A MOLECULAR AND BIOCHEMICAL ANALYSIS OF LIGNIN BIOSYNTHESIS IN PERENNIAL RYEGRASS

A Thesis submitted for the Degree of Doctor of Philosophy at The Australian National University

by

Fiona Margaret McAlister

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Certificate of Originality

This thesis contains no material which has been accepted for the award of any other degree or diploma in any University and, to the best of my knowledge, contains no material previously published or written by another person, except where due reference is made in the text.

Fiona Margaret McAlister
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ABBREVIATIONS

Amino acids: Alanine; Ala, A. Glycine; Gly, G. Valine; Val, V. Leucine; Leu, L. Isoleucine; Ile, I. Proline; Pro, P. Hydroxyproline; Hyp. Serine; Ser, S. Threonine; Thr, T. Lysine; Lys, K. Arginine; Arg, R. Histidine; His, H. Phenylalanine; Phe, F. Tyrosine; Tyr, Y. Tryptophan; Trp, W. Aspartate; Asp, D. Glutamate; Glu, E. Asparagine; Asn, N. Glutamine; Gln, Q. Cysteine; Cys, C. Methionine; Met, M.

ADH: alcohol dehydrogenase

ATP: adenosine triphosphate

bp: base pair

BSA: bovine serum albumin

CAD: cinnamyl alcohol dehydrogenase

cDNA: complementary DNA

Da: Dalton

DNA: deoxyribonucleic acid

dNTP: deoxyribonucleoside-5'-triphosphate

EDTA: ethylenediaminetetra-acetic acid

GLC: Gas liquid chromatography

IPTG: Isopropylthio-β-D-galactopyranoside

Km: Michaelis constant

M: Molar
MOPS: 3-[N-morpholino]propane-sulphonic acid

mRNA: messenger RNA

NAD+, NADH: nicotinamide adenine dinucleotide (reduced form)

NADP, NADPH: Nicotinamide adenine dinucleotide phosphate (reduced form)

PCR: Polymerase chain reaction

pI: isoelectric point

RNA: ribonucleic acid

RNase: ribonuclease

SDS: sodium dodecyl sulphate

SSC: standard saline citrate

Tris: Tris (hydroxymethyl) amino methane
The digestibility of ryegrass by ruminant animals is partly determined by the amount of cell wall lignification that has occurred during plant development. By manipulating lignin biosynthesis it may be possible to increase the digestibility, and hence, the nutritional value of important pasture plants. The research undertaken in this thesis focused on two key enzymes involved in lignin biosynthesis; cinnamyl alcohol dehydrogenase (CAD) and caffeic acid O-methyltransferase (COMT). Analysis of gene regulation and enzyme activity of cinnamyl alcohol dehydrogenase (CAD) and caffeic acid O-methyltransferase (COMT) was undertaken in order to determine their relationship with lignin content, the formation of hydroxycinnamic acid bridges and changes in cell wall digestibility. A perennial ryegrass (Lolium perenne L.) cDNA library was independently screened with two PCR-amplified gene fragments encoding regions of highly conserved amino acid sequences found in CAD or COMT. These gene fragments were generated from ryegrass cDNA template using degenerate oligonucleotides designed from the conserved amino acid sequences from partial or full-length CAD and COMT genes isolated from other monocot and dicot species. Full-length cad and comt cDNAs were isolated from the perennial ryegrass cDNA library and were identified by sequence analysis and comparisons with sequences from other species. The CAD-encoding cDNA, LpeCad1, is the first reported full-length cad isolated from a monocot. Activity of a functional COMT enzyme in bacteria expressing the comt cDNA was further verification of this clone’s identity. Northern blot hybridization analyses indicated strong levels of CAD gene expression in root, sheath and stem tissue of perennial ryegrass and lower levels of expression in young or mature leaves and flowers. The COMT gene showed strong levels of expression in stem tissue and very low levels of expression in other tissues. Southern blot hybridization analyses indicated that the CAD gene is a member of a small gene family containing two genes and the COMT gene is a member of a multigene family. Further northern blot hybridization analyses indicated that these genes are under
developmental control in leaf tissue with both CAD and COMT genes having high levels of expression early in leaf development. Physiological analyses of the developing perennial ryegrass leaf revealed that peaks of CAD and COMT enzyme activity were reached within the first 7 days of development. The CAD enzyme also showed increased affinity for coniferyl aldehyde as the substrate compared to sinapyl aldehyde. Correlated with peaks in enzyme activity was the rapid increase in leaf lignin content during the first 7 days of perennial ryegrass development. Gas liquid chromatography measured high amounts of ferulic acid ester-linked to cell wall polysaccharides which are further ether-linked to lignin. Physiological analyses of the leaf indicated a general 4% decrease in dry matter digestibility and a 10% decrease in cell wall digestibility during these periods of increases in enzyme activity, lignin content and hydroxycinnamic acid bridging. This suggests that lignin content, as well as its physical and chemical associations with cell wall polysaccharides may be involved in decreasing leaf tissue digestibility. Taken together, these observations suggest that genetic manipulation of these aspects of lignification will need to be targeted during the early stages in leaf development.
CHAPTER ONE

GENERAL INTRODUCTION AND LITERATURE REVIEW

1.1 General Introduction

Perennial ryegrass is one of the most valuable temperate pasture grasses in the world and approximately six million hectares of perennial ryegrass-based pastures are used for the sheep, dairy and meat industries in Australia (Cunningham et al., 1993). Perennial ryegrass was introduced into Australia from northern Europe in the 1850's after the collapse of the native pasture grazing systems and was the first grass to be sown in a pure stand (Kloot, 1983). Given suitable growing conditions perennial ryegrass at most times of the year, has the highest palatability, energy value and protein of all pasture grasses. It is popular because of its good herbage yields, persistence, grazing tolerance, ease of establishment, management and seed production (Wilkins, 1991). It is used widely in intensive, high-rainfall dairy and beef pastures and is naturalized throughout the higher rainfall temperate areas, extending into southern Queensland (Kloot, 1983). Dairy production in Australia relies almost entirely on grazing perennial ryegrass and white clover (Trifolium repens L.) pasture and therefore modifications in the quality of this feed source is likely to make a positive impact on animal production.

Dairying is a major Australian industry and is worth approximately $7 billion a year in terms of production, jobs, domestic economic activity and export sales. Milk production occurs in all states of Australia and across a wide range of climates, from cool temperate to semi-tropical, with Victoria accounting for sixty percent (Figure 1.1). Australia's climate and soil types are favourable to dairying based on year-round pasture grazing systems and in spring and early summer, excess pasture is conserved as silage and hay and fed out in the paddock during winter and autumn. Increasing attention is being paid to both pasture management and nutritional value. More sophisticated pasture and grazing
One of the major factors affecting the indoor production of a crop is the quality of the input materials. The Australian dairy regions and the state distribution of national production are shown in Figure 1.1.

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Figure 1.1 Australian dairy regions and the state distribution of national production.
management techniques are being developed, allowing farmers to stock more cows to the hectare with the potential to produce more milk from genetically improved animals.

It is important for the dairy industry to maintain the cost-effective edge of its pasture-based production systems. High-quality forages are a primary source of nutrients for ruminant animal production and they remain vital to the dairy industry. The development of pasture species optimized for difficult environments, with improved plant nutritive value, nutrient uptake, and protection against pests, diseases and soil acidification, could improve the dairy production system. Genetic engineering provides the techniques to improve the digestibility, palatability and nutritive value of pasture plants. Newly developed legume and grass species will increase the efficiency of livestock production by providing farmers with a pasture-based system that meets the nutritional requirement of the animals and maintains economic sustainability (Sullivan, 1994).

One of the major factors affecting ruminant productivity is the quality of the feed consumed. The low digestibility and high concentration of plant cell walls in forages, such as perennial ryegrass, particularly as they mature and reach flowering, often limits feed intake due to long rumen retention times, and reduces energy availability. As the genetic potential for production increases in ruminant livestock through breeding, the ability of contemporary pastures to provide sufficient nutrients and energy to realize that potential will not be sustained unless the quality of pastures can also be improved. The intake of forages and the digestibility of the cell wall may be improved by alterations in the concentration of cell-wall material or components associated with it.

1.2 Perennial ryegrass biology and agronomy

Perennial ryegrass (*Lolium perenne* L.) is a native of Europe, temperate Asia, and North Africa and occurs now both as a naturalized and as an extensively sown species in South Africa, North and South America, New Zealand, and Australia (Lamp *et al.*, 1990). It
occurs as a diploid (2n=14), wind pollinated, obligate outcrossing species but suffers serious inbreeding depression (Cunningham et al., 1993). Perennial ryegrass crosses freely with *L. multiflorum*, resulting in fully fertile hybrids (Kloot, 1983). Perennial ryegrass has a number of morphological forms but generally grows to a height of 90cm with acute, glabrous and shiny leaves (Figure 1.2). This species is separated from the other members of the *Lolium* genus by its perennial nature and distinctive spikelet characteristics (Kloot, 1983).

**Figure 1.2** A representation of a flowering perennial ryegrass plant divided into a number of tillers (eg. 1). Inset shows small tillers inside the leaf sheaths of the older flowering tillers and tillers with a partly emerged leaf.
The ryegrass plant appears as a clump in a perennial pasture and consists of a number of individual units called tillers (Figure 1.2). A mature ryegrass plant has approximately twenty-five tillers, with each tiller having its own leaves and root system, enabling the tiller to survive independently of the parent plant (Smith, 1993). All new growth arises from the production of new tillers. A mature tiller has only three healthy leaves, with leaf number one being the oldest and positioned at the base of the plant. Leaf number two is the next youngest leaf and its growth ceases once the third leaf appears at the top of the leaf sheath. Perennial ryegrass has two growth phases, commonly referred to as vegetative and reproductive. Plants in the vegetative stage are capable of producing leaves, roots and other tillers. As the plant matures it changes its growth patterns to producing seed heads. Reproductive activity is triggered by increases in day length and is evidenced by an acceleration in leaf appearance rate.

Perennial ryegrass is best suited to cooler regions that have higher rainfall with a growing season of at least eight months. The plant requires fertile, well-drained, preferably heavy textured soil and will not perform well if conditions are saline or the pH is lower than 4.8 (Smith, 1993). Perennial ryegrass pastures are generally sown in autumn to maximize soil moisture availability and temperature for successful germination and establishment.

A disadvantage of perennial ryegrass is its association with the seed-carried endophytic fungus, *Acremonium lolii* Latch. While seedling establishment, herbage yield, and persistence of perennial ryegrass are all increased by the presence of the endophyte (Cunningham et al., 1993) further research has shown that the fungus produces compounds which deter insects and nematodes from feeding on the grass (Smith, 1993). This association, however, also results in the well-documented neurotoxic disease of Ryegrass Staggers (Cunningham et al., 1993) which can affect the nervous system of livestock that graze on infected pastures. The fungus produces alkaloid toxins which affect the nervous system of grazing animals, causing tremors, spasms, loss of coordination and collapse. Losses of livestock from the disease are rare, however, stress-induced reductions in animal productivity can occur. Consequently, the grazing of
ryegrass pastures during the peak endophyte periods of late summer and early autumn must be carefully controlled.

Perennial ryegrass is a valuable forage grass which is used extensively in intensive high rainfall dairy and beef pastures. However, the quality of perennial ryegrass varies throughout the year due to changes in a number of nutritive characters indicating the potential for improvement. A number of these nutritive characteristics are amenable to genetic manipulation via traditional or molecular genetic approaches.

1.3 Factors affecting forage nutritive value

Grazing animals require a continuous supply of high-quality forage to sustain levels of high performance. Under Australian conditions of extensive grazing, variations in the quality and nutritive value of basal pasture can have a significant impact on animal production and production economics. Put simply, this means that improved feed quality improves animal performance. The nutritive value of a pasture grass refers to its capacity to meet the animal's energy and production requirements. Forage nutritive value can be evaluated in terms of both the voluntary intake of feed by the animal and the utilization of this feed to obtain energy resources (Stone, 1994). There are direct links between voluntary intake and feed utilization since some plant traits, such as digestibility, can affect both intake and utilization of nutrients (Stone, 1994).

Plant characteristics which affect voluntary intake are those such as palatability, leaf/stem ratios, plant habit, and the dimensions of leaves and stems. These characteristics involve both chemical determinants such as the presence of alkaloids, tannins, sugars and phytoestrogens, as well as plant physical and morphological characteristics such as green herbage availability, plant accessibility to the grazing animal and the resistance of the plant to mastication (Stone, 1994).
Plant characteristics which affect feed utilization are generally those involving cellular constituents and cell wall components. The most important herbage quality characters are high non-structural carbohydrate content and high dry matter digestibility (Wheeler and Corbett, 1989). High concentrations of water-soluble carbohydrates in forage grasses are positively related to efficient ruminant digestion. During maturation and senescence, the loss of easily-digestible cellular constituents such as water-soluble carbohydrates, minerals, and proteins is the major factor contributing to the reduction of in vitro dry matter digestibility (IVDMD) (Ballard et al., 1990). The factors that effect digestibility and nutritive value of plants during the earlier times of development, when water-soluble carbohydrates are not limiting, are those related to cell wall materials and structural carbohydrates. Cell walls represent between 30 and 80% of plant dry matter in grasses, and under some circumstances the majority of the carbohydrate fermented by rumen-microorganisms may be derived from the cell wall (Stone, 1994). Cell wall constituents such as wall polysaccharides, lignin, suberin, cutin, and lignin-polysaccharide complexes all play a role in determining the value of the feed under these conditions. For example, studies with Bermuda grass hybrids indicated that a 12% increase in digestibility due to more bioavailable plant cell wall (Akin et al., 1990) resulted in a 30% mean weight gain in cattle (Lowrey et al., 1968). There may be several opportunities for enhancing the nutritive value of pastures (see review by Stone, 1994), and factors that affect dry matter and cell wall digestibility may be of particular importance.

1.4 Structure of the plant cell wall

The importance of plant cell wall utilization by ruminants cannot be over estimated. Digestibility is influenced by cell wall width, rigidity, and chemical characteristics. Energy for animal production from most forages is obtained largely from fermentation of plant cell walls by rumen microorganisms. High live-weight gain or milk production by ruminants requires a high intake of forage and a high digestibility of the cell wall
component. There are important aspects of cell wall structure and development that may be related to the degradability of the wall to rumen microorganisms and hence, to the digestibility of the plant.

The cell wall in plants is composed of cellulose microfibrils embedded in a lignin-hemicellulosic matrix to which acetyl and phenolic acid groups are bound (Morrison, 1974). The growth and development of the cell wall can generally be divided into two phases: firstly, primary wall growth and secondly, secondary cell wall thickening. The primary cell wall constitutes the outermost layer of the wall of the cell. The primary wall, comprising a base structure of cellulose microfibrils, is laid down while cells are dividing and expanding (Wilson, 1993). At this stage, the cell wall is composed of polysaccharides, proteins and some phenolic acids including components such as pectins, xylans and hemicellulose. The cellulose microfibrils are bound to the hemicellulose polymers by hydrogen bonding (Morrison, 1979). In grasses, ferulic acid and a small amount of p-coumaric acid are esterified to arabinoxylan polymers which are laid down in the primary wall (He & Terashima, 1989).

The secondary cell wall is laid down inside the primary cell wall after cell expansion and elongation ceases (Wilson, 1993). During this phase, the cell wall becomes progressively thicker as it grows from the inner edge of the primary wall towards the centre of the plant cell (Bacic et al., 1988). The walls of cells with secondary thickening provide structural strength due to their ability to resist compressive forces. During the formation and thickening of the secondary cell wall, lignin is deposited in the middle lamella, extending into the secondary wall. The first stage of lignification occurs at the cell corners and middle lamella after the deposition of pectic substances is completed finished (Terashima et al., 1993). The second stage is a slow lignification stage, associated with further hemicellulose and xylan deposition. The major extent of lignification occurs in a third stage after the deposition of cellulose microfibrils has started.
1.5 Lignin as a component of plant cell walls

Lignin is a complex phenolic polymer composed of three phenylpropanoid subunits, \( p \)-coumaryl, coniferyl and sinapyl alcohols (Figure 1.3). The three monolignols, differ only in the pattern of aromatic ring substitution and give rise to \( p \)-hydroxyphenyl, guaiacyl and syringyl lignins, respectively. Most of the lignin synthesized in vascular plants is deposited in the cell walls of a few cell types such as sclerids, vessel elements, xylem and phloem fibres (Wilson, 1993) at the last stages of their differentiation. Lignin imparts rigidity to cell walls and assists in the conductance of solutes, by decreasing the permeability of cell walls in conductive xylem tissues (Higuchi, 1981). The deposition of lignin also occurs in response to wounding and pathogen attack, and may provide resistance to the penetration of plant tissues by fungal pathogens (Dixon and Paiva, 1995).

The lignified cell walls of plant tissue are the major cause of the low recovery of available energy from forages (Wilson, 1993). The lignin polymer is covalently linked to the cell wall and can be divided into “core” and “non-core” lignin components. Core lignin is a highly-condensed, high molecular weight polymer of the cinnamyl alcohols (Grisebach, 1981). Arabinoxylan ferulate esters found in the primary wall can be incorporated into the cross-linkages of the xylans to this core lignin (Iiyama et al., 1990). Non-core lignins are hydroxycinnamic acid monomers which usually form an ester linkage to either core lignin or hemicellulose. Non-core lignin units such as ferulic acid possess a second linkage to other cell wall components, particularly arabinoxylans, and act as a bridge, cross-linking core lignin and hemicellulose (Jung, 1989). Ferulate esters act as the initiation sites for these reactions and are thought to be mediated by a peroxidase-\( \text{H}_2\text{O}_2 \) formation of ether linkages between ferulic acid molecules and lignin phenolic monomers. Molecules of \( p \)-coumaric acid are also incorporated into the cell wall via ether linkages but they do not form cross-links to arabinoxylan (Lam et al., 1992).
Figure 1.3 Lignin monomer structures.

The three main lignin monomers, \textit{p}-coumaryl, coniferyl and sinapyl alcohols, are stored as glucosides and then transported to the cell wall by glucosyltransferase (GTF) and coniferin-specific 4-\textit{O}-glucosidase (C4G), where they are polymerized by peroxidase (PXD) or laccase (LAC) to form the \textit{p}-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) residues in lignin.
The monomer composition of lignin also changes as lignification of the cell wall proceeds from the primary cell wall into the thickening secondary wall. During lignin deposition the composition of the core lignin which is deposited changes from being composed mainly of guaiacyl units, derived from coniferyl alcohol, to lignin that becomes richer in syringyl units derived from sinapyl alcohol (Grisebach, 1981). Grasses also contain significant amounts of p-hydroxyphenyl units derived from p-coumaryl alcohol. These compositional changes in lignin during cell wall development of grasses are also associated with large increases in the amounts of p-coumarate and ferulate esters incorporated into the secondarily thickened cell wall (Ralph et al., 1994).

The architecture of the plant cell wall also differs with respect to the details of wall composition and structure among the major taxonomic groups of forage plant species. For example, leaves of legume species contain much less cell wall than do those of grasses, and do not exhibit the large increase in cell wall concentration which is typically associated with maturation in grass leaves (Wilman and Altimimi, 1982). The midrib of grasses is the major vein structure of the leaf and although in cross-sectional area it may be relatively small (6-13%) it can comprise 18-28% of the weight of the leaf and contain 14-25% of the lignified tissues in the leaf (Wilson, 1993). However, all forage species exhibit higher amounts of cell wall in stem material than leaves, with increases in the content of cell wall being common in maturing stems (Jung & Vogel, 1992). The cell walls of legumes are rich in pectins and contain higher amounts of cellulose, compared to xylans, than is observed for grasses (Aman, 1993).

1.6 Factors that affect cell wall digestibility

The cell wall is one of the greatest variables in feed utilization by ruminants. The basic limitations of rate and extent of digestion determine much of the cell wall matrix digestibility and utilization by all animals (Van Soest, 1993). The digestibility of the cell walls vary from 100% digestible, such as in mesophyll cells, to virtually 0% digestible,
such as in xylem (Akin, 1989). Variation in digestibility occurs among different tissues within a plant part and among similar tissues in different forage species for a number of reasons. One of the main reasons for these differences is the presence of lignin in the cell wall. Lignin has an affect on cell wall digestibility due to its concentration and composition. For example, the lignin content of legume cell walls is greater than that of grasses, however, grass lignin has a greater negative affect on cell wall digestibility than the lignin in legumes (Buxton and Russell, 1988). This suggests that not only lignin concentration *per se* but also lignin composition and other chemical associations may be involved in determining cell wall digestibility.

1.6.1 Lignin content and its effect on cell wall digestibility

Lignin is the major component of the cell wall that is recognized as limiting the digestion of cell wall polysaccharides in the rumen (Jung & Deetz, 1993). The diverse effects of lignin and its precursors on animal nutrition and forage digestibility have been recognized for many years. These effects are assumed to be due to a direct influence on cell wall digestibility, because lignin is a major component of the cell wall, rather than to an influence on total organic matter digestibility (Jung & Allen, 1995). Lignin shields the cell wall polysaccharides from enzymatic hydrolysis such that enzymes are unable to align themselves properly with the potential substrates for hydrolysis to occur. Mutant plants of maize, sorghum and pearl millet exhibit a brown-midrib phenotype associated with alterations in lignin content and composition and recent work on these mutants has shown that lignin not only reduces the extent to which cell wall polysaccharides are digested but possibly slows the rate of cell wall digestion (Cherney *et al.* 1986). Strong negative correlations between lignin and digestibility have consistently been found when analysing forages at a range of maturities and with different tissue fractions. Studies by Buxton and Russell (1988) and Van Soest (1964) show that both dry matter and cell wall digestibility of grasses decrease faster during maturation than that of legumes due to a more rapid accumulation of lignin in grass cell walls.
1.6.2 Lignin composition and its effect on cell wall digestibility

Variation in forage digestibility cannot be explained by lignin content alone. The composition and structure of lignin are also important factors in determining how efficiently structural polysaccharide in forages is digested. During maturation, cell wall lignin composition changes from mainly guaiacyl-type lignins to lignins rich in syringyl units. (Grisebach, 1981). Concurrently, the digestibility of these maturing cell walls becomes lower suggesting that lignin composition also affects digestibility of the cell wall. Additionally, the concentration of lignin in legumes is greater than in grasses. However, the lignin in grasses has a greater effect on reducing cell wall digestibility, further indicating that all lignins do not equally affect cell wall digestibility (Buxton & Russell, 1988).

The best evidence for the importance of concentration versus composition of core lignin is the data for the brown-midrib (bmr) mutants of the C₄ grasses. The bmr mutation does not always result in depressed concentrations of core lignin. Studies of the mutations in C₄ grasses such as sorghum and maize show that there is not only a reduction in lignin content (Chabbert et al., 1994a; 1994b) but also a change in lignin composition (Kuc et al., 1968, Akin et al., 1986; Lapierre et al., 1988). For example, even when bmr corn silage was not significantly lower in core lignin concentration, the in vivo digestibility was still greater and animal production was higher (Jung and Deetz, 1993). Taken together, these observations indicate that parameters other than lignin concentration may be responsible for differences in the way in which lignin affects cell wall digestibility. Possible explanations for this have been suggested that include: the increased levels of aromatic aldehydes incorporated in the lignin (Pillonel et al., 1991); decreased p-coumaric acid (Kuc et al., 1968; Chabbert et al., 1994b; Pillonel et al., 1991; Akin et al., 1986; Chereney et al., 1991); increased syringyl unit content and increased proportion of p-hydroxyphenyl units (Chabbert et al., 1994a; 1994b).
Further evidence for the effects of monomer composition on plant cell walls comes from the variation in lignin composition between gymnosperms and angiosperms. The guaiacyl-syringyl lignin, which occurs in dicotyledonous angiosperms, is more easily removed during wood pulping than the guaiacyl lignin typically found in gymnosperms (Chiang et al., 1988). The presence of methylated 5-OH groups in syringyl lignin is thought to decrease cross-linking opportunities in the lignin polymer, resulting in the syringyl lignin being easier to remove (Chiang et al., 1988). The differences seen between lignin monomer composition in angiosperms compared to gymnosperms and in the various mutant plants has been attributed to differences in lignin biosynthetic enzymes, their regulation and activity (Higuchi, 1985). The potential for altering cell wall digestibility of grasses can also be examined in terms of altering lignin composition.

1.6.3 Phenolic acids and their effect on cell wall digestibility

There have been numerous reports correlating phenolic acid content with digestibility (see review by Jung & Deetz, 1993). These phenolic acids are synthesized via the shikimic acid pathway and are the precursors for core lignin biosynthesis. Phenolic acids generally include ester-linked p-coumaric and ferulic acid. However, diferulic, sinapic, cinnamic and various benzoic acid derivatives are also esterified to forage cell walls (Jung, 1989).

Ferulic and p-coumaric acid are generally esterified to different components of the cell wall. Ferulic acid tends to be associated with the hemicellulose fraction whereas the majority of p-coumaric acid is esterified to core lignin (Iiyama et al., 1993). The negative effect of phenolic acids on cell wall digestibility is thought to correspond to the occurrence of ferulic acid, rather than p-coumaric acid, because ferulic acid forms ester-ether cross-links with arabinoxylan and lignin (Lam et al., 1994). p-Coumaric acid levels increase concurrently with lignin concentration during maturation and its esters-linkages are considered to be an indicator of the degree of lignification in a forage plant rather than exhibiting a direct influence on digestibility (Chesson, 1993). Although a small amount of p-coumarate esters are linked to arabinoxylan and interfere with digestion, the majority
of \( p \)-coumaric acid is esterified to lignin and is unlikely to directly affect digestibility (Jung & Deetz, 1993).

Ferulic acid esters linked to arabinoxylan are thought to interfere with digestion by hindering the alignment of xylanase to its polysaccharide substrate and preventing hydrolysis (Jung & Allen, 1995). These esterified ferulic acid links are only expected to reduce the rate of cell-wall digestion, rather than the extent, as phenolic esterases produced by rumen microorganisms, are ultimately capable of cleaving these bonds and removing the impediment to cell-wall digestion (Akin et al., 1993). Ferulic acid which is ester- and ether cross-linked between arabinoxylan and lignin, however, will not only affect the rate of cell-wall digestion but also the extent (Jung and Deetz, 1993). Ferulate esters are extensively cleaved by \textit{in vivo} ruminal fermentation (Jung and Fahey, 1983), however, when linked to arabinoxylan and lignin, the ester-linkage of the ferulate bridge is no longer susceptible to cleavage by ferulate esterase. The inhibition of esterase function is a physical one as the enzyme is unable to reach its target site due to the close proximity of the lignin polymer (Jung and Deetz, 1993).

Ether linkages are also inaccessible to enzyme degradation due to steric hindrance by the proximity of the lignin polymer, and there is no evidence to suggest that cleavage of ether linkages occurs in an aerobic environment. The biological depolymerization of lignin is an aerobic process (Kirk & Farrell, 1987), and is unlikely to occur in the rumen environment. Therefore, the effect of ferulate ethers on cell-wall digestibility is unlikely to be overcome by ruminal fermentation and is considered an undesirable characteristic of cell wall lignification in grasses.

Lignin content and composition, as well as the presence of phenolic acid precursors, are known to be involved in limiting grass cell wall digestibility in ruminants. The biosynthesis of lignin and its assembly into the cell wall are therefore a primary targets for genetic manipulation in order to increase cell wall digestibility. By elucidating and altering the key enzymes involved in the lignin biosynthetic pathway to regulate the flux
of metabolites, it may be possible to control lignin biosynthesis to enhance the cell wall digestibility of grasses and other economically important plants.

1.7 The lignin biosynthetic pathway

At the biochemical level, the overall synthesis of lignin involves a sequence of enzymatic reactions through the shikimic acid pathway, then via the common phenylpropanoid pathway from which the specific lignin biosynthetic pathway diverges (Figure 1.4). The general plant phenylpropanoid pathway involves the enzymes phenylalanine ammonia lyase (PAL), cinnamate-4-hydroxylase (C4H) and 4-coumarate-CoA ligase (4CL). PAL is the first enzymatic step of the general phenylpropanoid pathway which converts phenylalanine into derivatives of cinnamic acid. The pathway then diverges into three branches which lead to the formation of lignin, flavonoids and esters, respectively (Grisebach, 1981).

PAL first converts phenylalanine into trans-cinnamic acid, via deamination, and this product is in turn the substrate for C4H. The hydroxylase adds an -OH group to position 4 of the aromatic ring, utilizing O₂ and NADPH, to produce 4-hydroxycinnamic acid (Higuchi, 1981).

4-Hydroxycinnamic acid is converted into a number of different products by hydroxylation at the 3- and 5- positions by coumarate 3-hydroxylase (C3H) and ferulate-5-hydroxylase (F5H) and, in the case of the precursors used by the lignin and ester pathways, methylation of hydroxyl moieties occurs via caffeic acid O-methyltransferase (COMT), with S-adenosyl methionine as a co-factor. The next reaction step involves the conversion of the above precursor compounds into their corresponding CoA esters by 4CL, utilizing ATP and Mg²⁺, prior to being committed down one of the three branch pathways leading to either lignin, flavonoids or esters. An alternative methylation of the CoA ester precursors may occur at this position involving a cinnamoyl CoA O-methyltransferase (CCoAOMT) instead of COMT as reported in Zinnia (Ye et al., 1989).
Figure 1.4 The lignin biosynthetic pathway (drawn after Campbell and Sederoff, 1996).

The enzymes involved in lignin biosynthesis are: **PAL**, phenylalanine ammonia lyase; **C4H**, cinnamate 4-hydroxylase; **C3H**, 4-hydroxycinnamate-3-hydroxylase; **OMT**, S-adenosyl-methionine caffeate/5-hydroxyferulate-O-methyltransferase; **F5H**, ferulate 5-hydroxylase; **4CL**, hydroxycinnamate:CoA-ligase; **CCoA-3H**, 4-hydroxy cinnamoyl-CoA 3-hydroxylase; **CCoA-OMT**, S-adenosyl-methionine:caffeoyl-CoA/5-hydroxy-feruloyl-CoA-O-methyltransferase; **CCR**, cinnamoyl-CoA:NADPH oxidoreductase; **CAD**, cinnamyl alcohol dehydrogenase. Solid arrows mark reactions that have been demonstrated in vitro. Broken arrows are reactions that have been inferred by feeding studies. Corresponding EC numbers and detailed pathway discussion is outlined in the text.
The next two enzymes, cinnamoyl CoA reductase (CCR) and cinnamyl alcohol dehydrogenase (CAD) are specific to the lignin biosynthetic pathway. CCR converts the CoA esters into their corresponding aldehydes, using NADPH as a cofactor, while CAD converts the aldehydes to the respective p-coumaryl, coniferyl, and sinapyl alcohols exclusively utilizing NADP. These monolignol precursors, also known as p-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) units, are synthesized in the cytoplasm and then transported across the cell membrane (Campbell and Sederoff, 1996). As the hydroxycinnamyl alcohols have low water solubility (Iiyama et al., 1993), they are probably converted by specific transferases into the more soluble cinnamyl alcohol glucosides prior to transport out of the cell (Schmid and Grisebach, 1982; Schmid et al., 1982). Once outside the cytoplasm they may be released as alcohols by β-glucosidase prior to polymerization (Iiyama et al., 1993). Polymerization is generally thought to occur via a cell wall-bound peroxidase in the presence of the cofactor hydrogen peroxide (H$_2$O$_2$) (Iiyama et al., 1993). However, another enzyme, a polyphenol oxidase called laccase, has recently been implicated in polymerization via an alternative pathway (Bao et al., 1993). An alternative route for polymerization via O$_2$ and indole acetic acid has also been suggested (Ferrer et al., 1990).

