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Research Program: Development and Implementation of Candidate Gene-Based Markers in Outcrossing Forage Species

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1 Plain English Executive Summary

Perennial and short term ryegrasses account for 54% of all temperate pasture seed sales in Australia and 47% of all pasture-based seed sales (Gout and Jones, 2006[1]). For perennial ryegrass, the top 8 varieties capture 56% of the market share. The delivery of dramatically improved varieties of perennial ryegrass and the companion legume white clover to the dairy, beef and sheep meat and wool production industries has the potential to generate large-scale economic benefits for farmers, manufacturers and processors. Genetic improvement is designed to increase profitability through increasing productivity and quality of pasture plants, so that benefits are markedly higher than the costs of any additional inputs. The historical rate of progress in genetic improvement in pasture plant breeding is generally regarded as low, with estimates of up to 7% per decade having been made for perennial grasses. In order to 'future-proof' the feed-base of the pastoral industries, major advances are required in the rate of genetic gain and incorporation of novel traits for improved adaptation to a changing environment.

A funding consortium was established to enable the development of significant molecular genetic tools and resources in pasture plant breeding between the Geoffrey Gardiner Dairy Foundation (GGDF), Meat and Livestock Australia (MLA), Dairy Australia (DA), the Victorian Department of Primary Industries (VDPI) and the Molecular Plant Breeding Cooperative Research Centre (MPB CRC).The PMP project was an investment for a 6-year term, ending on 31st December 2009. The project was reviewed at the 2.5 and 5.5 year stages.

The objective of stakeholder investment in the **Pasture Markers Project** (**PMP**) has been to deliver tangible economic benefits to the Australian dairy, beef, sheep meat and wool production industries. The project aimed to achieve this outcome through development and implementation of molecular marker systems using state-of-the-art technologies in commercial pasture plant breeding programs, and to enable genetic improvement in cultivar production to deliver to industry, demonstrably superior plant genetics for the feed-base. The investment to date in the PMP can be classified as **pre-competitive investment** in molecular marker technologies, and it was envisaged that the resulting science outputs could be readily used by any or all of the PPB companies. The nature of the pasture plant genetic supply industry is such that innovative genetic products, with end-user benefit, must be brought to market through pre-commercial engagement with breeding companies, which consist of a small number of global players. One of the major challenges is to adapt these new technologies to long-standing breeding process and commercial practices used by the seed industry, which are essential for delivery of novel pasture plant genetics to end-users for industry benefit.

The technologies developed in this program have targeted key aspects of forage nutritive value and persistence in species that are adapted to cultivation throughout the southerly grazing regions of Australia. For instance, a review commissioned by Pastures Australia (Grains Research and Development Corporation [GRDC], MLA, Australian Wool Innovation [AWI], DA, Rural Industries Research and Development Corporation [RIRDC]) has estimated the gross value of pastures in southern Australia to be $8.6 billion *per annum* (p.a.), of which approximately $2 billion is derived from dairy production, $2.4 billion from beef, $3.7 billion from sheep (meat and wool), the remainder being obtained from fodder, seed and the benefit of biological nitrogen fixation for subsequent cropping rotations. Genetic improvement of key agronomic traits has been demonstrated to lead to large potential gains in the profitability of

[1] M. Gout and S. Jones (2006) Pastures Australia; Market analysis and complete workshop report, investment in pasture improvement,

grazing industries. As an example, improvement of forage quality through increasing digestibility by 5 percentage units over the summer period has been estimated to be capable of increasing the productivity of the Australian dairy industry by c. $50 million p.a., through increased energy availability from forage and reduced requirement for grain supplementation.

Although the common goal was to enable the strategic science program, each funding organisation had a specific focus:

- GGDF funds research and development for the benefit of the Victorian dairy industry and dairying communities, for the benefit of the wider community, with the aim of maximising profitability with sustainability and ensuring that the Victorian dairy industry is competitive internationally.

- Dairy Australia wishes to lead effective change and to improve the profitability and sustainability of the Australian dairy industry and the Australian meat and livestock industries, respectively.

- MLA aims to deliver world-class services and solutions in partnership with industry and government. Its core activities are building demand for Australian red meat, improving market access for products, conducting research and development to provide competitive advantages for the industry, and collaborating with its partners to build capability within the industry.

- MPB CRC, which invests in research, development and commercialisation in relation to molecular plant breeding, has capability for the development and delivery of projects and has proposed to undertake further research relevant to the priorities determined by GGDF, Dairy Australia and MLA.

The major desired outcome of the project is the availability to Australian farmers of dramatically improved grass cultivars to improve the feed base of the dairy and red-meat industries. This will deliver demonstrable benefits and real choice to the farming community.

The **general research objective** of the program, to enable the outcome, was to develop and implement molecular marker systems using state-of-the-art technologies that would develop science outputs capable of delivering a marketable proposition to the PPB companies. The companies would then be able to deliver improved perennial ryegrass and white clover cultivars for Australian agriculture, with increased rates of genetic gain within foreshortened timelines. It was envisaged that the new, genetically superior, varieties would be readily adopted by farmers and producers to enable efficient, flexible and responsive agricultural practices which would effectively 'future-proof' the feed-base for the livestock and dairy industries. The project also aimed to provide continuous technical innovation to pasture-based agriculture, along with training and skills development for future generations of pasture plant breeders.

In order to achieve this objective through program activities in the second phase of investment, the **specific objectives** of the PMP were:

- To continue the development and evaluation of molecular markers from genes considered candidates for beneficial characters that are associated with a range of prioritised output traits, and to evaluate the degree of genetic variation within both perennial ryegrass and white clover.

- To further develop and intensively phenotype a range of perennial ryegrass and white clover selected families for key agronomic traits associated with herbage yield, herbage quality and tolerance to stresses from biological (pests and pathogens) and non-biological (drought) sources.
- To develop and implement methods for the use of genetic markers to identify within- and between-population variation in pasture grasses and clover, and to discriminate between cultivars for establishment and protection of plant breeders' rights (PBR).

In addition to improved forage cultivars, the application of molecular genetic technologies into the programs of pasture plant breeding companies will permit for the first time a degree of quality assurance and certification (QA/QC) to be delivered around seed sales, so that the general farming community will be able to buy their feed base with a greater degree of confidence and assurance over the product that they are choosing.

Associated tasks to enable delivery of the specific objectives were developed, and progress against delivery of the tasks by the due milestone date are described in this final report.

All project milestones have been either achieved or over-achieved. Program activities have achieved the crucial 'proof-of-concept' demonstration that the developed molecular markers in selected genes can be associated with significant variation in target breeders' characters for the development of new superior cultivars. Dramatic developments in DNA-based technologies now offer the ability to extend and strengthen the resulting opportunity, within experimental and commercial germplasm for the benefit of Australian agriculture.

As a direct result of the specific project activities the following highlight science outputs and strategic positions have been achieved:

Perennial ryegrass molecular genetics:

- World-leading activities to obtain a substantial collection of single DNA base change (SNP) molecular genetic markers based on incremental gene-specific amplicon resequencing.

- Identification of molecular markers which are functionally associated with variation of herbage quality traits in perennial ryegrass, to enable molecular marker-assisted pasture plant breeding.

- Detailed genetic knowledge of the current elite cultivars available to the Australian livestock-based industries, including origins and degree of relatedness.

- A cohort of molecular markers (SNPs) capable of being formatted and validated for applications in the analysis of population structure. This panel will also be used for a cultivar identification test capable of 'turn-key' implementation in a commercial setting. Documentation for this purpose has been generated relating to models of service provision, scope of work and indicative costings. Development of the finalised product is to be completed later in 2010.

- Advanced discussions with PPB companies relating to commercial utilisation of program-generated technology.

- Analysis performed for environmental stress tolerance, with special reference to water logging and water deficit conditions. Key regions of DNA and specific germplasm adapted to Australian conditions have been identified.

White clover genetics and genomics

- World-leading research performed to determine relationships between the sub-genomes of allotetraploid white clover.

- Unique and World-leading resources of molecular markers (SNPs) generated and selectively described through publication.

- Enabling research performed for whole genome sequencing efforts to be considered and potentially scoped.

- Generation and phenotypic analysis of traits associated with tolerance to saline stress.

Under an agreement that will be executed through the MPB CRC office, the intellectual property (IP) generated from this project will be available for unhindered use for the benefit of the Australian dairy and red meat production industries. All access to IP will be enabled through direct contact with staff of the Biosciences Research Division, Department of Primary Industries, namely Prof. John Forster, Dr. Noel Cogan, Dr. Junping Wang and Prof. German Spangenberg.

A number of key recommendations have arisen from the outcomes of the Pasture Markers Program:

Project scientific activities and outputs have generated technology which has matured to the point at which utilisation by pasture plant breeding companies, to deliver outcomes for Australian agriculture is both feasible and desirable. A detailed investment strategy document has been completed on behalf of the funding consortium by the program management and commercialisation committee to describe the means by which such implementation may occur.

Investment by the PPB companies will generate a greater level of commitment and interest in the technology, and will improve the relevance and potential applicability of the work undertaken in any future project. A future pilot molecular breeding program, performed in collaboration with and tailored to the needs of a pasture plant breeding company, would be required to engender confidence in the value and application of the relevant technologies. In addition, elements of research outputs generated to date (such as the molecular marker 'tool-kit' for perennial ryegrass cultivar discrimination) need to be further developed for implementation in a service provision model.

To complement the pilot molecular breeding program, a parallel activity for continuous innovation in molecular marker technology would be required, to ensure that significant gains made in breeding activities are continuously reinforced. This strategic research project would entail the development of methods and tools for high-throughput DNA sequencing and genotyping, leading to comprehensive sets of molecular markers distributed across the whole genome (or gene-space), and linked to innovations in advanced automated phenotypic analysis.

On-going interest has been achieved from pasture plant breeding companies and co-investment has been secured for the delivery process. A project of facilitated adoption of the technology in the commercial setting is currently being scoped and will commence later in 2010.

2 Plain English Program Deliverables

- The development of a suite of reliable, gene based, state-of-the-art molecular marker systems suitable for high-throughput genetic analysis in perennial ryegrass and white clover (corresponding to over 400 characterised gene loci from each species).

- The definition of marker-trait associations based on co-location of candidate gene markers and quantitative trait loci (QTLs) for a range of key agronomic traits controlling herbage yield and quality, persistence, disease resistance and environmental stress tolerance in perennial ryegrass and white clover.

