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ORIGINAL RESEARCH

Efficient and Specific Modifications of the *Drosophila* Genome by Means of an Easy TALEN Strategy

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ABSTRACT

Technology development has always been one of the forces driving breakthroughs in biomedical research. Since the time of Thomas Morgan, Drosophilists have, step by step, developed powerful genetic tools for manipulating and functionally dissecting the *Drosophila* genome, but room for improving these technologies and developing new techniques is still large, especially today as biologists start to study systematically the functional genomics of different model organisms, including humans, in a high-throughput manner. Here, we report, for the first time in *Drosophila*, a rapid, easy, and highly specific method for modifying the *Drosophila* genome at a very high efficiency by means of an improved transcription activator-like effector nuclease (TALEN) strategy. We took advantage of the very recently developed "unit assembly" strategy to assemble two pairs of specific TALENs designed to modify the *yellow* gene (on the sex chromosome) and a novel autosomal gene. The mRNAs of TALENs were subsequently injected into *Drosophila* embryos. From 31.2% of the injected F_0 fertile flies, we detected inheritable modification involving the *yellow* gene. The entire process from construction of specific TALENs to detection of inheritable modifications can be accomplished within one month. The potential applications of this TALEN-mediated genome modification method in *Drosophila* are discussed.

KEYWORDS: TALEN; *Drosophila*; Genomic modification; Unit assembly; Reverse genetics

1. INTRODUCTION

To modify the *Drosophila* genome (to knock-out/delete, insert, or replace a piece of DNA), scientists have been developing technologies that are feasible at the bench to manipulate the genomic sequence, a possibility that is becoming more and more important, particularly for systematic functional studies of the *Drosophila* genome. The techniques thus far available include transposase-mediated deletion from the genome (Voelker et al., 1984; Daniels et al., 1985), homologous recombination (HR)-based knock-out and knock-in techniques (Rong and Golic, 2000, 2001; Xu et al., 2009; Chen et al., 2010; Du et al., 2010), phiC31-integrase-mediated site-specific recombination-related techniques (Bischof et al., 2007; Gao et al., 2008; Markstein et al., 2008; Huang et al., 2009), and zinc-finger nuclease (ZFN)-based genomic-editing techniques (Bibikova et al., 2002; Beumer et al., 2006, 2008). These techniques have contributed significantly to the functional dissection of the *Drosophila* genome, but current reverse genetic manipulation techniques are usually very time consuming, laborious, and/or expensive (e.g., the commercially available artificial ZFN system) (Maeder et al., 2008; Pearson, 2008; Ramirez et al., 2008; Kim

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et al., 2010; DeFrancesco, 2011). In addition, P element insertion, HR-based gene modification, and integrase-based gene editing have target DNA sequence preference (Yu and Jiao, 2010). The limitations of the ZFN method also stem from the recognition by each ZFN module of only a triplet of nucleotides in the genome (Segal et al., 1999; Dreier et al., 2001, 2005; Beumer et al., 2006).

The transcription activator-like effectors (TALEs) are a family of proteins, first discovered in the plant pathogen *Xanthomonas sp*., which contain variable N- and C-termini but a conserved central domain for specific DNA binding (Schornack et al., 2006; Boch et al., 2009; Boch and Bonas, 2010). The DNA-binding domain consists of a variable number of tandem repeats of a 34-amino-acid monomer, which specifies the DNA-binding sequence by its 12th and 13th repeat-variable di-residues (RVDs, which specifically recognize a single nucleotide) (Boch et al., 2009; Moscou and Bogdanove, 2009; Bogdanove and Voytas, 2011). The molecular and structural characteristics of the DNA-binding domain of this class of proteins have made them applicable for generation of designable proteins that can modify the genome at both the genetic and the epigenetic levels by coupling the DNA-binding domain to an effector domain (Miller et al., 2011; Zhang et al., 2011; Deng et al., 2012; Mak et al., 2012). In the past two years, a series of studies has demonstrated that, by generating a customized DNA-binding domain fused with the *Fok* I nuclease, scientists can successfully modify any DNA sequence in a variety of experimental systems including human cells, rats, zebrafish, worms, and plants (Cermak et al., 2011; Hockemeyer et al., 2011; Huang et al., 2011; Miller et al., 2011; Sander et al., 2011; Tesson et al., 2011; Wood et al., 2011).

