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CHARACTERIZATION OF OVERWINTERING PERENNIALITY IN NAPIERGRASS: TRADITIONAL AND MOLECULAR APPROACHES

A Dissertation

by

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ABSTRACT

Napiergrass (*Pennisetum purpureum* Schum.) is a tropical grass limited in adaptation to the southern United States, and efforts to expand its area of production as a quality forage crop as well as a high-biomass biofuel crop are needed. Phenotypic selection and screening of napiergrass S_1 inbred lines and F_1 hybrids for increased winter hardiness (USDA Winter Hardiness Zones 8b to 7b) demonstrated that napiergrass is more winter hardy than expected. Variation in hardiness zone 7b winter weather patterns at Vernon, TX, and Alma, AR, resulted in 40% survival across genotypes and no winter survival, respectively, even though the minimum temperatures at both locations were similar. Winter survival in this species is associated with rhizome development. Therefore, rhizome candidate gene expression assays using quantitative polymerase chain reaction (qPCR), on fall and spring sampled rhizomes for genes associated with overwintering, rhizome development, and rhizome proliferation resulted in two cloned genes, APETELA2 (AP2) and Rare Cold Inducible 1 (RCI1) conferring detectable changes in gene expression patterns. Spring sampled rhizomes showed the greatest fold change (FC), specifically in the zone of cell division across all genotypes. Attempts to transfer genes associated with cold-tolerance from oriental fountaingrass (*Pennisetum orientale* L.C. Rich.) and buffelgrass [*Cenchrus ciliaris* (L.) syn *Pennisetum ciliare* Link] into napiergrass via wide hybridization were not successful because no interspecific hybrids were recovered. Analysis of pollen tube growth and pollen-pistil interactions did not reveal barriers to these hybridizations, but post-

fertilization events may be the reason for the failure to recover hybrids and this needs to be investigated.

DEDICATION

I would like to dedicate this work to my parents, Charlie and Debbie Dowling, who have been a great reinforcement to me through the years. My dad told me to go to college if I did anything while he was lying on what we thought was his deathbed. Well, Dad, I did, and here I am 15 years later. Thank you for your prayers, emotional and financial support, as well as giving me the basic knowledge and skills to be successful in this world. I would also like to thank Grams Berit and Slim for all of their loving kindness and support as well. I could not have made it this far without your continuous cheer and encouragement.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER I

LITERATURE REVIEW

The Genus *Pennisetum*

The genus *Pennisetum* belongs to the Poaceae family as part of the multicellular eukaryotes within the angiosperms of the Plantae kingdom. The genus is a member of the Panicoideae subfamily and is one of the larger genera in the tribe Paniceae (Pilger, 1940). *Pennisetum* is part of a species complex within its own genus and the genera *Cenchrus* and *Setaria*, which are comprised of taxa with diverse morphology, methods of reproduction, and modes of pollination (Brunken, 1977; Jauhar, 1981). For example in only 56 years (from Linnaeus in 1753 to Willdenow in 1809), pearl millet (*Pennisetum glaucum* [L.] R. Br.) has been classified as a member of five different genera: *Panicum*, *Holcus*, *Alopecurus*, *Pennisetum*, and *Penicillaria* (Brunken, 1977). Many *Pennisetum* species are widely adapted to an array of tropical, subtropical, and temperate environments (Juahar, 1981). The genus consists of approximately 140 species, which has a range of base chromosome numbers (x=5, 7, 8, 9), ploidy levels (diploid to octoploid), methods of reproduction (sexual, obligate and facultative apomicts), and life cycles (annual, biennial, and perennial) (Martel et al., 1997). The genus is subdivided into five subsections, or subgenera, based primarily on differences in their floral morphology. These are *Gymnothrix*, *Eupennisetum*, *Penicillaria*, *Heterostachya*, and *Brevivalvula* (Stapf and Hubbard, 1934).

Pearl Millet

Pearl millet putatively originated in a transitional area of Sub-Saharan Africa called the Sahel Region that extends from Senegal to western Sudan (Harlan and de Wet, 1971). The species spread towards the Fertile Crescent and further east into India where it became naturalized. An annual species that is cultivated for both grain and forage, pearl millet is grown primarily as a grain crop for human consumption in India, Pakistan, and Africa. However, in the United States, Australia, and parts of South America, it is almost exclusively used as forage for livestock (Hanna et al., 2004a). In the United States, there has been increasing interest in growing pearl millet as a grain crop to provide feed for poultry and livestock (Hanna et al., 2004a). More than 25 million ha of pearl millet are cultivated in Africa and East Asia, where it is a staple grain crop providing essential carbohydrates, vitamins, minerals, and amino acids. Pearl millet can be productive on poor, droughty, infertile soils and will respond to supplemental fertility and moisture. Because of its wide range of adaptation, it has been referred to as the world's hardiest crop that will grow in arid environments and on marginal soils (ICRISAT, 2015). It tolerates low rainfall conditions in the Sahel region of western Africa with an annual precipitation of only 250 to 300 mm and is also productive on acidic soils (Bogdan, 1977; Hanna et al., 2004b). Even though inbred lines are available for hybrid seed production, pearl millet is naturally cross-pollinated because of its protogynous flowering behavior and is a diverse, heterozygous species (Bogdan, 1977; Hanna et al., 2004a).

Napiergrass

Napiergrass (*Pennisetum purpureum* Schum.), also referred to as elephantgrass, is native to the tropical regions of Africa and has become naturalized in the tropics throughout the world (Boonman, 1993; Hanna et al., 2004b). Its documented history is rather recent. The first report of its benefits as a forage was in 1905 when a Hungarian missionary, who was in what at that time was northwest Rhodesia, sent plant material to the Zurich Botanical Gardens (Melle, 1918). It was given the common name napiergrass or Napier grass in honor of Colonel Napier of Bulawayo, Rhodesia who brought this grass to the attention of officials with the Rhodesian Department of Agriculture. In Uganda, napiergrass grows in a humid, warm, fertile belt at an elevation ranging from less than 250 m to 1500 m near the northern and northwestern shores of Lake Victoria, on both sides of the Zaire-Uganda border and northeast of Mount Ruwenzori up to 2600 m in elevation. It competes well with forest vegetation of this area because of natural fire regimes as well as traffic and defoliation of tree saplings by elephants (Boonman, 1993). Some genotypes have sufficient cold-tolerance to survive and persist in subtropical regions of the world, including the southern United States. Normally the species occurs in areas that receive at least 1000 mm of rainfall $year^{-1}$, but it will grow and persist in areas that receive substantially less precipitation (Bogdan, 1977).

Because it is a C_4 perennial grass with robust, compact, and creeping rhizomes with dense clumps of basal buds giving rise to new tillers, napiergrass has been used in the tropics as cut and carry foraging systems for beef and dairy livestock (Bogdan,

1977). This leafy, hardy grass grows to a height ranging from 2 to 6 m. In general, napiergrass has desirable characteristics for a forage crop such as more drought tolerance, grows on low soil pH with increased levels of heavy metals, low nutrient requirements, more insect and pest resistance, a more rapid growth rate, and greater feeding value than other C_4 grasses. Napiergrass is documented of producing as much as 85 t dry wt. ha^{-1} y^{-1} of biomass, which is among the greatest for forage grasses, and it has one of the most rapid growth rates of higher plants (Orodho, 2006; 2011; Rengsirikul et al., 2013).

Napiergrass is cross-pollinated because of its protogynous flowering behavior, which results in a greater level of heterozygosity and extensive genetic diversity that can be utilized in breeding programs. However, it can tolerate some inbreeding without significant loss of vigor. This is true for the S_2 generation of plants in the Perennial Grass Breeding Program at Texas A&M University—College Station. Both napiergrass and pearl millet belong to the subgenus *Penicillaria* (Stapf and Hubbard, 1934). It is a short-day, photoperiodic species and typically does not flower during the peak growing season in most Texas and Arizona production areas (Osgood et al., 1997). Naturally occurring populations normally produce little or no seed, thus, uniform stands have to be established vegetatively. 'Merkeron' and 'Mott' are the only forage cultivars that have been released in the United States. Mott is a dwarf that was selected from a self-pollinated population of Merkeron. Merkeron is characterized by having improved pollen production, resistance to the eye spot disease caused by *Helminthosporium*

sachari B. de Haan, more winter hardiness, more leafiness, and greater yields (Burton, 1989; Sollengerger, 1989).

A desirable characteristic of napiergrass is its ability to persist and produce adequate forage on marginal lands (O'brien, 2012). Growing the grass for biofuels on marginal and abandoned lands rather than on fertile cropland minimizes food versus fuel competition by eliminating the need to compete for land normally used for crop production. Species capable of producing and assimilating biomass on marginal lands tend to have other beneficial characteristics. These include, but are not limited to, improved abiotic stress tolerance (e.g., drought, heat, and salinity), improved water-use efficiency (WUE), and improved nutrient-use efficiency (NUE). Napiergrass has all of these important traits.

Oriental Fountaingrass and Buffelgrass

Oriental fountaingrass (*Pennisetum orientale* L.C. Rich.) is a perennial forage species that is considered a moderately temperate, Mediterranean grass which will grow at elevations from 600 to 2700 m (Stewart, 1945). It has more cold-tolerance than any other *Pennisetum* species with the exception of flaccidgrass (*Pennisetum flaccidum* Griseb.). The grass is drought-tolerant, high in nutritive value, and palatable to livestock (Parihar and Tripathi, 1987). Oriental fountaingrass is a modestly rhizomatous species that grows from 1 to 1.5 m in height and is commonly found growing on low stony hills in the Western Himalayans (Koul et al., 1999). It has spread westward from India into

Asia Minor and as far west as Morocco in northern Africa (Stewart, 1945; Bor, 1960; Patil et al., 1962). Oriental fountaingrass produces forage when grown on productive soils in Morocco where it usually does not senesce and remains green throughout the year (Whyte et al., 1959). It also has potential as perennial forage in North Carolina (Chatterji and Timothy, 1969). As with most deep-rooted perennial grasses, this species controls erosion very well, especially on the mountain slopes of the Himalayas. Oriental fountaingrass contains about 7.5% crude protein, produces between 2 to 3 t of dry wt. ha^{-1} yr^{-1}, and has 45 to 58% dry matter digestibility (Sarwar et al., 2006).

