Transgenesis and Genomics in Molecular Breeding of Forage Plants

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Abstract

Forage plant breeding has been largely based on phenotypic selection following sexual recombination of natural genetic variation found between and within ecotypes. Advances in plant genetic manipulation over the last 15 years have provided convincing evidence that these powerful technologies can complement and enhance plant breeding programs. Significant progress in the establishment of the methodologies required for the molecular breeding of forage plants has been made. Examples of current products and approaches for the application of these methodologies to forage grass and legume improvement are outlined. Large-scale genomic analysis of many organisms is under way with human, arabidopsis and rice genome sequences almost completed. Forage plant breeding is just now entering the genome era. The plethora of new technologies and tools now available for high-throughput gene discovery and genome-wide gene expression analysis have opened up opportunities for innovative applications in the identification, functional characterisation and use of genes of value in forage production systems and beyond. Examples of these opportunities, such as 'molecular phenotyping', 'symbio-genomics' and 'xeno-genomics' are introduced.

Introduction

Grassland agriculture is highly dependent upon a reliable source of forage as the primary feed base for ruminant livestock (Barnes and Baylor, 1995). In most areas of the world, forage production is a low cash-input production system, and thus the most economical way to deliver advanced technology to forage producers is through the genetic improvement of forage plant cultivars (McKersie, 1997). Conventional forage plant breeding has been based on the use of natural genetic variation as found between and within ecotypes or created through sexual recombination (Van Wijk *et al.*, 1993). Biotechnology allows the generation of novel variability as well as more efficient use of existing genetic variability. It offers opportunities to enhance the sources of useful genes accessible for the development of new cultivars and to speed up genetic improvement programs.

The enabling methodologies for the application of molecular technologies to the improvement of key pasture grasses and legumes have been largely developed, and recently reviewed (McKersie and Brown, 1997; Spangenberg *et al.*, 1997; 1998; 2000a; Spangenberg, 1999; Forster and Spangenberg, 1999; Forster *et al.*, 2000). They include the establishment of efficient and robust plant regeneration systems from cells competent for genetic transformation; the combination of whole or partial genomes by somatic hybridization and cybridization through protoplast fusion; the production of transgenic forage plants mainly by *Agrobacterium*-mediated and biolistic transformation using selectable marker and reporter genes; and the establishment of highly informative co-dominant molecular marker systems and their use in the development of framework genetic maps.

Here we focus on describing current and future applications and impact of transgenesis and genomics in pasture plant improvement.

1. Forage Plant Transgenesis

Gene technology and the production of transgenic plants offers the opportunity to generate unique genetic variation, when the required variation is either absent or has very low heritability. In recent years, the first transgenic pasture plants with simple 'engineered' traits have reached the stage of field-evaluation (Austin and Bingham, 1997; Kalla *et al.*; 2000a; White *et al.*, 2000). While gaps in our understanding of the underlying genetics, physiology and biochemistry of many complex plant processes are likely to delay progress in many applications of transgenesis in forage plant improvement, gene technology is a powerful tool for the generation of the required molecular genetic knowledge. Consequently, applications of transgenesis to forage plant improvement are focussed on the development of transformation events with unique genetic variation and in studies on the molecular genetic dissection of plant biosynthetic pathways and developmental processes of high relevance for forage production.

Primary target traits for the application of transgenesis to forage plant improvement are forage quality, disease and pest resistance, tolerance to abiotic stresses, and the manipulation of growth and development. Some representative approaches and selected examples in forage grasses and legumes are discussed below.

1.1. Forage Quality

Molecular breeding based on transgenesis to overcome limitations in forage quality may be targeted to the individual subcharacters involved: dry matter digestibility, water-soluble carbohydrate content, protein content, secondary metabolites, alkaloids, etc. These molecular breeding approaches may include modification of the lignin profile to enhance dry matter digestibility, genetic manipulation of fructan metabolism to increase non-structural carbohydrate content, genetic manipulation of condensed tannin synthesis to develop 'bloat-safe' forages, and the expression of 'rumen by-pass' proteins to improve the supply of proteins and essential amino acids. Most quality or anti-quality parameters are associated with specific metabolic pathways or the production of specific proteins. This allows target enzymes or suitable foreign proteins to be identified, corresponding genes isolated, and their expression manipulated in transgenic forage plants.

1.1.1. Manipulation of Lignin Biosynthesis

Dry matter digestibility of forage plants declines markedly (>10%) as plants flower and senesce (Buckner *et al.*, 1967; Radojevic *et al.*, 1994; Stone, 1994). The changes in dry matter digestibility greatly contribute to the lowering of nutritive value of forage during summer (Stone, 1994). For example, increasing dry matter digestibility has been ranked as the most important goal in genetic improvement of nutritive value of forage grasses for dairy pastures (Smith *et al.*, 1997). However, since heritability of dry matter digestibility is low and a large number of genes control it, the potential for rapid genetic improvement by traditional methods is low (Barnes, 1990).

Lignification of plant cell walls has been identified as the major factor responsible for lowering digestibility of forage tissues as they mature (Buxton and Russell, 1988).

The inhibitory effects of lignin on forage digestibility depend on lignin monomer composition and functional groups, lignin content, and the extent of cross-linking to cell wall polysaccharides (Jung and Vogel, 1986; Sewalt et al., 1997; Casler and Jung, 1999; Casler and Kaeppler, 2000).

Small increases in digestibility are expected to have significant impact on forage quality and concomitantly on animal productivity. A 1% increase in *in vitro* dry matter digestibility has led to an average 3.2% increase in mean live-weight gains (Casler and Vogel, 1999).

Lignification is comprised of a highly coordinated and regulated set of metabolic events resulting in the biosynthesis of lignin precursors (monolignols) and lignins (Figure 1) (Whetten and Sederoff, 1995; Boudet and Grima-Pettenati, 1996; Boudet *et al*., 1996; Campbell and Sederoff, 1996; Dixon et al., 1996).

Molecular breeding for improved digestibility by down-regulating monolignol biosynthetic enzymes through antisense and sense suppression in transgenic forage plants are currently being explored. The main target enzymes being considered are caffeic acid *O*-methyltransferase (COMT), 4-coumarate:CoA ligase (4CL), cinnamoyl CoA reductase (CCR) and cinnamyl alcohol dehydrogenase (CAD). Experiments with the model plants tobacco and poplar have shown that the down-regulation of COMT, CAD and 4CL expression leads to altered lignin composition or reduced lignin content (Halpin *et al*., 1994; Higuchi *et al*., 1994; Ni *et al*., 1994; Hibino *et al*., 1995; Kajita *et al*., 1996; Boudet and Grima-Pettenati, 1996; Bernard Vailhé *et al*., 1996b; 1998; Baucher *et al*., 1996; 1999; Stewart *et al*., 1997). A significant improvement in dry matter digestibility of transgenic sense and antisense COMT tobacco plants showing reduced COMT activity has been demonstrated (Bernard Vailhé *et al*., 1996a; Sewalt *et al*., 1997). A decrease in syringyl lignin units (Bernard Vailhé *et al*., 1996a) or a reduction in lignin content (Dwivedi *et al*., 1994; Sewalt *et al*., 1997) was observed. Similarly, transgenic antisense CAD tobacco and alfalfa plants with down-regulated CAD activity have been reported to produce chemically more extractable lignin which was altered in composition or structure (Halpin *et al*., 1994; Higuchi *et al*., 1994; Hibino *et al*., 1995; Boudet and Grima-Pettenati, 1996; Bernard Vailhé *et al*., 1996b; 1998; Baucher *et al*., 1996; 1999). Furthermore, simultaneous down-regulation of CAD and CCR in transgenic tobacco led to a decrease in lignin content without the alteration in plant development observed in the CCR-down-regulated parental plants. The parental plants had a similar decrease in lignin content (50% of the control) which resulted in the reduction in size and collapse of xylem cells (Chabannes *et al*., 2000). These results indicate that improvement of dry matter digestibility by the introduction of chimeric sense and antisense lignin biosynthetic genes can be achieved apparently without impairing normal development of the plant.

While the basic effect of transgenically manipulating the expression of some enzymes in the monolignol pathway may be similar to that of native genes coding for decreased enzyme activity in brown mid rib (*bmr*) mutants (Casler and Kaeppler, 2000), transgenic approaches offer the potential for transgenic plants with highly-unusual novel lignins, higher frequencies of novel-lignin phenotypes compared to natural variation, effective simultaneous down-regulation of multiple enzymes, and highly targeted down-regulation of enzymes through the choice of cell type-specific and developmentally-regulated promoters.

Transgenic approaches to genetic manipulation of monolignol biosynthesis to enhance herbage quality are currently being explored in forage legumes (e.g. *Stylosanthes humilis*, *Medicago sativa*) and forage grasses (e.g. *Lolium perenne* and

Festuca arundinacea) (L. McIntyre, personal communication; Guo *et al.*, 2000; Spangenberg *et al.*, 2000; Z.Y. Wang, personal communication). Genes (cDNAs and genomic clones) encoding the key enzymes COMT, 4CL, CCR and CAD of perennial ryegrass (*L. perenne*) have been isolated, sequenced, characterised and used for the molecular genetic dissection of this biosynthetic pathway in grasses (Heath *et al.*, 1998; Lynch *et al.*, 2000; McInnes *et al.*, 2000; Spangenberg *et al.*, 2000b).

Once proof of concept and suitable transformation events in forage plants with down-regulation of individual enzymes or simultaneously for multiple enzymes of the monolignol biosynthetic pathway are obtained, a thorough agronomic assessment of these transgenic plants – particularly for vigour and stress tolerances - and hybridization to generate transgenic elite germplasm, with subsequent selection, will be required to produce marketable cultivars.

1.1.2. Manipulation of Fructan Metabolism

Fructans are polyfructose molecules produced by many grass species as their main soluble storage carbohydrate form. It has been shown that ryegrass lines which accumulate higher concentrations of soluble carbohydrates do not suffer as great a decline in digestibility during summer (Radojevic *et al.*, 1994). The increased level of soluble carbohydrates appears to offset the decline in digestibility due to lignification. In addition, herbage intake, protein capture in the rumen and live-weight gains may be improved by increasing the concentrations of non-structural carbohydrates in pasture plants (Michell, 1973; Jones and Roberts, 1991; Beever, 1993).

The introduction of the microbial fructosyltransferase *SacB* gene from *Bacillus subtilis* into fructan-devoid and starch-accumulating tobacco and potato plants led to the accumulation of considerable amounts of high molecular weight levan-type fructans (Ebskamp *et al.*, 1994; Van der Meer *et al.*, 1994). These results demonstrate that sucrose, the substrate for fructosyltranferase, can be efficiently routed into a new sink in non-fructan-accumulating species. Furthermore, transgenic tobacco plants that accumulate bacterial levan showed enhanced performance under drought stress (Pilon-Smits *et al.*, 1995).

