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Research

Proteomic-Based Approach to the Proteins Involved in 1-Deoxynojirimycin Accumulation in Silkworm *Bombyx mori* (Lepidoptera: Bombycidae)

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Abstract

1-Deoxynojirimycin (DNJ) is the most abundant poly-hydroxylated alkaloid in the latex of mulberry leaves and it protects mulberry from insect predation. However, silkworms can survive the poisoning effect of DNJ and accumulate DNJ by consumption of the mulberry leaves. In order to determine the molecular mechanism of DNJ accumulation in silkworm, comparative proteomic analysis was employed to evaluate protein expression in two groups of silkworm bodies (the third instar silkworm bodies had the maximum content of DNJ throughout life, and the newly hatched silkworm bodies had no DNJ). Our results indicated some differentially expressed proteins in the third instar silkworm involved in material metabolism, energy metabolism, oxidation-reduction, detoxification, immune, and transport regulation may correspond to the accumulation of DNJ. Furthermore, the expression levels of five selected differentially expressed protein-encoding genes namely heat shock cognate protein (Hsp 70), glutathione S-transferase sigma 1 (GST), serine protease precursor (Ser), hemolymph protein (30K), and thiol peroxiredoxin (TPx) were investigated by quantitative real-time PCR and the accumulation of DNJ was measured by HPLC. Correlation analysis showed that the expression levels of Hsp70 and Ser were negatively correlated to DNJ accumulation with weak correlation, while 30K, GST, andTPx genes had positive correlation with DNJ accumulation. The findings suggested that these three proteins were probably important in the physiological process of DNJ accumulation in silkworm.

Key words: Silkworm (*Bombyx mori*), 1-Deoxynojirimycin (DNJ), Proteomics, two-dimensional electrophoresis, quantitative real-time PCR

Silkworm is the larva of *Bombyx mori* (Lepidoptera: Bombycidae), and it belongs to the order Lepidopteran. It is an insect with a significant importance in science as a model for molecular genetics, structural, and functional genomics studies as well as an economically valuable insect involved in the production of silk (Goldsmith et al. 2005, Mang et al. 2015). 1-Deoxynojirimycin (DNJ) is the most abundant poly-hydroxylated alkaloid present in the milk of mulberry leaves, and it protects mulberry from insect predation (Yoshiaki and Hivonu 1976, Asano et al. 2001, Konno et al. 2006). This poly-hydroxylated alkaloid is very toxic to other insect caterpillars that are not hosted by mulberry, such as castor silkworm and *Plutella xylostella* larvae; however, DNJ and its derivatives have no toxicity on silkworm that parasitizes on mulberry leaves. It has been reported that silkworm has developed some kind of defense mechanism against the toxicity and chemical defense of mulberry leaves as a result of coexistence (Yoshiaki and Hivonu 1976, Despres et al. 2007, Hirayama et al. 2007). DNJ content in the leaves of mulberry is found to be higher than other plants (Kim et al. 2003, Kimura et al. 2007, Yin et al. 2010) and silkworm larvae have the ability to accumulate DNJ with a special mechanism when they are reared on young fresh mulberry leaves (Asano et al. 2001, Liu et al. 2013, Chen et al. 2014). DNJ is a potent antidiabetic, antiviral, and antioxidant agent (Hughes and Rudge 1994, Ryu et al. 1997, Pollock et al. 2008, Yatsunami et al. 2008). Nowadays, several products made from mulberry leaves and silkworm are very popular in South Korea, Japan, China, and other countries (Gui et al. 2004, Yang and Han 2006, Han et al. 2007, Lee et al. 2011, Lim et al. 2013).

However, there are very few reports on the molecular mechanism which has been adopted by silkworm in evading the chemical defense of mulberry thus achieving efficient enrichment of DNJ

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(Hirayama et al. 2007, Daimon et al. 2008). In order to study the mechanism of DNJ accumulation in silkworm, and further use silkworm as an important bioreactor for the production of natural DNJ by regulating expression of relevant proteins, we previously examined the DNJ content at different life stages of silkworm and found the third instar silkworm bodies had the highest content of DNJ, and the newly hatched silkworm bodies had no DNJ (Liu et al. 2013). Therefore, in this study, the third instar silkworm and newly hatched silkworm bodies were chosen for the comparative proteomics analysis. Furthermore, we screened five differentially expressed proteins in the third instar silkworm body, which may possibly relate to DNJ accumulation, using quantitative real-time PCR (qRT-PCR) to detect the expression levels of selected protein-encoding genes and further analyzing their correlation to DNJ accumulation.

