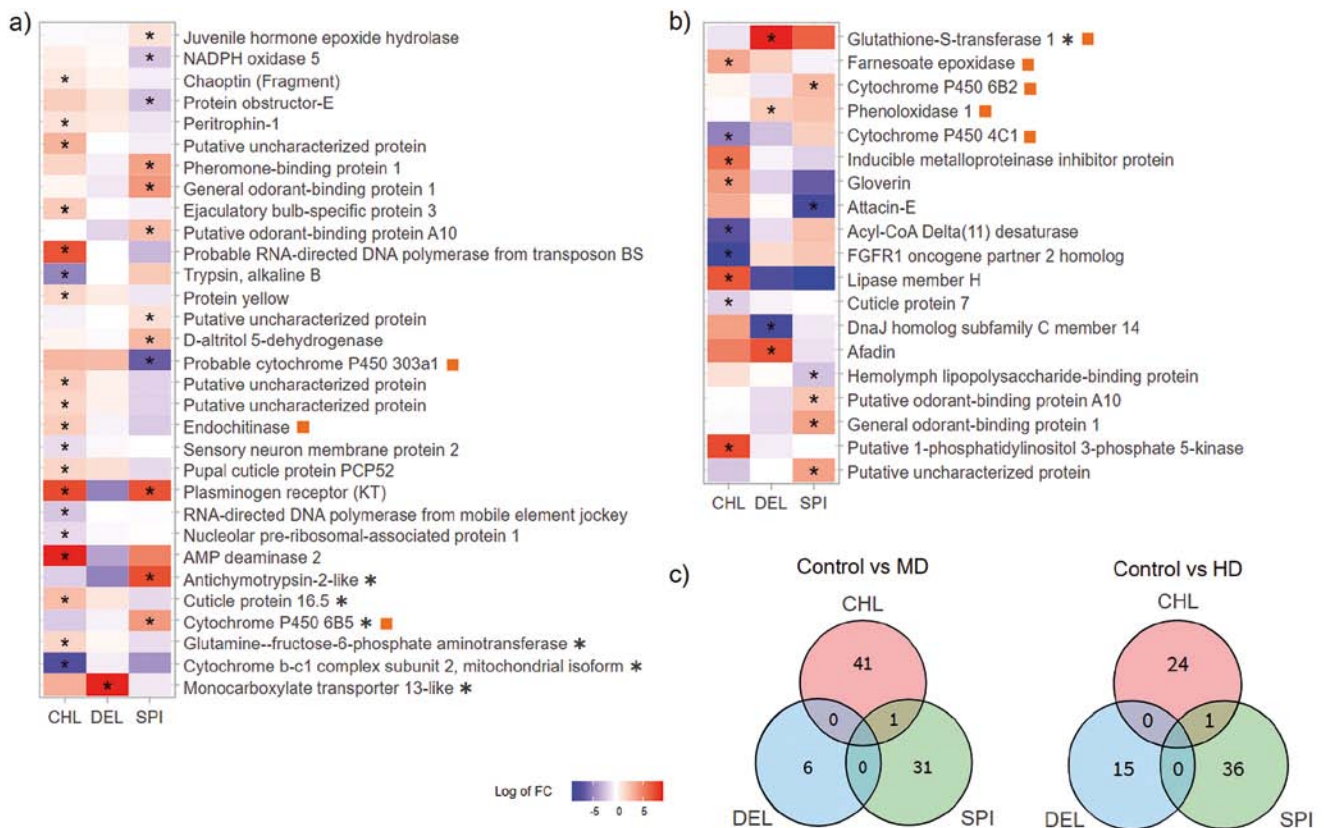


Fig. 2. Expression profiles of annotated genes associated with resistance in our insecticide-selected lines (CHL: chlorantranilprole-selected, DEL: deltamethrin-selected, SPI: spinosad-selected). All genes differentially transcribed in at least one selected sub-line are shown. Colour scale shows the mean Log₂ fold change between each selected sub-line and its control sub-line. Stars indicate a significant differential transcription ($|\log_2FC| > 1.5$ and $FDR < 0.05$). Genes marked with an orange square indicate their belonging to our database of candidate genes, genes indicated with an asterisk have had their annotation retrieved from the *nr* NCBI database. **a)** Between F9-10 control and medium LD (MD) sub-lines. **b)** Between F9-10 control and high LD (HD) sub-lines. **c)** Overview of the differentially expressed genes in common between the different insecticide-selected sub-lines.

3.4 Detection of transcript polymorphism

Overall, 592 polymorphisms were identified with filters based on the frequency of mutations: 156, 208 and 228 for the deltamethrin, chlorantranilprole and spinosad selected lines, respectively. None was common to the 3 insecticides (Fig. S3, Supplementary Information). Three were common to the deltamethrin and spinosad selected lines (including a CKLF-like MARVEL transmembrane domain-containing protein, cpo059390, which seems to play a role in the immune system) and 4 were common to the chlorantranilprole and spinosad selected-lines (Table S6, Supplementary Information).