Lignin heterogeneity in relation to quantity, quality and distribution probably results from the complexity of the pathway and the mechanisms involved in its regulation. Lignin heterogeneity may be due to: the flux of metabolites into the pathway controlled by entry point enzymes; the flux of metabolites through the pathway influenced by enzyme activity and metabolic channelling (Campbell and Sederoff, 1996); or combinations of each of these. The enzymes involved in the formation of lignin include those which are specific to lignification, such as CAD and CCR and those which are involved in other phenylpropanoid pathways or metabolic steps in the plant. Because of the complex nature of the pathway, there are many points at which alteration of lignin biosynthesis may be achieved. To prevent unwanted pleiotropic effects, approaches to modifying these enzymes will vary depending on their particular roles in phenylpropanoid metabolism,
whether they are members of multi-enzyme families, or whether they are single enzymes under tight regulatory control. Discussion of each of the main enzymes involved in lignin biosynthesis, and their potential for modification in order to alter lignin content and composition, are presented below.

1.7.1 Phenylalanine ammonia-lyase: PAL

PAL (EC 4.3.1.5) catalyses the first committed branch point in the biosynthesis of phenylpropanoid derivatives, which include lignin, suberin, flavonoid pigments and phytoalexins (Figure 1.4) (Hahlbrock and Scheel, 1989). The deamination of phenylalanine (or tyrosine in some grasses) to trans-cinnamic acid by PAL has been indicated as a key regulatory step in controlling the flux of metabolites through the phenylpropanoid pathway. PAL has been purified from a number of plant sources and is reported to exist as a monomeric form in some species and as a multimeric form in others. PAL apparently exists as a single isozyme in bamboo shoots (Chen et al., 1988), elicited pine suspension cultures (Campbell and Ellis, 1992b), sunflower hypocotyls (Jorrin et al., 1988), strawberry fruit (Given et al., 1988) and developing loblolly pine xylem (Whetten and Sederoff, 1992). Multiple isozymes of PAL have been found in rice (Minami et al., 1989), Arabidopsis (Ohl et al., 1990), potato (Joos and Hahlbrock, 1992), poplar (Subramaniam et al., 1993) and pea (Yamada et al., 1992). Multiple PAL cDNAs have been isolated from bean (Bolwell et al., 1985; Liang et al., 1989), parsley (Lois et al., 1989), and alfalfa (Gowri et al., 1991) whereas PAL appears to be encoded by a single gene in loblolly pine (Whetten and Sederoff, 1992).

During development, PAL enzymes are highly regulated by internal and external factors, such as substrates, products, inhibitors, hormones, light, carbohydrate levels, and wounding or infection, all of which affect both the synthesis and the activity of this enzyme (Gross, 1985). For example, rapid increase in the activity of a single PAL enzyme in response to elicitation has been reported in cell suspension cultures of jack pine (Pinus banksiana) (Campbell and Ellis, 1992b). This coincided with an increase in
lignification, with a preferential accumulation of guaiacyl lignin which appears to be a symptom of stress-induced lignification. Multiple PAL isozymes have been extensively studied in bean, parsley and alfalfa. Lignin heterogeneity could be explained in terms of different PAL isoforms being active at different times or locations and with different substrates during lignin biosynthesis. The characterized PAL isoforms may play different roles in the many aspects of phenylpropanoid metabolism. Because of the position of PAL in lignin biosynthesis, it may be possible to alter this enzyme to vary the flux of metabolites into the pathway, without altering the synthesis of other phenylpropanoid products, particularly if there are isozymes specific for lignin synthesis.

1.7.2 Hydroxylases: cinnamate-4-hydroxylase (C4H); coumarate 3-hydroxylase (C3H) and ferulate-5-hydroxylase (F5H)

These enzymes catalyze three types of aromatic ring hydroxylations resulting in the formation of hydroxycinnamic acids. The hydroxylase enzymes may be key branch points for determining the relative abundance of lignin monomer types. The first enzyme, cinnamate-4-hydroxylase (C4H; EC 1.14.13.11), catalyses the hydroxylation of cinnamic acid to generate p-coumaric acid, the second step in the general phenylpropanoid pathway. C4H has been purified to homogeneity from wounded *Helianthus tuberosus* tuber tissues (Gabriac et al., 1991) and Jerusalem artichoke (Benveniste et al., 1977). As with other enzymes in this pathway, C4H is highly inducible by different environmental cues. Recently, cDNAs coding for C4H have been characterized from Jerusalem artichoke (Teutsch et al., 1993), alfalfa (Fahrendorf and Dixon, 1993), mung bean (Mitzutani et al., 1993) and *Zinnia* (Ye, 1997). Analysis of these genes has indicated that C4H is a member of a new cytochrome P450 gene family (Fahrendorf and Dixon, 1993). It has been shown that there is an increase in C4H expression in *Zinnia* cell culture systems as tracheary elements begin to differentiate and this is specifically associated with the start of lignification (Ye, 1997). Recent studies have shown that the levels of the substrate and product of C4H are involved in transcriptional regulation of PAL and
chalcone synthase, indicating that C4H may play a regulatory role in the lignin pathway (Orr et al., 1993; Mavandad et al., 1990; Loake et al., 1991).

Coumarate-3-hydroxylase (C3H) and ferulate-5-hydroxylase (F5H) catalyze the hydroxylation of coumaric and ferulic acids to the substituted catechols caffeic and 5-hydroxyferulic acids, respectively. Little is known about C3H or the suggested alternative hydroxylation pathway involving coumarate CoA 3-hydroxylase (Kamsteeg et al., 1991). C3H is at a pivotal position in the pathway and may play an important role, not only in determining the rate of flux of metabolites through the pathway, but also in early pathway control of the ratios of monomers which are incorporated into lignin and/or the hydroxylation of products derived from p-coumaric acid. In some plants the hydroxylases which are active on p-coumarate are also active on p-coumaryl CoA, which suggests that a single enzyme could function with either substrate in vivo and potentially regulate lignin production at a number of points in the pathway.

F5H is also a member of the new cytochrome P450-linked monooxygenases (Meyer et al., 1997). A cDNA encoding F5H has recently been cloned from an Arabidopsis F5H mutant (fah1) (Chapple et al., 1994) by using a T-DNA tagged allele of fah1. The identity of the cloned gene was verified by complementation of the mutant phenotype using a wild-type version of the cloned gene. Assays of these hydroxylase enzymes in xylem and sclerenchyma enriched tissues from poplar stems, revealed differences in the ratio of each enzyme\(^2\)'s activity (Grand, 1984). These differences in relative hydroxylase activities may partially account for the variations in alcohol monomer composition of the lignin in various tissue types and at various stages of plant development. C3H may be important in regulating the ratio of p-hydroxyphenyl to guaiacyl and syringyl subunits, while F5H may be important for regulating the ratio of guaiacyl to syringyl subunits. The absence of syringyl units in conifers has been partly attributed to the absence, or low level expression of F5H (Meyer et al., 1997).
1.7.3 Caffeic acid O-methyl transferase; COMT

The penultimate end products of the lignin biosynthetic pathway are \( p \)-coumaryl, coniferyl and sinapyl alcohols (Figure 1.4). The relative proportions of these monomers in lignin varies from species to species and between cell types within an individual species (Lewis and Yamamoto, 1990). The differences in the ratios of these monomers incorporated into lignin is due partially to differences in substrate specificity between COMTs (EC 2.1.1.68) in different species. These methylating enzymes represent the third reaction type in the cinnamate pathway yielding ferulic acid and sinapic acid from the methylation of caffeic and 5-hydroxyferulic acids, respectively. cDNAs encoding COMT have been isolated from a number of dicotyledonous species such as poplar (Dumas et al., 1992; Hayakawa et al., 1996), aspen (Bugos et al., 1991; Tsai et al., 1995; Hu, 1996), alfalfa (Gowri et al., 1991), tobacco (Jaeck et al., 1992), Chrysosplenium (Gauthier et al., 1996), Eucalyptus (Poeydomenge et al., 1994), Stylosanthes (McIntyre et al., 1995), almond (Garcia et al., 1994), Zinnia (Ye and Varner, 1995), and loblolly pine (Zhang and Chiang, 1995). The isolation of genes encoding COMT from monocotyledonous species has been limited, with genes identified in maize (Collazo et al., 1992) and recently, a putative gene in barley (Lee et al., 1996). Investigations of the substrate specificities of COMTs reveal that the enzyme isolated from gymnosperms, such as pine, preferentially methylates caffeic acid and is almost inactive on 5-hydroxyferulic acid. In contrast, the analogous enzyme from angiosperms methylates both of these substrates at approximately equal rates (Bugos et al., 1992). These two enzyme activities are commonly known as mono- and bi-specific, respectively, and these substrate specificities may represent at least one biochemical basis for the known differences between the lignin from gymnosperms and angiosperms.

1.7.4 Caffeoyl-CoA O-methyltransferase; CCoA-OMT

CCoAOMT (EC 2.1.1.104) has been implicated in plant defence responses. A cDNA was isolated from Zinnia cell cultures which were undergoing tracheary element
differentiation (Ye et al., 1994). The encoded CCoAOMT activity was highly correlated with lignification in this system and its role as an alternative methylation pathway using caffeoyl CoA and 5-hydroxyferuloly-CoA as substrates has been hypothesized (Ye et al., 1994).

1.7.5 4-Coumarate-CoA ligase; 4CL

4-Coumarate-CoA ligase (4CL; EC 6.2.1.12) catalyses the formation of the CoA thioesters of 4-coumaric, caffeic or ferulic acid. The coenzyme A esters of 4-coumaric acid and other cinnamate derivatives are central intermediates in the synthesis of many phenylpropanoid derivatives in higher plants (Hahlbrock and Grisebach, 1979). These thiol esters are synthesized by 4CL which uses ATP, proceeding through acyl adenate intermediates to form the CoA thioester (Whetten and Sederoff, 1995). The products generated from this enzyme reaction represent branch points between the general phenylpropanoid metabolism and pathways leading to the synthesis of various end products including lignins, condensed tannins and flavonoids. It has been suggested that 4CL plays a pivotal role at a branch point in phenylpropanoid metabolism by regulating the flux of substituted cinnamate intermediates into subsequent products (Lee and Douglas, 1996). This regulatory role is supported by the existence of isoforms of 4CL with different substrate specificities and tissue distributions.

These isoforms of 4CL have been purified from several leguminous and woody species including soybean (Knobloch & Hahlbrock, 1975), petunia (Ranjeva et al., 1976), Forsythia (Gross and Zenk, 1974), pea (Wallis & Rhodes, 1977), poplar (Grand et al., 1983), and maize (Vincent and Nicholson, 1987). The partially-purified isoforms from soybean, petunia and poplar exhibit different substrate specificities towards the substituted cinnamic acids. Only a single isoform of 4CL has been found in spruce (Luderitz et al., 1982) and loblolly pine (Voo et al., 1995) and two slightly-different 4CL forms have been identified in parsley (Knobloch & Hahlbrock, 1977), both with similar substrate preferences.
The cDNA sequences encoding 4CL have also been isolated from parsley (Lozoya et al., 1988), potato (Bechor-Andre et al., 1991), soybean (Uhlmann and Ebel, 1993), loblolly pine (Voo et al., 1995), Arabidopsis (Lee et al., 1995), aspen (Kawaoka and Chiang, 1996) and tobacco (Lee and Douglas, 1996). Genomic clones have also been isolated from parsley (Douglas et al., 1987; Lozoya et al., 1988), rice (Zhao et al., 1990), potato (Becker-Andre et al., 1991) and loblolly pine (Zhang and Chiang, 1997). The isolation of these 4CL cDNAs and genes generally supports the hypothesis that in some species 4CL is encoded by multiple and divergent genes, which in others it is encoded by similar duplicated genes or by a single gene.

As with other phenylpropanoid enzymes, 4CL is developmentally regulated, is activated by environmental conditions, and exhibits different substrate specificities and catalytic properties. The gymnosperm 4CL, and some angiosperm isoforms, do not use sinapic acid as a substrate, again indicating that independent 4CL isozymes could play regulatory roles in providing precursors for specific phenylpropanoid pathways, or that different isoform activities could influence lignin monomeric composition (Grand et al., 1983).

1.7.6 Cinnamyl CoA reductase; CCR

The enzyme CCR (EC 1.2.1.44) catalyses the reduction of the hydroxycinnamoyl-CoA esters to their corresponding p-coumaryl, coniferyl and sinapyl aldehydes. This is recognized as the branch point enzyme into the lignin biosynthetic pathway and, as such, is considered to be a potential control point regulating the flux of phenylpropanoid metabolites towards the biosynthesis of lignins. CCR has been purified and partially characterized from a number of species including soybean cultures (Wegenmayer et al., 1976), spruce cambial sap (Luderitz & Grisebach, 1981), and poplar xylem (Sarni et al., 1984). A purified CCR protein from eucalypt xylem was sequenced and the corresponding cDNA and genomic clone have been obtained and characterized (Boudet et al., 1995; Lacombe et al., 1997). The identity of the cDNA clone was verified by functional expression of the recombinant protein in E. coli. The Eucalyptus CCR cDNA
was used to isolate corresponding cDNAs from tobacco, poplar and festuca, indicating that the gene is highly conserved among plant species. Sequence analysis shows that CCR shares an ancestral link with dihydroflavanol reductase (DFR), the first enzyme of the anthocyanin pathway and both the enzymes are considered to be new members of the plant dihydrophenol reductase superfamily (Grima-Pettenati et al., 1997; Lacome et al., 1997).

1.7.7 Cinnamyl alcohol dehydrogenase: CAD

CAD (EC 1.1.1.195) catalyses the final reductive step of the lignin biosynthetic pathway, producing the hydroxycinnamyl alcohols, \( p \)-coumaryl, coniferyl and sinapyl alcohol. These compounds are the monomeric precursors of lignins. CAD is considered to be specific for lignification as it occurs after the branch points for general phenylpropanoid metabolism. CAD has been purified from a number of species and has generally been shown to consist of two subunits. A single isoform is present in the gymnosperms loblolly pine, and it has been shown to be encoded by a single gene although allelic variants are known to exist (O'Malley et al., 1992). The situation is more complex, however, with angiosperms where single and multiple forms of the enzyme have been isolated. One of the most polymorphic species is wheat (Pillonel et al., 1992) in which up to 7 possible isoforms have been demonstrated in crude extracts.

To date, cDNAs encoding CAD have been cloned from a large number of dicotyledonous species including tobacco (Knight et al., 1992), Aralia (Hibino et al., 1993b), poplar (Van Doorsselaere et al., 1995a), alfalfa (Van Doorsselaere et al., 1995a), Eucalyptus (Grima-Pettenati et al., 1993), loblolly pine (O'Malley et al., 1992) and spruce (Galliano et al., 1993). The isolation of full-length DNA sequences encoding CAD from monocotyledonous species has not been reported, however, partial genomic sequences have been isolated from maize (GenBank Accession no. a24085) and rice (GenBank Accession no. d40685). These angiosperm and gymnosperm CAD genes have been shown to have a wide range of tissue expression patterns and genomic organisation.
ranging from single a gene, as in loblolly pine (O’Malley et al., 1992), to small gene families as in tobacco (Knight et al., 1992) and Aralia (Hibino et al., 1993b).

1.7.8 Peroxidase; PXD and/or Laccase; LAC

Polymerization of the monolignol precursors is the final step in the lignification sequence, and is initiated by the oxidation of their phenolic hydroxyl groups. It has been considered likely that a peroxidase (PXD; EC 1.11.1.7) functions as the natural catalyst of this reaction and evidence of peroxidase distribution in lignifying tissues (Harkin & Obst, 1973) further strengthens this view. Recently, however, a laccase (LAC; EC 1.10.3.2) was purified from differentiating xylem of loblolly pine (Bao et al., 1993); it is an oxidase that requires oxygen, instead of hydrogen peroxide. This purified enzyme has been suggested to be involved in lignification because of its association with lignification and its substrate specificity (Bao et al., 1993; O’Malley et al., 1993). Both PXD and LAC are involved in other phenylpropanoid pathways and perturbation of these enzymes may result in pleiotropic effects. The occurrence of isozymes of these enzymes, some of which may be specific for lignification, may provide an opportunity to alter their activity in a manner which specifically affects the polymerisation of lignin.

1.8 Mutants plants with altered lignin properties

Mutant plants with altered lignin content and/or composition have been characterized in maize, sorghum, pearl millet, Arabidopsis and loblolly pine (Table 1.1). The best known of these mutant plants are the C₄ grasses which exhibit a brown-midrib (bmr; bm) phenotype. The typical phenotype of these plants is the display of a red-brown midrib in the leaf and stem of the plant. The brown-midrib phenotype has been examined in a number of mutant lines and was found to result from various mutations in the lignin biosynthetic pathway.
Table 1.1 Summary of natural or chemically induced mutant plants with altered lignin properties.

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Species</th>
<th>Enzyme</th>
<th>Lignin (content)</th>
<th>Lignin (composition)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>bm1</td>
<td>Maize</td>
<td>?</td>
<td>-18%</td>
<td>-p-coumarate</td>
<td>Kuc et al., 1968</td>
</tr>
<tr>
<td>bm2</td>
<td>Maize</td>
<td>?</td>
<td>-30%</td>
<td>+S:G ratio</td>
<td>Chabbert et al., 1994b</td>
</tr>
<tr>
<td>bm3</td>
<td>Maize</td>
<td>-OMT</td>
<td>20-30%</td>
<td>-G units</td>
<td>Chabbert et al., 1994a</td>
</tr>
<tr>
<td>bm4</td>
<td>Maize</td>
<td>?</td>
<td>-8%</td>
<td>-S units</td>
<td>Kuc et al., 1968</td>
</tr>
<tr>
<td>bmr6</td>
<td>Sorghum</td>
<td>-CAD</td>
<td>5-51%</td>
<td>+aldehydes</td>
<td>Pillonel et al., 1991</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-G &amp; -S units</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-p-coumarate</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+ ferulate</td>
<td></td>
</tr>
<tr>
<td>bmr12/18</td>
<td>Sorghum</td>
<td>?</td>
<td>-S units</td>
<td>-p-coumarate</td>
<td>Akin et al., 1986</td>
</tr>
<tr>
<td>bmr</td>
<td>Pearl millet</td>
<td>?</td>
<td>-20%</td>
<td>+G units</td>
<td>Cherney et al., 1991</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-p-coumarate</td>
<td></td>
</tr>
<tr>
<td>fah1</td>
<td>Arabidopsis</td>
<td>-F5H</td>
<td>no change</td>
<td>-S:G ratio</td>
<td>Chapple et al., 1992</td>
</tr>
<tr>
<td>cad null</td>
<td>Loblolly pine</td>
<td>-CAD</td>
<td>-10%</td>
<td>+aldehyde</td>
<td>MacKay et al., 1997</td>
</tr>
</tbody>
</table>

* Items marked with a - indicate a decrease, items marked with + indicate an increase, items marked with ? are unknown. G identifies guaiacyl units, an S identifies syringyl units.
Four specific brown-mid rib mutations (bml-bm4) have been identified and characterized in maize (Kuc et al., 1968). Each one of these mutations results in reduced lignin content, increased digestibility, increased lodging, and a reduction in yield (Cherney et al., 1991). With the bm1 mutant of maize (Kuc et al., 1968), lignin content is reduced by up to 18% in mature plants compared to the wild-type. It has been hypothesized that the bm1 mutation affected the reduction of the phenolic acids (Gee et al., 1968). The bm2 mutant has been studied in more detail and exhibits 5-15% lower amounts of p-coumarate and a decrease in lignin content of up to 30%. The monomeric composition of the lignin is also altered with an increased S/G ratio due to a reduction in G units and an increase in S units (Chabbert et al., 1994a). The bm3 mutant is the best characterized of all four maize brown-midrib mutants as a biochemical and molecular explanation for this mutation has been elucidated. In maize lines carrying the bm3 mutation, the lignin content is reduced by 20-30% and the levels of COMT enzyme activity are as low as 10% of the wild-type plant (Grand et al., 1985). This mutation also results in an alteration of lignin composition evidenced by a decrease in the presence of guaiacyl units. The molecular basis for this mutation is a structural change in the gene encoding COMT (Vignols et al., 1995). The mutation in bm4 reduces the amount of lignin by up to 8% with a concomitant reduction in the presence of syringyl aldehyde (Kuc et al., 1968) but the biochemical and molecular lesion has not been defined.

The chemically-induced bmr mutants from sorghum (bmr6, bmr12, bmr18) share similar phenotypic and chemical characteristics as those of the bmr mutations in maize. The bmr6 mutation has been well characterized, with mutants showing between 5-51% reduction in lignin content in the stem and a 5-25% reduced lignin content in the leaves. This is correlated with an increase in dry matter digestibility of 33% and cell wall digestibility of 43% (Porter et al., 1978). Biochemically, the plants show a 6-fold reduction in CAD activity and an unexpected decrease in OMT activity (Bucholtz et al., 1980). The monomeric composition of lignin is altered in these mutants which have a 3-fold increase in aldehydes, reduced guaiacyl and syringyl units, decreased p-coumarate and increased
ferulate contents (Pillonel et al., 1991). The bmr12 and 18 mutants exhibit a reduced lignin content and compositional changes including reduced syringyl moieties and decreased p-coumarate. A bmr mutation has also been identified in pearl millet (Cherney et al., 1991) exhibiting a reduction in lignin content of approximately 20% and a lignin composed of more guaiacyl units and a 38% decreased p-coumarate content (Hartley et al., 1992).

Phenotypically the brown-midrib plants are similar as are the measured reductions in lignin content. However, lignin composition and the presence of hydroxycinnamic acids vary substantially between each line. This suggests that several different steps in the lignin biosynthetic pathway may be altered, offering a number of potential points for manipulation to attempt to increase cell wall digestibility in other species.

Mutations have been found in the dicotyledonous angiosperm, Arabidopsis (Chapple et al., 1992) and the gymnosperm, loblolly pine (McKay et al., 1997) with both of these mutants exhibiting variations in lignin composition. The lignin mutation of Arabidopsis (fah1) was found to affect the F5H enzyme in the lignin biosynthetic pathway. The lignin of this fah1 mutant resembled that of gymnosperms, containing only guaiacyl units rather than the guaiacyl-syringyl units found in the wild-type Arabidopsis. The identification of the Arabidopsis fah1 mutant supports the hypothesis that one of the differences between angiosperm and gymnosperm lignins is due to the absence of F5H in the gymnosperm lignin pathway (Campbell and Sederoff, 1996).

A mutant carrying a null allele for the gene encoding CAD has been identified in loblolly pine (MacKay et al., 1997). This null allele has a small recessive effect on lignin content, decreasing it to 90% of that of wild-type plants. The major effect of this null allele is reflected in the altered lignin composition, which resembles that of the bmr mutants of the C4 grasses. The wood of this CAD null is red-brown in colour and is enriched in coniferyl aldehyde. These compositional changes in lignin may cause the increased alkali and soda pulping extractability of the lignin reported in these trees (MacKay et al., 1997).
1.9 Transgenic plants with altered lignin properties

The effect of altering many of the enzymes involved in lignin biosynthesis has been examined by the construction of genetically engineered plants. These plants show how reductions or increases in the activity of these enzymes effect the lignin content or composition, plant morphology and cell wall digestibility. (Table 1.2)

To gain a greater understanding of the role of PAL in the regulation of phenylpropanoid metabolism, a PAL gene from bean (*Phaseolus vulgaris*) was introduced into tobacco to study the effects of over expression of the enzyme (Elkind et al., 1990). This resulted in some plants having a 2- to 5-fold increase in PAL activity with increased accumulation of chlorogenic acid and 4-coumaric acid. Interestingly, two plants from this initial transformation showed sense suppression of endogenous PAL at both the RNA and enzyme level (Elkind et al., 1990). The level of PAL activity in these lines ranged from 100% to 0.2% of wild-type. The plants were stunted in growth and in plants with PAL activity lower than 25% of wild-type levels, the content of lignin was 3- to 4-fold lower (Bate et al., 1994). Both the PAL over- and under-expression experiments suggest that this enzyme catalyses important rate-determining step regulating not only lignin deposition but the overall flux into the phenylpropanoid pathway. The effectiveness of modification of lignin by PAL transgenes will depend on the extent of inhibition of PAL activity, the activity of enzymes downstream in the pathway, and whether the suppression of early steps in the pathway will lead to pleiotropic effects (Campbell and Sederoff, 1996).

To clarify the role of 4-coumarate:CoA ligase (4CL) in the biosynthesis of the guaiacyl and syringyl lignin units found in angiosperms, *Arabidopsis* plants containing antisense 4CL transgenes were generated (Douglas et al., 1997). In these plants, varied decreases of 4CL activity, as low as 8% of wild-type levels, were recorded. Phenotypically, these plants were normal, however, they were found to have up to a 50% reduction in thioglycolic acid-derived lignin. The S:G ratio in these plants also increased due to a
Table 1.2 Summary of transgenic plants with altered lignin properties.

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Test species</th>
<th>Enzyme</th>
<th>Lignin (activity)</th>
<th>Lignin (content)</th>
<th>Lignin (composition)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>s-PAL</td>
<td>tobacco</td>
<td>0.2-100% decrease</td>
<td>no change</td>
<td>Bate et al., 1994</td>
<td></td>
<td></td>
</tr>
<tr>
<td>as-4CL</td>
<td><em>Arabidopsis</em></td>
<td>-8% -50%</td>
<td>+S:G ratio</td>
<td>Douglas et al., 1997</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ox-F5H</td>
<td>tobacco</td>
<td>+F5H no change</td>
<td>+18-27% in S</td>
<td>Meyer et al., 1997</td>
<td></td>
<td></td>
</tr>
<tr>
<td>as-COMT</td>
<td>tobacco</td>
<td>decrease no change</td>
<td>-S, +5OHG</td>
<td>Atassanova et al., 95</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>tobacco (P)</td>
<td>decrease no change</td>
<td>-S</td>
<td>Dwivedi et al., 1994</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>tobacco (A)</td>
<td>decrease decrease no change</td>
<td>-S</td>
<td>Ni et al., 1994</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Stylosanthes</em></td>
<td>-95% no change</td>
<td>-S</td>
<td>Rae et al., 1996</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Poplar</td>
<td>-50-95% no change</td>
<td>-S:G</td>
<td>Van Doorsselaere et al., 1995b</td>
<td></td>
<td></td>
</tr>
<tr>
<td>s-COMT</td>
<td>tobacco</td>
<td>decrease decrease</td>
<td>-S: +5OHG</td>
<td>Atanassova et al., 95</td>
<td></td>
<td></td>
</tr>
<tr>
<td>as-CCR</td>
<td>tobacco</td>
<td>-30% -10%</td>
<td>+S:G: -G</td>
<td>Grima-Pettenati et al., 1997</td>
<td></td>
<td></td>
</tr>
<tr>
<td>s-CCR</td>
<td>tobacco</td>
<td>-98% no change</td>
<td>-S:G: -S</td>
<td>Schuch et al., 1997</td>
<td></td>
<td></td>
</tr>
<tr>
<td>as-CAD</td>
<td>tobacco</td>
<td>-80-93% no change</td>
<td>+aldehydes</td>
<td>Halpin et al., 1994</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+S</td>
<td></td>
<td></td>
</tr>
<tr>
<td>as-PXD</td>
<td>tobacco</td>
<td>-44% no change</td>
<td>no change</td>
<td>McIntyre et al., 1996</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rib-PXD</td>
<td>tobacco</td>
<td>-52-60% no change</td>
<td>no change</td>
<td>McIntyre et al., 1996</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ox-PXD</td>
<td>tobacco</td>
<td>increase 2x increase</td>
<td>no change</td>
<td>Lagrimini et al., 1990</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Items marked with: s indicates sense constructs, as indicates antisense constructs, ox indicates over-expression, rib indicates ribozyme constructs. Tobacco marked with (A) indicates heterologous system with alfalfa cDNA, and (P) indicates heterologous systems with poplar cDNA. Items marked with - indicate a decrease and a + indicates an increase.
significant decrease in the guaiacyl units but no change in the proportion of syringyl units. This lack of change in syringyl lignin suggests that the 4CL enzyme may not be directly involved in activating the precursors for sinapyl alcohol synthesis and that an alternative pathway which has yet to be characterized, may exist in *Arabidopsis*. If this down-regulation of 4CL leads to a decrease in guaiacyl units this strategy may be useful in altering lignin composition to one that is less condensed and contains more syringyl units.

The over-expression of a previously-cloned ferulate-5-hydroxylase (F5H) cDNA (Chapple *et al.*, 1994) in tobacco and *Arabidopsis* has provided information on the potential role that F5H may play in altering lignin monomer composition. Over-expression of F5H in these transgenic plants resulted in significant increases (18-27%) in the syringyl content of lignin in both species (Meyer *et al.*, 1997). This alteration of lignin composition but not content, demonstrates that F5H may be a rate-limiting step in lignin biosynthesis. Compositional changes in lignin may be achieved without pleiotropic effects, and in a controlled way, when manipulating the expression of this gene.

Antisense and sense constructs of COMT have been transformed into a number of species and this has been effective in altering the lignin composition and content in these plants. The effect of antisense and sense COMT transgenes in poplar resembles that reported with the *bm3* mutation in maize. The plants have reduced COMT enzyme activity and a decrease in lignin syringyl units. The formation of a new monomer, 5-hydroxyguaiacyl (5 OH-G), was reported but there was no apparent change in total lignin content (Van Doorsselaere *et al.*, 1995b). Transgenic tobacco plants expressing sense and antisense mRNAs of COMT from tobacco (Attassanova *et al.*, 1995), lucerne (Ni *et al.*, 1994) and aspen (Dwivedi *et al.*, 1994) also have reported changes in lignin content and composition. Tobacco containing homologous partial sense and full-length antisense COMT gene constructs showed reductions in COMT activity. This was correlated with a drop in the syringyl unit content of the lignin and an increase in that of the new 5 OH-G moiety. However, no visible change in midrib colour was reported as seen in maize *bm3* mutants and in transgenic poplar (Van Doorsselaere *et al.*, 1995b). Tobacco containing
antisense poplar COMT also showed a decrease in COMT activity which was associated with a drop in syringyl units but no alternate moiety, such as 5-OH guaiacyl was formed. Contrary to this, tobacco containing antisense lucerne COMT showed the expected decrease in COMT activity, and a reduced lignin content rather than a change in lignin composition.

