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Targeted mutagenesis in the silkworm *Bombyx mori* using zinc finger nuclease mRNA injection

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ABSTRACT

Targeted mutagenesis is one of the key methods for functional gene analysis. A simplified variant of gene targeting uses direct microinjection of custom-designed Zinc Finger Nuclease (ZFN) mRNAs into *Drosophila* embryos. To evaluate the applicability of this method to gene targeting in another insect, we mutagenized the *Bombyx mori* epidermal color marker gene *BmBLOS2*, which controls the formation of uric acid granules in the larval epidermis. Our results revealed that ZFN mRNA injection is effective to induce somatic, as well as germline, mutations in a targeted gene by non-homologous end joining (NHEJ). The ZFN-induced NHEJ mutations lack end-filling and blunt ligation products, and include mainly 7 bp or longer deletions, as well as single nucleotide insertions. These observations suggest that the *B. mori* double-strand break repair system relies on microhomologies rather than on a canonical ligase IV-dependent mechanism. The frequency of germline mutants in G_1 was sufficient to be used for gene targeting relying on a screen based solely on molecular methods.

1. Introduction

The recent sequencing of several insect genomes for species of agricultural or medical interest, such as *Bombyx mori*, *Apis mellifera*, *Anopheles gambiae*, *Aedes aegypti*, and *Tribolium castaneum*, offers a wealth of genes potentially involved in key biological processes. Further progress in both basic and applied research is dependent on molecular and genetic methods, including transgenics and gene knockouts. Such technologies, initially developed for *Drosophila melanogaster*, are being gradually introduced and modified for other insects. Yet many methods remain to be adapted to non-drosophilid species.

The silkworm, *B. mori*, has been reared to produce silk for thousands of years. *Bombyx* genetics is well established with a great number of mutant strains and marker genes. Key molecular genetic methods have been successfully established for silkworm, including stable transgenesis of the germline (Tamura et al., 2000) targeted gene expression using the *GAL4/UAS* system (Imamura et al., 2003), and enhancer trap screening (Uchino et al., 2008). Several reports have involved RNAi gene silencing, including dsRNA injection into the silkworm embryo (Quan et al., 2002; Tomita and Kikuchi, 2009) and RNA hairpins expressed by recombinant Sindbis virus (Uhlirova et al., 2003) or in a *piggyBac* transgene (Isobe et al., 2004). Nevertheless, the knock-down of gene expression by RNAi has a serious limitation in the silkworm since gene silencing is incomplete. The absence of a general gene-targeting system to allow systematic reverse genetic studies has been a significant limitation in silkworm research. The first attempt to establish targeted mutagenesis in the silkworm was reported by Yamao et al. (1999), who induced a mutation into the *fib-L* gene by homologous recombination, using *Autographa californica nucleopolyhedrovirus* (AcNPV). However, the efficiency of this method was too low to be used as a standard technique.

Two methods of gene targeting were established for *Drosophila* by Rong and Golic (2000) and Bibikova et al. (2002). The first is based on a pair of site-specific DNA modifying enzymes from yeast, a recombinase and endonuclease that release a linear DNA fragment containing a modified sequence of the target gene in primordial germ cells. This method allows gene alteration by homologous recombination, but requires generation of transgenic fly strains expressing the yeast enzymes and a number of crossing steps to put a multitude of transgenes together. Thus the use of this technique in other insects has not been established yet. The other method is based on custom designed zinc finger nucleases (ZFNs), which are

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chimeric enzymes consisting of a zinc finger DNA recognition domain and the nonspecific nuclease domain of the Fok I restriction enzyme (Kim et al., 1996). This method allows a simple change in the target sequence by non-homologous end joining (NHEJ). Alternatively, specific alterations of the target region can be achieved by providing a donor plasmid with a mutated target sequence, which may recruit the homologous repair machinery. Whereas the original protocol describing ZFN mutagenesis also required transgenic flies and extensive crossing (Bibikova et al., 2002), a simplified variant of this method using direct embryo injection with mRNAs encoding ZFN was described recently in *Drosophila* (Beumer et al., 2008), zebrafish (Doyon et al., 2008) and rats (Geurts et al., 2009). This adapted method does not require ectopic expression of enzymes and avoids laborious genetic manipulations. The microinjected RNA is translated into a functional ZFN which induces double stranded breaks in a specific region of the genome. The free ends of the digested DNA then initiate a repair process, which can lead to mutations. As well, a plasmid with a mutated donor sequence can be coinjected to allow homologous recombination.

