

GENETIC ENGINEERING TO IMPROVE NUTRITIONAL QUALITY AND PEST  
RESISTANCE IN BAHIAGRASS (*Paspalum notatum* VAR. FLUGGE)

By

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To my family and friends  
To McNair, Carmen and Luca

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Bahiagrass (*Paspalum notatum* var. Flugge) is the predominant grass in forage pastures in tropical and subtropical regions worldwide. It supports the beef and dairy cattle industries in the state of Florida. To improve forage production and animal performance, the low nutritional quality and the insect pest susceptibility of this subtropical grass should be addressed. To improve nutritional quality, the *vspB* gene encoding a soybean VSP $\beta$  that is a lysine-enriched and rumen proteolysis-resistant protein seems to be a promising candidate. To improve insect pest resistance, the *cryIF* gene encoding a  $\delta$ -endotoxin from *Bacillus thuringiensis* that provides protection against Lepidopteran pests in commercial crops such as cotton and corn seems to be a suitable candidate. Our objectives were: to overexpress the *vspB* gene for improving nutritional in bahiagrass cv. "Argentine", and to express a synthetic *cryIF* gene for enhancing resistance against fall armyworm in bahiagrass cvs. "Argentine" and "Tifton 9." To overexpress the *vspB* gene, we cloned an expression vector containing a constitutive and strongly expressed promoter for monocots; a KDEL signal for ER-retention and a c-myc tag for antibody detection. We co-bombarded mature seed-derived calli from bahiagrass cv. "Argentine" with minimal constructs containing the *vspB* gene from pGL4 and pRSVP1 vectors and the *nptII* gene as selectable

marker from pJFNPTII vector. Following previous reports, we generated 91 *nptII*(+) plants from 110 analyzed plants. Based on the results from *nptII* expression detected by ELISA and VSP $\beta$  expression detected by western blots, 89% of the plants coexpressed the *nptII* and the *vspB* genes. Western blot analyses indicated that VSP $\beta$  expression levels varied among plants, tillers within plants and leaves within tillers. This is the first report on expression of the *vspB* gene in bahiagrass cv. "Argentine." The VSP $\beta$  expression levels observed in transgenic bahiagrass plants were low and similar to those previously reported in transgenic corn plants. Potentially, these VSP $\beta$  expression levels could be enhanced by coexpressing recombinant VSP $\beta$  and VSP $\alpha$  subunits, and/or by targeting the subunit to different cell compartments in transgenic bahiagrass plants. To express the synthetic *cryIF* gene, we co-bombarded mature seed-derived calli from bahiagrass with minimal constructs containing the synthetic *cryIF* gene from pHZCRY vector and the *nptII* gene as selectable marker from pHZ35SNPTII vector. Based on previous reports, we regenerated three paromomycin-resistant plants from bahiagrass cvs. "Argentine" and "Tifton 9." PCR and southern blots analyses indicated independent transgene integration, and RT-PCR analyses confirmed *cryIF* expression in all transgenic bahiagrass lines. Two immunoassays for *cryIF* gene expression indicated detectable cry1F levels in two bahiagrass lines from cv. "Tifton 9." Cry1F expression levels correlated well to resistance levels determined by insect bioassays. An average mortality rate of 83 % was observed when fall armyworm neonates were fed with transgenic leaves of the highest cry1F expressing line. These results indicated that high and stable *cryIF* expression levels can control fall armyworm in transgenic bahiagrass plants. The expression of the *cryIF* gene in plants of cv. "Tifton 9" enhanced the resistance against fall armyworm in insect bioassays indicating that these transgenic lines seem to be suitable candidates for field studies.

## CHAPTER 1 INTRODUCTION

### **Bahiagrass (*Paspalum notatum* var. Flugge)**

Bahiagrass (*Paspalum notatum* Flugge) is a perennial, warm-season grass widely grown in southern United States and other tropical and subtropical regions of the world. Also, it is one of the most important forage grasses for supporting beef and dairy cattle industries in Florida and other southeastern states such as Georgia, Alabama and over the Gulf Coastal Plain (Chambliss, 2002). Bahiagrass low management requirements and good yield under biotic stresses such as heavy grazing or frequent harvests and abiotic stresses such as drought and poor soils, make it the preferred forage grass by beef cattle producers in Florida (Smith et al., 2002; Chambliss, 2002; Blount et al., 2001).

The main cultivars grown in Florida are sexual diploids such as “Pensacola” and “Tifton 9”, and apomictic tetraploids such as “Argentine.” The cultivar “Argentine” is a wide-leaf, cold-susceptible and late-flowering cultivar mainly grown in South Florida for landscaping. It is commonly used as a turf grass in home lawns and along highways. The cultivar “Tifton-9” is a narrow-leaf, and early-flowering cultivar, which is less cold-susceptible than cv. “Argentine”; and it is used as forage grass in the Panhandle region (Chambliss, 2002).

### **Genetic Engineering for Crop Improvement**

Currently, genetic engineering combined with plant breeding programs has become a common and efficient tool for crop improvement. It is used not only for gene function and expression studies but also for a wide range of applications in plant breeding programs mostly focused in generating new cultivars with higher yields (Hansen and Wright, 1999). These biotechnological applications are mostly related to agriculture, industry and human health (Newell, 2000). In agriculture, genetic engineering studies have been used to improve from

simple agronomic traits such as herbicide, insect, disease and nematode resistance to more complex traits such as stress tolerance for improving crop yield (Dunwell, 2000; Newell, 2000; Bhalla, 2006). Pesticide-resistant crops are a remarkable example where these products resulted in economic and environmental benefits including higher crop yields, reduced economic losses and no adverse effects on the environment (Cannon, 2000). Also, genetic engineering has been used to improve nutritional quality of field crops or to produce biopharmaceutical products such as antibodies, vaccines or human therapeutic proteins (Newell, 2000; Horn et al., 2004). Nutritional deficiencies in animal and human diets could be compensated by improving lysine and threonine levels in cereals, methionine in legumes and vitamins A and E in crucifers and rice. These genetic improvements would have a strong impact since these crops represent the staple food for one-third of the world population (Job, 2002).

#### **Genetic Engineering to Improve Nutritional Quality: Vegetative Storage Proteins**

To improve nutritional quality, several molecular approaches were developed (Tabe and Higgins, 1998; Galili and Hoefgen, 2002; Sun and Liu, 2004). These approaches include: developing synthetic proteins, optimizing protein sequence, overexpressing proteins or regulating the free aminoacid pool or the sink demand (Sun and Liu, 2004). Two alternative strategies successfully enhanced nutritional quality of crops: to increase the free essential aminoacid pools or to increase the enriched-protein pools in the transgenic plants. The first strategy includes increasing the levels of free aminoacid pools by upregulating or downregulating the expression levels of those enzymes involved in aminoacid biosynthesis and/or degradation respectively. An enhanced production of free essential aminoacids may lead to an increase of total protein content and protein quality in plants (Galili et al., 2000; Habben et al., 1995). The second strategy includes increasing the levels of essential aminoacids-enriched proteins, mostly by overexpressing seed storage proteins (SSPs) or vegetative storage proteins

(VSPs). Early reports indicated that SSPs were efficiently degraded in vegetative tissues (Saalbach et al., 1994). Therefore, to prevent protein degradation two approaches were followed: to target the transgenic proteins to cell compartments avoiding cytoplasmic degradation (Khan et al., 1996; Tabe et al., 1995) or to use VSPs that were naturally accumulated in vegetative tissues. These storage proteins include plant proteins such as patatin from potato, sporamin from sweet potato, VSPs and lipoxygenase from soybean, crown storage proteins from alfalfa and lectin-like proteins from the bark of certain deciduous trees (Staswick, 1994; Cunningham and Volenec, 1996).

VSPs seem to be promising candidates for increasing protein content and quality because they are naturally accumulated in vegetative tissues. VSP- $\alpha$  and VSP- $\beta$ , reached 15% of the total soluble proteins in soybean paraveinal tissue (Grando et al., 2005) and high levels were associated with shoot regrowth after cutting and deppoding in soybean plants (Wittenbach et al., 1983). VSP- $\alpha$  and VSP- $\beta$  are polypeptides with 27-28 and 29-31 kD of molecular weight respectively. These glycoproteins share 80% aminoacid homology, have ER- and vacuolar targeting signals, form homo- or hetero-dimers when they are assembled, and have 7% lysine content (Mason et al., 1988; Staswick et al., 1988, 1989a, 1994).

### **Genetic Engineering to Enhance Insect Resistance: *Bacillus thuringiensis* Toxins**

To improve insect resistance, most integrated pest management strategies involved the use of pesticides. The indiscriminate use of pesticides produces adverse effects on human health and the environment including the development of insect resistance and the elimination of other beneficial insects (Ranjekar et al., 2003; Ferry et al., 2006). To overcome these problems, environmentally-friendly pesticides containing *Bt* (*Bacillus thuringiensis*) spores and crystals were developed. But these spray formulations were only partially effective because they did not reach burrowing insects and did not persist long enough in the environment. Therefore, *Bt*

transgenic crops expressing  $\delta$ -endotoxins were developed (Schnepf et al., 1998; Kaur, 2006). Currently, this technology is adopted worldwide resulting in 14 million hectares covered with *Bt* crops (James, 2005). In the USA, *Bt* crops represent almost 20% of the total cropping area and their use is directly linked to higher yields and profits in corn and cotton (Cannon, 2000).

These *cry*-encoded  $\delta$ -endotoxins are classified in four groups providing protection against four insect orders: *cry1* (Lepidoptera), *cry2* (Lepidoptera and Diptera), *cry3* (Coleoptera) and *cry4* (Diptera) (de Maagd et al., 2001; Ferre and Van Rie, 2002; Griffiths and Aroian, 2005). First, these crystal protoxins are solubilized and proteolitically activated in the insect midgut and second, the active toxins bind to specific receptors in the intestine epithelial cells leading to pore formation and cell death. The active toxins have a conserved structure formed by three domains and domains II and III determine host specificity because they have very high affinity to receptors located in the gut epithelium of different insect orders (Schnepf et al., 1998; Ranjekar et al., 2003; Abanti, 2004; de Maagd et al., 2001).

Based on laboratory studies on field-selected strains, the potential of resistance development exists in insect populations; however, only one case of field-developed resistance was reported (Ferre and Van Rie, 2002; Griffiths and Araoian, 2005). To delay insect resistance, different molecular and management approaches were developed. To boost *cry* gene expression, the use of *cry* truncated sequences expressing the active toxins, the use of *cry* codon-optimized sequences for enhanced plant expression, the stacking of *cry* genes with different binding sites and genes encoding proteins with different toxicity mechanisms are the most commonly used molecular approaches (Schnepf et al., 1998; Bohorova et al., 2001; Kaur, 2006; Ferry et al., 2006). However, high dose levels are still difficult to reach and *cry* expression patterns may be limited by external factors like nitrogen fertilization (Abel and Adamczyk, 2004).

## Objectives

Bahiagrass (*Paspalum notatum* Flugge) plays a key role supporting beef and dairy cattle industries in Florida and other southern states of the United States (Chambliss, 2002). Targets for bahiagrass genetic improvement include its low nutritive value and its susceptibility to insect pests like mole crickets and fall armyworm.

Naturally, subtropical bahiagrass cultivars have low protein content, ca. 11% protein content, which directly affects animal performance (Cuomo et al., 1996). This protein content decreases to 5-7% affecting cattle growth and reducing cattle weight during summer and fall (Grando, 2001). Therefore, high and stable expression levels of the *vspB* gene in transgenic bahiagrass plants would have two main advantages: rumen stability and enriched essential aminoacid composition. While most plant proteins are degraded by rumen proteolysis, bypass proteins remained intact and they are absorbed in ruminant intestines. It was previously observed that VSP $\beta$  not only contains ca.7% lysine (Mason et al., 1988; Staswick, 1988) but also behaves as a bypass protein being stable in the rumen and absorbed in the cattle intestines (Guenoume et al., 2002b). Previous reports indicated that VSPs contributed to the accumulation of high lysine levels in transgenic tobacco plants and, therefore they could compensate lysine deficiency in ruminant feeding (Guenoune et al., 2003).

Bahiagrass is susceptible to insect pests such as mole crickets (*Scapteriscus* spp) and fall armyworm [*Spodoptera frugiperda* (J. E. Smith)]. Fall armyworm (FAW) is one of the most important insect pests in the southeast of United States, causing seasonal economic losses in forage and turf grasses and field crops such as corn, rice and sorghum (Sparks 1979, Meagher and Nagoshi, 2004). Recently, field trials with corn hybrids expressing a full length *cryIF* gene (Herculex I) indicated that this gene provided protection against a wide range of insect pests

including FAW (EPA, 2001). However, there are no reports on transgenic insect resistance to insect pests in forage or turfgrasses.

The objectives of our research project were:

- To overexpress a soybean vegetative storage protein gene and to evaluate the effects of this gene on the nutritional quality of bahiagrass.
- To express a synthetic *cryIF* gene and to evaluate the effects of this gene on resistance to fall armyworm in bahiagrass.

## CHAPTER 2 LITERATURE REVIEW

### **Bahiagrass (*Paspalum notatum* var. Flugge)**

#### **General Description**

The genus *Paspalum* (Poaceae family) originated along the Parana River in the border between Brasil and Argentina. The notata group (Chase et al., 1929; Burton, 1967) was spread from this region to other subtropical and tropical regions. Phylogenetic and cytogenetic studies showed that the genus is characterized by a basic chromosomic number  $X=10$  but its species vary from diploids ( $2n = 2x = 20$ ) to pentaploids ( $2n = 2x = 50$ ) with sexual diploids ( $2n = 2x = 20$ ) and apomictic tetraploids ( $2n = 4x = 40$ ) being the most common forage species. During the evolution of *Paspalum* spp., the apomictic autotetraploid forms, that were more robust, fit and competitive, originated from the sexual diploid forms (Gates et al., 2004). Also, bahiagrass is a C4 plant with an efficient photosynthetic system which allows it to colonize new environments with high temperature, humidity and light intensity. It is important to note that C4 grasses have higher water use and nitrogen use efficiencies than C3 grasses indicating that C4 growth rate doubles C3 growth rate using the same water and nitrogen supplies (Gates et al., 2004; Moser et al., 2004).

Bahiagrass (*Paspalum notatum* Flugge) is a rhizomatous plant with short internodes that produce adventitious shoots and roots. It has a deep and well developed root system that supports several tillers with broad leaves and inflorescences that are panicles formed by two terminal racemes (Gates et al., 2004) (Figure 2-1).

The species is distributed in tropical and subtropical regions worldwide including Argentina, Australia, Belize, Benin, Bolivia, Brazil, Colombia, Costa Rica, Cuba, Ecuador, El Salvador, Gabon, Guatemala, Guyana, Honduras, Japan, Mexico, Nicaragua, Panama, Paraguay,

United States and Zambia. In the United States, bahiagrass was introduced as a forage grass in early 1900s. According to the PLANTS Database, generated by the Natural Resources and Conservation Services from the United States Department of Agriculture (USDA-NCRS, 2007), it is found in the states of Alabama, Arizona, California, Florida, Georgia, Louisiana, Massachusetts, North Carolina, South Carolina, Tennessee and Texas. Bahiagrass covers ca. 2.5 million hectares including the southeastern states in the Gulf Coastal Plain in the United States (Burton et al., 1997; Blount et al., 2001), and more than 1 million hectares of which ca. 70 % represent improved pastures in the state of Florida. As a forage crop, bahiagrass supports beef and dairy cattle industries in Florida and other southern states (Chambliss, 2002).

The main cultivars grown in Florida are sexual diploids like “Pensacola” and “Tifton 9,” and apomictic tetraploids like “Argentine” (Chambliss, 2002). According to the Bureau of Plant Industry-Office of Foreign Seed and Plant Introductions (BPI-OFSPI), the cultivar “Argentine” (Plant Introduction N° 148996) was introduced from Argentina in 1944 and released in the United States in 1950. It is a wide-leaf, cold-susceptible and late-flowering cultivar. It is mainly grown for landscaping in south Florida (Chambliss, 2002). The cultivar “Tifton-9” (Plant Introduction N° 531086) was released in 1987 by a breeding program at Tifton (Georgia, USA) after nine cycles of recurrent restricted phenotypic selection from the cultivar “Pensacola.” It is a narrow-leaf, cold-tolerant and an early-flowering cultivar mainly grown and used as forage in the states of Georgia, Alabama, Louisiana and north Florida (Cook et al., 2005).

### **Role of Bahiagrass as Subtropical Grass in Southeast United States**

In Florida, beef and dairy cattle industries are formed by 1.5 million cows and 140,000 cows respectively (Florida Department of Agriculture and Consumer Services, Division Animal Industry, 2007). These industries represent 85 and 8% of the livestock production respectively and they are mainly supported by bahiagrass pastures covering one million hectares in the state

(Chambliss, 2002). Bahiagrass is preferred by farmers and producers because it has low management requirements and very good persistence and yield (Smith et al., 2002; Chambliss, 2002; Blount et al., 2001). Among these advantages are (Cook et al., 2005):

- It is well adapted to sandy and light-textured soils, and not only tolerates drought but also its root system penetrates soil and improves water holding capacity and prevents nutrient leaching.
- It is well adapted to low fertility soils with marginal pH (i.e. 5.5-6.5). These deficiencies are partially compensated by root interactions with arbuscular mycorrhizal fungi and nitrogen-fixing bacteria like *Azotobacter paspali*.
- Once established, it spreads fast by stolons and is highly tolerant to overgrazing and high cutting frequencies during summer, these factors favors plant regrowth and maintains nutritional quality (Stewart et al., 2007).
- It is very tolerant to fungal diseases and insect pests. The most important fungal pathogens are the ergot (*Claviceps* spp) that reduces seed set and the leaf spot (*Helminthosporium* spp) that produces leaf lesions in cv. "Argentine." The most important insect pests are mole crickets that feed on the roots (*Scapteriscus vicinus*, *S. borellii* and *S. abbreviatus* commonly known as tawny, southern and short-winged mole crickets respectively) and fall armyworms (*Spodoptera* spp) that feed on the leaves (Burson and Watson, 1995; Chambliss, 2002).
- It reduces nematode populations when it is used in sod-rotation cropping systems and alternated with cotton, peanut and corn showed the same or increased yields (Gates, 2003; Wright et al., 2005).

However, it also has several physiological and nutritional limitations:

- It has a slow rate of seed establishment. It requires high sowing rates and good weed control because bahiagrass seedlings are weak and susceptible to the most commonly used post-emergency herbicides (Cook et al., 2005).
- Plant growth has a seasonal pattern which directly affects plant yield. So that, slower growth produces low yield during fall and winter, and faster growth produces higher yield during summer in subtropical bahiagrass cultivars (Cuomo et al., 1996).
- This seasonal pattern also affects plant nutritive value, because of the fast growth rate directly related to high temperatures which implies secondary growth and reproductive growth (Stewart, 2006). Specifically, subtropical grasses nutritional quality decreases with maturity (Johnson et al., 2001).

## **Traditional Breeding of Grasses**

Forage and turfgrasses contribute to support economical and sustainable agriculture systems that represent the basis of most economies in the world. Forage grasses are not commonly appreciated as a commodity because its value is measured indirectly as a feed cost for cattle production. Turfgrasses are mostly grown with recreational purposes on sport fields, parks, home lawns and roadsides (Wang et al., 2001).

Breeding of forage grasses is mainly focused on supporting ruminant feeding and includes increasing herbage production by increasing dry matter yield and different feeding value parameters. These parameters are in vitro dry matter digestibility (IVDMD), crude protein (CP) content, water-soluble carbohydrate (WSC) content. Also, there are antiquality factors which affect it, such as lignin and alkaloid contents (Wilkins and Humphreys, 2003). Therefore, the objectives of breeding programs for improving nutritional quality include increasing voluntary intake, dry matter yield, IVDMD, CP and WSC and decreasing lignin and alkaloid contents. Other breeding objectives include to enhance persistence, tolerance to environmental stresses such as cold, frost, heat and drought, resistance to insect pests and viral and fungal diseases, and to increase seed yield (Wilkins and Humphreys, 2003).

Most grasses are crosspollinated and most traits are quantitative including DMY, IVDMD, tolerance to stresses and resistance to pests and diseases. Before, breeding programs focused on identifying those natural populations with superior phenotypes (Wilkins and Humphreys, 2003). In the United States, some examples of those cultivars are ‘Kentucky-31’ tall fescue (*Festuca arundinacea*), ‘Linn’ perennial ryegrass (*Lolium perenne*), ‘Lincoln’ smooth brome grass (*Bromus inermis*), and ‘Merion’ Kentucky bluegrass (*Poa pratensis*) (Alderson and Sharp, 1994). Later, breeding programs focused on phenotypic selection and progeny tests including full-sib or half-sib family selection (Cunningham et al. 1994). Basically, traits such as DMY and

IVDMD, with broad-sense heritabilities ranking between 30–70%, showed an increased of 10 % decade, and this increase was almost doubled (Wilkins and Humphreys, 2003). Other classical breeding approaches such as gene introgression by backcrossing and chromosome doubling were reported with limited success. However, most forage grasses breeding programs are supported by seed companies and low seed value and long breeding cycles (2-3 years/each) limit their expansion (Wilkins and Humphreys, 2003).

Bahiagrass breeding efforts followed the same breeding approaches and focused in the same breeding objectives. Early on, sexual tetraploids were generated by chromosome doubling with colchicines from cv. “Pensacola” (Forbes and Burton 1961). Later on, cv. “Tifton 9” was generated by restricted recurrent phenotypic selection (Burton 1974). A breeding program was established at Tifton (Georgia) and starting material was selected from several farms. Based on herbage production, this program included breeding cycles where the best plants were selected and intermated in a polycross to produce seeds for the next selection cycle. At the ninth cycle, the cultivar “Tifton 9” was released (Burton, 1989). This cultivar not only showed 30 % more biomass production and more seedling vigour than cv. “Pensacola” but also the same IVDMD. Later reports indicated that early germination and reduced dormancy were related to higher yields (Gates and Burton 1998). Stronger and faster seed establishment makes bahiagrass cultivars more competitive against weeds in early stages and more productive extending the growing season. To increase DMY in bahiagrass subtropical cultivars, other factors such as cold resistance, photoperiod sensitivity and crown vigor had been considered. For example, day-neutral and cold-resistant plants with vigorous crown (expressed as fast growth and profuse tillering) will have an extended growing season and higher biomass production (Blount et al. 2001, 2003).

## **Genetic Engineering in Plants**

### **Plant Transformation**

In the last forty years, genetic engineering has overcome the basic problems concerning to the development of protocols for DNA transfer, tissue culture and selection in specific genotypes or cultivars. Genetic engineering is focused on crop improvement and a wide range of biotechnological applications in industry and human health (Hansen and Wright, 1999; Newell, 2000). In agriculture, genetic engineering and plant breeding has improved agronomic traits such as herbicide, insect, disease and nematode resistance (Dunwell, 2000; Newell, 2000; Bhalla, 2006). Improving yield and nutritional quality of field crops is more complicated because traits such as drought tolerance or essential aminoacid deficiencies are regulated in more complex ways. Increasing lysine and threonine levels in cereals, methionine in legumes and vitamins A and E in crucifers and rice could compensate nutritional deficiencies in animal and human diets (Job, 2002). Also, molecular farming can efficiently produce biopharmaceutical products. Some of these products, such as trypsin and aprotinin, have already reached the market. Other products, such as industrial enzymes (phytases, proteases, glycosidases and oxido-reductases) or monoclonal antibodies and antigens for edible vaccines are close to commercialization (Newell, 2000; Horn et al., 2004).

### **Tissue Culture Protocols**

Tissue culture protocols are a prerequisite for a successful plant transformation. However, monocotyledoneous plants were considered as recalcitrant species for being propagated in tissue culture. Early reports in wheat focused on identifying suitable explants for the induction of embryogenic callus like scutelli, mature and immature embryos; adjusting bombarding parameters and selection and regeneration protocols (Altpeter et al., 1996a, Rasco-Gaunt et al., 1999). Later reports focused on the screening of commercially important cultivars for tissue

culture and transformation experiments (Takumi and Shimada, 1997; Iser et al., 1999, Altpeter et al., 2001).

Early reports indicated that plant regeneration from bahiagrass (*Paspalum notatum* Flugge) and other *Paspalum* spp was possible through the use of young inflorescence-derived callus (Bovo and Mroginski, 1986, 1989). Bahiagrass regeneration using mature seeds via somatic embryogenesis was firstly described by Marousky and West (1990). They indicated that seeds from bahiagrass cv. “Pensacola” germinated and developed small callus at the basis of the coleoptile in Murashige and Skoog medium (MS, Murashige and Skoog, 1962) supplemented with 9  $\mu$ M 2,4-D. However, authors reported very low embryogenic callus and plant regeneration rates (12 and 29% respectively). Also, Akashi et al. (1993) regenerated bahiagrass plants from six bahiagrass genotypes using seed-derived callus cultured in the same medium. They observed that callus induction, callus proliferation and plant regeneration were influenced by genotype effects. Besides, cv. “Pensacola” was the best regenerant with 40% embryogenic callus formation and 74% plant regeneration. Later reports focused on regeneration of bahiagrass cv. “Tifton 9” that was derived from cv. “Pensacola” by restricted recurrent phenotypic selection and released to the market in 1989 (Burton, 1989). Shatters et al. (1994) investigated bahiagrass using leaf-stem cross sections cultured in a Schenk and Hildebrandt medium (Schenk and Hildebrandt, 1968) supplemented with 30  $\mu$ M dicamba. Authors reported 96% regenerant callus and indicated that regeneration ability declined with the longer subcultures in the earlier selected lines while remained steady until 10 months in the later ones. Grando et al. (2002) reported an optimized bahiagrass regeneration protocol based on the use mature seed-derived callus. Authors observed 66% germination and 21% embryogenic callus formation using MS medium supplemented with 30  $\mu$ M dicamba and 5  $\mu$ M BAP. Subsequently, Altpeter and Positano (2005)

produced 85% germination and 55% embryogenic callus rates in cultivar “Argentine” using 13.5  $\mu\text{M}$  dicamba and 5  $\mu\text{M}$  BAP. Currently, this bahiagrass regeneration protocol is a routine protocol used to generate stable transgenic plants for the different lines of research developed in our laboratory.

### **Transformation Methods**

To generate transgenic plants, efficient protocols for plant regeneration, DNA delivery, transgenic tissue selection and recovery of normal and fertile phenotypes are required. In addition, these protocols should be highly reproducible and efficient, so that they can be used in a large scale and a short time frame. Currently, three methods fulfill these criteria: protoplast transformation, biolistic transformation and *Agrobacterium*-mediated transformation (Hansen and Wright, 1999).

Protoplast transformation involves protoplast isolation from different callus lines derived from immature tissues like embryos, inflorescences, leaves and anthers. These young tissues can be dedifferentiated and more susceptible to DNA uptake. Therefore, protoplasts could be transformed by different methods such as electroporation, microinjection and polyethyleneglycol (PEG). These technologies were used in the absence of *Agrobacterium*-mediated transformation protocols for monocots, but they were highly genotype-dependent and they were not suitable for transforming most important agronomic crops (Hansen and Wright, 1999; Newell, 2000; Taylor and Fauquet, 2002).

*Agrobacterium* is a gram-positive, soil-borne bacterium that produces a crown-gall disease and naturally infects different dicotyledonous plants. This disease is characterized by the transfer of the Ti plasmid, i.e. specific DNA fragment with specific flanking regions, from *Agrobacterium tumefaciens* into the plant cells. The Ti plasmid contains genes encoding enzymes involved in the synthesis of growth regulators inducing plant cell growth and tumor

formation, and the production of opines that support bacterial growth. Initially, *Agrobacterium*-mediated transformation was very successful in dicotyledonous plants because they are the natural host range for the bacterium. To overcome the host-plant specificity, *Agrobacterium*-mediated transformation was optimized by the use of hypervirulent strains and the use of wounding methods to enhance bacterial infection. To improve transformation efficiency, specific protocols for bacterial infection, inoculation, and cocultivation; and plant selection and regeneration were developed according to the specific requirements of the bacterial strain and the plant host. Recently, several protocols bypassed tissue culture by using in vivo inoculations; however, these protocols are restricted to model species such as *A. thaliana* and *N. tabacum*. The use of this technology has several advantages: Transgene integration patterns showed fewer and intact copies after T-DNA transfer compared with those transgene integration patterns produced by biolistic experiments. Subsequently, *Agrobacterium* host range was extended to monocotyledonous plants such as corn, rice, wheat and barley (Hansen and Wright, 1999; Newell, 2000; Gelvin, 2003).

Biolistic or microparticle bombardment technology involves the acceleration of microprojectiles coated with foreign DNA into target plant tissues. These gold or tungsten particles pass through the plant cell wall and nuclear envelope to release and integrate the DNA into the plant genome. This technology allows a wide range of transformation strategies including transient and stable expression studies, chloroplast and mitochondrial transformation studies and also viral expression studies (Altpeter et al., 2005). Due to its physical nature, microparticle bombardment is not limited by the pathogen-host interaction observed in *Agrobacterium*-mediated transformation. Therefore, it is used in a broad range of targets including not only those groups considered as recalcitrant groups among plants such as cereals

and grasses, but also other living organisms such as bacteria, fungi, algae, insects and mammals (Hansen and Wright, 1999; Newell, 2000; Taylor and Fauquet, 2002, Altpeter et al., 2005).

### **Particle Bombardment Method**

Currently, particle bombardment is the most widely used and successful method for introducing genes into monocotyledonous plants (James, 2003; Altpeter et al., 2005). This technology is routinely used to improve agronomic traits such as crop yield and quality, resistance to biotic stresses like fungal diseases and insect pests, tolerance to abiotic stresses like drought and cold, and molecular farming (Newell, 2000; Dunwell, 2000; Job, 2002; Horn et al., 2004).

There are two important factors that determine the success of gene transfer by particle bombardment: the physical parameters of the bombardment process and the biological requirements of the plant tissues before, during and after bombardment. Transient studies with reporter genes such as GUS, luciferase and GFP genes were designed to optimize these physical parameters according to the specific needs of each genotype (Southgate et al., 1995; Taylor and Fauquet, 2002). However, the target tissues to produce transgenic plants need to be prepared to integrate the foreign DNA, to undergo selection and to regenerate normal and fertile plants. Hence, the challenge is to develop an efficient transformation protocol using embryogenic or meristematic tissues and shortening the tissue culture time for avoiding somaclonal variation and the development of aberrant or infertile phenotypes.