Fructan synthesis in grasses involves the concerted action of at least three enzymes; sucrose:sucrose 1-fructosyltransferase (1-SST); fructan:fructan 1-fructosyltransferase (1-FFT); and sucrose:fructan 6-fructosyltransferase (6-SFT) which synthesises the more complex mixed linkage fructans which prevail in grasses and cereals (Figure 2). A number of plant fructan metabolism related genes, such as barley 6-SFT, onion 6G-FFT and artichoke 1-SST, have been isolated in recent years; and have led to oligofructan accumulation when introduced into native fructan-devoid species, and to novel fructan production in native fructan-accumulating plants (Sprenger *et al.*, 1995; 1997; Hellwege *et al.*, 1997; Vijn *et al.*, 1997).

Transgenic approaches for the genetic manipulation of fructan biosynthesis to enhance herbage quality and tolerance to abiotic stresses are being explored in both forage legumes (e.g. *Trifolium repens*, *Medicago sativa*) and forage grasses (e.g. *Lolium perenne* and *Festuca arundinacea*) (Jenkins *et al.*, 2000; Johnson *et al.*, 2000; LePage *et al.*, 2000; Lidgett *et al.*, 2000; Luescher *et al.*, 2000; Terdich *et al.*, 2000; Ye *et al.*, 2000).

Transgenic Italian ryegrass (*L. multiflorum*) plants with altered fructan metabolism brought about by the expression of chimeric bacterial levansucrase genes have been generated (Ye *et al.*, 2000). cDNAs encoding perennial ryegrass (*L. perenne*)

fructosyltransferase homologues have been isolated, characterised and are being used for the systematic molecular genetic dissection of fructan biosynthesis in transgenic grasses (Johnson *et al*., 2000; Lidgett *et al*., 2000; Terdich *et al*., 2000). A cDNA encoding 1-SST from tall fescue (*F. arundinacea*) has been isolated and functionally characterised in transient assays with tobacco protoplasts and in methylotrophic yeast, *Pichia pastoris* (Luescher *et al*., 2000). Transgenic white clover (*T. repens*) plants expressing chimeric *B. subtilis SacB* genes for enhanced tolerance to drought have been produced (LePage *et al*., 2000). Transgenic lucerne (*M. sativa*) and white clover plants expressing a fructosyltransferase gene, derived from *Streptococcus salivarius*, have also been generated (Jenkins *et al*., 2000).

The molecular genetic dissection of fructan biosynthesis in key pasture grasses will enhance our knowledge of fructan metabolism and carbohydrate partitioning in grasses and clarify their functional role in tolerance to cold and drought. This knowledge will be instrumental in designing experimental approaches to produce transgenic forage plants with enhanced forage quality and tolerance to abiotic stresses.

1.1.3. Transgenic Expression of 'Rumen By-Pass' Proteins

Sulphur-containing (S-) amino acids, methionine and cysteine, are among the most limiting essential amino acids in ruminant animal nutrition (Ørskov and Chen, 1989). In particular, wool growth in sheep is frequently limited by the supply of S-amino acids under normal grazing conditions (Reis, 1979; Higgins *et al*., 1989). Rumen fermentation contributes partly to the S-amino acids deficiency, since rumen microflora degrade the feed protein and, in some circumstances, resynthesize proteins with a lower nutrient value (Rogers, 1990). Post-ruminal supplements of methionine and cysteine have been shown to result in a 16 -130% increase in the rate of wool growth (Reis and Schinckel, 1963; Langlands, 1970; Pickering and Reis, 1993). There have also been reports on positive effects of feeding protected methionine on milk production in dairy cows and growth rate in beef animals (Buttery and Foulds, 1988). Therefore, it is predicted that the ingestion of forage containing relatively rumen-stable proteins rich in S-amino acids would enhance the supply of limiting essential amino acids for ruminant nutrition and lead to increased animal productivity, particularly wool growth (Higgins *et al*., 1989; Rogers, 1990).

The production of transgenic forage legumes expressing genes encoding different 'rumen by-pass' proteins rich in S-amino acids, such as chicken ovalbumin, pea albumin and sunflower seed albumin, has been reported (Schroeder *et al*., 1991; Ealing *et al*., 1994; Tabe *et al*., 1995; Khan *et al*., 1996). Low expression levels were observed for the ovalbumin gene in transgenic lucerne (*M. sativa*) and the pea albumin gene in transgenic white clover, where accumulation of the proteins was less than 0.01% of total cell protein (Schroeder *et al*., 1991; Ealing *et al*., 1994). Accumulation of sunflower seed albumin up to 0.1% of soluble leaf protein was achieved in transgenic lucerne when the gene was driven by a *rbcS* promoter from *Arabidopsis thaliana* (Tabe *et al*., 1995). In transgenic subterranean clover (*T. subterraneum*), the accumulation of sunflower albumin increased with leaf age, with old leaves of the most highly expressing plants containing 1.3% of total extractable protein (Khan *et al*., 1996).

Transgenic tall fescue plants expressing chimeric genes carrying sunflower albumin SFA8 cDNA sequences (Kortt *et al*., 1991) with the endoplasmic reticulum retention signal KDEL (Wandelt *et al*., 1992), under control of different promoters were generated through biolistic transformation (Wang *et al*., 2000). Transgenic tall fescue

plants produced the expected transcript and accumulated the methionine-rich SFA8 protein at levels of up to 0.2% of total soluble protein (Figure 3). In order to achieve nutritionally useful levels, expression of the sunflower seed albumin may be required to reach 2 - 5% of total soluble protein. Strategies for increasing the accumulation levels of foreign proteins in the leaves of forage plants are required, if the full potential offered by transgenic approaches to create novel protein-phenotypes of forages is to be captured.

1.1.4. Manipulation of Condensed Tannin Biosynthesis

Condensed tannins (proanthocyanidins) are polymeric phenyl-propanoid-derived compounds synthesised by the flavonoid pathway. They are agronomically important in a range of forage legumes, where they are regarded either as beneficial or detrimental. While at levels above 4 - 5% dry weight, condensed tannins are generally considered to be nutritionally detrimental and act as antifeedants and antinutritional factors for grazing livestock (Barry and Duncan, 1984; Waghorn *et al.*, 1990; Morris and Robbins, 1997), moderate amounts (1-3%) improve herbage quality since they reduce bloat in grazing ruminants by disrupting protein foam, decrease the loss of dietary protein by microbial deamination and reduce parasitic load (Barry and Duncan, 1984; Howarth *et al.*, 1991; Tanner *et al.*, 1995; McMahon *et al.*, 2000).

Molecular genetic approaches for the manipulation of tannin biosynthesis have been mainly aimed at the introduction of condensed tannins in lucerne and white clover, and at the reduction of tannin content in high tanniniferous forage legumes. These transgenic approaches, including strategies for increasing tannin content, for modifying tannin structure, molecular weight and tissue distribution, and for novel tannin enzyme and gene discovery have been recently reviewed (Morris and Robbins, 1997; Gruber *et al.*, 2000).

1.2. Disease and Pest Resistance

Pathogen and pest infection can considerably lower herbage yield, persistency, nutritive value, and palatability of forage plants (Reed, 1994). An armory of genes and strategies for engineering disease and pest resistance in transgenic plants has been developed and tested over the last decade, including chitinases, glucanases, plant defensins, phytoalexins, ribosome-inactivating proteins, viral coat proteins, viral replicase, viral movement proteins, *Bt* toxins, proteinase inhibitors, and α-amylase inhibitors. Some of them have been applied to the development of pasture plants, mainly forage legumes, for enhanced disease and pest resistance (Hill *et al.*, 1991; Voisey *et al.*, 1994; 2000; Masoud *et al.*, 1996; Strizhov *et al.*, 1996; Garrett and Chu, 1997; Kalla *et al.*, 2000a).

1.2.1. Transgenic Approaches to Enhance Resistance to Fungal Diseases

Fungi attack leaves and root systems of forage plants causing leaf and root damage which results in poor establishment, reduced yield, lower quality and limited persistence. The constitutive, organ-specific or pathogen-inducible expression in transgenic plants of genes encoding antifungal proteins (AFPs) and acting either individually or in a concerted manner may confer useful novel types of resistance to fungal diseases. Transgenic approaches aimed at enhancing fungal disease resistance are being undertaken mainly in forage legumes, such as lucerne and subterranean clover.

Transgenic lucerne plants expressing a rice class I chitinase gene have been produced, however the resistance status of the transgenic lucerne plants to challenge with *Rhizoctonia solani* and *Sclerotium rolfsii* remains to be determined (Mizukami *et al*., 2000).

The fungus *Phytophthora clandestina* is recognised as the main cause of subterranean clover root rot in Australia. Estimated losses caused by this disease are in excess of 47% reduction of dry matter production. The discovery of new races of *P. clandestina* that are pathogenic on subterranean clover varieties previously shown to be resistant is of major concern. Outbreaks of clover scorch disease, caused by *Kabatiella caulivora*, have devastated over 1 million ha and seriously affected over 1.5 million ha of subterranean clover pastures in southern Australia in the 1960s and 1970s. Recently, breakdown of resistance to clover scorch has occurred in subterranean clover due to a new race of *Kabatiella*. Other root rot fungi affecting subterranean clover pastures are *Rhizoctonia solani* and *Fusarium* species. There are no sources of resistance in new releases of subterranean clover to all races of *Phytophthora* and none available to *Rhizoctonia* or *Fusarium*. Four different AFPs have been identified in *in vitro* assays as effective against the target pathogens, and corresponding chimeric genes have been used to generate transgenic AFP-expressing, phenotypically normal subterranean clover plants (Figure 4) (Aldao *et al*., 2000). While the resistance status of these transgenic forage legumes expressing individual AFP genes remains to be assessed, approaches for pyramiding different resistance transgenes may give substantially greater protection against fungal pathogens than the deployment of individual synthetic resistance genes.

1.2.2. Transgenic Approaches to Enhance Resistance to Viral Diseases

Viruses such as alfalfa mosaic alfamovirus (AMV), white clover mosaic potexvirus (WCMV) and clover yellow vein potyvirus (CYVV) have been found to have significant adverse effects on forage legumes (Guy *et al*., 1980; Garrett, 1991; Johnstone and Chu, 1992; Forster *et al*., 1997; Dudas *et al*., 1998). Each of these viruses individually infects a large number of plant species, has a worldwide distribution, and causes significant losses, especially in pasture and grain legumes. For example, it has bee estimated that controlling AMV, WCMV, and CYVV could increase profitability of Australian rural industries by over AUD 860 million (Kalla *et al*., 2000a). Most of the classical methods for preventing plant virus infections are laborious and economically unsustainable. While potential sources of AMV, WCMV and CYVV tolerance or resistance have been identified in lucerne and a few *Trifolium* species, there is no effective, durable, readily transferable natural resistance to these viruses which has been incorporated in forage legume cultivars. This makes the production of virus resistant and immune forage legumes through gene technology an attractive option. Gene technology offers the ability to overcome species-specific barriers, to develop multigenic resistance and to manipulate levels and sites of expression. Transgenic approaches have been successfully applied to develop effective and durable resistance to a diverse range of plant viruses (Beachy, 1997).

