Proteomic Analysis of Larval Integument in a Dominant Obese Translucent (Obs) Silkworm Mutant

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Abstract

The dominant obese translucent (Obs) mutant of the silkworm (Bombyx mori) results in a short and stout larval body, translucent phenotype, and abnormal pigmentation in the integument. The Obs mutant also displays deficiency in ecdysis and metamorphosis. In the present study, to gain an understanding of multiple Obs phenotypes, we investigated the phenotypes of Obs and performed a comparative analysis of the larval integument proteomes of Obs and normal silkworms. The phenotypic analysis revealed that the Obs larvae were indeed short and fat, and that chitin and uric acid content were lower but melanin content was higher in the Obs mutant. Proteomic analysis revealed that 244 proteins were significantly differentially expressed between Obs and normal silkworms, some of which were involved in uric acid metabolism and melanin pigmentation. Twenty-six proteins were annotated as cuticular proteins, including RR motif-rich cuticular proteins (CPR), glycine-rich cuticular protein (CPG), hypothetical cuticular protein (CPH), cuticular protein analogous to peritrophins (CPAPs), and the chitin_bind_3 motif proteins, and accounted for over 84% of the abundance of the total significantly differentially expressed proteins. Moreover, 22 of the 26 cuticular proteins were downregulated in the Obs mutant. Comparative proteomic analysis suggested that the multiple phenotypes of the Obs mutant might be related to changes in the expression of proteins that participate in cuticular formation, uric acid metabolism, and melanin pigmentation. These results could lay a basis for further identification of the gene responsible for the Obs mutant. The data have been deposited to ProteomeXchange with identifier PXD010998.

Key words: dominant obese translucent, uric acid, melanin pigmentation, cuticular formation, proteomics
shape mutants stony and Bo, respectively (Qiao et al. 2014, Xiong et al. 2017). So far, about 479 silkworm mutants have been obtained and maintained as genetic resources, >20 of which are related to body shape (http://shigen.nig.ac.jp/silkwormbase/ViewStrainGroup.do, last accessed October 15, 2018). The silkworm, therefore, represents an attractive model for studying body shape determination in insects.

Dominant obese translucent (Obs) is one of the body shape mutants of silkworm. The Obs mutant not only shows a short and stout larval body shape but also exhibits a translucent phenotype and suppression of melanin deposition in the larval, and crescent markings on the dorsal integument, as shown in Fig. 1. Moreover, abnormal phenotype is observed for Obs mutant in ec dysis and metamorphosis (Yoshimura et al. 1984). More than 30 mutants in silkworm exhibit the translucent phenotype (http://shigen.nig.ac.jp/silkwormbase/ViewStrainGroup.do, last accessed October 15, 2018), which results from the abnormal uric acid metabolism in the larvae (Tamura and Sakate 1983, Tamura and Akai 1990), and these mutants are termed as ‘translucent mutants’. Some proteins responsible for the unique phenotype of the translucent mutants have been identified, to be involved in the biosynthesis (Kōmoto 2002, Kōmoto et al. 2003, Fujii et al. 2016), transport (Kōmoto et al. 2009, Kiuchi et al. 2011, Wang et al. 2013b), and accumulation of uric acid (Ito et al. 2009; Fujii et al. 2010, 2012; Wang et al. 2013a; Zhang et al. 2017). The melanin pigmentation of insects has been widely studied. By the action of different enzymes, tyrosine is converted into melanogenic precursors, which are then transported from the epidermal cell into the developing cuticle, leading to the melanization of cuticles (Andersen 2010). More than 50 mutants in silkworm exhibit various larval pigmentation and patterns (Banno et al. 2010), and some of the responsible genes have been cloned (Dai et al. 2010, Futahashi et al. 2010, Ito et al. 2010, Liu et al. 2010, Zhan et al. 2010, Fujii et al. 2013, Yamaguchi et al. 2013, Yoda et al. 2014, Yuasa et al. 2016). It is notable that the content of uric acid is lower at the areas of melanin deposition in the larval integument of several lepidopteran species, and vice versa (Ninomiya et al. 2006, Hu et al. 2013). However, the biological mechanism of this phenomenon remains unknown. The Obs mutant is governed by a single gene but shows a pleiotropic phenotype, including abnormal body shape determination, uric acid metabolism, and melanin pigmentation (Yoshimura et al. 1984). Research on the Obs mutant may, therefore, provide new clues to understand the relationship among these processes in the insects.

