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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 accomplished several major goals:

- creeping bentgrass tissue culture and regeneration system
- successful biolistic and protoplast transformations of creeping bentgrass
- recovery of several cultivars of creeping bentgrass with resistance to two different herbicides
- field tests of clones of all herbicide-resistant creeping bentgrass
- turf quality (including disease resistance) study of herbicide-resistant transgenic creeping bentgrass
- a progeny analysis of Finale-resistant creeping bentgrass is underway.

We are making good progress in incorporating single gene traits for herbicide resistance and enhanced disease resistance in turfgrass. We have expanded the creeping bentgrass tissue culture and regeneration system from nine creeping bentgrass cultivars to several other elite cultivars including 'Penncross', 'Penneagle', 'Crenshaw', and 'A-1'. Transgenic creeping bentgrass clones of 'Cobra', 'Emerald', and 'Southshore' have been obtained from particle and protoplast transformation with resistance to the herbicide bialaphos. They are resistant to 5x the field rate in greenhouse herbicide spray tests and up to 3x the recommended rate in field herbicide applications of Finale™. Field tests of herbicide-resistant creeping bentgrass were conducted in 1994 and 1995. Transgenic creeping bentgrasses from the 1994 field test were vernalized in the field overwinter and returned to a containment greenhouse in the spring before flowering. At flowering they were cross-pollinated with wild type plants. Seeds were harvested and progeny analyses to determine heritability of the transgene will be performed in the fall of 1995. This will be the first study of transgene transmission in creeping bentgrass. Four field tests of herbicide-resistant creeping bentgrasses were conducted in 1995. The Cobra transgenic plants obtained from protoplast transformation showed a high level of herbicide tolerance, up to 3x the recommended field rate of Finale, like the transgenic plants tested in the 1994 field test. To enhance fungal disease resistance, we have performed greenhouse herbicide tests with putative transgenic plants that carry genes expressing bean chitinase, tobacco chitinase B and maize chitinase, and have started testing transgenic plants for resistance against *Rhizoctonia solani.*

I. Herbicide Resistance

A. *Bar* gene for bialaphos resistance

(1) Field test

1994 field test:

We completed the 1994 field test of Finale-resistant creeping bentgrass at Rutgers' Research and Development Center at Bridgeton, NJ in June 1995 by removing selected numbers of vernalized transgenic plants to a containment greenhouse in New Brunswick and destroying the remaining 1994 field plots as required by the USDA-APHIS permit.

Table 1 summarizes the number of plants harvested for progeny analysis of Finale-resistant creeping bentgrass. Fifteen Emerald and Southshore creeping bentgrass clones, representing 4 independent transformants were harvested from the 1994 field test. During the summer of 1995, transgenic plants were pollinated by the control plants in a containment greenhouse. Seeds have been harvested from transgenic mother plants and germination tests and progeny analyses of herbicide resistance are in progress.

1994 vernalization study in fenced greenhouse

As mentioned in our Nov '94 report, twenty-seven transgenic plants were placed in 10-inch pots in a trench outside, to see if cold treatment would provide sufficient vernalization for inflorescence formation. Table 2 summarizes the number of plants harvested for progeny analysis of Finale-resistant creeping bentgrass that were vernalized overwinter in a fenced greenhouse area. Twenty-four plants of Southshore and Cobra creeping bentgrass, from 2 bombardment and 2 protoplast transformations produced inflorescences and were moved back to a containment greenhouse for pollination before flowering. Since some 90% of plants produced inflorescences, cold treatment (in pots in a trench) thus appears to provide sufficient vernalization.

1995 field test

A renewed field test permit was obtained from USDA-APHIS to continue evaluation of Finale-resistance and seed fertility of transgenic creeping bentgrass plants in 1995 at Rutgers' Research and Development Center at Bridgeton, NJ. Transgenic plants tested were survivors of greenhouse herbicide tests of protoplast transformants and new transgenic plants from biolistic transformations. Herbicide was applied at two rates, 1x (0.75 lb AI/A) and 3x (2.25 lb AI/A) the label rate. There was also an untreated control. Each treatment had three replicates.

Vegetatively propagated plants, along with control plants germinated from seeds of Cobra, Emerald and Southshore, were planted on June 9, 1995. The plants were sprayed with the herbicide Finale at the two rates described above on July 26. Mean damage ratings were scored two weeks after herbicide application. Due to the dry summer weather, some transgenic plants showed discoloration before the herbicide treatment which is reflected in the damage ratings shown in Table 3. All plants that survived a 2 mg/ml rate in greenhouse tests were completely resistant to both 1x and 3x field rates. They remained green and unaffected like untreated plants in the control plot. No control plants (Cobra, Emerald, and Southshore plants from seeds) survived. More than 36 Cobra and Emerald creeping bentgrass lines are resistant to 3x field rate. These lines represent 3 independent transformants.

