



Published in final edited form as:

J Genet Genomics. 2014 April 20; 41(4): 215–219. doi:10.1016/j.jgg.2014.02.003.

Mutagenesis at Specific Genomic Loci of Amphioxus *Branchiostoma belcheri* Using TALEN Method

Guang Li^{a,e}, Jun Feng^a, Yong Lei^b, Jing Wang^a, Hui Wang^a, Lü-Ke Shang^a, Dong-Teng Liu^c, Hui Zhao^{b,f}, Yong Zhu^{c,d}, and Yi-Quan Wang^{a,e,*}

^aState Key Laboratory of the Cellular Stress Biology, School of Life Sciences, Xiamen University, Xiamen 361102, China

^bSchool of Biomedical Sciences, Faculty of Medicine, The Chinese University of Hong Kong, Shatin, New Territories, Hong Kong, China

^cState Key Laboratory of Marine Environmental Science, College of Ocean and Earth Sciences, Xiamen University, Xiamen 361005, China

^dDepartment of Biology, East Carolina University, Greenville, NC 27858, USA

^eShenzhen Research Institute of Xiamen University, Shenzhen 518058, China

^fShenzhen Research Institute, The Chinese University of Hong Kong, Shenzhen 518057, China

Amphioxus, also called lancelet or cephalochordate, is a promising model organism owing to its particularly evolutionary position, simple genome content and comparable body plan to that of vertebrates (Holland et al., 2004; Bertrand and Escriva, 2011). However, use of amphioxus as a model organism has been limited for many years because of lack of an efficient genomic modification method. Recently, several revolutionary gene targeting methods that could induce directed mutations, insertions and deletions at intended target sites, have been developed (Gaj et al., 2013). Among these methods, the transcription activator-like effector nuclease (TALEN) has drawn much interest because of its efficiency in generating target gene alterations, simplicity in vector design and nearly limitless in targeting range (Huang et al., 2011; Miller et al., 2011; Bedell et al., 2012; Lei et al., 2012). Up to date, this method has been shown to be effective in inducing mutations in a broad range of organisms including zebrafish, frog, rat, mouse and human (Tong et al., 2012; Gaj et al., 2013; Liu et al., 2013a), suggesting a great potential use for adopting it in amphioxus genome engineering. Here, we presented the first report of an effective TALEN-mediated genome editing method in Chinese amphioxus, *Branchiostoma belcheri*. We chose *B. belcheri* because it is the only amphioxus species which could spawn consecutively all year round (Li et al., 2012, 2013) and be raised through generations in captivity (Zhang et al., 2007). Besides, the species is one of the four amphioxus frequently used in evolutionary/developmental studies and its genome sequence (http://mosas.sysu.edu.cn/genome/gbrowser_wel.php) and embryo microinjection are available in our lab (Liu et al., 2013b).

*Corresponding author. Tel/fax: +86 592 218 4427. wangyq@xmu.edu.cn (Y.-Q. Wang).

SUPPLEMENTARY DATA

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.jgg.2014.02.003>.

To establish an efficient TALEN system suitable for genome editing in amphioxus, we examined the effectiveness of three TALEN backbone vector systems (namely, Goldy, HZ and BZ systems) in inducing mutations in amphioxus embryonic cells. These three systems have been optimized and demonstrated to be highly active in lots of model organisms (Huang et al., 2011; Bedell et al., 2012; Lei et al., 2012; Ma et al., 2013; Qiu et al., 2013; Xiao et al., 2013). Six TALEN pairs targeting the first coding exon of amphioxus *Pax1/9* gene were constructed using these three systems (two TALEN pairs for each system). Unexpectedly, among these six pairs, only the two pairs generated using the Goldy backbone could mutagenize the targeted loci with mutation ratio at 34.3% and 21.9%, respectively (Fig. 1A and Fig. S1A; Table 1). Goldy TALEN-induced mutations included small insertions or deletions (indels), which were the characteristics of non-homologous end joining (NHEJ) mediated repairs (Fig. 1A and Fig. S1A). Two additional TALEN pairs constructed using the HZ system targeting amphioxus *Pax3/7* and *Pax4/6* failed to induce indel mutations in amphioxus embryos. In contrast, 60% and 27.8% mutation frequencies were respectively obtained when the same loci were targeted using the Goldy TALEN system. The apparent failure of the HZ and BZ systems appeared unrelated to their translation based on the detection of immunoreactive TALEN protein on Western blots comparable to the levels translated from the positive control *DrAmmecl1* TALEN mRNAs (Fig. S1B). Mislocalization of TALEN protein was also unlikely since the backbone vectors of the two systems contained exactly the same nuclear localization signal peptide (PKKKRKV) in their N-termini as that of the Goldy vector (Fig. S2).

