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# Improved forage digestibility of tall fescue (*Festuca arundinacea*) by transgenic down-regulation of cinnamyl alcohol dehydrogenase

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## Summary

Lignification of cell walls during plant development has been identified as the major factor limiting forage digestibility and concomitantly animal productivity. cDNA sequences encoding a key lignin biosynthetic enzyme, cinnamyl alcohol dehydrogenase (CAD), were cloned from the widely grown monocotyledonous forage species tall fescue (*Festuca arundinacea* Schreb.). Recombinant tall fescue CAD expressed in *E. coli* exhibited the highest $V_{max}/K_m$ values when coniferaldehyde and sinapaldehyde were used as substrates. Transgenic tall fescue plants carrying either sense or antisense *CAD* gene constructs were obtained by microprojectile bombardment of single genotype-derived embryogenic suspension cells. Severely reduced levels of mRNA transcripts and significantly reduced CAD enzymatic activities were found in two transgenic plants carrying sense and antisense *CAD* transgenes, respectively. These CAD down-regulated transgenic lines had significantly decreased lignin content and altered ratios of syringyl (S) to guaiacyl (G), G to *p*-hydroxyphenyl (H) and S to H units. No significant changes in cellulose, hemicellulose, neutral sugar composition, *p*-coumaric acid and ferulic acid levels were observed in the transgenic plants. Increases of *in vitro* dry matter digestibility of 7.2–9.5% were achieved in the CAD down-regulated lines, thus providing a novel germplasm to be used for the development of grass cultivars with improved forage quality.

## Introduction

Forages are the backbone of sustainable agriculture and contribute extensively to world economy. On a world-wide basis, grassland acreage is estimated to be twice that of cropland (Jauhar, 1993). In the USA, forage grasses occupy more land area than any other crop and are of immeasurable value for livestock feed, especially ruminants (Asay and Sleper, 1989). The ruminant digestive systems and associated microbial populations are adapted for obtaining nutrients and energy from forage cell walls (Buxton and Redfearn, 1997). Feeding and grazing studies have shown that small changes in forage digestibility can have a significant impact on animal performance, i.e. beef and milk production (Casler and Vogel, 1999; Vogel and Sleper, 1994). Improvement in forage grass cell wall digestibility has become an important goal of many plant-ruminant animal research programmes (Morrison *et al.*, 1998).

The most important constraint on the digestion of plant cell walls is lignin (Buxton and Redfearn, 1997; Cherney *et al.*, 1991; Vogel and Jung, 2001). Lignification of forage tissues limits the amount of digestible energy available to livestock, resulting in an incomplete utilization of cellulose and hemicellulose by ruminant animals (Casler *et al.*, 2002). Lignins are complex phenolic heteropolymers associated with the polysaccharidic components of the wall in specific plant cells. Lignin in forage grasses comprises guaiacyl (G) units derived from coniferyl alcohol, syringyl (S) units derived from sinapyl alcohol, and *p*-hydroxyphenyl (H) units derived from *p*-coumaryl alcohol. Lignin is also an undesirable component in the pulp and paper industry because it must be removed from the wood fibres (Baucher *et al.*, 1996).

 

Because of the sizable economic benefits that might be achieved, considerable research effort has been made toward reducing lignin content (concentration) or modifying lignin composition in plants (Jung and Ni, 1998). However, reports on the transgenic modification of lignin biosynthesis have thus far been mainly on dicotyledonous (dicot) species, such as tobacco, *Arabidopsis*, alfalfa and poplar (reviewed by Baucher *et al.*, 1998; Dixon *et al.*, 2001; Grima-Pettenati and Goffner, 1999; Humphreys and Chapple, 2002). In monocotyledonous (monocot) species, there has been only one report on the down-regulation of caffeic acid *O*-methyltransferase (COMT) in maize (Piquemal *et al.*, 2002). Altered lignin and increased digestibility were found in brown-midrib (*bmr*) mutants of maize (Cherney *et al.*, 1991; Marita *et al.*, 2003), due to mutations in the *COMT* gene (Vignols *et al.*, 1995) or cinnamyl alcohol dehydrogenase (*CAD*) gene (Halpin *et al.*, 1998). Recently, an effective approach based on genome sequencing information and the screening of T-DNA lines was employed to identify *CAD* mutants in *Arabidopsis* (Sibout *et al.*, 2003).

Tall fescue is the predominant cool-season perennial forage species in the USA, where it is grown on an estimated 35 million acres (Sleper and West, 1996). It is also widely used in other parts of the world as a forage base for beef, wool and dairy production. Lignification in tall fescue has been shown to increase with plant development and is negatively associated with digestibility (Chen *et al.*, 2002). Many catalytic enzymes are involved in the lignification process, with CAD as one of the key enzymes solely dedicated to the lignin biosynthetic pathway (Baucher *et al.*, 1998; Halpin *et al.*, 1998). Here we report the cloning of *CAD* cDNAs from tall fescue, and the generation and analyses of sense and antisense transgenic tall fescue plants with reduced lignin content, altered lignin composition and increased *in vitro* dry matter digestibility. This is the first time that a widely grown forage grass species with modified lignin and improved digestibility that might benefit the livestock industry has been obtained by genetic engineering.

## Results

### Cloning of *CAD* cDNAs and expression of recombinant CAD in *E. coli*

We chose CAD as a target for transgenic down-regulation because this lignin-specific enzyme catalyses the last step in the biosynthesis of lignin precursors, which is the reduction of cinnamaldehydes to cinnamyl alcohols (Supplemental Figure 1). Four full-length cDNAs of *CAD* were isolated from a cDNA library constructed from 2-month-old tall fescue plants. The four cDNA clones all have an open reading frame (ORF) of 1086 bp, and have various lengths of different 5′ and 3′ untranslated regions. Among themselves, these four clones share about 97–99% identity in nucleotide (ORF region) and deduced amino acid sequences. They also share high amino acid sequence similarity to a perennial ryegrass *CAD* (McAlister *et al.*, 2001) (96–99% identity) and a maize *CAD* (Halpin *et al.*, 1998) (87–88% identity).

Because of the high similarity among the tall fescue cDNA clones, one of them, *FaCAD1b*, was used for further analyses and the construction of transgenes. To confirm that the cloned sequences encode a CAD enzyme and to investigate its kinetic properties and substrate specificity, the coding region of *FaCAD1b* was cloned into pET29a vector for the expression in *E. coli*. The purified recombinant CAD protein exhibited catalytic activity against coniferaldehyde, sinapaldehyde and *p*-coumaraldehyde, its three predicted substrates during monolignol biosynthesis (Table 1). Based on $V_{max}/K_m$ values, both coniferaldehyde and sinapaldehyde were excellent substrates for tall fescue CAD. These results confirm that *FaCAD1b* encodes a functional CAD enzyme capable of converting cinnamyl aldehydes into cinnamyl alcohols. The other two substrates, caffeoyl aldehyde and 5-hydroxyconiferaldehyde, showed the lowest $V_{max}/K_m$ ratio (Table 1).

