DEVELOPMENT OF A TISSUE CULTURE AND TRANSFORMATION PROTOCOL FOR SEASHORE PASPALUM (*Paspalum vaginatum* SWARTZ)

By

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To friends and family I relied on to keep me going.

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LIST OF ABBREVIATIONS

2,4-D	2,4-Dichlorophenoxyacetic acid
2,4,5-T	2,4,5-Trichlorophenoxyacetic acid
BAP	Benzylaminopurine
Bt	*Bacillus thuringiensis*
dicamba	3,6-Dichloro-2-mehthoxybenzoic acid
GFP	Green fluorescent protein
GUS	β–glucuronidase
ICP	Insecticidal crystal protein
NAA	α-Naphthaleneacetic acid
PCR	Polymerase chain reaction
PMI	Phosphomannose isomerase
SI1	Sea Isle 1

Abstract of Thesis Presented to the Graduate School
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Seashore paspalum (*Paspalum vaginatum* Swartz) is a salt tolerant, fine textured turfgrass used on golf courses in coastal, tropical and subtropical regions. Targets for genetic engineering of seashore paspalum include improved disease and insect resistance. However, a genetic transformation protocol for seashore paspalum is lacking. In this work, a callus induction, plant regeneration, and transformation protocol for this commercially important turfgrass species has been developed. Induction of highly regenerable callus with approximately 400 shoots per cultured immature inflorescence (1 cm in length) was achieved by culturing 0.2 cm segments on media with 3 mg L^{-1} 3,6-dichloro-2-mehthoxybenzoic acid (dicamba) and 0.1 or 1.0 mg L^{-1} benzylaminopurine (BAP). A multifactorial experiment showed that callus induction medium containing 3 mg L^{-1}dicamba and 1.0 mg L^{-1} BAP had a plant regeneration frequency that was 12 times higher than medium with 3 mg L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) alone, and 10 times higher than the combination of 3 mg L^{-1} 2,4-D and 1.0 mg L^{-1} BAP. The correlation between transient and stable transformation frequency in seashore paspalum was evaluated using callus derived from six different callus induction media for biolistic gene transfer of a constitutive *uidA* (GUS) reporter gene expression cassette or an *hph* selectable marker expression

cassette. Callus induction media using immature inflorescence segments as explants differed in auxin type (2,4-D or dicamba) and cytokinin concentration (0.0, 0.1 or 1.0 mg L^{-1} BAP). Media with 2,4-D supported significantly higher transient GUS expression. However, media containing dicamba showed higher plant regeneration frequencies. Transgenic plants regenerated on both 2,4-D- and dicamba-containing media. Transgenic plants grew vigorously and did not show phenotypic differences compared to nontransformed controls. This is the first report of the production of transgenic seashore paspalum plants.

CHAPTER 1
INTRODUCTION

Introduction

Fresh water availability is one of the many challenges facing society on a global scale. According to the UN, in 2025 nearly 3 billion people will face severe water shortages if water consumption continues at the current rate (Montaigne 2002). Thus for issues like turf management, it will become impractical to continue using fresh water for irrigation. Indeed, when water shortages do occur, the use of fresh water for turfgrass maintenance will be a low priority (Kjelgren et al. 2000). Besides fresh water shortages, currently many land areas face salinity issues. As of a decade ago, 10 to 20 Mha of irrigated land lost productivity from salinity related toxicity annually (Hamdy 1996; Choukr-Allah 1996).

To mitigate shortages of fresh water, states such as California and Arizona have passed laws requiring the use of saline sources for turfgrass irrigation (California State Water Resource Control Board 1993; Arizona Department of Water Resources 1995). These sources include sewage effluent or brackish water. Emphasis on better management of fresh water use is particularly important in coastal areas. These regions face unique challenges because overused aquifers fall victim to salt intrusion (Parker 1975; Murdock 1987). Therefore, it is not surprising that using recycled water as the primary source for irrigating golf courses in Florida has increased from only 8% in 1974 to nearly 50% in 2000 (Haydu and Hodges 2002). Although these practices may address issues pertaining to fresh water conservation, they do not alleviate saline stresses that would result from irrigating with these recycled sources. As fresh water becomes limiting, alternative flora with adaptation to saline conditions will be needed.

Using a salt tolerant turf would reduce poor performance of turf in saline conditions. Because plants are more susceptible to salt stress under hot dry conditions than cool humid ones

(Hoffman and Rawlins 1971), a turfgrass showing high levels of salt tolerance grown in tropical conditions would be economically advantageous to incorporate in areas where fresh water irrigation is no longer a viable option. Seashore paspalum (*Paspalum vaginatum* Swartz) is a turfgrass that has shown much promise for its excellent turf quality under saline conditions (Duncan and Carrow 2000a).

Seashore Paspalum

Seashore paspalum, *Paspalum vaginatum* Swartz, is also known as saltwater couch, sand knotgrass, or siltgrass. It is a halophytic warm season perennial grass highly adapted to saline, tropical environments and sandy soils (Malcom and Laing 1969; Morton 1973; review Duncan and Carrow 2000b). Seashore paspalum is native to coastal environments where it is able to grow directly on sand dunes adjacent to breaking waves (Duncan 1996).

Seashore paspalum has good potential for use in soil bioremediation in part due to its great adaptability to soil pHs ranging from 4.0 to 9.8, and its tolerance to a wide range of soil types (Duncan 1996), as well as to flooding (Malcom 1969; Morton 1973; Peacock and Dudeck 1985), and drought (Beard et al. 1991b). Being a warm season perennial suitable for golf, seashore paspalum is often compared to bermudagrass (*Cynodon dactylon* L.). A study comparing wear tolerance between bermudagrass and seashore paspalum found that wear tolerance of both species can be attributed to high shoot density and leaf moisture, suggesting that turfgrass managers can maintain high levels of wear tolerance in seashore paspalum like bermudagrass by avoiding drought stress and adjusting their cultural practice to maintain a high shoot density (Trenholm et al. 2000). Another study on the wear tolerance of seashore paspalum also found cultivars with high shoot densities to be equal to or better than tested bermudagrasses, while cultivars of seashore paspalum with low shoot densities had poor wear tolerance (Trenholm et al. 1999).

Much work has been done on screening for salinity tolerance in seashore paspalum (Dudeck and Peacock 1985; Lee et al. 2002; 2004a; 2004b). Seashore paspalum showed a greater salt tolerance than cultivars of bermudagrass (Lee et al. 2002). One cultivar of seashore paspalum 'Sea Isle 1' (SI1) was patentable because of its "distinguished high tolerance to salinity, dark green color, fine textured leaves, and high turf quality and density" (Duncan 2002). SI1 was selected with criteria important to golf managers looking for aggressive growth and tolerance to close mowing. It has unknown parentage from Argentina (Duncan 2002). It is also useful for erosion control because of its ability to tolerate waterlogged conditions and occasional mesosaline flooding (Duncan 2002).

A botanical description of seashore paspalum, subfamily Panicoideae, includes unmowed culms ranging in height from 8 to15 cm with glabrous nodes. It has fine textured, distichouss leaves up to 50 mm in length and 2 mm in width and lacking auricles. The lamina shape is linear-triangular and tapers to a narrow apex. Other features of the leaf include a prophyllum of 20 mm in length, a 1 mm ligule with a distinguishable pubescent collar, obscure venation, with smooth edges on the leaf blades. Internode lengths range from 7 to 9 mm and nodes are pubescent. The inflorescence has a digitate shape with two unilateral short spreading racemes 20 to 25 mm in length including 16 to 25 twin-rowed spikelets on each primary raceme. The spikelets are solitary, plano-convex, subsessile, elliptic, approximately 2.5 mm in length, and 0.9 to 1.5 mm wide. Anthers are 1.2 to 1.4 mm in length. Glumes are glabrous. It is a creeping, mat forming, short, species having both stolons and rhizomes. Vegetative cuttings are the primary method of propagation (Duncan 2002; Loch and Roche 2003).

Seashore paspalum is diploid (2n=2x=20) and highly self-incompatible (Burson 1981). Seashore paspalum can be found world-wide, but is mostly found in the tropical and subtropical east coast of the Americas.

Initially, seashore paspalum was not popular as turf due to incorrect management from high fertilizer rates which caused significant problems with thatch (Duncan 1996). The best way to maintain high quality seashore paspalum plants is to avoid excess nitrogen application, irrigation in the latter part of the day, scalping, shade, and areas with poor air circulation (Trenholm and Unruh 2002). Some seashore paspalum cultivars have shown high quality without any additional N fertilizer, a key factor in becoming a practical turfgrass for golf courses in subtropical areas (Beard et al. 1982; 1991b; Duble 1989). Also, some cultivars of seashore paspalum tolerate low mowing heights of less than 13 mm (Beard et al. 1991a; 1991b).

There are potential advantages to using seashore paspalum in home lawns, including good wear tolerance, low fertility requirements and the production of extensive root systems (Trenholm and Unruh 2002). However, this turfgrass has significant hurdles to overcome to be a success in the home landscape. Shaded turf area is estimated to be at 25% in the US (Beard 1973) and seashore paspalum has poor shade tolerance (Trenholm and Unruh 2002). Weed control is limited because seashore paspalum has poor tolerance to most conventional herbicides (Trenholm and Unruh 2002). It also performs best at consistent mowing heights of one to two inches. It is also important to not remove more than 1/3 of the leaf blade when mowing as this can lead to scalping and leave the grass susceptible to fungal and insect problems (Trenholm and Unruh 2002). As such, current cultivars of seashore paspalum do perform best as turf in a well maintained golf course.

In a study with two paspalum cultivars, two zoysiagrass (*Zoysia japonica* Steud.) cultivars, and two bermudagrass cultivars, resistance to fall armyworm was greatest in zoysiagrass and worst in the paspalum cultivars with SI1 being the most susceptible (Braman et al. 2004). Fall armyworm has been found to be one of the most serious pests in corn and grasses throughout the Americas (Ashley et al. 1989). Increasing resistance to fall armyworm in seashore paspalum, and particularly in SI1, may make it more popular as a turf. Other targets for genetic improvement of seashore paspalum include shade (Duncan 1996), cold (Ibitayo and Butler 1981) and enhanced drought tolerance, as well as disease resistance.

While genetic improvement by traditional plant breeding is complicated by the high self incompatibility and poor seed set of the species, genetic diversity for both cold tolerance and a certain level of tolerance to mole crickets and fall armyworm have been described (Duncan 1996). Other warm season turfgrasses have also shown genetic variability in pest tolerance (Quisenberry 1990; Braman et al. 1994; Shortman et al. 2002). Furthermore, in one study, a high level of variation was found in tolerance to drought for seashore paspalum suggesting that breeders can use these variations to improve cultivars (Carrow 2005). Genetic transformation allows for the introduction and expression of heterologous genes from genetically distant sources with the potential to significantly improve seashore paspalum. However, a genetic transformation protocol is not yet available for seashore paspalum. For genetic transformation to be successful, protocols for *in vitro* tissue culture, gene delivery, selection and regeneration of transgenic plants must be developed.

Objectives of the Research

Given the above background, the objectives of the research were to:

1) Enhance the regeneration response of seashore paspalum tissue culture through optimization of explant type and culture media composition;

2) Use highly regenerable tissues as targets for the co-transfer of a selectable marker gene and an insect resistance gene *cry1Fa* from *Bacillus thuringiensis*;

3) Optimize a protocol for the selection of transgenic events; and

4) Perform molecular analysis for evaluation of successful transgene integration and expression in seashore paspalum plants.

CHAPTER 2
LITERATURE REVIEW

Turfgrass Tissue Culture

There are 14,000 golf courses in the US and an additional 300 golf courses are being added annually (Lee 1996). Golf managers would prefer turf that requires less management and fewer pesticide applications (Lee 1996). Traditional plant breeding has made impressive progress in the development of advanced cultivars but faces the limitations given by natural hybridization barriers. Transgenic technology can overcome these limitations and contribute to accelerated development of genetically improved germplasm (Vasil 1995). This certainly would apply to seashore paspalum where progress by traditional plant breeding is limited by poor fertility, and genetic improvement for insect and disease resistance is highly desirable. Since a genetic transformation protocol for seashore paspalum is not available, its development was the focus of this research. As a prerequisite for genetic transformation of grasses, a protocol for efficient plant regeneration from undifferentiated tissues must be developed (Ritala et al. 1995).

Plant tissue culture and regeneration systems have been extended from major crop species to other species like turf and forage type grasses (Chai and Sticklen 1998). Progress with grasses, once considered extremely recalcitrant to tissue culture, can be attributed to 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; 1985; Lu et al. 1984).

Regenerable tissue cultures have been developed for forages (Chen et al. 1977; Lo et al. 1980; Baja et al. 1981; Songstad et al. 1983; Johnson and Worthington 1987; Metzinger et al. 1987; Straub et al. 1989; Franklin et al. 1990; Akashi and Adachi 1992a;1992b). However, progress in tissue culture of turfgrass has been slow because of the initial use of mature and

differentiated tissues as explants (Vasil and Vasil 1994). According to Vasil (1995), there are some strategies to successfully establishing regenerable callus cultures with turfgrass that involve choosing explant tissues made of meristematic tissues and undifferentiated cells and using culture media supplemented with strong auxins.

For the tissue culture phase to be successful, callus induction from the explant, induction of embryoids, and efficient regeneration of normal plants must be optimal (Street 1979). Genotypic diversity in tissue culture response has been reported in varying cultivars of selfing cereals (Lazer et al. 1983) like barley (*Hordeum vulgare*; Bregitzer 1992) and wheat (*Triticum* spp.; Fennell et al. 1996), and grasses (Altpeter and Posselt 2000). For different cereals, genotypic differences can affect callus initiation and plant regeneration in tissue culture (Dornelles et al. 1997). In one study, 25 tall fescue (*Festuca arundinacea* Schreb.) cultivars were tested and significant differences were found for callus formation ranging from 4.4% to 40.3% and for shoot regeneration ranging from 16.7% to 54.5% (Bai and Qu 2000).

One of the first observations of somatic embryogenesis in grass species was made using immature embryos of Italian ryegrass, *Lolium multiflorum* Lam. (Dale 1980). Many subsequent studies have successfully regenerated whole plants from callus initiated with immature inflorescences (Ahn et al. 1985; 1987; Artunduaga et al. 1988; 1989; Chaudhury and Qu 2000; Dale et al. 1981; Dale and Dalton 1983; Baja et al. 1981; Xu et al. 1984; Songstad et al. 1986; Van der Valk et al. 1989; George and Eapen 1990; Straub et al. 1992).

Immature inflorescences have been shown repeatedly to be one of the best explant sources in grasses to generate callus. The use of immature inflorescences for callus induction has been reported for common bermudagrass (Ahn et al. 1985; 1987), tall fescue (Eizenga and Dahleen 1990), pentaploid bermudagrass (2n=5X=45; Jain et al. 2005), perennial ryegrass (*Lolium*

perenne L.; Can et al. 2004), Kentucky bluegrass (*Poa pratensis* L.; Van der Valk et al. 1989), Chinese leymus (*Leymus chinensis*) (Caswell et al. 2000), species of *Paspalum* (Bovo and Mroginski 1985), and in seashore paspalum genotypes (Cardona and Duncan 1997). The maturity of the cultivated immature inflorescence is critical in obtaining regenerable callus with only inflorescences of specific lengths and a yellowish to pale green color being effective (Lu and Vasil 1982).

In many instances, 2,4-D (2,4-Dichlorophenoxyacetic acid) is the preferred auxin for callus induction in grasses (Bhaskaran and Smith 1990; Chaudhury and Qu 2000). Various concentrations of 2,4-D have shown positive response in embryogenic callus induction. Embryogenic callus was induced in buffelgrass (*Cenchrus ciliaris*) with 2,4-D (3 and 9 mg L^{-1} ; Colomba et al. 2006), tall fescue (9 mg L^{-1} 2,4-D; Bai and Qu 2000), and pentaploid bermudagrass (3 to 6 mg L^{-1} 2,4-D; Jain et al. 2005). Therefore, auxin concentration is an important factor to consider when optimizing a tissue culture protocol, but tissue can also have a different callus response based on the auxin source. There are alternative auxin sources that have been shown to be successful in the induction of somatic embryos in turfgrass species. In a comparative study of auxin types for induction of embryogenic callus of sorghum (*Sorghum bicolor*), 2,4-D was superior followed by α-Naphthaleneacetic acid (NAA), dicamba, picloram, and 2,4,5-Trichlorophenoxyacetic acid (2,4,5-T) (Jogeswar et al. 2007).

