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Research paper

Direct and indirect effects of RNA interference against pyridoxal kinase and pyridoxine 5′-phosphate oxidase genes in *Bombyx mori*

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ABSTRACT

Vitamin B_6 comprises six interconvertible pyridine compounds (vitamers), among which pyridoxal 5′-phosphate is a coenzyme involved in a high diversity of biochemical reactions. Humans and animals obtain B_6 vitamers from diet, and synthesize pyridoxal 5′-phosphate by pyridoxal kinase and pyridoxine 5′-phosphate oxidase. Currently, little is known on how pyridoxal 5′-phosphate biosynthesis is regulated, and pyridoxal 5′-phosphate is supplied to meet their requirement in terms of cofactor. *Bombyx mori* is a large silk-secreting insect, in which protein metabolism is most active, and the vitamin B_6 demand is high. In this study, we successfully down-regulated the gene expression of pyridoxal kinase and pyridoxine 5′-phosphate oxidase by body cavity injection of synthesized double-stranded small interfering RNA to 5th instar larvae of *Bombyx mori*, and analyzed the gene transcription levels of pyridoxal 5′-phosphate dependent enzymes, phosphoserine aminotransferase and glutamic-oxaloacetic transaminase. Results show that the gene expression of pyridoxal kinase and pyridoxine 5′-phosphate oxidase has a greater impact on the gene transcription of enzymes using pyridoxal 5′-phosphate as a cofactor in *Bombyx mori*. Our study suggests that pyridoxal 5′-phosphate biosynthesis and dynamic balance may be regulated by genetic networks.

1. Introduction

Vitamin B_6 is a generic term referring to pyridoxine (PN), pyridoxamine (PM), pyridoxal (PL), and their phosphorylated forms: pyridoxine 5′-phosphate (PNP), pyridoxamine 5′-phosphate (PMP) and pyridoxal 5′-phosphate (PLP). PLP is the catalytically active form of vitamin B_6, and PLP-dependent enzymes are not only involved in the synthesis, interconversion and degradation of amino acids but also play key roles in the metabolism of neurotransmitter, one-carbon units, biogenic amines, tetrapyrrolic compounds, amino sugars, modulation of steroid receptor-mediated gene expression and regulation of immune function (Di Salvo et al., 2011). All living beings rely on vitamin B_6 for their existence, however, only microorganisms and plants are able to synthesize it *de novo*. Humans and animals obtain PLP from B_6 vitamers acquired from diet and recycled in a "salvage pathway" involving phosphatases, an ATP-dependent pyridoxal kinase (PLK) (EC 2.7.1.3.5) and a FMN-dependent pyridoxine 5′-phosphate oxidase (PNPO) (EC 1.4.3.5) (McCormick and Chen, 1999). Since phosphorylated B_6 vitamers are not permeable to cell membranes, the phosphate group must be removed by phosphatase before entering the cells. Inside the cells, PLK phosphorylates the 5′ alcohol groups of PN, PL and PM to form PNP, PLP and PMP respectively. PNP and PMP are further oxidized to PLP by PNPO. On the other hand, PLP contains a very reactive aldehyde group at the 4′ position that easily forms aldimines with primary and secondary amines. The pool of free PLP *in vivo* is maintained at a very low level in the body, presumably to prevent toxic buildup (Di Salvo et al., 2011). Therefore, PLP production in cells by PLK and PNPO should be tightly regulated to meet the requirements for activating newly synthesized apo-PLP enzymes. Currently, the mechanism of PLP dynamic regulation is poorly understood and represents a very challenging research field.

Bombyx mori is a large silk-secreting insect in which protein metabolism is most active. Our previous study showed that the vitamin B_6 demand of *B. mori* is especially high, and the larva PLP level displays clear developmental cyclic changes (Huang et al., 1998). As an important base for nutritional physiology, the PLP level determines to a certain extent larva growth after molting and silk-protein synthesis (Huang et al., 1999; Zhang and Huang, 2003). Considering the importance of studying the mechanism of regulation of PLP level, we have cloned the cDNAs encoding *B. mori* PLK (BmPLK) and PNPO (BmPNPO) (GENBANK accession number: NM_001043975.1 and NP_001037442.1) and characterized their corresponding enzyme products (Shi et al., 2007; Huang et al., 2009, 2011, 2012). Phylogenetic analysis show that

Abbreviations: PN, pyridoxine; PM, pyridoxamine; PL, pyridoxal; PNP, pyridoxine 5′-phosphate; PMP, pyridoxamine 5′-phosphate; PLP, pyridoxal 5′-phosphate.