Pathogen-derived resistances have been used in the production of transgenic forage legumes for enhanced resistance to AMV, WCMV and CYVV. Coat protein mediated resistance to AMV has been reported in transgenic lucerne (Hill *et al*., 1991). AMV field-immune transgenic white clover plants expressing the AMV coat protein gene have been produced (Kalla *et al*., 2000a). Transgenic white clover plants expressing the coat protein gene of WCMV showed low levels of resistance to the virus

and delayed systemic infection (Kalla *et al*., 2000b; Voisey *et al*., 2000). High levels of resistance or immunity to WCMV were observed in transgenic white clover plants expressing a mutated form of the WCMV 13 kDa movement protein or the WCMV replicase gene, respectively (Voisey *et al*., 2000). Transgenic white clover plants carrying a chimeric CYVV coat protein gene showed resistance and immunity to the virus (Chu *et al*., 2000). Transgenic red clover (*T. pratense*) plants expressing AMV and WCMV coat protein genes for immunity and enhanced resistance to these viruses, respectively, have been generated (Figure 5) (Kalla *et al*., 2000b).

Barley yellow dwarf virus (BYDV) and ryegrass mosaic virus (RMV) are widely distributed in forage grasses, such as ryegrasses and fescues (Chapman, 1979; Catherall and Parry, 1987; Henry and Dedryver, 1991; Eagling *et al*., 1992). BYDV infection in ryegrasses reduces dry matter yield by as much as 24% (Catherall, 1966; Wilkins and Catherall, 1977). RMV infection in ryegrasses results in dry matter reductions of 5 - 50% (A'Brook and Heard, 1975; Jones *et al*., 1977; Eagling *et al*., 1992). RMV infection also reduces the competitiveness of perennial ryegrass, resulting in poor establishment and reduced persistence (Eagling *et al*., 1991).

The coat protein-mediated transgenic resistance approach has been applied to the development of transgenic perennial ryegrass plants expressing a chimeric RMV-coat protein gene, however, the RMV resistance status of these plants is yet to be determined (Altpeter *et al*., 2000).

1.2.3. Transgenic Approaches to Enhance Resistance to Pests

Pasture pests can damage plants directly by consuming foliage and roots or indirectly by transmitting plant pathogens (Allen, 1987). Dry matter production of pastures may be reduced by 20 - 40% in pastures infested by dense populations of several insect pests (East and Pottinger, 1984). A range of insect pests may cause significant damage to legumes and grasses in pastures, such as porina (*Wiseana* spp.), grass grub (*Costelytra zealandica*), black field cricket (*Teleogryllus commodus*), lucerne flea (*Sminthurus viridis*), weevils (*Sitona* spp.), case-bearer moths (*Coleophora* spp.), soldier fly (*Inopus rubriceps*) and aphids (*Acyrthosiphon* spp.) (Gaynor and Skipp, 1987).

Different approaches for the application of transgenesis to enhance resistance of forage plants to insect pests, notably *Bacillus thuringiensis* (*Bt*) insecticidal crystal proteins and proteinase inhibitors (PIs) are available (Burgess and Gatehouse, 1997). Expression of *Bt* toxins and PIs were shown to be effective against selected insect pests in transgenic forage legumes (Voisey *et al*., 1994; Strizhov *et al*., 1996).

Transgenic white clover plants expressing a chimeric *Bt* CryIBa gene, modified for a decreased A/T content, and accumulating the CryIBa δ–endotoxin in leaves up to 0.1% of soluble protein were produced (Voisey *et al*., 2000). *Wiseana* (porina) larval feeding assays using selected moderately *Bt* CryIBa-expressing white clover plants showed a significant larval feeding inhibition, a reduction in larval growth and enhanced larvae mortality compared to non-transgenic control plants (Voisey *et al*., 2000).

Proteinase inhibitors, such as the bovine pancreatic trypsin inhibitor or aprotinin, have also been evaluated in transgenic approaches to enhance insect pest resistance in forage plants. Transgenic white clover plants expressing aprotinin to 0.07% of total soluble protein in mature leaves led to reduced growth of *Wiseana* larvae in feeding assays (Voisey *et al*., 2000).

These approaches can be adopted for protection of forage grasses against target pests which can be controlled by *Bt* toxins and PIs. In addition to the production of transgenic grasses, surrogate transformation in grasses using transformed *Neotyphodium* endophyte (Murray *et al*., 1992; Tsai *et al*., 1992) may open up opportunities for using 'ruminant-safe' strains of the endophyte to express and secrete protective proteins such as *Bt* toxins and proteinase inhibitors in order to protect forage grasses from pest damage (Spangenberg *et al*., 1998).

1.3. Growth and Development

1.3.1. Manipulation of Pollen Allergens

Hayfever and seasonal allergic asthma due to grass pollen are environmental diseases that afflict up to 25% of the population in cool temperate climates around the world (Ong *et al*., 1993; Tamborini *et al*., 1995). Ryegrass pollen shows the greatest abundance of all types of grass pollen in cool temperate regions (Marsh, 1975; Smart *et al*., 1979) and is the major allergen for 49 - 67% of allergic patients (Ford and Baldo, 1986; Freidhoff *et al*., 1986; Knox *et al*., 1989).

The pollen of *L. perenne* contains at least four major classes of allergenic proteins, each composed of multiple, immunologically indistinguishable isoforms involving 17 allergens ranging in size from 12 to 89 kDa (Ford and Baldo, 1986; Singh *et al*., 1991; Sidoli *et al*., 1993; Knox and Suphioglu, 1996). At least one protein from each of these classes has been isolated and characterized in some detail. cDNA clones for the major ryegrass pollen allergens *Lol p* 1 (Perez *et al*., 1990), *Lol p* 2 (Sidoli *et al*., 1993) and *Lol p* 5 (Singh *et al*., 1991) have been isolated. A number of *Lol p* 1- and *Lol p* 2-related sequences exist in the genomes of both *L. perenne* and *L. multiflorum*. First transgenic perennial ryegrass (*L. perenne*) and Italian ryegrass (*L. multiflorum*) plants bearing antisense *Lol p* 1 and *Lol p* 2 genes under the control of a pollen-specific promoter for the down-regulation of these main ryegrass pollen allergens have been generated (Figure 6) (Wu *et al*., 1997). These plants will allow the study of the functional role *in planta* of these pollen allergens and to explore the potential for the generation of hypo-allergenic ryegrass cultivars (Wu et al., 1997; Spangenberg *et al*., 1998; Donato *et al*., 2000).

More recently, transgenic annual ryegrass plants (*L. rigidum*) carrying an antisense *Lol p* 5 chimeric gene for down-regulation of this important ryegrass pollen allergen have been reported (Bhalla *et al*., 1999).

1.3.2. Manipulation of Phase Change and Flowering

The decline of feeding value in some perennial forage species is associated with the onset of stem growth, flowering and senescence. Stopping the formation of the less digestible stems or delaying the senescence process is expected to increase herbage quality.

Large modifications of flowering time in transgenic plants caused by regulating the expression of floral meristem initiation genes have been reported. The constitutive expression of the flower-meristem-identity genes *LEAFY* or *APETALA1* of *Arabidopsis thaliana* has led to precocious flower development in transgenic aspen (Weigel and Nilsson, 1995) and *A. thaliana* (Mandel and Yanofsky, 1995; Weigel and Nilsson, 1995). *A. thaliana* plants carrying mutations in one or more flower-meristem-identity genes exhibit either incomplete transformation from flowers to inflorescences or

abolished flower development (Bowman *et al.*, 1993). Thus, opportunities exist to control or inhibit flowering in transgenic forages by down-regulating the expression of genes determining floral meristem initiation, such as *LEAFY* or *APETALA1* orthologs.

An additional target for manipulation of reproductive development in forages is the *INDETERMINATE1 (ID1)* gene. *ID1* plays an important role in controlling floral initiation as well as in maintaining the florally determined state in maize (Colasanti *et al.*, 1998). Its mutation is the only one known in monocots to specifically and severely block the transition to reproductive growth and to enhance leaf production. Recently, *ID1* homologue cDNA clones from perennial ryegrass were isolated and characterized (Liu and Spangenberg, 2000). It is expected that inhibition of the transition from vegetative growth to the formation of flowering stems and inflorescences in forage grasses will enhance herbage quality, and further lead to a reduction of airborne pollen allergens.

Controlled or inhibited flowering in transgenic forages through sense or antisense suppression of either *ID1* or *LEAFY* and *APETALA1* orthologs could lead to increased quality, improved seasonal growth patterns and would represent an approach for transgene containment. For seed production, the flowering block of the non-flowering lines needs to be reversed. An inducible promoter for controlling the suppression of *ID1*, *LEAFY* or *APETALA1* orthologs in the non-flowering transgenic forage plants, such as the copper-controllable gene expression system (Mett *et al.*, 1993; 1996), may need to be considered.

Different approaches on the manipulation of reproductive development and phase change which may lead to a block of flowering, to the development of apomixis and male sterility may provide opportunities for transgene containment of particular relevance for transgenic wind-pollinated out-crossing forages. Pollen dispersal is an important factor in the risk assessment of the release of genetically modified wind-pollinated grasses (Giddings *et al.*, 1997a, b; Giddings, 2000).

1.3.3. Manipulation of Plant Organ Senescence

Inhibition of leaf senescence has been achieved by the autoregulated production of cytokinin in transgenic plants (Gan and Amasino, 1995). The system is based on the use of the *A. thaliana* senescence-specific SAG12 promoter controlling the transgenic expression of the isopentenyl transferase gene (*ipt*) of *Agrobacterium tumefaciens*, which catalyzes the rate-limiting step in cytokinin biosynthesis. Delayed leaf senescence but no developmental abnormalities were observed in transgenic tobacco plants that expressed the chimeric SAG12-*ipt* gene (Gan and Amasino, 1995). Analogous chimeric *ipt* genes under control of developmentally regulated or senescence associated promoters have been tested in transgenic white clover plants and led to a significant delay in leaf senescence in otherwise phenotypically normal plants (Ludlow *et al.*, 2000).

1.4. Molecular Farming: Transgenic Forage Plants for Non-Forage Uses

Plants can be used to express recombinant heterologous proteins. Transgenic plants may be an attractive alternative to microbial systems for the production of certain biomolecules (Goddijn and Pen, 1995).

The perennial growth habit, the biomass production potential, the capacity for biological nitrogen fixation, and the ability to grow in marginal areas exhibited by

forage plants, particularly pasture legumes, make them potential suitable candidates for molecular farming. Advances in genetic manipulation technologies that allow high levels of transgene expression and transgene containment may, in the not too distant future, make it possible to exploit some forage plants as bioreactors for the production, among others, of industrial enzymes, pharmaceuticals, vaccines, antibodies and biodegradable plastics.