Here we report the first successful use of transcription activator-like effector nucleases (TALENs) to modify the *Drosophila* genome by generating small local indels (insertions and deletions, natural products of endogenous DNA repair of double-strand breaks in the genome) in specific target DNA sequences. We designed two pairs of TALENs that specifically recognize the *yellow* gene on the X chromosome and the *CG9797* gene on chromosome 3R. After injection of the mRNAs transcribed from the TALEN constructs *in vitro* into wild-type *Drosophila* embryos, efficiently inheritable modifications were revealed by genetic and molecular screens in the F_1 generations of both the *yellow* and the *CG9797* genes.

2. MATERIALS AND METHODS

2.1. Construction of the customized TALE repeats

The target DNA sequence selected for the left TALEN and the right TALEN to bind and the spacer DNA between the TALENs were predicted by TAL Effector Nucleotide Targeter software (Cermak et al., 2011). Several "rules" govern TALEN binding-site selection. 1) The length of TALEN-binding sequence should be 12−17 bp. Longer sequences are better but also entail more work and greater cost. 2) Binding sequences for the left and the right TALENs can be of different lengths. 3) The binding sequence usually starts after a T (not with a T) at the 5′ end. 4) The length of the spacer (between the left and right TALEN-recognition sequences) is usually 14−18 bp in length and may contain a restriction-enzyme cutting site (for molecular verification of the modifications). 5) For directed mutagenesis, the target site should usually be in the first 2/3 of the coding sequence, preferably in an important domain or at the intron-exon boundary. 6) The selected sequence should go through a "quality control" check at https://boglab.plp.iastate.edu/. For example, for *yellow*, 5′-GCCCCTATGCGGTAA-3′ was chosen as the spacer. The flanking sequences (always starting immediately after a T) 5′-ACCACCACTAATCCGT-3′ and 5′-GGTCAAGTCAAAGACAT-3′ (sequence complementary to 5′-ATGTCTTTGACTTGACC-3′) were then taken as the left and right TALEN binding sites.

The TALE repeats were constructed essentially according to the "unit assembly" strategy (Huang et al., 2011). Briefly, a tetrarepeat unit corresponding to "CCAC" was excised from the tetraunit plasmid pCCAC by a double digestion with *Spe* I and *Hin*d III before being ligated into an *Nhe* I- and *Hin*d III-linearized tetraunit, pACCA, to form the octaunit, pACCACCAC. Similarly a tetraunit, pTAAT, and a triunit, CCG, were ligated to form the heptaunit, pTAATCCG. Finally, the octa- and the heptaunits were joined to form the left TALE, pMD-TALE-yL. The right TALE repeats, pMD-TALE-yR, were assembled with pGGTC, pAAGT, pCAAA, and pGACA according to the same principle.

2.2. Construction of the TALENs and *in vitro* transcription

To generate the TALEN expression plasmids pCS2-TALEN-yL and pCS2-TALEN-yR, again for modifying *yellow*, we cut the left and right TALE sequences from the plasmids pMD-TALE-yL and pMD-TALE-yR, respectively, with a *Spe* I/*Nhe* I double digestion and subsequently cloned them into the *Nhe* I-linearized vectors of pCS2-PEAS and pCS2-PERR, both of which contain a similar, but not identical, *Fok* I coding sequence.

For *in vitro* transcription of these TALENs, pCS2-TALEN-yL and pCS2-TALEN-yR were linearized with *Not* I and recovered as corresponding transcription templates. The transcription was carried out with a Sp6 mMESSAGE mMACHINE Kit (Ambion, USA). The capped mRNAs encoding the left and the right TALENs for the *yellow* gene were purified before microinjection into the *Drosophila* embryos.

2.3. Germ-line transformation and modification analysis

The *in vitro* transcribed left and right TALEN mRNAs were mixed to a final concentration of about 0.5 mg/mL for microinjection according to standard methods (Song et al., 2007; Xu et al., 2009; Huang et al., 2010; Liu et al., 2011).

For modification of the *yellow* gene, the mixed mRNAs were injected into w^{1118} embryos (F_0). The F_0 adults were singly crossed to the *yw* flies of the opposite sex to produce the

F_1. The male F_0 parents were used to screen for mosaic *yellow* phenotypes and the adult *yellow* phenotype-scoring results were used for evaluation of somatic targeting efficiency.

For further analysis, the male F_0 that produced yellow female F_1 and the female F_0 that produced yellow progeny were both scored as indel-yielders. The genome of the yellow male F_1 produced by the female F_0 was extracted and sequenced to confirm the indels.

