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The NemaGENETAG initiative: large scale transposon insertion gene-tagging in *Caenorhabditis elegans*

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Abstract The nematode *Caenorhabditis elegans* is a widely appreciated, powerful platform in which to study important biological mechanisms related to human health. More than 65% of human disease genes have homologues in the *C. elegans* genome, and essential aspects of mammalian cell biology, neurobiology and development are faithfully recapitulated in this organism. The EU-funded NemaGENETAG project was initiated with the aim to develop cutting-edge tools and resources that will facilitate modelling of human pathologies in *C. elegans*, and advance our understanding of animal development and physiology. The main objective of the project involves the generation and evaluation of a large collection of transposon-tagged mutants. In the process of achieving this objective the NemaGENETAG consortium also endeavours to optimize and automate existing transposon-mediated mutagenesis methodologies based on the *Mos1* transposable element, in addition to developing alternatives using other transposon systems. The final product of this initiative—a comprehensive collection of transposon-tagged alleles—together with the acquisition of efficient transposon-based tools for mutagenesis and transgenesis in *C. elegans*, should yield a wealth of information on gene function, immediately relevant to key biological processes and to pharmaceutical research and development.

Keywords *Caenorhabditis elegans* · *Mariner* · *Mos1* · Gene tagging · Tc1 · Transposon

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Abbreviations

DNA	Desoxyribonucleic acid
DSB	Double strand break
*Mos*TIC	*Mos1* transgene induced conversion
PCR	Polymerase chain reaction
CGC	*Caenorhabditis* genetics center

Introduction

The new challenge for the scientific community is to make true sense out of the biological information presented as a massive amount of raw data generated by the genome sequencing projects. This is essential for understanding the delicate complexity of biological processes and their interactions, and ultimately for interfacing with approaches aiming to improve human health and quality of life. Therefore, considerable scientific efforts have been focused in the development of novel tools for determining gene function and genetic interactions. A starting point is the manipulation of the genome by tagging and mutating every gene separately. The use of transposable elements has had an enormous contribution in this field of genetic analysis, in many organisms. Insertion mutagenesis using transposable elements overcomes the need for genetic mapping and provides sequencing tags allowing the identification of the insertion site.

Since the completion of the *C. elegans* genome sequencing (The *C. elegans* Sequencing Consortium 1998), plans have been made towards the establishment of collections of identified mutants using non-directed, transposon-based insertional mutagenesis. The *C. elegans* genome contains active transposons which belong to the *Tc1/Mariner* family [reviewed in (Plasterk et al. 1999)]. The major drawback of the endogenous transposons is that

they are present in the genome in multiple copies and new insertions do not provide unique tags. In this respect, a major break-through has been the demonstration that the *Drosophila mariner* derived *Mos* transposon, a member of the *Tc1/Mariner* family, is active in *C. elegans*. Mobilization of the exogenous *Mos1* transposon, was accomplished in somatic cells and in the germ line of *C. elegans* (Bessereau et al. 2001). The transformation protocol has been optimized since, allowing a significant improvement of the mutagenesis efficiency (Williams et al. 2005). Presently the *Mos1* system is the most widely used, fully-controllable transposable element for functional genomics purposes in *C. elegans* (Bessereau 2006).

The NemaGENETAG consortium has built on the *Mos1* system to generate a large, ordered library of tagged nematode genes. To achieve this goal the consortium also focused on upgrading and automating existing transposon-mediated mutagenesis protocols by developing the appropriate platform technologies required. Participating research teams worked closely to coordinate and perform the individual tasks required for the successful completion of the project (Fig. 1; see also http://elegans.imbb.forth.gr/nemagenetag/). The sophisticated genetic tools and the resource of transposon-tagged mutants acquired during the course of the project are available to the scientific community and are already becoming an essential component of the *C. elegans* toolbox. In this review article, we present the NemaGENETAG project, its objectives and the outcome of activities carried out by the research teams comprising the NemaGENETAG consortium. The dedicated and persistent efforts of these teams, lead by Jean-Louis Bessereau (Ecole Normale Supérieure, Paris, France), Jonathan Ewbank (Centre d'Immunologie de Marseille-Luminy, Marseille, France), Johan Geysen (Maia Scientific, Geel, Belgium), Patricia Kuwabara (University of Bristol, Bristol, UK), Laurent Ségalat (Université Claude Bernard Lyon-1, Lyon, France) and N. T. (Foundation for Research and Technology-Hellas, Heraklion, Greece), brought the project to fruition.

