Identification of Four ATP-Binding Cassette Transporter Genes in *Cnaphalocrocis medinalis* and Their Expression in Response to Insecticide Treatment

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Abstract

The ATP-binding cassette (ABC) transporters belong to a superfamily of genes involved in the transport of specific molecules across lipid membranes, as well as insecticide resistance, present in all living organisms. In this study, we combined the *Cnaphalocrocis medinalis* transcriptome database with a bioinformatics approach to identify four *C. medinalis* ABCs (CmAABCs), including CmAABCG1, CmAABCG4, CmAABCC2 and CmAABCC3. Tissue expression analysis showed that these genes had a tissue-specific expression pattern. CmAABCG1 had significantly higher expression in the haemolymph and head compared to the other tissues. The expression of CmAABCG4, CmAABCC2 and CmAABCC3 was highest in the midgut, followed by expression in the fat body. The developmental stage expression analysis showed that CmAABCG1, CmAABCG4, CmAABCC2 and CmAABCC3 were mainly expressed in adults. The transcription of CmAABCG1, CmAABCG4 and CmAABCC2 was significantly induced by chlorpyrifos. Taken together, the results of our study provided useful information for understanding of the detoxification system of *C. medinalis*.

Key words: *Cnaphalocrocis medinalis*, ABC transporter, mRNA expression, insecticide detoxification

The rice leaffolder, *Cnaphalocrocis medinalis* (Lepidoptera: Pyralidae) is a major pest of rice crops, and is widely distributed in tropical and temperate rice-producing regions (Khan et al. 1988, Riley et al. 1995). The control of rice insect pests is mainly dependent on the extensive use of chemical insecticides. However, this results in disturbance of the environment and resistance of pests to insecticides (Abudulai et al. 2001, Huang et al. 2003). Metabolic resistance and target resistance are the two major mechanisms underlying insecticide resistance. Metabolic resistance relies on detoxification enzymes such as glutathione S-transferases (GSTs), carboxylesterases (CarEs) and cytochrome P450 monoxygenases (P450s) (Ranson et al. 2002). Liu et al. (2015b) identified 25 candidate GST genes and 36 putative P450 genes in *C. medinalis* by searching the transcriptome dataset, which may be involved in the insecticide detoxification displayed by these moths. The involvement of ABC transporters in xenobiotic resistance for arthropods was historically not well documented, but an increasing number of studies were focused on differentially gene expression analysis to show their importance (Dermauw and Lzeuwen 2014). ATP-binding cassette (ABC) transporters have been identified as important detoxification enzymes in *Plutella xylostella*, in addition to GSTs, CarEs and P450s (He et al. 2012). Epis et al. (2014) also identified ABC transporter genes from *Anopheles stephensi* based on transcriptome data, and the results confirmed the role of ABC transporter in insecticide defense. ABC transporters also play an important role in the detoxification of insecticides.

ABC transporters belong to a large superfamily responsible for the transport of specific molecules across lipid membranes, in addition to insecticide resistance, present in all living organisms (Higgins 1992). The multidomain integral membrane proteins of ABC transporters utilize the energy produced by ATP hydrolysis to translocate solutes across cellular membranes in all phyla (Jones and George 2004). ABC transporters have a modular structure, consisting of two nucleotide-binding domains (NBDs), which bind and hydrolyze ATP, as well as two transmembrane domains (TMDs), which are involved in the translocation of their respective substrates (Rees et al. 2009). The NBDs contain characteristic motifs, including Walker A, Walker B, D-loop, and Q-loop. ABC transporter genes also contain the signature C motif, located just upstream of the Walker B site (Dean et al. 2001). Sequence analyses of
eukaryotic ABC transporters revealed that they can be divided into eight subfamilies (ABCA-H) (Dean and Annilo 2005). In recent years, many more species genomes have been sequenced. Liu et al. (2010) and Xie et al. (2012) identified 51 putative ABC transporter genes in silkworms, classified into eight subfamilies (A-H) based on whole-genome analyses. Broehan et al. (2013) found 73 ABC transporter genes in the genome of Tribolium castaneum, which can also be grouped into eight subfamilies (ABCA-H). The ABC family of coleopterans is significantly larger than that reported for insects in other taxonomic groups. In Tetranychus urticae, 103 ABC transporter genes have been identified, which is the highest number discovered in a metazoan species to date (Dermauw et al. 2013). Furthermore, the different subfamilies of ABC transporters may be involved in different functions. The ABC transporter C subfamily is thought to be involved in drug resistance (Labbé et al. 2011). Gahan et al. (2010) reported an inactivating mutation of the ABC transporter ABC2 which was associated to Cry1Ac resistance, as a result of inhibiting the binding of Cry1Ac to membrane vesicles. In Drosophila melanogaster, the white, brown and scarlet genes encode proteins that transport guanine or tryptophan, all of which belong to the ABC transporter superfamily. These proteins play a major role in the absorption of pigment precursors during eye development (Mackenzie et al. 1999). However, no studies have investigated the ABC transporter genes in C. medinalis.

