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RNA investigation of *Oryzaephilus surinamensis* (L.) (Coleoptera, Silvanidae) resistant and susceptible strains to fenitrothion insecticide

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Abstract

Molecular techniques are useful tools to explain problems with insects resistant to insecticides. Three *Oryzaephilus surinamensis* strains, OS1 susceptible, OS4 moderately resistant and OS10 resistant to the organophosphorous insecticide fenitrothion were studied to verify the differential display of genes. The total RNA extracted from the insects was used for syntheses of cDNA (RT-PCR) and isolation of differentially expressed cDNAs from the resistant strain OS10. These cDNAs were sequenced to investigate resistant genes similarity with a genbank. The sequences obtained from fragments presented highly significant similarity with the *Bacteroides thetaiotaomicron* microorganism, an antibiotic resistant type, and with metionine aminopeptidase enzyme, which promotes protein degradation. The information generated by the differential display of RNA method application is important to study the genetic structure of pest strains in order to understand the resistance mechanism involved, and also as a detection tool for resistance management.

Key words: Oryzaephilus surinamensis, DNA sequence, complement DNA, pesticide resistance, organophosphorous.

Introduction

Organophosphorous insecticides have been widely used to control stored grain pests, however, its intensive use has lead to the development of insect resistance in many species and in many cases this represents a significant threat to the continuity of its effective use.

Oryzaephilus surinamensis (L.) populations have shown resistance levels to the organophosphorous pirimiphos-methyl, malathion, fenitrothion e clorpiriphos-methyl (Collins, 1985; 1986; Conyers et al., 1998) and, in most cases, it has been difficult to separate the effects of the many resistance mechanisms and their components (Dauterman, 1983), as when a single system can't provide a efficient level of protection, the resistance can be developed by a combination of mechanisms (Lockwood et al., 1984).

In the *O. surinamensis* metabolic resistance is an important mechanism which gives organophosphorous resistance and is characterized

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by a super-production of the detoxicating esterase enzyme, which promotes the degradation of the insecticide (Conyers et al., 1998). Considering that the action of many resistance factors are being explained, the knowledge of its origin and development is indispensable.

The mutations are being considered responsible for the resistance origin, however according to Oppenoorth (1984) comparisons between amino acid sequences from resistance and susceptible populations are necessary to validate this information. Recent studies report gene sequencing so as to study the insect's phylogeny (Howland and Hewitt, 1995) and also for studies related to insecticide resistance.

In studies by Conyers et al. (1998) with *O. surinamensis*, the esterase enzyme of a susceptible population and of a organophosphorous insecticide resistant population was purified and sequenced. The authors detected differences in the amino acid sequences and suggest that the purified enzyme of the two populations may be different.

In a review paper by Hemingway (2000), it is mentioned that the esterase, which grant resistance by the increase of the metabolism, occur because of structural gene mutations, although few were characterized at the nucleotides level. These mutations may significantly change the specification of the enzyme's substrate.

The agro-evolution, due to the insecticide selection pressure in many management strategies, shows many examples of the extreme adaptations a organism may achieve, making an understanding of the different aspects that characterize resistance necessary (Via, 1986). The investigation of these aspects require an understanding of the genetic structures of the pest populations through specific molecular methodologies (Brown et al., 1997).

The differential display of RNA – DD-RNA (Liang and Pardee, 1992) is a technique used with the purpose of identifying the differential display of genes from various eukaryote systems. The fragments (cDNAs) are amplified by PCR and settled in sequencing gel so as to verify the differentially displayed genes.

In the search for alternatives in the control of *O. surinamensis*, the knowledge of the insect-insecticide interaction on a molecular level may contribute to the understanding of the mechanisms by which the gene products are transcribed and their probable functions, and the knowledge of the insect genome sequences can lead to new strategies for the control of this species.

The aim of this study was to describe the cloning and sequencing of differentially displayed cDNA fragments from an organophosphorous fenitrothion resistant *O. surinamensis* population.

Material and methods

Strains of *O. surinamensis* and resistance bioassay

For the study of gene's differential display, three populations of *O. surinamensis* were selected: OS1, susceptible, OS4, moderately resistant and OS10, resistant to organophosphorous fenitrothion insecticide at 500 g i.a./L (Sumigran 500 CE).

Non-sexed, adult individuals, maintained in grinded wheat grain, were used. The insects from the OS1 and OS4 populations were from the tenth generation (F_{n+10}) and the ones from the OS10 population were collected in the field (F^0).

So as to compose the samples, a bioassay, where the populations were submitted to a fenitrothion treatment based on the previously determined CL_{50} of each population (Beckel et al., 2002) besides a control with no treatment, was done. The fenitrothion insecticide was diluted in petroleum ether so as to obtain the required concentrations; 1.0 mL of the concentration was distributed upon filter paper with 9 cm of diameter in Petri dishes, with four replicates for each population. After the solvent evaporation, 10 insect adults were released in the interior of each dish and, after 1, 6 and 24 hours of infestation, these individuals were immersed in liquid nitrogen, macerated and stored at – 80 ºC until the time of the tests.

