AN EXECUTIVE SUMMARY

DEVELOPMENT OF GENETICALLY ENGINEERED CREEPING BENTGRASS RESISTANT TO FUNGAL DISEASES

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(Virginia Polytechnic Institute and State University)

This project is designed to improve disease resistance of creeping bentgrass using genetic engineering. The objectives are 1) to develop efficient gene transfer systems in creeping bentgrass and 2) to develop genetically engineered creeping bentgrass with high expression of chitinase genes. Chitinase is one of the defense-response proteins induced in plants upon fungal infection. It is a lytic enzyme that catalyzes the hydrolysis of chitin, a cell wall component of many fungal pathogens. It was shown that constitutively high expression of this protein in genetically engineered tobacco resulted in enhanced resistance to fungal diseases.

For the first year of this project we focused our research efforts on developing genetic transformation systems in creeping bentgrass and isolating chitinase genes from Kentucky bluegrass.

To develop gene delivery systems in creeping bentgrass, we have tried two methods, particle bombardment using a gene gun and direct gene transfer into protoplasts (plant cells without cell walls) by electric discharges. Parameters affecting efficiencies of gene delivery for both methods have been optimized and transformed cells are now being selected. We have also identified strong regulatory sequences required for a high level of foreign gene expression in creeping bentgrass. Based on these results, transformation vectors that are more suitable for creeping bentgrass are being constructed.

A partial fragment of chitinase genes has been isolated from kentucky bluegrass and we are currently working on isolation of complete chitinase genes.

ANNUAL PROGRESS REPORT

(First Year)

DEVELOPMENT OF GENETICALLY ENGINEERED CREEPING BENTGRASS RESISTANT TO FUNGAL DISEASES

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FIRST YEAR PROGRESS REPORT

DEVELOPMENT OF GENETICALLY ENGINEERED CREEPING BENTGRASS RESISTANT TO FUNGAL DISEASES

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The project is designed to develop genetically engineered creeping bentgrass resistant to diseases (esp., brown patch) by introduction of a modified chitinase gene into the species. Initial funding for this project was delayed for two months due to the disagreement on a liability clause between Virginia Tech and US Golf Association. For the first year of this project we have focused our research efforts on developing genetic transformation systems for creeping bentgrass and isolating chitinase genes from Kentucky bluegrass. Research progress and results achieved during the first year are described below.

A. Development of embryogenic callus culture

Establishment of embryogenic cell culture is a prerequisite for the development of transgenic creeping bentgrass. We could obtain embryogenic calli from `Penncross' seeds using several different culture media (e.g., MS medium with 2,4-D, MS medium with Dicamba and Benzyl aminopurine, and B5-1 medium). After transferring these embryogenic calli onto the plant regeneration medium, we could regenerated whole plants from these callus cells.

B. Screening efficient selective agents

Efficient selection of transformed cells is the most critical step in transformation experiments. We have tested two antibiotics (kanamycin and hygromycin) and one herbicide (phosphinothricin: PPT) to determine appropriate concentrations of selective agents which suppress the growth of untransformed cells. Kanamycin was not effective to select transformed creeping bentgrass cells because untransformed cells could continuously grow after culture with up to 800 mg/l of kanamycin (Fig. 1). Hygromycin is more effective in suppressing the cell growth of creeping bentgrass than kanamycin . Hygromycin totally suppressed the cell growth after 3 weeks of culture at a concentration of 150 mg/l (Fig.2). PPT was the most effective selective agent for creeping bentgrass. The cell growth nearly stopped after 2 weeks of culture with 10 mg/l of PPT (Fig. 3).

C. Development of gene transfer systems in creeping bentgrass

1) Particle bombardment

To establish a genetic transformation system in creeping bentgrass, we initially used a gene gun (PDS-1000) which delivers into the cells DNA-coated particles accelerated by the explosion of gun powders. To quickly monitored the efficiency of this gene delivery device, we used the reporter GUS gene encoding beta-glucuronidase. The bombarded cells showing transient GUS expression can be easily detected by the blue color staining with the addition of the staining substrate, X-gluc. Although an

average of 230 blue spots per bombardment using PDS-1000 were obtained in tall fescue cells, only less than 10 blue spots were obtained in creeping bentgrass. This frequency was too low to obtain stable transformants for creeping bentgrass.

