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TALEN-mediated genome editing of the *ku80* gene in the silkworm *Bombyx mori*

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Genome editing is a powerful tool for the functional analysis of targeted genes. We have previously found that a novel knock-in system, precise integration into target chromosome (PITCh), allows the integration of a donor vector harboring the *hsp90* promoter and GFP into the silkworm *biogenesis of lysosome-related organelles complex 1, subunit 2* gene in a precise and efficient manner. Here, we examined whether this technique can be used for the knock-in of other silkworm genes. The *ku80* gene was selected as a target and was efficiently mutagenized using a pair of transcription activator-like effector nucleases (TALENs) that were constructed for its specific cleavage. Knock-in was then carried out using these TALENs. Microinjection of TALEN mRNAs mixed with the donor vector resulted in significant expression of the GFP marker in G0 larvae. Importantly, GFP expression was also detected in G1 individuals, suggesting that the integrated donor vector can be inherited in the next generation. Genotyping analysis showed that the donor vector was inserted into the targeted *ku80* locus in four individuals. It was further found to be inherited in the G2 generation in a Mendelian manner. We argue that the PITCh system offers a versatile tool for the knock-in of various genes, and should contribute to the further promotion of sericultural studies.

Key words: TALEN, genome editing, knock-in, PITCh, *ku80*, *Bombyx mori*

INTRODUCTION

Genome editing is a very powerful technique for the disruption or modification of targeted genes in various organisms. In the silkworm *Bombyx mori*, zinc finger nucleases (ZFNs) have been used to knockout the *biogenesis of lysosome-related organelles complex 1, subunit 2* (*BLOS2*) gene in an effective manner (Takasu *et al.*, 2010). More recently, transcription activator-like effector nucleases (TALENs) were developed as another type of programmable site-specific nuclease. Due to the simplicity of the DNA recognition code, this technique has become a major genome-editing tool in various organisms (Sun and Zhao 2013). The knockout efficiency of TALEN was initially similar to that of ZFN in *B. mori* (Ma *et al.*, 2013; Sajwan *et al.*, 2013), but modification of TALEN architecture greatly improved its efficiency in this species (Takasu *et al.*, 2013; 2014; 2016) and a number of knockout studies are ongoing (Yoda *et al.*, 2014; Enya *et al.*, 2015; Daimon *et al.*, 2015; Shiomi *et al.*, 2015; Osanai-Futahashi *et al.*, 2015). In contrast, editing using the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) system appears to be less effective in *B. mori* (Daimon *et al.*, 2014).

Knock-in is another significant application of genome editing. Integration of a large plasmid at a targeted genomic site permits a wide variety of usages, including fluorescent tagging of a specific gene and efficient screening of knockout individuals. Homologous recombination (HR)-mediated knock-in has been attempted in the silkworm, resulting in the integration of a donor vector into the targeted genome locus (Daimon *et al.*, 2014). However, the efficiency was extremely low, presumably due to the variable frequencies of HR across cell types and organisms. Recently we exploited a novel knock-in system known as PITCh (Precise Integration into Target Chromosome) (Nakade *et al.*, 2014). This system is based on microhomology-mediated end-joining (MMEJ), a double strand break-repair mechanism that is independent of the HR pathway. This method has been used in the successful integration of exogenous donor DNA into the targeted genomic site in human cells, frogs and the silkworm (Nakade *et al.*, 2014; Sakuma *et al.*, 2015; 2016). In the case of the silkworm, a ~10 kb donor vector carrying GFP and the *hsp90* promoter was integrated into the *BLOS2* locus via TALEN-based PITCh (Nakade *et al.*, 2014). Importantly, the PITCh system allows not only efficient but also precise knock-in, suggesting that this method could provide a standard and promising knock-in tool in various organisms (Nakade *et al.*, 2014). However, whether PITCh can be used for the knock-in of other silkworm genes has yet to be determined.

