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ENDOPHYTES OF TURFGRASSES: NEW TOOLS AND APPROACHES

Executive Summary

This project was proposed and initiated by Dr. Peter Day, AgBIOTECH Center and Dr. Reed Funk, Department of Crop Science, Rutgers. Dr. T.M.A. Wilson, AgBIOTECH Center, is responsible for supervision of the laboratory work of the project. Program goals are: (1) to produce a germplasm collection of fungal endophyte-infected grasses concentrating on *Poa* and *Agrostis* species; (b) to produce a collection of unifungal endophyte cultures for classical and molecular analysis; (c) to produce endophyte-specific DNA probes; (d) to use the probes to characterize endophyte variability and produce RFLP maps for taxonomy; (e) to develop gene transfer methods for fungal endophytes; and (f) to identify the genes responsible for insect repellent alkaloid biosynthesis and metabolism.

After extensive screening of turfgrass germplasm collections, particularly *Poa* and *Agrostis* species throughout the U.S.A. and Europe (in collaboration with Dr. Jim White, Auburn University, Alabama) we have obtained a limited number of fungal endophyte-infected grasses in these two genera. However, to date, the presence of fungal endophytes in Kentucky bluegrass and Creeping bentgrass continues to elude us. Recently acquired endophytes in other *Poa* and *Agrostis* species are being cultured with a view to introducing them into Kentucky bluegrass and Creeping bentgrass through germinating seedlings, hyphal macerates, etc. A collection of fungal endophyte cultures has been established on agar plates and contains representative isolates from a wide variety of turfgrass genera. Selected examples are being used for DNA cloning and genetic fingerprint analysis. Fungal endophyte-specific DNA probes have been produced by the polymerase chain reaction (PCR) and diagnostic fingerprints of DNA sequences generated by randomly amplified polymorphic DNA (RAPD)-PCR methods. The latter technique has demonstrated an exquisite sensitivity for variation in total genomic DNA sequences and in many cases has shown as much inter-isolate variation as inter-specific and inter-generic variation at the DNA level. This result highlights the primitive nature of fungal endophyte taxonomy as well as providing diagnostic banding patterns for a particular endophyte "species". We have therefore elected to provide more precise DNA sequence information to aid fungal endophyte taxonomy. This work is in collaboration with Dr. Christopher Schardl (University of Kentucky, Plant Pathology Dept.) and requires that we obtain the DNA sequences of the poorly conserved spacer regions between the more highly conserved ribosomal RNA genes. Schardl has already developed an evolutionary tree of turfgrass fungal endophytes of the *Acremonium* genus (anamorph *Epichloe*) from a limited number of grasses and our data will provide additional resolution to this taxonomic device. Work on the development of gene transfer methods for fungal endophytes has progressed in a closely parallel project through production of fungal protoplasts and attempts to introduce recombinant DNA plasmids containing convenient antibiotic resistance genes. In principle, the technique looks feasible, however some additional selectable marker genes must be sought as *Acremonium* spp. have a high endogenous resistance to hygromycin. Because of the absence of widely available, natural endophytes in *Poa* and *Agrostis* species of interest to the USGA, we have elected to take a different route toward production of insect-resistant or otherwise modified turfgrasses for golf courses. Since March 1991, the project has therefore focused on the development of techniques for *Poa* and *Agrostis* tissue culture and regeneration of mature culms from single cells or disorganized calli (grass tumors). This work has been extremely successful and highly regenerable embryogenic turfgrass tissue cultures have been developed. We have also investigated the possibility of introducing foreign genes into turfgrass cells by DNA particle bombardment techniques. To date, the level of transient reporter gene expression has been encouraging and we are currently selecting for stably transformed turfgrass cell lines which express a gene conferring resistance to the herbicide Bialaphos (Basta™). In parallel with Bialaphos resistance, we are negotiating with several commercial organizations for genes which might confer insect resistance, virus resistance, growth retardation, resistance to fungal and bacterial pathogens and a variety of other single-gene traits. We consider this to be a major technological breakthrough for the production of transgenic turfgrass with improved agronomic performance through insertion of one, or a few, desirable genes.

I. Turfgrass Germplasm and endophyte culture collection and screening for *Agrostis* and *Poa* endophytes.

During 1990, Dr. Jane Breen, the post-doctoral researcher, completed the initial phase of the collection and screening. This summer several individual field collected selections were added through the work of Dr. James White (Auburn University, Montgomery, AL) (See IV related projects) and Dr. Funk. Dr. Funk had collected endophyte-containing redtop (*Agrostis alba*) from Somerset, NJ and autumn bent (*Agrostis perrenans*) also from Somerset, NJ.

