IGTRCN Peer-to-Peer Training Fellowship Report

Rajendra VE Chilukuri

Host: Dr Chris Jiggins, Department of Zoology, University of Cambridge,UK

Project title: Acquiring microinjection skills and CRISPR/Cas9 construct designing expertise, in order to develop transgenics of commercial silkworm *Bombyx mori*

Objectives of the training

The domesticated silkworm *Bombyx mori*, a major source of commercial silk, is considered as one of the most economically important and beneficial insects. With the advent of advanced genome engineering techniques, it has now become highly feasible to develop transgenic animals with desired traits and outcomes. The purpose of acquiring the skills was to carry out studies that would apply advanced techniques like CRISPR/Cas9 system to develop disease resistant and highly productive silkworm strains. Also of interest is the efforts to introduce better silk producing genes, isolated from non Bombyx species such as *Anterea mylitta* and also other species such as spiders, into B. mori. These studies would require for me to carry out microinjection.

The traineeship was used to develop skills in two aspects

1) To acquire proper and efficient microinjection skills for developing transgenic insects.
2) To design and construct gene targeting guide RNAs (sgRNAs) for CRISPR/Cas9 based gene knockout studies in insects, and applying the same to silkworm.

Note: The host Laboratory at Department of Zoology, Cambridge, UK was of Dr Chris Jiggins. The team at Cambridge has demonstrated expertise in CRISPR/Cas9 gene knock out studies and have done so in the form of publication (Ref 1)

Approach and Objectives

Butterfly rearing and microinjection preparation:

The initial week in the lab was spent acquainting to the model system, the species of butterflies that were worked with at Dr jiggins lab are, *Heliconius molpomene*, *Heloconius erato*, *Heliconius microglia Heliconius sara*, I have successfully learned butterfly-rearing aspects to the basic requirement, from egg lay set up to changing plants and collection of the eggs. The microinjection reagents and paraphernalia were prepared including:

1) Making of microinjection needles
2) Preparation of eggs

3) Sterilization of the eggs/equipment and
4) Plating the eggs on the slide

Initiating the training on the second day of arrival, *Heliconius melepomene* and *Heliconius sara* eggs were prepared for injection, to go through the basic protocol, I along with Dr Richard Wallbank set the plants for fresh egg lay in the green house. After allowing an hour for females to lay eggs we have collected eggs off plants, plated the eggs and injection of sgRNA and Cas9 protein into the eggs was carried out.

Eggs were glued on to glass slide, and were set for injection under dissecting microscope, Borosilicate needles used for injection, these needles were prepared in house using a needle puller, which is set at 56^0C of heat and pulled at 800 pull speed. The needle is filled with about 4ul of mix of sgRNA and Cas9 protein, the needle was mounted on to the microinjector. The microinjector was adjusted at 40psi pressure for clear and injection was carried out around 11-18psi. Each embryo was injected with appropriate amount of sample, ensuring the yolk does not leak. After injection, the slides with embryos were placde in a petridish with dab of wet tissue and were left in controlled room at 25°C of temperature and 70% of relative humidity. Once the caterpillars emerge they were put in a cup with leafs which are the natural food source of the caterpillar.

Objective 2: Design and Construction of sgRNA for *WntA*, *cortex*. Cas9 protein was procured from commercial manufacturer.

I selected three individual sgRNA target sites to be tested individually. Three sgRNAs targeting sites were selected and guide RNAs were designed using geneious a molecular biology and NGS tool. Since the *Heliconius molpomene* total genome sequence is available, this software with whole genome sequence of *Heliconius* could be used for designing of guide RNA. Synthetic oligomers specific were designed for target genes of interest (these were already available for WntA with Dr Wallbank), he has demonstrated the process and we have designed primers for other targets, in order for me to go through the training. These oligomers were annealed and subcloned into a DR274 plasmid at the *Bsa*I cleavage site to construct. *In vitro* transcription was carried out to obtain the corresponding sgRNAs. The sgRNA-containing DR274 vector was linearized with *Dra*I, purified by phenol-chloroform extraction, and subjected to a T7 RNA polymerase. The resulting sgRNAs were further purified with acid phenol-chloroform and quantified. Quality and concentration of sgRNA was determined by obtaining OD and by gel electrophoresis. *In vitro* validation of the sgRNA and Cas9 efficiency was carried out by treating the PCR amplicon of gene of interest with the mix of sgRNA/Cas9, once cleavage is observed and efficiency is determined the construct were used for *in vivo* microinjection. For microinjection into embryos, ideally higher concentration of guide RNA ~2ug/ul and Cas9 1ug/ul is preferred, eventually after preparing the injection mixture 2ul gRNA (400ng/ul final) and 5ul Cas9 protein (500ng/ul final) were used. My initial injections were carried out using sgRNAs for WntA, I have carried 2 sets of injection, and roughly about 70 eggs were injected. Figure 1 shown below was a mutant obtained from my first injection itself.