For *CG9797*, the process was similar to that for *yellow*, except that the spacer containing *Bsp1286*I site was chosen for the convenience of molecular identification of indels (Fig. S1), in addition, the F_0 and F_1 were crossed to TM6B,Tb/TM2,y^+ double balancer flies (for details see Fig. 1C), and the mRNAs were injected into *yw* embryos. From each F_0 single cross, 5−6 progeny flies were taken for F_1 single crosses.

3. RESULTS

3.1. Essential principle and protocol of TALEN-mediated genome modification in *Drosophila*

In Fig. 1, we show schematically the principle and brief protocol of our TALEN strategy. Essentially, modification of a specific sequence in the *Drosophila* genome requires design of a pair of TALENs according to the local sequence. A single TALEN consists of an N-terminal domain that includes a nuclear localization signal; a central domain that is typically composed of 17.5 repeats, each of which differs at the RVDs (12th and 13th repeat-variable di-residues of the total 34 amino acids of each repeat) and thus recognizes a specific DNA sequence; and a C-terminal domain that is fused with a functional endonuclease *Fok* I (Fig. 1A). After assembly of the designed TALEN sequences and *in vitro* transcription, the TALEN mRNAs are injected into *Drosophila* embryos, where they are presumably translated into a pair of TALEN proteins. This pair binds to their targets, which are separated by spacer DNA (usually 14−18 bp). Their binding facilitates heterodimer formation of the attached *Fok* I endonucleases, which consequently cut in the spacer DNA region. The result is the desired double-stranded break (Fig. 1B). The break is repaired mainly through two pathways: nonhomologous end joining (NHEJ) and homologous recombination (HR) (when a proper template is available; Fig. 1B). During the repair processes, NHEJ largely produces small indels, whereas HR either precisely restores the wild-type sequence (e.g., when it uses the unbroken homologous chromosome as a template) or produces an artificial knock-in sequence when an artificial homologous template is provided. The genetic crossing scheme is shown in Fig. 1C (with the *CG9797* gene as an example). Flies from the injected embryos (F_0) are singly crossed to a double balancer stock. Some (5−6 from each of the F_0 single cross) of the F_1 flies are singly crossed to a double balancer to establish an F_2 stock, and after producing sufficient offspring, the F_1 flies are sacrificed for molecular identifications including enzyme digestion and sequencing for the possible indels. Recessive adult phenotypes including lethality can only be observed and assayed in the F_3 flies.