Materials and Methods

Silkworm Breeding and Sample Collection

Silkworm strains '7021' was obtained from the Sericultural Research Institute, Chinese Academy of Agricultural Sciences, Zhenjiang, China. Silkworms were reared under the conditions of temperature (25°C ± 1°C) and relative humidity (80% ± 2%). The silkworms were fed with the same fresh mulberry leaves (mulberry cultivar: Yu71-1 with 0.11% DNJ content, cultivated in mulberry field of Jiangsu University, Zhenjiang, China). The newly hatched and day 2 of the third instar silkworm bodies were collected, respectively, for the proteomics analysis. Besides, the silkworm bodies samples were collected in different life stages (from newly hatched to fourth instar) to detect the expression levels of selected genes, DNJ contents, and further correlation analysis. All silkworm bodies samples were starved for 24 h (except newly hatched silkworm bodies) and stored at −80°C until further use.

Protein Sample Preparation

All silkworm body samples were ground to powder in liquid nitrogen. Tris–HCl (pH 7.5) was used to extract proteins and was centrifuged at 15,000 rpm for 10 min. The supernatant was collected and precooled TCA/10% acetone (containing 0.07% (w/v) DTT) was added. The above mixture was precipitated for 2 h at 4°C and centrifuged at 15,000 rpm for 30 min. The pellet was washed twice with acetone (containing 0.07% (w/v) DTT), dissolved in lysis buffer (containing 7 M urea, 2 M thiourea, 4% (w/v) chaps, and 1% (w/v) DTT) and centrifuged at 15,000 rpm for 30 min. The supernatant used for protein analysis. The protein concentration was determined according to Bradford method (Bradford 1976).

Two-Dimensional Gel Electrophoresis Analysis (2-DE)

Isoelectric focusing (IEF) electrophoresis was performed with 17 cm (linear, pH 5–8) IPG strips at 20°C according to the manufacturer's instruction (Ettan DALT six system, GE Healthcare) at a sample loading level of 300 µg. IEF was performed as follows: 250 V for 30 min, 1,000 V for 1 h, 8,000 V for 5 h, linear, 8,000 V for a total of 50,000 V/h, 500 V for 12 h. The strips were equilibrated in equilibration buffer I (6 mol/l urea, 50 mmol/l Tris-HCL, 0.07% (w/v) SDS, 30% (w/v) glycerol, and 1% (w/v) DTT) for 15 min and then in buffer II (replaced 1% DTT with 2.5% (w/v) iodoacetamide) for another 15 min. The equilibrated gel strip was subjected to 12.5% SDS–PAGE and sealed with 0.5% (w/v) agarose. SDS–PAGE was performed at a constant power of 100 V per gel for 30 min, and switched to 200 V per gel until the bromophenol blue frontier reached the bottom of the gel. The gels were stained with 0.1% (w/v) coomassie brilliant blue G-250 and scanned with a high-precision scanner (Scan Maker 9700XL) at a resolution of 600 dpi. Image analysis was performed using PD Quest 2D software. The experiment was done in triplicate for each sample.

In-Gel Digestion and Mass Spectrometry Analysis

Protein spots were excised manually and transferred into 1.5 ml RNase-free centrifuge tubes. The spots were washed three times with double distilled water, de-stained by sonication in 50 mM ammonium bicarbonate, 50% (v/v) acetonitrile, dehydrated with acetonitrile and dried in a vacuum pump. The dried proteins spots were treated by 10 mM DTT for 1 h at 56°C, alkylated with 55 mM iodoacetamide for 45 min at room temperature, washed with 25 mM ammonium bicarbonate and 50% (v/v) acetonitrile, respectively. It was further dehydrated with acetonitrile and incubated with 10~15 µl trypsin solution (Promega, 10 µg/ml) at 37°C overnight.