### Generation of transgenic tall fescue plants with altered expression of *CAD*

Transformation vectors were constructed by placing the ORF of *FaCAD1b* in sense and antisense orientations under the control of the maize ubiquitin promoter (Figure 1A,B). Biolistic transformation of tall fescue was performed using single genotype-derived embryogenic suspension cells as bombardment targets and a PDS-1000/He device to deliver DNA-coated gold particles. Chimeric *CAD* gene constructs were co-transformed with a hygromycin phosphotransferase (*hph*) gene, which served as a selectable marker gene rendering

**Table 1** Kinetic properties of recombinant tall fescue CAD protein

| Substrate | $K_m$ (μM) | $V_{max}$ (nmol/min/μg) | $V_{max}/K_m$ (%) |
|---|---|---|---|
| Coniferaldehyde | 3.15 ± 0.54 | 0.32 ± 0.02 | 100 |
| Sinapaldehyde | 4.18 ± 0.78 | 0.27 ± 0.03 | 63.58 |
| *p*-Coumaraldehyde | 10.64 ± 1.37 | 0.23 ± 0.04 | 21.28 |
| Caffeoyl aldehyde | 62.78 ± 5.62 | 0.21 ± 0.03 | 3.29 |
| 5-Hydroxyconiferaldehyde | 37.68 ± 2.98 | 0.22 ± 0.02 | 5.74 |

Values are means ± SE.

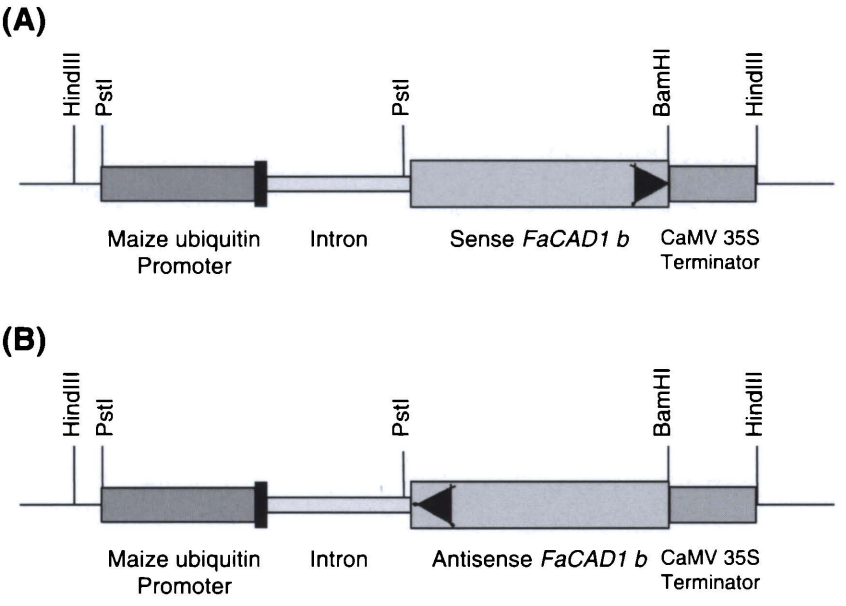


**Figure 1** Schematic maps of chimeric cinnamyl alcohol dehydrogenase (*CAD*) genes used for the genetic transformation of tall fescue (*Festuca arundinacea*). (A) Sense *FaCAD1b* gene under the control of maize ubiquitin promoter. (B) Antisense *FaCAD1b* gene under the control of maize ubiquitin promoter.

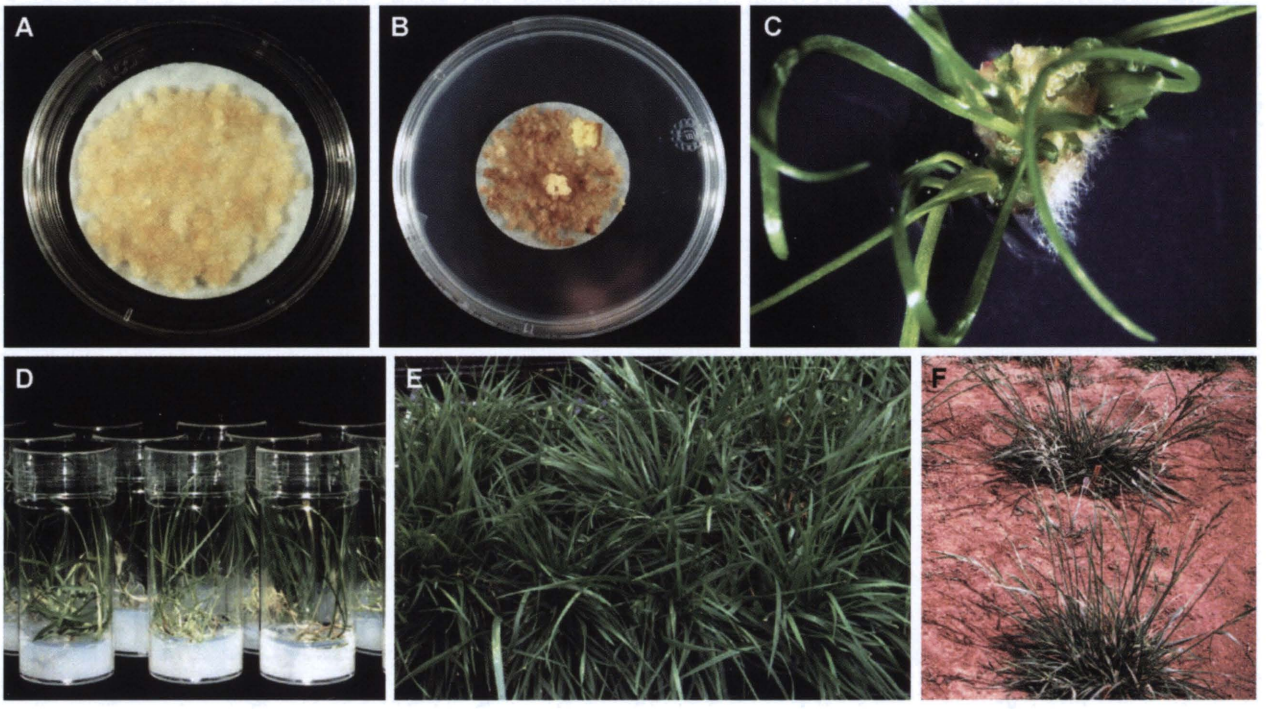


**Figure 2** Transgenic tall fescue (*Festuca arundinacea*) plants obtained by biolistic transformation of embryogenic suspension cells. (A) Suspension cells plated on filter paper disk prior to microprojectile bombardment. (B) Hygromycin resistant calluses obtained after microprojectile bombardment and selection of bombarded cells on a medium containing 250 mg/mL hygromycin. (C) Shoot differentiation of hygromycin resistant calluses 4 weeks after transfer on to regeneration medium. (D) *In vitro* transgenic plantlets recovered 4 months after microprojectile bombardment of embryogenic cells. (E) Greenhouse-grown transgenic plants 6 months after bombardment of embryogenic cells. (F) Field-grown transgenic tall fescue plants.

transformed cells resistant to hygromycin. The bombarded embryogenic suspension cells (Figure 2A) were subjected to hygromycin selection. Calluses resistant to hygromycin were obtained in 27% of the bombarded dishes after selection with high concentration of hygromycin (250 mg/L, Figure 2B). One-third of the hygromycin resistant calli differentiated multiple green shoots (Figure 2C) and allowed the establishment of corresponding *in vitro* rooted plantlets (Figure 2D). Soil-grown tall fescue plants were obtained within 6 months of microprojectile bombardment of suspension cells (Figure 2E).

