Balancer-assisted outcrossing to remove unwanted background mutations

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Abstract

Whole-genome sequencing analysis allows us to identify a large number of natural variants and genetic changes created by mutagenesis. For instance, the Million Mutation Project isolated many point mutant alleles, which are available from the Caenorhabditis Genetics Center. Although collections of such mutations are very useful for genetic studies, the strains are often sick because they have multiple other mutations than the mutation of interest. To utilize the strains, it is necessary to outcross with other strains to remove undesired mutations. We previously constructed an inversion balancer toolkit covering a large part of C. elegans genome. In contrast to classical translocation balancers that cover parts of two chromosomes, each balancer from the toolkit covers a part of a chromosome. We think this compactness is beneficial for outcrossing mutants containing multiple background mutations. Here, we show that the fluorescence inversion balancer can be practically useful for outcrossing in the case where researchers want to simply evaluate the phenotypes.
Figure 1. Outcrossing of a Million Mutation Project strain with the fluorescence balancer tmC18[tmIs1200].

(A) Dissecting microscope view of freshly starved wild type worms (top), gk837385 worms before outcross (VC40832, middle), and gk837385 worms after outcross (FX31729, bottom). The panel of the gk837385 original strain (middle) has many unhatched eggs (arrows). The panels of wild type (top) and the outcrossed strain (bottom) have many small larvae (arrowheads). Insets: enlarged images of the boxed area. (B) Genomic loci covered by tmC18. (C) The outcrossing scheme to segregate away unlinked mutations using the fluorescently labeled inversion balancer (shown by green). N2 males are first crossed to the fluorescently labeled inversion balancer strain to generate heterozygous tmC18[tmIs1200]/+ males (P0). Males resulting from this cross are then crossed to the ego-1 mutant hermaphrodites (F1). Venus-positive hermaphrodites resulting from the second cross are then self-fertilized (F2). Following self-fertilization, a non-Venus hermaphrodite is isolated (F3).

Description

In some situations, researchers may want to evaluate whether mutants of the Million Mutation Project (Thompson et al. 2013) show the intended phenotype or not. To utilize the strains, it is required to outcross with other strain to remove background mutations. In the case of deletion mutations, they can be identified by the difference in the size of the PCR bands when genotyping after outcrossing, which is relatively easy. However, the majority of the mutations in this collection are point
mutations, and genotyping them by Sanger sequencing and/or restriction fragment length polymorphism (RFLP) analysis are required (Robinson et al. 2017). Alternatively, genotyping can be done by using allele-specific PCR (Chen and Schedl 2021) or commercial kits, such as the High Resolution Melting (HRM) analysis (Doyle et al. 2021). It should be noted that regardless of the method, one needs to pick and genotype many candidate strains, unless the mutation linked to a visible phenotype. The fluorescent balancer can distinguish heterozygosity of alleles in the covered region: fluorescent marker-negative siblings from the parent bearing mutation over the fluorescent balancer are homozygotes for the mutation of interest because recombination near the locus of interest occurs very rarely (Edgley et al. 2006). Theoretically, outcrossing can be achieved by selecting worms without fluorescence for cleaning background mutations not covered by the balancer. Importantly, the balancer-assisted approach does not require genotyping many candidate strains and is most useful in the following situations: (a) the mutation of interest has no visible phenotype, (b) the strain has unlinked deleterious mutations, and (c) there are unlinked mutations that may modify the phenotype of mutation of interest. Indeed, a previous study applied this approach using the qC1 balancer and provided a good example of the situation (c), where three of daf-2 mutant strains from the Million Mutation Project also contained daf-18 mutations that suppress the daf-2 mutant phenotypes (Bulger et al. 2017). We experimentally tested how practically useful outcrossing with a structurally defined inversion balancer that we previously created by CRISPR/Cas9 gene editing (Dejima et al. 2018) could be and provide an example where the balancer-assisted approach works on the situation (b).

We focused on the ego-1 gene, which encodes an RNA-dependent RNA polymerase (Smardon et al. 2000). The null mutants for ego-1 show a sterile phenotype with a germline RNAi defect. When we ordered 23 mutants (see Reagents) from CGC, five strains (VC40084, VC40832, VC40259, VC20439, VC40175) were sick, and 3 of them (VC40084, VC40832, VC40259) were not able to be made frozen stocks on the first receipt. We ordered these three strains from CGC again. Two of the strains were frozen successfully, but one strain (VC40832) was difficult to freeze because it had mostly lethal embryos (Fig. 1A). We performed outcrosses of all strains once using a balancer tmC18[tmIs1200] that covers the ego-1 locus (Fig. 1B and C) (Dejima et al. 2018). For 22/23 strains, we obtained healthy worms by outcrossing. For example, for strain VC40832, the outcross improved its embryonic lethality (Fig. 1A, 99.37 ± 0.55 %, n = 20, and 0.18 ± 0.18 %, n = 9, for VC40832 and FX31729, the strain after outcross, respectively). However, for gk115395 a single outcross did not improve the small reduced brood size (303.8 ± 10.5, n = 4, and 67.6 ± 8.7, n = 4, for N2 and gk115395, respectively) suggesting that either additional outcrossing should be performed or that the mutation causing the decreased brood size is covered by the inversion balancer. Alternatively, the reduced brood size could be an ego-1 phenotype. Importantly, analysis by Sanger sequencing revealed the presence of mutations in all 23 outcrossed strains. Therefore, outcrossing with a fluorescence balancer is practically effective if researchers look for a simple screening method. Although we only tried tmC18 in this study, one can do the same with other loci and balancers. In addition, while we performed only a single outcross in this study and was able to prepare healthy worms for further experiments, it is important that the process should be repeated to further replace the mutagenized genome with wild type sequences. If the mutants of interest have invisible phenotypes such as behavior abnormality, experiments are greatly enhanced to work with recombination-free balancers as we presented here.