More genes were affected in the chlorantranilprole selected line ($N = 60$) compared to the deltamethrin and spinosad selected lines ($N = 38$ and 31 , respectively) and there was a similar proportion of missense variants considered to have a moderate impact in the final protein (Table 1).

For the deltamethrin-selected lines, 4 candidate genes were affected: 2 cuticle proteins (cpo098380 and cpo039980) were affected, as well as one NADPH-cytochrome P450 reductase (cpo060030) and one ATP-binding cassette (cpo136030), which presented 2 SNP next to each other (Table S7, Supplementary Information). Regarding the chlorantranilprole-selected lines, 4 candidate genes were affected by these mutations: 2 ATP binding cassettes (cpo053890 and cpo138210), one heat shock factor binding protein 1 (cpo066900) and one NADH dehydrogenase (cpo070030) (Table S7, Supplementary Information). Last, for spinosad, the candidate genes impacted were: an esterase B1 (cpo113930), a NADPH cytochrome P450 reductase (cpo060030), a multidrug resistance protein homolog (cpo124940), a heat shock protein (cpo057810) and a NADH dehydrogenase (cpo127980) (Table S7, Supplementary Information).

Table 1. Number of polymorphisms and their associated impact in our transcriptomic data from F9-10 individuals.

	Chlorantraniliprole lines	Deltamethrin lines	Spinosad lines
Total number of polymorphisms	82,172	96,144	87,463
Number of polymorphisms passing filter	208 (0.25%)	156 (0.16%)	228 (0.26%)
Number of genes affected	60 (4 candidate genes)	38 (4 candidate genes)	31 (5 candidate genes)
Synonymous variants	48 (23%)	29 (19%)	20 (9%)
Non-synonymous variants	10 (5%)	5 (3%)	12 (5%)
Upstream variants	16 (8%)	16 (10%)	14 (6%)
Downstream variants	79 (38%)	68 (44%)	121 (53%)
Variants in unreferenced transcripts	55 (26%)	38 (24%)	61 (27%)

4 Discussion

So far, the attention of most studies investigating *C. pomonella* resistances molecular mechanisms has been set on the effect of a few genes with major phenotypic effects (Bosch et al. 2018). However, with decreasing insecticide pressures, other mechanisms conferring resistance are evolving, with an accumulation of genes having a minor effect on the final resistant phenotype (Roush & Daly 1990). Associated with the democratization of NGS, this leads to a change of methodology in characterizing the underlying molecular mechanisms at play in resistance, as well in the development of new detection tools to assess resistance in field populations (Faucon et al. 2017; Ingham et al. 2021).

Our original hypothesis was that cross-resistance in our original population was due to NTSR and that detoxification enzymes would be involved, as it had been already observed in *C. pomonella* (Reyes et al. 2009; Wan et al. 2019). Surprisingly, out of the 157 differentially expressed genes, only 4 were detoxification genes and only one polymorphism was found in a detoxification gene. A large proportion of differentially expressed genes and polymorphisms associated with resistance were found in genes for which no annotation was available. Altogether, our results indicate a complex tangle of mechanisms involving efflux, cuticle thickening, metabolism and detoxification. The low number of genes and polymorphisms identified as involved in a response to selection could be related to the stringency of our approach that combined three biological replicates for each insecticide-selected line in our experimental design.

Results also support that multiple resistances rather than cross-resistances were more likely involved in the observed resistance phenotype to several insecticides in the initial population. Indeed, no shared differential gene expression or transcript polymorphism was observed among the three lines. Despite the original population being resistant to deltamethrin and spinosad, we did not find shared differential expression or polymorphisms common to the deltamethrin and spinosad-selected lines only. Hardly two genes with dif-

ferential expression (a plasminogen receptor, which seems to play a role in inflammatory responses, and a gene without annotation) and seven polymorphisms were common to chlorantraniliprole and deltamethrin or chlorantraniliprole and spinosad-selected lines, none was present in our candidate gene database.

Interestingly, while the resistance to deltamethrin had the highest resistance ratio in the original population, differential expression affected 3 times fewer genes and there were 40% fewer polymorphisms associated with resistance in the deltamethrin-selected lines. This might be due to the presence at a low frequency of the TSR *kdr* mutation whose frequency was estimated at 33 % in the original population (data not shown). We did not however find *kdr* mutation in transcripts of the selected lines.

Several genes were found to be specifically differentially expressed in each selected line. Among those genes, a number of them were from gene families already identified in the literature as being involved in detoxification mechanisms. We found two P450 genes differentially expressed, *CYP4C1* (cpo142370, in the chlorantraniliprole lines) and *CYP6B2* (cpo040630, in the spinosad lines), that were previously found positively selected in a pyrethroid and an organophosphate resistant strains *C. pomonella* (Dai et al. 2022). Interestingly, the pyrethroid-resistant strain was originating from the same area in the south of France as our initial population. However, the *CYP6B2* gene copy is different between the two studies. *CYP4C1* which was characterized by a high expression level in larvae in Dai et al. (2022) was found underexpressed in adults of our study. This underlines the interest in performing RNAseq on various life stages. We also found a GST gene, the glutathione-S-transferase-1 (cpo154510) overexpressed in the deltamethrin line. Other GST genes were previously found overexpressed in *C. pomonella* following exposure to several pyrethroids (Ju et al. 2021).

