Development of Genetic Transformation Systems
in Creeping Bentgrass (*Agrostis palustris* Huds.)

By

Lian Xiao

Dissertation submitted to the Faculty of the Virginia Polytechnic Institute and
State University in partial fulfillment of the requirements for the degree of

**DOCTOR OF PHILOSOPHY**

in

**CROP AND SOIL ENVIRONMENTAL SCIENCES (BIOTECHNOLOGY)**

Approved:

Dr. Sam-Bong Ha, Chairman

Dr. Richard E. Veilleux

Dr. Carl A. Griffey

Dr. M.A. Saghai Maroof

Dr. Asim Esen

December, 1994

Blacksburg, Virginia
Development of Genetic Transformation Systems
in Creeping Bentgrass (*Agrostis palustris* Huds.)

By

Lian Xiao

Committee Chairman: Sam-Bong Ha

Crop and Soil Environmental Sciences

(ABSTRACT)

As a first step toward improving creeping bentgrass (*Agrostis palustris* Huds.) via genetic engineering, this study was conducted to develop genetic transformation systems in creeping bentgrass.

Establishment of embryogenic cell cultures is a prerequisite for crop improvement via genetic engineering. A protocol for initiating and maintaining embryogenic callus and suspension cultures in creeping bentgrass was developed by substantially modifying and combining a few existing protocols. A high frequency of plant regeneration was obtained following this protocol.

Several factors affecting electroporation efficiency were studied using transient expression assay of the reporter *uidA* gene encoding β-glucuronidase (GUS). Increases in plasmid DNA resulted in increases in GUS activity. Maximal GUS activity was observed at field strength of 950 V/cm, protoplast density of 2 x 10⁶/ml, and KCl concentration of 125 mM in the electroporation buffer. Information obtained from this study facilitated optimization of electroporation conditions.

To identify a 5’ regulatory sequence conferring a high level of transgene
expression in creeping bentgrass, the effect of six different 5' regulatory sequences on transient gene expression was studied in electroporated creeping bentgrass protoplasts. The cauliflower mosaic virus (CaMV) 35S promoter was least active; whereas the rice actin 1 gene 5' sequence was most active among the six sequences tested. Ranked in order of activity (high to low), the other four 5' sequences were: 1) the CaMV 35S promoter plus the maize alcohol dehydrogenase 1 gene (Adh1) intron 6; 2) the 5' sequence of the maize ubiquitin gene (Ubi-1), 3) the maize Adh1 promoter and its intron 1, and 4) the 35S promoter plus the Adh1 intron 1.

Stable transformation of creeping bentgrass was conducted via particle bombardment and electroporation using a plasmid, pZO1052, containing the reporter β-glucuronidase (uidA) gene and the selectable marker hygromycin phosphotransferase (hph) gene under the control of CaMV 35S promoter plus the maize Adh1 intron 6. Putative transformants were selected by culturing cells on medium containing hygromycin. Transgenic plants and calli were obtained following particle bombardment. The frequency of putative transformants was 4.6 hygromycin-resistant colonies per bombardment. Integration of the transgenes was confirmed by Southern blot hybridization. A high frequency of escapes, however, occurred in the transformant selection following electroporation, which resulted in inefficient transformant recovery.

In this study, efficient genetic transformation systems using particle bombardment were established. Use of these systems will facilitate the improvement of creeping bentgrass.
To my husband Jielu Zhao and my son Yining Zhao
Acknowledgement

The author gratefully acknowledges the guidance and assistance given by the members of the doctoral committee -- Dr. Asim Esen, Dr. M. A. Saghai Maroof, Dr. Richard E. Veilleux, and Dr. Carl A. Griffey -- for their guidance, suggestions, support and encouragement toward completion of the study.

The author would like to express her deep thanks to the chairman of the doctoral committee, Dr. Sam B. Ha for his guidance, support and constructive criticism in all these years.

Special appreciation is given to Dr. Richard Veilleux and Dr. Carl Griffey for their time and efforts in improving my English writing throughout the dissertation process.

The author offers sincere thanks to her friends and colleagues -- Mr. Min Du, Dr. Pengyin Chen, Mr. Varma Penmetsa, Mr. Will Simmons, Ms. Tracy K. Thorne, Ms. Ping Zhao, Dr. Y. S. Chung, Dr. Modan Das, Dr. Xiaorong Zhang, Ms. Wendy Baur, -- for their support and assistance.

The author acknowledges the support from Dr. David C. Martens, Dr. Robert Q. Cannell, Dr. John R. Hall, and the Department of Crop and Soil Environmental Sciences.

Finally, the author would like to express her greatest appreciation and gratitude to her parents and parents-in-law, her sisters and brothers, her son and husband for their understanding, sacrifice, encouragement, and support.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>ii</td>
</tr>
<tr>
<td>DEDICATION</td>
<td>iv</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENT</td>
<td>v</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>ix</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>x</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>References</td>
<td>6</td>
</tr>
<tr>
<td><strong>CHAPTERS</strong></td>
<td></td>
</tr>
<tr>
<td>I.  PLANT REGENERATION OF CREEPING BENTGRASS (\textit{Agrostis palustris} Huds.) FROM SEED-DERIVED CALLI AND SUSPENSION-DERIVED PROTOPLASTS.</td>
<td>9</td>
</tr>
<tr>
<td>Abstract</td>
<td>10</td>
</tr>
<tr>
<td>Introduction</td>
<td>11</td>
</tr>
<tr>
<td>Materials and Methods.</td>
<td>13</td>
</tr>
<tr>
<td>Results and Discussion.</td>
<td>17</td>
</tr>
<tr>
<td>References</td>
<td>28</td>
</tr>
</tbody>
</table>

vi
II. EFFECTS OF DIFFERENT ELECTROPORATION CONDITIONS ON TRANSIENT GENE EXPRESSION IN CREEPING BENTGRASS

(Agrostis palustris Huds.) PROTOPLASTS. .............................. 30

Abstract ................................................................. 31

Introduction. ............................................................. 32

Materials and Methods. .................................................. 34

Results. ......................................................................... 37

Discussion. ...................................................................... 39

References. ....................................................................... 47

III. TRANSIENT GENE EXPRESSION DRIVEN BY DIFFERENT 5’ REGULATORY SEQUENCES IN ELECTROPORATED CREEPING BENTGRASS (Agrostis palustris Huds.) PROTOPLASTS. ................. 50

Abstract. ................................................................. 51

Introduction. ............................................................. 52

Materials and Methods. .................................................. 54

Results and Discussion. .................................................. 55

References. ....................................................................... 59
IV. STABLE TRANSFORMATION OF CREEPING BENTGRASS (*Agrostis palustris* Huds.) VIA PARTICLE BOMBARDMENT AND ELECTROPORATION. .......................... 62

Abstract. ........................................ 63

Introduction. .................................... 64

Materials and Methods. ........................ 66

Results and Discussion. ........................ 72

References. ....................................... 86

SUMMARY ........................................ 90

References ....................................... 94

VITA ............................................. 95
LIST OF TABLES

CHAPTER I.

Table 1. Effects of different media and age of calli on suspension culture initiation of creeping bentgrass ........................................... 22

Table 2. Effects of two concentrations of 2,4-D and two carbohydrate sources in liquid media on suspension cultures of creeping bentgrass ................. 23

Table 3. Effects of two concentrations of 2,4-D and conditioned medium on protoplast-derived cell formation of creeping bentgrass. ....................... 24

Table 4. Effects of two different plant regeneration media with two different carbohydrate sources on protoplast-derived cell regeneration of creeping bentgrass. .................................................. 25

CHAPTER III.

Table 1. Effects of different 5' regulatory sequences on transient gene expression in creeping bentgrass. .................................................... 58
LIST OF FIGURES

CHAPTER I.

Figure 1. Plant regeneration of creeping bentgrass from six-month old seed-derived calli. .......................................................... 26

Figure 2. Plantlet regeneration of creeping bentgrass from protoplast-derived calli (2.5 months after protoplast isolation) ...................... 27

CHAPTER II.

Figure 1. Transient GUS activity and protoplast viability as functions of field strength .......................................................... 43

Figure 2. Transient GUS activity as a function of plasmid DNA concentration .......................................................... 44

Figure 3. Transient GUS activity as a function of protoplast density ........... 45

Figure 4. Transient GUS activity and protoplast viability as functions of KCl concentration .......................................................... 46

CHAPTER IV.

Figure 1. A diagram of plasmid pZO1052 ................................. 79

Figure 2. Selection of transformed creeping bentgrass cells after particle bombardment .......................................................... 80

Figure 3. A transgenic creeping bentgrass plant growing in soil. ............. 81

Figure 4. PCR analysis of putative transgenic creeping bentgrass plants recovered after transformation via particle bombardment .............................. 82

Figure 5. Southern blot hybridization of creeping bentgrass transformants obtained via particle bombardment with the digoxigenin-labeled hph probe........ 83
Figure 6. Southern blot hybridization of creeping bentgrass transformants obtained via particle bombardment with the digoxigenin-labeled \textit{uidA} probe. \ldots 84

Figure 7. PCR confirmation of creeping bentgrass transformants obtained via particle bombardment \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots 85
Introduction

Creeping bentgrass (Agrostis palustris Huds.), an important turfgrass species widely used on golf, bowling, and tennis greens, is highly susceptible to several major turfgrass diseases such as smut (Ustilago trebouxi H.), brown patch (Rhizoctonia solani) and Helminthosporium leaf spot (Helminthosporium sorokinianum) (James, 1982; Couch, 1973). Disease control is a major problem in bentgrass management. In addition, pests, weeds and environmental stresses also threaten bentgrass. Development of cultivars with disease and pest resistance and other desirable traits, therefore, is greatly needed.

Genetic engineering is an important new tool for crop improvement. In principle, genetic engineering allows specific coding sequences of desirable genes to be transferred as single defined traits. Once a gene of interest has been cloned, the gene potentially can be introduced into any crop, regardless of the relationship between the donor and the recipient. Therefore, a large gene pool can be used for crop improvement via genetic engineering. In addition, useful traits can be introduced into plants independent of undesirable traits and consequently, backcrossing, a time consuming procedure, is not needed (for review, see Draper and Scott, 1991). Given these advantages, genetic engineering provides great potential for the improvement of creeping bentgrass.

Genetic engineering depends on the availability of gene transfer and plant
regeneration systems. Only when these systems are available for the crop of interest, can transgenic plants with improved traits be obtained.

At present, several gene transfer systems for higher plants have been used. The major ones include *Agrobacterium*-mediated transformation and direct gene transfer by chemically-facilitated DNA uptake, electroporation, and particle bombardment.