In this study, we identified four complete C. medinalis ABC transporters (CmABCs) based on a transcriptome database from our laboratory, which were named CmABCG1, CmABCG4, CmABCC2 and CmABCG3 (Yu et al. 2015). All sequences were analyzed by bioinformatic, and their molecular characteristics and functions were predicted. Real-time quantitative PCR (RT-qPCR) analysis was performed to determine their level of expression in different tissues and at different developmental stages, as well as their response to chlorpyrifos exposure. The results of our study will contribute to better understanding the detoxification systems of C. medinalis, in addition to assisting in the establishment of new target sites for insect control.

Materials and Methods

Insect Collection and Sample Preparation

Leaffolder larvae were collected from the rice paddy field at the Rice Institute of Anhui Academy of Agricultural Sciences and were reared in insectary for a few days based on previous method (Liao et al. 2012). The first instar, second instar, the first day of third instar, fourth instar, the first day of pupae and adults were collected. On the first day of fourth-instar, midgut, head, integument, fat body and haemolymph samples were obtained from C. medinalis larvae. All tissues samples were ground in liquid nitrogen and stored at −80°C.

Insecticide Treatment

In order to understand the function of ABC transporter genes relevant to the field of insect pest control, fourth-instar larvae of C. medinalis were treated with an insecticide. Chlorpyrifos was purchased from Biogen Crop Science Limited (Shanxi, Weinan, China). In order to simulate exposure of C. medinalis to insecticides in the field, chlorpyrifos was diluted to a working solution with sterile water (1:300). Fresh rice leaves were picked and placed in sterile plastic boxes, and each leaf was sprayed with 1 ml of the working chlorpyrifos solution (Goh et al. 2011, Painkra and Salam 2013). The boxes were covered with plastic netting to prevent C. medinalis escape (n = 20 in each group). Control insects were treated with sterile water only. Each treatment was performed 3 biological replicate and 10 larvae were treated in each replicate. Data was statistically analyzed by a paired Student’s t-test. All midgut samples were collected after 24 h, frozen in liquid nitrogen and stored at −80°C.

RNA Isolation and cDNA Synthesis

Total RNA was isolated from the integument, head, midgut, fat body and haemolymph of the larvae and adults using TRizol reagent, according to the manufacturer’s instructions. The A260/280 ratio and RNA concentration of the samples were detected using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, New York, NY). The total RNA samples were treated with PrimeScript™ RT kit with gDNA Eraser (TaKaRa, Dalian, China) to remove any genomic DNA, then the first strand cDNA was synthesized according to the manufacturer’s instructions. Briefly, 2.0 µl of 5× gDNA Eraser buffer, 1.0 µl of gDNA Eraser and 1.0 µg of total RNA were mixed in a 100-µl PCR tube, then RNase Free dH2O was added to reach 10 µl, which was then incubated at room temperature for 5 min. Afterwards, 5× PrimeScript buffer, 1.0 µl PrimeScript RT enzyme mix I and 1.0 µl of RT primer mix were added to the tube, which was made up to 20 µl with RNase Free dH2O. The mixture was incubated at 37°C for 15 min, and then incubated at 85°C for 5 s. The cDNA was stored at −20°C for later use.

Identification of ABC Genes From the Cnaphalocrocis medinalis Transcriptome

Transcriptome sequencing was performed on fourth-instar C. medinalis larvae using the Illumina pair-end sequencing method (Biomarker Technology Company, Beijing, China) to obtain the C. medinalis transcriptome database. The ABC transporter genes were identified from the dataset using the TBLASTN algorithm in Basic Local Alignment Search Tool (BLAST). The candidate ABC genes for C. medinalis were confirmed by searching the NCBI nonredundant protein database using BLASTX (cut-off E value of 10−5). The bioinformatic sequence analyses revealed that CmABCG1, CmABCG4, CmABCC2 and CmABCG3 had complete sequences.

Bioinformatic Analysis

The molecular mass and isoelectric point was predicted using the Compute pI and Mw tool (http://us.expasy.org/tools/pi_tool.html). The open reading frames (ORFs) of the ABC genes were predicted using ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). Conserved domains were determined using the NCBI conserved domain database (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). The homology of amino acid sequences was determined with DNAMAN 7.0 software (Lynnon Biosoft, Vandreuil, QC, Canada). The phylogenetic tree was constructed with MEGA 5.0 software using the neighbor-joining method with 1,000-fold bootstrap resampling (Tamura et al. 2011).