Recently, the production of stable transgenic plants from forage and turf grasses including tall fescue (*Festuca arundinacea* Schreb.), red fescue (*Festuca rubra* L.), ryegrass (*Lolium perenne* L.), bermudagrass [ *Cynodon dactylon* (L.) Pers.] and creeping bentgrass (*Agrostis palustris*). Specifically, earlier protocols indicated the successful transformation and regeneration of fescue using tall fescue protoplasts (Ha et al., 1992) and tall and red fescue

embryogenic cell suspensions (Spagenberg et al., 1995). Robust protocols with efficient selection systems allowed to generate large numbers of red fescue plants using the *nptII* gene and paromomycin as selective agent (Altpeter & Xu, 2000). Recently, tall fescue nutritional quality was improved by overexpressing a sulphur-rich sunflower albumin (SFA8) under the control of *cab* wheat promoter (Wang et al., 2001). Also, tall fescue forage digestibility was enhanced by downregulating the expression of cinnamyl alcohol dehydrogenase (CAD), an enzyme involved in lignin biosynthetic pathway, in transgenic plants containing sense and antisense constructs of the *cad* gene (Chen et al., 2003). Altpeter et al. (2000) reported a rapid and efficient protocol for generating perennial ryegrass plants by using an expression cassette with the ubiquitin promoter and the *nptII* gene and obtaining the highest transformation efficiencies (4-11%) using calli derived from immature inflorescences and embryos in 9-12 weeks. Recently, Hisano et al. (2004) reported an increased tolerance to freezing in perennial ryegrass overexpressing wheat fructosyltransferase genes, *wft1* and *wft2*, which encode sucrose-fructan 6-fructosyltransferase (6-SFT) and sucrose-sucrose 1-fructosyltransferase (1-SST), respectively, under the control of CaMV 35S promoter. These plants contained significantly higher fructan levels than wild type and tolerated freezing at cellular level. Reports on common and triploid bermudagrass (Li and Qu, 2004; Zhang et al., 2003) indicated variable success in the production of transgenic bermudagrass plants probably related to not very efficient transformation, regeneration and selection protocols. The production of stable transgenic plants from bahiagrass (*Paspalum notatum* Flugge) cv. "Tifton 7" was firstly reported by Smith et al. (2002). However, this apomictic tetraploid genotype is not a commercially used cultivar, and authors reported that the transgenic nature of most of the glufosinate resistant plant could not be confirmed by PCR analyses (Smith et al., 2002). Later, unpublished studies revealed that the glufosinate resistance

was conclusive to indicate the transgenic nature and that the PCR analyses had resulted in false negative events (Smith, personal communication). In this context, there was a need of developing efficient protocols for transformation and regeneration of bahiagrass commercial cultivars such as “Argentine” and “Tifton 9” that are preferred for commercial production.

### **Selectable Markers and Selection Protocols**

To generate transgenic plants, tissue culture and transformation protocols should be coupled to efficient selection protocols. These protocols are based on the use of selectable marker genes (SMGs) which encode enzymes that regulate the growth or death of the transformed tissues. These SMGs conferred resistance to agents such as antibiotics, herbicides, toxic metabolic intermediates, or non-toxic metabolic intermediates. However, all these systems depend on the application of a selection agent in the culture medium. Instead, new SMGs encode enzymes such as isopentyltransferases, histidine kinase homologues and hairy-root inducing genes that regulate and limit plant growth (Miki and McHugh, 2004).

Currently, kanamycin, hygromycin and phosphinothricin comprised more than 90% of the selectable markers in research studies and field trials for selection of yeast, plant and animal tissues (Miki and McHugh, 2004). The gene *nptII* encodes the neomycin phosphotransferase from *E. coli*, an ATP-dependent dephosphorylase that acts on several aminoglycosides including neomycin, kanamycin, gentomycin (G418) and paromomycin. The *nptII* gene is the most widely used in plants and its use includes model species of dicots such as *Arabidopsis* and tobacco (15 and 73% studies respectively) and monocots like rice and corn (4 and 33% respectively). The *hph* or *hpt* genes from *E. coli* encode the hygromycin B phosphotransferase which is an ATP-dependent phosphorylase that phosphorylates and inactivates hygromycin B (inhibitor of protein synthesis). The *pat* or *bar* genes confer resistance to the L-isomer of phosphinothricin (PPT). This enzyme transforms toxic ammonia radicals into glutamic acid in plant cells. The *bar* gene

(*S. higroscopicus*) and the *pat* gene (*S. viridochromogenes*) encode the phosphinothricin N-acetyltransferase that acetylates and inactivates PPT. Therefore, the toxicity of the commercial herbicides containing PPT such as Basta™, Ignite™ and Liberty™ is due to an increase in ammonia levels which leads to plant death. The *bar* gene is widely used in plants including important crops like corn, wheat, rice, and other species like conifers and orchids. Both SM systems, hygromycin and phosphinothricin based-systems, are widely used in 30% of the research studies (Miki and McHugh, 2004).

Public and regulatory concerns were raised about the risks of using antibiotic-resistant genes given that horizontal transfer to pathogenic soil or gastrointestinal bacteria could affect environment and human health. However, antibiotic resistance transfer from transgenic crops to animal or humans should fulfill several conditions including no DNA degradation in field conditions and the presence of a potential bacterial host for transgene integration and expression. In this context, Gay and Gillispie (2005) contrasted the potential increase in the antibiotic resistance reservoir created by plants with SMGs with the current situation created by medical antibiotic prescribing. Authors concluded that even though these SMGs could survive environmental conditions, the barriers to transfer, incorporation, and transmission indicated that SMGs contribution to antibiotic resistance is minimal compared with the contribution made by antibiotic prescription in clinical practice (Gay and Gillispie, 2005). Additionally, the horizontal transfer of herbicide-resistant genes from commercial crops to closely related weeds by cross-pollination could create new superweeds. Therefore, the use of selectable marker (SM)-free plants eliminates human and environment potential risks and favors public and governmental acceptance of transgenic crops (Sreekala et al., 2005; Darbani et al., 2007). To eliminate SMGs, several strategies were developed including the use of SMGs not based on antibiotic or

herbicide-resistant genes and the use of excision systems to eliminate the SMGs after regenerating the transgenic plants. The SM cassettes could be excised by cotransformation with both genes in different DNA fragments, the use of site-specific recombination systems transiently expressed like Cre/loxP, FLP/FRT and R/RS, transposon-based systems and interchromosomal recombination-based systems (Miki and McHugh, 2004; Darbani et al., 2007).

### **Transgene Integration and Expression Patterns**

Currently, *Agrobacterium*-mediated and microparticle bombardment are the most commonly used transformation methods to generate transgenic plants. In both cases, transgene integration plays a key role determining transgene stability and expression in primary transformants and subsequent generations. However, transgene integration and expression still remain as poorly understood phenomena (Kohli et al., 2003).

Traditionally, biolistic methods were used to generate transgenic plants from monocotyledonous plants, mostly cereals and grasses, which were not natural hosts for *A. tumefaciens*. The integration pattern observed involves a high number of transgene copies inserted in one single locus (Kohli et al., 2003; Latham et al., 2006). The locus structure varies from a single copy that could be intact, truncated or rearranged to several copies forming tandem or inverted repeats, concatemers or clusters with interspersed genomic DNA. Detailed studies on the structure of the junctions between the transgene and the genomic DNA suggested that the integration occurs by illegitimate recombination. These events are recognized due to the presence of microhomologies in the coding sequence of both recombinant DNAs, the presence of filler DNA, not belonging to either molecule, and similar motifs to those found in the topoisomerase I cleavage sites. Besides, vector backbone sequences seemed to have recombination hotspots that favored transgene rearrangements. Therefore, the use of minimal constructs (MCs) containing only the expression cassettes, instead of the complete plasmid

vectors, will enhance the expression levels in the transgenic plants (Altpeter et al., 2005). The proposed transgene integration models considered that transgene rearrangement and integration involves the participation of DNA repairing complexes generating a hotspot for further integration of other transgene copies in the genomic DNA. Authors suggested that these hotspots could be impediments for transcription complexes suppressing gene expression and leading to gene silencing (Kohli et al., 2003).

*Agrobacterium*-mediated methods are commonly used to generate transgenic plants mostly from dicotyledoneous plants. Recently, the method was improved and showed similar efficiencies to those obtained by particle bombardment protocols for cereals and other monocotyledonous plants. The transgene integration usually implies a lower number of transgene copies and the locus structure is less complex than those observed in the transgenic plants obtained by biolistic experiments. However, its complexity depends on several factors including the *Agrobacterium* strain, the transformation method, the plant species and the explant. Besides, the integration of vector sequences is a very common phenomenon. Also, it is interesting to notice that the integration occurs by illegitimate recombination and the integration process occurs in hotspots as those plants generated by microparticle bombardment (Kohli et al., 2003; Filipecki and Malepsky, 2006).

## **Future Prospects**

### **Tissue culture**

During tissue culture, plant tissues are exposed to different stress factors including wounding, desiccation, osmotic stress, limited access to nutrient supplies and high concentrations of growth regulators and antibiotics (Carman, 1995). These factors lead not only to plant dedifferentiation and regeneration but also to other uncontrolled results such as somatic recombination, chromosome rearrangements, ploidy changes, mutations, deletions and insertions

among other DNA rearrangements. These genetic changes induced by tissue culture conditions, called somaclonal variation, have direct effects on gene expression (Kaeppler et al., 2000). Besides, these changes accumulate with prolonged tissue culture (Fukui, 1983). Therefore, transformation methods should include the development of protocols reducing the tissue culture time (Altpeter et al., 1996a, Filipecki and Malepsky, 2006).

### ***Agrobacterium*-mediated transformation**

Currently, *Agrobacterium*-mediated transformation protocols were efficiently developed for most cereals including wheat (*Triticum aestivum* L.), rice (*Oryza sativa*), maize (*Zea mays* L.), barley (*Hordeum vulgare* L.), sorghum (*Sorghum bicolor* L.) and rye (*Secale cereale* L.) (*Agrobacterium* Protocols, Humana Press eds, 2006). Specifically, Wu et al. (2003) observed that factors such as embryo size, pre-culture, inoculation, and cocultivation times, acetosyringone and surfactant inclusions and selection time were important factors on transformation efficiencies (0.3-3%) obtained in four bread wheat cultivars. Recently, Toki et al. (2006) reported a successful rice transformation protocol based on the use of scutellum tissue obtained from one day pre-cultured seeds; the use of these explants enhanced further selection and shortened the tissue culture step avoiding somaclonal variation risks. Also, forage and turfgrasses were mostly transformed with scorable and selectable marker genes including tall fescue (*Festuca arundinacea* Schreb.), switchgrass (*Panicum virgatum* L.) and perennial ryegrass (*Lolium perenne* L.) (*Agrobacterium* Protocols, Humana Press eds, 2006). Recently, successful protocols were reported for perennial ryegrass (Altpeter et al., 2006; Bajaj et al., 2006) and zoysiagrass (Ge et al., 2006). To improve *Agrobacterium*-mediated transformation, a large repertoire of plasmids, bacterial strains and transformation protocols were developed. New approaches included transgene and bacterial or host factors cotransformation with the purpose of enhancing bacterial infection and transgene integration. It is expected that these factors will

broaden the host range and improve the transformation efficiencies of those still recalcitrant plant species (Tzfira and Citovsky, 2006; Lacroix et al., 2006).

### **Particle bombardment transformation**

Transgene integration, either by *Agrobacterium* or particle bombardment, plays a determining role not only in further expression of the transgene but also in other endogenous genes, being able to affect gene expression in all levels in the transgenic plants. Amongst the insertion effects, the most important ones include favoring new rearrangements and integration events, leading to silencing in the transcriptional or posttranscriptional levels. Transgenic RNAs could interact with endogenous RNA generating an iRNA response and shutting down some endogenous genes and/or transgenic polypeptide products could act as new sinks for endogenous free aminoacid pools or as new substrate for endogenous enzymes. In this context, transcript, protein and metabolic profiles are affected and the response could affect plant fitness and productivity. According to Filipecki and Malepsky (2006), these changes among different transgenic lines are minimal compared with those changes in profiles from plants belonging to different cultivars or ecotypes. However, particle bombardment generates very complex integration patterns with higher number of transgene copies and rearrangements (Latham et al., 2006). Therefore, transgene copy number affects transgene expression and could led to a wide range of expression levels from silencing to enhanced expression. Early reports in *Petunia* indicated that higher copy number lead to lower anthocyanin expression levels (Napoli et al., 1990; Jorgensen et al., 1996; Grant-Downton and Dickinson, 2005). Recently, a detailed screening of 132 *Arabidopsis* transgenic lines containing the GFP, GUS and SPT genes through generations indicated that plants containing one or two transgene copies expressed the reporter genes with twofold differences, while plants containing higher number of copies showed posttranscriptional gene silencing. In addition, transcriptional gene silencing could also occur

(Schubert et al., 2004; Filipecki and Malepsky, 2006). Besides, transgene expression could be naturally enhanced if the integration occurs within the minimal promoter sequence and is influenced by endogenous enhancers. Transgene expression and stability also could be enhanced by using matrix attachment regions as flanking sequences or by removing vector backbone sequences prior bombardment (Allen et al., 2000; Altpeter et al., 2005; Filipecki and Malepsky, 2006).

### **Selectable markers**

According to Darbani et al. (2007), the production of selectable marker-free transgenic plants could be approached by using different technologies. So far, cotransformation systems, site-specific recombination systems and positive markers based on non-toxic metabolites are the most widely used.

Specifically, Park and coworkers (2004) observed independent segregation of the transgenes and produced *nptII*-free transgenic tobacco plants by cotransforming two binary vectors containing the *nptII* and the *coda* genes in one T-DNA and the GUS gene in the other T-DNA. One alternative strategy involves the *dao1* gene encoding a D-aminoacid oxidase which shifts from a positive marker with the substrates D-alanine or D-serine to a negative marker with the substrates D-isoleucine or D-valine (Erikson et al., 2004).

Amongst the non-toxic metabolites, the phospho-mannose isomerase (PMI) is the most widely used positive SM included in the production of transgenic plants from sugar beet, canola, corn, wheat, rice and pearl millet (Darbani et al., 2007). Recently, O'Kennedy et al. (2004) reported stable integration and inheritance of the PMI gene and increased transformation efficiency using the PMI gene for selecting transgenic plants from pearl millet.

Amongst the site-specific recombination systems, the Cre/lox is the most exploited because of its precise, complete and stable SM removal which implies that the SMGs could be

recycled in a stacking gene engineering strategy. Recent reports indicated the successful production of SM-free transgenic plants in rice (Sreekala et al., 2005) and corn (Zhang et al., 2003). Interestingly, Zhang et al. (2003) observed that cotransformation of the SMG with the recombinase gene under the control of a heat shock promoter allowed the excision of both cassettes by using heat shocks in early stages of callus regeneration.

### **New strategies: Multigene engineering, chloroplast engineering and SM-free plants**

Currently, most genetic engineering studies use microparticle bombardment because it is a very versatile and efficient tool allowing for transgene integration and expression in transient expression studies with reporter genes, stable expression studies with genes integrated in the nucleus, chloroplasts and mitochondrias and host-pathogen interaction studies with virus (Altpeter et al., 2005). Several advantages contributed to the success of the microparticle bombardment including a large range of plant species and genotypes, a large range of target tissues and organs, the elimination of shuttle vectors and the stacking of multiple genes. It is not restricted by biological requirements being used in a wide range of plant species and genotypes. It could target a broad range of tissues and explants including embryos, seeds, shoot apices, leaf discs, callus, microspores, pollen grains and inflorescences generating an embryogenic or organogenic response. Also, shuttle vector is not required and vector backbone sequences (origin of replication, antibiotic gene and others) could be eliminated. It facilitates transgene stacking allowing for the integration of genes encoding different agronomic traits, multimeric proteins and several enzymes involved in a specific metabolic pathway. Recently, metabolic engineering studies in rice focused on the carotenoid pathway to increase provitamin A levels for preventing blindness, and the phenylpropanoid pathway to increase lignan levels for preventing different cancer types and coronary heart disease (Altpeter et al., 2005). Specifically, transgenic rice plants expressing three genes involved in  $\beta$ -carotene pathway in the plastids from rice endosperm

showed not only high levels of provitamin A but also a normal and fertile genotype (Ye et al., 2000; Datta et al., 2003).

Furthermore, microparticle bombardment combined with chloroplast engineering has shown several advantages including no transgene silencing, no position effects affecting transgene expression, high and uniform transgene expression levels, polycistronic translation allowing the expression of multiple genes under a common promoter, specific-site integration through homologous recombination and protein storage preventing cytoplasm degradation and transgene containment due to maternal inheritance (Heifetz, 200; Bock, 2001; Daniell et al., 2002; Daniell, 2006). Similarly to nuclear engineering, chloroplast engineering early efforts were focused in generating plants with traits such as herbicide tolerance, insect resistance and metabolite production. Early reports indicated that tobacco plants overexpressing the gene encoding 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) were tolerant to glyphosate (Roundup) (WO 00/03022 international patent application). Also, McBride et al. (1994) expressed the *cryIAC* gene in tobacco reaching 3-5% total soluble protein. Besides the simultaneous expression of multiple genes at high levels, chloroplast folding and assembly complexes assured the correct processing of proteins formed by single or multiple subunits making the system highly suitable for the production of biopharmaceuticals and vaccines (Daniell, 2006). Vaccine antigens against human diseases like cholera, anthrax, tetanus, plague or canine parvovirus were overexpressed reaching transgene expression levels of 4-31% of the total soluble protein. Also, therapeutic proteins like human serum albumin, somatotropin, interferon gamma and antimicrobial peptides were expressed at 6-21.5% of the total soluble protein. Chloroplast engineering allows oral delivery and an easy, efficient and economic protein

purification system (Daniell, 2006). Therefore, chloroplast engineering seems to be a very promising technology for crop improvement in the future.

### **Genetic Engineering for Crop Improvement**

According to the APHIS-USDA database (2007), genetic engineering coupled to breeding programs has generated more than 50 transgenic cultivars from the most important commercial crops since 1996 in the United States. Based on the number of approved releases for field tests, the dominant crops in the market are soybean, cotton, potato, tomato and wheat with 1104, 785, 769, 599 and 392 approved releases. Cereals are represented by wheat, rice and barley (with 392, 226 and 61 approved releases) and grasses are represented by creeping bentgrass, Kentucky bluegrass, tall fescue, St. Augustine grass, bermudagrass and bahiagrass (with 175, 35, 20, 17, 15 and 10 approved releases). Looking at the phenotype categories of these transgenic crops, research efforts are mostly focused in herbicide tolerance, insect resistance, product quality, agronomic properties and virus resistance represented by 4,064, 3,447, 2,917, 1,596 and 1,275 approved releases (26, 22, 18.6, 9.9 and 8.1% total releases, respectively)(Figure 2-2a). These efforts are further reflected by 33, 24, 14, 9 and 6 petitions for deregulation in the phenotypes for herbicide tolerance, insect resistance, product quality, virus resistance and enhanced agronomic properties respectively (Figure 2-2b). The states leading the field tests are Hawaii, Illinois, Iowa, California, Indiana and Florida, along with the Commonwealth of Puerto Rico, with 2,016, 1,926, 1,513, 1,311, 822, 737 and 1,675 issued or acknowledged field test locations in 2007 respectively (APHIS-USDA database, 2007) (Figure 2-3).

It is interesting to note that most crops were improved by using single gene traits like herbicide tolerance and insect resistance while few crops were improved for increasing yield by growth rate and photosynthetic efficiency because these traits are controlled in more complex ways (Dunwell, 2000).

## Genetic Engineering to Improve Nutritional Quality

Plant proteins provide the essential aminoacids required for a balanced diet and appropriate development of humans and animals. Cereals, grasses and legumes are the basis of human and animal diets. However, cereal proteins are deficient in lysine, tryptophan and threonine, while legume proteins are deficient in methionine and cysteine (Tabe and Higgins, 1998; Guenoune et al., 1999; Galili and Hoefgen, 2002; Sun and Liu, 2004).

To overcome the nutritional deficiencies of plant-based foods, two metabolic engineering approaches were considered: to increase the free aminoacid pools or to increase the essential aminoacid enriched-protein pools (Sun and Liu, 2004). Essential aminoacid pools could be regulated by modifying the expression levels of those enzymes involved in their biosynthetic or degradation pathways. The aspartate biosynthetic pathway leads to lysine formation on one branch and threonine, methionine and isoleucine formation on the other branch in the chloroplast (Galili, 1995). The expression of bacterial genes, encoding enzymes from the aspartate pathway that are insensitive to the plant feedback mechanisms, produced increased levels of free aminoacids and altered phenotype in cases like tobacco (Shaul and Galili, 1992b) but normal phenotypes in potato (Perl et al., 1992), *Arabidopsis* (Ben Tzvi-Tzchori et al., 1996) and alfalfa (Galili et al., 2000). Essential aminoacids-enriched protein pools could be modified by overexpressing SSPs or VSPs in cereal or legume crops. Early reports indicated the potential of corn zeins and soybean VSPs for being accumulated in transgenic tobacco leaves (Guenoune et al., 1999). Specifically,  $\beta$  and  $\delta$ -zein were expressed and stably accumulated but they showed low expression levels in tobacco leaves (Bagga et al., 1995, 1997; Sharma et al., 1998). Soybean VSPs were accumulated up to 2-6% total soluble protein in transgenic tobacco leaves (Guenoune et al., 1999; 2002a, 2003). Authors suggested that these high VSP expression levels in transgenic tobacco plants were probably due to the strong activity of the CAMV35S promoter and the lack

of those endogenous proteases founded in vacuoles of mature soybean leaf tissue (Guenoune et al., 1999). These high and stable transgene expression levels are necessary to assure an impact in animal feeding and performance.

From a nutritional standpoint, transgenic plants not only need to have higher and enriched-protein contents but also these proteins should be resistant to rumen degradation and absorbed in the intestines. Early studies indicated that nearly 40% of the plant proteins undergo rumen degradation in animals feeding on temperate pastures (Ulyatt et al., 1988). Methionine and lysine are the most limiting essential aminoacids for lactating (Rulquin and Verite, 1993) and growing animals (Merchen and Trigemeyer, 1992); therefore, they should be incorporated in bypass proteins and these proteins should be at least 30% to assure animal weight gain (NRC, 1989).

### **Vegetative Storage Proteins (VSPs)**

Vegetative storage proteins (VSP) are proteins which are accumulated at high levels in storage vacuoles of vegetative tissues (at least more than 5 % of total protein), used as temporary nitrogen reserves and without any other obvious enzymatic or metabolic role. So, these proteins regulate nitrogen availability according to the plant requirements, have a turnover rate controlled by the sink/source status of the storage organs and can be preferentially synthesized or degraded at different developmental stages of the plant (Staswick, 1994).

### **General characteristics**

First reports on VSPs described 27 and 29 Kda polypeptides in soybean plants (vsp- $\alpha$  and vsp- $\beta$  respectively) that were preferentially accumulated in young leaves reaching 6-15% of the total protein before flowering and declined to 1% during seed growth (Wittenbach, 1983). It was observed that VSP content increased with depodding, reaching 45% of the total protein in soybean leaves. Besides, it increased after petiole girdling in soybean cotyledons during seed germination (Wittenbach, 1983). This fact suggested that VSPs may also have a storage role in

young seedlings where the cotyledon changes from a storage organ to a photosynthetic organ involving degradation of storage vacuoles and releasing of protein content (Staswick, 1991, 1994).

Simultaneously, the paraveinal mesophyll (PVM) was characterized in soybean plants (Franceschi and Giaquinta 1982a, b, 1983a,b). This tissue consisted in one thick layer of larger cells interconnected by tubular arms that are wrapped around the phloem bundles. Ultrastructural and histochemistry studies showed that this tissue is directly involved in synthesis and degradation of proteins during the change of vegetative to reproductive growth in soybean plants. Later on, immunoblotting and immunocytochemical studies showed that three polypeptides of 27, 29 and 94 Kda were specifically accumulated in the PVM vacuoles and their turnover rate was regulated by sink/source status (i.e. high levels in young leaves and/or depodded plants)(Klauer et al., 1991). Furthermore, Klauer et al. (1996) observed the presence of these three polypeptides in the vacuoles of PVM in other legumes species. However, the 94 kda polypeptide was identified as a lipoxygenase (Tranbarger et al., 1991). Furthermore, DeWald et al. (1992) showed that VSPs had high sequence homology to a tomato acid phosphatase and low acid phosphatase activity. Later on, phylogenetic analyses showed that the loss of the catalytic site and activity is probably a requirement for changing to a storage function in legume species (Leelapon et al., 2004).

### **Genes and polypeptides**

VSPs and *vsp* genes were identified and characterized using different soybean tissues by different authors (Staswick, 1988; Mason et al., 1988; Rapp et al., 1991). Staswick (1988) detected the polypeptides VSP25 and VSP27 in leaves with high levels of mRNA of depodded soybean plants (*Glycine max* L. Merr. Cv. Williams 82). Simultaneously, Mason et al. (1988) detected the polypeptides VSP28 and VSP31 in stems of dark-grown seedlings from the same

soybean cultivar. This study showed that polypeptides and their encoding genes have 80% sequence homology. The pVSP28 and pVSP31 have N-terminal leader peptide sequences of 34 and 35 aminoacids respectively and their predicted cleavage sites are located after Gln18-Ala21 and Pro17-Gly20 respectively. These cleavages yielded 25 and 29 Kda mature products. PKSH5 and pKSH3 are the genomic clones encoding pVSP28 and pVSP31 respectively. Based on alignment studies, it was determined that pKSH3 and pVSP27 were the same transcript while pKSH5 and pVSP25 corresponded to different genes or they had alternative splicing (Staswick, 1988; Mason et al., 1988). Rapp et al. (1991) screened a genomic library of soybean leaves from depodded plants with the pVSP27 probe and detected both polypeptide products assembling and forming homo- or heterodimers. Specifically, these authors identified the putative CAAT box, the TATA box and a TGTTGT(A/T)(G/T) enhancer in the 5' flanking region and three exons and two introns in the coding sequence of pVSP29. Also, this coding sequence appeared as an inverted tandem repeat in one of the genomic clones indicating a recent duplication (Rapp et al., 1991). Currently, these genes are called *vspA* and *vspB* and their polypeptide products VSP $\alpha$  and VSP $\beta$ , respectively.

Homologous genes have been identified in other species such as *Atvsp* in *Arabidopsis thaliana*, and *Bspa* in hybrid poplar (*Populus alba* x *P. tremula*). The *Atvsp* gene encodes two polypeptide products of 29 and 30 kda proteins, is accumulated to high levels in hypocotyls, young leaves, flowers and pods and is regulated by sugars, jasmonates, wounding, light, phosphates and auxins (Berger et al., 1995). The *Bspa* gene encodes a bark storage protein of 32 kda, which accumulates in storage vacuoles of inner bark parenchyma and xylem rays reaching almost 50% storage proteins during fall (Zhu and Coleman, 2001). Three VSPs of 15, 19 and 32 kda were identified as a major component of the soluble proteins in the taproots of alfalfa. These

proteins are accumulated during fall, when the plants are dormant, and degraded during spring or after defoliation (Hendershot and Volenec, 1993). Thus, it was observed that their level was 28 % of the total soluble protein before defoliation, decreased after defoliation but it was recovered 30 days later (Avice et al., 1996).

### **Trafficking pathway**

Plant tissues usually store reduced nitrogen into proteins or polypeptides so-called storage proteins that are allocated into storage vacuoles. In this way, they are able to retain nitrogen and some essential aminoacids without creating an osmotic imbalance in the plant cell. These proteins are called seed or vegetative storage proteins according to the plant tissues where they were produced and stored (SSPs or VSPs respectively). SSPs include the albumins, globulins, prolamins and glutelins. For example, the prolamins include zeins from maize (*Zea mays* L.), gliadins from wheat (*Triticum sativum* L.) and hordeins from barley (*Hordeum vulgare* L.). VSPs were found in plants with storage organs such as tubers from potato (*Solanum tuberosum* L.) and sweet potato (*Ipomea batata* L.), taproots of alfalfa (*Medicago sativa*), paraveinal mesophyll tissue from soybean (*Glycine max*) and other legumes and bark tissues from trees such as poplar (*Populus* spp) or willow (*Salix* spp) (Muntz, 1998).

These soluble proteins could follow three different pathways (Vitale and Raikhel, 1999; Nehaus and Rogers, 1998; Muntz, 1998). Naturally, the default pathway implies that proteins are primarily processed in the endoplasmic reticulum (ER), further processed in the Golgi apparatus (GA) and secreted outside the cell. Some polypeptides are produced in the rough ER and accumulated into large vesicles attached to it. These vesicles are storage vacuoles (also called protein bodies) like those vacuoles commonly formed by prolamins in the endosperm cells from maize and rice. Finally, these storage vacuoles, coming either from the ER or the GA, could be

fused in the central vacuole or tonoplast like globulins from dicotyledonous plants and some prolamins from other cereals (Muntz, 1998).

In the latest pathway, the first processing step involves a specific docking mechanism at the ER with loss of the targeting peptide during the translation and incorporation of the polypeptides into the ER lumen for further modifications including glycosylation, formation of disulfide bonds, folding and polymerization. In the second processing step, ER, GA or tonoplast-derived vacuoles have other specific docking mechanism at the GA and this mechanism involves internal, carboxy-terminal or amino-terminal vacuolar targeting signals. These docking systems are highly conserved among kingdoms and they were better described in animal models. Some remarkable examples are the docking systems formed by BIP at the ER and BP-80 at the vacuoles (Vitale and Raikhel, 1999). In the final processing step, plant proteins lose the targeting peptides and acquire their final conformation as storage products at the vacuoles (Muntz, 1998).

According to VSP coding sequences, targeting peptides to the ER and the vacuole were predicted (Staswick, 1988, 1994; Mason et al., 1988). Later on, ultrastructural and immunocytochemical studies showed that VSPs were accumulated via RER and/or GA in the storage vacuole of the PVM from several legume species. It was reported that PVM cells were enriched with RER and AG and that VSP $\alpha$  and  $\beta$  were detected along the whole pathway in induced soybean plants where these proteins reached 50% of the total protein content (Klauer and Franceschi, 1997). Previous studies indicated that vacuoles were differentially labeled by antibodies against tonoplast intrinsic proteins (TIPs) which were indicators of the storage or lytic conditions of the vacuole ( $\gamma$  and  $\delta$ -TIPs respectively) (Jauh et al., 1998; Vitale and Raikhel, 1999). Recently, immunolabelling studies showed that these vacuoles are functionally flexible

and can be converted from storage to lytic forms or viceversa according to the soybean nitrogen requirements (Murphy et al., 2005).

### **Overexpression of *vspB* Gene to Improve Nutritional Quality in Bahiagrass**

Subtropical bahiagrass cultivars have a seasonal growth pattern which directly affects plant yield. Slower growth produces low yield during fall and winter, and faster growth produces higher yield during spring and summer in subtropical bahiagrass cultivars. Specifically, Cuomo et al. (1996) observed that biomass production varied between 2-3 Mg ha<sup>-1</sup> for cultivars “Pensacola”, “Argentine” and “Tifton 9” in late spring, while they reached 11-12 Mg ha<sup>-1</sup> in summer. Early on, Mislevy et al. (1990) indicated that cultivar “Tifton 9” had 30% more biomass production than cultivar “Pensacola” during early winter. Later on, cultivars “Pensacola”, “Tifton 9” and RRPS cycle 18 were evaluated for biomass production during the cool-season and authors observed that cultivar “Tifton 9” and RRPS cycle 18 doubled the yield of “Pensacola.”

This seasonal growth pattern also affects plant nutritive value, because of the fast growth rate directly related to high temperatures which implies secondary growth and reproductive growth (Stewart, 2006). Therefore, nutritional quality of subtropical grasses decreases with maturity during spring and summer. Specifically, Johnson et al. (2001) observed that dry matter yield increased while forage quality parameters such as digestibility and protein fractions decreased through successive cuttings during the summer in three subtropical grasses (bermudagrass cv. “Tifton 85”, stargrass cv. “Florona” and bahiagrass cv. “Pensacola”). This decrease in digestibility and soluble nitrogen fractions across summer could be partially compensated through nitrogen fertilization. However, this fertilization treatment reaches a threshold after midsummer, so that new dietary supplements need to be considered to support further animal performance.

