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PYRETHROID RESISTANCE AND P450 GENE REGULATION IN THE TARNISHED PLANT BUG *LYGUS LINEOLARIS* (HETEROPTERA: MIRIDAE)

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Abstract

Pyrethroid resistance in insects is associated with elevated P450 monooxygenase levels to detoxify pesticides. The objective of this study was to examine how resistant tarnished plant bugs, *Lygus lineolaris* (Palisot de Beauvois), tolerate insecticide toxicity through gene regulation. A cDNA, cloned from both pyrethroid-susceptible and -resistant strains of *L. lineolaris*, contained a 1548-nucleotide open reading frame encoding a 516 amino acid residue protein. Predicted cytochrome P450s from cDNAs were highly similar to several insect CYP6 P450 monooxygenases which are responsible for reduced sensitivity to pyrethroid insecticides. A total of 48 nucleotide substitutions were revealed between cDNAs of susceptible and resistant strains, 13 of which were observed on the cytochrome P450 protein coding region resulting in 12 silent substitutions and only one amino acid modification from Ser^{487} in the susceptible strain to Ala^{487} in the resistant strain. The resistant strain contained 2.1-fold higher P450 monooxygenase mRNA than the susceptible strain. Topical treatment with 10 ng permethrin elevated P450 monooxygenase mRNA levels by ~2-fold. Treatment with 10 µg piperonyl butoxide suppressed P450 monooxygenase mRNA levels by ~34%.

Introduction

The tarnished plant bug, *Lygus lineolaris* (Palisot de Beauvois), is an important field crop insect with both pyrethroid and organophosphate insecticide resistance. In the Mid-South area, control of the tarnished plant bug in cotton almost exclusively depends on insecticides and resistance to pyrethroids has been found in many tarnished plant bug populations (Snodgrass, 1996a; Snodgrass and Scott, 2000). Holloway et al. (1998) used synergists to study the nature of pyrethroid resistance in tarnished plant bugs. They concluded that pyrethroid metabolism mediated by monooxygenases probably played a significant role in the resistance.

Insect P450s metabolize synthetic organic insecticides, such as pyrethroids (Tomita and Scott, 1995) and cyclodienes (Feyereisen et al., 1995), and natural plant products, such as furanocoumarins (Hung et al., 1997; Ma et al., 1994). It is well established that elevated levels of cytochrome P450 monooxygenases are responsible for metabolic resistance in many insects (Brun et al., 1996; Dombrowski et al., 1998; Feyereisen, 1999; Kasai and Scott, 2000; Li et al., 2000; Liu and Scott, 1998; Ranasinghe et al., 1998; Scott et al., 1999; Tomita et al., 1995; Wang and Hobbs, 1995; Zhang and Scott, 1996). Knowledge of how gene regulation and biochemical modification produce resistance is highly desirable for monitoring, delaying, and management of this resistance. Here, we report the first pyrethroid-resistance related cytochrome P450 cDNA from both susceptible and resistant strains of the tarnished plant bug. Gene transcript expression levels were quantitatively analyzed, and mRNA expression locality, induction and inhibition were also examined.

Materials and methods

Clone and Sequence P450 cDNA

Total RNA was prepared from both susceptible and resistant strains of the tarnished plant bug and was used as a template for cDNA synthesis. Forward (P4501F: TTYTWYNBNGCNGGNTWYGARAC) and reverse (P4502R: CARTTNCKNKGNCCNDVNCCRAANGG) degenerate primers were designed based on conserved P450 monooxygenase sequence regions, F-F/Y-G/I/L/A/MA-G-F/Y-E-T and P-F-G-D/A/L-G-P/Q-R-–C, respectively. Polymerase chain reaction (PCR) was conducted to amplify the P450 monooxygenase cDNA fragment from RT-cDNA. cDNA fragments (~450 bp) were successfully amplified and cloned. After the partial sequence of P450 monooxygenase-like cDNA was obtained, forward and reverse primers were designed, and were used to obtain 5' and 3' ends of P450 monooxygenase-like cDNA. To obtain full P450 monooxygenase-like cDNA from both pyrethroid-susceptible and resistant strains of the tarnished plant bug, a forward primer P450F3 and a reverse primer P450R4 were synthesized and used in PCR reactions to flank the 5' and 3' non-coding regions, respectively. To eliminate potential error created by Taq DNA polymerase, a thermostable proof-reading Pfu DNA polymerase (Promega, Madison, WI) was used to reamplify and sequence a full length P450 monooxygenase cDNA from the RT-cDNAs of susceptible and resistant strains of the tarnished plant bug.

Quantitative Reverse Transcription (QRT) PCR Analysis of P450 mRNA Expression

Procedures for the quantitative determination of the expression levels of the cytochrome P450 mRNAs were modified from those used by Alexandre et al. (1998), Guenthner and Hart (1998), Igaz et al. (1998), Freeman et al. (1999), and Zhu et al. (2000). A homogeneous internal standard was developed to produce 254 bp deletion, and was subsequently used for QRT-PCR analysis of P450 monooxygenase-like mRNA expression.

