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Splicing and Expression Regulation of *fruitless* Gene in *Bemisia tabaci* (Hemiptera: Aleyrodidae)

Yating Liu [1], Jinxi Xie [1], Wenlu Wang [2], Yanyuan Lei [3], Xuguo Zhou [4], Youjun Zhang [2] and Wen Xie [2,*]

[1] School of Food Science and Engineering, Foshan University, Foshan 528225, China; liuyating@fosu.edu.cn (Y.L.); xiejinxi2023@163.com (J.X.)
[2] Department of Plant Protection, Institute of Vegetables and Flowers, Chinese Academy of Agricultural Sciences, Beijing 100081, China; wenluwang123@163.com (W.W.); zhangyoujun@caas.cn (Y.Z.)
[3] Guangdong Provincial Key Laboratory of High Technology for Plant Protection, Plant Protection Research Institute, Guangdong Academy of Agricultural Sciences, Guangzhou 510640, China; leiyanyuan@gdaas.cn
[4] Department of Entomology, University of Kentucky, Lexington, KY 40546-0091, USA; xuguozhou@uky.edu
* Correspondence: xiewen@caas.cn

Abstract: The *fruitless* (*fru*) gene is a key factor in controlling sexual behavior in insects. The homolog of *fru* has been identified in most insect species and exhibits conservation in the transcript architecture and regulation of male sexual behavior. However, the structure of *fru* transcripts in Hemiptera remains unknown. Here, we identified and characterized two *fru* transcripts in *Bemisia tabaci*, named *fru-a* and *fru-b*. *fru-a* contained a 1263 bp open reading frame (ORF) encoding for 420 amino acids, while *fru-b* contained a 1143 bp ORF encoding for 380 amino acids. These two proteins start with the same BTB domain and end with two different zinc finger domains, belonging to ZnA and ZnG, respectively. The expression of *fru-a* and *fru-b* differed significantly between females and males, and both were expressed at lower levels in males. We demonstrated the presence of multiple TRA/TRA-2 binding sites and alternative splicing in *fru-a* and *fru-b*. Moreover, the RNAi result provided evidence that *transformer* regulates the expression of *fru-a* and *fru-b*. These results promote the study of the sex determination cascade in *B. tabaci* and lay the foundation for the study of sexual behavior in this insect.

Keywords: Hemiptera; Aleyrodidae; haplodiploidy; sex determination; alternative splicing; reproductive biology

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1. Introduction

The mechanisms of sex determination in insects are diverse. Even among closely related species, the mechanisms for determining sex are different. Its divergent model comprises four levels of sex-determining regulators: primary signal > key gene > double-switch gene > sex-differentiation gene. The primary signal is variable among insect species, resulting in repression or activation of the key gene. The active state of this key gene is conveyed by alternative splicing of a conserved double-switch gene. This switch gene then transmits similar splicing information to the sex differentiation genes, which ultimately translate the molecular signal into a specific sexual phenotype. In *Drosophila melanogaster* (Diptera: Drosophilidae), the model is a well-characterized genetic hierarchy X:A > *Sxl* > *tra*/*tra2* > *dsx* and *fru* [1]. This cascade appears to control sex determination in all *Drosophila* species and is partially conserved in other insect species, especially the *tra* > *dsx* and *fru* regulatory modules [2].

Transformer (tra) is a master regulator of sex determination in many insects. It not only regulates its own sex-specific alternative splicing, but also controls the splicing of downstream target genes *dsx* and *fru* [3]. *Doublesex* (*dsx*) is a double-switch gene that controls sexually dimorphic characteristics at the bottom of the somatic sex determination cascade and is functionally conserved in many insect species [4]. *Fruitless* (*fru*) is the

downstream sex-differentiation gene that controls sexual behavior. It was originally found to be related to regulating the development of the muscle of Lawrence (MOL), a male-specific structure in the abdomen of *D. melanogaster* [5]. Later, it was found that *fru* controls the formation of sexual dimorphisms in neurons and regulates male courtship, copulation, and even aggressive behavior [6,7]. Now, it is clear that *fru* is the switch gene for sexual behavior in *Drosophila* and is involved in the regulation of almost all male sexual behaviors [8]. Recently, various *fru* homologs were identified in distantly related insect species, including *Aedes aegypti* (Diptera: Culicidae), *Anopheles gambiae* (Diptera: Culicidae), *Bactrocera dorsalis* (Diptera: Tephritidae), *B. correcta*, *Nasonia vitripennis* (Hymenoptera: Pteromalidae), *Bombyx mori* (Lepidoptera: Bombycidae), *Gryllus bimaculatus* (Orthoptera: Gryllidae), *Schistocerca gregaria* (Orthoptera: Acrididae), and *Blattella germanica* (Blattodea: Blattellidae) [9–15]. In all of these insects, *fru* has been shown to be the primary regulator of male sexual behavior. This suggests that, despite the evolution of insects, the regulation of sexual behavior by *fru* is conserved. Therefore, *fru* has become the starting point for understanding the genetic determination of sexual behaviors.

