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Hybrid Bermudagrass Improvement by Genetic Transformation

**2000 Annual Report
to
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by

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Participants and time devoted to the project:

Dr. Rongda Qu (PI)	10%
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Executive Summary

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The ultimate goal of this research project 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 overall recalcitrant for plant tissue culture. We mainly use the young inflorescences of hybrid cultivar 'Tifgreen' and a common bermudagrass cultivar 'Savannah' as tissue culture materials because their potential to regenerate. So far, we have spent substantial efforts to improve the tissue culture responses of bermudagrass. We have showed previously that by supplementing 6-benzylaminopurine (BAP) and abscisic acid (ABA) in the callus induction medium, we substantially improved somatic embryogenesis from bermudagrass calluses. Addition of gibberellic acid further improved the germination/regeneration of the embryos. This year, we repeated the experiments and confirmed our observation. We also observed secondary somatic embryogenesis in bermudagrass culture which contributed to the regeneration. Moreover, we found a better combination of phytohormones for common bermudagrass tissue culture medium.

We have spent most of our efforts in the transformation experiments with emphasis on *Agrobacterium*-mediated transformation. A gene expression vector pRQ219 was constructed and 8 *Agrobacterium* strains were made. We found that some strains are more infectious in bermudagrass than others, and some bermudagrass cultivars are more susceptible than other cultivars. We also identified the optimal time to perform the infection. We performed 28 batches of agro-transformation experiments using young inflorescences and their derived calluses. In addition, we established 5 suspension cell lines which have been used for transformation experiments. Using an intron-GUS reporter gene, we observed cell clusters showing GUS activity, indicating stable transformation of bermudagrass cells.

Bermudagrass 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. In the past year, we further improved bermudagrass tissue culture conditions and spent great efforts in exploring the transformation methods of bermudagrass.

I. Optimize Tissue Culture Conditions

A. Further confirm the roles of BAP, ABA and GA on bermudagrass somatic embryogenesis and regeneration

We have been using the young inflorescence of a hybrid cultivar 'Tifgreen' (*Cynodon transvaalensis* x *C. dactylon*) and a common bermudagrass (*C. dactylon*) cultivar 'Savannah' as explant tissue. In 1998, we discovered the enhancement of somatic embryogenesis by supplementing 6-Benzylaminopurine (BAP, 0.01 mg/L) to the callus induction medium. In 1999, we observed that addition of abscisic acid (ABA) to the medium further improved somatic embryogenesis, and that gibberellic acid (GA) enhanced germination /regeneration of bermudagrass somatic embryos. In 2000, we repeated these two experiments and further confirmed the beneficial roles of the three phytohormones in bermudagrass tissue culture. Supplement of 5 mg/L ABA to the callus induction medium (Table 1) and 0.2-0.5 mg/L GA (Table 2) to the regeneration medium seem to be the optimal concentrations for cultivar 'Tifgreen' (Figure 1).

In addition, we have found out that the above combination of BAP and ABA may not be the best for 'Savannah' (and maybe other common bermudagrass). We observed that higher concentration of BAP (0.2 mg/L) in combination with ABA (5 mg/L) induced substantially more somatic embryos from 'Savannah' young inflorescence culture (Table 3).

Moreover, we observed 'secondary' somatic embryogenesis on somatic embryos of bermudagrass, mainly from 'Savannah', i.e., formation of somatic embryos from original somatic embryos (Figure 2). There was little report on this phenomenon in the family of *Poaceae*. Our observation could be the first in the family. Secondary somatic embryogenesis may help improve the regeneration of bermudagrass.

B. Establish suspension cell cultures for genetic transformation of bermudagrass

So far most of the transformation work has been using young inflorescences and their derived calluses. We have started to establish suspension cell cultures as a new target

tissue for transformation because (1) suspension cells may be different in tissue structure from callus and have worked better as target tissue for other turfgrass (tall fescue, perennial ryegrass) transformation in the laboratory, and (2) will provide more research materials during winter period. We have selected embryogenic calluses from cultivars of 'Tifgreen', 'Savannah', 'Mirage', and 'J1224' young inflorescence cultures and established five suspension cell lines (Figure 3). We are currently trying to establish more such cultures and adjusting the supplement of phytohormones (2,4-D, BAP and ABA) to optimize the culture conditions.

