MOLECULAR IMPROVEMENT FOR FALL ARMYWORM RESISTANCE IN SUGARCANE, TIFEAGLE AND TIFTON 85

By

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In memory of my parents, Dr. D. V. Joshi and Sunita Joshi

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MOLECULAR IMPROVEMENT FOR FALL ARMYWORM RESISTANCE IN SUGARCANE, TIFEAGLE AND TIFTON 85

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Sugarcane (*Saccharum* spp. hybrids) is a tropical grass native to Asia. Florida is the largest producer of sugarcane in the U.S. followed by Louisiana, Hawaii, and Texas. TifEagle [*Cynodon dactylon* (L.) Pers. X C. *transvaalensis* Burtt Davy] is a warm season, triploid bermudagrass cultivar that provides high quality turf and is an ideal grass for golf course putting greens. Tifton 85 [*Cynodon dactylon* (L.) Pers. X C. *transvaalensis* Burtt Davy] is a warm season perennial forage grass with high yield and superior quality.

All of these grasses are susceptible to the insect pest, fall armyworm (FAW) *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae). Therefore, the goal of the present research was to incorporate resistance against the rice strain of FAW through the introduction and expression of a *Bacillus thuringiensis* (*Bt*) endotoxin gene via genetic engineering. This approach was chosen because traditional breeding is laborious and difficult for sugarcane, a highly aneu-polyploid crop, and impossible for TifEagle and Tifton 85 due to sterility.

A long-term goal is to produce transgenic sugarcane in a relatively short period of time by bypassing a callus phase. Therefore, an initail study was conducted to evaluate a direct regeneration tissue culture system using three cultivars, CP84-1198, CP88-1762 and CP89-2143.

Such as system has advantages over callus-based methods in that less time is required to produce transgenic plants and there is less of a dependency on tissue culture, thereby potentially reducing somaclonal variation. The importance of explant distance from the meristem and orientation of the explants was examined for induction of shoots and roots. The greatest number of shoots was produced by CP88-1762, followed by CP89-2143 and CP84-1198 from explants closest to the meristem that were oriented vertically on the medium. The most roots were produced by CP84-1198, followed by CP88-1762 and CP89-2143. Overall, CP88-1762 was the best cultivar among the three for the production of plantlets via direct organogenesis followed by CP89-2143 and CP84-1198. Therefore, CP88-1762 will be used in future experiments to optimize this tissue culture system in conjunction with an *Agrobacterium*-mediated transformation protocol.

To produce sugarcane transgenics with resistance to the rice strain of FAW, biolistics was used to introduce the *Bt cry1Fa* gene encoding a δ-endotoxin that has been effective in protecting against lepidopteran pests. Sugarcane embryogenic callus was co-bombarded with a *cry1Fa* gene cassette and an *nptII* gene cassette as the selectable marker. Four independent sugarcane transgenic lines of CP84-1198 were obtained showing stable integration of multiple copies of the *cry1Fa* gene. A qualitative immuno-chromatographic assay indicated that two of the four transgenic lines had Cry1Fa levels above the detection level of the assay. A semi-quantitative immunoassay, based on the QualiPlate™ kit for Cry1F (EnviroLogix™), showed that the same two lines had Cry1Fa levels equal to or more than the Cry1F levels found in Herculex I® corn or WideStrike® cotton , while the remaining two lines had lower levels of Cry1Fa. These transgenics were subjected to FAW laboratory bioassays over a 5-day period. Rice strain larvae showed intense feeding on wild type leaves along with high weight gains, while those fed leaves from the four transgenic lines showed significantly limited feeding and weight gain.

Additionally, the FAW larvae showed high mortality rates when fed the transgenic leaves particularly for the two lines that expressed the higher levels of Cry1Fa. This is the first report of transgenic *Bt* sugarcane conferring resistance to FAW, and future work will test their efficacy in the field.

For Tifton 85 and TifEagle, a successful direct regeneration protocol using stolon node explants was developed. This tissue culture system was used with *Agrobacterium* infection in transformation experiments. One transgenic TifEagle line containing *cry1Fa* was obtained using *Agrobacterium tumefaciens* strain AGLO, but this approach was unsuccessful for obtaining transgenic Tifton 85. The transgenic *cry1Fa* TifEagle line was subjected to FAW bioassays. However, no differences in FAW larval weight gain or mortality were found suggesting that the level of expression of *cry1Fa* in this line was insufficient to provide resistance to the rice strain of FAW.

CHAPTER 1
INTRODUCTION

Sugarcane (*Saccharum* spp. hybrids) is a tropical grass native to Asia. In Florida, sugarcane is grown near the southern and eastern shores of Lake Okeechobee. Palm Beach County accounts for approximately 70% of the acreage and 75% of the total harvested tonnage (Glaz et al., 2004). The remainder is grown in the adjacent counties of Hendry, Glades, and Martin. Florida is the largest producer of sugarcane in the U.S. followed by Louisiana, Hawaii and Texas.

'TifEagle' [*Cynodon dactylon* (L.) Pers. X C. *transvaalensis* Burtt Davy] is a warm season bermudagrass grown in tropical regions of the world normally providing high quality turf for golf course putting greens (Bunnell et al., 2005). It has a distinct genetic background, turf density, and an ability to tolerate extremely low cutting heights. TifEagle is grown throughout Florida on numerous golf courses (Bunnell et al., 2005).

There are approximately 3.5 million acres of improved perennial pasture grasses in Florida. Bermudagrass is the most important pasture grass in southern U.S. (Mitich, 1989; Burton and Hanna, 1995) covering an area of 12 million ha for grazing and hay production (Taliaferro et al., 2004). 'Tifton 85' [*Cynodon dactylon* (L.) Pers. X C. *transvaalensis* Burtt Davy] is a high quality forage grass producinghigh yields of hay and pastures resulting in large live weight gains in cattle. It was ranked first in forage production in 1992 (Burton et al., 1993). It has 34% higher dry matter yield, 47% higher digestible yield, and superior animal performance with growing steers compared to coastal bermudagrass (*Cynodon dactylon*), (Mandebvu et al., 1999). It has become the standard by which other bermudagrass varieties are compared (Hill et al., 2001; Evers et al., 2004).

All of the above grasses are susceptible to fall armyworm (FAW), *Spodoptera frugiperda* (J.E.Smith) (Lepidoptera: Noctuidae). Fall armyworm is one of the most devastating insect pests in the southeastern U.S. causing seasonal economic losses (Sparks, 1979; Meagher and Nagoshi, 2004). It prefers more than 60 host plants including corn (*Zea mays* L.), sorghum (*Sorghum bicolor* L.), and grasses such as common bermudagrass [Cynodon dactylon (L.) Pers.] (Luginbill, 1928).

Fall armyworm consists of two morphologically indistinguishable strains, a corn strain that prefers corn, cotton (*Gossypium hirsutum* L.) and sorghum, and a rice strain that prefers rice (*Oryza sativa* L.) and bermudagrass (Pashley, 1986; Pashley, 1988; Pashley et al., 1995). The rice strain is more specialized to preferred plant hosts than the corn strain. Previous research has also shown that rice strain larvae complete larval development and gain weight more rapidly on bermudagrass than on other grasses (Pashley et al., 1995).

The main goal of this research was to incorporate resistance against FAW into sugarcane, TifEagle, and Tifton 85 through genetic engineering. Developing FAW resistance through traditional breeding in sugarcane is time consuming and laborious due to its high ploidy level (2n = 36-170), low fertility, and high genotype by environment interaction (Gallo-Meagher and Irvine, 1996). Developing resistance against FAW in TifEagle and Tifton 85 is not possible through traditional breeding because both grasses are sterile.

Field trials with corn hybrids expressing a full length *cry1Fa* gene (Herculex I®), isolated from *Bacillus thuringeinsis aizawai,* indicated that this gene provides protection against FAW. The expressed Cry1Fa protein also effectively controls other lepidopteran insect larvae including European corn borer (ECB; *Ostrinia nubilalis* Hubner), southwestern corn borer (SWCB; *Diatraea grandiosella* Dyar), and black cutworm (*Agrostis ipsilon* Hufnagel) (EPA, 2001).

However, this gene has not been used to develop insect resistance in sugarcane, TifEagle, or Tifton 85. Therefore, genetic engineering of *cry1Fa* into these grasses offers a viable and attractive approach for incorporating FAW resistance into these grasses.

Specific objectives of the research were to:

- develop rapid and efficient tissue culture regeneration systems for sugarcane, TifEagle, and Tifton 85,
- introduce *cry1Fa* into the three grasses using biolistic or *Agrobacterium*-mediated transformation methods, and
- characterize transgenics at the molecular level and determine their resistance to the rice strain of FAW in laboratory bioassays.

CHAPTER 2
LITERATURE REVIEW

Sugarcane (*Saccharum* spp. hybrids)

Sugarcane records date back to 510 BC. At that time, 'reeds which produce honey without bees' was reported by Emperor Darius' soldiers near the river Indus. Sugarcane then spread westward with the conquest of India by Alexander the Great in 327 BC (Purseglove, 1972). Sugarcane was brought to Europe along with the Crusades in the 11th century, and in 1319 the first large shipment of sugar reached England. Sugarcane spread quickly in the 1400s, and in 1420 it reached the Canary Islands, and from there it was introduced to the New World by Columbus in 1493 (Cordeiro et al., 2007). Currently, sugarcane is the main sugar producing crop in the tropical and subtropical regions of the world (James, 2004).

Sugarcane belongs to the genus *Saccharum* L., first established by Linnaeus in *Species Plantarum* in 1753 with two species: *S. officinarum* and *S. spicalum*. The genus is in the tribe Andropogoneae in the grass family Poaceae. The tribe includes other tropical grasses such as sorghum and corn (Cordeiro et al., 2007). Very closely related to *Saccharum* are another four genera that readily interbreed, forming what is now commonly referred to as the *Saccharum* complex (Daniels and Roach, 1987). The other four genera are *Erianthus* section *Ripidum*, *Miscanthus* section *Diandra*, *Narenga* and *Sclerostachya*. All have a high level of polyploidy, and aneuploidy creating a challenge for taxonomists (Daniels and Roach, 1987; Sreenivasan et al., 1987).

There are now six species of *Saccharum* that have been revised from the original classification of Linnaeus: *S. officinarum*, known as noble cane; *S. spontaneum*, *S. robustum*, and *S. edule*, classified as wild species; and *S. sinense* and *S. barberi*, classified as ancient hybrids (Buzacott, 1965; Daniels and Roach, 1987; D'Hont and Layssac, 1998). *Saccharum officinarum* is

high in sucrose content and is primarily used for sugar production. It is characterized as being moderately tall, of various colors, with thick stalks and low fiber. It has a chromosome number of 2n = 80 with a basic chromosome number (x) of 10 (D'Hont et al., 1998). As mentioned above, *S. spontaneum* is a wild species. It has thinner canes, high variability in morphology, high fiber and relatively little sugar. Its chromosome number varies from 2n = 40 to 128 with x = 8 (D'Hont et al., 1998). It can be weedy and in countries such as Thailand, India, the Philippines, and Indonesia, it is considered a serious weed that competes with other crops (Holm et al., 1997).

In the early 19th century, clones of *S. officinarum* were crossed with *S. spontaneum* and backcrossed twice with *S. officinarum* as the recurrent parent to produce interspecific hybrids. *Saccharum officinarum* transmitted its somatic chromosome number during the first hybridization and the first backcross in contrast to the *S. spontaneum* clones. The chromosome number after the second backcross became normal (Bremer 1961). This process, referred to as 'nobilisation', has resulted in modern varieties with genomes that comprise multiple sets of homologous chromosomes derived from a single species (autopolyploid), as well as possessing two or more unlike sets of chromosomes (allopolyploid) (Sreenivasan et al., 1987), and having a high total chromosome number (2n = 36 to 170) with about 80% of the genome derived from *S. officinarum* and the remainder from *S. spontaneum*.

Bermudagrass (*Cynodon spp*.)

Bermudagrass (*Cynodon spp*.) is an important warm-season perennial grass that grows in the temperate and tropical regions of the world. Bermudagrass has a rhizomatous and stoloniferous growth habit (McCarty and Miller, 2002). It can grow in diverse soil and moisture conditions, withstanding drought well and also tending to eliminate other plants. Bermudagrass is a good grass to use for soil conservation because it has long runners that root at the node (Hu et al., 2005). Its origin can be traced back to sub-humid rangelands around the Indian Ocean from eastern Africa to

the East Indies. Bermudagrass was believed to have been imported to the U.S. around 1751 from Africa (McCarty and Miller, 2002).

Common bermudagrass [*Cynodon dactylon* (L.) Pers.] has 36 chromosomes and is adapted to most soils due to its fast growing habit. It is a coarse textured, dense grass that is favored for its tolerance of low mowing heights, soil salinity, traffic, drought tolerance, and rapid recovery from damage. However, it has very poor shade tolerance, a high nitrogen requirement, produces many seed heads, and it will go dormant (brown) when temperatures fall below 10 $^{\circ}$C (McCarty and Miller, 2002).

Hybrid bermudagrass, the result of an interspecific cross [*C. dactylon* (L.) Pers. X C. *transvaalensis* Burtt Davy], has 2n = 3x = 27 chromosomes (McCarty and Miller, 2002). These grasses have finer leaf textures, low growth habits and higher shoot densities. Hybrid cultivars are sterile, produce stolons and rhizomes, and have good winter color as compared to other grasses. These triploid grasses can be mowed at lower heights which recover quickly and provide a uniform consistent playing surface for golf. The first hybrid released in the U.S. was 'Tiffine' in 1953. Since then there have been numerous releases and today nearly all golf courses and sports fields use hybrids (McCarty and Miller, 2002; Unruh and Elliott, 1999).

Another hybrid 'Tifdwarf' was released in 1965 and quickly became a favorite among superintendents in the south (Burton, 1966). Tifdwarf is still used today to some extent and is a standard to which other putting green grasses are compared. In recent years several cultivars, including 'Champion', 'FloraDwarf', 'MiniVerde', 'MS Supreme' and 'TifEagle', have been released and they have shorter internode lengths, smaller leaf blades, and denser stands compared to Tifdwarf (Gray and White, 1999). These cultivars are referred to as "ultradwarfs."

The ultradwarf TifEagle was cooperatively released by the USDA-ARS and the University of Georgia Coastal Plain Experiment Station in 1997 (Hanna and Elsner, 1999). TifEagle has a dark green color, a prostrate growth habit, and can be maintained at very low mowing heights (0.30 cm) for short periods of time. TifEagle is a high maintenance grass that is prone to thatch development and requires full sun for at least six to eight hours for optimum growth. Ultradwarfs like TifEagle are best maintained on golf courses with sufficient labor and maintenance budgets (Gray and White, 1999; McCarty and Miller, 2002).

Bermudagrasses are superior pasture and fodder grasses, and they stay green during hot weather (Hu et al., 2005). 'Tifton 85' is a pentaploid hybrid forage bermudagrass (Burton et al., 1993). It is the most popular warm-season perennial grass for hay production and pastures in the southern U.S. (Taliaferro et al., 2004). It has thicker stems, broader leaves, and is sterile. It is taller than other bermudagrass cultivars, with a darker green color and very large, fast growing stolons (Burton et al., 1993). It has a higher nutritive content and is more drought resistant than coastal bermudagrass, but it is not more cold resistant. It produces best in deep, droughty sands and although it can grow in clay and backlands, they are not optimum sites. It out performs coastal bermudagrass and bahiagrass (*Paspalum notatum Fluegge*) in terms of dry matter yields (Burton et al., 1993).

Fall Armyworm, *Spodoptera frugiperda* (J.E. Smith)

Fall armyworm (FAW), *Spodoptera frugiperda* (J.E. Smith) is a significant economic pest in most of the continental U.S. It can cause substantial losses in maize (*Zea mays*), sorghum (*Sorghum bicolor*), forage grass, turf grass, rice (*Oryza sativa*), cotton (*Gossypium hirsutum* L.) and peanut (*Arachis hypogaea*) production (Luginbill, 1928; Sparks, 1979). Because it cannot survive prolonged freezing temperatures, it overwinters in southern Florida and southern Texas and then migrates annually to cause infestations in the continental U.S. (Barfield et al., 1980).

Knipling (1980) stated that if overwintering populations in Florida were the primary source of the infestations, a rigid suppression program in the overwintering areas would have a great impact on the FAW population throughout the southeastern and Atlantic coast regions.

Fall armyworm consists of two host-strains: the corn strain that feeds predominantly on corn (*Zea mays* L.), and the rice strain that feeds on smaller grasses such as rice (*Oryza sativa* L.) and bermudagrass (Pashley et al., 1985; Pashley, 1986). In Florida, insects collected throughout overwintering areas consist of both strains (Meagher and Gallo-Meagher, 2003, Meagher and Nagoshi, 2004, Nagoshi and Meagher, 2004). These two strains exhibit polymorphism at five allozyme loci (Pashley, 1986), in their mitochondrial DNA (mtDNA) (Pashley, 1989; Lu and Adang, 1996) and in their nuclear DNA (Lu et al., 1992). Recently, several restriction sites in the cytochrome oxidase 1 gene have been identified by sequence analysis and may potentially be specific to one of the two FAW host strains (Nagoshi et al., 2006).

Rice strain larvae appear to be more susceptible to transgenic *Bacillus thuringiensis* Berliner (*Bt*) cotton than corn strain larvae (Pashley et al., 1987; Adamczyk et al., 1997). Additionally, there have been distinct differences in feeding of bermudagrass genotypes in laboratory and field studies with rice strain larvae generally gaining more weight and consuming more plant material than corn strain larvae (Pashley et al. 1987; Quisenberry and Whitford 1988; Meagher et al., 2007).

Bacillus thuringiensis Berliner (*Bt*)

Bacillus thuringiensis Berliner (*Bt*) is a naturally occurring gram-positive rod shaped bacterium with pesticidal properties due to the production of toxic proteins (Glare and O'Callaghan, 2000). There are more than 60 serotypes and hundreds of different subspecies that have been described for *Bt* out of which *kurstaki* (*Btk*) is effective against lepidoptera and *israelensis* (*Bti*) is effective against Diptera. Some other species of *Bacillus* such as *popilliae* and *sphaericus* are effective against Coleoptera and Diptera, respectively (Nester et al., 2002).

During sporulation, *Bt* produces parasporal crystals that are comprised of one or more related insecticidal crystal proteins (ICPs) encoded by crystal (*cry*) and cytolytic (*cyt*) genes (Nester et al., 2002). Insect larvae feeding on plant surfaces ingest these parasporal crystals which are then dissolved in the juices of the midgut. The proteins are activated then by enzyme proteases in the juices which typically are alkaline. These activated proteins, also known as δ-endotoxins (or Cry proteins), bind to specific receptors on the insect midgut epithelium (Hofmann et al., 1988) causing a disruption in membrane integrity and ultimately death (Höfte et al., 1986).These specific receptors are responsible for the narrow host specificity (Höfte et al., 1986).