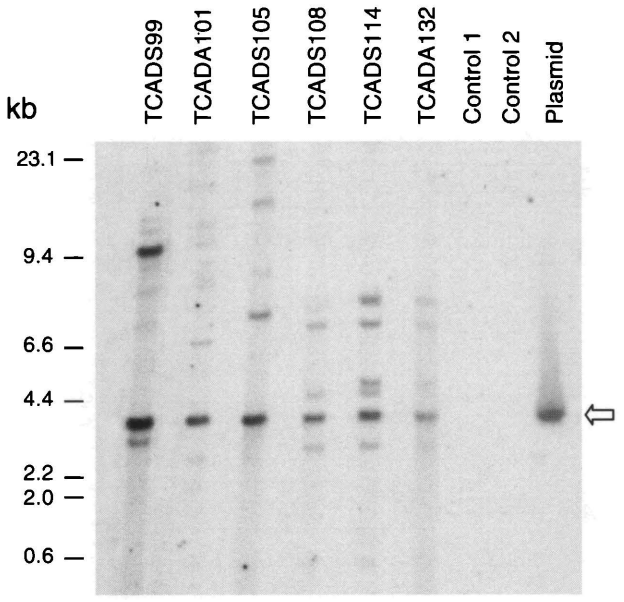


**Figure 3** Southern blot hybridization analysis of digested tall fescue genomic DNA hybridized with the radioactive labelled maize ubiquitin promoter sequence. Genomic DNA was isolated from transgenic and control plants and digested with *Hin*dIII, which cut out a ≈ 3.5 kb fragment containing the promoter and the *FaCAD1b* sequences. The probe was isolated by digestion of the plasmid DNA with *Pst*I, which cut out the ubiquitin promoter sequences. The arrow indicates the expected band after *Hin*dIII digestion and Southern hybridization.

PCR analyses were used to screen co-transformants with the *CAD* gene constructs (data not shown). Southern hybridization using an ubiquitin promoter as a probe confirmed the transgenic nature of these regenerated plants (Figure 3). Out of 28 hygromycin resistant plants, 21 contained the *CAD* transgene, and therefore the co-transformation frequency is estimated to be 75% for the transgenic tall fescue plants obtained.

Northern hybridization analyses using *FaCAD1b* as a probe were performed to detect changes in transcript levels of the transgenic plants. A sense co-suppressed plant TCADS105 and an antisense down-regulated plant TCADA101 had severe reductions in the accumulation of *CAD* transcript (Figure 4). No obvious changes in transcript levels were observed for other transgenic plants, including the sense transgenic lines (Figure 4). It has been observed in other studies that over-expressing sense *CAD* and *COMT* genes did not lead to a substantial or stable increase in transcript levels or enzyme activities (Baucher *et al.*, 1996; Guo *et al.*, 2001; Jouanin *et al.*, 2000).

Since maturity stage and environmental factors affect lignin and forage quality (Casler and Vogel, 1999; Chen *et al.*, 2002), vegetative tissues harvested from greenhouse-grown material of the same developmental stage were used for molecular and chemical analyses. It has been shown that a strong genetic correlation exists for forage quality traits measured at the vegetative stage and later developmental stages (Casler, 2001).

### CAD activity in transgenic tall fescue plants

CAD enzymatic activities were assayed using three substrates: coniferaldehyde, sinapaldehyde and *p*-coumaraldehyde, which lead to the formation of the lignin precursors coniferyl alcohol, sinapyl alcohol and *p*-coumaryl alcohol, respectively (Supplemental Figure 1). Compared with empty vector control plants and seed-derived control plants (Seed-ctrl), transgenic lines TCADA101 and TCADS105 showed significantly reduced CAD activities (Figure 5A,B). The magnitude of reduction was similar for the two transgenic lines, with 34% and 50% residual activity in TCADA101 and 38% and

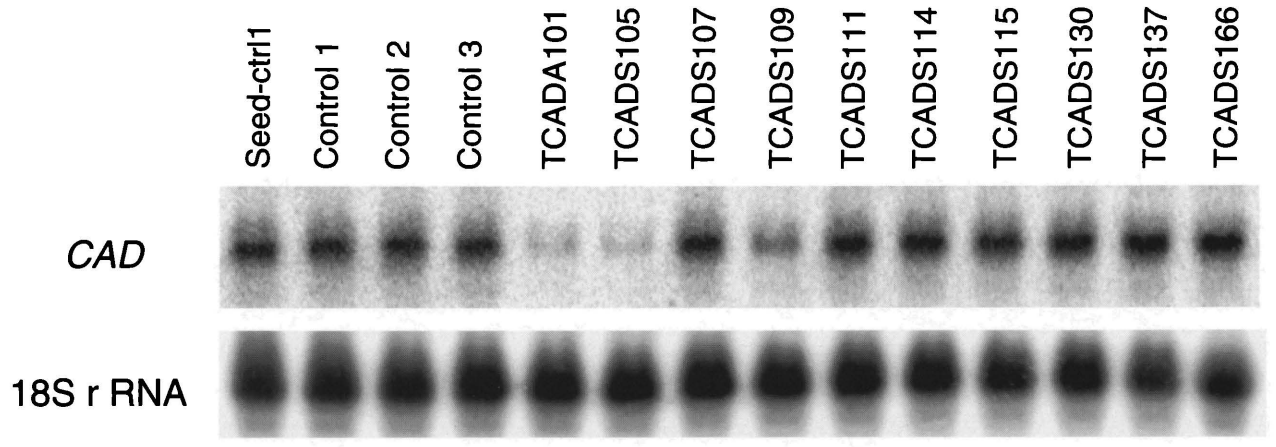


**Figure 4** Northern blot hybridization analysis using total cellular RNA isolated from tall fescue plants and hybridized with the *FaCAD1b* probe. Seed-ctrl: seed-derived control plants. Control: empty vector control plants.

