Lignin, one of the major components of plant cell walls, has negative impacts on forage digestibility and bioethanol production through fermentation. Understanding the regulatory mechanism of lignin biosynthesis is essential to developing plants with altered lignin contents. The sorghum (Sorghum bicolor (L.) Moench) brown-midrib-6 mutant (bmr-6) has reduced levels of several key enzymes in the lignin biosynthesis pathway. Among the genes encoding these enzymes, the gene for cinnamyl alcohol dehydrogenase (CAD; EC 1.1.1.195) contains a nonsense mutation (responsible for bmr-6) that results in reduced CAD gene transcript levels. We found that the CAD coding region was 4225 bp and consisted of four exons separated by three introns. Bioinformatic analysis of the 5′-flanking region identified several putative binding sites for transcription factors. Moreover, a cross-species comparison of CAD genomic DNA sequences revealed evolutionary and structural variation among plant taxa. The expression level of the CAD gene was much lower in bmr-6 than in normal isoline (N-6) in root, stem, leaf, and midrib. Expression levels of other lignin biosynthesis genes were comparable in bmr-6 and N-6. These results suggest that the depression of CAD enzymatic activity in bmr-6 could affect enzymatic activity and post-translational regulation of other enzymes related to lignin biosynthesis.

Key Words: bmr-6, brown midrib, cinnamyl alcohol dehydrogenase (CAD), lignin, sorghum.
forage crops and bioenergy feedstocks, it is essential to understand the molecular bases for regulation of lignin biosynthesis in grasses.

The brown midrib mutants (bm or bmr) existed in maize (Zea mays L.) and sorghum (Sorghum bicolor (L.) Moench) have been widely used for studies of the regulation of lignin biosynthesis in grasses. These mutants show a reddish-brown pigment in the leaf midrib and stem. This trait is inherited as a simple recessive trait and is closely associated with improved forage digestibility (Barrière and Argillier 1993, Bittinger et al. 1981). In sorghum, several distinct mutants were produced by chemical mutagenesis (Porter et al. 1978), of which three mutants, bmr-6, bmr-12, and bmr-18, affect lignin content and composition in cell walls, and appeared to be the most promising for improvement of forage quality (Cherney 1980, Pillonel et al. 2003). In addition, Saballos et al. (2009) and Sattler et al. (2009) reported that base substitutions resulting in premature stop codons were identified in the coding regions of the genes encoding CAD in sorghum bmr-6 mutants and the binding sites of the enzyme have been changed, which caused the loss of enzymatic activity. However, previous studies showed that PAL and COMT activities were also depressed simultaneously with CAD in the bmr-6 mutant (Bucholtz et al. 1980, Pillonel et al. 1991, Tsuruta et al. 2003).

Here, reduction of CAD activity of bmr-6 at the mature lignification stage, which strongly influences forage digestibility, appeared to be due to a loss of the functional protein caused by a premature termination codon, consistent with the results of Saballos et al. (2009) and Sattler et al. (2009). We describe a comparative analysis of the structural features of the CAD gene and the spatiotemporal expression patterns of PAL, COMT, and CAD genes in the bmr-6 mutant. The possible impact of the structure and function in CAD in regulation of lignin biosynthesis is discussed.

Materials and Methods

Plant materials and extraction of nucleic acids

The sorghum brown-midrib-6 mutant (bmr-6) chemically induced from grain sorghum line, 954114 and corresponding normal segregate (N-6) were utilized for this study (Porter et al. 1978). These seeds were kindly provided by Dr. H. Nakagawa, National Agriculture and Food Research Organization, Tsukuba, Ibaraki, Japan. Seeds were grown in separate plastic pots under greenhouse conditions, as described previously (Tsuruta et al. 2003). The leaves of young plants were collected 20 days after sowing. Genomic DNA was extracted by the CTAB (cetyltrimethylammonium bromide) method (Murray and Thompson 1980). Samples of roots, stems, leaves, and midribs were harvested 40 to 60 days after sowing for the extraction of total RNA. Following harvest, all tissues were immediately placed in RNAlater solution (Ambion, TX, USA), incubated overnight at 4°C, and then stored at −20°C until RNA extraction. Total RNA was extracted from each tissue using an RNaseasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions.

