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Transcriptomic dissection of sexual differences in *Bemisia tabaci*, an invasive agricultural pest worldwideSUBJECT AREAS:  
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Sex difference involving chromosomes and gene expression has been extensively documented. In this study, the gender difference in the sweetpotato whitefly *Bemisia tabaci* was investigated using Illumina-based transcriptomic analysis. Gender-based RNAseq data produced 27 Gb reads, and subsequent *de novo* assembly generated 93,948 transcripts with a N50 of 1,853 bp. A total of 1,351 differentially expressed genes were identified between male and female *B. tabaci*, and majority of them were female-biased. Pathway and GO enrichment experiments exhibited a gender-specific expression, including enriched translation in females, and enhanced structural constituent of cuticle in male whiteflies. In addition, a putative *transformer2* gene (*tra2*) was cloned, and the structural feature and expression profile of *tra2* were investigated. Sexually dimorphic transcriptome is an uncharted territory for the agricultural insect pests. Molecular understanding of sex determination in *B. tabaci*, an emerging invasive insect pest worldwide, will provide potential molecular target(s) for genetic pest control alternatives.

Gender is a strong determinant of transcript levels in comparison to animals' ontogeny<sup>1</sup>, age, and genotype<sup>2</sup>. Animals from a broad range of taxa show sex differences in development time, lifespan<sup>3</sup>, body size<sup>4</sup>, sex-biased gene and protein expression<sup>5,6</sup>, and sex chromosomes<sup>7</sup>. Sex-biased gene expression has been documented in multiple insect species including *Drosophila*<sup>2,8–10</sup>, *Anopheles gambiae*<sup>11–13</sup>, *Tribolium castaneum*<sup>14</sup>, *Daphnia pulex*<sup>15</sup>, and vespid wasp<sup>16</sup>. Among them, a rapid evolution at transcription level is evident in *Drosophila* and *An. Gambiae*<sup>5</sup>, in which a large proportion of the transcriptome is sex-biased<sup>2,8,17</sup>. Expression of male-biased genes had significantly faster rates of evolution than that in female-biased or unbiased genes in *D. melanogaster*<sup>18</sup>. Also, specific gene families were preferentially expressed in unique tissues. The *takeout* family, for example, was enriched in the head of male *D. melanogaster*<sup>19</sup>.

The sweetpotato whitefly, *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae), is an emerging agricultural pest that causes serious damage through feeding and vectoring plant viruses in protected crops and field crops worldwide<sup>20</sup>. More than 34 morphologically indistinguishable cryptic species have been identified through various techniques<sup>21–24</sup>. Among them, *Bemisia tabaci* B (also referred to as the Middle East-Asia Minor 1) has been determined as the most invasive and destructive biotype in many parts of the world<sup>22</sup>. In China, *B. tabaci* was first recorded in the late 1940s. However, *B. tabaci* did not cause serious losses in vegetable, fruit and ornamental crops until the introduction and establishment of invasive *B. tabaci* B in the mid-1990s<sup>25,26</sup>.

*Bemisia tabaci* has a haplo-diploid life cycle. The males, derived from unfertilized eggs, are haploid, and females are diploid<sup>27</sup>. The estimated nuclear DNA content of male and female *B. tabaci* B was 1.04 and 2.06 pg, respectively<sup>28</sup>. Regarding the biological traits, male and female *B. tabaci* are different in longevity<sup>29</sup>, body size<sup>30–32</sup>, secondary symbionts<sup>33</sup>, and efficiency of transmitting plant viruses<sup>34–39</sup>. For squash leaf curl virus, however, female and male *B. tabaci* has the same efficiency<sup>40</sup>. There were no differences in response between male and female whitefly when they were exposed to thiamethoxam, a widely used neonicotinoid insecticide<sup>41</sup>. Molecular basis of sex determination has been traditionally focused on model insects with readily available genomes, including *D. melanogaster*<sup>42,43</sup>, *Apis mellifera*<sup>44,45</sup>, *Nasonia*<sup>46</sup>, and *T. castaneum*<sup>47</sup>. A suite of genes, including *transformer* (*tra*), *transformer2* (*tra2*), *feminize* (*fem*), *complementary sex determination* (*csd*) and a key switch gene *doublesex* (*dsx*), are determined to play important role in sex determination. In other non-model insects,



Table 1 | Illumina sequencing metrics

	Caf*	Cuf	Tof	Cof	Cam	Cum	Tom	Com
Total Reads	36,616,870	43,576,274	41,959,788	65,050,162	53,477,310	19,373,358	40,522,552	41,192,154
Total Lengths (bp)	2,929,349,600	3,486,101,920	3,356,783,040	5,204,012,960	4,278,184,800	1,549,868,640	3,241,804,160	3,295,372,320

\*Ca, Cu, Co, and Tom are abbreviation for the respective host plants including Cabbage, Cucumber, Cotton and Tomato, while m and f denote males and females.

especially the ones without genome, such as haplo-diploid *B. tabaci*, molecular basis of sex determination remains largely unknown.

To reveal the gender differences at the transcription level, we carried out the largest *de novo* transcriptome sequencing in *B. tabaci* up-to-date (336 million reads, 27 Gb). Based on comparative analyses of eight *B. tabaci* transcriptomes generated from male and female whiteflies reside in four different host plants, genes and gene networks putatively involved in sex divergence were investigated. We also summarized the function and sequence divergence of genes with sexually enriched expressions to determine which genes, if any, are rapidly evolving and potentially involved in sex determination.

