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Short technical report

Transgenesis in the parasitic nematode *Strongyloides ratti*

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ABSTRACT

Strongyloides and related genera are advantageous subjects for transgenesis in parasitic nematodes, primarily by gonadal microinjection as has been used with *Caenorhabditis elegans*. Transgenesis has been achieved in *Strongyloides stercoralis* and in *Parastrongyloides trichosuri*, but both of these lack well-adapted, conventional laboratory hosts in which to derive transgenic lines. By contrast, *Strongyloides ratti* develops in laboratory rats with high efficiency and offers the added advantages of robust genomic and transcriptomic databases and substantial volumes of genetic, developmental and immunological data. Therefore, we evaluated methodology for transgenesis in *S. stercoralis* as a means of transforming *S. ratti*. *S. stercoralis*-based GFP reporter constructs were expressed in a proportion of F1 transgenic *S. ratti* following gonadal microinjection into parental free-living females. Frequencies of transgene expression in *S. ratti*, ranged from 3.7% for pAJ09 to 6.8% for pAJ20; respective frequencies for these constructs in *S. stercoralis* were 5.6% and 33.5%. Anatomical patterns of transgene expression were virtually identical in *S. ratti* and *S. stercoralis*. This is the first report of transgenesis in *S. ratti*, an important model organism for biological investigations of parasitic nematodes. Availability of the rat as a well-adapted laboratory host will facilitate derivation of transgenic lines of this parasite.

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Although stable transgenesis in *Caenorhabditis elegans* was achieved almost two decades ago [1–3], adaptation of this now standard method to animal parasitic nematodes has been difficult and relatively slow. Difficulties in accomplishing this stem mainly from the fact that most medically important parasitic nematodes are obligate parasites and cannot be cultured for a full generation outside their hosts. By contrast, members of the superfamily Strongyloidoidea, which resides in Clade IV of the contemporary nematode phylogeny [4,5], naturally undergo one or more generations of free-living adult development between parasitic generations [6]. The culture biologies and body plans of the free-living females of *Strongyloides* spp. and *Parastrongyloides* spp. are so similar to those of *C. elegans* hermaphrodites that it has been relatively straightforward to adapt the technique of gene transfer by gonadal microinjection to this group of parasitic nematodes. Consequently, heritable transgenesis using this method has been achieved for *Strongyloides stercoralis* [7–9] and *Parastrongyloides trichosuri* [10,11].

As research subjects, both of these parasites have distinct advantages, but also some disadvantages. *P. trichosuri* is the more genetically tractable of the 2 parasites. It can undergo repeated generations of free-living development under appropriate environmental conditions that may be simulated on laboratory culture plates [10]. This factor has allowed derivation of stable transgenic lines by culture passage in a manner similar to protocols for *C. elegans* [11]. Offsetting this advantage is the fact that *P. trichosuri* is a natural parasite of the Australian brush-tailed possum, *Trichosurus vulpecula*, an animal that is very difficult to obtain and maintain in the laboratory. Efforts to introduce *P. trichosuri* into more widely available non-marsupial species have failed thus far, but a smaller Australian marsupial, the sugar glider, *Petaurus breviceps*, is susceptible to infection and has proven a practical laboratory host for this parasite [12]. The sugar glider is commercially available through the pet trade in many parts of the world, but it is not, to our knowledge, bred for research, and so its use as a laboratory animal may be problematic in some settings.

As a parasite of humans, *S. stercoralis* is the most medically significant member of the Strongyloidoidea. It infects approximately 300 million people and, because of its unique capability to undertake autoinfection, can cause potentially fatal disseminated hyperinfection in persons immunocompromised as a result of HTLV-1 infection [13,14] or immunosuppressive drug therapy