Resistant transgenic plants will be vernalized in the field and moved to a containment greenhouse in spring 1996 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.

A second 1995 field test was planted on June 28, 1995 at Rutgers University Horticulture Farm II located at Ryders Lane, East Brunswick, NJ. The purpose is to evaluate the disease resistance of herbicide tolerant transgenic creeping bentgrass. The transgenic plants were vegetatively propagated, planted, and established and will be mowed and tested for disease resistance. Two transgenic lines (Emerald and Southshore) of herbicide tolerant creeping bentgrass from the 1994 field test and one Cobra transgenic line from protoplast transformation will be scored for reaction to natural infection by the turfgrass fungal pathogen *Rhizoctonia solani*. Herbicide treatments (at 0.1x, 0.5x, and 1x field rates) will be applied to see if the herbicide affects the development of the brown patch disease caused by *Rhizoctonia solani* .

The plot layout uses a split-plot design, for testing the three transgenic lines (side by side). Four treatments (0, 0.1x, 0.5x, and 1x field rates of Finale herbicide) will be used with three replicates per treatment. The Emerald and Cobra clones had formed a lawn in September 1995, however, the Southshore clone has grown very slowly. Due to the time needed for establishment, we did not observe natural infection in summer of 1995. We will continue our experiment next summer.

B. Glyphosate resistance*

(1) From Laboratory to field test

As described in the Nov '94 report, we have obtained glyphosate resistant creeping bentgrass lines of two cultivars, PennLinks and Cobra which survived glyphosate tests at 1x (1.5 lb AI/A) label rate. Table 4 shows that when tested with 3x rate, 103 plants of PennLinks survived. Of these, 87 plants survived a further 5x label rate treatment. Glyphosate resistant plants continued to grow normally while sensitive plants and controls were killed within 5-10 days after herbicide application. The glyphosate resistant transgenic plants are being field tested in 1995 at Rutgers' Research and Development Center at Bridgeton, NJ. The presence of transgenes had earlier been confirmed by molecular analysis (western blot hybridization as presented in the 1994 November report).

In the field test, each treatment has three replicates. Each main plot contains 70 one meter square subplots. All 4 test lines, together with control lines of the original turfgrass cultivars (plants germinated from seeds of Cobra and PennLinks), were grown in a randomized block design. Vegetatively propagated plants were planted in each plot on September 29, 1995. Herbicide treatments will be conducted after establishment of the transgenic plants. We will apply two rates (2 lb, and 6 lb AI/A) of glyphosate using a commercial product (Roundup™).

* We have been asked by Monsanto to keep our use of the glyphosate resistance gene confidential. This part of our report to USGA should therefore not be made public. The research contract between Monsanto and ourselves has not been renewed after Jan. 1995.

C. Pursuit resistance

Putative Pursuit resistant creeping bentgrass clones were produced by protoplast

transformation with a mutant acetohydroxy acid synthase (AHAS) gene obtained from American Cyanamid. Protoplasts were transformed using polyethylene glycol (PEG) and cultured using a feeder layer system. Selection with 1 μM Pursuit was initiated 16 days after protoplast isolation and transformation. Resistant colonies were visible 3 to 4 weeks after selection. Plants were regenerated on MS medium and shoots were transferred to Plantcons^{R} for rooting.

A commercial formulation of Pursuit was used in greenhouse herbicide tests. Herbicide rates were established using control plants based on the commercial rate of 0.7 oz AI/acre (1x the field rate). These putative transgenic plants survived 4x field rates.

We planted these transgenic plants September 29, 1995. Each treatment has three replicates. Six test lines, together with 3 control lines of the original turfgrass varieties (plants germinated from seeds of Cobra, Emerald, and Southshore), were planted in a randomized block design. Herbicide treatments will be conducted soon after establishment of the transgenic plants. with two rates of imidazolinone using a commercial product (Pursuit™).

Herbicide-resistant creeping bentgrass lines will be provided to interesting collaborators (grass breeders) to be used as parent in a traditional breeding program to generate resistant cultivars for commercial use.