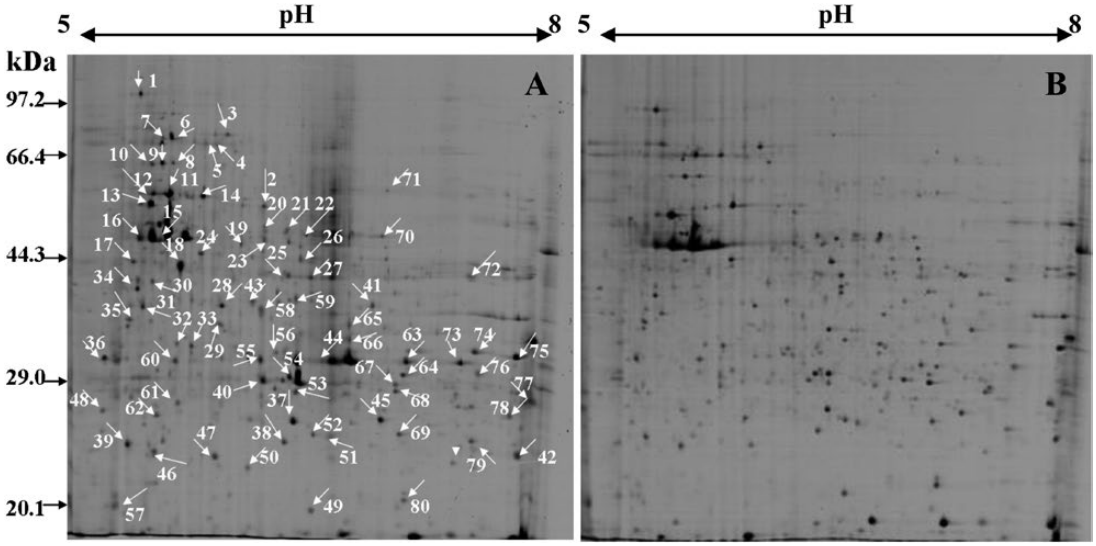

Fig. 1. 2-DE pattern of silkworm bodies proteins. The third instar silkworm bodies (A) and the newly hatched silkworm bodies (B). The identified differentially expressed protein spots in this study are indicated by arrows in the third instar silkworm bodies (A), the numbers corresponded to those are in Table 1.

Table 1. Differentially expressed proteins of the third-instar silkworm of '7021' strain identified by MALDI-TOF/TOF

Spot No.	Protein name	Accession No.	pI/ molecular weight (kDa)	Coverage (%)	Score	Biological function
1	HSC70	gil1495233	5.50/72.10	9	448	Molecular repair
2	Beta-1,3-glucan recognition protein 4 precursor	gil261245087	6.45/42.20	4	96	Signal transduction
3	Unknown	—	—			—
4	Vacuolar ATP synthase subunit B	gil148298717	5.25/54.70	21	729	Energy metabolism
5	Cytosolic aspartate aminotransferase	gil220684	6.73/46.63	5	121	Detoxification
6	Vacuolar ATP synthase subunit B	gil148298717	5.25/54.70	22	734	Energy metabolism
7	Heat shock cognate protein(Hsp70)	gil112982828	5.33/71.36	17	752	Molecular repair
8	Vacuolar ATP synthase catalytic subunit A	gil148298878	5.27/68.56	16	739	Energy metabolism
9	Vacuolar ATP synthase catalytic subunit A	gil148298878	5.27/68.56	16	704	Energy metabolism
10	Unknown	—	—			—
11	Vacuolar ATP synthase catalytic subunit A	gil148298878	5.27/68.56	16	762	Energy metabolism
12	Unknown	—	—			—
13	Cytosolic aspartate aminotransferase	gil220684	6.73/46.63	12	543	Detoxification
14	Vacuolar ATP synthase subunit B	gil148298717	5.47/42.21	15	548	Energy metabolism
15	Vacuolar proton pump subunit B	gil401326	5.26/55.10	21	659	Transportation
16	Vacuolar ATP synthase subunit B	gil148298717	5.25/54.70	22	734	Energy metabolism
17	H^+ transporting ATP synthase beta subunit isoform 1	gil114052072	5.26/55.01	22	826	Energy metabolism
18	Glyceraldehyde-3-phosphate dehydrogenase	gil109119903	7.70/35.50	16	417	Energy metabolism
19	Unknown	—	—			—
20	Beta-1,3-glucan recognition protein 4 precursor	gil261245087	6.45/42.20	16	405	Signal transduction
21	Mitochondrial aldehyde dehydrogenase	gil114052408	7.52/56.21	4	72	Energy metabolism
22	Unknown	—	—			—
23	Elongation factor 1 gamma	gil112983898	5.83/48.64	4	42	Cell function
24	Unknown	—	—			—
25	Arginine kinase	gil25453077	6.24/40.10	11	162	Energy metabolism
26	Translation elongation factor 2 isoform 1	gil112983010	6.23/98.20	7	387	Cell function
27	Translation elongation factor 2 isoform 1	gil112983010	6.23/98.20	3	184	Cell function
28	Keratin 1	gil7331218	8.16/66.15	4	204	Structure
29	Unknown	—	—			—
30	Unknown	—	—			—

