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The identification and characterisation of a new deltamethrin resistance-associated gene, *UBL40*, in the diamondback moth, *Plutella xylostella* (L.)

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ABSTRACT

Differential expression of ubiquitin was previously reported between *Plutella xylostella* strains that are resistant or susceptible to the pesticide deltamethrin (DM). This finding hinted at the potential involvement of ubiquitin in deltamethrin resistance, a theory that demanded further testing. Real-time PCR analyses revealed that one of the ubiquitin genes, *UBL40*, was overexpressed in the deltamethrin-resistant strain during the fourth instar. To investigate the functional relationship between this gene and deltamethrin resistance, RNA interference (RNAi) and cell transfection were utilised. *UBL40* knockdown was observed to significantly reduce the level of resistance in RNAi-treated larvae after 48 h. Conversely, overexpression of *UBL40* in *Drosophila* Kc cells conferred a degree of protection against deltamethrin. These results represent the first evidence that *UBL40* plays a role in the regulation of deltamethrin resistance in *P. xylostella*.

1. Introduction

The diamondback moth, *Plutella xylostella* (L.) has been the most destructive insect to Cruciferae crops worldwide, and the annual cost of control is extraordinary (Talekar and Shelton, 1993). At present, chemical control is the principal method employed to manage the insect pest. Synthetic pyrethroid insecticides are the most commonly used pesticides for both agricultural and household use because of their effectiveness, their limited persistence in soil, and their low toxicity to humans and other mammals (Sayeed et al., 2003; Soderlund et al., 2002; Yang et al., 2008). Unfortunately, following their long-term and widespread application, resistance to these insecticides has emerged, presenting a difficult obstacle for pest control (Moulton et al., 2000; Widawsky et al., 1998).

Resistance to pesticides involves a number of genes (Sayyed and Wright, 2004). Conventional resistance models can be explained by one of three mechanisms: reduced cuticular penetration (Noppun et al., 1989), target site resistance (due to mutation of target genes) (Soderlund and Knipple, 2003), or metabolic resistance. The voltage-sensitive sodium channel in nerve cell membranes is generally considered the primary target of pyrethroid insecticides and DDT; in fact, reduced channel sensitivity has been identified as one of the major mechanisms responsible for resistance to these compounds. This mode of resistance, which was first observed in *Musca domestica* (Busvine, 1951) and later recognised in *P. xylostella* (Schuler et al., 1998), was termed knockdown resistance (kdr). Previous studies have shown that metabolic resistance is closely associated with enhanced activity of P450 enzymes (Shen et al., 2003), nonspecific esterases (Hemingway et al., 2000; Paton et al., 2000) and glutathione S-transferases (Vontas et al., 2001). The identification and characterisation of additional pesticide resistance related genes continue (Pedra et al., 2004). Our previous studies showed that ubiquitin was differentially expressed in deltamethrin-resistant and deltamethrin-susceptible strains of *P. xylostella* (Cheng et al., 2005).

Ubiquitin is a highly conserved protein that plays an essential role in the intracellular turnover of proteins (Ozkaynak et al., 1987). There are two universally conserved types of ubiquitin genes. The first class codes for the polyubiquitin gene, which encodes a precursor protein containing head-to-tail tandem repeats that are posttranslationally cleaved into monomers. The second class, consisting of the *UBL40* and *UBS27* genes, encodes an N-terminal UBI protein fused to ribosomal proteins L40 and S27, respectively (Baker and Board, 1991; Finley et al., 1989; Mezquita et al., 1997; Ozkaynak et al., 1987). In mammals, polyubiquitin is induced by stress (Flick and Kaiser, 2012), and *UBS27* is overexpressed in response to growth (Wong et al., 1993). During tumour cell apoptosis,

Abbreviations: DM, Deltamethrin; PCR, Polymerase Chain Reaction; qPCR, quantitative real-time PCR; dsRNA, double-stranded RNA; RNAi, RNA interference; DDT, Dichlorodiphenyltrichloroethane; kdr, knockdown resistance; RH, relative humidity; LC_{50}, Lethal Concentration 50; es, elution solution; DMSO, dimethyl sulfoxide; CCK-8, Cell Counting Kit-8; SD, standard deviation; bp, base pair; kDa, kiloDalton.

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ubiquitin fusion proteins exhibit altered dynamics (Han et al., 2012). No previously published report has mentioned a possible role for UBI in pesticide resistance.

In this study, the polyubiquitin and hybrid ubiquitin genes from *P. xylostella* were cloned. Quantitative real-time PCR was utilised to establish the expression profiles of the three UBI genes in DM-resistant and DM-susceptible *P. xylostella*. RNAi and cell transfection studies were also conducted to investigate the role of ubiquitin in DM resistance.

2. Materials and methods

2.1. P. xylostella

The DM-susceptible strain of *P. xylostella* was originally collected from the Huaxi cabbage fields of Guiyang, China and toxicity evaluation showed that it was a deltamethrin-susceptible strain compared to the Wuhan susceptible strain. It was cultured in our laboratory for use as a standard susceptible strain.

The DM-resistant strain of *P. xylostella* was produced from its susceptible counterpart through selection in our laboratory (Liu et al., 1995) by continuous exposure of the susceptible strain to DM. After 180 generations of selection, the resulting resistant strain was maintained using previously described methods (FAO, 1979), while the susceptible strain was kept without exposure to any insecticide within the laboratory setting. Larvae from both strains were kept at 25 ± 1 °C on cabbage leaves and radish seedlings with a photoperiod of 16 h light/8 h dark and 75% relative humidity (RH).

