Rapid Creation of Forward-Genetics Tools for *C. briggsae* Using TALENs: Lessons for Nonmodel Organisms

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Abstract

Although evolutionary studies of gene function often rely on RNA interference, the ideal approach would use reverse genetics to create null mutations for cross-species comparisons and forward genetics to identify novel genes in each species. We have used transcription activator-like effector nucleases (TALENs) to facilitate both approaches in *Caenorhabditis* nematodes. First, by combining golden gate cloning and TALEN technology, we can induce frameshifting mutations in any gene. Second, by combining this approach with bioinformatics we can predict and create the resources needed for forward genetic analysis in species like *Caenorhabditis briggsae*. Although developing genetic model organisms used to require years to isolate marker mutations, balancers, and tools, with TALENs, these reagents can now be produced in months. Furthermore, the analysis of nonsense mutants in related model organisms allows a directed approach for making these markers and tools. When used together, these methods could simplify the adaptation of other organisms for forward and reverse genetics.

Key words: **evolution, nematode, TALENs, model organism, *C. briggsae*.**

Letter

Introduction

With the advent of genome sequencing, there has been an explosion in the number of organisms being used for research into the evolution of gene regulation and function (e.g., Emlen et al. 2005) or that might be adapted for these studies in the near future (e.g., Kiontke et al. 2011; Kanzaki et al. 2012). However, the only method available for analyzing gene function in many species is RNA interference. Despite the simplicity of this approach, there can be significant variations in the response to RNAi between species (Nuez and Felix 2012), which complicates evolutionary comparisons.

The analysis of null mutations would provide a better method for comparing the functions of genes across species, but creating these mutations can be laborious, and maintaining them often requires balancers. In addition, forward genetic screens would allow the unbiased identification of new genes or genes with unexpected functions, which can be critical for evolutionary analysis (Guo et al. 2009). However, the ability to carry out these screens depends on genetic markers, balancing chromosomes and tools that took decades to develop for *Drosophila melanogaster* or *Caenorhabditis elegans*. For example, the related nematode *C. briggsae* has been studied for years, but random screens have only produced convenient sets of markers for half of its chromosomes. We show that transcription activator-like effector nuclease (TALEN) technology can overcome this problem.

New Approaches

We used TALENs to help develop the model organism *C. briggsae* for genetic and evolutionary studies. TALENs can be designed to target any DNA sequence (reviewed by Sun and Zhao 2013), and were recently tested in both *C. elegans* and *C. briggsae* (Wood et al. 2011; Lo et al. 2013). However, each TALEN requires 15–20 repeat units to bind a unique target, so we adapted golden gate shuffling to streamline the process of making custom TALENs for nematodes. Furthermore, new mutations can be difficult to study unless other genetic resources are available, so we used information from a set of *C. elegans* null mutations to identify and target genes needed to create markers, balancers, and tools for *C. briggsae*. This approach could be adapted to develop many other species as genetic model organisms.

Results and Discussion

To simplify producing custom TALENs for *C. briggsae*, we adapted the golden gate shuffling method (Cermak et al. 2011) for use in nematodes (supplementary fig. S1, Supplementary Material online). Furthermore, to speed up the identification of new mutations, we screened for insertions or deletions by their altered size on 10% acylamide gels, eliminating the need for nuclease digestion (fig. 1*A*).

Using these approaches, we created hundreds of mutations in dozens of genes during the past 8 months (table 1, unpublished results). The efficiency of some TALENs was so high that many F_1 animals were heterozygous for two new mutations: one induced in the maternal genome and a second in the paternal genome after fertilization (fig. 1*A*). Although most of the mutations were small insertions or deletions, we occasionally recovered large deletions (fig. 1*B*). Thus, TALENs can produce a variety of mutations in nematode genes, and the procedure is rapid and efficient.