- Development and implementation of methodologies for population-based genetic analysis in pasture species, allowing the selection of individual plants with superior genes from germplasm collections.

- Development and implementation of methodologies for genetic marker-based cultivar identification and discrimination in pasture species.

- The provision of gene based markers and marker-trait associations for implementation into pasture plant breeding programs using novel strategies geared to existing commercial enterprises, leading to the development of world-leading germplasm and varieties with exceptionally high economic benefit.

The higher-level outcomes of the Pasture Markers Program have been:

- Efficient systems for single DNA base change (SNP – Single Nucleotide Polymorphism) molecular genetic marker discovery and validation in genes that are candidates for causal effect with traits of agronomic importance in perennial ryegrass
    - Continuously adapted to sequencing technology improvements for enhanced throughput
- Efficient systems for SNP discovery and validation in genes that are candidates for causal effect with traits of agronomic importance in white clover
    - Continuously adapted to sequencing technology improvements for enhanced throughput
    - General solution for other outbreeding polyploids e.g. tall fescue
- Knowledge and effective use of related model species that have already undergone whole genome sequencing efforts
- Enhanced knowledge of genetic control of key agronomic traits in perennial ryegrass and white clover through linkage mapping
    - Herbage quality, disease resistance, abiotic stress tolerance
- 'Proof-of-concept' for discovery of diagnostic marker-trait associations
    - SNPs/molecular markers in genes that are believed to be associated with increased herbage quality in perennial ryegrass
- Process for validation of diagnostic marker-trait associations
    - Ongoing evaluation of predicted markers in commercial germplasm
- Detailed knowledge of population structure in target species
    - Germplasm characterisation, discrimination and integrity surveillance

The higher-level outputs of the Pasture Markers Program have been:

- Catalogue of large-scale gene-based SNP molecular marker data for perennial ryegrass and white clover
    - DNA sequence data
    - Genetic map data
    - Assay conditions and details
- Methodology for sequence variant characterisation in outbreeding polyploid forage species
    - White clover
    - Tall fescue
- DNA based molecular markers for epidemiology of the crown rust pathogen
- Catalogue of marker-trait associations for perennial ryegrass and white clover
    - Herbage quality, disease resistance, abiotic stress tolerance
    - QTLs and associated linked markers on genetic maps
    - Predicted diagnostic SNP molecular markers for herbage quality in perennial ryegrass
- System for cultivar discrimination and identification in outbreeding forage species
    - Optimised analytical procedures

3 Expected Outcomes

- The delivery of dramatically improved varieties of perennial ryegrass and white clover to the dairy, beef and sheep meat and wool production industries has the potential to generate large-scale economic benefits for farmers, manufacturers and processors. The Victorian dairy industry, as a highly performing component of the pastoral production industry, is especially well placed to benefit. The technologies to be developed in this program have targeted key aspects of forage nutritive value and persistence in species that are adapted to cultivation throughout the southerly grazing regions of Australia. For instance, a review commissioned by Pastures Australia (Grains Research and Development Corporation [GRDC], MLA, Australian Wool Innovation [AWI], DA, Rural Industries Research and Development Coprporation [RIRDC]) has estimated the gross value of pastures in southern Australia to be $8.6 billion *per annum* (p.a.), of which approximately $2 billion is derived from dairy production, $2.4 billion from beef, $3.7 billion from sheep (meat and wool), the remainder being obtained from fodder, seed and the benefit of biological nitrogen fixation for subsequent cropping rotations. Genetic improvement of key agronomic traits has been demonstrated to lead to large potential gains in the profitability of grazing industries. As an example, improvement of forage quality through increasing digestibility by 5 percentage units over the summer period has been estimated to be capable of increasing the productivity of the Australian dairy industry by c. $50 million p.a., through increased energy availability from forage and reduced requirement for grain supplementation.

- The genetic supply industry for temperate, perennial pasture species such as perennial ryegrass and white clover is such that improvement of key agronomic traits must be delivered through elite varieties bred in commercial organisations and distributed as high-quality certified seed. This project has consequently focused on the development and validation of technologies for perennial ryegrass and (by extension, the closely related grass species) tall fescue, which are by far the major

temperate grass species sown in Australia (and world-wide). Approximately $35 million p.a. of the $93 million p.a. total grass seed market is comprised of cultivars of these species. White clover is the second most important species of perennial pasture legume ($4.2 million p.a.) behind lucerne ($14.7 million p.a.). White clover consequently represented the secondary target for this program. Although a number of cultivars of each target species are already available to the market, the dominant share resides with relatively few major cultivars. It is therefore possible to achieve good market penetration through the incorporation of high-value traits into these market-leading genetic backgrounds. Realisation of value on farm as a consequence of the proposed program is consequently dependent on the establishment of strong links with the private sector, and this was reflected in both the research plan and the extensive process of engagement with commercial partners.

4 General Experimental Approaches

Identification and validation of SNPs in selected candidate genes from perennial ryegrass and white clover, through *in silico* discovery (identification and validation of predicted SNPs from bioinformatic analysis of EST contigs) and *in vitro* discovery (amplicon PCR, cloning, sequencing and sequence alignment analysis).

Accelerated methods for targeted *in vitro* SNP discovery across multiple amplicons from multiple genes, through the use of the Roche GS FLX massively-parallel pyrosequencing technology.

Genotyping of validated gene-associated SNPs in pair-cross derived genetic mapping families of perennial ryegrass and white clover using, in the first instance, the single nucleotide primer extension (SNuPe) assay, and in the subsequent program phases, the Illumina GoldenGate™-VeraCode™-BeadXpress™ genotyping system, followed by genetic map construction in association with framework genetic markers.

Evaluation of perennial ryegrass and white clover pair-cross derived progeny sets for multiple phenotypic traits, with appropriate statistical analysis, followed by QTL detection and evaluation of candidate gene-based marker-trait QTL co-location. Methods for selective phenotyping based on selection of full-sib population sub-sets identified for maximal recombination events were implemented to refine this process.

Exploitation of data from model Poaceae species (rice, wheat etc.) and model Fabaceae species (*Medicago truncatula*, *Lotus japonicus*) for targeted analysis of perennial ryegrass and white clover candidate genes, respectively.

Evaluation of genetic diversity within and between populations and varieties of perennial ryegrass, white clover and the crown rust pathogen of perennial ryegrass, using SSR markers, as a support for development of strategies of association genetics analysis.

Determination of haplotypic variation for selected candidate genes in large-scale perennial ryegrass germplasm collections and in related *Lolium* species, and in large-scale white clover germplasm collections. The process of ‘allele panning’ was highly accelerated through the use of the Roche GS FLX massively-parallel pyrosequencing technology.

Development of strategies for evaluation of haplotype-specific gene expression and correlations between genotypic and phenotypic variation in perennial ryegrass, based on the most advanced methods for association genetics analysis with due compensation for population structure.

Development of strategies for identification and discrimination of pasture grass and clover varieties, based on SSR and SNP marker polymorphism and advanced statistical analytic techniques.

5 Delivery of Scientific Objectives

5.1 Quality and relevance of research

5.1.1 Research quality

Quality of the research within PMP Phase II can be assessed in terms of the following criteria: continuity with previously successful research, peer recognition and scientific publication outputs.

5.1.1.1 Continuity with previous research

PMP Phase II activities were based on knowledge, experience and technologies derived originally from research within the portfolios of the Cooperative Research Centre for Molecular Plant Breeding (CRC MPB) and VDPI from 1997-2003, and more significantly, the first phase of PMP activities from October 2003 to February 2006 (the period prior to the first GGDF-sponsored review). These activities established a world-leading position for MPB CRC and VDPI research, which was recognised through the GGDF review processes (in both 2006 and 2009) and an associated 'desk-top' review undertaken on behalf of DA by Dr. Michael Casler (University of Wisconsin, USA) and Dr. Charles Brummer (at that time employed by Iowa State University, USA, now at the University of Georgia, USA). The innovations in trait-specific population development and basic genetic linkage map construction (CRC MPB) were augmented by innovative and successful methods for SNP discovery and validation in PMP Phase I, leading to the enhanced methods for genetic polymorphism identification and SNP implementation for association genetics analysis characteristic of PMP Phase II. This seamless integration of research strategies and outputs, particularly across the period from 2006, was indicative of high-quality program design and implementation.

5.1.1.2 Peer recognition

The program leaders (Prof. John Forster and Prof. Kevin Smith) have been invited speakers at each of the five international symposia on Molecular Breeding of Forage and Turf Crops (MBFT) held to date. Prof. Forster presented plenary papers at the first, second, third and sixth MBFT symposia held in Nishinasuno, Japan in 1998; Lorne and Hamilton, Australia in 2000; Dallas, USA in 2003; and Buenos Aires, Argentina in 2010. Prof. Forster also presented major invited papers at the fourth and fifth MBFT symposia held in Aberystwyth, United Kingdom in 2005 and Sapporo, Japan in 2007. Prof. Smith presented a plenary paper at the fourth MBFT symposium and a major invited paper at the fifth MBFT symposium.

In addition to presentations at major conferences, both Prof. Forster and Prof. Smith have been invited speakers at the Plant and Animal Genome (PAG) conference series. Research seminars have been presented at national and international venues such as the Centre for Plant Conservation Genetics, Southern Cross University, Lismore, Australia (March 2006), the Centre for Legume Improvement in Mediterranean Agriculture (CLIMA), University of Western Australia, Perth, Australia (July 2006), the Australian Centre for Plant Functional Genomics (ACPFG), Adelaide, Australia (August 2008) and the Centre for Agricultural

Genomic Technologies (CAGT), University of Georgia, Athens, Georgia, USA (January 2009). Industry forum presentations have been made at the Australian Dairy Industry Conference Breakfast, Flemington, Victoria, Australia in November 2006 (Forster, J.W., Smith, K.F., Spangenberg, G. [2007] Gene technology for dairy pastures – what's new, what's next?) and the Australian Dairy Conference, Shepparton, Victoria, Australia in February 2007 (Smith, K.F., Forster, J.W., Spangenberg, G. [2007] Developing molecular markers for ryegrass breeding).

