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**Published in:**

G3: Genes, Genomes, Genetics

**Published:** 01/12/2022

**Document Version:**

Final Published version, also known as Publisher's PDF, Publisher's Final version or Version of Record

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**Publication record in CityU Scholars:**

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**Published version (DOI):**

[10.1093/g3journal/jkac254](https://doi.org/10.1093/g3journal/jkac254)

**Publication details:**

Ding, Q., Ren, X., Li, R., Chan, L., Ho, V. W. S., Bi, Y., Xie, D., & Zhao, Z. (2022). Highly efficient transgenesis with *miniMos* in *Caenorhabditis briggsae*. *G3: Genes, Genomes, Genetics*, 12(12), [jkac254].  
<https://doi.org/10.1093/g3journal/jkac254>

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# Highly efficient transgenesis with *miniMos* in *Caenorhabditis briggsae*

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## Abstract

*Caenorhabditis briggsae* as a companion species for *Caenorhabditis elegans* has played an increasingly important role in study of evolution of development and genome and gene regulation. Aided by the isolation of its sister species, it has recently been established as a model for speciation study. To take full advantage of the species for comparative study, an effective transgenesis method especially those with single-copy insertion is important for functional comparison. Here, we improved a transposon-based transgenesis methodology that had been originally developed in *C. elegans* but worked marginally in *C. briggsae*. By incorporation of a heat shock step, the transgenesis efficiency in *C. briggsae* with a single-copy insertion is comparable to that in *C. elegans*. We used the method to generate 54 independent insertions mostly consisting of a mCherry tag over the *C. briggsae* genome. We demonstrated the use of the tags in identifying interacting loci responsible for hybrid male sterility between *C. briggsae* and *Caenorhabditis nigoni* when combined with the GFP tags we generated previously. Finally, we demonstrated that *C. briggsae* tolerates the *C. elegans* toxin, PEEL-1, but not SUP-35, making the latter a potential negative selection marker against extrachromosomal array.

**Keywords:** *miniMos*, *Caenorhabditis briggsae*, heat shock, transgenesis

## Introduction

As a comparative model of *Caenorhabditis elegans*, *Caenorhabditis briggsae* shares a similar morphology, carries a genome of comparable size (Stein et al. 2003; Ross et al. 2011; Ren et al. 2018; Yin et al. 2018; Li et al. 2020) and adopts similar developmental pattern to that of *C. elegans* (Zhao et al. 2008). *Caenorhabditis elegans* is a model organism. Its genome has been subject to intensive manipulations with various tools, including random mutagenesis with ultraviolet light coupled with trimethylpsoralen (UV/TMP) (Thompson et al. 2013), low copy insertion of transgenes with biolistic bombardment (Praitis et al. 2001; Hochbaum et al. 2010; Radman et al. 2013), targeted single-copy insertion using transcription activator-like effector nucleases system (Wood et al. 2011; Lo et al. 2013), or using CRISPR/Cas9 system (Chiu et al. 2013; Cho et al. 2013; Waaijers et al. 2013; Dickinson et al. 2015; Dickinson and Goldstein 2016), Mos1-mediated single-copy insertion (MosSCI) (Frøkjær-Jensen et al. 2008) as well as random single-copy insertion with *miniMos* (Frøkjær-Jensen et al. 2014). Many of these tools have been adopted in *C. briggsae* for genome editing with a comparable or a much-reduced successful rate. For example, biolistic bombardment was successfully adopted in *C. briggsae* transgenesis with comparable efficiency, whereas single-copy insertion with *miniMos* demonstrated a much lower efficiency in *C. briggsae* than in *C. elegans* (Praitis et al. 2001; Semple et al. 2010; Zhao et al. 2010; Frøkjær-Jensen et al. 2014; Bi

et al. 2015). An efficient transgenesis method with single-copy insertion is essential for comparative functional study of biology between *C. elegans* and *C. briggsae*. Recent work in *Caenorhabditis* species has demonstrated that heat shock treatment or compromised function in heat shock pathway significantly increased the frequency of transposition (Ryan et al. 2016), raising the possibility of improving *miniMos*-based transgenesis in *C. briggsae* by incorporation of a heat shock step or inhibition of activities of heat shock proteins.

Isolation of *C. briggsae* sister species, *Caenorhabditis nigoni*, with which it can mate and produce viable progeny, paves the way of speciation study using nematode as a model for the first time (Woodruff et al. 2010; Kozłowska et al. 2012). To isolate postzygotic hybrid incompatible (HI) loci between *C. briggsae* and *C. nigoni*, a dominant and visible marker is required for targeted introgression, in which the genome of one species is labeled with the marker and repeatedly backcrossed into the other to isolate the HI loci. The marker greatly facilitates tracing of its linked genomic fragment during backcrossing and its associated HI phenotype. Given numerous such markers are required over a genome, generation of such markers using targeted single-copy insertion becomes an enormous burden. This is because that the efficiency for targeted single-copy insertion is relatively low. As an alternative, over 100 GFP markers were inserted into the *C. briggsae* genome with biolistic bombardment, which allowed genome-wide

Received: July 12, 2022. Accepted: August 29, 2022

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mapping of HI loci between the 2 species (Yan et al. 2012; Bi et al. 2015; Ren et al. 2018).