The abnormal phenotypes of the Obs mutant are related to the larval integument. In this study, to gain a better understanding of the molecular mechanism determining the Obs phenotypes, we performed a comparative analysis of the larval integument proteomes of the Obs and normal silkworms on day 3 of the fifth instar using shotgun liquid chromatography–tandem mass spectrometry (LC–MS/MS). The phenotypes of Obs mutant were investigated, and the proteins involved in cuticle formation, uric acid metabolism, and melanin synthesis were analyzed. Our results revealed the possible reasons for the abnormal phenotypes of the Obs mutant, thus enhancing current understanding of the processes of body shape determination, uric acid metabolism, and melanin pigmentation, and their inter-relationships in the synthesis of insect integuments, which will provide a reference for further identification of the gene responsible for the Obs mutant.

Materials and Methods

Silkworm Strains and Tissue Collection

The heterozygous Obs mutant strain o90 (Obs+/+) was obtained from the silkworm stock center at Kyushu University, Fukuoka, Japan. The wild-type strain p50T was obtained from the University of Tokyo. Individuals showing the Obs phenotype were maintained by hybridizing female offspring showing the Obs phenotype with p50T males (Obs+/♀×p50T♂). In the present study, all Obs and normal phenotype individuals were bred using this cross. All silkworm larvae were reared on fresh mulberry leaves at 25°C. The integuments of 3-day-old fifth-instar larvae exhibiting the normal and Obs phenotypes were dissected carefully on ice, and other tissues, including fat body, trachea, and head, were removed. The integuments were immediately washed with 0.7% normal saline, blotted on filter paper, and then stored at ~80°C until protein extraction was performed. Three individuals with the same phenotype were collected to be one unique sample, and there were three biological replicates for each sample.

Morphological Observations and Phenotype Analysis

The integuments of the larvae showing the Obs and normal phenotypes on day 3 of the fifth instar were carefully dissected and treated as described earlier, cleaned with ultrapure water, blotted on filter paper, spread on plastic film, and photographed. The lengths of inter-nodes, intersegmental folds, distances between spiracles, and circumferences at each spiracle were measured using Adobe Photoshop as described previously (Bradshaw et al. 2007).

Quantification of Chitin Content

The integuments of both Obs and normal phenotype larvae obtained prior to the fourth molt and on day 3 of the fifth instar were dissected and treated carefully as described earlier, cleaned with ultrapure water, and blotted on filter paper. The treated integuments and the cuticle shed during the fourth molt were dried at 60°C overnight.

Fig. 1. Phenotype of the normal silkworm and Obs larvae on day 3 of the fifth instar. The normal type is indicated on the upper row, with the Obs type on the lower row.
and then weighed on an analytical balance (Mettler-MS10SDU). Extraction and determination of chitin content were performed according to a previous protocol (Zhang and Zhu 2006). All evaluation was performed on three biological replicates for each sample.

**Measurement of Uric Acid**

Uric acid was extracted from integuments of 3-day-old fifth-instar larvae as described previously (Tamura 1977). The integuments were treated as described earlier, cleaned with ultrapure water, and blotted on filter paper, followed by drying at 90°C for 180 min and grinding with a pestle. Ground integument was boiled in water for 20 min; the samples were subsequently centrifuged at 12,100 × g for 30 min, and the supernatant was collected. The precipitate was further treated as described earlier. Supernatants were mixed for further analysis, and uric acid was measured using a BioAssay Systems uric acid assay kit according to the manufacturer’s instructions (BioAssay Systems, Hayward, CA).

**Quantification of Melanin Content**

The integuments of both the Obs and normal phenotype 3-day-old fifth-instar larvae were dissected and treated as described earlier, cleaned with ultrapure water, and blotted on filter paper. Extraction of melanin from the treated integuments was performed as described previously (Ito et al. 2016). Melanin content was estimated from a standard curve of synthetic melanin (Sigma, St. Louis, MO) at 405 nm absorbance as described previously (Sun et al. 2017). Four biological replicates of each sample were analyzed.

