Display female-specific doublesex RNA interference in early generations of transformed oriental fruit fly, Bactrocera dorsalis (Hendel)

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Abstract

BACKGROUND: The oriental fruit fly, Bactrocera dorsalis (Hendel), is one of the most destructive pests in many Asian countries. An effective strategy to reduce fly density in the field is urgently required. Recently, the doublesex of B. dorsalis (Bddsx*) has been cloned, and RNA interference (RNAi) indicates that it can reduce the offspring in vitro. In this study, a piggyBac-based construct that generates short hairpin RNA (shRNA) against the female-specific region of Bddsx was introduced into the pest to test the RNAi effects on reproductive functions in vivo.

RESULTS: After embryonic injection and backcross, 21 transgenic lines with germline transformation were identified. Genomic DNA analysis showed that the exogenous transgene including short hairpin Bddsx and a DsRed marker had integrated into the genomes of 11 transformed lines. Northern blot analysis indicated the presence of Bddsx transcript but also repressed expression of the downstream yolk protein gene (Bdyp1).

CONCLUSION: The results clearly indicate that RNAi is heritable through the expression of specific siRNA in early generations of transformed oriental fruit fly. These results can broaden the understanding of sex-related developmental mechanisms in the fly, and also offer a possible molecular approach for pest control in the future.

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Keywords: doublesex gene; Bactrocera dorsalis; RNA interference; short hairpin RNA; reverse transcription real-time PCR

1 INTRODUCTION

Bactrocera dorsalis (Hendel) is widespread in many Asian countries and has a broad host range of more than 150 fruit crops. After the female fly oviposits through the peel, the hatched larvae cause severe damage to fruits and lead to huge economic loss to farmers. Owing to the ability of this insect to adapt readily to its environment and its high reproductive potential, many pest management techniques, including methyl eugenol/protein baiting, the eradication-based sterile insect technique and insecticide spraying, have been applied in Taiwan over the past few decades, but these methods are unable to control the pest effectively. As the number of female flies is the major concern in pest management, it is crucial to develop a novel control technique to target the female flies.

Functional studies on the sex determination of dipteran insects were performed first in Drosophila melanogaster Meig. In D. melanogaster, the X/autosome ratio, Sex-lethal (Sxl), transformer (tra), transformer-2 (tra-2), doublesex (dsx) and fruitless (fru) in a cascade form the sexual determination hierarchy. In the regulatory process, the Sxl is the key switch on sex selection.1–3 However, the isolation of Sxl genes of Musca domestica and Ceratitis capitata breaks the rule, because Ccsxl is expressed equally in both sexes.4 Subsequently, the Cctra gene has been demonstrated to be the key gene for sexual fate determination.5 Because of the highly diversified sex fate determination in different dipteran insects, several upstream regulators have been proposed, involving an unidentified dominant male determinant M, which controls the expression of the key gene F.6,7 A general model for sex determination in tephritidae has also been reported in a study regarding a novel function performed by a Cctra-2 homologue.8 Recently, the F gene of housefly has been identified and characterised.9 In spite of the sex determination system diversity among dipteran insects, dsx are evolutionarily conserved both on structure and function.10 Both doublesex proteins (DSXs) are transcriptional regulators that derive from the same dsx gene

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doublesex RNA interference in B. dorsalis

as a result of sex-specific alternative splicing.\textsuperscript{11} They function as a double switch at the bottom of the sexual determination hierarchy to control somatic sexual differentiation.\textsuperscript{1} Structural analyses of \textit{dsx} homologues have been performed in many other species belonging to the Tephritidae family, such as \textit{C. capitata},\textsuperscript{12} \textit{Bactrocera tryoni},\textsuperscript{13} \textit{B. oleae}\textsuperscript{14} and \textit{Anastrepha} sp.\textsuperscript{15,16}

RNAi was first reported in \textit{Caenorhabditis elegans},\textsuperscript{17} and this post-transcriptional gene silencing technique has quickly become a powerful tool for function studies.\textsuperscript{18,19} Functional conservation of \textit{dsx} homologues has been shown by RNAi in \textit{Musca domestica}.\textsuperscript{10} Recently, the authors’ laboratory introduced \textit{in vitro} synthesised female-specific \textit{Bddsx} (Bddsxf\textsuperscript{7}) dsRNA to trigger sequence-dependent cleavage. The repressed expression of both the target gene and downstream yolk protein gene were observed.\textsuperscript{20} A single-dose dsRNA treatment can result in reduced reproductive ability and the appearance of a deformed ovipositor in treated female flies. This observation prompted the present authors to determine whether RNAi can be stably maintained through genetic manipulation and used as a novel genetic pest control approach.

