NOVEMBER 1994

DEVELOPMENT OF IMPROVED TURFGRASS WITH HERBICIDE RESISTANCE AND ENHANCED DISEASE RESISTANCE THROUGH TRANSFORMATION

Executive Summary

This project seeks to improve creeping bentgrass through transformation to provide golf course managers with more effective and selective weed control with herbicides and more environmentally sound and cost-effective control of plant diseases with reduced use of fungicides. We have reached several milestones; successful turfgrass transformation, efficient tissue culture and regeneration systems, recovery of several cultivars of creeping bentgrass with resistance to two different herbicides, and field tests of clones of Ignite-resistant creeping bentgrass. Transgenic bentgrass, a product of laboratory experimentation, shows promise as a useful tool for golf course management.

We are making good progress in incorporating single gene traits for herbicide resistance and enhanced disease resistance in turfgrass. We now have embryogenic callus lines and suspension cultures derived from them with high regeneration potential from nine creeping bentgrass cultivars. We have established both particle gun and protoplast transformation systems for creeping bentgrass and have obtained first generation stable transformants with resistance to the herbicide bialaphos. In tests of more than one thousand regenerants from transformed tissues for herbicide sensitivity in greenhouse, we have obtained over one hundred herbicide-resistant transgenic plants of 'Cobra', 'Emerald', and 'Southshore'. This summer, we conducted the first field test of herbicide-resistant creeping bentgrass in the USA, and showed that the transgenic plants were resistant to up to 3x (2.25 lb AI/A) the label rate (1.5 to four fluid ounces per gallon of water). To enhance fungal disease resistance in turfgrass, three chitinase gene constructs were obtained, adapted to our transformation vectors, and introduced into creeping bentgrass through particle gun and protoplast transformation. We have begun greenhouse herbicide tests with putative transgenic plants that carry genes expressing bean chitinase or tobacco chitinase B, and are preparing to test transgenic plants for disease resistance.

I. Herbicide Resistance

A. *Bar* gene for bialaphos resistance

(1) From laboratory to field test

One of our major accomplishments this year is the first field test of herbicide-resistant creeping bentgrass in the USA in the summer of 1994 at Rutgers' Research and Development Center at Bridgeton, NJ. Transgenic plants tested were survivors from greenhouse herbicide tests. The presence of transgenes had earlier been confirmed by molecular analysis such as PCR (polymerase chain reaction), and Southern, and Northern blot hybridizations as reported in the 1994 semi-annual report.

A field test permit was obtained from USDA-APHIS in May 1994 to evaluate the herbicide resistance and seed fertility of these transgenic creeping bentgrass plants. Seventy-two transgenic lines from 12 bombarded filters were planted along with control plants germinated from seeds of Emerald, Putter, and Southshore in a randomized block design. Herbicide was applied at two rates, 1x (0.75 lb AI/A) and 3x (2.25 lb AI/A) the label rate (1.5 to four fluid ounces per gallon of water). There was also an untreated control. Each treatment had three replicates.

Vegetatively propagated plants were planted on June 9, 1994. Six weeks later, the plants were sprayed with the herbicide Ignite at the two rates described above on July 21. Figure 1 shows an overview of the field plots three weeks after the first herbicide treatment. Herbicide effects were observed as early as 5 days after application. All plants that survived a 2 mg/ml rate in greenhouse tests were completely resistant to both 1x and 3x field rate. They remained green and unaffected like untreated plants in the control plot. No control plants (Emerald, Putter, and Southshore plants from seeds) survived. More than 30 Emerald and Southshore creeping bentgrass are resistant to 3x field rate (Table 1). These lines represent 7 independent transformants, using two single seedling derived clones of Emerald and one clone of Southshore.

Herbicide-resistant plants were again sprayed with Ignite on September 14 1994. All plants that survived the first spray (i.e. all plants that survived the 2 mg/ml greenhouse test) were completely resistant to the second application at both rates. Figure 2 shows a resistant plant (by the white pen) and control dead plant (by the red pen) two weeks after the second application.

Resistant transgenic plants will be vernalized in the field and moved to a containment greenhouse in spring 1995 for pollination and seed production to determine the inheritance of herbicide resistance. Suitable resistant clones will be used as parents in a traditional breeding program before a resistant cultivar is made available commercially.

(2) Other cultivars incorporating the *bar* gene

Thirty Cobra transgenic plants were obtained (3 independent transformants from 3 seedling clones) in 3 later bombardment experiments and from protoplast transformation. One hundred and fifty-three plants were regenerated from 2 resistant colonies obtained from protoplast transformation. All plants survived the 2 mg/ml herbicide spray in greenhouse tests and are resistant to 5x the field rate. Northern blot hybridization confirmed the expression of the transgene in Cobra transgenic plants obtained from protoplast transformation.

