Proof of principle for piggyBac-mediated transgenesis in the flatworm

Macrostomum lignano

Kirill Ustyantsev,* Jakub Wudarski,† Igor Sukhikh,* Filipa Reinoite,‡ Stijn Mouton,‡ Eugene Berezikov*,‡

*Institute of Cytology and Genetics SB RAS, Novosibirsk, 630090, Russia.
†Laboratory of Biological Diversity, National Institute for Basic Biology, Okazaki 444-8585 Aichi, Japan.
‡European Research Institute for the Biology of Ageing, University of Groningen, University Medical Center Groningen, Groningen, 9700AD, The Netherlands.

These authors contributed equally to this work.

Corresponding author: Institute of Cytology and Genetics SB RAS, Prospekt Lavrentyeva 10, Novosibirsk, 630090, Russia. E-mail: eberez@bionet.nsc.ru

ABSTRACT

Regeneration-capable flatworms are informative research models to study the mechanisms of stem cell regulation, regeneration, and tissue patterning. The free-living flatworm Macrostomum lignano is currently the only flatworm where stable transgenesis is available, and as such it offers a powerful experimental platform to address questions that were previously difficult to answer. The published transgenesis approach relies on random integration of DNA constructs into the genome. Despite its efficiency, there is room and need for further improvement and diversification of transgenesis methods in M. lignano. Transposon-mediated transgenesis is an alternative approach, enabling easy mapping of the integration sites and the possibility of insertional mutagenesis studies. Here, we report for the first time that transposon-mediated transgenesis using piggyBac can be performed in M. lignano to create stable transgenic lines with single-copy transgene insertions.

KEYWORDS piggyBac, transposons, transgenesis, flatworms, Macrostomum lignano
Introduction

*Macrostomum lignano* is a free-living flatworm that is gaining attention as a powerful model organism. Thanks to its high regeneration capabilities and the availability of a robust transgenesis method, it can be used as a testbed in many research areas, including stem cell and germline biology, regeneration, and ageing (Wudarski et al. 2020). Although the current published protocol for transgenesis by random integration of DNA constructs in *M. lignano* is easy to implement and efficient, it has several disadvantages such as high propensity to form tandem insertions, which are hard to map and can potentially affect the stability of the inserts due to recombination (Wudarski et al. 2017). Another drawback of the current random integration approach is the use of irradiation. Mild exposure to gamma rays causes double strand breaks in the DNA, stimulating the repair mechanisms of the cell, and is used to increase the efficiency of integration of transgenes in the genome (Wudarski et al. 2017). However, the damage inflicted to the DNA can introduce alterations in the genome that are difficult to detect and correct.

DNA transposons such as *Sleeping Beauty* (Aronovich et al. 2011; Song et al. 2012), *Tol2* (Urasaki et al. 2008), *Mos1* (Frokjaer-Jensen et al. 2014), and *piggyBac* (Yusa 2015) are widely used as vectors for nonviral gene delivery in diverse animal models. Compared to random integration methods, mainly single-copy transposon insertions are easily tractable and also reversible, i.e. can be removed afterwards if desired (Izsvák and Ivics 2004; Frøkjær-Jensen et al. 2008; Lacoste et al. 2009). Additionally, transposons offer opportunities for forward genetics studies, including insertional mutagenesis and trapping and mapping of functional DNA regulatory elements such as promoters, enhancers, and polyadenylation signals (Kawakami et al. 2004; Bonin and Mann 2004; Boulin and Bessereau 2007; Rad et al. 2010; Song et al. 2012; Casandra et al. 2018).

In this proof of principle study, we report transposon-mediated integration of *piggyBac*-derived genetic constructs in *M. lignano* using both the original and the hyperactive versions of the *piggyBac* transposase. We demonstrate that this method results in stable single-copy insertions with a frequency which is acceptable for practical applications.

