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Engineering a complex, multiple enzyme-mediated synthesis of natural plant pigments in the silkworm, *Bombyx mori*

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Plants produce various pigments that not only appear as attractive colors but also provide valuable resources in applications in daily life and scientific research. Biosynthesis pathways for these natural plant pigments are well studied, and most have multiple enzymes that vary among plant species. However, adapting these pathways to animals remains a challenge. Here, we describe successful biosynthesis of betalains, water-soluble pigments found only in a single plant order, Caryophyllales, in transgenic silkworms by coexpressing three betalain synthesis genes, cytochrome P450 enzyme *CYP76AD1*, *DOPA 4,5-dioxygenase,* and *betanidin 5-O-glucosyltransferase*. Betalains can be synthesized in various tissues under the control of the ubiquitous IE1 promoter but accumulate mainly in the hemolymph with yields as high as 274 μg/ml. Additionally, transformed larvae and pupae show a strong red color easily distinguishable from wild-type animals. In experiments in which expression is controlled by the promoter of silk gland-specific gene, *fibroin heavy-chain*, betalains are found predominantly in the silk glands and can be secreted into cocoons through spinning. Betalains in transformed cocoons are easily recovered from cocoon shells in water with average yields reaching 14.4 μg/mg. These data provide evidence that insects can synthesize natural plant pigments through a complex, multiple enzyme-mediated synthesis pathway. Such pigments also can serve as dominant visible markers in insect transgenesis applications. This study provides an approach to producing valuable plant-derived compounds by using genetically engineered silkworms as a bioreactor.

Bombyx mori | bioreactor | silk gland | betalains

Significance

Betalains are tyrosine-derived, water-soluble pigments found in Caryophyllales plants. Heterologous synthesis of betalains was attempted in a wide variety of organisms due to their attractive health-promoting properties and potential as a dominant genetic marker. We achieved multiple enzyme-mediated betalain production through heterologous expression of three betalain synthesis genes in a single transgenic silkworm line. Betalains were synthesized in whole bodies and silk glands using ubiquitous or tissue-specific gene promoters. Remarkably, betalains can accumulate in silk glands and be secreted into cocoons with high yields. This proof-of-principle study shows that a complex plant pigment synthesis pathway can be established in animals, offering an approach in the heterogenous biosynthesis of natural compounds.

The visually appealing colors of plant fruits and flowers result from a diverse array of naturally occurring pigments classified generally as chlorophylls, carotenoids, betalains, and anthocyanins (1, 2). Natural plant pigments have attracted both scientific and economic interest due to their complex biosynthetic pathways and health-promoting properties as food colorants (3, 4). However, whether plant pigment biosynthetic pathways can be established safely and productively in animals remains to be shown.

Betalains are naturally occurring water-soluble pigments derived from the aromatic amino acid, tyrosine, and comprise red–violet betacyanins and yellow betaxanthins. Betalains were thought to be restricted to plants belonging to Caryophyllales order, such as *Selenicereus costaricensis*, *Beta vulgaris*, and *Dianthus caryophyllus*. However, more recent studies show that they also exist in some species of fungi (*Amanita* and *Hygrocybe*) and a bacterium (*Gluconacetobacter diazotrophicus*) (5, 6). The betalain biosynthetic pathway has three main enzymatic steps in addition to a number of spontaneously occurring conversions. The pathway starts with the 3-hydroxylated conversion of tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA) catalyzed by cytochrome P450 enzymes (CYP76AD1/5/6) (Fig. 1). Subsequently, L-DOPA is oxidized further and cyclized to *cyclo*-DOPA by CYP76AD1. Alternatively, L-DOPA is converted to betalamic acid through a two-step reaction initiated by the ring-opening extradiol cleavage enzyme, DOPA 4,5-dioxygenase (DODA). After the spontaneous reaction, *cyclo*-DOPA and betalamic acid condense to betanidin. Finally, betanidin is glucosylated at the 5′O position by betanidin 5-O-glucosyltransferase (Glucosyltransferase) to form betanin (single-glucosylated betalain). There also is a variation of the core pathway in which *cyclo*-DOPA is first glycosylated by *cyclo*-DOPA-5-O-glucosyltransferase (cDOPA5GT) at the 5′O position to form *cycol*-DODA 5-O-glucoside, which spontaneously conjugates with betalamic acid to produce betanin. Betanin can undergo additional modifications including glycosylation and acylation to form complex betacyanins that contribute to the structural diversity of betalains. Betalamic acid can also spontaneously conjugate with amino acids or other amines, giving rise to the formation of betaxanthins (5–8). In addition to essential roles as visual attraction stimuli for pollinators and abiotic and biotic stress defense in the Caryophyllales, betalains also are reported to have potential beneficial roles as antioxidant, hypolipidemic, hepatoprotective, anti-inflammatory, and antidiabetic activities in humans (5, 9).