**Protein Isolation**

Samples prepared as described earlier were frozen in liquid nitrogen and ground with a pestle to produce a powder, to which an appropriate amount of SDT lysis buffer [containing 4% sodium dodecyl sulfate, 100-mM Tris/HCl pH 7.6, and 1-mM dithiothreitol (DTT)] was added. The resulting homogenate was incubated at 100°C for 15 min, followed by sonication, centrifugation (12,100 × g, 30 min, 4°C), and collection of the supernatant. This sodium dodecyl sulfate, dithiothreitol, and Tris/HisCl (SDT) extraction processes were repeated twice, and all resulting supernatants were mixed. To this supernatant preparation, 14-ml trichloroacetic acid (TCA)/acetone (1:9) was added and the mixture was maintained at −20°C overnight. The following day, the mixture was centrifuged at 7,000 × g for 30 min at 4°C and the supernatant was discarded. To the pellet, 13 ml of precooled acetone was added following centrifugation at 7,000 × g for 30 min. This acetone wash procedure was repeated three times and the final pellet was air-dried. An appropriate amount of SDT lysis buffer was added to the dried pellet, followed by mixing and boiling for 15 min. The preparation was centrifuged at 14,000 × g for 40 min and the supernatant was collected. Proteins in the supernatant were quantified using a BCA Protein Assay Kit (Bio-Rad, Hercules, CA). Remaining sample was stored at −80°C for further experiments.

**Protein Digestion**

Proteins purified above were digested according to the FASP procedure (Wisniewski et al. 2009). For each sample, 200 µg of protein solution was reduced by incubation with 100-mM DTT in a boiling water bath for 15 min, and cooled to room temperature. To this solution, 200-µL urea (UA) buffer (8M urea, 150 mM Tris−HCl, pH 8.0) was added, followed by thorough mixing. The mixture was then transferred to 30-kDa ultrafiltration centrifuge tubes (Wisniewski et al. 2009) and centrifuged at 14,000 × g for 15 min. After discarding the flow-through, 200-µL UA buffer was added, followed by centrifugation at 14,000 × g for 15 min, and again discarding the flow-through. Thereafter, 100-µL iodoacetamide (IAA) (50 mM IAA in UA) was added to alkylate the preparation, followed by oscillation at 600 rpm for 1 min. The preparation was then stored in the dark for 30 min at room temperature and centrifuged at 14,000 × g for 10 min. The ultrafiltration tube was washed twice with 100-µL UA buffer and then twice with 100-µL 25 mM NH₄HCO₃. Proteins were digested with trypsin (2-µg trypsin in 40-µL NH₄HCO₃ buffer) overnight at 37°C. Since the digested peptides were smaller than the bore diameter of the ultrafilter, tryptic peptides were collected by centrifugation at 14,000 × g for 10 min, after which the tryptic peptides were desalted on a C18-SD Extraction Disk Cartridge (66872-U Sigma). The peptide content was estimated by UV light spectral density at 280 nm.

**Liquid Chromatography-Tandem Mass Spectrometry Analysis**

Equal amounts of tryptic peptides for each example were separated using a Thermo Finnigan EASY-nLC 1000 system (Thermo Fisher Scientific, Waltham, MA). The peptide mixture was loaded into a Thermo EASY column SC001 trap (150 μm × 20 mm; RP-C₁₈) connected to a Thermo EASY column SC200 150 μm × 100 mm (RP- C₁₈) in mobile phase A (0.1% formic acid in 2% acetonitrile), and separated with a linear gradient of 0%–100% mobile phase B (0.1% formic acid in 84% acetonitrile) over 120 min at a flow rate of 400 nL/min. LC−MS/MS analysis was performed for 120 min using a Q-Exactive mass spectrometer (Thermo Fisher Scientific). MS data were acquired using a data-dependent top10 method, dynamically selecting the most abundant precursor ions from the survey scan (300–1,800 m/z) with a charge state of +2 to +5 for higher collisional dissociation (HCD) fragmentation. The duration dynamic exclusion was 60 s. Survey scans were acquired at a resolution of 70,000 at m/z 200 and the resolution for HCD spectra was set to 17,500 at m/z 200, and with an isolation width of 2 m/z. The normalized collision energy was 30 eV.

