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Designer Pasture Plants: From Single Cells to the Field

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Pasture occupies more land area than any other crop and it of tremendous value as livestock feed. Ryegrasses (*Lolium spp.*) and white clover (*Trifolium repens* L.) are among the most important forage plants in the world. The demand for high quality forages continues to grow. Elite pasture plants need to demonstrate tolerance to both biotic and abiotic stresses, including drought-stress, low temperatures, diseases and insect pests, without compromising their forage quality and productivity. Generation of this type of advanced forages is beyond the scope and speed of conventional plant breeding. Functional genomics has greatly increased our understanding of mechanisms that determine the genetic, molecular and biochemical basis of economically important traits in forage plants and allow plants to develop and adapt to a dynamic environment. In the post-genomics era, we need to convert this information into practical benefits for farmers and the agricultural sector. This has required multi-disciplinary approaches that exploit advances in molecular genetics, functional genomics and computational biology as well as close collaboration with plant breeders. This review discusses recent progress in finding the molecular and biochemical basis of quality traits in white clover and ryegrass which will enable developing transgenic plants with improved forage quality, yield and adaptation to the environment.

Keywords: *fructan, lignin, metabolites, organic acids, plant biotechnology, proanthocyanidin, ryegrass, white clover*

INTRODUCTION

White clover (*Trifolium repens* L.) and ryegrasses (*Lolium spp.*) are major components of temperate improved pastures, worldwide, and are key forage plants in countries with intensive livestock production systems (Forster and Spangenberg, 1999). These species are commonly used for forage throughout mainland Europe, the United Kingdom, New Zealand, Australia, USA and Japan. White clover has many benefits for grazing systems including symbiotic nitrogen fixation, the production of forage with high protein content and the accumulation of many natural products, namely, flavones, flavonols, methoxyflavonols, coumestans, isoflavans, anthocyanins and proanthocyanidins (condensed tannins). Most of these products are known to have important functions in plants, including the attraction of pollinators and agents of seed dispersal to flowers and fruit, pollen development, signalling associated with plant-microbe interactions, and the protection of plants from ultraviolet radiation, herbivores and pathogens (Dixon and Sumner, 2003). The most commercially-important ryegrasses are Italian or annual ryegrass (*L.multiflorum* Lam.) and perennial ryegrass (*L.perenne* L.). Perennial ryegrass has a number of attributes that have led to the widespread usage of the species as a forage crop. These include high digestibility, persistence in pastures with a high density of tillering, resistance to treading and a strong response to nitrogenous fertilisers (Jung et al., 1996).

Modification of proanthocyanidin (PA) biosynthesis in white clover. The agronomic importance of PAs lies in their ability to suppress bloat-inducing characteristics of some forage legumes, including white clover and alfalfa, by binding to dietary plant proteins. The large protein component in the leaves of these plants is rapidly fermented by rumen microorganisms, generating protein foams that can trap rumenal gases and lead to pasture bloat, a disease that is estimated to cost the Australian pastoral industry over $AU100 million each year. The presence of a low level of PAs (2-4% of dry weight) in forage can prevent bloat and improve the efficiency of protein uptake by ruminants, leading to increased milk, meat and wool production (Wang et al., 1996).

The forage value of white clover is compromised by the vegetative tissues having an insignificant level of PAs (Aerts et al., 1999). 4-dimethylaminocinnemal-dehyde (DMACA) staining has shown that Pas and/or their monomers are not detectable in white

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clover foliage outside of glandular trichomes. In contrast, PAs are produced at a high level in the floral organs of these plants. Since flowering is seasonal, the development of white clover germplasm that produces the optimal level of PAs in foliage for bloat safety is very desirable and at the same time a challenge for biotechnology.