We attempted direct ZFN mRNA injection to *B. mori* embryos to evaluate the applicability of this procedure to gene targeting in the silkworm. The reported success rate of simple assembly of characterized triplet binding ZFN modules is relatively low (Ramirez et al., 2008), and targeted mutagenesis usually works only for one out of four targets (Kim et al., 2010). We chose three target sites residing in two *B. mori* epidermal color marker genes in order to offset this relatively low success rate. Both genes control the formation of uric acid granules in the larval skin, and mutations lead to the visible phenotype of translucent epidermis.

2. Materials and methods

2.1. Silkworm strains

A nondiapausing strain (*pnd*), which is *wt* for *BmBLOS2* and *Bmwh3,* was used in all of the experiments. The *w-3^{ol}* strain is mutant for *Bmwh3* and was used as a tester. The *pnd* and *w-3^{ol}* strains were from silkworm collections maintained at the National Institute of Agrobiological Sciences (Tsukuba, Japan) and the Graduate School of Agriculture, Kyushu University (Fukuoka Japan), respectively. The larvae were reared on an artificial diet (Nihon Nosanko, Yokohama, Japan) at 25 °C.

2.2. Target selection, DNA constructs

Two *B. mori* epidermal color marker genes, *BmBLOS2* and *Bmwh3*, were chosen for experiments and their coding regions were surveyed for the best available ZFN targets, preferably sequences close to $(NNC)_3N_6(GNN)_3$, using the "Zinc Finger Tools" program (Mandell and Barbas, 2006) available on the Carlos Barbas laboratory website (http://www.scripps.edu/mb/barbas/zfdesign/zfdesignhome.php). The candidate sites were assessed according to a table of DNA triplets (Table S1A,B and C) using the specificity evaluation reported by Carroll et al. (2006). Two sequences in the first and third exons of *BmBLOS2* and one sequence from the second exon of *Bmwh3* gene were selected and designated as targets BL-2, BL-1 and BW-1, respectively (Fig. 1 and S1, S4 and S7).

The coding regions of zinc-finger proteins that specifically bind the target gene sequence were designed and synthesized from corresponding oligonucleotides (Fig. S2-S9) combined by PCR using the method of Carroll et al. (2006). The procedure therefore included the assembly of Sp1C frameworks (consensus-based protein derived from the middle zinc finger of human transcription factor Sp1) with the ZF coding sequences using the 7-primer procedure and subcloning the resulting 294-bp DNA fragments into the pENTR-NLS-ZFN gateway vector (Desjarlais and Berg, 1992; Carroll et al., 2006). The vector also contained a nuclear localization signal (NLS) and a Fok I nuclease domain-encoding sequence (Carroll et al., 2008). For *in vitro* transcription, the resulting DNA fragments encoding the complete ZFN with NLS were transferred to the destination vector pCS2-DEST (Fig. S10) via the Clonase reaction (Invitrogen, Carlsbad, USA).

2.3. mRNA synthesis

Template plasmids for *in vitro* transcription were purified with a Qiagen HiSpeed plasmid midi kit (Qiagen, Germany), digested completely with the *Not I* restriction enzyme and subjected to *in vitro* transcription using an mMESSAGE mMACHINE kit with SP6 polymerase (Ambion, USA) according to the manufacturer's protocol. RNA was precipitated with LiCl, washed with 70% ethanol three times and air-dried.

2.4. Microinjection

Pairs of mRNA samples were dissolved at a concentration of 0.1 μg/μl for each RNA in 0.5 mM phosphate buffer (pH 7.0) containing 5 mM KCl. RNA concentration was measured by absorbance at 260 nm. RNA solutions of 3–5 nl were injected into silkworm eggs 4–8 h after oviposition, which corresponds to the syncytial preblastoderm stage. Injections were performed through the chorion as previously described by Tamura et al. (2000). The injection opening was sealed with instant glue (Aron Alpha, Konishi Co, Osaka) and the embryos were allowed to develop at 25 °C. Mutations of the target gene in epidermal cells were detected during the final larval instar as a mosaic of translucent and normal (opaque) skin of injected individuals.