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the *BmPLK* and *BmPNPO* exhibit high homology with the human, cattle and mouse enzymes to form a group, leaving the microbial *PLK* and *PNPO* on another branch (Shi et al., 2007; Huang et al., 2009). The amino acid sequence of BmPNPO shares 51.44% identity with human PNPO, and that of BmPLK shares 48.6% identity with human PLK. Recently, we found that *BmPLK* and *BmPNPO* are regulated at the transcription level by larva development and are responsive to hormones (Huang et al., 2016). Molting hormone stimulates the expression of genes encoding BmPLK and BmPNPO, and juvenile hormone appears to work against molting hormone. Since the genes transcription responds to hormones in a similar way as that of amino acid metabolism (Cui and Zhang, 1993), we hypothesized that the gene expression of PLK and PNPO are coordinately regulated with that of enzymes using PLP as a cofactor.

RNA interference (RNAi) is specific mRNA degradation induced by exogenous or endogenous small interfering RNA (siRNA), which blocks the expression of specific genes in the body (Fire et al., 1998). RNAi has been successfully applied in lepidoptera (Terenius et al., 2011) and achieved great progress in the studies on gene functions in the egg, larval, and pupal stages. Methods for introduction of siRNA into the body of insects included injection, feeding, transgene and viral co-transfection. Researches on larvae have found that injection method is advantageous over feeding (Price and Gatehouse, 2008), indicating that the body cavity injection of siRNA is suitable for more applications. To prove the above-mentioned hypothesis, we used body cavity injection of synthesized double-stranded siRNA to treat *B. mori* 5th instar larvae to down-regulate the expression of *PLK* and *PNPO*, and then analyzed the gene transcription levels of PLP-dependent enzymes, phosphoserine aminotransferase (PSAT) and glutamic-oxaloacetic transaminase (GOT). Results show that the gene expression of PLK and PNPO has a greater impact on the gene transcription of enzymes using PLP as a cofactor in *B. mori*.

2. Materials and methods

2.1. B. mori strain and reagents

The strain of *B. mori* was Dazhao, fed with synthetic diet purchased from Shandong Province Institute of Silkworm, kept at 26 ± 1 °C. EASYpin Plus tissue RNA rapid extraction kit was purchased from Aidlab Biotechnology Co. Ltd. (Beijing, China). HiFiScript cDNA First-strand Synthesis Kit was purchased from CW Biotechnology Co. Ltd. (Beijing, China). SYBR ® Premix Ex TaqTM was purchased from TransGen Biotechnology Co. Ltd. (Beijing, China). DNA mark and DNase were purchased from Takara Biotechnology Co. Ltd. (Dalian, China).

2.2. siRNA design and synthesis

Sequences for double-stranded siRNAs were designed using the online tool (http://bioinfo.clontech.com/rnaidesigner/sirnaSequenceDesignInit.do) based on BmPLK (GENBANK accession number: NM_001043975.1) and BmPNPO (GENBANK accession number: NP_001037442.1) cDNA sequences respectively. Three different regions were targeted by siRNAs for each mRNA. The siRNAs were synthesized by GenePharma Technology Co. Ltd. (Shanghai, China) with 2′-O Me modification (addition of methyl group on 2′ carbon on pentose). Lyophilized siRNA was reconstituted with DEPC water to make 1 μg/μl solution for body cavity injection. Table 1 shows the double-stranded siRNA sequences, and Fig. 1 shows the targeting regions. The siRNAs 1, 2 and 3 were used for BmPLK, while the siRNAs 4, 5 and 6 for BmPNPO.