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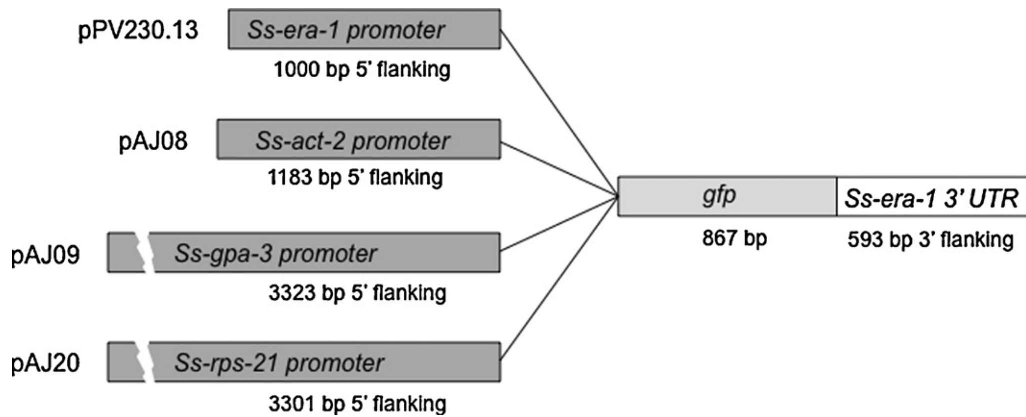

Fig. 1. Diagrams of constructs used to transform *Strongyloides ratti*. Each plasmid links a tissue specific plasmid upstream of the sequence encoding GFP (867 bp) and a multifunctional 3′ UTR comprising 593 bp of 3′ flanking sequence from *Ss-era*-1 [8]. Plasmid pPV230.13 incorporates a putative promoter comprising 1 kb of 5′ flanking sequence from *Ss-era*-1, which encodes an endoplasmic reticulum ATPase expressed in intestinal cells of *S. stercoralis* [8]. Plasmid pAJ08 contains a putative promoter (1183 bp of 5′ flanking sequence) from *Ss-act*-2, which encodes a body wall-specific cellular actin [9]. Plasmid pAJ09 contains a putative promoter comprising 3323 bp of 5′ flanking sequence from *Ss-gpa*-3, which encodes a Gα protein expressed in amphidial and phasmidial neurons [9], and plasmid pAJ20 contains a putative promoter comprising 3301 bp of 5′ flanking sequence from *Ss-rps*-21, which encodes the ubiquitously expressed ribosomal small subunit protein S21 in *S. stercoralis* [9]. GenBank accession numbers for the *Ss-era*-1, *Ss-act*-2, *Ss-gpa*-3 and *Ss-rps*-21 promoter sequences are DQ333398, EF587761, AF292562, EF589665, respectively. The GenBank accession number for the *Ss-era*-1 3 2 UTR is DQ333399. Plasmid details and distribution for all constructs described is available at http://www.addgene.org/James_Lok.

[15,16]. Like *P. trichosuri*, the life cycle of *S. stercoralis* contains free-living females and these provide a convenient target for gene transfer by gonadal microinjection [7]. However, *S. stercoralis* is capable of undergoing only one generation of free-living development, and all progeny of free-living males and females develop to infective third-stage larvae (L3i). Consequently, derivation of stable transgenic lines of *S. stercoralis* will necessitate host passage of F1 transformants. A natural parasite of humans and dogs, *S. stercoralis* will also establish patent infection in the Mongolian gerbil, *Meriones unguiculatus* [17], thus providing a small animal system for host passage. The infection efficiency of *S. stercoralis* in the gerbil is such that on average 6.5% (range = 0.6–27.6%; $n = 540$) of inoculated infective third-stage larvae (L3i) establish as adult parasitic females in the gerbil [9,17]. The frequency of germline transformation resulting from gonadal microinjection of DNA into parental free-living females in *S. stercoralis* has not yet been determined, but the corresponding value for F1 progeny of microinjected *C. elegans* hermaphrodites rarely exceeds 10% [18]. If it is assumed that this transformation frequency also applies to *S. stercoralis* (though this has not yet been determined), this means that derivation of a single germline transformed parasitic female *S. stercoralis* would require inoculation of 167 transgenic L3i into a gerbil. Although possible, such numbers are challenging to produce with current microinjection technology; the requisite number of 167 L3i for a single germline transformant would entail microinjection of 60 P0 free-living females in the *S. stercoralis* system. It is clear then that a more efficient host–parasite system is required for practical, routine generation of stable transgenic lines of *Strongyloides* spp.