The structure of the Fru protein is strikingly similar: it starts with a highly conserved protein–protein interaction module, the BTB domain, and ends with 1-4 exons encoding zinc finger domains [16]. In *D. melanogaster*, the *fru* gene forms a set of transcription factors by using four different promoters (P1–P4) and a large number of alternative splicing. Among them, the *fru* transcripts regulating male sexual behavior are directed from the most-distal P1 promoter. In females, *tra* and *tra2* splice P1-directed *fru* transcripts into female specific isoforms and prevent their translation. In males, due to the lack of active TRA protein, *fru* undergoes default splicing and translates into the FruM protein [17]. Neurons expressed by FruM connect with other neurons to form sexually dimorphic circuits that control male mating behavior [18]. Sex-specific splicing isoforms of *fru* have also been demonstrated in other non-*Drosophila* insects. In most insects, the sex-specific splicing of *fru* is regulated by upstream *tra* [16]. However, whether this mode is conserved in Hemiptera is not yet known.

The whitefly, *Bemisia tabaci* (Hemiptera: Aleyrodidae), is an important worldwide invasive pest that has a unique reproductive pattern. That is, the haplodiploid sex-determination model: males are haploid; females are diploid. The parthenogenesis and sexual reproduction of the whitefly coexist. Unmated females only give birth to haploid males, and mated females can give birth to both males and females [19]. Previously, we identified 26 sex-determining genes in *B. tabaci* and confirmed by RNAi the interaction between *Btdsx* and *Bttra2* and their important role in male genital formation, as well as the relationship between *Btix* and female fertility [20–23]. In addition, we identified a number of candidate genes for female mating response by transcriptomic analysis [24]. However, the mechanism of mating behaviors in *B. tabaci* males remains a mystery. In this paper, we characterized the *fru* gene in *B. tabaci*, explored the alternative splicing of *Btfru* in adults, and provide evidence that *Bttra* regulates the expression of *Btfru*. Our results deepen the understanding of sex-differentiation genes in *B. tabaci* and promote the study of sex-determination mechanisms in *B. tabaci*.

2. Materials and Methods

2.1. Insect Strains

The Q biotype *Bemisia tabaci* (Mediterranean, MED) strain used in this study was originally collected in Beijing, China, in 2009. Since then, the population has been reared on cotton in a naturally lit greenhouse with an ambient temperature of 25 ± 1 °C and a humidity of 70 ± 5%. Mitochondrial cytochrome oxidase I (*mtCOI*) markers were used every 2–3 generations to confirm population purity.

2.2. Sample Collection

Samples of eggs, 1st-, 2nd-, 3rd-, and 4th-instar nymphs and newly emerged females and males of the whitefly were collected, snap frozen in liquid nitrogen, and stored at −80 °C for subsequent experiments.

Clean, insect-free cotton was placed in an insect cage, and the plants were removed after 3 days of egg-laying. Then, the eggs were collected on this as egg-stage samples. The remaining egg-bearing cotton seedlings were placed in a clean insect-free cage and raised in a greenhouse. Five days later, when the whitefly had hatched, it was collected as a first-instar sample. Eight days later, it was collected as a second-instar sample. Twelve days later, it was collected as a third-instar sample, and sixteen days later, it was collected as a fourth-instar sample. When collecting the newly emerged female and male adults, the original adults on the leaves were first sucked away, and then, the emerged adults were collected in microscopic tubes (5.0 × 0.5 cm) after 1 h. Each tube contained 1 adult, and the sex of the insects was distinguished under a stereomicroscope. A total of 3 biological replicates were collected for each group of samples.

2.3. RNA Extraction and cDNA Synthesis

Total RNA was extracted by TRIzol reagent (Life Technologies). The purity and quantity of RNA were measured using NanoDrop 2000. RNA integrity was detected by 1% Tris/Borate/EDTA (TBE) agarose gel electrophoresis. The first strand of cDNA was synthesized with PrimeScript® RT Reagent Kit (TaKaRa Biotech, Kyoto, Japan), and the product was used immediately or stored at −20 °C for future use.

2.4. Gene Cloning and Splice-Variant Detection

The annotated sequence obtained previously was used to clone the *fru* gene in *B. tabaci* [21]. The full-length primers for the *Btfru* gene were designed with Primer Premier 5.0 software (Table 1). PCR reactions consisted of 12.5 µL Es-Taq MasterMix, 10.5 µL ddH_2O, 1 µL cDNA template, and 0.5 µL of each primer (10 mM). The reaction procedure was as follows: denaturation at 95 °C for 5 min; amplification, 95 °C for 30 s, 56 °C or 59 °C (depending on the primers) for 30 s, and 72 °C for 1 min (35 cycles); extension, 72 °C for 10 min. PCR products were purified by a DNA Gel Extraction Kit (NEB, Beijing, China), cloned into the pEASY-T1 vector (TransGen, Beijing, China), and sequenced. The analysis of alternative splicing was consistent with previous methods [22].

