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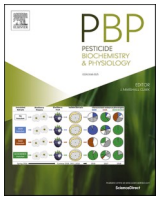

Bulk segregant mapping and transcriptome analyses reveal the molecular mechanisms of spinetoram resistance in *Spodoptera frugiperda*

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ABSTRACT

The evolution of resistance to insecticides poses a significant threat to pest management programs. Understanding the molecular mechanisms underlying insecticide resistance is essential to design sustainable pest control and resistance management programs. The fall armyworm, *Spodoptera frugiperda*, is an important insect pest of many crops and has a remarkable ability to evolve resistance to insecticides. In this study, we employed bulk segregant analysis (BSA) combined with DNA and RNA sequencing to characterize the molecular basis of spinetoram resistance in *S. frugiperda*. Analysis of genomic data derived from spinetoram selected and unselected bulks and the spinetoram-resistant and susceptible parental strains led to the identification of a three-nucleotide deletion in the gene encoding the nicotinic acetylcholine receptor α6 subunit (nAChR α6). Transcriptome profiling identified the upregulation of few genes encoding detoxification enzymes associated with spinetoram resistance. Thus, spinetoram resistance in *S. frugiperda* appears to be mediated mainly by target site insensitivity with a minor role of detoxification enzymes. Our findings provide insight into the mechanisms underpinning resistance to spinetoram in *S. frugiperda* and will inform the development of strategies to control this highly damaging, globally distributed crop pest.

1. Introduction

The fall armyworm, *Spodoptera frugiperda*, is one of the most damaging insect pests of cultivated crops. The importance of this pest has been increasing worldwide because it has relatively recently become an invasive pest in many countries of the African, Asian and Oceanian continents (Baloch et al., 2020; Goergen et al., 2016). Due to its high degree of polyphagia, this pest causes severe damage to numerous agricultural crops including maize, soybean, cotton, sorghum and rice (Montezano et al., 2018). The control of *S. frugiperda* has primarily relied on the intensive use of insecticides, which inevitably resulted in the evolution of resistance to several major groups of insecticides (Bolzan et al., 2019; Carvalho et al., 2013; Diez-Rodríguez and Omoto, 2001; Garlet et al., 2021; Lira et al., 2020; Muraro et al., 2021; Nascimento et al., 2022; Nascimento et al., 2016).

The ability of *S. frugiperda* to rapidly evolve insecticide resistance poses a threat to its sustainable control. Spinetoram is a spinosyn-based insecticide used for *S. frugiperda* control with neurotoxic effects that acts as an allosteric modulator of nicotinic acetylcholine receptors (Dripps et al., 2008; Salgado and Sparks, 2005). This insecticide is a mixture of synthetically modified metabolites (spinosyn J and L) of the actinomycete soil bacterium *Saccharopolyspora spinosa* and has positive toxicological attributes compared to its predecessor spinosad (Crouse et al., 2001; Dripps et al., 2008; Salgado and Sparks, 2005). Spinosyn insecticides have been an important tool in pest management programs due to their high efficacy against insect pests and low toxicity to beneficial and non-target organisms (Dripps et al., 2011; Salgado and Sparks, 2005). However, resistance cases to this group of insecticides have been reported for many insects pests (Sparks et al., 2012), including *S. frugiperda* (Lira et al., 2020; Okuma et al., 2018).

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The resistance mechanisms of spinosyn insecticides have been studied in several insect pests. Most studies have associated resistance to this insecticide class with mutations of the target site, the nicotinic acetylcholine receptor (nAChR) α6 subunit. Point mutations, deletions, exon skipping, mis-splicing and truncated proteins involving the nAChR α6 subunit have been reported in spinosad-resistant strains of *Drosophila melanogaster* (Perry et al., 2007), *Plutella xylostella* (Baxter et al., 2010; Rinkevich et al., 2010), *Frankliniella occidentalis* (Puinean et al., 2013; Wan et al., 2018), *Frankliniela intonsa* (Hiruta et al., 2018), *Thrips palmi* (Bao et al., 2014) *Ceratitis capitata* (Ureña et al., 2019) and *Tuta absoluta* (Berger et al., 2016; Grant et al., 2019; Silva et al., 2016). Moreover, some studies have shown that detoxification enzymes such as cytochrome P450 monooxygenases and esterases may also be involved in spinosad resistance (Bao et al., 2014; Herron et al., 2014; Rehan and Freed, 2014; Wang et al., 2009; Wang et al., 2009; Zhang et al., 2020). However, most of these studies have focused only on the sequencing analysis of the nAChR α6 gene and the use of synergists that inhibits detoxification enzymes to investigate the possible mechanisms associated with spinosyn resistance.

The recent advances in genome/transcriptome sequencing technologies have provided a valuable tool for a better understanding of the molecular basis of insect resistance (Pittendrigh et al., 2014). These technologies allow for the surveying of changes in the entire genome and transcriptome, enabling a more comprehensive analysis of the mechanisms responsible for insect resistance. Bulk segregant analysis (BSA) is a rapid approach that has been employed to identify insecticide resistance mechanism by using pooled sequencing progeny followed the application of a selection pressure (Kurlovs et al., 2019). In an attempt to elucidate the molecular mechanisms underlying spinosyn resistance in *S. frugiperda*, we employed bulk segregant analysis combined with DNA and RNA sequencing technologies to analyze and compare the changes at the genomic and transcriptomic levels between spinetoram-resistant and susceptible strains.

2. Material and methods

2.1. Insect strains

Two strains of *S. frugiperda* were used to conduct this study: the laboratory susceptible strain (SS-Lab) and the spinetoram-resistant strain (RR). The laboratory susceptible strain was collected in Sete Lagoas – MG and has been maintained without selection pressure from any insecticides and Bt proteins for >20 years. The spinetoram-resistant strain was selected under laboratory conditions from a field-collected population collected in São Desidério – BA in 2018 and presented a resistance ratio of 971-fold compared to the susceptible strain (Kanno et al., 2023). Reciprocal crosses between ♀ RR × ♂ SS-Lab and ♂ RR × ♀ SS-Lab were performed for bulk segregant analysis. The resulting F_1 progeny from both reciprocal crosses were inbred for 8 generations. All insects were reared on artificial diet (Kasten Jr et al., 1978) under laboratory conditions (25 ± 2 °C, 70% relative humidity and 14:10 h light/dark photoperiod).

A subset of larvae from the F_8 generation was selected at the discriminating concentration of 100 μg ml^{-1} of spinetoram (Lira et al., 2020) and the surviving individuals were used as the selected strain (Sel). The remaining F_8 larvae formed the unselected control strain (Unsel). The Sel and Unsel strains were reared for one generation and then the larvae from each strain were collected for DNA/RNA extraction. DNA was extracted from the Sel, Unsel, RR and SS-Lab strains, while RNA was extracted from the Sel, Unsel and SS-Lab strains.

2.2. Toxicological bioassays

The diet-overlay bioassay method was used to characterize the susceptibility of the Sel and Unsel strains to spinetoram. Six to eight logarithmically spaced concentrations were tested for each strain ranging from 1.8 to 180 μg ml^{-1} for the Unsel strain and 32 to 1800 μg ml^{-1} for the Sel strain. These different concentrations were obtained by the dilution of the formulated insecticide (Exalt® 120 g a.i. l^{-1}, Corteva Agriscience) in distilled water with the addition of 0.1% (*v*/v) of the surfactant Triton X-100 (Sigma Aldrich Brasil Ltda). The bioassays were conducted in 24-well acrylic plates with each well (1.9 cm^2 area) containing artificial diet. After dilution, 30 μl of the insecticide solution was applied in each well. One early third-instar larva was added to each well. Approximately three replicates of 24 larvae were used for each concentration. The bioassay plates were kept under controlled conditions of 25 ± 2 °C, 70% relative humidity and a photoperiod of 14:10 (L:D) h. Mortality was assessed after 48 h and larvae that did not showed coordinated movement when prodded were considered dead.