Buffelgrass [*Cenchrus ciliaris* (L.) syn *Pennisetum ciliare* Link] is a perennial C_4 grass that is native to tropical and subtropical Africa (Bogdan, 1977). It is an important pasture grass in the arid regions of the tropics and subtropics throughout much of the world. There have been extensive plant exploration and collection trips in eastern and southern Africa to locate buffelgrass ecotypes, and a number of selections were evaluated in Kenya and Tanzania by Sands et al. (1970) and Wigg (1973). Because of the morphological diversity in the ecotypes collected in the Republic of South Africa, Bashaw (1985) proposed that this area was probably the center of origin for the species. According to Bogdan (1977), buffelgrass spread north into dry and arid regions of subtropical and tropical Africa and eventually to Madagascar, the Canary Islands, Arabia, and finally to the arid regions of India and Pakistan. East Africa is generally considered a center of genetic diversity for buffelgrass (Boonman, 1993). The drought tolerance and forage quality of buffelgrass make it a desired pasture grass, and it is

grown on approximately 50 million ha worldwide (Hanselka et al., 2004). Both buffelgrass and oriental fountaingrass have a protogynous flowering behavior, which is characteristic of most *Pennisetum* species.

Cytogenetics of Pearl Millet, Napiergrass, Buffelgrass, and Oriental Fountaingrass

Members of the genus *Pennisetum* have been divided into three gene pools by Harlan and de Wet (1971) based on the reproductive isolation of pearl millet with its undomesticated relatives. The species are classified as being members of the primary, secondary, and tertiary gene pools. This taxonomic classification is based on the ability or inability of wild species to hybridize with the domesticated form of *Pennisetum*, which impacts the amount of gene flow that occurs between all the members of the gene pool (Robert et al., 2011). The primary gene pool is defined as taxa, predominantly pearl millet and undomesticated diploids that will successfully hybridize with one another as in the same species. The secondary gene pool consists of cenospecies that can exchange DNA, but they experience considerable difficulty with fertility when hybridized with a member of the primary gene pool. The tertiary gene pool consists of species that are difficult to hybridize, and when hybrids are recovered, gene transfer is a rare, spontaneous event. Pearl millet and napiergrass belong to the *Pennisetum* primary and secondary gene pools, respectively (Harlan and de Wet, 1971). Napiergrass is an allotetraploid (2n=4x=28) with the genome formula A'A'BB, and Jauhar (1981) concluded that the A' genome is homeologous with members of the A genome of pearl

millet, which has a genome formula AA (2n=2x=14). During meiosis in their interspecific hybrids, some members of the A' genome from napiergrass pair with members of the A genome from pearl millet and as many as seven bivalents have been observed (Muldoon and Pearson, 1979; Jauhar, 1981; Dujardin and Hanna, 1985). Members of the B genome are present as seven univalents. They tend to lag behind the dividing chromosomes of the A and A' genomes during anaphase I and II and often some or all of them are not incorporated into the developing nuclei which affects pollen viability and the fertility of the plant. Pearl millet chromosomes are about twice as large as those of napiergrass (4.02 μm vs. 2.00 μm) (Techio et al., 2010). It has been hypothesized that during the molecular and chromosomal evolution of pearl millet, ectopic recombination of DNA fragments in concert with the autonomous activity of *Ac/Ds* transposable elements, resulted in the expansion of the pearl millet chromosomes (Hutley et al., 1995; Martel et al., 1997).

Buffelgrass is a member of the tertiary gene pool. However, there is some uncertainty as to whether or not the species is a member of the genus *Pennisetum* or *Cenchrus*. The two genera are closely related and the primary characteristic that separates them is fusion or lack of fusion of bracts of the involucre (Hanslka, et al., 2004). The species has a base chromosome number of x=9, and tetraploidy is the most commonly reported chromosome number (2n-4x=36). However, pentaploids (2n=5x=45), hexaploids (2n=6x=54), septaploids (2n=7x=63), and a range of aneuploids have been reported for the species (Burson et al., 2012). The most common method of

reproduction is aposporous apomixis followed by pseudogamy (Fisher et al., 1954; Snyder et al. 1955). Buffelgrass was considered an obligate apomict until a sexual off-type plant was discovered (Bashaw, 1962). This sexual plant made it possible to develop improved types through hybridization (Taliaferro and Bashaw, 1966). Before this, all commercially available buffelgrass cultivars were superior apomictic ecotypes that were selected, increased, and released (Burson et al., 2012; Loch, 1999; Hanselka et al., 2004). Since the discovery of this sexual plant, additional sources of sexual germplasm have been discovered by different investigators, and the species is now considered a facultative apomict having varying levels of sexuality and apomixis (Bashaw and Johns, 1983; Bray, 1978; Sherwood, et al., 1980; Visser et al., 2000).

Oriental fountaingrass is also a member of the tertiary gene pool, and it is a polymorphic species consisting of several cytotypes. It is primarily considered a tetraploid species (2n=4x=36); however, Jauhar (1981) reported chromosome numbers of 2n=18, 27, 36, 45, and 54. Therefore, its ploidy number ranges from diploid to hexaploid. The morphological subsection of this species is *Heterostachya*. The polyploids reproduce primarily by aposporous apomixis. Chatterji and Timothy (1969) observed megasporogenesis and embryo sac development in five accessions, and they reported that all were obligate apomicts. However, Ramu (1994) examined 11 oriental fountaingrass accessions and reported all reproduced by facultative apomixis and one accession (PI 315867) was predominantly sexual (85.5%). All of these accessions were tetraploids, and two of them were included among the accessions studied by Chatterji

and Timothy (1969). The reproductive behavior of oriental fountaingrass is complex, but it appears to be a facultative apomict with varying levels of sexuality and apomixis.

Objectives

The objectives of this research are to:

1. Evaluate the efficacy of producing hybrids between napiergrass and both oriental fountaingrass and buffelgrass towards producing cold-tolerant F_1 hybrids. This includes investigating pollen-pistil interactions of interspecific crosses, using embryo rescue to recover hybrid seedlings from wide crosses, and pollinating targeted species in a greenhouse to produce interspecific hybrids.
2. Phenotypic screening of napiergrass germplasm in the Perennial Grass Breeding Program at Texas A&M University in conjunction with rhizome candidate gene expression assays. This includes the selection and advancement of elite experimental germplasm to screen for overwintering at Alma, AR and Vernon, TX (USDA Winter Hardiness Zone 7b). Assays of candidate gene expression [i.e. quantitative polymerase chain reaction (qPCR)] of fall and spring collected rhizomes for genes associated with overwintering, rhizome development, and rhizome proliferation of plants at College Station (zone 8b) and Commerce, TX (zone 8a).

CHAPTER II

INTERSPECIFIC HYBRIDIZATION BETWEEN NAPIERGRASS AND OTHER *PENNISETUM* SPECIES

Introduction

Plant breeders often produce novel genotypes by successfully introgressing traits from one species into another of the same genus or another genus (Clausen, 1952). Many wild relatives of cultivated crop species possess genes that can be utilized in a breeding program to improve traits of interest, and these traits include, but are not limited to, improving resistance to insects and pathogens, contribution of genes associated with increased yields and quality of fruit/grains, and resistance/tolerance to abiotic stresses such as drought, salinity, and cold (Harlan, 1976; Stalker, 1980; Hajjar and Hodgkin, 2007; Hajjar and Hodgkin, 2008; Dwivedi et al., 2008). This provides a useful source of genetic variation from which many important traits can be selected. The same is true for *Pennisetum* species. The *Pennisetum* branch of the monophyletic bristle clade of grasses consists of several important forage species, pearl millet a major grain crop, and at least 17 documented apomictic species (Doust and Kellogg, 2002; Ozias-Akin et al., 2003). Apomixis was traditionally seen as a barrier to hybridization until more recently, and the availability of sexual types is necessary to create succeeding generations (Bashaw, 1962). Hybridization of these species with napiergrass could incorporate desirable traits associated with tolerance to abiotic and biotic stresses. Traits such as drought tolerance, pest resistance, and improved winter hardiness can potentially

be transferred from oriental fountaingrass to napiergrass. Wide hybrids between pearl millet and oriental fountaingrass produced by Dujardin and Hanna (1987) demonstrated this is feasible within the *Pennisetum.* Wild *Pennisetum* species are used as forage grasses in the tropical and subtropical grasslands. Besides napiergrass, other non-cultivated grasses such as oriental fountaingrass, *P. squamulatum* Fresen., mission grass [*P. polystachion* (L.) Schult.], flaccidgrass, buffelgrass, African feather grass (*P. macrourum* Trin.), and Kyasuma grass (*P. pedicellatum* Trin.) have contributed to forage production in the humid and subhumid tropics (Robert et al., 2011).

Despite wide hybridization's potential for transferring important traits from one species to another, it is often unsuccessful due to reproductive barriers. These can result from pollen-pistil incompatibilities that prevent pollen tubes from reaching or entering female gametophytes. Even if pollen tubes enter the female gametophyte and the sperm nuclei are released, fertilization and/or postzygotic barriers can prevent the development of seed. When fertilization does occur, the developing embryo or endosperm often aborts during early seed development and viable seed are not produced. These reproductive barriers prevent natural gene flow between *Pennisetum* species of the tertiary gene pool with those in the primary and secondary gene pools regardless of their base chromosome number. However, wide hybrids have been reported between species of the primary, secondary, and tertiary gene pools. Regular hybridization techniques have been used to transfer rust resistance from a wild progenitor of the primary gene pool, *P. glaucum* (L.) R. Br. spp. *monodii* (Maire) Brunken into pearl millet (Hanna et

al., 1985). Two other wild, diploid relatives of pearl millet, *P. violaceum* (Lam.) Rich. and *P. mollissimum* Hochst, are sympatric in the wild with pearl millet, and they have produced natural hybrids with pearl millet (Brunken, 1977). Hybrids have been recovered from crosses between pearl millet and napiergrass (Burton, 1944), pearl millet and tetraploid oriental fountaingrass (2n=4x=36) (Hanna and Dujardin, 1982), pearl millet and diploid oriental fountaingrass (2n=2x=18) (Patil and Singh, 1964), pearl millet and *P. setacum* (Forssk.) Chiov. (2n=3x=27) (Hanna, 1979), pearl millet and *P. squamulatum* (2x=6x=54) (Dujardin and Hanna, 1983), pearl millet and *P. schweinfurthii* Pilger (2x=2x=14) (Hanna and Dujardin, 1986), pearl millet and buffelgrass (Read and Bashaw, 1974), pearl millet and *P. mezianum* Leek. (2n=4x=32) (Nagesh and Subrahmanyam, 1996), buffelgrass and birdwoodgrass (*Cenchrus setigerus* Vahl; 2x=4x=36) (Read and Bashaw, 1969; Bashaw and Hignight, 1990), buffelgrass and flaccidgrass (2n=4x=36) (Hussey et al., 1993), purple fountaingrass (2n=6x=54) (*P. setaceum* [Forsk.] Chiov.) and buffelgrass (Simpson and Bashaw, 1969), and flaccidgrass and *P. mezianum* (Bashaw et al., 1992). Hanna et al. (1989) also reported trispecific hybrids [(pearl millet x napiergrass) x *P. squamulatum*)], double-cross hybrids [(pearl millet x napiergrass) x (pearl millet x *P. squamulatum*)], and backcross hybrids [pearl millet x (pearl millet x *P. squamulatum*)]. Kaushal et al. (2010) reported novel trispecific *Pennisetum* double-cross hybrids after crossing a backcross hybrid between diploid pearl millet (GG) and diploid oriental fountaingrass (OO) (2n=3x=23, GGO) with a hexaploid F_1 hybrid between tetraploid pearl millet (2n=4x=28, GGGG) and *P.*

squamulatum (2n=8x=56, SSSSSSSS) (2n=6x=42, GGSSSS). All six double-cross hybrids had 44 chromosomes consisting of 21 from pearl millet, 14 from *P. squamulatum*, and 9 from oriental fountaingrass. Based on flow cytometric data and meiotic analysis, these hybrids resulted from the fertilization of unreduced egg cells with reduced male gametes (Kaushal et al., 2010). All hybrids were male sterile and reproduced by aposporous apomixis except for one plant that was male and female sterile. Intergeneric hybrids have been reported between rice (*Oryza sativa* L.) and a *Pennisetum* species (Wu and Tsai, 1963), hexaploid wheat (*Triticum aestivum* L.) and pearl millet (Ahmad and Comeau, 1990), and pearl millet and maize (*Zea mays* L.) (Nitsch et al., 1986).

