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Acetylcholinesterase point mutations putatively associated with monocrotophos resistance in the two-spotted spider mite

Deok Ho Kwon [a], Jae Seong Im [a], Jeong Joon Ahn [a], Joon-Ho Lee [a], J. Marshall Clark [b], Si Hyeock Lee [a,*]

[a] Department of Agricultural Biotechnology, Seoul National University, Seoul 151-742, Republic of Korea
[b] Department of Veterinary & Animal Sciences, University of Massachusetts, Amherst, MA 01003, USA

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ABSTRACT

Molecular mechanisms of monocrotophos resistance in the two-spotted spider mite (TSSM), *Tetranychus urticae* Koch, were investigated. A monocrotophos-resistant strain (AD) showed ca. 3568- and 47.6-fold resistance compared to a susceptible strain (UD) and a moderately resistant strain (PyriF), respectively. No significant differences in detoxification enzyme activities, except for the cytochrome P450 monooxygenase activity, were found among the three strains. The sensitivity of acetylcholinesterase (AChE) to monocrotophos, however, was 90.6- and 41.9-fold less in AD strain compared to the UD and PyriF strains, respectively, indicating that AChE insensitivity mechanism plays a major role in monocrotophos resistance. When AChE gene (*Tuace*) sequences were compared, three point mutations (G228S, A391T and F439W) were identified in *Tuace* from the AD strain that likely contribute to the AChE insensitivity as predicted by structure analysis. Frequencies of the three mutations in field populations were predicted by quantitative sequencing (QS). Correlation analysis between the mutation frequency and actual resistance levels (LC_{50}) of nine field populations suggested that the G228S mutation plays a more crucial role in resistance ($r^2 = 0.712$) compared to the F439W mutation ($r^2 = 0.419$). When correlated together, however, the correlation coefficient was substantially enhanced ($r^2 = 0.865$), indicating that both the F439W and G228S mutations may work synergistically. The A391T mutation was homogeneously present in all field populations examined, suggesting that it may confer a basal level of resistance.

1. Introduction

The two-spotted spider mite (TSSM), *Tetranychus urticae* Koch, is one of the most serious pests in ornamental plants and vegetable crops grown in greenhouses worldwide. TSSM has rapidly developed resistance to almost all types of acaricides, including organophosphates (OPs), due to its short life cycle, high biotic potential and arrhenotokous parthenogenesis reproduction [1,2]. OP-resistant TSSM populations were first found in rose greenhouses in the Eastern United States as early as 1948 and now exist in over 40 countries in both greenhouses and field crops [3,4].

OP resistance in TSSM is primarily due to acetylcholinesterase (AChE) insensitivity, which was first reported in a parathion-selected strain [5]. Similar AChE insensitivity mechanisms have also determined in other OP-resistant TSSM strains by biochemical studies [6–10]. Point mutations likely responsible for AChE insensitivity were identified by cloning and sequence comparisons of the AChE gene (*Tuace*) [11]. Among the seven single nucleotide polymorphisms (SNPs) identified, the F439C mutation, which occurred at one of the conserved aromatic residues lining the active-site gorge, was predicted as a major mutation conferring OP resistance. The S228G and D237E mutations, both located near the catalytic center, were also suggested to affect AChE activity. However, no direct correlations of any of these mutations have been made with actual levels of resistance.

The OP monocrotophos has been widely used to control TSSM since its registration in 1981 in Korea and field TSSM populations resistant to monocrotophos were first reported in 2002 [12]. Early resistance monitoring is essential for efficient resistance management and spray and leaf dipping methods have been used in traditional resistance monitoring of TSSM. These bioassay-based methods, however, require a large time commitment and are costly. In view of this, rapid detection of mutations responsible for AChE insensitivity in field populations of TSSM has been suggested as an alternative resistance monitoring method. Recently, a quantitative sequencing (QS) method was developed as a population genotyping method to detect pyrethroid resistance-associated mutations in human head louse populations [13].

Abbreviations: TSSM, two-spotted spider mite; AChE, acetylcholinesterase; *Tuace*, TSSM acetylcholinesterase gene; TuAChE, acetylcholinesterase in TSSM; QS, quantitative sequencing.

* Corresponding author. Fax: +82 2 873 2319.
E-mail address: shlee22@snu.ac.kr (S.H. Lee).

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In this study, we report additional AChE mutations putatively associated with resistance through extensive comparisons of the *Tuace* sequences among three different mite strains with different monocrotophos sensitivities. With this information, a QS protocol was developed for the prediction of AChE mutation frequencies in field populations of TSSM, and used to assess the functional importance of each mutation by correlating the actual resistance level (LC_{50}) to the mutation frequencies estimated by QS. Finally, we discussed the potential of QS as an alternative resistance monitoring tool for the estimation of resistance allele frequencies in the field mite populations.

2. Materials and methods

2.1. Mite strains

The insecticide-susceptible strain (UD) was collected from *Chelidonium majus* Linnaeus in June 2006 on Ulleung Island, South Korea, and has been reared on soybean plants at 25 ℃ and 50–60% humidity without exposure to any acaricides. A monocrotophos-resistant strain (AD) of TSSM was collected from an apple orchard in Andong in 2004 and have been selected six times using a LC_{75} concentration of monocrotophos (dimethyl phosphate of 3-hydroxy-N-methyl-cis-crotonamide). The UD strain possessed the exactly same morphological characters, including the number of sitae on the tibia and the shape of genitalia, but showed slightly different nucleotide sequences in mitochondrial cytochrome oxidase subunit I gene (mtCOI) and ribosomal internal transcribed spacer 2 (ITS2), when compared to all other TSSM strains including the AD strain [14]. Phylogenetic analysis based on the nucleotide sequences of mtCOI and ITS2 suggested that the UD strain was a relatively more ancient form belonging to the *T. urticae* complexes (unpublished data). A moderately insecticide-resistant strain (PyriF), which has the same genetic backgrounds to the AD strain, was obtained from National Academy of Agricultural Science (NAAS) of Rural Development Administration (Suweon, Korea). Nine additional field populations of TSSM were collected from apple orchards in three different regions in Korea and have been maintained in this laboratory as described above.

