Masculinizer and Doublesex as Key Factors Regulate Sexual Dimorphism in Ostrinia furnacalis

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Abstract: Sex determination is an important and traditional biological process. In Lepidoptera, Masculinizer (Masc) and Doublesex (dsx) genes are essential factors in sex determination and play critical roles in sexual differentiation and development. The functions of Masc and dsx have been characterized in several model insect species. However, the molecular mechanism and sex determination functions of Masc and dsx in Ostrinia furnacalis, an agricultural pest, are still unknown. Here, we successfully used the CRISPR/Cas9 genome editing system to knock out OfMasc and Ofdsx. Mutation of OfMasc induced male external genital defects and sterility. Disruptions of the Ofdsx common region caused sex-specific defects in the external genitals and adult sterility. In addition, we found that OfMasc and Ofdsx can regulate the pigmentation genes that control wing pigmentation patterns. These results demonstrate that the Masc and dsx genes are conserved factors in sexually dimorphic traits, and therefore represent potential target genes in the effort to control O. furnacalis and other lepidopteran pests.

Keywords: CRISPR/Cas9; Masculinizer; Doublesex; sexual dimorphism; Ostrinia furnacalis

1. Introduction

Sexually dimorphic traits are ubiquitous in plants and animals. Most animal species consist of two distinct sexes, and the differences between male and female animals are numerous and pronounced at the morphological, physiological, and behavioral levels [1]. However, sexual dimorphism presents a question: how can a genome largely shared between the sexes give rise to such different forms [2]? A compelling answer to this question is provided by the sex-specific expression of shared genes [3]. Sex determination is an essential and hierarchically regulated biological process with high diversity in different organisms, including insects [4–6]. Sexually dimorphic traits, including body size, pigmentation, external genitals, sex-specific behavior and physiology are prevalent across the
animal kingdom and especially in insect species [7]. Sexual dimorphism typically manifests in differences in body and wing color [1].

In animals, the *doublesex (dsx)* gene, the *mab-3* gene and the Dsx- and mab-3-related transcription factor 1 (*Dmrt1*) are three homological factors that, through female and male specific expression and splicing, play an important role in the regulation of sexually dimorphic traits in *Drosophila melanogaster*, *Caenorhabditis elegans*, and mammalian species [8–10]. In insect species, sex determination plays a key part in biological development and reproduction [5,11]. In *D. melanogaster*, the *Sex lethal (Sxl)* gene is initially regulated by the X:A ratio [12]. Then, the *dsx* gene, which is a conserved downstream gene involved in sex determination, regulates sexual differentiation [13,14].

In *Drosophila*, the HOX protein Abdominal-B (ABD-B) and the sex-specific isoforms of DSX directly regulate the *bab cis*-regulatory element (CRE) to induce sexually differentiated pigmentation on abdominal segments [15]. In dragonflies, there is sexually dimorphic coloration; for instance, in *Ischnura senegalensis*, females are orange and males are blue. However, the molecular mechanism that regulates this difference is unclear [16]. In locusts, such as the gregarious *Schistocerca gregaria*, the body color of males and females does not differ in the nymphal stage; when male locusts reach sexual maturity, however, their male abdomen and legs turn yellow [17]. However, despite the fact that sexually dimorphic coloration is widespread in insects, the molecular mechanisms that regulate it are not well understood, and it is also unclear how the sex determination pathway regulates pigmentation.

In Lepidoptera, studies of sex determination have mainly focused on the silkworm *Bombyx mori*, which is an important lepidopteran model insect [18–20]. A previous study showed that the *Masculinizer (Mas)* gene is repressed by *Fem* piRNA in female silkworm [21]. Moreover, the *Mas* gene controls the *Bmdsx* gene splicing in *B. mori* [22,23]. Mutation in *Mas* induces the appearance of female characteristics, including female-specific ventral chitin plates and genital papillae in male individuals [24]. Furthermore, disruption of the *Bmdsx* gene induces abnormal gonads and external genitalia, and sex-specific sterility [24,25]. In the Asiatic corn Borer, *Ostrinia furnacalis* (Lepidoptera: Pyralidae), which is one of the most destructive pests of corn, especially in China and northeast Asia [26,27], the *Mas* gene and the *dsx* gene have been described in previous studies [28–31]. The *OfMas* gene is regulated by the endosymbiotic bacterium *Wolbachia*; a failure dosage compensation induces male lethality [28,30]. However, the genetic and functional relationships between these genes in *O. furnacalis* sex determination and differentiation are still unclear.

