BERMUDAGRASS TISSUE CULTURE AND GENETIC TRANSFORMATION THROUGH *AGROBACTERIUM* AND PARTICLE BOMBARDMENT METHODS

By

ARNOLD SARNE PARCO

Bachelor of Science
University of the Philippines at Los Banos
Los Banos, Laguna, Philippines
1988

Master of Science
Oklahoma State University
Stillwater, Oklahoma
2000

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Thesis Approved:

Dr. Charles M. Taliaferro

Thesis Adviser

Dr. Michael P. Anderson

Dr. Bjorn C. Martin

Dr. Arron C. Guenzi

Dr. Ulrich K. Melcher

Dr. A. Gordon Emslie

Dean of the Graduate College

PREFACE

The primary objective of this dissertation was to establish and optimize methods for bermudagrass tissue culture and genetic transformation. Callus production and subsequent plant regeneration using different explant tissues were evaluated on solid MS nutrient media and the promotive effects of plant growth regulator supplements were studied (Chapter 2). Caryopsis-derived callus culture was developed to provide a regeneration system for bermudagrass in addition to the more commonly utilized young inflorescences and stolon nodes. The choice and preparation of suitable materials for the genetic transformation experiments such as plasmid vector, selectable marker, expression cassette for genes of interest, and *A. tumefaciens* strain are described in Chapter 3.

A practical objective of this dissertation was the production of transgenic bermudagrass plants over-expressing a phytochrome (*PHY*) gene which may potentially exhibit improved photomorphogenic characteristics particularly in response to shade conditions- a research proposal conceptualized a few years ago in Dr. Arron Guenzi's laboratory at the OSU Plant Transformation Facility. Phytochrome photoreceptors serve as light-switchable components of several transcriptional regulator complexes. Increasing levels of phytochrome activity can lead to beneficial outputs downstream of light signal perception pathways.

Chapter 4 deals with production and identification of hygromycin-resistant bermudagrass plants transformed with pCAMBIA1305.2 binary vector carrying the

Sorghum bicolor PHYC gene under control of the constitutive maize *Ubi-1* promoter. Seven transgenic plants were directly regenerated from stolon nodes of Guymon co-cultivated with *Agrobacterium EHA105* harboring the binary vector, while twenty-seven plants were obtained from caryopsis-derived callus tissues of Yukon subjected to particle bombardment. PCR analysis and preliminary Southern blot hybridization were performed on total plant genomic DNA. More detailed confirmation tests as well as morphological and physiological examination of transgenic plants with regards to *PHYC* over-expression will be addressed in future studies.

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TABLE OF CONTENTS

Chapter		Page
1	BERMUDAGRASS IMPROVEMENT PROGRAMS AND OBJECTIVES OF THIS DISSERTATION	
	Brief Botanical Descriptions	1
	Bermudagrass Breeding	2
	The Shade Tolerance Problem and Roles of Phytochrome Photoreceptors	4
	Objectives of this Dissertation	7
	References	8
2	REGENERATION OF BERMUDAGRASS FROM TISSUE CULTURE USING MS AGAR NUTRIENT MEDIA	
	Abstract	12
	Introduction	13
	Objectives	18
	Materials and Methods	18
	Results and Discussion	23
	Summary and Conclusions	44
	References	45
3	BINARY VECTOR, GENE CONSTRUCTS, *A. TUMEFACIENS* STRAIN, AND HYGROMYCIN SELECTION FOR GENETIC TRANSFORMATION OF BERMUDAGRASS	
	Abstract	48
	Introduction	49
	Objectives	51
	Materials and Methods	51
	Results and Discussion	58
	Summary and Conclusions	83
	References	84

4 GENETIC TRANSFORMATION OF BERMUDAGRASS THROUGH *AGROBACTERIUM* AND PARTICLE BOMBARDMENT METHODS

Abstract 86
Introduction 87
Objectives 91
Materials and Methods 91
Results and Discussion 96
Summary and Conclusions 104
References 105

Appendix A: Media Used in Bacterial Cultures 110

LIST OF TABLES

Table Page

2.1 Plant growth regulators used in plant tissue culture. 14

2.2 Previous reports of callus initiation (C), embryogenic callus formation (EC), and plant regeneration (R) in bermudgrass tissue culture. 17

2.3a Percent callus initiation (%C) and plant regeneration (%R) from mature caryopses of four seeded bermudagrass cultivars on MS agar medium under nine PGR supplementations. 24

2.3b Analysis of variance in callus initiation data given in Table 2.3a. 24

2.3c Analysis of variance in plant regeneration data given in Table 2.3a. 25

2.4 Percent callus initiation (%C) and plant regeneration (%R) from immature caryopses of five seeded bermudagrass cultivars on MS agar medium under nine PGR supplementations. 27

2.5. Numbers of callus tissues (C) and regenerated plants (R) obtained from young inflorescence explants of six bermudagrass cultivars on MS agar medium under nine PGR supplementations. 29

2.6. Numbers of callus tissues obtained from nodal explants of six bermudagrass cultivars on MS agar medium under nine PGR supplementations. 34

3.1 Full-length cDNAs that were subcloned in the binary plasmid vector pCAMBIA1305.2 and provided the *Ubi-1* promoter sequence. 52

3.2 PCR primers used to amplify fragment of *Agrobacterium virb1* and of genes carried by the pCAMBIA vector. 57

3.3 Growth of EHA101 and EHA105 in different antibiotics. 74

3.4 Numbers of dead seedlings of Guymon and Yukon after transplanting to MS agar media with different levels of hygromycin B. 79

4.1 Percentage of caryopsis-derived callus tissues of Yukon displaying transient GUS expression 5 days after bombardment with pCAMBIA1305.2 vector. 98

LIST OF FIGURES

Figure Page

2.1 Explant tissues of bermudagrass and callus formation. 20

2.2 Numbers of callus tissues obtained from nodal explants of six bermudagrass cultivars under nine PGR supplementations. 35

2.3 Seed germination and callus initiation in bermudagrass caryopses under different levels of 2,4-D. 40

2.4 Percent callus differentiation in caryopsis culture of twelve bermudagrass cultivars under four PGR regimens. 41

2.5 Growth and differentiation of caryopsis-derived callus tissues of Arizona Common on MS agar media under different PGR supplementations. 42

2.6 Caryopsis culture as regeneration system for bermudagrass genetic transformation. 43

3.1 Modification of pAHC17 cloning site and isolation of "*Ubi-1* promoter – cloning site – NOS polyA" fragment. 59

3.2 Ligation of "*Ubi-1* promoter – cloning site – NOS polyA" fragment of pAHC17 to pCAMBIA1305.2 binary vector. 65

3.3 Nucleotide sequence of *Ubi-SbPHYC-NOS* in truncated pCAMBIA1305.2. 68

3.4 Nucleotide sequence of *Ubi-CynCHT1-NOS* in truncated pCAMBIA1305.2. 71

3.5 PCR amplification of gene fragments from EHA105 and *E. coli* (DH5α) cells. 76

3.6 Death of Guymon and Yukon seedlings under different levels of hygromycin B. 80

3.7 Germination of Guymon and Yukon seeds under different levels of hygromycin B. 81

3.8 Death of callus-derived shoots of Guymon and Yukon under different levels of hygromycin B. 82

4.1 Transient GUS expression in caryopsis-derived callus tissues of Yukon bombarded with pCAMBIA1305.2. 98

4.2 PCR amplification of the *hptII* gene from total genomic DNA of hygromycin-resistant plants. 102

4.3 Southern hybridization of *Xho*I-digested genomic DNA of hygromycin-resistant plants with the *hptII* gene probe. 103

4.4 Stolon nodes of Guymon co-cultivated with *A. tumefaciens* EHA105 harboring pCAMBIA1305.2:*Ubi-SbPHYC*. 104

ABBREVIATIONS

2,4-D	2,4-dichlorophenoxyacetic acid
ABA	abscisic acid
bp	base pair
x *g*	centrifugal force
dNTPs	deoxynucleotides
g	gram
kb	kilobase
L	liter
Hg	mercury
M	molar, mole per liter
MS	Murashige and Skoog
OD	optical density
PGR	plant growth regulator
PCR	polymerase chain reaction
rpm	revolution per minute

CHAPTER 1

BERMUDAGRASS IMPROVEMENT PROGRAMS AND OBJECTIVES OF THIS DISSERTATION

BRIEF BOTANICAL DESCRIPTIONS

Bermudagrass (*Cynodon* species) is a sod-forming, highly variable perennial grass of African origin that is found in over 100 countries throughout tropical and warmer temperate climates (Duble, 1996). Low temperature (below -1°C) is the main environmental factor that limits its adaptability. Optimum growth is achieved when daily temperatures are between 35°C and 38°C. In the United States, bermudagrass is best adapted and most widely distributed in the southeast. It is widely used with irrigation in the southwest and is sparsely found in many northern states. Bermudagrass reproduces by seeds and asexually through above-ground stolons and underground rhizomes. The stolon nodes readily develop roots and lateral buds which produce erect or ascending stems reaching up to 40 cm. The sharply pointed leaves are 2 to 16 cm long, 1.5 to 5 mm wide, and are borne on stems with long internodes alternating with short internodes. Inflorescences are 3 to 10 cm long and often consist of 3 to 7 spikes in a single whorl. The root system is fibrous and perennial, and produces vigorous deep rhizomes.

Serious insect pests that feed on bermudagrass foliage and stems include armyworms, webworms, cutworms, mealybugs, and bermudagrass mites. Leaf spot, dollar spot, spring dead spot, and brownpatch are fungal diseases causing significant

damage to bermudagrass turf. Cultural, biological, and chemical methods have been applied to control these pests and diseases. Regarded as a durable, drought-resistant, and salt-tolerant turf and forage grass, bermudagrass is also considered in many countries as a serious weed of crops such as sugarcane, corn, cotton, and vineyards that are grown in high rainfall climates or irrigated areas.

Bermudagrass is utilized for pasture, hay production, turf, and erosion control on roadsides, ditch banks, and airfields. As turf, it is used in home lawns, sports fields, parks, and golf course greens and fairways. Among nine species of the genus *Cynodon*, the so-called common bermudagrass, *Cynodon dactylon* (L.) Pers ($2n = 4x = 36$), is most widespread being used both as turf and forage in temperate and tropical regions of the world. Other species were introduced in the U.S. for turf purposes including *C. transvaalensis*, Burtt-Davy, *C. magennisii* Hurcombe, and *C. bradleyi* Stent (Alderson and Sharp, 1995). Interspecific hybridization has been important in the development of improved varieties. The triploid ($2n = 3x = 27$) and vegetatively propagated hybrids of *C. dactylon* x *C. transvaalensis* provide high quality turf for lawns and sports fields as they possess fine leaf texture at high density and low growth profile.

BERMUDAGRASS BREEDING

Turf characteristics desired for bermudagrass include ability to survive high and low temperature extremes, enhanced resistance to diseases, insects, nematodes, and weed encroachment, less demand for mowing, irrigation, and fertilization, tolerance to acid, alkaline, or saline soil, shade tolerance, as well as stability of hereditary characteristics.

Superior strains of common bermudagrass, natural hybrids between *C. dactylon* and *C. transvaalensis*, and products of crosses from breeding programs have been released by state universities, the Crop Research Division of USDA, and by the U.S. Golf Association Green Section. The development of more cold-tolerant turf-type varieties such as U-3 and Midiron extended reliable use of bermudagrass in the U.S. Turf bermudagrass breeding at Oklahoma State University has been aimed at developing progressively improved and broadly adapted seeded cultivars and superior clonally-propagated hybrids for the U.S. transition zone. This program continuously evaluates *Cynodon* germplasm accessions for important descriptors such as seed production potential, cold tolerance, and other traits. Development of new bermudagrass turf cultivars remains a major objective of the U.S. Golf Association's Turfgrass and Environmental Research Program. The USGA project aims to improve turfgrasses for better tolerance to environmental stresses such as heat, extreme cold, poor soil quality, diseases, and pests, which would decrease reliance on pesticides and fertilizers and help conserve water resources (Keena, 1999; Nus *et al.*, 2003).

Cellular and molecular tools are becoming more extensively applied in non-cereal grasses such as turf and forage crops which generally have not been amenable to biotechnological techniques due to their genetic complexity and recalcitrance to *in vitro* manipulations. For instance, transgenic plants expressing novel genes or genes with altered properties are being utilized in breeding programs to develop improved and low input turf and pasture. Genetic engineering of bermudagrass has been initiated by different groups of researchers in order to address biotic and abiotic problems. Sting nematode resistance, drought tolerance, and herbicide resistance are useful traits being

engineered in bermudagrass (Qu, 1999; Zhang *et al*., 2003; Goldman *et al.,* 2004). In retrospect, further refinement of *in vitro* procedures for bermudagrass is needed to facilitate more efficient applications of gene transfer technology.

THE SHADE TOLERANCE PROBLEM AND ROLES OF PHYTOCHROME PHOTORECEPTORS

Poor shade tolerance limits utilization of turf bermudagrass even in geographic areas where it is otherwise well adapted (Beard, 1973; Peacock and Dudeck, 1993). Shades caused by trees and buildings result in rapid shoot growth and unsightly areas of discolored and deteriorating bermudagrass. Although given less priority in bermudagrass breeding programs, this problem entails huge costs due to yearly turf replacement and maintenance. At low light intensities (less than 60% of full sunlight), bermudagrass develops narrow elongated leaves, thin upright stems, long internodes, weak rhizomes, and very sparse turf. To control excessive shoot growth and maintain turf quality of bermudagrass and other turf species under reduced irradiance, application of gibberellic acid biosynthesis inhibitors such as trinexapac-ethyl, paclobutrazol, and flurprimidol has been a management practice (Watschke and DiPaola, 1995; Fagerness and Yelverton, 2000).

Shade tolerance refers to a suite of leaf-level traits that permit maximal net carbon fixation under low irradiances, and differences in leaf physiology account for variation in the strength of shade tolerance between plants (Bjorkman, 1981; Henry and Aarssen, 1997). Shade is phytochrome-mediated perception of enrichment of far-red light or

reduction in red:far-red ratio which elicits shade-avoidance responses in many plant species. These responses include enhanced vertical growth, reduced lateral branching, long internodes and petioles, increased length-to-width ratio of leaves, and decreased resource allocation to roots, leaves, and fruits due to carbohydrate depletion. Inappropriate expression of shade-avoidance traits is maladaptive (Pigliucci and Schmitt, 1999).

The phytochrome photoreceptors of plants absorb the red (600-700 nm) and far-red (700-750 nm) regions of the electromagnetic spectrum, and serve as light-switchable components of several transcriptional regulator complexes (Martinez-Garcia *et al*., 2000; Neff *et al.,* 2000). By sensing light wavelength, distribution, and periodicity, they control many downstream cellular and physiological processes that modulate plant adaptation to light and shade (Casal *et. al*, 1998; Smith, 2000).

A phytochrome molecule is a dimer of large polypeptides (~125 kilodaltons) each with a covalently bound linear tetrapyrrole chromophore. At least five phytochrome apoprotein genes, *PHYA* to *PHYE*, were found in a number of plant species (Mathews and Sharrock, 1996). *PHYB, D*, and *E* are evolutionary related and separated from *PHYA* and *C*. *PHY* genes display only minor differences in their expression patterns and appear to play distinct, redundant, or antagonistic roles during plant development (Somers and Quail, 1995; Goosey *et al*., 1997; Qin *et al*., 1997; Whitelam and Devlin, 1997; Kircher *et al*., 2002; Moller *et al*., 2002). *PHYA* was implicated in seed germination, perception of day length, and in many aspects of seedling de-etiolation. *PHYB* functions in seed germination and seedling de-etiolation, and has main roles in shade-avoidance response. *PHYD* and *E* are mediators of shade-avoidance responses such as petiole elongation and

flowering time, with *PHYE* having a specific role in controlling internode elongation. *PHYC* has similar photosensory specificity to *PHYB* but a distinctive capacity for enhancing leaf expansion. *PHYC* also functions in the perception of daylength but appears not to play a major role in responses to low red:far-red ratio. All five phytochrome apoproteins were shown to be imported into the nuclei of plants and their translocation is regulated differentially by light. This indicates that the nucleus is a hot-spot for early events in phytochrome signaling.

Studies involving transgenic plants have demonstrated that phytochrome over-expressors acquire altered photomorphogenic characteristics and developmental traits in response to light (Cherry *et al.,* 1991; Casal and Sanchez, 1994; Clough *et al.,* 1995; Halliday *et al*., 1997; Yanovsky *et al*., 1998; Thiele *et al.,* 1999; Garg *et al*., 2006). Notable consequences of increased phytochrome activity were suppression of internode elongation, reduction in petiole length, increased tillering, higher chlorophyll content, delayed leaf senescence, and higher yields of harvestable plant parts. These researches suggest that genetic engineering strategy may overcome the shade-avoidance responses of plants grown under reduced irradiation by altering the photosensory system. Thus, biotechnological application of phytochromes may provide a means for maintaining healthy and aesthetically pleasing turfgrasses in both high and low light environments with minimal environmental impact.

OBJECTIVES OF THIS DISSERTATION

Regeneration of bermudagrass from somatic embryo culture was first reported by Ahn *et al.* (1984) while a genetic transformation system was first described by Zhang *et al.* (2003). Succeeding studies (reviewed in Chapters 2 and 4) collectively suggest that outcomes of these *in vitro* procedures are dependent on plant genotypes, explant tissues, and composition of nutrient media. Tissue culture of bermudagrass has usually employed stolon nodes and young inflorescences. The potential of other tissues for somatic embryogenesis such as caryopses, shoots, and root tips, and their advantages as explant source have not been examined adequately. Gene transfer experiments have also used either nodal or inflorescence explants, and in four reported transformation studies only two bermudagrass genotypes (TifEagle and *C. dactylon* cv J1224) were used.

This study aimed to establish methods and obtain further information on bermudagrass tissue culture and genetic transformation. For this purpose, culture performance of caryopsis, young inflorescence, and nodal explants of selected bermudagrass cultivars was evaluated on MS agar nutrient media. The promotive effects of the plant growth regulators 2,4-D, kinetin, and ABA on callus production and somatic embryogenesis were studied. Suitable explant for use in genetic transformation experiments was identified, and the culture method was further developed.

Another objective of this research was to produce transgenic bermudagrass plants over-expressing a phytochrome gene for photomorphogenic studies particularly with regards to shade tolerance improvement. Full-length cDNAs of *PHYA, B,* and *C* genes from heterologous sources were subcloned into the T-DNA region of the binary plasmid vector pCAMBIA1305.2 and each was provided with the maize *Ubi-1* gene promoter. In

the present study, transfer of *Sorghum bicolor PHYC* (the first cDNA that was properly subcloned in the pCAMBIA vector) to *C. dactylon* cvs Guymon and Yukon was undertaken through *Agrobacterium tumefaciens* and microprojectile bombardment. PCR and preliminary Southern blot analyses were performed on total genomic DNA of hygromycin-resistant plants to demonstrate gene integration and to assess efficiency of the gene transfer methods used. To this extent, plant materials were made available for studying morphological and physiological consequences of a constitutively expressed phytochrome gene in bermudagrass.

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CHAPTER 2

REGENERATION OF BERMUDAGRASS FROM TISSUE CULTURE USING MS AGAR NUTRIENT MEDIA

ABSTRACT

Simple and reproducible tissue culture methods to regenerate bermudagrass plants through somatic embryogenesis will help facilitate and enhance the efficiency of genetic transformation experiments. In this study, regeneration potential of callus tissues derived from bermudagrass caryopses, young inflorescences, and nodal segments was evaluated on MS agar nutrient media supplemented with plant growth regulators (PGR). All three explant types were capable of callus production under 2,4-D supplementations, but no plants were regenerated from nodal segments. Callus induction and plant regeneration frequencies varied with cultivar and PGR treatment. The auxin 2,4-D was essential for callus formation but not kinetin and ABA. However, low levels (1-3 mg L^{-1}) of kinetin and/or ABA were found to have promotive effects on embryogenesis of caryopsis-derived callus tissues. Because caryopsis explants are easy to prepare and produce callus tissues within a relatively short time compared with young inflorescences, a protocol for culturing mature caryopses was further developed for use in genetic transformation experiments.

INTRODUCTION

In vitro plant regeneration is a crucial part of genetic and molecular improvement strategies for various cereal and perennial grass species (Ozias-Akins and Vasil, 1982; Chaudhury and Qu, 2000; Bradley *et al.*, 2001). Production of transgenic plants with any gene transfer method depends on having an efficient cell, tissue, or organ culture system. Plant regeneration from culture may occur either through somatic embryogenesis or organ differentiation. For the purpose of genetic transformation, somatic embryogenesis is preferred over organogenesis because embryos usually originate from single cells that are suitable targets for genetic manipulation. Resulting plants are uniformly transformed and are not chimeras. Embryos derived from somatic cells lack seed coats and endosperm but develop into mature plants in much the same way as their zygotic counterparts that originate from fertilized eggs. Poaceous tissue culture has utilized varied explants including shoot apices, root tips, young inflorescences, nodes, and seed scutella that produce unorganized, proliferated masses of callus cells which can later develop into somatic embryos (Hanning and Conger, 1982; Wernicke and Milkovits, 1986; Fei *et al.,* 2001). Apical meristems, nodal regions, and scutella contain cells that have the potential to develop into bud primordia or floral meristems during normal plant growth. Immature inflorescences have differentiated from leaf primordia to floral primordia which give rise to ovules and pollen.

Four major classes of plant growth regulators (PGR) or hormones are utilized in plant tissue culture: auxins, cytokinins, gibberellins, and abscisic acid (Table 2.1). Requirements for these hormones differ among genera, species, and cultivars (Brock and Kaufman, 1991). Auxins promote rapid cell growth, enlargement, and proliferation,

Table 2.1. Plant growth regulators used in plant tissue culture.

Range of working concentrations (mg L^{-1}) in plant tissue culture.*		Concentrations (mg L^{-1}) used in bermudagrass tissue culture.**	
		Callus Induction and Maturation	Plantlet Regeneration
Auxin	0.01 - 10	**2,4-D:** 0.01, 0.1, 0.25, 0.5, 1, 2, 3, 4, 5, 6, 7 **dicamba:** 6.5 **IAA:** 1 **NAA:** 1 **pichloram:** 1, 3	**2,4-D:** 0.13, 0.25, 0.5 **NAA:** 0.2
Cytokinin	0.1 - 10	**BAP:** 0.01, 0.2, 0.5, 1, 4.5 **thidiazuron:** 1, 3	**BAP:** 0.5, 1, 2, 2.5 **zeatin:** 1
ABA	0.1 - 10	0.5, 2.5	
GA_3	0.01 – 5		0.2, 0.5

* PhytoTechnology Lab. Handbook (Shawnee Mission, Kansas)
** Based on references listed in Table 2.2.

callus formation, lateral rooting, adventitious budding, and induction of somatic embryogenesis. High auxin levels or prolonged exposures result in suppressed morphogenesis. Several forms of auxin are known including 2,4-dichlorophenoxyacetic acid (2,4-D), indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), α-naphthalene-acetic acid (NAA), picloram, and dicamba.

Cytokinins regulate growth and morphogenesis, stimulate cell division, induce shoot and axillary shoot proliferation but inhibit root formation, and are known to activate RNA synthesis and enzyme activities. Kinetin, zeatin, 6-benzylaminopurine (BAP), thidiazuron (TDZ) are popular forms of cytokinins.

The most commonly used form of gibberellins is gibberellic acid (GA_3) which enhances cell enlargement and callus growth, promotes flowering and elongation of

shoots and stems, breaks dormancy of seeds, buds, corms, and bulbs, and elongates dwarfed or stunted plantlets.

Abscisic acid (ABA) promotes leaf abscission and dormancy development in embryos, buds, and bulbs, inhibits precocious germination of embryos but stimulates their maturation, enhances formation of shoots, buds, bulbs, and tubers, and either inhibits or stimulates callus growth depending on the plant species.

Nutrient media for grass tissue culture are often supplemented with auxin and low levels of cytokinin and ABA (Bhaskaran and Smith, 1990). Application of auxin such as 2,4-D is crucial for callus formation and embryogenesis. Endogenous levels of ABA were shown to be important for production of regenerable cultures in some monocots (Rajasekaran *et al.,* 1987), and therefore must be supplied exogenously when needed. Addition of cytokinin can lower regenerability if adequate amounts of the hormone are already present in the explant tissues. For instance, supplementation of zeatin in orchardgrass culture inhibited embryogenesis in genotypes with normally high capacity for zeatin regeneration (Wenck *et al.,* 1988). Essentially, the types and relative amounts of PGRs present during culture steps significantly influence the extent of morphogenesis and plant regeneration.

Perennial grasses are generally difficult to manipulate *in vitro* and regeneration potential is often restricted to few genotypes or cultivars. Regenerability was regarded to be genetically controlled and a dominant trait (Ma *et al*., 1987; Willman *et al*., 1989). However, it is also possible that the process is largely a physiological phenomenon that can be manipulated by exogenous administration of PGRs. Explant tissues taken from the same plant may not respond identically in culture due to varying gradients of endogenous

hormones (Wernicke and Brettell, 1982). Different organs differ in hormone metabolism and this can be further influenced by seasons or climate changes. Thus, the types and levels of supplemental PGRs necessary for optimal callus induction and plant regeneration need to be established for a cultivar of a species and for the explant tissues used.

Tissue culture methods were reported for some bermudagrass cultivars (Table 2.2). In most cases, plants were regenerated from cultured somatic embryos derived from tissues of young inflorescences (Ahn *et al*., 1985; Ahn *et al.,* 1987; Li and Qu, 2004; Jain *et al*., 2005) and stolon nodes (Croughan, 1985; Goldman *et al*., 2004). Culture performance of young inflorescences widely varies with cultivar, age of tissues, season, and medium composition (Ahn *et al.*, 1985; Artunduaga *et al*., 1988). Stolons are abundantly available but require cumbersome liquid culture phase to proliferate embryogenic callus cells. Moreover, regenerated plants often acquire somaclonal and ploidy level changes (Goldman *et al*., 2004). Plant regeneration using other explant tissues such as seeds or caryopses has not been studied adequately. Krans (1981) produced callus tissues from mature caryopses and only regenerated roots but not shoots. Ahn *et al.* (1984) produced callus tissues from mature caryopses, nodes, root tips, young leaves, and older inflorescences but did not regenerate any plant. Recently, high callus induction and plant regeneration frequencies were reported in common bermudagrass using mature seed explants (Salehi and Khosh-Khui, 2005).

Table 2.2. Previous reports of callus initiation (C), embryogenic callus formation (EC), and plant regeneration (R) in bermudagrass tissue culture.

(Values given are from best PGR supplementation when different media were tested; ns: percentage not explicitly specified by the authors)

References	Cultivar	Explant	%C	%EC	%R	Nutrient Medium
Ahn *et al.,* 1985	common bermudagrass	young inflorescences	ns	0-84	ns	NB, N_6, and MS agar
Ahn *et al.,* 1987	74 X 12-6 Tifton 44 Tifway common bermudagrass	young inflorescences young inflorescences young inflorescences young inflorescences	93 87 97 10	29 15 7 50	ns ns ns ns	N_6 agar N_6 agar N_6 agar N_6 agar
Artunduaga *et al*., 1988	A-10978b (90% of regenerants were albinos) A-12164 Brazos	young inflorescences young inflorescences young inflorescences	ns ns ns	50 43 42	ns ns 0	MS agar MS agar MS agar
Artunduaga *et al*., 1989	Zebra	young inflorescences	ns	ns	ns	MS agar
Croughan and Quisenberry, 1989	Brazos Grazer OSU LCB W26	nodes nodes nodes	ns ns ns	ns ns ns	ns ns ns	all on MS phytagel + suspension
Williams, 1994	Guymon Tifgreen	mature seeds young inflorescences	ns ns	ns ns	ns ns	MS agar MS agar
Chaudhury and Qu, 2000	Savannah Tifgreen Tifgreen	young inflorescences young inflorescences nodes	93 70 86-100	ns ns 0	41 24 0	MS phytagel MS phytagel MS phytagel
Qu and Chaudhury, 2001	Tifway	young inflorescences	53	ns	5.5	MS phytagel
Li and Qu, 2002	Savannah Tifgreen	young inflorescences young inflorescences	ns ns	ns ns	ns 85.3	MS phytagel MS phytagel
Zhang *et al.,* 2003	TifEagle	nodes	ns	ns	ns	MS phytagel + suspension
Goldman *et al*., 2002, 2004	TifEagle, TifSport	nodes	ns	36	ns	MS agar
Li and Qu, 2004	J1224 9 other *Cynodon. dactylon* cultivars	young inflorescences young inflorescences	ns ns	30-40 5-30	ns ns	MS phytagel MS phytagel
Jain *et al*., 2005	Tifton 85	young inflorescences	88	ns	>60	MS phytagel
Salehi and Khosh-Khui, 2005	common bermudagrass	mature seeds	100	ns	100	MS agar

OBJECTIVES

1. Examine callus initiation and plant regeneration frequencies in mature and immature caryopses, young inflorescences, and young shoots and nodal tissues from stolons of selected bermudagrass cultivars on MS agar nutrient media under different PGR supplementation.
2. Choose suitable bermudagrass cultivars and explant type for use in genetic transformation experiments, and further improve tissue culture procedures.

MATERIALS AND METHODS

Bermudagrass Plants

Six cultivars were initially used: Riviera, Yukon, Arizona Common, Tifway, PRC58, and Brazos. Vegetative cuttings were taken from field plots and grown in trays (53 X 38 X 10 cm) in the greenhouse for explant tissue collection. Riviera, Yukon, and Arizona Common are turf-type varieties of the common bermudagrass (*C. dactylon*). Tifway is an industry standard turf and interspecific triploid hybrid of *C. dactylon* and *C. transvaalensis*. PRC58 (Chinese accession having both turf and forage characteristics) and Brazos (hybrid forage bermudagrass) are *C. dactylon* that were previously regenerated from young inflorescence explants (Artunduaga *et al.,* 1988). Other seeded *C. dactylon* cultivars were used in succeeding experiments on mature caryopses: Wrangler, Princess, Mohawk, Guymon, Giant Bermudagrass, Cheyenne, PRC18, and CD194.

Explant Tissues

The following explants were used: mature and immature caryopses, young inflorescences, and young shoots and nodal segments from stolons (Figure 2.1). Dehulled seeds from germplasm collection provided ample supply of mature caryopses. The other explant tissues were obtained from plants grown in the greenhouse. Immature seeds were extracted by hand from inflorescences collected approximately 2 weeks after anthesis, and the hulls were removed to obtain the immature caryopses. Two developmental stages of immature caryopses were studied: younger pale green caryopses depicting swollen ovaries and the harder but still hydrated maturing caryopses (Figure 2.1D). Young inflorescences were taken from unopened boots identified by flag leaves (Figure 2.1A). Stolons were subdivided to obtain young shoots (tips of stolons) and several nodal segments 2-3 cm in length with a node at the middle (Figure 2.1G). The outer sheath at the tip of each stolon was peeled off to obtain the young shoot bearing a primordial node. Nodal segments were numbered from 1 to 10 starting from the youngest node. First and second nodes were pooled together, and so were the third and fourth, fifth and sixth, seventh and eight, and ninth and tenth, to represent five age groups.

