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DEVELOPMENT OF IMPROVED BENTGRASS CULTIVARS WITH HERBICIDE RESISTANCE, ENHANCED DISEASE RESISTANCE AND ABIOTIC STRESS TOLERANCE THROUGH BIOTECHNOLOGY

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Executive Summary

The goals of this project are to produce improved creeping bentgrass cultivars through a combination of genetic engineering and breeding. Our aim is to provide golf course managers with more effective and selective weed control with herbicides by developing herbicide-resistant cultivars. We are also attempting to produce cultivars with improved disease resistance and abiotic stress tolerance which can be maintained in a more environmentally sound and cost-effective manner.

Through the Rutgers-Scotts-Monsanto partnership the development of a herbicide resistant commercial cultivar is actively underway.

We currently have transgenic lines expressing one of five potential disease resistance genes in field tests. From ratings of a field test established in 1998, two lines show consistent enhanced dollar spot resistance relative to the average of the nontransgenic controls. We have established a new field test containing these and other transgenic lines. They will be evaluated in the summer of 2000 to determine if the resistance we observed this year is repeatable.

We are constructing a synthetic gene for delta-9-desaturase, a yeast gene which has conferred good disease resistance in dicots but which is not producing the active product in transgenic creeping bentgrass, a monocot. We will determine if the synthetic, optimized gene will perform in creeping bentgrass.

We have transgenic plants expressing the HVA1 gene, a potential drought and salinity tolerance gene. These plants are currently being tested for drought tolerance.

INTRODUCTION

The goals of this project are to produce improved creeping bentgrass cultivars through a combination of genetic engineering and breeding. Our aim is to provide golf course managers with more effective and selective weed control with herbicides by developing herbicide-resistant cultivars. We are also attempting to produce cultivars with improved disease resistance and abiotic stress tolerance which can be maintained in a more environmentally sound and cost-effective manner.

HERBICIDE RESISTANCE

An agreement between Rutgers, the Scotts seed company and Monsanto has been finalized and we are now actively pursuing herbicide resistant commercial cultivar development.

DISEASE RESISTANCE

We are currently working with five potential disease resistance genes. Four of these were developed by Rutgers faculty. Bacterio-opsin (BO) was developed by Eric Lam (Mittler et al., 1995). Pokeweed antiviral protein (PAP) and pokeweed antiviral protein II (PAPII) were developed by Nilgun Tumer (Hur et al., 1995; Wang et al 1998). One of the Rutgers genes we are working with has not yet been patented. In order to preserve the potential for patenting, in this report that gene will be called Gene B. Glucose oxidase (GO) was developed by scientists at Monsanto (Wu et al., 1995).

We have generated transgenic lines expressing these genes and are currently field testing them for dollar spot resistance.

Field Test #1

Ratings from the field test established in 1998 are encouraging regarding the potential of biotechnology to play a part in the development of disease resistant cultivars. There are two transgenic lines which showed consistently enhanced dollar spot resistance relative to the average of the nontransgenic controls. One of the transgenic lines contains the glucose oxidase gene and one contains Gene B.

This field test was established in May 1998. The field was inoculated with the dollar spot fungus in June 1998. The field was not inoculated with fungus in 1999. Dr. Bruce Clarke has found that the year following inoculation actually gives more natural disease pressure. This is the field that had experienced a toxic herbicide spray in 1998 and some of the plants were lost. Those that recovered were rated in 1999.

In Table 1 the overall ratings of the plants from that field are summarized. The ratings were done by Stacy Bonos, a graduate student with Dr. Bill Meyer. The ratings are the averages of the replicates over the four rating dates. In Table 2 the individual ratings of the top four lines are shown at each of the four rating dates.

Based on consistency of the replicates the two most promising lines are 11032 (Gene B) and 9963 (GO). All three replicates of the Gene B line showed similarly high ratings. For the GO line, 9963, two replicates have very high ratings but one replicate has low ratings. For lines 12426 and 11725, only one replicate has very high ratings.

In Figure 1 are photographs taken on Sept. 14, 1999 of representative transgenics and of an average control plant, one given a rating of 4 on Sept. 7.

