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Cloning, functional characterization, and expression profiles of NADPH-cytochrome P450 reductase gene from the Asiatic rice striped stem borer, *Chilo suppressalis* (Lepidoptera: Pyralidae)

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ABSTRACT

NADPH-cytochrome P450 reductase (CPR) is one of the most important components of the cytochrome P450 enzyme system. It catalyzes electron transfer from NADPH to all known P450s, thus plays central roles not only in the metabolism of exogenous xenobiotics but also in the regulation of endogenous hormones in insects. In this study, a full-length cDNA encoding of a CPR (named CsCPR) was isolated from the Asiatic rice striped stem borer, *Chilo suppressalis*, by using reverse transcription-polymerase chain reaction (RT-PCR) and rapid amplification of cDNA ends (RACE) methods. The cDNA contains a 2061 bp open reading frame, which encodes an enzyme of 686 amino acid residues, with a calculated molecular mass of 77.6 kDa. The deduced peptide has hallmarks of typical CPR, including an N-terminal membrane anchor and the FMN, FAD and NADPH binding domains. The N-terminal-truncated protein fused with a 6 × His·tag was heterologously expressed in *Escherichia coli* Rosetta (DE3) cells and purified, specific activity and the K_m values of the recombinant enzyme were determined. Tissue- and developmental stage-dependent expression of *CsCPR* mRNA was investigated by real-time quantitative PCR. The *CsCPR* mRNA was noticeably expressed in the digestive, metabolic, and olfactory organs of the larvae and adults of *C. suppressalis*. Our initial results would provide valuable information for further study on the interactions between CPR and cytochrome P450 enzyme systems.

1. Introduction

Cytochrome P450 monooxygenase systems in insects play important roles not only in the metabolism of a wide range of foreign compounds such as insecticides and plant secondary metabolites, but also in the regulation of endogenous substrates (Scott et al., 1998; Feyereisen, 1999). However, typical examples of P450 enzymes catalytic cycle require an electron donor, the NADPH-cytochrome P450 reductase (CPR) (EC 1.6.2.4) (Paine et al., 2005). CPR has several conserved functional domains, including the flavin mononucleotide (FMN), the flavin adenine dinucleotide (FAD) and the nicotinamide adenine dinucleotide phosphate (NADPH) binding regions, which are involved in the transfer of electrons from NADPH to the central heme-group of the P450s through a series of redox-coupled reactions (Feyereisen, 1999; Paine et al., 2005). CPR also shuttles electrons to other oxygenase enzymes including cytochrome b_5 (Schenkman and Jansson, 1999, 2003) and heme oxygenase (Wang and de Montellano, 2003) in most eukaryotes.

So far, multiple P450 gene families have been identified and functionally characterized from several insect species for which partial or completed genome sequences are available (Tijet et al., 2001; Ramsey et al., 2010; Xue et al., 2010; Ai et al., 2011; Zhu et al., 2013). However, there is typically only one CPR gene that exists in each insect genome (Nelson, 2009). Several literatures revealed that the addition of CPR to the lysates of cells heterologously expressing P450s results in an increased catalytic activity of plant allelochemicals (Mayer and Prough, 1977; Wen et al., 2003; Sasabe et al., 2004; Murataliev et al., 2008; Mao et al., 2009), and that knockdown of the CPRs of the malaria mosquito, *Anopheles gambiae* and the bed bug, *Cimex lectularius* by RNA interference (RNAi) leads to enhanced susceptibility to pyrethroids (Lycett et al., 2006; Zhu et al., 2012). In addition, abundant CPR transcripts were detected in the antennae of the fruit fly, *Drosophila melanogaster*, the cotton leafworm *Spodoptera littoralis* and the cabbage armyworm, *Mamestra brassicae*, indicating a putative function in olfactory clearance (Hovemann et al., 1997; Maïbèche-Coisne et al., 2005; Pottier et al., 2012). CPR mRNA was also expressed at a high level in the oenocytes of the fruit fly, and involved in the synthesis of the cuticular hydrocarbons (Qiu et al., 2012). These studies clearly demonstrated essential roles for insect CPRs involved in P450 system-mediated metabolism to insecticides, environmental xenobiotics and endogenous

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compounds, and validated its potential as a target for developing synthetic inhibitors to manage insect pests.

