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Designer Pasture Plants: From Single Cells to the Field

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ABSTRACT

Pasture occupies more land area than any other crop and it is of tremendous value as livestock feed. Ryegrasses (*Lolium* spp.) and white clover (*T. repens*) 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 these 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 bases of quality traits in white clover and ryegrass which will enable the development of transgenic 'designer' cultivars with improved forage quality, yield and stress tolerance.

Keywords: fructan, lignin, metabolites, organic acids, transgenic, proanthocyanidin, ryegrass, white clover
Abbreviations: Al, aluminum; **DMACA**, 4-dimethylaminocinnemaldehyde; **PA**, proanthocyanidin

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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).

Over the last decade, an intensive research effort has been focussed on determining the genetic, molecular and biochemical bases for economically-important traits in forage plants. This has required multi-disciplinary approaches that exploit advances in molecular genetics, functional genomics and computational biology, in close collaboration with plant breeders. This review covers recent progress in finding the molecular basis for a range of forage quality traits in pasture plants, with a focus on white clover and ryegrass. The forage improvement strategies described in this review are likely to become key technologies for the improvement of quality traits for other forage legumes and grasses.

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 micro-

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Invited Review

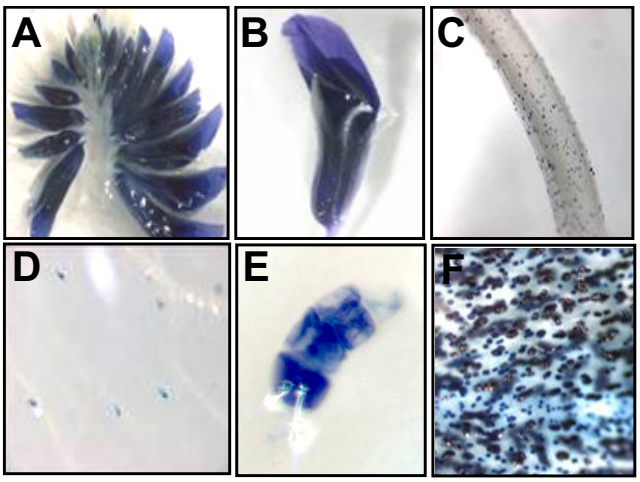

Fig. 1 Histochemical staining of proanthocyanidins with 4-dimethyaminocinnemaldehyde (DMACA) in different organs of white clover (A-D) and *Lotus corniculatus* plants (**F**): white clover inflorescence (**A**), flower (**B**), stolon (**C**), leaves (**D**), trichomes (**E**), *L. corniculatus* leaves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

organisms, 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-dimethylaminocinnemaldehyde (DMACA) staining has shown that PAs and/or their monomers are not detectable in white clover foliage outside of glandular trichomes (**Fig. 1**). In contrast, PAs are produced at a high level in the floral organs of these plants (Foo *et al*. 2000; **Fig. 1**). 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 reprogramming of flavonoid biosynthesis in order to divert intermediates from branches 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, molecular mechanisms underlying for the transport of PA monomers from the cytoplasm to the vacuole, and their polymerisation, are 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 catalyse consecutive steps in a metabolic pathway, within a multi-enzyme complex (Winkel 2004). 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 multi-enzyme 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 (Winkel 2004; 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 (Bradley *et al*. 1998; de Majnik *et al*. 2000; Bovy *et al*. 2002; Ray *et al*. 2003; 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 (Ray *et al*. 2003). Constitutive expression of *Sn*, another MYC-family factor from maize, in *Lotus corniculatus* increased the level of anthocyanin accumulation in leaf bases, midribs 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 (Sharma and Dixon 2005). 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 (Ma *et al*. 2001). 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 (Ridney *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 (Dolling *et al*. 2001). 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 cations than anions from the soil (Ridney *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 decrease (Dolling *et al*. 2001; Ridney *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 (von Uexkull and Mutert 1995). In acidic soils, Al and manganese ions, which are toxic to plants, are solubi-

lised 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 (Delhaize *et al*. 1993; 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 improve 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 (Ma *et al*. 2001; 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*) (Anoop *et al*. 2003), *Arabidopsis thaliana* (Koyama *et al*. 2000), tobacco (*Nicotiana tabacum*) (de la Fuente *et al*. 1997), and alfalfa (*Medicago sativa*) (Tesfaye *et al*. 2000) 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 Sasaki *et al*. 2004), which is associated with malate efflux and Al tolerance in wheat (Delhaize *et al*. 2004). Transgenic barley overexpressing *ALMT1* showed a high level of Al tolerance when grown in either hydroponic culture or acidic soil (Delhaize *et al*. 2004). 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 (**Fig. 2A-D**; Labandera *et al*. manuscript in preparation).

