RNA Interference Depletion of the Halloween Gene Disembodied Implies its Potential Application for Management of Planthopper Sogatella furcifera and Laodelphax striatellus

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Introduction

The white-backed planthopper Sogatella furcifera and the small brown planthopper Laodelphax striatellus are economically important pests of rice in China. They cause serious damage to rice plants by acting as vectors of several rice viruses, sucking the phloem sap and blocking the phloem vessels. Ecdysteroid hormone 20-hydroxyecdysone regulates insect development and reproduction. A cytochrome P450 monoxygenase CYP302A1 (22-hydroxylase), encoded by the Halloween gene disembodied (dib), plays a critical role in ecdysteroidogenesis. The objective of this study is to test whether dib genes are potential targets for RNA interference-based management of S. furcifera and L. striatellus. We cloned and characterized Sfdib and Lsdib. The open reading frame regions of dib genes were generated and used for designing and constructing dsRNA fragments. Experiments were conducted using oral delivery of dsdib to investigate the effectiveness of RNAi in S. furcifera and L. striatellus nymphs. Real-time quantitative reverse transcriptase-PCR analysis demonstrated that continuous ingestion of dsdib at the concentration of 0.01, 0.05 and 0.50 mg/ml diminished Sfdib expression levels by 35.9%, 45.1% and 66.2%, and ecdysone receptor (SfEcR) gene mRNA levels by 34.0%, 36.2% and 58.5% respectively in S. furcifera, and decreased Lsdib expression level by 18.8%, 35.8% and 56.7%, and LsEcR mRNA levels by 25.2%, 46.8% and 68.8% respectively in L. striatellus. The reduction in dib and EcR transcript abundance resulted in observable phenotypes. The development of nymphs was impaired and the survival was negatively affected. Our data will enable the development of new insect control strategies and functional analysis of vital genes in S. furcifera and L. striatellus nymphs.

Abstract

Sogatella furcifera and Laodelphax striatellus are economically important rice pests in China by acting as vectors of several rice viruses, sucking the phloem sap and blocking the phloem vessels. Ecdysteroid hormone 20-hydroxyecdysone regulates insect development and reproduction. A cytochrome P450 monoxygenase CYP302A1 (22-hydroxylase), encoded by the Halloween gene disembodied (dib), plays a critical role in ecdysteroidogenesis. The objective of this study is to test whether dib genes are potential targets for RNA interference-based management of S. furcifera and L. striatellus. We cloned and characterized Sfdib and Lsdib. The open reading frame regions of dib genes were generated and used for designing and constructing dsRNA fragments. Experiments were conducted using oral delivery of dsdib to investigate the effectiveness of RNAi in S. furcifera and L. striatellus nymphs. Real-time quantitative reverse transcriptase-PCR analysis demonstrated that continuous ingestion of dsdib at the concentration of 0.01, 0.05 and 0.50 mg/ml diminished Sfdib expression levels by 35.9%, 45.1% and 66.2%, and ecdysone receptor (SfEcR) gene mRNA levels by 34.0%, 36.2% and 58.5% respectively in S. furcifera, and decreased Lsdib expression level by 18.8%, 35.8% and 56.7%, and LsEcR mRNA levels by 25.2%, 46.8% and 68.8% respectively in L. striatellus. The reduction in dib and EcR transcript abundance resulted in observable phenotypes. The development of nymphs was impaired and the survival was negatively affected. Our data will enable the development of new insect control strategies and functional analysis of vital genes in S. furcifera and L. striatellus nymphs.

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Phenotypic analysis reveals that dib mutant embryos produce little or no cuticle, exhibit severe defects in many late morphogenetic processes such as head involution, dorsal closure and gut development, and finally die at the embryonic stage [18].

Furthermore, mostly based on sequence similarity, dib genes are described or predicted in hemipteran Acrithosiphon pisum [23] and Bemisia tabaci [24], in dipteran Aedes aegypti [23], in lepidopteran Spodoptera littoralis [26], and in hymenopteran Apis mellifera [27]. In addition to insects, a dib gene has been identified in the water flea Daphnia pulex [28].

In the present paper, we cloned and characterized dib genes in S. furcifera and L. striatellus. We demonstrated the insecticidal action of orally-delivered dsRNA (dsfurc) in S. furcifera and L. striatellus nymphs. We found that continuous ingestion of dsfurc delayed nymphal growth and caused lethality in both planthopper species. Our data will enable the development of new insect control strategies and functional analysis of vital genes.

Materials and Methods

Insect Culture

S. furcifera and L. striatellus were routinely reared on the rice (Oryza sativa) variety Taichung Native 1, using a protocol described recently [14,15]. In our laboratory, S. furcifera eggs hatched into nymphs within 7 days. Nymphs went through 5 instars, with the first-, second-, third-, fourth- and fifth-instars lasting 2.5, 2.0, 2.0, 3.0 and 3.0 days, respectively. L. striatellus eggs hatched into nymphs within 13 days. The first-, second-, third-, fourth- and fifth-instar nymphs spent an average of 6.0, 5.0, 5.0, 5.0 and 6.0 days, respectively.