Dicamba is an alternative auxin that has been used to induce embryogenic and somatic callus in monocot tissue culture. The use of dicamba instead of 2,4-D led to much higher quality callus across four genotypes of barley (Tiidema and Truve 2004). In one study on wheat, dicamba was more effective than 2,4-D in inducing embryogenic callus from cultivated mature embryos (Filippov et al. 2006). Varying levels of dicamba were tested with immature

inflorescences from perennial ryegrass and the highest generation of fresh weight was with dicamba at 7.5 mg L^{-1} while the highest shoot regeneration was with 5.0 mg L^{-1} dicamba (Can et al. 2004). Dicamba was also better at maintaining prolonged cell cultures in barley and bahiagrass (*Paspalum notatum* Flugge) than 2,4-D (Castillo et al. 1998; Altpeter and Positano 2005).

Supplementation of callus regeneration media with cytokinins like benzylaminopurine (BAP) also typically enhances formation of shoot primordia and plant regeneration. In a study with triploid bermudagrass, regeneration media lacking cytokinins had a significantly lower plant regeneration frequency than media containing cytokinins (Lu et al. 2006). Similarly, inclusion of the cytokinin BAP in the regeneration medium increased plant regeneration rates of St. Augustinegrass (*Stenotaphrum secundatum*; Li et al. 2006).

Low levels of a cytokinin in the callus induction media in combination with an auxin enhanced the embryogenic nature and plant regeneration of callus in several grasses including ryegrass (Altpeter and Posselt 2000; Bradley et al. 2001), bermudagrass (Chaudhury and Qu 2000), barley (Cho et al. 2000), tall fescue (Bai and Qu 2001), and bahiagrass (Altpeter and Positano 2005). The correct combination of auxin type to cytokinin type and the appropriate auxin and cytokinin concentration must be determined to optimize a tissue culture medium (Hagio et al. 1995; Altpeter and Positano 2005; Jain et al. 2005). Species-specific differences, however, cannot be ignored. A recent study on buffelgrass, a warm-season perennial forage grass, found that the inclusion of BAP with 2,4-D inhibited embryogenic callus growth and did not permit plant regeneration (Colomba et al. 2006). Similar findings have been found in tissue culture response of Kentucky bluegrass where 2,4-D in association with BAP led to lower frequencies of callus induction than 2,4-D alone (Ha et al. 2001).

Other medium components also affect tissue culture response. For example, higher levels of $CuSO_4$ have been shown to enhance tissue culture response in wheat (Purnhauser 1991), barley (Cho et al. 1998), and rye (Popelka and Altpeter 2001.)

Long tissue culture periods generally increase the risk of producing somaclonal variants and abnormal plants (Zaghmout and Torello 1992). Dicamba is thought to lead to higher rates of somaclonal variation than 2,4-D (Bregitzer et al. 1998). However, somaclonal variation can be an effective tool in contributing useful variations to populations that breeders could exploit. Breeders have used somaclonal variation to increase disease resistance in sugarcane (Saccharum spp.; Ramos Leal et al. 1996), and enhance drought tolerance in bermudagrass (Lu et al. 2006). Somaclonal variation has also been used in an attempt to improve resistance to fall armyworm in common bermudagrass (Croughan and Quisenberry 1989). However, the large number of undesirable somaclonal variation events typically outweighs the desirable events. Minimizing the period in tissue culture typically also contributes to higher reproducibility of genetic transformation protocols in recalcitrant species and would lessen the frequency of random somaclonal variations (Altpeter et al. 1996).

Gene Transfer to Grasses

The first report of a perennial transgenic grass was in 1988 using protoplast-mediated gene transfer into orchardgrass, *Dactylis glomerata* L. (Horn et al. 1988). Protoplast-derived generation of transgenic plants was also successful for red fescue (*Festuca rubra* L.) (Spangenberg et al. 1994) and zoysia (*Zoysia japonica* Steud.) (Inokuma et al. 1998).

Biolistic gene transfer (Sanford et al. 1993) is less genotype-dependent than protoplast-derived genetic transformation and allows for a shorter tissue culture period which reduces the chance of somaclonal variation and increases reproducibility of the genetic transformation protocol. Consequently, many turf and forage grasses have been genetically

transformed using biolistic gene transfer such as tall fescue (Wang et al. 1992; Ha et al. 1992), creeping bentgrass (*Agrostis stolinifera* L.) (Zhong et al. 1993; Hartman et al. 1994), perennial ryegrass (Spangenberg et al. 1995a; Altpeter et al. 2000), red fescue (Altpeter and Xu 2000), Kentucky blue grass (Ha et al. 2001), bahiagrass, (Smith et al. 2002; James et al. 2004; Gondo et al. 2005), bermudagrass (Zhang et al. 2003; Goldman et al. 2004; Li and Qu 2004), and zoysiagrass (Qi et al. 2006).

Agrobacterium-mediated transformation is the method of choice for obtaining transgenic plants with lower copy number and stable gene expression. However, compared to *Agrobacterium*-mediated gene transfer, biolistic gene transfer is more versatile in that it creates transgenic events with a wider range of transgene expression. Biolistic gene transfer is also the preferred method for the introduction of multiple transgene expression cassettes typically required for pathway engineering or gene stacking (reviewed in Altpeter et al. 2005).

In the past, *Agrobacterium*-mediated gene transfer was considered not applicable for monocot transformation because monocots are not natural hosts for *Agrobacterium tumefaciens* (De Cleene and Deley 1976). However, it was subsequently discovered that successful *Agrobacterium*-mediated transformation of grasses can be achieved and the transformation frequency can be enhanced by using hyper virulent strains, virulence gene inducing agents (i.e. acetosyringone), effective means for 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). *Agrobacterium tumefaciens* 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), tall fescue (Bettany et al.

2003), perennial ryegrass (Altpeter 2006), bermudagrass (Ge and Wang 2006) and orchardgrass (Lee et al. 2006).

Selectable and Scorable Markers Used for Grass Transformation

Herbicide and antibiotic selectable marker genes have been frequently used for the generation of transgenic grasses. The *bar* gene, conferring resistance to the herbicide phosphinothricin, was introduced into creeping bentgrass (Hartman et al. 1994), tall fescue (Dalton et al. 1995), zoysia (Toyama et al. 2003) and bahiagrass (Smith et al. 2002). The *nptII* gene was introduced into perennial ryegrass (Altpeter et al. 2000), red fescue (Altpeter and Xu 2000), and bahiagrass (James et al. 2004) for selection of transgenic plants with the antibiotic paromomycin. The *hph* gene in combination with hygromycin selection has been used widely for grass transformation and hygromycin was effective to suppress non-transgenic tissues and shoots at concentrations of 20 mg L^{-1} in orchardgrass (Horn et al. 1988) to concentrations as high as 250 mg L^{-1} for selection in transformed tall fescue (Wang et al. 1992; Ha et al. 1992; Dalton et al. 1995; Spangenberg et al. 1995b). As an alternative to herbicide or antibiotic selectable marker genes, an *Escherichia coli* phosphomannose isomerase (PMI) encoding gene was successfully used for the generation of transgenic creeping bentgrass (Fu et al. 2005), and sugarcane (Jain et al. 2007).

The *E. coli uidA* gene encoding β–glucuronidase (GUS) (Jefferson et al. 1987) has been used as a reporter gene for optimization of stable or transient expression in grasses and also to study expression patterns generated by different promoters (e.g. Toyama et al. 2003; review: Basu et al. 2004; Gondo et al. 2005; Lee et al. 2006). The gene encoding the green fluorescent protein (GFP) is an alternative reporter, which in contrast to the GUS reporter system, has the advantage of allowing non-destructive monitoring of reporter gene expression over time. It was

successfully used to establish genetic transformation protocols in bentgrass (Yu et al. 2000) and perennial ryegrass (Altpeter 2006).

Fall Armyworm [*Spodoptera frugiperda* (J. E. Smith)]

Fall armyworm is one of the most severe pests in the southeastern US, causing significant seasonal economic losses in forage and turf grasses and other crops (Sparks 1979; Meagher and Nagoshi 2004). This lepidopteran pest has two morphologically identical host strains differing in host preference and pesticide susceptibility (Nagoshi and Meagher 2004a; 2004b). The corn strain is specialized on maize and sorghum, while the rice strain is found predominately in turf grasses, forage grasses, and rice (*Oryza* spp.). To distinguish between the two strains, molecular methods are required (Meagher and Gallo-Meagher 2003; Nagoshi and Meagher 2004a; 2004b; Nagoshi et al. 2006).

Bacillus thuringiensis

Bacillus thuringiensis (Bt), a gram-positive spore-forming bacterium, is known to produce safe and effective insecticidal crystal proteins (ICPs), also known as δ-endotoxins (Dulmage 1981). *Bt* is a ubiquitous soil bacterium present in considerable variety at low levels world-wide (Bernhard et al. 1997). *Bt* ICP has been used for over 40 years in a spray form, but through genetic engineering has become more popular in modern day agriculture (Tabashnik et al. 1998). There are a number of ICPs encoded by various genes. At least 180 *cry* and *cryt* genes have been discovered (Crickmore et al. 2000). The *cry* gene products (ICPs) are harmless to humans, fish, wildlife, and agriculturally important insects (McClintock et al. 1995). The first step to activating the protoxin (encoded by the *cry* gene) is ingestion by the target, a phytophagous insect with an alkaline midgut. The ICP is then solubilized in the midgut of the pest and the protoxin is cleaved by proteases into active toxin (Gill et al. 1992). Midgut proteases process the protoxin into a protease-resistant core fragment making this compound toxic. This toxin then

passes through the peritrophic membrane and binds to the midgut epithelium receptors in the insect where it is effective (Hoffman et al. 1988a; Van Rie et al. 1990a; Van Rie et al. 1990b). Partial insertion of the toxin into the midgut cell occurs and this in connection with the protein binding leads to pore formation, ultimately leading to cell lysis and death of the insect (Gill et al. 1992; Schnepf et al. 1998).

Although *Bt* ICP as a biopesticide can be a useful alternative to commercial insecticides, it is limited in its field stability, its ability to reach insects parasitizing cryptic parts of the plant, and its narrow spectrum of activity (Ferré and Van Rie 2002). *Bt* ICPs produced in transgenic plants are more effective than a topical application of *Bt* ICP biopesticides. Biopesticides contain more toxins on a gram per hectare basis, however they are not ensured to be effective to control larvae which may occur several weeks after application or may feed inside the plant or on the roots of the plant. However, these larvae would be affected immediately when consuming transgenic *Bt* crops (Cannon 2000). *Bt* δ-endotoxins genes have been introduced into several crops such as cotton, potato, soybean and corn and extensively released in the US and other countries (James 2006). Currently, *Bt* transgenic crops are grown on more than 14 million hectares worldwide (James 2005). In the US, *Bt* crops are grown on approximately 20% of the crop acreage. The use of *Bt genes* has reportedly led to a reduction in the use of synthetic insecticides (Constable 1998; Roush 1997). Their use has been directly linked to higher yields and profits (Cannon 2000).

To remain effective against pests, crops need to express sufficient levels of the endotoxin to significantly reduce the fitness of the phytophagous pest. A high dose is defined as being capable of causing mortality of all heterozygous individuals that feed on the transgenic tissue (Alstad and Andow 1999). The EPA has endorsed a toxic concentration of *Bt* to be 25 times

more concentrated than the level required to kill 99% of the pests (Renner 1999). The expression of the *cry* gene can be affected by where in the genome the gene construct is inserted, the environment where the plant is grown, tissue age, and tissue type (Sachs et al. 1998; Greenplate 1999). *Bt* expression levels in transgenic plants were successfully increased by codon-optimization of *cry* sequences, the reduction of AT sequences, and the truncation of the native *cry* sequence (Schnepf et al. 1998; Bohorova et al. 2001; Kaur 2006). Stacking of different *cry* genes (Kaur 2006), expression of *Cry* fusion constructs (Bohorova et al. 2001), and pyramiding genes including *cry* genes with genes encoding proteins having alternative insect control mechanisms like vegetative insecticidal proteins or proteinase inhibitors is hypothesized to reduce the risk of insects developing resistance to *Bt* toxins (Ferry et al. 2006).

In 1985, the first development of resistance to *Bt* ICP was reported (McGaughey 1985) followed by additional reports (Bauer 1995; Ferré et al. 1995; Frutos et al. 1999; Schnepf et al. 1998; Tabashnik 1994; Van Rie and Ferré 2000). Interestingly, diamondback moth (*Plutella xylostella* L.) has developed resistance to four *Bt* toxins (Tabashnik et al. 1997b). Therefore, there is a possibility that pests can become resistant to a range of *Bt* endotoxins and thus measures must be taken to reduce resistance acquisition in these pests. Resistance management should rely on four key strategies, the diversification of lethal sources, the use of refuges to reduce selection pressure, prediction and monitoring of resistance, and policy implementation (Whalon and Norris 1996). Maintaining non *Bt* ICP crops in the retaining zone ensures a population of non-resistant pests and should slow down the evolution of resistance to *Bt* crops (Roush 1996).

To-date, three biochemical mechanisms for resistance have been found in resistant populations ranging from proteolytic processing of protoxins to repair of damaged midgut cells

and modification of affected binding sites (Ferré and Van Rie 2002). Unfortunately there is some overlap and occasionally resistance to one *Cry* protein will lead to resistance to several other *cry* gene products (Tabashnik et al. 1997a; 1997b). Still, there are many *cry* genes coding for different *Cry* proteins and although some insects have shown a buildup of resistance to certain *cry* genes, other *cry* genes oftentimes remain effective (Müller-Cohn et al. 1996; Tabashnik et al. 1997b). In most reported cases, resistance has been unstable (Ferré and Van Rie 2002). In several studies, selection has been removed after resistance was detected in a population, and in most instances, resistance was lost likely due to fitness costs (Tabashnik et al. 1994, Ferré and Van Rie 2002).

Extensive high dose feeding studies and field studies have shown no adverse affect to non-targets such as adult and larval honeybees, ladybird beetles, collembolan, parasitic wasps, and lacewigs (Armstrong et al. 2000; Sims and Martin 1997). The EPA has also concluded that tested *Cry* proteins are nontoxic to mammals (EPA 1998a; EPA 1998b). Receptors like those found in insects affected by *Cry* proteins have not been found in mammalian species (Hoffman et al. 1988b; Sacchi et al. 1986). Furthermore, *Cry* proteins are also known to degrade rapidly under natural conditions (Palm et al. 1993; Palm et al. 1994; Palm et al. 1996). Therefore, these *Cry* proteins do not adversely affect non-target pests, beneficial insects addressed or other animals.

Currently, marketed *Bt* ICP products include *Bt* corn containing the *cry1Ab*, *cry1Fa*, *cry3Bb1* and stacked *cry1Ab* and *cry3Bb1* genes for controlling European corn borer [*Ostrinia nubilalis* Hübner], southwestern corn borer [*Diatraea grandiosella* Dyar] and corn rootworm [Diabrotica barberi Smith and Lawrence], and *Bt* cotton containing *cry1Ac*, stacked *cry1Ac* and *cry2Ab2,* stacked *cry1Ac* and *cry1Fa* for controlling tobacco budworm [*Heliothis virescens*

Fabricius], cotton bollworm [*Helicoverpa zea* Boddie], and pink bollworm [*Pectinophora gossypiella* Saunders] (Castle et al. 2006). *Cry1Fa* has been reported to control fall armyworm in cotton (Adamczyk and Gore 2004) and bahiagrass (Luciani et al. 2007). Hence, the overall goal of this work was to develop a genetic transformation protocol for seashore paspalum to evaluate the expression of a synthetic *Cry1Fa* gene in transgenic seashore paspalum and its effect on resistance to fall armyworm.