Genome analysis

The genomic DNA of N-6 was used for construction of a genomic library in Lambda DASH II (Stratagene, CA, USA). Lambda DASH II vector arms were prepared by digestion with BamHI and NotI. The vector arms were ligated with genomic DNA fragments that had been partially digested with Sau3AI and packaged into phage particles using Gigapack III XL packaging extract (Stratagene). The amplified library was plated, and 2 x 10^6 recombinant phages were transferred onto duplicate Hybond N+ membranes (GE Healthcare Bio-sciences Corp., NJ, USA). The DNA was fixed to membranes with 0.4 M NaOH, and the membranes were hybridized with the CAD cDNA probe from sorghum in ECL hybridization buffer (GE Healthcare) at 42°C for 15 h. Following hybridization, the membranes were washed twice in washing buffer (6 M urea, 0.4% SDS, and 0.5× SSC) for 20 min at 42°C and twice in 2× SSC for 5 min at room temperature. Positive clones were analyzed by restriction enzyme mapping with two sorghum CAD cDNA clones (SbCAD IP2 and SbCAD 17) as probes (Tsuruta et al. 2007) and sequenced.

PCR amplification was used to determine the sequence of CAD genomic DNA from bmr-6 using primer pair gSbCAD-1 (5′-AAAGGAGGAGATGGGGAGCCT-3′/5′-GGGATGAGGTTGGAGC-3′) and gSbCAD-2 (5′-GCTCAAAAGATTCGCTCG-3′/5′-AAGGCATCCATACGCTGAC-3′) as probes (Tsuruta et al. 2007) and sequenced.

DNA gel blot analysis

Genomic DNA (2 μg) from N-6 and bmr-6 was digested
with BamHI, BglII, EcoRV, HindIII, Apal, DraI, EcoRI, and KpnI, fractionated by electrophoresis in a 0.6% TBE-agarose gel, and transferred onto Hybond N+ membrane (Roche Diagnostics, Mannheim, Germany) by alkaline transfer. The DNA was fixed to the membrane by baking at 120°C for 20 min, and the membrane was hybridized with the sorghum CAD cDNA probe in ECL hybridization buffer (GE Healthcare) at 42°C for 15 h. Following hybridization, the membrane was washed twice in washing buffer as described for genomic library screening (see “Genome analysis”, above).

Gene cloning

Sequences of cDNA encoding PAL (GenBank Accession Number AB565487; this paper), COMT (AY217766; Bout and Vermerris 2003), and CAD (AB288109; Tsuruta et al. 2007) in sorghum were used to design primers for expression analysis. A partial sequence of sorghum PAL cDNA was isolated by nested PCR with primers designed from the sequence of the maize PAL cDNA (L77912). First-strand cDNA was synthesized from total RNA extracted from stems of 50-day-old plants using the TaKaRa RNA PCR Kit (AMV) ver. 3.0 (TaKaRa). Reverse transcription was performed in a mixture containing 500 ng total RNA, 1× RT buffer, 1 mmol each dNTP, 5 mmol MgCl₂, 0.125 μmol oligo-dT primer, 1 unit of RNase inhibitor, and 0.25 units of AMV Reverse Transcript XL in a total reaction volume of 10 μl at 30°C for 10 min, 42°C for 30 min, and 99°C for 5 min. After cooling on ice, second-strand cDNA was synthesized by nested PCR with the first-strand cDNA as template. The first amplification was performed in a mixture containing 5 μl of RT product, 1× PCR buffer (TaKaRa), 2 pmol each dNTP, 2.5 mmol MgCl₂, 0.2 pmol each forward primer (MzPAL-F1: 5′-CTGCGAGGTGCTAAGCCGAAA-3′) and reverse primer (MzPAL-R1: 5′-GTTTTCCCA GTACGAGC-3′), and 0.5 units of Ex-Taq HS polymerase (TaKaRa) in a total reaction volume of 50 μl. The PCR cycle conditions were set at 95°C for 5 min, followed by 30 cycles of 95°C for 30 s, 58°C for 30 s, and 72°C for 1 min, in a TaKaRa PCR Thermal Cycler Dice. The amplification product was diluted 1:10 with sterile water, and 2 μl of the diluted cDNA was used as a template for the second amplification in a 20-μl reaction mixture. The second amplification was set up using primer MzPAL-F2 (5′-CTGCGACCCACAA GCTGAGCACC-3′) and M13 primer M4 with the same three-step program as for the first amplification. Amplification product was purified using a Quick Step 2 PCR Purification Kit (Edge BioSystems) for direct sequencing.