2.3. DNA/RNA extraction and sequencing

Total DNA and RNA was extracted from pools of ten fourth-instar larvae of *S. frugiperda* using the AllPrep DNA/RNA Mini Kit (Qiagen), according to the manufacturer's instructions. Four biological replicates were prepared for each strain. The integrity, quality and concentration of the DNA and RNA samples was checked using agarose gel electrophoresis and on a Qubit Fluorometer (Thermo Fisher Scientific). The prepared libraries were sequenced on an Illumina HiSeq 2500 platform using paired-end 150 bp reads.

2.4. Analysis of DNA reads and variant calling

The quality of the DNA reads was assessed with FASTQC (Andrews, 2010). Adapters and low-quality reads were removed using Trimmomatic v. 0.39 (Bolger et al., 2014). High-quality DNA reads were aligned to the reference genome of *S. frugiperda* (NCBI Accession Number PRJNA590312) using BWA with default parameters. The alignment files were processed using SAMTOOLS (Danecek et al., 2021). The SAM files were converted into BAM files and sorted using the *view* function. Then, the BAM files were sorted using the *sort* function and finally the BAM files from the same strain were merged using the *merge* function. The Picard software was used to add read groups and mark duplicates into merged BAM files. Variant calling analysis was performed using FreeBayes (Garrison and Marth, 2012). The variants were annotated using SnpEff (Cingolani et al., 2012). Several criteria were considered for variant calling analysis: to remove, i) reads with depth < 15; ii) heterozygous alleles in the susceptible sample; iii) intron variants; iv) intergenic regions; v) low impact effects.

To perform the bulk segregant analysis (BSA), the final filtered variant calling file with SS-Lab and Sel samples was used to estimate the SNP index (alternative allele reads/total read depth) in the Mutplot software (Sugihara et al., 2022). Variants with SNP index ≥0.90 were considered homozygous, following the criteria proposed by Abe et al. (2012). The variants associated with resistance were required to present a SNP index ≥0.90 in both RR and Sel samples.

2.5. Analysis of RNA reads

The quality of RNA sequencing reads was assessed using FASTQC (Andrews, 2010) and adapters were removed using Trimmomatic v. 0.39 (Bolger et al., 2014). The clean reads were directly mapped to the reference genome of *S. frugiperda* (NCBI Accession Number PRJNA590312) using the HISAT2 alignment software (Kim et al., 2019). The aligned reads were counted with *featureCounts* (Liao et al., 2014). Differentially expressed genes (DEGs) were identified using DESeq2 (Love et al., 2014) by adjusting *p*-value <0.05 and relative expression log2FoldChange > 2 for up-regulated genes and log2FoldChange < −2 for down-regulated genes. GO enrichment analysis of the DEGs was performed using the topGO package (Alexa and Rahnenführer, 2009) and Fisher exact test. KEGG enrichment analysis of the DEGs was performed using the *enrichKEGG* function from the clusterProfiler package

(Yu et al., 2012) based on a hypergeometric test. All analyses were performed in the R Software (R Core Team, 2022).

2.6. Synergist bioassays

Synergist bioassays were performed on the SS-Lab and RR strains to evaluate the effect of detoxification enzymes on spinetoram resistance. The synergists piperonyl butoxide (PBO, Sigma Aldrich), diethyl maleate (DEM, Sigma Aldrich) and S-S-S-tributyl phosphorotrithioate (DEF, Chem Service) were diluted in acetone, and 1 µl of the solution was applied onto the third instar larvae pronotum using a microapplicator (Bukard). The doses of synergists PBO, DEM and DEF were 0.1 µg, 1 µg, and 0.32 µg per larva, respectively (Muraro et al., 2021). The control treatment consisted of acetone alone. After 2 h of synergist application, the larvae were exposed to spinetoram with the diet overlay bioassay method described above. The tested concentrations of spinetoram ranged from 0.1 to 5.6 µg ml^{-1} for the SS-Lab strain and 180 to 5600 µg ml^{-1} for the RR strain.

2.7. Molecular analysis of a nAChR α6 deletion and its association with spinetoram resistance

To examine the association of the Y232del with spinetoram resistance in *S. frugiperda*, we designed crosses between the SS-Lab and the RR strain to examine the association of the mutation with survival to a discriminating concentration of spinetoram. Initially, a single couple were established by crossing one female individual from the SS-Lab strain with one male individual from the RR strain. Then, a backcross was established by crossing one male individual from the F_1 progeny with one female individual from the RR strain, which is the parental strain that was phenotypically more distinct from F_1 (Lira et al., 2020). A total of 72 early third-instar larvae from this backcross were submitted to diet overlay bioassays with a discriminating concentration of spinetoram (100 µg ml^{-1}). The evaluation of the bioassays was performed 48 h post-infestation to phenotypically identify the dead and alive individuals. Larvae that did not show coordinated movement were considered dead. The dead and alive larvae from the bioassays, as well as the adults involved in the crosses, were stored at −80 °C prior to DNA extraction.

DNA from individual dead and alive larvae from the bioassays, as well as from the adults of the SS-Lab strain, RR strain, and F_1 progeny from crosses, was extracted using a modified CTAB protocol (Marín et al., 2021). The quality of DNA extraction was verified through a 1.5% agarose gel electrophoresis reaction. PCR was performed with 2.5 µl of 10× PCR Buffer Mg^{2+} Free, 1.75 µl of $MgCl_2$ at 25 mM, 0.5 µl of dNTP Mix at 10 mM, 0.8 µl of forward and reverse primers at 10 uM, 0.3 µl of Taq DNA Polymerase at 5 units/µl and 2 µg of DNA, in a final volume of 25 µl. The forward (5' TTCACCATCATGATCAGGAGAC 3') and reverse (5' AGCGTGAGTTTCTCTCCG 3') primers were used for PCR to amplify a 129 bp region of the nAChR α6 gene of *S. frugiperda* (*Sf α6*). PCR amplification was performed using the following temperature cycling conditions: 1 cycle of 94 °C for 2 min followed by 35 cycles of 94 °C for 45 s, 58.5 °C for 30 s and 72 °C for 1 min, and a final step of 72 °C for 10 min. PCR products were purified using the ExoSAP-IT™ PCR Product Cleanup Reagent (Thermo Fisher Scientific) and the purified fragments were quantified by 3% agarose gel electrophoresis. The purified PCR fragments were sequenced using the Sanger method with the reverse primer detailed above. Sequencing was performed using the Applied Biosystems 3500 Genetic Analyzer at the Plant Breeding Laboratory - CENA/USP. The alignment of the sequences obtained from the Sanger sequencing was performed against the nAChR α6 gene reference sequence of *S. frugiperda* available on NCBI (Gene ID: LOC118270232). A chi-square test was performed to examine the association between Y232del and survival to spinetoram exposure.

2.8. Complementation test

A complementation test was performed to verify if the three-nucleotide deletion of nAChR α6 gene, found in the spinetoram-resistant strain, is also present in the spinosad-resistant strain. For this, we used the spinosad-resistant strain (Spin-res) established by Okuma et al. (2018), which presents a resistance ratio of 890-fold. Reciprocal crosses between ♀ Spin-res × ♂ RR and ♂ Spin-res × ♀ RR were performed, resulting in the S1 and S2 progenies, respectively. The S1 and S2 progenies were maintained in 100 ml plastic cups containing artificial diet. At least 192 early third-instar larvae from each parental strain, as well as from the S1 and S2 progenies, were exposed to both spinetoram and spinosad insecticides in the diet overlay bioassays. The bioassays were performed with the discriminating concentrations of 100 µg ml^{-1} and 1000 µg ml^{-1} of spinetoram and spinosad, respectively. The evaluation of the bioassays was performed 48 h post-infestation and larvae that did not show coordinated movement when prodded were considered dead. To confirm the presence of three-nucleotide deletion of nAChR α6 gene, 12 individuals from each of the S1 and S2 progenies and 10 individuals from each RR and Spin-res strains were submitted to DNA sequencing using the Sanger method following the described procedure in section 2.7.