Because a creeping bentgrass cultivar is a population of related but unique individuals, we had multiple control plants in the field. The transgenics were generated from callus derived from the cultivar Crenshaw. There were 31 individual Crenshaw plants, each started from a single seed and thus each is a unique individual. There were also control plants of another commercial cultivar, L93, included in the field test. The rating distributions of the Crenshaw controls on the four rating dates is shown in Figure 2. Because each control is unique, there is expectedly a range of disease severity at each date.

Field Test #2

In September of 1998 we also established another field plot which contained the following independent transgenic lines:

BO	11
PAP	10
GO	9
Gene B	12

Some of the above lines are the same as in the first field, along with additional lines. This field was inoculated with dollar spot fungus during the summer 1999. The combination of virulent inoculum and environmental conditions conducive to disease in the summer of 1999 simply overwhelmed all the plants. All of the inoculated plants were so badly diseased that it was not possible to obtain meaningful ratings. The field was sprayed with fungicide to allow the plants to recover. This field will be evaluated next year.

Field Test #3

The two previous field plots were established by plugging the creeping bentgrass into a fine fescue cover. This was done to fill in the area between the plants so the field could be mowed. The fine fescue has, however, inhibited the growth of the bentgrass plants. Recently Dr. Bill Meyer has started using a killed bentgrass field as the surface for tiller plots. In this method a bentgrass field is killed with Round-Up and the new plants are plugged into the dead surface. This method maintains a cover over the soil and a barrier for weeds, yet there is nothing to compete with the plants of interest.

In October 1999, we established a new trial with our transgenic lines in such a field. We anticipate that this plot design will provide better plant establishment for the transgenics. In this field we have the same transgenic lines which were in Field Test #2. In addition to the line of Gene B which showed enhanced performance in Field Test #1, we have an additional 12 independent lines containing Gene B. It will be important to confirm that the dollar spot resistance we observed this year is repeatable in the Gene B line 11032 and the GO line 9963. Because of the problem encountered with field test #2, we have not yet obtained evaluations of the BO lines and some of the PAP lines. These plants are in the new field test.

Also, since the last report we have obtained 6 independent lines expressing another potential disease resistance gene, PAPII. These plants are also in the new field test. Like PAP, PAPII is a ribosome inactivating protein from the pokeweed plant, *Phytolacca americana*. The protein sequence of PAPII is only 41% identical to that of PAP. When expressed in transgenic tobacco plants, PAPII showed resistance to *Rhizoctonia solani* (Wang et al., 1998).

Delta-9-Desaturase

Delta-9-desaturase is a yeast gene being developed for disease resistance by Chee-Kok Chin at Rutgers. Expression of this gene in transgenic tomato has shown dramatic disease resistance (Wang et al., 1998). Expression of this gene results in increased levels of unsaturated fatty acids which either directly or indirectly enhance the disease resistance of the plants. We have produced transgenic plants containing the delta-9-desaturase gene which produce messenger RNA for the gene. From feeding studies conducted in Dr. Chin's lab, however, there was no production of the unsaturated fatty acids in the creeping bentgrass transgenics. One possible reason for this is the plants may not be producing adequate levels of the delta-9-desaturase protein.

We are investigating the possibility that the codon usage in the yeast gene is suboptimal for adequate protein production in a monocot. To do this Dr. Zhenfei Guo is constructing a synthetic gene which codes for an identical protein but utilizes the monocot preferred codons in the DNA sequence. This is done by assembling synthetic oligonucleotides containing the desired sequences. Our strategy is to divide the gene into six segments which are each assembled separately. We now have three segments completed and anticipate finishing the last three shortly. When we finish the complete synthetic gene, we will construct the bentgrass expression vector and use it for transformations. The resulting transgenic creeping bentgrass plants will be assayed for production of the unsaturated fatty acids. If expression of the synthetic gene does result in the anticipated fatty acid profile, the plants will be field tested to determine if they have increased disease resistance.

STRESS TOLERANCE

In addition to herbicide resistance and disease resistance, we are also interested in approaches which may improve the abiotic stress tolerance of creeping bentgrass, in particular drought and salinity tolerance.

Dr. David Ho and colleagues have found that a barley protein, which they call HVA1, accumulates in developing barley seeds and in barley seedlings subjected to drought and salt stress (Hong et al., 1992). They found that when the HVA1 gene was expressed constitutively in transgenic rice, the plants were significantly protected from both drought and salt stress (Xu et al., 1996).