II. Development of Transformation Procedure for Bermudagrass

In 1999, we spent a great deal of efforts trying to use the biolistic method to bombard various tissues (young inflorescence fragments and induced calluses) at various stages and to select the transgenics with either hygromycin or bialaphos. However, we did not recover stable transformants. Thus, in 2000, we spent most of our efforts trying *Agrobacterium* transformation approach since our 1999 results showed that *Agrobacterium* can infect bermudagrass.

A. Construction of plasmid pRQ219 and new strains of *Agrobacterium* for bermudagrass transformation

Plasmid pRQ219 was constructed to include a *nptII* gene driven by *nos* promoter for kanamycin/G418 selection, a *bar* gene driven by CaMV 35S promoter for bialaphos selection and a *GUS* gene with an intron under control of the maize *ubi1* gene promoter as a reporter gene which is only expressed in the plant cells (Figure 4). We also obtained a plasmid pCAMBIA 1301 (Figure 4) from CAMBIA (Canberra, Australia) which provides kanamycin and hygromycin selection and has an intron-GUS gene driven by the 35S promoter. The two plasmids were introduced to various *Agrobacterium* strains by freeze-thaw method, and a total eight strains were obtained. They are 219 series (E219, ET219, L219 and LT219) and 1301 series (E1301, ET1301, L1301 and LT1301). All the strains were evaluated with transient GUS expression assays after infection of bermudagrass tissues. Most of the strains showed GUS activity indicating their capabilities to infect bermudagrass. It was found that the 219 series had stronger transient GUS expression than the 1301 series probably attributed to the *ubi1* promoter. Within the 219 series, it was found that ET219 and L219 often had more GUS expression spots and were chosen for further experiments.

B. Evaluation of susceptibility to *Agrobacterium* infection among bermudagrass cultivars

Hybrid bermudagrass cultivar 'Tifgreen' and 17 additional common bermudagrass cultivars in a NTEP field trial were tested for their susceptibility to *Agrobacterium* infection (strain ET219). Percentage of calluses that showed GUS activity was used to evaluate the cultivars and the results are given in Table 4. It was observed that substantial differences exist among the cultivars in their susceptibility to the infection. Among the best which had more than 50% calluses showing GUS activity were: J-1224, Mirage, Pyramid, and Yuma (Figure 5).

C. Evaluation of susceptibility of age of the cultured young inflorescence to *Agrobacterium* infection

During the experiments, we have found differences of culture age in susceptibility to agro-infection. It was found that the fresh collected young inflorescence tissue was susceptible to the infection (Figure 6). The susceptibility was reduced in the next couple of

days. From 4 -14 days in culture when callus started to form, the susceptibility was getting higher and went down again after two weeks in culture. The observation indicated the optimal time periods for *Agrobacterium* transformation.

D. Efforts to select stable transformants

In the past year, we have performed 28 agro-transformation experiments. Nearly 7,000 young inflorescences were sliced into small fragments and were cultured and inoculated with *Agrobacterium* in 465 culture plates. They are currently under bialaphos selection. In addition, we also performed 4 agro-transformation experiments using suspension cells as target tissue. So far the most promising signs came from the suspension cell transformation experiments. We have seen clusters of cells that showed strong GUS activity about a month after agro-infection and selection (Figure 7). We also saw something quite unique: it seems that the transformed cell and its daughter cells only divided in one direction and yielded a narrow (one cell in width) and long thread of cells with GUS activity (Figure 8). These cultures are still under selection.

III. Publications generated from the Project

Chaudhury, A. & R. Qu (2000) Somatic embryogenesis and plant regeneration of turf-type bermudagrass: effect of 6-benzyladenine in callus induction medium. *Plant Cell, Tissue and Organ Culture*, 60: 113-120.