Table 1. Primer sequences for full-length gene cloning, alternative splicing analysis, gene expression analysis, and RNA interference were used in this study.

Application of Primers	Gene Name	Primer Name	Primer Sequence (5′-3′)
Cloning and AS analysis	*fru-a*	fru6255-F	CGTCTCTCCCCCAACCAG
		fru-full-R	CCCTTAGCATCAATAGCGG
	fru-b	fru-F5-full	ATGGAGGAGGCATTTTGTTTGAAG
		fru-R5-full	TTATGTGTTGTGCTTGAGCCTGAAA
qRT-PCR analysis	*fru-a*	qfru-A-F1	AAGCAATCCGCAGCCGTT
		qfru-A-R1	CTGATGTCGTTGAGATACCGC
	fru-b	qfru-G-F2	ATGAAAAACCACTTCTTGACGC
		qfru-G-R2	TATGTGTTGTGCTTGAGCCTGA
	fru	BTB-qF	CATTCGTCAAGTTTTTCGGGTA
		BTB-qR	GGAAGGTCTCGCTCGCTAAA
	tra	dsTra-qF2	AAGTCCCTCTCCTCAGCCCA
		dsTra-qR2	GCCACGGGTTAGACCTTTGA
	SDHA	SDHA-qF	GCGACTGATTCTTCTCCTGC
		SDHA-qR	TGGTGCCAACAGATTAGGTGC

Table 1. *Cont.*

Application of Primers	Gene Name	Primer Name	Primer Sequence (5′-3′)
RNAi analysis	*EGFP*	dsEGFP-F	TAATACGACTCACTATAGGGAGACA GTGCTTCAGCCGCTAC
		dsEGFP-R	TAATACGACTCACTATAGGGAGAGTT CACCTTGATGCCGTTC
	tra	dsTra-F2	GGATCCTAATACGACTCAC TATAGGTTGAGACGAATCAG CAATCG
		dsTra-R3	GGATCCTAATACGACTCAC TATAGGGACCTTCGCAG GAACTTTTG

2.5. Phylogenetic Analysis

To analyze the evolutionary placement of *fru* zinc finger domains, we firstly collected the nucleotide sequences of known *fru* zinc fingers. Secondly, we aligned these sequences by CLUSTALW [25]. The phylogenetic tree was constructed with MEGA 6 software, using the neighbor-joining method with a p-distance model and pairwise deletion of gaps [26].

2.6. Real-Time Quantitative PCR

To distinguish the spliceosomes of *fru*, we designed quantitative primers in the BTB domain, the common region of *fru-a* and *fru-b*, and the ZnA and ZnG regions, respectively. The specific primers are shown in Table 1. Relative transcript levels of all *fru* isoforms, *fru-a* and *fru-b*, and the reference gene (*SDHA*) were assayed by real-time qPCR with the conditions described as follows [27]: denaturation at 95 °C for 10 min; amplification, 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s (40 cycles). Amplified products were identified using SuperReal PreMix Plus (SYBR Green) (Tiangen, Beijing, China). Three independent biological replicates were included for each stage. The relative differences in transcript levels were analyzed by the $2^{-\Delta\Delta Ct}$ method [28].

2.7. RNA Interference

To amplify dsRNA targeting *Bttra*, primers containing a T7 promoter sequence were designed (Table 1). The dsRNA for enhanced green fluorescent protein (EGFP) was used as the negative control. All dsRNAs were prepared using the T7 Ribomax™ Express RNAi system (Promega, Madison, WI, USA). RNAi was achieved by directly feeding a 0.20 mL drop of diet solution containing 5% yeast extract, 30% sucrose (wt/vol), and 100 ng dsRNA to *B. tabaci* adults in an incubated chamber [29,30]. Approximately 40 newly emerged mixed adults (the ratio of males to females was 1:1) were introduced into an environmental chamber at 25 °C under a photoperiod of L14:D10 and a relative humidity (RH) of 70%. Each sample was represented by three technical replicates. Mortality was recorded, and *B. tabaci* specimens were collected after two days of feeding.

