Development of genetic manipulation tools in Macrostomum lignano for dissection of molecular mechanisms of regeneration
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CHAPTER 7

Summary, Discussion and Future Perspectives
SUMMARY

Genetic manipulation has become one of the most essential tools used in biological research. It is widely used in forward and reverse genetics and was one of the most important missing techniques in the field of flatworm biology. This thesis aimed at filling in this gap by developing a stable, reproducible and reliable set of genetic manipulation tools in the flatworm *Macrostomum lignano*. We have also used the developed methods to address biological questions regarding stress response to temperature shock and differences in the transcriptomic profiles between neoblasts and germline cells.

Chapter 2 gives a general introduction to the model organism used in this thesis. It is a literature overview aimed at familiarizing the reader with the main topics and nomenclature used throughout the following chapters. Besides presenting the basics of development, reproduction and regeneration of *Macrostomum lignano*, Chapter 2 also gives an overview how areas such as stem cell biology, regeneration, rejuvenation, ageing and genome maintenance can benefit from using it as a model organism.

Chapter 3 is the main chapter of the thesis. It presents a robust and highly reproducible method enabling creation of stable transgenic lines of the flatworm *Macrostomum lignano*. We show that using microinjection into one-cell stage embryos allows for insertion of foreign genetic material into the genome of the flatworm. A series of optimization steps were taken to establish the most efficient approach towards the generation of transgenic animals. The form of the microinjected material was tested. The linear DNA was shown to have higher integration rate over the DNA in the form of a plasmid. Low dose irradiation was also introduced and shown to improve the integration efficiency. The microinjected material was generated based on the new genome and transcriptome and a set of *M. lignano* specific promoters was designed to drive the expression of fluorescent proteins. Apart from the ubiquitously expressed elongation factor alpha (EFA) promoter, the selection of the other promoters was based on their transcript levels in different tissues. This approach led to specific expression of the chosen marker genes in the ovaries (CABP7), testes (ELAV), gut (APOB) and muscles (MYH6). The positive results with the majority of tested promoters and the success rate of 1% to 5% of the total number of injected eggs for generating various transgenic lines was a confirmation of the robustness of the method and the quality of the new genome and transcriptome assemblies, which are also introduced in this chapter. A good quality genome assembly that is reliably annotated is of high importance when planning any genetic research, therefore this step was very important for the results obtained in the following chapters of the thesis. The improvements of the genome are clearly visible when comparing the N50 contig size values of the older assembly. While the previously published ML2 genome has N50 of 64.5 kb the one introduced in this chapter reached 215.2 kb. The transcriptome presented in this chapter is genome-guided and it is 98.1% complete. Findings presented here provide the strongest argument in favor of using *Macrostomum lignano* as an alternative or supplementary model organism in research areas described in Chapter 2.
Chapter 3 also introduces the molecular toolkit for genetic manipulation in *M. lignano* in the form of a collection of plasmids with *Macrostomum* codon-optimized fluorescent proteins, promoters and 3'UTR sequences. This toolkit is further expanded and tested in the subsequent chapters.

Chapter 4 further expands the genome manipulation toolkit developed for *M. lignano*. We focused on using the piggyBac transposon based system to enable stable and a more controlled integration of the desired DNA fragment into the genome of the flatworm. Transposons offer several advantages over the random integration method presented in Chapter 3. The possibility to more finely control the number of copies of the integrated fragment is one of them. The second major advantage is a much easier mapping of the insert location in the target genome. The third advantage is the potential of transposons to be excised from the previous integration site. In this chapter we present how piggyBac transposon can be used to create a stable transgenic line that expresses a fluorescent marker gene. We introduce a double marker plasmid that can be used to select for the transposon mediated transgenic animals, eliminating ones obtained through random integration events. We also provide a detailed characterization of the integration site of the insert using the Genome Walker approach. The proof-of-principle for the use of transposon mediated transgenesis further strengthens the findings from Chapter 3 and points the way for future improvements in the area of flatworm genetic manipulation.