The δ-endotoxins produced in *Bt* are packaged into parasporal inclusions in their tertiary conformation. This tertiary structure consists of three different domains, I, II and III. Domain I is made of seven α-helices, domain II comprises three antiparallel β sheets that are folded into loops, and domain III is made of a β sandwich of two antiparallel β strands (de Maagd et al., 2003). Domain I, due to its membrane spanning hydrophobic and amphipathic α helices, is capable of forming pores in the cell membranes of the larval midgut. Domain II basically determines the insecticidal specificity of the toxin as it is hyper variable in nature, and domain III is involved in a variety of functions like structural stability, ion channel gating, binding to the brush border membrane vesicles, and also insecticidal specificity (Li et al., 1991). These three domains interact closely to bring about insecticidal activity of the protein.

Cry Proteins

The Cry proteins disrupt the ion regulation of the insect midgut by increasing the potassium permeability and the toxins may also form non-specific pores. These non-specific pores are permeable to small ions and molecules and these pores will enlarge forming osmotic swelling and eventually cell death (Sacchi et al., 1986).

Different Cry proteins have different levels of toxicity for different insects. This may be due to a different three-dimensional structure of the Cry protein and therefore different binding sites and binding specificities depending upon the receptor proteins. However, binding of a Cry protein does not always indicate its toxicity. Some Cry proteins may have common binding sites due to similar structures and some may have different sites.

Different *cry* genes encode different Cry proteins. There are four different classes of *cry* genes which have retained the names they received under the system of Höfte and Whiteley (1989), but with a substitution of Arabic for roman numerals. The *cry1* genes encode proteins toxic to lepidopterans; *cry2* genes encode proteins toxic to both lepidopterans and dipterans; *cry*3 genes encode proteins toxic to coleopterans; and *cry4* genes encode proteins toxic to dipterans. Under the revised system, there are currently Cry1 through Cry22 proteins (Crickmore et al., 1998).

For the Cry1 toxins, the C-terminal portion of the 133-kDa protoxin is removed by proteolysis, leaving an active toxin of ca. 65 kDa. The active toxin, located in the N-terminal half of the protoxin, binds to the insect receptors (Knight et al., 1994; Sangadala et al., 1994). In corn, Herculex I® was developed in collaboration between Dow Agro Sciences LLC and Pioneer Hi-Bred International, Inc. and it contains the *cry1F* transgene. The *cry1F* gene expresses an insecticidal protein (Cry1F) derived from *Bacillus thuriengenesis aizawai*. Expression of the *cry1Fa* gene has been shown recently to enhance resistance against FAW in bahiagrass (*Paspalum notatum Fluegge*) (Luciani et al., 2007).

Tissue Culture Systems for Sugarcane and Bermudagrasses

Generally, tissue culture protocols are a pre-requisite for successful plant transformation. Plant tissue culture and regeneration systems have been extended from dicot crop plants to many monocot species. Substantial progress in tissue culture with previously recalcitrant grasses has been made through the examination of important factors like genotype (Maddock et al., 1983;

Krumbiegel-Schroeren et al., 1984), donor plant quality (Lu et al., 1984; Zimmy and Lörz, 1989), explant type, and media composition (Eapen and Rao, 1982; Lu et al., 1984).

Vasil (1987) reviewed and generalized the key strategies to establish regenerable cell cultures for grass species. They are as follows: (i) choose explants that have meristematic tissues and undifferentiated cells such as immature embryos or seeds, leaf base meristems, and meristematic segments of young inflorescences, (ii) use culture medium supplemented with high concentrations of strong auxins, such as 2,4-dichlorophenoxyacetic acid (2,4-D) and 3,6-dichloro-o-anisic acid (dicamba) for inducing embryogenic calli, and (iii) use embryogenic calli-derived cell suspensions for protoplast isolation. These strategies have led to the development of successful regeneration systems for sugarcane and all major turfgrass species.

Among monocotyledonous crops, sugarcane was one of the first to be successfully used in tissue culture (Barba and Nickell, 1969), plant regeneration (Heinz andMee, 1969), and protoplast isolation (Nickell and Heinz, 1973). Regeneration through somatic embryogenesis or organogenesis has been well characterized (Ho and Vasil, 1983; Guiderdoni and Demarly, 1988; Taylor et al., 1995). Studies have been conducted to assess the extent of variability arising from *in vitro* regeneration and its transmission into successive generations via vegetative propagation (Lourens and Martin, 1987; Burner and Grisham, 1995). These investigations demonstrated that large amounts of somaclonal variability occur in *in vitro*-derived propagules, irrespective of the method of regeneration.

For small grass species, Dale (1980) did a pioneer study in plant regeneration with the establishment of an embryogenic culture of Italian ryegrass (*Lolium multiflorum* Lain.) from immature embryos. Even though direct somatic embryogenesis was achieved in orchardgrass (*Dactylis glomerata* L.) from mesophyll cells (Harming and Conger, 1982; Conger et al., 1983)

plant regeneration from embryogenic callus is the single most important path for turfgrass, as many major bermudagrass species have been regenerated in this way.

Bermudagrasses have similar culture medium requirements as those for other grass species. Generally high salt nutrient solutions such as the Murashige and Skoog (MS) medium, the most frequently used medium for bermudagrass, and modified CC-medium with 3 to 12% sucrose, as well as high concentrations of strong auxins such as 2, 4-D and dicamba have been used (Murashige and Skoog, 1962; Potrykus et al., 1979; Nielsen and Knudsen, 1993). To promote callus initiation in turf grass species, cytokinins such as 6-benzylaminopurine (6-BA) and N-(2-furanylmethyl) - lH-purin-6-amine (kinetin), at low concentration, in combination with auxins is used. Addition of casein hydrolysate to the culture medium was found to be beneficial for embryogenic callus initiation (Artunduaga et al., 1988). Shetty and Asano, (1991) showed that proline and glutamine have stimulatory effects on callus induction among the organic N compounds tested in *Agrostis alba* L.

Callus induction and plant regeneration from young inflorescences of common and hybrid bermudagrass was first reported by Ahn et al. (1985). Although calli have been readily induced from vegetative tissues such as nodal segments of some turf type bermudagrass varieties (Chaudhury and Qu, 2000), regenerable callus mostly has been obtained from immature inflorescence culture (Ahn et al., 1985, 1987; Artunduaga et al., 1988, 1989; Chaudhury and Qu, 2000; Li and Qu, 2002). Albinism has also been encountered during plant regeneration (Artunduaga et al., 1988). Artunduaga et al. (1989) reported an improved tissue culture response of the common bermudagrass cultivar Zebra by using a combination of 13.6 µM 2, 4-D and 200 mg l^{-1} casein hydrolysate in the culture medium. Chaudhury and Qu (2000) substantially improved green plantlet regeneration of turf-type bermudagrass by lowering the level of 2, 4-D (4.5 µM) and

adding BAP (0.04 µM) to the MS (Murashige and Skoog 1962) callus induction medium. Li and Qu,(2002) showed that somatic embryo formation of the hybrid bermudagrass variety Tifgreen can be further improved by including ABA in the callus induction medium and the germination/regeneration of somatic embryos was accelerated by supplementing Gibberellic acid in the regeneration medium. They also observed repetitive somatic embryogenesis in tissue culture of common bermudagrass variety Savannah and showed that somatic embryogenesis is a major route for plant regeneration in bermudagrass revealed through scanning electron microscopic studies.

A callus-free method for the production of transgenic plants from bermudagrass (*Cynodon* spp.) and creeping bentgrass (*Agrostis stolonifera*) was developed by Wang and Ge (2005). They used stolon nodes as explants and successfully bypassed the callus formation phase by direct infection of stolon nodes with *Agrobacterium* followed by rapid regeneration of transgenic plants. Also in Zoysiagrass (*Zoysia japonica* steud.), direct shoot formation from stolons was used to develop an efficient transformation method using *Agrobacterium* (Ge et al., 2006).

Biolistics and *Agrobacterim*-mediated Transformation

To develop transgenic crops, a foreign gene(s) must stably be integrated into the genome. In order to do that, usually an efficient protocol for plant regeneration has to be established, as mentioned above, as well as effective DNA delivery, transgenic tissue selection, and recovery of normal, fertile phenotypes. These protocols should be highly reproducible so that they can be used on a large scale and in a short time frame.

In monocot transformation, the genes encoding hygromycin phosphotransferase (*hpt*), phosphinothricin acetyltransferase (*pat* or *bar*), and neomycin phosphotransferase (*npt II*) are the most commonly used as selectable markers. These genes driven by constitutive promoters such as the cauliflower mosaic virus (CaMV) 35S promoter or the maize ubiquitin promoter, work

effectively for selection of transformed cells (Cheng et al., 2004). Currently, two DNA delivery methods are the most widely employed: biolistic transformation, and *Agrobacterium*-mediated transformation.

Biolistic or microprojectile bombardment technology involves propelling high velocity micron sized tungsten or gold particles coated with foreign DNA into target plant tissues. These microprojectiles pass through the plant cell wall and nuclear envelope by simple diffusion mechanism to release and integrate the foreign DNA into the plant genome. The original observation was by Klein et al. (1987) indicating that tungsten particles could be used to introduce macromolecules into epidermal cells of onion (*Allium cepa*) with subsequent transient expression of enzymes encoded by these compounds. Shortly thereafter, Christou et al. (1988) demonstrated that this process could be used to deliver biologically active DNA into living cells and produce stable transformants.

The foreign DNA used in biolistic experiments consists of a plant expression cassette inserted into a vector such as a high copy number bacterial cloning plasmid. Either the entire plasmid or the minimal expression cassette without vector backbone can be delivered to the target tissues. DNA integration is in two stages: DNA transfer followed by DNA integration into the genome. DNA integration is less efficient than DNA transfer because DNA may enter the cell and be expressed for a short time (transient expression), but never integrate into the host genome and eventually it will be degraded by nucleases. So transient expression following microprojectile bombardment with a reporter gene such as *gusA* or *gfp* often is used to optimize gene transfer parameters or compare different expression constructs (Altpeter et al., 2005).

Microprojectile bombardment has several advantages over *Agrobacterium*-mediated gene transfer. It provides a wide range of transformation strategies, and no vector sequences are

required for efficient delivery of foreign DNA (Altpeter et al., 2005). Also, due to its physical nature, microprojectile bombardment is not limited by a pathogen-host interaction that characterizes *Agrobacterium*-mediated transformation. Therefore, it is used on a broad range of targets including not only those plants considered as recalcitrant 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). Addtionally, different cell types can be targetted by microprojectile bombardment and multiple gene constructs can be delivered simultaneously. According to a review by Dafny-Yelin and Tzfira (2007), 12 different plasmids were used to deliver 12 different gene constructs in soybean (*Glycine max* L.) embryogenic suspension cultures (Hadi et al., 1996), and there has been successful integration of 11 different transgenes with a mixture of 14 different plasmids in rice embryogenic cultures (Chen et al., 1998).

Investigations of microprojectile-mediated transformation by Franks and Birch (1991) in Australia led to the development of the first transgenic sugarcane plants from a commercial cultivar in 1992 (Bower and Birch, 1992). Subsequently, microprojectile-mediated transformation of several commercially cultivated sugarcane genotypes has been reported from a number of laboratories worldwide (e.g. Birch and Maretzki, 1993; Gallo-Meagher and Irvine, 1996; Bower et al., 1996; Birch, 1997; Irvine and Mirkov, 1997; Joyce et al., 1998a, b; Nutt et al., 1999; Jain et al., 2007). Among the different explants used, embryogenic callus appears to be the preferred target due to its high transformability and regenerability. Additionally, immature leaf whorls and inflorescences have been successfully used for microprojectile-mediated sugarcane transformation (Elliott et al., 2002; Lakshmanan et al., 2003).

The production of stable transgenic forage and turf grasses through microprojectile bombardment has also been achieved and include tall fescue (*Festuca arundinacea* Schreb.) (Takamizo et al., 1995; Ha et al., 1992), red fescue (*Festuca rubra* L.) (Spagenberg et al., 1995), ryegrass (*Lolium perenne* L.) (Ye et al., 1997), bermudagrass [*Cynodon dactylon* (L.) Pers.] (Zhang et al., 2003) and creeping bentgrass (*Agrostis palustris*) (Xiao and Ha, 1997). Specifically, earlier studies 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). Altpeter and Xu (2000) developed robust protocols with efficient selection systems to generate large numbers of red fescue plants using the *nptII* gene and paromomycin as the selective agent. 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 obtained the highest transformation efficiencies (4-11%) using calli derived from immature inflorescences and embryos in nine to 12 weeks.

Production of transgenic common and triploid bermudagrass via biolistics has met with variable success probably due to rather inefficient transformation, regeneration and selection protocols (Li and Qu, 2004). A successful transformation system for TifEagle was established using embryogenic callus as recipient material producing homogenously transgenic plants showing stable transcription of the *hpt* gene following biolistics (Zhang et al., 2003). A herbicide resistance protocol was also developed for biolistic transformation of TifEagle using the *bar* gene producing 89 transgenic plants (Goldman et al., 2004b).

Agrobacterium tumefaciens is a gram-negative, soil phytopathogen that produces a crown-gall disease and naturally infects different dicotyledonous plants. This disease is characterized by the tumorous growth of plant tissues in the stem due to the transfer of the T-DNA region of the

tumor-inducing (Ti) plasmid. The T-DNA contains genes encoding enzymes involved in the synthesis of growth regulators that induce plant cell growth and tumor formation, and the production of opines that support bacterial growth (Zupan et al., 2000).

The first achievement in *Agrobacterium* transformation was the removal of wild type T-DNA from Ti plasmids to generate 'disarmed strains' (Hoekema et al., 1983). Initially, *Agrobacterium*-mediated transformation was only successful in dicots because they are the natural hosts for the bacterium. Therefore, to overcome the host-plant specificity, *Agrobacterium*-mediated transformation was optimized through the use of hypervirulent strains, virulence gene inducing agents (i.e. acetosyringone), effective elimination of *Agrobacterium* after cocultivation, and antioxidants and cysteine in the co-culture medium (Hiei et al. 1994; Ishida et al. 1996; Tingay et al. 1997; Frame et al. 2002). Additionally, to improve transformation efficiency, specific protocols for bacterial infection, inoculation, co-cultivation, plant selection and regeneration were developed according to the specific requirements of the bacterial strain used and the plant host to be transformed. Consequently, the *Agrobacterium* host range has been extended to important crop plants such as corn, rice, wheat (*Triticum aestivum*), barley (*Hordeum vulgare*), sugarcane and other grasses (Hansen and Wright, 1999; Newell, 2000; Gelvin, 2003).

Although efforts were made initially by Birch and Maretzki (1993) to produce transgenic sugarcane plants using *Agrobacterium*, Arencibia et al. (1998) were the first to regenerate normal transgenic sugarcane plants from co-cultivation of calluses with LBA 4404 and EHA 101 strains. Their success was based on the use of young regenerable callus as target tissue and pre-induction of organogenesis and somatic embryogenesis to increase the T-DNA transfer process. Enriquez-Obregon et al. (1998) reported the production of transgenic sugarcane plants resistant to phosphinothricine (PPT), the active compound of the herbicide BASTA using the meristematic

region of sugarcane treated with antinecrotic compounds such as silver nitrate and abscorbic acid. More recently, transgenic plants carrying *bar* and *gus* under control of the CaMV 35S promoter and *npt*II driven by the *nos* (nopaline synthase) promoter were generated with a high efficiency (~50%) using axillary meristems of sugarcane inoculated with either *Agrobacterium* LBA 4404 or EHA 105 strains (Manickavasagam et al., 2004).

A. tumefaciens also has been used successfully to transform perennial grasses like creeping bentgrass (Yu et al. 2000), switchgrass (*Panicum virgatum* L.; Somleva et al. 2002), zoysiagrass (Toyama et al., 2003; Ge et al., 2006), tall fescue (Bettany et al., 2003), perennial ryegrass (Altpeter, 2006; Bajaj et al., 2006), bermudagrass (Ge and Wang, 2005; Hu et al., 2005) and orchardgrass (Lee et al., 2006). Wang and Ge (2005) made substantial improvements in the *Agrobacterium*-mediated transformation of bermudagrass and creeping bentgrass (*Agrostis stolonifera*) by using stolon nodes to produce transgenic green shoots. This method bypassed the callus formation phase thereby saving time, and resulted in transgenic plantlets within seven weeks with a transformation efficiency of 4.8 % - 6.1 % for bermudagrass and 6.3 % - 11.3 % for creeping bentgrass. Ge et al. (2006) also used stolon node explants to produce transgenic zoysiagrass (*Zoysia japonica* Steud.) plants within 10-12 weeks with a transformation efficiency of 6.8 %. Other *Agrobacterium*-mediated transformation protocols that bypass tissue culture by using *in vivo* inoculations have been developed in the model species *Arabidopsis thaliana* using the 'floral dip' method (Clough and Bent, 1998), *Medicago truncatula* via infiltration of seedlings (Wang et al., 1996), and *Nicotiana tabacum* (Dasgupta et al., 2001). All of these methods have the added advantage that generally transgene integration patterns are simpler compared with those transgene integration patterns produced following tissue culture and transformation.

CHAPTER 3
COMPARATIVE ANALYSIS OF DIRECT PLANT REGENERATION FROM YOUNG LEAF SEGMENTS OF THREE DIFFERENT SUGARCANE CULTIVARS

Introduction

Sugarcane (*Saccharum* spp. hybrid) is a complex aneu-polyploid hybrid of noble sugarcane *S. officinarum* (2n = 70-122) and *S. spontaneum* (2n = 36-128). Due to its adaptability to both tropical and subtropical conditions, sugarcane is considered one of the most efficacious biomass crops and is now being targeted for use in biofuel production along with its traditional use as a sugar crop (Nonato et al., 2001; McQualter et al., 2004 Lakshmanan et al., 2005). The complex ploidy level and low fertility of sugarcane make breeding for improved cultivars difficult; therefore it is a superior candidate for improvement through genetic engineering.

Much research has been done to develop efficient genetic transformation systems for sugarcane (e.g. Chen et al., 1987; Bower and Birch, 1992; Rathus and Birch, 1992; Birch and Maretzki, 1993; Gambley et al., 1993, 1994; Birch, 1997; Arencibia et al., 1998; Enriquez-Obregon et al., 1998). Different transformation techniques using electroporation (Rathus and Birch, 1992), polyethylene glycol (PEG) treatment (Chen et al., 1987), microprojectile bombardment (e.g. Franks and Birch, 1991) and *Agrobacterium*-mediated transformation (Arencibia et al., 1998; Elliott et al., 1998; Manickavasagam et al., 2004; Santosa et al., 2004; Zhangsun et al., 2007) have been used to introduce transgenes into sugarcane cells and callus. However to-date, most transgenic sugarcane has been produced using microprojectile bombardment with traits such as herbicide resistance (Chowdhury and Vasil, 1992; Gallo-Meagher and Irvine, 1996; Falco et al., 2000; Leibbrandt and Snyman, 2003) and insect resistance (Sétamou et al., 2002; Falco and Silva-Filho, 2003; Weng et al., 2006; Christy et al., 2009).