Multidisciplinary efforts will, however, be needed to identify the most feasible targets, to generate transgenic plants with suitable expression levels, and to develop efficient downstream processing technology that could adapt transgenic forage plants for non-forage uses and make them a cost-effective alternative for molecular farming. Significant progress achieved in recent years in the production of value-added proteins in transgenic lucerne has been recently reviewed (Austin-Phillips and Ziegelhoffer, 2000).

1.5. Field Evaluation of Transgenic Forage Plants

Small scale planned releases of transgenic plants are required to assess the stability of transgene expression and the novel phenotypes under field conditions and to identify transformation events suitable for transgenic germplasm and cultivar development. Only after the transformation events have been thoroughly evaluated for the stability of the novel phenotype outside of the controlled environment in a glasshouse would it be advisable to continue to integrate these in molecular breeding programs for the development of transgenic cultivars.

An illustrative example of design features of such a small scale field trial can be found in a recent field trial of alfalfa mosaic virus (AMV) immune transgenic white clover plants (Kalla *et al*., 2000a; Spangenberg and Chu, 2000). Important biosafety features incorporated in the design of this trial included a 2 hectare buffer-zone sown with forage legumes known not to inter-breed with white clover. The use of forage legumes such as red clover, Persian clover and lucerne in the buffer zone sown in alternating strips ensured that there were a large number of flowering non-transgenic legumes present in the trial at the critical time of flowering of the transgenic test plants (Figure 7). The dimensions of the buffer zone were based upon considerations on bee behaviour as white clover pollinator, pollen dispersal, and gene flow determinations using the easily scorable *Feathermark* dominant marker gene in white clover (Woodfield *et al*., 1995). In order to assess transgene flow, two trap rows of non-transgenic white clover clover were included in the field trial design surrounding the perimeter of the trial and the central plots with the transgenic test plants. Seeds harvested from the white clover plants in the trap rows were screened by a combination of antibiotic resistance (G418 resistance mediated by the *npt2* gene carried on the T-DNA integrated into the genome of transgenic plants) and PCR analysis for the presence of the selectable marker gene. Results from this analysis confirmed the suitability of the field trial design.

1.6. Integrating Transgenic Forage Plants in Breeding Programs and Development of Transgenic Cultivars

As outlined in the previous sections, a range of transformation events in forage legumes and grasses with proof of concept for the technology under containment conditions are being developed. The challenge now is how to best deploy these

molecular technologies and tools to evaluate their full potential based on the transgenic transfer of single and multiple valuable genes, to generate novel genetic variability and novel elite transgenic germplasm, and to efficiently incorporate these factors into breeding programs for the development of improved cultivars.

Efficient strategies for the introgression of transgenes into elite parents for the subsequent production of synthetic cultivars have been developed ensuring stable and uniform transgene expression in all plants in the population (Spangenberg *et al*., 2000a). Figure 8 illustrates the strategy as applied to the production of AMV immune transgenic elite white clover plants homozygous for the transgenes. It involves initial top crosses of transformation events chosen after their field evaluation with elite non-transgenic white clover parental lines (step 1); selecting for progeny from the harvested seed carrying the transgene and its linked selectable *npt2* marker gene by antibiotic selection or PCR screening followed by diallel crosses between the T_1 progeny (step 2). The T_2 offspring plants homozygous for transgenes can be directly identified by high-throughput quantitative PCR transgene detection (step 3) or alternatively following a test cross (Figure 8) (Spangenberg *et al*., 2000a). The elite white clover plants homozygous for the transgenes are then planted in a selection nursery together with elite non-transgenic parental lines for identification of the new parents of transgenic experimental synthetic cultivars and their subsequent multisite evaluation.

2. Forage Plant Genomics

Plant biology has entered the genome era. Genome research has been driven primarily by the human genome project and its spin-offs. Telomere-to-telomere genome sequences for the model plants *Arabidopsis thaliana* and rice are close to completion. Tens of thousands of plant expressed sequence tags (ESTs) are providing the starting point for elucidating the function of thousands of plant genes. Plant genome analysis will provide insights into all aspects of plant growth, development, differentiation and responses to biotic and abiotic stresses. Plant genome research will thus revolutionise the improvement of plants and their products.

Genomics refers to structural and functional genome analyses and uses high throughput large scale experimental methodologies (i.e. high throughput DNA sequencing and microarray technology with chip-reading robots) combined with computational biological analysis (bioinformatics) leading to the 'industrial scale' discovery of genes and their functions.

The creation of EST databases of the world's major crops has become a top priority for agricultural plant genomics. Facilitated by extensive public and private sector funding, plant genome projects have extended well beyond the initial emphasis on structural genomics of rice and *Arabidopsis*, to include a range of plant species worldwide.

Forage plant breeding is just now entering the genome era. The plethora of new technologies and tools now available for high-throughput gene discovery and genome-wide expression analysis have opened up opportunities for innovative applications in the identification, functional characterisation and use of genes of value in forage production systems and beyond. Examples of these opportunities, such as 'molecular phenotyping', 'symbio-genomics' and 'xeno-genomics' have been reviewed (Spangenberg *et al*., 2000a) and are briefly presented below.

2.1. Forage Plant Gene Discovery and Microarray-Based Analysis of Plant Gene Expression

Plant genomics projects with a major focus on EST discovery are currently being undertaken for two model forage legumes, *Lotus japonicus* and, particularly, *Medicago truncatula* (Cook *et al*., 1997; Covitz *et al*., 1998; Cook, 1999; Cook and Denarie, 2000). Approximately 80,000 ESTs from *M. truncatula* have been generated by international genomics projects supported by the French Centre National de Sequencage, the International Human Frontier Science Program Organization, the Samuel Roberts Noble Foundation, Stanford University, the US National Science Foundation Plant Genome Program, and the US Department of Energy Biosciences Program with estimated 100,000 sequences to be available by December 2000 (G. May and K. VandenBosch, personal communication).

A joint Pasture Plant Genomics Program undertaken by Agriculture Victoria-DNRE and AgResearch Limited (New Zealand) has generated approximately 100,000 ESTs from the key forage crops of temperate grassland agriculture, perennial ryegrass (*L. perenne*) and white clover (*T. repens*) using high-throughput sequencing of randomly selected clones from cDNA libraries representing a range of plant organs, developmental stages, and experimental treatments. 49,503 perennial ryegrass DNA sequences were generated, analysed by BLAST searches and categorised functionally (Figure 9) (Spangenberg *et al*., 2000a).

Within the joint Pasture Plant Genomics Program, high density spotted cDNA microarrays (with 4,000 – 5,000 spots/array) have been established as a main screening tool for novel ryegrass and clover sequences of unknown function (Figure 10) (Spangenberg *et al*., 2000a). EST-based plant microarrays thus allow the global analysis of gene expression patterns as a main approach for functional genomics and other applications (Richmond and Somerville, 2000). Novel applications of EST-based forage plant arrays including 'molecular phenotyping', i.e. the analysis of global or targeted gene expression patterns using complex hybridisation probes from contrasting genotypes or populations and contrasting environments, are now conceivable to integrate microarray data with current conventional phenotypic selection approaches used in forage plant improvement (Spangenberg *et al*., 2000a).

Comparative sequence and microarray data analyses from ryegrass and clover with data from complete genome sequencing projects in *Arabidopsis* and rice as well as from extensive EST discovery programs in the model forage legume *M. truncatula* will provide insight into conserved and divergent aspects of grass and legume genome organization and function.

2.2. Forage Plant Symbio-Genomics and Patho-Genomics

Forage legumes and grasses offer unique and exciting opportunities in genome research to study plant-pathogen interactions, legume/nitrogen-fixing bacteria symbiosis, legume/mycrorrhiza associations, and grass/endophyte endosymbiosis, as well as to the application of the knowledge gained from these studies to develop resistance to pathogens and improved beneficial associations in forages.

Gene discovery efforts in *M. truncatula* currently target plant response to and characterization of different pathogen systems, including fungal pathogens such as *Colletotrichum trifolii* and *Phytophthora medicaginis* and bacterial pathogens such as *Xylella fastidiosa* and *Xanthomonas alfalfae* (Cook, 1999; Cook and Denarie, 2000). By

31 July 2000, the US *M. truncatula* Functional Genomics Project with a primary focus on legume-microbe interactions had generated over 27,000 DNA sequences including 2,828 ESTs from *Colletotrichum*-infected leaves; 2,462 ESTs from *Phytophthora*-infected leaves; 3,259 from mycorrhizal roots and over 9,500 sequences from roots at different time-points post-inoculation with *Sinorhizobium meliloti* and from mature and senescent nodules (VandenBosch *et al.*, 2000; K. VandenBosch, personal communication). An integrated functional genomics and genetics approach particulary targeted to understand the legume's function in symbiotic nodulation using *L. japonicus* as model (Gresshoff *et al.*, 2000) is expected to complement current efforts in *M. truncatula* genomics. These and other genomics resources in *M. truncatula* and *L. japonicus* will significantly contribute to research in and enhance the understanding of pathogen and stress responses, and rhizosphere interactions in forage legumes.

A Grass Endophyte Genomics Program undertaken by Agriculture Victoria-DNRE within the CRC for Molecular Plant Breeding has targeted grass-endophyte gene discovery in the tall fescue association with the endophyte, *Neotyphodium coenophialum* (Spangenberg *et al.*, 2000a). Approximately 8,000 *N. coenophialum* DNA sequences were generated, analysed by BLAST searches and categorised functionally (Figure 11). The program is focused on the discovery of genes involved in host colonization, nutrient supply to the endophytic fungus, and the biosynthesis of active pyrrolopyrazine and pyrrolizidine secondary metabolites (e.g. the insect deterrents peramine and N-formylloline, respectively) and their regulation. It will provide insight into the molecular genetics of the grass endophyte/host interaction as well as into the physiological mechanisms leading to the increased plant vigour and enhanced stress tolerance (Spangenberg *et al.*, 2000a). These genomic tools and knowledge will underpin the development of technologies to manipulate grass/endophyte associations for enhanced plant performance, improved grass tolerance to biotic and abiotic stresses, and altered grass endophyte host specificity, to the benefit of the grazing and turf industries. Progress in the isolation and characterization of genes involved in the biosynthesis of the indole-diterpenes and ergopeptines responsible for animal toxicosis in the perennial ryegrass-*N. lolii* association has been recently reviewed (Scott, 2000).

2.3. Xeno-Genomics

Comparative genomics based on the analysis of ESTs from a range of abiotic stress-tolerant plants is expected to allow for the identification of common gene networks associated with environmental stresses, such as high salinity, drought and low-temperature, and the assessment of conserved plant stress response pathways (Cushman and Bohnert, 2000).

Genome research with exotic plant species, i.e. 'xeno-genomics', includes gene discovery by high-throughput EST sequencing and large-scale simultaneous gene expression analysis with EST-based microarrays (Spangenberg *et al.*, 2000a). Xeno-genomics has opened up opportunities for a 'genomic bio-prospecting' of key genes and gene variants from exotic plants. This approach is particularly suited for the discovery of novel genes and the determination of their expression patterns in response to specific abiotic stresses.