To confirm the versatility of the PITCh system, we here investigated whether this technique could be applied for the knock-in of silkworm genes other than *BLOS2*. We selected the *ku80* gene as a target and found that our designed TALEN caused the knockout of this gene efficiently. We subsequently attempted knock-in and found that the donor vector was integrated into the *ku80* locus in an effective manner. Sequencing analysis revealed that the in-

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tegration occurred in a precise manner, suggesting that the PITCh system could provide a versatile tool for the precise and efficient knock-in of various genes. We argue that this technique can be used for a wide variety of purposes, including gene functional analysis and recombinant protein production, and thus should promote sericultural studies.

MATERIALS AND METHODS

Silkworm strains

Silkworms were reared on an artificial diet (Nihon Nosan Kogyo, Yokohama, Japan) at 25°C under a 12-hours light/dark photoperiod. The *w1-pnd* (non-diapausing) strain was used for the knockout and knock-in experiments. The established knock-in strain was crossed with the *w-c* (diapausing) strain to maintain the stocks.

Database search and alignment

The silkworm *ku80* homologue was retrieved from the silkworm genomic database (KAIKOBase, The International Silkworm Genome Consortium 2008; Suetsugu *et al.*, 2013) using *Drosophila melanogaster ku80* sequence (Genbank no. AF23772) as a query. Monarch butterfly (*Danaus plexippus*) *ku80* homologue was identified via the NCBI database search (http://www.ncbi.nlm.nih.gov/). The alignments were carried out using CLC Sequence Viewer software (CLC bio, Aarhus, Denmark).

Construction of TALENs and PITCh vector

TALENs were constructed as described in Takasu *et al.* (2014). The target site was selected by manual search and the sequence around the target site was determined in the *w1-pnd* strain. TALEN assembly was conducted using the Golden Gate TALEN and TAL Effector Kit (Addgene, Cambridge, USA) and the TALEN backbone vector pBlue-TAL (Takasu *et al.*, 2013). *In vitro* mRNA synthesis was carried out using the mMESSAGE mMACHINE T7 kit (Ambion, Carlsbad, USA).

For PITCh vector construction, the TALEN recognition and microhomology sequence was inserted upstream of the SV40 sequence in the pBachsp90GFP-3xP3DsRed plasmid (Tsubota *et al.*, 2014; Nakade *et al.*, 2014). The inverse PCR was carried out using primers 5'-TAGGTCC AAATACCCATTTCATCCTATAGCACCACCTGTTCCT GTAG-3' and 5'-TTCAAGTCCACTGAGGTCAGAATTC CACTCGAATTAGATCTTTGG-3', and the PCR product was self-ligated. The inserted sequence was checked using Applied Biosystems 3130*xl* (Life Technologies) after cycle sequencing with BigDye Terminator V3.1 (Life Technologies).

Microinjection

Microinjection was carried out following Tamura *et al.* (2000). TALEN mRNAs with or without the PITCh vector were injected into the *w1-pnd* embryos at the syncytial preblastoderm stage. The concentration of TALEN mRNA was 125 ng/μl in the knockout experiment and 25 ng/μl in the knock-in experiment. The PITCh vector concentration was 500 ng/μl.

Determination of ku80 knock-out efficiency

Knock-out efficiency was measured following Takasu *et al.* (2014). TALENs were injected into 40-50 embryos and the genomic DNA was extracted three days later using DNAzol (Life Technologies, Carlsbad, USA). The target region was amplified by PCR using primers 5'-TGCAAG GCTGTTTGTTGAAC-3' and 5'-CTTCTTTGTCCACTT GCCAAC-3'. The amplicon was subjected to TA cloning using TArget Clone Plus (Toyobo, Osaka, Japan) and subsequent colony PCR using 33 colonies. The PCR products were digested with *Bsp*EI and electrophoresis was used to check the band size.

Detection of GFP fluorescence

Expression of the fluorescent protein GFP was observed by stereomicroscopy using model MZ16FA (Leica, Solms, Germany) for the G0 larvae and model SZX16 (Olympus, Tokyo, Japan) for the G1 embryos. A GFP long pass filter (excitation: 440/80 nm, emission: 510 nm or excitation: 460/90 nm, emission: 510 nm) was used for the observation. Images were captured using the DFC300FX (Leica) or DP71 (Olympus) systems.

