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TECHNOLOGY REPORT

Molecular Cloning of a Dominant Roller Mutant and Establishment of DNA-Mediated Transformation in the Nematode *Pristionchus pacificus*

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Summary: We report the molecular cloning of a dominant Roller mutant of *Pristionchus pacificus*, which encodes a cuticle collagen. We use the mutant locus as a marker to develop transgenic technique by generating complex arrays and present flourescent-protein based transcriptional reporter constructs for *P. pacificus*. genesis 47:300–304, 2009. © 2009 Wiley-Liss, Inc.

Key words: *Pristionchus pacificus*; DNA-mediated transformation; collagen; nematodes; parasite

Transgenes are an important tool for experimental biology. The methods of transformation differ significantly between established model organisms, but all available procedures rely on markers that allow the identification of transgenic individuals. Those markers lead to visible phenotypes that are easy to score and are specific for a given organism. Hence, for every new model-organism, new ways of transformation and new marker constructs have to be developed.

Pristionchus pacificus has been established as a model organism in two areas of evolutionary biology (Hong and Sommer, 2006). In evolutionary developmental biology, *P. pacificus* is compared with *C. elegans* and the availability of forward and reverse genetic tools and the genome sequence of *P. pacificus* provide mechanistic insight into the evolution of developmental processes (Dieterich *et al*., 2008; Schlager *et al*., 2006; Tian *et al*., 2008). In evolutionary ecology, the species-specific association of *Pristionchus nematodes* with scarab beetles provides a system for studying the genetic basis of species interactions in the environment (Herrmann *et al*., 2007; Hong *et al*., 2008). Although several genetic and genomic tools are available, no method for transgenic technology had been developed for *Pristionchus* so far, due to the lack of a good transformation marker.

The most frequently used method to transform *C. elegans* is microinjection. A dominant allele (*su1006*) of the gene *rol-6* served as the original transformation marker (Mello *et al*., 1991). Worms, which carry and express a copy of this gene, show altered locomotion and "roll" or helically twist around their antero-posterior axis. Transgenes created by microinjection very rarely integrate into the chromosomes but rather form so-called extrachromosomal arrays. Extrachromosomal arrays are characterized by two main features. First, they are seemingly random concatemers of injected DNA molecules. Second, they are less faithfully segregated than endogenous chromosomes and are frequently lost during meiosis resulting in nonmendelian segregation (Mello *et al*., 1991). The mechanisms of array formation are poorly understood and only very few enzymes have been shown to be necessary. Simplifying, one can think of extrachromosomal arrays as small, highly repetitive, and artificial nematode chromosomes.

Here, we present the molecular cloning of the *P. pacificus* roller mutant *Ppa-prl-1*. We show that *Ppa-prl-1* encodes a cuticle collagen and that the dominant mutation *Ppa-prl-1*(*tu92*) is caused by an amino acid substitution identical to the ones found in dominant alleles of *C. elegans rol-6* and *sqt-1*. We use the *Ppa-prl-1*(*tu92*) locus to develop transgenic technique for *P. pacificus*.

THE DOMINANT ROLLER MUTANT *prl-1* ENCODES A CUTICLE COLLAGEN

We have previously identified an EMS-induced dominant Roller mutant, *Ppa-prl-1*(*tu92*) (*P*ristionchus *R*oller *l*ike-1) (Fig. 1A). Rough mapping has placed this mutant between the SNP markers S80 and S28 on chromosome I (Kenning *et al*., 2004). In *C. elegans*, only two genes have been found to be mutable to a dominant Roller phenotype, *rol-6* and *sqt-1*, respectively. Both encode nematode cuticle-collagen proteins, a family of nematode-specific collagen proteins that contain a characteristic N-terminal domain (Kramer and Johnson, 1993). Assuming

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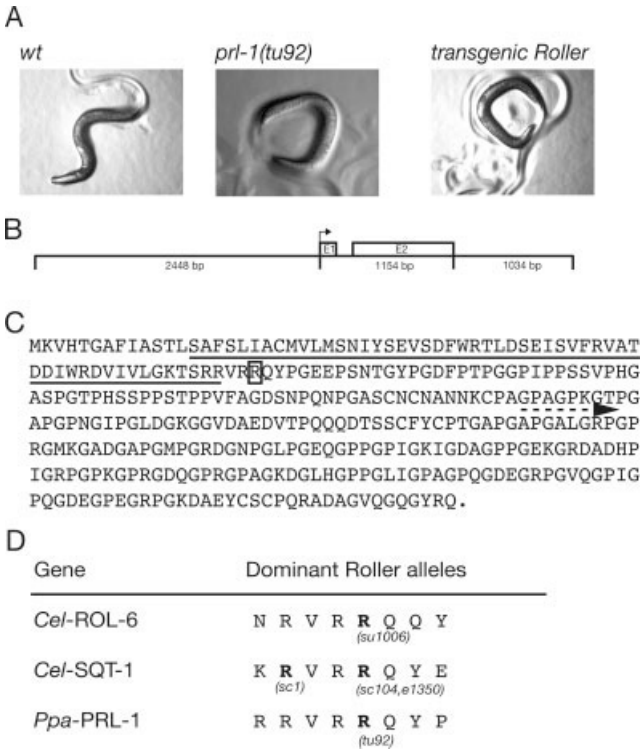

