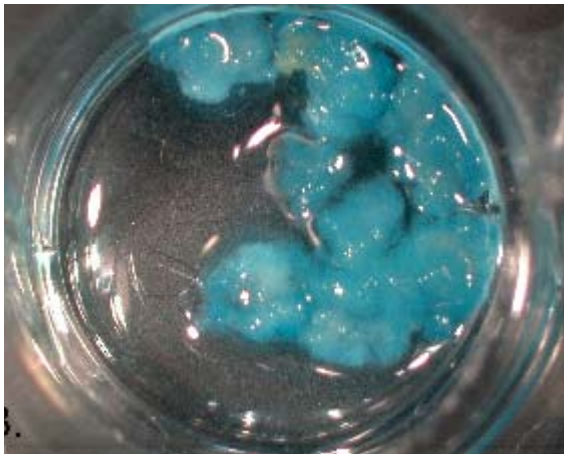
Turfgrass and Environmental Research Online

...Using Science to Benefit Golf



Michigan State University researchers used genetic engineering techniques, including the use of a reporter gene (gus) to indicate successful transformation (shown in the callus culture above), to create transgenic lines of creeping bentgrass that are resistant to glufosinate (a broad-spectrum herbicide) and infection by *Rhizoctonia solani* (Brown Patch).

Volume 2, Number 21
November 1, 2003

PURPOSE

The purpose of *USGA Turfgrass and Environmental Research Online* is to effectively communicate the results of research projects funded under USGA's Turfgrass and Environmental Research Program to all who can benefit from such knowledge. Since 1983, the USGA has funded more than 290 projects at a cost of $25 million. The private, non-profit research program provides funding opportunities to university faculty interested in working on environmental and turf management problems affecting golf courses. The outstanding playing conditions of today's golf courses are a direct result of ***using science to benefit golf***.

Editor

Jeff Nus, Ph.D.
904 Highland Drive
Lawrence, KS 66044
jnus@usga.org
(785) 832-2300
(785) 832-9265 (fax)

Research Director

Michael P. Kenna, Ph.D.
P.O. Box 2227
Stillwater, OK 74076
mkenna@usga.org
(405) 743-3900
(405) 743-3910 (fax)

Genetic Engineering Turfgrasses for Pest Resistance

S. B. Maqbool and M. B. Sticklen

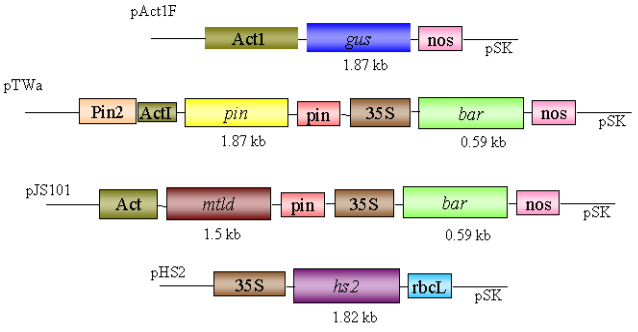
SUMMARY

Genetic engineering of crops is known to complement crop breeding for development of genotypes that are impossible to develop through breeding practices only. Michigan State University researchers:

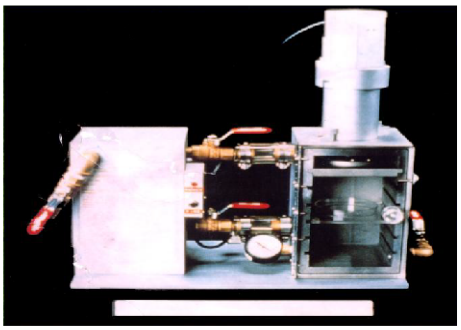
- Developed an efficient cell and tissue culture turfgrass regeneration system that is the prerequisite for genetic transformation.
- Used embryogenic turfgrass callus (non-differentiated cell lines) to recover genetically altered turfgrass and developed a genetic engineering system for creeping bentgrass using a reporter (gus) blue gene to determine successful gene incorporation into plants.
- Successfully incorporated a gene (bar) for resistance to glufosinate (FinaleTM or bialaphos, a non-selective herbicide) and developed transgenic turfgrass plants resistant to this herbicide.
- Discovered that bialaphos also has fungicidal in addition to herbicidal properties. As a result, they were 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.
- Cloned and characterized a full-length chitinase gene (hs2) that contains the necessary chitin-binding domain from a Dutch elm disease resistant American elm (*Ulmus Americana*).
- Constructed a mini-gene containing this chitinase gene and successfully inserted this chitinase gene into creeping bentgrass. Fungal bioassays utilizing chitinase expressing transgenic plants were conducted at laboratory and greenhouse levels in collaboration with Dr. Joseph Vargas. These bioassays showed that two out of five independently transgenic turfgrass lines were 3-5 fold resistant to *R. solani*.

Due to its tolerance of low mowing heights, density, and other turf quality characteristics, creeping bentgrass (*Agrostis stolonifera* L.) is an excellent species for use on golf courses throughout most of the United States. However, creeping bentgrass is greatly affected by pathogens, insects and environmental stresses associated with drought and temperature extremes. Furthermore, weeds are major problems because they are the hosts for pathogens and insects and compete for water and nutrients with turfgrass.

The best approach to combat these stresses is through the development of transgenic plants, followed by a breeding program. Transgenic plants are created by the introduction



(a)



(b)

Figure 1. (a) Plasmid constructs used in transformation experiments. Act1: rice actin gene promoter; Pin2: pin2 gene promoter; 35S: CaMV 35S promoter; Act1: intron from rice actin gene promoter; gus: coding sequence of GUS gene; pin: coding sequence of pin2 gene; mtld: mannitol 3'-phosphate dehydrogenase gene; bar: coding sequence of bar gene; hs2: the elm chitinase gene; nos: transcription terminator; pin: pin transcription terminator; rbcL: rbcL transcription terminator. (b) Biolistic GunTM used for DNA delivery to turfgrass tissues via bombardment.

S. B. MAQBOOL, Ph.D., Research Associate Postdoc, and M. B. Sticklen, Ph.D., Professor, Cereal and Turfgrass Genetic Engineering Laboratory, Department of Crop and Soil Sciences, Michigan State University, East Lansing, MI.

of genes (fundamental units of heredity) into the nuclear deoxyribonucleic acid (DNA), the primary carrier of genetic information.