In research papers presented at MBFT2005, MBFT2007 and MBFT2010, the program leaders established priority for internationally-leading outcomes in SNP discovery, validation, LD analysis and implementation for both perennial ryegrass and white clover. Prof. Forster also presented a major summary of progress and outcomes from the perennial ryegrass component of the program in the International *Lolium* Genome Initiative (ILGI) workshop at PAGXVII in January 2009, and a review of developments in temperate pasture plant genetics over the preceding decade at PAGXVIII in January 2010. Prof. Forster was invited to present a keynote presentation on association genetics of pasture grasses at the EUCARPIA symposium in La Rochelle, France in September 2008, but was unable to attend due to other commitments. The evidence from all of these community meetings was that the program established and maintained a world-leading position in forage genetics, relative to the activities in other organisations such as the European Union (EU) Framework VI consortium termed GRASP (Grass Allele-Specific Polymorphisms), which has now concluded, and the New Zealand-based Pastoral Genomics consortium involving AgResearch New Zealand and Vialactia Biosciences Ltd.

Specific recognition has been provided through the leadership role taken by the program leaders in the establishment of the International *Trifolium* Network (ITN) as a coordinatory body for molecular breeding of clover species and translation of information from model legumes such as *Medicago truncatula*. ITN was founded following a workshop held at the Institute of Grasslands and Environmental Research (IGER), Aberystwyth, UK following MBFT2005, under the international coordination of Dr. Michael Abberton (IGER). Prof. Forster was confirmed as coordinator of ITN Working Group 1, with responsibility for issues related to genetic map construction, trait-dissection and genome nomenclature. In this area, Prof. Forster successfully conducted a detailed audit of existing structured genetic mapping populations and marker resources throughout the community, arranged for release of c. 200 genomic DNA-derived simple sequence repeat (SSR) primer pairs from the VDPI program prior to 2003 as a community resource (to date, the most significant action of this kind from any ITN partner) and has promulgated a fully rationalised nomenclature system for white clover linkage groups based on homoeologous group numbering with reference to *M. truncatula* counterparts, and sub-genome designation based on putative progenitor genome relationships.

The program has continued to incorporate collaborative elements with a number of international groups, such as those of Prof. Toshihiko Yamada (University of Hokkaido, Sapporo, Japan), Dr. Michael Abberton (Institute for Grassland and Environmental Research, Aberystwyth, United Kingdom) and Jaime Garcia (INIA, Uruguay). Dr. E. Charles Brummer (University of Georgia, USA), the leading alfalfa molecular breeder in North America, and also works with white clover, spent three months on sabbatical in VDPI from October-December 2007, based at both DPI-Bundoora and DPI-Hamilton, and made very significant contributions to elements of the program, especially for the white clover component.

The PMP research program is part of a wider portfolio of activities within the Biosciences Research Platform of the VDPI Biosciences Research Division (BRD) based at both DPI-Bundoora and DPI-Hamilton, under the overall leadership of Prof. German Spangenberg. Large components of this portfolio are within the MPB CRC, of which Prof. Spangenberg is Chief Scientist and Research Director-Transgenic Technologies, and will transfer into the

Dairy Futures CRC during the period from 1st January – 30th June 2010. The VABC-based activities involve world-leading activities in computational biology, structural and functional genomics and gene technology for temperate pasture species, in addition to the activities in molecular marker technology activities described here. VABC is equipped with state-of-the-art facilities for bioinformatics, biorobotics (Beckman Biomek FX), DNA sequencing (ABI3730xl, MegaBACE 4000, Roche GSFLX Titanium, Illumina GA2) DNA genotyping (Illumina BeadXpress™, Illumina iSCAN™) and transcriptomics (Agilent, Affymetrix and CombiMatrix platforms).

BRD staff members, especially Profs. Forster and Spangenberg and Prof. Smith, have been involved in international leadership of the forage and turf genetics community, as shown by the organisation and hosting of the MBFC2000 symposium in Lorne and Hamilton in November 2000, with Prof. Spangenberg chairing the MBFT International Organising Committee since its inception in 2000 over a decade, and roles in planning for the MBFT symposium held in Buenos Aires, Argentina in March 2010.

5.1.1.3 Scientific publication outputs

The following publications were directly attributable to outcomes of PMP research: 20 peer-reviewed manuscripts published, 1 accepted subject to revision and 1 submitted; 64 conference proceedings published; 12 book chapters published and 1 in an advanced stage of preparation. As an indication of the impact of the PMP on international conferences, 14 poster abstracts were prepared for the MBFT2007 symposium and 3 book chapters were derived from oral presentations presented at that conference. A total of 12 abstracts were prepared for the MBFT2010 symposium.

5.1.2 Relevance to international scientific developments

International developments in plant molecular genetics and molecular breeding within the duration of PMP Phase II have been dominated by several trends: an increasing emphasis on the use of non-structured or quasi-structured genetic populations for association analysis of marker-trait correlation; major activities in large-scale SNP discovery and validation; continued use of comparative genomics from species such as *Oryza sativa* and *Brachypodium distachyon* (Poaceae) and *Lotus japonicus* and *Medicago truncatula* (Fabaceae) for computational interpretation of species-specific genomics; fine-mapping activities for integration of genetic and physical maps; and the aggressive use of platform technologies for high-throughput genetic analysis, including DNA sequencing (Roche GS, Illumina GA, ABI SoLiD) and DNA genotyping (Illumina BeadXpress™). Activities in PMP during the review period have aligned with all of these trends and facilitated early-adoption of relevant approaches and technologies. This strategy has retained a leading international position for the program. The program has also greatly benefited from interactions with parallel programs in animal molecular genetics (especially bovine and ovine studies) within VDPI, including methods for obtaining and interpreting high-density SNP chip data to allow whole-genome selection.

In future, the use of whole-genome sequencing (WGS) information, not only for reference genome characterisation, but also for large-scale sequence polymorphism identification on an individual basis, will be the key trend in molecular genetics. Strategies for this development in the PMP mandate species have been progressed during Phase II through 'proof-of-concept' activities in pooled amplicon sequencing and the generation and analysis of reduced complexity representations (through construction of hypomethylated sequence libraries) to access a large proportion of the 'gene-space'. Sequencing activities based on the use of heterozygous genotypes immediately allows access to SNP variation. Regular distribution of SNP loci across the genome has been assisted by the implementation of

'virtual' physical mapping with reference to model species genomes. 'Proof-of-concept' for detailed comparative genetic and physical fine mapping has been obtained for the *S* and *Z* gametophytic self-incompatibility genes of perennial ryegrass in the Ph.D. project of Hiroshi Shinozuka, which operates within MPB CRC parallel to PMP. In addition, international developments in phenomics are being enabled within VDPI through the integration of transcriptomics, proteomics and metabolomics capabilities in a single Discovery Technologies sub-platform at VABC, and the planned acquisition of a LemnaTec automated glasshouse and imaging system later in 2010.

5.2 Summary of Research Progress

5.2.1 Sub-program 1: Staged implementation of validated functionally-associated markers in perennial ryegrass breeding programs

Sub-program 1: Staged implementation of validated functionally-associated markers in perennial ryegrass breeding programs			
1-1	Completion of residual tasks from PMP phase one, continuity for on-going tasks common between PMP phases one and two, initiation of preliminary tasks for PMP phase two [Core activities: Continuation of haplotype-phenotype correlation experiments; continuation of white clover genetic map construction and trait-dissection; continuation of genetic analysis of the perennial ryegrass-crown rust pathogen interaction; initiation of candidate gene identification and *in vitro* SNP discovery for abiotic stress tolerance in perennial ryegrass and white clover]	31st December 2006 (+1)	Progress reported in *Interim Technical Report* for the period October – December 2006, indicating status and continuity of research, as appropriate
1-2	Validation of existing marker-trait associations in elite breeder's germplasm of perennial ryegrass	31st December 2007 (+5)	Positive associations between diagnostic markers for herbage quality and phenotypic variation identified ready for exploitation
1-3	Completion of experimental work and analysis for the AMP field experiment, permitting consolidation of set of predicted diagnostic SNPs for herbage quality.	31 December 2009 (+13)	NIRS phenotyping completed, SNP genotyping completed, full data analysis performed and interpreted
1-4	Completion of experimental work and analysis for the SNP validation panel (SVP) Phase I experiment, and actions performed for implementation of SVP Phase II	31 December 2009 (+13)	SSR and SNP genotyping performed and interpreted, populations selected and established for SVP Phase II
1-5	Technology 'tool-box' consolidated and scientific/commercialisation strategies in place for commercial interactions	31 December 2009 (+13)	SSR and SNP genotyping systems fully evaluated or in advanced design phase, modes of engagement scoped, costed and prioritised

Task 1-1: Completion of residual tasks from PMP phase one, continuity for on-going tasks common between PMP phases one and two, initiation of preliminary tasks for PMP phase two [Core activities: Continuation of haplotype-phenotype correlation experiments; continuation of white clover genetic map construction and trait-dissection; continuation of genetic analysis of the perennial ryegrass-crown rust pathogen interaction; initiation of candidate gene identification and* in vitro *SNP discovery for abiotic stress tolerance in perennial ryegrass and white clover] (Due date: December 31st 2006)

The requisite tasks in this category were designed to permit continuity between PMP Phases I and II, and were all successfully achieved by the due date.

Task 1-2: Validation of existing marker-trait associations in elite breeder's germplasm of perennial ryegrass (Due date: 31st December 2007)

All candidate gene SNP genotyping, SNP haplotype reconstruction and SSR genotyping, phenotypic analysis and preliminary detection of genotype-phenotype correlations were completed by the due date. Highly indicative relationships between specific SNP loci in candidate genes known to be functionally correlated with target traits of interest were identified, and were progressed for validation by specific field trial analysis. Novel methods for sequence haplotype correlation were also developed and successfully implemented.

Task 1-3:* Completion of experimental work and analysis for the AMP field experiment, permitting consolidation of set of predicted diagnostic SNPs for herbage quality *(Rescheduled due date: 31st December 2009)

Phenotypic evaluation was performed by assessing a field trial of association mapping panel genotypes that were attributed to a 'meta-population' with minimal intrapopulation differentiation. The meta-population consisted of a total of 220 plants (diverse germplasm [171 genotypes] and two ecotypes - Kangaroo Valley [28 genotypes] and Victorian [21 genotypes]). The field trial was established as a row-column design (11 x 20) with 4 clones per plot and 3 replicates, totalling 12 clones per genotype. Samples were obtained from both vegetative and reproductive stages for NIRS analysis. The NIRS-based predictions of phenotypic values were improved by analysis of a sub-set of samples by 'wet' chemistry protocols. Significant variation was detected, with ranges for fibre content from c. 30g/100g to c. 60g/100g observed from different genotypes. Initial analysis has identified a collection of 27 SNP markers that are significantly associated with phenotypic variance (V_p) for key herbage quality traits, each SNP accounting for between 5.2 -11.9% of V_p. Additional experimentation in the application of the associated markers is required to convert them from the output of a research project to a fully validated turn key solution for pasture plant breeders. This will involve the application of a pilot breeding nursery to perform test crosses to demonstrate the applicability of the approach.