PA production in leaves might be enhanced by increasing the expression domain of flavonoid pathway enzymes that are normally active in white clover flowers, but not in foliage. An alternative approach involves metabolic reprogrammimg of the flavonoid pathway that produce other molecules in leaves, such as anthocyanins, to the PA-specific branch. However, these strategies are complicated by a lack of detailed information about PA biosynthesis and its regulation in forage legumes and factors that limit the rate of PA production in white clover foliage. For example, the molecular basis for the transport of PA monomers from the cytoplasm to the vacuole, and their polymerisation is poorly understood. To complicate matters further, some flavonoid pathway enzymes are encoded by multigene families. This suggests that specific isoforms may be involved in the biosynthesis of particular flavonoids.

Metabolic channelling involves the direct transfer of intermediates between enzymes that form consecutive steps in a metabolic pathway, within a multi-enzyme complex. In theory, the compartmentalisation of biochemical reactions increases the local concentrations of intermediates and prevents unstable intermediates from reacting with other components of the cell. The regulation of genes encoding components of specific multienzyme complexes, or metabolons, in response to stress or developmental cues could explain why particular flavonoids, including PAs, have a restricted pattern of production in plants (Dixon et al., 2005). From another perspective, the mutually exclusive assembly of metabolons could allow cell-type-specific biosynthesis of particular flavonoids, such as PAs, anthocyanins or isoflavonoids, from common intermediates in the phenylpropanoid pathway (Jorgensen et al., 2005).

Several transcription factors have been tested for their ability to influence the accumulation of flavonoids, anthocyanins or PAs when expressed in heterologous plant systems (Robbins et al., 2003). Expression of *Lc*, a maize MYC-family transcription factor, in alfalfa enhanced the production of anthocyanins and PA under conditions of high light intensity and low temperature. Constitutive expression of *Sn*, another MYC-family factor from maize, in *Lotus corniculatus* increased the level of anthocyanin accumulation in leaf mid-ribs, leaf bases and petioles and enhanced PA accumulation in leaf tissues known to synthesize PAs (Robbins et al., 2003). Simultaneous overexpression of *TT2*, *PAP1* and *Lc*, three key transcription factors involved in both anthocyanin and PA biosynthesis, resulted in proanthocyanidin synthesis throughout young leaves and cotyledons of transgenic *Arabidopsis* plants, followed by death of the plants 1-2 weeks after germination. It was interesting that combined overexpression of PAP1 and anthocyanidin reductase (BANYULS) in tobacco leaves resulted in the production of (epi)-flavan-3-ols (epicatechins), which are PA monomers produced by anthocyanidin reductase activity (Xie et al., 2006). The accumulation of epicatechin and gallocatechin monomers, and a mixture of dimers and oligomers consisting primarily of epicatechin units have been detected in these transgenic plants. Overexpression of the BANYULS gene in leaves of the forage legume *Medicago truncatula*, in which anthocyanin pigmentation is visible, resulted in the production of a specific subset of PA oligomers (Xie et al., 2006).

Aluminium tolerance in white clover. Acidic soils have been estimated to occur on more than 40% of the Earth's land area and restrict the growth of many agriculturally important plant species. Soils may be naturally acidic or may become acidic due to human activity, including certain farming practices or acid rain as a consequence of industrial processes. Australian soils are acidic because they are geologically old and have been leached of most of their minerals apart from silicates and metal oxides. In Victoria (Australia) 35% of agricultural soils has a surface pH of less than 4.8 (Hamblin, 2001). The problem is compounded by these soils having low levels of phosphorus, which is an essential nutrient for crops and improved pastures.

On a small-scale, the addition of calcium carbonate (lime) to soils is the only effective way to increase the pH. However 'liming' is costly where large areas of land are affected and is ineffective at countering subsoil acidity (Ridley et al., 2001).

Soil acidification is caused by the decomposition of organic matter and the leaching of nitrates through the soil profile. In Australia, the use of legumes in pasture improvement has greatly increased the level of organic matter in underlying soils. Furthermore, symbiotic nitrogen fixation by legumes grown as food crops or for animal fodder is associated with nitrate leaching. In general, plants take up more cation nutrients than anions from the soil (Ridley et al., 2001). In farming systems, the loss of plant products from a cropping site of growth or the concentration of animal waste because of the behaviour of grazing animals results in a depletion of cation nutrients and a pH increase (Ridley et al., 2001). Hence, the use of legumes and symbiotic nitrogen

fixation, key to the success of dryland agriculture, also leads to soil acidification in the long-term.

