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Heritable genetic transformation of *Strongyloides stercoralis* by microinjection of plasmid DNA constructs into the male germline

Hongguang Shao, Xinshe Li, James B. Lok *

Department of Pathobiology, School of Veterinary Medicine, University of Pennsylvania, 3800 Spruce Street, Philadelphia, PA 19104, USA

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ABSTRACT

Heretofore, transgenesis in the parasitic nematode genus *Strongyloides* has relied on microinjecting transgene constructs into gonadal syncytia of free-living females. We now report transgenesis in *Strongyloides stercoralis* by microinjecting constructs into the syncytial testes of free-living males. Crosses of individual males microinjected with a construct encoding GFP with cohorts of 12 non-injected females produced a mean of 7.28 ± 2.09 transgenic progeny. Progeny of males and females microinjected with distinct reporter constructs comprised 2.6% ± 0.7% of individuals expressing both paternal and maternal transgenes. Implications of this finding for deployment of CRISPR/Cas9 mutagenesis in *Strongyloides* spp. are discussed.

Transfer of plasmid-encoded transgenes by microinjection into the syncytial gonads of phenotypically female hermaphrodites became the primary means of gene transfer in the model nematode *Caenorhabditis elegans* when this method was pioneered in the 1980s (Kimble et al., 1982; Stinchcomb et al., 1985; Fire, 1986), and it has remained so to date. Infusing solutions of plasmid-based vectors into the gonadal syncytium results in transformation of a substantial proportion of oocyte nuclei, with the majority of plasmid-encoded transgenes being incorporated into tandem multi-copy arrays that are transmitted as episomes in the F1 generation of progeny and beyond (Mello and Fire, 1995; Evans, 2006). To our knowledge there has been no focused effort to introduce transgenes into *C. elegans* via the male germline.

Heritable transgenesis in animal parasitic nematodes was first achieved in *Strongyloides stercoralis* and *Parastrongyloides trichosuri* (Grant et al., 2006; Li et al., 2006). In contrast to the majority of other animal parasitic nematodes whose adult stages are confined to the definitive host, *Strongyloides* spp. and related genera have one or more generations of free-living males and females that exchange genetic material during sexual crosses (Viney and Lok, 2015). The body plans of free-living strongyloidoid females are strikingly similar to those of *C. elegans* hermaphrodites (Lints and Hall, 2009) with amphidelphic ovaries that have syncytial zones in their distal ends (Schad, 1989; Kulkarni et al., 2016), and this has permitted adaptation of microinjection techniques for gene transfer in *C. elegans* to strongyloidoid parasites with few modifications (Lok and Massey, 2002; Grant et al., 2006; Lok, 2007, 2012; Lok et al., 2017). The result has been a reliable system for introducing transgenes via the female germlines of *S. stercoralis* (Li et al., 2006; Junio et al., 2008) and *Strongyloides ratti* (Li et al., 2011) that are expressed in a promoter-regulated fashion in F1 progeny, and ultimately may be integrated into the chromosomes of *S. ratti* and inherited and expressed indefinitely through host and culture passage (Shao et al., 2012).

We recently used this system for transgenesis in *S. stercoralis* to express the Cas9 endonuclease and appropriate small guide RNAs to create a precise CRISPR-induced insertional mutation designed to disrupt a selected target gene, *Ss-daf-16* (Lok et al., 2017). Given that these CRISPR/Cas9 elements were expressed in pre-fertilization oocytes, we consider it likely that the mutations we confirmed in the F1 progeny derived from these were heterozygous. Using this approach for introducing CRISPR/Cas9 elements into *S. stercoralis*, creating homozygous mutations would require rearing F1 progeny to infective L3s, infecting a susceptible host to create a patent infection with parthenogenetic parasitic females, collecting their progeny from host feces and engineering crosses between heterozygous free-living males and females in culture to obtain a proportion of homozygous third generation mutants. While theoretically possible, this approach would be difficult to impractical, considering the small numbers of F1 mutant worms that can be produced in a short timeframe and the lack of a well-adapted small animal host for *S. stercoralis*. We therefore sought a method of generating *Strongyloides* spp. with disrupting

* Corresponding author.
E-mail address: jlok@vet.upenn.edu (J.B. Lok).