Spot No.	Protein name	Accession No.	pI/ molecular weight (kDa)	Coverage (%)	Score	Protein function
31	Thioredoxin	gil114052058	5.11/32.20	25	280	Oxidation-reduction
32	Thioredoxin	gil114052058	5.11/32.20	14	158	Oxidation-reduction
33	Coatomer protein complex subunit delta	gil289629220	5.91/56.70	12	142	Transportation
34	Chaperonin	gil120444903	5.40/59.60	15	402	Molecular repair
35	Thioredoxin	gil114052058	5.11/32.20	25	278	Oxidation-reduction
36	ATP synthase β subunit	gil287945	5.19/53.50	11	358	Energy metabolism
37	Glutathione S-transferase sigma 1 (GST)	gil112983028	5.98/23.60	25	335	Oxidation-reduction
38	Serine protease precursor (Ser)	gil112984052	10.19/30.05	30	525	Energy metabolism
39	Serine protease precursor (Ser)	gil112984052	10.19/30.05	41	783	Energy metabolism
40	Hemolymph protein (30K)	gil187281703	6.23/29.78	28	581	Immune
41	Proteasome alpha 3 subunit	gil114051245	5.27/28.40	12	208	Molecular repair
42	Unknown	—	—			—
43	Hemolymph protein (30K)	gil187281703	6.23/29.78	28	528	Immune
44	Proteasome subunit alpha type-4-like	gil156552814	6.36/28.90	11	237	Molecular repair
45	Voltage-dependent anion-selective channel	gil328670887	6.96/30.13	8	79	Transportation
46	Unknown	—	—			—
47	Unknown	—	—			—
48	Heat shock protein hsp21.4	gil112983414	5.79/21.40	25	332	Molecular repair
49	Actin-depolymerizing factor 1	gil153792659	6.17/17.20	22	170	Structure
50	Triosephosphate isomerase	gil187281708	5.67/26.90	16	215	Energy metabolism
51	Unknown	—	—			—
52	Proteasome subunit alpha type-4-like	gil156552814	6.36/28.90	24	423	Molecular repair
53	Heat shock protein hsp21.4	gil112983414	5.79/21.40	25	332	Molecular repair
54	Thiol peroxiredoxin (TPx)	gil112982996	6.09/22.07	20	318	Oxidation-reduction
55	Keratin 1	gil7331218	8.16/66.15	4	160	Structure
56	Proteasome subunit alpha type-4-like	gil156552814	6.36/28.90	11	235	Molecular repair
57	Unknown	—	—			—

(continued)

Table 1. Continued

Spot No.	Protein name	Accession No.	pI/ molecular weight (kDa)	Coverage (%)	Score	Biological function
58	Hemolymph protein (30K)	gil187281703	6.23/29.78	16	452	Immune
59	Cytosolic malate dehydrogenase	gil114052561	6.85/35.61	15	389	Energy metabolism
60	Keratin 10	gil21961605	5.09/59.00	7	159	Structure
61	Keratin 1	gil7331218	8.16/66.15	4	96	Structure
62	Unknown	—	—			—
63	Glutathione S-transferase sigma 1 (GST)	gil112983028	5.98/23.60	25	343	Oxidation-reduction
64	Thiol peroxiredoxin (TPx)	gil112982996	6.09/22.07	20	304	Oxidation-reduction
65	Heat shock protein hsp21.4	gil112983414	5.79/21.40	25	332	Molecular repair
66	Voltage-dependent anion-selective channel	gil328670887	6.96/30.13	12	214	Transportation
67	Unknown	—	—			—
68	Sorbitol dehydrogenase	gil95103082	6.31/39.20	24	538	Energy metabolism
69	Beta-1,3-glucan recognition protein 4 precursor	gil261245087	6.45/42.20	4	96	Signal transduction
70	Beta-1,3-glucan recognition protein 4 precursor	gil261245087	6.45/42.20	16	405	Signal transduction
71	Heat shock cognate protein(Hsp70)	gil112982828	5.33/71.36	17	752	Molecular repair
72	Fructose 1,6-bisphosphate aldolase	gil148298685	8.38/39.97	16	473	Energy metabolism
73	Glyceraldehyde-3-phosphate dehydrogenase	gil109119903	7.70/35.50	16	417	Energy metabolism
74	Mitochondrial aldehyde dehydrogenase	gil114052408	7.52/56.21	4	72	Energy metabolism
75	Unknown	—	—			—
76	Fructose 1,6-bisphosphate aldolase	gil148298685	8.38/39.97	16	473	Energy metabolism
77	Glyceraldehyde-3-phosphate dehydrogenase	gil109119903	7.70/35.50	16	417	Energy metabolism
78	Hemolymph protein (30K)	gil187281703	6.23/29.78	28	581	Immune
79	Bombyrin precursor	gil112983654	7.04/22.80	5	98	Structure
80	Signal sequence receptor beta subunit precursor	gil114052941	6.90/20.90	13	142	Signal transduction