Developmental and Tissue-Specific Expression Profiles of the CmABCs

In order to detect the expression of CmABCG1, CmABCG4, CmABCC2 and CmABCG3 in different tissues (midgut, head,
integument, fat body and haemolymph) and at different developmental stages (first instar, second instar, the first day of third instar, fourth instar, the first day of pupal and adult), four pairs of RT-qPCR primers were designed, including the forward and reverse primers of *CmA BC G1*, *CmA BC G4*, *CmA BC C2* and *CmA BC C3* (Table 1). The 25-μl reaction mixture for RT-qPCR contained 12.5 μl SYBR II, 9.5 μl ddH₂O, 1.0 μl forward primer, 1.0 μl reverse primer and 1.0 μl cDNA template. The thermal cycle profile began with an incubation at 50 °C for 2 min, then denaturation for 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Each gene was assayed in three replicates. The reactions were performed in 96-well plates using the Multicolor RT-PCR Detection System (Bio-Rad, Hercules, CA). The *CmA C tin* gene (GenBank: JN029806.1) served as the internal control. The relative expression levels were calculated using the 2^ΔΔCt method, following the protocol described by Livak and Schmittgen (2001). Data was statistically analyzed by ANOVA, and Fisher's least significant difference (LSD post hoc) test was used to determine between group differences. All statistical analyses were performed using SPSS software (Version 19).
Expression Patterns of CmABC at Different Developmental Stages

In order to determine the expression of CmABCs at different developmental stages, we performed RT-qPCR with cDNA from the first instar, second instar, the first day of the third instar and fourth instar larval stages, as well as the pupal and adult stages. The expression levels of CmABCG1, CmABCG4, and CmABCC2 were low in the early instar larvae (first, second, third and fourth stages) when compared to the late instar stages (pupae and adult). The expression increased sharply from pupae to adult. Interestingly, the relative expression of CmABCG1 and CmABCC2 showed an increasing trend during the second instar. The expression of CmABCC3 fluctuated considerably from first-instar larvae to adults. CmABCC3 was expressed highly in the second instar, fourth instar and adult stages, but expression was low in pupae.

Gene Expression Profile After Insecticide Treatment

The results obtained for CmABC expression showed that most genes had high expression in the midgut, except CmABCG1. Therefore, the fourth-instar larval midgut was selected as the target tissue for insecticide exposure. In previous studies, the organophosphate insecticide chlorpyrifos has been effectively used for the control of a wide range of pests, including Chilo suppressalis, planthoppers and C. medinalis (Zheng et al. 2011, Zhang et al. 2014). The expression of the four genes was compared between chlorpyrifos-treated and untreated control insects. We found that the expression of CmABCG1, CmABCG4 and CmABCC2 increased remarkably 24 h after chlorpyrifos exposure, indicating that these genes are likely to be involved in the insecticide detoxification process. However, there was no significant difference in CmABCC3 expression between the chlorpyrifos-treated and untreated control insects.

Discussion

Insect ABC transporters can be classified into eight subfamilies (ABCA-H), according to phylogenetic tree analysis (Walker et al. 1981). In this study, we identified four ABC transporters by transcriptome analysis, and analyzed their conserved domains. These results are consistent with that previously reported for a member of the silkworm G subfamily (Liu et al. 2011). Additionally, some ABC transporters of members of the G subfamily have been demonstrated to confer resistance to xenobiotics, including insecticides (Labbé et al. 2011). The amino acid sequences of CmABCC2 and CmABCC3 were found to contain two ABC_membrane and two AAA domains.
Fig. 3. Phylogenetic relationships of ATP-binding cassette (ABC) transporters from five insect species, Cnaphalocrocis medinalis (Cm); Bombyx mori (Bm), Drosophila melanogaster (CG), Tribolium castaneum (Tc) and Tetranychus urticae (Tu). The phylogenetic tree was constructed with MEGA5 software using the neighbor-joining method. Bootstrap values indicate the analysis of 1,000 replicates. The ABC transporters of C. medinalis are highlighted in red.

Fig. 4. Expression of CmABC genes in different larval tissues. The statistical analysis used the lowest expression level as a reference, and the significant differences are indicated by * (P < 0.05) or ** (P < 0.01).
metals via metal-responsive transcription factor 1, and is involved in the biochemical detoxification of zinc and copper (Yepiskoposyan et al. 2006). The results obtained from the structural domain analysis, combined with that previously reported by others, suggests that \textit{CmABCG1}, \textit{CmABCG4}, \textit{CmABCC2} and \textit{CmABCC3} are likely to be involved in transmembrane transport of xenobiotics. However, its functions were required for further study.