2.9. Statistical analysis

The mortality data from concentration-response curves were analyzed by Probit analysis using a generalized linear model with binomial distribution. The LC_{50}s and the respective confidence intervals were estimate using the function *dose.p* from the MASS package (Venables and Ripley, 2002). The resistance ratio was calculated dividing the LC_{50} value of the tested strain by the LC_{50} value of the SS-Lab strain. Synergistic ratios were calculated by dividing the LC_{50} value of the control (insecticide alone) by the LC_{50} value of the insecticide plus synergist treatment. All statistical analyses were performed using R Software (R Core Team, 2022).

3. Results

3.1. Toxicity of spinetoram to S. frugiperda strains

Insecticide bioassay of the SS-Lab strain estimated an of LC_{50} of 0.81 µg ml^{-1} for spinetoram, whereas the RR and Sel strains presented a LC_{50} of 776.9 and 499.53 µg ml^{-1}, respectively, resulting in a high resistance ratio of 971.12-fold for the RR strain and a resistance ratio of 624.37-fold for the Sel strain. The Unsel strain exhibited an LC_{50} of 6.53 µg ml^{-1} for spinetoram, resulting in a resistance ratio of 8.12-fold (Table 1).

Table 1
Susceptibility of *Spodoptera frugiperda* strains to spinetoram.

Strain	n[a]	Slope (± SE)	LC_{50} (CI 95%)[b]	χ^2 (df)	Resistance ratio[c]
SS-Lab*	648	2.4 ± 0.2	0.8 (0.7–0.9)	9.1 (5)	–
RR*	693	2.6 ± 0.2	776.9 (685.7–880.3)	9.8 (5)	971.12
Sel	480	4.4 ± 0.6	499.5 (431.5–578.2)	9.6 (4)	624.37
Unsel	552	1.8 ± 0.3	6.5 (4.4–9.6)	17.7 (5)	8.12

[a] number of larvae tested.
[b] lethal concentration (µg ml^{-1}) of applied insecticide solution that kills 50% of the individuals.
[c] Resistance ratio: LC_{50} of the tested strain/LC_{50} of the susceptible reference strain.
* data from Kanno et al. (2023).

3.1.1. Identification of SNPs and Indels associated with spinetoram resistance in Spodoptera frugiperda

Genome resequencing data was obtained by sequencing four biological replicates of the SS-Lab and RR strains and two replicates of the Sel and Unsel bulk strains. After removing adapter and low quality sequences, a total of 222 million to 610 million reads were obtained for each strain, representing an average genome sequence coverage of 98.57×. The percentage of DNAseq reads mapping to the reference genome of *S. frugiperda* ranged from 75.15 to 76.52% (Table S1). The identification of genetic variants in the four strains using the FreeBayes software resulted in the identification of 13,558,008 SNPs and 2,015,795 Indels in the SS-Lab strain, 13,286,753 SNPs and 1,995,161 Indels in the RR strain, 14,067,279 SNPs and 2,120,392 Indels in the Sel strain and 14,114,545 SNPs and 2,096,308 Indels in the Unsel strain. The combined variant calling file resulted in 29,163,352 variants, including 17,520,073 SNPs and 11,643,279 Indels. We started the filtering process by removing 3,301,834 genetic variants with read depth < 15. Following this, 335,782 genetic variants were removed due their representation as heterozygous alleles in the susceptible sample. Subsequently, we removed 13,261,977 located in intronic regions and 6,459,261 genetic variants in intergenic regions. After removing 1,115,142 genetic variants with low impact effects, the variant calling file presented a total of 203,201 genetic variants, including 131,501 SNPs and 71,700 Indels. The SNP index was then estimated using these 203,201 genetic variants.

Only genetic variants with SNP index ≥0.90 in both RR and Sel samples were selected. This resulted in nine SNPs and Indels that lead amino acid substitutions or deletions (Table 2). These SNPs occur in 9 genes of which 8 have functional annotation. Among these variants, the strongest candidate for a role in resistance was a 3 bp deletion in the spinetoram target-site, the nAChR α6 subunit gene, resulting in an amino acid deletion (Y232del). However, a nonsynonymous SNP (A243S) was also identified in a gene annotated as UGT39B42 (following the UGT Nomenclature Committee). UDP-glucuronosyltransferases (UGTs) have been shown to be involved in resistance to insecticides in other insects (Chen et al., 2019; Du et al., 2023; Grant et al., 2023; Li et al., 2018; Tian et al., 2019; Yang et al., 2023).

Good sequencing coverage of the region encompassing the nAChR deletion site was obtained for all sequenced *S. frugiperda* samples, allowing us to estimate the frequency/genotype of this mutation in the experimental strains. The SS-Lab strain presented a homozygous genotype for the reference (wildtype) allele (SNP index of 0). Both the RR and Sel strains presented homozygous alternative (mutated) alleles (a SNP index of 0.98 and 0.97, respectively). The Unsel strain appeared to be heterozygous for the mutation, presenting a SNP index of 0.52. The deletion occurs in exon 7 and results in the loss of a tyrosine amino acid at the 232 position of nAChR α6 gene protein. The alignment of this region with the encoded amino acid sequence of nAChR α6 genes of other insect species demonstrated that the deletion of the tyrosine amino acid occurs in a high conserved region across a diversity of insect species (Fig. 1).

In the case of UGT39B42, the nonsynonymous mutation occurs in exon 2 causing an alanine to serine substitution at position 243. The SS-Lab strain presented a homozygous genotype for the reference (wildtype) allele, with a SNP index of 0.06. In contrast, both the RR and Sel strains presented a SNP index of 0.90 and 0.91, respectively, indicating a homozygous mutated allele in these strains. Additionally, the Unsel strain demonstrated heterozygosity for the mutation, as reflected by a SNP index of 0.70 (Fig. S1).

Table 2
Non synonymous SNPs and Indels with SNP index ≥0.90 identified in the RR and Sel strains of *Spodoptera frugiperda*.

ID	Reference	Alternative	Amino acid modification	Description
LOC118270232	GTACTACT	GTACT	Y232del	neuronal acetylcholine receptor subunit alpha-7-like
LOC118274764	CG	GA	R108S	trypsin, alkaline B-like
LOC118270596	C	T	R544K	uncharacterized LOC118270596
LOC118277248	A	C	K153N	zinc finger protein OZF-like
LOC118277371	A	T	R31Y	serine/threonine-protein kinase atg1-like
LOC118275334	TGA	TA	T92fs	keratin, type I cytoskeletal 10-like
LOC118269227	G	T	A243S	UGT39B42
LOC118275724	C	A	P493T	bile salt-activated lipase-like
LOC118278816	T	A	E208D	neurofilament heavy polypeptide-like

3.2. Identification of differentially expressed genes associated with spinetoram resistance

Following RNA sequencing of the SS-Lab, Sel and Unsel strains of *S. frugiperda,* a mean of 17,925,160 clean reads were obtained for each library after removing adapter and low quality sequences (Table S2). Due to the high resistance ratio of the resistant strains (RR and Sel), we decided to sequencing the RNA only for the Sel strain. After processing, 85.54 to 86.76% of the clean reads mapped to reference genome of *S. frugiperda* (NCBI Accession Number PRJNA590312). Differential gene expression analyses of the 20,637 genes of the reference genome of *S. frugiperda* were conducted by making comparisons of SS-Lab vs Sel, SS-Lab vs Unsel and Sel vs Unsel. Compared to the SS-Lab transcriptome, a total of 2688 differentially expressed genes (DEGs) were identified in the Sel strain, of which 1470 were up-regulated and 1218 were down-regulated (Fig. 2A and B). A similar number of DEGs were found in the comparison of the SS-Lab vs Unsel transcriptomes, a total of 2565 DEGs were identified in the Unsel strain, including 1376 up-regulated and 1189 down-regulated (Fig. 2C and D). However, only 16 DEGs were found in the comparison of Sel vs Unsel, of which 9 were up-regulated and 7 were down-regulated in the Sel strain. (Fig. 2E and F).

