Hybrid Bermudagrass Improvement by Genetic Transformation

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by Dr. Rongda Qu
Department of Crop Science
North Carolina State University
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Participants and time devoted to the project:

Dr. Rongda Qu (PI)	**10%**
Dr. Liangcai Li (Visiting Scientist)	**80%**

Collaborators:

Dr. Eric Davis, Assistant Professor, Plant Pathology, NCSU

Dr. Karel Schubert, Professor, Botany, University of Oklahoma

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Executive Summary

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The ultimate goal of this research direction is to improve turf-type bermudagrass cultivars for the golf courses through biotechnology. The specific goals of the project include: to optimize tissue culture conditions for inducing embryogenic calli and regenerating plantlets of bermudagrass, to develop procedures to transform bermudagrass, and to obtain transgenic bermudagrass plants that express potential nematode resistant genes.

The turf-type bermudagrass is particularly recalcitrant for plant tissue culture. We mainly use the young inflorescences of hybrid cultivar 'Tifgreen' and a common bermudagrass cultivar 'Savannah' as research matertials. So far, we have spent substantial efforts to improve the tissue culture responses of bermudagrass. Last year, we showed that by adding 0.01 mg/L 6-benzylaminopurine (BAP) in the callus induction medium (MS with 1 mg/L 2,4-D), we were able to induce embryogenic structures from otherwise very fluffy callus and the bermudagrass regeneartion was substantially improved. A manuscript dealing with this work has been accepted by a journal. This year, we further tested a total of 65 combinations of plant growth regulators at various concentrations in the culture medium in 52 batches of experiments. It was observed that a combination of BAP and abscisic acid (ABA) can further improve the callus quality and increase the occurrence of the embryogenic structures. It was also observed that inclusion of gibberellic acid (GA) in the regeneration medium effectively improved the regeneration of the embryogenic calli.

Another hybrid cultivar, 'Tifway', was even more recalcitrant. By pre-treating the young inflorescences with 0.2% ascorbic acid, we have been able to induce calli from 'Tifway' tissue culture and to recover regenerated plants.

We have spent substantial efforts in the transformation experiments. By histologically examining the callus structure, we have focused on the very young, undifferentiated calli as target tissue for transformation. We have performed 15 batches of transformation experiments using the biolistic method and bialaphos selection. Although some calli and plantlets survived the selection, we have no strong evidence to show they are transgenic. In searching for an alternative to the herbicide selection, we also identified G418 as a potential selection agent. In addition, we explored the possibility to transform bermudagrass with agrobacterium. Transient expression assays of an intron-GUS reporter gene suggested that agrobacterium can infect bermudagrass and thus may be used to transform bermudagrass.

Hybrid bermudagrass (*Cynodon transvaalensis x C. dactylon*) is an important warm-season grass species for the greens, tees and fairways of golf courses in the South of the United States. The ultimate goal of the research direction is to improve bermudagrass cultivars for the golf courses through biotechnology. The specific goals of this project are as the following:

1. To develop and optimize tissue culture conditions in order to obtain embryogenic calli and to regenerate plantlets from bermudagrass;

2. To develop procedures to transform bermudagrass and to recover transgenic plants;

3. To obtain transgenic plants of bermudagrass that express potential nematode resistant genes.

This project was initiated in 1997 in my laboratory and has been supported by a USGA grant since July 1, 1998. Since bermudagrass, particularly the turf-type varieties, is recalcitrant in *in vitro* plant regeneration, we have spent a great deal of efforts in optimizing tissue culture conditions. In the past year, we also spent a substantial effort in developing

