

Breaking through the feed barrier: options for improving forage genetics

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Abstract. Pasture based on perennial ryegrass (*Lolium perenne* L.) and white clover (*Trifolium repens* L.) is the foundation for production and profit in the Australasian pastoral sectors. The improvement of these species offers direct opportunities to enhance sector performance, provided there is good alignment with industry priorities as quantified by means such as the forage value index. However, the rate of forage genetic improvement must increase to sustain industry competitiveness. New forage technologies and breeding strategies that can complement and enhance traditional approaches are required to achieve this. We highlight current and future research in plant breeding, including genomic and gene technology approaches to improve rate of genetic gain. Genomic diversity is the basis of breeding and improvement. Recent advances in the range and focus of introgression from wild *Trifolium* species have created additional specific options to improve production and resource-use-efficiency traits. Symbiont genetic resources, especially advances in grass fungal endophytes, make a critical contribution to forage, supporting pastoral productivity, with benefits to both pastures and animals in some dairy regions. Genomic selection, now widely used in animal breeding, offers an opportunity to lift the rate of genetic gain in forages as well. Accuracy and relevance of trait data are paramount, it is essential that genomic breeding approaches be linked with robust field evaluation strategies including advanced phenotyping technologies. This requires excellent data management and integration with decision-support systems to deliver improved effectiveness from forage breeding. Novel traits being developed through genetic modification include increased energy content and potential increased biomass in ryegrass, and expression of condensed tannins in forage legumes. These examples from the wider set of research emphasise forage adaptation, yield and energy content, while covering the spectrum from exotic germplasm and symbionts through to advanced breeding strategies and gene technologies. To ensure that these opportunities are realised on farm, continuity of industry-relevant delivery of forage-improvement research is essential, as is sustained research input from the supporting pasture and plant sciences.

Additional keywords: endophyte, genetic modification, genomic selection, inter-specific hybridisation.

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Introduction

Industry competitiveness and farm productivity are strongly influenced by genetics. The rate of genetic improvement is a key performance indicator for breeding and underpinning sciences. Historically, rates of genetic gain in forage species have been low relative to other agricultural plant and animal species (Woodfield 1999; Brummer and Casler 2014). Challenges in achieving and sustaining specific trait improvements in forage species have been noted (Parsons *et al.* 2011). Furthermore, grass genetic gain has been evaluated in small-plot monocultures, despite their ultimate application in mixed species swards, and therefore published rates may not accurately reflect performance gain realised in pastures. Research to assess impact of genetic improvement of forage species at the farmlet scale in New Zealand have had mixed results (Crush *et al.* 2006). Given the importance of forage in farm systems, substantial research effort is focussed

on developing better breeding strategies to lift the rate of genetic gain in pasture species, and to deliver step changes in key traits. These are long-term research projects, guided by emerging industry-led forage trait prioritisation and cultivar benchmarking (Chapman *et al.* 2012).

Improved productivity from forage offers ongoing opportunities to pasture-based dairying, providing options to lift production efficiency as measured through economic and environmental metrics. Several factors contribute to the urgency to improve the genetic potential of forage. The established rate of gain in the genetic potential of dairy animals is in itself a driver for improved performance from forage, so as to meet these animals' genetic potential. There is also the need to cater to intensively managed systems with higher stocking rates, and for greater cost efficiency through increased plant nutrient-use efficiency and improved seasonal growth. While supplementary feeds offer flexibility, they carry economic, biosecurity and

market-availability risks, and cannot provide the major feed source in cost-competitive pasture-fed dairy systems.

Perennial ryegrass (*Lolium perenne* L.) and white clover (*Trifolium repens* L.) are the cornerstone species supporting pasture-based dairy systems in New Zealand and Australia. While perennial ryegrass provides most of the feed base, white clover is a valuable pasture component that enhances animal intake and performance, contributes to seasonal feed supply, fixes atmospheric nitrogen and improves soil health (Jahufer *et al.* 2012). In addressing the need for improved performance from pasture species, the forage value index initiative (Chapman *et al.* 2012) is a critical factor in defining and prioritising breeding targets, and in benchmarking cultivar performance in economic terms using trials that reflect on-farm conditions. While there are notable recent breakthroughs in improvement of allied forage species such as red clover (Ford and Barrett 2011), our objective is to identify research opportunities in these two species that will improve their value to the Australasian dairy industry. To that end, we highlight progress and opportunities in the following four distinct research areas: harnessing forage genetic resources through wide hybridisation and pre-breeding, advances in plant fungal-endophyte research, genomic selection in forage breeding, and progress towards step changes in forage traits through genetic modification.