2.5. The crossing scheme and screening strategy

Since the *BmBLOS2* gene is located on the *Z* chromosome, we took advantage of female hemizygosity (Fig. S11). In our screen, mutagenized G_0 males were crossed to wild-type females and G_1 females were checked for the *oily* phenotype (Fig. 2). Since the nonmosaic oily phenotype is well visible even in the first instar larvae, the cost of the screening was quite low.

The second gene used in this study, *Bmwh3,* is an autosomal mutation, which also causes the *oily* phenotype, for which a homozygous mutant tester strain *w-3^{ol}*/*w-3^{ol}* was available. In the pilot experiment to investigate the appearance of somatic mutations, we scored the fifth instar larvae of the microinjected heterozygous *w-3^{ol}*/+ individuals (Fig. S12A). For the detection of germline mutants, we scored the first instars of the F_1 progeny from a cross between the microinjected G_0 *pnd* individuals and *w-3^{ol}*/*w-3^{ol}* tester silkworms (Fig. S12B).

2.6. Sequence analysis

Genomic DNA of all mutant G_1 individuals was extracted using the Blood & Tissue Genomic DNA Extraction Miniprep System (Viogene, Sijhih, Taiwan) according to the manufacturer's instructions. About 30 mg of larval, pupal, or moth tissue were used per DNA extraction to obtain 3–15 μg of DNA. Fragments flanking the target region were amplified by PCR using primers 1F and 1R (Fig. 1). The PCR product was gel purified and sequenced using the same primers. Some DNA samples contained a larger deletion, and thus three sets of more distal primer pairs were used for PCR. All PCR reactions were carried out using Ex Taq DNA polymerase (Takara-bio, Kyoto, Japan). Sequencing reactions were performed with a BigDye terminator cycle sequence ready reaction kit version 3.1 and resolved on an ABI Prism 377 capillary sequencer (Applied Biosystems).

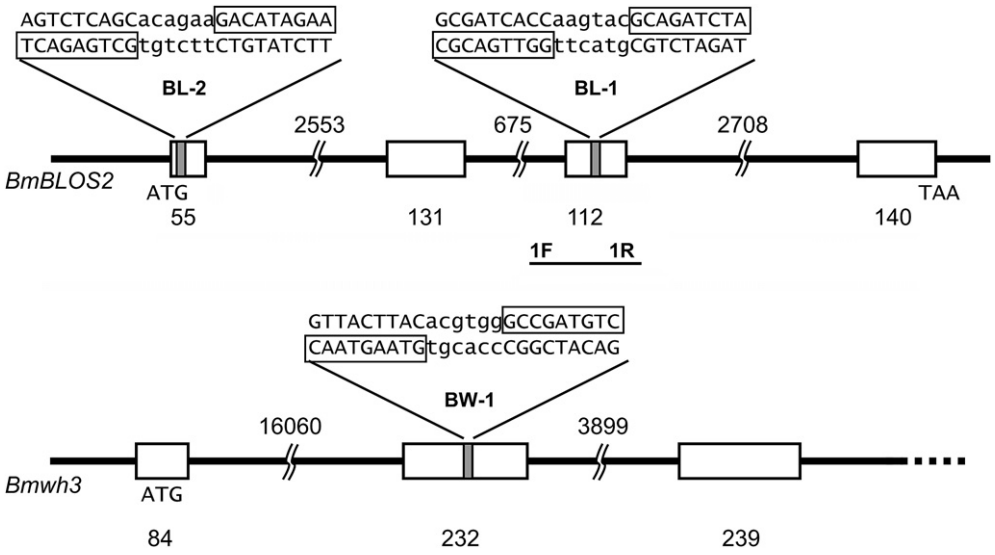

Fig. 1. Structure of the *BmBLOS2* and *Bmwh3* genes. Open boxes on the lines represent exons. The ZFN target sites are indicated as gray boxes with their sequences depicted above (boxed letters identify nine nucleotide motifs recognized by zinc fingers), two of them in the first and third exons of *BmBLOS2* gene (BL-2 and BL-1, respectively) and one in the second exon of the *Bmwh3* gene. The sizes of exons and introns (in bp) are indicated below and above the map. The pair of primers 1F-1R amplified a 683 bp fragment, including the BL-1 target site. More information on ZFN target sites is shown in Fig. S1, S4 and S7.