Analyses of the suppression of COMT activity in *Stylosanthes humilis* by antisense has included investigations on its effect on lignin and the digestibility of this important forage species (Rae et al., 1996). Results showed that in the most affected lines, COMT activity was suppressed by more than 95% compared to the wild-type. Overall lignin content in these lines was unchanged; however, histochemical tests revealed low levels of syringyl units present in the lignin. The correlation between change in lignin composition and digestibility was evidenced by the 10% increase in the digestibility of antisense plants compared to the controls. This is the first report of the successful molecular improvement of the digestibility of a pasture plant (Rae et al., 1996). These findings suggest that down regulating the expression of the genes encoding COMT may lead to modification of the lignin content or composition of forage plants and thereby to increased fodder digestibility.

Antisense and partial sense RNA constructs of previously-isolated tobacco cDNAs encoding CCR (Boudet et al., 1995) have been transformed into tobacco under constitutive and tissue-specific control (Grima-Pettenati et al., 1997; Schuch et al., 1997). A 30% decrease in CCR activity was recorded in antisense plants while activity in sense-suppressed plants were as low as 1.2% of wild-type activity. The lignin content in the antisense plants was also reduced from 19% in the wild-type plant to between 17% and 10% in the transgenic lines. Lignin compositional changes in these transgenic lines were also recorded with antisense plants exhibiting an increased S/G ratio due to a decrease in the presence of guaiacyl but not syringyl residues. In contrast to this, the sense suppressed plants showed a decrease in the S/G ratio due to a decrease in syringyl units. Pleiotropic effects associated with the decrease in CCR activity were varied with some of
the antisense plants exhibiting darker green leaves, a brown pigmented xylem tissue which showed evidence of collapsed xylem vessels, and increased amounts of cellulose and hemicellulose. Similarly, the plants containing the partial CCR sense RNA constructs also exhibited a range of morphological changes such as stunted growth and brown coloration of xylem tissue. These plants apparently exhibited an increase in cellulose fibre width and flexibility and up to a 40% increase in plant biomass when the plants were grown to maturity (Schuch et al., 1997).

Tobacco plants transformed with an antisense CAD construct showed varying degrees of reduction in CAD activity (80-93%) and modification of lignin composition (Halpin et al., 1994). Plants with a 93% reduced CAD activity showed changes in monolignol composition which included increases in the ratios of aldehyde- to alcohol-derived lignin and a preferential use of syringyl subunits. Plants with 80% reduced CAD activity did not show any changes in monolignol composition. However, both lines showed an increase in the content of alkali-extractable phenolic components, suggesting an alteration in the proportion of phenolic residues bound by ester linkages to the cell wall. These lines also showed an increase in thiglycolic acid-extractable lignin, suggesting that the types of bonds formed between phenolic acids, monolignols and other cell wall polymers depend upon both the concentrations and types of different subunits available (Whetten and Sederoff, 1995).

Transgenic tobacco have been produced, that over-express an anionic peroxidase which is believed to be involved in lignification (Lagrimini et al., 1990) or that are suppressed in peroxidase activity (McIntyre et al., 1996). In the over-expressing plants, the percentage of lignin and lignin-related polymers in the cell walls of pith tissue was 2-fold greater when compared to that of wild-type plants. A browning of the tissue and an increase in peroxidase activity was recorded as a response to wounding, with wound-induced lignification beginning earlier in the over-expressing plants compared to the control plants (Lagrimini, 1991). While transgenic tobacco carrying peroxidase antisense or ribozyme constructs had peroxidase enzyme levels reduced to 44% and 52-60% of wild-type.
respectively, these plants showed no significant change in lignin content or composition (McIntyre et al., 1996).

1.10 Aims of this thesis

The main objective of this work was to examine the nature of lignin biosynthesis in the developing perennial ryegrass plant in order to determine the correlations between lignification and decreases in digestibility in this agronomically important species. The first approach taken in this developmental study involved the isolation and characterization of cDNAs which encode cinnamyl alcohol dehydrogenase (CAD) and caffeic acid O-methyltransferase (COMT). The isolation of full-length cDNA sequences for CAD from perennial ryegrass would provide the first molecular data on genes encoding this enzyme from a monocotyledonous species. Developmental northern blot hybridization analyses would be important in determining the relationship of gene expression and regulation to enzyme activity during perennial ryegrass leaf development. Southern blot hybridization analyses would provide information on the genomic organization of genes encoding this enzyme.

The isolation of genes encoding COMT from perennial ryegrass is important in terms of providing the molecular information about monocotyledonous genes which encode potentially lignin-specific forms of the COMT enzyme which are expressed in lignified tissue such as leaf, sheath and stem. Developmental characterization of these COMT-encoding genes by northern blot hybridization analysis would proved information on the regulation of these genes. Southern blot hybridization would be useful in determining the number of genes in this species, which may encode different forms of COMT and be used as candidates for controlled manipulation of lignin biosynthesis.

The second approach to this study involves the biochemical analysis of CAD and COMT enzyme activities during perennial ryegrass development, as this had not been established
in any monocotyledonous species prior to this work. A characterization involving reaction kinetics and substrate affinities throughout the period of leaf development would indicate if either of these enzymes were appropriate targets for attempts to manipulate lignin biosynthesis in this monocotyledonous species.

A third approach to the study of lignin biosynthesis in perennial ryegrass involves correlating the CAD and COMT enzyme activities and gene expression with changes in content, potential changes in composition, and the lignin cross-linking interactions with the cell wall. These studies had not been done in young developing pasture grass species and would provide further indications on the interaction of lignin biosynthesis and alterations in the digestibility of the plant cell wall.

A final analyses on the changes in the in vitro digestibility of total dry matter and the cell wall was undertaken in order to study the physiological response caused by changes in CAD and COMT activity, lignin content and lignin-polysaccharide cross-linking as the perennial ryegrass plant develops. See Addendum
CHAPTER 2

ISOLATION AND CHARACTERIZATION OF A RYEGRASS CAD cDNA

2.1 INTRODUCTION

Lignin monomers are elaborated from phenylalanine and the first three steps, which lead to the synthesis of 4-coumaryl-CoA, are common to the synthesis of a wide range of phenylpropanoid products including, in addition to lignin, flavonoid pigments, isoflavonoid phytoalexins and hydroxycinnamic acid esters (Lewis and Yamamoto, 1990). After this branch point, production of the lignin monomers involves two reduction steps catalyzed by cinnamoyl-CoA reductase (CCR; EC 1.2.1.44) and cinnamyl alcohol dehydrogenase (CAD; EC 1.1.1.195). These enzymes are specific to the synthesis of lignin and related wall-bound phenolic material, making them the most obvious targets for the manipulation of lignin (Boudet et al., 1995). CAD catalyses the reduction of the cinnamyl aldehydes; p-coumaryl, coniferyl and sinapyl aldehyde to their corresponding cinnamyl alcohols which are the monomeric precursors of lignin. CAD is a unique member of the alcohol dehydrogenase family of enzymes, due to its absolute requirement for NADPH as a co-factor, in contrast to other members of the alcohol dehydrogenase family which use NAD.

The difference in substrate affinities of CAD enzymes from angiosperms and gymnosperms may play a role in controlling the formation of different types of lignin (Kutsuki et al., 1982). CAD preparations from gymnosperms are more active on coniferyl aldehyde, whereas angiosperm CADs show equal activity for both coniferyl and sinapyl aldehydes (Gross, 1985). Isoforms of CAD with markedly different substrate specificities have been detected in soybean (Wyrambik and Grisebach, 1975), wheat (Pillonel et al., 1992), Eucalyptus (Goffner et al., 1992), and Salix (Mansell et al., 1974) while other species such as loblolly pine (O'Malley et al., 1992; MacKay et al., 1995) contain only a single form of the enzyme encoded by a single gene.
CAD has been a target for genetic engineering because of its committed involvement in lignin biosynthesis. Evidence for the effectiveness of manipulating CAD in altering the lignin state of plants comes from both natural mutants and transgenic plants. Biochemical studies of brown-midrib (bmr) mutants of sorghum, in particular those lines carrying the bmr6 mutation have significantly reduced CAD activity (Bucholtz et al., 1980). The bmr6 mutation leads to a 25-51% reduction in the lignin content in leaf and stem tissue. The monomeric composition of the lignin is also altered, being composed of more aldehydes and less guaiacyl and syringyl units. These changes in lignin content and composition result in a 33% increase in digestibility of stem and leaf tissue (Porter et al., 1978).

Similarly, tobacco plants transformed with an antisense CAD construct show varying degrees of reduction (80-93%) in CAD activity and modification of lignin composition (Halpin et al., 1994). Plants with a 93% reduced CAD activity showed changes in lignin composition which included increases in the ratios of aldehyde- to alcohol-derived lignin and a preferential incorporation of syringyl subunits. Plants with 80% reduced CAD activity, however, did not show any changes in lignin composition. Both lines showed an increase in the content of alkali-extractable phenolic components, suggesting that there was an alteration in the proportion of phenolic residues bound by ester linkages to the cell wall (Halpin et al., 1994). These lines also showed an increase in thioglycoic acid-extractable lignin, suggesting that the types of bonds formed between phenolic acids, monolignols and other cell wall polymers depend upon both the concentrations and types of different subunits available (Whetten and Sederoff, 1995). These findings indicate that down regulating the expression of the genes encoding CAD can lead to modifications in the lignin content and composition of forage plants and possibly to increased fodder digestibility.

To date, cDNAs encoding CAD have been cloned from a large number of dicotyledonous species including tobacco (Knight et al., 1992), Aralia (Hibino et al., 1993b), poplar (Van Doorsselaere et al., 1995a), alfalfa (Van Doorsselaere et al., 1995a), Eucalyptus (Grima-Pettenati et al., 1993), loblolly pine (O’Malley et al., 1992) and spruce (Galliano
et al., 1993). The isolation of full-length DNA sequences encoding CAD from monocotyledonous species has not been reported, however, partial genomic sequences have been isolated from maize (GenBank Accession no. a24085) and rice (Genbank Accession no. d40685). These angiosperm and gymnosperm CAD genes have been shown to have a wide range of tissue expression patterns and genomic organisation ranging from a single gene, as in loblolly pine (O’Malley et al., 1992), to small gene families as in tobacco (Knight et al., 1992) and Aralia (Hibino et al., 1993b).

To fully understand the function and regulation of the CAD enzymes in perennial ryegrass, a molecular characterization of the corresponding genes must first be completed, including the isolation and sequencing of cDNAs. A common practice in molecular biology is to use recently cloned genes from other species in order to facilitate the isolation of the corresponding cDNA in the target species. Full-length sequences encoding CAD have only been isolated from several dicotyledonous species. Codon usage in monocotyledonous species has evolved to be very different from that of other higher plants. Monocotyledonous species show an extreme bias towards the use of G or C at the third position of the codon (Campbell and Gowri, 1990), which may limit the utility of dicotyledonous hybridization probes for the isolation of ryegrass cad cDNAs.

This chapter details the experimental approach used in the identification, isolation and cloning of a full-length CAD cDNA from perennial ryegrass using an alternative strategy. This approach involved the use of degenerate oligonucleotide primers for PCR-amplification of homologous gene fragments for subsequent library screening. This chapter further details the characterization of the isolated cad cDNA from perennial ryegrass by Southern and northern blot hybridization analysis in order to ascertain the complexity of the gene family and the expression pattern of this gene in lignifying tissue.
2.2 MATERIALS AND METHODS

2.2.1 Plant material

Seeds of perennial ryegrass (*Lolium perenne* cv. Yatsyn), were treated to remove the resident endophyte fungus (*Acremonium lolii* Latch) following the methods of Latch (1993). Seeds were surface sterilized by soaking for 5 h in 1% (v/v) sodium hypochlorite, rinsing thoroughly in sterile water and drying on sterile filter paper in a laminar flow cabinet. After treatment, the seeds were placed into sterile Petri dishes in bell jars containing water to a depth of 1 cm. These containers were stored in the dark, at 37°C for 3 weeks. The treated seeds were germinated on sterile 1% Bactoagar at 24°C for 1 week and were then planted into 10 cm diameter pots of soil and maintained in the glass house, under natural light conditions (16 h day and 8 h night). When the seedlings were six weeks old they were checked for the presence of endophyte fungus. A leaf sheath was taken from each plant, an epidermal strip was removed, stained in lactophenol blue following the methods of Harvey et al. (1982) and examined under a light microscope for the presence of *Acremonium* mycelium between the plant cells.

2.2.2 Isolation of RNA for cDNA library construction

Total RNA for use in cDNA library construction was isolated using a Total RNA Isolation Kit (Advanced Biotechnologies Ltd) that is based on a 14 M solution of guanidine salts and urea. All glassware and solutions were DEPC treated and/or autoclaved where possible before use. Plant material at various vegetative stages of development was harvested, wrapped in foil and immediately frozen in liquid nitrogen. From 100 to 500 mg of pooled, frozen tissue was ground while thawing under 5 mL of RNA extraction reagent (1 mL reagent / 100 mg tissue) with acid-washed sand until a fine homogeneous slurry was formed. This was poured into 15 mL Corex tubes and left at 4°C for 5 min. A 1/5th volume of chloroform (0.2 mL chloroform/ 1 mL reagent) was added to the Corex tube, vortexed for 15 s and incubated on ice at 4°C for 5 min. After centrifugation at 12,000xg at 4°C for 15 min, the aqueous phase was carefully transferred to a fresh 15 mL Corex tube and an equal volume of isopropanol was added. After incubation at 4°C
for 10 min, samples were centrifuged at 12,000xg at 4°C for 10 min. The supernatant was removed and the RNA pellet was washed twice with 75% ethanol by vortexing and subsequent centrifugation at 7,500xg at 4°C for 5 min. The RNA pellet was dried in a 37°C incubator for 10 min and dissolved in sterile DEPC-treated water (400 µL/g original tissue). The concentration and purity of the RNA was determined spectrophotometrically at 260 and 280 nm (assuming OD 1.0 at 260 nm = 40 µg RNA/mL). The RNA quality was checked by electrophoresis of 5 µg of RNA on a 1.4% agarose-formaldehyde gel.

2.2.3 Isolation of poly-A⁺ RNA
The polyadenylated RNA (poly-A⁺ RNA) was isolated using oligo-dT magnetic beads from DYNAL®. The DYNABEADS® mRNA purification kit was used to isolated 5 µg of whole plant poly-A⁺ RNA from perennial ryegrass.

2.2.4 Construction of the cDNA library
The whole plant cDNA library was constructed directionally using cDNA synthesis and lambda ZAP II cDNA cloning kits from Stratagene. First-strand cDNA was synthesized from 5 µg of whole plant (leaf and stem) poly-A⁺ RNA, using oligo-dT primers containing an XhoI restriction site and StrataScript RNase H-deficient Moloney Murine Leukemia Virus reverse transcriptase (MMLV-RT) enzyme which was incubated at 37°C for 60 min. Double-stranded cDNA was synthesized by the addition of RNase H and DNA polymerase I and incubation at 16°C for 150 min. Blunting of cDNA termini was done by treatment with cloned Pfu DNA polymerase at 72°C for 30 min. The resulting cDNA was phenol-chloroform (1:1) extracted twice and precipitated overnight at -20°C.

For cloning, the blunt ends of the cDNA were ligated with EcoRI adaptors consisting of phosphorylated 9-mer and dephosphorylated -13mer oligonucleotides which are complementary to each other and create an EcoRI cohesive end. After adaptor ligation, the cDNA was phosphorylated using T4 polynucleotide kinase and the resulting cDNA was digested with Xho I. The adapted "directional" cDNA was size fractionated (above
using a Sephacryl S-400 spin column, phenol-chloroform extracted and precipitated prior to ligation with EcoRI-Xho I digested Uni-ZAP XR vector arms. After vector arm ligation, the recombinant phage were packaged using Gigapack II Gold packaging extract and plated on the E. coli host strain XL1-Blue MRF'[Δ(mcrA)183 Δ(mcrCB-hsdSMR-mrr)171 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac [F' proAB lacI4ZAM15 Tn 10 (Tet R)]] from Stratagene.

The cDNA library was amplified from approximately 10^6 individual phage plaques by plating ~50,000 bacteriophage per plate with 600 µL of host cells. These plates were incubated for 8 h at 37°C, then overlayed with 10 mL of SM buffer (100 mM NaCl; 8 mM MgSO_4·7H_2O; 50 mM Tris-HCl, pH 7.5; 0.01% (w/v) gelatin) and gently rocked at 4°C overnight. The bacteriophage suspension was recovered from each plate, pooled and incubated for 15 min at room temperature with 5% chloroform. This suspension was centrifuged for 10 min at 500xg and the supernatant was recovered and stored in 1 mL aliquots with 0.3% v/v chloroform at 4°C (short term) and 7% DMSO at -80°C (long term).

### 2.2.5 Template preparation for PCR amplification

A 1 mL aliquot of amplified cDNA library was incubated with 50 µL of Proteinase K (10 mg/mL) at 37°C for 60 min. EDTA was added to a final concentration of 20 mM and incubated at 65°C for 60 min. The reaction was extracted three times with an equal volume of phenol:chloroform (1:1) and the aqueous phase was ethanol precipitated. The cDNA was pelleted by centrifugation and dissolved in 20 µL of sterile distilled water (dH_2O). The concentration and purity of the cDNA was determined spectrophotometrically at 260 and 280 nm (assuming OD 1.0 at 260 nm = 50 µg DNA/mL). The cDNA quality was checked by electrophoresis of 1 µg of cDNA on a 1.0% agarose gel.
2.2.6 Design of degenerate CAD oligonucleotide PCR primers

In the absence of a full-length monocotyledonous CAD sequence for heterologous probing of the perennial ryegrass cDNA library, available partial CAD sequences from monocotyledonous species were utilized in the design of the oligonucleotides used as PCR amplification primers. Oligonucleotide primers corresponding to conserved regions of partial monocotyledonous and dicotyledonous CAD amino acid sequences were designed. Table 2.1 lists each of the CAD oligonucleotides designed and used in this work, including orientation, length, degeneracy and nucleotide sequence. Degenerate 27-mer forward and reverse oligonucleotide primers were synthesized based on the comparison of amino acid sequences of cad.Ms.1, an alfalfa cad cDNA (Van Doorselaere et al. 1995a), pZCAD1, a genomic fragment from maize (GenBank Accession no. a24085)) and a genomic fragment from rice (GenBank Accession no. d40685). Inosine residues were included at the third base position of each codon when codon degeneracy was high. Figure 2.1 shows the location of these CAD oligonucleotides relative to the full-length alfalfa and partial maize and rice CAD peptide sequences.

2.2.7 PCR amplification of CAD-encoding DNA fragments

100 ng of cDNA previously isolated from the phage lysate of the amplified cDNA library, was used as a template in PCR reactions with cad primers 1 and 2. 10 µL of a PCR cocktail (1xBoehringer-Mannheim buffer; 200µM dNTPs; 50 ng of forward primer; 50ng of reverse primer; 0.2U/µL Boehringer-Mannheim Thermus aquaticus [Taq] DNA polymerase) was added to 100ng of cDNA and PCR amplification was carried out using a Corbett FTS-IS Capillary Thermal Sequencer. The program was as follows: [94°C/4 min]x1 cycle; [94°C/30 s, 55°C/30 s, 72°C/150 s]x35 cycles; [25°C/60 s]x1 cycle. A single PCR product of the expected size of 150bp was obtained and shown, by dye terminator sequencing (Applied Biosystems 373A DNA Sequencing System Users Manual), to include the conserved region of the alfalfa cad cDNA and partial genomic sequences from maize and rice, used in the primer design (Figure 2.1).
Table 2.1 Degenerate oligonucleotides used as PCR primers to amplify homologous *cad* gene fragments from perennial ryegrass.

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**Figure 2.1** CAD oligonucleotide primer design based on the alignments of a full length alfalfa cDNA and partial genomic sequences from maize and rice. The forward primer (Primer 1) is highlighted in red and the reverse primer (Primer 2) is highlighted in blue.
2.2.8 Nucleotide sequence analysis of the CAD-encoding PCR fragment

The PCR-amplified CAD-encoding DNA fragment was sequenced in both directions using the Taq DyeDeoxy™ Terminator Cycle Sequencing Kit from Applied Biosystems. The sequencing reaction products were purified using a Wizard PCR purification column (Promega) and 500 ng of DNA was used as template for the sequencing reaction. The sequencing reaction was carried out as outlined by the manufacturer, using the degenerate cad oligonucleotides described above to prime forward and reverse sequencing reactions.

2.2.9 Preparation of ³²P-labelled DNA probes

Gel-purified or PCR-amplified DNA fragments were used as templates for radioactive probe synthesis. Double-stranded DNA probes were made using a random-priming method modified from Sambrook et al. (1989). Approximately 100 - 500 ng of template DNA was digested with HaeIII for 20 min at 37°C prior to probe synthesis. Random primers were added to the digested DNA and the mixture was boiled for 2 min and then placed on ice. Probes were made using 100-500 ng of digested template DNA, HaeIII buffer (8 mM MgCl₂; 10 mM mercaptoethanol; 10 mM Tris-HCl, pH 7.4), 200 µM dNTPs, 5 U Klenow enzyme, and 40 µCi of α³²P-dCTP. After incubation for 60 min at 37°C, the reaction was terminated by the addition of 5 µL of 0.25M EDTA and 100 µL of TES (10 mM Tris-HCl, pH 8.0; 1mM EDTA; 100 mM NaCl). Unincorporated nucleotides were removed using a Sephadex G-50 spin column. Probes were boiled for 3-5 min before addition to hybridization buffers.

2.2.10 Preparation of plaque lifts

Approximately 10⁶ phage were plated on twenty-four 15 cm diameter plates using the E. coli host strain XL1-Blue MRF'. Plates were incubated overnight at 37°C and then cooled at 4°C for 30 min. The first lift was made, as described by Sambrook et al. (1989), by leaving nitrocellulose (Schleicher and Schuell, BA85) filters in contact with the agar surface for 2 min. The second (duplicate) lift was left in contact for 4 min. The DNA was fixed onto the membranes by leaving them for 5 min on 3MM paper soaked in
0.4 M NaOH, then transferring for 5 min to a 5M NaCl/2M Tris-HCl solution and finally transferring to 2x SSC for 5 min. The filters were baked under vacuum at 80°C for 2 h and then washed in 3x SSC with 0.1% SDS at 65°C for 5 h and air dried.

### 2.2.11 Screening the cDNA library

The duplicate plaque lifts of the perennial ryegrass cDNA library were pre-hybridized in Southern buffer (4xSSC; 0.1% (w/v) SDS; 0.2% (w/v) Ficoll; 0.2% (w/v) BSA; 0.2% (w/v) PVP; 100 µg salmon sperm DNA /mL) at 55°C for 12 h in a circular plastic container. The filters were then placed in fresh Southern buffer (at 55°C) and the radioactive probe was added after being boiled for 2 min. The filters were hybridized at 55°C with gentle rocking for 12 h. The filters were washed once in 2x SSC at room temperature for 10 min, twice in 2x SSC at 65°C for 60 min, and once in 1xSSC/0.1%SDS at 65°C for 60 min. The filters were placed on wet 3MM paper and exposed to Kodak XAR film at -80°C for 24 h, with an intensifying screen. Plaques showing positive hybridization were isolated from the plates and placed in 1 mL of SM buffer and 20 µL of chloroform.

For second and third-round screening, approximately 200 phage were plated with the E. coli host strain XL1-Blue MRF' on 9cm diameter Petri dishes. Plaques were lifted, in duplicate, onto nitrocellulose filters as described above. The filters were then washed in 3x SSC with 0.1% SDS at 65°C for 12 h. The filters were pre-hybridized in Southern buffer for 12-18 h, and hybridized with the PCR-generated cad probe at 65°C for 12 h. Plaques that consistently hybridized were selected and stored in 1 mL of SM buffer containing 10% chloroform at 4°C.

### 2.2.12 In vivo excision of the pBluescript phagemid from the Uni-Zap vector

The method for phagemid excision was as outlined in the Stratagene Zap-cDNA Synthesis Kit instruction manual. Overnight cultures of the E. coli hosts XL1-Blue MRF' and
SOLR were grown in LB broth at 30°C. For each strain, 50 mL of LB broth was inoculated with 500 µL of an overnight culture, and grown at 37°C with shaking for 2-3 h to mid-log phase (OD_{600} = 0.2-0.5). The XL1-Blue MRF' cells were pelleted by centrifugation at 1500xg for 5 min and were resuspended in 10 mM MgSO_4 to an OD_{600} of 1.0. The SOLR cells were grown to a final OD_{600} of 0.5-1.0 and left at room temperature. For phagemid excision, 200 µL of XL1-Blue-MRF' cells, 250 µL of phage stock, and 1 µL (10^9 pfu) of ExAssist helper phage (Stratagene) were incubated at 37°C for 15 min. Following incubation, 3 mL of LB broth was added and the incubation was continued at 37°C for 2 h. The reaction was terminated by heating at 70°C for 15 min and the mixture was centrifuged at 4000xg for 15 min. The supernatant, which contained the excised pBluescript phagemid, was decanted into a sterile tube and stored at 4°C. To plate the excised phagemids, 200 µL of freshly-grown SOLR cells and 100 µL of phage supernatant were added to a 1.5 mL microcentrifuge tube and incubated at 37°C for 15 min. After incubation, 50 µL of the cells were plated on LB-ampicillin (50 µg/mL) agar plates and incubated overnight at 37°C.

2.2.13 Size estimation of putative cad cDNA inserts using PCR amplification

The cDNA inserts of the pBluescript phagemids were amplified by PCR to select for possible full-length cDNA clones. *E. coli* transductants containing the excised phagemids were plated as described above and bacterial colonies were picked and streaked on LB-ampicillin agar plates. A sterile Gilson pipette tip was touched onto a single bacterial colony and the adhering cells were resuspended in 20 µL of sterile dH_2O. The cloned cDNA inserts were directly PCR amplified from 1 µL of this bacterial cell suspension in a 10 µL reaction using universal M13 forward and reverse primers. The PCR cocktail included 1x Boehringer-Mannheim buffer, 200 µM dNTPs, 50 ng of forward primer, 50 ng of reverse primer, and Boehringer-Mannheim *T. aquaticus* (Taq) DNA polymerase (0.2 U/µL). The inserts were PCR amplified using a Corbett FTS-IS Capillary Thermal Sequencer and the program was as follows: [94°C/60 s]x1 cycle; [94°C/30 s, 58°C/30 s,
72°C/60 s]x30 cycles; [25°C/60 s]x1 cycle. These reactions were electrophoresed on a 1% agarose gel and isolates yielding PCR fragments of 1 kb, or larger, were retained for further analysis.

2.2.14 Small-scale plasmid preparations

Small-scale plasmid preparations were based on the lysozyme boiling method of Holmes and Quigley (1981). Aliquots of 5 mL of LB broth were inoculated with a fresh single colony of E. coli isolates and incubated at 37°C, 200rpm for 3-4 h. Approximately 1.5 mL of cells were pelleted using an Eppendorf centrifuge (10,000rpm; 30 s). The pellet was resuspended in 40 µL of STET buffer (8% sucrose; 5% Triton X-100; 50 mM EDTA; 50 mM Tris-HCl, pH8.0), and 5 µL of fresh lysozyme (10 mg/mL in 250 mM Tris-HCl, pH 8.0) was added, and the mixture was then boiled for 40 s. After centrifugation (10,000rpm; 10 min), 40 µL of the supernatant was removed and the nucleic acids were precipitated with an equal volume of isopropanol. After centrifugation (10,000 rpm; 10 min) the pellet was air dried and dissolved in 30-50 µL of dH2O. This DNA was suitable for restriction analysis and radioactive probe preparation.

2.2.15 Isolation of perennial ryegrass RNA

Total RNA was isolated as described by Higgins et al. (1976) with some modifications. All glassware and solutions were autoclaved where possible before use. Root, shoot, sheath, stem, leaf, and flower material from perennial ryegrass plants grown in the glass house, as described above, were harvested and immediately frozen in liquid nitrogen. Plant tissue (200-500 mg) was ground under liquid N2 until a fine powder was formed. A 600 µL aliquot of NTES extraction buffer (0.1 M NaCl; 10 mM Tris-HCl, pH 8.0; 1mM EDTA; 1.0% w/v SDS) (2 mL/g tissue) and 800 µL of phenol-chloroform (1:1) was immediately added and grinding was continued while thawing. The slurry was placed into a 1.5 mL Eppendorf tube, and vortexed for 1 min. After centrifugation at 10,000xg for 10 min, the aqueous phase was removed, mixed with an equal volume of 4 M LiCl and maintained for at least 2 h at 4°C. After centrifugation at 10,000xg for 10
min the resulting pellet was dissolved in 400 µL of 0.2M Na-acetate, pH 5.8 and recentrifuged for 2 min. The supernatant was transferred to a fresh tube and the RNA was precipitated by adding 1 mL of 100% ethanol and storing at -80°C for 1 h. After precipitation, and centrifugation at 10,000xg for 10 min, the pellet was washed with 70% ethanol, air dried and dissolved in 20 µL of sterile dH₂O. The concentration and purity of the RNA was determined spectrophotometrically at 260 and 280 nm (assuming OD 1.0 at 260 nm = 40 µg RNA/mL).

2.2.16 Formaldehyde-agarose gel electrophoresis
RNA was electrophoresed using denaturing formaldehyde-agarose gels prepared essentially as described by Sambrook et al. (1989). Gels were cast on glass plates with 1.4% agarose w/v, 5% v/v formaldehyde in MOPS buffer (20 mM MOPS, pH 7.0; 5 mM Na-acetate; 1 mM EDTA). RNA samples were denatured at 65°C for 5 min in a solution of 50% v/v formamide; 17.5% v/v formaldehyde; MOPS buffer and 10 µg ethidium bromide/mL. RNA size markers (BRL; Bethesda Research Laboratories) were used as standards. Gels were run submerged in MOPS buffer for 4 h at 80 V or with wicks for 12 h at 40 V.

2.2.17 Blotting of RNA onto nylon membranes
RNA was capillary blotted onto Hybond-N+ membrane (Amersham) and fixed with 50 mM NaOH following the manufacturer’s instructions. Transfer was for 6 h and the membrane was washed in 2x SSC for 2 min and air dried.

2.2.18 Northern blot hybridization analysis
RNA blots were prehybridized and hybridized in Southern buffer at 65°C for 12 h. The blots were washed once in 2X SSC at room temperature, once in 2X SSC at 65°C for 1 h, once in 1X SSC/0.1% SDS at 65°C for 1 h and then once in 0.5X SSC at 65°C for 1 h. Blots were placed on wet 3MM paper and exposed to Kodak BioMax film at -80°C, with an intensifying screen, for 6 - 24 h.
2.2.19 Preparation of overlapping deletions for DNA sequencing

DNA template for deletion reactions was prepared as described above for small-scale plasmid DNA preparations. The method for the preparation of overlapping deletions was based on the Promega Erase-a-Base System described in the Promega Protocols and Applications Guide (1991). Restriction enzyme analysis of cDNA inserts was done to determine appropriate combinations of enzymes for use in the preparation of deletions. Approximately 20 µg of plasmid DNA was restricted with the appropriate enzymes to allow exonuclease III (Promega) digestion of DNA from a 5'-protruding or blunt end, while leaving a 3'-protruding end intact. The CAD cDNA was sequenced on both strands by deleting one orientation of a cloned insert from opposite directions.