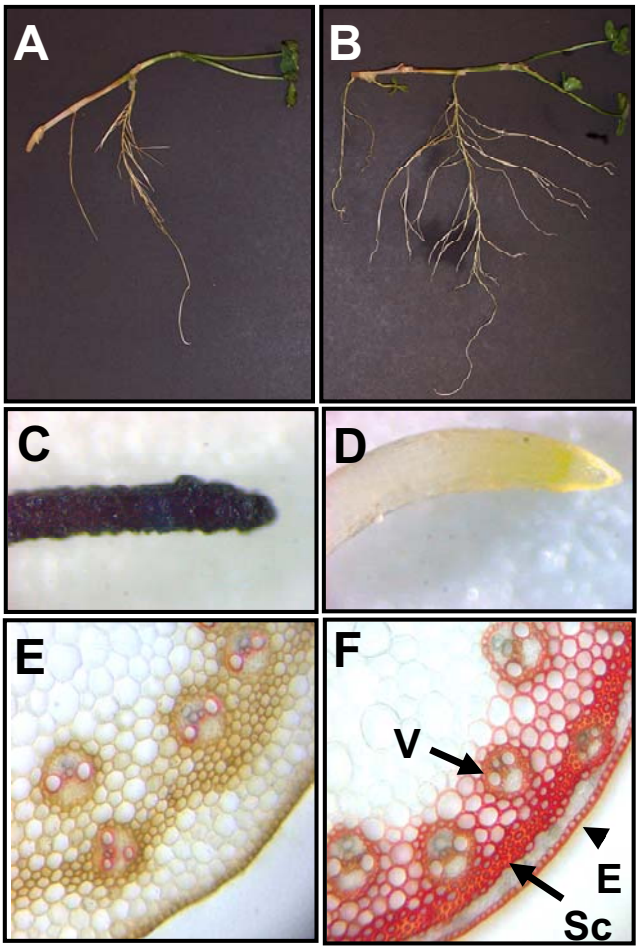

Fig. 2 (**A-D**) Aluminium-tolerant phenotypes of white clover plants expressing a transgene encoding a clover homologue of the nodule-enhanced malate dehydrogenase (*TrneMDH*) under the control of a constitutive (2x35S) or root-specific (*PT1*) promoter. Root growth in wild-type (**A**) and transgenic plants expressing the 2x*35S::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 $AlCl_3$ (**E, F**). Histochemical (Mäule) 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.

MODIFICATION OF LIGNIN BIOSYNTHESIS IN GRASSES

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 plants (Casler *et al*. 2002). 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% (Smith *et al*. 1998).

Lignin is a complex phenolic polymer synthesized exclusively by plants and is the second most abundant terrestrial biopolymer after cellulose (Campbell and Sederoff 1996). 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 (Bird 1988). 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 (**Fig. 2E, 2F**). H, G and S lignin are produced at different stages of development during the vegetative-floral transition in ryegrasses. H and G lignin accumulate during the vegetative stages. Transition to flowering is associated with heavy deposition of S lignin within sclerenchyma cells.

Lignin biosynthesis has been successfully downregulated in both monocotyledons and dicotyledons by targeting various genes in the monolignol pathway using cosuppression, antisense and double-stranded interfering RNA (dsRNAi) approaches (Humphreys and Chapple 2002; Chen *et al*. 2003; Gressel and Zilberstein 2003). In monocotyledons, downregulation of the gene encoding cinnamoyl alcohol dehydrogenase (CAD) in tall fescue plants (*Festuca arundinacea* Schreb.) increased the dry matter digestibility by 7.2% to 9.5% (Chen *et al*. 2003). Transgenic maize plants,

in which a gene encoding O-methyltransferase (OMT) was downregulated, had an average of 20% less lignin in stems and 12% less lignin in leaves than controls (Piquemal *et al*. 2002; He *et al*. 2003). On a whole-plant basis, the total lignin level of transgenic plants was reduced by an average of 17%, and by up to 31%, compared to wild type 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; Inoue and Kasuga 1989, Pedersen *et al*. 2005). 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 decreased 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 (Chen *et al*. 2003). 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 (Pedersen *et al*. 2005). 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 (Pedersen *et al*. 2005). Since *COMT* and *CAD* are encoded by multigene families, these data may suggest that different isoforms have distinct 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 determined.

