PAST, PRESENT, AND FUTURE OF TURFGRASS BIOTECHNOLOGY

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Turfgrrass improvement traditionally has relied on conventional breeding methods, in which the accessible genetic material is restricted by sexual reproduction. During the past two decades, substantial progress has been made in applying modern biotechnology in turfgrass genetic improvements. Applications of biotechnology have assisted turfgrass breeding programs in several different ways. These are: applications of molecular markers to assist breeding practices, *in vitro* culture (tissue culture), genetic engineering, the use of fungal endophytes to improve turfgrass performance, and recently the use of DNA microarray technology to discover important genes in turfgrass.

The turfgrass industries identify cultivars and breeding lines in order to control the quality of germplasm and protect breeder's rights. On the other hand, the paucity of available genetic markers has constrained efforts for detailed analysis of turfgrass genomes, and therefore, has hampered progress in cultivars improvement. Two types of molecular markers have been used in turfgrass: protein-based and DNA-based markers. Cultivars of bentgrass (*Agrostis* spp.) and Kentucky bluegrass (*Poa pratensis* L.) were distinguished on the basis of electrophoretic separation of leaf proteins on polyacrylamide gels. Peroxidase was the first isozyme marker used in turfgrasses for the identification of creeping bentgrass and Kentucky bluegrass. Other isozyme markers including esterases and phosphoglucomutases have been successfully tested for red fescus (*Festuca rubra* L. subsp. *Rubra*) and Kentucky bluegrass. Isozyme markers provide a convenient and inexpensive tool for turfgrass genotyping. Nevertheless, the lack of polymorphism, variations in the quality and quantity of isozymes and proteins due to plant growth and development, and environmental effect makes isozyme markers unsuitable for resolving closely related cultivars or breeding lines.

DNA-based markers have revolutionized genetic characterization of organisms. Restriction Fragment Length Polymorphisms (RFLPs) assay was developed to detect DNA restriction fragment of different lengths. RFLPs are enormous in number because any change to the DNA sequence generates polymorphisms. In addition to RFLPs of low-copy number sequences, some repetitive DNA sequences could be used to study genome evolution and species divergence at the molecular level. Despite the important utilities of RFLPs in turfgrass studies, generation of these markers requires prior DNA sequence knowledge for making proper probes. However, Random Amplified Polymorphic DNA (RAPD) has remarkable potential for investigating genetic variations in unknown sequences. The RAPD markers were used in Kentucky bluegrass in determining the genetic origins of aberrant plants derived from facultative aposporous apomixes. RAPD-based DNA analysis would be of great value for almost any research or breeding program in which monitoring, identification, and genetic mapping of cultivars are involved. Simple Sequence Repeat (SSR) polymorphism is another class of PCR-based DNA markers. Applications of SSRs in plants have been focused on linkage mapping. SSR markers reveal high allelic variation throughout the entire genomes. Nevertheless, the development of SSR markers is considered time consuming and expensive. There is another novel PCR-based assay, Amplified Fragment Length Polymorphism (AFLP) was developed to

selectively amplify and detect the restriction fragments from endonuclease digestion of the genomic DNA. AFLP assays take less time and need no prior sequence knowledge and it generates higher polymorphisms than that of RFLPs. Therefore, AFLPs are promising for rapid identification and mapping in plant species like turfgrasses, for which little sequence knowledge is afforded by their lower priorities in the economy.

An efficient *in vitro* regeneration is a necessary step to recover genetically altered material. *In vitro* regeneration is also used for somaclonal variations, haploid development, as well as for micropropagation and aseptic storage of valuable germplasm. Somaclonal variations in turfgrass species could be useful for generating superior genotypic characteristics for production of new commercial genotypes. In our laboratory, we were able to produce a dwarf variety in turfgrass due to somaclonal variations through *in vit*ro cultures of turfgrass (Figure 1). Efforts have been made to establish regenerable *in vitro* cultures from diverse turfgrass explant material. Plant regeneration from embryogenic callus is the single most important path for turfgrass improvement, as many major turfgrass species have been regenerated in this way. Embryogenic turfgrass callus (undifferentiated cells in process of differentiating into embryos) are produced from *in vitro* cultures of cells obtained from asexual turfgrass organs. Embryogenic callus is characteristically friable, somewhat organized, and generally white to light yellow in color. Also, regenerable cell suspensions are established using embryogenic callus. These cells too, provide totipotent cells (i.e. cells capable of producing whole plants). *In vitro* regeneration has been reported in several major turfgrass species including creeping bentgrass, Kentucky bluegrass, and Italian ryegrass using cell suspension cultures. Although protoplasts (i.e. naked cells or cells without cell walls) are useful for multiple manipulations in turfgrass biotechnology, though, this technology is still considered the most difficult method from which to recover plantlets. In studies of turfgrass, plant regeneration has also been achieved using shoot apices and anther cultures. However, the use of these specific procedures for turfgrass regeneration is genotype dependent. Single-genotype-derived *in vitro* cultures have improved the efficiency of *in vitro* manipulations, which has helped to evaluate and optimize other culture condition factors.

Progress in turfgrass genetic transformation has been made in exploring and optimizing transformation systems that have been used for other grass species. These systems include *Agrobacterium*-mediated transformation, Protoplast transformation, and Biolistic^{TM} Gun-mediated transformation. Transgenic plants were obtained in tall fescue, red fescue, orchardgrass, creeping bentgrass, and redtop grass using protoplast-mediated gene transfer technology. Although protoplast-mediated transformation has been successful in some turfgrass species, plant regeneration from protoplasts is still difficult to achieve due to such uncontrollable parameters as genotype-dependent competence for regeneration, infertility problems, and unwanted somaclonal variations. Whereas, Biolistic^{TM} Gun-mediated transformation in turfgrass have achieved great success during the last few years. This method circumvents the host range limitations of *Agrobacterium* and also eliminates the need for tedious plant regeneration from protoplasts.

