2021-22 Annual Update / Final Report

Client report summary:

Key:	CONT-47267-CRFRP-AGR C10X1603-CR-6
Project:	Forages with Elevated Photosynthesis and Growth
Contract ID:	C10X1603
Investment process:	CRFRP 2016 Contestable Research Fund - Research Programmes
Organisation:	AGR AgResearch Limited
IMS assigned to:	Alison Slade
Reporting period:	01/07/2021 to 30/06/2022
Contract total value:	$11,500,000.00
Team:	

Annual Update

2021-22 Annual Update

As this is the final report, this annual update will cover a brief history of the basis for this contract and the science that led up to the start. The significance of each of the five key impact areas will be described and then how this research progressed. Finally, the outputs of the research and their significance will be discussed, including seven peer reviewed scientific publications and the major findings as well as areas yet to be published. Importantly this contract has had strong stakeholder support and the significance of this to the contract and to the stakeholders will be summarized.

A Brief Science History.

The genesis for this outstanding science originally came from research to increase the energy density of forages in the early 2000s. The team were developing a fatty acid expression and protection system in the model species Arabidopsis (Winichayakul *et al.,* 2013). They successfully doubled leaf fat levels and a serendipitous discovery opened the door to a wide range of applications in crops.

During analysis of the transgenic Arabidopsis, it was observed that the plants grew 50% faster than control plants. The team identified that these plants had elevated photosynthesis. This was subsequently demonstrated in forage perennial ryegrass. More recently (from research in this contract), it has been discovered that the main mechanism for enhanced photosynthesis is a reduction in the negative feedback based on the carbohydrate status of the plant (Beechy-Gradwell *et al.,* 2020). It should be noted that over the last two decades, enormous investment has been made internationally toward improving this complex process in plants. Before this discovery, while incremental improvements were made, other research efforts were limited by this negative feedback mechanism plants use to regulate photosynthesis.

The discovery of enhanced photosynthesis led to the recognition that this novel technology had applications in multiple crops. Over the last 14 years, the team developed new partnerships, progressed the science in other crops, and contributed to building novel commercialization models. Within a few years, this important technology is expected to benefit farmers and consumers in several countries (soybean in 2026).

During presentations to Dairy NZ, PGG-Wrightsons Seeds, Agriseeds and Grasslanz Technology in 2015 it was recognized that a joint MBIE-Industry funded research programme could progress the development of HME ryegrass to a stage where it became a commercialization programme. There were important fundamental questions on the plant response to nitrogen, its water use, the mechanism for enhanced photosynthesis and the challenges of breeding in PC2 containment to be answered. The need for HME ryegrass field trials and animal nutrition trials to establish the value proposition for New Zealand was recognized. As New Zealand had not adopted genetically modified crops the social license and farmer engagement was another important activity.

Initiation of the Research Programme for Contract C10X1603.

The research was divided into five key areas: Carbon Dioxide Recycling, Nitrogen Utilisation; Nitrogen and Water Use Efficiency; Breeding in Containment; Increasing Farmer Awareness and understanding of HME Forages. The industry co-funding supported some of the five key areas and in addition helped support the USA based field trials. The plan was to progress to an animal nutrition trial in dairy cows but in 2021/2022 the strategy for this changed due to a new understanding that the USA climate was not suitable and the emerging opportunity of the Australian market.

Carbon Dioxide Recycling.

High metabolizable energy (HME) ryegrass plants have increased levels of lipids stored in the green tissues in micro-organelles (Winichayakul *et al.,* 2013; Roberts *et al.,* 2010, 2011; Beechy-Gradwell *et al.,* 2020). These organelles are stable within the leaf and remain during the ensiling process (Winichayakul *et al.,* 2020). The early Arabidopsis research published in 2013 (Winichayakul *et al.,* 2013) speculated that CO_2 recycling led to the enhanced photosynthesis. However, as it has turned out from research in this contract, there is another very novel and important mechanism and this has been one of the most important discoveries from this research.