In our study, we used the CRISPR/Cas9 genome editing system to generate somatic mutations in the *Mas* and *dsx* genes in the sex determination pathway of *O. furnacalis*. The *OfMas* and *Ofdsx* genes are structural orthologs of the key sex regulation factors in *B. mori*. Mutation of the *Ofdsx* gene induced abnormal external genitalia, adult sterility, and sex reversal of sexually dimorphic traits, including wing pigmentation, gene expression patterns, and *dsx* sex-specific splicing. These results demonstrate that the *Mas* and *dsx* genes are the conserved factors in sexually dimorphic traits, and therefore represent potential target genes for research into the control of *O. furnacalis* and other lepidopteran pests.

2. Materials and Methods
2.1. Insect Strains and Rearing

A laboratory strain of *O. furnacalis* was reared on an artificial diet (Table 1) under standard conditions in an incubator, at a temperature of 25 °C and with a 16:8 h light:dark cycle [32]. *O. furnacalis* pupae were sexed, and the emerging adults were mixed in transparent air-filled plastic bags to mate with each other and lay eggs [26].
Table 1. The components of the artificial diet given to the laboratory strain of *Ostrinia furnacalis*.

| Components                  | Weight (g) or Volume (mL) |
|-----------------------------|---------------------------|
| Wheat germ powder           | 150                       |
| Yeast extract powder        | 40                        |
| Agar strip                  | 14                        |
| Sucrose                     | 5                         |
| Vitamin C                   | 4                         |
| Sorbic acid                 | 4                         |
| Methyl p-hydroxybenzoate    | 4                         |
| Linoleic acid               | 0.5                       |
| purified water              | 900                       |

Preparation method: Boil 900 mL of water mixed with agar, add other ingredients, mix well, and store in the refrigerator after cooling.

2.2. Phylogenetic Analysis

Phylogenetic relationships were determined based on sequence alignment (DNAMAN 8.0 software) and phylogenetic analysis using Mega 5 [33,34]. All ambiguous positions were removed for each sequence pair. The neighbor-joining method was used to create a tree from 9 available MASC protein sequences, and the reliability of the tree was tested by bootstrap analysis with 1000 replications. The GenBank accession numbers and references of the protein sequences are as follows: *B. mori* (BAO79517.1), *Trilocha varians* (BAS02075.1), *Helicoverpa armigera* (QCD63870.1), *Agrotis ipsilon* [35], *Plutella xylostella* [36], *O. furnacalis* (BAS02074.1), *Ephestia kuehniella* (QXE45293.1), *Artemia franciscana* (ARB66312.1), *Artemia parthenogenetica* (ARB66313.1).

2.3. Quantitative Real-Time PCR (qRT-PCR)

For qRT-PCR analyses, total RNA was extracted from *O. furnacalis* larvae and adults using Trizol reagent (Invitrogen, Carlsbad, CA, USA) and treated with RNase-free DNase I (Ambion, Austin, TX, USA), according to the manufacturer’s instructions. cDNAs were synthesized using the Omniscript Reverse Transcriptase kit (Qiagen, Hilden, Germany) in a 20 µL reaction mixture containing 1 µg total RNA. qRT-PCR analysis for *Of Masc* and *Of dsx* mutants was performed using a SYBR Green Real-Time PCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA) on an Eppendorf Real-Time PCR System. The PCR conditions were as follows: initial incubation at 95 °C for 5 min, 40 cycles at 95 °C for 15 s and 60 °C for 1 min. *O. furnacalis actin* was used as an internal control [32]. The gene-specific primers used for qRT-PCR are listed in Table 2.

