EVALUATION OF TRANSGENIC STRATEGIES TO ENHANCE TURF QUALITY OF
BAHIAGRASS (*Paspalum notatum* FLÜGGE)

By

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To my family and friends for their unconditional love and support… And in loving memory of my grandfather
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Bahiagrass (Paspalum notatum Flügge) is a popular forage and turf species in the southeastern US due to its persistence under low-input conditions. However, the turf quality of bahiagrass is limited by its open growth habit and prolific production of long inflorescences. We successfully improved turf quality in bahiagrass following reduction of bioactive gibberellic acid, by over-expressing GA-2-oxidase-1 (AtGA2ox1) or ATHB16 from Arabidopsis. Here, evaluation of turf quality, performance and AtGA2ox1 or ATHB16 transgene expression of these plants under field conditions is reported.

Transgenic bahiagrass and wild-type controls plants were established in 1 x 1 m² plots under USDA-APHIS permits 05-364-01r and 06-219-01r at the UF-IFAS Plant Research and Education Center in Citra, Florida. Turf quality and field performance were evaluated in two field studies.

Bahiagrass over-expressing ATHB16 or ATGAox1 produced significantly more tillers than wild-type bahiagrass. Transgenic plants also showed decreased stem length while root and rhizome biomass as well as drought tolerance and low input characteristics were not
compromised. Most of the $AtGA2ox1$ expressing lines displayed improved tolerance and recovery from drought, whereas some of the $ATHB16$ expressing lines exhibited improved recovery from drought. Delayed and reduced flowering and reduced inflorescence stem length was observed on $ATHB16$ and $ATGAox1$ expressing lines. Some of the bahiagrass lines expressing $ATHB16$ also exhibited a proportional semi-dwarfing (shorter tillers and finer leaves). Our data on the $AtGA2ox1$ suggests that GA affects flowering, outgrowth of axillary buds and apical dominance in bahiagrass. Reduced levels of GA may also contribute to improved drought stress response in bahiagrass. The data presented on the $ATHB16$ expressing lines indicates that expression of this gene in bahiagrass significantly changes plant architecture of this species. Our findings are consistent with the proposed function of $ATHB16$ as repressor of cell expansion.
CHAPTER 1
INTRODUCTION AND RATIONALE

Turfgrasses are grown and maintained around the world. They can increase the value of a
property and add aesthetic value. Turf can also prevent erosion, reduce noise and air pollution,
generate oxygen, serve as fire retardant, filter groundwater and moderate temperature. In
addition, natural turf serves as surface for many athletic and recreational activities. In the US
alone, the turf industry is worth $40 billion annually (National Turfgrass Federation, 2003). It is
estimated that in the US turfgrasses cover 20 million hectares (National Turfgrass Federation,
2003). Despite its many benefits, the increasing use of turf is also creating challenges. Turf
requires water, fertilizer, and pesticides for establishment and maintenance. This raises many
environmental concerns including water conservation.

With water resources available for irrigation becoming increasingly scarce due to global
warming and increased populations (Breshears et al., 2005; Zhang and Wang, 2007),
environmental enhancement and water conservation is becoming a common goal of our society.
Thus increasing the demand for high quality, low maintenance turfgrasses with the ability to
survive periods of environmental constraint. Water use in turf and ornamentals in urban areas in
the US was estimated to account for 9% of the total annual water consumption (Landry, 2000;
Huang, 2008). In 2005, withdrawals of freshwater for agricultural use constituted 40% while
recreational irrigation used another 5% of total withdrawals in Florida (Borisova and Carriker,
2009). Haley et al. (2007) reported that 64% of potable water was used for landscape irrigation in
the central Florida Ridge area.

In Florida irrigation is necessary due to its sandy soils with lower water holding capacity,
dry spring weather and sporadic rainfall events experienced. Turfgrass plants, like all green
plants, require water for survival and growth. According to Beard (1973), drought stress remains
the most important environmental factor limiting growth of turfgrass. However, growing concern
for depletion of our fresh water resources has motivated the state of Florida to develop strategies
to reduce water consumption. In 2001, after a record drought for the state was experienced, the
Florida Department of Environmental Protection (FDEP) developed the Water Conservation
Initiative (WCI) to find ways to improve water efficiency. One of their recommendations was to
regulate irrigation practices (FDEP, 2002). Another strategy being explored by the state is
passing ordinances that limit available water use for landscapes and restricting the use of the
state’s turf industry standard, St. Augustinegrass. Hence, the development and utilization of more
drought tolerant turfgrass species for Florida is warranted. Turfgrass grown in Florida must be
adapted to an array of environments and several abiotic and biotic stresses, which can hinder the
performance of any given variety (Kenworthy et al., 2007).

Bahiagrass’ (*Paspalum notatum* Flügge), tolerance to drought, heat, minimal fertility soil,
overgrazing, long lived stands and resistance to most disease and pests makes it a prime low-
input turf species for Florida. Its root system is more extensive than any other turfgrass (Busey,
2003), which serves to support its drought tolerance and reduces the impact of nematode damage
(Trenholm et al., 2003). Along with deeper rooting, better recovery also grants this species with
the highest level of drought survival of any sod-forming turfgrass (Busey, 2003). Bahiagrass can
survive without much water by retarding its growth when water is limiting, but recovers rapidly
when it receives water (Trenholm and Uruh, 2006). Native to South America, bahiagrass has
widely adapted to the southern Coastal Plain region of the US since its introduction in 1912
(Scott, 1920). This creeping, warm-season perennial species is grown extensively throughout
Florida and the southern US and is commonly used for forage and utility turf along highways and
roadsides. All these qualities make bahiagrass a prime low-input turf species for the state.
However, bahiagrass use as a turfgrass has been limited to roadsides, along highways, road medians and for large lawn areas due to its poor turf quality. Factors contributing to its poor turf quality include the prolific production of tall (60 cm) inflorescences during the summer months and its poor stand density. Not only do these problems affect visual quality but also increase mowing requirements and weed encroachment respectively. Plant growth retardants (PGRs) have been used to suppress inflorescences and leaf growth due to rising mowing costs (Unruh and Brecke, 2006). However, applications of PGRs are associated with phytotoxicity, reduced recuperative potential from physical damage on treated turf and increased weed pressure due to reduced competition from treated plants (Unruh and Brecke, 2006).

An alternative would be the development of genetically improved bahiagrass cultivars. ‘Argentine’ a tetraploid, is commonly used as low maintenance turf due to its darker green color, and reduced period of flowering compared to other cultivars such as diploid ‘Pensacola’ (Trenholm et al., 2003). However, the improvement of tetraploid cultivars by traditional breeding methods is limited due to their genome complexity and asexual apomictic reproduction lacking female meiotic recombination during seed production. The methodology for breeding these asexual tetraploids involves chromosome doubling of diploid cultivars, thus generating sexual tetraploids (Burton and Forbes, 1960). These sexual autotetraploids generated are then used as female parents in crosses with the apomictic tetraploid males to produce segregating populations (Hanna and Burton, 1986). This induced polyploidy makes it possible then to overcome sterility associated with apomixis. An alternative to traditional breeding is the use of genetic transformation technology to introduce desired traits into the tetraploid cultivar ‘Argentine’. This technology allows direct introductions of desired traits and offers perhaps the least time consuming alternative with the greatest potential value.
An effective tissue culture and transformation protocol for apomictic bahiagrass cultivar ‘Argentine’ was recently developed by Altpeter and James (2005) and Altpeter and Positano (2005). The apomictic nature of this cultivar was expected to result in uniform transgenic progenies and may reduce risk of unintended transgene dispersal. However, Sandhu (2008) recently reported transgene flow between ‘Argentine’ and non-transgenic bahiagrass under greenhouse and field conditions. Thus using a highly apomictic cultivar like ‘Argentine’ does not provide absolute transgene containment. Nevertheless, this transformation protocol allowed for the introduction of transgenes that successfully improved reduced bioactive gibberellic acid, by constitutive expression of \textit{AtGA2ox1} (Agharkar et al., 2007) or \textit{ATHB16} (Zhang et al., 2007) from Arabidopsis. \textit{GA-2-oxidase-1} encodes a gibberellin-catabolizing enzyme, whereas \textit{ATHB16} encodes a transcription factor functioning as a repressor of cell expansion.

Changing the plants architecture might compromise the low input characteristics. Also stability of transgene expression is not always guaranteed under field conditions. Therefore, field-testing of these transgenic plants under different low-input and management conditions is very important. The aim of this study was to comparatively evaluate the turf performance of transgenic bahiagrass plants overexpressing \textit{AtGA2ox1} or \textit{ATHB16} and evaluate stable transgene expression under field conditions.
CHAPTER 2
LITERATURE REVIEW

Bahiagrass (*Paspalum notatum* Flügge)

The Genus *Paspalum* L.

Members of the genus *Paspalum* share various physical and genetic characteristics. This genus is characterized by their raceme-like inflorescences with plano-convex spikelets, with the lower glume generally absent (Clayton and Renvoize, 1986). Most *Paspalum* species have a chromosome base number $x = 10$. Polyploidy and apomictic reproduction are features that occur frequently among the *Paspalum* species (Jarret et al., 1998). Many *Paspalum* species are highly self-pollinating (Burson, 1987; Burson and Young, 2000).

*Paspalum* L., member of the Paniceae tribe, is one of the largest genera within the Poaceae family (Watson and Dallwitz, 1994; Souza-Chies et al., 2006). This genus contains 300 to 400 species most of which are endemic to tropical and subtropical regions of the New World (Barkworth et al., 2007). Several species are economically important as forage and turf grasses (Burson and Bennet, 1971), including *Paspalum notatum* Flügge, *P. fasciculatum*, *P. dilatatum* Poir, *P. atratum* Swallen, *P. nicorae* Parodi, *P. vaginatum* Swartz, *P. plicatulum* Michx, and *P. guenoarum* Arech. However, only a few of these species have been included in selection programs: *P. notatum*, *P. dilatatum*, *P. plicatulum* and *P. guenoarum* *P. atratum*, and *P. vaginatum* (Valls, 1992; Kretschmer et al., 1994; Duncan and Carrow, 2000; Pozzobon et al., 2008).

Importance and Use

Bahiagrass is one of the most widely grown grasses in the southeastern United States where it is estimated to cover more than 2.4 million hectares (Burton et al., 1997). In the United States, bahiagrass is used for forage, turf, crop rotations, and erosion control. In Georgia,
Alabama, and Florida bahiagrass is the predominant forage grass utilized by the beef cattle industry (Blount et al., 2001). In Florida, an estimated that 2 million hectares of grasslands are planted in bahiagrass alone, as reported by the Florida Agricultural Statistics in 1997 (Blount, 2004). It comprises approximately 85% of the improved pastures in Florida (Kretschmer and Hood, 1999; Kretschmer and Pitman, 2000). The diploid cultivar, ‘Pensacola’, is the predominant pasture grass in the southeastern US covering an estimated 1.2 million hectares in Florida alone (Nordie, 2008). As a turf, Bahiagrass accounts for 19% of total turfgrass area in the state occupying an estimated 0.30 million hectares (Hodges et al., 2004). This creeping perennial is an excellent turf former (Rather, 1942) and widely used as a seeded lawn grass (Janick et al., 1969a). In 2003, bahiagrass comprised 24% (8,892 hectares) of the total sod production in the state (Haydu et al., 2005). The apomictic tetraploid cultivar ‘Argentine’ is more commonly used as low maintenance turf due to its darker green color, and reduced period of flowering compared to other cultivars such as ‘Pensacola’ (Trenholm et al., 2003). Bahiagrass is also utilized in crop rotations to interrupt disease cycles and improve soil quality in cash crop systems (Katsvairo et al., 2006).

**Origin and Distribution**

The first intentional introduction of *Paspalum notatum* into the US was in 1912 (Scott, 1920) from southern Brazil, Uruguay, northeastern Argentina, and Paraguay, the presumed center of origin is presumed to be from of the species (Parodi, 1937). It has been introduced since the late 1800s to the United States, Africa, Asia, Australia and Europe (Busey, 2003). In the United States, bahiagrass can be found from southern California to eastern Texas, from Florida to New Jersey, and from central Tennessee to Arkansas (Chase, 1929; Watson and Burton, 1985). Occurrences of bahiagrass in the US have been reported in Alabama, Arkansas, California, Florida, Georgia, Hawaii, Illinois, Louisiana, Missouri, North Carolina, New Jersey,
Oklahoma, South Carolina, Tennessee, Texas, Virginia, Puerto Rico, and the Virgin Islands (USDA Plants database 2009).

**Botanical Characteristics**

A botanical description of Bahiagrass is found in Busey et al. (2003) and is summarized here. Bahiagrass is a warm season perennial grass that spreads by stout runners covered with dead persistent sheaths of previous leaves. These runners can be found in the literature referred to as rhizomes or stolons or both. For sake of consistency they will be referred to as rhizomes thorough this thesis. Its leaf blades are erect or decumbent and pointed. As with most of the *Paspalum* species, its inflorescences are racemose, generally with 2 racemes and the first glume is absent. Solitary plano-convex spikelets are found in two rows on each raceme. The floret contains three anthers and two stigmas, both generally purple in color. Flowering occurs soon after sunrise beginning at the top of the inflorescence.

**Ploidy and Cytology**

The base chromosome number of bahiagrass is x=10. This species is highly diverse, containing races with various ploidy levels. Bahiagrass cytotypes have 20, 30, 40, and 50 chromosomes (Gould, 1975; Trischler and Burson, 1991; Burson and Young, 2000). Out of which the most common are diploids (2n=2x=20) and tetraploids (2n=4x=40) (Burton, 1946; Saura, 1948; Gould, 1966). The diploid races belong to *Paspalum notatum* var. saurae, commonly known as Pensacola bahiagrass. These are sexual and cross pollinating due to self-incompatibility (Quarin et al, 2001). Generally referred to as Common bahiagrass, the apomictic tetraploids are the most abundant cytotypes found in South America nears its center of genetic diversity (Burson and Watson, 1995; Pozzobon and Walls, 1997). These tetraploids are obligate apomicts that reproduce through apospory and pseudogamy (Burton, 1948). Apomixis via apospory is when unreduced embryo sacs develop from nucellar somatic cells (Martinez et al.,
Artificially induced tetraploid plants have been produced by doubling the chromosome number of diploid races with colchicine (Burton and Forbes, 1960), an alkaloid derived from *Colchicum autumnale* (Janick et al., 1969b). Natural or induced tetraploid plants may usually be distinguished from diploid by plants by their larger, thicker leaves and organs. Bahiagrass is not an exception to this rule. Broad leaves, strong roots and stout rhizomes characterize common tetraploid bahiagrass. In contrast, the diploid Pensacola type is taller, with longer and narrower leaves and smaller spikelets.

**Agronomic Attributes**

Bahiagrass is a creeping perennial, relatively easy and inexpensive to establish from seeds, sod, springs, or plugs. Its abundant seed production makes it easy and inexpensive to propagate (Trenholm et al., 2003). Bahiagrass also has a large and extensive root system (Blue and Graetz, 1977; Impithuksa and Blue, 1978). Its roots can penetrate through the compaction zone (Elkins et al., 1977) which aids it under drought conditions. Bahiagrass’ root system also reduces the impact of nematode damage (Trenholm et al., 2003). It has the highest level of drought survival of any sod-forming turfgrass attributed to better recovery and deeper rooting (Busey, 2003).

Bahiagrass is persistent under a variety of soils and conditions including low fertility, drought and flooding. It grows on upland well-drained sands, as well as on moist, poorly drained flatwood soils, typical of peninsular Florida (Chambliss and Adjei, 2002). It displays good resistance to most diseases and pests. Bahiagrass tolerates intense clipping or over-grazing (Sampaio and Beaty, 1976).

Day-length plays an important role in the growth of bahiagrass (Blount et al., 2001). It was previously demonstrated that forage yield in short-day months is restricted by photoperiod in bahiagrass (Sinclair et al., 2001; Sinclair et al., 2003). An overall forage yield increase was observed during short-day months when bahiagrass plots were artificially treated with 15-h
photoperiods (Sinclair et al., 2001; Sinclair et al., 2003). Bahiagrass is also strongly photoperiod dependent for flowering induction (Knight and Bennett, 1953). With a flowering threshold of 13-h daylength, it can flower in 14-h but not 12-h days. Flowering under short-days was initiated whenever the night period was interrupted by red or far-red light (Marousky and Blondon, 1995).

Targets for improvement of bahiagrass include its turf quality, shade tolerance, susceptibility to high soil pH, salinity, mole crickets (Scapteriscus spp), and dollar spot (Sclerotinia homoeocarpa). Also, bahiagrass does not tolerate freezes and ceases to grow in late fall and winter period (Blount et al., 2001).

