Executive summary of research-in-progress for the transformation of creeping bentgrass with the pHS2 chitinase gene.

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More than one thousand creeping bentgrass plants have been putatively transformed for herbicide and insect resistance with the *bar* and *pinII* genes. They exhibit resistance to Ignite herbicide (1.2% commercial product containing 200g/L active ingredient of glufosinate ammonium) foliar spray. Molecular analysis of these plants is in progress.

The pHS2 chitinase gene cloned from elm chitinase has been successfully manipulated to express in *E. coli*. When induced with IPTG, *E. coli* cells with the pGEX-4T-1-pHS2 (plasmid + gene) produced the GST-chitinase fusion protein. This was determined by CDNB assay. The 58kD fusion protein was visible on Coomassie stained SDS Laemli gels. The uninduced cultures containing pGEX-4T-1-pHS2 and both induced and uninduced cultures without pGEX-4T-1-pHS2 did not produce the GST-chitinase fusion protein.

Attempts to purify the GST-chitinase fusion protein by adsorption to glutathione-agarose beads were not successful. Therefore, experiments were set up to collect fractions of the bacterial extract at several steps during the purification process. These experiments resulted in discovering that the GST-chitinase fusion protein was not soluble in the buffer used. This, again, was confirmed using SDS Laemli gels. In an effort to remedy this situation, two steps have been taken. They are both currently underway. The first step is to remove the signal peptide currently located at the amino terminal of the protein. This amino acid sequence is 16 amino acids in length and is highly hydrophobic which may be contributing to the insolubility of the GST-chitinase fusion protein. A PCR primer has been designed which will be used to produce a PCR product containing the chitinase gene without the hydrophobic signal peptide. This PCR product will be cloned into a bacterial expression vector. The construct will be used to transform *E. coli*. Bacterial cultures containing the construct will be induced with IPTG and tested to determine if they contain soluble chitinase with antifungal activity.

The second step underway is transformation of tobacco with the pHS2 chitinase gene. Tobacco is relatively quick and easy to transform via *Agrobacterium*. This successful transformation will give us information about the plant's ability to produce active chitinase from pHS2. If tobacco can successfully produce pHS2 chitinase with antifungal activity, this would indicate that bentgrass plants will likely do the same. At that point it is expected that no further manipulation of the pHS2 chitinase gene will be necessary before the transformation of bentgrass plants. Upon successful transformation of tobacco, leaf extracts will be used in bioassays to test for antifungal activity.

Antifungal bioassays have been attempted. However, due to the insolubility of the GST-chitinase fusion protein produced in bacteria, no activity has yet been observed.

Chitinase Research Update

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I. Transformation of creeping bentgrass for herbicide and insect resistance.

Creeping bentgrass was transformed by particle bombardment with both the *bar* and *pin II* genes. The *bar* gene conferred resistance to the herbicide Ignite (active ingredient glufosinate ammonium). The *pin II* gene coded for a protease inhibitor from potato which has the potential to inhibit insect growth by inhibiting the insects' digestion of protein by chymotrypsin and trypsin.

More than one thousand putative transgenic creeping bentgrass plants were sprayed with 1.2% Ignite (commercial product containing 200 g/L of active ingredient glufosinate ammonium). The putative transgenic plants were unaffected by the herbicide treatment. They were similar in appearance and size to control plants which were sprayed with water only. Untransformed plants sprayed with the same amount of Ignite turned chlorotic and died. Molecular analysis of the putative transgenic plants for the *bar* and *pin II* genes is in progress.

II. Expression of cloned chitinase in *Escherichia coli.*

The first attempt to express a cloned elm chitinase gene in *E. coli* was made using the pHS2 clone of elm chitinase, which had been partially characterized previously. The pHS2 clone contained a 1.2 kb cDNA obtained from an elm cDNA library constructed from elm NPS 3-487. The 1.2 kb cDNA from pHS2 was inserted into pKK223-3 (Pharmacia, Piscataway, New Jersey) (Brosius and Holy, 1984), an expression vector for overexpression of proteins in *E. coli*. This plasmid construct