2.4. In Vitro Transcription of Cas9 mRNA and sgRNA

We selected two 23-bp sgRNAs targeting *Of Masc* and one sgRNA targeting *Of dsx*. The sgRNAs were sub-cloned into the 500-bp linearized CloneJet PJET1.2-T vector (Thermo Fisher Scientific) upstream of the protospacer adjacent motif (PAM) sequence, to allow sgRNA expression under the control of the T7 promoter. The sgRNAs were synthesized in vitro using a MEGAScript T7 kit (Ambion), according to the manufacturer’s instructions. Cas9 mRNA was synthesized in vitro using the mMESSAGE T7 Kit (Ambion) and a PTD1-T7-Cas9 vector as the template [35], according to the manufacturer’s instructions.

2.5. Microinjection of Embryos

Mated female *O. furnacalis* moths were allowed to lay eggs on transparent plastic bags. A previously reported microinjection method was employed [37]. Within 1 h of oviposition, the eggs were injected on the lateral side with a mixture containing 300 ng/µL of Cas9 mRNA and 150 ng/µL sgRNA. After injection, the eggs were incubated in a humidified chamber at 25 °C for 4 days until hatching.
### Table 2. Primers used in PCR amplification and mutant detection.

| Primer Name | Primer Sequence (5′-3′) | Primer Purpose |
|-------------|--------------------------|----------------|
| Masc-sgF1   | TAATACGACTCACTATAGGGTTTGTAGGGATTACGTTGTTTTAGAGCTAGAAATAGCAA | Preparation of sgRNA templates |
| Masc-sgF2   | TAATACGACTCACTATAGGGCGACATGAAGCTCACGGAGTTTTAGAGCTAGAAATAGCAA | Identification of somatic mutations |
| Dsx-sgF1    | TAATACGACTCACTATAGGTGTCCCTAGAAACCCTGGGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAG | Identification of somatic mutations |
| sgRNA-R     | AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACG GACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAAC | Identification of somatic mutations |
| Masc-F1     | ACATAGTGAACAAAATGGCCGCCAC | Identification of somatic mutations |
| Masc-R1     | TTGAGGTGGTGGTGCTGAAACAGAA | Identification of somatic mutations |
| Dsx-DC-F1   | AAACGCTTTTATTTAGAGGTTAAGAGGG | Identification of somatic mutations |
| Dsx-DC-R1   | GCTGAAATGATGATGATGATCCAAA | Identification of somatic mutations |
| Dsx-RTPCR-F | AAGTTTCCACTATCTTCTGGGAG | qRT-PCR for genes |
| Dsx-RTPCR-R | AGCACACGATGAGAGGAGAG | qRT-PCR for genes |
| Actin-qF    | CGTGCTCTCCTGACGACGAGGGTC | qRT-PCR for genes |
| Actin-qR    | CTTAACAAATCCGGCATTG | Identification of somatic mutations |
| Vg-qF       | GGTGTGGGAACACCATCTCCCG | Identification of somatic mutations |
| Vg-qR       | GAAAGCGGTGGTGGTGATGGGCAACG | Identification of somatic mutations |
| OR53-qF     | GGACCTTATCTACGTAAGGAGGAG | Identification of somatic mutations |
| OR53-qR     | TTAACGCGAGCTTACGGTGATGAC | Identification of somatic mutations |
| PBP2-qF     | ATGGTGGTCGATGACGAGGG | Identification of somatic mutations |
| PBP2-qR     | CTTGGATGAAACGCAAGAGG | Identification of somatic mutations |
| PBP3-qF     | AAGACGCTTGTTGACACCATGGGCA | Identification of somatic mutations |
| PBP3-qR     | CATGAGTTGATATCGAGGG | Identification of somatic mutations |
| Optix-qF    | GGCACATTACGGAAGGAGGA | Identification of somatic mutations |
| Optix-qR    | CAGTCCTCTCTTCTTGTGGC | Identification of somatic mutations |
| 206617-qF   | ATGGATACGAGGCAAAAGCG | Identification of somatic mutations |
| 206617-qR   | GAGGATGACCTGAGGCAACG | Identification of somatic mutations |
| APA-qF      | TATGCGGTGAGGACACCTTGG | Identification of somatic mutations |
| APA-qR      | GAAAGCGATGACGATCGTGTA | Identification of somatic mutations |
| Ddc-qF      | TTGGTGTCCTTCTGACGCTTT | Identification of somatic mutations |
| Ddc-qR      | CATTATGCGCTTACAAACAA | Identification of somatic mutations |
| Tan-qF      | CTATCGGGAAGCAGATGCTAAC | Identification of somatic mutations |
| Tan-qR      | ATGGTCTCAAATCTGGTCTGTC | Identification of somatic mutations |
| Ebony-qF    | CGTCTGACCTATTTACGAC | Identification of somatic mutations |
| Ebony-qR    | CACCAAGCTTCTGGACCTGCTC | Identification of somatic mutations |
| Yellow-qF   | TGTTGGAATACCGGATCTCTC | Identification of somatic mutations |
| Yellow-qR   | ACCGGACCGCTTAAATTCCTG | Identification of somatic mutations |