Cloning and Sequencing of Full-length Lsdib cDNA

We obtained SfDib and Lsdib unigenes from the corresponding transcriptome datasets of S. furcifera and L. striatellus recently constructed in our laboratory [14,15]. The resulting sequences were authenticated by polymerase chain reaction (PCR) using specific primers (Table 1), the same thermal cycling conditions and PCR reaction system as described recently [14,15]. The full-length SfDib and Lsdib cDNAs were obtained by 5’- and 3’-RACE, using antisense and sense gene-specific primers (Table 1) and the universal primers in the SMART® RACE kit, and the standard manufacturer-recommended components and thermal cycling conditions. The PCR products were then cloned into pGEM-T easy vector (Promega), and sequenced at both strands. After full-length cDNAs were obtained, two primer pairs were designed (Table 1) to verify the sequence. Open reading frames were predicted using the ediseq program of DNASTar (http://www.dnastar.com). The nucleotide sequences were finally annotated by Dr. David Nelson in accordance with the CYP nomenclature committee convention (http://drnelson.uthsc.edu/DrDaveN/CytochromeP450.html) [29]. The resulting sequences were submitted to GenBank (SfDib, KC579455; Lsdib, KC579460).

Multiple Sequence Alignment and Phylogenetic Analysis

The annotated Dib proteins from a spider species Tetranychus urticae and 17 insect species were aligned with the predicted SfDib and Lsdib sequences using ClustalW2.1 [30]. The alignment was used to construct the maximum-likelihood (ML) trees using RAxML version 7.26 [31] to select the best-fitting model (LG+I) after estimation by ProtTest [32]. The reliability of the ML tree topology was evaluated by bootstrapping a sampling of 1000 replicates.

Bioassays Using dsRNA, 20E and their Mixtures

DNA samples for the production of SfDib, Lsdib and enhanced green fluorescent protein (egfp, control) were synthesized by PCR, using a 458 bp SfDib cDNA, a 406 bp Lsdib cDNA and a 414 bp egfp fragments, and gene-specific primers (Table 1) incorporating the T7 RNA polymerase promoter sequence (5’-taatagcactata-tagge-3’) [14,15]. PCR products were purified using the Wizard HS Gel and PCR Clean-Up System (Promega, Madison, WI, USA) and used for dsRNA synthesis using the T7 Ribomax TM Express RNAi System (Promega, Madison, WI, USA). The synthesized dsRNAs were respectively isopropanol-precipitated, resuspended in nuclease-free water, quantified by a spectrophotometer (NanoDrop TM 1000, Thermo Fisher Scientific, USA) at 260 nm, and kept at ~70°C until use.

A dietary dsRNA-introducing procedure previously reported [8,13] was used to feed the insects, with glass cylinders (12 cm in length and 2.8 cm in internal diameter) as feeding chambers. Twenty S. furcifera first-instar nymphs or L. striatellus third-instar nymphs were carefully transferred into each chamber and pre-reared for one day to the next instar, on liquid artificial diet between two layers of stretched Parafilm M (Pechiney Plastic Packaging Company, Chicago, IL, USA) that were placed at both ends of the chamber. The artificial diet containing one of the dsRNAs was then used to feed the nymphs. The diet was changed and dead nymphs were removed daily.

Two bioassays were carried out for each planthopper species, with its corresponding dsfurc (dsSfDib for S. furcifera nymphs, dsLsdib for L. striatellus nymphs). The first experiment had five treatments including non-dsRNA diet (blank control), the diet containing dsegfp at the concentration of 0.50 mg/ml (negative control), and the diets containing dsfurc at the concentration of 0.05, 0.01 and 0.50 mg/ml. The second bioassay was a rescue experiment. 20E (Sigma, purified by reverse-phase HPLC before experiments) in ethanol was mixed with the diet to obtain a final concentration of 0.50 mg/ml diet, a dose that has no obvious negative effects on planthopper species [14,15]. There were four treatments: non-dsRNA+ethanol diet (blank control), 0.50 mg/ml of dssegfp+ethanol diet (negative control), 0.50 mg/ml of dsfurc+ethanol diet, and 0.50 mg/ml dsfurc+0.5 mg/ml of 20E diet. All treatments in the experiments were replicated 25 times (25 chambers), and a total of 250 nymphs in each treatment were used (100 nymphs for bioassays and 150 nymphs for q-PCR).

The experiments lasted 6 days for S. furcifera, and 5 days for L. striatellus nymphs. Mortality was recorded daily. After each experiment, the surviving nymphs were transferred to rice to measure the development delay. The instars were identified by the same method described recently [14,15]. Mortalities from the termination of experiment to adults emergence were also calculated.