Of four transposable elements available, \textit{piggYBac} has been successful in transforming \textit{B. dorsalis},\textsuperscript{21} and its broad host range makes it likely to become the most widely used system.\textsuperscript{22} In the present study, a \textit{piggYBac}-based vector was constructed for expression of \textit{Bddsxf}\textsuperscript{7} shRNA under the control of the U6 promoter. After microinjection and selection, several transformed lines were established. After screening and identification, integration of the transgene was confirmed by genomic PCR analysis, and northern blot analysis demonstrated the existence of small interfering RNA (siRNA). In addition, specific inhibition of the expression of the downstream yolk protein gene (\textit{Bdyp1}) and the significant reduction in offspring number in transformed fruit flies indicated the RNAi effects of the introduced transgene.

2 MATERIALS AND METHODS

2.1 Origin of insects

A laboratory strain of \textit{B. dorsalis} that had been raised in the laboratory for over 7 years was used in the experiments. The strain was kept at 28 °C in a 12:12 h light:dark photoperiod. Adult flies were housed in cages for mating and egg laying. Hatched larvae were cultured by periodic transfer to an artificial diet.

2.2 \textit{Bddsxf\textsuperscript{7}} shRNA expression vector construction

Based on the \textit{Drosophila melanogaster} U6 promoter sequence (GenBank accession number M24606), two primers, U6.2-\textit{F} (5′-TGGATATCTTGGTTCGACTTGCAG-3′) and U6.2-\textit{R} (5′-GTAAGCTTGAAGTATTGAGGAAAACATACC-3′), were designed and synthesised. After PCR amplification, a 415 bp corresponding to the \textit{B. dorsalis} U6 (BdU6) promoter (GenBank accession number FJ668652) was isolated and subcloned into the \textit{EcoR}\textsuperscript{V} and \textit{Hind} III sites of pBluescript II KS\textsuperscript{+} (pBluescript II KS\textsuperscript{+}-BdU6). To make the \textit{Bddsxf\textsuperscript{7}} shRNA, a 22 bp inverted repeat specific to \textit{Bddsxf\textsuperscript{7}} (GenBank accession number AFY669318, nt. 1039F to 1060F), separated by a small loop sequence, was constructed as shown in Fig. 1A. Quantities of 20 pmol of each oligonucleotide Bddsf\textsuperscript{7}-F(HindIII), 5′-TCAAGCTTCTGATCGAAGATCTCCGGTCTGTGTCGTGCC-3′, and Bddsf\textsuperscript{7}(ClaI), 5′-GCCCATGATAGGAACTGCGAAAAGAACACTCCGGCGGACGACACAC-3′, which contained the three underlined mutations, were annealed and used in the subsequent extension reaction including 1X EcoPol reaction buffer [10 mm Tris-HCl (pH 7.5), 5 mm MgCl\textsubscript{2}, 7.5 mm dithiothreitol, 0.2 mm concentration of dNTPs] and 1 U Klenow fragment (3′ → 5′ exo\textsuperscript{-}) (New England Biolabs, Inc., Beverly, MA). The reaction was incubated at 37 °C for 30 min, and the Klenow fragment was inactivated at 80 °C for 20 min. The Klenow extension step generated the sense-loop–antisense-terminal signal plus the corresponding cloning sites. Five units of Hind III and Cla I (NEB) were directly added into the reaction. After complete digestion at 37 °C for 2 h, the DNA fragments were purified by GeneClean\textsuperscript{R} III (QiBioGene, Solon, OH) and ligated into the pBluescript II KS\textsuperscript{+}-BdU6. Positive clones were confirmed by automated sequencing (DNA Sequencing Core Facility, Biotechnology Centre of National Chung-Hsing University).

