An antibiotic selection marker for nematode transgenesis

Rosina Giordano-Santini[1], Stuart Milstein[2–4], Nenad Svrzikapa[2–4], Domena Tu[5], Robert Johnsen[5], David Baillie[5], Marc Vidal[2–4] & Denis Dupuy[1]

We have developed a nematode transformation vector carrying the bacterial neomycin resistance gene (*NeoR*) and shown that it could confer resistance to G-418 on both wild-type *Caenorhabditis elegans* and *C. briggsae*. This selection system allows hands-off maintenance and enrichment of transgenic worms carrying non-integrated transgenes on selective plates. We also show that this marker can be used for Mos1-mediated single-copy insertion in wild-type genetic backgrounds (MosSCI-biotic).

© 2010 Nature America, Inc. All rights reserved.

Antibiotic-resistance genes are commonly used as markers to monitor the introduction of exogenous genetic material into cells. Although they are widely used for genetic manipulation of cultured eukaryotic cells, yeast and bacteria[1], antibiotic selection systems have not so far been used for nematode transgenesis. Most genetic markers for nematode transgenesis have been based on easy-to-score phenotypes[2–5]. With a few exceptions[3,5], these markers do not provide a selective advantage, making the enrichment and maintenance of a transgenic population time-consuming. Moreover, with the exception of flurorescence-based methods, these systems rely on the use of mutant strains that are scarce in nematode species other than *C. elegans*[6–8].

We tested five nematode species and found that they were sensitive to G-418, a drug that inhibits protein synthesis in eukaryotes and prokaryotes and that is commonly used as a selective agent for eukaryotic cells[1]. Young larvae were more sensitive to G-418 than adults, probably because they are more dependent on their protein synthesis machinery for development. For each species, we determined a critical G-418 concentration, corresponding to the optimal selective conditions, at which young larvae were unable to develop and died after a few days, whereas young adults lived and were able to lay eggs (**Fig. 1a**).

We developed nematode transformation vectors carrying *NeoR* under the control of the ubiquitous promoter of *C. elegans* ribosomal protein gene *rps-27* (**Fig. 1b**,**c**). *NeoR* is widely used to confer resistance to G-418 to eukaryotic cells[1]. We injected wild-type *C. elegans* and *C. briggsae* with pDD04Neo also carrying the fluorescent reporter *C.e.*$_p$*myo-2::gfp*. We then placed single injected P_0 parents onto nematode growth medium plates containing G-418 at the critical concentration (selective plates) and allowed them to lay eggs at 20 °C. After 3 or 4 d, we screened plates for F_1 adults and we isolated them onto selective plates. We obtained several independent extrachromosomal G-418-resistant lines for both species. It was possible to identify at a glance plates from the F1 generation in which transmission of the array occurred, as only transgenic worms could give rise to mixed-stage populations of worms. These results showed that pDD04Neo confers resistance to G-418 on wild-type *C. elegans* and *C. briggsae* and allows hands-off selection of transgenic worms in the presence of the drug. To facilitate cloning experiments, we designed pdestDD04Neo (**Fig. 1b**) as a destination vector