Since the last report we have identified eleven transgenic creeping bentgrass lines expressing the HVA1 gene. George Zhang, a graduate student with Bill Meyer, is currently testing the plants for drought resistance in the greenhouse. Preliminary results look promising. In this experiment, the HVA1 transgenics and 100 individual Crenshaw conrol plants were subjected to drought stress by withholding water. At 13 and 16 days following the initiation of drought, the plants were rated. At 16 days one of the HVA1 lines is outperforming all of the controls. This experiment is still is progress and the plants will continue to be rated for probably one more week. We will send an update on this experiment once it has been completed.

We are also currently initiating a test of the HVA1 plants for resistance to salt stress.

SUMMARY

During the past year we have obtained promising results from the field test for disease resistance. Two transgenic lines, one containing Gene B and one containing the glucose oxidase gene, appear to have good resistance to dollar spot. We have established a new field test containing these lines and also all our transgenic lines.

If the positive results for Gene B obtained in 1999 are repeatable in 2000, then we would pursue patenting the gene for use in conferring disease resistance.

In dicots the yeast delta-9-desaturase gene appears to be an extremely promising gene for conferring disease resistance. From our creeping bentgrass transgenic lines containing this gene it is clear that there is a difference between dicots and monocots in activity of the gene. During the coming year we will be focusing on producing a codon optimized synthetic gene to try to obtain transgenic bentgrass plants with the disease resistance seen in tomato.

During the coming year we will also be focusing on evaluating our HVA1 plants for drought and salinity stress resistance.

References

Hong, B., Barg, R., Ho, T-HD. (1992) Developmental and organ-specific expression of an ABA- and stress-induced protein in barley. Plant Mol. Biol. 18:663-674.

Hur, Y., Hwang, D-J., Zoubenko, O., Coetzer, C., Uckun, F.M., and Tumer, N.E. 1995. Isolation and characterization of pokeweed antiviral protein mutations in *Saccharomyces cerevisiae*: Identification of residues important for toxicity. Proc Natl Acad Sci USA 92:8448-8452.

Mittler, R., Shulaev, V., and Lam, E. 1995. Coordinated activation of programmed cell death and defense mechanisms in transgenic tobacco plants expressing a bacterial proton pump. Plant Cell 7:29-42.

Wang, C., Chin, C.-K., and Chen, A. (1998) Expression of the yeast delta-9 desaturase gene in tomato enhances its resistance to powdery mildew. Physiological and Molecular Plant Pathology 52: 371-383.

Wang, P., Zoubenko, O., and Tumer, N.E. (1998) Reduced toxicity and broad spectrum resistance to viral and fungal infection in transgenic plants expressing pokeweed antiviral protein II. Plant Molecular Biology 38: 957-964.

Wu, G., Shortt, B.J., Lawrence, E.B., Levine, E.B., Fitzsimmons, K.C., and Shah, D.M. 1995. Disease resistance conferred by expression of a gene encoding H_2O_2-generating glucose oxidase in transgenic potato plants. Plant Cell 7:1357-1368.

Xu, D., Duan, X., Wang, B., Hong, B., Ho, T.-H., and Wu, R. (1996) Expression of a late embryogenesis abundant protein gene, HVA1 from barley confers tolerance to water deficit and salt stress in trangenic rice. Plant Physiol. 110:249-257.

TABLE 1 OVERALL AVERAGE RATINGS OF TRANSGENIC LINES

Line #	Transgene	N	Mean
11032	Gene B	3	7.75
11725	GO	2	6.5
9963	GO	3	6.4
12426	GO	3	6.17
10907	PAP	2	5.37
10009	GO	3	5.33
Crenshaw	Control	31	4.76
10649	GO	2	4.75
10912	PAP	3	4.5
11615	GO	2	4.33
L93	Control	9	4.11
10017	PAP	3	4.08
10099	GO	3	4.08
12401	GO	3	4.00
11139	GO	2	3.87
9776	GO	2	3.5
10710	GO	2	2.5
12521	PAP	3	2.5