The establishment of creeping bentgrass protoplast transformation system is another major accomplishment.

This fall, some of the Cobra transgenic plants, in 10-inch pots, were placed in a trench outside, to see if cold treatment would provide sufficient vernalization for inflorescence formation. They will be moved inside in the spring of 1995.

B. [redacted] resistance

(1) Greenhouse tests

Table 2 summarizes the herbicide sensitivity test of regenerants from four bombardment experiments using a [redacted] resistance gene construct. Embryogenic suspension cell cultures, or embryogenic callus cultures, of creeping bentgrass were used as the target tissues. A total of 241 plants were regenerated. Fifty-nine plants from two experiments (3 bombarded filters) of two cultivars, PennLinks and Cobra survived the [redacted] tests at 1x (1.5 lb AI/A) label rate. [redacted] resistant plants continued to grow normally while sensitive plants and controls were killed within 5-10 days after herbicide application (Fig. 3). The [redacted] resistant plants are being tested with a 3 times higher rate.

(2) Molecular analysis

The expression of the [redacted] resistance gene in transgenic plants was confirmed by western blot hybridization. Total soluble proteins were extracted from herbicide resistant plants and protein concentrations determined by BCA protein assay (Pierce Co.). SDS-polyacrylamide gel electrophoresis was used to separate total soluble proteins which were then blotted from the gel to a nitrocellulose membrane and hybridized to antibody against the [redacted] resistance gene product. Figure 4 shows the immunoblot of [redacted] resistant plants.

Obtaining [redacted] resistant creeping bentgrass is our third major accomplishment.

[redacted]

II. Enhanced disease resistance

To enhance fungal disease resistance in turfgrass we obtained chitinase genes from three sources: a maize chitinase A cDNA clone (from Monsanto); a tobacco chitinase B cDNA clone (from Dr. Ward, Ciba-Geigy); and a bean chitinase gene construct (from Dr. Richard Broglie, DuPont).

We have completed construction of the two cDNA clones in a plant gene expression vector with the *bar* gene as a selectable marker. Several bombardment experiments are in progress.

A. Bean chitinase

The bean chitinase gene construct (pK35CHN) was co-transformed with a *bar* gene construct in bombardment experiments and protoplast transformations. Table 3 summarizes the list of putative bean chitinase transgenic plants. The co-transformation frequency varies with the constructs and plant species. We obtained antibody against bean chitinase from DuPont, and are analyzing expression of this chitinase in putative transgenic creeping bentgrass by western blot hybridization.

B. Tobacco chitinase/bar construct

Table 4 summarizes the regeneration of one bombardment experiment that introduced tobacco chitinase/bar (TbCHN) or pBARGUS into creeping bentgrass suspension cell cultures. To date, a total of 463 regenerants are growing in soil. More regenerants will be planted later. These regenerants are ready to be tested for herbicide sensitivity in the greenhouse. We have obtained transformants in most bombardment experiments with pBARGUS, thus, using pBARGUS and TbCHN constructs in the same experiment will help us evaluate the tobacco chitinase/bar construct in creeping bentgrass transformation.

C. Maize chitinase/bar construct

Three bombardment experiments and two protoplast transformation experiments using the maize chitinase/bar construct are in selection with bialaphos, or in regeneration.

To analyze expression of the chitinase construct in putative transgenic plants, molecular analyses such as PCR, southern, western, and northern blot hybridizations will be performed when sufficient plant materials are obtained. As mentioned in the 1994 semi-annual report, two other assays can also be tested: a) the growth of putative transgenics in soil carrying a fungal pathogen; b) plate assay of leaf lesions caused by fungal pathogen inoculation. These assays will be done after confirmation

of the incorporation of chitinase constructs into the plant genomes. Candidate fungal pathogens of turfgrasses which contain chitin include *Rhizoctonia solani* (brown patch), *Sclerotinia homoeocarpa* (dollar spot), and *Gaeumannomyces graminis* (take-all patch).

We would like to acknowledge the help of Drs. John Grande, Steve Johnston, Jim Murphy, and C. Reed Funk in field tests of herbicide-resistant creeping bentgrass.

III. Related project

A. Endophytes of turfgrasses: new tools and approaches (USGA 1990-Feb. 1993)

Plants derived from an Emerald bentgrass clone obtained from AS4 endophyte inoculation were sent to Dr. William A. Meyer of Pure-Seed Testing last year. Those plants are growing well in their nursery and still contained endophytes. In August 1994, we learned that it caused choke formation, although, there was late seed head formation. The seeds harvested will be tested for the presence of endophyte.

