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 using genetic engineering. The main objectives are 1) to develop a gene transfer system in creeping bentgrass and 2) to develop genetically engineered creeping bentgrass with overexpression of chitinase genes.

We have developed an efficient gene delivery system for creeping bentgrass using particle bombardment and selecting transformed calli with hygromycin.

We have also isolated three genomic clones of chitinase genes (*chi1, chi2, chi3*) from Kentucky bluegrass using adaptor-ligation polymerase chain reaction (PCR). *chi1* and *chi2* encode full length chitinases of 340 and 320 amino acids, respectively. *chi3* appears to encode a truncated chitinase (49 amino acids) due to the presence of a stop codon in the coding region. Using reverse transcription and PCR, we found that both *chi1* and *chi2* were induced by ethylene, strongly indicating both genes were involved in plant defense responses.

We subcloned each of these two genes (*chi1* and *chi2*) into the expression vector and transferred the plasmids containing the Kentucky bluegrass chitinase genes to creeping bentgrass by particle bombardment. Transformed calli have been selected on the medium containing hygromycin and we are currently regenerating transgenic plant from these calli. Once transgenic plants are developed, those which exhibit a high level of chitinase expression will be screened and tested for the resistance to fungal pathogens.

THIRD 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 overexpression of a chitinase gene (encoding an antifungal protein) into the species. For the third year of this project we have focused our research efforts on isolating and characterizing chitinase genes from Kentucky bluegrass and transferring these genes to creeping bentgrass.

A. Development of a gene transfer system for creeping bentgrass

As described in the second year progress report, we have established a gene delivery system for creeping bentgrass using particle bombardment. A hygromycin phosphotransferase (*hph*) gene conferring antibiotic (hygromycin) resistance to the transformed cells was delivered into creeping bentgrass cells. Five days after bombardment, the bombarded cells were transferred on the medium containing 150 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. 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. The integration of the *hph* gene into the genome of creeping bentgrass was confirmed by Southern blot hybridization in all 13 transgenic plants, whereas no hybridization band was detected in the untransformed control.

B. Isolation of chitinase genes from Kentucky blue grass

To develop transgenic creeping bentgrass resistant to fungal diseases (esp., brown patch) we originally planned to use a bean chitinase gene isolated by Dr. Richard Broglie at Du Pont. However, we could not obtain the gene from Du Pont because Virginia Tech could not indemnify the company for the work involved in the use of the gene following the guideline from the Virginia's Attorney General. Therefore, we decide to isolate chitinase genes from Kentucky bluegrass in our laboratory. By comparing amino acid sequences of chitinase genes from tobacco, potato, bean, and rice, we identified several conserved regions within the chitinase gene (Fig.1). These regions are believed to be conserved during the evolution presumably due to their functional and

structural importance to this enzyme. Using primers covering two conserved regions (1 and 4 in Fig.1) in polymerase chain reaction (PCR), we successfully amplified a 710 bp DNA fragment from Kentucky bluegrass genomic DNA. The PCR-amplified fragment was subcloned into pCR-Script SK(+) and sequenced. The nucleotide sequence of this fragment showed a high degree of similarity to the sequence of a rice basic chitinase gene, strongly indicating that the amplified fragment was a part of a Kentucky bluegrass chitinase gene.

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 adaptor-ligation PCR to amplify the 5' and 3' unknown regions flanking the partial fragment of the Kentucky bluegrass chitinase gene (Fig.2). After Kentucky bluegrass genomic DNA 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 from the first round of PCR in a second round of PCR using a new pair of primers (C2 & S2), specific DNA

bands were amplified. Adaptor-ligation PCR is a very powerful technique for the rapid isolation of a gene when partial sequence information of the gene is available. After spending a considerable time on optimizing this method, we have isolated three chitinase genes from Kentucky bluegrass genomic DNA. We designated these three chitinase genes as *chi1, chi2 and chi3*. *chi1* and *chi2* encode chitinases of 340 and 320 amino acids (molecular weights, 36 and 34 KD), respectively. *chi3* appears to encode a truncated chitinase due to the presence of a stop codon of TGA at 49 amino acid downstream of translation initiation codon ATG. Southern hybridization analysis indicates there are at least four to five chitinase genes in Kentucky bluegrass.