Biolistic bombardment using *cbr-unc-119* (Zhao et al. 2010; Bi et al. 2015) generates low copy number of transgene into genome but requires tedious animal preparations. It also needs to wait for several weeks before screening for transformed animal. In addition, presumably due to dramatic mechanical shearing, the transgenic animals often suffer from chromosomal rearrangements (Bi et al. 2015; Ren et al. 2018; Tyson et al. 2018; Ding et al. 2022). Importantly, the insertion site cannot be precisely determined due to unknown copy number of the transgene and its arrangement within host genome. As such, the transgene insertion sites were estimated by genotyping introgression boundary with PCR using species-specific primers (Yan et al. 2012; Bi et al. 2015). Unfortunately, the divergence between the *C. briggsae* and *C. nigoni* genomes is too far to allow efficient recombination during backcrossing, resulting in a poor mapping resolution of the insertion site (Yan et al. 2012; Bi et al. 2015, 2019).

We have recently demonstrated that inter-chromosomal interactions are involved in hybrid male sterility between *C. briggsae* and *C. nigoni* (Li et al. 2016; Bi et al. 2019). For example, a *C. briggsae* X chromosome fragment in an otherwise *C. nigoni* background as an introgression leads to male sterility. Hybrid F1 male sterility can be rescued by the presence of the introgression and another *C. briggsae* genomic fragment on its Chromosome II. We speculate that such interacting loci might be common in producing HI. Availability of the dual-color labeling system makes it possible for systematic mapping such interacting loci. This is because it facilitates tracking of cosegregation of the 2 loci. It is worth noting that most existing markers in the *C. briggsae* genome were derived from GFP, preventing effective screening for such loci. Therefore, *C. briggsae* animals bearing a visible marker other than the GFP are desired.

Transposon-mediated single-copy insertion at random genomic position with high efficiency has been successfully developed in *C. elegans* (Frøkjær-Jensen et al. 2014). A fly transposon *Mos1* was truncated with minimal transposon sequences, termed as *miniMos* cargo vector, in order to boost its capacity of carrying foreign DNA (Frøkjær-Jensen et al. 2014). Single-copy of transgene can be inserted into host genome at high frequency via coinjecting a transposase-expressing vector and *miniMos* cargo vector into *C. elegans* gonad. The method was also adopted in *C. briggsae* but with a much lower efficiency with unknown reasons (Frøkjær-Jensen et al. 2014). Attempts have been made to improve insertion frequency in *C. briggsae*. For example, the promoter (*cel-Peft-3*) driving transposase expression was substituted with *C. briggsae* promoter *cbr-Peft-3* or *cbr-Ppie-1* in order to boost transposase expression. However, the insertion frequency did not improve as expected. A highly efficient method for inserting single-copy transgene has yet to be established in *C. briggsae*. To facilitate screen for single-copy insertion, a negative selection marker *cel-Phsp-16.41::peel-1* was co-injected into animals to kill extrachromosomal array-bearing worms during screening (Frøkjær-Jensen et al. 2012). However, the killing efficiency of the negative selection marker *peel-1* has not been investigated in *C. briggsae*.

This study established a highly efficient methodology for inserting single-copy transgene into the *C. briggsae* genome based on *miniMos*. Negative selection markers against transgene existing only in extrachromosomal array were also explored with limited success.

## Materials and methods

### Nematode strains

*Caenorhabditis elegans*, *C. briggsae*, and *C. nigoni* wild isolates used in this study were N2, AF16, and JU1421, respectively. The *C. briggsae*

*dpy-5*(*zzy0580*) knockout strain ZZY0580 was generated using CRISPR/Cas9 with AF16 followed by backcrossing to AF16 for 3 generations. Introgression strains used were ZZY10330 (*X* [*cbr-myo-2p::gfp*, *cbr-unc-119(+)*]), AF16>JU1421 (Bi et al. 2015), ZZY10377 (*zzyIR10377* (*X* [*cbr-myo-2p::mCherry*, *neoR*]), AF16>JU1421) from *C. briggsae* transgenic strain ZZY0777 (Supplementary Table 1). Introgression strains ZZY10353 (*zzyIR10353* (*II* [*cbr-myo-2p::gfp*, *cbr-unc-119(+)*]), AF16>JU1421) (Bi et al. 2015) and ZZY10382 (*zzyIR10382* (*II* [*cbr-myo-2p::mCherry*, *neoR*]), AF16>JU1421) were generated from *C. briggsae* transgenic strain ZZY0782 (Supplementary Table 1). Details for all the transgenic strains generated in this study were included in Supplementary Table 1. All the *C. elegans* and *C. briggsae* lines were maintained on 1.5% rose nematode growth medium (NGM) seeded with *E. coli* OP50 at room temperature and in a 25°C incubator, respectively, unless specified otherwise.