The *Agrobacterium*-mediated transformation system has proven to be successful for many dicot plants (Klee et al., 1987) and a few monocot plants (Hernalsteens et al., 1984; Hooykaas-Van et al., 1984). However, due to the limited host range of the bacterium, this system does not work efficiently in most graminaceous monocots, including turfgrasses and many other important cereals such as wheat, rice and corn. Many efforts, therefore, have been made to introduce foreign genes into these graminaceous monocots by direct gene transfer approaches such as polyethylene glycol (PEG)-facilitated DNA uptake (Uchimiya et al., 1986; Werr et al., 1986), electroporation (Fromm et al., 1985; Ou-Lee et al., 1986), and particle bombardment (Klein et al., 1987). Electroporation has been considered to be one of the more efficient direct gene transfer methods (Dhir et al., 1991). Successful genetic transformation by electroporation has been accomplished in rice (*Oryza sativa*) (Toriyama et al., 1988; Zhang et al., 1988; Shimammoto et al., 1989), maize (*Zea mays*) (Rhodes et al. 1988), *Brassica napus* (Guerche et al., 1987a), and tall fescue (*Festuca arundinacea* Schreb) (Ha et al., 1992). In addition to stable
transformation, electroporation has been widely used for assays of transient gene expression for many purposes, such as promoter analysis (Hauptmann et al., 1988; Werr et al., 1986) and optimization of DNA delivery conditions (Dhir et al, 1991; Okada et al., 1986). However, electroporation generally requires protoplasts as transfer targets. Plant regeneration from protoplasts of many monocots has proven to be difficult and time consuming (Wang et al., 1988). An alternative strategy for gene transfer, especially for species in which the Agrobacterium-mediated transformation system does not work efficiently and/or plant regeneration from protoplasts is difficult, is particle bombardment. Particle bombardment can deliver foreign DNA into intact cells which may be more easily regenerated. Transformation via particle bombardment has succeeded in several species such as maize (Zea mays) (Gordon-Kamm et al., 1990), wheat (Triticum aestivum L.) (Vasil et al., 1992), and soybean (Glycine max Merrill.) (Finer and McMullen, 1991).

To establish an efficient gene transfer system in creeping bentgrass, both electroporation and particle bombardment have been used in this study. Electroporation efficiency is affected by many factors such as the electrical field strength, protoplast density and the amount of input DNA. Optimal conditions for these factors are highly species-dependent (Rouan et al., 1991; Dhir et al., 1991; Guerche et al., 1987b) and, therefore, must be defined for each species prior to genetic transformation. The parameters used in particle bombardment influence the efficiency of DNA delivery. The major parameters of particle bombardment on
creeping bentgrass have been defined in our preliminary experiments by histochemically staining for transient gene expression. Some other parameters were determined based on the work in tall fescue previously conducted in our laboratory (Du and Ha, unpublished data).

Successful transformation of plants depends not only on gene transfer efficiency but also the expression level of transgenes which is generally correlated to the strength of 5’ regulatory sequences. The cauliflower mosaic virus (CaMV) 35S promoter, a promoter widely used in dicot transformation systems, has been reported to be less active in monocot species (Fromm et al., 1985; Hauptmamm et al., 1987). Search for strong 5’ regulatory sequences, therefore, has become a high priority for monocot transformation. The strengths of several plant gene promoters and 5’ introns, such as rice actin 1 gene promoter and its intron 1, or maize alcohol dehydrogenase 1 gene promoter and its intron 1 have been studied in rice (Zhang and Wu, 1988; McElroy et al., 1990), maize (Callis et al., 1987; Reggiardo et al., 1991) and tall fescue (Ha et al., 1995). These 5’ regulatory sequences showed higher activity than CaMV 35S promoter. However, no information as such is available in creeping bentgrass.

Development of embryogenic cell cultures is a prerequisite for crop improvement by genetic engineering. Transformation without plant regeneration has limited value. Callus cells have been used as gene transfer targets in particle bombardment. For electroporation, protoplasts are the commonly used targets.
Therefore, embryogenic callus cultures and suspension cultures suitable for protoplast isolation need to be established.

Embryogenic callus of creeping bentgrass has been obtained by Krans et al. (1982) and Zhong et al. (1991). However, there were some inconsistent results between their work. Development of embryogenic suspension cultures of creeping bentgrass was shown to be difficult in early studies (Blanche et al., 1986). In 1992, Terakawa et al. reported successful establishment of embryogenic suspension culture, yet we failed to obtain similar results using the same protocol.

Our long-term goal is to develop transgenic creeping bentgrass with desirable traits such as disease resistance. Establishment of genetic transformation systems for creeping bentgrass is the first step to achieve this goal and, therefore is the primary objective of this study. The following work has been conducted to accomplish this objective: (1) development of embryogenic callus and suspension cultures; (2) optimization of electroporation conditions via transient gene assay; (3) determining the strengths of different 5’ regulatory sequences via transient gene assay following electroporation; and (4) stable transformation via particle bombardment and electroporation.
References


Chapter I

Plant regeneration of creeping bentgrass

(Agrostis palustris Huds.) from seed-derived calli

and suspension-derived protoplasts
Abstract

The purpose of this study was to develop embryogenic cell cultures for creeping bentgrass (Agrostis palustris Huds.). By substantially modifying and combining a few published protocols, a new protocol leading to plant regeneration of creeping bentgrass from seed-derived calli or protoplast-derived calli was developed. Embryogenic callus was induced and maintained on Murashige and Skoog (MS) medium supplemented with 1 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D). Cell suspensions, suitable for protoplast isolation, were initiated using this callus and cultured in MS liquid medium containing 1 mg/l 2,4-D and 3% maltose. Plant regeneration from seed-derived calli was induced on MS medium with 0.01 mg/l 2,4-D, 0.1 mg/l kinetin and 3% maltose. Plant regeneration from protoplast-derived calli was induced on half-strength MS medium with 1.5% maltose. Maltose was shown to be superior to sucrose for the maintenance of embryogenic potential in creeping bentgrass. An important change of existing protocols was the substitution of sucrose with maltose as a carbohydrate source in both suspension culture and plant regeneration media. From this study, an efficient method was developed for establishment of embryogenic suspension cultures in creeping bentgrass. High frequency of plant regeneration from either seed-derived calli or protoplast-derived calli was obtained in this study.
Introduction

Development of embryogenic cell cultures is an important prerequisite for crop improvement by gene transfer techniques because gene transfer without plant regeneration has limited value. An attempt was made to develop a transformation system for the improvement of creeping bentgrass (*Agrostis palustris* Huds.) via either particle bombardment or electroporation. In particle delivery systems, intact cells (e.g., callus cells) can be used as gene transfer targets. For electroporation, protoplasts isolated from suspension-cultured cells are commonly used as transfer targets. Therefore, embryogenic callus cultures and suspension cultures suitable for protoplast isolation need to be developed in creeping bentgrass.

Embryogenic calli of ‘Penncross’ creeping bentgrass have been obtained previously in other laboratories. The work by Krans et al. (1982) showed that embryogenic callus could be induced and maintained on Murashige and Skoog (MS) (1962) medium supplemented with 1 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D). Zhong et al. (1991) reported that embryogenic callus was obtained on MS medium with 30 μM 3,6-dichloro-o-anisic acid (dicamba) and 2.25 μM 6-benzyladenine (BA). They also observed that calli produced on MS medium with different concentrations of 2,4-D (0.5, 1, 2 or 4 mg/l) exhibited low embryogenic potential. This is somewhat inconsistent to the results of Krans et al. (1982).

Embryogenic cell suspensions of grasses are, in general, extremely difficult to
establish and maintain in culture (Vasil and Vasil, 1992). Creeping bentgrass is no exception. It was shown that plantlet formation in creeping bentgrass was inhibited significantly due to prior culture in suspension (Blanche et al., 1986). In 1992, an advance in the development of embryogenic suspension cultures of creeping bentgrass was made by Terakawa et al. (1992). They reported that embryogenic suspension cultures of 'Pencross' creeping bentgrass were established on B5 medium (Gamborg et al., 1968) containing 2 mg/l 2,4-D and 0.2% casamino acids and that plants were regenerated from protoplasts isolated from the suspensions. However, we failed to obtain similar results using the same protocol. When calli induced on B5-1 medium (Terakawa et al., 1992) were transferred into B5-2 medium in our repeated experiments, these calli gradually turned brown and died.

In order to develop a protocol for initiating and maintaining embryogenic callus and suspension cultures of creeping bentgrass, we studied the effects of several different media on callus induction, maintenance, plant regeneration, and suspension culture initiation and maintenance. The media tested in this study were primarily based on the work by Krans et al. (1982) and Zhong et al. (1992). Optimal conditions for protoplast culture and plant regeneration from protoplasts were also determined in this study.
Materials and Methods

*Callus induction.* Mature seeds of ‘Penncross’ (Tee-2-Green Corp., OR) creeping bentgrass (*Agrostis palustris* Huds.) were used for callus induction. The seeds (caryopses) were surface-sterilized in 70% ethanol for 5 min and then in 40% commercial bleach with 0.1% ‘Tween 20’ for 20 min on a shaker at low speed. This was followed by four rinses in sterile, distilled water. About 1,000 sterilized seeds were placed in 100x15 mm Petri dishes containing 25 ml of (1) Krans medium -- MS medium containing 3% sucrose, 0.5 mg/l nicotinic acid, 0.5 mg/l pyridoxine HCl, 0.1 mg/l thiamine HCl, 2 mg/l glycine, 1 mg/l 2,4-D, and 1.0% agar, pH 5.6 (Krans et al., 1982), or (2) MS medium containing 3% sucrose, 500 mg/l casamino acids, 6.6 mg/l dicamba, 0.5 mg/l BA, and 0.7% agar, pH 5.8 (Zhong et al., 1991). The media used were autoclaved at 120° C, 1.1 kg/cm² for 20 min. The Petri dishes containing seeds were sealed with parafilm and incubated at 26°C in dark for 4 weeks (case 1) or 8 weeks (case 2) in a walk-in growth chamber for callus induction.

*Callus maintenance.* After callus induction, embryogenic calli (opaque, yellowish and compact, Lu and Vasil, 1981) were transferred to 100x15 mm Petri dishes containing 25 ml autoclaved Krans medium. Calli were then subcultured every three or four weeks and incubated in the dark at 26°C in a walk-in growth chamber.
**Plant regeneration from seed-derived callus.** For plant regeneration, about 2 g of calli from each plate was transferred, respectively, to 100x20 Petri dishes containing 25 ml of plant regeneration (PR) medium. The composition of the PR medium was identical to Krans medium except it contained 3% maltose, 0.01 mg/l 2,4-D, and 0.1 mg/l kinetin. The calli were incubated at 26° C in the light for 16 h/day in a walk-in growth chamber.

**Suspension culture initiation.** About 0.7 g fast-growing embryogenic calli from cultures initiated on MS medium with 1 mg/l 2,4-D or from cultures initiated on MS medium with 6.6 mg/l dicamba, 0.5 mg/l BA and 500 mg/l casamino acids was transferred separately to 125 ml flasks containing 10 ml of various liquid media to initiate suspension cultures. About 25 pieces of fresh callus induced from mature seeds on the two media mentioned above were also transferred into liquid media for suspension culture initiation. Four different liquid media were used for suspension cultures; these consisted MS media containing a combination of 1 or 3 mg/l 2,4-D and 3% maltose or sucrose (Table 2). The media used were autoclaved at 120° C, 1.1 kg/cm² for 20 min. Cell suspensions were maintained in the laboratory at 26° C on a rotary shaker set at 150 rpm, and subcultured weekly by transferring 6-8 ml of cell suspensions to 25 ml fresh medium in 125 ml flasks. The suspensions to be subcultured were obtained by the following steps: 1) whirling the flask for 5 times; 2) letting the flask stand still for 1 min; and 3) taking the suspensions in the middle
portion of the medium where most of the cell clusters consisted of small, round, densely cytoplasmic cells.