The first step towards genetic improvement of turfgrass is to produce an efficient turfgrass *in vitro* (cell and tissue culture) regeneration system using embryogenic non-differentiated cell lines called callus. The second important step is to establish the most efficient and reproducible gene transfer system, where a reporter gene (showing a color) is successfully integrated into the plants. Once these steps are achieved, genes of interest such as those responsible for disease resistance, insect resistance, environmental stress resistance and/or genes of other important traits could be transformed into turfgrass species. Therefore, we first established an efficient and reproducible *in vitro* plant regeneration system for turfgrass using embryogenic callus produced from mature seeds, and then developed the transformation system for turfgrass with beta-glucuronidase or blue gene (gus) using the Biolistic Gun™ purchased from DuPont Company.

Bialaphos is a non-selective and broad-spectrum contact herbicide also known as glufosinate (18) or Finale™. Bialaphos resistance has been achieved by introducing a gene (bar) coding for a detoxifying enzyme, phosphinothricin acetyl transferase (PAT), into turfgrass and several other plant species (3, 9, 16). It has been shown that spraying bialaphos while using the bar gene expressing transgenic rice (*Oryza sativa*) plants could prevent the fungal infection (sheath blight) caused by *R. solani* (19).

Since *R. solani* is also the cause of brown patch, a severe foliar fungal disease of creeping bentgrass, we attempted to determine if glufosinate and bialaphos could be used for the protection of the bialaphos- and glufosinate-resistant transgenic creeping bentgrass from these fungal pathogens. The success of this approach would provide a novel and economical usage for the herbicide to control weeds, as well as reduce the level of fungal infections in turf area with bialaphos- or glufosinate-resistant creeping bentgrass.

Chitinases are antifungal proteins found in a wide variety of plants (4). Chitinases in plant tissues combat invasions by pathogens (10,11, 13). Plant chitinases attack pathogens directly by degrading chitin (a fungal cell wall component) to confer disease resistance. Chitinases have been purified from many plant species and tested against pathogens (4).

We isolated a chitinase cDNA (hs2) from an American elm selected by the National Park Service in Washington, D. C. found to be resistant to Dutch elm disease caused by the fungal pathogen *Ophiostoma ulmi* using field inoculation techniques (15). The hs2 was suggested to be a good candidate to produce fungal pathogen resistant variety of creeping bentgrass.

This paper reports the production of whole plants from embryogenic callus using mature turfgrass seeds, and genetic transformation of creeping bentgrass with gus, bar, and hs2 genes and the subsequent prevention of fungal diseases with simultaneous control of weeds.

Materials and Methods

Mini-genes

Mini-gene (plasmid) Act1-F containing a useful reporter blue gene (gus) and plasmid TW-a containing the herbicide resistance (bar) gene were used for the establishment of the most efficient and reproducible gene transformation system (Fig. 1a).

The plasmid pHS2 (Fig. 1a) carrying the elm chitinase gene (hs2) was constructed. This plasmid and another plasmid called JS101 (Fig. 1a) carrying the bar and the mannitol-1-phosphate dehydrogenase gene (mtlD) were used for co-transformation of creeping bentgrass.

Embryogenic callus induction and maintenance of creeping bentgrass

Embryogenic callus was initiated from mature seeds of creeping bentgrass as follows: mature seeds were surface sterilized using 50% ethanol for 5 minutes and then 40% commercial bleach (cloroxTM) containing 0.1% Tween 20 for 15 minutes. Surface sterilized seeds were cultured in Petri dishes containing 20 mL of MS (12) basal

medium supplemented with 500 mg/L enzymatic casein hydrolysate (Sigma), 3 % sucrose, and different concentrations of 2, 4-D (2.25, 4.5, 9 and 18 µM), dicamba (5, 10, 30 and 60 µM) or combinations of 30 µM dicamba and BA (2.25, 4.5, 9 and 18 µM), phytager (Gibco/BRL), pH 5.8. Cultures were incubated at 24 ± 2 ºC in a culture room in the dark. Callus was subcultured at 2-week intervals. Suspension cultures were initiated by transferring 2 g of one-month-old friable callus into a 225 ml flask containing 50 ml of the liquid callus induction medium (1). Both callus and suspension cultures were incubated at 25° C in the dark. Suspension cultures were kept on a rotary shaker at 150 rpm. The suspension culture medium was changed every three days.

Genetic transformation via bombardment

About 200 mg of friable and yellowish embryogenic callus (2-3 mm in diameter; 40-50 pieces/dish) or cells from suspension cultures were used for bombardment. A total of 25 µg plasmid DNA (pAct1-F or pTW-a or a mixture of pJS101 and pKYLX71-pHS2 in a 1:1 molar ratio) in 20 µl were adsorbed onto tungsten particles (1.2 µm; GTE Sylvania) with $CaCl_2$ and spermidine (free base, tissue culture grade, Sigma Chemical Co.) following a modified protocol from Klein et al. (7). Callus or cells from suspension cultures were placed on the surface of agar medium in Petri dish and bombarded twice with the DNA adsorbed tungsten particles using a Biolistic® particle acceleration device PDS-100/He system (Fig. 1b; Bio-Rad Laboratories, Inc.).

Selection of putative transgenic callus cultures

The bombarded callus or cells were cultured and selected on a callus induction medium containing 5 mg/L bialaphos (Meiji Seika Kaisha of Japan), or 15 mg/L glufosinate ammonium (a commercially available synthetic herbicide) as the selective agent for transformed cultures expressing the bar gene. To recover transgenic embryogenic cultures from the majority of non-transgenic culture population, callus culture media containing 5 mg/L and 10 mg/L bialaphos were used for ten weeks followed by selection medium containing 15 mg/L bialaphos for another six weeks of *in vitro* selection.