Real-time Quantitative PCR

For S. furcifera, total RNA samples were prepared from whole bodies of the second-instar (Day 1, 2 in the second instar, I2D1 and I2D2), third-instar (I3D1 and I3D2) and fourth-instar (I4D1, I4D2 and I4D3) nymphs on rice, from the head, thorax and abdomen of I4D3 nymphs, and from nymphs subjected to the 6-day bioassays. For L. striatellus, total RNA samples were prepared from I1D1 to I4D5, and I5D1 nymphs on rice, from the head, thorax and abdomen of I4D5 nymphs, and from whole bodies of nymphs which had been exposed to dsRNA for 5 days. Each sample contained 5–10 nymphs and repeated in biological triplicate. All RNAs were isolated using SV Total RNA Isolation System Kit (Promega). The expression levels of SfDib and Sfegfp in each S. furcifera nymphal sample were measured by qRT-PCR as
Table 1. Primers used in RT-PCR, 5’ and 3’ RACE, synthesizing dsRNA, and performing qRT-PCR.

| Primer                  | Sequence (5’ to 3’)          | Amplicon size (bp) |
|-------------------------|------------------------------|--------------------|
| Primers used in RT-PCR  |                              |                    |
| SfdibFp                 | GCCCCAGGTCTTTCCTCGTT         | 300                |
| SfdibRp                 | GGTCCGTGTTGAGCGCGAG         |                    |
| LsdibFp                 | AAATGGGGAAAGCTGATTGT       | 1431               |
| LsdibRp                 | TTATACCGAGGCGAGGC          |                    |
| Primers used in 5’-RACE |                              |                    |
| SfdibGSP                | ATCCCGATTGACCCGATGTTA       |                    |
| SfdibNGSP               | TCGGGATTTGGAAAGGTGTTC       |                    |
| LsdibGSP                | TAGCACCCCTCCATGCGATG        |                    |
| LsdibNGSP               | CCGGGTGTCTGAGAAGTTGTC       |                    |
| Primers used in 3’-RACE |                              |                    |
| SfdibGSP                | TGCGCATCGGAATGAGG           |                    |
| SfdibNGSP               | ACAGCCTTCTCCGCAAGGGG        |                    |
| LsdibGSP                | TCAGCGATGCGAATCCACC         |                    |
| LsdibNGSP               | GTCTCGCAAGACAAAGTGCGCT      |                    |
| Primers used in PCR for End to End |          |                    |
| SfdibFp                 | GTTGGGATGCTGCCCTTAA         | 2888               |
| SfdibRp                 | ATAGCCTATGCTACCTAAGG        |                    |
| LsdibFp                 | GTAAATCGTGATCATCATT         | 1682               |
| LsdibRp                 | TTACGGTTCAAGCTTG           |                    |
| Synthesizing the dsRNAs |                              |                    |
| SfdibFd                 | taatacgactcactataggg TCGAGGAAGTTGCACTG | 458              |
| SfdibRd                 | taatacgactcactataggg ACAGCGACCTTGGTACTT |            |
| LsdibFd                 | taatacgactcactataggg ACATCATTTGCTGCACTT | 406          |
| LsdibRd                 | taatacgactcactataggg TGAGCTCATACTGAGGTATTT |      |
| egfpup                  | taatacgactcactataggg AAGTTAGCGTGCTCG | 414            |
| egfpdown                | taatacgactcactataggg CTTGCCGTAATGCTAC |            |
| Performing the qPCR     |                              |                    |
| SfdibFq                 | TCGGACAAATCTGTAGGGAAGAC     | 116                |
| SfdibRq                 | GACTGTCGCGTCGCTTGAGG        |                    |
| SfEcRFq                 | AATGAGTTCGACCAAGCTTGGA      | 129                |
| SfEcRRq                 | AATGAGTTCGACCAAGCTTGGA      |                    |
| SfRPL9Fq                | TGTGTAAGCACCAGAGAAACTCA     | 131                |
| SfRPL9Rq                | AGCAGTGGCTCCTGTGCTCTT       |                    |
| SFARFq                  | CACAATATGACCCGACTTTGGTACGG | 141                |
| SFARFrq                 | CACAGTCAGCCAGCTCCTCCTC      |                    |
| LsdibFq                 | ATGTCTAGAAGGTGCTGAACATCG   | 132                |
| LsdibRq                 | TGGTGATCTGCTGCTGGTAAACG    |                    |
| LsEcRFq                 | AACTGTTGCGGAGGACCAAATC     | 187                |
| LsEcRRq                 | AGAATCGTCAAGAGCTGGTGCGACCA |            |
| LsEF-1Fq                | CCTTACCATGTTGGAGTCTTATT    | 95                 |
| LsEF-1Rq                | TGCTTCCCTGGCTCCTCCTC       |                    |
| LsARFq                  | TCGGACAGTATCAAGACCATC      | 104                |
| LsARFrq                 | GCAGCAATCTCATCAAAAGC       |                    |

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Halloween Gene Disembodied in Two Planthoppers