To improve transformation frequency, we have recently obtained an upgraded gene gun (PDS-1000/He) which utilize pressured helium gas for particle delivery. We optimized bombardment conditions for the new device using tall fescue cells. Various helium pressures (900, 1100, 1300 and 1,550 psi) did not influence the efficiency of gene delivery. However, higher pressures often caused more damaged area. In our experiments, 1100 psi was routinely used. Particle material and size were important factors for efficient gene delivery (Fig. 4). Gold particles were better than tungsten particles. One micron size of gold particles was more efficient for gene delivery than 1.6 micron size of gold particles because bigger particles often aggregated easily. The addition of up to 4% mannitol to the cells before bombardment increased the number of stained cells by two and half times compared to control, probably because more damaged cells could recover in the presence of mannitol (Fig. 5). With these optimized conditions and further improvement of staining procedures, we could now obtain maximal 10,000 blue spots from tall fescue cells per each bombardment.

For creeping bentgrass, now we could also obtain more than 2,000 blue spots from fine suspension cells and about 1,000 blue spots from compact embryogenic cells. These figures were dramatically improved over those obtained by the old device.

Selection of stably transformed creeping bentgrass cells are in progress using the optimized conditions described above. Transformed cells are being selected on the medium containing 150 or 200 mg/l hygromycin 10 days after bombardment. Now, we have some putative transformants that are continuously growing on the selection medium.

2) Direct gene transfer into protoplasts by electroporation

We have also tried another transformation technique, direct gene transfer into protoplasts (plant cells without cell walls) using electroporation. In this system foreign genes are introduced into protoplasts through reversible pores formed on the plasma membranes by electric discharges. Electroporation has been successfully used to develop transgenic tall fescue (Ha et al. 1992). In addition to bombardment, electroporation can be an alternative transformation technique for creeping bentgrass since whole plants can now be regenerated from protoplasts (Terakawa et al., 1992).

The efficiency of foreign gene transfer into protoplasts during electroporation is dependent on several factors including field strength (voltage/cm), protoplast density, and amount of DNA. We studied the effects of these factors on transient gene expression to rapidly optimize transformation conditions in creeping bentgrass. In order to determine the effect of field strength on protoplast viability and gene expression, protoplast viability and transient GUS expression were measured at field strengths of 500, 650, 800, 950 and 1100 V/cm (Fig. 6).

Protoplast viability was inversely influenced with the increase of field strength. Increases in field strength through 500, 650, 800 and 950 V/cm resulted in progressively higher transient GUS expression with maximal activity at 900 V/cm. Further increase in field strength to 1100 V/cm led to a sharp reduction in the GUS expression because most protoplasts could not survive under this condition. The effect of DNA concentration on transient gene expression was studied after protoplasts were electroporated in the presence of different concentrations (2, 8, 20, 80, 140 and 200 ug/ml) of plasmid DNA. Increases in the amount of plasmid DNA to 2, 20 and 80 ug/ml led to a sharp increase in GUS activity and further increase to 140 or 200 ug/ml did not significantly increase in GUS expression. The effect of protoplast density on transient gene expression was tested using different protoplast densities of 0.5, 1, 2, 4 and 6 x 10^6/ml. GUS activity increases with increases in the density of protoplasts up to a density of 4 x 10^6/ml. Using these optimized electroporation conditions, we are currently selecting stably transformed cells.

D. The selection of strong 5' regulatory sequences for a high level of transgene expression

In plant genetic engineering, the successful transfer of new traits largely depends upon the expression level of newly introduced genes (transgenes). The level of transgene expression is generally proportional to the strength of 5' regulatory sequences including promoters that turn on the transcription of a gene. In many monocot species, some introns (untranslated DNA

sequences of a gene) also significantly enhance the gene expression by increasing splicing efficiency and mature messenger RNA. In order to identify strong promoters and introns in creeping bentgrass, we have tested several promoter/intron constructs in creeping bentgrass protoplasts using transient assays. We monitored the strengths of these regulatory sequences by measuring the reporter GUS enzyme activities. As shown in Fig. 7, cauliflower mosaic virus (CaMV) 35S promoter alone (pPBI221) or with maize alcohol dehydrogenase 1 (*adh1*) intron 1 (pDPG208) and the maize *adh1* promoter with its own intron 1 (pBARGUS) were very weakly active. GUS expression was dramatically increased when the maize *adh1* intron 6 was added to the CaMV 35S promoter (pZO1052). The highest GUS expression was obtained from the promoter of rice actin 1 (*act1*) gene and its first intron (pAct1-D). Therefore, rice act1 promoter and its first intron appears to be the 5' regulatory sequence of choice for a high level of transgene expression in creeping bentgrass.