The allocation of different sources of carbon (sugars and fat) in different tissues is altered, leading to reduced negative feedback of photosynthesis (Beechy-Gradwell *et al.,* 2020; Cooney *et al.,* 2020). This enables the overall plant energy to be increased due to greater fixation of atmospheric CO_2. Increased plant growth rates are also observed, although the rate of increase is affected by competition for light in densely packed sward conditions.

International research on photosynthesis over the last 30 years has focussed on step-by-step incremental improvements of this complex 156 step set of interacting biochemical pathways. Progress was made in various steps but there were two overarching negative feedback mechanisms based on the carbohydrate status of the plant and the plants carbon:nitrogen balance. These negative feedbacks often limited the progress made. The discovery by the AgResearch team from research in this contract identified that one of the negative feedbacks (based on the plant carbohydrate status) was overcome, this has been a major step forwards. While there are conditions where HME ryegrass can have significantly greater growth rates, under competition for light and nutrients this diminishes and the plant allocates the extra photosynthate into greater energy density. The same is seen in soybean with the same technology where occasionally the plants have greater yields, but in general the enhanced photosynthesis leads to increased energy density stored in the seed through increased oil and protein composition.

Nitrogen Utilisation.

The plant nitrogen status was linked to photosynthesis. The nitrogen cycle is an important pathway linked to photosynthesis in that it supplies the reductant needed to drive the cycle. It was important to understand the plant response to different forms of nitrogen. It had been observed that the plant responds differently to reduced nitrogen compared to controls. For forages, this links into the farm nitrogen cycle which includes added and recycled nitrogen.

Research in this area is split into grasses and legumes with grasses including ryegrass and rice (as a model species), and legumes including alfalfa and now soybean. The overall goal of this research has been to understand the nitrogen requirement of different species and their responses to different nitrogen forms, nitrate, ammonia and urea. We made significant progress in ryegrass and this was published in 2018 (Beechy-Gradwell *et al.,* 2018). The key findings were that HME ryegrass utilised all three forms of nitrogen, but the greatest growth responses were to reduced forms of nitrogen (ammonia and urea).

Research on soybean has focussed on nitrogen levels within field grown plants and we have demonstrated that plants have some increased leaf nitrogen but not throughout the early reproductive stages and this appears to be then transferred into the seed which may account for the increased seed protein in some lines. It appears the legume symbiosis is sufficient to obtain competitive yields and addition of nitrogen (anhydrous ammonium) only benefits if soil N levels are low.

Nitrogen and Water Use Efficiency.

The aim of this research area was to determine if HME trait expression in transgenic plants alters plant nitrogen metabolism. This goal is different from the research in described above on nitrate utilization as it is more encompassing and focuses on overall plant nitrogen metabolism. We are also examining water use efficiency and other stress responses such as light and temperature.

We performed controlled environment experiments on HME ryegrass event ODR4501 and looked at its ability to utilize nitrate, ammonium, and urea. HME ryegrass shoot dry weight increased across the entire nitrogen supply range regardless of nitrogen form, whereas the non-GM control ryegrass shoot dry weight did not significantly increase beyond 7.5 mM nitrogen supply. At 10 mM nitrogen supply, HME ryegrass shoot dry weight was 27-34% greater and root dry weight was 25-45% greater than in the non-GM control ryegrass. Total plant percent nitrogen and the shoot to root ratio was lower for plants supplied with nitrate than with ammonium or urea but did not differ between the non-GM control and HME ryegrass. This suggested that HME ryegrass has a similar nitrogen utilisation efficiency and biomass partitioning.

Of particular interest is the legume species alfalfa and soybean. As these species can form symbioses with the nitrogen fixing bacterium Rhizobium, they are provided with a source of nitrogen in the form of ammonium. Research on soybean has focussed on nitrogen levels within field grown plants and some of this is ongoing and being conducted by our partner ZeaKal. This links to the observation mentioned previously that plants have some increased leaf nitrogen throughout the early reproductive stages and this appears to be then transferred into the seed which may account for the increased seed protein in some lines. It suggests that alfalfa and white clover are similarly likely to benefit.