3. Results

3.1. The test system

We selected *B. mori* as a new insect model species for establishing ZFN genetic targeting because of its well-developed genetics and large number of characterized markers (http://www.shigen.nig.ac.jp/silkwormbase/index.jsp), as well as the availability of a microinjection system for silkworm embryos (Tamura et al., 2000).

Previous experiments with ZFN mRNA injections into *Drosophila* embryos revealed that the usage of the *yellow* (*y*) marker, influencing epidermal color, allowed the detection of somatic mosaics in the G_0 generation (Beumer et al., 2008). The somatic *y* mosaics were observed in adult flies and had no practical use in experiments. The occurrence of somatic mosaics might, however, be potentially informative about the efficiency of microinjection and function of the introduced ZFNs.

Among the best characterized genes involved in the determination of epidermal color in silkworms are the *BmBLOS2* and *Bmwh3* genes, for which null mutations are recessive. Mutant larvae display translucent skin, also known as an "*oily*" phenotype (Fujii et al., 2008), since the gene products are involved in the accumulation of urate granules in the epidermis (Tamura and Sakate, 1983). There are approximately 25 mutant loci in this biosynthetic pathway (http://www.shigen.nig.ac.jp/silkwormbase/ViewStrainGroup.do), although few genes have been identified thus far.

The *BmBLOS2* gene is located on the *Z* chromosome (Fujii et al., 2008), which allows the detection of germline mutants in hemizygous G_1 females. *BmBLOS2* is a relatively small gene consisting of four exons and encoding a protein of 145 amino acids. There were no high affinity $(NNC)_3N_6(GNN)_3$ ZF binding sites within the coding sequence; therefore, we had to use targets containing two non-GNN triplets (Fig. 1). The best available target (designated BL-1) was located in exon 3 (Fig. 1) and contained two out of six triplets of the CNN type. As shown in Table 1, only two triplets in the BL-1 target were rated as "+++" by the specificity evaluation algorithm developed by Carroll et al. (2006). The second target from the *BmBLOS2* (designated BL-2) is located in exon 1, which also exhibits two out of six triplets of the non-GNN type.

The second *Bombyx* marker used for target site selection, *Bmwh3*, is an ABC transporter gene homologous to the *Drosophila white* locus (Abraham et al., 2000) and involved in urate granule deposition. *Bmwh3* mutants also display translucent larval skin and white eggs. Since we did not find a bona fide $(NNC)_3N_6(GNN)_3$ ZF binding site within this gene either, we also selected a target site with two non-GNN triplets (Fig. 1). One of the triplets, AGT, had a low rating of a single "+" (Table 1 and S1C).

We designed open reading frame (ORF) sequences of all three ZFN pairs appropriate for targeting the previously described sequences. We then assembled the appropriate synthetic oligonucleotides corresponding to the sequences of DNA binding domains within the Sp1C ZFN framework (see Figs. S2, S3, S5, S6, S8, S9 and Materials and Methods). Finally, we synthesized the ZFN mRNAs *in vitro* and injected them into silkworm eggs at the syncitial preblastoderm stage as described in Materials and Methods (Fig. S10, S11, S12).

3.2. Mutagenesis of the BL-1 target site

The hatchability of eggs injected with the ZFN specific for the BL-1 target was 51%. Approximately 72% of the fifth instar G_0 larvae of this group showed a mosaic pattern of translucent and normal skin (Fig. 2A, B), thus representing somatic mutants. The patches of translucent epidermis appeared in the ventral side of the body. As expected, the proportion of mosaic epidermis in the larvae was higher in females than in males (70 *vs.* 56 percent, respectively), with a majority of females (80%) expressing *oily* skin extending over more than three segments. In addition, the *oily* skin areas were smaller in mosaic males. Approximately 58% of males and 20% of females displayed "small" *oily* areas, distributed over only one to three larval segments. The relatively high percentage of male mosaics showed that this ZFN was efficient enough to create somatic mutations in both chromosomal alleles.

