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TRANSGENICS WITH GONADAL MICROINJECTION IN DIOECIOUS *CAENORHABDITIS* NEMATODES

Le Tho Son✉, Nguyen Thi Thu

College of Forestry Biotechnology and F-School, Vietnam National University of Forestry, Hanoi, Vietnam

✉To whom correspondence should be addressed. E-mail: sonlt@vnuf.edu.vn

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ABSTRACT

Biological research with nematode models is increasing to understand the phenomenon of molecular genomes, genetics, and biological comparison. Different methods have been developed to produce genetically modified organisms that have the desired characteristics. Found in 1985, the gonadal microinjection in *Caenorhabditis elegans* has been obtained greatly effective for transformations. To other nematodes, this transformation methodology could play an important role in studying genetics and genomics within and among the species. Research methods with several other non-*C. elegans* round nematodes were adopted the microinjections to do transformation. In this research of transformation, we report the results of transgenics for different plasmids in eight dioecious species (*C. portoensis*, *Caenorhabditis* sp. 33, *C. brenneri*, *C. nigoni*, *C. sinica*, *C. imperialis*, *C. nouraguensis*, and *C. remanei*). We gained the stable transgenes of the first four species and failed the last four.

Keywords: androdioecious, *Caenorhabditis*, fluorescent protein, microinjection, *myo-2*, *sur-5*, transformation.

INTRODUCTION

New species of *Caenorhabditis* nematodes are being discovered every year (Kiontke *et al.*, 2011; Felix *et al.*, 2014; Dieter Slot, 2017; Kanzaki *et al.*, 2018). A detailed study of their biology can be fruitful, especially for comparative questions in evolutionary biology with the *Caenorhabditis* nematodes. In this respect, *C. briggsae* has been the main model (Gupta *et al.*, 2007), partly because it is also a hermaphroditic species and was the second nematode to be sequenced (Stein *et al.*, 2003). Inclusion of other species has increased over time, and their study has covered topics such as evolutionary developmental biology (Kiontke *et al.*, 2007; Braendle, Felix, 2008; Huang *et al.*, 2014), sperm competition and sperm size evolution (LaMunyon, Ward, 1999; Vielle *et al.*, 2016), speciation and hybrid incompatibility (Woodruff *et al.*, 2010; Ross *et al.*, 2011; Ting *et al.*, 2014; Bi *et al.*, 2015), and genome evolution (Le *et al.*, 2017; Yin *et al.*, 2018).

Many of the comparative studies would benefit from suitable tools to conduct genetic manipulations. As satellite species to

the well-known model *C. elegans*, other *Caenorhabditis* species can adapt many pre-existing tools to engineer the genome, for example, transgenesis (Rieckher *et al.*, 2009), CRISPR/Cas9 (Clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9) (Lo *et al.*, 2013), TALENS (transcription activator-like effector nucleases) (Lo *et al.*, 2013; Wei *et al.*, 2014a; Wei *et al.*, 2014b), ZFNs (zinc-finger nucleases) (Wood *et al.*, 2011), *Mos*TIC (*Mos1* excision-induced transgene-instructed gene conversion) (Robert, Bessereau, 2007), and MosSci (Mos1-mediated Single Copy Insertion) (Frokjaer-Jensen *et al.*, 2008).

Transgenesis in *C. elegans* is typically achieved through gonadal microinjection (Stinchcomb *et al.*, 1985; Evans, 2006) or microparticle bombardment (Praitis *et al.*, 2001). Although both techniques are used in nematode species (Hashmi *et al.*, 1995; Higazi *et al.*, 2002; Grant *et al.*, 2006; Li *et al.*, 2006; Semple *et al.*, 2010; Cinkornpumin, Hong, 2011; Shao *et al.*, 2012), microinjection is more regularly used because microparticle bombardment needs a good selectable marker, requires large numbers of nematodes (Praitis *et al.*, 2001), and thus is more costly (Praitis, 2006).

