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journal homepage: www.elsevier.com/locate/bbrepLocus-specific integration of extrachromosomal transgenes in *C. elegans* with the CRISPR/Cas9 systemSawako Yoshina^a, Yuji Suehiro^a, Eriko Kage-Nakadai^{a,b}, Shohei Mitani^{a,c,*}^a Department of Physiology, Tokyo Women's Medical University School of Medicine, Tokyo, Japan^b The OCU Advanced Research Institute for Natural Science and Technology, Osaka City University, Osaka, Japan^c Tokyo Women's Medical University Institute for Integrated Medical Sciences, Japan

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ABSTRACT

We established a method to generate integration from extrachromosomal arrays with the CRISPR/Cas9 system. Multi-copy transgenes were integrated into the defined loci of chromosomes by this method, while a multi-copy transgene is integrated into random loci by previous methods, such as UV- and gamma-irradiation. The effects of a combination of sgRNAs, which define the cleavage sites in extrachromosomes and chromosomes, and the copy number of potential cleavable sequences were examined. The relative copy number of cleavable sequences in extrachromosomes affects the frequency of fertile F1 transgenic animals. The expression levels of the reporter gene were almost proportional to the copy numbers of the integrated sequences at the same integration site. The technique is applicable to the transgenic strains abundantly stored and shared among the *C. elegans* community, particularly when researchers use sgRNAs against common plasmid sequences such as β -lactamase.

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1. Introduction

To perform molecular genetic analyses using a model organism, techniques for generating transgenic animals are essential. In *C. elegans* transgenic animals are often obtained using DNA micro-injection into the hermaphrodite gonad, which generates multi-copy extrachromosomal (Ex) arrays [1,2]. Extrachromosomal arrays can then be integrated into random loci of chromosomes by gamma-ray or ultraviolet irradiation [3,4], so that the integrated arrays contain a high copy-number of transgenes. A technique termed Mos1-mediated single-copy insertion (MosSCI) is often the method of choice to insert a single-copy transgene into a defined location in the chromosomes of *C. elegans* [5,6]. Extrachromosomal arrays can also be integrated into random loci of chromosomes by using UV/TMP, benzimidazole and temperature selection [7,8]. In these cases, integrated transgenes contain single/low-copy transgenes and are useful for genes with low expression levels. Recently, the CRISPR/Cas9 system, which can induce double-strand breaks at specific loci in genomic DNA using specific sgRNAs, has been successfully applied to *C. elegans* [9]. The system has been widely used to produce small indels [9]. In addition, the CRISPR/Cas9 system allows homologous recombination-mediated insertion of sequence tags such as GFP [10–12].

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In the present study, we developed a technique using the CRISPR/Cas9 system to produce integrations from extrachromosomal arrays. This method allows the insertion of multi-copy transgenes at desired loci in chromosomes. In addition, we also demonstrate transgene integration of a relatively small number of copies using the CRISPR/Cas9 system together with benzimidazole and temperature selection.

2. Materials and methods

2.1. Nematode strains

C. elegans strain N2 worms were used as the wild-type animals. Worms were grown at 20 °C under well-fed conditions using the standard methods [13]. Strains carrying the following mutations were obtained from a UV/TMP-mutagenized library as described previously [14] and identified by PCR amplification with primers spanning the deletion region of *ben-1(tm234)III* and *vps-45(tm246)X*, as described previously [14,15]. The primers used for PCR genotyping were as follows: *tm234_1stround*, 5'-ACGTGGAATGGAACCATGT-3', 5'-TCTCCATTTCTCTCTCC-3'; *tm234_2ndround*, 5'-CTCCGCATTTGTAACGGAA-3', 5'-CCCTCCATTTGAAAGAGTCC-3'; *tm246*, 5'-CGCAATTGGATACTACTTGT-3', 5'-TCTCTGCTCTACTTCTGCT-3'. The mutants were backcrossed four times with N2 before use.