Strongyloides ratti is a natural parasite of the rat and has been used as a subject for studies into a wide range of topics including host–parasite relationships [19], genetic determinants of developmental fate [19–21], ageing [22,23], the genetics of anthelmintic resistance [24], and global patterns of gene expression that differentiate parasitic from free-living nematodes [25,26]. The availability of a well-adapted conventional laboratory animal host, the rat, has greatly facilitated many of these studies. In contrast to the relative inefficiency of the *S. stercoralis*–gerbil system, the rat allows from 50% to 100% of inoculated *S. ratti* L3i to develop to reproductively active parasitic females in a period of about five days, compared to a prepatent period of 10 days, with 21 days until peak adult worm establishment, for *S. stercoralis* in the gerbil [27,28]. This represents an infection efficiency that is 8- to 15-fold higher than is seen for *S. stercoralis* in the gerbil [9,17]. We have confirmed this efficiency estimate for *S. ratti* in the course of the present study by generating patent infection in 6 rats by inoculation of 10 or fewer L3i. Indeed one patent infection followed injection of a single *S. ratti* L3i. Coupled with an estimated stable germline transformation rate of 10% (above), this infection efficiency could enable derivation of a single germline transformant F1 from inoculation of only 20 *S. ratti* L3i into a rat. In addition to its other merits as an important model for parasitological research, this 8-fold higher probability of isolating stable transgenic parasites from a population of transiently transformed F1s makes *S. ratti* an attractive model for work towards a robust system for transgenesis in a parasitic nematode. To this end, we investigated whether *S. ratti* could be transformed by methods already developed for *S. stercoralis*.

Laboratory strains of *S. stercoralis* (UPD) and *S. ratti* (ED321) [20] were maintained and cohorts of free-living females were isolated from fecal culture [29,30] and microinjected intragonadally with plasmid vectors as described previously [7–9] with the exception that as a precaution against its lower tolerance for injection trauma,

Table 1
Expression frequencies of *Strongyloides stercoralis*-based reporter constructs in F1 transgenic *S. ratti* and *S. stercoralis*.

Construct	Parasite	No. P0 females microinjected	No. F1 progeny screened	No. (%) F1 progeny expressing GFP
pAJ08	*S. ratti*	60	207	16 (7.4)
	S. stercoralis	42	838	123 (14.6)
pPV230.13	*S. ratti*	55	323	17 (5.3)
	S. stercoralis	37	373	40 (10.7)
pAJ20	*S. ratti*	45	384	26 (6.8)
	S. stercoralis	57	609	204 (33.5)
pAJ09	*S. ratti*	48	163	6 (3.7)
	S. stercoralis	43	356	20 (5.6)

only 1 of the 2 gonadal arms was injected in *S. ratti*. The plasmid vectors all encoded reporter constructs in which the sequence encoding GFP was cloned downstream of one of four putative tissue-specific promoters from *S. stercoralis* and upstream of the *Ss-era*-1 3′ UTR from *S. stercoralis* (Fig. 1). Microinjected female worms were pooled with equal numbers of uninjected free-living males on NGM plates with lawns of *Escherichia coli* OP50 bacteria and incubated at 22 °C. At 48 and 72 h following microinjection, F1 progeny

