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Lignin Content and Digestibility in Transgenic Bahiagrass (*Paspalum notatum* Flügge) Obtained by Genetic Manipulation of Cinnamyl Alcohol Dehydrogenase Gene

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Abstract: Lignin is generally known as one of the major impediments for utilizing the potential of many forage plants because it limits digestibility and concomitantly, livestock productivity. Warm-season grasses yield high biomass but the digestibility is low due to their high lignin content. Cinnamyl alcohol dehydrogenase, a key enzyme that catalyzes the last stage in the lignin biosynthesis pathway, has been genetically altered in few grasses to increase their digestibility and forage quality. The aim of this study was to reduce the lignin content in a warm-season grass, bahiagrass (*Paspalum notatum* Flügge), by suppression of cinnamyl alcohol dehydrogenase gene expression. Using particle bombardment, cinnamyl alcohol dehydrogenase gene constructs with the antisense and RNAi vector under the control of the maize ubiquitin promoter were introduced into bahiagrass calli. The lignin content in most of the transgenic lines was significantly reduced, although the agronomic characteristics (plant height, leaf length, leaf width, tiller number, heading tiller and dry matter) differed between individuals. The *in vitro* dry matter digestibility of four of the nine transgenic plant significantly increased by 5.6-10.4% units. These results suggest that the molecular modification of the cinnamyl alcohol dehydrogenase gene function in the monolignol pathway significantly improved the feeding characteristics of the bahiagrass and that this approach could be used to improve the forage quality of other warm-season grasses. By utilizing their potential, novel cultivars could be developed that are amenable for intensified and sustainable grass forage production.

Key words: Bahiagrass, cinnamyl alcohol dehydrogenase, digestibility, lignin, transgenic plant

INTRODUCTION

Forages are vital for sustainability in agricultural enterprises and contribute greatly to world economy. Grasses supplies the major feedstuffs for ruminants and on a worldwide basis, it accounts for up to 60-90% for ruminant feed requirements (Wilkins and Humphreys, 2003). By 2050, total meat and milk consumption in developing countries is expected to at least double (FAO, 2006). About 40% of the world's agricultural areas are covered with grasslands (Suttie *et al.*, 2005) but the increasing global population, urbanization and increasing incomes are expected to further demands for livestock products (Thornton, 2010). Therefore, for efficient forage production, there is a concern to intensify production practices. However, animal production is hampered in many countries by the low quality of available forages. Bahiagrass is a warm-season grass that is a widely grown forage crop, particularly in tropical and subtropical regions whose mode of reproduction is either

diploid-sexual or obligate apomictic in tetraploid genotypes (Burton, 1946; Hirata *et al.*, 2003). The grass performs well under severe drought and grazing stress; however, one of the major limitations for cattle production on bahiagrass and other warm season forage grasses, is the poor forage quality in comparison to most temperate grasses or non-grass forage (Akin and Burdick, 1975; Reid *et al.*, 1990). Known for their high biomass yields, one major problem with warm-season grasses is their high lignin content, which negatively correlates to intake, digestibility and animal performance. Improved forage quality of these high-yielding forage grasses could be augmented through plant breeding efforts to maximize their potentials.

Lignin is an integral component for structural dynamics in plant growth and development. The most important function of lignin is to provide strength and rigidity to cell wall, plant defense and provides hydrophobicity for water and solute transport in the vascular system (Rogers and Campbell, 2004;

Vanholme *et al.*, 2008). With regards to forage use, lignin is considered an anti-quality component in forages by limiting digestibility (Moore and Hatfield, 1994). Approximately one third of the cell wall of a forage grass was estimated to be undigested (Wilson and Mertens, 1995) as accessibility of plant cell wall polysaccharides to enzymatic and microbial digestion in the rumen is often limited by the presence of the phenylpropanoid polymer lignin in the vascular tissues of forages walls (Jung *et al.*, 2012).

With the advancement of biotechnology, there has been rapid progress in understanding lignin biosynthesis and its molecular regulations (Vanholme *et al.*, 2008, 2010). Biosynthesis of lignin components includes hydroxycinnamyl alcohols, coniferyl alcohol and sinapyl alcohol, with trace amounts of p-coumaryl alcohol. Current understanding on monolignol biosynthesis involves 10 enzymes (Boerjan *et al.*, 2003). Among these, (hydroxyl) cinnamoyl CoA reductase (CCR; E.C. 1.2.1.44) and (hydroxy) cinnamyl alcohol dehydrogenase (*CAD*; E.C. 1.1.1.195) are specific for lignification and have been employed as target enzymes for the manipulation of lignin content or composition by genetic engineering (Baucher *et al.*, 2003).