The results obtained from this study also warrant some precautions when choosing plasmid vectors for transformation experiments in creeping bentgrass. Since many currently available transformation vectors have been constructed for transformation of other plant species, they contain selectable marker genes (antibiotic or herbicide resistance genes) under the control of the CaMV 35S promoter or with maize *adh1* intron 1 that is weakly active in creeping bentgrass cells. Low expression levels of selectable marker genes generally result in low transformation frequency. Therefore, we are currently

constructing a transformation vector containing a selectable marker gene (*bar* gene providing resistance to the herbicide PPT or bialaphos) under 5' regulatory sequences that are strongly active in creeping bentgrass.

F. The isolation of chitinase genes from Kentucky bluegrass

To develop transgenic creeping bentgrass resistant to brown patch disease, we originally planned to use a chitinase gene isolated from bean by Dr. Richard Broglie at Du pont. The negotiation with Du Pont to obtain the gene was not successful because Virginia Tech could not indemnify the company for the work involved in the use of the gene according to the guideline from the Virginia's Attorney General. Therefore, we decided to isolate chitinase genes from Kentucky bluegrass in my lab. Kentucky bluegrass was chosen because brown patch is not a serious problem in this turfgrass species. Although several chitinase genes have been isolated and characterized from a number of dicotyledonous species, there is little information available on structure and expression of chitinase genes in monocotyledonous species, with the exception of rice. By comparing amino acid sequences of chitinase genes from tobacco, potato, bean and rice, we identified several conserved regions within the genes. These regions are believed to be maintained during the evolution probably due to their functional and structural importance to the enzyme. Our hypothesis was that chitinase genes from Kentucky bluegrass also have these conserved regions. Using two nucleotide sequences (5'-AGACGTCCCACGAGACCAC-

3' and 5'-AGCCCGCCGTTGATGATGTT-3') covering these regions as primers for DNA amplification, we successfully amplified and cloned about 420 bp fragment of chitinase genes from Kentucky bluegrass genomic DNA after running polymerase chain reaction (PCR). Using this partial fragment of Kentucky bluegrass chitinase gene as a probe, we could detect several DNA bands from Kentucky bluegrass genomic DNA which hybridized to this probe. This indicates that there are several copies of chitinase genes in Kentucky bluegrass. Constructing genomic DNA library of Kentucky bluegrass is in progress for the isolation of a full length of chitinase genes.

G. Summary of research progress

We have determined optimal concentration of selective agents for selecting transformed cells. We have also identified strong regulatory sequences for high levels of transgene expression in creeping bentgrass. Based on these results, transformation vectors that are more suitable for creeping bentgrass are being constructed. For gene transfer, we have employed direct gene transfer into protoplasts by electroporation as well as particle bombardment described in the proposal. Using transient gene expression assays, we have determined optimal conditions for efficient gene delivery by bombardment and electroporation. Optimization of efficient procedures for the selection of transformed cells is in progress. Now, we have some putative transformants that are continuously growing on the selection medium. We have also cloned partial fragmnents of Kentucky

bluegrass chitinase genes and are making progress on the isolation of complete chitinase genes.

H. The proposed research plan for the coming year

For the coming year, we will establish an efficient genetic transformation system by using either particle bombardment or electroporation. Full sizes of chitinase genes will be isolated fron genomic library of Kentucky bluegrass and DNA sequencs will be determined. The chitinase genes will be subcloned into gene expression vectors which contain strong 5' regulatory sequences (e.g., rice actin promoter and its first intron). If everything goes well as planned, we will be able to introduce these constructs into embryogenic cells to develop transgenic creeping bentgrass expressing constitutively high levels of chitinase.

References

Ha, S. B., Wu, F, S. and T. K. Thorne. 1992. Transgenic turf-type tall fescue (*Festuca arudinacea* Schreb.) plants regenerated from protoplasts. Plant Cell Rep. 11:601-604.

Terakawa, T., Sato, T. and M. Koike. 1992. Plant regeneration from protoplasts isolated from embryogenic suspension cultures of creeping bentgrass (*Agrostis palustris* Huds.). Plant Cell Rep. 11: 457-461.