A Xeno-Genomics Program undertaken by Agriculture Victoria-DNRE is focussed on selected Australian native and exotic grasses and legumes that show unique adaptation to extreme environmental stresses (Spangenberg *et al.*, 2000a). We are currently isolating and characterising genes which allow certain plant species to tolerate

extreme abiotic stresses including drought, salinity and low fertility soils (Figure 12) (Spangenberg *et al*., 2000a). The targeted species in the xenogenomic EST discovery program include Australian native grasses, such as the halotolerant blown-grasses (*Agrostis adamsonii* and *A. robusta*) and the aluminium-tolerant weeping grass (*Microlaena stipoides*); as well as exotic species such as antarctic hair-grass (*Deschampsia antarctica*), one of only two vascular plant species native to Antarctica (Figure 12) (Spangenberg *et al*., 2000a).

The discovery of novel genes and their functional genomic analysis will facilitate the development of effective molecular breeding approaches to enhance abiotic stress tolerance in forages and other crops.

3. Summary and Conclusions

Significant progress has been made in the establishment of the methodologies required for the molecular breeding of forage plants. A number of biotechnological approaches currently being tested include nutritional improvements through altered biosynthesis of lignin, soluble carbohydrates and proanthocyanidins, the regulated expression of 'rumen by-pass' proteins rich in essential amino acids, protection against pathogens and pests through engineered virus resistance and regulated expression of antifungal and pesticidal proteins, as well as the manipulation of growth and development aimed at improved persistence, improved tolerance to abiotic stresses, delayed senescence, non-flowering phenotypes and down-regulation of pollen allergens. First transgenic forage plants have passed field-trial evaluations, and selected transformation events have been used in the production of elite transgenic germplasm for cultivar development.

Research tools in structural and functional genomics promise to close gaps in our understanding of the underlying genetics, physiology and biochemistry of many complex plant processes and thus speed-up progress in applying gene technology-based approaches to forage plant improvement.

The application of molecular methodologies and tools in forage plant improvement will greatly enhance current empirical phenotype-based selection with more directed and predictable genotype-based approaches. However, these molecular approaches show promise only when considered as a part of plant improvement programs. The most successful improvement programs are expected to be those that build on multidisciplinary teams including plant breeders, molecular and cell biologists, biochemists, plant pathologists, agronomists and animal scientists, and that efficiently embrace appropriate new gene technology, functional genomics, bioinformatics and high-throughput genotyping tools and apply these in a sensible manner. The effective integrated effort of such teams will be critical to the competitive development of marketable forage plant cultivars from molecular breeding programs and for the development of forage plants for non-forage uses.

Plant genomics will play a vital role in accelerating the application of biotechnology to forage and grassland agriculture. Genome research in forage plants will allow the development of technologies with applications well beyond forage production systems thus significantly increasing the value of seeds and agricultural products. Large pools of plant genes will be invaluable resources for insertion into a wide range of crop plants using gene technology and for their deployment as markers in new and innovative ways to enhance efficiency and efficacy of plant improvement.

