AN EXECUTIVE SUMMARY

DEVELOPMENT OF GENETICALLY ENGINEERED CREEPING BENTGRASS RESISTANT TO FUNGAL DISEASES

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This project is designed to improve disease resistance of creeping bentgrass via genetic engineering. The objectives are 1) to develop efficient gene transfer systems in creeping bentgrass and 2) to develop transgenic creeping bentgrass resistant to fungal diseases by overexpession of a chitinase gene. Chitinase is one of anti-fungal proteins produced in plants upon fungal infection. This enzyme catalyzes the hydrolysis of chitin, a cell wall component of many fungal pathogens. It was shown that constitutive overexpression of the chitinase gene in transgenic tobacco plants resulted in enhanced resistance to fungal diseases.

For the second year of this project we focused our research efforts on developing a gene transfer system for creeping bentgrass and isolating a chitinase gene from Kentucky bluegrass.

We have developed an efficient gene transfer system for creeping bentgrass using particle bombardment. A hygromycin resistance gene was transferred into embryogenic creeping bentgrass cells by particle bombardment and transformed cells were selected on the medium containing 150 or 200 mg/l hygromycin. A total of 124 transformed calli were obtained from 27 bombarded plates, with an average of 4.6 hygromycin-resistant colonies per bombardment. Thirteen transgenic plants were regenerated from the resistant colonies. Southern blot analysis confirmed the integration of the transgene into the genome of the transgenic plants.

Using a PCR *in vitro* cloning method, we have isolated a chitinase gene from Kentucky bluegrass. We are currently determining the sequence of this gene.

SECOND YEAR PROGRESS REPORT

DEVELOPMENT OF GENETICALLY ENGINEERED CREEPING BENTGRASS RESISTANT TO FUNGAL DISEASES

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This project is designed to develop genetically engineered creeping bentgrass resistant to fungal diseases (esp., brown patch) by introduction of a modified chitinase gene (encoding an antifungal protein) into the species. For the second year of this project we have focused our reseach efforts on developing an efficient genetic transformation system for creeping bentgrass and isolating a chitinase gene from Kentucky bluegrass. Research progress and results achieved during the second year are described below.

A. Development of a gene transfer system for creeping bentgrass

As described in the first year progress report, we have established embryogenic callus cultures from the seeds of `Penncross' creeping bentgrass. To develop a gene delivery system for creeping bentgrass, we have employed two methods, particle bombardment and direct gene transfer into protoplasts by electroporation.

For particle bombardment experiments, a hygromycin resistance (*hph*) gene was delivered into creeping bentgrass cells using a biolistic gun, PDS-1000/He (Du Pont). Five days after bombardment, the bombarded cells were transferred on the medium

containing 150 or 200 mg/l hygromycin and cultured in dark. Five weeks after selection, hygromycin-resistant colonies continued to grow, whereas no resistant colonies were observed on the control plates (Fig. 1). A total of 124 transformed calli were obtained from 27 bombarded plates in three independent experiments, with an average of 4.6 resistant colonies per bombardment. The resistant colonies were further transferred to the plant regeneration medium containing 200 mg/l hygromycin. Thirteen putative transgenic plants were regenerated from hygromycin-resistant colonies. To confirm the integration of the *hph* gene into the genome of creeping bentgrass, Southern blot hybridization was performed. All 11 transgenic plants and 8 out of 10 transformed calli tested showed hybridization with the digoxigenin-labelled *hph*-specific probe. Figure 2 shows Southern blot data of two transgenic plants (lanes 2 and 3), five transformed cell lines (lanes 4 to 8), and one control (lane 1). No hybridization band was detected in the untransformed control. All transformants except for the sample in lane 7 showed multiple bands, indicating that multiple insertion events occurred during gene transfer in most transformants. One of the transgenic plants growing in soil is shown in Fig. 3.