## Results

**Illumina sequencing and *de novo* assembly.** We constructed 8 cDNA libraries derived from B-biotype (Middle East-Asia Minor 1) *B. tabaci* female or male adults reared on four different host plants: cabbage, cucumber, tomato and cotton. Each sample resulted in a library containing an average of 42 million reads equal to 3.4 Gb and a total data of 27 Gb were generated (Table 1). For further analysis, two different strategies (separate or pool assembly) were carried out to guarantee the amount of sequence data for each assembly. First, eight libraries were separately assembled by using Trinity with default parameters<sup>48</sup>, and 34,055–77,065 long transcripts were generated with lengths longer than 200 bp (Table 2). Second, pooled reads covering all 8 libraries were assembled with the same method above; the length distribution and length statistic were shown in Table 2. Pooled assembly was used as the final result, and it generated 93,948 long transcripts with a length longer than 200 bp (Table 2). The total length of assembled long

transcripts was approximately 79.7 Mb and the N50 size was 1,853 bp with 21,273 sequences longer than 1 kb. In comparison to previously published transcriptome data of *B. tabaci*<sup>49–51</sup>, pooled assembly was better in data volume and in length (Table 2).

**Functional annotation.** For annotation, the pooled assembled unigenes were searched using blastx against the non-redundant (nr) NCBI protein database, SWISS-PROT, COG and KEGG all with an e-value cut-off of  $10^{-5}$ . As shown in Table 3, 25,554 genes (27.2% of all unigenes sequences) in nr, 20,003 genes in SWISS-PROT, 1656 genes in COG, and 5,548 genes in KEGG returned above the cut-off BLAST hits. This annotation rate is consistent with the previously reported B-type whitefly<sup>49,50</sup> and Q-type whitefly<sup>51</sup>. Gene Ontology terms were assigned by Blast2GO through a search of NR database. InterPro was searched by InterProScan. Using this approach, in total, 12,222 unigenes were assigned to one or more Gene Ontology (GO) terms. The Kyoto Encyclopedia of Genes and Genomes (KEGG) metabolic pathway analysis revealed that 5,548 unigenes could be assigned to 774 predicted pathways using *Acyrtosiphon pisum* as KEGG organism.

**Cluster correlation analysis.** To measure the correlation of any two samples in all eight libraries, read number of each sample was used and calculated according to the Spearman correlation coefficient. In this study, gene expression differences between the sexes are much greater than those among the host plants (Figure 1). On the other hand, we analyzed the possible reciprocal best matched sequences (RBMs) pairs between transcripts from each separated and pooled transcriptomes using bidirectional best hit<sup>49,52,53</sup>. With this method, we identified 31658, 35692, 31560, 36318 and 42150, 17929, 26169,

Table 2 | *Bemisia tabaci* transcriptome resources used in this study

	<i>B. tabaci</i> B <sup>50</sup>	<i>B. tabaci</i> Q <sup>51</sup>	<i>B. tabaci</i> B <sup>49</sup>	Caf <sup>a</sup>	Cuf	Tof	Cof	Cam	Cum	Tom	Com	Pooled
<b>Length distribution (bp)</b>												
0.1–0.5 k	151,375	151,360	40,254	30,416	31,089	32,175	31,152	44,478	23,187	41,068	25,051	55,584
0.5–1.0 k	22,546	12,949	13,007	11,259	12,885	12,599	11,234	15,864	6,934	13,808	6,127	17,091
1.0 k–2.0 k	4,046	3,929	4,070	7,622	8,935	7,905	6,060	9,829	3,204	8,390	2,195	10,992
>2.0 k	702	662	410	6,443	7,144	4,684	2,967	6,894	890	5,384	682	10,281
Total	178,669	168,900	57,741	55,740	60,053	57,363	51,413	77,065	34,215	68,650	34,055	93,948
<b>Sequencing statistics (bp)</b>												
Total (Mb)	62.9	42.9	26.4	48.4	53.5	43.0	33.8	62.0	17.8	49.8	15.9	79.7
Mean	368	266	478	911	934	786	689	843	543	761	490	889
Median	327	172	355	444	478	435	403	417	331	388	335	392
Min	102	100	200	201	201	201	201	201	201	201	201	201
Max	5,872	7,297	5,936	16,744	19,356	16,173	17,748	24,983	12,022	19,970	9,551	27,456
N50	423	331	552	1,757	1,710	1,294	1,023	1,503	723	1,320	561	1,853
<b>Comparative analysis</b>												
Platform	454	Illumina	Illumina	Illumina	Illumina	Illumina	Illumina	Illumina	Illumina	Illumina	Illumina	Illumina
Assembly	gsAssembler	SOAPdenovo	SOAPdenovo	Trinity	Trinity	Trinity	Trinity	Trinity	Trinity	Trinity	Trinity	Trinity
Hit <sup>b</sup>	72.5%	77.7%	93.7%									

<sup>a</sup>Ca, Cu, Co, and Tom are abbreviation for the respective host plants including Cabbage, Cucumber, Cotton and Tomato, while m and f denote males and females.

<sup>b</sup>Hit: Blastn against the pooled transcriptome dataset using an e-value cutoff of  $1e-5$ .