### 2.6. Genomic DNA Extraction and Identification of Mutagenesis

The genomic DNA was extracted from the newly hatched larvae, incubated with proteinase K, and purified via a standard phenol:chloroform extraction and isopropanol precipitation extraction, followed by RNase A treatment. A PCR was carried out to identify *OfMasc* and *Ofdsx* mutant alleles using primers F1 and R1 (Table 2) spanning the target site in *OfMasc* and *Ofdsx*. The PCR conditions were as follows: 98 °C for 2 min, followed by 35 cycles of 94 °C for 10 s, 55 °C for 30 s, and 72 °C for 1 min, followed by a final extension period of 72 °C for 10 min. The PCR products were sub-cloned into the CloneJet PJET1.2-T vectors (Thermo Fisher Scientific) and sequenced. The PCR products were also used for the T7 endonuclease I (T7EI) assay as previously described [38]. The mutants were photographed with a digital stereoscope (Nikon AZ100, Tokyo, Japan).

### 2.7. Hatchability Assays

In order to evaluate the hatchability of *Masc* and *dsx* mutants, the males and females with *OfMasc* and *Ofdsx* mutations were crossed with mutant moths and virgin wild type male and female moths. Five pairs of moths were collected for one group. Hatchability assays of each group were repeated 3 times. After female moths laid eggs for two days, the eggs of each pair were collected and incubated in a humidified chamber at 25 °C for 4 days until hatching. The hatching rates were analyzed.
2.8. Statistical Analysis of Data

The data were analyzed using GraphPad Prism (version 5.01) with one-way analysis of variance, the Dunnett post hoc test and Bonferroni analysis. Error bars stand for the means ± SEM, and three asterisks stand for \( p < 0.001 \).

3. Results

3.1. Phylogenetic Analysis of MASC and DSX Proteins in O. furnacalis

The phylogenetic tree was constructed using sequences of OfMASC and MASC protein sequences from six different lepidopteran insects, namely H. armigera, B. mori, T. varians, A. ipsilon, P. xylostella, and E. kuehniella, and two other species, namely A. franciscana and A. parthenogenetica (Figure S1B). The phylogenetic tree showed that OfMASC was closest to P. xylostella and E. kuehniella MASC, suggesting a conserved function. Subsequently, the amino acid sequence of the OfMASC protein was compared with the other lepidopteran MASC proteins. The analyzed multiple alignment results show that the OfMASC protein has two tandem CCCH-type zinc finger (ZF) domains, a bipartite nuclear localization signal (bNLS), and a masculinization domain (MD) (Figure S1A). Then, we used the NCBI BLAST program to find the amino acid sequences of DSX proteins in the NCBI database and constructed the phylogenetic tree of DSX (Figure S2). The analyzed results showed that the OfDSX protein was closest to Galleria mellonella, a moth of the same superfamily Pyraloidea as O. furnacalis, and clustered with the DSX proteins of other lepidopteran moth insects, suggesting a conserved function.

3.2. CRISPR/Cas9-Mediated Mutagenesis of OfMasc and Ofdsx

In order to investigate the function of these two sex determination genes, the high-efficiency genome editing system CRISPR/Cas9 was used to disrupt the OfMasc and Ofdsx genes. Following the GGN_19GG rule for sgRNA design [35], we designed two sgRNAs targeting the OfMasc and one sgRNA targeting the Ofdsx gene. Two targeted sgRNAs were at the exon 1 of the OfMasc gene locus (Figure 1A), and one targeted sgRNA was at the exon 2 of the common region of Ofdsx female and male transcript isoforms (Figure 1B). The fresh eggs, which were not more than 1 h older, were collected for microinjection. The Cas9 mRNA mixed with OfMasc or Ofdsx sgRNAs transcripted by a T7 promoter, was prepared according to previous reports [39]. To identify the mutated alleles of the OfMasc and Ofdsx genes, genomic DNA was extracted as phenotypic expression involved in mutagenesis was shown. The results of the genome sequences indicated that the successful deletion of sequences had taken place between the two target sites in the OfMasc gene and deletion in the Ofdsx gene (Figure 1).