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The authors declare no competing interest.

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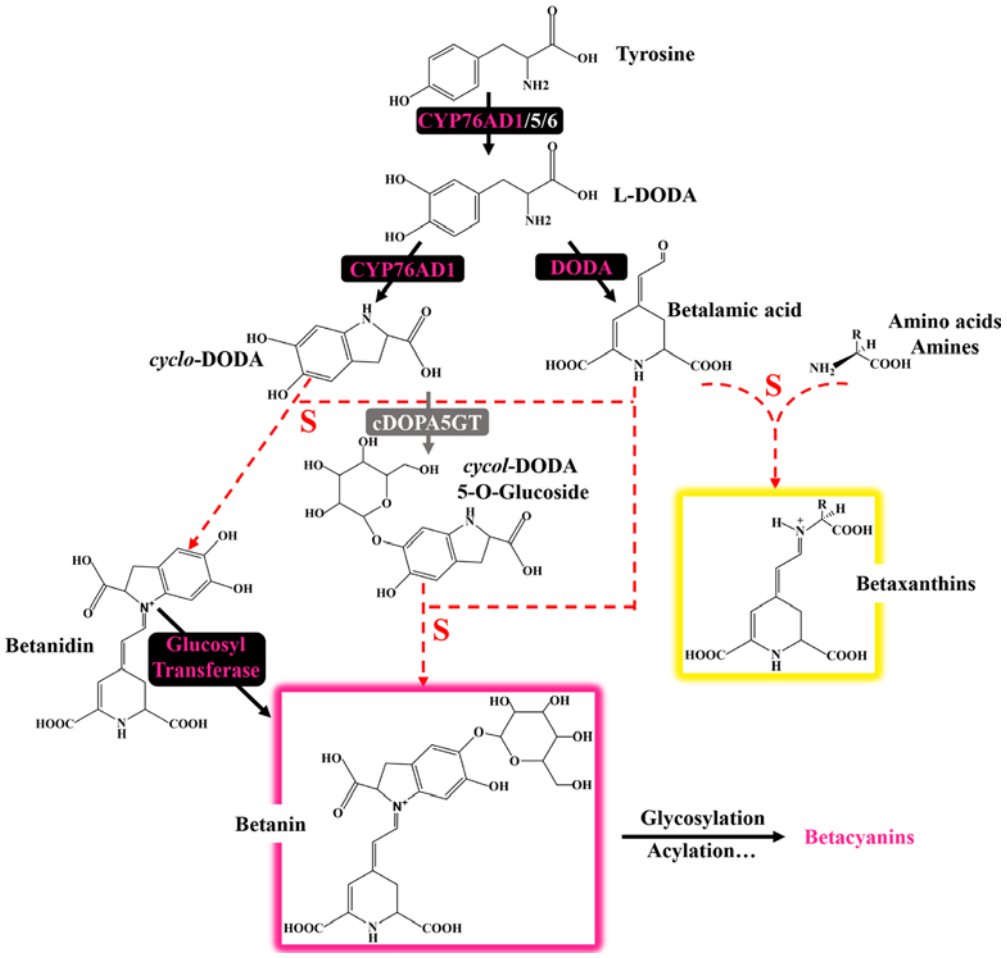

Fig. 1. A simplified schematic representation of the main enzymatic and spontaneous reactions for betalain biosynthetic pathway. The solid arrows indicate enzymatic reactions, while the dotted arrows and "S" represent spontaneous reactions.