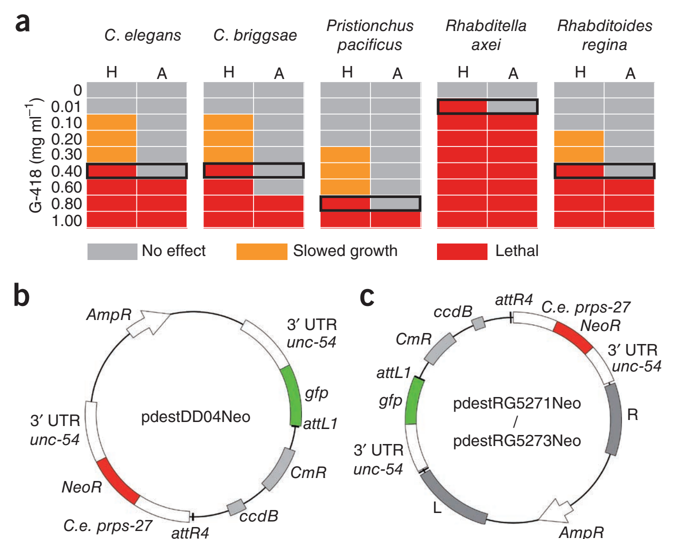

Figure 1 | *NeoR* as a genetic marker for nematode transgenesis. (**a**) Sensitivity of wild-type nematodes to G-418. Gravid adults (A) or hatchlings (H) were placed on selective plates with the indicated drug concentrations and growth was monitored for 1 month. Black frames indicate critical concentrations that were lethal for larvae but not for adults. (**b**) The pdestDD04Neo vector carries the bacterial neomycin resistance gene (*NeoR*) under the control of the *C. elegans rps-27* promoter. (**c**) The pdestRG5271Neo and pdestRG5273Neo MosSCI-biotic destination vectors carry the *NeoR* selection cassette and a Promoterome-compatible Gateway cassette upstream of *gfp*, both flanked by homologous regions corresponding to the left (L) and right (R) flanking sides of the Mos-1 insertion site of strains EN5271 and EN5273, respectively.

[1]Genome Regulation and Evolution, INSERM U869, Institut Européen de Chimie et Biologie, Pessac, France. [2]Center for Cancer Systems Biology, Dana-Farber Cancer Institute, Boston, Massachusetts, USA. [3]Department of Cancer Biology, Dana-Farber Cancer Institute, Boston, Massachusetts, USA. [4]Department of Genetics, Harvard Medical School, Boston, Massachusetts, USA. [5]Department of Molecular Biology and Biochemistry, Simon Fraser University, Burnaby, British Columbia, Canada. Correspondence should be addressed to D.D. (d.dupuy@iecb.u-bordeaux.fr).