After choosing the Goldy TALEN system, we conducted a series of experiments to establish optimum conditions for mutagenizing amphioxus embryos by testing different concentrations of TALEN transcripts and sampling times using *Pax3/7-Fw1/Rv1* TALEN vectors. Injection solutions containing 50 ng/μL, 260 ng/μL, 600 ng/μL and 1.3 μg/μL of the TALEN mRNAs were tested. We observed the ratios of normally developing embryos were between 39.7% and 43.5% after the microinjections with TALEN transcripts over a concentration range of 50–600 ng/μL. This is comparable to the proportion of normally developing embryos (47%) observed in the control microinjections with 200 mmol/L KCl solution (Fig. S3A). A high concentration of TALEN transcripts (1.3 μg/μL) resulted in a relatively high ratio of death and deformation (67.4%), indicating a modest deleterious effect of the increasing TALEN mRNAs on amphioxus development (Fig. S3A). Importantly, a much higher somatic mutation ratio (48.5%) was obtained in the embryos microinjected with 1.3 μg/μL of the TALEN mRNAs than those injected with 600 ng/μL (29.9%), 260 ng/μL (11.2%) or 50 ng/μL (1.6%) of the TALEN mRNAs, which were estimated by loss of *BamH* I recognition site in the spacer region (Fig. S3B). Since the DNA sequencing revealed a high mutation ratio (12 of 20 alleles) in 1.3 μg/μL injected embryos (Fig. S3D), we adopted 1.3 μg/μL as an optimal mutagenizing concentration of injection solution for all TALEN transcripts in subsequent experiments unless indicated otherwise. We also examined the temporal mutagenic activity of *Pax3/7-Fw1/Rv1* TALEN vector in amphioxus embryos collected at 40 min post-fertilization (just before the first cleavage), blastula, gastrula, early neurula and late neurula stages. The mutations, which inactivated the restriction site, were detectable at the one cell stage and increased throughout the development process, reaching an apparent plateau by the early neurula stage (Fig. S3C).

To examine the variation of gene-specific activity of Goldy TALEN, we assembled seven additional pairs of TALENs targeting six endogenous amphioxus genes (*Pax2/5/8*, *Pax4/6*, *Bra1*, *Bra2*, *Fgf8/17/18* and *En*). It should be noted that Bra-Fw1/Rv1 and Bra-Fw2/Rv2 were designed to target conserved sequences found both in *Bra1* and *Bra2* genes. Thus each of the TALEN pairs could induce mutations at both *Bra1* and *Bra2* gene loci concurrently. We found efficient genome modifications in the embryos injected with *in vitro* synthesized mRNAs transcribed from these seven pairs of TALENs. The somatic mutation frequencies ranged from 22.2% to 70% (Table 1 and Fig. S4). As expected, the two TALEN pairs (Bra-Fw1/Rv1 and Bra-Fw2/Rv2) targeting *Bra1* and *Bra2* genes could mutagenize the two genes simultaneously. It should be noted that the mutation frequencies determined by direct DNA sequencing were lower than those estimated by the analysis of restriction enzyme digestion for some target sites (e.g., Bra-Fw1/Rv1 and Bra-Fw2/Rv2) (Table 1). It was caused by sequence polymorphisms existing in the cleavage site of restriction enzyme.