Clarification of the core enzymatic steps provides the possibility of engineering betalains biosynthetic pathway in other organisms. Heterologous production of betalains has been reported in a wide variety of organisms including plant model organisms (*Arabidopsis* and tobacco), important crops (rice, tomato, potato, aubergine, and cotton), and microbes (*Escherichia coli* and *Saccharomyces cerevisiae*) (10–19). The bright color and simplicity of biosynthesis make betalains valuable as dominant visible genetic marker. A betalain-based system, MycoRed, in which expression of betalain synthesis genes are controlled by Arbuscular mycorrhiza-responsive promoters, has been used to show the occurrence and progression of AM symbiosis (20). Additionally, betalains have been used as biosensors for monitoring metabolite yields and heavy metal detection. However, betalain biosynthesis has yet to be achieved in animals (21–23).

The silkworm, *Bombyx mori*, is the most important economic insect in the silk industry and serves as a powerful lepidopteran model organism for genetic, physiological, neurobiological, and molecular studies. The high capacity for protein biosynthesis in silk glands and efficient genetic manipulation technologies, including germ-line transformation and genome editing, makes the silkworm ideal as a bioreactor for producing exogenous proteins (24–26). *B. mori* has been used to express exogenous proteins with potential biomedical applications, such as the human platelet-derived growth factor, epidermal growth factor, and fibroblast growth factor, as well as those with commercial applications including improving silk fiber mechanical properties through coexpression of spider silk proteins which enhance the tensile strength of silk fibers (27–32). Until now, the majority of silkworm silk gland bioreactors were restricted to the expression of a single protein. It is important to develop the expression capabilities for exogenous synthetic pathways to achieve mass production of beneficial biomolecules.

We show here the ability to produce betalains in transgenic silkworms by ectopic expression of three genes in the betalain synthesis pathway. Betalains are secreted into cocoon shells and easily extracted without affecting economically beneficial characteristics of the resulting silk. Thus, we not only verify the feasibility of synthesizing valuable heterogenous biomolecules produced by multiple-component biosynthetic pathways but also provide an approach for producing colorful silk fibers.

Results

Plasmid Construction and Establishment of Betalain Synthesis Pathway Strains. Two transgene plasmids, IE1-TG and FH-TG, were constructed comprising the three betalains synthesis genes, *CYP76AD1*, *DODA,* and *Glucosyltransferase*, under the control of either the constitutive, ubiquitously active IE1 gene promoter or the tissue- and temporally specific posterior silk gland (PSG) gene *fibroin heavy-chain* (*FibH*) promoter, FH-P (*SI Appendix*, Fig. S1 *A* and *B*). Expression by the single promoter of all three open-reading frames of the composite transgenes was achieved by removing the *CYP76AD1* and *DODA* stop codons and linking the coding sequences with a short sequence encoding the self-cleaving peptide, P2A. *piggyBac*-mediated germline transformation was used

to introduce the transgenes through microinjection in a bivoltine diapausing industrial silkworm strain, *Jingsong*. The embryos were treated with acid (15% HCl) before microinjection to terminate diapause. A total of 480 preblastoderm eggs in each group were microinjected with a mixed solution of expression and helper plasmids. Eight EGFP-positive IE1-TG G_1 broods were obtained from the 72 G_0 families set up, and six DsRed2-positive FH-TG G_1 broods were obtained from 61 families (*SI Appendix*, Table S1). The transformation efficiency per founding family, ~10%, is comparable to that of laboratory silkworm strains reported in previous studies (33, 34).

Expression Profile of Betalain Synthesis Pathway Genes. All transgenic progeny appeared to thrive normally, and no deleterious phenotypes were evident (*SI Appendix*, Fig. S2*A*). Additionally, fecundity and hatching of transgenic silkworms were comparable with WT animals (*SI Appendix*, Fig. S2 *B* and *C*). Two independent lines were selected from both IE1-TG and FH-TG groups for further analysis. Inverse PCR followed by sequencing was used to confirm that transgene cassettes were inserted into different intergenic regions of the genome (*SI Appendix*, Fig. S1 *C* and *D*). The accumulation levels of betalain synthesis pathway gene transcripts in different tissues and stages were evaluated by quantitative real-time PCR (qPCR). The three genes in IE1-TG strains were expressed ubiquitously in all sampled tissues with peak levels of accumulation in midguts (Fig. 2 *A*–*C*). The three genes in FH-TG strains were expressed predominantly in the PSG and the relative accumulation levels increased gradually from the early fifth larval stage to later wandering stage, mimicking the expression pattern of the endogenous *FibH* gene (Fig. 2 *D* and *E*). Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS/PAGE) analyses confirmed that betalains synthesis proteins were expressed highly in the PSG (Fig. 2*F*). The relative *CYP76AD1*, *DODA*, and *Glucosyltransferase* mRNA levels showed progressive but minor reductions consistent with their 5′ to 3′ ordered linkage to the single promoter.