We crossed the mutagenized BL-$1G_0$ males with *wt* females in order to test for germline mutants, which entailed a total of 71 crosses (Fig. S11). We obtained progeny from $55G_0$ males yielding a total of $16350G_1$ offspring. Among these, we detected 46 larvae, presumably females, which displayed translucent skin (Fig. 2E,F). These G_1 mutants came from at least five different G_0 males,

Fig. 2. Silkworms with somatic and germline mutations. Mutagenesis in epidermal cells of G_0 silkworms was detected by screening for *oily* mosaics in 5th instar larvae, which appeared predominantly on the ventral side (A), and rarely on the dorsal side (B). Ventral and dorsal sides of silkworms with normal epidermis are shown for comparison (C and D, respectively). Germline mutations were detected by the presence of a complete *oily* phenotype. G_1 mutants among 1st and 2nd instar larvae are indicated by arrows (E and F, respectively), and mutant and normal larvae in the final instar are shown in G and H, respectively.

meaning that more than 9% of fertile males yielded mutants. Eight of the G_1 mutant larvae died before metamorphosis, while the rest grew to the pupal stage, when they were confirmed to be females.

3.3. Mutagenesis of BL-2 and BW-1 target sites

The hatchability of the eggs injected with target BL-2 ZFNs (*BmBLOS2* gene) was very low. We injected 480 eggs, but only 18 of them hatched and we observed no somatic mosaics or germline G_1 individuals (Table 3). Similarly, the results for the BW-1 target site from the *Bmwh3* gene showed low hatchability. Among 144 injected eggs, 41 hatched and 27 reached the last larval instar. We observed six somatic mosaics among the G_0 individuals (22%), but no germline mutants in more than 4000G_1 larvae (see Fig. S13 for crossing schemes).

3.4. Verification of Mendelian segregation

Thirty-four of the G_1 female moths emerged from the BL-1 *oily* pupae and were crossed with male moths of the wild-type nondiapausing *pnd* strain; 25 of them laid fertile eggs. All of the G_2 males were heterozygotes showing the normal *wt* phenotype. Eleven G_2 males were crossed with females of the *pnd* strain and the G_3 larvae confirmed the expected 3:1 ratio of phenotype

Table 1
Target triplets and corresponding ZFN sequences. One, two or three "+"s rate relative specificity of triplets for ZFN targeting according to the system of Carroll et al. (18). See Tables S1A,B and C for more information.

ZFN	DNA triplet			Recognition sequence/quality rating		
	F1	F2	F3	F1	F2	F3
BL-1R	CTA	GAT	GCA	QNSTLTE++	TSGNLVR+++	QSGDLTR++
BL-1L	CGC	GAT	GGT	HTGHLLE++	TSGNLVR+++	TSGHLVR++
BL-2R	GAA	ATA	GAC	QSGNLAR++	QKSSLTA+	DRSNLTR+
BL-2L	ACT	GAG	GCT	THLDLTR+++	RSDNLAR+++	QSSDLTR+++
BW-1R	GTA	AGT	AAC	QSSSLVR+++	HRTTLTN+	DSGNLRV++
BW-1L	GCC	GAT	GTC	DCRDLAR+++	TSGNLVR+++	DPGALVR++

segregation (50% of female progeny) supported by a χ^2 statistical test and only one G_2 male offspring line (line number 65) showed marginally significant difference from the expected ratio (Table S2).