To establish transgenic \textit{B. dorsalis}, pBac(PUB-DsRed), a \textit{piggYBac}-based vector with DsRed (a kind gift of Dr AM Handler), was used to construct the shRNA expression vector, pBac(U6-shdsxf\textsuperscript{7}). The \textit{EcoR}\textit{V}/\textit{Cla I} fragment of pBluescript II KS\textsuperscript{+}-BdU6-dsxf containing the sh\textit{Bddsxf\textsuperscript{7}} expression cassette was used to replace the \textit{Hpa I}/\textit{Cla I} portion of pBac(PUB-DsRed). pBac(U6-shdsxf\textsuperscript{7}) was used to perform the following transformation experiments.

2.3 Microinjection and transgenic line establishment

The procedure for embryonic injection was modified from the standard \textit{Drosophila} procedures\textsuperscript{23} and the \textit{B. dorsalis} transformation protocol.\textsuperscript{24} Newly laid eggs (less than 3 h old) were collected, rinsed several times with 0.02% Triton X-100, placed on double-stick tape on a glass slide and desiccated under room air for 8–10 min. The desiccated eggs were then dechorionated with the sharp tips of a pair of pincers. Subsequently, these eggs were lined up on the second glass slide and further desiccated for several minutes. The eggs were covered with a layer of Halocarbon 700 oil (Sigma-Aldrich, St Louis, MO) before injection. Injections were performed with mixed pBac(PUB-DsRed)-based plasmid and the transposase source, phBac helper (under 500:300 ng µg\textsuperscript{-1}), in injection buffer (5 mm KCl, 0.1 mm sodium phosphate, pH 6.8). After injection, the eggs were placed in a humidified tissue culture chamber at 28 °C, followed by heat shock treatment at 37 °C for 1 h within 2–3 h post-injection. The surviving larvae were collected and transferred to a larval diet after hatching. The G\textsubscript{0} and G\textsubscript{1} adults were backcrossed individually to wild-type flies. Each individual was moved into a separate box on day 8 and supplied with three wild-type flies for mating. On day 14 after eclosion, eggs were collected and counted. The emergence rate, sex ratio and morphological changes were checked and recorded periodically. The offspring number of each generation was counted and statistically analysed by one-way ANOVA. The pBac(PUB-DsRed) vector without modifications was also injected into embryos as a control.

2.4 Genomic DNA isolation and PCR analysis

Genomic DNA was isolated from the heads of the transformed G\textsubscript{1} fruit flies according to the method reported by Walsh et al.\textsuperscript{24} Briefly, each head was placed into 1.5 mL Eppendorf tubes containing 150 µL of 5% Chelex 100. After thorough vortexing, the tubes were incubated at 55 °C for 60 min and then boiled in a water bath for 8 min. Following centrifugation at 15 000 × g for 5 min, the precipitate containing the chelating resin and cell debris was discarded. Approximately 100 µL of the supernatant that contained the DNA was collected as a template for the subsequent PCR. Primers F620 (5′-CGAGCTCAATAATCCGAATCGAGGAG-3′) and Bddsf\textsuperscript{7}-R620 (5′-GAGACAGACACAGACGGGGAGATCTCC-3′) were used to confirm the presence of the BdU6 promoter and the inserted \textit{Bddsxf\textsuperscript{7}}.
Figure 1. Diagram of Bddsxf shRNA expression cassette construction and identification of transformed *Bactrocera dorsalis* flies with red fluorescence. (A) A pair of inverted repeats of the target sequence separated by a loop was synthesised by primer extension of the annealed primer set, Bddsxf-F(Hind III) and Bddsxf-R(Cla I). The DNA fragment with appropriate cloning sites was inserted into the position immediately after the U6 promoter in pBluescript II KS+; the EcoRV/Cla I fragment harbouring the U6 promoter and the Bddsxf shRNA expression cassette was used to replace the Hpa I/Cla I portion of pBac(PUb-DsRed) to generate pBac(U6-shdsxf). (B) The left panels show DsRed gene expression under the fluorescence microscope. (a) The G1 transformed fly (right) showed red fluorescence in various tissues compared with the wild-type fly (left). (b) A close view of the abdomen of a G1 female near the ovipositor; the left fly carrying the DsRed gene had bright fluorescence; the wild-type female is shown (right) as control. (c) The whole body of the transformed G1 larva gave very bright fluorescence, and the top wild-type control larva can be seen in the dark. (d) The pupae of transformed G1 (above) showed strong fluorescence expression in the whole body, where this is a signal of germline transformation. A wild-type pupa is shown below as a control. The panels on the right (a’, b’, c’ and d’) show the same samples under visible light.
2.5 Northern blot analysis for short interfering RNA detection