2.2. Plasmid construction

We used site-directed mutagenesis to insert the guide sequences into a Cas9-sgRNA (single guide RNA) expression vector (pDD162) containing both sgRNA and Cas9 protein expression units, obtained through Addgene [11]. *dpy-3* was targeted for Cas9 cleavage using the guide sequence (TCACCGTCCAGTCTGCTAC) inserted into pDD162. *ben-1* was targeted for Cas9 cleavage using the guide sequence (AAGCAACTGCAGAGGAAGA) inserted into pDD162. The β -lactamase (ampicillin resistance) gene, which is contained in commonly used plasmids, was targeted for Cas9 cleavage using the guide sequence (TTAATAGACTGGATGGAGG) inserted into pDD162. We searched “CRISPR Design” (<http://crispr.mit.edu>) for potential off-target sequences [16]. The guide sequence of *dpy-3* had 1 candidate of off-target sites (0 are in genes, 4 mismatches (MMs), score of off-target: 0.0). The guide sequence of *ben-1* had 32 candidates of off-target sites (0 are in genes, 4 of 3MMs and 28 of 4MMs, max score of off-target: 1.5). The guide sequence of the β -lactamase gene had 7 candidates of off-target sites (0 are in genes, 7 of 4MMs, max score of off-target: 0.8). To generate a *dpy-3* genome fragment plasmid, pPD95.79_ *dpy-3*, a part of the *dpy-3* genome (491 bp), was amplified by PCR using the primers 5'-CGACTCTAGAGGATCCGAA-CAGTGAAGTAGACTTCTGCAAG-3' and 5'-CGCTCAGTTGGAATTC-CATCTTGCTGATGTACCGGCAT-3' with the N2 genome as a template. The fragment was inserted into the plasmid pPD95.79 using the *EcoRI* and *BamHI* sites.

The plasmids for transgenic markers, *myo-2p::Venus* and *dpy-7p::DsRed*, were generated as outlined previously [17]. The plasmids, *pGEMT_ben-1(+)*, *pFX_HBG_Lw_dpy-30p::NLS::GFP* and *eft-3p::vps-45* were constructed as described in a previous study [7].

2.3. Transgenic lines

Plasmids were injected using the standard *C. elegans* micro-injection procedures [1]. To generate *tmEx4252*, *tmEx4253* and *tmEx4277* transgenic animals, the *pPD95.79_dpy-3* genome was injected with *myo-2p::Venus* as an injection marker into N2 worms. The injection ratios of the mixed plasmids and the selection markers are listed in Table 1. We used the same *tmEx3199 [eft-3p::vps-45, pGEMT_ben-1(+), pFX_HBG_Lw_dpy-30p::NLS::GFP]* transgenic animals that were used in a previous study [7]. Images were taken with a BX-51 microscope (Olympus).

2.4. Generating integration lines using the CRISPR/Cas9 system

We injected Cas9-sgRNA plasmids with a selection marker, *dpy-7p::DsRed* or *myo-2p::DsRed*, into the extrachromosomal transgenic animals. The target genes for the CRISPR/Cas9 system and injected animals are listed in Table 1 and Supplementary Fig. 2B.

2.5. Quantitative PCR

Genomic DNA was isolated from adult animals using the DNeasy Tissue & Blood kit (QIAGEN). Quantitative PCR was performed in a 7500 Real-time Thermal cycler (Applied Biosystems) using the Power SYBR master mix (Applied Biosystems) with the following parameters: 95 °C for 10 min and 40 cycles of 95 °C for 5 s, 55 °C for 10 s and 72 °C for 34 s. All data were normalized to the *ama-1* gene. The primers used are listed in Supplementary Table 1.

2.6. Isolation of integration lines using the CRISPR/Cas9 system and the Dpy phenotype

Schematic diagram of the multi-copy integration is shown in the Fig. 1. Ex line marker (Venus/GFP)-positive worms (P0) were

Table 1
Summary of the multi-copy integration efficiencies into the *dpy-3* locus by using the CRISPR/Cas9 system.

Experiment no.	Parent strain	Cas9 target	No. injected ^a	total F1 ^b	No. F1 producing (viability %) ^c	No. F1 producing Dpy F2 ^d	No. producing Is (Allele name) ^e	Frequency (Is lines/P0 animals) %
Exp. 1	<i>tmEx4252[dpy-3 genome : myo-2p::Venus = 9 : 1]</i>	<i>dpy-3</i>	55	189	97 (51.3%)	34	2 (<i>tmIs1149, tmIs1151</i>)	3.6
Exp. 2	<i>tmEx4277[dpy-3 genome : myo-2p::Venus = 1 : 1]</i>	<i>dpy-3</i>	45	123	69 (56.1%)	15	2 (<i>tmIs1155, tmIs1156</i>)	4.4
Exp. 3	<i>tmEx4253[dpy-3 genome : myo-2p::Venus = 1 : 9]</i>	<i>dpy-3</i>	43	94	73 (77.6%)	27	4 (<i>tmIs1152, tmIs1154, tmIs1158, tmIs1162</i>)	9.3
Exp. 4	<i>tmEx4252[dpy-3 genome : myo-2p::Venus = 9 : 1]</i>	<i>dpy-3, β-lactamase gene</i>	40	35	14 (40.0%)	1	1 (<i>tmIs1150</i>)	2.5
Exp. 5	<i>tmEx3199[pFX_HBG_Lw_dpy-30p::NLS::GFP, pef1-3::vps-45, pGEMT_ben-1(+)]</i>	<i>dpy-3, β-lactamase gene</i>	50	65	48 (73.8%)	8	4 (<i>tmIs1157, tmIs1161, tmIs1163, tmIs1164</i>)	8.0
Exp. 6	<i>tmEx3199[pFX_HBG_Lw_dpy-30p::NLS::GFP, pef1-3::vps-45, pGEMT_ben-1(+)]</i>	<i>dpy-3</i>	32	114	82 (71.9%)	30	0	0.0

^a The number of injected parent Ex strains.