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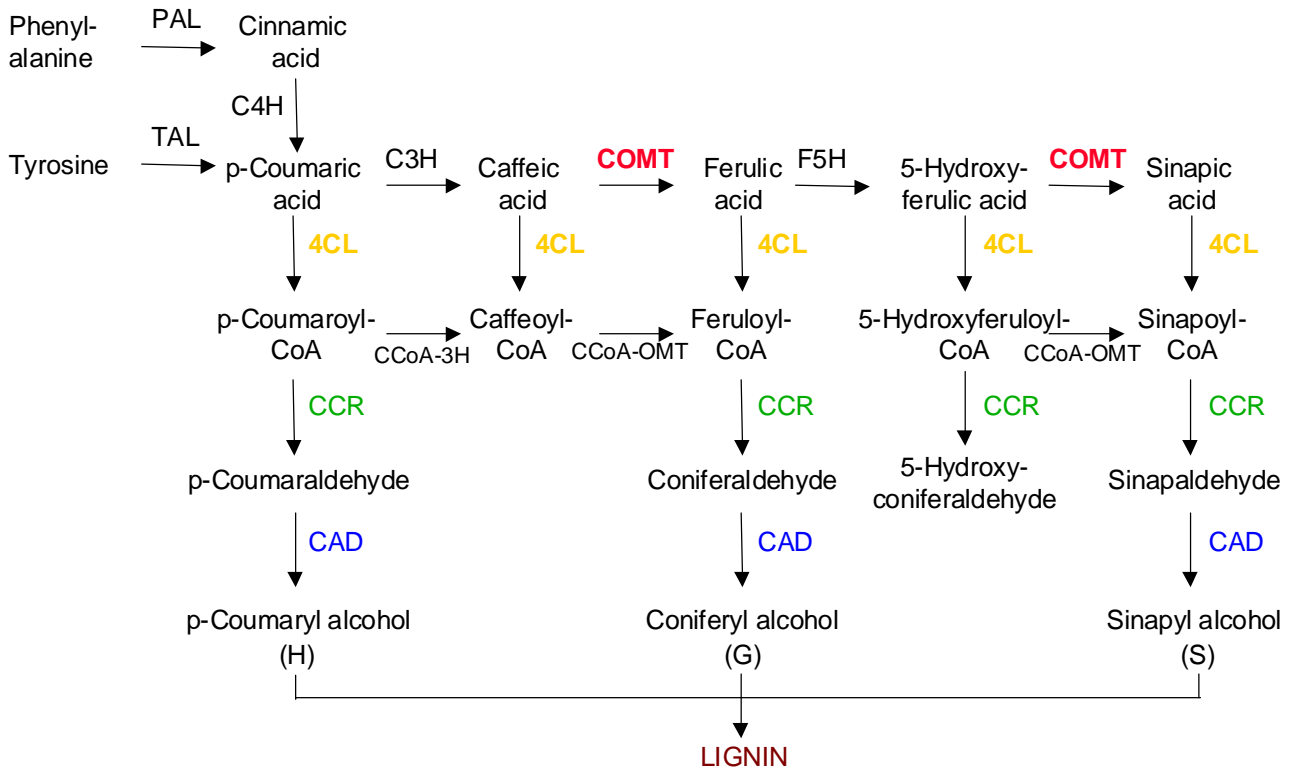
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Figure 1. The lignin biosynthetic pathway. The lignin biosynthetic enzymes are: PAL phenylalanine ammonia-lyase; TAL tyrosine ammonia-lyase; C4H cinnamate 4-hydroxylase; C3H 4-hydroxycinnamate 3-hydroxylase; COMT caffeic acid 3-O-methyltransferase; F5H ferulate 5-hydroxylase; 4CL 4-coumarate: CoA ligase; CCoA-3H coumaroyl-coenzyme A 3-hydroxylase; CCoA-OMT caffeoyl-coenzyme A O-methyltransferase; CCR cinnamoyl-CoA reductase; and CAD cinnamyl alcohol dehydrogenase (Modified from Boudet *et al*., 1996; Campbell and Sederoff, 1996).
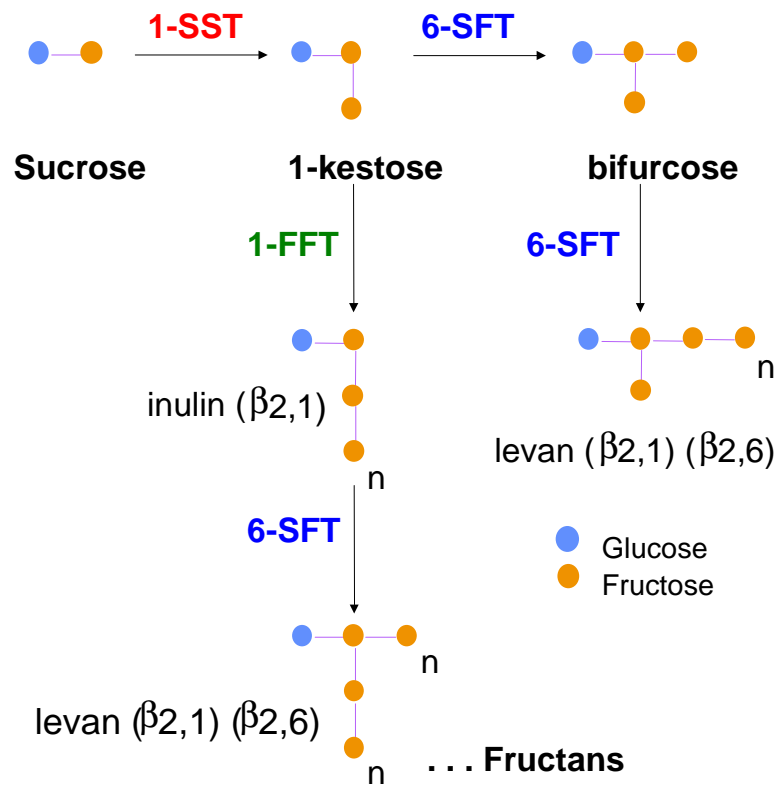
Figure 2. The fructan biosynthesis pathway in grasses (Spangenberg et al., 2000a). The fructan biosynthetic enzymes are: 1-SST sucrose:sucrose 1-fructosyltransferase; 1-FFT fructan:fructan 1-fructosyltransferase; and 6-SFT sucrose:fructan 6-fructosyltransferase.
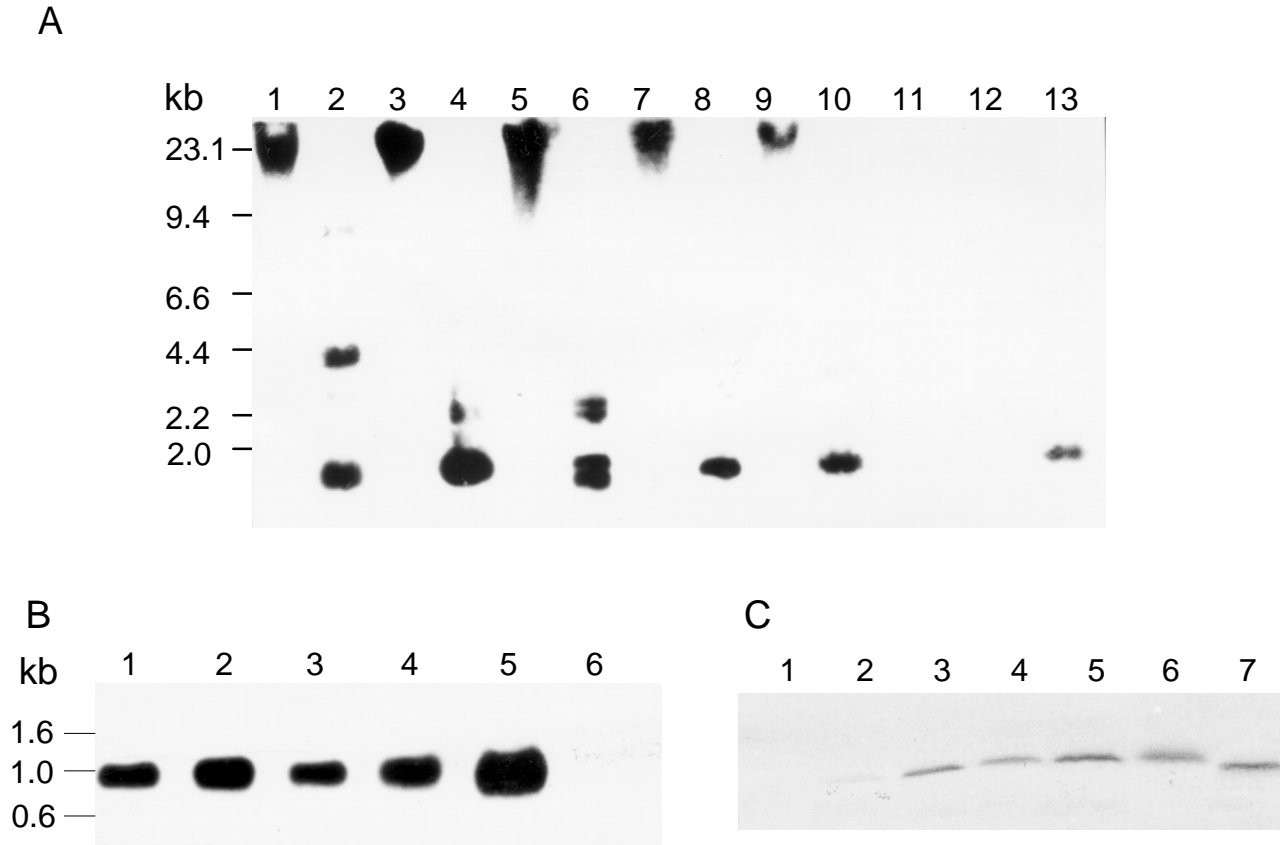
Figure 3. Expression of a chimeric sunflower albumin gene in transgenic tall fescue (*Festuca arundinacea*) plants (Wang *et al*., 2000). **A** Southern hybridisation analysis using undigested (odd-numbered lanes) and *Eco*RI-digested (even-numbered lanes) DNA samples from p35Ssf-transgenic tall fescue plants. Lanes 11 and 12 represent an untransformed tall fescue