The solubilisation of toxic cations is an important indirect consequence of soil acidity. Aluminum (Al) is the most abundant metal in the earth's crust and comprises some 7% of its mass, but free aluminium ions (Al^{3+}) are toxic to plants. In acidic soils, Al and manganese ions, which are toxic to plants, are solubilised, but calcium and magnesium salts, especially phosphates, are less soluble. Al^{3+} toxicity inhibits cell elongation and division, and causes plants to develop stunted root systems with a limited capacity for water and nutrient uptake (Lazof et al., 1994). If soils are acidic, phosphorus applied to soils as fertilisers is rapidly bound in a complex with Al^{3+} ions and cannot be utilised by plants, limiting productivity. Since white clover is particularly sensitive to Al^{3+} toxicity, germplasm with elevated Al^{3+} tolerance could increase the productivity of pasture in areas affected by soil acidity.

It is now clear that organic acid metabolism forms the basis for Al tolerance in many plant species. Some organic acids are able to chelate Al^{3+}, rendering it non-toxic to plant cells. Hue et al. (1986) assessed the ability of a range of organic acids to protect plant roots from Al toxicity in hydroponic culture. They found that organic acids with hydroxyl and carboxyl groups, which can form stable ring structures with Al^{3+} consisting of 5- or 6-bonds, confer the greatest protection from Al^{3+} toxicity. Citric, oxalic and malic acids are commonly found in plants and fit this criterion. Al-tolerant genotypes of many plant species exude these organic acids from roots in response to Al and subsequent chelation of Al^{3+} ions at the root-soil interface is believed to be the basis for Al-tolerance by external detoxification (Ryan et al., 2001).

A number of research groups have modified the expression of enzymes involved in organic acid biosynthesis in order to improve the Al-tolerance of crop plants. The Al^{3+} tolerance of canola (*Brassica napus*), *Arabidopsis thaliana*, tobacco (*Nicotiana tabacum*) and alfalfa (*Medicago sativa*) has been reported to be enhanced by the overexpression of citrate synthase (CS) or malate dehydrogenase (MDH) genes derived from plants or bacteria. Other strategies include the overexpression of the ALMT1 gene encoding a malate transporter, which is associated with malate efflux and Al tolerance in wheat (Delhaize et al., 1993, 2004). Transgenic barley overexpressing ALMT1 showed a high level of Al tolerance when grown in either hydroponic culture or acidic soil. Transgenic white clover plants expressing an endogenous nodule-enhanced MDH gene (TrneMDH) gene under the control of constitutive and root tip-specific promoters were highly Al-tolerant and haematoxylin staining of the root tips provided evidence for an Al exclusion mechanism (Figure 1A-D; Labandera et al., manuscript in preparation).

Modification of lignin biosynthesis in ryegrass. Temperate grasslands support most of the world's milk and meat production. Presently grass and forage account for 75% of feed requirements, although this varies from 60% of the feed for some dairy cows to up to 90% for sheep (Wilkins and Humphreys, 2003). The digestibility of forage grasses has a great impact on animal nutrition and the productivity of ruminant livestock. One of the most important factors affecting grass-based forage quality is the level and composition of lignin, a structural phenolic compound produced by. In general, the level of lignin in forage is inversely proportional to digestibility, since the cell walls of highly lignified plants are resistant to degradation by rumen bacteria and the nutrients are inaccessible to the animal. For example, a 5-6% increase in the level of digestibility of perennial ryegrass was predicted to increase summer milk production in southern Australia by 27%.