**cv ‘Argentine’**

‘Argentine’ is a selection that was introduced from Argentina in 1944 (Chambliss and Adjei, 2002). In contrast to ‘Pensacola’, this apomictic tetraploid (2n=4x=40), has wider leaf blades, produces fewer inflorescences that emerge later in the season (Trenholm et al., 2003) and has a lower cold tolerance (Chambliss and Adjei, 2002). It is widely used in lawns and for solid sodding on highways, roadsides and large residential areas. It is considered to be better for turf than other cultivars since it produces fewer inflorescences (Trenholm et al., 2003) and has a darker green color. ‘Argentine’ also has a higher root dry weight than ‘Pensacola’ (Busey, 1992).

**Strategies for Improvement of the Turf Quality of Bahiagrass**

**Traditional Breeding of Bahiagrass**

Bahiagrass breeding is a multi-state effort between University of Florida faculty and USDA scientists from Georgia and Florida (Blount et al., 2003). Traditional breeding for bahiagrass, based on recurrent selection, has primarily focused on improved forage yield by improving establishment, seedling vigor, cold tolerance, reduced photoperiod sensitivity, seasonal distribution of forage production, forage quality, and insect and nematode and disease resistance. ‘Recurrent Restricted Phenotypic Selection’ in diploid cytotypes for improved forage
yield resulted in plants with more upright growth and reduced rhizome development (Blount et al., 2001). Although traditional breeding has proven successful in breeding sexual diploid types, breeding of tetraploid apomictic cultivars has been difficult.

Breeding of sexual diploid cultivars is complicated by self incompatibility and the abundance of cross-pollinating pollen sources. Therefore such cultivars are typically lacking the uniformity desired for turf applications. In contrast, an apomictic cultivar, reproducing asexually, produces uniform seed progeny that is a tremendous advantage if uniform turf quality needs to be achieved. However, improvement of apomictic cultivars is limited due to the lack of genetic recombination during asexual seed production (Blount et al., 2001). Applying transgenic approaches may help to overcome these limitations.

**Genetic Transformation of Turf and Forage Grasses**

Transgenic strategies offer opportunities to introduce novel genes into plants, thus offering new opportunities for molecular breeding of grass (Poaceae) species. It allows the introduction of heritable traits between unrelated species thus amplifying the genetic resources and variability beyond the possibilities within traditional breeding. The most popular methods used to generate transgenic plants are *Agrobacterium*-mediated and microprojectile bombardment genetic transformation. *Agrobacterium tumefaciens* is a phytopathogenenic bacteria that contains a Tumor-inducing (Ti) plasmid that contains a transfer (T) DNA that can insert into the chromosome of cells at the wound site on the root of the plant and cause crown gall disease. In *Agrobacterium*-mediated transformation, the bacterium’s T-DNA is replaced with the desired gene to be introduced into the plant’s genome. DNA within the T-DNA will be transferred to the plant and integrated into the plant nuclear DNA. Using recombinant DNA methods, the tumor-causing genes are deleted from the T-DNA. Until recently, *Agrobacterium*-mediated transformation was thought to be limited to dicotyledons since most monocot species are not
natural hosts for the bacterium. However, Hiei et al. in 1994 described efficient transformation of rice by Agrobacterium. Following, there have been reports for numerous monocot species including commercially important crops like maize, barley and wheat (Ishida et al., 1996; Tingay et al., 1997; Cheng et al., 1997).

Currently, particle bombardment is the most widely used and successful method for introducing genes into monocotyledonous plants (James, 2003; Altpeter et al., 2005). Microprojectile bombardment or biolistic is the direct gene transfer with DNA-coated particles into embryogenic cells. This is achieved with a so called “gene gun” or "Biolistic Particle Delivery System" invented by Dr. Jonhn C. Sanford. Plasmid DNA carrying promoter sequence, selectable marker and/or reporter genes in addition to the desired gene(s) is precipitated onto micron-sized tungsten or gold microcarrier particles. DNA-coated microcarriers are accelerated with helium pressure into the nucleus within the cells where the DNA may be integrated into the plant’s genome. Due to the physical nature of this system, microparticle bombardment is not limited by the pathogen-host interaction observed in Agrobacterium-mediated transformation. Transgene integration through either method occurs through illegitimate recombination (Kohli et al.1999; Zhang et al. 2008; Sandhu et al., 2008). Following, transformed tissues are selected for the expression of the selectable marker gene and transgenic plants are regenerated and evaluated for integration and expression of the transgene(s).

It is very important for the transgenic plants generated to stably express the transgene over successive generations. Major problems associated with the productions of stable transgenic plants include gene expression variability and silencing (De Wilde et al., 2000; Kohli et al., 2003; Chawla et al., 2006). Variation in expression levels is influenced by a multitude of factors including transgene copy number, positional effects, transgene structure, and gene silencing. The
two mechanisms recognized in gene silencing of transgenics are transcriptional gene silencing (TGS) and post-transcriptional gene silencing (PTGS) (Vaucheret et al., 1998; Lechtenberg et al., 2003; Tang et al, 2007). Both mechanisms of silencing have been associated with multiple inserts of the transgene (Tang et al, 2007). Gene copy number rarely correlates to level of expression (Spencer et al., 1990; Pawlowski and Somers, 1996, Maqbool and Christou, 1999). In transformation the integration of only one transgene copy is believed to be desirable to avoid the possibility of co-suppression of expression caused by multiple gene integrations (Matzke et al., 1994; Gondo et al., 2009). Single copy integration of transgenes has been shown in transgenic grasses obtained by microprojectile bombardment, but the number of transgene copies integrated usually varies resulting in complex transgene integration patterns (Spangenberg et al., 1995a, b; Ye et al., 1997; Dalton et al., 1999; Richards et al., 2001; Wang et al., 2001, 2003b; Wang and Ge, 2006). It is known that Agrobacterium-mediated transformation generally results in a lower copy number and an improved stability of gene expression than bombardment methods. However, complex transgene integration patterns have also been reported in Agrobacterium-mediated transformation (Kononov et al. 1997; Zhang et al. 2008; Sandhu and Altpeter, 2008). It has been reported that transgenic perennial ryegrass lines with five or more transgene copies exhibited gene silencing after sexual and vegetative reproduction but that an estimated 50% of these high-copy-number lines still stably expressed the transgene (Altpeter et al. 2004; Altpeter et al., 2005). Positional effects, such as integration site have also been proposed to influence transgene expression (Kohli et al., 1999). Also transgene structure influenced expression. Rearranged copies of the transgene may result in silencing if other copies are intact and functional (Kohli et al., 1999; Altpeter et al., 2005). These rearrangements can take place before or during integration into the host genome (Altpeter et al., 2005).
Transgenic Turf and Forage Grasses

Particle bombardment has been used for many years in the transformation of many graminaceous species including tall fescue (*Festuca arundinacea* Schreb.) (Wang et al., 1992), creeping bentgrass (*Agrostis palustris* Huds.) (Zhong et al., 1993), red fescue (*Festuca rubra* L.) (Spangenberg et al., 1995a), perennial ryegrass (*Lolium perenne* L.) (Spangenberg et al., 1995b), Italian ryegrass (*Lolium multiflorum*) (Ye et al., 1997), orchardgrass (*Dactylis glomerata* L) (Denchev et al., 1997), wimmera ryegrass (*Lolium rigidum*) (Bhalla et al., 1999), Kentucky bluegrass (*Poa pratensis* L.) (Ha et al., 2001), switchgrass (*Panicum virgatim*) (Richards et al., 2001), bahiagrass (*Paspalum notatum* Flügge) (Smith et al., 2002), blue grama grass (*Bouteloua gracilis*) (Aguado-Santacruz et al., 2002), bermudagrass (*Cynodon* spp) (Zhang et al., 2003), *Dichanthium annulatum* (Dalton et al., 2003), Russian wildrye (*Psathyrostachys juncea*) (Wang et al., 2004), and buffalograss (*Buchloe dactyloides*) (Fei et al., 2005).

More recently, *Agrobacterium*-mediated transformation has been successfully used in grasses such as creeping bentgrass (Yu et al., 2000), switchgrass (Somleva et al., 2002), Italian ryegrass (Bettany et al., 2003), tall fescue (Bettany et al., 2003), zoysiagrass (Toyama et al., 2003), colonial bentgrass (Chai et al., 2004), bermudagrass (Hu et al., 2005), and perennial ryegrass (Wu et al., 2005).

Transgenic Bahiagrass

Biolistic transformation of bahiagrass has been reported for the T7 genotype (Smith et al, 2002), and cultivars ‘Argentine’ (Altpeter and James, 2005; Altpeter and Positano, 2005) and ‘Pensacola’ (Gondo et al., 2005; Luciani et al., 2007). This technology has allowed the introduction of transgenes that successfully improved turf quality (Agharkar et al., 2007; Zhang et al., 2007), abiotic stress tolerance (James et al., 2008), insect (Luciani et al., 2007) and herbicide resistance (Sandhu et al., 2007). In this study, two different approaches to alter the
phenotype and improve the turf quality of bahiagrass were evaluated. The first involved over-expression of a gene coding an enzyme involved in gibberellin metabolism. Plant hormones control plant growth and development by affecting the division, elongation, and differentiation of cells. Thus, a change in their level can cause a multitude of effects. Hormones therefore provide a tool for manipulating plant processes and their phenotype. The second involved the manipulation of a regulatory gene. Transcription factors are regulatory proteins that control the expression of specific genes (Broun, 2004) with some of them regulating plant development and response to environmental conditions (Zhang, 2003). Since transcription factors tend to control a multitude of genes, they are powerful tools for the manipulation of metabolic pathways in plants (Broun, 2004). When transcription factors are over-expressed, changes such as altered plant architecture and improved stress tolerance have been reported (Zhang, 2003).

**Transgenic Strategies for Improvement of Bahiagrass Turf Quality**

*AtGA2ox1*

Gibberellins (GAs) are plant growth hormones that control various developmental processes including stem elongation, leaf expansion, seed germination, floral induction, fruit development, and apical dominance (Harberd et al, 1998). They mediate these physiological responses in response to environmental signals such as photoperiod and light (Hedden and Phillips, 2000). Several studies have made the association between GAs and flowering in monocotyledons (Evans, 1964; Pharis and King, 1985; King and Evans 2003; King et al., 2001, 2006; MacMillan et al., 2005; King et al., 2008; Ubeda-Tomás et al., 2006; Gallego-Giraldo et al., 2007). In ryegrass GA has been suggested as a leaf-derived long distance signaling molecule for floral transition (King et al, 2006; Colassanti and Coneva, 2009). Bioactive GAs have been proposed to suppress tiller bud outgrowth, enhance apical dominance and promote flowering in
Grasses under long day (LD) (Lester et al., 1972; Johnston and Jeffcoat, 1977; Agharkar et al., 2007).

Gibberellin-2-oxidases (GA2ox) are a family of enzymes involved in the degradation of bioactive GAs rendering them inactive. They are 2-oxoglutarate-dependent dioxygenases (2ODDs), which hydrolyze the C-2 of active GAs (Martin et al, 1999; Thomas et al, 1999; Sakamoto et al., 2001).

*ATHB16*

*ATHB16* (Arabidopsis thaliana Homeobox 16) is an HDZip gene involved in the control of cell expansion (Wang, et al., 2003a). Members of the HDZip family of plant transcription factors encoded by HDZip genes are characterized by the presence of a DNA binding homeodomain and an adjacent Leu zipper motif from which their name originates. They are involved in the plant’s developmental processes (Henriksson et al., 2005). The transcription factor encoded by *ATHB16* functions as a repressor of cell elongation independent of the GA signal transduction (Wang et al., 2003a). In Arabidopsis, transgenic over-expression of *ATHB16* resulted in plants with reduced stem length due to reduced leaf expansion, increased number of shoots and reduced sensitivity of flower induction to photoperiod (Wang et al., 2003a). The exact mechanism for the altered phenotype is unknown. However, Wang et al. (2003a) suggested that ATHB16 transcription factor might regulate plant development as a mediator of blue-light based photomorphogenesis.
CHAPTER 3
MATERIALS AND METHODS

Transgenic Lines and Experimental Controls

Transgenic lines selected for the field studies were generated and analyzed for transgene integration and expression (\textit{AtGA2ox1} or \textit{ATHB16}) and evaluated under greenhouse conditions as described by Agharkar et al. (2007) and Zhang et al. (2007). GA catabolizing \textit{AtGA2ox1} was subcloned under the control of the maize \textit{ubiquitin} promoter and Nos 3'UTR. Whereas \textit{ATHB16} was subcloned under the control of the CaMV 35S promoter and Nos 3'UTR. Minimal \textit{AtGA2ox1} or \textit{ATHB16} expression cassettes lacking vector backbone sequences were stably introduced into apomictic bahiagrass by biolistic gene transfer.

Two non-transgenic controls were included in our study, wild-type ‘Argentine’ bahiagrass (WT) and wild-type St. Augustinegrass cultivar ‘Floratam’ (SA) as the turf industry standard. Transgenic \textit{AtGA2ox1} and \textit{ATHB16} lines along with wild-type controls were established and evaluated simultaneously in field study I (2006) and field II (2007, 2008 and 2009).

Field Evaluation

Field Study I

Propagation, Establishment and Field Site

Field study I was conducted in 2006. Plants from five transgenic lines expressing \textit{AtGA2ox1} (B3, B6, B7, B9 and B11) and three transgenic lines expressing \textit{ATHB16} (I4, I10 and I32) were propagated under greenhouse conditions along with experimental controls from single rooted tillers in steam-sterilized soil from the field site (Figure. 3-1A). Transgenic and control plants were established on 11 July 2006 at the G.C. Horn Turfgrass Research Facility, located at the UF-IFAS Plant Research and Education Center (PSREU) in Citra, Florida (USDA permit 05-364-01r) in 1 x 1 m$^2$ plots using four 8x8x7cm$^3$ grass plugs (representative images shown in
Figure 3-1B; Figure 3-1C). The experimental design was a randomized block design with a total of 24 replications. The soil near Citra, FL is hyperthermic, uncoated Quartzipsamments of the Candler series (Thomas et al., 1979). Summer solstice at the field site occurred on June 21, 2006 with a day length of 14-h.

**Fertility and Management**

All plots were treated equally and irrigated after transplanting to prevent severe moisture stress on the young transplants. After transplanting plants were allowed to grow without being mowed for four weeks. Plants were mowed the second time on 3 October 2006, when a weekly mowing regime was established. Plants were fertilized with 0.23 kg of N 100 m\(^{-2}\) using a complete 18-3-18 NPK fertilizer on 26 July 2006. Prowl (Pendimethaline) herbicide was applied as a pre-emergent to all plots at 7.4 x10\(^{-3}\) kg 100 m\(^{-2}\) on 1 August 2006.

**Evaluation Techniques**

Turf quality was evaluated by recording density, mowing quality, aboveground biomass, establishment and development of inflorescences. Turf density of transgenic plants was evaluated by counting the number of tillers in a randomly selected 10 × 10 cm\(^2\) (100 cm\(^2\)) area of each plot and with visual ratings. To evaluate aboveground biomass, plots from different environments were mowed at 8 cm using a Rotary Mower HRX217TDA (American Honda Motor Co., Inc. Alpharetta, GA) equipped with a blade brake clutch that allows removing the bag to collect clippings while the engine is running. Clippings were harvested from each plot and their dry weights were measured after a week at 80\(^{\circ}\)C. Development of inflorescences was monitored by counting total number of inflorescences per plot. Length of the inflorescence stems was also evaluated. Turf quality was evaluated using visual ratings for mowing quality, density and establishment. Visual ratings were assigned following National Turfgrass Evaluation Program (NTEP) guidelines (Morris and Shearman, 2006). Visual ratings were based on a 1-9
rating scale, 1 representing the poorest and 9 the best or highest overall rating of the parameter being evaluated within the study.

**Statistical Analysis**

Statistical analysis was performed according to the randomization structure using the ANOVA-procedure of SAS version 9.2 (SAS Institute Inc. Cary, North Carolina, USA). Means were compared by the t-test ($\alpha = 0.05$).