sample, lane 13 represents 10 pg of plasmid p35Ssf. **B** Northern hybridisation analysis. Lane 6 represents an untransformed tall fescue sample. **C** Western blot analysis using a goat anti-sunflower albumin antibody. Lane 7 represents 80 µg of SFA8 protein.
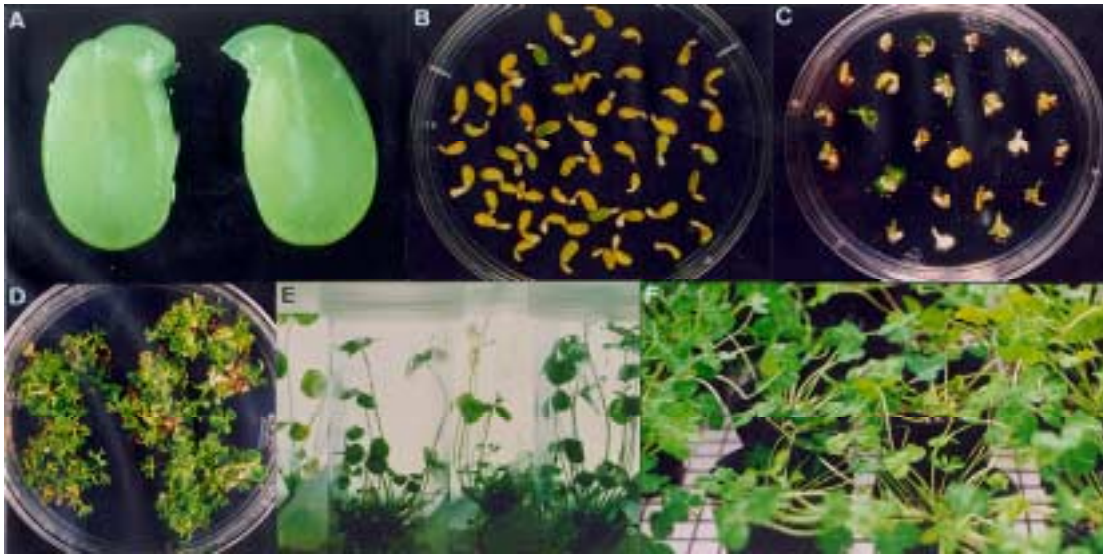
Figure 4. Production of transgenic subterranean clover (*Trifolium subterraneum*) plants for root rots and clover scorch resistance by *Agrobacterium*-mediated transformation using cotyledonary explants, a chimeric *npt2* gene as selectable marker, and different chimeric antifungal protein genes (Spangenberg *et al*. 2000a).
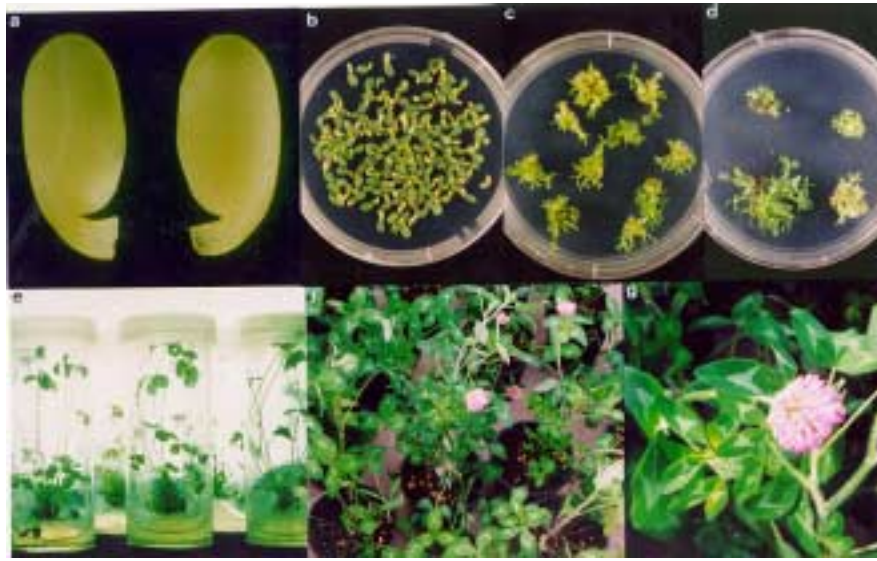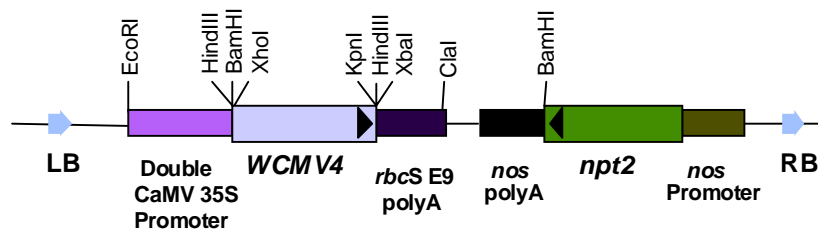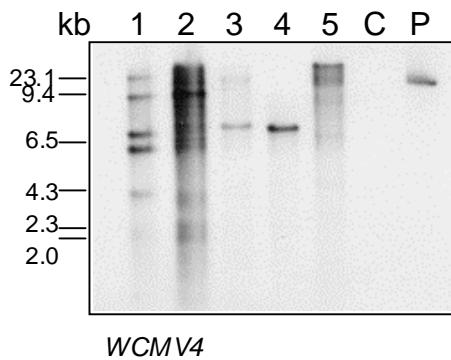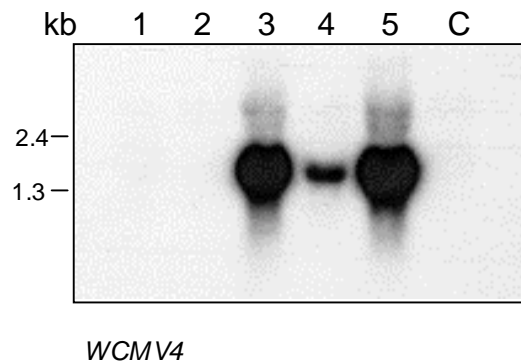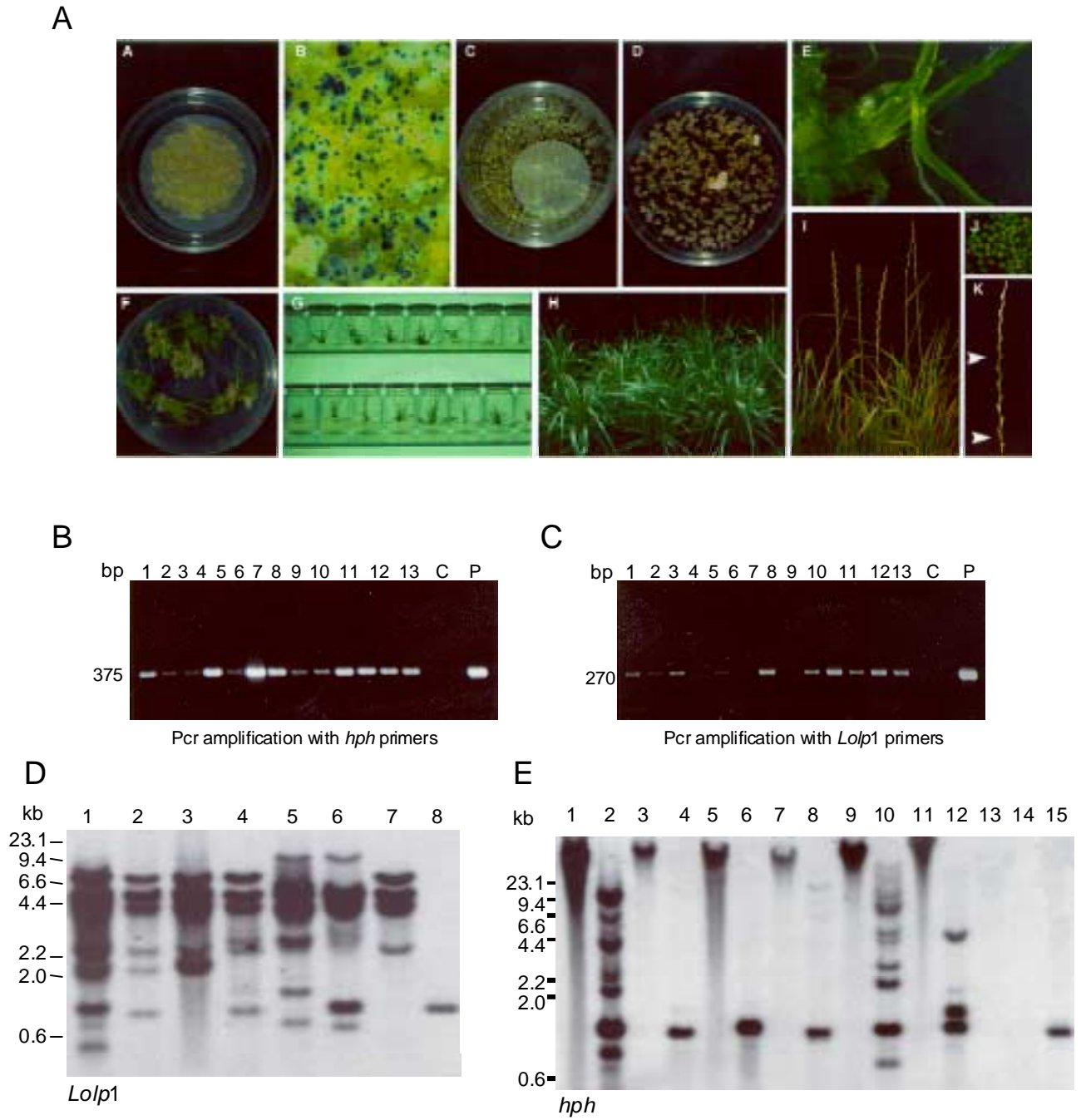
**Figure 5. A** Production of transgenic red clover plants (*Trifolium pratense*) for alfalfa mosa
virus (AMV) and white clover mosaic virus (WCMV) virus resistance by *Agrobacteriu*
mediated transformation using cotyledonary explants and chimeric *npt2* gene as selectab
marker. **B** T-DNA of binary vector containing the chimeric *npt2* gene and the WCMV co
protein gene (*WCMV4*). **C** Southern hybridisation analysis of transgenic red clover plan
using *WCMV4* probe. **D** Northern hybridisation analysis of transgenic red clover plan
expressing chimeric *WCMV4* gene.