Total RNA from three female adults was isolated by TRI reagent (Molecular Research Center Inc., Cincinnati, OH) and purified with chloroform and ethanol. A quantity of 20 µg of total RNA was run on a 1.2% agarose gel. Lane 1 is a DNA size marker. Odd lanes show the transformed female (G1 number 24) that only had shdsxf transgenes. The results from lanes 8 and 9 also show the shdsxF - and DsRed -harbouring both shdsxf and DsRed. Lanes 2 and 3 show the transformed female (G1 number 21) harbouring both shdsxF and DsRed. Lanes 4 and 5 show the transformed female (G1 number 24) that only had DsRed (shdsxF had been lost). Lanes 6 and 7 are the results from an untransformed female fly as controls. Lanes 8 and 9 show that the transformed male (G1 number 3) had fully functional transgenes. The results from lanes 8 and 9 also show the shdsxF -lost male fly (G1 number 5). In the last two lanes, both shdsxF and DsRed were undetectable in a wild-type male, as expected.

2.6 Reverse transcription real-time PCR (RT real-time PCR)

Fat bodies dissected from abdomens of B. dorsalis were homogenised using TRI reagent following the manufacturer’s instructions. A quantity of 5 µg of total RNA was reverse transcribed by SuperScript reverse transcriptase II supplied by the SuperScript Preamplification System for First-Strand cDNA Synthesis kit (Invitrogen Corporation, CA). Real-time PCR was performed using the iCycler IQ system; primers were designed with Beacon Designer 7 (PRIMER Biosoft International, Palo Alto, CA), and reactions were performed in 96-well plates with a SYBR® GREENER® qPCR SuperMix for iCycler® (Invitrogen Corporation, CA). To eliminate the possibility of genomic DNA contamination, the primers designed for Bddy1 amplification expanded a 76 bp intron as an internal control. The protocol for cDNA amplification was one cycle of 50 °C for 2 min, one cycle of 95 °C for 8 min 30 s and 50 cycles of 95 °C for 15 s, then 57.5 °C for 1 min, followed by melting curve analysis to detect specific product amplification. The real-time PCR primers were as follows: Bddy1 forward primer (5′-TCAACACCTCAATCTCCATCGAC-3′, position 516–530 of Bddy1, Genbank accession number AF368053); reverse primer (5′-GATCACCAGGCCAACACCC-3′, position 919–930 of Bddy1); BddsxF forward primer (5′-TCTCACAATCACTCCATCGCC-3′, position 1464–1487 of BddsxF; Genbank accession number NY669318); reverse primer (5′-GTGGCAT TAACTCCATGCG-3′, position 1599–1618 of BddsxF); Bdactin forward primer (5′-AAGTCGTACCACTCTTTCAC-3′, position 2236–2256 of Bdactin; Genbank accession number L12254; He and Haymer, 1994); reverse primer (5′-ATCTGCGGTCACTACATACAC-3′, position 2323–2342 of Bdactin). Each sample was analysed in triplicate and normalised to the actin expression of B. dorsalis as internal control. Real-time data were collected by the Bio-Rad iQ5 2.0 Standard Edition Optical System Software v.2.0.