RECEIVED 18 DECEMBER 2009; ACCEPTED 14 JUNE 2010; PUBLISHED ONLINE 22 AUGUST 2010; DOI:10.1038/NMETH.1494

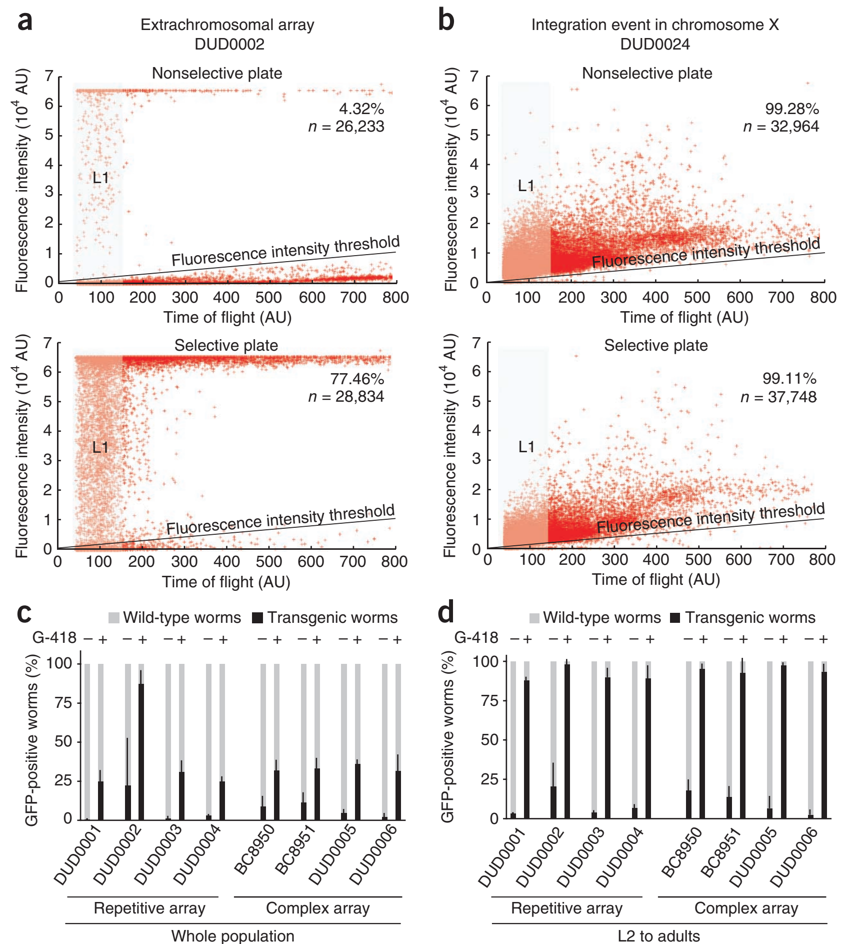

Figure 2 | Enrichment of NeoR transgenic lines in the presence of G-418. (**a**,**b**) Scatter plots from COPAS Profiler sorting of transgenic populations grown on nonselective (top) or selective (bottom) plates for a line carrying a repetitive extrachromosomal array (**a**, DUD0002) or a single-copy insertion of the transgene on chromosome X (**b**, DUD0024). Each data point represents one worm; fluorescence intensity in arbitrary units (AU) is plotted against time of flight (that is, the number of data points collected by the sensor, which is proportional to the size of the worms). The indicated fluorescence intensity threshold separates transgenic from wild-type worms. The percentage of GFP-positive worms relative to the total population and the total number of worms analyzed are shown. (**c**,**d**) Percentage of enrichment of NeoR transgenic worms. The percentage of GFP-positive worms for the indicated strains are plotted (black bars). They are calculated relative to the whole analyzed population ($n \approx 5{,}000$ worms; **c**) or population excluding L1 larvae on the basis of size (**d**). Error bars, s.d.; $n = 3$.

for introducing promoters from the Promoterome resource[9] upstream of *gfp*[10].

To assess the enrichment we could achieve using *NeoR*, we grew extra-chromosomal G-418-resistant lines of *C. elegans* and *C. briggsae* on selective plates. Just before starvation, we measured the percentage of transgenic worms in the populations, on the basis of GFP expression, using a COPAS Profiler (a fluorescence-assisted nematode sorter, Union Biometrica) (**Fig. 2**). When worms were grown on nonselective plates, transgenic worms represented 6.82% of the total population on average owing to the instability of the array[3] (**Fig. 2c**). When worms were grown at the critical G-418 concentration, the mean enrichment percentage reached 37.68% (three independent experiments with six *C. elegans* and two *C. briggsae* stable lines each). As the sorter also counted arrested or dead wild-type larvae, this value underestimates the actual enrichment. When we calculated enrichment percentages excluding L1 larvae on the basis of size, worms expressing GFP represented 93.09% of worms from L2 to adults, on average (**Fig. 2d**). The percentage of transgenic worms could reach up to 99.5% and was independent of species and transmission rate. We also carried out preliminary selection experiments in liquid medium (M9 buffer), in which we obtained 25–125-fold enrichment of transgenic worms, after a 1:10,000 dilution with wild-type worms, in one generation (4 d) (**Supplementary Table 1**). To our knowledge, this is the first evidence of a nematode transformation marker allowing near-perfect enrichment of non-integrated transgenic populations.

We obtained G-418-resistant lines by injection of either circular pDD04Neo $_p$*myo-2::gfp* (yielding transgenic worms with repetitive extrachromosomal arrays) or a complex mixture of linear plasmid and digested conspecific genomic DNA (yielding transgenic worms with complex extrachromosomal arrays). Some progeny of *C. elegans* lines with repetitive extrachromosomal arrays showed germline morphological defects and lower fertility even in the absence of the drug (data not shown), although the lines could still be efficiently propagated on selective plates over many generations without manual maintenance. This phenotype is unlikely to be a side effect of the expression of *NeoR* itself as extra-chromosomal arrays are silenced in the germline[3]. Conversely, silencing of the pDD04neo array might lead to a co-suppression effect[11]. This hypothesis is supported by the observation that both *C. elegans* and *C. briggsae* NeoR transgenic lines carrying complex arrays did not show this phenotype. Array composition did not have an effect on the efficiency of G-418 selection (**Fig. 2c**,**d**).

In the course of this work we have maintained more than 12 strains on selective media for several months without observing any adverse effect on the resistant worms other than the accumulation of dead eggs and arrested larvae due to the instability of the extrachromosomal array. There is no indication that G-418 causes harm to non-mosaic transgenic worms that are properly protected by the transgene. However it remains possible that the presence of G-418 in the medium affects the outcome of certain types of experiment. For such cases the use of integrated lines or preselected resistant individuals should overcome the problem.