FIG. 1. Molecular cloning of the dominant Roller mutant *Ppa-prl-1(tu92)*. (**A**) Three photomicrographs showing representative phenotypes of a *P. pacificus* wildtype (wt), *Ppa-prl-1*(*tu92*) mutant and a transgenic Roller animal. The transgenic Roller is an animal carrying a complex array. (**B**) Structure of the pRL3 marker plasmid. Predicted exons of the *Ppa-prl-1* gene are indicated as boxes above the line. Numbers below the line indicate length in bp of upstream sequence, coding sequence (including the single intron of *Ppa-prl-1*) and 3′ sequence. The genomic mutant locus was cloned into TOPO-TA 2.1. (**C**) Predicted amino acid sequence of *Ppa*-PRL-1, a nematode cuticle collagen. The "Nematode cuticle collagen N-terminal domain" is underscored. The boxed Arginine residue is mutated to Cysteine in *Ppa-prl-1*(*tu92*). The dashed arrow indicates the start of typical G-X-Y collagen triple helix repeats. (**D**) Partial amino acid alignment between *Cel-rol-6*, *Cel-sqt-1* and *Ppa-prl-1*. The amino acids shown are the so-called Homology Domain A as defined in (Kramer and Johnson, 1993). The first amino acids shown are at position 67 (ROL-6), 65 (SQT-1) and 65 (PRL-1) of these proteins. Amino acids that have been found to be mutated in dominant Roller mutants are printed bold. All are Arginine to Cysteine mutations. Alleles are indicated in parentheses.

that a homologous gene could have been mutated in the *Ppa-prl-1*(*tu92*) mutant, we looked for cuticle-collagen encoding genes in the genomic vicinity of the known linked markers of *prl-1*. SNP marker S80 lies on Supercontig 27 of the most recent assembly of the *P. pacificus* genome sequence. BLAST searches showed that Contig 27.20 contains a gene that encodes a cuticle collagen with high sequence similarity to both, *rol-6* and *sqt-1*. We sequenced PCR products containing the entire coding sequence and flanking regions of this gene from both wildtype and *prl-1* worms and found that the *prl-1*(*tu92*) mutant contained a point mutation in the predicted coding sequence. The mutation is nonsynonymous and alters a conserved arginine codon to code for a cysteine (CGT → TGT) (Fig. 1B–D). A homologous arginine residue is mutated to cysteine in three of the known dominant Roller alleles of *C. elegans*: *rol-6*(*su1006*) and *sqt-1*(*sc1204,e1350*) (Fig. 1D) (Kramer and Johnson, 1993). The predicted amino acid sequence of *Ppa*-PRL-1 is most similar to *Cel*-SQT-1, but orthology is difficult to assign for a large and diverse gene family like the cuticle collagens. We, therefore, do not rename the *P. pacificus* gene, but continue to refer to it as *Ppa-prl-1*.