Project objectives

The NemaGENETAG initiative has three clear objectives. Specifically:

Objective 1. Optimization/automation of the *Mos1*-based system for large-scale mutagenesis

The *Mos1* system has already been established as an efficient tool for gene-tagging in *C. elegans* (Boulin and Bessereau 2007). It is based on the generation of transgenic worm strains carrying two independent extrachromosomal arrays, one that encodes the *Mos1* transposase under the control of an inducible promoter, and another that includes copies of the *Mos1* transposon (Bessereau et al. 2001). Induction of transposase expression leads to the mobilization of the transposon and its integration within the *C. elegans* genome. The extrachromosomal arrays are not inherited in a Mendelian fashion and can be easily lost in subsequent generations, resulting in the stabilisation of the *Mos1* insertion. The first objective of the NemaGENETAG project was to characterize this system in terms of insertion bias and mutagenicity. Through such detailed characterization, we have sought to optimize *Mos1* tools and reagents for high-throughput screenings. Scaling up transposon-mediated gene-tagging to the whole-genome level requires considerable investment in the development of technology platforms that will allow automation and streamlining of various processes.

Objective 2. Development of novel transposon-based systems for mutagenesis, transgenesis and genome engineering in *C. elegans*

Development of other transposons systems is important for two reasons. First, all transposons have preferential insertion sites in genomes. We predict that even after isolating a very large number of *Mos1* insertions, specific regions of the genome will be under-sampled. Another transposon would have a distinct insertion bias and would provide a way to target the genes that are found in such regions. Second, transposons can be used to introduce foreign sequences into the host genome. This feature is widely used for enhancer-trap systems or tissue-specific expression systems (Asakawa et al. 2008; Awazu et al. 2007; Balciunas et al. 2004; Bonin and Mann 2004; Faucheux et al. 2001; Geurts et al. 2006; Korswagen et al. 1997; Nagayoshi et al. 2008; Parinov et al. 2004; Sasakura et al. 2008; Score et al. 2006). *Mos1* can accommodate exogenous DNA but the frequency of transposition decreases exponentially with the size of the insert, such that a maximum of only 300 bp of exogenous DNA can be included in recombinant *Mos1* transposons (Bessereau et al. 2001). To circumvent these issues we aim to develop alternative transposon systems in *C. elegans* based on the well-characterized and widely used *Minos* and *piggyBac* transposable elements.

Localized transposon insertions also represent an entry point to further manipulate the locus where they are inserted. First, imprecise excision of *Mariner* transposons causes various types of gene lesions such as gene deletions. *Mos1* re-mobilization followed by imprecise repair can be achieved in *C. elegans* (Robert and Bessereau 2007). By reintroducing a *Mos1* transposase expression transgene, it is possible to identify excision events that cause deletions or small insertion footprints in the gene that is tagged with *Mos1* insertion. Experiments indicate that *Mos1* re-excision