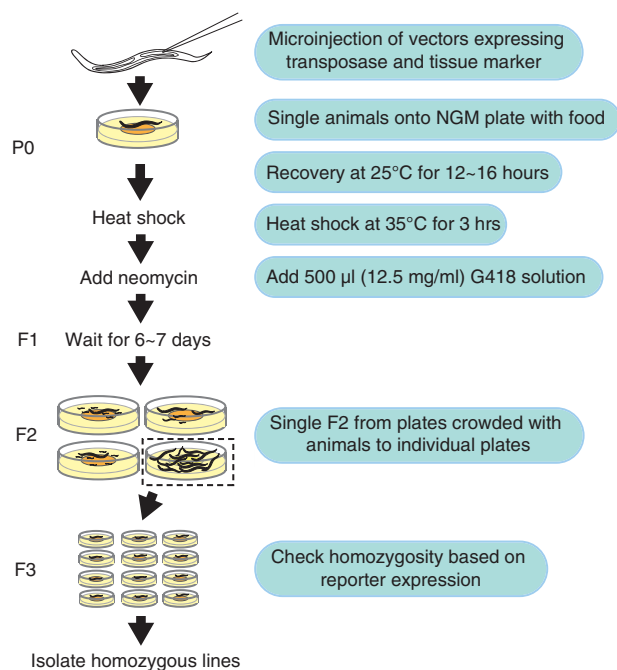
### Molecular cloning

pZZ203 was derived from pZZ0031 (Yan et al. 2012) by removing *cbr-Pmyo-2::gfp::his-72* UTR by cutting using KpnI and ApaI (NEB). The digested pZZ0031 backbone carrying *cbr-unc-119(+)* was gel purified, blunt end repaired, and self-ligated to give rise to pZZ203. pZZ160 and pZZ161 was derived from pCFJ910[NeoR] and pCFJ909[*cbr-unc-119(+)*], respectively, by inserting both *cbr-Pmyo-2::gfp::his-72* UTR and *cbr-dpy-5(+)* into minimal *Mos1* transposon. pZZ184 was derived from the pZZ160 by replacing *gfp::his-72* UTR and *cbr-dpy-5(+)* with *mCherry::unc-54* UTR from pGH8 (Frøkjær-Jensen et al. 2008). The plasmids pZZ185 and pZZ196 for negative selection were derived from pCFJ601[*Peft-3::Mos1* transposase:*tbb-2* UTR] by replacing the *Mos1* with *sup-35* (Ben-David et al. 2017) and *peel-1* (Seidel et al. 2008, 2011), respectively. The plasmid kit containing pCFJ601, pCFJ909, pCFJ910, pGH8, and pMA122 was acquired from Addgene (cat# 1000000031). Vector pZZ113 containing sgRNA expression cassette against *cbr-dpy-5* was derived from PU6:*unc-119\_sgRNA* (Addgene plasmid # 46169) as described (Friedland et al. 2013). The *cbr-dpy-5* gRNA target sequence is GGAGCCCCAGGAGAGCCAGG. Primers used for construct building were listed in Supplementary Table 2. An overview of plasmid compositions was shown in Supplementary Fig. 1.

Plasmids for microinjection were extracted using the PureLink HQ Mini Plasmid Purification Kit or HiPure Plasmid Midiprep kit (Invitrogen). Genomic DNAs were extracted from mix-staged animals with PureLink Genomic DNA Mini Kit (Invitrogen). Injection mixture for transformation consisted of *miniMos* cargo vector at 20 ng/μl, *Mos1* transposase-expressing plasmid at 50 ng/μl, red or green fluorescent coinjection markers each at 10 ng/μl. pZZ203 was added to 80 ng/μl with a total DNA concentration of 170 ng/μl. Plasmids pMA122, pZZ185, and pZZ196 were built to test their use as a negative selection marker. Neomycin stock solution of 12.5 mg/ml was made with G418 powder (ThermoFisher) in water.

### Transgenesis

Animal injected with pZZ160 (GFP) or pZZ184 (mCherry) and pCFJ601 was individually placed on a 55-mm crossing plate seeded with OP50 (~6.7 ml NGM per plate), and allowed to recover at 25°C overnight (roughly 12–16 h) (Fig. 1). For heat shock treatment, plates with injected animals were sealed with parafilm and incubated on the water bath preheated to 35°C for 3 h. Remove parafilm and any water droplet on the inner lid. Incubate plates at room temperature (~22°C) for half an hour. Add 0.5 ml 12.5 mg/ml neomycin (G418) solution prewarmed up at room temperature onto each plate. Gently rotate plates to allow neomycin solution to fully cover plate surface. Air-dry the plates and incubate the dried plates in a 25°C incubator for 6–7 days. Due to the presence of selection marker NeoR in the



**Fig. 1.** Schematics of optimized protocol for *miniMos*-based transgene insertion in *C. briggsae*. Young adult animals were injected with transposase-expressing vector, i.e. pCFJ601, along with a cargo vector, i.e. pZ2160 (*Pmyo-2::GFP*) or ZZ184 (*Pmyo-2::mCherry*). The expressed transposase is expected to cut the *myo-2* promoter fusion DNA fragment and randomly insert into the host genome. A heat shock was included to increase transgenesis efficiency. A neomycin resistance gene, NeoR, was included in the cargo vector so that only transgenic animals carrying NeoR as either extrachromosomal array or single-copy transgene can grow on the NeoR<sup>+</sup> plates. The categories of transgenic animals can be roughly differentiated by population growth rate on NeoR<sup>+</sup> plates. As an option, a second visible marker, for example, a second fluorescence marker with different color, can be included in the injection mixture to distinguish the array-containing animals from those without array.

injected *miniMos* plasmids (pZ2160 and pZ2184), single 10–12 survived L4 or young adult animals from the plates crowded with animals that do not express coinjection marker onto individual plates to be incubated at 25°C. After 2–3 days, harvest animals with homozygous transgene based on the reporter expression and lack of expression of visible coinjection marker for genotyping.

### Quantification of P<sub>0</sub> insertion frequency

Three days after microinjection, plates without F<sub>1</sub> transgenic progeny were discarded and excluded from the number of injected P<sub>0</sub> animals. The F<sub>2</sub> animals were screened for *miniMos* insertion under stereo-microscope based on reporter expression or phenotypic rescue, and the absence of coinjection markers. Animals confirmed with transgene insertion from the same P<sub>0</sub> plate were treated as an independent single-copy insertion. The P<sub>0</sub> insertion frequency was measured through dividing the number of independent insertions by the total number of injected P<sub>0</sub> animals. Mapping of insertion site was performed as described (Frøkjær-Jensen et al. 2014). The *C. briggsae* cb4 genome (Ross et al. 2011) was used to report the chromosomal coordinates.