Callus growing on selection medium was developed into plantlets using regeneration medium (half strength MS basal medium). Regeneration of plantlets was carried out for four weeks on the regeneration medium containing 15 mg/L bialaphos under continuous light for 24 hours at 24 ± 2 ºC. After shoot and root development, the transgenic plants were transferred into clay pots containing a 1:1 mixture of sand:Bacto Pro Plant Mix (Michigan Peat Co.) and maintained in a greenhouse. The regenerated plants were propagated by separation of their stolons.

Histochemical assay for GUS (blue protein) activity in transgenic plants

Tissues from transgenic and control plants were used for GUS (blue protein) activity. Samples were incubated for 24 hours in a filter-sterilized GUS staining solution containing X-gluc (5-bromo-4-chloro-3-indolyl glucuronide) in the dark at 37º C or at room temperature. Green tissues were incubated in 75% ethanol for one hour to remove the green pigment before photography.

DNA isolation and Southern blot analyses

Genomic DNA from transgenic and non-transgenic plants was isolated using a modified C-TAB method (14). Southern blot hybridization was performed according to Southern et al. (17). Briefly, 20 micrograms of genomic DNA from each sample was restriction digested, electrophoresed and transferred onto a piece of pre-wetted Hybond N+ nylon membrane (Amersham). Gene specific probes: a EcoR1-NotI digested 3.2 Kb gus fragment from pAct1-F, smaI digested 0.6 Kb bar, HindIII-BamHI digested 1.5 Kb mtld fragment from pJS101 and a PstI digested 1.2 Kb hs2 fragment from pHS2 were used in Southern hybridization. Hybridization was performed at 65º C for 16 hours. Membranes were then washed and autoradiographed on Kodak X-OMAT film.

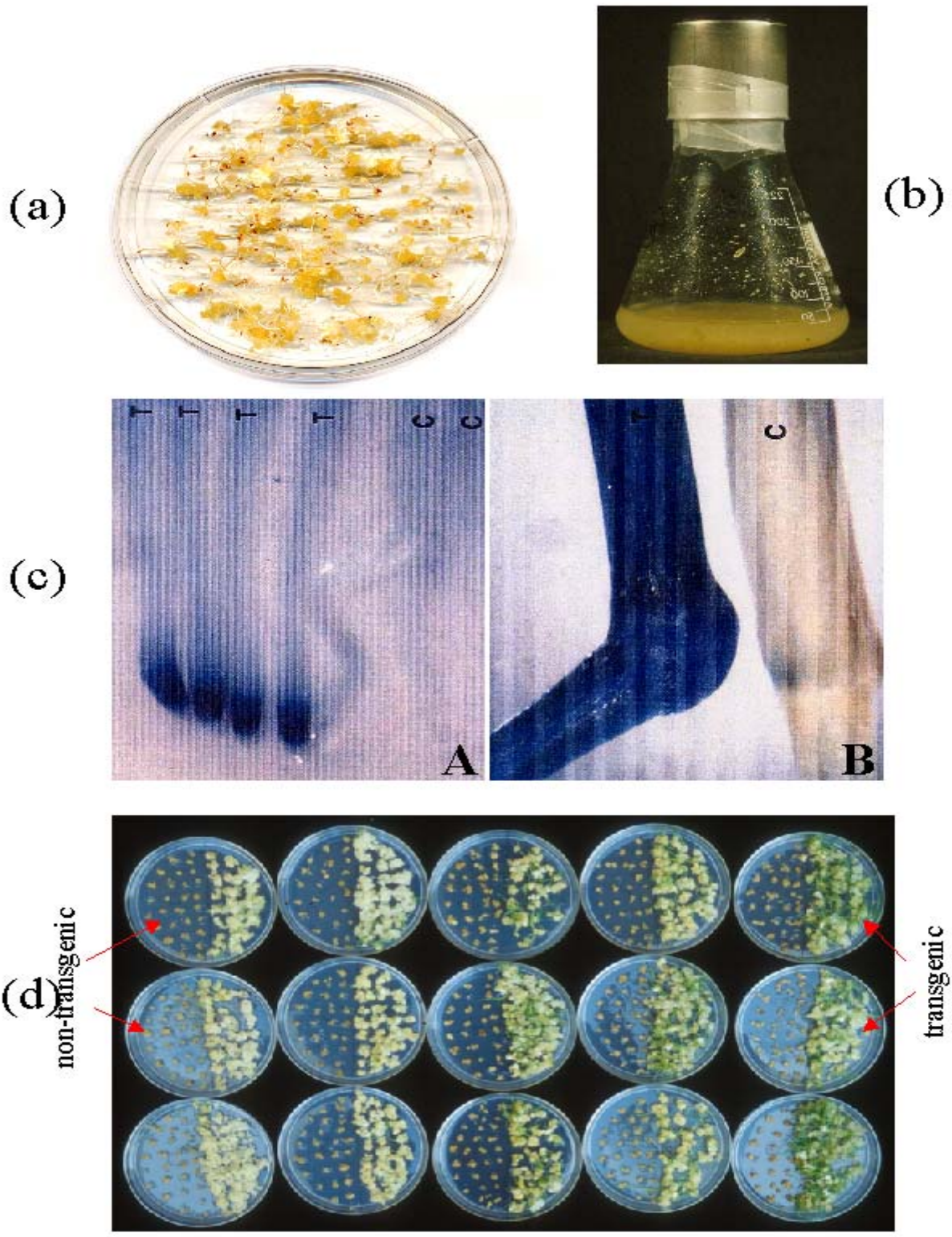


Figure 2. (a) Embryogenic callus from mature seeds of turfgrass. (b) Cell suspension culture of turfgrass callus. (c) Histochemical assay of GUS (blue marker) activity in transgenic bentgrass plants (A: roots and B: stems). T: transgenic plant; C: control (non-transgenic) plants; (d) Selection and regeneration of transgenic callus on bialaphos (selection marker) containing medium.

Greenhouse test for herbicide and fungal disease resistance

To investigate the nature of herbicide resistance in transgenic plants expressing the bar gene, a greenhouse test was performed. Regenerated and greenhouse growing non-transgenic and transgenic plants were sprayed with 2.4 g/L of glufosinate-ammonium in a spray volume of 150 L/ha. The sprayed plants were examined after two weeks.