Table 2. Selected five genes and their primers for qRT-PCR

Gene name	Forward primer (5′-3′)	Reverse primer (5′-3′)
Heat shock cognate protein(Hsp70)	CCCCTTTCCCTCGGTATT	TCCCGGTCAGCTCGAATT
Glutathione S-transferase sigma 1 (GST)	TGCATGATATTCGCGCCA	CTGGTGAACACGAAATCA
Serine protease precursor (Ser)	CAGAAGAGCCCATCGAACTC	CGATCACAAGTCCAGCAAGA
Hemolymph protein (30K)	GCCATCAAACTCGGTGCT	CACCATCCGTACCCGTTC
Thiol peroxiredoxin (TPx)	TGACCAAACCCGCTCCCC	CAGCCGATCTTGCGGAAC
Housekeeping gene, actin-3 (Act-3)	AATGGCTCCGGTATGTGC	TTGCTCTGTGCCTCGTCT T

The resulting peptides were collected and mixed with an equal volume of 10 mg/ml matrix solution (a-cyano-4-hydroxycinnamic acid), saturated with 0.1% (v/v) trifluoroacetic acid in 50% (v/v) acetonitrile, spotted on an MTP Anchor Chip (Bruker, Germany), and analyzed by matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS, Bruker, Germany) to acquire spectra with a mass range from 500 to 6,000 Da. External calibration was performed with standard peptides. The matrix and autolysis of trypsin were served as internal standards. The mass spectra were interpreted using the Mascot peptide mass fingerprint engine available on the website (http://www.matrixscience.com) for protein identification.

Quantitative Real-Time PCR

All silkworm bodies (about 200 mg) for expression level analysis were ground with a mortar and pestle in liquid nitrogen. Total RNA was extracted using Trizol reagent (Invitrogen), subsequently, total RNA was reversely transcribed into cDNA using PrimeScriptTM RT reagent Kit (TaKaRa, RR047A). All the PCR reactions were carried out using SYBR Premix Ex TaqTM kit (TaKaRa, Kyoto, Japan) on a Mx3000PTM system. Reaction system was 25.0 µl with 2.0 µl cDNA, 0.5 µl forward primer (20 mM), and 0.5 µl reverse primer. PCR amplification procedure was performed using the following program: 30 s at 95°C; 40 cycles of 5 s at 95°C, 20 s at 55°C; 40 s at 72°C. All samples were run in triplicate for each gene. The expression level of each gene was quantified relative to silkworm housekeeping gene (actin-3), and comparison of Ct value method was used to quantify gene expression level.

HPLC Determination of DNJ Content in Silkworm Bodies From Different Life Stages

All silkworm bodies samples for DNJ analysis were lyophilized, smashed, and passed through a 80-mesh screen. Dried silkworm body powder of 0.1 g was taken and transferred into 1.5 ml centrifuge tube, 10 ml hydrochloric acid (0.05 M) was added and mixed evenly. The solution was centrifuged at 12,000 rpm for 15 min, and the supernatant was obtained. The residue was extracted one more time as above. The two extraction supernatants were merged in a

volumetric flask, and distilled water was added to a total volume of 50 ml, then the resulting extraction solution was made. Resulting extraction solution 10 µl was treated with 10 ml borate saline buffer (pH 8.5), 5 mM FMOC-Cl solution (20 µl), mixed thoroughly and placed in 20°C water bath for 20 min, and subsequently, 10 µl of 0.1M glycine was added to quench the remaining FMOC-Cl. Finally, 950 µl of 0.1% (v/v) acetic acid was added to stabilize the DNJ-FMOC formed in the reaction (Yin et al. ,2010). The sample was analyzed for DNJ measurement with the aid of RP-HPLC. The HPLC analysis was performed with a JASCO LC-1500 instrument (JASCO, Japan) with a reversed-phase column HiQSiL C18 (5 µm, 250 mm × 4.6 mm). The column was eluted with a mobile phase of acetonitrile: 0.1% aqueous acetic acid (55:45, v/v) at 1.0 ml/min. Analysis was monitored using a UV detector (254 nm) throughout the entire run. A sample of 10 µl was injected to the column. A two-point external standard method was used to calculate the concentration of DNJ in silkworm samples. All samples were performed in triplicate.

Statistical Analysis

Data are presented as mean ± standard error of three independent experiments. Statistical analysis was carried out using SPSS version 16.0 software (SPSS Inc, Chicago, IL). One-way analysis of variance followed by Fisher's post hoc analysis. Differences were considered statistically significant at $P < 0.05$.