Several different types of explant material have been used successfully in turfgrass to recover transgenic plants after Biolistic bombardment. Genetic transformation via the Biolistic delivery has been reported in creeping bentgrass, tall fescue and red fescue, and perennial

ryegrass from different laboratories. Our laboratory developed the Biolistic transformation in turfgrass using embryogenic callus and cell suspension cultures, transferred multi-gene in plants, and tested transgenic plants for herbicide and disease resistance. Initially, we engineered creeping bentgrass, using a marker i.e. called *gus* gene and the Liberty herbicide (bialaphos and glufosinate) resistant *bar* gene (Figure 2). Through extensive studies using bialaphos and glufosinate, we also discovered that this herbicide also has fungicidal properties. We observed that herbicidal spray on herbicide resistant transgenic creeping bentgrass could prevent simultaneously the growth of weeds as well as the fungal infection caused by *Rhizoctonia solani* (brown patch, Figure 3) and *Sclerotinia homoecarpa* (dollar spot).

In nature, fungal pathogens have evolved to protect themselves from external damages through the chemical "chitin" that covers the fungal hyphae. On the other hand, certain plants have evolved protecting themselves from fungal attacks through excretion of an enzyme called chitinase, which degrades fungal chitin. We cloned and characterized a series of seven antifungal chitinase genes from a Dutch elm disease resistant Americana elm (*Ulmus Americana*) in our laboratory. We also genetically transferred one of these chitinase genes (*hs2*) into creeping bentgrass (Figure 4). Expression of this chitinase gene (*hs2*) in creeping bentgrass was expected to promote disease control against fungal pathogens via chitin degradation.

As mentioned above, the bialaphos herbicide provides a means for the simultaneous control of weeds and fungal pathogen in turf areas with transgenic bialaphos resistant creeping bentgrass. In combination with the chitinase gene transferred into turfgrass, transgenic herbicide resistant plants may provide significant protection against fungal infections in grasses. Further research on genetic transformation of turfgrass species using insect resistant and cold resistant genes is in progress in our laboratory.

A recently developed highly powerful technology, DNA Microarray, has the potential to revolutionize the future of turfgrass in biotechnology. Although a very expensive and highly technological, microarray technology has the potential to be highly effective for discovering large number of turfgrass genes with a wide variety of functions such as genes for developmental regulation, genes controlling plant morphology, abiotic stress-induced genes and genes for disease and insect resistance. There are two fold uses for these novel bentgrass genes to be discovered via microarray technology. First, these genes could be used as actual turfgrass defensive genes to genetically engineer susceptible turfgrasses, and second, these novel genes can be used as molecular probes to track their presence in other turfgrasses in breeding programs.

We, with the collaboration of other laboratories, are in the process to identify such genes in turfgrass whose expressions are playing a specific role in disease resistance using microarray technology (Figure 5). These genes will be isolated from turfgrass cDNA libraries and characterized. Identified genes, as being potential candidates for disease or environmental stress resistance will be used for future studies in developing genetically engineered turfgrass varieties. Also, these genes will become available to turfgrass breeders who wish to use them as probes (indicators) to track the presence of corresponding genes in their breeding programs.

We would like to acknowledge the support of the United States Golf Association and Michigan Turfgrass Foundation for this new line of research.

Fig. 1: Production of a dwarf variety in turfgrass due to somaclonal variations through *in vitro* cultures.

Fig. 2: Transgenic plants and untransformed control plants 2 wk after foliar spray of glufosinate.

Fig. 3: The application of bialaphos for prevention of infection by *R. solani* in transgenic, bialaphos-resistant creeping bentgrass 1 wk after application. The plants were inoculated with about 500 mg of wheat seed inoculums (fungal cultures were grown on wheat seeds) and were sprayed with 200 mg/L of bialaphos 3 h before pathogen inoculation. Transgenic plants not sprayed with bialaphos showed severely damage by pathogen infection.

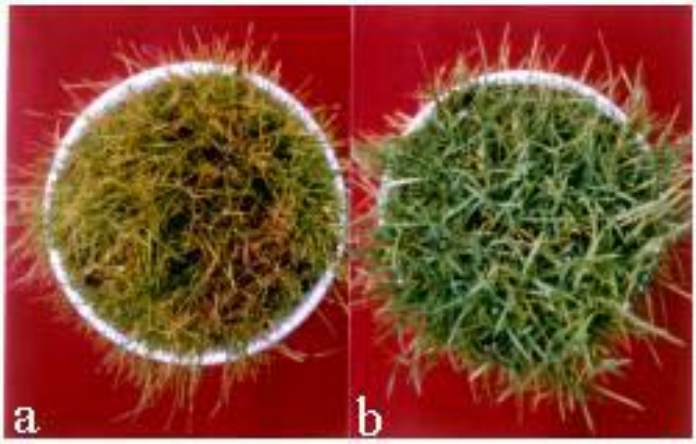

Fig. 4. The whole plant bioassays: transgenic creeping bentgrass expressing chitinase (*hs2*) and *bar* genes 7 days after inoculation with spores of *R. solani*. a: Non-transgenic plants; b:Transgenic plants

Fig. 5. Microarray Laboratory: printing array on glass slides using robotic DNA printer.