Ratings based on a 1-9 scale with 9 = least diseased

TABLE 2 RATINGS OF THE TOP FOUR TRANSGENIC LINES

	Aug. 23	Sept. 7	Sept. 15	Sept. 22
Gene B (11032)				
rep 1	9	8	8	7
rep 2	8	8	6	6
rep 3	9	8	8	8
GO (11725)				
rep 1	7	4	4	4
rep 2 died				
rep3	8	8	8	9
GO (9963)				
rep 1	9	8	7	7
rep 2	9	9	9	8
rep 3	2	3	3	3
GO (12426)				
rep 1	9	9	9	8
rep 2	9	7	5	3
rep 3	4	4	4	3

Ratings based on a 1-9 scale with 9 = least disease

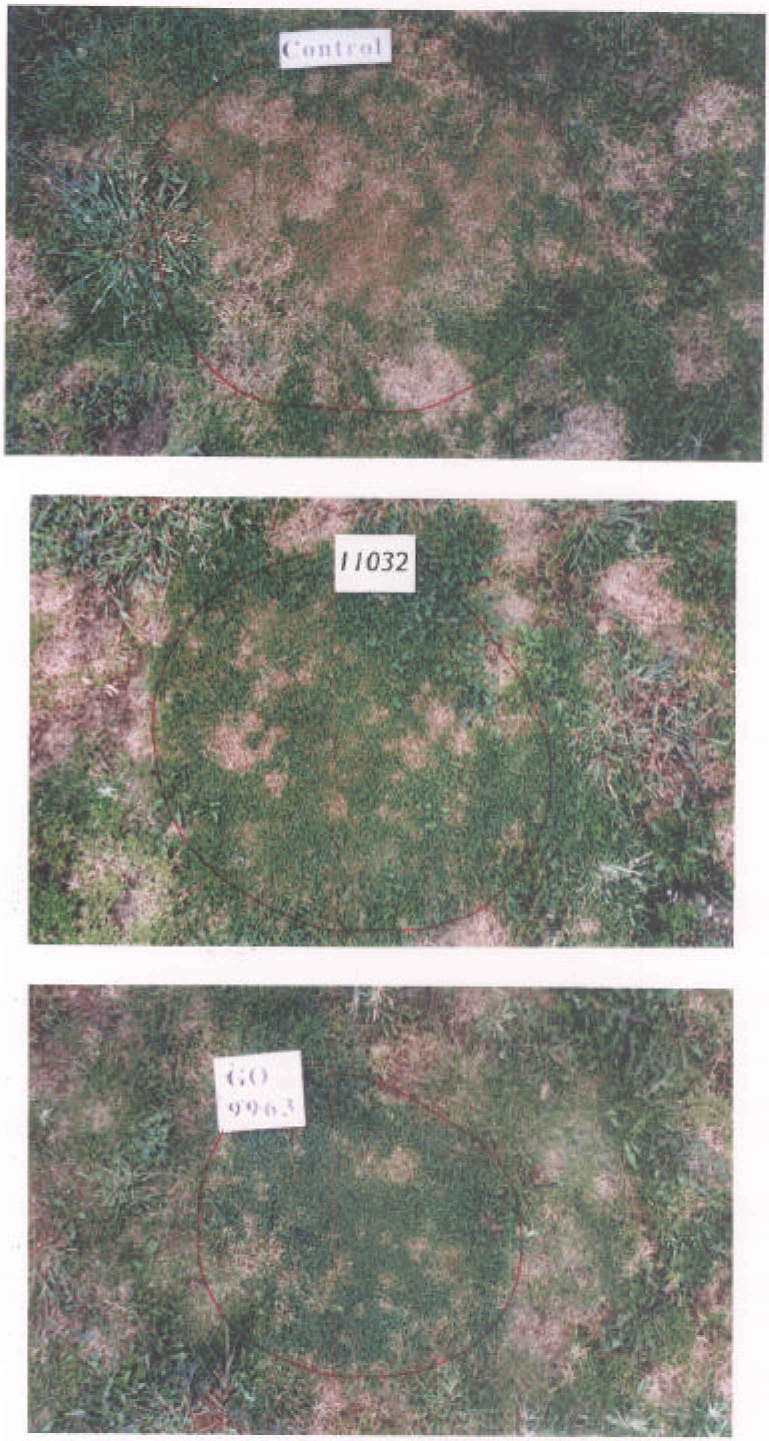

Figure 1. Representative plants of control and transgenic lines 9963 and 11032 photographed on Sept. 14, 1999. The control is a plant, which had received the average rating of 4 on Sept. 7.