3.5. Sequence analysis of representative BmBLOS2 alleles

We extracted genomic DNA from 46 *BmBLOS2* BL-1 mutants representing at least five independent sibling groups. We amplified the areas surrounding the mutated target points by PCR and determined the target region sequences. They showed a pattern of small deletions and insertions (Fig. 3) which are characteristic for NHEJ junctions (Beumer et al., 2006; Bibikova et al., 2002). We were able to distinguish several classes of mutations. The first class contained small 7 and 8 bp deletions. The second class contained an 18 bp deletion, which caused a loss of six amino acids. The third class had two types of insertions of a single adenosine at the target sites, causing frame shifts. A fourth class encompassed uncharacterized mutations that we were unable to amplify and most probably representing large deletions. The last class was represented by one large substitution, which occurred in brood 19, in which a 4.5 kb region of the *BmBLOS2* locus was replaced with a 596 bp fragment from chromosome 8, bearing significant homology with 131 bp *Bm1* element within the 5' upstream region of *BmBLOS2* gene (Fig. S13). Sibling groups 9, 19 and 69 included at least two types of mutations. This meant that NHEJ occurred in more than two germline cells in the G_0 embryo. The most frequent changes were small 7 and 8 bp deletions (63%) and small insertions (11%). The individuals carrying the 7 and 8 bp deletions originated in the same brood and may each represent progeny of a single germ line cell.

4. Discussion

Our work demonstrates that ZFN targeting can be successfully used to generate germline mutations in an insect species other than