Tissues were surface sterilized to eliminate bacterial and fungal contaminants. Chemicals and components of nutrient media were purchased from Sigma (St. Louis, MO) unless otherwise specified. Sterilization was performed following these sequential steps: washing with running distilled water in a strainer, gentle shaking in 0.5% Triton X-100 detergent, rinsing with running distilled water, shaking in 10% Daconil fungicide (GroTech, Inc., Madison, GA), rinsing with distilled water, shaking in 25% household bleach solution, rinsing three times with sterile distilled water, soaking in 70% ethanol

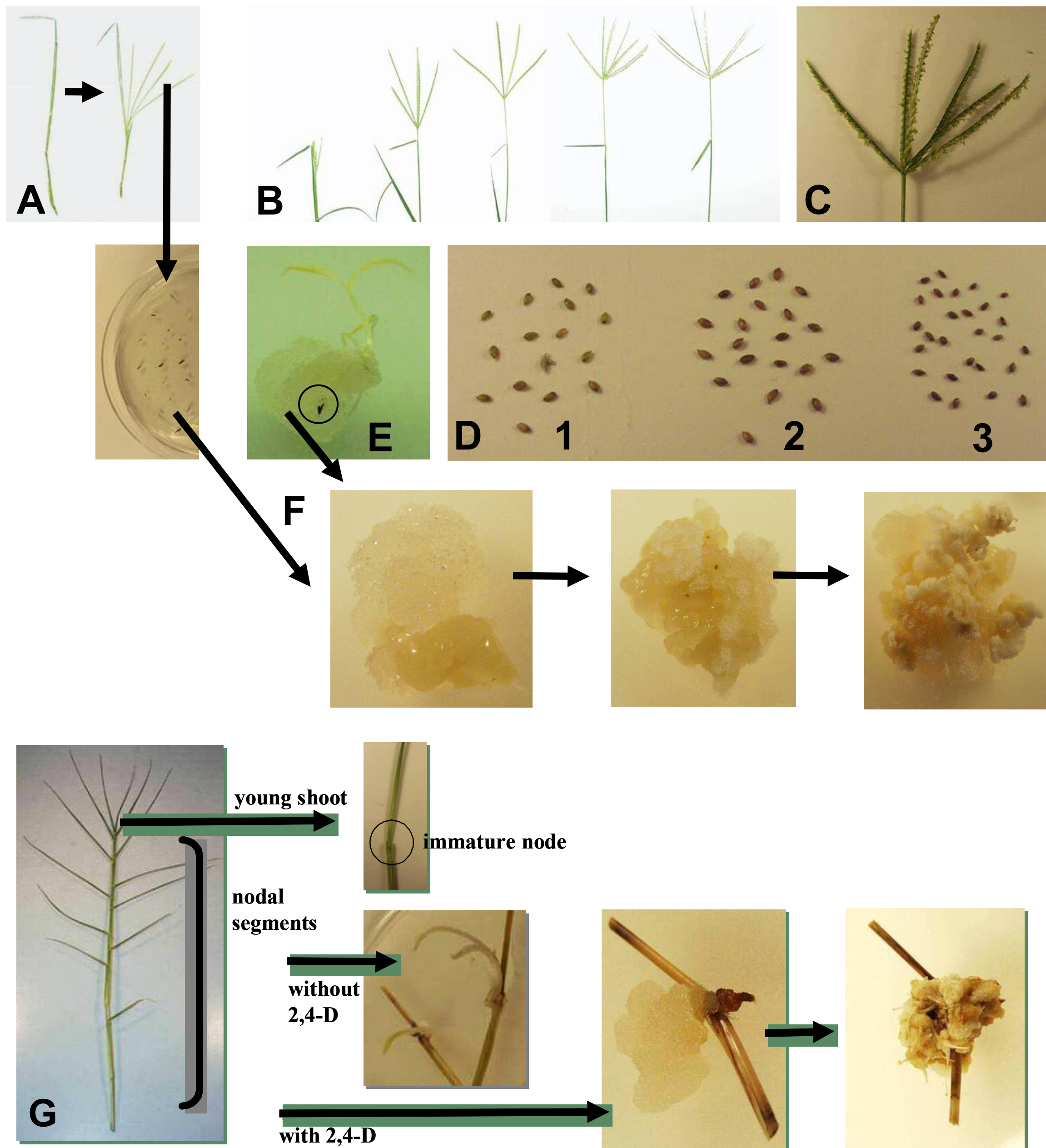


Figure 2.1. Explant tissues of bermudagrass and callus formation. A) Young inflorescence taken out from the closed boot (flag leaf is at the tip of the boot). An inflorescence consists of spikes which vary in number. Inflorescence tissues were cut into 2-3 mm pieces and plated on nutrient medium. B) Opening of the inflorescence and ascent above the flag leaf. Pollination and seed development follow. C) Pollen shedding. D) 1- younger immature caryopses that are soft and pale green depicting swollen ovaries, 2- older immature caryopses that are brown and harder compared to 1 but still hydrated, 3- mature and dehydrated caryopses. E) Seed germination and callus formation both occurring in a caryopsis explant. F) Calli derived from inflorescence and caryopsis tissue share same morphology. The callus is initially soft and white, and more compact and yellowish regions later develop. G) Stolon was stripped of leaves and cut to obtain several nodal segments as well as the young shoot at the tip. Nodal segments plated on nutrient medium without 2,4-D developed shoots. With 2,4-D, callus tissues were formed which differ in morphology compared with those derived from inflorescences and caryopses.

for 1 minute, and rinsing with sterile distilled water. Durations of treatment with detergent, fungicide, and bleach solution varied with the type of tissues: 15-30 minutes in each solution for caryopses and mature nodal sections, and as short as 1 minute for delicate tissues such as inflorescences, shoots, and younger nodal segments. Immature caryopses and young inflorescences were sterilized while inside the hulls and the boots, respectively. After surface sterilization, tissues were soaked for 30 minutes to a few hours in 2% PPM (Plant Preservative Mixture, Plant Cell Technology, Inc., Washington, DC) with 50 mg L^{-1} $MgCl_2$.

Solid Nutrient Media and Culture Steps

All culture media were based on MS formulation (Murashige and Skoog, 1962) with 30 g L^{-1} sucrose as carbon source. Media were prepared by dissolving 4.3 g of 10X MS Basal Salt Mixture and 30 g sucrose in 1 L of water. The pH was brought to 5.8, 7 g agar was added, and the solution was autoclaved at 121°C under 40 inches of Hg for 20 minutes. One milliliter of premixed 1,000X MS Vitamin Solution and specific amounts of PGRs were added to the cooled solution just before pouring into Petri plates or Magenta boxes. Filter-sterilized concentrated stock solutions of PGRs were prepared such that for any working concentration planned, 500 µL of stock had to be added to 1 L of medium. To do this, a series of less concentrated stocks were made by diluting the most concentrated stock. Aliquots of stock solutions were stored in 1.5-mL tubes and frozen at -20°C.

Callus initiation, growth, and differentiation were carried out in 100 X 15-mm Petri plates under nine different PGR supplementations (described below) at 28°C in the

dark inside a growth chamber. Initiated calli at least 5 mm in length were transferred into fresh media with same PGR supplements, and subcultured approximately every 2 weeks. At any time, calli that developed hard and opaque tissue mass characteristic of embryogenic cells were transferred to shooting medium. Remaining calli that appeared non-embryogenic were placed on shooting medium after five subcultures.

Shoot formation was carried out in 100 X 20-mm Petri plates at 28^oC under 16-hour photoperiod. Illumination was provided by four 40-watt long fluorescent lights: two Cool White Plus and two Gro-Lux Wide Spectrum bulbs (Osram Sylvania Ltd., Ontario, Canada). A single formulation of PGRs was used in the shooting medium: 0.01 mg L^{-1} BAP, 0.1 mg L^{-1} 2,4-D, and 0.2 mg L^{-1} GA_3. Calli that developed green shoots were counted as regenerated plants, and were transferred to rooting medium. Calli that did not form shoots were maintained for up to 6 weeks. Shoots were rooted in Magenta boxes under 16-hour photoperiod on MS medium without PGRs. Shoots developed vigorous root systems after a few days in the rooting medium.

PGR Treatments

Nine different PGR supplementation treatments were evaluated for their effects on callus initiation, growth, and differentiation: 1, 3, and 5 mg L^{-1} 2,4-D (treatments 1, 2, and 3, respectively), 1, 3, and 5 mg L^{-1} 2,4-D each in combination with 1 mg L^{-1} kinetin (treatments 4, 5, and 6, respectively), and 1, 3, and 5 mg L^{-1} 2,4-D each in combination with 1 mg L^{-1} kinetin and 3 mg L^{-1} ABA (treatments 7, 8, and 9, respectively). Medium not supplemented with PGR served as control. As mentioned, media used during shoot and root development were the same for all callus tissues.

RESULTS AND DISCUSSION

Mature Caryopses

Callus tissues initiated from bermudagrass caryopses were initially soft, smooth, and white to pale yellow in color (Figure 2.1). Callus formation was visible as early as five days after plating of caryopses on medium. More compact and yellowish sectors developed in some calli after a few days. Development of somatic embryos was characterized by clumping of cells forming mounds that impart a rough appearance to the previously smooth callus. After transfer to shooting conditions, a single shoot or multiple shoots emerged from the embryogenic cells. In a few cases, roots developed prematurely and this prevented shoot development. Other callus tissues remained undifferentiated after subcultures. These non-embryogenic calli were fast growing, soft and spongy, lacked compact and rough sectors, and did not form shoots. Caryopsis-derived calli of Brazos, a robust forage cultivar, were strikingly different in appearance compared with those of other cultivars. Callus tissues of Brazos were relatively larger, fast growing, and devoid of yellowish sectors although capable of forming somatic embryos.

The frequencies of callus initiation and plant regeneration were influenced by cultivar, PGR treatment, and cultivar X PGR interaction (Table 2.3a). These three factors were significant at $P < 0.0001$ (Tables 2.3b and 2.3c). Brazos was the best performer among the four cultivars studied. Media supplemented with 2,4-D alone (treatments 1, 2, and 3) generally promoted higher callus initiation frequencies compared with media that were additionally provided with kinetin or kinetin and ABA (treatments 4 to 9). Under all PGR treatments, only a fraction of initiated calli ranging from 0% to about 50%

regenerated into plantlets. For instance, 61 calli were initiated from a total of 320 caryopses of Riviera under treatment 6, 34 of which were regenerated (Table 2.3a).

Table 2.3a. Percent callus initiation (%C) and plant regeneration (%R) from mature caryopses of four seeded bermudagrass cultivars on MS agar medium under nine PGR supplementations. (Both %C and %R were based on number of explants used.)

		Arizona Common 40 seeds/plate 8 plates/trt 320 seeds/trt		**Brazos** 40 seeds/plate 3 plates/trt 120 seeds/trt		**Riviera** 40 seeds/plate 8 plates/trt 320 seeds/trt		**Yukon** 40 seeds/plate 8 plates/trt 320 seeds/trt	
Trt	**PGR(s)**	**% C**	**% R**	**% C**	**% R**	**% C**	**% R**	**% C**	**% R**
1	1 mg L^{-1} 2,4-D	20.6	6.2	59.2	5.8	19.4	6.2	37.2	1.9
2	3 mg L^{-1} 2,4-D	23.4	6.6	57.5	4.2	22.5	6.2	36.3	0
3	5 mg L^{-1} 2,4-D	18.1	5.9	39.2	0	25.3	3.8	36.3	0.6
4	1 mg L^{-1} 2,4-D + 1 mg L^{-1} kinetin	21.6	4.7	55.8	14.2	14.7	7.2	35.0	3.4
5	3 mg L^{-1} 2,4-D + 1 mg L^{-1} kinetin	20.3	9.1	33.3	5.8	19.7	7.5	30.0	0.3
6	5 mg L^{-1} 2,4-D + 1 mg L^{-1} kinetin	12.8	3.8	43.3	8.3	19.0	10.6	33.8	0
7	1 mg L^{-1} 2,4-D + 1 mg L^{-1} kinetin + 3 mg L^{-1} ABA	0	0	26.7	7.5	0.3	0	21.9	8.1
8	3 mg L^{-1} 2,4-D + 1 mg L^{-1} kinetin + 3 mg L^{-1} ABA	0	0	59.2	22.5	0	0	23.8	5.0
9	5 mg L^{-1} 2,4-D + 1 mg L^{-1} kinetin + 3 mg L^{-1} ABA	0	0	55.8	0.8	0.6	0	0	0

Table 2.3b. Analysis of variance in callus initiation data given in Table 2.3a.

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Cultivar	3	3.17	1.057	328.98	< 0.0001
PGR Treatment	3	1.82	0.227	70.78	< 0.0001
Cultivar * PGR Treatment	24	0.81	0.033	10.45	< 0.0001

Table 2.3c. Analysis of variance in plant regeneration data given in Table 2.3a.

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Cultivar	3	0.06	0.021	16.88	< 0.0001
PGR Treatment	3	0.07	0.008	7.00	< 0.0001
Cultivar * PGR Treatment	24	26.6	0.011	8.78	< 0.0001

Germination of caryopses (emergence of radicle and coleoptile) occurred in the control medium that was not provided with PGR and no callus tissues were produced. In the presence of 2,4-D, seed germination as well as callus formation took place, with callus formation apparently occurring mostly in germinated caryopses. Callus tissues must have been initiated primarily via seed germination which brings out actively dividing cells where undifferentiated cell growth may commence when auxin is present. In succeeding experiments using different seed lots, it was clear that percent germination correlates with percent callus initiation. Seed lots with 0% to very low percentages of germination (tested using germination paper) had very low frequencies of callus initiation on MS agar medium even when supplied with high 2,4-D levels (up to 10 mg L^{-1}). Vernalization, pre-treatment with GA_3, removal of seed coats, and chopping the caryopses sometime improved callus initiation in dormant bermudagrass seeds (data not shown).

Kinetin when supplied with 2,4-D (treatments 4, 5, and 6) caused pronounced seed germination and fast growing coleoptiles at the expense of callus growth. Some calli contained hard lumps of tissues and displayed browning. However, recovered calli under kinetin supplementation especially at lower 2,4-D levels differentiated faster compared

with calli under treatments of 2,4-D alone. ABA in combination with 2,4-D and kinetin (treatments 7, 8, and 9) imparted unfavorable effects both on seed germination and callus formation. The caryopses, initiated calli, as well as the culture medium displayed browning and callus growth was stunted. The extent of adverse effect of ABA varied with cultivars and 2,4-D levels. For instance, seed germination and callus induction were totally inhibited in caryopses of Arizona Common but not in caryopses of Brazos (Table 2.3a). In a follow-up experiment, caryopses were plated on media supplied with 3 mg L^{-1} of either kinetin or ABA. Neither of these PGRs induced callus formation in the absence of 2,4-D. ABA inhibited seed germination and caused browning of caryopses while kinetin enhanced seedling growth (but not percent germination) as compared with those in control plates not supplied with any PGR. These observations substantiated the results of the previous experiments involving nine PGR treatments and are consistent with known effects of kinetin and ABA on plant cells (Brock and Kaufman, 1991). To conclude, the data given in Table 2.3a especially for Brazos suggest that kinetin and ABA have promotive effects on callus morphogenesis and plant regeneration. However, these two PGRs should be withheld during callus formation in bermudagrass caryopses.

Immature Caryopses

The frequencies of callus induction in immature caryopses depended on PGR treatment and cultivar (Table 2.4). Supplementation of kinetin with 2,4-D (treatments 4, 5, and 6) resulted in more conspicuous seed germination in terms of radicle emergence but not of coleoptile growth. Incorporation of 3 mg L^{-1} ABA (treatments 7, 8, and 9) suppressed callus growth, and resulted in the browning of caryopses and callus tissues.

The developmental stage of immature caryopses as well as kinetin supplementation appeared to influence culture response but the total number of explants used (24 explants per treatment) was small to support statistical analysis.

The highest percentage of callus initiation in immature caryopses was obtained at 37.5% in Brazos (Table 2.4) compared with 59.2% in mature caryopses (also in Brazos, Table 2.3a). The highest percentage of plant regeneration from immature caryopses based

Table 2.4. Percent callus initiation (%C) and plant regeneration (%R) from immature caryopses of five seeded bermudagrass cultivars on MS agar medium under nine PGR supplementations. (A: younger immature caryopses, B: older immature caryopses; Twenty-four caryopses were used for each PGR treatment. Both %C and %R were based on 24 explants used.)

			Arizona Common		Brazos		PRC58		Riviera		Yukon	
Trt	PGR(s)		%C	%R	%C	%R	%C	%R	%C	%R	%C	%R
1	1 mg L^{-1} 2,4-D	A B	0 0	0 0	33.3 20.8	20.8 12.5	0 8.3	0 4.2	0 0	0 0	12.5 0	12.5 0
2	3 mg L^{-1} 2,4-D	A B	8.3 12.5	4.2 0	25 29.2	12.5 8.3	12.5 16.7	12.5 8.3	0 4.2	0 0	20.8 4.2	16.7 0
3	5 mg L^{-1} 2,4-D	A B	4.2 0	4.2 0	29.2 29.2	16.7 8.3	0 16.7	0 4.2	0 0	0 0	8.3 16.7	8.3 0
4	1 mg L^{-1} 2,4-D + 1 mg L^{-1} kinetin	A B	25 0	16.7 0	29.2 25	20.8 12.5	0 16.7	0 8.3	0 0	0 0	0 0	0 0
5	3 mg L^{-1} 2,4-D + 1 mg L^{-1} kinetin	A B	0 4.2	0 0	37.5 29.2	16.7 8.3	4.2 8.3	4.2 0	4.2 0	4.2 0	8.3 4.2	4.2 0
6	5 mg L^{-1} 2,4-D + 1 mg L^{-1} kinetin	A B	33 4.2	12.5 4.2	20.8 33	8.3 20.8	4.2 8.3	0 8.3	0 12.5	0 0	8.3 12.5	8.3 8.3
7	1 mg L^{-1} 2,4-D + 1 mg L^{-1} kinetin + 3 mg L^{-1} ABA	A B	0 0	0 0	8.3 0	0 0	0 0	0 0	0 0	0 0	4.2 4.2	0 4.2
8	3 mg L^{-1} 2,4-D + 1 mg L^{-1} kinetin + 3 mg L^{-1} ABA	A B	0 0	0 0	16.7 8.3	4.2 4.2	0 4.2	0 4.2	0 4.2	0 4.2	8.3 4.2	0 0
9	5 mg L^{-1} 2,4-D + 1 mg L^{-1} kinetin + 3 mg L^{-1} ABA	A B	0 0	0 0	16.7 12.5	8.3 0	0 0	0 0	0 16.7	0 0	0 0	0 0

on total number of explants used was obtained at 20.8% in Brazos (Table 2.4) compared with 22.5% from mature caryopses (in Brazos, Table 2.3a). Callus initiation frequencies in immature caryopses were generally low possibly because of reduced germination. Control plates without PGRs correspondingly showed low germination counts. As with mature caryopses, callus initiation in immature caryopses appeared to be promoted by seed germination which brings out actively dividing cells.

Because of low callus initiation frequencies, use of immature caryopses was not pursued in succeeding experiments. In addition, they are also tedious to prepare. Newly opened inflorescences of plants in the greenhouse need to be marked and the dates for harvesting the immature caryopses should be noted. Removal of seed coats to obtain the soft immature caryopses is tedious and has to be done carefully using forceps.

Young Inflorescences

Each whole inflorescence was chopped into 2-3 mm pieces and ten pieces of tissue were plated in each of nine PGR treatments and a control plate without PGR. Callus induction took 3-4 weeks in several plates provided with PGR(s), while no callus was produced in control plates, as expected. The callus tissues resembled those obtained from caryopses although somewhat more dense and compact at early growth stage. The frequencies of callus formation ranged from 0% to about 50% depending on PGR treatment, cultivar, and specific inflorescence (Table 2.5, data for first 6 inflorescences are shown although as many as 12 inflorescences were used for some cultivars). In most experiments, only a fraction of initiated callus tissues regenerated into plants.

Table 2.5. Numbers of callus tissues (C) and regenerated plants (R) obtained from young inflorescence explants of six bermudagrass cultivars on MS agar medium under nine PGR supplementations. (Tissue of each whole inflorescence was divided equally among the nine PGR treatments: 10 pieces of tissue per treatment).

Arizona Common		Inflor. 1		Inflor. 2		Inflor. 3		Inflor. 4		Inflor. 5		Inflor. 6	
Trt	**PGR(s)**	**C**	**R**	**C**	**R**	**C**	**R**	**C**	**R**	**C**	**R**	**C**	**R**
1	1 mg L^{-1} 2,4-D	1	0	0	0	2	1	1	1	1	1	0	0
2	3 mg L^{-1} 2,4-D	4	2	2	2	6	4	2	2	3	1	0	0
3	5 mg L^{-1} 2,4-D	3	3	3	1	5	4	4	2	2	2	1	0
4	1 mg L^{-1} 2,4-D + 1 mg L^{-1} kinetin	1	0	1	1	0	0	2	1	0	0	0	0
5	3 mg L^{-1} 2,4-D + 1 mg L^{-1} kinetin	3	2	2	1	1	1	3	3	2	2	2	1
6	5 mg L^{-1} 2,4-D + 1 mg L^{-1} kinetin	2	2	2	2	1	1	2	2	2	1	1	0
7	1 mg L^{-1} 2,4-D + 1 mg L^{-1} kinetin + 3 mg L^{-1} ABA	1	1	0	0	1	0	2	1	0	0	0	0
8	3 mg L^{-1} 2,4-D + 1 mg L^{-1} kinetin + 3 mg L^{-1} ABA	2	0	1	0	1	0	1	0	0	0	0	0
9	5 mg L^{-1} 2,4-D + 1 mg L^{-1} kinetin + 3 mg L^{-1} ABA	0	0	0	0	0	0	0	0	0	0	0	0

Brazos		Inflor. 1		Inflor. 2		Inflor. 3		Inflor. 4		Inflor. 5		Inflor. 6	
Trt	**PGR(s)**	**C**	**R**	**C**	**R**	**C**	**R**	**C**	**R**	**C**	**R**	**C**	**R**
1	1 mg L^{-1} 2,4-D	0	0	1	1	0	0	2	2	0	0	0	0
2	3 mg L^{-1} 2,4-D	3	3	5	3	4	2	6	4	2	2	3	2
3	5 mg L^{-1} 2,4-D	6	2	4	4	3	2	5	5	4	4	2	1
4	1 mg L^{-1} 2,4-D + 1 mg L^{-1} kinetin	0	0	3	3	1	0	2	1	1	1	1	0
5	3 mg L^{-1} 2,4-D + 1 mg L^{-1} kinetin	2	2	4	2	2	2	4	3	1	1	2	2
6	5 mg L^{-1} 2,4-D + 1 mg L^{-1} kinetin	3	1	2	2	3	3	3	2	0	0	0	0
7	1 mg L^{-1} 2,4-D + 1 mg L^{-1} kinetin + 3 mg L^{-1} ABA	0	0	1	1	0	0	0	0	0	0	0	0
8	3 mg L^{-1} 2,4-D + 1 mg L^{-1} kinetin + 3 mg L^{-1} ABA	0	0	1	1	0	0	1	0	0	0	0	0
9	5 mg L^{-1} 2,4-D + 1 mg L^{-1} kinetin + 3 mg L^{-1} ABA	1	0	2	1	0	0	0	0	0	0	0	0

Table 2.5 continued

PRC58		Inflor. 1		Inflor. 2		Inflor. 3		Inflor. 4		Inflor. 5		Inflor. 6	
Trt	PGR(s)	C	R	C	R	C	R	C	R	C	R	C	R
1	1 mg L^{-1} 2,4-D	2	2	3	2	1	1	0	0	1	1	1	0
2	3 mg L^{-1} 2,4-D	3	2	2	2	4	2	2	1	1	1	0	0
3	5 mg L^{-1} 2,4-D	7	4	3	3	6	3	2	2	3	2	2	0
4	1 mg L^{-1} 2,4-D + 1 mg L^{-1} kinetin	2	2	1	0	1	0	0	0	1	1	0	0
5	3 mg L^{-1} 2,4-D + 1 mg L^{-1} kinetin	2	2	1	1	3	3	1	1	1	0	1	1
6	5 mg L^{-1} 2,4-D + 1 mg L^{-1} kinetin	1	1	1	0	2	1	0	0	1	0	0	0
7	1 mg L^{-1} 2,4-D + 1 mg L^{-1} kinetin + 3 mg L^{-1} ABA	0	0	1	1	1	0	2	1	0	0	0	0
8	3 mg L^{-1} 2,4-D + 1 mg L^{-1} kinetin + 3 mg L^{-1} ABA	0	0	0	0	2	1	0	0	0	0	0	0
9	5 mg L^{-1} 2,4-D + 1 mg L^{-1} kinetin + 3 mg L^{-1} ABA	0	0	0	0	1	0	0	0	0	0	0	0

Riviera		Inflor. 1		Inflor. 2		Inflor. 3		Inflor. 4		Inflor. 5		Inflor. 6	
Trt	PGR(s)	C	R	C	R	C	R	C	R	C	R	C	R
1	1 mg L^{-1} 2,4-D	0	0	0	0	0	0	0	0	0	0	0	0
2	3 mg L^{-1} 2,4-D	1	0	2	0	0	0	2	0	0	0	0	0
3	5 mg L^{-1} 2,4-D	2	2	1	0	1	0	3	1	0	0	2	1
4	1 mg L^{-1} 2,4-D + 1 mg L^{-1} kinetin	0	0	1	1	0	0	0	0	0	0	1	0
5	3 mg L^{-1} 2,4-D + 1 mg L^{-1} kinetin	2	0	0	0	0	0	0	0	0	0	0	0
6	5 mg L^{-1} 2,4-D + 1 mg L^{-1} kinetin	1	1	1	1	0	0	1	1	0	0	3	1
7	1 mg L^{-1} 2,4-D + 1 mg L^{-1} kinetin + 3 mg L^{-1} ABA	0	0	1	0	0	0	0	0	0	0	0	0
8	3 mg L^{-1} 2,4-D + 1 mg L^{-1} kinetin + 3 mg L^{-1} ABA	0	0	0	0	0	0	0	0	0	0	0	0
9	5 mg L^{-1} 2,4-D + 1 mg L^{-1} kinetin + 3 mg L^{-1} ABA	0	0	0	0	0	0	0	0	0	0	0	0

Table 2.5 continued

Tifway		Inflor. 1		Inflor. 2		Inflor. 3		Inflor. 4		Inflor. 5		Inflor. 6	
Trt	**PGR(s)**	**C**	**R**	**C**	**R**	**C**	**R**	**C**	**R**	**C**	**R**	**C**	**R**
1	1 mg L^{-1} 2,4-D	0	0	0	0	0	0	0	0	0	0	0	0
2	3 mg L^{-1} 2,4-D	0	0	1	0	1	0	0	0	2	1	0	0
3	5 mg L^{-1} 2,4-D	1	1	2	1	1	0	1	1	3	0	1	0
4	1 mg L^{-1} 2,4-D + 1 mg L^{-1} kinetin	0	0	0	0	0	0	0	0	2	1	0	0
5	3 mg L^{-1} 2,4-D + 1 mg L^{-1} kinetin	0	0	2	0	1	1	0	0	1	1	0	0
6	5 mg L^{-1} 2,4-D + 1 mg L^{-1} kinetin	1	0	0	0	1	0	0	0	1	0	0	0
7	1 mg L^{-1} 2,4-D + 1 mg L^{-1} kinetin + 3 mg L^{-1} ABA	0	0	0	0	0	0	0	0	0	0	0	0
8	3 mg L^{-1} 2,4-D + 1 mg L^{-1} kinetin + 3 mg L^{-1} ABA	0	0	0	0	0	0	0	0	0	0	0	0
9	5 mg L^{-1} 2,4-D + 1 mg L^{-1} kinetin + 3 mg L^{-1} ABA	0	0	0	0	0	0	0	0	0	0	0	0

Yukon		Inflor. 1		Inflor. 2		Inflor. 3		Inflor. 4		Inflor. 5		Inflor. 6	
Trt	**PGR(s)**	**C**	**R**	**C**	**R**	**C**	**R**	**C**	**R**	**C**	**R**	**C**	**R**
1	1 mg L^{-1} 2,4-D	2	2	1	0	1	1	4	2	5	3	3	1
2	3 mg L^{-1} 2,4-D	6	3	3	1	4	1	5	1	3	2	5	2
3	5 mg L^{-1} 2,4-D	3	3	2	0	6	2	3	1	4	3	5	2
4	1 mg L^{-1} 2,4-D + 1 mg L^{-1} kinetin	1	0	2	1	3	1	1	0	2	1	3	1
5	3 mg L^{-1} 2,4-D + 1 mg L^{-1} kinetin	4	2	5	2	5	3	3	0	2	1	3	0
6	5 mg L^{-1} 2,4-D + 1 mg L^{-1} kinetin	3	1	5	3	4	1	3	2	3	2	2	2
7	1 mg L^{-1} 2,4-D + 1 mg L^{-1} kinetin + 3 mg L^{-1} ABA	1	0	1	1	1	1	0	0	0	0	1	0
8	3 mg L^{-1} 2,4-D + 1 mg L^{-1} kinetin + 3 mg L^{-1} ABA	2	1	1	1	3	1	0	0	2	0	1	1
9	5 mg L^{-1} 2,4-D + 1 mg L^{-1} kinetin + 3 mg L^{-1} ABA	0	0	0	0	1	0	0	0	0	0	1	0

Administration of 2,4-D alone at 3 and 5 mg L^{-1} (treatments 2 and 3) generally promoted higher callus initiation in young inflorescences than other treatments. Kinetin at 1 mg L^{-1} seems permissible if not actually beneficial for callus induction especially when 2,4-D was supplied at more than 1 mg L^{-1} (treatments 5 and 6). Use of 3 mg L^{-1} ABA or all three types of PGRs at the same time (treatments 7, 8, and 9) was not beneficial for callus induction especially when 2,4-D was supplied at 5 mg L^{-1}. Compared with caryopses, inflorescence explants and the callus cells derived from them appear to be intolerant to relatively high levels of supplemental kinetin and ABA. Highly regenerable inflorescence cultures of hybrid bermudagrasses were obtained using 1-4 mg L^{-1} 2,4-D together with the cytokinin BAP at a very low concentration of 0.01 mg L^{-1} (Chaudhury and Qu, 2000; Qu and Chaudhury, 2001; Jain *et al.*, 2005).