was designated pSoleman. pKK223-3 contains the *tac* promoter, which is regulated by the *lac* repressor and induced by the addition of isopropyl-b-D-thiogalactoside (IPTG) to the growth medium. After induction of the bacterial cultures containing pSoleman, no chitinase protein was produced. A previous report indicated that the start codon of the insert should be located 5-9 nucleotides from the ribosome-binding site encoded in pKK223-3 in order to obtain expression of the inserted gene (Kozak, 1983). The pSoleman clone contained approximately 100 nucleotides between the ribosome-binding site and the start codon. These extra nucleotides were suspected to be at least partially responsible for the lack of expression. In order to make a construct without the extra nucleotides, it was necessary to insert a restriction site immediately in front of the start codon. Polymerase chain reaction (PCR) would be used to create an Eco RI site. In order to properly design the primer necessary for the PCR reaction, an accurate sequence of pHS2 was required. Although preliminary sequencing of pHS2 had already been accomplished, the previous sequence was not accurate enough for PCR primer design. Therefore, pHS2 was sequenced accurately in both directions by Sanger dideoxy sequencing (Sanger, 1977). The accurate sequence corrected approximately 10% of the nucleotides of the previous preliminary sequence. Using the corrected sequence, a primer was designed to insert an Eco RI site immediately before the initiation codon of pHS2. The primer sequence, designated MB45 was AAGAATTCATGAGGTTTTGGGCATTG. The PCR reaction was done using the MB45 primer and a -20 primer which would anneal to a sequence in the multicloning site of the pBluescript SK- vector (Stratagene, La Jolla, CA). The PCR product was 1.1 kb in size as expected (fig. 1). The PCR reaction product was

restricted with Eco RI and Xho I, and ligated into Eco RI and Xho I cut pGEX-4T-1 (Pharmacia), a procaryotic gene fusion vector. The resulting construct was designated pGEX-4T-1-pHS2. When induced with IPTG, *E. coli* containing the construct should express a fusion protein consisting of glutathione-S-transferase (GST) and chitinase. The pGEX-4T-1-pHS2 construct was introduced into *E. coli* HB101 by $CaCl_2$ mediated transformation. Upon induction with IPTG, the bacterial cultures containing pGEX-4T-1-pHS2 did successfully express the GST-chitinase fusion protein. The 58 kd fusion protein was clearly visible on Coomassie stained SDS Laemli gels of the induced bacteria (fig. 2). Uninduced cultures containing pGEX-4T-1-pHS2 or uninduced or induced cultures of *E. coli* without pGEX-4T-1-pHS2 did not produce the 58 kd GST-chitinase fusion protein. Time course experiments demonstrated that increasing amounts of the GST-chitinase fusion protein were produced up to four hours after induction (fig. 3).

III. Purification of chitinase expressed in bacteria.

Attempts to purify the GST-chitinase fusion protein by adsorption to glutathione-agarose beads were not successful. Therefore, experiments were done to determine the location of the GST-chitinase during the purification process. Procedures were done according to the "GST Gene Fusion System, 1993" instruction manual provided by the supplier (Pharmacia). *E. coli* HB101 containing pGEX-4T-1-pHS2 were grown overnight in 2XYT-G medium (16 g/l tryptone, 10 g/l yeast extract, 5 g/l NaCl, 2% glucose, pH 7.0) with 100 mg/l ampicillin. The overnight culture was

diluted 1:10 into 500 ml of fresh 2XYT-G medium and incubated for one hour before induction with IPTG to a final concentration of 0.1 mM. Incubation was continued at 37C with 200 rpm shaking for an additional four hours. Bacteria from induced cultures were collected by centrifugation and resuspended in phosphate buffered saline (PBS) (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3) with 0.2 mM 4-(2-aminoethyl)-benzenesulfonylfluoride, HCl (AEBSF) and 1 mM ethylenediaminetetraacetic acid, sodium salt (EDTA) as proteinase inhibitors. Lysozyme (0.1 volume of a 10 mg/ml solution in 25 mM tris(hydroxymethyl)aminomethane-HCl (Tris-HCl) , pH 8.0) was added. After incubation at 37C for 30 min., the bacteria were lysed by sonication. Microscopic examination revealed that the lysis was satisfactory. Triton X-100 (20%) was added to a final concentration of 1%, and the lysate was incubated at room temperature for 30 min. The lysate was clarified by centrifugation, and the pellet was resuspended in an equal volume of the same solution. Enzyme assays were done on four separate fractions: 1) The culture medium left after pelleting of the bacterial cells, 2) the bacterial lysate before the second centrifugation, 3) the supernatant after the second centrifugation, 4) the resuspended pellet after the second centrifugation. Results indicated that from cultures from bacteria containing pGEX-4T-1 alone without chitinase, which expressed GST alone without chitinase, the GST was mostly contained in fraction 3, the supernatant of the second centrifugation. But from cultures expressing GST-chitinase, virtually all of the fusion protein was contained in fraction 4, the pellet after the second centrifugation. This indicated that the GST-chitinase fusion protein was insoluble in the buffer used. SDS Laemli gels confirmed