**Field Study II**

**Propagation, Establishment and Field Site**

Field study II was conducted from 2007 to 2009. Plants from four transgenic lines expressing *AtGA2ox1* (B3, B7, B8 and B10) and four transgenic lines expressing *ATHB16* (I12, I23, I28 and I32) were propagated under greenhouse conditions along with experimental wild-type controls from single rooted tillers in steam-sterilized soil from the field site (a representative image is shown in Figure. 3-1A). Transgenic and experimental control plants were established on 10 July 2007 at the G.C. Horn Turfgrass Research Facility, located at the UF-IFAS Plant Research and Education Center in Citra, Florida, USA (USDA permit 06-219-01r) in 1 x 1 m$^2$ plots using four 8x8x7 cm$^3$ grass plugs (Figure 3-1B; Figure 3-1C). Summer solstice at the field site for the corresponding evaluation years occurred at 21 June 2007 and 20 June 2008 both with a day length of 14-h. Field experiment treatments were arranged as split-split plots in a randomized complete block with four replications. Six main plots were assigned one of three irrigation treatments (full irrigation, moderate irrigation or non-irrigated). Main plots were split in half and one of two mowing frequencies (weekly or biweekly) was assigned randomly to each split-plot. Two replications from each genotype (transgenic lines and wild-type controls) were randomly sited within each split-plot (Figure 3-2).
Fertility and Management

All plants were subjected to the same fertility and management aside from the irrigation and mowing treatment differences. Plants were fertilized with a 15-5-15 NPK fertilizer with iron at a rate of 0.25 kg m\(^{-2}\) on 10 August 2007, with a 7-7-7 NPK fertilizer at 0.5 kg m\(^{-2}\) on 24 October 2007, with a 18-3-18 NPK fertilizer at 0.5 kg m\(^{-2}\) on 6 March 2008 and with a 15-15-15 NPK fertilizer at 0.5 kg m\(^{-2}\) on 4 August 2008. Soar micronutrient mix was applied on 6 June 2008 at a rate of 4.7 L ha\(^{-1}\). To reduce weed pressure, Prowl (Pendimethaline) herbicide was applied as pre-emergent to all plots at rate of 1.5 L ha\(^{-1}\), on 7 August 2007, on 2 October 2007 at 3.5 L ha\(^{-1}\), on 28 May 2008 at 4.7 L ha\(^{-1}\) and on 17 February 2009 at 4.7 L ha\(^{-1}\). Prodiamine herbicide was also applied as a pre-emergent on 3 March 2008 at a rate of 1.1 kg - ha\(^{-1}\), on 30 July 2008 at 1.7 kg ha\(^{-1}\) and on 21 October 2008 at 1.7 kg ha\(^{-1}\). Halosulfuron herbicide was applied to control sedge on 7 August 2007 at 92 g ha\(^{-1}\), 22 August 2007 at rate of 92 g ha\(^{-1}\), on 27 September 2007 at 71 g ha\(^{-1}\), on 14 May 2008 at 71 g ha\(^{-1}\) and on 28 August 2008 at 92 g ha\(^{-1}\). Acephate insecticide was applied to control insects on 28 August 2007 at 1.8 kg A\(^{-1}\), on 16 November 2007 at 4.5 kg ha\(^{-1}\) and on 4 June 2008 at 3.4 kg ha\(^{-1}\). Chlorothalonil fungicide was applied on 4 June 2008 at rate of 23.7 L ha\(^{-1}\) to control infection on the St. Augustinegrass control plants.

Mowing and Irrigation Treatments

During the establishment period, all transgenic and wild-type control plants were grown under the same conditions and management. All equal irrigation to prevent moisture stress on the young transplants. The plots were mowed for the first time 4 weeks after transplanting. From then onwards, two alternative mowing schedules were compared (weekly and biweekly).

Irrigation treatments were initiated at the end of March 2008 with the onset of a seasonal drought period experienced in April and May 2008 (Figure 3-3). Irrigation was controlled using
soil moisture sensors coupled to a Campbell Scientific data logger controlling solenoid valves to mini-wobblers sprinkler system. Full irrigation plots were irrigated to prevent visual moisture stress in wild-type St. Augustine grass. The system was calibrated to override scheduled irrigation once specific volumetric water content (VWC) was detected. Fully irrigated plots, received approximately 1.25 cm of water twice a week (Figure 3-3). If approximately 1.25 cm of rainfall or more were received just prior to the scheduled irrigation, the VWC would trigger the system to overwrite the scheduled irrigation. Moderate irrigation plots were irrigated to prevent visual moisture stress in the best transgenic lines. For moderately irrigated plots the system was set to approximate 1.25 cm of water once a week (Figure 3-3). Non-irrigated plots received no additional water to that provided by rainfall (Figure 3-3). Irrigation events were scheduled in the early mornings to minimize negative impact of wind and high temperatures on irrigation success.

**Evaluation Techniques**

Turf quality was evaluated by assessing turf density, establishment, spring green-up, weed encroachment, drought tolerance and recovery, above- and below-ground biomass, development of inflorescences, and length of inflorescence stems. Turf density of transgenic plants was evaluated by counting the number of tillers in a randomly selected $10 \times 10$ cm$^2$ (100 cm$^2$) area of each plot and with visual ratings. Density, establishment, spring green-up, weed encroachment, and drought tolerance and recovery were evaluated using visual ratings. Visual ratings were assigned following National Turfgrass Evaluation Program (NTEP) guidelines (Morris and Shearmen, 2006). Visual ratings were based on a 1-9 rating scale, 1 representing the poorest and 9 the best overall quality of the parameter being evaluated within the study.

To evaluate aboveground biomass all environments were mowed at 8-cm cutting height production using a Rotary Mower HRX217TDA (American Honda Motor Co., Inc. Alpharetta, GA) equipped with a blade brake clutch that allows removing the bag to collect clippings while
the engine is running. Clippings were harvested from individual plots and the dry weight of the clippings was assessed after a week at 80°C. To compare biomass production from the different mowing treatments the dry weight data was analyzed as the total dry weight of clippings during a two-week period. Weekly plots were harvested once a week for two consecutive weeks and the sum of the dry weight for those two weeks was the value used. Biweekly plots were allowed to grow for these same two weeks but only harvested during the second week.

Below-ground biomass was evaluated by recording root and rhizome dry weight. Rhizomes and roots samples were collected using a 10-cm wide, 5-cm across, and 50-cm long plant root sampler (Eijkelkamp’s 0508). Samples were taken from each individual field plot, soil was washed off and roots and rhizomes separated into different bags. Dry weight was assessed after 3 days at 80°C. In the case of St. Augustinegrass, rhizome dry weight was assessed instead of rhizome.

Development of inflorescences was monitored throughout the flowering season by counting total number of inflorescences per field plot on a regular basis. Inflorescence length was measured without the racemes, which were cut regularly to prevent pollen formation.

Besides using visual ratings to evaluate drought tolerance and recovery, SPAD readings and maximum quantum yield of photosystem II were measured during and after the drought event. The maximum quantum yield was measured using dark-adapted leaves. Measurements were made at night using Pulse Amplitude Modulated Fluorometry (PAM 2100, Heinz Walz GmbH, Germany). The measured photosynthetically active quantum flux density (PAR) was maintained between 5 and 8 µmol quanta m⁻²s⁻¹ during the measurements. Standard instrument settings were used and the measurements were made using the saturation pulse mode. Maximum quantum yield (Fv/Fm) data were obtained by using the pre-programmed ‘run 2’ mode. The third
fully expanded leaf from two different tillers per plot was used for the measurements. SPAD measurements were made on the third fully expanded leaf from three different tillers per plot using a SPAD meter (Minolta SPAD 502 Meter, Spectrum Technologies, Inc., East-Plainfield, Illinois, USA).

**Statistical Analysis**

Statistical analysis was performed according to the randomization structure using the restricted maximum likelihood (REML) method with the MIXED-procedure of SAS version 9.2 (SAS Institute Inc. Cary, North Carolina, U.S.A.). Genotypes, irrigation and mowing were considered fixed, while replicates and interactions were considered random. When analyzing the data no significant difference was observed between mowing treatments so data presented was analyzed by removing mowing from the model. Means were compared by the t-test ($\alpha = 0.05$).

**Regulatory Compliance**

In order to ensure the containment of the transgenic material, a strict protocol was followed to fulfill the field trial requirements under the USDA permits 05-364-01r and 06-219-01r. The field, laboratory and greenhouse sites used for this study were regularly inspected by USDA-APHIS and the institutional biosafety committee. All equipment used in the study, including the mower, was kept on-site in a shed. All transgenic plant material was transported in double containers in a well-contained state vehicle (Figure 3-4). Clippings along with any plant material were autoclaved prior to discarding. Bahiagrass plants in the field plots were not allowed to produce pollen, by scheduled mowing and regular hand cutting of the inflorescence racemes. A 10 m fallow area surrounded the plots. Diploid bahiagrass growing around our field was also controlled with plant growth retardants and weekly mowing. Upon termination of the field study, plants will be killed with herbicide application and the area monitored.
Evaluation of Stable Transgene Expression of Field Grown Vegetative Progeny

Transgene expression under field conditions was confirmed by RT-PCR results. After approximately 20 months in the field, including periods of drought and freezing, 100 mg of young leaf tissue was used to extract total RNA using the RNeasy® Plant Mini Kit (Qiagen Inc., Valencia, CA) followed by RNase-free DNase I (Qiagen Inc., Valencia, CA) treatment to eliminate genomic DNA contamination. For cDNA synthesis via reverse transcription, 500 ng of total RNA was used with the iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories, Inc., Hercules, CA) in a reaction volume of 20 μl. In detecting the *AtGA2ox1* or *ATHB16* gene transcripts by PCR, 2 μl of cDNA synthesized and 1 μl of the plasmid were used as template for PCR in an Eppendorf Mastercycler (Eppendorf, Westbury, NY, USA) using the HotStarTaq® DNA Polymerase (Qiagen Inc., Valencia, CA). For the *ATGA20x1* lines the primer pair with sense pair with sense 5'- GAACACGAGACCGTCGATTT -3’ and antisense 5’- GGAGGGACAGAGATCCATGA -3’ was designed to amplify a 516-bp fragment for RT-PCR. For the *ATHB16* lines the primer pair with sense pair with sense 5'- TGGGTCTATCGGAGAAGAAG -3’ and antisense 5’- TTGGAGAAGGGAATCATTGT -3’ was designed to amplify a 278-bp fragment for RT-PCR. Samples were denatured at 95° C for 15 min; followed by 30 cycles at 95°C for 30 s, 60°C for 30 s, 72°C for 1 min, and final extension at 72° C for 10 min. PCR products were analyzed by electrophoresis in a 1.2% agarose gel at 100V for 30 minutes.
Figure 3-1. Propagation and field establishment. (A) Propagation of plants under greenhouse conditions from single rooted tillers. (B) Establishment of field plots using four 8x8x7 cm$^3$ grass plugs. (C) Fully-established 1 x 1 m$^2$ plots.
Figure 3-2. Field study layout. (A) Irrigation and mowing treatment assignment to split-split plots. (B) Overview of the six experimental plots at the UF-IFAS Plant Research and Education Center in Citra, Florida.
Figure 3-3. Total monthly rainfall and irrigation received by ‘full’, ‘moderate’ and ‘no’ irrigation plots during seasonal drought in (A) 2008 and (B) 2009.

Figure 3-4. State vehicle loaded with transgenic plant material enclosed in double container.
RESULTS AND DISCUSSION

\textit{ATGA2ox1} Expressing Lines (B Lines)

Results

Field Study I

\textit{ATGA2ox1} expressing lines B3 and B9 consistently displayed the highest turf density with the most number of tillers produced among all transgenic lines and the wild-type. Eight weeks after establishment, B3 and B9 had 23\% and 16\% significantly more tillers than the wild-type bahiagrass (Figure 4-1A). Four weeks later line B11, B3, B7 and B9 had 25\%, 44\%, 22\% and 40\% more tillers than the wild-type, respectively (Figure 4-1B). The higher number of tillers in the transgenic plants resulted in denser turf as represented by our density ratings (Figure 4-1C) and a more erect growth pattern compared to wild-type with more prostrate and open growth habit (Figure 4-1D).

The faster production of tillers by the transgenic lines B3 and B9 also resulted in a better field establishment than the other lines and wild-type bahiagrass. Most of the lines (B3, B7 and B9) that produced more tillers than the wild-type also received higher establishment ratings than the wild-type and St. Augustinegrass (Figure 4-2A). Line B6 also displayed better field establishment than the wild-type. These lines spread faster inside the field plots than the wild-type bahiagrass and St. Augustinegrass (Figure 4-2B).

The higher number of tillers, faster establishment and erect growth of the transgenic lines contributed to 52\% (B3), 34\% (B7), and 62\% (B9) more clippings compared to the wild-type bahiagrass, following four weeks of growth after establishment (Figure 4-3). Consistent with the data on tiller number, density and establishment, lines B9 and B3 had the highest clipping dry...
weight at this timepoint. Line B6, displaying the most dwarf phenotype, produced 44% significantly less clippings than the wild-type bahiagrass.

The denser more upright (bunchtype appearance) phenotype of the transgenic lines also resulted in higher ratings for mowing quality for lines B3, B6, B7 and B9 compared to the wild-type bahiagrass (Figure 4-4A). Consistent with the density ratings, lines B3 and B9 had the highest ratings for mowing quality among all lines and wild-type (Figure 4-4A). This is reflected in the comparison of freshly mowed transgenic line B3 to wild-type bahiagrass (Figure 4-4B). Line B3 displayed a high turf density with no visible rhizomes or gaps in contrast to wild-type (Figure 4-4B).

Lines B3, B7 and B9 consistently produced less inflorescences than the wild-type and the other transgenic lines (Table 4-1). On 8 August the number of inflorescences production by transgenic lines B3, B7 and B9, ranging from 0 to .08, was significantly lower than the production by the wild-type (1.46) (Table 4-1). On 29 August, lines B3, B7 and B9 produced 14%, 22% and 15% less inflorescences than the wild-type bahiagrass, respectively.

The inflorescence stems of most lines was 13% (B11), 16% (B3), 31% (B6), and 11% (B7) shorter than that of the wild-type bahiagrass (Figure 4-5). Inflorescence stem length of line B9 was not significantly different to the wild-type or most transgenic line (B11, B3, B7). Line B6 displayed the shortest inflorescence stems of all lines and wildtype.

Field Study II

In Field study II, conducted in 2007, 2008 and 2009 most of the transgenic lines produced significantly more vegetative tillers than the wild-type bahiagrass. Lines B3 and B7 consistently produced the highest amount of tillers among all lines and the wild-type. During establishment in September 2007, AtGA2ox1 expressing lines B3, B7 and B8 produced 41%, 35% and 27% more vegetative tillers than the wild-type, respectively (Figure 4-6A). Line B10 was the only
transgenic line that did not produce significantly more tillers than the wild-type (Figure 4-6A). In measurements taken in May 2008, transgenic lines again produced 26% (B10), 36% (B3), 38% (B7) and 17% (B8) more tillers than the wild-type bahiagrass (Figure 4-6B). Whereas in September 2008, the transgenic lines produced 21% (B10), 43% (B3), 48% (B7) and 30% (B8) more tillers than the wild-type bahiagrass (Figure 4-6C). In May 2009 the transgenic lines produced 29% (B10), 44% (B3), 47% B7 and 28% (B8) more tillers than the wild-type bahiagrass (Figure 4-6D).

Increased tillering of the transgenic lines in comparison to the wild-type bahiagrass was consistent under all three irrigation treatments in May 2008 (Figure 4-6E) and 2009 (Figure 4-6F). Number of tillers was significantly reduced in non-irrigated plots for all lines and wild-type bahiagrass during the droughts in May 2008 (Figure 4-6E) and 2009 (Figure 4-6F). Bahiagrass transgenic plants and the wild-type produced on average 35% and 27% more tillers under full irrigation than in non-irrigated plots during the drought period in May 2008 (Figure 4-6E) and 2009 (Figure 4-6F), respectively. This was the only timepoint with a significant interaction between irrigation regime and production of tillers.

The higher number of tillers in transgenic lines (B3, B7, B8) resulted in significantly higher turf density as confirmed by our density ratings taken in September 2007 (Figure 4-7A), in May 2008 (Figure 4-7B) and 2009 (Figure 4-7C). Line B3 consistently displayed the greatest density among all lines and wild-type. In all three assessments line B10 was the only transgenic line that did not display greater turf density than the wild-type (Figure 4-7). This is consistent with tiller data for September 2007 (Figure 4-6A). As previously observed in field study I, higher number of tillers for AtGAoxl lines was associated with a denser, more erect growth in the
transgenic lines as compared with the wild-type bahiagrass with a more open growth habit and prostrate growth (Figure 4-8).

As in Field Study I, the faster production of tillers by the transgenic lines was associated with quicker field establishment. All transgenic lines exhibited faster establishment than the wild-type four weeks after transplanting as revealed by our ratings (Figure 4-9A). Eight weeks after transplanting, all transgenic lines (B3, B7, B8), except B10, received higher establishment ratings than the wild-type (Figure 4-9B). This is consistent with line B10 producing the least tillers and exhibiting the lowest density out of all the transgenic lines evaluated in this study. Lines B3 and B7 received the highest ratings among all lines and wild-type. The faster establishment was associated with the transgenic lines increased tillering and therefore spreading and establishing faster than the wild-type within the field plots (Figure 4-9C).