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| LsdibRp                 | TTATACCGAGGCGAGGC          |                    |
| Primers used in 5’-RACE |                              |                    |
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| SfdibNGSP               | TCGGGATTTGGAAAGGTGTTC       |                    |
| LsdibGSP                | TAGCACCCCTCCATGCGATG        |                    |
| LsdibNGSP               | CCGGGTGTCTGAGAAGTTGTC       |                    |
| Primers used in 3’-RACE |                              |                    |
| SfdibGSP                | TGCGCATCGGAATGAGG           |                    |
| SfdibNGSP               | ACAGCCTTCTCCGCAAGGGG        |                    |
| LsdibGSP                | TCAGCGATGCGAATCCACC         |                    |
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| Performing the qPCR     |                              |                    |
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| SfEcRFq                 | AATGAGTTCGACCAAGCTTGGA      | 129                |
| SfEcRRq                 | AATGAGTTCGACCAAGCTTGGA      |                    |
| SfRPL9Fq                | TGTGTAAGCACCAGAGAAACTCA     | 131                |
| SfRPL9Rq                | AGCAGTGGCTCCTGTGCTCTT       |                    |
| SFARFq                  | CACAATATGACCCGACTTTGGTACGG | 141                |
| SFARFrq                 | CACAGTCAGCGTCCGCTCCTC      |                    |
| LsdibFq                 | ATGTCTAGAAGGTGCTGAACATCG   | 132                |
| LsdibRq                 | TGGTGATCTGCTGCTGGTAAACG    |                    |
| LsEcRFq                 | AACTGTTGCGGAGGACCAAATC     | 187                |
| LsEcRRq                 | AGAATCGTCAAGAGCTGGTGCGACCA |            |
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| LsEF-1Rq                | TGCTTCCCTGGCTCCTCCTC       |                    |
| LsARFq                  | TCGGACAGTATCAAGACCATC      | 104                |
| LsARFrq                 | GCAGCAATCTCATCAAAAGC       |                    |

desccribed previously [14,15]. The S. furcifera ADP-ribosylation factor (ARF) and ribosomal protein RPL9 were used as internal controls. The mRNA abundance of Lsdib and LsEcR in each L. striatellus nymphal sample was measured using the internal control genes ADP-ribosylation factor (ARF) and elongation factor-1 (EF-1) (primers for these genes are listed in Table 1). Each sample was repeated in technical triplicate. Data were analyzed by the 2−ΔΔCt method [33], using the geometric mean of the two internal control genes.
for normalization according to the strategy described previously [33,34].

Data Analysis

The data were given as means ± SE, and were analyzed by ANOVA or a repeated measures ANOVA followed by the Tukey-Kramer test, using SPSS for Windows (SPSS, Chicago, IL, USA).

Results

Molecular Cloning and Sequence Analysis

Two cDNAs of the putative Halloween gene dib (disembodied, cyp302a1) were obtained. Sfdib from S. furcifera contained a complete coding sequence and encoded a 547-amino-acid protein. Similarly, Lsdib from L. striatellus had a complete open reading frame which encoded for a putative 22-hydroxylase of 550 amino acid residues (Fig. 1).

The deduced amino acid sequences of Sfdib and Lsdib share 95% identity. Moreover, Sfdib and Lsdib have 30% and 30%, 57% and 55%, 56% and 53%, and 54% and 54% of identities with the homologues from A. pisum, Tribolium castaneum, M. sexta and D. melanogaster. Both Sfdib and Lsdib have five conserved motifs typical of insect P450s, i.e., WxxxR (Helix-C), GxE/DTT/S (Helix-I), ExxR (Helix-K), PxxFxPE/DRF (PERF motif) and PFxxGxRxCxG/A (heme-binding domain), where ‘x’ means any amino acid [35,36] (Fig. 1).

Insect Dib belongs to the mitochondrial P450 family. The N-termini of Sfdib and Lsdib have a typical mitochondrial targeting sequence, consisting of several charged residues. Moreover, both Sfdib and Lsdib possess two positive charged residues near heme-binding domain, another character of typical mitochondrial P450 enzymes (Fig. 1).

The evolutionary relationship of Dib-like proteins derived from a spider mite species and 19 insect species was evaluated (Fig. 2). Those from 19 insects were distant from that came from the spider mite Tetranychus urticae. In 19 insect species, the Dib-like proteins formed a Hymenoptera clade, a Diptera clade and a Lepidoptera clade. Among the Hymenopteran clade, three sub-clades were formed by proteins derived from Apidae, Formicidae and Pteromalidae, supported by 100% 96% and 98% of bootstrap.

Figure 1. Amino acid sequence alignment of Dib-like proteins. ClustalX (2.1) multiple sequence alignment program were used. CYP302A1 (Disembodied, Dib) originates from Spodoptera littoralis (Sfdib, ACM46003), Anopheles gambiae (AgDib, NP_524810), Nasonia vitripennis (NvDib, XP_001601675), Sogatella furcifera (Sfdib) and Laodelphax striatellus (Lsdib) respectively. Amino acids with 100%, 80%, and 60% conservation are shaded in black, dark grey and light grey, respectively. The characteristic P450 structure, a mitochondrial targeting segment, Helix C, Helix I, Helix K, PERF motif and Heme-binding domain are indicated above the alignment. Two positively charged residues, a signature for mitochondrial enzyme, are indicated with triangles.