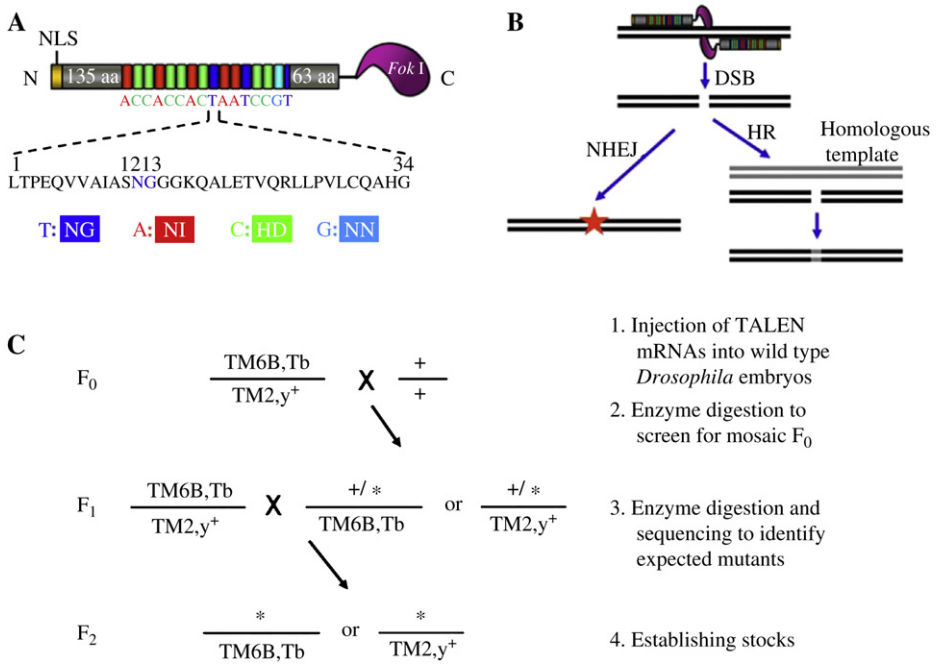

Fig. 1. Schemes of the principle and protocol of TALEN-mediated genomic modifications.
A: protein structure of the left TALEN that targets *yellow* with its binding sequence depicted below in 4 different colors. NLS, nuclear localization signal. Central repeats consisted of 15.5 repeats (naturally 17.5) of a 34-amino-acid unit (colored box) that differed in the 12th and 13th amino acids, which could be NG (recognizing T), NI (recognizing A), HD (recognizing C), or NN (recognizing G), for each repeat. *Fok* I endonuclease (purple) was fused to the C-terminal domain. **B:** double-strand breaks (DSBs) that resulted from the cut by *Fok* I were repaired either by nonhomologous end joining (NHEJ) to yield indels or by homologous recombination (HR) to restore the wild-type. The red star represents an indel. **C:** crossing scheme, with the legends to the right. F_0 (single crosses), injected, parental generation. F_1 (single crosses to establish), may carry a copy of the inheritable indel indicated by an asterisk (*). F_2, established stock.

3.2. Generation of null mutants of the *yellow* gene with customized TALENs

To establish the feasibility of TALEN-mediated genomic modifications in *Drosophila*, we chose to modify the easy-to-follow *yellow* gene, which is located on the 1st chromosome (X chromosome). The questions that we wished to address included: 1) Does the TALEN-mediated genomic modification work in flies as it does in other organisms such as mammals, fish, worms, and plants? 2) What is the efficiency with which inherited modifications are obtained? 3) Do males and females differ in this efficiency? 4) Are some sites in the genome (on different chromosomes) better targets than others?

The TALENs that we designed for modification of the *yellow* gene are shown in Fig. 2. A 16-nucleotide DNA sequence (boxed) immediately after a T was chosen for binding by the left TALEN and another 17-nucleotide sequence (boxed) that also follows a T in the 5′→3′ direction on the complementary strand was selected for binding by the right TALEN (Fig. 2A). A 15-bp DNA spacer separating the left and the right TALEN-binding sequences, is the region where the *Fok* I nuclease cuts. This TALEN region resides in the second exon of the *yellow* gene (Fig. 2A).

After injection of the mRNAs transcribed from the TALEN constructs into the w^{1118} embryos, subsequent crosses, and molecular identifications, we successfully obtained the expected mutations of the *yellow* gene. The results are shown in Fig. 2B and Table 1. Nine indels and the wild-type sequence are shown in Fig. 2B. The nine indels, which include both in-frame and frame-shift insertions and/or deletions, are from 15 sequencing samples, so some identical indels come from independent modification events. The modifications that occurred in the F_0 somatic cells can already be observed by the mosaic pigmentation in the abdominal region of the injected male flies (Fig. 3), although the inheritable indels did not necessarily arise from the F_0 that exhibited mosaic *yellow* phenotypes. The yellow patches are variable in size (Fig. 3B−D), and the wild-type flies do not have any yellow patches on the abdomen (Fig. 3A). In summary, 43.8% of F_0 males were somatic mosaics, as judged by *yellow* phenotype, whereas 17.2% of the F_0 males (injected) and 9.7% of the X gametes from F_0 males (as judged by yellow appearance in F_1 females) yielded germ-line indels.

To address the question whether the TALEN-mediated genomic modification technique is applicable to genomic sequences other than the *yellow* locus, we chose to modify a novel gene, *CG9797*, which is located on chromosome 3R. After the similar procedure used for *yellow*, but with TALENs specific to *CG9797*, we also successfully modified the genomic sequences of *CG9797*. As shown in Table 1, in F_0 male somatic cells, the frequency of modification events (as judged by loss of enzyme digestion, Fig. S1) is higher than that for *yellow* (Table 1). In the male germ lines, this difference in efficiency is even greater; with 66.7% of the injected *CG9797* F_0 males yielded TALEN-mediated alterations (Table 1), but in the F_0 females, we observed lower frequency of germ-line modifications in the *CG9797* locus than in the *yellow* locus (Table 1).

We have successfully established a TALEN-mediated genomic modification technique in *Drosophila*. The entire procedure, from the design of TALENs to identification of modification events, can be accomplished within one month.