Digestibility and voluntary intake studies indicated that crude protein content and *in vitro* organic matter digestibility (IVOMD) decreased in four tropical grasses (bermudagrass cv. “Tifton 85”, stargrass cv. “Florona” bahiagrass, cv. “Pensacola” and limpograss cv. “Floralta”) between 4-week and 10-week harvests during fall. However, the feeding value of “Pensacola” bahiagrass, represented by *in vitro* and *in vivo* OMD, remained steady while feeding values of “Tifton 85” bermudagrass and “Florona” stargrass decreased through the 6 weeks. Therefore, bahiagrass seems to retain its forage quality through fall season compared with other tropical grasses (Arthington and Brown, 2005). Therefore, nitrogen fertilization increases biomass production and crude protein content in tropical grasses in general and in bahiagrass in particular, and these increases could be reflected in animal performance (Stewart, 2006).

In summary, bahiagrass shows a seasonal growth pattern that is reflected not only in biomass production but also in nutritional quality. According to Cuomo et al. (1996), Jhonson et al. (2001), Arthington and Brown (2005) and Stewart (2006), this seasonal pattern involves an increase in biomass production and decrease in nutritional quality during the warm-season which directly limits animal growth and performance. This loss could be partially compensated by nitrogen fertilization and dietary supplies. However, the use of the *vspB* gene, encoding the VSP $\beta$ , presents two main advantages: rumen stability and enriched aminoacid composition. While most plant proteins are degraded by rumen proteolysis, bypass proteins remained intact and they are absorbed in ruminant intestines. Also, engineered plants containing VSps accumulated high levels of lysine in heterologous plants (Guenoune et al., 2003). Therefore, soybean VSP $\beta$  seems to be a promising candidate to enhance the nutritional quality of bahiagrass and it could affect total protein and essential aminoacid contents.

## Genetic Engineering to Enhance Pest Resistance

### Development of *Bt* crops

Currently, insect pests cause 10-20% of crop losses and are a major limiting factor in crop production (Ferry et al., 2006). Traditionally, these pests were controlled by Integrated Pest Management (IPM) strategies involving the use of pesticides. However, the indiscriminate use of pesticides can produce adverse effects on human health and the environment, including the development of insect resistance and the elimination of other beneficial insects (Ranjekar et al., 2003; Ferry et al., 2006). To overcome these problems, environmentally-friendly pesticides containing *Bt* (*Bacillus thuringiensis*) spores and crystals were developed. But these spray formulations were partially effective because they did not reach burrowing insects and did not persist long enough in the environment. Therefore, *Bt* transgenic crops expressing  $\delta$ -endotoxins were developed (Schnepf et al., 1998; Ferre and Van Rie, 2002; Ranjekar et al., 2003; Kaur, 2006).

Currently, this technology has been adopted not only in the United States but also in the rest of the world including developing countries such as China and India, where it was easily adopted by small farmers because it has a direct economic impact due to the reduction of pesticide applications and the increase of crop yields (Huesing and English, 2004).

The first generation of *Bt* crops include several commercial products expressing *cryIAb* and *cryIF* genes for protecting crops like cotton and corn against different insect pests like european corn borer (*Ostrinia nubilalis* Huebner), southwestern corn borer (*Diatraea grandiosella* Dyar) and corn earworm (*Helicoverpa zea* Boddie) that were released since 1996 in the United States (Mendelsohn et al., 2003). These examples include marketed products expressing the *cryIAb* gene like YieldGard™ corn hybrids (events MON810 and BT11 from

Monsanto and Syngenta respectively). Later, HerculexI corn expressing a full length *cryIF* gene was produced by Pioneer Hi-Bred International and Dow Agrosiences / Mycogen.

In cotton, the second generation of *Bt* products is already in the market. These products included Bollgard II, expressing the *cry2Ab* and *cryIAc* genes (from Monsanto) and WideStrike expressing the *cryIAc* and *cryIF* genes (from Dow AgroSciences). These products offer a broader spectrum of protection against insect pests (Bates et al., 2005).

### ***Bacillus thuringiensis* toxins**

Delta-endotoxins protect plants against nematodes and a large group of insects including Lepidoptera (butterflies and moths), Diptera (flies and mosquitoes), Coleoptera (beetles and weevils) and Hymenoptera (wasps and bees)(de Maagd et al., 2001; Ferre and Van Rie, 2002; Griffiths and Aroian, 2005). These crystal protoxins are solubilized and proteolytically activated in the insect midgut where the active toxins bind to specific receptors in the intestine epithelial cells leading to pore formation and cell death. The active toxins have a conserved structure formed by three domains. Based on conformational studies, domain I showed hydrophobic helices indicating that it could be responsible for pore formation, while domain II showed external loops in the  $\beta$ -sheets suggesting that it is involved in receptor binding and therefore host specificity, similar to those specific binding mechanisms observed in immunoglobulin-antigen binding reactions (Schenpf et al., 1998). Also, domain III is also involved in both phenomena because it has very high affinity to receptors located in the gut epithelium of different insect orders (Schenpf et al., 1998; Ranjekar et al., 2003; Abanti, 2004; de Maagd et al., 2001). These  $\delta$ -endotoxins are classified into four groups providing protection against four insect orders: *cry1* (Lepidoptera), *cry2* (Lepidoptera and Diptera), *cry3* (Coleoptera) and *cry4* (Diptera). So far, more than 100 *cry* genes had been already identified. Besides these crystal proteins produced during sporulation, other bacterial proteins with insecticidal activities produced during vegetative

growth such as the vegetative insecticidal proteins (*vips*) are under study (Schenpf et al., 1998; de Maagd et al., 2001; Bates et al., 2005).

### **Strategies to increase *cry* genes expression levels**

Currently, only one case of field-developed resistance was reported but laboratory studies on field-selected strains indicated the potential of resistance development in different insect populations (Ferre and Van Rie, 2002). According to Bates et al. (2005), this delay in resistance development under field trials could be explained by several factors including resistant individuals with higher fitness costs than the susceptible ones, a low frequency of resistant alleles, a dilution of resistant alleles in susceptible individuals feeding on non-transgenic plants, and high toxin doses in the transgenic plants. Originally, the use of transgenic crops expressing moderate  $\delta$ -endotoxin levels to ensure the survival of susceptible individuals in the insect population seemed to be the best strategy. But, this strategy was influenced by environmental conditions producing a small delay and affecting crop yield. Currently, the use of transgenic crops expressing high toxin levels to ensure the death of the heterozygous individuals for the resistance gene (autosomal, recessive), produces a longer delay and maintains the crop damage below an economic threshold (Bates et al., 2005).

Therefore, to increase *Bt* expression levels different molecular approaches were taken: the use of *cry* truncated sequences only expressing the active toxins, the use of *cry* codon-optimized sequences for enhanced plant expression and the reduced use of AT sequences for eliminating alternative splicing sites and polyadenylation signals (Schenpf et al., 1998; Bohorova et al., 2001; Kaur, 2006). Other strategies involve creating fusion constructs (Bohorova et al., 2001), stacking *cry* genes with different binding sites (like hybrid or site-directed mutagenesis-generated genes)(Kaur, 2006), and pyramiding genes encoding proteins with different toxicity mechanisms like vegetative insecticidal proteins (*vip*) or proteinase inhibitors (PI) (Ferry et al., 2006). To

delay insect resistance development, these molecular strategies were combined with insect pest management (IPM) strategies including the use of refuges formed by non-transgenic plants (Cannon, 2000; Ranjekar et al., 2003; Bates et al., 2006).

### **Expression of *cryIF* Gene to Enhance Insect Resistance in Bahiagrass**

Bahiagrass (*Paspalum notatum* var. Flugge) is the predominant grass in forage in the southeast of the United States and it plays a key role supporting beef and dairy cattle industries in North Florida (Chambliss, 2002). Even though bahiagrass withstands most plant diseases, it is susceptible to insect pests such as mole crickets (*Scapteriscus* spp) and fall armyworm (*Spodoptera frugiperda* J. E. Smith). Fall armyworm (FAW) is one of the most important insect pests in the southeast of United States, causing seasonal economic losses in field crops such as sweet and field corn, forage and turf grasses, other cereals like rice and sorghum, and other crops like cotton and peanut (Sparks 1979, Meagher and Nagoshi, 2004). In the 1970s, these outbreaks resulted in economic losses of 30-60 million dollars (Sparks, 1979, Meagher, personal communication). Initially, it was observed that *cryIAb* and *cryIF* genes provided good protection levels against different insect pests like European corn borer, southwestern corn borer and Corn Earworm in cotton and corn crops (Mendelsohn et al., 2003). Amongst these pests, the ECB is considered the most important insect pest in the Midwestern and northeastern, while FAW and CEW are the most important pests in the southeastern United States. In southeastern U.S., the subtropical weather allows double cropping if the tropical crops like corn and cotton are resistant enough to overcome summer pests and diseases. Further studies showed that all *Bt* events expressing the *cryIAc* gene (YieldGard corn hybrids) were effective in controlling whorl damage by FAW and ear infestation and damage by CEW at normal and late planting dates (Williams et al., 1997; Buntin et al., 2004; Wiatrak et al., 2004). However, Abel and Adameczyk (2004) suggested that differential *cryIAb* expression patterns could be limited by reduced

nitrogen supply or photosynthesis rate in green tissues. Recently, field trials with corn hybrids expressing a full length *cryIF* gene (Herculex I) indicated that this gene provided protection against a wide range of insect pests including FAW (EPA, 2001). However, these reports are limited to commercial products from crops like corn and cotton and there are no reports on the effects of *cry* genes against insect pests in forage or turfgrasses. Therefore, the expression of a synthetic *cryIF* gene encoding a  $\delta$ -endotoxin was evaluated as a strategy to enhance resistance against FAW in bahiagrass.

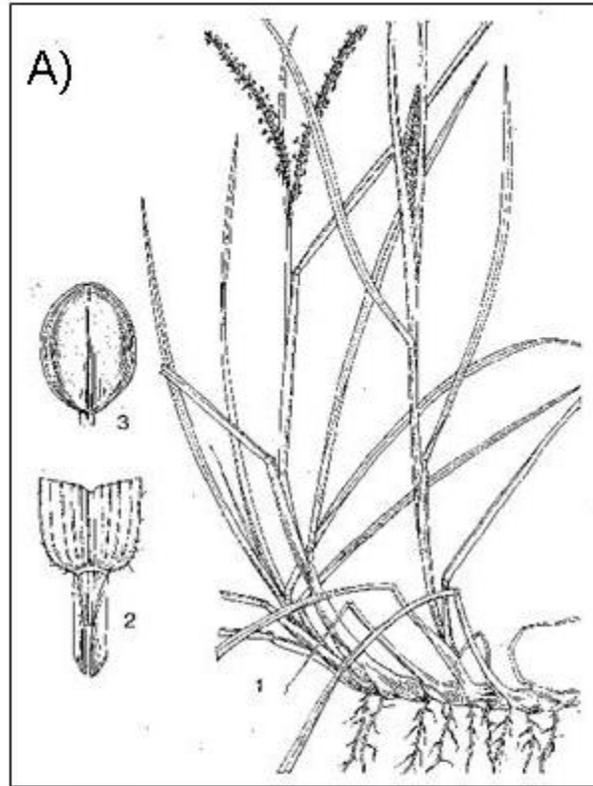


Figure 2-1. Bahiagrass (*Paspalum notatum* Flugge). A) erect or semiprostrate plant growth habit indicating aboveground stolons, underground rhizomes, aerial stems and roots with details of the ligule and spikelet [http://www.tropicalforages.info/key/Forages/Media/Html/Paspalum\\_notatum.htm](http://www.tropicalforages.info/key/Forages/Media/Html/Paspalum_notatum.htm). B) bahiagrass pasture with inflorescences <http://plant-materials.nrcs.usda.gov/gapmc/photo.html> . C) cattle grazing on bahiagrass pastures in Florida <http://www.animal.ufl.edu/facilities/sbru/> .

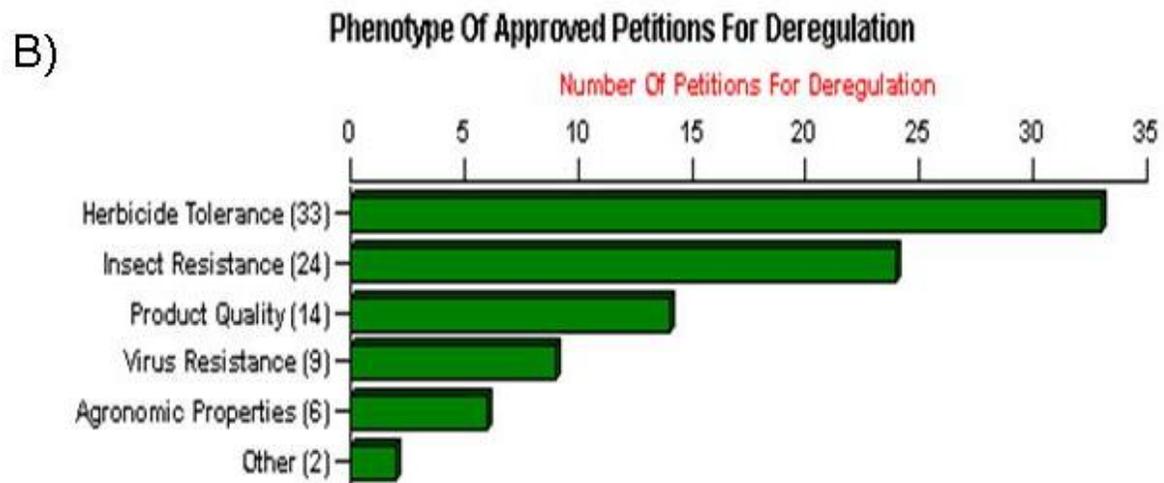
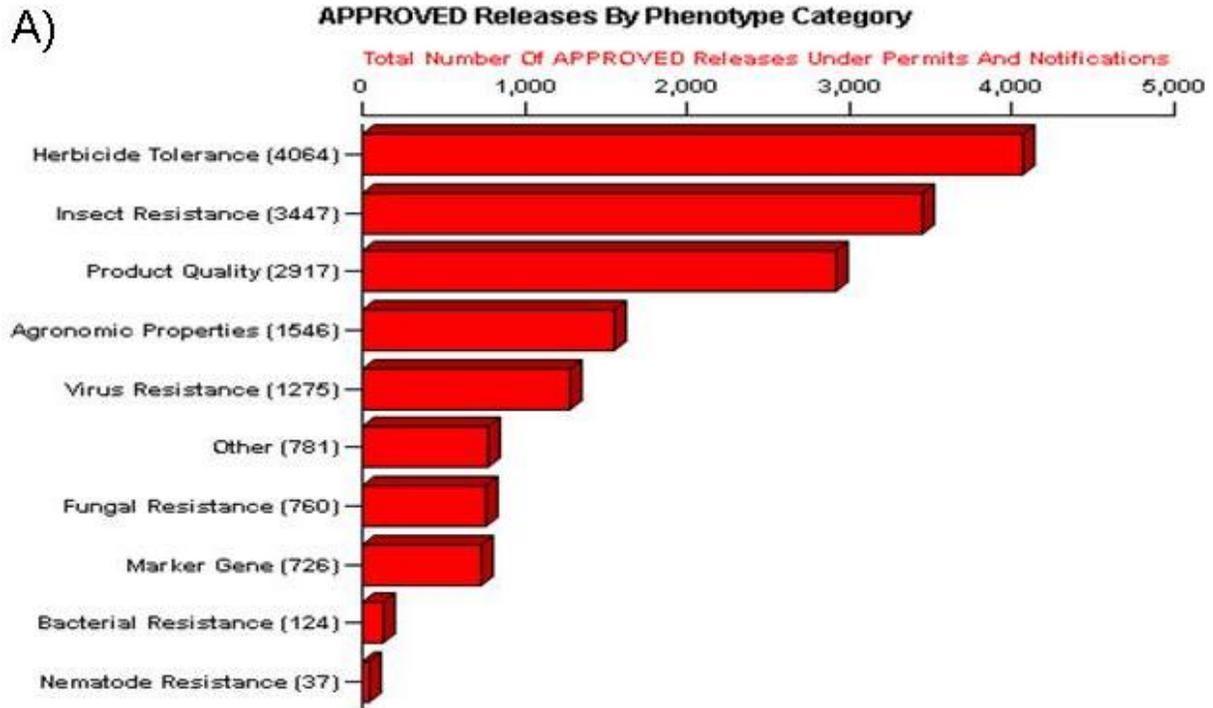


Figure 2-2. Transgenic crops under field trials and deregulation process in the United States (APHIS-USDA database <http://www.nbiap.vt.edu/>, last accessed May, 2007). A) Approved field releases by phenotypes, B) Approved deregulation petitions by phenotypes

## Total Number of Issued or Acknowledged Field Tests by U.S. State

(No number indicates a value of 0)

**1987-Present** (February 21, 2007)

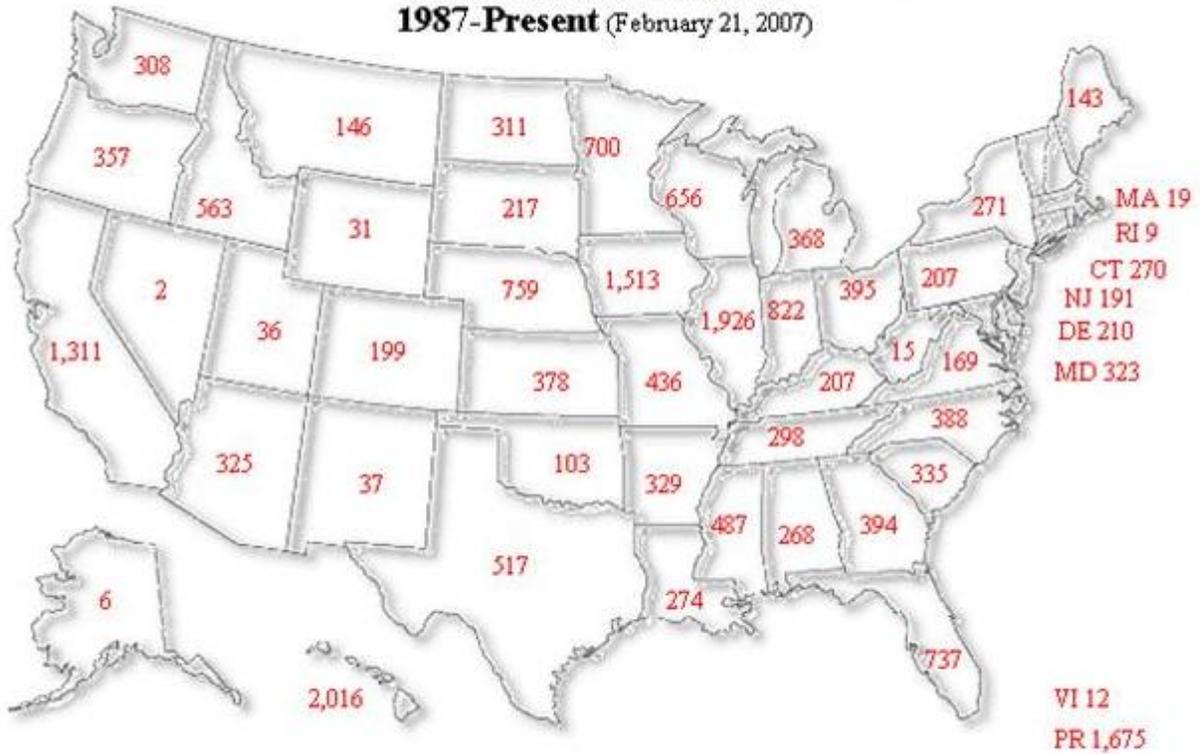


Figure 2-3. Total number of issued or acknowledged field tests of transgenic crops sorted by state in the United States (<http://www.isb.vt.edu/cfdocs/fieldtests1.cfm>, last accessed May, 2007).

CHAPTER 3  
OVEREXPRESSION OF THE *vspB* GENE FROM SOYBEAN TO ENHANCE  
NUTRITIONAL QUALITY IN BAHIAGRASS (*Paspalum notatum* VAR. FLUGGE)

**Introduction**

Bahiagrass (*Paspalum notatum* var. Flugge) is the predominant grass in forage pastures in tropical and subtropical regions and it supports ruminant production in these areas worldwide. This subtropical grass is a C4 plant, like corn and sorghum, which implies that it has higher water and nitrogen use efficiencies and performs better than C3 plants under high temperature, moisture and light intensity conditions (Gates et al., 2004; Moser et al., 2004). In Florida, bahiagrass is the primary forage crop supporting beef and dairy cattle industries and covering 2.5 million hectares (Chambliss, 1996, 2002; Burton et al., 1997; Blount et al., 2001). It is preferred by farmers and producers because it has low management requirements and very good persistence and yield under different environmental stresses. It is tolerant to poor soils including light-textured, low fertility and marginal pH soils. It is tolerant to drought stress because it has a well-developed root system that helps to improve water holding capacity and to reduce nutrient leaching in soils. It is tolerant to most fungal diseases and insect pests, except for ergot (*Claviceps* spp), dollar spot (*Sclerotinia* spp), mole crickets (*Scapteriscus* spp) and fall armyworms (*Spodoptera* spp). Moreover, it is tolerant to overgrazing and high cutting frequencies which favors plant regrowth and maintains nutritional quality during summer (Chambliss, 2002; Blount et al., 2001; Stewart, 2006). However, it requires high sowing rates and is susceptible to common herbicides. Most C4 tropical grasses, including bahiagrass, have a low protein content which affects animal performance. Bahiagrass protein content reaches 11% during spring and decreases to 5-7% during summer and fall. This decrease in protein content with an increase in fiber content reduces not only bahiagrass nutritional value but also animal performance giving reduced average daily gain (Cuomo et al., 1996; Gates et al., 2004).

Genetic engineering and breeding efforts has been focused in crop improvement. Most transgenic crops are improved by incorporating traits like herbicide tolerance and insect resistance while few crops are improved for increasing crop yield and quality because these traits are controlled in more complex ways (Dunwell, 2000). Specifically, grass pastures have low nutritive value compared with legume based-pastures because grasses have low protein content and are deficient in essential aminoacids like lysine, tryptophan and threonine (Sun and Liu, 2004). Nutritional quality is a complex trait because it requires the regulation of some metabolic pathways without affecting the metabolic profile of the plants.

To improve nutritional quality new molecular approaches were developed (Tabe and Higgins, 1998; Galili and Hoefgen, 2002; Sun and Liu, 2004). These molecular studies are focused on up- or down-regulating expression levels of enzymes involved in the aminoacid synthesis or degradation pathways so that an enhanced production of free essential aminoacids may lead to an increase of total protein content and protein quality in plants (Galili et al., 2000; Habben et al., 1995). Also, they are focused on increasing the levels of essential aminoacids-enriched proteins, mostly by overexpressing storage proteins in forage crops.

To prevent heterologous protein degradation, two approaches were followed: producing recombinant proteins including endoplasmic reticulum (ER)-retention signals like lys-asp-glu-leu (KDEL) (Khan et al., 1996; Tabe et al., 1995; Wandelt et al., 1992) or using those storage proteins that are naturally accumulated in vegetative tissues (VSPs).

Several VSPs were identified, isolated and used for increasing protein content and quality in forages. Vegetative storage proteins were found in plants with storage organs such as tubers from potato (*Solanum tuberosum* L.) and sweet potato (*Ipomea batata* L.), taproots of alfalfa (*Medicago sativa*), paraveinal mesophyll tissue from soybean (*Glycine max*) and other legumes

and bark tissues from trees such as poplar (*Populus* sp) or willow (*Salix* sp) (Staswick, 1994; Muntz, 1998). Although soybean-derived VSPs have high homology with a tomato acid phosphatase (DeWald et al., 1992), they have very low acid phosphatase activity probably due to the loss of the catalytic site (Leelapon et al., 2004). These proteins have a turnover rate controlled by the sink/source status of the storage organs and they could be preferentially synthesized or degraded at different developmental stages of the plant. Originally, VSP $\alpha$  and VSP $\beta$  and their encoding genes, *vspA* and *vspB*, were characterized from soybean tissues (Staswick, 1988; Mason et al., 1988; Rapp et al., 1991). Later, ultrastructural and immunocytochemical studies showed that VSPs were accumulated via rough ER (RER) and/or golgi apparatus (GA) in the storage vacuole of the paraveinal mesophyll (PVM) from several legume species. It was reported that PVM cells were enriched with RER and GA and that *vsp* $\alpha$  and  $\beta$  were detected along the whole pathway in induced soybean plants where these proteins reached 50% total protein content (Klauer and Franceschi, 1997). Recently, immunolabelling studies showed that these vacuoles are functionally flexible being converted from storage to lytic forms and viceversa according to the soybean nitrogen requirements (Murphy et al., 2005).

The use of the *vspB* gene, encoding the soybean VSP $\beta$ , presents two main advantages: rumen stability and enriched aminoacid composition. While most plant proteins are degraded by rumen proteolysis, bypass proteins remained intact and they are absorbed in ruminant intestines. It was previously observed that VSP $\beta$  behaves as a bypass protein being stable in the rumen and absorbed in the cattle intestines (Guenoume et al., 2002). Besides, VSPs contain ca.7% lysine (Mason et al., 1988; Staswick, 1988). Engineered plants containing VSPs accumulated high levels of lysine in heterologous plants (Guenoune et al., 2003). Because *vsp* $\beta$  is a rumen proteolysis-resistant (Galili et al., 2002) and lysine-enriched protein (Mason et al., 1988;

Staswick, 1988), it is a promising candidate to improve plant nutritional quality in bahiagrass for ruminant feeding. Therefore, the objective of this work was to overexpress a soybean vegetative storage protein gene and to evaluate the effects of this gene on the nutritional quality of bahiagrass (*Paspalum notatum* Flugge).

## **Materials and Methods**

### **Soybean *vspB* Gene**

The *vspB* gene and VSP $\beta$  polypeptide from different soybean tissues were molecularly characterized by different authors (Staswick, 1988; Mason et al., 1988; Rapp et al., 1991). Specifically, they identified two cDNA clones that were highly homologous: pVSP27 (Staswick, 1988) and pKSH3 (Mason et al., 1988). Further studies using genomic libraries identified the putative CAAT box, the TATA box and a TGTTGT(A/T)(G/T) enhancer at the 5' flanking region and three exons and two introns in the coding sequence of *vspB* gene (Rapp et al., 1991). The *vspB* gene from the *vsp27* cDNA clone, as reported in the NCBI database (M20038), has a coding sequence that is 762 bp in length (Figure 3-1). The VSP $\beta$  polypeptide is 254 aa length and contains a 35 residue ER-signal peptide (Mason et al., 1988) and a putative FPLR vacuolar-transit peptide (DeWald, 1992) in the NH<sub>2</sub>-terminal region. The soybean *vspB* coding sequence in the pKSH3 vector was kindly provided by Dr. R. Shatters (ARS-USDA, Ft. Pierce, Fl). To enhance *vspB* expression, the pKSH3 vector was used in further cloning strategies.

### **Construction of Expression Vectors for the *vspB* Gene**

A precise description of the cloning strategy to generate the expression vectors and respective cassettes is described in Figure 3-2 and the specific cloning protocols and vector maps are described in Appendix 1 and 2 respectively.

The complete soybean *vspB* sequence was amplified from the plasmid pKSH3 and introduced in the pGEM-T Easy Vector (Promega). For amplifying the *vspB* coding sequence,

we used the forward primer: 5' *CGTCTAGAACGCGT*  
ATGAAGTTGTTTGTTCCTTTGTTGC including the *XbaI* and *MluI* sites and the reverse  
primer: 5' *CGGCGGCCGCACTAGTCTCAATGTAGTACATGGGATTAGGAAG* including  
the *NotI* and *SpeI* sites. A 25 µl PCR reaction was set up using the PCR Core System II  
(Promega). The PCR product was used in a ligation reaction 1:1 (insert:vector) with the  
LigaFast™ Rapid DNA Ligation System (Promega) and incubated overnight at 4°C. The  
ligation products were transformed into commercial chemical competent cells (Life  
Technologies), plated in Luria Broth (LB) medium with 7 µl IPTGX, 40 µl X-gal and 100 µg/ml  
ampicillin and incubated overnight at 37°C. Primary colony screening identified four white  
colonies. These colonies were grown in 4 ml LB medium containing ampicillin overnight at 37°C  
and 220 rpm. Plasmid DNA was isolated using the QIAprep Spin Miniprep Kit (QIAGEN) and  
plasmid quantity and quality was confirmed by running a 0.8% agarose gel. Further screening by  
PCR and by restriction digestions with *XbaI* and *NotI*, *MluI* and *SpeI* and *XbaI*, *NotI* and *BamHI*  
indicated that all colonies contained the 712 bp amplified fragment and all the restriction sites  
were functional. According to the sequencing results, both colonies were completely  
homologous to the *vspB* sequence. The intermediate vector was called pGFL1 (Figure2).

The *vspB* sequence was excised as an *XbaI-NotI* fragment from the intermediate vector  
pGFL1 and cloned into the plasmid pCMYCKDEL (kindly provided by Dr. Altpeter, Agronomy  
Department, University of Florida). The plasmid pCMYCKDEL contained the 35S CAMV  
promoter, the c-myc tag, the ER-retention signal KDEL and the 35S CAMV polyadenylation  
signal. The KDEL is a carboxy-terminal tetrapeptide that allows the retention of a family of  
soluble proteins in the ER (Munro & Pelham, 1987). The c-myc tag is a synthetic peptide formed  
by ten aminoacid residues corresponding to the coding sequence between the 408-439bp of

human c-myc gene product (EQKLISEEDL from the 9E10 hybridoma, Evan et al., 1985).

Epitope tagging is widely used for different protein studies like protein expression, localization, purification, topology, dynamics, interactions, functional analysis, and discovery (Jarvik and Telmer, 1998).

Plasmid DNA was isolated using the QIAprep Spin Miniprep Kit (QIAGEN) and plasmid concentration and quality were evaluated by running a 0.8% agarose gel. Plasmid backbone was prepared for ligation by 3' end dephosphorylation with shrimp alkaline phosphatase (SAP, Promega) and cleaned with spin columns from the QIAquick Gel Extraction Kit (QIAGEN). The *XbaI-NotI* insert vector was gel-extracted and cleaned with spin columns from the QIAquick Gel Extraction Kit (QIAGEN). Both fragments were used in a ligation reaction 1:1 (insert:vector) incubated overnight at 4°C. The ligation products were transformed into electrocompetent cells of *E. coli* DH5 $\alpha$ . Six colonies were screened by PCR. Four positive colonies were further checked by restriction digestions with *XbaI*, *XbaI* and *NotI* and *PstI* showing the right size fragments. Two colonies were sequenced and one colony was homologous to the *vspB* sequence. Besides, the translation frame of *vspB* coding sequence was aligned with the c-myc tag and the ER-retention signal KDEL, and the CAMV 35S terminator from the pCMYCKDEL vector. This plasmid was called pGL2 (Figure 3-2).

From the pGL2, a fragment containing the *vspB* sequence, the c-myc tag, the ER-retention signal SKDEL and the 35S CaMV terminator was amplified. A forward primer: 5' **CGGGTACCATGAAGTTGTTTGTTCCTTTGTTGC** including the *KpnI* and a reverse primer: 5' **CGGAGCTCGTTTAAACGCATGCCTGCAGGTC**ACTG including the *SacI* and *PmeI* sites were used. The 25  $\mu$ l PCR reaction was set up using the PCR Core System II (Promega). PCR product was cleaned with spin columns from the QIAquick Gel Extraction Kit (QIAGEN).