^b The number of Ex marker (*myo-2p::Venus* or *dpy-30p::NLS::GFP*)-positive and injection marker (*dpy-7p::DsRed* or *myo-2p::DsRed*)-positive F1 animals.

^c The number of F1 animals producing viable *DsRed*(+) or *DsRed*(-) F2 animals. The viability (No. F1 producing F2 / total F1) is described in parentheses.

^d The number of F1 animals which laid GFP/Venus(+) or GFP/Venus(-) Dpy animals in F2 progeny.

^e The number and the names of produced Is lines.

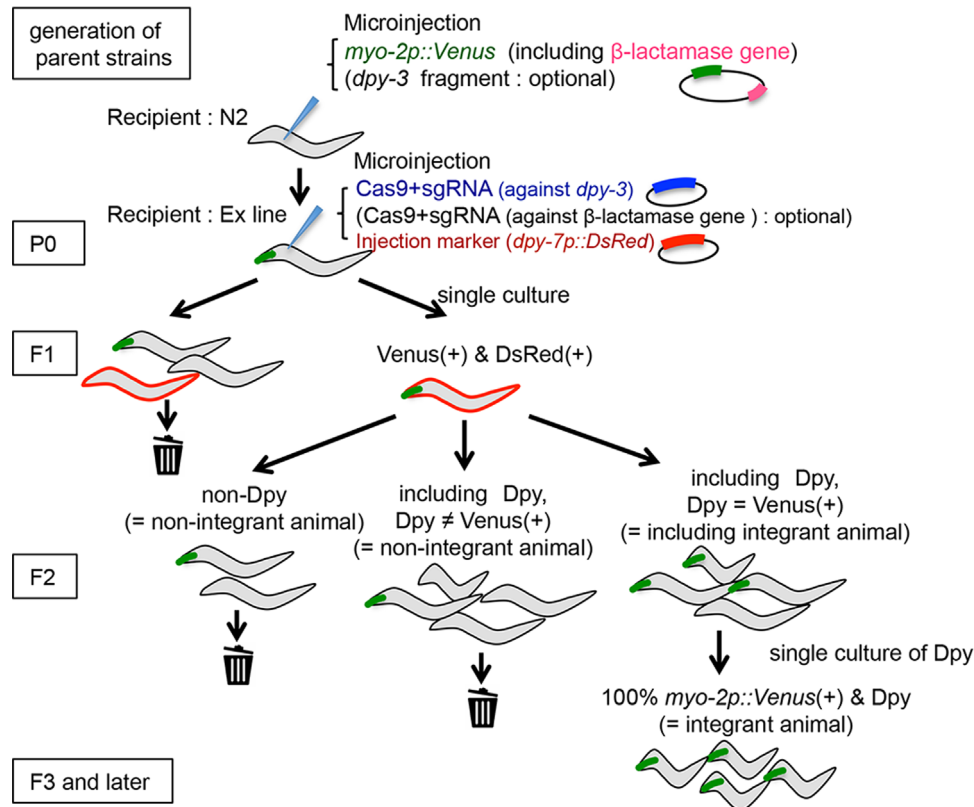


Fig. 1. Overview of multi-copy integration experiments by using the CRISPR/Cas9 system. An injection marker (*myo-2p::Venus* including β -lactamase gene) and a portion of the *dpy-3* genome sequence (optional) were co-injected into the N2 worms to generate transgenic strains carrying Ex arrays. Ex line marker (Venus)-positive worms (P0) were injected with the Cas9-sgRNA plasmid(s), against the *dpy-3* and/or β -lactamase gene(s), with an injection marker (*dpy-7p::DsRed*). Cas9-sgRNA plasmid(s) were designed to break extrachromosomal arrays and the integration locus. Both the parent Ex marker- and injection marker-positive F1 worms were singled to new plates. The lines that expressed Venus in all Dpy animals were selected as candidates for integrant (Is) strains. F2 Dpy worms in Is candidate strains were singled to new plates. Dpy worms that had the parent Ex marker in all the F3 or later progeny were selected as Is lines.

injected with the Cas9-sgRNA plasmid(s), against the *dpy-3* and/or β -lactamase gene(s), with another injection marker, *dpy-7p::DsRed* or *myo-2p::DsRed*. After a few days, both the parent Ex marker- and injection marker-positive F1 worms were singled to new plates. We selected the lines that expressed Venus/GFP in all Dpy animals. The strains harboring inserted parental extrachromosomal arrays into the genomic location of the *dpy-3* gene were selected in this process. We selected Dpy worms as insertion (Is) lines that had the parent Ex marker in all the F3 or later progeny. We confirmed that the Dpy phenotype did not segregate with fluorescence, and confirmed that transgenes were integrated at the *dpy-3* gene by PCR and sequencing (Supplementary Fig. 1). We performed PCR using purified genomic DNA of each Is strain as a template. We designed primer sets located within the *dpy-3* gene, which is not included in the extrachromosomal arrays, and the vectors, which is included only in the extrachromosomal arrays. The primers used in this experiment are listed in Supplementary Table 2. High transmitting Ex lines were produced in this experiment, but it was possible to exclude these Ex lines by our method.