Co-injection of mRNAs encoding two pairs of TALENs that target adjacent regions within the same chromosome would generate two tandem double-stranded breaks (DSBs). These two DSBs could then be fused *via* NHEJ-mediated repair concomitant with the deletion of the intervening region (Carlson et al., 2012; Ma et al., 2012; Gupta et al., 2013; Xiao et al., 2013). We used this strategy to determine the utility of TALEN-mediated segment deletion in amphioxus. Transcripts of six TALEN pairs targeting the amphioxus *Pax1/9* and *Pax2/5/8* loci and the tail-to-tail amphioxus *Bra1* and *Bra2* gene loci, which were expected to generate deletions of 249 bp, 5.6 kb, 16.3 kb and 16.4 kb, were used in this analysis (Fig. S5A). We detected the expected lengths of segmental deletions in the embryos treated with all four TALEN injections (Fig. 1B and Fig. S5B). Based on the intensity of bands amplified from the modified region and the control region (full length amplicon or unmodified genomic DNA), we estimated deletion ratios at the *Pax1/9*, *Pax2/5/8* and two *Bra* loci were 7.39%, 5.34%, 7.33% and 5.83%, respectively. Sequence analysis of the PCR amplicons further confirmed the deletion of the genomic fragments between the TALEN target sites and revealed a set of distinct junction sequences (Fig. 1B and Fig. S5B). Notably, the sequence outside TALEN binding sites was also deleted from four of five sequenced alleles from embryos injected with the Bra-Fw2/Rv2 mRNAs (Fig. S5B).

Both indel mutations and segmental deletions induced by TALEN were generally produced *via* NHEJ of DSBs. Nevertheless, the TALEN-induced DSBs could also be repaired *via* homology-directed repair (HDR) if a donor template was provided. Using this principle, Bedell et al. (2012) and Zu et al. (2013) successfully inserted exogenous DNA sequences including an *EcoR* V restriction site, a *LoxP* site and a *GFP* gene into zebrafish genome. To determine whether TALEN would induce HDR in amphioxus genome, we designed and synthesized two distinct ssDNA oligonucleotides (oligo 1 and oligo 2) that included a modified *LoxP* (*mLoxP*) site in the middle and two 25-bp homology arms of TALEN-targeted gene at both 3'- and 5'-ends (Fig. S6A). Two highly active TALENs (*Pax3/7-Fw1/Rv1* and *Pax2/5/8-Fw1/Rv1*) were applied in the study. After co-injection of mRNAs of each TALEN pair and their corresponding ssDNAs (Fig. S6B), somatic insertions of the *mLoxP* sequence could be detected in the injected embryos using PCR detection (Fig. 1C and Fig. S6C). However, when we selected about 100 positive colonies containing the target

region from injected embryos, which were generated by PCR plus TA-cloning, for further detection, no clone including the *mLoxP* sequence was found, indicating that the insertion ratio was less than 1%. Therefore, to determine whether the *mLoxP* sequence was correctly inserted into the targeted sites, we directly amplified the target segment from the DNA template of the injected embryos using gene-specific forward and *mLoxP* reverse primers. The PCR products were then cloned into the pGEM-T vector. Among six identified clones, we observed three precise homologous recombinations that resulted in *mLoxP* insertion at the 3'-end of the *Pax2/5/8* locus (Fig. S6C). The remains included small indels at the modification site. We also observed a similar result in the embryos co-injected with *Pax3/7-Fw1/Rv1* mRNA and its oligo 1 (Fig. 1C). Based on these results, we concluded that the Goldy TALEN system could induce precise fragment insertions at 5'-end of a targeted site. Taken together, our results suggest that Goldy TALEN system could induce precise genome insertions of a small DNA sequence in amphioxus *via* co-injection of TALEN mRNAs and ssDNA donor templates.