**Table 3 | Annotation of a pooled assembly, representing male and female *B. tabaci* transcriptomes on different host plants**

Database	Number of Transcripts <sup>a</sup>
Nr	25,554
SWISS-PROT	20,003
COG	1,656
KEGG	5,548
InterPro	23,466
GO	12,222

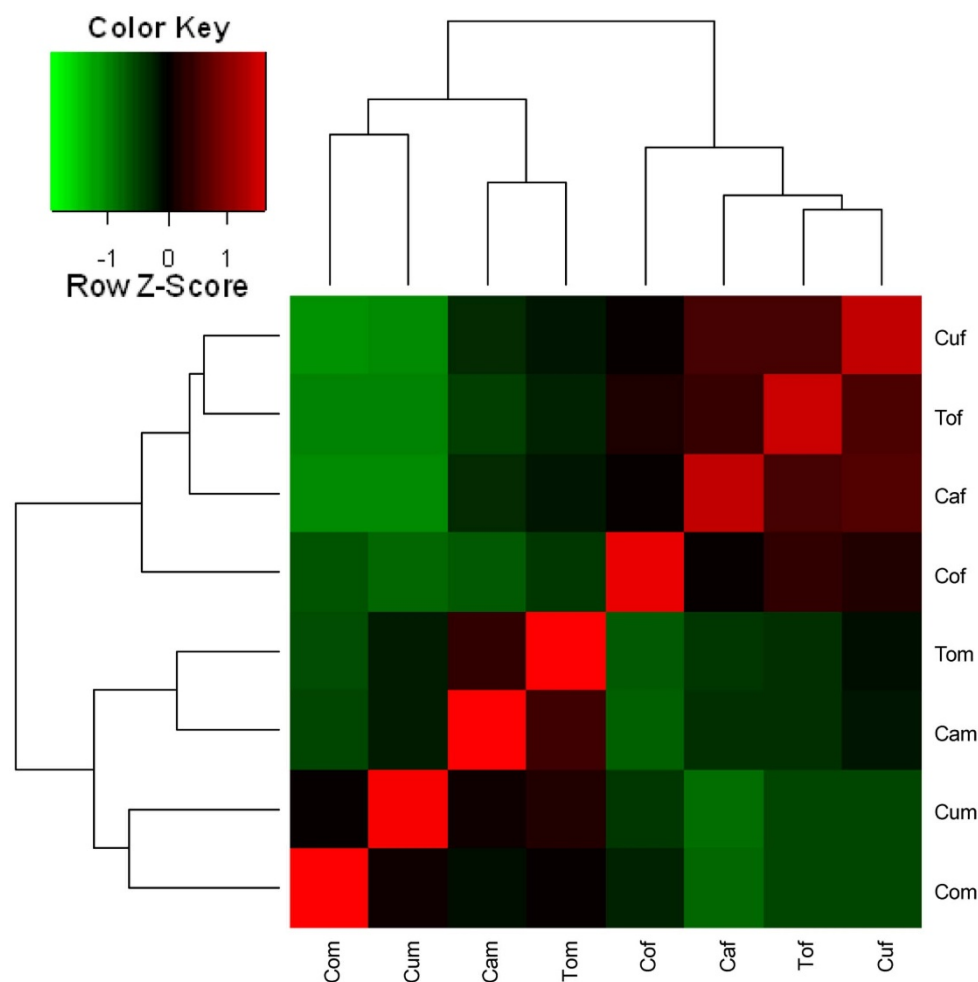
<sup>a</sup>Transcripts with a length greater than 200 bp were subjected to annotation.

17110 pairs of putative orthologs, respectively, in female *B. tabaci* on cabbage (Caf), cucumber (Cuf), tomato (Tof), cotton (Cof), and male *tabaci* on cabbage (Cam), cucumber (Cum), tomato (Tom), and cotton (Com) corresponding to the pooled transcripts (Table S3).

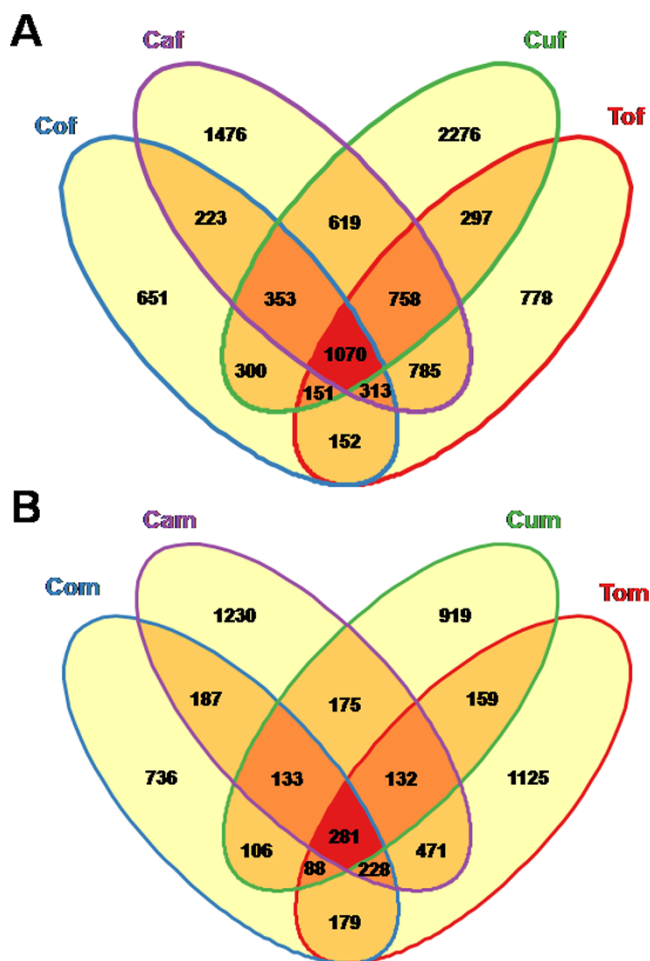
**Global expression profiles between sexes, and sex biased genes.** To specifically identify sex-biased genes that might also affect the sex difference process, we performed a series of expression profiling to examine gene activity changes between sexes. By comparing female to male, differentially expressed transcripts (DETs) ( $q\text{-value} < 0.05$  and  $\log_2(\text{Fold change}) > 1$  or  $\log_2(\text{Fold change}) < -1$ ) were generated in four host strains (Table S4). This resulted in 8,434 differentially expressed transcripts between Caf and Cam, 7817