Results of this experiment indicated that fairly good callus initiation and plant regeneration can be achieved with young inflorescences using MS agar medium supplemented with 2,4-D. Like caryopses, the culture response of inflorescences depends on genotype. Among the six cultivars studied, inflorescences of Riviera and Tifway showed relatively poor culture response (Table 2.5). If follow-up studies will be conducted to determine whether kinetin and ABA have significant promotive effects on somatic embryogenesis of bermudagrass inflorescence cultures, these PGRs should be administered at concentrations much lower than 1 mg L^{-1}.

Young inflorescences are commonly used explants for tissue culture of vegetatively propagated bermudagrass cultivars. Inflorescences are not available throughout the year and their culture performance is known to vary widely with genotype, age of tissue, season of the year, and place of collection (tissues collected from

the field often perform better than those from the greenhouse) (Ahn *et al.*, 1985; Artunduaga *et al.,* 1988 and 1989; Jain *et al*., 2005). Maintaining highly regenerable callus lines is a means to conveniently use inflorescence explants, although extended culture period may result in somaclonal changes.

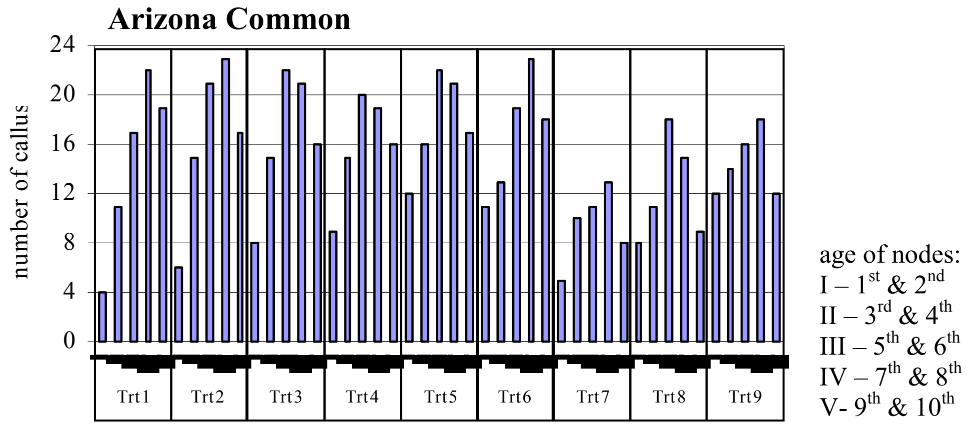
Young Shoots and Nodal Segments

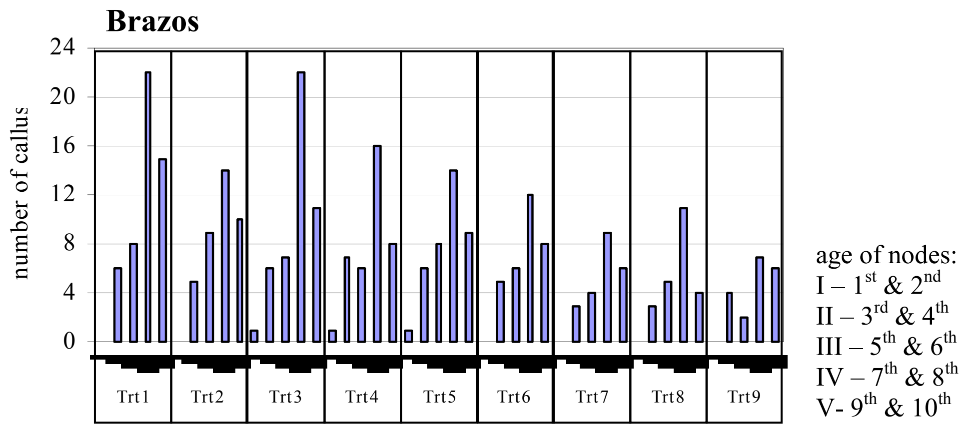
Young shoots obtained from tips of bermudagrass stolons browned and deteriorated on the MS agar medium and no callus tissues were produced. Nodal segments in the control plates without PGRs did not produce callus tissues but many of them had shoot and root growth originating from the nodal buds. Under all PGR treatments, round callus tissues with diameter of 5 mm or larger were produced about 10 days after plating of nodal segments on the medium. Node-derived calli had different morphology compared with those produced from caryopses and young inflorescences (Figure 2.1). They were off-white to pale yellow in color, and relatively dry and spongy during the first few weeks of culture. The frequencies of callus initiation were generally higher in more mature and larger nodes (5th, 6th, 7th, 8th, 9th, and 10th), and were dependent on genotype (Table 2.6 and Figure 2.2). Among the six bermudagrass cultivars studied, nodal explants of PRC58 and Yukon performed poorly. Very few nodes of these two cultivars grew shoots and roots in the control plates.

Callus induction from nodal explants was attributed to 2,4-D supplementation. Callus size was positively correlated with 2,4-D level, especially when only 2,4-D was supplied. Calli under treatment 1 were notably smaller than those under treatments 2 and 3. Kinetin, which promotes cell division, appeared to enhance callus formation and

Table 2.6. Numbers of callus tissues obtained from nodal explants of six bermudagrass cultivars on MS agar medium under nine PGR supplementations. (Nodal segments from each stolon were grouped based on age wherein the 1st and 2nd nodes were the youngest. Three replicate plates each containing eight nodal segments were prepared for each PGR treatment X age group combination, and values shown were obtained from a total of 24 explants. No significant variations were observed among replicate plates.)

Trt	PGR(s)	Age of Nodes	Arizona Common	Brazos	PRC58	Riviera	Tifway	Yukon
1	1 mg L^{-1} 2,4-D	1st & 2nd	4	0	0	2	2	0
		3rd & 4th	11	6	0	12	5	0
		5th & 6th	17	8	0	19	4	1
		7th & 8th	22	22	1	21	6	2
		9th & 10th	19	15	0	22	7	1
2	3 mg L^{-1} 2,4-D	1st & 2nd	6	0	2	3	3	0
		3rd & 4th	15	5	1	8	7	1
		5th & 6th	21	9	1	17	8	2
		7th & 8th	23	14	2	18	9	3
		9th & 10th	17	10	0	15	6	2
3	5 mg L^{-1} 2,4-D	1st & 2nd	8	1	0	3	1	0
		3rd & 4th	15	6	0	10	4	0
		5th & 6th	22	7	1	16	3	1
		7th & 8th	21	22	1	19	5	1
		9th & 10th	16	11	0	13	7	1
4	1 mg L^{-1} 2,4-D + 1 mg L^{-1} kinetin	1st & 2nd	9	1	2	6	1	0
		3rd & 4th	15	7	1	14	2	0
		5th & 6th	20	6	3	23	5	2
		7th & 8th	19	16	2	18	5	4
		9th & 10th	16	8	1	12	9	1
5	3 mg L^{-1} 2,4-D + 1 mg L^{-1} kinetin	1st & 2nd	12	1	2	7	3	0
		3rd & 4th	16	6	1	8	5	2
		5th & 6th	22	8	1	18	5	3
		7th & 8th	21	14	4	23	7	2
		9th & 10th	17	9	2	16	8	2
6	5 mg L^{-1} 2,4-D + 1 mg L^{-1} kinetin	1st & 2nd	11	0	3	5	2	0
		3rd & 4th	13	5	3	7	3	0
		5th & 6th	19	6	4	14	4	1
		7th & 8th	23	12	1	17	5	3
		9th & 10th	18	8	0	13	11	1
7	1 mg L^{-1} 2,4-D + 1 mg L^{-1} kinetin + 3 mg L^{-1} ABA	1st & 2nd	5	0	2	3	1	0
		3rd & 4th	10	3	0	4	0	0
		5th & 6th	11	4	1	9	3	0
		7th & 8th	13	9	0	12	11	1
		9th & 10th	8	6	0	15	5	0
8	3 mg L^{-1} 2,4-D + 1 mg L^{-1} kinetin + 3 mg L^{-1} ABA	1st & 2nd	8	0	3	1	0	0
		3rd & 4th	11	3	2	3	2	0
		5th & 6th	18	5	2	6	3	2
		7th & 8th	15	11	1	11	9	0
		9th & 10th	9	4	0	14	7	0
9	5 mg L^{-1} 2,4-D + 1 mg L^{-1} kinetin + 3 mg L^{-1} ABA	1st & 2nd	12	0	2	0	0	0
		3rd & 4th	14	4	1	2	0	0
		5th & 6th	16	2	0	7	2	1
		7th & 8th	18	7	0	13	11	0
		9th & 10th	12	6	0	15	4	0





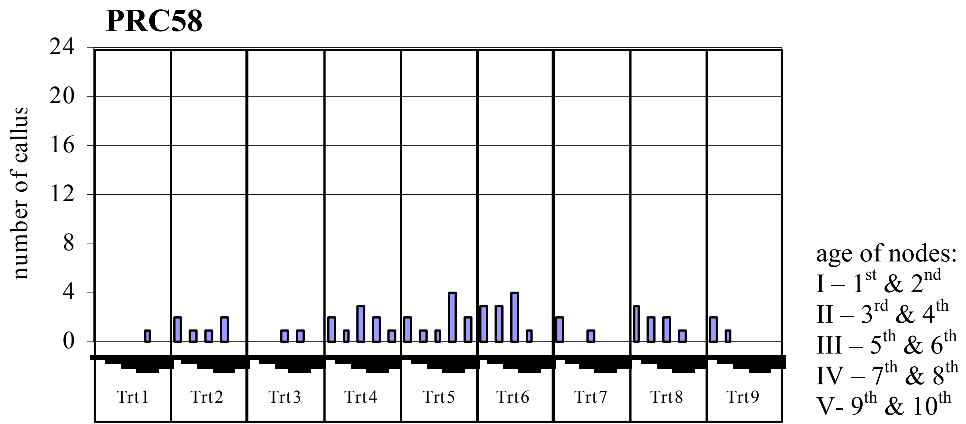


Figure 2.2, continued next page

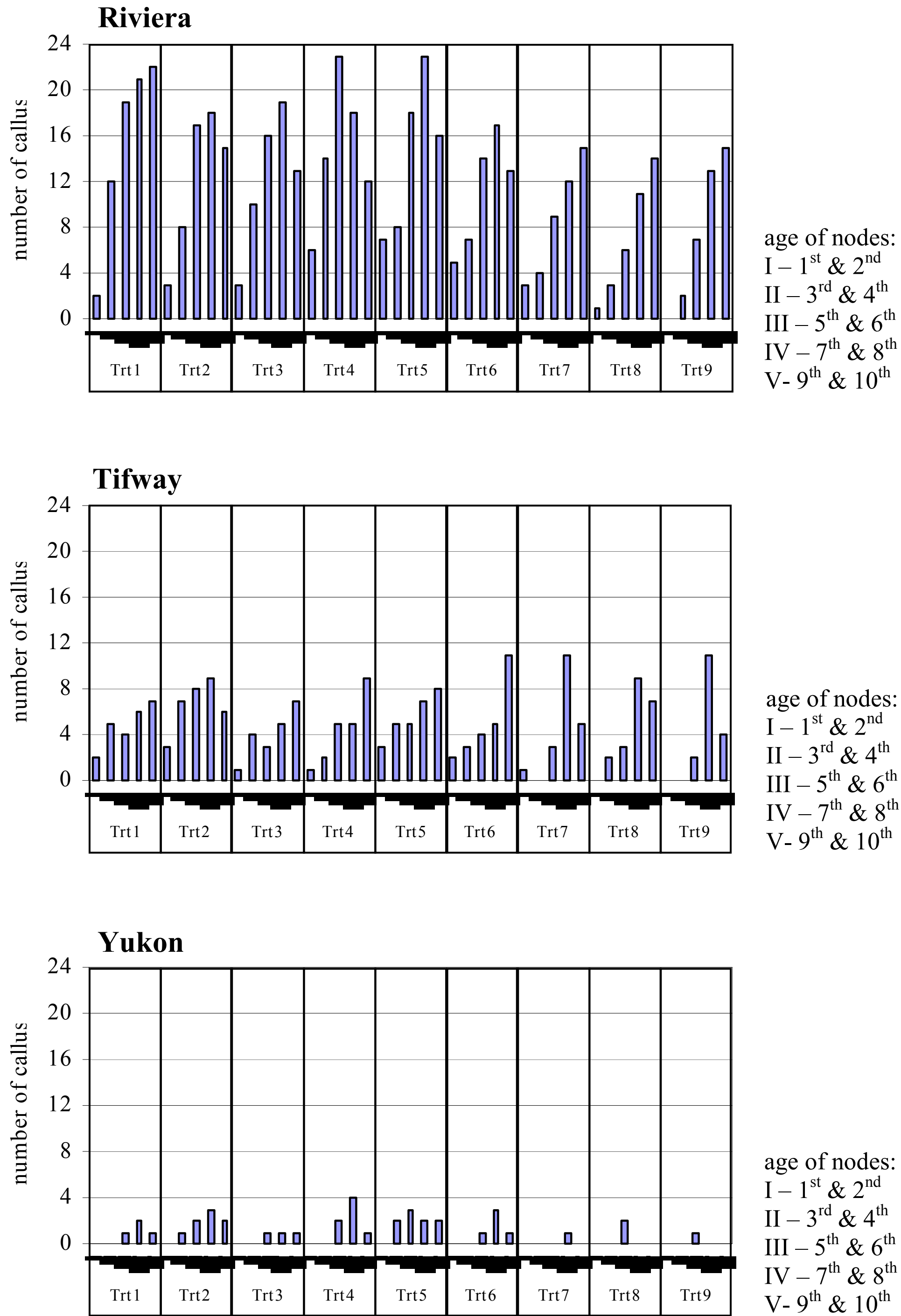


Figure 2.2. Numbers of callus tissues obtained from nodal explants of six bermudagrass cultivars under nine PGR supplementations. (Values were taken from Table 2.6.)

growth at 1 mg L^{-1}. Shoot growth from the nodes was also promoted by kinetin and competed with callus proliferation particularly under treatment 4 where kinetin and 2,4-D were both supplied at 1 mg L^{-1}. Addition of ABA at 3 mg L^{-1} caused slight browning of calli and explants, and sometimes arrested callus growth, particularly at higher 2,4-D levels (treatments 8 and 9). No enhancement of callus initiation by ABA was noted.

The callus tissues were subsequently excised from nodal explants and subcultured under same PGR treatments to allow further growth and maturation. Clumps of cells formed mounds on top of the previously smooth tissues. Node-derived calli were dry and hard at later stages of culture. Although roots often developed, no shoots and plantlets were obtained after extended culture under conditions that promote shoot development. It is notably difficult to regenerate plants from callus tissues derived from vegetative cells of bermudagrass (Chaudhury and Qu, 2000; Zhang *et al.,* 2003; Jain *et al*., 2005). Regeneration from nodal explants often requires a liquid culture phase to increase callus tissue mass and promote embryogenesis. Such liquid cultures are lengthy and induce somaclonal variations, and are purposely utilized to produce desirable mutants (Croughan and Quisenberry, 1989). Goldman *et al*. (2004) obtained genetically transformed bermudagrass plants from nodal cultures that had increased ploidy levels. The authors concluded that susceptibility to ploidy changes may be inherent in nodal explants. It is also possible that suspension cultures promote susceptibility to the effect of 2,4-D of inducing polyploidization or susceptibility to selection of polyploid cell growth. Nodes are desirable explants because they provide a ready source of tissues for both seeded and vegetatively propagated plants. However, simpler (only require solid medium) and shorter culture methods that do not increase somaclonal variations need to be established.

Optimization of Mature Caryopsis Culture

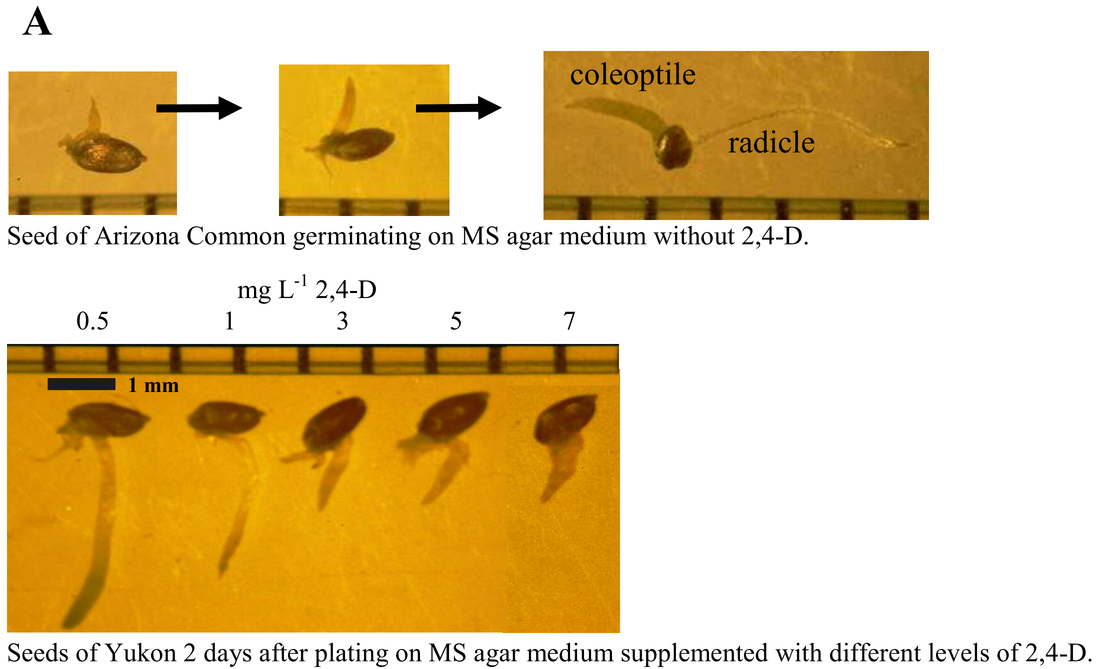
Because of abundant availability, ease of preparation, and short duration of callus induction, mature caryopses were further studied to optimize plant regeneration frequencies. Using twelve *C. dactylon* cultivars, a range of 2,4-D concentrations was tested during callus initiation (Figure 2.3). Levels from 3 to 7 mg L^{-1} were found to be appropriate for obtaining robust and viable callus tissues that have minimal coleoptile growth. Beyond 7 mg L^{-1}, 2,4-D imparted toxicity to the callus tissues which displayed stunted growth and desiccation. Callus initiation was then routinely carried out at 5 mg L^{-1} 2,4-D, followed by subcultures at lower 2,4-D levels. In all cultivars examined, supplementation of kinetin and/or ABA at 1 to 3 mg L^{-1} during subcultures significantly enhanced callus differentiation into embryogenic tissue which was characterized by formation of compact yellow sectors (Figures 2.4 and 2.5). When only 2,4-D was supplied during subcultures, somatic embryogenesis occurred at lower frequencies.

Based on the results of optimization experiments, a standardized protocol for regenerating bermudagrass plants from MS agar culture of caryopsis-derived callus tissues was developed for employment in gene transfer experiments (Figure 2.6). The turf-type cultivars Yukon and Guymon were selected for transformation studies because of availability of seed lots that consistently showed high culture responsiveness following this protocol. Supplementation of 1 mg L^{-1} ABA at early stage of callus growth was crucial for obtaining high percentages of regenerable callus tissues. This tissue culture procedure normally takes 6 weeks from plating of caryopsis explants to regeneration of shoots. However, longer time is needed when transformation experiment is performed. Callus tissues require time to recuperate from the stress imparted by the gene transfer

method. Selection agents such as hygromycin also slow down callus differentiation and plant regeneration.

Other factors were optimized to improve the culture method. The MS nutrient medium was enhanced by addition of 0.5 g L^{-1} casein enzymatic hydrolysate (*Phyto*Technology Lab., Shawnee Mission, KS). The anti-oxidant poly(vinypoly-pyrrolidone) (PVPP, cross-linked preparation, Sigma, St. Louis, MO) was added to all types of media (callus, shooting, and rooting media), and this reduced browning of explants and callus tissues. There was noticeable reduction in tissue browning when PVPP was increased from 3 g L^{-1} to 5 g L^{-1} of medium. Levels of PGRs in the shooting medium were modified: 1 mg L^{-1} GA_3, 0.5 mg L^{-1} BAP, and 0.25 mg L^{-1} 2,4-D, in order of presumed importance. No significant enhancement was observed when amount of sucrose was doubled (from 30 to 60 g L^{-1}) during callus initiation and differentiation.

Timely subcultures and maintenance of sterile conditions have been important for obtaining healthy and viable callus tissues. Pretreatment of explants with 2% PPM and addition of 0.05% PPM in the nutrient medium helped minimize growth of microbial contaminants. Hydration of callus tissues on the agar medium often resulted in browning and arrested growth. To prevent this, excess moisture was blotted dry with sterile tissue paper and the plates were air-dried inside the laminar flow hood for a few hours after each subculture.



Seed of Arizona Common germinating on MS agar medium without 2,4-D.

Seeds of Yukon 2 days after plating on MS agar medium supplemented with different levels of 2,4-D.

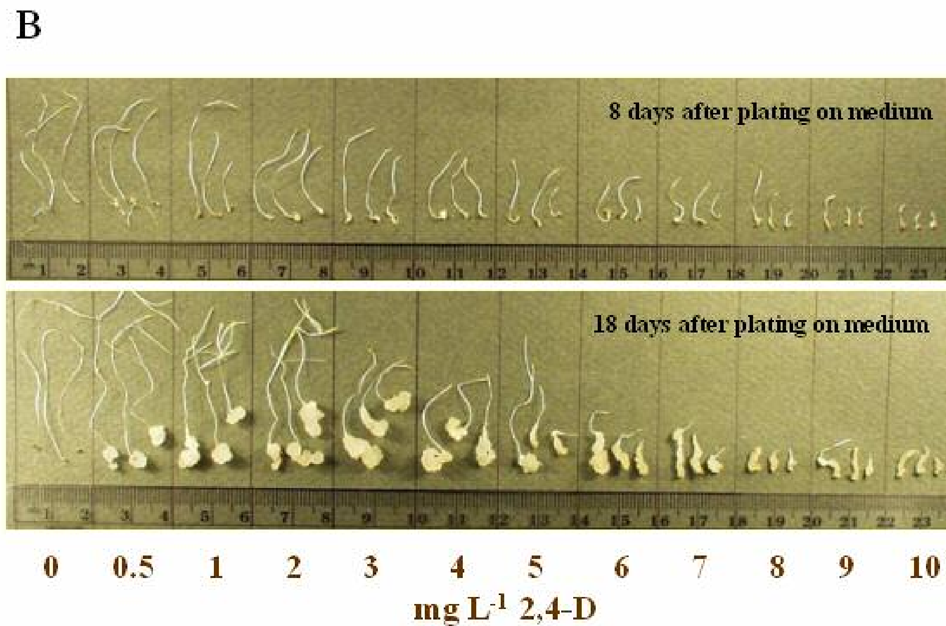


Figure 2.3. Seed germination and callus initiation in bermudagrass caryopses under different levels of 2,4-D. A) Close-up view. Callus tissues are formed at the base of coleoptiles. At higher 2,4-D levels, callus initiation is more pronounced, coleoptile growth is suppressed, and the radicle may not emerge. B) Typical culture response of caryopsis explants (12 bermudagrass cultivars were examined) on MS agar medium under different 2,4-D levels. Shown are callus tissues initiated from caryopses of the cultivar Princess. Three calli were pictured for each 2,4-D level to show the range of callus size and extent of coleoptile growth. As low as 0.05 mg L^{-1} 2,4-D promoted callus initiation (not shown).

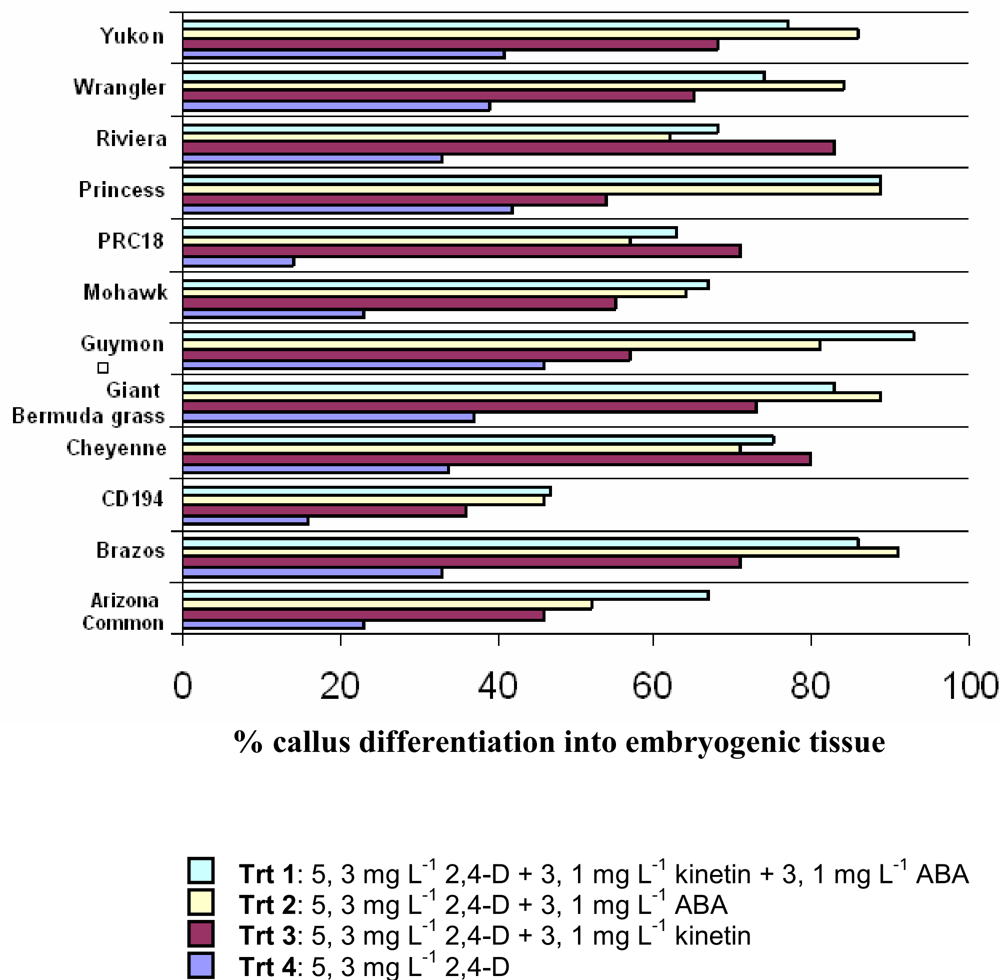
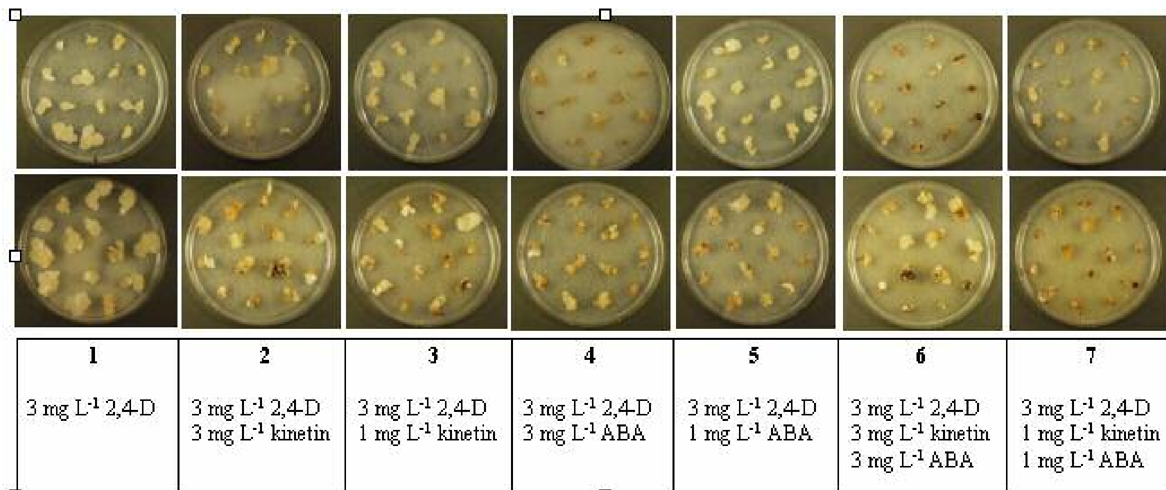


Figure 2.4. Percent callus differentiation in caryopsis culture of twelve bermudagrass cultivars under four PGR regimens.
Total number of callus tissues examined differed among cultivars. Calli were initiated in 5 mg L^{-1} 2,4-D and subcultured under specific PGR treatments twice. PGR concentrations were lowered at second subculture. For example under treatment 3, initiated calli were subcultured in 5 mg L^{-1} 2,4-D + 3 mg L^{-1} kinetin for 2 weeks, and then subcultured in 3 mg L^{-1} 2,4-D + 1 mg L^{-1} kinetin for another 2 weeks.



1 Low frequencies of callus differentiation and somatic embryogenesis. Most calli were fast growing.
2 Controlled callus growth and higher occurrence of embryogenic callus sectors than in treatment **1**. Pronounced growth of coleoptiles.
3 Resembles treatment **2**, but less pronounced coleoptile growth and slightly lower occurrence of embryogenic sectors.
4 Browning and stunted growth of callus tissues during early stage of culture. Generally high occurrence of embryogenic sectors.
5 Best treatment for many bermudagrass cultivars. Resembles treatment **4**, but less browning and growth stunting of callus tissues.
6 More severe browning and growth stunting than in treatment **4**, but also high frequencies of somatic embryogenesis.
7 Less browning and growth stunting than in treatment **6**, but lower frequencies of somatic embryogenesis.

Figure 2.5. Growth and differentiation of caryopsis-derived callus tissues of Arizona Common on MS agar media under different PGR supplementations. Callus tissues were initiated in 5 mg L^{-1} 2,4-D and subcultured for 2 weeks twice under specific PGR treatments. Top plates: after 2 weeks of culture. Bottom plates: after 2 more weeks of culture.