MODIFICATION OF FRUCTAN METABOLISM IN RYEGRASS

The availability of water-soluble carbohydrate that can easily be fermented in the rumen is an important aspect of nutritional value for ruminants. 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 changes when plants experience drought stress (Spollen and Nelson 1994; Thomas and James 1999; de Roover *et al*. 2000). Transgenic tobacco (*Nicotiana tabacum*) and sugar beet (*Beta vulgaris*) plants that accumulated higher fructan levels than the wild type had slightly elevated drought tolerance, when compared to control plants (Pilon-Smits *et al*. 1995, 1999). Some researchers have proposed that fructan may directly stabilize membranes under stress conditions (Demel *et al*. 1998; Vereyken *et al*. 2001; 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*. 2001a, 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 (Lidgett *et al*. 2002; Chalmers *et al*. 2003; Johnson *et al*. 2003; Gallagher *et al*. 2004; Hisano *et al*. 2004a, 2004b, 2004c; Chalmers *et al*. 2005a, 2005b).

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*) (Sévenier *et al*. 1998) and petunia (*Petunia hybrida*) (van der Meer *et al*. 1998) 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 (Hisano *et al*. 2004c).

TRANSGENIC TECHNOLOGIES

Crop improvement by genetic engineering requires the delivery and integration of defined exogenous genes into suitable explants in tissue culture, followed by regeneration of plants expressing the transgenes. Although methods have been established for the delivery and insertion of DNA into the nucleus of a plant cell, the level of transgene expression is often dependent on other factors, such as transgene copy number, position effect and the possibility of sense-suppression caused by host-specific silencing machineries. Exogenous DNA within the genome of a transformed cell needs to display mitotic and meiotic stability, in order to generate a transgenic plant. Consequently, just a small proportion of integrated transgenes result in a desirable phenotype and are stably inherited.

Over the last two decades a variety of approaches have

been used to introduce exogenous DNA into plants, including microinjection, electroporation, polyethyleneglycol (PEG)- and WHISKERS-mediated approaches, biolistics and *Agrobacterium*-mediated transformation (*A. tumefaciens* and *A. rhizogenes*). The last two approaches are most frequently used for the transformation of a wide range of species (Jackson and Linskerns 2004).

The principle of microprojectile bombardment-mediated transformation is based on physical penetration of the cell nucleus by exogenous DNA, where it is integrated into the genome (Klein *et al*. 1987). This technology is able to deliver exogenous DNA into regenerable cells, tissues or organs, and appears to achieve truly genotype-independent transformation by overcomming most regeneration problems associated with tissue culture (Christou *et al*. 1997; Luthra *et al*. 1997). Furthermore, multiple plasmids can be used to integrate genes of interest at a single locus in the genome of a transgenic plant allowing, for example, the stacking of genes encoding a biosynthetic pathway and avoiding the possibility of segregation in future generations (Hadi *et al*. 1996; Chen *et al*. 1998). Large fragments of exogenous DNA, including yeast artificial chromosomes, can also be introduced into plant genomes using particle bombardment (van Eck *et al*. 1995). The biolistic method of transformation has been used to generate transgenic pasture plants with improved forage quality traits, including a higher level of metabolizable energy, improved digestibility and stress tolerance (Dixon and Reddy. 2003, Ye *et al*. 2001, Chen *et al*. 2003, Hisano *et al*. 2004), and health-related improvements, such as reduced allergenicity (Petrovska *et al*. 2004).