The overall nitrogen utilisation of HME ryegrass is relevant to on farm models currently being used to help establish the value in farming systems. As the program moves into the commercialisation phase from 2022 and beyond, we are repeating our farm and financial models informed from the US based field trial data and recent work on methane mitigation experiments from in vitro methane assays.

Breeding in Containment and the Ryegrass Endophyte.

The novel breeding approach based in containment facilities the team has developed in collaboration with their seed company partners has enabled seed to be developed for 5 years of overseas field trials and has set the programme up for much larger scale planting for animal nutrition trials. The breeding occurs in crossing cages in PC2 containment and takes 9-10 months per cycle. Once sufficient homozygous familied have been developed and characterised it is possible to scale up to an open flowering and pollination system.

Two methods of plant transformation were used with gene gun plants produced prior to the start of this contract and Agrobacterium plants produced during the contract. We found the Gene Gun plants had multiple loci of integration of the transgene gene cassette and these became problematic during the breeding phase as they segregated and we ended up with partial HME phenotypes. The Agrobacterium system produced a favourable frequency of single copy, single locus integrations and we were able to recreate many HME ryegrass plants that performed much better in the breeding phase. To ensure we has intact single copy integrations of the T-DNA we performed whole genome sequence analysis (an innovation that became an option in 2018/2019). This enabled us to map the integrations to the genome assemble and identify genic and intragenic insertions. Not all events were easy to map as the genome is not complete and therefore, we adopted a long-read sequencing approach which is still to be fully completed for some events.

The ryegrass endophyte that lives intercellularly within the plant leaves and stem provides protection from various insect predators of ryegrass. As the transgenic HME ryegrass material was developed out of the plant transformation process it was endophyte free (to prevent contamination in tissue culture). The AR1 and AR37 endophytes were introduced in the breeding process and an important question was how this symbiotic fungus would survive in a HME ryegrass plant. This has been answered in containment and field-based experiments and we have found no difference in transmission from seed generation to seed generation, no difference in fungal biomass and a minor reduction of some of the protective alkaloids, although the levels still remain within the seasonal variation range. This will be submitted for publication in 2022.

The proposed animal nutrition trials require one ha of HME ryegrass and one ha of a null control line. This needs 10 to 20 kg of seed for each treatment to be produced in PC2+ containment. Additionally, the three to four cycles of breeding need to be performed in elite industry ryegrass germplasm. We developed a unique system in the new PC2+ containment glasshouses at the Palmerston North Campus. Using a rapid homozygous breeding protocol that minimises in breeding depression while minimising the generations required, we have developed the first batch of seed and by February 2023 we will have produced the 10-15 kg of seed for use in a trial in Australia. This enables both in and off season seed production. Analysis of seed quality indicated it passes the phytosanitary requirements for shipping to Australia. Protocols were co-developed with an industry advisory group and containment measures supervised by the Ministry of Primary Industries. This is the first time this volume of seed has been produced in containment from a genetically modified wind pollenated grass species and highlights the unique facility and capability that has been developed.

Increasing Farmer Awareness and Understanding of HME Forages.

In collaboration with NZIER, AgResearch conducted a survey on farmer decision making and the key decision timeframes for a GM HME ryegrass. Key findings were:

- Some farmers are interested in the potential benefits of HME ryegrass. They may adopt it within a year or two of its commercial release. However, they want to know more about how it might perform.
- They are likely to want information early, through many channels. The best time to provide information will be after the field trials and before product release. Some farmers want information even earlier. Given the number of information sources that farmers use, and the time frames for decision making, the information should be available in many formats through many channels.
- The use of genetic modification (GM) technology creates additional complexity. Some farmers will not adopt HME ryegrass because it is GM. Other farmers would adopt HME ryegrass but recognise that GM technology is an issue with consumers and in their supply chains.
- HME ryegrass would likely be a minority of total pasture. Among potential adopters, HME ryegrass would likely be one of several cultivars used.