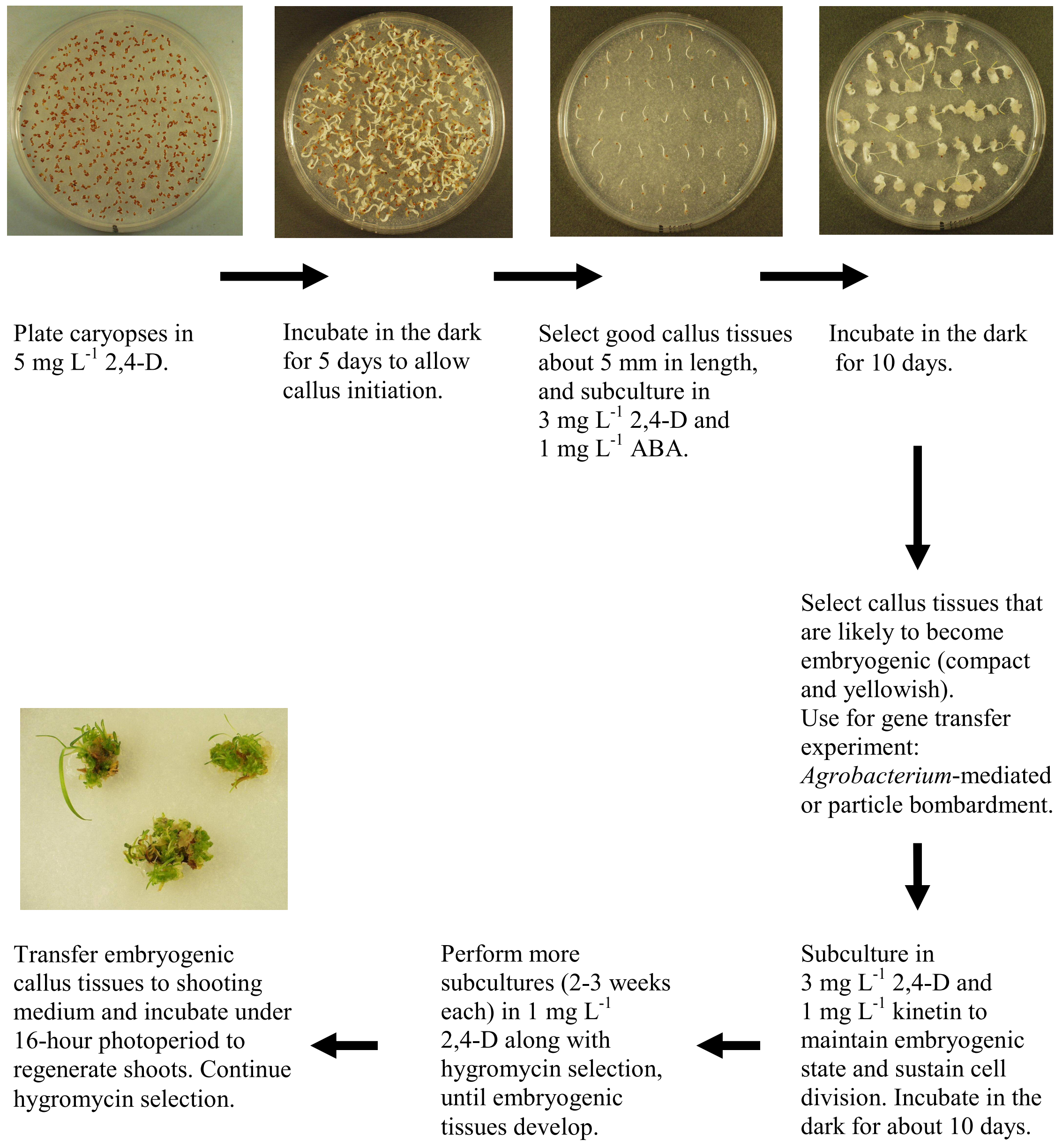


Figure 2.6. Caryopsis culture as regeneration system for bermudagrass genetic transformation.

SUMMARY AND CONCLUSIONS

In order to establish an efficient tissue culture method for bermudagrass using MS agar medium, plant regeneration from callus cultures derived from caryopses, young inflorescences, and stolon nodes was evaluated. PGR supplementation treatments consisting of 2,4-D, kinetin, and ABA were tested to determine the effects of these PGRs on callus production and plant regeneration. Promotion of callus initiation by 2,4-D in all explant types was correlated to the level of 2,4-D supplied. Optimal callus induction was generally achieved at 3 and 5 mg L^{-1} 2,4-D depending on cultivar. Kinetin and ABA when administered at 1 and 3 mg L^{-1}, respectively, often had unfavorable effects on callus induction by 2,4-D, and these PGRs must be withheld during this step. Callus tissues from nodal explants were not regenerated on the MS agar medium, while those from caryopses and young inflorescences regenerated fairly good numbers of plants depending on cultivar. Mature caryopses provide a convenient explant source for seeded bermudagrass cultivars because of their abundant availability, ease of preparation, and short duration of callus induction. These advantages have not been adequately exploited in bermudagrass tissue culture.

Optimization of mature caryopsis culture was carried out in this study. It was found that kinetin and ABA at concentrations of 1-3 mg L^{-1} have significant promotive effects on somatic embryogenesis of caryopsis-derived callus tissues. A standardized protocol for mature caryopsis culture was developed for use in genetic transformation experiments. The turf-type bermudagrass cultivars Yukon and Guymon were selected for transformation studies.

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CHAPTER 3

BINARY VECTOR, GENE CONSTRUCTS, *A. TUMEFACIENS* STRAIN, AND HYGROMYCIN SELECTION FOR GENETIC TRANSFORMATION OF BERMUDAGRASS

ABSTRACT

Full-length cDNAs of *Sorghum bicolor PHYA* and *C*, *Arabidopsis thalina PHYA, B,* and *C*, and of *Cynodon dactylon CynCHT1* were provided with the maize constitutive *Ubi-1* promoter and NOS terminator. The gene constructs were subcloned into the binary plasmid vector pCAMBIA1305.2 for use in present and future genetic transformation studies in bermudagrass. Nucleotide sequences of constructs for *Sorghum bicolor PHYC* and for *CynCHT1* were verified. In preparation for gene transfer experiments, *Agrobacterium tumefaciens* strain EHA105 was transformed with pCAMBIA1305.2 and versions carrying the gene constructs. PCR primers to amplify the selectable marker *hptII* gene in the pCAMBIA vector were made for screening transformed plants. The toxic effect of hygromycin B on seed germination, seedling viability, and growth of callus-derived shoots of the bermudagrass cultivars Guymon and Yukon was evaluated. Results indicated that the antibiotic can effectively eliminate non-transformed plant materials within 2-3 weeks of selection at concentrations of 50-75 mg L^{-1}.

INTRODUCTION

To initiate a research project on phytochrome over-expression in bermudagrass, this dissertation aimed to establish gene transfer methods and to secure suitable materials that are crucial for carrying out genetic transformation experiments. These include primarily the plasmid vector that will carry reporter and selectable marker genes and the expression cassette for the gene of interest. Binary plasmid vectors are a practical choice of transformation vector because many of them are designed for use in both *Agrobacterium*-mediated and particle bombardment methods of plant transformation. Modular sets of binary vectors such as the pBIN19 (Bevan, 1984), pBECK2000 (McCormac *et al.,* 1997), and pCAMBIA series (Roberts *et al*., 1998) were created to suit specific needs of a plant transformation project.

In the binary vector system the two essential parts of the large Ti plasmid of Agrobacteria, the T-DNA and the *vir* regions, are segregated into two smaller plasmids. One of these plasmids is a wide-host range small replicon having an origin of replication that allows maintenance in a wide range of bacteria. This recombinant plasmid contains the left and right T-DNA borders, a gene for selection in bacterial hosts, and the DNA to be transferred to plants in place of the T-DNA. Use of this smaller recombinant plasmid promotes high transfer efficiency to Agrobacteria from *E. coli* where initial gene cloning work is performed. The other plasmid, called the helper Ti plasmid, lacks the entire T-DNA but contains an intact *vir* region. Helper plasmids determine virulence of experimental *A. tumefaciens* strains. For instance, the super-virulent strains A281, EHA101, EHA105, and their derivatives are most effective with plant species that are recalcitrant to genetic transformation.

Constitutive gene promoters have been isolated to over-express genes of interest in monocot plant transformation. The rice *Act-1* and maize *Ubi-1* promoters were shown in cereal transformation studies to be more active than the CaMV 35S promoter that works well in dicots (McElroy *et al.,* 1990; Christensen *et al.,* 1992). Activity of the maize *Adh-1* promoter appears to be restricted to specific plant tissues (Kyozaka *et al*., 1991).

Reporter and selectable marker genes are employed to initially verify transformation events and to favor survival and proliferation of transformed plant materials. These genes are provided with constitutive promoters so that they are expressed in most tissues at all times. *UidA* which codes for β-glucoronidase or GUS has been a popular reporter gene used to optimize parameters in gene transfer experiments (Spangenberg *et al*., 1995). Selectable marker genes are usually based on resistance to antibiotics or herbicides (Fraley *et al.,* 1986). Examples of antibiotics employed are kanamycin (resistance conferred by *nptII* gene), streptomycin/spectinomycin (*spt* gene), and hygromycin (*hpt* or *hph* gene). Examples of herbicides are glyphosate or Round-up (*epsp* synthase gene) and glufosinate ammonium or Liberty (*pat* and *bar* genes).

Hygromycin B selection was first employed in rice transformation to obtain fertile transgenic plants (Shimamoto *et al.,* 1989) and was subsequently applied in some non-cereal grasses. Li and Qu (2004) observed that common bermudagrass has natural resistance to hygromycin such that relatively high concentrations (150-250 mg L^{-1}) are necessary for selecting transgenic callus tissues. However, plantlets appear to be more sensitive to hygromycin than callus tissues, and 50-100 mg L^{-1} may be sufficient for selection at this stage.

OBJECTIVES

1. Subclone specific *PHY* genes into the T-DNA region of the binary plasmid vector pCAMBIA1305.2, along with the maize *Ubi-1* promoter and NOS terminator.
2. Introduce pCAMBIA1305.2 carrying *Ubi-1* gene construct into EHA105 cells in preparation for *Agrobacterium*-mediated bermudagrass transformation.
3. Assess the potency of hygromycin B on wild type plant materials of Guymon and Yukon to determine appropriate levels to use for selecting transformed plants.

MATERIALS AND METHODS

Gene Constructs

Five full-length cDNA clones of phytochrome genes were obtained from different providers (Table 3.1): *PHYA*, *PHYB*, and *PHYC* of *Arabidopsis thaliana* (Sharrock and Quail, 1989) and *PHYA* and *PHYC* of *Sorghum bicolor* (Childs *et al*., 1997). These phytochrome genes and the bermudagrass chitinase gene *CynCHT1* from *Cynodon dactylon* (de los Reyes *et al*., 2001) were subcloned in pCAMBIA1305.2 binary vector. *CynCHT1* has been implicated in bermudagrass cold acclimation and was also aimed for constitutive expression in bermudagrass.

The transformation vector chosen, pCAMBIA1305.2, belongs to a set of modular binary vectors for use in both *Agrobacterium*–mediated and direct DNA uptake gene transfer methods (Roberts *et al.,* 1998) distributed by the Center for the Application of Molecular Biology to International Agriculture (CAMBIA, Canberra, Australia). *Ubi-1* promoter and NOS terminator sequences were taken from pAHC17, a cloning vector

Table 3.1. Full-length cDNAs that were subcloned in the binary plasmid vector pCAMBIA1305.2 and provided the *Ubi-1* promoter sequence.

Gene Name, Size; Source Plant Species; Original Cloning Vector	Provider	Name of Resulting Gene Construct
AtPHYA, 3.5 kb; *Arabidopsis thaliana*; pBluescript KS+	Peter H. Quail, University of California, Berkeley	pCAMBIA1305.2:*Ubi*-*AtPHYA*
AtPHYB, 3.5 kb; *Arabidopsis thaliana*; pBluescript II KS+	Enamul Huq, University of Texas at Austin	pCAMBIA1305.2:*Ubi*-*AtPHYB*
AtPHYC, 3.3 kb; *Arabidopsis thaliana*; pBluescript KS+	Peter H. Quail, University of California, Berkeley	pCAMBIA1305.2:*Ubi*-*AtPHYC*
SbPHYA, 3.8 kb; *Sorghum bicolor*; pBluescript KS+	Kevin L. Childs, Texas A&M University	pCAMBIA1305.2:*Ubi*-*SbPHYA*
SbPHYC, 3.7 kb; *Sorghum bicolor*; pBluescript KS+	Kevin L. Childs, Texas A&M University	pCAMBIA1305.2:*Ubi*-*SbPHYC*
CynCHT1, 2.1 kb; *Cynodon dactylon*; pBluescript II SK+	Michael P. Anderson, Oklahoma State University	pCAMBIA1305.2:*Ubi*-*CynCHT1*

intended for high-level expression of marker genes in monocotyledonous plants (Christensen and Quail, 1996). Subcloning of cDNAs was facilitated using duplex oligonucleotide linkers that were synthesized by Integrated DNA Tech., Inc. (Coralville, IA).

Subcloning in *E. coli*. Plasmids received from providers and newly constructed plasmids from ligation reactions were introduced to DH5α chemically competent cells (Invitrogen Corp., Carlsbad, CA) or to SCS110 competent cells yielding Dam or Dcm methylation-free plasmids (Stratagene, La Jolla, CA) in order to obtain sufficient DNA materials and secure glycerol stocks of bacterial cells harboring the plasmids. About 10

ng of plasmid DNA or 25 ng of ligation product was incubated with 50-100 µL of competent cells on ice for 30 minutes, at 42°C for 45 seconds, and then on ice for 5 minutes. Cells were rescued by adding 900 µL of SOC medium (Appendix A) followed by incubation at 37°C for 2 hours with gentle agitation. Aliquots of SOC culture were plated on LB agar medium (Appendix A) containing appropriate antibiotic. Cells transformed with pBluescript and pAHC17 were selected on agar plates with 100 µg mL^{-1} ampicillin while cells harboring pCAMBIA1305.2 were selected on plates with 75 µg mL^{-1} kanamycin. A single bacterial colony was picked with a sterile toothpick, inoculated to 750 µL of liquid LB medium with antibiotic, and grown at 37°C for 4-5 hours with gentle agitation. From this pre-culture, 50 µL was inoculated to 50 mL of liquid LB medium with antibiotic and grown for 15-20 hours. Cells were harvested to isolate plasmid DNA and to make 50% glycerol stocks of transformed cells for storage at -80°C. Subsequent cultures were made using 1 µL of glycerol stock per milliter of culture medium.

DNA preparation. Plasmid DNA was isolated from *E. coli* using the QIAprep Spin Miniprep Kit (QIAgen Inc., Valencia, CA). Fresh bacterial culture was dispensed in several 1.5-mL tubes and spun at 10,000 rpm for 1 minute in a table-top microcentrifuge. Pelleted cells were resuspended in buffer solution and purified by the alkaline lysis method following the manufacturer's instructions. Plasmid DNA was solubilized in low-salt buffer solution and eluted from the QIAprep spin columns. DNA concentration was estimated using a NanoDrop ND-1000 Spectrophotometer (Coleman Technologies Inc., Glen Mills, PA).

Restriction endonuclease digestion. Restriction of DNA was performed overnight using enzymes and buffer solutions purchased from Invitrogen Life Tech.(Carlsbad, CA), New England Biolabs Inc. (Beverly, MA), and Promega Corp. (Madison, WI) by following the manufacturers' instructions. Digests involving two restriction endonucleases were carried out in two steps. The first digest was cleaned with QIAquick Nucleotide Removal Kit (QIAGEN Inc., Valencia, CA) to eliminate previously used enzyme and salts before doing the second digest. Partial digests were obtained after incubating reactions for 3-10 minutes at optimum temperature using 1 unit of restriction enzyme for each microgram of DNA. To anticipate conflicts regarding endonuclease recognition sites, the nucleotide sequences of cDNAs, pAHC17, pCAMBIA1305.2, and oligonucleotide linkers were scanned for recognition sites using the software provided at http://www.in-silico.com (Bikandi *et al*., 2004). Partial sequences of pBluescript plasmids were also obtained using T3 and T7 universal primers to examine restriction sites present between each cDNA insert and to confirm orientation of cDNA reading frames.

Agarose gel electrophoresis and isolation of DNA fragments. Products of endonuclease digestion were separated and visualized in 0.8-1% ethidium bromide-stained, routine-grade agarose gel (Sigma Chemical Co., St. Louis, MO) using 1X TAE electrophoresis buffer (0.04 M Tris-acetate, 0.001 M EDTA, pH 8.0). To isolate specific DNA fragments for ligation reactions, products of endonuclease digestion were separated in low-melting point agarose with 1 mM guanosine added to gel and tank electrophoresis buffers to minimize DNA damage by UV light (Grundemann and Schomig, 1996). The gel was visualized over low-intensity UV light and small pieces of gel containing desired

DNA fragments were obtained using a scalpel. The DNA was then extracted from agarose using the QIAquick Gel Extraction Kit (QIAGEN Inc., Valencia, CA).

DNA ligation. Vector and insert DNA forming circular products were mixed at 1:5 molar ratio using 10 ng of DNA per microliter of ligation reaction. DNA fragments for concatamer ligation were mixed at 1:1 molar ratio using 100 ng of DNA per microliter of reaction. Ligation was carried out by T4 DNA ligase (Promega Corp., Madison, WI) at 16°C for five hours for cohesive-end ligation or up to ten hours for blunt-end ligation.

Sequencing of gene constructs. Nucleotide sequencing of gene constructs for *Sorghum bicolor PHYC* and *CynCHT1* was performed by the OSU Recombinant DNA/Protein Resource Facility, and the sequence outputs were compared with reference sequences. Sequencing primers for pCAMBIA1305.2 and for the cDNAs were designed from sequences reported at NCBI while those for pAHC17 were based on an unpublished sequence provided by Dr. Kay Scheets (Department of Botany, Oklahoma State University). Alignment of gene constructs with reference sequences was performed using the DNA alignment tool provided at http://insilico.ehu.es/align.

A. tumefaciens Cells

Supervirulent *A. tumefaciens* strains EHA101 and EHA105 were provided by Dr. Elizabeth Hood. Competent cells were prepared by growing in YEP medium (Appendix A) at 28°C with moderate shaking for 15-20 hours or until OD_{600} = 0.5 – 1.5. Cells were chilled on ice for 30 minutes, pelleted at 5,000 rpm (Beckman JA-17 rotor) for 10 minutes at 4°C, and resuspended in ice cold 1 mM HEPES (pH 7.0). Centrifugation and

resuspension of pellet were repeated three more times while gradually decreasing the volume of HEPES used. Cells were finally suspended in ice cold 10% glycerol at 1/30 of original volume of culture, dispensed into 45 µL aliquots, frozen in dry ice for 5 minutes, and stored at -80°C.

Binary vector pCAMBIA1305.2 and versions carrying *Ubi-1* gene construct were introduced into competent EHA105 cells by electroporation using the Bio-Rad Gene Pulser Apparatus (Bio-Rad Lab., Richmond, CA). About 45 µL of cells mixed with 2 µL of plasmid DNA (50-100 ng) was placed in a 0.1-cm cuvette, subjected to 12.5 kv cm^{-1}, immediately added to 500 µL of YEP medium, and incubated at 28°C for four hours with gentle shaking. Aliquots of rescued cells were plated on agar YEP medium with 75 µg mL^{-1} kanamycin and incubated at 28°C for up to two days. A single bacterial colony was inoculated to liquid YEP medium with kanamycin, and resulting culture was used to prepare glycerol stocks of transformed *Agrobacterium*.

A fragment of the *gus, hptII, SbPHYC*, and *CynCHT1* genes was PCR amplified from EHA105 and *E. coli* (DH5α) cells to verify introduction of the pCAMBIA vector and gene constructs. Amplification of fragment of the *Agrobacterium virb1* gene was also attempted to determine its presence in EHA105. PCR primers for *virb1* were based on sequence obtained from plasmid Ti of *A. tumefaciens* strain C58 (NCBI Acc. NC_003065). Primer sequences were selected using the Web Primer Tool at http://seq.yeastgenome.org and synthesized by Integrated DNA Tech., Inc. (Coralville, IA) (Table 3.2). DNA template was prepared by boiling a diluted sample of bacterial cells for 5 minutes. PCR reactions were prepared in a 50-µL volume with sterile water and contained 5 uL of boiled bacterial cells, 1X PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM each of

four dNTPs (Fisher Scientific, Pittsburgh, PA), 0.4 μM of forward and reverse PCR primers, 1 unit of Mango-Taq polymerase (Bioline USA Inc., Randolph, MA), and 10% glycerol. Amplification was carried out with the following thermocycler program: 1) hot start at 95°C for 5 minutes, 2) denaturation at 95°C for 1 minute, 3) annealing at 55-60°C for 90 seconds, 4) primer extension at 72°C for 90 seconds, 5) 34 cycles of steps 2 to 4, and 5) further primer extension at 72°C for 10 minutes. PCR products were resolved on 1.75% ethidium-bromide stained agarose gel using 1X TAE electrophoresis buffer.

Table 3.2. PCR primers used to amplify fragment of *Agrobacterium virb1* and of genes carried by the pCAMBIA vector.

Gene Amplified	Primer Sequence	Expected Size of Amplification Product (bp)
virb1	Forward: 5’ TGGGGAATGTTGAAGGCAA 3’ Reverse: 5’ ATTGCGGACCTCCTTGATTT 3’	742
gus	Forward: 5’ AAGGGCGGATTCCTGCCATT 3’ Reverse: 5’ TTTCCTGGGCAACGCTTGTT 3’	1,187
hptII	Forward: 5’ TGATCGAAAAG TTCGACAGCG 3’ Reverse: 5’ CGATTTGTGTACGCCCGACA 3’	885
*SbPHYC**	Forward: 5’ TGTTGATGCGGGTTTTACTGA 3’ Reverse: 5’ TAGGCGAGCAACGTGAAGGTG 3’	1,159
*CynCHT1**	Forward: 5’ TGTTGATGCGGGTTTTACTGA 3’ Reverse: 5’ AGGTGTAGAACCCCTTTGCCG 3’	837

* The same forward primer was used to amplify fragment of *SbPHYC* and of *CynCHT1*, and this was based on the 3’ end of the *Ubi-1* promoter sequence joining the genes. The primer pairs were intended for screening transformed bermudagrass plants which have endogenous phytochrome and chitinase genes.

Hygromycin “Kill” Experiments

Hygromycin “kill” experiments were performed to test the toxicity of different levels of the antibiotic to non-transformed bermudagrass plant materials, and thus identify levels of hygromycin that will be appropriate for selecting transformed plants and callus tissues. Seed germination, seedling growth, and survival of callus-derived shoots of *C. dactylon* cvs Guymon and Yukon were evaluated on MS agar medium with 30 g L^{-1} sucrose and containing 0, 10, 20, 30, 40, 50, 60, 70, or 80 mg L^{-1} hygromycin B.

RESULTS AND DISCUSSION

Gene Constructs

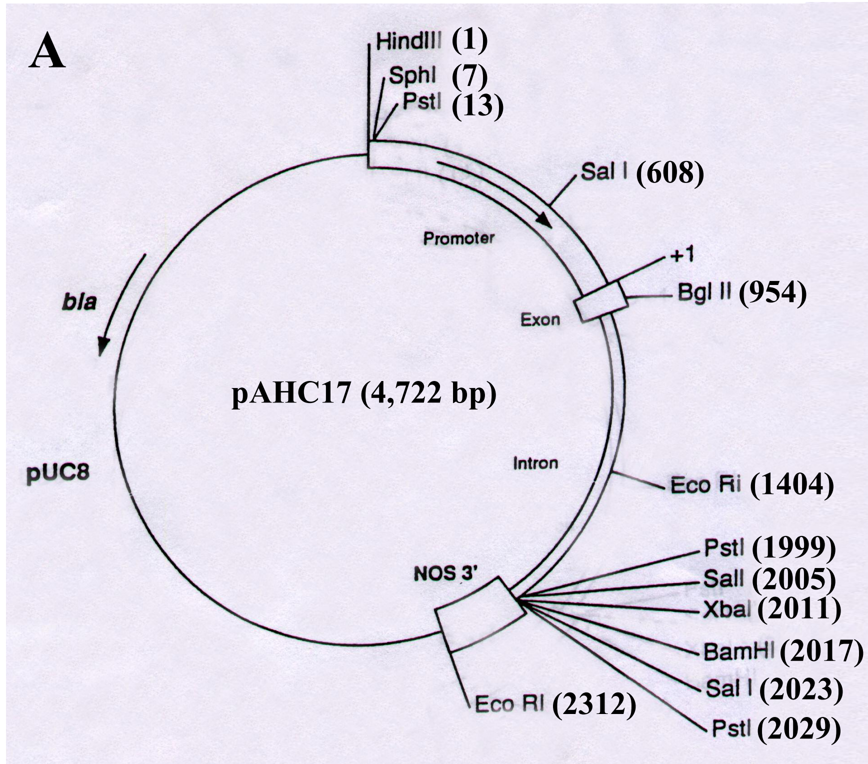
The genes intended for over-expression in bermudagrass were provided with the strong promoter of the maize *Ubi-1* gene. The maize *Ubi-1* promoter and NOS terminator were taken from pAHC17, a plasmid derived from pUC8 carrying ampicillin resistance for bacterial selection (Figure 3.1A). The promoter sequence includes the 5’ untranslated exon and the first intron of *Ubi-1* for enhanced promoter activity. About 250 bp of nopaline synthase (NOS) 3’ untranslated sequence and its polyadenylation signals are located downstream of the polylinker or cloning site. Very few restriction sites are present in the polylinker and only *Bam*HI has a unique site in the entire plasmid. To place each cDNA between *Ubi-1* promoter and NOS polyA with sense directionality, the original cloning site was expanded by inserting a duplex oligonucleotide linker containing several restriction endonuclease recognition sequences. Details of this procedure are shown in Figure 3.1B, C, and D. A pair of unique restriction sites was

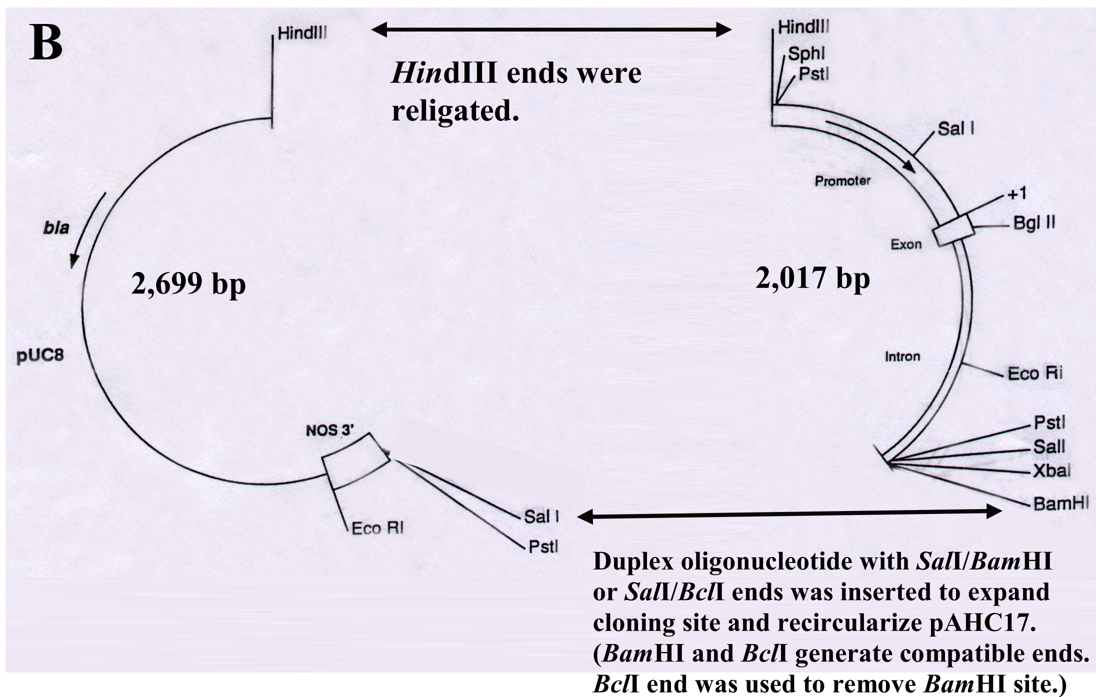
identified to excise each cDNA clone from its pBluescript cloning vector. These restriction sites were used in subsequent subcloning steps. "*Ubi-1* promoter – modified cloning site - NOS polyA" fragment was isolated from pAHC17 by digestion with *Hin*dIII and *Eco*RI and inserted in pCAMBIA1305.2 as described further below. Presence of an internal *Eco*RI site necessitated partial digestion to isolate the entire fragment (Figure 3.1D). Because the cDNA clones often have *Hin*dIII and *Eco*RI sites, genes were subcloned after placement of pAHC17's "*Ubi-1* promoter – modified cloning site - NOS polyA" fragment in pCAMBIA1305.2.

Figure 3.1. Modification of pAHC17 cloning site and isolation of "*Ubi-1* promoter – cloning site - NOS polyA" fragment.

(A) Map of pAHC17.
(B) The plasmid was restricted with *Hin*dIII and *Bam*HI (both unique sites) to isolate 2,017-bp fragment, as well as with *Hin*dIII and *Sal*I to isolate 2,699-bp fragment that has no internal *Sal*I site. Plasmid was reconstituted after rejoining *Hin*dIII ends of the two isolated fragments and after ligation of a duplex oligonucleotide linker to *Sal*I and *Bam*HI ends.
(C) Nucleotide sequence of inserted linker showing restriction endonuclease sites. Arbitrary nucleotides were added to increase distances between restriction sites. The 5' ends had overhangs for cohesive-end ligation. Two versions of the linker were made to facilitate subcloning of six cDNAs with directionality.
(D) Agarose gel electrophoresis showing steps leading to isolation of "*Ubi-1* promoter – modified cloning site – NOS polyA" fragment of pAHC17 in preparation for ligation to pCAMBIA1305.2 binary plasmid vector.

Continued





Duplex oligonucleotide with *Sal*I/*Bam*HI or *Sal*I/*Bcl*I ends was inserted to expand cloning site and recircularize pAHC17. (*Bam*HI and *Bcl*I generate compatible ends. *Bcl*I end was used to remove *Bam*HI site.)