I. Optimizing Tissue Culture Conditions

A. Further improvement of young inflorescence culture of hybrid and common bermudagrass

We have been using a hybrid cultivar 'Tifgreen' and a common bermudagrass cultivar 'Savannah' as experimental materials. Our experiments in 1998 suggested that explant tissue plays a very important role in the success of bermudagrass tissue culture. Although we have intensively tested the media and medium supplements, it was extremely difficult to obtain regenerable callus from culture of vegetative tissue, such as nodal segment. On the other hand, we greatly improved the callus regeneration ability of young inflorescence culture in 1998 by reducing 2,4-D (an auxin used in the medium to induce callus formation) to 1 mg/L in the callus induction MS medium and by supplementing 0.01 mg/L 6-benzylaminopurine (BAP) in the same medium. The resulting medium is called 'MS1' for the convenience in the context. Very compact, nodular, embryogenic structures were formed on the surface of approximately 20% of the calli induced on MS1. The embryogenic structures had high regeneration potential. The regeneration rates of the calli with embryogenic structures, when transfgerring to the regeneration medium (MS medium with 2.5 mg/L BAP) could reach as high as 50 to 80%.

However, we have observed variation in the realized regeneration of the embryogenic structures from batch to batch. In addition, this embryogenic structure is too differentiated to be directly used as the target tissue for genetic transformation. Thus, one goal for our 1999 experiments is to continue to improve tissue culture response to: (1) further increase the percentage of callus with embryogenic structures and to increase and to stabilize the regeneration rate of such structures; and (2) provide suitable target tissues for the transformation experiments.

A total of 52 batches of tissue culture experiments were performed in 1999, which included 37 batches of 'Tifgreen' and 15 batches of 'Savannah'. Approximately 7,000 young inflorescences were cultured in these experiments. In experiments to compare effects of various BAP concentrations, it was observed that 0.005-0.01 mg/L BAP was the

optimal concentration for inducing the embryogenic structures. Higher concentration of BAP (0.1 and 1 mg/L) was generally detrimental. Subsequently, a total of 65 combinations of various plant growth regulators at different concentrations were tested. In most of the cases, plant growth regulators were added to MS1 to test their effects.

It was observed that embryogenic structures were increased by supplementing abscisic acid (ABA) in the MS1 medium. ABA reduced the growth rate of the callus and made the callus more compact. The optimal concentration of ABA is 5 mg/L for 'Tifgreen'. At this concentration, addition of ABA yielded 60 to 100% more embryogenic structures in 'Tifgreen' young inflorescence culture (Table 1). Common bermudagrass seemed to have a slightly different requirement to achieve the optimum. A combination of a higher ABA concentration (10 mg/L) plus a higher BAP level (0.2 mg/L) was the best and yielded over 130% more embryogenic structures than MS1 only for 'Savannah' (Table 2).

In regeneration experiments, it was observed that addition of gibberellic acid (GA, at 0.2 mg/L), a phytohormone, to the regeneration medium stably and effectively improved the regeneration of the calli bearing the embryogenic structure (Table 3, Figure 1). It is probably more precise to state that addition of GA promoted germination of the somatic embryos.

B. Regeneration of hybrid bermudagrass cultivar 'Tifway'

'Tifway' young inflorescence is extremely recalcitrant in callus induction mostly due to the browning of the explants in culture medium. Based on our experience in 1998, all the segments of the young inflorescences were pre-treated in MS liquid medium supplemented with 0.1% ascorbic acid (Vc, an anti-oxidant) for 1-3 hrs before they were cultured. Forty-five 'Tifway' young inflorescences were cultured on four callus induction media. A total of 63 pieces of callus were induced. The medium supplemented with PVPP (polyvinylpolypyrrolidone, 6 g/L) yielded more calli (average 3 calli per inflorescence) with an overall better quality (more compact). Among the 63 calli transferred to the regeneration medium, plantlets were regenerated from two calli (Table 4, Figure 2). Probably still due to the browning problem (phenolic compound formation), the calli had to be transferred twice on the regeneration medium to regenerate, and the regenerants twice on the rooting medium to develop roots and to grow. In the future, a combination of Vc pretreatment and supplements of PVPP and low concentration of BAP (e.g. 0.01 mg/L) to the callus induction may further imporve the regeneration of 'Tifway'.