### Selection marker for transformation

For selection with Neomycin (NeoR), injected animals were allowed to recover overnight (12–16 h) followed by heat shock treatment on a 35°C water bath for 3 h. The heat shock treated

plates were incubated at room temperature for half hour to recover. Then 500 µl of 12.5 mg/ml G418 solution was added to each plate. Plates were incubated at 25°C for 6–7 days. Twelve F<sub>2</sub> animals were singled onto a new NGM plate from the P<sub>0</sub> plates crowded with animals. Homozygosity was checked based on reporter expression in F<sub>3</sub>.

For selection with *cbr-dpy-5*, all rescued *dpy-5(+)* F<sub>1</sub> animals were singled onto individual plates. Twelve F<sub>2</sub> animals were singled onto new plate from each F<sub>1</sub> plate in which over 50% animals were *dpy-5(+)* ones. F<sub>2</sub> plates with 100% *dpy-5(+)* animals that did not express visible coinjection markers were kept for genotyping.

### Development of negative selection marker for extrachromosomal array

A fusion PCR product was made between a cassette consisting of *peel-1::tbb-2* UTR or *gfp::tbb-2* UTR and the *C. briggsae* syntenic region of *hsp-16.41* promoter with the primers listed in Supplementary Table 2. Additional details of *C. briggsae* syntenic region of *hsp-16.41* promoter sequence screening are available in Supplementary Fig. 2. Injection mixture was made with pMA122, pZ2185, pZ2196, or the fusion PCR product at 70 ng/µl, fluorescent coinjection marker vector pZ2161 at 20 ng/µl, pGH8, pCFJ90, pCFJ104 at 10 ng/µl each, and pZ2203 at 50 ng/µl.

To examine the lethality in *C. briggsae* after heat shock, an array-bearing line was generated and embryos harvested for synchronization. The synchronized L1 animals were divided into 2 groups, one was subjected to heat shock treatment at 35°C for 3 h before transferring into 25°C incubator, and the other was incubated at 25°C without heat shock treatment as a control. *Caenorhabditis elegans* heat shock treatment was performed at 34°C for 3 h. The ratio of surviving adults carrying an array out of all progeny were calculated in both control and heat-shock treated animals.

### Introgression

Introgression was performed as described (Bi et al. 2015). Specifically, *C. briggsae* transgenic strains, ZZY0777 and ZZY0782 each expressing a single-copy of *Pmyo-2::mCherry* located on the X and chromosome II, respectively, were backcrossed to *C. nigoni* (JU1421) for 15 generations to give rise to ZZY10377 and ZZY10382. Introgression boundaries were mapped as described (Yan et al. 2012).

## Results

### Heat shock treatment significantly increased the efficiency of transgene insertion

To improve the transgenesis efficiency using *miniMos* in *C. briggsae*, we started the transgenesis by repeating the steps basically as described previously (Frøkjær-Jensen et al. 2014), i.e. injection of a vector carrying *cbr-Pmyo-2::gfp* along with a transposase-expressing vector into *cbr-unc-119* deletion mutant strain RW20000 (Zhao et al. 2010). One of major complications associated with the *cbr-unc-119* as an injection selection marker was the low transformation efficiency even for the formation of extrachromosomal array. This was the case using the rescuing fragment from either *C. elegans* or *C. briggsae* (data not shown). We speculated that the low insertion rate associated with the selection marker could be related to the low transformation efficiency of single-copy insertion.

To improve transformation efficiency, we generated another injection selection marker *cbr-dpy-5* using CRISPR/Cas9. This was based on the observation that *dpy-5* had been used as a very

efficient selection marker in *C. elegans* microinjection (Zhao *et al.* 2004; Hunt-Newbury *et al.* 2007). As expected, *cbr-dpy-5* worked as an effective selection marker for microinjection. We successfully obtained 3 independent transgenic lines with single-copy insertion using the marker after injecting 35 animals. However, due to lack of negative selection marker against extrachromosomal array, it is tedious to screen for the rescued animals out of all progeny.

We then performed *miniMos*-based single-copy insertion using neomycin as a selection marker as described (Giordano-Santini *et al.* 2010; Frøkjær-Jensen *et al.* 2014). The method provided a key advantage in simplifying selection of the transformed animals because all unrescued animals were killed, but the overall efficiency of obtaining single-copy insertion was not improved compared with previous studies (Fig. 2).

Given that heat shock treatment significantly increased transposition frequency (Ryan *et al.* 2016), we reasoned that inclusion of a heat shock step might boost the successful rate of transgenesis in *C. briggsae* based on *miniMos*, which is a modified transposon. As expected, inclusion of a step of heat shock treatment, i.e. 35°C for 3 h, we were able to significantly increase the frequency of single-copy insertion of *Pmyo-2::gfp* or *Pmyo-2::mCherry* (Figs. 1 and 2). Insertion frequency increased from lower than 5% without heat shock to ~25% with heat shock for both constructs, indicating that the heat shock treatment was essential for increasing transposition rate.

## A large collection of single-copy insertions expressing *Pmyo-2::mCherry* were generated in *C. briggsae*

To complement the genetic resources mainly consisting of GFP insertions over the *C. briggsae* we generated previously (Bi *et al.* 2015), we decided to generate a cohort of insertions randomly distributed over the *C. briggsae* genome with a focus on generation of insertion expressing a fluorescent protein other than GFP, i.e. *Pmyo-2::mCherry*. This would be particularly useful for isolation of interacting loci responsible for hybrid male sterility as detailed below. To this end, we generated a total of 54 insertions

consisting of 9 *Pmyo-2::gfp* and 45 *Pmyo-2::mCherry* that were able to be uniquely mapped to the *C. briggsae* genome (Fig. 3; Supplementary Table 1). The markers show roughly random distribution over the *C. briggsae* genome.