Utilising genetic resources

Plant genetic diversity is a fundamental driver of genetic improvement. Both ryegrass and white clover have widespread genetic resources *in situ*, radiating from their Old World centres of diversity (Abberton and Thomas 2011). There are also extensive curated *ex situ* seed collections, including the Margot Forde Forage Germplasm Centre in New Zealand. Over 15 000 accessions have been added to the Centre over the past 3 years from ongoing collection trips to centres of diversity, and from research population development. White clover, a stoloniferous perennial legume, and related *Trifolium* species provide an excellent example of how knowledge and utilisation of wider genetic resources can contribute to agriculture in Australasia. Although our pastures are dominated by grass species, the contribution of white clover to feed quality and nitrogen fixation, as well as to animal intake and productivity, means that it continues to offer value as a key component of grass-based grazing systems. White clover is genetically variable, but this variation is insufficient to derive high-yielding cultivars adapted to intermittent moisture stress or soil phosphate deficiencies. For example, there is variation for drought tolerance within white clover, possibly influenced by specific metabolites (Ballizany *et al.* 2012a, 2012b), but this variation is limited. Rather than selecting within only the white clover genome for these traits, an alternative approach is to introduce new diversity from close but predominantly undomesticated wild relatives, using the proven technique of interspecific hybridisation.

Relationships among species contributing to this wider gene pool were identified as part of a molecular-based phylogeny of the genus *Trifolium* (Ellison *et al.* 2006). In addition to direct crosses with white clover, other hybrid combinations between these related species provide a valuable source of new forage germplasm. They can also be used as genetic bridges through

which new traits can be introduced into white clover (Williams *et al.* 2011). Re-introducing genomes that have long been separated, and subsequent genetic recombination in these interspecific hybrids, are both options to increase the potential for useful variation. Transgressive segregation is a common feature of wide crosses, indicating relevant agronomic trait values outside the parental range are possible.

Species closely related to white clover possess a range of desirable characteristics, including drought tolerance, salt tolerance, tolerance to viruses and nematodes, prolific flowering, increased seed production, deep root systems and rhizomes (Williams *et al.* 2012; Nichols *et al.* 2014c). Through introgression of these traits, some of the limitations to white clover agronomic performance may be overcome. For example, growth and persistence of white clover is severely constrained by soil moisture deficit. Backcross hybrids between white clover and *T. uniflorum* are markedly less affected by drought stress than are white clover cultivars (Nichols *et al.* 2014b). Under controlled field conditions, total shoot dry weight under water stress decreased ~20% less in first-generation backcross hybrids than in white clover. Traits likely to contribute to this drought tolerance have been identified, including stolon morphological characteristics such as leaf size and internode length, senescence, root biomass and diameter, and production of protective biochemical compounds. Similar responses have been observed in hybrids between white clover and the progenitor species *T. occidentale*. In backcrossed hybrids introgressing the *T. occidentale* parent into a white clover background, shoot dry weight decreased 15% less under moisture stress than in white clover, and root biomass increased by 70% (Hussain and Williams 2013).

A similar opportunity related to nutrient-use efficiency is also being investigated. Under glasshouse conditions, some *T. repens* × *T. uniflorum* first-generation backcross hybrids had greater growth than did white clover under low external phosphate supplies in soil and sand culture, including low to intermediate soil Olsen phosphorus (P) levels (Nichols *et al.* 2014a, 2014d). For example, one hybrid family had shoot dry weights that were, on average, 17% higher than for white clover at Olsen soil P levels of 9–20. This may be due in part to differences in internal phosphate-use efficiency. Root characteristics associated with increased P acquisition, such as a highly flexible root : shoot ratio and increased root branching, have also been observed (Nichols *et al.* 2014a). According to current knowledge, hybrids created with close relatives of white clover nodulate freely with *Rhizobium* strains effective on white clover. Nichols *et al.* (2014a) found evidence that nitrogen fixation did not differ between *T. repens* × *T. uniflorum* hybrids and white clover in the field. Even so, ongoing monitoring of nitrogen-fixation capacity would be prudent with this novel germplasm. Further screening for rhizobia strains compatible with specific hybrid combinations for agronomic potential may also be valuable.