The higher number of tillers, erect growth and faster establishment in the transgenic lines contributed to greater dry weight of clippings in transgenic lines compared to the wild-type bahiagrass plants for the establishment period in 2007 (Figure 4-10A). Transgenic lines that consistently produced more tillers and received higher density and establishment ratings produced 47%, 42%, 37% (B3); 51%, 58%, 50% (B7); 57%, 42%, 40% (B8) significant greater clipping weight than the wild-type for August, September and October, respectively (Figure 4-10A). Line B7 consistently produced the greatest amount of clippings for this season. For these same months, clipping dry weight for B10 did not differ from the wild-type.

Differences in clipping dry weight between transgenics and the wild-type were not as significant in the in the 2008 growing season as they had been in during establishment in 2007. In March 2008 there was no significant difference between transgenic and wild-type bahiagrass for clipping dry weight (Figure 4-10B). In April, line B7 produced significantly more clippings
than the wild-type bahiagrass over all treatments (Figure 4-10B). During the months of May, June, July, and August 2008 transgenic lines B10 and B3 produced fewer clippings than the wild-type bahiagrass over all treatments (Figure 4-10B). They produced 39%, 44%, 21% (B10) and 32%, 30%, 19% (B3) less clippings than the wild-type for the months of May, June and August 2008, respectively (Figure 4-10B). In September 2008, lines B7 and B8 produced 25% and 20% respectively more clippings than the wild-type bahiagrass (Figure 4-10B). In October 2008, line B3 produced 36% more clippings than the wild-type bahiagrass (Figure 4-10B). It is interesting to note that lines B7 consistently produces more clippings than the wildtype early (April) and late (September and October) in the 2008 growing season (Figure 4-10B). Line B3 also produces more clippings than the wild-type late (October) in the 2008 growing season (Figure 4-10B).

In dry weight of clippings data for March 2009, only line B7 was significantly different than the wild-type; producing 32% more clippings Figure 4-10C). This is consistent with the greater production of clippings observed by B7 early in the 2008 growing season. No significant differences were observed for clipping weight between the transgenic lines and the wild-type bahiagrass in the April 2009 data (Figure 410C). Line B10 produced less clippings than lines B7 and B8 in both April and May 2009 (Figure 4010C). Moreover, there was no significant interaction between any of our treatments and clipping weight in the 2009 data.

Weed encroachment ratings are based visual estimate of the amount of weeds per plot. These were taken in March when the highest incidence of weed emergence was observed. Ratings were assigned using a 1 to 9 scale, with 9 being no weeds and 1 being all covered in weeds. In ratings taken in March 2008 transgenic lines B3, B7, B8 that produced more tillers and had a more dense habit, also had significantly fewer weeds than the wild-type (Figure 4-11A). In
2009, significant differences between transgenic lines and wild-type bahiagrass were only observed for full irrigation plots with all transgenic lines having fewer weeds than the wild-type (Figure 4-11B).

Spring green-up ratings were also used to evaluate performance. Green-up is a measure of the transition from winter dormancy to active spring growth. It is based on plot color not genetic color. The visual rating of spring green-up is based on a 1 to 9 rating scale with 1 equaling straw brown and 9 equaling dark green. Winter temperatures in 2008 were not severe enough to turn all leaf tissue brown (Figure 4-12A). By March 2008 lines B3, B7 and B8 had recovered their whole plot color faster as observed in the higher visual ratings compared to the wild-type (Figure 4-12B). Line B7 received the highest ratings in 2008, consistent with the higher clipping dry weight observed early in the growing season that year. In 2009 more extreme temperatures were experienced (Figure 4-12A) and by January 2009 all plots were completely brown. Green-up ratings in March 2009 identified transgenic line B8 as recovering faster from winter dormancy (Figure 4-12C). There was a significant interaction between irrigation regime and spring green-up ratings for 2009. As expected plants that had received full irrigation the previous season hence less stress had higher green-up ratings.

In 2008 a seasonal drought period was experienced for the months of April and May (Figure 3-3A). Drought tolerance ratings were based on leaf firing, wilting, and whole plot color. In April at the beginning of the drought, transgenic lines B3 and B7 received higher drought tolerance ratings than the wild-type under moderate and non-irrigated respectively (Figure 4-13A). The same two lines produced 64% (B3) and 70% (B7) significant higher clipping weight than the wild-type under moderate irrigation for April (Figure 4-13B). The improved biomass production and higher visual ratings of these two lines compared to the wild-type may reflect a
better tolerance to drought than the wild-type. Figure 4-13C displays differences observed in April 2008 between line B7, wild-type bahiagrass and St. Augustinegrass in non-irrigated plots. Visual ratings for drought tolerance were repeated in May 2008. Under moderate irrigation all lines displayed better tolerance to drought than the wild-type while only B7 had better tolerance in non-irrigated plots as revealed by our ratings (Figure 4-14A). However, no significant difference was observed in regards to dry weight of clippings between the transgenic lines and the wild-type in the month of May (Figure 4-14B). Results for drought ratings are reflected in the comparison of line B7, wild-type bahiagrass and St. Augustinegrass in May 2008 in non-irrigated plots (Figure 4-14C). The interaction was also significant between irrigation regime and dry weight of clippings for the same two months where drought was experienced. However, no individual lines displayed a significant difference in dry weight between moderate and non-irrigated for the month of April (Figure 4-13B). In May, most lines and wild-type bahiagrass with the exception of line B10 and St. Augustinegrass produced significantly less clippings in non-irrigated plots than in moderate irrigation in May (Figure 4-14B). St. Augustinegrass received the lowest visual ratings both in April and May (Figure 4-13A;B). It also produced significantly less clippings under moderate and non-irrigated plots in April 2008 and under moderate irrigation in May 2008 (Figure 4-14B).

In June, as the plants were recovering from the drought, all transgenic lines received significantly higher ratings than the wild-type under moderate irrigation (Figure 4-15A). Lines B3, B7 and B8 continue to have higher ratings than the wild-type in non-irrigated plots (Figure 4-15A). Significant interaction was found between irrigation regime and drought recovery ratings taken in June. All lines and wild-type bahiagrass and St. Augustinegrass obtained higher recovery ratings under moderate irrigation as compared to non-irrigated (Figure 4-15A). No
significant difference was observed in regards to dry weight of clippings between most transgenics and the wild-type (Figure 4-15B). Transgenic line B10 produced lower dry weight of clippings than the wild-type under moderate and non-irrigated (Figure 4-15B). Being that this line had good visual ratings the difference in dry weight of clippings could be associated more with phenotypic difference. Line B10 displayed the most dwarfing and the least tillering between the transgenic lines. No significant interaction was found between irrigation regime and dry weight of clippings for the month of June. Line B8 was the only line to produce fewer clippings in non-irrigated plots as compared to moderate irrigation (Figure 4-15B). St. Augustinegrass received the lowest visual ratings for recovery under moderate and non-irrigated plots Figure 4-15A). It also produced the least amount of clippings at this time (Figure 4-15B). Figure 4-14C and Figure 4-15D displays differences observed between transgenic lines and wild-type bahiagrass and St. Augustinegrass during recovery in June 2008.

In 2009 a seasonal drought period was experienced for the months of March and April (Figure 3-3B). In contrast to 2008, the drought was experienced earlier at which point the plants were still in the process of greening-up from winter dormancy. In drought ratings taken in April 2009, lines B10, B3 and B8 displayed better tolerance to drought than the wild-type under moderate irrigation (Figure 4-16A). St. Augustinegrass displayed the least tolerance to drought (Figure 4-16A). However, no significant difference was observed in regards to dry weight of clippings between the transgenic lines, the wild-type or St. Augustinegrass in April 2009 (Figure 4-16B). In non-irrigated plots, line B8 produced significantly more clippings than B10 and B3 but not than the wild-type. There was no significant difference in clipping weight between irrigation frequency within any transgenic line or wild-type bahiagrass.
Performance during drought and recovery may suggest that some of the transgenic lines have a better tolerance to lower irrigation than the wild-type and that they recover faster from drought. However, maximum quantum yield of photosystem II using dark-adapted leaves and SPAD measurements taken during the drought and recovery periods displayed no significant difference. Results for maximum quantum yield of photosystem II using dark-adapted leaves taken during the drought in May 2008 revealed no significant difference between any transgenic lines and the wild-type under full and non-irrigated plots; under moderate irrigation only line B10 was significantly lower than the wild-type (Figure 4-17A). Moreover, no significant difference was observed in any line or wild-type between irrigation treatments for this measurement (Figure 4-17A). However, line B7 displayed significantly better readings than St. Augustinegrass under moderate and non-irrigated plots (Figure 4-17A). In June, during drought recovery, no significant difference was observed between irrigation treatments or between lines and wild-type for maximum quantum yield of photosystem II (Figure 4-17B). In June St. Augustinegrass received lower readings than most lines and the wild-type bahiagrass under moderate irrigation (Figure 4-17B). No significant interaction was observed between irrigation regime and maximum quantum yield of photosystem II for either measurement time point, May or June 2008.

SPAD measurement results for May reveal lines B3 and B7 having lower values than the wild-type under full irrigation (Figure 4-18A). Wild-type bahiagrass had lower SPAD readings than all the transgenic lines in non-irrigated plots (Figure 4-18A). In June, during drought recovery, only line B8 showed lower SPAD readings than the wild-type in non-irrigated plots (Figure 4-18B). Under moderate irrigation, line B10 had higher SPAD readings than the wild-type and the other transgenic lines (Figure 4-18B). There was significant interaction between
SPAD readings and irrigation regime both in May and in June. We obtained higher readings for all lines in non-irrigated plots as compared to full or moderate irrigation in May and in June (Figure 4-18). St. Augustinegrass received the lowest SPAD readings in May under full and moderate irrigation (Figure 4-18A) and in June under moderate and non-irrigated plots (Figure 4-18B). In contrast to visual scores, tiller numbers and clipping weight data, chlorophyll fluorescence and SPAD measurements are point measurements which are subject to great variability within a single plant. Moreover, only two leaves from two separate tillers were measured per plot. Taking more measurements per plot might have had an effect on reducing the variability within these measurements.

Root and rhizome dry weight was also evaluated. In non-irrigated plots, the transgenic lines B10 and B7 produced 64% and 66% respectively significantly higher dry weight of rhizomes than the wild-type (Figure 4-19). No significant difference was observed in root dry weight between the transgenics and the wild-type (Figure 4-20).

We previously evaluated drought stress response of the \textit{AtGA2ox1} lines under greenhouse conditions. This evaluation also revealed, that the change in plant architecture did not compromise the drought tolerance of bahiagrass (Agharkar, 2007).

Flowering in all of the transgenic lines was reduced as compared to the wild-type in 2007 (Figure 4-21A), 2008 (Figure 4-21B) and 2009 (Figure4-21C) during onset of flowering and throughout most of the flowering season of bahiagrass. In 2007 all lines produced fewer inflorescences than the wildtype in July through end of August (Figure 4-21A). Towards the end of the flowering season (October) only line B10 produced more inflorescences than the wild-type both in 2007 (Figure 4-21A). In 2008 lines B3, B7 and B8 consistently produced fewer inflorescences than the wild-type (Figure 4-21B). Line B10 did not display a significant
difference in inflorescence production to the wild-type in June and July (Figure 4-21B). As observed in 2007, line B10 produced more inflorescences than the wild-type and all other transgenic lines at the end of the flowering season in 2008 (Figure 4-21B).

Consistent with measurements taken in 2007 and 2008, early in the 2009 flowering season all lines produced fewer inflorescences than the wild-type.

Despite line B10 producing the most inflorescences at the end of the flowering season, it along all other lines produced significantly less total inflorescences than the wild-type in 2007 (Figure 4-22A), 2008 (Figure 4-22B), and 2009 (Figure 4-22C). In 2007, transgenic lines B10, B3, B7, and B8 produced 16%, 58%, 58%, and 36% less inflorescences than the wild-type bahiagrass respectively (Figure 4-22A). In 2008, transgenic lines produced 14% (B10), 76% (B3), 71% (B7) and 41% (B8) less total inflorescences than the wild-type bahiagrass (Figure 4-22B). As plants in the field started flowering in May 2009, transgenic lines produced 86% (B10), 99% (B3), 99% (B7) and 77% (B8) significantly fewer inflorescences than the wild-type bahiagrass (Figure 4-22C). Production of inflorescences was not significantly affected by mowing frequency; no significant interaction was found between total inflorescences produced and mowing frequency in any year. Significant interaction was found between total number of inflorescences produced in 2008 and irrigation regime. Lines B10, B8 and wild-type bahiagrass produced significantly more inflorescences in 2008 under moderate irrigation compared to full or moderate irrigation. Line B3 produced significantly more inflorescences under moderate irrigation as compared to full irrigation only. Length of inflorescence stems of the transgenic lines was on average 8-cm shorter than the wild-type. Transgenic lines B10, B3, B7 and B8 produced inflorescence stems on average 10-cm, 13-cm, 4-cm and 4-cm shorter than the wild-type respectively (Figure 4-23).
Reverse transcription PCR (RT-PCR) analysis (Figure 4-24) confirmed the presence of the *AtGA2ox1* transcripts in all lines after approximately 20 months of growing in the field in non-irrigated plots.

**Discussion**

We previously reported that over-expression of *AtGA2ox1* in bahiagrass resulted in reduction of bioactive GA levels with production of semi-dwarf plants with reduced stem length, increased number of vegetative tillers, delayed flowering and shorter inflorescences (Agharkar et al., 2007). The results presented here confirm our previous findings and demonstrate that field performance is not compromised and even improved in some of our transgenic lines.

The main characteristic of GA-deficient mutant or transgenic plants described in the literature is reduced height, associated with shorter internodes. GA-deficient mutants exhibiting dwarf or semi-dwarf phenotypes have been identified and described in several plant species including pea, (Brian and Hemming, 1955), maize (Phinney, 1956), Arabidopsis (Koornneef and van der Veen, 1980; Sun et al., 1992). Transgenic plants expressing a GA2-oxidase and exhibiting reduced height have also been described in several species including rice (Sakamoto et al., 2001; Sakamoto et al., 2003), Arabidopsis (Schomburg et al., 2003; Radi et al., 2006), tabacco (Schomburg et al., 2003; Biemelt et al., 2004; Ubeda-Tomás et al., 2006; Gallego-Giraldo et al., 2007), wheat (Hedden and Phillips, 2000; Appleford, 2007), poplar (Busov et al, 2003), *Nicotiana sylvestris* (Lee and Zeevaart, 2005; Kourmpetli et al., 2009), *Poa pratensis* L. (Blume et al., 2008), *Solanum nigrum* (Kourmpetli et al., 2009).

Interestingly, none of the abovementioned reports describe an enhanced number of vegetative tillers we consistently documented in our study with data over three years. Nevertheless, bioactive GAs have been proposed to suppress tiller bud outgrowth and enhance apical dominance in grasses (Lester et al., 1972; Johnston and Jeffcoat, 1977; Agharkar et al.,
Increased tillering was reported following applications of Trinexapac-ethyl, inhibiting synthesis of bioactive GAs (Ervin and Koski, 1998). Increased tillering ability of transgenic lines may be attributed to the reduction of apical dominance due to a reduction in bioactive GAs. Expression of ATH1 in ryegrass resulted in plants with more vegetative tillers and delayed or non-flowering (van der Valk et al., 2004). ATH1, the Homeobox transcription factor from Arabidopsis, is a negative regulator in the light-regulated gibberellins biosynthesis pathway (Quaedvlieg et al., 1995; Garcia-Martinez and Gil, 2001). Tiller number and plant height exhibit a highly negative correlation in several plant species including rice (Yan et al., 1998; Iwata et al., 1995; Li et al., 2003; Ishikawa et al., 2005) and in Arabidopsis and (Beveridge et al., 1996; Booker et al., 2004; Sorefan et al., 2003; Zou et al., 2006). Zou et al. (2006) proposed that dwarfing in GA mutants was a secondary effect to the increased formation of tillers partly due to the reduced apical dominance (Zou et al., 2006; McSteen, 2009).