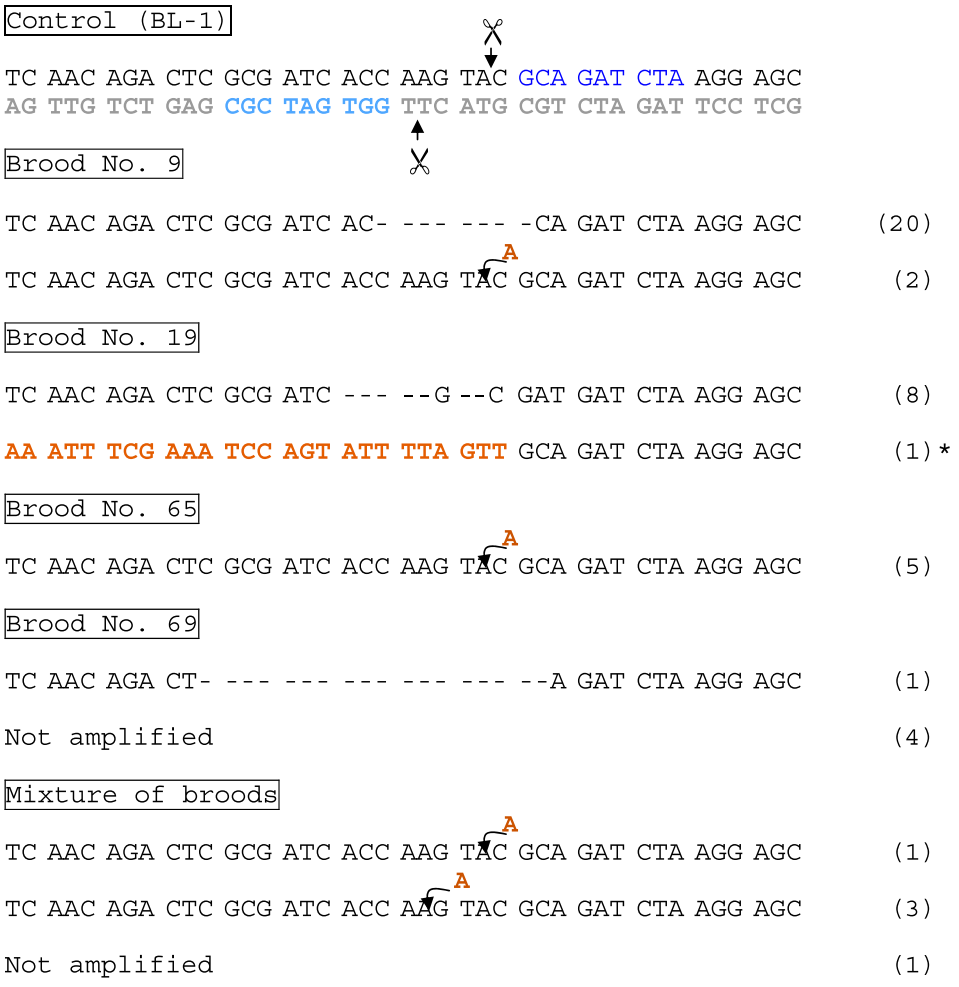

Fig. 3. Sequence analysis of novel *BmBLOS2* alleles. The sequence of the BL-1 site (both strands) is shown at the top for comparison. G_1 mutants were obtained from at least five broods, four of which (No. 9, 19, 65, and 69) were reared separately; and the remainder ("mixture of broods") came from a separate mix of several moths reared together, thus preventing the determination of the lineage of transformants. Each genotype was determined by the sequencing of PCR fragments as described in Materials and Methods. Dashes indicate deletions of residues and orange letters are inserted or substituted residues. The brood numbers with sequences of mutants are shown together with the number of individuals bearing a certain genotype (indicated in parenthesis on the right). "*" indicates that this insertion matches the sequence from chromosome 8 (see also Fig. S13).

Drosophila. The direct microinjection of mRNA into embryos allowed us to avoid the laborious construction of transgenic strains and genetic manipulations needed for the proper expression of DNA-modifying enzymes. The use of an epidermal marker gene allowed easy detection of somatic mutations and monitoring of the efficiency of microinjection. The amount of RNA microinjected into preblastoderm embryos, as well as the conditions used for microinjections, were similar to those used earlier in *Bombyx* by Uchino et al. (2007) with *Minos* mRNA.

We performed ZFN targeting of three target sites from two loci and were able to obtain germline mutants for only one target site. Our success rate was higher than the reported average for ZFNs designed by the modular assembly approach (Ramirez et al., 2008). Our target sites did not contain optimal target sequences composed solely of GNN triplets or triplets with the high "+++" rating (Beumer et al., 2006) and each of them contained two non-GNN triplets (Table 1). The results for particular targets differed significantly. The hatchability of eggs microinjected with the BL-1 target ZFNs mRNAs was 51%, which lies well within the standard range of 30–70% for injected silkworm eggs (Table 2). Also the number of fertile adults that emerged from the microinjected individuals was quite high, suggesting that the microinjection procedure worked well. In contrast, the injections of ZFN mRNAs for the BL-2 and BW-1 targets resulted in high embryo mortality, which may have been due to the lower specificity of the BL-2- and BW-1-ZFNs, which both contained triplets rated as only "+" (Table 1, S1B and S1C).

Although the hatchability of eggs was an important indicator of the possible toxicity of injected molecules, it still did not provide any information about the function of the microinjected product. The occurrence of somatic mosaics, however, indicated whether the engineered ZFN in question was or was not functioning (Table 3). Whereas we observed a high number (72%) of somatic mosaics for BL-1 target mutagenesis (*BmBLOS2*), we observed only 22% with the BW-1 target (*Bmwh3*) ZFNs and none in the BL-2 (*BmBLOS2*) targeting experiment. These results indicated that the choice of the epidermal markers for the *Bombyx* targeting experiment was important, since there was a correlation between the frequency of induced NHEJ somatic mutations with the number of germline mutants detected. The frequency of BL-1 specific somatic mosaics was comparable with the 46-80% mosaics described for the experiment with the *Drosophila yellow* gene, in which the flies were mutagenized by ZFNs using a heat-shock protocol (Bibikova et al., 2002).

We screened 16350 G_1 larvae for the *oily* phenotype and detected a total number of 46 BL-1 *BmBLOS2* target female mutants, or 0.28 percent of the total G1 progeny. The true number of mutations was probably twice as much since our phenotypic screen did not allow the detection of heterozygous male mutants. This number would still be more than an order of magnitude less than the percentage of mutants reported in a *Drosophila rosy* gene mutagenesis screen (Beumer et al., 2008); (Table 3), suggesting that the specificity of BL-1 ZFN might be lower despite its high efficiency in generating somatic mosaics. Alternatively, the lower efficiency of microinjected constructs into the silkworm germline compared to similar experiments in *Drosophila* may be a species-specific feature (T. Tamura, personal communication). This disparity may be due to differences in germ cell formation between the two species as well as the formation of the pole plasm, which is at the posterior end in *Drosophila* and at the ventral midline in silkworm (Nakao, 2009).