Legume germplasm with improved drought resistance and tolerance of low soil P may have greater resilience during drought events, and lower P-fertiliser input requirements. This could have environmental and economic benefits associated with improved productivity per unit of irrigation and fertiliser. Improvements in white clover productivity and persistence

under moisture- and P-limiting conditions would also maintain the legume component of the sward over the longer term, and could increase clover content where it is currently limited by the environment or input requirements. This would have positive impacts on the direct and indirect contributions of white clover to pasture productivity and animal performance.

Interspecific hybrid combinations are expected to deliver value to the pastoral sector through the development of white clover cultivars with improved performance for one or more key traits. Further elucidation of the genetic and physiological basis of these traits may be aided by the use of genomics tools such as linkage maps (Griffiths *et al.* 2013) and reference genome information to guide the introgression effort. This approach will aid selection against undesirable traits present in some of these wild species.

Building on progress in legume breeding via wide hybridisation, there are parallel opportunities to access variation for traits related to adaptation and yield in pasture grass species, where inter-specific hybrids can be made. The *Festuca-Lolium* complex and their hybrids (King *et al.* 2013) are the most widely studied system. Amphiploids combining all traits, or targeted trait introgression via backcrossing offer flexibility in the ways these grasses are combined and manipulated to improve traits in breeding populations (Thomas *et al.* 2003). With the potential to expand adaptation, improve resource-use efficiency, enhance legume compatibility, and improve adaptation to climate change, these hybrids warrant increased research activity in Australasia, given the dominant role of grass in the dairy pasture feed base.

In addition to the substantial presence in temperate regions, dairy production from pasture in subtropical climates is a component of the Australasian industry. Dairying in these northern regions of New Zealand and Australia, where pasture can be dominated by C₄ grass species such as kikuyu (*Pennisetum clandestinum*) would benefit from genetic improvement of tropical and subtropical grass species (Crush and Rowarth 2007; García *et al.* 2014). A farmer-led action group has investigated means to improve production on kikuyu-based pastures in the Northland region of New Zealand since 1999, demonstrating the importance of the species in such regions and local interest in regionally tailored forage options. Improvement of tropical forage can have a substantial impact, as demonstrated by the economic analysis of development of *Brachiaria* cultivars with drought and pest tolerance via inter-specific hybridisation and selection (Rivas and Holmann 2005; Miles *et al.* 2006). This emphasises the high value and long-term nature of research to deliver traits from inter-specific hybrids, and, additionally, suggests that there may be value in pre-breeding research to support improvement of subtropical forage species for Australasia.

Grass fungal endophyte

One of the key microbial interactions in pastures of Australia, New Zealand and North America involves a fungal symbiont of cool-season grasses, the Calvepitalean fungi *Epichloë*. The *Epichloë* forms endo-symbioses with grasses in the subfamily Pooideae, including important forage species such as perennial ryegrass, tall fescue (*Festuca arundinacea*) and meadow fescue

(*F. pratensis*). It plays a key role in pasture, primarily through the production of secondary metabolites, including alkaloids that protect the host plant from biotic and abiotic stress (Johnson *et al.* 2013; Scharld *et al.* 2013).

The wild-type endophyte *Epichloë festucae* var. *lolii* (= *Neotyphodium lolii* = *Acremonium loliae*) found in ryegrass, also known as standard endophyte (SE), produce lolitrem B, ergovaline and peramine. Peramine has effects on the pasture pest Argentine stem weevil (*Listronotus bonariensis*), with no concomitant animal toxicity (Popay *et al.* 1990; Rowan *et al.* 1990; Pownall *et al.* 1995). Ergovaline has effects on African black beetle (*Heteronychus arator*) and increases vasoconstriction in ruminants (Klotz *et al.* 2007). Lolitrem B reduces growth of Argentine stem weevil larvae (Prestidge and Gallagher 1988) and is the causal agent of ryegrass staggers in ruminants (Fletcher and Harvey 1981), while epoxy-janthitrems, produced by certain endophyte strains, have broad anti-insect effects, with the possibility of a weak ryegrass staggers effect (Fletcher 2005; Fletcher and Sutherland 2009; Popay and Thom 2009). Studies have shown that both lolitrem B and epoxy-janthitrems can be detected in milk from cows grazing endophyte-infected grass. Studies of these compounds in milk indicate that they do not accumulate to biologically meaningful levels, and are unlikely to pose a threat to human health (Finch *et al.* 2007, 2013).