Protoplast isolation. Protoplasts were isolated from three- or four-day-old cultures of suspension cells as described by Terakawa et al. (1992). Suspension cells (1-1.5 g) were placed in a 100x20 mm Petri dish containing 20 ml filter-sterilized enzyme solution [2% (w/v) Cellulase Onozuka RS (Yakult Honsha Co., LTD), 0.3% Macerozyme R-10 (Yakult Honsha Co., LTD), 0.1% Pectolysase Y-23 (Kikkoman Corp.), 10 mM CaCl₂·H₂O, 0.1% MES, 3% sucrose, and 8% mannitol, pH 5.8]. Following enzyme digestion for 3-5 h, the released protoplasts were filtered through a series of sterile 105, 53 and 21 μm nylon meshes. The filtered protoplast suspension was centrifuged for 7 min at 71 x g. The protoplast pellets were washed once in KMC solution (Harms and Potrykus, 1978) and then once in protoplast culture medium (MS liquid medium containing 0.4 M glucose and 2.4-D at 0.1 or 0.5 mg/l, pH 5.8; Terakawa et al., 1992) via centrifugation for 7 min at 71 x g.

Protoplast cultures. The protoplasts (2 x 10⁶ per 35 x 10 mm Petri dish) were cultured in 2 ml protoplast culture medium containing 2.4-D at 0.1 and 0.5 mg/l concentrations. When conditioned medium was used, 1 ml of the protoplast medium was mixed with 1 ml conditioned medium. The medium was filter sterilized. The conditioned medium was obtained through the following steps: 1) centrifuging cell
suspensions which had been cultured in MS basal medium for five to seven days; 2) transferring the supernatant to a Petri dish; 3) adding and dissolving glucose in the supernatant to get 0.4 M glucose in the medium; and 4) sterilizing the medium through a 0.2 μm filter. Protoplasts were incubated in the dark at 26°C on a rotary shaker set at 40 rpm. One month after incubation, the effects of different concentrations of 2,4-D and use of conditioned medium on protoplast-derived callus formation were evaluated by visually counting the number of cell colonies. The protoplast-derived calli were then transferred to maintenance medium (MS medium containing 0.4 M glucose and 0.1 mg/l 2,4-D with 1.25% SeaPlaque agarose, Terakawa et al., 1992).

*Plant regeneration from protoplast-derived cells.* One month after culture on the maintenance medium, protoplast-derived calli were transferred to plant regeneration medium. Two different PR media with sucrose or maltose as the carbohydrate source were used for protoplast-derived cell regeneration: 1) MS medium supplemented with 0.5 mg/l nicotinic acid, 0.5 mg/l pyridoxine HCl, 0.1 mg/l thiamine HCl, 2 mg/l glycine, 0.01 mg/l 2,4-D, 0.1 mg/l kinetin and 3% sucrose or maltose (modified from Krans et al., 1982), and 2) half-strength MS medium supplemented with 250 mg/l casamino acids and 1.5% sucrose or maltose (modified from Zhong et al., 1991).
Results and Discussion

Callus induction

Two media, MS medium with 1.0 mg/l 2,4-D (Krans et al., 1982) and MS medium with 6.6 mg/l dicamba and 0.5 mg/l BA (Zhong et al., 1991), were compared for their effects on embryogenic callus induction. The experiments showed that calli induced on either induction medium exhibited high embryogenic potential. High frequency of plant regeneration (more than 50 shoots from 2 g callus) was obtained after transferring the calli onto plant regeneration medium (Fig. 1). Therefore, either medium can be used for embryogenic callus induction.

Suspension culture initiation and maintenance

Calli induced from the two different induction media mentioned above were highly embryogenic. However, they exhibited a difference when used for suspension culture initiation. The suspension cultures initiated from calli induced on MS medium with 1 mg/l 2,4-D showed good quality whereas, the quality of suspension cultures initiated from calli induced on MS medium with 6.6 mg/l dicamba and 0.5 mg/l BA was poor (Table 1). The calli induced on MS medium with 1 mg/l 2,4-D, therefore, was used for suspension culture initiation. It was observed that calli cultured on the callus maintenance medium for more than two months were better for suspension initiation than freshly induced calli excised from the mature seeds. It took less time
for establishment of suspension cultures suitable for protoplast isolation when the maintained calli were used for suspension culture initiation (Table 1).

Among the media tested for suspension cultures (Table 2), those with lower concentrations of 2,4-D (1 mg/l) were better than those with higher concentrations (3 mg/l) for developing better and more embryogenic cultures; at a lower concentration of 2,4-D, the medium with maltose was better than that with sucrose. It has been reported that a lower concentration of 2,4-D is beneficial for maintenance of embryogenic potential and for plant regeneration (Krans et al., 1982; Lowe and Conger, 1979). The results of our experiments is in agreement with these reports. The effect of different carbohydrate sources on maintenance of embryogenic potential and on plant regeneration is highly species dependent (Fleihinghaus et al., 1991). Maltose was found to be superior to sucrose as a carbohydrate source in wheat (*Triticum aestivum*) (Last and Brettell, 1990), potato (*Solanum tuberosum* L.) (Batty and Dunwell, 1989) and rye (*Secale cereale* L.) (Fleihinghaus et al., 1991). Opposite results were reported in maize (*Zea mays*) (Deimling et al., 1990) and cucumber (*Cucumis sativus* L.) (Ladyman and Girard, 1992). Comparison of the effect of sucrose and maltose on maintenance of embryogenic suspensions in this study showed that maltose was superior to sucrose in creeping bentgrass. Consequently, maltose was used in subsequent suspension culture medium.

Rapidly growing, embryogenic cell suspensions suitable for protoplast isolation were established two months after culture initiation under optimal conditions (i.e.,
calli induced on MS medium with 1 mg/l 2,4-D and cultured for more than two months as the material for suspension culture initiation, and MS medium with 1 mg/l 2,4-D and 3% maltose as suspension culture medium). High embryogenic potential of the suspensions could be maintained for about 6 months. After then, it gradually decreased. The same phenomena was also found in barley (*Hordeum vulgare* L.) (Luhrs and Lorz, 1988). This problem could be overcome by continuous re-establishment of new suspensions.

**Protoplast culture**

Protoplast isolation and culture were carried out according to Terakawa et al. (1992). Several conditions were re-tested to verify their results. As shown in Table 3, greatest colony formation was obtained at a lower concentration of 2,4-D (0.1 mg/l) with use of conditioned medium. This is consistent with the result obtained by Terakawa et al. (1992).

**Plant regeneration from protoplast-derived callus**

Essentially two kinds of media (Krans et al, 1982; Zhong et al, 1991) were tested for plant regeneration from protoplast-derived callus. Since maltose and sucrose had a different effect on creeping bentgrass suspension cultures, the effect of PR media containing maltose or sucrose on protoplast-derived callus regeneration also was included in this study. The experiments indicated that a higher frequency
of plant regeneration was obtained on medium containing maltose as the carbohydrate source compared to the medium containing sucrose (Table 4). The medium adapted from Krans et al. (1982) and from Zhong et al. (1991) had no obviously different effect on protoplast-derived cell regeneration, except that some calli turned brown on the medium adapted from Krans et al. (1982). Therefore, half-strength MS medium containing maltose, modified from Zhong et al. (1991), was subsequently used as the PR medium for creeping bentgrass protoplast-derived cell regeneration. Fig. 2 shows plantlets regenerated from protoplast-derived calli with high efficiency.

Conclusions

This study indicated that embryogenic callus can be induced from mature seeds of creeping bentgrass on either MS medium with 1 mg/l 2,4-D (Krans et al., 1982) or MS medium with 6.6 mg/l dicamba and 0.5 mg/l BA (Zhong et al., 1991). However, only the callus induced on MS medium with 1 mg/l 2,4-D was suitable for suspension culture initiation. The use of calli cultured on maintenance medium for more than two months reduced the time required for establishment of suspension cultures for protoplast isolation. Maltose was superior to sucrose for the maintenance of embryogenic potential in creeping bentgrass. In this study, a protocol for initiation and maintenance of embryogenic callus and suspension cultures in creeping bentgrass was developed. A high frequency of plant regeneration from
either seed-derived calli or suspension-derived protoplasts was obtained following this protocol.
Table 1. Effects of different media and calli age on suspension culture initiation of creeping bentgrass\(^1\)

<table>
<thead>
<tr>
<th>Callus induction medium</th>
<th>Age of callus</th>
<th>Suspension culture quality(^2)</th>
<th>Time for establishing suspension(^3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS, 1 mg/l 2,4-D</td>
<td>Freshly induced</td>
<td>++ +</td>
<td>4-5 months</td>
</tr>
<tr>
<td></td>
<td>Maintained for over two months</td>
<td>++ +</td>
<td>2-3 months</td>
</tr>
<tr>
<td>MS, 6.6 mg/l Dicamba, 1 mg/l BA</td>
<td>Freshly induced</td>
<td>-</td>
<td>Not available(^4)</td>
</tr>
<tr>
<td></td>
<td>Maintained for over two months</td>
<td>-</td>
<td>Not available(^4)</td>
</tr>
</tbody>
</table>

\(^1\) The observations were based on three replications for each condition.

\(^2\) Suspension cultures were rated visually for quality where ++ + designates cultures composed of small cells (mean cell size = 19.8 x 19.8 \(\mu\)m) and small aggregates, the medium remained clear over time in culture, ++ designates cultures composed of a greater frequency of larger cells and cell aggregates, and the medium became slightly viscous over time in culture, + designated cultures with more than 50% large cells (mean cell size = 60.5 x 30.3 \(\mu\)m), - designates cultures composed primarily of large elongated cells and the culture medium became viscous over time in culture.

\(^3\) This refers to the time from suspension initiation to the time when the cell suspension was suitable for protoplast isolation.

\(^4\) The quality of the cultures was too poor for continued maintenance.
Table 2. Effects of two concentrations of 2,4-D and two carbohydrate sources in liquid MS media on suspension cultures of creeping bentgrass

<table>
<thead>
<tr>
<th>2,4-D (mg/l)</th>
<th>Sugar</th>
<th>Culture quality^2</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>sucrose</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>maltose</td>
<td>+++</td>
</tr>
<tr>
<td>3</td>
<td>sucrose</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>maltose</td>
<td>+</td>
</tr>
</tbody>
</table>

^1 Each treatment was replicated three times.

^2 See footnote 2 under Table 1.
Table 3. Effects of two different concentrations of 2,4-D and conditioned medium on protoplast-derived cell formation of creeping bentgrass

<table>
<thead>
<tr>
<th>2,4-D (mg/l)</th>
<th>Conditioned medium</th>
<th>No. of cell colonies/million mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>Yes</td>
<td>1810 ± 161</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>330 ± 240</td>
</tr>
<tr>
<td>0.5</td>
<td>Yes</td>
<td>320 ± 190</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>2 ± 1</td>
</tr>
</tbody>
</table>

1 Each treatment was replicated three times.
Table 4. Effects of two different plant regeneration media with two different carbohydrate sources on protoplast-derived cell regeneration of creeping bentgrass

<table>
<thead>
<tr>
<th>Medium</th>
<th>2,4-D (mg/l)</th>
<th>Kinetin (mg/l)</th>
<th>Sugar</th>
<th>PR ²</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS (Krans et al., 1982)</td>
<td>0.01</td>
<td>0.1</td>
<td>sucrose</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>maltose</td>
<td>+++ ³</td>
</tr>
<tr>
<td>1/2 MS (Zhong et al., 1992)</td>
<td>0</td>
<td>0</td>
<td>sucrose</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>maltose</td>
<td>+++</td>
</tr>
</tbody>
</table>

¹ The observations were based on three duplications at each condition.