**Protein Identification and Label-Free Quantification**

Six raw LC−MS/MS files obtained from the above process were analyzed using MaxQuant version 1.3.0.5 (Max Planck Institute of Biochemistry, Martinsried, Germany) (Cox and Mann 2008). The MaxQuant searches were performed against the Silkworm Genome Database (SilkDB) (http://silkworm.swu.edu.cn/silkdb/, last accessed October 15, 2018), National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/, last accessed October 15, 2018), and KAIKObase (http://sgp.dna.affrc.go.jp/KAIKObase/, last accessed October 15, 2018) databases. Peptide searches were executed using Andromeda search algorithms (Cox et al. 2011). Main search and MS/MS tolerance precursors were set to 6 and 20 ppm, respectively. Two missed cleavages were allowed. Methionine oxidation and N-terminal acetylation were used as the variable modifications, and carbamidomethylation of cysteines was considered a fixed modification. For all searches, the false discovery rate of peptide and protein identifications was 1%. The minimum number of quantified peptides per protein was one unique peptide. Proteins that were detected in two or more biological replicates were considered as representing reliable results. The data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD010998. The identified peptides and proteins are listed in Supp Tables 1 and 2 (online only), respectively. For the following analyses, all common contaminants were removed.
For comparison of protein abundances across different samples, the label-free quantification (LFQ) algorithm was used, which compares the intensities of the same peptides detected in different samples (Luber et al. 2010). In contrast, for comparison of abundances of different proteins within a sample, the intensity-based absolute quantification (iBAQ) algorithm was used (Schwanhäusser et al. 2011). The ratio of LFQ intensity of the Obs type compared with that of the normal type was set at two times as the criteria for screening significantly differential proteins with a statistically significant difference ($P$-value < 0.05). Statistical differences were evaluated using a $t$-test.

**Results**

**Characterization of the Obs Mutant**

Obs larvae have many different phenotypic characteristics when compared with the normal silkworm phenotype, including a short and stout larval body shape, translucent phenotype, abnormal melanin pigmentation, a shorter tail angle and abdominal appendage, and a lighter eye pattern (Fig. 1). Both Obs and normal-type larvae have 3 thoracic segments and 10 abdominal segments. By measuring the lengths of the internodes, intersegmental folds, and spiracles spacing in abdominal segments according to Supp Fig. 1 [online only], we found that the body shape of Obs larvae is consistently shorter (Fig. 2A–C). By measuring the circumference at each spiracle, we found that the circumference at the third, eighth, and ninth spiracles of Obs larvae was significantly greater than that of the normal type, although the circumference at the other spiracles did not differ significantly between the two types (Fig. 2D). This helps explain why Obs larvae appear slightly fatter than the normal-type larvae. These findings indicate that Obs larvae are indeed short and fat.

**Determination of Chitin, Uric Acid, and Melanin Content**

Chitin is a principal cuticle component and is crucial for the growth and development of insects. Chitin content was, therefore, determined in the larval integuments before the fourth molt, on day 3 of the fifth instar, and in the cuticle shed during the fourth molt. The results indicated that the content of chitin was significantly lower during these three periods in Obs larvae (Fig. 3A). Although Obs is regarded as a translucent mutant, Obs individuals lack the appearance of the classical translucent mutants of silkworm described in previous studies (Kiuchi et al. 2011, Wang et al. 2013a,b). By determining the uric acid content in the larval integument of Obs and normal-type larvae, we found that the content

Fig. 2. Characterization of the Obs mutant. (A) Length of internodes in the abdominal segment of the normal type and Obs type. 1st indicates the first abdominal internode, 2nd indicates the second abdominal internode, and so on. The eighth, ninth, and tenth internodes were measured as one internode because it is difficult to measure them separately. $n = 15$. (B) Length of intersegmental folds in the abdominal segment of the normal type and Obs type. 1st indicates the first intersegmental fold, 2nd indicates the second intersegmental fold, and so on. $n = 15$. (C) Length between spiracles of the normal type and Obs type. “1st–2nd” indicates between the first and second spiracles, “2nd–3rd” indicates between the second and third spiracles, and so on. $n = 15$. (D) Circumference at each spiracle of the normal type and Obs type. 1st indicates the first spiracle, 2nd indicates the second spiracle, and so on. $n = 15$. Data are presented as means ± SD. Student’s $t$-test, $^* P$ < 0.05 (A–D).
was significantly lower in the larval integument of Obs (Fig. 3B), indicating that Obs is a true translucent mutant.

The body color of Obs larva appears darker than that of the normal type. We speculated that there was a difference in the melanin content in integuments of Obs and normal-type larvae on day 3 of the fifth instar, and the results showed that the melanin content in the larval integument of Obs was significantly higher than that of the normal type (Fig. 3C). This may be the reason why the body color of Obs becomes darker than that of normal-type larva.