In summary, we successfully established a Goldy TALEN-mediated genome editing method for amphioxus. The method could efficiently induce somatic mutations, segmental deletions and insertions in amphioxus genome. So far, the germline transmission of TALEN-mediated genome modifications was successfully carried out in all examined model animals (Bedell et al., 2012; Carlson et al., 2012; Lei et al., 2012; Qiu et al., 2013; Xiao et al., 2013; Zu et al., 2013). We undoubtedly expected to create genome modified amphioxus immediately by germline transmission after this successful application of TALEN technique. However, several factors hinder us to obtain this amphioxus at the moment. The first restricting factor is the long generation time of amphioxus *B. belcheri*. The neonatal amphioxus needs about one year for growing and developing before its sexual maturity (Zhang et al., 2007). Secondly, the reproduction of amphioxus follows a strict r-selection strategy: a single mature female releases several thousands of eggs in one spawning, but over 90% of them die before metamorphosis (Zhang et al., 2007). Therefore, optimizing animal rearing method for shortening amphioxus generation time and increasing their surviving ratio is under urgent requirement.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We wish to express our great appreciation to Prof. Bo Zhang at Peking University for her kindly providing the plasmids and useful suggestions, and to Dr. Edmund J Stellwag at East Carolina University for his editing the manuscript. We also thank Dr. Anlong Xu for his kindly providing access to the unpublished genome sequence of *B. belcheri*. This work was supported by the National Natural Science Foundation of China (Nos. 31071110, 30830023 and 31101631) and the Scientific and Technical Innovation Committee of Shenzhen, China (No. CXZZ20120614164555920).

References

Bedell VM, Wang Y, Campbell JM, Poshusta TL, Starker CG, Krug2nd RG, Tan W, Penheiter SG, Ma AC, Leung AY, Fahrenkrug SC, Carlson DF, Voytas DF, Clark KJ, Essner JJ, Ekker SC. *In vivo*