Genotyping of the knock-in individuals

Genomic DNA was extracted using the DNeasy kit (QIAGEN, Hilden, Germany) for each G1 adult from the crosses, as well as several individuals that died at the pupal stage. PCR amplification was then carried out using primers 5'-TGCAAGGCTGTTTGTTGAAC-3' and 5'-ATTTG TTGGCAGCACTGCTT-3' for the 5' junction and 5'-ATA ACGACCGCGTGAGTCAA-3' and 5'-CTTCTTTGTCCA CTTGCCAAC-3' for the 3' junction. The amplified fragments were sequenced using Applied Biosystems 3130*xl* after cycle sequencing with BigDye Terminator V3.1.

RESULTS

Construction and activity analysis of TALENs for *Bombyx ku80* gene

ku80 is a gene that plays critical roles in the non-homologous end joining (NHEJ) process. Its protein product binds to the DNA ends in complex with Ku70 and recruits and activates protein kinase (Kotnis *et al.*, 2009). Knock-out of *ku80* may increase the frequency of homologous recombination-mediated knock-in, as reported for *ku70* (Bertolini *et al.*, 2009; Fattah *et al.*, 2008; Näätsaari

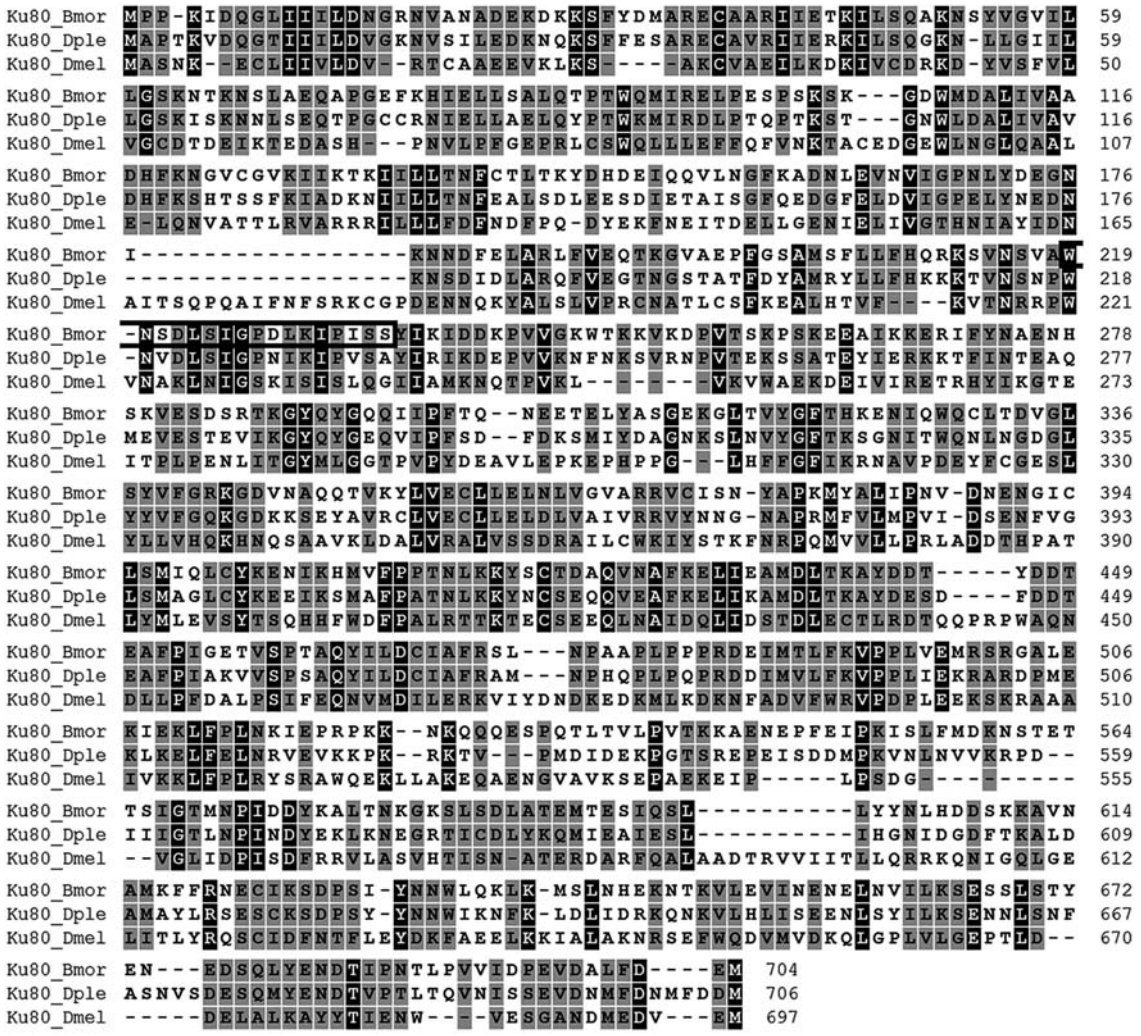

Fig. 1. Alignment of Ku80 proteins. Bmor indicates *B. mori*, Dple *D. plexippus* and Dmel *D. melanogaster*. The amino acid sequence of *B. mori* Ku80 is a prediction from cDNA clone fphe26N20. Amino acids conserved in all three species are colored black and those shared by two species are colored gray. Amino acid residues encoded by the TALEN target site are enclosed by a box.

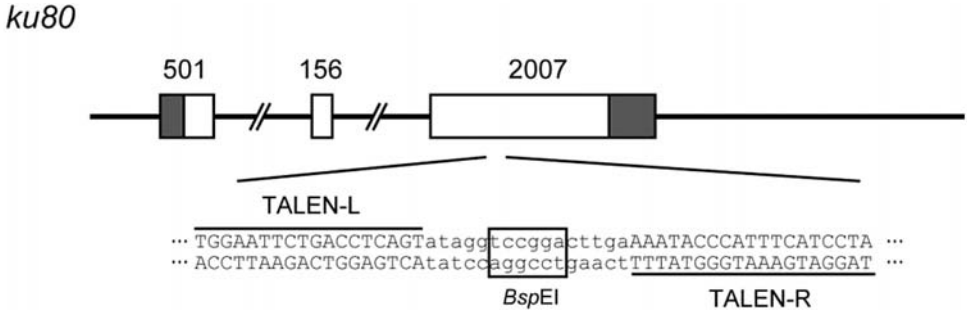