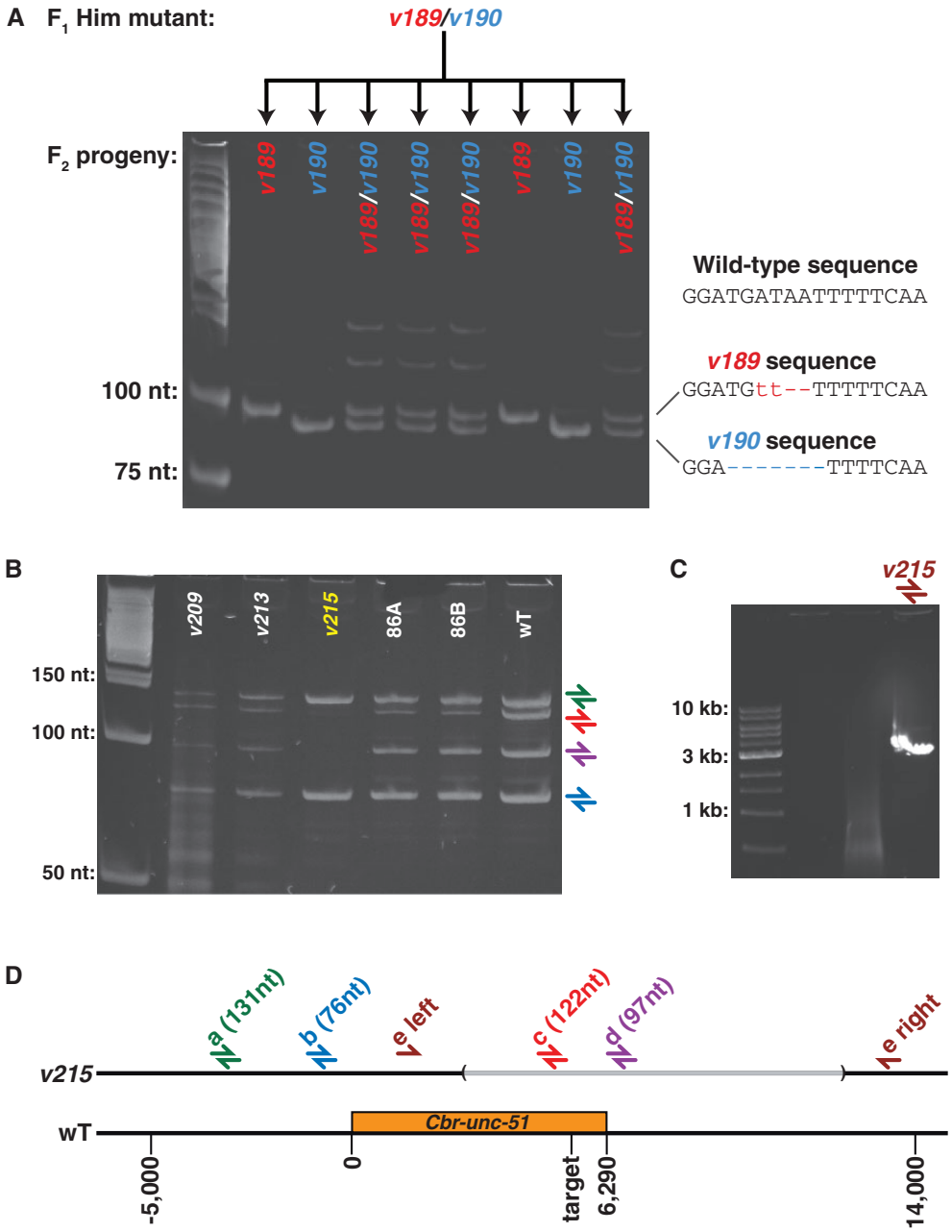

Fig. 1. Isolation and characterization of TALEN-induced mutations in *C. briggsae*. (*A*) Many F_1 animals were heterozygous for two distinct mutations. Following injection of TALENs targeting the *Cbr-him-8* gene, we isolated an F_1 animal with the Him phenotype (high incidence of male progeny). DNA from eight F_2 self-progeny was amplified by the polymerase chain reaction (PCR) and separated on a 10% acrylamide gel, revealing the presence of two different alleles. The sequence of each lesion is shown at the right. (*B*) TALENs can produce large deletion mutations in nematodes. DNA from potential *Cbr-unc-51* mutants was amplified using four sets of primer pairs (a–d). The size of each expected fragment is indicated in (*D*). Mutant *Cbr-unc-51(v215)* lacked DNA in the region of primer pairs c and d. (*C*) The primer pair e could amplify a single band of approximately 3 kb from *v215* DNA, indicating that most of this region was deleted. (*D*) Map of the region, showing the extent of the 9423 bp *v215* deletion, determined by sequencing the fragment shown in (*C*). Positions on the map are related to the start of *Cbr-unc-51*.