3. Results

3.1. Characterization of Btfru

Previously, we successfully identified a *fru* homolog in *B. tabaci* [21]. In this study, we found two *fru* transcripts in our transcriptome data, named *fru-a* and *fru-b*. *fru-a* contains a 1263 bp open reading frame (ORF) encoding 420 amino acids with a predicted molecular weight (Mw) of 46.67 kDa and an isoelectric point (pI) of 6.47. *fru-b* contains a 1143 bp ORF encoding for 380 amino acids with a predicted Mw of 42.33 kDa and a pI of 6.43. Genomic structural analysis showed that *fru-a* and *fru-b* were located in the same scaffold, with *fru-a* containing 7 exons and 6 introns and *fru-b* containing 6 exons and 5 introns (Figure 1). The BTB domain and C_2H_2 zinc finger domain were identified in the predicted *fru-a* and *fru-b* amino acid sequences (Figure 1). The two transcripts both begin with an exon linked to the

BTB-coding exon and end with alternative C_2H_2 zinc finger domain coding exons. We also searched for the presence of Tra/Tra2 binding sites in the *Btfru* gene and found two in *fru-a* and one in *fru-b*, respectively (Figure 1).

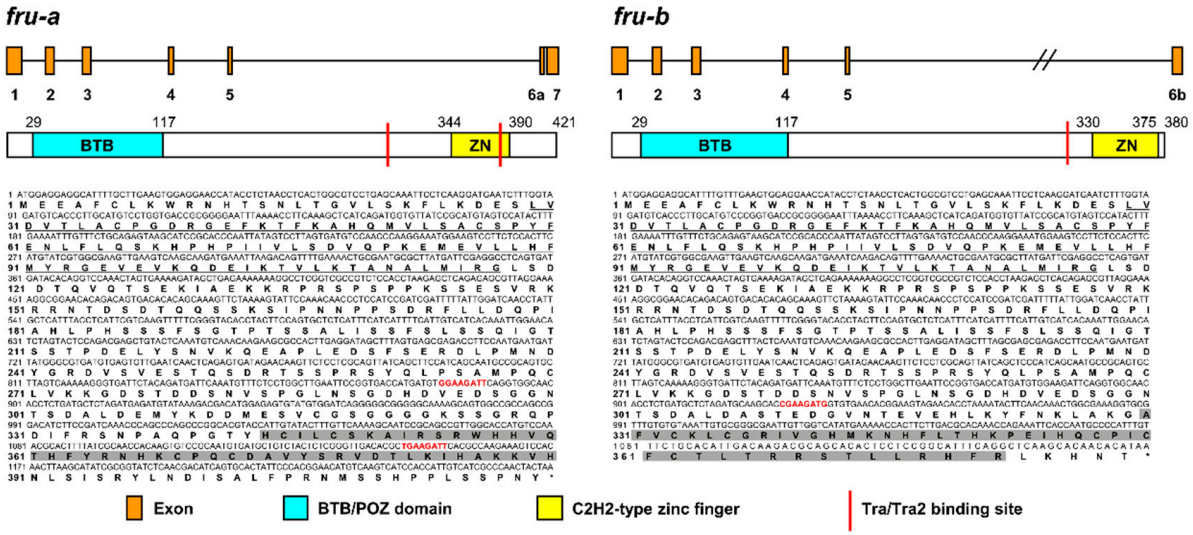

Figure 1. The gene structure, deduced protein conserved domains, nucleotides, and deduced amino acid sequences of *fru-a* and *fru-b*. The orange boxes and lines between them represent exons and introns, respectively. The sizes of the exons and introns are plotted in proportion. The BTB domain is highlighted with an underline; the zinc finger domain is highlighted in dark grey; the TRA/TRA2 binding sites are highlighted in red bold characters.

3.2. Phylogenetic Analysis of Btfru

Phylogenetic analysis showed that Fru proteins were clustered within each insect order, and two BtFru were closely related to that of *Lygus hesperus* (Figure S1). In order to reveal the evolutionary origin of *fru-a* and *fru-b*, the zinc finger domains of both were analyzed in depth. By searching the literature, the zinc finger sequences of the reported insect *fru* gene were collected, and the phylogenetic tree was constructed with *fru-a* and *fru-b*. The result is shown in Figure 2. The zinc fingers of *fru-a* and *fru-b* belong to ZnA and ZnG, respectively (Figure 2). Multi-alignment analysis of the ZnA and ZnG sequences of known insects revealed high similarity in each species (67% and 71%, respectively), as shown in Figure S2. From the evolutionary analysis, it is known that ZnF and ZnB, ZnA and ZnC are likely to be derived from the same ancestor. Phylogenetic analysis also showed that not all insect FRUs contain all known zinc finger domains; for example, *Drosophila fru* has only ZnA, ZnB, and ZnC; *Nasonia fru* has ZnA, ZnB, ZnC, ZnF, and ZnG; the whitefly *fru* has only ZnA and ZnG.

In addition, we found that the C_2H_2 zinc finger domains of *fru* have the same consistent sequence, CX2CX9HX3HX6CX2CX5RXDX4HX4H, where C and H are cysteines and histidines, while X represents an unconserved amino acid. If the H sequences of the zinc finger domains are excluded, there are three additional amino acid residues (underlined) that are conserved in all *frus*: CX2CX<u>K</u>X<u>V</u>X5HX3HX6CX2CX5RXDX4HX3<u>K</u>H (Figure S2). This should be a typical feature of the *fru* zinc finger domains.