Fig. 2. The gene structure of *ku80*. Boxes indicate exons together with their lengths. The gray regions indicate the UTRs. The TALEN-binding sequences are indicated by horizontal lines, with the naturally occurring *Bsp*EI site enclosed by a box.

et al., 2012; Qi *et al.*, 2013). We first investigated whether TALEN-mediated knockout could be achieved for this gene. The *B. mori* genome database revealed a putative *ku80* homologue with the full-length cDNA fphe26N20 encoding a protein homologous to *D. melanogaster* Ku80 (Fig. 1). A search of the NCBI database resulted in the identification of *ku80* homologous genes in the Monarch butterfly *D. plexippus* (Fig. 1). TALENs were designed against the coding region of *ku80* in *B. mori* (Fig. 2) and were microinjected into silkworm embryos. The embryonic

assay showed that efficient knockout was occurring (Table 1; see Materials and Methods), suggesting that our assembled TALENs are functional.

Knock-in of *ku80*

We next attempted knock-in using the TALENs described above. TALEN-mediated PITCh (TAL-PITCh) has previously been used for the integration of a donor vector harboring the *hsp90* promoter and GFP into the *BLOS2* locus (Nakade *et al.*, 2014). The *hsp90* promoter induces gene expression strongly and ubiquitously in various silkworm developmental stages (Tsubota *et al.*, 2014) and thus the use of this vector allows the easy detection of knock-in (Nakade *et al.*, 2014). It therefore makes a suitable vector for the *ku80* knock-in experiment.