In both Australasia and the Americas, the issue of endophyte toxicities has been addressed by identifying non-toxic and low-toxicity endophyte strains, and co-selecting them within improved, locally adapted temperate grass populations. These strains have been isolated from grasses derived primarily from Europe, where a diversity of chemo-types occur (Tapper and Latch 1999). Strains have been isolated from this primary germplasm, cultured in the laboratory, then inoculated into elite pasture germplasm (Simpson *et al.* 2012; Johnson *et al.* 2013). The development of selected endophytes for use in grass-based forage systems have made substantive contributions to the pastoral sector; the non-toxic strain AR1 and subsequent strains constitute the single biggest development in the improvement of perennial ryegrass pastures in the past 50 years (Williams *et al.* 2007).

The interaction of the fungus and the host grass determines functional characteristics of the symbiotum, including drought-stress tolerance, plant growth and seed yield. These effects are affected by the specific environment of the symbiotum (Hesse *et al.* 2003, 2004). The biological system is complex, representing a genotype (fungus) × genotype (grass) × environment interaction with trophic effects via biotic interactions with ruminants, invertebrates and microbes (Müller and Krauss 2005). Sown pastures will reflect this biological complexity due to the diversity of host genotypes, while persistence of specific symbiota is mediated through both biotic and abiotic conditions. Thus, there is potential for ongoing breeding and selection for symbiota better adapted to agricultural systems (Easton 2007).

Studies of the effects of endophyte in dairying systems have been conducted in south-eastern Australia. Ryegrass staggers were observed on 33–43% of dairy farms surveyed in south-western Victoria (Reed *et al.* 2004). In South Australia, 4–12% reductions in milk volume were observed when animals were

rotationally grazed on SE-infected irrigated perennial ryegrass pastures, as compared with production from a low-endophyte pasture (Valentine *et al.* 1993). In coastal New South Wales, a case study examined the effects of ergovaline ingestion on the health and productivity of lactating dairy cows. This showed a loss of body condition, a decrease in milk production and an increase in somatic cell counts when consuming ryegrass silage with high ergovaline concentrations, compared with ryegrass pasture with total or partially mixed rations (Lean 2001). Work by Moate *et al.* (2012) in south-eastern Victoria compared milk production from systems using perennial ryegrass infected with SE, AR1 and AR37 endophyte. In this trial, both AR1- and AR37-infected pasture had no effect on milk production compared with SE-infected pasture, and generally did not cause ryegrass staggers, while SE-infected pastures caused staggers in one of the years of the trial (Moate *et al.* 2012). However, this trial included a high level of supplementary feeding, which substantially diluted endophyte toxins in times of peak alkaloid concentration. Consequently, the results cannot be compared directly to studies involving pasture-only diets. These studies suggest that effects can vary among years and seasons and with levels of supplementary feed. However, research findings, in general, support the consistent message that it benefits the pasture to have an endophyte, and that selected, non-toxic strains have benefits for the grazing animal.

For *Epichloë* endophytes to be deployed in farming systems, strains with desirable chemical profiles must first be identified from natural grass populations and the strain must then be inoculated into adapted grass germplasm for evaluation. This, in conjunction with an established method for infecting new populations, has allowed the production of grass cultivars with no or low mammalian toxicity while retaining invertebrate antibiosis properties. Current research into the genetic diversity of endophyte strains and their characteristics in novel associations supports this effort to identify useful endophytes for deployment in agriculture (Ekanayake *et al.* 2012, 2013; Tian *et al.* 2013a, 2013b). Possibilities for the future include utilising genetic technologies that knock out metabolite genes and eliminate toxins and/or accumulate desirable early pathway metabolites. Alternatively, new strains can be developed by protoplast fusion of selected strains, with a view to combining, in one strain, metabolites that do not otherwise occur together or, for example, to producing lolines in perennial ryegrass *Epichloë* strains. Currently, efforts are being made to synthetically produce symbioses between *Epichloë* and cereal grasses such as rye (*Secale cereale*) (Simpson *et al.* 2014). These will have application in forage systems, in addition to possibilities for conferring insect-pest protection and enhanced adaptation to crops grown for human consumption.

In aggregate, research findings demonstrate the importance of the *Epichloë*-grass symbiosis in pastoral agriculture and, specifically, in Australasian dairy production systems. The impact of these symbioses on dairy production is generally positive, but varies depending on the environment, pasture cultivar, endophyte strain and the management system of the production unit. The strategy of discovery and deployment of novel strains that produce targeted metabolites offers a solution to the issue of pasture-grass protection from predation by insect

pests. From a dairy production point of view, there are benefits in production and profitability to be harnessed by deploying these selected endophyte strains, and evidence suggests human consumption of the products of animals ingesting *Epichloë* metabolites presents no risk to either market acceptance or human health.