For most grasses like sugarcane, callus induction and plant regeneration from the induced callus is not only time consuming but can cause somaclonal variation (Spangenberg et al., 1998;

Choi et al., 2000; Goldman et al., 2004b). As discussed in Lakshmanan et al. (2006), the first report of large variations in both chromosome number and morphological traits in sugarcane plants regenerated from callus was by Heinz and Mee (1971). Since then, there have been frequent reports of genetic variability in tissue-cultured sugarcane (Lourens and Martin, 1987; Burner and Grisham, 1995; Taylor et al., 1995; Hoy et al., 2003), and numerous studies to assess the extent of variability arising from *in vitro* regeneration and its transmission into successive generations via vegetative propagation (Lourens and Martin, 1987; Burner and Grisham, 1995). These investigations demonstrated that large amounts of somaclonal variability occur in *in vitro*-derived propagules, irrespective of the method of regeneration. Gilbert et al. (2009) reported the first successful gene transfer of sugarcane yellow leaf virus resistance in sugarcane and also the first report of variations in microsatellite repeat number associated with regeneration from embryogenic callus. Consequently, in sugarcane there is a need to develop a genotype-independent explant source that can be used for *Agrobacterium*-mediated transformation and form shoots directly in a manner similar to that reported in Lakshmanan et al. (2006). Such a system has been used successfully for grasses other than sugarcane thereby saving time while also increasing transformation efficiency and decreasing somaclonal variation (Ge et al., 2006; Wang and Ge, 2005).

In this study, three sugarcane cultivars, CP84-1198, CP88-1762 and CP89-2143, were compared for their ability to undergo direct shoot and root regeneration. The efficiency of their direct regeneration response was examined relative to the explant source and its orientation on the culture medium.

Materials and Methods

Preparation of Explants and Culture Conditions

Shoot tops of four- to eight-month-old, field-grown sugarcane cultivars, CP84-1198, CP88-1762 and CP89-2143, were obtained from the U.S. Sugarcane Field Station (USDA-ARS, Canal Point, Florida). After removing the outer mature leaves, the shoot tops were cut just below the first node and a 9 cm segment was treated with absolute ethanol for 1 min followed by immersion in 20% Clorox solution (6% sodium hypochlorite as active ingredient) containing two drops of Tween-20 for 20 min followed by four to five washes in sterile distilled water for 10 min each. From a single top, five different explants numbered 1 to 5, with 1 being the explant closest to the first node and 5 being the farthest from the meristem, were examined for direct regeneration (Figure 3-1A). Each explant was 0.5 cm in length and 1 cm in diameter. The five explants were placed on the same Petri plate (100 mm × 25 mm) containing a medium reported in Gill et al. (2006) that consisted of Murashige-Skoog (MS) basal salts (Sigma, St. Louis, MO) supplemented with 5 mgL^{-1} naphthalene acetic acid and 0.5 mgL^{-1} kinetin along with 2% (w/v) sucrose plus 5.6 mgL^{-1} Agargel ^{TM} (Sigma, St. Louis, MO). The pH of the medium was adjusted to 5.8 before autoclaving. All plates were incubated at 26 °C under a 16 hr photoperiod provided by cool white florescent tubes with a photon flux density of 5170 lux.

For each experiment, 24 shoot tops per cultivar were sectioned into 120 explants with 60 placed vertically and 60 placed horizontally (Figure 3-1B) onto the medium (Figure 3-1C). The effect of explant orientation was examined as well as the direct regeneration response of explants 1-5. Visual observations were taken every week and regeneration rates were determined. At the end of four weeks, the number of shoots per explant, the number of shoots greater than 1 cm per explant, and the number of roots per explant were obtained. The complete experiment was repeated three times on different starting dates. Data were analyzed with the SAS PROC MIXED

procedure using the LSMeans statement modified with Tukey's adjusted t-test to separate treatment means of main effects (Littell et al., 1996; SAS Institute, 2002). Unless otherwise specified, a probability of 1% was used to determine statistical differences.

Results

Shoot Production

There were significant differences in shoot production among the three cultivars ($P < 0.0001$; Table 3-1). CP88-1762 produced the most shoots (50; Table 3-1; Figure 3-2A), followed by CP89-2143 (18; Table 3-1; Figure 3-2B), and CP84-1198 (9; Table 3-1). With regard to position of the explants from the first node, all cultivars displayed a similar linear trend with explant 1 (closest to the meristematic region) producing the most shoots (34), and explant 5 (19) producing the fewest shoots (Table 3-1). However, there was a significant interaction between explant orientation and cultivar ($P < 0.0015$), and explant position and cultivar ($P < 0.0001$) with regard to the number of shoots produced. Because of these interactions, separate analyses for each cultivar was done for the number of shoots produced by explant orientation and position.

In terms of explant orientation for the least responsive cultivar CP84-1198, the horizontal orientation (10) produced more shoots per explant than the vertical orientation (8; Table 3-2). Likewise for CP89-2143, the horizontal orientation (20) was better at producing shoots than the vertical orientation (16; Table 3-2). Conversely, for the most responsive cultivar CP88-1762, the vertical orientation produced more shoots (56) than the horizontal orientation (45; Table 3-2).

In terms of explant position, all cultivars produced more shoots from explant 1 than from explant 5. However, CP88-1762 produced 26 more shoots from explant 1 versus explant 5 compared to only 8 more for CP84-1198 and 13 more for CP89-2143 (Table 3-2). This indicates that CP88-1762 is more responsive to explant distance from the shoot than other cultivars.

Shoot Elongation

The results for shoot elongation paralleled the results for shoot induction with significant differences among the three cultivars ($P < 0.001$; Table 3-1). CP88-1762 produced the most shoots greater than 1 cm (36) followed by CP89-2143 (12) and CP84-1198 (6; Table3-1). With regard to the position of the explants, explant 1 produced the most elongated shoots (24), while explant 5 produced the least number of elongated shoots (12; Table3-1). However there was a significant interaction between the orientation and the cultivar ($P < 0.0001$) and the explant position and the cultivar for the number of shoots greater than 1cm produced per explant ($P < 0.0001$). Consequently, analysis for each cultivar was done for the number of shoots greater than 1cm per explant. These two-way interactions are described in the next two paragraphs.

The horizontal explant orientation, produced more shoots greater than 1 cm (7) than the vertical orientation in CP84-1198 (5; Table 3-3) but only slightly. Conversely the vertical orientation produced 11 more elongated shoots (42) than horizontal orientation (31) in CP88-1762. Explant orientation made no difference in the number of elongated shoots produced from CP89-2143,

Regarding explant position, the results were similar for all three cultivars with explants at position 1 producing more elongated shoots than the explants at position 5 (Table 3-3). However, the magnitude of the difference between the number of elongated shoots produced from explant 1 versus explant 5 varied among the cultivars indicating a difference in response to explant position among cultivars. CP88-1762 produced more than 46 elongated shoots from explant 1 and only 26 shoots from explant 5, a difference of 20 shoots (Table 3-3). In contrast, CP89-2143 produced only 10 more shoots greater than 1 cm at position 1 (18) than at position 5 (8; Table 3-3). Similarly, CP84-1198 produced only 4 more elongated shoots at position 1 (8) compared to position 5 (4; Table 3-3).

Root Induction

There were dramatic differences in root production among the three cultivars. CP84-1198 produced the most roots (41; Figure 3-2C), followed by CP88-1762 (28) and CP89-2143 (9; Table 3-1). With regard to explant position, explant 1 produced the most roots (30) as compared to explant 5 (21) which produced the fewest roots per explant (Table3-1). However, there was a significant interaction between explant orientation and cultivar ($P < 0.0001$) and explant position and cultivar on the number of roots produced per explant ($P < 0.0001$). Due to this, analysis by cultivar was done for the number of roots per explant.

For all three cultivars, the vertical orientation produced more roots than the horizontal orientation (Table 3-4). However, vertically oriented explants of CP84-1198 produced 52, more roots than horizontally oriented explants whereas CP88-1762 produced only 12 more and CP89-2143 produced only 2 more than the vertical position (Table 3-2).

Regarding the effect of explant position on root production, the results were similar for CP94-1198 and CP88-1762 with explants at position 1 producing more roots than explants at position 5 in a general trend of decreasing root production as the distance from the meristem increased. However, CP89-2143 produced almost the same number of roots regardless of explant position (Table 3-4).

Discussion

Sugarcane biotechnology research began in the 1960s and it was one of the first plant species to be successfully cultured and regenerated *in vitro* (Barba and Nickell, 1969; Heinz and Mee, 1969). It has been studied extensively with regard to the development of efficient procedures for both micro propagation and transformation (e.g. Ho and Vasil, 1983; Lee, 1987; Burner and Grisham, 1995; Chengalrayan and Gallo-Meagher, 2001). Previous research has shown that it takes eight weeks to produce regenerable callus (Burner and Grisham, 1995) and 10 weeks to

produce shoots from sugarcane callus on medium containing thidiazuron (Chengalrayan and Gallo-Meagher, 2001). Also a great deal of time and effort is required to maintain the callus (Lee, 1987; Burner and Grisham, 1995; Garcia et al., 2006).

Consequently, there has been considerable effort to develop direct plant regeneration methods for sugarcane. Successful attempts have been made using a transverse thin cell layer culture system to produce plantlets that bypass the callus phase (Lakshmanan et al., 2006). In their study, explants that were 1cm in diameter and 1-2 mm in length produced more shoots compared to explants that were 5 mm in length. Their results showed that the potential of leaf tissue to form shoots is greatly influenced not only by the size of the explants, but also by tissue polarity. The basal end which is closer to the shoot apical meristem was significantly more prolific than the middle and the distal segments. Similar results were found in the present study as the explants closer to the meristem or the first node produced more shoots than those farthest from the first node. Lakshmanan et al. (2006) also showed that shoot regeneration was significantly reduced when the explants were cultured with their proximal sides in direct contact with the medium due to possible oxygen tension developing in the meristematic cells at the explant-medium interface. Due to their results, we maintained our explants with their distal ends in contact with the medium. Lakshmanan et al. (2006) also found that NAA was the best among a number of auxins tested for sugarcane shoot production. In the present work, 5 mgL^{-1} NAA along with 0.5 mgL^{-1} of kinetin was used and these hormone concentrations were previously shown to produce large numbers of shoots and roots for different sugarcane cultivars (Gill et al., 2006).

Among the three cultivars used in this study, CP88-1762 was the most responsive to the tissue culture system (Table 3-1). It produced the highest number of shoots and the most elongated shoots, along with a sufficient number of roots to produce viable plantlets that were easily

transferred to small pots within eight weeks. Therefore, of the three cultivars tested, CP88-1762 is the best candidate for an *Agrobacterium*- mediated transformation system using this direct regeneration approach. Although CP89-2143 took longer to regenerate and produced fewer shoots and roots, it did make viable plantlets and could also be used for transformation. However, under the conditions used in this study, CP84-1198 could produce roots but directly from the explants and not from any shoots that formed, therefore viable plantlets were difficult to obtain (Table 3-1).

There was production of phenolics by all cultivars, but CP84-1198 produced more phenolics than the other two cultivars and that may have contributed to its poor shoot induction. Phenolics are intermediates of phenylpropanoid metabolism (Cvikrova et al., 1998) and precursors of lignin (Lewis and Yamamoto, 1990). Their deposition in cell walls is an important defense mechanism after pathogen infection (Bolwell et al., 1985). Although CP84-1198 is prolific at producing embryogenic callus under different media formulations (e.g. Chengalrayan and Gallo Meagher, 2001), it is not effective at direct regeneration under the culture conditions used in this study.

From this work, a general recommendation for direct regeneration of sugarcane, when using the culture conditions described, would be to select shoot explants that are close to the meristem, orient them vertically with their distal ends in contact with the medium. However, cultivars should be independently evaluated because there can be significant genotype effects. Future work will employ CP88-1762 for *Agrobacterium*-mediated transformation using this direct regeneration protocol.

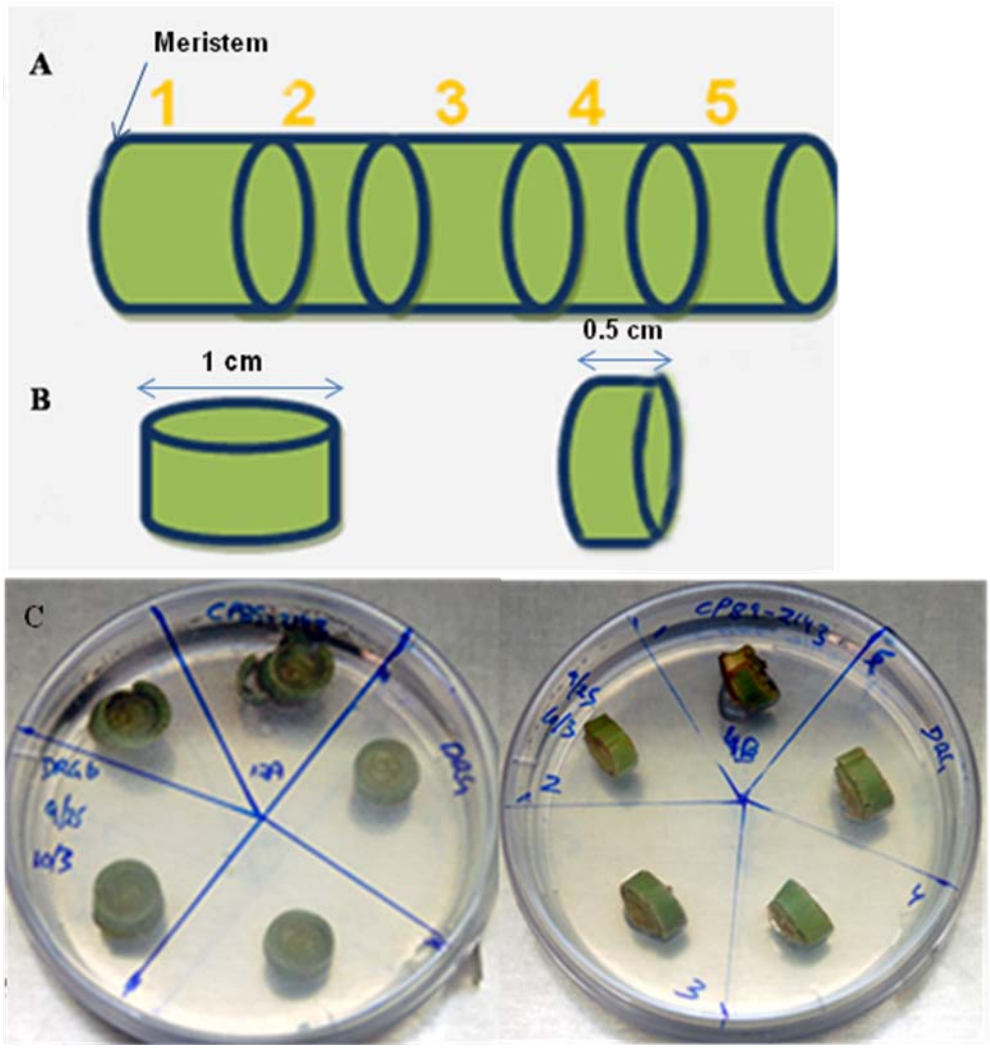


Figure 3-1. Sugarcane explants used for direct regeneration. A) Shoot section indicating the position of explants 1-5, B) Vertical orientation (left, 1 cm diameter) and horizontal orientation (right, 0.5 cm length), and C) Explants 1-5 placed in a vertical, left) or a horizontal (right) orientation.

Table 3-1. Number of shoots, number of shoots > 1cm, and number of roots per explant for three different sugarcane cultivars, five explants and two explant orientations.

	Number of Shoots	Number of Shoots > 1 cm	Number of Roots
Cultivar			
CP84-1198	9 [c]	6 [c]	41 [a]
CP88-1762	50 [a]	36 [a]	28 [b]
CP89-2143	18 [b]	12 [b]	9 [c]
Explant Position			
1	34 [a]	24 [a]	30 [a]
2	29 [b]	21 [b]	28 [b]
3	25 [c]	17 [c]	26 [c]
4	22 [d]	15 [d]	25 [c]
5	19 [e]	12 [e]	21 [d]
Orientation			
Horizontal	25 [b]	17 [a]	15 [b]
Vertical	26 [a]	19 [a]	37 [a]

Same letters in a column within a category are not significant at $P = 0.05$ using the Tukey-Kramer adjusted t-test.

Table 3-2. Number of shoots per explant analyzed for each sugarcane cultivar.

	CP84-1198	CP88-1762	CP89-2143
Orientation			
Horizontal	10 [a]	45 [b]	20 [a]
Vertical	8 [b]	56 [a]	16 [b]
Explant Position			
1	13 [a]	64 [a]	26 [a]
2	11 [b]	56 [b]	19 [b]
3	9 [bc]	49 [c]	16 [bc]
4	8 [c]	44 [c]	15 [c]
5	5 [d]	38 [d]	13 [c]

Same letters in a column within a category are not significant at $P = 0.05$ using Tukey-Kramer adjusted t-test.

Table 3-3. Number of shoots > 1cm per explant analyzed for each sugarcane cultivar.

	CP84-1198	CP88-1762	CP89-2143
Orientation			
Horizontal	7 [a]	31 [b]	13 [a]
Vertical	5 [b]	42 [a]	12 [a]
Explant Position			
1	8 [a]	47 [a]	18 [a]
2	7 [ab]	42 [a]	13 [b]
3	6 [bc]	36 [b]	10 [bc]
4	5 [c]	30 [c]	11 [bc]
5	4 [d]	26 [d]	8 [c]

Same letters in a column within a category are not significant at $P = 0.05$ using the Tukey-Kramer adjusted t-test.

Table 3-4. Number of roots per explants analyzed for each sugarcane cultivar.

	CP84-1198	CP88-1762	CP89-2143
Orientation			
Horizontal	15 [b]	22 [b]	8 [b]
Vertical	67 [a]	34 [a]	10 [a]
Explant Position			
1	48 [a]	33 [a]	10 [a]
2	44 [b]	31 [ab]	10 [a]
3	41 [bc]	29 [b]	7 [c]
4	38 [c]	26 [c]	11 [a]
5	33 [d]	23 [cd]	8 [abc]

Same letters in a column within a category are not significant at $P = 0.05$ using the Tukey-Kramer adjusted t-test.