between Cuf and Cum, 6967 between Tof and Tom and 5151 between Cof and Com (Table S4). These results suggested that majority of the differentially expressed transcripts between sexes were female-biased. To reduce the DETs between female and male whiteflies, intersection elements of female (Figure 2A) and male (Figure 2B) specific genes were determined. Among them, 1070 female and 281 male specific genes were annotated (Table S1 and S2). The analysis of the transcriptome dynamics was shown in Figure 3. Both the female and male biased genes were enriched with members of distinct KEGG pathways and GO categories. Pathway enrichment tests revealed that pyrimidine metabolism, ribosome, mRNA surveillance pathway, ribosome biogenesis in eukaryotes, RNA polymerase, RNA degradation, DNA replication and RNA transport were enriched in female whitefly. In the case of male whitefly, starch and phototransduction, lysosome, nitrogen metabolism, glycerophospholipid metabolism and phagosome were enriched (Figure S1 and S2). The enriched GO analysis for female and male biased genes were shown in Figure S3, which indicated that many enriched GO categories in female included only extracellular region while structural constituent of cuticle and hormone activity were predominant in male (Figure S3).

**qRT-PCR validation.** Only 52% (33/64) of the genes exhibited significantly different expression profiles between female and male whiteflies (Table S6). For the 48 female enriched genes, 18 of them were over-expressed in the female compared to male (expression



**Figure 1 | Pairwise correlation between all of the samples based on the Spearman correlation coefficient.** Based on the read counts measurements, the heatmap exhibits a hierarchical clustering of 8 transcriptome samples (sex\*host plant). The inserted color key depicts the value of Spearman correlation coefficient, ranging from green (normalized expression of  $-1$ ) to red (normalized expression value of  $1$ ).



**Figure 2** | Venn diagram demonstrating sex-specific gene expression in *B. tabaci*. (A). Female specific gene expression in different host plants, including cabbage (Caf), cotton (Cof), cucumber (Cuf), and tomato (Tof). (B). Male specific gene expression in different host plants, including cabbage (Cam), cotton (Com), cucumber (Cum), and tomato (Tom).

ratio of female/male  $> 1$  and significant based on *t* test), whereas 15 out of 16 were over-expressed in the male compared to female. The comparative analysis of qRT-PCR and RNA sequencing of selected sex enriched genes revealed that the qRT-PCR results were, for the most part, consistent with the sequencing analysis results, especially to the male enriched genes. For instance, among the differentially expressed genes from qRT-PCR analysis, 85% (28/33) exhibited significant difference at a fold change of above 2 (female enriched genes) or below 2 (male enriched genes) (Table S6).

**Characterization of *Bt-tra2* gene.** Two different splice variants (*Bt-tra2*<sup>266</sup> and *Bt-tra2*<sup>143</sup>) were identified that were not sex-specific in embryos, larvae or adults by verifying their amplicons in all developmental stages by sequencing (Figure 4A, 4B). High expression abundance in eggs and L4 compared to L1-L2, L3 and adults stages was also found by semi-quantitative transcriptional profile analysis of *Bt-tra2* transcripts at different developmental stages (Figure 4B). Because of evolutionary constrain in RRM (RNA binding domain or RNA-recognition motif), we only unambiguously aligned the amino acid sequences with those of hymenopteran (*N. vitripennis*, *A. mellifera* and *A. echinior*), lepidopteran (*B. mori*), coleopteran (*T. castaneum*) and dipteran (*D. melanogaster*, *L. cuprina* and *M. domestica*) that shared structural features for RRM domain in above insects (Figure 4C). Results indicated that the RRM amino acid sequence diverges in

relation to phylogenetic distance. The RRM domains of the hymenopterans (*A. mellifera*, *N. vitripennis* and *A. echinior*) show a pairwise sequence identity of 81 to 85%, and 68 to 80% for the dipterans (*L. cuprina*, *M. domestica* and *D. melanogaster*), whereas RRMs of *B. tabaci* and hymenopterans or dipterans have pairwise sequence identity of 70 to 78%, and 61 to 68%, respectively. Within the RRM of *Bt-Tra2* protein, there are two putative RNP (ribonucleoprotein consensus peptide) elements (Figure 4C), which can directly interact with the *dsx* pre-mRNAs in *D. melanogaster*. In comparison to *D. melanogaster*, three amino acid differences were identified in the eight-amino acids-long RNP1 sequence element of *B. tabaci*, whereas, only one amino acid differences exhibited in the six-amino-acid long RNP2 sequence element.