Upon injection of the modified vector into silkworm embryos in a mixture with TALEN mRNAs (Fig. 3), around 90 % of hatched larvae showed patchy expression of GFP (Fig. 4A, Table 2). We speculate that such GFP expression would be occurring in cells in which the donor vector was integrated into the genome stably; in a previous analysis somatic mutation was observed in a mosaic pattern in G0 individuals injected with TALEN mRNAs and a GFP-expressing donor plasmid (Daimon *et al.*, 2014). These individuals were reared until adult and crossed with non-injected individuals, and GFP expression was examined in their progenies. Three broods included embryos with strong and ubiquitous GFP expression (Fig. 4B-D'), with a total of 26 GFP-positive individuals (Table 2). This suggests that the donor vector was integrated into the germ line, thus allowing the mutation to be transmitted to the next generation.

Table 1. Determination of knock-out efficiency for *ku80* by embryonic assay

mutant clones	wildtype clones	clones with a mixture of wildtype and mutant	efficiency (%)
24	5	4	79

Genotyping of *ku80* knock-in individuals

Genotyping was used to determine whether the knock-in was occurring in a precise manner as intended. Genomic DNA was extracted from each GFP-positive G1 individuals, followed by PCR amplification of the 5' and 3' junc-

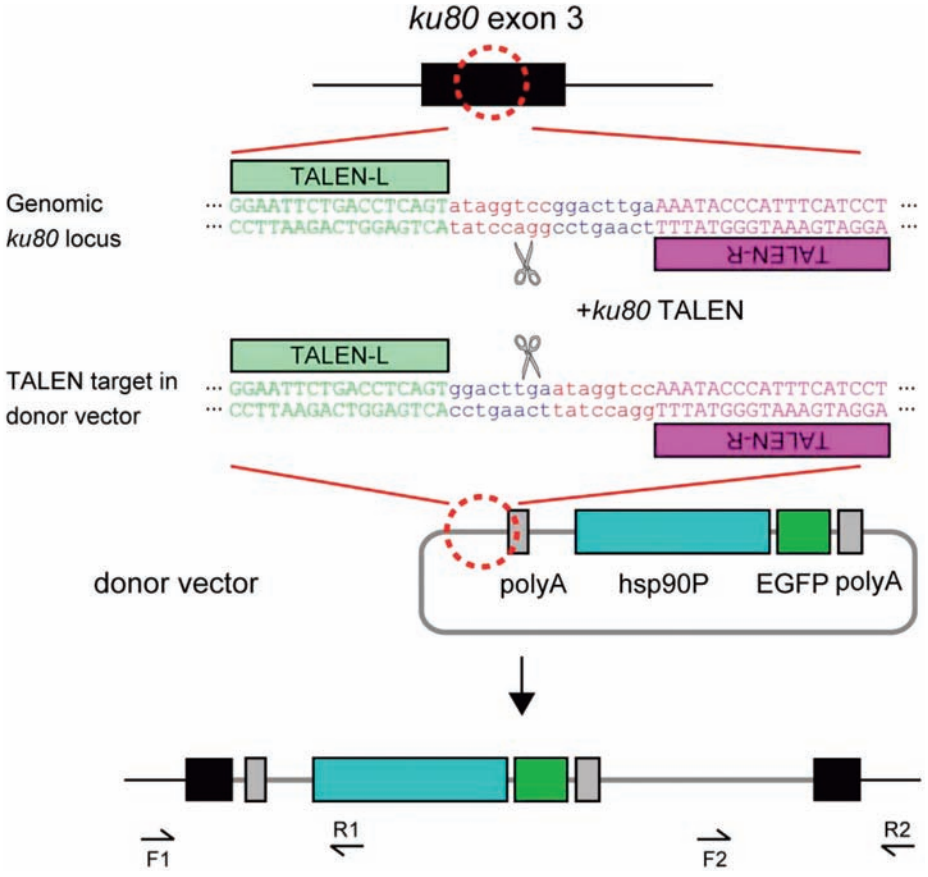

Fig. 3. Schematics of the *ku80* knock-in experiment. Nucleotides shown in green and purple are the left and right TALEN recognition sequences. Nucleotides shown in red and blue indicate the microhomology sequences designed for donor integration. The length of the microhomology sequence is 8 bp each in this experiment. Within the donor vector, hsp90P indicates the *hsp90* promoter and EGFP is the enhanced green fluorescent protein gene. F1, F2, R1 and R2 depict the position of each primer used for the genotyping.