the presence of insoluble GST-chitinase in fraction 4 (fig. 4).

IV. Removal of the chitinase signal peptide.

An examination of the deduced amino acid sequence of pHS2 revealed that the amino terminal 16 amino acids comprise a highly hydrophobic signal sequence. This sequence may function to facilitate the transport of the protein to the vacuole in plant cells. It is possible that this sequence must be cleaved from the chitinase before the protein becomes soluble and enzymatically active. In order to test this hypothesis, a PCR primer was designed to create a Bam HI site in pHS2 between the hydrophobic signal sequence and the start of the chitin binding domain. The sequence of the primer was TGTCCGGATCCCAAGGAGGCTGGGCAG. This primer, along with a -20 primer will be used to produce a PCR product which will contain the chitinase gene minus the hydrophobic signal peptide. The PCR product will be cloned into pGEX-4T-1 and introduced into *E. coli*. These bacteria will be induced with IPTG as before and lysed. The lysates will be tested for the presence of soluble, enzymatically active chitinase, and for antifungal activity.

V. Expression of chitinase in plants.

In order to test the ability of the pHS2 chitinase to express well in plants and to inhibit growth of pathogenic fungi *in vivo*, an experiment was initiated to express the chitinase in tobacco. Tobacco was chosen because it is relatively fast and simple to

transform. Successful inhibition of fungal pathogens by pHS2 chitinase in tobacco would demonstrate the potential for antifungal activity of pHS2 chitinase in turfgrass. In order to transform tobacco, it was necessary to construct a plasmid containing chitinase for use in *Agrobacterium*-mediated transformation. The Eco RI to Xho I fragment of pHS2 containing the chitinase gene was ligated into pKYLX71 (Schardl. et al., 1987) cut with Hind III and Xho I. The Eco RI and Hind III sites had been filled in with the Klenow fragment of DNA polymerase I to create compatible blunt ends. The resulting plasmid was designated pKYLX71-pHS2. The pKYLX71-pHS2 plasmid was introduced into *Agrobacterium tumefaciens* LBA4404 by triparental mating. The *Agrobacteria* containing pKYLX71-pHS2 were used for transformation of tobacco leaf discs. The leaf discs were incubated on MS medium to allow them to regenerate. When they have regenerated, they will be assayed for the presence of the chitinase gene, and for expression of chitinase. They will also be assayed for enhanced resistance to plant pathogenic fungi.

VI. Chitinase antifungal bioassay.

Preliminary experiments in which pHS2 chitinase from *E. coli* is tested in plate bioassays to determine its antifungal activity against *Rhizoctonia solani, Sclerotinia homoeocarpa,* and *Pythium aphanidermatum* are currently underway. In addition, when the tobacco transformations are completed, extracts of tobacco will be tested *in vivo* for antifungal activity to help determine whether the pHS2 chitinase is folded properly within the plant cells. Initially, the method used will be similar to that of

Huynh, et al (Huynh, et. al. 1992) in which the chitinase will be added to sterile filter paper disks on agar plates with a plug of fungal mycelia in the center of the plate. Adaptations will be made as warranted.

Figure 1: PCR product used to make pGEX-4T-1-pHS2.

Lane 1: l DNA restricted with Bst EII; Lane 2: PCR product containing the pHS2 chitinase gene without the approximately 100 nucleotide 5' leader.