## Dual-colored marking system facilitates screen for interacting loci responsible for HI loci

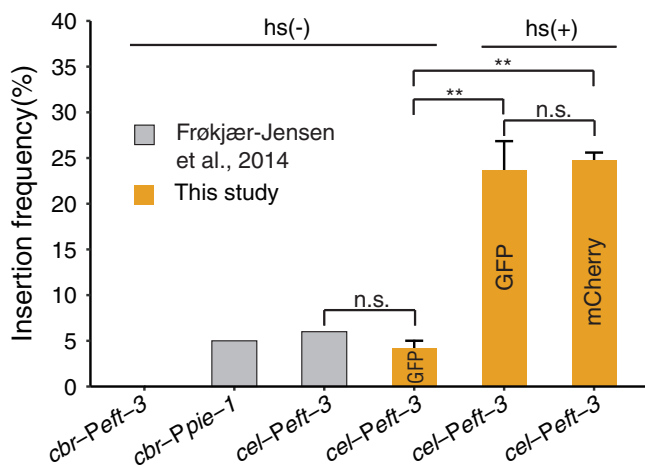
We demonstrated that genetic interaction between X Chromosome and autosome was essential for hybrid male sterility between *C. briggsae* and *C. nigoni* (Bi *et al.* 2019). Identification of such interacting loci is challenging without proper markers on interacting chromosomes. We showed that the interaction between independent GFP-labeled *C. briggsae* introgression fragments on 2 different chromosomes was essential for male fertility in *C. nigoni*, but it was difficult to pinpoint these interacting loci systematically. Availability of such dual-color labelled strains would greatly facilitate the identification of such interactions. To help illustrate this point, we tried to recapitulate the interaction we identified earlier by using 2 *C. nigoni* strains ZZY10377 and ZZY10382 that carry a mCherry-labeled introgression fragment derived from the right arm of the *C. briggsae* X and chromosome II (Fig. 4), respectively, between which an interaction was found (Bi *et al.* 2019). We crossed 2 introgression strains, one labeled with GFP and the other with mCherry in reciprocal way. We then examined the fertility of the males that simultaneously carried both introgressions. We found that the males carrying both loci were mostly fertile, whereas the males carrying a single introgression of GFP or mCherry inserted on the X chromosome were sterile, indicating that the dual-color labeled introgressions did recapitulate the interaction. Presence of the dual-colored markers paves the way for genome-wide identification of any other interacting loci responsible for the hybrid incompatibilities.

## Caenorhabditis briggsae appeared to develop native immunity against PEEL-1

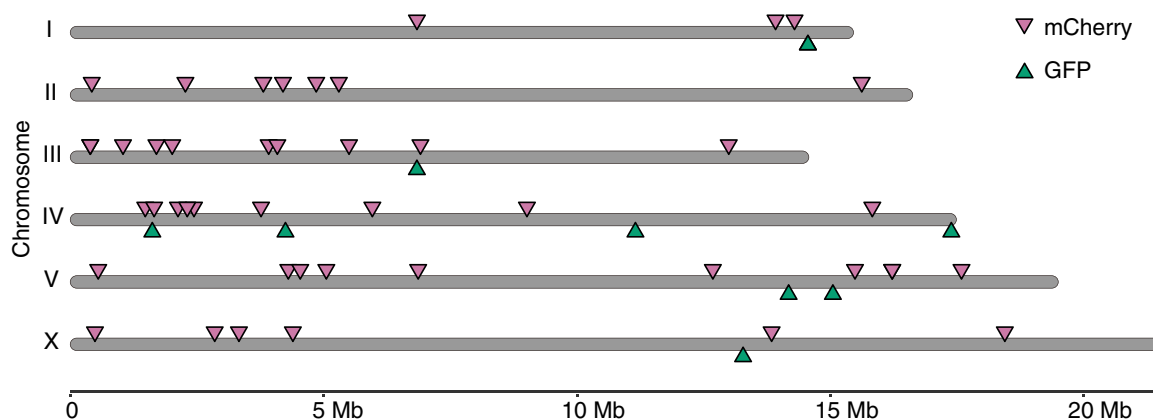
For transgene insertion using *MosSCI* or *miniMos* in *C. elegans*, a sperm-derived toxin gene, *peel-1*, is used as a negative selection marker to effectively kill extrachromosomal array-bearing worms (Seidel *et al.* 2011; Frøkjær-Jensen *et al.* 2012, 2014), greatly reducing the burden of screening for transgenic strains carrying an insertion out of all transgenic animals, including those carrying an extrachromosomal array.

To adopt the negative selection marker in *C. briggsae*, we first compared the killing effect of PEEL-1 in *C. elegans* and *C. briggsae* through its forced expression driven by a *C. elegans* heat shock promoter, *Phsp-16.41*. We generated transgenic lines carrying extrachromosomal array consisting of the PEEL-1-expressing vector in both *C. elegans* and *C. briggsae*. The synchronized L1 animals were subjected to heat shock at 34°C and 35°C for *C. elegans* and *C. briggsae*, respectively. The killing effect in *C. elegans* was comparable to that reported previously (Seidel *et al.* 2011), but no apparent killing was observed in *C. briggsae* after heat shock treatment (Fig. 5a).

To further investigate what caused the failure of PEEL-1 to kill *C. briggsae*, we replaced *C. elegans* heat shock promoter, *Phsp-16.41*, with its *C. briggsae* equivalent (Supplementary Fig. 2) and generated the transgenic lines. Again, we did not observe significant increase in killing (Fig. 5a). We reasoned that the *C. briggsae* heat shock promoter might not be a functional equivalent of *Phsp-16.41*. To examine whether the *C. briggsae* syntenic heat shock promoter responds to heat shock treatment, we generated transgenic lines carrying extrachromosomal array consisting of a fusion between the *C. briggsae* heat shock promoter and GFP. We did see induced GFP expression in the transgenic animals after



**Fig. 2.** Comparison of insertion frequencies in *C. briggsae* between this and previous study (Frøkjær-Jensen *et al.* 2014). Y-axis denotes insertion frequency in  $P_0$  animals. X-axis indicates various promoters used in the previous and this study that drive *Mos1* transposase expression. Each fluorescent marker in this study was tested for at least 3 replicates. Animals with or without heat shock are indicated with hs(+) and hs(-), respectively. Student's t-test was performed for comparison between animals or without heat shock. Error bars represent SE.