IV. Future directions

We will continue to work on (1) analyses of putative transgenics by herbicide tests, polymerase chain reaction (PCR), western, northern, and southern blot hybridizations, (2) evaluation of progeny of transformants via field testing for inheritance of herbicide resistance, (3) analyses of putative transgenics containing chitinase gene constructs by molecular analysis, soil tests, and plate assays, (4) transformation by particle gun bombardment and protoplast transformation of turfgrass with agronomically important genes.

V. Publications

Lee, L., Laramore, C., Day, P.R., and Tumer, N.E. (1994) Plant regeneration and transformation from protoplasts of creeping bentgrass suspension cultures. (in preparation)

Hartman, C.L., Lee, L., Day, P.R., and Tumer, N.E. (1994) Herbicide resistant turfgrass (*Agrostis palustris* Huds.) by biolistic transformation. Biotechnology 12: 919-923.

Lee, L., Hartman, C.L., Laramore, C., Tumer, N.E., and Day, P.R. (1994) Herbicide-resistant creeping bentgrass. USGA Green Section *RECORD* (submitted).

Table 1. List of transgenic creeping bentgrass lines produced by particle bombardment

Cultivar	Particle bombardment number		2.0 mg/ml Herbiace	Tissue* clone	Phenotype
Emerald	1	14-3	3	EB.5	erect
	2	14-4	1	EB.5	erect
	3	14-18	14	EB.5	erect
	4	16-27	-		
	5	19-15	5	EBmm	creeping**
Southshore	1	16-24	4	SSB.2	petite
	2	19-25	8	SSB.2	petite
	3	19-28	20	SSB.2	petite

* Each clone derived from one seedling.
** Creeping phenotype is equivalent to w.t. of two cultivars.

Table 2. Summary of herbicide sensitivity of regenerants of bombardment experiments using glyphosate resistance gene construct.

Exp.	Cultivar	nice & green	Dead
30	Southshore	0	25
31	Pennlinks callus	0	42
32	Pennlinks callus	1	88
40	Pennlinks	56	7
		0	6
40	Cobra callus	2	14

Table 3. List of putative transgenic plants containing bean chitinase gene construct.

Cultivar	Plant No.	Transformation	Protein content (mg/ml)
Cobra	4131	Protoplast	4.44
	4132		4.09
	4134		5.16
	4135		4.65
	4137		6.64
	4140		5.03
	4141		6.72
	4142		5.40
Cobra	4149	Protoplast	4.78
	4154		5.75
	4161		6.13
	4165		5.67
	4169		5.38
Cobra	4594	Bombardment	7.10
	4595		6.59
	4596		4.87
	4598		4.49
	4599		5.73
	4603		10.27
SR1020	4076-4082	Bombardment	
PennLinks	5545-5607	Bombardment	
Cobra	5608-5623	Bombardment	
PennLinks	5624-5640	Bombardment	

Table 4. Summary of plant regeneration of bombardment experiment of tobacco chitinase/bar construct (TbCHN) or pBARGUS.

Cultivar	Filter No.	Number of regenerants in soil	
		TbCHN	pBARGUS
Cobra	#2	126	na
Southshore	#3	55	na
Cobra	#6	na	21
Cobra	#8	na	19
Southshore	#9	na	58
Southshore	#10	na	158