Figure 1. Association mapping panel (AMP) plants established as a field trial at DPI-Hamilton.

Task 1-4: Completion of experimental work and analysis for the SNP validation panel (SVP) Phase I experiment, and actions performed for implementation of SVP Phase II (Rescheduled due date: 31st December 2009)

Phase I of the SNP validation panel experiment consisted of the genotypic description of a collection of 8 cultivars, with 96 genotypes sampled from within each cultivar. The plants were assessed for population structure using the same collection of 60 SSR markers that had been used to describe population structure in the AMP study. The data identified significant population structure in the samples analysed, and all of the genotypes from each of the cultivars could be grouped together and unambiguously attributed to a source cultivar. Cultivars Aberdart and Abermagic originate from the Germinal Holdings breeding activity and have the most distinctive genotypic profile. There is significant overlap of many of the other cultivars that originate from New Zealand Agriseeds or PGG Wrightsons, which indicates a high degree of common germplasm usage within the breeding activities of the two companies, and the prevalence of selections arising from a specific cultivar or by combination of germplasm from several cultivars. Detailed discussions with stakeholders and PPB company representatives on implementation of SVP Phase II has led to the scoping of a more substantial pilot project aimed at integrating technology with applied breeding and providing the basis for PPB company interaction and utilisation. Full details of this pilot project are provided in the investment strategy document (Appendix 1).

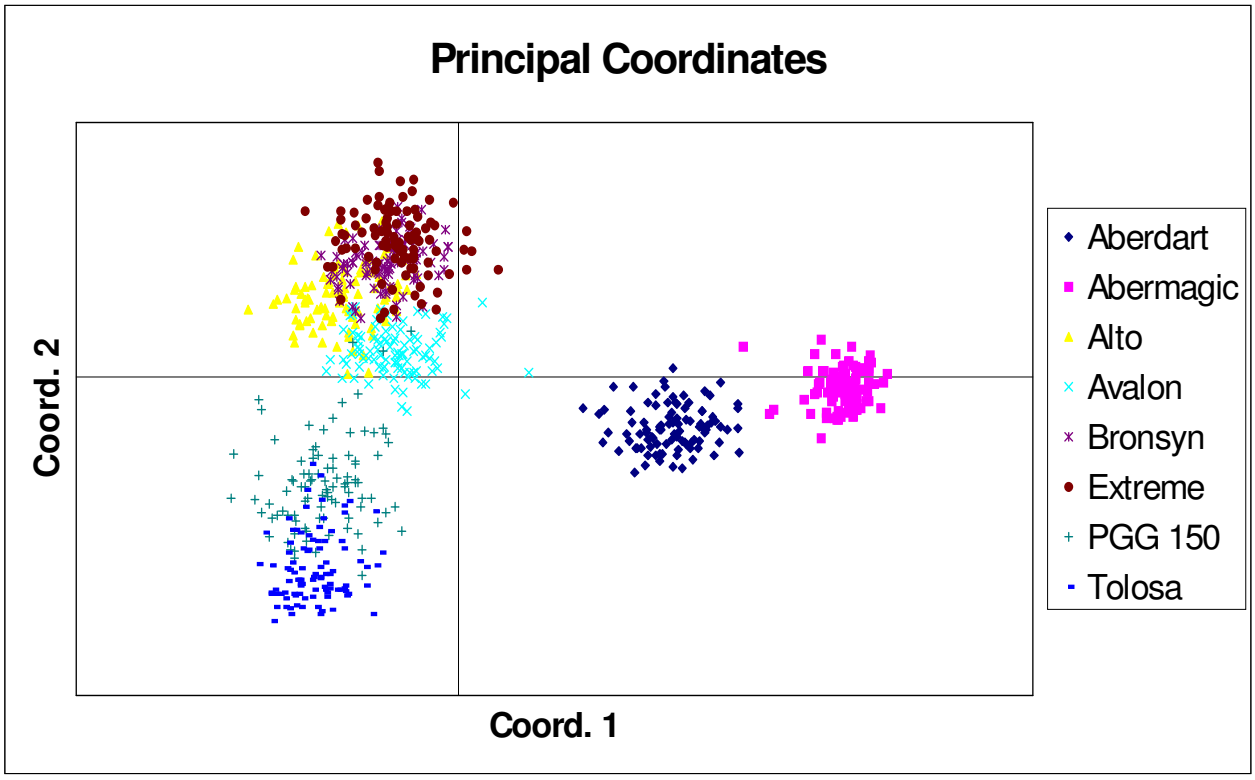

Figure 2. Principal coordinate analysis of the genotypes analysed for SSR variation within the SVP Phase II experiment.

Task 1-5: Technology 'tool-box' consolidated and scientific/commercialisation strategies in place for commercial interactions (due date: 31st December 2009)

To enable utilisation of PMP-derived technology, significant time and resource investments have been devoted to interactions with the PPB companies to generate engagement. This has led to the production of separate documentation for the investment strategy and for utilisation (Appendices 1, 2). Although substantial progress has been made, there has been a realisation that to achieve effective uptake, a pilot project would need to be scoped to specifically fit the requirements of the PPB companies, in order to engender confidence in program technology. In addition, advanced designs for background genome diversity

assessment using Illumina GoldenGate™ OPA SNP genotyping technology has been progressed, along with initial costing models for cultivar identification service delivery.

The outputs of sub-program 1 have led to the following position being established for ryegrass molecular genetics:

- **Establishment of a world-leading collection of perennial ryegrass SNP markers that are functionally associated with variation for herbage quality traits, to enable molecular genetic marker-assisted pasture plant breeding.**
- **Detailed genetic knowledge of the current elite cultivars available to the Australian livestock-based industries, including origins and degree of relatedness.**
- **Methodologies for cultivar identification, along with documentation relating to models for service provision, scope of work and indicative costings.**
- **Advanced discussions with PPB companies relating to commercial utilisation of program technology.**

5.2.2 Sub-program 2: New genetic knowledge and novel breeding methodologies for ryegrasses and fescues

Sub-program 2: New genetic knowledge and novel breeding methodologies for ryegrasses and fescues			
2-1	Identification of candidate genes associated with abiotic stress tolerance traits in pasture grasses	30th June 2007 (+3)	30-50 genes identified and prioritised based on informatics and empirical analysis
2-2	Development and implementation of phenotypic analysis protocols for abiotic stress tolerance traits in pasture grasses	31st December 2007 (+5)	Effective multi-level phenotypic protocols developed and validated
2-3	SNP haplotype structure determined for candidate genes associated with abiotic stress tolerance in pasture grasses	30th June 2008 (+7)	SNP variation, detected and quantified, and SNPs assigned to genetic linkage maps
2-4	Establishment of marker-trait associations for abiotic stress tolerance in pasture grasses	31st March 2009 (+10)	Linkage and LD mapping used to detect positive associations
2-5	Development and implementation of methodologies for high-throughput screening for superior allele content at validated candidate genes in pasture grass germplasm collections I: Genotyping	30th June 2009 (+11)	Next-generation genotyping platforms exploited to screen for pre-characterised polymorphism.
2-6	Development and implementation of methodologies for high-throughput screening for superior allele content at validated candidate genes in pasture grass germplasm collections II: Sequencing	31st December 2009 (+13)	Next generation sequencing platforms exploited to screen for uncharacterised polymorphism.

Task 2-1: Identification of candidate genes associated with abiotic stress tolerance traits in pasture grasses (Due date: 30th June 2007)

This task was achieved, with representatives of major gene classes identified in excess of expectation. In addition, microarray based gene expression identified a collection of c. 700 genes that were up-regulated as a result of abiotic stress application. Genes from the collection that was differentially expressed were prioritised through the process of sequence annotation, and a subset of 100 genes was chosen for sequencing analysis and SNP discovery.

Task 2-2: Development and implementation of phenotypic analysis protocols for abiotic stress tolerance traits in pasture grasses (Due date: 31st December 2007)

Optimised protocols were established based on the use of sealed sandpots for abiotic stress tolerance phenotyping. The use of sandpots enables the assessment of both above- and below-ground growth characteristics for phenotypic evaluation under both waterlogged and water deficit conditions. The sandpots were c. 50 cm in length and contained a sealed plastic sleeve that was filled with washed river sand. A single tiller of each ryegrass plant for assessment was transplanted to the sandpot and grown under favourable conditions for 4 weeks for establishment, and subsequently, treatments are imposed for a duration of not less than 4 weeks. Upon completion of the stress treatment, the plastic sleeve was removed and the whole plant was extracted for measurement of shoot and root traits.

Figure 3. Abiotic stress tolerance phenotypic trial using sandpots, each containing a single tiller of a unique individual perennial ryegrass plant. A red edge to the sandpot indicates a water deficit treatment, a blue edge indicates waterlogging treatment, and no colour indicates control conditions.

Task 2-3: SNP haplotype structure determined for candidate genes associated with abiotic stress tolerance in pasture grasses (Due date: 30th June 2008)

SNP haplotype structures were fully determined for selected genes processed through incremental *in vitro* gene discovery, and novel methods for accelerated SNP discovery using massively-parallel DNA sequencing technology were developed and implemented with large cohorts of genes (>100) with annotations for known abiotic stress related genes, or genes identified by transcriptome analysis. The derived sequences were assembled into contigs

and assessed for the presence of sequence variation through manual and automated pipelines. Sets of putative SNP markers were assembled and assessed for design potential in high-throughput genotyping systems using the Illumina GoldenGate™ SNP genotyping technology. Extension of activities in molecular breeding for improved herbage quality were also prioritised as the most likely category for commercial implementation. Additional candidate genes associated with herbage quality (oligosaccharide biosynthesis and cell wall metabolism) were identified and have been progressed for SNP discovery and validation, effectively doubling the number of genes analysed for associations with variation for NIRS-calibrated herbage quality characters.