2.2.20 Preparation of DNA template for sequencing

Double-stranded (ds) DNA template was prepared following the small-scale plasmid preparation protocol of Holmes and Quigley (1981) as described above. Plasmid DNA preparations were further purified by treatment with RNaseA (10 µg/µl) for 60 min at 37°C, followed by two phenol:chloroform (1:1) extractions and precipitation with 0.3M Na-acetate, pH 5.2 and 100% ethanol. The DNA pellet was washed in 70% ethanol, vacuum dried and dissolved in 20 µL of dH2O. The DNA concentration was estimated by comparison with a known amount of pBluescriptII SK DNA standard on an agarose gel.

2.2.21 DNA sequencing reactions

Approximately 1 µg of ds DNA was used for sequencing. The DNA template was sequenced using *T. aquaticus* (Taq) DNA polymerase (Boehringer-Mannheim), fluorescently-labelled M13 forward and reverse primers (Applied Biosystems) and an automated sequencing instrument (Applied Biosystems, Model 373A) using the dideoxy-termination method of Sanger *et al.* (1977), modified appropriately (Applied Biosystems manual). The sequencing reactions were prepared as a cocktail. The PCR cycle
sequencing program was as follows: [95°C/90 s]x1 cycle; [95°C/20 s; 55°C/20 s; 72°C/60 s]x15 cycles; [95°C/20 s; 70°C/60 s]x15 cycles; [25°C/60 s]x1 cycle. The reactions for each DNA sample were pooled and precipitated using 0.3M Na-acetate, pH 5.2 and 100% ethanol at -80°C for 30 min. The pellet was washed by vortexing in 70% ethanol, vacuum dried and stored at -20°C until run on a sequencing gel.

2.2.22 Sequence compilation and analysis

Sequence compilation and analysis was done on a VAX computer using GCG sequence analysis programs (Devereux et al., 1984). Overlapping deletions of DNA fragments were compiled using the "SEQED" program. Optimum alignments of CAD translations were obtained using the "PILEUP" program. All other DNA homologies were determined using the "GAP" program.

2.2.23 Isolation of ryegrass genomic DNA

Genomic DNA isolation was based on a CTAB extraction procedure described in Ausubel et al. (1987). Approximately 1-5 g of leaf material was ground to a fine powder under liquid nitrogen using a mortar and pestle. The powder was transferred to a 30 mL Corex tube containing 25 mL of CTAB extraction buffer (50 mM Tris-HCl, pH 7.5; 35 mM sorbitol; 5 mM EDTA; 10% (w/v) PEG; 5% (w/v) PVP; 2% (v/v) mercaptoethanol; 0.2% (w/v) spermidine; 0.1% (w/v) BSA) and gently vortexed to maximize contact with the buffer. After centrifugation at 10,000 rpm for 20 min at 4°C, the pellet was gently resuspended in 5 mL of ice-cold CTAB wash buffer (50 mM Tris-HCl, pH 7.5; 35 mM sorbitol; 5 mM EDTA; 0.4% (v/v) mercaptoethanol). After the addition of 600 µL of 10% (w/v) sarkosyl, 800 µL of 5M NaCl and 650 µL of 8.6% (w/v) CTAB/0.7M NaCl, the mixture was incubated at 65°C for 20 min. The solution was extracted three times with an equal volume of phenol:chloroform (1:1). The supernatant was then transferred to a fresh Corex tube and the DNA was precipitated by the addition of 0.6 x volume of isopropanol. The DNA was spooled onto a Pasteur pipette and redissolved in 500 µL of
sterile dH2O. The concentration and purity of the genomic DNA was estimated by spectrophotometry at 260 and 280nm (where an OD260 of 1.0 = 50 µg DNA/mL).

2.2.24 Southern blot hybridization analysis of genomic DNA

Genomic DNA was restricted with a series of enzymes that do (BglII, Scal and Xhol) or do not (BamHI, EcoRI, HindIII and Nsil) cut the cad cDNA internally. The DNA fragments were separated by electrophoresis on a 1% TAE agarose gel. The gels were blotted onto Hybond-N+ membrane and fixed using 0.4M NaOH for 6 h as outlined by the manufacturer (Amersham) and then washed briefly in 2xSSC and air dried. The DNA blots were prehybridized and hybridized in Southern buffer and probed with the full-length radioactively-labelled cad cDNA (previously boiled for 5 min) at 65°C for 12 h. The blots were washed once in 2xSSC at room temperature for 10 min, once in 2xSSC at 65°C for 60 min, once in 1xSSC/0.1%SDS at 65°C for 60 min and then once in 0.2xSSC/0.1%SDS at 65°C for 60 min. Autoradiography of the Southern blots was with Kodak BioMax film and an intensifying screen at -80°C for 6-48 h.

2.3 RESULTS

2.3.1 PCR amplification of a perennial ryegrass CAD gene fragment

A single, reproducible 150-bp PCR product was amplified using the degenerate primers designed from the predicted peptide sequences of partial CAD-encoding genomic sequences from rice and maize and a full-length cDNA sequence from alfalfa. This product was of the expected size, based on the minimum separation of the oligonucleotides in the alfalfa sequence. The identity of the PCR product was verified by dye terminator DNA sequencing and the resultant sequence was translated in all three reading frames and compared to other CADs in the data base. Figure 2.2 shows the alignment of the maize, rice and alfalfa predicted peptides with the predicted amino acid sequence encoded by the cad ryegrass PCR product. Table 2.2 lists the percentage similarities of the perennial ryegrass cad gene fragment at the nucleotide and predicted
amino acid sequence levels, with the corresponding maize, rice and alfalfa sequences. The perennial ryegrass cad fragment shows 74% and 77% homology, respectively, at the nucleotide level to the maize and rice sequences and 73% with that of alfalfa. Higher levels of homology are seen at the amino acid sequence level for maize (86%), alfalfa (89%) and rice (90%). The amino acid sequence encoded by the cad PCR product spanned the consensus regions involved in zinc active site binding and zinc structural binding which are found in all alcohol dehydrogenase enzymes (Branden et al., 1975), and also included two amino acid residues (shown in Figure 2.4) which are believed to be involved in the cinnamyl aldehyde substrate specificity of CAD (McKie et al., 1993).

Table 2.2  Sequence comparison of the PCR-amplified perennial ryegrass cad gene fragment, and its predicted amino acid sequence, with the corresponding partial sequences from maize and rice and the full-length sequences from alfalfa.

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**Figure 2.2** Alignment of the predicted amino acid sequence of the perennial ryegrass PCR-amplified CAD gene fragment with the predicted amino acid sequences of the alfalfa cDNA and the partial maize and rice genomic sequences. Regions of homology between all the sequences are highlighted in blue, forward and reverse primers sites are highlighted in green.
2.3.2 Isolation of a full-length ryegrass cad cDNA

A full-length cDNA sequence encoding CAD was isolated from perennial ryegrass by screening a λZAP II cDNA library with the homologous PCR-amplified cad gene fragment. Thirty-four positively-hybridizing plaques were detected after the primary screening of approximately 10⁶ recombinant phage from the amplified library. These plaques were isolated and screened through several rounds of purification. Nine isolates showed consistently strong hybridization and were selected for further analysis. After in vivo excision of the pBluescript plasmids and estimation of insert sizes by PCR amplification, one of these clones was found to contain an insert of approximately 1.4kb, suggesting that it was a full-length cDNA. The DNA sequence of this insert was determined on both strands by dye primer sequencing reactions. The sequence was translated three reading frames and the identity and correct reading frame confirmed by homology with other CAD sequences. The complete nucleotide and deduced amino acid sequence of this perennial ryegrass clone, designated LpeCad1, are shown in Figure 2.3.

2.3.3 Nucleotide sequence analysis

The isolated LpeCad1 cDNA is 1382 nucleotides in length with an open reading frame (ORF) of 1085 nucleotides (Figure 2.3). This region is flanked by a 5' untranslated region of 80 nucleotides and a 3' untranslated region of 215 nucleotides. The ORF has a 65% G+C content and it encodes a predicted protein of 262 amino acids with a predicted molecular weight of 38,818 Da and a pI of 5.83. These values are consistent with the relative mass of each subunit of CAD as estimated by Hibino et al. (1993a). There is a putative translation start site (AAGAGAATGGG) which has 64% homology (underlined nucleotides) to the consensus sequence of plants (Joshi, 1987). The cad cDNA has three possible polyadenylation signals: AATAG (nucleotides 1312-1316); AAGAAA (nucleotides 1318-1323), and AATAAT (nucleotides 1345-1350). The nucleotide sequence of the ORF show 66% similarity with that of the alfalfa clone (Van Doorselaere et al., 1995a) and 89% and 88% with that of the partial maize and rice genomic sequences, respectively, used to design the degenerate oligonucleotide primers.
Figure 2.3. The nucleotide and predicted amino acid sequence of the \textit{LpeCad1} cDNA isolated from perennial ryegrass. The putative translation start site is underlined and the ATG start codon is marked in bold. The three putative polyadenylation signals are boxed.
2.3.4 Identification of important CAD structural and functional elements

The protein encoded by the perennial ryegrass LpeCad1 cDNA can be classified as a member of the alcohol dehydrogenase (ADH) family of enzymes. This is based on alignments with predicted CAD amino acid sequences from the data base (Figure 2.4) which reveal high conservation of many key residues involved in the structure and function of alcohol dehydrogenase enzymes. These regions are: 1) a Zinc1-binding ligand and neighbouring residues; 2) Zinc2-binding lobes; 3) the Rossmann nucleotide-binding domain; 4) a co-factor determining polar residue and 5) a catalytic site for substrate specificity. These structural features identified in alcohol dehydrogenases (Jornvall et al., 1987), are also found in CAD enzymes isolated from a number of species (Van Doorselaere et al., 1995a; MacKay et al., 1995; Baucher et al., 1995; Grima-Pettaniti et al., 1993; Knight et al., 1992). The identity of the protein encoded by LpeCad1 can be inferred from the presence of these structural and functional features.

There are two zinc-binding domains in alcohol dehydrogenases, one of which is involved in the catalytic function (Zn1) of the enzyme while the other is part of a lobe involved in the structural stability of the enzyme (Zn2). In the perennial ryegrass LpeCad1 predicted amino acid sequence, the catalytic zinc consensus sequence GHE(X)2G(X)5G(X)2V is located between residues 69-83. This region complexes with invariant cysteine and histidine residues found at positions C-48, C-164 and H-49. Amino acids involved in structural zinc binding (Zn2) are contained within the consensus sequence GD(X)10C(X)2C(X)2C(X)7C and this motif is also contained in the predicted perennial ryegrass CAD protein, between residues 89-115. The deduced amino acid sequence from cad1 also contains the Rossmann fold motif, an amino acid structure comprising the glycine-rich sequence GXGGXG (Rossmann et al., 1974). This motif is an important region involved in nucleotide-binding enzymes, including alcohol dehydrogenases, and is located between residues 190-195 in the predicted amino acid sequence encoded by LpeCad1. In all CAD sequences, this motif also contains highly-conserved leucine and valine (GLGGVG) residues which are also in the perennial ryegrass sequence.
**Figure 2.4** Comparison of predicted amino acid sequences of plant CAD enzymes.

Highly-conserved residues are highlighted in red and marked with a *, the blue box marks a Zn-1 binding domain, the green box marks a Zn-2 binding domain, the pink box marks the Rossman fold, the light blue boxes marks coenzyme binding specificity residues and red boxes mark the substrate specificity residues, acidic amino acid residues with important binding properties are highlighted in purple and marked with a *. Dots indicate amino acid homology with the perennial ryegrass sequence and dashes indicate gaps introduced to maintain the sequence alignments. A black * indicates a stop codon.
Three acidic amino acid residues (A-51, E-71, and A-90), important for binding interactions, are also present (Jornvall et al., 1987). All the known CAD enzymes are NADP-dependent dehydrogenases. A serine at position 213 and a lysine (K-218) are highly conserved between CAD sequences and are considered important in identifying the co-factor specificity as that of NADP and not NAD+ which is used by other members of the alcohol dehydrogenase family (Knight et al., 1992; Goffner et al., 1992). The amino acids which are thought to be responsible for aromatic alcohol substrate specificity (McKie et al., 1993) are present in the predicted perennial ryegrass CAD sequence as residues Y-114, I-119, W-120 and F-301.

2.3.5 Comparison of CAD amino acid sequences

The nucleotide and predicted amino acid sequence of the perennial ryegrass LpeCad1 were used to search databases (EMBL and Genbank). Comparison of the translated sequence of LpeCad1 with other CAD amino acid sequences reveals extensive homology (Table 2.3; Fig 2.4). The highest level of amino acid similarity (94.9%) is with the predicted amino acid sequence of the partial genomic cad sequence from maize (GenBank Accession no. a24085). The dicotyledonous angiosperm predicted CAD sequences showed high levels of similarity to that of the perennial ryegrass CAD1 protein in the range of 85-87%. The highest level of similarity (87%) with dicotyledonous angiosperm species was with the proteins encoded by the tobacco cad gene, CAD14 (Knight et al., 1992) and the cadac1 gene isolated from Aralia (Hibino et al., 1993b). Comparable levels of similarity were seen with other dicotyledonous angiosperm CADs, ranging from 86% with poplar (Van Doorsselaere et al., 1995a), Arabidopsis (Somers et al., 1995), and the protein encoded by the second tobacco cad gene, CAD19 (Knight et al., 1992) and 85% with Eucalyptus gunnii (Fuillet et al., 1993), E. botryoides (Hibino et al., 1994) and alfalfa (Van Doorsselaere et al., 1995a). The gymnosperm sequences from pine (MacKay et al., 1995; Wagner et al., 1996) and spruce (Galliano et al., 1993) showed slightly lower levels of similarity, 82% and 81%, respectively, to the perennial ryegrass CAD amino acid sequence.
Table 2.3 The percentage similarity, accession number, and reference for the predicted amino acid sequences of CADs and putative CADs isolated from plants.

<table>
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<th>Accession</th>
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<th>Reference</th>
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<th>PmcC</th>
<th>PmcB</th>
<th>PmcA</th>
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<th>T 14</th>
<th>E. gunnii</th>
<th>E. botryoides</th>
<th>Maize</th>
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The class of genes suggested to be involved in elicitor responses, possibly involving lignification, had lower levels (65%-69%) of similarity when compared to the ryegrass and other CAD sequences. The comparison of an alcohol dehydrogenase (ADH) amino acid sequence from maize, with the other CAD sequences reveals low levels of similarity in the range of 44%-51%. The ryegrass CAD protein has only 47% similarity to this enzyme at the amino acid sequence level, presumably due to the conserved structural and functional features common to all members of the alcohol dehydrogenase family.

2.3.6 Phylogenetic analysis of CAD amino acid sequences

The aligned CAD amino acid sequences were examined for their phylogenetic relationships with other members of the alcohol dehydrogenase family (Figure 2.5). The resultant dendrogram clusters into 2 main groups (clades) each with smaller sub-groups branching from the main cluster. The first main clade consists of all the angiosperm and gymnosperm CAD sequences while the second clade contains the putative CAD-like elicitor (Eli) induced sequences. Within the first clade, the first sub-group consists of the sequences from pine and spruce. This grouping of the gymnosperm conifers separate from the other CADs probably reflects the evolutionary divergence between gymnosperms and angiosperms. The second sub-group contains the only full-length monocotyledonous cad sequence, isolated from perennial ryegrass, and the partial genomic sequence from maize. The representation of the monocotyledonous sequence as a distinct branch off the angiosperm clade is a reflection of the codon bias between monocotyledonous and dicotyledonous species and the evolutionary differences between these groups. The third sub-group consists of all of the known CAD sequences from dicotyledonous angiosperms, including the CAD sequences from alfalfa, tobacco (14 and 19), Aralia, poplar, Arabidopsis and Eucalyptus. The second major clade consists of the sequences encoding enzymes which are active during elicitor responses in plants and which have been suggested to be CAD-like in structure and possibly function (Boudet et al., 1995). Completely distant from all of these CAD and CAD-like sequences is the third clade containing the maize alcohol dehydrogenase amino acid sequence.
Figure 2.5  Dendrogram showing the phylogenetic relationship of the predicted amino acid sequence of *LpeCad1* to other plant CAD and ADH sequences. The monocot CAD sequences are highlighted in red, dicot angiosperms are highlighted in green, the gymnosperms are highlighted in blue, the elicitor-induced sequences are in pink and the maize alcohol dehydrogenase is in purple.
2.3.7 Genomic organisation of cad genes

The arrangement of cad gene(s) in perennial ryegrass was examined by Southern blot hybridization analysis to determine whether cad was part of a multigene family or existed as a single copy (Figure 2.6). Restriction analysis of the LpeCad1 cDNA, revealed that there were no internal sites for the restriction enzymes BamHI, EcoRI, HindIII, and NsiI and one site each for BglII, ScaI and XmnI (data not shown). The restriction fragments generated by each of these enzymes, were separated by electrophoresis, and probed with the full-length LpeCad1 cDNA. After high stringency washing, the hybridization patterns seen in Figure 2.6 suggest a low level of gene complexity.

Hybridization patterns of genomic DNA restricted with the enzymes which do not cut the LpeCad1 cDNA show two bands of similar intensity and some minor bands of varying intensity. Hybridization patterns of genomic DNA restricted with enzymes that cut the LpeCad1 cDNA once, show at least three or four major hybridizing bands. This suggests that cad genes in perennial ryegrass may be organized into a small gene family consisting of two members. A similar situation has also been suggested for cad genes from Aralia (Hibino et al., 1993) and tobacco (Knight et al., 1992).

2.3.8 Organ specificity of CAD gene expression

Expression of the perennial ryegrass CAD-encoding cDNA was further analysed by northern blot hybridization. Equal amounts of total RNA isolated from different plant tissues were probed with the full-length LpeCad1 cDNA. Figure 2.7 shows that LpeCad1 is preferentially expressed in roots, sheaths and stems with lower levels of expression in shoots, leaves, and flowers. In all tissue types, only a single mRNA species was detected. The LpeCad1 mRNA is approximately 1400 nucleotides in length, which is similar in size to the known 1382-bp size of the corresponding cDNA.
Figure 2.6 Southern blot hybridization analysis of genomic DNA (20 ug per lane). Blots were probed with the full-length LpeCad1 cDNA. Restriction enzymes used were BamHI (Ba), BglII (Bg), EcoRI (Ec), HindIII (Hi), Nsil (Ns), Scal (Sc) and XmnI (Xm). Asterisks indicate enzymes that do not cut the LpeCad1 cDNA. Size markers are shown on the left.
Figure 2.7 Northern blot analyses of total RNA (10 ug per lane) from root (R), 7 day old shoot (S), sheath (Sh), mature leaf (L), stem (St) and flower (F) using the full-length *LpeCad1* cDNA as a probe. The ethidium bromide stained gel is included to indicate even RNA loadings.
High levels of cad gene expression in stem tissue have been reported in loblolly pine (MacKay et al., 1995), Aralia (Hibino et al., 1993b), poplar (Grima-Pettenati et al., 1993), and tobacco (Knight et al., 1992) which is consistent with the occurrence of CAD in tissue that becomes lignified. Expression of cad genes is also found in non-lignified tissues (MacKay et al., 1995) suggesting that CAD enzymes may have different roles in different tissues. The presence of the LpeCad gene mRNA in tissues which are not strongly lignified, such as roots and flowers, may be a reflection of the different roles that CAD enzymes play in plant development.

2.4 DISCUSSION

A cDNA clone encoding a cinnamyl alcohol dehydrogenase (CAD) enzyme was isolated from a perennial ryegrass λZAPII cDNA library using a 150-bp PCR-amplified homologous cad gene fragment as a probe. To increase the probability of isolating a CAD-encoding cDNA, the cad probe was amplified from a region spanning the length of the highly-conserved Zn1-binding domain present within all the reported alcohol dehydrogenase enzymes (Jornvall et al., 1987). The probe was amplified from ryegrass cDNA template using degenerate primers designed specifically from the predicted amino acid sequence of the alfalfa CAD and the partial cad genomic sequences from maize and rice. By including monocotyledonous sequences in the primer design, and using a homologous monocotyledonous probing system, the probability of encountering problems due to codon bias was reduced. The resultant full-length clone was verified as a CAD-encoding cDNA by the analysis of its nucleotide and amino acid sequences.

The perennial ryegrass LpeCad1 cDNA has high sequence similarity (95%) with the maize partial genomic clone. The maize clone is 180 bp in length and its predicted amino acid sequence shows strong homology with the structural and active site zinc-binding regions shown to be highly conserved in all alcohol dehydrogenases (Jornvall et al., 1987). This maize cad clone shows only 50.8% amino acid sequence homology to that of a maize alcohol dehydrogenase, indicating that the levels of similarity between the maize
CAD and the ryegrass CAD are not only due to these alcohol dehydrogenase consensus sequences but also to shared amino acid similarity which is specific to monocotyledonous CAD sequences. The perennial ryegrass LpeCad1 cDNA encodes a predicted protein which has 84-87% amino acid sequence similarity to those of the dicotyledonous angiosperm species (Table 2.4). The slightly lower levels of similarity to the pine A (82.1%), pine B (81.8%), pine C (82.3%) and spruce (81.2%) CAD amino acid sequences is a reflection of the different phylogenetic origin of these plants and may also be a reflection of the different substrate specificities suggested for CAD isoforms isolated from angiosperms and gymnosperms (Kutsuki et al., 1982).

The predicted amino acid sequence of the perennial ryegrass CAD protein shows lower homology with the plant defence-related cDNAs Eli3, Eli3-1, and Eli3-2 from parsley and Arabidopsis (Kiedrowski et al., 1992; Somssich et al., 1989). Although these cDNAs, encoding elicitor-responsive proteins, have not been shown to encode CAD proteins, subsequent comparisons with the amino acid sequences encoded by other cad genes, reveals strong similarities (Table 2.3). While the function of the proteins encoded by these cDNAs has yet to be determined, it has been hypothesized that they may encode ADHs (Baucher et al., 1995) or CAD isoforms which are preferentially induced in plant defence responses (Boudet et al., 1995). Consistent with CAD being a unique member of the ADH family, the amino acid sequence similarity between CAD and elicitor-induced sequences with an ADH from maize, are low. For example, the predicted rye grass CAD shows only 47% amino acid sequence similarity while other CAD sequences and the elicitor-induced sequences show between 45% and 51% similarity with that of the maize ADH. These levels of similarity presumably reflect the only conserved structural and functional elements found in all alcohol dehydrogenases.

Regions of homology between all the CAD clones isolated have been previously identified (Jornvall et al., 1987), and are present within the perennial ryegrass CAD protein sequence (Figure 2.4). These include the two zinc-binding domains which are involved
in catalytic function (Zn1) and structural stability (Zn2) of the enzyme, and the Rossmann fold which is thought to have a role in ATP binding. Other highly-conserved residues are also present throughout the ryegrass CAD protein sequence including the amino acids important in defining the NADP-dependant co-factor specificity (Knight et al., 1992) of all CADs and their aromatic alcohol substrate specificity (McKie et al., 1995).

A phylogenetic analysis (Figure 2.5) of 20 different plant CAD sequences and a maize alcohol dehydrogenase, show that the ryegrass LpeCad1 clone comprises a distinct sub-group within the angiosperm CAD grouping that contains only monocotyledonous CAD sequences. The difference in codon usage between monocotyledonous and dicotyledonous species presumably accounts for the separation of these monocotyledonous sequences into a separate group within the angiosperm clade. This suggests that the success of sense and antisense suppression of lignin genes in transgenic plants using heterologous systems will depend on the levels of gene sequence similarity between the target plant and the plant from which the constructs are derived. The identities of the elicitor-inducible sequences contained within the second major clade of the phylogenetic tree are not known, however, their homology to the angiosperm and gymnosperm sequences of the CAD clade, supports the inclusion of this group of sequences in the phylogeny as related enzymes, or possibly, as a different class of CAD enzymes.

It has been proposed that CAD enzyme activity is an indicator of lignin biosynthesis because of its committed and penultimate position in the lignin biosynthetic pathway (Grand et al., 1987). The expression of the LpeCad1 gene in tissues of perennial ryegrass which undergo lignification, such as the stem, support this hypothesis. However, studies by O'Malley et al. (1992) and Hawkins and Boudet (1994) showed that expression of CAD in cells other than those making lignin suggests that there may be differential regulation of CAD during development. The induction of cad mRNA in response to pathogen elicitation and ozone stress has also been recorded (Galliano et al.
The presence of detectable levels of *LpeCad1* mRNA in all tissues (Figure 2.7) may be a reflection of the different roles that CAD enzymes play in plant development, or that there is cross-hybridization with other *cad* gene transcripts which may be present in perennial ryegrass. The presence of multiple CAD enzymes or isoforms in perennial ryegrass would suggest that they are encoded by separate genes and may be regulated by different developmental and environmental cues. However, the detection of a single sized message in the northern hybridization analysis suggests that if there is more than one gene, then they have very similarly-sized coding regions but may be differentially regulated. Southern blot hybridization data are consistent with the conclusion that there may be two *cad* genes in perennial ryegrass. Isoforms of CAD with different substrate specificities have been detected in wheat (Pillonel *et al.*, 1992) and *Eucalyptus* (Goffner *et al.*, 1992), however, the number of genes responsible for encoding these isozymes has yet to be deduced.

In conclusion, a perennial ryegrass cDNA encoding cinnamyl alcohol dehydrogenase has been cloned and its DNA sequence determined. This is the first reported of a full-length cDNA encoding CAD to be isolated from a monocotyledonous species. The patterns of expression of the corresponding gene resemble those from other species, in which gene expression is associated with lignifying tissue. However, as in some other species *cad* gene expression in perennial ryegrass is also found in non lignifying tissues. The *LpeCad1* gene may be a member of a small gene family. This cloned perennial ryegrass *LpeCad1* gene may be a useful tool for the modification of lignin biosynthesis to improve the digestibility and nutritive value of this important pasture plant.
3.1 INTRODUCTION

Caffeic acid: S-adenosyl-L-methionine O-methyltransferase (OMT; EC 2.1.1.6) catalyses the methylation of caffeic acid (CA) and 5-hydroxyferulic acid (5HFA) to ferulic and sinapic acids, respectively. These compounds are known as hydroxycinnamic acids and are the early precursors of the alcohol monomers of lignin, p-coumaryl, coniferyl, and sinapyl alcohols. The chemical structures of these monomeric lignin precursors differ only in the number of associated methoxyl groups, and therefore methylation of the 3- and 5-hydroxyl groups of the hydroxycinnamic acids is an important step influencing lignin composition. The monomeric composition of lignin differs between angiosperm and gymnosperm species and the ratio of methylated lignin monomers incorporated into the lignin polymer is regarded as the key factor responsible for these differences (Chiang et al., 1988). Gymnosperm lignin is composed mainly of the monomethoxylated guaiacyl units which form a highly condensed (cross-linked) lignin while angiosperm lignin contains guaiacyl units and the dimethoxylated syringyl units which form less cross-linkages (Chiang et al., 1988). Presumably due to this different composition, wood from angiosperms has been shown to have better pulping characteristics than that derived from gymnosperms. The methylation of aromatic lignin precursors is regarded as one of the key steps responsible for these differences, as the presence of methylated 5-OH groups in syringyl lignin is thought to decrease cross-linking in the lignin polymer. Reduced cross-linking results in lignin which is less condensed and easier to remove (Chiang et al., 1988). The differences in caffeic acid O-methyltransferase (COMT) substrate specificities in angiosperms and gymnosperms may account for some of the chemical differences in lignin structure between these two groups. COMTs isolated from dicotyledonous angiosperms are considered to be bifunctional as the same enzyme is able to methylate both CA and 5HFA (Bugos et al., 1992). In contrast, COMT from gymnosperm species
are only able to efficiently methylate CA (Kuroda et al., 1975; Kuroda et al., 1981) although crude extracts show some activity with 5HFA (Higuchi, 1981).

Evidence for the potential manipulation of COMT to alter lignin content and composition comes from studies of natural and transgenic COMT mutants. Brown-midrib (bmr) mutant lines of maize, sorghum, and pearl millet have been shown to have reduced lignin content and altered lignin composition, leading to increased digestibility (Cherney et al., 1991). In particular, the bm3 mutant of maize has only 10% of the COMT activity of wild-type due to alterations in the gene encoding COMT (Vignols et al., 1995). This mutation leads to a 30% reduced lignin content and the lignin contains increased proportions of p-coumaryl alcohol monomers. These changes in lignin content and composition result in a 50% increased digestibility of leaf and stem material.

Similarly, antisense and sense constructs of COMT transformed into a number of species, have been effective in altering the lignin composition and content of these plants. In poplar, the effect of antisense and sense COMT transgenes resembles that reported with the bm3 mutation in maize. The transgenic plants have reduced COMT enzyme activity and a decrease in lignin syringyl units. The formation of a new unit, 5-hydroxyguaiacyl (5 OH-G) was noted but there was no change in total lignin content (Van Doorsselaere et al., 1995b). In transgenic tobacco plants expressing sense and antisense mRNAs of COMT from either tobacco (Attassanova et al., 1995), alfalfa (Ni et al., 1994) or aspen (Dwivedi et al., 1994), changes in lignin content and composition have also been reported. The use of COMT suppression to alter lignin biosynthesis and increase dry matter digestibility has been recorded in the forage species Stylosanthes humilis (Rae et al., 1996). A 95% reduced COMT activity compared to the wild-type was measured, however, overall lignin content in these lines was unchanged. Histochemical tests revealed low levels of syringyl units present in the lignin and the digestibility of these antisense plants increased by 10% compared to the controls. This is the first report of the successful molecular improvement of the digestibility of a pasture plant (Rae et al., 1996).
These findings indicate that down regulating the expression of the genes encoding COMT can lead to modification of the lignin content or composition of forage plants and possibly to increased fodder digestibility.

Genes encoding COMT have been isolated from a large number of dicotyledonous species such as poplar (Dumas et al., 1992; Hayakawa et al., 1996), aspen (Bugos et al., 1991; Tsai et al., 1995; Hu, 1996), alfalfa (Gowri et al., 1991), tobacco (Jaeck et al., 1992), Chrysosplenium (Gauthier et al., 1996), Eucalyptus (Poeydomenge et al., 1994), Stylosanthes (McIntyre et al., 1995), almond (Garcia-Mas et al., 1995), Zinnia (Ye and Varner, 1995) and loblolly pine (Zhang and Chiang, 1995). The isolation of genes encoding COMT from monocotyledonous species has been limited, with genes identified in maize (Collazo et al., 1992) and recently, a putative gene in barley (Lee et al., 1996). These COMT genes have been shown to have a wide range of tissue expression patterns and genomic organization ranging from a single gene, as in maize (Collazo et al., 1992), to a multigene family as in poplar (Hayakawa et al., 1996).