II. DNA Fingerprinting

Intergenic Transcribed Spacer sequences (ITS-1) (1) between the 5.8S ribosomal RNA gene and the 18S ribosomal RNA gene were cloned from our standard 12 endophyte isolates (Table 1) by the polymerase chain reaction (PCR). Direct sequencing of purified ITS-1 DNA fragments from PCR products was not successful. We thus inserted the ITS-1 DNA fragments from our PCR reactions into a plasmid vector pCR™1000 using a commercial "TA cloning™" system. Sequencing of the ITS-1 sequences from *Acremonium typhinum* and the anamorph *Epichloe typhina* PP17, LF2, and 1210-1 with the pCR™1000 plasmid is in progress. Comparisons of sequence data of the ITS-1 fragments of our endophyte isolates from *Agrostis* spp. and *Poa* spp. with sequence data from *Festuca* spp. (in collaboration with Dr. Christopher L. Schardl, University of Kentucky; letter attached) will further establish the molecular taxonomy of fungal endophytes.

The RAPD-PCR method (2) was used with four ten-base random DNA primers (60-70% GC) (Fig. 1) to study 11 turfgrass fungal DNA isolates and 1 plant DNA (isolated from a Black Mexican Sweet maize suspension culture) as control (Table 2, Fig. 2). Many major bands were detected from the PCR products. Different isolates of the same species of *Acremonium* with the same PCR primer showed some common bands and some different bands. This preliminary screening showed that significant genome heterogeneity may exist between species as well as in endophytes classified in the same species (isolate variation). Further screening with more primers may be helpful. We are also testing the possibility of detecting endophytes in infected plant tissues using the RAPD-PCR technology (plus-and-minus screening of total turfgrass DNA from the leaf bases). The major PCR products from turfgrass fungal DNAs can be used not only diagnostically as molecular markers but also can be used as molecular probes to produce RFLP maps as taxonomic aids.

III. Tissue Culture

Callus induction from mature seeds of Kentucky bluegrass (*Poa pratensis*) and Creeping bentgrass (*Agrostis palustris*) was initiated in April, 1991. Several callus culture initiation media with different concentrations of 2,4-D or dicamba were used. Successful regeneration of callus cultures from six cultivars of Kentucky bluegrass (*Poa pratensis*) and three cultivars of Creeping bentgrass (*Agrostis palustris*) is shown in Table 3. Two varieties of Kentucky bluegrass (Baron and Midnight) and all three varieties of Creeping bentgrass (Penncross, Emerald, and Putter bentgrass) yielded mature plants growing very nicely in the growth

chamber. Two embryogenic callus lines of Putter bentgrass were identified and used to initiate embryogenic suspension cultures. These embryogenic callus lines showed extremely high regeneration potential. Figure 3 shows regeneration of about 40 mg. of embryogenic callus in turfgrass regeneration medium which was slightly modified from Lee et al. (3) by replacing 0.2% "Gelgro"™ with agarose. The embryogenic suspensions were also highly regenerable when plated directly onto regeneration medium, approximately 150 plantlets were regenerated from 1 ml packed volume of cells.

We have started to test the possibility of transforming grass with foreign DNA using the Biolistic (particle bombardment) approach. Both embryogenic callus and embryogenic suspension cells were used as targets. We used the E. coli β-glucuronidase (GUS) gene (4) as the scorable marker, to assay transient expression of an added gene in the grass cells after bombardment. Although there is no clear correlation between high transient expression and stable transformation frequency, in general, higher transient expression may indicate a better transformation frequency. Our initial bombardment experiments (No. 1, 2, & 3) gave very low transient expressions of the GUS gene. However, the most recent bombardment experiment showed that a GUS construct with the maize alcohol dehydrogenase first intron, which is reported to elevate expression in monocot cells, improved transient expression by about two orders of magnitude (Table 4).

The bar gene which confers resistance to the herbicide "*Bialaphos*"™ in the transgenics has been used as a selectable marker. Several attempts to stably transform Creeping bentgrass (Putter) to become bialaphos resistant are in progress. Negotiations with DNAP (Cinnaminson) Monsanto (St. Louis) and Diatech Ltd. (J. Sainsbury, plc; U.K.) are underway to bring this technology to market rapidly and to acquire other agronomically important genes (e.g. for glyphosate resistance, insect resistance, virus resistance (Poa mosaic virus CP)).