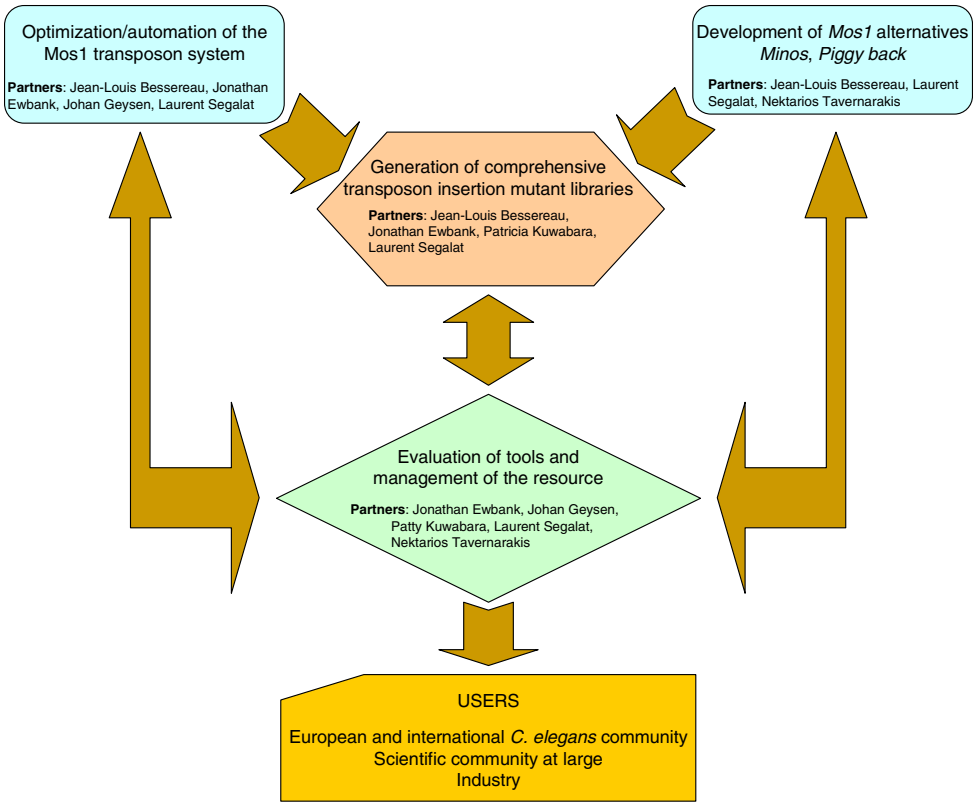

Fig. 1 NemaGENETAG project organization. The work plan is broken down to activities that partially or totally depend on each partner, and is further dissected into work packages, which correspond to major subdivisions of the program. The structure of the project reflects the complementarity of the approaches and of the expertise of the individual participants. Hence, each participant contributes to several work packages

is very efficient. After excision, the double-strand break is repaired from homologous sequences. This repair mechanism regenerates a *Mos1* copy at the site of excision. However, repair is inhibited when chromosome pairing is disrupted. These features of *Mos1* re-excision can be used to recover imprecise excision events at high frequency. This tool combined with a comprehensive library of *Mos1* insertions would provide a general resource to knock-out most of the *C. elegans* genes at low cost.

Gene conversion following transposon excision has been exploited to copy information into a genome in a site-specific manner (Robert et al. 2008). If exogenous sequences could be introduced efficiently into the *C. elegans* genome via *Mos1*-mediated transgene-instructed gene conversion, a library of strains containing *Mos1* insertions in most genes would provide a feasible alternative to homologous recombination techniques that are not currently available in *C. elegans*.

Objective 3. Construction of a large, ordered library of transposon-tagged *C. elegans* genes

Our aim is to use the tools and technologies described above to generate a comprehensive collection of transposon-tagged nematode genes. Such a mutant collection is an extremely valuable resource because it will accelerate our understanding of gene function. Since, ~50% of human genes have a *C. elegans* homologue and more than 65% of human disease genes are represented in the nematode genome, a library of transposon-tagged genes will provide ready-made models and reagents to tackle human pathologies. Mutant strains will be maintained and distributed to interested colleagues all over the world. *C. elegans* strain stocks can be kept frozen and thawed easily, which greatly facilitates the conservation and distribution of such mutant collections (Brenner 1974).

Work plan

The main objective of the NemaGENETAG research programme is the generation of a comprehensive collection of *C. elegans* strains carrying transposon-tagged genes. The use of heterologous transposable elements instead of resident Tc transposons allows for rapid and controllable genetic and molecular manoeuvres, such as gene identification and cloning, and when necessary, efficient generation of knock out alleles. This collection will greatly facilitate functional genomics approaches to disease mechanisms, and gene function, in an organism with exceptional experimental advantages.