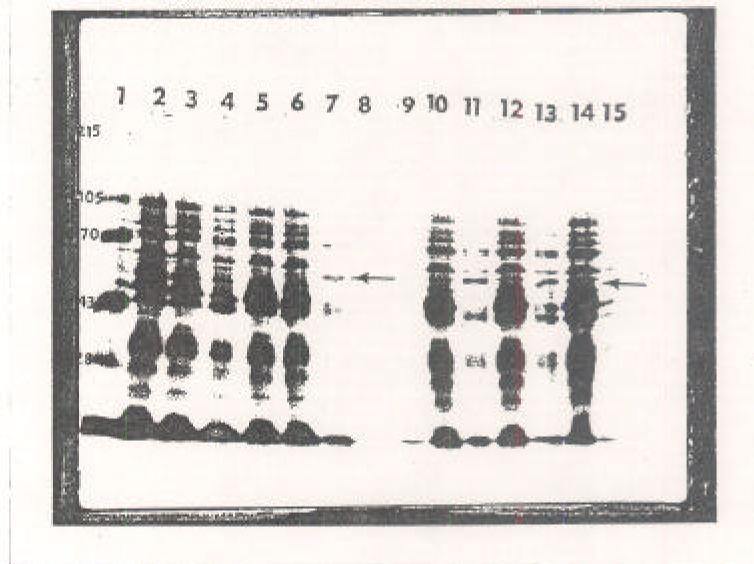

Figure 2: Expression of pHS2 in *E. coli*.

E. coli JM105 containing no plasmid, pSoleman, or pGEX4T-1-pHS2 were grown in 2YT medium at 37 C with 200 rpm shaking. Induction was with 1 mM IPTG. Samples were harvested 4 hours after induction and electrophoresed on a polyacrylamide gel. The gel was stained with Coomassie brilliant blue. Lane 1: Molecular weight markers; Lanes 2, 4, 6, 8, 10, 12, 14: Not induced; Lanes 3, 5, 7, 9, 11, 13, 15: induced with IPTG; Lanes 2, 3: No plasmid; Lanes 3, 4: pSoleman; Lanes 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15: pGEX4T-1-pHS2.

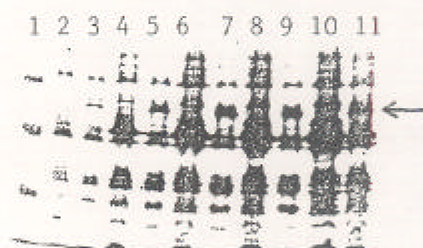

Figure 3: Expression of GST-chitinase in *E. coli*; time course experiment.

Lane 1: Molecular weight markers; lanes 2, 4, 6, 8, 10: not induced; lanes 3, 5, 7, 9, 11: induced with 0.1 mM IPTG; lanes 2, 3: 1 hr after induction; lanes 4, 5: 2 hr after induction; lanes 6, 7: 3 hr after induction; lanes 8, 9: 4 hr after induction; lanes 10, 11: 5 hr after induction.

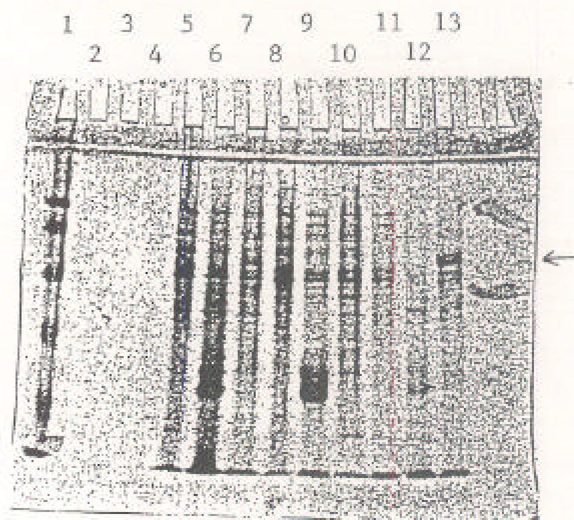

Figure 4: Partial purification of GST-chitinase.

Lane 1: molecular weight markers; lanes 2, 5, 8, 11: *E. coli* with no plasmid; lanes 3, 6, 9, 12: *E. coli* with pGEX-4T-1; lanes 4, 7, 10, 13: *E. coli* with pGEX-4T-1-pHS2; lanes 2-4: supernatant after cells were pelleted; lanes 5-7: lysate before second centrifugation; lanes 8-10: supernatant after second centrifugation; lanes 11-13: pellet after second centrifugation resuspended in an equal volume of buffer.

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