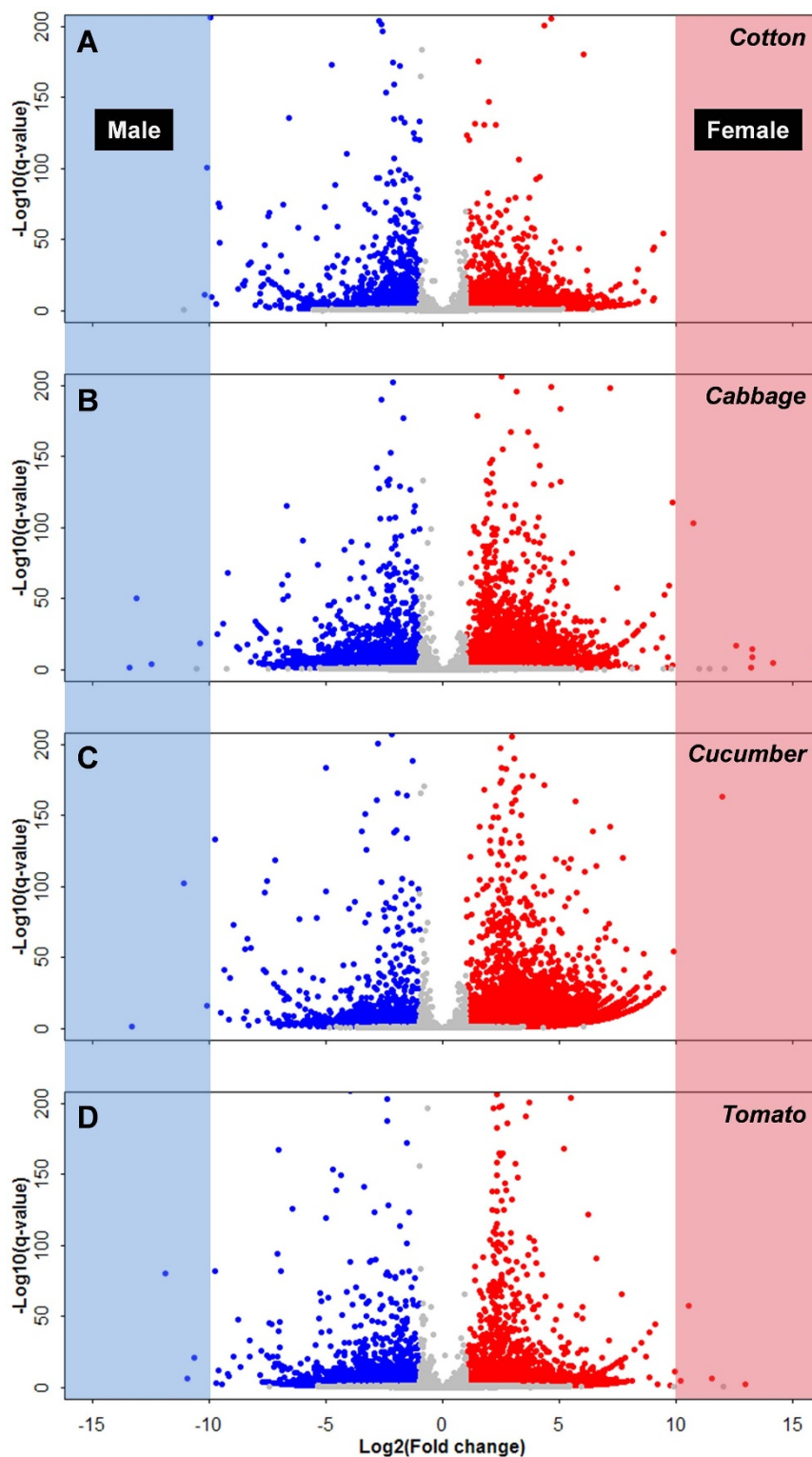
## Discussion

In this study, eight paired end libraries, each containing an average of 42 million reads, were constructed individually using the Trinity method<sup>48</sup>, representing the largest *de novo* transcriptome assembly in whiteflies (27 Gb)<sup>49–51</sup>.

Sexual dimorphism represents phenotypic differences between males and females of the same species. Differences between males and females can be resolved by the evolution of differential gene expression in the two sexes<sup>5</sup>. Features of *B. tabaci* make it particularly interesting for a comparative study of sex-biased genes. *B. tabaci* just like thrips (Thysanoptera), beetles (Coleoptera) and bees (Hymenoptera), have haplodiploid sex determination; males are haploid, develop from unfertilized eggs and only inherit a maternal genome, whereas females are diploid, develop from fertilized eggs and inherit a paternal and a maternal genome. Thus in *B. tabaci*, we expect to find the patterns of sex-biased gene expression common or specific to the other arthropods. Previous experiments have found that a large portion of the transcriptome is differentially expressed between the sexes<sup>11,14,54,55</sup>. However, most of these studies have focused on model organisms such as *Drosophila*, *An. gambiae* and *T. castaneum*, with few studies on non-model species due to the lack of genome sequences.

Our comparative transcriptome analyses result in 1351 sex biased genes, in which 1070 are female specific and 281 are male specific. It is worth noting that without replications, the intersection of four experiments involving gender and host plants was fairly conservative in detecting the differentially expressed genes. Nevertheless, we do find a large number of genes to be female-biased, which is consistent with *Tribolium castaneum*<sup>14</sup> and *Anopheles gambiae*<sup>13</sup>. In contrast, Eads et al (2007)<sup>15</sup> reported that majority of the sex-biased genes were male specific in *Daphnia*. The discrepancy reflects the species-specific nature of gender-associated genes. On the other hand, the intrinsic genetic differences between haploid male and diploid female might contribute to such inconsistency as well. Recent studies in other haploid/diploid organisms, including *Nasonia*, beetles and honeybee<sup>44–47</sup>, indicate that the *transformer (tra) -doublesex (dsx)* axis is conserved among all insects and plays a pivotal role in sex determination mechanisms.

