Application of Molecular Technologies in Forage Plant Breeding

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Key points

1. A range of molecular breeding technologies have been developed for forage plant species including both transgenic and non-transgenic methodologies.
2. The application of these technologies has the potential to greatly increase the range of genetic variation that is available for incorporation into breeding programs and subsequent delivery to producers in the form of improved germplasm.
3. Further developments in detailing the phenotypic effect of genes and alleles both through research in target species and through inference from results from model species will further refine the delivery of new forage cultivars.

Keywords: plant breeding, molecular markers, forage, grasses, clovers, transgenic

Introduction

The application of molecular breeding technologies in forage plant breeding offers the potential for more accurate development of cultivars with broader adaptation within a shorter generation time. These benefits are already being realised in other plant species, as shown by the development of genetically modified canola (*Brassica napus*) varieties, and the use of molecular markers for trait selection in Australian barley (*Hordeum vulgare*) (Langridge and Barr, 2003) and wheat (*Triticum aestivum*) (Marshall *et al.*, 2001) breeding programs.

In recent years, a concerted research effort has led to the development of tools for the implementation of molecular breeding technologies in forage species. This paper will discuss the application of both molecular marker-based and transgenic technologies in forage plant breeding, using specific examples from our research programs.

Molecular marker technologies in forage plants

Molecular maps have been described for key forage grass species such as perennial ryegrass (*Lolium perenne* L.) (Hayward *et al*., 1998; Armstead *et al*., 2002; Jones *et al.*, 2002a, 2002b; Faville *et al.,* 2004), meadow fescue (*Festuca pratensis* Huds.) (Alm *et al.*, 2003), tall fescue (*Festuca arundinacea* Schreb.) (Xu *et al*., 1995; Saha *et al*., 2005), as well as pasture legumes such as white clover (*Trifolium repens* L.) (Jones *et al*., 2003; Barrett *et al*., 2004), red clover (*Trifolium pratense* L.) (Isobe *et al*., 2003) and alfalfa/lucerne (*Medicago sativa* L.) (Diwan *et al*., 2000).

The use of these maps to identify quantitative trait loci (QTL) controlling the expression of key agronomic traits is described elsewhere in these proceedings (Yamada and Forster, 2005). However, it is worth noting that in a relatively short time, QTLs have been identified for a large number of traits and this has led to a large increase in the available information on the genomic location of genes controlling key forage species traits. In perennial ryegrass for

instance, QTLs have been identified for resistance to the crown rust pathogen (*Puccinia coronata* f.sp. *lolii*) (Dumsday *et al*., 2003); various aspects of flowering time and reproductive development (Armstead *et al*., 2004; Yamada *et al*., 2004; Jensen *et al*., 2005); and forage quality traits (Cogan *et al*., 2005). Some of the identified QTLs have only accounted for relatively small proportions of the phenotypic variation for the relevant quantitative traits. However, in several instances perennial ryegrass QTLs have been identified in regions of conserved synteny with known QTLs or genes for equivalent traits in other Poaceae species. Syntenic relationships have been inferred between a heading date QTL region in perennial ryegrass and the *Hd3* locus in rice (Armstead *et al*., 2003); a region containing a ryegrass forage digestibility QTL cluster and several lignin biosynthetic genes from wheat (Cogan *et al*., 2005); and a vernalisation response QTL from perennial ryegrass and the *VRN1* locus from wheat (Jensen *et al*., 2005). This co-location information helps to assess the likely biological basis and significance of QTL data derived from forage species, and will be crucial for the choice of QTLs for practical implementation in forage programs. More importantly, the co-location of QTLs that are robust across multiple environments with candidate genes involved in physiological processes correlated with target phenotypes will facilitate the identification of gene-associated single nucleotide polymorphisms (SNPs). Development of SNP markers diagnostic for associated with favourable alleles will permit marker assisted selection in outcrossing forage species free of the complexities associated with use of linkage markers (Spangenberg *et al*., 2005). It is interesting to note that although relatively few studies have been published on QTL locations in forage species, a number of instances of candidate gene-QTL co-location have already been observed. For perennial ryegrass, these include the co-location of the *VRN1* gene with a vernalisation response QTL on LG4 (Jensen *et al*., 2005); the co-location of a casein protein kinase (*Lpck2a-1*) gene (Shinozuka *et al*., in preparation) with a flowering time QTL on LG4 identified by Yamada *et al.* (2004); and the co-location of several lignin biosynthetic genes (Heath *et al*., 1998; Lynch *et al*., 2002; McInnes *et al*., 2002) with QTLs for forage digestibility (Cogan *et al*., 2005). While more extensive research is required to validate positive associations between haplotype and phenotype for putative candidate genes that map to QTL locations, proof-of-concept for this approach is currently being developed for the abiotic stress tolerance gene *Lp*ASRa1 (Spangenberg *et al*., 2005)