Phenotypic Characterization of Transgenic Silkworms. A strong red color easily distinguishable from wild-type (WT) coloration was observed in all IE1-TG silkworms at different developmental stages (Fig. 3 *A* and *B* and *SI Appendix*, Fig. S3*A*). The hemolymph displayed a bright red color in dissected first-day, larval-stage transgenic silkworms, contrasting sharply with that seen in WT controls (Fig. 3*C*). Liquid chromatography–mass spectrometry (LC-MS) analysis was used to verify the observed red color resulted from the exogenous synthesis of betalains. Betanin content in transgenic silkworms was as high as 274 μg/mL in hemolymph (Fig. 3*D* and *SI Appendix*, Fig. S4). In comparison, no conspicuous red pigment was observed in other tissues of the transgenic silkworms, except for a faint amount visible in the testes (*SI Appendix*, Fig. S3 *B*–*E*). In addition, the internal surface of transgenic silkworm cocoons displayed faint red color, indicating that betalains can be secreted into the cocoon (*SI Appendix*, Fig. S3 *F* and *G*).

The whole-body red color seen in the IE1-TG silkworms was restricted to the silk glands in FH-TG transgenic insects and deepened progressively from the first day of the fifth larval stage to wandering stage (Fig. 3*E*). No obvious red color was seen in other tissues, including the hemolymph, fat body, and gonads, supporting the conclusion that betalains were synthesized specifically in the silk glands (*SI Appendix*, Fig. S5). Remarkably, betalains can be secreted into cocoons through silk spinning. FH-TG cocoons evidenced a strong red color and their internal surfaces appeared red-violet, indicative of a high yield of betalains (Fig. 4*A*). Secretion and incorporation of betalains into silk fibers was confirmed by unreeling the threads (Fig. 4*B*). Furthermore, FH-TG cocoons emit bright green fluorescence under ultraviolet light indicating the presence of betaxanthins (Fig. 4*C*) (35). Anticipating one potential beneficial role of betalains, we showed that IE1-TG silkworms have slightly increased resistance to starvation compared to WT controls (*SI Appendix*, Fig. S6).