Continued

C

pAHC17 linker 1: *Sal*I/*Bam*HI-ended linker (56 bp):

```
5' GATCC TGT GAGCTC GGTACC TGA GGGCCC TGA TGATCA ACA ACTAGT GTTAGA CCGCGG G         3'
3'         G ACA CTCGAG CCATGG ACT  CCCGGG ACT ACTAGT TGT TGATCA CAATCT GGCGCC CAGCT 5'
  BamHI           SacI      KpnI          ApaI           BclI          SpeI                 SacII     SalI
```

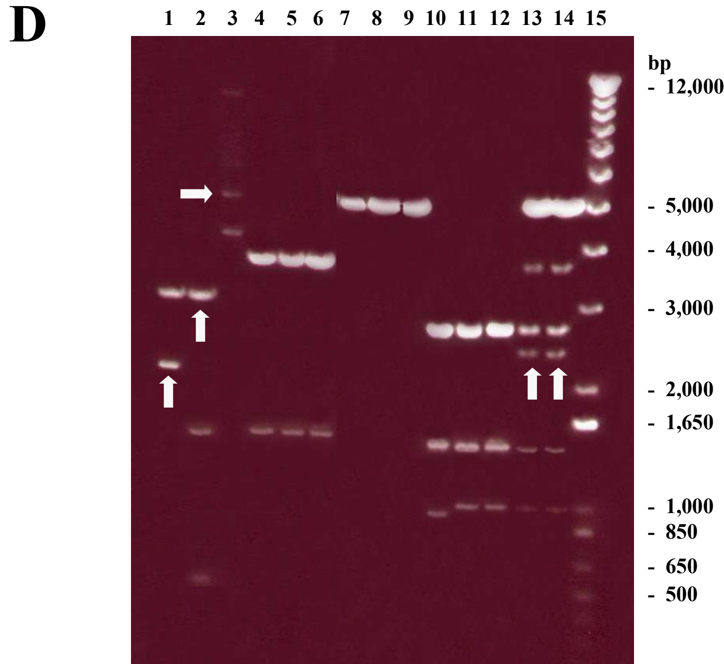
pAHC17 linker 2: *Sal*I/*Bcl*I-ended linker (51 bp):

```
5' GATCA GGTACC GAGCTC TCTAGA ACTAGT GGGCCC GGATCC GTTAGGA CCGCGG G         3'
3'          T CCATGG CTCGAG AGATCT TGATCA CCCGGG CCTAGG CAATCCT GGCGCC CAGCT 5'
    BclI     KpnI      SacI      XbaI      SpeI      ApaI    BamHI               SacII      SalI
```

Enzyme pair used to excise cDNA from pBluescript vector:			Linker used to subclone cDNA:
	5'	**3'**	
AtPHYA	***Sac*I**	***Apa*I**	**linker 1**
AtPHYB	***Kpn*I**	***Bcl*I**	**linker 1**
AtPHYC	***Xba*I**	***Bam*HI**	**linker 2**
SbPHYA	***Bam*HI**	***Sac*II**	**linker 1**
SbPHYA	***Spe*I**	***Sac*II (alternative)**	
SbPHYC	***Kpn*I**	***Spe*I**	**linker 1**
SbPHYC	***Apa*I**	***Spe*I (alternative)**	
CynCHT1	***Bam*HI**	***Kpn*I**	**linker 1**

Continued

D



Agarose gel (0.8%) electrophoresis. DNA fragments shorter than 100 bp were not resolved.
Lanes 1 and 2: Fragments generated from pAHC17 after *Hin*dIII and *Bam*HI digestion (2,705, 2,017 bp) and after *Hin*dIII and *Sal*I digestion (2,699, 1,397, 608, 18 bp), respectively. The 2,017 and 2,699-bp fragments (arrows) were isolated to reconstruct pAHC17 and modify cloning site.
Lane 3: Ligation of *Hin*dIII ends of fragments isolated from lanes 1 and 2. Ligation products were digested with *Bam*HI to lessen undesired concatamers. Arrow points to desired ligation product (4,716 bp) with *Sal*I and *Bam*HI ends that was isolated from gel and ligated to linker.
Lanes 4, 5, and 6: *Sal*I restriction test on original pAHC17 (3,307, 1,397, 18 bp), on pACH17 with linker 1 (3,307, 1,397, 68 bp), and on pAHC17 with linker 2 (3,307, 1,397, 63 bp), respectively. (Restriction tests were also performed on each endonuclease site along linkers 1 and 2, not shown).
Lanes 7, 8, and 9: *Hin*dIII restriction test on original pAHC17 (4,722 bp), on pAHC17 with linker 1 (4,772 bp), and on pAHC17 with linker 2 (4,767 bp), respectively.
Lanes 10, 11, and 12: *Hin*dIII plus *Eco*RI restriction test on original pAHC17 (2,410, 1,404, 908 bp), on pAHC17 with linker 1 (2,410, 1,404, 958 bp), and on pAHC17 with linker 2 (2,410, 1,404, 953 bp), respectively.
Lanes 13 and 14: *Hin*dIII digestion followed by partial *Eco*RI digestion of pAHC17 with linker 1 and of pAHC17 with linker 2, respectively. Partial *Eco*RI digest resulted in 1) intact *Hin*dIII fragment, 2) *Eco*RI digest at position 1404, see Figure 3.1A, 3) *Eco*RI digest at position 2312, and 4) *Eco*RI digest at both positions 1404 and 2312. Six expected fragments were obtained. From pAHC17 with linker 1: 4,772, 3,368, 2,410, 2,362 (desired fragment), 1,404, and 958-bp fragments. From pAHC17 with linker 2: 4,767, 3,363, 2,410, 2,357 (desired fragment), 1,404, and 953-bp fragments. Arrows point to desired *Hin*dIII/*Eco*RI fragments containing "*Ubi-1* promoter – modified cloning site - NOS polyA" sequence that were ligated to pCAMBIA1305.2.
Lane 15: 1 Kb Plus DNA Ladder.

The binary vector pCAMBIA1305.2 has the pBR322 *ori* and *bom* sites for high copy replication in *E. coli* and transmission by mating or electroporation, and contains the broad host range pVS1 *rep* and *sta* for low copy and stable replication in Agrobacteria (Figure 3.2A). This plasmid also carries kanamycin (*nptII*) and hygromycin (*hptII*) resistance genes for bacterial and plant selection, respectively. Also present is the *Staphylococcus* GUS*Plus* reporter gene that was provided the rice glycine-rich signal peptide sequence (GRP) for secretion to the apoplastic space. Researchers may add a gene of interest with its own promoter and polyA signal via the polylinker. Alternatively, the gene of interest may replace the GRP-GUS*Plus* and be inserted between the existing CaMV 35S promoter and NOS polyA.

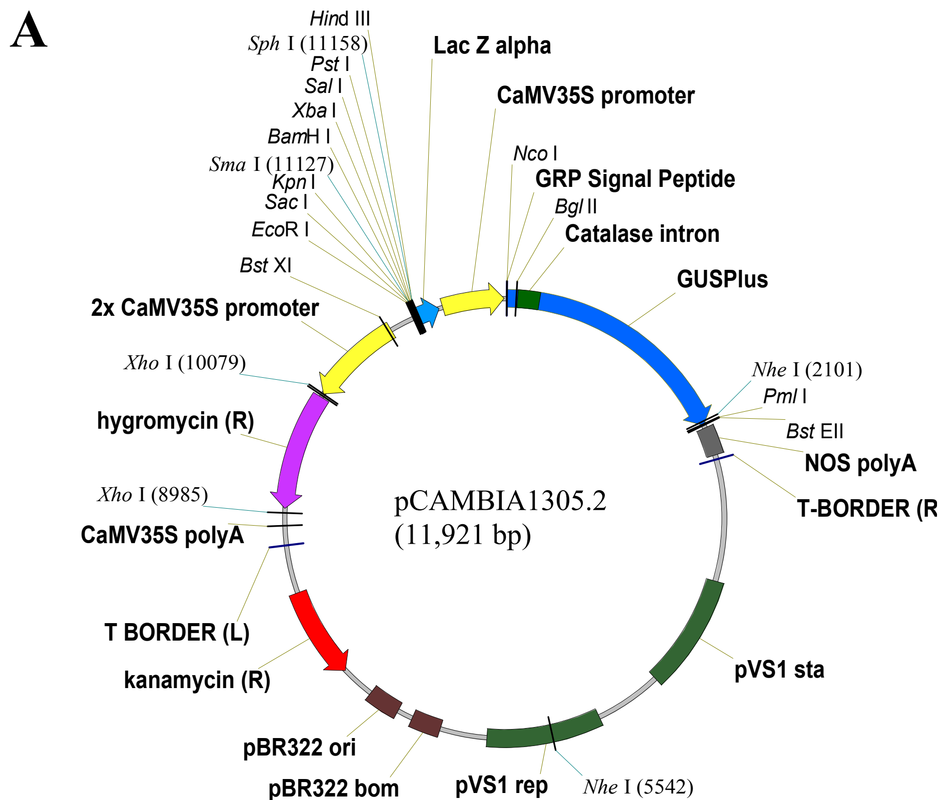
Complete and truncated versions of pCAMBIA1305.2 carrying the "*Ubi-1* promoter – cloning site – NOS polyA" fragment of pAHC17 were constructed (Figure 3.2B and C). For the complete version, the pAHC17 fragment was inserted at pCAMBIA's polylinker between *Eco*RI and *Hin*dIII sites. For the truncated version, portion of pCAMBIA1305.2 bounded by *Eco*RI and *Pml*I sites containing the polylinker and GUS*Plus* cassette was deleted to minimize the final size of transformation vector. A 25-bp duplex oligonucleotide with sticky *Eco*RI and blunt *Pml*I ends was ligated to recircularize the truncated plasmid. The pAHC17 fragment was then inserted between *Eco*RI and *Hin*dIII sites along the 25-bp linker. Another reason for deleting the GUS*Plus* cassette was to anticipate the problem of interference among strong promoters which may occur when several of them are found within a short stretch of DNA. In either complete or truncated version, the *Ubi-1* promoter is positioned far from the two copies of CaMV 35S promoter driving expression of the hygromycin resistance gene.

The *Ubi-1* gene constructs were subcloned in the truncated pCAMBIA vector. In the future, subcloning can be done using the complete version of the vector to test promoter interference and if inclusion of the *gus* reporter gene is desired. The resulting plasmid after inserting a gene of interest for over-expression through the *Ubi-1* promoter was referred to as pCAMBIA1305.2:*Ubi*-gene name (for example, pCAMBIA1305.2: *Ubi*-*SbPHYC*).

The nucleotide sequences of constructs for *SbPHYC* and *CynCHT1* were determined (Figure 3.3 and Figure 3.4, respectively). These genes were properly subcloned in the pCAMBIA vector early during the course of this study. A few initial sequencing primers did not work, possibly due to secondary structures along the plasmid. These problem sites include the 5' end of *SbPHYC* and the portion of pCAMBIA joining the *Ubi* promoter. The pAHC17 reference sequence provided by Dr. Kay Scheets turned out to be taken from a modified version which lacked the internal *Eco*RI site between the *Ubi* promoter and NOS terminator. This modification must have been made to easily isolate the expression cassette from pAHC17, a task that required several steps in this experiment. *CynCHT1* has one base deletion while *SbPHYC* has one base insertion, one deletion, and three substitutions when aligned with respective reference sequences. These mismatches can be verified by resequencing the constructs. Sequencing primers for *SbPHYA* and *AtPHYB* constructs have been prepared.

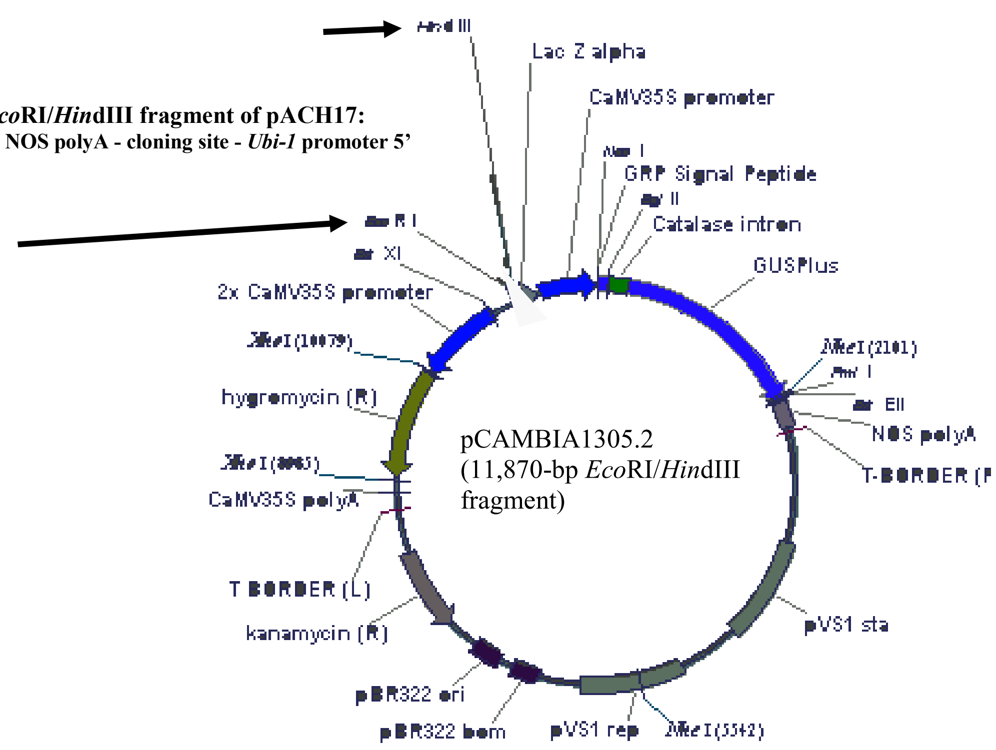
Figure 3.2. Ligation of "*Ubi-1* promoter – cloning site – NOS polyA" fragment of pAHC17 to pCAMBIA1305.2 binary vector.

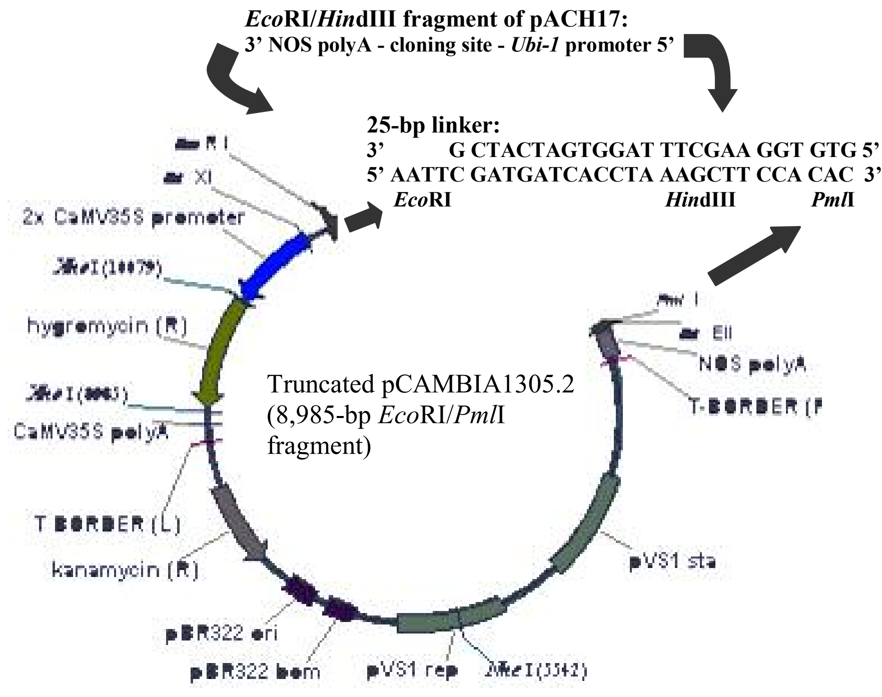
(A) Map of pCAMBIA1305.2.
(B) Use of complete pCAMBIA vector (top diagram): *Hin*dIII/*Eco*RI fragment of pACH17 containing "*Ubi-1* promoter – cloning site – NOS polyA" was inserted at the polylinker between *Hin*dIII and *Eco*RI sites.
Use of truncated pCAMBIA vector (bottom diagram): Fragment containing the polylinker, *Lac Z* alpha, and GRP-GUS cassette was deleted by digestion with *Eco*RI and *Pml*I. Plasmid was recircularized by ligating a 25-bp oligonucleotide linker. The pAHC17 fragment was then inserted between *Hin*dIII and *Eco*RI along the 25-bp linker.
(C) Agarose gel electrophoresis showing ligation of *Hin*dIII/*Eco*RI pAHC17 fragment to complete and truncated pCAMBIA vector.



Continued

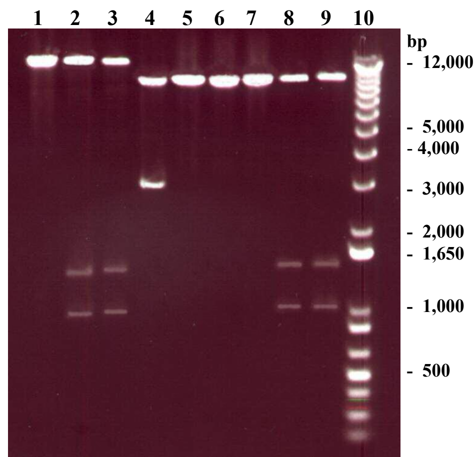
B





Continued

C



Agarose gel (0.8%) electrophoresis.

Ligation of *Hin*dIII/*Eco*RI pAHC17 fragment (approx. 2,360 bp, containing linker 1 or linker 2 as described in Figure 3.1) to pCAMBIA vector.
Lane 1: Digestion of pCAMBIA1305.2 by *Hin*dIII and *Eco*RI yielding 11,870-bp and 51-bp (not resolved) fragments.
Lanes 2 and 3: *Hin*dIII plus *Eco*RI restriction test on pCAMBIA with pAHC17 fragment containing linker 1 and linker 2, respectively. Restriction produced 11,870-bp *Hin*dIII/*Eco*RI pCAMBIA fragment, 1,404-bp *Hin*dIII/*Eco*RI pAHC17 fragment, and approx. 956-bp *Eco*RI/*Eco*RI pAHC17 fragment.

Ligation of *Hin*dIII/*Eco*RI pAHC17 fragment to truncated pCAMBIA vector.
Lane 4: Digestion of pCAMBIA1305.2 by *Eco*RI and *Pml*I yielding 8,985 and 2,936-bp fragments. The 8,985-bp fragment was circularized by ligating a 25-bp linker.
Lanes 5, 6, and 7: *Eco*RI, *Hin*dIII, and *Pml*I digestion, respectively, confirming ligation of 25-bp linker. Truncated plasmid vector (9,010 bp) was then ligated to pAHC17 fragment via *Hin*dIII and *Eco*RI sites.
Lanes 8 and 9: *Hin*dIII plus *Eco*RI restriction test on truncated pCAMBIA with pAHC17 fragment containing linker 1 and linker 2, respectively. Restriction produced 8,996-bp *Hin*dIII/*Eco*RI pCAMBIA fragment, 1,404-bp *Hin*dIII/*Eco*RI pAHC17 fragment, and approx. 956-bp *Eco*RI/*Eco*RI pAHC17 fragment.
Lane 10: 1 Kb Plus DNA Ladder.

Figure 3.3. Nucleotide Sequence of *Ubi-SbPHYC-NOS* in Truncated pCAMBIA1305.2. Below is the contiguous nucleotide sequence of *Ubi-SbPHYC-NOS* construct subcloned in truncated pCAMBIA1305.2 plasmid vector (pages 68-70, top sequence joins following sequence below). Arrows indicate sequencing primers used. Restriction sites are italicized and named. Mismatches after aligning gene construct with reference sequences are indicated by shaded boxes and include base substitution, insertion, and deletion.

portion of pCAMBIA1305.2 aligned with reference sequence (NCBI Acc. AF354046.1 bases 2,763 - 2,127):

5’TAATGTCTCCGGTTCTAGTCGCAAGTATTCTACTTTATGCGACTAAAACACGCGACAAGAAAACGCCAGGAAAAGGGCAGGGCGGCAGCCTGTCGCGTAACTTAGGACTTGTGCG 3’

5’ ACATGTCGTTTTCAGAAGACGGCTGCACTGAACGTCAGAAGCCGACTGCACTATAGCAGCGGAGGGGTTGGATCAAAGTACTTTGATCCCGAGGGGAACCCTGTGGTTGGCATGC3’
Sph I

5’ ACATACAAATGGACGAACGGATAAACCTTTTCACGCCCTTTTAAATATCCG_TTATTCTAATAAACGCTCTTTTCTCTTAGGTTTACCCGCCAATATATCCTGTCAAACACTGATAGT 3’
(A inserted in reference sequence) right T-border (italicized)

5’TTAATTCCCGATCTAGTAACATAGATGACACCGCGCGCGATAATTTATCCTAGTTTGCGCGCTATATTTTGTTTTCTATCGCGTATTAAATGTATAATTGCGGGACTCTAATCATAAAAACCCAT3’
NOS poly A (italicized)

5’ CTCATAAATAACGTCATGCATTACATGTTAATTATTACATGCTTAACGTAATTCAACAGAAATTATATGATAATCATCGCAAGACCGGCAACAGGATTCAATCTTAAGAAACTTTATTGCCAAATGT3’

5’ TTGAACGATCGGGGAAATTCGAGCTGGTCACCAATTCA 3’
*Bst*E II

portion of linker used to create truncated pCAMBIA1305.2:

5’ CACGTGTGG 3’
Pml I

***Ubi-1* promoter-exon-intron aligned with pAHC17 sequence provided by Dr. Kay Scheets:**

5’ AAGCTTGCATGCCTGCAGTGCAGCGTGACCCGGTCGTGCCCCTCTCTAGAGATAATGAGCATTGCATGTCTAAGTTATAAAAAATTACCACATATTTTTTTTGTCACACTTGTTTGAA 3’
Hind III *Sph* I *Pst* I

5’ GTGCAGTTTATCTATCTTTATACATATATTTAAACTTTACTCTACGAATAATATAATCTATAGTACTACAATAATATCAGTGTTTTAGAGAATCATATAAATGAACAGTTAGACATG 3’

5’ GTCTAAAGGACAATTGAGTATTTTGACAACAGGACTCTACAGTTTTATCTTTTTAGTGTGCATGTGTTCTCCTTTTTTTTTGCAAATAGCTTCACCTATATAATACTTCATCCATTTT 3’

5’ ATTAGTACATCCATTTAGGGTTTAGGGTTAATGGTTTTTATAGACTAATTTTTTTAGTACATCTATTTTATTCTATTTTAGCCTCTAAATTAAGAAAACTAAAACTCTATTTTAGTTTT 3’

5’TTTATTTAATAATTTAGATATAAAATAGAATAAAATAAAGTGACTAAAAATTAAACAAATACCCTTTAAGAAATTAAAAAAACTAAGGAAACATTTTTCTTGTTTCGAGTAGATAA 3’

5’TGCCAGCCTGTTAAACGCCGTCGACGAGTCTAACGGACACCAACCAGCGAACCAGCAGCGTCGCGTCGGGCCAAGCGAAGCAGACGGCACGGCATCTCTGTCGCTGCCTCTGGA 3’
Sal I

5’ CCCCTCTCGAGAGTTCCGCTCCACCGTTGGACTTGCTCCGCTGTCGGCATCCAGAAATTGCGTGGCGGAGCGGCAGACGTGAGCCGGCACGGCAGGCGGCCTCCTCCTCCTCTCA 3’

5’ CGGCACCGGCAGCTACGGGGGGATTCCTTTCCCACCGCTCCTTCGCTTTCCCTTCCTCGCCCGCCGTAATAAATAGACACCCCCTCCACACCCTCTTTCCCCAACCTCGTGTTGTTC 3’
(C and G deleted in reference sequence)

5’ GGAGCGCACACACACACACAACCAGATCTCCCCCAAATCCACCCGTCGGCACCTCCGCTTCAAGGTACGCCGCTCGTCCTCCCCCCCCCCC__TCTCTACCTTCTCTAGATCGGCGTTC 3’
Bgl II (two Cs inserted in reference sequence)

5’ CGGTCCATGGTTAGGGCCCGGTAGTTCTACTTCTGTTCATGTTTGTGTTAGATCCGTGTTTGTGTTAGATCCGTGCTGCTAGCGTTCGTACACGGATGCGACCTGTACGTCAGACAC 3’

5’ GTTCTGATTGCTAACTTGCCAGTGTTTCTCTTTGGGGAATCCTGGGATGGCTCTAGCCGTTCCGCAGACGGGATCGATTTCATGATTTTTTTTGTTTCGTTGCATAGGGTTTGGTTTG 3’

5’ CCCTTTTCCTTTATTTCAATATATGCCGTGCACTTGTTTGTCGGGTCATCTTTTCATGCTTTTTTTTGTCTTGGTTGTGATGATGTGGTCTGGTTGGGCGGTCGTTCTAGATCGGAGTA 3’

5’ GAATT____CTGTTTCAAACTACCTGGTGGATTTATTAATTTTGGATCTGTATGTGTGTGCCATACATATTCATAGTTACGAATTGAAGATGATGGATGGAAATATCGATCTAGGAT 3’
*Eco*R I (reference sequence lost the *Eco*R I site *GAATTC* due to insertion of four contiguous bases, AATT)

5’ AGGTATACATGTTGATGCGGGTTTTACTGATGCATATACAGAGATGCTTTTTGTTCGCTTGGTTGTGATGATGTGGTGTGGTTGGGCGGTCGTTCATTCGTTCTAGATCGGAGTAGA 3’

5’ ATACTGTTTCAAACTACCTGGTGTATTTATTAATTTTGGAACTGTATGTGTGTGTCATACATCTTCATAGTTACGAGTTTAAGATGGATGGAAATATCGATCTAGGATAGGTATACA 3’

5’TGTTGATGTGGGTTTTACTGATGCATATACATGATGGCATATGCAGCATCTATTCATATGCTCTAACCTTGAGTACCTATCTATTATAATAAACAAGTATGTTTTATAATTATTTTGA 3’

5’TCTTGATATACTTGGATGATGGCATATGCAGCAGCTATATGTGGATTTTTTTAGCCCTGCCTTCATACGCTATTTATTTGCTTGGTACTGTTTCTTTTGTCGATGCTCACCCTGTTGTT 3’

5’TGGTGTTACTTCTGCAGGTCGACTCTAGAGGATCC 3’
Pst I *Sal* I *Xba* I *Bam*H I (original restriction sites in pAHC17)

portion of pAHC17 linker 1:

5’TGTGAGCTCGGTACC3’
Sac I *Kpn* I

pBluescript cloning site:

5' GGGCCCCCCCTCGAGGTCGACGGTATCGATAAGCTTGATATCGAATTCGCGGCCG 3'

SbPHYC **cDNA aligned with reference sequence (NCBI Acc. SBU56731 bases 1 – 3,755):**

5' CTGCCCCGCGCAAGCCG_CTCGCTGTCCCACTCGCGCGCCACGCCTGACGCCTCGGACCCGGAGGTGTATTCCCTCTTCTTCCCCCGTGCTTATCCGCGGCGGAATCCAATCCCC 3'
(A replaced by G) ■ ■ (G inserted in reference sequence)

5' TCCCTCATCACGTCCTTCTCCCAGGCAAAGCCCCCTCTTTTTGGGCGTATGCCCCCGCTGGCGCGCCTATGATTCCCGCCGATTCATCCGCCCGCTCGGTCCAGCAACCCTTGCTGC 3'
■ (C deleted in reference sequence)

5' TGCAGTGCTGCGGCTAGTTCGGTGGCGGGGAAGGAGGAGGAGGAGGGATCTTGCAGTGGGGCGAAGCGGAGATGTCGTCGCCGTTGAACAACCGGGGGACGTGCTCCCGGAGC 3'

5' AGCTCTGCGCGGTCCAGGCACAGCGCGCGGGTGGTGGCGCAGACGCCCGTGGACGCGCAGCTGCACGCCGAGTTCGAGAGCTCCCAGCGCAACTTCGACTACTCCTCGTCGGTG 3'

5' AGCGCCGCCATCCGACCGTCGGTCAGCACCAGCACCGTCTCCACCTACCACCAGACCATGCAGCGGGGCCTCTACATCCAGCCCTTCGGCTGCCTGCTCGCCGTCCACCCGGACA 3'

5' CCTTCACGTTGCTCGCCTACAGCGAGAACGCGCCGGAGATGCTCGACCTCACGCCACACGCGGTCCCCACCATCGACCAGCGGGACGCGCTCGCCGTCGGCGCCGACGTGCGCA 3'

5' CGCTCTTCCGCTCGCAGAGCTCCGTCGCGCTGCACAAGGCCGCCACCTTCGGGGAGGTCAACCTGCTCAACCCCATCCTCGTGCATGCCAGGACGTCGGGGAAGCCCTTCTACGC 3'

5' CATATTGCACCGGATCGACGTCGGCCTTGTCATCGACCTTGAGCCGGTCAACCCAGTTGACGTGCCAGTCACTGCTGCGGGTGCGCTTAAGTCGTACAAGCTCGCCGCCAAGGCC 3'

5' ATCTCCAGGCTGCAGTCGCTGCCCAGCGGGAACCTGTCGCTGCTGTGCGATGTGCTTGTCCGTGAGGTGAGCGAGCTCACGGGCTATGACCGGGTCATGGCGTACAAGTTCCATG 3'

5' AGGATGAGCATGGTGAGGTCATTTCCGAGTGCAGGAGGTCTGATCTGGAGCCGTATCTTGGCCTGCACTACCCAGCCACCGACATCCCGCAGGCGTCCAGGTTCTTGTTTATGAA 3'

5' GAACAAAGTGAGGATGATATGTGATTGCTCTGCCACTCTGGTGAAGATCATTCAGGATGATAGCCTAGCACAGCCTCTCAGCCTCTGTGGTTCCACCCTCAGGGCTTCCCATGGTT 3'

5' GCCATGCACAGTACATGGCAAACATGGGTTCTGTTGCATCGCTTGTGATGTCAGTGACTATAAGCAATGATGAGGAGGAAGATGTTGATACCGGGAGTGACCAACAACCGAAAG 3'

5' GCAGGAAACTGTGGGGGCTGGTCGTCTGCCATCATACAAGCCCGAGGTTCGTCCCTTTCCCACTAAGGTACGCTTGCGAGTTTCTCTTGCAAGTATTTGGCATACAGCTAAACAAG 3'

5' GAGGTGGAATTGGCTGCTCAGGCAAAGGAGAGGCACATCCTCAGAACGCAAACCCTTCTTTGTGATATGCTCCTGCGGGATGCTCCTGTTGGGATATTTACCCAGTCACCTAATG 3'
■ (T replaced by G in reference sequence)

5' TGATGGATCTAGTAAAGTGCGATGGAGCTGCATTGTATTACCAGAACCAGCTTTTGTTGCTCGGATCAACACCCTCCGAGTCAGAGATAAAGAGCATTGCCACATGGCTGCAGGA 3'
■ (C replaced by T in reference sequence)

5' GAACCATGATGGTTCAACTGGGCTGAGTACTGACAGCTTAGTGGAAGCAGGTTATCCTGGTGCTGCTGCACTTCGTGAAGTTGTGTGTGGCATGGCGGCTATAAAGATCTCTTCCA 3'