Task 2-4: Establishment of marker-trait associations for abiotic stress tolerance in pasture grasses (Rescheduled due date: 31st March 2009)

Framework genetic linkage mapping was performed in a large customised full-sib population to enable fine structure analysis for candidate gene identification. Based on the genetic map structure, an initial cohort of individuals was selected that displayed maximal complementary recombination across the genome, and would hence provide the most informative individuals for phenotypic analysis. Initial phenotypic screens were conducted for plant growth under waterlogging and control conditions. QTLs for morphological traits relevant to survival under Australian field conditions were identified on several linkage groups. A second phase of phenotypic evaluation was then performed to fine-map specific target QTLs using selected genotypes that were maximally recombinant in the relevant genomic regions, in order to prioritise selection of candidate genes through comparative genomics alignment with the genomes of model species such as rice and *Brachypodium distachyon*. The selection of phenotypically divergent individuals in this second round of analysis also permits identification of potentially valuable germplasm capable of vegetative yield under conditions of environmental stress.

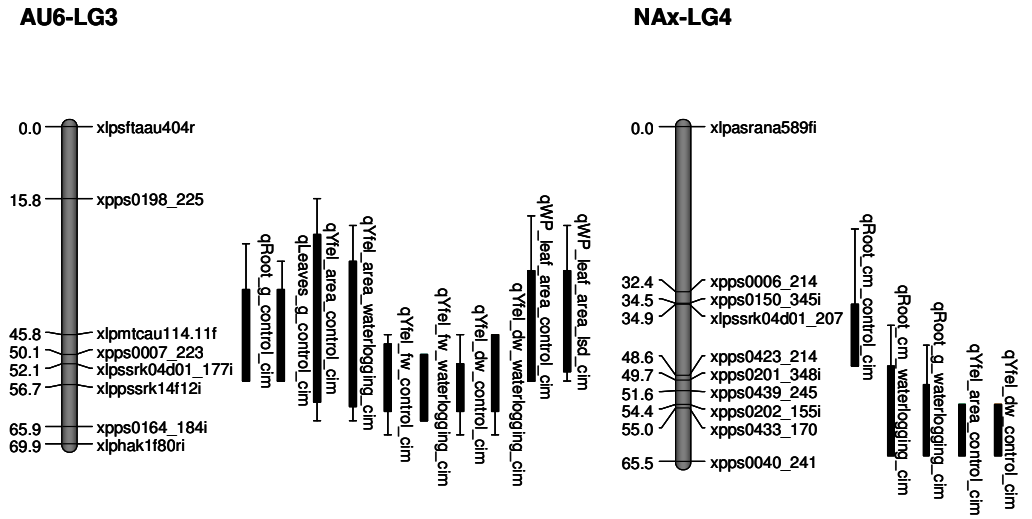

Figure 4. QTLs on linkage groups (LGs) 3 and 4 of the parental genetic maps of the $F_1(NA_x \times AU_6)$ fine-structure mapping population, identified from phenotypic analysis of the sandpot-based abiotic stress tolerance evaluation experiment.

Task 2-5: Development and implementation of methodologies for high-throughput screening for superior allele content at validated candidate genes in pasture grass germplasm collections I: Genotyping (Due date: 30th June 2009)

A preliminary set of 384 perennial ryegrass SNPs was formatted for multiplexed genotyping using the Illumina GoldenGate™-VeraCode™-BeadXpress™ platform, the equipment platform was established, and a substantial proportion of the candidate SNPs were fully

validated to establish capability for multiplexed genotyping. Previously validated SNPs which were not suitable for this format were assembled into panels for lower-multiplex implementation using the allele-specific primer extension (ASPE) SNP genotyping assay chemistry. The attrition rates observed during the design and validation process provided insight into input requirements for different desired genotyping outcomes, which will permit large sets of SNP markers to be routinely developed and implemented.

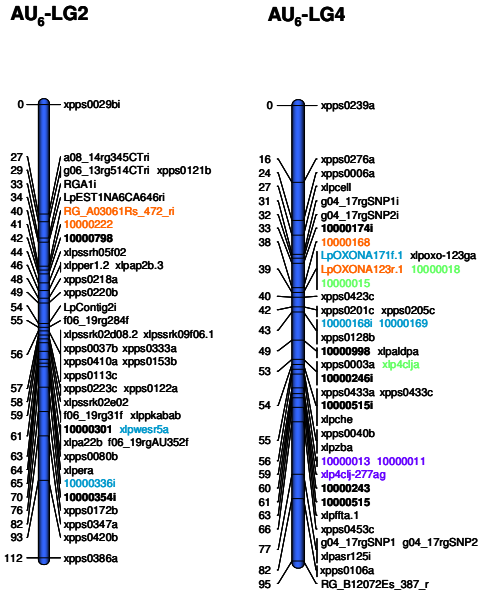

Figure 5. Genetic map location of SNP markers mapped using both SNuPe and Illumina GoldenGate™ technology.

Task 2-6: Development and implementation of methodologies for high-throughput screening for superior allele content at validated candidate genes in pasture grass germplasm collections II: Sequencing (Due date: 31st December 2009)

'Proof-of-concept' for this activity was obtained through massively-parallel DNA sequencing of selected amplicons from multiple perennial ryegrass herbage quality genes pooled from multiple individuals ('allele panning'), permitting identification of novel SNPs and SNP haplotypes, as well as accelerated SNP discovery performed by pooling of amplicons from multiple (c. 150) candidate genes. These technology products have been combined with the capability for multiplexed SNP genotyping in Task 2-5. In addition, exploitation of comparative genomics strategies using information on deletion bin mapping of wheat ESTs and the recently sequenced genome of *Brachypodium distachyon*identified blocks of macrosynteny that could be used to predict gene order and content in perennial ryegrass, allowing targeted gene template selection, amplicon generation and sequencing to be performed to support region-specific marker discovery. Preliminary work on genome complexity reduction was performed using methylation-sensitive restriction enzymes, to produce a representation enriched for genic portions of the genome suitable for survey sequencing. Initial data from analysis of a single perennial ryegrass genotype generated more than 90 Mb of sequence, and tentative prediction of a total of greater than 10,000 putative SNP markers.

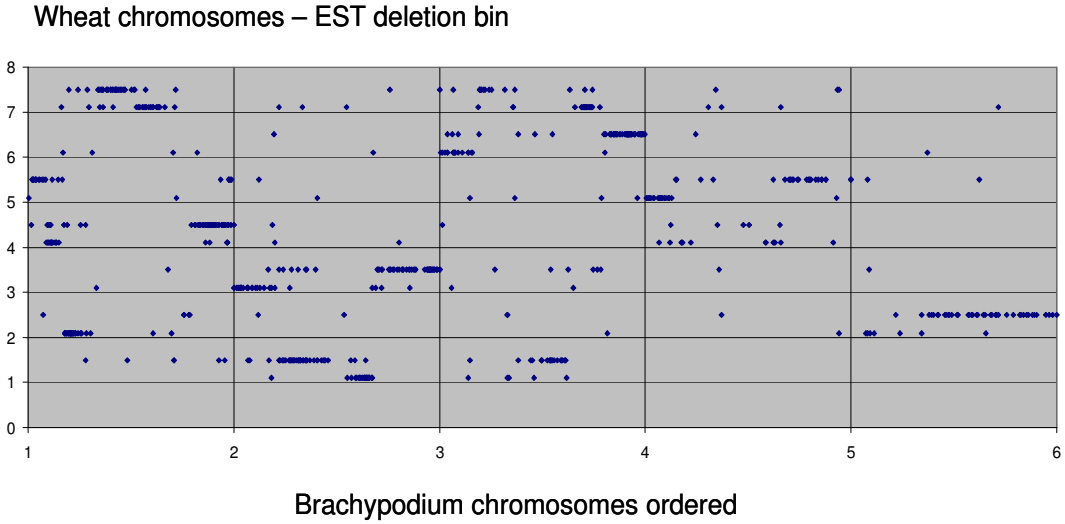

Figure 6. Comparative genome analysis of wheat and *Brachypodium distachyon* by BLAST comparison. Multiple matches indicate conserved macrosynteny between the two species that can inform predictions of gene order and content.

The outputs of sub-program 2 have led to the following position being established for ryegrass molecular genetics:

- **World-leading application of highly-multiplexed Illumina GoldenGate™ OPA SNP genotyping technology for ryegrass species.**
- **Novel application of comparative genomics for exploitation of the genome sequences of model species genome sequence to predict gene content and order in perennial ryegrass, as a support for candidate gene identification and structured SNP discovery.**
- **GSFLX-based sequencing performed to generate a collection of more than 20,000 putative genic SNPs, based on novel approaches using pooled amplicons and complexity reduction representations.**
- **QTL analysis performed for tolerance to abiotic stress traits such as water logging and water deficit conditions, leading to identification of key genomic regions for selection and specific adapted germplasm relevant to Australian agriculture.**
- **Suite of SNP markers suitable for formatting and validation as a 384-plex assay to determine population structure and permit cultivar discrmination tests as a 'turn-key' solution for service delivery.**

5.2.3 Sub-program 3: Genetic analysis of white clover

Sub-program 3: Genetic analysis of white clover			
3-1	Identification of candidate genes associated with abiotic stress tolerance traits in pasture clovers	30th June 2007 (+3)	30-50 genes identified and prioritised based on informatics and empirical analysis
3-2	Development and implementation of phenotypic analysis protocols for abiotic stress tolerance traits in pasture clovers	31st December 2007 (+5)	Effective multi-level phenotypic protocols developed and validated
3-3	Development and implementation of tools and strategies for rapid genetic and genomic data translation from model legumes to white clover I	30th June 2008 (+7)	Strategies developed and implemented for exploitation of microsynteny and genome-specific sequence variation
3-4	SNP haplotype structure determined for candidate genes associated with abiotic stress tolerance in pasture clovers	31st December 2008 (+9)	SNP variation, detected and quantified, and SNPs assigned to genetic linkage maps
3-5	Establishment of marker-trait associations for abiotic stress tolerance in pasture clovers	31st March 2009 (+10)	Linkage and LD mapping used to detect positive associations
3-6	Development and implementation of tools and strategies for rapid genetic and genomic data translation from model legumes to white clover II	30th June 2009 (+11)	Strategies developed and implemented for exploitation of physical mapping and BAC sequence data
3-7	Development and implementation of methodologies for high-throughput screening for superior allele content at validated candidate genes in pasture clover germplasm collections	31st December 2009 (+13)	Next generation genotyping and sequencing platforms exploited to screen for pre-characterised and uncharacterised polymorphism.