CHAPTER 3
MATERIALS AND METHODS

Development of a Tissue Culture Protocol for Seashore Paspalum

Plant Material

Seashore paspalum cultivar SI1 was obtained from Turfgrass America, Houston, TX. Plant material was propagated in Fafard #2 soil mix (Fafard, Inc., Apopka, FL) in an airconditioned greenhouse at 30ºC day / 25ºC night temperature. The photoperiod was extended to a 16 h day length using 1000 watt sodium vapor lights. Miracle-Gro Bloom Booster (Scotts Miracle-Gro Products Inc, Marysville, OH) was applied biweekly at the recommended rate to enhance flowering. Automatic, daily irrigation was achieved with an ebb and flow irrigation system.

Preparation of Explants

Shoots with immature inflorescences were collected at the end of June, 2006. Excess leaf material was removed before surface sterilization with 70% ethanol for 4 min followed by a 10% sodium hypochlorite solution with 0.01% Tween 20® for 20 min. After sterilization, tissue was rinsed five times with sterile distilled water with the last two rinses including a 10 min soaking period. Immature inflorescences were then excised with a sterile scalpel in aseptic conditions and inflorescences of 1 cm in length were cut into five 0.2 cm segments and placed onto the culture medium. Inflorescence segments were numbered 1 to 5, with 1 being the apex of the inflorescence and 5 being the base just above the immature peduncle.

Experimental Design

The experimental design consisted of four randomized blocks, each represented by six media treatments. Each replication was represented by 10 inflorescences cut into 50 segments and cultured on five plates with the same medium. In total, 240 immature inflorescences were

cut into 1200 segments before culture initiation. Callus was maintained at 25ºC in an incubator (Percival I36LLVL, Percival Scientific Inc, Perry IA) with 30 μE $m^{-2}s^{-1}$ light and 16 h light / 8 h dark photoperiod. The six treatments included one of the two auxin types (2,4-D or dicamba) at 3 mg L^{-1} with the cytokinin BAP at 0, 0.1 or 1.0 mg L^{-1}.

Callus Induction

Callus induction media components (PhytoTechnology Laboratories, Shawnee Mission, KS,) consisted of 4.3 g L^{-1} MS salts (Murashige and Skoog 1962) supplemented with 12.45 mg L^{-1} CuSO4 and 20 g L^{-1} sucrose. Before the addition of 3 g L^{-1} phytagel, the pH was adjusted to 5.8. Media was autoclaved at 121ºC and 120 kPa for 20 min. MS vitamins (Murashige and Skoog 1962), auxins and BAP were added from concentrated and filter sterilized stock solutions after autoclaving. Callus was sub-cultured weekly and callus induction was recorded weekly for 56 d. The time of callus induction was determined to be when undifferentiated cells were observed on the inflorescence segment. Callus fresh weight was measured 10 wk after culture initiation.

Plant Regeneration

Two wk after assessing callus fresh weight, callus was transferred to plant regeneration media under 150 μE$m^{-2}s^{-1}$ light at 25ºC with a 16 h light / 8 h dark photoperiod. Composition of regeneration media was the same as the callus induction media, except that neither auxin nor cytokinin were included. Number of shoots was determined at 0, 48, and 94 d after transfer to regeneration medium.

Statistical Analysis

Statistical analysis was performed according to the randomization structure using the GLM-procedure of SAS version 9.1 (SAS Institute Inc. 2005). Means were compared by the t-test (LSD, $p< 0.05$).

Transient Transformation of Sea Isle 1 Callus by Biolistic Gene Transfer

Plant Propagation and Preparation of Explants

Seashore paspalum cultivar SI1 plant propagation and preparation of explants were all done as described earlier in the chapter: The Development of a Tissue Culture Protocol for Seashore Paspalum.

Tissue Culture Composition

Callus induction media were made as described earlier. Auxins and BAP were added as concentrated and filter sterilized stock solution after autoclaving. Sorbitol media for osmotic pre-bombardment treatment was made by the addition to the respective experimental media of 72.85 mg L^{-1} of sorbitol before sterilization.

Transient Reporter Gene Expression

Callus initiated from four randomized blocks with all six treatments of media (2,4-D or dicamba with 0, 0.1 or 1.0 mg L^{-1} BAP) was used for bombardment 15 wk after callus initiation to determine biolistic bombardment efficiency with a GUS reporter gene assay (Jefferson 1987). Non-embryogenic or necrotic callus was not included in the experiment. The vector pUbiGUS (Fig. 3-1) containing the maize ubiquitin promoter with first intron (Christensen et al. 1992), the *uidA* gene (Novel and Novel 1973), and the *nos* terminator (Bevan 1984; Fraley et al. 1983) was used for bombardment.

DNA gold solutions were made with 1µm gold (BioRad Laboratories, Hercules, CA) coated with 1 µg of pUbiGUS following the protocol described by Somers et al. (1992). To generate four independent replications of each treatment, plasmid was precipitated on gold particles in four independent reactions. Each reaction was used for six shots, one shot for callus on each media treatment resulting in a total of 24 shots for all four replications. Four h before bombardment, callus was placed within a circle of 18 mm in diameter on its respective culture

media supplemented with the addition of sorbitol evenly and without gaps between callus pieces. Using a PDS-1000/He Particle Delivery System (Biorad), callus was placed 6 cm below a 1100 psi rupture disc and a vacuum of 27.5" Hg was pulled before helium pressure was released once 1100 psi was reached.

After bombardment, callus was transferred to callus induction medium and propagated an additional 4 d under the same conditions it was cultivated on before exposure to GUS substrate (Jefferson et al 1987). Immediately after immersing callus in the GUS assay substrate, the callus was placed in a vacuum of 29" Hg for 10 min. Callus exposed to substrate was then incubated for 16 h at 37ºC in the dark. After incubation, GUS transient expression was determined by counting the number of blue foci observed using a S4E (Leica Microsystems) dissecting scope. Each blue focus was interpreted as an individual transformation event (Fig. 4-6, 4-7).

Stable Genetic Transformation of Seashore Paspalum by Biolistic Gene Transfer

Tissue Culture Composition

Callus induction media were made as described earlier. Sorbitol media for osmotic pre-bombardment treatment were made with the addition to the respective experimental media of 72.85 mg L^{-1} of sorbitol before sterilization in an autoclave. Callus selection media were identical to callus induction media except for being supplemented with 30 mg L^{-1} hygromycin added as concentrated and filter sterilized stock solution after autoclaving and 6 mg L^{-1} agarose. Regeneration selection media were identical to callus induction media except that they lacked auxins and cytokinins and were supplemented with 6 mg L^{-1} agarose and hygromycin at either 10 or 20 mg L^{-1}. For the first two months on regeneration media, hygromycin was added at concentrations of 20 mg L^{-1} followed by a reduction of the hygromycin concentration to 10 mg L^{-1}.

Stable Genetic Transformation

Callus was induced on six different media (2,4-D or dicamba with 0, 0.1 or 1.0 mg L^{-1} BAP) and bombarded 15 wk after callus initiation to evaluate stable transformation using the *hph* selectable marker gene. Non-embryogenic or necrotic callus was not included in the experiment. SI1 callus was arranged within a circle of 25 mm in diameter on callus induction medium containing sorbitol and bombarded with microprojectiles coated with linearized phphcry1Fa plasmid, containing a constitutive *cry1Fa* cassette linked to a constitutive *hph* selectable marker expression cassette (Fig. 3-2). A total of 114 Petri dishes of SI1 callus were bombarded. From media containing dicamba and BAP (0, 0.1 or 1.0 mg L^{-1}) 9, 23, and 25 Petri dishes were bombarded, respectively, while from media containing 2,4-D and BAP (0, 0.1 or 1.0 mg L^{-1}) 16, 22, and 19 petri dishes were bombarded, respectively.

Selection schemes were consistent for the different media treatments. Callus bombarded with *hph* was allowed to recover after bombardment on its respective media without any selection pressure for seven d and then transferred to selection media containing 30 mg L^{-1} hygromycin and the same composition as the callus induction medium. Calli were subcultured on selection media every two wk for a period of three months with 30 μE $m^{-2}s^{-1}$ light and 16 h light / 8 h dark photoperiod and 25ºC. Then callus was transferred every three wk to media without growth regulators and 20 mg L^{-1} hygromycin with 150 μE$m^{-2}s^{-1}$ illumination and a 16 h light / 8 h dark photoperiod at 25ºC. Two months later, the hygromycin concentration was reduced to 10 mg L^{-1} hygromycin. After two to three subcultures on this selection medium, regenerated plants were transferred to soil and grown under the same greenhouse conditions as the original explant material described.

Evaluation of *cry1Fa* Integration and Expression

Genomic DNA was extracted using the CTAB method (Doyle and Doyle 1987; as modified by Cullings 1992). After extraction, DNA concentration was measured with a ND-1000 spectrometer (NanoDrop technologies, Wilmington, DE) and concentrations were adjusted to 50 ng μl^{-1} for the polymerase chain reaction (PCR). PCR was carried out using the PCR core system II (Promega; Madison, WI) in an iCycler thermocyler (BioRad)

Using HotStarTaq® DNA Polymerase (Qiagen), the putative transgenic DNA samples (50 ng), nontransformed SI1 negative control DNA (50 ng), negative PCR control (no DNA), and a positive control (50 pg, phphcry1Fa) were used for PCR. All samples were mixed well by tapping, and then spun briefly (5 s) in a table top centrifuge before undergoing PCR. The PCR program was first set for 15 min at 95ºC to activate the HotStarTaq® DNA polymerase. To amplify *hph,* a forward primer of 5' - CCC GAT ATG AAA AAG CCT GA -3' and a reverse primer of 5' – GAT GTT GGC GAC CTC GTA TT -were used resulting in a PCR fragment of 889 bases. Thirty cycles were used with 1 min at 95ºC, 1 min at 48ºC, and 1 min at 72ºC. After the 30th cycle, the samples were held at 72ºC for 5 min and then held at 4ºC until use. To amplify cry1Fa in phphcry1Fa, the same procedure was done with a different forward primer of 5' – CCG GGA CCA TTG ACT CTC TA -3' and a reverse primer of 5' – CAC TTC GTT GCC TGA ACT GA -3' resulting in a PCR fragment of 581 bases. PCR amplification was verified by electrophoresis with a 0.8% agarose gel.

Immunochromatography

Testing for *Cry1Fa* was done with the commercially available immunochromatographic Cry1Fa QuickStix test (EnviroLogix; 500 Riverside industrial parkway, Portland, ME 04103) To conduct the immunochromatographic Cry1Fa QuickStix test, four 3 cm segments of leaf tissue were ground with a sterile micro-pestle in a 1.5 mL eppendorf tube with 125 µL of protein

extraction buffer, provided by the manufacturer. After centrifugation for 5 s with a table top centrifuge, the supernatant was transferred to a fresh eppendorf tube and the QuickStix strip was then placed into the protein extract solution. Approximately 10 min later, results were visible (Figure 4-9).

Statistical Analysis

Statistical analysis for the GUS assay was performed according to the randomization structure using the GLM-procedure of SAS version 9.1 (SAS Institute Inc. 2005). Means were compared by the t-test (LSD, $p < 0.05$).

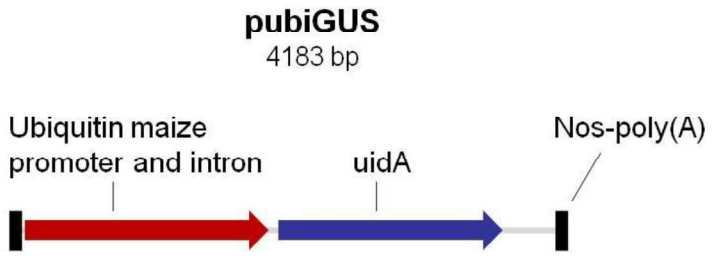


Figure 3-1. The pUbiGUS expression cassette. Plasmid map showing the uidA (GUS) gene, the ubiquitin promoter, the nos-poly(A) region.

Figure 3-2. The pHZubicry1Fa expression cassette. Plasmid map showing the synthetic *cry1Fa* gene, the constitutive promoter (CaMV35S), the ubiquitin promoter, the nos-poly (A) region, the hygromycin phosphotransferase gene (hph) for hygromycin resistance.

CHAPTER 4
RESULTS

Development of a Tissue Culture Protocol for Seashore Paspalum

Callus Induction

Preliminary experiments confirmed that immature inflorescences of a size of 1 cm were best suited for induction of embryogenic, regenerable callus. While all segments of these immature inflorescences were able to induce callus, the callus induction frequency differed significantly by segment position in the inflorescence (Table 4-1). Twenty-one d after culture initiation, 60% of the cultured basal inflorescence segments and 35% of the cultured apical inflorescence segments had formed embryogenic callus (Fig. 4-2).

The auxin type showed significant effects for all evaluated parameters. Callus induction percentages were calculated by averaging the rate of callus induction per inflorescence across all five segments of the original immature inflorescence. Significant auxin effects on callus induction were recorded after 14 d of culture initiation (Table 4-2) with callus induction being highest on 2,4-D containing media (Table 4-2, 4-3). At 21 d after culture initiation, 2,4-D induced callus from 54% of the cultured inflorescences, while dicamba supported callus induction from 42% of the cultured inflorescences (Table 4-3).

The different BAP concentrations had no significant effect on callus induction (Fig. 4-1, 4-2).

Callus Fresh Weight

Callus fresh weight gain was significantly higher only for segment 2 (31.7 ± 7.3 mg) which was not significantly different than the fresh weight gained for segment 1 (27.3 ± 7.3 mg) despite the lower callus induction frequency. Average fresh weight was lowest for segment 4, at an average of 22.3 ± 7.3 mg of callus generated (Table 4-1).

Auxins tested had a clear significant effect on callus fresh weight gain. Dicamba was significantly higher with 92.5 mg of average weight gained per inflorescence compared to 40.4 mg of average weight gained per inflorescence on 2,4-D (Fig. 4-2).

Auxin and cytokinin interaction also led to significant differences in the accumulated callus fresh weight. Callus generated on dicamba with 0 and 0.1 mg L^{-1} BAP had significantly higher average callus fresh weight per inflorescence than all callus induced on media with 2,4-D regardless of BAP concentration. However, callus induced on dicamba with 1.0 mg L^{-1} BAP had a fresh weight average that was not significantly different than callus generated on 2,4-D with 0.1 mg L^{-1} BAP. Callus generated on dicamba with 0.1 mg L^{-1} BAP average fresh weight of induced callus per inflorescence was 102.0 mg, while that of callus generated on 2,4-D with no BAP was only 23.6 mg (Fig. 4-2).

Shoot Regeneration

After callus induction on dicamba containing media, callus rapidly produced a large number of shoot primordia. In contrast, calli initiated on 2,4-D containing media remained mostly undifferentiated at 48 d on regeneration media (Fig. 4-5). The formation of shoot primordia was more pronounced on media supplemented with BAP (Fig. 4-2). After a cultivation period of 48 d on regeneration media, callus generated on dicamba had an average of 336.6 shoots per inflorescence while callus generated on 2,4-D had an average of only 35.3 shoots per inflorescence, a highly significant difference ($Pr>F <0.0001$; Fig. 4-1, 4-2). The effect of cytokinins on shoot formation was less drastic but still significant ($\alpha=0.05$; Fig. 4-1, 4-2). While calli induced on BAP free media produced on average 133.7 shoots per inflorescence, those induced on 0.1or 1.0 mg L^{-1} BAP produced 207.1 or 223.2 shoots per inflorescence, respectively (Fig. 4-2). A significant auxin type × BAP concentration interaction was also observed ($Pr>F <0.0659$; Fig. 4-1). Addition of BAP to 2,4-D-containing media had no

significant effect on shoot regeneration compared to 2,4-D-containing media without BAP. However, shoot regeneration almost doubled when 0.1or 1.0 mg L^{-1} BAP was added to dicamba-containing media, compared to dicamba-containing media without BAP (Fig. 4-2).

Auxin type and BAP concentration did not significantly affect the proportion of cultured inflorescences that had the ability to regenerate at least one shoot (Fig. 4-1, 4-2).