5' AAGATTTTATCTTCTGGTTCCGATCGCACACAACAAAGGAGATCAAGTGGGGTGGGGCTAAGCATGAACCGGTTGACGCAGATGACAATGGCAGGAAGATGCATCCACGATCTT 3'

5' CATTCAAGGCCTTCTTGGAGGTGGTTAAATGGAGAAGTGTTCCCTGGGAGGATGTTGAAATGGATGCTATTCATTCTTTGCAGTTAATATTACGTGGCTCCCTGCAAGATGAAGAT 3'

5' GCCAACAGAAACAATGTAAGGTCCATTGTAAAAGCTCCACCTGATGATACGAAGAAGATACAGGGGCTACTTGAACTAAGAACAGTTACAAACGAGATGGTCCGCTTAATAAGC 3'

5' TCCACCTGATGATACGAAGAAGATACAGGGGCTACTTGAACTAAGAACAGTTACAAACGAGATGGTCCGCTTAATTGAGACAGCAACCGCCCCTGTCTTGGCTGTCGACATTGCC 3'

5' GGTAACATAAATGGATGGAACAATAAAGCTGCAGAACTAACAGGGTTACCTGTAATGGAAGCCATAGGGGGCCTCTGATAGATCTTGTTGTTGTTGATTCTATTGAAGTGGTTAA 3'

5' GCGGATTTTGGACTCAGCTTTACAAGGAATTGAAGAGCAAAATCTGGAAATCAAGCTTAAAGCATTCCATGAACAGGAATGCAATGGTCCAATAATCTTGATGGTTAACTCCTGC 3'

5' TGTAGTCGGGACCTTTCAGAGAAAGTCATTGGAGTTTGCTTTGTAGGACAAGATTTGACCACGCAGAAGATGATTATGGATAAGTATACTAGGATACAAGGAGACTATGTTGCCA 3'

5' TAGTAAAGAACCCCAGTGAGCTCATCCCTCCCATATTTATGATCAATGATCTTGGTTCCTGCTTAGAGTGGAATAAAGCTATGCAGAAGATTACCGGTATACAGAGGGAAGATGT 3'

5' GATAGATAAGTTGTTAATTGGGGAGGTCTTCACCCTTCATGATTATGGCTGTAGGGTGAAAGATCATGCTACTCTAACGAAACTTAGCATACTGATGAATGCAGTGATTTCTGGTC 3'

5' AGGATCCCTGAGAAGCTCCTTTTTGGTTTCTTCGACACAGATGGGAAGTATATTGAATCCTTGCTGACAGTGAACAAGAGAATAAATGCTGAGGGTAAGATCACTGGCGCTATTT 3'

5' GCTTTCTGCATGTGGCCAGTCCAGAGCTTCAGCATGCTCTCCAGGTGCAGAAAATGTCTGAACAAGCTGCCACAAACAGTTTTAAGGAATTAACTTACATTCATCAAGAATTAAG 3'

5' GAACCCACTCAATGGCATGCAATTTACTTGCAACTTATTGGAGCCTTCCGAATTGACAGAGGAGCAGAGGAAACTTCTTTCATCTAATATTCTCTGTCAGGACCAGCTGAAAAAG 3'

5’ ATTTTACATGACACTGATCTTGAAAGCATTGAACAGTGCTATATGGAGATGAACACAGTAGAGTTCAACCTTGAGGAAGCTCTTAATACGGTCCTAATGCAAGGCATTCCTTTGG 3’

5’ GCAAGGAAAAGCGAATTTCTATTGAACGTGATTGGCCGGTGGAAATATCACGCATGTACCTTTACGGGGACAATTTAAGGCTTCAGCAGGTCCTAGAACACAGTAGAGTTCAACC 3’

5’ TTGAGGAAGCTCTTAATACGGTCCTAATGCAAGGCATTCCTTTGGGCAAGGAAAAGCGAATTTCTATTGAACGTGATTGGCCGGTGGAAATATCACGCATGTACCTTTACGGGGA 3’

5’ CAATTTAAGGCTTCAGCAGGTCCTAGCAGACTATCTGGCATGCGCCCTTCAATTCACACAACCAGCTGAAGGACCTATCGTGCTCCAGGTCATTCCCAAGAAGGAAAACATTGGG 3’

5’ TCTGGCATGCAGATTGCTCATTTGGAGTTCAGGATTGTCCATCCAGCTCCAGGCGTCCCCGAGGCCCTGATACAGGAGATGTTCCGGCACAACCCAGAGGTGTCCAGGGAGGGCC 3’

5’ TCGGCCTGTACATATGCCAGAAGCTGGTGAAAACGATGAGTGGCACGGTACAGTACCTACGAGAAGCCGATACCTCATCGTTCATCATCCTGATAGAGTTCCCAGTCGCCCAGCT 3’

5’ CAGCAGCAAAGCGGTCCAAGCCTTCGACGAGTAAATTCTGACACTCATGCTCCTGGAACTGCAGTGAGAGTTCGGGCGGCCGCGAA 3’

pBluescript cloning site:

5’ TTCCTGCAGCCCGGGGGATCC 3’

portion of pAHC17 linker 1:

5’ *ACTAGT*GTTAGA*CCGCGG* 3’
Spe I *Sac* II

NOS **terminator aligned with pAHC17 sequence provided by Dr. Kay Scheets:**

5’ *GTCGACCTGCAG*ATCGTTCAAACATTTGGCAATAAAGTTTCTTAAGATTGAATCCTGTTGCCGGTCTTGCGATGATTATCATATAATTTCTGTTGAATTACGTTAAGCATGTAATAA 3’
Sal I *Pst* I (original restriction sites in pAHC17)

5’ TTAACATGTAATGCATGACGTTATTTATGAGATGGGTTTTTATGATTAGAGTCCCGCAATTATACATTTAATACGCGATAGAAAACAAAATATAGCGCGCAAACTAGGATAAATT 3’

5’ ATCGCGCGCGGTGTCATCTATGTTACTAGATCCGATGATAAGCTGTCAAACATGA*GAATTC* 3’
(C substituted by G in reference sequence) *Eco*R I (reference sequence lacked 21 bases 5’ of *Eco*R I site)

portion of pCAMBIA1305.2 aligned with reference sequence (NCBI Acc. AF354046.1 bases 11,107 – 11,035, additional bases are shown at 3’ end to include priming site):

5’ GTAATCATG_TCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGG 3’
(G inserted in reference sequence)

Figure 3.4. Nucleotide Sequence of *Ubi-CynCHT1-NOS* in Truncated pCAMBIA1305.2. Below is the contiguous nucleotide sequence of *Ubi-CynCHT1-NOS* construct subcloned in truncated pCAMBIA1305.2 plasmid vector (pages 71-72, top sequence joins following sequence below). Arrows indicate sequencing primers used. Restriction sites are italicized and named. Mismatches after aligning gene construct with reference sequences are indicated by shaded boxes and include base substitution, insertion, and deletion.

portion of pCAMBIA1305.2 aligned with reference sequence (NCBI Acc. AF354046.1 bases 2,761 - 2,127):

5' ATGTCTCCGGTTCTAGTCGCAAGTATTCTACTTTATGCGACTAAAACACGCGACAAGAAAACGCCAGGAAAAGGGCAGGGCGGCAGCCTGTCGCGTAACTTAGGACTTGTGCGAC 3'

5' ATGTCGTTTTCAGAAGACGGCTGCACTGAACGTCAGAAGCCGACTGCACTATAGCAGCGGAGGGGTTGGATCAAAGTACTTTGATCCCGAGGGGAACCCTGTGGTTG*GCATGC*AC 3'
Sph I

5' ATACAAATGGACGAACGGATAAACCTTTTCACGCCCTTTTAAATATCCGATTATTCTAATAAACGCTCTTTTCTCTTAG*GTTTACCCGCCAATATATCCTGTC*AAACACTGATAGTTT 3'
■ (A deleted in reference sequence) right T-border (italicized)

5' AATT*CCCGATCTAGTAACATAGATGACACCGCGCGCGATAATTTATCCTAGTTTGCGCGCTATATTTTGTTTTCTATCGCGTATTAAATGTATAATTGCGGGACTCTAATCATAAAAACCCATCT* 3'
NOS poly A (italicized)

5' *CATAAATAACGTCATGCATTACATGTTAATTATTACATGCTTAACGTAATTCAACAGAAATTATATGATAATCATCGCAAGACCGGCAACAGGATTCAATCTTAAGAAACTTTATTGCCAAATGTTT* 3'

5' *GAACG*ATCGGGGAAATTCGAGCT*GGTCACC*AATTCA 3'
*Bst*E II

portion of linker used to create truncated pCAMBIA1305.2:

5' *CACGTG*TGG 3'
Pml I

Ubi-1 **promoter-exon-intron aligned with pAHC17 sequence provided by Dr. Kay Scheets:**

5' *AAGCTTGCATGCCTGCAG*TGCAGCGTGACCCGGTCGTGCCCCTCTCTAGAGATAATGAGCATTGCATGTCTAAGTTATAAAAAATTACCACATATTTTTTTTGTCACACTTGTTTGAA 3'
Hind III *Sph* I *Pst* I

5' GTGCAGTTTATCTATCTTTATACATATATTTAAACTTTACTCTACGAATAATATAATCTATAGTACTACAATAATATCAGTGTTTTAGAGAATCATATAAATGAACAGTTAGACATG 3'

5' GTCTAAAGGACAATTGAGTATTTTGACAACAGGACTCTACAGTTTTATCTTTTTAGTGTGCATGTGTTCTCCTTTTTTTTTGCAAATAGCTTCACCTATATAATACTTCATCCATTTT 3'

5' ATTAGTACATCCATTTAGGGTTTAGGGTTAATGGTTTTTATAGACTAATTTTTTTAGTACATCTATTTTATTCTATTTTAGCCTCTAAATTAAGAAAACTAAAACTCTATTTTAGTTTT 3'

5' TTTATTTAATAATTTAGATATAAAATAGAATAAAATAAAGTGACTAAAAATTAAACAAATACCCTTTAAGAAATTAAAAAAACTAAGGAAACATTTTTCTTGTTTCGAGTAGATAA 3'

5' TGCCAGCCTGTTAAACGCC*GTCGAC*GAGTCTAACGGACACCAACCAGCGAACCAGCAGCGTCGCGTCGGGCCAAGCGAAGCAGACGGCACGGCATCTCTGTCGCTGCCTCTGGA 3'
Sal I

5' CCCCTCTCGAGAGTTCCGCTCCACCGTTGGACTTGCTCCGCTGTCGGCATCCAGAAATTGCGTGGCGGAGCGGCAGACGTGAGCCGGCACGGCAGGCGGCCTCCTCCTCCTCTCA 3'

5' CGGCACCGGCAGCTACGGGGGATTCCTTTCCCACCGCTCCTTCGCTTTCCCTTCCTCGCCCGCCGTAATAAATAGACACCCCCTCCACACCCTCTTTCCCCAACCTCGTGTTGTTC 3'
■ ■ (C and G deleted in reference sequence)

5' GGAGCGCACACACACACAACC*AGATCT*CCCCCAAATCCACCCGTCGGCACCTCCGCTTCAAGGTACGCCGCTCGTCCTCCCCCCCCCCC_ _TCTCTACCTTCTCTAGATCGGCGTTC 3'
Bgl II ■ (two Cs inserted in reference sequence)

5' CGGTCCATGGTTAGGGCCCGGTAGTTCTACTTCTGTTCATGTTTGTGTTAGATCCGTGTTTGTGTTAGATCCGTGCTGCTAGCGTTCGTACACGGATGCGACCTGTACGTCAGACAC 3'

5' GTTCTGATTGCTAACTTGCCAGTGTTTCTCTTTGGGGAATCCTGGGATGGCTCTAGCCGTTCCGCAGACGGGATCGATTTCATGATTTTTTTTGTTTCGTTGCATAGGGTTTGGTTTG 3'

5' CCCTTTTCCTTTATTTCAATATATGCCGTGCACTTGTTTGTCGGGTCATCTTTTCATGCTTTTTTTTGTCTTGGTTGTGATGATGTGGTCTGGTTGGGCGGTCGTTCTAGATCGGAGTA 3'

5' *GAATT*_ _ _ _*C*TGTTTCAAACTACCTGGTGGATTTATTAATTTTGGATCTGTATGTGTGTGCCATACATATTCATAGTTACGAATTGAAGATGATGGATGGAAATATCGATCTAGGAT 3'
*Eco*R I ■ (reference sequence lost the *Eco*R I site *GAATTC* due to insertion of four contiguous bases, AATT)

5' AGGTATACATGTTGATGCGGGTTTTACTGATGCATATACAGAGATGCTTTTTGTTCGCTTGGTTGTGATGATGTGGTGTGGTTGGGCGGTCGTTCATTCGTTCTAGATCGGAGTAGA 3'

5' ATACTGTTTCAAACTACCTGGTGTATTTATTAATTTTGGAACTGTATGTGTGTGTCATACATCTTCATAGTTACGAGTTTAAGATGGATGGAAATATCGATCTAGGATAGGTATACA 3'

5' TGTTGATGTGGGTTTTACTGATGCATATACATGATGGCATATGCAGCATCTATTCATATGCTCTAACCTTGAGTACCTATCTATTATAATAAACAAGTATGTTTTATAATTATTTTGA 3'

5' TCTTGATATACTTGGATGATGGCATATGCAGCAGCTATATGTGGATTTTTTTAGCCCTGCCTTCATACGCTATTTATTTGCTTGGTACTGTTTCTTTTGTCGATGCTCACCCTGTTGTT 3'

5' TGGTGTTACTT*CTGCAGGTCGACTCTAGAGGATCC* 3'
Pst I *Sal* I *Xba* I *Bam*H I (original restriction sites in pAHC17)

Cyn CHT1 **genomic DNA aligned with reference sequence (NCBI Acc. AF105425 bases 574 – 1,859):**

5' *GCTCATCAGCGATAGCCACCTCTATCTATATAACGCACGTCCATGCGCACATGCCGC*TCCAGAACTCCTTCCATATTCAACACAAGCCGTACGCTACCGCCAAGTAGCAAGTGCGATC 3'
CHT1 promoter (italicized)

5' GCCACTTGGTTGCGTCGACGATGGCATATTCCGACGCATTATTGTTCGCCGTCACGGCTGTTGCTTCCCTGGTCACTTCCGGCGGCTTCTTCGCGGAGGCGCGGTGGTACGGCCCC 3'

5' GGCGGGAAGTGCAGCTCCGTGGAGGCGCTCGTGAGCGAGCGGCTGTACAACTCGTTGTTCCTGCACAAGGACGACCCGGCCTGCCCGGCAAAGGGGTTCTACACCTACTCGTCCT 3'

5' T_CATCCAGGCCGCCCGCGCCTTCCCCAAGTTCGCCGGCACCGGCGACCTTGCCACCCGCAAGCGCGAGCTCGCCGCCTTCTTCGCGCAAATCTCCCACGAGACCACAGGTAGGT 3'
■ (T inserted in reference sequence)

5' AGGAGGCACGGTAGTAGAACAGATGATCAAACAATGAATCTTGTTCAAATCATCAATGGATATGACGTTTAATTTGTGACAAAACGCTCGATTTTCTGCAAGGCGGCTGGGCGAC 3'

5' GGCTCCGGACGGCCCGTACTCGTGGGGCCTGTGCTACAAGGAGGAGATCAGCCCGGCGAGCAACTACTGCGACGCCACGGACAAGCAGTGGCCGTGCTACCCGGGCAAATCCTA 3'

5' CCACGGCCGGGGCCCCATCCAGCTCTCGTGGAACTTCAACTACGGGCCGGCGGGGCAGGCGCTGGGCTTCGACGGCCTGCGTAACCCGGAGATCGTAGCCAATTGCTCCGACAC 3'

5' GGCTCCGGACGGCCCGTACTCGTGGGGCCTGTGCTACAAGGAGGAGATCAGCCCGGCGAGCAACTACTGCGACGCCACGGACAAGCAGTGGCCGTGCTACCCGGGCAAATCCTA 3'

5' CCACGGCCGGGGCCCCATCCAGCTCTCGTGGAACTTCAACTACGGGCCGGCGGGGCAGGCGCTGGGCTTCGACGGCCTGCGTAACCCGGAGATCGTAGCCAATTGCTCCGACAC 3'

5' GGCGTTCCGGACGGCGCTCTGGTTCTGGATGACGCCGCGGAGACCCAAGCCGTCGTGCCACGAGGTCATGGTCGGGGAGTACCGACCCACCGCCACCGATGTTGCGGGGAACCG 3'

5' GATGCCCGGGTTCGGGCTCGTCACCAACATCGTCAACGGCGGCCTCGAGTGCAACCGCACGGACGACGCGCGCGTGAACAACCGCATCGGGTTTTACCGACGGTACTGCCAGAT 3'

5' TTTCAACGTCGACACCGGGCCCAACCTCGATTGCGCGCACCAGCAACCGTACTAGCAAGTGATCAGCGCGTGGGTCGTATATAGTGCCCAAGTTTAGATGATACAAATGCCATTG 3'

5' ATCTTGAGCTGTCAATGTGTAGATTCAATCCTTAAAAACTTTACTAAGTGTAAAATTTAGGAGAGATGTTACGGTGCTTGGAGTAAATAAGCAAAAAGCTCTAAATCAAGGTGAT 3'

5' GGTCGCTCCACTCGTGTTGCC 3'

portion of pAHC17 linker 1:

5' *GGTACC*TGA*GGGCCC*TGA*TGATCA*ACA*ACTAGT*GTTAGA*CCGCGG* 3'
Kpn I *Apa I* *Bcl I* *Spe* I *Sac* II

NOS **terminator aligned with pAHC17 sequence provided by Dr. Kay Scheets:**

5' *GTCGACCTGCAG*ATCGTTCAAACATTTGGCAATAAAGTTTCTTAAGATTGAATCCTGTTGCCGGTCTTGCGATGATTATCATATAATTTCTGTTGAATTACGTTAAGCATGTAATAA 3'
Sal I *Pst* I (original restriction sites in pAHC17)

5' TTAACATGTAATGCATGACGTTATTTATGAGATGGGTTTTTATGATTAGAGTCCCGCAATTATACATTTAATACGCGATAGAAAACAAAATATAGCGCGCAAACTAGGATAAATT 3'

5' ATCGCGCGCGGTGTCATCTATGTTACTAGATCCGATGATAAGCTGTCAAACATGA*GAATTC* 3'
(C substituted by G in reference sequence) ■ *Eco*R I (reference sequence lacked 21 bases 5' of *Eco*R I site)

portion of pCAMBIA1305.2 aligned with reference sequence (NCBI Acc. AF354046.1 bases 11,107 – 11,035, additional bases are shown at 3' end to include priming site):

5' GTAATCATG_TCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGG 3'
■ (G inserted in reference sequence)

A. tumefaciens Cells

A. tumefaciens EHA101, a derivative of strain A281, has a C58 chromosome background and the disarmed pTi Bo542 as the virulence helper plasmid wherein the entire T-DNA was replaced by a *nptII* gene for bacterial selection (Hood *et al*., 1986). To permit use of binary vectors that also contain kanamycin resistance for selecting bacteria, the *nptII* gene was eliminated from the helper plasmid of EHA101 resulting in EHA105 (Hood *et al.,* 1993). Thus, EHA105 was used in this study to harbor pCAMBIA1305.2 which carries *nptII*.

There is no antibiotic selection suggested to maintain EHA105. Initial preparation of EHA105 showed slight resistance to kanamycin (compared to full resistance of EHA101 based on density of bacterial growth) likely due to contamination with EHA101 cells since the two strains were grown at the same time. This posed difficulty in selecting EHA105 cells transformed with the pCAMBIA1305.2 vector. The problem was corrected after going back to the original agar culture sent by the provider, which resulted in EHA105 preparation that was completely sensitive to kanamycin. Resistance of the *Agrobacteria* to other antibiotics was tested (Table 3.3) by growing 50 µL of glycerol stocks in 50 mL of liquid YEP medium at 28°C. Resistance was based on turbidity of medium after 20 hours of culture. EHA101 and EHA105 are known to have chromosome-based resistance to rifampicin and nalidixic acid as conferred by the parent strain A281. These antibiotics at 50 ug mL^{-1} permitted very minimal growth of the *Agrobacteria*. The medium did not turn turbid but some bacterial cells were pelleted upon centrifugation. Except for kanamycin resistance, no other differences are expected between EHA101 and EHA105. Interestingly, the tests performed here indicated that

EHA105 but not EHA101 has strong resistance to 75 ug mL^{-1} spectinomycin and 100 ug mL^{-1} ampicillin.

Table 3.3. Growth of EHA101 and EHA105 in different antibiotics.

	EHA101	EHA105	EHA105 harboring pCAMBIA1305.2	*E. coli* strain SCS110 not harboring any plasmid
without antibiotic	normal growth	normal growth	normal growth	normal growth
75 µg mL^{-1} kanamycin	normal growth (resistant)	no growth (sensitive)	normal growth (resistant)	no growth (sensitive)
75 µg mL^{-1} chloramphenicol	no growth (sensitive)	no growth (sensitive)	no growth (sensitive)	no growth (sensitive)
75 µg mL^{-1} spectinomycin	no growth (sensitive)	normal growth (resistant)	normal growth (resistant)	no growth (sensitive)
100 µg mL^{-1} ampicillin	minimal growth (low resistance)	normal growth (resistant)	normal growth (resistant)	no growth (sensitive)
50 µg mL^{-1} rifampicin	minimal growth (low resistance)	minimal growth (low resistance)	minimal growth (low resistance)	no growth (sensitive)
50 µg mL^{-1} nalidixic acid	minimal growth (low resistance)	minimal growth (low resistance)	minimal growth (low resistance)	no growth (sensitive)

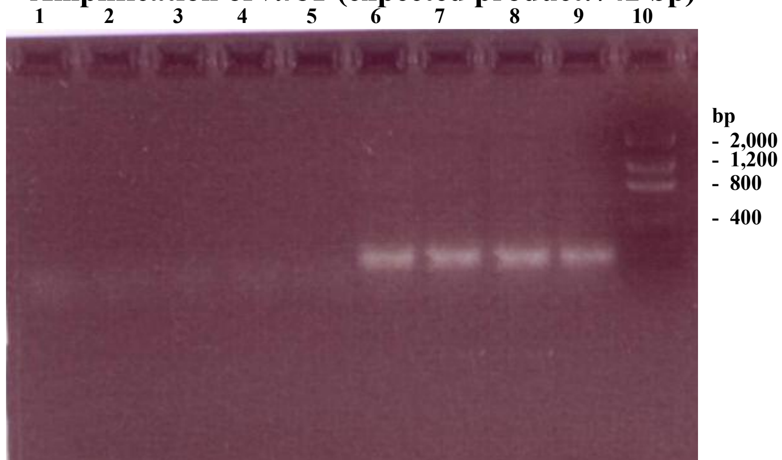
To further examine the *Agrobacterium* preparation and rule out possible contamination with *Escherichia* species, the 3-ketolactose test (Bernaerts and de Ley, 1967) was performed. EHA101 and 105 colonies grown on 3-keto medium were somewhat shiny and seemed to have the expected yellow rings of Cu_2O after being

flooded with Benedict reagent although the yellow stain was very pale. The rings were not observed in the control *E. coli* DH5α colonies which were smaller in diameter compared with the *Agrobacteria*. Furthermore, EHA101 and 105 grew optimally at 28°C and very poorly at 37°C which is characteristic of Agrobacteria.

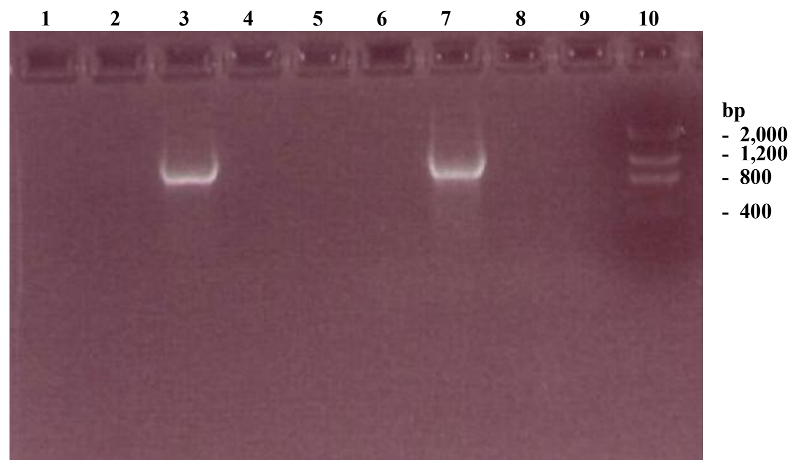
A fragment of the *gus, hptII*, *SbPHYC, CynCHT1*, and *virb1* was PCR amplified from EHA105 and *E. coli* cells (Figure 3.5). The *virb1* primer pairs were based on nucleotide sequence of the Ti plasmid of *A. tumefaciens* strain C58 (expected size of amplification product was 742 bp). These primers amplified a single band (<400-bp) from EHA105 suggesting presence of the virulence gene sequence in its engineered helper plasmid. Expected fragment size for *gus, hptII, SbPHYC*, and *CynCHT1* was amplified from transformed EHA105 and *E. coli* cells indicating successful introduction of specific plasmid vectors. Amplification of the *hptII* gene produced a single specific band and this PCR assay was designated for screening transformed plants. Amplification products for *SbPHYC* and *CynCHT1* were designed to include a short sequence at the 3' end of the *Ubi* promoter. However, the PCR primers used produced primer dimers and non-specific amplification products. New primers need to be designed to improve the PCR assay and to effectively use this scheme for screening transformed bermudagrass plants that have endogenous phytochrome and chitinase genes.

EHA105 cells transformed with pCAMBIA carrying *PHY* genes grew much slower in YEP medium compared with cells transformed with the native pCAMBIA vector. This must be due to the increased size of the vector after inserting a phytochrome cDNA (average size of 3.5 kb). During plant transformation, this would mean that the *Agrobacterium* will take longer time to generate a copy of the T-DNA.

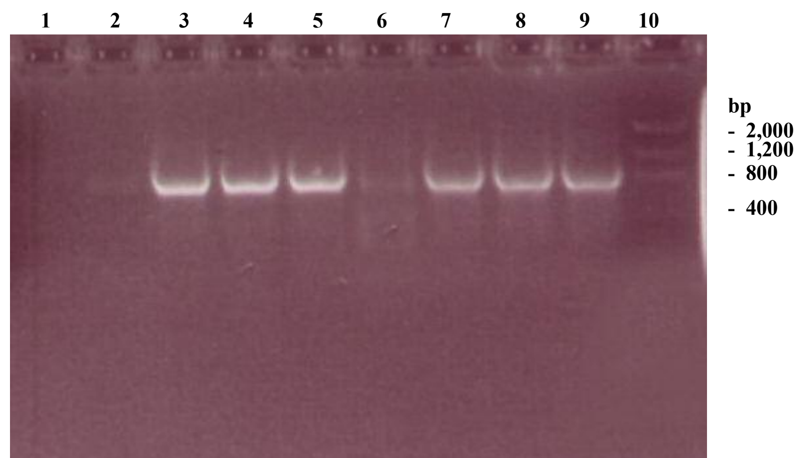
Amplification of *virb1* (expected product:742 bp)



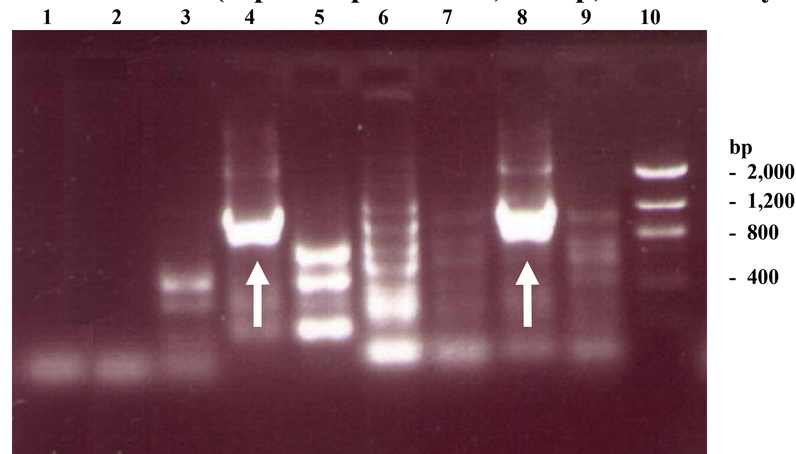
Amplification of *gus* (expected product: 1,187 bp)



Amplification of *hptII* (expected product: 885 bp)



Amplification of *Ubi-SbPHYC* (expected product: 1,159 bp, indicated by arrows)



Amplification of *Ubi-CynCHT1* (expected product: 837 bp, indicated by arrows)

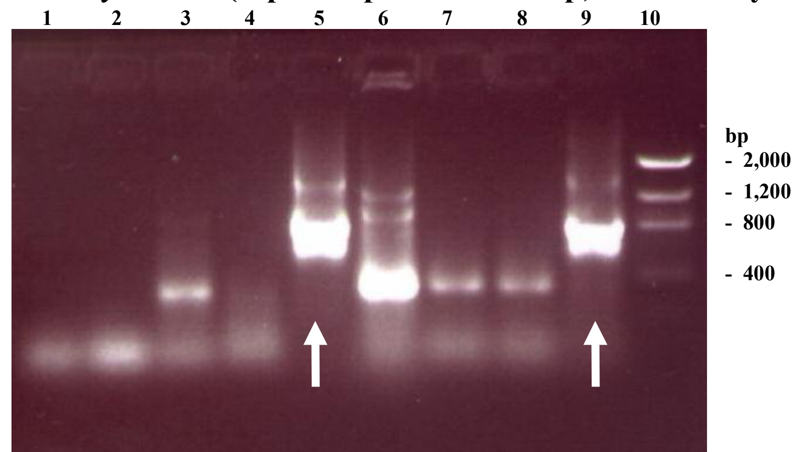


Figure 3.5. PCR amplification of gene fragments from EHA105 and *E. coli* (DH5α) cells. DNA templates were prepared by boiling diluted samples of bacterial cells.