A *PstI* fragment containing *ubiI* promoter and first intron (Christensen and Quail, 1992) from pJFNPTII (Altpeter, 2000) was cloned into the pCAMBIA2300 vector. The pCAMBIA2300 contained a selectable marker cassette formed by a double 35S promoter, the *nptII* gene and a 35S terminator (Figure 3-2). The *PstI* insert was gel-extracted and cleaned using the spin columns from the QIAquick Gel Extraction Kit (QIAGEN). The pCAMBIA2300 backbone was digested with *PstI*, dephosphorylated with SAP (QIAGEN) and cleaned with spin columns from the QIAquick Gel Extraction Kit (QIAGEN). Both fragments were used in a 1:1 ligation reaction incubated overnight at 4°C. Ligation products were electroporated into electrocompetent cells of *E. coli* DH5 $\alpha$ . Four colonies were screened. These colonies were grown in 4 ml cultures overnight in LB medium and kanamycin at 37°C and 220 rpm. Plasmid DNA was isolated using the QIAprep Spin Miniprep Kit (QIAGEN). Further screening by restriction digestions with *XhoI*, *EcoRI* and *PstI* indicated that the insert was correctly oriented in one colony. This intermediate vector was called pGFL2 and used as recipient vector for further cloning steps (Figure 3-2).

The PCR fragment from pGL2 was cloned into the pGFL2 vector to produce the expression vector pGL4. Insert and vector were digested with *SacI* and *KpnI*. The pGFL2 vector was further dephosphorylated with SAP (Promega) and cleaned with spin-columns from the QIAquick Gel Extraction Kit (QIAGEN). The 3:1 (insert:vector) ligation reaction was set up using LigaFast™ Rapid DNA Ligation System (Promega), and incubated overnight at 4°C. The ligation products were transformed into electrocompetent cells. Five colonies were screened by PCR and restriction digestions. These colonies were grown in 4 ml LB medium and ampicillin overnight at 37°C and 220 rpm. Plasmid DNA was isolated with QIAprep Spin Miniprep Kit (QIAGEN) and plasmid quantity and quality were confirmed by 0.8% agarose gel. PCR and

restriction digestions with *PmeI* and *SacI*, *KpnI* and *SacI*, *PmeI* and *SacII* and *HindIII* indicated the presence of the insert. Two colonies were sequenced. One colony was completely homologous to the *KpnI-SacI* fragment sequence and all the restriction sites were functional (Figure 3-2).

### **Other Expression Vectors and Cassettes**

The vectors pJFNPTII and pHZ35SNPTII were used as selectable markers. The pJFNPTII vector contains a selectable marker cassette formed by maize ubiquitin promoter and first intron (Christensen and Quail, 1992), the *nptII* coding sequence (Bevan, 1984) and the CAMV35S polyadenylation signal (Dixon et al., 1986). The pHZ35SNPTII vector contains a selectable marker cassette formed by the CAMV35S promoter (Odell et al., 1985) and hsp70 intron (Rochester et al., 1986), the *nptII* coding sequence (Bevan, 1984) and the CAMV35S polyadenylation signal (Dixon et al., 1986). The vector pRSVP1 (provided by Dr. R. Shatters, ARS-USDA, Ft. Pierce, Fl) contains a *vspB* expression cassette formed by maize *ubiI* promoter and Adh intron, the *vspB* coding sequence and a 3' *nos* polyadenylation signal (Grando, 2001; Grando et al., 2005).

Plasmid DNA was isolated using the QIAprep Midiprep Kit (QIAGEN). Fu et al. (2000) indicated that the use of minimal constructs (MCs) containing only the expression cassettes, instead of the complete plasmid vectors, enhanced the expression levels in the transgenic plants. Therefore, plasmids were digested, their respective fragments were gel-extracted and fragment quality and concentration were confirmed by a 0.8% agarose gel. The *vspB* cassette was excised as a *PvuII* fragment (3.4kb) from the pGL4 vector and as an *EcoRI* fragment (1.9kb) from the pRSVP1 vector. The *nptII* cassette was excised as an *I-SceI* fragment (3kb) from the pJFNPTII vector and as *AlwNI-NotI* fragment (2.6kb) from the pHZ35SNPTII. These expression cassettes were isolated by gel electrophoresis, and the corresponding band was excised and purified using

the Wizard SV Gel and PCR cleanup system (Promega, Madison, WI) to remove vector backbone sequences. These expression cassettes were used for the biolistic gene transfer (Figure 3-3).

### **Transformation and Regeneration Protocols**

Mature seeds were used for generating embryogenic calli from bahiagrass (*Paspalum notatum* Flugge) cultivar “Argentine” (Altpeter and Positano, 2005). Plates with embryogenic calli were cobombarded with microparticles coated with the MCs from the pGL4, pRSVP1, pJFNPTII and pHZ35SNPTII vectors. Four independent gene transfer experiments were carried out. Specifically, 49 petri dishes, with approx. 30 callus pieces each, were cobombarded with MCs from pGL4 and pJFNPTII, 23 petri dishes with MCs from pGL4, pRSVP1 and pJFNPTII, and 19 petri dishes with MCs from pGL4 and pHZ35SNPTII. Transgenic calli expressing the *nptII* gene were identified during subsequent subcultures in a medium containing 50 mg L<sup>-1</sup> paromomycin sulfate as a selective agent (Altpeter and James, 2005). Transgenic plants were grown under 16 h light photoperiod at 26-29°C in growth chambers and transferred to the greenhouse after two or three weeks.

### **Molecular Studies**

Transgene integration was evaluated by molecular studies including polymerase chain reaction (PCR) analyses while transgene expression was evaluated by enzyme linked-immunoadsorbent assays (ELISA) for the *nptII* gene and western blots for the *vspB* gene. Recombinant VSP $\beta$  was isolated by co-immunoprecipitation using anti c-myc antibodies.

### **Enzyme linked-immunoadsorbent assays (ELISA)**

A rapid qualitative assay was performed to evaluate the expression of the *nptII* marker gene in leaf tissue from bahiagrass transgenic lines. Pieces of young leaf tissue were ground with a disposable pestle in an ependorf tube and protein extracts were obtained in a phosphate saline

buffer (PBS). Protein concentration was estimated with the Coomassie Plus Protein Assay reagent and Coomassie standards (Pierce Biotechnology, Inc.) at OD<sub>595</sub> with a spectrophotometer (BioRad Laboratories). Twenty micrograms of total protein were loaded in each well and wild type, positive and negative controls were included. ELISA assays were performed according to the protocol suggested by the Agdia kit (Agdia, Elkhart, IN) and colorimetric data were captured with a digital camera.

### **Polymerase chain reaction (PCR)**

PCR analyses were performed to confirm the integration of the *vspB* gene into the bahiagrass transgenic lines. Genomic DNA from the transgenic lines was extracted following a modified Dellaporta method (1983). Three sets of primers were used for the screening. The forward primer 5'***CGGGTACCATGAAGTTGTTTGT***TTTCTTTGTTGC and the reverse primer 5'***CGGAGCTCGTTTAAAC*** GCATGCCTGCAGGTCACTG were designed for amplifying a 1kb fragment containing the coding sequence of the *vspB* gene, the c-myc tag, the SKDEL signal and the 35S polyadenylation signal. PCR was performed using the PCR Core System II (Promega). The cycling conditions were 95°C 2 min initial denaturation, 30 cycles of 95°C 1 min, 50°C 1 min, 72°C 1min and 72°C 5 min final extension. Also, two sets of primers were used according to Grando (2001). The first pair included the forward primer P1 5'GTTCTTCGGAGGTAAAAT and the reverse primer P2 5' TTCGCCTCTGTGGT and it amplified a 611 bp fragment. The second pair included the forward primer P3 5'GCAGGCTACCAAAGGT and the reverse primer P4 5'TAGGTGACTTACCCACAT which amplified an 843 bp fragment. Both fragments contained the *vspB* coding sequence. PCR was performed using the PCR Core System II (Promega). The cycling conditions were 95°C 15 min initial denaturation, 35 cycles of 95°C 1 min, 57°C 1 min, 72°C 1min and 72°C 15 min final extension. The PCR products were electrophoresed in 0.8% agarose gel, stained with ethidium

bromide and digital images were captured with the QuantityOne Software (BioRad Laboratories).

### **Sodium dodecyl sulphate polyacrilamide gels (SDS-PAGE)**

Five hundred milligrams of leaves were ground with liquid nitrogen and 1 ml of extraction buffer (100 mM Tris.HCl (pH 8.0), 200 mM NaCl, 1Mm EDTA, 5% glycerol, 0.2% TritonX100, 1mM  $\beta$ -mercaptoethanol or DTT) supplemented with 32  $\mu$ l/ml buffer of the Protease Inhibitor Cocktail (Sigma). Two successive centrifugation steps for 20 and 10 min at 4000 rpm (Sorwall SS-34) were done to obtain a clear supernatant. Protein concentration was estimated with the Coomasie Plus Protein Assay reagent and Coomasie standards (Pierce Biotechnology, Inc.) at OD<sub>595</sub> with a spectrophotometer (BioRad Laboratories). Fifteen or twenty micrograms of total protein were separated on 12% Sodium Dodecyl Sulphate Polyacrylamide Gels (SDS-PAGE). Two molecular markers were used: Prestained SDS-PAGE standards, low range (Bio-Rad Laboratories) and Magic Mark XP Western protein standards (In vitrogen). Gels were electrophoresed in Mini PROTEAN 3 Electrophoresis Cell (Bio-Rad Laboratories) at 125 v and room temperature for 75 min. Gels were either stained with Coomasie blue (Sigma R-250, Sigma) or further used for western blot analysis.

### **Western blot analysis**

SDS-PAGE gels were transferred to nitrocellulose or PVDF membranes using a Mini Trans-blot Electrophoretic Transfer Cell and the recommended protocols (Bio-Rad Laboratories). VSP was detected with VSP $\beta$  antibodies (provided by Dr. S. Galili, Agronomy and Natural Resources Department, The Volcani Center, Israel) or c-myc antibodies (anti c-myc antibody, Sigma). Protocols were adjusted for VSP $\beta$  antibodies with soybean leaves and for c-myc antibodies with bahiagrass leaves. Parameters evaluated were different leaves, extraction buffers, protein concentrations, primary and secondary antibody dilutions and incubation times.

For VSP $\beta$  antibody detection, a tissue sample of soybean leaves was used as starting material, 15  $\mu$ g total protein were loaded in the SDS-PAGE, and the VSP $\beta$  antibody was incubated in a 1:1000 dilution in orbital shaker for 4 hr. For c-myc antibody detection, a tissue sample of bahiagrass leaves was used as starting material, 20  $\mu$ g total protein were loaded in the SDS-PAGE gel and the c-myc antibody was incubated in a 1:1000 dilution in orbital shaker for 2 hr. Secondary antibodies were incubated in a 1:50,000 dilution in orbital shaker for 2 hr. Detection was performed using a very sensitive chemiluminescent substrate (Supersignal West Femto Maximum Sensitivity Substrate, Pierce Biotechnology, Inc.). Western blots with c-myc antibodies were performed in bahiagrass samples to evaluate the VSP $\beta$  expression levels variation from different leaves, tillers, and plants. To compare different leaves, four samples were taken from the the youngest to the oldest leaf and they were labeled consecutively as 1, 2, 3 and 4. Highest expression was observed in third and four bahiagrass leaves. Therefore, to compare different plants and tillers within plants, two samples from the third fully emerged leaf were taken.

### **Co-immunoprecipitation of recombinant VSP $\beta$**

The Pro-Found™ c-myc Tag IP/Co-IP kit and application set (Pierce Biotechnology, Inc.) was used to purify the VSP $\beta$  recombinant protein. Five grams of leaf tissue were ground with liquid nitrogen, and 15 ml extraction buffer (100 mM Tris.HCl (pH 8.0), 200 mM NaCl, 1Mm EDTA, 5% glycerol, 0.2% TritonX100, 1mM  $\beta$ -mercaptoethanol or DTT) was added. Two successive centrifugation steps for 20 and 10 min at 4000 rpm (Sorwall SS-34) were done to obtain a clear supernatant. Acetone precipitation was done to concentrate the protein extracts. Protein concentration was estimated with the Coomassie Plus Protein Assay reagent and Coomassie standards (Pierce Biotechnology, Inc.) at OD<sub>595</sub> with a spectrophotometer (BioRad Laboratories). Ten microliters of anti c-myc agarose with 2 mg protein sample and with 50  $\mu$ l c-

myc tagged control were incubated overnight in an orbital shaker at 4C. The mixture was loaded into the spin-columns, washed three times with TBS-T buffer, and eluted with Elution buffer according to the instructions of the manufacturer (Pierce Biotechnology, Inc.). The elutants of the three steps (i.e. loading, washing and eluting) were recovered. To evaluate the quality and quantity of the recombinant VSP $\beta$ , twenty microliters samples were loaded into a SDS-PAGE gel and western blots were performed.

## **Results**

### **Transformation and Regeneration Protocols**

Four independent gene transfer experiments produced 214 regenerated plants from 91 petri dishes with an average of 2.31 regenerated plants/plate (Figure 3-4). Specifically, these experiments included 156 regenerated plants with MCs from pGL4 and pJFNPTII, 16 regenerated plants with MCs from pGL4, pRSVP1 and pJFNPTII, and 42 regenerated plants with MCs from pGL4 and pHZ35SNPTII.

### **Molecular Studies**

#### **Enzyme linked-immunoadsorbent assays**

The screening of the putative transgenic plants by *nptII* ELISA indicated that 91 plants expressed the *nptII* gene from 110 plants analyzed. Specifically, 83 *nptII*(+)/102 analyzed plants with MCs from pGL4 and pJFNPTII, 8 *nptII*(+)/8 analyzed plants with MCs from pGL4, pRSVP1 and pJFNPTII. Figure 3-5C shows the ELISA plate arrangement for one set of transgenic bahiagrass lines, including negative and positive controls from *nptII* protein and a control of transgenic bahiagrass.

#### **Polymerase chain reaction**

The screening of the putative transgenic bahiagrass plants by PCR with the three primers sets confirmed the presence of the *vspB* gene in 34 transgenic plants (Figure 3-5A). These plants

include 28 transgenic plants with MCs from pGL4 and pJFNPTII, 6 transgenic plants with MCs from pGL4, pRSVP1 and pJFNPTII, and no transgenic plants with MCs from pGL4 and pHZ35SNPTII. Figure 3-5B shows the 1 kb fragment amplified with the first set of primers in gDNA from the transgenic bahiagrass lines.

### **Sodium dodecyl sulphate polyacrilamide gels**

According to the SDS-PAGE gels, VSPs were detected in the total protein profile from three soybean samples. In the soybean profile, VSP $\beta$  appeared as a strong 32kda band with variable concentration ranking between 10-15% total protein/plant (Figure 3-6A). A weak 32kda band (31.5kda predicted size, Gasteiger et al., 2003) corresponding to the recombinant VSP $\beta$  was detected in the total profile from several transgenic bahiagrass lines. However, it was not clear if this band corresponded to the recombinant VSP $\beta$  or to some other endogenous protein (3-6B).

### **Western blot analysis**

Western blots with VSP antibodies indicated that VSP $\beta$  levels were directly related to total protein levels in the range of 15 to 60  $\mu$ g total protein in soybean. In order to estimate the sensitivity of the VSP antibodies, a calibration curve with a range from 0.5 to 15  $\mu$ g total protein was established using the soybean samples. Based on this curve, the VSP antibodies were able to detect less than 50 ng soybean VSPs. To determine the detection limit of VSP $\beta$  antibodies in bahiagrass, a calibration curve in the range of 15 to 75  $\mu$ g total protein was established using bahiagrass samples. Irrespective of the strong band in the soybean control and the corresponding bands in the bahiagrass samples observed in the SDS-PAGE gels, the recombinant VSP $\beta$  was not detected in bahiagrass samples by VSP antibodies (data not shown).

Western blots with more sensitive c-myc antibodies indicated that VSP $\beta$  levels varied among different plants (Figure 3-7A,B), different tillers within plants (Figure 3-7C) and different leaves within tillers (Figure 3-7D). The highest VSP $\beta$  levels were observed in third and four

leaves from the tillers and in AT22-4 and AT22-7 among bahiagrass plants. According to the sensitivity limit for Supersignal West Femto Maximum Sensitivity Substrate, the substrate detects between pico and femtomoles per microgram of loaded protein, therefore the bands obtained from bahiagrass leaf samples corresponded to 0.001-0.01% total proteins.

### **Co-immunoprecipitation of recombinant VSP $\beta$**

Based on the co-immunoprecipitation kit protocol three fractions were recovered for the bahiagrass protein extracts and the c-myc control. These fractions corresponded to unbound proteins from the original protein extract, washing buffer and elution buffer containing the c-myc antibodies bound to the recombinant VSP $\beta$ . The SDS-PAGE gels containing these fractions indicated no degradation in the total proteins from bahiagrass samples. The western blots with the anti c-myc antibodies indicated the presence of the recombinant VSP $\beta$  (32kda) in the elution fraction and of a large polypeptide (approx. 50kda) in the unbound fraction from bahiagrass leaf samples. The control fractions showed the presence of the c-myc control in the elution fraction indicating that co-immunoprecipitation was efficient; however, it seemed to be an excess of c-myc protein because a small band was detected in the washing fraction (Figure 3-8).

### **Discussion**

We designed an expression vector including a constitutive and strongly expressed promoter for monocots; a KDEL signal for ER-retention and a c-myc tag for antibody detection to enhance the expression of the *vspB* gene in the transgenic bahiagrass plants. The maize *ubil* promoter is one of the strongest promoters in monocot transgenic plants. Transgene expression is normally enhanced by including its first intron (Christensen et al., 1992; Christensen and Quail, 1996; McElroy and Brettell, 1994). Previous molecular farming studies indicated that KDEL ER-retention signal increased expression levels of functional single-chain antibodies (Schouten et al., 1996) and monoclonal antibodies (Ramirez et al., 2003). Also, sorting signals such as the ER-

signal peptide (Mason et al., 1988) and a putative FPLR vacuolar-transit peptide (DeWald, 1992) in the NH<sub>2</sub>-terminal from the VSP $\beta$  enhanced expression of a hepatitis B antigen in transgenic potatoes for oral immunization studies (Richter et al., 2000). To increase transformation efficiency and transgene expression and stability, we also used MCs in the biolistics experiments because its use should generate simple integration patterns with low number of transgene copies and rearrangements and less chances of silencing events (Fu et al, 2000).

Based on bahiagrass transformation, selection and regeneration protocols previously established (Altpeter and Positano, 2005; Altpeter and James, 2005), we generated 91 *nptII*(+) plants from 110 analyzed plants. Based on the results from *nptII* expression detected by ELISA and VSP $\beta$  expression detected by western blots, 89% of the plants coexpressed the *nptII* and the *vspB* genes. Also, VSP $\beta$  expression levels varied among different plants, different tillers within plants and different leaves within tillers having the highest expression levels in third and four bahiagrass leaves. However, VSP $\beta$  expression levels in bahiagrass leaves corresponded to 0.001-0.01% total proteins. Currently, VSPs have a controversial role as storage proteins in soybean plants because even though they represented 10-15% total protein in young soybean leaves (Grando et al., 2005) and increased to 45% total soluble protein after deppoding; VSP expression levels could be reduced without affecting plant yield. These results indicated that VSPs do not play a key role in seed protein accumulation and seed production (Staswick et al., 2001). Nevertheless, transgenic tobacco studies indicated that high VSP levels were stored in tobacco leaves. Specifically, VSP $\alpha$  was stably expressed reaching 2-6% total proteins (Guenoune et al., 1999) while VSP $\beta$  only reached 2% total protein and it was degraded with leaf age (Guenoune et al., 2002b). To enhance these expression levels, authors co- targeted VSPs to the vacuole and the chloroplast (Guenoune et al., 2002a) or coexpressed both soybean VSPs or VSP $\beta$  and DHPS

reaching ca. 4% total protein in leaves (Guenoune et al., 2003). In contrast, we observed VSP $\beta$  expression levels corresponded to 0.001-0.01% total proteins in bahiagrass leaves from preflowering plants. These levels were similar to those reported in corn by Grando et al. (2005). Specifically, these studies indicated low VSP $\beta$  expression levels showing 0.5% total soluble protein and still lower 0.03% in leaves of preflowering plants from primary transformants and their progeny (Grando et al., 2005). Based on low VSP $\beta$  expression levels observed in corn and bahiagrass, it seems that transgenic proteins are more difficult to express in monocotyledoneous plants probably because their reduced translation or their degradation by endogenous proteases (Grando et al., 2005). Particularly, Bellucci et al. (2000) coexpressed  $\delta$  and  $\beta$ -zeins containing the KDEL signal in tobacco for enhancing ER accumulation (Wandelt et al., 1992). Authors observed that the  $\delta$ -zein was able to undergo posttranslational changes and form protein bodies while the  $\beta$ -zein remained accumulated into the ER lumen indicating that the KDEL signal could have interfered in molecule steric conformation. Hence, this is the first report on expression of the *vspB* gene in bahiagrass cv. "Argentine." It may be possible to increase VSP $\beta$  expression levels in transgenic bahiagrass plants by coexpressing recombinant VSP $\beta$  and VSP $\alpha$  subunits, and/or by targeting the subunit to different cell compartments. However, these increased levels may not likely enhance the total protein content of transgenic plants.



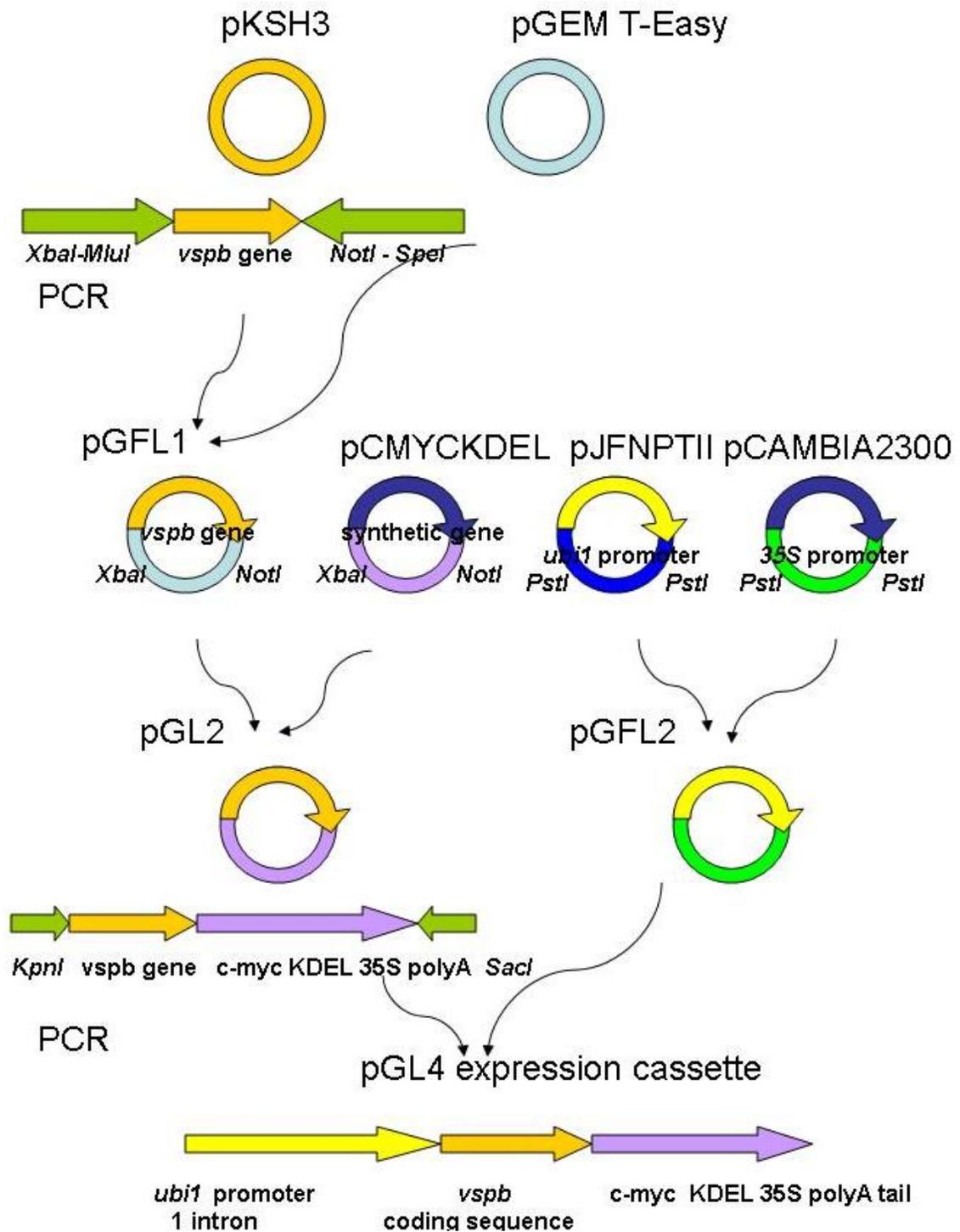
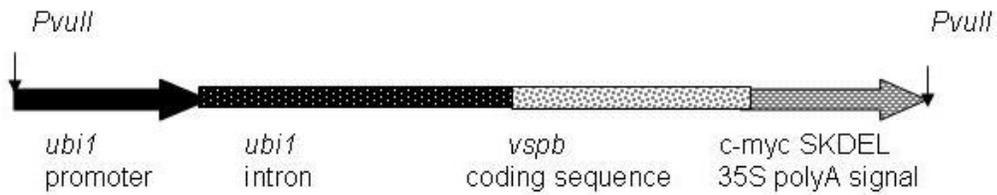
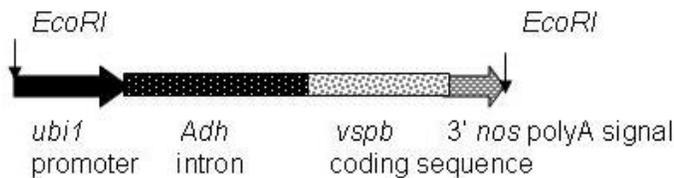


Figure 3-2. Cloning strategy for incorporating the *vspB* sequence from pKSH3 vector; the c-myc tag, KDEL ER-signal and 3'35S polyadenylation tail from pCMYCKDEL, and the *ubi1* promoter and first intron from pJFNPTII into the vector pGL4.

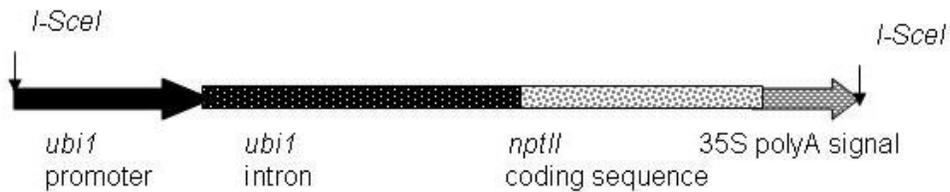
A) *vspb* expression cassette (3.4kb)



B) *vspb* expression cassette (1.9kb)



C) *nptII* expression cassette (3.0kb)



D) *nptII* expression cassette (2.5kb)

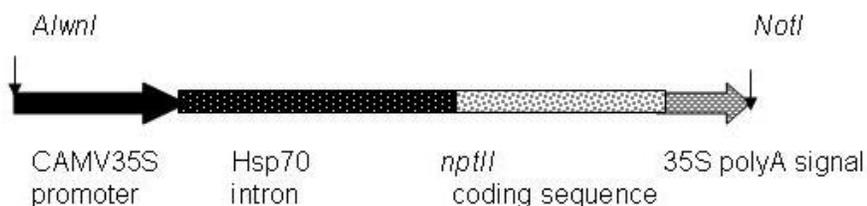


Figure 3-3. Minimal constructs used in the biolistic experiments to generate transgenic lines from bahiagrass (*Paspalum notatum* var. Flugge) cv. "Argentine". A) *PvuII* fragment (3.4kb) containing MC from pGL4 vector B) *EcoRI* fragment (1.9kb) containing MC from pRSVP1 vector C) *I-SceI* fragment (3.0kb) containing MC from pJFNPTII D) *AlwNI-NotI* fragment (2.5kb) containing MC from pHZ35SNPTII vector.

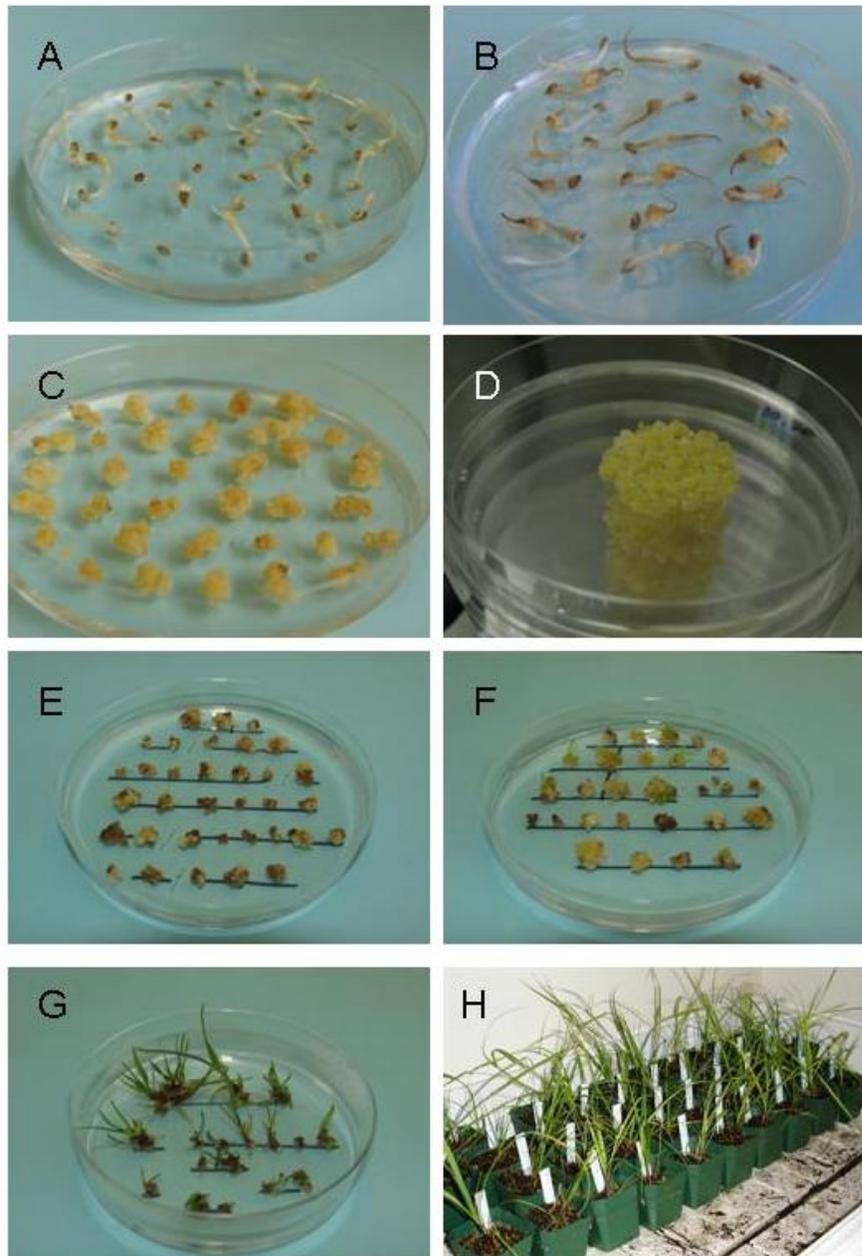


Figure 3-4. Transformation and regeneration protocols for bahiagrass (*Paspalum notatum* Flugge) cultivar “Argentine” . Different stages during the tissue culture: a) two-week seedlings, b) four-week seedlings, c) six-week calli, d) seven-week calli plate ready for bombardment, e) after 4 weeks of selection, f) after 4 weeks in regeneration with  $50 \text{ mg L}^{-1}$  paromomycin, g) after 2 weeks in root medium, and h) after 2 weeks in pots.