3.3. Disruption of OfMasc and Ofdsx Genes Induced Abnormal External Genitalia and Pigmentation

The mutants displayed some abnormal phenotypes of external genitalia in the pupal stage. In the wild type, females and males have distinct gonopore characteristics in pupa morphology, which are key to distinguishing between females and males. Female pupae have an X-shaped line and a small crevice in the eighth abdominal segment, whereas male pupae develop two prominent points at the abdomen end of the ninth abdominal segment (Figure 2). Because of the key role of sex determination genes in regulating sexual dimorphic traits, the mutant sex determination genes result in abnormal female and male morphological characteristics and sex reversal [40]. For OfMasc mutant pupae, we found that there were some abnormal phenotypes, such as deformed gonopores, but only in the male mutants (Figure 2). Moreover, in mutant 2 (M2) and M3, there were some female specific characteristics similar to the X-shaped line. The gender of these male mutants was identified after eclosion. In the Ofdsx mutant pupae, we found there were three types of abnormal phenotypes. The Ofdsx female mutant had an abnormal X-shaped line, similar to M4, M5, and M6 (Figure 2), and the Ofdsx male mutant had defective gonopores, such as in M10, M11 and M12 (Figure 2). Some Ofdsx mutant pupae, such as M7, M8 and M9, also
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had two gonopore characteristics that differed between females and males. We named this mutant type DSX-FM (Table S1).

![Diagram of CRISPR/Cas9-mediated mutations in the OfMasc and Ofdsx genome loci.](image)

**Figure 1.** CRISPR/Cas9-mediated mutations in the OfMasc and Ofdsx target sites. (A) The two target sites of the OfMasc genome locus focused on the first exon. (B) The target site of the Ofdsx genome locus focused on the second exon of the common region in the female and male spliced variants. (C) T7 endonuclease I treatment of extracts of wild-type (WT) and mutant (M) pupae of OfMasc and genomic sequencing demonstrate alterations at the target site. (D) Sequencing chromatogram of the Ofdsx mutants. (E) OfMasc mutations detected by sequencing. (F) Ofdsx mutations detected by sequencing. The PAM sequence is in red. The black line represents the target site.

When the mutant pupae entered the adult stage, we found that there were some instances of abnormal external genitals. In the wild type, adult male external genitalia mainly consist of a harpago, some uncuses and an aedeagus. Female external genitalia mainly consist of a genital papilla and a ventral plate. In OfMasc mutants, the external genitals were normal in the females and abnormal in the males, which presented with a shorter aedeagus and an abnormal harpago (Figure 3M6). In the female Ofdsx mutants, there were some defective genital papillae and aedeagi (Figure 3M1,M2); abnormal harpago and female-specific genital papillae appeared in male Ofdsx mutants (Figure 3M4,M5).

We also found some other sexual dimorphism trait changes in the adult stage. In the wild type, the wing color of males is deeper than that of females. In the OfMasc mutants, however, the wing color of males was weaker than that of wild-type males and similar to that of wild-type females (Figure 4). In the Ofdsx mutants, we found the DSX-FM mutants showed more pronounced wing color and stripes than those of wild-type females, but weaker than those of wild-type males (Figure 4). The results suggest that the dsx gene regulates pigmentation in O. furnacalis.
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Figure 2. The morphologies of wild type compared with OfMasc and Ofdsx mutant pupae. In the wild type, the females and males have different gonopore characteristics in pupa morphology; these variations are key to differentiating males from females. Female pupae have an X-shaped line and small crevice in the eighth abdominal segment, whereas male pupae develop two prominent points at the abdomen end in the ninth abdominal segment. The OfMasc and Ofdsx mutants showed abnormal or defected morphologies. M1–M3 are the male mutants of Masc in O. furnacalis. In the dsx mutants, M4–M6 are the female mutants; M7–M9 are the intersex mutants; M10–M12 are the male mutants. Scale bar: 1 mm.