2.7. Isolation of integration lines using the CRISPR/Cas9 system with the aid of benzimidazole, temperature and PCR selection

Schematic diagram of the integration is shown in the Supplementary Fig. 2A. *ben-1(tm234); vps-45(tm246); tmEx3199 [eft-3p::vps-45, pGEMT_ben-1(+), pFX_HBG_Lw_dpy-30p::NLS::GFP]* (P0) were injected with the Cas9-sgRNA plasmid, which targeted to the *ben-1* gene, and an injection marker, *myo-2p::DsRed*. After a few days, both the parent Ex marker (*dpy-30p::NLS::GFP*)- and the

injection marker-positive F1 worms were singled to new plates and incubated at 20 °C, while the F3 animals hatched. The F3 animals were selected for integrant strains by benzimidazole and temperature by using a modified version of a previously described method [7,8]. Briefly, F3 animals were plated onto NGM agar plates containing 10 μ g/ml benzimidazole (Wako) (approximately 50 animals/9-cm plate) and were cultured at 20 °C for 7–10 days. F5 animals were plated onto NGM agar plates (approximately 100 animals/9-cm plate) and cultured at 20 °C for 3 days. In this process, the integrant animals carrying the *vps-45* transgene but not carrying the complete *ben-1* transgene were selected. Surviving animals were singled and examined by PCR. The primers used are listed in Supplementary Table 3. We confirmed that the integration of transgenes at the *ben-1* gene by PCR and sequencing (Supplementary Fig. 1). We performed PCR using purified genomic DNA of each Is strain as a template. We designed primer sets located within the *ben-1* gene, which is not included in the extrachromosomal arrays, and the vectors, which is included only in the extrachromosomal arrays. The primers used in this experiment are listed in Supplementary Table 2.

3. Results and discussion

3.1. Transgene integration using the CRISPR/Cas9 system

We investigated whether the CRISPR/Cas9 system is able to generate integrated transgenic worms, and how the number of Cas9 cleavage sites in extrachromosomes affects the frequency of isolation of transgenic animals. We first attempted to determine

whether a single sgRNA could cleave both extrachromosomal arrays and the genomic target site by using an sgRNA targeting *dpy-3* as the simplest experimental design (Fig. 1). Because *dpy-3* mutants show a highly penetrant visible and viable recessive phenotype termed “Dpy”, we inferred that integration of an extrachromosomal transgene into the *dpy-3* locus would yield Dpy and fluorescence-positive integrant animals. Integrant Dpy lines can be distinguished easily from parental extrachromosomal transgenic animals without the Dpy phenotype. We selected Dpy worms as Is lines that had the parent Ex marker (Venus) in all the F3 or later progeny. To investigate the effect of the number of cleavage sites in extrachromosomal array, we generated 3 parent Ex lines that expressed the *myo-2p::Venus* together with a portion of the *dpy-3* genome sequence (491 bp) at three different concentration ratios (Table 1, exp. 1–3). The *dpy-3* genome fragment served as the target for sgRNA against the *dpy-3* locus, as shown in Fig. 1 and Table 1.

