Insect Biochemistry and Molecular Biology 47 (2014) 12–22

Contents lists available at ScienceDirect

Insect Biochemistry and Molecular Biology

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

Identification of conserved and novel microRNAs in *Manduca sexta* and their possible roles in the expression regulation of immunity-related genes☆

Xiufeng Zhang [a], Yun Zheng [b], Guru Jagadeeswaran [c], Ren Ren [d], Ramanjulu Sunkar [c], Haobo Jiang [a,*]

[a] Department of Entomology and Plant Pathology, Oklahoma State University, Stillwater, OK 74078, USA
[b] School of Life Science and Technology, Kunming University of Science and Technology, Kunming, Yunnan 650500, China
[c] Department of Biochemistry and Molecular Biology, Oklahoma State University, Stillwater, OK 74078, USA
[d] School of Life Sciences, Fudan University, Shanghai 200433, China

ARTICLE INFO

Article history:
Received 8 December 2013
Received in revised form
22 January 2014
Accepted 26 January 2014

Keywords:
Posttranscriptional regulation
Illumina sequencing
Lepidoptera
Insect immunity
Target site prediction

ABSTRACT

The tobacco hornworm *Manduca sexta* has served as a model for insect biochemical and physiological research for decades. However, knowledge of the posttranscriptional regulation of gene expression by microRNAs is still rudimentary in this species. Our previous study (Zhang et al., 2012) identified 163 conserved and 13 novel microRNAs in *M. sexta*, most of which were present at low levels in pupae. To identify additional *M. sexta* microRNAs and more importantly to examine their possible roles in the expression regulation of immunity-related genes, we constructed four small RNA libraries using fat body and hemocytes from naïve or bacteria-injected larvae and obtained 32.9 million reads of 18–31 nucleotides by Illumina sequencing. Mse-miR-929 and mse-miR-1b (antisense microRNA of mse-miR-1) were predicted in the previous study and now found to be conserved microRNAs in the tissue samples. We also found four novel microRNAs, two of which result from a gene cluster. Mse-miR-281-star, mse-miR-965-star, mse-miR-31-star, and mse-miR-9b-star were present at higher levels than their respective mature strands. Abundance changes of microRNAs were observed after the immune challenge. Based on the quantitative data of mRNA levels in control and induced fat body and hemocytes as well as the results of microRNA target site prediction, we suggest that certain microRNAs and microRNA*s regulate gene expression for pattern recognition, prophenoloxidase activation, cellular responses, antimicrobial peptide synthesis, and conserved intracellular signal transduction (Toll, IMD, JAK-STAT, MAPK-JNK-p38, and small interfering RNA pathways). In summary, this study has enriched our knowledge on *M. sexta* microRNAs and how some of them may participate in the expression regulation of immunity-related genes.

Abbreviations: Alk, anaplastic lymphoma kinase; AMP, antimicrobial peptides; ANKRD54, ankyrin repeat domain 54; Aop, anterior open; aPKC, atypical protein kinase C; AtgX, autophagy-related protein X; βGRP, β-1,3-glucan recognition protein; CF, IF, CH and IH, control (C) and induced (I) fat body (F) and hemocytes (H); CTL, C-type lectin; Dscam, Downs syndrome cell adhesion molecule; ECSIT, evolutionarily conserved intermediate in Toll pathway; ERK, extracellular signal regulated kinase; HAIP, hemocyte aggregation inhibitor protein; Hem, hemipterous; HP, hemolymph proteinase; IAP, inhibitor of apoptosis; IKK, IκB kinase; IMD, immune deficiency; IML, immulectin; JAK-STAT, Janus kinase-signal transducer and activator of transcription; JNK, Jun N-terminal kinase; Jra, Jun related antigen; MAPK, mitogen-activated protein kinase; MASK, multiple ankyrin repeats single KH domain; MEKK, MEK kinase; MKK, MAP kinase kinase; MLK, mixed-lineage kinase; PAP, prophenoloxidase activating proteinase; PGRP, peptidoglycan recognition protein; PIAS, protein inhibitor of activated STAT; PO and PPO, phenoloxidase and its precursor; PPBP, paralytic peptide binding protein; PRR, pattern recognition receptors; PSP, plasmatocyte spreading peptide; Pvr, PDGF/VEGF receptor; serpin, serine proteinase inhibitor; SOCS, suppressor of cytokine signaling; SPH, serine proteinase homolog; Spz, spatzle; TAK, transforming growth factor β-activated kinase; TEP, thioester-containing protein; Tollip, toll interacting protein; Ubc, ubiquitin-conjugating domain.