Total Proteins and Differential Protein Expression

The Obs mutant not only exhibits an abnormal body shape but also shows reduced accumulation of uric acid and abnormal pigmentation in the larval integuments. To elucidate the cause of multiple phenotypes, we performed LC–MS/MS analyses on the total proteins of Obs and normal-type integument on day 3 of the fifth instar. Taking all data together, we identified 16,010 tryptic peptides (Supp Table 1 [online only]). These peptides were assembled into 2,131 proteins (Supp Table 2 [online only]), among which 1,981 proteins were common to Obs and normal-type larvae, whereas only 117 and 33 were identified exclusively in Obs and normal type larvae, respectively (Fig. 4, Supp Table 3 [online only]). In total, 1,380 proteins were differentially expressed between Obs and normal-type larvae (Fig. 4, Supp Table 4 [online only]), among which 53 were significantly upregulated and 41 were significantly downregulated in the Obs larvae compared with the normal-type larvae (Fig. 4, Supp Table 5 [online only]). Overall, 244 proteins showed significantly differential expression between Obs and normal-type larvae, and the following analyses were performed on the basis of these differentially expressed proteins.

Classification of Significantly Differentially Expressed Proteins

The 244 significantly differentially expressed proteins between Obs and normal-type larvae were classified into 10 categories based on their annotated molecular function: enzymes (109), cuticular proteins (26), cytoskeleton-related proteins (20), small molecule- and ion-binding and transport proteins (21), protein synthesis-related proteins (15), vesicular transport-related proteins (12), nutrient storage proteins (7), protease inhibitors (5), immune-related proteins (11), and nonclassified and unknown-function proteins (18) (Fig. 5A; Supp Table 6 [online only]). The detected enzymes included six subgroups (EC1−6), of which oxidoreductases (EC1, 27%), transferases (EC2, 22%), and hydrolases (EC3, 27%) comprised 76% of the total number of enzymes. The cuticular proteins are mainly chitin-binding structural proteins. The cytoskeleton-related proteins are actin, tubulin, and their binding proteins. The small molecule- and ion-binding and transport proteins are involved in the interaction with carbohydrates, lipids, ions, and other small molecules. The category of protein synthesis-related proteins consists of replication-, transcription-, and translation-related proteins. The vesicular transport-related proteins are involved in processes mediated by transport vesicles. The nutrient storage proteins mainly belong to the low molecular mass proteins.
30-kDa lipoproteins. The identified protease inhibitors are classified into the serpin family and cysteine-rich protease inhibitor families.

Analysis of Chitin-Binding Proteins

Chitin-binding proteins play key roles in the structural and functional integrity of the cuticle. Figure 3A shows that the content of chitin was significantly reduced in Obs at the sampling time points employed in the present study. Therefore, we analyzed differences in the expression of chitin-binding proteins between Obs and normal-type larvae. According to a previous report (Dong et al. 2016), chitin-binding proteins mainly include cuticular proteins, 30-kDa lipoproteins, enzymes, and some small molecule-binding and transport proteins. Here, in our work, 59 proteins of the 244 significantly differentially expressed proteins were identified as chitin-binding proteins (Fig. 5B and Supp Table 7 [online only]). Among these, 21 proteins were significantly upregulated and 38 were significantly downregulated in the Obs larvae compared with the normal-type larvae. Of these 59 proteins, the highest proportion (42%) was cuticular proteins.

In the present study, the expression of 26 cuticular proteins was identified to be significantly altered between Obs and normal-type larvae, with four being upregulated and 22 being downregulated in Obs (Table 1). According to the classification of cuticular proteins (Dong et al. 2016), 16 out of the 26 cuticular proteins belonged to the RR motif-rich cuticular proteins (CPR), 5 were glycine-rich cuticular proteins (CPG), 2 were hypothetical cuticular proteins (CPH), and 2 were CPAP families, and 1 was chitin_bind_3 motif, as shown in Table 1. With the exception of one CPG motif protein, BGIBMGA010654, other cuticular proteins were identified as chitin-binding proteins. Furthermore, we estimated the protein abundance using the iBAQ algorithm (Supp Tables 8 and 9 [online only]). The results showed that the abundance of the 26 cuticular proteins and the total proteins were reduced in Obs larvae, and the abundance of these cuticular proteins in Obs larvae represented only 56.5% of that in the normal type larvae (Fig. 6). In addition, we found that the abundance of cuticular proteins accounted for 84.3% and 94.0% of the total proteins in Obs and normal type larvae, respectively (Fig. 6).

Analysis of Proteins Related to the Translucent Mutants in Silkworm

To date, the genes responsible for 10 translucent mutants in silkworm have been cloned as described in the Introduction. In the present study, we analyzed the proteins encoded by these 10 genes. The results showed that only the protein responsible for the os translucent mutant, BGIBMGA003864, was significantly downregulated in Obs individuals (Kiuchi et al. 2011; Supp Fig. 2A [online only]). In addition, the protein responsible for another silkworm translucent mutant, ow (BGIBMGA000173) (Ito et al. 2009), was downregulated by approximately 1.90-fold (Supp Fig. 2B [online only]); however, it did not meet the criterion (ratio: >2 or <0.5) used when screening for significantly differentially expressed proteins.