To determine if bar gene would be useful in the prevention of fungal diseases caused by *R. solani*, *S. homoecarpa*, and *Pythium aphanidermatum*, bioassays were conducted using non-transgenic and bar gene-expressing transgenic plants. The fungal cultures for *R. solani*, *S. homoecarpa*, and *P. aphanidermatum* to use for inoculum on turfgrass plants were prepared using wheat seeds. Actively growing mycelium of *R. solani*, *S. homoecarpa*, and *P. aphanidermatum* from potato dextrose agar Petri dishes were aseptically transferred to 12 g of sterilized wheat seeds. The fungal cultures were incubated under light at 25º C for one week after the inoculation. Five hundred milligrams of wheat seed inoculum was placed on each pot of three-month old non-transgenic and transgenic plants.

Different concentrations of bialaphos from 200 to 2,400 mg/L were applied to the plants either three hours before or two days after fungus inoculation. After the bialaphos spray, plants were covered with vented plastic bags to create 100% humidity. Transgenic and non-transgenic plants that were not sprayed with bialaphos were also used for fungal inoculation. There were 10 replications in each experiment. Plant damage due to fungal infection was visually rated on a 0-10 scale (0= no damage, 10= completely dead). Data were analyzed using the F-test at P=0.05 and Tukey's Honestly significant test at P=0.05.

Bioassay of transgenic plants expressing chitinase (hs2) gene against *R. solani*

Fungal bioassays were conducted to assess levels of resistance among the chitinase (hs2) expressing transgenic lines towards *R. solani* compared with non-transgenic plants. Prior to fungal inoculation, pots containing creeping bentgrass were mowed at a 2.5 cm height, and foliar coverage was visually estimated as the percent soil coverage per pot. The center of each pot was then inoculated with approximately 3 g of inoculum colonized by *R. solani*. Inoculated plants from five transgenic lines were sealed in plastic bags to maintain 100 % relative humidity. Plastic bags containing inoculated bentgrass plants were placed inside a growth chamber under14-hour light and 10-hour dark at 25º C. Sixteen replicates of each transgenic line or non-transgenic plants were screened in a randomized complete block design with four to five blocks over time. Disease severity was visually estimated at three, five, and seven days post-inoculation using the Horsfall-Barrett scale (6).

Results

Somatic embryogenesis

The somatic embryogenesis using mature seeds of turfgrass (creeping bentgrass) varied significantly with the kind and concentration of growth regulators used in the callus induction medium. All 2,4-D concentrations induced callus formation but 9 µM 2,4-D resulted into high embryogenesis compared to other concentrations. However, the embryogenesis was significantly high in the medium using dicamba compared to 2,4-D. It was 57 % on 5 µM and 79 % on the medium containing 10 µM dicamba. Increases in dicamaba concentrations up to 30 or 60 µM reduced embryogenesis. This inhibitory effect of 30 µM dicamba on somatic embryogenesis was counteracted with the addition of BA to the medium.

Optimum embryogenesis was achieved using MS basal medium containing 30 µM dicamba plus 2.25 µM BA (Fig. 2a and b). The embryogenic callus was capable of efficiently producing somatic embryos even after eight months of maintenance. More than 80% of the embryogenic callus produced on media containing 30 µM dicamba and any concentration of BA (especially 2.25 µM), were able to produced shoots and roots three

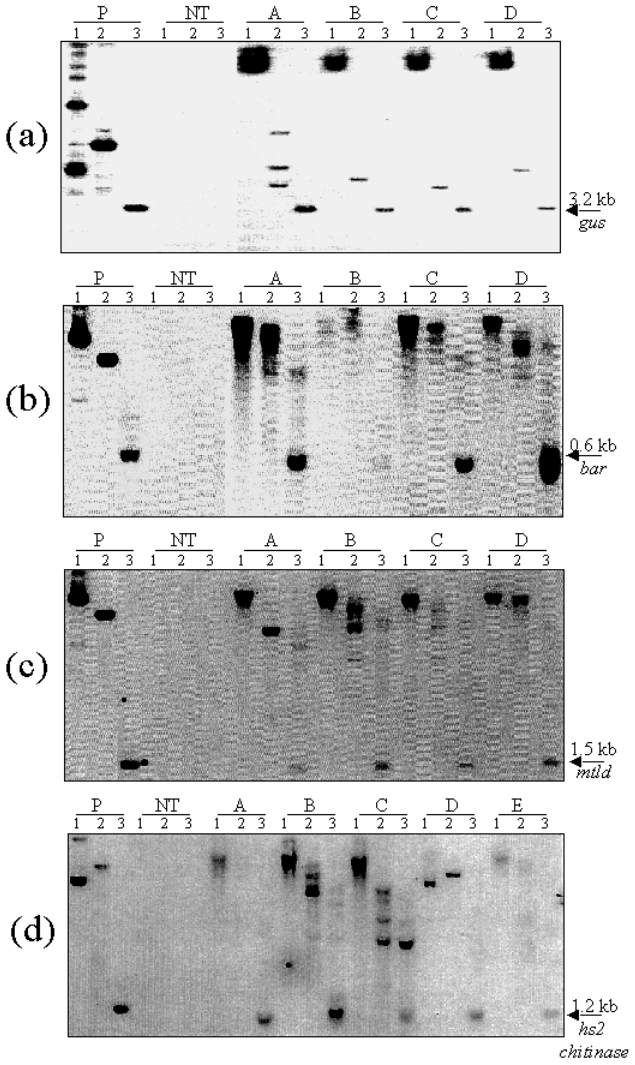


Figure 3. Southern blot analyses of turfgrass plants transformed with (a) gus (b) bar (c) mtld and (d) chitinase hs2 genes. P: plasmid (a) pACT1-F (b) pTWa (c) pJS101 (d) pHS2; NT; non-transgenic turfgrass plants; A-E: transgenic turfgrass plants. 1: undigested DNA; 2: single digested DNA ; 3: double digested DNA.

weeks after their transfer to regeneration medium and exposure to light. When transferred to a soil mix, about 95% of these plantlets survived in the greenhouse conditions and developed into healthy plants. (For details see 21).