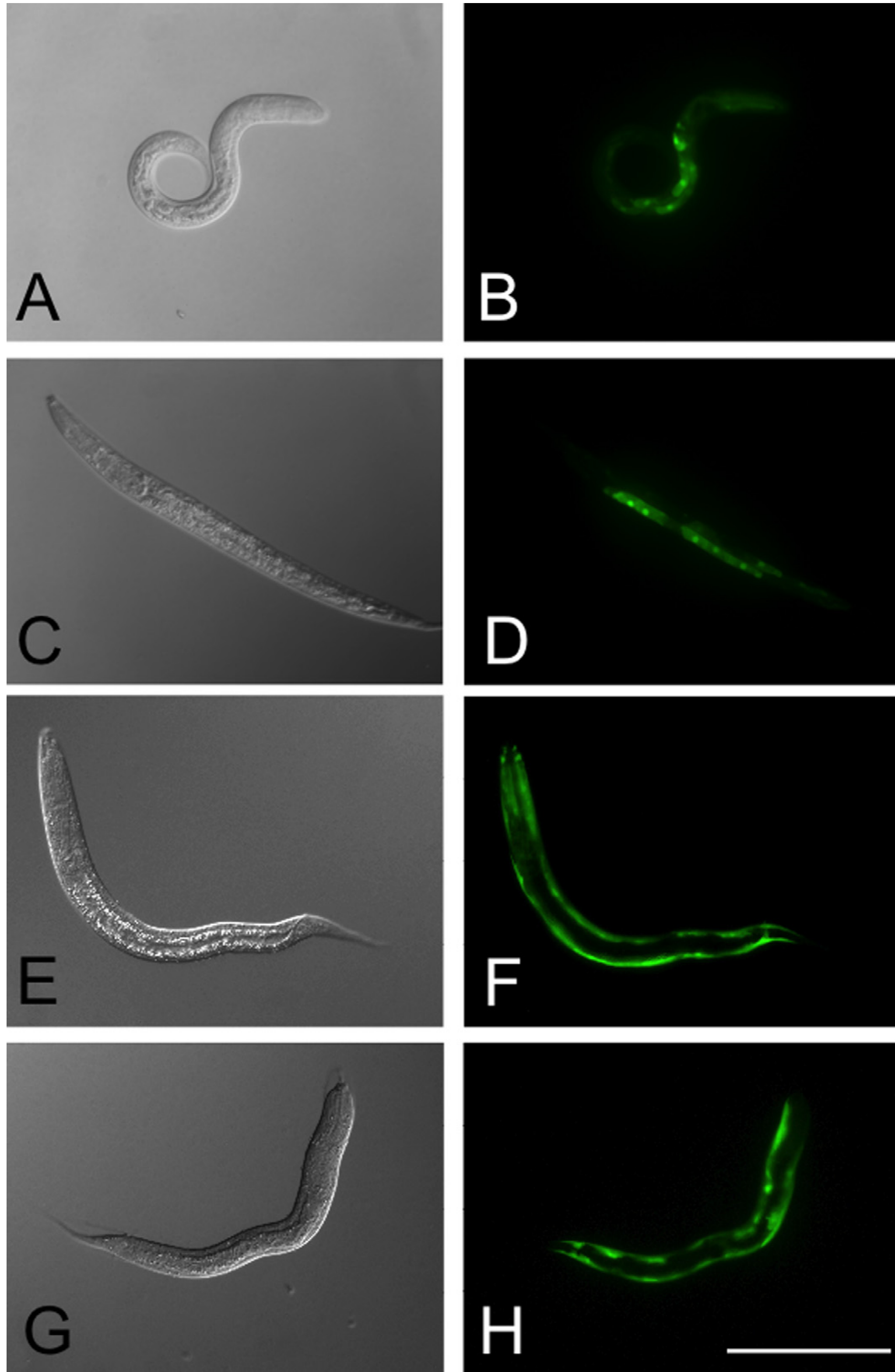

Fig. 2. Expression patterns of intestine- and body-wall-specific GFP reporter constructs containing regulatory sequences from *Strongyloides stercoralis* in transgenic first-stage larvae of *Strongyloides ratti* and *S. stercoralis*. In all constructs the *gfp* coding sequence is flanked by the *Ss-era*-1 3′ UTR and the putative promoter from one of the following genes: *Ss-era*-1 (plasmid pPV230.13), *Ss-act*-2 (plasmid pAJ08). (A, B) DIC and fluorescence images, respectively, of pPV230.13 expression in *S. stercoralis*. (C, D) Expression of pPV230.13 in *S. ratti*. Note similar patterns of expression in intestinal cells of both parasites. (E, F) Expression of pAJ08 in *S. stercoralis*. (G, H) Expression of pAJ08 in *S. ratti*. Note virtually identical patterns of expression in body walls of both parasites. Scale bar = 200 μm.

