Control of Bentgrass Pathogenic Fungi Dollar Spot, Brown Patch and Pythium Blight using Chitinase

Annual Report

to

The United States Golf Association

from

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I. Executive Summary of the Proposal

Dollar spot (*Sclerotinia homoeocarpa*), brown patch (*Rhizoctonia solani*) and Pythium blight (*Pythium aphanidermatum)* are major pathogenic diseases of turfgrass. All of these pathogens contain chitin in their cell walls, and therefore may be susceptible to chitinases [1]. Also, all of these pathogens contain proteinases which are essential for the survival of the pathogenic fungi. The Sticklen laboratory team has cloned and characterized a full length chitinase gene which contains the necessary chitin-binding domain ([2; 3; 4] Gene Bank Number L22032). This laboratory also has constructed several plasmids containing a potato proteinase inhibitor II controlled by different (monocot-specific, wound-inducible, and constitutive) promoters.

During the first year of our USGA-supported project, we expressed this chitinase gene in *E. coli*, extracted chitinas-containing slurry, and confirmed the expression of this plant gene in *E. coli.* Dr. Vargas's laboratory examined the effect of the recombinant chitinase on turfgrass pathogenic fungus, *R. solani*.. Due to the hydrophobic (water insoluble) nature of the expressed chitinase, bioassay did not provide us with accurate results. Work is in progress to remove the hydrophobic portion of this gene, and express the modified gene in *E. coli* again.

We also expressed this chitinase gene, in a homozygous state, in second generation of tobacco plants. Dr. Vargas's laboratory is testing these plants against *R. solani.* Work is in progress to express this chitinase gene in creeping bentgrass.

In addition, during the first year of the project we expressed the potato proteinase inhibitor II and the *bar* (Ignite herbicide resistance) genes in creeping bentgrass. We developed thousands of pots of transgenic turfgrass expressing these two genes. To date, thirty seven independent groups of transgenic creeping bentgrass (with different sites of gene insertion) have been identified. Professor Donald Penner, the MSU herbicide physiologist, has confirmed the degree of resistance of these transgenic plants to ignite herbicide. Several of three independent transgenic plants have been transferred to the field. Further analysis against both Ignite herbicide and turfgrass pathogenic fungi will be performed on these plants during Spring and Summer of 1996.

II. Annual Report

A. Expression of a plant chitinase gene in *E. coli*, extraction and purification of the chitinase protein (objective 1):

The Sticklen research team cloned and characterized a defense gene (pHS2) that is believed to lyse the chitin containing-cell walls of pathogenic fungi [1]. We sequenced this gene using the MSU Automated DNA Sequencing Services. Since certain restriction enzymes did not cut our pHS2 chitinase gene, we concluded that the service has not been very accurate. Therefore, we manually sequenced both strands of the pHS2 chitinase gene, found mistakes done by the automated service, and confirmed the exact sequence of this gene by restriction digestions.

Using our previously developed method [5], we expressed this gene in *E. coli* and extracted the protein following our previous research [5]. Since this chitinase gene was found to be hydrophobic (water insoluble), pathogen bioassay did not provide an accurate result. Therefore, we conclude that we must (1) use the gene directly to transform plants (a model plant to obtain second generation homozygous seeds and creeping bentgrass) for bioassay, and (2) to remove the DNA sequences coding for a hydrophobic signal peptide, to make the chitinase water soluble. Then transform E. coli, extract soluble chitinase, and perform the bioassay against turfgrass pathogenic fungi.

B. Construction of plasmids containing the chitinase (pHS2) gene for plant transformation:

We constructed a plasmid containing the pSH2 chitinase gene controlled by the 35S constitutive promoter. This plasmid was to be co-transferred to turfgrass with the *bar* ignite herbicide resistance gene in turfgrass. However, since we planned to transform tobacco with this gene first and quickly obtain second generation homozygous seeds, we included the kanamycin resistance gene controlled by a second constitutive promoter in our construct.

C. Transformation of tobacco and development of second generation homozygous transgenic tobacco expressing our chitinase gene

Tobacco plants were genetically engineered using our plasmid containing the pHS2 chitinase gene and the kanamycin resistance gene. Integration and expression of these genes were confirmed via Southern and northern blots. Second generation homozygous transgenic plants were developed and provided to Dr. Vargas's laboratory for evaluation against *R. solani*.

D. Analysis of second generation transgenic tobacco using turfgrass pathogenic fungi, *R. solani*

Screening is currently underway in the greenhouse. The second generation transgenic tobacco seedlings are being screened against *R. solani*. This R. *solani* was isolated from ryegrass. Preliminary tests on untransformed tobacco plants showed that this isolate is capable of infecting tobacco seedlings. Test is in progress to screen transgenic plants.

E. Transformation of creeping bentgrass with pHS2 chitinase gene and the ??? drought resistance genes.

We have initiated transferring plasmids containing the pHS2 chitinase gene and the ??? drought resistance genes in microcallus lines of creeping bentgrass. This was also performed following our previous method of turfgrass transformation and regeneration [6; 7]. We also have included the *bar* herbicide resistance gene, as a selectable marker gene, in our studies for co-transforming creeping bentgrass. Since we already completed the kill curve studies on the PPT chemical, we used the optimal level in our selecting transgenic callus lines.

F. Transformation and regeneration of creeping bentgrass using a herbicide resistance gene, the potato proteinase inhibitor II gene, and the chitinase gene

Development of a kill curve using the selectable marker chemical PPT
We have used several concentrations of PPT herbicide to determine the minimum level that can kill creeping bentgrass microcallus lines and regenerated plants. These levels were chosen to screen for transgenic callus lines and transgenic in vitro regenerated plantlets.