Results and Discussion

Comparison of Protein Patterns

Determination of expressed proteins was achieved with the aid of 2-DE separation using Coomassie Blue G-250. Prominent differences were observed between the 2D-PAGE patterns of proteins from the third instar silkworm and newly hatched silkworm bodies. There were 961 individual protein spots detected in the third instar silkworms, the molecular weight ranged from 22 to 97 kDa, while the pI (isoelectric point) was from 5.0 to 8.0 and 1,060 protein spots were identified in newly hatched silkworm with a molecular weight range from 25 to 100 kDa, with pI range of 5.0–7.5. Out of these, 499 protein spots matched, with a matching ratio of 49.53% (Fig. 1A and B).

Mass Spectra Analysis

Eighty differentially expressed protein spots with high abundance from the third instar silkworms were excised from 2-DE gels and subjected to in-gel trypsin digestion and subsequent MALDI-TOF/TOF mass spectrometry. Out of these spots, 64 spots were successfully identified (Fig. 1A; Table 1). All identified proteins were classified into nine categories: 22 spots were related to energy metabolism (vacuolar ATP synthase subunit B, vacuolar ATP synthase catalytic subunit A, 34.38%); 11 spots for molecular repair (such as HSC70 and Hsps, 17.19%); seven spots for oxidation-reduction

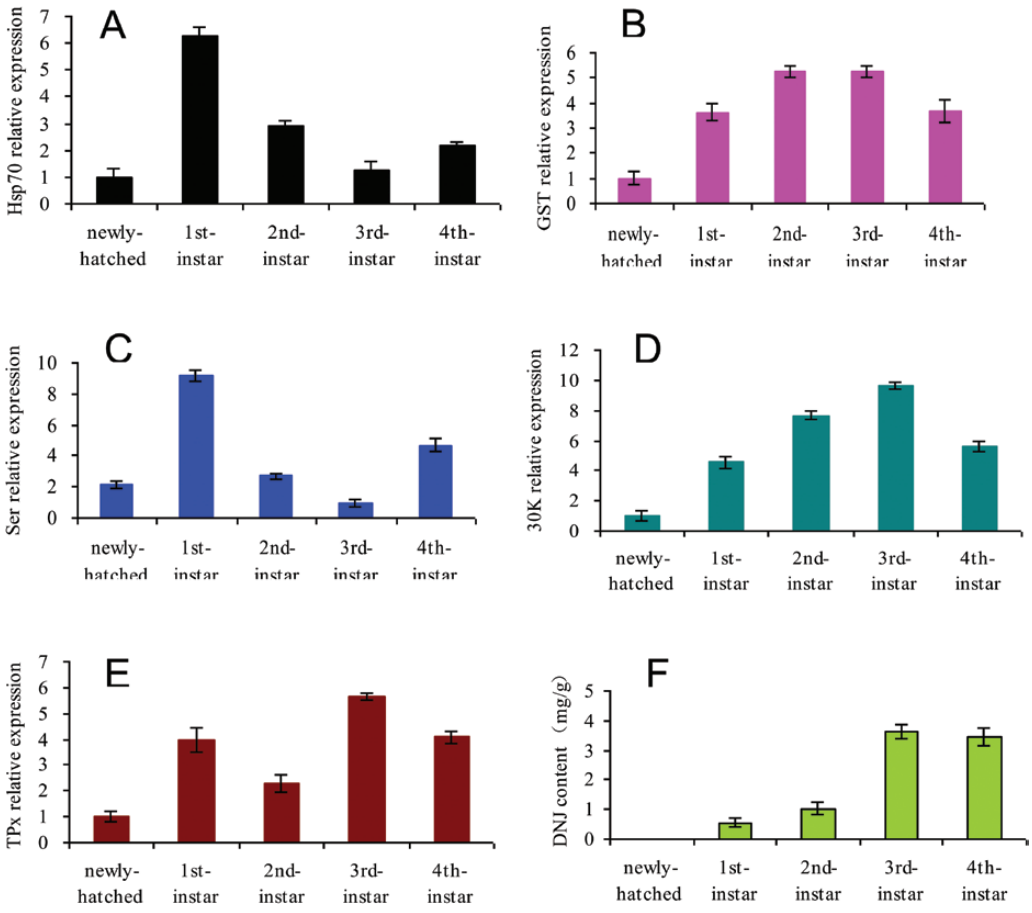

Fig. 2. Relative expression of selected five protein-encoding genes and DNJ content in silkworm bodies at different silkworm life stages. qRT-PCR technique was used to detect the relative expression levels of five genes, and HPLC was used to measure DNJ content in silkworm bodies from newly hatched to fourth-instar silkworm. (A: Hsp 70; B: GST; C: Ser; D:30K; E:TPx and F: DNJ content).