More Recently a Survey of Gatekeepers and New Zealand Agrifood Exports (Kaye-Blake *et al*., 2022) was conducted to understand:

- Whether there is significant gatekeeping behavior in New Zealand export supply chains.
- Where in the supply chains gatekeeping occurs?
- Whether GM technology might be expected to trigger gatekeeping behavior.

Interviews and an online survey were conducted with respondents involved in export supply chains for meat and milk products from New Zealand. The focus was on perceptions of gatekeeping behaviour and the impact of private standards on the ability to sell GM food to overseas consumers. Gatekeeping and private standards are methods by which companies can exert influence on global value chains. The growth in private standards in particular has been advanced as a limitation on producers and their ability to innovate. Although results did suggest that there is gatekeeping in the export supply chains, they provide little support for the idea that either gatekeeping or private standards significantly impede New Zealand's ability to market GM food to overseas consumers. Instead, government regulation and non-GM demand by some consumers were more important factors. It is planned to publish this research in the journal New Biotechnology.

New Zealand Based Nutrition Trial.

There is potential to perform a sheep nutrition trial to assess the methane mitigation effects of HME ryegrass from ensiled HME and Control lines grown in PC2 containment. We focussed on methane reductions as published nutritional studies of ruminants suggest a 5% reduction in methane emissions for every 1% increase in dietary fat. We aim to have experimental resolution to measure a 10% reduction in methane emissions.

Previous research on the stability of the fatty acids stored in the leaf micro-organelles (MSc Thesis Beechy-Gradwell, 2015) and more recently in the publication by Winichayakul *et al.,* (2020) looking at in vitro methane assays using ensiled ryegrass, suggested ensiling was a viable storage method.

We developed a novel system to grow and ensile ryegrass in batches and store the material for up to 18 months until we have approximately 1000 kg of dry matter needed for a trial. AgResearch funded the project that will be conducted over the next 18-24 months. This system enables us to generate enough dry matter for both HME and control ryegrass to conduct the trial in sheep at the AgResearch Palmerston North Campus. This means the first animal nutrition study of this technology will be complete by the end of 2024.

USA Field Trials.

AgResearch was able to partner with the University of Missouri in a five year collaborative program to conduct field trial based assessment of HME ryegrass in regulated field trials. The robust but user friendly regulatory system in the USA enabled analysis of genetically modified ryegrass with two restrictions, no seed planted in the field and no plant reproduction. The first two years involved assessing the environmental conditions and developing protocols for transplantation of glasshouse germinated seedlings, comparing space plants *vs*. sward growth conditions and prevention of reproduction. We initially assessed gene gun derived HME ryegrass which turned out to have problematic multi-loci integrations of the HME gene cassette that segregated during the breeding. Therefore in 2019-2021 we used the superior Agrobacterium derived HME ryegrass. The recent publication from the team (Beechy-Gradwell *et al.,* 2022) shows the primary benefits for HME ryegrass (elevated leaf fats and gross energy) reliably translate from the laboratory to the field, as demonstrated across two field seasons and under realistic sward-like growing conditions.

Across the 2019 and 2020 field seasons, HME ryegrass displayed a 25–34% increase in FA delivering up to 0.5 MJ kg^{-1} DW higher gross energy and no penalty to biomass production. If successfully converted to ME, this energy gain is 250% greater than the total ME gain achieved over the last 4 decades by traditional genetic selection. Consequently, these increases are predicted to deliver 30 kg MS cow^{-1} season^{-1} (based on FARMAX modelling) and 10% less methane for the New Zealand farmer.

The translation of the secondary benefits for HME technology, elevated photosynthesis, and growth from the laboratory to the field were also examined in this paper. The team showed that HME ryegrass could deliver 18% greater carbon assimilation and up to 13% higher growth, but only in spaced pots, when light competition was low. When grown in dense swards, in either the laboratory or the field, HME ryegrass displayed no increase in photosynthesis or growth rate. Consequently, this paper provides a comprehensive assessment of the reliability of HME technology's primary and secondary benefits, what traits translate to the field (increased fatty acids and gross energy), and which do not (increased growth and photosynthesis).