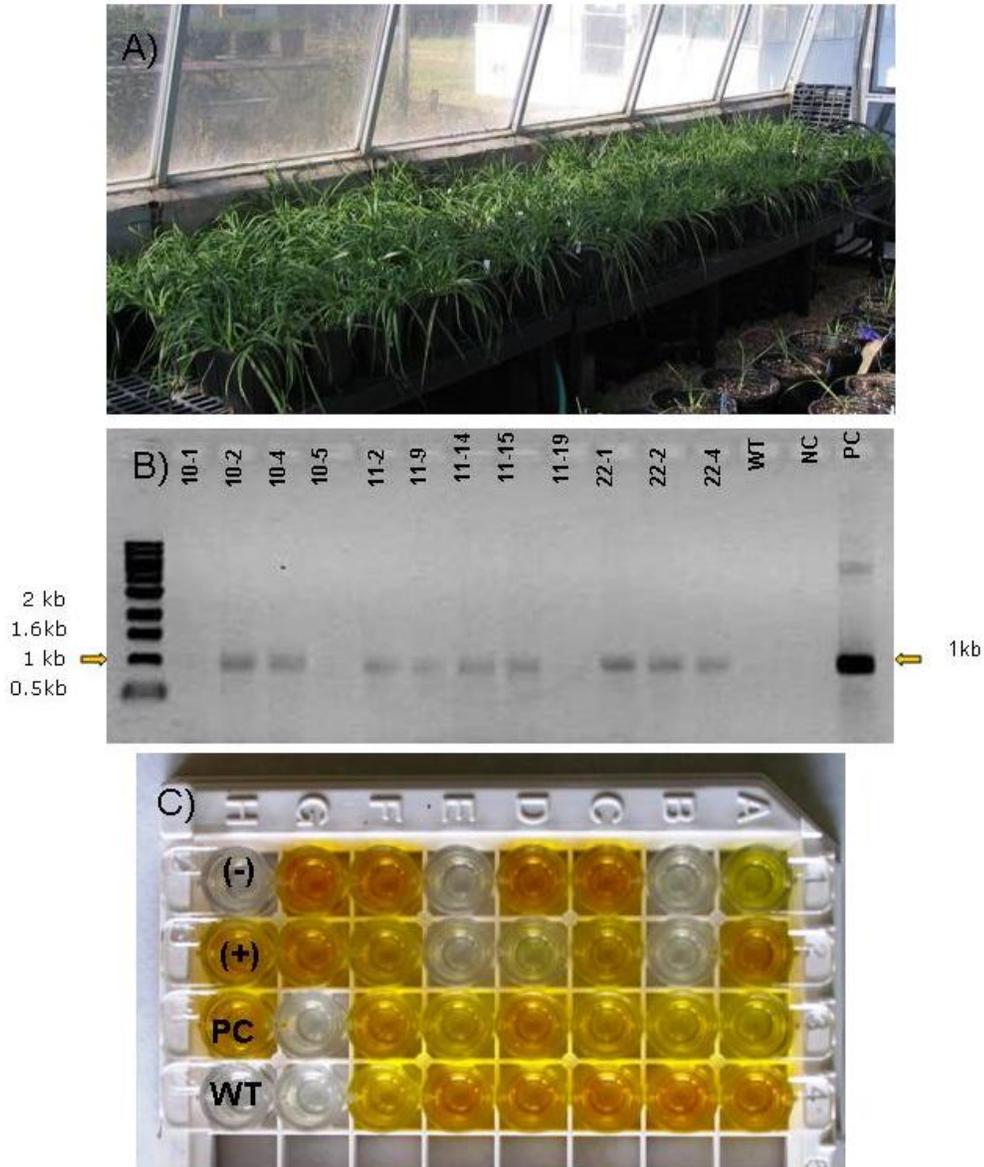


Figure 3-5. Transgenic plants from bahiagrass (*Paspalum notatum* Flugge) cv. “Argentine” . A) Transgenic bahiagrass lines in the greenhouse. B) PCR analyses for the *vspB* gene. A 1kb fragment was amplified using the forward primer 5'**CGGGTACCATGAAGTTGTTTGTTCCTTTGTTGC** and the reverse primer 5'**CGGAGCTCGTTTAAACGCATGCCTGCAGGTC**ACTG. The cycling conditions were 95°C 2 min initial denaturation, 30 cycles of 95°C 1 min, 50°C 1 min, 72°C 1min and 72°C 5 min final extension. AT10-1 to AT22-4, transgenic bahiagrass lines; WT, wild type bahiagrass; NC, negative control; PC, positive control; 1kb ladder. C) ELISA assays for the *nptII* gene (Agdia kit). A-G columns: AT10-1 to AT11-22 bahiagrass samples. H column: (-) negative control, (+) *nptII* positive control, PC transgenic positive control and WT wild type.

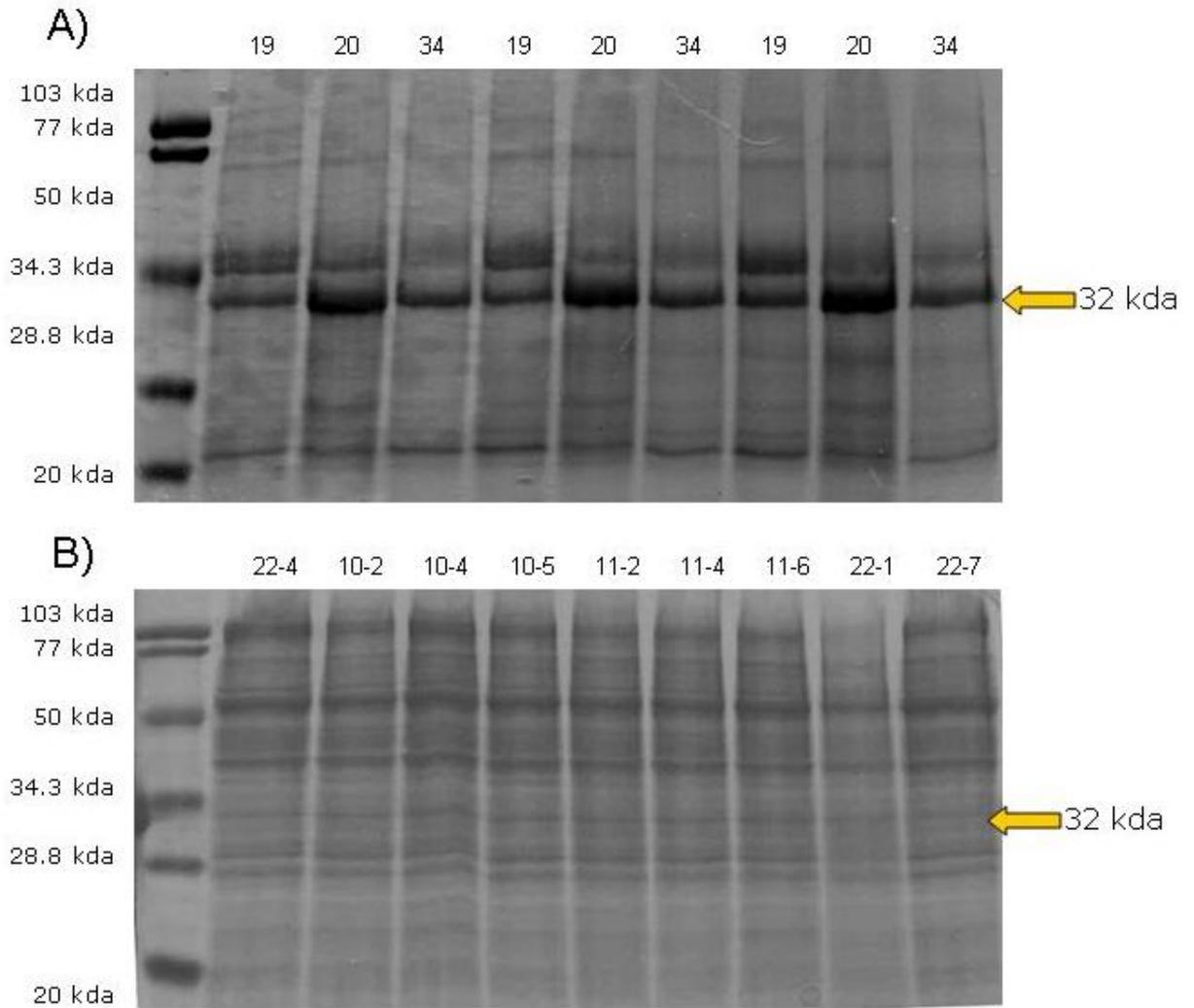


Figure 3-6. SDS-PAGE gels for soybean (*G. max*) and transgenic bahiagrass plants (*P. notatum* var. Flugge) cv. “Argentine”. A) Soybean plants (three different protein extracts of lines 19, 20, 34). B) Transgenic bahiagrass plants (lines 10-2, 10-4, 10-5, 11-2, 11-4, 11-6, 22-1, 22-4, 22-7). Arrows indicate the 32 kda band corresponding to VSP $\beta$ .

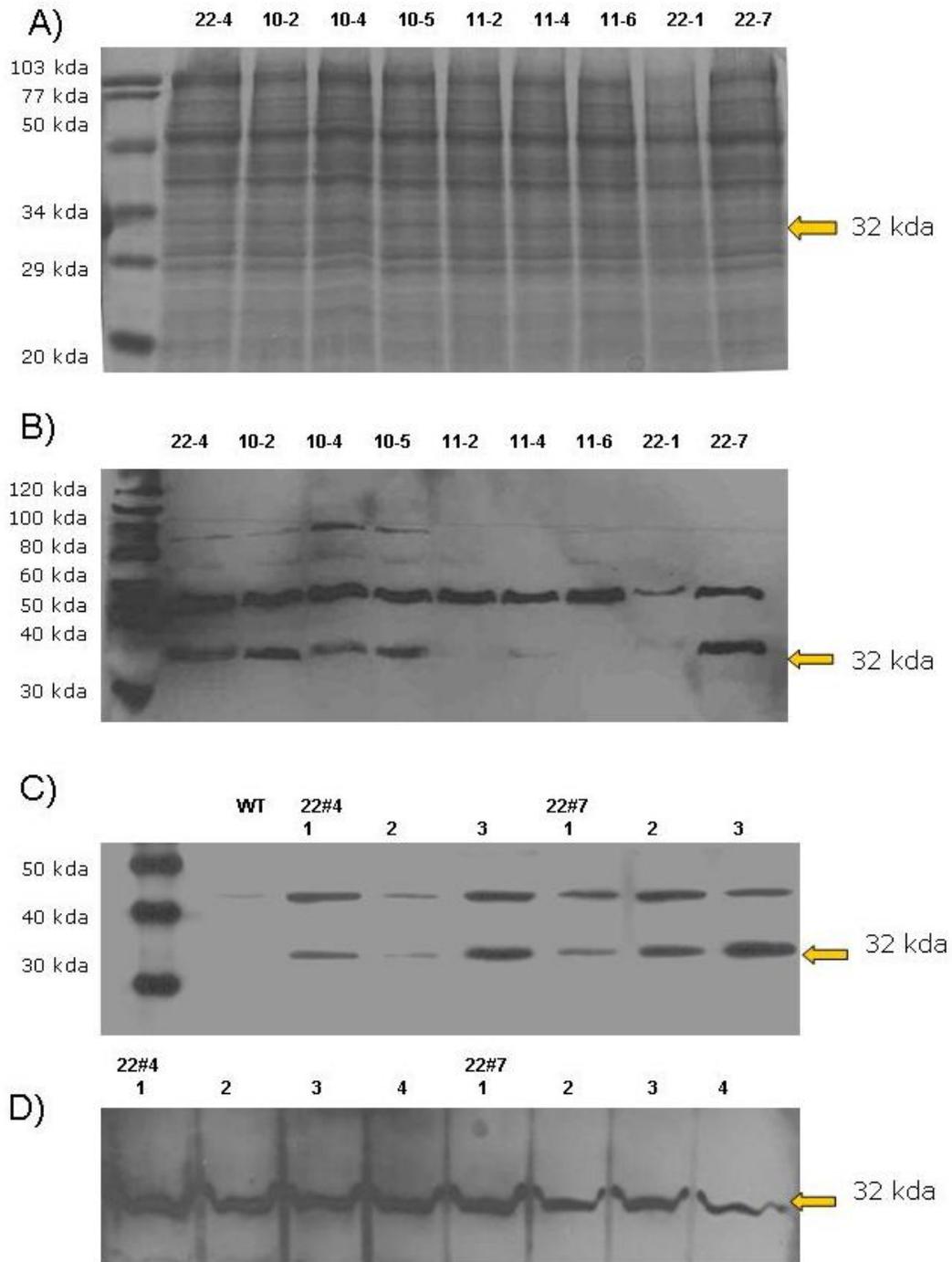


Figure 3-7. Transgenic bahiagrass (*Paspalum notatum* Flugge) cv. “Argentine”. A) SDS-PAGE of different transgenic bahiagrass lines. B) Western blots with c-myc antibodies from different transgenic bahiagrass lines. C) Western blot of three different tillers (#1,2,3) within lines (lines 22-4 and 22-7). D) Western blot of four different leaves (#1,2,3,4) within lines (lines 22-4 and 22-7).

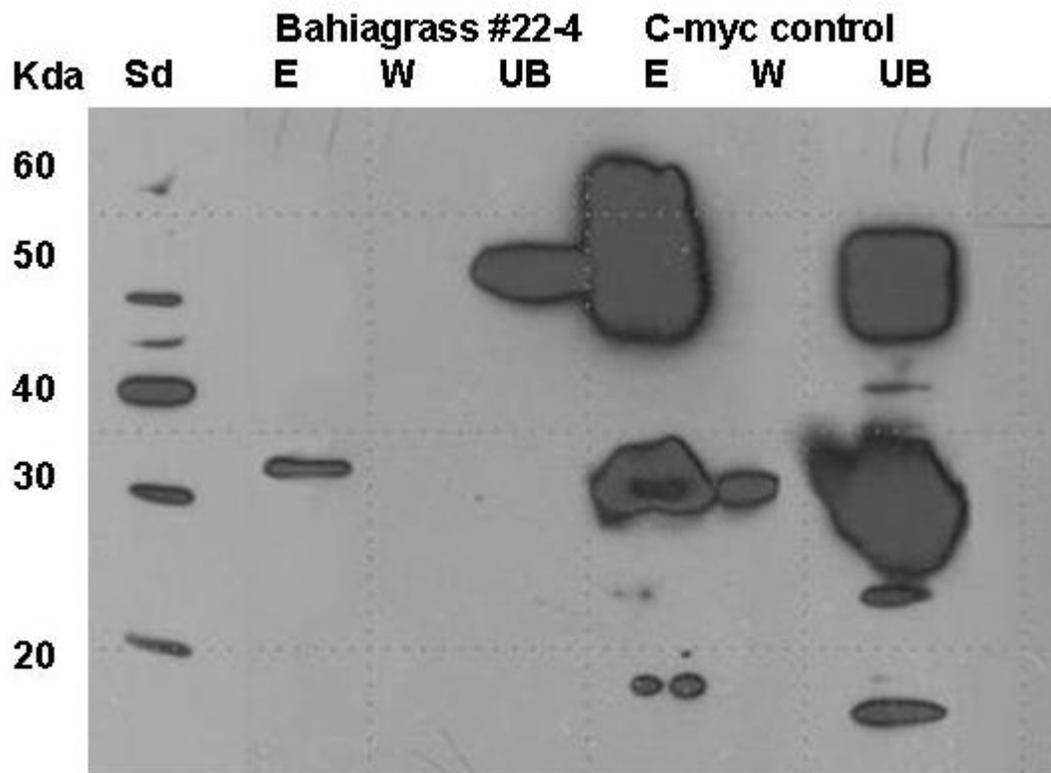


Figure 3-8. Co-immunoprecipitation of recombinant VSP $\beta$  from transgenic bahiagrass (*Paspalum notatum* Flugge) cv. "Argentine". Western blot with c-myc antibodies indicating three fractions: unbound fraction, wash fraction and elutant fraction with the recombinant VSP $\beta$  from plant 22-4 and anti c-myc (+) control.

CHAPTER 4  
EXPRESSION OF A SYNTHETIC *cryIF* GENE FROM *Bacillus thuringiensis* TO ENHANCE  
RESISTANCE AGAINST FALL ARMYWORM IN BAHIAGRASS (*Paspalum notatum* VAR.  
FLUGGE)

**Introduction**

Bahiagrass (*Paspalum notatum* var. Flugge) is an important forage grass in tropical and subtropical regions around the world. It is grown on 2.5 million hectares in the southern United States (Burton et al., 1997; Blount et al. 2001). Its popularity is based on low maintenance requirements and tolerance to drought, heat, many diseases and overgrazing (Chambliss, 2002). However, bahiagrass is susceptible to two major insect pests: mole crickets (*Scapteriscus* spp.) and fall armyworm (*Spodoptera frugiperda* J. E. Smith). Fall armyworm is one of the most important insect pests in the southeastern U.S., causing significant seasonal economic losses in forage and turf grasses and many other crops (Sparks, 1979; Nagoshi and Meagher, 2004; The bugwood network, 2007).

Traditionally, many insect pests are controlled using Integrated Pest Management (IPM) strategies involving the use of pesticides with resistant varieties or biological control agents. However, the indiscriminate use of pesticides produces adverse effects on human health and the environment including the development of insect resistance and the elimination of other beneficial insects (Ranjekar et al., 2003; Ferry et al., 2006). Transgenic crops expressing *Bacillus thuringiensis* (*Bt*)  $\delta$ -endotoxins were a natural choice for controlling insects since *Bt* crystal protein (Cry) and spore formulation products have been successfully used for many years (Schnepf et al., 1998; Ferre and Van Rie, 2002; Ranjekar et al., 2003; Kaur, 2006).

The crystal protoxins require solubilization, followed by proteolytic activation in the insect midgut. The activated toxins bind to specific receptors in the intestine epithelial cells leading to pore formation and cell death. Based on amino acid sequence homologies and phylogenetic

relationships, these crystal endotoxins are classified in four groups providing protection against four insect orders: *cry1* (Lepidoptera), *cry2* (Lepidoptera and Diptera), *cry3* (Coleoptera) and *cry4* (Diptera) (de Maagd et al., 2001; Ferre and Van Rie, 2002; Griffiths and Aroian, 2005). The active toxins have a conserved structure formed by three domains, and domains II and III are involved in the specificity to particular insects through receptor binding (Schenpf et al., 1998; de Maagd et al., 2001; Ranjekar et al., 2003; Abanti, 2004).

Insect resistance to *Bt* toxins in targeted populations arises through different mechanisms and/or at different levels (Ferre and Van Rie, 2002; Griffiths and Araoin, 2005). Laboratory bioassays indicated that cross-resistance occurs in populations of diamondback moth (*Plutella xylostella* L.) which were resistant to four closely-related  $\delta$ -endotoxins with highly homologous binding sites (Tabashnik et al., 1997a, 2001). To date, only one case of field-developed resistance has been reported, but laboratory studies on field-selected strains indicated the potential for resistance development in different insect populations (Ferre and Van Rie, 2002).

To increase *Bt* expression levels in transgenic plants, codon-optimization of *cry* sequences, the reduction of AT sequences and the truncation of the native *cry* sequence have been successfully used (Schenpf et al., 1998; Bohorova et al., 2001; Kaur, 2006). Elimination of vector backbone sequences and biolistic transfer of minimal transgene expression constructs (Fu et al., 2000) also supported high transgene expression levels (Agrawal et al., 2005). Stacking of different *cry* genes (Kaur, 2006), expression of *cry* fusion constructs (Bohorova et al., 2001), and pyramiding genes including *cry* genes with genes encoding proteins having alternative insect control mechanisms like vegetative insecticidal proteins or proteinase inhibitors, will reduce the risk of insects developing resistance to *Bt* toxins (Ferry et al., 2006).

In the U.S., growers must conform to the high-dose refuge strategy to delay insect resistance development. The first component of this strategy is to express toxins in plants at a high enough level to kill heterozygotes in the insect population. The second component is to provide structured refuges. Refuges are small areas cultivated with non-transgenic crops which are interspersed with the transgenic crop. Mating of susceptible adults that developed from non-transformed plants with those from transgenic plants allow for the elimination of homozygous resistant individuals and the reduction of resistant alleles (Cannon, 2000; Ranjekar et al., 2003).

Currently, *Bt* transgenic technology is adopted worldwide and *Bt* crops are grown on more than 14 million hectares (James, 2005). In the U.S., *Bt* crops are grown on approximately 20% of the crop acreage and their use is directly linked to higher yields and profits and reduced pesticide application (Cannon, 2000). Currently, marketed products include *Bt* corn containing the *cry1Ab*, *cry1F*, *cry3Bb1* and stacked *cry1Ab* and *cry3Bb1* genes for controlling European corn borer [*Ostrinia nubilalis* Hübner], southwestern corn borer [*Diatraea grandiosella* Dyar] and corn rootworm [*Diabrotica barberi* Smith and Lawrence], and *Bt* cotton containing *cry1Ac*, stacked *cry1Ac* and *cry2Ab2*, stacked *cry1Ac* and *cry1F* for controlling tobacco budworm [*Heliothis virescens* Fabricius], cotton bollworm [*Helicoverpa zea* Boddie], and pink bollworm [*Pectinophora gossypiella* Saunders] (Castle et al., 2006). *Cry1F* has been reported to control fall armyworm in cotton (Adamczyk and Gore, 2004). However, there are no previous reports on *Cry* proteins expressed in forage and turf grasses and their effects against fall armyworm. Transgenic plants of the non-commercial apomictic genotype “Tifton 7”, diploid bahiagrass cultivar “Pensacola”, and apomictic cultivar “Argentine” have been recently reported (Smith et al., 2002; Gondo et al., 2005; Altpeter and James, 2005). These genetic transformation protocols allow the introduction of exogenous insect resistance genes into bahiagrass. Hence, the objective of this

work was to evaluate the expression of a synthetic *cryIF* gene in transgenic bahiagrass and its effect on resistance to fall armyworm.

## **Materials and Methods**

### **Minimal Transgene Expression Constructs**

Based on the *cryIF* gene sequence available in the NCBI database (M73254), a codon-optimized sequence for the  $\delta$ -endotoxin was generated. The synthetic *cryIF* gene (1863 bp) was synthesized and subcloned into a pPCR-Script vector by Geneart (Regensburg, Germany).

*BamHI* and *HindIII* sites were introduced 5' and 3' of the *cryIF* coding sequence respectively (Figure 1), to facilitate subcloning of *cryIF* under transcriptional control of the maize ubiquitin 1 promoter and first intron (Christensen and Quail, 1992) and the CaMV 35S polyadenylation signal (Dixon et al., 1986) (pHZCRY vector, Figure 2A).

The pHZ35SNPTII selectable marker cassette contains the neomycin phosphotransferase II (*nptII*) coding sequence (Bevan, 1984) under transcriptional control of the CaMV 35S promoter (Odell, 1985) and hsp70 intron (Rochester, 1986), and the CaMV 35S polyadenylation signal (Dixon et al., 1986) (Figure 2A). Following the strategy described by Fu et al. (2000), minimal transgene expression constructs (MCs) containing only the expression cassettes without vector backbone were used for biolistic gene transfer. The *nptII* and *cryIF* gene expression cassettes were excised from their plasmids by restriction digestion with *NotI* resulting in a 2.55 kb or 4.15 kb fragments, respectively (Figure 2A). Transgene expression cassettes were isolated by gel electrophoresis, and the corresponding band was excised and purified using the Wizard SV Gel and PCR cleanup system (Promega, Madison, WI) to remove vector backbone sequences.

### **Tissue Culture, Transformation and Regeneration of Bahiagrass**

Embryogenic callus was induced from mature seeds of bahiagrass cultivars “Tifton 9” and “Argentine” following a protocol previously described by Altpeter and Positano (2005). The

callus induction medium (CIM) consisted of 4.3 g L<sup>-1</sup> MS salts (Murashige and Skoog, 1962), 30 g L<sup>-1</sup> sucrose, 1.1 mg L<sup>-1</sup> 6-Benzylaminopurine (BAP), 3 mg L<sup>-1</sup> 3,6-Dichloro-2-methoxy benzoic acid (dicamba) and 6 g L<sup>-1</sup> Agarose (Sigma, St. Louis, MO), supplemented with filter sterilized MS vitamins (Murashige and Skoog, 1962) which were added after the medium was autoclaved for 20 min. Calli were kept in darkness at a temperature of 28°C and subcultured to fresh CIM biweekly. Embryogenic calli were placed on CIM medium supplemented with 0.4 M sorbitol, for 4-6 h prior to gene transfer. The *nptII* and *cryIF* gene expression cassettes were used in a 1:2 molar ratio and co-precipitated on 1.0 µm diameter gold particles (Altpeter and James, 2005). The BioRad PDS-1000 / He device (BioRad Laboratories Inc., Hercules, CA) was used for biolistic gene transfer at 1100psi and 28mm Hg. Bombarded calli were transferred to fresh CIM following gene transfer, and kept in the dark for 10 days before being transferred to low light conditions (30µEm<sup>-2</sup>s<sup>-1</sup>), with 16h/8h light/dark photoperiod, at 28°C, on selection CIM containing 50 mg/l of paramomycin. After four weeks, calli were subcultured on shoot regeneration medium, similar to CIM but containing 0.1 mg/l BAP and no dicamba, and transferred to high light (150 µEm<sup>-2</sup>s<sup>-1</sup>) intensity with a 16h/8h light/dark photoperiod at 28°C. After two weeks, calli were transferred to hormone-free CIM to induce root formation. After four to six weeks, regenerated plantlets were transplanted into Fafard 2 mix (Fafard Inc., Apopka, FL) and acclimatized in growth chambers at 400 µEm<sup>-2</sup>s<sup>-1</sup> light intensity with a 16h/8h light/dark photoperiod at 28°C / 20°C day / night. Two weeks later plants were transferred to an air-conditioned greenhouse at 30°C / 20°C day / night and natural photoperiod. Plants were fertilized bi-weekly with Miracle Grow Lawn Food (Scotts Miracle-Gro, Marysville, OH) at the recommended rate.

## **Polymerase Chain Reaction, Reverse Transcriptase Polymerase Chain Reaction and Southern Blot Analysis**

Genomic DNA was extracted from bahiagrass transgenic and wild type lines as described by Dellaporta et al. (1983). The forward primer 5'ATGGTTTCAACAGGGCTGAG3' and the reverse primer 5'CCTTCACCAAGGGAATCTGA3' were designed for amplifying a 570 bp fragment internal to the coding sequence of the *cryIF* gene. Approximately 100 ng genomic DNA was used as template for PCR in a BioRad Icycler (BioRad Laboratories Inc., Hercules, CA). PCR was performed using the HotStart PCR kit (Qiagen, Valencia, CA). The cycling conditions were 95°C for 15 min initial denaturation, 35 cycles of 95°C for 1 min, 57°C for 1 min, 72°C for 1 min and 72°C for 15 min final extension. PCR products were analyzed by electrophoresis on a 0.8% agarose gel.

Total RNA was extracted from emerging young leaves using the RNeasy Plant Mini Kit (Qiagen, Valencia, CA), followed by RNase free DNase I (Qiagen, Valencia, CA) treatment to eliminate genomic DNA contamination. Total RNA (500 ng) was used for cDNA synthesis via reverse transcription with the iScript cDNA Synthesis kit (Bio-Rad Laboratories Inc., Hercules, CA) in a reaction volume of 20 µl. cDNA (2 µl) was used as a template to detect the transcripts of the *cryIF* gene by PCR with the same primer pair as described above for PCR from genomic DNA. PCR products were analyzed by electrophoresis on a 0.8% agarose gel.

For Southern blot analysis, genomic DNA from bahiagrass transgenic and wild type lines was isolated using the CTAB method (Murray and Thompson, 1980). Genomic DNA (15 µg) was digested with *BamHI* and fractionated on a 1% agarose gel, transferred onto a nitrocellulose membrane (Hybond, Amersham BioSciences, Piscataway, NJ) and hybridized using the complete *cryIF* coding sequence (1.8kb) as a probe, labeled with P<sup>32</sup> using the Prime-a-Gene kit

(Promega). Hybridization and detection were performed according to the instructions of the manufacturer.

### **Immunological Assays**

Qualitative expression of the *cryIF* endotoxin in leaf tissue of the transgenic lines from cultivars “Tifton 9” and “Argentine” was evaluated using the Quickstix™ kit for *cryIF* (EnviroLogix™, Portland, ME), originally developed for Herculex I corn, and following the recommendations of the manufacturer. Relative levels of expression of the *cryIF* endotoxin in leaf tissue were estimated by using the ELISA QualiPlate™ kit for *cryIF* (EnviroLogix™) originally developed for Herculex I corn. Following eight months of vegetative propagation of the primary transformants, protein extracts were obtained from wild type and three transgenic lines from cv. “Tifton 9” including three different vegetative clones per line and three different replicates per clone. Protein extracts from *cryIF* expressing corn grain were quantified and used as a positive control in a dilution series. Protein concentration of the extracts was determined using the Bradford assay (Bradford, 1976) and absorbance was measured at 595 nm. BSA was used to prepare a standard curve ( $R^2$  value of 96%). Ten micrograms of total protein were loaded per well. The immunoassay was performed according to the instructions of the manufacturer. Reaction kinetics was recorded at 450 nm using an ELISA microplate reader (Bio-Rad Laboratories Inc., Model 680). Optical density (OD) values for each line were compared within the linear range of the reaction kinetics after addition of the ELISA substrate. OD data from bahiagrass transgenic and wild type lines were analyzed by Proc ANOVA and means were separated according to Tukey’s test ( $P < 0.05$ ) (Littell et al., 1996, SAS Institute, 2002).

### **Insect Bioassays**

Insecticidal activity of the transgenic lines was evaluated by following a modified version of the protocol described by Adamczyk and Gore (2004). Fall armyworm neonates (rice host

strain) were obtained from egg masses hatched the same day, placed in Petri dishes and fed on four leaf pieces of 2 cm length of the third fully emerged leaf. A completely randomized experimental design was used. There were 10 replications per transgenic line represented by individual Petri dishes with leaves and larvae, and the experiment was repeated four times. For estimating fall armyworm resistance, neonate mortality rates from bahiagrass transgenic and wild type lines were evaluated after five days of feeding. Fall armyworm mortality rates, expressed as a percentage, were analyzed by Proc Mixed and means were separated according to Fisher's protected LSD (Littell et al., 1996, SAS Institute, 2002).

## **Results**

### **Generation of Transgenic Bahiagrass Lines**

Co-bombardment experiments with the MCs from the pHZCRY vector and the pHZ35SNPTII vector were done (Figure 4-2A). Three hundred calli were obtained from ten shots and three transgenic lines from each cultivar, cvs. "Argentine" and "Tifton 9", were generated in four months of tissue culture (4-2B,C). These six transgenic lines were transferred to small pots under controlled growing conditions and later to larger pots under greenhouse conditions (4- 2D,E).

### **Polymerase Chain Reaction, Reverse Transcriptase Polymerase Chain Reaction and Southern Blot Analysis**

PCR analyses showed that the six transgenic lines from cultivars "Tifton 9" and "Argentine" amplified the 570 bp internal fragment of the synthetic *cryIF* gene confirming the gene integration into the plant genome. Also, this 570bp fragment was amplified in the plasmid control (PC) and not amplified in the wild type bahiagrass (WT)(Figure 4-3A, 4-4A).

RT-PCR analyses also showed that the six bahiagrass lines amplified the same 570 bp internal fragment of the *cryIF* confirming gene expression at the RNA level in the transgenic plants (Figure 4-3B, 4-4B).