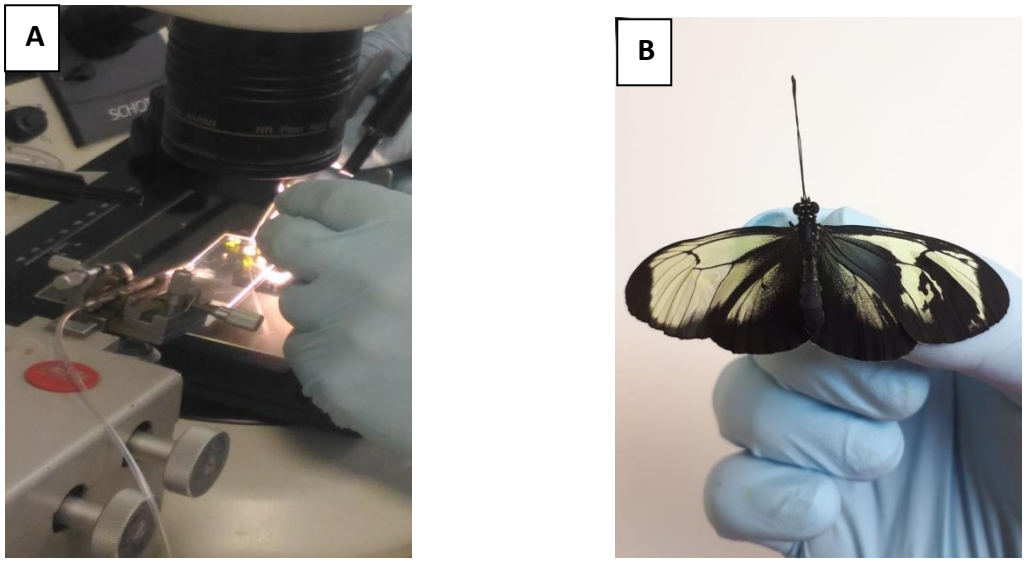

Figure 1: Panel A shows the setup and the arrangement of eggs for microinjection, Panel B is the WntA mutant obtained from CRISPR/Cas9 based gene knockout system. The wing pattern on the is normal is on the right and the mutant pattern is seen on the left (Ref-1 shows the published image for similar experiment)

<u>After visit experimental activity</u>:

Right after my return, I have initiated CRISPR/Cas9 gene knockout experiments ate CDFD, India. Using the same protocol as learnt at Cambridge, I have designed sgRNA for BmBLOS, this is a skin phenotype gene, giving mosaic phenotype on knockout. This gene was selected just as to get a hold of the protocol and further studies will be carried out once protocol is standardised for silkworm.

Note of Thanks: I extended my gratitude to Dr David O'Brochta and the IGTRCN peer-to-peer training fellowship for providing me with a tremendous opportunity to learn and execute insect transgenic experiments. Dr Chris Jiggins laboratory at department of Zoology, University of Cambridge is an expert lab in lepdopteran studies and their expertise is manifested from the publication records and achievements.

I personally want to thank Mrs Valeria Saffer for her support and help all through.

References:

1) Macro evolutionary shifts of WntA function potentiate butterfly wing-pattern diversity.Mazo-Vargas A, Concha C, Livraghi L, Massardo D, Wallbank RWR, Zhang L, Papador JD, Martinez-Najera D, Jiggins CD, Kronforst MR, Breuker CJ, Reed RD, Patel NH, McMillan WO, Martin A.Proc Natl Acad Sci U S A. 2017 Oct 3;114(40):10701-10706.