IV. Related Projects

(1) A complementary project, "Screening for extrachromosomal genetic elements in *Acremonium* endophytes," was funded by the Rutgers Turf Research Program. A Ph.D. student, Chan-Seok Oh, has screened 24 different isolates of *Acremonium* for the presence of double-stranded RNA viruses and DNA plasmids. Neither of these extrachromosomal elements has yet been found in *Acremonium*. At the suggestion of Dr. James White, *Atkinsonella hypoxylon*, a choke-causing epiphyte that occasionally grows endophytically and grows significantly faster in culture than *Acremonium*, was examined and revealed the presence of both dsRNA molecules and dsDNA plasmids. The dsRNA molecules, presumably of viral origin, have been cloned and partially sequenced. Single sporing experiments aimed at curing the fungus of the dsRNA elements or dsDNA plasmid resulted in three colony types, which are currently under further investigation. Attempts at stable introduction of foreign DNA into the fungal genome have begun with the successful production of regenerable protoplasts. Hygromycin resistance was investigated as a selectable marker, but both *Acremonium* and *Atkinsonella* spp. were found to be highly resistant to the drug. Other drugs will be screened until a suitable selectable marker for which a resistance gene is available is found. Fungal sensitivity to copper ions may be exploited using a copper chelating thionin gene.

(2) Screening for *Agrostis* and *Poa* endophytes.

Dr. James White from Auburn University, Alabama, who worked with us last summer in screening for endophytes in *Agrostis* and *Poa* spp. visited the Sports Turf Research Institute in Bingley, Yorkshire, England this summer. He examined many of their *Agrostis* cultivar trials and found that all were endophyte-free. In the field, he commonly located *Acremonium typhinum* in *Agrostis alba*, *Agrostis tenuis* and *Agrostis stolonifera*. He also located endophytes in *Festuca pratensis, F. arundinacea, F. rubra, F. ovina and Bromus ramosus.* Still, he has not located endophytes in *Poa pratensis.*

V. Future directions

The collections of *Acremonium typhinum* on *Agrostis stolonifera* (from Dr. James White) and endophyte-containing redtop (*Agrostis ulba*) (from Dr. Funk) may provide *Agrostis* compatibility factors that will help us develop fungal transfer methods between different turfgrasses. For these choke-forming fungi, the development of vegetative propagation through tissue culture may be useful.

We will continue to work on (1) characterization of endophyte variability and taxonomy, (2) development of gene transfer methods for fungal endophytes as well as ways to introduce new endophytes into turfgrass, (3) transformation of turfgrass with agronomically important genes.

Table 1
Endophyte isolates used for ITS-1 DNA cloning

Fungal	Turfgrass	Isolate/code
A. coenophialum	*Poa autumnalis*	
A. huerfanum	*Festuca arizonica*	
A. typhinum	*Poa palustris*	pp10
		pp17
	Festuca rubra	blue 4
A. starrii	*Festuca rubra*	Longfellow 2 (LF2)
		Longfellow 3 (LF3)
		Longfellow 4 (LF4)
	Festuca longifolia	ST3
Epichloe typhina	*Festuca rubra*	1209-7 50% choke
		1210-1 No choke
Atkinsonella hypoxylon	*Danthonia spicata*	1-low choke