3.3. GO and KEGG enrichment analysis of DEGs

Enrichment analysis was used to identify gene ontology (GO) terms that were enriched in the DEGs identified by transcriptome profiling of resistant and susceptible strains of *S. frugiperda*. The 10 most enriched GO terms of the DEGs of each comparison (Sel vs SS-Lab, Unsel vs SS-Lab and Sel vs Unsel) are shown in the Fig.S2. The DEGs of Sel vs SS-Lab and Unsel vs SS-Lab comparisons were enriched in almost the same GO terms. For biological process, the most enriched terms include chitin-based cuticle development (GO:0040003), carboxylic acid metabolic process (GO:0019752) and small molecule metabolic process (GO:0044281). In the molecular function category, the most enriched terms were related to cuticle structure (GO:0005214 - structural constituent of chitin-based cuticle, GO:0042302 – structural constituent of cuticle and GO:0008010 – structural constituent of chitin-based larval cuticle), oxidoreductase activity (GO:0016491) and catalytic activity (GO:0003824). The most enriched terms in the cellular component category were extracellular region (GO:0005576), extracellular matrix (GO:0031012) and extracellular space (GO:0005615).

The DEGs of the Sel vs Unsel comparison were enriched in terms of the biological process category that includes the cellular response to interferon-gamma (GO:0071346 and GO:0034341) and fibroblast

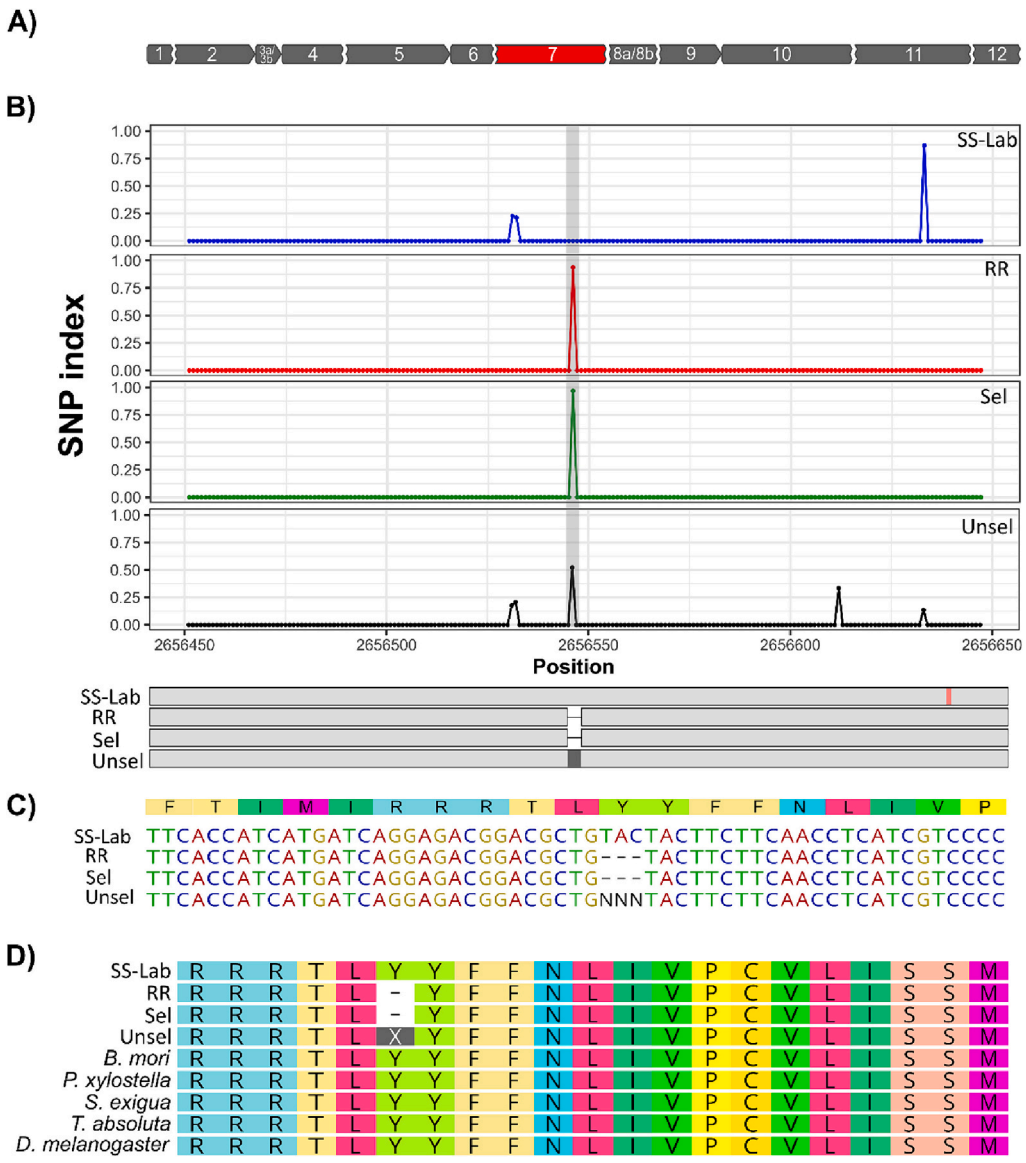

Fig. 1. Characterization of a three nucleotide deletion in the nAChR α6 gene of *Spodoptera frugiperda* associated with spinetoram resistance. A) Exon structure of the nAChR α6 gene highlighting exon 7 where a mutation was identified. B) SNP index in exon 7 of the nAChR α6 gene for the SS-Lab, RR, Sel and Unsel strains. C) A triplet deletion presented in RR and Sel strains results in the deletion of an amino acid (Y232del). D) Alignment of the amino acids sequences of nAChR α6 of *S. frugiperda* strains with amino acids sequences of nAChR α6 from other insect species. The accession numbers of the shown sequences are: *Bombyx mori* (NP_001091842.2), *Plutella xylostella* (ADD69773.1), *Spodoptera exigua* (QIC53910.1), *Tuta absoluta* (ALM23508.1) and *Drosophola melanogaster* (NP_723494.2).

proliferation (GO:0048144). In the molecular function category, the DEGs were enriched in signaling receptor activator activity (GO:0030546), receptor regulator activity (GO:0030545), oxidoreductase activity, acting on NAD(*P*)H (GO:0016651) and structural constituent of ribosome (GO:0003735). The most enriched terms in cellular component category were lysosomal lumen (GO:0043202), organellar ribosome (GO:0000313) and mitochondrial ribosome (GO:0005761) (Fig. S2).

Based on the KEGG enrichment analysis, both DEGs of Sel vs SS-Lab and Unsel vs SS-Lab were enriched in almost the same pathways (Fig. S3). The majority of DEGs were enriched in metabolism pathways. The most enriched pathways included biosynthesis of secondary metabolites, microbial metabolism in diverse environments and carbon metabolism. Pathways of biosynthesis of amino acid and ether lipid metabolism are also present in the top 10 enriched pathways. Antigen processing and presentation was the only enriched KEGG pathway found in the DEGs of Sel vs Unsel.

3.4. Expression patterns of insecticide detoxification related genes

The DEGs encoding potential enzymes involved in insecticide detoxification, which include the cytochrome P450 monooxygenases (P450), carboxylesterases (CarE), glutathione S-transferases (GST), ATP-binding cassette (ABC) transporter and UDP-glycosyltransferases (UGT), were obtained from the transcriptomes of Sel, Unsel and SS-Lab strains. Upon analyzing the DEGs, 86 P450s (Fig. 3), 18 CarEs (Fig. 4A), 19 GSTs (Fig.4B), 5 ABC transporters (Fig. 4C) and 29 UGTs (Fig. 4D) were identified. The number of DEGs of each detoxification enzyme are presented in Table 3.