Qu, R. & A. Chaudhury (2001) Improved Young Inflorescence Culture and Regeneration of 'Tifway' Bermudagrass (*Cynodon transvaalensis* x *C. dactylon*). (submitted)

Li, L. and R. Qu: Somatic embryogenesis in bermudagrass (in preparation)

IV. Proposed Research Schedule for Next Year

Bermudagrass is a recalcitrant species for tissue culture as well as for genetic transformation. In this project, we have improved bermudagrass tissue culture results, particularly the regeneration ability, profoundly by supplementing, and adjusting the levels of, various phytohormones in the culture medium. So far, young inflorescence tissue and the derived callus were the main tissues used for transformation efforts. In 1999, intensive transformation efforts were spent using these tissues and the biolistic approach without much positive results. In 2000, we put our emphasis on *agrobacterium* approach and expand the tissues to suspension culture cells. We also tested a wide range of bermudagrass cultivars and found some more susceptible cultivars to agro-infection. For unknown reason, it seems suspension cells may have a better chance to be stably transformed. Thus, our research focus of next year will be to obtain stable transformants by the following approaches:

- (1) Using selective calluses induced from young inflorescences to establish more suspension cell lines;
- (2) Try to obtain and to improve regeneration of the suspension cells;
- (3) Using both agro-infection and the biolistic methods to perform transformation experiments with the suspension cells;
- (4) Continue transformation efforts using young inflorescences, with emphasis on more susceptible cultivars.

Table 1 Effect of ABA on embryogenesis of 'Tifgreen' (results of 2000)

ABA (mg/L)	No. of young inflo- rescence cultured	No. of somatic embryo clusters formed
0	15	20
0.5	15	28
2	15	38
5	15	41
10	15	31

Note: The callus induction medium was MS medium with 1 mg/L 2,4-D and 0.01 mg/L BAP. The young inflorescences used were about 1 cm in length.

Table 2 Effect of GA supplement on somatic embryo germination of 'Tifgreen'

GA (mg/L)	No. of somatic embryo clusters cultured	No. of embryo clusters develop to plants
0	18	4
0.2	19	18
0.5	18	15

Note: The basal regeneration medium was MS medium with 1 mg/L BAP. The somatic embryos were about 1 month old.

Table 3 Effect of BAP and ABA on somatic embryogenesis of 'Savannah'

Treatment (mg/L)	No. of young inflo- rescence cultured	No. of somatic embryo clusters formed
BAP 0.01	30	0
BAP 0.01 + ABA 5	30	6
BAP 0.2 + ABA 5	30	40

Note: The callus induction medium was MS medium with 1 mg/L 2,4-D. The cultured young inflorescences were 0.75-2 cm in length.

Table 4. Response of bromegrass cultivars to *Agrobacterium tumefaciens* (strain 'ET219') infection

Callus with GUS activity (%)	Cultivars
50-80%	J-1224, Mirage, Pyramid, Yuma
20-50%	Blackjack, Blue-Muda, Savannah, Shangri La, Sundance, Sundevil II, SW1-7, Tifgreen
<20%	Cheyenne, Jackpot, OKS 95-1, Princess, Sunstar, Zebra