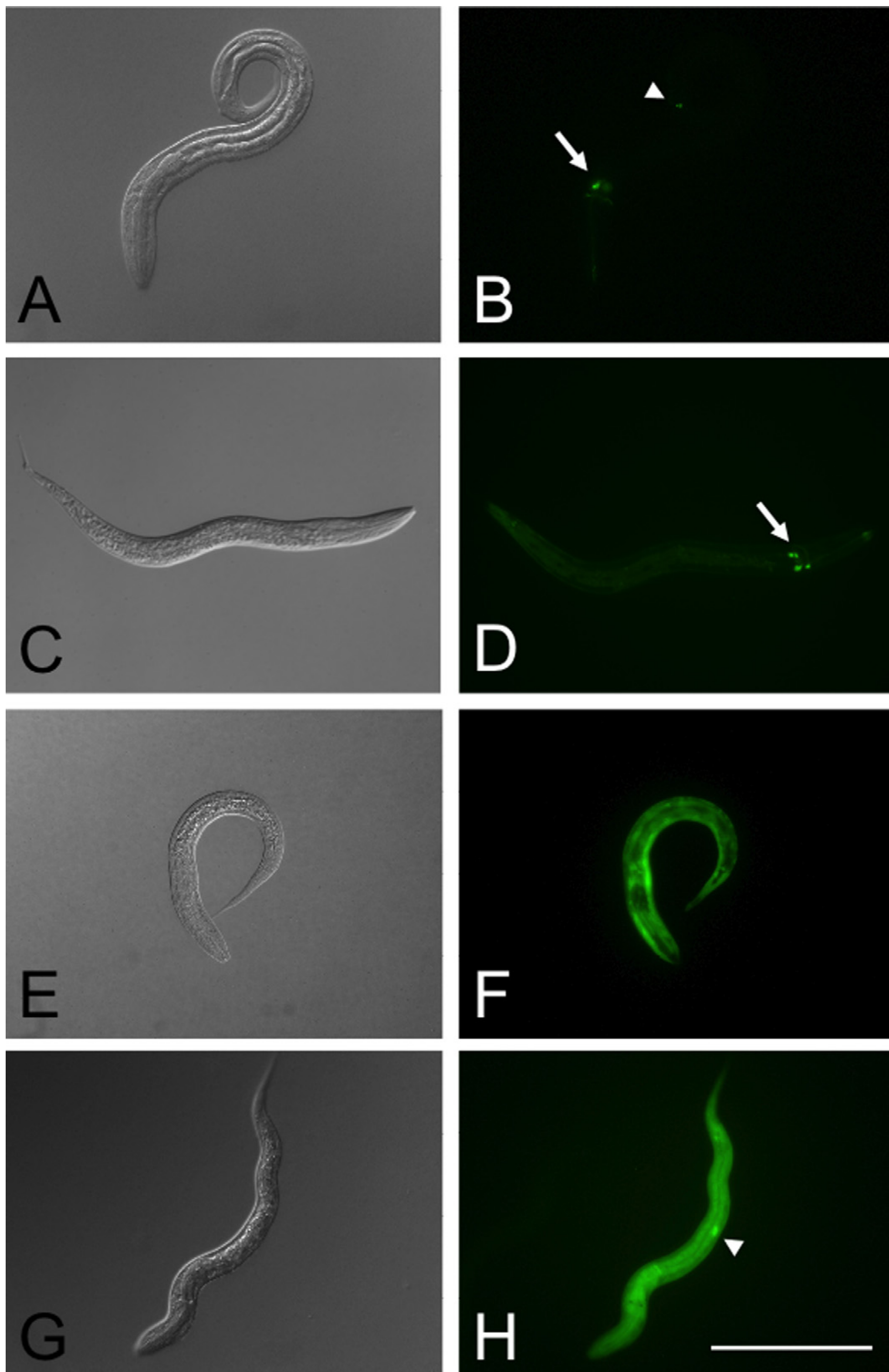

Fig. 3. Neuron-specific and ubiquitous expression of GFP reporter constructs containing regulatory sequences from *Strongyloides stercoralis* in transgenic first-stage larvae of *Strongyloides ratti* and *S. stercoralis*. In all constructs the *gfp* coding sequence is flanked by the *Ss-era*-1 3′ UTR and the putative promoter from one of the following genes: *Ss-gpa*-3 (plasmid pAJ09) or *Ss-rps*-21 (plasmid pAJ20). (A, B) Expression of plasmid pAJ09 in *S. stercoralis*. (C, D) Expression of pAJ09 in *S. ratti*. Note expression in cell bodies of amphidial neurons in both parasites (arrows). *S. stercoralis* also shows expression of this construct in a phasmidial neuron (arrow head). (E, F) Expression of construct pAJ20 in *S. stercoralis*. (G, H) Expression of pAJ20 in *S. ratti*. Note ubiquitous expression of this construct in both parasites. *S. ratti* shows an intense locus of expression in the genital rudiment (arrow head). Scale bar = 200 μm.

were screened for GFP fluorescence as first-stage larvae, and digital images were captured.