THE *Ppa-prl-1*(*tu92*) MUTANT LOCUS CAN BE USED AS A TRANSFORMATION MARKER

The plasmid pRF4 is a genomic clone of the dominant Roller allele *rol-6*(*su1006*) and was the first marker for microinjection-based transformation techniques in *C. elegans* (Mello *et al.*, 1991). However, this plasmid cannot induce Roller phenotypes when injected into *P. pacificus* (data not shown). We wished to determine whether the *Ppa-prl-1*(*tu92*) locus could be used as a dominant marker for the development of transgenic technology in *P. pacificus*. We cloned the entire *Ppa-prl-1*(*tu92*) locus, containing 2.5 kb of 5′ and 1 kb of 3′ sequence, into the TOPO-TA 2.1 plasmid and injected the resulting clone (pRL3) as purified circular plasmid DNA into the syncytial gonad of adult *P. pacificus* PS312 hermaphrodites. We then screened the F1 progeny of injected animals for the occurrence of the Roller phenotype. DNA concentrations between 40 and 200 ng/µl repeatable gave strongly rolling F1 animals (Fig. 1C, Table 1). The methods we used are exactly as those used to generate "extrachromosomal arrays" in *C. elegans* (Fire, 1986; Mello *et al.*, 1991). We routinely obtained between 10 and 20 F1 Rollers per 40 injected P0s (Table 1). This equals roughly 0.1 transformed F1 animal per injected P0, which is substantially lower than the transformation efficiency in *C. elegans*. We think the different efficiency is at least partly due to anatomical differences. The gonad of *P. pacificus* has a more complex shape than that of *C. elegans* and the *rhachis* is more difficult to inject (Rudel *et al.*, 2005). Taken together, we conclude that the *Ppa-prl-1*(*tu92*) locus can be used as a dominant marker for transformation by microinjection and that the transformation efficiency is lower than in *C. elegans*.

TRANSFORMATION WITH CIRCULAR PLASMIDS IS NOT HERITABLE IN *P. pacificus*

Microinjection of circular plasmid DNA into *C. elegans* can lead to formation of extrachromsomal arrays. On average, about 90% of F1 Rollers do not transmit the transgene to the next generation. This might have one of three reasons: Either the F1 animal does not form an array and expresses the mutant ROL-6 protein from the plasmid/s (transient transformation) or the array is not present in the germline. Alternatively, the array is initially generated, but then silenced by a process called transgene silencing (Kelly *et al.*, 1997). While trying to

Table 1
Transformation Experiments in Pristionchus pacificus

	Plasmids						Lines
Exp. no.	pRL3	A second plasmid	Carrier (genomic DNA)	Conc. (ng/μl)[a]	No. injected	F1 Hermaphodites	Arrays
1	Circular	Circular	–	100/50/0	>3,000	>500	1
2	HindIII	HindIII	*Ppa* gDNA (HindIII)	1/1/60	103	10	6
3	PstI	PstI	*Ppa* gDNA (PstI)	1/1/60	100	7	3
4	PstI	PstI	*Ppa* gDNA (PstI)	1/10/60	37	2	1
5	PstI	PstI	*Ppa* gDNA (PstI)	1/0.1/60	40	2	2
6	PstI	HindIII	*Ppa* gDNA (PstI/HindIII)	1/0.01/60	85	7	4
7	HindIII	HindIII	*Ppa* gDNA (PstI)	1/1/60	60	3	0
8	PstI	PstI	*Ppa* gDNA (HindIII)	1/1/60	51	1	0
9	PstI	PstI	*Ppa* gDNA (NotI)	1/1/60	40	3	0
10	PstI	PstI	Samon sperm (Sheared and PstI)	1/1/60	50	1	0
11	PstI	PstI	Zebrafish (PstI)	1/1/60	70	2	1
12	PstI	PstI	*Arabidopsis lyrata* (PstI)	1/1/60	46	1	0
13	HindIII	HindIII	*Drosophila* (HindIII)	1/1/60	40	0	0
14	PstI	PstI	*C. elegans* N2 (PstI)	1/1/60	40	0	0
15	PstI	PstI	Samon sperm (Sheared)	1/1/60	110	7	0

Experiment 1 contains the pooled data from several experiments (ca. 50) that aimed to transform *P. pacificus* with circular plasmids. Sometimes, but not always, a second plasmid was coinjected (usually a flourescent reporter constructs). As discussed in the text, only one case of transmission was found from more than 500 F1 Rollers. **Experiments 2-15** are individual experiments aimed at transforming *P. pacificus* with "complex array" mixtures of linearized plasmid DNA and digested or sheared genomic DNA from various species. **Experiments 2-6** Plasmid and carrier were digested with the same restriction enzyme/s. **Experiments 7-9** Plasmid and carrier were digested with different enzymes. **Experiments 10-15** Plasmid was mixed with carrier DNA from species other than *P. pacificus*. Comparing **Experiment 2-6** with **Experiments 7-15** shows that plasmid and carrier have to be digested with the same enzyme to lead to efficient transformation and establishment of transgenic lines and that using *P. pacificus* genomic DNA is important for transformation efficiency. Foreign DNA also led to strong developmental retardation of F1 animals after injection.
[a]Concentrations are in ng/μl and in the following order: pRL3, second plasmid, and carrier DNA.

transform *P. pacificus* with circular plasmids, we have injected >3000 P0 animals and have observed more than 500 F1 Rollers. However, we found only one case of transmission to the F2 and formation of an extrachromsomal array (Table 1). PCR testing of 96 non-Rol F2 progeny, all of which derived from F1 Rol animals, indicated that the transgenes were not transmitted to the F2 generation (data not shown). In combination with the low transformation rates, this poses a severe limitation in using circular plasmids to transform *P. pacificus*.