We expressed Cas9, the *dpy-3*-specific sgRNA and a vector driving the expression of *dpy-7p::DsRed* to label transformed F1 progeny from the parent transgenic worms, *tmEx4252*, *tmEx4277* or *tmEx4253*. We isolated DsRed- and Venus-positive worms and screened their F2 progeny for Dpy phenotypes (Fig. 1). The integrated transgenic strains in the progeny were easily identified by *myo-2p::Venus* fluorescence and Dpy (Table 1, column “No. producing Is”). We obtained integrant strains at frequencies of 3.6%, 4.4% and 9.3% from *tmEx4252*, *tmEx4277* and *tmEx4253*, respectively. Decreasing the number of *dpy-3* sequence in extrachromosomal arrays induced higher efficiency of generating integrant strains (Supplementary Table 4, exp. 1–3). Notably, we obtained F2 animals with the Dpy phenotype without Venus fluorescence (Table 1, column “No. F1 producing Dpy F2”). This suggests that not all of the animals with a double-strand break at the *dpy-3* locus accepted the extrachromosomal DNA fragments but were instead self-repaired with some changes (small deletion and/or insertion) at the locus, as found in many CRISPR/Cas9-induced knockout strains [9]. Candidates of integrated strains with Dpy and Venus-positive phenotypes did not segregate Dpy and Venus negative nor non-Dpy and Venus-positive in the present experiments. We confirmed that the transgenes were integrated at the *dpy-3* locus by PCR and sequencing (Supplementary Fig. 1). Thus, integration into off-target loci is rare if not at all. Practically Venus fluorescence alone is enough to isolate integrated strains. We conclude that extrachromosomal arrays can be integrated into chromosomal loci of interest by using the CRISPR/Cas9 system (Fig. 2). The frequencies of isolation of integrant strains were between 3.6% and 9.3% of P0 strains, which is comparable to or a little higher than the integration method by short wave-length UV irradiation, which yields integrant strains of up to 5% of P0 animals [3]. Our results suggest that a higher concentration of recognition sequences for sgRNAs in extrachromosomal arrays results in a lower frequency of producing transgenic animals. To further examine this hypothesis, we microinjected *tmEx4252* transgenic worms with two vectors encoding Cas9, *dpy-3*-, and β -lactamase-specific sgRNAs together with an injection marker, *dpy-7p::DsRed*. One integrant strain was isolated from 40 injected P0 worms, suggesting that use of the β -lactamase-specific sgRNA yielded integrated strains at a comparable or slightly lower frequency (2.5%, Table 1, exp. 4) than that of injection of the *dpy-3* sgRNA alone (3.6%; Table 1, exp. 1). This result supports the hypothesis that too many fragmented extrachromosomal arrays may reduce the frequency of producing transgenic animals.

3.2. Homologous sequences in extrachromosomal arrays are dispensable for generating integrant strains

There are abundant extrachromosomal transgenic strains shared by the *C. elegans* research community stored in the CGC (*Caenorhabditis* Genetics Center; <http://www.cbs.umn.edu/research/resources/cgc/strains>) and many individual laboratories.

Thus, we next investigated whether it is possible to add another sgRNA to target a sequence that is common among most extrachromosomal transgenic animals, to investigate whether such biological materials could be re-used as parent strains for integration into a locus of interest. We used sgRNA against the β -lactamase gene, which is present in all parent strains in the present experiments and is likely to be present in most of the existing extrachromosomal transgenic strains, since the β -lactamase (ampicillin resistance) gene is contained in general plasmid vectors. We attempted to integrate extrachromosomal transgenes by using another strain, *tmEx3199*, which lacks the *dpy-3* sequence in the extrachromosomal arrays. We injected sgRNAs against both the β -lactamase and *dpy-3* genes as above and obtained 4 integrant strains from 50 P0 parent animals (8.0%, Table 1, exp. 5). By contrast, we could not generate any Is line by injecting *tmEx3199* with sgRNA against only the *dpy-3* gene and Cas9 (Table 1, exp. 6, Supplementary Table 4). We conclude that fragmentation of the array, in addition to the cleavage of the genome, is important for efficient integration.

Because the probability of integration with the present method is high enough to generate integrant lines, we next evaluated how the integration processes are affected by the conditions used. The mechanism of this phenomenon is not currently clear but is likely due to DNA repair processes such as homologous recombination (HR) and nonhomologous end-joining (NHEJ) [18] because Cas9 causes double-strand breaks. If the HR system mainly functions in the mechanism of integration, integrated strains should be generated more efficiently by *tmEx4252*, which has a larger number of homologous sequences (Table 1, exp. 1, Supplementary Table 4), than by *tmEx4277* or *tmEx4253* (Table 1, exp. 2, 3, Supplementary Table 4). This was not the case. We obtained integrant strains at frequencies of 3.6%, 4.4% and 9.3% from *tmEx4252*, *tmEx4277* and *tmEx4253*, respectively. These results suggest that the HR system may not play the main role for integration (Fig. 3C, case 1). This idea is compatible with the fact that Cas9 plasmids are injected into the germ cells after meiosis which do not have sister chromosomes. If the NHEJ system mainly functions in the mechanism of integration, even *tmEx3199* can generate the integrant strains. This was the case. We obtained integrant strains at a frequency of 8.0% from *tmEx3199*, which does not have the *dpy-3* sequence in the extrachromosomal array (Table 1, exp. 5). This result suggests that the NHEJ system may play the main role for integration (Fig. 3C, case 2).