To test whether this selection system was compatible with the recently described Mos1-mediated single-copy insertion method (MosSCI)[12], we selected two strains with an intergenic Mos1 insertion (EN5271, EN5273). For each of them we built a repair template vector containing the *NeoR* cassette and a $_p$*myo-2::gfp* transgene between ~1.4 kb of homologous chromosomal DNA from each

© 2010 Nature America, Inc. All rights reserved.

flanking side of the Mos1 element insertion site, as described[12] (**Fig. 1c**). We co-injected these vectors together with a vector encoding a transposase under the control of a heat-shock promoter (pJL44) and a vector carrying $_p$*rgef-1::DsRed2* (pCB101). As expected, the obtained extrachromosomal array strains were resistant to G-418 and expressed GFP in the pharynx and DsRed2 in the nervous system. After exposing the worms to heat shock[13], we allowed their progeny to proliferate and screened for integration events by identifying G-418-resistant individuals that expressed GFP in the pharynx and did not express DsRed2 (ref. 12). We confirmed insertion events by PCR (**Supplementary Fig. 1**) and checked that the worms carried a single copy of the inserted transgene by quantitative PCR (qPCR) (**Supplementary Figs. 2** and **3**). Thus, we demonstrated that our antibiotic selection system could be used in the context of MosSCI (**Fig. 2b**). This combined MosSCI-biotic method can be used directly on any strain from the NemaGENETAG[14] collection without the need to introduce a mutant *unc-119*, which is typically used as a co-insertion marker for MosSCI, into the genetic background[12] (**Supplementary Table 2**).

Another commonly used method for *C. elegans* transformation is microparticle bombardment[3]. We did not succeed in using G-418 selection with this technique as clear-cut selection cannot be obtained in excessive crowding conditions and ballistic transformation requires a very large population of target worms.

Antibiotic selection has several advantages over commonly used nematode transformation markers. First, rescue of nonlethal mutations such as *unc-119* or *dpy-5* requires the use of specific mutant backgrounds[3,4] that might not be appropriate for some biological studies; by contrast, antibiotic resistance can be used with any genetic background including transgenic or mutant strains of interest. Second, maintenance and enrichment of nonintegrated transgenic lines using existing markers is done by manually picking transgenic worms. This process is time consuming and may limit experiments that need many transgenic worms.

It is also possible that antibiotic resistance markers might prove useful for nematodes other than *C. elegans* and *C. briggsae*. Commonly used markers rely on mutant strains or dominant alleles, which are not always available for other nematode species, although some genes such as *C.e.rol-6(su-1006)* and *C. elegans* fluorescent reporters have been shown to work in other *Caenorhabditis* species[8]. Although many efforts have been made to apply *C. elegans* transgenesis methods to other species, the lack of a convenient selection system remains a limitation for comparative and evolutionary studies[6–8]. Here we have shown that five nematode species are sensitive to G-418; it should therefore be possible to use a common G-418 resistance marker for transgenesis in other species as well.

Finally, the use of antibiotic resistance markers for nematode transgenesis can be expanded to other drugs[15], which should enable the development of a wide range of powerful applications.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturemethods/.

Note: Supplementary information is available on the Nature Methods website.

ACKNOWLEDGMENTS

Supported by the Program INSERM "Avenir" (D.D.), la Fondation Bettencourt-Schueller (D.D.), le Conseil Régional d'Aquitaine (D.D.), la Fondation pour la Recherche Médicale (D.D.), Natural Science and Engineering Research Council of Canada (D.B.) and le Ministère Français de l'Enseignement de la Recherche et des Technologies (R.G.-S.). We thank I.A. Hope, J. Ewbanks and J.L. Bessereau for discussions and access to facilities; and T. Leste-Lasserre and G. Drut for discussion about qPCR. EN5271 and EN5273 and MosSCI related plasmids were provided by J.L. Bessereau (INSERM U1024, Institute of Biology of the École Normale Supérieure). The *Caenorhabditis* Genetics Center, which is funded by the National Center for Research Resources of the US National Institutes of Health, provided the nematode species.