In sharp contrast, in bahiagrass a more erect growth was observed in transgenic lines over-expressing AtGA2ox1 and displaying increased tillering. Increased tillering of the transgenic lines and more upright growth resulted in higher dry weight of clipping than the wild-type. Gates et al. (1999) speculated that morphological changes accompanying numerous selection cycles for higher yields in ‘Pensacola’ bahiagrass resulted in taller more erect plants with fewer rhizomes (Werner and Burton, 1991; Pedreira and Brown, 1996a,b; Gates et al., 1999). Pedreira and Brown (1996b) suggested that taller more erect growth in selected ‘Pensacola’ bahiagrass resulted in higher percentage of biomass being harvested by mowing rather than actual higher biomass yields. This is consistent with the observations for dry weight of clippings obtained in this study.
Several studies have made the association between GAs and flowering in monocotyledons (Evans, 1964; Pharis and King, 1985; King and Evans 2003; King et al., 2001, 2006; MacMillan et al., 2005; King et al., 2008; Ubeda-Tomás et al., 2006; Gallego-Giraldo et al., 2007). In ryegrass GA has been suggested as a leaf-derived long-distance signaling molecule for floral transition (King et al, 2006; Colassanti and Coneva, 2009). Consistent with our findings, in all years (2006-2009) delayed flowering has been consistently observed in transgenic plants over-expressing a GA2-oxidase (Hedden and Phillips, 2000; Sakamoto et al., 2001; Schomburg et al., 2003). However, ectopic expression of Ga2-oxidase gene resulted in plants with normal flowering (Hedden, 2003; Sakamoto et al., 2003).

Bahiagrass’ popularity is largely owed to its extensive root system, which confers great drought tolerance. Evaluation of AtGA2ox1 transgenics under greenhouse conditions revealed that the change in plant architecture did not compromise the drought tolerance of bahiagrass (Agharkar, 2007). The phenotypical changes observed in our transgenic lines did not compromise root biomass or drought tolerance. Moreover, performance during drought and recovery suggests that some of the transgenic lines have a better tolerance to lower irrigation than the wild-type and that they recover faster from drought. It has previously been reported that plants treated with gibberellin (GA)-biosynthesis inhibitors are usually more tolerant to range of environmental stresses (Rademacher 1997; Vettakkorumakankav et al., 1999; Magome et al., 2004; Sarkar et al, 2004). Vettakkorumakankav et al. (1999) demonstrated that GA levels play a key role in heat stress response in barley and suggested the same is true for other abiotic stresses. Other studies have suggested the involvement of GA in stress response of Arabidopsis (Achard et al., 2006; Magome et al., 2004). Magome et al. (2008) describe how salt reduces bioactive GA contents via an increase in a GA2oxidase transcript levels in Arabidopsis. Under salinity stress
these plants highly express *DWARF AND DELAYEDFLOWERING1 (DDF1)*, encoding an AP2 transcription factor closely related to the CBF/DREB1 family, which binds to the DRE-like motifs present in the *GA2ox7* promoter, which reduced bioactive GA levels (Achard and Genschik, 2009). This reduction in bioactive GA levels resulting from salt stress improved abiotic stress protection (Magome et al., 2004, 2008; Achard et al., 2006; Achard and Genschik, 2009). The dehydration responsive element/C-repeat (DRE/CRT) *cis*-acting element and the AP2 transcription factors DREB/CFB (DRE binding protein/CRT binding factors) play an important role in the ABA-independent stress response pathway (Shinozaki and Yamaguchi-Shinozaki, 2000). The overexpression of their genes improved drought and salt tolerance in different plants (Jaglo-Ottosen et al. 1998; Liu et al. 1998; Kasuga et al. 1999) including some grass species (Pellegrineschi et al., 2004; Oh et al., 2005; James et al., 2008).

Magome et al. (2008) propose that the GA-dependent growth retardation provided by *GA2ox* is an important mechanism for stress adaptation. In contrast to these findings overexpression of *AtGA2ox1* resulted in increased clipping weights and rhizome biomass in bahiagrass along with moderately improved drought, suggesting that growth retardation may not be the main mechanism supporting drought tolerance in this case. To better understand this mechanism activation or suppression of genes by GA suppression should be explored further in transgenic bahiagrass with a global gene expression profiling. It has been suggested that faster recovery of *Andropogon gerardii* (big bluestem grass) could be attributed to greater leaf turnover after water stress, which leads to rapid recovery of photosynthesis post-stress, more allocation to roots and reduced allocation to flowering.
Reduced levels of gibberellins lead to a decrease in cell division and elongation at the apical meristem of the shoot, but have little effect on root growth (Giafagna, 1995). This is consistent with our findings. None of the transgenic lines produced less roots than the wild-type.

Although clear trends were observed in transgenic bahiagrass lines expressing \textit{AtGA2ox1}, some line-to-line variation in phenotype and performance was also observed. Variation observed between lines may be due to variation in transgene copy number, expression levels or interaction with other protein complexes, somaclonal variation, constitutive expression of transcription factors or their transgene integration site.

Transgene silencing can occur through epigenetic mechanisms resulting from exposure to stress (Meng et al., 2006). Various plant stresses high light and temperature, and insecticide treatment have been associated with silencing in several species (Meng et al., 2006). Plant stresses, abiotic or biotic, can especially interfere with transgene stability when conducting field trials where the environment is not controlled (Meyer et al., 1992; Dorlhac de Borne et al., 1994; Brandle et al., 1995; De Wilde et al., 2000). Our analysis confirmed transgene expression stability of our \textit{AtGA20x1} lines following various cycles of vegetative propagation under controlled environment conditions and almost two years in the field where they were exposed to stresses such as dehydration, freezing stress and mowing.

\textit{ATHB16} Expressing Lines (I Lines)

Results

Field Study I

In field study I conducted in 2006 transgenic \textit{ATHB16} bahiagrass plants displayed increased number of vegetative tillers or a proportional dwarfing with shorter tillers and finer leaves. \textit{ATHB16} expressing line I10 displayed the highest turf density as a result of producing
21% and 19% more vegetative tillers than the wild-type eight (Figure 4-25A) and twelve weeks (Figure 4-25B) after transplanting, respectively. The higher number of tillers per area of line I10 resulted in a more compact more dense growth as compared to the wild-type bahiagrass (Figure 4-25C). Transgenic lines I32 and I4 produced shorter tillers and narrower leaves than those of the wild-type bahiagrass. Line I32 produced tillers 14% shorter (Figure 4-22D) and leaves 8% narrower (Figure 4-25E) than those of the wild-type bahiagrass. Line I4 produced tillers 13% shorter (Figure 4-25D) and leaves 12% narrower (Figure 4-25E) than those of the wild-type. This resulted in the lines displaying proportional dwarfing (Figure 4-25F).

The faster production of tillers by transgenic line I10 resulted in a better field establishment as confirmed by our establishment ratings (Figure 4-26A). Line I10 showed overall faster establishment than other lines and wild-type bahiagrass and St. Augustinegrass (Figure 4-26A). This line spread faster than the wild-type and St. Augustinegrass inside the field plots (Figure 4-26B). St. Augustinegrass displayed a slower establishment than all of our bahiagrass plants (Figure 4-26).

As we observed in the \textit{AtGA2ox1} lines, a higher turf density and more vegetative tillers was associated with a more upright growth. The erect growth pattern and the higher number of tillers in the transgenic line I10 contributed to 41% significantly higher clipping weight compared to the wild-type, following four weeks of growth after establishment (Figure 4-27). Lines I32 and I4, displaying, a more dwarf phenotype, produced 31% and 57% respectively less clippings than the wild-type (Figure 4-27).

All transgenic lines had significantly higher visual rating for mowing quality as compared to the wild-type bahiagrass (Figure 4-28A). Transgenic line I10 displayed a more compact growth with no visible rhizomes or gaps in contrast to wild-type (Figure 4-28B).
The number of inflorescences in 8 August 2006, during establishment, produced by transgenic lines I10 and I32, ranging from 0.67 to 0.88, was significantly lower than wild-type (1.46) (Table 4-2). In 29 August lines I10 and I4 produced 31% and 45% less inflorescences than the wild-type, respectively. The length of inflorescence stems of all lines was 8% (I10), 17% (I32) and 28% (I4) shorter than those of the wild-type bahiagrass (Figure 4-29).

**Field Study II**

During field study II, conducted in 2007, 2008 and 2009, transgenic bahiagrass lines expressing *ATHB16* produced significantly more vegetative tillers than the wild-type bahiagrass. Lines I12 and I28 consistently produced the highest number of tillers in all dates and under all irrigation treatments (Figure 4-30). During establishment in September 2007, *ATHB16* expressing lines produced on average 44% (I12), 19% (I23) and 39% (I28) more tillers than the wild-type bahiagrass (Figure 4-30A). Line I32 was the only transgenic line that did not produce significantly more tillers than the wild-type (Figure 4-30A). In measurements taken on May 2008, transgenic lines I12 and I28 produced 33% and 31% more tillers than the wild-type, respectively (Figure 4-30B). Whereas in September 2008, the transgenic lines produced 49% (I12), 32% (I23), 48% (I28), and 26% (I32) more tillers than the wild-type (Figure 4-30C). We found significant interaction between tillers produced and irrigation regimes for May 2008. In May 2009 transgenic lines produced 45% (I12), 26% (I23), 44% (I28) and 22% (I32) more tillers than the wild-type (Figure 4-30D). In contrast to *AtGA2ox1* data from the same time point, increased tillering of the transgenic lines in comparison to the wild-type bahiagrass was not consistent for all lines under all three irrigation treatments in May 2008 (Figure 4-30E). In May 2008 number of tillers was significantly reduced in non-irrigated plots for all lines and wild-type bahiagrass during the drought (Figure 4-30E). During the drought, transgenic plants and wild-type produced under full irrigation 27% (I12), 24% (I23), 31% (I32), and 40% (WT) more tillers.
than in non-irrigated plots (Figure 4-30E). The exception was line I28, producing the most tillers under moderate irrigation, 47% more tillers than in non-irrigated plots (Figure 4-30E). Under full irrigation all lines except I23 produced more tillers than the wild-type bahiagrass (Figure 4-30E). While under moderate irrigation, I32 did not produce more tillers than the wild-type (Figure 4-30E). In non-irrigated plots, all lines produced more tillers than the wild-type (Figure 4-30E). Tiller production was reduced in non-irrigated plots for all lines and wild-type bahiagrass in May 2009 (Figure 4-30F). Transgenic plants and wild-type produced under full irrigation 25% (I12), 10% (I23), 13% (I28), 25% (I32) and 43% (WT) more tillers than in non-irrigated plots (Figure 4-30F). Bahiagrass transgenic plants and wild-type produced on average 30% and 23% more tillers under full irrigation than in non-irrigated plots in May 2008(Figure 4-30D) and 2009 (Figure 4-30F), respectively. These were the only time-points when there was a significant interaction between irrigation regime and production of tillers.

The higher number of tillers in transgenic lines (I12, I23, I28) resulted in significantly higher turf density as confirmed by our density ratings taken in September 2007 (Figure 4-31A), May 2008 (Figure 4-31B) and May 2009 (Figure 4-31C). On all dates where density was evaluated, I32 was the only transgenic line that did not display greater turf density than the wild-type (Figure 4-31). This is consistent with tiller data for September 2007 (Figure 4-31A) and May 2008 tiller data Figure (4-31B).

Consistent to findings from field study I, *ATHB16* bahiagrass plants displayed a proportional dwarfing with shorter tillers and finer leaves. Transgenic lines produced 21% (I12), 14% (I23), 11% (I28) and 5% (I32) shorter tillers than those of the wild-type bahiagrass (Figure 4-32A). The same lines produced 14% (I12), 9% (I23), 9% (I28) and 7% (I32) narrower leaves than those of the wild-type bahiagrass (Figure 4-32B). Narrower leaves of the transgenic lines
resulted in finer leaf texture in all our lines confirmed by our visual ratings (Figure 4-32C). Visual ratings for leaf texture were assigned using a 1 to 9 scale, where 1 is the coarsest (widest) and 9 is the finest leaf texture.

As previously mentioned, higher number of tillers was associated with a denser, more compact and erect growth in the transgenic lines as compared with the wild-type bahiagrass, which exhibited a sparser looking and prostrate growth (Figure 4-33).

Four weeks after transplanting, faster production of vegetative tillers by the transgenic lines I12 and I28 resulted in a better, faster field establishment as compared to the wild-type (Figure 4-34A). The faster establishment was associated with the transgenic lines having increased tillering (Figure 4-34B). Eight weeks later (twelve weeks after transplanting), lines I12 and I32 had lower establishment ratings than the wild-type (Figure 4-34C). This was a result of the denser, more compact and erect growth. The higher number of tillers of this line was very concentrated without much lateral spread twelve weeks after transplanting. This growth is evident in comparing growth between line I12 and wild-type bahiagrass (Figure 4-33).

The higher tiller number and erect growth of transgenic lines I12 and I28 contributed to higher dry weight of clippings compared to the wild-type bahiagrass plants all months of the establishing period in 2007 (Figure 4-35A). Transgenic line I12 produced 73%, 57% and 60% greater clipping weight than the wild-type for the months of August, September and October 2007 (Figure 4-35A). Line I28 produced 58%, 54% and 52% greater clipping weight than the wild-type for the months of August, September and October 2007 (Figure 4-35A). Line I23 produced 13% more clippings than the wild-type in August 2007 (Figure 4-35A). No significant interactions or other significant differences between transgenic lines and wild-type were observed for clipping dry weight in 2007.
In 2008, some significant differences between dry weight of clipping of transgenic lines and wild-type (Figure 4-35B). Line I28 produced 58%, 51% and 25% more clippings than the wild-type in March, April and September respectively (Figure 4-35B). Whereas, line I12 produced 34%, 46%, 36% and 40% less clippings than the wild-type for the months of April, May, June, July and August, respectively (Figure 4-35B). Line I23 produced 29% and 24% less clippings than the wild-type in May and June and 22% more clippings than the wild-type in September (Figure 4-35B). Line I32 produced 35% and 42% less clippings than the wild-type in May and August (Figure 4-35B). In March and April 2009 only line I28 produced more clippings than the wild-type bahiagrass, 39% and 43% more, respectively (Figure 4-35C). Moreover, there was no significant interaction between any of our treatments and clipping weight in March or April 2009.

Some lines displayed reduced vigor as indicated by our establishment and dry weight of clippings data. Twelve weeks after establishment of field plots, lines I12 and I32 received lower establishment ratings (Figure 4-34C). The altered phenotypes observed in these lines affected their establishment rate. Lines I12, I23 and I32 produced less clippings than the wild-type at various time-points. Line I12, spreading the slowest, produced less clippings than the wild-type during most of the summer months in 2008 (Figure 4-35B). Line I23, produced less clippings than the wild-type during the drought and recovery months specifically, which could indicate reduced vigor when exposed to drought (Figure 4-35B). Line I32 also produced less clipping than the wild-type in May, during drought (Figure 4-35B). This could indicate that the changed observed by the over-expression of \textit{ATHB16}, improve the appearance but may hinder the plant vigor of some of our lines. Specifically, line I12, which displayed the most extreme phenotype and reduced vigor.
Weed encroachment ratings were taken in March when the highest incidence of weed emergence was observed. Ratings were assigned using a 1 to 9 scale, with 9 being no weeds and 1 being all covered in weeds. In ratings taken in March 2008 transgenic lines I12, I23, I28 and St. Augustinegrass received higher ratings for resistance to weeds (Figure 4-36A). Line I12, produced more tillers and had a denser habit, which suppressed weed encroachment. In 2009, significant differences between transgenic lines and wild-type bahiagrass were observed for full irrigation plots with lines I28, I32 and St Augustinegrass having fewer weeds than the wild-type (Figure 4-36B). There was a positive interaction between weed encroachment and irrigation with all lines and wild-types being more susceptible to weeds under full irrigation (Figure 4-36B).

Spring green-up ratings were also used to evaluate performance. Green-up is a measure of the transition from winter dormancy to active spring growth. It is based on plot color not genetic color. The visual rating of spring green-up is based on a 1 to 9 rating scale with 1 equaling straw brown and 9 equaling dark green. Winter temperatures in 2008 where not severe enough to turn all leaf tissue brown (Figure 4-37A). By March 2008 lines I12, I23 and I28 had almost recovered their whole plot color. Faster recovery from winter temperatures than the wild-type are described by higher visual ratings (Figure 4-37B.) More extreme winter temperatures were experienced in 2009 (Figure 4-37A). By January 2009 all plots were completely brown. Green-up ratings in March 2009 show one of the transgenic lines, I28, recovering better from winter temperatures than the wild-type (Figure 4-37C). There was a significant interaction between irrigation regime and spring green-up ratings for 2009. As expected plants that had received full irrigation the previous season displayed higher green-up ratings. All lines and wild-types received significantly higher green-up ratings under full irrigation compared to no or moderate irrigation.
Flowering in transgenic lines I12, I23 and I28 was reduced as compared to the wild-type during onset and throughout the flowering season in 2007 (Figure 4-38A), 2008 (Figure 4-38B) and 2009 (Figure 4-38C). In 2007, lines I12 and I28 consistently produced the least amount of inflorescences (Figure 4-38A). Line I23 and I32 produced less inflorescences than the wild-type in all dates measured except in 13 August (Figure 4-38A). In 2008, lines I12 and I28 continued to produce the least amount of inflorescences all through the flowering season (Figure 4-38B). Lines I23 and I32 produce less inflorescences than the wild-type early in the season but display no difference to the wild-type in 24 July, 28 August and 29 September (Figure 4-38B). Early in the 2009 flowering season all lines produce significantly less inflorescences than the wild-type (Figure 4-38C).

