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Heritable and inducible gene knockdown in *C. elegans* using Wormgate and the ORFeome

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Abstract

Double-stranded RNA (dsRNA) mediated gene silencing (RNA interference; RNAi) is a powerful tool for investigating gene function. It is usually performed in *Caenorhabditis elegans* via the injection or oral delivery of dsRNA, but an alternative approach, the expression of RNA hairpins from introduced DNA (hairpin RNAi; hpRNAi) has several advantages: (1) it can be induced systemically or in a tissue-specific manner; (2) because it is heritable, it allows consistent RNAi silencing across a whole population of genetically identical animals; and (3) it can be applied in refractory tissue such as neurons. hpRNAi has not been widely used to investigate gene function because a number of steps are relatively inefficient and labour-intensive. We describe Wormgate, a new cloning system, which facilitates the efficient high-throughput production of hpRNAi constructs using clones from the *C. elegans* ORFeome library. The combined use of pWormgate2 and the ORFeome library, with a recently developed particle bombardment transformation system, expedites hpRNAi gene silencing. This will be particularly useful for studying those genes that are refractory to the effects of injected or fed dsRNA, such as neural genes. We report the efficient production of hpRNAi constructs using pWormgate2 and also the knockdown of selected genes, including neurally expressed genes that have previously been refractory to RNAi. Further, when combined with the *rrf-3* RNAi hypersensitive strain, the Wormgate approach delivered a highly penetrant knockdown phenotype in nearly 100% of worms for a gene that was completely refractory to other RNAi delivery methods.

Keywords: Gateway; *unc-119*; hpRNAi; Hairpin

1. Introduction

Interfering RNA is usually delivered to the nematode *Caenorhabditis elegans* by injection of double-stranded RNA (dsRNA) (Fire et al., 1998), orally by soaking nematodes in a concentrated solution of dsRNA (Tabara et al., 1998; Maeda et al., 2001) or by feeding worms bacteria engineered to produce dsRNA (Timmons and Fire, 1998). Microinjection of dsRNA is effective at inducing RNA interference (RNAi), but it is labour-intensive, requiring high levels of skill and is not well suited for large-scale screens. In contrast, oral delivery is quick and easy to perform. Indeed, many studies have used the feeding approach to screen nearly 78% of the 19,920 *C. elegans* genes (worm sequence release WS112; www.wormbase.org; Kamath et al., 2003; Rual et al., 2004) resulting in RNAi phenotypes for 12.8% of tested genes (Fraser et al., 2000; Gönczy et al., 2000; Piano et al., 2000; Hanazawa et al., 2001; Maeda et al., 2001; Zipperlen et al., 2001; Ashrafi et al., 2003; Kamath et al., 2003; Lee et al., 2003; Pothof et al., 2003; Simmer et al., 2003; Vastenhouw et al., 2003). Despite the value of these screens and others like them, the

Abbreviations: BLAST, basic local alignment search tool; ddH_2O, double distilled water; hpRNAi, hairpin RNA interference; L1, larval stage one; L4, larval stage four; RNAi, RNA interference.

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functions of over 80% of *C. elegans* genes remain unknown and complementary approaches are required to probe their functions.

One limitation of RNAi delivered orally or by microinjection is that neuronal genes are at least partially, and in many cases entirely, refractory to these techniques (Tavernarakis et al., 2000; Kamath et al., 2003). For example, known loss-of-function phenotypes were replicated in the genome-wide RNAi-feeding screen for only approximately 12% of neuronal genes, whereas loss-of-function phenotypes were observed for 71% of all genes with lethal phenotypes (Kamath et al., 2000, 2003). Some neurons may be refractory to RNAi because they lack the ability to transport dsRNA into the cell. Consistent with this, the putative dsRNA transporter SID-1, which is required for systemic RNAi, is absent from almost all neurons (Winston et al., 2002). The presumed inability of neurons to import dsRNA has been overcome by transforming worms with transgenes that express an endogenous hairpin dsRNA within the cell (Tavernarakis et al., 2000), so-called hairpin RNAi (hpRNAi). This has additional advantages over oral delivery or microinjection of dsRNA, in that by expressing the hairpin under the control of an inducible promoter, RNAi can be initiated at any stage of development. In addition, RNAi can be evoked in large populations of genetically identical worms, which can improve the consistency of the RNAi phenotype and provide enough RNAi nematodes for biochemical analysis (Tavernarakis et al., 2000).