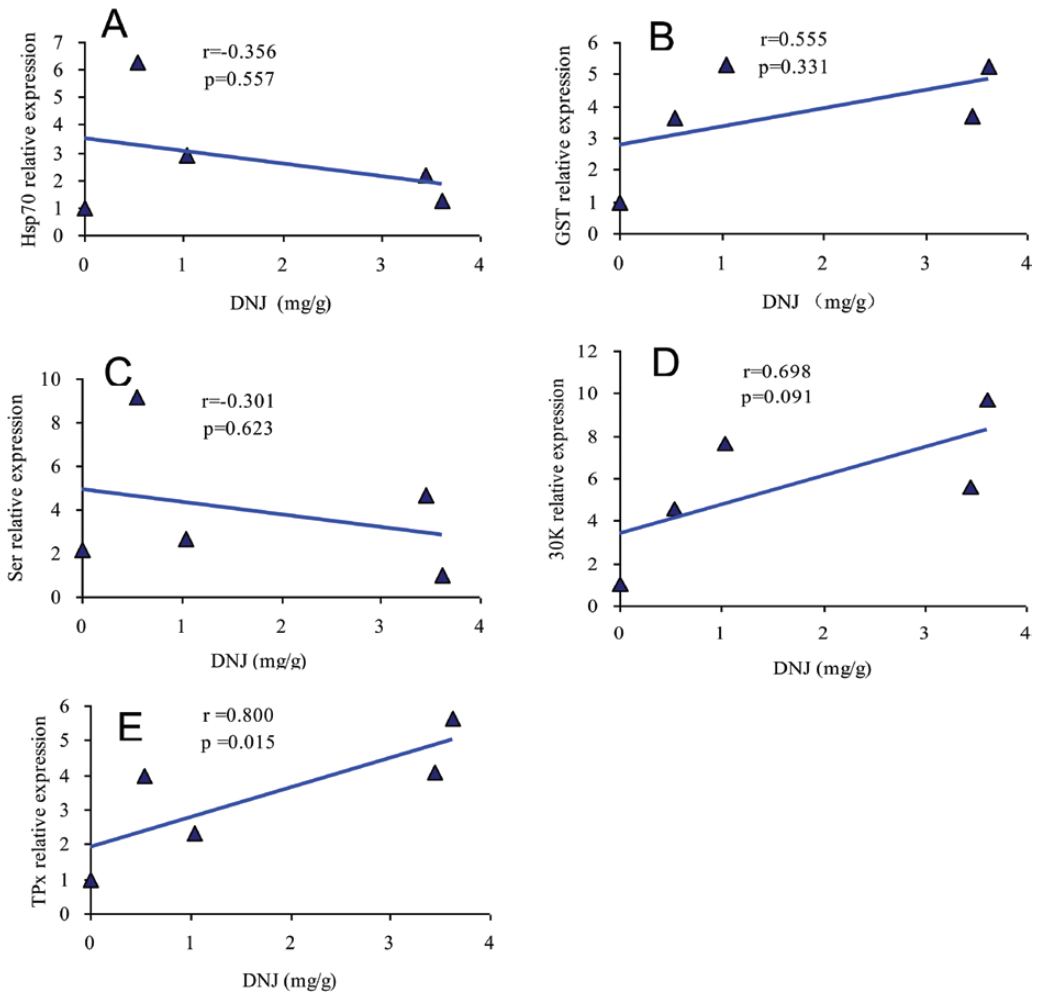

Fig. 3. Correlation between DNJ content and the expression levels of selected five protein-encoding genes. (A: Hsp70-DNJ; B: GST-DNJ; C: Ser-DNJ; D: 30K-DNJ and E: TPx-DNJ).

(thioredoxin, 10.94%); six spots for structure (keratin 1 and keratin 10, 9.38%); five spot were involved in signal transduction (signal sequence receptor beta subunit precursor, 7.81%); four spots for immune system (hemolymph protein [30K], 6.25%); four spots for transportation (vacuolar proton pump subunit B, 6.25%); three spots for cell function (like elongation factor 1 gamma, 4.69%); two spots for detoxification (cytosolic aspartate aminotransferase, 3.13%). This result suggested that silkworm could probably accumulate DNJ by feeding on mulberry leaves through the processes of molecular repair, energy consumption, detoxification, autoimmunity, and oxidation resistance.