**Fig. 3.** Genomic position of mapped insertions of visible marker *Pmyo-2::gfp* or *Pmyo-2::mCherry* over *C. briggsae* genome (cb4). Insertions landed onto contigs unassigned to a specific chromosome were not shown. Insertions of individual GFP (green) or mCherry (purple) marker are indicated with inverted triangle along the genome in scale, detailed insertion information is listed in [Supplementary Table 1](#). Insertion site was determined using 2 rounds inverse PCR as described previously.

heat shock treatment in both *C. elegans* and *C. briggsae* (data not shown), suggesting that *C. briggsae* somehow develops immunity against PEEL-1. To further investigate *C. briggsae*'s immunity against PEEL-1, we generated transgenic *C. briggsae* animals expressing *peel-1* driven by an effective promoter, *Peft-3*, which was known to be able to drive expression in *C. briggsae* (Frøkjær-Jensen et al. 2012, 2014). Again, we did not observe a significant killing effect (Fig. 5b). Taken together, it seems that *C. briggsae* develops native immunity against PEEL-1, preventing it from being used as a negative selection marker against extrachromosomal array.

### Limited success of using *sup-35* as a negative selection marker for extrachromosomal array

In addition to *peel-1*, a maternal-effect toxin, SUP-35, that kills developing embryos, has recently been identified in *C. elegans* (Ben-David et al. 2017). To test the killing efficiency of SUP-35 in *C. briggsae*, we made a *sup-35*-expressing vector driven by the *C. elegans* *eft-3* promoter, *Peft-3* (Supplementary Fig. 1). We injected the construct along with fluorescence coinjection markers into both *C. elegans* and *C. briggsae*. As expected, nearly all F<sub>1</sub> embryos expressing the fluorescence markers arrested at late embryogenesis or as early larvae for both *C. elegans* and *C. briggsae* (Fig. 5c), indicating that SUP-35 is an effective toxin in *C. briggsae* and has a potential to be developed as a negative selection marker against extrachromosomal array in *C. briggsae*. To this end, we generated transgenic strains in *C. elegans* and *C. briggsae* that carry an extrachromosomal array, which consists of the *sup-35*-expressing vector driven again by *C. briggsae* equivalent of *hsp-16.41* promoter (Supplementary Fig. 2). However, we did not observe any significant killing effect in *C. elegans* after heat shock treatment (Fig. 5d). *Caenorhabditis elegans* expresses both PEEL-1 and its antidote ZEEL-1 only transiently in embryo, but expresses SUP-35 and its antidote PHA-1 throughout its life cycle (Gerstein et al. 2010). It is possible that the postembryonic PHA-1 is sufficient to neutralize the toxicity of SUP-35 induced by heat shock treatment in *C. elegans*. It is also possible that the *C. briggsae* heat shock promoter may not respond to heat shock as effectively as its *C. elegans* equivalent in *C. elegans*. Consistent with this, the heat shock treatment showed a significant killing effect in *C. briggsae* carrying the same construct, i.e. from 66.4% to 39.6% though it is not efficient enough to serve as a negative selection marker.