CAD catalyzes the reduction of the monolignol aldehydes, sinapaldehyde, coniferaldehyde and p-coumaraldehyde into alcohols prior to their incorporation into lignin polymers (Mansell *et al.*, 1974). Down-regulation of *CAD* could increase digestibility on dry weight basis, alter cell wall architecture, reduce lignin level and incorporate phenolic aldehydes into lignin in brown midrib mutants in sorghum and maize (Sattler *et al.*, 2010). Transgenic analyses confirmed that *CAD* can be a target enzyme for improvement of *in vitro* dry matter digestibility (IVDMD) in alfalfa (Inoue *et al.*, 1998), switchgrass (Fu *et al.*, 2011a), tall fescue (Chen *et al.*, 2002, 2003) and sugarcane (Jung *et al.*, 2013). However, there were only few reports of transgenic analyses that exist for this category. Most of the studies involved in the molecular targeting of lignin biosynthesis genes for the improvement of forage digestibility has been reported on dicots, whereas only few reports exist in warm-season forage grasses (Jung *et al.*, 2012). Transgenic approaches have the potential for adding new useful metabolic pathways to targeted plants. We have established an efficient transformation system in bahiagrass (Gondo *et al.*, 2005). Therefore, we considered that it would be possible to create novel transgenic bahiagrass plants with reduced lignin and improved forage digestibility.

In this study, our aim was to reduce lignin content and improve forage digestibility in bahiagrass by

repressing *CAD* activity using antisense and RNAi gene silencing approaches. The main goal of this study was to improve forage quality of bahiagrass for livestock production.

MATERIALS AND METHODS

Formation of embryogenic callus: Embryogenic calli were induced as previously described (Gondo *et al.*, 2005). Briefly, seeds of diploid bahiagrass (*Paspalum notatum* Flügge cv. Pensacola) with lemmas and paleas removed were surface-sterilized in 70% (v/v) ethanol for 1 min and 2% (v/v) sodium hypochlorite for 15 min, followed by three washings with sterile water. Seeds were cultured at 80-90 seeds per 90 mm petri dish on filter paper (Advantec Toyo Co., Japan) and 5 mL liquid MS medium (Murashige and Skoog, 1962) supplemented with 2.0 mg L⁻¹ 2, 4-dichlorophenoxyacetic acid (2, 4-D), 0.1 mg L⁻¹ 6-benzylaminopurine (BAP) and 50 μM CuSO₄ (DBC medium). All media were adjusted to pH 5.6-5.8 prior to autoclaving at 121 °C for 15 min and all cultures were incubated at 31 °C in the light (30 μmol m⁻² sec⁻¹). After 14 days of culture, primary calli were transferred to fresh DBC medium solidified with 0.3% Gelum gum (Wako, Japan). Single seed-derived, yellowish-white and friable callus, presumed to be embryogenic was selected and small callus clumps were subcultured every 14 days onto the same medium.

Vector construction: One *CAD* antisense vector and four RNAi vectors were constructed, using either a full-length *SbCAD1098* or a *SbCAD687* bp 3'-fragment of sorghum *CAD* cDNA (GenBank Accession No. AB288109; Tsuruta *et al.*, 2007), that were assembled in opposite direction, driven by the maize ubiquitin-1 (Ubi) promoter and the first intron and terminated by the nos terminator from pAHC25 (Christensen and Quail, 1996) (Fig. 1). A *CAD* antisense vector (pCADanti) was constructed by insertion of *SbCAD1098* to the expression vector. RNAi vectors were constructed using *SbCAD1098* and *SbCAD687* in opposite orientations separated by a linker sequence derived from the *uidA* gene as shown in Fig. 1.