We also tested whether the chitinase genes that we cloned were involved in plant defense responses by examining the expression of the chitinase genes in ethylene-treated Kentucky bluegrass seedlings by reverse transcriptase and PCR (RT-PCR). Ethylene is a plant hormone that is involved in induction of defense genes. Two-week old Kentucky bluegrass seedlings treated with ethylene for 0, 2 and 4 days were used for RNA extraction and RT-PCR. RT-PCR result showed that both *chi1* and *chi2* were induced by ethylene treatment, indicating that both *chi1* and *chi2* were involved in defense responses. From the RT-PCR experiments, we also isolated four cDNA clones: BCHC1, BCHC2, BCHC3, and BCHC4 (Fig.3). The nucleotide sequences of BCHC1 and BCHC2

were identical to those of *chi1* and *chi2*, respectively, indicating that *chi1* and *chi2* had no introns in the coding region. BCHC3 and BCHC4 were identical to BCHC1 and BCHC2, respectively, except that both cDNAs had a deletion of 366bp (122 amino acids) at the same amino acid position. In conclusion, we have isolated three genomic clones and four cDNA clones of chitinase genes from kentucky bluegrass. To our knowledge, these Kentucky bluegrass chitinase genes are the first genes isolated from turfgrass species.

C. Development of transgenic creeping bentgrass overexpressing a chitinase gene

Since *chi1* and *chi2* genes encode complete chitinases and are involved in defense responses, we subcloned each of these two genes into the expression vector which contained 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 was the most active regulatory sequence for a high level of transgene expression among the constructs we tested in creeping bentgrass. We transferred the plasmids containing the Kentucky bluegrass chitinase genes into creeping bentgrass by particle bombardment. Transformed calli have been selected on the medium containing 150 mg/l hygromycin and we are currently regenerating plants from these transformed calli. Once transgenic plants are developed, we will screen for those which exhibit a high level of chitinase gene expression.

Transgenic creeping bentgrass plants producing high levels of chitinase will be inoculated with fungal pathogens and their resistance to these pathogens will be determined.

BUDGET SUMMARY (4/92-10/95)

	Budget	Spending	Balance
Wage budget	$41,589	$43,635	$ 2,046 (-)
material & Supplies	$25,353	$19,301	$ 5,559
Scientific meetings	$ 1,000	$ 507	$ 493
Indirect cost	$10,871	$ 8,444	$ 2,427
Total	$78,813	$72,380	$ 6,433

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                  10                  30                  50
RICE      MRALAVVAMVARPF.....LAAAVHAEQCGSQAGGAVCPNCLCCSQFGWCGSTSDYCGAG
TOBACCO   ............SL      LLSAS           R ASG    K  1  N N    P
POTATO1   ....TIFSLLFSLL      LN SGSN..VVHRPD L APG    K     N N    P
POTATO2     RHKE NF  YLLFSLLV VS AL QN    G  KA ASGQ   K     N N    S
BEAN      IWSVG  W L...L      VGGSYG    R     L  GGN           T    P

                  70                  90                 110
RICE      .CQSQCSRLRRRRPDASGGGGSGVASIVSRSLFDLMLLHRNDAACPA.SNFYTYDAFVAA
TOBACCO   N     P......GGPTPP  GDLG  I S M  Q  K    N  QG KG  S N   IN
POTATO1   N     P........GGP PSGDLGGVI N M  Q  N    N  QGKN   S N   IS
POTATO2   N     P.....GGGPGP P GDLG AI N M  Q  K   ENS QG K   S N   IN
BEAN            ........GGPSPAPTDLSALI   T  Q  K    G     KG        I

                 130                 150                 170
RICE      ASAFPGFAAAGDADTNKREVAAFLAQTSHETTGGWATAPDGPYTWGYCFKEENGGAGPDY
TOBACCO    RS    GTS  TTAR   I   F    2              A    WLR Q SP .
POTATO1    GS    GTT  ITAR   I               PS      A     LR Q SP .
POTATO2    RS    GTS  INAR   I   F            S      A     LR R NP .
BEAN       K Y S GNT  TA R   I    G                  A     VR RNPST..