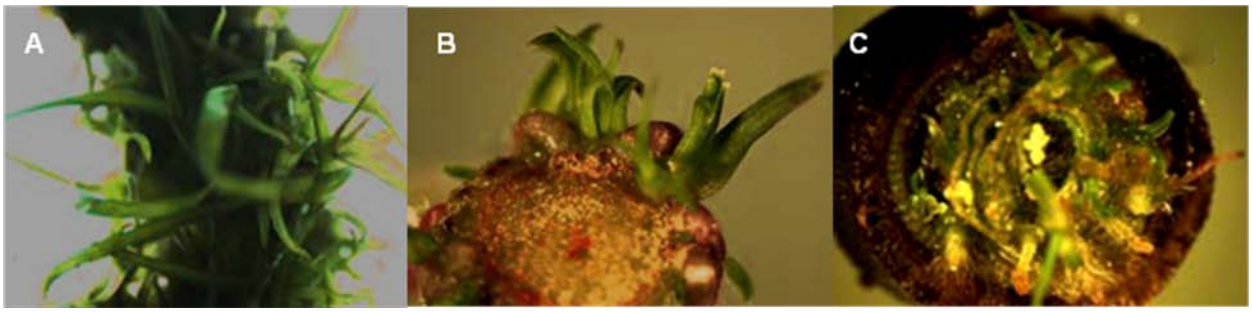


Figure 3-2. Direct organogenesis from sugarcane leaf roll explants. A) CP88-1762 with a large number of shoots, B) CP89-2143 showing direct shoot organogenesis, and C) CP84-1198 displaying both shoot and root organogenesis.

CHAPTER 4
EXPRESSION OF *CRY1Fa* TO ENHANCE FALL ARMYWORM RESISTANCE IN SUGARCANE

Sugarcane (*Saccharum* spp. hybrid) is one of the most photosynthetically efficient plants cultivated in over 120 countries in both the tropics and subtropics, and covers a land area of more than 32 million acres (Cordeiro et al., 2001). It is also one of the oldest crops known to mankind and provides over 70% of the sugar produced in the world. In 2008, sugarcane in the U.S. was grown and harvested on approximately 869,000 acres with a production of 29,855,000 tons. Florida produced the most sugarcane (15,600,000 tons), followed by Louisiana, Hawaii and Texas (http://www.nass.usda.gov/QuickStats/PullData_US.jsp). Both sugar production and consumption in the U.S. have increased steadily by about 2.3% annually since 1994 (Haley and Bolling, 2005). Currently, sugarcane in the U.S. is being considered as a promising biofuel crop.

In order to maintain high yields, sugarcane must be resistant to a number of insect pests. One of these is the noctuid moth fall armyworm (FAW), *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae), a significant economic pest in most of the continental U.S. Because it cannot survive prolonged freezes, FAW overwinters in southern Florida and southern Texas and then migrates annually to cause infestations in the continental U.S. (Barfield, 1980).

There are two host-strains of FAW, the corn strain that feeds predominantly on corn (*Zea mays* L.), and the rice strain that feeds on smaller grasses such as rice (*Oryza sativa* L.) and bermudagrass (*Cynodon dactylon* L.) (Pashley et al., 1986; Pashley et al., 1988). In Florida, insects collected throughout the overwintering areas are of southern Florida are of both strains (Meagher and Gallo-Meagher, 2003; Nagoshi and Meagher, 2004).

Conventionally, integrated pest management strategies that are used to control insect pests such as FAW do so through the combined use of biological control agents that include predators, parasitoids and pathogens, along with pesticides and resistant varieties. Biological control agents

kill or debilitate their host and are relatively specific to certain insect groups. On the other hand, the extensive use of pesticides produces adverse effects on human health as well as the surrounding environment including the development of insect resistance and eradication of other beneficial insects (Ranjekar et al., 2003; Ferry et al., 2006). Another management strategy for controlling pests such as FAW is by producing transgenic plants expressing a *Bacillus thuringenesis* (*Bt*) δ-endotoxin. These *Bt* δ-endotoxins are also known as insecticidal crystal proteins or the *Bt* crystal protein (Cry) (Dulmage, 1981). On the basis of amino acid sequence homologies and phylogenetic relationships, the Cry proteins are classified into four groups providing protection against three insect orders: Cry1 (Lepidoptera), Cry2 (Lepidoptera and Diptera), Cry3 (Coleoptera) and Cry4 (Diptera).

Crops such as corn, cotton (*Gossypium hirsutum* L.), and soybean (*Glycine max* L.) expressing *Bt* δ-endotoxin genes (*cry*) have been extensively released in the U.S. and other countries (James, 2005). Commercially available *Bt* corn contains *cry1Ab*, *cry1Ac*, or *cry3 Bb1*, and stacked *cry 1Ab* and *cry3B1* genes for controlling European corn borer [*Ostrinia nubilalis* Hübner], southwestern corn borer [*Diatraea grandiosella* Dyar] and corn rootworm [*Diabrotica barberi* Smith & Lawrence]. *Bt* cotton contains *cry1Ac*, stacked *cry1Ac* and *cry2Ab2*, and stacked *cry1Ac* or *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 also been reported to control FAW in cotton (Adamczyk and Gore, 2004). Transgenic FAW-resistant plants expressing Cry proteins in forage and turf grasses also have been reported to reduce larval weights in the diploid bahiagrass cultivar “Pensacola”, (*Paspalum notatum* var. saurae) (Luciani et al., 2007), and seashore paspalum (Altpeter et al., 2009).

The objective of this research was to introduce *cry1Fa* into sugarcane, to characterize transgenics at the molecular level, and to determine their resistance to the rice strain of FAW. Consequently, transgenics produced were evaluated for transgene integration and expression, as well as resistance to FAW in laboratory bioassays under controlled environmental conditions.

Materials and Methods

Plant Material

Shoot tops of four- to eight-month-old, field-grown sugarcane cultivars CP84-1198, CP88-1762 and CP89-2143 were obtained from the U.S. Sugarcane Field Station (USDA-ARS, Canal Point, Florida). After removing the outer mature leaves, shoot tops were cut slightly below the first node and a 9 cm segment was treated with absolute ethanol for 1 min to remove outer contamination followed by immersion in 20% Clorox (sodium hypochlorite 6% as an active ingredient) containing two drops of Tween-20 for 20 min with continous stirring. Following sterilization, explants were washed four to five times in sterile distilled water for 10 min each.

Transgene Expression Constructs

The selectable marker cassette in pHZ35SNPTII contains the neomycin phosphotransferase II (*nptII*) coding sequence (Beck et al., 1982) under the transcriptional control of the CaMV 35S promoter (Odell et al., 1985) along with the heat shock protein 70 (*hsp* 70) intron (Rochester et al., 1986), and the CaMV 35S polyadenylation signal (Dixon et al., 1986; Figure 4-1A).

Based on the *cry1Fa* sequence available in the NCBI database (M73254), a codon optimized sequence for the δ-endotoxin was generated. The synthetic *cry1Fa* (1863 bp) was made and sub-cloned into a pPCR-Script vector by Geneart (Regensburg, Germany). *BamHI* and *HindIII* sites were introduced 5' and 3' to the *cry1Fa* coding sequence, respectively, to facilitate sub-cloning of *cry1Fa* into pHZCRY under the transcriptional control of the maize ubiquitin I promoter and 1st

intron (Christensen etal., 1992), and the *nos* 3' untranslated region (Fraley et al., 1983; Figure 4-1B).

Tissue culture, Transformation and Regeneration of Sugarcane

From each top, 0.5 cm explants were taken and placed onto a Petri plate (100 mm x 20 mm) containing Murashige and Skoog (MS) basal medium supplemented with 20 gL^{-1} sucrose and 13.6 µM 2,4-dichlorophenoxy acetic acid (2,4-D) with the pH adjusted to 5.6-5.8 (CI_3 medium; Chengalrayan and Gallo-Meagher, 2001). Explants were subcultured every two weeks. Callus induction occurred between two to four weeks and embryogenic callus was formed on the same medium after four to six weeks (Figure 4-2A). The embryogenic calli were placed on CI_3 medium supplemented with 0.4 M sorbitol for four to six hr prior to bombardment (Figure 4-2B). The *nptII* and *cry1Fa* plasmids were used in a 1:2 molar ratio and co-precipitated onto 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 1100 psi and 28 mm Hg. Bombarded calli were transferred onto fresh CI_3 medium and kept at 28 ^oC under a 16 hr/8hr light/dark photoperiod provided by cool white florescent tubes with a photon flux density of 5170 lux for one week. The calli were then transferred onto CI_3 selection medium containing 25 mg L^{-1} geneticin (Tadesse et al., 2003). Optimal antibiotic concentration for calli growth and inhibition of plant regeneration from non-transformed material was determined previously (data not shown). After two weeks, the geneticin concentration was increased to 50 mg L^{-1}. After three to four weeks, the surviving calli were transferred onto shoot initiation medium containing MS medium supplemented with 2.5 µM thidiazuron (TDZ) (Chengalrayan et al., 2005) with the same concentration of selection agent (Figure 4-2C). Following two to four weeks on shooting medium, regenerated shoots were transferred onto rooting medium supplemented with 19.7 µM indole-3-butyric acid (IBA) (Gallo-Meagher et al., 2000) with the same concentration of geneticin (Figure

4-2D). After four to six weeks, the regenerated plantlets were transplanted into Fafard 2 mix 9 (Fafard Inc., Apopka, FL) and acclimatized in growth chambers with a 16hr/8rh light/dark photoperiod, at a light intensity of 5170 lux, and a 29 ^{o}C /20 ^{o}C day/night cycle (Figure 4-2E). Plantlets were fertilized bi-weekly with Miracle Grow Lawn Food (Scotts Miracle-Gro, Marysville, OH) at the manufacturer's recommended rate.

Polymerase Chain Reaction, Southern Blot Analysis, and RT-PCR

Genomic DNA was extracted from leaves as described by Dellaporta et al. (1983). A forward primer 5'ATGGTTTCAACAGGGCTGAG3' and a reverse primer 5'CCTTCACCAAGGGAATCTGA3' were designed to amplify a 570 bp fragment internal to the coding sequence of *cry1Fa* as described by Luciani et al. (2007). Approximately 100 ng genomic DNA was used as a template for PCR using the Mastercycler Gradient (Eppendorf Laboratories Inc., Hamburg, Germany). PCR was performed using the HotStart PCR kit with 25 µl reaction (Qiagen, Valencia, CA). The cycling conditions were 95 ºC for 15 min initial denaturation, 35 cycles of 95 ºC for 1 min, 57 ºC for 1 min, 72 ºC for 1 min and 72 ºC for 15 min final extension. PCR products were analyzed by electrophoresis on a 0.8% agarose gel. A transgenic bahiagrass line containing *cry1Fa* was used as a positive control (Luciani et al., 2007).

For Southern blot hybridization, genomic DNA was extracted from putative sugarcane transformants, the *cry1Fa* transgenic bahiagrass, and wild type sugarcane plants as described above. Approximately 10 μg of DNA was digested with *EcoRV* and fractionated on 0.8% agarose gels. DNA was transferred onto Hybond N membranes (Amersham BioSciences, Piscataway, NJ) using the alkaline transfer protocol, as well as, prehybridization and hybridization reactions according to standard procedures (Sambrook and Russel, 2001). The complete *cry1Fa* coding sequence (1.8 kb) was used as a probe and labeled with α-(^{32}P)-dCTP using the Prime-a-Gene Labelling System (Promega, Madison WI).

Total RNA was isolated from young leaves using the RNeasy plant mini kit (Qiagen, Valencia, CA). Total RNA (100 ng) was used for cDNA synthesis via reverse transcription using the Superscript® III First-Strand cDNA synthesis kit (Invitrogen, Carlsbad,CA) in a reaction volume of 30 µl. cDNA (8µl) was used as a template to detect transcripts of *cry1Fa* by RT-PCR with the same primer pair as described above. RT-PCR products were analyzed by electrophoresis on a 0.8% agarose gel.

Immunological Assays

Qualitative expression of Cry1Fa in leaf tissue of the regenerated plants was evaluated using the QuickStix™ kit for Cry1F (EnviroLogix™, Portland, ME), originally developed for Herculex I® corn, and following the recommendations of the manufacturer. Relative levels of expression of Cry1Fa in leaf tissue were estimated by using the ELISA QualiPlate™ kit for Cry1F (EnviroLogix) also originally developed for Herculex I® corn. Following six to seven months of vegetative propagation of the primary transformants, protein extracts were obtained from wild type and four putative transgenic sugarcane lines. Cry1F that came with the kit was used as a positive control. Protein concentrations of the extracts were determined using the Bradford assay (Bradford, 1976) and absorbance was measured at a wavelength of 595 nm. Bovine serum albumin was used to prepare a standard curve (R^2 value of 98%) according to the manufacturer's instructions. Reaction kinetics were recorded at 450 nm using an ELISA microplate reader (Bio-tek 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 the tested plants were analyzed by Proc ANOVA and means were separated according to Duncan's multiple range test (Littell et al., 1996, SAS Institute, 2002).

Insect Bioassays

Fall armyworm rice strain adults were placed in cylindrical screen cages (28 cm height, 21 cm diameter) and supplied with distilled water and a 2% sugar-honey solution for nourishment. Paper towels were stretched at the tops of the cages as an oviposition substrate. Emerging neonates (first instar larvae) were placed in rearing tubs (Rubbermaid No. 4025, 9.1 l, Fairlawn, OH) that had plastic grids (29 x 17.5 cm) on the bottom. Larvae were raised on a pinto bean (*Phaseolus vulgaris* L.) artificial diet according to the procedures of Guy et al. (1985). After appriximately 23 days, pupae were removed from the tubs, sexed, and emerged adults were placed in the screen cages. Larvae and adults were reared in incubators or large rearing units at ~ 23º C, 70% RH, and 14:10 photoperiod.

Bioassays were conducted over a five-day period. On day 1, two blades of sugarcane (fifth leaf down from the shoot tip) were placed with 0.3 ml distilled water on a 42.5 mm filter paper (Whatman, Maidstone, England) within a tight-fitting petri dish (50 x 9 mm, Falcon® 1006, Becton Dickinson, Franklin Lakes, NJ). Three day old rice strain 2nd instar larvae (Meagher and Gallo-Meagher, 2003; Nagoshi et al., 2006) were placed singly into the petri dishes. Larval weights were taken on day five. Five replications of five larvae each (n = 25) were tested per line.

Data were subjected to an ANOVA (PROC Mixed, SAS 9.1), where replication was the random variable (Littell et al. 1996). If the treatment variable produced a significant F value, treatment means were separated using simple effect differences of the least square means.

Results

Generation of Transgenic Sugarcane Lines and Molecular Characterization

Co-bombardment of 1660 sugarcane calli (956 of CP84-1198, 484 of CP88-1762, and 220 of CP89-2143) with the pHZCRY expression cassette harboring the *cry1Fa* gene and the pHZ35SNPTII expression cassette harboring the *nptII* gene, followed by geneticin selection,

resulted in the regeneration of 246 plantlets with a total regeneration efficiency of 14.81% (131 of CP84-1198, 77 of CP88-1762, and 38 of CP89-2143) in approximately six months following tissue culture and transformation (Table 4-1). PCR analysis for the presence of *cry1Fa* was used for the initial screening of these plantlets. Of the 246 regenerated plantlets, only four of CP84-1198 amplified the expected 570 bp internal *cry1Fa* fragment. This fragment was correctly amplified from pHZCRY and the transgenic bahiagrass positive control line, but was not amplified from the negative control which was wild type sugarcane (Figure 4-3A). These four putative *cry1Fa* transgenics were initially transferred to soil and then placed under controlled growth conditions and later moved to greenhouse conditions.

Southern blot analyses of *EcoRV* digested genomic DNA showed independent and complex transgene integration patterns for the four transgenic lines (Figure 4-3B). Line 3 showed the most hybridization bands with 5, followed by lines 1 and 2 that displayed four hybridization bands, and line 4 with only 3 hybridization bands. Sizes ranged from 23130 bp to 310 bp. RT-PCR was done on these four lines and *cry1Fa* transcripts were detected in all of them. Lines 1 and 2 in this non-quantitative experiment appeared to have less *cry1Fa* expression compared to lines 3 and 4 (Figure 4-3C).

Immunological Assays

A qualitative immuno-chromatographic assay indicated that transgenic lines 3 and 4 had Cry1Fa levels above the detection level of the kit. However, Cry1Fa was not detected in protein extracts from lines 1 and 2 (Figure 4-4A). A semi-quantitative immunoassay based on the QualiPlate™ kit for Cry1F (EnviroLogix™) showed that lines 1 and 2 had low levels of Cry1Fa as compared to the Cry1F levels found in the positive control provided with the kit (Herculex I® corn or WideStrike® cotton), while lines 3 and 4 had Cry1Fa levels equal to or greater than the Cry1F levels found in Herculex I® corn or WideStrike ® cotton (Figure 4-4B). These observations

correlated with the OD_{450} values obtained for the transgenic lines. The four transgenic lines had OD_{450} values significantly different from each other ($P < 0.0001$; Figure 4-4C). The level of Cry1Fa in line 1 was not significantly different from wild type sugarcane. Lines 2, 3, and 4 displayed levels of Cry1Fa that were approximately 3-, 11-, and 8-fold higher than those measured for line 1, respectively (Figure 4-4C). The Cry1Fa levels found in lines 3 and 4 were almost double that of the Cry1F positive control ($P = 0.05$).

Insect Bioassays

Fall armyworm larvae fed leaves from the transgenic lines showed higher mortality rates than feeding on wild type leaves with the highest levels of mortality occurring for lines 3 and 4 (80% and 90%, respectively; Table 4-2). Larvae showed intense feeding on the wild type lines along with high weight gains, while larvae on lines 1, 2, 3 and 4 showed significantly reduced weight gains ($P < 0.0001$; Table 4-2).

Discussion

The transformation protocol used in this study was only successful in producing transgenics from one of the cultivars tested, CP84-1198. This cultivar is known for a high degree of embryogenic callus formation and regeneration (Chengalrayan and Gallo-Meagher, 2001, Chengalrayan et al., 2005). A differential response of sugarcane cultivars to tissue culture has also been noted by Lourens and Martin (1987), Hoy et al. (2003), and Gilbert et al. (2005). Compared to the other two cultivars, CP84-1198 produced more embryogenic callus in a shorter period of time and consequently the number of CP84-1198 calli bombarded was 2x and 4x higher than for CP88-1762 and CP89-2143, respectively (Table 4-1). Obtaining four transgenic CP84-1198 plants from 956 bombarded calli reflects a transformation frequency of only 0.4%. Given that same frequency, only two transgenics would have been expected for CP88-1762 and none would have been expected for CP89-2143. Additionally, recovering a total of 131 plants of CP84-1198

following bombardment and selection represents a 97% escape rate. All of the 115 plants regenerated from CP89-2143 and CP88-1762 were escapes. A major reason for these large number of escapes could be low or insufficient selection pressure. In this study, selection with 25 mgL^{-1} geneticin was initiated one week after bombardment and lasted for two weeks followed by an increase to 50 mgL^{-1}. This selection regime was similar to that used by Tadesse et al. (2003) for the production of transgenic sorghum. However, in a recent report by Wang et al. (2009), 50 mgL^{-1} geneticin was used immediately after bombardment to produce transgenic sugarcane plants. Consequently, a higher selection pressure initiated soon after a short resting period may have decreased the number of escapes and possibly increased the transformation rate. Also, various bombardment parameters such as microprojectile size, rupture pressure, rupture disc to macrocarrier distance, stopping screen to target cell distance, and chamber evacuation are critical in determining the efficiency of transformation via biolistics (Lerche and Hallmann, 2009). Additionally, target quality is a major factor in transformation efficiency, and as mentioned above, CP84-1198 produced higher quality embryogenic callus compared to the other cultivars.