The above demonstrates cross-compatibility between some species in the genus *Pennisetum*, but for most hybrids pearl millet was the maternal parent. However, for most species interspecific crossability is low and sporadic (Dujardin and Hanna, 1989). Hybrids have not been produced between napiergrass and other members of the tertiary gene pool. The only reported interspecific hybrids involving napiergrass are with pearl millet and the double-cross and trispecific hybrids reported by Hanna et al. (1989). The reasons for low crossability between napiergrass and species of the tertiary gene pool are unknown and warrant investigation.

Materials and Methods

Interspecific Hybridizations

Pearl millet accession PEGL09TX04 and two napiergrass genotypes (the cultivar Merkeron and a male-sterile accession PEPU09TX01) were used as checks in this experiment because napiergrass x pearl millet and napiergrass x napiergrass hybrids are reported to be easily produced (Burton, 1944, Dowling et al. 2013). In addition, PEPU09TX01 was self-pollinated to determine the amount of self-compatibility in this male-sterile accession and to compare it with other intra- and interspecific pollinations. Table 1 list the buffelgrass, oriental fountaingrass, pearl millet, and napiergrass genotypes used as pollen parents in all pollinations. Only one napiergrass genotype (PEPU09TX01) was used as the maternal parent because it expresses environmentally-modulated, genetic male sterility in the greenhouse in College Station, TX. This male sterility and the plant's protogynous flowering behavior made it possible to make a large number of pollinations without emasculating the female florets because the chances of self-pollination were greatly reduced.

Controlled pollinations were made between napiergrass and oriental fountaingrass and buffelgrass under greenhouse conditions at College Station, TX, during the winter months as described by Dowling et al. (2013) to produce more winter hardy interspecific hybrids. Inflorescences of the female plants were enclosed in glassine bags prior to stigma exertion. When the stigmas of PEPU09TX01 began to protrude from the florets, inflorescences of male parents that were undergoing anthesis

were gently agitated above a glass Petri dish and the pollen was collected in the dish. Then the glassine bags were removed from the inflorescences of the female parents and the pollen in the Petri dishes was dusted onto the stigmas. Following pollination, the napiergrass inflorescences were again enclosed in glassine bags and the bags remained on the inflorescences until maturity to prevent seed loss from shattering. The enclosed inflorescences were collected 4-6 wk after pollination and kept in cold storage until the florets were threshed and germinated. For germination, florets were evenly distributed over the surface of flats filled with potting soil and then additional soil was lightly sprinkled over the florets to cover them. The flats were then watered as needed and monitored daily for emerging seedlings.

Table 1. Plant materials used as pollen parents in wide crosses and for pollen tube growth studies.

Species	Identification	Origin	Ploidy, Base Chromosome Number	Reproduction
Pennisetum glaucum	PEGL09TX04	College Station, TX	2n=2x=14; x=7	Sexual
Pennisetum ciliare	cv. Common	South Africa	2n=4x=36; x=9	Facultative apomict
	cv. Frio	College Station, TX	2n=5x=45; x=9	Facultative apomict
Pennisetum purpureum	cv.Merkeron	Tifton, GA	2n=4x=28; x=7	Sexual
Pennisetum orientale	cv. Cowboy	Kennedy, TX	2n=4x=36; x=9	Facultative apomict
	PI 315867	Czechoslovakia	2n=4x=36; x=9	Facultative apomict
	433	ICRISAT, India	2n=4x=36; x=9	Facultative apomict
	PI 314994	India	2n=4x=36; x=9	Facultative apomict
	PI 269961	Pakistan	2n=4x=36; x=9	Facultative apomict
	PEOR09TX01	College Station, TX	2n=4x=36; x=9	Unknown
	PI 271595	India	2n=4x=36; x=9	Facultative apomict
	PI 271596	India	2n=4x=36; x=9	Facultative apomict

Embryo rescue techniques were used to increase chances of recovering hybrid seedlings. Pollinated inflorescences were removed from the maternal parent 2-3 d after controlled pollinations were made. Individual spikelets were removed from the inflorescences, placed into glass vials containing distilled water, and then taken into the laboratory. The fertile floret was dissected from each spikelet using a dissecting microscope, and then the pistils were removed. The pistils were placed in sterilized filtered water until enough were collected to place them on a medium. This was done under a laminar flow hood that has been thoroughly sterilized with 70% alcohol, UV-A and UV-B light, and the pistils were handled with autoclaved utensils. The pistils were then sterilized in a sterilization series of 50% alcohol for 1-3 min followed by a double rinse in autoclaved and filtered double distilled water. They were finally placed on a Murashige and Skoog medium in Petri dishes. Pistils were kept at various light regimens to optimize the chances for embryo growth. These include 24 h of light, 16 h of light, or complete darkness. All ovaries were placed in a 16 h light regimen after 2 or 3 wk.

Pollen Stainability

Florets were collected just prior to anthesis while the anthers were still enclosed and the anthers were dissected from the floret. Pollen stainability of each genotype was determined by placing a drop of potassium iodide (I_2KI) and the anthers on a glass microscope slide and the anthers were macerated in the solution to expose the pollen grains to the stain. After the anther tissue was removed from the slide, a cover slip was

gently placed over the solution and the pollen grains were observed with a compound light microscope under 100X magnification using bright field illumination. The number of stained and non-stained pollen grains was recorded.

In Situ Pollen-Pistil Interactions

Pollen-pistil interaction studies were conducted to obtain more insight into the actual crossability between the species. The same genotypes that were used as parents in the crosses (Table 1) were pollinated in the same manner as is outlined above for the interspecific crosses attempted. At different time intervals following pollination, 15 spikelets were removed from the pollinated napiergrass (PEPU09TX01) inflorescence and fixed in formaldehyde-acetic acid-alcohol (FAA) solution for 24 h. They were then transferred into and stored in 70% alcohol until they could be prepared for analyses. Pistils were dissected from the fixed florets under a dissecting microscope. The pistils were prepared for examination with fluorescent microscopy using a modified version of the technique described by Kho and Baer (1968). Pistils were placed into a 1N NaOH solution for 2 to 4 h depending on the time required to adequately soften the tissue, and then transferred into a solution of 0.1 N potassium phosphate (K_3PO_4) with 0.1% aniline blue for a minimum of 15-20 min before they were placed on a microscope slide. They were examined with a Zeiss Axiophot microscope equipped with an Epi-fluorescence condenser illuminated with a mercury arc bulb. Percent pollen germination was determined by counting the total number of germinated and non-germinated pollen grains on the stigmas. Pollen grains were deemed as germinated once a visible pollen

tube had emerged from the grains. Pollen tube growth into the stigma, style, and ovary was observed. The following information was collected: total number of pollen grains present on each stigma; number of grains that germinated (percent germination was calculated from this information); number of pollen tubes penetrating the stigma branches and had grown into the central axis of the stigma; number of pollen tubes that had grown into the style, ovary, and to the micropyle at the base of the ovule. Pollen tubes were considered to have entered the style once they grew beyond the lower most stigma branches on the central axis. This information was used to determine if there were any incompatibilities preventing the tubes from reaching the ovary and the micropyle

Results and Discussion

Wide Hybridizations with Napiergrass

Pollen was collected in bulk from the eight stainable oriental fountaingrass genotypes from Table 1 and used to pollinate the napiergrass stigmas. This aided in increasing the pollen load onto the napiergrass stigmas in hopes of producing an interspecific hybrid. One hundred and ten napiergrass inflorescences were pollinated with an estimated 25,600 total florets. No confirmed hybrids were recovered from these crosses. Only one seedling germinated, but it ceased growth at the first true leaf stage and died. Because of its rapid decline and small size, sufficient tissue could not be collected to determine if it was an interspecific hybrid based on its DNA content or by

utilizing oriental fountaingrass-specific EST-SSR markers that had been developed in the Perennial Grass Breeding Program at Texas A&M University. Its small size and abnormal growth habit implies it was possibly an interspecific hybrid because this type of hybrid weakness has been reported (Rieseberg and Carney, 1998). Interspecific hybrids can vary greatly in their vigor and a wide spectrum of positive to negative heterosis has been reported for several different species (Stebbins, 1958; Levin 1979; Grant, 1981; Jackson, 1985). Similar behaviors have been observed in pearl millet x napiergrass and their reciprocal F_1 hybrids in the Texas A&M Perennial Grass Breeding Program as well as in the first reports of these interspecific hybrids by Burton (1944) where progeny derived from the same maternal parent showed a drastic variation in phenotypes—from frail and chlorotic types to robust and hearty ones.

Pollen Stainability

Because only one purported napiergrass x oriental fountaingrass F1 hybrid was recovered, efforts were directed at determining why the two species did not hybridize. The pollen stainability of all of the oriental fountaingrass accessions used to pollinate napiergrass was determined. The majority of the accessions had a moderate and acceptable degree of stainable pollen. However, the stainability (2.07%) of one accession (PI 219610) was sufficiently low that it was not a factor in the pollinations, but the remaining accessions appeared to produce adequate amounts of stainable pollen (Table 2 and Figure 1). Even though ovules were not examined to determine the level of apomixis in this accession, it may be conclude from these observations that PI 219610 is

an obligate apomict that produces seed autonomously without pseudogamy or is pseudogamous when pollinated with pollen from another oriental fountaingrass accession. The female, napiergrass PEPU09TX01, is predominantly male-sterile under greenhouse conditions during the experiment induced by uncharacterized cues. When this occurred, florets on the inflorescences did not produce any anthers, which made it easy to visually identify male sterile inflorescences. However, on rare occasions, an inflorescence produced some anthers which explain the pollen stainability data in Table 2. Pollen stainability data for all accessions are presented in Table 1. Figure 1 shows stained and non-stained pollen of the oriental fountaingrass cultivar Cowboy in the same field of view of a microscope.