² Protoplast-derived calli were rated for plant regeneration on a visual scale of "+++", for those calli from which more than 50 shoots per plate were regenerated, "++" for those from which 10-50 shoots per plate were regenerated, "+" for those giving less than 10 shoots per plate, "-" for those with no shoot formation.

³ Some calli turned brown.
Fig 1. Plant regeneration of creeping bentgrass from six-month old seed-derived calli. The photograph was taken six weeks after the calli were transferred on PR medium.
Fig 2. Plantlet regeneration of creeping bentgrass from protoplast-derived callus (2.5 months after protoplast isolation).
References


Last DI, Brettell RIS (1990) Embryo yield in wheat anther culture is influenced by the choice of sugar in the culture medium. Plant Cell Rep. 9:14-16

Lowe KW, Conger BV (1979) Root and shoot formation from callus cultures of tall fescue. Crop Sci. 19:397-400


28


Chapter II

Effects of different electroporation conditions on transient gene expression in creeping bentgrass 

(*Agrostis palustris* Huds.) protoplasts
Abstract

Electroporation efficiency is affected by many factors such as field strength, amount of DNA, electroporation buffer, and density of protoplasts. In order to determine optimum electroporation conditions for gene transfer in creeping bentgrass (*Agrostis palustris* Huds.), these factors were studied using transient gene expression assay after the reporter *uidA* gene encoding β-glucuronidase (GUS) was introduced by electroporation. GUS activity peaked at a field strength of 950 V/cm. Increases in the amount of plasmid DNA led to increases of GUS activity within the plasmid DNA concentrations tested (0 to 200 µg/ml). GUS activity increased in response to increases in protoplast density up to $2 \times 10^6$/ml, and plateaued thereafter. GUS activity peaked when the electroporation buffer contained 125 mM KCl. Information obtained from this study facilitated setting of optimal conditions for electroporation in creeping bentgrass.
Introduction

Creeping bentgrass (*Agrostis palustris* Huds.) is the most commonly used turfgrass species for golf course greens in North America (Zhong et al., 1993). In addition, it is widely used as turf for bowling and tennis greens. However, this important turfgrass species is not only affected by environmental stresses, but also threatened by diseases, pests and weeds (Zhong et al., 1993). Improvement of creeping bentgrass, therefore, is of great value.

Genetic engineering is a relatively new tool for crop improvement, which facilitates gene transfer between unrelated species. Consequently, a large gene pool can be used for crop improvement. Given this advantage, genetic engineering provides great potential for improvement of creeping bentgrass.

Electroporation is one of the more reliable and efficient gene transfer techniques used for genetic engineering of monocots (Dhir et al., 1991). Successful genetic transformation via electroporation has been accomplished in several species such as rice (*Oryza sativa*) (Toriyama et al., 1988; Zhang et al., 1988;), maize (*Zea mays*) (Rhodes et al. 1988), and tall fescue (*Festuca arundinacea* Schreb) (Ha et al., 1992).

Electroporation efficiency is affected by factors such as electric field strength, buffer composition and protoplast density. These factors are highly species dependent (Rouan et al., 1991; Dhir et al., 1991; Guerche et al., 1987). The optimal
conditions for these factors must be defined for each species.

A transient gene expression assay following gene introduction by electroporation has been widely used in various studies such as analysis of the strengths of 5' regulatory sequences (Hauptmann et al., 1988; Kuozuka et al., 1990; Mascarenhas et al., 1990), and optimization of gene transfer conditions (Dhir et al., 1991; Lindsey and Jones, 1987; Prols et al., 1988). A transient gene expression assay can be defined as an assay for monitoring the expression of a reporter gene shortly (a few hours to a few days) after introduction of the gene into cells. The reporter gene can be expressed using the transcriptional and translational machinery of the host cells even though it may not be integrated into the host genome. Since non-integrated DNA is eventually degraded by nucleases, the expression of the reporter gene is temporary. The transient expression of non-integrated DNA is free from position effects that have been observed in stable transformants (in which introduced DNA has been integrated into the host genome) (Horsch et al., 1985; Jones et al., 1985). In addition, the short time interval between gene transfer and transient gene expression assay makes the transient expression system practical and advantageous.

To optimize electroporation conditions in creeping bentgrass, several factors affecting electroporation efficiency were studied using transient gene expression assay following the introduction of the reporter β-glucuronidase gene.
Materials and Methods

Suspension culture initiation and maintenance. Suspension cultures were initiated from seed-derived callus. Callus was induced from mature seeds of ‘Penncross’ creeping bentgrass on Krans medium (Krans et al., 1982) as described on page 13 of Chapter I. Callus maintenance and suspension culture initiation and maintenance were also carried out as described on pages 13-15 of Chapter I. MS liquid medium supplemented 1 mg/l 2,4-D and 3% maltose was used for suspension cultures. Rapidly growing, embryogenic cell suspensions suitable for protoplast isolation were established in two months after the suspension culture initiation.

DNA preparation. The plasmid pZO1052 (Ha et al., 1992) containing the β-glucuronidase reporter gene (uidA) was used in all experiments. The plasmid was propagated in the E. Coli strain, MC 1000, and isolated by the alkaline lysis method of Birnboim and Doly (1979). Plasmid DNA was purified by cesium chloride density gradient ultracentrifugation. The DNA was quantified by measuring absorbance at 260 nm with a spectrophotometer. Carrier DNA was prepared by digesting calf thymus DNA (Sigma) with restriction enzyme EcoRI. The restriction enzyme was then inactivated by heating at 65°C for 20 min. The digested calf thymus DNA was sterilized by filtering through a sterile 0.2 µm nylon filter.
Protoplast isolation. Protoplast isolation was carried out as described on page 15 of Chapter 1.

Electroporation. Protoplasts (4 x 10⁶/ml, unless otherwise mentioned) were suspended in electroporation buffer (10 mM Hepes, 125 mM KCl unless otherwise mentioned, 5 mM CaCl₂, 0.4 M mannitol, pH 5.8). Aliquots (0.5 ml suspension) were pipetted into sterile 1.25 ml cuvettes and mixed with 50 µg carrier DNA and, unless otherwise mentioned, 40 µg plasmid DNA pZO1052. The cuvettes were kept on ice for 10 min prior to electroporation. Electroporation was carried out at 950 V/cm field strength (unless otherwise mentioned) with a 400 µF capacitance. A BTX-300 Transfector (Biotechnologies and Experimental Research, Inc.) was used for electroporation. The protoplasts were kept on ice for 10 min after electroporation and then cultured in 35x10 mm Petri dishes containing 1 ml PC4 medium (MS salts, 6.84% glucose, 5% mannitol, 0.1 mg/l 2,4-D, and 0.1 mg/l 6-benzyladenine, pH 6.0; Dalton, 1988).

Protoplast viability test. Protoplast viability was determined according to Widholm (1972) by staining with fluorescein diacetate (FDA).

Protein extraction and quantification. Electroporated protoplasts were harvested in a 1.5 ml microcentrifuge tube 22 h after incubation at 26° C, and then centrifuged at 9,000 rpm for 1 min. The pelleted protoplasts were resuspended in GUS
Extraction Buffer (50 mM NaPO₄, pH 7.0, 10 mM Na₂EDTA, 0.1% sodium lauryl sarcosin, 0.1% Triton-X, 10 mM β-mercaptoethanol) and then disintegrated on ice by two bursts (6 sec/10 sec interval/6 sec) sonication (50 Microsonic Distruptor, Tekmar). After centrifugation at 14,000 rpm for 4 min, the supernatant containing total protein fraction was transferred to new tubes and protein concentration was measured according to Bradford (1976).

**GUS assay.** Transient GUS activity was measured 22 h after electroporation according to Jefferson (1987). The reaction was carried out by adding 40 or 50 μg total protein to 100 μl of GUS assay buffer [1 mM 4-methyl umbelliferyl β-D-glucuronide (4MU) in GUS Extraction Buffer] and incubating at 37°C. At two time intervals, 15 and 30 min after reaction initiation, 50 μl of the assay mixture was transferred to 500 μl GUS stop buffer (0.2 M Na₂CO₃) to terminate the reaction and thereby obtain two sets of reaction points. Aliquots of 100 μl of each terminated reaction mixture were measured on a fluorometer (TKO-100, Hoefer Scientific). GUS activity for each sample was calculated based on the slope of the two sets of time-point data. All tests for GUS transient assay were replicated three times.
Results

Effect of field strength on transient GUS expression and protoplast viability

Transient GUS activity and protoplast viability were measured at field strengths of 500, 650, 800, 950 and 1100 V/cm with 400 uF capacitance 22 h after electroporation (Fig. 1). GUS activity increased with increases in the field strength until it peaked at 950 V/cm. At the field strength above 950 V/cm, GUS activity dramatically decreased. The viability of unpulsed protoplasts was 83.8%. As the field strength increased from 500 V/cm to 650 V/cm and 800 V/cm, viability of pulsed protoplasts decreased gradually to 76.6, 73 and 64%, respectively. With the increase of field strength from 800 V/cm to 950 V/cm, protoplast viability was considerably reduced from 64% to 40.9%. Thereafter, it dropped to about 1% at 1100 V/cm.

Effect of plasmid DNA concentration on transient GUS expression

The effect of the amount of plasmid DNA on transient GUS expression was studied after protoplasts were electroporated in the presence of different concentrations of plasmid DNA (0, 20, 80, 140 and 200 µg/ml) and a constant amount (100 µg/ml) carrier DNA (Fig. 2). GUS activity continued to increase with each subsequent increase in a plasmid DNA concentration. No plateau was reached within the range of plasmid DNA concentrations tested (from 0 to 200 µg/ml). However, the slope of the increase in GUS activity tended to decline gradually.
**Effect of protoplast density on transient GUS expression**

The effect of protoplast density on transient GUS expression was studied by measuring transient GUS activity after electroporation was conducted with different protoplast densities of 0.5, 1, 2, 4 and $6 \times 10^6$/ml. GUS activity increased rapidly with an increase in protoplast density up to $2 \times 10^6$/ml and plateaued thereafter (Fig. 3).

**Effect of KCl concentration on transient GUS expression**

An optimal molarity of KCl in electroporation buffer was determined by measuring transient GUS activity and protoplast viability after electroporation using buffers with different concentrations (75, 100, 125, 150, and 175 mM) KCl (Fig. 4). GUS activity and protoplast viability were extremely low after electroporation when the buffer contained 75 or 100 mM KCl. When the concentration of KCl increased from 100 to 125 mM, GUS activity and protoplast viability dramatically increased. While GUS activity peaked at 125 mM and decreased greatly at higher concentrations (150 and 175 mM) of KCl, the protoplast viability continued to increase up to KCl concentrations of 150 mM.

The above results were reproducible with variation of overall GUS activity among experiments. This phenomenon was also mentioned by Penmetsa and Ha (1994) and Callis et al. (1987). The variation among all experiments was less than threefold.
Discussion

Several factors affecting transient gene expression following electroporation were studied in creeping bentgrass protoplasts. They were electric field strength, plasmid DNA concentration, protoplast density, and KCl concentration in electroporation buffer.