A

B

Pcr amplification with *hph* primers

C

Pcr amplification with *Lolp*1 primers

D

*Lolp*1

E

hph

Figure 6. A Production of transgenic perennial ryegrass (*Lolium perenne*) plants for down-regulation of main pollen allergens by biolistic transformation of embryogenic suspension cells with a vector bearing a chimeric antisense *Lolp*1 gene. **B-C** Screening of putative perennial ryegrass transgenic plants by PCR with *hph* primers (B) and *Lolp*1 primers (C). **D-E** Southern hybridisation analysis of transgenic perennial ryegrass plants using a *Lolp*1 hybridisation probe (D) and *hph* hybridisation probe (E).
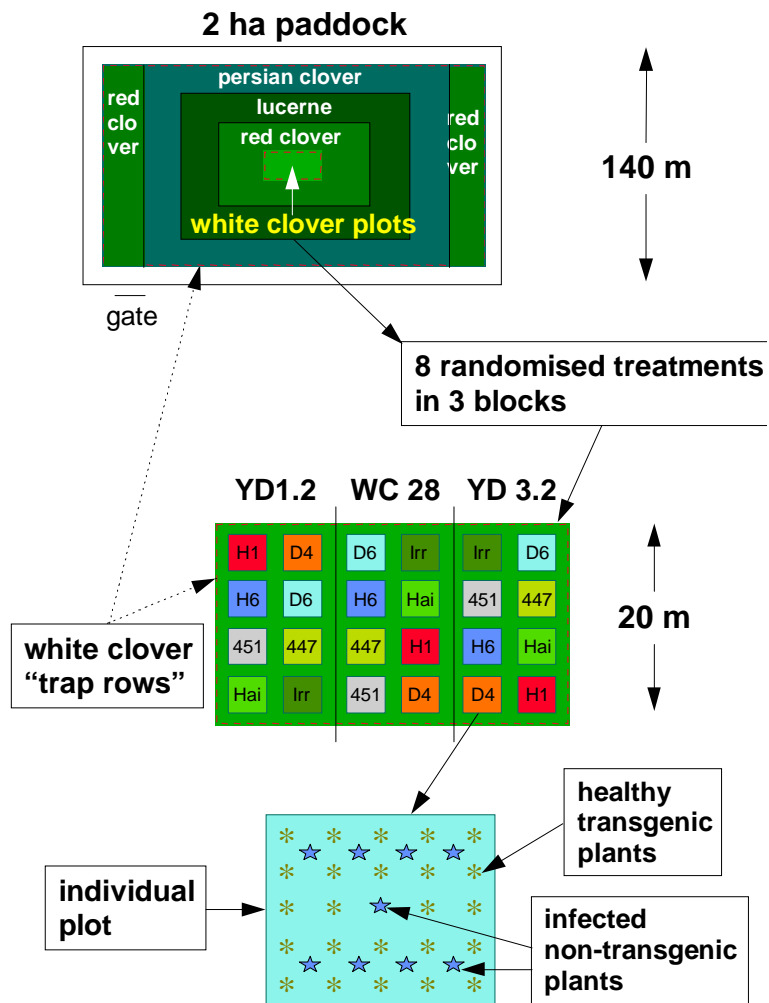
Figure 7. Experimental design of small scale planned field release for the evaluation of world's first AMV immune transgenic white clover (*Trifolium repens*) plants (Kalla *et al.*, 2000a).
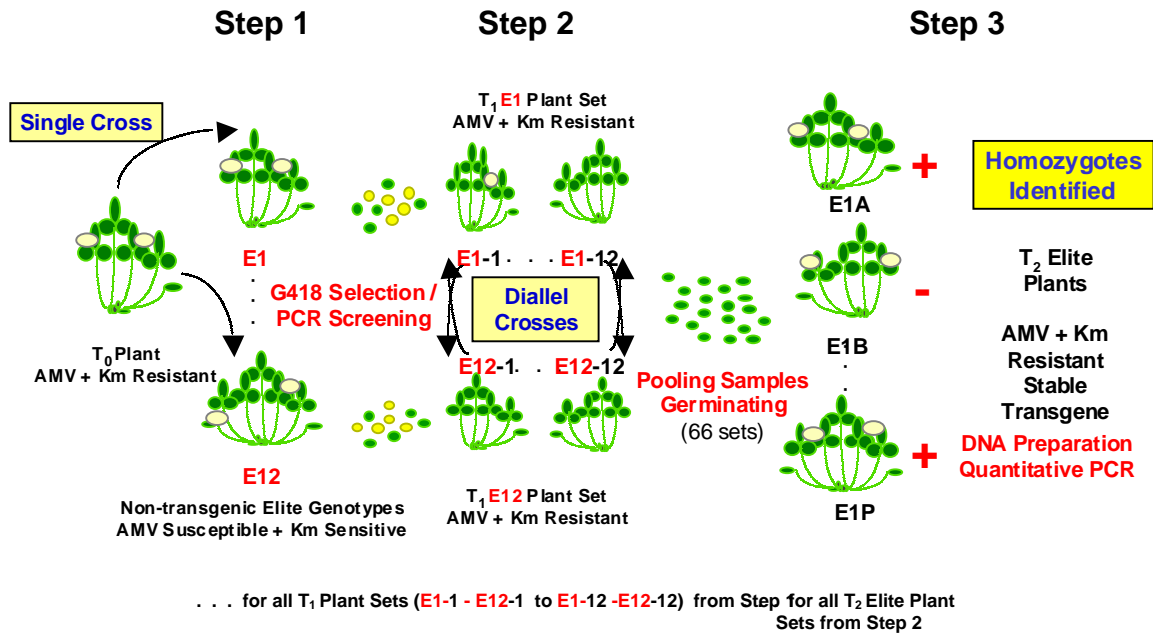
Figure 8. Strategy for the development of elite transgenic AMV immune white clov (*T. repens*) germplasm based on high-throughput quantitative PCR detection for th identification of plants homozygous for the transgenes (*npt2* and *AMV4*) (Spangenbe *et al.*, 2000a).
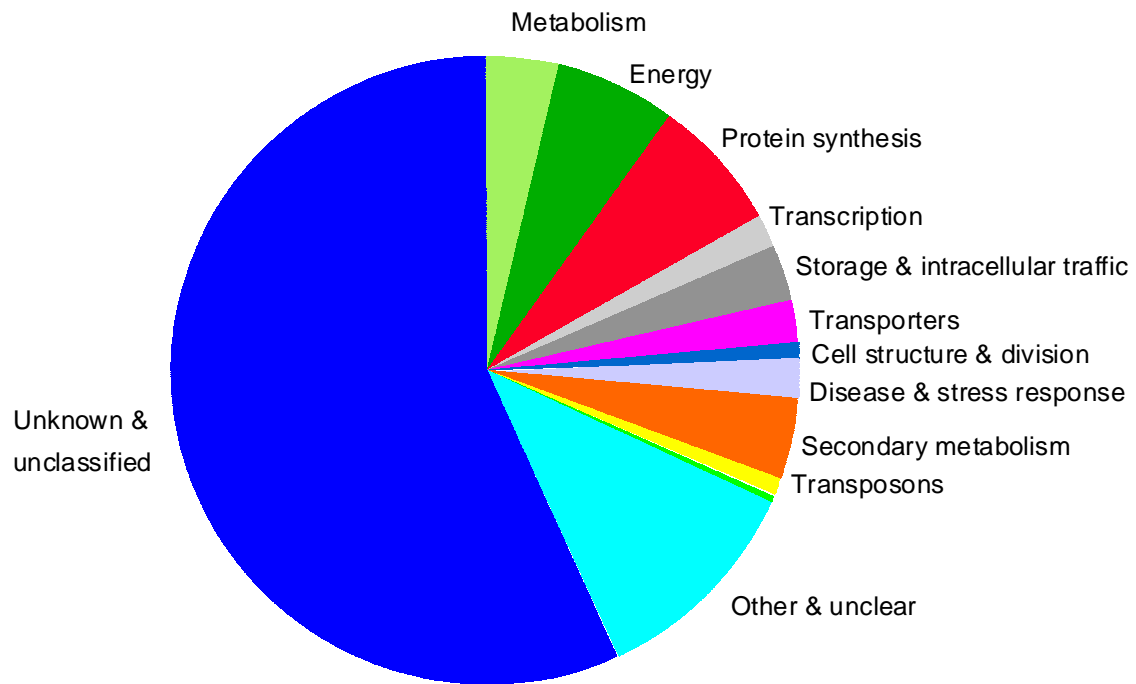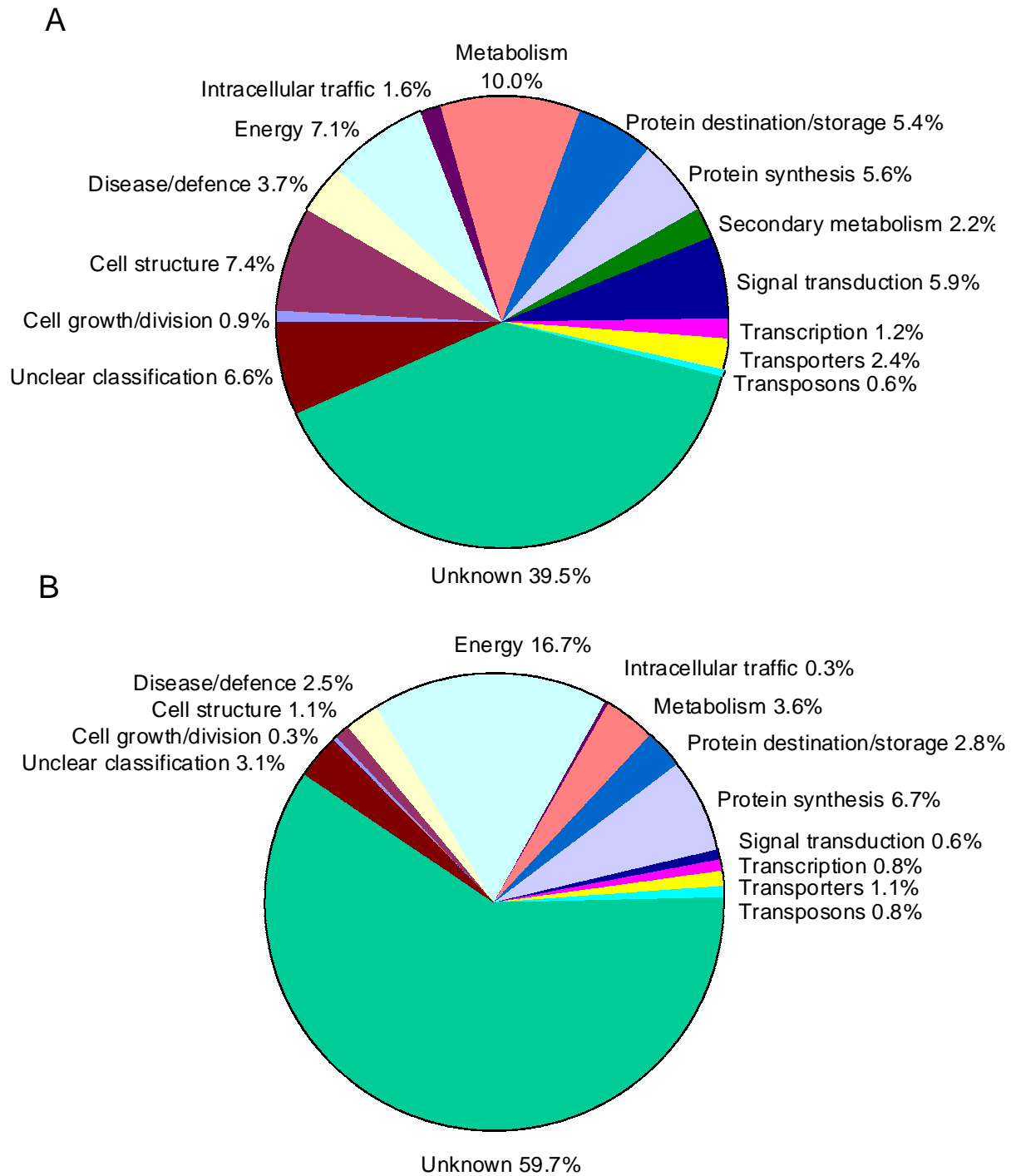
Figure 9. Functional categorisation of expressed sequence tags from perennial ryegrass (*Lolium perenne*) (Spangenberg *et al.*, 2000a).
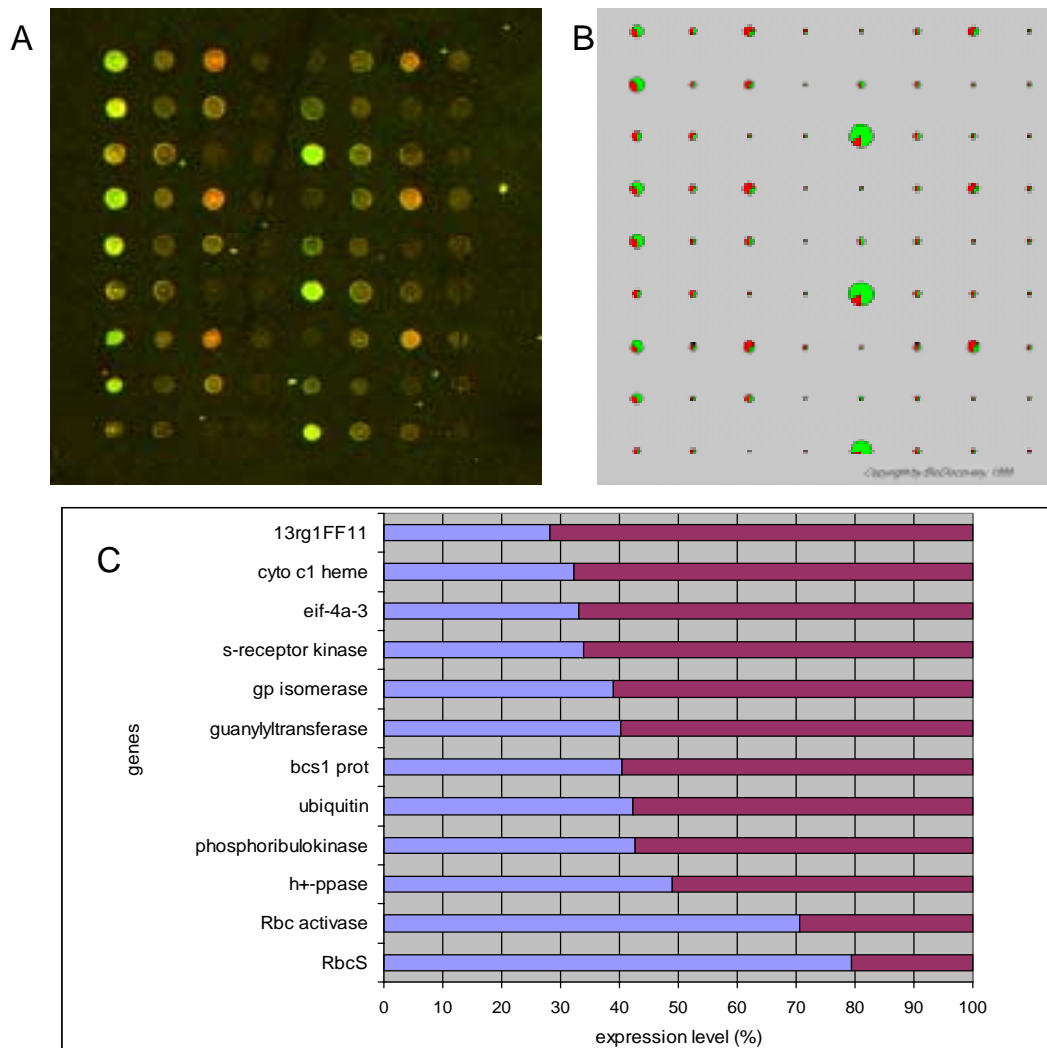
Figure 10. Microarray analysis of expressed sequence tags from perennial ryegrass (*L. perenne*) (Modified from Spangenberg *et al*., 2000a). **A** Subgrid (9x8) of a perennial ryegrass cDNA microarray hybridised with labelled total RNA from ryegrass seedlings grown under light (Cy3-label) and dark (Cy5-label) conditions for 2 days. Perennial ryegrass genes with expression up-regulated under light conditions (green), up-regulated under dark conditions (red), and in both light and dark conditions (yellow) are shown. **B** GenePie diagram showing relative expression levels of perennial ryegrass genes under light (green sector) and dark (red sector) generated by BioDiscovery ImaGene software. The size of the pies reflect the level of gene expression. **C** Bar diagram showing relative level of expression of selected perennial ryegrass genes from A-B with relative expression levels shown for ryegrass seedlings grown under light (purple) and dark (burgundy) conditions. Average data from three replicates are shown.
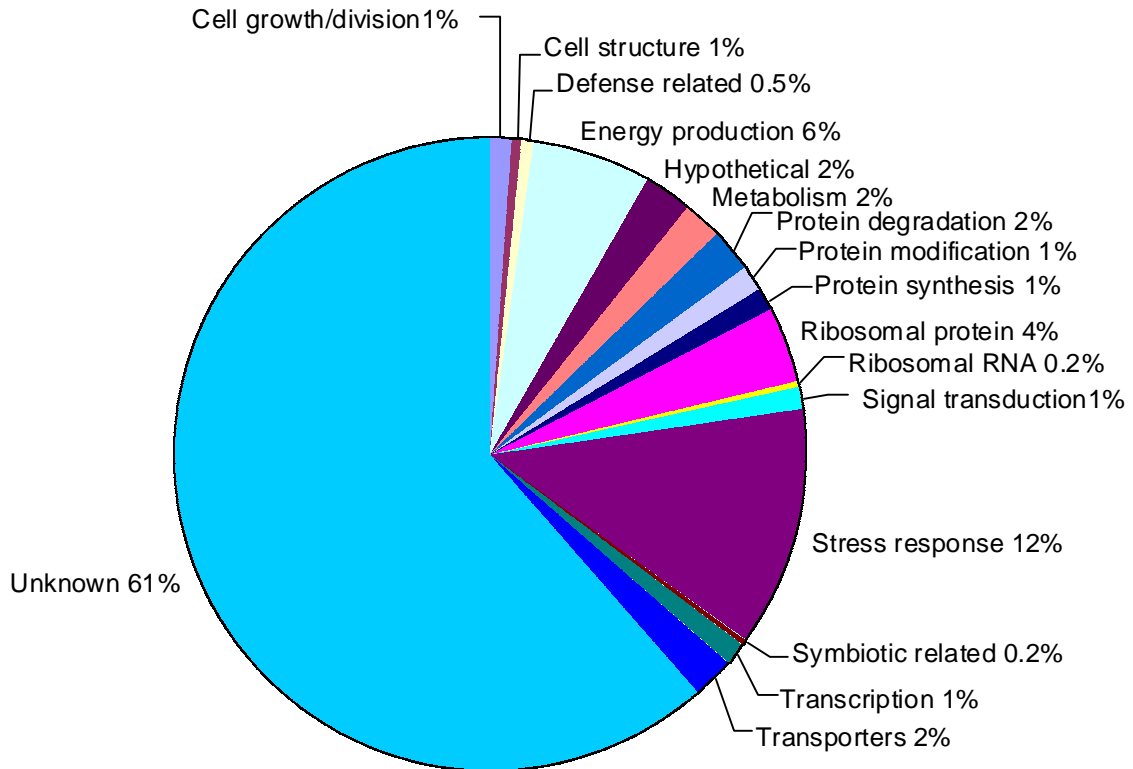
Figure 11. Grass endophyte 'symbio-genomics' (Modified from Spangenberg *et al.*, 2000a). Functional categorisation of expressed sequence tags from the tall fescue (*F. arundinacea*) endophyte *Neotyphodium coenophialum* grown *in vitro*.

A

B

Figure 12. 'Xeno-genomics' (Spangenberg *et al*., 2000a). Functional categorisation of expressed sequence tags from **A** salt-stressed roots from the halotolerant Australian native grass, *Agrostis robusta*; and **B** cold-acclimated leaves from the antarctic hairgrass, *Deschampsia antarctica*.