Transient Expression of *uidA* in Seashore Paspalum Callus

Transient expression was found to be significantly higher when using 2,4-D with average expression rates of 334.92 ± 155.9 blue foci, while dicamba had an average rate of 49.17 ± 22.5 blue foci. BAP levels also had a significant effect on transient GUS expression. Average transient expression was highest in callus originating from media with 2,4-D and 1.0 mg L^{-1} BAP (Fig. 4-6, 4-7). The next transient expression level was observed in calli derived from media with 0.1 or 0.0 mg L^{-1} BAP and 2,4-D. While all callus derived from dicamba with (0, 0.1, 1.0 mg L^{-1}) showed significantly lower transient expression (Fig. 4-6).

Stable Transformation

Stable transformed lines had vigorous shoot and root growth while on selection medium (Fig. 4-8). Transgenics were recovered, showing a strong Cry1Fa-specific signal in the immunochromatographic analysis suggesting high level expression of *cry1Fa* (Fig. 4-9). Four *Cry1Fa* expressing lines were generated, one from callus induction media originally containing 2,4-D with 0 mg L^{-1} BAP and one from callus induction media originally containing dicamba with 0.1 mg L^{-1} BAP. Two transgenic lines were generated from media originally containing 2,4-D with 0.1mg L^{-1}. There were 214 inflorescences used to induce callus for this experiment with callus generated on dicamba × BAP (0, 0.1, 1.0 mg L^{-1}) originating from 19, 40, and 41 inflorescences respectively, while callus originating on media with 2,4-D × BAP (0, 0.1, 1.0 mg L^{-1}) originating from 39, 38, and 37 inflorescences respectively. Callus initiated on

dicamba media had a 1.75% and 1% transformation efficiency from bombarded Petri dish and cultured immature inflorescences respectively. Callus initiated on 2,4-D media had a 5.26% and 2.63% transformation efficiency from bombarded Petri dish and cultured immature inflorescences respectively (Table 4-4). Transgenic plants did not show any phenotypic difference from wildtype (Fig 4-5). PCR confirmed the presence of the *cry1Fa* gene in genomic DNA extracts of transgenic lines (Fig. 4-10).

Table 4-1. Callus induction and regeneration in response to the original position of immature inflorescence segments

	Induction of callus (%)[a]	Callus fresh weight (mg)[b]	Number of shoots per segment[c]
Segment 1	34.9^{C}	27.3^{AB}	33.3^{C}
Segment 2	46.6^{B}	31.7^{A}	101.2^{A}
Segment 3	52.9^{AB}	24.5^{B}	75.9^{AB}
Segment 4	48.9^{B}	22.3^{B}	60.4^{BC}
Segment 5	60.2^{A}	22.9^{B}	47.5^{BC}

[a] = 21 d after initial platting on callus induction media. [b] = 10 wk after initial platting on callus induction media. [c] = 48 d after platting of callus onto regeneration media. Same *letters* in a *column* are not significant at α=0.05 using LSD test. Immature inflorescences 1cm in size were cut into 5 mm segments. The segments are numbered in order with the apical segment is segment 1 the basal segment is segment 5.

Table 4-2. Callus induction and regeneration as influenced by the immature inflorescence segments, auxin type, BAP concentration, and interaction of auxin type × BAP concentration.

	Induction of callus in % of cultured inflorescences after culture initiation			Callus fresh weight (mg) per inflorescence[a]	Number of shoots per inflorescence[b]	% of cultured inflorescences regenerating shoots[c]
	7 d	14 d	21 d			
Segment (df = 4)						
MS	0.9	2.3	1.9	1777.7	23595.6	
F-Value	6.3*	10.8***	9.4***	2.2ns	3.57**	
P-Value	<0.0001	<0.0001	<0.0001	0.0664	0.0070	
Treatment (df=5)						
MS	2.5	5.4	5.1	37748.3	54112.9	
F-Value	1.4ns	2.0ns	1.8ns	7.1***	19.1***	
P-Value	0.2179	0.0843	0.1059	<0.0001	<0.0001	
[A] Auxin (df=1)						
MS	3.6	21.5	20.9	162921.9	236464.9	
F-Value	1.8ns	6.7**	5.7*	25.2***	80.2**	
P-Value	0.1798	0.0103	0.0177	<0.0001	<0.0001	
[C] Cytokinin (df=2)						
MS	1.7	1.8	1.7	8732.6	9732.6	
F-Value	0.9ns	0.6ns	0.5ns	1.4ns	3.3*	
P-Value	0.4132	0.5728	0.6319	0.2609	0.0414	
A × C (df=2)						
MS	3.1	0.8	0.6	4177.2	8269.7	0.8
F-Value	1.6ns	0.3ns	0.2ns	0.7ns	2.8ns	5.7**
P-Value	0.2124	0.7824	0.8530	0.5248	0.0659	0.0048

[a] = 10 wk after originally platting callus on callus induction media. [b] and [c] = 48 d after transferring callus to regeneration media. *,**,*** indicate significance at α=0.05, 0.01, and 0.001 respectively using SAS proc glm. Immature inflorescences 1com in size were cut into 5 mm segments. The most apical segment is segment 1 the most basal segment is segment 5. ns = non-significant

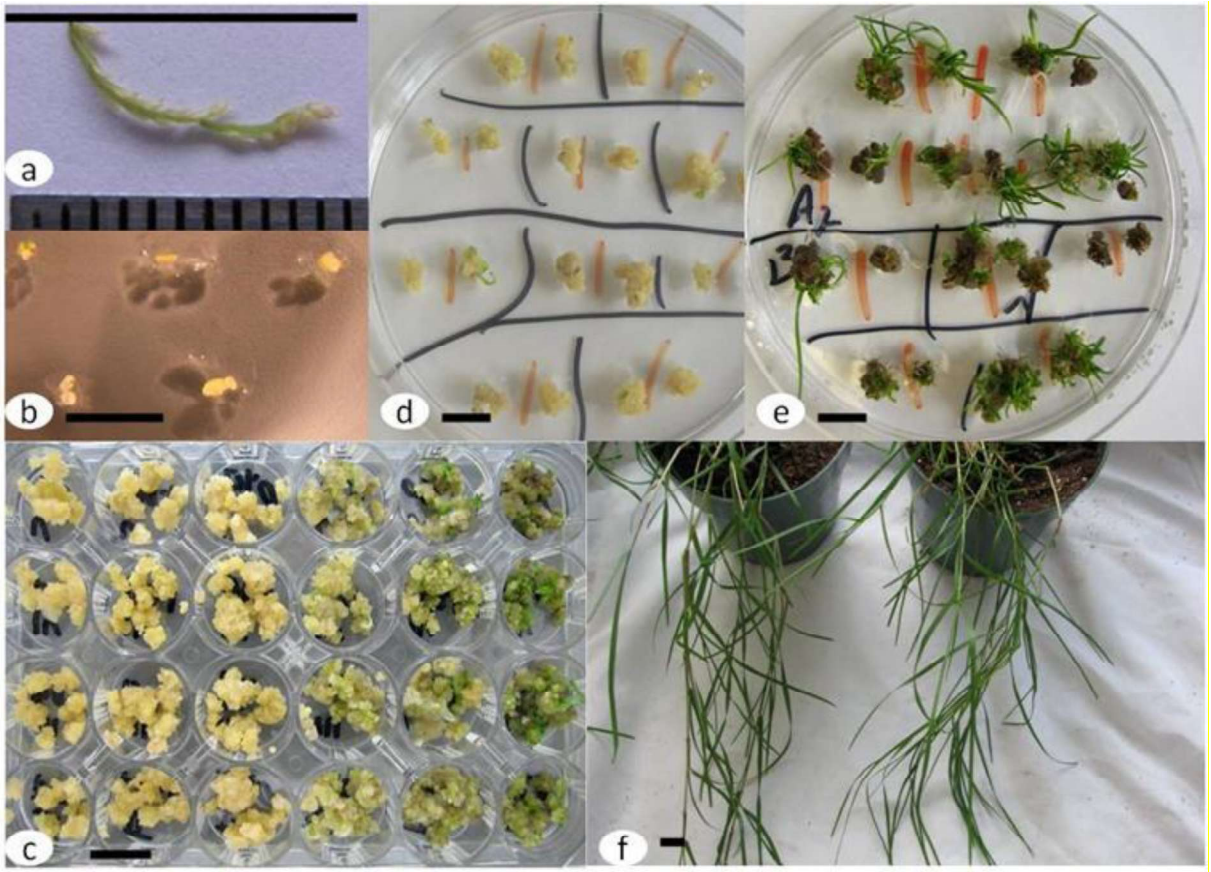
Table 4-3. Callus induction and regeneration as influenced by auxin type BAP concentration and interaction of auxin type and BAP concentration

	Induction of callus in % of cultured inflorescences after culture initiation			Callus fresh weight (mg) per inflorescence[a]	Number of shoots per inflorescence[b]	% of cultured inflorescences regenerating shoots[c]
	7 d	14 d	21d			
Dicamba	20.0^{A}	34.0^{B}	42.0^{B}	92.5^{A}	336.6^{A}	87.5^{A}
2,4-D	16.0^{A}	46.0^{A}	54.0^{A}	40.4^{B}	35.3^{B}	73.9^{A}
BAP 0 mg L^{-1}	16.0^{A}	40.0^{A}	50.0^{A}	57.7^{A}	133.7^{B}	73.3^{A}
BAP 0.1 mg L^{-1}	20.0^{A}	44.0^{A}	50.0^{A}	78.0^{A}	207.1^{AB}	87.5^{A}
BAP 1.0 mg L^{-1}	22.0^{A}	36.0^{A}	44.0^{A}	63.6^{A}	223.2^{A}	81.3^{A}
Dicamba × BAP 0 mg L^{-1}	18.0^{AB}	34.5^{B}	45.0^{AB}	91.8^{A}	221.3^{B}	100.0^{A}
Dicamba × BAP 0.1 mg L^{-1}	26.7^{A}	35.0^{B}	43.5^{AB}	102.0^{A}	383.0^{A}	100.0^{A}
Dicamba × BAP 1.0 mg L^{-1}	20.5^{AB}	33.0^{B}	38.5^{B}	83.7^{AB}	405.5^{A}	62.5^{B}
2,4-D × BAP 0 mg L^{-1}	14.0^{B}	46.5^{AB}	52.8^{AB}	23.6^{C}	33.7^{C}	85.7^{AB}
2,4-D × BAP 0.1 mg L^{-1}	13.5^{B}	51.0^{A}	58.0^{A}	54.0^{BC}	31.2^{C}	75.0^{B}
2,4-D × BAP 1.0 mg L^{-1}	23.0^{AB}	41.0^{AB}	51.6^{AB}	43.5^{C}	40.9^{C}	62.5^{B}

[a] = 10 wk after originally platting callus on callus induction media. [b] and [c] = 48 d after transferring callus to regeneration media. Same *letters* in a *column* are not significant at α=0.05 using the LSD test

Table 4-4. Transformation efficiencies

	Number of bombarded Petri Dishes	Number of cultured immature inflorescences	Transgenic events	Efficiency of gene transfer. Transgenic lines in % of	
				bombarded Petri Dishes	cultured inflorescences
Dicamba	57	100	1	1.75	1
2,4-D	57	114	3	5.26	2.63
BAP 0 mg L^{-1}	25	58	1	4	1.72
BAP 0.1 mg L^{-1}	45	78	3	6.67	3.85
BAP 1.0 mg L^{-1}	44	78	0	0	0
Dicamba × BAP 0 mg L^{-1}	9	19	0	0	0
Dicamba × BAP 0.1 mg L^{-1}	23	40	1	4.35	2.5
Dicamba × BAP 1.0 mg L^{-1}	25	41	0	0	0
2,4-D × BAP 0 mg L^{-1}	16	39	1	6.25	2.56
2,4-D × BAP 0.1 mg L^{-1}	22	38	2	9.09	5.26
2,4-D × BAP 1.0 mg L^{-1}	19	37	0	0	0



Figures 4-5. Seashore paspalum response to composition of tissue culture media. (a) Sterile immature inflorescence of SI1 (1 cm in length). (b) Segments from immature inflorescence (1 cm in size) 7 d after culture initiation on callus induction medium. (c) Callus 15 wk after culture of inflorescence segments on different media, from left to right- 2,4-D (0; 0.1; 1.0 mg L^{-1} BAP) and dicamba (0; 0.1; 1.0 mg L^{-1} BAP). (*d*) Regeneration of callus following initiation on medium supplemented with 2,4-D (3 mg L^{-1}) and BAP (0.1 mg L^{-1}) and after being cultivated on hormone free shoot regeneration media for 48 d. (*e*) Regeneration of callus following initiation on medium supplemented with dicamba (3 mg L^{-1}) and BAP (0.1 mg L^{-1}) after being cultivated on hormone free shoot regeneration media for 48 d. (f) On the left, a SI1 transgenic plant regenerated from callus induction media with 2,4-D 3 mg L^{-1} with no BAP and on the right, a SI11 non transformed plant. bar equal to 1 cm.

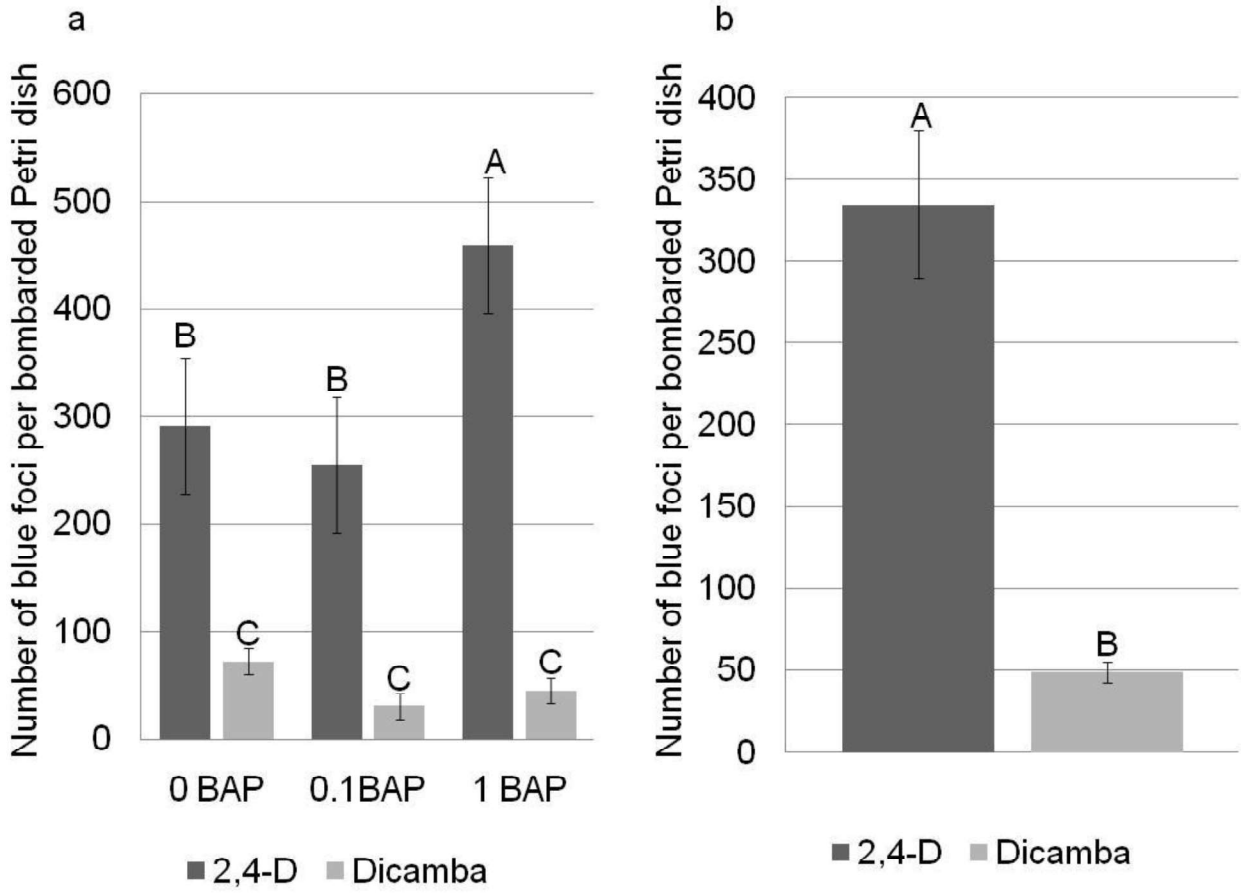


Figure 4-6. Transient GUS gene expression in callus induced on different culture media and following biolistic transfer of the uidA gene. (a) Average number of blue foci resulting from callus induced on six different media. Four petri dishes with callus placed in ring of 18 mm in diameter were bombarded from each media. (b) Average transient expression of blue foci from callus induced on 2,4D or dicamba containing media. Twelve petri dishes with callus placed in ring of 18 mm in diameter were bombarded from each media.