To fully understand the role of COMT enzymes in perennial ryegrass a molecular characterization of the genes which encode this enzyme must be completed. To study the regulation and effect of these genes in perennial ryegrass lignin biosynthesis requires the identification, isolation and cloning of cDNA sequences. This chapter details the experimental approach used in the isolation and identification of a stem-abundant COMT-encoding cDNA and describes the expression pattern and genomic organization of the comt gene(s) from perennial ryegrass. This chapter further outlines the characterization of this stem-abundant cDNA by expression in E. coli and analysis of the functional protein for COMT activity.
3.2 MATERIALS AND METHODS

All methods were as described in the previous chapters, in addition to those given below.

3.3.1 Design of degenerate COMT oligonucleotide primers for PCR amplification

The availability of a full-length monocotyledonous sequence for OMT from maize simplified the design of oligonucleotide primers for PCR amplification of homologous perennial ryegrass sequences. PCR oligonucleotide primers corresponding to conserved regions of monocotyledonous and dicotyledonous OMT amino acid sequences were designed. Table 3.1 lists each of the COMT oligonucleotides used in this work, including orientation, length, degeneracy and nucleotide sequence. Two degenerate forward (24- and 21-mer) and reverse (24- and 27-mer) primers were synthesized based on a comparison of the amino acid sequences of COMTl, an alfalfa omt cDNA (Gowri et al., 1991) and MC1, a maize omt cDNA (Collazo et al., 1992). Inosine residues were included at the third base position of each codon, when codon degeneracy was high. Figure 3.1 shows the location of these COMT oligonucleotides relative to the full-length maize (Collazo et al., 1992) and alfalfa (Gowri et al., 1991) COMT protein sequences.

3.2.2 PCR amplification of OMT-encoding DNA fragments

The cDNA template was prepared as described in Chapter 2. This cDNA was used in PCR reactions with the four possible comt primer combinations of 1 or 2 with 3 or 4. The expected size of the amplification products with the various primer combinations are listed in Table 3.2. The PCR reaction mix was as described in Chapter 2 and the program was as follows: [94°C/4 min]x1 cycle; [94°C/30 s, 55°C/30 s, 72°C/90 s]x35 cycles; [25°C/60 s]x1 cycle. A single PCR product of the expected size (600bp) was obtained using primers 1 and 4 and was shown, by dye terminator sequencing (Applied Biosystems 373A DNA Sequencing Systems Users Manual), to correspond to the conserved regions of the maize MC1 and alfalfa COMT1 cDNAs used in the primer design.
Table 3.1 Degenerate oligonucleotides used as PCR primers to amplify homologous *comt* gene fragments from perennial ryegrass.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Amino Acid and Nucleotide Sequence</th>
<th>Length</th>
<th>Degeneracy</th>
<th>Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>( M \quad N \quad Q \quad D \quad K \quad V \quad L \quad M )</td>
<td>24-mer</td>
<td>32-fold</td>
<td>Forward</td>
</tr>
<tr>
<td></td>
<td>5' ATG AAT CAA GAT AAA GTC ACT ATG 3'</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td>( F \quad N \quad K \quad A \quad Y \quad G \quad M )</td>
<td>21-mer</td>
<td>16-fold</td>
<td>Forward</td>
</tr>
<tr>
<td></td>
<td>5' TTT AAT AAA GCT TAT GGC ATG 3'</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td>( M \quad K \quad W \quad I \quad L \quad H \quad D \quad W )</td>
<td>24-mer</td>
<td>16-fold</td>
<td>Reverse</td>
</tr>
<tr>
<td></td>
<td>3' TAC TTT ACC TAI GAI GTA CTA ACC 5'</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.</td>
<td>( M \quad L \quad A \quad H \quad N \quad P \quad G \quad G \quad K )</td>
<td>27-mer</td>
<td>8-fold</td>
<td>Reverse</td>
</tr>
<tr>
<td></td>
<td>3' TAC GAI CGI GTA TTA GGI CCI CCI TTT 5'</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 3.1  OMT oligonucleotide primer design based on alignments of the predicted amino acid sequences from full-length alfalfa and maize cDNAs. The forward primers (1 and 2) are highlighted in blue and the reverse primers (3 and 4) are highlighted in red.

Table 3.2  Summary of the expected sizes (in base pairs) of PCR amplification products when using various combinations of *comt* forward primers 1 and 2 with *comt* reverse primers 3 and 4.

<table>
<thead>
<tr>
<th></th>
<th>Primer 3</th>
<th>Primer 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primer 1</td>
<td>426</td>
<td>600</td>
</tr>
<tr>
<td>Primer 2</td>
<td>357</td>
<td>525</td>
</tr>
</tbody>
</table>
3.2.3 Screening the cDNA library

The PCR-amplified *comt* DNA fragment was used as a template for probe synthesis. A radioactive double-stranded (ds) DNA probe was made using a random-priming method modified from Sambrook *et al.* (1989) as outlined in Chapter 2. Duplicate plaque lifts of the perennial ryegrass cDNA library were made as described in Chapter 2 and pre-hybridized in Southern buffer at 55°C for 12 h in a circular plastic container. The filters were then placed in fresh Southern buffer containing 10% dextran sulphate and the radioactive probe (previously boiled for 5 min) was added and the filters were hybridized at 55°C with gentle rocking for 12 h. The filters were washed once in 2x SSC at room temperature for 10 min, twice in 2x SSC at 65°C for 60 min, once in 1xSSC/0.1%SDS at 65°C for 60 min and once in 0.5xSSC/0.1% SDS at 65°C for 60 min. The filters were placed on wet 3MM paper and exposed to Kodak XAR film at -80°C for 24 h, with an intensifying screen. Plaques showing hybridization were isolated from the plates and placed in 1 mL of SM buffer and 20 µL of chloroform.

For second and third round screening, approximately 250 phage were plated with the *E. coli* host strain XL1-Blue MRF' on 9 cm Petri dishes. Plaques were lifted, in duplicate, onto nitrocellulose filters and then washed in 3x SSC with 0.1% SDS at 65°C for 12 h. The filters were pre-hybridized in Southern buffer for 12-18 h, and hybridized with the radioactive probe at 65°C for 12 h. Plaques that consistently hybridized with the *comt* probe were selected and stored in 1 mL of Phage Storage Buffer containing 10% chloroform at 4°C.

3.2.4 Estimation of *comt* cDNA insert sizes using PCR amplification

The sizes of cloned DNA inserts in purified recombinant plaques were estimated by PCR amplification. Aliquots of phage lysates (5 µL) were diluted into 20 µL of dH2O and heated to 65°C for 5 min. Two µL of the diluted lysate was used as template for PCR. The amplification mixture and program were as previously described for phagemid amplification in Chapter 2. The PCR products were separated on 1% (w/v) agarose gels.
and isolates with cDNA inserts of 1kb or larger were chosen for plasmid excision. The method for plasmid excision was as outlined in the Stratagene ZapII-cDNA Synthesis Kit instruction manual.

3.2.5 RNA isolation

Perennial ryegrass plants were grown under glass house conditions as outlined in Chapter 2. Root, shoot, sheath, stem, leaf and flower tissue were harvested for RNA isolation. Total RNA was isolated essentially as described by Higgins et al. (1976) and electrophoresed and blotted onto Hybond-N+ membrane as outlined in Chapter 2. The RNA blots were pre-hybridized and hybridized in Southern buffer and probed with the full-length comt cDNA at 65°C for 12 h. The blots were washed and autoradiography conditions were as outlined in Chapter 2.

3.2.6 DNA sequence analysis

The perennial ryegrass comt cDNA was sequenced on both strands by deleting one orientation of a cloned insert from opposite directions. Restriction analysis of the comt cDNA insert was done to determine appropriate combinations of enzymes for use in the preparation of deletions. A deletion series in the 5' to 3' direction was made while the comt cDNA was cloned in pBluescript II SK. The cDNA was recloned into pGEM7Zf(+) (Promega) for deletions in the opposite direction due to the lack of appropriate restriction sites in the pBluescriptIIISK vector. Overlapping deletions of each DNA strand were prepared following the Erase-a-Base System described in the Promega Protocols and Application Guide (1992). Approximately 20 µg of comt DNA, cloned in pBluescriptII or pGEM7Zf(+), was restricted with either SacII and XbaI or NsiI and HindIII, respectively, to allow exonuclease III digestion of DNA from a 5' protruding or blunt end, while leaving a 4-base 3' protruding end intact. Approximately 1µg of double-stranded DNA was used for sequencing. The cDNA deletion series were sequenced as described in the Applied Biosystems 373A DNA Sequencing System Users Manual using
dye primers. Sequence compilation and analysis were carried out using the Genetics Computer Group (GCG) version 8 (1996) sequence analysis programs.

3.2.7 Southern blot hybridization analysis

Genomic DNA was isolated from ryegrass leaves using a CTAB extraction procedure described by Ausubel et al. (1987). The DNA was restricted with enzymes that do (BamHI and Xho I) or do not (BglII, EcoRI, HindIII, NsiI and ScaI) cut the comt cDNA internally. The DNA fragments were separated by electrophoresis on 1% (w/v) agarose gels, blotted onto Hybond-N+ membrane and fixed with 0.4M NaOH for 6 h as previously outlined in Chapter 2. The DNA blots were pre-hybridized and hybridized in Southern buffer and probed with the full-length comt cDNA at 65°C for 12 h. The blots were washed and autoradiography conditions were as outlined in Chapter 2.

3.2.8 Expression of the comt cDNA in E. coli

Expression of the perennial ryegrass comt clone in either the E. coli strain XL1-blue MRF' (Stratagene) or BL21 (Novagen) was based on the methods of Collazo et al. (1994). A 50 mL volume of LB was inoculated with 500 µL of an overnight culture and incubated at 37°C for 2 h. Expression of the comt cDNA in E. coli was induced by the addition of IPTG to a final concentration of 2 mM and the cells were incubated for a further 2 h. Cells were pelleted by centrifugation and resuspended in 5 mL of COMT extraction buffer (100 mM Tris-HCl, pH 7.5; 10% (v/v) glycerol; 50 mM mercaptoethanol). The cells were sonicated and centrifuged at 14,000 rpm for 10 min. The supernatant was collected and used for protein content determination and enzyme assays as outlined below.

3.2.9 Crude plant COMT enzyme extracts

One gram of young leaf tissue was ground to a fine powder with a mortar and pestle in the presence of liquid nitrogen. COMT extraction buffer (4mL) was added to the powder and ground thoroughly while thawing. The crude extract was then centrifuged at
14,000 rpm for 10 min at 4°C. The supernatant was transferred to a precooled microfuge tube and kept on ice for protein determination and enzyme assays.

### 3.2.10 Protein determination

Protein content was determined with a prepared dye-binding reagent (Bio-Rad) using bovine serum albumin (BSA) as the standard and following the manufacturer’s protocols.

### 3.2.11 COMT assays

The COMT enzyme assay conditions were based on a previous protocol by Kuroda et al. (1981) and were optimized for perennial ryegrass COMT. The reaction mixture (200 µL) contained 50 mM Tris-HCl, pH 8.0; 10 mM MgCl₂; 1mM phenylpropanoid substrate (routinely caffeic acid) and enzyme solution equivalent to 500 µg of bacterial protein or 100 µL of crude plant extract, in a 1.5 mL microfuge tube. The reaction mixture was preincubated for 5 min at 30°C, and then diluted ¹⁴C-labelled S-adenosyl methionine (SAM-¹⁴Me; µCi) was added to a final concentration of 0.5 mM and the reaction was incubated for 2 h. [Diluted SAM-¹⁴Me was prepared by adding 4.9mg SAM.HSO₄ (Boehringer) to 100 µL of SAM-¹⁴Me (54.0 mCi/mmol) (Amersham) and 900 µL of distilled water]. The reaction was terminated by the addition of 40 µL of 1M HCl. The ¹⁴Me-hydroxycinnamic acid products were extracted from the unincorporated SAM-¹⁴Me with 1 mL of ether, vacuum dried and resuspended in 10 µL of 100% ethanol. The standards used for chromatographic mobility markers were prepared by dissolving 1 mg of either caffeic, p-coumaric, ferulic, sinapic or 3,4-dimethoxycinnamic acid in 500 µL of 100% ethanol. Reaction products and 10 µL of standards were applied to thin layer chromatography (TLC) silica plates (Merck 60F-254, pre-coated plates), developed in toluene: acetic acid: distilled water (5:5:1, upper phase), and autoradiography was conducted for 5 days on Kodak BioMax film at -80°C with an intensifying screen.
3.3 RESULTS

3.3.1 PCR amplification of a perennial ryegrass OMT gene fragment

A single, 600-bp PCR product was successfully amplified using the oligonucleotide primer combination 1 and 4. This product was of the expected size based on the primer separation in the maize and alfalfa sequences. The identity of the PCR product was verified by using oligonucleotides 1 and 4 to prime dye terminating sequence reactions. The resulting sequence was translated in three reading frames and its identity was verified by homology to other OMT sequences in the data base and the presence of the internal sequences of primers 2 and 3. Figure 3.2 shows an alignment of the maize and alfalfa peptides with the translated amino acid sequence for the ryegrass PCR fragment. Table 3.3 lists the similarity and longest stretches of nucleotide and encoded amino acid sequence homology between the PCR cont fragment and the corresponding maize and alfalfa sequences. The PCR cont fragment is 83% and 89% similar to the maize nucleotide and amino acid sequences, respectively. The sequence similarity is lower when compared to alfalfa, with only 61% nucleotide and 79% amino acid sequence homology, respectively. The high level of similarity between the monocotyledonous sequences is also reflected in continuous runs of nucleotide homology, of up to 44 base pairs, compared with a maximum of only 11 for the alfalfa sequence (Table 3.3).

Table 3.3 Sequence similarities of the PCR-amplified perennial ryegrass cont gene product with the full-length amino acid and nucleotide sequences from alfalfa and maize.

<table>
<thead>
<tr>
<th>Source</th>
<th>Nucleotide Similarity (%)</th>
<th>Nucleotide Length of run (base pairs)</th>
<th>Amino Acid Similarity (%)</th>
<th>Amino Acid Length of run (amino acids)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maize</td>
<td>82.8</td>
<td>44</td>
<td>89.4</td>
<td>15</td>
</tr>
<tr>
<td>Alfalfa</td>
<td>61.4</td>
<td>11</td>
<td>79.3</td>
<td>12</td>
</tr>
</tbody>
</table>
Maize | Alfalfa | Rye PCR
--- | --- | ---
1 | Mgstagd vaavdeac myamqlasss ilpmlknai elgllelvqk | ...
50 | Mgstgetqit pthisdeean lfamqlasas vlpmlksal eldlleiaiak | ...........
100 | Eagggkaala peevarmpa apsdfaaaa mvdrmlrla sydvvrcqm. | ...........
150 | Agpg...aquis pteiasolpt tnpd...afv mlrmlrla cyiiltcsvr | ...........
200 | Edrogryerr ysaapvckwl tpnedgsvma alalmnqdkv lmeswyylkd | ...........
250 | Avldggipfn kaygtmtafev hgtdarfnv fnegmknhsv iitkldlfy | ...........
300 | Hfgqsiadiavv gtvavgggat vsgvfnftqf yisepffpgv | ...........
350 | Rhvggdmfas vpgddalmk wihdwdshah catllknvad apfengkviv | ...........
400 | Ehvggdmfvs ipkadafvmn wihdwsdeh clkflknvye apfdngkviv | ...........
450 | Gfkayiyans awaielik* | ...........
500 | Gfkhcnapi tyimeflkkv*. | ...........

**Figure 3.2** Alignment of the predicted amino acid sequence of the PCR-amplified perennial ryegrass *com* gene fragment with the predicted amino acid sequence of the full-length maize and alfalfa cDNAs. Amino acid homology between all three species is highlighted in blue. Forward and reverse primers used to amplify the gene fragment are highlighted in green.
3.3.2 Isolation of a full-length ryegrass comt cDNA

A perennial ryegrass λZAPII cDNA library was screened with the 600-bp PCR-amplified ryegrass comt cDNA probe in order to isolate a full-length COMT-encoding cDNA. Thirty-six positively-hybridizing plaques were detected after low-stringency hybridization of approximately 1 X 10^6 plaque-forming units from the amplified library. These plaques were picked and screened through several rounds of stringent purification. Twelve of these isolates consistently showed varying intensities of hybridization with the homologous ryegrass probe and these were selected for further analysis. After in vivo excision of the pBluescript II SK plasmid and estimation of insert sizes by PCR amplification, four of the strongest-hybridizing isolates were found to contain similarly-sized inserts of approximately 1.5 kb. These four cloned inserts also had identical restriction maps and initial 5' DNA sequence analysis of each of these positive clones revealed that they were identical (data not shown). The insert was sequenced on both strands to ensure accurate calling of all bases in the sequence. Analysis of the DNA sequence revealed an open-reading frame which encoded an amino acid sequence with homology to those of known COMT proteins in the Genbank database. The complete nucleotide sequence and deduced amino acid sequence of one of these perennial ryegrass clones, designated LpeComt1, are shown in Figure 3.3.

3.3.3 Nucleotide sequence analysis

The insert of LpeComt1 is 1475 nucleotides in length with one major open reading frame (ORF) of 1083 nucleotides (Figure 3.3). This region is flanked by 5' and 3' untranslated regions of 70 and 321 nucleotides, respectively. The ORF has a G+C content of 65.5% and encodes a predicted protein of 361 amino acids with a predicted molecular weight of 38,666 Da and a pl of 5.43. The most prevalent amino acids are alanine and leucine which comprise 11.9% and 10.3% of the amino acid mole fraction, respectively. There is a putative translation start site (TAGATCGATGGGCT) which has 50% homology (underlined bases) to the consensus sequence of plants (Joshi, 1987). A putative polyadenylation signal, AATAAC, is located 90 nucleotides upstream of the poly(A) tail.
Figure 3.3 The nucleotide and predicted amino acid sequence of the *LpeComtl* cDNA isolated from perennial ryegrass. The putative translation start site is underlined and the ATG start codon is marked in bold. The putative polyadenylation signal is boxed.
GGCACGAGCTGCTCACACCAAAATCGCCCACCAGCACCAGCATCTCTCGATCGGCAGAC
M G S T A A D M A A S A D E D V A
ACTGTTCGCCCTCAGCTCGCTTCCTCTCAGCTCGAGCTGAGACGTCGC
L F A L Q L A S S V L P M T L K N A I
CGAGCTTGGCCTCCTGGAGATCCTGGTGGCCGCCGGCGGCAAGTCGCTGACCCCGACCGA
E L G L L E I L V A A G G K S L T P E
GTCGCCATTAGATCAGATCCGCAAGGAGGGCCCGCTTACACCGTACCGAGGAG
V A A K L P S A V N P E A P D M V D R I
ACTCCGGCTGCTCCGTCGATCAAGCTGTCGTGAGAGGAGGCAAGAGGAG
L R L L A S Y N V T C L V E E G K D G
CCGCTTCCCGGAGCTAGGCGCGCCCGGCGCGTGCAGATTCGCTACCCCGACGAGGA
R L S R S Y G A P V C K F L T P N E D
CCGCTTCCCGAGCGCGCTGCTGGCTGTATAGGAAGCTCCGGCTATGAGGAGTG
G V S M A A L A L M N Q D K V L M E S W
GTACTACCTCTCAAGAGCGCAGTCGCTTTCGACGGCGACCTCCCCTCTTCCACAGGCTATGCT
Y V L K D A V D G C I P F N K A Y G M
GCTGCGATTACGAGTACGCGGCAGGAGGGCCCGCTTACACCGTACCGAGGAG
S A F E Y H G T D P R F N R V F N E G M
GAGAACCTACTCATATATCACCAGGAGGATCCTCAGCAGCTTCCAGGGG
K N H S I I I I T K K L L E L Y H G F Q G
CTCAGGCTACCCCTGTCAGTGGGGGCGGCGCTTACCGTGCTGCCATACCGCGG
L G T L V D V G G G V G A T V A A I A A
CCACTACCCCACATCACAGGGCTCACTTGCACCTCCCACACAGCTATCGCGAGGCGCC
H Y P A I K G V N F D L P H V I S E A P
GACTTCCCCGCGCTACCCCAAGTGGGAGACATGTCTAAGGAGGTGCGCTCCGGGAG
Q F P G V U H V G G D M F K E V P S G D
TGGCATCTCTTCGAGATCGATCTCCAGCAGCAGCAGCTCCGCCACGCTGGCT
A I L M K W I L H D W S Q H C AT L L
GGAGAACGTCTACGAGGCGTGGCCGCGCTTACCGTGCTGCCATACCGCGG
K N C Y D A L P A N G K V L V L E C I L
GCCGCACTCACCAGGAGCCCAATTTCCCGATGCGACGGGCTTCTCGAGCTGCTGATGATCAT
P V N P E N P S Q G V F H D M I M
GTCGCAGCAACCACCGGCGGCTACCCGGAGAAGGATCGATCGAGGGGATCTCCGGCTCGCAAG
A L H N P G R E V R E F Q A L A R
GGAGCCGGAGTCGCGCGGCTACCCGGAGAAGGATCGATCGAGGGGATCTCCGGCTCGCAAG
G A C F T G V K S T Y I Y A N A W A I E
GTTGAAAGGAATGGGAGAACGTCTCCCAAGAGATCACCAGAGCAGCAGATCGGCAAGGAGA
F T K *
ACGATCGTGTCGATGTCGTCGGTGGCGCTCGGCTGTGCAGATGATGATGACAGGAGG
120
CTGCTGTTGGTTGTCTCTTCTGGGGGCTGGCTGCTCTCTGCTGATGATGATGACAGGAGG
1320
TACATGTTATGTTATGTTATGTTATGTTATGTTATGTTATGTTATGTTATGTTATGTTATGTTATGTTAT
1440
The nucleotide sequence of the ORF shows 82% similarity with that of the maize clone, MC1 (Collazo et al., 1992) and 57% similarity with that of the alfalfa clone COMT1 (Gowri et al., 1991). Both of these sequences have been shown to encode a caffeic acid O-methyltransferase and their predicted amino acid sequences were used to design the degenerate PCR primers.

### 3.3.4 Identification of key COMT structural and functional elements

The protein encoded by the *LpeComt1* cDNA can be classified within the family of methyltransferase enzymes. This is based on the presence of five regions of homology which have previously been identified as being conserved between all the OMTs isolated (Bugos et al., 1992). These regions (I-V) are present between the amino acids 212-227, 228-245, 276-288, 294-305 and 342-348, respectively, of the sequence alignment and are highlighted in Figure 3.4. Of the five regions outlined, three (I, III and IV) are conserved between all enzymes which use S-adenosyl methionine as a co-factor (Ingrosso et al., 1989). The deduced amino acid sequence from *LpeComt1* also contains a putative ATP-binding site, with the consensus sequence GXGXXG, between positions 358-364 (in Figure 3.4). It is possible that this consensus sequence is involved in S-adenosyl methionine binding. Two invariant glycine residues (G-218 and G-220), which may be involved in protein folding and function (Ingrosso et al., 1989), are also conserved within the predicted *LpeComt1*-encoded protein sequence. In addition to these conserved regions, the predicted protein contains COMT-specific protein motifs (COMT I and COMT II), between residues 157-169 and 170-198, reported by McIntyre et al. (1995) and these are highlighted in Figure 3.4. Interestingly, the *LpeComt1*-encoded protein differs from all other COMTs at positions 19, 20, 302 and 345.

### 3.3.5 Amino acid sequence analysis and comparisons

The nucleotide sequence and its predicted amino acid sequence were used to search databases (EMBL and GenBank) and comparison of the translated sequence of *LpeComt1* with other published COMT sequences reveals extensive homology (Table 3.4; Fig 3.4).
Table 3.4 The percentage similarity, accession number, and reference of amino acid sequences of COMT and other OMTs isolated from plants.

<table>
<thead>
<tr>
<th>Plant</th>
<th>Accession</th>
<th>Clone</th>
<th>Reference</th>
<th>Wht</th>
<th>Pars</th>
<th>Bl</th>
<th>M2</th>
<th>M3</th>
<th>M4</th>
<th>Lob</th>
<th>Zin</th>
<th>Alf</th>
<th>Alf</th>
<th>Asp1</th>
<th>Asp2</th>
<th>Pop3</th>
<th>Pop4</th>
<th>Pop5</th>
<th>Pop6</th>
<th>Stylo</th>
<th>Fuc</th>
<th>Chry</th>
<th>Tob</th>
<th>M1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ryegrass</td>
<td>M73235</td>
<td>comtI</td>
<td>Collazo <em>et al.</em> 1992</td>
<td>41</td>
<td>52</td>
<td>54</td>
<td>64</td>
<td>65</td>
<td>65</td>
<td>74</td>
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<td>75</td>
<td>75</td>
<td>75</td>
<td>75</td>
<td>75</td>
</tr>
<tr>
<td>Maize</td>
<td>M84411</td>
<td>OMT1</td>
<td>Jaek <em>et al.</em> 1992</td>
<td>38</td>
<td>49</td>
<td>53</td>
<td>65</td>
<td>63</td>
<td>75</td>
<td>76</td>
<td>77</td>
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% Amino Acid Similarity
Figure 3.4 Comparison of predicted amino acid sequences of plant COMTs.

Previously identified regions of homology (I-V) are boxed in red, two invariant glycines are boxed in green, the Rossman fold is boxed in light blue, the ryegrass variable residues are marked in blue with an * and the COMT specific regions are boxed in purple.
The highest level of similarity (88%) is with the amino acid sequence of MCI, a caffeic acid OMT isolated from maize (Collazo et al. 1992). The highest level of similarity (79%) with a dicotyledonous species is with a tobacco caffeic acid methyltransferase gene, OMT1 (Jaeck, et al. 1992). Comparable levels of similarity were seen with the other dicotyledonous COMTs ranging from 77% to Chrysosplenium (Gauthier et al., 1996) and Eucalyptus (Peydomenage et al., 1994), 76% to Stylosanthes (McIntyre et al., 1995) and poplar (Hayakawa et al., 1996), 75% to the remaining poplar (Dumas et al., 1992) and aspen (Bugos et al., 1991; Tsai et al., 1995) sequences and 74% to the COMT sequence isolated from Zinnia (Ye and Varner, 1995). The COMT isolated from a gymnosperm, loblolly pine, shows a low level of similarity (65%) to the perennial ryegrass COMT amino acid sequence. Neither the ryegrass nor the maize amino acid sequences show high levels of similarity to that of a putative COMT from barley (Lee et al., 1996) with only 64% and 65% similarity, respectively. Lower levels of similarity to OMTs isolated from maize roots (54%) (Held et al., 1993), and fungal-infected barley leaves (52%) (Gregerson et al., 1994) are also recorded. The regions of homology outlined in Figure 3.4 are also present in other genes encoding OMTs of different biochemical function. Even with these conserved regions, the ryegrass COMT is only 52% similar to a parsley caffeoyl CoA-O-methyltransferase (CCoAOMT) (Schmitt et al., 1991) and 41% similar to L-isoaspartyl-O-methyltransferase (IOMT) from wheat (Mudgett and Clarke, 1993).

3.3.6 Phylogenetic analysis of COMT amino acid sequences

The aligned COMT amino acid sequences were examined for their phylogenetic relationships with each other and with other members of the methyltransferase enzyme family (Figure 3.5). The dendrogram in Figure 3.5 shows complex branching patterns, displaying a number of specific groupings (clades) of sequences. The first clade, contains the methyltransferase sequences obtained from maize roots and barley leaves infected with a fungal pathogen. The second clade, is the largest and consists of confirmed COMT-encoding sequences from a wide range of angiosperms and one
Figure 3.5 Dendrogram showing the phylogenetic relationship of the putative \textit{LpeComt1} amino acid sequence to other COMT and OMT amino acid sequences from plants. The monocot COMT sequences are highlighted in \textcolor{red}{red}, dicot angiosperms are highlighted in \textcolor{green}{green}, the gymnosperms are highlighted in \textcolor{blue}{blue}, the alternative monocot sequences are highlighted in \textcolor{lightblue}{light blue}, the \textit{CCoAOMT} is highlighted in \textcolor{orange}{orange} and the IOMT is in \textcolor{purple}{purple}. 
gymnosperm species. This major clade is separated into distinct branches which divides the angiosperm species into sub-clades of related phyla. The first sub-clade contains the monocotyledonous COMT-encoding sequences isolated from maize and ryegrass. The next sub-clade consists of the herbaceous angiosperms, alfalfa and *Stylosanthes* with the woody angiosperms, poplar, almond and *Eucalyptus* grouped together in a specific sub-clade. The sequences from *Chrysosplenium*, tobacco and *Zinnia* are within the main angiosperm clade, but form individual sub-clades. The sequence isolated from loblolly pine, forms a distinct branch off the major COMT clade as does the putative COMT isolated from barley. Completely distant from all of these COMT sequences are the clades containing the parsley CoAOMT and the wheat IOMT sequences.

### 3.3.7 Organ specificity of COMT gene expression

Expression of the perennial ryegrass COMT-encoding cDNA was further analysed by northern blot hybridization analysis. Equal amounts of total RNA isolated from different plant tissues were probed with the full-length *LpeComtl* cDNA. Figure 3.6 shows the higher accumulation of the *LpeComtl* transcript in stem tissue with lower levels of expression in roots, shoots, leaves, sheaths and flowers. In all tissue types, only a single mRNA species was detected. The *LpeComtl* mRNA is approximately 1.4kb in length which corresponds to the known 1475 bp size of the encoding cDNA. High levels of *comtl* gene expression in stem tissue have also been reported in poplar (Hayakawa *et al.*, 1996), *Stylosanthes* (McIntyre *et al.*, 1995), *Zinnia* (Ye and Varner, 1995), aspen (Bugos *et al.*, 1991) and alfalfa (Gowri *et al.*, 1991) which is consistent with the occurrence of COMT in tissue that becomes lignified.

### 3.3.8 Genomic organisation of ryegrass COMT genes

To determine the number of genes coding for COMT in perennial ryegrass, genomic DNA was digested with restriction endonucleases and analyzed by Southern blot hybridization (Figure 3.7). Restriction analysis of the *LpeComtl* cDNA (data not shown) indicated that
Figure 3.6 Northern blot hybridization analyses of total RNA (10μg per lane), from root (R), stem (St), 7 day old shoot (S), mature leaf (L), sheath (Sh) and flower (F) using the full-length LpeComt1 cDNA as a probe. The ethidium bromide stained gel is included to indicate even RNA loadings.
Figure 3.7 Southern blot hybridization analyses of genomic DNA (20µg per lane). Blots were probed with the full-length *LpeComt1* cDNA. Restriction enzymes used were *BamHI* (Ba), *BgIII* (Bg), *EcoRI* (Ec), *HindIII* (Hi), *NsiI* (Ns), *ScaI* (Sc) and *XhoI* (Xh). Asterisks indicate enzymes which do not cut the *LpeComt1* cDNA. Size markers are shown on the left.
there were no internal sites for the restriction enzymes BglII, EcoRI, HindIII, NsiI and ScaI, and one site for BamHI and XhoI. In all cases, several DNA fragments of varying sizes and intensities were revealed after hybridization to the full-length LpeComtl probe. These data suggest that COMT genes in perennial ryegrass may be organized in a small multigene family of at least two members or that there are allelic variants of the gene.