We note that there is a limitation of the balancer-assisted approach: the mutation of interest needs to be covered by one of the existing inversion balancers. The approach is most appropriate for removing unlinked mutations as unwanted mutations that are not covered by the balancer will be removed.

Methods

- Outcrossing with a balancer

The original ego-1 mutants (VC strains) were provided by the Caenorhabditis elegans Genetics Center, which is supported by the National Institutes of Health National Center for Research Resources. To outcross the strains, males of heterozygotes for tmC18[tmIs1200] were crossed with each ego-1 mutant strain. Venus+ F2 hermaphrodites were singled, then their Venus- F3 progeny was further singled and propagated. To confirm the mutations are present in the outcrossed strains, Sanger sequencing was performed. The primers used for PCR amplification and sanger sequencing are listed in the ego-1 mutant strain list (see Reagents).

Reagents
Balancer strain:
FX30167: tmC18[tmls1200(Pmyo-2::Venus)] I

-ego-1 mutant strains:

| Original strain | allele  | position | Outcrossed strain | Primers (PCR, Sanger seq.) |
|-----------------|---------|----------|-------------------|--------------------------|
| VC40259         | gk540555 | 7650738  | FX31713           | F26A3#F37R40, R40        |
| VC40886         | gk864727 | 7650888  | FX31714           | F26A3#F37R41, R41        |
| VC20206         | gk115390 | 7651213  | FX31715           | F26A3#F38R42, F38        |
| VC20319         | gk317493 | 7651478  | FX31716           | F26A3#F39R43, F39        |
| VC30058         | gk115391 | 7651882  | FX31717           | F26A3#F40R21, F40        |
| VC40244         | gk532049 | 7652169  | FX31718           | F26A3#F41R21, F41        |
| VC40660         | gk749674 | 7652199  | FX31719           | F26A3#F41R21, F41        |
| VC41006         | gk925207 | 7652313  | FX31720           | F26A3#F41R21, F41        |
| VC20618         | gk357146 | 7652424  | FX31721           | F26A3#F41R21, F41        |
| VC40613         | gk721963 | 7653391  | FX31722           | F26A3#F19R21, F44        |
| VC40920         | gk882383 | 7653502  | FX31723           | F26A3#F19R1, F44         |
| VC40140         | gk481348 | 7653535  | FX31724           | F26A3#F19R1, F44         |
| VC20439         | gk115393 | 7653805  | FX31725           | F26A3#F19R1, F45         |
| VC40951         | gk896494 | 7653809  | FX31726           | F26A3#F19R1, F45         |
| VC30158         | gk426642 | 7654398  | FX31727           | F26A3#F19R1, R1          |
| VC20474         | gk115394 | 7654992  | FX31728           | F26A3#F18R44, R44        |
| VC40832         | gk837385 | 7655076  | FX31729           | F26A3#F18R45, F18        |
| VC40084         | gk115395 | 7655149  | FX31730           | F26A3#F18R46, R46        |
| VC20545         | gk115397 | 7655230  | FX31731           | F26A3#F18R47, R47        |
- Oligonucleotides:

| Primer name | Sequence 5’ > 3’                  |
|-------------|-----------------------------------|
| F26A3#F18   | AAGCTCCACGAACTGTCCATC             |
| F26A3#F19   | AGGTGGAATCTATTTCCGCCAG           |
| F26A3#F37   | GTTCCGACCAGACGAGGAGT             |
| F26A3#F38   | CTACCAGGTGAGATGAAACC             |
| F26A3#F39   | GTAGTAGGTTTTGAGTGCGG             |
| F26A3#F40   | CGTGGCCATTGCTCTAACAT             |
| F26A3#F41   | TTACTATCAGTCGGAACCGG             |
| F26A3#F42   | CGACGTCTACGGTCGATCCC             |
| F26A3#F43   | ACCTGCTGCAGTCGACACCTCA           |
| F26A3#F44   | CCAAGCATTGACGGCGCTC             |
| F26A3#F45   | GAAAGCATTTGTCTGTCCAG             |
| F26A3#R1    | GGAATATGGCACCAGTTCT             |
| F26A3#R12   | GTCACGTTCTGTCTCATCT             |
| F26A3#R21   | TCGCCAGTTCCAGTGGCATT            |
| F26A3#R40   | GTTCCATCGCAAGCTGGTAG            |
| F26A3#R41   | CCGCACTCAAACCCCTAC              |
Acknowledgments: We thank the Mitani lab members for their support, the Million Mutations Project for generating gk mutants and the Caenorhabditis Genetics Center for strains.

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Funding: This work was supported by The National BioResource Project, Ministry of Education, Culture, Sports, Science, and Technology of Japan Grants-in-Aid for Scientific Research to KD (20K06561) and SM (20H03422), and a grant from the Takeda Science Foundation.

Author Contributions: Katsufumi Dejima: writing - original draft, funding acquisition, investigation. Shohei Mitani: writing - review editing, conceptualization, funding acquisition.

Reviewed By: Anonymous
History: Received March 17, 2022 Revision Received Invalid Date Accepted April 27, 2022 Published April 28, 2022

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Citation: Dejima, K; Mitani, S (2022), Balancer-assisted outcrossing to remove unwanted background mutations. microPublication Biology. 10.17912/micropub.biology.000561