To investigate the relationship between the numbers of fragmented extrachromosomal array and the efficiency of generating transgenic animals, we compared the results of exp. 1 and exp. 4 in the Table 1 and Supplementary Table 4. Increasing the cleavage sites in extrachromosomal arrays induced higher rates of sterile or larval arrest phenotypes in F1 animals. The same tendency was observed in exp. 1, exp. 2 and exp. 3 (Table 1, exp. 1–3, Supplementary Table 4). Thus, a higher concentration of DNA fragments might result in the ligation of chromosomal break points to the incorrect arms by NHEJ, leading to abortive DNA fragments from extrachromosomal arrays and causing aneuploidy (Fig. 3C, case 3).

Researcher may wonder which types of extrachromosomal arrays should be used. Extrachromosomal arrays with or without homologous sequences to insertion sites may be used. However, it may be easier to use already existing extrachromosomal transgenic animals. The Cas9-sgRNA (for β -lactamase) expression vector is available upon request.

3.3. The expression of reporter genes is proportional to the copy number of the construct at the same integration locus

To determine the copy number of insertions, we performed quantitative PCR using purified genomic DNA as a template. We designed primer sets located within the promoter of the *myo-2* gene and the *dpy-3* gene that were contained in the extrachromosomal

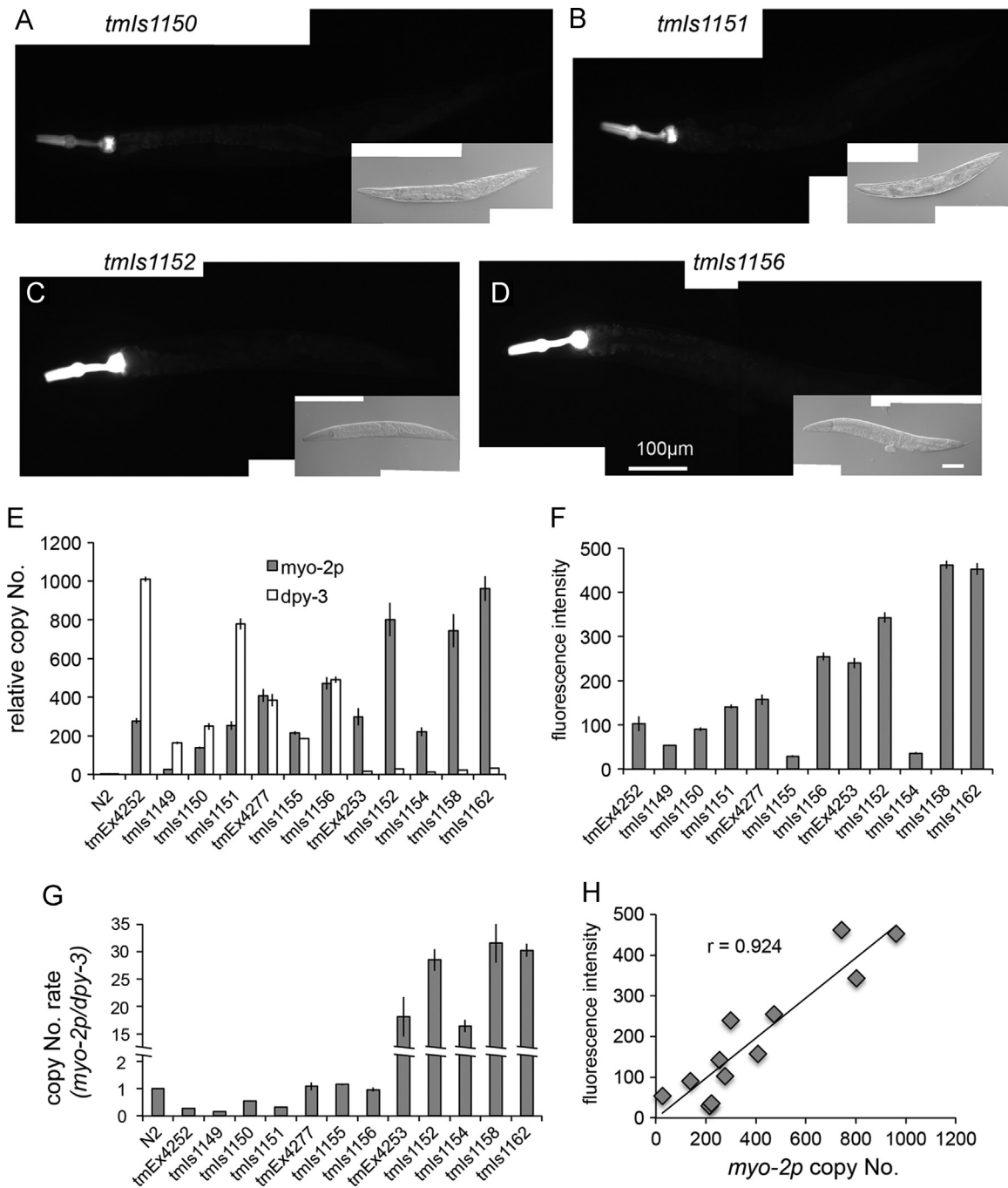


Fig. 2. Expression of *myo-2p::Venus* in the multi-copy integrated strains (A–D) Venus protein expressed in pharyngeal muscle cells. Scale bar = 100 μ m. (400 msec. exposure time) (E) Relative amounts of the *myo-2* promoter and the *dpy-3* genome as determined by quantitative PCR. The data were normalized to the *ama-1* gene. The copy numbers are presented as a ratio to the wild-type N2. Bars represent the mean \pm SE of three independent