Pollen-Pistil Interactions

Pollen tube growth was observed in napiergrass pistils following intra- and interspecific pollinations involving four different species and 13 differing accessions that were sampled at 1 h intervals up to 24 h following pollination. Only one napiergrass genotype (PEPU09TX01) was used as the maternal parent for all pollinations because of its environmentally-modulated, genetic male sterility expressed in the greenhouse. The mechanisms controlling this behavior have not been determined, and there were a very low number of inflorescences that produced anthers over three consecutive years in the greenhouse.

Table 2. Pollen stainability of napiergrass, oriental fountaingrass, and buffelgrass genotypes.

Species/Genotype	Total No. Pollen Grains Obs.	Total No. Pollen Grains Stained	Total No. Pollen Grains Not Stained	% Stainable Pollen
Napiergrass				
PEPU 09TX01	1,177	515	662	43.75
Oriental				
'Cowboy'	538	238	345	44.24
PI 315867	612	417	195	68.13
433	695	255	440	36.69
PI 314994	1,504	712	792	47.34
PI 269961	580	342	238	58.97
PEOR 09TX01	745	364	381	48.86
PI 271595	903	436	467	48.28
PI 271596	759	342	417	45.06
PI 219610	580	12	568	2.07
Buffelgrass				
Common	2,940	2,184	756	74.29
Frio	2700	1,096	1,604	40.59

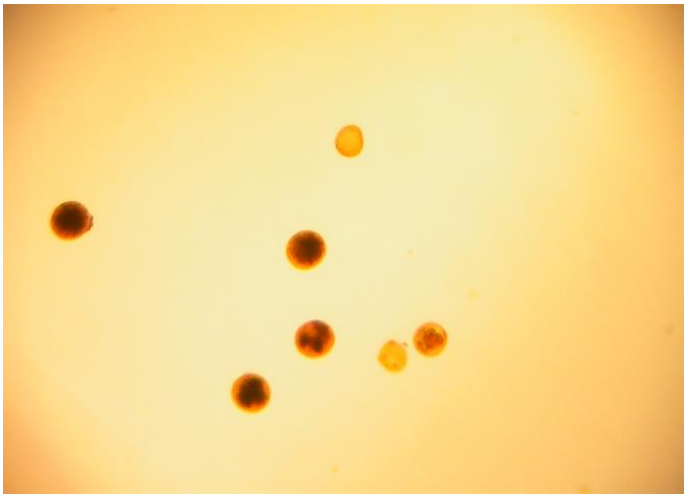

Figure 1. Pollen stainability of oriental fountaingrass 'Cowboy'. On the left to left-center are four stained pollen grains and on the right center are three empty or non-stained grains.

Tables 8 through 20 (Appendix A) providedetailed information regarding the time required for pollen grains from each paternal genotype to germinate and the pollen tubes to grow into the stigma, through the style and ovary, and to the micropyle of the pollinated napiergrass pistils. For the interspecific pollinations, pollen tubes from most genotypes grew into the ovary within 3 to 6 h after pollination; however, for one genotype, the tubes did not enter the ovary until 6 h after pollination. The pollen tubes of oriental fountaingrass accession 433 had not grown beyond the stigmas even after 24 h following pollination.

When self-pollinated, napiergrass accession PEPU09TX01, had a limited number of tubes to the ovary within 12 h following pollination, and an even fewer number reached the micropyle by 24 h.

The position of pollen grains on the napiergrass stigma and distance the pollen tubes grew down into the stigma branch or central axis was generally similar across all genotypes, species, and time points. Pollen grains landing higher on the stigma had tubes that either grew a short distance into the stigma branch or into the central axis but stopped growth before reaching the style. Tubes from pollen grains attached to the lower stigma branches or central axis tended to grow to the ovary. Reductions in the length of pollen tubes and incompatibilities brought about by genetic and biochemical incongruences including the lack of cytosolic Ca^{2+} and K^{+} accumulation or the great distance they had to travel to enter the ovary could be a factor in these crosses (Mouline et al, 2002). Because napiergrass' stigmas are very long, usually several millimeters

longer than the fertile floret (4.5-7 mm) from which it emerged, pollen tubes are presented with increased chance of failure (Gould and Shaw, 1983).

Although rarely developed, about half (44%) of the pollen produced by PEPU09TX01 was stainable. This indicates that its viability may be low. This accession was self-pollinated to obtain a better understanding of its actual pollen viability. Its rate of pollen tube growth when self-pollinated is in Table 8 of Appendix A. Its pollen required 6 h to reach the style (1.1% of pollen tubes at 6 h), 12 h to proceed from the style into the ovary (0.8% of pollen tubes at 12 h). No tubes were observed in proximity of the micropyle until 24 h following pollination (1.5% of pollen tubes at 24 h). These percentages are slightly higher at 24 h because fewer pistils (13) and pollen grains were observed (753) and lower pollen germination (50.98%). These findings are not surprising considering the limited number of anthers produced by this accession and since napiergrass is considered to be a highly cross-pollinated species.

Pollen germination on napiergrass stigmas was the highest for the pearl millet accession, PEGL09TX04 (83%), which was expected because it is a meiotically stable diploid (Table 9). PEGL09TX04 pollen tubes grew more rapidly into the ovary than tubes from any of the other intra- or interspecific pollinations. Twenty-two pearl millet pollen tubes grew into the napiergrass ovary within only 1 h after pollination (1.5%) and many tubes had grown to the micropyle within 2 h following pollination (5.1%). These growth rates were even more rapid than those reported for other pearl millet x pearl millet pollinations (Kaushal and Sidhu, 2000; Mohindra and Minocha, 1991). Kaushal

and Sidhu (2000) reported it took 3 h for them to reach the ovary; whereas, Mohindra and Minocha (1991) indicated it required 6 h.

Merkeron napiergrass pollen germination was essentially the same as the best oriental fountaingrass at 76% (Table 10). Its germination was expected to be greater because this was an intraspecific pollination. Merkeron tubes were inside the ovary of PEPU09TX01 within 2 h after pollination (0.3%), and they were well inside the ovary and close to the micropyle within 4 h following pollination (1.9% in ovary; 0.6% to micropyle). More than 100 PEPU09TX01 x Merkeron F_1 hybrids were produced in the Texas A&M Perennial Grass Breeding Program during the winter of 2010 and 2011 from a limited number of pollinations; therefore, this appears to be a compatible cross.

Common buffelgrass tubes grew quite rapidly into the napiergrass pistils in that a single tube was observed in the ovary only 1 h after pollination (0.1%) and 24 tubes were present 2 h following pollination (3.2%) (Table 11). The napiergrass x Frio buffelgrass pollinations were similar to most of the napiergrass x oriental fountaingrass pollinations (Table 12), but the Frio tubes did not grow as rapidly as those of Common buffelgrass. The growth of Common buffelgrass tubes in napiergrass was not surprising when considering the findings reported by Bartek et al. (2012). They determined that Common buffelgrass tubes grew into the ovaries of a sorghum [*Sorghum bicolor* (L.) Moench] line with the inhibition of alien pollen (*iap/iap*) allele. This indicates Common buffelgrass pollen has a broad affinity for alien pistils. Chaix and Marchais (1996) reported pollen incompatibilities between napiergrass and buffelgrass with the percentage of ovaries

without pollen tubes at the micropyle ranged from 66 to 92% of all gynoecia. This disagrees with the finding from this study. However, Chaix and Marchais (1996) pollinated a napiergrass accession from the République du Bénin with pollen from a buffelgrass accession from Agadés, Niger, and their findings differed, possibly because of difference source locations of the germplasm. This further demonstrates the likelihood of genotype-specific affects in the napiergrass x buffelgrass pollinations. While Read and Bashaw (1974) have reported a pearl millet x buffelgrass hybrid, the rate of pollen tube growth shown here for napiergrass x buffelgrass is much faster than the pearl millet x buffelgrass pollinations reported by Mohindra and Minocha (1991). They reported the time required for the pollen tubes to enter the ovary was approximately six hours.

Oriental fountaingrass genotypes PEOR09TX01 and PI 269961 had the highest pollen germination (77%) of all genotypes, and the germination of the remaining accessions ranged from 74% (PI 314994) to 51% (433). These findings are similar to those reported in other studies involving interspecific pollen tube growth in *Pennisetum* (Mohindra and Minocha, 1991; Kaushal and Sidhu, 2000). Pollen tubes of all genotypes used in the napiergrass x oriental fountaingrass pollinations reached the ovary and micropyle region between 3 and 6 h after pollination with the exception of accession 433, which never grew into the ovary. It was the only genotype that exhibited a definite pollen-pistil incompatibility. Because its tubes were never observed in the napiergrass styles, this barrier is probably a stylar incompatibility. Data for the napiergrass x oriental

fountaingrass pollinations (PEPU09TX01 x PEOR09TX01, PEPU09TX01 x PI 269961, PEPU09TX01 x PI 314994, PEPU09TX01 x PI 271595, PEPU09TX01 x PI 271596, PEPU09TX01 x PI 315867, PEPU09TX01 x Cowboy, and PEPU09TX01 x accession 433) are in Tables 13, 14, 15, 16, 17, 18, 19, and 20, respectively. In the PEPU09TX01 x PEOR09TX01 pollination, more pollen tubes (1.5%) were in the napiergrass ovaries within 3 h after pollination (Table 13) than in any of the other crosses. Pollinations using PI 269961 (0.8%), PI 314994 (0.4%), and PI 315867 (0.5%) had tubes in the ovary 3 h after pollination, but a lower percentage of the tubes present in the pistils had entered the ovaries (Tables 14, 15, 18). Pollen tubes of PI 271595 and Cowboy were in the ovary 4 h after pollination (Tables 16 and 19, respectively). The pollen tubes of PI 271596 were in the ovary 6 h following pollination (Table 17). Of the genotypes examined, these findings partially eliminate prezygotic incompatibilities between oriental fountaingrass pollen and the napiergrass gynoecium, except for accession 433. The results from accession 433 pollinations show there are genetic differences between it and the other oriental fountaingrass accessions (Table 20).

Pollen tubes of several accessions of oriental fountaingrass, both buffelgrass cultivars, and Merkeron napiergrass grew in a disoriented manner when attempting to penetrate and grow through the stigma. The tubes of oriental fountaingrass accession 433 exhibited the greatest amount of disorientation and the least amount of penetration into the stigma branches. Coiling of the pollen tube apex during unsuccessful attempts to penetrate the stigma was also observed. Kaushal and Sidhu (2000) reported a similar

behavior when they pollinated pearl millet with oriental fountaingrass pollen. Figure 2 shows a pollen tube from accession 433 in a napiergrass (PEPU09TX01) stigma axis 24 h after pollination. It stopped elongating shortly after germinating and entering the stigma. Figure 3 shows a series of images of pollen tubes from napiergrass and oriental fountaingrass with irregular growth and disorientation once they entered the stigma axis or branch.