Bombardment with particle inflow gun: The self-built particle bombardment apparatus (spray gun) was constructed as described in a previous report (Akashi *et al.*, 2002). Four hour prior to bombardment, embryogenic calli were transferred to MS-DBC medium supplemented with 0.3 M mannitol and 0.3 M sorbitol were left on this medium for 16 h after bombardment for osmotic post-treatment. The plasmid pDM302 (Cao *et al.*, 1992), containing the bialaphos resistance gene (*bar*)

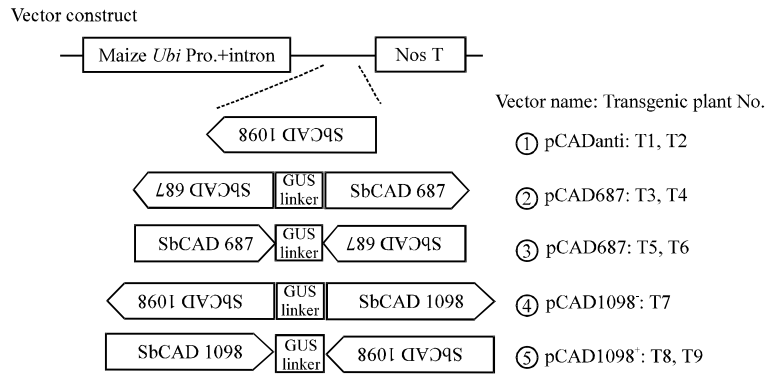


Fig. 1: Schematic diagram of sorghum *CAD* (*SbCAD*) antisense vector, RNAi vector and transgenic bahiagrass lines, each with their respective vectors. *SbCAD1098*: Full-length sorghum, *CAD*: cDNA, *SbCAD687*: 3'-fragment of sorghum *CAD* cDNA, GUS linker: Internal 370 bp sequence of *uidA* gene, Maize *Ubi* pro+intron: Maize ubiquitin promoter and first intron, Nos T: Nopaline synthase terminator. Each vector name (pCADanti, pCAD687⁻, pCAD687⁺, pCAD1098⁻ and pCAD1098⁺) and transgenic plant number (T1-T9) represented, respectively

under the control of the rice actin promoter (*Act1*) was co-transformed with each antisense vector and RNAi vector (Fig. 1), respectively. Plasmid DNA was precipitated onto gold particles (1.5-3.0 μm diameter; Aldrich, USA) as described by Gondo *et al.* (2005). Bombardment was carried out at a reduced air pressure of -0.1 MPa, a target distance of 9.6 cm, helium pressure of 5 kg cm^{-2} and single shots per plate.

Selection and regeneration of transgenic plants:

Following bombardment and osmotic post-treatment, calli were placed on DBC medium for 3 days and subsequently sub-cultured several times for 14 day periods on the same medium containing 5 mg L^{-1} bialaphos (Wako). After 60-70 days of subculture under selective conditions, bialaphos-resistant calli were transferred to hormone-free MS medium for shoot bud induction. After 20-30 days of culture, calli were transferred to MS medium with 2 mg L^{-1} BAP and 0.01 mg L^{-1} 1-Naphthaleneacetic acid (NAA) for shoot growth. All regenerated shoots were transferred to hormone-free $\frac{1}{2}$ MS medium. After 20-30 days, rooted plants were analyzed by Polymerase Chain Reaction (PCR) for the presence of *CAD* vectors. PCR-positive plants were transferred to soil and grown to maturity in the greenhouse where temperatures were dependent on the climatic conditions in Miyazaki, Japan.

DNA isolation, PCR and DNA gel blot analysis: For PCR, plant genomic DNA was extracted from the leaf tissue (0.1-0.5 g) of rooted plants using the Isoplant Isolation Kit (Nippongene, Tokyo, Japan). Primers for the internal 370 bp GUS gene linker fragment of RNAi vectors were 5'-CGTGGTGATGTGGAGTATTGC-3' and

5'-GCACACTGATACTCTTCACTCC-3' and primers for the internal 687 bp *CAD* gene fragment of an antisense vector were 5'-AAGAGGAGAATGGGGAGCCT-3' and 5'-AACGTTG CACGCCGTCAGT-3'. The reaction mixture (20 μL) contained 250 ng DNA, 200 μM dNTPs, 50 nM of each primer, 0.5 units *Taq* polymerase and *Taq* polymerase buffer (Takara Bio, Otsu, Japan). Samples were first heated to 94°C for 5 min and then subjected to 40 cycles of the following temperature conditions: 30 sec at 94°C, 30 sec at 58°C and 1 min at 72°C for *uidA* gene primers and 30 sec at 94°C, 30 sec at 64°C and 1 min at 72°C for *CAD* gene primers. Finally, samples were subjected to 72°C for an additional 7 min and stored at 4°C until used. PCR products were separated by electrophoresis in 1% agarose gel electrophoresis. The DNA samples were stained with by EZ-Vision Three dye (Amresco, Solon, OH, USA) before loading on agarose gels. DNA fragments were visualized under ultraviolet light and were photographed using a bio imaging system (Gene Genius 2; Syngene, Cambridge, UK).