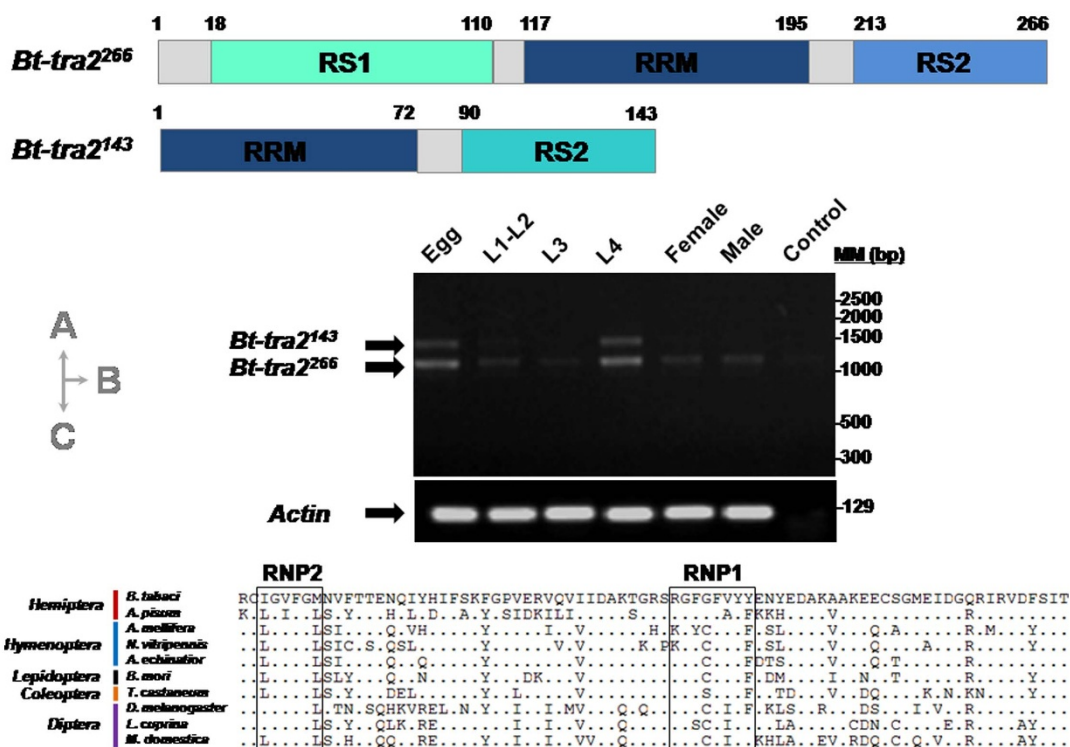
Functional categories enriched in female *B. tabaci* were associated with translation, pyrimidine metabolism, ribosome function and DNA replication. Previous report indicated that proteins involved in translation and DNA packaging were predominantly female-biased in expressions among flies<sup>9</sup>, mosquitoes<sup>11</sup> and *Daphnia*<sup>15</sup>. One of the enriched functional categories in *B. tabaci* is the structural constituent of cuticle, which is consistent with a previous report in *Daphnia*<sup>15</sup>, suggesting that structural difference (e.g., cuticle composition) and increased cuticle metabolism in males might be a result of gender differences. Previous report indicated that diet could affect lifespan and reproduction, and favor different type of nutrient intake in males and females<sup>56,57</sup>. During starvation, neurons from males are readily undergoing autophagy and dead, whereas neurons from females mobilize fatty acids, accumulate triglycerides, form lipid



**Figure 3** | Volcano plot illustrating *B. tabaci* gender differences at transcription level. The x-axis represents the relative expression of transcript for female and male *B. tabaci*, respectively. The color-shaded regions depict significantly female-biased genes [red,  $\text{Log}_2(\text{fold change}) > 1$ ,  $q\text{-value} < 0.05$ ], and male-biased genes [blue,  $\text{Log}_2(\text{fold change}) < -1$ ,  $q\text{-value} < 0.05$ ] among cotton (A), cabbage (B), cucumber (C), and tomato (D).

droplets, and survive longer<sup>58</sup>. Differential immune responses between the sexes were also observed in an autumnal moth, *Epirrita autumnata*<sup>59</sup>. In whiteflies, functional categories such as nutrient-related (nitrogen metabolism and glycerophospholipid metabolism) and immune-related (lysosome and phagosome) pathways were enriched in males.

The accuracy of the corresponding genes of most enriched pathway and GO terms was confirmed by the qRT-PCR analysis, with 52% of the selected ESTs showed significant differences. Similar to other microarray or RNA-seq analysis, this method tends to generate false positives. Consequently, results from both analyses need to be validated by qRT-PCR. Whitefly female and male biology difference



**Figure 4** | Characterization of *Bt-tra2*, a putative sex determination gene in *B. tabaci*. (A). The schematic drawing shows structural features of the two predicted *Bt-tra2* isoforms (RS, arginine/serine rich domain; RRM, RNA binding domain). (B). Semi-quantitative transcriptional profile of *Bt-tra2* transcripts throughout different developmental stages, including L1–L2 (1–2<sup>nd</sup> instar larvae), L3 (3<sup>rd</sup> instar larvae), L4 (4<sup>th</sup> instar larvae), female and male adults. The *actin* was used as a loading control. (C). The phylogenetic relationship of Tra2 RRM domains among different insect orders, including Hemiptera (*B. tabaci* and *Acyrtosiphon pisum*), Hymenoptera (*Apis mellifera*, *Nasonia vitripennis*, and *Acromyrmex echinator*), Lepidoptera (*Bombyx mori*), Coleoptera (*Tribolium castaneum*), and Diptera (*Drosophila melanogaster*, *Lucilia cuprina*, and *Musca domestica*). Dots indicate amino acids identical to the predicted *Bt-Tra2* RRM of the whitefly. RNP sequence elements (RNP1 and RNP2) are highlighted in boxes.

were obvious in previous report including some virus transmission and biological characters and so on, but seemly are not distinctly and necessarily associated with sexual divergence in this study. The public release of the assembled whitefly genome, combined with genetic mapping studies, should provide new insight into the roles of sex-biased genes.