## Discussion

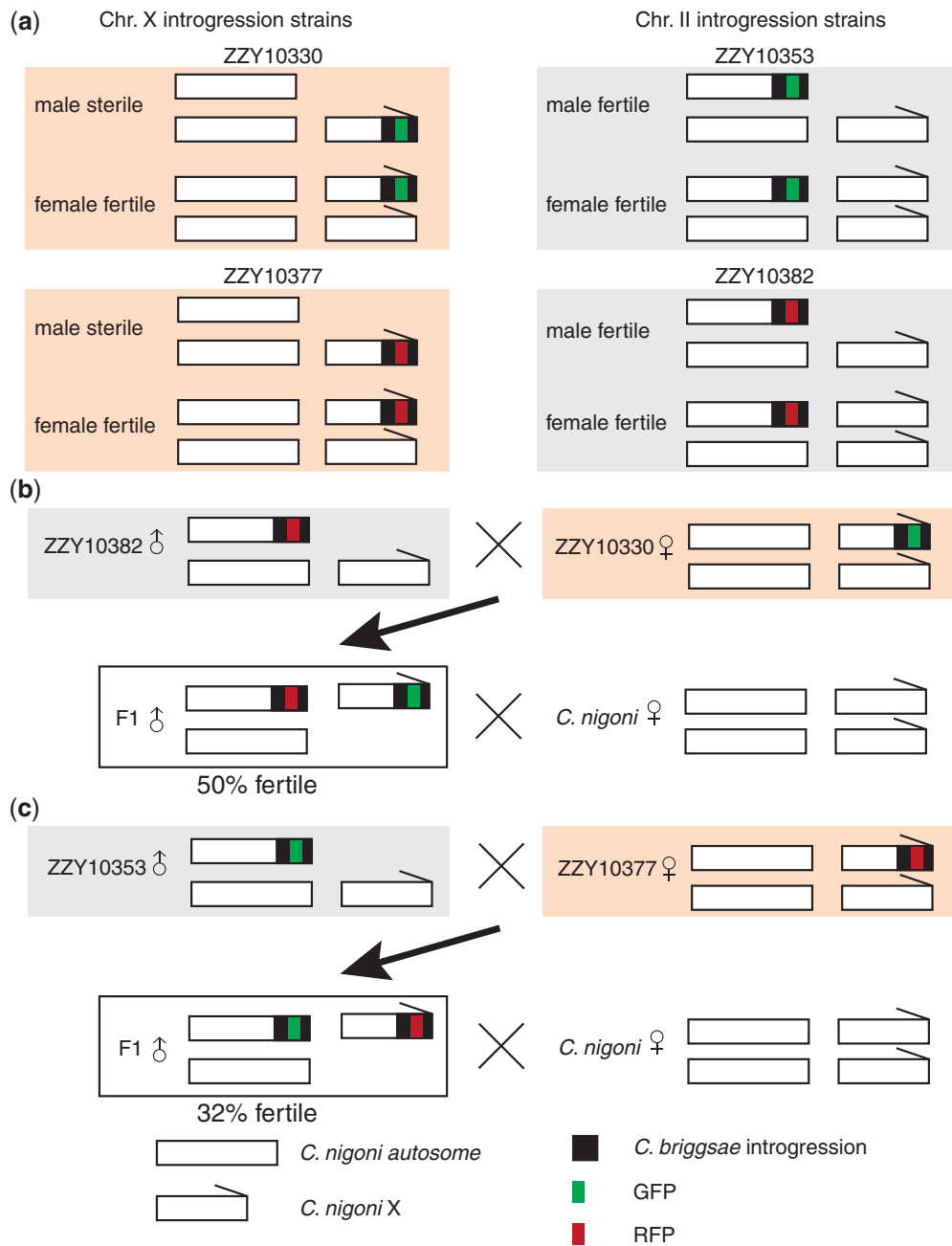
Given the similar morphology, physiology and developmental program between *C. elegans* and *C. briggsae*, methods for *C. elegans* transgenesis are expected to be transferrable to *C. briggsae* with minimal modification. However, there is an exception to this, which is the case for *miniMos* mediated single-copy insertion (Frøkjær-Jensen et al. 2014).

### Potential cause of low efficiency of transgenesis in *C. briggsae* mediated by *miniMos*

It has been well established that transposon mobility can be significantly increased across species after exposure to biotic or abiotic stress (Liu et al. 1995; Walbot 1999; Bouvet et al. 2008). Heat shock proteins serve as molecular chaperone to facilitate folding of other proteins into their appropriate conformations following heat exposure, thus buffering the subsequent phenotypic changes (Erlejman et al. 2014). Perturbation of heat shock protein also increases transposition frequency in a similar way to that of heat treatment itself. It is possible that under optimal growth condition, *C. briggsae* has a more robust system to maintain genome integrity in its germline than *C. elegans*, which prevents its genome from environment-induced damage or editing. Improvement of transgenesis efficiency by heat shock treatment or inhibition of heat shock pathway could be at the cost of compromised genome integrity. One important way to preserve genome integrity in the germline is through Piwi-interacting RNA (piRNA), which constitutes one of the major regulatory molecules that curb the transposon activities (Ishizu et al. 2012). Consistent with this, functional perturbation of Hsp90 attenuates the piRNA silencing mechanism, leading to transposon activation and the induction of morphological mutants in *Drosophila* (Specchia et al. 2010). It is unclear in *C. elegans* whether the heat shock treatment leading to increased transposon mobilization. This is because the PRG-1 piRNA mutant does not show increased transposon mobilization even after many generations (Wahba et al. 2021).

### Differential responses to PEEL-1 and SUP-35 between *C. elegans* and *C. briggsae*

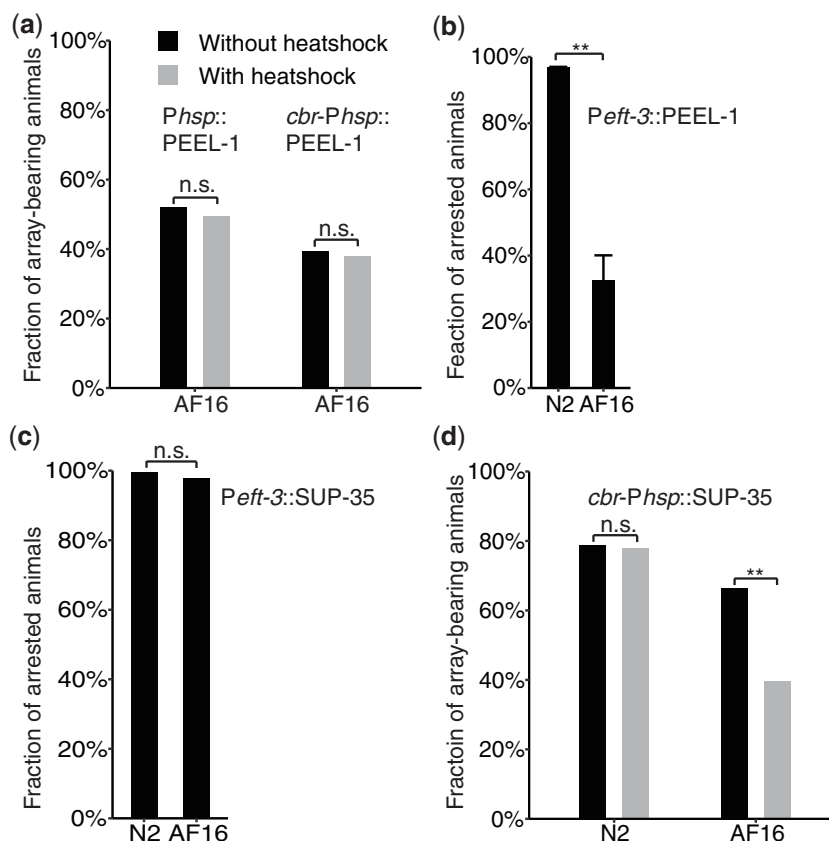
It is intriguing that *C. briggsae* responds differentially to the overexpressed paternal toxin PEEL-1 and maternal toxin SUP-35 from *C. elegans*. In *C. elegans*, PEEL-1 was suspected to function as a calcium pump on the cell membrane by generating a membrane pore, which leads to the release of intracellular calcium (Seidel