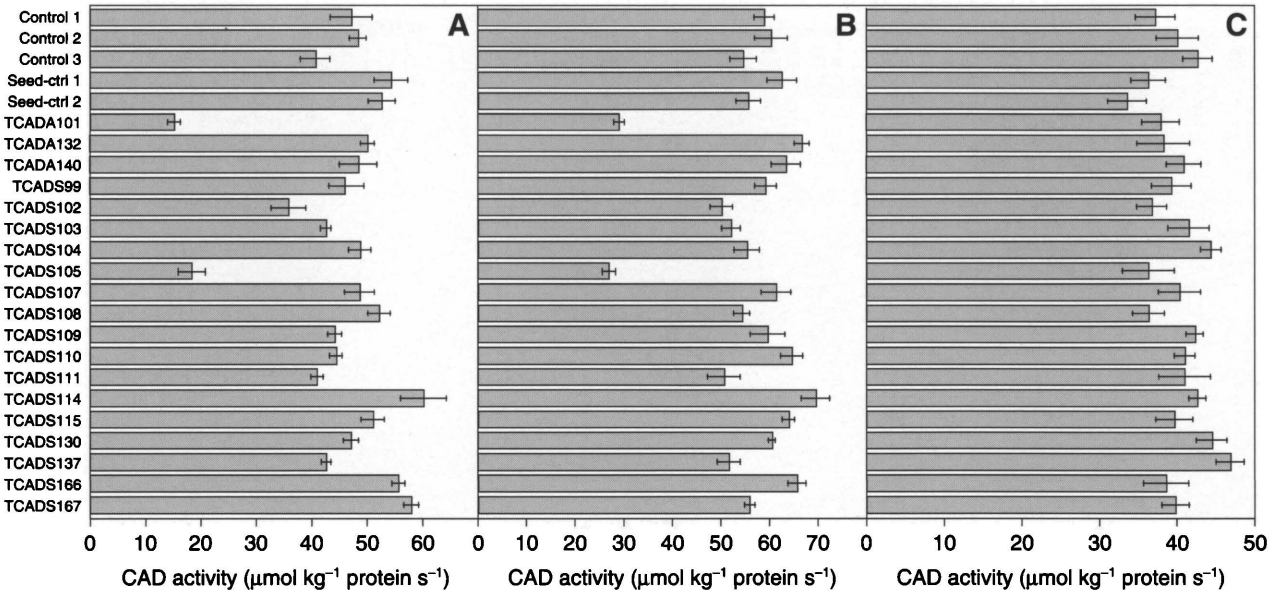


**Figure 5** Cinnamyl alcohol dehydrogenase (CAD) activity assay in transgenic tall fescue plants. CAD enzyme activities of transgenic and control plants were measured against: (A) coniferaldehyde, (B) sinapaldehyde, and (C) 4-coumaraldehyde. Seed-ctrl: seed-derived control plants. Control: empty vector control plants.

**Table 2** Lignin content and composition of transgenic tall fescue with modified expression of *CAD*

| Plant line | Klason lignin (% dry matter) | S lignin (μmol/g dry CW) | G lignin (μmol/g dry CW) | H lignin (μmol/g dry CW) | S/G ratio | G/H ratio | S/H ratio |
|---|---|---|---|---|---|---|---|
| Control 1 | 7.13 ± 0.27 | 115.84 ± 7.62 | 159.04 ± 10.26 | 11.47 ± 1.46 | 0.73 | 13.87 | 10.10 |
| Control 2 | 7.29 ± 0.24 | 107.64 ± 8.80 | 159.33 ± 9.37 | 11.35 ± 0.80 | 0.68 | 14.04 | 9.48 |
| Control 3 | 7.25 ± 0.31 | 124.68 ± 8.57 | 162.37 ± 11.47 | 12.34 ± 1.23 | 0.77 | 13.16 | 10.10 |
| Seed-ctrl 1 | 7.94 ± 0.36 | 134.66 ± 8.26 | 171.58 ± 11.30 | 13.67 ± 1.51 | 0.78 | 12.55 | 9.85 |
| Seed-ctrl 2 | 7.34 ± 0.38 | 124.60 ± 6.38 | 162.38 ± 10.29 | 12.55 ± 0.98 | 0.77 | 12.94 | 9.93 |
| TCADA101 | 6.18 ± 0.29* | 68.71 ± 7.58* | 122.20 ± 7.82* | 12.48 ± 1.04 | 0.56 | 9.80 | 5.51 |
| TCADA132 | 7.24 ± 0.11 | 125.39 ± 7.70 | 168.71 ± 9.66 | 11.58 ± 1.23 | 0.74 | 14.57 | 10.83 |
| TCADA140 | 7.18 ± 0.28 | 119.87 ± 7.08 | 158.60 ± 11.52 | 12.57 ± 1.07 | 0.76 | 12.62 | 9.54 |
| TCADS99 | 7.11 ± 0.29 | 109.30 ± 7.05 | 160.73 ± 10.97 | 11.76 ± 1.47 | 0.68 | 13.67 | 9.29 |
| TCADS105 | 6.13 ± 0.22* | 71.27 ± 6.74* | 122.67 ± 6.88* | 11.69 ± 1.25 | 0.58 | 10.49 | 6.10 |
| TCADS107 | 7.13 ± 0.38 | 122.30 ± 8.74 | 153.63 ± 10.55 | 11.71 ± 1.13 | 0.80 | 13.12 | 10.44 |
| TCADS108 | 7.07 ± 0.27 | 106.29 ± 6.98 | 148.54 ± 9.07 | 10.91 ± 1.56 | 0.72 | 13.62 | 9.74 |
| TCADS109 | 6.57 ± 0.45 | 84.32 ± 5.32 | 141.54 ± 12.37 | 10.62 ± 1.23 | 0.60 | 13.33 | 7.94 |
| TCADS110 | 7.18 ± 0.32 | 119.55 ± 6.38 | 160.39 ± 8.60 | 11.50 ± 1.40 | 0.75 | 13.95 | 10.40 |
| TCADS111 | 6.73 ± 0.12 | 94.80 ± 6.08 | 130.78 ± 8.90 | 11.62 ± 0.97 | 0.72 | 11.25 | 8.16 |
| TCADS114 | 7.54 ± 0.30 | 131.28 ± 7.18 | 168.33 ± 12.45 | 13.48 ± 1.69 | 0.78 | 12.49 | 9.74 |

CW: Cell wall. Control: Empty vector control. Seed-ctrl: Seed-derived control plants. Values are means ± SE.

47% residual activity in TCADS105, when coniferaldehyde (Figure 5A) or sinapaldehyde (Figure 5B) were used as substrates, respectively. Other transgenics showed largely similar levels of enzyme activities to control plants. No significant differences in CAD activity were found between transgenic and control plants when *p*-coumaraldehyde was used as the substrate (Figure 5C).