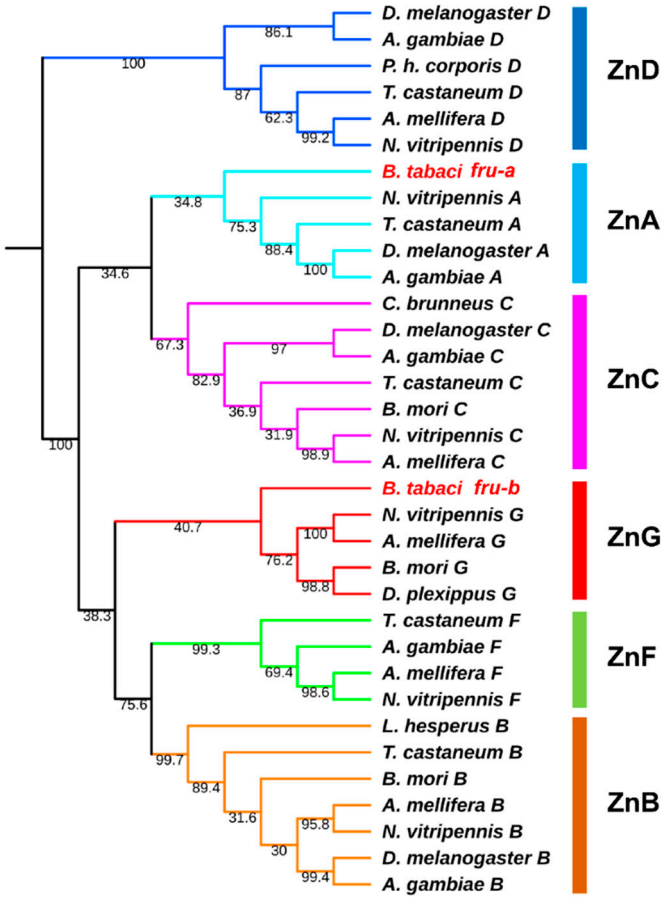

Figure 2. Phylogenetic tree of *fru* zinc finger nucleotide sequences in different insects. The sequences of *B. tabaci* were determined in the current study, and sequences for the other insects were obtained from the GenBank database. ZnD was set as the outgroup. The full species names are *Drosophila melanogaster*, *Anopheles gambiae*, *Pediculus humanus corporis*, *Tribolium castaneum*, *Apis mellifera*, *Nasonia vitripennis*, *Bemisia tabaci*, *Chorthippus brunneus*, *Lygus hesperus*, *Bombyx mori*, and *Danaus plexippus*.

3.3. Developmental Expression of Btfru

In order to reveal the expression patterns of *fru-a* and *fru-b* in different developmental stages of *B. tabaci*, quantitative primers specific to the *fru-a* and *fru-b* zinc fingers were designed, and the cDNA samples of different developmental stages of *B. tabaci* were used as the templates for qRT-PCR. The results showed that both *fru* transcripts were highly expressed in the egg stage, followed by the third-instar larvae, with the lowest expression in males and significant differences between males and females (Figure 3).

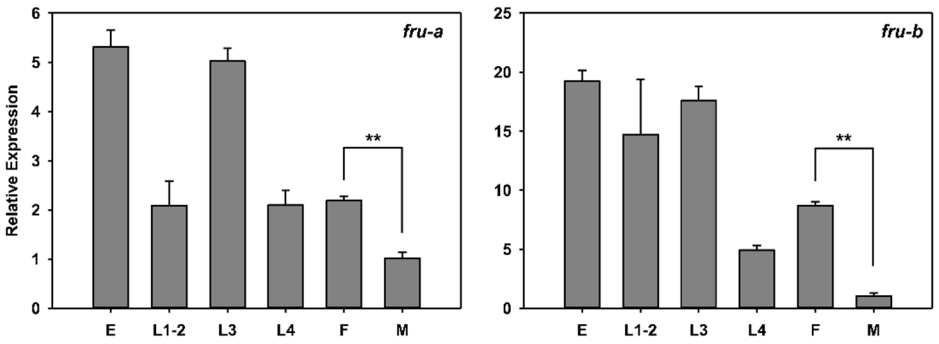

Figure 3. Expression profiling of two *fru* transcripts in *Bemisia tabaci* at different developmental stages. E, egg; L1-2, 1st- and 2nd-instar nymphs; L3, 3rd-instar nymph; L4, 4th-instar nymph; F, newly emerged females; M, newly emerged males. ** represents highly significant differences ($P < 0.01$).