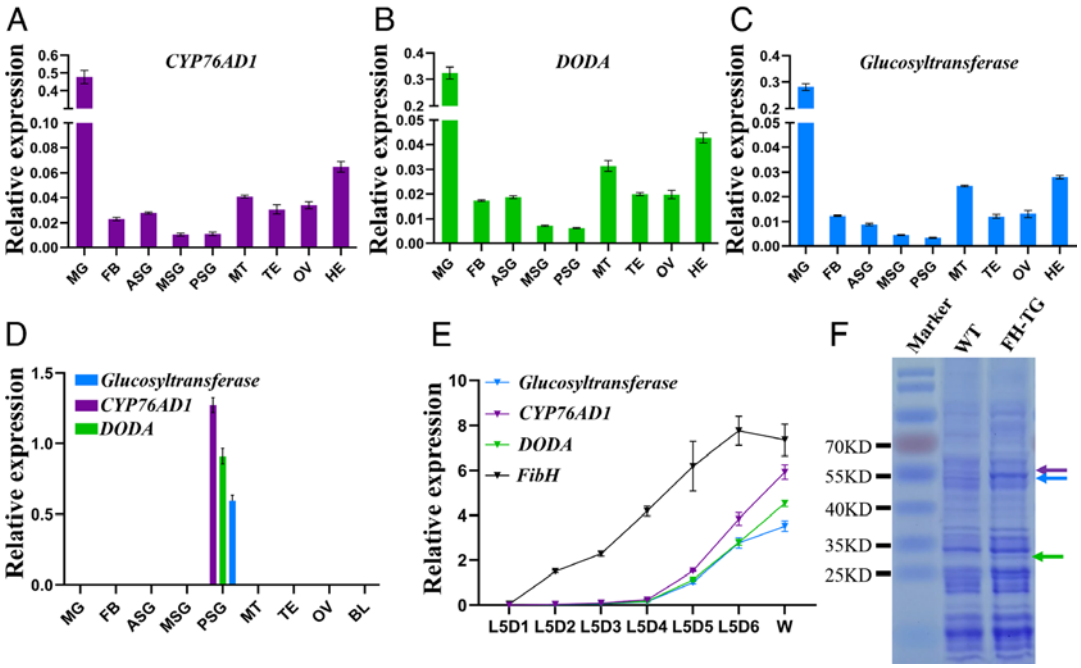

Fig. 2. Expression profiles of betalains synthetic pathway genes in transgenic silkworms. (*A*–*C*) Relative mRNA levels of *CYP76AD1* (*A*), *DODA* (*B*), and *Glucosyltransferase* (*C*) in different tissues of IE1-TG silkworms. Tissues tested were midgut (MG), fat body (FB), anterior silk gland (ASG), middle silk gland (MSG), PSG, *Malpighian* tubule (MT), testis (TE), ovary (OV), and hemolymph (HE). (*D*) mRNA of betalain synthetic pathway genes accumulating specifically in the PSGs of FH-TG silkworms. (*E*) Relative mRNA levels of betalain synthesis genes from the first day of fifth larval stage (L5D1) to wandering stage (W) in FH-TG silkworm. (*F*) The SDS/PAGE analyses of PSG from WT and FH-TG animals. Purple, blue, and green arrows indicate CYP76AD1, Glucosyltransferase, and DODA, respectively.

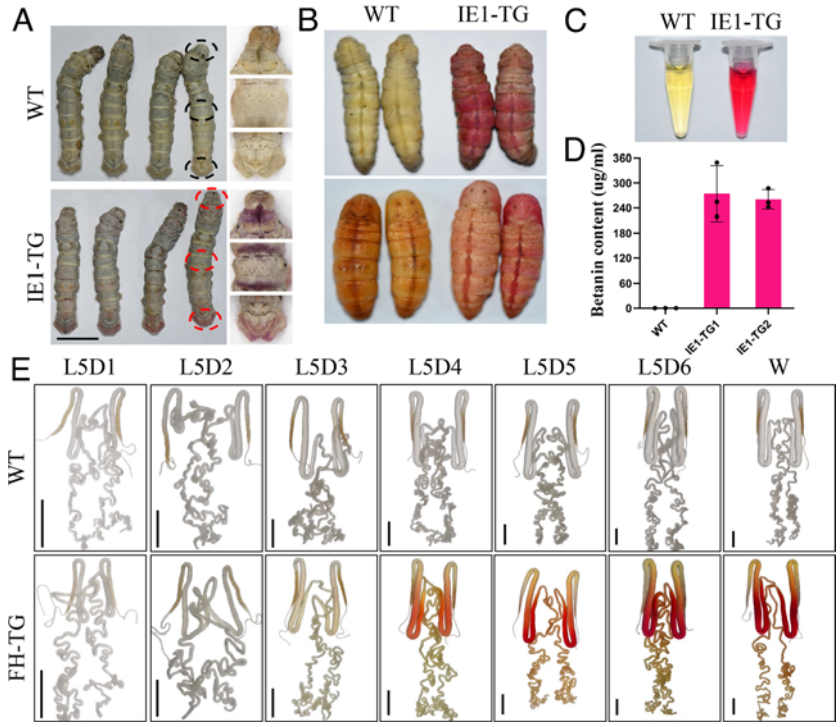

Fig. 3. Betalains can be synthesized in the silkworm. (*A* and *B*) Representative images of WT and IE1-TG transgenic silkworms at different developmental stages including larval (*A*), prepupal (*B*, top lane), and pupal stage (*B*, bottom lane). (Scale bar: 1 cm.) (*C*) Hemolymph of WT and IE1-TG animals. (*D*) Betanin content in the hemolymph of WT and IE1-TG animals. (*E*) Silk glands of WT and FH-TG animals from the first day of fifth larval stage (L5D1) to wandering stage (W). (Scale bar: 1 cm.)