Molecular marker technologies in *Neotyphodium* species

Many of the pasture and turf grasses within the *Lolium-Festuca* complex are hosts to symbiotic fungi of the genus *Neotyphodium* (Christiansen *et al*., 1993). Although this symbiosis provides positive agronomic benefits to the plant through the mitigation of nutrient and water stress, the fungi also produce a range of alkaloid compounds that are toxic to grazing herbivores, leading to the disorders known as ryegrass staggers and fescue toxicosis. Recently, a number of endophyte strains have been identified that do not produce certain toxins and these are being marketed in both perennial ryegrass (e.g. AR1, AR6, NEA2) and tall fescue (MaxP, ArkPlus) varieties. SSR markers have been developed to detect genetic variability within and between *Neotyphodium* species (van Zijll de Jong *et al*., 2003) and this technology has been extended to allow *in planta* detection and co-genotyping of *Neotyphodium* strains (van Zijll de Jong *et al*., 2004). As would be expected for an asexually propagated species, the majority of the variation was present between rather than within species (van Zijll de Jong *et al*., 2003). However, 'novel' endophyte strains were clearly distinguishable from many of the wild-type variants (Figure 1) (van Zijll de Jong *et al*., 2005).

Table 1 The identity and incidence of endophyte strains in perennial ryegrass seed lots classified on the basis of endophyte SSR polymorphism

	Endophyte Inoculated	Number of plants	Number infected	AR1	Strain B	Strain C	Unclassified[a]
Line 1	AR1	95	75	75	0	0	0
Line 1	Strain B	90	82	0	79	0	3
Line 2	Strain B	90	67	0	43	23	0

[a] Unable to discriminate endophyte strain.

Development of transgenic technologies in forage plants

The use of biolistic or *Agrobacterium*-mediated transformation allows for the targeted up- or down-regulation of genes coding for individual enzymes in complex biochemical pathways, such as those involved in fructan metabolism or lignin biosynthesis (Spangenberg *et al*., 1998; 2001), or induction of plants to produce novel compounds through the transfer of genes from unrelated organisms, such as the introduction of a bacterial gene coding for fructan production into Italian ryegrass (Ye *et al*., 2001). Genetic modification is particularly useful for elucidation of the role of enzymes in key biosynthetic pathways, and for the modification of traits for which there is no known genetic variation, or that have proven difficult to manipulate through selection and crossing. The number of genes available to plant breeders has rapidly increased with the advent of large-scale gene discovery programs such as those based on expressed sequence tags (ESTs) from relevant target species like perennial ryegrass and white clover (Sawbridge *et al*., 2003a, 2003b) or the whole genome sequencing of model species such as barrel medic (*Medicago truncatula* L.) (Kulikova *et al*., 2004), *Lotus japonicus* L. (Stougaard, 2001) and rice (*Oryza sativa* L.) (Goff *et al*., 2002; Yu *et al*., 2002). The limitation to adoption of gene technologies in breeding programs is not, therefore, the isolation of the genes themselves, but rather the functional annotation of these genes and the 'proof-of-phenotype' in target species.

This paper highlights the use of transgenic technologies in forage plant breeding using two examples: first, manipulation of forage quality in grasses through the modification of lignin biosynthesis is described, and second, improvement of biotic stress tolerance in white clover through the development of plants immune to infection by alfalfa mosaic virus (AMV) using virus coat protein-mediated resistance.