For electroporation experiments, protoplasts(plant cells without cell walls) were isolated from 3- to 4-day old suspension subcultures. The electroporation was conducted in the presence of the *hph* gene for all samples except for the control in which electroporation was conducted without the gene. The electroporated protoplasts were cultured in the liquid culture

medium without selection. One month after the culture, the electroporated protoplasts formed visible cell colonies. The protoplast-derived cells were transferred to solid medium containing 100 mg/l hygromycin. In our preliminary experiments, no resistant colonies were grown on the medium when 150 mg/l hygromycin was used. Therefore, 100 mg/l hygromycin was used for the selection of transformed cells. However, under this selection condition, some resistant colonies were always observed in control plates. The failure of the clear-cut selection was probably due to the lack of an optimal selection conditon. For effective selection of transformed cells following electroporation, the concentration of hygromycin and the application time needed to be further optimized. However, since plant regeneration from bombarded cells was easier and less time consuming than that from electroporated protoplasts, and a relatively high frequency of resistant colonies per bombardment was obtained in a clear-cut selection, no further optimization of selection conditions for electroporated protoplasts were attempted.

B. Isolation of a chitinase gene from Kentucky blue grass

To develop transgenic creeping bentgrass resistant to fungal diseases (esp., brown patch) we originally planned to use a gene isolated from bean by Dr. Richard Broglie at Du Pont. The negotiation with Du Pont to obtain the gene was unsuccessful because Virginia tech could not indemnify the company for the work involved in the use of the gene according to the guideline

from the Virginia's Attorney General. Therefore, we decide to isolate a chitinase gene from Kentucky bluegrass in our laboratory. By comparing amino acid sequences of chitinases from tobacco, potato, bean, and rice (Fig. 4), we identified several conserved regions in the chitinase genes. These regions are believed to be conserved during the evolution presumably due to their functional and structural importance to this enzyme. Using two nucleotide sequences (5'-AGACGTCCCACGAGAACCAC-3' AND 5'-AGCCCGCCGTTGATGATGTT-3') covering these regions (i.e., two boxed regions in Fig. 4) as primers for DNA amplification, we successfully amplified and cloned a partial fragment (420 bp) of a chitinase gene from Kentucky bluegrass genomic DNA after running polymerase chain reaction (PCR).

To isolate a full length chitinase gene (an estimated size of 1,000 bp), we first tried to construct a genomic library from Kentucky bluegrass and to screen for the genomic clone containing the full length gene. This approach, however, was unsuccessful. Therefore, we used a PCR *in vitro* cloning technique to amplify the unknown regions flanking the partial fragment of the Kentucky bluegrass chitinase gene (see Fig. 5.). After the genomic DNA of Kentucky bluegrass was digested with various restriction enzymes, a DNA cassette containing two common primers (C1 & C2) was attached to the end of digested genomic DNA. We designed two specific primers (S1 & S2) from the sequence of a partial chitinase fragment. After running a first round of PCR using a pair of primers (C1 and S1), we didn't usually obtain a specific band. However, when we used the amplified PCR product as a

template in a second round of PCR using a new pair of primers (C2 & S2), a specific DNA band was amplified. This technique is very powerful for the rapid isolation of a gene when partial sequence information of the gene is available. After optimizing conditions for this method, we have isolated the full length chitinase gene from Kentucky bluegrass. To our knowledge, this is the first gene isolated from turfgrass. We are currently determining the sequence of this gene and the preliminary result indicates that this is a real chitinase gene.

C. Summary of research progress

To develop a gene delivery system for creeping bentgrass, we have employed two methods, particle bombardment and direct gene transfer into protoplasts by electroporation. We have developed an efficient gene transfer system for creeping bentgrass using particle bombardment. A hygromycin resistance gene was transferred into embryogenic creeping bentgrass cells by particle bombardment and transformed cells were selected on the medium containing 150 or 200 mg/l hygromycin. A total of 124 transformed calli were ontained from 27 bombarded plates. Thirteen transgenic plants were regenerated from the resistant colonies. The Southern blot analysis confirmed the integration of the transgeneinto the genome of the transgenic plants and transformed cells. However, attempts to development of transgenic plants via electroporation was unsuccessful primarily due to the ineffective selection.

