Genetic Engineering for the Improvement of Forage Grass Quality

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The forage quality of warm season perennial grasses grown in the Southern States, subtropics, and the tropics is often inadequate and is a major limitation to satisfactory animal performance. Quality improvement of those warm season grasses has been a high priority research objective in breeding programs. Hammond (1987) pointed out that C_4 tropical and subtropical grasses generally have lower quality than the C_3 temperate grasses. A major problem with those grasses is that they have proportionately higher cell wall constituents than cool season grasses, and this generally correlates negatively to intake (Waldo 1985), digestibility and animal performance (Moore and Mott, 1973; Van Soest, 1978; Morris, 1984). Cell wall digestibility decreases rapidly with increasing maturity and is reduced further by higher growth temperatures as encountered in the south and the tropics (Morris, 1984). It is also well established that forage grass leaves have a much higher digestibility than stems.

The major antiquality components of grass cell walls are lignin and phenolic monomers which esterify with cell wall polysaccharides. Lignin negatively affects cell wall digestibility by providing a physical barrier to microbial attack, formation of lignin-polysaccharide complexes, and by the antimicrobial effects of lignin precursors and degradation products (Jung and Fahey, 1983). In addition to a higher proportion of cell walls, subtropical and tropical grasses have higher lignin content than do temperate grasses (Morris, 1984). Lignin content increases with increased maturity and higher growth temperatures (Van Soest, 1978).

Genetic variation for quality exists in grass species and traditional breeding programs have been using that variability to improve forage quality. Although progress has been made, it has been slow and limited. Developments in genetic engineering now offer additional ways to improve forage quality. Methods are now available to down-regulate lignin biosynthetic genes to reduce lignin and/or change its type to improve forage digestibility. The lignin biosynthetic pathway and several key enzymes are reasonably well understood. Additionally, "brown midrib" mutants in maize and sorghum with defective O-methyltransferase (OMT) and cinnamyl alcohol dehydrogenase (CAD) lignin biosynthetic genes have been shown to produce modified as well as reduced amounts of lignin compared to normal plants. In each case where measured, those mutants have significantly improved forage digestibility and animal performance (Lechtenberg et al., 1972; Cherney et al., 1986; Fritz et al., 1981) .

Our research has focused on using the maize (*Zea mays* L.) model system to evaluate antisense technology in down-regulating OMT activity and lignin biosynthesis, and improving forage digestibility. Since a forage grass system that was easily transformed and regenerated had not been developed, we selected the easily regenerated maize Hi-II system to avoid regeneration problems that could interfere with the successful evaluation of antisense technology in down-

regulating OMT and the subsequent modification of lignin biosynthesis. That system worked well and we established a repeatable monocot transformation system for the antisense OMT evaluation.

Antisense constructs of OMT were assembled using recombinant DNA technologies requiring several steps with components from three sources: 1) the maize Ubi promoter and intron (from plasmid AHC17, obtained from Dr. Peter Quail, Plant Gene Expression Center, Albany, CA 94710) to drive the transcription of antisense OMT; 2) the sorghum OMT gene cloned in the antisense direction (orientated in the reverse direction relative to the promoter) and; 3) the *bar* selectable marker gene, which gives resistance to glufosinate herbicide, (from plasmid AHC20, also from Dr. Quail) driven by the 35s promoter (a commercial formulation of glufosinate is Finale™). We isolated the sorghum OMT and CAD cDNAs previously from our sorghum cDNA library. We also wanted to compare the relative effectiveness of maize (homologous) and sorghum (heterologous) antisense OMTs in reducing maize OMT activity, but were unable to secure permission to use the maize OMT clone.

Transformation was accomplished by microprojectile bombardment of type II maize callus with the antisense construct. The maize cultures were subjected to selection for six cycles (1-2 weeks/cycle) on media containing 2-5 mg/L glufosinate, then regenerated into plants. Of the 350 plants regenerated, 55% were resistant to applications containing 1% glufosinate. Verification of the transgenic origin of plants was done by PCR, and by Southern and northern analyses. The heterologous sorghum antisense construct functioned well in maize and OMT activity was found to be reduced ($p=0.05$) in transgenic plants with some transgenic plants showing a reduction of as much as 60% compared to control plants. In addition, about 10% of the transgenic plants expressed the "brown midrib" phenotype similar to natural "brown midrib" mutants with modified lignin and increased digestibility. Both reduced OMT activity and the brown midrib phenotype were heritable based on expression in self pollinated progeny (T_1).

Transgenic plants with down-regulated OMT activity had improved ($P=0.05$) *in vitro* organic matter digestibility (IVOMD) as compared to controls, with stems the most improved (7.4%), followed by sheaths (5.2%) and leaves (1.9%). Lignin content was significantly reduced with stem lignin reduced 19.8%, sheath lignin reduced 18.8% and leaf lignin reduced 11.8%. Since most of the nutrients in forages are required for animal basal metabolism, increases in digestibility of this magnitude can potentially produce very significant improvements in animal performance. This genetic engineering technology applied to tropical forages could make significant improvement in human nutrition through increased animal products.

The success of this research, recent reports of successful culture and regeneration of bahiagrass (*Paspalum notatum* Fluegge), and the importance of bahiagrass as a forage prompted us to initiate genetic engineering in that grass. The heterologous sorghum antisense OMT construct worked well in maize, however, the sorghum OMT and maize OMT genes were closely related, having about 90% similarity. Since no sequence data was available, we estimated the relatedness of the sorghum OMT gene with that of bahiagrass by comparing the hybridization rates of

sorghum and bahiagrass transcripts on a northern blot probed with the sorghum OMT cDNA. The strong and similar hybridization of those transcripts suggested that the two OMT genes were also closely related, hopefully, closely enough for the heterologous antisense OMT construct to function in down-regulating OMT in bahiagrass.

Two bahiagrass cultivars, `Tifton-9', a sexual diploid, and `Tifton-7', an apomictic tetraploid, are being used. It is unknown what effect the ploidy levels will have on the down-regulation of OMT, however, since there are no known sexual tetraploid bahiagrasses, any introduced genes in the tetraploid cultivar would assure that the transgenic genes would be unable to escape to other bahiagrasses or weedy species.

Bahiagrass embryogenic calli were initiated using germinating seedlings as explants. Preliminary experiments were conducted defining suitable conditions for breaking bahiagrass seed dormancy, seed sterilization, embryogenic callus initiation, and regeneration. A kill curve (50%) was generated to determine suitable glufosinate selection strategy. Transformation was by microprojectile bombardment in a similar way to that of maize mentioned above. During the first cycle of microprojectile bombardment, selection and regeneration, many problems were encountered that required refinement of techniques. Most of those problems were solved and several transgenic plants have been regenerated as defined by positive PCR amplification of the *bar* gene. Another set of glufosinate resistant calli are on regeneration medium and should yield additional transgenic plants. As adequate plant tissue becomes available they will be characterized further, including lignin down-regulation, and digestibility improvement.

We expect these studies to yield glufosinate-resistant and higher quality bahiagrasses, as well as insights into the molecular mechanisms of lignin biosynthesis. This paper has centered on improving forage grass digestibility, and to introducing herbicide resistance, however, other transgenic traits are being introduced for the improvement forages include increased forage protein content and disease resistance, especially those caused by viruses.

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