Analysis of Proteins Related to Melanin Synthesis

The Obs mutant is characterized by abnormal pigmentation on the lunar and crescent markings, and eye pattern as shown in Fig. 1. **Fig. 5.** Analysis of significantly different proteins. (A) Protein categorization based on annotated molecular function. (B) Chitin-binding protein categorization based on annotated molecular function. Binding and transport proteins indicate small molecule- and ion-binding and transport proteins. The abscissa (x-axis) shows the number of proteins mapped to each categorization. Bars indicate the number of proteins.
Figure 3C also showed that the melanin content in the integument of Obs larvae was significantly higher than that of the normal type. Analysis of the proteins related to melanin synthesis revealed that BGIBMGA003199 and gi|827552989|, which were annotated as dopa decarboxylase and phenoloxidase subunit 1-like, respectively, appeared to be significantly upregulated (Supp Fig. 2C and D [online only]).

Analysis of Obs Mutant Candidate Genes

The Obs mutant is governed by a single gene, which has been mapped at 6.2 cM on chromosome 18 (Yoshimura et al. 1984). In this study, the 244 significantly differentially expressed proteins were detected between the Obs mutant and normal silkworm, indicating that the mutation of the Obs gene could alter the expression of these proteins. To identify the possible candidate gene, the loci of genes encoding the 244 significantly differentially expressed proteins were analyzed according to reference genomes (http://silkworm.swu.edu.cn/silkdb/ and http://silkbase.ab.a.u-tokyo.ac.jp/cgi-bin/index.cgi, last accessed October 15, 2018) (Supp Tables 3 and 5 [online only]). The results indicated that nine genes encoding significantly differentially expressed proteins were located on chromosome 18 (Supp Table 10 [online only]) and were, therefore, potential candidate genes for the Obs mutant. Among these nine genes, BGIBMGA008213 and BGIBMGA008255 encode cuticular proteins. The proteins encoded by AK383678 and gi|827551453| are methylmalonate-semialdehyde dehydrogenase and 4-coumarate-CoA ligase 1-like, respectively. The protein encoded by gi|512914799| was annotated as an odorant-binding protein, which has been shown to be essential for a normally functioning olfactory system (Brito et al. 2016). BGIBMGA008477 is a silkworm homologue of the gene encoding 39S ribosomal protein L17, which is known to be involved in the process of translation, and has been reported to be a noninvasive biomarker with potential value in the differential diagnosis of minimal change disease and primary

Table 1. Cuticular proteins differentially expressed significantly between the Obs and normal-type larvae

| Protein IDs | LFQ intensity average of Obs type | LFQ intensity average of normal type | Ratio (Obs/normal) type | P-value | Cuticular protein family |
|-------------|----------------------------------|-------------------------------------|------------------------|---------|------------------------|
| BGIBMGA010500 | 3.77E+08 | 1.09E+08 | 3.47 | 5.43E−03 | CPR |
| BGIBMGA010913 | 2.47E+10 | 1.18E+10 | 2.10 | 6.11E−05 | CPR |
| BGIBMGA000339 | 3.00E+07 | 0 | 0 | CPR |
| BGIBMGA012605 | 5.73E+07 | 0 | 0 | CPR |
| BGIBMGA000249 | 7.81E+09 | 1.58E+10 | 0.49 | 2.60E−04 | CPR |
| BGIBMGA013163 | 1.45E+09 | 3.44E+09 | 0.42 | 9.72E−03 | CPR |
| BGIBMGA000324 | 6.61E+09 | 2.39E+10 | 0.28 | 7.39E−03 | CPR |
| BGIBMGA012594 | 7.05E+09 | 1.61E+09 | 0.44 | 3.83E−03 | CPR |
| BGIBMGA002349 | 1.50E+10 | 4.90E+10 | 0.31 | 2.60E−03 | CPR |
| BGIBMGA000332 | 2.80E+09 | 6.12E+10 | 0.46 | 2.48E−03 | CPR |
| BGIBMGA000329 | 6.07E+10 | 1.70E+11 | 0.36 | 8.25E−04 | CPR |
| BGIBMGA000333 | 1.34E+10 | 3.98E+10 | 0.39 | 2.52E−04 | CPR |
| BGIBMGA000338 | 8.78E+08 | 2.06E+09 | 0.43 | 2.51E−04 | CPR |
| BGIBMGA012596 | 6.94E+08 | 2.87E+09 | 0.24 | 1.11E−04 | CPR |
| BGIBMGA0005278 | 8.71E+07 | 5.10E+10 | 0.33 | 1.82E−02 | CPR |
| BGIBMGA014015 | 0 | 1.55E+07 | 0 | CPR |
| BGIBMGA000330 | 1.22E+09 | 2.68E+09 | 0.46 | 2.62E−05 | CPR |
| BGIBMGA002385 | 5.32E+08 | 1.95E+09 | 0.27 | 3.05E−03 | CPR |
| BGIBMGA008213 | 5.92E+08 | 1.87E+09 | 0.21 | 4.40E−03 | CPR |
| BGIBMGA008255 | 6.99E+09 | 2.74E+10 | 0.25 | 2.26E−04 | CPR |
| BGIBMGA010654 | 0 | 7.41E+07 | 0 | CPR |
| BGIBMGA011141 | 0 | 1.61E+08 | 0 | CPR |
| BGIBMGA014292 | 9.91E+09 | 2.28E+10 | 0.43 | 2.16E−03 | CPR |
| gi|223670968| 3.92E+09 | 8.60E+09 | 0.46 | 3.98E−03 | CPR |
| BGIBMGA007901 | 1.76E+08 | 4.72E+08 | 0.37 | 9.00E−03 | CPAP |
| BGIBMGA007677 | 7.55E+08 | 1.76E+10 | 0.43 | 9.06E−04 | CPAP |