For the isolation of a chitinase gene from Kentucky bluegrass, we have used a PCR *in vitro* cloning method. Using this new technology, we have isolated a full length chitinase gene from Kentucky bluegrass. We are currently determining the sequence of this gene.

D. The proposed research plan for the coming year

For the coming year, we will subclone the chitinase gene into the expression vector, pCOR, which contains the 5' region (promoter and intron 1) of the rice actin gene 1 (*Act1*) for a high level of transgene expression. The 5' region of the rice *Act1* gene is the most active regulatory sequence for a high level of transgene expression among the constructs we tested in creeping bentgrass. We will transfer the plasmid containing the Kentucky bluegrass chitinase gene into creeping bentgrass by the gene transfer system using particle bombardment established in our laboratory. Once transgenic plants are developed, we will screen for those which exhibit high level expression of the chitinase gene. Transgenic creeping bentgrass plants producing high levels of chitinase will be inoculated with fungal pathogens in the greenhouse and their resistance to these pathogens will be determined.

BUDGET SUMMARY (4/93-10/93)

	Budget	Spending	Balance	
Wage budget	$ 27,066	$ 25,994	$ 1,072	
Materials & Supplies	$ 16,615	$ 17,512	$ 897	(-)
Scientific meetings	$ 500	$ 372	$ 128	
Indirect cost	$ 7,069	$ 4,167	$ 2,902	
Total	$ 51,250	$ 48,045	$ 3,205	

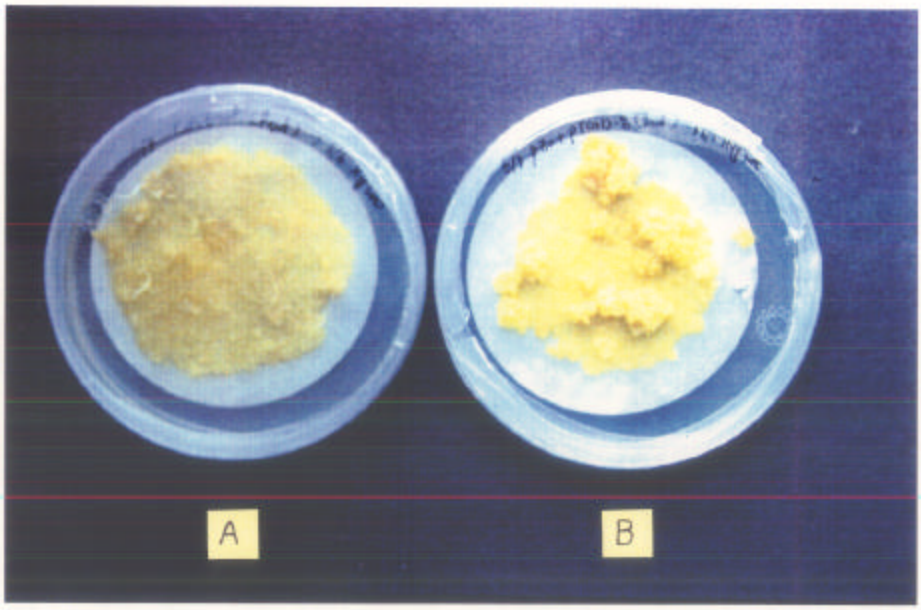

Fig. 1. Selection of transformed creeping bentgrass cells after bombardment. A: untransformed control; B: hygromycin-resistant colonies five weeks after selection.