Figure 3. The external genital morphology of the wild type compared with OfMasc and Ofdsx mutants. In the wild type, adult male external genitalia mainly consist of a harpago, some uncuses and an aedeagus. Female external genitalia mainly consist of a genital papilla and a ventral plate. The male-specific external genitalia in OfMasc and Ofdsx mutant males exhibited severe structural defects, and the genital papilla and ventral plate were not present and ectopic in Ofdsx mutant females. Scale bar: 0.5 mm.
3.4. OfMasc and Ofdsx Mutations Induce Sterility in O. furnacalis

We then analyzed the fertility data of mutants. We found that deletion of the OfMasc gene induced male lethality at the embryonic stage. We collected data concerning sex ratios in the adult stage three times. The percentages of sex ratios of female adults in the total population were about 78%, 85%, and 92% (Figure S4A). In order to analyze the physiological changes in OfMasc and Ofdsx mutants, we investigated the reproductive ability and hatching rate of the embryos produced when the mutants mated with each other. Because of the defects in the OfMasc male mutants’ external genitals, these males could not mate with wild-type females or with OfMasc female adults, and no eggs were hatched (Figure S4B). In the Ofdsx mutants, both the \( \triangle \text{DSX-F} \) and \( \triangle \text{DSX-M} \) individuals all had abnormal external genitals; as such, the Ofdsx mutants had no reproductive ability and no next-generation eggs were hatched (Figure S4C).

3.5. Detection of Sex-Specific Gene Expression in OfMasc and Ofdsx Mutants

In order to explain these mutant phenotypes, we used the RT-PCR to determine the Ofdsx gene expression. In the wild type, the Ofdsx female-specific isoform was longer than the male-specific isoform; the specific bands present the female or male Ofdsx expression. In the mutants, however, we found that the OfMasc male mutants had two bands in one lane, and the Ofdsx mutants also had non-single bands in corresponding lanes (Figure S3). These results demonstrate that the mutation of the OfMasc gene induces the appearance of female-specific Ofdsx isoforms in males.

To investigate whether the disruption of sex-specific OfMasc and Ofdsx transcripts influences the expression of known sex-biased genes in O. furnacalis, we examined the female-biased Vitellogenin (OfV\( \text{g} \)) and the Olfactory Receptor 53 (OfOR53) genes, which encode a protein essential for oogenesis and the reception of outside information; we also examined two male biased genes, Pheromone Binding Protein 2 (OfPBP2) and Pheromone Binding Protein 3 (OfPBP3) [32]. Compared with the wild-type males, the relative mRNA expression levels of OfV\( \text{g} \) and OfOR53 were significantly up-regulated in OfMasc and Ofdsx male mutants (Figure 5A,B,E,F); in Ofdsx mutant females, meanwhile, the levels of OfV\( \text{g} \) and OfOR53 were significantly decreased (Figure 5E,F). The relative mRNA expression levels of OfPBP2 and OfPBP3 were significantly down-regulated in OfMasc and Ofdsx male mutants (Figure 5C,D,G,H), but were significantly increased in Ofdsx female mutants (Figure 5G,H). These results demonstrate that OfV\( \text{g} \), OfOR53, OfPBP2, and OfPBP3 are direct or indirect targets of dsx in O. furnacalis, which is consistent with previous reports [24,41,42].

Figure 4. The pigmentation of the wild-type individuals compared with the OfMasc and Ofdsx mutants. In the wild type, the wing color of males is deeper than that of females. In the OfMasc mutant males, the wing color was weaker than in wild-type males. In the Ofdsx mutants, the wing color had a level of pigmentation between that of the wild-type males and females. Scale bar: 2 mm.
** and *** represent significant differences at the 0.05, 0.01 and 0.001 levels.

