Final Report
to
The United States Golf Association

A Multigene-Transfer Strategy to Control Pathogens and Enhance Environmental Stress Tolerance in Creeping Bentgrass

From

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Note: Dr. James Baird was one of the Co-PIs of the proposal. However, he has not been involved with this final report because he is no more at MSU.

Executive Summary

Previously, Sticklen's research team developed creeping bentgrass clones that contain the glufosinate ammonia (Liberty or FinaleTM) resistant herbicide, a chitinae gene, a proteinase inhibitor gene, and a drought and salt tolerance mannitol dehydrogenase (*mt1D*) gene. Studies have shown that the chitinase genes can make transgenic plants resistant to pathogenic fungi such as *R. solani*, etc..

Our research team confirmed that glofosinate ammonia has fungicidal as well as herbicidal properties. Therefore, we have been able to simultaneously control weeds as well as turfgrass pathogens (mainly *Sclerotinia ulnocarpal* and *Rhizoctonia solani*) by spaying this herbicide on transgenic creeping bentgrass expressing the bar gene under greenhouse conditions.

Research by Dr. Vargas laboratory has shown two out of 44 independently transformed lines of our transgenic creeping bentgrass, transcribing the elm chitinase gene, has improved resistance of plants to R. *solani* under controlled environmental conditions.

Experiments performed by Dr. Baird's laboratory has shown no translation of our transcribed *mtlD* gene in certain lines of transgenic turfgrass. Of course, none of these transgenic lines tested for western blotting also showed accumulation of mannitol, as a sign for drought and/or salt tolerance. With no surprise, none of these transgenic lines tested showed any drought and/or salt tolerance either. Since we made synthetic peptides and produced extra antibodies to the MTLD protein, we plan to test the rest of *mtlD*-transcribed transgenic lines for expression of MTLD protein by western blotting. If positive, then once again we will perform mannitol test and will also test plants for drought and or salt tolerance. Should MSU have a new turfgrass physiologist in place soon. this work will be performed with collaboration of the new physiologist.

Introduction

Creeping bentgrass (*Agrostis stolonifera* L.) is a desirable species for use on golf courses throughout most of the United States due to its tolerance of low mowing heights, density, and other turf quality characteristics that enhance the game of golf. Whether or not it is grown within or beyond its zone of adaptation, creeping bentgrass is limited by environmental stresses associated with drought and temperature extremes, and by pathogenic diseases that prey on stressed turf.

The most promising approach to combating the major biotic and abiotic stresses associated with creeping bentgrass and other turfgrasses is through the development of transgenic plants. These plants are created by the introduction of genes (fundamental units of heredity) into existing deoxyribonucleic acid (DNA), the primary carrier of genetic information. Thus, it would be advantageous to insert genes into creeping

bentgrass that express greater resistance to stresses induced by extreme environmental conditions and pathogens.

Multi-Gene Transformation Studies

Initially, Dr. Sticklen's laboratory developed a genetic engineering system for creeping bentgrass using a marker (*gus*) gene to determine success of gene incorporation (Zhong et al., 1991; Zhong et al., 1993) into turfgrass. Then, under the financial support of the United States Golf Association, her team successfully incorporated a gene for resistance to glufosinate (FinaleTM), a non-selective herbicide (Liu, 1996). Additional research conducted showed that glufosinate has fungicidal, in addition to herbicidal, properties. As a result, we have been able to simultaneously control weeds and diseases caused by the pathogenic fungi *Rhizoctonia solani* (brown patch) and *Sclerotinia homoeocarpa* (dollar spot) by spraying the herbicide on transgenic creeping bentgrass expressing this gene (Liu et al., 1998).

Our next challenge was to insert a chitinase gene cloned and characterized in Sticklen's laboratory into creeping bentgrass. Chitinases are enzymes (proteins) that degrade chitin, a structural polysaccharide of fungal cell walls and insect exoskeletons. Since fungi cause the major pathogenic diseases of turfgrasses, expression of the chitinase gene in creeping bentgrass is expected to promote disease control via chitin degradation. Studies have shown that chitinase genes can make transgenic plants resistant to pathogenic fungi such as R. solani (Graham and Sticklen, 1994). The Sticklen laboratory team cloned and characterized a full-length chitinase gene which contains the necessary chitin-binding domain from American elm (*Ulmus americana*) (Hajela and Sticklen, 1993; Sticklen et al., 1993; Hajela et al., 1993). Then our team constructed a plasmid containing this chitinase gene regulated by the 35S promoter, and successfully inserted this chitinase gene into creeping bentgrass (Chai, 1997). The, collaboration was made with Dr. Vargas for laboratory and greenhouse levels inoculation studies of transgenic plants. These studies showed that two out of 44 independently transgenic turfgrass genetic lines were resistant to *R. solani*. The studies were repeated at the laboratory and the greenhouse levels, and results were submitted to Phytopathology as two out of 44 transgenic lines showed 3 to 5 fold resistance to *R. solani* (Chai et al, 2000; Green et al, 2000). A more detailed report of the screening of transgenic plants is shown below.