Betalains Are Extracted Easily from Cocoon Shells. Betalains are water soluble and can be extracted at room temperature from FH-TG cocoons. A 100 mg cocoon shell was cut into pieces and dissociated with 4 mL nuclease-free water for 2 h with intermittent stirring. Betalains were successfully dissolved in the water, which displayed bright red color (Fig. 4*D*). The yield of soluble betanin using LC-MS analysis can reach 14.4 μg/mg in cocoons (Fig. 4*E* and *SI Appendix*, Fig. S7). This extraction method is more

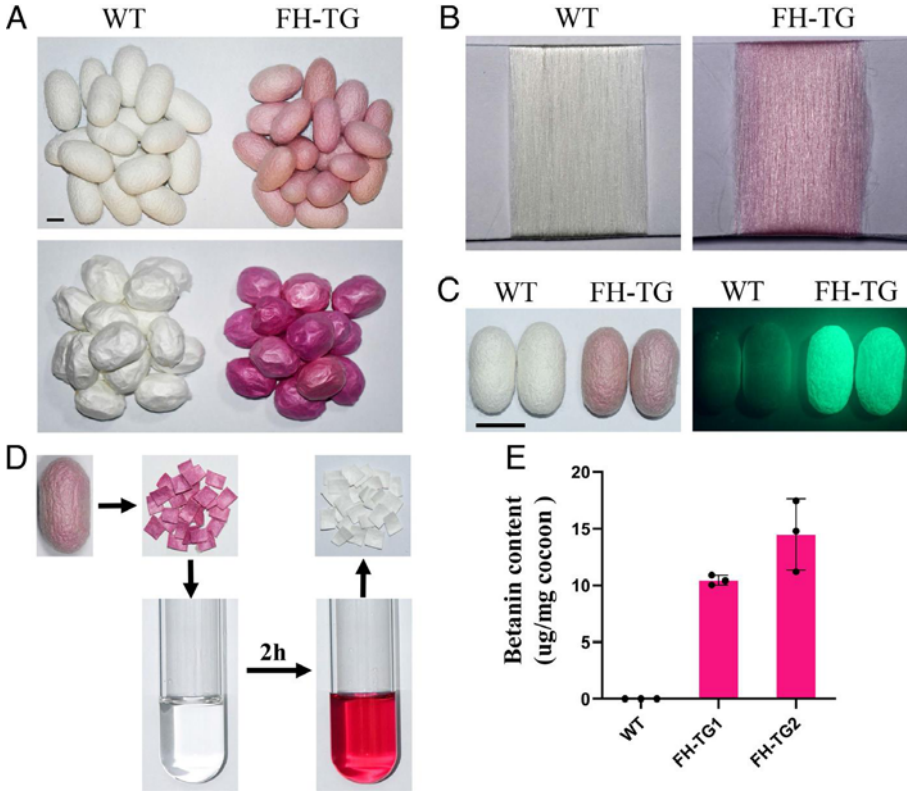

Fig. 4. Betalains can be recovered easily from cocoon shells. (*A*) Cocoons of WT and FH-TG animals. (*B*) Silk fibers of WT and FH-TG animals. (*C*) Cocoons of WT and FH-TG animals under brightfield (*Left*) and fluorescent excitation light (*Right*). (*D*) Betalains can be extracted from cocoons using water at room temperature. (*E*) Betanin content in cocoons of WT and FH-TG animals.

convenient than the canonical strategy using plants as raw material (36). The mechanical properties of the single silk fibers from WT and FH-TG silkworms were evaluated to determine if the presence of the pigments affects cocoon quality. The results support the conclusion that all measured properties, including average cross-sectional area, breaking stress, breaking strain, Young's modulus, and breaking energy, were equivalent between WT and FH-TG animals (*SI Appendix*, Fig. S8*A* and Table S2). In addition, FH-TG silkworms developed normally with no deleterious phenotypes detected, and the cocoon shell ratio (cocoon shell weight/total cocoon weight) also showed no significant difference compared to WT animals (*SI Appendix*, Fig. S8*B* and Table S3).