II. Development of Transformation Procedure for Bermudagrass

In 1999, we have initiated efforts to develop procedures for bermudagrass transformation. The efforts include: identifying suitable target tissue for transformation, searching for suitable selection agent(s), and testing both biolistic and agrobacterium methods for bermudagrass transformation.

A. Identifying suitable target tissue for transformation

In general, genetic transformation requests non-differentiated, actively-dividing meristematic cells for DNA delivery and integration. As mentioned above, the embryogenic structure is too differentiated for transformation purpose. On the other hand, the long-tubular cells found on surface of fluffy callus of bermudagrass are not suitable either because they do not divide any more although transient GUS reporter gene expression still can be observed from these cells after microprojectile bombardment. A histological analysis of bermudagrass grown-up callus indicated that the meristematic cell

clusters, which are in charge of the callus growing, are buried inside the callus. Only calli at very early stage mostly consist of the meristematic cells and are likely to be the tissue used for transformation efforts.

B. Searching for suitable selection agent(s)

Bermudagrass seems to have natural resistance to antibiotic and herbicide selection agents. The concentration of kanamycin and hygromycin needs to be in the range of 350-400 mg/L to completely inhibit the callus growth. We have been using bialaphos as the selection agent. It seems even at 7 mg/L selection strength (most of cereals use 5 mg/L for selection), the survivors are more likely the escapes rather than true transformants. Thus, we recently tested G418, an antibiotic, for suitability as a selection agent. It was observed that the growth was reduced to half at 50 mg/L G418, and was completely inhibited when G418 was at 250 mg/L. We are currently trying G418 selection for bermudagrass transformation.

C. Biolistic transformation efforts

So far, we have performed 15 batches (150 plates) of biolistic transformation experiments. We have used different ages of calli ranging from 1 d to 3 wks, and more compact calli yielded from ABA-containing media. We also tried to dissect the calli so to have the inside meristematic cells exposed to the bombardment. Good transient GUS reporter gene expression was obtained (Figure 3). We have been mostly using bialaphos as the selection agent so far. In bialaphos selection, we used a step-up selection strategy, starting at 3 mg/L, changed to 5 mg/L after 2-3 wks, and to 7 mg/L after another 2-3 wks. Some calli survived the selection but grew slowly in the selection media. Two plantlets were regenerated in the regeneration medium containing 3 mg/L bialaphos but they started turning yellow at 5 mg/L bialaphos. Although we have not yet performed the molecular assays, the survived calli and plantlets were more likely the escapes. This reflects the difficulty of bermudagrass transformation. We are currently testing G418 selection.

D. Agrobacterium transformation efforts

As an alternative to the biolistic approach, we also explored the possibility to transform bermudagrass with agrobacterium. Five strains of *Agrobacterium tumefeciens* was tested, each having an intron-GUS reporter gene. Infection of bermudagrass calli was evaluated by the transient GUS gene expression. Ten batches of experiments have been performed. Transient GUS expression was observed (Figure 4) from calli in approximately 1/3 to 1/2 plates, indicating that bermudagrass is susceptible to agrobacterium infection, and that there is a possibility to transform bermudagrass with agrobacterium.

III. Publication from the Project

A. Chaudhury & R. Qu: Somatic embryogenesis and plant regeneration of turf-type bermudagrass: effect of 6-benzyladenine in callus induction medium. Plant Cell, Tissue and Organ Culture (accepted).

IV. Proposed Research Schedule for Next Year

A. Biolistic and agrobacterium transformation

We feel that we have optimized the tissue culture conditions of bermudagrass. Thus, next year we will concentrate on transformation efforts using both biolistic and

agrobacterium approaches. The key components will be the tissues used for the transformation and the selection method. We are currently evaluating G418 selection and will try hygromycin selection too. As for the target tissues, we will focus on the early age calli. We will try to use suspension cell cultures too for the transformation experiment. In addition to the cultured calli, we consider to use apical meristem tissue of bermudagrass plants as a target, particularly for agrobacterium transformation. The general idea is to take the advantage of vegetative proliferation of bermudagrass. The plant branches will be collected, and the apical meristems will be sliced open and exposed to the agrobacterium bearing the herbicide-resistant *bar* gene. The branches will be grown in the culture medium and then soil. The newly grown branches will be tested for resistance to the herbicide glufosinate ('Finale').