Production of transgenic plants containing blue marker (gus) gene

For a rapid and sensitive method of detecting blue gene (gus) expression, different parts of transgenic and non-transgenic plants were tested for blue color formation (i.e. histochemical staining for GUS). Tissues from non-transformed plants showed no GUS expression, while tissues from transgenic plants showed GUS activity (Fig. 2c). All root tips from transgenic plants showed intense GUS staining in the root hair zone and root cap (Fig.2c-A). Similarly, GUS staining was intense in the stem nodes, leaves, and internodes (Fig.2c-B). Southern analysis confirmed the integration of gus in transgenic plants (Fig.3a). (For details see 22).

Herbicide resistance in transgenic lines

Bialaphos- or glufosinate-resistant independent creeping bentgrass lines were produced three months after the bombardment of embryogenic callus with the selectable marker bar gene (Fig. 2d). Southern analysis confirmed the integration of the bar in all independent herbicide-resistant transgenic plant lines (Fig. 3b).

We determined the nature of herbicide resistance in transgenic lines by applying glufosinate spray in a greenhouse test. Transgenic and non-transgenic plants two weeks after the foliar spray of glufosinate are shown in Fig. 4a. All transgenic plants were growing normally while non-transgenic plants were completely dead. (For details see 8).

Prevention of fungal diseases using bialaphos- and glufosinate-resistant transgenic lines

Various concentrations of bialaphos were applied to the transgenic creeping bentgrass expressing the bar gene to assess the effects of bialaphos on development of three different fungi (*R. solani*, *S. homoecarpa*, and *P. aphanidermatum)*. A significant ($p<0.05$) effect of bialaphos was observed on the suppression of *R. solani* development one week after fungal inoculation (Fig. 4b-A and B).

Transgenic plants not treated with bialaphos showed typical disease symptoms and had significant amounts of plant damage. There was very little plant damage one week after inoculation of *R. solani* on transgenic plants sprayed with bialaphos three hours before inoculation (Table 1). Bialaphos was also very effective in inhibiting fungal growth and development of

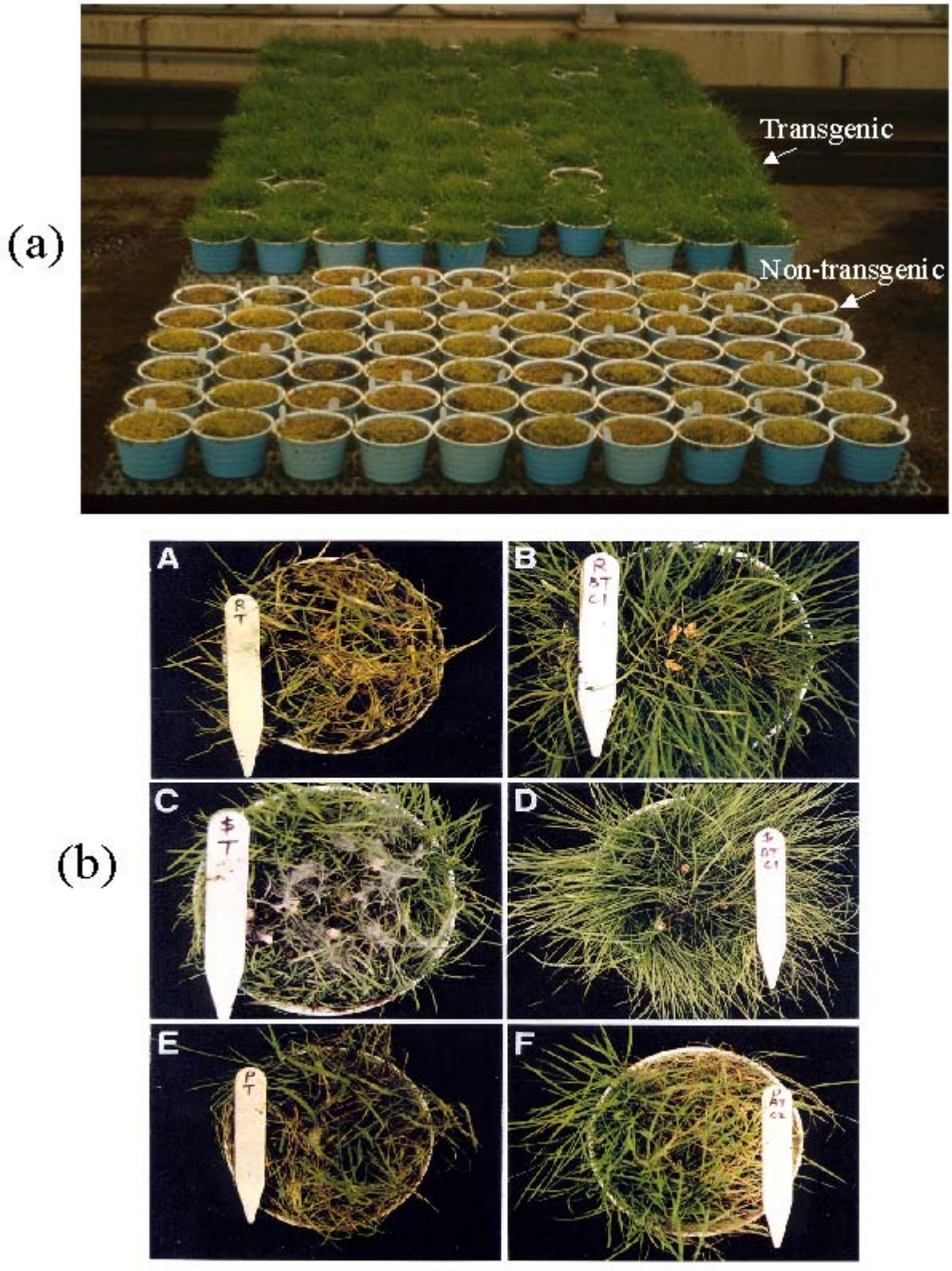


Figure 4. (a) Herbicide resistance in turfgrass plants transformed with bar gene. Turfgrass plants transgenic (upper portion) and non-transgenic (lower portion) two weeks after the foliar spray of glufosinate. (b) The application of bialaphos for prevention of fungal infection in transgenic bialaphos-resistant creeping bentgrass. Transgenic plants inoculated with *Rhizoctonia solani* (A and B), *Sclerotinia homoeocarpa* (C and D), and *Pythium aphanidermatum* (E and F). A, C and E: transgenic plants not sprayed with bialaphos. B and D: transgenic plants sprayed with 200 mg/l of bialaphos three hours before pathogen inoculation; F: transgenic plant sprayed with 400 mg/l of bialaphos two days after pathogen inoculation.

brown patch disease on transgenic plants when sprayed two days after pathogen inoculation.