AUTHOR CONTRIBUTIONS

S.M. and N.S. performed preliminary experiments under the supervision of M.V. D.D. designed and supervised the project and constructed the pDD04neo vector with N.S. Microinjections were performed by R.G.-S. and D.T. under the supervision of D.D., D.B. and R.J. R.G.-S. constructed MosSCI-biotic related vectors, characterized the transgenic worms and wrote the manuscript with D.D.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

Published online at http://www.nature.com/naturemethods/.
Reprints and permissions information is available online at http://npg.nature.com/reprintsandpermissions/.

1. Colbere-Garapin, F., Horodniceanu, F., Kourilsky, P. & Garapin, A.C. *J. Mol. Biol.* **150**, 1–14 (1981).
2. Mello, C.C., Kramer, J.M., Stinchcomb, D. & Ambros, V. *EMBO J.* **10**, 3959–3970 (1991).
3. Praitis, V., Casey, E., Collar, D. & Austin, J. *Genetics* **157**, 1217–1226 (2001).
4. Thacker, C., Sheps, J.A. & Rose, A.M. *Cell. Mol. Life Sci.* **63**, 1193–1204 (2006).
5. Granato, M., Schnabel, H. & Schnabel, R. *Nucleic Acids Res.* **22**, 1762–1763 (1994).
6. Schlager, B., Wang, X., Braach, G. & Sommer, R.J. *Genesis* **47**, 300–304 (2009).
7. Zhao, Z. *et al. Genetics* **184**, 853–863 (2010).
8. Streit, A. *et al. Genetics* **152**, 1573–1584 (1999).
9. Dupuy, D. *et al. Genome Res.* **14**, 2169–2175 (2004).
10. Fire, A., Kondo, K. & Waterston, R. *Nucleic Acids Res.* **18**, 4269–4270 (1990).
11. Dernburg, A.F., Zalevsky, J., Colaiacovo, M.P. & Villeneuve, A.M. *Genes Dev.* **14**, 1578–1583 (2000).
12. Frokjaer-Jensen, C. *et al. Nat. Genet.* **40**, 1375–1383 (2008).
13. Robert, V.J., Katic, I. & Bessereau, J.L. *Methods* **49**, 263–269 (2009).
14. Bazopoulou, D. & Tavernarakis, N. *Genetica* **137**, 39–46 (2009).
15. Semple, J.I., Garcia-Verdugo, R. & Lehner, B. *Nat. Methods* advance online publication, doi:10.1038/nmeth.1495 (22 August 2010).

© 2010 Nature America, Inc. All rights reserved.

ONLINE METHODS

Worm strains. Repetitive extrachromosomal array lines were obtained after injection of 100 ng μl^{-1} pDD04Neo $_p$*myo2::gfp* into *C. elegans* N2. *C. briggsae* carrying complex extrachromosomal arrays were obtained after injection of 0.8 ng μl^{-1} pDD04Neo $_p$*myo2::gfp* and 60 ng μl^{-1} conspecific genomic DNA, both digested with SmaI. *C. elegans* carrying complex extrachromosomal arrays were obtained after injection into CB907 of 0.4 ng μl^{-1} pDD04Neo $_p$*myo2::gfp*, 30 ng μl^{-1} conspecific genomic DNA, both digested with SmaI and pCeh361 at 40 ng μl^{-1}. For MosSCI experiments, lines were obtained after injection of 30 ng μl^{-1} pRG5271Neo $_p$*myo-2::gfp* (or pRG5273Neo $_p$*myo-2::gfp*), 30 ng μl^{-1} pCB101 and 30 ng μl^{-1} pJL44 into EN5271 (or EN5273). Injected P_0 parents were moved onto selective plates at the critical concentration of G-418 (0.4 mg ml^{-1}) and allowed to proliferate. Plates were screened between 4 d and 1 week for resistant F_1 progeny. Once lines were established, they were maintained on 13-cm selective plates by chunking or by picking individuals (**Supplementary Table 3**).