B. Test the effects of the proposed anti-nematode gene in transgenic tobacco plants

We are in the process to reach a MTA so to acquire transgenic tobacco seeds from Dr. K. Schubert of Univ of Oklahoma. The tobacco plants transgenic of Pentin 3, a trypsin inhibitor gene isolated from a tropical rain forest species was resistant to root knot nematode and free living nematode, *C. elegans* (personal communication with Dr. Schubert). We will evaluate its effects on the growth of sting nematodes in collaboration with Dr. E. Davis.

Table 1. ABA effects on 'Tifgreen' embryogenesis

Experiment	Supplement	No. of young inflo-rescence cultured	No. of embryogenic structure obtained	%
I	none	20	58	100
	ABA 2 mg/L	20	81	140
	ABA 5 mg/L	20	93	160
	ABA 10 mg/L	20	86	148
II	none	15	39	100
	ABA 2 mg/L	15	89	228
	ABA 5 mg/L	15	84	215

medium: MS1 (MS, 1mg/L 2,4-D, 0.01 mg/L BAP)

Table 2. ABA effects on 'Savannah' embryogenesis

BAP (mg/L)	ABA (mg/L)	No. of young inflo-rescence cultured	No. of embryogenic structure obtained	%
0.01	none	20	40	100
0.01	5	20	30	75
0.01	10	20	6	15
0.2	10	20	93	232

medium: MS with 1 mg/L 2,4-D

Table 3. Effects of GA and NAA on 'Tifgreen' regeneration

Supplements		Embryo structure	Embryo structure regenerated	
GA (mg/L)	NAA (mg/L)	cultured	No.	%
0	0	48	7	14.6
0	0.2	43	6	14
0.2	0	93	48	51.6
0.2	0.2	95	56	58.9

regeneration medium: MS with 1 mg/L BAP

Table 4. Tissue culture results of 'Tifway'

Medium	Supplement	No. of young inflo-rescence cultured	No. of callus induced	No. of callus regenerated
MS	none	11	20	1
MS	PVPP			
	6 mg/L	11	33	1
MS	Ascorbic acid			
	1 g/L	12	0	0
NB	none	11	10	0

culture medium has 1 mg/L 2,4-D

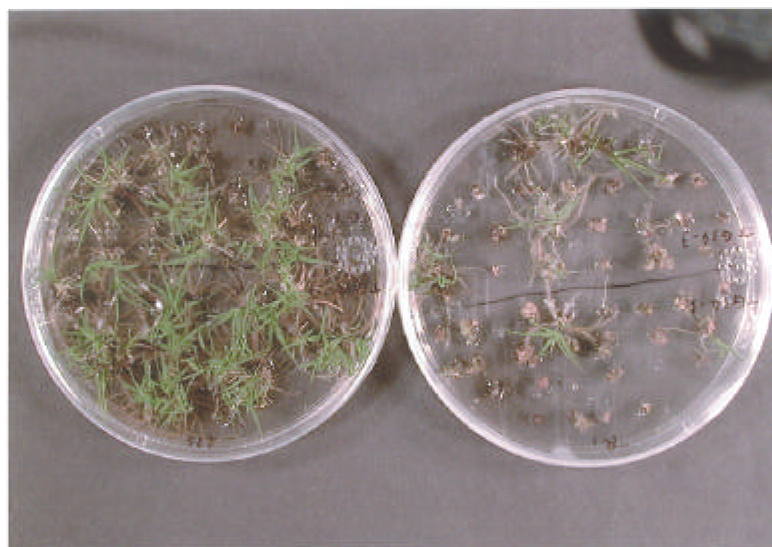

Figure 1. Supplement of GA (left dish) enhances regeneration of embryogenic calli.

Figure 2. Regenerated plantlet of 'Tifway'.