Lane 1: water (control template)
Lane 2: untransformed *E. coli* (DH5α)
Lane 3: *E. coli* (DH5α) harboring native pCAMBIA1305.2
Lane 4: *E. coli* (DH5α) harboring truncated pCAMBIA1305.2:*Ubi-SbPHYC*
Lane 5: *E. coli* (DH5α) harboring truncated pCAMBIA1305.2:*Ubi-CynCHT1*
Lane 6: untransformed EHA105
Lane 7: EHA105 harboring native pCAMBIA1305.2
Lane 8: EHA105 harboring truncated pCAMBIA1305.2:*Ubi-SbPHYC*
Lane 9: EHA105 harboring truncated pCAMBIA1305.2:*Ubi-CynCHT1*
Lane 10: Low DNA Mass Ladder

Hygromycin "Kill" Experiments

Surface-sterilized seeds of Guymon and Yukon were germinated in the dark on germination paper. Seedlings with shoots measuring 0.5-1 cm were transplanted to MS agar medium containing 0, 10, 20, 30, 40, 50, 60, 70, or 80 mg L^{-1} hygromycin B, and grown for 15 days under 16-hour photoperiod at 25^oC nighttime and 28^oC daytime temperatures. Numbers of dead seedlings were counted at regular intervals during the 15-day culture (Table 3.4, Figure 3.6). Seedling viability was impaired by hygromycin manifested as suppressed shoot growth and root development as well as yellowing of shoots. These toxic effects were evident at a low concentration of 10 mg L^{-1}. After 15 days, nearly 100% death of Guymon seedlings occurred at 20 mg L^{-1} hygromycin (average of 14.6 dead plants out of 15) and of Yukon seedlings at 50 mg L^{-1} hygromycin (average of 14.6 dead plants out of 15). All seedlings including controls (0 mg L^{-1} hygromycin) displayed initial weakening due to transplanting stress.

To eliminate transplanting stress, seeds were directly germinated on the MS agar medium and incubated in the dark for 9 days. Inhibition of seed germination by hygromycin was manifested as lower germination counts and shorter shoot growth compared with controls (0 mg L^{-1} hygromycin) (Figure 3.7). Suppressed germination of Guymon seeds was evident starting at 20 mg L^{-1} hygromycin while germination of Yukon was suppressed starting at 60 mg L^{-1}. While germination counts in Yukon were not significantly reduced by increasing hygromycin levels, shoot growth was adversely affected. These results agree with the first set of data which indicated that level of natural hygromycin resistance varies with genotype or cultivar.

Table 3.4. Numbers of dead seedlings of Guymon and Yukon after transplanting to MS agar media with different levels of hygromycin B. (There were five replications for each level of hygromycin, and fifteen seedlings in each replicate.)

mg L^{-1} hygromycin	Day After Transplanting													
	Guymon Seedlings							Yukon Seedlings						
	3rd	5th	7th	9th	11th	13th	15th	3rd	5th	7th	9th	11th	13th	15th
	0	0	0	0	0	1	1	0	0	0	0	0	1	1
	0	0	0	0	0	0	0	0	1	1	1	1	2	2
0	0	0	0	0	1	1	1	0	0	0	0	0	0	0
(control)	0	0	0	0	0	1	1	0	0	0	0	0	0	0
	0	0	1	1	2	2	2	0	1	1	1	1	1	1
Ave.	0	0	0.2	0.2	0.6	1	1	0	0.4	0.4	0.4	0.4	0.8	0.8
	0	1	1	2	5	6	8	0	0	1	2	2	2	2
	0	2	3	6	9	11	12	2	2	3	3	3	3	4
10	0	0	1	8	10	11	11	0	1	1	1	2	2	4
	0	2	2	9	12	13	13	0	1	2	2	2	3	3
	0	0	0	5	9	10	13	0	0	0	0	0	0	2
Ave.	0	1	1.4	6	9	10.2	11.4	0.4	0.8	1.4	1.6	1.8	2	3
	0	2	2	11	14	15	15	0	1	2	4	6	8	9
	0	3	3	11	12	14	15	0	1	3	5	7	9	9
20	0	4	6	12	15	15	15	0	1	1	4	4	6	6
	0	0	7	8	13	13	13	0	1	1	2	3	5	6
	1	1	7	11	13	14	15	1	4	5	5	7	9	9
Ave.	0.2	2	5	10.6	13.4	14.2	14.6	0.2	1.6	2.4	4	5.4	7.4	7.8
	1	4	11	14	15	15	15	1	1	1	3	4	11	11
	1	3	12	15	15	15	15	0	1	3	5	7	10	12
30	0	5	12	15	15	15	15	0	1	3	3	4	9	9
	0	2	6	13	14	15	15	0	1	3	7	8	8	12
	0	1	6	12	14	15	15	1	2	4	6	7	9	10
Ave.	0.4	3	9.4	13.8	14.6	15	15	0.4	1.2	2.8	4.8	6	9.4	10.8
	0	3	6	13	15	15	15	0	2	3	5	8	8	12
	2	4	8	14	15	15	15	1	2	4	5	6	9	10
40	0	4	13	15	15	15	15	0	2	2	5	6	9	11
	0	5	9	14	15	15	15	0	0	2	3	6	9	11
	0	4	8	14	15	15	15	1	3	3	5	7	11	13
Ave.	0.4	4	8.8	14	15	15	15	0.4	1.8	2.8	4.6	6.6	9.2	11.4
	0	2	5	14	15	15	15	0	2	3	8	11	12	14
	0	1	7	15	15	15	15	0	3	4	8	12	14	14
50	0	0	4	15	15	15	15	0	2	5	6	11	13	15
	0	3	10	15	15	15	15	0	3	3	8	13	15	15
	0	1	7	15	15	15	15	0	2	3	6	13	14	15
Ave.	0	1.4	6.6	14.8	15	15	15	0	2.4	3.6	7.2	12	13.6	14.6

Continued

mg L^{-1} hygromycin	Day After Transplanting													
	Guymon Seedlings							Yukon Seedlings						
	3rd	5th	7th	9th	11th	13th	15th	3rd	5th	7th	9th	11th	13th	15th
	1	5	9	15	15	15	15	1	1	1	8	9	12	15
	0	3	11	14	15	15	15	0	1	2	6	11	15	15
60	0	3	9	14	15	15	15	1	1	2	8	13	14	15
	0	6	11	15	15	15	15	2	5	5	5	13	14	15
	1	2	11	15	15	15	15	0	3	3	8	10	15	15
Ave.	0.4	3.8	10.2	14.6	15	15	15	0.8	2.2	2.6	7	11.2	14	15
	2	4	7	15	15	15	15	3	6	7	7	12	14	15
	1	5	11	15	15	15	15	1	2	2	10	10	13	15
70	1	4	6	15	15	15	15	1	3	4	4	7	15	15
	1	3	9	15	15	15	15	0	4	4	6	12	15	15
	1	4	8	14	15	15	15	1	3	5	5	13	15	15
Ave.	1.2	4	8.2	14.8	15	15	15	1.2	3.6	4.4	6.4	10.8	14.4	15
	3	7	9	12	15	15	15	1	2	4	9	13	13	15
	3	5	6	15	15	15	15	2	3	3	8	12	15	15
80	4	7	9	15	15	15	15	1	1	2	7	13	15	15
	1	4	6	15	15	15	15	2	2	3	6	12	15	15
	2	6	10	15	15	15	15	3	3	3	7	9	15	15
Ave.	2.6	5.8	8	14.4	15	15	15	1.8	2.2	3	7.4	11.8	14.6	15

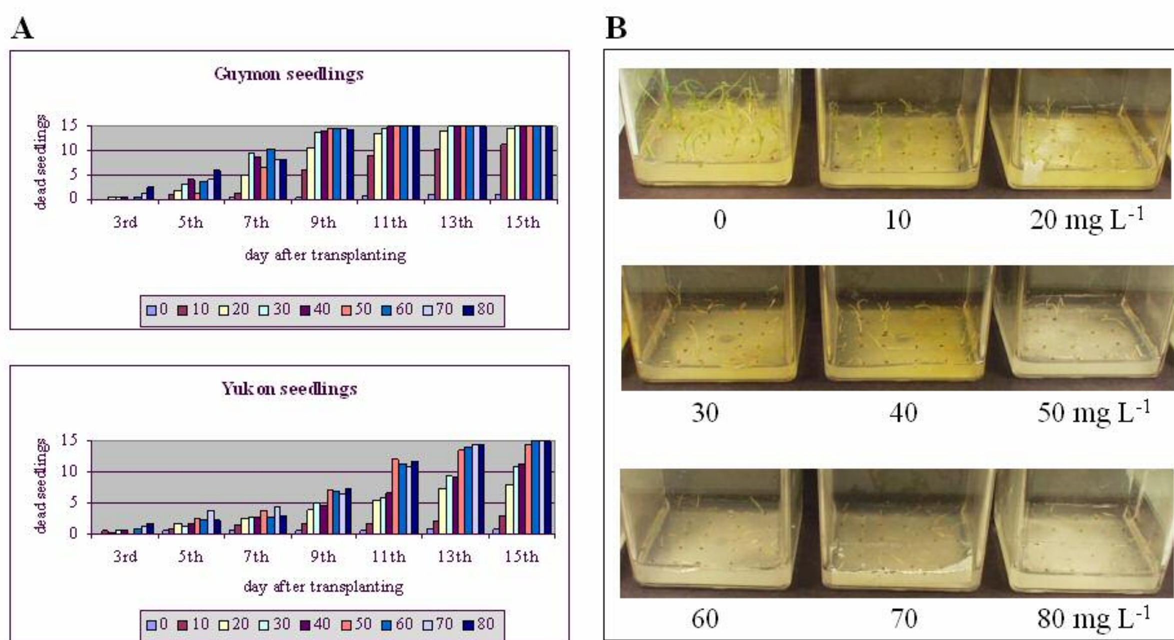


Figure 3.6. Death of Guymon and Yukon seedlings under different levels of hygromycin B. (A) Graphical presentation of the data given in Table 3.4. Averages for five replicate boxes for each level of hygromycin were plotted in the graphs. (B) Magenta boxes pictured at the end of the 15-day culture. Each box contained 15 seedlings of Guymon (left half) and 15 seedlings of Yukon.

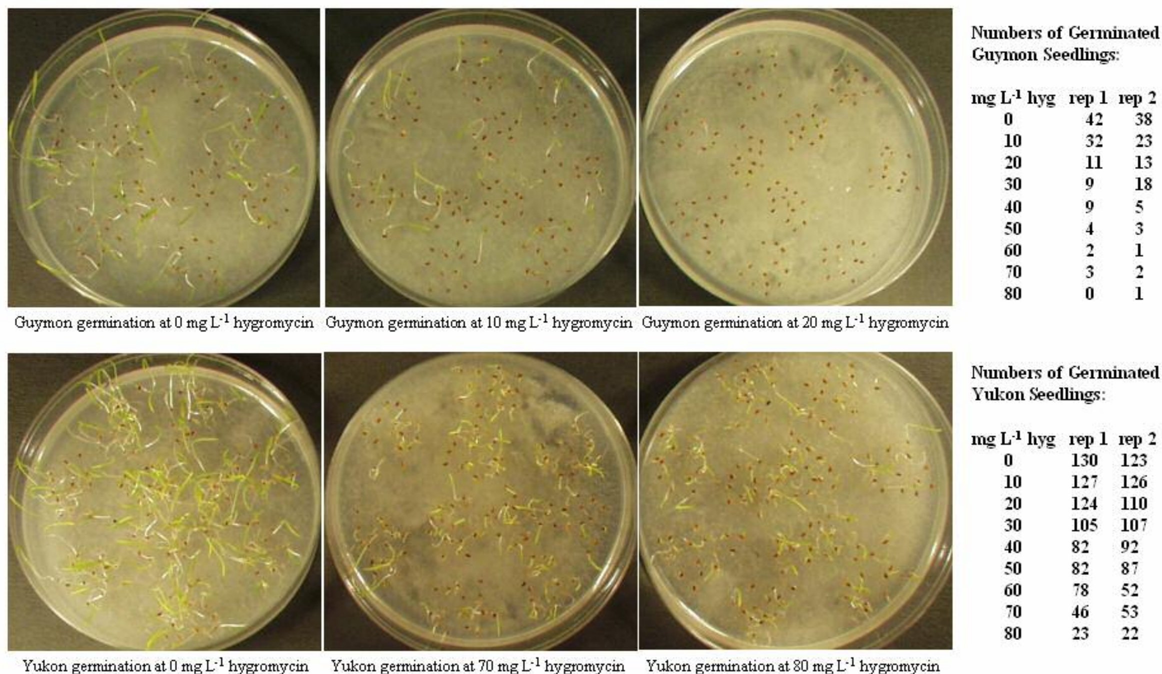
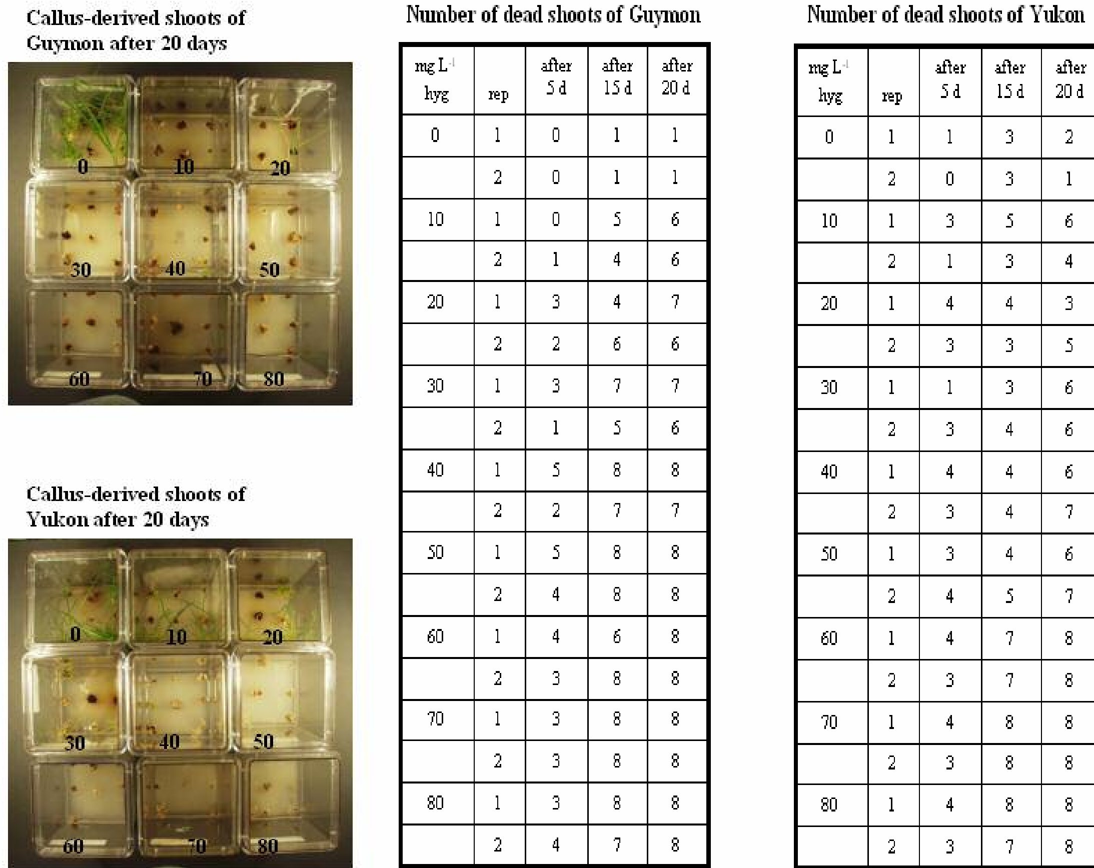


Figure 3.7. Germination of Guymon and Yukon seeds under different levels of hygromycin B. Total of 120 Guymon seeds and 150 Yukon seeds were germinated on each plate of MS medium for 9 days. Two replicate plates were prepared for each level of hygromycin.

The third experiment was performed on shoots regenerated from cultured embryogenic callus tissues. Newly shooted calli were subdivided into approximately same sizes and randomly distributed among hygromycin treatments. Shoots were placed on MS agar medium and grown under 16-hour photoperiod. Death of shoots was counted after 5, 15, and 20 days (Figure 3.8). After 5 days, browning of shoots was generally observed starting at 20 mg L^{-1} hygromycin and more adverse effects including death were observed starting at 40 mg L^{-1}. After 15 days, most surviving shoots at higher hygromycin levels were brown and stunted although patches of green tissues still persisted. On the 20th day when control shoots had grown and extensively rooted, 100% shoot death was generally observed at around 50 mg L^{-1} both in Guymon and in Yukon.



Number of dead shoots of Guymon

mg L^{-1} hyg	rep	after 5 d	after 15 d	after 20 d
0	1	0	1	1
	2	0	1	1
10	1	0	5	6
	2	1	4	6
20	1	3	4	7
	2	2	6	6
30	1	3	7	7
	2	1	5	6
40	1	5	8	8
	2	2	7	7
50	1	5	8	8
	2	4	8	8
60	1	4	6	8
	2	3	8	8
70	1	3	8	8
	2	3	8	8
80	1	3	8	8
	2	4	7	8

Number of dead shoots of Yukon

mg L^{-1} hyg	rep	after 5 d	after 15 d	after 20 d
0	1	1	3	2
	2	0	3	1
10	1	3	5	6
	2	1	3	4
20	1	4	4	3
	2	3	3	5
30	1	1	3	6
	2	3	4	6
40	1	4	4	6
	2	3	4	7
50	1	3	4	6
	2	4	5	7
60	1	4	7	8
	2	3	7	8
70	1	4	8	8
	2	3	8	8
80	1	4	8	8
	2	3	7	8

Figure 3.8. Death of callus-derived shoots of Guymon and Yukon under different levels of hygromycin B. Eight bunches of shoots were transplanted in each Magenta box. Two replicate boxes were prepared for each level of hygromycin B.

The hygromycin "kill" experiments were performed to determine appropriate levels of the antibiotic to use for selecting plant materials transformed with the selectable marker *hptII* gene carried by the pCAMBIA1305.2 vector. Results of these experiments suggest that hygromycin B can effectively eliminate non-transformed bermudagrass plant materials within 2-3 weeks of selection at concentrations of 50-75 mg L^{-1}. Therefore, levels of 150-200 mg L^{-1} implemented in other studies were excessively high.

Other stresses may aggravate the toxic effects of hygromycin such as transformation procedures involving *Agrobacterium* infection and microprojectile bombardment. Thus, newly transformed plant materials need a few days of recovery before implementing hygromycin selection. Negative selection by hygromycin exerts stress on transgenic cells because of the release of phenolic substances from non-transformed dying cells. Positive selection such as that based on growth in normally non-metabolizable sugars like mannitol may serve as a better alternative.

SUMMARY AND CONCLUSIONS

Full-length cDNA clones of phytochrome genes (*PHYA*, *PHYB*, and *PHYC* of *Arabidopsis thaliana* and *PHYA* and *PHYC* of *Sorghum bicolor*) and *CynCHT1* were subcloned in truncated pCAMBIA1305.2 binary vector and were provided the maize *Ubi-1* promoter for constitutive expression studies in transgenic bermudagrass. The efficiency of the cloning strategy employed was validated by the nucleotide sequences obtained from *Ubi-SbPHYC* and *Ubi-CynCHT1* constructs. *A. tumefaciens* strain EHA105 was transformed with pCAMBIA1305.2 and versions carrying *Ubi-1* gene constructs, in preparation for gene transfer experiments. A PCR assay based on amplification of the selectable marker *hptII* gene in the pCAMBIA vector was designed for screening transgenic bermudagrass plants. Results of hygromycin "kill" experiments indicated that concentrations of 50-75 mg L^{-1} hygromycin are sufficient for selecting transformed callus tissues and regenerated plants.

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CHAPTER 4

GENETIC TRANSFORMATION OF BERMUDAGRASS THROUGH *AGROBACTERIUM* AND PARTICLE BOMBARDMENT METHODS

ABSTRACT

Gene transfer methods utilizing *Agrobacterium* and particle bombardment were applied in bermudagrass. Caryopsis-derived embryogenic callus tissues of *C. dactylon* cv Yukon were bombarded with pCAMBIA1305.2:*Ubi-SbPHYC* using the PDS-1000 He device. A total of 27 plants that showed resistance to 75 mg L^{-1} hygromycin and positive PCR amplification of the selectable marker *hptII* gene were obtained from 11 bombarded plates of callus tissues. *A. tumefaciens* strain EHA105 harboring pCAMBIA1305.2:*Ubi-SbPHYC* was co-cultivated with 756 stolon nodes of *C. dactylon* cv Guymon. Seven among 13 plants that were directly regenerated from nodes under 75 mg L^{-1} hygromycin selection showed PCR amplification of the *hptII* gene, indicating transformation efficiency of 0.9%. *Xho*I-digested genomic DNA of transgenic plants obtained from both transformation methods showed the expected 1-kb band in Southern hybridization with the *hptII* probe. Outcomes of these experiments provided groundwork for screening and characterizing bermudagrass plants that constitutively express the sorghum *PHYC* gene, and for transforming bermudagrass with the other *PHY* gene constructs prepared in this study.

INTRODUCTION

Early genetic transformation experiments with monocot plant species have used protoplast cells subjected to direct gene transfer either by electroporation or with the aid of polyethylene glycol (Fromm *et al.,* 1985; Shillito *et al.,* 1985). Transformation efficiencies were generally low and few plant species such as rice and maize were suitable for protoplast-based manipulations. Plant transformation mediated by the soil-borne pathogen *Agrobacterium tumefaciens* was previously thought to be limited to dicotyledonous species, in particular to the *Solanaceae* (Horsch *et al.* 1984). However, utilization of *Agrobacterium* was later extended to cereals and non-cereal grasses (Hiei *et al*., 1994; Komari *et al.,* 1998; Yu *et al.,* 2000). Potential benefits of *Agrobacterium*-mediated transformation include simplicity of integration pattern and low-copy number of insertion events, preferential transfer of transgenes into transcriptionally active regions of chromosomes, low occurrence of rearrangements, transfer of large DNA fragments, and exclusion of non-relevant vector sequences (Roberts *et al*., 1998; Dai *et al*., 2001).

Agrobacterium tumefaciens is a Gram-negative soil bacterium that genetically transforms dicotyledonous plants by infecting wound sites and integrating its own DNA into the host cell chromosome where it is expressed (Zupan and Zambryski, 1995). The transferred DNA or T-DNA contains loci regulating plant hormone levels and synthesis of bacterial metabolites (opines) in crown gall tumors that are formed in the process (Chilton *et al.,* 1977; Garfinkel *et al*., 1981). The 35-kb virulence (*vir*) region of the Ti plasmid located outside the T-DNA consists of several loci, *virA, virB, virC, virD, virE, virG,* and *virH*, encoding for virulence proteins that are inducible by plant signals. These proteins direct the specific events in plant cell recognition, generate a copy of the T-

DNA, and mediate its transfer to the host chromosomes. Host cell factors involved in *Agrobacterium*-mediated plant transformation are less understood. Wound sites of some plants release low molecular weight phenolic compounds and sugars to which the *Agrobacterium* responds chemotactically (Huang *et al.,* 1990). Virulence-inducing phenolic compounds such as acetosyringone appear to be limited to Solanaceous plants and are supplied when doing genetic transformation experiments to enhance induction of *vir* genes. Inhibition of purine biosynthesis in host cells appears to modulate and increase gene transfer efficiency (Roberts *et al*., 2003). Other virulence-inducing factors are low pH, relatively low temperatures (below 26°C), and high osmotic pressure.

Invention of the biolistic or microprojectile bombardment technique provided alternative means to genetically engineer plants and eliminated difficulties associated with protoplast-based methods and host-range limitations of *Agrobacterium* (Klein *et al.,* 1987; Sanford, 1988). Stably transformed plants were reported for diverse species including forage and turfgrasses (Zhong *et al*., 1993; Spangenberg *et al*., 1995; Xiao and Ha, 1997; Dalton *et al.,* 1999; Richards *et al*., 2001). Particle bombardment has been widely used because it is less tedious and is applicable to a variety of plant species and cell types. Its main disadvantages are high cost and the complexity of transgene integration patterns.

Certain parameters need to be optimized to operate the helium-driven bombardment device (McCabe *et al*., 1993; Sanford *et al.,* 1993). These include vacuum pressure within the chamber, acceleration rate, target distance, size and density of microcarriers, DNA amount per bombardment, as well as durations of preculture and osmotic treatment of plant tissues. For transient gene expression, a variety of plant

materials were used including nonmorphogenic cell suspensions (Hensgens *et al.,* 1993). Transient expression studies were usually conducted to optimize bombardment parameters and to test utility of gene constructs. Production of transgenic plants necessitated the use of embryogenic cultures consisting of proembryogenic cell clusters and meristematic cells rich in cytoplasm (Zhong *et al*., 1993; Ye *et al.,* 1997; Spangenberg and Wang, 1998). Particle bombardment can have severe effects on the regeneration response of plant tissues. Both single copy and complex transgene integration patterns were observed in transgenic grasses produced by the biolistic method (Spangenberg *et al.,* 1995; Richards *et al.,* 2001).

Less frequently adopted DNA delivery protocols include microinjection, electrophoresis, and use of silicon carbide fibers (Griesbach, 1985; Akohas, 1989; Kaeppler *et al*., 1992). These methods have been moderately effective due to limitations imposed by a number of physical and chemical factors. In general, plant genetic transformation remains a difficult procedure in agricultural biotechnology, especially for recalcitrant species of the *Graminae*. The suitability of a gene transfer method primarily depends on the plant species, target tissues, and regeneration system. Ideal methods must be simple, relatively efficient, and preferably inexpensive.

A transformation system for bermudagrass was first reported for the sterile triploid cultivar TifEagle based on biolistic bombardment of embryogenic callus tissues from nodes of stolons (Zhang *et al*., 2003). Using the selectable marker *hpt* gene under control of the rice actin gene promoter, 75 hygromycin-resistant transgenic lines were obtained from 18 bombarded dishes. Most lines were multiple-copy transformants showing obvious somaclonal variations, and this was attributed to long periods of tissue

culture. Goldman *et al.* (2004) also transformed stolon-derived callus tissues of TifEagle by microprojectile bombardment. TifEagle was transformed with the *bar* gene driven by maize *Ubi-1* promoter in order to confer herbicide resistance and serve as a weed management tool. A total of 89 phosphinothricin-resistant plants (not all of them were transgenics) were obtained from six separate bombardments and from at least nine independent transformation events as suggested by identical restriction patterns in Southern hybridization. Most plants had multiple copies of the inserted gene, and flow cytometry indicated that 82 plants were hexaploids and the remaining seven were triploids. Recovery of hexaploid plants was attributed to the combined effects of the tissue culture system and use of 2,4-D. Time spent from tissue bombardment to production of rooted plants ranged from three to six months, which was shorter than that reported by Zhang *et al.* (2003) due to elimination of a lengthy liquid culture phase.

Li and Qu (2004) reported transformation of common bermudagrass (*Cynodon dactylon* var. J1224) by bombardment of six long-term cultures of highly regenerable callus lines derived from young inflorescence explants. Five independent integration events of *bar* and *hph* genes under control of maize *Ubi-1* and rice *Act1* promoters, respectively, were obtained from one callus line only. Four green plants having multiple transgene copies were recovered and three of them actually displayed resistance to glufosinate conferred by the *bar* gene.

Agrobacterium-mediated transformation of TifEagle was reported by Wang and Ge (2005). Sixty one hygromycin-resistant transgenic plants were produced directly from 1,127 nodal segments that were co-cultivated with *Agrobacterium* EHA105 or LB4404. Three binary vectors were used collectively in separate experiments: pCAMBIA1301,

pCAMBIA1304, and pTOK233 which all carried the *gus* and *hph* genes. The method did not involve callus culture steps and took 7 weeks from infection of nodes to production of plantlets.

OBJECTIVES

1. Transform *C. dactylon* cv Yukon with pCAMBIA1305.2:*Ubi-SbPHYC* by particle bombardment of caryopsis-derived embryogenic callus tissues.
2. Transform *C. dactylon* cv Guymon with pCAMBIA1305.2:*Ubi-SbPHYC* by directly regenerating plants from stolon nodes co-cultivated with *A. tumefaciens* EHA105 harboring the plasmid.
3. Perform PCR and Southern analyses of hygromycin-resistant plants to demonstrate transgene integration events.

MATERIALS AND METHODS

Particle Bombardment

The improved MS agar nutrient media described in Chapter 2 were used in tissue culture steps for both bombardment and *Agrobacterium* methods of genetic transformation. Callus tissues of Yukon were prepared from caryopsis explants as described in Figure 2.6. Calli were bombarded with truncated pCAMBIA1305.2 (*gus* gene deleted) carrying the *Ubi-SbPHYC* construct. To perform GUS assay and optimize bombardment parameters, the original pCAMBIA1305.2 vector carrying the *gus*

expression cassette was used in initial experiments. Six hours before bombardment, callus tissues were placed with close spacing on the center of Petri plates containing MS agar medium with 0.6 M mannitol for osmotic treatment. Each plate was bombarded twice at 6 and 9-cm target distances using the PDS-1000 He System (Bio-Rad, USA) by a mixture of 5 μg DNA and 1 mg gold particles delivered at 1,100 psi acceleration pressure and under 28 inches of Hg vacuum pressure.

Gold microcarriers were coated with DNA according to the method of Weeks *et al.* (1993) with modifications. Gold particles of 1 μm diameter were washed thrice with 500 μL absolute ethanol by vortexing for a few minutes and pelleting by centrifugation. The gold was washed twice with 500 μL sterile distilled water and finally suspended in water at 0.05 mg μL^{-1}. DNA and ethanol-washed gold particles were combined in a solution containing 1 M $CaCl_2$ and 0.01 M spermidine, and the mixture was gently shaken in TOMY microtube mixer (TOMY Tech USA, Inc., CA) for 30 minutes at 4^oC. The mixture was spun down, the supernatant removed, and the pellet was rinsed with 500 μL absolute ethanol before finally suspending in ethanol at a concentration of 5 μg DNA and 1 mg gold per 20 μl. Twenty microliters of the mixture were spread out on each piece of macrocarrier disc.

After bombardment, callus tissues were left on the osmotic medium for four hours, and then subcultured on MS medium with 3 mg L^{-1} 2,4-D and 1 mg L^{-1} kinetin for about 10 days in the dark at 28^oC daytime temperature and 25^oC nighttime temperature. Calli were further subcultured on MS medium with 3 mg L^{-1} 2,4-D and 50 mg L^{-1} hygromycin B for a few weeks to permit development and selection of hygromycin-resistant embryogenic tissues. Shoot development was induced under 16-hour

photoperiod on MS medium supplemented with 0.25 mg L^{-1} 2,4-D, 0.5 mg L^{-1} BAP, 1 mg L^{-1} GA_3, and 75 mg L^{-1} hygromycin. Rooting was performed in 75 mg L^{-1} hygromycin on MS medium without PGR supplement. Plantlets were transferred to potted soil in the greenhouse.