The majority of the detoxification genes were found to be down-regulated in the Sel and Unsel strains compared to the SS-Lab strain. Among the P450 genes, 22 genes were up-regulated in the Sel and Unsel strains compared to the SS-Lab strain, while 60 and 61 genes were down-regulated in the Sel and Unsel strains, respectively. Most of the GST and CarE DEGs were down-regulated in Sel and Unsel strains compared to the SS-Lab strain, with only 1 CarE and 3 GST genes up-regulated in the

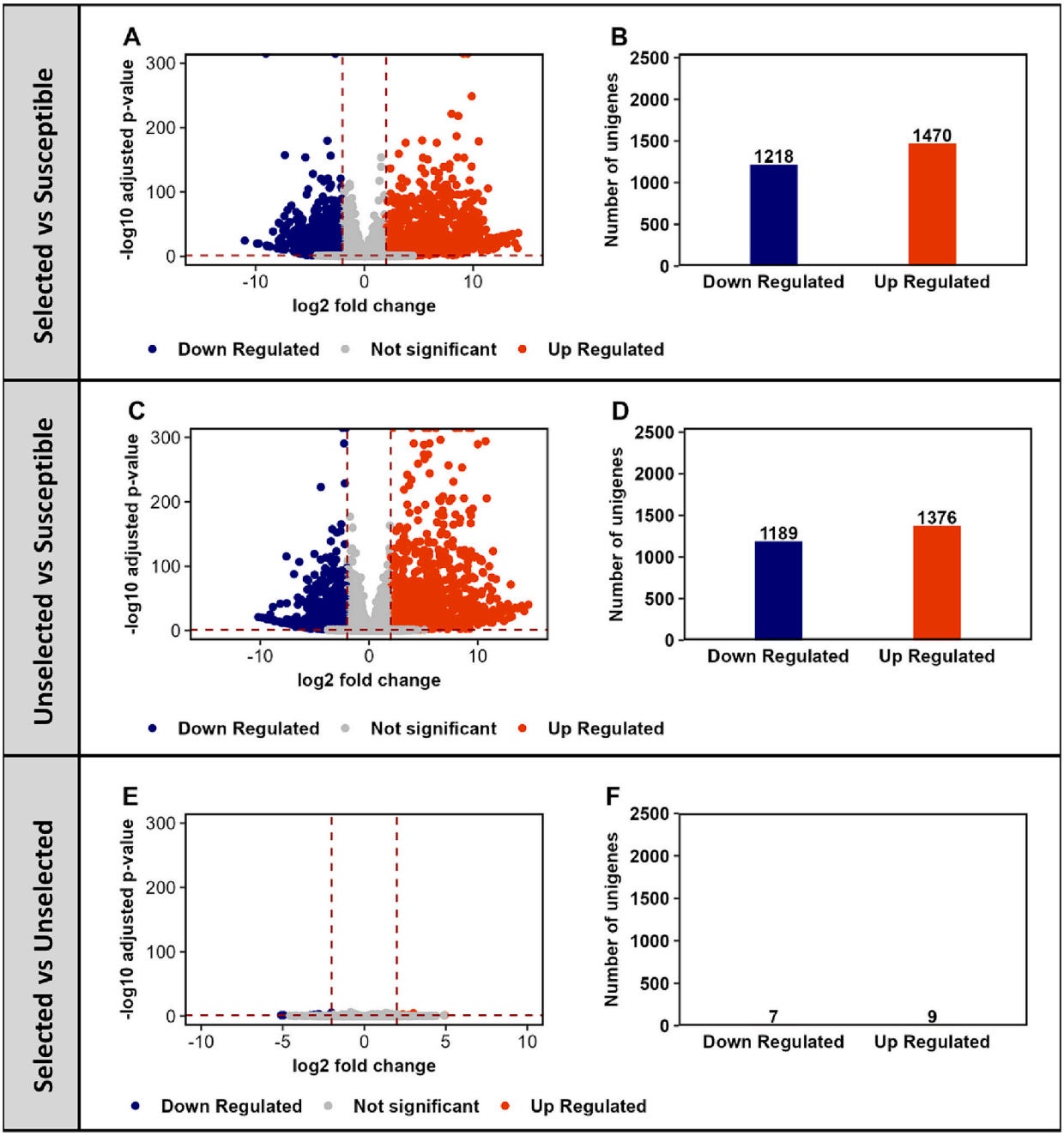

Fig. 2. Differentially expressed gene (DEG) analysis in comparisons of the susceptible (SS-Lab), Selected (Sel) and Unselected (Unsel) strains of *Spodoptera frugiperda*. A) Volcano plot of the DEGs for Sel vs SS-Lab; B) Number of up- and down-regulated DEGs for Sel vs SS-Lab; C) Volcano plot of the DEGs for Unsel vs SS-Lab; D) Number of up- and down-regulated DEGs for Unsel vs SS-Lab; E) Volcano plot of the DEGs for Sel vs Unsel; F) Number of up- and down-regulated DEGs for Sel vs Unsel.

Sel and Unsel strains, respectively. Additionally, 5 and 3 ABC transporter genes were up-regulated in the Sel and Unsel strains, respectively, compared to the SS-Lab strain. As for the UGT DEGs, 4 genes were up-regulated in Sel and Unsel strains compared to the SS-Lab strain. No detoxification genes were found to be significantly differentially expressed in the Sel vs Unsel comparison.

3.5. Synergist bioassays

To further investigate the role of detoxification enzymes in spinetoram resistance in *S. frugiperda,* insecticide bioassays were conducted on the parental SS-Lab and RR strains in the presence of inhibitors of the three superfamilies of detoxification enzymes, P450s, esterases and glutathione S-transferases, most frequently implicated in resistance. The P450 inhibitor PBO, the glutathione S-transferase inhibitor DEM and the esterase inhibitor DEF did not increase the toxicity of spinetoram against either the SS-Lab or RR strains. The LC_{50} values of the SS-Lab strain with spinetoram plus the synergist treatment ranged from 0.68 to 0.72 μg ml^{-1}, whereas the LC_{50} value of spinetoram alone was 0.86 μg ml^{-1}. It resulted in a synergistic ratio of 1.19, 1.06 and 1.26-fold for PBO, DEM and DEF, respectively. For the RR strain, the LC_{50} values of spinetoram plus the synergist treatment ranged from 538.99 to 650.29 μg ml^{-1}, whereas the LC_{50} value of spinetoram alone was 727.97 μg ml^{-1}. The synergistic ratios for RR strain were 1.35, 1.11, 1.17-fold for PBO, DEM and DEF, respectively (Table 4). These findings suggest that spinetoram resistance in the RR strain is not mediated by the action of common