                 190                 210                 230
RICE      CQQSAQWPCAAGKKYYGRGPIQLSYNFNYGPAGQAIGADLLGDPDLVASDATVSFDTAFW
TOBACCO    TP G     P R  F      I H Y    C R   V   NN     T PVI  KS L
POTATO1    TP S     P R  F      I H Y    C R   V   NN     T SVI  KS I
POTATO2    PP S     P R  F      I H Y    C R  AV   NN     T PVI  K  L
BEAN       SATP F   P QQ        I W Y   QC R   V   NK     T SVI  KS L

                 250                 270                 290
RICE      FWMTPQSPKPSCNAVATGQWTPSADDQRAGRVPGYGVITNIINGGLECGHGEDDRIADRI
TOBACCO      3        HD II R Q  SA RA N L  F       4      R T S VQ
POTATO1               HD I  R Q  GA  A N    F                S S VQ
POTATO2               HD II R N  SA RA N L  F              R T N VQ
BEAN          A      SHD I SR    SA VA R L    TV           R Q S VQ

                 310                 330
RICE      GFYKRYCDILGVSYGANLDCYSQRPSAPP....KLRLPSFHTVINNH*
TOBACCO      R   S     P D    GN  SFGNGLLVDTM*
POTATO1      R   G     P D    GN  SFGNG    L ....VD  *
POTATO2      R   S    TP D    VN  WFGNALL  ..VDTL*
BEAN        F     L   G  N       T FGNS    L . SDLV SQ*
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Fig.1. Comparison of the amino acid sequences of chitinases from rice and dicotyledonous plants. For chitinases of dicotyledonous plants, only amino acid sequences differing from the rice chitinase are shown. Underlined areas were used to design PCR primers for amplification of a partial chitinase gene from Kentucky bluegrass. Dots indicate gaps; asterisks indicate the stop codon.