We confirmed the BL-1 target *BmBLOS2* mutants by molecular analysis of the targeted regions. Each brood contained mostly the same type of mutation, which suggests that numerous individuals may have originated from the same mutated germ line progenitor cell.

We did not observe 4 bp deletions or insertions, which would be expected from loss and fill in, respectively, of the 5′ overhang left by ZFN cleavage (Beumer et al., 2008; Smith et al., 2000). The nucleotide sequence pattern of mutations was therefore slightly different from the NHEJ results with wild-type flies. The silkworm mutations we observed were instead reminiscent of those obtained in flies deficient in *lig4* (DNA ligase IV) or in *Caenorhabditis elegans*, in which double-stranded repair involves microhomologies (Bozas et al., 2009; Morton et al., 2006). Perhaps the generation of the deletions observed in our *BmBLOS2* mutants is also driven by a microhomology-induced mechanism. The most common 8-bp deletion (Fig. 3) is defined by a 2-bp microhomology (CA). It would be interesting to see if this phenomenon is commonplace upon the generation of more mutants. Interestingly, the 7-bp deletion is probably a deletion-insertion, and it is not flanked by a microhomology (Fig. 3). Another unique deletion-insertion mutant recovered from brood 19 contained repair product accompanied by the insertion of extra, "captured" DNA at the breakpoint, which showed homology with *Bm1* element (Fig. S13). The 600 bp inserted fragment most probably originated from chromosome 8 (Fig. S13) and might occur by synthesis-dependent strand annealing (Kurkulos et al., 1994; Nassif et al., 1994).

Our goal is to knockout genes with unknown phenotypes. Thus, we must rely on the efficiency of the method employed and the detection of mutations based solely on molecular assays. The critical step is the design of new zinc finger combinations directed to chosen DNA sequences. The design of ZFNs is improving dramatically with a number of efficient zinc-finger combinations described (Fu et al., 2009; Maeder et al., 2008). Alternatively, several candidate target sites from the same locus can be chosen together with ZFNs designed by modular assembly, and the most efficient enzyme could be selected by a simple yeast-based assay (Doyon et al., 2008) or a CEL-I Nuclease Mismatch Assay (Miller et al., 2007). The ZFNs chosen in this fashion could then be introduced via mRNA injection to the embryos with more confidence. Non-invasive genotyping could be used for G_1 mutant screens, based on DNA obtained from a single drop (6–10 μl) of hemolymph (by pricking individual fifth instar larvae), followed by PCR amplification together with fragment length analysis and sequencing. If the percentage of G_1

Table 2
Comparison of the survival of microinjected *Bombyx* embryos used for ZFN-NHEJ mutagenesis and microinjections with *Minos* vector RNA.

Experiment	Number of injected embryos	% Hatched	% Fertile adults	Reference
BL-1 *BmBLOS*	480	51	36	This study
BL-2 *BmBLOS*	480	4	N.D.	This study
BW-1 *Bmwh3*	144	28	18[a]	This study
Pmia3A	1888	40.3	26	(21)
Pmia3B1	894	35.3	23.9	(21)
Pmia3B2	678	44.5	27.1	(21)
Pmia3B3	2288	40.0	37.7	(21)

[a] These individuals were kept only until the last larval instar.

Table 3
The Efficiency of ZFN-NHEJ mutagenesis with direct embryo injection in *Bombyx (B.m.)* and *Drosophila (D.m.)*.

Experiment	Number of embryos used	Yielders	% Somatic mosaics	% Germline NHEJ mutants	Non-GNN triplets in the target	Reference
B.m. BL-1	480	5–9	72	0.28[a]	2	This study
B.m. BL-2	480	0	0	0	2	This study
B.m. BW-1	144	0	22	0	2	This study
D.m. pask	14	5	N.D.	6	1	(12)
D.m. rosy	99	41	N.D.	8.2	0	(12)
D.m. coil	45	5	N.D.	5–8	1	(12)

[a] Only females were detected by the phenotypic screen.

germline mutants is about the same as described in this paper, we should be able to obtain several positive individuals per 1000 treated silkworms. Screening this number of larvae is well within the scale of a high-throughput silkworm laboratory.

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Appendix. Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.ibmb.2010.07.012.

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