**Fig. 4.** Use of dual-color fluorescent markers in mapping interacting loci responsible for rescue of HI phenotype. a) Schematics of introgression strains expressing GFP marker (green) generated previously with biolistic bombardment or mCherry marker (red) generated with *miniMos* in this study. Strains ZZY10330 and ZZY10377 carry an X-linked introgression derived from *C. briggsae* that produces GFP or RFP expression, respectively, and HI phenotype in an otherwise *C. nigoni* background, i.e. hybrid male sterility. Strains ZZY10353 and ZZY10382 carry a Chromosome II-linked introgression derived from *C. briggsae* that produces GFP or RFP expression, respectively, and HI phenotype in an otherwise *C. nigoni* background, i.e. homozygous inviable (data not shown). b) Schematics of crossing strategy in mapping interacting loci between X chromosome and Chromosome II. We previously demonstrated that presence of the introgression fragment from ZZY10353 rescued the male sterility of ZZY10330. However, this demands tedious genotyping of ZZY10353 to ensure its presence because it is impossible to distinguish the 2 introgressions both expressing GFP. Substitution of ZZY10353 with ZZY10382 expressing RFP greatly facilitated the process for screening for simultaneous presence of the 2 introgressions. c) Reciprocal crossing between strains with autosome- and X-linked introgressions expressing GFP and RFP, respectively, serves as the same purpose as in (b).

*et al.* 2011). The maternal-effect toxin, SUP-35, kills developing embryos, but the molecular mechanism of its toxicity remains unclear (Ben-David *et al.* 2017). Our data showed that *C. briggsae* somehow develops native immunity against PEEL-1 but not SUP-35 (Fig. 5). However, forced expression of SUP-35 driven by the *C. briggsae* heat shock promoter did not mediate complete killing of

*C. briggsae* after heat shock treatment (Fig. 5d). Two possible reasons account for the ineffective killing. First, time windows for SUP-35 killing seem to be narrow, i.e. at certain stage of embryogenesis. This is why a constitutive promoter, i.e. *eft-3* promoter is able to drive SUP-35 expression and mediate complete killing. For the heat shock promoter, its response to heat shock may be more



**Fig. 5.** Comparison of killing efficiencies of PEEL-1 (a and b) and SUP-35 (c and d) in *C. elegans* (N2) and *C. briggsae* (AF16). a) Fraction of surviving array-bearing animals with (grey) and without (black) heat shock in *C. briggsae* (AF16), which carries a *peel-1* driven by heat shock promoter as an extrachromosomal array as indicated. Note, no significant change in survival using *hsp-16.41* promoter of from *C. elegans* and CBG19187(*cbr-hsp-16.60*) promoter from *C. briggsae*. b) Fraction of arrested F<sub>1</sub> animals presumably carrying an injection marker derived from P<sub>0</sub>, which was injected with *peel-1* driven by a constitutively expressing promoter, *Peft-3*. Note the incomplete killing of PEEL-1 expressing animals in *C. briggsae*. c) Fraction of arrested F<sub>1</sub> animals presumably carrying a co-injection marker derived from P<sub>0</sub>, which was injected with *sup-35* driven by *Peft-3*. Note the complete killing of *sup-35* expressing animals in both *C. elegans* and *C. briggsae*. d) Fraction of surviving array-bearing animals with (grey) and without (black) heat shock in *C. elegans* (N2) and *C. briggsae* (AF16), which carries a *sup-35* driven by a heat shock promoter from CBG19187(*cbr-hsp-16.60*) as an extrachromosomal array as indicated. Note, no significant change in survival in *C. elegans* and a significant drop in *C. briggsae* survival after heat shock.

effective during larval development than embryogenesis. Second, heat-shock induced expression of SUP-35 may not provide enough dosage to kill *C. briggsae* larvae. Further optimization of heat shock condition or choice of a different heat-shock promoter is necessary to develop an effective negative selection marker against extrachromosomal array in *C. briggsae*.

## Data availability

Strains and plasmids are available upon request. [Supplementary Figure 1](#) contains configurations of plasmids used in the study. [Supplementary Figure 2](#) shows the schematics of steps in identifying *C. briggsae* heat shock promoter and the sequence of *C. briggsae* heat shock promoter tested in this study. [Supplementary Table 2](#) contains sequence information of all primer sequences used in genotyping and molecular cloning. [Supplementary Table 1](#) lists transgene insertion site within the *C. briggsae* genome.

[Supplemental material](#) is available at G3 online.

## Acknowledgments

We thank Ms Cindy Tan for logistic support and members of Zhao's lab for helpful comments. Some strains were provided by the CGC,

which is funded by NIH Office of Research Infrastructure Programs (P40 OD010440).

## Funding

This work was supported by General Research Funds (N\_HKBU201/18, HKBU12100118, HKBU12101520, HKBU12101522) from Hong Kong Research Grant Council, and Initiation Grant for Faculty Niche Research Areas RC-FNRA-IG/21-22/SCI/02 from Hong Kong Baptist University to ZZ.

## Conflicts of interest

None declared.

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Communicating editor: M.-A. Félix