2.2. RNA extraction and cDNA synthesis

Total RNA was extracted from 10 fourth instar *P. xylostella* larvae using the RNeasy Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. The extracted RNA was subjected to 1% agarose gel electrophoresis to evaluate RNA integrity. RNA concentration and purity were measured using a NanoDrop 2000 spectrophotometer (Thermo Scientific, USA). First-strand cDNA was reverse-transcribed using the PrimeScript® RT Reagent Kit (Takara, Japan) according to the manufacturer's protocol.

2.3. Cloning and sequencing of UBL40

To clone the full-length coding sequence of *UBL40*, a pair of degenerate primers was used (forward primer 5′-ATGCAGATCTTYGTGAARACC-3′, reverse primer 5′-YTAYTTSAVCTTCTTCTTGGG-3′). The PCR program was as follows: initial denaturation at 95 °C for 3 min, followed by 30 reaction cycles (denaturation at 95 °C for 30 s, annealing at 45 °C for 30 s and extension at 72 °C for 1 min) and a final extension at 72 °C for 10 min. cDNA fragments were separated by 1% agarose gel electrophoresis, and the *UBL40* PCR product was purified using the Nucleic Acid Purification Kit (Axygen, USA). The purified PCR product was ligated into the pMD 19-T vector (Takara, Japan) at 16 °C for 30 min, and then the ligation mixture was transformed into *Escherichia coli* DH5α competent cells, which were subsequently cultured on an LB plate containing ampicillin (100 mg/ml), IPTG (24 mg/ml) and X-Gal (20 mg/ml). White colonies were picked and subjected to colony PCR to confirm the presence of the desired product. Plasmid DNA was purified using the AxyPrep Plasmid Miniprep Kit (Axygen, USA) and sequenced using an automated DNA Sequencer (Applied Biosystems, USA).

2.4. Quantitative real-time PCR analysis

The relative expression levels of *UBL40* in DM-resistant and DM-susceptible *P. xylostella* larvae in the fourth instar were determined by quantitative real-time PCR. Total RNA was isolated separately from 10 fourth instar larvae for each of the two strains using the RNeasy Mini Kit. First-strand cDNA synthesis was carried out using the PrimeScript® RT Reagent Kit. The cDNA mixture was diluted at 1:10 and stored at −70 °C until qRT-PCR analysis. The LightCycler PCR and detection system (Roche, Switzerland) was used for amplification and real-time quantification, utilising the housekeeping gene *GAPDH* as an internal control. The primers used for qRT-PCR were designed to target conserved sequences of *P. xylostella UBL40*. Two pairs of specific primers were designed using Primer Premier 5 software (Lalitha, 2000). For *UBL40*, the forward and reverse primers were 5′-TTGGTGTTGAGGC TTAGAGGAGGTA-3′ and 5′-GGCGGAGGTTGTTGGTGTGTC-3′, respectively; for *GAPDH*, the forward and reverse primers were 5′-TGGA AGGTGGTGCCAAGAA-3′ and 5′-AAGGGGAGCGAGGCAGTTAG-3′, respectively. Amplification was carried out in 20-μl reactions containing 10 μl of 2 × SYBR Premix Ex Taq (Takara, Japan), 2 μl of cDNA, 0.4 μl (each) of 10 μM forward and reverse primers, and 7.2 μl of ddH_2O. The real-time PCR program was as follows: initial denaturation at 95 °C for 30 s, followed by 45 cycles of denaturation at 95 °C for 5 s, annealing at 56 °C for 10 s and extension at 72 °C for 15 s. A melting curve analysis was conducted immediately following completion of the PCR program with denaturation at 95 °C for 5 s, annealing at 65 °C for 60 s and 95 °C continuously. All analyses were based on normalised Ct values, which were read automatically with the included software. Each sample was analysed in triplicate, and Ct values were averaged. The relative expression levels of *UBL40* in the two *P. xylostella* strains were analysed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The expression level of *UBL40* in the susceptible strain was designated as the background level and normalised to 1. To ensure reproducibility of the results, the experiment was performed three times with independently purified RNA samples.

2.5. UBL40 gene silencing

2.5.1. dsRNA synthesis

A double-stranded RNA corresponding to a portion of *UBL40* was synthesised using the MEGAscript RNAi Kit (Ambion, USA) with cDNA and specific primers including a T7 recognition region (in bold): sense primer 5′-**TAATACGACTCACTATAGGG**CGAAAATGTCAAAGCC-3′ and antisense primer 5′-**TAATACGACTCACTATAGGG**GGAGGTTGTTGGTGTG-3′. The dsRNA was dissolved in the elution solution (es) provided with the MEGAscript RNAi Kit, quantified using a spectrophotometer and stored at −20 °C until use.

2.5.2. Injection of dsRNA

Injection into fourth instar *P. xylostella* larvae of the DM-resistant strain was performed under a dissecting microscope (Olympus, Japan). Larvae were anaesthetised with CO_2 for 2–3 min and were then aligned on the microscope slides. We microinjected either 160 ng of *UBL40* dsRNA or an equal volume of elution solution as a negative control into the dorsal side of the thoracic segment of each larva using a standard micromanipulator (WPI, USA). The larvae were allowed to feed in glass petri dishes at 25 ± 1 °C, 75% RH and 16: 8 h (light:dark). Larvae were considered to be alive when movement occurred in response to touch.

2.5.3. qPCR verification

At 48 h post-injection, total RNA was extracted from ten *UBL40*-treated and control larvae, and first-strand cDNA was reverse-transcribed as previously described. Rotor-Gene Q (Qiagen, Germany) was utilised and real-time PCR was conducted for knockdown efficiency analysis. The experiment was repeated three times.

2.6. Bioassay

The toxicity of DM to fourth instar larvae, which had been subjected to either RNAi or elution solution (buffer) injection conditions, was assessed using the standard procedure for LC_{50} determination. A topical application method was employed and five different concentrations of