The Asiatic rice striped stem borer, *C. suppressalis* (Lepidoptera: Pyralidae) is a notorious rice lepidopteran pest in East Asian countries, causing great economic losses to cultivated rice crops (Zhu et al., 2007). For a long time, control of the insect pest mainly relies on insecticide application in various geographical areas. However, indiscriminate usage of pesticides has resulted in resistance during the past two decades (He et al., 2007, 2008), some of the chemical insecticides have become ineffective to control the pest even at relatively high rates (Qu et al., 2003; He et al., 2008). In this case, alternative management strategies are sorely needed. P450s participate in the metabolism of agrochemicals in all kingdoms of agricultural pests (Li et al., 2007b), and CPR plays important roles in the P450-mediated catalytic reactions by supplying electrons (Paine et al., 2005). Therefore, CPR can be a novel target for the development of environmental friendly pest management strategies.

We are interested in exploring the *C. suppressalis CPR* (*CsCPR*) as a potential target to manage the rice pest, as well as related species in the Order Lepidoptera, which includes many of the most disruptive agricultural pests (Xia et al., 2004). In the present study, we report: (1) cloning and sequence analysis of *CsCPR*; (2) activity and kinetic assays of *CsCPR*; and (3) tissue, developmental and sexual expressions of *CsCPR*. The preliminary results will provide a solid foundation for future functional research of insect CPRs.

2. Materials and methods

2.1. Insects

The *C. suppressalis* colony used in this study was originated from a field population collected in the China National Rice Research Institute, Fuyang, China. Insects were reared on rice variety Taichung Native 1 (TN1, susceptible to almost all herbivores of rice) in an insectary at 26 ± 1 °C, 75% relative humidity under a 16:8 h light:dark cycle.

2.2. RNA extraction and cDNA synthesis

Total RNA was extracted from *C. suppressalis* third-instar larvae by using a TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and was treated with RNase-free DNase I (Takara, Dalian, China) to avoid genomic DNA contamination. The quality and concentration of total RNA sample was determined by a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and gel electrophoresis. 1 μg of the total RNA was reverse transcribed to cDNA using the PrimeScript 1st Strand cDNA Synthesis Kit (Takara, Dalian, China), according to the manufacturer's manual.

2.3. Molecular cloning of CsCPR

Degenerate primers were designed according to known moth CPR cDNA sequences obtained from the GenBank: forward: 5′-GAAGAATGY GATRTGGARGAACT-3′, reverse: 5′-GGGAAWGGATGYTTYTTGCT-3′, and were used to amplify the partial *CsCPR* fragment. Polymerase chain reaction (PCR) was performed in a Bio-Rad C1000 thermal cycler using KOD FX DNA polymerase (Toyobo, Osaka, Japan). The thermal cycling condition was as follows: one cycle at 94 °C for 2 min, followed by 35 cycles at 98 °C for 30 s, 55 °C for 30 s and 68 °C for 1 min. The PCR product was gel-purified, ligated into the pGEM-T easy vector (Promega, Madison, WI), transformed into *Escherichia coli* DH5α competent cells and sequenced in both directions (Sunny Biotech., Shanghai, China).

To obtain the complete open reading frame (ORF) of *CsCPR*, the rapid amplification of cDNA ends (RACE) method was performed by using the 5′-Full RACE Kit and 3′-RACE Core Set Ver. 2.0 (Takara), in accordance with the manufacturer's instructions. Gene-specific primers for RACE were designed according to the previous isolated partial *CsCPR* fragment: 5-race: 5′-CACCCTCACCGTAAGTTGCCAT-3′, 3-race: 5′-GCAAGGCTTCACA CCTATCAAGT-3′. Finally, a cDNA fragment including complete ORF of *CsCPR* was amplified by using a pair of primers: forward: 5′-GTGTTGTG CTCGCAATAG-3′, reverse: 5′-GCCTAACTCCACACGTCT-3′, and was confirmed to be the target gene by DNA sequence.