Task 3-1: Identification of candidate genes associated with abiotic stress tolerance traits in pasture clovers (Due date: 30th June 2007)

This task was achieved by the due date, with representatives of major gene classes already identified in excess of expectation (c. 200), and permitting effective subsequent progress towards achievement of Task 3-4. The successful implementation of a strategy based on comparison with amplicons derived from putative progenitor species made a major contribution to gene-specific marker development, and was highly influential in progressing international conventions on genetic map nomenclature. The output of this milestone was used as the template for development of technology applicable to achievement of task 3.7.

Task 3-2: Development and implementation of phenotypic analysis protocols for abiotic stress tolerance traits in pasture clovers (Due date: 31st December 2007)

Optimised protocols were developed for growth characteristics under control and saline stress conditions in the F_1(Haifa$_2$ x LCL$_2$) and F_1(S184$_6$ x LCL$_6$) genetic mapping families. Optimisation was particularly focused on the application of stress conditions in hydroponics and subsequent regulation of pH value in the growth medium, along with normalisation of the size and morphology of input plant material. Optimal salt concentrations for stress treatment were also determined for the two mapping populations under investigation and were specified as 50 mM and 75mM, respectively.

Task 3-3: Development and implementation of tools and strategies for rapid genetic and genomic data translation from model legumes to white clover I (Due date: 30th June 2008)

To complement the rationalised, empowered SNP discovery pipeline, comparative genomics analysis was developed with the two model legume species *Medicago truncatula* and *Lotus*

japonicus, in order to exploit the available sequence data from these two species for rapid candidate gene identification. Sequence of a sub-set of mapped white clover SSR-containing ESTs were compared to the *L. japonicus*, *M. truncatula* draft genomes. A total of 6 of the 8 homoeologous groups (HGs) show a predominant match to single *Mt* chromosome. There was some evidence to support the presence of a reciprocal translocation event involving *M. truncatula* chromosomes 2 and 6. All of the white clover HGs were numbered on the basis of the majority of matches to *M. truncatula*.

The HG nomenclature system for white clover was described as part of as a peer-reviewed publication in *Genome* (George *et al.* 2008), hence fully delivering the requisite information for completion of task 3-3.

Novel methods for identification of sub-genome-specific sequence variation based on progenitor comparisons were successfully implemented, underpinned by gene selection informed by knowledge of comparative relationships between white clover and model legume genomes. In addition, knowledge of comparative relationships was implemented for selective enhancement of the white clover genetic linkage maps.

Task 3-4: SNP haplotype structure determined for candidate genes associated with abiotic stress tolerance in pasture clovers (Due date: 31st December 2008)

SNP haplotype structures have been fully determined for selected genes processed through incremental *in vitro* gene discovery, and novel methods for accelerated SNP discovery using massively-parallel DNA sequencing technology have been developed and implemented. Resulting data was analysed to allow unambiguous resolution of haplotype structure.

A key limitation, identified as a result of the Reeves-Gough review conducted on behalf of GGDF at the 2.5 year time-point, was the problematic nature of SNP discovery in white clover, and the associated high attrition levels experienced during the validation process. During the second phase of the program, research was conducted to address this problem. Amplicon sequencing at greater depth was performed, along with the addition of potential diploid progenitors of white clover (*Trifolium occidentale* D.E. Coombe*, Trifolium pallescens* Schreber) to resolve haplotypic ambiguity.

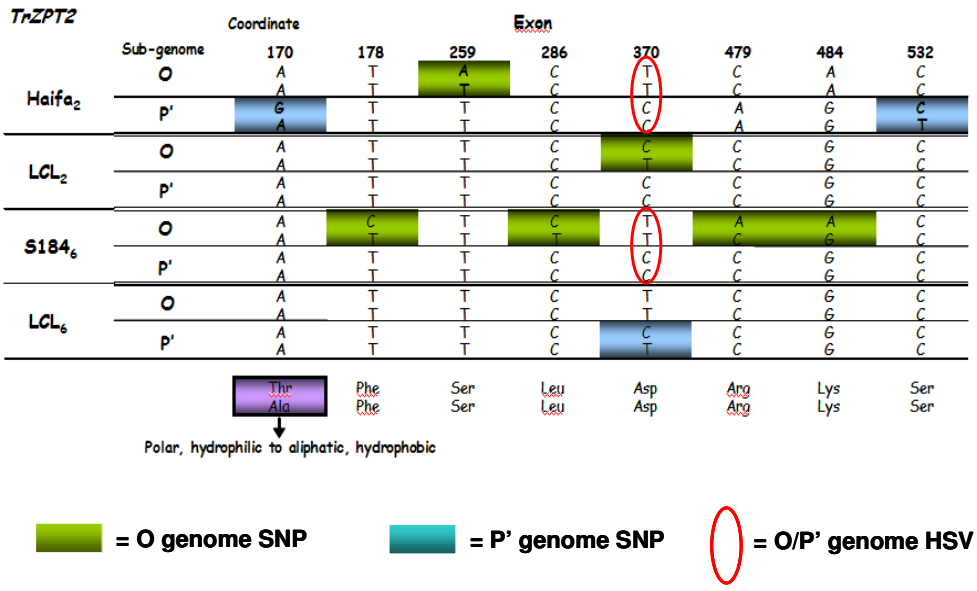

Figure 7. SNP haplotype classification within the white clover *TrZPT2* gene, with base variants assigned to specific sub-genomes.

By comparative sequencing of amplicons derived from the contemporary taxa proposed to be most closely related to the putative diploid progenitors of white clover, the haplotypic complexity observed in contigs could be decomposed into homologous **SNPs** within sub-genomes**,** homoeologous sequence variants **(HSVs)** between sub-genomes and paralogous sequence variants **(PSVs)** within and between sub-genomes. Through this process, the two proposed progenitor species were examined in depth. *Trifolium occidentale* exhibited close affinity to two of the identified haplotypes from contemporary white clover (corresponding to allelic variants), which were therefore assigned to the O sub-genome. The second pair of haplotypes from white clover displayed weaker affinity with *Trifolium pallescens*, and was actually more closely related to *T. occidentale*, leading to designation as the P' sub-genome.

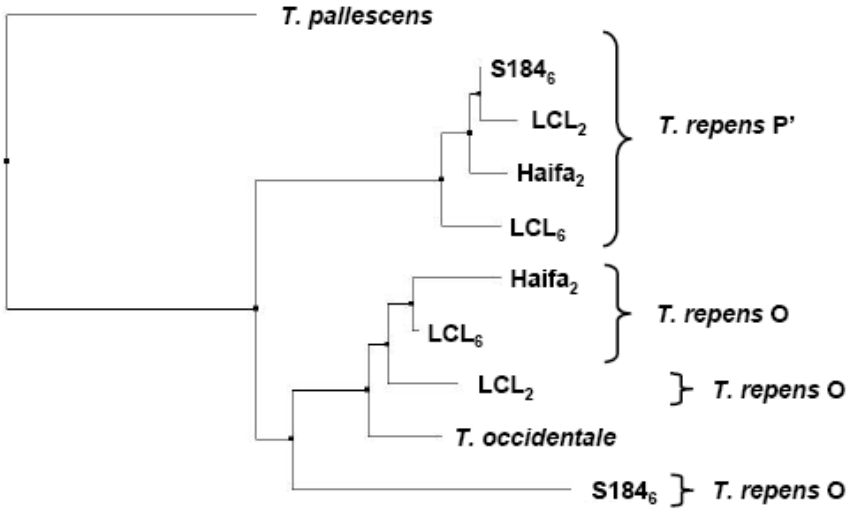

Figure 8. NJ dendrogram representing levels of nucleotide similarity between white clover O and P' sub-genome consensus sequences for each mapping family parent and both *T. occidentale* and *T. pallescens* reference sequences. Relative branch lengths from nodes represent percentage nucleotide identities for pair-wise comparisons between TrZPT2 haplotypic sequences.

Through discrimination of SNPs from HSVs and PSVs, allelic variants can be assigned to specific genetic map locations on homoeologous linkage groups attributable to one or other sub-genome. This process allows white clover genetic maps to be, for the first time, completely described in terms of homoeologous structure. A total of 9 genes were intensively analysed in this manner, generating a total of 7,290 bp of DNA sequence. A total of 134 SNPs and 274 HSVs were identified, with SNPs identified at 1 per 87 bp in the O sub-genome and 1 per 97 bp in the P' sub-genome. Of the identified SNPs, 57% were coincident with HSVs. HSVs were identified at a frequency of 1 per 27 bp, on average, and 28% of the HSVs also were coincident with SNPs, explaining the high attrition levels experienced in the initial efforts of SNP discovery.

The improved SNP discovery system for white clover was described in a peer-reviewed publication in *Molecular Genetics and Genomics* (Hand *et al.* 2008), hence fully delivering the requisite information for completion of task 3-4.

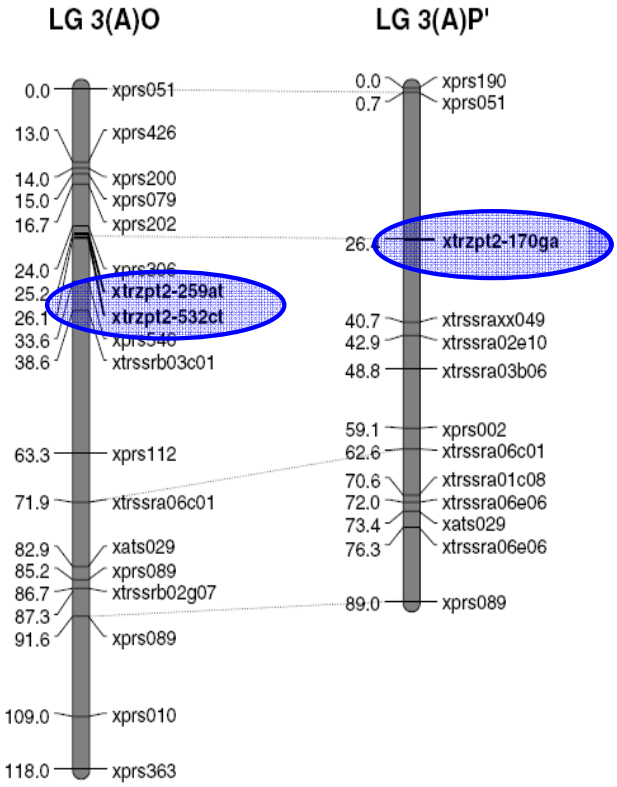

Figure 9. Location of *TrZPT2* SNP loci on homoeologous linkage groups of the Haifa$_2$ parental genetic map. HG designation is defined by origin of the SNP loci, defining the identity of the O and P' sub-genomes.