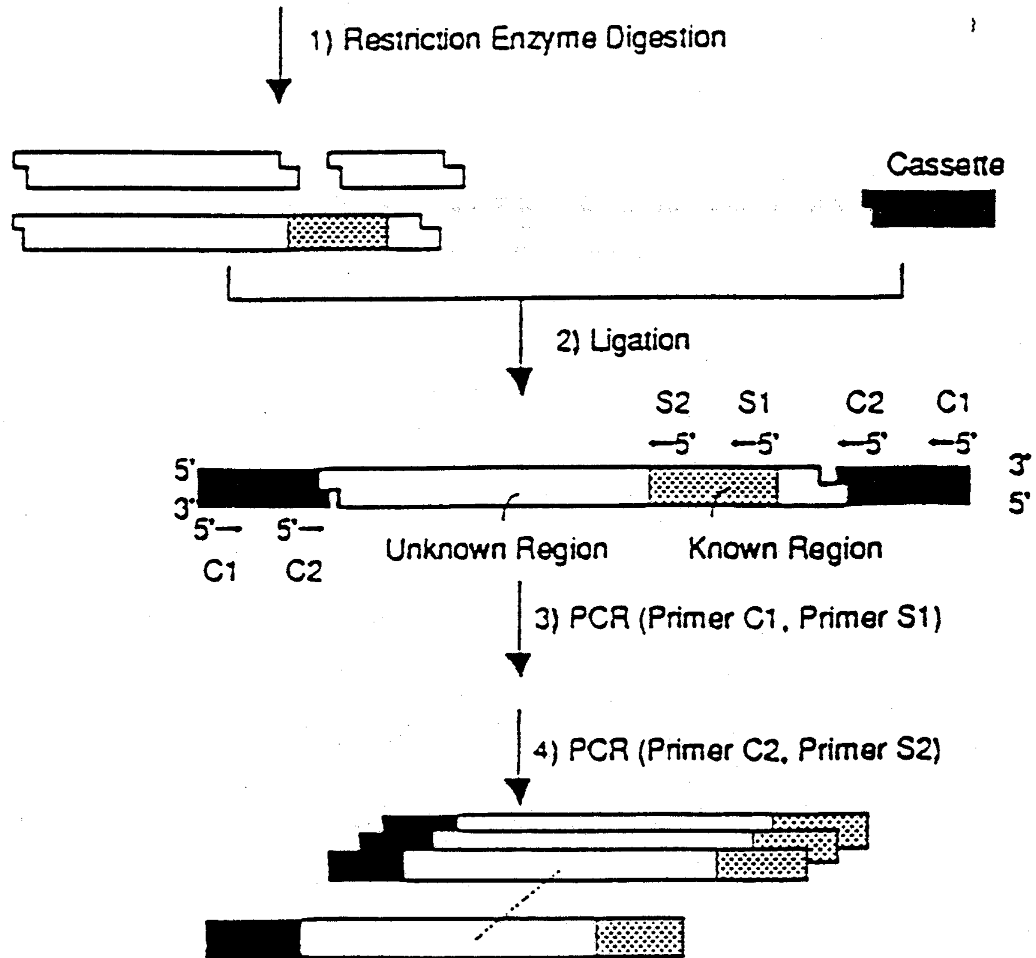

Fig.2. Schematic diagram describing amplification of unknown regions of a gene using the adaptor-ligation PCR method.

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BCHC1  MRGLVVVAILVAAFAVSAHAEQCGSQAGGATCPNCLCCSKFGFCGNTSDYCGT  53
BCHC2  *******T************************************T********  53
BCHC4  ********V**********QG************************D*****..  53
BCHC3  ***************************************************..  53

BCHC1  GCQSQCNGCSGPTPVTPTPSGGGGVSSLVSQSLFEQMLLHRNDPSCQANGFYT 106
BCHC2  ***************************I***************AA*L*K**** 106
BCHC4  ..................................................... 106
BCHC3  ..................................................... 106

BCHC1  YKAFIAAANSFAGFGTTGSTDVRKREVAAFLAQTSHETTGGWPTAPDGPYSWG 159
BCHC2  *N*************************************************** 159
BCHC4  ..................................................... 159
BCHC3  ..................................................... 159

BCHC1  YCYKQEQGATSDYCSPSSQWPCAPGKKYFGRGPIQISFNYNYGPAGQAIQTDL 212
BCHC2  **F**********************************Y*************** 212
BCHC4  ..............***********************Y**************K 212
BCHC3  ..............*************************************** 212

BCHC1  LNNPDLVASNATVSFRTALWFWMTAQSPKPSSHAVITGQWSPSSADQAAGRVP 265
BCHC2  ********TDP****************************************** 265
BCHC4  ********TDP****K************************************* 265
BCHC3  ***************************************************** 265

BCHC1  GYGVITNIINGGLECGKGQDNRVAVADRIGFYKRYCDLLGVSYGDNLDCYNES 318
BCHC2  ************************..******************N*****SQR 318
BCHC4  ************************..******************N*****SQS 318
BCHC3  ***************************************************** 318

BCHC1  PFGSSAKRNIKRNIYYVSCFPT 340
BCHC2  **** 322
BCHC4  **** 322
BCHC3  ********************** 340
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Fig.3. Deduced amino acid sequences of four Kentucky bluegrass cDNAs: BCHC1, BCHC2, BCHC3, and BCHC4.