BUDGET SUMMARY (4/93-10/93)

	Budget	Spending	Balance
Graduate assistantship	$ 13,203	$ 6,073	$ 7,129
Material & supplies	$ 7,849	$ 7,790	$ 59
Scientific meetings	$ 500	0	$ 500
Indirect cost	$ 3,448	$ 1,396	$ 2,053
Total	$ 25,000	$15,259	$ 9,741

Fig. 1. KN TEST ON BENTGRASS (I)
(Kanamycin)

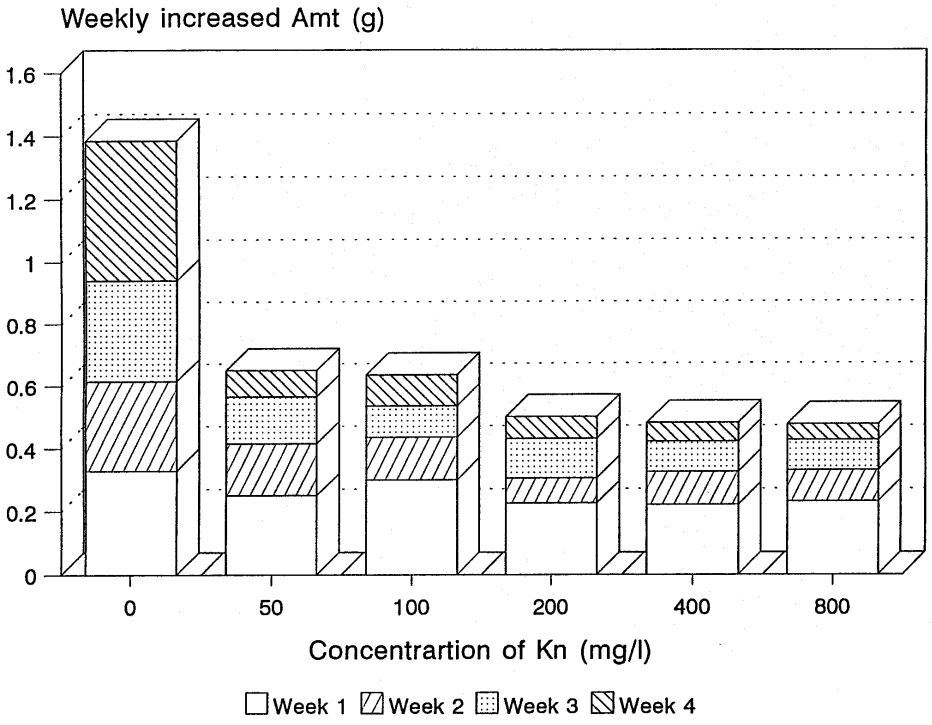

Fig. 2. HYG TEST ON BENTGRASS (I)

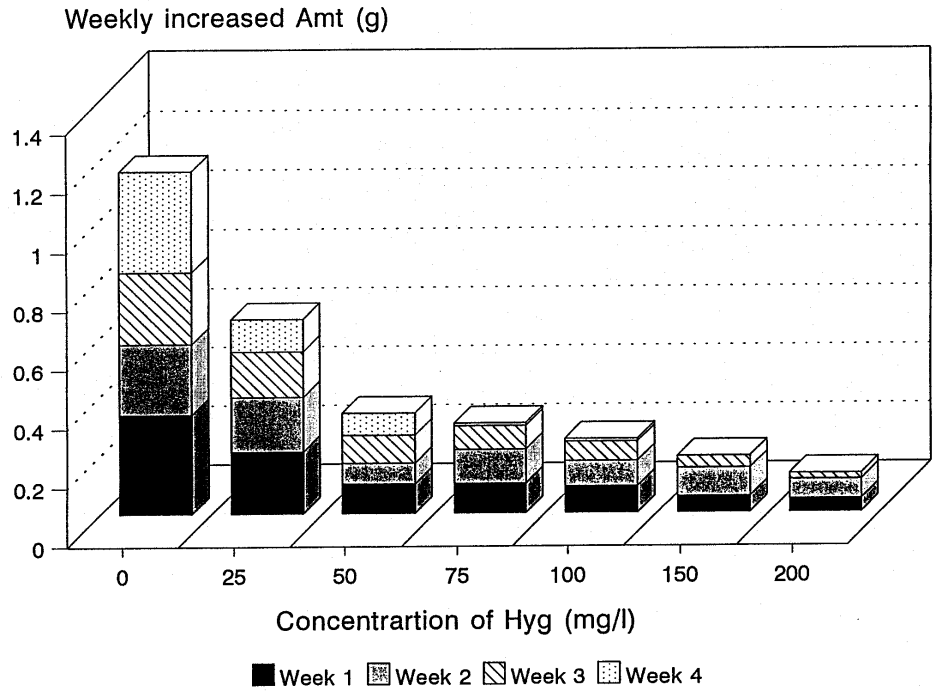

Fig. 3. PPT TEST ON BENTGRASS (I)