Expression Levels of Selected Protein-Encoding Genes, DNJ Accumulation, and Their Correlations

After analyzing and comparing all identified proteins functions (Table 1), we chose these five differentially expressed protein-encoding genes (Table 2) which may play a major role in DNJ accumulation process and investigated their expression levels related to DNJ accumulation. The five genes relative mRNA levels from newly hatched to fourth instar silkworm bodies were detected with the aid of qRT-PCR analysis. Silkworm actin-3 (Act-3) gene was used as housekeeping gene, and their primers were designed using Primer 5.0 software. The primer sequences were listed in Table 2. Simultaneously, DNJ content from newly hatched silkworm to fourth instar silkworm bodies was determined too. The changes observed in the expression levels of the five genes mentioned above and DNJ content were shown in Fig. 2, the analyzed correlations between DNJ content and expression levels of the five genes were shown in Fig. 3.

As shown in Fig. 2A and C, the relative expression levels of Hsp70 and Ser genes were observed to be significantly high in the first instar silkworm as compared to the second and third instar which had very low expression levels of these two genes. In contrast, there was a gradual increase in the relative expression of glutathione S-transferase sigma 1 (GST) and 30K genes from the newly hatched to the third instar. The third instar had the highest relative expression of these two genes, and a slight decline in the relative expression was observed for the fourth instar (Fig. 2B and D). As indicated in Fig. 2F, DNJ content was also observed to obviously change at different silkworm life stages. There was a gradual increase in DNJ content from the newly hatched to the third instar. The highest DNJ content was accumulated in the third instar stage of the silkworm. Correlation analysis also showed that there was a weak negative correlation between DNJ content and the expression level of genes Hsp70 and Ser (Fig. 3A and C), suggesting that these two genes might not play any significant role in DNJ enrichment. However, the relative expression levels of GST, 30K, and thiol peroxiredoxin

(TPx) genes had strong positive correlation to DNJ content (Fig. 3B, D, and E), which suggested that these three genes may be most likely involved in DNJ accumulation.

GST gene is one of the three enzymatic superfamilies which functions as a detoxifier (Liu et al. 2010), scavenge oxygen free radicals and catalyzes the conjugation of electrophilic compounds to glutathione, thus playing a critical role in cell survival and is an integral component of the antioxidant defense against reactive oxygen species (Hayes et al. 2005, Yamamoto et al. 2006, Yamamoto et al. 2009). It is also involved in intracellular transport, hormone synthesis, and protection of cell injury caused by oxidative stress (Board et al. 2000). The 30K gene is one of the essential energy storage proteins during the growth and development process of silkworm, and it plays important roles in the embryonic development of silkworm through the formation of vitellogenin and material transport (Ujita et al. 2002, Zhong et al. 2005). The 30K protein has also been reported to display a strong resistance to apoptosis (Yu et al. 2011). TPx, an antioxidant protein, functions in the removal of reactive oxygen free radicals. External stimulus and pathogenic micro-organisms infection usually induce TPx protective mechanism against their harmful effects. Previous reports have indicated that upon a viral infection, the silkworm will induce the production of TPx to reduce oxidative damage and enhance the activity of its natural killer cells (Lee et al. 2005).

DNJ, a potent glucosidase inhibitor, is an important defense mechanism for mulberry. Yet, silkworm can enrich itself with DNJ from mulberry leaves. Daimon et al. (2008) found out that the expression of β-fructosyltransferase in silkworm was abnormal and its activity was not influenced by DNJ, which partially explained the adaptation of silkworm to mulberry-defense mechanism. In this study, we further discovered that the expression levels of GST, 30K, and TPx genes specifically increased along with the enrichment of DNJ in different life stage of silkworm bodies. This indicated that these three genes in silkworm may play very important roles in the physiological process of DNJ accumulation to adapt to mulberry-defense mechanism. From this, we can reasonably extrapolate that DNJ enrichment in silkworm or its adaptation to mulberry chemical defense was a very complex physiological processes with a lot of proteins involved.

Conclusion

In this study, the third instar silkworm and the newly hatched silkworm bodies were used to evaluate protein expression, and 64 differently expressed proteins spots were identified from the third instar silkworm bodies. Our result indicated that silkworm could probably accumulate DNJ by regulating the processes of substances metabolism, energy consumption, detoxification, autoimmunity, and oxidation resistance. The expression levels of five selected protein-encoding genes and their correlation to DNJ accumulation suggested that GST, 30K, and TPx genes were more likely involved in the process of DNJ accumulation. Thus, our findings provided a scientific basis for further study to reveal the mechanism of DNJ accumulation in silkworm and use silkworm as a bioreactor to produce natural DNJ by regulating the expression of genes related to DNJ accumulation.

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Author Contribution Statement

Z.O. conceived the experiments and provided the main experimental financial and technical support; H.C. and Y.L. carried out the experimental procedures; W.W. and H.C. performed qRT-PCR experiment; G.P. provided the silkworm variety "7021", H.C. and O.J.O. performed data analysis and wrote the article. All authors approved the final version.

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