Chapter 5 is focused on how the temperature influences the basic characteristics of *Macrostomum lignano*, namely: development, reproduction and regeneration. By investigating the development speed at different temperatures we propose a method for easier production and storage of the flatworm eggs. This finding improved the microinjection procedure by enabling more efficient use of picked eggs and accelerating the hatching of the microinjected eggs. The comparison of different temperatures pointed to the optimal conditions that can be used when culturing the worms for different purposes. Keeping the worms at 4°C can be used for more cost efficient storage of multiple lines. Temperatures above the standard 20°C increase the fertility and save time when establishing new transgenic lines. We pointed out to 25°C as the most reasonable choice when the fast development of the worms is required, because temperatures above 30°C induce stress response. We used qRT-PCR together with the analysis of the expression of a fluorescent marker driven by the heat shock 20 promoter to fully characterize the heat shock activated stress response. We also used one of the previously established transgenic lines to more reliably measure the influence of the temperature on the regeneration process of cut worms. The ELAV line expresses GFP specifically in the testes and sperm cells. We analyzed the appearance of fluorescent signal in testes and the seminal vesicle and used it to compare regeneration speed across different temperatures. This chapter illustrates how the transgenic lines established using the molecular toolkit introduced in this thesis can improve and supplement research on *Macrostomum lignano*.

Chapter 6 presents how tissue specific expression of fluorescent proteins can be used
to obtain reliable information regarding gene expression. We used FACS to isolate cell populations expressing GFP in a tissue-specific manner. We sequenced the sorted cells using the CEL-Seq protocol and analyzed the generated transcriptomes. The results supported the specificity of the isolated tissue cells and had their tissue specific genes upregulated. We also used the testes (ELAV) and ovary (CABP7) lines to correct the available neoblast stringent gene list, by eliminating the transcripts enriched in the germline. The work presented in this chapter highlights the advantage of tissue-specific marker genes in transcriptome analysis of different cell types.

**DISCUSSION AND FUTURE PERSPECTIVES**

The thesis is centered on the development and use of genetic manipulation tools for the flatworm *Macrostomum lignano*. We present how access to transgenic techniques can facilitate the research in the field of flatworm biology.

The introduced molecular toolkit was used to establish a series of transgenic lines that express fluorescent marker protein under the control of tissue specific promoters. We also presented how these lines allowed for FACS isolation and sequencing of flatworm cell populations. The generated RNA-seq data improved the currently existing transcriptomic profiles of neoblasts expanding our knowledge of the stem cell system of the flatworms. An important follow up will be finding neoblast-specific marker genes. A transgenic line expressing fluorescent protein in the stem cells would enable live imaging, lineage tracing and FACS, leading to a more complete characterization of the neoblast system. This could be achieved by either finding a neoblast-specific promoter or tagging a neoblast-specific gene with the help of genome engineering tool such as CRISPR/Cas9.

In chapter 5 we introduced heat shock inducible transgenic line, a valuable addition to the *Macrostomum* genetic manipulation toolkit that is being presented in this thesis. We used Hsp20 promoter to drive the expression of a fluorescent marker. The protein was detectable only when the worms were subjected to elevated temperatures. In this thesis the line was used as an indicator of the heat shock response, but the potential applications of this promoter are broader, such as inducible expression of various proteins, overexpression or protein interaction studies.

The work presented in this thesis opens a range of future opportunities in the fields of flatworm biology, stem cell research and ageing. One of the important factors to keep in mind is that the method presented is by large a proof-of-principle work. While the delivery method has been refined and optimized, the genetic manipulation tools can still be improved and expanded. A clear next step is the introduction of the CRISPR/Cas9 system. It will enable precise genome engineering, knock-ins and knock-outs, and will complete the genetic manipulation toolkit for *Macrostomum lignano*. 