2.4. Analyses of cDNA and its deduced amino acid sequences

The theoretical isoelectric point and molecular mass of the deduced *CsCPR* protein were calculated using the ExPASy ProtParam tool (http://web.expasy.org/protparam/). Sequence identities and searching for orthologs were performed using BLASTp (http://blast.ncbi.nlm.nih.gov/). Transmembrane domain and N-terminal signal peptide were predicted by using TMHMM (http://www.cbs.dtu.dk/services/TMHMM/) and SignalP (http://www.cbs.dtu.dk/services/SignalP/), respectively. Deduced amino acid sequences of CPRs from *C. suppressalis* and other insect species were aligned by using ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/), a phylogenetic tree was constructed by the neighbor-joining method (1000 bootstrap replications) using the MEGA 5.0 software (Tamura et al., 2011).

2.5. Prokaryotic expression and purification of CsCPR

Protein expression was performed by following the procedure described previously (Lamb et al., 2001). An N-terminal-truncated *CsCPR* cDNA fragment was amplified by KOD Plus DNA polymerase (Toyobo) using a set of primers: forward: 5′-GGTACCTCCAGGAAAGAAAATAAGA-3′, reverse: 5′-GGATCCCTAACTCCACACGTCTGAT-3′, and subcloned into the *Kpn* I/*BamH* I sites of the pET30a vector, which was used to transform *E. coli* Rosetta (DE3) cells.

For protein expression, a single colony was used to inoculate the liquid Luria–Bertani (LB) medium (with 50 μg/ml final concentration of kanamycin) and grown on a shaker at 37 °C, 220 rpm until OD_{600} was 0.6, then isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to 0.3 mM final concentration. After growing at 24 °C, 180 rpm for 6 h, cells were harvested by centrifugation at 3000 ×*g* for 10 min at 4 °C, washed twice with 50 mM Tris·HCl pH 7.4, 500 mM NaCl, resuspended and lysed by sonication on ice. After centrifuging at 15,000 ×*g* at 4 °C for 20 min, the supernatant was loaded on a HisTrap column (GE Healthcare) and eluted with a linear gradient of 0–200 mM imidazole in 50 mM Tris·HCl pH 7.4, 500 mM NaCl. Purified protein was desalted by the Centricon centrifugal filter device YM-30 (30,000 MWCO) (Millipore, Ireland) using 50 mM Tris·HCl pH 7.4 as mobile phase. The purity of recombinant *CsCPR* was analyzed by 12% (w/v) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the concentration of the protein was measured with a BCA protein assay kit (Thermo Scientific).

2.6. Enzyme assays

The activity of recombinant *CsCPR* was assayed by monitoring the increase of absorbance at 550 nm when cytochrome *c* was reduced (Yang et al., 2010). Briefly, the 1 mL reaction mixture contained 1 μg protein preparation and 50 μM cytochrome *c* (horse heart, extinction coefficient of 21.4 mM^{-1} cm^{-1}) in 50 mM Tris·HCl pH 7.4 at 25 °C. The reaction was initiated by adding 10 μL 5 mM NADPH (final concentration, 50 μM). The time-dependent absorption increase was monitored on an Eppendorf Biophotometer Plus instrument (Eppendorf, Hamburg, Germany). Each sample was carried out in triplicate, enzyme activity was expressed as micromoles of reduced cytochrome *c* produced per min per mg of enzyme (Guengerich et al., 2009).

Methods for estimating kinetic parameters of recombinant *CsCPR* were described previously by Zhou et al. (2011). Briefly, all assays and incubations were carried out in 50 mM Tris·HCl pH 7.4 at 25 °C. The kinetic parameter for cytochrome *c* was determined by adding 50 μM NADPH to the reaction mixtures containing varying concentrations of