The tight integration of individual contributions and activities was important for the successful completion of the project (Fig. 1). Consortium activities were categorized

into two major types, exemplified in the form of five distinct Workpackages:

- Research activities, which are distributed into four work packages as follows:
  - A. Optimization/automation of *Mos1* transposon-based technologies.
  - B. Development of alternative systems for mutagenesis and transgenesis in *C. elegans* based on the *Minos* and *piggyBac* transposons.
  - C. Generation of a comprehensive, ordered library of tagged nematode genes.
  - D. Case-studies/evaluation of the resource.
- Technological development, innovation and demonstration of related activities:
  - E. Development/deployment of platform technologies and infrastructure required to achieve the main objective and to manage and maintain a large resource.

Below we outline these activities, explain the structure of the plan and present the overall methodology used to achieve the objectives.

Workpackage 1: optimization/automation of Mos1 transposon-based technologies

The goal of this Workpackage was to validate the utility of the *Mos1* system at the whole genome scale and to generate new tools to engineer the *C. elegans* genome. It breaks into three major points:

- Evaluation of a pilot collection of *Mos1* insertions including localization and statistical analysis of insertion distribution. A subset of insertions was selected and analyzed for phenotypic alterations.
- Development of a strategy for efficient recovery of gene deletions after *Mos1* excision.
- Development of a strategy for engineering mutations by transgene-instructed double strand DNA break repair following *Mos1* excision.

Workpackage 2: development of alternative systems for mutagenesis and transgenesis in *C. elegans* based on the Minos and piggyBac transposons

The objective of this Workpackage was to develop alternative systems to the *Mos1* system, which is presently the only fully-controllable transposable element which can be used in *C. elegans* to construct libraries of tagged genes. *Minos* and *piggyBac* were chosen for the following reasons: (1) they have a large spectrum of hosts; and (2) they can support extensive modification of their internal sequences without hampering the ability to transpose (Bonin and Mann 2004; Catteruccia et al. 2000; Ding et al. 2005; Drabek et al. 2003; Grossman et al. 2000; Handler and McCombs 2000; Handler et al. 1998; Hediger et al. 2001; Horn et al. 2003; Li et al. 2001; Lobo et al. 1999, 2001; Lorenzen et al. 2003; Loukeris et al. 1995a, b; Matsuoka et al. 2004; Morales et al. 2007; Nolan et al. 2002; Pavlopoulos et al. 2004; Peloquin et al. 2000; Perera et al. 2002; Ren et al. 2006; Rodrigues et al. 2006; Sasakura et al. 2003; Shinmyo et al. 2004; Sumitani et al. 2003; Tamura et al. 2000; Thibault et al. 2004; Uchino et al. 2007; Wang et al. 2008; Zagoraiou et al. 2001; Zhang et al. 2002). Efficient transposition based on these systems would not only facilitate the construction of the insertion library (Workpackage 3), but would also benefit the *C. elegans* community as a new tool that would pave the way for a much needed enhancer trap system.

Workpackage 3: generation of a comprehensive, ordered library of tagged nematode genes

The objective of this Workpackage was to generate an ordered collection of *C. elegans* strains carrying transposon-tagged genes. Transposon insertions are localized by sequencing transposon insertion alleles.

Workpackage 4: case-studies/evaluation of the resource

The objective of this Workpackage was to evaluate, and to provide added value to, the bank of *C. elegans* transposon-tagged genes generated in Workpackage 3 using forward genetic screens.

Two main forward genetic screens were planned:

- Screens to identify genes required for nicotinic neurotransmission (Boulin et al. 2008; Eimer et al. 2007; Gally et al. 2004). These genes will represent candidate drug targets, especially for the treatment of nicotine addiction.
- Screens to identify genes required for necrosis and neurodegeneration in the nematode. Functional characterization of such genes will elucidate mechanisms of inappropriate cell death and reveal targets for therapeutic intervention aiming to counter neurodegeneration.