Sequence analysis

Sequencing reactions were performed with the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) and run on an ABI 3100 sequencer (Applied Biosystems). The computer software Genetyx ver. 12 (Genetyx Corporation, Tokyo, Japan) was used for bioinformatic analysis of the DNA sequences. Analysis of the promoter region to search for sequence motifs found in plant cis-acting elements was performed using the Plant Cis-acting Regulatory DNA Elements (PLACE) database (http://www.dna.afrc.go.jp/htdocs/PLACE/; Higo et al. 1999). Nucleotide sequences were compared using online Basic Local Alignment Search Tool (BLAST) programs (http://blast.ncbi.nlm.nih.gov/).

Semi-quantitative RT-PCR analysis

Total RNA samples from root, stem, leaf, and midrib at several developmental stages of 40 to 60 days after sowing were prepared and used for semi-quantitative RT-PCR analysis. For cDNA synthesis, each 2 μg of total RNA was reverse-transcribed by using the SuperScript III First-Strand Synthesis System (Invitrogen, CA, USA) following the manufacturer’s directions. The first-strand cDNA products were used as a template for PCR amplification with gene-specific primers SbPAL (5′-ACCTCGAGGAACCTC AAG-3′/5′-ACTCCTTGAGGCACGTGAG-3′), SbOMT (5′-CCATCTCTCATAGTGTGATTCC-3′/5′-CATGTATG TACGGACATCGG-3′) and SbCAD (5′-GACTCTGGT GACCTAC-3′/5′-GACCATCTTCAACTCATCTG-3′). All primers were designed from the 3′ end of the coding region of each gene at a standard melting temperature (Tm) of 56–58°C and amplification products ranging from 500 to 600 bp. The sorghum actin gene (X79378; Zhang et al. 2007) was used for the normalization of RNA quantity. Two microlitres of the RT mixture was used as a template for PCR in a 20-μl reaction mixture containing 1× PCR buffer, 0.2 mM each dNTP, 2.5 mM MgCl₂, 0.5 pmol each primer, and 0.5 units of Ex-Taq DNA polymerase. The PCR cycle conditions were set at an initial 94°C for 5 min, then 22 cycles of 40 s at 94°C, 40 s at 56°C or 58°C (SbPAL and SbOMT), and 40 s at 72°C. The number of PCR cycles was optimized by preliminary experiments so that the product formation was terminated before saturation. Negative controls with no reverse transcriptase were always included to ensure that product formation was not due to cross-contamination by genomic DNA. The analysis was repeated at least three times. Amplification products were separated in 1.5% agarose gel and stained with ethidium bromide. The gel images were digitally captured, and the band intensities were quantified by densitometry using the QuantiScan software (Biosoft, Cambridge, UK). The values were normalized against those of the corresponding actin gene reaction, and the means and standard deviations were calculated.