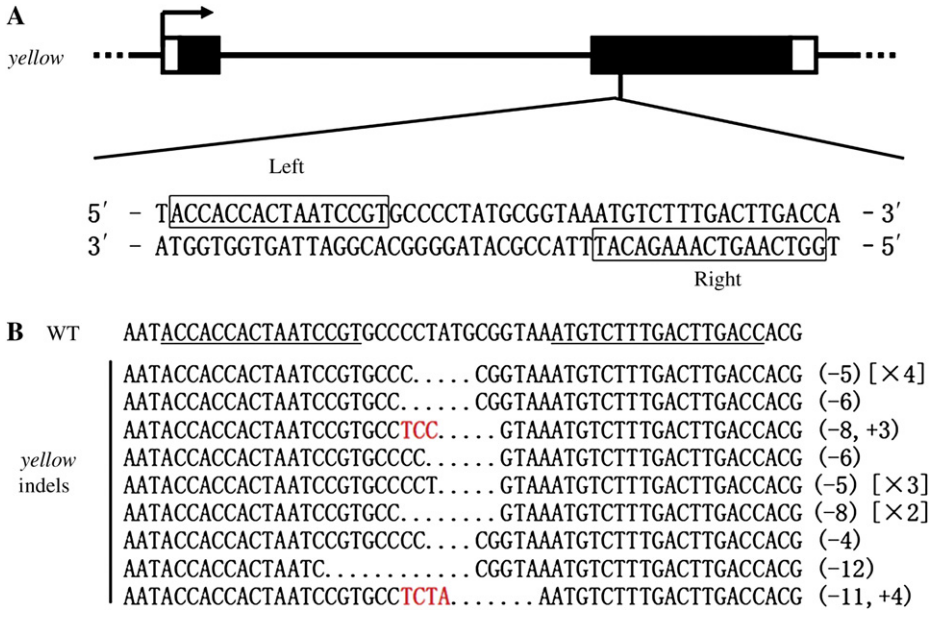

Fig. 2. Generation of null mutants of the *yellow* gene by TALEN-mediated genomic modifications.

A: genomic organization of the *yellow* gene. Boxes are transcribed regions, filled parts are coding regions. The turning arrow indicates the transcriptional start site. The sequences for our designed TALENs are shown below. Left, left TALEN-binding sequence. Right, right TALEN-binding sequence. **B:** wild-type (WT) sequence in the target region of the *yellow* gene and nine typical indels obtained from our TALEN-mediated genomic modifications. Deletions are indicated by dotted lines and inserted nucleotides are in red. $-n$ signifies deletion of n nucleotides, $+n$ insertion of n nucleotides. $\times n$ indicates detection of the same indel n times in our screen.

Table 1
Statistics of TALEN-induced genomic modifications of the genes *yellow* and *CG9797*

Gene	Sex of F_0	Indel-yielding F_0/total fertile F_0 (%)	Detected mutants/total F_1 progeny (%)	Mosaic F_0/total F_0 examined (%)
yellow	Male	10/58 (17.2)	60/622 (9.7)[a]	32/73 (43.8)[b]
	Female	23/51 (45.1)	175/424 (41.3)	—
CG9797	Male	10/15 (66.7)[c]	—	21/36 (58.3)[d]
	Female	10/29 (34.5)[c]	—	—

[a] The female progeny of F_1 were counted as total progeny; [b] Mosaic F_0 as judged by yellow patches on the abdomen region; [c] "Indel-yielding F_0" is the number of F_0 that bears a lethal mutation in the germ line; [d] mosaic F_0 were determined by enzyme digestion.

The frequency of modified genomic sequences in the germ-line cells is from 17.2% to 66.7%, independent of either the location of the genomic target sequences or the sex of the animals.

4. DISCUSSION

Our extension of the TALEN-mediated genomic modification technique to *Drosophila* is a big step for all *Drosophila* scientists toward the modern reverse-genetic studies in the post-genomic era involving high-throughput genomic modification techniques. Its advantages include the following: 1) It is the fastest, most efficient, and most specific method so far for modification of the *Drosophila* genome and permits modification of a large fraction, if not all, of the genome. 2) It permits tagging of endogenous genes when an appropriate homologous donor template DNA can be provided at the step of injection of TALEN mRNAs. Researchers can thus trace any proteins in a living organism, and express any exogenous genes in the same spatiotemporal pattern as a given endogenous target gene. 3) It has the advantage over other reverse-genetic mutagenesis methods that one can directly inject the TALEN mRNAs into an appropriate flippase recombination enzyme (FRT) stock and omit the step of combining a particular mutation with a FRT chromosome to make somatic clones. This ability is particularly useful for those genes that are difficult to combine with FRT chromosomes, usually because they are very close to centromeres. 4) Cell- or tissue-specific knock-out of particular genes are much more feasible when two FRTs are easily knocked in the start and stop regions of this gene through TALEN-mediated homologous recombination. 5) A specific TALE in combination with (fused to) a particular modification effector, such as a transcriptional-activation domain of a transcription factor or a chromatin-modification enzyme, can generate very useful tools for study of activities and/or functions of these effectors at the chromatin level. This ability could open a very important research path for epigenetic scientists (Huang and Jiao, 2012).