In microinjection of the *Caenorhabditis* nematodes species, one or several plasmids (or DNA molecules) are co-injected into the syncytial gonads of hermaphrodites in androdioecious species (*C. elegans*, *C. briggsae*, and *C. tropicalis*) or females in dioecious species (*C. brenneri*, *C. angaria*, and *Caenorhabditis* sp. 2) (Fire, 1986; Fire *et al.*, 1990; Fire *et al.*, 1991; Kennedy *et al.*, 1993; Fire *et al.*, 1998; Evans, 2006; Nuez, Felix, 2012). The plasmids in the cell assemble into presumably linear extrachromosomal arrays. The formation of arrays seems to be enhanced by overlapping homologous sequences although such homology is not necessary (Stinchcomb *et al.*, 1985; Fire, 1986; Mello *et al.*, 1991). The gonadal microinjection is often aided by the co-injection of a gene rescue or dominant-phenotype markers such as *unc-119* (Maduro, Pilgrim, 1995), *rol-6* (*su1006*) (Kramer *et al.*, 1990; Mello *et al.*, 1991), *unc-22* (Fire *et al.*, 1991), green fluorescent proteins (GFP), red fluorescent protein (RFP), or yellow fluorescent protein (YFP) (Chalfie *et al.*, 1994; Gu *et al.*, 1998; Miller *et al.*, 1999; Hutter, 2003; Wenick, Hobert, 2004).

The transformation rate varies in F1 generations from low to high, often less than 2% of progeny (Fire, 1986), although rarely as high as 90% (Kadandale *et al.*, 2009). This was found to simultaneously depend on multiple factors, including the sequences, concentration, and size of the injected constructs as well as the practical skill (Fire, 1986; Fire *et al.*, 1990; Mello *et al.*, 1991). The gonadal microinjection method is likely a hallmark of research because it allows any exogenous DNA constructs to be introduced into the organisms. However, the method is technically and biologically challenging and requires excessive effort. This research reports the transgenesis to generate the transgenes for studying transformation ability, the evolution of genome sizes (Le *et al.*, 2017), and the pharynx in eight dioecious *Caenorhabditis* nematodes.

MATERIALS AND METHODS

Nematode strains: *C. brenneri* (CB5161), *C. nigoni* (BRC10093), *C. imperialis* (EG5716), *C. nouraguenis* (JU2079), *C. portoensis* (EG4788), *C.* sp. 33 (BRC10016), *C. remanei* (PB4641), and *C. remanei*

(BRC310). Each species was genetically identified by ourselves or kindly provided by collaborators (Le *et al.*, 2017). The worms were regularly cultured repeatedly on nematode growth media seeded with *Escherichia coli* OP50 strains.

Plasmids: pDD04Neo [*C. elegans myo-2::GFP*]; pPD158.87 [*C. elegans sur-5::GFP*]; pCFj104 [*Pmyo-3::mCherry::unc-54*], and pPD135.83 [pLET::GFP].

General microinjection: Transformation by gonadal microinjection was adapted from androdioecious *Caenorhabditis* nematodes (Evans, 2006; Kanzaki *et al.*, 2018) for dioecious species. Briefly, female worms at the young adult stage were mounted on the microscopic pad. They were injected bilaterally with one or two drops of the injection mixture into the gonads. In detail, 20 ng/µL pDD04Neo with and without 100 ng/µL pPD158.87 was injected in *C. portoensis*; 5, 10, and 20 ng/µL of pDD04Neo in *Caenorhabditis* sp. 33; 20 ng/µL pDD04Neo with 100 ng/µL pPD158.87 in *C. brenneri*; 20 ng/µL pDD04Neo with and without 100 ng/µL pPD158.87; and 150 ng/µL of *hsp16/4* and *hsp16/2* in *C. nigoni*.

After, the injected worms were recovered with a sugar solution on *E. coli* OP50-seeded plates before three to five L4-stage or young males were added. We looked for the transgenic F1 offspring expressing the fluorescence marker, i.e. GFP, under microscopes in the next days until the injected worm stopped laying eggs. Each transgenic F1 individual was picked onto a single new *E. coli* OP50-seeded plate, on which three to five L4 of the other sex were added to reproduce the F2 progeny. Beyond F2s, each transgene was transferred in chunks to a new *E. coli* OP50-seeded plate every two weeks.

RESULTS

We tested the transgenic ability for eight dioecious species, which were *C. portoensis*, *C.* sp. 33, *C. brenneri*, *C. nigoni*, *C. sinica*, *C. imperialis*, *C. nouraguensis*, and *C. remanei*, with different transgenic plasmid components (Table 1). We conducted every injection trial until getting one transgene for each nematode species.

