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Characterization of acephate resistance in the diamondback moth *Plutella xylostella*

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ABSTRACT

Decreased acetylcholinesterase (AChE) sensitivity and metabolic detoxification mediated by glutathione *S*-transferases (GSTs) were examined for their involvement in resistance to acephate in the diamondback moth, *Plutella xylostella*. The resistant strain showed 47.5-fold higher acephate resistance than the susceptible strain had. However, the resistant strain was only 2.3-fold more resistant to prothiofos than the susceptible strain. The resistant strain included insects having the A298S and G324A mutations in AChE1, which are reportedly involved in prothiofos resistance in *P. xylostella*, showing reduced AChE sensitivity to inhibition by methamidophos, suggesting that decreased AChE1 sensitivity is one factor conferring acephate resistance. However, allele frequencies at both mutation sites in the resistant strain were low (only 26%). These results suggest that other factors such as GSTs are involved in acephate resistance. Expression of GST genes available in *P. xylostella* to date was examined using the resistant and susceptible strains, revealing no significant correlation between the expression and resistance levels.

1. Introduction

The diamondback moth *Plutella xylostella* is a major pest of brassicas worldwide [1]. Resistance to various kinds of insecticides including organophosphates (OPs) has been reported in *P. xylostella*. Two major molecular mechanisms—changes in sensitivity of the target site [2] and increased metabolic detoxification [3–5]—have been proposed as conferring resistance to OPs in *P. xylostella*.

Acetylcholinesterase (AChE) catalyzes the hydrolysis of the neurotransmitter, acetylcholine, to terminate the influx transmission at cholinergic synapses. The OPs bind to the active site of AChE and inhibit activity by phosphorylating a serine residue in the enzyme's catalytic center [6], leading to repetitive firing of the postsynaptic nerve, to desensitization of the nervous system, and eventually to death [7]. In *P. xylostella* and some other insects, AChEs (AChE1 and AChE2) of two types have been identified, which differ in their substrate and inhibitor specificity [8–11]; *Drosophila melanogaster* has only one AChE gene encoding AChE2. Lee et al. [12], using three-dimensional modeling and functional expression in the *Sf9* cells, showed that two amino acid mutations (A298S and G324A) in AChE1 are involved in target-insensitive resistance to prothiofos in *P. xylostella*. However, no mutations putatively associated with resistance to OPs have been identified in *AChE2* [13].

Resistance to OPs in *P. xylostella* is also conferred by enhanced activity of metabolic detoxification systems including glutathione *S*-transferases (GSTs) [4,5]. In fact, GSTs are a family of proteins involved in detoxification of endogenous and xenobiotic compounds in vertebrates and invertebrates [14]. They have the capacity to conjugate reduced glutathione on the thiol of cysteine to various electrophiles and to bind with high affinity to various hydrophobic compounds [14]. Furthermore, some GSTs catalyze a dehydrochlorination reaction using reduced glutathione as a cofactor rather than a conjugate [15]. Chiang and Sun [4] and Ku et al. [16] showed that GSTs are involved in the degradation of and resistance to some OPs in *P. xylostella* through purification and characterization of four GST isozymes from its larvae. Among the isozymes, PxGSTe (formerly denoted as GST-3 by Chiang and Sun [4]) showed distinct substrate preference for some OPs such as parathion, methyl parathion, and paraoxon [4].

In the present study, the full-length cDNA sequences of *AChE1* and *AChE2* were compared between *P. xylostella* strains with different sensitivities to acephate. Results suggest that previously identified mutations (A298S and G324A) in AChE1 are involved in acephate resistance, but some other factors are also involved in the resistance. Then, four GST genes including a novel GST gene (*PxGSTd*) were examined for their involvement in the acephate resistance using the strains. No significant correlation between the expression and resistance levels was found for any GST gene, which suggests that some unidentified enzyme or mechanism is involved in the acephate resistance shown by *P. xylostella*.