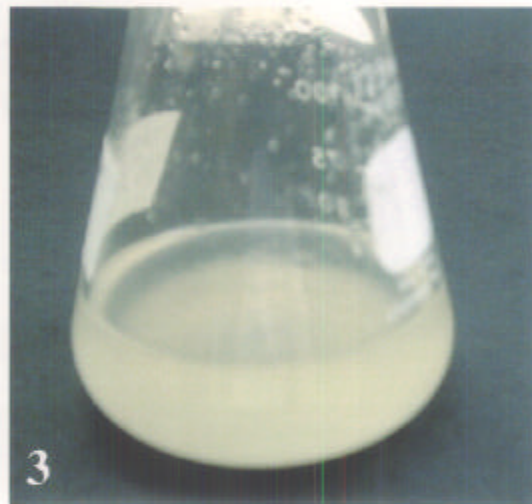
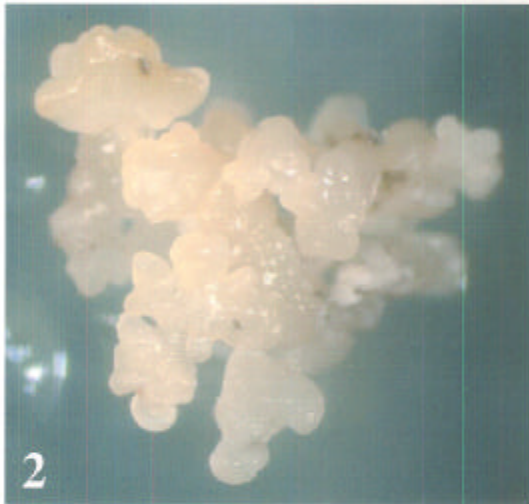
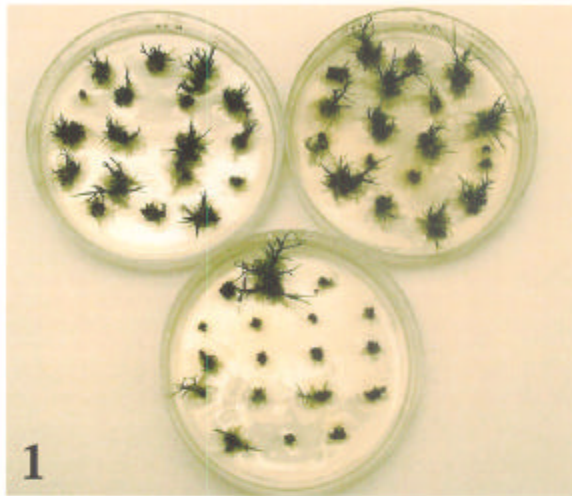


Fig. 1 Effect of GA on germination of bermudagrass somatic embryos. Upper left: 0.2 mg/L; upper right, 0.5 mg/L; bottom, 0 mg/L.

Fig. 2 Secondary somatic embryogenesis in bermudagrass tissue culture.

Fig. 3 An established bermudagrass suspension cell culture.