Materials and Methods

*M. lignano lines and culture conditions*

The wild-type NL12 line was previously described (Wudarski et al. 2017). Animals were cultured in laboratory conditions in plastic Petri dishes (Greiner), filled with nutrient-enriched artificial
sea water (Guillard’s f/2 medium). Worms were fed ad libitum with the unicellular diatom *Nitzschia curvilineata* (Heterokontophyta, Bacillariophyceae) (SAG). Climate chamber conditions were set on 20 °C with constant aeration and a 14/10 h day/night cycle. Cultures designated for microinjection experiments were prepared as previously described (Wudarski *et al.* 2017). To speed-up the development of transgenic lines, microinjected eggs and the subsequent progeny were kept at 25 °C under otherwise the same conditions (Wudarski *et al.* 2019).

### mRNA synthesis and preparation of transgenic constructs

Codon-optimized sequences of the original (PBase) and the hyperactive (hyPBase) *piggyBac* transposases were designed using the previously established codon optimization algorithm (Wudarski *et al.* 2017) and the published sequences (Cary *et al.* 1989; Yusa *et al.* 2011). The designed sequences were commercially synthesized as gBlocks (IDT) and cloned into the pGEM-T-Easy backbone (Promega) under the *M. lignano* HSP20 promoter and followed by the *M. lignano* EF1a 3' UTR. The resulting plasmids JP4 and JP5 (Figure S1) can in principle be used to generate transgenic *M. lignano* lines with inducible transposase expression, similar to the previous heat shock inducible *M. lignano* constructs (Wudarski *et al.* 2019), but were only used in this study as PCR templates for *in vitro* synthesis of transposase mRNA. During the PCR, the T7 promoter sequence was added to the forward primer, and the product was used as a template for *in vitro* transcription. The reaction was carried out using the HiScribe T7 ARCA mRNA Kit with tailing (NEB) according to the manufacturer’s instructions.

A plasmid containing *piggyBac* transposon 5' and 3' termini was made by cloning the commercially synthesized termini sequences (gBlocks, IDT) into the pGEM-T-Easy backbone (Promega). Two donor plasmid constructs were generated by cloning *(long)EFa::mNeonGreen* (JW88), and *(short)EFa::mNeonGreen* (KU75) fragments between the *piggyBac* termini. For the JW88 plasmid, a negative selection *DLG4::mScarlet-I* cassette was additionally cloned upstream of the transposon sequence in the *NcoI* site. See Figure S1 for full transgene sequences and annotations.

### Microinjections, PCR screening, and insertion site identification

All microinjections were carried out following the previously published protocol (Wudarski *et al.* 2017). Only fresh, single-cell stage *M. lignano* embryos were used. Micromanipulations were done using either a microinjection stage equipped with AxioVert A1 inverted microscope (Carl Zeiss), PatchMan NP2, TransferMan NK2, FemtoJet express, and PiezoXpert (Eppendorf) or a microinjection...
stage equipped with PrimoVert inverted microscope (Carl Zeiss), Narishige MO-202 micromanipulators, and OpenSpritzer in-house build microinjector (Forman et al. 2017).

We co-injected PBase mRNA with the JW88 donor plasmid (molar ratio 10:1, final concentrations 50 ng/μl and 15 ng/μl respectively) or hyPBase mRNA with the KU75 donor plasmid (molar ratio 2:1, final concentrations 45 ng/μl and 20 ng/μl respectively) into single-cell stage M. lignano embryos. The resulting hatchlings were screened for the presence of fluorescent signal. In case of the KU75 plasmid, all hatchlings positive for mNeonGreen expression were selected. For the JW88 plasmid, which contains the additional negative selection marker DLG4::mScarlet-I, only mNeonGreen-positive/mScarlet-I-negative worms were selected, while double-positives animals were discarded. The selected hatchlings (P0) were paired with single wild-type NL12 worms that were raised in the same conditions. The worm pairs were transferred to fresh food every 2 weeks. For each cross, mNeonGreen-positive F1 animals were selected and put together on fresh food, transferred to separate dishes, and allowed to propagate. The F2 populations were selected bi-weekly and only the mNeonGreen-positive worms were kept. When 200 positive worms were obtained, half of each population was sacrificed for genomic DNA extraction using the QIAamp DNA Mini kit (QIAGEN) needed for subsequent PCR screens. The rest of the worms were kept to establish stable cultures.