II. Enhanced disease resistance

To enhance fungal disease resistance in turfgrass we have constructed and transformed three chitinase genes: a maize chitinase A cDNA clone (from Monsanto) linked with the *bar* gene; a tobacco chitinase B cDNA clone (from Dr. Ward, Ciba-Geigy) linked with the *bar* gene; and a bean chitinase gene construct (from Dr. Richard Broglie, DuPont).

A. Bean chitinase

The bean chitinase gene construct (pK35CHN) was co-transformed with a *bar* gene construct in bombardment experiments and protoplast transformations. A Henry Rutgers Scholar, Evelyn Icasiano, analyzed the expression of bean chitinase in putative transgenic plants. Three assays were performed : a) western blot hybridization with antibody against bean chitinase (obtained from DuPont); b) plate assay of leaf lesions caused by inoculation with *Rhizoctonia solani* ; c) plate assay of crude protein extracts of transgenic plants against *R. solani.*

Table 5 summarizes her analysis with western blot hybridization. Of the 6 transformation groups (all herbicide resistant) she analyzed, only one group of transgenic plants regenerated from protoplast transformation seems to express a unique protein band not present in western blots of control plants. Black Mexican Sweet (BMS) maize transformed callus lines also produced a similar bean chitinase band. However, when Ms. Icasiano used crude protein extracts in plate assays, the result was not obvious. Plate assays of leaf lesions caused by *R. solani* inoculation did not show clear differences between transgenic and control plants.

To further confirm the expression of bean chitinase in these transgenic plants, molecular analysis by southern hybridization was attempted. Again, we did not obtain a positive hybridization band to confirm the incorporation of bean chitinase in plant genome. Using chitin plate assay with M9 minimal medium to check chitinase activity with crude protein extract from transgenic plants is in progress.

B. Tobacco chitinase/bar construct

Table 6 summarizes the herbicide sensitivity of regenerants from one bombardment experiment that introduced tobacco chitinase/*bar* (TbCHN) into creeping bentgrass suspension cell cultures. Of a total of 181 plants tested, only three Cobra plants were resistant to 1.5 mg/ml Herbiace. When tested with 2 mg/ml Herbiace, all three died. These probably are low expressors of the *bar* gene. Southern blot hybridization showed some weak hybridization bands to the probe containing the *bar* coding region. The three putative resistant plants will be tested by chitin plate assay to check chitinase activity. Another transformation experiment, with 5 filters of Penncross and 5 filters of Putter, is in regeneration stage. Two more experiments, co-transformed, either with a bO/*bar* gene or a pokeweed antiviral protein mutant gene, are in selection on plates.

C. Maize chitinase/bar construct

We constructed a plant expression vector with the maize chitinase and the *bar* gene which has been used for three bombardment experiments and two protoplast transformation experiments. Table 7 shows that only two putative transgenic herbicide resistant plants were obtained from 2 bombardment experiments. Molecular analyses, southern and northern blot hybridizations, and chitin plate assays and assay of leaf lesions following fungal inoculation will be carried out to confirm transgene expression. Candidate fungal pathogens of turfgrasses which contain chitin include *Rhizoctonia solani* (brown patch), *Sclerotinia homoeocarpa* (dollar spot), and *Gaeumannomyces graminis* (take-all patch).

We acknowledge the help of Drs. John Grande, Stephen Johnston, Brad Majek, Jim Murphy, C. Reed Funk and Willam Dickson in the field tests of herbicide-resistant creeping bentgrass.

III. Related project

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

As mentioned in the 1994 November report, plants derived from an Emerald bentgrass clone obtained from AS4 endophyte inoculation were sent to Dr. William A. Meyer of Pure-Seed Testing in 1993. We learned that this caused choke formation in August 1994. Although, there was late seed head formation, the seeds harvested did not germinate. However, the endophyte is still associated with this Emerald bentgrass clone and reisolated endophyte still showed the same RAPD profile.

B. Improvement of disease resistance of Kentucky bluegrass (Sanford Scientific Inc. April 1995-March 1996)

We obtained a research contract with Sanford Scientific to work on the improvement of disease resistance in Kentucky bluegrass through biolistic transformation. Gene constructs were provided by Sanford and hygromycin was used for the selection in biolistic transformation. Selection and regeneration of several transformation experiments is in progress.

IV. Future directions

We would like to continue to work on the development of improved turfgrass with herbicide resistance and enhanced disease resistance. 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 the 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 such as genes that may improve drought or stress tolerance. A detailed proposal is attached as an appendix.