### Effects of down-regulation of CAD on lignin content and composition

The measurement of Klason lignin is probably the best of many methods available for estimating lignin content (Jung, 1997). As shown in Table 2, Klason lignin levels of control lines averaged 7.22% of dry matter. This value was reduced to

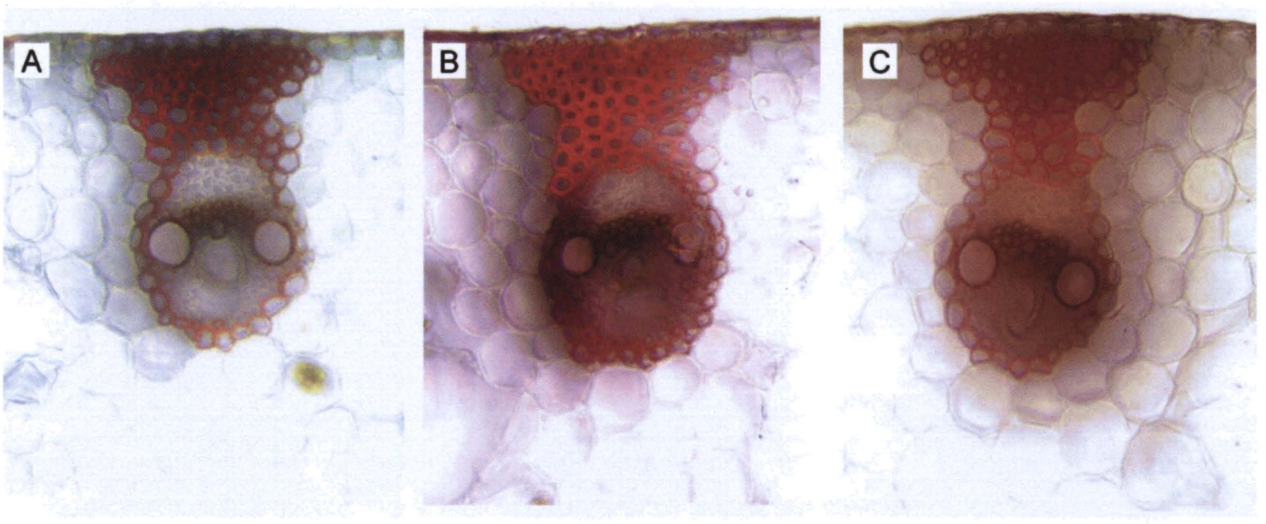


**Figure 6** Weisner staining of cross sections of transgenic tall fescue plants with modified expression of *CAD*. (A) Control plant, (B) transgenic plant TCADS105, (C) transgenic plant TCADA101.

6.13% and 6.18% in the respective transgenic lines TCADS105 and TCADA101. The levels of reduction (14–15%) in Klason lignin content were significant for both of the CAD down-regulated plants (Table 2).

Lignin composition was revealed by gas chromatography/mass spectrometry (GC/MS) after thioacidolysis, which has been commonly used for degrading lignin polymer to reveal monomer composition (Lapierre *et al.*, 1985). Significant reduction of both G and S lignin content was observed in the two transgenic lines, while H lignin content did not change significantly (Table 2). The data indicate that CAD down-regulation affects the amounts of both G and S lignin, but not H lignin in the cell wall. When comparing the relative ratios of the lignin units, the percentage of H unit in total lignin increased in both of the transgenic lines. Ratios of S/G, S/H and G/H declined in the two transgenic lines when compared with the control plants (Table 2).

Histochemical staining by the Weisner method further confirmed the results regarding lignin alteration in the transgenic plants. Cross-sections of tall fescue plants were stained with acid phloroglucinol (Figure 6), a reagent which is traditionally used to detect cinnamyl aldehyde and lignin (Halpin *et al.*, 1998). Staining of cuttings from leaf sheath of transgenic plants displayed intense red coloration in the vascular tissue and the lignified tissues between vascular bundle and epidermal cells (Figure 6B,C), whereas corresponding tissues in the control plants showed much less intensity of staining (Figure 6A). The colour difference indicates increased aldehyde content in the transgenic tissues. Reddish coloration has been observed in xylem tissues of CAD down-regulated tobacco and poplar plants and *bm1* mutant of maize (Baucher *et al.*, 1996; Halpin *et al.*, 1994, 1998; Higuchi *et al.*, 1994), mainly due to the accumulation of cinnamaldehyde groups in lignin (Halpin *et al.*, 1998; Hibino *et al.*, 1995; Higuchi *et al.*, 1994).

### Effects of down-regulation of CAD on cell wall polysaccharides and hydroxycinnamic acids

Changes in cell wall polysaccharides can affect forage quality (Aman, 1993). In order to investigate if there are any changes in cell wall polysaccharides of the transgenic tall fescue plants, hemicellulose, cellulose and pectin fractions of cell wall polysaccharides were prepared and evaluated on the basis of dry cell wall mass (Supplemental Table 1). No differences were found between transgenic and control plants regarding the contents of the two major polysaccharides, hemicellulose and cellulose. Only an increase in pectin content was observed in one of the transgenic plants, TCADS105. Being a small fraction of the plant cell wall, pectin may only have limited influence on dry matter digestibility.

Neutral sugar composition of the non-cellulosic polysaccharides was further investigated. The major components of hemicellulose and pectin revealed by GC analysis were glucose, xylose, arabinose and galactose. No significant differences were found between transgenic and control plants regarding these components of hemicellulose and pectin (Supplemental Table 2).

Hydroxycinnamic acids, *p*-coumaric acid and ferulic acid play important roles in cell wall matrix interactions and may limit cell wall digestion (Hatfield *et al.*, 1999). HPLC analysis of wall-bound hydroxycinnamic acids revealed no significant difference in the levels of *p*-coumaric acid, ferulic acid and

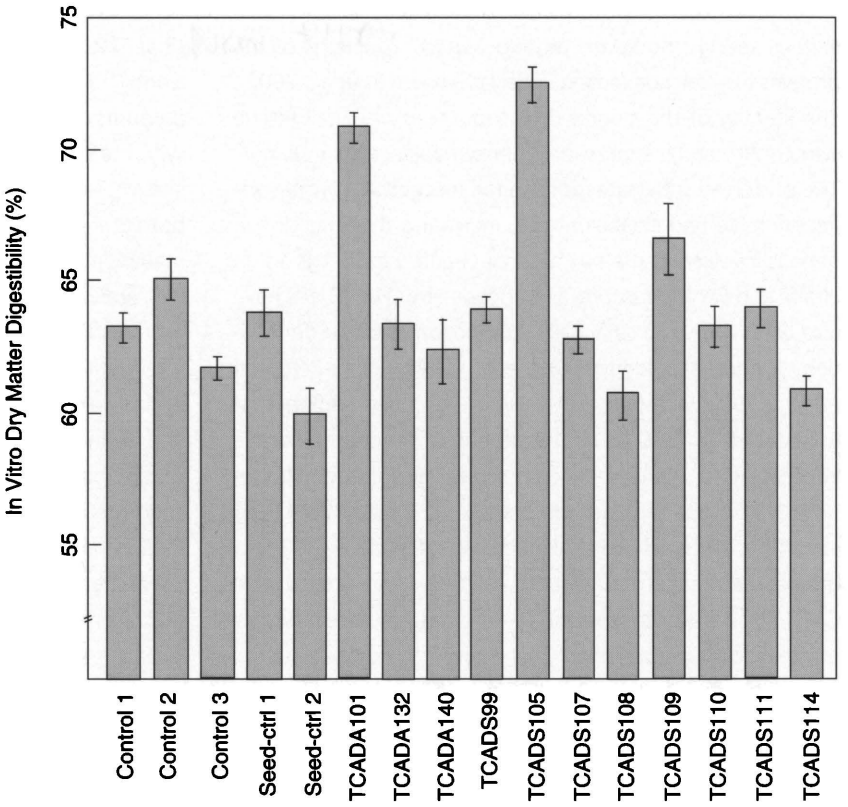


**Figure 7** *In vitro* dry matter digestibility of transgenic tall fescue plants with modified expression of *CAD*.

diferulic acid between the control and transgenic plants (Supplemental Table 3). This suggests that CAD down-regulation does not affect the major wall-bound phenolic acids in the plants.