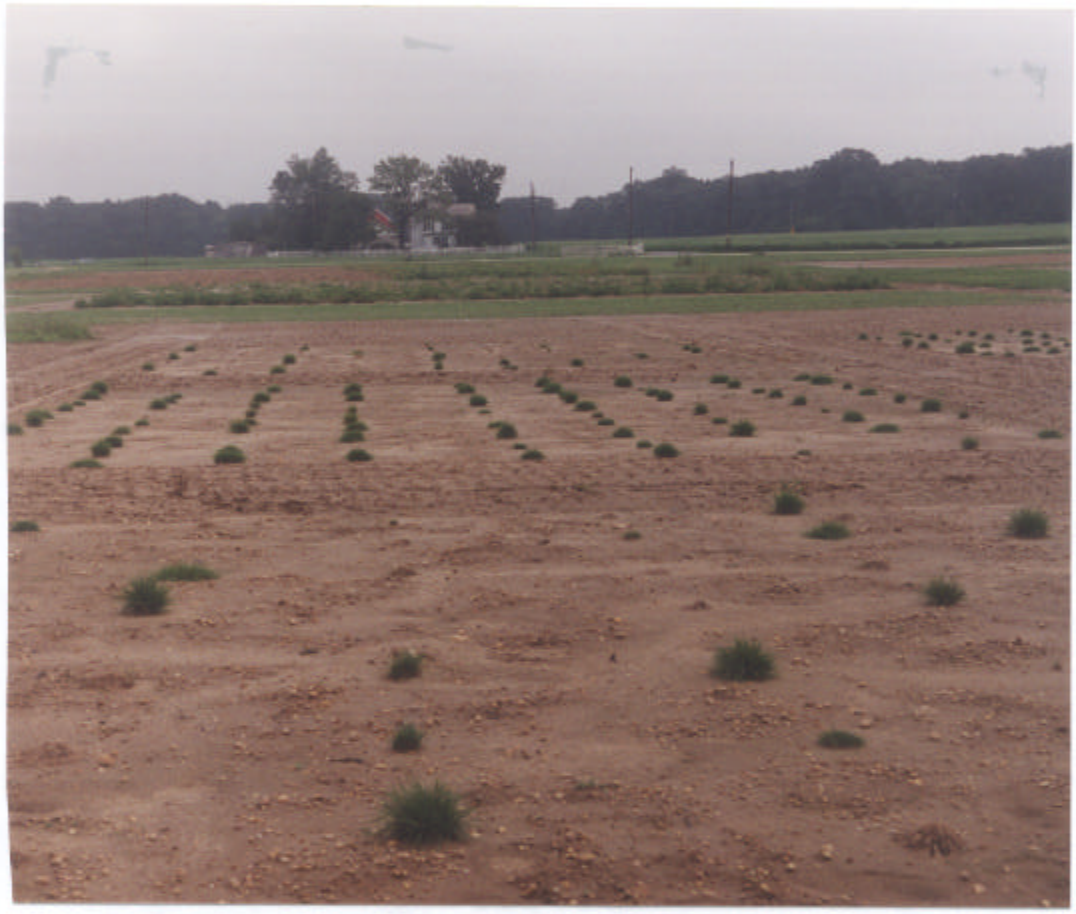

Figure 1. Field test of Ignite-resistant creeping bentgrass showing overview of the field plots. Picture was photographed 3 weeks after first herbicide application.

Figure 2. Field tested herbicide-resistant creeping bentgrass (by the white pen) and control (by the red pen), two weeks after second application of 3x (2.25 lb AI/A) rate of Ignite™.

Figure 3. [redacted] resistance of transgenic plants and controls at application rate of 1.5 lb AI/A. Plants were photographed three weeks after herbicide application. Resistant plants remained green, while others and controls were killed.

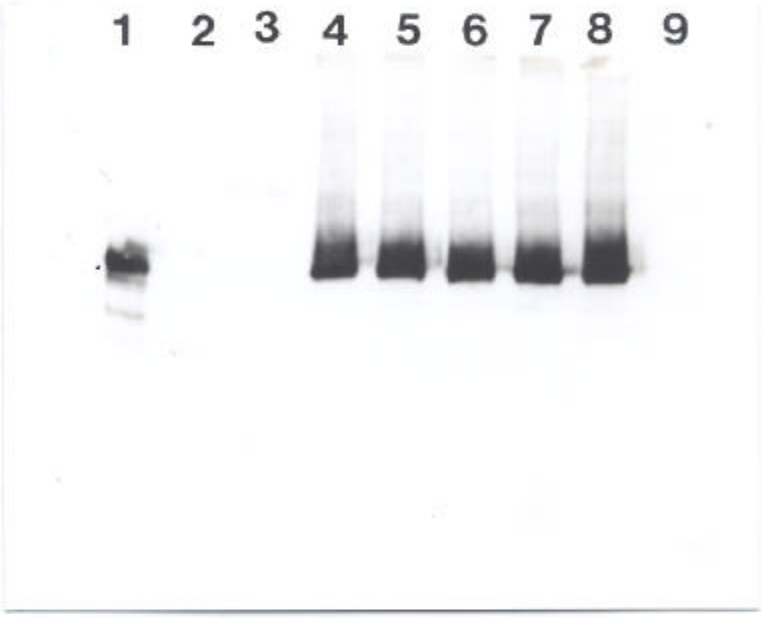

Figure 4. Immunoblot of total soluble proteins separated on a SDS-polyacrylamide gel and hybridized with the antibody against [redacted] resistance gene product. Lane 1: potato positive control, Lane 3 : prestained protein markers, Lanes 4 to 8: transgenic plants, Lane 9: untransformed control plants.