Lignin is a complex phenolic polymer synthesized exclusively by plants and is the second most abundant terrestrial biopolymer after cellulose. Lignin provides strength and rigidity to cell walls in plant tissues including xylem, which transports water throughout the plant, and sclerenchyma and bundle sheath cells, which form a natural barrier to microbial pathogens. In grasses, lignin is composed of three main monomer species, termed p-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) subunits, which differ in the number of methoxyl groups on the aromatic ring. A high level of S subunits can reduce digestibility because of cross-linking between highly methoxylated S subunits and arabinoxylans, another major cell wall component (Pond et al., 1987). In ryegrasses, lignin accumulation has been detected in three groups of cells, epidermal cells, sclerenchyma ring cells and vasculature (Figure 1E, 2F). H, G and S lignin differentially accumulate at different stages of ryegrasses development. H and G lignin accumulate at early, vegetative stages. Transition to flowering is associated with strong accumulation of S lignin within sclerenchyma group of cells.

Lignin biosynthesis has been successfully downregulated in both monocotyledons and dicotyledons by targeting various genes in the biosynthetic pathway using cosuppression, antisense and double-stranded interfering RNA (dsRNAi) approaches (Gressel and Zilberstein, 2003). In monocotyledons, downregulation of the gene encoding cinnamoyl alcohol dehydrogenase (CAD) in tall

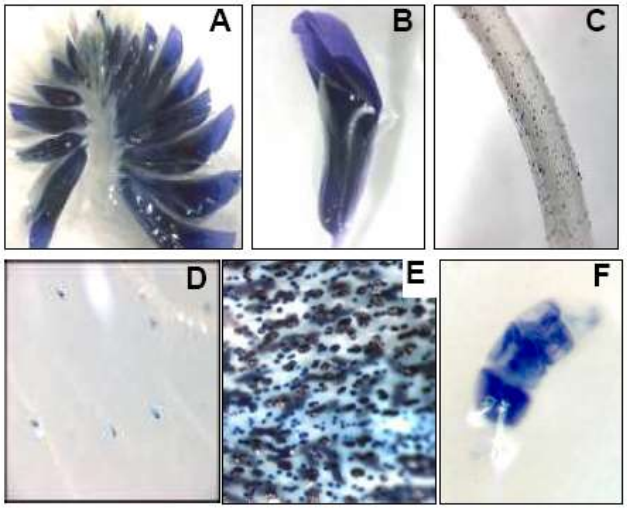

Figure 1. Histochemical staining of proanthocyanidins with 4-dimethyaminocinne-maldehyde (DMACA) in different organs of white clover (A-D) and *Lotus corniculatus* plants (F): white clover inflorescence (A), flower (B), stolon (C), leaves (D), trichomes (E), *L.corniculatus* leaves (F).

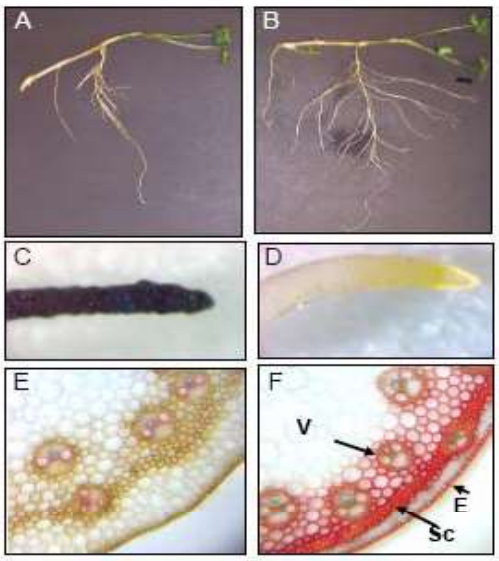

Figure 2. (A-D) Aluminium-tolerant phenotypes of white clover plants expressing a transgene encoding a clover homologue of the nodule-enhanced malate dehydrogenase (TrneMDH) under the control of a constitutive (2x35S) or root-specific (PT1) promoter. Root growth in wild-type (A) and transgenic plants expressing the 2x35S::Tr neMDH transgene in solid media containing 500 µM $AlCl_3$ (B); hematoxylin staining of aluminium in the roots of wild-type (C) and transgenic PT1:Tr neMDH plants (D) grown in the presence of 500 µM AlCl3 (E, F). Histochemical (Mdule) staining of G (brown) - and S (red) - lignin in perennial ryegrass plants at different stages of development: elongation stage (E) and reproductive stage (F). E, epidermal cells, Sc, sclerenchyma cells, V-vascular cells.