The final 2021 HME ryegrass field trial in the USA analysed the performance of homozygous families and the assessment of primary and secondary traits. Building on the 2019 and 2020 trials that utilised hemizygous families we were able to demonstrate that gross energy and plant fatty acid composition translated from lab to field. In this line we observed a yield penalty over the season however it is unclear if it was a feature of this line or the propagation conditions. Our initial plan had been to perform animal nutrition studies in the USA, however the environmental risk of the hot summers led to a new strategy.

Row Crop Science Innovation.

In addition to the breakthroughs in forages, significant innovation in soybeans has led to major benefits.

Translating from a model species and grass into a seed-producing legume required new ways to control gene expression. The timing of gene expression has been critical, as the goal is to provide the plant with sufficient photosynthate for efficient seed development. This has been achieved with increased seed oil and protein composition and an overall increase in both co-products per acre over multiple seasons and sites across the USA. More recently, the ZeaKal team have shown that PhotoSeed™ plants can outperform their controls in non-irrigated settings. About

75% of U.S. soybeans are propagated in non-irrigated conditions. A recent innovation is the use of genes and regulatory elements derived solely from soybean which will assist the deregulation process.

The ZeaKal team has also branched into hemp and developed hemp plants with increased leaf oil. This potentially adds a new application to this crop, one that has lacked the plant breeding developments of other crops due to prohibition. The team has also branched into corn with the potential for greater oil yields per acre.

Intellectual Property.

A critical component for raising investment in biotech is a robust and investible patent portfolio. Since 2009, the team has developed a portfolio of 13 patents around increasing plant oils, protection of plant oils, enhancing photosynthesis, modifying plant architecture and reducing nitrous oxide emissions from crops. These are granted in multiple (92) jurisdictions. Part of the strategy has been extending the life of the patent portfolio by filing more recent methods patents and new applications. To date, this portfolio has led to the raising of nearly $100M of government, industry, and venture capital investment. There is potential that HME ryegrass may provide farmers with a tool for both methane and nitrous oxide reductions and this could be utilised alongside other mitigation measures to make significant reductions to Australasia's greenhouse gas inventory. The new patent filing extends the protection period for this technology well beyond the 2029 expiry date for the cysteine oleosin patent.

Social and Environmental Benefits.

HME ryegrass represents a unique opportunity to improve both the productivity and environmental impact of pastoral farming. Exemplification of the stable translation in the USA field trials of HME was a major step towards commercialising this technology and delivering these benefits to farmers both domestically and abroad.

Improving the nutritional composition of forages through the accumulation of fatty acids in the grazed portion of the plant provides significant potential productivity and environmental benefits. A forage with increased metabolizable energy provides farmers the opportunity to maintain productivity using less land and potentially fewer animals. There also potential benefits of reduced methane emissions, supported by evidence from several animal nutritional studies and from in vitro methane assays. The goal is to achieve a minimum of a 10% reduction in methane emissions.

The improved nutritive quality is expected to reduce nitrogen excretion in ruminants leading to reduced nitrate leaching and nitrous oxide emissions. Recently, evidence from controlled environment experiments suggests another significant reduction in nitrous oxide emissions can be directly derived by the altered plant metabolism and morphology. This can drive up farm profitability and provide farmers with additional tools to manage environmental impacts from pastoral grazing systems. Experiments in controlled environment chambers on HME ryegrass and control mesocosms designed to measure nitrous oxide emissions from plants treated with bovine urine have indicated a novel mitigation potential. Two HME ryegrass events were tested in different industry cultivars. In one case a significant 50% reduction in nitrous oxide emissions was observed over the course of the experiment in cultivar Impact and in the second more modern line there was a clear trend for reduction. We anticipate that in most modern cultivars the reduction would be lower than the 50% seen in CV Impact. This would need to be verified in field conditions and may comprise part of the Australian science plan.

Future Commercialisation Options Beyond the Contract Completion.

We are now developing a commercialisation and science plan to progress in Australia in 2023 and are about to seek regulatory approval from the Office of the Gene Technology Regulator. The Australian market is a good opportunity as there is already GM crop production and there is a significant ryegrass market for both the dairy, sheep and beef industries. Australia can become a market leader to inform New Zealand on the benefits of HME ryegrass in pastoral agriculture.