- genome editing using a high-efficiency TALEN system. *Nature*. 2012; 491:114–118. [PubMed: 23000899]
- Bertrand S, Escriva H. Evolutionary crossroads in developmental biology: amphioxus. *Development*. 2011; 138:4819–4830. [PubMed: 22028023]
- Carlson DF, Tan W, Lillico SG, Stverakova D, Proudfoot C, Christian M, Voytas DF, Long CR, Whitelaw CB, Fahrenkrug SC. Efficient TALEN-mediated gene knockout in livestock. *Proc Natl Acad Sci USA*. 2012; 109:17382–17387. [PubMed: 23027955]
- Gaj T, Gersbach CA, Barbas CF 3rd. ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering. *Trends Biotechnol*. 2013; 31:397–405. [PubMed: 23664777]
- Gupta A, Hall VL, Kok FO, Shin M, McNulty JC, Lawson ND, Wolfe SA. Targeted chromosomal deletions and inversions in zebrafish. *Genome Res*. 2013; 23:1008–1017. [PubMed: 23478401]
- Holland LZ, Laudet V, Schubert M. The chordate amphioxus: an emerging model organism for developmental biology. *Cell Mol Life Sci*. 2004; 61:2290–2308. [PubMed: 15378201]
- Huang P, Xiao A, Zhou M, Zhu Z, Lin S, Zhang B. Heritable gene targeting in zebrafish using customized TALENs. *Nat Biotechnol*. 2011; 29:699–700. [PubMed: 21822242]
- Lei Y, Guo X, Liu Y, Cao Y, Deng Y, Chen X, Cheng CH, Dawid IB, Chen Y, Zhao H. Efficient targeted gene disruption in *Xenopus* embryos using engineered transcription activator-like effector nucleases (TALENs). *Proc Natl Acad Sci USA*. 2012; 109:17484–17489. [PubMed: 23045671]
- Li G, Yang X, Shu Z, Chen X, Wang Y. Consecutive spawnings of Chinese amphioxus, *Branchiostoma belcheri*, in captivity. *PLoS ONE*. 2012; 7:e50838. [PubMed: 23251392]
- Li G, Shu ZH, Wang YQ. Year-round reproduction and induced spawning of Chinese amphioxus, *Branchiostoma belcheri*, in laboratory. *PLoS ONE*. 2013; 8:e75461. [PubMed: 24086537]
- Liu J, Li C, Yu Z, Huang P, Wu H, Wei C, Zhu N, Shen Y, Chen Y, Zhang B, Deng WM, Jiao R. Efficient and specific modifications of the *Drosophila* genome by means of an easy TALEN strategy. *J Genet Genomics*. 2013a; 39:209–215. [PubMed: 22624882]
- Liu X, Li G, Feng J, Yang X, Wang YQ. An efficient microinjection method for unfertilized eggs of Asian amphioxus *Branchiostoma belcheri*. *Dev Genes Evol*. 2013b; 223:269–278. [PubMed: 23584404]
- Ma S, Zhang S, Wang F, Liu Y, Liu Y, Xu H, Liu C, Lin Y, Zhao P, Xia Q. Highly efficient and specific genome editing in silkworm using custom TALENs. *PLoS ONE*. 2012; 7:e45035. [PubMed: 23028749]
- Ma AC, Lee HB, Clark KJ, Ekker SC. High efficiency *in vivo* genome engineering with a simplified 15-RVD GoldyTALEN design. *PLoS ONE*. 2013; 8:e65259. [PubMed: 23734242]
- Miller JC, Tan S, Qiao G, Barlow KA, Wang J, Xia DF, Meng X, Paschon DE, Leung E, Hinkley SJ, Dulay GP, Hua KL, Ankoudinova I, Cost GJ, Urnov FD, Zhang HS, Holmes MC, Zhang L, Gregory PD, Rebar EJ. A TALE nuclease architecture for efficient genome editing. *Nat Biotechnol*. 2011; 29:143–148. [PubMed: 21179091]
- Qiu Z, Liu M, Chen Z, Shao Y, Pan H, Wei G, Yu C, Zhang L, Li X, Wang P, Fan HY, Du B, Liu B, Liu M, Li D. High-efficiency and heritable gene targeting in mouse by transcription activator-like effector nucleases. *Nucleic Acids Res*. 2013; 41:e120. [PubMed: 23630316]
- Tong C, Huang G, Ashton C, Wu H, Yan H, Ying QL. Rapid and cost-effective gene targeting in rat embryonic stem cells by TALENs. *J Genet Genomics*. 2012; 39:275–280. [PubMed: 22749015]
- Xiao A, Wang Z, Hu Y, Wu Y, Luo Z, Yang Z, Zu Y, Li W, Huang P, Tong X, Zhu Z, Lin S, Zhang B. Chromosomal deletions and inversions mediated by TALENs and CRISPR/Cas in zebrafish. *Nucleic Acids Res*. 2013; 41:e141. [PubMed: 23748566]
- Zhang QJ, Sun Y, Zhong J, Li G, Lu XM, Wang YQ. Continuous culture of two lancelets and production of the second filial generations in the laboratory. *J Exp Zool B Mol Dev Evol*. 2007; 308:464–472. [PubMed: 17497691]
- Zu Y, Tong X, Wang Z, Liu D, Pan R, Li Z, Hu Y, Luo Z, Huang P, Wu Q, Zhu Z, Zhang B, Lin S. TALEN-mediated precise genome modification by homologous recombination in zebrafish. *Nat Methods*. 2013; 10:329–331. [PubMed: 23435258]