Results

Structure of the sorghum CAD gene

A genomic library of sorghum N-6 was screened by plaque hybridization with a CAD cDNA probe from sorghum. The screening of 2 × 10⁵ recombinant clones resulted in a single positive clone (gSbCAD1) that hybridized to the sorghum CAD probe. The gSbCAD1 clone was digested with several restriction enzymes, and restriction maps were constructed by DNA gel blot analysis using sorghum CAD probes (data not shown). The result revealed that the inserted
Characterization of the CAD gene in the sorghum bmr-6 mutant

DNA fragment was approximately 15 kbp in length. The sequence of the CAD genomic DNA was 4225 bp from the translation initiation codon to the termination codon and consisted of four exons (89, 114, 229 and 665 bp) separated by three introns (1761, 644 and 722 bp) (Fig. 1A). The combined sequence of the four exons was identical to the sequence of the full-length sorghum CAD cDNA. Amino acid sequences involved in the zinc binding regions are found in exons II and III, and a binding site for NADP (rather than NADPH) is found in exon IV.

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The nucleotide sequence of the 5'-flanking region (1.1 kbp) of the sorghum CAD gene is presented in Fig. 1B. A TATA-like motif was located at position 855–860 bp (Fig. 1A). The combined sequence of the four exons was identical to the sequence of the full-length sorghum CAD cDNA. Amino acid sequences involved in the zinc binding regions are found in exons II and III, and a binding site for NADP (rather than NADPH) is found in exon IV.

The nucleotide sequence of the 5'-flanking region (1.1 kbp) of the sorghum CAD gene is presented in Fig. 1B. A TATA-like motif was located at position 855–860 bp. The nucleotide sequence motifs for putative binding sites of transcription factors were searched against the PLACE database (Higo et al. 1999). One such motif, AAAG, was found at positions 19–22 bp and 180–183 bp. The sequence was identified as a core motif required for binding of Dof proteins, which are suggested to play a regulatory role in the gene expression in the maize α-zein promoter (Vincente-Carbajosa et al. 1997). Furthermore, a motif of six nucleotides (CCTACC) identified as the core of the consensus maize P (MYB homologue) binding site (MYB PZM), is located at position 493–498 bp (Grotewold et al. 1994).

Comparison of CAD genomic DNA structure across taxa

The coding region structures of CAD genes from several gymnosperms, dicots, and monocots are presented in Fig. 2. The CAD genes of sorghum N-6 and Oryza sativa were approximately 3.6 and 4.2 kbp in length, whereas those of
gymnosperms and dicots were 1.5 to 3.4 kbp (mean value of 2.2 kbp). In addition, the exon-intron structure in the 3’ part of the gene differed among taxa: In the case of the sorghum and O. sativa CAD genes, the polypeptide was encoded by four exons, whereas the polypeptides of gymnosperms and dicots were encoded by six and five exons, respectively.

Structure of CAD mutant allele in bmr-6

The CAD genomic DNA sequence of the sorghum bmr-6 mutant was obtained by PCR amplification with three sequence-specific primer combinations. A comparison of the sequences derived from N-6 and bmr-6 revealed a C-to-T transition in the third exon of bmr-6, leading to a single amino acid alteration (glutamine to termination codon), as described in recent studies of bmr-6 (Saballos et al. 2009, Sattler et al. 2009). To characterize the genomic organization of bmr-6, DNA gel blot analysis was conducted using genomic DNA from sorghum N-6 and bmr-6. The genomic DNAs were digested with eight restriction enzymes and hybridized with a sorghum CAD cDNA probe. The hybridization pattern revealed one or two strong bands in each digest (Fig. 3). The patterns suggest that CAD copy number is consistent between the two genotypes, with a single copy in each, and that no large structural mutation (e.g., a deletion or insertion) distinguishes the CAD gene of bmr-6 from that of N-6 (Fig. 3).