3 RESULTS

3.1 Microinjection and germ-line transformation identification

A transgenic approach was used to test whether BddsxF-specific dsRNA produced in vivo can lead to RNAi, as has been shown in vitro. The U6 promoter is an RNA polymerase III promoter that is generally used for U6 RNA synthesis and is suitable to produce shRNA in vivo. Based on the information on D. melanogaster, the corresponding promoter was successfully isolated, as the sequence similarity between the U6 promoter of B. dorsalis and D. melanogaster is over 99%. For in vivo expression of siRNA, an expression cassette including two inverted BddsxF-specific repeats separated by an eleven-nucleotide spacer and ending with five thyminides that serve as a RNA polymerase III terminator was generated by primer extension as shown in Fig. 1A. The authors chose 22 bases (nt. 1039F to 1060F of BddsxF gene) near the junction between the common and female-specific
4.70

<p>| Table 1. The integration of transgene in transformed G1 and their offspring after cross-back to wild-type flies |
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region as the target. Cleavage at the coding region can assure the loss of the regulatory function of BDDSXf. Although shRNA with complete homology usually has the most potent RNAi, constructing an expression vector carrying the 100% matched target sequence proved difficult. It is possible that the ‘palindromic’ structure of complete homology causes problems in subcloning and sequencing. As it has been reported that the introduction of G–U base pairs within the recognition sequence has little effect in both the *Drosophila* S2 cell system and mammalian cells, three mutations were introduced into the synthesised Bddsxf (ClaI) primer, and the expected plasmid was obtained after primer extension and subcloning. Subsequently, a piggyBac-based construct, pBac(U6-shdsxf), was generated by replacement of the *Hpa I/Cla I* portion of pBac(PUb-DsRed) by an *EcoR V/Cla I* fragment containing a Bddsxf-specific siRNA expression cassette. After transformation, the inserted transgene predicted to produce the RNA transcript was able to fold into a shRNA in vivo.

A total of 733 embryos were injected with the pBac(U6-shdsxf)/helper mixture; 156 larvae hatched and 55 emerged as adults. Among these surviving flies, five flies died before sex maturation, and the rest of the flies were individually backcrossed to wild-type flies. Only the transformed flies gave progeny with red fluorescence expression in their whole body at all developmental stages, suggesting germline transformation (Fig. 1B). A total of 21 G1 lines with germline transformation were observed, as shown in Table 1. Transformation experiments were also performed with the original piggyBac vector, pBac(PUb-DsRed), as a control. Of 548 embryos injected, six transformed flies were found, and they transmitted the DsRed transgene to their offspring. Offspring from the red fluorescence control line had no significant developmental, morphological or behavioural differences compared with wild-type flies. These results indicated that, as a screening marker, the DsRed protein is not harmful and is suitable for developmental studies of the oriental fruit fly.

3.2 Incorporation of the Bddsxf siRNA transgene in the genome represses female reproductive ability

To examine the integration of the *Bddsxf* siRNA transgene and the *DsRed* gene, genomic analysis was conducted. Two specific primer sets were used, and their locations are indicated in Fig. 2A. After PCR using genomic DNA from individual flies, 391 bp and 280 bp DNA fragments were the expected sizes for the *Bddsxf* siRNA transgene and *DsRed* gene respectively. In Fig. 2B, the flies with both inserted fragments, only *DsRed* insertion and without exogenous insertions can be clearly distinguished. In 12 transformed females (G1), eight female flies bore both *Bddsxf* siRNA transgene and *DsRed*, and the remaining four lines had only *DsRed* (Table 1). In the transformed males (G1), three lines carried both *Bddsxf* siRNA and *DsRed*, and the remaining six only had *DsRed* (Table 1).

Based on the above genotype results, the eight female lines carrying both *Bddsxf* siRNA and *DsRed* were individually backcrossed to three wild-type flies to examine whether there were any effects on reproductive ability. Comparing the number of progeny (G2) from the cross between a wild-type fly and a transformed female (G1), as expected owing to RNAi, the average number of offspring was significantly reduced to 36% compared with those of wild-type female flies. The bars on the histogram represent the standard deviation. The low P-values indicate significant differences among different flies.

