TILLING: EMS mutagenesis in *Epichloë* endophytes and mutation screening using High Resolution Melting analysis and Next Generation Sequencing

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ABSTRACT

Epichloë are fungal endophytes (family Clavicipitaceae) of pasture grasses of the sub family poöideae. These endophytes live in symbiotic association with their hosts and confer resistance to insect and animal herbivory through the production of bioactive secondary metabolites (alkaloids) that are produced *in planta*. For a number of years endophyte research has been focused at manipulating fungal genes responsible for production of alkaloids which have toxic effects on livestock. However, the techniques used to date involve genetic modification to delete genes responsible for alkaloid production and strict regulations around genetically modified organisms in New Zealand prevent commercialisation of these organisms. Traditional mutagenesis was not practical. To find mutations in secondary metabolite pathways, the mutants had to be inoculated back into plants, which would have been a laborious and time-consuming process. The aim of my research was to develop Targeting Induced Local Lesions In Genomes (TILLING) methodology in *Epichloë* to disrupt fungal secondary metabolite genes using Ethyl methanesulfonate (EMS) and screen for mutations using high throughput screening techniques such as High Resolution Melting (HRM) analysis and whole genome sequencing, MiSeq.

In order to carry out the mutagenesis, uninucleate propagules would be preferred but as most of the filamentous fungi (including *Epichloë*) are multinucleate in nature, spores were thought to be an ideal alternative for mutagenesis. However, many of the commercially used *Epichloë* strains, such as AR1 and AR37, do not readily produce spores. Therefore an alternative mutagenesis system using fungal protoplasts was investigated and employed.

EMS mutagenesis showed that the number of colonies derived from protoplasts after mutagenesis declined steadily at a reproducible rate as measured by time-course of 0, 15, 30, 45 and 60 minutes to give LD50 values. At 60 minutes there was decline in the number of colonies to the levels of 10% of the initial number. To determine the effectiveness of EMS as a mutagen positive selection, using 5-Fluoroorotic acid (5-FOA), was also performed on the mutagenized protoplasts to derive the mutation frequency of 6 mutations per 1000 mutants compared to 0.002 mutations per 1000 for non-mutagenized protoplasts. This suggests a 3000-fold EMS-induced increase in the frequency of mutations.

Having established mutation frequency from the 5-FOA, positive selection and steady decline in number of colonies from EMS mutagenesis, an EMS mutant library was screened using next generation sequencing. . However, high throughput whole genome sequencing (MiSeq) led to the detection of only three verifiable point mutations (1 in 10Mb). Microscopic observations revealed that while individual protoplasts were largely (85%) uninucleate, protoplasts typically formed clumps containing 15-30 protoplasts. In theory, multiple nuclei would lead to an overestimation of the number of mutations since each nucleus would accumulate different SNPs. However, MiSeq sequencing did not detect this, probably due to being filtered out during bioinformatics processing. Thus if methods can be devised for plating single protoplasts, EMS mutagenesis should be applicable to this system. TILLING technology can be used to reduce the time for endophyte discovery and improvement. My research demonstrated that this procedure, although very promising in terms of benefit to fungal improvement, carries certain difficulties with it that we had to address such as mutagenesis using protoplasts and subsequent mutation discovery. I succeeded in establishing TILLING methodology for mutagenesis of *E. festucae* strain Fl1 as well as optimising protocols to screen mutants.

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ABBREVIATIONS

°C	Degree Celsius
bp	Base pairs
DAPI	4'-6'- diamidino-2-phenylindole
dHPLC	Denaturing high performance liquid chromatography
DNA	Deoxyribonucleic acid
dNTPs	Dinucleotide triphosphates
ds	Double stranded
EMS	Ethyl methanesulfonate
ENU	Ethyl nitrosourea
YFP	Yellow fluorescent protein
5-FOA	5-Fluoroorotic acid
HRM	High Resolution Melting
IGV	Integrative Genomics Viewer
ltm	Lolitrem
µL	Microliter
mM	Millimolar
NCBI	National Center for Biotechnology Information
NGS	Next generation sequencing
OM	Osmotic Medium
OMP	Orotidine-5'-monophosphate decarboxylase
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PDB	Potato dextrose broth
perA	Peramine
Q value	Quality value
RG	Regeneration agar
RNA	Ribonucleic acid
SNP	Single nucleotide polymorphisms
TAE	Tris-acetate-EDTA-buffer
Taq polymerase	*Thermus aquaticus* DNA polymerase
TGGE	Temperature gradient gel electrophoresis
TILLING	Targeting Induced Local Lesions In Genomes
T_m	Melting temperature

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