Field strength greatly affects transient gene expression following electroporation. The influence of field strength on transient gene expression after electroporation is mainly based on its effect on: 1) the number, size and duration of pores produced by the electric pulse; and 2) protoplast viability after electroporation (Lindsey and Jones, 1987). Maximal transient gene expression occurs under the condition where there is an optimal compromise between electro-permeation of DNA and protoplast viability. Below this condition, the field strength may be insufficient for induction and/or maintenance of electropores. Above this condition, larger and/or more pores can be induced but the concomitant damage to the plasma membrane would be too great for protoplasts to retain viability. Maximum GUS activity was observed at a field strength 950 V/cm with a 400 uF capacitance in this study. Under this condition, about 40% of the protoplasts survived (Fig. 1).

The amount of plasmid DNA used in electroporation influences transient gene expression. The general trend of transient gene expression in response to increased amounts of plasmid DNA in previous studies was a rapid increase at lower
concentrations of DNA and a plateau at higher concentrations of DNA (Nyman and Wallin, 1992; Dhir et al., 1991; Penmetsa and Ha, 1994). In the present study, no plateau was reached within the range of plasmid DNA concentrations tested (from 0 to 200 μg/ml). However, the increase in GUS activity tended to decline gradually with higher concentrations of plasmid DNA. With a further increase in the plasmid DNA amount beyond 200 μg/ml, a plateau is expected. Since the increases in GUS activity were much smaller beyond 80 μg/ml plasmid DNA, this concentration of plasmid DNA was subsequently used in all experiments to obtain relatively high GUS activity and to minimize the consumption of plasmid DNA.

The minimal amount of plasmid DNA needed to reach a plateau level differs for each protoplast system. For instance, it is 30 μg/ml DNA for soybean (Glycine max) protoplasts (Dhir et al., 1991), 140 μg/ml DNA for tall fescue (Festuca arundinacea Schreb.) (Penmetsa and Ha, 1994), and 200 μg/ml DNA for strawberry (Fragaria x ananassa Duch.) (Nyman and Wallin, 1992). Differences in the minimal amount of plasmid DNA needed to reach a plateau level may result from the genotypic or physiological status of target protoplasts, the form of DNA used (linear vs. circular), or the variation in size among plasmid constructs which may result in differences in the molar concentrations used (Penmetsa and Ha, 1994).

A roughly linear relationship exists between transient gene expression and protoplast density within a certain range of protoplast density, after which a plateau occurs (Lindsey and Jones, 1987; Nyman and Wallin, 1992). Results from the present
study (Fig. 3) are consistent with these reports. According to Lindsey and Jones (1987), this phenomenon between transient gene expression and protoplast density may be due to the fact that the proportion of permeabilized protoplasts in the pulsed population is lower at higher density versus lower density.

The salts in an electroporation buffer are mainly involved in conductivity (Guerche et al., 1987). At a low concentration of KCl, the protoplasts are more conductive (ionic) than the medium. The current of the electric pulse passes from one electrode to the other chiefly through the cell membranes, resulting in a bursting of the protoplasts. By increasing the concentration of KCl, the medium itself becomes more electrically conductive. When a pulse is given in this medium, more current of the pulse passes from one electrode to the other through the medium. Consequently, the protoplasts are exposed to less electric current, resulting in higher protoplast viability. However, an excessive concentration of KCl should be avoided because cell machinery and enzymes do not work efficiently at high salt concentrations (Dr. James A. Saunders, personal communication). The optimal amount of a salt in the electroporation buffer should be determined at a concentration where a maximal level of gene expression is obtained. In our system, it was 125 mM KCl.

In conclusion, the optimal electroporation conditions for transient gene expression were: field strength at 950 V/cm; protoplast density at 2 x 10⁶/ml; KCl concentration in the electroporation buffer at 125 mM. The plasmid DNA
concentration used in our experiments was 80 μg/ml to obtained relatively high level of gene expression and to minimize the consumption of the DNA. The optimized conditions based on the transient expression assay can be extended to stable transformation (Guerche et al., 1987), although factors like field strength need further confirmation by stable transformation where protoplast viability may be more critical. The transient assay system established in the current study can be applied in studies for selection of strong regulatory sequences and for rapid analysis of gene constructs in creeping bentgrass.
Fig 1. Transient GUS activity and protoplast viability as functions of field strength. Closed circles represent GUS activity. Open circles represent protoplast viability. Bars represent standard deviations of three replicates.
Fig 2. Transient GUS activity as a function of plasmid DNA concentration. Bars represent standard deviations of three replicates.
Fig 3. Transient GUS activity as a function of protoplast density. Bars represent standard deviations of three replicates.
Fig 4. Transient GUS activity and protoplast viability as functions of KCl concentration. Closed circles represent GUS activity. Open circles represent protoplast viability. Bars represent standard deviations of three replicates.
References

Birnboim HC, Doly J (1979) A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. 7:1513-1523


48
CHAPTER III

Transient gene expression driven by different
5’ regulatory sequences in creeping bentgrass

(Agrostis palustris Huds.) protoplasts
Abstract

To identify efficient 5' regulatory sequences for high level expression of transgenes in creeping bentgrass (*Agrostis palustris* Hud.), effects of six different 5' regulatory sequences on gene expression were studied using a transient gene expression assay after the reporter *uidA* gene encoding β-glucuronidase (GUS) was introduced into bentgrass protoplasts via electroporation. The cauliflower mosaic virus (CaMV) 35S promoter exhibited very weak activity. Adding the maize alcohol dehydrogenase 1 gene (*Adh1*) intron 1 to this promoter did not significantly increase the activity. However, about a 37-fold increase was obtained by addition of the maize *Adh1* intron 6 to the CaMV 35S promoter. A combination of the maize *Adh1* promoter and its intron 1 produced a seven-fold increase in GUS expression when compared with the CaMV 35S promoter alone. The maize ubiquitin gene (*Ubi1*) promoter plus its first intron was 20 times more active than the CaMV 35S promoter. The most active 5' sequence tested in this study was the 5' region of the rice actin 1 gene (*Act1*). It was 72 times more active than the CaMV 35S promoter and would be the best choice for high level expressions of transgenes in creeping bentgrass.
Introduction

Successful transformation of plants via genetic engineering depends not only on gene transfer efficiency, but also the expression level of transgenes. In general, the expression level of a transgene is correlated to the strength of its 5' regulatory sequences which are involved in transcription initiation. The cauliflower mosaic virus (CaMV) 35S promoter, a promoter widely used for transgene expression in transgenic dicotyledonous plants, was 1/10 to 1/100 times as active in graminaceous monocots than in dicot species (Fromm et al., 1985; Hauptmann et al., 1987). Identification of strong 5' regulatory sequences for transgene expression in graminaceous monocots, therefore, is critical for transformation of monocot species.

The expression of a chimeric chloramphenicol acetyltransferase gene under the maize alcohol dehydrogenase 1 gene (Adh1) promoter was enhanced 100-fold by the addition of the Adh1 intron 1 in maize (Zea mays) (Callis et al., 1987). Introns (noncoding regions which are removed from the nascent mRNA in splicing) are known to play an important role in increasing the diversity of gene products produced from a single locus. They may also contain transcriptional regulators such as enhancer elements that are involved in the control of gene expression. Studies in animals have shown that introns can be important components for normal expression of some genes (Hamer and Leder, 1979; Gruss et al., 1979; Gasser et al., 1982). In plants, however, early studies indicated that introns were not important for gene expression (Chee et al., 1986). The intron-mediated enhancement of gene expression
observed in maize by Callis et al. (1987) led to reconsideration of the role of introns in plant gene expression. In recent years, intron-mediated enhancement of gene expression in plants has been observed by many other scientists, for instance, Kyozuka et al. (1990) in rice (Oryza sativa), Mascarenhas et al. (1990) and Luehrsen and Walbot (1991) in maize (Zea mays). Such studies on intron-mediated enhancement of gene expression have broadened the search for efficient 5' regulatory sequences. Recent studies on identification of strong 5' regulatory sequences have been conducted on both promoters and various combinations of promoters and introns. It was found that the 5' region (promoter plus intron) of the maize alcohol dehydrogenase 1 gene (Adh1) was 10- to 20-fold more active than the CaMV 35S promoter in rice (Oryza sativa) (Zhang and Wu, 1988). The 5' region of the rice Act1 gene was 5- to 10-fold more active than the 5' region of the maize Adh1 gene in rice (McElroy et al., 1990). The strength of 5' regulatory sequences is species dependent. The 5' region of the maize ubiquitin gene (Ubi1) was 10 times more active than the CaMV 35S promoter in maize (Christensen et al., 1992), whereas it was 94 times more active in sugarcane (Saccharum spp. hybrids) (Gallo-Meagher and Irvine, 1993). The strength of 5' regulatory sequences, therefore, needs to be evaluated in the species of interest. Studies on the effects of various promoters and introns on gene expression have been conducted in maize, rice, wheat (Triticum aestivum), barley (Hordeum vulgare) (Taylor et al., 1993), and tall fescue (Festuca arundinacea Schreb.) (Ha et al., 1995). However, similar studies have not been conducted in creeping
bentgrass (*Agrostis palustris* Hud.).

In this study, the CaMV 35S promoter and five other promoter/intron constructs were studied to identify efficient 5’ regulatory sequences for high level expression of transgenes in creeping bentgrass.

**Materials and Methods**

*Suspension culture initiation and maintenance.* Suspension cultures were initiated from seed-derived callus and callus was induced from mature seed of ‘Penncross’ creeping bentgrass (*Agrostis palustris* Hud.). Suspension culture initiation and maintenance were carried out as described on page 34 of Chapter II.

*Plasmids.* Six plasmids containing different 5’ regulatory sequences connected to the coding region of reporter *uidA* gene were used in this study: pBI221 (Jefferson, 1987), pDPG208 (Gordon-Kamm et al., 1990), pZO1052 (Ha et al., 1992), pAct1-D (McElroy et al., 1990), pNG1 (Klein et al., 1989), and pAHC25 (Christensen et al., 1992). The 5’ regulatory sequences in these plasmids are described in Table 1.

*DNA preparation.* Plasmid DNA and carrier DNA were prepared as described on page 34 of Chapter II.
Protoplast isolation. Protoplasts were isolated as described on page 15 of Chapter I.

Electroporation. Electroporation was conducted as described on page 35 in Chapter II.

Protein extraction, quantification, and GUS assay were carried out as described on pages 35-36 of Chapter II.

Results and Discussion

The effects of six different 5' regulatory sequences on gene expression in creeping bentgrass were studied via transient GUS assay following electroporation. When the reporter gene was driven by the CaMV 35S promoter alone (pBI221), the transient GUS expression was very weak (Table 1). This result is consistent with previous reports that CaMV 35S promoter did not work efficiently in monocots (Fromm et al., 1985; Hauptmann et al., 1987; Christensen et al., 1992; Ha et al., 1995).

The level of GUS expression was not significantly increased by the addition of the maize Adh1 intron 1 to the CaMV 35S promoter (pDPG208). A similar result was obtained in tall fescue (Ha et al., 1995), but it differed from the observation in maize where Callis et al. (1987) reported a 100-fold increase in a chimeric reporter
gene expression by the addition of the Adh1 intron 1.

When the Adh1 intron 6 was added to the CaMV 35S promoter (pZO1052), the GUS gene expression was enhanced by about 37-fold compared to that of pBI221. Similar results were obtained in tall fescue (Ha et al., 1995) and maize (Mascarenhas et al., 1990).