3.4. Analyses of Alternative Splicing Variants of Btfru

To analyze the spliceosomes of *Btfru*, we performed PCR amplification with full-length primers using newly emerged males and females as templates (Figure S3). All *Btfru* isoforms obtained from this analysis are listed in Table 2. In our study, 21 isoforms were found in *fru-a*, and amongst them, only 3 contained both the BTB and Zn finger domains (Nos. 1, 18, 21). No. 1 occurred in both females and males; No. 18 occurred only in males; No. 21 occurred in females. In addition, six isoforms were found in *fru-b*. No. 1 and No. 6 contain both BTB and Zn finger domains. No. 6 occurred only in females.

Table 2. Alternative splicing isoforms of *fru-a* and *fru-b* in *Bemisia tabaci*.

Type	Variants	Exons Included	Size (bp)	Female (3) [a]	Male (3) [a]	Domain
fru-a	1	1,2,3,4,5,6a,7	1263	3	3	BTB + Zn
	2	$1_{-198,160-}$7	278	1	0	BTB
	3	$1_{-169,178-}$7	289	1	0	BTB
	4	$1_{-210,110-}$7	316	1	0	
	5	$1_{-194,88-}$7	354	1	0	BTB
	6	$1_{-308,48-}$6a,7	357	1	0	Zn
	7	$1_{-200,78-}$7	358	1	0	BTB
	8	$1_{-279,71-}$6a,7	363	0	1	Zn
	9	$1_{-161,85-}$7	390	1	0	BTB
	10	$1_{-197,38-}$7	401	1	0	BTB
	11	$1_{-132,98-}$7	406	1	0	BTB
	12	$1_{-346,55-}$5,6a,7	444	1	0	Zn
	13	$1_{-131,9-}$7	496	1	0	BTB
	14	$1_{-305,37-}$5,6a,7	503	1	0	
	15	1,2,3$_{-107,244-}$7	667	1	0	BTB
	16	1,2,3$_{-7,79-}$7	895	0	1	BTB
	17	1,2,3,4,5$_{-86,163-}$7	935	1	0	BTB
	18	1,2,3,4$_{-73,21-}$6a,7	1074	0	1	BTB + Zn
	19	1,2,3,4,5,6a$_{-66,82-}$7	1114	1	0	BTB
	20	1,2,3,4,5$_{-56,}$7	1130	1	0	BTB
	21	$1_{-10,98-}$2,3,4,5,6a,7	1155	1	0	BTB + Zn
fru-b	1	1,2,3,4,5,6b	1143	3	3	BTB + Zn
	2	$1_{-185,155-}$6b	253	1	0	BTB
	3	$1_{-284,}$5,6b	348	1	0	
	4	$1_{-292,}$5,6b	396	1	0	BTB
	5	1,2$_{-81,178-}$6b	517	0	1	BTB
	6	$1_{-51,}$2,3,4,5,6b	1090	1	0	BTB + Zn

a: Numbers in parentheses indicate the number of biological replicates, with 40 females or males assessed as a group per replicate.

The number of *fru* isoforms obtained from PCR assays was numerous, and their differences between males and females were enormous. To verify that this result was real and not caused by sequencing, we designed quantitative primers in the BTB domain and the ZnA and ZnG regions, respectively. The results are shown in Figure S4. Quantitative primers stuck in the BTB domain reflected the expression of all *fru* isoforms, and there was no significant difference between male and female expression at this time, indicating that most isoforms retained the BTB domain, which was consistent with the PCR sequencing results. The quantitative primers stuck in the ZnA and ZnG regions responded to the expression levels of specific zinc finger domains, which showed highly significant differences between males and females, with the expression of females in ZnG reaching 8.7-times higher than that of males. This result suggests that it is the use of different zinc finger domain endings that leads to the creation of the *fru* sex-specific isoforms.

Although we know that *frus* have sex-specific alternative splicing in *B. tabaci*, we did not find any sex-specific exons or stable sex-specific variants in *fru-a* and *fru-b*. All of the sex-specific variants were found in only one biological replicate. We tried many times, but

failed to find a marker that could mark the sex-specific *fru* spliceosome. This is probably because the spliceosome of *fru* is too complex and variable.

At the same time, we also performed the *fru* spliceosome analysis of a single whitefly, and the results were even more complex, which further indicated that the population of the whitefly is too heterozygous and has too much individual variation.

3.5. Analysis of the Interaction between Bttra and Btfru

Comparative analysis of *fru* sequences of *N. vitripennis*, *Apis mellifera*, and *B. tabaci* revealed the presence of a short repeat sequence (T/G/C)GAAGAT(T/A) in all three genes (Figure 4A). These repeats are thought to be TRA/TRA2 binding sites in hymenopterans and are conserved in the *dsx* and *fru* genes of *N. vitripennis* and *A. mellifera* [9]. This finding indicates that the splicing of *Btfru* is also mediated by *tra*, as in other insect species.