Genomic DNA samples from the F2 mNeonGreen-positive worms were first screened by PCR to check for the retention of the plasmid backbone flanking the transposon cassette on both sides. In cases where no plasmid backbone was detected, we proceed to map the insertion sites.

Genomic locations and flanking sequences of the inserted piggyBac-derived constructs were obtained using Palindromic sequence-targeted (PST) PCR for the NL30 line and by inverse PCR for the NL31 and NL32 lines following the published protocols (Frokjaer-Jensen et al. 2014; Kalendar et al. 2019). Sanger sequencing of the gel-purified PST-PCR/inverse PCR products was done either by an external company (Eurofins, Ebersberg, Germany) or using the Big Dye Terminator V. 3.1. Cycle Sequencing Kit (Applied Biosystems). Products of sequencing reactions were analyzed using the ABI 3130xl Genetic Analyzer (Genomics Core Facility, ICBFM SB RAS, Novosibirsk, Russia), and the resulting sequences were mapped to the Mlig_3_7 M. lignano genome assembly (Wudarski et al. 2017). The insertion locations were additionally verified by PCR using primers specific for the M. lignano genomic DNA and the piggyBac transposon termini. Sequences of all primers used in the study are provided in Table S1.
**Microscopy and imaging**

Selection of positive transgenic worms and all imaging was performed using a Zeiss Axio Zoom V16 microscope with an HRm digital camera and Zeiss filter sets 38HE (FITC) and 43HE (dsRed) at the Joint Center for Microscopy of Biological Objects, of the Institute of Cytology and Genetics, SB RAS (Novosibirsk, Russia). For the imaging, worms were first starved for 48 hours, and then relaxed in 7.14% MgCl₂ * 6H₂O solution in Guillard’s f/2 medium until they stopped moving. To bring all the organs to the focus, the relaxed worms were put in a drop of the MgCl₂ solution on the bottom of a plastic Petri dish, and the liquid was slowly removed until the worms became gently squeezed. The images were arranged for publication using ImageJ v. 1.53c and GIMP v. 2.10.18.

**Results and Discussion**

Using the microinjection approach, we delivered two sets of piggyBac-derived genetic constructs, JW88 and KU75, together with PBase or hyPBase mRNA into single-cell stage eggs of *M. lignano* (Table 1). Both constructs contained the sequence coding the positive selection transgenic marker **EF1a::mNeonGreen** between the piggyBac termini (Figure 1A). In these constructs, **mNeonGreen** is expressed under the control of a ubiquitous promoter of the *M. lignano* elongation factor alpha 1 gene (**EF1a**) with its 5'UTR, and followed by the **EF1a** 3'UTR sequence. The two constructs differed as follows: (1) JW88 had a longer, 1309 bp, version of the **EF1a** promoter region together with the 5'UTR, (**long**)EF1a, as originally described (Wudarski et al. 2017), while for the KU75 plasmid it was shortened to 465 bp, (**short**)EF1a; (2) JW88 had a negative selection cassette **DLG4::mScarlet-I** cloned outside of the transposon terminal repeats (Figure 1A). The negative selection cassette in JW88 served as a control to discriminate between actual piggyBac-mediated transposition and random integration in our initial experiments (Figure S2A). The KU75 (Figure 1A, Figure S1) construct was made as an alternative to JW88 to decrease both the cargo and overall plasmid size, which resulted in its easier propagation in *E. coli*, and also lowered the extent of homology to the *M. lignano* genome, reducing potential interference.

Microinjection of 436 *M. lignano* eggs with the JW88/PBase mix over the course of 4 weeks resulted in a single green-only germline transmitting P0 worm (31 worms simultaneously positive for red and green were excluded from subsequent crossing experiments). Microinjection of 393 eggs with the KU75/hyPBase mix within 3 weeks resulted in 4 **mNeonGreen** positive transmitting founders.
Offspring of one of the KU75-based founders were visibly segregated by brightness of the green signal and were therefore split in two groups, resulting in a total of five mNeonGreen KU75 positive lines. Subsequent PCR screening of the F2 progeny for the retention of the plasmid sequences flanking the transposon insertions showed that two KU75-based lines had negative PCR results. The three other lines were positive (Table 1) and hence likely derived from random integrations of the KU75 plasmid (Figure S2B).