With this method in hand, we asked whether TALENs could be used to speed up the development of genetic model organisms, using *C. briggsae* as a test case. The first mutations in this species were isolated more than 60 years ago (Nigon and Dougherty 1950), but three of the six chromosomes still lacked sets of mapped marker genes to simplify genetic analysis. These three chromosomes (LG *I*, *V*, and *X*, www.briggsae.org, last accessed November 19, 2013) contain about 50 Mbp of DNA.

Fortunately, bioinformatic resources for *C. briggsae* are extensive. The genome sequence is nearly complete (Stein et al. 2003), and single nucleotide polymorphisms between the AF16 and HK104 strains have been used to create a rough linkage map (Hillier et al. 2007; Koboldt et al. 2010; Ross et al. 2011). These steps are critical for work in new species, but further progress depends on genetic resources. Unfortunately, forward screens have produced few visible markers, and new mutations take time to place on the genetic map. Reverse

Table 1. Comparison of *Caenorhabditis elegans* and *C. briggsae* Mutations.

Gene	LG	Protein	*Cel* Allele	*Cel* Phenotype	*Cbr* Allele	*Cbr* Phenotype	# *Cbr* Alleles
unc-40	*I*	Netrin receptor (1,415 aa; Chan et al. 1996)	*e1430* R157stop	Weak kinker Unc, slightly Dpy	*v248*fs (8 bp Δ)	Kinker Unc, slightly Dpy	2
dpy-5	*I*	Procollagen (284 aa; Thacker et al. 2006)	*e61* G203stop	Strong Dpy	*v234*fs (8 bp Δ/6 bp ins)	Strong Dpy	28
smg-5	*I*	Novel (549 aa; Anders et al. 2003)	*r860* Q17stop	NMD defective, pVul	*v246*fs (8 bp Δ)	NMD defective, pVul	4
him-8	*I*	Zinc fingers (361; MacLeod et al. 1981)	*tm611* deletion	High Incidence of males	*v188*fs (7 bp Δ)	High incidence of males	9
unc-54	*I*	Myosin heavy chain (1,963 aa; MacLeod et al. 1981)	*e1092* Q1072stop	Paralyzed Unc	*v139*fs (11 bp Δ) *v138* (6 bp Δ)	Paralyzed, lethal Paralayzed Unc	2
unc-34	*V*	Enabled/VASP (468 aa; Yu et al. 2002)	*gm104* W10stop	Coiler Unc	*v255*fs (11 bp Δ)	Coiler Unc	7
dpy-11	*V*	Thioredoxin-like (246 aa; Ko and Chow 2002)	*e207* R4stop	Dpy	*v241* (6 bp Δ)	Dpy	11
unc-51	*V*	Protein kinase (856 aa; Ogura et al. 1994)	*ks38* Tc1 insertion	Paralyzed and Dpy	*v204*fs (5 bp Δ)	Paralyzed and Dpy	14
unc-1	*X*	Stomatin-like (289 aa; Rajaram et al. 1998)	*e719*fs	Kinker Unc	*v236*fs (2 bp Δ)	Kinker Unc	4
dpy-8	*X*	Collagen (452 aa; McMahon et al. 2003)	*e130* unknown	Dpy	*v262* (15 bp Δ)	Dpy	8
unc-7	*X*	Innexin (522 aa; Krishnan et al. 1993)	*e5* Q96stop	Kinker Unc	*v272*fs (11 bp Δ)	Kinker Unc	3

Note.—If a gene produces more than one transcript, only the size of the largest product is listed. A representative null alleles or strong loss-of-function allele is shown for each gene. "fs" indicates a frameshift mutation, and "Δ" indicates a deletion. "*Cel* allele"—putative null alleles of each *C. elegans* gene except *dpy-8*, which lacked data. "*Cbr* allele"—reference alleles isolated in *Caenorhabditis briggsae* using TALENs. Most are null. "# *Cbr* alleles"—the number of alleles isolated in the TALEN screen. Some were not saved.

genetic approaches might solve this problem, but directed screens for large deletions have been slow and expensive (Hill et al. 2006). Thus, we applied TALEN technology.