DNA (2 μg) for DNA gel blot hybridization was extracted from leaf tissue of greenhouse-grown plants by the CTAB method. The DNA was *SacI*-digested, separated on a 0.8% agarose gel and transferred to Hybond nylon membranes (Roche, Mannheim, Germany). Membranes were hybridized using 687 bp fragment of *CAD* from antisense vector labeled with a PCR DIG probe synthesis kit (Roche, Mannheim, Germany). Hybridization signals were visualized on FUJI X-ray film (Fuji, Tokyo, Japan).

Morphological characteristics and dry matter yield: To further evaluate the impact of *CAD* down-regulation on

agronomic traits of transgenic bahiagrass, young transgenic plants were grown in the greenhouse in April 26, 2011. Five transgenic plants of the same line were planted in one pot and were replicated three times per each line. Transformed plants were compared to the control plants in terms of morphological characteristics and dry matter yield. Five agronomic traits include plant height, leaf blade length, leaf blade width, number of tillers and heading tillers. Plant height, leaf blade length and leaf blade width were analyzed during flowering time, while number of tillers and heading tillers were analyzed in August 2, 2011. Subsequently, samples were harvested to determine dry matter yield. Yield measurements were obtained from each pot with three replications and their corresponding mean was analyzed. The mean from these measurements was used to define the morphological traits described in this study.

Lignin and digestibility measurements: Whole plant samples were collected and dried for 24 h at 70°C using an air-forced oven. After drying, samples were ground with a hammer mill and were sieved through a 1 mm mesh for

the determination of Acid Detergent Lignin (ADL) and *in vitro* digestibility (IVDMD). The ADL procedure was determined using the methods of Goering and Van Soest (1970) and IVDMD was determined using the pepsin-cellulase method (Goto and Minson, 1977). To obtain mean values for both ADL and IVDMD, three plant samples were analyzed from each pot with three replications each.

The replicated data was tested for the significance using Tukey's test.

RESULTS AND DISCUSSION

Transformation of bahiagrass: Calli resistant to bialaphos were obtained in 1.7% (104 resistant calli/6240 bombarded calli) of the bombarded calli and co-transformation with *CAD* construct was confirmed in half of the 52 calli by PCR. However, down-regulation of *CAD* hindered plant regeneration in many of the transformed calli and the total of 9 transformed lines was obtained. The presence of *SbCAD* gene in 9 transgenic lines was confirmed by Southern hybridization analysis (Fig. 2). The control plant