Transformation of creeping bentgrass
While we were sequencing the pHS2 for confirmation and during the time that we were constructing plasmids containing the pHS2, we initiated the transformation of creeping bentgrass with the potato proteinase inhibitor II and the *bar* (Ignite herbicide resistance) genes. This was performed following our previous method of turfgrass transformation and regeneration [6; 7] . Bentgrass micro-callus pieces were bombarded with a plasmid containing the above two gene. The bombarded calli were gently transferred onto our turfgrass regeneration medium [6] containing the desired concentrations of PPT selectable chemical. Plants were regenerated from the selected tissue material and greenhouse grown transgenic plants were produced. Confirmation of gene integration (at the genomic level) and expression (at the mRNA and protein level) followed our routine work [7].

Confirmation of gene integration at the genomic level:
To confirm the integration of the above two genes in the putatively transgenic plants, genomic DNA was isolated from putatively transgenic and control (untransformed) plants, and then Southern analysis was performed following Sticklen's [8; 9; 7] modification of Southern's procedure.

Confirmation of gene expression at the mRNA level:
Total cellular RNA was isolated from plant tissues using the Killer Buffer procedure. The foreign mRNAs (bar and proteinase inhibitor II) were detected by northern blot hybridization using the complementary DNA fragments labeled by random primed labeling as probes. The mRNAs were electrophoresed on denaturing formaldehyde agarose gels, transferred to nitrocellulose filters, hybridized with the probe, then exposed to X-ray film. After exposure of the probed northern filter to X-ray film, the density of specific bands in untransformed and transformed plant lanes provided levels of mRNAs present in these tissues.

Confirmation of gene expression at the protein level:
To quantitate the levels of foreign protein present in transformed tissues, polyclonal antibodies (provided by Dr. C. Ryan, the developer of proteinase inhibitor II gene)) was used for western analysis adapted in Sticklen's laboratory [9; 7] . Protein

preparations were electrophoresed on polyacrylamide gels and electroblotted onto nitrocellulose. Following incubation of the filter with the primary antisera, bound rabbit anti-target protein was detected using goat anti-rabbit alkaline phosphatase conjugated secondary antisera. The bound secondary antisera was then visualized using the substrates nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolylphosphate (BCIP).

Transformation using the chitinase gene
The work on transferring our chitinase gene in creeping bentgrass has just initiates. several callus lines have been bombarded with this gene and the bar selectable marker gene. Results will be obtained during the second year of this project.

G. Field testing of transgenic creeping bentgrass

We produced over 1,000 greenhouse-grown transgenic creeping bentgrass plants expressing the herbicide resistance and the proteinase inhibitor II genes. We obtained a permit from APHIS/USDA for field trial of these transgenic plants. We designed an experiment containing 50 pots of each of the three independent transgenic plants which expressed both the herbicide resistance gene and the proteinase inhibitor II genes at the greenhouse level, and were all resistant to an above-average concentration of ignite herbicide.

Further analysis of these plants for herbicide resistance and pathogen resistance will be initiated during spring 1996. The herbicide resistance test will be performed by Dr. Donald Penner and the pathogen assay will be performed by Dr. Joseph Vargas.

H. Research initiated beyond the scope of the proposed work

Recently, we initiated an independent transformation studies on creeping bent grass. Genes used for transformation studies included the *bar* herbicide resistance gene (as selectable marker), and a drought resistance gene. This drought resistance gene has been previously expressed in tobacco and in rice. Both transgenic plants have become resistant to water stress. One reason for expressing the drought resistance gene in turfgrass is to increase resistance of turfgrass to water deficits. The second reason is the fact that excess of irrigation water enhances pathogen growth, and therefore plants resistance to drought does not require much water.

I. Generated publications

1. M. Sticklen, 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. **Springer Verlag, Publs.** Berlin, Germany. In press.

2. Chien-An Liu (1995). Engineering herbicide resistance in creeping bentgrass and its potential application on the prevention of fungal diseases. **Ph. D. Thesis**. Michigan State University.

3. Chien-An Liu, Joseph Vargas, and Mariam Sticklen (1995). Evaluation of biolophos on the prevention of fungal diseases in transgenic creeping bentgrass. In preparation for **Bio/Technology**.

III. References Used in the Report

[1] Graham L. and M. B. Sticklen (1993). Plant Chitinases. Can. J. Bot. Submitted Invited review paper.

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

[3] 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. In press.

[4] 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. In press.

[5] Bolyard M. G. and M. B. Sticklen (1992). Expression of a recombinant Dutch elm disease toxin in *Escherichia coli.* Mol. Plant-Microbe Interaction (MPMI). 5(6): 478-482.

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

[7] Zhong H., Bolyard M. G., Srinivasan C., and Sticklen M. B. (1992c). Transformation of creeping bentgrass and regeneration of mature transgenic plants. Plant Cell Reps. 13 (1): 1-6.

[8] Sticklen M. B. (1990). Stable transformation of rice followed by regeneration of fertile plants. In: D. Schumacher (ed.). Proc. MidWest Plant Biotechnology Consortium. Indianapolis, IN. Oct. 7-8, 1990. 191-198.

[9] Cheng J., M. G. Bolyard, R. Saxena, and M. B. Sticklen (1992). Insect resistant transgenic potato expressing a *Bacillus thuringiensis* var kurstaki gene. Plant Science. 81(1): 83-91.