Because TALENs usually produce small deletions, they often create null mutations. Thus, we tested two criteria for selecting potential marker genes in *C. briggsae*: 1) They must be orthologs of *C. elegans* genes with viable null mutations and 2) the *C. elegans* mutants must have visible phenotypes. For example, *C. elegans unc-32(e189)* is a widely used marker for LG*III*, but *unc-32* null alleles are lethal (Pujol et al. 2001), so we did not target its *C. briggsae* ortholog. Similarly, we avoided genes with wild-type null phenotypes, such as *unc-93* (Greenwald and Horvitz 1980). After selecting potential markers, we targeted each gene with TALENs, to see whether these guidelines allowed an accurate prediction of phenotypes in *C. briggsae* (table 1). This effort represents one of the largest cross-species comparisons of animal gene function done using targeted knockouts.

Using this approach, we rapidly isolated morphological mutants for the remaining *C. briggsae* chromosomes (fig. 2). The phenotypes of most mutants strongly resemble those of their *C. elegans* orthologs, including *unc-40* and *dpy-5* on LG*I*, *unc-34* and *unc-51* on LG*V* and *unc-1*, *dpy-8*, and *unc-7* on *X* (table 1). However, the phenotypes of two genes were more severe—*C. briggsae unc-54*(*v139*fs) and *dpy-11* null mutants were barely viable and could not be maintained, whereas the corresponding *C. elegans* mutants were much healthier. In these two cases, we also recovered partial loss-of-function mutations that were viable and easily scored. Overall, our approach predicted the null phenotypes for seven of nine genes and produced useful mutations for all nine. Moreover, each new mutation was already positioned in the *C. briggsae* genome, providing an instant link between the physical and genetic maps (fig. 2).

To see if this method could be extended to other species, we chose *C. sp. 11* (Kiontke et al. 2011), another hermaphroditic nematode. After identifying the *C. sp. 11 unc-23* gene (supplementary fig. S2, Supplementary Material online), we used TALENs to induce *v277* and *v279*, two frameshift mutations that are null alleles. The mutant animals have difficulty moving normally, often coil, and show progressively greater defects with age, because the head bends to one side. These phenotypes are very similar to those of *C. elegans unc-23* mutants, which cause fragile muscle attachments (Plenefisch et al. 2000).

Although mutants with visible phenotypes can be used as balancers or to mark chromosomes in crosses, other genetic tools are also important. First, many TALEN mutants produce truncated proteins because of frameshifting mutations. If these transcripts are not eliminated by the nonsense-mediated decay (NMD) surveillance system (Weischenfeldt et al. 2005), they could be used to map functional domains in proteins. Thus, we used TALENs to knock out *C. briggsae smg-5*, which encodes a component of the NMD system (Anders et al. 2003). As predicted, this mutation restores partial activity to stop mutants in other genes (manuscript in preparation).