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value respectively. In the Apidae sub-clade, two Dibs from the genus Bombus (B_ter and B_imp) and two from the genus Apis (A_mel and A_flo) clustered together, supported by 100% and 99% of bootstrap values. Among the Dibs of the dipteran clade, two from A. aegypti (A_aeg) and A. gambiae (A_gam) formed a mosquito sub-clade with 100% bootstrap support, and then the mosquito sub-clade and that from D. melanogaster (D_mel) joined together to form the dipteran clade, supported by 100% of bootstrap values. However, four Dib-like proteins from Hemiptera formed two clades. Three putative Dib proteins from S. furcifera (S_fur), L. striatellus (L_str) and A. pisum (A_pis) formed one clade, which was well segregated from a putative Rhodnius prolixus Dib. Moreover, the placements of Dibs from T. castaneum (T_cas) and Pediculus humanus (P_hum) were also not well consistent with the traditional view of insect phylogeny (Fig. 2).

Temporal and Spatial Transcript Profiles

For S. furcifera, second-, third- and fourth-instar nymphs lasted an average of 2.0, 2.0 and 3.0 days. Sfdib showed three expression peaks at day 2 of the second-instar (I2D2), day 2 of the third-instar (I3D2) and day 3 of the fourth-instar nymphs (I4D3). In contrast, the expression levels were lower in the newly-molted second (I2D1), third (I3D1) and fourth (I4D1)-instar nymphs. An ANOVA analysis (P<0.05, df=6,14) showed that the three peaks were significantly higher than the three troughs (Fig. 3A).

In our rearing protocol, L. striatellus fourth-instar nymphs lasted an average of 3.0 days. Lsdib showed two expression peaks, with the first at day 2 and the second at day 4–5 in fourth-instar nymphs. In contrast, the expression levels were lower and formed two troughs in the newly-molted fourth- and fifth-instar nymphs. An ANOVA analysis (P<0.05, df=5,12) revealed that the two peaks were significantly higher than the two troughs (Fig. 3B).

Figure 2. A phylogenetic tree of Dib-like proteins from representative insect species. An unrooted phylogenetic tree was constructed by the maximum-likelihood method based on the protein sequence alignments. Dib-like proteins from the spider mite Tetranychus urticae (T_urt, JGI_V11_204789), and 19 insect species are shown. Bombus terrestris (B_ter, XP_003394738), B. impatiens (B_imp, XP_003492183), Apis florea (A_flo, XP_003695126), A. mellifera (A_mel, XP_001122832), Camponotus floridanus (C_flo, EFN63712), Acromyrmex echinatior (A_ech, EGI60082), Nasonia vitripennis (N_vit, XP_001601675), Anopheles gambiae (A_gam, ABU42523), Aedes aegypti (A_aeg, AAEB5205), Drosophila melanogaster (D_mel, NP_524810), Manduca sexta (M_sex, ABC96069), Bombyx mori (B_mori, NP_001036953), Spodoptera littoralis (S_lit, ACM460003), Tribolium castaneum (T_cas, XP_974252), Pediculus humanus (P_hum, XP_002426284), Acyrthosiphon pisum (A_pis, XP_001948299), Rhodnius prolixus (R_pro, RPRC011595-RA), Sogatella furcifera (S_fur) and Laodelphax striatellus (L_str), respectively. The percentiles of bootstrap values (1,000 replicates) are indicated. The scale bar represents the amino acid divergence.

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Figs. 3C and 3D showed the spatial distribution of Sfdib and Lsdib on day 3 and day 5 of S. furcifera and L. striatellus fourth-instar nymphs. Both Sfdib and Lsdib are expressed at a higher level in the thoraces where prothoracic glands were located. Moreover, trace amounts of transcripts were found in the heads and abdomens. An ANOVA analysis (P < 0.01, df = 2, 6) demonstrated that the expression levels in thoraces were significantly higher than the mRNA levels in heads and abdomens.

**Figure 3. Relative transcript levels of dib genes.** The mRNA levels were measured in S. furcifera second through fourth instars (I2D1, I2D2, I3D1, I3D2, I4D1, I4D2 and I4D3) (A) and in L. striatellus fourth (I4D1 to I4D5) and fifth instars (I5D1) (B) at 24 h intervals, and in the head, thorax and abdomen of S. furcifera I4D3 (C) and L. striatellus I4D5 (D) nymphs. For each sample, 3 independent pools of 5–10 nymphs were measured in technical triplicate using qRT-PCR. The values were calculated using the 2 ^ -ΔΔCt method. The relative expression levels were the ratios of relative copy numbers in individuals of specific developmental stage or specific body part to that in S. furcifera I2D1, L. striatellus I4D1 or the head of corresponding species. The bars represent averages with vertical lines indicating SE. Bars with the same lowercase or uppercase letters are not statistically different at P = 0.05 or 0.01.