**Figure 3.** Effect of *in vivo* RNAi on the average offspring numbers from transformed G1 flies. The relative number of G2 to wild-type females is indicated. *Bddsxf* RNAi indicates transformed flies harbouring pBac(U6-shdsxf), Bds-DsRed is transformed flies with pBac(PUb-DsRed) and wild type is untreated flies. In *Bddsxf* RNAi female G1 flies, their average offspring numbers only reach 36% compared with those of wild-type female flies.
Figure 4. Northern blot analysis for the detection of in vivo synthesised siRNA. After RNA isolation from three fat bodies, northern blot analysis was performed with a biotin-labelled probe, Bddsxf. Under denaturing conditions, only a 25 nucleotide signal appeared in the RNA sample from transformed Bddsxf RNAi flies, as shown in the middle lane, and full-length shRNA (55–60 nucleotides) was not detected. In the wild-type control, no signal was detectable. A quantity of 1 pmol of biotin-labelled T25 primer was used as a size marker (right-hand lane).

compared with the wild-type and pBac(PUb-DsRed) transformed control respectively (Fig. 3).

It was observed that the Bddsxf RNAi transformed female flies (G2) exhibited changes in mating behaviour and delays in egg maturation during the developmental process. Normal flies start to mate 6 days after eclosion, and eggs can be collected 2–4 days after mating. By contrast, the transformed flies showed delayed mating to 18–21 days after eclosion, and eggs required as long as 25 days for maturation instead of 8–10 days under normal conditions. These results are consistent with a previous in vitro Bddsxf RNAi study.20 The three Bddsxf RNAi transformed males obtained in this study gave various offspring numbers, as shown in Table 1.

3.3 In vivo synthesised Bddsxf siRNA inhibit Bddsxf and Bdyp1 expression

To determine the effect of Bddsxf RNAi more clearly, northern blot analysis was used to confirm the existence of in vivo synthesised siRNA. To enrich the effect of RNAi, the transgenic G2 flies carrying Bddsxf RNAi transgenes were self-crossed to obtain G3. Total RNA from G3 transformed flies were subjected to northern blot and RT real-time PCR analyses. Total RNA was separated on 15% denaturing polyacrylamide and transferred to a positively charged nylon membrane first. The biotin-labelled antisense probe, Biotin-Bddsxf portion and the loop, was used for siRNA detection. As shown in Fig. 4, a specific signal of approximately 25 nucleotides close to the size marker was observed. This result demonstrated the expression of the inserted Bddsxf siRNA transgene in the transformed flies. As no hairpin RNA molecule of 55–60 nucleotides was observed under denaturing condition, the authors believe that the shRNA was processed by Dicer after synthesis in vivo.

To evaluate the direct effects of the expressed siRNA on Bddsxf transcripts, total RNA was extracted from the fat body of G3 flies
from day 6, and RT-real-time PCR was performed. The present data indicated that the expression of the Bddsxf siRNA transgene prevents translation/stabilisation of the dsxf mRNA, lowering the amount of this mRNA in transformed flies (Fig. 5A). Three independent experiments showing similar results provided direct evidence that the RNAi could mediate the sequence-dependent repression of Bddsxf transcript in vivo. Consequently, the level-down of functional DSX protein led to the reduced expression of the downstream Bdyp1 gene (Fig. 5B). The observations provided additional evidence for the regulatory interaction between Bddsxf and Bdyp1.

4 DISCUSSION

To gain more information about the sex determination pathway, and to identify a novel strategy to control the oriental fruit fly, experiments were performed using both transgenic and RNAi approaches. RNA polymerase III promoters are usually used to synthesise short RNAs under natural conditions and give relatively higher transcriptional levels compared with RNA polymerase II promoters. When designing a suitable siRNA expression vector, the U6 small nuclear RNA promoter was the preferential choice. Therefore, the B. dorsalis U6 promoter was isolated and used for the subsequent transformation experiments. The present data demonstrated that the isolated U6 promoter was functional, and siRNA could be detected in the transformed flies (Fig. 4). Owing to the limited information about the optimised loop sequence within shRNA, the sequence containing the relatively effective loop 7 was used in this study and, it seems, functioned well in the given expression system.