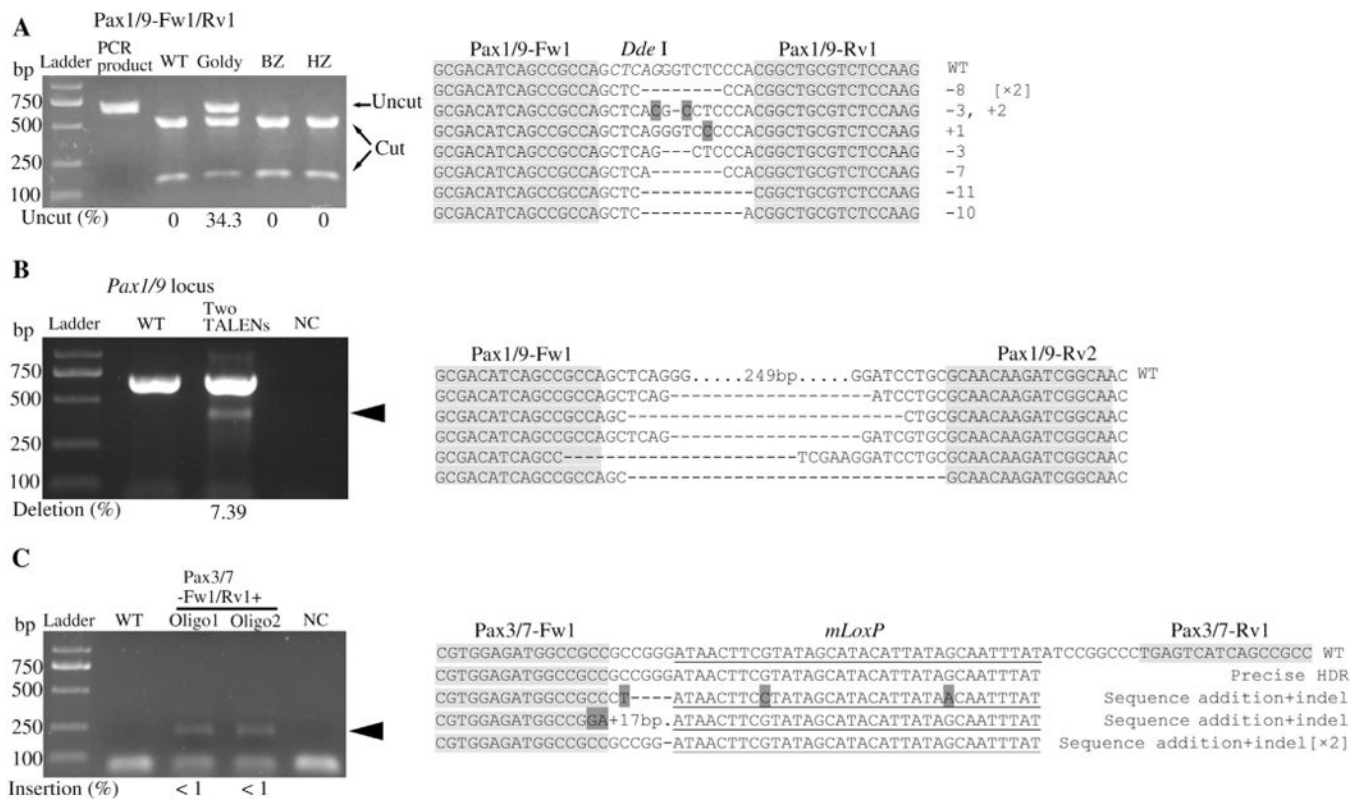


Fig. 1. Goldy TALEN-mediated genome editing in amphioxus embryos

A: Activities of three TALEN systems at amphioxus *Pax1/9* locus targeted by TALEN pair Pax1/9-Fw1/Rv1. The induced mutation ratios (estimated as percentages of uncut PCR products) are labeled under the gel image. **B:** Goldy TALEN-induced large DNA fragment deletions at amphioxus *Pax1/9* locus by injecting two TALENs. PCR products amplified from the genomic DNA extracted from injected and un-injected (WT) embryos, and their sequences are shown. No DNA template was added in the negative control (NC). PCR products marked by black arrowheads would be obtained when the targeted deletion occurred. The left half sequence of the first TALEN and the right half sequence of the second TALEN are shown in the first line (WT) in each alignment, with TALEN site shaded in grey and spacer unmarked. **C:** Goldy TALEN-mediated homology-directed repair (HDR) at amphioxus *Pax3/7* locus. PCR products amplified from the genomic DNA extracted from injected (Pax3/7-Fw1/Rv1 mRNAs + ssDNA oligonucleotides) and un-injected (WT) embryos, and their sequences are shown. TALEN binding sites are shaded in grey and the *mLoxP* site is underlined. PCR was conducted using a forward gene-specific primer and a reverse *mLoxP* primer. PCR amplicons marked by black arrowheads would be obtained when HDR and *mLoxP* insertion occurred, while no PCR products would be obtained when no *mLoxP* insertion happened. The amplicons amplified from genomic DNA of the embryos injected with TALEN mRNAs and oligo1 were cloned into pGEM-T vector, and five or six selected clones were sequenced for each locus. No DNA template was added in NC (negative control). Abbreviations: WT, wild type; Goldy, Goldy TALEN system; HZ, Hui Zhao's TALEN system; BZ, Bo Zhang's TALEN system; NC, negative control.