In the wild type, the wing pigmentation of males is deeper than that of females, meaning that some pigmentation genes have different expression patterns for males and females. Through the qRT-PCR analysis, we found there were some highly expressed genes in males, including optix, 20661, aperiodous A (AP-A), Ddc, and Tan. High expression caused a deepening of pigmentation in males. In the OfMasc male mutants, however, we found that these genes, including optix, 20661, AP-A and Ddc, were down-regulated compared to wild-type males (Figure 6A). Moreover, in the Ofdsx mutants, the expressed pattern was similar to that of OfMasc male mutants (Figure 6B). These results demonstrate that disruption of OfMasc and Ofdsx induces a sex reversal of pigmentation phenotypes, and also that the expression of some genes was up- or down-regulated by the Ofdsx gene, either through direct or non-direct effects.

Figure 5. qRT-PCR analysis of the putative downstream genes of Ofdsx in the OfMasc and Ofdsx mutants. (A–D) Relative mRNA expression levels of OfVg, OfOR53, OfPBP2, and OfPBP3 in OfMasc mutants. (E–H) Relative mRNA expression levels of OfVg, OfOR53, OfPBP2, and OfPBP3 in Ofdsx mutants. Three individual biological replicates were performed using qRT-PCR. Error bar: SD; *, ** and *** represent significant differences at the 0.05, 0.01 and 0.001 levels (t-test) compared with the control.

Figure 6. The relative expression of pigmentation genes in OfMasc male mutants (A) and Ofdsx FM mutants (B). Three individual biological replicates were performed using qRT-PCR. Error bar: SD; n.s., *, ** and *** represent significant differences at the 0.05, 0.01 and 0.001 levels (t-test) compared with the control.
4. Discussion

In this study, we focused on the sex determination genes OfMasc and Ofdsx. The phylogenetic analyses of the OfMasc and Ofdsx genes showed high homology with other insect species (Figures S1 and S2). Using the CRISPR/Cas9 genome editing system, we successfully knocked out these two genes, which are crucial elements of the sex determination pathway (Figure 1). In OfMasc mutants, this induced abnormal external genitals in pupal and adult males (Figures 2 and 3), which led to the sterility of these males and an imbalance in the sex ratio (Figure S4). Disruption of the Ofdsx common region induced the malformation of female and male external genitals (Figures 2 and 3), which led to the sterility of male and female adults (Figure S4). Regarding sexual dimorphism, mutated OfMasc and Ofdsx caused a weakening of pigmentation and down-regulation of the pigmentation genes both for the OfMasc males and the Ofdsx males, while the Ofdsx female mutants showed enhanced pigmentation and up-regulation of pigmentation genes (Figures 4 and 6). RT-PCR results showed the expression of female and male specific Ofdsx isoforms in the OfMasc-M mutants, and in the Ofdsx-F and Ofdsx-M mutants (Figure S3). The qRT-PCR results demonstrated disruption of OfMasc and Ofdsx influenced the expression of sex-biased genes (Figure 5). Our study provides direct evidence that OfMasc regulates the expression of the Ofdsx gene, and that the Ofdsx gene regulates the sexual dimorphism of O. furnacalis, including characteristics such as pigmentation, external genitals, sex-biased genes, and fertility. As such, OfMasc and Ofdsx constitute potential target genes in research aimed at controlling O. furnacalis and other lepidopteran pests (Figure S4).

The key masculinization factor played an important role and appears to have a conserved function in lepidopteran insects [21–24,30,36,43,44]. In B. mori, the Masc gene has two CCCH zinc finger domains and is regulated by Fem piRNA in order to control the male-specific dsx isoform expression, which is consistent with our results (Figures S1 and S3) [21,22,24]. In A. ipsilon and P. xylostella, the Masc gene was identified and shown to control masculinization through regulating the expression of dsx [36,44]. In previous studies, the OfMasc gene was cloned and shown to be regulated by Wolbachia to induce female-specific strains [30,31]. In our research, we used the CRISPR/Cas9 genome editing system to knock out the OfMasc gene, and then demonstrated that the OfMasc gene controls sexual dimorphism by regulating the expression of the dsx gene in O. furnacalis (Figures 1 and 4).