V. Publications

Lee, L., Laramore, C., Day, P.R., and Tumer, N.E. (1995) Transformation and regeneration of creeping bentgrass (*Agrostis palustris* Huds.) protoplasts. Crop Sci. (in print)

Lee, L., Hartman, C.L., Laramore, C., Tumer, N.E., and Day, P.R. (1995) Herbicide-resistant creeping bentgrass. USGA Green Section *RECORD*. March/April, 16-18.

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. (1995) Turfgrass Biotechnology. Plant Sci. (invited review)

Table 1. Summary of numbers of plants harvested for progeny analysis of herbicide resistant creeping bentgrass of the 1994 field test.

Cultivar	Filters	Numbers of Plants	Plant No.	Tissue clone
Emerald	3	2	82, 95	EB.5
	18	2	363, 364	EB.5
	15	5	616, 617, 619 620, 790	EBmm
Southshore	25	6	803, 805, 811 857, 872, 885	SSB.2

Table 2. Summary of numbers of plants harvested for progeny analyses of herbicide resistant creeping bentgrass clones vernalized in the fenced greenhouse area.

Cultivar	Filters	Number of Plants	Plant No.
Southshore	25	2	811*, 864
	28	4	899, 938, 955, 1009
Cobra	protoplast 31.2	8	4131, 4132, 4137, 4139 4143, 4144, 4146, 4366
	protoplast 31.3	10	4151, 4161, 4165, 4370 4931, 4951, 5018, 5024 5048, 5061

* Field tested in 1994.

Table 3. Mean damage rating for 3 replicates of creeping bentgrass plants showing high resistance to the herbicide Finale in the 1995 field test.

Cultivar	Filters	Plant No.	Control	1x	3x
Cobra	protoplast 31.2	4131	1	1.7	2
		4132	1	0.7	1.7
		4133	1.3	0.3	1.7
		4138	1.3	0.7	2
		4139	1	2	2.3
		4140	1	1.3	1.7
		4141	2	1	3.3
		4142	0.3	2	1.7
		4143	1	2	2.7
		4144	1.7	0.7	2
		4145	0.3	1.3	2.7
		4147	2	0.7	2
Cobra	protoplast 31.3	4149	1.3	0	0.3
		4151	1	0.7	0.7
		4152	1.3	0.70	0
		4153	0.3	0.30	0
		4157	1.0	0	0
		4162	0	0	1
		4166	1	0.3	0
		4167	1.3	0.3	0
Cobra	protoplast 31.2	4365	0.7	0.3	2
		4366	0.3	1.3	2
		4367	1.7	1.7	2
Cobra	protoplast 31.3	4369	1	0.3	0.3
		4370	1	0.3	0
Cobra	bombardment	4475	0.7	0.3	0
		4480	1	0.7	0
		4481	0.3	0.3	0
Cobra	protoplast 31.3	4931	1	0	0
		4941	0.3	0	0
		4981	1	0.3	0
		5002	0.7	0	0.3
		5012	1	0.3	0
		5036	0.7	0	1
		5048	1	0	0
		5061	1.3	0.3	0.3
Cobra			0.3	5	5
Emerald			0.3	5	5
Southshore			0.3	5	5

Ratings were as follows:

0. No visible plant injury.
1. Leaf damage 10-24%
2. Leaf damage 25-39%
3. Leaf damage 40-59%
4. Leaf damage 60-99%
5. Total plant death.

Table 5. Results of western hybridization for expression of bean chitinase in putative transgenic plants.

Experiment	No. of plants analyzed	No. of positive
HB035-16	7	0
HB040-3	9	0
HB032-1	3	0
HB043 (BMS)	5 *	5 *
PR031.2	5	0
PR031.3	7	5

* These are transformed BMS calli.

Table 4. Summary of tests for glyphosate resistance in transgenic creeping bentgrass produced by biolistic transformation.

Cultivar	Filters	Numbers of Plants Glyphosate rate 1x	3x	5x	Sensitive
PennLinks	31-12	10	10	0	42
callus	31-14	6	6	0	0
	32-1	6	0	0	89
PennLinks	40-3	54	54	54	9
	40-3	33	33	33	4
Cobra	40-14	2	0	0	14

Table 6. Herbicide sensitivity of regenerants from bombardment experiment using a tobacco chitinase/*bar* construct.

Cultivar	Filters	No. of Regenerants	Herbicide Resistant
Cobra	2	126	3
Southshore	3	55	0

Table 7. Herbicide sensitivity of regenerants from bombardment experiments using a maize chitinase/*bar* construct.

Cultivar	Filters	No. of Regenerants	Herbicide Resistant
Cobra	3	64	0
SR1020	6	43	1
PennLinks	2	77	1
PennLinks	4	3	0