experiments. (F) Quantification of the fluorescence in pharyngeal muscle cells. The graph represents the intensity of fluorescence of pharyngeal muscle cells ($n > 30$ animals per strain). Fluorescence intensity was examined using ImageJ (NIH, Bethesda, MD). Bars represent the mean \pm SE. (G) The copy number ratio of the *myo-2* promoter to the *dpy-3* genome in the 3 parent Ex lines and the 9 Is lines. Bars represent the mean \pm SE of three independent experiments. (H) The scatter plot of the number of *myo-2* promoter (x-axis) against fluorescence intensity (y-axis) in the 3 Ex lines, tmEx4252, tmEx4253 and tmEx4277, and the 9 Is lines, tmls1149, tmls1150, tmls1151, tmls1155, tmls1156, tmls1152, tmls1154, tmls1158 and tmls1162.

array and in the *C. elegans* genome. Four Ex lines and 13 integrant strains were tested and compared to wild-type N2 (Fig. 2E and Supplementary Fig. 4C). As a result, the *myo-2p*-to-*dpy-3*, *dpy-30p*-to-*ben-1* or *vps-45*-to-*ben-1* ratio in each integrant strain was similar to each parent Ex strain (Fig. 2G, Supplementary Fig. 4D).

To compare the copy number of the *myo-2* promoter sequences with fluorescence intensity, the integrant strains were photographed under the same exposure time (Fig. 2A–D and F). Fluorescence intensity was highly correlated with the copy number of the *myo-2* promoter (correlation coefficient was 0.924; Fig. 2H).

This suggests that the expression of transgenic genes in the integrant transgenes is nearly proportional to the copy numbers of the genes among strains with the same integration locus.

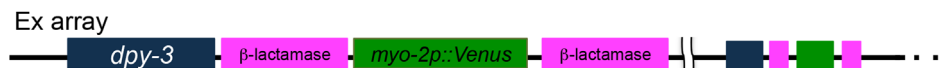
3.4. Transgene integration using the CRISPR/Cas9 system, benzimidazole and temperature selection

We previously demonstrated that single/low-copy integrant strains can be isolated using positive (temperature-sensitive lethal phenotype rescue) and negative (benomyl-sensitive phenotype