Results

P450 Monooxygenase cDNA Sequences and Deduced Protein Sequences

Pairwise alignment of cDNAs from susceptible and resistant strains revealed that cDNA from the susceptible strain had 1917 (accounting 97.5%) nucleotides identical with the cDNA from the resistant strain. Although the two insect strains had the same length coding region, there were 13 nucleotide differences on open reading frames, twelve of which were silent mutations coding for the same amino acids as in the susceptible strain. However, at position 1578, a nucleotide thymine in the susceptible strain was replaced by a guanine in the resistant strain. The codon change from TCT for the susceptible strain to GCT for the resistant strain changed the amino acid residue from serine (susceptible strain) to alanine (resistant strain). The 1548-bp open reading frame from both insect strains coded 516 amino acid residues. Homology search of GenBank revealed that deduced 516-residue proteins were similar to several insect CYP6 P450 monooxygenases which were responsible for reduced sensitivity to pyrethroid insecticides.

Sequence Difference and Gene Frequency

cDNAs amplified with both Taq DNA polymerase and Pfu DNA polymerase included a nucleotide substitution at position 1578 resulting in a serine residue in the susceptible strain and an alanine residue in the resistant strain. This modification was confirmed by PCR amplification of specific allele (PASA) of group and individual RT-cDNA and genomic DNA. Selective amplification of the Ala^{487}-allele indicated that this gene allele existed only in the resistant strain. The failure of amplification of the Ala^{487}-allele fragment in the susceptible strain indicated that this colony was fixed for the Ser^{487} allele. However, amplification of both Ala^{487}-allele and Ser^{487}-allele from the resistant strain revealed that both alleles were present in the resistant strain.

PASA amplification of RT-cDNA prepared from RNA of individual insects showed that the Ser^{487}-allele fragment was amplified from every individual insect RT-cDNA of the 10 susceptible and the 10 resistant tarnished plant bugs. The cDNA fragment of the resistant Ala^{487}-allele was amplified in only 3 of the 10 resistant insect RT-cDNAs, and there was no amplification of the resistant Ala^{487}-allele from any of the susceptible insect RT-cDNAs. The individually extracted genomic DNA was also subjected to PASA amplification. The resistant Ala^{487}-allele fragment was amplified in 8 of the 23 resistant insect DNAs, and no such amplification was achieved from any of susceptible insect DNAs.

P450 Monooxygenase mRNA Expression Levels

By using the specific primers P450F1 and P450R4, the RT-PCR was successful in simultaneously amplifying a 771-bp P450 monooxygenase cDNA fragment from the target cDNA template and a 517-bp cDNA fragment from the internal standard, and both were clearly separated on agarose gel. Results showed that the untreated resistant strain contained 2.1-fold higher P450 monooxygenase mRNA per microgram of total RNA than the untreated susceptible strain. The ten ng permethrin treatment increased P450 monooxygenase mRNA levels by 2.2-fold for the susceptible strain and 1.6-fold for the resistant strain. The ten μg PBO treatment decreased P450 monooxygenase mRNA levels by 33.3% for the susceptible strain and 35.53% for the resistant strain.

Discussion

The tarnished plant bug is becoming an increasingly important insect pest on genetically-modified and conventional varieties of cotton. Because of the ineffectiveness of the Bt transgene for plant bug control and reduced foliar applications in addition to the development of insecticide resistance, control of the tarnished plant bug has become difficult. This study initiated an investigation to understand resistance gene regulation. A resistance related cytochrome P450 cDNA was sequenced which showed structural and expression level differences between susceptible and resistant strains. Future study will include molecular cloning of other forms of cytochrome P450s and the determination of enzyme activity through recombinant protein expression system. Genetic linkage will be established, and molecular markers will be developed to monitor resistance gene frequency in field populations to provide information for developing pest management strategies.

References Cited

Alexandre, I., Zammatteo, N., Ernest, I., Ladriere, J.M., Le, L., Hamels, S., Chandelier, N., Vipond, B., Remacle, J., 1998. Quantitative determination of CMV DNA using a combination of competitive PCR amplification and sandwich hybridization. BioTechniques 25, 676-683.

Brun, A., Cuan, A., Le, M.T., Berge, J., Amichot, M., 1996. Inducibility of the *Drosophila melanogaster* cytochrome P450 gene, CYP6A2, by phenobarbital in insecticide susceptible or resistant strains. Insect Biochem. Molec. Biol. 26, 697-703.

Dombrowski, S.M., Krishnan, R., Witte, M., Maitra, S., Diesing, C., Waters, L.C., Ganguly, R., 1998. Constitutive and barbital-induced expression of the Cyp6alpha2 allele of a high producer strain of CYP6A2 in the genetic background of a low producer strain. Gene Amsterdam. 221, 69-77.

Feyereisen, R., Andersen, J.F., Carino, F.A., Cohen, M.B., Koener, J.F., 1995. Cytochrome p450 in the house fly: structure, catalytic activity and regulation of expression of CYP6A1 in an insecticide-resistance strain. Pestic. Sci. 43, 233-239.