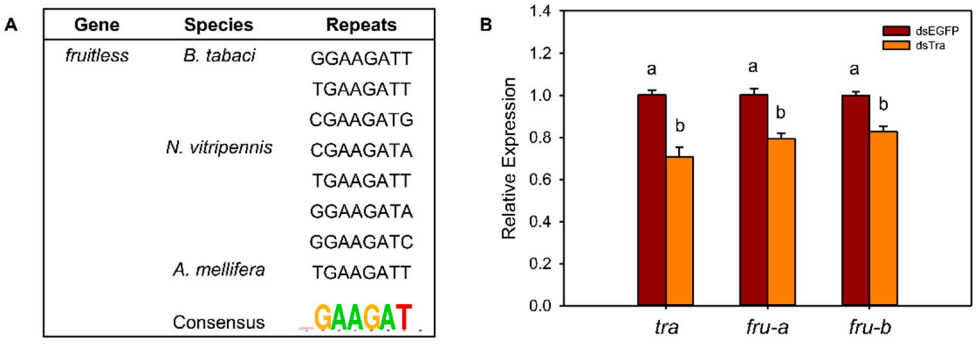

Figure 4. (**A**) Short repeats found in the *fru* genes of *B. tabaci*, *N. vitripennis* and *A. mellifera*. (**B**) Relative transcript levels of *tra*, *fru-a*, and *fru-b* in control and *Bttra* dsRNA-fed *B. tabaci*. Different lowercase letters indicate significant differences ($p < 0.05$).

Previously, we successfully identified a *tra* homolog in *B. tabaci*, named *Bttra* [21]. After silencing *Bttra* in *B. tabaci* adults, both *fru-a* and *fru-b* expression decreased significantly compared with the dsEGFP control groups (Figure 4B). This result suggests that *Bttra* regulates the expression of *Btfru*.

4. Discussion

The *fruitless* gene performs multiple functions during *Drosophila* development. In recent years, *fru* homologues have been identified in most insect species. These Frus are conserved both in transcript architecture and in the regulation of male sexual behavior. However, the status of Hemiptera *fru* remains unknown. Here, we conducted a detailed analysis of *fru* transcripts in *B. tabaci*.

The *fru* transcripts generally begin with a broad-complex, tramtrack, and bric-a-brac (BTB) domain and end with one alternative C_2H_2 zinc finger domain [31]. The BTB domain is involved in protein oligomerization and recruitment of transcriptional corepressors. It is the most-conserved feature of all Fru proteins [32,33]. The BTB domain found in *B. tabaci* *fru* transcripts is encoded by a single exon (Figure 1), which is the same as *N. vitripennis*, but different from that of *D. melanogaster* [9]. In *Drosophila*, the BTB domain is encoded by three different exons [34]. Multi-alignment analysis of the BTB domains revealed a high identity rate (80%) (Figure S5), further illustrating the high conservation of the BTB domain in insect Fru proteins.

The zinc finger domains in insect *fru* genes are diverse and less conserved than those in the BTB domain. The *fru* gene in Diptera encodes three C_2H_2-type zinc finger isoforms identified as A, B, and C. However, in *Nasonia*, the *fru* gene encodes five C_2H_2-type zinc fingers termed the A, B, C, F, and G exons [8,9,35–37]. In this study, we found two C_2H_2-type zinc finger isoforms (A and G) in *B. tabaci* (Figure 2). Of the species that have been reported so far, only *N. vitripennis* and *B. tabaci* possess both A and G, while other species

have either A or G. The specific function of these two zinc fingers remains for further experimental study.

By alternative splicing, the *fru* gene encodes a set of transcription factors in *D. melanogaster*. The transcript begins with one of four promoters (P1–P4) and ends with one of four final exons (A–D). Only those transcripts produced by the most-terminal P1 promoter are spliced in a sex-specific way [31,38]. *Nasonia fru* transcripts derive from at least six different promoters. As in *Drosophila*, the transcripts common to both sexes arise from the promoters closest to the BTB exon (P2-P3-P4-P5-P6 promoters). In contrast, sex-specific transcripts are derived from promoters further upstream in the *fru* locus (P0-P1) [9]. In addition to *Drosophila* and *Nasonia*, sex-specific isoforms were observed in *A. mellifera*, *A. gambiae*, *Ceratitis capitata*, *Aedes aegypti*, *Musca domestica*, and *Blatella germanica* [10,35–37]. In this paper, we did not find any stable sex-specific variants in two *fru* transcripts. This may be partly due to the fact that *B. tabaci* is a rapidly evolving complex species with large individual differences [39]. On the other hand, it may be because we used the whole body instead of the head to analyze the *fru* transcripts. After all, in both *Nasonia* and *Drosophila*, sex-specific *fru* transcripts driven by P1 promoters are expressed primarily in the head [9]. Furthermore, the male-specific *Ceratitis fru* is expressed only in male head samples [40]. Therefore, we may be able to obtain some new *fru* transcripts by collecting head tissues from both males and females for transcriptome sequencing.