These results agree with the consensus that napiergrass is cross-pollinated, and though individuals have been recovered that were self-pollinated, it is apparent the pollen tubes of Merkeron would outcompete those of PEPU09TX01 when growing in the same pistil. This is in agreement with reports by Viands et al. (1988) where pollen tubes from a different individual outcompeted pollen tubes from self-pollinating in alfalfa, a well-known cross-pollinated species that is known to allow selfing to occur though at the expense of intense inbreeding depression.

Overall, findings by Kaushal and Sidhu (2000) reflect what is reported here. Even though pearl millet x buffelgrass, pearl millet x oriental fountaingrass hybrids have been produced by Hanna and Dujardin (1982), no interspecific hybrids were recovered from napiergrass x oriental fountaingrass crosses. The growth rate of pollen tubes in the napiergrass x oriental fountaingrass crosses (1 to 3 h) were faster than in the pearl millet x oriental fountaingrass crosses (7 to 10 h) reported by Kaushal and Sidhu (2000).

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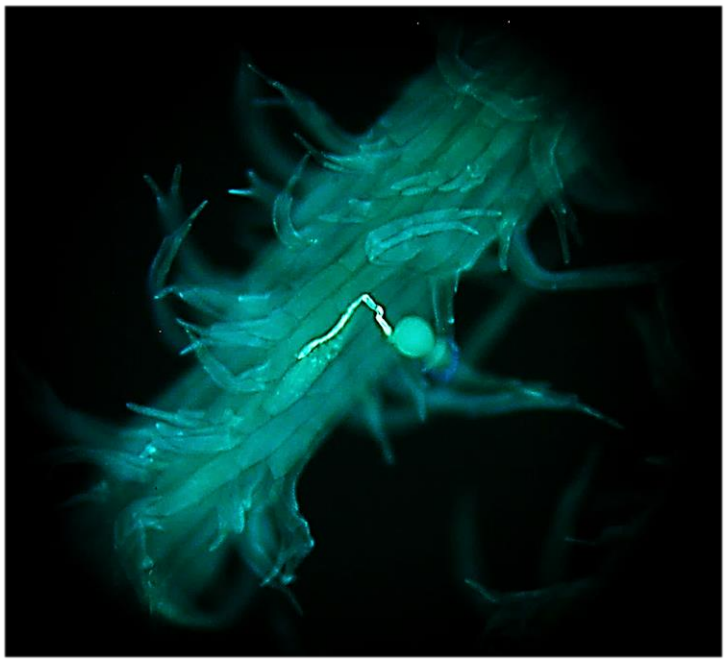
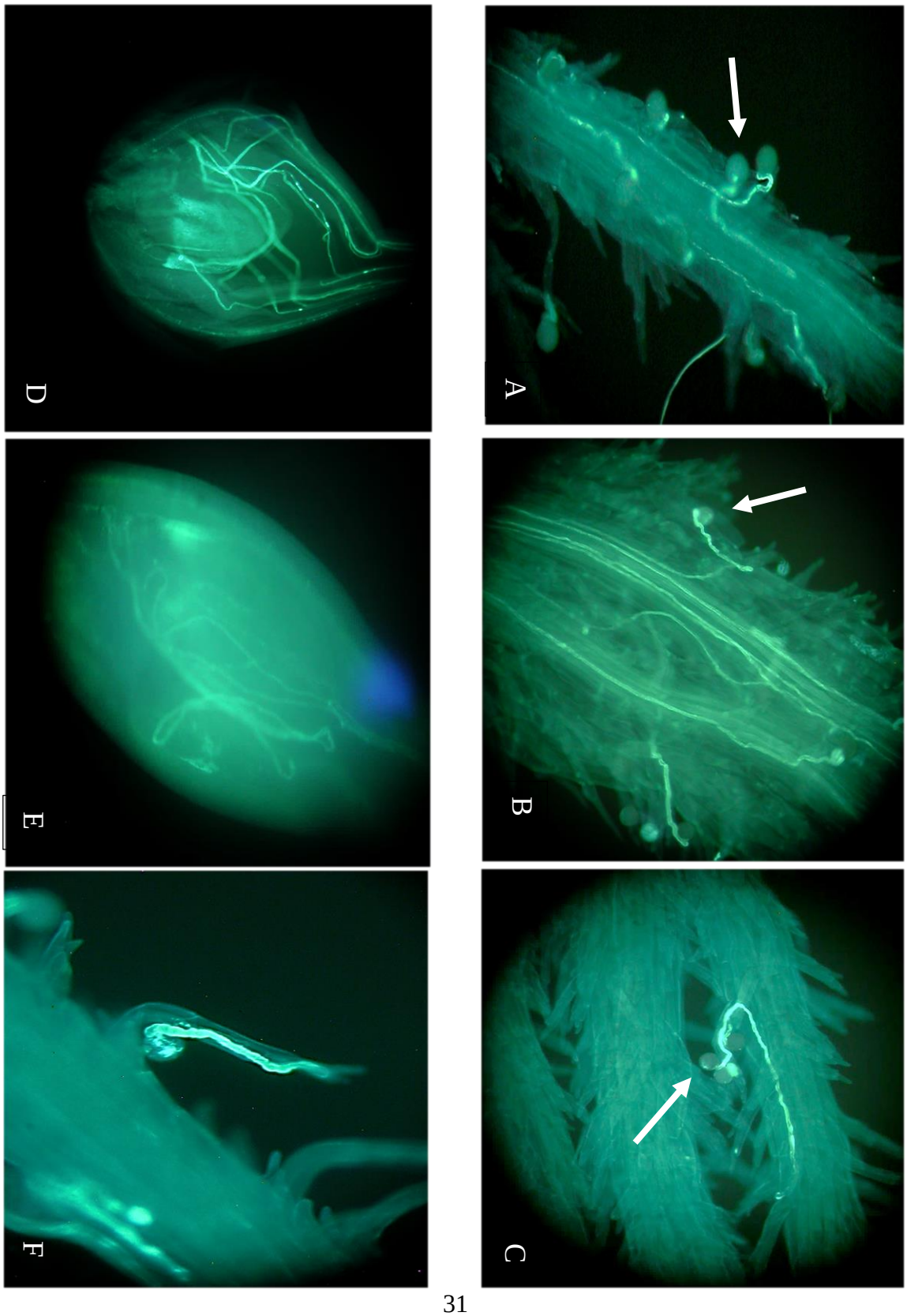


Figure 2. Pollen tube of oriental fountaingrass accession 433 that stopped growing within 12 hours after pollination.

These findings generally agree with those of previous studies when pearl millet was used as the maternal parent as well as the napiergrass x buffelgrass pollinations. The conclusions drawn by Chaix and Marchais (1996) regarding the compatibility between pearl millet and oriental fountaingrass cannot be compared to napiergrass even though seven of the eight oriental fountaingrass pollinators were compatible with napiergrass, at least from a prezygotic standpoint. Apparently some postzygotic barriers are preventing the development of napiergrass x oriental fountaingrass hybrids.

Figure 3. Images of irregular pollen tube growth in pistils from intra- and interspecific pollinations. (A) A pollen tube of the napiergrass cv. Merkeron growing backwards in a napiergrass PEPU09TX01 stigma two hours after pollination; (B) A pollen tube of the oriental fountaingrass cv. Cowboy growing backwards in a napiergrass PEPU09TX01 stigma three hours after pollination; (C) A pollen tube of oriental fountaingrass accession PI 271596 growing backwards four hours after pollination; (D) Pollen tubes of PEOR09TX01 growing in a disoriented manner in an ovary of napiergrass PEPU09TX01 four hours after pollination; (E) Pollen tubes of oriental fountaingrass accession PI 314994 growing in a disoriented manner in an ovary of napiergrass PEPU09TX01 six hours after pollination; (F) A pollen tube of oriental fountaingrass accession 433 growing backwards in a stigma branch of napiergrass PEPU09TX01 six hours after pollination. White arrows point to the pollen grain of interest in each image. All photos were taken at magnifications from 100x to 200x.


A
B
C
D
E
F

Pollen-pistil interactions and prezygotic barriers for, either self- or cross-incompatible mechanisms, can influence pollen germination and tube growth of certain genotypes. These include, but are not limited to, the composition of exudates on the stigma surface that can selectively degrade pollen tube-wall components and affect pollen-tube adhesion and orientation, the water gradient potential within the stigma, the concentration of lipids, proteins, and γ–aminobutyric acid in the style, and Ca^{2+} ion concentration gradients (Swanson and Edlund, 2004; Gao, et al., 2010; Rejón et al., 2013). Post-fertilization barriers sometimes occur in crosses between parents that have different ploidy levels, meiotically irregular progeny, and genetic differences that can prevent zygote formation or results in the breakdown of the developing endosperm. Abnormal endosperm development leading to abnormal seed development or abortion is a frequent occurrence in interploidy crosses (Whitmire, 2011; Kuligowska et al., 2015). However, hybrids between species with different base chromosome numbers and ploidy levels are reported in *Kalanchoë*, *Salvia*, *Brassica*, *Chrysanthemum* and *Dianthus* (Kuligowska et al., 2015; Tychonievich and Warner, 2011; Cheng et al., 2010; FitzJohn et al., 2007; Gatt et al., 1998). The primary explanation for this behavior, or lack thereof, is imbalanced maternal:paternal ploidy levels and endosperm:embryo ploidy levels. However, more recent findings suggest that imbalances in epigenetic modification and subsequent expression patterns of imprinted genes can also affect endosperm development in crosses of distantly related species (Kinoshita, 2007).

Conclusions

This research revealed there are overall no negative pollen-pistil interactions occurring in napiergrass x buffelgrass and napiergrass x oriental fountaingrass crosses with the exception of accession 433. Therefore, analysis of additional pollen tube samples of different buffelgrass and oriental fountaingrass genotypes could increase the likelihood of recovering wide hybrids. While many disoriented pollen tubes were observed for all pollinations, excluding napiergrass x pearl millet, 12 out of 13 of them did reach the micropyle indicated the possibility of fertilization in many of the observations. The lack of apparent prezygotic barriers indicates the possibility of postzygotic barriers in these crosses, especially since no hybrids were recovered after several thousand pollinations were made. Embryo rescue and culture efforts were unsuccessful in recovering hybrid seedlings. While interspecific hybrids between these species would create novel plant forms with potential for harboring desirable traits, more research is needed to determine the exact mechanisms preventing interspecific hybridization. More research is needed to investigate the precise embryological, genetics, and biochemical mechanisms creating post-fertilization barriers in these interspecific pollination events.