Both southern blot analyses, i.e. with *BamHI* and *EcoRI* gDNA digestions, showed an independent integration pattern for each transgenic line and the transgene integration varied from simple to more complex integration patterns in cv. “Tifton 9.” Specifically with *BamHI*, Line 1 showed seven hybridization bands, while Lines 2 and 3 showed two and four hybridization patterns respectively (Figure 4-3C). Instead, the three transgenic lines from cv. “Argentine” showed complex integration patterns with more than two hybridization bands in the three transgenic lines (Figure 4-4C).

### **Immunological Assays**

A qualitative immunoassay, the Quickstix™ kit for *cryIF* (EnviroLogix™), indicated that bahiagrass Lines 2 and 3 from cv. “Tifton 9” contained detectable levels (Figure 4-5A), while the same assay showed no detectable levels of *cryIF* in those lines from cv. “Argentine.” A semiquantitative immunoassay, based on the QualiPlate™ kit for *cryIF* (Envirologix™), showed that Line 1 had a low expression level only detected by the plate reader whereas Lines 2 and 3 had higher expression levels that were easily detected by naked eye in cv. “Tifton 9” (Figure 4-5B). However, low expression levels were detected in the three lines from cv. “Argentine.” These observations were correlated to the OD<sub>450</sub> values obtained for the six transgenic lines. The OD<sub>450</sub> values indicated that Line 1 produced relatively low levels of Cry1F, while Lines 2 and 3 displayed *cryIF* expression levels that were 4 and 12-fold higher than those levels, respectively ( $P < 0.05$ )(Figure 4b). Also, no significant differences in *cryIF* expression between clones of the same line were found ( $P < 0.05$ ; data not shown). Cry1F protein levels in bahiagrass leaves were estimated by comparison with a crude Cry1F protein standard supplied by the manufacturer and

were approximately 1.4 and 4.5  $\mu\text{g protein/ g fresh weight}$  for Lines 2 and 3 from cv. “Tifton 9” respectively.

### **Insect Bioassays**

Fall armyworm mortality rate showed differences between the wild type and the bahiagrass transgenic lines from cultivar “Tifton 9.” Lines 1, 2 and 3 produced 35, 65 and 83% neonate mortality rate respectively (Figure 4-6A). Wild type and Line 1 showed intense feeding while Lines 2 and 3 showed limited feeding indicating early larvae death (Figure 4-6B). Instead, survival rate was no different between the wild type and the three transgenic low expressing lines from cultivar “Argentine” after five days of feeding (Figure 4-6C).

### **Discussion**

This is the first report of stable, transgene expression of a Bt crystal protein gene that enhances insect resistance in a forage and turf grass. Bahiagrass (*Paspalum notatum* Flugge) is an important subtropical forage grass that is also used as low input turf. Constitutive over-expression of the *cryIF* gene in bahiagrass resulted in increased resistance to fall armyworm (*Spodoptera frugiperda* J.E. Smith).

The transformation protocol for the apomictic tetraploid bahiagrass cultivar “Argentine” (Altpeter and James, 2005; Sandhu et al., 2007) was successfully applied to generate transgenic, sexual, diploid bahiagrass plants of cultivars “Tifton 9” and “Argentine.” The transformation efficiency for “Tifton 9” (1%) was similar to those earlier reported for the diploid sexual bahiagrass cultivar “Pensacola” (2.2%) (Gondo et al., 2005). However, 10% transformation efficiency was reported for the apomictic cultivar “Argentine” (Sandhu et al., 2007). Genotypic differences in tissue culture response and transformation efficiency have been reported previously. The production of stable transgenic plants from bahiagrass (*Paspalum notatum* Flugge) cv. “Tifton 7” was firstly reported by Smith et al. (2002). However, authors reported that

the transgenic nature of most of the glufosinate resistant plant could not be confirmed by PCR analyses (Smith et al., 2002). Later, unpublished studies revealed that the glufosinate resistance was conclusive to indicate the transgenic nature and that the PCR analyses had resulted in false negative events (Smith, personal communication). In contrast, following co-bombardment of the target gene and *nptII* gene and selection using paromomycin, all of the regenerated plants in this study and in a previous bahiagrass transformation study (Sandhu et al., 2007) were transgenic as indicated by Southern blot analysis. Genotypic differences and alterations in tissue culture and selection protocols may have contributed to the lack of escapes in these more recent bahiagrass studies.

Analysis of the complexity of minimal transgene expression construct (MC) integration patterns has resulted in controversial results in the past. Fu et al. (2000) described that biolistic transfer of MCs resulted in simpler integration patterns and lower copy numbers than plasmids. In contrast, no differences between the two DNA forms were reported by Breitler et al. (2001) and Romano et al. (2003). Southern blot analysis of the three transgenic bahiagrass lines transformed with MC's of the *cryIF* gene showed multiple transgene copies in all lines with line 1 displaying the most complex transgene integration pattern. This complex transgene integration pattern following biolistic transfer of MC's into bahiagrass is in agreement with findings of Breitler et al. (2001) and Romano et al. (2003). It suggests that the complexity of transgene integration is more likely dependent on factors intrinsic to the plant rather than on the form of DNA as proposed by Agrawal et al. (2005). Nevertheless, elimination of vector backbone integration through MC technology is important for increasing transgene expression stability and to remove prokaryotic antibiotic expression constructs to obtain regulatory approval for commercial release. Clean DNA technology by employing the use of MC's for biolistic

transformation is capable of producing similar or higher transformation and expression efficiencies than whole plasmids (Castle et al., 2006). In the present study, two bahiagrass lines from cultivar “Tifton 9” expressed the *cryIF* transgene in vegetative progeny at a high enough level to control FAW neonate larvae.

Transgenic corn expressing the *cryIAb* gene was first commercially released in 1996. Constitutive *cryIAb* expression of 3.3 or 10.3 µg/g fresh weight of leaves resulted in 50-75% or 98% control of the European corn borer (*O. nubilalis*) in corn field trials (Mendelsohn et al., 2003). This pest is considered the most important corn insect pest in the midwestern and northeastern regions of the U.S. (Wiatrak et al., 2004); while fall armyworm is the most important pest on grasses and other crops in the southeastern U.S. Recently, corn expressing the *cryIF* gene (Herculex I) was commercially released by Pioneer Hi-Bred International and Dow Agrosiences (Events TC1507 and DAS-06275-8). Field trials indicated that these transgenic corn lines effectively controlled multiple insect pests like *O. nubilalis*, *D. grandiosella*, *H. zea*, *S. frugiperda*, *A. ipsilon* and *R. albicosta* (US EPA, 2001). In cotton, fall armyworm bioassays indicated that neonate mortality was significantly higher when larvae were fed on leaves expressing *cryIF* (80%) compared with non-transgenic leaves (48%) or leaves expressing *cryIAc* (45%) (Adamczyk and Gore, 2004). CryIF concentrations were estimated at 1.4 and 4.5 µg/g fresh weight in bahiagrass transgenic lines 2 and 3, respectively. These expression levels were associated with 65% and 83% neonate mortality rates, respectively, while wild type and transgenic bahiagrass with barely detectable *cryIF* expression showed a significantly lower fall armyworm mortality rate. These results indicate the potential of *cryIF* to control fall armyworm in accordance with the results reported for *cryIF* expressing cotton (Adamczyk and Gore, 2004) and corn (US EPA, 2001). In conclusion, stable expression of minimal synthetic *cryIF*

expression constructs in bahiagrass enhanced resistance to the difficult to control, and important insect pest, fall armyworm.



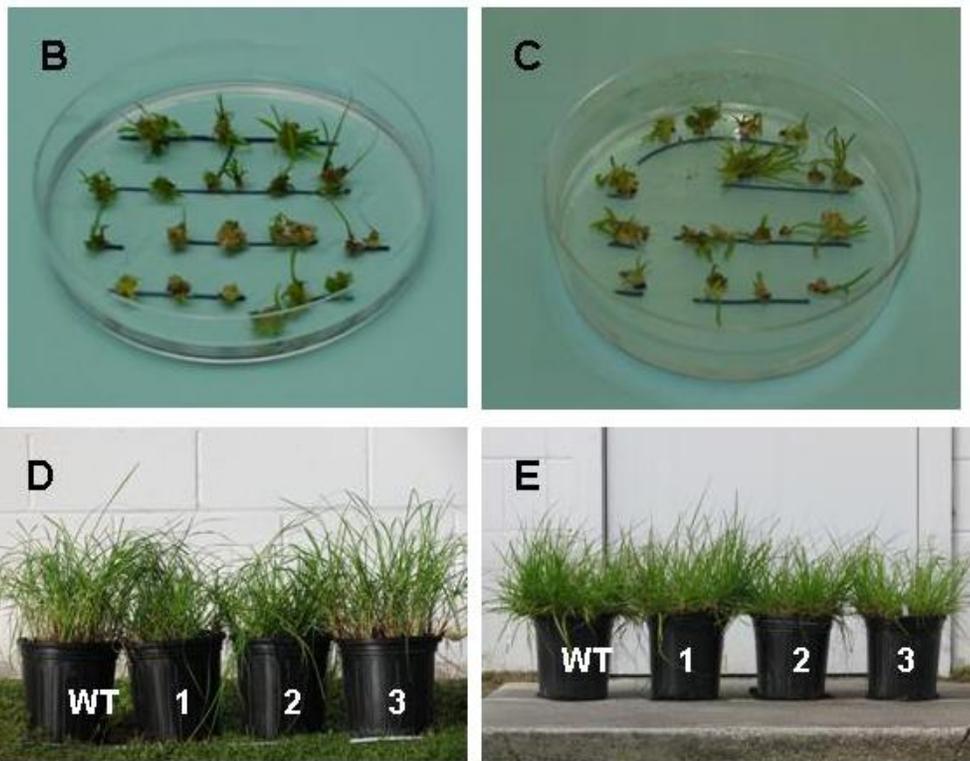
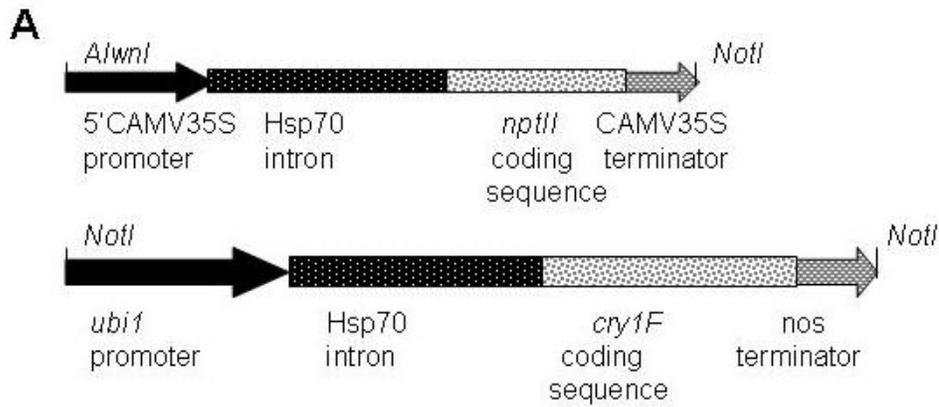


Figure 4-2. A) The expression cassettes for synthetic *cryIF* gene (4155bp) from vector pHZCRY and *nptII* gene (2554bp) from vector pHZ35SNPTII used for biolistics experiments. B) and C) *In vitro* plants under selection with paromomycin from bahiagrass (*Paspalum notatum* Flugge) cvs. “Tifton 9” and “Argentine” respectively. D) and E) Transgenic plants obtained from bahiagrass (*Paspalum notatum* Flugge) cvs. “Tifton 9” and “Argentine” respectively. Lines 1, 2, 3 and wild type (WT).

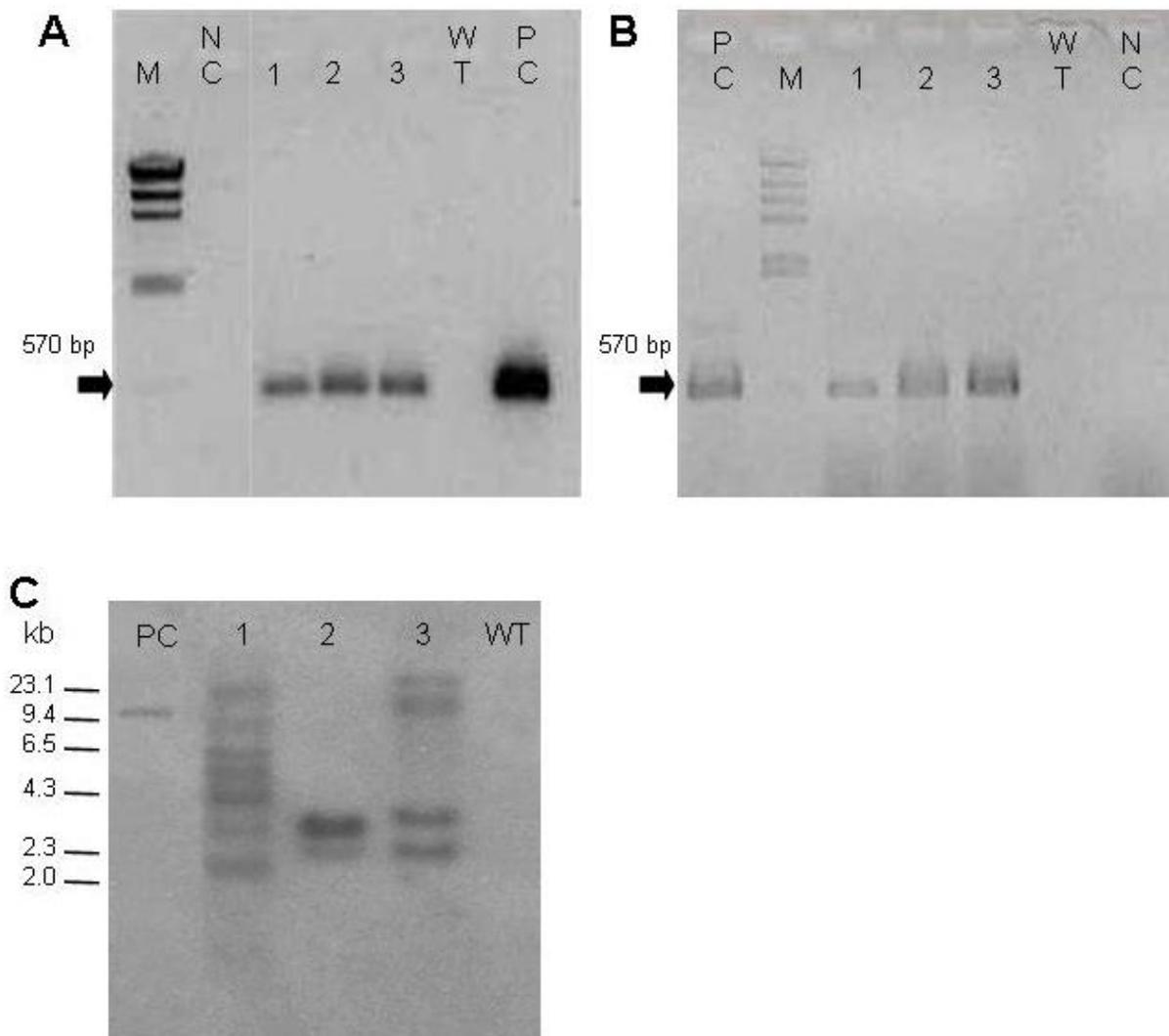


Figure 4-3. A 570 bp fragment was amplified by PCR using the forward primer 5'atggttcaacagggtgag and the reverse primer 5'ccttcaccaagggaatctga. The cycling conditions were 95°C 15 min initial denaturation, 35 cycles of 95°C 1 min, 57°C 1 min, 72°C 1min and 72°C 15 min final extension. A) PCR, B) RT-PCR, C) Southern blot of genomic DNA digested with *Bam*HI of transgenic bahiagrass lines from cv. "Tifton 9" (1, 2, 3). Wild type bahiagrass (Wt), negative control (NC), positive control (PC).

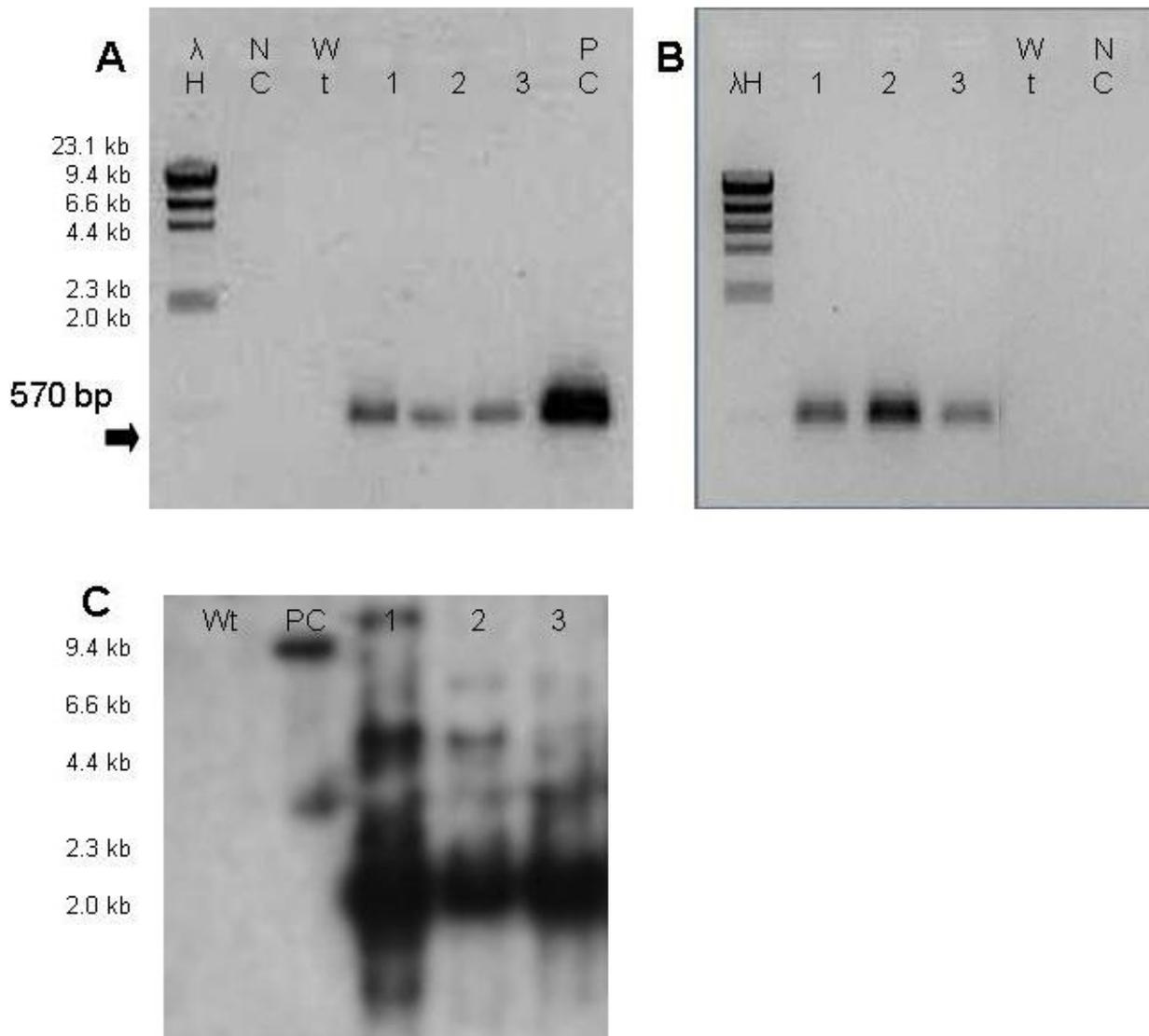


Figure 4-4. A 570 bp fragment was amplified by PCR using the forward primer 5'atggttcaacagggtgag and the reverse primer 5'ccttcaccaagggaatctga. The cycling conditions were 95°C 15 min initial denaturation, 35 cycles of 95°C 1 min, 57°C 1 min, 72°C 1min and 72°C 15 min final extension. A) PCR, B) RT-PCR, C) Southern blot of genomic DNA digested with *Bam*HI of transgenic bahiagrass lines from cv. "Argentine" (1, 2, 3). Wild type bahiagrass (Wt), negative control (NC), positive control (PC).

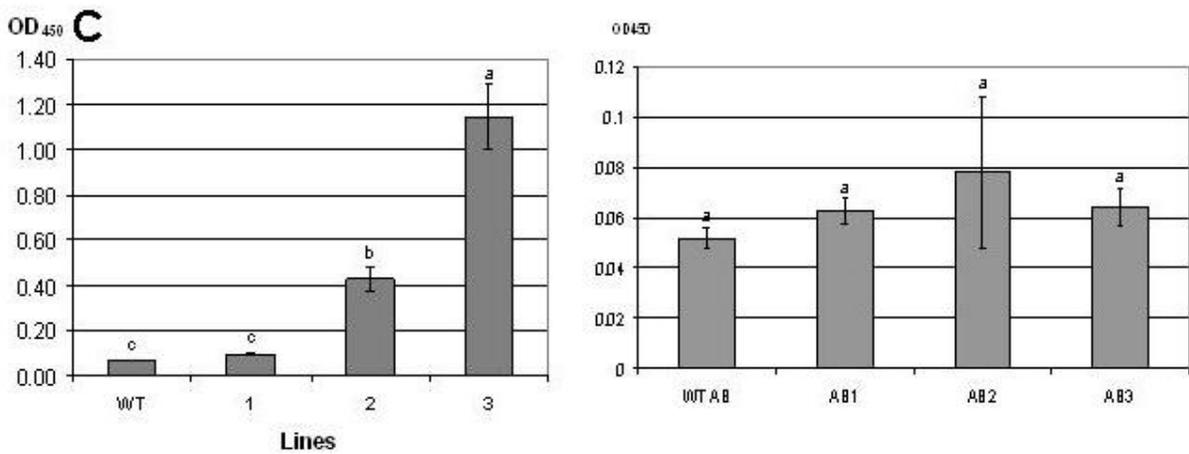
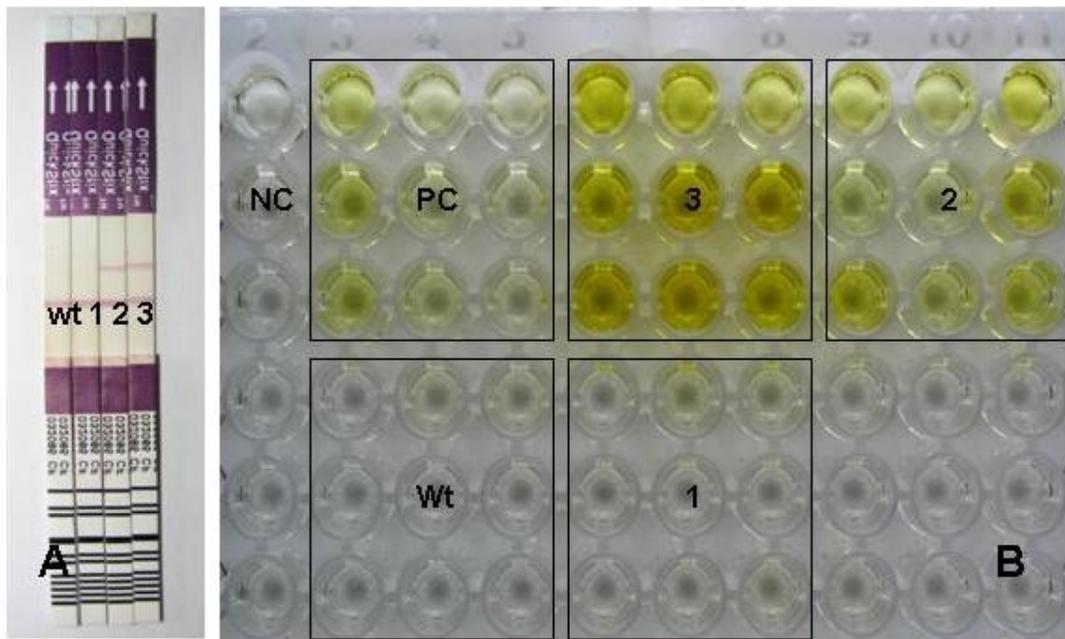


Figure 4-5. Levels of expression in leaves of transgenic lines from bahiagrass (*Paspalum notatum* var. Flugge). A) Lines from cv. "Tifton 9" tested with Quickstix™ kit for the *cryIF* gene (Enviroligix™). B) Lines from cv. "Tifton 9" tested with the QualiPlate™ kit for the *cryIF* gene (Enviroligix™). C) Levels of expression represented by OD<sub>450</sub> values in lines from cv. "Tifton 9". D) Levels of expression represented by OD<sub>450</sub> in lines from cv. "Argentine" values. Wild type bahiagrass (Wt), transgenic lines (1,2,3), negative control (NC), positive control (PC).

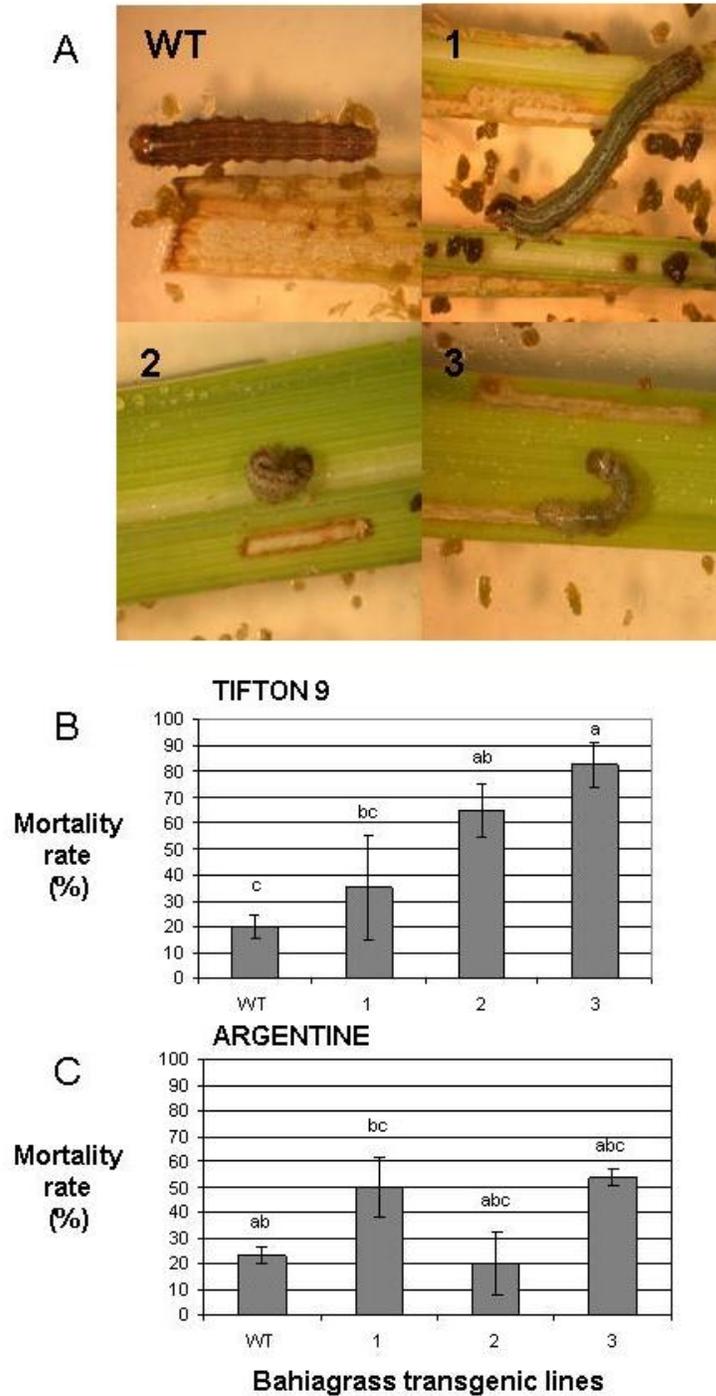


Figure 4-6. Insect bioassays with fall armyworm larvae feeding on leaves from bahiagrass (*Paspalum notatum* var. Flugge) cv. “Tifton 9” after 5 feeding days. A) Feeding pattern from wild type and three transgenic lines. B) Mortality rate (%) for neonates in wild type and three transgenic lines from cv. “Tifton 9” (WT and Lines 1,2,3). C) Mortality rate (%) for neonates in wild type and three transgenic lines from cv. “Argentine” (WT and Lines 1,2,3).

## CHAPTER 5 SUMMARY AND CONCLUSIONS

### **Overexpression of the *vspB* Gene to Improve Nutritional Quality in Bahiagrass**

Amongst vegetative storage proteins, soybean VSP $\beta$  is a lysine enriched-protein resistant to rumen proteolysis (Mason et al., 1988; Staswick, 1994; Galili et al., 2002). The objective of this work was to overexpress this soybean vegetative storage protein gene in bahiagrass and to evaluate the effects of this gene on the nutritional quality of bahiagrass.

To enhance the expression of the *vspB* gene in transgenic plants, we designed an expression vector including a constitutive and strongly expressed promoter for monocots; a KDEL signal for ER-retention and a c-myc tag for antibody detection. To enhance transformation efficiency and transgene expression and stability (Fu et al, 2000), we used minimal constructs containing only the transgene expression cassettes without vector backbone sequences during the biolistic experiments. We co-bombarded mature seed-derived calli from bahiagrass cv. Argentine with minimal constructs containing the *vspB* gene from pGL4 and pRSVP1 vectors and the *nptII* gene as selectable marker from pJFNPTII vector.

Based on bahiagrass transformation, selection and regeneration protocols previously established (Altpeter and Positano, 2005; Altpeter and James, 2005), we generated 172 paromomycin-resistant bahiagrass plants. An ELISA screening confirmed the expression of the *nptII* gene detecting 83% of these plants. Western blots confirmed the expression of the *vspB* gene in 89% of the plants expressing the *nptII* gene. Also, Western blot analyses indicated that VSP $\beta$  expression levels varied among different plants, different tillers within plants and different leaves within tillers having the highest expression levels in third and four bahiagrass leaves. According to previous reports, VSPs represented between 10-15% total protein in young leaves of soybean plants and VSPs were degraded during plant growth (Staswisck et al., 2001).

Transgenic studies in tobacco indicated that VSP $\alpha$  was stably expressed reaching 2-6% total proteins (Guenoune et al., 1999) while VSP $\beta$  reached a maximum of 2% total protein in young leaves and it was degraded with leaf age (Guenoune et al., 2002b). These expression levels were enhanced by targeting the heterologous protein to the vacuole and the chloroplast simultaneously (Guenoune et al., 2002a) or enhanced by co-expressing both soybean VSPs or VSP $\beta$  and dihydrodipicolinate synthase reaching ca. 4% total protein in leaves (Guenoune et al., 2003). In contrast, corn transgenic studies showed that VSP $\beta$  was accumulated to 0.03-0.004% total protein in leaves (Grando et al., 2001). Similarly, VSP $\beta$  expression levels accumulated to 0.001-0.01% of the total proteins in bahiagrass leaves indicating that high VSP $\beta$  expression levels are difficult to reach in monocot plants such as cereals and grasses. Hence, this is the first report on expression of the *vspB* gene in bahiagrass cv. "Argentine." The VSP $\beta$  expression levels observed in transgenic bahiagrass plants were low and similar to those previously reported for corn plants. These VSP $\beta$  expression levels in transgenic bahiagrass plants could be enhanced by coexpressing recombinant VSP $\beta$  and VSP $\alpha$  subunits, and/or by targeting the subunit simultaneously to different cell compartments. However, these improvements may not enhance the total protein content of transgenic bahiagrass significantly.