The maize Adh1 promoter plus its intron 1 (pNG1) was about seven times more active than the combination of the CaMV 35S promoter and the Adh1 intron 1 (pDPG208). A similar result was obtained in tall fescue (Ha et al., 1995). The 5' region of the Adh1 was reported to be active in maize (Callis et al., 1987) and 10 to 20 times more active than the CaMV 35S promoter in rice (Zhang and Wu, 1988; McElroy et al., 1990). However, this 5' regulatory sequence was usually induced by anaerobic stress (McElroy et al., 1990).

The activity of the maize Ubi1 promoter and its first intron (pAHG25) was 20 times higher than that of the CaMV 35S promoter in creeping bentgrass. Compared to the CaMV 35S promoter, this 5' region produced a 10-fold higher activity in maize (Christensen et al., 1992) and a 94-fold higher activity in sugarcane (Gallo-Meagher and Irvine, 1993).

Among the six 5' regulatory sequences tested in this study, the most active sequence was the 5' region of the rice Act1 gene (pAct1-D). This sequence was about 72-fold more active than the CaMV 35S promoter. The 5' region of rice actin 1 gene is also very active in maize, barley, wheat, sorghum (Sorghum vulgare)
(McElroy et al., 1992), and tall fescue (Ha et al., 1995). In addition, this region is likely to confer constitutive expression of transgenes in plants (Zhang et al., 1991).

In this study, the strengths of six different 5' regulatory sequences were evaluated using transient GUS activity as a criterion. The CaMV 35S promoter was least active whereas the 5' sequence of the rice actin 1 gene was most active among the six 5' sequences. Ranked in order of activity (high to low), the other four 5' sequences were: 1) the combination of the CaMV 35S promoter and the maize alcohol dehydrogenase 1 gene (Adh1) intron 6; 2) the 5' sequence of the maize ubiquitin gene (Ubi-1); 3) the maize Adh1 promoter and its intron 1; and 4) the combination of the 35S promoter and the Adh1 intron 1. Since the 5' region of the rice actin 1 gene (Act1) is the most active 5' sequence among the six sequences tested and likely confers constitutive expression of transgenes, this 5' regulatory sequence would be advantageous for the development of an efficient transformation system in creeping bentgrass.

Acknowledgements. The author thanks Dr. M. E. Fromm for providing the plasmid pNG1, Dr. P. G. Lemaux for pDPG208, Dr. R. Sinibaldi for pZO1052, Drs. D. McElroy and R. Wu for pAct1-D, and Dr. P. H. Quail for pAHC25.
Table 1. Effects of different 5' regulatory sequences on transient gene expression in creeping bentgrass

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>5' regulatory sequences(^1)</th>
<th>GUS activity (pmol 4MU·mg protein(^{-1})·min(^{-1})) Mean ± SD</th>
<th>Relative strength</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBI221</td>
<td>P(_{35S})</td>
<td>32 ± 1</td>
<td>1</td>
</tr>
<tr>
<td>pDPG208</td>
<td>P(_{35S}) &amp; Adh IN(_1)</td>
<td>38 ± 13</td>
<td>1</td>
</tr>
<tr>
<td>pZO1052</td>
<td>P(_{35S}) &amp; Adh IN(_6)</td>
<td>1206 ± 100</td>
<td>37</td>
</tr>
<tr>
<td>pNG1</td>
<td>P(_{Adh}) &amp; Adh IN(_1)</td>
<td>246 ± 150</td>
<td>7</td>
</tr>
<tr>
<td>pABC25</td>
<td>P(_{Ubi}) &amp; Ubi IN(_1)</td>
<td>636 ± 240</td>
<td>20</td>
</tr>
<tr>
<td>pAct1-D</td>
<td>P(_{Act}) &amp; Act IN(_1)</td>
<td>2306 ± 300</td>
<td>72</td>
</tr>
</tbody>
</table>

\(^1\) P\(_{35S}\) represents the cauliflower mosaic virus (CaMV) 35S promoter; P\(_{Act}\), the rice actin 1 gene (Act1) promoter; P\(_{Adh}\), the maize alcohol dehydrogenase 1 gene (Adh1) promoter; P\(_{Ubi}\), the maize ubiquitin gene (Ubi1) promoter; Adh IN\(_1\), the maize Adh1 intron 1; Adh IN\(_6\), the maize Adh1 intron 6; Act IN\(_1\), the rice Act1 intron 1; Ubi IN\(_1\), the maize Ubi1 intron 1.
References


Hamer DH and Leder P (1979) Splicing and the formation of stable mRNA. Cell 18:1299-1302


Chapter IV

Stable transformation of creeping bentgrass

(*Agrostis palustris* Huds.) via particle bombardment and electroporation

62
Abstract

To establish an efficient genetic transformation system for creeping bentgrass (*Agrostis palustris* Huds.) improvement, particle bombardment and electroporation were used for transformation of creeping bentgrass. The transformation was performed using the plasmid pZO1052 which contains the reporter β-glucuronidase (*uidA*) gene and the selectable marker hygromycin phosphotransferase (*hph*) gene. Transgenic plants and calli were obtained via particle bombardment followed by transformant selection on hygromycin-containing medium. A high frequency of putative transformants (4.6 resistant colonies per bombardment) was obtained. Southern analyses confirmed the integration of foreign genes and showed that the transformant selection following bombardment was highly effective (19 transformants of 21 putative transformants tested). A high frequency of escapes, however, occurred in transformant selection after electroporation of protoplasts. Viable colonies in controls occurred at nearly the same frequency as in treated samples. The ineffective selection resulted in an inefficient recovery of transformants. Of eight resistant colonies tested, only one was demonstrated to be truly transformed in Southern analysis. This study demonstrated that the genetic transformation systems using particle bombardment were efficient. Use of these systems will facilitate the improvement of creeping bentgrass.
Introduction

Creeping bentgrass (*Agrostis palustris* Huds.) is a turfgrass species widely used on golf, bowling and tennis greens. Because of its importance, there is an increasing demand for new cultivars with desirable traits such as disease and pest resistance.

Genetic engineering has been employed as a new tool for crop improvement in modern agriculture. This technology facilitates gene transfer between unrelated species, which greatly expands the gene pools available for crop improvement.

Plant genetic engineering has succeeded largely via the *Agrobacterium*-mediated transformation systems (Potrykus et al., 1991). However, the application of this system has been restricted mainly to dicot plants. Most monocots have been recalcitrant to *Agrobacterium*-mediated transformation due to the host-range of *Agrobacterium* (Weising et al., 1988) and the lack of proper wound response in many monocots (Potrykus, 1990). The limitation of *Agrobacterium*-mediated transformation for most monocot species has been circumvented by direct gene transfer techniques such as chemically-facilitated DNA uptake, electroporation, and particle bombardment. Direct gene transfer theoretically can introduce foreign genes into any plant. Among the direct gene transfer methods, electroporation has been considered to be one of the more efficient ones (Dhir et al., 1991; Klein et al., 1988a). Successful stable transformation by electroporation has been accomplished in *Brassica napus* (Guerche et al., 1987), maize (*Zea mays*) (Rhodes et al. 1988), rice (*Oryza*
(Zhang et al., 1988; Shimamimoto et al., 1989), and tall fescue (Festuca arundinacea Schreb) (Ha et al., 1992). However, electroporation generally requires protoplasts as a transfer target. Plant regeneration from protoplasts of many monocots has proven to be very difficult and time consuming (Wang et al., 1988). An alternate strategy of direct gene transfer is particle bombardment. Particle bombardment can deliver foreign DNA into intact cells which can be more easily regenerated. Transformation via particle bombardment has been successful in several species such as maize (Zea mays) (Gordon-Kamm et al., 1990), soybean (Glycine max Merrill.) (Finer and McMullen, 1991), and wheat (Triticum aestivum L.) (Vasil et al., 1992).

Stable transformation of creeping bentgrass via particle bombardment has been recently reported by Zhong et al. (1993). However, the selection efficiency of transformants was low (4 transformants of 15 putative transgenic plants tested) in their experiments due to the lack of selectable marker genes. In the present study, transformation was performed with the pZ01052 plasmid. This vector contains the reporter β-glucuronidase (uidA) gene and the selectable marker hygromycin phosphotransferase (hph) gene both of which are driven by the cauliflower mosaic virus (CaMV) 35S promoter plus the maize alcohol dehydrogenase 1 gene (Adh1) intron 6 (Fig. 1). Although the study in Chapter III indicated that the most active 5’ regulatory sequence among the six sequences tested in creeping bentgrass was the rice 5’ regulatory region, the available vector (pAct1-D) containing the rice 5’
regulatory region has no selectable genes. Since the pZO1052 plasmid confers a relatively high level of gene expression and contains both reporter and selectable genes, it presently is the best choice available for transformation in creeping bentgrass. Transformant selection has been applied in this study since the hph gene confers hygromycin resistance to transformed cells.

The long-term goal of our research is to develop transgenic creeping bentgrass with useful new traits via genetic engineering. The objective of this study is to establish efficient transformation systems in creeping bentgrass via particle bombardment or electroporation.

Materials and Methods

Callus and cell suspension. Callus and suspension culture initiation and maintenance were performed as described on page 34 of Chapter II.

Protoplast isolation. Protoplast isolation were carried out as described by Terakawa et al. (1992).

DNA preparation. Plasmid DNA and carrier DNA were prepared as described on page 34 of Chapter II.
**Electroporation and protoplast culture.** Electroporation was conducted as described on page 35 of Chapter II except that after electroporation the protoplasts were cultured in protoplast culture medium (MS medium supplemented with 0.4 M glucose, 0.1 mg/l 2,4-D, pH 5.8) which was mixed with an equal volume of conditioned medium as described on pages 15-16 of Chapter I. The protoplasts were incubated in the dark at 26°C on a rotary shaker set at 40 rpm.

**Particle bombardment.** The plasmid (pZO1052) DNA was coated on gold particles (1 μm in diameter) according to the manufacturer’s manual for a Biolistic Particle Delivery System, PDS-1000/He (DuPont). The gold microcarriers were prepared as follows: Sixty mg of gold particles was placed in 1 ml of 100% ethanol in a 1.5 ml microcentrifuge tube and vortexed on high for 1 to 2 min. The tube was centrifuged at 10,000 rpm for 5 sec in an Eppendorf centrifuge followed by removal of the supernatant. The microcarriers were washed by adding 1 ml sterile distilled water, resuspending, centrifuging, and removing the supernatant. These procedures were repeated once. The gold microcarriers were then resuspended in 1 ml sterile distilled water. Fifty μl suspension was aliquoted into microtubes while vortexing the suspension. The aliquots were stored at 4°C if they were not used immediately. To each aliquot, 5 μl DNA (1 μg/μl), 50 μl 2.5 M CaCl₂ and 20 μl 0.1 M spermidine (free base, tissue culture grade) were added in order, under continuous vortexing. The vortexing was continued for 3 min followed by centrifuging of the microcarriers.
at 10,000 rpm for 5 sec in an Eppendorf centrifuge. The supernatant was removed as much as possible. The microcarriers coated with DNA were washed with 250 μl 100% ethanol by vortexing briefly, centrifuging, and removing the supernatant. Finally, the microcarriers were resuspended in 60 μl 100% ethanol. For each bombardment, about 1.5 to 2 g callus cells grown on agar medium or 1 to 1.5 g suspension cells were placed on a filter paper in a 60x15 mm Petri plate as a thin cell layer. The plate was placed in the vacuum chamber of the Biolistic Delivery System (PDS-1000/He) at 11 cm from the stopping plate. Eight μl gold microcarriers coated with plasmid pZO1052 were loaded for each bombardment. The helium pressure was set at 7500 KPa with a partial vacuum of 700 mm Hg. After bombardment, the filter paper carrying the cells was transferred to callus culture medium.

