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Molecular cloning of *Bombyx mori* cytochrome P450 gene and its involvement in fluoride resistance

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ABSTRACT

To investigate the effects of fluorosis on development and gene expression profiles of silkworm, highly resistant silkworm strain 441, and highly susceptible silkworm strain 440 were treated with 200 ppm fluoride (designated as 440F and 441F) and water (designated as 440DZ and 441DZ). Fluorotic silkworm showed body color and behavior changes. Statistical analysis indicated that growth index of 440F was lower than 440DZ, 441DZ, and 441F. The mortality of 440F was higher than others. Fluorescent differential display enabled us to obtain a differentially expressed cDNA. Bioinformatics analyses indicated that it belonged to cytochrome P450 family, denoted *Bmcyp306a1*, which contained seven exons and six introns. Phylogenetic tree showed BmCYP306A1 had high homology with *Manduca sexta*' P450 protein. Expression analysis indicated that *Bmcyp306a1* was exclusively expressed in 441DZ and 441F and was down-regulated under fluoride treatment. The tissue-specific expression indicated *Bmcyp306a1* had high-expression level in midgut and ovary in 441F. The data revealed that there was obvious dose–effect and times relationship with the pathological changes and gene expression. Expression profiles of *Bmcyp306a1* suggested that P450 gene was crucial to physiological modification and might be involved in fluoride resistance.

1. Introduction

As a major pollutant in industrial areas, fluoride is present in varying amount in the air, water, and in some of the agricultural products produced in the polluted areas. Fluoride of high concentration has toxic effects on both humans and animals, causing toxification such as endemic fluorosis [1] and industrial fluorosis [2,3]. Fluorosis causes damage to many animal and human organs [1,4,5], predominately the skeletal systems and teeth. Simultaneously, the structures and functions of the non-skeletal systems such as brain, liver, kidney, and spinal cord are also damaged [6–8]. Previous studies have demonstrated that the damage caused by fluoride intoxication is mainly mediated through lipid peroxidation. Yet little is known about the mechanism of detoxification.

Many studies have proved that cytochrome P450 was involved in the production and metabolism of many molecules with important physiological functions and was believed to play important roles in detoxification. Currently, the cytochrome P450 superfamily consists of over 5500 designated sequences, in which approximately 1300 sequences were found in animals (http://drnelson.utmem.edu/cytochrome p450.html) [9]. Many P450 genes have multiple functions while a vast majority of cytochrome P450 have unknown functions. In human, 15 cytochrome P450 proteins can metabolize xenobiotics and all of them are from *CYP1*, *CYP2* ,and *CYP3* families [10]. Due to involvement of the cytochrome P450 in physiological functions, many of them have been studied in detail in different tissues and cell types. The temporal and tissue-specific expression and regulation are very important to cytochrome P450 involved in steroid and eicosanoid biosynthesis and catabolism of vitamins [11]. It may be valuable to predict the function of cytochrome P450 on tissue- and cell-specific expression.

Silkworm is one of the economically important insects. Silk industry plays an important role in China. However, silk production has been seriously affected by fluoride pollution. In this study, highly resistant silkworm strain 441 [12] and highly susceptible silkworm strain 440 were used as materials to analyze differential expression of related genes and to find out whether the crucial genes were related to fluoride detoxification. Using FDD (fluorescent differential display) and *in silico* cloning methods, a full-length cDNA sequence encoding the putative P450 protein was obtained and named as *Bmcyp306a1*. Expression analysis suggested that *Bmcyp306a1* gene might play an important role in resistance to fluoride. Our study provides new information on the involvement of P450 protein in fluoride detoxification.

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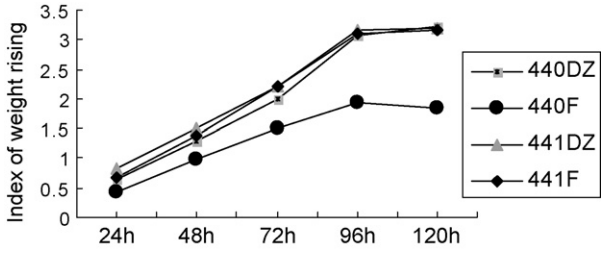

Fig. 1. The change of body weight. According to data in Table 1, the growth index of body weight was analysed.

2. Materials and methods

2.1. Fluoride

Sodium fluoride (NaF): 200 ppm.

2.2. Silkworm strains

Two silkworm strains were used. Strain 440 was highly susceptible to fluoride. Strain 441 was highly resistant to fluoride. Strain 441 was maintained in our laboratory through adding fluoride in its fodder. Its fluoride-tolerance ability was 200 ppm at the time of experimentation. All larvae were fed with mulberry leaves three times a day at 25 ± 2 °C under a 12 h light/12 h dark cycle and were maintained in two separate groups. All larvae were raised up to the fifth instar. Starting from the fifth instar, the larvae were fed with mulberry leaves treated with either clean water (control) or with 200 ppm NaF solution for 5 min. Then, 50 larvae of similar size were taken for investigating their physiological changes, respectively. Larvae of strains 440 and 441 feeding on leaves treated with water were designated as 440DZ and 441DZ, respectively. Those feeding on leaves treated with NaF were designated as 440F and 441F.