In *D. melanogaster*, sex-specific splicing of *fru* is regulated by *tra* and its cofactor *tra-2* [17,31,38]. The pattern of *tra*/*tra2* co-regulation of *fru* splicing is widely conserved in Hymenoptera, Coleoptera, and Diptera [40,41]. For example, injection of *tra* dsRNA in the early embryonic stage of *B. dorsalis* caused fruM to appear in pseudomales [14]. Similarly, by embryonic injection of *tra2* dsRNA in *C. capitata*, male-specific *fru* mRNA was detected in XX-pseudomales [40]. Recently, researchers found that the expression of *Drosophila* male-specific *fru* in the gonads is regulated by *dsx* and is independent of *tra*. This led to the inference of a novel mechanism regulating sex-specific splicing of *fru*. This mechanism is regulated by *dsx* at the transcriptional level and is not mediated by splicing of *tra* [42]. In this study, the presence of the TRA/TRA-2 binding sites in *Btfru* and the expression changes induced by *Bttra* RNAi suggest that, like most insects, *fru* is regulated by *tra* in *B. tabaci* (Figure 4).

Functional studies of the *Btfru* gene can be complex. This is because the number of *fru* splice variants is large and varies greatly between individuals. Thus far, we have not found stable *fru* sex-specific variants in *B. tabaci*. Both *Btfru* transcripts were found to be highly expressed in females (Figure 3), indicating that the two *Btfru* transcripts may function in females, but not in males. The function of *fru* in males has been widely reported. In *D. melanogaster*, *fru* mutant males were unable to complete mating and exhibited courtship behavior towards both females and males. This confirmed that FruM proteins determine male courtship and orientation [8,43]. In addition, the functions of *Drosophila* FruM include influencing the formation of sexual dimorphisms in neurons and the formation of male-specific MOL, as well as controlling the differentiation of imaginal discs [16,18]. In *Bombyx*, mating behavior is independently regulated by *fru*. Loss of *Bmfru* completely prevents mating, but males can still exhibit courtship behavior [15]. RNAi knockdown of *fru* in *S. gregaria* prevents successful mating and affects male fertility [11]. Silencing the cockroach *fru*, males no longer showed courtship behavior [10]. All these experiments confirmed the ancestral function of *fru* in male sexual behavior. Its function in females, however, is rarely reported. Actually, female mate choice plays a key role in species reproduction. It affects both sexual selection within species and reproductive isolation between species. In 2019, a non-sex-specifically spliced *fru* transcript was revealed to influence female rejection behavior. *fru* mutant females not only did not show acceptance of copulation, but also actively rejected courting males, as evidenced by extrusion of the ovipositor, kicking and/or wing clipping, and actively moving away from courting males [44]. It was the first implication of *fru* in female behavior. Therefore, we still have much work to perform in the functional verification of *Btfru*.

With the in-depth study of the mechanism of insect sex determination, people began to try to conduct genetic manipulation of sex in some insects, for example RNAi knockdown of *tra* and *tra2* to generate male-only progeny in *B. dorsalis* [45]. CRISPR-Cas9-targeted *A. gambiae dsxF* did not affect male development or fertility, whereas females showed an intersex phenotype and complete sterility [46]. Disruption of *Osp* in *B. mori* and *Spodoptera litura* results in female sterility, while male fertility is not affected [47]. The success of these experiments is driving the process of genetic manipulation of gender. Likewise, the possibility of the manipulation of the sex determination pathway opens up a new opportunity for pest control. In this study, we identified multiple spliceosomes of the sex-differentiation gene *fru*. Later, we can target specific spliceosomes for interference or gene editing, which is expected to realize the genetic regulation of sex in the whitefly.

5. Conclusions

In the present study, we cloned and characterized the *fru* gene in *B. tabaci* and explored the alternative splicing of *fru* in *B. tabaci* adults. Furthermore, we confirmed by RNAi that *fru* expression is regulated by *tra*. Our results suggest that *tra* is functionally conserved in controlling downstream sex-differentiation gene expression. Meanwhile, the sex-differentiation gene *fru* is far more complex in whiteflies than we thought. Future in-depth studies of the mechanism of sex determination in whiteflies may reveal the function of this gene in whiteflies.

Supplementary Materials: The following Supporting Information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae9090962/s1, Figure S1: The phylogenetic tree of known *fru* genes in insects, Figure S2: Conservation of zinc finger domains among insect species, Figure S3: PCR cloning of *fru-a* and *fru-b* in *B. tabaci* females and males, Figure S4. Expression levels of *fru* transcripts in *B. tabaci* adults, Figure S5: Conservation of BTB domains among insect species.

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Data Availability Statement: The sequences have been uploaded to the NCBI database, and the accession numbers are *fru-a*: OP868816 and *fru-b*: OP868817.

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