Insect Biochemistry and Molecular Biology 99 (2018) 54–62

Contents lists available at ScienceDirect

Insect Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/ibmb

Cap 'n' collar C regulates genes responsible for imidacloprid resistance in the Colorado potato beetle, *Leptinotarsa decemlineata*

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ARTICLE INFO

Keywords:
Insecticide resistance
RNA sequencing
RNA interference
Xenobiotic transcription factor
ABC transporter
P450

ABSTRACT

The Colorado potato beetle (CPB), *Leptinotarsa decemlineata* developed resistance to imidacloprid after exposure to this insecticide for multiple generations. Our previous studies showed that xenobiotic transcription factor, cap 'n' collar isoform C (CncC) regulates the expression of multiple cytochrome P450 genes, which play essential roles in resistance to plant allelochemicals and insecticides. In this study, we sought to obtain a comprehensive picture of the genes regulated by CncC in imidacloprid-resistant CPB. We performed sequencing of RNA isolated from imidacloprid-resistant CPB treated with dsRNA targeting CncC or gene coding for green fluorescent protein (control). Comparative transcriptome analysis showed that CncC regulated the expression of 1798 genes, out of which 1499 genes were downregulated in CncC knockdown beetles. Interestingly, expression of 79% of imidacloprid induced P450 genes requires CncC. We performed quantitative real-time PCR to verify the reduction in the expression of 20 genes including those coding for detoxification enzymes (P450s, glutathione S-transferases, and esterases) and ABC transporters. The genes coding for ABC transporters are induced in insecticide resistant CPB and require CncC for their expression. Knockdown of genes coding for ABC transporters simultaneously or individually caused an increase in imidacloprid-induced mortality in resistant beetles confirming their contribution to insecticide resistance. These studies identified CncC as a transcription factor involved in regulation of genes responsible for imidacloprid resistance. Small molecule inhibitors of CncC or suppression of CncC by RNAi could provide effective synergists for pest control or management of insecticide resistance.

1. Introduction

Insects exhibit a wide range of insecticide resistance mechanisms including enhanced metabolism mediated by detoxification enzymes, target site insensitivity, reduced penetration and behavioral resistance. Previous studies have shown that the Colorado potato beetle (CPB), *Leptinotarsa decemlineata* is one of the most successful insects to evolve insecticide resistance as its genome codes for a sophisticated xenobiotic metabolic enzyme network for detoxification of plant toxins and insecticides (Schoville et al., 2018; Zhu et al., 2016). For instance, this pest has developed resistance to imidacloprid within two years after the introduction of this insecticide for its control (Alyokhin et al., 2008). Moreover, 55 insecticides spanning the most classes of insecticides fail to control this pest due to resistance development (Insecticide Resistance Action Committee, http://www.irac-online.org/). Therefore, this pest may serve as a good model to elucidate mechanisms associated with insecticide resistance.

Insects cope with insecticides through coordinated transcriptional regulation of genes coding for detoxification enzymes that metabolize insecticides (Feyereisen, 2012). Binding of xenobiotic transcription factors (XTFs) to cis-regulatory elements such as xenobiotic response elements, result in the regulation of expression of detoxification genes (Liska, 1998). In mammals, bZIP (basic leucine zipper) transcription factors, Nrf2 (NF-E2-related factor 2) and Keap1 (Kelch-like ECH-associated protein 1) play critical roles in the induction of xenobiotic or oxidative responses in order to protect from multiple diseases (Slocum and Kensler, 2011; Sykiotis and Bohmann, 2010). In invertebrates, the homolog of Nrf2, cap 'n' collar isoform C (CncC) plays a vital role in defending the organism against oxidative stress by regulating many stress-responsive genes and also contributes to resistance against insecticides/xenobiotics. Under normal conditions, its negative regulator, Keap1, sequesters CncC. However, under oxidative stress, CncC binds to

Abbreviations: CPB, Colorado potato beetle; CncC, Cap 'n' collar isoform C; XTFs, Xenobiotic transcription factors; Nrf2, NF-E2-related factor 2; P450, Cytochrome P450; GSTs, Glutathione-S-transferases; Maf, muscle aponeurosis fibromatosis; AREs, Antioxidant response elements; dsCncC, dsRNA of CncC; dsGFP, dsRNA of green fluorescent protein; RNAi, RNA interference; RT-qPCR, Real-time quantitative PCR

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https://doi.org/10.1016/j.ibmb.2018.05.006
Received 24 April 2018; Received in revised form 20 May 2018; Accepted 24 May 2018
Available online 28 May 2018
0965-1748/