Genetic modification of forage quality in grasses

To date, most of the functional effects of altering the expression of genes involved in lignin biosynthesis have been described in model plant species such as Arabidopsis thaliana L. or tobacco (Nicotiana tabacum L.), the results of which have been reviewed by Casler (2001). In summary, modification of the expression of genes coding for enzymes early in the lignin synthesis pathway such as phenylalanine ammonialyase (PAL) led to a wide range of negative phenotypes, along with general reductions in lignin concentration (Elkind *et al*., 1990) and improved digestibility in tobacco plants with down-regulated PAL activity (Sewalt *et al*., 1997). The results of manipulating the expression of down-stream enzymes in the phenylpropanoid or monolignol pathways have been more promising, with down-regulation of expression of the key enzymes caffeic acid O-methyl transferase (OMT) and cinnamyl

alcohol dehydrogenase (CAD) leading to favourable changes in lignin composition and digestibility (Bernard Vailhé *et al*., 1996; Sewalt *et al*., 1997). Casler (2001) noted that the phenotypic expression of these changes was similar to that obtained with natural variants selected for altered lignin concentration or digestibility, but that the extreme phenotypes were relatively common compared to the rarity of natural variants. The other aspect of transformation-generated variation is that it will allow the targeted manipulation of combinations of enzymes in complex pathways, to facilitate the development of extreme phenotypes that are outside the range of natural variation.

Recently, perennial ryegrass homologues of 3 key genes in the monolignol biosynthesis pathway have been cloned and characterised: CAD (Lynch *et al*., 2002), 4-coumarate:CoA-ligase (4CL) (Heath *et al*., 1998) and cinnamyl CoA reductase (CCR) (McInnes *et al*., 2002). Transgenic perennial ryegrass plants with sense and anti-sense regulation of these genes are currently being generated, and will provide the opportunity of assessing phenotypic changes in digestibility of perennial ryegrass. The role of down-regulating OMT in altering the digestibility phenotype of transgenic tall fescue plants has already been demonstrated in tall fescue (Chen *et al*., 2004) with digestibility increased by approximately 10% in some plants, although variation of the effect on increased in digestibility was observed. Further understanding of the cause of this variation will lead to optimisation of transgenic breeding strategies and aid the development of routine phenotypic screening programs for the deployment of transgenic technologies to improve grass digestibility.

Development of AMV resistant white clover

Our most advanced application of molecular breeding of forages using genetic modification is the development of white clover that is immune to alfalfa mosaic virus (AMV) (Emmerling *et al*., 2004). AMV, white clover mosaic virus (WCMV) and clover yellow vein virus (CYVV) are members of the *Bromoviridae*, potexvirus group and *Potyviridae* respectively, and are estimated to cause combined losses to the Australian rural industries of more than $A800 million per year. Infections with these viruses result in reduced foliage yield, reduced nitrogen-fixing capacity and reduced vegetative persistence and can affect the production potential of white clover pastures by up to 30% (Campbell and Moyer, 1984; Dudas *et al*., 1998; Garrett, 1991; Gibson *et al*., 1981; Latch and Skipp, 1987; Nikandrow and Chu, 1991).

Even though potential sources of tolerance or resistance to AMV, CYVV or WCMV have been described in *Trifolium* species and *Medicago sativa* L. (Barnett and Gibson, 1975; Crill *et al*., 1971; Gibson *et al*., 1989; Martin *et al*., 1997; McLaughlin and Fairbrother, 1993), conventional breeding programs have not been successful. This is mostly due to limitations imposed by virus strain variability, lack of durability of natural resistance and barriers to interspecies sexual and/or somatic hybridisation. Chemical control of insect, fungal or nematode vectors is environmentally unacceptable and economically non-viable for forage legumes.

White clover was transformed with a binary vector carrying a chimeric gene for expression of a cDNA corresponding to AMV RNA4 using *Agrobacterium* mediated transformation (Ding *et al*., 2003). After selection, putative transgenic plants were analysed for the presence and copy number of the transgene by Southern hybridisation as well as levels of expression of the transgene by northern and western hybridisation analyses. The plants were clonally propagated and evaluated in a field trial in Hamilton, Victoria. Over a 2-year period, the plants proved to be immune to heavy natural aphid-mediated AMV challenges. White clover

plants originating from 2 independent transformation events, H1 and H6, were chosen for the development of elite germplasm due to their high level of expression of the transgene (see Figure 2) and the high titre of the AMV coat protein (AMV-CP; data not shown) as well as their AMV immunity phenotype and agronomical performance during the field trials.