### *In vitro* dry matter digestibility of CAD down-regulated transgenic tall fescue plants

Digestibility is one of the most important characteristics of a forage (Casler, 2001). The development of *in vitro* dry matter digestibility analysis has been considered to be the most important single event in the history of breeding for improved forage quality in grasses (Casler and Vogel, 1999). Forage samples collected from the two CAD down-regulated transgenic plants, empty vector control plants and seed-derived plants, as well as some other transgenics were analysed for their *in vitro* dry matter digestibility (IVDMD). When compared with the average of empty vector control plants, the IVDMD of TCADA101 and TCADS105 increased by 7.2 and 9.5% units (Figure 7). The digestibility of the CAD down-regulated plants was also significantly higher than that of the seed-derived or the rest of transgenic plants (Figure 7).

Transgenic and control plants were transferred to the field to evaluate their phenotype and morphology (Figure 2F). Both transgenic and control plants grew well and were phenotypically normal. No significant difference was found between the transgenic and control plants regarding major agronomic traits, including heading date, anthesis date, height, growth habit, number of reproductive tillers and seed yield. Neither lodging nor abnormal plant architecture was observed. No changes were observed concerning the incidence of pest or pathogens in the transgenic plants.

## Discussion

The *CAD* cDNA sequences we isolated from tall fescue were highly homologous with each other and showed a very high similarity to those of other related monocot species such as perennial ryegrass. The coding region of one of the tall fescue *CAD* cDNAs (*FaCAD1b*) was used to construct sense and antisense chimeric transgenes, which were introduced back to tall fescue by biolistic transformation. Consistent and closely related molecular and biochemical data demonstrated that two transgenic lines (TCADA101 and TCADS105) were down-regulated in their lignin biosynthesis. The two transgenic lines showed substantially reduced levels of transcripts, significantly reduced enzymatic activities, significantly decreased lignin content and apparently altered lignin composition. When considered for the purpose of forage improvement, these CAD down-regulated plants had significant increases in their *in vitro* dry matter digestibility.

Putative *CAD* cDNA sequences have been isolated from various species; however, the biochemical functions of the proteins they encode remain largely unknown (Li *et al.*, 2001). The identity of the cloned *CAD* sequences from tall fescue was confirmed by expression of enzymatic activity in *E. coli*. The preferred substrates for the tall fescue CAD were coniferaldehyde and sinapaldehyde, indicating that the cloned gene is involved in the synthesis of G and S monolignols. In alfalfa and *Eucalyptus*, proteins encoded by *CAD* cDNAs have also been shown to catalyse the reduction of both coniferaldehyde and sinapaldehyde (Brill *et al.*, 1999; Grima-Pettenati *et al.*, 1993). In aspen, however, it has been shown that aspen CAD (*PtCAD*) is coniferaldehyde specific, whereas another gene, *PtSAD* (encoding sinapyl alcohol dehydrogenase, SAD), is sinapaldehyde specific (Li *et al.*, 2001). The enzymatic efficiency of PtSAD for sinapaldehyde was 60-fold greater than that of PtCAD (Li *et al.*, 2001). For the tall fescue *CAD*, in addition to enzyme kinetic data of recombinant protein, down-regulation of *CAD* expression in transgenic tall fescue revealed a reduction in CAD enzymatic activities when coniferaldehyde and sinapaldehyde were used as substrates, thus further confirming that the tall fescue CAD is involved in the biosynthesis of both G and S lignin.

The transgenic manipulation of CAD by either an antisense or sense strategy has been reported in several dicot species. In most of the cases, a reduction of CAD activity led to changes in lignin composition or structure, while lignin content remained unchanged. Suppression of CAD activity led to significant decreases in S/G ratios in tobacco (Halpin *et al.*, 1994; Yahiaoui *et al.*, 1998) and alfalfa (Baucher *et al.*, 1999). In poplar, although the S/G ratio was not significantly modified in CAD down-regulated plants, increases in the number of aldehyde units in lignin (Baucher *et al.*, 1996) or free phenolic groups in lignin (Lapierre *et al.*, 1999; Pilate *et al.*, 2002) were found. Increases of cinnamaldehyde groups in lignin have also been observed in transgenic tobacco with reduced CAD activity (Hibino *et al.*, 1995; Higuchi *et al.*, 1994). Some of the changes in lignin composition or structure resulted in increased lignin extractability or degradability (Baucher *et al.*, 1996; Baucher *et al.*, 1999; Bernard-Vailhe *et al.*, 1996; Yahiaoui *et al.*, 1998). Only a slight reduction in lignin content was observed in transgenic poplar (Lapierre *et al.*, 1999; Pilate *et al.*, 2002).

In the present study, the CAD down-regulated tall fescue plants showed a reduced S/G ratio, this result is consistent with some of the reports in tobacco and alfalfa (Baucher *et al.*, 1999; Halpin *et al.*, 1994; Yahiaoui *et al.*, 1998). However, in contrast to the unchanged lignin quantity reported in some dicot species (Baucher *et al.*, 1996, 1999; Halpin *et al.*, 1994; Hibino *et al.*, 1995; Higuchi *et al.*, 1994; Yahiaoui *et al.*, 1998), the transgenic tall fescue plants showed significantly reduced Klason lignin content as well as reduced amounts of G and S lignin monomers. This result is consistent with the enzyme activity data, in which the cloned *CAD* was shown to be responsible for catalysing the synthesis of both G and S lignin. Due to a lack of reports on the transgenic manipulation of *CAD* in monocot species, the only comparable case we can find in a monocot species is the natural *bm1* mutants in maize. A decrease in CAD activity (by 60–70%), a reduction of Klason lignin content (by 20%), a reduction in the yield of G and S lignin monomers after thioacidolysis and an increase in digestibility were observed in maize *bm1* mutant plants (Halpin *et al.*, 1998). Mapping studies strongly suggested that maize *bm1* directly affects expression of the *CAD* gene (Halpin *et al.*, 1998).

The transgenic tall fescue plants with a 14–15% reduction in Klason lignin content were phenotypically normal. The level of reduction of lignin content in tall fescue was rather modest when compared with the 50% reduction in lignin in transgenic tobacco obtained by the simultaneous down-regulation of CAD and cinnamoyl CoA Reductase (CCR) (Chabannes *et al.*, 2001). The transgenic tobacco plants were able to tolerate such a severe reduction in lignin content and displayed normal development and maintained structural integrity of the vessels (Chabannes *et al.*, 2001). In transgenic aspen, down-regulation of 4-coumarate:CoA ligase (4CL) or a combinational modification of both 4CL and coniferaldehyde 5-hydroxylase (CAld5H) led to a drastic reduction of lignin, but this was compensated by a large increase in cellulose, and growth of the plants was substantially enhanced (Hu *et al.*, 1999; Li *et al.*, 2003). However, the increased cellulose production and enhanced growth of transgenic aspen were questioned (Anterola and Lewis, 2002). In the CAD down-regulated transgenic tall fescue plants, no significant changes in cellulose and hemicellulose contents were found.