COMPLEX ARRAYS ARE STABLY PROPAGATED IN *P. pacificus*

A method to evade the problem of transgene silencing in *C. elegans* is the use of so-called complex arrays. Rather than injecting circular pure plasmid as used for standard arrays, linearized plasmids at low concentrations are mixed with a higher-concentration of sheared or digested genomic DNA. The resulting extrachromosomal arrays are not silenced in F1 animals, presumably because they are less repetitive (Kelly *et al*., 1997).

We prepared complex array mixtures of the linearized pRL3 plasmid and *P. pacificus* genomic DNA and injected them into *P. pacificus* hermaphrodites. We obtained F1 Rollers at a frequency of one to three animals per 40 injected P0s (Table 1). We found that 18 out of 46 F1 Rollers transmitted the Roller phenotype to the F2. From the F2 generation onwards, the Roller phenotype was stably expressed for >10 generations (Table 1). Stable transgenic lines were only observed when the complex array was formed with genomic DNA of *P. pacificus* that had been digested with the same restriction enzyme as the plasmid DNAs (Table 1). In total, we obtained 16 stable lines from 365 injected P0 animals. When using genomic DNA of different species, we observed only a few transgenic F1 animals and only one line from 356 injected animals (Table 1). Together, these experiments indicate the first feasible method for generating transgenic lines in *P. pacificus*.

A SET OF FLOURESCENT REPORTER CONSTRUCTS FOR *P. pacificus*

Next, we aimed to generate transcriptional reporter constructs for *P. pacificus*. Several publications point at the importance of endogenous splicing and polyadenylation signals for the robust expression of transcriptional reporter constructs in nematodes (Li *et al*., 2006; Okkema *et al*., 1993). Therefore, we made reporter constructs with *P. pacificus*-specific splicing and adenylation sequences (Fig. 2A). Specifically, we made one GFP and one TurboRFP based reporter. The GFP construct contains a nuclear localization sequence (NLS) and three artifical introns in the GFP coding sequence, the TurboRFP construct contains neither of those. Both, the GFP and RFP construct contain an upstream splice acceptor sequence, which conforms to the *P. pacificus* splice acceptor consensus sequence, and contain the 3′UTR of the *P. pacificus rpl-23* gene (Fig. 2A). The artifical introns of the GFP construct are based on those of