Figure 1. Random ten-base primers used in RAPD-PCR

	5' to 3'	GC
A-01	CAGGCCCTTC	70%
A-02	TGCCGAGCTG	70%
A-03	AGTCAGCCAC	60%
A-04	AATCGGGCTG	60%

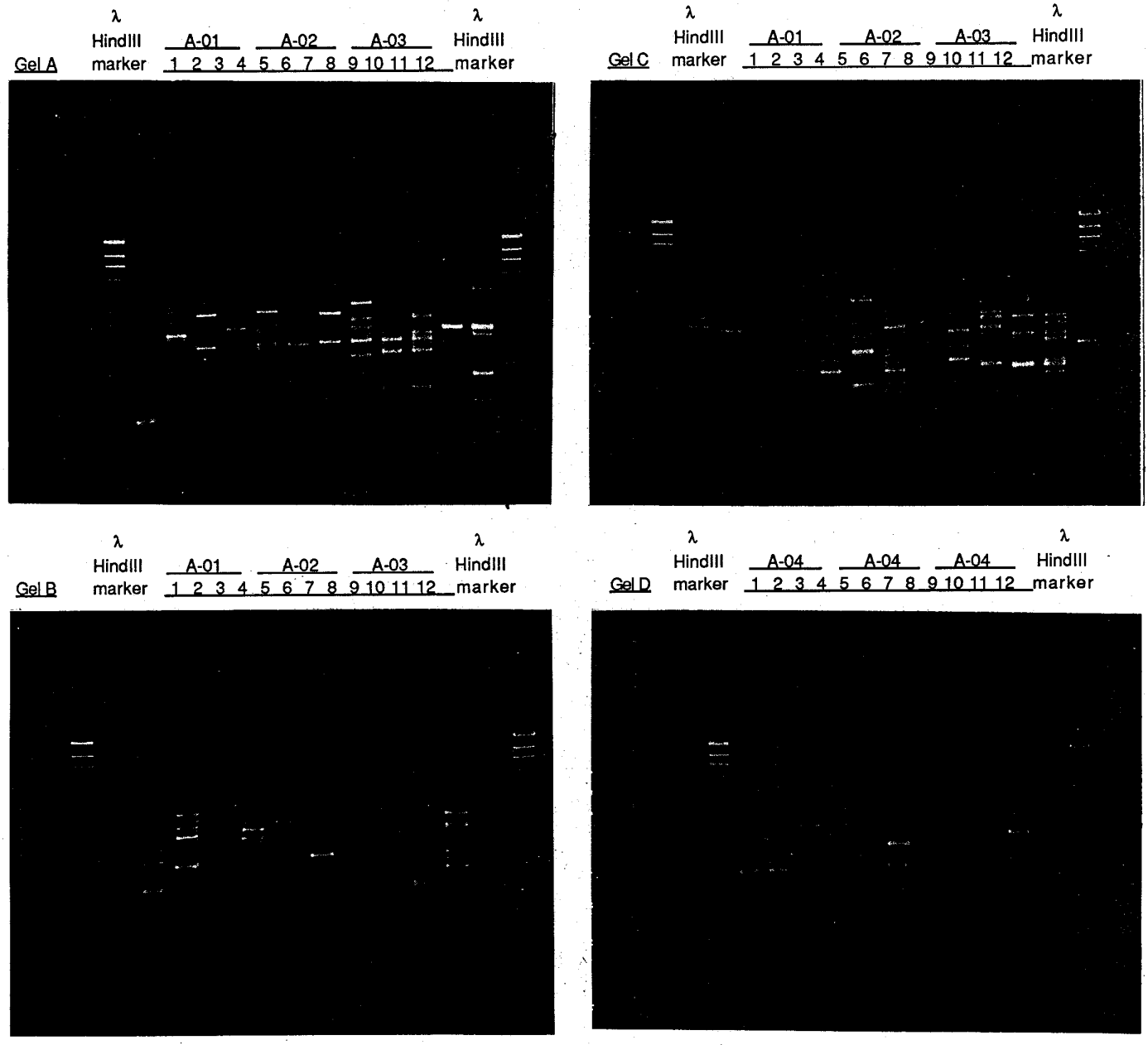

Figure 2. Amplification of 11 isolates and BMS DNAs with primers A-01, A-02, A-03 and A-04.

Table 2

RAPD-PCR of 11 fungal isolates and BMS DNAs with four ten-base random DNA primers

Fungal	Turfgrass	Isolate	Position in the Figure 2					
Acremonium					A-01	A-02	A-03	A-04
typhinum	*Festuca rubra*	ST1	Gel B,	Lane	2	6	10	Gel D Lane 5
		ST3	Gel B,	Lane	3	7	11	Gel D Lane 6
		ST4	Gel B,	Lane	4	8	12	Gel D Lane 7
		1204	Gel A,	Lane	3	7	11	Gel D Lane 3
		1209-7	Gel A,	Lane	1	5	9	Gel D Lane 1
		1210-1	Gel A,	Lane	2	6	10	Gel D Lane 2
		1211	Gel A,	Lane	4	8	12	- -
A. coenophialum	*Poa autumnalis*		Gel C,	Lane	3	7	11	Gel D Lane 10
	Triumph		Gel C,	Lane	4	8	12	Gel D Lane 11
Atkinsonella hypoxylon	*Danthonia spicata*	1-low choke	Gel C,	Lane	1	5	9	Gel D Lane 8
		2-high choke	Gel C,	Lane	2	6	10	Gel D Lane 9
BMS		maize suspension	Gel B,	Lane	1	5	9	Gel D Lane 4

Table 3

Regeneration of callus cultures from six cultivars of Kentucky bluegrass (*Poa pratensis*) and three cultivars of Creeping bentgrass (*Agrostis palustris*)

Kentucky bluegrass	Regeneration
Able I	-
Alene	-
Baron	+
Midnight	+
MN2405	-
Ram-I	-
Creeping Bentgrass	
Penncross	+ +
Emerald bentgrass	+ +
Putter bentgrass	++++