The experiment was maintained in a room

with 25 ± 0.5 ºC of temperature and 65 ± 5 % of relative air humidity.

RNA isolation and tests

The total RNA isolating was based on the methodology described by Lângaro (2002), adapted from Liang and Pardee (1992). So as to obtain the differential RNA fragments, the protocol described by Averboukh et al. (1996) and adapted by Lângaro (2002) was used.

Reverse RNA-PCR transcription

The isolated *O. surinamensis* RNAs were submitted to the reverse RNA transcription technique, followed by the amplifying of the complementary DNA fragments (cDNA) (RT-PCR), using the initiating oligonucleotides (primers): oligo 1(dTC), 5' AAG CTT TTT TTT C 3' and oligo 2 (dTG), 5' AAG CTT TTT TTT TTT G 3'.

RT-PCR products electrophoreses

For the RT-PCR, the initializing oligonucleotides oligo 1 – oligo 2 combinations were selected, according to Lângaro (2002).

For the RT-PCR products separation, a "differential display" reaction was done, in which the cDNA samples were settled. The acrilamida gel at 6 % was prepared as described in Promega (1993) and remained at 50 W (pre-corrida) until its temperature stabilized at 45 ºC. The cDNAs were previously incubated at 70 ºC for 2 minutes and settled in the gel for approximately 3 h and 30 min. The molecular weight was compared to the standard 1 Kb (Biogen). After the settling of the samples, the gel was treated was silver coloring (Silver Sequence™, (Promega, 1993) and then photographed (Kodak Digital Science 1 D™).

RT-PCR product extraction and amplification

The differentially displayed cDNAs were isolated from the gel by using the band excision technique based on the PCR (Wilton et al., 1999), in the same conditions as described previously.

Cloning, plasmodium isolation and sequencing

For the cloning, the cDNAs excised from the gel and PCR amplified were used in a bonding reaction with the top vector TA®, followed by the transformation into *Escherichia coli* cells (Invitrogen, 2001). The plasmidial DNA was extracted according to the protocol described by Brasileiro and Carneiro (1998). The sequencing cycle of the fragments, amplified by the oligo 1 e oligo 2 beginners, was done by PCR using the ABI PRISM® "Big Dye Terminator™ Cicle Sequencing Ready" (Applied Biosystems) reaction kit and the primers from M13 vector "forward and reverse". The sequenced data was collected and analyzed using ABI Prism® 3100 "Genetic Analyzer" sequencer (Applied Biosystems). The sequences were aligned and compared with the help of the DNA Star – "Module SeqMan contained in the program Lasergene" software (DNASTAR, Inc.) and later compared to the sequences of other species, deposited in a gene bank (GenBank), so as to identify similarities in the resistance genes.

Results and discussion

The cDNA fragments display standard was verified in a differential display reaction. After the electrophoreses in acrilamida gel, the differentially accumulated cDNAs were selected as to fenitrothion insecticide exposure and, the differential interaction between the insect's genotypes and the insecticide's CLs_{50} in the treatments, became evident by the presence or absence of fragments in the gel.

In the differential display reaction, in which the RT-PCR samples, obtained from RNA extraction, were settled, it was observed that there was a larger accumulation of RNA at 1, 6 and 24 h after the infestation, with or without insecticide

exposure, in the fenitrothion resistant *O. surinamensis* population (OS10). Two differentially displayed cDNAs were selected: Band 2 and Band 4, these were extracted from the gel and re-amplified as described in the methodology (Wilton et al., 1999).

For the transformation, cloning and plasmid extraction stages, four replicates were done for each cDNA, and only in the 2.4C replicate (Band 2) and in the 4.3C, 4.3D and 4.3E replicates (Band 4) the plasmidial DNA was visualized in agarose gel at 0.8 %.

The 2.4C, 4.3C, 4.3D and 4.3E (redundant) partial sequences were obtained, these where compared with sequences deposited in the data bank of Genbank (National Center for Biotechnology Information, 2003). The sequences displayed in the *O. surinamensis* – fenitrothion interaction were compared with the gene sequences available in the mentioned data bank by using the BLAST tool (Altschul et al., 1990), by which we can obtain, among other information, the similarity "*I*" (Table 1) between the sequences and the "*E*" value, parameter that describes the number of times that a particular sequence can be found in a data bank. The identity between the sequences was separated, according to the "*E*" value, into significant ($E \geq e^{-19}$) and moderately significant ($e^{-3} \leq E \leq e^{-19}$) (Kim et al., 2001).

The 2.4C cDNA partial sequence did not show significant homology with the sequences deposited in the GenBank data bank, and might be part of an unknown gene. This and other sequences might be obtained in later studies with this insect and should then be tested with a wider cDNA library, with the intention of validating the resistance relation – treatment relation.