Note: All cuticular proteins except BGIBMGA010654 have chitin-binding ability.

Figure 6. Abundance analysis of significantly differentially expressed proteins. Total proteins indicate the total significantly differentially expressed proteins. The data are presented as means ± SD. (n = 3), Student’s t-test, **P < 0.01, * P < 0.05.

Figure 3C also showed that the melanin content in the integument of Obs larvae was significantly higher than that of the normal type. Analysis of the proteins related to melanin synthesis revealed that BGIBMGA003199 and gi|827552989|, which were annotated as dopa decarboxylase and phenoloxidase subunit 1-like, respectively, appeared to be significantly upregulated (Supp Fig. 2C and D [online only]).
focal segmental glomerulosclerosis (Pérez et al. 2017). The protein encoded by BGIBMGA008526 is a protein of unknown function.

Discussion

OBS is a pleiotropic mutant of silkworm showing multiple phenotypes. The body of mutant larvae is short, stout, and translucent. Furthermore, there is an absence of melanin pigmentation on the larval and crescent markings on the second- and fifth-abdominal segments, respectively. OBS larvae experience deficiency in complete ecdysis and metamorphosis (Yoshimura et al. 1984). In this study, we observed that the eye pattern color of OBS larvae was lighter, and the tail angle and abdominal appendages were shorter than those of the normal-type larvae. In order to gain an understanding of the molecular processes underlying these abnormalities in the OBS mutant, we analyzed differences in the proteomes of OBS and normal-type larval integuments on day 3 of the fifth instar using LC−MS/MS, thus expanding current knowledge of the body shape determination, the metabolism of uric acid, and integument pigmentation in the silkworm.

The body shape of OBS larvae is shorter and stouter than that of the normal-type larvae. The OBS mutant gene has been located on Chromosome 18 (Yoshimura et al. 1984). In this study, 26 cuticular proteins were significantly differentially expressed between OBS and the normal-type larvae. Two of these are encoded by BGIBMGA008213 and BGIBMGA008255, which are located on chromosome 18. The silkworm genome contains about 220 genes encoding cuticular proteins (Futahashi et al. 2008). Until now, the mutation of only two cuticular protein genes has been known to produce mutated body shape in silkworm. For example, dysfunction in BmorCP2 arising from a frame shift mutation in the silkworm stony mutant, results in an abnormal body shape with the limitation of cuticle extension and an aberrant ratio between inter-nodes and intersegmental folds. At the same time, reduced chitin content in larval cuticle was discovered in the stony mutant (Qiao et al. 2014). Bo, another mutant, exhibits a bamboo-like body shape because of a 5-bp deletion in the second exon of BmorCPH24 that results in a frame shift mutation (Xiong et al. 2017). Furthermore, in Drosophila, cuticular proteins, TweedleD and Obstructor-E, have been found to be responsible for body shape (Guan et al. 2006, Tajiri et al. 2017). These findings indicate that gene mutation of cuticular proteins can result in body shape mutations in insects. Thus, we infer that the unique phenotype of the OBS mutant might be caused by one of the two cuticular protein genes. Regarding the high number of cuticular proteins differentially expressed in the OBS mutant, we speculated that the mutation in a cuticular protein gene might cause the abnormal formation of the OBS larval cuticle, which influenced the expression of other cuticular proteins. Alternatively, the mutation of other genes, such as those encoding transcription factors or signaling molecules, may lead to changes in downstream genes encoding cuticular proteins, thus resulting in the OBS mutant phenotypes.