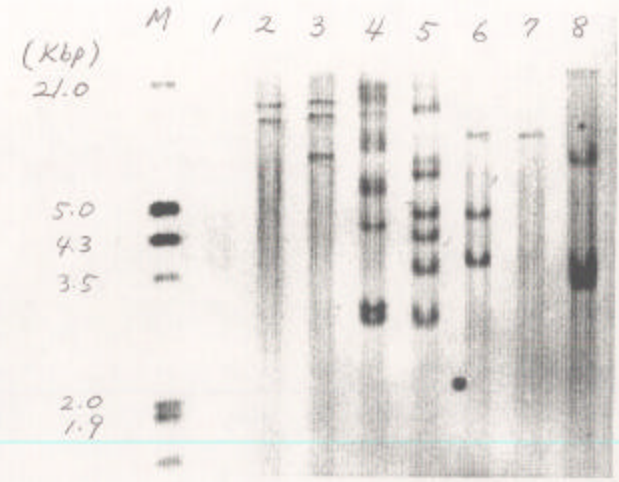

Fig. 2. Southern blot hybridization of creeping bentgrass transformants obtained via particle bombardment with the digxogenin-labeled *hph* probe. M, DNA size marker; lane 1, untransformed control; lane 2 & 3, transgenic plants; lane 4 to 8, transformed calli.

Fig. 3. A transgenic creeping bentgrass plant growing in soil.

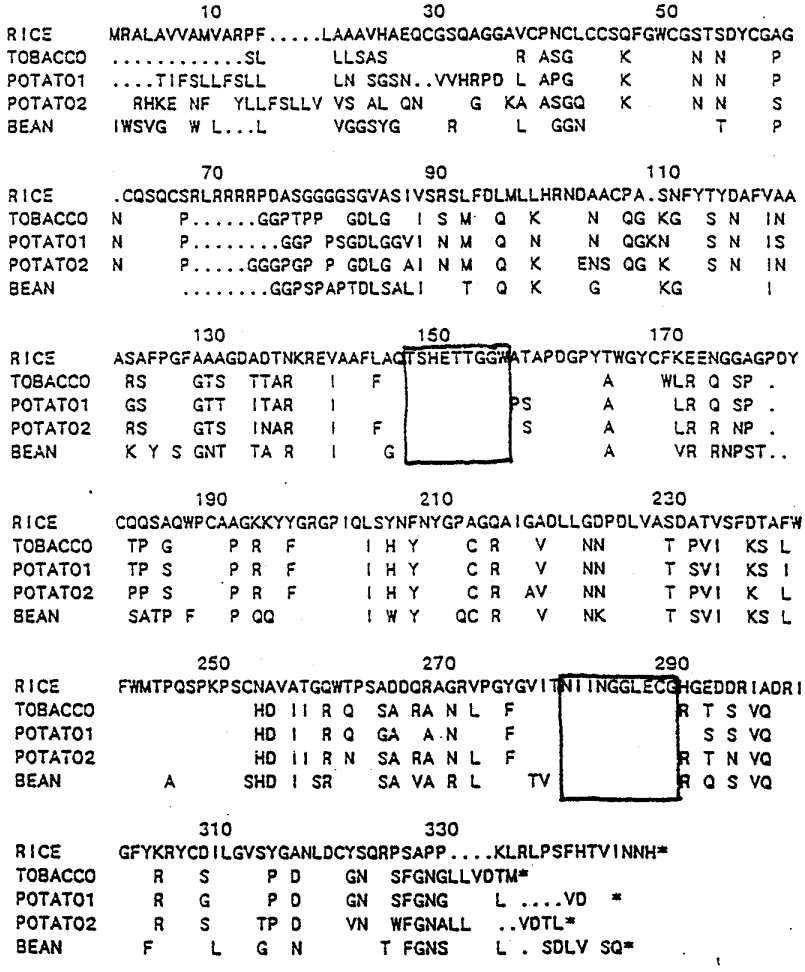

Fig. 4. Comparison of the amino acid sequences of chtinases from rice and dicotyledonous plants. Amino acid sequences of tobacco, potato, and bean chitinases are aligned with the rice chitinase sequence. Only amino acids differing from the rice chitinase are shown. Boxed areas were used to design PCR primers for amplification of a partial chitinase gene from Kentucky bluegrass.Dots indicates gaps; asterisks indicate the stop codon.