3.3.9 COMT activity in E. coli extracts

The LpeComtl cDNA was cloned into both pBluescriptII SK (Stratagene) and pET-28a(+) (Novagen) using the unique EcoRI and HindIII restriction sites located at the 5' and 3' ends of the insert, respectively. In both expression constructs, the LpeComtl cDNA is in frame with respect to the coding sequence of the phage T7 gene 10 or lacZ gene in the pET-28a(+) and pBluescript vectors, respectively. These constructs were introduced into the E. coli host strain BL21 which lacks the outer membrane proteases that can degrade proteins during purification (Groberg and Dunn, 1988). The methylation activity of protein from induced E. coli extracts is demonstrated by TLC analysis the extracted reaction products (Figure 3.8).

Bacterial extracts (lanes 7,10,11 & 20) and crude plant extracts (lanes 12-14) were tested for COMT activity. Specific meta-methylation was demonstrated by the production of ferulic acid in both types of extracts using caffeic acid as a substrate. No additional non-specific methylation of the 4-OH leading to 3,4-dimethoxycinnamic acid was observed. Enzyme activity was significantly higher in the pET-28a(+) construct than with the pBluescript construct. This is consistent with the expected stronger expression of comt from the bacteriophage T7 promoter in pET-28a(+) than from the lac promoter in pBluescript (Studier et al., 1990).

No enzymatic activity was observed in control extracts from bacteria without (lane 5) or with (lane 6) the pET28-pha(+) vector, or with a pET-28a(+) construct containing an unrelated cDNA insert (lane 8). Similarly, bacterial extracts without reaction mix (lanes
and reaction mix without extracts (lane 4) showed no COMT activity. An extract from an uninduced culture containing the pET-28a(+)*comt* construct (lane 19) showed no enzyme activity indicating that the COMT activity observed in the corresponding induced cultures (lanes 7, 10, 11) was directed by the phage T7 expression system in pET-28a(+).

Methylation was not observed in the absence of caffeic acid (lane 9) or when ferulic, sinapic, *p*-coumaric or 3,4-dimethoxycinnamic acids are used as substrates (lanes 15-18), thus confirming that *LpeComtl* encodes a COMT enzyme.

Lane 21 shows the chromatographic mobility of the SAM-14Me and lanes 22-26 show the chromatographic mobilities of the caffeic, ferulic, sinapic, *p*-coumaric and 3,4-dimethoxycinnamic acids, used as markers as visualized under UV light.

### 3.4 DISCUSSION

A COMT-encoding cDNA clone (*LpeComtl*) was isolated from a whole plant perennial ryegrass λZAPII cDNA library using a homologous 600-bp PCR product as a probe. The PCR-generated homologous probe spanned regions which contain portions of the highly-conserved domains present within all the reported COMTs (Bugos *et al.*, 1992). Monocotyledonous species show an extreme codon bias towards the use of G or C at the third position which may limit the utility of dicotyledonous hybridization probes for the isolation of ryegrass *comt* cDNAs. To increase the probability of isolating a COMT from ryegrass, a probe was amplified from ryegrass cDNA template using degenerate primers designed specifically from the conserved amino acid sequences of the maize and alfalfa COMT enzymes. Verification of the perennial ryegrass *comtl* as a COMT-encoding cDNA was established by the analysis of its nucleotide and predicted amino acid sequences and the specific COMT enzyme activity in extracts from bacteria expressing the protein encoded by the cDNA.
**Figure 3.8** Autoradiography of thin layer chromatography analysis of COMT activity in *E. coli* extracts with 0.25µCi of S-adenosyl-L-(14C-methyl)-methionine and 1.0 mM caffeic acid.

| Lane | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 |
|------|---|---|---|---|---|---|---|---|---|----|----|----|----|----|----|----|----|----|----|----|
| Bacterial extract | + | + | + | - | + | + | + | + | + | - | - | - | + | + | + | + | + | + | + | + |
| Insert | - | - | cont | - | - | cont | cont | cont | cont | - | cont | cont | cont | cont | cont | cont | cont | cont | cont | cont | cont |
| Plant extract | - | - | - | - | - | - | - | - | - | + | + | + | - | - | - | - | - | - | - | - | - |
| IPTG | - | - | - | + | + | + | + | + | + | - | - | - | + | + | + | + | - | + | - | + |
| 14Me-SAM | - | - | - | + | + | + | + | + | + | - | - | - | + | + | + | + | + | + | + | + | + |
| Buffer | - | - | - | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Caffeic acid | - | - | - | + | + | + | + | + | + | - | 0.5 | 2.0 | 0.5 | 1.0 | 2.0 | - | - | - | - | - | - |
| Other substrate | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | FA | SA | pCA | 3,4D |

* CA - caffeic acid; FA- ferulic acid; SA- sinapic acid; pCA-p-coumaric acid; 3,4D- 3,4-dimethoxycinnamic acid

** Lanes 22-26 show the chromatographic mobilities of CA-caffeic acid, SA, FA, pCA and 3,4-D under UV light
The nucleotide composition of the coding region of LpeComtl is GC-rich with 65.5% G+C and 34.5% A+T, a feature which is common in many monocotyledonous genes (Campbell and Gowrie, 1990) and one that is also reflected in the maize comtl gene, MC1 (Collazo et al., 1992). The perennial ryegrass comtl cDNA has high homology to the MC1 clone both at the nucleotide (80%) and amino acid levels (88%). The enzyme from maize, encoded by MC1, has been shown to possess caffeic acid O-methyltransferase (COMT) activity by the expression of a functional enzyme in bacteria containing the cloned cDNA (Collazo et al., 1992). Extracts from bacteria transformed with the perennial ryegrass comtl cDNA, cloned in the appropriate orientation in pBluescriptII SK or pET-28a(+), also show activity in the methylation of caffeic acid to ferulic acid. Similar results have been described for genes isolated from aspen (Bugos et al., 1991), and alfalfa (Gowri et al., 1991). The translation of the functional COMT in the maize, alfalfa and aspen systems was suggested to be occurring due to the presence of a potential ribosome binding-site within the respective cloned cDNAs rather than the ribosome-binding sites contained in the plasmid as the inserts were out-of-translational frame with respect to the vector lacZ coding sequence. Because the ryegrass LpeComtl is in-frame with respect to the lacZ or T7 gene coding region in the pBluescript and pET vectors, respectively, and is only expressed after bacterial induction with IPTG, translation of the functional ryegrass COMT enzyme is most likely directed from ribosome binding sites within each of the vectors.

At high substrate concentrations, only the highly-specific meta-O-methylation of caffeic acid to produce ferulic acid, was detected for the ryegrass COMT. This is in contrast to the bacterially-expressed enzyme activity previously reported from maize by Collazo et al. (1992) where at high substrate concentrations both the specific meta- and non-specific para-O-methylation of caffeic acid were detected. The inability of the ryegrass COMT enzyme to catalyze non-specific para-methylation of caffeic acid is further evidence that the LpeComtl cDNA encodes an enzyme which is directly involved in the methylation of caffeic acid to ferulic acid in the lignin biosynthetic pathway. The non-specific para-O-
methylation of caffeic acid, seen with bacterial extracts expressing a functional COMT protein from the maize MC1 clone (Collazo et al., 1992), suggests that this root-preferentially expressed maize enzyme may also be involved in the methylation of hydroxycinnamic acids involved in another branch of the phenylpropanoid pathway as well as in lignin biosynthesis.

In angiosperms, COMT is a bispecific enzyme due to its ability to catalyse the S-adenosyl-L-methionine (SAM)-dependent methylation of 3,4-dihydroxycinnamic acid (caffeic acid), and 5-hydroxyferulic acid to form 3-methoxy,4-hydroxycinnamic acid (ferulic acid) and 3,5 dimethoxy,4-hydroxycinnamic acid (sinapic acid), respectively. Bispecific COMT activity has been demonstrated for purified enzymes from alfalfa (Gowri et al., 1991) and aspen (Bugos et al., 1991) and is assumed, due to cDNA sequence homology, for those found in maize (Collazo et al., 1992), Stylosanthes (McIntyre et al., 1995) and poplar (Dumas et al., 1992). The high levels of amino acid sequence similarity between the LpeComtl-encoded enzyme and these other sequences, particularly that of maize, suggests that it may be bispecific also. Secondary structure predictions for the perennial ryegrass COMT also show a high probability of helix formation in various parts of the sequence indicating that it may be a globular polypeptide, a characteristic of proteins which have enzymatic activity. These COMTs, also have similar molecular weights (Ryegrass COMT, 38666Da; Maize MC1, 39572 Da; Alfalfa COMT, 39964 Da; Aspen Ptomt1, 39802 Da; poplar pPCL4, 39720 Da) and deduced pl values between 5.43 and 6.03, where the predicted pl for the ryegrass enzyme is 5.43.

The perennial ryegrass LpeComtl encodes a predicted protein which has amino acid sequence homology in the range of 74-79% with the dicotyledonous angiosperm COMTs (Table 3.4). The lower levels of sequence homology of the maize and ryegrass cDNAs with the other COMT sequences may reflect the evolutionary divergence between dicotyledonous and monocotyledonous species. Lower levels of similarity of the dicotyledonous angiosperm COMTs to the loblolly pine COMT sequence (64%) may be a
reflection of the different phylogenetic origin of these plants and hence the different substrate specificities reported for COMT isolated from angiosperms and gymnosperms (Chiang et al., 1988).

The predicted amino acid sequences of the ryegrass and maize COMT proteins do not show high levels of similarity to a putative COMT isolated from barley (Lee et al., 1996). Both the ryegrass and maize cDNAs have relatively low nucleotide and amino acid sequence similarities to that of the barley clone being the lowest levels of homology observed when all COMT sequences are compared. This suggests that the barley COMT may be more related to methyltransferases involved in other branches of the phenylpropanoid pathway rather than to those involved in lignin biosynthesis. However, the observation that the barley sequence does encode some of the conserved amino acid motifs present in the other COMTs suggests that it could be an allelic variant or an alternate gene for COMT, for which no counterpart has been isolated from other species.

Consistent with their different metabolic functions, the amino acid sequence similarity between the COMTs and other forms of OMT are low. For example, ryegrass COMT shows only 52% amino acid sequence similarity to a parsley caffeoyl-CoA-O-methyltransferase, an enzyme which catalyses the conversion of coumaryl-CoA to feruloyl-CoA and is involved in the disease resistance response (Pakusch et al., 1989). Although this enzyme is structurally unrelated to COMTs, its possible role in an alternative methylation pathway in the lignin biosynthetic pathway has been suggested (Ye et al., 1994). Similarly, the L-isoaspartyl methyltransferase isolated from wheat, postulated to function in the repair or the catabolism of damaged proteins (Mudgett and Clarke, 1993), shows little similarity (42%) to the ryegrass COMT. The similarities of the OMT sequences from these species to the COMTs may simply reflect the conserved structural and functional elements found in all methyltransferases (Ingrosso et al., 1989).
Regions of homology (I-V) between all the COMT genes isolated have been previously identified (Bugos et al., 1991) (highlighted in Figure 3.4.) and are present in the perennial ryegrass COMT protein sequence. Three of the five regions (I, III and IV) are conserved between all enzymes which utilize SAM as a co-factor (Ingrosso et al., 1989). Two invariant glycine residues, found in the highly conserved region I, have been postulated to be involved in protein folding and function (Ingrosso et al., 1989). Additionally, a structure found in many nucleotide-binding enzymes is the Rossmann fold (GXGXXG) which is a series of interconnecting α-helices and β-sheets (Rossmann et al., 1974). This motif is present in the perennial ryegrass and other COMT protein sequences and is thought to have a role in ATP and SAM binding (Bugos et al., 1991). The two other amino acid sequence motifs, shown by McIntyre et al. (1995) to be highly conserved in the COMTs, are also present in the sequence isolated from perennial ryegrass.

The phylogenetic analysis (Figure 3.4) of 22 different plant OMT sequences shows that the maize and ryegrass enzymes comprise a distinct sub-group within the COMT clade. This presumably reflects the evolutionary separation between the dicotyledonous and monocotyledonous plants. The other OMTs of known function included in the dendrogram appear further distant from COMT, consistent with their different metabolic roles.

Southern blot hybridization analysis of ryegrass genomic DNA provided evidence for the existence of a small gene family containing at least two genes. In contrast, the data for maize suggests the presence of only one gene (Collazo et al., 1992). Gene families, consisting of at least two genes, have been reported in alfalfa (Gowri et al., 1991) and a multigene family has been reported in aspen (Bugos et al., 1991). The complex hybridization patterns for perennial ryegrass, and the variation in number and intensity of bands with different restriction enzymes, suggests the presence of a number of introns. Alternatively, these data may also indicate that COMT in perennial ryegrass, which is an outcrossing species, may be encoded by a number of allelic variants.
Northern blot hybridization analysis shows that the \textit{LpeComtl} mRNA is preferentially expressed in stem tissue, which is the major site of lignin deposition. This is consistent with the involvement of the enzyme encoded by \textit{LpeComtl} in lignin biosynthesis. Lower level expression of \textit{comtl} is seen in roots, shoots, leaves, sheaths and flowers and may reflect the less-vascular, and hence less-lignified nature of these tissue types. By contrast, the \textit{MC1} gene from maize (Collazo et al., 1992) shows strongest expression in roots, with very low levels of expression in other tissues including leaves, seeds and flowers. The COMT-encoding genes from alfalfa (Gowri et al., 1991), tobacco (Jaeck et al., 1992) and Stylosanthes (McIntyre et al., 1995) all show strong expression in stem tissue. In poplar (Hayakawa et al., 1996), two genes for COMT have been isolated and, although they are highly homologous, their expression patterns are different in different tissues. The poplar gene \textit{homt1} shows strong expression in stem tissue, while \textit{homt2} shows preferential expression in young leaves suggesting that there may be a different COMT for lignin biosynthesis in these different tissues. In perennial ryegrass, the detection of low levels of \textit{comt} in other tissues, and the complex Southern blot hybridization results, may be a reflection of cross hybridization between members of a COMT multigene family in this species.

In conclusion, a caffeic acid \textit{O}-methyltransferase cDNA from ryegrass has been cloned and its DNA sequence determined. The pattern of predominantly stem-abundant expression of the corresponding gene closely resembles that of COMT-encoding genes from a number of dicotyledonous species thought to be involved in lignin biosynthesis. The perennial ryegrass \textit{comtl} is, however, the first stem-abundantly expressed \textit{comt} cDNA to be isolated from a monocotyledonous plant. This perennial ryegrass \textit{comt} gene may be a useful tool for the modification of lignin biosynthesis to improve the digestibility and nutritive value of this important pasture plant.
CHAPTER 4
DEVELOPMENTAL STUDY OF COMT AND CAD ENZYME ACTIVITY

4.1 INTRODUCTION
A pre-requisite to directed genetic manipulation of lignin is a more thorough understanding of its biosynthesis. Lignin content and composition varies with species, tissue type, developmental stage and subcellular location (Lewis and Yamamoto, 1990). Environmental cues such as wounding or pathogen attack can also induce the deposition of lignins with a monomeric composition different from that of constitutive lignins (Ride et al., 1975). This raises important questions about specific functions for the different lignin compositions. Analytical investigations have shown that the composition and structure of lignin from gymnosperms is different from that of angiosperms (Chiang et al., 1988). Gymnosperm lignin is composed mainly of coniferyl alcohol (guaiaacyl lignin), whereas lignin from dicotyledonous angiosperms originates from coniferyl and sinapyl alcohol (guaiaacyl-syringyl lignin). The monocotyledonous angiosperms incorporate all three of the monolignols; p-coumaryl, coniferyl and sinapyl alcohols, into their lignin, forming a p-hydroxyphenyl-guaiacyl-syringyl lignin (Higuchi 1981). The different lignin compositions potentially may be controlled by the activities of different lignin biosynthetic enzymes or enzyme isoforms.

The difference in lignin composition between angiosperms and gymnosperms may be a reflection of the substrate specificities of enzymes such as cinnamyl alcohol dehydrogenase (CAD) and caffeic acid methyltransferase (COMT), which are involved in the biosynthesis of the lignin precursors. For example, COMTs from angiosperms are considered bi-functional as they catalyse the methylation of both caffeic and 5-hydroxyferulic acid to produce the lignin precursors ferulic and sinapic acid, respectively. In contrast, COMTs from gymnosperms appear to only efficiently methylate caffeic acid (Kuroda et
al., 1975; 1981). COMTs catalyse important steps in lignin precursor synthesis as they control the degree of methylation of the monolignols and hence the degree to which lignin condenses and cross links (Chiang et al., 1988).

CAD catalyses the reduction of the cinnamyl aldehydes to their corresponding cinnamyl alcohols (Kutsuki et al., 1982). CAD is considered as an ideal target enzyme for manipulation of the lignification pathway because it is specific for lignin biosynthesis and it is the enzyme that catalyses the final step in the biosynthesis of the monolignols. The difference in substrate affinities of CAD enzymes from angiosperms and gymnosperms may play an important role in controlling the formation of different types of lignin (Kutsuki et al., 1982). CAD preparations from gymnosperms are more active on coniferyl aldehyde whereas angiosperm CADs generally show equal affinity for both coniferyl and sinapyl aldehydes (Gross, 1985).

The biological significance and regulation of the chemical differences seen between lignin from different tissues and in different plants are yet to be elucidated. The role of CAD and COMT as control points in lignin biosynthesis has been addressed by studies of lignification mutants such as the brown-midrib mutants of maize (Pillonel et al., 1991) and sorghum (Akin et al., 1986) and various transgenic plants (Dwivedi et al., 1994; Ni et al., 1994; Halpin et al., 1994; Van Doorselaere et al., 1995b; Attassanova et al., 1995; Rae et al., 1996). The variability in both lignin content and composition in association with varied levels of enzyme suppression suggests complex regulatory controls of the lignin biosynthetic pathway. The expression of genes encoding these lignin biosynthetic enzymes has been indicated to be regulated by developmental and environmental cues and, therefore, may be influential in the timing and localization of lignification (Campbell and Sederoff, 1996).

The studies described in this chapter were aimed at determining the crucial times of CAD and COMT enzyme activity during the development of perennial ryegrass leaves. The
relationship of these enzyme activities with gene expression was examined in order to
determine the basis for the potential use of CAD and COMT as control points for
manipulation of lignin biosynthesis in perennial ryegrass. Enzyme substrate specificities
were analyzed to further investigate the possible presence of multiple CAD or COMT
enzymes or enzyme isoforms and the role these may play in determining the changes in
lignin content and composition during development.

4.2 MATERIALS AND METHODS

4.2.1 Plant material

Clones of a single endophyte-free perennial ryegrass plant (Lolium perenne cv. Yatsyn),
were grown in a glass house. Cloned plants were vegetatively propagated and cultivated
in growth chambers under controlled environmental conditions. These plants were
maintained at 16 h days (300µE m⁻² s⁻¹ light) at 24°C and 8 h nights at 16°C for a period
of 60 days. Non-destructive measurements of increases in the length of the third leaf
were recorded over a 30 day period and from this data, eight leaf lengths relating to
important growth time points were determined, and material harvested.

4.2.2 Harvesting

Leaf blades from the third leaf of the plants grown under controlled conditions were
harvested for analysis. The leaf blade was designated as that part of the leaf which
extended from the ligule to the leaf tip. To obtain a range of different age leaf material,
the leaves were harvested (on ice) and pooled according to the length categories of 0-30,
30-60, 60-100, 100-140, 140-180, 180-220, 220-260 mm and leaves which had reached
220-260 mm were grown for a further 10 days. Approximately 1 g fresh weight, replicate
samples were wrapped in foil packs, frozen in liquid nitrogen and stored at -80°C.

4.2.3 Enzyme extraction

Collected leaf tissue was ground to a fine powder with a mortar and pestle in the presence
of liquid nitrogen. Extraction buffer (100mM Tris, pH 7.5; 10% glycerol; 50mM
mercaptoethanol) was added to the powder (4mL buffer per 1 g tissue) and grinding was continued until the tissue had thawed. The crude extract was centrifuged at 14,000 rpm for 10 min at 4°C. The supernatant was transferred to a pre-cooled microfuge tube and endogenous salts and substrates were removed by passage through a Sephadex G-25 column (5mL column per 1 mL extract) which was pre-equilibrated with 15 mL of extraction buffer. The crude enzyme preparation was loaded onto the column, the excluded protein front was collected and either kept on ice or frozen in liquid nitrogen and stored at -80°C for later protein determination and enzyme assays.

4.2.4 Protein determination
The protein content in the desalted enzyme extracts were determined with a prepared dye-binding reagent (Bio-Rad) using BSA as the standard and following the manufacturer’s protocol.

4.2.5 Cinnamyl alcohol dehydrogenase (CAD) enzyme assays
The CAD enzyme assays were based on previous protocols by Mansell et al. (1974) and Luderitz and Grisebach (1981) and refined for perennial ryegrass. CAD enzyme activity can be assayed spectrophotometrically by measuring either the oxidation of cinnamyl alcohols in the presence of NADP (reverse direction) or the reduction of cinnamyl aldehydes in the presence of NADPH (forward direction) (Figure 4.1). The reverse reaction was monitored by following the change in absorbance due to aldehyde formation at 400nm. In the forward direction assay, decreases in the concentration of NADPH were monitored at 340nm. The reverse reaction (total volume 1 mL) was composed of 50 µL of enzyme extract in 0.1M Tris-HCl (pH 8.8) and 0.2 mM NADP. The reaction was initiated by the addition of 1 mM coniferyl alcohol in a solution of 50% methanol and 100mM Tris-HCl, pH 8.8. Reactions were run in triplicate at 400 nm using a Varian CARY 210 Ultraviolet Spectrophotometer. The molar coefficient (E400) of coniferyl aldehyde was 18.5x10^6 cm^2 mol^-1 in a Tris-HCl buffer pH 8.8 (Mansell et al., 1974).
Figure 4.1 CAD enzyme activity assay outline.

The forward reaction (total volume 1 mL) consisted of 50 µL of the enzyme preparation in 0.1M potassium phosphate buffer (pH 7.0) and 0.3 mM NADPH. The reaction was initiated by the addition of 50 µM coniferyl or sinapyl aldehydes in a solution of 50% methanol and 100 mM potassium phosphate buffer, pH 7.0. The conversion of coniferyl and sinapyl aldehydes to corresponding alcohols was recorded as a decrease in absorbance due to the loss of NADPH at 340nm. CAD activity was calculated on a fresh weight basis, defined as mU of enzyme activity per gram of fresh weight (mU/g fr wt) where a mU is the amount of enzyme required to oxidize or reduce 1 nmol of alcohol or aldehyde, respectively, per minute at 30°C. This corresponds to a change in absorbance at 400nm of 0.035/min for alcohol oxidation and 0.016/min at 340nm for aldehyde reduction (Mansell et al., 1974).
4.2.6 Caffeic acid \(O\)-methyltransferase (COMT) enzyme assays

The COMT enzyme assay was based on a previous protocol by Kuroda et al. (1981). COMT enzyme activity was measured by recording the incorporation of \(^{14}\text{C}\)-labelled \(S\)-adenosylmethionine (SAM-\(^{14}\text{Me}\)) methyl group into caffeic acid to produce a \(^{14}\text{C}\)-labelled ferulic acid end product (Figure 4.2). Enzyme reaction conditions were optimized for perennial ryegrass leaf tissue by determining the appropriate substrate, co-factor and enzyme concentrations, pH, buffer type and reaction times (see Results). All assays were performed in triplicate.

\[
\begin{align*}
\text{Caffeic Acid} & \quad \text{or} \quad \text{5-hydroxyferulic Acid} \\
\text{COMT} & \\
\text{14C-SAM} & \quad \text{SAH} \\
\text{acidify with HCl} & \quad \text{and ether extract} \\
\text{14C-Ferulic Acid} & \quad \text{or} \quad \text{14C-Sinapic Acid}
\end{align*}
\]

**Figure 4.2** COMT enzyme activity assay outline

\(^{14}\text{C}\)-SAM, \(S\)-adenosyl methionine; SAH, \(S\)-adenosyl homocystine

The reaction mixture (200 µL) contained 50 mM Tris-HCl, pH 8.0; 10 mM MgCl\(_2\); 1 mM phenylpropanoid substrate (routinely caffeic acid) and 100 µL of enzyme solution in a 1.5 mL microfuge tube. The reaction mixture was preincubated for 5 min at 30\(^\circ\)C, and diluted SAM-\(^{14}\text{Me}\) (0.25 µCi µmol\(^{-1}\)) was added to a final concentration of 0.5 mM and the reaction was incubated for 15 minutes at 30\(^\circ\)C. The diluted SAM-\(^{14}\text{Me}\) was prepared
by adding 4.9 mg SAM.HSO₄ (Boehringer) to 100µl of SAM-¹⁴Me (54.0 mCi mmol⁻¹) (Amersham) and 900 µL of dH₂O. The reaction was terminated by the addition of 40 µL of 1M HCl. The ¹⁴Me-hydroxycinnamic acid products were separated from the unincorporated SAM-¹⁴Me by a single extraction with 1 mL of ether. The ether phase was removed and reduced in volume under vacuum to 100 µL, spotted onto glass fibre filters (Whatman GFA) and dried. The filters were placed in scintillation vials containing 5mL of a toluene based scintillant (6g PPO, 0.6g POPOP, 670mL Toluene, 330mL Teric X-10, 200 mL ethanol) and left to equilibrate for at least 10 min before counting. Results are expressed as mU of enzyme activity per gram fresh weight (mU/g fr wt) where a mU is the amount of enzyme required to methylate 1nmol of caffeic acid per minute at 30°C.

4.2.7 Northern analysis of developmental expression of cad and omt genes
Total RNA was isolated from samples of the pooled leaf tissues as used to analyse enzyme activity at 8 different developmental stages, following the methods outlined previously in Chapter 2. The RNA was electrophoresed and analysed for mRNA transcripts by probing with either the full-length LpeCad1 or LpeCmt1 cDNAs, previously isolated from perennial ryegrass. Hybridization and autoradiography conditions were as previously described (Chapter 2).

4.3 RESULTS

4.3.1 Changes in leaf length during development
Figure 4.3 shows the increase in length of the third leaf of perennial ryegrass during a 30 day period of development. This figure shows a rapid seven-fold increase in leaf length within the first 7 days of leaf development. After this time period the leaf only increases in length by a further 20% and at day 16 is considered fully expanded. At this time, cell expansion has ceased and the leaf is fully mature. The graph provides a basis for using leaf length as a measurement of the age and developmental stage; leaves that are pooled into length categories are representative of a similar developmental age.
Figure 4.3 Increase in leaf length during the development of the third leaf of a perennial ryegrass plant. Mean values from 3 replicates are plotted and where no error bar is present, errors were smaller than the size of the symbol.

4.3.2 Extraction and stability of the CAD and COMT enzymes

Cinnamyl alcohol dehydrogenase (CAD) and caffeic acid O-methyltransferase (COMT) were measured in extracts from leaves of perennial ryegrass plants at eight stages of development. The crude enzyme preparations were desalted by size exclusion chromatography through a Sephadex G-25 column to remove endogenous substrates, cofactors and possible enzyme inhibitors. Preliminary experiments indicated that the inclusion of 10% glycerol and 50 mM β-mercaptoethanol in the extraction and column elution buffers maintained enzyme stability and prevented rapid oxidation of extracts (data not shown). The desalted enzyme extracts were stored on ice for immediate use or snap frozen in liquid nitrogen and stored at -80°C. Under these conditions, enzyme activity was maintained with both methods of storage, showing no appreciable loss of activity after 48 hours on ice, or 3 months at -80°C.
4.3.3 Protein levels

The amount of extractable protein per gram of leaf tissue was measured (Figure 4.4). The results show a gradual increase in extractable protein content of the leaf from 0-18 days. Little increase in protein content per µL of extract is observed after day 18.

![Figure 4.4 Increase in the extractable protein content of the developing perennial ryegrass leaf. Mean values from 3 replicates are plotted and where no error bar is present, errors were smaller than the size of the symbol.](image)
4.3.4 Optimizing the CAD enzyme assay conditions

Substrate and cofactor concentrations

Using extract from 7 day old tissue substrate concentration curves approximating hyperbolic responses were obtained for both the alcohol and aldehyde substrates in CAD activity assays. Figure 4.5(A) indicates that 1mM coniferyl alcohol is sufficient for maximum enzyme activity, and the apparent $K_m$ was 0.17mM. No activity was recorded at any concentration when sinapyl alcohol was used as the substrate. The concentrations of both coniferyl and sinapyl aldehyde required for maximum CAD enzyme activity were 50µM (Figure 4.5B). The lower apparent $K_m$ values of 12 µM and 6 µM for coniferyl and sinapyl aldehydes, respectively, compared to the observed in vitro apparent $K_m$ value for the alcohol substrate (Table 4.1), are consistent with the CAD enzyme utilizing the aldehyde substrates preferentially in vivo. The extract showed no reaction with the aliphatic alcohols methanol, ethanol or isopropanol (data not shown).

The effect of varying co-factor concentrations for CAD activity from a 7 day old leaf enzyme preparation are shown in Figure 4.6 (A and B). Under standard assay conditions (see Materials and Methods) the maximum activity for alcohol oxidation or aldehyde reduction was reached with co-factor concentrations of 300 µM NADP and 200 µM NADPH, respectively. The apparent $K_m$ values for NADP with 1mM coniferyl alcohol was 45 µM while for NADPH apparent $K_m$ values of approximately 25 and 55 µM with either coniferyl or sinapyl aldehyde (50 µM), respectively, were observed (Table 4.1).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Optimal Concentration</th>
<th>Apparent $K_m$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coniferyl Alcohol</td>
<td>1 mM</td>
<td>170 µM</td>
</tr>
<tr>
<td>- NADP</td>
<td>300 µM</td>
<td>45 µM</td>
</tr>
<tr>
<td>Coniferyl Aldehyde</td>
<td>50 µM</td>
<td>12 µM</td>
</tr>
<tr>
<td>- NADPH</td>
<td>200 µM</td>
<td>25 µM</td>
</tr>
<tr>
<td>Sinapyl Aldehyde</td>
<td>50 µM</td>
<td>6 µM</td>
</tr>
<tr>
<td>- NADPH</td>
<td>200 µM</td>
<td>55 µM</td>
</tr>
</tbody>
</table>

Table 4.1 CAD enzyme kinetics
Figure 4.5 Effect of substrate concentration on CAD activities in perennial ryegrass leaf extracts. A- Coniferyl alcohol (-●-). B- Coniferyl aldehyde (-△-) and sinapyl aldehyde (-■-). Mean values from 3 replicates are plotted and where no error bar is present, errors were smaller than the size of the symbol.
Figure 4.6 Effect of CAD co-factor concentration on enzyme activity in perennial ryegrass leaf extracts. **A** - NADP co-factor concentration curve for CAD activity with coniferyl alcohol (-●-). **B** - NADPH co-factor concentration curve for CAD activity with coniferyl aldehyde (-▲-) and sinapyl aldehyde (-■-) substrates. Mean values from 3 replicates are plotted and where no error bar is present, errors were smaller than the size of the symbol.
Buffer composition and pH

The effects of two buffers and pH on enzyme activity for both alcohol oxidation and aldehyde reduction are shown in Figure 4.7 (A-C). The oxidation of coniferyl alcohol is optimal with Tris-HCl buffer at pH 8.8 (Figure 4.7A). Activity declined sharply at lower pH with about half the maximum activity at pH 8.5 and negligible activity at pH 7.0. In contrast, optimal reduction of coniferyl and sinapyl aldehydes by CAD were observed with potassium phosphate buffer at a pH of 6.5-7.0 (Figure 4.7B and 4.7C).