Agrobacterium-mediated transformation is a simple, low cost and highly efficient alternative to direct gene delivery methods. The main advantage of using the *Agrobacterium*-based system is the insertion of a defined segment of DNA into a plant genome. Furthermore, several researchers have taken advantage of the frequency of multi-locus insertion events to integrate the transgene of interest and the selectable marker gene at different loci and then recover marker-free transgenic plants by segregation. *Agrobacterium*-mediated transformation has been successfully adapted to a large number of dicotyledon and monocotyledon plant species (Ding *et al*. 2003; Bettany *et al*. 2003; Wu *et al*. 2005). A highly reproducible, robust and genotype-independent protocol for genetic transformation and regeneration of forage legumes has been developed in our group (Ding *et al*. 2003). It has been successfully applied to *Trifolium* species and cultivars including white clover (*T. repens*, cvs. 'Haifa', 'Huia', 'Irrigation' and 'Mink'), red clover (*T. pratense* cvs. 'Astred', 'Colenso', 'Cherokee', 'Quinequeli', 'Redquin' and 'Renegade'), subterranean clover (*T. subterraneum* ssp. *brachycalycinum* cv. 'Clare', ssp. *subterraneum* cvs. Denmark and Woogenellup and ssp. *yanninicum* cvs. Larisa and Trikkala), *T. michelianum* and *T. isthmocarpum*. This methodology has also allowed the successful transformation of *Medicago* spp. including alfalfa (*M. sativa*), *M. polymorpha*, *M. truncatula*, *M. litoralis* and *M. tonata*. A further development of this methododology, the 'isogenic transformation' approach, provides transgenic and untransformed control plants with the same genetic background (Ding *et al*. 2003).

Rapid and simple *in planta* transformation methods have been described for *M. truncatula* (Trieu and Harrison 1996; Trieu *et al*. 2000). One approach involved the vacuum-infiltration of flowering plants. This procedure had been used previously in *Arabidopsis*. A second approach involved vacuum-infiltration of young seedlings with *Agrobacterium*. Although high transformation efficiencies were reported, ranging from 4.7-76% and 2.9-27.6% for flower infiltration and seedling infiltration respectively, these results have been difficult to reproduce and the methods have not been widely used for forage legume transformation.

Public policy on the development and commercialisation of genetically modified organisms (GMOs) has focused on effective management of environmental and health risks. For example, the persistence of genetic sequences encoding selectable marker genes in commercial field crops is now considered undesirable by most biosafety regulation authorities.

Both biolistic and *Agrobacterium*-mediated transformation strategies are based on the integration of an exogenous gene of interest into the plant genome. These genes could be potentially 'contaminated' with flanking sequences that belong to transformation vector backbones, selectable markers and even short recombination sites introduced into GATEWAY-enabled vectors. Interestingly, detailed molecular analyses of transgenic plants have shown that integration of the vector backbone sequences is associated with both biolistic and *Agrobacterium*-mediated transformation methods (Kononov *et al*. 1997; Wenck *et al*. 1997; DeBuck *et al*. 2000; Yin and Wang 2000).

The integration of vector-derived bacterial sequences, including the backbone, selectable marker and origin of replication, into plant genomes may be a major reason for transgene instability because AT-rich prokaryotic sequences may be recognized by plants as non-self DNA, leading to transgene inactivation by methylation, acetylation or post-transcriptional gene silencing (Jakowitsch *et al*. 1999; Matzke *et al*. 2000). To overcome this potential problem, plants have been bombarded with expression cassettes to avoid the use of plasmid vectors (Fu *et al*. 2000; Breitler *et al*. 2002; Vidal *et al*. 2006).

Generation of selectable marker-free transgenic plants through the *Agrobacterium*-mediated transformation can be achieved by three methods: (i) co-transformation of plant cells using two different *Agrobacterium* strains with different binary vectors containing either the gene of interest or the selectable marker (McKnight *et al*. 1987; DeBlock and Debrouwer 1991; DeNeve *et al*. 1997); (ii) Transformation of plant cells using a single *Agrobacterium* strain containing two separate binary vectors or one binary vector with two T-DNAs (Komari *et al*. 1996, Daley *et al*. 1998), and (iii) sequential transformation of plant cells with two *Agrobacterium* strains containing different vectors (Rommens *et al*. 2004). Marker-free plants could be generated by the segregation of selectable marker and transgene loci by breeding and 'clean' events identified by screening transgene loci for flanking vector-derived sequences.

CHALLENGES AND FUTURE DEVELOPMENTS

The agricultural 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 stress factors 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 in which 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-regulated metabolons, are an important step towards the metabolic re-programming 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 types of plant cell. 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, laser-capture 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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