☆ While *Manduca sexta* miRNAs and miRNA*s are still used, their new names with -5p or -3p are posted on our website (http://entoplp.okstate.edu/profiles/profiles/jiang.htm).

* Corresponding author. Tel.: +1 405 744 9400.
E-mail addresses: xiufeng.zhang@okstate.edu (X. Zhang), zhengyun5488@gmail.com (Y. Zheng), guruswa@okstate.edu (G. Jagadeeswaran), renren@fudan.edu.cn (R. Ren), ramanjulu.sunkar@okstate.edu (R. Sunkar), haobo.jiang@okstate.edu (H. Jiang).

http://dx.doi.org/10.1016/j.ibmb.2014.01.008
0965-1748/

1. Introduction

MicroRNAs (miRNAs) are non-coding RNAs, generally between 20 and 22 nucleotides (nt) in length. Their precursors, from either primary transcripts or intron lariats, are transported into the cytoplasm for processing (Asgari, 2011). The RNase III-type enzyme Dicer 1 trims the loop to generate miRNA:miRNA* duplexes, of which the mature miRNA strands are usually incorporated into the RNA-induced silencing complex (RISC) to initiate target mRNA translational repression or degradation, mostly binding to 3′-untranslated regions (3′-UTRs) of the mRNAs. The passenger strands (miRNA*s) are usually disposed of rapidly and detected at lower levels by high-throughput sequencing. In some cases, however, miRNA*s are maintained at high levels (Jagadeeswaran et al., 2010; Kato et al., 2009; Zhang et al., 2012). The dominant usage of 5′ or 3′ arms of miRNA precursors is considered to be a possible mechanism for insect miRNA evolution (Marco et al., 2010). After their discovery, miRNAs were found to regulate diverse physiological processes, including insect development, host defense, and metabolism (Asgari, 2011; Baker and Thummel, 2007; Chawla and Sokol, 2011).

Insects only possess innate immunity. As a lepidopteran model species, *Manduca sexta* has contributed significantly to biochemical research on insect antimicrobial defense (Jiang et al., 2010). Hemocytes and fat body are major sources of plasma proteins. Upon exposure to bacteria and fungi, various recognition proteins interact with pathogen-associated molecular patterns to stimulate cellular and humoral immune responses. Phagocytosis, nodule formation, and encapsulation are early hemocyte responses aimed at eliminating the invading pathogens. Pathogen recognition initiates a serine proteinase cascade to activate prophenoloxidase (PPO) for melanization, pro-Spätzle for Toll pathway activation, and paralytic peptide precursor for plasmatocyte spreading. Melanization entraps and kills pathogens (Cerenius et al., 2008; Nappi and Christensen, 2005). A superfamily of plasma serine proteinase inhibitors (serpins) modulates the serine proteinase cascade by specifically inhibiting various pathway members (Jiang et al., 2010). The Toll pathway, together with the immune deficiency (Imd) pathway, is important for induced production of antimicrobial peptides (AMPs) (Lemaitre and Hoffmann, 2007). Highly conserved JNK, JAK-STAT, and MAPK pathways in the insect cells also assist in host defense against pathogens (Bond and Foley, 2009; Goto et al., 2010; Ragab et al., 2011).