### **Expression of the *cryIF* Gene to Enhance Pest Resistance in Bahiagrass**

*Bt* (*Bacillus thuringiensis*) transgenic crops expressing  $\delta$ -endotoxins were developed to control different insect and nematodes (Schnepf et al., 1998; Ranjekar et al., 2003; Kaur, 2006). Currently, *Bt* crops cover 14 million hectares and represent 20% total for crops like cotton and corn (Cannon, 2000). Marketed products include *Bt* corn containing the *cryIAb*, *cryIAc*, *cry9C* genes for controlling European corn borer populations and *Bt* cotton containing *cryIAc*, *cryIAc* and *cryIF* for controlling bollworm (Mendelsohn et al., 2003). However, there are no previous reports on *cryIF* proteins expressed in forage and turf grasses and their effects against fall

armyworm. Hence, the objectives of this work were to generate transgenic lines expressing a synthetic *cryIF* gene and to evaluate the effects of this gene on resistance against fall armyworm in subtropical bahiagrass cultivars “Tifton 9” and “Argentine.”

For enhancing transformation efficiency and transgene expression stability (Fu et al, 2000), we co-bombarded mature seed-derived calli from bahiagrass with minimal constructs containing the synthetic *cryIF* gene from pHZCRY vector and the *nptII* gene as selectable marker from pHZ35SNPTII vector. Based on previously reported protocols (Altpeter and Positano, 2005; Altpeter and James, 2005) for bahiagrass transformation and regeneration, we generated six paromomycin-resistant bahiagrass plants (three from cv. “Argentine” and three from cv. “Tifton 9”). PCR analyses confirmed the presence of the *cryIF* gene and Southern blots analyses indicated independent transgene integration in all transgenic bahiagrass lines. Transgene integration varied from simple to more complex integration patterns in cv. “Tifton 9” while showed complex integration patterns in cv. “Argentine.” RT-PCR analyses confirmed *cryIF* expression in all transgenic bahiagrass lines. A qualitative immunoassay for *cryIF* gene expression (Quickstix<sup>TM</sup> kit, EnviroLogix<sup>TM</sup>) indicated detectable levels in two bahiagrass lines from cv. “Tifton 9” and no detectable levels in one line from cv. “Tifton 9” and three lines from cv. “Argentine.” A semiquantitative immunoassay for *cryIF* gene expression (QualiPlate<sup>TM</sup> kit, Envirologix<sup>TM</sup>) showed that Line 1 had a low expression level only detected by the plate reader while Lines 2 and 3 had higher expression levels that were easily detected by naked eye in cv. “Tifton 9.” Low expression levels were detected in the three lines from cv. “Argentine.” According to fresh weight and protein concentration of the samples, the estimated *cryIF* expression levels were 1.4 and 4.5 µg cry toxin/ g fresh weight in Lines 2 and 3 from cv. “Tifton 9.” Insect bioassays indicated significant differences between wildtype and transgenic lines in cv.

“Tifton 9” showing 35, 65 and 83% mortality rates in Lines 1, 2 and 3. Instead, no differences were observed between wild type and transgenic bahiagrass lines from cultivar “Argentine.” The *cryIF* expression levels in bahiagrass lines from cv. “Tifton 9” were similar to those *cryIAb* levels reported for commercial corn lines (Abel and Adamczyk, 2004). Besides, the protection levels against FAW neonates were similar to those observed in *cryIF* cotton lines (Adamczyk and Gore, 2004). Hence, we cloned a codon-optimized version of the *cryIF* gene under the control of the *ubi1* promoter and first intron in two bahiagrass cultivars. In cultivar “Tifton 9”, results indicated that the expression levels of the *cryIF* gene detected by immunological assays and the protection levels detected by insect feeding bioassays were positively correlated. Moreover, the *cryIF* expression and protection levels were similar to those levels detected for commercial crops in corn and cotton. Therefore, the expression of the *cryIF* gene in bahiagrass cultivar “Tifton 9” enhanced the resistance against fall armyworm in insect bioassays indicating that these transgenic lines seem to be suitable candidates for further field studies.

APPENDIX A  
LABORATORY PROTOCOLS

**Protocols for Molecular Cloning**

**Preparation of Electrocompetent *E.coli***

1. Grow o/n DH5 $\alpha$  culture in 5ml LB.
2. Inoculate 2 x 250ml LB in 1L flasks with 2ml o/n culture. Incubate shaking at 37°C until OD<sub>600</sub> = 0.6-0.8 (Approx 5.5 hours).
3. Pellet cells for 15 mins, 4000g at 4°C (Sorvall with GSA rotor, 3x 250ml bottles).
4. Pour off supernatant and resuspend in an equivalent volume of ice-cold sterile water (3x 165ml). Handle cells gently at all times.
5. Centrifuge 15 mins, 4000g, 4°C.
6. Pour off supernatant and resuspend in 0.5 volume of ice-cold sterile water (3x 83ml). Divide cell culture between 2 bottles.
7. Centrifuge 15 mins, 4000g, 4°C.
8. Pour off supernatant and resuspend in 0.5 volume of ice-cold sterile water (2x 125ml).
9. Centrifuge 15 mins, 4000g, 4°C.
10. Pour off supernatant and resuspend in 0.02 volume of ice-cold sterile water (2x 5ml). Transfer cell culture to 30ml centrifuge tube.
11. Centrifuge 15 mins, 4000g, 4°C (Sorvall, SS-34 rotor).
12. Pour off supernatant and resuspend in 0.02 volume ice-cold sterile 10% glycerol (1.2ml).
13. Transfer 40 $\mu$ l aliquots to sterile 1.5ml eppendorf tubes with holes pierced in the lids using a sterile needle. Quick-freeze in liquid nitrogen and store at -70°C.

**Electroporation of *E.coli***

1. Chill 0.1cm gap cuvettes (BioRad) on ice.
2. Place DNA for transformation (usually 1-2 $\mu$ l – 10-15ng ligation) in 1.5ml eppendorf tubes on ice.
3. Thaw required number of 40 $\mu$ l aliquots of electrocompetent cells on ice.
4. Set BioRad micropulser to Ec1 (1.8kV, 5msec, 0.1cm gap).
5. Add cells to DNA. Leave on ice 1 min.
6. Transfer cells to cuvette. Tap suspension to bottom.
7. Place cuvette in slide and push slide into chamber ensuring that cuvette is seated between contacts in the base of the chamber.
8. Pulse once by pressing pulse button. Alarm signifies pulse is complete.
9. Remove cuvette and immediately add 360 $\mu$ l SOC medium to the cuvette. Quickly but gently transfer cells to a 2ml tube. Time is critical.
10. Check and record pulse parameters (time constant should be close to 5msec).
11. Incubate cells at 37°C shaking for 1 hour before plating on appropriate antibiotic medium (50 $\mu$ l for circular plasmid, 100-200 $\mu$ l for ligation product).

**Plasmid DNA Extraction Method (modified Dellaporta et al., 1983)**

1. Harvest one length eppendorf tube of leaf material.
2. Add 300  $\mu$ l Extraction Buffer D and grind with micropestle until the buffer is green.
3. Add 20  $\mu$ l 20% SDS. Vortex.
4. Incubate at 65C, 10 minutes.
5. Add 100  $\mu$ l 5 M potassium acetate. Shake vigorously.
6. Incubate on ice (or -20C), 20 minutes.
7. Centrifuge at maximum speed, 20 minutes and transfer supernatant to new tube.
8. Add 450  $\mu$ l isopropanol.
9. Incubate on ice (or -20C), 30 minutes.
10. Centrifuge at maximum speed, 15 minutes, pour off the supernatant, air-dry the pellet.
11. Resuspend pellet in 100  $\mu$ l TE.
12. Add 10  $\mu$ l 3 M sodium acetate and 220  $\mu$ l 100% ethanol.
13. Centrifuge at maximum speed, 10 minutes, pour off the supernatant, add 500  $\mu$ l 70% ethanol.
14. Centrifuge at maximum speed, 10 minutes, pour off the supernatant, air-dry pellet for 10 minutes.
15. Resuspend pellet in 150  $\mu$ l TE buffer.
16. Use 1  $\mu$ l for PCR reaction.

## Polymerase Chain Reactions

### To amplify fragment from pCMYCKDEL vector

PCR kit	PCR Core System II(Promega)
Primer sequences	Forward 5' <i>CGTCTAGAACGCGT</i> ATGAAGTTGTTTGTTTTCTTTGTTGC
	Reverse 5' <i>CGGCGGCCGCACTAGTCTCAATGTAGTACAT</i> GGGATTAGGAAG
PCR program	Gaby1 2' 95C for initial denaturation, 25 cycles with 1' 95C for denaturation, 1' 48C for annealing and 1' 72C for extension, 5' 72 for final extension and hold at 4C
PCR reaction	
DNA template	0.5-1.5 $\mu$ l
Forward primer	1 $\mu$ l
Reverse primer	1 $\mu$ l
PCR Buffer 10 X	2.5 $\mu$ l
DNTPs	0.5 $\mu$ l
Taq polymerase	0.125 $\mu$ l
Dd water	19-18.375 $\mu$ l
Final volume	25 $\mu$ l

### To amplify fragment from pGL2 vector

PCR kit	PCR Core System II(Promega)
Primer sequences	Forward 5' CGGGTACCATGAAGTTGTTTGTTCCTTTGTTGC Reverse 5' CGGAGCTCGTTTAAACGCATGCCTGCAGGTCACTG
PCR program	Gaby4 2' 95C for initial denaturation, 25 cycles with 1' 95C for denaturation, 1' 48C for annealing and 1' 72C for extension, 5' 72 for final extension and hold at 4C
PCR reaction	
DNA template	2 µl
Forward primer	1 µl
Reverse primer	1 µl
PCR Buffer 10 X	2.5 µl
DNTPs	0.5 µl
Taq polymerase	0.125 µl
Dd water	17.875 µl
Final volume	25 µl

### Restriction Digestions

#### To digest pCMYCKDEL vector

Restriction Enzymes	<i>NotI</i> and <i>XbaI</i> (Promega)
Digestion reaction	
pCMYCKDEL Vector (3 ug)	12 µl
Restriction Buffer D 10X	3.5 µl
BSA 10X	3.5 µl
<i>XbaI</i>	3.5 µl
<i>NotI</i>	3.5 µl
Dd water	9 µl
Final volume	35 µl

### To digest pGFL1 vector

Restriction Enzymes	<i>NotI</i> and <i>XbaI</i> (Promega)
Digestion reaction	
pGFL1 Vector (4 ug)	12 $\mu$ l
Restriction Buffer D 10X	4 $\mu$ l
BSA 10X	4 $\mu$ l
<i>XbaI</i>	4 $\mu$ l
<i>NotI</i>	4 $\mu$ l
Dd water	7 $\mu$ l
Final volume	40 $\mu$ l

### To digest pJFNPTII vector

Restriction Enzymes	<i>PstI</i> (Promega)
Digestion reaction	
pJFNPTII Vector (5 ug)	20 $\mu$ l
Restriction Buffer H 10X	4 $\mu$ l
BSA 10X	4 $\mu$ l
<i>PstI</i>	4 $\mu$ l
Dd water	7 $\mu$ l
Final volume	40 $\mu$ l

### To digest pCAMBIA2300 vector

Restriction Enzymes	<i>PstI</i> (Promega)
Digestion reaction	
pCAMBIA2300 Vector (8.5 ug)	3 $\mu$ l
Restriction Buffer H 10X	1.5 $\mu$ l
BSA 10X	1.5 $\mu$ l
<i>PstI</i>	7 $\mu$ l
Dd water	2 $\mu$ l
Final volume	15 $\mu$ l

### To digest pGFL2 vector

Restriction Enzymes	<i>KpnI</i> and <i>SacI</i> (Promega)
Digestion reaction	
pGFL2 Vector (5.5 ug)	14 $\mu$ l
Multicore Buffer 10X	3 $\mu$ l
BSA 10X	3 $\mu$ l
<i>KpnI</i>	4.3 $\mu$ l
<i>SacI</i>	2.2 $\mu$ l
Dd water	3.5 $\mu$ l
Final volume	30 $\mu$ l

### To digest PCR fragment from pGL2 vector

Restriction Enzymes	<i>KpnI</i> and <i>SacI</i> (Promega)
Digestion reaction	
PCR fragment	28 $\mu$ l
Multicore Buffer 10X	4 $\mu$ l
BSA 10X	4 $\mu$ l
<i>KpnI</i>	3 $\mu$ l
<i>SacI</i>	1 $\mu$ l
Dd water	0 $\mu$ l
Final volume	40 $\mu$ l

### Ligation Reactions

#### To ligate PCR fragment from pKSH3 vector

Ligation kit	LigaFast™ Rapid DNA Ligation System (Promega)
Ligation reaction	
pGEM-T Easy Vector (50 ng)	1 $\mu$ l
PCR product (14 ng)	0.65 $\mu$ l
Ligation Buffer 2 X	5 $\mu$ l
T4 ligase	1 $\mu$ l
Dd water	2.35 $\mu$ l
Final volume	10 $\mu$ l

**To ligate PCR fragment from pCMYCKDEL vector**

Ligation kit	LigaFast™ Rapid DNA Ligation System (Promega)
Ligation reaction	
pCMYCKDEL Vector (100 ng)	1 µl
PCR product (25 ng)	0.25 µl
Ligation Buffer 2 X	5 µl
T4 ligase	1 µl
Dd water	2.75 µl
Final volume	10 µl

**To ligate *Pst*I fragments from pJFNPTII and pCAMBIA2300 vectors**

Ligation kit	LigaFast™ Rapid DNA Ligation System (Promega)
Ligation reaction	
pJFNPTII insert	0.8 µl
pCAMBIA2300 backbone	2 µl
Ligation Buffer 2 X	5 µl
T4 ligase	1 µl
Dd water	1.2 µl
Final volume	10 µl

## **Protocols for Bahiagrass Transformation and Regeneration**

### **Protocol for Bahiagrass Seed Sterilization (modified from Smith et al., 2002)**

1. Sort out good seeds. Remove any broken or black seeds (check both sides of seed). Put into tube to fill 1/3 full (approx 500 seeds).
2. Prepare 3-4 1L beakers of DI H<sub>2</sub>O plus 1 empty beaker and a 2-ply square of cheesecloth for each tube.
3. In fume hood, add concentrated sulphuric acid to ½ full and shake well to mix.
4. Leave for 16 minutes (may vary between 15 – 20 mins depending on seed).
5. Using half a large beaker of DI H<sub>2</sub>O, wash seeds into a second large beaker of DI H<sub>2</sub>O. Stir seeds with glass rod to wash.
6. Pour off the liquid and floating debris.
7. Add half a beaker of DI H<sub>2</sub>O to the seeds and stir.
8. Pour off floating debris.
9. Strain seeds through cheesecloth using a little more water.
10. Squeeze seeds in cloth and wash into a clean beaker with the next half beaker of DI H<sub>2</sub>O.
11. Repeat 2x leaving seeds in cheesecloth after 3<sup>rd</sup> wash.
12. Allow seeds to dry partially on the cheesecloth (15-20 mins).
13. Transfer seeds to a deep petri dish.
14. Fill a small beaker with 20ml Clorox bleach (5.25% sodium hypochlorite) and 10ml glacial acetic acid. Place in bottom of glass desiccator in fume hood.
15. Place open petri dish of seeds and lid into desiccator. Leave 1 hour.
16. Add small amount of sterile dH<sub>2</sub>O, seal plate and leave to soak for 1 hour.
17. Transfer to IF medium, 20 seeds per plate.
18. Incubate at 28°C in the dark.

### **Protocol for Bahiagrass Particle Bombardment (Altpeter and James, 2005)**

1. Gold stocks (60mg/ml): Weigh 12mg gold into an eppendorf tube. Wash several times in Absolute EtOH by vortexing and centrifuging briefly. Resuspend in 200µl ddH<sub>2</sub>O.
2. Gold preparation: Mix 15µl 0.75µm gold, 15µl 1µm gold and 30µl DNA by vortexing 1 min. Add 20µl 0.1M spermidine and 50µl 2.5M CaCl<sub>2</sub> while vortexing. Keep vortexing for 1 min. Centrifuge briefly to settle gold. Wash in 250µl Absolute EtOH by vortexing. Spin and remove supernatant. Resuspend gold in 180µl Absolute EtOH. (or resuspend in 90µl for more gold per shot). Use 5µl per shot to deliver 50µg gold. Enough for 25 shots.
3. Sterilization of gun components: Autoclave 5 macrocarrier holders, stopping screens and macrocarriers. Lay out in laminar flow hood to dry. Sterilize 1100psi rupture discs by dipping in Absolute EtOH and allowing to dry in flow hood. Place all sterile components in sealed petri dishes. Clean gun chamber, assembly and flow hood thoroughly with EtOH and allow to dry half and hour before use.
4. Bombardment: Turn on gun, vacuum pump and helium. Place macrocarriers into holders. Spread 5µl gold prep evenly onto macrocarriers and allow to dry briefly. Place rupture disc into holder and screw tightly into place. Place stopping screen into shelf assembly and put inverted macrocarrier assembly on top. Place shelf at highest level. Place tissue culture plate

on shelf 2 levels below gold. Close door and switch on vacuum to reach 27in Hg. Press fire button and check that disc ruptures at 1100psi. Vent vacuum and remove sample. Dismantle assembly and set up for next shot. Use stopping screen 4-5 times.

### **Protocol for Bahiagrass Regeneration (Altpeter and Positano, 2005)**

1. Culture of mature seeds in Callus Induction Medium (CIM, IF medium with 400µl MS vitamins, 600µl dicamba, 440µl BAP), in darkness at 28C for two weeks.
2. Culture of seedlings in CIM under the same conditions for two weeks.
3. Culture of callus CIM under the same conditions for two weeks.
4. Transfer of callus to fresh CIM one week before bombardment.
5. Prebombardment treatment in CIM supplemented with 0.4 M sorbitol for 4-6 h.
6. Bombardment.
7. Culture of callus in no selection medium for one week.
8. Culture of callus in Selection Medium (SM, IF medium supplemented with with 400µl MS vitamins, 600µl dicamba, 440µl BAP, 50 mgL<sup>-1</sup> paromomycin) in darkness at 28C for two weeks.
9. Culture of callus in SM under 30 µEm<sup>-2</sup>s<sup>-1</sup> light at 28C for two weeks.
10. Culture of callus with shoots in Regeneration medium (ReM, IF medium supplemented with 400µl MS vitamins, 40µl BAP, 50 mgL<sup>-1</sup> paromomycin) under 150 µEm<sup>-2</sup>s<sup>-1</sup> light at 28C for two weeks.
11. Culture of callus with shoots in ReM under 150 µEm<sup>-2</sup>s<sup>-1</sup> light at 28C for two weeks.
12. Culture of callus with shoots in Rooting Medium (RoM, IF medium supplemented with 400µl MS vitamins, 50 mgL<sup>-1</sup> paromomycin) under 150 µEm<sup>-2</sup>s<sup>-1</sup> light at 28C for two weeks.
13. Culture of callus with shoots in RoM under 150 µEm<sup>-2</sup>s<sup>-1</sup> light at 28C for two weeks.
14. Transfer of in vitro plants to small pots with Farfard#2 potting mix into the growth chamber.

### **Protocols for Molecular Techniques**

#### **Plasmid DNA Extraction Method (QIAprep Miniprep kit, Qiagen)**

1. Grow over-night cultures each from a single colony of bacteria in 5 ml LB broth containing 50 µg/ml kanamycin at 37°C with constant shaking.
2. Add the provided RNase A solution to Buffer P1, mix well by inverting and store at 4°C.
3. Add 100% ethanol (volume provided on the bottle label) to the Buffer PE concentrate to prepare the working solution.
4. Centrifuge the cultures at 13,200 rpm for 1 min to pellet the bacterial cells. Remove the supernatant by pipetting and autoclave before disposing.
5. Resuspend the bacterial cells in 250 µl Buffer P1 by vortexing and transfer the suspension to a sterile 1.5 ml microcentrifuge tube.
6. Add 250 µl Buffer P2 and mix well by inverting the tube gently 4-6 times.
7. Add 350 µl Buffer P3 and mix immediately but gently by inverting the tube 4-6 times.
8. Centrifuge the samples at 13,000 rpm for 10 min. A compact white pellet will form.

9. Pipet the supernatant onto QIAprep Spin Columns and centrifuge for 1 min at 13,000 rpm. Discard the flow-through.
10. Pipet 750  $\mu$ l Buffer PE onto the columns and centrifuge for 1 min at 13,000 rpm to wash the columns.
11. Discard the flow-through and centrifuge for 1 min to remove all traces of the wash buffer (Buffer PE).
12. Place the QIAprep columns in clean 1.5 ml microcentrifuge tubes. Add 50  $\mu$ l Buffer EB to the center of each QIAprep column to elute DNA. Let stand for 1 min and centrifuge at 13,000 rpm for 1 min.
13. Estimate the DNA concentration using a spectrophotometer or by running on a gel.
14. Store the samples at -20°C.

### **Plasmid DNA Extraction Method (Plasmid Midi Kit, Qiagen)**

1. Inoculate a starter culture from either a single colony or a glycerol stock of the bacteria in 5 ml LB broth containing 50  $\mu$ g/ml kanamycin. Grow the culture at 37°C for 8 hours with constant vigorous shaking.
2. Prepare Buffer P1 by adding the provided RNase A solution, mix well by inverting and store at 4°C.
3. Prepare 250 ml flasks containing 25 ml LB broth, one per midi prep. Sterilize the broth by autoclaving the flasks for 20 min. Cool the broth before use.
4. Prepare 50 ml corning tubes (3 per sample) by autoclaving for 20 min followed by drying at 60°C.
5. After cooling add kanamycin to each flask to get a final concentration of 50  $\mu$ g/ml kanamycin (25  $\mu$ l) in a clean bench. Add 250  $\mu$ l of the actively growing starter culture to each flask (1:1000 dilution) and incubate for 16 hr at 37°C with constant vigorous shaking.
6. Transfer each culture to a sterile 50 ml corning tube and centrifuge at 6000 x g for 15 min at 4°C.
7. Place Buffer P3 in ice.
8. Resuspend the bacterial pellet in 4 ml Buffer P1 by vortexing the samples until no clumps are visible.
9. Add 4 ml Buffer P2 and mix well by gently inverting 4-6 times. Incubate the samples at room temperature for 5 min.
10. Add 4 ml of chilled Buffer P3 and mix immediately but gently by inverting the tube 4-6 times. Incubate the samples in ice for 20 min.
11. Centrifuge at 20,000 x g for 30 min at 4°C. Immediately transfer the supernatant to a new tube by pipetting.
12. Centrifuge the supernatant again at 20,000 x g for 15 min at 4°C. Transfer the supernatant to a new tube by pipetting immediately.
13. Apply 4 ml Buffer QBT to a QIAGEN-tip 100 for equilibration and allow the column to empty by gravity flow.
14. Apply the supernatant from Step 12 immediately to the equilibrated tip and allow it to enter the resin by gravity flow.
15. After all the liquid has entered the column wash the QIAGEN-tip 2x with 10 ml Buffer QC.

16. Add 5 ml Buffer QF to the column to elute the DNA. Collect the eluate in a sterile 50 ml tube.
17. Add 3.5 ml (0.7 volumes) room-temperature isopropanol to the eluted DNA to precipitate it. Mix well and centrifuge immediately at 15,000 x g for 30 min at 4°C. Carefully decant the supernatant taking care not to disturb the pellet.
18. Wash the DNA pellet with 2 ml room-temperature ethanol and centrifuge at 15,000 x g for 10 min. Remove the supernatant by pipetting.
19. Air-dry the pellet by inverting the tube on absorbent paper for 5-10 min.
20. Add 200 µl TE to the tube and rinse the walls of the tube thoroughly to resuspend the DNA.
21. Leave resuspending over-night at 4°C. Transfer to a sterile microcentrifuge tube, estimate concentration and store at -20°C.

### **Plasmid DNA Gel-Extraction Method (QIAquick Gel Extraction kit, Qiagen)**

1. Prepare Buffer PE by adding 40 ml 100% ethanol to the provided concentrate.
2. Excise the DNA fragment precisely from the agarose gel using a clean sharp scalpel. Avoid excess agarose.
3. Weigh the gel slice in a colorless sterile microcentrifuge tube. Restrict the volume of gel in each tube to 400 mg or less.
4. Add 3 volumes of Buffer QG to 1 volume of gel.
5. Incubate at 50°C in a heating block for 10 min or until the gel has completely dissolve. Vortex every 2 min during incubation to ensure complete dissolution of gel.
6. Following incubation check that the color of the mixture remains similar to Buffer QG (yellow), indicating that the pH has not changed.
7. Add 1 gel volume of isopropanol to the mixture and mix by inverting the tube several times.
8. Place a QIAquick spin column in a provided 2 ml collection tube and apply the sample to the column. Centrifuge at 13,200 rpm for 1 min. Repeat this step if the volume of the mixture is more than 800 µl, the maximum capacity of the QIAquick column.
9. Discard the flow-through and place the column in the same collection tube.
10. Wash the QIAquick column by adding 750 µl Buffer PE to the column and centrifuging for 1 min at 13,000 rpm.
11. Discard the flow-through, place the column back in the same collection tube and spin for an additional 1 min at 13,000 rpm to remove residual ethanol from Buffer PE.
12. Place the QIAquick column in a clean, sterile 1.5 ml microcentrifuge tube.
13. Add 30 µl Buffer EB (10 mM Tris-Cl, pH 8.5) to the center of the QIAquick membrane to elute DNA, let stand for 1 min and then centrifuge for 1 min at 13,000 rpm.
14. Store DNA at -20°C.

### **Genomic RNA Extraction Method ( RNeasy Plant Mini Kit,Qiagen)**

1. Prepare enough Buffer RLT for use by adding 10 µl β-Mercaptoethanol (Sigma) per 1 ml of buffer.
2. Add 44 ml of 100% ethanol to the RPE buffer concentrate to prepare the working solution.
3. Harvest 100 mg young leaves and freeze immediately using liquid nitrogen.
4. Grind the sample to a fine powder using a sterile (Autoclaved for 30 min) mortar and pestle. Transfer the ground sample to a sterile, liquid-nitrogen cooled 2 ml microcentrifuge tube.

5. Add 450  $\mu$ l prepared Buffer RLT to the powder and vortex vigorously.
6. Pipet the lysate onto a QIAshredder spin column placed in a 2 ml collection tube and centrifuge for 2 min at maximum speed (13,200 rpm). Transfer the supernatant of the flow-through to a new sterile microcentrifuge tube taking care not to disturb the pellet of cell-debris. Estimate the approximate volume of the supernatant.
7. Add 0.5 volume (225  $\mu$ l) ethanol (200 proof) to the collected supernatant and mix immediately by pipetting.
8. Immediately transfer sample, including any precipitate formed, to an RNeasy mini column placed in a 2 ml collection tube. Close the tube gently and centrifuge for 15s at 10,000 rpm. Discard the flow-through.
9. Perform DNase treatment using the RNase-Free DNase Set (Qiagen).
10. Transfer the RNeasy column to a new 2 ml collection tube. Wash the column by pipetting 500  $\mu$ l Buffer RPE onto it and centrifuging for 15 s at 10,000 rpm. Discard the flow-through.
11. To dry the RNeasy silica-gel membrane, pipette another 500  $\mu$ l of Buffer RPE onto the column and centrifuge for 2 min at 10,000 rpm. Discard the flow-through.
12. Transfer the column to a new sterile 1.5 ml collection tube supplied with the kit. To elute RNA, pipet 30  $\mu$ l RNase-free water directly onto the RNeasy silica membrane in the center of the column. Close the tube gently and centrifuge for 1 min at 10,000 rpm.
13. Estimate concentration and use 1 $\mu$ g RNA immediately to prepare cDNA.
14. Store remaining RNA at -80°C.

#### **DNase Treatment using the RNase-Free DNase Set (Qiagen)**

1. Dissolve the solid DNase I (1500 Kunitz units) in 550  $\mu$ l of the provided RNase-free water to prepare the DNase I stock solution. Mix gently by inverting the vial. Store the solution at -20°C.
2. Wash the RNeasy column by pipetting 350  $\mu$ l Buffer RW1 onto the column and centrifuging for 15 s at 10,000 rpm. Discard the flow-through.
3. Add 10  $\mu$ l DNase I stock solution to 70  $\mu$ l Buffer RDD for each sample and mix by inverting gently.
4. Pipet the 80  $\mu$ l DNase I mixture onto the RNeasy silica-gel membrane and incubate on the bench (20-30°C) for 15 min.
5. Wash the RNeasy column again using 350  $\mu$ l Buffer RW1 and centrifuge for 15 s at 10,000 rpm.
6. Continue with step 10 of the total RNA isolation protocol.

#### **Large-Scale Genomic DNA Extraction Method (CTAB method, Murray and Thompson, 1980)**

1. Sterilize mortar, pestles and spatulas by autoclaving for 20 min and drying in 60 °C oven.
2. Pipet 15 ml of CTAB buffer (5 ml/gram leaf material) into 50 ml sterile disposable polypropylene tubes, one for each sample. Add 30  $\mu$ l  $\beta$ -Mercaptoethanol to each tube and mix well. Heat to 65°C in a water bath.
3. Harvest 3 g young leaf material and store on ice or freeze in liquid nitrogen.

4. Cool mortar and pestle by adding liquid nitrogen. Chop fresh leaf into liquid nitrogen using scissors. Grind to a fine powder using nitrogen approximately three times.
5. Add the frozen leaf powder to the pre-heated buffer and mix well to remove lumps using a spatula or glass rod.
6. Incubate at 65 °C for 1 hour. Mix the contents 2-3 times during the incubation. Cool to room temperature.
7. Add equal volume (15 ml) chloroform/isoamyl alcohol (24:1) and mix gently by hand. Then place on a shaker with gentle mixing for 30 min to form an emulsion. Mix samples by hand once half-way through the incubation.
8. Centrifuge at 4,000 rpm for 1 min. Discard lower layer.
9. Centrifuge at 4,000 rpm for 10 min. Transfer the top layer to a new sterile 50 ml tube.
10. Add 2/3 volumes (10 ml) isopropanol and mix gently by inverting.
11. Use a sterile blue pipette tip to hold the spool of DNA and pour away the liquid. Transfer the DNA to a sterile 2 ml microcentrifuge tube.
12. Wash the DNA 2x in 70% ethanol, while holding the DNA inside the tube.
13. Centrifuge at 13,200 rpm for 1 min and remove the supernatant.
14. Air-dry the pellet for 15 min in a clean bench and resuspend in 500 µl TE.
15. Add 2 µl RNase A (30 mg/ml) and incubate at 60°C over-night.
16. Add 300 µl phenol/chloroform (25:24:1 Phenol:Chloroform:Isoamylalcohol) and mix by inverting and tapping to form an emulsion.
17. Centrifuge at 13,200 rpm for 5 min at 4°C. Remove the top layer and transfer to a new sterile 2 ml microcentrifuge tube.
18. Repeat the phenol/chloroform extraction.
19. Add 300 µl chloroform/isoamylalcohol (24:1) and mix well to form an emulsion.
20. Centrifuge at 13,200 rpm for 5 min at 4°C. Remove the top layer and transfer to a new sterile 2 ml microcentrifuge tube.
21. Repeat the chloroform extraction.
22. Add 1/10 volume (50 µl) 3M sodium acetate and 2 volumes (1 ml) 100% ethanol.
23. Spin for 1 min at 13,200 rpm and remove the supernatant by pipetting.
24. Wash 2x in 70% ethanol. After the first wash pour off the supernatant and after the second wash spin for 1 min at 13,200 rpm and remove supernatant by pipetting. This allows faster drying of the DNA pellet.
25. Dry the pellet for 15-25 min (depending on the size of the pellet) in a vacuum rotatory evaporator (Speed-Vac) with the heating on. Do not over-dry the pellet as this may compromise the solubility of the DNA.
26. Resuspend in 200 µl T1/10E. Incubate at 60°C over-night to help resuspension.
27. Make 10X dilutions for all samples. Use these samples to estimate DNA concentration using a spectrophotometer and by running on a gel.
28. Store the dilutions and stocks at 4°C.