Genomic selection

Conventional approaches to forage plant breeding using phenotypic or genotypic selection are limited in their capacity to improve a range of low- and moderate-heritability quantitative traits, including nutritive value and yield, primarily due to cost and logistics constraints. Molecular markers are effective in identifying genome regions influencing trait variation in complex populations (Maureira-Butler *et al.* 2007; Barrett *et al.* 2008; Faville *et al.* 2012). However, factors including low throughput of some marker technologies, the need to re-estimate marker-trait associations in each target breeding population, lack of monogenic traits of economic relevance, the limited proportion of genetic variance captured by single or few markers for complex traits, and small market size have limited the use of markers by forage breeders in Australasia. Genomic selection (GS) may offer a viable alternative. In general, GS enables a plant breeder to use a comprehensive DNA fingerprint to assess the genetic potential of an otherwise untested individual, and thereby make a genomic prediction for use in selection. This creates options to shorten the breeding cycle and/or improve accuracy of selection, the potential to improve low-heritability traits, and increase the rate of genetic gain in forage species (Heffner *et al.* 2010; Resende *et al.* 2014). It may also address some previously intractable challenges, such as grass-legume co-selection, which are currently heavily constrained by resource and logistic limitations.

Emerging theoretical (Heffner *et al.* 2010; Hayes *et al.* 2013; Resende *et al.* 2014) and empirical (Poland *et al.* 2012; Pryce *et al.* 2014) evidence indicates that GS can provide a substantial increase in rate of genetic gain versus conventional, phenotypic selection, and can be integrated with existing plant breeding systems. The GS theoretical framework developed over a decade ago (Meuwissen *et al.* 2001) is now enabled by efficiency gains in DNA marker (Davey *et al.* 2011; Elshire *et al.* 2011; Poland and Rife 2012) and, more recently, plant phenotypic (White and Conley 2013) data generation, management and analysis; and offers proven value in economic plant species (Massman *et al.* 2013; Spindel *et al.* 2015).

The emergence of high-capacity-marker platforms with improved flexibility and lower costs, such as genotyping by sequencing (GBS; Elshire *et al.* 2011), further improves the efficiency of genotyping. This allows breeders to develop GS models that deliver genomic estimated breeding values for selection candidates, which better access and harness the many genes of small effect throughout the genome. This approach is particularly attractive for complex forage traits such as yield, nutritive value and persistence that are all influenced by large numbers of loci, and are subject to environment and symbiont interactions. Genomic selection may be augmented by candidate gene-marker haplotypes, where the marker effect is verified for

stability across environmental and genetic backgrounds, and of proven value to the predictive model.

In evaluating an advanced breeding strategy such as GS, it is important to understand the context and ensure the fundamentals of forage plant improvement are attended to. These include breeding program logistics, accurate phenotypes, understanding the species and patterns of diversity, genotype \times environment interactions, and trait prioritisation. Currently, some breeding programs are resource limited to the point that theoretically optimal conventional breeding strategies that are expected to deliver enhanced genetic gain, such as recurrent selection among and within families (Casler and Brummer 2008), are rarely implemented in a comprehensive way. Fortunately, GS addresses this limitation, for example, by providing a cost-efficient means for accessing within-family variation, as well as providing a method for implementing selection for long-term traits such as plant persistence within an annual selection cycle (Resende *et al.* 2014). The definition of trait targets and assigning economic weights for use in a standardised evaluation system is a recent initiative that will help define targets for advance breeding methodologies, in addition to providing unbiased and in-depth information to farmers regarding current cultivar performance (Chapman *et al.* 2012).

The cost of linking plant phenotypes to genotypes has been markedly affected by reduction in the cost of high-density DNA-marker genotyping. However, plant phenotyping technology has advanced comparatively slowly over the past three decades (White *et al.* 2012). This is the major bottleneck for plant-improvement strategies, and is, consequently, a major research focus. In recent years, there has been a burgeoning array of in-field sensor options and deployment platforms (e.g. hand-held or vehicle-mounted) proposed, tested or actively deployed for major crop species (White *et al.* 2012; Andrade-Sanchez *et al.* 2014; Araus and Cairns 2014).