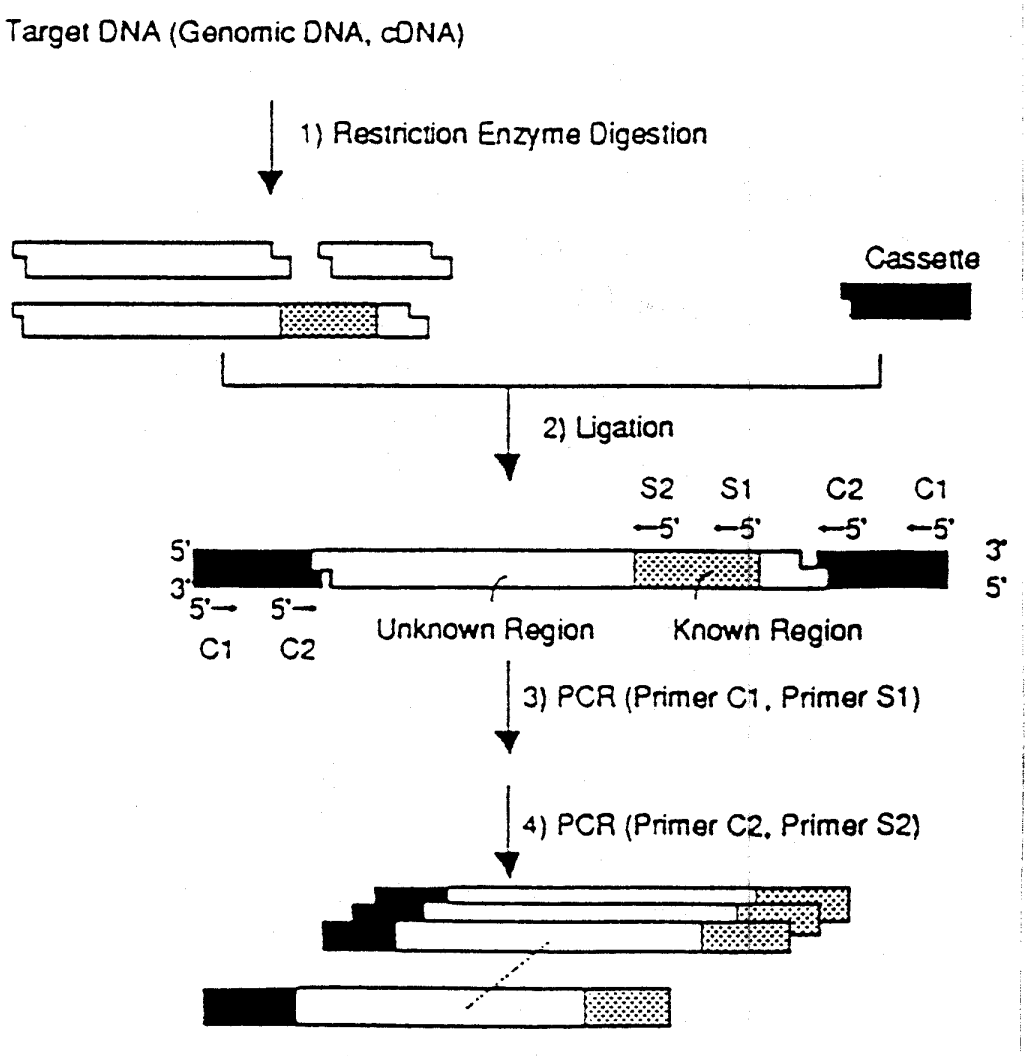

Fig. 5. The specific amplification of unknown region of a gene using the PCR *in vitro* cloning method.