Current activities include developing the costed draft science plan, identifying the ideal business model from four possible options, bringing in new partners and fund raising.

References.

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Publicly Available Information.

High Metabolisable Energy (HME) Ryegrass is being developed as an option for farmers in New Zealand, and temperate climates overseas. HME has the potential to increase farm productivity while reducing livestock's environmental impacts, for example nitrogen leaching and methane emissions. Many of the environmental impacts occur because the proportion of protein in NZ's forage plants is far in excess of the energy available to livestock. This means that there is an excess of nitrogen from plant proteins, which is excreted in urine and subsequently lost from the farm through nitrate leaching. In addition, the greenhouse gas methane is produced by methanogenic microbes in the rumen (stomach) of livestock as they digest the forage.

To improve the nutritional balance and increase the overall amount of energy available to livestock on each hectare of pasture the Team has produced genetically modified (GM) plants that has specialised microscopic oil micro-organelles in the leaves of ryegrass. This extra oil, while only being a small proportion (2 - 3.3%) of the plant's dry matter, delivers up to a 10% increase in the amount of energy available to an animal eating the plant. This means that an animal can eat less grass to obtain the energy it needs, then along with that - lower intake means less excess protein/nitrogen in the urine.

This project was started almost 20 years ago and since then the amount of methane produced by NZ's livestock has come under national and international scrutiny due to its impact on increasing global warming. However, along with reducing nitrogen losses, HME Ryegrass also has the potential to reduce methane production. Studies have shown that livestock diets with higher amounts of oils in their diets produce lower amounts of methane. By matching the level of oil in our plants against the results from those other studies, it appears that HME Ryegrass has the potential to reduce methane emissions by 10 - 17%. This may not be the 'silver bullet' but when combined with other products in development it can become part of the bigger solution to global warming and climate change.

Serendipitously we also found that under good growing conditions HME Ryegrass has enhanced photosynthesis and growth. All plants have exquisite control mechanisms that allow them to effectively 'snack' on light as needed, where one of these control mechanisms is the rate of carbohydrate/sugar formation going on in the plant. By using the carbon-based molecules, typically used to produce carbohydrates, to produce additional oils instead, it appears that the plant overcompensates, capturing over 20% more carbon dioxide to and converting it into more plant biomass and energy.

While it was possible to apply to undertake field trials with the GM HME Ryegrass in NZ, it was decided that the programme would be able to generate the information it required within a shorter timeframe by conducting trials in the mid-west of the USA. In 2020 we demonstrated that key characteristics of heterozygotes (one copy of the HME Ryegrass transgenes) were similar when measured in PC2 containment growth chambers and glasshouses or the field. In 2021 we demonstrated that homozygotes (two copies of the HME ryegrass transgenes) and again shown that the even greater increase in oil in homozygous plants (compared to heterozygous plants) translated from lab to field for gross energy and total fatty acid content.

Results of the research has been published in seven peer reviewed scientific journal articles between 2018 and 2022.

The program is now focussing on commercialisation opportunities in Australia.

Key Achievements.

1. Scaled-Up Breeding and Seed Production of GM grasses in PC2+ Containment.

Conducting a meaningful nutrition study focussed on methane emissions from large ruminants consuming genetically modified High Metabolizable Energy (HME) ryegrass requires one ha of HME ryegrass and one ha of a null control line. This needs 10 to 20 kg of seed for each treatment to be produced in PC2+ containment. Additionally, the three to four cycles of breeding need to be performed in elite industry ryegrass germplasm. We developed a unique system in the new PC2+ containment glasshouses at the Palmerston North Campus. Using a rapid homozygous breeding protocol that minimises in breeding depression and the generations required, we are close to achieving 10-15 kg of seed needed for a trial in Australia. This enables both in and off season seed production. Analysis of seed quality indicated it passes the phytosanitary requirements for shipping to Australia.