Discussion

We show here that plant pigments resulting from a multistep biosynthetic pathway can be synthesized in animals and produce high yields of extractable products. Betalain synthesis genes were expressed ubiquitously in IE1-TG silkworms in all detected tissues and conspicuous betalain production was evident as a red color found only in the hemolymph. This likely results from betalains being water soluble, and all betalains produced by different tissues accumulate in the hemolymph through physiological circulation since all tissues are immersed in this aqueous medium. In contrast, the strong red color appeared only in the larval silk glands of FH-TG silkworms following betalain production in the PSG, and the predominant accumulated accumulation was observed in the PSG and MSG (middle silk gland). Furthermore, betalains produced in silk glands can be transferred into cocoons and into the final fibers during spinning. We investigated the content of betanin, the single-glucosylated betalain, using LC-MS analysis in IE1-TG silkworm hemolymph and FH-TG silkworm cocoons. Considering the structural diversity of various betalains, total yields of betalains produced in transgenic silkworms are likely to be much higher than what we detected. Betaxanthins can emit green fluorescence (35) and a strong signal was detected in FH-TG silkworm cocoons, indicative of the presence of betaxanthins. This confirms that betalains can be used as a dominant marker in insect genetic transformation since it can show both visible red color and green fluorescence.

Growth and development of transgenic silkworms were not affected by expression of exogenous betalain synthesis genes and subsequent accumulation of betalains. Economic characteristics such as cocoon weight and mechanical properties of the single silk fibers of FH-TG silkworms were altered minimally. These results support the conclusion that betalains can be a benign coproduct in the silk industry. Furthermore, recovering betalains from cocoons is more convenient than other methods that use plants as raw materials (36). In addition to their attractive colors, betalains are noted for their potential beneficial properties such as antioxidative, hypolipidemic, anti-inflammatory, and antidiabetic activities (5, 9). It will be of great interest to investigate the possibility of promoting disease resistance of beneficial transgenic animals expressing betalains.

Another potential application of this expression system is to produce predyed colorful silk since betalains can be secreted into cocoons. However, betalains are hydrophilic, and no pigments would be left in silk fiber during silk reeling from cocoons. Thus, creating predyed and free-of-degumming colorful silk is still challenging. Nevertheless, our successful study demonstrating a heterologous biosynthetic pathway comprising three enzymatic steps and several spontaneously occurring reactions introduced into the transgenic silkworm provides support for further work in this area. This is the basis for potential mass production of natural plant compounds using genetically engineered silkworms, providing an avenue for the biosynthesis of other valuable compounds. We expect that the yield of betalains can be increased significantly using targeted gene replacement systems reported in our previous study (31). In summary, our study provides the proof of principle that betalains can be synthesized in animals and offers experimental evidence for the possibility of introducing other complete heterologous biosynthetic pathways into the silkworm.

Materials and Methods

Silkworm Material and Growth Conditions. The bivoltine diapausing industrial silkworm strain, *Jingsong*, was used for all experiments. The larvae were reared on fresh mulberry leaves at 25 °C under standard conditions as described (33).

Plasmid Construction. The protein sequences of the three betalain synthesis genes were downloaded from the National Center for Biotechnology Information, and the GenBank accessions are as follows: CYP76AD1 (AET43289.1), DODA (AET43293.1), and Betanidin-5-O-Glucosyltransferase (CAB56231.1). The coding sequences were optimized for better protein expression in *B. mori* by removing the *CYP76AD1* and *DODA* stop codons and linking all three using a sequence encoding the self-cleavage peptide, P2A (GSGATNFSLLKQAGDVEENPGP), forming a betalain production open-reading-frame (ORF) (11, 16). This was synthesized commercially by Sangon Biotech (Shanghai). The ORF was subcloned into the pBac-IE1-EGFP vector to generate pBac-IE1-EGFP-IE1-ORF-sv40 for ubiquitous expression. The ORF was subcloned into pBac-3×P3-dsRed2 vector to form pBac-3×P3-dsRed2-FibH-P-NTD-ORF-CTD for PSG-specific expression. The composition of NTD and CTD was described previously (31).