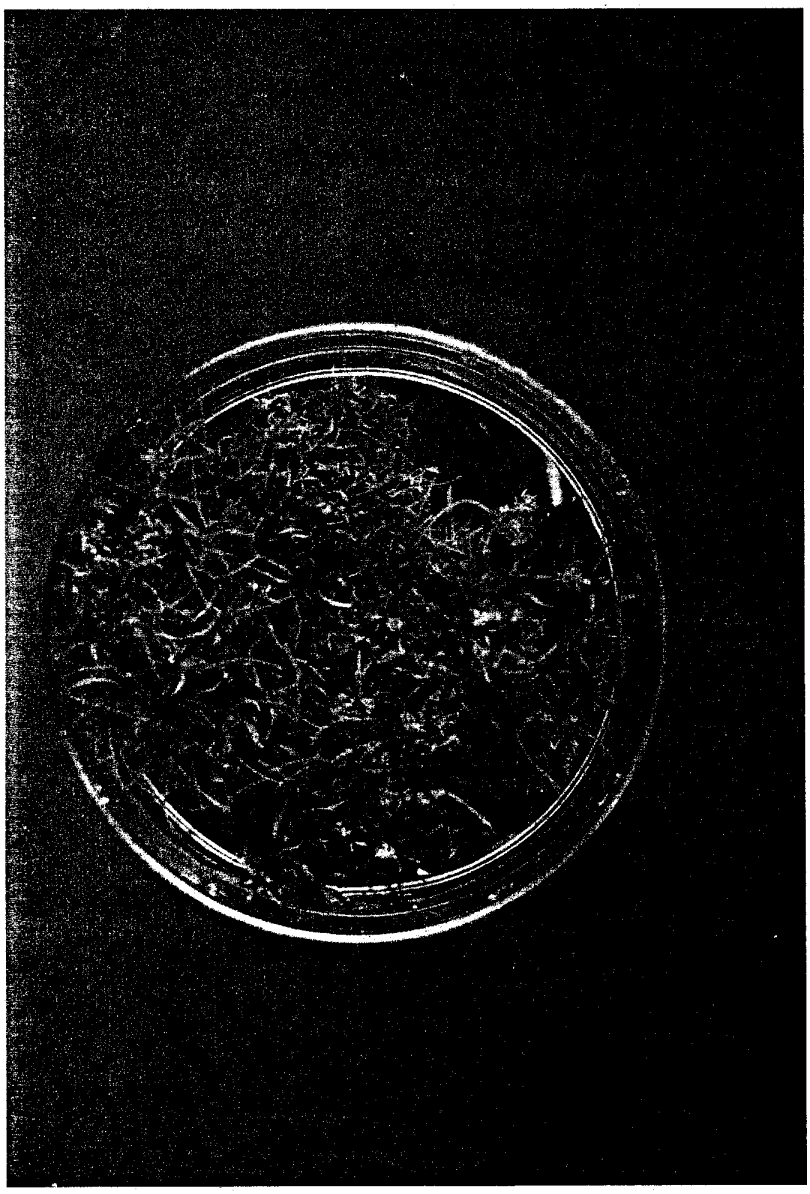

Figure 3. High efficiency regeneration of the embryogenic callus of Putter bentgrass.

Table 4. Transient expression of 35S-GUS gene constructs with or without the *Adh* intron in embryogenic suspensions of Creeping bentgrass expressed as total number of blue spots after DNA bombardment and X-Gluc staining.

	Blue Spots (Number)		
Experiment Number	Intron-GUS(pPHI460)	GUS(pAGUS1)	GUS(pFF19)
1	NA	2	NA
2	NA	2	NA
3	3	0	NA
4	212	13	21

pPHI460 was obtained from Dr. Dorothy Pierce of Pioneer Hi-Bred.
pFF19 was obtained from Dr. Pal Maliga, Waksman Institute, Rutgers University.
pAGUS1 was obtained from Dr. Ray Gesteland, Utah University.

Table 5. Presentations and Meetings Attended (1991)

1. ISPMB Tucson - October 6-12th - Poster/Discussions (L. Lee)
2. DNAP (Nov. 14th) - Presentation (TMAW and L. Lee)
3. Rutgers University Turfgrass Field Day - Nov. 16 (Presentation L. Lee)
4. New Jersey Turfgrass Association - Dec. 4 (Presentation TMAW).

References

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2. Williams, J.G.K., A.R. Kubelik, K.J. Livak, J.A. Rafalski, and S.V. Tingey. (1990) DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res.* **18**:6531-6535.

3. Lee, L., R.E. Schroll, H.D. Grimes, and T.K. Hodges. (1989) Plant regeneration from Indica rice (*Oryza sativa* L.) protoplasts. *Planta* **178**:325-333

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