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Effect of Dietary Introduction of dsRNA on the Expression of dib and EcR Genes

Dietary introduction of dsSfdib reduced dib transcript levels. In S. furcifera, a 6 day period of continuous ingestion of the diet containing dsSfdib at the concentration of 0.01, 0.05 and 0.50 mg/ml diminished Sfdib expression level by 35.9%, 45.1% and 66.2% respectively. The three expression levels were significantly lower than those in the blank control (Fig. 4A). Similarly, after 5-day ingestion of dsLsdib at the concentration of 0.01, 0.05 and 0.50 mg/ml, the mRNA levels of Lsdib in treated L. striatellus nymphs were reduced by 18.8%, 35.8% and 56.7% respectively, with the last two significantly lower than that in blank control (Fig. 4B). In contrast, Sfdib and Lsdib expression levels in dsegfp-exposed planthoppers were not changed (Fig. 4A and 4B).

Since Dib is expected to affect the expression of genes involved in the ecdysteroid-signaling pathway, the effects of Sfdib and Lsdib knockdown were examined on the transcript levels of ecdysone receptor genes SfEcR and LsEcR. EcR constitutes one of the two subunits of the 20E heterodimeric nuclear receptor. EcR is also known to be regulated by 20E through a positive feedback loop directly [37] or indirectly [38] in D. melanogaster. As expected, SfEcR expression levels in S. furcifera nymphs that had ingested dsSfdib at the concentration of 0.01, 0.05 and 0.50 mg/ml significantly decreased by 34.0%, 36.2% and 58.5% respectively, when compared with that in blank control (Fig. 4C). Similarly, LsEcR expression levels in L. striatellus nymphs that had ingested dsLsdib at the concentration of 0.01, 0.05 and 0.50 mg/ml reduced by 25.2%, 46.8% and 68.8% respectively, with the last two concentrations significantly lower than that in blank control (Fig. 4D). In contrast, Sfdib and Lsdib expression level in dsegfp-fed planthoppers was not different than insects in the blank control (Fig. 4C and 4D).

Effects of Ingestion of dsRNA on Nymph Survival

Continuous ingestion of dssegfp-containing diet had no significant impact on nymphal survival relative to blank controls in S. furcifera and L. striatellus. However, when S. furcifera and L. striatellus nymphs had been fed on diets containing dsSfdib at concentrations of 0.01,
0.05 and 0.50 mg/ml, significant impacts on survival were observed (Fig. 5A and 5B). After the experiment, the surviving nymphs were transferred to rice until adult emergence. The mortalities from the termination of experiment to adults emergence were 8.4% (±1.3%, SE), 7.6% (±1.4%), 15.3% (±1.5%), 17.4% (±1.8%), and 21.5% (±2.2%) respectively for S. furcifera on normal, egfp-dsRNA-, and 0.01, 0.05 and 0.50 mg/ml dsDib-containing diets; and were 9.1% (±1.5%), 11.8% (±1.3%), 14.9% (±1.6%), 20.2% (±1.7%), and 28.9% (±2.3%) respectively for L. striatellus. For both planthopper species, the mortalities at 0.50 mg/ml were significantly higher than those on normal and egfp-dsRNA-containing diets (one-way ANOVA analysis, P < 0.05, df = 4, 20).

Ingestion of dsRNA on Nymph Development

The development of S. furcifera and L. striatellus nymphs that were fed dsDib-containing diet was significantly delayed. More than 90% surviving planthoppers on normal and egfp-dsRNA-containing diets developed to the fourth (for S. furcifera) or fifth (for L. striatellus) instar. In contrast, only 83.7%, 64.6% and 41.3% of S. furcifera survivors reared on diets containing Sfdib-dsRNA at concentrations of 0.01, 0.05 and 0.50 mg/ml reached the fourth instar (Fig. 5C). Likewise, 74.3%, 63.2% and 37.8% of L. striatellus individuals reared on diets containing Lsdib at concentrations of 0.01, 0.05 and 0.50 mg/ml reached the fifth instar (Fig. 5D). In S. furcifera, the time required to develop from the 2nd to the 4th instar on normal diet, dsegfp-diet and diets containing Sfdib at the concentration of 0.01, 0.05 and 0.50 mg/ml were 7.0 (95% confidence interval 6.2–7.8), 6.7 (5.7–7.7), 8.0 (7.3–8.7), 8.4 (7.5–9.3) and 9.2 (8.3–10.1) days respectively. Nymphs fed the normal or dsegfp-containing diets had significantly shorter developmental times than nymphs fed with 0.50 mg/ml dsDib (P < 0.05, df = 4, 20) (Fig. 5E). Similarly, the average duration of L. striatellus fourth-instars on normal, dsegfp- and the diets containing dsLsdib at the concentration of 0.01, 0.05 and 0.50 mg/ml were 4.9 (4.6–5.2), 5.0 (4.4–5.6), 5.6 (5.0–6.2), 5.8 (4.8–6.8) and 6.5 (5.8–7.2) days respectively. Nymphs fed with diet containing 0.50 mg/ml of dsDib took significantly longer to develop than nymphs fed either normal or dsegfp-diet (P < 0.05, df = 4, 20) (Fig. 5F).

Rescue Experiment

Ingestion of 20E did not affect the expression level of dib in either S. furcifera or L. striatellus nymphs. In contrast, 20E ingestion restored the mRNA expression levels of SfEcR and LsEcR similar to those of controls (Fig. 6C and 6D). Moreover, 20E ingestion almost completely overcame the negative effects on the nymphal survival (Fig. 6E and 6F) and development in dsdib-treated S. furcifera and L. striatellus nymphs (Fig. 6G to 6J).