Significant reduction in plant damage was observed on transgenic plants using 200 mg/L bialaphos (about one-tenth of the recommended herbicide rate to kill common weeds) (Fig. 4b; Table 1). The untreated transgenic plants were severely damaged (Fig. 4b-A, C and E). The grass blades of untreated plants at first became water soaked and dark, but soon became dry and withered and turned brown due to brown patch infection. The disease was able to develop continuously even under low humidity conditions and many of the untreated plants died two to three weeks after pathogen inoculation. There was no significant ($P>0.05$) difference observed between two different timings of bialaphos application for brown patch control.

Bialaphos applied either three hours before or two days after pathogen inoculation was also very effective in preventing development of *S. homoecarpa* on transgenic creeping bentgrass plants. The disease damage on transgenic, bialaphos-resistant plants after bialaphos application was significantly ($p<0.05$) less than on transgenic plants not treated with bialaphos (Fig. 4a-C and D). However, there was more plant damage caused by the infection of *S. homoecarpa* when bialaphos was applied to transgenic plants two days after pathogen inoculation (Table 1).

Bialaphos also showed some growth inhibition of *P. aphanidermatum*, though not as effective as for *R. solani* and *S. homoecarpa* (Fig. 4b-E-F). The timing of bialaphos application was also important in suppressing disease symptoms of *P. aphanidermatum*. Greater reduction in plant damage was obtained when bialaphos was applied three hours before pathogen inoculation. (For details see 8).

Production of chitinase (hs2) expressing transgenic lines

Embryogenic suspension and callus cultures were bombarded with the Biolistic® Gun using pHS2 and pJS101 (Fig. 1). Independent transgenic lines were produced after the selection of bombarded cultures and regenerated plants that survived on bialaphos-containing medium. Southern blot analysis revealed the integration of bar, mtld and hs2 (Fig. 3c and d). The unique banding pattern in lanes containing EcoRI-digested DNA (single digest) indicated that the hs2 transgenic lines have originated from independent transformation events. The appearance of 1.2 Kb

		Disease rating[a]					
		Bialaphos concentration (mg/L)				Untreated[b]	
Pathogen	Spray time	200	400	600	800	Transgenic	Non-tansgenic
R. solani	3 h before	0.4	0.3	0.2	not applied	7.8	8.2
	2 d after	0.5	0.5	0.2	not applied		
S. homoeocarpa	3 h before	0.6	0.4	0.2	0.1	5.2	6.3
	2 d after	1.3	0.9	0.7	0.5		
P. aphanidermatum	3 h before	4.3	3.6	3.4	3.2	8.3	9.0
	2 d after	5.8	5.6	5.1	4.8		

[a]Visual rating of plant damage on a 0-10 scale (0= no damage, 10= all dead).

[b]Untreated means that bialaphos was not sprayed either before or after the pathogen inoculation.

Table 1. Effect of bialaphos on the development of fungal pathogens applied three hours before or two days after the pathogen inoculation in the transgenic creeping bentgrass