Because the dicot angiosperm lignin contains two major monomer species, G and S units, it is common to use the S/G ratio to describe lignin composition in these species. Lignin from monocot plants also contains a significant proportion of H units derived from coumaryl alcohol. Therefore, in addition to the S/G ratio, we also compared S/H and G/H ratios. Because the H lignin content did not change, while both G and S lignins decreased in the transgenic plants, a reduction in the ratios of S/G, S/H and G/H was observed in the transgenic plants. There has been very little information available about the biosynthesis of H lignin. In our transgenic tall fescue plants, when measured against *p*-coumaraldehyde, CAD activity did not show significant change. This is not

surprising because the enzymatic activity of the recombinant CAD protein against *p*-coumaraldehyde was much lower than corresponding values measured against coniferaldehyde and sinapaldehyde. These results indicate that there may be a more specific enzyme involved in the synthesis of H lignin, or that CAD activity is not rate limiting in H lignin biosynthesis.

The transgenic expression of lignin genes has provided a powerful tool for elucidating lignin biosynthetic pathways in dicot plants. This is not yet the case in monocot species. Pathways of lignin biosynthesis in monocot species have been adopting the schemes developed based on studies in dicot plants, even though it is known that there may be some differences between dicot and monocot plants, at least concerning H lignin biosynthesis. To date, the only report on genetic manipulation in a monocot species is the down-regulation of COMT in maize, where a transgenic plant showed decreased COMT activity, a strong decrease in Klason lignin content, a decrease in S unit and a lower p-coumaric acid content (Piquemal *et al.*, 2002). The genetic manipulation of other lignin genes (e.g. *CAD*) has not been reported in monocots. In forage grasses, although *CAD* and *COMT* have been cloned from perennial ryegrass (McAlister *et al.*, 1998, 2001), no information on transgenic manipulation is available in these species. The lack of reports on the successful modification of lignin in forage grasses is mainly due to the difficulties of obtaining transgenics and identifying transgenic plants expressing changes in lignin. We established an efficient plant regeneration and transformation system for tall fescue, and for the first time in a major forage grass species, produced transgenic plants with altered lignin and an increased digestibility of 7.2–9.5%.

Genetic manipulation for increased *in vitro* dry matter digestibility of forage grasses can lead to rapid financial benefits to the agricultural community and society (Casler and Vogel, 1999). For beef cattle, a 1% increase in dry matter digestibility generally leads to a 3.2% increase in average daily live-weight gains (Casler and Vogel, 1999). It has also been estimated that in the US dairy industry, a 10% increase in wall digestion would result in an additional $380 million in milk and meat sales while reducing manure solids by 2.3 million megagrams and grain input into the diet by 3.0 million megagrams (Hatfield *et al.*, 1999). Conventional breeding by phenotypic recurrent selection has resulted in the release of grass cultivars with improved dry matter digestibility. However, continued selection for increased dry matter digestibility might affect plant fitness (Casler *et al.*, 2002). The genetic engineering of a lignin biosynthetic pathway may offer a more effective approach to improve the forage digestibility of grasses. The successful approach of this study could be readily applied to other grass species, such as bermudagrass and perennial ryegrass, in which forage quality improvement has also been an important goal. The transgenic tall fescue plants will be evaluated over a few years regarding their agronomic and animal performance and will be incorporated into our grass breeding programme.

## Experimental procedures

### Plant material

The most widely grown tall fescue (*Festuca arundinacea* Schreb.) cultivar, Kentucky-31, was used throughout the study. The seeds and plants of Kentucky-31 used for the experiments were endophyte-free.

### Isolation of *CAD* cDNA sequences and expression of recombinant CAD in *Escherichia coli*

A cDNA library was constructed from mRNA isolated from 2-month-old tall fescue plants using the ZAP express cDNA synthesis kit (Stratagene, La Jolla, CA). The library was screened with a [$^{32}$P] dCTP-labelled perennial ryegrass *CAD* probe, kindly provided by J. Watson and F. McAlister (McAlister *et al.*, 2001). Excised and cloned cDNA inserts were obtained using the ExAssist helper phage with SOLR strain, as described by the manufacturer (Stratagene). Eight clones were analysed and four different full-length *CAD* cDNA clones were identified (sequences of the four cDNAs were deposited in GenBank with the accession numbers AF188292, AF188293, AF188294 and AF188295). In order to express and purify the recombinant protein, the coding sequences of *FaCAD1b* were amplified by polymerase chain reaction (PCR) to introduce a *Bam*HI site at the 5′ end and 3′ end, and the PCR product was cloned into pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA). Plasmid DNA prepared from the TOPO-*FaCAD* construct was digested by *Bam*HI and the isolated *FaCAD* fragment was cloned into a pET-29a(+) vector containing the histidine tag (Novagen, Madison, WI). After sequence confirmation, the engineered pET-29a(+) construct was transferred into the host bacterial strain BL21(DE3). The induction of bacterial cells with isopropyl β–D-thiogalactoside and the purification of CAD protein using a His·Bind Quick Column were performed according to the manufacturer's instructions (Novagen). To determine the $K_m$ and $V_{max}$ values of the recombinant CAD protein, *p*-coumaraldehyde, coniferaldehyde, sinapaldehyde, caffeoyl aldehyde and 5-hydroxyconiferaldehyde were used as substrates. The enzyme reaction and kinetic analysis

was carried out following the procedures described by Li *et al.* (2001).

### Generation of transgenic tall fescue plants

The open reading frame of *FaCAD1b* was PCR amplified to create new restriction sites, and was placed in sense and antisense directions under the control of a maize ubiquitin promoter, kindly provided by P. Quail (Christensen and Quail, 1996). Sterilized seeds/caryopses of Kentucky-31 were used as explants to induce callus. Embryogenic calluses derived from single seeds/caryopses (representing individual genotypes) were individually transferred to a liquid culture medium to establish single genotype-derived cell suspension cultures (Wang *et al.*, 1994). Cell clusters from an embryogenic suspension line were used as direct targets for biolistic transformation to generate transgenic plants. The use of single genotype-derived cell suspensions allowed the generation of transformants from the same genotype and excluded genotypic effects in the regenerants (Wang *et al.*, 2001). Empty vector control plants were from the same genotype used for transformation. A chimeric hygromycin phosphotransferase (*hph*) gene under control of the rice actin-1 5′ regulatory sequences was used as the selectable marker (Wang *et al.*, 2003a). Equal molar amounts of plasmid DNA of the *CAD* transgene and the *hph* selectable marker gene were mixed and used to co-transform tall fescue. Previously described biolistic transformation parameters were used (Wang *et al.*, 2003a). Hygromycin-resistant calli were obtained after microprojectile bombardment of suspension cells and subsequent selection in medium containing 250 mg/L hygromycin. Transgenic tall fescue plants were regenerated from the hygromycin resistant calli and later transferred to the greenhouse (390 $\mu E/m^2/s$, 16 h light).