Expression of all *S. stercoralis*-based reporter transgene constructs was observed in a proportion of F1 progeny of microinjected free-living *S. ratti* females (Table 1). Free-living females of *S. ratti* tolerated microinjection somewhat less well than those of *S. stercoralis*, and resulting mortality among these worms is reflected in a comparatively lower yield of total F1 larvae per microinjected female in this initial trial (Table 1). In general, the anatomical patterns of expression for a given reporter construct in *S. ratti* closely resembled the pattern observed here and previously for the same construct in *S. stercoralis* (Fig. 2) [8,9]. The same intestinal cell-specific pattern of GFP expression under the *Ss-era*-1 promoter in plasmid pPV230.13 was observed in both *S. stercoralis* [8] (Fig. 2A and B) and in *S. ratti* larvae (Fig. 2C and D). Similarly, the *S. stercoralis* actin promoter in plasmid pAJ08 drove GFP expression in the same body wall-specific pattern in *S. ratti* larvae (Fig. 2G and H) as it did in *S. stercoralis* larvae observed here (Fig. 2E and F) and previously [9]. The *Ss-gpa*-3 promoter in plasmid pAJ20 drove GFP expression in amphidial neurons of transgenic *S. stercoralis* (Fig. 3A and B) and *S. ratti* (Fig. 3C and D) larvae. Expression of GFP from plasmid pAJ09 was also seen in phasmidial neurons of some *S. stercoralis* larvae [9] (Fig. 3B) but not in the transgenic *S. ratti* larvae observed in this study (Fig. 3D). This character is variable in *S. stercoralis*, and not all larvae expressing the transgene in the amphidial neurons do so in phasmidial neurons [9]. Finally, the same ubiquitous pattern of GFP expression under the *S. stercoralis Ss-rps*-21 promoter in plasmid pAJ20 was seen in transgenic *S. stercoralis* (Fig. 3E and F) [9] and *S. ratti* (Fig. 3G and H). An intense locus of expression in the genital rudiment of *S. ratti* L1 transformed with plasmid pAJ20 (Fig. 3H) has also been reported for *S. stercoralis* [9].

Thus, we have achieved transgenesis in *S. ratti* using the same method of gonadal microinjection that we adapted from *C. elegans* methodology for *S. stercoralis* [7]. We have also found that the functions of gene regulatory sequences from *S. stercoralis*, comprising four putative promoters and a single multi-functional 3′ UTR, are sufficiently conserved in *S. ratti* to give virtually identical anatomical and cellular patterns of GFP reporter expression in the 2 parasites. The frequencies of transformation among F1 progeny of microinjected females were higher in *S. stercoralis* than in *S. ratti* for all constructs tested. For three of the four transgene constructs tested (pAJ08, pPV230.13 and pAJ09) this disparity can be explained by the facts that only one gonadal arm was injected in *S. ratti*, and observed frequencies in that parasite are roughly half those observed in *S. stercoralis* (Table 1) where both arms were injected. The larger disparity observed in the case of pAJ20 may reflect the heterologous nature of this *S. stercoralis*-based construct in *S. ratti*, notwithstanding conservation of the anatomical expression pattern, and might be overcome by the use of a construct with homologous regulatory elements. The robust nature of the EST database for *S. ratti* [31] and the imminent completion of a full draft genome for this parasite [32] will greatly facilitate the creation of such homologous constructs. In general we found *S. ratti* to be somewhat less tolerant of microinjection than *S. stercoralis* and this was reflected in a higher mortality among parental females in the first 24 h following DNA transfer and a consequently lower total of F1 progeny per female microinjected in *S. ratti* than in *S. stercoralis*. Nevertheless, we believe that when we attempt to derive stable lines of transgenic *Strongyloides* spp. by host passage, these disparities will be more than offset by the 8- to 15-fold higher infection efficiency seen in the *S. ratti*-rat system compared to *S. stercoralis*-gerbil system. As discussed above, the ability of 50–100% of inoculated *S. ratti* L3i to establish as reproductively competent parasitic females in the rat [27,28] will greatly increase the probability of capturing the expected 1–10% of F1 transformants that carry transgene sequences in their germlines and thus constitute founders of transgenic lines. Transgenesis in *S. ratti* is a significant development given the relatively highly developed nature of genetics, and developmental and evolutionary biology in this parasite. These areas are accompanied by increasingly robust genomic and transcriptomic data sets, and transgenesis in a tractable parasite like *S. ratti* will be an asset in assigning function to genes and transcripts that are parasite specific and therefore encode potential targets for drug or vaccine intervention.

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