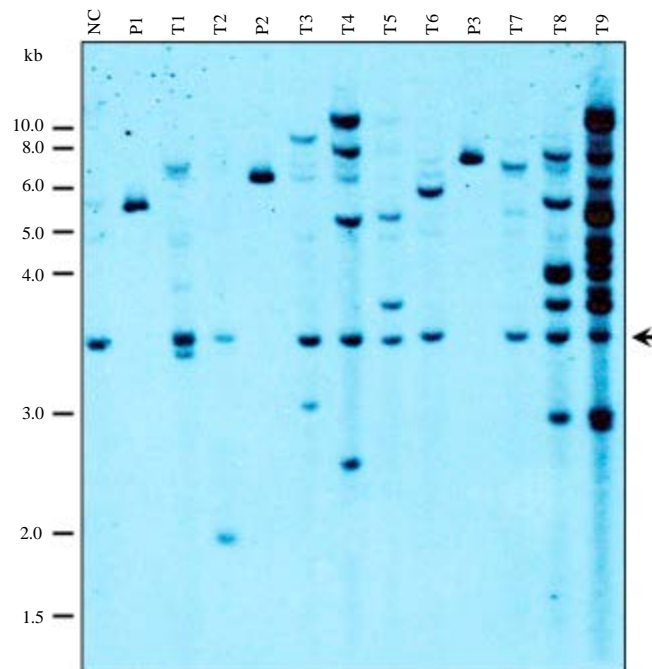


Fig. 2: DNA gel blot analysis of transgenic bahiagrass plants. Two microgram genomic DNA, isolated from leaf tissue of non-transformed (NC) and 9 independent transformed lines (T1-T9), were digested with *SacI*. Hybridization was carried out with a DIG-dUTP-labeled *SbCAD* gene probe. P1, P2 and P3 are positive control of 5 pg *SacI*-digested plasmid pCADanti, pCAD687, pCAD1098, respectively. The arrow indicates the endogenous band derived from bahiagrass *CAD* gene. Transgenic plants (T1-T9) are linked to the transgenic lines of Fig. 1

showed only the 3.5 kbp-hybridized bands, which was derived from the endogenous *CAD* gene in bahiagrass. The *SbCAD* gene, which was derived from sorghum, was integrated at different sites and various copy numbers ranged from 1 to 13 per genome.

In this study, only 9 transgenic lines were obtained from 52 bialaphos-resistant calli co-transformed with *CAD* constructs. Many shoots showed reddish to brown pigmentations and the shoots died in the process of regeneration. It is conceivable that the strong down-regulation of the *CAD* gene may hinder plant regeneration by the high aldehyde pigmentation because the *ubi* promoter was strong and constant in regulating the expression of *CAD* in all bahiagrass tissues. Therefore, the selection of a promoter that is time-specific and tissue-specific to regulate expression are important factors for the effective down-regulation of lignin without affecting plant growth. Positive results were obtained when *Adh1* (alcohol dehydrogenase promoter) was used for COMT down-regulation in maize regulating expression in vascular and schlerenchyma tissues (Piquemal *et al.*, 2002). Recently, Yang *et al.* (2013) reported narrowed down lignin biosynthesis in C4H by using a vessel-specific promoter for C4H in *Arabidopsis thaliana*, which generated plants with phenotypes similar with those of wild-type plants.

Lignin content and *in vitro* dry matter digestibility in transgenic bahiagrass: To analyze the effect of suppressing *CAD* on lignin content and forage digestibility, we measured the ADL (as percentage dry matter) and IVDMD of transgenic and control lines. Plant samples utilized in both analyses were taken from the first cutting in August 2, 2011 with three samples per each transgenic line. As shown in Fig. 3, lignin contents of all transgenic lines except for T2 and T4 lines were significantly lower than seed-derived control plants ($p < 0.05$). Whole plant mean ADL% of lignin content was 4.7 and that of the control was 5.6, which represents a 16% reduction in lignin content. Among transgenics, T1, T4 and T6 lines had significantly higher level of IVDMD ($p < 0.01$) (Fig. 3). Especially, T1 line showed the highest IVDMD (63.4%) in all transgenic lines and the digestibility increased 19.6% compared with control level.

Digestibility is an essential characteristic of good forage quality but is often hampered by lignin, especially in warm season grasses (Akashi *et al.*, 2003). Genetic engineering is a key to reducing lignin content and increasing digestibility as reported in dicot, forage crops and temperate grasses. However, there were few reports on warm-season grasses that indicate high lignin content on low digestibility. With the present study, suppression