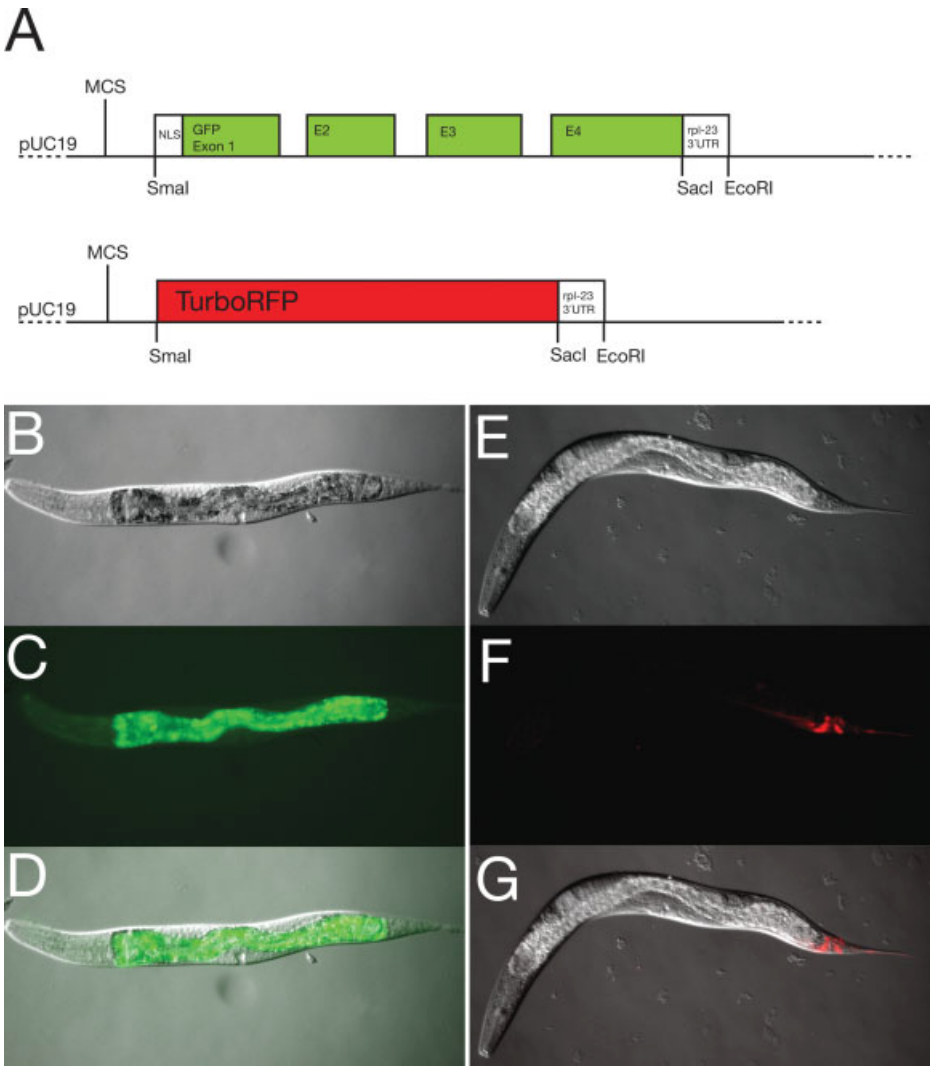

FIG. 2. Flourescent protein reporter constructs for *P. pacificus*. (**A**) Schematics of the GFP and TurboRFP reporter constructs used in this study. Both are based on pUC19. Intron-exon structure is indicated and drawn to scale. In both constructs the coding sequence of flourescent proteins is followed by the 3′ UTR of *Ppa-rpl-23*, a small-subunit ribosomal protein. Detailed maps, sequences and plasmids are available upon request. MCS multiple cloning site. NLS nuclear localization signal. (**B**–**G**) DIC and flourescent micrographs of transgenic *P. pacificus* animals carrying complex arrays containing one of two reporters. (B–D) A young L4 animal expressing *Ppa-sur-5*::GFP. This construct contains 2 kb of promotor sequence and the first two exons of *Ppa-sur-5* (a partial translational fusion). Expression is strongest in intestinal cells but also detectable in hypodermal cells. (E–G) A young adult expressing *Ppa-egl-20*:TurboRFP. This construct contains 2.1 kb of promotor sequence of *Ppa-egl-20* (a transcriptional fusion). Expression is restricted to several cells in the tail. (B, E) DIC micrographs (C, F) Flourescent micrographs (D, G) Overlays of B+C and E+F.

the *C. elegans* reporter construct pPD95.73 but were changed to contain the *P. pacificus* splice acceptor.

We cloned several *P. pacificus* promoters into these constructs and tested them by generating stable lines containing complex arrays. A *Ppa-sur-5*::GFP construct expressed in the gut and hypodermis (Fig. 2B–D). *Ppa-egl-20*:RFP expressed in several cells of the tail, consistent with the previously published in situ hybridization expression pattern (Fig. 2E–G) (Tian *et al*., 2008).

Although we did not explicitly test the contribution of individual *P. pacificus* signal sequences to detectable expression levels, we made one observation that leads us to believe they are important: the *Ppa-egl-20* promoter is unable to drive expression from an RFP plasmid that contains no upstream splice acceptor and the SV40 3′ UTR. When the *P. pacificus* splice acceptor and *rpl-23* 3′ UTR were introduced this promoter was able to drive robust expression of RFP (Fig. 2E–G).

TRANSGENESIS IN NEMATODES OTHER THAN *Caenorhabditis*

The nematodes are a biologically diverse phylum with many animal and plant parasitic species (Blaxter *et al*.,

1998). Although the last decade has seen a vast expansion of genomic tools in some of the parasites with agricultural and medical importance, transgenic methods are largely restricted to *C. elegans* and some of its close relatives. Exceptions to this have been the human parasite *Strongyloides stercoralis* (Li *et al*., 2006) and the Australian possum parasite *Parastrongyloides trichosuri* (Grant *et al*., 2006), for which transgenic technologies have been developed. However, expression of transgenes in *S. stercoralis* has been restricted to F1 transgenic animals. No expression in later generations has been reported presumably because of transgene silencing (Li *et al*., 2006). The complex array approach described in this study for *P. pacificus* might provide a technique to overcome transgene silencing and might become a general strategy for nematode transgenesis.

In conclusion, two features were crucial for the development of a transformation system in *P. pacificus*. First, the development of a suitable transformation marker, a feature that might remain a major obstacle for transgenic techniques in other nematodes. Second, inheritable transgenes required the establishment of complex arrays, a method that could be transferred to other nematodes.

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