From the present results it is clear that the Bddsxf RNAi effect can be stably maintained through germline transformation. Repressed reproductive activity and delayed egg maturation in transformed females both resulted from the inhibition of endogenous Bddsxf and Bdyp1 gene expression. These phenomena were quite similar to the effects of in vitro RNAi treatments. Less than half the G2 offspring (36 or 45%) (Fig. 3) show the adverse effects in the transformed females both resulted from the inhibition of endogenous Bddsxf and Bdyp1 gene expression. These phenomena were quite similar to the effects of in vitro RNAi treatments. 20 Less than half the G2 offspring (36 or 45%) (Fig. 3) show the adverse effects in the transformed females both resulted from the inhibition of endogenous Bddsxf and Bdyp1 gene expression. These phenomena were quite similar to the effects of in vitro RNAi treatments. 20

There were no expected deformed ovipositors, as shown in a previous in vitro study, or other morphological changes detected in the transformed flies. It was hypothesised that this might be for the following reasons. First, it is possible that a single high dose (1 μg per female) abdominal injection in vitro might dramatically reduce Bddsxf mRNA levels at a critical developmental point and lead to the formation of a deformed ovipositor. Second, the Bddsxf dsRNA (270 bp) used for abdominal injection is much longer than the in vivo synthesised shRNA. The long dsRNA is expected to be degraded by Dicer to generate various guide sequences for target mRNA degradation. It is reasonable that more guide sequences from the injected dsRNA can result in higher silencing effects on the Bddsxf mRNA compared with the relatively short shRNA. However, more experimental evidence is necessary before drawing any clear conclusions.

Unexpectedly, some G1 offspring carrying only the DsRed gene and without the Bddsxf shRNA transgene were detected. The loss of the shRNA transgene in several transformed lines indicated the occurrence of DNA recombination during germ cell formation. The results indicated that screening of transformed lines cannot solely depend on a marker gene, and genotype examination will be necessary to confirm the existence of the Bddsxf shRNA transgene. In addition, the stability of piggyBac-mediated transformation needs to be confirmed before practical application. In the mosquito species, piggyBac-mediated transgenes had no somatic or germline remobilisation. In contrast, D. melanogaster treated with the same piggyBac vector revealed somatic remobilisation. 31 The present authors will determine the transgene insertion sites and analyse the hetero- or homoygous status in order to assess the stability of the RNAi effects in oriental fruit fly.

Genes involved in the sex determination of Bactrocera pests have been suggested as the potential targets for developing new genetic control strategies. 2 Based on the results from this study, a potential application in B. dorsalis management through sequence-mediated RNAi may be possible. In theory, the female-specific region used in the study can only interrupt the mRNA of Bddsxf without a cleavage effect on the transcript of Bddsxf. 12 Three transformed male flies were collected in this study, and their backcross G2 progeny showed a variation from 0 to 136 (Table 1). G1 number 3 revealed normal reproductive ability with a compatible number of offspring close to the normal fly, as expected, but G1 number 18 was sterile. It is known that transgenic animals are typically weaker than wild-type animals owing to insertion mutations, and usually less fecund on average. Regarding the extreme case of G1 number 18, the insertion site of the exogenous transgene might be an interference to the normal gene function(s) that are essential for fertility.

In summary, several transformed lines of B. dorsalis have been successfully generated using a piggyBac-based vector. Insertion and expression of the Bddsxf shRNA transgene was confirmed by genomic DNA PCR and northern blot analysis. Specific repression of the Bddsxf and Bdyp1 transcripts owing to RNAi was demonstrated by RT real-time PCR experiments. The results clearly indicate that the Bddsxf RNAi effect can be detected in following generations after genetic transformation. The apparent changes, including mating behaviour, reproductive activity and delayed egg maturation in the transformed female, are RNAi related. These results are consistent with previous inhibitory effects of in vitro dsRNA injections.

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doublesex RNA interference in B. dorsalis