To map genomic sites of transgene insertions in the candidate piggyBac-derived lines (NL30, NL31 and NL32), we next used a combination of PST-PCR (Kalendar et al. 2019) and inverse PCR (Frokjaer-Jensen et al. 2014). We obtained the genomic sequences flanking the transgene insertions in all the transgenic lines, and mapped transgene insertion sites in the M. lignano genome assembly (Figure 2). In all three cases, we observed insertion patterns consistent with single-copy integration of the piggyBac-derived transgenes in M. lignano (Figure 2, Figure S3). For the NL31 and NL32 lines (KU75 with hyPBase) the insertion site and the target site duplications (TSDs) sequences flanking the transposon insertion were the canonical TTAA (Yusa 2015), while in the line NL30 (JW88 with PBase) the insertion site was ATAT, indicating a non-canonical insertion (Figure 2). These analyses confirm that the established transgenic lines are indeed the result of piggyBac transposon activity. Furthermore, the inserted transgenes are stably transmitted through the germline, since their expression has remained stable for more than 10 generations for the NL30 line, and for more than 3 generations for the NL31 and NL32 lines.

Interestingly, the non-canonical target site duplication in the NL30 line is also asymmetric, with TTAA at the 5’ end and ATAT at the 3’ end (Figure 2). The observed target site duplication asymmetry can be explained if the mismatch at the 5’ end is repaired to the canonical piggyBac TTAA site while the 3’ end mismatch is repaired to the host genome variant ATAT (Figure S4). Whether this is a frequent or rare integration site in the case of piggyBac activity in M. lignano is still to be determined. Previous research in human embryonic stem cells showed that non-canonical insertions of piggyBac can happen in 2% of the integration cases, and that the mismatch in the sequence is repaired by the repair mechanism of the host cell (Li et al. 2013). Deviation from the canonical insertion pattern might indicate that some M. lignano cellular factors can somehow intervene with the transposition process through interaction with the transposase/transposon nucleoprotein complexes (Feschotte 2006; Kolacsek et al. 2014).
All three piggyBac-derived lines express mNeonGreen at visibly lower levels than the NL31a line resulted from random integration, which corresponds to the expected low number of transgene copies integrated by transposition. Importantly, there was no significant difference in either brightness or expression pattern between the NL30 and NL32 lines, which are derived from different donor constructs (Figure 1B). This suggests that the shorter version of the EF1a promoter has all required regulatory elements and can thus be used instead of the longer version. The third piggyBac-based line, NL31, showed an overall lower expression level of mNeonGreen and lacks expression in the ovaries and developing eggs when compared to NL32, which is based on the same KU75 construct (Figure 1B). This difference in the expression patterns is most likely explained by the insertion position effect and emphasizes the need of generating multiple transgenic lines when investigating expression patterns of different promoters.

Here, we showed that both variants of the codon-optimized piggyBac transposases PBase (the original insect sequence) and hyPBase (the artificial variant with 7 amino acid mutations) (Cary et al. 1989; Yusa et al. 2011) are active in M. lignano (Figure 1). Based on the previous studies in mammals, hyPBase should have demonstrated several fold higher excision and integration efficiencies compared to PBase (Yusa et al. 2011; Burnight et al. 2012). However, we did not observe significantly higher number of piggyBac-derived transgenic M. lignano worms with hyPBase compared to PBase (Table 1). Therefore, we cannot conclude that hyPBase has higher efficiency compared to PBase in our setting, although optimization of transposase to transposon ratio might be the issue (Wu et al. 2006) and will be the subject of further optimization.