*Transformant selection and plant regeneration.* In particle bombardment experiments, the cells were transferred five days after bombardment to callus maintenance medium containing 200 mg/l hygromycin. About five weeks later, resistant cell colonies were transferred to the fresh medium containing 200 mg/l hygromycin. All cultures were kept in the dark at 26°C. For plant regeneration, resistant calli were transferred to plant regeneration medium A (MS medium supplemented with 3% maltose, 0.01 mg/l 2,4-D, 0.1 mg/l kinetin, 0.5 mg/l nicotinic acid, 0.5 mg/l pyridoxine HCl, 0.1 mg/l thiamine HCl, 2 mg/l glycine, and 1.0% agar, pH 5.6) containing 200 mg/l hygromycin. The cultures were incubated under light at 16 h/day and 26°C.
In electroporation experiments, protoplast-derived calli were transferred one month after electroporation to MS medium supplemented with 0.1 mg/l 2,4-D, 0.4 M glucose, 1.25 % (w/v) Seaplaque agarose and 100 mg/l hygromycin. About five weeks later, resistant colonies were transferred to fresh medium containing 150 mg/l hygromycin. All cultures were kept in the dark at 26°C. For plant regeneration, 50 resistant cell colonies were transferred to plant regeneration medium B (half-strength MS medium supplemented with 250 mg/l casamino acids, 1.5% maltose and 0.7% agar) with 150 mg/l hygromycin under light at 16 h/day and 26°C.

Untransformed controls were included in all the selection procedures in both particle bombardment and electroporation experiments on hygromycin-containing media and media without hygromycin.

_Histochemical staining for GUS activity._ Histochemical staining of putative transformed cells or plants for GUS expression was carried out according to Jefferson (1987).

_PCR and Southern blot hybridization._ Calli or leaf tissues from hygromycin-resistant and control materials were frozen in liquid nitrogen and ground to fine powder in a mortar using a pestle. Total DNA was extracted from the powder according to Saghai-Maroot et al. (1984). Polymerase chain reaction (PCR) was carried out in 50 µl of Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 200 µM of dATP, dTTP, dGTP,
dGTP, 1.25 units AmpliTaq DNA polymerase, 200 ng genomic DNA or 2 ng plasmid DNA, and 100 ng of each oligonucleotide primer. The primers (5'-ACAGCGTCTCCGACCCGATGCA-3' and 5'-AGTCAATGACCGCTGTATGCG-3', Genosys Biotechnologies, Inc.) amplified an internal 590 bp fragment of the *hph* gene. The primers (5'-TGGTGGGAAAAGCGCGTTACAAG-3' and 5'-GTCTTACGCGTTGCTTCGCGCCAG-3', Genosys Biotechnologies, Inc.) amplified an internal 1200 bp fragment of the *uidA* gene. The amplification reaction was performed in a DNA thermal cycler (PERKIN ELMER CETUS) under the following conditions: denaturation temperature 94°C, 1 min; annealing temperature 62°C, 2 min; extension temperature 72°C, 2 min. For the PCR product analysis, the amplification products were electrophoresed in 1% agarose gel. For the Southern analysis, the total DNA (10 μg) from each sample was digested with the EcoRI restriction enzyme (6 units/μg DNA), since pZO1052 plasmid has a unique EcoRI site (Fig. 1), for 22-24 h at 37°C. The digested DNA was then electrophoresed in 0.9% agarose gel in tris-borate electrophoresis (TBE) buffer at 22 volt for 14 h and transferred to a positively charged nylon membrane (Boehringer Mannheim Biochemicals). It was prehybridized in standard prehybridization buffer (750 mM NaCl, 75 mM sodium citrate, pH 7.0, 0.1% N-lauroylsarcocine, 0.02% sodium dodecyl sulfate, and 1% blocking reagent for nucleic acid hybridization: the Genius System, Boehringer Mannheim Biochemicals) at 65°C for 2 h and then hybridized with the digoxigenin (DIG)-labeled *hph*-specific probe for 22-24 h followed by post-
hybridization washing and detection of DIG-labeled probe using chemiluminescent substrate Lumigen PPD. Thereafter, the membrane was exposed to a Kodak XAR5-X-ray film. Duration of exposure to the film varied from 1-4 h depending on the signal strength. After the hybridization with hph-specific probe and detection, the membrane was stripped in alkaline probe-stripping solution at 37°C for 30 min. Then, it was reprobed with the uidA-specific probe beginning with the prehybridization step. Southern blot hybridization, detection of DIG-labeled probe, stripping and reprobing were carried out according to the Genius System manual (Boehringer Mannheim Biochemicals). The hph probe was prepared by PCR amplification using primers designed to amplify an internal 590 bp fragment within hph coding region as mentioned above and the pZO1052 plasmid as the template. The PCR-amplified fragments of the hph gene were purified using the GENECLEAN II Kit (BIO 101 Inc.). The probe was then labeled with digoxigenin-11-dUTP using the random primed method according to the Genius System manual (Boehringer Mannheim Biochemicals). The probe specific for uidA gene was prepared in the same way except that the uidA probe was amplified using primers designed to amplify an internal 1200 bp fragment within the uidA coding region.
Results and Discussion

Gene transfer by particle bombardment and selection of transformants

Embryogenic calli one- to two-weeks after subculture or suspension cells 3- to 4-days after subculture were bombarded with the plasmid pZO1052 DNA. Calli or suspension culture cells bombarded without plasmid DNA were included in all experiments as a control. The hph gene carried by plasmid pZO1052 confers hygromycin resistance to transformed cells. Therefore, after being bombarded and kept on callus maintenance medium for five days, the cells were then transferred to callus maintenance medium containing 200 mg/l hygromycin for transformant selection. Five weeks after selection, hygromycin-resistant colonies continued to grow while no resistant colonies were observed on control plates in any experiment (Fig. 2). One hundred and twenty-four resistant colonies were obtained in 27 plates from three separate experiments, with an average of 4.6 resistant colonies per bombardment. This frequency was comparable to that (three resistant colonies per bombardment) reported in soybean (Glycine max Merrill.) by Finer and McMullen (1991) and significantly higher than those reported in sugarcane (Saccharum species hybrid, CP72-1210) (six resistant colonies from 24 plates bombarded in four independent experiments; Chowdhury and Vasil, 1992) and in sorghum (Sorghum vulgare) (eight from 10 plates and three from five plates; Hagio et al, 1991). The resistant cell colonies were subsequently transferred to fresh callus maintenance
medium containing 200 mg/l hygromycin and to plant regeneration medium containing 200 mg/l hygromycin. Thirteen putative transgenic plants were regenerated from resistant cell colonies in one plate. One of the thirteen plants transferred to soil is shown in Fig. 3.

Transformation of protoplasts via electroporation and transformant selection

Protoplasts used for electroporation were isolated from suspensions 3- or 4-days after subculture which corresponded to suspension cultures at an exponential stage (Penmetsa and Ha, 1994). Electroporation was conducted in the presence of the plasmid pZG1052 for all samples except for controls in which electroporation was conducted without the plasmid. One month after electroporation, the protoplasts formed visible cell colonies. The protoplast-derived cells were transferred to agar-solidified MS medium containing 100 mg/l hygromycin for selection. In preliminary experiments, no further growth was observed when cells were transferred to a medium containing 150 mg/l hygromycin, which implied that the selection stringency might be too high. Therefore, a lower concentration of hygromycin, 100 mg/l, was used for selection. However, under this selection condition, a few (one to seven) viable colonies were observed in control plates, demonstrating incomplete selection. The failure of effective selection may be due to two facts: 1) the concentration of hygromycin used was too low; and 2) the time of application of hygromycin was not optimal. For effective transformant selection following electroporation, the
concentration of the selective agent and the application time need to be further optimized. However, since plant regeneration from callus which was used in particle bombardment was easier and faster than from protoplasts used in electroporation, and because a relatively high frequency of resistant colonies per bombardment was obtained with effective selection, no further optimization of selection conditions for electroporated protoplasts was attempted.

Albinism was encountered in this study. After protoplast-derived calli of untransformed controls were transferred onto plant regeneration medium B, only albino plantlets were regenerated from the calli. Seven of 50 resistant colonies obtained from transformation experiments gave rise exclusively to albino plantlets on plant regeneration medium B containing 150 mg/l hygromycin. These plantlets, however, did not continue to grow, indicating that they were not truly transformants. Although albinism is a common problem in cell culture of grasses (Takamizo et al., 1990), in the study described in Chapter I, only green plants were regenerated from protoplasts. Albinism in cell culture has been attributed to temperature (Takamizo et al., 1990; Van der Valk et al., 1988), the age of suspension cultures at the time of protoplast isolation (Takamizo et al., 1990; Srinivasan and Vasil, 1986), and 2,4-D concentration in the media (Creemers-Molenaar et al., 1988). Recovery of only albino plantlets from protoplasts isolated from older suspension cultures was reported in sugarcane (*Saccharum officinarum*) by Srinivasan and Vasil (1986). However, age-related albinism would not appear to be relevant in the present study because the
suspension cultures were newly established (about two months after suspension culture initiation). Suspension culture initiation and maintenance, as well as protoplast culture and regeneration were conducted as in Chapter I except that the temperature was much higher (33°C) than normal (26°C) for about one week. This was due to a breaking down of the air conditioning systems during suspension culture initiation. This may have caused albinism in the present study.

_Histochemical GUS expression analysis_

Of the 95 putative transformants tested (fifty-four resistant calli and 11 resistant plants from bombardment, and 30 resistant calli from electroporation), only one callus line showed blue cells when assayed histochemically for GUS expression. Similar results were found in wheat (*Triticum aestivum* L.) (three of 82; Vasil et al., 1992) and sugarcane (none in all cell lines tested, Chowdhury and Vasil, 1992; Rathus and Birch, 1992). The lack of GUS expression in most transformants is unclear. It might be due to rearrangement of the transgene, position effect on the transgene (Fang et al., 1989), and copy number-related methylation (Matzke et al., 1989).