Comparisons of disease resistance in different independent lines of transgenic creeping bentgrass transcribing the chitinase gene

Seven transgenic lines (711, 7204, 7205, 7208, 815-1, 815-7, 9106, 910-10, 9601, 9603, & 9606) of *A. palustris* carrying a selectable marker for bialophos resistance (*bar* gene), and the class I basic elm chitinase (pHS2) were screened for resistance to *Rhizoctonia solani* AG 1-Ia (casual agent of brown patch) and *Sclerotinia homoeocarpa* (casual agent of dollar spot) under controlled environmental conditions. Parental cultivars of *A. palustris*, Penncross and Putter, and a transgenic line of each parental cultivar containing only the *bar* gene were included as controls. Growth, color, and turfgrass quality varied within the eleven transgenic creeping bentgrass lines examined. Only lines 711, 815-1,

9603, 9606, and 9604 were equivalent in turfgrass quality compared to their parental cultivars. Coarse texture or low shoot density resulted in poor turfgrass quality in the other transgenic lines.

Brown patch screen. Controlled environment experiments were conducted 15 May through 27 July, 1998 to assess levels of *R. solani* resistance among the transgenic lines and their parental cultivars as discussed by (Green et al., 2000). To summarize our findings, two transgenic lines 711 and 9603 had significantly ($P \leq 0.01$) improved resistance to *R. solani* as compared to their parental cultivar, Penncross, and the Penncross derived *bar*-only line 9604. Transgenic lines 711 and 9603 were found to have approximately a 3- and 1-fold improved level of resistance to *R. solani* AG 1-Ia, respectively, when compared to Penncross. Other transgenic lines derived from the creeping bentgrass cultivars Penncross and Putter did not provide significantly improved resistance to *R. solani*.

Dollar spot screen. Controlled environment experiments were conducted 12 February through 15 May 1999 to assess levels of *S. homoeocarpa* resistance among the transgenic creeping bentgrass lines and their parental cultivars. None of the transgenic lines containing the chitinase gene showed improved levels of resistance to the dollar spot pathogen as compared to their parental cultivars. Whereas, four of the transgenic lines carrying the chitinase gene (910-12, 9601, 9603, 9606) showed reduced levels of resistance to *S. homoeocarpa* as compared to their parental cultivar. Failure of the pHS2 chitinase gene to provide improve resistance to this ascomycete fungus, agrees with other literature where differences in fungal cell wall composition appear to inhibit the effective suppression of some fungal pathogens by the chitinase enzyme . These reports suggest that variation in carbohydrates and proteins on the fungal cell wall surface protect the chitin in the cell wall from the chitinase enzyme.

Testing drought tolerance of transgenic creeping bentgrass

In addition to our herbicide and pathogen resistance transformation studies, we successfully incorporated one drought and salt resistance gene [mannitol 1-phophate dehydrogenase (*mtlD*)] regulated by a monocot specific promoter and intron (*Act1* promoter) into creeping bentgrass (Chai,1997). This portion of the project was in collaboration with Dr. James Baird. the former faculty member of the Department of Crop and Soil Sciences at Michigan State University. The *mtlD* gene is associated with drought and salinity tolerance in plants (Tarczynski et al., 1992, 1993). Dr. Sticklen's laboratory also confirmed that this gene (*mtlD*) too was incorporated and transcribed in transgenic creeping bentgrass. Since Dr. Wu laboratory at Cornell University transferred this *mtlD* gene regulated by the same promoter (rice *Act*1) into rice and confirmed that transgenic rice had become tolerant to both drought and salt (Xu et al., 1996), we conducted studies to see whether our transgenic turfgrass transcribing the mtlD gene were tolerant to drought or salt. In our studies, Dr. James Baird's laboratory tested several of our transgenic lines, and found no resistance of these lines to drought or salt. We also

designed synthetic peptides and produced antibodies against the MTLD protein. Dr. Baird's student conducted few western blots from our transgenic lines in which tall showed not translation of our transcribed *mtlD* gene in transgenic turfgrass. Interestingly, none of these transgenic lines tested for western blotting showed accumulation of mannitol, as a sign for drought and/or salt tolerance. Since our northern blots clearly show the transcription of *mtlD* gene in several of our transgenic lines, we plan to use the rest of antibodies and test other transgenic clones that have transcribed the *mtlD* gene to see whether any of the other lines show the translation of the *mtlD* gene.