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2. Materials and methods

2.1. Insects

An OP-susceptible (SS) strain was obtained from Sumitomo Chemical Co. Ltd., Osaka, Japan, in 2004. The SS strain was collected at Katano City, Osaka Prefecture, to initiate a laboratory culture in 1970, where it has since been maintained without insecticide selection. A multi-resistant (MR) strain was provided by Nihon Nohyaku Co., Ltd., Tokyo, Japan, in 2007; it has been selected with several insecticides including acephate (300 ppm). The MR strain was collected at Kishiwada City, Osaka Prefecture, to initiate a laboratory culture in 1997. Insects were maintained with radish seedlings at 25 °C under a long photoperiod (16L: 8D).

2.2. Bioassay

Acephate (orthene 15% liquid formulation) and prothiofos (tokuthion 45% E.C.) were purchased, respectively, from Sumitomo Chemical Garden Products Co. Ltd., Tokyo, Japan and Bayer Crop Science, Tokyo, Japan. Susceptibilities to acephate and prothiofos were determined using newly molted fourth-instar larvae. The leaf-dipping bioassay technique was used. Six concentrations plus control were used for bioassay. After 48 h, the numbers of dead or moribund larvae and surviving larvae were recorded. The LC_{50} value was estimated for each strain using probit analysis [17]. For acephate-induction of GST genes, insects of the MR strain treated with 300 ppm of acephate for 12 h were used.

2.3. RNA extraction, cDNA synthesis and amplification of AChE1 and AChE2

Total RNA extraction from *P. xylostella* and first-strand cDNA synthesis were performed as described in Sonoda et al. [18]. The PCR primers for *AChE1* and *AChE2* were designed based on the nucleotide sequences of *P. xylostella* reported by Lee et al. [12] (GenBank/EMBL/DDBJ Accession No. AY970293) and Ni et al. [13] (Accession No. AY061975), respectively, and used to amplify cDNA fragments.

The partial clone of *AChE1* was amplified by PCR using the primers ace1-L-5′ (5′-accggcaagaaggtcgacgc-3′) and ace1-3′ (5′-cgcagacactcgatcatagg-3′). Three and five clones from the SS and MR strains were sequenced, respectively. For 3′-rapid amplification of cDNA ends (RACE), PCR was performed using primers ace1-5′ (5′-gcggaaaccctcacaatgtc-3′) and M4 (Takara Bio Inc., Ohtsu, Japan). Subsequently, nested PCR was performed using primers ace1-5′-2 (5′-atgcaatctgcagccgcatc-3′) and M4. Three clones were sequenced for each strain. For 5′-RACE, PCR was conducted using the 5′-full RACE core set (Takara Bio Inc.) following the manufacturer's instructions: 1st PCR was conducted using the primers ace1-5′ and ace1-3′-3 (5′-tcgcgttgaggatctcatcg-3′), followed by 2nd PCR using the primers ace1-5′-2 (5′-atgcaatctgcagccgcatc-3′) and ace1-3′-2 (5′-aggtcgccgatgggtttctg-3′). Three and four clones were sequenced, respectively, from the SS and MR strains.

The partial clone of *AChE2* was amplified using PCR with the primers ace-5′ (5′-catatcggaggattgcctctatttgaac-3′) and ace2-3′-3 (5′-aagactgcatgatatgcgcg-3′). To amplify the 3′-end of *AChE2*, PCR was conducted with primers ace2-5′-4 (5′-acgagatggagtacgtgttc-3′) and ace2-R51 (5′-gaatgtgtctatctccgtcttc-3′). Subsequently, nested PCR was conducted with primers ace2-5′-5 (5′-cgctgaacatgtctcttcag-3′) and ace2-R51. The 5′-end of *AChE2* was amplified using PCR with the primers ace2-5′ (5′-gtcggactagagagccattg-3′) and ace-3′ (5′-ctggagacaacatgtgtagactcacgc-3′).