Based on the phylogenetic tree analysis, the ABC transporters can be widely classified into two subfamilies (C and G). In \textit{Tetranychus urticae}, the ABCC subfamily consists of 39 transporters, and phylogenetic analysis showed that 23 of the ABCCs of \textit{T. urticae} were clustered with amino acid sequences of CG6214 and Dappul347281 from \textit{D. melanogaster} and \textit{Daphnia pulex}, respectively (Dermauw et al. 2013). Sturm et al. (2014) reported the presence of seven ABCC family transporters in \textit{D. pulex}. The phylogenetic tree of \textit{Daphnia}, human, worm and \textit{Drosophila} ABCC proteins is characterized by comparatively low support of the more basal nodes, which most probably reflects the heterogeneity of the ABCC subfamily. Our results showed that \textit{CmABCC2} amino acid sequence is closely related to \textit{TcABCC-4A}, and \textit{CmABCC3} is closely related to \textit{TcABCC-9A}. Within the G subfamily, \textit{CmABCG1} appears to be most closely related to the \textit{Drosophila} ABCC CG7346, and \textit{CmABCG4} is most closely related to \textit{BmABC010557}. These results are suggestive of similar physiological function and evolutionary relatedness between insect species.

The expression of the ABC transporters differ in different tissues and at different developmental stages, depending on the species. \textit{CmABCG4}, \textit{CmABCC2} and \textit{CmABCC3} were found to be tissue-specific ABC genes. They were more highly expressed in the midgut and fat body than the other tissues evaluated. The insect midgut is a
major tissue for the metabolism of various chemicals from food. The midgut epithelium is the first physical barrier after oral intake, and contains abundant digestive enzymes needed to obtain nutrients from food, and also contributes to the detoxification of insecticides (Smagghe and Tirry 2001). In silkworms, BmABC005226, BmABC005203 and BmABC010555 were shown to be exclusively expressed in the midgut, as determined by RT-qPCR. However, we reported that CmABCG1 is highly expressed in the haemolymph and head. Insect haemolymph contains a variety proteins involved in insecticide detoxification. Therefore, CmABCG4, CmABCC2 and CmABCC3 may play an important role in detoxification, with the midgut as a target. The expression analysis at different developmental stages showed that CmABCG1, CmABCG4 and CmABCC2 were highly expressed in adults, with lower expression levels observed in the early instar larvae (first, second, third and fourth instar). In a previous study, Guo et al. (2015) found that expression of PxABCH1 was highest in the head of adult males, as determined by spatial-temporal expression analysis. We speculated that C. medinalis had a greater detoxification ability in adults when compared to larvae. Interestingly, our RT-qPCR results also showed that CmABCG1 expression was up-regulated by 11.8 times from the fourth instar larvae to the pupal stage (Fig. 5). In silkworm, BmABC010555 was reported to be highly expressed during the wandering and prepupal stages. However, the function of CmABCG1 in the pupae was unknown. CmABCC3 expression showed considerable fluctuation from first instar to adult, and expression was lowest on the first day of the third instar stage. We considered that the first day of the third instar was at molting stage, which was regulated by edcsyne and juvenile hormone. In an unpublished study of D. melanogaster, we found that several ABCG transporters were exclusively expressed in two important endocrine organs, the corpus allatum, which produces juvenile hormone, and the prothoracic gland, which produces edcsyne (the immediate precursor to 20E).

In recent years, ABC transporters have been identified as detoxification enzymes, in addition to GSTs, CarEs and P450s. Chlorpyrifos is a broad-spectrum organophosphate insecticide. This compound can bind to the active site of cholinesterase (ChE), resulting in accumulation of acetylcholine in the synaptic cleft (Karanth and Pope 2000). In this study, we found that expression of CmABCG1, CmABCG4 and CmABCC2 was induced after chlorpyrifos application, and therefore, these genes are possibly involved in the detoxification of insecticides. The expression of several insecticide genes can be induced by xenobiotics, and these are known to be involved in the detoxification of insecticides. Epis et al. (2014) reported that AnstABCG4 expression was threefold higher in permethrin-treated Anopheles stephensi compared to the untreated control insects after 24 h. In pyrethroid-resistant Culex pipiens, CpGSTD1 was shown to be able to metabolize 1,1,1-trichloro-2,2-bis (4-chlorophenyl) ethane (DTT) (Samra et al. 2012). However, we found no significant differences in the expression of CmABCG3 between chlorpyrifos-treated and untreated control insects. Therefore, we speculate that CmABCG3 is mainly associated with C. medinalis development.

In conclusion, we identified four ABC transporter genes in C. medinalis for the first time, in addition to analyzing their structure and expression patterns, based on bioinformatic and RT-qPCR approaches. These results will assist in establishing new target sites for insect control, and provide a theoretical basis for the development of biopesticides.

Supplementary Data

Supplementary data are available at Journal of Insect Science online.

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