Critical G-418 concentration and enrichment experiments. Selective plates were made as follows: G-418 (Gibco, Invitrogen) from a 50 mg ml^{-1} solution in water was added to standard nematode growth medium (NGM) before pouring (50–55 °C). Plates were left for 1 d at room temperature (20–22 °C). We use a 5× concentrated *E. coli* OP50 culture to seed the plates as the bacteria lawn does not develop in the presence of G-418. Before seeding, plates were allowed to dry for 20–30 min under a sterile hood. We added 1 ml 5× concentrated bacteria culture to a 13-cm plate, 300 μl to a 5-cm plate and 50 μl to a 2.5-cm plate. The bacteria lawn was allowed to dry under the sterile hood. Plates were stored at 20–22 °C for immediate use or at 15 °C when used in the following weeks (we stored plates at 15 °C for up to 3 weeks). We took all precautions to avoid bacterial contamination on selective plates as resistant bacteria seem to degrade G-418 and protect worms from the drug. To test the sensitivity of wild-type nematodes to G-418, we put ten hatchlings or ten gravid adults onto 5-cm selective plates at different concentrations from 0.01 mg ml^{-1} to 2 mg ml^{-1}. Their growth was monitored for up to 1 month. The critical concentration corresponds to the G-418 concentration at which hatchlings die while young adults live and can lay eggs. For enrichment experiments (**Fig. 2c**,**d**), three individuals were moved onto 5-cm selective plates or NGM plates (nonselective) and allowed to proliferate at 20 °C. To generate scatter plots (**Fig. 2a**,**b**), three individuals were moved onto 13-cm selective plates or nonselective plates and allowed to proliferate at 20 °C. Upon exhaustion of the bacteria lawn the mixed-stage population was analyzed using a COPAS Profiler (Union Biometrica). At this point, populations were composed of several thousand worms. For all the experiments, selective plates were rejected if contaminated with resistant bacteria.

For enrichment experiments in liquid media, we used synchronized populations of L1 larvae. L1 larvae were obtained by hypochlorite treatment of gravid adults followed by incubation of the released eggs overnight in M9 buffer. We mixed a synchronized population of wild-type *C. elegans* L1 larvae and 10 L1 larvae carrying a single copy insertion of the transgene (DUD0029) into M9 supplemented with 0.4 mg ml^{-1} G-418 without food (bacteria). Worms were incubated at 20 °C under agitation. By day four, worms were harvested and plated onto NGM plates. After 2 d, when living worms had reached adulthood, we counted wild-type and transgenic worms based on the presence or absence of GFP (**Supplementary Table 1**).

MosSCI-biotic. The presence of Mos1 insertions in lines EN5271 and EN5273 was verified by PCR with primers annealing inside (oJL115) and outside Mos1 (oVR261 and oVR266, respectively). Repair templates were built following the MosSCI protocol[12] and contained $_p$*myo-2::gfp* and $_p$*rps-27::NeoR::3′UTR unc-54* flanked by the right and left sides of the Mos1 insertion locus (**Supplementary Table 4**). We established stable lines *kr5271 I;Ex[pCB101;pRG5271Neo* $_p$*myo-2::gfp;pJL44]* and *kr5273 X;Ex[pCB101;pRG5273Neo* $_p$*myo-2::gfp;pJL44]* that could properly be propagated on selective plates (0.4 mg ml^{-1} G-418). As expected, all of these lines were resistant to G-418, and expressed GFP in the pharynx and DsRed2 in the nervous system. Selective plates containing mainly young adults were heat-shocked in a water bath for 1 h at 33 °C, allowed to recover for 1 h at 15 °C and heat-shocked again for 1 h at 33 °C (ref. 13). This treatment triggered the expression of the transposase and consequently the mobilization of Mos1. After one night at 15 °C, pools of 20 young adults were transferred to fresh selective plates and allowed to proliferate at 20–22 °C. When worms were about to be starved, a quarter of each plate was chunked to a new selective plate. Worms were allowed to starve at 20–22 °C again. At this point, a small chunk was transferred to a new plate and worms were screened under a fluorescence scope. Worms expressing GFP in the pharynx but lacking DsRed2 expression in the nervous system were retained as integration events for further analysis. Because this experiment leads to excessive crowding conditions, some plates contained escaper worms lacking GFP and DsRed2 expression. To confirm that these worms did not represent complex insertion events, we isolated some of them on selective plates. None of the isolated escapers gave rise to resistant progeny. From this point we only considered as potential integration events resistant worms expressing GFP in the pharynx.