2.3. siRNA body cavity injection and tissue sampling

The third day larvae of 5th instar were used for the RNAi experiment. Each larva of the siRNA group was injected with 4 μl of siRNA solution (1 μg/μl). Larvae in the control group were injected with the same dose of negative control siRNA. Each treatment was triplicated, each replication 20 larvae. For ease of operation, the larvae were first chilled in an ice box for a short time, and then injected with siRNA through the membrane between segments in the abdomen (Price and Gatehouse, 2008). The wound was disinfected with alcohol. After injection, the larvae were daily dissected to obtain posterior silk gland, fat body and midgut, and the samples were kept in −70 °C refrigerator for analysis of gene transcription levels.

Table 1
Sequences of double stranded siRNA against *BmPLK* and *BmPNPO*.

Name	Sense strand	Antisense strand
siRNA1	GCCAGAUUCUCUCAGAGAATT	UUCUCUGAGAGAAUCUGGCTT
siRNA2	GCAUUCCAAUGAAGGAUCUTT	AGAUCCUUCAUUGGAAUGCTT
siRNA3	UCAUGUUACAAGAUCCCAATT	UUGGGAUCUUGUAACAUGATT
siRNA4	CCUUGUAUCUAAAGAACCUTT	AGGUUCUUUAGAUACAAGGTT
siRNA5	GCAUGUGCAAGCUACCAAATT	UUUGGUAGCUUGCACAUGCTT
siRNA6	GGCUUCAUGAUCGCAUCAATT	UUGAUGCGAUCAUGAAGCCTT
N-control	UUCUCCGAACGUGUCACGUTT	ACGUGACACGUUCGGAGAATT

2.4. Transcription level analysis

Total RNA was extracted with EASYpin plus RNA Fast Extraction Kit, treated with DNase to remove the genome DNA, examined for integrity using 1.0% agarose gel electrophoresis (Fig. 2-A), and measured with an UV spectrophotometer for purity and concentration. Total RNA (1 μg) was reverse transcribed with the HiFiScript cDNA synthesis Kit to make the first strand cDNA, according to the instructions of the kit. The concentration of cDNA was determined and diluted to 100 ng/μl.

Real-time fluorescence quantitative PCR (RT-qPCR) was employed to analyze the transcription levels of *PLK*, *PNPO*, *PSAT* and *GOT*. A housekeeping gene *Actin 3* (GENBANK accession number: AAC47446.1) was used as the reference gene. The primers used in RT-qPCR were designed with Primer 5.0 software based on the cDNA sequences of BmPLK, BmPNPO, Actin 3, *B. mori* PSAT (BmPSAT) (GENBANK accession number: DQ311395.1) and *B. mori* GOT (BmGOT) (GENBANK accession number: DQ443125.1), tested with DNAman software, and then synthesized by the Yingjun Biotechnology Co. Ltd. (Shanghai, China). Table 2 lists the sequences of the primers. RT-qPCR was done with a

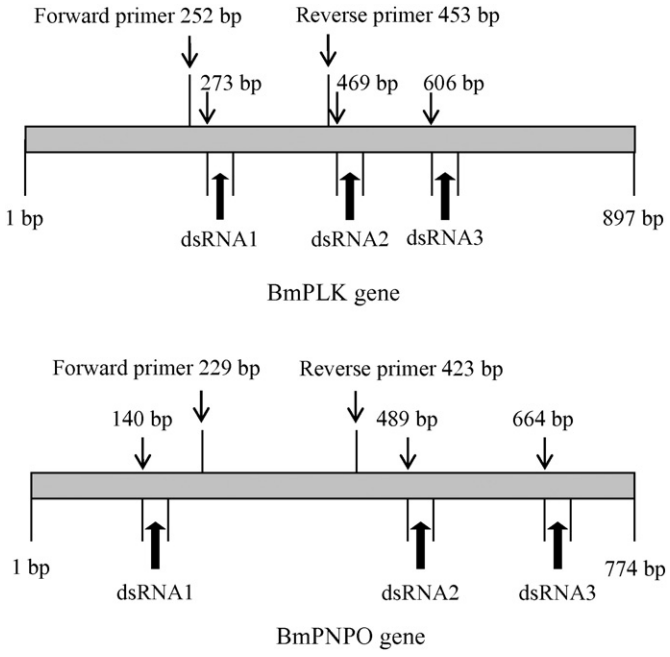

Fig. 1. Schematic representation of BmPLK and BmPNPO genes, showing siRNA target sites and primer pairs for RT-PCR.