Exploring genetic engineering strategies to enable heterologous monoterpenoid production in model microalgae, *Chlamydomonas reinhardtii* and *Phaeodactylum tricornutum*

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Certificate of Original Authorship

I, Jestin George, declare that this thesis is submitted in fulfilment of the requirements for the award of Doctorate of Philosophy, in the Faculty of Science at the University of Technology Sydney. This thesis is wholly my own work unless otherwise referenced or acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis. This document has not been submitted for qualifications at any other academic institution. This research is supported by the Australian Government Research Training Program.

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Thesis Abstract

This thesis focuses on next generation engineering strategies for *Chlamydomonas reinhardtii* and *Phaeodactylum tricornutum*, exploring aspects at the genomic and phenotypic level, to understand the biochemical implications and potential of heterologous monoterpenoid production in microalgae.

Chapter 1 outlines the ecological, and biotechnological relevance of microalgae, in the context of genetic engineering strategies for heterologous monoterpenoid production.

Chapter 2 investigates different strategies for delivering CRISPR-Cas9 ribonucleoprotein (RNP) into *C. reinhardtii* for targeted genome editing. This study highlighted major bottlenecks in CRISPR-Cas9 genome editing in this species, specifically low delivery efficiencies and unreliable endogenous markers.

Chapter 3 explores extrachromosomal expression (EE) and randomly integrated chromosomal expression (RICE) strategies to genetically engineer *P. tricornutum* to express *Catharanthus roseus* geraniol synthase (GES) for production of the monoterpenoid, geraniol. We identified superior RICE geraniol-yielding strains by developing a high-throughput phenotyping analysis and used long-read whole genome sequencing to interrogate the genomes of highly expressing cell lines. This revealed precise integration locations and unexpectedly large concatenated arrangements. We also demonstrated that exogenous DNA designed for EE does not inadvertently integrate into the nuclear genome.

Chapter 4 investigates CRISPR-Cas9 mediated targeted integration (TGI) for geraniol production in *P. tricornutum* in the genomic loci identified in Chapter 3. We showed that CRISPR-Cas9 RNP delivery is still inefficient in this species and that the recently

described endogenous marker gene uridine-5'-monophosphate synthase *(UMPS)* is unreliable in *P. tricornutum*, due to the highly mutagenic effect of 5-fluoroorotic acid, the selectable agent required to screen *UMPS* knock-out mutants.

Chapter 5 explores metabolic engineering approaches for increasing heterologous geraniol production in *P. tricornutum*. We fused two genes encoding adjacent enzymes in geraniol biosynthesis pathway, *GES* and *Abies grandii* geranyl pyrophosphate synthase, and showed that this approach decreased geraniol production, while constitutive expression of *GES* using a strong promoter resulted in a three times increased geraniol production. We used these strains to demonstrate that heterologous geraniol production in *P. tricornutum* did not perturb the native biosynthesis of major sterols and pigments.

Chapter 6 discusses why these findings are important for (1) providing insight as to why CRISPR-Cas9-based editing is still difficult to achieve in microalgae (2) improving *P. tricornutum's* status for heterologous terpenoid production with regard to its metabolic flexibility and capacity for high geraniol accumulation (3) characterising both well-established and new genetic engineering tools, including uncovering putative safe harbour loci for TGI required for developing more complex synthetic biology approaches in *P. tricornutum*.