Targeted introduction of point mutations in *Epichloë festucae*

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AgResearch is a world leader in the commercial utilisation of endophytes in forage grasses. These fungal endophytes have a symbiotic relationship with their host grass and and have traditionally been an agricultural double edged sword. On one hand, they increase plant yield, provide resistance to abiotic stress, and produce insecticidal secondary metabolites. However, they also produce toxic mammalian alkaloids that seriously affect the health of the grazing livestock. AgResearch has identified and commercialised endophyte strains (AR1, AR37 and MaxQ/MaxP) that lack the capacity to produce two of the main classes of toxic mammalian alkaloids. In addition to the successful commercial strains, AgResearch has characterised a number of strains with potentially beneficial traits that were not placed into a commercial development pipeline because they would lead to negative effects on livestock (e.g. lolitremB producers). As part of our strategy for developing the next generation of endophytes, we are looking for strains with novel activities against some of the insect pests that are not deterred by the current commercial strains. It is likely that some of these strains will be lolitrem B or ergovaline producers and therefore some means of eliminating these pathways is desirable.

The majority of the genes and enzymes that produce mammalian toxic alkaloids in the endophyte have now been characterised (Young *et al.* 2005; Young *et al.* 2006). We can routinely create mutant strains in these biochemical pathways in the laboratory using gene replacement or gene disruption technology (Young *et al.* 2005; Young *et al.* 2006). Generally these genetically modified mutant strains contain a selectable marker gene such as the hygromycin or neomycin resistance gene. Ideally we would hope to identify a naturally occurring mutant strain, or develop a mutant strain in the laboratory in which the target gene has been mutated and rendered inactive. Such strains would not contain transgenes and would not be considered to be genetically modified. This is a similar principle to the approach of many plant breeders who have used chemical or radiation mutagenesis to create novel traits in plants, many of which have been bred into commercial cultivars. The California based company Cibus LLC has a proprietary technology called Rapid Trait Development System (RTDS™) for selective point mutagenesis of targeted genes. In RTDS™ conversions, one transforms an oligonucleotide that hybridises with the gene of interest but contains a single base mismatch. The cell then precisely converts the corresponding base in the endophyte genome into the desired based. This technique is attractive to agricultural biotechnology because it avoids the untoward changes of random mutagenesis and the regulatory issues of transgenics.

We plan to change a single base in one of the genes responsible for synthesis of mammalian toxic alkaloids in endophyte strains that have otherwise beneficial properties. The single base change will introduce a premature stop codon, which prevents expression of enzymes that produce the toxic alkaloid.

There are fundamental unanswered questions about the cellular mechanism of RTDS™ (Igoucheva *et al.* 2004). It is known that recombination and DNA repair enzymes are necessary, but the details are largely unknown. RTDS™ conversions have been successfully performed in plants such as wheat (Dong *et al.* 2006) and maize (Zhu *et al.* 2000), mammalian cells (Bartlett *et al.* 2000), and bacteria with varying success. In plants, only 0.0002% of protoplasts that take up the oligonucleotide acquire the desired mutation. The rate is between 1% and 25% for *E. coli*. This will be the first use of RTDS™ in a multicellular fungus. We have several strategies for increasing the conversion rate should it be below 0.1%. Various chemical treatments have been shown to increase RTDS™ efficiency by causing DNA damage (Ferrara & Kmiec 2004; Ferrara *et al.* 2004), opening the chromatin (Parekh-Olmedo *et al.* 2003), or slowing down DNA replication (Wu *et al.* 2005). Overexpression of the protein RAD51 has been shown to increase conversion rates by up to 100 fold (Liu *et al.* 2004). Unfortunately the effectiveness of these strategies has not been compared in a single experiment. Furthermore, the synergistic effect of employing more than one of these strategies has not been investigated. We plan to conduct this experiment with the hope of dramatically increasing the efficiency of conversion.

We will initially perform experiments in the *Epicholë festucae* strain FL1 because of its good protoplasting and transformation properties. We have selectable mutations planned in two separate genes. In the first, we will introduce a premature stop codon into the gene for orotidine-5'-monophosphate decarboxylase (pyr4) (Collett *et al.* 1995). Cells carrying the functional enzyme are poisoned by 5-fluoro-orotic acid supplementation in the media. The second mutation we would like to attempt is the correction of a mutated hygromycin resistance gene (hph). We are planning to put a single copy of the hph gene (with a premature stop codon) into the endophyte genome. We will then use RTDS™ to change the stop codon to the native codon and restore hygromycin resistance. These conversions will also be carried out in *E. coli* model systems to verify that the oligonucleotides are functional.

Following the development work, our primary objective is to eliminate mammalian toxic alkaloids from an otherwise commercially viable strain. An important first step is to identify the strains and mutations that will result in the most attractive final product. The initial research will be vital for success in the commercial strain for two reasons. First, it is possible that the conversion rate will be lower in strains that have worse protoplasting and transformation properties than the model strain *E. festucae* Fl1.

Second, mutations in secondary metabolite pathways are not selectable like mutations in genes such as pyr4 or hph. The development on the pyr4 mutation is applicable to the development of a co-conversion strategy in the endophyte. We plan to introduce two different oligonucleotides into the endophyte. One will target the pyr4 gene, and the other will target a gene of interest. Selection of cells with the pyr4 mutation enriches the population for ones that have the nonselectable mutation. This co-conversion strategy has been shown to decrease the number of transformants that must be genotyped (van Brabant *et al.* 2004). We plan to isolate DNA from the transformed cells and use allele-specific PCR to identify cells that have the mutation in the target gene.

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