***Agrobacterium*-mediated Transformation**

The method described by Wang and Ge (2005) was followed with some modifications. Nodal segments of Guymon were surface sterilized and cut at the nodes to expose the meristems that were targeted for *Agrobacterium* infection. Nodal segments were immersed in inducted cells of *A. tumefaciens* EHA105 harboring pCAMBIA1305.2: *Ubi-SbPHYC*. *Agrobacteria* harboring the original pCAMBIA1305.2 vector which carries the *gus* expression cassette were used to perform GUS assay on a few nodal tissues. Vacuum pressure of 15 inches of Hg was applied for 10 minutes and excess bacterial cells were removed by blotting the nodes onto sterile paper towel. Nodes were then co-cultivated with *Agrobacteria* on MS agar medium containing 1 mM sodium thiosulfate, 3.5 mM L-cysteine, 1.5 mg L^{-1} BAP, and 1.5 mg L^{-1} kinetin for three days at room temperature under a low light condition. Co-cultivation was performed in 6 X 8-inch glass trays and the nodal segments were planted vertically in the agar medium with the cut nodal ends partially submerged to about 5-mm depth. Nodes were decontaminated by rinsing with a solution of 500 µg mL^{-1} carbenicillin and 250 µg mL^{-1} cefotaxime, and subcultured vertically in Magenta boxes containing MS agar medium supplemented with 1.5 mg L^{-1} BAP, 1.5 mg L^{-1} kinetin, 250 µg mL^{-1} carbenicillin, 250 µg mL^{-1} cefotaxime, and 75 mg L^{-1} hygromycin. The nodal cultures were incubated under a 16-hour

photoperiod (28°C daytime temperature and 25°C nighttime temperature) for several weeks. Hygromycin-resistant shoots that developed from the nodes were subcultured on MS medium without PGRs and containing 75 mg L^{-1} hygromycin to permit root development. Well-rooted plantlets were transferred to potted soil in the greenhouse.

EHA105 cells harboring the pCAMBIA vector were grown to saturation in AB-sucrose minimal liquid medium (Chilton *et al*., 1974; Appendix A) with 50 µg mL^{-1} kanamycin at 28°C for 3 days. The cells were pelleted by centrifugation at 10,000 x *g* and resuspended in an equal volume of induction medium (Hei *et al.,* 1994; Appendix A) containing 500 µM of acetosyringone (*Phyto*Technology Lab., Shawnee Mission, KS). Cells were inducted by gently shaking the suspension at room temperature for 1-2 hours, and were immediately used to inoculate nodal segments.

GUS Assay

GUS histochemical assay was performed on callus tissues bombarded with the original pCAMBIA1305.2 vector and on nodal segments co-cultivated with *Agrobacteria* harboring this plasmid, based on the method of Rueb and Hensgens (1989). Plant tissues were incubated in 0.1 M phosphate buffer (pH 6.8) with 0.1% Alconox pH-neutral detergent (Alconox, Inc., New York, NY) at 37°C for at least an hour, followed by rinsing with the phosphate buffer to remove the detergent. Tissues were then immersed in assay buffer (0.05 M NaH_2PO_4, 0.05 M Na_2HPO_4, 0.5 mM $K_3Fe(CN)_6$, 10 mM Na_2EDTA, pH 6.6) containing 50 mM X-Gluc (Sigma Chemicals, St. Louis, MO), subjected to a vacuum pressure of 15 inches of Hg for 10 minutes, and incubated at 37°C for 2-4 days. Stained tissues were stored in 70% ethanol.

Isolation of Plant Genomic DNA

Total plant genomic DNA for PCR and Southern analyses was isolated based on the method of Dellaporta *et al.* (1983). About 5 mL of leaf tissue ground with liquid nitrogen were added to 15 mL of extraction buffer (100 mM Tris, 50 mM EDTA, 500 mM NaCl, 1.25% SDS, pH 8.0) and the mixture was incubated in a 65°C-water bath for 20 minutes. Five milliliters of 5 M potassium acetate was added, the mixture shaken in an ice bath for 20 minutes, spun at 10,000 x *g* for 15 minutes, and filtered through a layer of miracloth to obtain a cleared supernatant. DNA was precipitated by adding 2/3 volume of isopropanol, pelleted by centrifugation at 10,000 x *g*, and washed with 70% ethanol. The DNA pellet was suspended in 1 mL of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0), incubated with 10 µL of 10 mg mL^{-1} RNaseA at 37°C for 15 minutes, reprecipitated by adding 1 /10 volume of 3 M sodium acetate and 2 volumes of absolute ethanol, and finally suspended in 500 µL of TE buffer. DNA concentration was determined using the NanoDrop ND-1000 Spectrophotometer (Coleman Technologies Inc., Glen Mills, PA).

PCR Analysis

Total genomic DNA of hygromycin-resistant plants was tested for amplification of the *hptII* gene carried by the pCAMBIA1305.2 plasmid vector. Primer pairs for *hptII*, components of the PCR reaction, thermocycler program, and agarose gel electrophoresis were as described in Chapter 3. Fifty to one hundred nanograms of plant genomic DNA were used as template for 50 µL PCR reaction, and 5-10 ng of pCAMBIA plasmid were used as positive control template.

Southern Blot Analysis

Genomic DNA (10-15 μg) of hygromycin-resistant plants was digested with *Xho*I enzyme, electrophoresed through an agarose gel (0.9%) in 1X TAE buffer, blotted onto Hybond-N membrane (Amersham Biosciences Corp., Piscataway, NJ), and hybridized to the *hptII* gene probe by following standard procedures of Sambrook *et al.* (1989). The *hptII* gene was isolated from the pCAMBIA1305.2 plasmid by digestion with *Xho*I yielding a 1,094-bp fragment (Figure 3.2A). This fragment was radiolabeled by the random hexamer priming method using [α-^{32}P]dCTP and Ready-To-Go DNA Labelling Beads (both from Amersham Biosciences Corp., Piscataway, NJ). Blots of restricted plant genomic DNA were hybridized to labeled *hptII* probe at 65°C for 10 hours, and then washed sequentially for 1 hour each at 65°C in 2X, 1X, and 0.25X SSC solution each containing 0.1% SDS. The SSC solution was prepared from 20X stock (pH 7.0) consisting of 3 M sodium chloride and 0.3 M sodium citrate. The hybridization buffer contained 5X SSC, 5X Denhardt's solution, 0.5% SDS, and 10 mg mL^{-1} sheared salmon sperm DNA that served as blocking agent. Membranes were exposed to autoradiography films provided with intensifying screens at -80°C for 2 days.

RESULTS AND DISCUSSION

Particle Bombardment

Newly initiated callus tissues of Yukon were routinely collected 5 days after plating of caryopsis explants on MS nutrient medium supplemented with 5 mg L^{-1} 2,4-D. Calli about 5 mm in length were subcultured in medium with 3 mg L^{-1} 2,4-D and 1 mg

L^{-1} ABA. At least two weeks of subculture were necessary to obtain calli at an optimal growth stage for bombardment. At this stage, somatic embryos are not yet formed and the callus tissues (about 1 cm in length) have sufficient mass to withstand stress caused by osmotic treatment and particle bombardment. Osmotic treatment is crucial for increasing transformation efficiencies (Vain *et al.,* 1993). When target cells undergo plasmolysis, they are less likely to extrude their protoplasm following penetration of the microprojectiles. Osmotic treatment by mannitol resulted in mucilaginous growth of caryopsis-derived callus tissues of Yukon, and this step was shortened to 6 hours before bombardment and 4 hours after bombardment. Use of younger callus tissues including newly initiated ones was attempted to limit the number of untransformed cells that need to be eliminated during hygromycin selection. However, the bombardment procedure rendered these smaller calli nonviable for further growth and morphogenesis.

Bombardment parameters were optimized by examining transient GUS expression in callus tissues bombarded with the original pCAMBIA1305.2 vector (Figure 4.1). The number of callus tissues on each plate showing blue GUS stain was counted and the frequency of blue spots in individual tissues was noted. In early experiments, less than 10% of callus tissues displayed GUS activity. Much higher percentages (59-72% of callus tissues showing multiple blue spots, Table 4.1) were obtained after adjustment of the following parameters: duration of pre-culture (no earlier than 10 days after callus initiation), osmotic treatment before bombardment (at least 6 hours), number of shots per plate (2 shots at 6 and 9-cm distances), and amount of DNA per shot (5 µg). The manner by which the gold microcarriers were coated with DNA and how the gold-DNA mixture

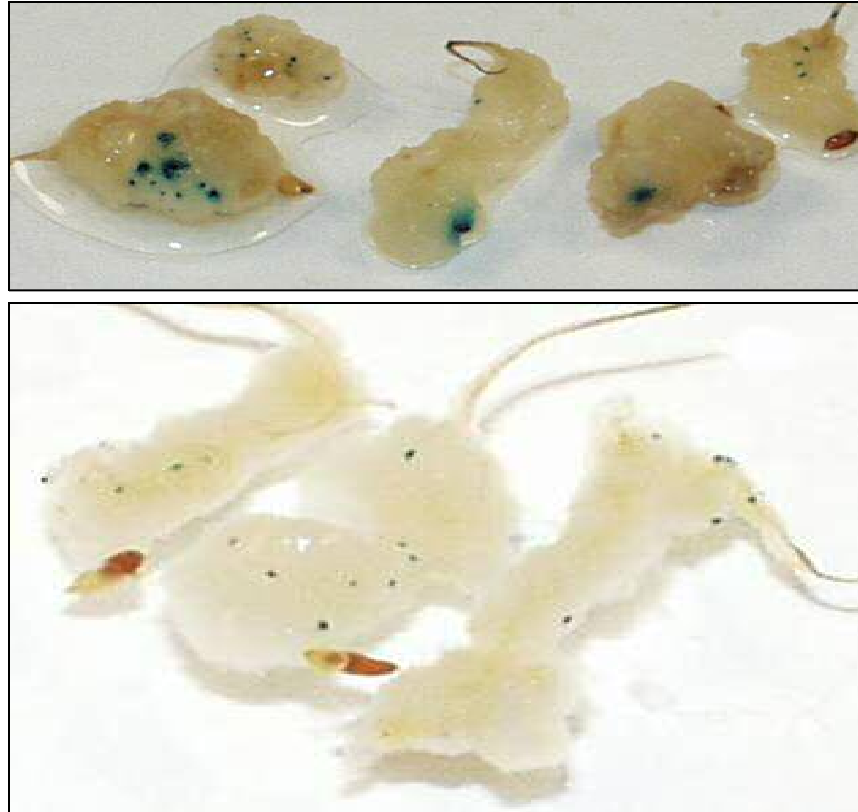

Figure 4.1. Transient GUS expression in caryopsis-derived callus tissues of Yukon bombarded with pCAMBIA1305.2. Top photo: 5 days after bombardment. Bottom photo: 3 days after bombardment.

Table 4.1. Percentage of caryopsis-derived callus tissues of Yukon displaying transient GUS expression 5 days after bombardment with pCAMBIA1305.2 vector.

	Total number of callus on plate	Number of callus showing GUS stain	Percent of callus showing GUS stain
pCAMBIA1305.2	23	16	69%
	25	18	72%
	27	16	59%
	29	21	72%
Unbombarded Control	28	0	0%
Truncated pCAMBIA1305.2: *Ubi-SbPHYC*	22	0	0%
Truncated pCAMBIA1305.2: *Ubi-CynCHT1*	22	0	0%

was spread out on the macrocarrier disc were also considered important. GUS activity was absent in unbombarded control tissues. When truncated pCAMBIA1305.2 plasmid (*gus* gene deleted) carrying either *Ubi-SbPHYC* or *Ubi-CynCHT1* construct was used, GUS stain was also not observed. This ruled out the possibility of endogenous GUS activity being present in the callus tissues used or of endogenous GUS activity being induced by the act of bombardment.

Bombardment of callus tissues with pCAMBIA1305.2:*Ubi-SbPHYC* produced a total of 27 hygromycin-resistant plants from 11 bombarded plates prepared on separate occasions. Shoots were obtained 6-9 weeks after bombardment. All 27 regenerated plants showed positive PCR amplification of fragment of the *hptII* gene (Figure 4.2, lanes 12-19 and 21-39). PCR assays were performed three times and the results were reproducible. The hygromycin-resistant plants appeared morphologically normal and resembled non-transformed parental plants. Hygromycin selection pressure of 50 mg L^{-1} during callus stage and 75 mg L^{-1} at shooting and rooting stages was sufficient to prevent occurrence of escapes or recovery of non-transformed plants. *Xho*I-digested genomic DNA of 10 randomly selected hygromycin-resistant plants hybridized to the 1,094-bp *Xho*I-ended *hptII* probe (Figure 4.3, lanes 2-11). As expected regardless of number of integration events, a single band of about 1 kb was detected on the Southern.

***Agrobacterium*-mediated Transformation**

Caryopsis-derived callus tissues of Yukon and Guymon were previously used in gene transfer experiments with *A. tumefaciens* EHA105. Plant regeneration occurred at very low frequencies because the callus tissues suffered from hydration stress and ceased

to grow and differentiate after immersion in *Agrobacterium* solution. Modification in the inoculation procedure is being worked out to overcome this difficulty. Interestingly, the cultivar Yukon appeared to have some resistance to *Agrobacterium* infection. Callus tissues of Yukon showed very minimal bacterial overgrowth after co-cultivation with the bacteria compared with tissues of Guymon.

Direct plant regeneration of transgenic bermudagrass from stolon nodes co-cultivated with *Agrobacterium* has been reported by Wang and Ge (2005). This callus-free transformation procedure is a significant improvement for genetic transformation of monocot species because it requires a short tissue culture step. In this study, EHA105 harboring pCAMBIA1305.2:*Ubi-SbPHYC* was co-cultivated with stolon nodes of Guymon (Figure 4.4). Samples of bacterial overgrowth were collected after the 3-day culture. Presence of kanamycin resistance and PCR amplification of *hptII* gene from these bacterial samples indicated that the *Agrobacterium* cells retained the pCAMBIA plasmid during co-cultivation. Transient GUS expression was assayed in nodes co-cultivated with EHA105 harboring the original pCAMBIA1305.2 plasmid carrying the *gus* gene. However, high endogenous GUS activity was similarly observed in non-inoculated control nodes making the assay difficult.

Thirteen shoots were obtained from a total of 756 nodal segments inoculated and co-cultivated with EHA105 harboring pCAMBIA1305.2:*Ubi-SbPHYC* after 6-7 weeks of subculture under 75 mg L^{-1} hygromycin selection. The shoots were rooted under the same level of hygromycin. Out of 13 plants established, only 7 showed positive PCR amplification of the *hptII* gene (Figure 4.2, lanes 4-10). This suggests that hygromycin levels higher than 75 mg L^{-1} can be applied to select for transformed nodal cells. Southern

hybridization with the *hptII* probe indicated transgene integration in the 7 hygromycin-resistant plants (Figure 4.3, lanes 13-19). Transformation efficiency was 0.9% compared with up to 6% that was reported for *Agrobacterium*-mediated transformation of TifEagle stolon nodes (Wang and Ge, 2005).

Agrobacterium-mediated gene transfer is more difficult to optimize compared with the microprojectile bombardment method because of the interaction between the bacteria and plant cells. Regarding the use of nodal tissues for *Agrobacterium*-mediated transformation, some points were noted that may be important for improving the method followed in this study. The nodal segments must be loosely planted in the agar medium to permit aerobic growth of *Agrobacteria* at the target plant tissues during co-cultivation. When the nodal ends were tightly planted, bacterial growth was restricted on the surface of the agar medium (Figure 4.4). Longer nodal segments with 1 or 2 intact nodes along their lengths must be used as they have greater viability during subculture. In this study, inducted *Agrobacteria* were used to inoculate nodal tissues. Induction by phenolic compounds such as acetosyringone requires prior growth in minimal medium to be effective. Non-inducted *Agrobacteria* grown in rich medium such as YEP have been successfully used by other researchers to infect experimental plant materials. It is worthwhile to test whether this preparation will increase transformation frequencies in nodal cells of bermudagrass.

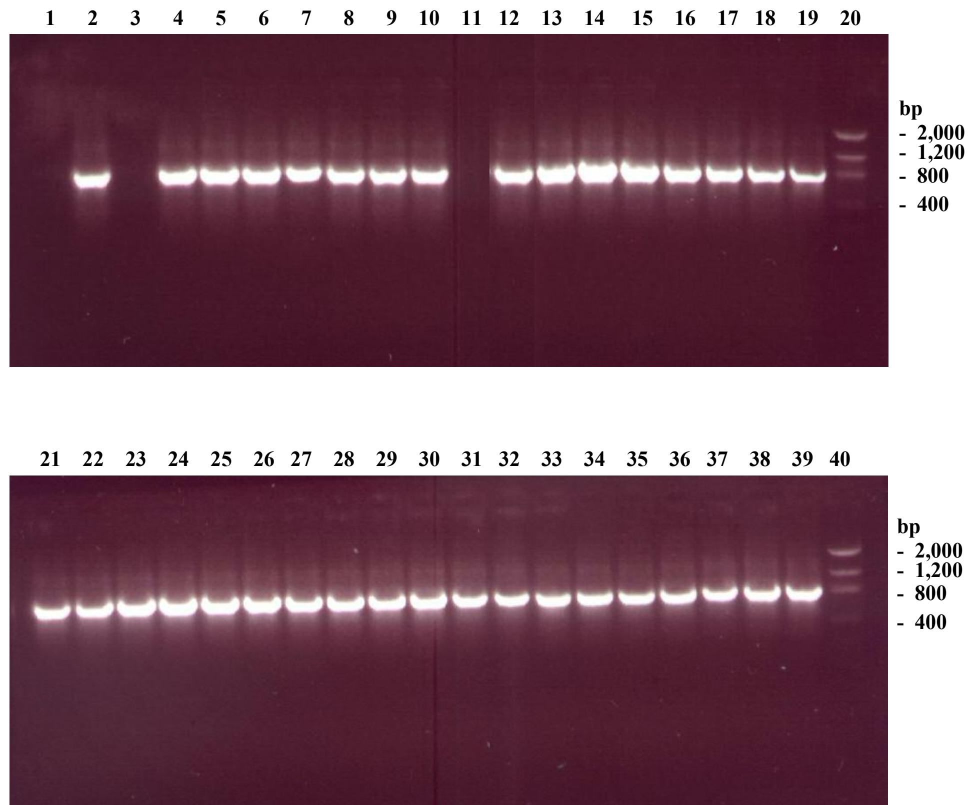

Figure 4.2. PCR amplification of the *hptII* gene from total genomic DNA of hygromycin-resistant plants.

Lane 1: Water (negative control template)
Lane 2: pCAMBIA1305.2:*Ubi-SbPHYC* plasmid (positive control template)
Lane 3: Genomic DNA isolated from pooled leaf tissues of 5 non-transformed Guymon plants grown from individual seeds (negative control template)
Lanes 4-10: Genomic DNA templates isolated from leaf tissues of 7 hygromycin-resistant Guymon plants obtained from nodal tissues co-cultivated with *Agrobacterium* EHA105 harboring pCAMBIA1305.2:*Ubi-SbPHYC*
Lane 11: Genomic DNA isolated from pooled leaf tissues of 5 non-transformed Yukon plants grown from individual seeds (negative control template)
Lanes 12-19 and 21-39: Genomic DNA templates isolated from leaf tissues of 27 hygromycin-resistant Yukon plants regenerated from callus tissues bombarded with pCAMBIA1305.2:*Ubi-SbPHYC*
Lanes 20 and 40: Low DNA Mass Ladder

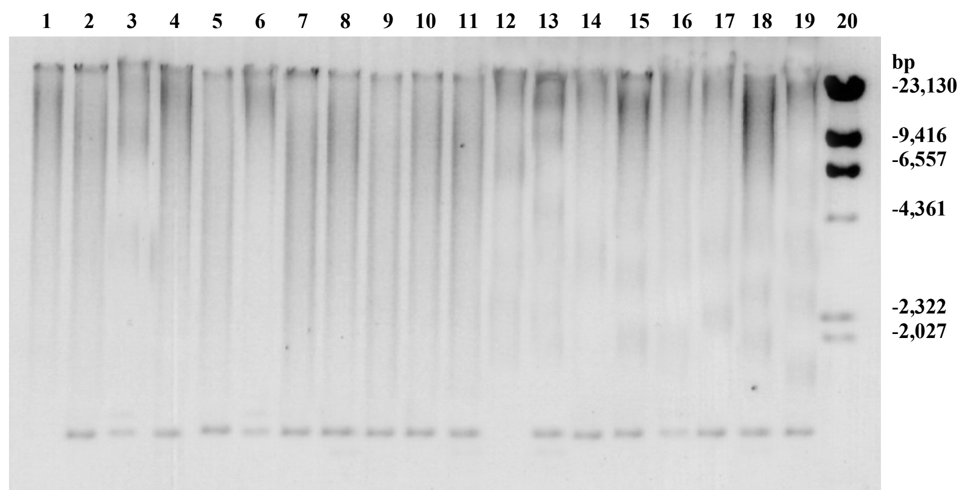

Figure 4.3. Southern hybridization of *Xho*I-digested genomic DNA of hygromycin-resistant plants with the *hptII* gene probe.

Lane 1: Genomic DNA isolated from pooled leaf tissues of 5 non-transformed Yukon plants grown from individual seeds (negative control)

Lanes 2-11: Genomic DNA isolated from leaf tissues of 10 hygromycin-resistant Yukon plants regenerated from callus tissues bombarded with pCAMBIA1305.2:*Ubi-SbPHYC*

Lane 12: Genomic DNA isolated from pooled leaf tissues of 5 non-transformed Guymon plants grown from individual seeds (negative control)

Lanes 13-19: Genomic DNA isolated from leaf tissues of 7 hygromycin-resistant Guymon plants obtained from nodal tissues co-cultivated with *Agrobacterium* EHA105 harboring pCAMBIA1305.2:*Ubi-SbPHYC*

Lane 20: λ *Hind*III size marker

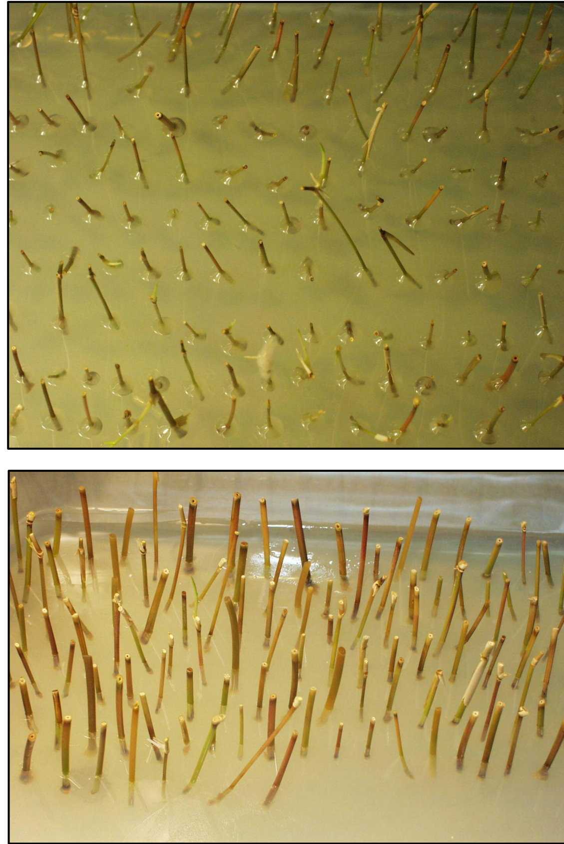

Figure 4.4. Stolon nodes of Guymon co-cultivated with *A. tumefaciens* EHA105 harboring pCAMBIA1305.2:*Ubi-SbPHYC.* Top photo: Inoculated nodes with bacterial overgrowth. Bottom photo: Non-inoculated control nodes.

SUMMARY AND CONCLUSIONS

Cynodon dactylon cvs Yukon and Guymon were transformed with pCAMBIA1305.2:*Ubi-SbPHYC*. Through an optimized particle bombardment method, 27 transformed plants of Yukon were obtained from 11 bombarded plates of callus tissues. This experiment has demonstrated the competency of caryopsis-derived embryogenic callus of bermudagrass for genetic transformation and regeneration under hygromycin selection. Seven transgenic plants of Guymon were directly regenerated from stolon nodes using *A. tumefaciens* strain EHA105, with transformation efficiency of

0.9%. Further improvement of this *Agrobacterium* method is needed to increase transformation efficiencies in future experiments.

Further analyses will be done on plants that were transformed with pCAMBIA:*Ubi-SbPHYC*. The number of insertion events will be determined to identify desirable low-copy transformants. This can be carried out through Southern hybridization of the *hptII* probe with plant genomic DNA restricted by enzymes other than *Xho*I that do not cut along the probe sequence. Constitutive expression of the sorghum *PHYC* gene will be established in selected transformants by Northern hybridization or RT-PCR analysis of transcribed mRNA and by spectrophotometric quantitation of phytochrome protein activity in leaf tissues. Following this, greenhouse and field experiments will be conducted to examine the morphological and physiological consequences of *PHYC* over-expression and its bearing on the shade-tolerance response of bermudagrass.

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APPENDIX A

Media Used in Bacterial Cultures

SOC Medium: The following were dissolved and brought to 100 mL with distilled water: 2 g tryptone, 0.5 g yeast extract, 1 mL of 1 M NaCl, and 1 mL of 1 M KCl. The pH was adjusted to 7.0 and the solution was sterilized by autoclaving. To this solution, 1 mL of each of the following filter-sterilized solutions was added: 1 M $MgCl_2•6H_20$, 1 M $MgSO_4•7H_20$, and 2 M glucose (final concentration of Mg^{2+} and glucose was 20 mM). The complete medium was passed through 0.2 μ filter unit.

LB Medium: The following were dissolved and brought to one liter with distilled water: 10 g tryptone, 5 g yeast extract, and 10 g NaCl. The pH was adjusted to 7.0 and the solution was sterilized by autoclaving. The medium was cooled to 60°C before adding antibiotic. When making solid medium, 15 g of agar per liter of solution was added before autoclaving.

YEP Medium: The following were dissolved and brought to one liter with distilled water: 10 g peptone, 10 g yeast extract, and 5 g NaCl. The pH was adjusted to 7.0 and the solution was sterilized by autoclaving. The medium was cooled to 60°C before adding antibiotic. When making solid medium, 15 g of agar per liter of solution was added before autoclaving.

AB-Sucrose Minimal Medium, pH 7.0: The following were mixed, brought to one liter with distilled water, and sterilized by autoclaving: 50 mL of 20X AB buffer 1, 50 mL of 20X AB buffer 2, 50 mL of 20X AB salts (pH 7.0), and 5 g sucrose. The medium was stored in dark container.

20X AB Buffer 1: 78.6 g L^{-1} K_2HPO_4
20X AB Buffer 2: 23 g L^{-1} NaH_2HPO_4
20X AB Salts: The following were dissolved in one liter of distilled water and the pH of solution was brought to 7.0: 0.05 g $FeSO_4•7H_20$, 0.07 g $Na_2EDTA•2H_2O$, 20 g NH_4Cl, 6 g $MgSO_4•7H_2O$, 3 g KCl, and 0.3 g $CaCl_2•2H_2O$. The solution was sterilized by autoclaving and stored in dark container.

Induction Medium, pH 5.6: The following were mixed, brought to one liter with distilled water, and sterilized by autoclaving: 50 mL of 20X AB salts, 20 mL of 0.1 M Na_2HPO_4, 50 mL of 1 M MES (pH 5.6), and 5 g glucose. The medium was stored in dark container.

VITA

Arnold Sarne Parco

Candidate for the Degree of

Doctor of Philosophy

Thesis: BERMUDAGRASS TISSUE CULTURE AND GENETIC TRANSFORMATION THROUGH *AGROBACTERIUM* AND PARTICLE BOMBARDMENT METHODS

Major Field: Plant Science

Biographical:

Education: Received a Bachelor of Science degree in Biology from the University of the Philippines at Los Banos in March 1988, and a Master of Science degree with a major in Plant and Soil Science from Oklahoma State University in May 2000. Completed the requirements for the Doctor of Philosophy degree with a major in Plant Science at Oklahoma State University in May 2007.

Work Experiences: Research Assistant in the Department of Plant Breeding, Genetics, and Biochemistry at the International Rice Research Institute from 1990 to 1997. Graduate Assistant in the Department of Plant and Soil Sciences at Oklahoma State University from 1998 to 2006.

Professional Membership: Crop Science Society of America

Name: Arnold Sarne Parco		Date of Degree: May, 2007

Institution: Oklahoma State University		Location: Stillwater, Oklahoma

Title of Study: BERMUDAGRASS TISSUE CULTURE AND GENETIC TRANSFORMATION THROUGH *AGROBACTERIUM* AND PARTICLE BOMBARDMENT METHODS

Pages in Study: 110		Candidate for the Degree of Doctor of Philosophy

Major Field: Plant Science

Scope and Method of Study: The primary objective of this dissertation was to develop and optimize methods for bermudagrass tissue culture and genetic transformation. Callus production and subsequent plant regeneration using different explant tissues of selected bermudagrass cultivars were evaluated on solid MS nutrient medium and the promotive effects of plant growth regulator (PGR) supplements were studied. Gene transfer methods utilizing *Agrobacterium* and particle bombardment were respectively applied to bermudagrass cultivars Guymon and Yukon.

Findings and Conclusions: Caryopsis, young inflorescence, and nodal explants were capable of callus production under 2,4-D supplementations, although no plants were regenerated from nodal segments. Callus induction and plant regeneration frequencies varied with explant type, cultivar, and PGR treatment. The auxin 2,4-D was essential for callus formation but not kinetin and ABA. However, low levels (1-3 mg L^{-1}) of kinetin and/or ABA were found to enhance somatic embryogenesis in caryopsis cultures. Culture of mature caryopses was further developed for utilization in genetic transformation experiments. Evaluation of the toxic effect of hygromycin B on seed germination and growth of seedlings and callus-derived shoots of Guymon and Yukon, indicated that this antibiotic can effectively eliminate untransformed plant materials within 2-3 weeks of selection at concentrations around 50 mg L^{-1}. Seven hygromycin-resistant plants were directly regenerated from stolon nodes of Guymon co-cultivated with *Agrobacterium EHA105* harboring pCAMBIA1305.2:*Ubi-SbPHYC* plasmid, while twenty-seven hygromycin-resistant plants were obtained from caryopsis-derived embryogenic callus tissues of Yukon bombarded with the plasmid. Transgene integration was verified by PCR amplification of the selectable marker *hptII* gene and by Southern hybridization of total plant genomic DNA with the *hptII* probe. Further optimizations of the *Agrobacterium* method were suggested. This study has yielded transgenic bermudagrass plants that will be screened for constitutive expression of the *Sorghum PHYC* gene and used in photomorphogenic studies.

ADVISER'S APPROVAL: DR. CHARLES M. TALIAFERRO