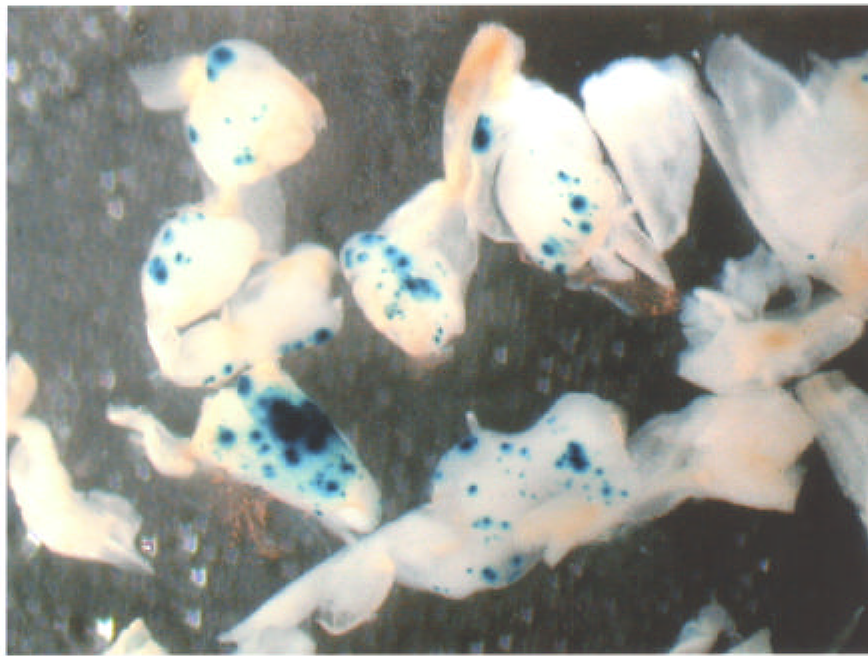

Figure 3. Transient GUS expression after bombardment of bermudagrass calli.

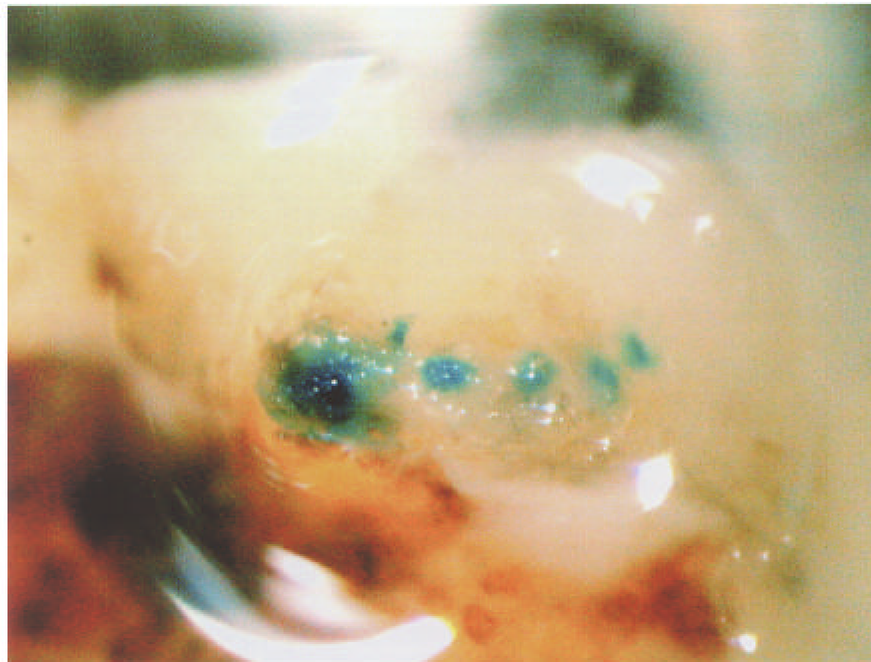

Figure 4. Transient expression of an intron-GUS gene after agrobacterium transformation of a bermudagrass callus.