The 4.3C and 4.3D cDNA sequences showed more then a hundred significant alignments with sequences available in the GenBank data bank.

A highly significant similarity between the 4.3C cDNA, displayed in the *O. surinamensis* –

Table 1. Isolated cDNA fragments form a resistant *Oryzaephilus surinamensis* population(OS10) exposed to the fenitrothion insecticide, and its similarities with the sequences available at a gene bank (GenBank). Passo Fundo, RS, 2003.

DS	Access	Source (cDNA)	I	Blast[e]
4.3.C	M62807	rRNA - Myroides odoratus	88/96	3*e*-24
	AE016937	*Bacteroides thetaiotaomicron* VPI-5482	107/122	4*e*-23
	AE012963	*Chlorobium tepidum* TLS	83/92	6*e*-22
	M62805	rRNA *Chlorobium limicola*	78/86	1*e*-20
	X68427	rRNA *Streptococcus oralis*	60/64	4*e*-17
	AY155590	rRNA - *Bacteroides caccae* 23S	78/89	2*e*-15
4.3.D	AE016937	*Bacteroides thetaiotaomicron*	54/71	2e-22
	AE016953	*Enterococcus faecalis* V583	54/69	1e-21
	AE017178	*Porphyromonas gingivalis* W83	51/69	4e-19
	AE006294	*Lactococcus lactis*	49/68	5e-19
	NP811618	Metionina aminopeptidase		
		B. thetaiotaomicron	58/77	4e-19
	AE008472	*Streptococcus pneumoniae*	47/67	4e-18

DS – displayed sequences (cDNAs) in the resistant *O. surinamensis* population (OS10) – fenitrothion insecticide interaction.

I – displayed sequences similarities in the resistant *O. surinamensis* population (OS10) – fenitrothion insecticide interaction with the gene sequences available at the GenBank.

e – *E* value: parameter that describes the expected number of times that a particular sequence can be found in a data bank. Significant ($E \geq e^{-19}$) and moderately significant ($e^{-3} \leq E \leq e^{-19}$).

fenitrothion interaction, and the rRNA cDNAs of the *Myroides odoratus*, *Chlorobium limicola*, *Streptococcus oralis* and *Bacteroides caccae* 23S microorganisms (accesses M62807, *e* = -24; M62805, *e* = -20; X68427, *e* = -17 e AY155590, *e* = -15) (Table 1) was verified. In the same manner, the result of the comparison between the 4.3D cDNA sequences verified a high homology with the *Bacteroides thetaiotaomicron* microorganism and with the metionina aminopeptidase enzyme of this same microorganism (accesses AE016937, *e* = -22 e NP811618, *e* = -19), as well as with other microorganisms (Table 1).

The genome of the anaerobic gran-negative bacteria *B. thetaiotaomicron*, dominant member of the human intestine, was recently sequenced (Xu et al., 2003). The bacteria are amongst the anaerobics most resistant to antibiotics and *B. thetaiotaomicron* contains 60 proteins that are components of the efflux drugs system, this possibly being the main antibiotic resistant mechanism in this microorganism. Considering these evidences and the highly significant homology between cDNA partial sequences from a fenitrothion resistant *O. surinamensis* population with those of *B. thetaiotaomicron*, we suggest further studies so as to investigate a possible resistance mechanism linked to both species.

The high homology with the microorganism metionina aminopeptidase enzyme was another expressive result found (*e* = -19) (Table 1). This enzyme belongs to the hydrolases class and its main function is to remove the metionina amino-terminal (N-terminal) from the newly formed proteins (Kyoto Encyclopedia of Genes and Genomes, 2003). This characteristic has an important influence on the protein's stability, which frequently resists hydrolyses because it has a chemically (usually acetylation) modified N-terminal region, in this way fulfilling an important role in the protection against degradation (Alberts et al., 1994).

The information obtained on the function of the metionina aminopeptidase enzyme are already very expressive, however, studies related with the possible involvement of this enzyme with insect's defense responses are still necessary.

It is suggested that one should correlate the *O. surinamensis* isolated cDNAs differential display with insecticide resistance using techniques such as the "*Northern Blot*" (Guerrero, 2000) or the "*Macroarray*" (Nogueira et al., 2003).

In the last decade, great advances in the understanding of the insecticide resistance molecular base were observed. Structural genes that encode enzymes and that occur in great quantity in many insect species have been cloned and characterized. The understanding of how these genes are regulated will be another great advantage for the elucidation of these systems, allowing the management of pest insect species so as to restore their insecticide susceptibility (Hemingway, 2000).

This study is a pioneer in the stored grain pest insecticide resistance area, associating the pest species – insecticide interaction. Even though the data obtained are not conclusive, the displayed sequences can, at least partly, represent the molecular processes that occur during the *O. surinamensis* – fenitrothion interaction and contribute to future studies that approach the genetic structures of pest populations with the intention of developing new control methods.

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