In forages, a significant proportion of phenotypic data is acquired routinely via semiquantitative visual-scoring approaches (Walter *et al.* 2012). Traits that require tissue harvest and laboratory measurement, such as nutritive-quality measures, are not routinely screened due to cost or logistic issues. Development of in-field phenotyping tools for forages, therefore, has the potential to enhance accuracy for traits routinely measured by semiquantitative means, as well as supporting extension of phenotyping to traits previously not accessible. This process will involve evaluation of proximal sensing and imaging options as well as deployment platforms that are appropriate for the estimation of targeted traits and in relevant trial formats (e.g. individual plant or sown plots). Comprehensive sensor calibration, referenced to appropriate laboratory assays, will be required and calibration models will need to be built and validated, accounting for different environments and seasons to ensure applicability across Australasian conditions. Recently, this has been demonstrated in forages in the context of pasture agronomy (Pullanagari *et al.* 2013), indicating the potential for utilisation in plant breeding.

Use of an indirect selection method such as GS, which may be trained using indirect phenotypic trait measures such as data acquired via sensors, offers some reason for caution in development of a genomic prediction capability. This may be

compounded in the case of traits such as mixed sward yield, which rely on measurements in imperfectly correlated environments such as single plants or rows. These biological complexities are a primary challenge in harnessing this technology for driving improvement of key economic traits. Substantial work to generate relevant, accurate genomic prediction equations for key traits is essential to avoid pitfalls and realise the potential this technology offers.

In New Zealand, genomic selection for forage yield and nutritive-value traits is being empirically tested at a proof-of-concept scale, using a training population of replicated perennial ryegrass families evaluated in multiple environments. Application of GS at the larger, industry-wide scale, encompassing multiple breeding programs, presents research challenges (Crossa *et al.* 2014) including genomics applications development; standardising, aggregating and prioritising phenotypic records; assessing and harnessing genotype \times environment interactions (Lado *et al.* 2013); in-field phenotyping precision and efficiency; data optimisation and integration; and optimisation for prediction accuracy (Riedelsheimer and Melchinger 2013). In meeting these challenges, statistical, bioinformatic, genomic and field-evaluation processes must be improved to ensure GS in forages delivers value at an industry scale, presenting a planning and logistics challenge to the research community. Scale up for GS in forage breeding will be guided by a quantitative genetic model that is linked with economic and biophysical models, to ensure that traits targeted are adaptive within on-farm environmental and management constraints.

Evidence of efficacy for any novel breeding strategy must include assessment of rate of genetic gain against historic baselines and other breeding strategies (Resende *et al.* 2013). This includes empirical and modelled evidence to assess comparative efficiencies per unit resource (Massman *et al.* 2013; Resende *et al.* 2013) across the range of traits under selection.

Gene technology

Genetically modified (GM) crops have become well established globally, with 18 million farmers in 27 countries planting 175 million hectares of GM crops in 2013 (James 2013). This represents an area 6.6 times the total land area of New Zealand or almost a quarter of the total land area of Australia. However, commercial application of GM technology is limited to a few widely grown crops such as soybean, cotton, maize and canola. These GM crops are often used for animal feed. Meta-analysis of over 100 billion animals, including several long-term (>90-day) and multi-generational studies pre- and post-introduction of GM feeds, has revealed no adverse effects on ruminant productivity or health attributable to GM (Van Eenennaam and Young 2014). However, the adoption of GM in forage grasses and legumes lags behind that in crops, and is limited to herbicide-resistant lucerne (*Medicago sativa*) (James 2013) and, more recently, a low-lignin trait in lucerne. However, there is substantial interest in output traits for animal production (Van Eenennaam and Young 2014).

For genetic modification of forage grasses and legumes to deliver substantive improvements in animal production, it is

essential to develop forages with marked improvements in genetically tractable output traits such as yield and forage quality, so as to justify the investment associated with GM development and deregulation. Forage quality is a major constraint on productivity. The main components of forage nutritive value include protein, water-soluble carbohydrates (WSC) as the major component of non-fibrous carbohydrate, neutral detergent fibre digestibility, and lipid contents. Several research groups internationally have attempted to increase the concentration of fructans, the main WSC fraction in perennial ryegrass. Conventionally bred high-sugar grasses produce 25–50% more total WSC than do conventional cultivars; however the full expression of this trait is regulated by a gene-by-environment interaction, requiring prolonged exposure to short days and temperature under 10°C (Rasmussen *et al.* 2013, 2014). Perennial ryegrass with genetic modifications to the fructan biosynthetic pathway has demonstrated increases in WSC similar to the concentrations seen in conventionally bred high-sugar grasses. The main benefit from genetic modification in this case is that the gene-by-environment interaction is overcome (Rasmussen *et al.* 2013).