Improving the Expression of Genes in Creeping Bentgrass

We constructed a plasmid containing the chitinase gene and the rice *Act1* promoter and intron. The objective of this part of the proposal was to see whether we could improve the level of expression chitinase gene in turfgrass, as compared to the level of its expression under control of the 35S promoter. Then, we genetically engineered creeping bentgrass with the plasmid containing the rice *Act1* promoter. To date, we have not found a clear result showing that there are much expression level differences between these two promoters in creeping bentgrass. Of course, we have produced and tested a small number of transgenic turfgrasses with the plasmid containing the rice *Act1* promoter.

Cross Breeding of Certain Lines of Transgenic Turfgrass

Michigan State University and Pure Seed Testing, Inc. entered into a license agreement whereby the Oregon Research Corporation conducted further testing of transgenic plants developed at Michigan State University. This private sector also cross bred our transgenic lines with their inbred lines and produced hybrid seeds. The Pure Seed Testing, Inc. also studied the distance needed to be kept between transgenic and the non-transformed turfgrass fields in order to avoid transfer of transgenic pollen grains to the surrounding fields. It was unfortunate that anti-biotechnologists destroyed the Pure Seed Testing, Inc. transgenic fields.

Personnel Trained Under the Financial Support of the USGA

1. Dr. David Green	Postdoctoral Associate	Dr. Vargas's Lab.
2. Ms. Susan Redwine	MS Student	Dr. Baird's Lab.
3. Dr. Chien-An Liu	Ph. D. Student	Dr. Sticklen's lab.
4. Dr. Benli Chai	Ph. D. Student	Dr. Sticklen's Lab.
5. Mrs. Robabn Sabzikar	Technician	Dr. Sticklen's Lab.

Please note that partial salaries of the above personnel were paid through the USGA grant.

Patents or Provisional Patent Applications Filed

1. U.S. Patent in progress: MSU 4.1-153, Serial # 08/036,056, March 23, 1993.Method for isolating a grass plant with foreign DNA. On appeal.

2. U.S. Patent in progress: MSU 4.1-315, Serial # 60/015,485, Apr. 15, 1996.Simultaneous control of weeds and turfgrass diseases with spray of Bialaphos on genetically engineered turfgrass. Provisional filed on Apr. 15, 1997.

3. U.S. Patent in progress: MSU 4.1-395, ID 98-031, May 26, 1998.Disease resistant transgenic turfgrass containing a chitinase gene. Filed in 1998.

Conclusions

At conclusion, we developed a very reliable system of genetic engineering for turfgrass, transferred multi-genes in plants, and tested transgenic plants for herbicide, disease and drought tolerance. Cross breeding was performed by a private sector that confirmed the integration and expression of transgenes into the company's commercial lines.

It is ashamed that biotechnology researchers and biotechnology private sectors have been disrespectfully blamed for their research programs. We believe that it is only a matter of time to educate public in understanding and appreciating the applications of biotechnology, especially the application of biotechnology to improve a non-food/non-feed crop such as turfgrass.

Acknowledgement

At the end, we would like to sincerely appreciate the financial and moral supports of the USGA. This support resulted in training of several graduate students, technicians, and postdoctoral associates at Michigan State University.

References

Chai B., Green D., Sabzikar R., Vargas J., and **Sticklen M. B.** (2000). Brown patch resistance in transgenic creeping bentgrass with an elm chitinase gene. Phytopathology. Submitted.

Chai and **M. B. Sticklen** (1998). Application of biotechnology in turfgrass improvement. Crop Sci. 38: 1320-1338.

Chai B. 1998. Transgenic creeping bentgrass expressing the elm chitinase and the mannitol-1-phosphate dehydrogenase drought tolerance genes. Ph.D. Dissertation. Michigan State University.

Graham L. and **M.B. Sticklen.** 1994. Plant Chitinases. Can. J. Bot. 72: 1057-1083.

Green II D. E., Vargas Jr. J. M., Chai B., Dykema N. M., and **Sticklen M. B.** (1999). The use of transgenic plants to confer resistance to brown patch caused by *Rhizoctonia solani* in Agrostris *palustris*. In: The fate and management of turfgrass chemicals. J. Clark ed.