CHAPTER III

IMPROVED OVERWINTERING IN NAPIERGRASS

Introduction

The growth and development of organisms is affected to some degree by cold temperature stress. Plants adjust to this environmental change physiologically by the slowing of biochemical reactions rates, fluctuating the fluidity of cell membranes, modifications of protein conformation, changing ion potential, and other processes that may give selective advantages towards cold-tolerance (Catalá and Salinas, 2010). Plants are limited in their geographical distribution largely because of the constraints placed on them by near- or below-freezing temperatures. One of the most important reactions associated in response to lower temperatures and increased winter hardiness is cold acclimation. Species adapted to temperate and arctic climates have evolved this phenomenon after repeated exposure to low nonfreezing temperatures prior to the exposure to freezing temperatures (Levitt, 1980). However, the ability to resume growth in the spring after periods of below freezing temperatures is not only associated with the minimum temperature these plants can survive but also the processes of integrated genetic encoding and gene expression.

It is important to distinguish between cold acclimation and actual overwintering, and while they are similar, cold acclimation is only one factor contributing to the ability to overwinter. Overwintering is a complex process that involves the biological evolution of gene functionalization, the development of responses to local environmental cues and

syncing induced responses with the local climate, maintaining cellular function at intervals of severe stress (cold), and the recognition of reduced stress that commences spring growth (Guy, 1990).

The ability of a plant to acclimate to cold temperatures is an important component of winter hardiness, and two major functions regulate this process. These are the widespread adjustment of cellular functions that influence the metabolic response to physical constraints imposed by low non-freezing temperatures, and the initiation of the induction of freezing tolerance (Guy, 1990). The first differentiates individuals that are chilling-sensitive from those that are chilling-tolerant, and the second differentiates chilling-tolerant but freezing-sensitive plants from freezing-tolerant plants (Guy, 1990). It is inconclusive at this time whether both of these functions of cold acclimation are required, or required in succession, for the development of improved overwintering and tolerance to below freezing temperatures (Guy, 1990).

Exposure to low temperatures over time, i.e. cold acclimation, is not the only way that tolerance to freezing temperatures is assimilated. Studies have reported increased tolerance to freezing in plants that were treated with exogenous abscisic acid (Reaney and Gusta, 1987). It is well-known that abscisic acid is the major phytohormone induced by plant stress, especially desiccation and/or drought. Abscisic acid-dependent and -independent regulatory pathways have been discovered in response to low temperatures and desiccation, and they overlap in genes involved in regulation and function. This research is not focused on the role of abscisic acid in winter hardiness,

but Yang et al. (2005) wrote a thorough review regarding the cold-response network including the role of abscisic acid.

The regulation of the variation in enzyme activation and protein content has been correlated with cold acclimation and freezing tolerance, and thus, winter hardiness. The accumulation of soluble proteins during the fall and their rapid decline during the spring has been characterized as a general response to cold acclimation. Several studies have reported the presence of new protein species in cold-acclimated plants and freezing-tolerant plants in comparison to non-acclimated plants, and differences in protein content between these two phenotypes has been observed (Kacperska-Palacz, et al., 1977; Rosas, et al., 1986).

Pathways of signal transduction exist for cellular responses to many, if not all, external mechanisms, and the recognition of these signals by receptors is the first step in any signal transduction pathway. For plants cells, acclimation to cold is thought to occur at the onset of changes in cell membrane fluidity, which acts as a receptor to changes in temperature (Örvar et al., 2000; Sangwan et al., 2002). This is only a hypothesis and has not been subjected to the rigor of scientific scrutiny, however. In addition, there is increasing evidence that indicates the role of protein phosphorylation, a common mechanism initiating signal transduction, in plant cold acclimation.

Short-term mechanisms of temperature variation and fluctuations in seasonal and diurnal photoperiods affect both plant dormancy responses and overwintering potential. This potential is regulated by plant nutrient cycling, hormone signaling, protein and lipid

metabolism, carbohydrate composition, and regulation of gene expression (Guy, 1990). These complex biochemical and physiological responses to cold are mainly considered to be controlled by regulation at the transcriptional level; however, more recent discoveries implicate the importance of regulation at the levels of posttranscription, translation, and posttranslation (Mazzucotelli et al., 2006; Zhu et al., 2007; Catalá and Salinas, 2008).

A continuous array of phenotypes can exist because of the heterozygous nature of napiergrass, not only for winter hardiness, but also for plant height, architecture, robustness, and other desirable traits. It is a common observation in several species where hybrid crosses give a range of progeny across the extremes of the two parents in regards to winter hardiness, and this has led to the conclusion that it is a quantitatively inherited trait (Nilsson-Ehle, 1912; Worzella, 1935; Rudolph and Nienstaedt, 1962; Law and Jenkins, 1970; Parodi et al., 1983; Norell et al., 1986). These observations also demonstrate how the tolerance to cold may remain hidden in the genetic background. It is of particular importance to understanding the evolutionary magnitude and breeding possibilities of polyploid species in regards to the inheritance and expression of cold-tolerance where genetic buffering is prominent (Worzella, 1935; Limin and Fowler, 1988; Chapman et al., 2005). In particular, non-additive allelic effects can result in the expression of overdominance. While epistasis in cross-pollinated and hybrid plants may alter gene expression and inheritance of genes leading to the stable offspring phenotypes. These types of patterns are prevalent in regard to abiotic stress tolerances as well as the manifestation of heterosis (Song, et al., 2012). In addition and of particular importance

regarding polyploids like napiergrass, allele dosage coupled with allele-specific gene regulation has a large impact on expression patterns. This makes inheritance and fixation of alleles for cold-tolerance more difficult, especially in polyploids, and it validates the need for marker-assisted breeding and other molecular genetic techniques to target desirable genes.

Transgressive segregation for winter hardiness has been reported for several crop species, which supports the quantitative inheritance observed for overwintering capacity (Nilsson-Ehle, 1912; Reid, 1965; Davis and Gilbert, 1970; Hummel et al., 1982). In addition, winter hardiness likely behaves in a quantitative fashion given that complex morphological, developmental and physiological processes, and environmental cues drive the capacity for cold-tolerance (Levitt, 1980). A few studies have attempted to describe the inheritance of cold acclimation as well as the ability to overwinter with contrasting conclusions of partial dominant and recessive gene action (Worzella, 1935; Rohde and Pulham, 1960; Law and Jenkins, 1970; Gullord et al., 1975; Burke et al., 1976; Rehfeldt, 1977; Sutka, 1981; Liesenfeld et al., 1986; Laroche and Hopkins, 1987). There has been no correlation of cold-tolerance or winter hardiness to maternal inheritance in studies involving reciprocal crosses to date (Harris, 1965; Wilner, 1965; Muehlbauer et al., 1970; Sutka, 1981; Hummel et al., 1982).

Nearly all tropical and subtropical plant species are poorly suited to low, non-freezing temperatures, meaning that they are chilling sensitive, and they may incur extensive injury or even death when exposed to these temperatures (Levitt, 1980). While

napiergrass is fairly tolerant to these conditions, it is not well adapted to prolonged periods of near or below freezing temperatures. Increasing winter hardiness in the species would extend its range of adaptation into more temperate environments. This would expand napiergrass' production area as a dual-use biomass feedstock including other valuable co-products that can be derived from the grass. It is important to consider all of the genetic, molecular, biochemical, and physiological pathways and mechanisms that are responsible for increasing winter hardiness in napiergrass, including cold acclimation, when screening genotypes and making selections. Assessment of napiergrass' range of adaptation in Texas and the southern United States is warranted for several reasons. Increasing its range of adaptation will expand areas for napiergrass biomass production that were previously unavailable. As a dual-use biomass crop, napiergrass can be the source of many bio-based, value-added products such as biofuels, cosmetics, plastics, protein supplements, vitamin and mineral supplements. Another important trait of napiergrass is its ability to phytoremediate soils contaminated with petroleum and heavy metals (Holm, 2010). Sustainable production of coproducts from napiergrass could stretch the market for them as well as alleviate the need for strictly petroleum-based products. In addition, analysis of napiergrass rhizome gene expression can reveal and confirm physiological, cellular, molecular, and genetic mechanisms controlling both overwintering and acclimation to cold temperatures in non-dormant, tropical species such as napiergrass.

Material and Methods

Phenotyping Napiergrass for Overwintering

Traditional approaches were utilized to select napiergrass genotypes adapted to more temperate climates. Selections were made from a nursery at College Station, TX consisting of more than 1,000 diverse S_1 and F_1 individuals. Individuals with traits associated with overwintering were selected. These plants were in year two of growth and had survived the winter of 2011. Phenotypic selection criteria for improved overwintering were based on the number of rhizome-derived shoots, tiller number, rhizome distance from crown, tiller density, and overall plant robustness. The individuals selected were planted into an evaluation nursery at Commerce, TX during the summer of 2012 and were evaluated for winter survival following regrowth during the spring of 2013. The surviving genotypes were vegetatively increased and planted into a completely randomized design with five replications at both Vernon, TX and Alma, AR in June 2013. Table 3 gives descriptions of the three sites used to screen napiergrass for cold-tolerance. Multiple phenotypic parameters were characterized prior to senescence in late fall 2013 at Vernon and Alma to identify any traits that might contribute to a selective advantage for overwintering. These included plant height, tiller number, rhizome-derived shoots, incidence of flowering, and overall plant architecture. The napiergrass cultivar Merkeron was used as a check as its range of adaptation has prior documentation in comparison to the experimental lines in this experiment (Burton, 1989; Woodard and Sollenberger, 2011).

Table 3. Location information for analysis of overwintering in napiergrass

Location	Latitude and longitude	Elevation (ft)	Soil type	Ave. annual precipitation (in.)	Extreme annual temperature (max. / min °F)
Commerce, TX	33°11'51.6"N 95°55'33.2"W	550	Crockett loam	42.80	105 / 19
Vernon, TX	34°05'10.5"N 99°21'51.6"W	1,184	Miles fine sandy loam	27.99	107 / 7
Alma, AR	35°22'50.7"N 94°14'03.9"W	433	Roxana silt loam	45.46	93 / 8

Napiergrass Rhizome Candidate Gene Expression

Rhizomes from winter hardy napiergrass genotypes growing in field trials at Commerce and College Station, TX, were used for the gene expression studies. These rhizomes were from plants that survived winters in the field and not from plants exposed to short-term cold stress in controlled growth chambers or under laboratory conditions. This provides a more realistic assessment of the gene expression profile of field-grown plants when exposed to prolonged chilling temperatures and photoperiodic cues (d to wk) rather than just short-term cold exposure (min to h). Rhizome tissue was collected from plants at both College Station and Commerce during the fall of 2013 when they were nearing complete senescence and also during the spring of 2014 after some aboveground regrowth was visible. Rhizome tissues were separated into the areas where cell division and cell elongation were occurring and were independently screened for differential gene expression. The area of cell division was established as a 2.5 cm region in the apex of the rhizome, which included some sheath material as rhizomes are modified stems, and the area of cell elongation was established as a 4-5 cm region

contiguous to the area of cell division. The plants were subjected to minimally destructive sampling, especially during the fall.