Fall armyworm is the most important insect pest of grasses and other crops in the southeastern U.S. Corn expressing the *cry1F* gene (Herculex I®) was commercially released by Pioneer Hi-Bred International and Dow Agrosciences (Events TC 1507 and DAS-06275-8). Field trials showed that these transgenic corn lines effectively controlled multiple insects including FAW (EPA, 2001). In cotton, FAW bioassays indicated that neonate mortality was significantly higher when larvae were fed leaves expressing cry1F (80%) compared with non-transgenic leaves (48%) or leaves expressing cry1Ac (45%) (Adamczyk and Gore, 2004). However, in these studies the FAW populations tested were probably corn strain (most studies do not identify strain, but use colonies collected from corn). Although, previous research has shown that strain-specific

physiological differences in *Bt* susceptibility can occur between strains (Adamczyk et al. 1997), little is known about the response of rice strain to most *Bt* endotoxins. Additionally, Meagher and Gallo-Meagher (2003) showed that rice strain adults were more predominant on grasses in south Florida including in sugarcane in the Everglades Agriculture Area. Consequently in the current work, bioassays were conducted with the rice strain of FAW. Two of the four transgenic lines obtained expressed the *cry1Fa* transgene at a high enough level to significantly affect mortality and larval weight in insect bioassays. This is the first report of transgenic *Bt* sugarcane conferring resistance to the difficult to control, and important insect pest, FAW. Future work will focus on evaluating FAW resistance using this transgenic strategy in sugarcane under field conditions.

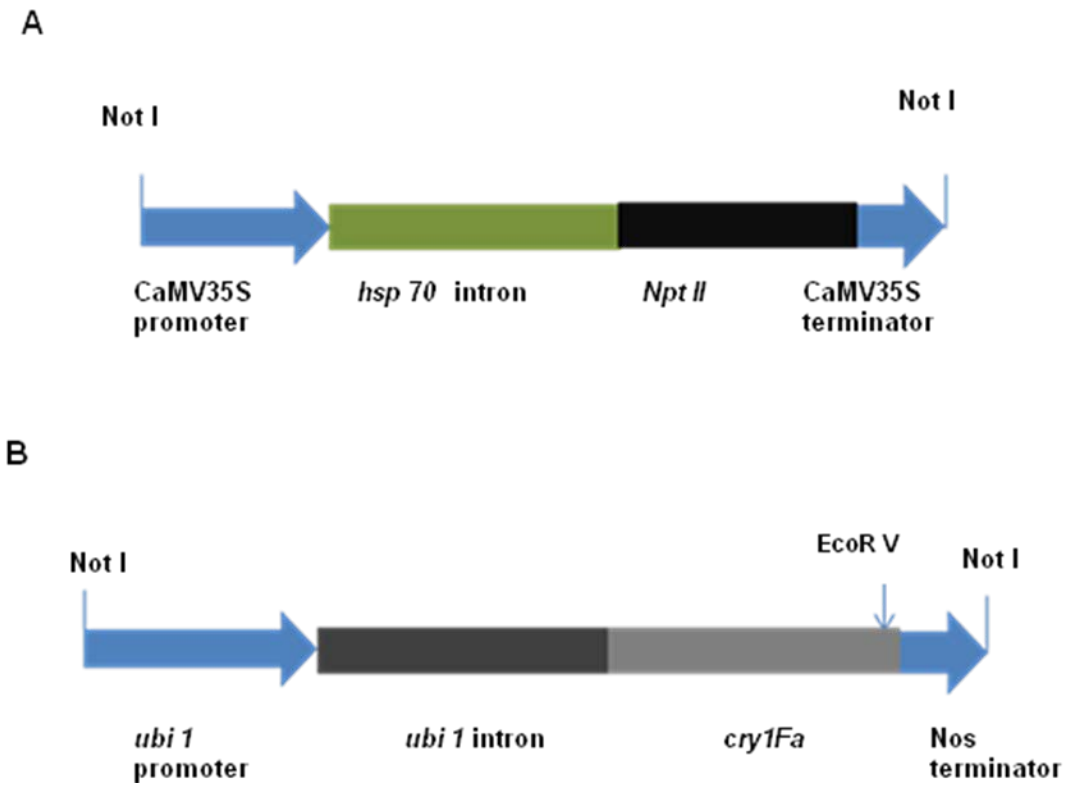


Figure 4-1. Expression cassettes used for sugarcane bombardment**.** A) portion of pHZ35SNPTII containing the *nptII* selectable marker gene, and B) portion of pHZCRY containing the *cry1Fa* gene.

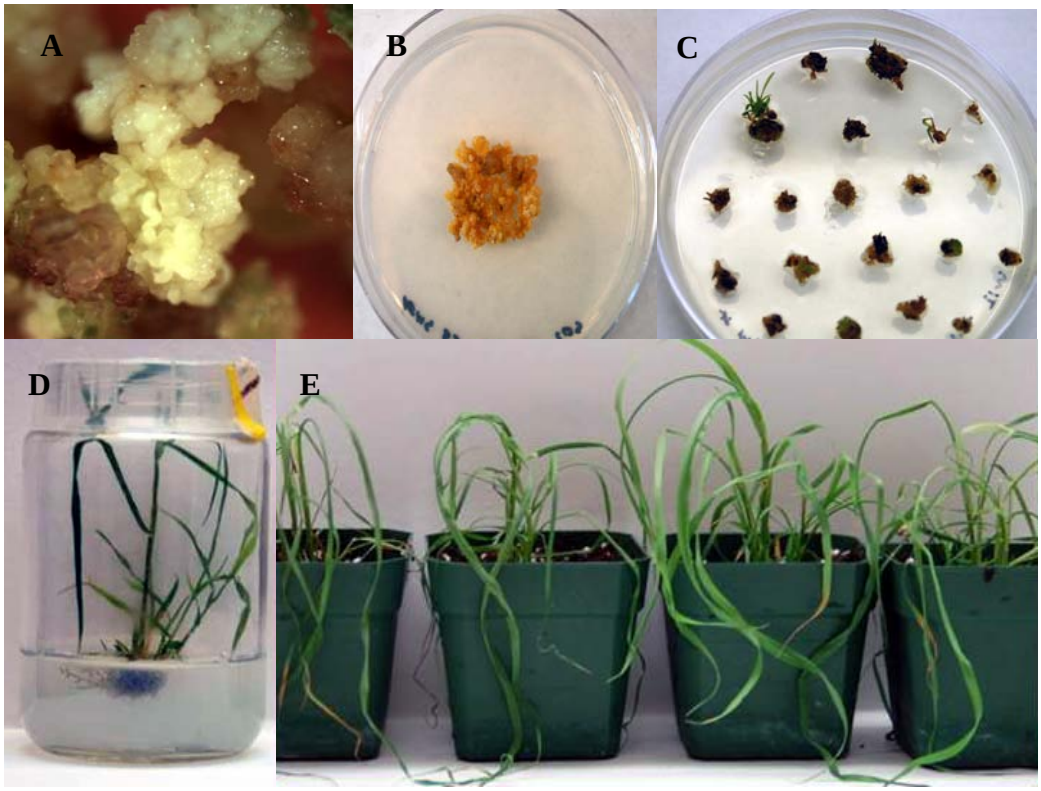


Figure 4-2. Sugarcane tissue culture and regeneration following bombardment of CP84-1198. A) embryogenic callus, B) callus arranged in a circle on 0.4 M sorbitol containing medium ready for bombardment, C) sugarcane callus producing shoots under geneticin selection, D) regenerated sugarcane plantlet in a jar containing 19.7 µM IBA medium, and E) regenerated sugarcane plantlets in small square pots (3.5") with soil.

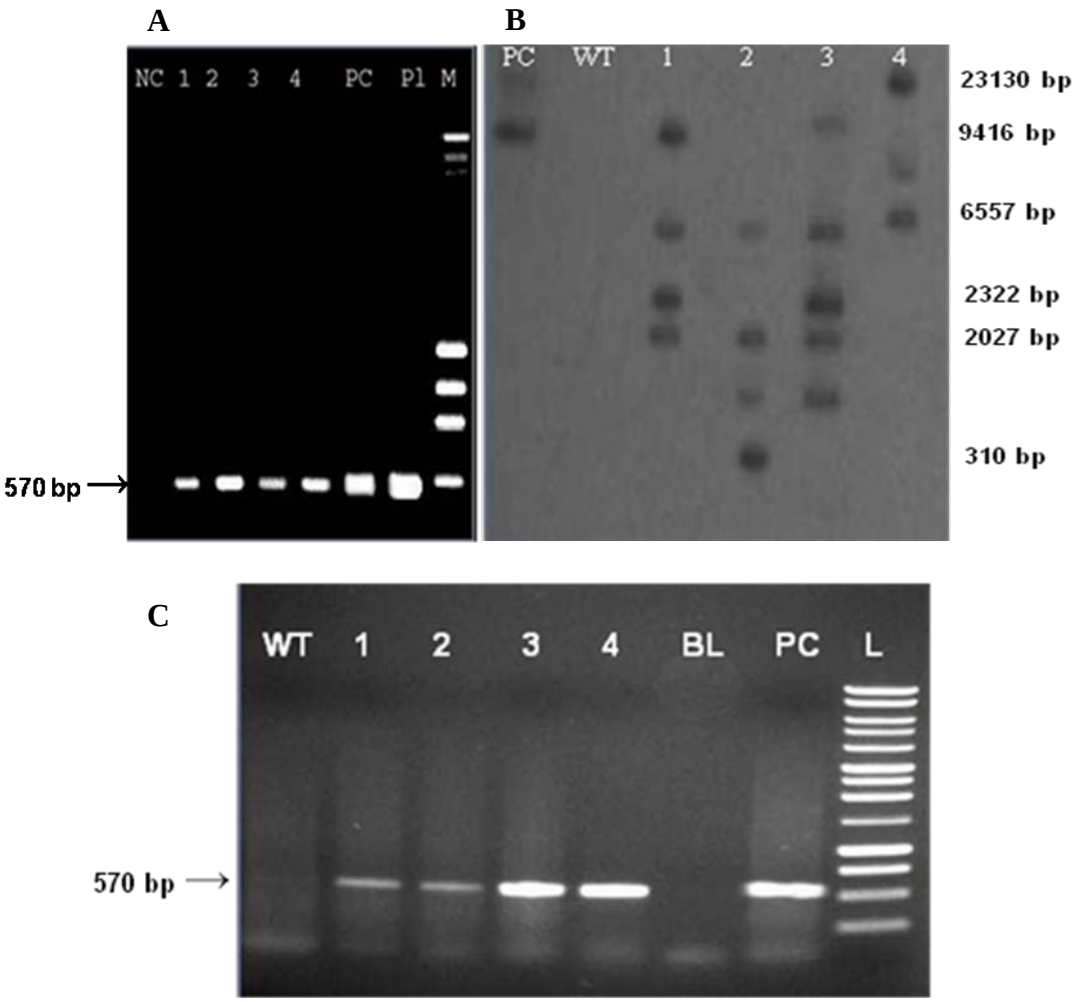


Figure 4-3. Molecular analyses of transgenic sugarcane containing *cry1Fa*. A) A 570 bp pair *cry1Fa* fragment amplified from genomic DNA of transgenic sugarcane lines (1, 2, 3 and 4) in comparison to wild type sugarcane (NC), a *cry1Fa* transgenic bahiagrass line (PC), and pHZCRY (Pl) using forward 5'ATGGTTTCAACAGGGCTGAG3' and reverse 5'CCTTCACCAAGGGAATCTGA3' primers. M is the λΦχ marker. B) Southern blot of genomic DNA digested with *EcoRV* from independent transgenic lines (1, 2, 3 and 4) in comparison to a *cry1Fa* transgenic bahiagrass line (PC) and wild type sugarcane (WT) following hybridization with a ^{32}P-labeled *cry1Fa* coding sequence probe, and C) Presence of *cry1Fa* transcripts following RT-PCR of sugarcane lines (1, 2, 3, and 4) along with wild type sugarcane (WT), a *cry1Fa* transgenic bahiagrass line (PC) and 1kb ladder (L). The lane designated BL is the negative control, water only sample.

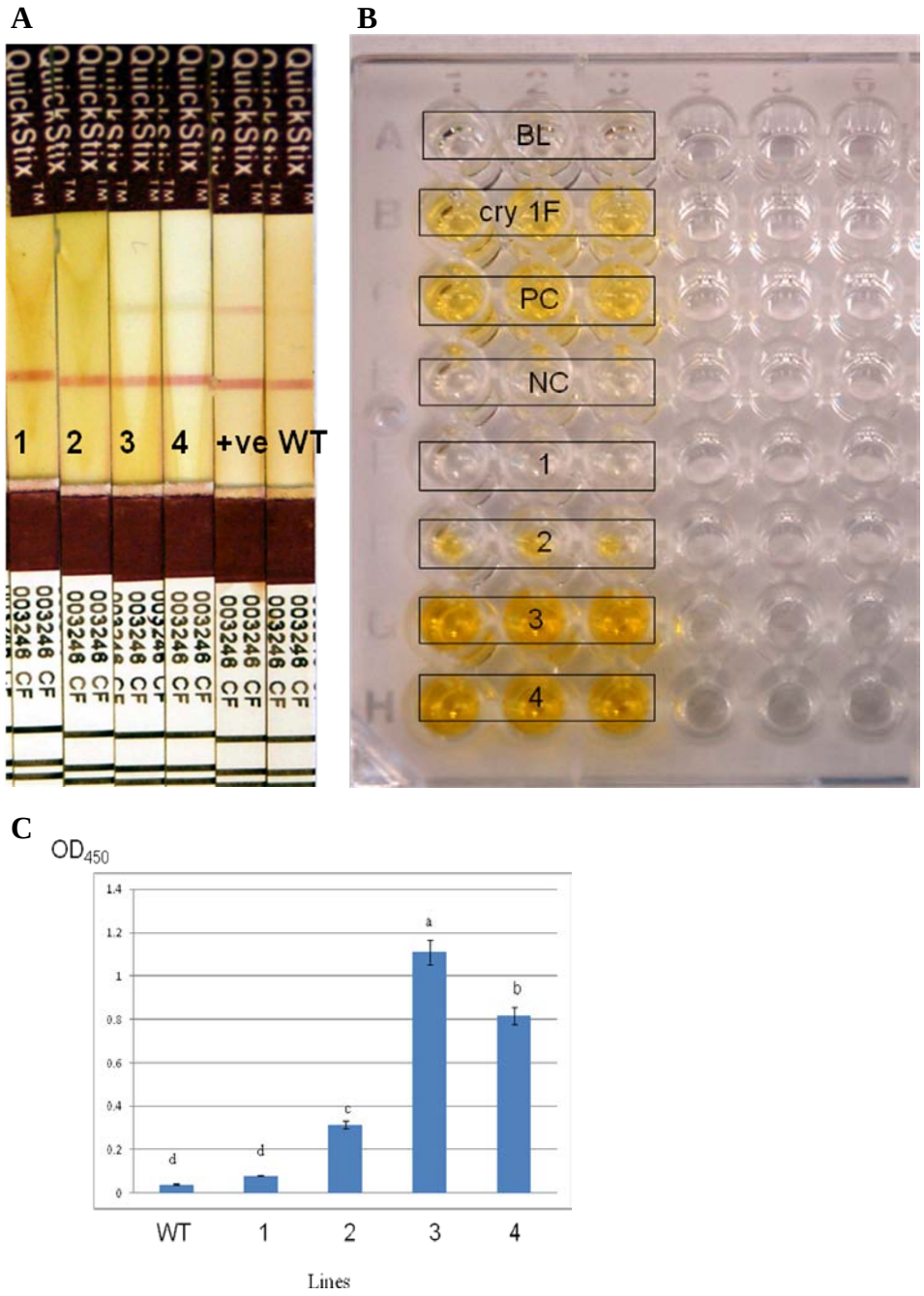


Figure 4-4. Cry1Fa levels in leaves of transgenic sugarcane lines and wild type. A) Immuno-chromatographic (Cry1Fa QuickStix ™, EnviroLogix ™) screening of protein extracts of transgenic sugarcane lines (1, 2, 3 and 4), a *cry1Fa* transgenic bahiagrass line (+ve), and wild type sugarcane (WT), B) An ELISA Qualiplate ™ kit for the Cry1Fa (Environlogix ™) with blank (BL), pure Cry 1F protein (cry 1F), a *cry1Fa* transgenic bahiagrass line (PC), wild type sugarcane (NC), and the four transgenic sugarcane lines (1,2,3,and 4), and C) Optical densitometry at 450 nm (OD_{450}) of Cry1Fa ELISA of protein extracts from wild type sugarcane (WT) and transgenic sugarcane lines (1, 2, 3 and 4). Bars represent standard error. Different letters indicate differences by Duncan's multiple range test ($P < 0.0001$).

Table 4-1. Summary of biolistic gene transfer experiments with three different cultivars, CP84-1198, CP89-2143 and CP88-1762.

Experiment Number	Cultivar	Number of Bombarded Plates	Number of Calli Bombarded	Number of Plantlets Regenerated	Regeneration Efficiency (%)
1	CP84-1198	24	280	25	8.92
2	CP89-2143	24	220	38	17.27
3	CP88-1762	24	265	27	10.18
4	CP84-1198	30	381	52	13.64
5	CP88-1762	24	219	50	22.83
6	CP84-1198	24	295	54	18.3
Total		150	1660	246	14.81

Table 4-2. Fall armyworm bioassays with 2nd instar rice strain larvae feeding on leaves of wild type and transgenic sugarcane lines showing mortality rates and percent weight increases[1].

Line	Mortality (%)	% Weight Increase ±SE
WT1	0	1921.0±112.3a
WT2	0	1983.3±141.6a
1	30	62.0±7.5b
2	40	164.5 ± 85.0 b
3	80	25.2 ± 4.2 b
4	90	80.0 ± 0 b

[1]Data were obtained after five days of feeding. WT1 and WT2 are wild type sugarcane plants. 1-4 are transgenic sugarcane lines; means with the same letter are not significantly different. ($P < 0.0001$)

CHAPTER 5
EVALUATING *AGROBACTERIUM* VACUUM INFILTRATION OF STOLON NODES FOR THE PRODUCTION OF TRANSGENIC BERMUDAGRASS

Introduction

Bermudagrass is widely used as a warm-season turf and forage grass in the tropical and lower parts of the temperate regions of the world. It is the most important pasture grass in the southern U.S. (Mitich, 1989; Burton and Hanna, 1995), and it covers an area of 12 million ha for livestock grazing and hay production (Taliaferro et al., 2004).

'Tifton 85' [*Cynodon dactylon* (L.) Pers. X *C. transvaalensis* Burtt Davy] is a high quality forage bermudagrass. It is one of the higher yielding hybrids available for hay and pasture production and produces high weight gains in cattle. It has 34% higher dry matter yield, 47% higher digestible yield, and superior animal performance with growing steers compared with coastal bermudagrass (Mandebvu et al., 1999). It has become a new standard for comparing bermudagrass varieties, due to its superior production and quality (Hill et al., 2001; Evers et al., 2004). Tifton 85 is completely male and female sterile and therefore genetic improvement through classical breeding is impossible.