The OBS mutant not only shows a short and stout larval body shape but also experiences deficiency in completing ecdysis and metamorphosis (Yoshimura et al. 1984). Chitin is one of the main components of the cuticle and is essential for the structural and functional integrity of cuticle (Moussian et al. 2005a). For example, chitin deficiency leads to a weakened cuticle in Tribolium castaneum embryos (Arakane et al. 2008). In Drosophila embryos, deficiency of chitin also causes the softening of cuticle (Moussian et al. 2005a). In this study, the reduction of chitin was detected in the larval cuticle of OBS mutant. It is possible that the cuticle structure in OBS mutant was disrupted due to a shortage of chitin, which resulted in inability of ecdysis and metamorphosis. In addition, the deacetylation of chitin is required for the cross-linking of proteins via amino groups, which renders the insect cuticle rigid and compact (Zhu et al. 2016, Qu et al. 2017). In this study, 59 significantly differentially expressed proteins were identified to have chitin-binding abilities according to a previous report (Dong et al. 2016). Two of these, gi|160333138| and BGIBMGA006214, are the homologous proteins of chitin deacetylase 1 and 2 in silkworm (Supp Fig. 2E and F [online only]). It has been reported that the downregulation of chitin deacetylase 1 and 2 resulted in an inability to shed the old cuticle; consequently, the mutant were found trapped in their exuviae, in the case of T. castaneum (Arakane et al. 2009, Zhu et al. 2016). It is, therefore, possible that the downregulation of these two proteins may cause a reduction in chitin deacetylation in OBS larvae, leading to the difficulty in completing ecdysis and metamorphosis.

The silkworm translucent mutant os is known to be defective in a gene encoding a hypothetical uric acid transporter in epidermal cells (Kiuchi et al. 2011). In the present study, BGIBMGA003864, the protein responsible for os, was significantly downregulated in OBS (Supp Fig. 2A [online only]) and may contribute to the translucent phenotype of OBS. In addition, another silkworm translucent mutant, ow, is controlled by Bmvarp, which is homologous to the vacuolar protein for sorting domain-containing proteins in mammals (Ito et al. 2009). In this study, BGIBMGA000173, which is responsible for ow, was downregulated by approximately 1.9-fold (Supp Fig. 2B [online only]). The downregulation of this protein may also contribute to the translucent phenotype of OBS.

Although our results showed that the melanin content in OBS larvae was significantly higher than that in normal-type larvae, light eye spots were observed, and crescents and star spots were barely visible in OBS. It has been reported that the pigmentation of larval cuticle markings in the swallowtail butterfly, Papilio xuthus, is closely related to the structures of the cuticle (Futahashi et al. 2012). Similarly, a correlation between the scale structure and pigmentation in butterfly wings has been reported (Janssen et al. 2001). This work showed that the reduction of chitin could disrupt the structural integrity of cuticle in the OBS mutant. It is possible that the defective structure of the larval cuticle may be an important reason for the abnormal melanin pigmentation in OBS larvae. In addition, in P. xuthus, several cuticular proteins display pigment-marking specificity and have been speculated function in transporting or maintaining specific cuticular pigments (Futahashi et al. 2012). Some of the downregulated cuticular proteins in OBS may, therefore, be involved in the transportation of the melanin or maintenance of melanin pigmentation.

Conclusions

The OBS mutant is a dominant mutant controlled by a single gene and is associated with multiple phenotypes. In this study, we investigated the phenotypes of OBS and analyzed the differential protein expression between the larval integument of OBS and normal silkworms. Comparative proteomic analysis suggested that the multiple phenotypes of OBS were related to the changes in the expression of proteins that participate in cuticle formation, uric acid metabolism, and melanin pigmentation. These observations may substantially contribute to understanding the molecular mechanisms underlying the OBS phenotypes.

Supplementary Data

Supplementary data are available at Journal of Insect Science online.
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Competing interests

All authors declare that they have no conflict of interest.

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