Task 3-5: Establishment of marker-trait associations for abiotic stress tolerance in pasture clovers (Rescheduled due date: 31st March 2009)

Genetic linkage maps have been constructed for both of the mapping populations, with SNP and SSR markers. For the parental map of Haifa$_2$ a total of 17 LGs were constructed totalling 1973cM. The genetic map of LCL$_2$ covers 19 LGs and cumulative length of 1838cM. Whenever possible, LGs were attributed to specific sub-genomes through assignment of homoeologous SNP markers to the framework map. Phenotypic evaluation was performed following the parameters identified from research undertaken for delivery of task 3.2. Hydroponic trials were conducted in nutrient solution culture under glasshouse conditions, with a total of 6 replicates in an incomplete block design at two NaCl concentration levels (0 and 75 mM) and three replicates per combination genotype-treatment, resulting in a total of 1,404 plants. A total of 51 putative QTLs for the 11 traits assessed under control and salt-stressed conditions were identified, 30 on the Haifa$_2$ map and 21 on the LCL$_2$ map. Coincident QTLs provide independent estimations of the effect of the same genomic region, as many of these traits are highly correlated. A total of 8 genomic regions were identified on the Haifa$_2$ genetic map and 6 regions were identified on the LCL$_2$ genetic map. QTLs were identified for a range of traits encompassing growth in control and under stress conditions. The range of phenotypic variation (V_p) accounted was from 5 to 17%, with a mean of 8% across both genetic maps.

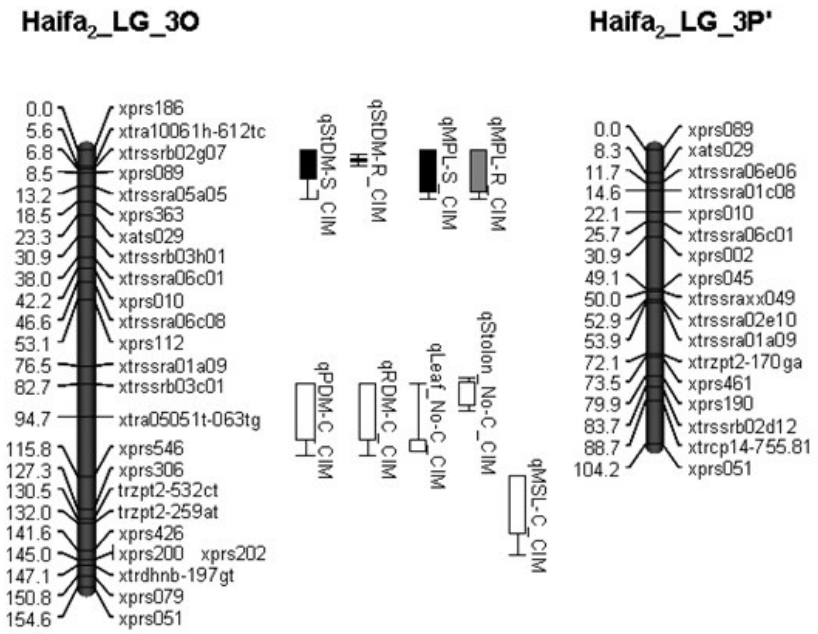

Figure 10. QTLs for growth characteristics identified under control and stress conditions and attributed to a specific homoeologous linkage group of the white clover genetic map.

The QTL mapping study was described in peer-reviewed publication and has been published in *Theoretical and Applied Genetics* (Wang *et al.* 2010), hence fully delivering the requisite information for completion of task 3-5.

Task 3-6: Development and implementation of tools and strategies for rapid genetic and genomic data translation from model legumes to white clover II (Due date: 30th June 2009)

Research was performed to describe the overall affinity of white clover sub-genomes at the nucleotide level in broader terms than previously attempted, and to compare the sequence data with the genome drafts of model legume species to increase the resolution of conserved synteny comparisons to the microscale level. From activities to deliver task 3-4, small-scale sequencing demonstrated that the two sub-genomes of white clover have a high degree of sequence similarity in transcribed genic regions (98.65% exon and 72.44% intron nucleotide identity). However, similarity had not been examined in regulatory regions, and conservation of gene order at the microscale level was not examined. The chief purpose of this study was to assist in development of strategies for high-throughput SNP discovery in the whole gene-space of the white clover genome, and to test the feasibility of whole genome sequencing for an outbreeding allopolyploid species.

A set of four homoeologous BAC pairs were identified and sequenced using Sanger chemistry. A total of 173 kb of overlapping sequence was assembled from the four pairs. Similar levels of sequence similarity to those already seen in the transcribed portions of the sequence were observed (exon 97.18% and intron 88.93% nucleotide indentity). Calibration of sequence divergence rates in comparison to the equivalent values for the model legume species, and the inferred time of evolutionary divergence between *L. japonicus* and *M. truncatul*a, suggested that the divergence time for the two sub-genomes is c. 4.2 million years. The two sub-genomes share conserved gene orders in the areas under examination and comparison to the genome drafts of the model legume species, for which syntenic

regions had been sequenced, showed high levels of gene order conservation. The current data will contribute to further assessment of sub-genome-specific expression and QTL regulation, as well as permitting refinement of targeted gene orthologous prediction and evaluation of QTL location conservation in comparison to the genomes of model species.

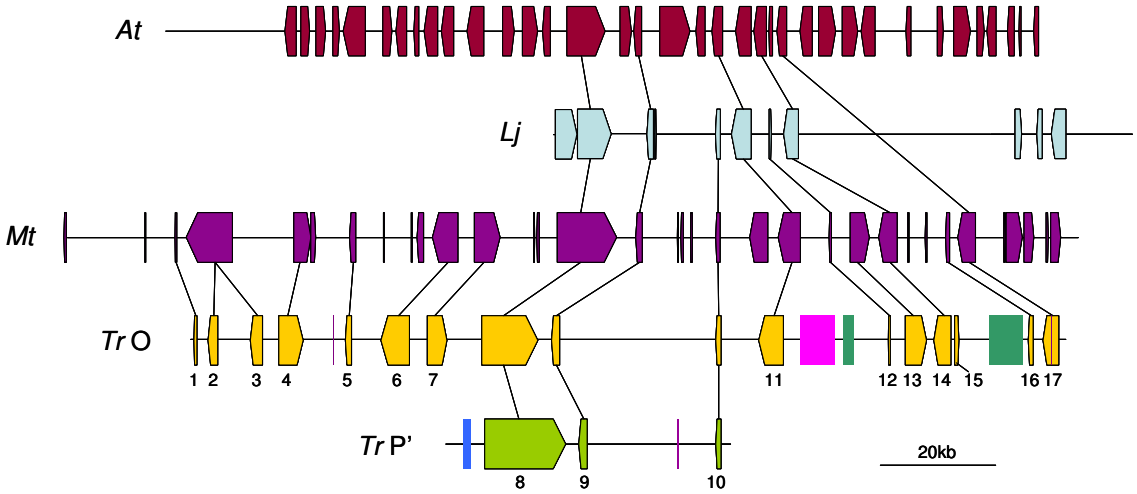

Figure 11. Comparative gene order for the O and P' sub genomes of white clover in comparison to *M. truncatula* and *L. japonicus* and *A. thaliana*.

The enhanced sub-genome sequence characterisation for white clover has been described as a publication which is currently in an advanced state of preparation for submission to the journal *BMC Plant Biology*, hence fully delivering the requisite information for completion of task 3-6.

Task 3-7: Development and implementation of methodologies for high-throughput screening for superior allele content at validated candidate genes in pasture clover germplasm collections (Rescheduled due date: 31st December 2009)

Following the initial in-depth SNP discovery process for 9 candidate genes, all with sequence annotations associated with abiotic stress tolerance, the experience was applied to a 'reduction-to-practice' for SNP discovery on a larger scale. A collection of c. 200 genes was assembled that covered a range of gene classes, 100 of which were related to abiotic stress tolerance, as well as a small collection of other agronomically important gene classes. **Assembly of this collection of genes delivered task 3-1.** Two rounds of GSFLX-based sequencing were performed on the amplicons derived from the gene collection. Analysis of base variant frequency should correctly categorise SNPs based on prior knowledge, both types of variant (independently located, and coincident with HSV) being potentially useful. A pipeline of sequence assembly developed in-house using Mosaik and SNP detection using 454-polybayes was used to identify a total of 9,610 variant bases. The identified variant bases were submitted to Illumina to assess feasibility of design for a GoldenGate™ SNP OPA genotyping panel and a total of 3317 variant bases were identified.

Sequence characterisation and SNP identification activities for white clover have been fully documented and options for publication are under consideration. The activities described here fully deliver the requisite information for completion of task 3-7.

The outputs from sub-program 3 have lead to the following position being established for white clover genetics and genomics:

- **World-leading research has been performed to dissect relationships between sub-genomes of white clover.**

- **A unique resource, in international terms, of SNP markers has been generated and described.**

- **Enabling research for whole-genome sequencing characterisation has been performed.**

- **Phenotypic analysis protocols have been optimised for assessment of saline stress tolerance in two genetic mapping populations.**

- **QTLs have been identified for vegetative growth under saline-stressed and control conditions, and have in some instances been assigned to sub-genome of origin.**

6 Progress towards utilisation of project technology

6.1 Progress relative to specified timetable for securing commercial involvement

The time-table for commercial engagement was evolved in concert with the deliberations of the Program Management Commercialisation Committee (PMCC) over the life-time of the program, and all agreed actions in respect of commercialisation of activities were met, as specified in the minutes of the relevant PMCC meetings.

The outputs of the research must be taken up by the pasture plant breeding companies to have any impact for the levy-payers. The PPB companies are very supportive of the strategic science that underpins the development of new plant breeding technologies and perceive that there is potential for these newer technologies to accelerate genetic gain in pasture plant breeding.

6.2 Progress in development of specified commercialisation plan

6.2.1 Knowledge of target markets and commercial players

Background

Throughout the first two years (2003-2005) of the research program, informal contacts were made with the commercial managers and breeders within the major commercial players in the Australasian market. A detailed position paper describing the major commercial players in the breeding of ryegrasses and clovers on an international basis was prepared by Dr. Peter Fennessy, Prof. John Forster and Prof. Kevin Smith in August 2005.

6.2.2 Applicability of project technology to market and community needs

Commercial Market Needs

The genetic supply industry for temperate, perennial pasture species such as perennial ryegrass and white clover is such that improvement of key agronomic traits must be delivered through elite varieties bred in commercial organisations and distributed as high-quality certified seed, *supporting the corresponding productivity benefits obtained by Australian dairy farmers*.