fescue plants (*Festuca arundinacea* Schreb.) increased the dry matter digestibility by 7.2% to 9.5%. Transgenic maize plants in which an O-methyltransferase (OMT) gene had been downregulated had an average of 20% less lignin in stems and 12% less lignin in leaves than controls. On a whole-plant basis, lignin was reduced by an average of 17%, but by up to 31%, compared to controls. The digestibility of leaves and stems of transgenic plants was 2% and 7% higher, respectively, than that of wild type plants. On average, whole transgenic plants were 4% more digestible than controls.

The impact of reduced lignin levels on plant fitness has been tested in a range of natural mutants and in transgenic plants where lignin biosynthesis was modified. Increased levels of lodging, reduced yields and pathogen and insect susceptibility were expected to correlate with reduced lignin levels. It was interesting that several studies did not find a high rate of lodging in the maize brown midrib mutants, bm1, bm2, bm3 and bm4 (Weller et al., 1985). After analysis of 15 bm3 lines and isogenic wild type lines, Lee and Brewbaker (1984) showed that the bm3 gene was not linked to genes associated with yield reduction and reduced photosynthesis, which are pleiotropic effects of bm3. To date, a deleterious effect of lignin content on plant fitness has not been documented in forage grasses. Downregulation of the CAD gene in tall fescue de-

creased lignin content, but no differences between control and transgenic plants were observed in terms of time to plant maturity, height,
growth habit, tillering, seed yield, lodging, and pest or pathogen susceptibility. In some cases a reduced level of lignin had a neutral or even a positive effect on agricultural fitness. For example, Hu et al. (1999) showed that leaf, stem and root growth were enhanced in transgenic poplar (*Populus tremuloides* Michx.) that had 45% less lignin than the wild type.

Although a low level of caffeic acid O-methyltransferase (COMT) activity in the bm3 maize mutant affected grain and dry matter yields, the sorghum COMT mutations, bmr-12, bmr-18, did not significantly affect plant fitness. Reduced CAD activity in the sorghum bmr-6 mutant affected the dry matter yield, height, tillering and abiotic stress resistance of plants. In contrast, a maize CAD mutation, bm1, had no phenotype apart from a reduction in the days to flowering. Since COMT and CAD are encoded by multigene families these data may suggest that the isoforms of these enzymes have different roles in lignin biosynthesis. Interactions between modified genes, the plant genome and the environment may be another explanation for the unexpected results. Hence, it is important that genetic modifications affecting lignin content are evaluated in a range of genetic backgrounds and environments, and that the roles of multiple isoforms of lignin-related enzymes are evaluated.

Modification of fructan metabolism in ryegrass. One important aspect of nutritional value for ruminants is the availability of watersoluble carbohydrate that can easily be fermented in the rumen. In addition to starch, the major form of carbon storage in plants, 12-15% of higher plants produce fructan, an alternative storage polysaccharide that is essentially a water-soluble polymer of fructose derived from sucrose (Turner et al., 2006). Fructan, in combination with sucrose, glucose, raffinose and myoinositol, could also have a role in tolerance to abiotic stress. High levels of fructan accumulate in ryegrasses and fescues in response to drought stress and cold treatment (Amiard et al., 2003). In several species, fructan level and composition change when plants experience drought stress (de Roover et al., 2000). Transgenic tobacco (*Nicotiana tabacum*) and sugar beet (*Beta vulgaris*) plants that accumulated higher fructan levels than the wild type displayed slightly elevated drought tolerance, when compared to control plants (Pilon-Smits et al., 1999). Some researchers have proposed that fructan may directly stabilize membranes under stress conditions (Hincha et al., 2002).

Fructan molecules have a wide range of branched forms and have chain lengths of between three and a few hundred fructose units. The fructan accumulated by the temperate forage grasses and cereals is structurally distinct from the other fructan classes (Pollock and Cairns, 1991). Fructan metabolism is limited by the availability of the sucrose precursor molecule, its conversion to fructose by invertases (INV) and the balance between the activities of fructosyltransferase (FT) and fructan exohydrolase (FEH) enzymes that catalyse fructan biosynthesis and degradation, respectively (reviewed by van den Ende et al., 2004). The regulation of fructan metabolism in grasses is still poorly understood.