Molecular marker development and candidate gene selection relied on specific strict criteria. Preliminary candidate genes were chosen from a range of cultivated, closely associated, and model species to represent and target a wide array of potential molecular genetic mechanisms. These species include: thale cress [*Arabidopsis thaliana* (L.) Heynh.]; alfalfa (*Medicago sativa* L.); rice (*Oryza sativa* L.); sweet potato [*Ipomoea batatas* (L.) Lam.]; tomato (*Lycopersicon esculentum* Mill.); sorghum [*Sorghum bicolor* (L.) Moench.]; and switchgrass (*Panicum virgatum* L.). The initial candidate genes chosen were identified as upstream in cold response pathways, associated with stress tolerance or overwintering mechanisms, and present in *S. bicolor* since it is the closest relative with a sequenced genome. Targeted candidate genes conferring functional annotation as well as transcription factors (TFs) and master regulators of cold-responsive gene expression were analyzed by blasting these sequences into the Plant Transcription Factor Database (Jin, et al., 2014). This provided a detailed report regarding the conserved nature and function of the target genes within and across documented species. Blasting these candidate genes in Phytozome to investigate colocalization with sorghum QTLs associated with rhizome number, rhizome length, subterranean rhizomes, overwintering, and the Rhz2/Rhz3 QTLs in rice provided additional information on the functional annotation of the candidates genes selected for analysis. Previous publications have reported colocalization of rhizome-specific or rhizome-enriched genes

from *S. bicolor* onto QTLs responsible for rhizomatousness in *S. halepense* L. Pers. and *S. propinquum* (Kunth) Hitchc. (Jang et al., 2006; Jang et al., 2009; Washburn et al., 2013; Zhang et al., 2013). Overlapping QTLs for rhizomes and overwintering from closely related species were also used as potential candidates.

Genomic primers were designed using Primer3 and tested for presence or absence of the gene of interest, allele dosage, and product size in a napiergrass bulk utilizing polyacrylamide gel electrophoresis. Candidates chosen for gene expression analysis were based on the aforementioned criteria, had only a single or double dose of the gene of interest to avoid repetitive elements, and were between 100-250 bp. TaqMan® gene expression primers were designed using Beacon Designer® 8.0 and only the coding sequence of the genes of interest were used in primer design and not 5' or 3' untranslated regions. The resulting suitable probes were then designated as PPGE, for "*Pennisetum-purpureum*-Gene-Expression".

RNA from the rhizome tissue was prepared using a RNA MicroPrep™ kit by Zymo Research. Eluted, concentrated RNA samples were distributed as 2 μL aliquots into 7 μL DEPC-treated H_20 for dilution to working concentrations. Thereafter, confirmation of RNA quality and yield was conducted on the Agilent 2100 Bioanalyzer using the Agilent RNA 6000 Nano Kit and the 2100 Expert software. The 2100 Bioanalyzer provided RNA information in nanograms per microliter (ng μL^{-1}) for yield, quality, and integrity as the RNA integrity number (RIN) and 28S:18S ribosomal RNA (rRNA) ratios. The RIN number provided by the instrument is on a scale of 1 to 10, with

one being the most degraded and 10 being the most intact sample, and the rRNA ratio is also included in this algorithm. Only samples with a RIN of seven or greater were used to synthesize double-stranded complementary DNA (cDNA). Purified RNA samples were stored at -80 °C until cDNA strands were synthesized. RNA was added to each cDNA synthesis reaction at a concentration of 200 ng μL^{-1} to standardize cDNA concentrations for qPCR reactions. The first strand cDNA production was completed by using the AffinityScript™ cDNA synthesis kit by Agilent Technologies. A cDNA reaction was 20 μL in total with the following reagents per reaction: 2 μL DEPC-treated H_20; 10 μL master mix; 2 μL oligo-dT primer; 1 μL random primer; 1 μL RNase Block; and 4 μL RNA (800 ng RNA per 20 μL cDNA reaction). Succeeding cDNA synthesis was analysis of gene expression via the MxPro 3005P™ qPCR instrument by Agilent Genomics, and data analysis of the subsequent gene expression occurred in the MxPro™ software. The qPCR reactions were performed using the Brilliant III Ultra-Fast QPCR Master Mix® kit by Agilent Technologies. There were 20 μL per reaction and each reaction contained 6.7 μL of DEPC-treated H_20, 10 μL of 2x qPCR master mix, 1 μL of Taqman® probe, 0.3 μL of passive reference dye diluted 1:50 in PCR-grade H_20, and 2 μL of cDNA. An elongation factor 1-alpha (EF1A) and an actin2 (ACT2) from buffelgrass were used as endogenous controls based on the results of qPCR stability in different tissues, organs, and developmental stages obtained by Simon, et al. (2013). In addition, preliminary analysis confirmed the stability of fluorescence and threshold cycle in napiergrass rhizome and leaf tissue.

Results and Discussion

Phenotyping Napiergrass for Overwintering

Eighteen napiergrass selections made during the summer of 2012 after the plants had demonstrated the ability to overwinter during the winter of 2011-2012 at College Station, TX. The criteria for selection are listed in the materials and methods. These plants were established in two replications at Commerce, TX, during the summer of 2012 and allowed to grow to maturity prior to winter. None of the plant material was harvested prior to winter senescence. Of the 18 selections from College Station, 10 of them overwintered during the winter of 2012-2013 at Commerce with between 50-100% survival rates (Table 4). This demonstrated that napiergrass has sufficient genetic diversity to screen and select for winter hardiness and improved overwintering ability. The 10 individuals that survived the winter at Commerce were vegetatively increased and planted at both Vernon, TX, and Alma, AR, in five replications during June 2013. Table 5 lists the napiergrass selections, their genetic background, and averages for the abovementioned traits used for evaluation of overwintering. Unfortunately, neither the number of tillers nor the number of rhizome-derived shoots was correlated with overwintering in these individuals. This is in contrast to previous reports that plant morphology including the number of rhizomes can affect winter survival in perennial grasses (Bashaw, 1980). It appears mechanisms involved in freezing tolerance and cold acclimation may be more important for overwintering than plant morphology. Teutonico et al. (1993) concluded this as well. Overwintering observations were made after soil

temperatures reached 60°F for at least 2 to 3 wk in order to allow the plants to exhibit new aboveground growth. Table 6 shows the percentage of survivors from the five cloned genotypes at Vernon, TX, and Alma, AR during the winter of 2013. While no plants survived at Alma, eight of the 11 selections planted at Vernon showed regrowth during the spring of 2014. These plants did not exhibit any abnormal phenotypes from the changes in latitude or the winter and were similar in size and maturity during the 2014 growing season as during the establishment year.

Table 4. Percentage of plants surviving the 2012-2013 winter at Commerce, TX.

Identification†	Plant ID	Overwintered (%)
Merkeron S1:	PEPU12TX05	0
Merkeron S1:	PEPU12TX06	0
Merkeron S1:	PEPU12TX07	0
Merkeron S1:	PEPU12TX08	0
Merkeron S1:	PEPU12TX09	100
Merkeron S1:	PEPU12TX10	100
PEPU 09FL01 S1:	PEPU12TX11	0
PEPU 09FL01 S1:	PEPU12TX12	0
PEPU 09FL01 S1:	PEPU12TX13	50
PEPU 09FL02 S1:	PEPU12TX14	50
PEPU 09FL02 S1:	PEPU12TX15	100
PEPU 09FL02 S1:	PEPU12TX16	0
PEPU 09FL03 S1:	PEPU12TX17	100
PEPU 09FL03 S1:	PEPU12TX18	100
PEPU 09FL03 S1:	PEPU12TX19	100
Mott x Merkeron:	PEPU12TX20	50
PEPU 09TX01 O.P.:	PEPU12TX21	100
PEPU 09TX01 x Merkeron:	PEPU12TX22	50

†Each ID is representative of two plants each in a completely randomized design and denotes the parental genotype.

Table 5. Phenotypic data collected in the fall of 2013. Numbers represent an average across Vernon, TX, and Alama, AR, locations (n=10).

Source	ID	Ave. Height (cm)	Average Tiller No.	Rhizome –Derived Shoots (%)	Flowering (%)
cultivar	Merkeron	192	18	60	0
Merkeron S_1	12TX09	196.9	28.6	60	20
Merkeron S_1	12TX10	217.5	15.7	80	0
PEPU09FL02 S_1	12TX14	214	24.6	70	10
PEPU09FL02 S_1	12TX15	212.6	14.6	80	0
PEPU09FL03 S_1	12TX17	204.6	22	80	0
PEPU09FL03 S_1	12TX18	211.2	17.2	60	0
PEPU09FL03 S_1	12TX19	203.4	25.6	30	0
Mott x Merkeron	12TX20	199.6	17.3	20	0
PEPU09TX01 O.P.	12TX21	195.5	24.7	50	20
PEPU09TX01 x Merkeron	12TX22	256.2	21.5	40	60

Table 6. Comparison of napiergrass survival across both locations during the winter of 2013-2014. Each location had five clonal replicates of each plant genotype (ID) per location. Zero out of 55 plants exhibited the ability to overwinter at Alma, AR.

ID	Location Survival (%)	
	Alma, AR	Vernon, TX
Merkeron	0	80
12TX09	0	40
12TX10	0	0
12TX14	0	80
12TX15	0	0
12TX17	0	40
12TX18	0	20
12TX19	0	0
12TX20	0	20
12TX21	0	20
12TX22	0	20

The differences in plant survival may be partially associated with the difference in the rate of increase in the soil temperature during the spring at these two locations. During March and early April, flash freezes accompanied by precipitation repeatedly occurred at Alma during critical times of regrowth, and precipitation events occurred during the winter when ambient temperatures were below freezing for multiple days. Comparing the minimum temperatures at Alma and Vernon from October 2013 through April 2014 revealed they were similar with 78 and 79 days below freezing, respectively. The main difference was the extended freezing periods at Alma where 38 of 65 days were below freezing in addition rain and/or snow events occurred 17 times while the temperature was below freezing. In contrast, Vernon only had a single rain event recorded when temperatures were below freezing from October 2013 through April 2014. Damage may have occurred to the rhizomes during the spring when rising soil temperatures initiated cell expansion and differentiation prior to a cold and wet event. For napiergrass, winter hardiness does not appear to be influenced by photoperiod because Vernon and Alma are relatively similar in latitude (34°10'N and 35°29'N, respectively). This hypothesis agrees with previous findings by Johnston and Dicken (1976). They determined that freezing tolerance was not affected by photoperiodic treatments (6, 12, and 18 h) and temperature regimes (23°C max., 4°C min. and 23°C constant) in centipedegrass (*Eremochloa ophiuroides* [Munro] Hackel), a warm-season, perennial turfgrass. However, the difference in the minimum winter temperatures between 34°10'N and 35°29'N may be the critical latitudinal threshold for napiergrass

winter adaptation in the southern United States. It is likely other environmental factors such as the rate of soil warming, timing of flash freezes during early spring, and prolonged wet and cold winter conditions at Alma may be a major factor affecting meristem mortality.