2.3. Pathological change investigation

Pathological changes were investigated from fifth instar to pupal stage. The observed items included body weight, body color, behavior, individual size, and mortality. The body weight was measured at 7:00 a.m. before they were fed with mulberry leaves. Growth index was calculated by using the body weight at the test time to divide the body weight at the beginning of experiment. For example, if the weight of a larva is 2 g at 7:00 a.m. of the first day of fifth instar and the same larva weighs 3 g at 7:00 a.m. of the second day, the growth index will be 1.50 which is 3 divided by 2.

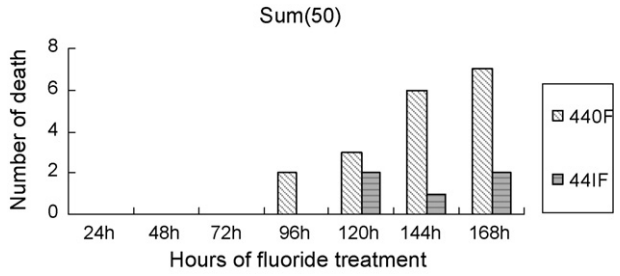

Fig. 2. Statistics of silkworm mortality. Larvae in groups 440DZ and 441DZ grew normally and did not die. Those of group 440F were found to die from 96 to 168 h with the average number of death increasing from 2 to 7, while those of group 441F was found to die from 120 h, with the number of death being below 2.

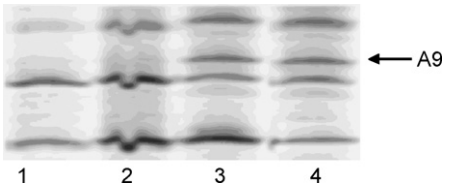

Fig. 3. Result of the FDD analysis from only one pair of arbitrary primers. Lane 1, 440DZ; lane 2, 440F; lane 3, 441DZ; lane 4, 441F; lane 1 and lane 3, from midguts after fluoride treatment for 48 h; lane 2 and lane 4, from midguts after water treatment for 48 h. The differential expression band A9 is indicated by an arrow. A9 was expressed in fluoride resistant strain 441.

2.4. Total RNA extraction and reverse transcription

At 48 h of the fifth instar, 10 silkworms were sampled from each group for isolating total RNAs. Total RNAs were isolated from the midguts using Trizol (Invitrogen) reagent. The total RNAs were treated with RNase-free DNase (Promega, WI). The RNA precipitates were dissolved in 50 mL of 10 mM Tris/1 mM EDTA buffer (pH 8.5) and spectrophotometrically quantified at 260 nm. The integrity of the isolated RNA was examined on an ethidium bromide stained, denaturing 1% agarose gel. Single stranded cDNAs for PCR were synthesized from total RNAs (2 μg for all tissues) with MMLV reverse transcriptase (Promega, WI).

2.5. Fluorescent differential display analysis

For FDD–PCR (RNAspectra^{TM} Kit, GenHunter), the total RNAs (2 μg) were reverse-transcribed with MMLV reverse transcriptase. 3′-Anchored oligo-T11A (FH-T11A) primer and arbitrary primers (HAP9-HAP21) were used. For each PCR, 2-μL first-strand cDNA was added into a mixture containing 1 unit of Taq polymerase (TaKaRa, Dalian), 50 μmol/L of each dNTP, 10 pmol of arbitrary primers,10 pmol of fluorescein *iso*-thiocyanate labeled 3′-anchored oligo-T11A (FH-T11A) primer, and 2.0 μL of 10× PCR buffer. FDD–PCR was performed using the following thermal cycling conditions: 94 °C for 3 min, then 40 cycles of 94 °C for 30 s, 40 °C for 2 min and 72 °C for 2 min, followed by a final extension at 72 °C for 5 min. Each PCR product was electrophoresed in a 6% denaturing polyacrylamide gel in the 1× TBE buffer. The FDD gel was scanned with the FMBIO II (Hitachii Genetic System). The differential bands of interest were cut and extracted to recover cDNAs. Re-amplified cDNA products were cloned into pM18-T vectors (Takara) and then transformed into *E. coli* strain DH5α followed by clone selection based α-complementation. The plasmids were purified using the MiniBEST Plasmid Purification Kit (TaKaRa, Dalian) and were sequenced using CEQ8000 (Beckman).

2.6. Molecular cloning

Blast searches in NCBI were used to show that it had high similarity to ESTS (GenBank no. AV404609) of *B. mori*. Using *in silico* cloning method, its cDNA sequence was obtained. According to the sequence, specific primers were designed as follows: cross-primer pair 1 (sense primer 5′-GTCGACTATCAAGTAATATGG ACC-3′ and anti-sense primer 5′-CTTCA GTCGGAATAAGAGTA-3′) and cross-primer pair 2 (sense primer 5′-TGGCAATGGTTTCGTCAA-3′ and anti-sense primer 5′-CTCGAGTTAA ATTGGTTCGCAAT AG-3′). Both primer pairs were used to re-amplify the target sequence.

2.7. Bioinformatic analysis

A homology search of the DNA sequences against GenBank (http://www.ncbi.nlm.nih.gov/) was performed. The cDNA sequence was compared with the silkworm genomic