Discussion

Oral delivery of dsRNA has a potential practical application as an insecticide in the field. Moreover, it is more convenient and easy to manipulate than microinjection [8]. Oral delivery of
in vitro-synthesized dsRNA has previously been used to induce gene silencing in a variety of pest insects in Lepidoptera [39–42], Coleoptera [43–47], Hymenoptera [48], Diptera [44,49–53], Hemiptera [8–13,16,44,54–56], Orthoptera [57] and Isoptera [58]. In the present paper, we cloned and characterized putative disembodied (dib) genes in two planthoppers S. furcifera and L. striatellus, and tested whether oral delivery of dsdib effectively down-regulated the target genes.

Characterization of Sfdib and Lsdib Proteins

We provided here three lines of experimental evidence to support the hypothesis that the putative Sfdib and Lsdib encoded functional Dib proteins. Firstly, predicted SfDib and LsDib showed typical protein domains, and temporal and spatial expression patterns associated with ecdysteroid biosynthesis. SfDib and LsDib have five insect conserved P450 motifs, i.e., Helix-C, Helix-I, Helix-K, PERF and heme-binding motifs. Similar structural characters have been documented in Dib-like proteins from other insect species of diverse orders [17–27]. Moreover, the N-termini of SfDib and LsDib have a mitochondrial targeting sequence and possess two positively charged residues near the heme-binding domain, two important features of mitochondrial P450 enzymes. Consistent with the structural characters, mitochondrial localization of Dib was observed in dib transfected Drosophila S2 cells [59]. In addition, 22-hydroxylase activities are detected in mitochondria prepared from Locusta [60] and Manduca [61].
Figure 6. Effects of dsdib, 20E, and 20E+dsdib ingestion on S. furcifera (A) and L. striatellus (B). The nymphs were continuously exposed to dsRNA, 0.5 mg/mL 20E and 0.5 mg/mL 20E+dsRNA. The relative expression levels of dib and EcR genes, mortality and duration were measured. The values represent averages with vertical lines indicating SE. Lines with the same lowercase letters are not statistically different at P = 0.05.

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Our qPCR results revealed that \textit{Sf\textsubscript{dib}} and \textit{Ldib} showed expression peaks in late instar stages. In contrast, their expression levels were lower in the newly-molted nymphs. In \textit{N. lugens} \cite{62} and \textit{S. littoralis} \cite{26}, the level of E and 20E showed a peak in the later instar stage. Thus, it can be speculated that the temporal expression pattern of \textit{Sf\textsubscript{dib}} and \textit{Ldib} coincides with the changes in E and 20E titers in the haemolymph. Similarly, the variations of expression levels of \textit{Bndib} and \textit{Mbdib} were correlated with the changes in E and 20E titers in the haemolymph of \textit{B. mori} \cite{20} and \textit{M. sexta} \cite{21}. Moreover, we found that \textit{Sf\textsubscript{dib}} and \textit{Ldib} were expressed at higher levels in the thoraces where prothoracic glands were located, compared to the mRNA levels in corresponding heads or abdomens. The spatial expression patterns of \textit{Sf\textsubscript{dib}} and \textit{Ldib} are consistent with their function in the paired prothoracic glands. Similarly, \textit{dib} genes were observed to be expressed in the prothoracic glands of various insect species such as \textit{D. melanogaster} \cite{18,19}, \textit{B. mori} \cite{20} and \textit{M. sexta} \cite{21}. In addition, \textit{Sf\textsubscript{dib}} and \textit{Ldib} were slightly but distinctly expressed in the head and abdomens. Similarly, even though \textit{dib} had highest expression level in the prothoracic gland of \textit{M. sexta} fifth-instar larvae, substantial expression was observed in the fat body, midgut, ganglia, Malpighian tubules, and epidermis, in descending order. Moreover, \textit{dib} was also expressed in several pupal tissues and in the adult ovaries of \textit{M. sexta} \cite{21}. In \textit{S. littoralis}, \textit{dib} was also found in midgut, brain, fat body, epidermis and Malpighian tubules in the sixth instar larvae \cite{26}. These tissues may operate as secondary sources of ecdysteroids by catalyzing terminal hydroxylation from late intermediates, and may provide extra-prothoracic gland ecdysteroids for local effects such as paracrine and autocrine during larval development and metamorphosis.