Expression of genes encoding CAD and other enzymes in lignin biosynthesis

To characterize the expression profile of the CAD gene in bmr-6, the accumulation of CAD transcripts in N-6 and bmr-6 was compared in different tissues at several developmental stages (Fig. 4). Transcript accumulation was measured by semi-quantitative RT-PCR of total RNAs extracted from root, stem, leaf, and midrib. An example of a single replication of this analysis is shown in Fig. 4A; quantitation across three replicates is shown in Fig. 4B. In N-6, expression in root and midrib peaked in 50-day-old plants and then decreased, and expression in stem was highest in 40- to 60-day-old plants. Expression in N-6 leaf gradually decreased during development. The tissue-specific patterns of expression in CAD gene correlate to the accumulation of lignin and the variation of the enzymatic activity during developmental stages (Tsuruta et al. 2003). In contrast, the CAD expression pattern in bmr-6 showed no significant change in any of the tissues at any developmental stages, and was lower in all tissues than in N-6 (Fig. 4B).
Characterization of the CAD gene in the sorghum bmr-6 mutant

In several previous studies, bmr-6 appeared to have reduced activities of several lignin biosynthesis enzymes, including PAL, COMT, and CAD (Palmer et al. 2008, Pillonel et al. 1991, Tsuruta et al. 2003). To examine the effect of transcript accumulation on activities of these enzymes, we compared transcript levels in N-6 and bmr-6 stems during several developmental stages by using semi-quantitative RT-PCR (Fig. 5). Primers for PAL gene expression analysis were designed from the sequence of sorghum PAL cDNA. The sorghum PAL cDNA sequence was partially cloned by PCR with primers designed from maize PAL cDNA sequence and analyzed for homology by BLAST. The partial sorghum PAL cDNA sequence obtained in this study was highly homologous to maize in the 3' coding region (88.3% nucleotide identity). High levels of sequence similarity were also found with Saccharum and rice (95.4% and 91.1%, respectively). Primers for COMT gene expression analysis designed from the sorghum COMT cDNA sequence were identified by Bout and Vermerris (2003). All specific primers were designed for the 3' end of the coding region of each gene, and semi-quantitative RT-PCR analyses were performed for all genes. The results showed that expression levels of PAL and COMT were not significantly reduced in bmr-6 relative to normal isolate N-6, whereas expression of the CAD gene was significantly reduced in the bmr-6 mutant.

Fig. 4. Semi-quantitative RT-PCR expression analysis of CAD genes from sorghum N-6 and bmr-6. (A) A single representative experiment showing expression of the CAD gene in four different organs at three developmental stages. Total RNA isolated from root, stem, leaf, and midrib was subjected to RT-PCR semi-quantitative analysis using the sorghum actin gene as an internal control. RNA samples without reverse transcriptase (CAD RT [−]) were amplified as controls for DNA contamination. N, N-6; B, bmr-6; C, SbCAD cDNA; M, 500-bp ladder marker. (B) Change of mRNA accumulation in four different organs at three developmental stages. Amplification products were quantified by densitometry using QuantiScan software. The sorghum actin gene (X79378) was used as a control for RNA input, and expression levels of each gene were adjusted by the corresponding amount of actin gene mRNA. The analysis was repeated at least three times. The values are the average of three replications; error bars indicate standard deviation. ○, N-6; ●, bmr-6.
CAD is a key enzyme in lignin biosynthesis and has been targeted for manipulation of lignin content and composition by genetic engineering. As part of an effort to understand the molecular basis for the regulation of lignin biosynthesis in grasses, we isolated and characterized normal CAD genomic DNA sequence from sorghum, including the coding region and the 5′-flanking region (1064 bp). Bioinformatic analysis of the 5′-flanking region suggested the presence of several putative binding sites for transcription factors. These same motifs were also binding sites for transcription factors in the promoter region of CCR (cinnamoyl CoA reductase), another gene involved in lignin biosynthesis (Larsen 2004). It is therefore likely that the genes encoding CCR and CAD have a common regulatory mechanism. The structure and function of the CAD gene promoter sequence in warm-season grasses are currently unknown, although the transcriptional regulation of CAD was investigated with promoter-GUS gene fusions to the CAD genes in poplar (Feuillet et al. 1995) and rice (Tobias and Chow 2005). A study of the simultaneous regulation of lignin biosynthesis genes in sorghum may provide valuable information on the mechanism of lignification in other warm-season grasses.