Another approach has been to modify cell-wall biosynthesis, as recently reviewed by Zhao and Dixon (2014). The main goal of international research efforts has been to improve the access of enzymes to the major cell-wall polysaccharides cellulose and hemicellulose. This has been achieved through the genetic modification of the lignin biosynthetic pathway by either reducing lignin concentrations or altering lignin composition. These modifications have improved forage digestibility *in vitro* (Getachew *et al.* 2011). The most successful approach has been to alter lignin content rather than lignin composition in alfalfa (Zhao and Dixon 2014).

Two major research projects at AgResearch have made significant progress on two important forage-quality targets and have the potential for step-changes in animal performance. These target major changes in forage quality by increasing energy via production of foliar lipids in grass, and slowing the rate of protein breakdown via production of condensed tannins in forage legumes. The genes required to meet both these challenges are either not known to exist, or do not exist in the target species.

High-energy forages

Forages with high energy can be developed by increasing the lipid concentration in vegetative tissue. Under normal circumstances, plants store neutral lipids only in seeds and pollen, as a source of essential energy for subsequent germination. The majority of leaf lipid is found in membranes, where it makes up ~3.5% of dry matter (DM). Lipids have twice the caloric value of the other main sources of energy in forage leaves, namely, WSC and protein. The goal is to double the level of leaf lipids to ~7–8% of DM. At this level, it is expected to obtain a 10% increase in metabolisable energy and the concentration of lipids would not be high enough to cause milk fat depression in grazing dairy cows (Flowers *et al.* 2008). Supplementary feeding trials in sheep have demonstrated that increasing the level of dietary fat to 8% DM led to a 30% increase in feed conversion efficiency (Cosgrove *et al.* 2004). There is minimal genetic variation in leaf lipid concentrations

and, therefore, the opportunity to rapidly and substantially change leaf lipid concentrations through conventional plant breeding is limited (Palladino *et al.* 2009; Glasser *et al.* 2013; Hegarty *et al.* 2013).

A technology to increase plant lipid concentrations and plant biomass has been recently reported (Winichayakul *et al.* 2013). A synthetic gene encoding a novel lipid-encapsulation protein, cysteine oleosin, was constitutively co-expressed with a gene encoding diacylglycerol acyltransferase (DGAT1), to increase energy density and reduce photorespiration of the model species *Arabidopsis thaliana* via the production and encapsulation of neutral lipids (Winichayakul *et al.* 2013). The leaf lipid concentration was doubled to ~8% DM, and lipid concentration in the plant root also increased to 8% of the DM. The 24% decrease in photorespiration is due to recycling of CO₂ in the chloroplast and results in a 50% increase in plant growth rates and corresponding increases in plant biomass (Winichayakul *et al.* 2013).

The same genes have been co-expressed in perennial ryegrass under the control of light-regulated, green tissue-specific promoters derived from the rice chlorophyll *a/b*-binding protein and ribulose biphosphate carboxylase genes (Sakamoto *et al.* 1991; Jang *et al.* 1999). When compared with a control population in containment glasshouse conditions with non-limiting water and nutrient supply, these GM plants have a 100% increase in leaf lipid concentration as determined by analytical chemistry of samples taken from mechanical defoliation trials as described below. They also have substantially increased (25%) growth rates, with corresponding increases in leaf and root biomass (G. T. Bryan, unpubl. data). These high-energy perennial ryegrass plants are yet to be field tested; however, they have the potential to provide a significant step change for pastoral farmers. This phenotype is stable under a mechanical defoliation regime in glasshouse-propagated plants. The plants have been defoliated with 4–5-week regrowth intervals over 30 times, with no negative impact on the plant (G. T. Bryan, unpubl. data). This technology is potentially applicable to all plant species with C₃ photosynthesis, and, therefore, can be applied to other forages such as white clover and lucerne.