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mutations in both alleles of the target gene in a single generation after delivery of transgenes encoding the CRISPR/Cas9 elements. We reasoned that if the targeted heterozygous mutations could be created in germ cells of both male and female parents, then their F1 progeny should contain a proportion of worms that are trans-heterozygous for the two mutant alleles. We would expect such trans-heterozygous worms to be phenotypically mutant and therefore useful for genetic analysis. To this end, we investigated the possibility of introducing transgenes via the male germline of *S. stercoralis* using microinjection of the distal testis as the method of DNA transfer.

The UPD strain of *S. stercoralis*, originally isolated from naturally infected dogs in 1976 (Schad et al., 1984), was used for all experiments. This strain was maintained in immunosuppressed dogs and free-living adults were reared and isolated as previously described (Schad et al., 1984; Lok, 2007). Dogs used to maintain *S. stercoralis* were purpose-bred, mixed breed animals that were handled according to protocols 804798 and 804883 approved by the University of Pennsylvania Institutional Animal Care and Use Committee (IACUC), USA. All IACUC protocols, as well as routine husbandry care of the animals, were conducted in strict accordance with the "Guide for the Care and Use of Laboratory Animals" of the National Institutes of Health, *USA*.

Two transgene constructs, pPV529 and pAJ50-2 (Fig. 1A), were designed to express the GFP and a red fluorescent protein (mRFPmars), respectively, under the promoter for *Sr-eft-3* in *S. ratti*. Just as regulatory sequences from *S. stercoralis* give active transgene expression in *S. ratti* (Li et al., 2011), the *Sr-eft-3* promoter drives a high level of reporter expression in *S. stercoralis* from transgenes delivered via the female germline (Fig. 1E). Cloning steps in the preparation of these constructs appear in the legend to Fig. 1.

Free-living male *S. stercoralis* were immobilized on dry agarose pads on 25 × 50 mm coverslips overlain with paraffin oil and were observed using an inverted microscope with differential interference contrast (DIC) optics at 400X magnification. Vector plasmids were microinjected into the testicular syncytium, which is located in the distal gonad just posterior to the junction between pharynx and intestine (Fig. 1B), using finely drawn glass capillary tubing pressurized with nitrogen and positioned with a micromanipulator. Microinjected males were demounted immediately by gentle prodding with a 36 gauge platinum "worm pick" and transferred to standard 60 mm Nematode Growth Medium (NGM) plates with lawns of *Escherichia coli* OP50 (Stiernagle, 2006). Bacterial lawns were confined to a central spot approximately 1 cm in diameter in the center of each plate to facilitate mating and assessment of reporter transgene expression in F1 progeny as described below. Transgene constructs were delivered to free-living female *S. stercoralis* by microinjection into the ovarian syncytium using techniques adapted from *C. elegans* methodology (Mello and Fire,