Although using a plasmid construct with a negative selection marker (like JW88) can potentially save a lot of time on the subsequent screening of transposon-derived insertions, the approach has several caveats. Apart from the more difficult propagation of plasmids of larger size in E. coli and the potential interference between regulatory elements, there is no guarantee that the plasmid will not be linearized somewhere in the negative selection marker sequence, which would lead to the absence of its expression and, thus, to false positive conclusion that the event was piggyBac-derived. It also appears that it is possible to distinguish a high-copy random integration event from a single-copy transposon insertion by eye, as the latter appears evidently dimmer (Figure 1B). Therefore, shorter donor vectors like KU75 may be more beneficial for future applications of piggyBac-based transgenesis in M. lignano.
Availability of data and materials

All data generated or analyzed during this study are included in this published article and its supplementary information files. All plasmids and *M. lignano* worm lines generated in this study are available from the corresponding author on reasonable request.

Funding

The work of Kirill Ustyantsev and Eugene Berezikov on construction of the KU75-based piggyBac-derived transgenic constructs, generation, and the analysis of transgenic worms was supported by the Russian Science Foundation grant № 20-14-00147 and performed in Institute of Cytology and Genetics SB RAS. General maintenance of worm cultures was performed in Institute of Cytology and Genetics SB RAS by Igor Sukhikh and supported by the State budget project 0259-2021-0013. The work on generating JW88-based transgenic animals was performed at the European Institute for the Biology of Ageing and supported by NWO ENW KLEIN grant (OCENW.KLEIN.054) to Eugene Berezikov. Filipa Reinoite was supported by the University of Groningen GSMS Fellowship.

Literature Cited

Aronovich E. L., R. S. McIvor, and P. B. Hackett, 2011 The *Sleeping Beauty* transposon system: a non-viral vector for gene therapy. Human molecular genetics 20: R14-20. https://doi.org/10.1093/hmg/ddr140

Bonin C. P., and R. S. Mann, 2004 A piggyBac transposon gene trap for the analysis of gene expression and function in Drosophila. Genetics 167: 1801–1811. https://doi.org/10.1534/genetics.104.027557

Boulin T., and J.-L. Bessereau, 2007 Mos1-mediated insertional mutagenesis in *Caenorhabditis elegans*. Nat Protoc 2: 1276–1287. https://doi.org/10.1038/nprot.2007.192

Burnight E. R., J. M. Staber, P. Korsakov, X. Li, B. T. Brett, *et al.*, 2012 A Hyperactive Transposase Promotes Persistent Gene Transfer of a piggyBac DNA Transposon. Molecular Therapy - Nucleic Acids 1. https://doi.org/10.1038/mtna.2012.12

Cary L. C., M. Goebel, B. G. Corsaro, H.-G. Wang, E. Rosen, *et al.*, 1989 Transposon mutagenesis of baculoviruses: Analysis of *Trichoplusia ni* transposon IFP2 insertions within the FP-locus of nuclear polyhedrosis viruses. Virology 172: 156–169. https://doi.org/10.1016/0042-6822(89)90117-7
Casandra D., J. Oberstaller, R. H. Y. Jiang, I. F. Bronner, J. H. Adams, et al., 2018 Uncovering the essential genes of the human malaria parasite *Plasmodium falciparum* by saturation mutagenesis. Science 360: eaap7847. https://doi.org/10.1126/science.aap7847

Feschotte C., 2006 The piggyBac transposon holds promise for human gene therapy. PNAS 103: 14981–14982. https://doi.org/10.1073/pnas.0607282103

Forman C. J., H. Tomes, B. Mbobo, R. J. Burman, M. Jacobs, et al., 2017 Openspritzer: an open hardware pressure ejection system for reliably delivering picolitre volumes. Scientific Reports 7: 2188. https://doi.org/10.1038/s41598-017-02301-2

Frøkjær-Jensen C., M. Wayne Davis, C. E. Hopkins, B. J. Newman, J. M. Thummel, et al., 2008 Single-copy insertion of transgenes in *Caenorhabditis elegans*. Nature Genetics 40: 1375–1383. https://doi.org/10.1038/ng.248