PCR analysis of MosSCI-biotic insertion events. To confirm that the transgene was inserted in the right locus and that these worms had lost the extrachromosomal array, we performed single-worm PCR with two pairs of primers (**Supplementary Fig. 1**). For insertion events in line EN5271, primers org115 (anneals in *unc-54* 3′ UTR downstream of *NeoR*) and org146 (anneals in the genome outside the right recombination region) give a PCR product of 1.4 kb if the transgene is inserted in the right locus. Primers M13F (anneals in the repair template backbone) and org99 (anneals in the left recombination region) give a PCR product of 1.5 kb if the extrachromosomal array is present. For insertion events in line EN5273, primers org115 and org145 (anneals in the genome outside the right recombination region) give a PCR product of 1.7 kb if the transgene is inserted in the right locus. Primers M13F and org103 (anneals in the left recombination region) give a PCR product of 1.5 kb if the extrachromosomal array is present (**Supplementary Table 5**).

Quantitative PCR analysis of MosSCI-biotic insertion events. All of the lines obtained by MosSCI-biotic were resistant to G-418, as expected. To confirm that resistance was conferred

© 2010 Nature America, Inc. All rights reserved.

doi:10.1038/nmeth.1494

by a single copy of *NeoR* and to exclude the possibility of a complex insertion event leading to more than one copy of the gene, we analyzed genomic DNA of insertion lines by qPCR. Genomic DNA were extracted using Genomic DNA Mini spin kit NucleoSpin Tissue (Macherey-Nagel). Aliquots of genomic DNA were subjected to PCR amplification on a DNA Engine Opticon2 fluorescence-detection system (MJ Research/Bio-Rad) with specific forward and reverse oligonucleotide primers (**Supplementary Fig. 2** and **Supplementary Table 6**). The DyNAmo SYBR Green qPCR kit (Finnzymes) was used with the following PCR amplification cycles: initial denaturation at 95 °C for 15 min, followed by 40 cycles with denaturation at 95 °C for 20 s and annealing-extension at 61 °C for 35 s. A dissociation curve was generated to verify that a single product was amplified. For each line, the cycle threshold (C(t)) of each gene was normalized against that of *unc-119*. For all integrated strains we obtained nearly identical C(t) values for all genomic controls and the tested transgenes, as would be expected with a single copy integration event (**Supplementary Fig. 3** and **Supplementary Table 7**). No amplification of the transgene was obtained from the untransformed receptor strains and a large number of copies were detected in the intermediate extrachromosomal arrays before integration.

Molecular cloning. For the construction of pdestDD04Neo, NeoR was amplified from pCDNA-dest53.

pRG5271Neo was built as follows: a 1.5-kb genomic region from the left side of *kr5271* was PCR-amplified from genomic DNA using oligonucleotides org98 and org99 (**Supplementary Table 5**) and used to replace the KpnI and ApaI fragment of pdestDD04Neo (p71L). A 1.3-kb genomic region from the right side of *kr5271* was PCR-amplified from genomic DNA using oligonucleotides org113 and org114. The 1.3-kb insert was cloned into p71L using the SmaI and NotI restriction enzymes.

pRG5273Neo was built following the same protocol: a 1.4-kb genomic region from the left side of *kr5273* was PCR-amplified from genomic DNA using oligonucleotides org102 and org103 and used to replace the KpnI and ApaI fragment of pdestDD-04Neo (p73L). A 1.6-kb genomic region from the right side of *kr5273* was PCR-amplified from genomic DNA using oligonucleotides org111 and org112. The 1.6-kb insert was cloned into p73L using the NotI and SmaI restriction enzymes.

A Gateway cassette flanked by *attR4* and *attL1* sites upstream of *gfp* was PCR-amplified from pdestDD04neo using primers org119 and org120. The cassette was cloned into pRG5271Neo and pRG5273Neo using ApaI to create pdestRG5271Neo and pdestRG5273Neo, respectively (**Supplementary Tables 4** and **5**).

npg © 2010 Nature America, Inc. All rights reserved.