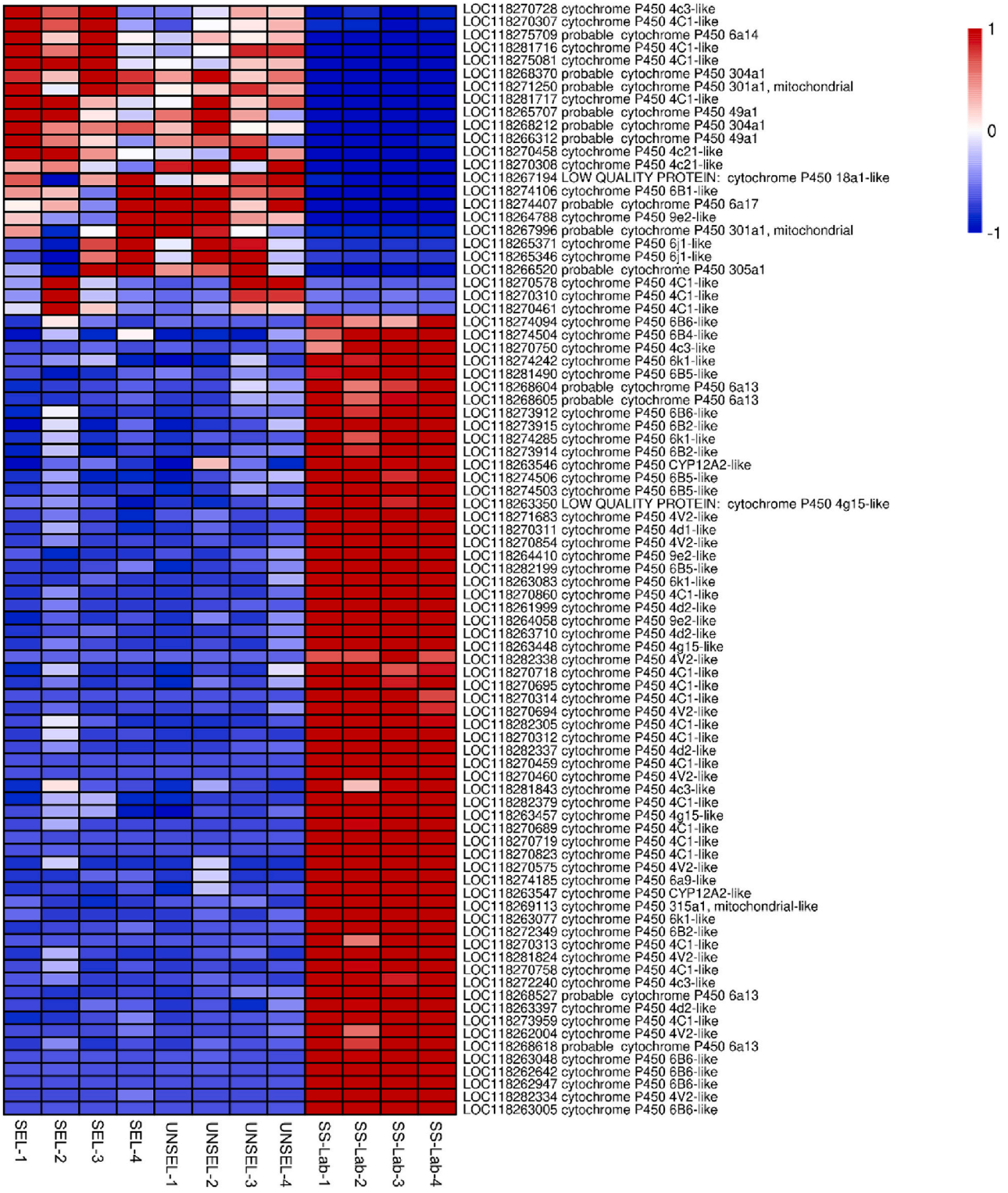

Fig. 3. Heatmap of the expression of cytochrome P450 genes in the susceptible (SS-Lab), Selected (Sel) and Unselected (Unsel) strains of *Spodoptera frugiperda.*

superfamiles of detoxification enzymes. Given this finding, we focused subsequent analysis on investigation of the role of target-site mutation in resistance to spinetoram.

3.6. Association of Y232del with spinetoram resistance in S. frugiperda

Sanger sequencing of the nAChR α6 gene confirmed the presence of the three-nucleotide deletion Y232del in the homozygous form in individuals of the RR strain. The DNA sequence of individuals of the SS-Lab strain did not carry Y232del and were homozygous wildtype. As expected, sequenced individual from the F_1 progeny of these strains was found to be heterozygous for the mutation as revealed by double peaks in sequence chromatograms of the region encompassing the mutation (Fig. 5). To investigate the causal link between Y232del and spinetoram resistance, we examined the association of the mutation presence with survival to a discriminating concentration of spinetoram. All 42

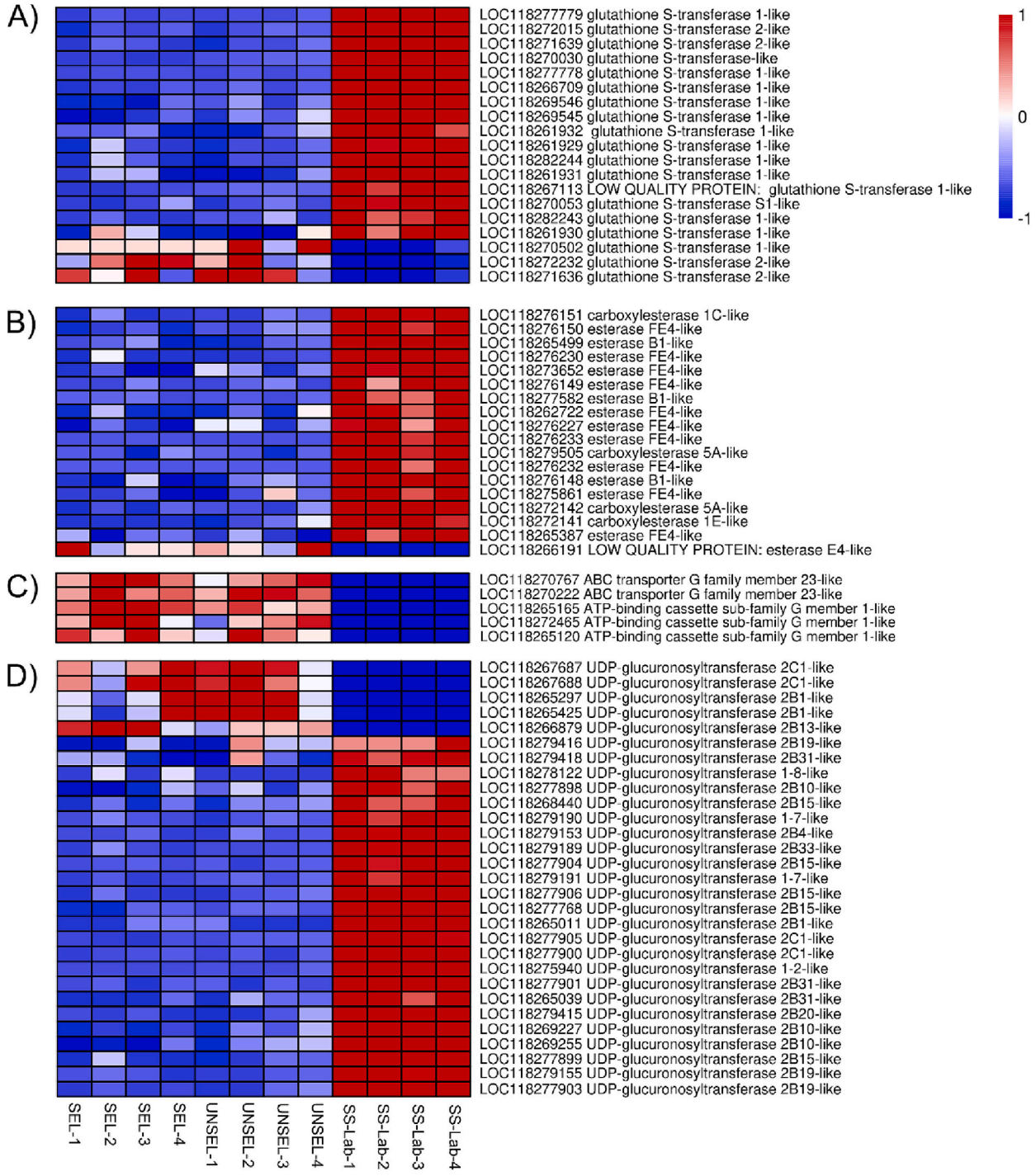

Fig. 4. Heatmap of the expression of A) Glutathione-S-transferases, B) esterases, C) ABC transporters and D) UDP-glycosyltransferases genes in susceptible (SS-Lab), Selected (Sel) and Unselected (Unsel) strains of *Spodoptera frugiperda.*

Table 3
Differentially expressed genes encoding detoxification enzymes in the comparison of the transcriptomes of the susceptible (SS-Lab), Selected (Sel) and Unselected (Unsel) strains of *Spodoptera frugiperda.*

DEGs of detoxification enzymes	Sel vs SS-Lab		Unsel vs SS-Lab		Sel vs Unsel	
	Up-regulated	Down-regulated	Up-regulated	Down-regulated	Up-regulated	Down-regulated
P450 (86)	22	60	22	61	0	0
CarE (18)	1	17	1	13	0	0
GST (19)	3	14	3	16	0	0
ABC transporter (5)	5	0	3	0	0	0
UGT (29)	4	23	4	20	0	0

Table 4
Effect of the synergists PBO, DEM and DEF on the toxicity of spinetoram to the spinetoram susceptible (SS-Lab) and resistant (RR) strains of *Spodoptera frugiperda*.