The sorghum CAD gene contains four introns and three exons, corresponding to the structure found in rice (Tobias and Chow 2005). Our comparison of CAD gene sequences from different species revealed important variations in their size and structure among plant taxa. This result is in contrast to the gene structure of CCR, the other enzyme in the monolignol-specific branch of lignin biosynthesis, whose intron/exon structure is conserved across *Lolium perenne* (monocot), *A. thaliana*, and *E. gunnii* (McInnes et al. 2002). Removal of introns over evolutionary time has occurred in several genes such as *rbcS* (Wolter et al. 1988) and that for triosephosphate isomerase (Marchionni et al. 1986). These findings suggest that the presence of multiple introns plays an important role in the acquisition of gene function, such as the incorporation of new functional domains; these introns are gradually lost during evolution. Studies of COMT genes in different species have indicated that the size and number of introns varies considerably among species (Besse et al. 2009, Bout and Vermerris 2003, Guillet-Claude et al. 2004).

The CAD gene has lost introns during the evolutionary process, so we speculate that the presence of these introns in ancestral species was essential for the CAD gene to acquire new functional properties. Phylogenetic analysis of CAD amino acid sequences supports this hypothesis. In many studies of the molecular evolution of CAD, phylogenetic analysis classified the gymnosperms and angiosperms into two major groups, reflecting the ancient divergence of the phylogenetic clusters. MacKay et al. (1995) reported that the branch lengths, which indicate the degrees of substitution of amino acid sequences, were longer and more variable within the angiosperms than within the gymnosperms. It is possible that the high level of variability of the CAD coding region caused its gene structure to differentiate among plant species. It will be necessary to conduct further comparative analyses of the CAD gene across large numbers of species to reveal whether the loss of introns is a general tendency.

**Fig. 5.** Comparison of PAL, COMT, and CAD expression at different developmental stages of N-6 and bmr-6 using semi-quantitative RT-PCR. Amplification products were quantified by densitometry using QuantiScan software. The sorghum actin gene (X79378) was used as a control for RNA input, and expression levels of each gene were adjusted by the corresponding amount of actin gene mRNA. The values are the average of three replications; error bars indicate standard deviation. Light gray and dark gray bars show the values for N-6 and bmr-6, respectively.

**Discussion**

CAD is a key enzyme in lignin biosynthesis and has been targeted for manipulation of lignin content and composition by genetic engineering. As part of an effort to understand the molecular basis for the regulation of lignin biosynthesis in grasses, we isolated and characterized normal CAD genomic DNA sequence from sorghum, including the coding region and the 5′-flanking region (1064 bp). Bioinformatic analysis of the 5′-flanking region suggested the presence of several putative binding sites for transcription factors. These same motifs were also binding sites for transcription factors in the promoter region of CCR (cinnamoyl CoA reductase), another gene involved in lignin biosynthesis (Larsen 2004). It is therefore likely that the genes encoding CCR and CAD have a common regulatory mechanism. The structure and function of the CAD gene promoter sequence in warm-season grasses are currently unknown, although the transcriptional regulation of CAD was investigated with promoter-GUS gene fusions to the CAD genes in poplar (Feuillet et al. 1995) and rice (Tobias and Chow 2005). A study of the simultaneous regulation of lignin biosynthesis genes in sorghum may provide valuable information on the mechanism of lignification in other warm-season grasses.