tions of the integrated donor vector within the *ku80* genomic locus (Fig. 3). For the 5' junction, four individuals showed a band of the size expected for the PCR product of the knock-in genome (1-3, 1-9, 1-11 and 1-12; Fig. 5A; Table 2). Sequencing revealed that the donor vector was inserted into the *ku80* locus (Fig. 5B). Importantly, no insertion or deletion of nucleotides was found around the 5' junction site for any of these individuals (Fig. 5B), suggesting that the knock-in occurred in a very precise manner. A nucleotide substitution was found within the TALEN recognition sequence (1-3, 1-9, 1-11 and 1-12; Fig. 5B), but this was probably due to natural polymorphism and not a mutation caused during the knock-in process, because the identical substitution was present in individuals in which the knock-in failed and also in the cDNA clone of this gene (data not shown). The 3' junction of the integrated donor was PCR-amplified from six individuals, among which four (1-3, 1-9, 1-11, 1-12) also gave a PCR product expected for the targeted integration for the 5' site, whereas two others (3-1 and 3-2) did not (Figs. 5A and 5C). Sequencing analysis revealed that the donor vector was integrated together with the insertion of 21 extra nucleotides for 1-3, 1-9, 1-11 and 1-12, and precisely for 3-1 and 3-2 (Fig. 5D). A polymorphism-like DNA substitution was also found within the TALEN recognition site (Fig. 5D).

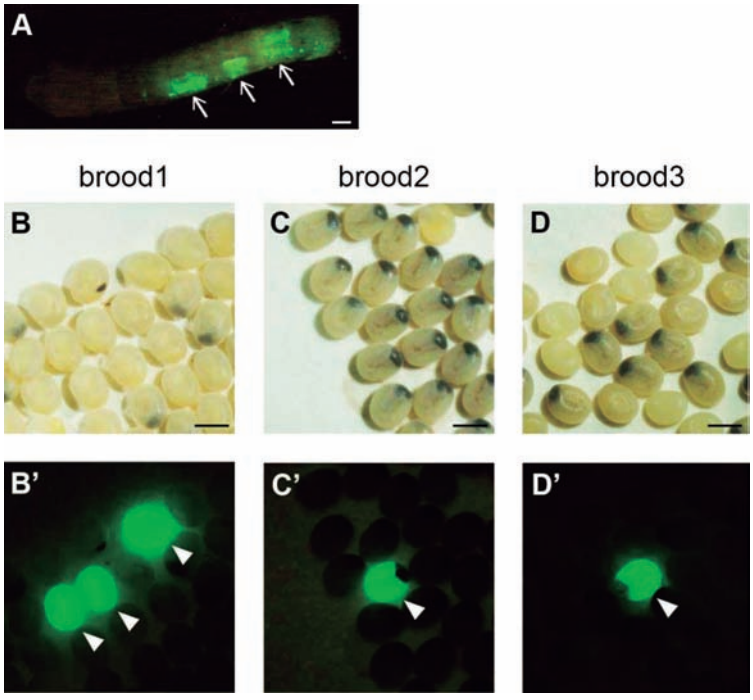

Fig. 4. (A) GFP expression in a G0 larva from the *ku80* knock-in experiment. The arrows indicate the GFP-positive patches. Scale bar = 1 mm. (B-D') GFP expression in the G1 embryos. (B, C, D) show white light images and (B', C', D') show fluorescent images of embryos from broods 1, 2, and 3 in each case. The GFP-positive embryos are marked by arrowheads. Scale bar = 1 mm.