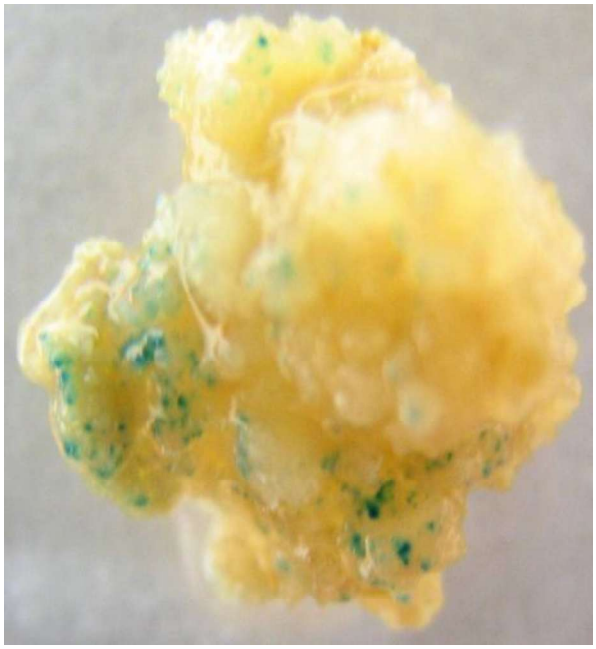


Figure 4-7. Transient GUS expression in seashore paspalum callus. Transient GUS expression on callus induced on media supplemented with 2,4-D and BAP 1.0 mg L^{-1}.

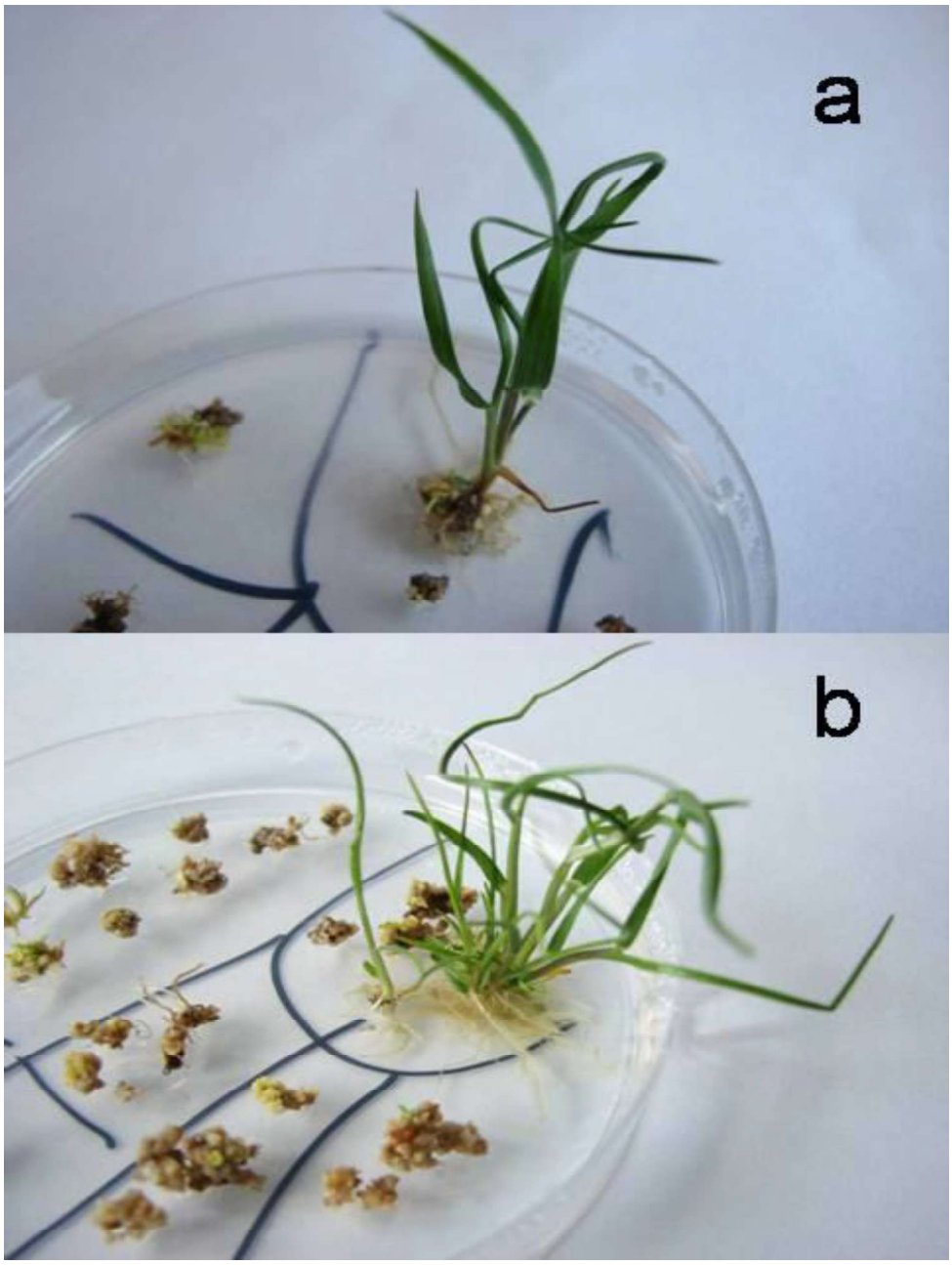


Figure 4-8. Transgenic seashore paspalum plants co-transformed with cy1Fa and hph, selected on callus induction media with 30 mg L^{-1} hygromycin and regenerating on media containing 10 mg L^{-1} hygromycin. (a) Induced on callus induction media with dicamba (3 mg L^{-1}) and BAP (0.1 mg L^{-1}). (b) Induced on callus induction media with 2,4-D (3 mg L^{-1}) and no BAP.

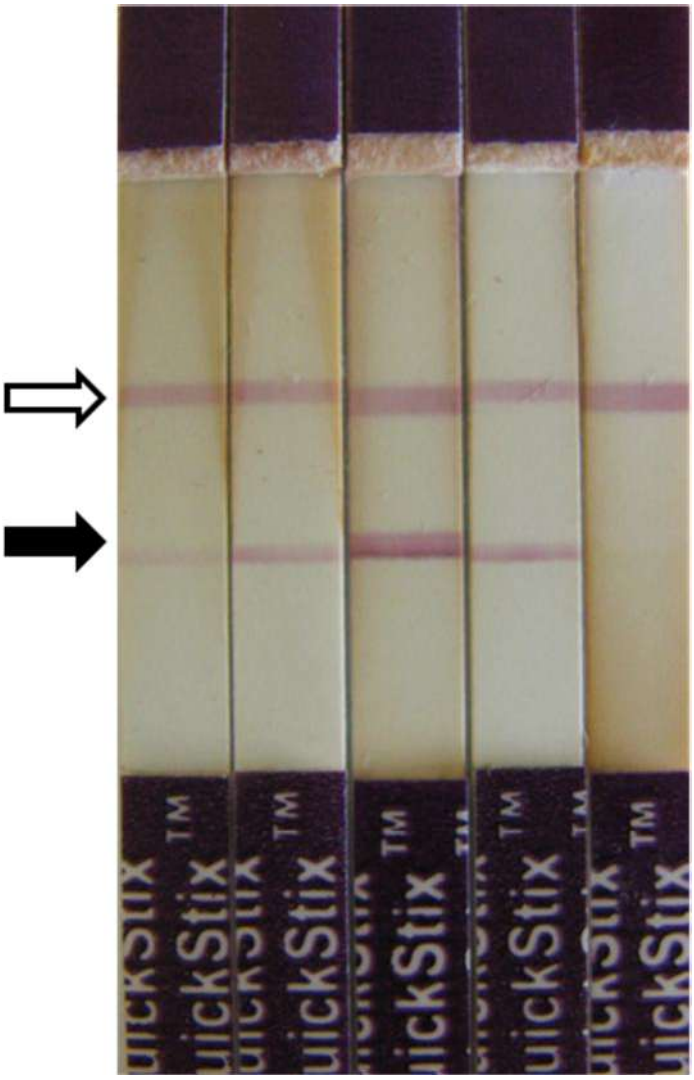


Figure 4-9. The Cry1Fa protein expression detected by the immunochromatographic Cry1Fa QuickStix Test. Samples were taken from SI1 Wt and SI1 lines that had gone through a tissue culture phase involving the induction of callus, transformation with the *cry1Fa* and *hph* gene, callus subculture under selection and shoot regeneration under selection with 30 mg L^{-1} hygromycin and 10 mg L^{-1} hygromycin used for selection respectively. The lines represented above were induced under different tissue culture media describe as follows from left to right: 2,4-D and no BAP, 2,4-D and 0.1 mg L^{-1}BAP, dicamba and 0.1 mg L^{-1} BAP, (d) 2,4-D and 0.1 mg L^{-1}BAP. The white arrow points to the line representing the control line indicate the functional integrity of the strip. While the black arrow points to the line indicating the presence of the Cry1Fa endotoxin in the sample.

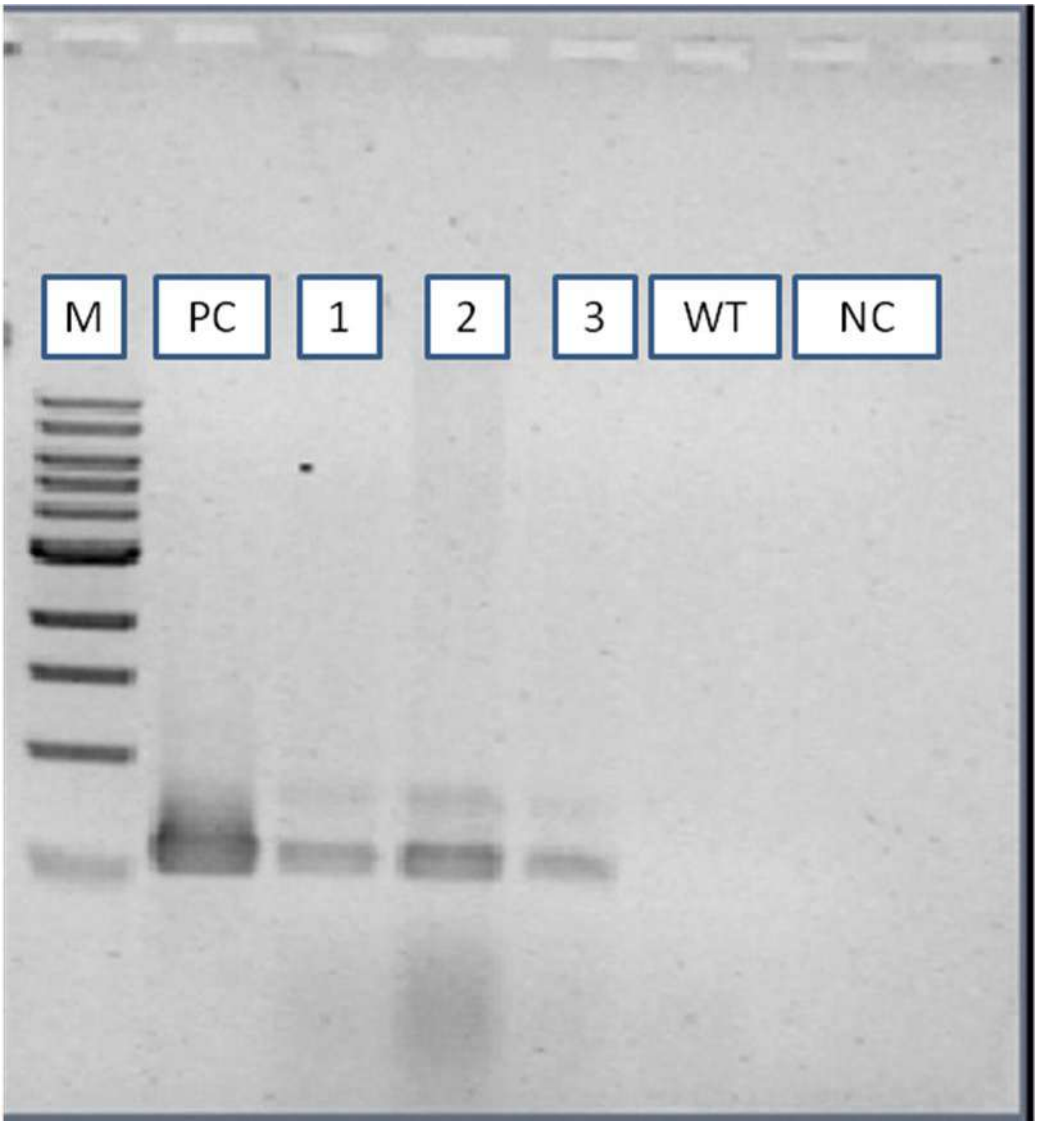


Figure 4-10. The PCR of genomic DNA from seashore paspalum lines suggesting the presence of *Ubicry1Fa*. "M" is the 1 kb ladder with the lowest band being equal to 500 base pairs, "PC" refers to the positive control which is an amplification of 50 pg of phphcry1Fa with fragment size of 581 base pairs, Lanes 1, and 2 indicate the presence of the cry1F gene in confirmed cry1Fa transgenic bahiagrass (*Paspalum notatum*) used as a positive control plant in this assay. Lane 3 represents a PCR amplification product from genomic DNA of transgenic seashore paspalum SI1. WT and NC refer to SI1 wildtype, and buffer control respectively.

CHAPTER 5
DISCUSSION AND CONCLUSIONS

Developing a Tissue Culture Protocol

Type and concentration of plant growth regulators like auxins and cytokinins are critical components in callus induction and plant regeneration. For the generation of regenerable callus from seashore paspalum, 2,4-D in combination with BAP was used earlier (Cardona and Duncan 1997). However, a comparison of different auxin types was not reported. Here it is shown that the use of dicamba drastically increases plant regeneration frequency from immature inflorescence-derived callus of the commercially important seashore paspalum cultivar SI1. Compared to an equimolar application of 2,4-D, dicamba increased callus average fresh weight two times and the number of average regenerated plants per explant 10 times. The use of dicamba was also identified as the most important factor in optimizing the tissue culture and stable genetic transformation response of barley (Trifonova et al. 2001; Castillo et al. 1998). Dicamba was also superior to 2,4-D in generation of regenerable callus in rye (Zimmy and Lörz 1989). However, 2,4-D was superior to dicamba for induction of embryogenic callus in pentaploid bermudagrass, sorghum and millets (Jain et al. 2005; Rao et al. 1995; Jogeswar et al. 2007; Kavi Kishor et al. 1992).

Seashore paspalum plants regenerated faster from dicamba-containing media and grew more vigorously when transferred to soil, similar to nontissue cultured plants. Different auxin types might have a different impact on the frequency of undesirable somaclonal variation (Deambrogio and Dale 1980; Murata 1989). A reduction in the tissue culture period has been reported as an effective means to reduce somaclonal variation in other grasses (Altpeter et al. 1996; 2000).