### **Protocol for Southern Blot (Sambrook and Russell, 2001)**

1. Turn on the incubator and the water bath at 65°C, remove P32 and salmon sperm from the freezer and placed the closed P32 behind the plastic shield to let them thaw. Take the hybridization buffer and placed it on the hot water bath.

2. Pre-wet the blots: Assemble needed hybridization tubes in the rack, place blots inside the tubes, wrap top with teflon tape (to avoid leaking), and prewet blots with 5X SSC solution. Thaw the Prime-a-gene kit (Promega) components and keep Klenow on ice. Thaw your probe, prepare the dNTP mixture (10  $\mu$ l TTP, ATP and GTP) and use screw cap eppendorf tubes for the probes.
3. Prepare the probe adding DNA and water (30  $\mu$ l total volume). Prepare 500  $\mu$ l salmon sperm. To denature, place both screw-cap tubes in a beaker with boiling water for 5 min. Then, place them back on ice for 5 min.
4. To pre-hybridize the blots: Take the hybridization tubes, eliminate the 5X SSC solution and add 20 ml of hot 1X SSC (65°C, water bath), add 500  $\mu$ l salmon sperm, cap tightly, and place the tubes in opposite directions inside the incubator. Close the door, turn on the rotator and check for leaky lids and proper rotation.
5. Probe labeling: Add 10  $\mu$ l buffer, 2  $\mu$ l BSA, 2  $\mu$ l dNTPs and 1 $\mu$ l Klenow to the probe tube working on ice and outside the yield. Move behind the yield, add 5  $\mu$ l P32, cap tightly, mix lightly, and place them in the rack (50  $\mu$ l total volumen).
6. Let the hybridization tubes (incubator) and probe tubes (radioactive bench) incubate for 3-4 hours.
7. Behind the yield, dilute the probe with 1/10 TE buffer (1 ml) and add 500  $\mu$ l salmon sperm. Denature the mix in boiling water for 5 min. Remove the hybridization tubes from the incubator, eliminate the pre-hybridization solution and place them in the rack.
8. Blot hybridization: Add 10 ml hybridization solution, add probe-salmon sperm mix, taking care that the pippette dispenses the hot probe in the hybridization solution and not on the blots. Recap the tubes and place them back in the incubator. Close the door, turn on the rotator and check for leaky lids and proper rotation. Allow to incubate overnight.
9. Washing blots: Next day, eliminate the probe solution in the radiactive waste container, wash the blots in the tubes with a hot solution (65°C) of 0.1X SSC and 0.1% SDS. Do three washes of 15 ml each: one quick wash and two 20 min washes in the incubator. All the washing solutions is pour into the radioactive waste container.
10. Behind the yield, remove the blots from the tubes and wrap them with Saran wrap. Check for radioactivity with the Geiger counter and place the blots into the film cassettes. Add the film and place the cassettes in the freezer at -80°C for 2-3 days.
11. Develop the film in the darkroom.

### **Protocol for Enzyme Linked Immunadsorbent-Assay for the *nptII* gene (Agdia kit)**

1. Dilute the PEB1 buffer concentrate (10x) to 1x using sterile ddH<sub>2</sub>O. Prepare enough to use 600  $\mu$ l per sample plus a little extra (for use as negative control).
2. Harvest 3 eppendorf lengths of leaf, always choosing the youngest full expanded leaf. Store samples on ice.
3. Add a pinch of polyvinyl pyrrolidone PVP and 600  $\mu$ l PEB1 buffer to each sample. Grind the leaf materials as much as possible using a sterile blue micropestle. Keep the samples on ice.
4. Centrifuge the samples at 14,000 rpm at 4°C for 15 min. Transfer the supernatant to a new microcentrifuge tube by pipetting and store on ice.
5. If the samples contain a lot of debris, centrifuge again.
6. Turn on spectrophotometer half an hour before use.

7. Dilute Bradford's reagent 1:5 using sterile ddH<sub>2</sub>O. Prepare enough to use 1 ml per sample including standards and blank.
8. Prepare a dilution series using BSA for use as standards.
9. Add 1 ml diluted Bradford's reagent to each cuvette. Add 10 µl of sample to each cuvette and mix by pipetting.
10. To ensure complete mixing, immediately invert the cuvette using a piece of parafilm. Leave to incubate at room-temperature while preparing the remaining samples. Use a new piece of parafilm for each sample to prevent contamination.
11. Measure OD<sub>595</sub> of each sample (ideally these should be between 0.2 and 0.8).
12. Plot a standard curve using BSA and use it to estimate protein concentration of the samples.
13. Calculate the volume of each sample required to get 15 µg.
14. Prepare the samples, including wild-type, in new tubes using 15 µg protein and the volume of buffer PEB1 required to make the total volume 110 µl.
15. Prepare standards as follows: 110 µl buffer PEB1 (negative control) and 110 µl of the provided positive control. Keep all prepared samples on ice.
16. Prepare a humid box by putting some damp paper towel in a box with a lid.
17. Add 100 µl of each prepared samples into the test wells, making note of the sequence in which the samples were applied. Also add 100 µl of the prepared standards.
18. Place the plate in humid box and incubate for 2 hours at room temperature.
19. Prepare the wash buffer PBST by diluting 5 ml to 100 ml (20x) with dd H<sub>2</sub>O.
20. Prepare the enzyme conjugate diluent by mixing 1 part MRS-2 with 4 parts 1x buffer PBST. Make enough to add 100 µl per well.
21. A few minutes before the incubation ends, add 10 µl from bottle A and 10 µl from bottle B per 1 ml of enzyme conjugate diluent to prepare the enzyme conjugate.
22. When incubation is complete, remove plate from humid box and empty wells into the sink by flipping quickly while squeezing the sides of the frame.
23. Fill all wells to over-flowing with 1x buffer PBST and then quickly empty them again. Repeat 5x.
24. After washing tap the frame firmly upside down on paper towels to dry the wells.
25. Add 100 µl of prepared enzyme conjugate into each well.
26. Place the plate in humid box and incubate for 2 hours at room temperature.
27. Measure out sufficient TMB substrate solution for 100 µl per well into a clean container. Allow to warm to room temperature during the 2 hour incubation.
28. When the incubation is complete, wash the plate with 1x buffer PBST as before.
29. Add 100 µl of room temperature TMB substrate solution to each well and place the plate in humid box for 15 min.
30. Add 50 µl 3M sulphuric acid (stop solution) to each well. The substrate color will change from blue to yellow.
31. The results must be recorded within 15 min after addition of the stop solution otherwise the reading will decline.

## High sensitivity Protocol for Enzyme Linked Immunadsorbent-Assay for the *cryIF* gene (QualiPlate™ Kit for *CryIF*, EnviroLogix)

### Buffer, control and leaf sample preparation for ELISA

1. Prepare washing buffer as follows: Add the contents of the packet of Wash Buffer Salts (phosphate buffered saline, pH 7.4 - Tween 20) to 1 liter of distilled or deionized water and stir to dissolve. Store refrigerated when not in use; warm to room temperature prior to assay. If more wash buffer is needed, order item # P-3563 from Sigma Chemical Co. ( St. Louis, MO), or prepare the equivalent.
2. Prepare Extraction Buffer: Add 0.5 ml Tween-20 to 100 ml of prepared Wash Buffer, and stir to dissolve. Store refrigerated when not in use; warm to room temperature prior to assay.
3. Prepare Positive and Negative Control ground corn extracts: Extracts of these controls must be run in every assay. To extract, add 5 ml of Extraction Buffer to each tube containing 2 grams of ground Control corn. Cap and shake vigorously by hand or vortex for 20-30 seconds. Let stand at room temperature for 1 hour to extract. Mix again at the end of the hour, then clarify by allowing to settle 10 minutes or by centrifuging 5 minutes at 5000 x g. The High Sensitivity Protocol requires that the Positive Control ground corn extract be diluted 1:3 in Negative Control ground corn extract (mix 100 µl Positive extract plus 200 µl Negative extract) prior to use.
4. Prepare leaf samples from the third fully emerged leaf: Take two leaf segments from the second and third uppermost leaves of length equivalent to that of a 1.5ml eppendorf tube. Mash the leaf tissue with a pestle matched to the micro-tube, or with a disposable pipette tip, or a Hypure cutter (HCT-200, PerkinElmer) in a 96-well. Add 0.25 ml of Extraction Buffer per leaf punch. Mix for at least 30 seconds, then allow particles to settle. Take extreme care not to crosscontaminate between leaf samples. Dispensing particles into the test plate can cause false positive results.

### ELISA

1. Dilute the Positive Control ground corn extract 1:3 in Negative Control ground corn extract for this protocol.
2. Add 50 µl of Extraction Buffer Blank (BL), 50 µl of Negative Control (NC) ground corn extract , 50 µl of diluted Positive Control (PC) ground corn extract, and 50 µl of each sample extract (S) to their respective wells.
3. Thoroughly mix the contents of the wells by moving the plate in a rapid circular motion on the benchtop for a full 20-30 seconds. Be careful not to spill the contents!
4. Cover the wells with tape or Parafilm to prevent evaporation and incubate at ambient temperature for 30 minutes. If an orbital plate shaker is available, shake plate at 200 rpm.
5. Add 50 µl Cry1F-enzyme Conjugate to each well. Thoroughly mix the contents of the wells, as in step 2.
6. Cover the wells with tape or Parafilm to prevent evaporation and incubate at ambient temperature for 90 minutes. If an orbital plate shaker is available, shake plate at 200 rpm.
7. After incubation, carefully remove the covering and vigorously shake the contents of the wells into a sink or other suitable container. Flood the wells completely with Wash Buffer, then shake to empty. Repeat this wash step three times.
8. Add 100 µl of Substrate to each well.

9. Thoroughly mix the contents of the wells, as in step 2. Cover the wells with new tape or Parafilm and incubate for 30 minutes at ambient temperature. Use orbital shaker if available. Caution: Stop Solution is 1.0N Hydrochloric acid. Handle carefully.
10. Add 100  $\mu$ l of Stop Solution to each well and mix thoroughly. This will turn the well contents yellow NOTE: Read the plate within 30 minutes of the addition of Stop Solution.

**Protocol for immunochromatography strip test for *cryIF* gene (Quickstix<sup>TM</sup> kit for *cryIF*, EnviroLogix)**

**Sample Preparation**

1. Prepare leaf samples from the third fully emerged leaf: Take two leaf segments from the second and third uppermost leaves of length equivalent to that of a 1.5ml eppendorf tube. Mash the leaf tissue with a pestle. Sample identification should be marked on the tube with a waterproof marker.
2. Insert the pestle into the tube and grind the tissue by rotating the pestle against the sides of the tube with twisting motions. Continue this process for 20 to 30 seconds or until the leaf tissue is well ground.
3. Add 0.25 mL of extraction Buffer into the tube. Repeat the grinding step to mix tissue with Extraction Buffer. Dispose of the pestle(do not re-use pestles on more than one sample to avoid cross-contamination).

**QuickStix Strip Test**

1. Allow refrigerated canisters to come to room temperature before opening. Remove the QuickStix Strips to be used. Avoid bending the strips. Reseal the canister immediately.
2. Place the strip into the extraction tube. The sample will travel up the strip. Use a rack to support multiple tubes if needed.
3. Allow the strip to develop for 10 minutes before making final assay interpretations. Positive sample results may become obvious much more quickly.
4. To retain the strip, cut off the bottom section of the strip covered by the arrow tape.
5. Development of the Control Line within 10 minutes indicates that the strip has functioned properly. Any strip that does not develop a Control Line should be discarded and the sample re-tested using another strip.
6. If the sample extract contained Cry1F endotoxin, a second line (Test Line) will develop on the membrane strip between the Control Line and the protective tape, within 10 minutes of sample addition. The results should be interpreted as positive for Cry1F endotoxin expression. Any clearly discernible pink Test Line is considered positive.
7. If no Test Line is observed after 10 minutes have elapsed, the results should be interpreted as negative, meaning that the sample contained less Cry1F endotoxin than is typically expressed in the tissues of Bt-modified plants.

## **Protocol for co-immunoprecipitation of recombinant VSP $\beta$ (ProFound™ c-Myc Tag IP/Co-IP Kit, Pierce Biotechnology)**

### **Material Preparation:**

1. Prepare the extraction and TBS buffers.
2. Prepare leaf samples: Weight and freeze 5 grams of young leaf tissue. Use mortar and pestle to grind the leaves with liquid nitrogen. Add 15 ml extraction buffer (1:3 weight: volumen ratio). Centrifugate tube samples for 20 minutes at 4000 rpm and 4°C (Sorwall SS-34). Take the supernatant and centrifugate samples for 10 minutes at 4000 rpm and 4°C. Take the supernatant.
3. Concentrate the protein extract by acetone precipitation: Cool the acetone required volume to -20°C. The final volume is four times the volume of your protein extract (ex. 15 ml extract needs 60 ml acetone). Place protein sample a polypropylene tube (split the extract in two tubes before adding the acetone because polypropylene tubes hold 30 ml). Add four times the sample volume of cold acetone to the tube. Vortex tube and incubate for 60 minutes at -20°C. Centrifuge 10 minutes at 13,000-15,000 rpm (Sorwall). Decant and properly dispose of the supernatant, being careful to not dislodge the protein pellet.
4. Add resuspension buffer (4 ml/tube) and resuspend the pellet using an orbital shaker.
5. Estimate protein concentration with the Coomassie Plus Protein Assay reagent and Coomassie standards (Pierce Biotechnology, Inc.) at OD<sub>595</sub> with a spectrophotometer. Protein concentration should be approx. 1 mg/ml.

### **Immunoprecipitation/Co-immunoprecipitation (IP/Co-IP) of c-Myc-tagged protein**

1. Set up a positive control: Use 50  $\mu$ l c-Myc-tagged Positive Control diluted in 150  $\mu$ l TBS.
2. Thoroughly resuspend the anti-c-Myc agarose by inverting the vial several times immediately before dispensing (do not vortex!). Dispense 10  $\mu$ l anti-c-myc agarose slurry (5  $\mu$ g anti-c-Myc antibody) into each tube containing 1ml of protein extract using a wide-bore pipette tip. Close the tube.
3. Incubate the tube with an orbital shaker at 4°C overnight (in the fridge).
4. Next day, get the spin column and remove the bottom plug. Put a collection tube under the column, add 0.5 ml of the mix slurry-protein extract and pulse for 10 seconds at maximum speed in microcentrifuge. Save the flow-through for future analysis. Repeat this step because the maximum volume for the spin column is 850  $\mu$ l.
5. Prepare a wash solution of TBS plus 0.05% Tween20 (TTBS). For each spin column prepare approximately 3 ml of wash solution. Add 0.5 ml TTBS to each column. Loosely screw on the cap and gently invert the column with the collection tube 2-3 times. Pulse centrifuge for 10 seconds. Save the wash for future analysis. Repeat this step two additional times.
6. Elution of c-myc-tagged protein: Place the spin column in a new collection tube. Add 10  $\mu$ l elution buffer to the anti-c-myc agarose, loosely screw on the cap and gently tap the tube to mix. Pulse centrifuge for 10 seconds. (It is not necessary to place the bottom plug on the spin column for this step).
7. Repeat this step two additional times. The three elutions may be recovered and pooled in one collection tube. Neutralize the elutant immediately by adding 1  $\mu$ l of 1 M Tris, pH 9.5 per 20  $\mu$ l of Elution Buffer.

8. To prepare the sample for reducing SDS-PAGE and western blot, take 25  $\mu$ l sample and add 2-3  $\mu$ l of 1 M DTT or 1-2  $\mu$ l of mercaptoethanol and loading buffer. The sample is ready to load.
9. Usually, the eluted c-myc-tagged positive control can be detected by coomassie or silver staining. But, for more sensitive detection methods such as Western blotting, dilute the control 10- to 50-fold (0.2-0.5  $\mu$ l elution is sufficient for analysis).

## Protocols for Western Blots

### Western blots with VSP $\beta$ antibodies

1. Prepare samples: Grind 500 mg leaf tissue (including leaves from all stages) with liquid nitrogen in a 2 ml tube. Add 1 ml extraction buffer (Bellucci et al., 2000). Vortex and place it on ice until finish processing all the samples. Centrifuge at 14,000 g 20 minutes. Take supernatant. Centrifuge at 14,000 g 10 minutes. Take supernatant.
2. Estimate protein concentration: Prepare calibration curve with a range between 0.5 and 5 mg/ml. Make 10X dilutions of the samples (5  $\mu$ l sample: 45  $\mu$ l ddH<sub>2</sub>O). Take OD595 readings with the spectrophotometer. Estimate sample concentrations.
3. Prepare loading samples: Add 15  $\mu$ g total protein per sample. Add 5  $\mu$ l loading buffer per sample. Incubate the samples at 100C 10 minutes. Store at -20C or place them on ice until use.
4. Prepare SDS-PAGE gels. Load molecular markers: 10  $\mu$ l Prestained SDS-PAGE standards, low range (Bio-Rad Laboratories) and 1  $\mu$ l MagicMark™ (Qiagen). Load 20  $\mu$ l plant samples.
5. Run SDS-PAGE gels in electrophoretic tank at 150 v, 1 hr 15 minutes.
6. Prepare fresh transfer buffer and keep it on ice or in the fridge (-20C). Place SDS-PAGE gels and nitrocellulose membrane (NC-membrane, Bio-Rad Laboratories) in transfer buffer; and rinse them 5 minutes twice.
7. Prepare transfer sandwich keeping all the pieces wet in transfer buffer. Place the transfer cell in the electrophoretic tank and run the transfer while stirring and keeping the tank cold with ice or inside the fridge, at 150 v, 1 hr 15 minutes.
8. To confirm protein transfer, stain gels with Coomassie blue (Sigma R-250, Sigma) or NC-membrane with Ponceau solution. Rinse nitrocellulose membranes with Tween Tris Buffer (TTBS) twice, 5 minutes.
9. To avoid unspecific binding, incubate NC-membrane in a solution with TTBS and 5% non-fat dry milk (Bio-Rad Laboratories) (0.75 g milk and 15 ml TTBS) in orbital shaker at room temperature. Rinse NC-membrane with TTBS buffer twice, 5 minutes.
10. Incubate NC-membrane in a solution with TTBS, 5% non-fat dry milk and 1:1000 VSP $\beta$  antibody dilution (10  $\mu$ l batch#73, 0.75 g milk and 15 ml TTBS) in orbital shaker at room temperature, 4 hr. Rinse NC-membrane with TTBS buffer twice, 5 minutes.
11. Incubate NC-membrane in a solution with TTBS, 5% non-fat dry milk and 1:50,000 secondary antibody dilution (1.2  $\mu$ l Pierce, 0.75 g milk and 15 ml TTBS) in orbital shaker at room temperature, 2 hr. Rinse NC-membrane with TTBS buffer twice, 5 minutes.

12. Incubate NC-membrane in a 1:1 solution with TTBS : chemiluminescent substrate (Supersignal West Femto Maximum Sensitivity Substrate, Pierce Biotechnology, Inc.) according to Pierce recommendations, and develop in dark room after 5-15 minutes.

### **Western blots with c-myc antibodies**

1. Prepare samples: Grind 500 mg leaf tissue (including leaves from all stages) with liquid nitrogen in a 2 ml tube. Add 1 ml extraction buffer (Bellucci et al., 2000). Vortex and place it on ice until finish processing all the samples. Centrifuge at 14,000 g 20 minutes. Take supernatant. Centrifuge at 14,000 g 10 minutes. Take supernatant.
2. Estimate protein concentration: Prepare calibration curve with a range between 0.5 and 5 mg/ml. Make 10X dilutions of the samples (5  $\mu$ l sample: 45  $\mu$ l ddH<sub>2</sub>O). Take OD595 readings with the spectrophotometer. Estimate sample concentrations.
3. Prepare loading samples: Add 15  $\mu$ g total protein per sample. Add 5  $\mu$ l loading buffer per sample. Incubate the samples at 100°C 10 minutes. Store at -20°C or place them on ice until use.
4. Prepare SDS-PAGE gels. Load molecular markers: 10  $\mu$ l Prestained SDS-PAGE standards, low range (Bio-Rad Laboratories) and 1  $\mu$ l MagicMark™ (Qiagen). Load 20  $\mu$ l plant samples.
5. Run SDS-PAGE gels in electrophoretic tank at 150 v, 1 hr 15 minutes.
6. Prepare fresh transfer buffer and keep it on ice or in the fridge (-20°C). Place SDS-PAGE gels and nitrocellulose membrane (NC-membrane, Bio-Rad Laboratories) in transfer buffer; and rinse them 5 minutes twice.
7. Prepare transfer sandwich keeping all the pieces wet in transfer buffer. Place the transfer cell in the electrophoretic tank and run the transfer while stirring and keeping the tank cold with ice or inside the fridge, at 150 v, 1 hr 15 minutes.
8. To confirm protein transfer, stain gels with Coomassie blue (Sigma R-250, Sigma) or NC-membrane with Ponceau solution. Rinse nitrocellulose membranes with Tween Tris Buffer (TTBS) twice, 5 minutes.
9. To avoid unspecific binding, incubate NC-membrane in a solution with TTBS and 5% non-fat dry milk (Bio-Rad Laboratories) (0.75 g milk and 15 ml TTBS) in orbital shaker at room temperature. Rinse NC-membrane with TTBS buffer twice, 5 minutes.
10. Incubate NC-membrane in a solution with TTBS, 5% non-fat dry milk and 1:1000 *c-myc* antibody dilution (Sigma, 0.75 g milk and 15 ml TTBS) in orbital shaker at room temperature, 2 hr. Rinse NC-membrane with TTBS buffer twice, 5 minutes.
11. Incubate NC-membrane in a solution with TTBS, 5% non-fat dry milk and 1:50,000 secondary antibody dilution (1.2  $\mu$ l Pierce, 0.75 g milk and 15 ml TTBS) in orbital shaker at room temperature, 2 hr. Rinse NC-membrane with TTBS buffer twice, 5 minutes.
12. Incubate NC-membrane in a 1:1 solution with TTBS : chemiluminescent substrate (Supersignal West Femto Maximum Sensitivity Substrate, Pierce Biotechnology, Inc.) according to Pierce recommendations, and develop in dark room after 5-15 minutes.

## Protocol for Insect Bioassays

### Feeding Experiments (Adamczyk and Gore, 2004)

1. Prepare Petri dishes containing 1g/l agar medium.
2. Harvest bahiagrass plants. Collect the third fully emerged leaf from different tillers within the same plant. Cut bahiagrass leaves discarding the top and the bottom of the leaf and using intermediate pieces of 2 cm length. Place four leaf pieces with the abaxial surface in contact with the medium on each Petri dish.
3. Collect fall armyworm neonate larvae from egg masses (hatching the same day) with a camel-hair brush and place on the top of the leaves. Place one larvae per Petri dish and seal the dish. Prepare 10 dishes per treatment and replicate.
4. Place Petri dishes in the incubator at 26-28°C. Leave the dishes for five days.
5. Then, open the Petri dish and look for the neonate larvae under the dissectoscope. Larvae were considered alive if coordinated movement was observed. Record mortality data.

### Buffers and Reagents

#### Bacterial Growth

##### SOC medium

SOB: 4g tryptone, 1g yeast extract and 0.1g NaCl. Dissolve in 180ml dH<sub>2</sub>O. Add 2ml 250mM KCl. Adjust to pH7 with 5N NaOH. Make up to 200ml. Just before use, add 10µl 1M MgCl<sub>2</sub> and 20 µl 1M glucose per 1ml SOB.

##### Antibiotics

- Ampicillin: Weigh 100 mg ampicillin. Dissolve in 2 ml of ddH<sub>2</sub>O. Filter sterilize into autoclaved Eppendorf tubes. Freeze at -20° C. Stock concentration: 50 mg/ml. Use 2 µl (100 µg)/ml LB.
- Kanamycin: Weigh 100 mg kanamycin. Dissolve in 10 ml of ddH<sub>2</sub>O. Filter sterilize into autoclaved Eppendorf tubes. Freeze at -20° C. Stock concentration: 10 mg/ml. Use 5 µl (50 µg)/ml LB.
- Tetracycline: Weigh 50 mg tetracycline. Dissolve in 10 ml of 100% ethanol. Aliquot into autoclaved Eppendorf tubes. Freeze at -20° C. Stock concentration: 5 mg/ml. Use 2 µl (10 µg)/ml LB.
- Paromomycin: Weigh 500 mg paromomycin. Dissolve in 10 ml of ddH<sub>2</sub>O. Filter sterilize into autoclaved Eppendorf tubes. Freeze at -20° C. Stock concentration: 50 mg/ml. Use 1 ml (50 mg)/ 1 IF.

- Rifampicin: Weigh 100 mg rifampicin. Dissolve in 4 ml of DMSO. Filter sterilize into autoclaved Eppendorf tubes. Wrap tubes in aluminum foil. Freeze at -20° C. Stock concentration: 25 mg/ml. Use 6 µl (150 µg)/ml LB.

### **Glycerol stocks**

Incubate the *E.coli* culture containing your plasmid overnight at 37C. Add 0.85 ml bacterial culture. Add 0.15 ml glycerol (previously autoclaved and at room temperature). Vortex for mixing. Freeze in liquid nitrogen. Store -80C. Prepare several tubes for plasmid. Avoid successive freeze-thaw cycles.

### **Ethanol precipitation**

Add 0.1 volumen 3 M sodium acetate and 2 volumes 100% ethanol to plasmid DNA solution. Place -20C for 15-30 minutes. Spin 12,000 g 10 minutes. Remove supernatant. Add 200 ul 70% ethanol. Spin 12,000 g 10 minutes. Remove supernatant. Air-dry or speed-vaccum the pellet. Resuspend in ddH<sub>2</sub>O or TE buffer.

### **Plasmid DNA Extraction**

- Extraction buffer (Buffer D): Add 4.44 g Tris.HCl, 2.65 g Tris base, 9.3 g EDTA, 7.3 G NaCl. Add ddH<sub>2</sub>O up to 500 ml.
- Sodium Dodecyl Sulphate stock (20% SDS): Add 100 g SDS in 450 ml ddH<sub>2</sub>O with heat. Adjust pH=7.2 with concentrated HCl. Add ddH<sub>2</sub>O up to 500 ml.
- Potassium acetate stock (5 M): Add 29.44 g potassium acetate, 11.5 ml glacial acetic acid, and make volume up to 100 ml ddH<sub>2</sub>O.
- Sodium acetate stock (3 M): Add 0.82 g sodium acetate to 5 ml ddH<sub>2</sub>O. adjust pH=5.2 with glacial acetic acid and make volume up to 10 ml with ddH<sub>2</sub>O.

### **Bahiagrass Tissue Culture Medium**

#### **IF Medium**

Add 1.72g MS salts (Murashige and Skoog, 1962), 40µl CuSO<sub>4</sub> (12.45mg/ml), 1.2g Phytigel and 8g Sucrose to 400 ml ddH<sub>2</sub>O. Adjust pH to 5.8 with KOH. Autoclave 20 mins.

Add 600µl dicamba (2mg/ml), 440µl BAP (1mg/ml) and 400µl MS vitamins (1000x) in sterile conditions.

#### **DICAMBA (3,6-dichloro-2-methoxybenzoic acid)**

Weigh 100 mg dicamba. Dissolve in 0.5 ml of 100% ethanol with heat. Add 49.5 ml ddH<sub>2</sub>O with heat. Filter-sterilize the stock solution. Aliquot into autoclaved Eppendorf tubes. Freeze at -20° C. Stock concentration: 2 mg/ml. Use 600 µl (1200 mg)/400 ml IF.

#### **BAP (6-benzylaminopurine)**

Weigh 825 mg BAP. Dissolve in 0.5 ml of NaOH (1N). Add 19.5 ml ddH<sub>2</sub>O. Filter-sterilize the stock solution. Aliquot into autoclaved Eppendorf tubes. Freeze at -20C. Stock concentration: 1mg/ml. Use 440 µl (1200 mg)/400 ml IF.

#### **Western bBots**

##### **Extraction buffer (Bellucci et al., 2000)**

	100 ml
100 mM Tris	1.21 g
200 mM NaCl	1.17 g
1 mM EDTA	29.2 mg
10% glycerol	10 ml
0.2% Triton100X	0.2 ml
4% β-mercaptoethanol	4 ml

Adjust final volume with ddH<sub>2</sub>O. Add fresh β-mercaptoethanol before use. Reduce to 1,2 mM β-mercaptoethanol and 5% glycerol to use co-immunoprecipitation columns.

##### **Loading buffer**

	10 ml
40% glycerol	4 ml
0.5 M Tris.HCl (pH=6.8)	2.5 ml
8% SDS	800 mg
0.24% Bromophenolblue	12 mg

Adjust final volume with ddH<sub>2</sub>O. Aliquot 200 µl and add 50 µl fresh β-mercaptoethanol before use.

##### **Electrophoresis buffer (Tris-glycine-SDS 10X)**

	1000 ml
25 mM Tris	30 g
192 mM glycine	144 g
0.1% SDS	10 g

Adjust final volume with ddH<sub>2</sub>O. Add components in order and wait until they are dissolved. pH is adjusted naturally (pH=8.3).

### **Destaining solution**

	500 ml
30% methanol	150 ml
10% acetic acid	50 ml
60% ddH <sub>2</sub> O	300 ml

Rinse in fume hood.

### **Tris base buffer (TBS)**

	1000 ml
Tris base	24.22 g
NaCl	87.66 g

Adjust pH=7.5 by adding 15-20 ml 1N HCl. To prepare TTBS, add 0.1% Tween 20 before use.

### **Transfer buffer**

	1000 ml
48 mM Tris	5.82 g
39 mM glycine	2.93 g
SDS	2.5 mg
20% methanol	200 ml
ddH <sub>2</sub> O	800 ml

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## BIOGRAPHICAL SKETCH

Gabriela Fabiana Luciani, the oldest daughter of Beatriz Marta Tica de Luciani and Alberto Daniel Luciani, was born in Bahia Blanca, Buenos Aires Province, Argentina, on October 7, 1968. In 1986, Gabriela started her college studies in biology, oriented to ecology, at the Facultad de Ciencias Naturales y Museo, Universidad Nacional de La Plata, La Plata; where she received her degree in biology in 1993. During this time, she was actively involved in the academic life as a teaching assistant in different classes.

In 1996, she was hired by Comision de Investigaciones Cientificas (CIC), to develop tissue culture techniques to improve garlic micropropagation. In 1997, she was hired by Comision Nacional de Investigaciones Cientificas y Tecnologicas (CONICET), to continue her studies focused on eliminating a viral complex during garlic tissue culture. After a car accident and more than a year in rehabilitation, and encouraged by her supervisor, Dr. Nestor Curvetto, she was admitted to pursue a M. Sc. degree in agronomy at the Facultad de Agronomia, Universidad Nacional del Sur, Bahia Blanca, in 1999. Encouraged by friends, she applied for a Fulbright-LASPAU fellowship to pursue her Ph. D. program in the United States. She received her M.Sc. degree in Agronomy in 2001, and moved immediately to Gainesville, to begin her program at the Agronomy Department, University of Florida.