Table 1

Assembled TALENs, TALEN-targeted genes, their binding sequences, and TALEN-induced mutation ratios estimated by direct DNA sequencing or restriction enzyme (RE) analyzing in *B. belcheri*

TALEN name	Target gene	Target site sequence	Lengths of left arm/spacer/right arm (bp)	TALEN system used	Mutation rate via sequencing (%) ^a	Mutation rate via RE reaction (%)	RE used for efficacy evaluation
Pax1/9-Fw1/Rv1 ^b	<i>Pax1/9</i>	TGCGACATCAGCCGCCAGCTCAGGGTCTCCACGGCTGCCGTCTCCAAGA	16/15/16	Goldy, HZ and BZ	40.0	34.3	<i>Dde</i> I
Pax1/9-Fw2/Rv2	<i>Pax1/9</i>	TGCCCTCGGTCTCGTCCATCTCCGGGATCTCGGCAACAAGATCGGCAACA	16/17/16	Goldy, HZ and BZ	25.0	21.9	<i>Bam</i> HI
Pax2/5/8-Fw0/Rv0	<i>Pax2/5/8</i>	TCGGCGCGCTTACGTCAACGGCCGCCCTCCCTGATGTGGTTCGGCA	16/15/16	Goldy	22.2	21.9	<i>Eag</i> I
Pax2/5/8-Fw1/Rv1	<i>Pax2/5/8</i>	TACAAGCGACAGAACCsACCATGTTCCGATGGAGATCAGAGACAGGCTA	16/17/16	Goldy	60.0	NA	NA
Pax3/7-Fw1/Rv1	<i>Pax3/7</i>	TCGTGGAGATGGCCGCCCGGGGATCCGGCCCTGCGTCAATCAGCCGCCA	16/15/16	Goldy and HZ	60.0	48.5	<i>Bam</i> HI
Pax4/6-Fw0/Rv0	<i>Pax4/6</i>	TCGGGGGGGTGTTCTGTGAACGGCCGCCCTCCCGATTCCACGGGGCAGAA	17/17/16	Goldy and HZ	27.8	NA	<i>Eag</i> I
Bra-Fw1/Rv1	<i>Bra1</i> & <i>Bra2</i>	TTCCCCGTGCTGAAGGTGAACGTGTCCGGACTGGACCCTAACGCCATGTA	16/16/16	Goldy	35.0 & NA	52.6 & 50.3	<i>Bsp</i> E I
Bra-Fw2/Rv2	<i>Bra1</i> & <i>Bra2</i>	TACTCTTCTCTGCTGGACTTCA CGGCCCGCCGACAAACCACCGCTGGAAGTA	16/16/16	Goldy	NA & 21.1	55.0 & 42.0	<i>Eag</i> I
Fgβ/17/18-Fw1/Rv1	<i>Fgβ/17/18</i>	TGCGATGCTTGCCTTTGTTACGGCTCGTCCCTCTGTAAGGACAAAGTTTGTGA	16/17/16	Goldy	70.0	64.1	<i>Bss</i> S I
En-Fw1/Rv1	<i>En</i>	TTCCGGCGCTCGCAAGAGAGACAGTAAAAGTTGTGACTCTCTCCCGTGTCCA	17/16/17	Goldy	29.4	NA	NA

^a 20 individual colonies were sequenced for each target site.

^b Pax1/9-Fw1 was the name of an assembled TALEN vector bound to the left side targeted sequence of *Pax1/9* gene in red color of target site sequence, whereas Pax1/9-Rv1 was the name of an assembled TALEN vector bound to the right side targeted sequence in blue color. Restriction enzyme recognition sequences are underlined. NA, no available.