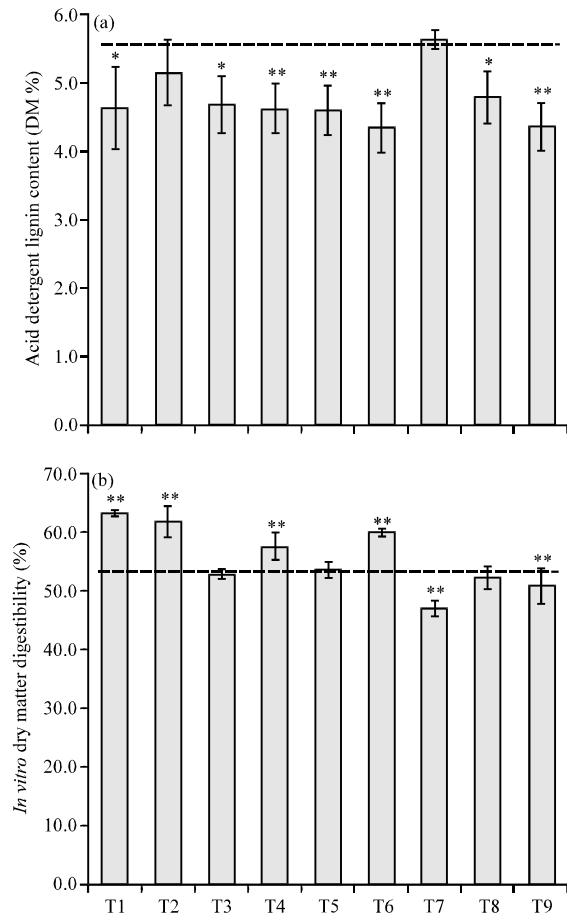


Fig. 3(a-b): (a) Acid detergent lignin content and (b) *In vitro* dry matter digestibility in transgenic bahiagrass. The horizontal line indicates the relative level of the control plants. Transgenic plants as represented (T1-T9), *,**Statistically significant difference between control and transgenic plants at $p < 0.05$, 0.01 level, respectively by T-test

of *CAD* significantly led to reduction of lignin content in almost all bahiagrass transgenic lines. In addition, 4 out of 7 transgenic lines achieved significantly higher digestibility than that of the control lines. Our reports are consistent with other reports involving transgenic plants belonging to Graminae where *CAD* suppression distinctively showed increase in digestibility due to lowered lignin quantity (Saathoff *et al.*, 2011). Down-regulation of other genes in the lignin pathway was reported in other grass species. *O*-methyltransferase decreased by 13% in lignin content with increased digestibility without affecting dry matter yield in maize (He *et al.*, 2003) and down-regulation of COMT activity



Fig. 4(a-d): Phenotypes of transgenic bahiagrass plants. Pot size was 25 cm in diameter. (a) NC (non-transgenic plant) derived from a seed. T1, T4, T6 represents transgenic plants with high digestibility in Fig. 3, (b) T1 (creeping phenotype), (c) T4 (dwarf phenotype) and (d) T6 (high biomass phenotype)

had similar positive results in switchgrass (Chen *et al.*, 2004; Fu *et al.*, 2011b) and sugarcane (Jung *et al.*, 2013).

RNAi gene silencing vectors have usually been constructed using homozygous target gene. In maize, *OMT* down-regulation used heterozygous sorghum gene constructs and full length or 5'-fragment *OMT* gene in antisense direction were introduced (He *et al.*, 2003). Lignin contents decreased in transgenic maize plants with 5'-antisense *OMT* fragment. Similar results were also identified in our experiment. Transgenic bahiagrass plants with heterozygous sorghum *CAD* gene assembled in antisense direction had reduced lignin content and 687 bp 3'-*SbCAD* fragment indicated the same effect as that of the full length fragment using RNAi vector. It is considered that sorghum 687 bp 3'-*SbCAD* fragment has high homology with other forage grasses, especially with C4 grasses.

Agronomic traits of transgenic bahiagrass: Transgenic plants were further evaluated for agronomic performance by means of biomass metrics among which includes plant height, leaf blade length, leaf blade width, number of

tillers, heading tiller and dry matter yield. Transgenic plants showed tendency of high leaf blade length, low number of tillers and percentage of heading tiller. The T1, T2 and T4 lines increased digestibility and showed tendency of small phenotype, low tiller number and percentage of heading tiller (Fig. 4). However, T6 line showed significant increase in plant height, leaf blade length and dry matter yield. This line also indicated increasing digestibility and biomass production.

The extent for which lignin content is reduced typically has impact on the plant's overall development (Vanholme *et al.*, 2008) and this modification is dependent on the type of gene down-regulated (Hisano *et al.*, 2009). Suppression of enzymatic activity in the earlier stage of the lignin biosynthetic pathway showed severe lignin reductions with concomitant perturbations in plant growth and development such as short stature and less total biomass yield (Elkind *et al.*, 1990; Van der Rest *et al.*, 2006; Do *et al.*, 2007; Leple *et al.*, 2007; Derikvand *et al.*, 2008). However, *CAD* and *COMT*, which are enzymes in the last stage of the lignin pathway, did not affect plant growth and biomass yield after their down-regulation in

transgenic tall fescue and switchgrass (Chen *et al.*, 2003; Fu *et al.*, 2011a; Jung *et al.*, 2012). As for our results, *CAD* down-regulation had affected the growth of some transgenic plants, although 3 lines of transgenic plants (T3, T5 and T6) show high yield remarkably (Fig. 5). Especially, T6 line indicated high digestibility and increased biomass production.