The third line of experimental evidence was that RNAi-mediated depletion of \textit{Sf\textsubscript{dib}} and \textit{Ldib} caused phenotypic defects similar to insects whose ecdysteroid synthesis had been disturbed \cite{14,15,24} or whose ecdysteroid-mediated signaling had been inhibited \cite{63,64}. RNAi-mediated knockdown of \textit{dib} reduces ecdysone receptor gene \textit{EcR} expression at mRNA levels in both \textit{S. furcifera} and \textit{L. striatellus} nymphs. Since \textit{EcR} is a nuclear receptor of ecdysteroids \cite{65}, our results raise the possibility that dietary introduction of \textit{Ldib}-RNA inhibit ecdysteroid signaling pathway. Since mutations in and RNAi against \textit{EcR} cause phenotypic defects and lethality in \textit{T. castaneum} \cite{63}, and in \textit{L. striatellus} and \textit{N. lugens} \cite{64}, we observed the influence of \textit{Ldib}-dsRNA ingestion on nymphal development to test the possibility. As expected, RNAi-mediated depletion of \textit{dib} genes caused nymphal lethality and developmental delay in both \textit{S. furcifera} and \textit{L. striatellus} species.

The third line of evidence was that ingestion of 20E almost completely rescued \textit{EcR} expression, and overcame the negative effects on the survival and the development in \textit{dsdb}-treated \textit{S. furcifera} and \textit{L. striatellus} nymphs. This suggested that \textit{dsdb} ingestion by \textit{S. furcifera} and \textit{L. striatellus} negatively affects ecdysteroidogenesis, and subsequently down-regulates \textit{EcR} expression in the two planthoppers. Similarly, knockout and knockdown of \textit{Halloween} genes caused a decrease in ecdysteroid titers in other insect species \cite{18,19,66-70}. Thus, we provided the third line of evidence to support the hypothesis that putative \textit{Sf\textsubscript{dib}} and \textit{Ldib} genes encode functional \textit{Dib} proteins that play a critical role in ecdysteroidogenesis in \textit{S. furcifera} and \textit{L. striatellus}.

Ingestion of dsRNA Showed Potential Application of Pest Management

In this study, the effects of orally delivered dsRNA on \textit{dib} and \textit{EcR} transcript levels in \textit{S. furcifera} and \textit{L. striatellus} were dose-dependent, with higher concentrations of \textit{dsdb} causing higher negative effects. Similar results have also been documented in \textit{Aedes aegypti} \cite{51} and \textit{N. lugens} \cite{71}. In previous experiments in \textit{D. melanogaster} and three other non-dipteran insect species, it was found that oral delivery of dsRNA at doses higher than 0.5 mg/ml was no more effective at inducing RNAs and killing the target insects \cite{44}. Similarly, several studies have also observed that increasing the concentration beyond an optimal dose does not improve the extent of RNAi, although the optimal concentration may vary for the species, mode of delivery, and the life stage \cite{55,57,72}. Thus, in the present paper, we tested the negative effects of dietary ingestion of \textit{dsdb} at the concentration of 0.01, 0.05 and 0.50 mg/ml. We did not find the optimal concentration of \textit{dsdb} in the present paper, and RNAi-mediated knockdown of \textit{db} genes was thus incomplete in \textit{S. furcifera} and \textit{L. striatellus}. Even so, our data were comparable to gene silencing levels observed in many other insects that have been fed dsRNA, where the extent of RNAi-induced silencing typically ranged between 40% and 60% \cite{9,41,49,51,54,50}. Thus, our results in this study offer two possible applications. Firstly, an oral dsRNA delivery method in pest will facilitate the development of higher throughput RNAi screens in \textit{S. furcifera} and \textit{L. striatellus}. Such high throughput screens may identify new targets for control technologies. Secondly, the RNAi-induced effects in planthoppers could serve as a possible method for pest control.

It is widely recognized that two factors are critical for dsRNA-based pesticides, namely effectiveness and dsRNA delivery method \cite{73}. As for effectiveness of dsRNA, our results showed that 5- or 6-day’s ingestion of \textit{dsdb} by second-instar \textit{S. furcifera} and fourth-instar \textit{L. striatellus} nymphs killed approximately 15%–25% planthoppers during the experiments, and caused a further 15%–20% of individual to die after the feeding experiments. RNAi depletion of \textit{db} gene should also affect embryonic and nymphal development, and adult reproduction \cite{18}, however, here we only tested the negative effect of \textit{dsdb} ingestion on nymphal development. We need to perform further experiments in future to evaluate the influence of \textit{dsdb} on adult reproduction and developing embryos.

As for dsRNA delivery, it has been reported that transgenic rice expressing insect-specific dsRNAs mediate RNAi-based knockdown of the target genes in \textit{N. lugens} insects, although the exact dsRNA concentration in rice was not measured \cite{10}. \textit{S. furcifera} and \textit{L. striatellus} deposit their eggs in the leaf sheaths of rice plants, and both nymphs and adults feed on rice phloem sap. Considering that a transgenic rice may provide the insecticidal dsRNA to pests in a stable and potent form \cite{10,40,43,74}, it can be expected that the transgenic rice may act as a potential ovicide through planthopper embryonic development stage, as well as a stomach toxin to whole nymphs and adults. Thus, the transgenic rice expressing dsRNAs of \textit{Halloween} genes such as \textit{dib} provides a potential application for planthopper control and deserves further investigations.

Author Contributions

Conceived and designed the experiments: GQL. PJW. Performed the experiments: PJW SJ NI JMF. Analyzed the data: PJW. Contributed reagents/materials/analysis tools: PJW. Wrote the paper: GQL.
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