Insect dsx genes are the downstream genes of the sex determination pathway; these genes are very conservative, and exhibit sex-specific splicing to generate male- (dsxM) and female-specific (dsxF) isoforms that control separate but corresponding sex-specific dimorphic traits [4,9,15,29,45]. In our study, we used the CRISPR/Cas9 genome editing system to disrupt the Ofdsx common region, which induced the inversion of sexual dimorphism in areas including pigmentation, the external genitals, and sex-biased genes (Figures 1–6). In B. mori, dsx is an important transcription factor that regulates sexually dimorphic differentiation. Mutation of Bmdsx induced abnormal external genitals and led to female and male sterility; as such, it could act as a targeted gene for sterile insect technologies (SIT) [24,25,46]. In other lepidopteran pests, including O. scapulalis [47], A. ipsilon [48], P. xylostella [41] and Hyphantria cunea [42], dsx gene function is conservative and regulates sexual dimorphism. These previous reports have shown that, in lepidopteran insects, the dsx gene has high homology and controls sexual dimorphism through the sex determination pathway.

Dsx is a mimicry supergene [49,50]. In O. furnacalis, wing pigmentation shows sexual dimorphism: pigmentation is deeper in males than it is in females. In our study, we knocked out the OfMasc and Ofdsx genes, which caused abnormalities in the sexually dimorphic traits; moreover, there was a reversal in sex-specific wing pigmentation patterns. qRT-PCR showed some pigmentation genes were down-regulated in male mutants and up-regulated in female mutants (Figures 4 and 6). These results demonstrate that the dsx gene can affect the expression of the genes that control sexual dimorphism in wing pigmentation patterns. In butterflies, a previous study found that females showed female-limited Batesian mimicry and displayed wing pattern polymorphism [51]. In Papilio polytes, this polymorphism is
controlled by a single autosomal locus, dominant locus H, which consists of a series of genes that affect color patterns [52]. Moreover, a recent study of *P. polytes* has shown that the mimetic phenotype is controlled by the *dsx* gene [49,50]. SiRNA-mediated down-regulation of the *dsx* gene induced the severe repression of red spots and white pigmentation in female wing patterns [50]. In *O. scapulalis*, Wolbachia-infected females showed sexual mosaics, which were composed of male (darker) and female (lighter) sectors; both male and female *dsx* isoforms were also expressed in these individuals [47]. These results demonstrate that the *Osdsx* gene can control the sexual dimorphism of wing pigmentation patterns in *O. scapulalis*.

In summary, we used the CRISPR/Cas9 genome editing system to disrupt the functions of the sex determination genes *Masc* and *dsx*. The results of our study demonstrate that *OfMasc* can regulate the expression of male *dsx* isoforms and induce male sexual phenotypes. *Dsx* performs essential functions in sexual dimorphism, and is involved in determining the morphology of external genitals and wing pigmentation patterns in *O. furnacalis*. The disruption of *Masc* induced a sex ratio imbalance and male sterility. Knocking out the *dsx* common region induced female and male sterility. These results demonstrate that *Masc* and *dsx* are potential target genes for efforts to control *O. furnacalis* and some other lepidopteran pests.

5. Conclusions

In this study, we investigated the function of *OfMasc* and *Ofdsx* in the lepidopteran agricultural pest *O. furnacalis*. We used the CRISPR/Cas9 genome editing system to successfully knock out *OfMasc* and *Ofdsx*. Mutation of *OfMasc* induced defects in the male external genitals, a sex ratio imbalance and male sterility. Disruptions of the *Ofdsx* common region caused sex-specific defects in the external genitals and adult sterility. In addition, we found that *OfMasc* and *Ofdsx* can regulate pigmentation genes to control wing pigmentation patterns. These results demonstrate that *OfMasc* and *Ofdsx* play key roles in sex determination and in the regulation of sexually dimorphic traits in *O. furnacalis*, and have the potential to be used in the genetic control of pests such as *O. furnacalis*.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/cells11142161/s1, Figure S1: Phylogenetic analysis of the *OfMasc* gene. Figure S2: Phylogenetic relationship of insect *dsx* genes, generated using NCBI BLAST program. Figure S3: The splicing patterns of *Ofdsx* were examined by RT-PCR in wild-type and mutant insects. Figure S4: The sex ratio difference in *OfMasc* mutants and the fertility of *OfMasc* and *Ofdsx* mutants. Table S1: Mutagenesis of *OfMasc* and *Ofdsx* induced by Cas9/sgRNA.

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