To achieve uniform and comparable growth stages for the molecular, biochemical and digestibility analyses, plants (in triplicates) were cut back and allowed to re-grow for 3 weeks before sampling. Whole plant tissue 5 cm above soil surface was harvested and immediately frozen in liquid $N_2$ and stored at –80 °C until use. Such cuttings and re-growth were repeated four times and forage samples were collected and analysed each time. Transgenic and control plants were transferred to the field in the autumn of 2002 to evaluate their phenotype and agronomic performance. The small scale field trial of transgenics was carried out under USDA regulations. Because tall fescue requires strong vernalization to flower (Wang *et al.*, 2003b), major phenology and morphology traits were measured in the spring of 2003. The agronomic characteristics evaluated were: heading date, anthesis date, height, growth habit, number of reproductive tillers and seed yield.

### Molecular characterization of transgenic plants

The isolation of total genomic DNA, gel electrophoresis and DNA blotting were carried out following standard protocols (Lichtenstein and Draper, 1985; Sambrook *et al.*, 1989). The hybridization probe was [$^{32}$P] dCTP-labelled using the RadPrime DNA Labelling System (Invitrogen, Carlsbad, CA), and the unincorporated nucleotides were removed by passing through ProbeQuant™ G-50 Micro Columns (Amersham Pharmacia Biotech, Piscataway, NJ). Southern hybridizations were performed using QuikHyb® Hybridization Solution (Stratagene, La Jolla, CA), according to the manufacturer's specifications.

Total RNA was isolated using TRI reagent (Molecular Research Center, Cincinnati, OH) and RNA gel blotting was carried out according to standard protocols (Sambrook *et al.*, 1989). Northern hybridizations were performed using the [$^{32}$P] dCTP-labelled *CAD* cDNA as probe following the QuikHyb® Hybridization protocols.

### CAD activity assay in transgenic plants

The assay of CAD activity essentially followed the procedures described by Morrison *et al.* (1994). CAD activity was determined by reacting it in enzyme extracts with 4-coumaraldehyde, coniferaldehyde and sinapaldehyde, to produce 4-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol via the oxidation of NADPH. The reaction mixture (3 mL) contained 2.4 μg of protein in 1750 μL 0.1 M $NaH_2PO_4/Na_2HPO_4$ buffer at pH 7.6, 60 μL of 1.0 mM aldehyde substrate, 600 μL of 1.0 mM NADPH, 50 μL of 20 mM $ZnSO_4 \cdot 7H_2O$. The oxidation of NADPH caused a linear decrease in absorbance, which was recorded spectrophotometrically at 340 nm initially (0 min), and after 10 min of incubation at 30 °C.

### Determination of lignin content and composition

The content and composition of lignin were determined according to previously described procedures (Chen *et al.*, 2002; Guo *et al.*, 2001). Two hundred milligrams of dried samples were used for lignin analysis, and Klason lignin content was calculated as weight percentage of the extract-free sample. Lignin composition was determined after thioacidolysis and Raney nickel desulphurization. Lignin-derived monomers were identified and quantified by gas chromatography (GC)/mass spectrometry (MS) (Chen *et al.*, 2002).

### Histochemical staining of lignin

The Weisner staining method (Dean, 1997; Halpin *et al.*, 1998) was used for the histochemical characterization of transgenic plants. Sections of tall fescue leaf sheath and stem were hand-cut with a vibratome (Series 1000. Ted Pella Inc., Redding, CA) and stained in 1% phloroglucinol : 70% ethanol (5 min). Excess phloroglucinol was removed and replaced with 18% HCl. Photographs were taken using an Olympus SZX stereomicroscope system with a SPOT RT colour camera.

### Fractionation of cell wall polysaccharides

The collected samples were boiled for 15 min in methanol at 70 °C, and then washed and stored in methanol until use. Cell wall polysaccharides were fractionated into pectin, hemicellulose and cellulose by the method of Kamisaka *et al.* (1990).

For the determination of non-cellulosic sugar compositions of the cell wall, 0.5 mg of pectin and hemicellulose were hydrolysed for 1 h at 121 °C with 1 mL of 2 N trifluoroacetic acid, the liberated sugars were then reduced with an excess amount of sodium borohydride, followed by acetylation in the presence of acetic anhydride at 121 °C for 3 h. The amounts of resulting acetylated alditols were determined using a Hewlett Packard 5890 series II gas chromatograph with a 5971 series mass selective detector (column: HP-1, 60 m × 0.25 mm × 0.25 μm film thickness), and the mass spectra were recorded in electron impact mode (70 eV) with a 60–650 *m/z* scanning range.

### Determination of wall-bound phenolics

The measurement of phenolic compounds essentially followed the procedure described by Guo *et al.* (2001). Residues from the extractions of soluble phenolics were washed three times with absolute ethanol, dried under $N_2$, and subjected to base hydrolysis in 1 N NaOH at room temperature. After centrifugation, the supernatant was removed, acidified to pH 3.0 with HCl, and extracted three times with an equal volume of ethyl acetate. The organic phases were combined, taken to dryness by $N_2$, and resuspended in HPLC-grade methanol to a final concentration equivalent to 1 g dry weight of original plant tissue per milliliter methanol. Five microlitres of solution was analysed by HPLC monitored at 235, 270 and 310 nm.

### *In vitro* dry matter digestibility

The filter bag method developed by Ankom Technology Corporation (Fairport, NY) was used to determine the *in vitro* dry matter digestibility (IVDMD) of collected forage samples. Independent research has shown that the Ankom Technology filter bag method produces results similar to the conventional IVDMD method and has distinct advantages in comparison to conventional systems of analysis (Vogel *et al.*, 1999).

### Statistical analysis

Triplicate samples were collected for individual plants during each harvest, and the mean values of each of the four harvests were used for statistical analyses. Thus, all the quantitative data have four replications, with each harvest as one replicate. Data from each trait (chemical component, digestibility) were subjected to one-way analysis of variance. The significance of treatments was tested at the $P = 0.05$ level. Standard errors are provided in all tables and figures as appropriate.

## Supplementary material

Supplemental Figure S1 and Tables S1–3 can be accessed through the journal's website at http://www.blackwellpublishing.com/products/journals/suppmat/PBI/PBI040/PBI040sm.htm.

**Figure S1** Partial scheme for the likely pathways of lignin biosynthesis in tall fescue. PAL, phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; C3H, 4-hydroxycinnamate 3-hydroxylase; COMT, caffeic acid 3-O-methyltransferase; F5H, ferulate 5-hydroxylase; 4CL, 4-coumarate:CoA ligase; CCoA-3H, coumaroyl-coenzyme A 3-hydroxylase; CCoA-OMT, caffeoyl-coenzyme A O-methyltransferase; CCR, cinnamoyl-CoA reductase; CAD, cinnamyl alcohol dehydrogenase. **Table S1.** Cell wall polysaccharide content of transgenic tall fescue with modified expression of *CAD*. **Table S2.** Neutral sugar composition in hemicellulose and pectin of transgenic tall fescue with modified expression of *CAD*. **Table S3.** Phenolic compounds in transgenic tall fescue with modified expression of *CAD*.

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