Linearity of activity with increasing CAD enzyme concentration

Figure 4.8 confirms the linear relationship between the amount of CAD and the observed enzyme rate under standard conditions. In addition, the relative rates obtained with different substrates can be compared. When coniferyl and sinapyl aldehydes are used as substrates, the rates of reduction are almost identical at 0.012 and 0.011 nM min\(^{-1}\) µL\(^{-1}\) of enzyme extract, respectively. In the reverse reaction the rate of coniferyl alcohol oxidation to coniferyl aldehyde is approximately 2.6 times slower (0.0046 nM min\(^{-1}\) µL\(^{-1}\)) than the forward reaction indicating that the forward reaction is the most efficient under optimized conditions. As noted earlier, no activity was observed with sinapyl alcohol.

4.3.5 Optimizing the COMT enzyme assay conditions

Substrate and co-factor concentration curves

A hyperbolic substrate concentration curve was obtained when caffeic acid was used as a substrate to measure in vitro COMT activity. From Figure 4.9 it can be seen that the maximum enzyme activity was obtained with the caffeic acid concentration of 1 mM, with a calculated apparent K\(_m\) of 75 µM.
Figure 4.7 Dependence of CAD activity on pH.

The oxidation of coniferyl alcohol (A), or the reduction of coniferyl aldehyde (B) and sinapyl aldehyde (C) were measured at various pH values in either 0.1M Tris-HCl buffer (-○-) or 0.1M potassium phosphate buffer (-▲-). Mean values from 3 replicates are plotted and where no error bar is present, errors were smaller than the size of the symbol.
Figure 4.8 Linearity of observed CAD activity with varying enzyme levels. Measuring the rate of oxidation of coniferyl alcohol (-●-) or the reduction of coniferyl aldehyde (-▲-) and sinapyl aldehyde (-■-). Mean values from 3 replicates are plotted and where no error bar is present, errors were smaller than the size of the symbol.

Figure 4.9 Effect of caffeic acid concentration on COMT activities in perennial ryegrass leaf extracts. Mean values from 3 replicates are plotted and where no error bar is present, errors were smaller than the size of the symbol.
The effect of varying concentration of SAM co-factor required for COMT activity is shown in Figure 4.10. Near maximum enzyme activity was achieved when the methylation conditions included a SAM concentration of 0.5 mM. At a caffeic acid concentration of 1mM, the apparent $K_m$ for SAM was 10 µM.

![Figure 4.10](image.jpg)

**Figure 4.10** Effect of $S$-adenosyl methionine co-factor concentration on COMT activity in perennial ryegrass leaf extracts. Mean values from 3 replicates are plotted and where no error bar is present, errors were smaller than the size of the symbol.

**pH optimum**

The COMT enzyme activity at different pH values was measured using 0.05M Tris-HCl or 0.05M potassium phosphate buffer with caffeic acid as substrate (Figure 4.11). High enzyme activity was achieved in either buffer over a wide pH range from 7.5 to 8.5. However, activity was highest at pH 8.0.
Figure 4.11 The dependence of COMT activity on pH.

The methylation of caffeic acid was measured at varying pH in either 50 mM Tris-HCl buffer (-○-) or 50 mM potassium phosphate buffer (-▲-). Mean values from 3 replicates are plotted and where no error bar is present, errors were smaller than the size of the symbol.

Volume of enzyme and length of reaction time

Figure 4.12 confirms the linear relationship between the amount of COMT and the observed enzyme rate under standard conditions. The COMT enzyme assay reaction conditions used in the following developmental analysis were standardized by using 100 µL of extract for a fifteen minute incubation period at 30°C.
Figure 4.12 The effect of COMT enzyme concentration and length of reaction incubation on the overall COMT enzyme specific activity. The amounts of enzyme in a 200µl reaction were: 25µl (-●-), 75µl (-○-), 100µl (-■-), 125µl (-□-). Mean values from 3 replicates are plotted and where no error bar is present, errors were smaller than the size of the symbol.

4.3.6 Developmental CAD enzyme assays and cad gene expression
The changes in perennial ryegrass CAD enzyme activity seen over a 28-day developmental period are shown in Figure 4.13. CAD activities were measured in both the forward direction, monitoring the reduction of coniferyl and sinapyl aldehydes, and the reverse direction which records the oxidation of coniferyl and sinapyl alcohol. Under standard reaction conditions, the CAD activity in perennial ryegrass is greatest with coniferyl aldehyde throughout development. CAD enzyme activity observed with
coniferyl alcohol as a substrate shows a similar profile, but at levels lower than with coniferyl aldehyde. No oxidative activity was recorded with sinapyl alcohol as the reaction substrate. The CAD activities recorded with sinapyl aldehyde also parallel\ the activities with coniferyl substrates. With each substrate, CAD activity increased during the first 2.5 days of leaf development reaching specific activity maxima of 110, 75 and 70 mU/g fr wt for the coniferyl aldehyde, coniferyl alcohol and sinapyl aldehyde substrates, respectively. The activity then declined until day 5 where basal activity levels of 90, 53 and 48 mU/g fr wt for coniferyl aldehyde, coniferyl alcohol and sinapyl aldehyde, respectively, were measured for the remaining time period.

Figure 4.13 CAD enzyme activities in perennial ryegrass leaf extracts over a 28 day developmental period. Enzyme activity was assayed with coniferyl alcohol (-○-), coniferyl aldehyde (-△-), and sinapyl aldehyde (-■-) as substrates. Mean values from 3 replicates are plotted and where no error bar is present, errors were smaller than the size of the symbol.
Total RNA was also isolated from developing perennial ryegrass leaves in order to examine the relationship between gene expression and the developmental activities of encoded enzymes. Northern blot hybridization analysis of the full-length *LpeCad1* cDNA to total RNA revealed strong levels of expression between days 0.5 - 4, after which accumulation of mRNA decreased slightly and then remained at a steady level for the remaining time period (Figure 4.14). Only a single sized transcript was detected at all stages of leaf development. This pattern of mRNA accumulation correlates well with the high initial level of CAD enzyme activity (day 2) followed by slight reductions of activity which then remains unchanged for the remaining time period. This may indicate that alterations in *LpeCad1* gene expression would potentially effect CAD enzyme activity, as there is no evidence of post-translational modification or regulation of the enzyme.

### 4.3.7 Developmental COMT assays and *LpeComt1* gene expression

The changes in COMT enzyme activity during the development of the perennial ryegrass leaf, over a 28 day period, can be seen in Figure 4.15. Total COMT activity initially increased during the first 2.5 days of leaf development by 25%, reaching a maximal enzyme activity of approximately 15 mU/g fr wt. The enzyme activity decreased steadily over a period of 12 days, reaching the lowest activity of 4 mU/g fr wt at day 14. This low level of activity was maintained until day 28.

The total RNA isolated from developing leaves of perennial ryegrass was probed with the full-length *LpeComt1* cDNA in order to examine the relationship between COMT gene expression and enzyme activity during development. Northern blot hybridization analysis revealed a single mRNA species at all stages of development. COMT mRNA is evident at all the time periods of development except for day 28 where no mRNA signal was evident (Figure 4.16). Strong levels of COMT gene expression were seen between days 0.5-2.5. After this time period the levels of expression decrease. The developmental patterns of *comt1* gene expression approximately correlate with enzyme activity levels, with both showing peak accumulation or activity at day 2.5.
Figure 4.14 Northern analysis of *LpeCad1* gene expression over different time periods during leaf development. A) Equal amounts of total RNA (10µg per lane) were loaded. B) Northern blot where numbers relate to 0.5, 2.5, 3.0, 4.5, 6.5, 14, 18 and 28 days after leaf initiation.
**Figure 4.15** COMT enzyme activity in perennial ryegrass leaf extracts over a 28 day developmental period. Enzyme activity was assayed under optimized conditions with caffeic acid (-•-) as the substrate. Mean values from 3 replicates are plotted and where no error bar is present, errors were smaller than the size of the symbol.

**4.4 DISCUSSION**

From an analysis of the developmental patterns of CAD and COMT expression and activity, it may be possible to determine their role in lignification of the developing perennial ryegrass leaf and also to identify critical time points for manipulation of lignin biosynthesis. Optimization of assays for CAD and COMT enzymes in extracts from perennial ryegrass was necessary in order to study the developmental activities of these enzymes. Optimal CAD and COMT enzyme reaction conditions were determined by examining substrate specificities and concentrations, co-factor requirements, pH, optimal amount of enzyme and, for COMT, reaction incubation times.
**Figure 4.16** Northern analysis of *LpeComt1* gene expression over different time periods during development of the third leaf. **A**) Equal amounts of total RNA (10µg per lane) were loaded. **B**) Northern blot where numbers represent 0.5, 2.5, 3.0, 4.5, 6.5, 14, 18, and 28 days after leaf initiation.
**CAD enzyme activity**

The crude perennial ryegrass enzyme extracts showed activities which catalysed the reduction of cinnamyl aldehydes to the corresponding alcohols. The optimized assay conditions resulted in perennial ryegrass CAD enzyme activities that are comparable with those obtained from other species (Mansell et al., 1974). In the monocotyledonous species for which CAD activity has been assayed, the highest activity of 56 mU/g fr wt was found in 7 day old leaves of *Zea mays* using coniferyl alcohol. CAD in perennial ryegrass extracts showed levels of specific activity as high as 90 mU/g fr wt with coniferyl alcohol (Figure 4.5), 75 mU/g fr wt with sinapyl aldehyde and 110 mU/g fr wt for coniferyl aldehyde (Figure 4.13). In general, the highest levels of CAD activity have been found in the gymnosperms such as *Picea abies* (1300 mU/g fr wt) with the highest angiosperm enzyme activity recorded in the cambial regions of *Acer campestre* (247 mU/g fr wt) (Mansell et al., 1974). Differences in substrate K_m values have been recorded for CAD between other species (Kutsuki et al., 1982) and this may account, in part, for the differences seen in the lignin composition between members of the angiosperm and gymnosperm phylogeny. The lower K_m values for the cinnamyl aldehydes compared with the cinnamyl alcohols (Table 4.1) are consistent with the role of CAD enzymes in the reduction of cinnamyl aldehydes under physiological conditions. This is similar to observations with CAD purified from other species and supports the view that this enzyme catalyses the last reaction in the pathway leading from L-phenylalanine to substituted cinnamyl alcohols, the precursors for lignin (Hahlbrock and Grisebach, 1979).

CAD activity was assayed in extracts from the leaves of perennial ryegrass at different developmental stages. The results showed that in perennial ryegrass, CAD activity is greater with coniferyl aldehyde as a substrate compared to sinapyl aldehyde at all stages of development. The profiles, throughout development, of the enzyme activities with each substrate are similar, consisting of a rapid increase in enzyme activity within the first 2.5 days of leaf development followed by a decrease in enzyme activity to a lower level,
reached at day 5. Enzyme activity with all three substrates decreased to a basal level of approximately 88 mU, 45 mU and 54 mU/g fr wt for coniferyl aldehyde, sinapyl aldehyde and coniferyl alcohol, respectively, within 2-3 days of maximal activity levels being reached. These similar activity profiles with each substrate could be explained by two possibilities. First, a single enzyme may be active with each substrate and one substrate (coniferyl aldehyde) preferentially binds to the active site of the enzyme. Alternatively, two or more separate CAD enzymes, each with a preferred substrate specificity, may be active and are regulated by the same cues during leaf development or different developmental cues. In perennial ryegrass, the lower levels of enzyme activity with sinapyl aldehyde during normal leaf development may also be due to the presence of two or more forms of CAD that under different environmental conditions are altered in their activity. In wheat, multiple forms of the constitutive CAD have been recorded (Pillonel et al., 1992) and lignin heterogeneity in this species has been suggested to be due to the differential expression of CAD isoforms with different substrate specificities (Mansell et al., 1974). For example, healthy wheat leaves predominantly contain guaiacyl lignin, whereas lignin in wounded tissues is mainly composed of guaiacyl-syringyl lignin with a substantial proportion of $p$-hydroxyphenyl residues (Mitchell et al., 1994). These different lignin compositions may be controlled by the activities of different CAD enzymes. Multiple forms of CAD have also been recorded in dicotyledonous species such as *Eucalyptus* (Goffner et al., 1992), and soybean (Wyrambrik and Grisebach, 1975).

In perennial ryegrass, the maximum activity for CAD with both sinapyl and coniferyl aldehyde, was reached early in development. The CAD activity with sinapyl aldehyde, the precursor to syringyl lignin, was 68% of the rate with coniferyl aldehyde, the guaiacyl lignin precursor. Later in development, the enzyme activity with sinapyl aldehyde decreased to 50% of that of coniferyl aldehyde (Figure 4.13). This is consistent with the hypothesis that there may be two forms of CAD, each with a different substrate specificity, and regulated at different times of development. This change in enzyme
activity ratios may be due to a reduction in the activity or production of a sinapyl aldehyde-specific CAD isoform. This hypothesis would suggest that as the leaf develops, reduced amounts of syringyl units may be produced, presumably leading to an overall increase in the proportion of guaiacyl units incorporated into the lignin. Because angiosperm lignins are generally composed of guaiacyl-syringyl units, they are less condensed and easier to remove than the primarily guaiacyl lignin of gymnosperms (Chiang et al., 1988). The absence of syringyl lignins in gymnosperms has been partly attributed to the lower affinity of gymnosperm CAD to sinapyl aldehyde, leading to a lignin which is highly condensed. Similarly, in perennial ryegrass, if there is an increase in the proportion of guaiacyl units, lignin produced later in development may have compositional changes which decreases digestibility as much as, or more than, those effects seen for increases in lignin content.

**COMT enzyme activity**

An enzyme active in catalyzing the methylation of caffeic acid to ferulic acid, using S-adenosyl-L-methionine as a methyl donor, was measured in extracts from the leaves of perennial ryegrass. Under optimized conditions, this COMT enzyme had activity levels similar to COMT enzymes from other species such as bamboo (7.9 mU/g fr wt) (Shimada et al., 1973). The COMT from perennial ryegrass, with caffeic acid, had a maximum specific activity of 14.8 mU/g fr wt (Figure 4.14). The apparent $K_m$ value for caffeic acid was 75µM (Table 4.1) and is similar to $K_m$ values for COMT enzyme from other species such as Spinach (68 µM) (Poulton and Butt, 1975) and bamboo (50 µM) (Shimada et al., 1973).

COMT activity in perennial ryegrass leaves at different developmental stages was assayed with caffeic acid as the substrate (Figure 4.14). The activity of COMT reached a maximum on day 2 followed by a levelling of activity between days 4 and 7, before decreasing to a steady rate after day 14. The pattern of changes in the activity of COMT in developing perennial ryegrass is very similar to that seen with wheat seedling primary
cell walls (Lam et al., 1996a) and developing primary leaves of oat (Knogge and Weissenbock, 1986). Both of these tissues showed high levels of COMT activity early in leaf development (days 3 to 5) followed by a second, but lower, peak or levelling of activity between days 7 and 9.

The activity of COMT measured in the early stages of wheat leaf development shows a preference for caffeic acid as a substrate (Lam et al, 1996a). The substrate specificity of the wheat COMT enzyme changes, however, to one which uses 5-hydroxyferulic acid later in leaf development. This shift in substrate specificity has been attributed to the presence of early and late forms of the COMT enzyme (Lam et al., 1996a). It has been suggested that the early COMT in wheat may be involved in the synthesis of ferulic acid destined for esterification of cell wall arabinoxylans (Lam et al., 1996a) and that the late COMT is involved in lignin deposition as vascular tissue and secondary wall thickening begins.

These peaks of COMT activity with caffeic acid as a substrate in perennial ryegrass and the other species, may indicate time points in leaf development in which there are large increases in the production of ferulic acid. The production of large amounts of hydroxycinnamic acids, mainly p-coumaric and ferulic acids, early in plant development has previously been reported in forage grasses (Hartley, 1972). These phenolic acids have been implicated, through their structural role in cell walls, as having an influence on forage digestibility (Jung and Fahey, 1983). Ferulic acid may influence digestibility by the formation of ester-ether bridges between lignin and polysaccharides in cell walls, as has been suggested in wheat and phalaris (Lam et al., 1992; 1994). In these species, the bridges probably involve arabinoxylans linked to ferulic acid ester which are in turn joined to lignin monomers through ether linkages. The hydroxycinnamic acid ester bridges are suggested to be formed early in leaf development while the ether linkages form once lignin deposition has begun (Lam et al., 1996a). The observation of high levels of COMT activity early in perennial ryegrass leaf development suggests that these
hydroxycinnamic acid bridges, particularly those involving ferulic acid, may also be formed in this species (see Chapter 5 for further discussion).

The postulated early and late forms of the COMT enzyme in wheat have been indicated as being encoded by separate genes (Lam et al., 1996a). The complex Southern blot hybridization patterns reported in Chapter 3 using the \textit{LpeComtl} gene isolated from perennial ryegrass, may also indicate the presence of more than one gene encoding COMT. The level of \textit{comt} gene expression recorded in northern analysis, over the developmental time period, parallels COMT enzyme activity measured in perennial ryegrass. The gene is strongly expressed at the early time periods corresponding to high levels of enzyme activity, suggesting that this \textit{comt} gene may be a useful target for manipulation of COMT activity early in the perennial ryegrass leaf. No increase in transcript message due to the presence of a possible second form of COMT, was detected later in perennial ryegrass leaf development. The lack of cross-hybridization of the \textit{LpeComtl} cDNA to a possible second mRNA species may be due to lower levels of sequence similarity between these two sequences, although the complex cross-hybridization seen in Southern analyses suggests that this is not the case. It may be that in perennial ryegrass a single COMT enzyme is involved in both the production of ferulic acid destined for esterification and lignin deposition, and that both these processes occur at the same time in leaf development. Alternatively, it may be that two forms of COMT are present in perennial ryegrass and that both enzymes are active at the same time and are encoded by similar sized genes. Further analysis of COMT enzyme activity in perennial ryegrass using 5-hydroxyferulic acid as a substrate may further elucidate this hypothesis.

Earlier studies on the \textit{p}-coumaric acid and ferulic acid content of cell walls of Italian ryegrass showed highly significant correlations between hydroxycinnamic acid content and the digestibility of the cell walls (Hartley 1972). This suggests that manipulation of the COMT in perennial ryegrass, which is active early in leaf development, may be effective in increasing digestibility by modifying the formation of hydroxycinnamic acids and the
deposition of lignin in cell walls during leaf development. The relationship between hydroxycinnamic acid bridges, lignin content and digestibility in the developing perennial ryegrass leaf has been examined and is the subject of the following chapter.

The transcription of genes that encode lignin biosynthetic enzymes has been indicated as being important in the temporal as well as spatial control of lignification, and in regulating the quantity and composition of lignin (Campbell and Sederoff, 1996). Based on the key roles of COMT and CAD in supplying precursors for the synthesis of lignin monomers required in development, expression of the ryegrass COMT and CAD genes was presumed to be under early developmental regulation. The studies reported in this chapter support this view.

In conclusion, the developmental activities of both CAD and COMT in perennial ryegrass show similar patterns, in which early stages of leaf development are critical time periods of enzyme activity. For the COMT enzyme, activity levels peak early and then proceed to drop rapidly; this may indicate the presence of a COMT enzyme which is active in producing both precursors for the cross-linking of phenolic acids to hemicellulose as well as lignin deposition, or that two forms of COMT are active at the same time. The CAD enzyme activity showed a more complex interaction with the varying substrates tested. Not only did enzyme activity peak early in development, indicating that this was an important time period in lignin precursor formation, but changes in the ratio of substrate specificities during development also suggest that CAD may be a control point for lignin compositional changes that occur later in leaf development. The strong correlation between enzyme activities and the corresponding gene expression, suggest that CAD and COMT-encoding genes are developmentally regulated in this species, which may be important if manipulation of lignin biosynthesis during perennial ryegrass leaf development is to be successful.
CHAPTER 5
A DEVELOPMENTAL STUDY OF CHANGES IN DIGESTIBILITY, LIGNIN CONTENT AND HYDROXYCINNAMIC ACID COMPOSITION

5.1 INTRODUCTION
The digestibility of forage grass cell walls decreases as they mature and this is associated with changes in their chemical composition (Ballard et al., 1990). Analyses of the relationship between chemical composition and digestibility have led to the conclusion that lignin (Jung and Fahey, 1983) and phenolic acid (Hartley, 1972; Jung and Fahey, 1983) components of the wall are influential in determining digestibility. For ruminant animals, the covalent attachment of lignin to wall polysaccharides limits overall polysaccharide digestibility and leads to significant amounts of undigested fibre (Jung and Deetz, 1993). The biological function of the phenolic acids in lignin is still a matter of debate. However, in some grasses at least, there is increasing evidence that ferulic acid ester-ether bridges may, through a structural role, contribute to the resistance of the lignified walls of grasses to digestion by rumen microorganisms (Lam et al., 1992). In these species, the bridges probably involve ferulic acid ester linkages to arabinofurans that are in turn joined to lignin monomers through ether linkages. There is also increasing evidence from studies on the brown-midrib mutants of sorghum, pearl millet and maize (Lam et al., 1996b), that the chemical composition, rather than the quantity, of lignin may be more important in reducing digestibility. Some of the brown-midrib mutant plants, in addition to exhibiting lower lignin contents compared to normal lines, also have lower ratios of syringyl to guaiacyl units in their lignin (Kuc and Nelson, 1964; Chabbert et al., 1994a; 1994b) and/or the inclusion of 5-hydroxyguaiacyl units (Lapierre et al., 1988). The increased digestibility of these mutant plants tissues could be explained by lignin with lower degrees of polymerization. The lower content of ferulic acid bridges between lignin and cell wall polysaccharides in bmr mutants may also contribute to the elevated digestibilities of their stems.
As forage grasses mature and senesce, declines in digestibility are often associated with the reduction in water-soluble carbohydrates and protein constituents in the cell (Ballard et al., 1990). During early phases of plant growth, however, changes in dry matter and cell wall digestibility are less influenced by losses of water soluble constituents and more by physical and chemical changes within the cell wall. In this chapter, the changes in lignin content and hydroxycinnamic acid content and linkages were measured in the leaves of perennial ryegrass during their development. These components of the perennial ryegrass leaf were measured in order to determine what effect they have, if any, on total dry matter and cell wall digestibility of the developing perennial ryegrass leaf.

5.2 MATERIALS AND METHODS

5.2.1 Plant material

The developing third leaf of perennial ryegrass plants (cv. Yatsyn) was used for all of the following experiments. The leaf material was grown and collected as outlined in Chapter 4. Harvested material was frozen in liquid nitrogen and immediately freeze dried. The samples were lyophilized for 3 days, ground in a ball mill to pass a 1mm sieve and stored in air tight screw-capped vials in a desiccator.

5.2.2 Neutral Detergent Fibre

Neutral detergent fibre (NDF) content of leaf dry matter (DM) was measured using a modification of the method by Goering and Van Soest (1970). Triplicate samples (100 mg) of tissue were weighed into 200 mL Quickfit refluxing flasks containing 50 mL of neutral detergent solution. Neutral detergent solution was made in three parts. Solution A contained 186.1 g of EDTA and 68.1 g of sodium tetra-borate dissolved in 1.5 L of dH2O. Solution B contained 45.6 g of Na2HPO4 in 1 L of dH2O and Solution C, 300 g of sodium lauryl sulphate was dissolved in 1.5 L of dH2O. These solutions were combined with 6 L of dH2O in the following order: Solution C; 100 mL of 2-ethoxyethanol; Solution A and then Solution B. This final combined neutral detergent solution was adjusted to pH 6.9-7.1. These samples were heated under reflux conditions...
for 60 min and filtered through glass fibre filters (Whatman GF/A) using a Millipore vacuum filtration system. The residues were washed three times with 50 mL of hot water each time and the filters were then dried overnight at 100°C and re-weighed. The NDF was calculated as a percentage of the dry weight of the initial starting material. Neutral detergent solubles (NDS) were calculated as the percentage difference between the weight of the initial starting material and the final NDF weight.

5.2.3 \textit{In vitro} digestibility of dry matter and NDF

The micro pepsin-cellulase \textit{in vitro} digestibility of both total dry matter (DM) and neutral detergent fibre (NDF) was based on the methods of McLeod and Minson (1978; 1980). Triplicate amounts (100 mg) of each leaf sample were weighed into 50 mL Falcon tubes. An acid-pepsin solution was prepared by dissolving 24 mg of pepsin in 5 mL of 0.125 M HCl for each sample and heating gently without exceeding 50°C. To each tube, 5 mL of the acid-pepsin solution was added and the tube was incubated with shaking at 50°C for 72 h. After incubation, 300 µL of a 1 M sodium carbonate solution was added to adjust the pH to 4.5 - 4.7. A cellulase-buffer solution was prepared by adding 60 mg of cellulase (Amersham) to 10 mL of a 50 mM sodium acetate solution (pH 4.6), for each sample and heating gently without exceeding 50°C. This cellulase-buffer solution (10 mL) was added to the pH-adjusted samples and incubated for a further 48 h at 50°C. The samples were then filtered through Guech crucibles of porosity size 1 and the residue was washed with hot water. The residues were dried at 100°C overnight, cooled in a desiccator and weighed.

5.2.4 Lignin content

The lignin content in the leaves of perennial ryegrass was analysed using three methods, a thioglycolic acid method (Campbell & Ellis, 1992a), an acetyl bromide method (Iiyama and Wallis, 1990) and a Klason lignin method (Kaar \textit{et al.}, 1991) and these are described below.
**Thioglycolic Acid Method (TGA)**

Triplicate samples (30 mg) of the freeze-dried, ball-milled leaf tissue, described above, were extracted to remove soluble carbohydrates and proteins. The first extraction was in 10 mL toluene:ethanol (1:2) at 80°C for 2 h, followed by extraction with 10 mL of 100% ethanol for 2 h at 80°C and a final extraction in 10 mL of dH₂O for 2 h at 80°C. Samples were vacuum filtered and dried at 50°C overnight and re-weighed. Triplicate samples (10 mg) of extracted tissue were weighed into 2 mL screw-capped microcentrifuge tubes. After the addition of 750 µL of distilled water, 250 µL of concentrated HCl, and 100 µL of thioglycolic acid, the contents were mixed and incubated at 80°C for 3 h. The tubes were centrifuged at 14,000 rpm for 5 min and the supernatant was discarded. The pellet was resuspended in 1 mL of dH₂O, vortexed and centrifuged at 14,000 rpm. The pellet was resuspended in 1 mL of 1M NaOH and agitated gently overnight at 25°C in the dark. The samples were centrifuged for 5 min at 14,000 rpm and 200 µL of concentrated HCl was added to the supernatant. The tubes were incubated on ice for 1 h, centrifuged as previously described and the pellet was resuspended in 1 mL of 1M NaOH. The absorbance of a 50-fold dilution in 1M NaOH was measured at 280 nm. The concentration of TGA-derived material was expressed as the absorbance at 280 nm per gram of dry matter or NDF.

**Acetyl Bromide (AcBr)**

Triplicate samples (10 mg) of neutral detergent fibre (NDF) were placed in 15 mL glass reaction vials with 3 mL of a solution containing 23% (w/w) acetyl bromide (AcBr), 73% glacial acetic acid and 4% perchloric acid. The bottles were sealed with a teflon-coated silicone cap and placed in an oven at 70°C for 90 min. The bottles were shaken at 10 min intervals to promote dissolution of the sample. After digestion, the mixture was cooled and 100 µL was transferred to a clean glass reaction vial containing 10 mL of 0.6M NaOH in 70% glacial acetic acid solution. The lignin content of the samples was determined by measuring the absorbance at 280nm and using the specific absorption coefficient for lignins of 20 g L⁻¹ cm⁻¹ (Iiyama and Wallis, 1990).
Klason Lignin (KL)

Triplicate samples, consisting of 100 mg of NDF suspended in 1 mL of 72% H$_2$SO$_4$ in 50 mL screw-capped Falcon tubes, were incubated in a water bath at 30°C for 1 h. The initial hydrolysis was followed by dilution to 4% H$_2$SO$_4$ by the addition of 17 mL dH$_2$O, and autoclaving at 121°C for 1 h. The acid hydrolysis mixture was passed through a glass-fibre filter (Whatman grade GF/C, 1.6µm particle retention) in a Millipore vacuum funnel. The residue was washed with a 20 mL volume of hot distilled water and the filter was oven dried at 105°C overnight, cooled in a desiccator, weighed and corrected for ash content to give acid insoluble Klason lignin.

5.2.5 Hydroxycinnamic acid measurements

The concentrations of esterified and total (esterified plus etherified) hydroxycinnamic acids released from the perennial ryegrass leaf samples were determined following the methods of Lam et al. (1990) and Iiyama et al. (1990). The starting material was freeze dried, ball-milled leaf tissue prepared as described previously. The samples were pre-extracted three times at 80°C, for 1 h each time, with 10 mL of 80% (v/v) aqueous ethanol to remove water-soluble carbohydrates, proteins, and free hydroxycinnamic acids. The residues were dried in a vacuum oven overnight, at 55°C, and re-weighed. To saponify the esterified p-coumaric acid (p-CA) and ferulic acid (FA), the ethanol-extracted tissue was suspended in 4 mL of 1 M NaOH in 12 mL screw-capped glass tubes. After flushing with nitrogen gas, the tubes were incubated with shaking at room temperature for 16 h. The hydrolysate was removed with a Pasteur pipette, and the residue was washed twice with 1 mL of distilled water. The combined extract and washings were acidified with 6 M HCl to pH 1 and 25 µL of a 10 mM solution of caffeic acid (Sigma) was added as an internal standard. The reaction mixture was extracted twice with 20 mL of dichloromethane, and twice with 20 mL of diethyl ether. The combined organic phases were extracted with water (30 mL) to remove the hydrochloric acid. The organic phases were dried with anhydrous sodium sulphate and evaporated to dryness on
a rotary evaporator. The samples were resuspended in 1 mL of diethyl ether, transferred to small GC septum vials and evaporated to dryness under nitrogen gas. The samples were then derivatized with 100 µL of \(N,O\)-bis(trimethylsilyl) acetamide at 90°C for 10 min.