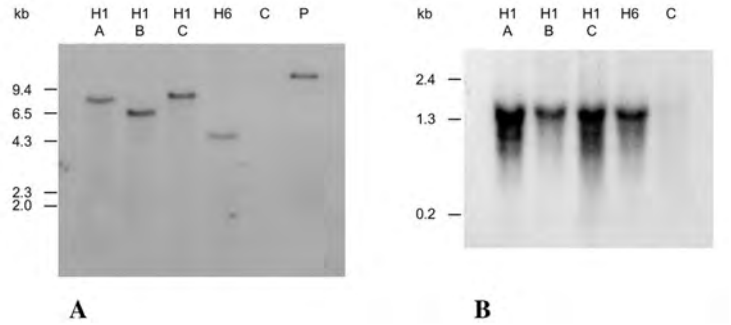

Figure 2 Molecular analysis of T_0 AMV-CP transgenic plants. A) Southern hybridisation analysis with genomic DNA isolated from white clover plants obtained from 4 independent transformation events. C indicates wild type (negative) control, P indicates plasmid (binary vector) control. B) Northern hybridisation analysis with RNA isolated from leaves of the same white clover plants. C indicates wild type (negative) control. Both blots were hybridised with an AMV CP cDNA probe.

The two selected transgenic AMV-resistant white clover lines, H1 and H6, were crossed with the parents of the white clover cultivar "Mink" and subjected to an elite germplasm development strategy designed to bring the transgene to homozygosity while minimising inbred depression (Kalla *et al*., 2000). More than 8,000 T_2 offspring of these crosses were analysed by real time-PCR (RT-PCR), and a total of 1,300 plants homozygous for the AMV-CP transgene were identified, 888 derived from the H1 event, 412 from the H6 event.

A spaced plant field trial was subsequently established in Hamilton, Victoria, to evaluate the 1,300 transgenic white clover T_2 progeny (see Figure 3). The plants were assessed for virus infection with AMV four and five months after being established in the field. None of the transgenic plants showed any sign of virus infection whereas 28% of the non-transgenic wildtype control plants were infected with AMV.

An initial selection of agronomically superior plants comprised 179 H1-derived and 104 H6-derived elite transgenic clover plants. The selection was based on the basis of plant height, stolon density, leaf length, internode length, flower number, summer growth and survival, and autumn and spring vigour. During the second growth season, a further selection out of the initially selected plants led to the identification of 21 H1-derived and 16 H6-derived elite plants. These plants, resulting from the world's first breeding nursery for white clover, are the Syn_0 parents for the production of agronomically superior transgenic AMV-immune white clover elite cultivars. We are currently working to transfer this AMV immunity into other backgrounds, and to combine AMV immunity with non-transgenic sources of resistance to CYVV.

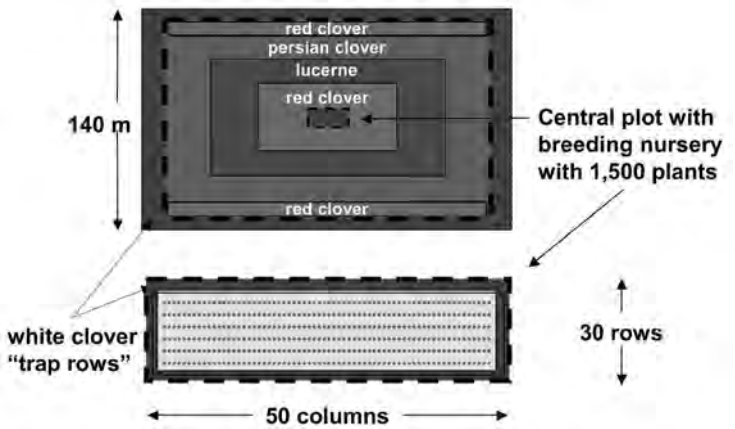

Figure 3 Layout of the spaced plant breeding nursery established in Hamilton, Victoria, to evaluate T_2 of spring of elite transgenic white clover lines homozygous for the AMV-CP transgene (GMAC PR64X2). Checks of non-transgenic control plants (cv. "Mink", total of 200 plants) are uniformly distributed among the 1,300 transgenic T_2 white clover plants.

Conclusions

The application of molecular breeding technologies in forage plant breeding is beginning to deliver on the promises of delivering novel genetic variation (e.g. AMV immune white clover) and more precise understanding of the nature of the genetic variation underlying key phenotypic effects (e.g. genetic variation in *Neotyphodium*, co-location of candidate genes and QTLs for forage quality and flowering time). Recent developments in genomics will greatly increase this genetic knowledge and provide candidate genes that are available for deployment in forage plant breeding. The development of robust phenotypic assays and molecular breeding strategies will ensure that these advances are efficiently captured and utilised to develop improved forage cultivars for the benefit of industry.

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