Strain	Treatment	n[a]	Slope (± SE)	LC_{50} (CI 95%)[b]	χ^2 (df)	SR[c]	Resistance ratio[d]
SS-Lab	Spinetoram	452	3.10 (± 0.38)	0.86 (0.72–1.02)	8.91 (4)	–	–
	Spinetoram + PBO	503	1.94 (± 0.19)	0.72 (0.59–0.87)	6.88 (5)	1.19	–
	Spinetoram + DEM	498	2.19 (± 0.15)	0.81 (0.69–0.96)	4.63 (5)	1.06	–
	Spinetoram + DEF	431	2.99 (± 0.43)	0.68 (0.55–0.85)	9.38 (4)	1.26	–
RR	Spinetoram	525	2.50 (±0.26)	727.97 (604.42–876.77)	10.33 (5)	–	846.47
	Spinetoram + PBO	576	3.08 (±0.27)	538.99 (473.38–613.68)	6.30 (5)	1.35	748.59
	Spinetoram + DEM	451	2.75 (±0.40)	650.29 (512.26–825.51)	10.74 (4)	1.11	802.82
	Spinetoram + DEF	472	3.21 (±0.28)	621.62 (535.13–722.10)	4.71 (4)	1.17	914.14

[a] number of larvae tested.
[b] lethal concentration (μg ml^{-1}) of applied insecticide solution that kills 50% of the individuals.
[c] Synergistic ratio: LC_{50} of tested strain with insecticide alone/LC_{50} of the same strain with insecticide plus the synergist.
[d] Resistance ratio: LC_{50} of the RR strain/LC_{50} of the SS-Lab strain.

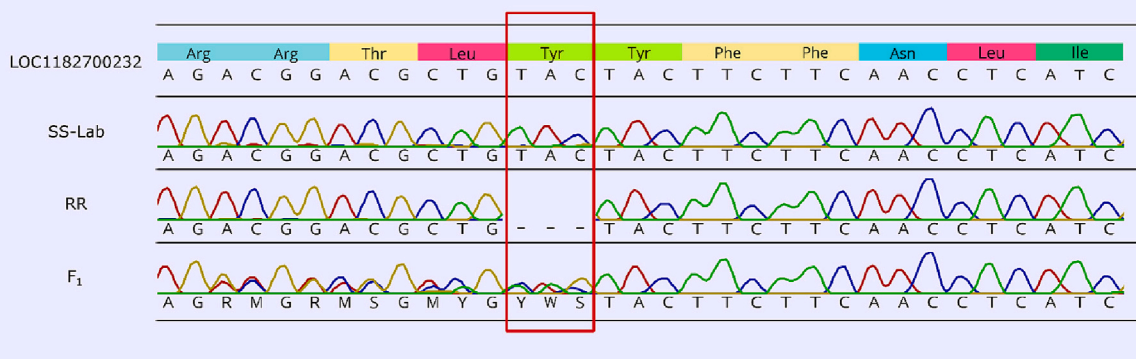

Fig. 5. Representative chromatograms from Sanger sequencing of the nAChR α6 PCR product from individuals of the susceptible (SS-Lab), spinetoram-resistant (RR) and F_1 crosses of these strains. The location of the Y232del is indicated with a red box. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

individuals (21 dead and 21 alive) from bioassays conducted with a discriminating concentration of spinetoram were successfully genotyped. A significant association was observed between the insect genotype and its phenotype ($\chi^2 = 28.1$, df = 1, $p < 0.001$). All individuals surviving the discriminating concentration of spinetoram presented a DNA sequence with the three-nucleotide deletion in homozygosis, while 85.71% of the dead individuals presented a DNA sequence with the deletion of three-nucleotide in heterozygosis (Fig. 6).

Fig. 6. Frequency of the Y232del mutation in alive and dead *Spodoptera frugiperda* individual larvae following exposure to a discriminating concentration of spinetoram. *n* dead = 21, *n* dead = 21. A significant association was observed between the insect genotype and its phenotype by chi-square test ($\chi^2 = 28.1$, df = 1, $p < 0.001$).

3.7. Complementation tests

A complementation test was performed to verify if the Y232del mutation, found in the spinetoram-resistant strain, is also present in a spinosad-resistant strain (Spin-res). All strains showed a high survival rate in spinetoram and spinosad bioassays performed for complementation tests. No significant differences were observed between the survival rates of the Spin-res and RR strains or reciprocal crosses of these strains (S1 and S2) (F = 2.12; df = 3,72; $p = 0.10$ for strain; F = 1,54; df = 1,71; $p = 0.21$ for insecticide; F = 2.36; df = 3,68; $p = 0.07$ for interaction). The survival rate observed in spinetoram bioassays was 91.2, 96.6, 95.8 and 92.5% for Spin-res, RR, S1 and S2, respectively. The survival rate observed in spinosad bioassays was 98.1, 95.8, 96.2 and 92.92% for Spin-res, RR, S1 and S2, respectively (Fig. S4A). Sanger sequencing demonstrated that all sequenced individuals of the RR, Spin-res, S1 and S2 strains that survived exposure to the two insecticides were homozygous for the three nucleotide deletion in the nAChR α6 gene (Fig. S4B).

4. Discussion

Understanding the molecular mechanisms underlying insecticide resistance is critical for the development and implementation of sustainable pest control methods and resistance management strategies. Here, we employed a bulk segregant analysis (BSA) approach in combination with DNA and RNA sequencing to characterize the molecular basis of spinetoram resistance in the global crop pest *S. frugiperda*. Our data provide evidence that resistance to spinetoram in this species is conferred by a mutation in the target-site of this insecticide class. This mechanism may act in concert with other mechanisms such as enhanced activity or expression of detoxification enzymes.

BSA was first employed in the characterization of the mechanisms of

pesticide resistance in arthropods in a study of the resistance of the two-spotted spider mite *Tetranychus urticae* to the acaricide etoxazole (Van Leeuwen et al., 2012). Leveraging this approach in our study identified nine variants, including SNPs and Indels, with a non-synonymous effect that were associated with spinetoram resistance. Those variants are found in genes related to cellular component organization, transferase and hydrolase activity and proteolysis. Among these, the most promising candidate for a role in resistance was a triplet deletion (Y232del) found in the nicotinic acetylcholine receptor subunit α6 that results in the loss of a tyrosine amino acid in exon seven. Spinosyn insecticides including spinetoram act on the insect nervous system by targeting the α6 subunit of the nAChR and mutations in this gene have been associated with spinosyn resistance in numerous other insect species. For example, point mutations in the nAChR α6 were associated with spinosad resistance in *D. melanogaster* (Perry et al., 2007), *P. xylostella* (Rinkevich et al., 2010), *F. occidentalis* (Puinean et al., 2013), *T. palmi* (Bao et al., 2014), *T. absoluta* (Silva et al., 2016), *F. intonsa* (Hiruta et al., 2018) and *C. capitata* (Ureña et al., 2019). Furthermore, mis-spliced transcripts of nAChR α6 producing truncated protein were reported for resistant strains of *P. xylostella* (Baxter et al., 2010) and truncated transcripts of nAChR α6 were also observed in resistant *Bactrocera dorsalis* (Hsu et al., 2012), *F. occidentalis* (Wan et al., 2018) and *R. dominica* (Wang et al., 2018). Finally, exon skipping and deletions were also associated with spinosad resistance in *T. absoluta* (Berger et al., 2016; Grant et al., 2019). Most relevant to our study, Grant et al. (2019) found a similar triplet deletion, F238del, in exon 7 of *Taα6* in a spinosad resistant strain of *T. absoluta* and demonstrated its causal association with resistance. Intriguingly, the mutation identified in our study is just three amino acids away from the F238del mutation identified by Grant et al. (2019). These mutations occur in, or immediately adjacent to, the first α-helical transmembrane domain (TM1) of the α6 subunit, in a region that is highly conserved across the Arthropoda. This suggests that this region is under strong functional constraint in arthropods, providing evidence that the Y232del mutation identified in our study is likely to be a bona fide resistance mutation rather than a natural variant segregating in *S. frugiperda* populations. The role of transmembrane regions in pentameric ligand-gated ion channels in insecticide binding has been revealed by structural studies of the glutamate-gated chloride channel (GluCl), which exhibts close structural similarity to nAChRs. Crystal structures of the GluCl bound to ivermectin, another macrocyclic lactone with structural similarity to spinetoram (Hibbs and Gouaux, 2011), revealed that ivermectin makes direct associations (by hydrogen bonding and van der Waals interactions) with TM1, TM2 and TM3 of the GluCl. Thus, the repeated finding of amino acid deletions in TM1 of the nAChR α6 subunit associated with spinosyn resistance provides additional evidence that spinosyns act on an allosteric site distinct from the conventional agonist binding site, likely formed, at least in part, from transmembrane regions of the receptor.