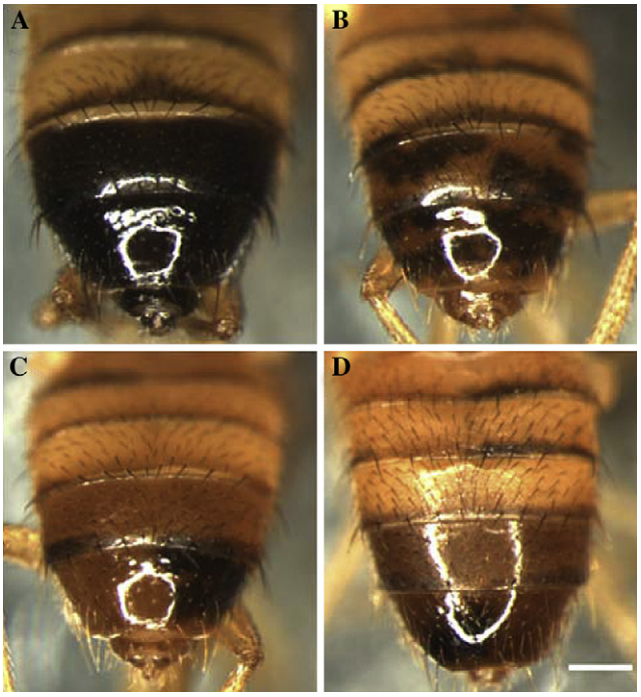

Fig. 3. Mosaic *yellow* phenotypes of the male F_0 that were injected with TALENs.
A: wild-type abdomen showing very dark (y^+) posterior region. **B–D:** abdomens of the male flies that were injected with TALENs directed toward *yellow*. The light-colored patches (variable in size) indicate the regions where, in the somatic cells, the *yellow* gene has been interrupted. Scale bar = 200 μm.

To make all these advantages practically useful, however, several remaining issues need to be addressed. 1) In the case of the modification of *yellow*, our results indicate that efficiency of generation of TALEN-mediated genomic modifications is lower in males than that in females, in contrast to reports of ZFN-mediated genomic modifications (Bibikova et al., 2002; Beumer et al., 2006, 2008). We do not yet know whether, in our examples, the DNA repairs leading to the final indels were all through NHEJ, producing the sex differences in modification efficiency. We nevertheless conclude, on the basis of our observations, that the efficiency of TALEN-mediated genomic modifications does not depend significantly on the location of the target sequence or the sex of the injected embryos. 2) To make the HR repair pathway more effective than the NHEJ pathway after the generation of double-strand breaks by the TALENs, for example, to promote knock-in efficiency, one might use NHEJ-defective fly stocks for the injection of TALENs. This is the basis for TALEN-mediated HR for modification of specific genomic targets. It is currently under investigation in our lab; we hope to optimize conditions for its efficient use in *Drosophila* by testing different forms (linear and circular, double-stranded and single-stranded, etc.) and different sizes (from tens of base pairs to several kilobases) of donor DNA in different genetic backgrounds (wild-type and pro-HR, e.g., ligase IV mutant, background).

Last, but not least, TALENs have at least the following advantages over ZFNs: 1) TALEN is more specific because it binds to about 16 nucleotides, whereas ZFN usually binds to 9. 2) TALEN is less toxic to the cells than ZFN, probably because ZFN may result in unspecific binding and subsequent cutting. Thus far, no apparent toxicity has been reported for TALEN in any model system. 3) The construction of TALENs by "unit assembly" strategy is easier than constructing ZFN. 4) Not all zinc fingers that recognize the 64 triplets have been well characterized, nor have all the combinations of different fingers been proven to work properly. In contrast, a designed TALEN should always work because, in principle, specific TALENs bind equally well to their corresponding targets. With TALEN, therefore, one should theoretically be able to choose any sequence in the genome as the modification target. 5) All units for the "unit assembly," used to construct customized TALENs, are freely available for academic researchers by means of a material transfer agreement with Peking University, China (forms available from Dr. Bo Zhang's laboratory).

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SUPPLEMENTARY DATA

Fig. S1. Molecular identification of the *CG9797* locus of injected F_0 somatic cells.

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jgg.2012.04.003.

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