'TifEagle' [*Cynodon dactylon* (L.) Pers. X *C. transvaalensis* Burtt Davy] is a warm season turf grass and normally provides high quality turf for golf course putting greens in the subtropical and tropical regions of the world (Bunnell, 2005). It has a distinct genetic background, turf density, and an ability to tolerate extremely low heights. TifEagle is grown on golf courses throughout Florida (Bunnell, 2005). Like Tifton 85, it is sterile.

Some bermudagrasses have been genetically engineered by either biolistics or *Agrobacterium*-mediated transformation. TifEagle has been transformed via biolistics with the *hpt* gene (Zhang et al., 2003) and the *bar* gene (Goldman et al., 2004b) and via *Agrobacterium* to produce resistantance to the herbicide Liberty (glufosinate ammonium) (Hu et al., 2005).

Transgenic turf type common bermudagrass with resistance to the herbicide glufosinate has been obtained through biolistics (Li and Qu, 2003), and Li et al. (2005) successfully transformed common bermudagrass variety 'J1124' with *Agrobacterium*-mediated transformation showing stable expression of the *hph* gene. *Agrobacterium*-mediated transformation is generally preferred over bioloistics because it usually allows for the stable integration of a defined segment of DNA into the plant genome and results in lower copy number, fewer rearrangements and improved stability of expression over generations (Smith and Hood, 1995; Dai et al., 2001).

In transformation experiments, even with *Agrobacterium*, callus cultures have been an unavoidable step (Vasil et al., 1992; Hartman et al., 1994; Wan and Lemaux, 1994; Spangenberg et al., 1995; Cho et al., 2001; Sallaud et al., 2003; Li and Qu, 2004; Wang et al., 2004; Wang and Ge, 2005). But callus induction and plant regeneration is not only time consuming and laborious, but can cause somaclonal variation. Consequently, callus-free methods of transformation have been explored (Wang and Ge, 2005; Spangenberg et al., 1998; Choi et al., 2000; Goldman et al., 2004a).

In this study, a callus-free method similar to that used by Wang and Ge (2005) was evaluated for the incorporation of *cry1Fa* into Tifton 85 and TifEagle. The objectives of the study were to:

(1) produce an efficient regeneration protocol for Tifton 85 and TifEagle from stolon nodes,

(2) introduce the c*ry1Fa* gene into their genomes via *Agrobacterium*-mediated vacuum infiltration of stolon nodes, followed by molecular characterization, and

(3) determine resistance to the rice strain of FAW in laboratory bioassays under controlled environmental conditions.

Materials and Method

Plant Material

Bermudagrass cultivars Tifton 85 and TifEagle were grown in the greenhouse with 16h/8h light /dark photoperiod provided by cool white florescent lights with a photon flux density of 5170 lux. Stolon segements 2-3 cm in length containing one node were sterilized with 100% ethanol for 1 min and 50% bleach with two drops of Tween-20 for 20 min and then rinsed with sterile water 4-5 times for10 min each. The segments were cut in half (0.5-1cm) and contained a cut node, these were directly used for *Agrobacterium*-mediated transformation.

Plantlet Regeneration

A preliminary experiment was carried out to determine the level of 2,4-D and kinetin that produced the most plantlets from stolon explants of TifEagle and Tifton-85 based on the BG-A2 medium used by Wang and Ge (2005). Five different levels of 2, 4-D ranging from 1 µM to 1.8 µM, and kinetin ranging from 3.0 µM to 5.0 µM were used in the matrix experiment with 25 different treatments and 3 replications each. Each treatment consisted of 2 Petri plates (100 mm X 25 mm) with 5 explants each of Tifton 85 and TifEagle for a total of 10 explants per cultivar. The plates were kept under a 16 hr/8hr light/dark photoperiod provided by cool white florescent tubes with a photon flux density of 5170 lux at 28 ^{o}C. The explants began producing shoots within 1-2 weeks of culture initiation and roots appeared in 3-4 weeks. Data were collected after 4 weeks of the culture period. For both TifEagle and Tifton 85, the most plantlets were produced on medium containing 1.8 µM 2, 4-D and 4.5 µM kinetin (data not shown).

Infection and Co-cultivation of Stolon Nodes with *Agrobacterium tumefaciens*

The *Agrobacterium tumefaciens* strains GV3101 and AGLO were used for transformation. Both strains harbored pWBVec10aCry1Fa (Figure 5-1). pWBVec10aCry1Fa contains the *hpt* selectable marker gene under the control of the CaMV 35S promoter and the synthetic *cry1Fa*

gene under the control of maize ubiquitin promoter and first intron (Altpeter et al., 2009). *Agrobacterium* cultures were grown at 28 ^{o}C in liquid YEP medium overnight with shaking at 175 rpm until an OD_{600} of 1.0-1.2 was reached. The cells were pelleted by centrifugation at 2400 g for 15 min and resuspended in liquid BG-A1 medium (Wang and Ge, 2005) containing half-strength MS medium (Murashige and Skoog, 1962) supplemented with 1.8 µM 2,4-D, 4.5 µM kinetin, 3.3 mM L-cysteine, 1 mM dithiothreitol, 1 mM Na-thiosulphate and 2% (w/v) sucrose. The OD_{600} of the resuspended *Agrobacterium* was adjusted to approximately 1.0, and 100 µM acetosyringone was added to 20 ml of the *Agrobacterium* suspension. The stolon nodes were immersed in the *Agrobacterium* suspension and a vacuum was drawn for 10 min. After the vacuum was released, the stolon nodes were incubated with *Agrobacterium* for 50 min with gentle shaking at 120 rpm. Excess bacteria were removed from the stolons after incubation under sterile conditions and the stolon nodes were transferred onto agar-solidified BG-A1 medium and placed in the dark at 25 ^{o}C for co-cultivation for two days.

Selection and Recovery of Transgenic Plants

Two days after co-cultivation, the infected stolon nodes were transferred onto selection medium (MS medium supplemented with 1.8 µM 2, 4-D, 4.5 µM kinetin, 2% (w/v) sucrose, 250 mgL^{-1} cefotaxime and 75 mgL^{-1} hygromycin). Regenerated green shoots following selection (Figure 5-2A and B) were transferred to Petri plates containing hormone-free half-strength MS medium for rooting (Figure 5-2C and D). All the regenerating cultures were kept at 25 ^{o}C in fluorescent light at a photoperiod of 16h in a growth chamber provided by cool white florescent tubes with a photon flux density of 5170 lux. Plantlets with well developed roots were transferred to soil in small plastic pots (3.5") and grown in the greenhouse with a 16hr/8rh light/dark photoperiod, at a light intensity of 5170 lux, and a 29 ^{o}C /20 ^{o}C day/night cycle.

Plantlets were fertilized bi-weekly with Miracle Grow Lawn Food (Scotts Miracle-Gro, Marysville, OH) at the manufacturer's recommended rate (Figure 5-2E).

Molecular Characterization of Transgenic Plants

Total genomic DNA was isolated from leaves using the method described by Dellaporta et al. (1983). A forward primer 5'ATGGTTTCAACAGGGCTGAG3' and a reverse primer 5'CCTTCACCAAGGGAATCTGA3' were designed for amplifying a 570 bp fragment internal to the coding sequence of the *cry1Fa* gene. Approximately 100 ng genomic DNA was used as a template for PCR using the Mastercycler Gradient (Eppendorf Laboratories Inc., Hamburg, Germany). PCR was performed using the HotStart PCR kit with a 25 µl reaction (Qiagen, Valencia, CA). The cycling conditions were 95 ℃ for 15 min initial denaturation, 35 cycles of 95 ℃ for 1 min, 57 ℃ for 1 min, 72 °C for 1 min and 72 ℃ for 15 min final extension. PCR products were analyzed by electrophoresis on a 0.8% agarose gel.

For Southern blot analysis, genomic DNA was isolated using the same extraction method referenced above and 10 µg was digested with *EcoRI*, fractionated on a 0.8% agarose gel, transferred onto a nitrocellulose membrane (Hybond, Amersham BioSciences, Piscataway, NJ) and hybridized using the complete *cry1Fa* coding sequence (1.8kb) as a probe, as described in the previous chapter.

Total RNA was isolated from young leaves using the RNeasy plant mini kit (Qiagen, Valencia, CA). Total RNA (100 ng) was used for cDNA synthesis via reverse transcription using the iScript cDNA synthesis kit (Bio-Rad laboratories Inc., Hercules, CA) in a reaction volume of 20 µl. cDNA (2µl) was used as a template to detect the transcript of *cry1Fa* gene by RT-PCR with the same primer pair as described above. RT-PCR products were analyzed by electrophoresis on a 0.8% agarose gel.

Immunological Assays

Qualitative expression of Cry1Fa in leaf tissue of the regenerated plants was evaluated using the QuickStix™ kit for Cry1F (EnviroLogix™, Portland, ME), originally developed for Herculex I® corn, and following the recommendations of the manufacturer. Following six to seven months of growth, leaf tissue was taken from the fifth leaf from the top of each plant and sandwiched between the cap and the body of the extractor tube and pushed down with a tooth pick, extraction buffer (0.5 ml) was added, and the tissue was ground with the help of small plastic pestles, and the test strip was placed into the tube for 5 min.

Insect Bioassays

Fall armyworm [*Spodoptera frugiperda* (J. E. Smith)] rice strain adults were placed in cylindrical screen cages (28 cm height, 21 cm diameter) and supplied with distilled water and a 2% sugar-honey solution for nourishment. Paper towels were stretched at the top of the cages as an oviposition substrate. Emerging neonates (first instar larvae) were placed in rearing tubs (Rubbermaid No. 4025, 9.1 l, Fairlawn, OH) that had plastic grids (29 x 17.5 cm) on the bottom. Larvae were raised on a pinto bean (*Phaseolus vulgaris*) artificial diet according to the procedures of Guy et al. (1985). After about 23 days, pupae were removed from the tubs, sexed, and emerged adults were placed in the screen cages. Larvae and adults were reared in incubators or large rearing units at ~ 23º C, 70% RH, and 14:10 photoperiod.

Bioassays were conducted over a 9-day period. On day 1, two small blades of grass (fifth leaf down from the shoot tip) were placed with 0.3 ml distilled water onto a 42.5 mm filter paper (Whatman, Maidstone, England) within a tight-fitting petri dish (50 x 9 mm, Falcon® 1006, Becton Dickinson, Franklin Lakes, NJ). Rice strain neonates (Meagher and Gallo-Meagher 2003; Nagoshi et al. 2006) were placed singly into the petri dishes. On day 5, more tissue was added. Larval weights were taken on day 9. Five replications of five larvae each (n = 25) were

tested per grass line. Data were subjected to an ANOVA (PROC Mixed, SAS 9.1), where replication was the random variable (Littell et al. 1996).

Results

Green shoots were directly obtained from infected stolons after four to five weeks of selection on hygromycin. Rooted *in vitro* plantlets were obtained after two weeks. Soil-grown bermudagrass cultivars were established in the greenhouse within nine weeks of initiating the *Agrobacterium*-mediated transformation experiments.

For Tifton 85, 370 explants infected with GV3101 resulted in 13 plants for a regeneration frquency of 3.5%, and 480 explants infected with AGLO resulted in 23 plants for a regeneration frequency of 4.7% (Table 5-1). For TifEagle, 580 explants infected with GV3101 resulted in 28 plants for a regeneration frequency of 4.8%, and 680 explants infected with AGLO resulted in 48 plants for a regeneration frequency of 7.0% (Table 5-1).

To determine whether the 112 regenerated plants were stably transformed, each was first screened by PCR for the presence of *cry1Fa*. Only three of the 36 Tifton 85 plants (8%) and three of the 76 TifEagle plants (4%) amplified *cry1Fa* (Figure 5-3). Based on these results, Southern blot analysis was performed on these six putative transformants. However, only one TifEagle plant from an AGLO transformation experiment showed integration of the *cry1Fa* gene into the host genome (Figure 5-3). As expected, expression analysis using RT-PCR showed that only that TifEagle transgenic plant expressed *cry1Fa* transcripts (Figure 5-3).

Qualitative expression of Cry1Fa in leaf tissue of the six plants was evaluated using the Quickstix™ kit for Cry1F (EnviroLogix™, Portland, ME), originally developed for Herculex I® corn, following the recommendations of the manufacturer. None of the plants, including the TifEagle transfomant, had discernable Cry1Fa levels (Figure 5-4).

Fall armyworm bioassays were conducted over a 9-day period for the one transgenic TifEagle plant and the two non-transformed TifEagle plants. There was no significant difference between the larval weights and mortality of the rice strain FAW fed on these plants as compared to wild type TifEagle, confirming that the level of Cry1Fa in the transgenic TifEagle plant was too low to affect feeding and cause mortality (Table5-2).

Discussion

Direct regeneration from stolon explants can significantly reduce the time required to regenerate plantlets. In previous biolistic transformation studies of bermudagrass, callus induction and maintenance took six weeks and resistant calli were recovered after eight weeks (Zhang et al., 2003; Li and Qu, 2004). Additionally, the total time from callus initiation to plantlets for bermudagrass transformation experiments was at least 20 weeks. The method reported here is based on a protocol originally developed by Wang and Ge (2005) for TifEagle. It produced rooted plantlets of both Tifton 85 and TifEagle in only approximately seven weeks which was the same timeframe that Wang and Ge (2005) reported.

However, the transformation protocol used in the present study was only successful in producing one TifEagle transgenic. This single transgenic TifEagle line expressed *cry1FA* transcripts, as determined by RT-PCR (Figure 5-3), but had low levels of expressed protein, as indicated by immunochromatography (Figure 5-4). This low Cry1Fa expression also had no affect on FAW rice strain mortality. Low levels of expression are not unusual in transgenics and could be due to a number of factors including transgene silencing (Matzke and Matzke, 1998). Transgene silencing may be due to DNA methylation, position effects, gene interactions and recognition of foreign DNA due to imcompatabilities in base composition.

Obtaining one transgenic TifEagle from 1260 infected stolon nodes is a transformation frequency of only a mere 0.08%, which is in stark contrast to the 4.8% - 6.1% reported by Wang

and Ge (2005). No transgenic Tifton 85 plants were recovered. As reviewed by Jian et al. (2009), T-DNA delivery into plant cells is a complex process which is influenced by many parameters such as explant type (Chen et al., 2008), *Agrobacterium* strain (Crane et al., 2006), preculture duration (Chen et al., 2008), temperature (Kereszt et al., 2007; Baron et al., 2001), and co-cultivation duration (Chen et al., 2008). Therefore, what parameters could have been altered in this study to improved transformation? Both co-cultivation temperature (25 ^{o}C) and period (two days) were most likely not factors for TifEagle transformation since higher temperatures would probably have resulted in decreased T-DNA transfer (Kereszt et al., 2007), and Wang and Ge (2005) used those exact parameters successfully in transforming TifEagle stolon explants. However, it is unclear whether those parameters were optimal for the transformation of Tifton 85 stolons.

The *Agrobacterium* strains used in this study (GV3101 and AGLO) probably had a major affect on transformation frequency. It is well known that different strains have varying virulence for different genotypes. Preliminary, transient expression assays were not conducted with these two strains to see if they could efficiently infect TifEagle and Tifton 85 stolons. In the two reports where transgenic TifEagle were produced, EHA105 (Hu et al., 2005; Wang and Ge, 2005) and LBA4404 were used (Wang and Ge, 2005). These strains carrying plasmids containing *cry1Fa* should be examined to obtain more TifEagle transgenic plants, and to develop an effective *Agrobacterium*-transformation method for Tifton 85. Only with the production of an adequate number of *cry1Fa* bermudagrass transgenics can the effectiveness of this strategy be evaluated as a control measure for FAW.

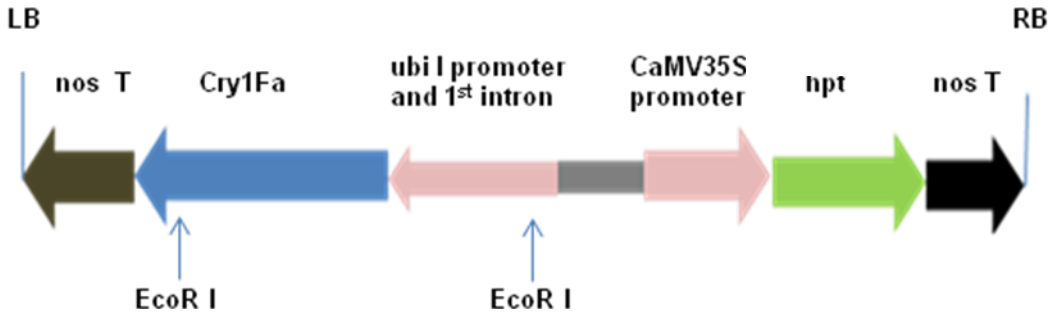


Figure 5-1. The gene cassette between the left (LB) and the right border (RB) in pWBVec10aCry1Fa used in *Agrobacterium* vacuum infiltration of stolon explants of Tifton 85 and TifEagle.

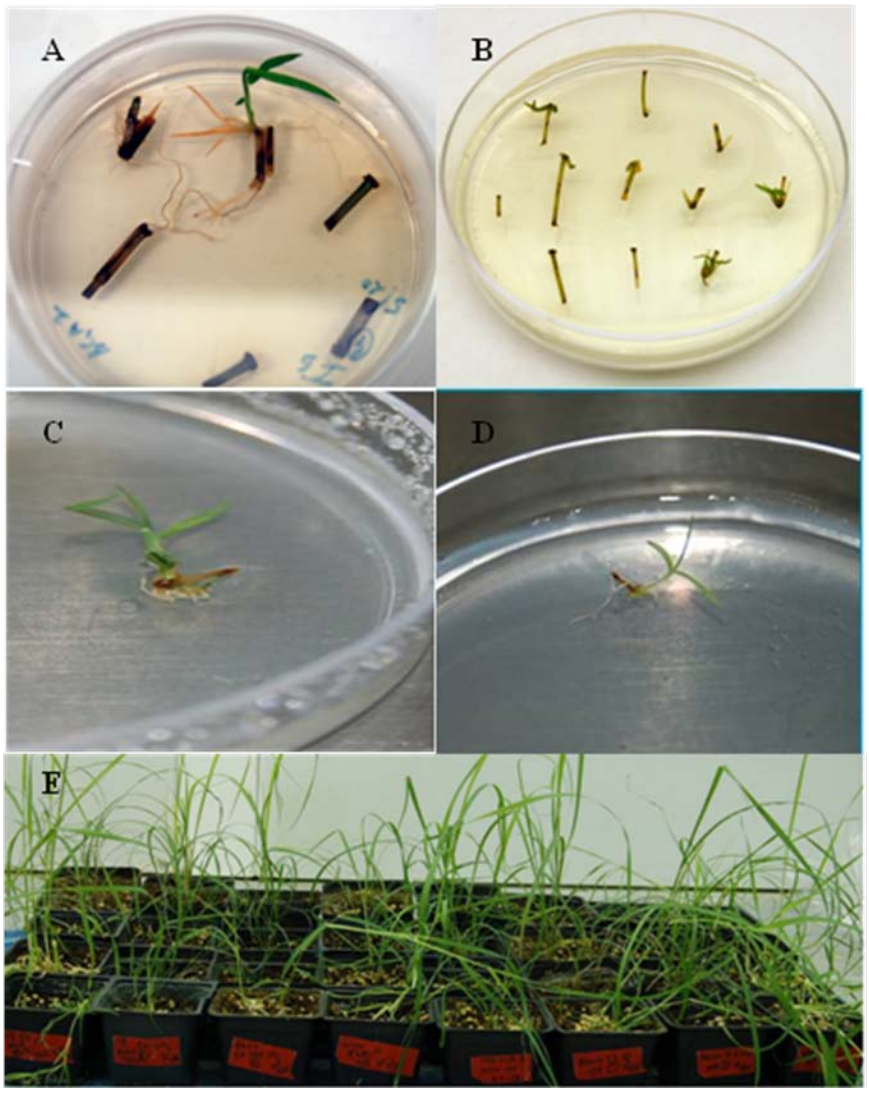


Figure 5-2 Tifton 85 and TifEagle tissue culture and regeneration following *Agrobacterium*-mediated transformation. A) A shoot emerging from a Tifton 85 stolon explant following hygromycin selection, B) Shoots emerging from TifEagle stolon explants following hygromycin selection, C) A regenerated Tifton 85 plantlet, D) A regenerated TifEagle plantlet, and E) TifEagle plants following selection, regeneration and transfer into pots.