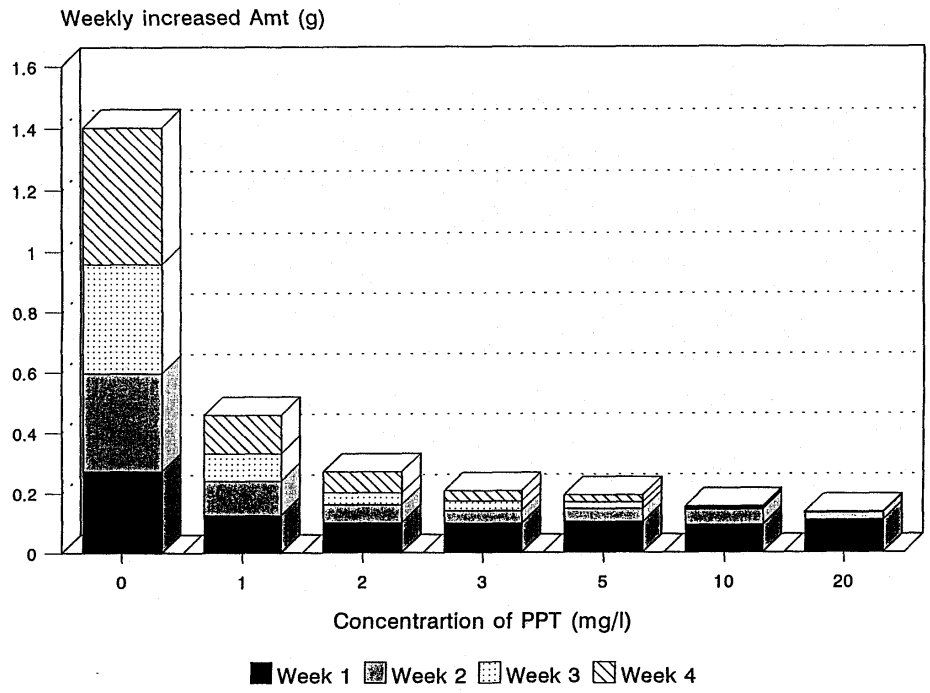

Fig. 4. The effect of particle type and size on transient GUS expression

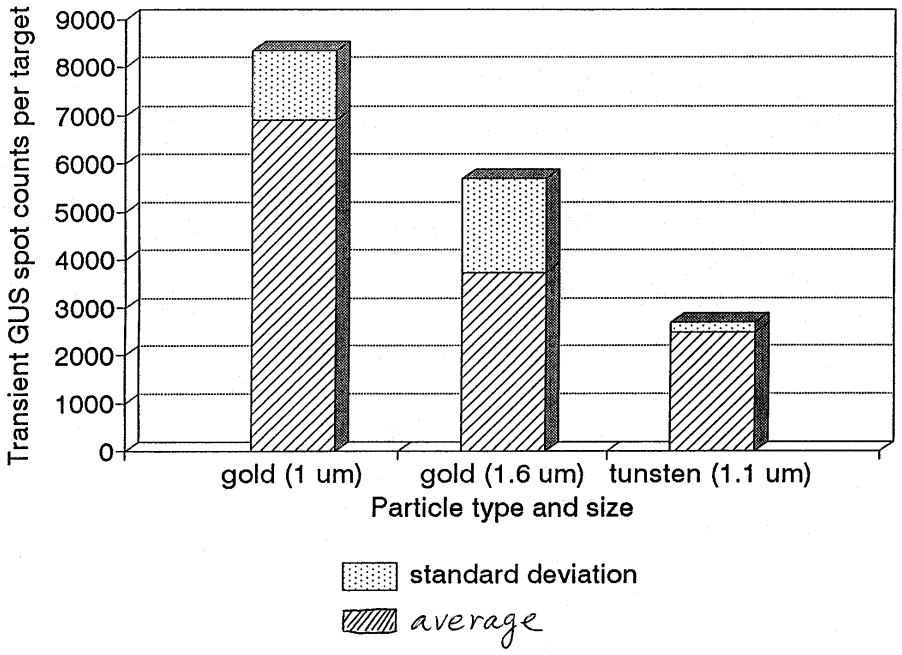

Fig. 5. The effect of mannitol on transient GUS expression

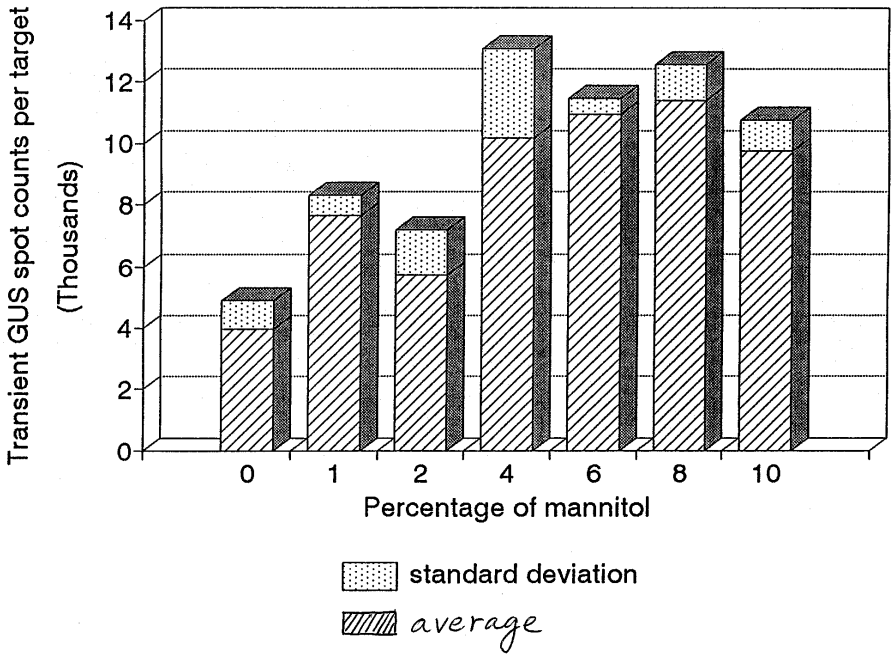