The fructan profile of perennial ryegrass includes inulin series, inulin neoseries and levan neoseries fructans (Pavis et al., 2001a, 2001b). The most abundant trisaccharides present in perennial ryegrass are 1-kestose and 6G-kestose, with 6-kestose being present in significantly smaller amounts (Pavis et al., 2001b). It has been proposed that at least four enzymes are required to produce this complement of fructan: sucrose:sucrose 1-fructosyltransferase (1-SST), fructan:fructan 1-fructosyltransferase (1-FFT), 6-glucose fructosyltransferase (6G-FT) and sucrose:fructan fructosyltransferase (6-SFT) (Pavis et al., 2001b). Complementary DNA sequences encoding a vacuolar invertase, a cell wall invertase, 1-SST, 1-FFT, 6G-FT and FEH have been isolated and partially characterised in perennial ryegrass (Chalmers et al., 2005).

Conventional plant-breeding approaches have successfully produced high-sugar ryegrasses that improve protein utilization by ruminants, and boost milk and meat production whilst reducing nitrogen losses in waste products (Miller et al., 2001). Greater understanding of the underlying regulation of water-soluble carbohydrate content and the importance of fructan will benefit future breeding programmes.

Transgenic plants expressing genes encoding plant-derived fructosyltransferases showed changes in the level and composition of fructan. Examples include the expression of the barley 6-SFT gene in tobacco (*Nicotiana tabacum*) and chicory (Sprenger et al., 1997), expression of the Jerusalem artichoke 1-SST and/or 1-FFT gene in sugarbeet (*Beta vulgaris*) and petunia (*Petunia hybrida*) and expression of the globe artichoke 1-SST and/or 1-FFT gene in potato (*Solanum tuberosum*) (Hellwege et al., 1997). Transgenic perennial ryegrass (*Lolium perenne*) plants that overexpress wheat genes encoding sucrose:fructan 6-fructosyltransferase (6-SFT) and sucrose:sucrose 1-fructosyltransferase (1-SST), showed significant increases in fructan levels and increased tolerance to freezing. Transgenic perennial ryegrass plants with enhanced level of fructan grown under field conditions in Australia is shown in Figure 3.

Challenges and future developments. The agri-

Figure 3. Field trial for transgenic perennial ryegrass plants with modified level of lignin in Australia.

cultural importance of white clover and ryegrasses make them an attractive target for multi-disciplinary research and metabolic engineering. This has led to substantial progress in our understanding of complex pathways that protect these plants from a wide spectrum of biotic and abiotic stresses and determine forage quality. However, the complexity of these pathways and our incomplete understanding of them is a significant challenge to metabolic engineering. PA, lignin and organic acids are produced by complex, branched pathways where some enzymes are represented by multigene families. Studies aiming to provide a better understanding of pathway regulation and to determine whether these metabolites are produced by differentially assembling metabolons, is an important step towards the metabolic reprogramming of pasture plants. This work is likely to be aided by recent improvements in structural analyses of enzymes and the measurement of RNA expression, protein production and metabolite accumulation in single cells or cell-layers.

The biosynthesis of some metabolites is restricted to specific plant cell types. Bulk sampling methods cause a loss of spatial resolution, but there has been progress in the development of methods for the analysis of specialised plant cells. For example, lasercapture micro-dissection (Schad et al., 2005) and the extraction of cell contents from single cells (Brandt et al., 1999) have recently been modified for the collection of material from plant sections. Coupling of these sampling methods to transcriptomic, proteomic and metabolomic techniques should soon allow the nano-level identification of cell-type-specific transcription factors, enzymes and metabolites. Hence, the integration of technologies from a wide range of disciplines should greatly enhance our ability to convert interesting discoveries about forage plants into practical solutions that will improve the productivity, economic value and environmental sustainability of pasture-based agriculture.

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