Genetic regulation of sex determination was focused initially on model insects *D. melanogaster* and *Caenorhabditis elegans*<sup>42</sup>, and recently expanded to other insects including Hymenoptera (*A. mellifera* and *Nasonia*) and Coleoptera (*T. castaneum*)<sup>46,47,60,61</sup>. In most insects, the sex-determining pathway consists of the basal switch *doublesex* that is acting as a conserved major switch at the downstream of the sex-determining cascade<sup>62</sup>. In *Drosophila melanogaster*, protein-protein interactions between the female-biased *transformer* (*tra*), the splicing regulator of *doublesex*, and *transformer2* (*tra2*), a non-sex-specific auxiliary splicing factor, are essential to promote female sexual differentiation<sup>63</sup>. In hemipterans, such as aphid and whitefly, regulation mechanism of sex determination are largely unknown. In this study, a gene encoding *transformer2* was cloned from the whitefly (*Bt-tra2*) and its expression profiles were documented. Not surprisingly, *Bt-tra2* did not display any detectable sexual dimorphic differences in expression or splicing patterns (Figure 4). Sex-specific splicing of *tra* as an important regulatory mechanism was described in the fruitfly, wasp<sup>46</sup> and red flour beetle<sup>47</sup>. However, non-sex-specifically spliced *tra2* from honeybee also act as an essential regulator of the sex determination hierarchy<sup>45</sup>. In addition, sex-specific *doublesex* (*Bt-dsx*) was also found and cloned from our other EST database (unpublished data). Functional characterization studies are currently underway to test the hypothesis that *Bt-tra2* affects embryonic viability and female splicing of *Bt-dsx* transcripts. In addition, a recent report showed that

endosymbionts can actively manipulate the sex determination mechanism of their host, *Ostrinia scapularis*<sup>64,65</sup>. This discovery can shed new light on the sex determination mechanism of lepidopteran insects and other herbivores. Thus, in the near future, a study may also be focused about the *Bt-tra2* splicing patterns in females with different types and densities of the bacterial symbionts.

## Methods

**Insect rearing and sample preparation.** The four *B. tabaci* host strains (cabbage, cucumber, tomato and cotton) were the same populations as described previously<sup>66</sup>. For sample collection, only 2-day-old adults were collected from each host strain using a glass tube (5 × 0.5 cm) and the sex was determined under a stereo microscope. Then adults of the same sex were pooled into a plastic tube using an aspirator. Finally, these different host strain female and male samples were respectively collected, snap frozen in liquid nitrogen, and transferred to a –80°C freezer for the long-term storage (Figure S4). Before sample collection, the populations had been completely separated and reared for at least five years, and all identified as B-type (the Middle East-Asia Minor 1) using mtDNA COI marker in the laboratory every 2–3 generations.

**RNA isolation, cDNA library construction, and illumina sequencing.** RNA from each female or male sample group was extracted with Trizol reagent (Invitrogen) according to the manufacturer's instructions, and purity and degradation were checked on 1% agarose gels. RNA integrity was further confirmed using the 2100 Bioanalyzer (Agilent Technologies) with a minimum RNA integrated number value of 8. Poly (A)-containing RNA was then separated from total RNA using the Dynabeads<sup>®</sup> mRNA purification kit (Invitrogen) and the quality was verified on a denaturing gel. Then, the eight paired-end cDNA libraries were all prepared using Illumina's protocols following manufacturer's recommendations and sequenced on the Illumina GAIPlatform. Briefly, the mRNA was fragmented into small pieces using divalent cations under elevated temperature and the cleaved RNA fragments were used for first strand cDNA synthesis using reverse transcriptase and random primers. This was followed by second strand cDNA synthesis using DNA polymerase I and RNaseH. These cDNA fragments were subjected to end repair process and ligation of adapters. These products were purified and enriched with PCR to create the final cDNA library.



**Sequence preprocessing, assembly, and functional annotation.** The 8 cDNA library was separately sequenced on the Illumina sequencing platform (GAII). The insert size of each library is approximately 500 bp and both ends of the cDNA are sequenced. Image deconvolution and quality value calculations were performed using the Illumina GA pipeline 1.3. The raw reads were filtered by removing adaptor sequences, low quality sequences (containing reads with unknown sequences 'N' and lower than 10 of mean phred scores). The reads obtained from each library were assembled separately, and all pooled reads of 8 libraries were also assembled using Trinity (version r2011-08-20) with default parameters<sup>48</sup>. To annotate the pooled assembly transcriptome, we performed a BLAST search against the non-redundant (NR) database in NCBI, SWISS-PROT, KEGG and COG with an e-value cut-off of 1e-5. Gene Ontology terms were assigned by Blast2GO<sup>67</sup> through a search of the NR database.

**Identification of reciprocal best matched sequences (RBMs).** With each library as an independent sample, identification pipeline of RBMs genes was done similar to the methods described in Wang et al. (2011)<sup>49</sup>. Briefly, the bidirectional best hit method (BBH) was considered in BLAST search (blastn, e-value cutoff is 1e-10) to identify genes that are putatively pairs of reciprocal best matched sequences between the pooled assembly and 8 separate assembly results. Pairs of sequences that were each other's best hit and longer than 200 bp were retained.