Stable transgenes with fluorescent expression

Caenorhabditis portoensis: 50 P0 females were injected with two mixtures of constructs (either 20 ng/µL pDD04Neo with or without 100 ng/µL pPD158.87). Two transgenic F1s were produced from each dose. The unique F1 individual transferred with pDD04Neo produced many transgenic generations that had GFP expression, suggesting this is a stable transgene (Table 1).

***Caenorhabditis* sp. 33**: 104 P0 females were injected with 5, 10, and 20 ng/µL of pDD04Neo. All three injected groups produced 318 transgenic F1s. One F1 transgenic individual induced by the highest construct concentration produced the next transgenic generations, indicating that it is a stable transgene (Fig. 1a and Table 1).

Caenorhabditis brenneri: 110 P0 females were injected with 20 ng/µL pDD04Neo and 100 ng/µL pPD158.87. Nighty-four F1s inherited the constructs and produced 24 stable lines (Table 1).

Caenorhabditis nigoni: 193 P0 females were injected with three mixtures of constructs (20 ng/µL pDD04Neo with and

without 100 ng/µL pPD158.87, and 150 ng/µL of *hsp16/4* and *hsp16/2*). The two first injected groups produced 25 transgenic F1s. Among the F1s, 24 of the first group were reproductive for transgenes in many generations, suggesting they were stable transgenic lines (Table 1).

In our research, we successfully conducted stable transgenes for four dioecious species (*C. portoensis*, *C.* sp. 33, *C. brenneri*, and *C. nigoni*). They expressed GFP in the pharynx, suggesting that *myo-2::GFP* in pDD04Neo was expressed. Previously, the same transformation was reported for the five dieocious species (*C. brenneri*, *C. angaria*, *C. sinica*, *C. nigoni*, *C. remanei*, *C.* sp. 2, and *C. inopinata* (Nuez, Felix, 2012), and *C. inopinata* (Kanzaki *et al.*, 2018)). In our knowledge, we proved the first transformation for *C. portoensis* and *Caenorhabditis* sp. 33, indicating that they are accessible to exogenous vectors by the gonadal microinjection (Table 1). We assumed that the pDD04Neo is sensitive to the pharyngeal expression in different *Caenorhabditis* species.

Non-stable transgenes without fluorescent expression

Caenorhabditis sinica: 115 P0 females were injected with the same construct mixtures to *C. nigoni*. Four F1s were transformed for the injected vectors but none of them were producible for the next transgenic generations (Table 1).

Caenorhabditis imperialis: 28 P0 females were injected with 20 ng/µL pDD04Neo and 100 ng/µL pPD158.87 for 28 P0 females. None of the F1s and F2s express the transformation (Table 1).

Caenorhabditis nouraguensis: Fourteen P0 females were injected with 20 ng/µL pDD04Neo plus 100 ng/µL pPD158.87 for 14 P0 females. A single F1 was transgenic but did not continue the transgenes in any progeny generations (Table 1).

Caenorhabditis remanei: 378 P0 females of two strains were injected with nine mixtures of constructs (pDD04Neo with and without pCFj104) that had little changes in vector concentrations. 135 F1 offspring were transgenic but none of them were reproductive for the transgenic F2s (Table 1).

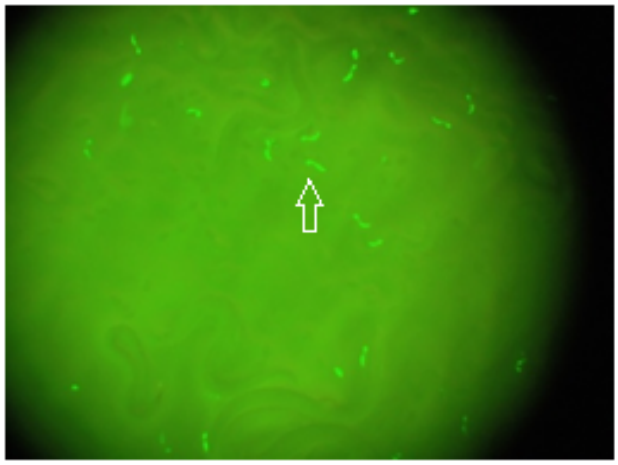

Figure 1. Larvae of *C.* sp. 33 (*myo-2::GFP*) expressing GFP in the pharynx of the F2 offspring generation (white arrow).