The PCR conditions were 1 cycle of 3 min at 94 °C, followed by 30 cycles of 30 s at 94 °C, 1 min at 60 °C and 1 min at 72 °C, finishing with final extension at 72 °C for 7 min.

2.4. Amplification of a GST gene belonging to the delta class (PxGSTd)

The initial clone of *PxGSTd* was amplified using PCR with degenerate primers i-gst-5′ (5′-aarhtgaayccycaacacac-3′) and i-gst-3′ (5′-ggwacardgtkccgatrtcraa-3′). The primers were designed based on nucleotide sequences of *Bombyx mori* (Accession No. AB176691) and *Choristoneura fumiferana* (Accession No. EF370473). The 5′ and 3′ segments of *PxGSTd* were amplified using RACE. For 3′-RACE, PCR was performed against the cDNA using the primer set i-gst-5′ and M4 adaptor primer (Takara Bio Inc.). Subsequently, nested PCR was performed using the primer set i-gst-5′-1 (5′-catcctcacatacctcgtcaac-3′) and M4. For 5′-RACE, cDNA was constructed separately from 0.5 μg of total RNA using a smart RACE cDNA amplification kit (Clontech, Palo Alto, CA, USA) and PCR was performed against the cDNA using the primer set i-gst-3′ and the 10× universal primer (10× UPM) (Clontech). Subsequently, nested PCR was performed using the primer set i-gst-3′-1 (5′-gttgacgaggtatgtgaggatg-3′) and the nested universal primer (NUP) (Clontech).

The PCR conditions were 1 cycle of 3 min at 94 °C, followed by 30 cycles of 30 s at 94 °C, 1 min at 60 °C and 1 min at 72 °C, finishing with final extension at 72 °C for 7 min.

2.5. Cloning and nucleotide sequencing

The PCR-amplified fragments were cloned into pGEM-T Easy (Promega Corp., Madison, WI, USA). The obtained clones were sequenced using a dye terminator cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) with M13 forward and reverse primers and applied to a DNA sequencer (3100 Avant Genetic Analyzer; Applied Biosystems).

Obtained sequences were analyzed using software (Genetyx ver. 9; Software Development Co. Ltd., Tokyo, Japan).

2.6. Direct sequencing

Nucleotide sequences involving the A298S and G324A mutations were examined by direct sequencing using cDNA and genomic DNA. Genomic DNA extraction was performed as described in Sonoda et al. [18]. The cDNA and genomic fragments containing both mutations sites were amplified individually from the SS and MR strains using the primers ace1-5′-4 (5′-tggttcggcatcccctacgc-3′) and ace1-3′; then they were sequenced directly using the primer ace-seq2 (5′-ggccacgctagatgtttatg-3′).

2.7. Inhibitory effect of methamidophos on AChEs

The inhibitory effect of methamidophos, the putative bioactive-metabolite of acephate, on AChEs was measured using the method described by Ellman et al. [19] with a minor modification. Fifteen 4th instar larvae were homogenized in 50 mM Tris–HCl buffer (pH 7.5) containing 0.1% Triton X-100; the homogenate was centrifuged at 5000 *g* for 10 min. The supernatant was diluted 10 times with 50 mM Tris–HCl buffer (pH 7.5) as enzyme source. Then 40 μl of enzyme source was added to each well of a microtiter plate containing 100 μl of 50 mM Tris–HCl buffer (pH 7.5) and 20 μl of DTNB solution (0.4 mM DTNB + 1.5 mM $NaHCO_3$). Then 40 μl of various concentrations of methamidophos was added to each well and incubated at 30 °C for 10 min. After adding 40 μl of substrate, 1.5 mM acetylthiocholine (ATCh) in 50 mM Tris–HCl buffer (pH 7.5), the enzyme mixture was incubated at 30 °C for 30 min. The OD at 405 nm was measured using a microplate reader (model