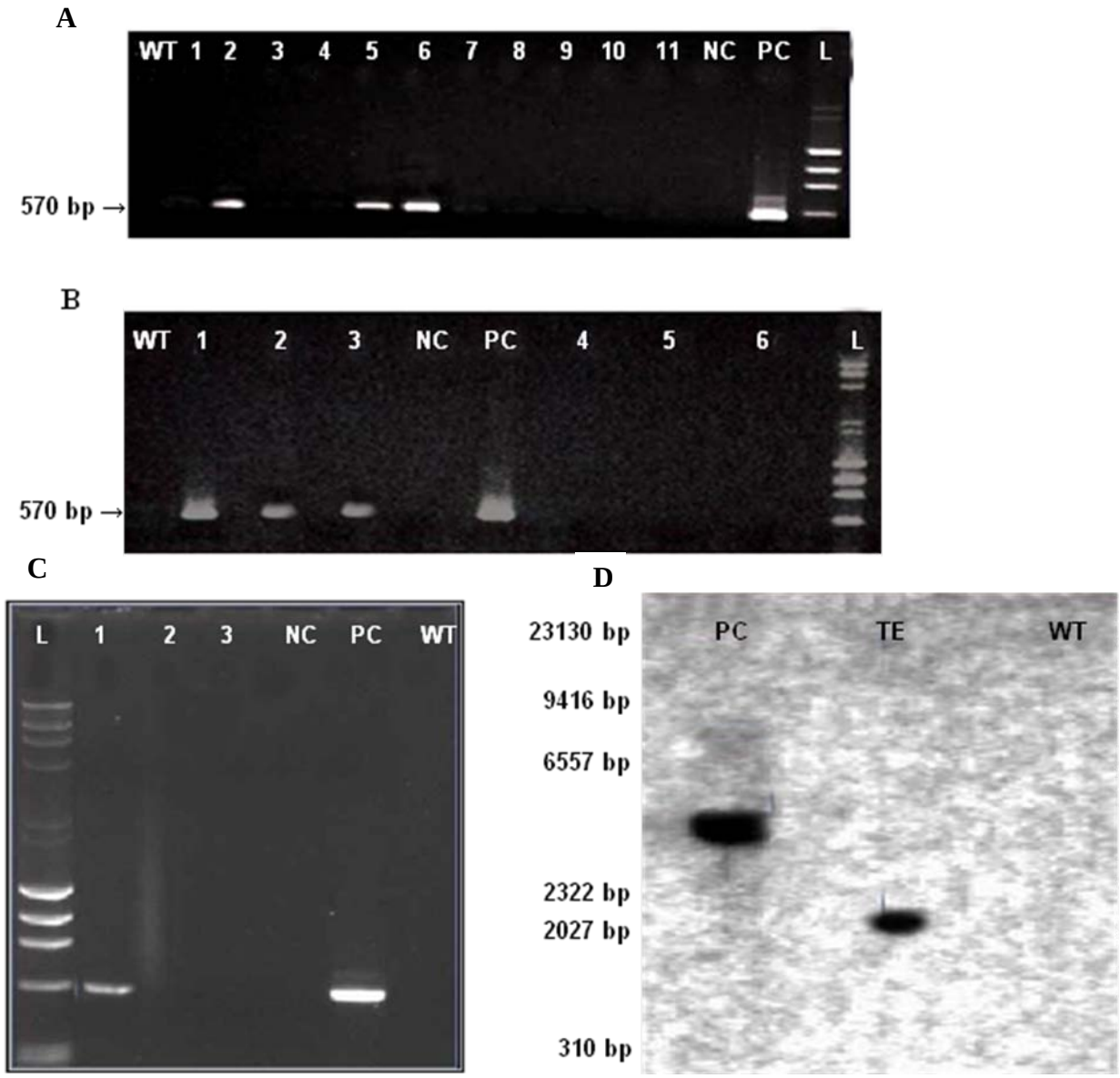


Figure 5-3. Molecular analyses of TifEagle and Tifton 85 plants obtained following *Agrobacterium* vacuum infiltration of stolon nodes. A 570 bp *cry1Fa* fragment amplified from genomic DNA of A) putative transgenic TifEagle plants (2,5, and 6), in comparison to wild type TifEagle (WT), non-transformed TifEagle plants (1,3,4, and 7-11), water (NC), a *cry1Fa* transgenic bahiagrass line(PC), and a 1kb ladder (L),and B) putative transgenic Tifton 85 plants (1,2, and 3), in comparison to wild type Tifton 85 (WT), water (NC), a *cry1Fa* transgenic bahiagrass line (PC), non-transformed Tifton 85 plants (4-6), and a 1 kb ladder (L). C) Presence of *cry1Fa* transcripts following RT-PCR of the *cry1Fa* transgenic TifEagle plant (1), and a *cry1Fa* transgenic bahiagrass line (PC) in comparison to non-transformed TifEagle plants (2 and 3), water (NC) and wild type TifEagle (WT). D) Southern blot of genomic DNA digested with *EcoRI* of a *cry1Fa* transgenic bahiagrass line (PC) and the *cry1Fa* transgenic TifEagle plant in comparison to wild type TifEagle (WT) following hybridization with a ^{32}P-labeled *cry1Fa* coding sequence probe.

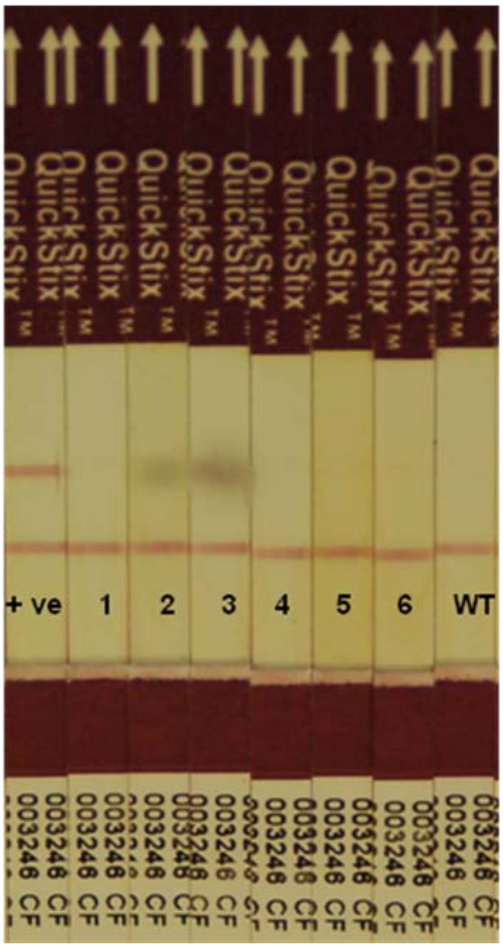


Figure 5-4. Cry1Fa levels in leaves of regenerated TifEagle and Tifton 85. The immunochromatographic (Cry1Fa Quickstix™, Envirologix ™) screening of protein extracts of TifEagle (1, 2, and 3), Tifton 85 (4, 5, and 6), a *cry1Fa* transgenic bahiagrass line (+ve), and wild type TifEagle (WT).

Table5-1. Summary of *Agrobacterium*-mediated transformation experiments using pWBVec10aCry1Fa with stolon nodes of Tifton 85 and TifEagle.

Cultivar	*Agrobacterium* Strain	Number of Stolon Nodes Infected	Number of Plantlets Regenerated	Regeneration Efficiency (%)
Tifton 85	GV3101	370	13	3.5
Tifton 85	AGLO	480	23	4.7
Total		850	36	4.2
TifEagle	AGLO	580	28	4.8
TifEagle	GV3101	680	48	7.0
Total		1260	76	6.0

Table5-2. Fall armyworm bioassays with neonate rice strain larvae feeding on leaves of TifEagle plants following *Agrobacterim*-mediated transformation experiments and wild type showing larval weight and percent mortality[1].

TifEagle Line	Larval Weight	Percent Mortality
1	18.4 ± 1.0 a	20.04 ± 6.0 a
2	18.1 ± 1.8 a	20.0 ± 9.0 a
3	15.4 ± 2.1 a	0.0 ± 0 a
wild type	13.4 ± 1.6 a	16.0 ± 4 a
	$P = 0.1684$	$P = 0.0951$

[1] Data were obtained after nine days. Plants 1, 2 and 3 were positive for *cry1Fa* by PCR and line 2 was positive for the transgene on Southern blots as well as RT-PCR; means with the same letter are not significantly different.

CHAPTER 6
SUMMARY AND CONCLUSIONS

Comparative Analysis of Direct Plant Regeneration of Three Sugarcane Cultivars

There has been considerable effort to produce direct plant regeneration methods for monocots, especially sugarcane. The objective of this study was to compare three different cultivars CP84-1198, CP89-2143 and CP88-1762 for rapid and efficient direct regeneration. The cultivars responded differently to the same culture conditions. CP88-1762 was the most responsive to the tissue culture system tested, with explants producing the most shoots and the most elongated shoots, along with a sufficient number of roots to produce viable plantlets that were easily transferred to small pots. Therefore, of the three cultivars tested, CP88-1762 is the best candidate for future *Agrobacterium*-mediated transformation using this direct regeneration approach. Additionally, this study will help to inform future work on rapid callus-free regeneration of sugarcane that also may be useful for reducing somaclonal variation.

Expression of *cry1Fa* to Enhance FAW Resistance in Sugarcane using Biolistics

Bacillus thuringenesis (*Bt*) δ-endotoxins are also known as insecticidal crystal protein (ICPs) or the *Bt* crystal protein (Cry) (Dulmage, 1981). These *Bt* crystal proteins have been used in spore formulations for over 40 years (Tabashnik et al., 1997), however their limited field stability, lack of capacity to reach cryptic insects, and narrow spectrum of activity, *Bt* sprays represent only a minor proportion of the insecticide market (Ferre and Van Rie, 2002). Therefore, one of the best approaches to control insect pests such as FAW is the use of transgenic plants expressing a *Bt* δ-endotoxin (Schnepf et al., 1998; Ferre and Van Rie, 2002; Griffits and Aroian, 2005). Hence the objective of the present work was to produce transgenic sugarcane lines expressing the *cry1Fa* gene and to evaluate their ability to confer resistance to FAW.

Four transgenic sugarcane lines were generated and molecular analyses confirmed the presence and integration of *cry1Fa* into the genome. Transgene integration varied from simple to more complex, and RT-PCR and immunoassays showed that the transgene was expressed in all four lines. The expression of the *cry1Fa* gene increased resistance to the rice strain of FAW in laboratory bioassays indicating that these transgenics may be suitable candidates for future field studies and provide another strategy that can be implement in an integrated pest management program.

Evaluating *Agrobacterium* Vacuum Infiltration of Stolon Nodes for the Production of Transgenic Bermudagrass

Bermudagrass cultivars such as Tifton 85 and TifEagle are commonly used as warm season forage and turf grasses, respectively, in temperate and tropical parts of the world. Both cultivars are susceptible to FAW and are sterile, so the main objective of the study was to produce a rapid regeneration protocol that bypassed the callus phase in order to introduce *cry1Fa* via *Agrobacterium* vacuum infiltration of stolon nodes. Southern blot analysis confirmed the integration of *cry1Fa* in only one TifEagle plant and no Tifton 85 plants. Although *cry1Fa* transcripts could be detected in RT-PCR analysis of the TifEagle transgenic, it did not express it at a high enough level in leaf tissue to affect FAW weight gain or mortality. Further transformation experiments must be conducted to generate more transgenic TifEagle and Tifton 85 lines to properly evaluate this approach for FAW resistance.

APPENDIX A
LABORATORY PROTOCOLS

Molecular Techniques

Genomic DNA Extraction (modified from Dellaporta et al., 1983)

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

20% SDS (500 ml)
100 g SDS in 450 ml DD H_20.
Adjust to pH 7.2 with concentrated HCl.
Bring up to 500 ml.

0.5 M EDTA (pH 8) (50 ml)
9.35 g EDTA disodium salt dissolved in 40 ml dd H_2O.
Adjust to pH 8 with NaOH pellets.
(EDTA will not dissolve at the wrong pH)
Bring up to 50 ml.

5 M Potassium Acetate (100 ml)
29.44 g in up to 60 ml dd H_2O.
11.5 ml glacial acetic acid.
Bring up to 100 ml.

Extraction Buffer D (500 ml)
4.44 g Tris-HCl
2.65 g Tris base

9.3 g EDTA
7.3 g NaCl

3 M Sodium Acetate (10 ml)
0.82 g NaOAc in 10 ml dd H_2O.
Adjust to pH 5.2 with glacial acetic acid.

1 M Tris-HCl (pH 8) (200 ml)
24.22 g Tris base dissolved in 160 ml dd H_2O. Adjust to pH 8 with concentrated HCl.
Bring up to 200 ml.

TE (pH 8) (50 ml)
500 µl 1 M Tris-HCl, 100 µl 0.5 M EDTA. Bring up to 50 ml with sterile dd H_2O.

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

1.Prepare enough Buffer RLT for use by adding 10 µl ß-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 (autoclave for 30 min) mortar and pestle. Transfer the ground sample to a sterile, liquid-nitrogen cooled 2 ml microcentrifuge tube.
5. Add 450 µl 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 µl) ethanol (200 proof) to the collected supernatant and mix immediately by pipetting.
8. Immediately transfer sample, including any precipitant formed, to an RNeasy mini column placed in a 2 ml collection tube. Close the tube gently and centrifuge for 15 s 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 µl 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 µl 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 µl 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 1µg RNA immediately to prepare cDNA.

14. Store remaining RNA at -80 ºC.

iScript cDNA Synthesis Reaction

Component	**Volume per reaction**
Water	14 µl
5x iScript	4 µl
reaction mix	
Reverse	1 µl
transcriptase	
RNA template	1 µl
Total	20 µl

Incubate complete reaction mix

5 min at 25 ^{o}C
30 min at 42 ^{o}C
5 minutes at 85 ^{o}C
Hold at 4 ^{o}C (optional)

SuperScript ^{TM} III First-Strand Synthesis System for RT-PCR

The following procedure is designed to convert 1 pg to 5 µg of total RNA or 1 pg to 500 ng of poly(A) + RNA into first-strand cDNA:

1. Mix and briefly centrifuge each component before use.
2. Combine the following in a 0.2- or 0.5-ml tube:

Component	**Amount**
Upto 5 µg total RNA	n µl
Oligo dt	1 µl
10 mM dNTP mix	1 µl
DEPC treated water	to 10 µl

3. Incubate at 65°C for 5 min, then place on ice for at least 1 min.
4. Prepare the following cDNA Synthesis Mix, adding each component in the indicated order.

Componenet	**1 Rxn**
10 X RT buffer	2 µl
25 mM $MgCl_2$	4 µl
0.1 m DTT	2 µl

RNase OUT™ (40 u/ µl)	1 µl
Superscript™ III RT (200 u/ µl)	1 µl

5. Add 10 µl of cDNA Synthesis Mix to each RNA/primer mixture, mix gently, and collect by brief centrifugation. Incubate as follows.
Oligo(dT)20 or GSP primed: 50 min at 50°C
Random hexamer primed: 10 min at 25°C, followed by
50 min at 50°C
6. Terminate the reactions at 85°C for 5 min. Chill on ice.
7. Collect the reactions by brief centrifugation. Add 1 µl of RNase H to each tube and incubate for 20 min at 37°C.
8. cDNA synthesis reaction can be stored at -20°C or used for PCR

Protocol for Biolistic Gene Transfer (Altpeter and James, 2005)

1. Gold stocks (60 mg/ml): Weigh 12mg gold into an eppendorf tube. Wash several times in Absolute EtOH by vortexing and centrifuging briefly. Resuspend in 200µl ddH_2O.
2. Gold preparation: Mix 15 µl 0.75 µm gold, 15 µl 1µm gold and 30µl DNA by vortexing 1 min. Add 20 µl 0.1 M spermidine and 50 µl 2.5 M $CaCl_2$ while vortexing. Keep vortexing for 1 min. Centrifuge briefly to settle gold. Wash in 250 µl Absolute EtOH by vortexing. Spin and remove supernatant. Resuspend gold in 180 µl Absolute EtOH. (or resuspend in 90µl for more gold per shot). Use 5 µl 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 5 µl 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 27 in 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.

Southern Blotting

10 x nucleic acid loading dye mix
To 40 mg bromophenol blue,40 mg xylene cyanol, and 2.5 g Ficoll™ 400, add approximately 8 ml of distilled water. Mix to dissolve. Make up to a final volume of 10 ml. Store at room temperature for up to three months.

50 x TAE (DNA electro-phoresis buffer)

242 g Trizma™ base. 18.6 g ethylenediaminetetra-acetic acid (EDTA), sodium salt. Add approximately 800 ml of distilled water. Mix to dissolve. Adjust to pH 8 with glacial acetic acid (~57 ml/l). Make up to a final volume of 1000 ml. Store at room temperature for up to 3 months.

Depurination solution

11 ml HCl, 989 ml distilled water. Mix. Store at room temperature for up to 1 month.

Denaturation buffer

87.66 g NaCl, 20 g NaOH,Add approximately 800 ml of distilled water. Mix to dissolve. Make up to a final volume of 1000 ml. Store at room temperature for upto 3 months.

Neutralization buffer

87.66 g NaCl, 60.5 g Trizma base,Add approximately 800 ml of distilled water. Mix to dissolve. Adjust to pH 7.5 with concentrated hydrochloric acid. Make up to a final volume of 1000 ml. Store at room temperature for up to 3 months.