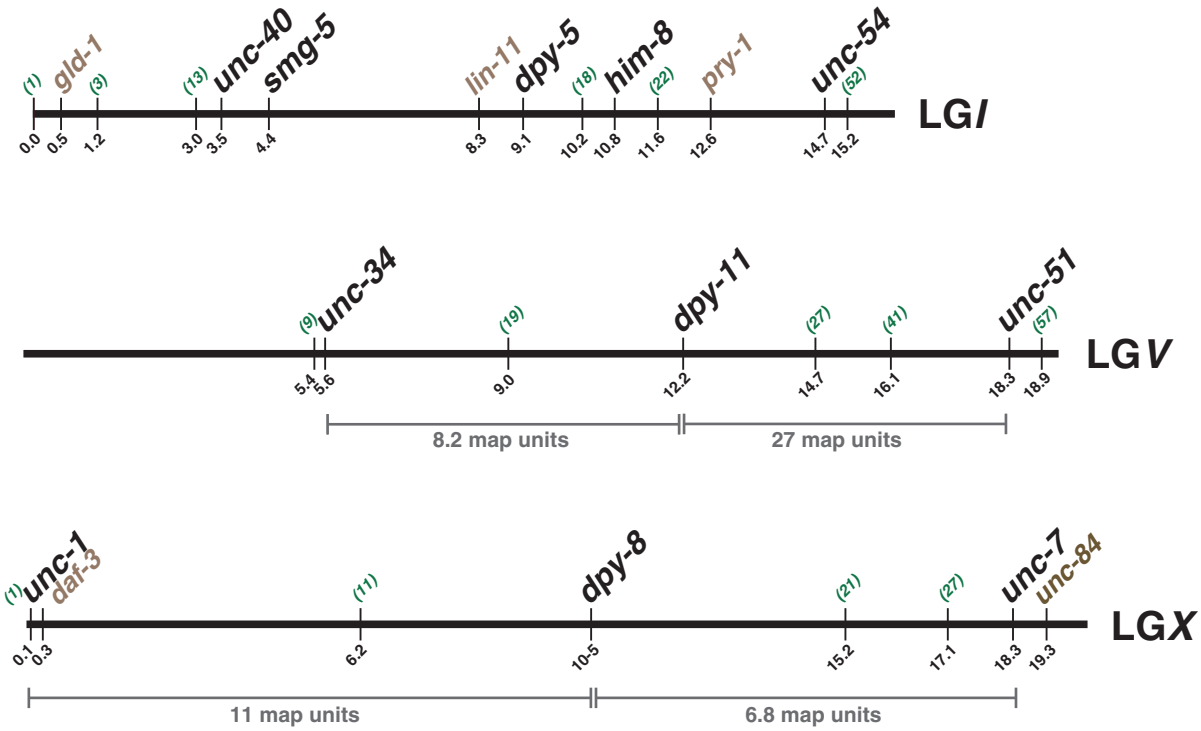

Fig. 2. Engineering *C. briggsae* chromosomes for genetic analysis. Maps of the three *C. briggsae* chromosomes used in this project. Positions in megabases are shown below each line, as listed in Wormbase. The approximate positions in centimorgans of SNPs mapped by bulk segregant analysis are shown above each line in parentheses (Koboldt et al. 2010). Genes with mutations isolated in this paper are in large, bold letters. The locations of mutations in genes that affect vulval development, fertility, or dauer formation are shown in gray (Inoue et al. 2007; Seetharaman et al. 2010; Beadell et al. 2011; Sharanya et al. 2012).

Second, hermaphroditic populations of nematodes reproduce by self-fertilization, but males are essential for crosses or phenotypic analysis. Thus, we created mutations in *C. briggsae him-8*, which controls *X*-chromosome pairing (Phillips and Dernburg 2006). Following self-fertilization, 35% of the *Cbr-him-8* progeny were *XO* males and only 2% died as embryos ($n = 498$). These phenotypes are similar to those for *C. elegans him-8* (Hodgkin et al. 1979).

Taken together, our results demonstrate a new use for TALEN or CRISPR (Damian and Porteus 2013) technologies—developing new species as genetic model organisms. This approach should be particularly valuable for species that are easily cultured in the laboratory and have closely related model organisms to guide the work, like members of *Caenorhabditis, Pristionchus, Drosophila,* or *Tribolium*. However, even distant model organisms might be helpful, because the proteins of *C. elegans* and *C. briggsae* are different from each other as those of humans and mice (Stein et al. 2003). Thus, information about *Tribolium* might aid in the development of more distant beetles as genetic model organisms, and zebrafish might be used for other fishes. However, there are some limits. For example, *Xenopus laevis* is polyploid, so it would be unsuitable for many genetic experiments. By contrast, TALENs work in the diploid frog *X. tropicalis* (Lei et al. 2013), so these techniques might facilitate its development as a model system.