Izsvák Z., and Z. Ivics, 2004 *Sleeping Beauty* Transposition: Biology and Applications for Molecular Therapy. Molecular Therapy 9: 147–156. https://doi.org/10.1016/j.ymthe.2003.11.009

Kalendar R., A. V. Shustov, M. M. Seppänen, A. H. Schulman, and F. L. Stoddard, 2019 Palindromic sequence-targeted (PST) PCR: a rapid and efficient method for high-throughput gene characterization and genome walking. Scientific Reports 9: 17707. https://doi.org/10.1038/s41598-019-54168-0

Kawakami K., H. Takeda, N. Kawakami, M. Kobayashi, N. Matsuda, et al., 2004 A Transposon-Mediated Gene Trap Approach Identifies Developmentally Regulated Genes in Zebrafish. Developmental Cell 7: 133–144. https://doi.org/10.1016/j.devcel.2004.06.005

Kolacsek O., Z. Erdei, Á. Apáti, S. Sándor, Z. Izsvák, et al., 2014 Excision Efficiency Is Not Strongly Coupled to Transgenic Rate: Cell Type-Dependent Transposition Efficiency of *Sleeping Beauty* and piggyBac DNA Transposons. Hum Gene Ther Methods 25: 241–252. https://doi.org/10.1089/hgtb.2013.149

Lacoste A., F. Berenshteyn, and A. H. Brivanlou, 2009 An Efficient and Reversible Transposable System for Gene Delivery and Lineage-Specific Differentiation in Human Embryonic Stem Cells. Cell Stem Cell 5: 332–342. https://doi.org/10.1016/j.stem.2009.07.011

Li M. A., S. J. Pettitt, S. Eckert, Z. Ning, S. Rice, et al., 2013 The piggyBac transposon displays local and distant reintegration preferences and can cause mutations at noncanonical integration sites. Molecular and cellular biology 33: 1317–30. https://doi.org/10.1128/MCB.00670-12

Rad R., L. Rad, W. Wang, J. Cadinanos, G. Vassiliou, et al., 2010 PiggyBac transposon mutagenesis: a tool for cancer gene discovery in mice. Science 330: 1104–1107. https://doi.org/10.1126/science.1193004

Song G., Q. Li, Y. Long, Q. Gu, P. B. Hackett, et al., 2012 Effective Gene Trapping Mediated by Sleeping Beauty Transposon. PLOS ONE 7: e44123. https://doi.org/10.1371/journal.pone.0044123

Downloaded from https://academic.oup.com/genetics/advance-article/doi/10.1093/genetics/iyab076/6276877 by guest on 21 May 2021
Urasaki A., K. Asakawa, and K. Kawakami, 2008 Efficient transposition of the Tol2 transposable element from a single-copy donor in zebrafish. PNAS 105: 19827–19832. https://doi.org/10.1073/pnas.0810380105

Wu S. C.-Y., Y.-J. J. Meir, C. J. Coates, A. M. Handler, P. Pelczar, et al., 2006 piggyBac is a flexible and highly active transposon as compared to Sleeping Beauty, Tol2, and Mos1 in mammalian cells. PNAS 103: 15008–15013. https://doi.org/10.1073/pnas.0606979103

Wudarski J., D. Simanov, K. Ustyantsev, K. de Mulder, M. Grelling, et al., 2017 Efficient transgenesis and annotated genome sequence of the regenerative flatworm model Macrostomum lignano. Nature Communications 8: 2120. https://doi.org/10.1038/s41467-017-02214-8

Wudarski J., K. Ustyantsev, L. Glazenburg, and E. Berezikov, 2019 Influence of temperature on development, reproduction and regeneration in the flatworm model organism, Macrostomum lignano. Zoological Letters 5: 7. https://doi.org/10.1186/s40851-019-0122-6

Wudarski J., B. Egger, S. A. Ramm, L. Schärer, P. Ladurner, et al., 2020 The free-living flatworm Macrostomum lignano. EvoDevo 11: 5. https://doi.org/10.1186/s13227-020-00150-1