Nucleic acid transfer buffer (20 x SSC)

88.23 g Tri-sodium Citrate, 175.32 g NaCl, Add approximately 800 ml of distilled water. Mix to dissolve. Check the pH is 7–8. Make up to a final volume of 1000 ml. Store at room temperature for up to 3 months.

TE buffer

1.21 g Trizma base, 0.372 g EDTA, sodium salt, Add approximately 800 ml of distilled water. Mix to dissolve. Adjust to pH 8 with concentrated hydrochloric acid. Make up to a final volume of 1000 ml. Store at room temperature for up to 3 months.

Protocol for capillary blotting

1. Cut a sheet of membrane to an appropriate size.
2. Half fill a tray or glass dish of a suitable size with the transfer buffer. Make a platform and cover with a wick made from three sheets of Hybond blotting paper saturated in transfer buffer.
3. Place the treated gel on the wick platform. Avoid trapping any air bubbles between the gel and the wick. Surround the gel with cling film to prevent the transfer buffer being absorbed directly into the paper towels.
4. Place the membrane on top of the gel. Avoid trapping any air bubbles.
5. Place three sheets of Hybond blotting paper cut to size and saturated in transfer buffer, on top of the membrane. Avoid trapping any air bubbles.
6. Place a stack of absorbent towels on top of the Hybond blotting paper at least 5 cm high.
7. Finally, place a glass plate and a weight on top of the paper stack. Allow the transfer to proceed overnight. The weight should not exceed 750 g for a 20 x 20 cm gel.
8. After blotting, carefully dismantle the transfer apparatus. Before separating the gel and membrane, mark the membrane to allow identification of the tracks with a pencil or chinagraph pen.
9. Fix the nucleic acid to the membrane by baking at 80°C for 2 hours or by using an optimized UV crosslinking procedure.
10. Blots may be used immediately. Blots must be thoroughly dried if storage is required.

Sothern blotting – Gel treatment

1. Separate the DNA samples on a suitable neutral agarose gel.
2. Following electrophoresis visualize the DNA samples in the gel with UV light and photograph.
3. Process the gel for blotting, between each step rinse the gel in distilled water.
4. Depurination. Place in 0.125 M HCl so that the gel is completely covered in the solution. Agitate gently for 10–20 minutes. During this time the bromophenol blue dye present in the samples will change colour.
5. Denaturation. Submerge the gel in sufficient denaturation buffer. Incubate for 30 minutes with gentle agitation. During this time the bromophenol blue dye will return to its original colour.
6. Neutralization. Place the gel in sufficient neutralization buffer to submerge the gel. Incubate for 30 minutes with gentle agitation

General procedure for radioactive hybridizations (Prime-a-gene® Labelling system, Promega).

1. All radioactive work must be done behind the shield and all users must have a film badge to handle any isotope.
2. Early in the day, prior to beginning, turn on the incubator and 65 ºC water bath, remove P^{32} and salmon sperm DNA needed from freezer. Place closed P^{32} container behind the plastic shield to thaw. Take hybridization buffer solution out of refrigerator and place in 65 ºC water bath.
3. Assemble large hyb tubes needed in rack, place blots in tubes, wrap top with Teflon tape, and pre-wet with 5X SSC solution. Heat 1 liter or larger beaker with water to boiling outside of shield area.
4. Thaw Prime-a-gene kit components except keep Klenow enzyme on ice. Thaw probe DNA, prepare dNTP mixture, and use screw cap Eppendorf tubes for probes.
5. Write out Hybridization solution reaction components and add water and probe DNA amount needed to screw cap tubes. Place the tubes in boiling water and heat to denature for 5 minutes. Also place a screw cap tube of Salmon sperm DNA to denature at the rate of 500 ul for each Hybridization tube. After boiling, place the tubes on ice for 5 minutes.
6. After the 5 minutes on ice, drain the 5X SSC from large Hyb tubes into sink, add 20 ml of fully heated 65 ºC Hybridization solution to each tube, add 500 ul of de-natured Salmon sperm DNA to each tube, cap tightly, but not too tight, and place into rack inside fully heated incubator, balancing tubes across from one another. Close door, turn on rotator and check for leaky lids and proper rotation.
7. Add buffer, BSA, dNTPs, and Klenow to probe DNA tubes on ice outside of shield. Place tubes on ice behind shield to add P^{32}. Cap tightly, mix lightly, and place in rack behind shield at room temperature for 3-4 hours.
8. After 4 hours, start boiling water in beaker behind shield.

9. When water is boiling, incubation is over so place Hyb probe screw cap tube along with 500ul per tube of Salmon sperm into tube float and boil to denature for 5 minutes.
10. After four minutes boiling, remove large Hyb tubes from incubator, drain pre-hyb solution into sink and place in rack.
11. Right before five minutes add 10ml hot Hyb solution to each tube, turn off burner, and add the probe/Salmon sperm mixture to the Hyb solution at the bottom of each tube, taking care to be sure the pipette dispenses radioactively 'hot' probe solution directly into Hyb solution and not onto blots in tube.
12. Work quickly to recap tubes and place back into incubator. Turn on rotator and check for leaky caps.
13. Allow to incubate overnight.
14. The next day pour off the probes into rad waste container and wash blots in the tubes with 0.1X SSC, 0.1% SDS solution heated to 65 ºC one quick rinse pouring waste into rad containers, then for 20 minutes two additional washes.
15. Remove blots from tubes behind shield and wrap with Saran wrap.
16. Check for activity with Geiger counter and place into film cassette.
17. In darkroom, add number of X-ray films needed, close cassette.
18. Place in -80o freezer for number of days needed.
19. Develop film in darkroom.

Protocol for Immunochromatography strip test for *cry1F* gene (Quickstix^{TM} kit for *cry1F*, EnviroLogix)

Sample Preparation

1 Prepare leaf samples from the fifth fully emerged leaf: Take two leaf segments 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
2. QuickStix Strips to be used. Avoid bending the strips. Reseal the canister immediately.
3. Place the strip into the extraction tube. The sample will travel up the strip. Use a rack to support multiple tubes if needed.
4. Allow the strip to develop for 10 minutes before making final assay interpretations. Positive sample results may become obvious much more quickly.
5. To retain the strip, cut off the bottom section of the strip covered by the arrow tape.
6. 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.

7. 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.
8. 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.

Quick start™ Bradford Protein Assay

1x Dye Reagent:

1 L of dye solution containing methanol and phosphoric acid. One bottle of dye reagent is sufficient for 200 assays using the standard 5 ml procedure or 4,000 assays using the microplate procedure.

BSA standard:

Provided in 2 ml tubes.

Standard Protocol

1. The standard protocol can be performed in three different formats, 5 ml and a 1 ml cuvette assay, and a 250 µl microplate assay.
2. The linear range of these assays for BSA is 125–1,000 µg/ml, whereas with gamma-globulin the linear range is 125–1,500 µg/ml.
3. Remove the 1x dye reagent from 4 °C storage and let it warm to ambient temperature. Invert the 1x dye reagent a few times before use.
4. If 2 mg/ml BSA or 2 mg/ml gamma-globulin standard is used, refer to the tables in theappendix as a guide for diluting the protein standard. (The dilutions in the tables are enough for performing triplicate measure-ments of the standards.) For the diluent, use the same buffer as in the samples.
5. Protein solutions are normally assayed in duplicate or triplicate. For convenience, the BSA or gamma-globulin standard setscan be used, but blank samples (0 µg/ml) should be made using water and dyereagent.
6. Pipet each standard and unknown sample solution into separate clean test tubes or microplate wells (the 1 ml assay may be performed in disposable cuvettes). Add the 1x dye reagent to each tube (or cuvette) and vortex (or invert). For microplates, mix the samples using a microplate mixer. Alter-natively, use a multichannel pipet to dispense the 1x dye reagent. Depress the plunger repeatedly to mix the sample and reagent in the wells. Replace with clean tips and add reagent to the next set of wells.
7. Incubate at room temperature for at least 5 min. Samples should not be incubated longer than 1 hr at room temperature.
8. Set the spectrophotometer to 595 nm. Zero the instrument with the blank sample.
9. Measure the absorbance of the standards and unknown samples.

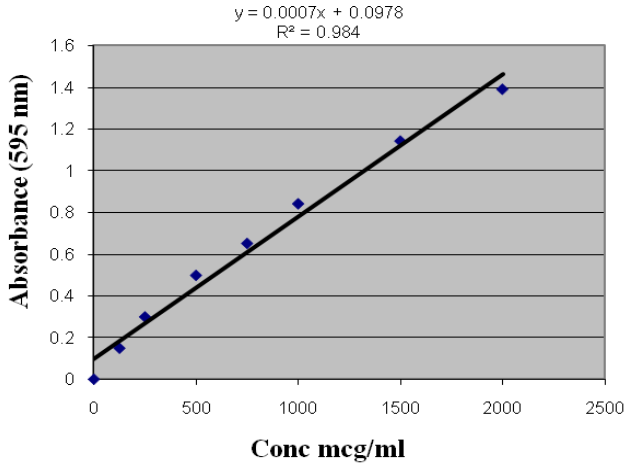
Assay	Volume of Standard and Sample	Volume of 1x Dye Reagent
5 ml	100 µl	5 ml
1 ml	20 µl	1 ml
Microplate	5 µl	250 µl

Data analysis

1. If the spectrophotometer or microplate reader was not zeroed with the blank, then average the blank values and subtract the average blank value from the standard and unknown sample values.
2. Create a standard curve by plotting the 595 nm values (y-axis) versus their concentra-tion in µg/ml (x-axis). Determine the unknown sample concentration using the standardcurve. If the samples were diluted, adjust thefinal concentration of the unknown samples by multiplying by the dilution factor used.

5 ml Standard Assay

Tube #	Standard Volume (µl)	Source of Standard	Dilutent Volume (µl)	Final Protein (µg/ml)
1	300	2 mg/ml stock	0	2000
2	375	2 mg/ml stock	125	1500
3	325	2 mg/ml stock	325	1000
4	175	Tube 2	175	750
5	325	Tube 3	325	500
6	325	Tube 5	325	250
7	325	Tube 6	325	125
8 (Blank)	-	-	325	0



High sensitivity Protocol for Enzyme Linked Immunadsorbent-Assay for the cry1F gene

(QualiPlate™ Kit for Cry1F, EnviroLogix)

Wash Buffer: Add the contents of the packet of Buffer Salts (phosphate buffered saline, pH 7.4 - Tween 20) to 1 liter of distilled or deionized water and stir to dissolve.

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.

Cry1F Positive Control: The Positive Control is used as provided in the Rapid Protocol, but must be diluted 1:4 for use in the High Sensitivity Protocol. Prepare this dilution just prior to running the assay: mix 50 µL Cry1F Positive Control with 150 µL extraction Buffer for each set of duplicate wells to be filled.

HIGH SENSITIVITY PROTOCOL

The High Sensitivity Protocol will detect 0.17% Herculex I corn or WideStrike cotton in ground grain/seed, and requires two hours of total assay incubation time. Dilute the Cry1F Positive Control 1:4 in Extraction Buffer for this protocol.

1. Add 50 µL of Extraction Buffer Blank (BL), 50 µL of diluted Positive Control (PC), and 50 µL of each sample and user-prepared control extract (S) to their respective wells, as shown in the Example Plate Layout. NOTE: In order to minimize setup time it is strongly recommended that a multi-channel pipette be used in steps 1, 5, 8, and 10.

2. 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!

3. 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.

4. 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. Alternatively, perform these four washes (300 µL/well) with a microtiter plate or strip washer. Slap the inverted plate on a paper towel to remove as much liquid as possible.

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 1 hour. If an orbital plate shaker is available, shake plate at 200 rpm.

7. Wash the plate as described in step 4.

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 1.0 N hydrochloric acid. Handle carefully.

10. Add 100 µL 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.

Calculate the 'Positive Control Ratio: Divide the OD of each sample extract by the mean OD of the Positive Control wells. This number is the "Positive Control Ratio".

Interpretation of Qualitative results (Leaf samples)
If the Positive Control Ratio calculated for a sample is less than 1.0, the sample does not contain Cry1F at the levels normally found in Herculex I corn or WideStrike cotton. If the Positive Control Ratio of a sample is greater than or equal to 1.0, the sample is Herculex I corn or WideStrike cotton.

	R1	R2	R3		
BL	0.038	0.035	0.038	0.037	
1	0.492	0.493	0.471	0.485333	Cry1f positive
2	0.686	0.678	0.61	0.658	plant positive
3	0.041	0.043	0.041	0.041667	Wild type
4	0.077	0.08	0.079	0.078667	1
5	0.322	0.315	0.305	0.314	2
6	1.122	1.098	1.109	1.109667	3
7	0.801	0.821	0.834	0.818667	4

PCR reaction components (25 µl reaction)

Water	16.3µl
Buffer	2.5 µl
$MgCl_2$	1.0 µl
dNTP	1.0 µl
Forward Primer	1.0 µl
Reverse Primer	1.0 µl
Taq	0.2 µl
DNA	2.0 µl

Total	25 µl

Hot Start PCR reaction components (25 µl reaction)

Water	17.25 µl
Buffer	2.5 µl
dNTP	1.0 µl
cry forward primer	1.0 µl
Cry reverse primer	1.0 µl
Hot start Taq	0.25 µl
DNA	2.0 µl
Total	25

Stocks and solutions

Acetosyrignone Stock (10 mg/ml)
Put 50 mg acetosyrignone in a tube, add 1 ml of 70% ethanol and mix (vortex) then Make the volume to 5 ml. Filter sterilize.

Hygromycin stock (10 mg/ml)
Dissolve 500 mg in 20 ml distilled water. Bring up to 50 ml.

Na-thiosulphate stock (10 mg/ml)
Dissolve 400 mg in 1 ml distilled water, mix and vortex and add up to 40 ml water. Filter sterilize and store at 4 ^{o}C.

DL-dithiothreitol stock (10 mg/ml)
Add 400 mg in a tube and dissolve in 1-2 ml distilled water while shaking on vortex Make up to 40 ml and filter sterilize.

Cefotaxime (10 mg/ml)
Dissolve 500 mg cefotaxime in 5 ml of distilled water. Mix while vortexing. Bring up to 50 ml.

IBA stock (1 mg/ml)
Dissolve 50 mg in 2-3 ml of 1 N Koh and then complete the volume to 50 ml with distilled water. Filter sterilize.

Geneticin Stock (50 mg/ml)
Add 750 mg in 1 ml distilled water and dissolve it and add 14 ml water. Bring up to 15 ml. Filter sterilize and store at 4 ^{o}C.

YEP medium (1 liter)
10 g peptone, 10 g yeast extract, 5 g NaCl, pH 7.0-7.2, autoclave and store at 4 ^{o}C.

10 X TBE /l
Add in order, Tris Base 108 g, boric acid 55 g, and 40 ml 0.5 M EDTA.

0.1M Spermidine
Add 986 µl of distilled water and to it add 14 µl spermidine.

$CaCl_2$ (2.5 M)
36.75 g dissolved in 100 ml distilled water. Autoclave to sterilize.

BAP (1 mg/ml)
0.025 g powder dissolved in 500 µl 1N KoH. Make up to 25 ml with distilled water. Filter sterilize and store at -20 ^oC.

2, 4-D (2 mg/ml)
0.1g dissolved in very little 1N KoH. Make up the volume with 50 ml distilled water. Filter sterilize and store in aliquots at -20^oC.

Gold Stock (60 mg/ml)
Weigh 12 mg gold. Weigh eppendorf tube, put tube on balance, make it zero, add gold to tube. Wash three times with absolute alcohol by vortexing and centrifuging briefly. Resuspend in 200 µl distilled water.

L-Cysteine (10mg/l)
Mix 500 mg L-cysteine in 1 ml water, mix and add 49 ml water.

Kanamycin stock (10 mg/ml)
Dissolve 500 mg kanamycin monosulphate in 20 ml water, bring up to 50ml with distilled water. Store at -20^oC.

Spectinomycin stock (10 mg/ml)
Dissolve 500 mg in 20 ml distilled water. Bring up to 50 ml with distilled water. Store at -20 ^oC.

BG-A1 Media (1 liter) solid.

1 Half strength MS media – 2.2g
2 Kinetin (4.5 µM) - 1ml/l (1mg/ml stock).
3 2,4-D (1.8 µM) - 39 µl/l (10mg/ml stock)
4 L-cysteine (3.3mM) - 400 mg/l
5 Dithiothreitol (1mM) - 15.4 mg/l
6 Na-thiosulphate (1mM) - 15.8 mg/l
7 Sucrose - 20 g/l
8 Agargel - 5.6 g/l
9 pH -5.8 to 5.85
Autoclave for 20 minutes

BG-A2 Media (1 Liter) Selection Media

1 MS Basal Medium - 4.4 g/l
2 Kinetin (4.5 µM) - 1 ml/l (1 mg/ml stock)
3 2,4-D (0.2 µM) - 4 µl/l (10 mg/ml stock)
4 PPM - 750 µl
5 Sucrose - 20 g/l
6 Hygromycin - 75 mg/l
7 Cefotaxime - 250 mg/l
7 pH -5.8 to 5.85
8 Agargel - 5.6g/l

DRG Media (1 liter)
1 MS Basal salts - 4.4 g
2 Sucrose - 20 g
3 PPM - 750 µl
4 pH - 5.8 to 5.85
5 Agargel - 5.6 g
Autoclave
6 Filter sterilized NAA (5 mg/l) - 500 µl (10 mg/ml)
7 Filter sterilized Kinetin (0.5 mg/l) – 500 µl (1 mg/ml)

Rooting Media (IBA) (500 ml)
1 MS Basal Medium - 2.2 g
2 PPM - 350 µl
3 Sucrose - 10 g
4 pH - 5.8 to 5.85
5 Agargel - 2.3g
Autoclave
6 Filter sterilized (IBA) - 2 ml (1mg/ml)

TDZ Media (Shoot initiation media) (1 liter)
1 MS Basal salts - 4.4 g/l
2 Sucrose - 20 g/l
3 PPM - 750 µl
4 pH -5.8 to 5.85
5 Agargel - 5.6 g
Autoclave
6 Filter sterilized TDZ - 550 µl (1mg/ml)

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BIOGRAPHICAL SKETCH

Sunil Joshi, the youngest and only son of Dr. D.V. Joshi was born in Ludhiana, Punjab, India on January 6th, 1976. In 1995, Sunil started his college studies in Agriculture at Punjab Agricultural University, Ludhiana; Punjab where he received his four years honors bachelors' degree in Agriculture in 1999 with an elective in crop science. After that, he joined Masters Degree in 1999 in Plant Breeding with research focusing on 'Genetic studies on leaf blight resistance in durum wheat (*Triticum turgidum*) L. var. Durum'. After his M.S. in 2002, he joined as Senior Research Fellow working on projects 'Development of hybrid rapeseed and mustard' and 'Breeding designer Brassica in rapeseed and mustard' till December 2004. After that he moved to Gainesville, Florida to begin his Ph.D. program at the Agronomy Department, University of Florida in 2005.