Although miRNAs extensively modulate insect immunity against viruses and apicomplexan parasites (Asgari, 2011; Fullaondo and Lee, 2012b; Hakimi and Cannella, 2011), knowledge is limited on miRNA-regulated reactions against pathogenic bacteria and fungi. As detected by microarray using 455 arthropod mature miRNAs as probes, abundances of 59 miRNAs in *Tribolium castaneum* changed after injection of peptidoglycan (PG) from *Micrococcus luteus* (Freitak et al., 2012). Out of the 59, fourteen were previously identified in *Tribolium castanuem* and the others are either conserved or novel miRNAs in other arthropods. While peptidoglycans initiate strong immune responses, differences exist in PGs from Gram-positive (G+) and Gram-negative (G-) bacteria, and PGs induced somewhat different responses as compared with whole bacteria (Sumathipala and Jiang, 2010). In *Drosophila melanogaster*, let-7 directly interacts with the 3′-UTR of an AMP gene *diptericin* and miR-8 negatively regulates the basal expression of *diptericin* and *drosomycin* without pathogen stimulation (Choi and Hyun, 2012; Garbuzov and Tatar, 2010). An *in silico* screening method was developed to predict miRNAs which may regulate *D. melanogaster* immune responses (Fullaondo and Lee, 2012a). However, there are no miRNA expression profiles presented and their abundances, based on the premise of expression co-regulation, were deduced from the microarray expression data of their adjacent genes. Differential regulation of *D. melanogaster* AMP genes in S2 and *Sf*9 cell lines implied that intracellular immune signaling pathways involve species-specific regulators (Rao et al., 2011). Upon encountering *Serratia marcescens* or *M. luteus*, *Apis mellifera* workers mounted immune responses and, among the thirteen miRNAs predicted to regulate immunity in the honeybee, only two exhibited significant changes at 6 h after *S. marcescens* infection (Lourenco et al., 2013). This result also suggests some miRNAs act differently in various insects and experimental data on levels of miRNAs and transcripts of their putative target genes are both needed to establish regulatory relationships. In the transcriptome analysis (Zhang et al., 2011; Gunaratna and Jiang, 2013), we determined the transcript levels of 232 putative immunity-related genes in *M. sexta*, which increased or decreased in fat body and/or hemocytes 24 h after injection of a mixture of bacteria and curdlan into the 5th instar larvae. Nevertheless, there is no report on related miRNA level changes and more efforts are needed to explore the expression regulation of *M. sexta* immunity-related genes by miRNAs.

In this work, we used the same total RNA samples from fat body (F) and hemocytes (H) of control (C) and bacteria-induced (I) *M. sexta* 5th instar larvae (Zhang et al., 2011) to prepare four small RNA libraries (CF, IF, CH and IH) for Illumina sequencing. Due to their spatiotemporal expression specificity, we were able to identify additional miRNAs identified from four developmental stages of *M. sexta* (Zhang et al., 2012). Numbers of miRNA reads were normalized and compared (CF vs. IF; CH vs. IH) to assess miRNA regulation upon pathogen invasion. We predicted miRNA target sites in 3′-UTRs of the 232 mRNAs that encode pathogen recognition proteins, hemolymph proteinases (HPs), serpins, AMPs, and members of the Toll, Imd, JNK, JAK-STAT and MAPK pathways. By correlating the miRNA and corresponding transcript levels (Zhang et al., 2011; Gunaratna and Jiang, 2013), we explored possible regulatory pairs of miRNA:mRNA for future research on *M. sexta* miRNA functions.

2. Materials and methods

2.1. Pathogen injection, total RNA extraction, and small RNA library construction

The same four total RNA samples (CF, IF, CH, IH) as used previously (Zhang et al., 2011) were used for small RNA library construction. Briefly, a mixture of *E. coli*, *M. luteus*, and curdlan was injected into day 2, 5th instar larvae (60) to induce immune responses. After 24 h, hemolymph was collected for hemocyte preparation and RNA isolation. Fat body was dissected from the induced larvae for RNA isolation. Similarly, hemocytes and fat body tissue were collected from day 3, 5th instar naïve larvae (60) for preparing control hemocyte and fat body RNA. The small RNA libraries were constructed for Illumina sequencing at National Center for Genome Resources (Santa Fe, NM) as described previously (Zhang et al., 2012).

2.2. Sequence analysis and identification of microRNAs

The analysis procedures were described previously (Zhang et al., 2012). Briefly, reads were first removed if they had no perfect match to 3′-adaptor sequence. Repeats, known noncoding RNAs (rRNAs, tRNAs, snRNAs, snoRNAs, etc.), mitochondrial nucleotide sequences were filtered out according to respective online databases. Compared to the *M. sexta* hemocyte-fat body EST dataset (http://ftp.genome.ou.edu/pub/for_haobo/manduca/fourlibrariesassembly/), *M. sexta* midgut EST dataset (http://rfc.ex.ac.uk/iceblast/iceblast.php) and *M. sexta* Cufflink RNA-Seq