Preliminary experiments also show the size of the inflorescence as a critical factor in induction of highly regenerable callus. This has been shown in other grasses (Cai and Butler 1990; Lu and Vasil 1982; Qu and Chaudhury 2001; Poeaim et al. 2005). However, when segmenting immature inflorescences it was not clear which effect the original position of the segment had on callus quality. Although the segment below the apical segment produced three times more shoots than the apical segment and two times more shoots than the basal segment, it seems reasonable to include all segments of the immature inflorescence for callus induction due to the limited seasonal availability of immature inflorescence and the cumbersome process involved in surface sterilizing, and isolating these explants.

While BAP did not have a significant effect on callus induction or callus fresh weight, both 0.1or 1.0 mg L^{-1} BAP increased the number of shoots per inflorescence 1.7- or 1.8-fold, respectively. Surprisingly, this BAP effect was only significant for callus induction media containing dicamba, but not for media containing 2,4-D. Earlier reports described that the cytokinin BAP has a positive impact on the induction and maintenance of embryogenic callus from grasses (Altpeter and Posselt 2000; Chaudhury and Qu 2000; Cho et al. 2000; Bradley et al. 2001). These earlier reports did not describe a significant auxin × cytokinin interaction. In tissue culture with pentaploid bermudagrass, Jain et al. (2005) found that using 2,4-D and BAP to be the only auxin/cytokinin combination effective for callus induction, growth, and establishment of regenerable callus; and not dicamba or IAA with any combination of BAP. This is suggestive of an important auxin × cytokinin interaction dependent on the genotype being induced to form callus.

Transient Transformation of Seashore Paspalum

The *E. coli* β–glucuronidase (GUS) encoding gene uidA (Jefferson et al. 1987) has been extensively used in earlier studies as a reporter gene for optimization of stable and transient

expression in grasses and also to study expression patterns of different promoters (Toyama et al. 2003; review: Basu et al. 2004; Gondo et al. 2005; Lee et al. 2006). Seashore paspalum callus initiated and maintained on 2,4-D media produced six times more transient GUS expression events than callus initiated on dicamba media. However, transient reporter systems may not translate directly to stable transgene expression. There is a positive correlation between the amount of particles used in biolistic gene transfer and transient expression rates (Chibbar et al 1991; Finer et al. 1992). However, plant regeneration potential may become limiting and can be partially compromised by tissue damage caused by particle-mediated gene transfer (Popelka et al. 2003; Castillo et al. 1994). For example, a study in apple cultivars found very high transient transformation events, but low stable transformation events, as transformed callus were not able to form leaf primordia (Maximova et al. 1998). Similar findings have been reported in wheat (Becker 1994) and barley (Hardwood et al. 2000), where higher gold concentrations led to greater tissue damage and less stable transformation events.

Interestingly, in the present study, dicamba supported significantly higher shoot regeneration while 2,4-D supported significantly higher transient reporter gene expression. Therefore, these results need to be followed up with stable transformation experiments to clarify which factor has a greater influence on stable integration of transgenes in seashore paspalum.

Stable Genetic Transformation of Seashore Paspalum

In order to maximize stable transformants, several parameters need to be optimized. One critical aspect in generating transgenic plants is that a selection scheme suppresses non-transgenic events from callus propagation and plant regeneration (Wang and Ge 2006). Species, genotypic, and tissue-specific difference were observed regarding tolerance to antibiotics that are used in the selection of transgenic events (Lee 1996). Hygromycin has been used effectively at concentrations of 20 mg L^{-1} in protoplast derived cell cultures of orchardgrass

(Horn et al. 1988) and has been used in concentrations as high as 250 mg L^{-1} for selection in transformed callus of tall fescue (Wang et al. 1992; Ha et al. 1992; Spangenberg et al. 1995a; Spangenberg et al. 1995b; Dalton et al. 1995). The selection scheme used in the present study with 30 mg L^{-1} hygromycin created a relative low selection pressure and allowed the regeneration of nontransgenic escape plants (94% escapes). Despite the low selection pressure, one stable transgenic plant per 29 shots or one transgenic plant per 56 cultured inflorescences was obtained. Although a more rigorous selection scheme using a higher concentration of hygromycin could have been implemented to reduce the number of escapes, in doing so there is a risk of reducing the number of transgenic plants. Several parameters, such as microcarrier concentrations, particle acceleration, alternative promoters and selection protcols should be investigated to achieve higher transformation efficiencies. Alternative genotypes of seashore paspalum are available and genotypic differences can contribute to significant improvement of transformation efficiency (Popelka and Altpeter 2001). Biolistic gene transfer could be improved by optimized procedures for particle coating. For example, Able et al. (2001) found that reporter gene expression levels were reduced 44% and 71% after using spermidine stored at -20ºC for 21 and 90 d, respectively. Since calli induced on dicamba-containing media produce a large number of plants it is less likely that regeneration potential becomes a limiting factor in these cultures. Therefore something like the ratio and final concentration of DNA and microcarrier particle or other biolistic gene transfer parameters affecting particle penetration into tissues should be further explored.

While further optimization of the described protocols are desirable, this research achieved, for the first time, stable genetic transformation of seashore paspalum and the stable expression of a promising insect resistance gene shown in expressing lines planted to soil from tissue culture.

This is expected to exemplify the potential of genetic transformation for the improvement of seashore paspalum.

CHAPTER 6
CONCLUSION

Golf managers would prefer turf that requires less herbicide and fungicide applications (Lee 1996). Increasing seashore paspalums resistance to pests like fall armyworm, would make seashore paspalum a more popular turf. Due to the limited availability of insect resistance genes within the species, heterologous genes from non-related sources are most promising for improvement of insect resistance in seashore paspalum. For example it is very desirable to introduce crystal protein encoding genes from *Bacillus thuringiensis* (*Bt Cry)*, as effective control against Lepidoptera. However, a genetic transformation protocol was lacking for seashore paspalum. Seashore paspalum is a vegetatively propagated turfgrass, assuring uniformity and avoiding variation caused by segregation of transgene loci. However, before this turf can be bioengineered a tissue culture regime needed to be established.

Here an efficient tissue culture protocol is presented, and for the first time, genetic transformation in the species seashore paspalum is achieved. The Cry1Fa endotoxin was detected in with a commercially available immuno-chromathoraphic strip indicating transgenic plants of the commercially important SI1cultivar expressed high levels of the synthetic *Bt* c*ry1Fa* gene. This is expected to confer resistance to fall armywormas it has already been demonstrated earlier for other transgenic grasses including bahiagrass (Luciani et al. 2007).

A factor to take into consideration when developing a successful transformation protocol is the mutagenic effect that extended periods in tissue culture might have (Zaghmout and Torello 1992). Different auxin types might have different impacts on the frequency of undesirable somaclonal variation (Deambrogio and Dale 1980; Murata 1989). A reduction in the tissue culture period has been reported as an effective means to reduce somaclonal variation in other

grasses (Altpeter et al. 1996; 2000). Seashore paspalum plants regenerated faster from dicamba-containing media and grew more vigorously when transferred to soil, similar to wildtype. However, dicamba is thought to lead to higher rates of somaclonal variation than 2,4-D (Bregitzer et al. 1998) and some somaclonal variation was observed after extended periods in tissue culture.

This work has successfully shown that transformation of seashore paspalum can be achieved following callus induction on media containing dicamba or 2,4-D. Because of the presence of stable transformation events with SI1 plants induced to form callus with both 2,4-D and dicamba, it is not clear as yet whether one of these growth regulators is superior to the other in terms of their use in a stable transformation system. This is not surprising since significantly higher numbers of shoots regenerated per callus initiated with dicamba containing media, but significantly higher levels of transient reporter gene expression events were observed with callus generated on 2,4-D media. These results confirm that transient expression and stable transformation results are not highly correlated due to factors such as tissue damage from gold penetration which can affect plant regeneration response from callus and differences in tissue firmness. Additionally, the finding that the medium with the far superior plant regeneration response did not promote higher transformation efficiencies than alternative media suggests that other factors such as genotype and selection might offer targets for further improvement of transformation efficiency. Enhancement of commercially important seashore paspalum by genetic transformation is now achievable and is expected to contribute to genetic improvement of this environmentally friendly turf.

APPENDIX
LABORATORY PROTOCOLS USED FOR VECTOR CONSTRUCTION, TISSUE CULTURE AND MOLECULAR ANALYSIS OF TRANSFORMED SEASHORE PASPALUM PLANTS

Protocols For Molecular Cloning

Preparation of Electrocompetent *E. coli*

- Always sterilize LB broth, containers used to grow cultures, pipette tips, and eppendorf tubes before use in an autoclave at 121°C and 120kPa for 20 min
- Each centrifuge step was done with a Sorvall with GSA rotor unless otherwise noted.

1. Using a sterile glass tube at least 20 mL in volume, incubate a DH5α culture in 5 mL LB broth at 37°C and grow overnight with shaking at 220 rpm on an orbital shaker with 1" orbit.
2. Inoculate 2 250 mL LB broth in 1L sterile flasks with 2 mL culture. Incubate at 37ºC shaking at 220 rpm on an orbital shaker with 1" orbit until OD600 = 0.6-0.8 (Approx 5-6 h).
3. Pellet cells for 15 min, 4000g at 4ºC (3x 250 mL bottles).
4. Pour off supernatant and resuspend in an equivalent volume of ice-cold sterile water (3x 165 mL). Handle cells gently at all times.
5. Centrifuge 15 min, 4000g, 4ºC.
6. Pour off supernatant and resuspend in 0.5 volume of ice-cold sterile water (3x 83 mL). Divide cell culture between 2 bottles.
7. Centrifuge 15 min, 4000g, 4ºC.
8. Pour off supernatant and resuspend in 0.5 volume of ice-cold sterile water (2x 125 mL).
9. Centrifuge 15 min, 4000g, 4ºC.
10. Pour off supernatant and resuspend in 0.02 volume of ice-cold sterile water (2x 5 mL). Transfer cell culture to 30 mL sterile centrifuge tube.
11. Centrifuge 15 min, 4000g, 4ºC (Sorvall, SS-34 rotor).
12. Pour off supernatant and resuspend in 0.02 volume ice-cold sterile 10% glycerol (1.2 mL).
13. Transfer 40µl aliquots to sterile, chilled 1.5 mL eppendorf tubes with holes pierced in the lids using a sterile needle. Quick-freeze in liquid nitrogen and store at –70ºC.

Electroporation of *E. coli*

1. Chill 0.1 cm gap cuvettes (BioRad; 1000 Alfred Nobel Drive, Hercules, CA 94547, US) on ice.
2. Place DNA for transformation (usually 1-2 µl – 10-15 ng ligation) in 1.5 mL sterile eppendorf tubes on ice.
3. Thaw 40µl aliquots of electrocompetent *E. coli* cells on ice.
4. Set BioRad MicroPulser Electroporator to Ec1 (1.8kV, 5 msec, 0.1 cm gap).
5. Add DNA to chilled competent *E. coli* cells.
6. Immediately transfer competent *E. coli* cells with DNA to cuvette, being careful to evenly fill the gap of the cuvette with competent cells.
7. Place cuvette in slide and push slide into chamber ensuring that cuvette is seated between contacts in the base of the chamber of the BioRad MicroPulser Electroporator.
8. Pulse once by pressing pulse button. Alarm signifies pulse is complete.

9. Check and record pulse parameters (time constant should be close to 5 msec, if it's not repeat procedure with a fresh batch).
10. Remove cuvette and immediately add 400 µL SOC medium to the cuvette. Quickly transfer cells to a 2 mL eppendorf tube. Time is critical.
11. Incubate cells at 37ºC shaking at 220 rpm on an orbital shaker with 1" orbit for 1 h before plating on appropriate antibiotic medium (50µl for circular plasmid, 100-200µl for ligation product).
12. To plate transformed *E. coli*, use a blunt instrument that has been flame sterilized and cooled to spread bacteria evenly on a plate of freshly made LB agar with the appropriate antibiotic. (Depending on the antibiotic LB agar media can be stored at 4°C between 1 wk and 1 m).

Glycerol Stocks

1. Grow *E. coli* culture containing desired plasmid overnight at 37ºC in 2 mL sterile LB broth containing the appropriate antibiotic at 37ºC with constant shaking at 220 rpm on an orbital shaker with 1" orbit.
2. Add 0.85 mL of the bacterial culture and 0.15 mL glycerol (previously autoclaved and cooled to room temperature) into a sterile eppendorf tube with a hole pierced in the lid.
3. Quickly mix by vortexing and immediately freeze in liquid nitrogen. Store -80ºC. Prepare several tubes for plasmid. Avoid successive freeze-thaw cycles.

Amplification and Purification of Plasmid DNA Using QIAprep® Miniprep Kit

(Qiagen; 27220 Turnberry Lane, Suite 200, Valencia, CA 91355, US)

1. Add the provided RNase A solution to Buffer P1, mix well by inverting and store at 4ºC.
2. Add 100% ethanol (volume provided on the bottle label) to the Buffer PE concentrate to prepare the working solution.
3. To dispose of any waste autoclave for 60 min (121°C, 15psi).
4. To amplifying a large number of DNA plasmid one can grow plasmid in 10 to 40 mL sterile LB broth using 1 to 4 spin columns. Add a maximum of 10 mL of LB broth to each spin column. Procedure remains the same except for doubling the amount of P1, P2, and P3 buffers used (500, 500, and 750 µL respectively mixed in a sterile 2 mL eppendorf tube. Also in the final step incubate the spin column after it has been washed at 65°C in a heat block and add 50 µL of heated sterile ddH_20 at 65°C. Incubate an additional min at 65°C before spinning into a sterile eppendorf tube. In the final step spin DNA plasmid from multiple columns into the same sterile eppendorf tube.
5. Precipitation in Buffer P2 can occur in cold temperature, if precipitation occurs, warm to room temperature before use.
6. Grow over-night cultures each from a single colony of bacteria in 5 mL LB sterile broth containing the appropriate antibiotic at 37ºC with constant shaking at 220 rpm on an orbital shaker with 1" orbit.
7. Centrifuge the cultures at 16,100g for 1 min to pellet the bacterial cells. Decant off the supernatant.
8. Resuspend the bacterial cells in 250 µL Buffer P1 by vortexing and transfer the suspension to a sterile 1.5 mL eppendorf tube (Shake Buffer P1 well before use)

9. Add 250 µL Buffer P2 and mix well by inverting the tube gently 4-6 times, incubate for 4 min at room temperature (in some kits the mixture will now turn an even blue color)
10. Add 350 µL Buffer P3 and mix immediately but gently by inverting the tube 4-6 times, a white cloudy precipitate should form (if previously mixture was blue the addition of Buffer P3 should make the mixture clear again, mix until there is no blue color left).
11. Centrifuge the samples at 15,700g for 10 min. A compact white pellet will form.
12. Decant the supernatant onto QIAprep Spin Columns and centrifuge for 1 min at 15,700g using a table top centrifuge. Discard the flow-through.
13. Pipette 750 µL Buffer PB onto the columns and centrifuge for 1 min at 15,700g to wash the columns (although this step is optional in the handbook we always do it)
14. Pipette 750 µL Buffer PE onto the columns and centrifuge for 1 min at 15,700g to wash the columns
15. Discard the flow-through and centrifuge for 1 min at 15,700g to remove all traces of the wash buffer (Buffer PE).
16. Place the QIAprep columns in clean 1.5 mL eppendorf tubes. Add 50 µL Buffer EB to the center of each QIAprep column to elute DNA. Let stand for 1 min and centrifuge at 15,700g for 1 min.
17. Estimate the DNA concentration using a spectrophotometer or by running on a 0.8% agarose gel.
18. Store the samples at -20ºC and avoid successive freeze thaw periods.