Yusa K., L. Zhou, M. A. Li, A. Bradley, and N. L. Craig, 2011 A hyperactive piggyBac transposase for mammalian applications. PNAS 108: 1531–1536. https://doi.org/10.1073/pnas.1008322108

Yusa K., 2015 piggyBac Transposon. Microbiol Spectr 3: MDNA3-0028–2014. https://doi.org/10.1128/microbiolspec.MDNA3-0028-2014

### Tables

#### Table 1

| Construct/Transposase                  | Number of batches\(^a\) | Eggs injected | Positive founders | Randomly integrated\(^c\) | piggyBac-derived |
|---------------------------------------|--------------------------|---------------|------------------|---------------------------|-----------------|
| (long) EF1α::mNeonGreen (JW88)/PBase  | 4                        | 436           | 1                | -                         | 1               |
| (short) EF1α::mNeonGreen (KU75)/hyPBase | 3                        | 393           | 4\(^b\)          | 3\(^b\)                    | 2\(^b\)         |

\(^a\) one batch corresponds to one week of injections with ~80-150 eggs injected per week.

\(^b\) there was segregation by the transgene expression pattern and brightness in positive offspring of one of the founders.
for JW88/PBase, progeny positive for DLG4::mScarlet-I (Figure S2A) were not analyzed for the transgene transmission. For the KU75/hyPBase, the number is based on the PCR screening for the KU75 plasmid retention (Figure S2B).

**Figure Legends**

**Figure 1.** PiggyBac-mediated transgenesis in *M. lignano*. (A) Schematics of the piggyBac-derived donor plasmids JW88 and KU75 used in this study. Blocks with thin arrows above denote the promoters with 5'UTR regions, and the directions of the arrows reflect the orientation of a gene cassette in the plasmids. 3'UTR regions are not shown. Orange arrow-shaped blocks correspond to the 5' and 3' piggyBac termini. Full plasmid structures and sequences can be found in Figure S1. (B) Differences in mNeonGreen fluorescent signal pattern and intensity between piggyBac-derived transgenic lines and a random integration-derived line. Three channels are shown - brightfield, FITC (green), and their combination. Exposure time for the FITC channel was set to 100 ms in all the cases except for the random integration-derived line NL32a (25 ms, marked with asterisk). NL12 - non-transgenic wild type line. All pictures were taken on the same day under the same magnification, differences in size are due to variation in squeeze preparations and age of the worms. Scale bar is 100 μm.

**Figure 2.** Genomic locations, flanking sequences, and target site duplications (TSDs) of the piggyBac-derived transgene insertions. Partial Sanger sequence traces of PST-PCR/inverse PCR products are shown with annotations on top of the sequences. Wave-shaped lines correspond to the genomic sequences flanking the insertion sites. Mlig_3_7 genomic coordinates are given on the top of the wave-shaped lines, and orientations of the insertions are indicated in parentheses.
A  

JW88 donor plasmid (7413 bp)

- 5' pBac
- (long)EF1a
- mNeonGreen
- 3' pBac

mScarlet-I  
DLG4  

KU75 donor plasmid (5019 bp)

- 5' pBac
- (short)EF1a
- mNeonGreen
- 3' pBac

B  

| piggyBac-derived | rand. int. |
|------------------|------------|
| JW88            | KU75       |
| NL30            | NL31       | NL32 | NL32a | NL12 |
|                 |            |      |       |      |

Downloaded from https://academic.oup.com/genetics/advance-article/doi/10.1093/genetics/iyab076/6276877 by guest on 21 May 2021
NL30
scaf1687:202592 - 202611 (+)

TSD

5' piggyBac

GAGTTAGATTAAGACTGGCT

3' piggyBac

TSD

scaf1687:202616 - 202639 (+)

NL31
scaf224:187249 - 187269 (-)

TSD

5' piggyBac

TTAGAATGACAATTACG

3' piggyBac

TSD

scaf224:187223 - 187245 (-)

NL32
scaf808:297777 - 297796 (+)

TSD

5' piggyBac

GATCAGTACAGAAGCT

3' piggyBac

TSD

scaf808:297801 - 297818 (+)