Table 1. Transgenesis in dioecious *Caenorhabditis* nematodes.

Species (strain)	Injection	Plasmid	Trial	P0	F1	F2	Expression
C. portoensis (EG4788)	1st	20 ng/µL pDD04Neo + 100 ng/µL pPD158.87	1	25	1	0	None
	2nd	20 ng/µL pDD04Neo	1	25	1	many	Pharynx†
C. sp. 33 (BRC10016)	1st	5 ng/µL pDD04Neo	1	40	5	0	None
	2nd	10 ng/µL pDD04Neo	1	28	13	0	None
	3rd	20 ng/µL pDD04Neo	1	36	300	1	Pharynx†
C. brenneri (CB5161)	1st	20 ng/µL pDD04Neo + 100 ng/µL pPD158.87	4	110	94	24	Pharynx, Intestine†
C. nigoni (BRC10094)	1st	150 ng/µL (hsp16/4 + hsp16/2)	1	25	0	0	None
	2nd	20 ng/µL pDD04Neo	2	87	1	0	None
	3rd	20 ng/µL pDD04Neo + 100 ng/µL pPD158.87	4	81	24	many	Pharynx, Intestine†
C. sinica (BRC10093)	1st	150 ng/µL (hsp16/4 + hsp16/2)	1	25	0	0	None
	2nd	20 ng/µL pDD04Neo	1	50	1	0	None
	3rd	20 ng/µL pDD04Neo + 100 ng/µL pPD158.87	1	40	3	0	None
C. imperialis (EG5716)	1st	20 ng/µL pDD04Neo + 100 ng/µL pPD158.87	1	28	0	0	None
C. nouraguensis (JU2079)	1st	20 ng/µL pDD04Neo + 100 ng/µL pPD158.87	1	14	1	0	None
C. remanei (PB4641)	1st	200 ng/µL pDD04Neo	1	30	6	0	None
	2st	10 ng/µL pDD04Neo	1	65	36	0	None
	3rd	7.5 ng/µL pDD04Neo	1	25	0	0	None
	4th	100 ng/µL pDD04Neo + 100 ng/µL pCFj104	1	20	2	0	None
	5th	2.5 ng/µL pDD04Neo + 125 ng/µL pCFj104	1	44	1	0	None
C. remanei (BRC310)	1st	20 ng/µL pDD04Neo	1	42	42	0	None
	2nd	10 ng/µL pDD04Neo	1	35	8	0	None
	3rd	5 ng/µL pDD04Neo	1	55	5	0	None
	4th	20 ng/µL pDD04Neo + 50 ng/µL pPD135	2	62	35	0	None

†The expression was already published (Le et al., 2017).

In our research, we failed to make transgenic lines that were stable for four species. *C. imperialis* did not produce any transgenic F1. In contrast, three other species (*C. sinica*, *C. nouraguensis*, and *C. remanei*) produced many transgenic F1s and no transgene in the next generations, suggesting that the *myo-2::GFP* constructs were expressed in the *Caenorhabditis* nematodes but the extrachromosomal arrays might not be inherited in germlines (Mello *et al.*, 1991; Rieckher *et al.*, 2009). However, for a better chance of getting a stable transgene, the number of P0 injections should be increased, resulting in numerous transgenic F1s by which a few stable transgenic F2 offspring are reproduced. For example, in this research, *C. brenneri* and *C. nigoni*, each after four subsequent trials, were effective. Other time-consuming methods would be initiated with new construct designs with the precise understanding of genome sequences.

CONCLUSION

Eight species (*C. portoensis*, *Caenorhabditis* sp. 33, *C. brenneri*, *C. nigoni*, *C. sinica*, *C. imperialis*, *C. nouraguensis*, and *C. remanei*) were injected with different plasmid mixtures. The first four of them were successfully transformed and well expressed the *myo-2::GFP* in pDD04Neo and *sur-5::GFP* in pPD158.87 in the pharynx through multiple generations. Among these, *C. portoensis* and *Caenorhabditis* sp. 33 were the first successful for microinjection. The last four of the tested species were failed for transformation.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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