For each new species, the critical steps are as follows: 1) Sequence the genomes of at least two inbred wild-type strains and identify SNPs; 2) develop a genetic map using a technique like advanced intercross recombinant inbred lines (Darvasi and Soller 1995); 3) use TALENs to create sets of genetic markers for each chromosome. Related species should provide a reliable guide, because most genes with nonsense mutations in *C. elegans* produced similar phenotypes when knocked out in *C. briggsae*; and 4) knock out additional genes to make genetic tools. Some tools would be determined by technical considerations, such as NMD mutations for analyzing truncations. Some would be determined by species, such as Him mutants for work with hermaphroditic nematodes, and others would be determined by the biological problem.

These steps are now rapid and inexpensive, usually less than $80 per gene for reagents. Opening up new species for sophisticated genetic analyses should revolutionize evolutionary developmental biology, because the use of null mutants provides a reliable way to compare gene function across species and nonbiased screens give a method for identifying evolutionary novelties (Guo et al. 2009).

Materials and Methods

Genetics

Caenorhabditis briggsae mutants were derived from the wild isolate AF16 (Fodor et al. 1983) and *C. sp. 11* from JU1373 (Kiontke et al. 2011). Two-factor mapping was done as described by Brenner (1974). From *dpy-8 unc-7*/++ mothers,

we saw 448 wild type, 19 Dpy, 22 Unc, and 131 Dpy Unc progeny. From *dpy-11 unc-51*/++, we observed 485 wild type, 96 Dpy, 105 Unc, and 72 Dpy Uncs. From *unc-1 dpy-8*/++, we observed 740 wild type, 63 Dpy, 62 Unc, and 333 Dpy Uncs, and from *unc-34 dpy-11*/++, we observed 1,008 wild type, 54 Dpy, 53 Unc, and 249 Dpy Uncs.

Procedures for Generating TALEN Knockout Mutants

First, pairs of custom TALENs were designed with TALE-NT 2.0 software (https://tale-nt.cac.cornell.edu/ [last accessed November 19, 2013]; Doyle et al. 2012), using a separation of 17 nt between binding sites. Each target sequence was 15–20 nt long and tested by Blast to avoid repetitive regions.

Second, each set of TALEN repeats was built using a golden gate assembly protocol (Cermak et al. 2011). Plasmids for the initial steps were purchased from Addgene (http://www.addgene.org/TALeffector/goldengateV2/, last accessed November 19, 2013). In the final step, the repeat sequences were cloned into the destination vector pRE189 (supplementary fig. S1, Supplementary Material online), rather than into one of the pTAL1-4 backbone vectors (Cermak et al. 2011). pRE189 combines sequences developed by Wood et al. (2011) with golden gate cloning sites and is optimized for use in *Caenorhabditis* nematodes.

Third, each plasmid was linearized by digestion with *Hind*III, treated with 100 μg/ml Proteinase K and 0.5% sodium dodecyl sulfate (SDS) for 30 min at 50 °C, and purified on a QIAquick column (Qiagen). We then synthesized mRNA using the SP6 mMessage Machine (Ambion) and purified it on MegaClear columns (Ambion). We precipitated each mRNA and dissolved it in water to give a final concentration of 4–6 μg/μl. Because pRE189 contains a transcribed polyA tail of 30 nt, we did not further polyadenylate the messages.

Fourth, the TALEN mRNAs were combined to produce a solution that was 2–3 μg/μl for each message. This solution was injected into the gonad of adult hermaphrodites (Wood et al. 2011), using methods developed by Evans et al. (1994). After injection, animals were soaked in recovery buffer (Evans 2006) and picked onto individual plates.

At 20 °C, the F_1 progeny from a 6- to 32-h time window following the injection was singled to new plates, and F_2 animals that carried mutations were identified by phenotype or by PCR analysis of the target site. Generally, we amplified fragments that were 60–100 nt long and separated them by size on 10% polyacrylamide gels (fig. 1*A*). Small insertions or deletions were obvious. More subtle changes were detected by the loss of a restriction site or the use of *Cel*1 nuclease (Wood et al. 2011).

Supplementary Material

Supplementary figure S1 is available at *Molecular Biology and Evolution* online (http://www.mbe.oxfordjournals.org/).

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