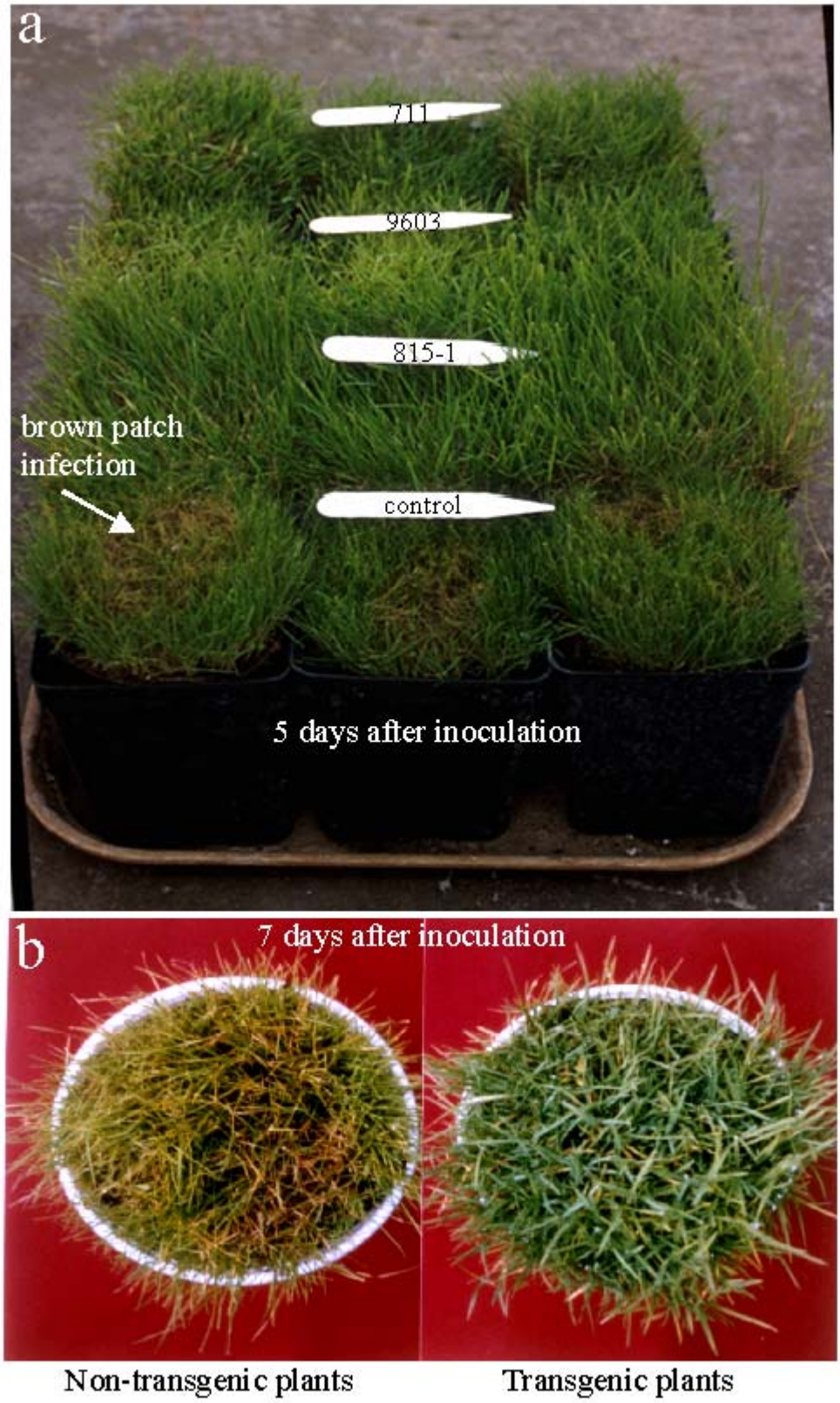


Figure 5. Whole plant bioassays of transgenic turfgrass plants expressing chitinase (hs2) and bar genes (a) five days and (b) seven days after inoculation with spores of *R. solani*.

Line	Disease severity (%)[1] 3 days[2]	5 days[2]	7 days[2]
non-transgenic	38.9 ± 0.98 a[3]	75.9 ± 0.55 a	78.1 ± 0.68 a
9604 (bar-only)	24 ± 1.05 ab	62 ± 0.99 ab	69.1 ± 0.88 a
9606	25.2 ± 0.90 ab	63 ± 0.98 ab	69.3 ± 1.44 a
9603	13 ± 0.5 b	41 ± 0.67 b	41.9 ± 0.90 b
9601	21.1 ± 1.18 ab	55.6 ± 0.9 ab	62.5 ± 0.52 a
815-7	36.9 ± 0.99 a	69.4 ± 0.65 a	74.2 ± 0.8 a
711	6.8 ± 0.65 c	27 ± 0.88 c	29.3 ± 0.99 c

1 Based on mid-point transformations of visual severity estimates using the Horsfall-Barrett Scale.

2 Days post-inoculation.

3 Letter designates significant differences between treatment means according to Dunnett's two-tailed t-test.

Table 2. Comparison of mean brown patch severity caused by *Rhizoctonia solani* on transgenic creeping bentgrass lines containing the hs2, and the bar-only line and the non-transgenic plants.

fragment in all PstI digested (double digest) transgenic lines showed the presence of hs2 gene in its intact form. (For details see 2).

Bioassay of transgenic creeping bentgrass against *R. solani*

At all three sampling periods, significant ($P<0.01$) differences were observed in the susceptibility to *R. solani* between the transgenic creeping bentgrass line 711 and the transgenic line containing the bar gene only (bar-only line 9604) and the non-transgenic plants. The transgenic creeping bentgrass line 9603 was also found to have significantly ($P<0.05$) greater levels of resistance to *R. solani* compared to the bar-only line 9604 and the non-transgenic plants, based on disease severity at all three sampling dates (Table 2; Fig.5).

The other three transgenic lines, 815-7, 9601, and 9606, did not show differences in susceptibility to *R. solani* compared to bar-only transgenic line 9604. However, the line 9601 and 9606 showed non-significant differences in their susceptibility to *R. solani* compared to non-transgenic plants at three and fives days after inoculation (Table 2). (For details see 2).

Discussion

Plant regeneration from *in vitro* cultures of creeping bentgrass opens the possibility to genetically modify this important turf species using gene transfer technology. High velocity microprojectile bombardment using Biolistic Gun™ to deliver DNA into embryogenic callus or suspension culture cells of most cereal crops has been used successfully (3). The regeneration of the bombarded embryogenic callus with a high level of expression of the transgenes has highly contributed to the recovery of transgenic turfgrass.

To improve transformation frequency and recover putative independent transformants, selectable marker genes have proven to be very useful. We showed the efficient recovery of transgenic turfgrass using only a reporter blue gene (gus) without any selection pressure, and also using herbicide resistance the selectable marker bar gene. Results further showed that using multiple genes in one bombardment experiment (our study with bar, mtld, and hs2) could also be of great success. Stable integration and expression of each transgene via molecular analysis were confirmed. This gene transfer strategy has great potential in terms of producing herbicide-, drought- ,and disease-resistant turfgrass plants in a short time and with less efforts than conventional breeding and selection methods.

Because of the antifungal activity of herbicide bialaphos and glufosinate, the herbicide-resistant transgenic turfgrass would provide simultaneous control of weeds, as well as fungal

pathogens. Our greenhouse bioassay experiments demonstrated that bialaphos applied to transgenic bialaphos-resistant creeping bentgrass suppressed disease development. Bialaphos was most significant in suppressing brown patch caused by *R. solani*. Treating with bialophos either three hours before or two days after inoculation provided significant suppression of *R. solani*.

Bialaphos applications either before or after pathogen inoculation also provided greater resistance to dollar spot caused by *S. homoecarpa*, although not as effective as for *R. solani*. Bialaphos applications inhibited pythium blight caused by *P. aphanidermatum* too, but not to the same extent as they did for *R. solani* and *S. homoecarpa*. The lowest concentration of bialaphos (200 mg/L) was sufficient to suppress the disease development of brown patch and dollar spot applied either three hours before or two days after inoculation. This concentration when applied three hours before pathogen inoculation also showed the suppression of pythium blight to some extent. However, greater disease control was achieved when bialaphos spray was made two days after inoculation with bialaphos concentrations up to 800 mg/L.

We tested the ability of the elm hs2 (Dutch elm isolated cDNA clone encoding a chitinase protein) to confer fungal resistance in transgenic creeping bentgrass. Bioassays revealed that two lines 711 and 9603 of the five tested transgenic lines improved levels of resistance ($p<0.01$) to *R. solani* as compared to the bar-only line and the non-transgenic plants.

Bialaphos, a broad-spectrum contact herbicide has been used as a selective agent in plant transformation experiments. However, the use of bialaphos as an effective selecting agent in transformation studies of *Cercospora kikuchii*, a fungal pathogen of soybean has been previously reported (20). These and our results suggested that bialaphos could be used as an efficient fungicide for a variety of fungal pathogens.