**Expression profiling pipeline.** *Gene expression value measurement and differentially expressed genes.* Based on the number of assembled genes, trinity pooled transcripts and raw reads for each sample were analyzed using RSEM software (v1.1.15)<sup>68</sup>. Second, in order to calculate the normalized read counts for each library, RNA composition bias and normalization factors were taken into account and calculated by edgeR<sup>69</sup>. Read counts for each sample is also used to calculate spearman correlation coefficient of two selected samples with *cor* function in R package, and based that heat map of spearman correlation coefficient for all samples was generated with *heatmap.2* in R package<sup>70</sup>. Then, the normalized count was directly used for subsequent differential expression gene analyses. DEGseq packages<sup>71</sup> were used to identify differentially expressed genes using MARS model (Q-value < 0.05 and Fold Change > 2)<sup>71,72</sup>.

**Enrichment analysis.** GOSeq (Version 1.8.0)<sup>73</sup> is used for GO enrichment analysis, which takes into account the gene selection bias due to difference in gene lengths and thus numbers of overlapping sequencing reads. Pathway mapping (used *Acyrtosiphon pisum* as KEGG organisms) and further KEGG enrichment analysis was carried out with local server KOBAS 2.0<sup>74</sup>. The Q-value < 0.05 is chosen as significant cutoff for enriched GO and pathway.

**Quantitative real time PCR (qRT-PCR) analysis.** *Sample preparation.* Female adult (Caf) and male adult (Cam) from cabbage strain were determined as described previously<sup>66</sup>. A total of 300 newly emerged and virgin Caf and Cam, respectively, were collected, snap frozen in liquid nitrogen, and stored at -80°C for the subsequent qRT-PCR analysis.

*qRT-PCR.* Total RNA was extracted from Caf and Cam, respectively, using Trizol (Invitrogen) following the manufacturer's protocols. The total RNA obtained was resuspended in nuclease-free water and the concentration was measured using Nanodrop (Thermo Scientific Nanodrop 2000). About 0.5 µg of total RNA was used as template to synthesize the first-strand cDNA using a PrimerScript RT reagent Kit (TaKaRa) following the manufacturer's protocols. The resultant cDNA was diluted to 0.1 µg/µl for further analysis in the qRT-PCR (ABI 7500) using an SYBR Green Realtime PCR Master Mix (TaKaRa).

qRT-PCR primers (Table S5) for the selected 64 unique genes, corresponding to the most enriched pathways, such as the pyrimidine metabolism in females (Figure S1) and the phototransduction in males (Figure S2), and the most enriched GO terms, including the structural constituent of ribosome, ribosome and translation in females, and the extracellular region, structural constituent of cuticle and hormone activity in males (Figure S3), were designed using the Primer Express 2.0 software. The cycling parameter was 95°C for 30 s followed by 40 cycles of 95°C for 5 s and 62°C for 34 s, ending with melting curve analysis (65°C to 95°C in increments of 0.5°C every 5 s) to check for nonspecific product amplification. Relative gene expression was calculated by the 2<sup>-ΔΔC<sub>t</sub></sup> method using housekeeping genes *RPL29*<sup>75</sup> as the reference to eliminate sample-to-sample variations in the initial cDNA samples.

**Characterization of *Bt-tra2*.** To determine the entire sequences of the *Bt-tra2* transcripts, we analyzed in detail the EST sequence (annotated transformer-2 sex-determining protein in Table S1) from the female biased genes relative to male. Based on this EST, combined with other insect EST annotated *tra2* in NCBI, the complete open reading frame (ORF) sequence of *Bt-tra2* was cloned and both-strands sequenced. We translated the mRNA sequences into the amino acid sequence, and we predicted the protein domains by the similarity to domains in the PROSITE database (<http://www.expasy.org/prosite/>). The GenBank accession numbers are KC333625 (*Bt-tra2*<sup>266</sup>) and KC333626 (*Bt-tra2*<sup>143</sup>).

***Bt-tra2* distribution throughout development.** Total RNA was extracted from male and female eggs, larvae (L1–L2, L3 and L4 instar larvae), female and male adult. We amplified cDNA fragments using oligonucleotides primers (forward primer, 5'-CCCCACTGAATCCTGTT-3' and reverse primer,

5'-CGGTAATGAGTGGAGAAGA-3') that span the complete open reading frame (ORF) of these two *Bt-Tra2* splice variants. The identity of the amplicons of all developmental stages was verified by sequencing.

**Phylogenetic analysis.** We utilized nine *Tra2* amino acid sequences from different insect species with ClustalW alignment through Molecular Evolutionary Genetic Analysis software version 5 (MEGA 5) to compare the phylogenetic and molecular relationships: *Acyrtosiphon pisum* (GenBank: XP\_001944825), *Apis mellifera* (GenBank: NP\_001252514), *Nasonia vitripennis* (GenBank: XP\_001601106), *Acromyrmex echinator* (GenBank: EGI70155), *Bombyx mori* (GenBank: NP\_001119709), *Tribolium castaneum* (GenBank: XP\_968550), *Drosophila melanogaster* (GenBank: NP\_476765), *Lucilia cuprina* (GenBank: ACS34688), *Musca domestica* (GenBank: AAW34233).

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## Author contributions

Conceived and designed the experiments: W.X., L.T.G., X.G.Z. and Y.J.Z. Performed the experiments: W.X. and L.T.G. Analyzed the data: W.X. and L.T.G. Contributed reagents/materials/analysis tools: W.X., L.T.G., X.G.J., N.N.Y., X.Y., Q.J.W., S.L.W., X.G.Z. and Y.J.Z. Wrote the paper: W.X., L.T.G., Q.J.W., S.L.W., X.G.Z. and Y.J.Z. All authors reviewed the manuscript.

## Additional information

**Data Deposition** The Illumina reads of *Bemisia tabaci* of eight libraries were submitted to the NCBI Sequence Read Archive under the accession number SRA050082.1-SRA050089.1.

**Supplementary information** accompanies this paper at <http://www.nature.com/scientificreports>

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