As reviewed recently, the labour intensive nature of the hpRNAi technique, compared with oral delivery methods, has prevented its common application (Sugimoto, 2004). Construction of hpRNAi genes by traditional recombinant techniques requires multiple steps and is a low efficiency process due to formation of hairpin structures that interfere with cloning. Using these techniques, typically only 1–5% of screened bacterial colonies contain the required hpRNAi construct, even when using *E. coli* strains that have an increased tolerance for hairpin constructs such as the SURE strain (Tavernarakis et al., 2000). The Gateway cloning system (Invitrogen) employs recombination rather than restriction/ligation based cloning and has been adapted to produce plant hpRNAi constructs with greatly increased efficiency (Wesley et al., 2001). An additional advantage of the Gateway system is that it is compatible with the *C. elegans* ORFeome library. The ORFeome library comprises the open-reading frames (ORFs) of *C. elegans* genes cloned into the Gateway shuttle vector pDONR201, which allows them to be easily transferred to a variety of Gateway destination vectors, including a modified version of the RNAi-feeding vector pL4440 (Reboul et al., 2003; Rual et al., 2004). The current version of the *C. elegans* ORFeome (v3.1) contains approximately 12,500 full-length ORFs and efforts are continuing to clone every *C. elegans* ORF (Lamesch et al., 2004). This paper describes a new Gateway destination vector, pWormgate2, which utilizes the *C. elegans* ORFeome library to produce constructs for hpRNAi in *C. elegans*.

2. Materials and methods

2.1. Nematode strains

N2 Bristol, CB4845 *unc-119(e2498)*, DA465 *eat-2(ad465)* and NL2099 *rrf-3(pk1426)* strains were from the *Caenorhabditis* Genetics Center and maintained using standard techniques (Brenner, 1974).

2.2. Genetic crosses

A strain, XA4261, which contains the integrated hpRNAi-*unc-4* transgene, *qaIs4254*, from XA4254 and *rrf-3(pk1426)*, was generated by mating NL2099 *rrf-3(pk1426)* males with XA4254 [hpRNAi-*unc-4(qaIs4254)*] hermaphrodites using standard techniques. The presence of both alleles was confirmed by PCR.

2.3. Plasmid construction

Standard recombinant DNA cloning methods were used to construct Wormgate plasmids (Sambrook et al., 1989). The plasmids were derived from pPD49.78 (Fire et al., 1990). The annotated sequence of pWormgate2 was lodged with Genbank, with accession number AY551267. pWormgate2 and its intermediates were maintained in DB3.1 *E. coli*, which are tolerant of the *ccdB* selectable marker gene. Gateway entry vectors that are not available in the current version of the ORFeome library were cloned by PCR and inserted into pDONR201 (Invitrogen) via the BP Gateway™ reaction as described in the Gateway instruction manual. For pENTR-*gfp* the *gfp* insert was amplified from pPD95.75; pENTR-*unc*-22 from pLT61; and pENTR-*pos*-1, pENTR-*deg-3* and pENTR-*eat-2* from cDNA clones yk117h11, yk131g9 and yk108h12, respectively. Structures of pENTR and hpRNAi constructs were confirmed by restriction digest and sequence analysis following standard protocols. hpRNAi constructs for *unc-4, unc-17, unc-25, unc-47, unc-76, unc-119, acr-5, acr-7, acr-9, acr-12*, F21A3.7, T01H10.1 and R03E1.3 were generated from clones of the ORFeome library. The Spearman rank test (InStat version 2.0) was performed to determine a correlation between ORF size and hpRNAi cloning efficiency.

2.4. Nematode transformation

Transformation by micro-particle bombardment of *unc-119* mutants was performed with the Biolistic PDS-1000/He with Hepta adapter (Bio-Rad). The bombardment protocol was based on that previously described (Praitis et al., 2001) with the addition of the Hepta adapter (Berezikov et al., 2004). Bombardment settings were: 1100 psi rupture disks;