2.2. Bioassay

Monocrotophos (24% SL, Dongbu HiTek, South Korea) was serially diluted with deionized water to a final concentration range of 0.15–960 ppm. Diluted solutions were applied as a spray for 10 s using a spray gun (Solo Spraystar 460, Newport News, VA) to 30 female mites that were placed on the top surface of a 2.5-cm diameter soybean leaf disc, which was placed on a water-soaked cotton pad. Each bioassay was replicated five times and mortality determined at 48 h post-treatment. LC_{50} values were calculated from log concentration versus probit mortality regression lines using the POLO plus program (LeOra Software Company, Petaluma, CA).

2.3. Enzyme preparation

Female mites (ca. 30 mg) were homogenized in 300 μl extraction buffer (0.1 M Tris–HCl, pH 7.8) using a 2-ml glass–glass tissue grinder (Radnoti, Monorovia, CA) and the homogenate centrifuged at 10,000*g* for 15 min. The supernatant was used as the enzyme source for the esterase (EST), glutathione-*S*-transferases (GST), cytochrome P450 monooxygenase (P450) and AChE activity determinations. The enzyme source for AChE activity determination was prepared as above except that the extraction buffer contained 0.1% Triton X-100. Protein concentrations were determined using the Bradford reagent (Sigma, St. Louis, MO) with bovine serum albumin as the standard protein.

2.4. Enzyme activity assays

2.4.1. EST

Enzyme source (5 μg protein) was incubated with 0.2 mM 1-naphthyl acetate (1-NA, Sigma) for 5 min at 25 °C. The reaction was terminated by adding 50 μl stop solution (0.2% fast blue RR salt and 0.2% sodium dodecyl sulfate) and the optical density measured at 595 nm using Bio-Rad 550 microplate reader (Bio-Rad, Hercules, CA). The same amount enzyme was also incubated with either 0.2 mM 4-nitrophenyl acetate (4-NPA) or 0.2 mM 4-nitrophenyl butyrate (4-NPB) for 10 min at 25 °C and the optical density measured at 405 nm as above.

2.4.2. GST

GST activity assay was conducted using the method of Yu with a slight modification [15]. Enzyme source (5 μg protein) was incubated with 20 mM 1-chloro-2.4-dinitrobenzene (CDNB) and 10 mM GSH (reduced glutathione) for 10 min at 25 °C and optical density measured at 355 nm using a Victor3™ plate reader (Perkin–Elmer, Waltham, MA).

2.4.3. P450

Enzyme source (20 μg protein) was incubated with 0.4 mM 7-ethoxycoumarin (7-EC) and 1 mM NADPH for 30 min at 30 °C in the wells of a 96-well microtiter plate. Fluorescence of NADPH was removed oxidizing it to the non-fluorescent NADP+ with the addition of 10 μl of oxidized glutathione (100 mM in water) and 20 μl glutathione reductase (1 U) [16]. After 10 min at room temperature, the reaction was stopped with 125 μl 50% acetonitrile in 50 mM Tris buffer (pH 10). The amount of 7-hydroxycoumarin released during incubation was quantified using the SPECTRAmax GEMINI XS spectrofluorometer (Molecular Device, Inc., Sunnyvale, CA) at 390 nm excitation and 465 nm emission wavelengths.

2.4.4. AChE

AChE activity was measured by the Ellman method with slight modification [17]. For the calculation of activities, the enzyme source (ca. 4.5 μg protein) was incubated with 125 μM 5,5-dithio-bis(2-nitrobenzoic acid) (DTNB, Sigma) and 100 μM acetylthiocholine iodide (ATChI) at 25 °C for 30 min. The AChE activity was measured at 415 nm using Victor3™ plate reader (Perkin–Elmer). Monocrotophos (ChemService, PA, USA) inhibition assays for the calculation of median inhibition concentrations (IC_{50}) was conducted by the preincubating the enzyme source with monocrotophos (0.1–100 μM) for 40 min prior to the addition of 100 μM ATChI and 125 μM DTNB. The remaining AChE activity was measured as described above.

2.5. cDNA cloning of Tuace

Total RNA was extracted from female mites (ca. 20 mg) with 400 μl TRI reagent (Molecular Research Center, Inc., Cincinnati, OH) according to manufacturer's instructions, and cDNA synthesized from the total RNA with Superscript cDNA synthesis kit (Invitrogen, Carlsbad, CA). The *Tuace*-specific primers were designed from previously reported sequences (GenBank Accession No. AAO73450). cDNA fragments, which contained complete open reading frames (ORFs), were PCR-amplified (10 μM primers, 250 μM dNTP, 1 U TAKARA Ex Taq™ DNA polymerase) from the UD, PyriF and AD strains using 35 thermal cycles of 94 °C/30 s, 56 °C/30 s and 72 °C/150 s. The ORF sequence was determined using a primer walking method with a series of primers (Table 1) by an ABI prism 3730 DNA sequence analyzer (PE Applied Biosystem, Foster City, CA).