_Confirmation of the transformation by PCR and Southern blot hybridization_

Putative transgenic plants obtained via particle bombardment were initially confirmed by PCR using primers specifically amplifying an internal 590 bp fragment of the *hph* and an internal 1.2 kb fragment of the *uidA* gene, respectively. All the 11
putative transgenic plants tested showed the presence of a band of the expected size for the hph fragment (Fig. 4 a) and a band of the expected size for the uidA fragment (Fig. 4 b). These bands were absent in the untransformed creeping bentgrass (lanes 3 in Fig. 4 a and b). The integration of foreign DNA (the pZO1052 plasmid) into the plant genome was further confirmed by Southern blot hybridization with the hph-specific and the uidA-specific probes. All of the eleven putative transgenic plants showed hybridization with both probes, demonstrating the presence of transgenes in the chromosomes of creeping bentgrass. However, only two hybridization patterns were observed among the eleven plants, indicating that these plants were probably derived from two cell lines. Southern blot hybridization with the hph-specific and the uidA-specific probes was also performed using total DNA from 10 callus lines which were randomly picked up from resistant colonies obtained from particle bombardment experiments. Of the 10 putatively transformed callus lines, eight showed hybridization with both the hph probe and the uidA probe, and two showed no hybridization with either probe. In all cases, no hybridization was observed in the controls. Figures 5 and 6 show Southern blot hybridization of creeping bentgrass transformants obtained via particle bombardment with the hph probe (Fig. 5) and the uidA probe (Fig. 6) for two representative transgenic plants (lanes 3 and 4), five transformed callus lines (lane 5 to 9), and an untransformed control (lane 2). In Fig. 5 and 6, multiple bands indicating multiple insertion events were observed in all transformants. Similar band patterns in different transformants were also observed.
Similar hybridization patterns in different individuals are commonly observed in Southern blot analysis of plant transformants, particularly when transformants were obtained via direct gene transfer methods (Klein et al., 1988b; Hayashimoto et al., 1990; Hagio et al., 1991; Wang et al., 1992). This type of pattern is likely attributed to favored sites for the insertion of foreign DNA into the recipient genome (Hess et al., 1990). In addition, different band intensity was observed within same individuals. This phenomenon is usually interpreted as single copy integration of a foreign gene in some sites vs. a comcatemeric rearrangement of multiple copy integration of the gene in some other sites (Czernilofsky et al., 1986). PCR amplification was performed using the DNA samples used in Southern analysis mentioned in Fig. 5 and 6. The plasmid pZO1052 was included in the PCR study as positive control. Figure 7 shows the presence of the bands of the expected size for the 590 bp fragment of the *hph* gene in positive control (lane 2) and in the transformants (lanes 4-10), and its absence in untransformant bentgrass (lane 3). This figure also shows the presence of the bands of the expected size for the 1200 bp fragment for the *uidA* gene in positive control (lane 11) and in the transformants (lanes 13-19), and its absence in untransformed bentgrass (lane 12). In lane 10 of Fig. 7, a band with an unexpected size was observed. This might be caused by the rearrangement of integrated foreign DNA with the recipient genome, but not by a non-specific amplification. This is because the stringency for PCR conducted in this study was high (annealing temperature was 62°C) and a non-specific amplification is not likely to occur under
a high stringency.

Southern blot hybridization was conducted for eight putative transformed cell lines derived from electroporated protoplasts. Only one sample showed the hybridization with the *hph* - and the *uidA*-specific probes (data not shown).

**Conclusions**

Two gene transfer techniques, particle bombardment and electroporation, were used for stable transformation of creeping bentgrass. A high frequency of hygromycin-resistant colonies was obtained in creeping bentgrass using particle bombardment and a rigorous transformant selection system. Among the 11 hygromycin-resistant plants and the 10 resistant calli tested, only two cell lines were escapes, indicating that transformant selection following bombardment was highly effective. However, in transformant selection after electroporation, a high frequency of escapes was observed due to the lack of efficient selection, resulting in inefficient recovery of transformants. This study demonstrated that the particle bombardment transformation system established here was efficient for improvement of creeping bentgrass via genetic engineering.
Fig 1. A diagram of plasmid pZO1052 (8.1 kb). The plasmid contains a uidA gene expression cassette which consists of the CaMV 35S promoter (35S, 0.5 kb), the maize Adh1 intron 6 (Io, 0.5 kb), uidA coding region (uidA, 1.8 kb), and nopaline synthase terminator (nos, 0.25 kb). The hph gene expression cassette of this plasmid consists of the same components except for the hph coding region (hph, 1 kb). These cassettes are in pUC19 (single line). Amp′ indicates the ampicillin resistance gene. The closed circle shows the origin of replication. The restriction sites illustrated are R, EcoRI; B, BglII; H, HindIII.
Fig 2. Selection of transformed creeping bentgrass cells after particle bombardment. Right: hygromycin-resistant colonies after five weeks of selection. Left: untransformed control.
Fig 3. A transgenic creeping bentgrass plant growing in soil.
Fig 4. PCR analysis of putative transgenic creeping bentgrass plants recovered after transformation via particle bombardment. (a) PCR was performed using primers amplifying an internal 590 bp fragment of the hph gene; (b) PCR was performed using primers amplifying an internal 1200 bp fragment of the uidA gene. In both (a) and (b): lane 1, λ HindIII DNA size marker; lane 2, plasmid pZO1052; lane 3, untransformed creeping bentgrass; lanes 4-14, putative transgenic creeping bentgrass plants.
Fig 5. Southern blot hybridization of creeping bentgrass transformants obtained via particle bombardment with digoxigenin-labeled *hph* probe. Genomic DNA was digested with EcoRI restriction enzyme. lane 1, digoxigenin-labeled DNA size marker; lane 2, untransformed control; lanes 3 and 4, transgenic plants; lanes 5 to 9, transformed calli.
Fig 6. Southern blot hybridization of creeping bentgrass transformants obtained via particle bombardment with digoxigenin-labeled *uidA* probe. Genomic DNA was digested with EcoRI restriction enzyme. Lane 1, digoxigenin-labeled DNA size marker; lane 2, untransformed control; lanes 3 and 4, transgenic plants; lanes 5 to 9, transformed calli.
Fig. 7. PCR confirmation of creeping bentgrass transformants obtained via particle bombardment. Lane 1, λ HindIII DNA size marker; lanes 2 and 11, plasmid pZO1052; lanes 3 and 12, untransformed creeping bentgrass; lanes 4 and 5, and lanes 13 and 14, transgenic plants; lanes 6-10 and lanes 15-19, transformed calli. Lanes 2-10, PCR was performed using primers amplifying an internal 590 bp fragment of the hph gene. Lanes 11-19, PCR was performed using primers amplifying an internal 1200 bp fragment of the uidA gene.
References


87


Summary

Establishment of genetic transformation systems for use in the development of transgenic creeping bentgrass (*Agrostis palustris*) with useful new traits was the goal of this study. The research conducted included the following work: 1) development of embryogenic callus and suspension cultures; 2) optimization of electroporation conditions; 3) evaluation of strengths of several 5' regulatory sequences on transient gene expression in transformed protoplasts; and 4) stable transformation of creeping bentgrass via particle bombardment and electroporation.

Development of embryogenic cell cultures is a prerequisite for crop improvement via genetic engineering. Embryogenic callus cultures were established in creeping bentgrass by Krans et al. (1982) and Zhong et al. (1991), and embryogenic suspension cultures were obtained by Terakawa et al. (1992). However, some of the results obtained by Krans et al. (1982) and by Zhong et al. (1991) were inconsistent. In addition, we could not obtain embryogenic suspension cultures using protocol developed by Terakawa et al. (1992). Therefore, development of a reliable protocol for initiating and maintaining embryogenic callus and suspension cultures in creeping bentgrass was necessary. By substantially modifying and combining the procedures developed by Krans et al. (1982), Terakawa et al. (1992) and Zhong et al. (1991), such a protocol was developed. Embryogenic callus was induced and
maintained on Murashige and Skoog (MS) medium supplemented with 1 mg/l 2,4-
dichlorophenoxyacetic acid (2,4-D). Cell suspensions, suitable for protoplast isolation,
were initiated using this callus and cultured in MS liquid medium containing 1 mg/l
2,4-D and 3% maltose. Plant regeneration from seed-derived calli and protoplast-
derived calli was induced on MS medium with 0.01 mg/l 2,4-D, 0.1 mg/l kinetin and
3% maltose, and on half-strength MS medium with 1.5% maltose, respectively. A
high frequency of plant regeneration was obtained from both seed-derived calli and
suspension-derived protoplasts was obtained following this protocol.

Several factors affecting electroporation efficiency were studied using transient
gene expression assay following the introduction of the reporter uidA gene encoding
β-glucuronidase (GUS). In a field strength study, GUS activity peaked at 950 V/cm.
Increases in the amount of plasmid DNA resulted in increases in GUS activity. No
plateau was reached within the plasmid DNA concentrations tested (0 to 200 μg/ml).
However, the slope of the increase in GUS activity gradually declined. GUS activity
also increased in response to increases in protoplast density up to 2 x 10⁶/ml; thereafter it plateaued. Maximal GUS activity was obtained when 125 mM KCl was
used in the electroporation buffer. This study facilitated setting the optimal
electroporation conditions which are essential not only for efficient transient gene
assay but also for successful transformation in creeping bentgrass.

An important factor affecting transformation is the expression level of the
transgene. In general, the expression level of a transgene is proportional to the
strength of its 5' regulatory sequences which are involved in transcription initiation. The effects of six different 5' regulatory sequences on gene expression in creeping bentgrass were studied using transient gene expression assay following electroporation. Among the six 5' regulatory sequences tested, the cauliflower mosaic virus 35S promoter was the least active and the rice actin 1 gene promoter plus its intron 1 was the most active. Ranked in order of activity (high to low), the other four 5' sequences were: 1) the combination of the CaMV 35S promoter and the maize alcohol dehydrogenase 1 gene (Adh1) intron 6; 2) the 5' region of the maize ubiquitin gene; 3) the maize Adh1 promoter plus its intron 1; and 4) the combination of the 35S promoter and the Adh1 intron 1. This study provides important information concerning the choice and design of transformation vectors for creeping bentgrass as well as for other monocot species.

To establish an efficient gene transfer system in creeping bentgrass, both particle bombardment and electroporation were examined. Transgenic plants and calli were obtained via particle bombardment. A relatively high frequency of putative transformants with an average of 4.6 resistant colonies per bombardment was obtained. Southern analyses confirmed the integration of foreign genes in all of the 11 putative transgenic plants tested and in eight of 10 putative transformed calli tested, thus showing that transformant selection following bombardment was highly effective. However, a high frequency of escapes occurred in transformant selection following electroporation. Viable colonies were observed in controls at nearly the
same frequency as observed in transformed samples on hygromycin-containing medium. Therefore, the selection was not complete and ineffective which resulted in an inefficient recovery of transformants. Only one of eight resistant colonies test in Southern analysis was demonstrated to be truly transformed. Albinism was encountered in experiments for stable transformation of bentgrass via electroporation. Albino plantlets were exclusively given rise from protoplast-derived calli. This may be attributed to the higher temperature (33°C) compared to the normal temperature (26°C) in the laboratory due to the breaking down of the air conditioning system during suspension culture initiation.

In this study, efficient genetic transformation systems using particle bombardment were established. Use of these systems will facilitate the improvement of creeping bentgrass. In addition, an assay for transient gene expression following electroporation of protoplasts was developed. This system can be used for rapid testing of vectors and for studies of cis-acting regulatory elements of plant genes in creeping bentgrass.
References


VITA

The author was born in Chongqing Municipality, Sichuan Province, People’s Republic of China, on August 3, 1956. In January of 1982, she graduated from the Department of Biology at Southwest China Normal University with a Bachelor’s degree in Science.

From 1982 to 1987, the author taught and researched as an assistant professor in the Department of Biology at Southwest China Normal University. In 1987, she was promoted to the position of lecturer which is higher than assistant professor and lower than associate professor in China’s higher education system. In September of 1989, she came to the U.S. as a visiting scholar to the Virginia Polytechnic Institute and State University and researched in the Department of Biochemistry and Nutrition.

In 1990, the author was enrolled in a Ph.D program in the Department of Crop and Soil Environmental Sciences at the Virginia Polytechnic Institute and State University.