Feyereisen, R., 1999. Cytochrome P450-mediated metabolic resistance to insecticides in the house fly, *Musca domestica*. Nippon, N.K., 73, 1043-1051.

Freeman, W.M., Walker, S.J., Vrana, K.E., 1999. Quantitative RT-PCR: pitfalls and potential BioTechniques 26, 112-125.

Guenthner, P.C., Hart, C.E., 1998. Quantitative, competitive PCR assay for HIV-1 using a microplate-based system. BioTechniques 24, 810-816.

Holloway, J.W., Leonard, B.R., Ottea, J.A., Pankey, J.H., Graves, J.B., 1998. Insecticide resistance and synergism of pyrethroid toxicity in the tarnished plant bug, *Lygus lineolaris*. Proc. Beltwide Cotton Conf. pp. 947-949.

Hung, C.F., Bernebaum, M.R., Schuler, M.A., 1997. Isolation and characterization of CYP6B4, a furanocoumarin-inducible cytochrome P450 from a polyphagous caterpillar (Lepidoptera: Papilionidae). Insect Biochem. Molec. Biol. 27, 377-385.

Igaz, P., Fejér, G., Szalai, C., Tóth, S., Falus, A., 1998. Development of competitive mRNA PCR for the quantification of interleukin-6-responsive *junB* oncogene expression. BioTechniques 24, 854-859.

Kasai, S., Scott, J.G ., 2000. Overexpression of cytochrome P450 CYP6D1 is associated with monooxygenase-mediated pyrethroid resistance in house flies from Georgia. Pestic. Biochem. Physiol. 68, 34-41.

Li, X., Berenbaum, M.R., Schuler, M.A., 2000. Molecular cloning and expression of CYP6B8: a xanthotoxin-inducible cytochrome P450 cDNA from *Helicoverpa zea*. Insect Biochem. Molec. Biol. 30, 75-84.

Liu, N., Scott, J.G., 1998. Increased transcription of CYP6D1 causes cytochrome P450-mediated insecticide resistance in house fly. Insect Biochem. Molec. Biol. 28, 531-535.

Ma, R., Cohen, M.B., Berenbaum, M.R., Schuler, M.A., 1994. Black swallowtail (*Papilio polyxenes*) alleles encode cytochrome P450s that selectively metabolize linear furanocoumarins. Arch. Biochem. Biophys. 310, 332-340.

Ranasinghe, C., Campbell, B., Hobbs, A.A., 1998. Over-expression of cytochrome P450 CYP6B7 mRNA and pyrethroid resistance in Australian populations of *Helicoverpa armigera* (Hubner). Pestic. Sci. 54, 195-202.

Scott, J.G., Liu, N., Wen, Z., 1998. Insect cytochromes P450: Diversity, insecticide resistance and tolerance to plant toxins. Comp. Biochem. Physiol. C. Pharmacol. Toxicol. Endocrinol. 121, 147-155.

Scott, J.G., Liu, N., Wen, Z., Smith, F.F., Kasai, S., Horak, C.E., 1999. House-fly cytochrome P450 CYP6D1: 5' flanking sequences and comparison of alleles. Gene Amsterdam 226, 347-353.

Snodgrass, G.L., 1996. Insecticide resistance in field populations of the tarnished plant bug (Heteroptera: miridae) in cotton in the Mississippi Delta. J. Econ. Entomol. 89, 783-790.

Snodgrass, G.L., Scott, W.P., 2000. Seasonal changes in pyrethroid resistance in tarnished plant bug (Heteroptera: miridae) populations during a three year period in the Delta area of Arkansas, Louisiana, and Mississippi. J. Econ Entomol. 93, 441-446.

Tomita, T., Liu, N., Smith, F.F., Sridhar, P., Scott, J.G., 1995. Molecular mechanisms involved in increased expression of a cytochrome P450 responsible for pyrethroid resistance in the housefly, *Musca domestica*. Insect Mol. Biol. 4, 135-140.

Tomita, T., Scott, J.G., 1995. cDNA and deduced protein sequence of CYP6D1: the putative gene for a cytochrome P450 responsible for pyrethroid resistance in house fly. Insect Biochem. Molec. Biol. 25, 275-283.

Wang, X.P., Hobbs, A.A., 1995. Isolation and sequence analysis of a cDNA clone for a pyrethroid inducible cytochrome P450 from *Helicoverpa armigera*. Insect Biochem. Molec. Biol. 25, 1001-1009.

Zhang, M., Scott, J.G., 1996. Cytochrome b_5 is essential for cytochrome P450 6D1-mediated cypermethrin resistance in LPR house flies. Pestic. Biochem. Physiol. 55, 150-156.

Zhu, Y.C., Kramer, K.J., Dowdy, A.K., Baker, J.E., 2000. Trypsinogen-like cDNAs and quantitative analysis of mRNA levels from the Indianmeal moth, *Plodia interpunctella*. Insect Biochem. Molec. Biol. 30, 1027-1035.