Protocols were co-developed with an industry advisory group and containment measures supervised by the Ministry of Primary Industries. This is the first time this volume of seed has been produced in containment from a genetically modified wind pollenated grass species and highlights the unique facility and capability that has been developed.

2. Completion of USA HME Ryegrass Trials and Commercialisation Strategy for Australia.

The final 2021 HME ryegrass field trial in the USA analysed the performance of homozygous families and the assessment of primary and secondary traits. Building on the 2019 and 2020 trials that utilised hemizygous families we were able to demonstrate that gross energy and plant fatty acid composition translated from lab to field. In this line we observed a yield penalty over the season however it is unclear if it was a feature of this line or the propagation conditions. Our initial plan had been to perform animal nutrition studies in the USA, however the environmental risk of the hot summers led to a new strategy.

We are now developing a commercialisation and science plan to progress in Australia in 2023 and are about to seek regulatory approval from the Office of the Gene Technology Regulator. The Australian market is a good opportunity as there is already GM crop production and there is a significant ryegrass market for both the dairy and sheep & beef industries. Australia can become a market leader to inform New Zealand on the benefits of HME ryegrass in pastoral agriculture.

3. Identification and Patenting of a Novel Approach to Reduce Agricultural Nitrous Oxide Emissions.

Experiments in controlled environment chambers on HME ryegrass and control mesocosms designed to measure nitrous oxide emissions from plants treated with bovine urine have indicated a novel mitigation potential. Two HME ryegrass events were tested in different industry cultivars. In one case a significant 50% reduction in nitrous oxide emissions was observed over the course of the experiment and in the second line there was a clear trend for reduction. This would need to be verified in field conditions and may comprise part of the Australian science plan. We filed a provisional patent in 2022 on the use of this technology for reducing nitrous oxide emissions in farming systems. We have successfully obtained funding for a PhD studentship to further elaborate the mechanisms for nitrous oxide reductions. There is potential that HME ryegrass may provide farmers with a tool for both methane and nitrous oxide reductions and this could be utilised alongside other mitigation measures to make significant reductions to Australasia's greenhouse gas inventory. The new patent filing extends the protection period for this technology well beyond the 2029 expiry date for the cysteine oleosin patent.

4. Development of Forage Ensiling System in PC2 Containment and its Use in Animal Trials.

There is potential to perform a sheep nutrition trial to assess the methane mitigation effects of HME ryegrass from ensiled HME and Control lines grown in PC2 containment. We focussed on methane reductions as published nutritional studies of ruminants suggest a 5% reduction in methane emissions for every 1% increase in dietary fat. We aim to have experimental resolution to measure a 10% reduction in methane emissions.

We developed a novel system to grow and ensile ryegrass in batches and store the material for up to 18 months until we have the 1000 kg of dry matter needed for a trial. AgResearch funded the project that will be conducted over the next 18-24 months. This system enables us to generate enough dry matter for both HME and control ryegrass to conduct the trial in sheep at the AgResearch Palmerston North Campus. This means the first animal nutrition study of this technology will be complete by the end of 2024.

5. New Knowledge on Plant DGAT1 Enzymes Patented, Published and In Commercialisation.

The enzyme diacylglycerol O-acyltransferase 1 (DGAT1) is ubiquitous in all eukaryotic organisms and has been well studied in animals where there is a clear model for its function, structure and topology as a membrane bound enzyme. This contract has supported research investigating the function of both monocotyledonous and dicotyledonous plant DGAT1s and the team made a breakthrough understanding the topology of plant DGATs and their function. This has added significantly to the understanding of plant DGAT1s which has previously been thought to have different topology to animal DGAT1s. DGAT1 is a fundamental component of the HME technology in ryegrass and it also has value in increasing oil seed composition. The team has recently filed a new provisional patent on a novel mechanism and uses of plant DGAT1s and published this in Frontiers in Plant Science. The IP developed in a family of four patents is currently in commercialisation in the USA by AgResearch's biotechnology start-up partner ZeaKal Inc. One version may be in the US market in soybean in 2026 once regulatory approval has been obtained.