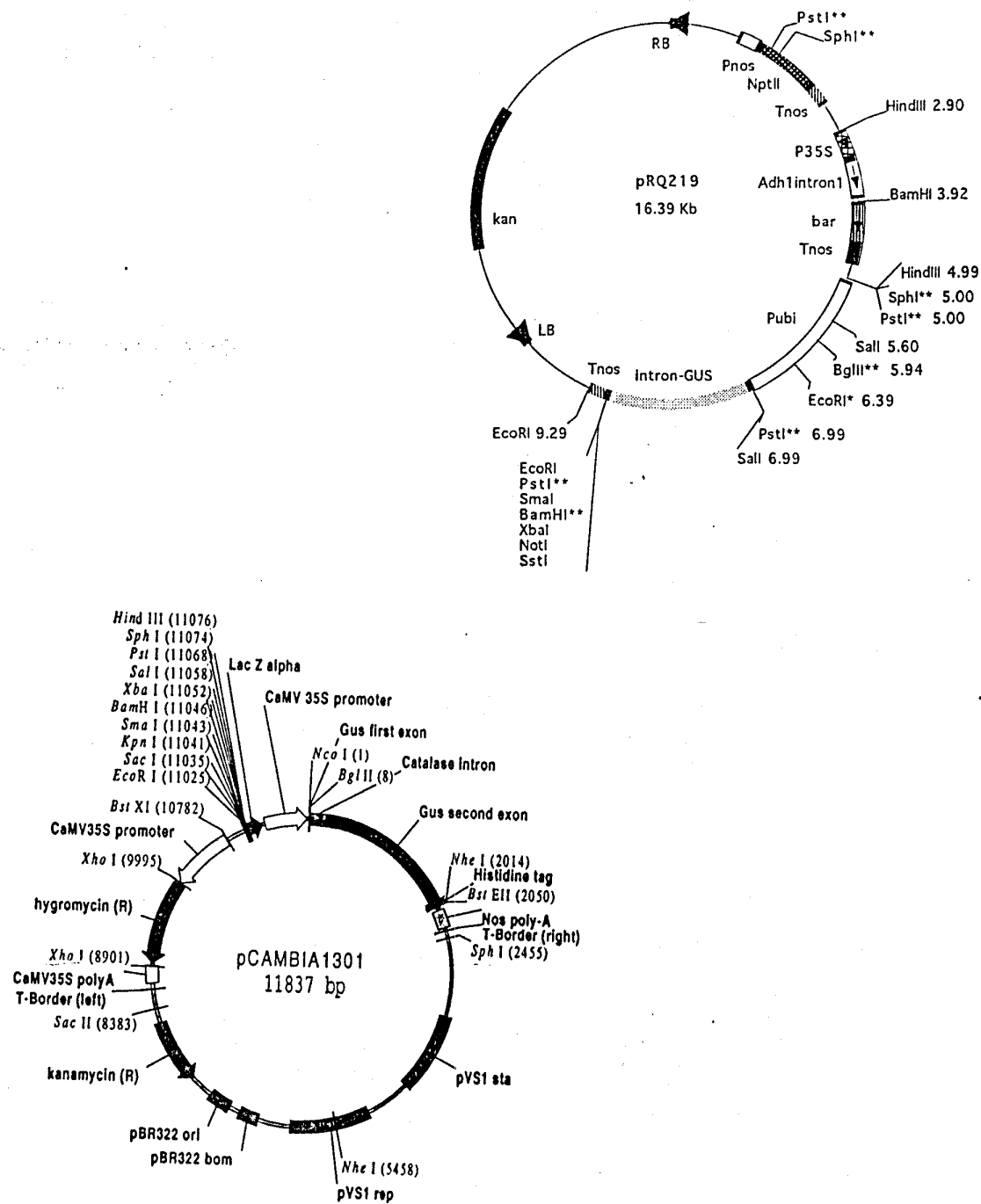


Fig. 4 Plasmids pRQ219 and pCAMBIA1301

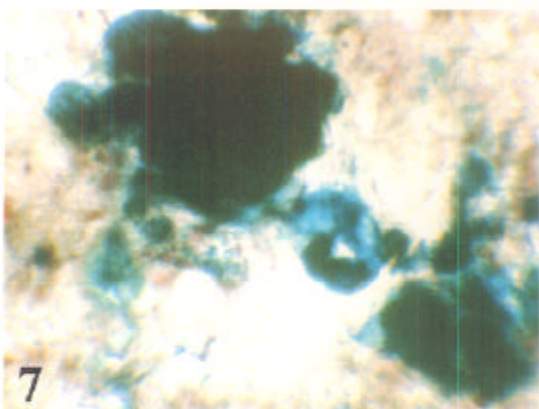
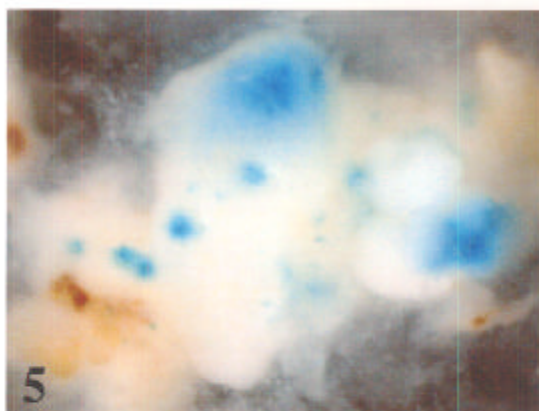


Fig. 5 Transient GUS expression after agro-infection of callus.

Fig. 6 Transient GUS expression after agro-infection of young inflorescence.

Fig. 7 Cell clusters showing GUS expression a month after agro-infection.

Fig. 8 A line of cells showing GUS expression a month after agro-infection.