Experience gained through interaction with the breeding companies as part of the PMP, and also the parallel discussions within Pastures Australia for establishment of a genetic improvement and evaluation method (GIEM) process (a form of ‘reconnaissance-in-force’), has permitted clearer definition of the key requirements for the companies, and the opportunities for effective engagement.

A detailed document describing the most likely path to utilisation of the technology developed during the course of the project was essential for future project adoption. Extensive consideration was given to the requirements of the PPB companies, along with the possible application of the technologies and nature of models for service level agreements.

6.2.3 Strategy for IP management and protection

A detailed audit of PMP-generated IP has been performed. For the ongoing application and use of the IP generated from this project, under an agreement that will be executed through the MPBCRC office, the IP will be available to the original funding consortium to be used unhindered for the benefit of the Australian dairy and red meat industries. All access to IP will be via direct contact with staff of the Biosciences Research Division, Department of Primary Industries, namely Prof. John Forster, Dr. Noel Cogan, Dr. Junping Wang or Prof. German Spangenberg.

7 Publications and Presentations

7.1 Program-related publications

7.1.1 Peer-reviewed journal articles

(20 published or in press; 1 accepted subject to revision; 1 submitted)

Cogan, N.O.I., Smith, K.F., Yamada, T., Francki, M.G., Vecchies, A.C., Jones, E.S., Spangenberg, G.C., Forster, J.W. (2005) QTL analysis and comparative genomics of herbage quality traits in perennial ryegrass (*Lolium perenne* L.). *Theoretical and Applied Genetics* 110: 364-380.

Yamada, T., Forster, J.W., Humphreys, M.W., Takamizo, T. (2005) Genetics and molecular breeding in the *Lolium*/*Festuca* pasture grass species complex. *Grassland Science* 51: 89-106.

Shinozuka, H., Hisano, H., Ponting, R.C., Jones, E.S., Cogan, N.O.I., Forster, J.W., Yamada, T. (2005) Molecular cloning and genetic mapping of perennial ryegrass casein protein kinase 2α-subunit genes. *Theoretical and Applied Genetics* 112: 167-177.

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(64 published)

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7.4 Public talks and seminars

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Forster, J.W., Smith, K.F., Spangenberg, G.C. 'Plant Biotechnology for the Pastoral Industries'. Public presentation at BioFestival04 conference, Melbourne Convention Centre, 11th August 2004.

Forster, J.W. (2004) Resources and strategies for association genetics studies in outcrossing forage species. Research seminar presented at State Agricultural Biotechnology Centre, Murdoch University, Perth, October 2004.

Forster, J.W. (2004) Plant biotechnology for the pastoral industries. Research seminar presented at the Western Australian Herbicide Resistance Initiative (WAHRI), University of Western Australia, Perth, October 2004.

Forster, J.W. (2004) Developing candidate gene-based marker systems for forage plants. PIRVic Plant Genetics and Genomics Platform Annual Research Conference, DPI-Hamilton, November 2005.

Forster, J.W. (2006) Functionally-associated genetic markers for temperate pasture plant improvement. Invited seminar, University of California Davis, USA, 20th January 2006.

Forster, J.W. (2006) Strategies and technology platforms for plant genotyping in PIRVic Plant Genetics and Genomics. Advances in Plant Genotyping Workshop, Centre for Plant Conservation Genetics, Southern Cross University, Lismore, NSW, 24th March 2006.

Forster, J.W. (2006) Functionally-associated genetic markers for improvement of white clover (*Trifolium repens* L.). Primary Industries Research Victoria-Plant Genetics and Genomics Annual Research Conference, Horsham, Victoria, Australia, November 2006.

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Forster, J.W., Smith, K.F., Spangenberg, G. (2007) Gene technology for dairy pastures – what's new, what's next? Australian Dairy Industry Conference Breakfast, Flemington, Victoria, November 2006.

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Forster, J.W., Smith, K.F., Spangenberg, G.C. (2007) Molecular breeding of pastures for improved pasture production. Dairy Science 2007: Meeting the Challenges for Pasture-based Dairying. University of Melbourne, Parkville, Victoria, 19th September 2007.

Smith, K.F. (2007) Molecular breeding of forages. Research seminar at Buenos Aires Province Agronomists Society, Argentina, May 2008.

Smith, K.F. (2008) Molecular breeding of pasture plants. Hawkesdale Branch of the Victorian Farmer's Federation, May 2008.

Forster, J.W. (2008) Molecular polymorphisms for temperate pasture plant improvement. Australian Centre for Plant Functional Genomics (ACPFG) seminar, Adelaide, South Australia, June 2008.

Forster, J.W. (2008) Molecular polymorphisms for temperate pasture plant improvement. Molecular Plant Breeding Cooperative Research Centre (MPB CRC) Annual Research Meeting, Creswick, Victoria, July 2008.

Forster, J.W., Cogan, N.O.I, (2008) Genome analysis for food quality and safety: technology platforms and applications. 'Farm to Fork' Workshop, Agilent Technologies, Victorian AgriBiosciences Centre, Bundoora, Victoria, July 2008.

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Forster, J.W. (2008) Molecular genetics: toys, orphans and school-leavers. DPI-Biosciences Research Division seminar, Bundoora, Victoria, September 2008.

Forster, J.W. 'Molecular polymorphisms for temperate pasture plant improvement' Centre for Agricultural Genetic Technologies, University of Georgia, Athens, Georgia, USA, January 2009.

7.5 Student dissertations

George, J. (2005) M.Sc.-Ph.D. conversion document, July 2005

Dracatos, P.M. (2006) M.Sc.-Ph.D. conversion document, October 2006.

Dracatos, P.M. (2008) Molecular genetic analysis of the crown rust (*Puccinia coronata* f.sp. *lolii*) – perennial ryegrass (*Lolium perenne* L.) interaction. Ph.D. thesis, La Trobe University, Bundoora, Victoria, Australia, awarded in January 2009.

Hogg, A.S. (2009) M.Sc.-Ph.D. conversion document, February 2009.

8 Program Personnel

Name	Substantive Role	Location
Prof. John Forster	State-Wide Leader and Principal Research Scientist (Molecular Genetics), Biosciences Research Division, Victorian Department of Primary Industries	DPI-Bundoora
Prof. Kevin Smith	State-Wide Leader and Principal Research Scientist (Molecular Plant Breeding), Biosciences Research Division, Victorian Department of Primary Industries	DPI-Hamilton
Prof. German Spangenberg	Executive Director, Biosciences Research Division, Victorian Department of Primary Industries	DPI-Bundoora
Dr. Noel Cogan	Senior Research Scientist (Molecular Genetics), Biosciences Research Division, Victorian Department of Primary Industries	DPI-Bundoora
Dr. Nathaniel Bannan	Senior Research Scientist (Molecular Plant Breeding), Biosciences Research Division, Victorian Department of Primary Industries	DPI-Hamilton
Dr. Mark Dobrowolski	Senior Research Scientist (Molecular Plant Breeding), Biosciences Research Division, Victorian Department of Primary Industries	DPI-Hamilton
Dr. Champa Bandaranayake	Senior Research Scientist (Molecular Plant Breeding), Biosciences Research Division, Victorian Department of Primary Industries	DPI-Hamilton
Dr. Junping Wang	Senior Research Scientist (Molecular Plant Breeding), Biosciences Research Division, Victorian Department of Primary Industries	DPI-Hamilton
Michelle Drayton	Junior Research Scientist (Molecular Genetics), Biosciences Research Division, Victorian Department of Primary Industries	DPI-Bundoora
Rebecca Baillie	Junior Research Scientist (Molecular Genetics), Biosciences Research Division, Victorian Department of Primary Industries	DPI-Bundoora
Melanie Hand	Junior Research Scientist (Molecular Genetics), Biosciences Research Division, Victorian Department of Primary Industries	DPI-Bundoora
Anita Vecchies	Junior Research Scientist (Molecular Genetics), Biosciences Research Division, Victorian Department of Primary Industries	DPI-Bundoora
Kahlil Lawless	Junior Research Scientist (Molecular Genetics), Biosciences Research Division, Victorian Department of Primary Industries	DPI-Bundoora
Jean Lamb	Technical Assistant (Molecular Plant Breeding), Biosciences Research Division, Victorian Department of Primary Industries	DPI-Hamilton
Mary Knight	Technical Assistant (Molecular Plant Breeding), Biosciences Research Division, Victorian Department of Primary Industries	DPI-Hamilton
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Darren Pickett	Technical Assistant (Molecular Plant Breeding), Biosciences Research Division, Victorian Department of Primary Industries	DPI-Hamilton
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Allison Hogg	Ph.D. Student (Molecular Genetics), Biosciences Research Division, Victorian Department of Primary Industries	DPI-Bundoora

9 Collaborations

Dr. Michael Abberton
Department of Plant Breeding, Institute of Grassland and Environmental Research (IGER), Aberystwyth, Wales, United Kingdom
(Genetic map construction and QTL analysis in white clover)

Prof. Charles Brummer
Centre for Applied Genetic Technologies, Crop and Soil Science Department, University of Georgia, USA
(Association genetics and genomics of white clover)

Dr. Michael Francki
Department of Agriculture and Food Western Australia (DAFWA) and State Agricultural Biotechnology Centre (SABC), Murdoch University, Western Australia and Molecular Plant Breeding Cooperative Research Centre
(Comparative genomics of wheat, rice and perennial ryegrass)

Dr. Masahiro Fujimori
Yamanashi Prefectural Dairy Experiment Station (YPDES), Yamanashi, Japan
(Genetic diversity of the crown rust pathogen)

Dr. Jaime Garcia
Instituto Nacional de Investigacion Agropecuaria (INIA), Uruguay
(QTL analysis in perennial ryegrass)

Dr. Benjamin Hayes
Victorian Department of Primary Industries, Biosciences Research
(Linkage disequilibrium genetics and marker implementation simulation modelling)

Prof. Michael Goddard
Victorian Department of Primary Industries, Biosciences Research
(Linkage disequilibrium genetics)

Dr. Alan Stewart
PGG Wrightson Seeds, Christchurch, New Zealand
(Genetic diversity of the crown rust pathogen)

Prof. Toshihiko Yamada
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(Genetic map construction and QTL analysis in perennial ryegrass; Genetic diversity of the crown rust pathogen)