The association between the Y232del mutation and resistance of *S. frugiperda* to spinetoram was determined by performing backcrosses and genotyping individuals surviving or dying following exposure to a discriminating dose of spinetoram. The results from Sanger sequencing demonstrated that the Y232del co-segregates with spinetoram resistance in our bioassays, providing evidence of the causal role of this mutation in resistance. Complementation tests also suggest that both spinosad and spinetoram resistance in *S. frugiperda* are conferred by the alleles at the same locus, and the Y232del was also found to present in the spinosad-resistant strain, confirming the presence of cross-resistance between these two insecticides (Lira et al., 2020).

Target-site modification are often associated with fitness costs, as they can potentially alter the function of typically highly conserved proteins and their interaction with other molecules (Kliot and Ghanim, 2012). A varying degree of fitness costs have been associated with spinosyn resistance (Sparks et al., 2012). Significant fitness costs were observed in a spinosad-resistant strain of *S. frugiperda* (Okuma et al., 2018), while the fitness of the spinetoram-resistant strain (RR) used in our study varied depending on the host plant the strain was reared on, showing that the Y232del mutation could impact the ability of *S. frugiperda* to adapt to certain host plants (Kanno et al., 2023). A more precise method to determine the effect of the Y232del mutation on spinosyn resistance in *S. frugiperda* would be to introduce the mutation into a susceptible strain using genome editing techniques. Functional validation using CRISPR-Cas9 editing tools will be conducted in our future studies to determine the impact of this deletion on spinosyn susceptibility and whether it incurs any fitness costs in *S. frugiperda*.

The involvement of detoxification enzymes in the resistance of *S. frugiperda* to spinetoram was also investigated by performing an RNA-Seq analysis. In order to identify the DEGs that could possibly be involved as a resistance mechanism, we selected the annotated DEGs that are associated with the three phases of detoxification of xenobiotics. These group of enzymes included P450s, CarEs, GSTs, and UGTs (Feyereisen, 2012; Ffrench-Constant, 2013; Li et al., 2007; Lu et al., 2020; Nagare et al., 2021; Pavlidi et al., 2018). In *S. frugiperda*, several studies have shown the involvement of these enzymes in the detoxification process of insecticides and plant allelochemicals (Bai-Zhong et al., 2020; Carvalho et al., 2013; Giraudo et al., 2015; Hafeez et al., 2021; Israni et al., 2020; Nascimento et al., 2023; Nascimento et al., 2015; Silva-Brandão et al., 2021; Yu et al., 2003). For spinosyn resistance, some studies have associated the overexpression of detoxification enzymes with spinosad resistance in *S. litura* (Rehan and Freed, 2014), *S. exigua* (Wang et al., 2006), *Helicoverpa armigera* (Wang et al., 2009), *F. occidentalis* (Herron et al., 2014) and *T. palmi* (Bao et al., 2014). Our transcriptome analyses demonstrated that some P450 genes of the CYP4, CYP6 and CYP9 families were overexpressed in spinetoram-resistant insects (Sel strain) when compared to the susceptible strain. Cytochrome P450s are typically involved in phase I of xenobiotic detoxification in insects and have been shown to mediate resistance to a wide variety of insecticides (Feyereisen, 1999; Lu et al., 2020; Nauen et al., 2022; Stanley, 2017). Some proteins related to phase II of detoxification such as GSTs and UGTs were also up-regulated in larvae resistant to spinetoram. Furthermore, proteins like ABC transporter C and G, responsible for the transportation and elimination of products of metabolization of xenobiotics (phase III), were overexpressed in the resistant strain (Sel strain) compared to the susceptible strain. However, the majority of the differentially expressed genes encoding these enzymes in our study are down-regulated in Sel and Unsel strains compared to the SS-Lab strain. This could be related with the fact that insects may evolve alternative mechanisms for dealing with insecticides that are more efficient and less costly than maintaining high levels of detoxification gene expression (Kliot and Ghanim, 2012; Samantsidis et al., 2020).

In addition to quantitative changes in their expression, detoxification genes may confer resistance as a result of qualitative changes in their coding sequence that enhance their activity against insecticides (Li et al., 2023; Pym et al., 2023; Zimmer et al., 2018). In this regard, it is notable that a mutation (A243S) was also identified by BSA in a gene annotated as UGT39B42. Overexpression of UGTs have been shown to be involved in resistance to insecticides in several other insects (Chen et al., 2019; Du et al., 2023; Grant et al., 2023; Li et al., 2018; Tian et al., 2019; Yang et al., 2023). Furthermore, nonsynonymous mutations in UGT genes have been linked to resistance to pyrethroid insecticides in the mosquito *Anopheles funestus* (Al-Yazeedi et al., 2023). Thus, further investigation of the functional capacity of UGT39B42 of *S. frugiperda* with and without the A243S substitution to metabolize spinosyns or their metabolites is warranted.

The results discussed above suggest that target-site and metabolic mechanisms may act in concert to confer resistance to spinosyns in *S. frugiperda*. This raises a question as to the relative contribution of these mechanisms to the resistance phenotype. Our synergism bioassays using inhibitors of the three main superfamilies of detoxification enzymes did not substantially enhance the sensitivity of *S. frugiperda* to spinetoram. Furthermore, a CRISPR-mediated knockout of nAChR α6

subunit in *S. frugiperda* and *S. exigua* exhibited a high levels of resistance to spinosad and spinetoram (Shi et al., 2022; Zuo et al., 2020). The resistance levels of RR and Sel strains are very close to those caused by α6 deletion by CRISPR/Cas9. This suggests that the up-regulated genes encoding for P450s, GSTs and esterases identified in our transcriptome analysis play a secondary role in conferring spinetoram resistance in relation to target-site mutation in *S. frugiperda*.

In conclusion, this study represents a crucial initial step towards improving our understanding of the molecular mechanisms of spinetoram resistance in *S. frugiperda*. We demonstrate that mutation of the nAChR α6 gene plays a key role in resistance and provide initial evidence that quantitative changes in the expression of some detoxification genes and qualitative changes in their coding sequence may contributed to resistance in a supplementary manner. The findings of this study will provide a basis for the development of molecular markers to detect spinetoram resistance in field populations of *S. frugiperda* in order to implement effective resistance management strategies.

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CRediT authorship contribution statement

Rubens H. Kanno: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Antonio R.B. Nascimento:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Carolina P. Monteiro:** Writing – review & editing, Methodology. **Fernando S.A. Amaral:** Writing – review & editing, Methodology, Data curation. **Kumar S. Singh:** Writing – review & editing, Methodology. **Bartlomiej J. Troczka:** Writing – review & editing, Methodology. **Chris Bass:** Writing – review & editing, Project administration, Methodology, Funding acquisition, Conceptualization. **Fernando L. Cônsoli:** Writing – review & editing, Methodology, Funding acquisition, Conceptualization. **Celso Omoto:** Writing – review & editing, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no conflict of interests.

Data availability

Illumina data will be publicly available at the NCBI BioProject PRJNA1103734, PRJNA1104133, PRJNA110461 and, PRJNA1104299. The data that support the findings of this study are available from the corresponding author upon reasonable request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pestbp.2024.105921.

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