Fig. 6. Effect of Electrical Field Strength on Transient GUS Expression (400uF with different voltages)

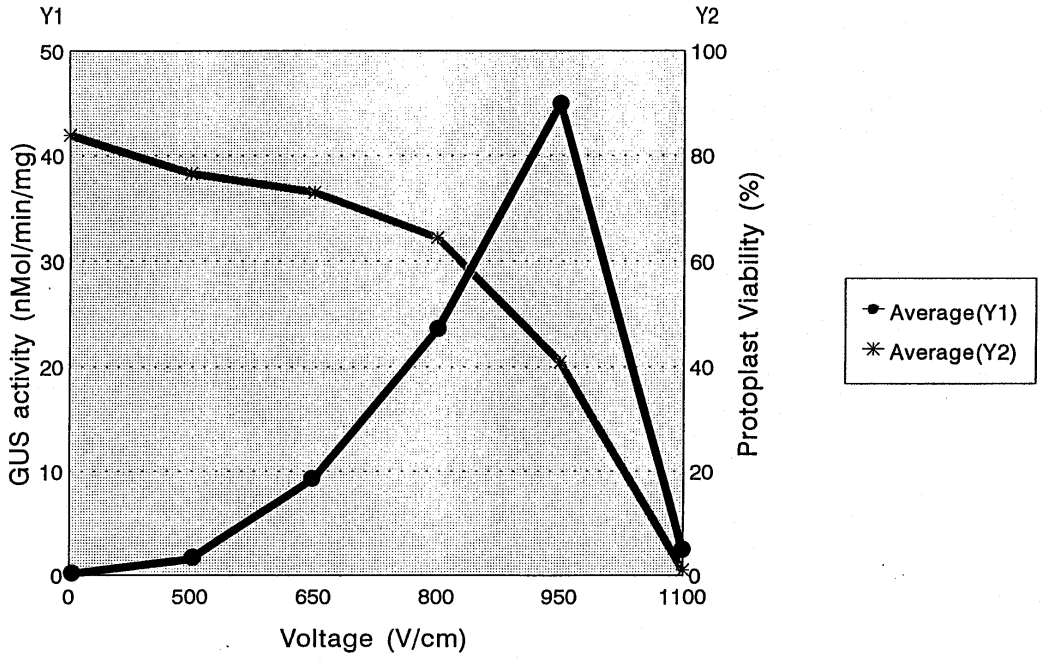

Fig. 7. Effect of Different Promotor Constructs on Transient GUS Expression