Surprisingly, the majority of genes that were differentially expressed (110 out of 151) were not included in our candidate genes database. This result supports our strategy to

explore the full genome of *C. pomonella* rather than focus on a list of candidate genes. It also suggests that our understanding of the molecular mechanisms at play in insect resistances might be still limited. Genes involved in stress and immune response were particularly represented in the pool of differentially expressed genes for the three insecticides. Pesticides can alter several different pathways and components in the different immunity responses in insects (James & Xu 2012). In our study, an inducible metalloproteinase inhibitor protein, involved in humoral immune response was overexpressed. Interaction between insecticides and some components of the humoral immune response has been observed, where the insecticide amplifies gene expression (Dimarcq et al. 1997). A phenoloxidase, which plays a role in the melanisation process, was also underexpressed in the deltamethrin selected line. The phenoloxidase cascade activity has already been documented as being affected in the presence of insecticides (James & Xu 2012), and the exposition of sublethal doses of deltamethrin on *Spoptera littoralis* individuals caused an underexpression of phenoloxidase. (Lalouette et al. 2016). Genes involved in the olfactory system were also differentially expressed. Interestingly, insecticide exposure has been shown to directly affect odorant-binding proteins, where exposure to a high dose of permethrin resulted in overexpression of an odorant-binding protein in *P. xylostella* (Bautista et al. 2015).

It is also worth noting that the differentially expressed genes in medium- and high-LD sub-lines were not the same. This result suggests that the nature of differentially expressed genes might change with the intensity of selective pressure. The fact that the biological responses differ according to the intensity of insecticide pressure is already known. It can even lead to an improvement in fitness at very low doses, a phenomenon known as hormesis (Cutler & Guedes 2017).

None of the identified polymorphisms of interest was located in the 74 genes found to be differentially expressed. Mutations affecting transport proteins and intermediary metabolism (ATP binding cassettes, NADH dehydrogenases, NADPH-cytochrome P450 reductase) were found to be associated with resistance to the three insecticides. ATP-binding cassettes have already been described as involved in chlorantraniliprole resistance in several insect species (Peng et al. 2021; Shan et al. 2021), as well as in deltamethrin resistance in *C. pomonella* (Dai et al. 2022). In the deltamethrin and spinosad selected lines, we detected polymorphisms located in the same gene coding for a NADPH-cytochrome P450 reductase, which has already been described as involved in deltamethrin resistance in *Cimex lectularius* (Zhu et al. 2012). SNPs were also present on several cuticle proteins in the deltamethrin-selected lines. This has already been described in deltamethrin-resistant mosquitoes (Bonizzoni et al. 2015). The role of cuticle proteins in insecticide resistance in *C. pomonella* has also already been suggested in

several studies (Dai et al. 2022; Wan et al. 2019). Also, a multidrug resistance protein was affected by polymorphisms in the spinosad-selected lines. The implication of this category of protein has been shown in several resistant phenotypes: in organophosphate and carbamate-resistant *Helicoverpa armigera* individuals (Akbar et al. 2014) and in deltamethrin-resistant *Trichoplusia ni* individuals (Simmons et al. 2013). A SNP has also been located in a gene coding for an esterase in our deltamethrin-selected lines, which was also described in *Anopheles gambiae* as involved in pyrethroid resistance (Weetman et al. 2018).

This study contributes to a better understanding of the complex mechanisms associated with insecticide resistance and provides resistance-related candidate markers that might be used to develop new resistance detection tools. Since it is generally conducted on small laboratory populations, artificial selection is expected to be biased towards the selection for polygenic resistance rather than a rare monogenic one (French-Constant 2013; Georgiou 1990; Roush & McKenzie 1987). However, we used a wild population that has been exposed to pesticides for many generations in the field and has evolved resistance to several insecticides prior to being artificially selected on the very same insecticides. We also made sure our initial population and the experimental lines' population sizes were as large as possible to mitigate this potential bias (Table S2, Supplementary Information). It would also have been interesting to conduct this experiment on a greater number of generations, even though our goal was not to create lines with high levels of resistance but rather to create a gradient of resistance level amongst the final generation of our sub-lines.

Furthermore, to better characterize their implication in resistant phenotypes, functional validation of these markers would be needed. A complementary approach to this work would be to carry on a DNA-sequencing-based study. Indeed, combining genomic and transcriptomic studies provides a broader view of the mechanisms at work in insecticide resistance, and might help to identify the underlying causes of differential gene expression involved in resistance (Faucon et al. 2017; Ingham et al. 2021).

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Table S1–7, Figure S1–S3, Appendix S1–S3