A Plasmid construction



B Extrachromosomal array



C Interaction of Ex array and genomic DNA

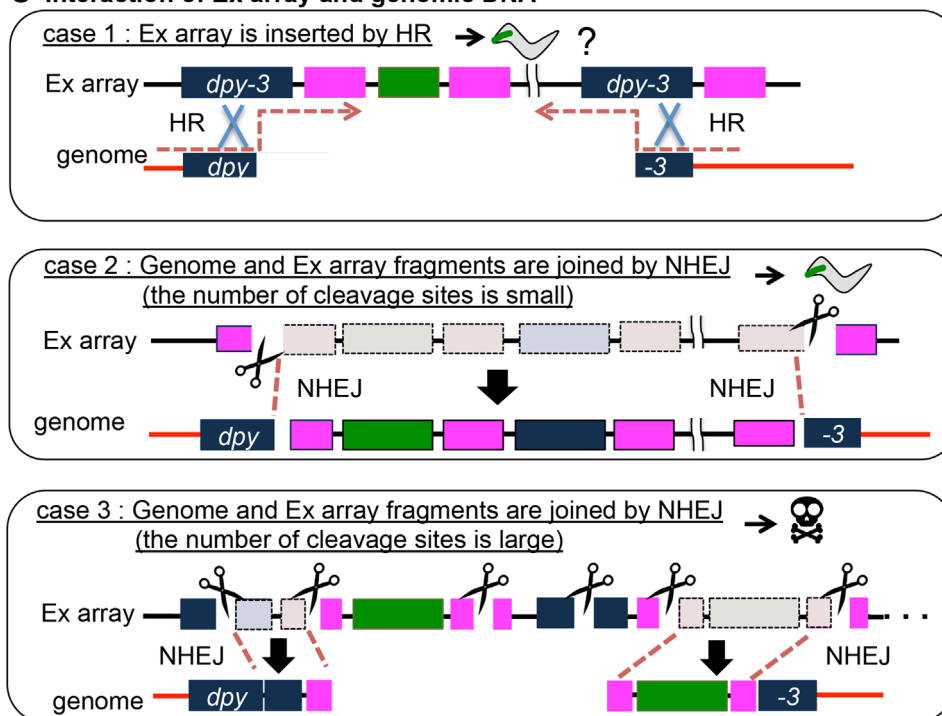


Fig. 3. Schematic diagram of Ex array insertion into the genome by the CRISPR/Cas9 system. (A) Plasmid structure used in the experiment. (B) Schematic diagram of Ex array. (C) In case 1, cleaved *dpy-3* sequences in *C.elegans* genome are ligated by homologous recombination (HR) to the intact extrachromosomal (Ex) array. In case 2, a fragmented Ex array is inserted to the chromosomal break point by nonhomologous end-joining (NHEJ), yielding an Is line. In cases 3, many fragmented Ex arrays are ligated by NHEJ to chromosomal break points but *C.elegans* genome are not joined each other, yielding aneuploidy.

rescue) selection [7,8]. We next investigated whether a similar selection also works using the CRISPR/Cas9 system to isolate smaller-copy integrations from extrachromosomal arrays. Mutants of *ben-1*, which encodes a β -tubulin gene of *C. elegans*, are resistant to an anti-tubulin drug benzimidazole [19]. Wild-type *C. elegans* worms are severely unhealthy, dumpy, and uncoordinated on benzimidazole-containing selection media [19]. Thus, *ben-1* mutants not carrying the *ben-1* gene or carrying an incomplete *ben-1* gene in the transgene predominantly grow and reproduce on benzimidazole-containing media. Temperature selection was based on rescue of the *vps-45* mutant phenotype. The *vps-45* mutants are unable to grow and reproduce at 20 °C [15]. *vps-45* mutants carrying the *vps-45* mini gene grow and reproduce, allowing for easy identification of the transformants. To insert the fragments of extrachromosomal transgenes into the chromosomes, *ben-1(tm234)*; *vps-45(tm246)*; *tmEx3199* animals were microinjected with vectors encoding Cas9, a *ben-1*-specific sgRNA and an injection marker. The progeny of microinjected animals were cultured on benzimidazole-containing NGM plates, and the animals that grew and reproduced on benzimidazole-containing media were cultured on NGM plates at 20 °C. We performed PCR selection to amplify the GFP sequence, using animals that survived

this selection as templates (Supplementary Fig. 2A). As a result, 2 integrant strains, *tms1159* and *tms1160*, were isolated from 54 injected worms (Supplementary Figs. 2B, 3A and B). This method produced a much higher frequency (3.7%) than the previous UV/TMP protocol (0.033%) [7]. We confirmed that the transgenes were integrated at the *ben-1* locus in *tms1159* and *tms1160* by PCR and sequencing (Supplementary Fig. 1).

To determine the copy number of insertions, we performed quantitative PCR using primer sets for the *dpy-30p*, *vps-45* and *ben-1* genes using purified genomic DNA as templates. *tms1159* and *tms1160* were tested for the copy number of these sequences and compared to wild-type N2 (Supplementary Fig. 3C and D). *tms1159* carried approximately 35, 22 and 22 copies of the *dpy-30p*, *vps-45* and *ben-1* genes, respectively. *tms1160* carried approximately 27, 16 and 10 copies of the *dpy-30p*, *vps-45* and *ben-1* genes, respectively (Supplementary Fig. 3C). This result indicates that the CRISPR/cas9 system is also useful for positive/negative selection via benzimidazole and temperature selection. *tms1159* and *tms1160* contained a slightly larger number of copies than the previously isolated single/low-copy transgenic strains [7,8]. Because the integrant strains isolated in the present study were resistant to benzimidazole, it is conceivable that the residual *ben-1*

genes in the integrant strains were disrupted through cleavage by the *ben-1* sgRNA and Cas9. Thus, the negative selection by benzimidazole was not as strong as before. We expected that the experimental conditions including the combination of sgRNA against the β -lactamase sequence for extrachromosomes and the *dpy-3* sequence for the chromosomal integration site could yield integrants of single/low-copy, as before. GFP Fluorescence was not observed in the germ cells (data not shown), suggesting intermediate-copy number transgenes might be silenced [20].

In conclusion, we developed an efficient method for the locus-specific integration of extrachromosomal arrays into chromosomes using the CRISPR/Cas9 system, while extrachromosomal arrays are integrated into random loci by previous methods. To generate integrant strains efficiently, it is important to cut extrachromosomal arrays in a moderate number of fragments. Although it has been a concern that expression levels are not reproducible depending on the integration site, researchers can now easily compare the expression and functions of various constructs at defined integration loci. Moreover, when using multiple mutants and integrant transgenics, it is apparently beneficial to use transgenic strains with convenient integration sites.

Conflict of interest

The authors declare no conflicts of interests.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.bbrep.2015.11.017>.

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