Transgenic lines had 61% (I12), 16% (I23) and 47% (I28) less total inflorescences than the wild-type in 2007, which was statistically significant (Figure 4-39A). In 2008 transgenic lines produced 77% (I12), 17% (I23) and 65% (I28) fewer inflorescences than the wild-type (Figure 4-39B). In 2009 lines produced 99.6% (I12), 68% (I23), 97% (I28) and 79% (I32) fewer inflorescences than the wild-type (Figure 4-39C). We did find significant interaction between total number of inflorescences produced in 2008 or 2009 and irrigation regime. Lines I23, I28, I32 and wild-type bahiagrass produced significantly more inflorescences in 2008 under moderate irrigation compared to full or non-irrigated. Length of inflorescence stems of the transgenic lines was 21% (I12) and 13% (I32) shorter than that of the wild-type (Figure 4-40).

In 2008 a seasonal drought period was experienced for the months of April and May (Figure 3-3A). Drought tolerance ratings were based on leaf firing, wilting, and whole plot color. In April at the beginning of the drought, there was no significant difference between our transgenic lines and wild-type (Figure 4-41A). All lines and wild-type received significant lower
ratings in non-irrigated plots as compared to moderate irrigation (Figure 4-41A). St. Augustinegrass received the lowest ratings under both irrigation treatments at this time (Figure 4-41A). Significant difference in dry weight of clippings for that same month shows line I28 producing more clippings under moderate irrigation than other lines and wild-type (Figure 4-41B). Visual ratings for drought tolerance were repeated in May 2008. Under moderate irrigation lines I23, I28 and I32 displayed better tolerance to drought than the wild-type (Figure 4-42A). There was no significant difference between transgenic and wild-type bahiagrass in non-irrigated plots (Figure 4-42A). Again, all lines and wild-type received significant lower ratings in non-irrigated plots as compared to moderate irrigation (Figure 4-42A). In May, no significant difference was observed in regards to dry weight of clippings between the transgenic lines and the wild-type (Figure 4-42B). Only line I28 and the wild-type produce significantly less clippings in non-irrigated plots as compared to moderate irrigation (Figure 4-42B). Figure 4-42C displays visual differences observed in May 2008 resulting from drought stress between transgenic line I28 and wild-type.

In June, as the plants were recovering from the drought, lines I23, I28 and I32 received higher ratings than the wild-type under moderate irrigation (Figure 4-43A). Lines I23 and I28 also had higher ratings than the wild-type in non-irrigated plots (Figure 4-43A). Significant interaction was found between irrigation regime and drought recovery ratings taken in June. All lines and wild-type bahiagrass and St. Augustinegrass obtained higher recovery ratings under moderate irrigation as compared to non-irrigated (Figure 4-43A). In regards to dry weight of clippings, line I28 produced higher clippings than the wild-type under moderate irrigation (Figure 4-43B). Figure 4-43C displays visual differences observed in June 2008 during the drought recovery period between transgenic line I28 and wild-type.
In 2009 a seasonal drought period was experienced for the months of March and April (Figure 3-3B). In contrast to 2008, the drought was experienced earlier at which point the plants were still in the process of greening-up from winter dormancy. In drought ratings taken in April 2009, lines I23, I28 and I32 displayed better tolerance to drought than the wild-type under moderate irrigation (Figure 4-44A). Line I28 produced more clipping weight than the wild-type under moderate irrigation in April 2009(Figure 4-44B).

Performance during drought and recovery indicates that all transgenic lines perform at least as well as the wild-type under drought. While line I28 appeared to perform better under the drought conditions than the other transgenic lines and the wild-type in. Nevertheless, maximum quantum yield of photosystem II using dark-adapted leaves and SPAD measurements taken during the drought and recovery periods displayed no significant difference. Results for maximum quantum yield of photosystem II using dark-adapted leaves taken during the drought in May 2008 revealed no significant difference between any transgenic lines and the wild-type under full and non-irrigated (Figure 4-45A). Moreover, no significant difference was observed in any line or wild-type between irrigation treatments for this measurement (Figure 4-45A). In June, during drought recovery, no significant difference was observed between irrigation treatments or between lines and wild-type for maximum quantum yield; only line I32 had lower values than the wild-type in non-irrigated plots (Figure 4-45B). No significant interaction was observed between irrigation regime and maximum quantum yield of photosystem II for either measurement timepoint, May or June 2008.

SPAD measurement results for May reveal line I32 having higher SPAD readings than wild-type and other lines under all three irrigation regimes (Figure 4-46A). Line I28 had higher readings than wild-type under moderate irrigation (Figure 4-46A). Line I23 had lower readings
than the wild-type in non-irrigated plots (Figure 4-46A). In June, during drought recovery, lines I23 and I32 have higher SPAD readings than the wild-type under moderate irrigation (Figure 4-46B). Line I28 showed lower SPAD readings than the wild-type under full irrigation (Figure 4-46B). There was significant interaction between SPAD readings and irrigation regime both in May and in June. A higher SPAD reading was recorded for all lines in non-irrigated plots as compared to full or moderate irrigation (Figure 4-46B). We also looked into root and rhizome dry weight to determine if the change in aboveground plant architecture had affected belowground organs. In non-irrigated plots, transgenic line I12 produced 69% higher dry weight of rhizomes than the wild-type (Figure 4-47). No significant difference was observed in root dry weight between the transgenics and the wild-type (Figure 4-48).

We also evaluated drought stress response of the ATHB16 lines under controlled environment conditions in comparison to wild-type bahiagrass plants and St. Augustinegrass (unpublished, 2006). We found that all lines evaluated performed at least as well as the wild-type under drought conditions, with line I32 recovering better.

Reverse transcription PCR (RT-PCR) analysis (Figure 4-49) confirmed the presences of the ATHB16 transcripts in all lines after approximately 20 months of growing in the field in non-irrigated plots.

**Discussion**

It was previously reported that over-expression the Arabidopsis ATHB16 in bahiagrass resulted in proportional dwarfing of the plant with shorter, finer leaves, increased tillering and reduced and delayed flowering compared to wild-type plants (Zhang et al., 2007). The results presented here confirm these findings and demonstrate that field performance is not compromised and even improved in some of the transgenic lines.
Over-expression of *ATHB16* in Arabidopsis resulted also in reduced leaf expansion and shoot elongation and with the proposed function for this gene as a suppressor of cell expansion (Wang et al., 2003a). In Arabidopsis, the rosette leaf area of transgenic lines was 30 % less than the wild-type resulting from the over-expression of the *ATHB16*. As a result, these plants appeared more compact and smaller when compared to the wild-type, as did our bahiagrass lines.

Delayed and reduced flowering was also observed in transgenic bahiagrass lines. Transgenic expression of *ATHB16* in Arabidopsis also resulted in plants with altered flowering time (Wang et al., 2003a). Arabidopsis plants over-expressing *ATHB16* also displayed significant delayed flowering and reduced inflorescence formation under long days (Wang et al., 2003a).

Increased tillering was another phenotype observed within the *ATHB16* bahiagrass lines. Over-expression of *ATHB16* in Arabidopsis also resulted in enhanced formation of vegetative tillers (Wang et al., 2003a). Expression of *ATH1* in ryegrass resulted in plants with more vegetative tillers and delayed or non-flowering (van der Valk et al., 2004). Another Arabidopsis homeobox transcription factor, *ATH1* gene is a negative regulator in the light-regulated gibberellins biosynthesis pathway (Quaedvlieg et al., 1995; Garcia-Martinez and Gil, 2001). Over-expression of *ATH1* in perennial ryegrass resulted in decreased levels of GA1 and was accompanied by the outgrowth of normally quiescent lateral meristems into extra leaves and delayed development of inflorescences (van der Valk et al., 2004). Tiller number and plant height exhibit a highly negative correlation in several plant species including rice (Ishikawa et al., 2005; Iwata et al., 1995; Li et al., 2003; Yan et al., 1998) and in Arabidopsis and (Beveridge et al., 1996; Booker et al., 2004; Sorefan et al., 2003; Zou et al., 2006). Zou et al. (2006) proposed that dwarfing in GA mutants was a secondary effect to the increased formation of tillers partly due to the reduced apical dominance (Zou et al., 2006; McSteen, 2009). However,
ATHB16 represses cell expansion independent of the GA signal transduction pathway (Wang et al., 2003a).

HDzip genes like ATHB16 represent a large gene family with 42 members in Arabidopsis. Proteins encoded by these genes are characterized by the presence of a DNA binding homeodomain and an adjacent Leu zipper motif; they are involved in developmental processes (Henriksson et al., 2005). Different members of HDzip proteins may form heterodimers and then bind to regulatory DNA region of downstream genes (Sessa et al., 1993; Wang, 2001). Hence, ATHB16 could interact with other HDzip proteins such as ATHB6 (Wang, 2001). ATHB6 is known to be upregulated in response to water-deficit conditions and to treatment of abscisic acid and has been proposed to function as a regulator of growth and development in response to limited water conditions (Söderman et al. 1999; Himmelbach et al. 2002). This implies that over-expression of ATHB16 may in concert with ATHB6 enhance the drought tolerance of transgenic plants over-expressing ATHB16. Target genes downstream of ATHB16 still have to be identified to fully understand its function.

Levels of expression of transcription factor encoding genes do not always correlate to the target gene expression and phenotype (Cao et al. 2006). This may be due to regulation by target gene expression by other mechanisms like posttranslational modification, protein-protein interaction and other signaling pathways (Gu et al. 2000; Chakravarthy et al. 2003). Pleiotrophic effects can also be caused by somaclonal variation, constitutive expression of transcription factors or their transgene integration site. Somaclonal variation is not likely to contribute to line to line variation, since the autotetraploid genome of bahiagrass cultivar ‘Argentine’ is buffered against mutations.
Our analysis confirmed transgene expression stability of our *ATHB16* lines following various cycles of vegetative propagation under controlled environment conditions and almost two years in the field where they were exposed to stresses such as dehydration, freezing stress and mowing.
Figure 4-1. Density of *AtGA2ox1* expressing bahiagrass lines (B11, B3, B6, B7, B9) and wild-type bahiagrass (WT) and St. Augustinegrass (SA). (A) Number of tillers produced in a 100 cm² area following eight weeks of growth after transplanting. (B) Number of tillers produced by in a 100 cm² area following twelve weeks of growth after transplanting. (C) Visual ratings for density eight weeks after transplanting (D) Comparison of *AtGA2ox1* expressing line B3 and wild-type bahiagrass (WT) 4 weeks after establishment of field plots. *Capital letters at the bottom of each graph indicate significant difference between lines at α = 0.05. Error bars indicate standard error of the means.*
Figure 4-2. Field establishment of \textit{AtGA2ox1} expressing lines (B11, B3, B6, B7, B9) and wild-type bahiagrass (WT) and St. Augustinegrass (SA). (A) Visual ratings for establishment of field plots four weeks after transplanting. (B) Comparison of \textit{AtGA2ox1} expressing line B3 and wild-type bahiagrass (WT) and St. Augustinegrass (SA) 4 weeks after establishment of field plots. \textit{Capital letters at the bottom of the graph indicate significant difference between lines at } \alpha = 0.05. \textit{Error bars indicate standard error of the means.}
Figure 4-3. Dry weight of clippings produced under weekly mowing four weeks after establishment by *AtGA2ox1* expressing lines (B11, B3, B6, B7, B9) and wild-type bahiagrass (WT) and St. Augustinegrass (SA). Capital letters at the bottom of the graph indicate significant difference between lines at $\alpha = 0.05$. Error bars indicate standard error of the means.

Figure 4-4. Mowing quality of *AtGA2ox1* expressing lines (B11, B3, B6, B7, B9) and wild-type bahiagrass (WT). (A) Visual ratings for mowing quality eight weeks after transplanting. (B) A freshly mowed *AtGA2ox1* expressing line B3 in comparison to wild-type bahiagrass (WT) following weekly mowing at 8 cm cutting heights and 14 week after establishment of field plots. Capital letters at the bottom of the graph indicate significant difference between lines at $\alpha = 0.05$. Error bars indicate standard error of the means.
Table 4-1. Emergence of inflorescences in *AtGA2ox1* expressing lines (B11, B3, B6, B7, B9) and wild-type bahiagrass (WT) under field conditions. Capital letters below each entry indicate significant difference between lines at each timepoint at $\alpha = 0.05$.

<table>
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<th>B3</th>
<th>B6</th>
<th>B7</th>
<th>B9</th>
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<td></td>
<td></td>
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<tr>
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</tr>
<tr>
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<td>C</td>
<td>A</td>
<td>BC</td>
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<td>A</td>
</tr>
<tr>
<td>August 29, 2006</td>
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<td>B</td>
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</tbody>
</table>

Figure 4-5. Length of fully expanded inflorescence stems (without racemes) of *AtGA2ox1* expressing lines (B11, B3, B6, B7, B9) and wild-type bahiagrass (WT). Capital letters at the bottom of the graph indicate significant difference between lines at $\alpha = 0.05$. Error bars indicate standard error of the means.
Figure 4-6. Number of tillers produced in a 100 cm² by AtGA2ox1 expressing lines (B10, B3, B7, B8) and wild-type bahiagrass (WT) in (A) September 2007, (B) May 2008, (C) September 2008, (D) May 2009, (E) May 2008 by irrigation, (F) May 2009 by irrigation. Capital letters at the bottom of each graph indicate significant difference between lines at α = 0.05. Lowercase letters above bars indicate significant difference between irrigation treatments within each line at α = 0.05. Error bars indicate standard error of the means.
Figure 4-7. Density of *AtGA2ox1* expressing bahiagrass lines (B10, B3, B7, B8) and wild-type bahiagrass (WT) and St. Augustinegrass (SA) in (A) September 2008, May 2008 (B) and 2009 (C). Capital letters at the bottom of each graph indicate significant difference between lines at $\alpha = 0.05$. Error bars indicate standard error of the means.
Figure 4-8. Comparison of fully established *AtGA2ox1* lines (B3, B7) and wild-type (WT).