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promoters of the CAD promoter region are transcription factor binding sites that may direct the timing and localization of lignin biosynthesis genes are transcriptionally regulated, and the genes involved in the phenylpropanoid pathway and monolignol biosynthesis are under some form of coordinate control. Reduced enzymatic activities of PAL, COMT, and CAD have been observed in crude extracts of whole plants and stems of bmr-6 (Palmer et al. 2008, Pillonel et al. 1991, Tsuruta et al. 2003). Many of the genes involved in the phenylpropanoid pathway and monolignol biosynthesis are transcriptionally regulated, and promoters of some of these genes contain common sequence elements that may direct the timing and localization of lignification (Zhong and Ye 2007). Several motifs found in the CAD promoter region are transcription factor binding sites that appear in the promoter region of CCR. Analysis of the promoters of the Eucalyptus gunnii CCR (EgCCR) and CAD (EgCAD2) genes expressed in Arabidopsis indicated that both promoters drove expression in a coordinated manner in both primary and secondary xylem tissues in stems and roots (Baghdady et al. 2006). Because expression of lignin biosynthesis genes is coordinate regulated by transcriptional regulators, including these binding sites (Rogers and Campbell 2004), mutation in transcription factors and their recognition sites could affect expression of some genes in this pathway. One member of the LIM family of transcriptional regulators has been shown to have the capacity to regulate the expression of some genes in phenylpropanoid metabolism, suggesting that the genes encoding lignin biosynthesis enzymes are under some form of coordinate control (Kawaoka et al. 2000). However, the expression levels of the PAL and COMT genes were relatively similar in both bmr-6 and N6, whereas expression of the CAD gene was strongly reduced in the bmr-6 mutant. These data suggest that some form of feedback system or post-transcriptional modification (PTM) could operate in lignifying cells, interrupting monolignol synthesis and transport by the inactivated enzymes. Several studies of the control of PAL gene expression have indicated that flux into the phenylpropanoid pathway is controlled, at least in part, by feedback regulation or PTM (Allwood et al. 1999, Blount et al. 2000, Cheng et al. 2001). PAL enzyme activity was also shown to be inhibited by cinnamaldehyde (Fujita et al. 2006). If our prior assumption is correct, regulation of lignin biosynthesis may be achieved by accurately adjusting the activity of the CAD enzyme. It will be necessary to verify the effect of reduced CAD activity on the flux in the phenylpropanoid pathway of bmr-6 mutant plants. This would provide additional insight into the control of metabolic flux in lignification.

Although many of the valuable forage and biomass crop species are grasses, considerable research on modification of lignin content and composition has been done on dicot species (Wang and Ge 2005). Here, expression analysis of lignin biosynthesis genes suggests that the absence of a functional CAD enzyme may affect flux into the phenylpropanoid pathway. These observations support the conclusion that CAD has a significant impact on lignin biosynthesis and could be a target enzyme for manipulation of lignin content and composition in grasses. The discovery of the molecular basis of the mutation in bmr-6 provides a new strategy for the regulation of lignin biosynthesis using transgenic approaches such as antisense and RNAi methods. The plasmids used for these approaches have usually been constructed using full-length CAD cDNA sequences. However, a short segment of exon IV may be useful as the target region, because the base substitution in bmr-6 is positioned at the 3′-end of exon III. In the case of grasses, this region is not expected to be separated by the introns and is relatively conserved among species; therefore, it should be able to be amplified from genomic DNA by using homologous primers across several grass species. There is still only limited information on the genomics and genetics of grasses used for forage and bioenergy. Additional knowledge of lignin biosynthesis genes in bmr mutants would facilitate studies of functional correlates of individual genes in the phenylpropanoid and monolignol biosynthesis pathways. In turn, this knowledge could contribute to the improvement of processing efficiency of grass feedstocks for forage digestibility and bioethanol production through fermentation.

Acknowledgements

We thank Dr. Yoshiaki Nagamura of the National Institute of Agrobiological Sciences, Tsukuba, Ibaraki, Japan for his technical support with genomic analysis. This work was supported by the Japan Livestock Technology Association, Tokyo, Japan.
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