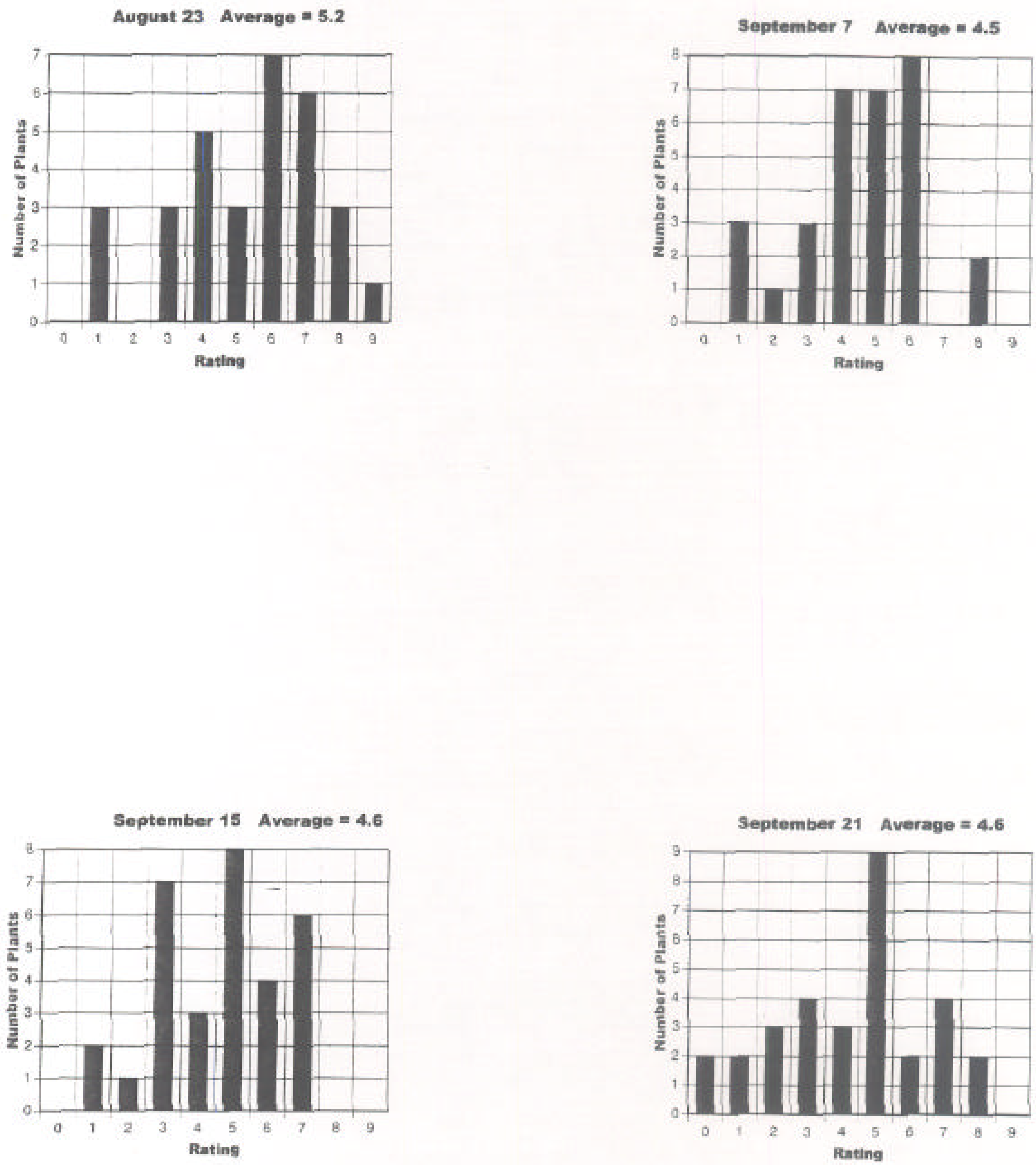

Figure 2. Rating distribution of the Crenshaw control plants at the four rating dates.