Figure 4-9. Field establishment of *AtGA2ox1* expressing bahiagrass lines (B11, B3, B6, B7, B9) and wild-type bahiagrass (WT) and St. Augustinegrass (SA). (A) Visual ratings for establishment of field plots four weeks after transplanting. (B) Visual ratings for establishment of field plots eight weeks after transplanting. (C) Comparison of *AtGA2ox1* expressing lines B3 and B7 and wild-type bahiagrass (WT) 4 weeks after establishment of field plots. Capital letters at the bottom of the graph indicate significant difference between lines at $\alpha = 0.05$. Error bars indicate standard error of the means.
Figure 4-10. Dry weight of clippings produced by *AtGA2ox1* expressing lines (B11, B3, B6, B7, B9) and wild-type bahiagrass (WT) and St. Augustinegrass (SA) in (A) 2007, (B) 2008 and (C) 2009. Lowercase letters at the bottom of the graph indicate significant difference between lines within each month and year at $\alpha = 0.05$. Error bars indicate standard error of the means.
Figure 4-11. Visual ratings for resistance to weed encroachment of \( AtGA2ox1 \) expressing lines (B11, B3, B6, B7, B9) and wild-type bahiagrass (WT) and St. Augustinegrass (SA) in March (A) 2008 and (B) 2009. Capital letters at the bottom of each graph indicate significant difference between lines at \( \alpha = 0.05 \). Lowercase letters above bars indicate significant difference between irrigation treatment within each line at \( \alpha = 0.05 \). Error bars indicate standard error of the means.
Figure 4-12. Spring green-up of *AtGA2ox1* expressing lines (B11, B3, B6, B7, B9) and wild-type bahiagrass (WT) and St. Augustinegrass (SA). (A) Minimum temperatures experienced in winter months. Visual ratings for green-up in March (B) 2008 and (C) 2009. Capital letters at the bottom of each graph indicate significant difference between lines at α = 0.05. Error bars indicate standard error of the means.
Figure 4-13. Drought tolerance of \textit{AtGA2ox1} expressing lines (B11, B3, B6, B7, B9) and wild-type bahiagrass (WT) and St. Augustinegrass (SA). (A) Visual ratings for drought tolerance in April 2008. (B) Dry weight of clipping under moderate and non-irrigated regimes in April 2008. (C) Comparison of line B7 and wild-type (WT) and St Augustinegrass (SA) during the onset of drought in April. \textit{Capital letters at the bottom of each graph indicate significant difference between lines at} \( \alpha = 0.05 \). \textit{Lowercase letters inside the bars indicate significant difference between irrigation treatment within each line at} \( \alpha = 0.05 \). \textit{Error bars indicate standard error of the means.}
Figure 4-14. Drought tolerance of *AtGA2ox1* expressing lines (B11, B3, B6, B7, B9) and wild-type bahiagrass (WT) and St. Augustinegrass (SA). (A) Visual ratings for drought tolerance in May 2008. (B) Dry weight of clipping under moderate and non-irrigated regimes in May 2008. (C) Comparison of line B7 and wild-type (WT) and St Augustinegrass (SA) during the drought in May. Capital letters at the bottom of each graph indicate significant difference between lines at $\alpha = 0.05$. Lowercase letters above bars indicate significant difference between irrigation treatment within each line at $\alpha = 0.05$. Error bars indicate standard error of the means.
Figure 4-15. Drought tolerance of *AtGA2ox1* expressing lines (B11, B3, B6, B7, B9) and wild-type bahiagrass (WT) and St. Augustinegrass (SA). (A) Visual ratings for drought recovery in June 2008. (B) Dry weight of clipping under moderate and non-irrigated regimes in June 2008. (C) Comparison of line B7 and wild-type (WT) during the drought recovery in June. (D) Comparison of lines B3, B7, B10 and St Augustinegrass (SA) during the drought recovery in June. Capital letters at the bottom of each graph indicate significant difference between lines at *α* = 0.05. Lowercase letters above bars indicate significant difference between irrigation treatment within each line at *α* = 0.05. Error bars indicate standard error of the means.
Figure 4-16. Drought tolerance of *AtGA2ox1* expressing lines (B11, B3, B6, B7, B9) and wild-type bahiagrass (WT) and St. Augustinegrass (SA). (A) Visual ratings for drought tolerance in April 2009. (B) Dry weight of clipping under moderate and non-irrigated regimes in April 2009. *Capital letters at the bottom of each graph indicate significant difference between lines at* $\alpha = 0.05$. *Lowercase letters above bars indicate significant difference between irrigation treatment within each line at* $\alpha = 0.05$. *Error bars indicate standard error of the means.*

Figure 4-17. Maximum quantum yield of dark-adapted leaves of transgenic lines (B10, B3, B7, B8), St. Augustinegrass (S) and wild-type bahiagrass (WT). (A) During drought in May 2008 (B) In June 2008, during recovery from drought. *Capital letters at the bottom of each graph indicate significant difference between lines at* $\alpha = 0.05$. *Lowercase letters above bars indicate significant difference between irrigation treatment within each line at* $\alpha = 0.05$. *Error bars indicate standard error of the means.*
Figure 4-18. SPAD meter readings of transgenic lines (B10, B3, B7, B8), St. Augustinegrass (SA) and wild-type bahiagrass. (WT). (A) During drought in May 2008 (B). In June 2008, during recovery from drought. Capital letters at the bottom of each graph indicate significant difference between lines at $\alpha = 0.05$. Lowercase letters above bars indicate significant difference between irrigation treatment within each line at $\alpha = 0.05$. Error bars indicate standard error of the means.
Figure 4-19. Dry weight of rhizomes produced by *AtGA2ox1* expressing lines (B11, B3, B6, B7, B9) and wild-type bahiagrass (WT) and St. Augustinegrass (SA) in non-irrigated plots. *Capital letters at the bottom of each graph indicate significant difference between lines at* $\alpha = 0.05$. *Error bars indicate standard error of the means.*

![Dry Weight of Rhizomes under Non-irrigated Conditions](image)

Figure 4-20. Dry weight of roots produced by *AtGA2ox1* expressing lines (B11, B3, B6, B7, B9) and wild-type bahiagrass (WT) and St. Augustinegrass (SA) in non-irrigated plots. *Capital letters at the bottom of each graph indicate significant difference between lines at* $\alpha = 0.05$. *Error bars indicate standard error of the means.*

![Dry Weight of Roots under Non-irrigated Conditions](image)
Figure 4-21. Inflorescences produced by AtGA2ox1 expressing lines (B11, B3, B6, B7, B9) and wild-type bahiagrass (WT) over time in (A) 2007, (B) 2008 and (C) 2009. Lowercase letters at the bottom of each graph indicate significant difference between lines within each time entry at $\alpha = 0.05$. Error bars indicate standard error of the means.
Figure 4-22. Total inflorescences produced by *AtGA2ox1* expressing lines (B11, B3, B6, B7, B9) and wild-type bahiagrass (WT) in (A) 2007, 2008 (B), and (C) 2009. Lowercase letters at the bottom of each graph indicate significant difference between lines at within each time entry $\alpha = 0.05$. Error bars indicate standard error of the means.
Figure 4-23. Average length of inflorescence stems without racemes per field plot produced by \textit{AtGA2ox1} expressing lines (B11, B3, B6, B7, B9) and wild-type bahiagrass (WT). Capital letters at the bottom of each graph indicate significant difference between lines at \( \alpha = 0.05 \). Error bars indicate standard error of the means.

Figure 4-24. RT-PCR analysis for expression of the \textit{AtGA2ox1} gene using specific primers for amplification of cDNA from transgenic lines (B11, B3, B6, B7, B9) [516 bp] compared to WT and plasmid \textit{UbiGAox1} [652 bp].
Figure 4-25. Phenotypic differences observed between *ATHB16* expressing lines (I4, I10, I32) and wild-type bahiagrass (WT). (A) Number of tillers produced by in a 100 cm² area following eight weeks of growth after transplanting. (B) Number of tillers produced in a 100 cm² area following twelve weeks of growth after transplanting. (C) Comparison between transgenic line I10 and wild-type bahiagrass (WT). (D) Length of the tiller measured from the crown to the tip of the leaf. (E) Leaf width. (F) Comparison between transgenic line I32 and wild-type bahiagrass (WT). *Capital letters at the bottom of each graph indicate significant difference between lines at α = 0.05. Error bars indicate standard error of the means.*
Figure 4-26. Field establishment of *ATHB16* expressing bahiagrass lines (I4, I10, I32) and wild-type bahiagrass (WT) and St. Augustinegrass (SA). (A) Visual ratings for establishment of field plots four weeks after transplanting (B) Comparison line I10 and wild-type bahiagrass (WT) and St. Augustinegrass (SA) 4 weeks after establishment of field plots. Capital letters at the bottom of the graph indicates significant difference between lines at $\alpha = 0.05$. Error bars indicate standard error of the means.

Figure 4-27. Dry weight of clippings produced under weekly mowing four weeks after establishment by *ATHB16* expressing lines (I0, I32, I4) and wild-type bahiagrass (WT) and St. Augustinegrass (SA). Capital letters at the bottom of the graph indicate significant difference between lines at $\alpha = 0.05$. Error bars indicate standard error of the means.
Figure 4-28. Turf mowing quality of *ATHB16* expressing lines (I0, I32, I4) and wild-type bahiagrass (WT). (A) Visual ratings for mowing quality. (B) Freshly mowed transgenic line I10 compared to wild-type bahiagrass (WT) following weekly mowing fourteen weeks after transplanting. *Capital letters at the bottom of the graph indicate significant difference between lines at α = 0.05. Error bars indicate standard error of the means.*
Table 4-2. Emergence of inflorescences of ATHB16 transgenic lines (I10, I32, I4) compared to wild-type bahiagrass (WT). Capital letters below each entry indicate significant difference between lines at each timepoint at $\alpha = 0.05$.

<table>
<thead>
<tr>
<th>Record / Line</th>
<th>I10</th>
<th>I32</th>
<th>I4</th>
<th>WT</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>8th August 2006</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Avg. No. of inflorescences per plot</td>
<td>0.67 ± 0.23</td>
<td>0.88 ± 0.23</td>
<td>1.08 ± 0.26</td>
<td>1.46 ± 0.33</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>B</td>
<td>AB</td>
<td>A</td>
</tr>
<tr>
<td><strong>29th August 2006</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Avg. No. of inflorescences per plot</td>
<td>43.25 ± 7.26</td>
<td>64.13 ± 6.43</td>
<td>34.63 ± 6.48</td>
<td>62.88 ± 3.82</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>A</td>
<td>B</td>
<td>A</td>
</tr>
</tbody>
</table>

Figure 4-29. Length of fully expanded inflorescence stems (without racemes) of $ATHB16$ expressing bahiagrass lines (I10, I4, I32) and wild-type bahiagrass (WT). Capital letters at the bottom of the graph indicate significant difference between lines at $\alpha = 0.05$. Error bars indicate standard error of the means.
Figure 4-30. Number of tillers produced in a 100 cm² by *ATHB16*-expressing lines (I12, I23, I28, I32) and wild-type bahiagrass (WT) in (A) September 2007, (B) May 2008, (C) September 2008, (D) May 2009, (E) May 2008 by irrigation, (F) May 2009 by irrigation. Capital letters at the bottom of each graph indicate significant difference between lines at $\alpha = 0.05$. Lowercase letters above bars indicate significant difference between irrigation treatments within each line at $\alpha = 0.05$. Error bars indicate standard error of the means.
Figure 4-31. Density of \textit{ATHB16} expressing lines (I12, I23, I28, I32) and wild-type bahiagrass (WT) and St. Augustinegrass (SA) in (A) September 2008, (B) May 2008 and (A) 2009. \textit{Capital letters at the bottom of each graph indicate significant difference between lines at } \alpha = 0.05. \textit{Error bars indicate standard error of the means.}
Figure 4-32. Phenotypic differences observed between *ATHB16* expressing lines (I12, I23, I28, I32) and wild-type bahiagrass (WT). (A) Length of the tiller measured from the crown to the tip of the leaf. (B) Leaf width (C) Visual ratings for leaf texture. *Capital letters at the bottom of each graph indicate significant difference between lines at α = 0.05. Error bars indicate standard error of the means.*
Figure 4-33. Comparison of *ATHB16* lines (I28, I12) and wild-type (WT).

Figure 4-34. Field establishment of *ATHB16* expressing bahiagrass lines (I12, I23, I28, I32) and wild-type bahiagrass (WT) and St. Augustinegrass (SA). (A) Visual ratings for establishment of field plots four weeks after transplanting (B) Visual ratings for establishment of field plots twelve weeks after transplanting (C) Comparison of *ATHB16* expressing lines I12, I28 and wild-type bahiagrass (WT) 4 weeks after establishment of field plots. Capital letters at the bottom of the graph indicates significant difference between lines at α = 0.05. Error bars indicate standard error of the means.
Figure 4-35. Dry weight of clippings produced by *ATHB16* expressing lines (I12, I23, I28, I32) and wild-type bahiagrass (WT) and St. Augustinegrass (SA) in (A) 2007, (B) 2008 and (C) 2009. Lowercase letters at the bottom of the graph indicate significant difference between lines within each month at $\alpha = 0.05$. Error bars indicate standard error of the means.
Figure 4-36. Visual ratings for resistance to weed encroachment of *ATHB16* expressing lines and wild-type bahiagrass (WT) and St. Augustinegrass (SA) in March 2008 (A) and March 2009 (B). Capital letters at the bottom of each graph indicate significant difference between lines at $\alpha = 0.05$. Lowercase letters above bars indicate significant difference between irrigation treatment within each line at $\alpha = 0.05$. Error bars indicate standard error of the means.
Figure 4-37. Spring green-up of *ATHB16* expressing lines (I12, I23, I28, I32) and wild-type bahiagrass (WT) and St. Augustinegrass (SA). (A) Minimum temperatures experienced in winter months. (B) Visual ratings for green-up in March 2008 and (C) 2009. Capital letters at the bottom of each graph indicate significant difference between lines at $\alpha = 0.05$. Error bars indicate standard error of the means.
Figure 4-38. Inflorescences produced by *ATHB16* expressing lines (I12, I23, I28, I32) and wild-type bahiagrass (WT) over time (A) 2007, (B) 2008 and (C) 2009. Lowercase letters at the bottom of each graph indicate significant difference between lines at within each time entry $\alpha = 0.05$. Error bars indicate standard error of the means.
Figure 4-39. Total inflorescences produced by *ATHB161* expressing lines (I12, I23, I28, I32) and wild-type bahiagrass (WT) in (A) 2007, (B) 2008 and (C) 2009. Capital letters at the bottom of each graph indicate significant difference between lines within each timepoint at $\alpha = 0.05$. Error bars indicate standard error of the means.
Figure 4-40. Average length of inflorescence stems of *ATHB16* expressing lines (I12, I23, I28, I32) and wild-type bahiagrass (WT). *Capital letters at the bottom of each graph indicate significant difference between lines at within each time entry α = 0.05. Error bars indicate standard error of the means.*

![Average Length of Inflorescence Stems per Field Plot](image)

Figure 4-41. Drought tolerance of *ATHB16* expressing lines (I12, I23, I28, I32) and wild-type bahiagrass (WT) and St. Augustinegrass (SA) in April 2008. (A) Visual ratings for drought tolerance. (B) Dry weight of clipping under moderate and non-irrigated regimes. *Capital letters at the bottom of each graph indicate significant difference between lines at α = 0.05. Lowercase letters above bars indicate significant difference between irrigation treatment within each line at α = 0.05. Error bars indicate standard error of the means.*
Figure 4-42. Drought tolerance of *ATHB16* expressing lines (I12, I23, I28, I32) and wild-type bahiagrass (WT) and St. Augustinegrass (SA) in May 2008. (A) Visual ratings for drought tolerance. B) Dry weight of clipping under moderate and non-irrigated regimes. (C) Comparison of line I28 and wild-type (WT) during the drought in May. Capital letters at the bottom of each graph indicate significant difference between lines at $\alpha = 0.05$. Lowercase letters above bars indicate significant difference between irrigation treatment within each line at $\alpha = 0.05$. Error bars indicate standard error of the means.
Figure 4-43. Drought recovery of *ATHB16* expressing lines (I12, I23, I28, I32) and wild-type bahiagrass (WT) and St. Augustinegrass (SA) in June 2008. (A) Visual ratings for drought recovery. (B) Dry weight of clipping under moderate and non-irrigated regimes. (C) Comparison of line I28 and wild-type (WT) and St Augustinegrass (SA) during the drought recovery period in June. Capital letters at the bottom of each graph indicate significant difference between lines at $\alpha = 0.05$. Lowercase letters above bars indicate significant difference between irrigation treatment within each line at $\alpha = 0.05$. *Error bars indicate standard error of the means.*
Figure 4-44. Drought tolerance of *ATHB16* expressing lines (I12, I23, I28, I32) and wild-type bahiagrass (WT) and St. Augustinegrass (SA) in May 2009 (A) Visual ratings for drought tolerance. (B) Dry weight of clipping under moderate and non-irrigated regimes. (C) Comparison of line I28 and wild-type (WT) and St Augustinegrass (SA) during the drought in May. *Capital letters at the bottom of each graph indicate significant difference between lines at $\alpha = 0.05$. Lowercase letters above bars indicate significant difference between irrigation treatment within each line at $\alpha = 0.05$. Error bars indicate standard error of the means.*
Figure 4-45. Maximum quantum yield of dark-adapted leaves of transgenic lines (I12, I23, I28, I32), St. Augustinegrass (S) and wild-type bahiagrass (WT). (A) During drought in May 2008 (B) In June 2008, during recovery from drought. Capital letters at the bottom of each graph indicate significant difference between lines at $\alpha = 0.05$. Lowercase letters above bars indicate significant difference between irrigation treatment within each line at $\alpha = 0.05$. Error bars indicate standard error of the means.
Figure 4-46. SPAD meter readings of transgenic lines (I12, I23, I28, I32), St. Augustinegrass (SA) and wild-type bahiagrass (WT). (A) During drought in May 2008 (B) In June 2008, during recovery from drought. Capital letters at the bottom of each graph indicate significant difference between lines at $\alpha = 0.05$. Lowercase letters above bars indicate significant difference between irrigation treatment within each line at $\alpha = 0.05$. Error bars indicate standard error of the means.
Figure 4-47. Dry weight of rhizomes produced by *ATHB16* expressing lines (I12, I23, I28, I32) and wild-type bahiagrass (WT) and St. Augustinegrass (SA) in non-irrigated plots. Capital letters at the bottom of each graph indicate significant difference between lines at $\alpha = 0.05$. Error bars indicate standard error of the means.