Hajela R.K., L.S. Graham and **M.B. Sticklen** (1993). Nucleotide sequences of a cDNA encoding a chitinase like polypeptide from American elm (Ulmus americana). Announcement Section. Plant Mol. Biol. 23:915.

Hajela R.K. and **M.B. Sticklen** (1993). Cloning of pathogenesis-related genes from Ulmus americana. In: M.B. Sticklen and J.L. Sherald (eds.). Dutch Elm Disease Research: Cellular and Molecular Approaches. Springer-Verlag New York, Inc. pp. 193-207.

Liu, C-A. (1996). Genetic engineering of creeping bentgrass for resistance to the herbicide glufosinate ammonia. Ph.D. Dissertation. Michigan State University.

Liu C-A, H. Zhong, J. Vargas, D. Penner, and **M. B. Sticklen** (1998). Prevention of fungal diseases in transgenic bialaphos and glufosinate-resistant creeping bentgrass (*Agrostis palustris*). Weed Science 46: 139-146.

Sticklen M.B., D. Warkentin, C-A Liu, R.K. Hajela, L. Graham, H. Zhong, B. Peterson, J. Vargas, and B. Branham. 1995. Genetic engineering in Agrostis palustris Huds. (creeping bentgrass). In: Y.P.S. Bajaj (ed.). Biotechnology in agriculture and forestry. Plant protoplasts and genetic engineering of plants. Vol. 38. Springer- Verlag, Publ. Berlin, Germany. pp. 151-162.

Sticklen M.B., R. Hajela, M. Bolyard, and L. Graham. 1993. Advances in gene cloning and genetic engineering of elms. In: Plant Protoplasts and genetic engineering of plants. Vol. 29. Springer-Verlag Publs. Berlin, Germany.

Sticklen M.B. (1991). Genetic engineering of plants: An alternative to pesticides and a new component of integrated pest management. In: D.L. Weigmann (ed.). Pesticides for the Next Decades: The Challenges Ahead. VPI Publ. Blacksburg, VA. pp. 522-566.

Sticklen M. B., D. Warkentin, C-A Liu, R. K. Hajela, L. Graham, H. Zhong, B. Peterson, J. Vargas, and B. Branham (1995). Genetic engineering in *Agrostis palustris* Huds. (creeping bentgrass). In: Y. P. S. Bajaj (ed.). Biotechnology in agriculture and forestry. Plant protoplasts and genetic engineering of plants. Vol. 38 . **Springer Verlag, Publs.** Berlin, Germany. pp. 151-162.

Sticklen M. and M. Kenna (1998). Cellular and Molecular Genetics Approaches to Turfgrass Improvement. **Ann Arbor Press.** Ann Arbor, MI. 256 P.

Tarczynski M.C., R.G. Jensen, and H.J. Bohnert. 1993. Stress protection of transgenic tobacco by production of the osmolyte mannitol. Science. 259: 508-510.

Tarczynski M.C., R.G. Jensen, and H.J. Bohnert. 1992. Expression of mtlD gene in transgenic tobacco leads to production and accumulation of mannitol. Proc. Natl. Acad. Sci. USA. 89: 2600-2604.

Warkentin D., B. Chai, C.-A. Liu, R. K. Hajela, H. Zhong, and **M. B. Sticklen** (1998). Development of transgenic creeping bentgrass (Agrostis palustris Huds.) for fungal disease resistance. In: M. B. Sticklen and M. Kenna (eds). Turfgrass Biotechnology: Cellular and Molecular Genetic Approaches to Turfgrass Improvement. Ann Arbor Press. pp. 153-161.

Xu D., Duan X., Wang B., Hang B., Ho TH-D., and Wu R. (1996). Expression of a late embryogenesis abundant protein gene , Hval, from barley confers tolerance to water deficit and salt stress in transgenic rice. Plant Physiol. 110: 249-257.

Zhong, H. and **M. B. Sticklen** (1993). Transgenic plants of turfgrass (*Agrostis palustris Huds*) from microprojectile bombardment of embryogenic callus. Plant Cell Rep. 13: 1-6.

Zhong H. and M. B. Sticklen (1991). Plant regeneration via somatic embryogenesis in creeping bentgrass (*Agrostis palustris Huds*). Plant Cell Rep. 10: 453-456.

H. Zhong, C.-A. Liu, J. Vargas, D. Penner, and **M. B. Sticklen** (1998). Simultaneous control of weeds, dollar spot, and brown patch diseases in transgenic creeping bentgrass. In:

M. B. Sticklen and M. Kenna (eds). Turfgrass Biotechnology: Cellular and Molecular Genetic Approaches to Turfgrass Improvement. Ann Arbor Press. pp. 203-210.