Workpackage 5: platform technology development/ deployment

Workpackage 5 incorporates the development of platform technologies required for the timely and successful completion of the project. The aim was to introduce a new ultra sensitive automated microscopic imaging device (MIAS-2)

into the consortium and to apply it to the advantage of its core objective: genome-wide application of current and novel transposon technologies. In addition to providing automated image acquisition and analysis, it is expected that the very high sensitivity in low light conditions of the MIAS-2 microscopic reader will increase the success rate of the project by investigating very low expression levels of reporter genes in the nematode body.

Optimization and automation of the *Mos1*-based transposon insertion system for large-scale mutagenesis

The *Mos1* system has already been established as an efficient tool for gene-tagging in *C. elegans*. One of the main objectives of the NemaGENETAG consortium was the characterization of this system in terms of insertion bias and mutagenicity. Through such detailed investigation, we sought to optimize *Mos1* tools and reagents for high-throughput screenings. Through a thorough analysis of multiple parameters (cultivation and heat-shock conditions, nematode sorter transfer protocols, PCR detection protocols) and after a number of further modifications that were made to the initial protocol (generation and frequent re-crosses of new double transgenic strains in which the transposon and transposase extrachromosomal arrays were associated with fluorescent reporters of different colours), the necessary automation of the process was successfully put in place (Duverger et al. 2007; Granger et al. 2004). The combined effect of all these improvements was an efficiency of more than 90% in the recovery of viable *Mos1*-mutagenized worms and a capacity to process close to 6,000 individual clones per week. The level of production reached a peak number of 5,000 *Mos1*-positive strains per month.

After mapping insertions by sequencing and curation of the strains, ~13,000 transposon insertion alleles are available, representing tags in 25–30% of the *C. elegans* genes (http://elegans.imbb.forth.gr/nemagenetag/). Analysis of chromosomal distribution shows that insertions are disperced with relatively weak bias throughout the genome, in line with previously published observations (Granger et al. 2004). Information on transposon insertions is publicly available on both the *C. elegans* database Wormbase and the website of the NemaGENETAG consortium (http://elegans.imbb.forth.gr/nemagenetag/). This collection has become a major genetic resource, which complements already existing resources for the study of gene function, which include the CGC collection of mutants, various other sources of deletion alleles and transposon mutations and RNAi libraries (Antoshechkin and Sternberg 2007).

Development of novel transposon-based systems for mutagenesis, transgenesis and genome engineering in *C. elegans*

The objective of this activity is to develop alternative transposon systems for manipulating the *C. elegans* genome, such as *Minos*, *piggyBac* and *Sleeping Beauty*, which have been tested extensively in multiple organisms. Similarly to *Mos1*, these transposons can be used as tools for mutagenesis, providing additional genome coverage by complementing *Mos1* insertion preference. Insertion bias is common among transposable elements and combining more than one would facilitate the construction of a more representative library of transposon-tagged genes.

Furthermore such transposon systems can potentially overcome a significant shortcoming of current transgenesis approaches in *C. elegans*, which is the lack of tools and methodologies for straightforward integration of single copy exogenous DNA in the genome. For example, the *Minos* transposon has been shown to act as an efficient carrier of exogenous DNA which can be introduced in the genome along with *Minos* element, in many species (Catteruccia et al. 2000; Drabek et al. 2003; Loukeris et al. 1995b; Pavlopoulos et al. 2004; Zagoraiou et al. 2001; Uchino et al. 2007). Such a feature can be used to develop transposon-based enhancer-trap vectors which would allow the development of genome-wide screens for gene regulatory regions in *C. elegans*. Integration of heterologous sequences in the host genome is also a feature of the *Mos1* system, but in this case, as the size of the cargo sequence increases, the transposition efficiency decreases dramatically such that a maximum of only 300 bp of exogenous DNA can be included in recombinant *Mos1* transposons.