Table 2. Summary of *ku80* knock-in experiments

G0					
Eggs injected	Hatched larvae	Larvae for which GFP expression was observed	GFP-positive larvae	Adults crossed with non-injected individuals	
264	126	104	91	69	
G1					
Broods with GFP-positive embryos	Total number of GFP-positive embryos	Individuals surviving to pupal stage	Genotyped individuals	Knock-in into *ku80* locus (5' junction)	Knock-in into *ku80* locus (3'junction)
3	26	18*	18	4	6

*Including 4 individuals that died during the pupal stage.

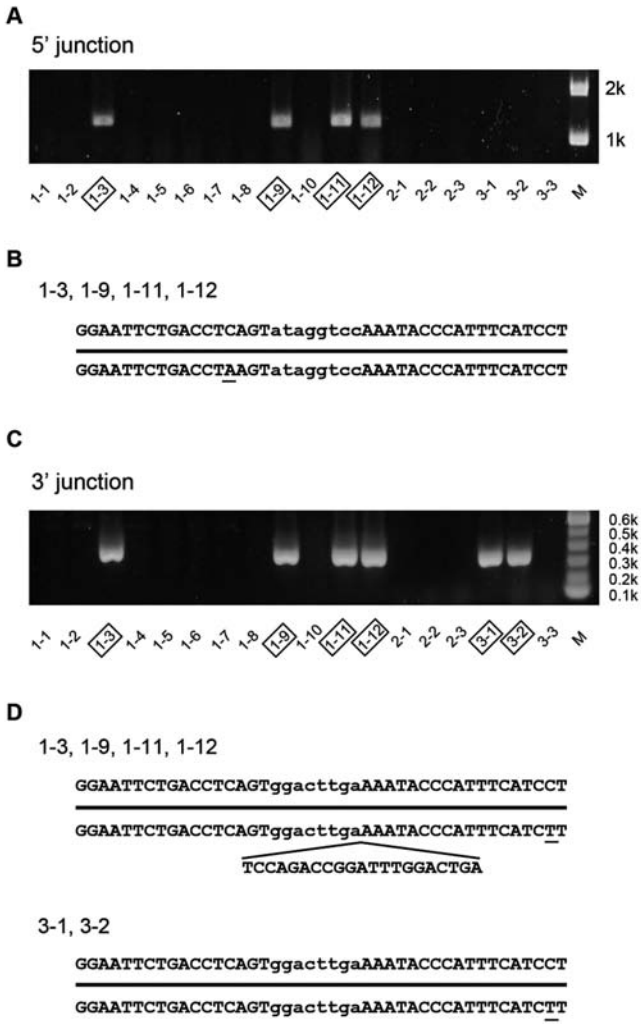

Fig. 5. Genotyping of *ku80* knock-in individuals. (A, B) Analysis of the 5' junction. (A) Gel image after electrophoresis. The PCR product was detected for individuals 1-3, 1-9, 1-11 and 1-12 (marked by boxes). Individuals 1-1 ~ 1-12 are derived from brood 1, 2-1 ~ 2-3 are from brood 2 and 3-1 ~ 3-3 from brood 3. M indicates the size standard. (B) Sequencing analysis of the knock-in individuals. The predicted sequence is shown above the bar and the determined sequence is shown below. Note that precise knock-in is occurring. The putative polymorphic nucleotide is underlined. (C, D) Gel electrophoresis (C) and sequencing analysis (D) of the 3' junction. In this case, the PCR product was detected for individuals 1-3, 1-9, 1-11, 1-12, 3-1 and 3-2 (C). Insertion of an extra 21 nucleotides was detected for 1-3, 1-9, 1-11 and 1-12, whereas for 3-1 and 3-2 precise knock-in was occurring (D). The putative polymorphic nucleotide is underlined.