Plant breeding and animal trials have been reported to demonstrate that improving forage digestibility while maintaining or improving forage yield significantly improved animal performance, which had increased economic gains for livestock production systems (Mitchell *et al.*, 2005). It has been reported that a 1% increase in *in vitro* dry matter digestibility (IVDMD) in

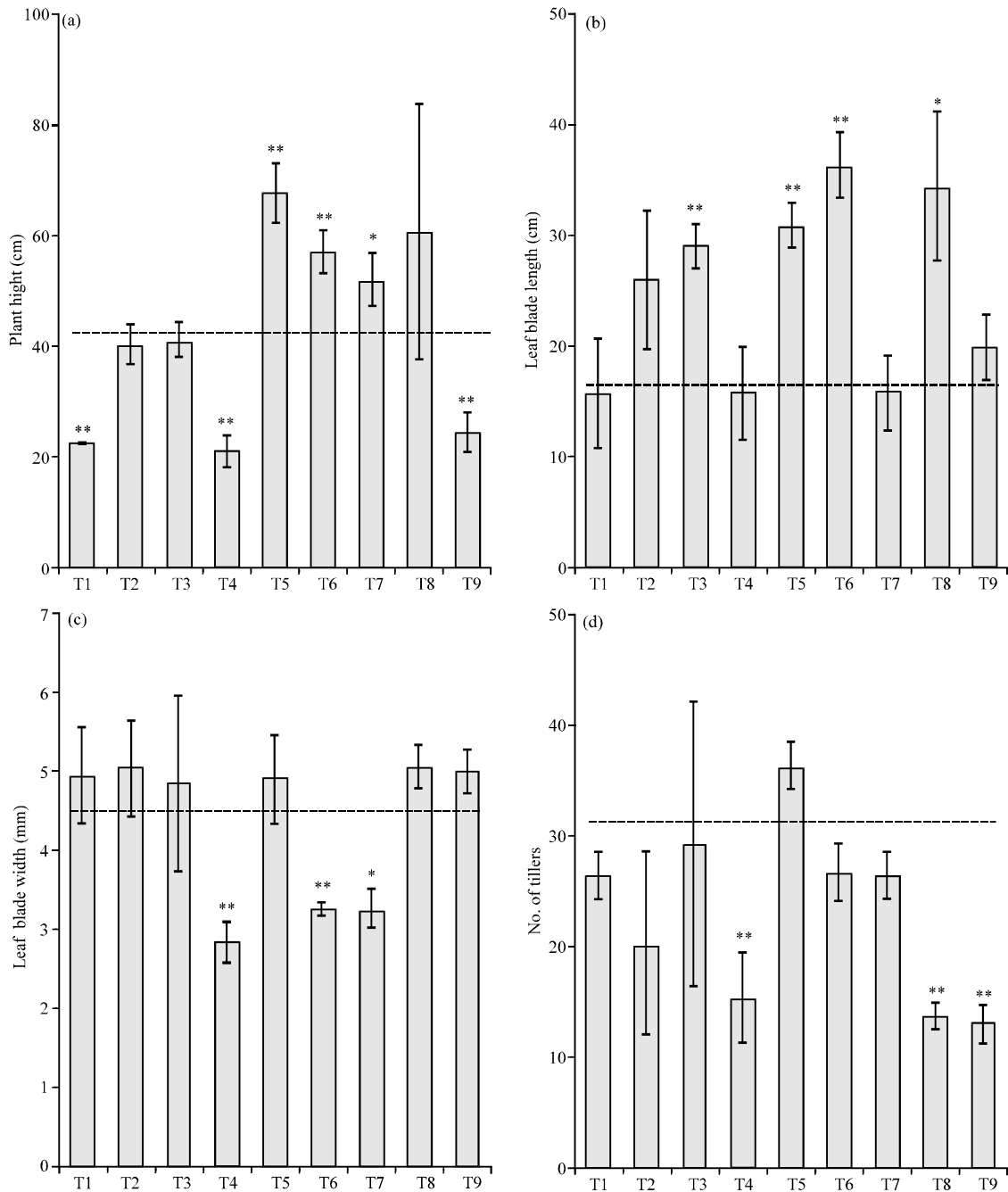


Fig. 5(a-f): Continue

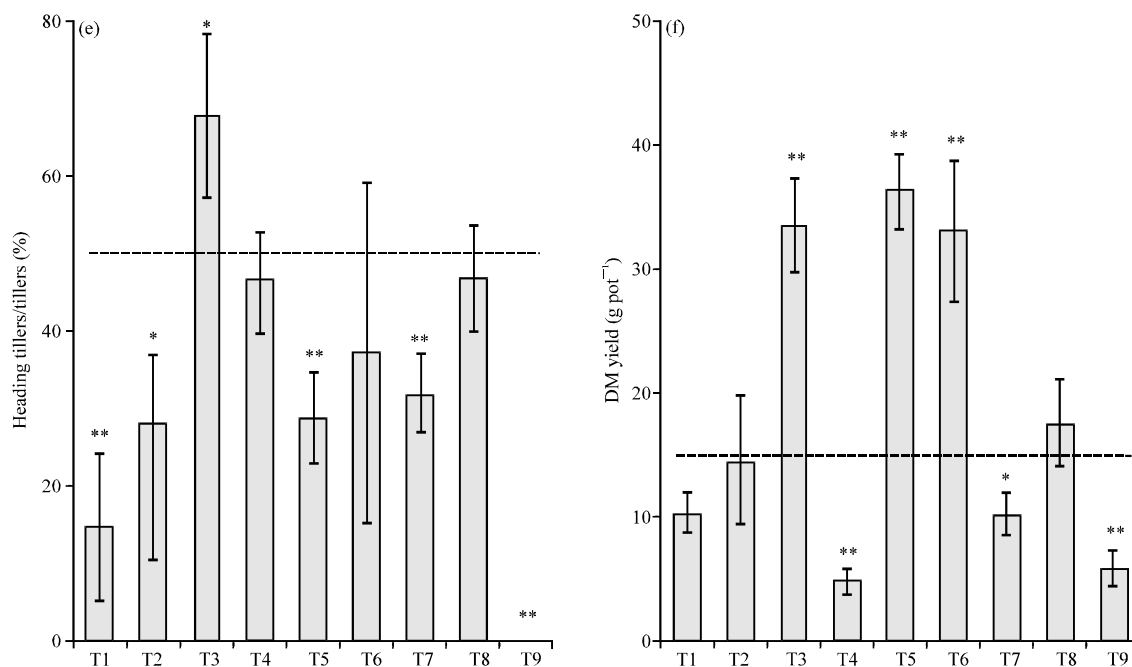


Fig. 5(a-f): Agronomic characteristics of transgenic bahiagrass, which indicates (a) Plant height, (b) Leaf blade length, (c) Leaf blade width, (d) No. of tillers, (e) Heading tillers and (f) Dry matter yield. The horizontal lines indicate the relative level of the control plants. *,** Statistically significant difference between control and transgenic plants at $p < 0.05$, 0.01 level, respectively by T-test

grasses generally leads to a 3.2% increase in average daily gains of beef cattle (Casler *et al.*, 2002). With the notable 10.4% increase in digestibility, transgenic bahiagrass has high potential to contribute to the livestock industry. Thus, genetic engineering proves to be a useful tool in manipulating the genes involved in the monolignol pathway in an effort to loosen the cell wall complex to reduce lignin for the improvement of forage digestibility in bahiagrass. On the other hand, C4 grasses has been a focus of bioenergy projects primarily because of its highly productive and perennial nature and because it does not compete with other food crops. Since the reduction of lignin directly impacts enzymatic saccharification in a parallel manner to the effects of forage digestibility (Reddy *et al.*, 2005; Chen and Dixon, 2007), our present result also contribute as a potential source for feedstock with improved processing efficiency for the biofuel production.

This understanding offers new opportunities to develop C4 forages with greater cell wall digestibility to enhance ruminant productivity for economic gains. Furthermore, the application of this breeding technology will reinforce strategic utilization for this species and to other dedicated warm-season biomass in the biofuel industry.

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