Minos, a mobile element discovered in *Drosophila hydei*, belongs to the family of *Tc1-mariner* transposable elements. Transposition of *Minos* only requires the presence of a transposase, encoded by *Minos* itself (Franz and Savakis 1991). We chose to investigate the transposition capacity of *Minos* in *C. elegans*, given that it has been successfully mobilized in several diverse organisms including insects (Catteruccia et al. 2000; Klinakis et al. 2000; Loukeris et al. 1995a; Pavlopoulos et al. 2004), crustaceans (Pavlopoulos and Averof 2005), ascidians (Sasakura et al. 2003; Matsuoka et al. 2004) and mammalian cells and tissues (Drabek et al. 2003; Zagoraiou et al. 2001).

The strategy that was successfully utilized to establish and optimize the *Mos1*-based transposon system was also adapted for the *Minos* element. However, while *Minos* transposition events could be detected in somatic tissues of *C. elegans*, repeated attempts to mobilize *Minos* in the germline failed. This could be due to insufficient expression of the *Minos* transposase, or to a low frequency of

transposition or both. We are currently exploring the transposable elements *Sleeping Beauty* and the *piggyBac* that similarly to *Minos* have been used extensively in multiple organisms, as alternatives to *Mos1* for mutagenesis and transgenesis in *C. elegans*.

The availability of a comprehensive library of *Mos1* insertions in *C. elegans* can be combined with alternative approaches to disrupt or modify *C. elegans* genes. These approaches include engineering of gene knock-outs and other chromosomal mutations at high frequency, following imprecise excision of *Mos1*, using transgene-instructed gene conversion (Robert and Bessereau 2007; Robert et al. 2008). The methodology is called *Mos*TIC for *Mos1* excision-induced Transgene Instructed gene Conversion and allows the introduction of homologous sequences from an extrachromosomal array into the genome at the native chromosomal locus. The extrachromosomal arrays used as repair templates for *Mos1* excision-induced double-strand break repair can also carry exogenous sequences and genomic modifications, such as point mutations, deletions and tags that will eventually be introduced into the *C. elegans* genome. A detailed description of this approach is provided in the article by Jean Louis Bessereau and Valérie Robert in this issue.

Development of platform technologies for automated analysis of nematode mutants

The NemaGENETAG participant MAIA Scientific (team lead by Johan Geysen) successfully introduced a new ultra sensitive automated microscopic imaging device (MIAS-2) that can analyze small model organisms, cells and tissue sections in multiple brightfield and fluorescent microscopy modes in parallel. The accompanying eaZYX software enables automated plate-handling, object recognition, autofocusing, image capture, storage and analysis. In addition to providing this automation, MIAS-2 microscopic reader is highly sensitive, allowing the investigation of low expression levels of reporter proteins in the nematode body. Furthermore, the MAIA Scientific team achieved the physical integration of two complementary platforms, the COPAS Biosort nematode sorter and MIAS-2 into a single robot station. The COPAS Biosort large particle flow sorter allows automated identification and sorting of *C. elegans* strains based on animal size and fluorescence signals. COPAS is equipped with robotics that are able to sort and dispense worms into multi-well plates at hundreds per hours. A COPAS integrated unit, the Profiler, can identify axial fluorescent profiles, allowing a worm population to be sorted based on positional changes or intensity of cells in the animals. This combined master station has the potential to speed up drug target identification and validation analysis through the advanced automation and handling capacity of the system. Both the COPAS and MIAS-2 platforms have contributed to the success of the NemaGENETAG project by accelerating the analysis of large collections of transposon insertion mutants.

Conclusions

The NemaGENETAG project has made a significant contribution to the field of genome-wide application of transposon technology. The successful efforts of the research teams involved resulted in the generation and evaluation of a large collection of *Mos-1* transposon-tagged mutants, with the ultimate aim to cover the complete genome of C. *elegans*. Furthermore, solutions for the automation and streamlining of the processes involved in generating the libraries of mutant strains were developed. The infrastructure necessary for managing and maintaining this resource was also established. The outcome of the NemaGENETAG project is already a great value to the scientific community. Many laboratories around the world have requested and obtained available tools and strains generated by the consortium and are exploiting them to study individual genes and biological phenomena of particular importance. The availability of such resources increases the versatility and usefulness of *C. elegans* as a platform for investigating gene function and modelling human diseases.

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