Amplification and Purification of Plasmid DNA Using the QIAGEN® Plasmid Midi Kit

- QIAGEN plasmid purification protocol as provided by the manufacturer of the kit
- All buffers (P1, P2, P3, QBT, QC, QF, TE) are included in the QIAGEN kit

1. Pick a single colony from a freshly streaked selective plate and inoculate a starter culture of 2-5 mL LB medium containing the appropriate selective antibiotic. Incubate for ~8h at 37°C with vigorous shaking (~220 rpm on an orbital shaker with 1" orbit)**.** Use a tube or flask with a volume of at least 4 times the volume of the culture
2. Dilute the starter culture 1/500 to 1/1000 into selective LB medium. For high-copy plasmids inoculate 25 mL medium. For low-copy plasmids, inoculate 100 mL medium. Grow at 37°C for 12-16 h with vigorous shaking (~220 rpm on an orbital shaker with 1" orbit). Use a flask or vessel with a volume of at least 4 times the volume of the culture.
3. Harvest the bacteria cells by centrifugation at 6000 g for 15 min at 4°C. Remove all traces of supernatant by inverting the open centrifuge tube until all medium has been drained.
4. Resuspend the bacterial pellet in 4 mL or 10 mL Buffer P1. The bacteria should be resuspended completely by vortexing or pipetting up and down until no cell clumps remain.
5. Add 4 mL Buffer P2, mix gently but thoroughly by inverting 4-6 times and incubate at room temperature for 5 min. Do not vortex.
6. Add 4 mL of chilled buffer P3 (chill on ice), mix immediately but gently by inverting 4-6 times, and incubate on ice for 15 to 20 min. After the addition of buffer P3, a fluffy white material forms and the lysate becomes less viscous. If the material still appears viscous and brownish, more mixing is required to completely neutralize the solution.
7. Centrifuge at 20,000g for 30 min at 4°C. Remove supernatant containing plasmid DNA promptly. Before loading the centrifuge, the sample should be mixed again

8. Centrifuge the supernatant again at 20,000g for 15 min at 4°C. Remove supernatant containing plasmid DNA promptly.
9. Equilibrate a QIAGEN-tip 100 by applying 4 mL Buffer QBT, and allow the column to empty by gravity flow.
10. Apply the supernatant from step 8 to the QIAGEN-tip and allow it to enter the resin by gravity flow.
11. Wash the QIAGEN-tip with 2 × 10 mL Buffer QC.
12. Elute DNA with 5 mL buffer QF. Collect the eluate in a 10 mL tube.
13. Precipitate DNA by adding 3.5 mL of room-temperature isopropanol to the eluted DNA. Mix and centrifuge immediately at 1500 g for 30 min at 4°C. Carefully decant the supernatant. All solutions should be at room temperature in order to minimize salt precipitation.
14. Wash DNA pellet with 2 mL of room temperature 70% ethanol, and centrifuge at 1500 g for 10 min. Carefully decant the supernatant without disturbing the pellet.
15. Air-dry the pellet for 5-10 min , and redissolve the DNA in a 200 µL TE buffer (Allow the pellet to remain in contact with the TE buffer overnight at 4°C before transferring to a sterile eppendorf tube)

Gel Extraction Using QIAquick® Gel Extraction Kit

- Prepare Buffer PE by adding 40 mL 100% ethanol to the provided concentrate.
- Always use protective wear to protect skin and eyes from harmful UV radiation.
- When running a gel ensure that all buffers were made fresh, when staining a gel make sure to use a fresh batch of ethidium bromide so as to not contaminate your plasmid with foreign DNA or DNase.

1. Place stained gel onto a clear plastic sheet on top of an ultraviolet light source to accurately identify where plasmid DNA is located.
2. Excise the DNA fragment precisely from the agarose gel using a clean sharp scalpel. Avoid excess agarose.
3. Weigh the gel slice in a clear sterile microcentrifuge tube. Restrict the volume of gel in each 2 mL eppendorf tube to 400 mg or less.
4. Add 3 volumes of Buffer QG to 1 volume of gel.
5. Incubate at 50ºC in a heating block for 10 min or until the gel has completely dissolve. Vortex every 2 min during incubation to ensure complete dissolution of gel.
6. After incubation check that the color of the mixture remains similar to Buffer QG (yellow), indicating that the pH has not changed.
7. Add 1 gel volume of isopropanol to the mixture and mix by inverting the tube several times.
8. Place a QIAquick spin column in a provided 2 mL collection tube and apply the sample to the column. Centrifuge at 16,100g for 1 min with a table top centrifuge. Repeat this step if the volume of the mixture is more than 800 µL, the maximum capacity of the QIAquick column.
9. Discard the flow-through and place the column in the same collection tube.
10. Wash the QIAquick column by adding 750 µL Buffer PE to the column and centrifuging for 1 min at 15,700g.
11. Discard the flow-through, place the column back in the same collection tube and spin for an additional 1 min at 15,700g to remove residual ethanol from Buffer PE.
12. Place the QIAquick column in a clean, sterile 1.5 mL microcentrifuge tube.

13. Add 30 µL Buffer EB (10 mM Tris-Cl, pH 8.5) to the center of the QIAquick membrane to elute DNA, let stand for 1 min and then centrifuge for 1 min at 15,700g.
14. Store DNA at -20ºC.

Vector Construction

Digestion of pUbiGUS

Restriction Enzyme	*HindIII* (Promega; 2800 Woods Hollow Road, Madison, WI 53711, US)
pUbiGUS (5 µg)	16.5 µL
Restriction Buffer 2	2 µL
HindIII	1.5 µL
Final volume	20 µL

Digest overnight at 37ºC.

Ligation of backbone fragment from <u>HindIII</u> digested pUbiGUS

Ligation kit	LigaFast™ Rapid DNA Ligation System (Promega)
pUbiGUS	1 µL
Ligation Buffer 2X	5 µL
T4 ligase	1 µL
ddH_20	2.35 µL
Final volume	10 µL

Ligate overnight at room temp

Linearization of <u>HindIII</u> digested pUbiGUS and self-ligation prevention

1. Digest 5µg pUbiGUS plasmid with Not1 overnight at 37ºC
2. Add 1µl CIP directly to the Not1 digestion mixture then incubate for 2 h at 37°C.

3. Add 1.5µl 0.5M EDTA (ph8.0) and incubate at 65°C for 1hr to inactivate CIP.
4. Add 30 µL phenol:chloroform:isoamyl alcohol 25:24:1. Mix by tapping and inverting.
5. Centrifuge at 3,200g at 4°C for 1 min. There will now be three layers. The top layer contains DNA, the middle layer contains precipitates, and the bottom layer contains phenol. Pipette out the bottom layer and discard into a waste bottle.
6. Spin at 3,200g at 4°C for 5 min. Take the supernatant to a fresh 1.5 mL eppendorf tube.
7. Add 1/10 volume (5 µL) 3M NaOAc, mix by tapping
8. Add 2 × volume (100 µL) chilled absolute ethanol. Mix. DNA precipitation may become visible.
9. Wash the DNA pellet by adding 200 µL chilled 75% ethanol and tap the tube to wash thoroughly
10. Spin at 3,200g at 4°C for 5 min. Take the supernatant out carefully by pipetting. Don't disturb the DNA pellet.
11. Dry the DNA pellet by putting the tube on a clean bench for 10 min.
12. Add 20 µL ddH_20 to dissolve the DNA pellet.

Restriction Digestions

The pHZubicry1Fa digestion

Restriction Enzyme	*NotI* (Promega)
pHZubicry1Fa Vector (3 µg)	12 µL
Restriction Buffer D 10X	3.5 µL
BSA 10X	3.5 µL
NotI	3.5 µL
DdH2O	12.5 µL
Final volume	35 µL

Digest overnight at 37ºC

Ligation of the ubiquitin promoter and *cry1Fa* coding sequence from pHZubicry1Fa into the pUbiGUS linearized backbone

Ligation kit	LigaFast™ Rapid DNA Ligation System (Promega)
Ligation reaction	
pUbiGUS backbone	1 µL
cry1Fa insert	0.5 µL
T4 ligase	1 µL
DdH2O	6.5 µL
Final volume	10 µL

Ligate overnight at room temperature

Protocols For Seashore Paspalum Callus Induction, Transformation, And Regeneration

Protocol for Seashore Paspalum Immature Inflorescence *In Vitro* Cultivation

- We maintained high quality donor plant tissue with 16hr photoperiods using 1000 Watt sodium vapor lights. Greenhouse temperature was controlled with air conditioning to approximately 30ºC during day and 25ºC during night. An ebb and flow system for irrigation was used, irrigating 5 min every morning and fertilization was done every two wk using the recommended rate of Bloom Booster plant food from Miracle-Gro (P.O. Box 606 Marysville, OH 43040). Plants were maintained in 6 inch pots with Fafard No2 soil.

1. Remove immature inflorescences before or just upon the emergence of the flag leaf. Cut the stem of the grass two to three nodes down from the uppermost node
2. Maintain harvested tissue in a moist environment before sterilization, moisten a cloth or paper towel and place in a bag with harvested tissue
3. Before surface sterilization, remove excess leaf, root, or soil material from the harvested immature inflorescence still wrapped in the sheath
4. Place up to 30 stalks into a 50 mL tube and fill tube with 70% Ethanol
5. Shake for 4 min by hand
6. Remove excess Ethanol and add pool bleach with active ingredient 10% sodium hypochlorite
7. Add 0.01% tween and stir 20 min at a low stir setting on any shaker
8. After sterilization tissue should be rapidly rinsed with sterile distilled water three time followed by two rinses with emersion periods in sterile distilled water for 10 min all in a clean bench. (When rinsing recap the 50 mL tube to ensure dilution of active ingredients on the lid itself)
9. The immature inflorescences can now be excised from the surface sterilized sheath and inflorescences of up to 1 cm can be cut and placed onto callus induction media.

Tissue Culture Conditions

- Tissue culture was initiated on either IF medium or Treatment Medium and transferred every 7-15 d to fresh medium.
- Tissue Culture was maintained with Low light intensity (30 $\mu Em^{-2}s^{-1}$ light) at 25ºC with a 16 h photoperiod in a Percival incubator.
- Shoot regeneration was done on Regeneration Medium at a high light intensity (150 $\mu Em^{-2}s^{-1}$ light) at 25ºC with a 16 h photoperiod. Tissue culture was maintained on fresh media biweekly to monthly depending on growth stage of newly formed shoots. Regenerated shoots were transferred to fresh medium less frequently than callus that had not yet formed shoots.

Protocol for Particle Bombardment

Gold stock (60 mg mL^{-1}) preparation

1. Weigh 30 mg 1.0 µg gold into a sterile 1.5 mL eppendorf tube.
2. Add 1 mL of 70% ethanol. (Prepare 70% ethanol with autoclaved sterile ddH_20)
3. Vortex for 3-5 min.
4. Incubate at room temperature for 15 min.

5. Pellet the microparticles using a table top centrifuge for 5 s.
6. Discard supernatant then wash three times by adding 1 mL sterile ddH_20.
7. Vortex for 1 min and let particles settle an additional min.
8. Pellet the microparticles with a brief 5 s spin in a table top centrifuge then remove supernatant.
9. After third wash add 500 µL sterile 50% glycerol. Store at -20°C.

Sterilization of Biolistic Gene Delivery Device (PDS 1000, BioRad) Components

1. Autoclave macrocarrier holders, stopping screens, macrocarriers, and a device capable of securing macrocarriers into the macrocarrier holders.
2. Lay out in laminar flow hood to dry.
3. Sterilize 1100psi rupture discs by dipping in Absolute Ethanol and dry in flow hood on the rim of a sterile petri dish.
4. Clean biolistic gene delivery device (PDS 1000, BioRad) chamber, assembly and flow hood thoroughly with 70% ethanol and thoroughly dry before use.
5. For large scale bombardments allow time to resterilize the biolistic gene delivery device (PDS 1000, BioRad) and its components with 70% ethanol to reduce risk for contamination.

Preparation of DNA Coated Microparticles

1. Mix 30µl 1µm gold stock and 30µl DNA by vortexing 1 min.
2. Add 20µl 0.1M spermidine and 50µl 2.5M $CaCl_2$ consecutively and immediately while continuing to vortex for an additional min.
3. Centrifuge briefly to settle gold.
4. Wash in 250µl Absolute Ethanol without disturbing the pellet.
5. Spin and remove supernatant.
6. Resuspend gold in 90µl Absolute Ethanol by sonication (Branson 2200, Branson Ultrasonics, Danbury, CT 06813-1961, US) for 2 s.
7. Ensure there are no large clumps of DNA by placing eppendorf tube against a light source and tapping the tube.
8. Use 5µl per shot enough for 12 shots (Upon placing DNA solution onto macrocarriers be sure that DNA and gold are in solution by rubbing briefly against an empty pipette rack)

Biolistic Bombardment

- Use the same stopping screen for 15-20 shots.

1. Turn on PDS-1000/He Particle Delivery System and vacuum pump, than increase helium pressure to the tube attached to the biolistic gene delivery device (PDS 1000, BioRad) to just slightly over 1100psi.
2. Place macrocarriers into holders securely using a sterile blunt object.
3. Spread 5µl gold prep evenly onto macrocarriers and dry briefly (don't use more than five macrocarriers at a time as to minimize DNA degradation from exposure to the air).
4. Place rupture disc into holder and screw tightly into place.

5. Place stopping screen into shelf assembly and put inverted macrocarrier assembly on top.
6. Place shelf containing the macrocarrier at the highest level.
7. Place tissue culture plate on shelf 2 levels below gold or 6 cm below the macrocarrier shelf.
8. Pull a vacuum to 27.5 Hg then press and hold the fire button until the disc ruptures at 1100psi.
9. Vent vacuum and remove petri dish.
10. Dismantle assembly and set up for next shot.

The GUS Assay

(Jefferson et al. 1987)

Solution 1

1. Add 70 mg X-gluc to 2 mL DMSO in a small beaker (cover beaker in foil to minimize exposure to light)

Solution 2

2. Mix 150 mL of 100mM Na_3Po_4 to 5 mL of 0.5M EDTA and add 200 µl Trinton X-100.
3. Mix solution 1 and 2 and make final volume up to 200 mL with ddH_20 and aliquot in 15 mL tubes and store at -20°C in the dark (wrap in foil).

The GUS stock solutions

- 100mM Na_3PO_4 (pH 7): Dissolve 7.602 g $Na_3PO_4 \cdot 12H_20$ and adjust pH to 7 with concentrated HCl
- 0.5M EDTA (pH 8): Dissolve 46.525 g EDTA disodium salt in 200 mL ddH_20. Adjust pH to 8 with NaOH and make up to 250 mL. Sterilize by autoclaving.

The GUS Assay Procedure

1. Bombard tissue using procedure described previously for biolistic transformation of callus.
2. Allow tissue to recover on fresh callus induction media after bombardment for 3 to 4 d.
3. Place bombarded tissue into a 24 well microplate and place plate into an empty pipette box to prevent spilling of assay into biolistic gene delivery device (PDS 1000, BioRad) or incubator.
4. Add enough GUS assay mix to completely cover the callus material in the microplate and place into the biolistic gene delivery device (PDS 1000, BioRad) to draw a vacuum for 10 min.
5. Afterwards incubate for 16hrs at 37ºC in the dark.
6. Blue foci should be visible on callus and can be counted using a microscope.

Buffers And Medium

Bacterial Growth

The SOC medium

- SOB: 4 g tryptone, 1 g yeast extract and 0.1 g NaCl. Dissolve in 180 mL ddH_2O. Add 2 mL 250mM KCl. Adjust to pH7 with 5N NaOH. Make up to 200 mL. Just before use, add 10 µL 1M $MgCl_2$ and 20 µL 1M glucose per 1 mL SOB.

Antibiotics

- Ampicillin: Weigh 100 mg ampicillin. Dissolve in 2 mL of ddH_2O. Filter sterilize into autoclaved eppendorf tubes. Freeze at -20ºC. Stock concentration: 50 mg mL^{-1}. Use 2µl (100 mg mL^{-1}) LB.
- Kanamycin: Weigh 100 mg kanamycin. Dissolve in 10 mL of ddH_2O. Filter sterilize into autoclaved eppendorf tubes. Freeze at -20ºC. Stock concentration: 10 mg mL^{-1}. Use 5µl (50 mg mL^{-1}) LB.
- Rifampicin: Weigh 100 mg rifampicin. Dissolve in 4 mL of DMSO. Filter sterilize into autoclaved eppendorf tubes. Wrap tubes in aluminum foil. Freeze at -20ºC. Stock concentration: 25 mg mL^{-1}. Use 6µl (150 mg mL^{-1}) LB.
- Spectinomycin: Weigh 100 mg spectinomycin. Dissolve in 10 mL of ddH_2O. Filter sterilize into autoclaved eppendorf tubes. Freeze at -20ºC. Stock concentration: 10 mg mL^{-1}. Use 5µl (50 mg mL^{-1}) LB.