Biophysical modelling (W. McG King, R. E. Vibart, unpubl. data) indicates that perennial ryegrass with these lipid attributes may enable farmers to have more efficient pasture utilisation, leading to increased farm profitability. A dairy cow model (Bryant *et al.* 2008) was used to estimate the change in feed intake with an increase in lipid content from 3.9% to 8%. Results suggested a reduction in feed intake of 30%, with no change in the production of milk solids. However, on the basis of a supplemental feeding study (Cosgrove *et al.* 2004) that showed a reduction in intake by sheep of 16% (with 8% total dietary lipid intake), we took a conservative approach and used 15% in all simulations. FarmaxDairy (Bryant *et al.* 2010) was used in three scenarios with different stocking rates (base, medium and high) using a model of Category 3 Waikato and Canterbury dairy farms. These scenarios were based on existing FarmaxDairy simulations developed using data from DairyBase and other DairyNZ sources and were considered to be typical for each province. On the basis of this modelling, the potential benefits include more efficient pasture utilisation, an increase in

the production of milk solids by 6–12%, up to a 17% reduction in greenhouse gas (GHG) emissions per kilogram of milk solids through a reduction in nitrous oxide emissions, and approximately NZ\$900/ha increase in farm revenue (e.g. in the Waikato scenario, farm revenues increased from NZ\$4222/ha to NZ\$5165/ha).

The GHG reduction is predominantly calculated on the basis that the cysteine oleosin containing pasture would help address the imbalance of energy sources in the feed, leading to a reduction of ammonia excreted by the animal and, therefore, a reduction in nitrous oxide emissions. On the basis of the data from a meta-analysis of the effects of dietary fat on methane emissions in cattle (Grainger and Beauchemin 2011), utilising a forage cultivar with double the lipid concentrations (8% DM), it may be possible to achieve a 10–17% reduction in methane emissions. This relatively wide range is due to the seasonal variation in lipid concentrations already seen in forage plants. While these plant-lipid data and range of model outputs are limited and reliant on several assumptions, they all consistently indicate a beneficial trend with regards to production, footprint and profit, which may be substantial in some cases.

Condensed tannins in forage legumes

Condensed tannins are associated with improved animal health and production due to a reduction in protein degradation and increased bypass to the animal gut (Aerts *et al.* 1999; Douglas *et al.* 1999; McMahan *et al.* 2000), especially in pastoral systems based on high legume content. Lucerne and white clover are very rich in proteins that are rapidly fermented in the rumen of grazing animals. This results in gas and foam formation in the rumen and leads to a potentially lethal condition known as pasture bloat (McMahan *et al.* 2000). Condensed tannins bind excess dietary proteins and bacterial enzymes, substantially reducing the level of protein degradation in the rumen. This increases protein bypass to the gut of the grazing animal, leading to improved absorption of essential amino acids and increased milk and meat production (McMahan *et al.* 2000). The decreased protein degradation in the rumen also decreases methane production and ammonium excretion in urine, which can contribute to significant reductions in emission of the potent GHGs methane and nitrous oxide from pastures (Smith *et al.* 2008). The presence of condensed tannins in animal diets has also been associated with reduced intestinal parasite load in ruminants (Aerts *et al.* 1999).

Lucerne and white clover contain negligible concentrations of condensed tannins in foliage. The opportunity to develop forage cultivars containing condensed tannins is, therefore, of considerable interest to the pastoral sector. The genes encoding the enzymes involved in the biochemical pathway to condensed tannins are present in lucerne and white clover; however, these genes are not sufficiently expressed in these species to permit accumulation of condensed tannins. In contrast to white clover, rabbit's foot clover (*T. arvense*) does accumulate significant concentrations of condensed tannins in leaves. We have identified *TaMYB14* as the regulatory gene from rabbit's foot clover responsible for turning on the genes along the pathway for production of condensed tannins. Silencing the *TaMYB14*

gene in transgenic plants of rabbit's foot clover blocked the accumulation of condensed tannins (Hancock *et al.* 2012). More importantly, the transgenic transfer of *TaMYB14* to white clover and lucerne activated production of condensed tannins in leaves (Hancock *et al.* 2012). The production of genetically modified clover and lucerne cultivars expressing *TaMYB14* may provide a valuable approach for a bloat-safe, highly productive pastoral agriculture with reduced GHG emissions.

Conclusions

Guidance by industry-agreed breeding priorities, rate of genetic gain as a key performance indicator, and step changes in specific traits provide a framework for improvement of pasture species of value to the Australasian dairy industry. In addressing these opportunities, progress on introgression of new genetic diversity for key traits and research in endophyte discovery and biology are imperative, in addition to the wider pasture- and plant-science disciplines supporting the pastoral sector. Genomic selection and improved field-based phenotyping technology are two near-term opportunities to improve forage breeding outcomes for Australasian dairy farmers. The need for relevant phenotypic records underscores the necessity for capability depth and expertise in field biology and breeding. Keeping options open for genetic modification offering step changes in performance is also essential. Continuity of applied-science delivery will improve the value of forage and provide options, ultimately contributing to an efficient and sustainable Australasian dairy industry.

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