The concentration of total (ester and ether-linked) \(p\)-CA and FA were determined by the alkali treatment of ethanol-extracted material at high temperature. Approximately 100 mg of tissue was added to 5 mL of 4 M NaOH in a 10 mL stainless steel pressure vessel, and incubated in an oil bath at 170°C for 2 h. The contents were collected, and the vessel was rinsed three times with 1 mL of 0.2 M NaOH. These samples were extracted and processed as for the room temperature saponified extracts described above. The trimethylsilyl-derivatized hydroxycinnamic acids were analyzed by gas-liquid chromatography (GLC) on a wall-coated open-tubular bonded phase fused silica capillary column (50 m x 0.25 mm internal diameter) (Gasakuro Kogyo Inc., Tokyo) in a gas chromatograph (Perkin Elmer) equipped with a flame ionization detector. The GLC conditions applied were: injection temperature, 280°C; oven temperature, 180°C for 5 min, then programmed temperature increase at 5°C/min to 280°C, held for 5 min. The linear flow rate of helium through the column was 30 mL/min. The yields of hydroxycinnamic acids were determined by comparisons of internal standard peaks and survival factors of 0.32 for \(p\)-CA and 0.22 for FA as described in Lam et al. (1990).

5.3 RESULTS

5.3.1 Leaf growth

The stages of leaf development measured during this study spanned early to mature vegetative growth. The increase in length of the third leaf of perennial ryegrass in relation to time is shown in Figure 5.1. These results, previously presented in Chapter 4, are presented again to allow comparisons with changes in lignin and hydroxycinnamic acid concentrations and dry matter and cell wall digestibility during leaf development. The
third leaf elongated and expanded from a length of 10 mm to a maximum length of 220 mm in 18 days. The first 7-9 days was a period of rapid leaf expansion and elongation with rates of leaf growth increasing at approximately 20 mm per day. The rate of increase from day 9 to day 18 was significantly lower at only 3 mm per day after which the leaf was considered fully expanded and no changes in leaf length were recorded.

![Graph showing leaf length vs time](image)

**Figure 5.1** Increase in leaf length during the development of the third leaf of a perennial ryegrass plant. Mean values from 3 replicates are plotted and where no error bar is present, errors were smaller than the size of the symbol.

**5.3.2 In vitro dry matter and NDF digestibility**

The neutral detergent soluble fraction (NDS), which contains mainly water-soluble carbohydrates and protein, was the major component of the leaf blade at all times, but decreased as a proportion of the leaf dry matter from 67% to 62% during the 28-day developmental period analysed (Figure 5.2). During this same time period, the proportion of NDF of the leaf tissue, which is mainly composed of cell wall materials, increased as the leaf matured. This increase in the proportion of NDF is constant during the first 14 days of development, after which the increase in NDF level remains constant.
The change in digestibility of total dry matter (DM) and of the cell wall (NDF) was analysed over this 28-day period of leaf development. The *in vitro* DM digestibility and the NDF digestibility decreased steadily following a logarithmic pattern ($R^2=0.9$) from early in leaf development (Figure 5.3). A gradual 4% reduction in *in vitro* DM digestibility was recorded over the 28 days of leaf development, while the *in vitro* digestibility of the cell wall showed a 10% reduction over the same 28-day period. The initial rate of decline in digestibility of the cell wall was greater during the first 7-9 days of development, with a gradual slowing of the rate of decline after this time period.

Calculations, based on the assumption that dry matter digestibility equals the sum of the digestibility of the cell wall (NDF) and the digestibility of the soluble cell contents (NDS), showed that the predicted *in vitro* digestibility of the NDS component remained between 99 - 100% over the 28-day developmental period (data not shown). These calculations also predicted that 71% of the decrease in dry matter digestibility was due to a decrease in cell wall digestibility while the remaining 29% was due to a loss in the concentration of NDS (data not shown).
5.3.3 Measurement of lignin content in the developing leaf

To account for the limitations of various lignin analytical procedures, the changes in lignin content during perennial ryegrass leaf development (Figure 5.4) were measured based on three commonly used methods; acetyl bromide (AcBr) (Iiyama and Wallis, 1990), Klason (KL) (Kaar et al., 1991) and thioglycolic acid (TGA) (Campbell and Ellis, 1992a). Lignin was not detected in leaf tissue, by either the KL or AcBr methods, in the first 3-4 days of leaf development (Figure 5.4A). Material absorbing at 280nm was detected at all stages of development using the TGA method (Figure 5.4B). Between days 4 and 7, the lignin concentration in the leaf tissue increased rapidly for all lignin determination methods. Between days 7 and 28, the rate of deposition of lignin in the leaf decreased significantly and almost negligible increases in lignin content were recorded from day 18 onwards.
Figure 5.4 The increase in lignin content of the third leaf of perennial ryegrass during development. A as measured by the Klason lignin (\(\bullet\)), acetyl bromide (\(\triangle\)) methods and B as measured by the thioglycolic acid method and expressed as absorbance per gram of total dry matter (\(\square\)) or cell wall (\(\triangledown\)). See Addendum.
5.3.4 Ester- and ether-linked hydroxycinnamic acids

Figure 5.5 shows representative gas chromatograms of the resolution of ester-linked hydroxycinnamic acids products liberated from the room temperature saponification of leaf tissue (Figure 5.5A) and the total (ester- and ether-linked) hydroxycinnamic acids liberated from the high temperature saponification procedure (Figure 5.5B). Three major peaks (labelled 1-3) were detected in the room temperature saponified samples (Figure 5.5A) and their corresponding retention times indicated that peak 1 represented p-coumaric acid (p-CA), peak 2 corresponded to the 3,4-dimethoxycinnamic acid internal standard (IS), and peak 3 represented ferulic acid (FA). A number of major peaks were detected in the high-temperature saponification samples (Figure 5.5B). Peaks 1, 2 and 4 corresponded to p-CA, IS and FA, respectively, while peak 2 corresponded to liberated coniferyl alcohol (CA) and peak 5 represented diferulic acid (DFA). The liberation of more phenolic compounds, and hence more complex GC peaks, is characteristic of high-temperature saponification of leaf tissue (Lam et al., 1990).

Gas chromatograms were produced for leaf tissue at each stage of development and the amounts of ester- and ether-linked hydroxycinnamic acids as a proportion of DM were calculated. The total amount of p-CA and FA found in the developing leaves of perennial ryegrass is shown in Figure 5.6. For each stage of development, the total amount of covalently bound FA was always significantly higher than total p-CA (Figure 5.6). The changes in the proportion of these hydroxycinnamic acids that are ester- or ether-linked to cell wall polysaccharides and lignin were also analysed and the results are shown in Figure 5.7. The proportion of FA and p-CA involved in ester-linkages to cell wall polysaccharides was highest in the early stages of development (day 3), after which it declined until a steady level was reached around days 7-9. The amount of FA and p-CA forming ether-linkages increased rapidly between days 3 and 7 and only gradual increases in the proportion of FA and p-CA involved in ether-linkages were recorded after day 7.
### Figure 5.5

Representative gas chromatograms for room temperature (A) and high temperature (B) saponification of leaf tissue from perennial ryegrass. Peaks identify *p*-coumaric acid (*p*-CA), ferulic (FA), internal standard (IS), coniferyl alcohol (CA), and diferulic acid (DFA).
Figure 5.6 Content of total amounts of bound ferulic acid (-■-) and p-coumaric acid (-○-) from the developing third leaf of perennial ryegrass. Mean values from 3 replicates are plotted and where no error bar is present errors were smaller than the symbol.

Figure 5.7 Content of ester- and ether-linked hydroxycinnamic acids in the third leaf of developing perennial ryegrass. Ester-linked ferulic acid (-■-); ether-linked ferulic acid (-□-); ester-linked p-coumaric acid (-○-) and ether-linked p-coumaric acid (-○-). Mean values are plotted and where no error bar is present, errors were smaller than the symbol. See Addendum.
5.4 DISCUSSION

In this present study, lignin content and the presence of hydroxycinnamic acid ester- and ether-linkages presumed to occur between lignin and cell wall polysaccharides, were analysed in developing perennial ryegrass in order to determine whether these factors are influential in affecting leaf digestibility. The *in vitro* digestibility of leaves of perennial ryegrass decreased at a steady rate during early development, slowing once the leaf reached full expansion (Figure 5.3). The 4% measured drop in dry matter digestibility may be caused by the interaction of losses of water-soluble cell components (NDS), decreases in the digestibility of the NDS, increases in the amount of cell wall (NDF) and decreases in the NDF digestibility. In perennial ryegrass, the decrease in digestibility was partially due to changes in the proportion of NDS, however, decreases in the concentration of NDS at these early stages of development were small (Figure 5.2) and calculations showed that the NDS remained almost 100% digestible during this time period. Calculations also indicated that the decrease in NDS concentration only contributes to 29% of the total decrease in dry matter digestibility during development of the leaf. The remaining 71% of the loss in digestibility can be attributed to changes in the cell wall, indicating that factors influencing cell wall digestibility are crucial in determining the overall digestibility of leaf dry matter.

The *in vitro* digestibility of the cell wall of perennial ryegrass leaves, decreased by 10% during the 28-day developmental period, with the greatest rate of decline measured in the first 7 days of leaf growth (Figure 5.3). Declines in digestibility of forage grasses as they reach anthesis (Terry and Tilley, 1964; Pritchard *et al.*, 1963) or as they senesce (Ballard *et al.*, 1990; Armstrong *et al.*, 1992) have been well studied and are principally associated with dramatic reductions in the content of water-soluble carbohydrate and protein constituents in the cell. This results in a reduced digestibility due to the increased proportion of neutral detergent fibre (NDF) in the leaf. Less is known about the subtle changes that occur in the cell wall that affect its digestibility early in leaf development,
well before the loss of NDS becomes the critical factor. Although the decreases in digestibility of perennial ryegrass during early development are more subtle compared to the dramatic changes seen as the plant matures and senesces (Ballard et al., 1990), small changes in NDF digestibility have been suggested to be important in relation to whole plant digestibility (Ballard et al., 1990). Animal feeding experiments have shown that small increases of only 1-3% in perennial ryegrass digestibility improved the live weight gain of lambs by up to 22% (Walters et al., 1985).

It is important to note that factors affecting this cell wall digestibility are those which are determined early in development. Increases in the physical and chemical associations between cell wall polymers, especially between polysaccharides and lignin, may contribute to the observed changes in NDF digestibility and hence, in vitro dry matter digestibility in perennial ryegrass leaves during the early phases of development. This is consistent with observed correlations between digestibility, lignin content and high ratios of pCA and FA in other species (Hartley, 1972; Burritt et al., 1984). These characteristics of the perennial ryegrass leaf were examined in order to investigate their possible roles in altering cell wall digestibility in this species.

In relation to lignin content, a few grasses and cereals have been well studied and show substantial differences ranging from 1.2% in canary grass (Burritt et al., 1984) to 26% for bamboo stems (Higuchi et al., 1967). After a 28-day period of development, perennial ryegrass had a final lignin concentration between 3.2 and 4.2% of dry matter, depending on the analytical procedure used for quantification (Figure 5.4). The methods available for determining lignin content in plants fall into two basic categories: 1) analytical procedures that remove all of the cell wall constituents except for lignin, and 2) oxidative procedures that liberate the lignin polymer from the cell wall matrix (Hatfield et al., 1994). The Klason lignin procedure (KL) results in an insoluble lignin residue by using sulphuric acid for hydrolysis of the cell wall polysaccharides after removal of proteins and water-soluble carbohydrates. The acetyl bromide (AcBr) and thioglycolic
acid (TGA) methods result in solubilization of the lignin in the sample, and content can be estimated by UV spectrophotometry. The AcBr values measured in perennial rye grass were comparable with the KL values (Figure 5.5A) but were slightly higher, possibly due to contaminating hydroxycinnamic acids. The TGA method, while showing a similar pattern of lignin accumulation as that determined by the AcBr and KL methods, not only estimates lignin but also TGA-derivable material (Figure 5.4B), including hydroxycinnamic acids. Absorbance measurements may not, however, detect the presence of highly condensed lignin due to incomplete solubilization. All three lignin measurement methods have their limitations (Hatfield et al., 1994) and these limitations must be carefully considered when drawing correlations between lignin content, hydroxycinnamic acid bridging and their effects on digestibility.

All three lignin measurement methods revealed similar profiles of lignin accumulation and, given the limitations of each method, this suggests that there is very little lignification of leaf tissue until day 3 to 4, after which lignin deposition occurs rapidly (until day 7-8), followed by a significantly slower rate of deposition (until day 17-18), after which there was only a negligible increase in lignin deposition (Figure 5.4). These changes in the rate of lignin deposition correlate well with the high rates of leaf growth early in development, followed by a slowing of elongation and expansion before leaf growth cessation. Lignification is generally regarded as a relatively late event in cell wall differentiation, temporally correlated with the appearance of secondary wall layers in aging tissues such as xylem or sclerenchyma (Iiyama et al., 1993).

This view that lignin is restricted to tissues which have ceased to grow is in contrast to the increase in lignification that is measured in expanding and elongating cell walls of developing rye grass leaves. This phenomenon has also been documented in other developing tissues such as maize coleoptiles (Musel et al., 1997). These increases in lignin content correlated well with the measured decreases in digestibility because the digestibility of the leaf declined over the same time period when lignin content increased.
(Figure 5.3 and Figure 5.4). These results, however, do not indicate to what extent the changes in digestibility of the cell walls are due to changes in the proportion of lignin content \textit{per se}. Chemical associations and physical inter-relationships of lignin and other phenolic materials are also thought to be important in affecting digestibility (Lam \textit{et al.}, 1993) and these characteristics of leaf tissue were also analysed.

The leaves of perennial ryegrass and similarly, the primary cell walls of other grasses have been shown to contain significant amounts of phenolic acids such as ferulic acid (FA) and \textit{p}-coumaric acid (\textit{p}-CA) (Figure 5.7) (Harris and Hartley, 1976). The ferulic acid in perennial ryegrass, like that in other grasses, presumably forms ester-links to cell wall matrix polysaccharides (Harris and Hartley, 1976). A significant proportion of the ferulic acid formed in perennial ryegrass leaf development is also in ether-bound forms (Figure 5.7), and probably participates in ester and ether bridges between lignin and polysaccharides. Ester-linked ferulic acid and polysaccharides and subsequent ether-linkages to lignin have also been reported in the cell walls of wheat and \textit{Phalaris} stem internodes (Lam \textit{et al.}, 1992; Lam \textit{et al.}, 1994).

The correlative increases of ferulic acid and lignin content are consistent with the hypothesis that ferulic acid ester-linked to cell wall polysaccharides provides points of growth for the lignin polymer (Lam \textit{et al.}, 1992), and that the leaf cell wall may contain carbohydrate and phenolic materials which are cross-linked by ferulic acid bridges (Iiyama \textit{et al.}, 1990; Lam \textit{et al.}, 1990). The presence of ester-ether bridges during early leaf growth also correlated well with the steady decrease in the \textit{in vitro} dry matter and NDF digestibility recorded during the 28 days of perennial ryegrass leaf development (Figure 5.3). The initial decreases in digestibility early in development may, therefore, be due to increases in lignin content and the early formation of the hydroxycinnamic acid ester-linkages to polysaccharides. The continued gradual decrease in digestibility may also be due to ferulic acid ether-links between the cell wall polysaccharides and recently deposited lignin.
The higher proportion of FA ester and ether linkages in perennial ryegrass compared to those involving p-CA, suggests that FA is the major hydroxycinnamic acid associated with cell wall polysaccharides and lignin in this species. FA is the major hydroxycinnamic acid associated with arabinoxylans in the cell walls of wheat tissues (Lam et al., 1993), and it is likely that the ester-linked ferulic acid in perennial ryegrass also arises from the FA-arabinoxylan complex that is deposited prior to secondary cell wall formation and lignin deposition. These FA-linkages have been suggested to be the precursors of the ether-linked FA involved in polysaccharide-lignin ester-ether bridges in wheat (Lam et al., 1992). Ether linkages of both FA and p-CA increased after lignin deposition began (compare Figures 5.4 and 5.7). A parallel increase in lignin content and p-CA and FA ether-linkages has also been observed in wheat coleoptile cell walls (Harris and Hartley, 1980). Similar results were also seen in the primary cell walls of wheat root, shoot and leaf tissue (Lam et al., 1996a).

From the results presented in this chapter it can be concluded that there are a number of phases during perennial ryegrass leaf development which are critical in terms of biochemical and physiological change. The first seven days in perennial ryegrass leaf development are periods where rapid increases in leaf growth, lignin deposition, and ester and ether linked hydroxycinnamic acids are occurring and these factors are presumably interacting and contributing to the decline in cell wall and hence, dry matter, digestibility. The second phase of development is characterized by a slower rate of leaf expansion and elongation, lignin deposition, formation of ester and ether linkages between cell wall polysaccharides and lignin, and decline in digestibility. This present investigation provides evidence that lignin is deposited early in ryegrass leaf development and that interactions between this lignin and hydroxycinnamic acids may be influencing the cell wall digestibility.
Perennial ryegrass is an important forage species in temperate dairy pastures of Australia. Under optimal growing conditions, it provides large amounts of nutritious, high-energy feed. The nutritive value of perennial ryegrass, however, decreases as the plant matures due to cellular changes involving water-soluble carbohydrate content and cell wall composition. Under certain growing conditions, structural carbohydrates in the cell wall are the major source of energy for ruminant animals therefore the developmental stage and the components which influence cell wall digestibility are important factors for consideration in any approach to genetically manipulate pasture plants, such as perennial ryegrass, for improved digestibility. Lignification of the cell wall is known to be associated with decreases in cell wall digestibility, particularly at times when pasture nutritive value is low. Enzymes which are specifically involved in lignin biosynthesis are therefore obvious targets for genetic manipulation aimed at improving cell wall, and thereby, pasture digestibility.

In the course of this study, cDNAs encoding cinnamyl alcohol dehydrogenase (CAD) and caffeic acid O-methyltransferase (COMT) enzymes involved in lignin biosynthesis were isolated from perennial ryegrass. These cDNAs were characterized and their use as a means to manipulate lignin biosynthesis, and hence dry matter digestibility, was investigated by analyzing changes in CAD and COMT enzyme activities, lignification and digestibility of the developing perennial ryegrass leaf.

In Chapter 3, the cDNA designated \textit{LpeComt1} was identified as encoding a COMT based on the high homology of its predicted amino acid sequence with those of other COMTs from plants, and its lower amino acid sequence homology with the sequences of metabolically-unrelated OMTs. Genes encoding COMT have been isolated from a number of dicotyledonous species, however, only one other COMT-encoding gene has
been identified from a monocotyledonous species, namely maize. The \textit{LpeComtl} cDNA from perennial ryegrass is the first reported stem-abundantly expressed, possibly lignin-specific COMT-encoding gene from a monocotyledonous species. The high levels of homology between the ryegrass COMT and those isolated from other species, particularly maize, are due not only to the conserved domains which are important in the general structure and function of methyltransferases (Ingrosso \textit{et al.}, 1989), but also to the presence of regions which are specific to methyltransferases involved in lignin biosynthesis which use caffeic acid as a substrate (McIntyre \textit{et al.}, 1995).

The identity of this \textit{LpeComtl} cDNA was confirmed by the expression of a functional COMT enzyme in \textit{E. coli} extracts (Chapter 3). The translated protein catalyzed the highly-specific meta-\textit{O}-methylation of caffeic acid to produce ferulic acid. The inability of this enzyme to catalyze non-specific para-\textit{O}-methylation of caffeic acid to form 3,4-dimethoxycinnamic acid is further evidence that the \textit{LpeComtl} cDNA encodes an enzyme which is directly involved in the methylation of caffeic acid to ferulic acid in the lignin biosynthetic pathway. The non-specific para-\textit{O}-methylation of caffeic acid seen with bacterial extracts containing a functional COMT protein from the maize MC1 clone (Collazo \textit{et al.}, 1992), suggests that this root-preferentially expressed maize enzyme may be involved in the methylation of hydroxycinnamic acids involved in another branch of the phenylpropanoid pathway as well as in lignin biosynthesis. Further work may include the examination of the \textit{LpeComtl}-encoded enzyme in a bacterial system, with 5-hydroxyferulic acid as the substrate, to determine if the encoded enzyme is bi-functional, or if it is a member of a COMT family in which separate genes encode enzymes with different substrate specificities.

The complex Southern blot hybridization patterns observed using \textit{LpeComtl} as a probe under stringent conditions suggest the presence of more than one \textit{comt} gene in perennial ryegrass (Chapter 3). The variation in plaque hybridization intensity in the initial library screening also suggests the presence of more than one form of COMT-encoding gene in
this species. Future work may involve isolating and characterizing other cDNAs in order to identify alternative COMT-encoding genes.

The presence of at least two comt genes, which are expressed at different developmental time, and each of which encode a COMT enzyme with a different substrate affinity, has been suggested from studies of COMT enzyme activity in developing wheat leaf tissue (Lam et al., 1996a). These studies suggest that there is an early and a late form of the enzyme, which use either caffeic acid or 5-hydroxyferulic acid, respectively, as a substrate. These early and late forms of COMT have also been postulated to have different roles in leaf development, either in ferulic acid synthesis for ester-linkages to cell walls or in lignification, respectively (Lam et al., 1996a). In perennial ryegrass, COMT activity with caffeic acid as a substrate, is high during early leaf development. This suggests that the enzyme is producing high amounts of ferulic acid which may be channelled through the lignin biosynthetic pathway towards the production of lignin precursors or that it may act as the nucleation sites for ester- and ether-linkages between cell wall polysaccharides and lignin.

The decrease in COMT activity following this early time period may indicate that as the leaf develops less enzyme is being translated, which suggests that expression of the gene may be under developmental regulation. Although enzyme activity with 5-hydroxyferulic acid was not measured, due to the difficulty in obtaining the substrate, the direct correlation between the expression of the LpeComt1 gene and COMT activity does suggest that there are no later peaks of enzyme activity, as would possibly be expected if a second (later) form of the enzyme were expressed (Chapter 4). The northern blot hybridization data only indicate high levels of gene expression early in development suggesting that there is only one enzyme which is involved in the synthesis of ferulic acid for ester-links and for lignification. A COMT utilizing 5-hydroxyferulic acid which is active later in development may not be detected if its encoding gene has low sequence similarity to the LpeComt1 probe. Alternatively, the northern analysis may also suggest
that, if there are two *comt* genes in perennial ryegrass, as the Southern blot data suggests, then they have the same transcript size and encode COMT isoforms which are active at the same time in development, concurrently producing ferulic acid for esterification and for lignin biosynthesis. This is in direct contrast to the results obtained by Lam *et al.* (1996a) where ferulic acid ester-bridges and lignification are suggested to occur as sequential events.

If, in perennial ryegrass, only a single caffeic-acid specific COMT isoform is present then it may be possible that caffeic acid, and some of the ferulic acid synthesized by the single COMT form, are converted by 4CL to the corresponding CoA thioesters, which are in turn methylated by an alternative pathway involving an enzyme such as CCoAOMT (see Introduction). This could explain for how perennial ryegrass, containing only a single form of COMT responsible for synthesizing ferulic acid involved in ester bridging, and no later (5-hydroxyferulic acid-specific) form of COMT involved in lignification, would have the ability to form bridges and synthesize the guaiacyl and syringyl precursors of lignin at the same time in development.

The results of lignin analyses in perennial ryegrass indicate that both the ferulic acid ester- and ether-bridging and lignin deposition are occurring at the same time in leaf development (Chapter 5). This provides support for the hypothesis that, in perennial ryegrass, either a single form of the enzyme is involved in both roles, or that two forms of the enzyme are present and active at the same time in development. Further analysis of this hypothesis will require assaying COMT enzyme activity, using 5-hydroxyferulic acid as a substrate to determine the times during development of the leaf in which activity with this substrate is highest. If activity with 5-hydroxyferulic acid is detected early in development, this would be supportive of the hypothesis that either a single form, or more than one form, are active early in development. A northern blot analysis, using *LpeComt1* as a probe, on developing wheat leaf tissue, could be done in order to determine whether the transcript for the later form of COMT is detectible, through cross
hybridization. If a second *comt* transcript is detected, this would provide further support for the validity of the perennial ryegrass developmental northern blot data in suggesting the presence of a single COMT form. Alternatively, if the second form is detected in wheat and is of the same transcript size as the early form, then the hypothesis that the perennial ryegrass northern blots are detecting a second form expressed early in development would also be supported.

The cDNA designated *LpeCad1* was initially identified as encoding a CAD enzyme on the basis of the high homology of its predicted amino acid sequence with those of other CAD sequences from plants and its lower homology to the amino acid sequences of other alcohol dehydrogenases (Chapter 2). Genes encoding CAD have been isolated from a number of dicotyledonous species, however, *LpeCad1* is the first full-length CAD-encoding cDNA isolated from a monocotyledonous species. The similarity of the predicted amino acid sequence encoded by *LpeCad1* to those of other plant CADs is due to the presence of important structural and functional domains, present in all members of the alcohol dehydrogenase family, as well as to unique motifs which are highly conserved among CAD enzymes with specific cinnamyl alcohol substrate specificities and NADP co-factor dependence.

High-level expression of the *LpeCad1* gene in perennial ryegrass tissue which is highly lignified, such as sheath and stem, suggests that this cDNA encodes a CAD which may be involved in lignification. The lower levels of expression of *LpeCad1* in less-lignified tissues suggest that CAD enzymes may have different roles in different tissues, or that *LpeCad1* is cross-hybridizing to another form of *cad* gene which is expressed in these tissues. The Southern blot hybridization analysis using the *LpeCad1* as a probe indicates the presence of two genes (Chapter 2). In wheat, the role of multiple forms of CAD in altering the monomer composition of lignin synthesized in response to wounding has been reported (Mitchell *et al.*, 1994), and it has been suggested that the changes in CAD activity may be due to the regulation of separate *cad* genes (Boudet *et al.*, 1995). This
hypotheses could be examined in perennial ryegrass by attempting to isolate these separate *cad* genes from different tissues at different developmental time periods or under different environmental conditions.

In perennial ryegrass, CAD enzyme assays show a peak activity in the early stages of leaf development (Chapter 4). Northern blot analysis using the *LpeCad1* cDNA as a probe, shows high expression early in development, which is consistent with the peak in enzyme activity. The increased CAD enzyme activity with coniferyl aldehyde, compared to sinapyl aldehyde as a substrate, and a change in the ratio of CAD enzyme activity with sinapyl aldehyde later in development, may be due to the presence of another form of CAD. The enzyme assay suggests that there is a preferential production of guaiacyl units, based on the fact that the CAD enzyme is more active with coniferyl aldehyde, which is the precursor of guaiacyl lignin units. The highly-condensed nature of lignin in a number of plant species has been attributed in part to the presence of high amounts of guaiacyl units (Chiang *et al.*, 1988). The guaiacyl lignin units are believed to form more covalent attachments to the cell wall polysaccharides than do syringyl units, thereby decreasing digestibility of the cell wall by rumen microorganisms.

Future work with this species should include a more detailed analysis of lignin compositional changes during leaf development related to CAD enzyme substrate specificity. Expression of the *LpeCad1* cDNA in *E. coli* may reveal whether the encoded CAD enzyme has broad substrate specificity or if it is a single form of CAD with a strict substrate specificity. Comparison of the perennial ryegrass CAD sequence with CAD sequences isolated from other species in which a single form of CAD has been shown to have equal affinity with all three aldehyde substrates, may also indicate whether *LpeCad1* is likely to be a single gene, or a member of a small gene family. In addition it would be worthwhile to look for other perennial ryegrass cDNAs which might encode CAD enzymes with different substrate specificities, or with different developmental patterns of expression. The outcomes from these studies may allow a more detailed investigation
into CAD enzyme activity, and its relationship to lignification and dry matter digestibility during the development of perennial ryegrass leaves.

The lignin content of the leaf, and its effect on the digestibility of cell wall polysaccharides, become increasingly important during later times in the pasture growing season when the cell wall component of fodder provides most of the energy resources for ruminant animals. Developmental studies undertaken in this thesis indicate that lignin in perennial ryegrass is laid down early in development and that the formation of ferulic acid ester- and ether-linkages between lignin and cell wall polysaccharides also occurs in these early stages of development (Chapter 5). The effect that increased lignin content and cross-linking has on the digestibility of perennial ryegrass leaves was seen by the steady decline of in vitro dry matter and cell wall digestibility during the earlier phases of leaf growth (Chapter 5). At this time in leaf development the losses of NDS were minimal, and therefore the decline in perennial ryegrass digestibility was more likely to be correlated with increases in lignin content and cross-linking.

Increases in CAD and COMT enzyme activity have been correlated with the observed increases in lignin and cross-linking. Gene expression has also been related to enzyme activity (Chapter 4) and the cDNAs isolated from perennial ryegrass show characteristic developmental regulation. This provides evidence that the down-regulation of CAD, and in particular COMT, in perennial ryegrass using the corresponding LpeCad1 and LpeComt1 genes may be effective in altering a number of the ways in which lignin effects cell wall digestibility in this species. These effects of lignin content and cross-linking on the digestibility of perennial ryegrass may also be associated with compositional changes in the lignin as it is deposited and future work may need to include an in-depth analysis of the composition of lignin in the perennial ryegrass plant as it develops.

Variable responses, in terms of alterations in lignin content and composition and the resultant effect on wood pulpability or cell wall digestibility, have been seen in transgenic
or mutant plants in which the activity of lignin biosynthetic enzymes, such as CAD and COMT, have been decreased (see Introduction). In the case of transgenic plants, constitutive gene promoters are generally used to drive the antisense and sense constructs. Lignin is known to play a role in plant defence and in the mechanical strength of tissues; therefore, constitutive alterations in the activities of lignin biosynthetic enzymes may not be ideal. It may be more beneficial to target the changes in lignification to specific cell types in which lignin accumulation causes the greatest problems. In perennial ryegrass, as in other species, particular cell types such as xylem, become more lignified than others. The isolation of tissue- and cell-specific promoters will allow the modification of lignin deposition to be directed to the appropriate tissues. Promoters which determine the tissue specific and developmental regulation of LpeComt1 and LpeCad1 could obviously be useful in the finer control of alterations in lignin biosynthesis in perennial ryegrass.

A reduction in lignin content, or a change in its composition, may be beneficial in changing the carbon status of the plant. Studies on transgenic perennial ryegrass, and other forage species, may be useful in determining if and how an increase in carbon channelled through other biosynthetic pathways will affect the plant. Investigations on whether excess carbon will affect the carbohydrate partitioning of the plant may be important in determining whether changes in the digestibility of transgenic forages is not only due to decreased lignin content, composition or cross-linking but also to a higher proportion of more digestible carbohydrates.

Perennial ryegrass is one of Australia’s most important temperate pasture grasses and the potential increases in its digestibility that may be achieved through genetically modifying the lignin content, composition or cross-linking will be beneficial in terms of animal production and the maintenance of a low cost pasture-based production system. The research in this thesis has led to the major conclusion that in perennial ryegrass, alterations in both lignin content and cross-linking early in development would be a major advantage in attempts to improve pasture digestibility, which may have larger benefits
later in the growing season when the grazing animal's main energy source is cell wall polysaccharides. Results from this thesis indicate that antisense, sense or ribozyme constructs of the \textit{LpeCad1} or \textit{LpeComt1} genes may be useful tools for such strategies, now that tissue culture and transformation procedures for this monocotyledonous species \textit{(G. Spangenberg, personal communication)} have been developed to alter lignin composition, content or cross-linking and thereby the digestibility of this important pasture species.
REFERENCES