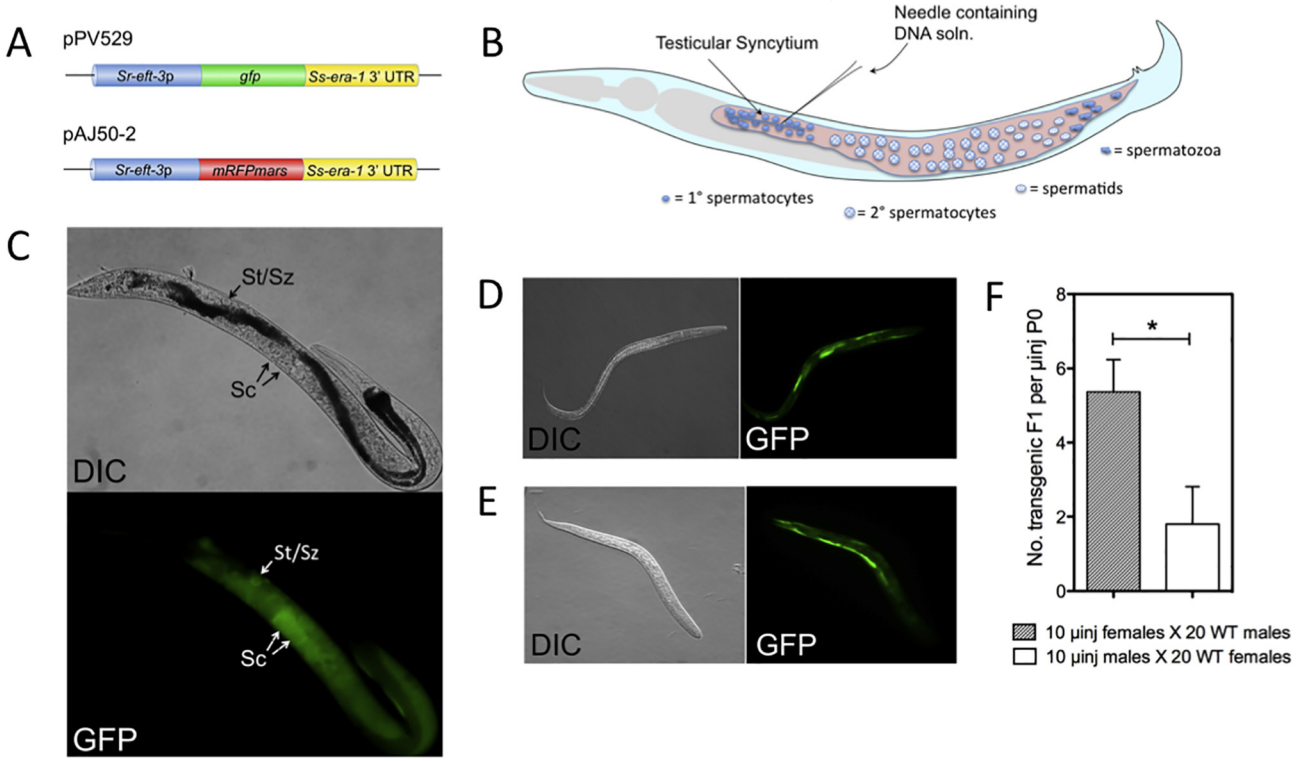

Fig. 1. Transgene constructs microinjected into the free-living male germline of *Strongyloides stercoralis* are incorporated into sperm and transmitted to F1 progeny following mating with non-transgenic free-living females. (A) Transgene constructs pPV529 and pAJ50-2 encoding GFP and mRFPmars, respectively. Construct pPV529 (GenBank Accession number KY852488) was prepared by digesting pPV254 (GenBank Accession number JX013636) with *Hind*III and *Age*I to excise the *Ss-act-2* promoter. The digest was then ligated with a 1163 bp fragment generated from the *Sr-eft-3* promoter region in *Strongyloides ratti* by PCR cloning. Construct pAJ50-2 (GenBank Accession number KY852489) was prepared by digesting the mRFPmars expression vector pAJ50 with *Hind*III and *Age*I to excise the 1295 bp *Ss-act-2* promoter fragment. The pAJ50 vector backbone was then re-ligated with the *Sr-eft-3* promoter fragment to yield pAJ50-2. In preparing both vectors, restriction digested fragments were resolved by agarose gel electrophoresis and purified from gel bands with the MinElute Gel Extraction Kit (Qiagen, USA, Cat. No. 28604). (B) Schematic of the male gonad of *S. stercoralis*, depicting the point of microinjection into the syncytial testis. (C) Differential interference contrast and fluorescence (GFP) images of a parental male expressing pPV529 throughout the gonad and in spermatocytes (Sc) in the distal testes and spermatids or spermatozoa (St/Sz) in the proximal testes. (D) Differential interference contrast and GFP images of a transgenic F1 larva produced by mating microinjected males with wild-type females. (E) Differential interference contrast and GFP images of a transgenic F1 larva produced by mating microinjected females with wild-type males. (F) Relative efficiencies of transgenesis deriving from microinjection of 10 parental (P0) females or 10 parental males. Data are means, and error bars represent S.E.M. for three biological replicates; statistical probabilities derived by Student's *T*-test. $^{*}P \leq 0.05$. soln, solution; UTR, untranslated region.

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