Bialaphos and glufosinate dissipate very rapidly in soil and surface water after the application (5). Their active ingredient and transitory metabolites are readily soluble in water, and do not evaporate or accumulate in fatty tissue of other animals. Application of 200 mg/L of bialaphos, which is about the one-tenth the recommended concentration to kill non-transgenic turfgrass plants, was enough to significantly reduce plant damage due to brown patch and dollar spot.

Conclusions

We developed a reliable system of genetic engineering for turfgrass, transferred multi-genes in plants, and tested transgenic plants for herbicide and disease resistance. Using bar- and hs2 (chitinase)-expressing transgenic bentgrass lines, one can spray bialaphos and simultaneously control weeds, as well as brown patch and dollar spot diseases of creeping bentgrass.

Acknowledgement

We would like to gratefully acknowledge the financial support of the National Park Service, the United States Golf Association (USGA) and the Michigan Turfgrass Foundation (MTF) for this research.

.

References

1. Benhamou, N., K. Broglie, I. Chet, and R. Broglie. 1993. Cytology of infection of 35S-bean chitinase transgenic canola plants by *Rhizoctonia solani*: cytochemical aspects of chitin breakdown *in vivo*. *Plant J.* 4:295-305.

2. Chai, B., S. B. Maqbool, R. K. Hajela, D. Green, J. M. Vargas Jr., D. Warkentin, R. Sabzikar, and M. B. Sticklen. 2002. Engineering fungal resistance in creeping bentgrass (*Agrostis palustris* Huds.) with a chitinase like cDNA clone from Dutch elm disease resistant triploid elm (*Ulmus Americana*) NPS-3-487. *Plant Science* 163:183-193.

3. Christou, P., T. L. Ford, and M. Kofron. 1991. Production of transgenic rice (*Oryza sativa* L.)

plants from agronomically important Indica and Japonica varieties via electric discharge particle acceleration of exogenous DNA into immature zygotic embryos. *Bio/Technology* 9:957-962.

4. Graham, L. S., and M. B. Sticklen. 1994. Plant chitinase. *Can. J. Bot.* 72:1057-1083. (TGIF Record 71481)

5. Hoerlein, G. 1994. Glufosinate (phosphinothricin), a natural amino acid with unexpected herbicidal properties. Pages 73-145. *In:* G. W. Ware (ed.). Reviews of Environmental Contamination and Toxicology. Springer-Verlag, New York. (TGIF Record 49718)

6. Horsfall, J. G., and E. B. Cowling. 1978. Pathometry: the measurement of plant disease. Pages 120-136. *In:* J. G. Horsfall and E. B. Cowling (eds.). Plant Disease: An Advanced Treatise, Vol. II, How Disease Develops in Populations. Academic Press, New York.

7. Klein, T. M., E. D. Wolf, R. Wu, and J. C. Sanford. 1987. High velocity microprojectiles for delivering nucleic acids into living cells. *Nature* 327:70-73.

8. 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. (TGIF Record 42291)

9. Lee, L., C. Hartman, C. Laramore, N. Tumer, and P. Day. 1995. Herbicide-resistant creeping bentgrass. *USGA Green Section Record* 33:16-18. (TGIF Record 33422)

10. Meins, F., and P. Ahl. 1989. Induction of chitinase and beta-1, 3-glucanase in bean leaves. *Plant Cell* 1:447-457.

11. Métraux, J. P., and T. Boller. 1986. Local and systemic induction of chitinase in cucumber plants in response to viral, bacterial, and fungal infections. *Physiol. Mol. Plant Path.* 28:161-169.

12. Murashige, T., and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant* 15:473-497.

13. Rasmussen, U., H. Geise, and J. D. Mikkelsen. 1992. Induction and characterization of chitinase in *Brassica napus* L. *spp. oleifera* infected with *Phoma lingam*. *Planta* 187:328-334.

14. Rogers, S. O., and A. J. Bendich. 1985. Extraction of DNA from milligram amounts of fresh, herbarium, and mummified plant tissues. *Plant Mol. Biol.* 5:69-76.

15. Sherald, J. L., F. S. Santamour, R. K. Hajela, N. Hajela, and M. B. Sticklen. 1994. A Dutch elm disease resistant triploid elm. *Can. J. Forest Res.* 24:647-653.

16. Somers, D. A., H. W. Rines, W. Gu, F. Kaeppler, and W. R. Bushnell. 1992. Fertile, transgenic oat plants. *Bio/technology* 10:1589-1594.

17. Southern, E. M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* 98:503-517.

18. Suzuki T., C. Moriya, and J. Yoshida. 1973). Isolation and physico-chemical and biological characterization of SF-1293 substance. *Science reports of Meiji Seika Kaisha* 13:34-41; *Chemical Abstracts* 1974, 81:89705b.

19. Uchimiya, H., M. Iwata, C. Nojiri, P. K. Samarajeewa, S. Takamatsu, S. Ooba, H. Anzai, A. H. Chiristensen, P. H. Quail, and S. Toki. 1993. Bialaphos treatment of transgenic rice plants expressing a bar gene prevents infection by the sheath blight pathogen (*Rhizoctonia solani*). *Bio/Technology* 11:835-836.

20. Upchurch, R. G., M. J. Meade, R. C. Hightower, R. S. Thomas and T. M. Callahan. 1994. Transformation of the fungal soybean pathogen *Cercospora kikuchii* with the selectable

marker bar. *Appl. Environ. Microbiol.* 60:4592-4595.

21. Zhong, H., C. Srinivusan, and M. B. Sticklen. 1991. Plant regeneration via somatic embryogenesis in creeping bentgrass (*Agrostis palustris* Huds). *Plant Cell Rep.* 10: 453-456. (TGIF Record 28289)

22. Zhong, H., M. G. Bolyard, C. Srinivasan, and M. B. Sticklen. 1993. Transgenic plants of turfgrass (*Agrostis palustris* Huds.) obtained by microprojectile bombardment of embryogenic callus. *Plant Cell Rep.* 13:1-6. (TGIF Record 40357)