Inheritance of the integrated donor vector in the G2 generation

GFP expression was examined in G2 individuals to determine whether the integrated donor vector is maintained stably. G1 adults 1-3 and 1-9 were crossed with *w-c* females and GFP expression was examined in their progenies. Both broods included embryos with strong and ubiquitous GFP expression, and importantly, the GFP-positive and negative embryos segregated in a ratio close to 1:1 (Table 3). Thus, the integrated donor vector can be maintained stably and inherited in a Mendelian manner.

Table 3. Ratio of GFP-positive and negative embryos in G2

brood name	GFP positive embryos	GFP negative embryos
1-3	27	37
1-9	57	78

DISCUSSION

In this study we found that the TAL-PITCh system can induce the targeted integration of a donor vector into the *ku80* locus. This technique was previously shown to allow the efficient knock-in of the *BLOS2* gene (Nakade *et al.*, 2014), and thus this is the second successful use of PITCh in the silkworm. The knock-in efficiency appears to be comparable for the two genes; for *BLOS2* the microinjection of 181 embryos resulted in the establishment of six knock-in individuals, whereas for *ku80* we obtained four individuals from the injection of 264 embryos (Table 2; Nakade *et al.*, 2014). The obtained *ku80* knock-in strain can possibly be utilized for the functional analysis of this gene, for example, whether its mutation can contribute to the increase of the efficiency of homologous recombination-mediated knock-in. We consider that the PITCh system is applicable to a wide variety of research objectives. Insertion of a marker gene during the production of the knockout individuals would make the keeping of the strains much more efficient. Without such markers, genotyping by PCR is required in every generation when the strains have to be kept as heterozygotes, as is the case when disruption of the target gene causes lethality. Knock-in of a reporter gene, such as GFP, in frame with the endogenous gene permits the easy monitoring of the gene of interest. The technique can also be applied to practical aims such as the optimization of recombinant protein production, by inserting a gene into a highly active locus such as *sericin* or *fibroin*. The PITCh system occasionally causes the insertion of extra nucleotides, especially at the 3' junction (Fig. 5D; Nakade *et al.*, 2014; Sakuma *et al.*, 2015), but this does not pose a problem for such applications. Currently the reason why the extra nucleotide insertion is frequently observed just for the 3' junction is unknown. In this study, the *hsp90* promoter-GFP was used as a marker, which allowed the detection of knock-in as early as the G0 larvae (Fig. 4A). Thus, the use of such a marker makes it possible to know in advance whether the integration is induced efficiently.

We and other groups have previously succeeded in the site-specific integration of a transgene via the phiC31-integrase-mediated technique (Yonemura *et al.*, 2012, 2013; Long *et al.*, 2013; Yin *et al.*, 2014). In this system, the tar-

get site 'attP' is pre-integrated into the host genome and a donor vector harboring 'attB' sequence can then be inserted at this site. Compared to this method, PITCh is advantageous in that the pre-integration step is not necessary. This offers a reduction in the labor necessary to generate and maintain the attP strain, which is a significant benefit in silkworm studies. On the other hand, the phiC31-integrase method has the following two merits: firstly, it allows the integration of a large-sized vector such as the bacterial artificial chromosome (Bac) (Venken *et al.*, 2006), and secondly, it offers cassette exchange integration that permits donor vector incorporation without inserting the vector backbone (Long *et al.*, 2013). The latter can also be accomplished by CRISPR/Cas9-mediated PITCh (Nakade *et al.*, 2014), but whether TAL-PITCh can also mediate this reaction has yet to be confirmed. Therefore, we consider that both the PITCh and integrase techniques should be improved further for use in sericultural studies.

We have here demonstrated the versatility of TALEN-mediated knock-in in the silkworm. Nevertheless, the CRISPR/Cas9 system offers advantages due to its simplicity, and thus we are now in the process of developing CRISPR/Cas9-mediated knock-in in this species.

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