Figure 4-48. Dry weight of roots produced by *ATHB16* expressing lines (I12, I23, I28, I32) and wild-type bahiagrass (WT) and St. Augustinegrass (SA) in non-irrigated plots. Capital letters at the bottom of each graph indicate significant difference between lines at $\alpha = 0.05$. Error bars indicate standard error of the means.
Figure 4-49. RT-PCR using analysis for expression of the *ATHB16* gene using specific primers for amplification of cDNA from transgenic lines (B11, B3, B6, B7, B9) [278 bp] compared to WT and plasmid.
CHAPTER 5
SUMMARY AND CONCLUSIONS

Bahiagrass (*Paspalum notatum* Flügge) is a popular forage and turf species in the southeastern US due to its persistence under low-input conditions. However, the turf quality of bahiagrass is limited due to its open growth habit and prolific production of long inflorescences. Two transgenic strategies to alter bahiagrass plant architecture and improve its turf quality we evaluated. The gibberellin catabolizing enzyme gene *ATGA2ox1* was subcloned under control of the constitutive maize *ubiquitin* promoter and co-transferred to the bahiagrass genome with the *nptII* selectable marker by biolistic gene transfer (Agharkar et al. 2007). The Arabidopsis *ATHB16* transcription factor was subcloned under the CaMV 35S promoter (Zhang et al., 2007). We recently reported successful improved turf quality in bahiagrass following reduction of bioactive gibberellic acid, by constitutive expression of *GA2oxidase1* (*AtGA2ox1*) or *ATHB16* from Arabidopsis. The goal of this study was to comparatively evaluate the turf quality and performance of transgenic bahiagrass plants overexpressing *AtGA2ox1* or *ATHB16* and evaluate stable transgene expression under field conditions.

In field study I several superior *ATGAox1* lines were identified. *ATGAox1* expressing lines produced significantly more tillers than wild-type bahiagrass. Specifically lines B3 and B9 consistently produced the most tillers and hence displayed the greatest density. The increased number of tillers resulted in a faster establishment for *ATGAox1* lines. Increased tillering was also associated with a more upright growth and less visible rhizomes. Higher density, more tillers, faster establishment and upright growth resulted in greater production of clippings when mowing the plants. Transgenic *ATGAox1* plants also showed reduced flowering. Specifically this was significant for lines B3, B7 and B9. Inflorescence stem length was also reduced significantly in lines B11, B3, B6 and B7. Lines B3 and B9 were the most similar *ATGAox1* lines in this
study. Based on the results of this study, lines B3 and B9 having improved density and fewer inflorescences, display an overall improved turf quality to the wild-type

In field study II, two superior ATGAox1 lines, in addition to B3 were identified. ATGAox1 expressing lines B10, B3, B7 and B8 produced significantly more tillers than wild-type bahiagrass. Furthermore, lines B3 and B7, in Field Study I, consistently produced the most tillers and hence displayed the greatest density. The increased number of tillers resulted in a faster establishment for ATGAox1 lines B3, B7 and B8. Line B7 displayed improved Spring green-up associated with higher clipping production early in the growth season. Interestingly, line B3, B7 and B8 also displayed higher clipping production at the end of the growing season. Line B7 also displayed improved drought tolerance and recovery. Transgenic ATGAox1 plants also showed reduced flowering. Specifically this was significant for lines B3, B7 and B8. Inflorescence stem length was also reduced significantly in lines B10, B3, B7 and B8. Lines B3 and B8 were the most similar ATGAox1 lines in this study. Based on the results of this study, these lines display an overall improved turf quality to the wild-type. In addition line B7 displays improved drought tolerance. Showing the best turf quality and the most consistent results, lines B3 and B7 would be a better choice for turf than the commercially available cultivars.

Data on the AtGA2ox1 transgenics suggest that GAs affect flowering, outgrowth of axillary buds and apical dominance in bahiagrass. Reduced levels of GAs may also contribute to improved drought stress response in bahiagrass.

For ATHB16 lines, a superior line was identified in field study I. Line I10 produced significantly more tillers than wild-type bahiagrass thus displaying greater density. As observed with the AtGA2ox1 lines, this increased tillering was associated with a more upright growth, faster establishment and greater clipping production Transgenic ATHB16 line I10 also showed
reduced flowering. Although all lines displayed some improved characteristics, line I10 showed the most comprehensive positive results with the most improved overall turf quality.

In field study II, several *ATHB16* lines display improved turf characteristics. Lines I12 and I28 consistently produced more tillers than wild-type bahiagrass, thus displaying the greatest density. The increased number of tillers resulted in a faster establishment for line I28. Increased tillering, faster establishment and upright growth resulted in line I28 consistently producing the most clippings. Both lines also displayed a proportional dwarning with shorter tillers and narrower leaves than the wild-type. Line I12 displays more dwarning than I28. This and the difference in establishment explains the difference in clipping production between this lines. Line I28 also displayed higher clipping production during the onset of a drought period as well as better recovery from drought. Transgenic *ATHB16* plants also showed reduced flowering. Lines I12 and I28 consistently produced fewer inflorescences than the wild-type. Inflorescence stem length was also reduced significantly in line I12. Based on the results of this study, these lines display an overall improved turf quality to the wild-type. However, line I28 displays better establishment and field performance. Showing the best turf quality, the most consistent results, and best field performance line I28 would be a better choice for turf than the commercially available cultivars.

The data presented on the *ATHB16* lines, indicate that over-expression of this transcription factor in bahiagrass significantly changes plant architecture and performance of this species. These findings are consistent with the proposed function of *ATHB16* as suppressor of cell expansion.

Introduction of *ATHB16* and *AtGA2ox1* genes altered bahiagrass’ plant architecture and field performance, hence improving its turf quality. Moreover, the phenotypes observed in the
transgenic lines could also proof beneficial for forage since total biomass production does not seem to be negatively impacted and even improved significantly in some of the transgenic bahiagrass plants. Late-season increased biomass production, as observed in some of our $AtGA2ox1$ lines would be of great benefit to the forage industry. Production of denser, faster tillering plants with more leaf tissue and fewer stems and gaps as observed in our transgenic plants can be desirable for forage plants. Reduction in flowering can also be of benefit for forage since floral stems contain low digestible compounds such as lignin and cell wall compounds cross-linked with lignin accumulation, which reduce the palatability and therefore fodder quality of the grass. Digestibility, palatability, protein and productivity would have to be analyzed using field grown transgenic bahiagrass to further explore this potential.

The mechanisms behind the transgene induced changes observed in our $AtGA2ox1$ and $ATHB16$ plants could be explored further in transgenic bahiagrass with a global gene expression profiling. Quantitative expression analysis and further exploring integration sites could also be interesting. To confirm that the phenotypes observed are a result of our transgenes chemical mutation targeting these proteins could be performed. In the case of the $AtGA2ox1$ lines exogenous application of GA could reverse the phenotype and confirm its nature.

Findings in this study confirm the great potential of transgenic technology for breeding of bahiagrass. However, the labor and expenses involved in the de-regulation of transgenic crops currently impedes the release of these plants for commercial purposes. Hybridization of transgenic crops with wild relatives is a public and environmental concern. And with the reported data by Sandhu (2007) we know that the apomictic nature of ‘Argentine’ is not an absolute barrier against this phenomenon. Another option that can prove useful in improving the time and efficiency in breeding bahiagrass is mutation breeding. Random mutagenesis is
currently being evaluated in our laboratory for application in bahiagrass breeding for forage and turf. Various interesting phenotypes have already been identified. (unpublished). PCR based screening can aid in identifying point mutations in key genes including those involved in gibberellin metabolism, cell expansion, stress response, etc. in the aims of improving turf and/or forage quality of this species. This technique would not require all the de-regulation involved in the release of genetically modified crops for commercial purposes.
APPENDIX A
PROTOCOLS USED FOR FIELD EVALUATION OF TRANSGENIC BAHIA GRASS
Measuring Maximum Quantum Yield of Dark-Adapted Leaves with PAM 2100
(PAM 2100, Heinz Walz GmbH, Germany)

Taking Measurements

1. Charge PAM prior to taking measurements.
2. Connect necessary cables to PAM:
   a. Fiber Optics
   b. Leaf clip
3. Turn on PAM-2000. A green light will flash regularly when on.
4. Insert the fiber optic cable into the leaf clip.
5. Press ‘COM’ key.
6. Use arrow keys to place cursor over ‘Mode Selection’ press ‘RTRN’ key.
7. Place cursor over ‘Saturation Pulse Mode’ and select using the ‘RTRN’ key.
8. Now on the main screen, the one with the boxes use the arrow up or arrow down keys
   and move cursor over to the box labeled ‘Run’ on the right of the screen. The box where
   the cursor is will be indicated by a dashed line instead of a solid line.
9. Select ‘run 2’ by either pressing the <-> or <+> buttons.
10. Open leaf clip and carefully place leaf inside.
11. Press red button on side of the clip to take measurement.
12. Measurements will be saved in the order taken.

Exiting Program and Turning Off

1. Press ‘COM’ key.
3. Pam will shut down.

Transferring Data to the Computer

1. Press ‘COM’ key.
2. Use arrow keys to place cursor over ‘Mode Selection’ press ‘RTRN’ key.
3. Place cursor over ‘Continuous Mode’ and select using the ‘RTRN’ key
4. From the Main Menu, choose ‘Data’ with cursor and press ‘RTRN’ key.
5. Select ‘Transfer Files’ and press ‘RTRN’ key. This will prompt the message ‘data ready’.
6. Connect Pam to RS-232 cable, already connected to the computer.
7. Open PamWin 2100 program on the computer and select ‘Com 1’ and press ‘Enter’ key.
8. Data will be downloaded.
APPENDIX B
LABORATORY PROTOCOLS FOR EVALUATION OF STABLE TRANSGENE
EXPRESSION OF TRANSGENIC BAHIAGRASS VEGETATIVE PROGENY UNDER
FIELD CONDITIONS

Purification of Total RNA using the RNeasy® Plant Mini Kit from Qiagen

1. Harvest 100 mg of tissue from young leaves and freeze immediately using liquid
   nitrogen. Store tissue in -80°C if not going to be used immediately.
2. Add 10-μl β-mercaptoethanol per 1 ml Buffer RLT.
3. Add 44 ml of 100% ethanol to the RPE buffer concentrate to obtain a working solution.
4. Place tissue in liquid nitrogen and grind into fine powder with a previously autoclaved
   mortar and pestle.
5. Decant ground sample to a sterile, RNase-free 2 ml microcentrifuge tube previously
   cooled with liquid nitrogen.
6. Add 450 μl Buffer RLT (with the β-ME) to the tube and vortex vigorously.
7. Pipette the lysate onto a QIAshredder spin column (lilac) placed in a 2 ml collection tube
   and centrifuge for 2 min at full speed in a bench-top centrifuge.
8. Transfer the supernatant of the flow-through to a new sterile microcentrifuge without
   disturbing the pellet.
9. Estimate the approximate volume of the supernatant and add 0.5 volume 100% ethanol
   to the collected supernatant and mix immediately by pipetting.
10. Transfer entire sample, including any precipitate formed, to an RNeasy mini column
    (pink) placed in a 2 ml collection tube.
11. Close the tube gently and centrifuge for 15s at 9,300g. Discard the flow-through solution.
12. Perform DNase treatment using the RNase-Free DNase Set Qiagen® (see below).
13. Transfer the RNeasy column to a new 2 ml collection tube.
14. Add 500 μl Buffer RPE into the column and centrifuging for 15 s at 9,300g. Discard the
    flow-through solution.
15. Add another 500 μl of Buffer RPE onto the column and centrifuge for 2 min at 9,300g.
    Discard the flow-through solution.
16. Place column in a new sterile 1.5 ml collection tube supplied with the kit.
17. To elute the RNA, pipette 30 μl RNase-free water directly onto the RNeasy silica
    membrane in the center of the column. Close the tube gently and centrifuge for 1 min at
    9,300g.
18. Estimate concentration using the Nanodrop spectrophotometer (see below).
19. Store RNA at -80°C.
**DNase Treatment using the RNase-Free DNase Set from Qiagen**

1. Dissolve the solid DNase I (1500 Kunitz units) in 550 μl of the provided RNase-free water to prepare the DNase I stock solution. Mix gently. Store the solution at -20ºC. for up to 9 months.
2. Add 10 μl DNase I stock solution to 70 μl Buffer RDD for each sample and mix by inverting gently.
3. Add 350 μl Buffer RW1 to the column and centrifuging for 15 s at 9,300g. Discard the flow-through.
4. Pipette the 80 μl DNase I-RDD buffer mixture onto the RNeasy silica-gel membrane and incubate at room temperature for 15 min.
5. Add another 350 μl Buffer RW1 to column and centrifuge for 15 s at 9,300g. Discard the flow-through.
6. Continue with step 13 of the total RNA isolation protocol.

**Using the Nanodrop ND-1000 Spectrophotometer**

(Nanodrop Technologies, Wilmington, DE, USA)

1. Pipette 2 μl a drop of water on the upper and lower pedestals and wipe off using a soft laboratory wipe.
2. Pipette 1 μl water on the lower pedestal, close the sampling arm.
3. Open Nanodrop software on the computer.
4. Select the ‘Blank’ option on the computer screen to set the blank.
5. Open the sampling arm and wipe the pedestals using a laboratory wipe.
6. Pipette 1 μl sample onto the lower pedestal and close the sampling arm.
7. Initiate the measurement using the computer software.
8. On completion of the measurement, open the sampling arm and wipe the sample from both the upper and lower pedestals using a laboratory wipe.
9. Repeat from step 5 for subsequent samples.
10. Upon completion of measurements, clean the pedestals as described in step 1.

**cDNA Synthesis using the iScript™ cDNA Synthesis Kit from Bio-Rad**

1. Estimate the volume of each sample required to get 1 μg RNA using Nanodrop concentrations.
2. Set up the reaction mix as follows:
   5x iScript Reaction Mixture 4 μl
   iScript Reverse Transcriptase 1 μl
   Nuclease-free water x μl RNA template (1 μg RNA) x μl
   Final volume 20 μl
3. Use the following cycling conditions:
   5 min at 25ºC
   30 min at 42ºC
   5 min at 85ºC
   Hold at 4ºC
4. Store the cDNA at 4ºC. 5.
Basic RT-PCR Set-up using the HotStarTaq® DNA Polymerase from Qiagen

1. Prepare a master-mix for all the samples, including controls, as follows
   10x Buffer 2 μl
   5x Q solution 4μl
   50x dNTP mix 0.4 μl
   10 μM Forward primer 1 μl
   10 μM Reverse primer 1 μl
   Sterile ddH₂O 9.5 μl
   HotStarTaq® 0.1 μl (add last)
   Final volume 18 μl
2. Keep master mix on ice until used.
3. Label RT-PCR tubes.
4. Dispense 2 μl of cDNA to the samples, 2 μl of sterile ddH₂O to the negative control and 2 μl of plasmid (50 pg/μl) to the positive control tubes.
5. Add 18 μl of the master mix into each tube.
6. Spin briefly and start the PCR program.
   **Note:** Remember to start the PCR program with 15 min at 95°C to activate the HotStarTaq®

**PCR cycling parameters:**

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Time</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>95°C</td>
<td>15 min</td>
<td>HotStarTaq®</td>
</tr>
<tr>
<td>95°C</td>
<td>30 sec</td>
<td>Initial denaturing</td>
</tr>
<tr>
<td>58°C</td>
<td>30 sec</td>
<td>Denaturing</td>
</tr>
<tr>
<td>72°C</td>
<td>1 min</td>
<td>Annealing</td>
</tr>
<tr>
<td>72°C</td>
<td>10 min</td>
<td>Extension</td>
</tr>
<tr>
<td>4°C</td>
<td>hold</td>
<td>Final extension</td>
</tr>
</tbody>
</table>

{30 cycles}


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BIOGRAPHICAL SKETCH

Paula Noemí Lomba Otero was born in 1984 in Bayamón, Puerto Rico, to Lilia Otero and Esteban Lomba. She grew up in San Juan, PR where she attended elementary, middle, and high school. She graduated from University of Puerto Rico High School on May 2002. After high school, she received an academic scholarship to attend University of Florida in Gainesville, FL from where she received her Bachelor of Science in biology on May 2006. While completing her undergraduate degree, she started working in Dr. Fredy Altpeter’s Laboratory of Molecular Plant Physiology. Paula began her graduate career in the Agronomy Department at the University of Florida in August 2006 under the supervision of Dr. Fredy Altpeter.