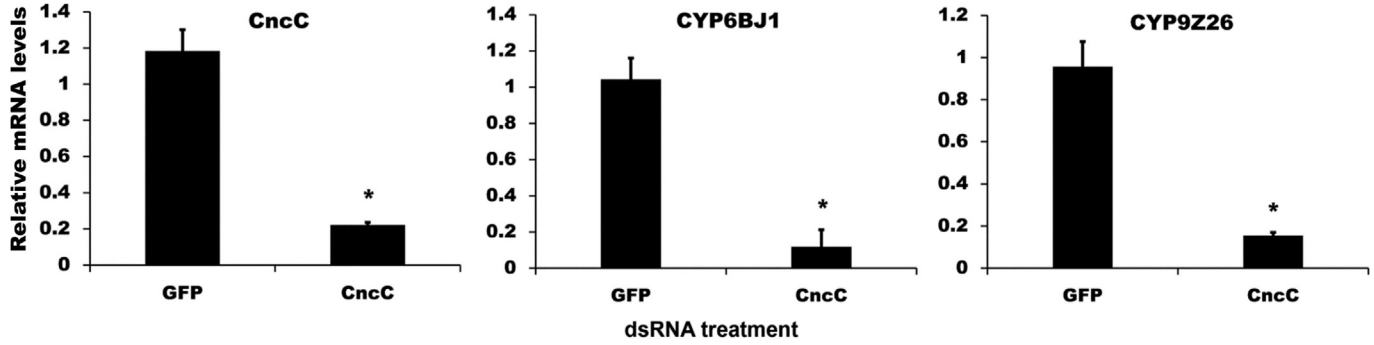

Fig. 1. Knockdown of CncC results in downregulation of CYP6BJ1, and CY9Z26 genes in imidacloprid-resistant CPB.
The beetles were injected with 5 μg dsCncC or dsGFP (control). On the sixth day after dsRNA injection, the total RNA was isolated, converted to cDNA and used in the RT-qPCR analysis. The housekeeping gene, RP18 was used as a reference gene for normalization of target gene expression. Error bars represent standard error mean of the three biological replicates. Data were analyzed using student's t-test; '*' denotes the significant differences in the expression levels of the tested genes between dsCncC treatment and control at a $p < 0.05$.

Table 1
Summary statistics of *L. decemlineata* transcriptome analysis.
Table shows read statistics after Illumina Hi-seq 4000 sequencing. Total number of raw reads after de-multiplexing of six samples from a single lane (containing a pool of six libraries). The last row shows the percent high-quality reads (after trimming) unambiguously mapped to reference *L. decemlineata* transcriptome.

Sample	dsGFP			CncC knockdown		
	Control_1	Control_2	Control_3	CncC_1	CncC_2	CncC_3
Total number of reads	23,469,816	20,639,073	21,415,149	13,932,633	14,732,375	43,610,287
Reads after trimming	23,187,967	19,355,634	19,976,706	13,697,715	14,615,770	43,466,897
Percentage trimmed	98.8	93.78	93.28	98.31	99.21	93.28
Reads mapped to reference	18,175,285	14,889,612	15,917,119	11,470,875	12,048,372	37,302,365
Mapping percentage	78.38	76.93	79.68	83.37	82.43	85.82

its partner Maf (muscle aponeurosis fibromatosis), then the heterodimer binds to antioxidant response elements (AREs) present in the promoter of genes coding for detoxification enzymes (cytochrome P450s) and induces the expression of these genes (Hirotsu et al., 2012; Kalsi and Palli, 2015, 2017a; b; Misra et al., 2011; Wan et al., 2013). For example, the expression levels of CYP6G1, CYP6A2, and CYP12D1 were suppressed by altering the expression of CncC in the fruit fly, *Drosophila melanogaster* (Maitra et al., 2000). Previous studies have shown that the expression levels of P450s are directly correlated with insecticide resistance levels, and resistant populations overexpressed many P450s genes belonging to CYP4, CYP6, CYP9, and CYP13 families (Li et al., 2007). The molecular mechanisms associated with over-expression of P450 genes or other detoxification genes in insecticide resistant populations are not fully understood. Therefore, understanding the regulatory network that coordinates expression of detoxification response genes is needed to break down the resistance.

Previously, we reported differential expression of cytochrome P450 genes between the susceptible and imidacloprid-resistant CPB and identified multiple P450s that are involved in detoxification of both insecticide and plant allelochemicals (Zhu et al., 2016). In a subsequent study, we employed RNAi to identify four cytochromes P450 genes (CYP6BJa/b, CYP6BJ1v1, CYP9Z25, and CYP9Z29) that are required for resistance against both insecticide and plant allelochemicals (Kalsi and Palli, 2017b). These studies also showed that CncC regulates the expression of these P450 genes by binding to target sites present in the promoters and induces their expression (Kalsi and Palli, 2017b). However, the comprehensive picture of CncC contribution to imidacloprid resistance in CPB remains elusive. Hence, in this study, we employed RNAi and RNA sequencing to identify the targets of CncC in order to understand its contribution to imidacloprid resistance in CPB. Data included in this paper showed that the CncC regulated genes code for not only metabolic enzymes but also ABC transporters that are required for imidacloprid resistance in CPB. Unraveling the identity and function of CncC regulated genes in insecticide resistance could be crucial as they could serve as potential targets for RNAi or CRISPR/Cas mediated management of insecticide resistance.

2. Materials and methods

2.1. Insects

The imidacloprid-resistant strain of CPB was collected from a potato field in Long Island, New York and the susceptible strain of CPB was obtained from the Department of Agriculture, New Jersey. Both strains were reared in a greenhouse for several generations on Dark Red Norland potato plants. The beetles were kept in cages (BugDorm-2120 Insect Tent, MegaView Science Co., Ltd) with potato plants at 25 ± 5 °C under a light: dark regime of 16:8 h. To avoid cannibalism, egg masses were collected and transferred to a separate cage containing potato plants.

2.2. CncC knockdown, total RNA extraction, and RT-qPCR

The template for dsRNA synthesis was obtained by PCR amplification using gene-specific primers containing T7 promoter sequence at the 5′ end (Table S1) and cDNA as a template. The dsRNA was synthesized using purified PCR product and MEGAscript RNAi kit by following the manufacturer's protocol (Ambion™). Three CPB adults were injected with five μg/μl of CncC dsRNA (dsCncC) each and controls were injected with the same amount of GFP dsRNA (dsGFP). For RNA sequencing and to verify the RNA sequencing data, the total RNA was extracted from three beetles on the sixth day after dsCncC or dsGFP (control) injection. For differential expression analysis of ABC transporters, the total RNA was isolated from the imidacloprid-resistant and susceptible strains of CPB and different tissues such as the brain, fat body, Malpighian tubules and the rest of the body of the imidacloprid-resistant CPB. To verify the knockdown of ABC transporter genes, the total RNA was extracted from three beetles on the sixth day after dsRNA