Germline Transformation. Embryos (G_0) were collected within 4 h after oviposition. After acid treatment (15% HCl) for 1 h at room temperature, embryos were washed using running water. Subsequently, a mixture of transformation expression plasmids (400 ng/μL) and helper plasmids (200 ng/μL) were microinjected into G_0 embryos (33). All treatments were completed within 8 h after oviposition. Injected embryos then were incubated at 25 °C in a humidified chamber for ~10 d until larvae hatched. The G_0 larvae were reared to the adult stage and mated to WT moths. G_1 progeny were screened for the presence of the fluorescent marker using a Nikon SMZ1270 fluorescence microscope.

Quantitative reverse-transcription polymerase chain reaction (qRT-PCR). qRT-PCR analyses were performed using SYBR Green Real-time PCR Master Mix (TOYOBO). Fluorescence signals were recorded using the CFX connect qRT-PCR instrument (Bio-Rad). PCR conditions were those given in the instructions for use of SYBR Green Real-time PCR Master Mix. *B. mori ribosomal protein 49* was used as internal reference. Three independent biological replicates were performed for each qRT-PCR analysis. All oligonucleotide primers are listed in *SI Appendix*, Table S1.

Inverse PCR. The inverse PCR assay was performed as described previously to confirm insert regions of transgene cassettes in the genome (33). Briefly, genomic DNA was extracted from transgenic moths and digested with *Bsp*143I (Thermo Fisher Scientific) and circularized with T4 ligase (Thermo Fisher Scientific) overnight at 22 °C. The circularized products were purified and served as templates for further PCR assays. Two rounds of PCR assays were performed and products of the first PCR assay was used as the template for the second PCR assay. Primer sequences were listed in *SI Appendix*, Table S1. Amplified products were extracted and cloned into the pJET-1.2 vector (Thermo Fisher Scientific) and sequenced.

Betanin Extraction and LC-MS Analysis. Individual hemolymph samples were collected from three IE1-TG silkworms. For FH-TG silkworms, a 100 mg cocoon shell was cut into pieces and dissociated with 4 mL nuclease-free water for 2 h, and each sample was collected from the solute of three cocoons. 20 μL of each sample was treated with 140 μL solvent [water: methanol: acetonitrile, 1: 2: 2 (v/v/v)], and the supernatant used for detection after centrifugation. All detection was performed on a Ultra-High Performance Liquid Chromatography instrument combined with a QTRAP® 6500+ MS system equipped with an electrospray ionization (ESI) source (AB SCIEX). Instrument control and data acquisition were performed using Analyst 1.6.3 software (AB SCIEX), and data processing was performed using MultiQuant 3.0.2 software (AB

SCIEX). The compounds were separated with an Agilent Poro shell 120 SB-Aq column (2.7 μm 3.0*100 mm) at 40 °C using eluants A (2 mM ammonium acetate in water) and eluants B (2 mM ammonium acetate in methanol). The optimized ESI operating parameters for positive mode were as follows: IS, 5500 V; CUR, 35 psi; TEM 500 °C; GS1, 55 psi; and GS2, 55 psi. Betanin content was identified and quantified based on authentic standards (Sigma).

Mechanical Testing of Silk Fibers. Single silk fibers of the WT and FH-TG silkworms were subjected to mechanical tests. The sericin layer of silk fibers was removed by gentle stirring in 0.05% (wt/vol) $NaHCO_3$ for 60 min at 100 °C. The degummed silk fibers were washed with water at 80 to 90 °C with careful stirring followed by distilled water washing at room temperature for six times. Individual fibers were tested for breaking stress, breaking strain, Young's modulus, and breaking energy (31). The cross-sectional areas for fibers were determined by taking an average of 15 measurements using a freezing microtome and confocal microscopy (Nikon C2). Single-fiber testing was performed with the MTS C42 mechanical testing instrument (at 20 to 25 °C and 40 to 45% relative humidity; gauge length: 20 mm; cross-head speed: 5 mm/min) with a cell load of 5 N. Each group has at least 45 fibers derived from five different individual cocoons.

Data, Materials, and Software Availability. All study data are included in the article and/or *SI Appendix*.

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