Seashore Paspalum Tissue Culture Medium

The IF medium

1. Add 4.3 g L^{-1} MS salts, 12.45 mg L^{-1} CuSO4, 20 g L^{-1} sucrose.
2. Adjust pH to 5.8.
3. Add 3 g L^{-1} phytagel.
4. Media was made with a final volume of 400 mL per bottle.
5. Sterilize at 121°C and 120kPa in an autoclave for 20 min.
6. Before the media became solid add 3 mg L^{-1} Dicamba, 103.2 mg L^{-1} MS 1000X vitamins, and 1.1 mg L^{-1} BAP (thoroughly mix all stock solutions before use)

Treatment medium

- 6 treatments made just like IF media except for the auxin used and BAP level. Media was made with either 2,4-D or dicamba both at 3 mg L^{-1} and of the two auxin types levels of BAP were either 0, 0.1, or 1.0 mg L^{-1} BAP.

Sorbitol medium

- Medium was made with the same constituents of the medium callus was induced on with the inclusion of sorbitol at 72.85 gL^{-1}. i.e. IF medium with 72.85 g L^{-1} sorbitol added before sterilization in autoclave.

Selection medium

- Hygromycin at 40 mg L^{-1} was added to both IF medium and the six treatment medium and 6 g L^{-1} of the gelling agent agarose was used instead of phytagel when callus was under selection. All medium made with hygromycin must be stored in the dark.

Regeneration medium

- Shoot regeneration medium was made following the protocol for IF or any of the six treatment medium with the exclusion of any auxin or cytokinin. Selection on regeneration medium was done at both 20 and 10 mg L^{-1} hygromycin again with the substitution of 6 g L^{-1} agarose instead of phytagel.

Stock Solutions for Tissue Culture

The 2,4-dichlorophenoxyacetic acid (2,4-D)

- Weigh out 0.1 g 2,4-D. Dissolve in very little 1N K0H and then add 50 mL ddH_20. Filter sterilize and store in 1 mL aliquots at -20°C.

The 3,6-dichloro-2-methoxybenzoic acid (dicamba)

- Weigh 100 mg dicamba. Dissolve in 0.5 mL of 100% ethanol with heat. Add 49.5 mL ddH_2O with heat. Filter sterilize the stock solution and aliquot into autoclaved eppendorf tubes and freeze at -20ºC. Stock concentration: 2 mg mL^{-1}. Use 600 µL (1200 mg)/400 mL IF.

The 6-benzylaminopurine (BAP)

- Weigh 825 mg BAP. Dissolve in 0.5 mL of NaOH (1N). Add 19.5 mL ddH_2O. Filter sterilize the stock solution and aliquot into autoclaved eppendorf tubes, then freeze at -20ºC. Stock concentration: 1 mg mL^{-1}. Use 440 µL (1200 mg)/400 mL IF.

Hygromycin

- Dissolve hygromycin in ddH_20 making final volume 125 mg mL^{-1}. Filter sterilize and store in aliquots at -20°C in the dark.

Paromomycin

- Weigh 0.5 g paromomycin in 10 mL ddH_20. Filter sterilize and store in 1 mL aliquots in eppendorf tubes at -20°C.

Molecular Techniques used in the Confirmation of Stable Transformants

Immunochromatographic Cry1Fa QuickStix Test

- EnviroLogix (500 Riverside industrial parkway, Portland, Maine 04103-1486, US) QuickStix Kit for Cry1Fa protocol as provided by the manufacturer of the kit

1. Sandwich a section of leaf tissue between the cap and body of the disposable tissue extractor tube (We used 4 leaf segments all equal in length to a 1.5 mL eppendorf tube. Push the leaf punches down into the tapered bottom of the tube with the 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 s or until the leaf tissue is well ground.
3. Uncap the bottle of extraction buffer and invert it directly over the tissue extractor tube. Carefully squeeze 10 drops (we used 5 drops) into the tube containing leaf sample.
4. Repeat the grinding step to mix tissue with extraction buffer. Dispose of the pestle (do not re-use pestles on more than one sample).
5. Allow refrigerated canisters to come to room temperature before opening. Remove the QuickStix Strips to be used. Avoid bending the strips. Reseal the canister immediately.
6. Place the strip into the extraction tube. The sample will travel up the strip. Use a rack to support multiple tubes if needed.
7. Allow the strip to develop for 10 min before making final assay interpretations. Positive sample results may become obvious much more quickly. Development of the control line within 10 min indicates that the strip has functioned properly. If the sample extract contained Cry1Fa endotoxin, a second line (test line) will develop on the membrane strip between the control line and the protective tape, within 10 min of sample addition.

The ELISA for Presence of Cry1Fa Protein

- *EnviroLogix QualiPlate™ Kit for* Cry1Fa *high sensitivity protocol as provided by the manufacturer of the kit*
- Will detect 0.17% Herculex I corn in ground grain/seed, and requires 2.5 h of total assay incubation time.
- Dilute the positive control ground corn extract 1:3 in negative control ground corn extract for this protocol.

1. Add 50µL of extraction buffer blank (BL), 50µL of negative control (NC) ground corn extract, 50µL of diluted positive control (PC) ground corn extract, and 50µL of each sample extract (S) to their respective wells.
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 s. 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 min. If an orbital plate shaker is available, shake plate at 200rpm.
4. Add 50µL *Cry1F*-enzyme conjugate to each well. Thoroughly mix the contents of the wells, as in step 2.
5. Cover the wells with tape or parafilm to prevent evaporation and incubate at ambient temperature for 90 min. If an orbital plate shaker is available, shake plate at 200 rpm.

6. 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.
7. Add 100µL of substrate to each well.
8. Thoroughly mix the contents of the wells, as in step 2. Cover the wells with new tape or parafilm and incubate for 30 min at ambient temperature. Use orbital shaker if available. Caution: stop solution is 1.0N hydrochloric acid. Handle carefully.
9. Add 100µL of stop solution to each well and mix thoroughly. This will turn the well contents yellow NOTE: Read the plate within 30 min of the addition of stop solution.

Interpretion of results (Spectrophotometric measurements and general test criteria)

1. Set the wavelength of the microtiter plate reader to 450 nm (If it has dual wavelength capability, use 600, 630 or 650 nm as the reference wavelength.)
2. Set the plate reader to blank on the extraction buffer blank wells (this should automatically subtract the mean optical density (OD) of the blank wells from each control and sample OD). If the reader cannot do this, it must be done manually.

- The mean OD of the blank wells should not exceed 0.2.
- The mean, blank-subtracted OD of the positive control wells should be at least 0.15.
- The coefficient of variance (%CV) between the duplicate positive control wells should not exceed 15%:%CV = std. deviation of OD's × 100 mean pos.ctl. OD
- 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".
- Single corn leaf and seed samples: If the positive control ratio calculated for a sample is less than 0.5, the sample does not contain Cry1Fa at the levels normally found in Herculex I corn. If the positive control ratio of a sample is greater than or equal to 0.5, the sample does contain Cry1Fa at the levels normally found in Herculex I corn.

Preparation of Solutions for ELISA

Wash buffer

- Add the contents of the packet of wash buffer salts (phosphate buffered saline, pH 7.4 - tween 20) to 1 L of distilled or deionized water and stir to dissolve. Store refrigerated when not in use; warm to room temperature before assay. If more wash buffer is needed, order item # P-3563 from Sigma Chemical Co. (St. Louis, MO 63118), or prepare the equivalent.

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 before assay.

Sample preparation

- Positive and negative control ground corn extracts

Extracts of these controls must be run in every assay. To extract, add 5 mL of extraction buffer to each tube containing 2 g of ground control corn. Cap and shake vigorously by hand or vortex for 20-30 s. Let stand at room temperature for 1 h to extract. Mix again at the end of the hour, then clarify by settling 10 min or by centrifuging 5 min at 5000g.

The high sensitivity protocol requires that the positive control ground corn extract be diluted 1:3 in negative control ground corn extract (mix 100µL positive extract plus 200µL negative extract) before use.

Leaf testing

1. Take a single leaf punch of approximately 5 mm diameter (We used two leaf segments from the second and third uppermost leaves of length equivalent to that of a 1.5 mL eppendorf tube), using a micro-tube cap or a paper punch. Mash the leaf tissue with a pestle matched to the micro-tube, or with a disposable pipette tip, or a Hypure cutter (HCT-200; PerkinElmer, 940 Winter Street, Waltham, Massachusetts 02451, US) in a 96-well plate (Costar #3370; Corning life Sciences, Tower 2, 4th floor, 900 Chelmsford street, Lowell, Massachusetts 01851, US; or equivalent).
2. Add 0.25 mL of extraction buffer per leaf punch. Mix for at least 30 s, then allow particles to settle. Take extreme care not to cross contaminate between leaf samples. Dispensing particles into the test plate can cause false positive results.

The DNA Extraction Using the CTAB Method

Procedure

- Sterilize mortar and pestle and eppendorf tubes in an autoclave for 20 min before use.

1. Collect 0.1 g of young leaf tissue
2. Freeze and grind the sample to a fine powder in liquid nitrogen (grind three times, grind when nitrogen has evaporated to prevent loss of sample)
3. Add freshly ground sample to 500 µL CTAB buffer in a 1.5 mL eppendorf tube. Mix by vortexing briefly.
4. Add 3µl RNase
5. Incubate at 65ºC for 1 h mixing every 15 min.
6. Cool to room temperature then add an equal volume (500 µL) chloroform /isoamylalchohol 24:1. (Work in a fume hood)
7. Mix Thoroughly
8. Shake for 30 min gently
9. Spin at 3,200g at room temperature for 5 min
10. Transfer Supernatant to fresh tubes
11. Add equal volume (500 µL) chloroform /isoamylalchohol 24:1. (Work in a fume hood)
12. Mix thoroughly
13. Shake gently for 15 min
14. Spin 3,200g at 4ºC for 5 min
15. Discard the bottom chloroform layer into the waste bottle in the fume hood
16. Spin 3,200g at 4ºC for 5 min
17. Carefully take the supernatant and transfer to fresh 1.5 mL eppendorf tubes.
18. Add 2/3 volume isopropanol
19. Mix thoroughly, should be able to visually see DNA

20. Wash 2X with 70% ethanol spin 3,200g at 4ºC for 5 min
21. Dry with a vacuum centrifuge for five min
22. Dissolve the DNA in 50 µL sterile ddH_20

The CTAB buffer (500 mL)

- 50 mL 1M Tris-HCl (100mM final)
- 20 mL 0.5M EDTA (disodium salt) (20mM final)
- 140 mL 5M NaCl (1.4M final)
- 10 g CTAB (2% w/v final)

The PCR Reactions

Basic PCR set-up using HotStarTaq® DNA Polymerase (Qiagen)

1. Determine the number of samples to be used for PCR analysis. Also include the wild-type, negative control (reaction with all components but no template) and positive control (reaction containing the plasmid as template).
2. Prepare a master-mix for all the samples together as follows:

10x Buffer	2.50 µL
5x Q solution	5.00 µL
50x dNTP mix	0.50 µL
50x $MgCl_2$	0.50 µL
10 µM Forward primer	1.00 µL
10 µM Reverse primer	1.00 µL
HotStarTaq	0.15 µL
Sterile ddH_2O	13.35 µL
Final volume	24.00 µL

3. Label the tubes for PCR and dispense 24 µL of the master mix into each tube.
4. Add 1 µL DNA to the samples (50 ng), 1 µL sterile ddH_2O to the negative control and 1 µL plasmid (50 pg μL^{-1}) to the positive control. Mix well by pipetting, spin briefly and start the PCR program.
   Note: Remember to start the PCR program with 15 min at 95ºC to activate the

HotStarTaq® polymerase.

Amplification of *hph* in modified phphcry1Fa

PCR kit	PCR Core System II (Promega)	
Primer sequences	Forward	5' - CCC GAT ATG AAA AAG CCT GA -3'
	Reverse	5' – GAT GTT GGC GAC CTC GTA TT -3'
PCR program	Isaac *hph*	2' 95ºC for initial denaturation, 30 cycles with 1' 95ºC for denaturation, 1' 48ºC for annealing and 1' 72ºC for extension, 5' 72ºC for final extension and hold at 4ºC
PCR reaction		
DNA template	1.0 µL	
PCR Buffer 10 X	2.0 µL	
Q Buffer	4.0 µL	
DNTPs	0.4 µl	
Forward primer	1.0 µL	
Reverse primer	1.0 µL	
Taq polymerase	0.1µl	
DdH_2O	10.5µl	
Final volume	20.0 µL	

Amplification of *cry1F* in modified phphcry1Fa

PCR kit	PCR Core System II (Promega)	
Primer sequences	Forward	5' – CCG GGA CCA TTG ACT CTC TA -3'
	Reverse	5' – CAC TTC GTT GCC TGA ACT GA -3'
PCR program	Isaac *cry*	2' 95ºC for initial denaturation, 30 cycles with 1' 95ºC for denaturation, 1' 48ºC for annealing and 1' 72ºC for extension, 5' 72ºC for final extension and hold at 4ºC
PCR reaction		
DNA template	1.0 µL	
PCR Buffer 10 X	2.0 µL	
Q Buffer	4.0 µL	
DNTPs	0.4 µl	
Forward primer	1.0 µL	
Reverse primer	1.0 µL	
Taq polymerase	0.1µl	
DdH_2O	10.5µl	
Final volume	20.0 µL	

Agarose Gel Electrophoresis

Procedure

1. Prepare a 0.8% agarose gel by weighing 0.4 g agarose. Transfer this to a 250 mL Erlenmeyer flask. Add 50 mL 0.5x TBE and boil in a microwave oven for 30 s followed by stirring and boiling another 30 s.

2. Allow to cool to 55-60°C.
3. Get a gel mold and seal both ends in the holder. Place in a level platform and attach a comb.
4. Pour agarose into the gel mold and solidify.
5. Pour 0.5 × TBE buffer into the electrode tank. Mount the gel mold on to the electrode tank with the comb oriented toward the cathode end. Remove bubbles with a pipette tip.
6. Gently remove the comb.
7. Load 10 µL 1 Kb ladder on the first well and load 12 µL each of the 3different concentrations of lambda DNA (100, 50, 25 ng μL^{-1}) on the next 3 wells.
8. Apply sample in other wells with each well having a final volume if 12µl 2 of which are a 6X loading dye. Load the mixtures in the succeeding wells.
9. Close the tank and attach the electrodes to the power supply. Run at 90 V for 45 min.
10. After the run, turn off the electric current and remove the gel mold from the tank. Transfer the gel in a staining tray with 100 µL of EtBr solution in a 1000 mL ddH_2O. Stain for 15-20 min.
11. Photograph the gel under UV light and estimate each DNA sample by comparing with the lambda DNA made to concentrations of 25, 50, and 100 ng μL^{-1}

The TBE stock

5x stock TBE: 54 g Tris base

27.5 g boric acid

20 mL 0.5M EDTA (pH8)

Make up to 1L with ddH_2O

Dilute stock to 0.5x for use in gels and running buffer. Allow 500 mL for small gel tank

Using the Nanodrop ND-1000 Spectrophotometer

(NanoDrop Technologies, 3411 Silverside Rd Bancroft Building, Wilmington, DE 19810)

1. Pipette 2µl of water on the upper and lower pedestals and wipe off using a soft laboratory wipe to clean the pedestals.
2. Pipette 1 µL water on the lower pedestal, close the sampling arm, initialize the instrument using the operating software and then set the blank.
3. Open the sampling arm and wipe the two pedestals using a laboratory wipe.
4. With the sampling arm open, pipette 1 µL sample onto the lower pedestal (ensure complete covering of the lower pedestal)
5. Close the sampling arm and initiate the measurement using the operating software.
6. On completion of the measurement, open the sampling arm and wipe the sample from both the upper and lower pedestals using a laboratory wipe.
7. Repeat from step 5 for subsequent samples.
8. Clean the pedestals as described in step 2 after completion of all measurements.

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