Napiergrass Rhizome Candidate Gene Expression

Utilizing the candidate gene approach of screening species with functionally annotated targets, 115 potential candidate genes were identified. These targets met the specific criteria set for napiergrass rhizome gene expression analysis mentioned above and all had their gene functions and sequences fully available. While these are too numerous to mention individually, it is important to know where these 115 candidates originated because of their potential as cold-responsive targets. Of the 115 candidate genes, 54 were from *Arabidopsis*, 11 were from alfalfa, 18 were from rice, four were from sweet potato, three were from tomato, 17 were from *S. bicolor* directly, and eight were from switchgrass.

For genomic DNA-based marker screening, 91 of these 115 candidates were chosen because they met the *S. bicolor* colocalization criteria as well as having important functional annotation associated with overwintering and/or cold-responsiveness. Multiple superfamilies, i.e. gene families, were represented from the candidate genes including APETALA2/ethylene-responsive element binding factor (AP2/ERF), myeloblastosis (MYB), no apical meristem (NAM), 14-3-3 regulatory molecules, major facilitators, basic helix-loop-helix (bHLH), leucine rich repeats (LRR), EF-hand motifs,

calcium-dependent protein kinases (CDPK), glycosyl hydrolases, and dehydrins. These are important because many TFs and major gene families have been proven to participate in conservation of gene function and coding through subfunctionalization and/or superfunctionalization following speciation events. This allows plants' enhanced adaptation to adverse environmental conditions (Paterson et al., 2006). These families are also known to initiate and/or control various stimuli-induced genes across all multicellular organisms and can provide further insight into the complex networks of regulatory genes and how they function and are involved with abiotic stress responses (Shinozaki et al., 2003). Most importantly in this context is tolerance to cold. In addition, previous reports of rhizome-enriched gene expression displayed a range of functionality, which supports the hypothesis of gene specialization in conjunction with gene duplication and speciation events for these major gene families (Paterson et al., 2003, 2004).

No candidates were from *S. halapense* or *S. propinquum* because they did not meet all the criteria for gene expression analysis, and they did not meet the requirement of gene dosage. This is not unexpected for *S. halapense* since it is a polyploid. Other explanations for this are that these genes are present as repetitive elements in napiergrass or there has been extensive divergence in sequence and function of *Pennisetum* and *Sorghum* orthologs brought about by gene duplication or rearrangement of the genome by transposable elements after speciation events. After screening the 91 candidates using genomic markers, only 13 met the criteria of having a single or double dose of the allele.

Table 7 provides details regarding the 13 candidate Taqman® probes assayed for differential rhizome gene expression and the two reference genes included as endogenous controls. They were unique in their primer and amplicon sequence while some overlapped in gene function.

From these 13 probes, only two showed detectable changes in gene expression. Preliminary analysis of the 13 genes confirmed that their sequences exist in napiergrass. However, subsequent qPCR revealed no detectable changes in transcript levels. This could be an artifact from spatiotemporal variation in gene expression. Assays of *in situ* hybridization or immunohistochemistry were not performed to confirm the presence of these patterns; it is a likely phenomenon hindering this step of the experiment. The two sequences of importance and were used in analysis of differential expression of rhizome tissue were PPGE 53 and PPGE 73. PPGE 53 encodes an AP2 DNA-binding TF similar to an ERF DNA-binding TF and is DNA-dependent.

Table 7. Details and description of the rhizome candidate and reference genes designed for Taqman® molecular beacons

Sequence ID	Phytozome or NCBI Accession	Clone description/name	Gene function	Forward/Reverse Primers	Product length
PPGE 2	Sb10g028550	Xyloglucan endo-transglycosylase C-terminus/XET C-terminus	Xyloglucan metabolism	CAGTCCAAGTCCGAGTAC/ GAGGTAGAAGGTGGTGAC	93
PPGE 3	Sobic.006G184800	C-repeat binding factor/CBF	Regulation of transcription	CGACTTCGCTTACTACCC/ GCTCCAAAGACTGACCTC	145
PPGE 12	Sobic.002G419000	Lipopolysaccharide kinase/LPSK	Protein phosphorylation	CCAGATCAAACTCTATGTG/CC TGTAAGCATTTCAAAGA	124
PPGE 27	Sobic.001G261400	Leucine-rich repeat kinase/LRR	Protein phosphorylation	TCTCCATCTATAATTCACAAG A/GGCACTATAACCAGAACC	147
PPGE 53	Sobic.002G212000	APETALA2 DNA-binding TF/AP2	Regulation of transcription	GGAGATAGACCTACTGCTA/ GAGCTTCAGCATCGTATG	181
PPGE 62	Sobic.006G184800	APETALA2/AP2	Regulation of transcription	CGACTTCGCTTACTACCC/ GCTCCAAAGACTGACCTC	145
PPGE 64	Sobic.002G269400	APETALA2/AP2, similar to *S. bicolor* CBF6	Regulation of transcription	GGGAGTCCTCTGAATCTG/ GAAGCCTTCGTCCATGAA	164
PPGE 67	Sobic.003G396600	zinc finger, C2H2 protein/ZFP	DNA, RNA, protein, lipid binding	CCACCAAATTCCAAGAAA/ ACCAGAATCATCACTAGAA	184
PPGE 68	Sobic.004G309600	Basic Leusine Zipper protein/bZIP	Regulation of transcription	GGGAAGATGGAAGGAGAC/ ATGCCTGTTTCCTTTGAC	178
PPGE 69	Sb02g036730	EF-hand; calcium-dependent protein kinase/EF-hand CDPK	Protein phosphorylation	AGAGGTTATCAATGCCATTA/C GCAAACTCATCATAGCTA	73
PPGE 73	Sobic.009G025500	Low temp. & salt responsive hydrophobic protein/RCI2	Proteolipid membrane modulator	GAGACGTTCCTGGAGATC/ GGATGTATCCCAGGATGG	118
PPGE 75	Sobic.010G041900	Inducer of CBF expression1/ICE1	Osmotic and cold response	GATGGAGAAGATCAAGGA/ CCTTGATTTTCTCCATGAA	182
PPGE 85	Sb06g032280	similar to Transcription factor ICE1-like/ICE1	Regulation of transcription	CCATCAACACCAACATTG/ GTGGATATTGACTGCATG	135
EF1A	EB672663.1	Elongation factor 1-alpha/EF1A	Translational	GCTCATTACCAAGATCGA/ GTGGGAATCATCTTCACC	99
ACT2	EB670295.1	Actin2/ACT2	Cytoskeleton	GACAGCTTGATGAAGATC/ GCTCATAACTCTTCTCCA	166

It is located within 7 Mbp (mega base pairs) of a known QTL for subterranean rhizomes on chromosome 2 of *S. bicolor*, and it occurs between 60,418,543 and 60,422,020 bp (Phytozome). In addition, colocalizing around this QTL for subterranean rhizomes are QTLs on rice chromosome 7 that are associated with rhizome branching degree, average rhizome length, average rhizome internode length, ratooning ability, and rhizome number per plant (Jang, et al., 2006). PPGE 73 encodes a proteolipid membrane potential modulator, which is a hydrophobic protein given the name rare cold inducible 2 (RCI2). It is located within 6 Mbp of a different subterranean rhizome QTL on *S. bicolor* chromosome 9, and it occurs between 2,276,598 and 2,277,493 bp (Phytozome). Jang et al. (2006) also reported this subterranean rhizome QTL on *S. bicolor* chromosome 9 to colocalize with rice QTLs on chromosome 4 associated with several traits including rhizome branching degree, average rhizome length, average rhizome internode length, average rhizome internode number, and rhizome number per plant. These RCI2 genes show homology with the 14-3-3 proteins that have been described in mammals to be involved with the regulation of multifunctional protein kinases, i.e. protein phosphorylation (Aitken et al., 1992; Jarillo et al., 1994). Interestingly, Jarillo et al. (1994), who discovered and cloned these genes, reported that RCI2 was not developmental stage or organ specific but was inducible independent of abscisic acid and was not transiently expressed in *Arabidopsis*. While the RCI1 clone had returned to normal expression levels within 24 h of deacclimation, RCI2 transcript levels were still elevated after three days of deacclimation. In addition, RCI2 did not accumulate to

substantial levels until day three of cold treatment indicating an important role in cold acclimation over longer periods of time.

Analysis of rhizome gene expression revealed there were no apparent differences in relative expression levels for the regions of cell division (D) and cell elongation (EL) across all four genotypes and at either location. However, there was a noticeable increase in the relative expression levels in the spring compared to the fall across all genotypes as well as cell types (D and EL). Only two genotypes were analyzed from Commerce, PEPU12TX09 and PEPU12TX10, and generally, the fold change (FC) was higher for spring samples in comparison to fall samples (Figures 4 and 5). AP2 expression was significantly higher in the spring compared to the fall for 12TX09 for both D and EL cell types. The FC in 12TX09 spring-sampled rhizomes was roughly five and 11 for EL and D, respectively, but EL and D of 12TX09 in the fall did not demonstrate a two-FC in expression, which is a general threshold for declaring there is differential expression. While 12TX10 showed similar results for AP2, the FC demonstrated less differentiation, most notably for D compared to EL. While the 12TX10 EL region showed differential expression in the fall and spring for AP2, the 12TX10 D region did not show differential expression in the fall and barely reached the two-FC mark in the spring.

For RCI2 at Commerce, there was also a significant increase in spring versus fall differential expression levels in 12TX09 for both D and EL with a FC of roughly 6.5 and 4, respectively. Again, the trending results were lower levels of expression for 12TX10

in fall and spring samples for RCI2 as was shown for AP2. The only treatment showing at least a two-FC and differential expression was fall EL. The increase of 12TX10 EL differential expression in fall and spring samples for AP2 and in the fall for RCI2 leads to the hypothesis that 12TX10 may acclimate adequately to cold temperatures at the onset of winter but lacks the appropriate deacclimation response in the spring to confer true overwintering capacity. This leads to the conclusion that, at least for these two genes, 12TX10 is less responsive to the warming temperatures in the spring and may be less winter hardy than 12TX09. This is supported by the fact that 40% of the 12TX09 clones survived the winter of 2013 at Vernon while none of the 12TX10 clones overwintered. Jang et al. (2006) also reported similar findings in comparing what they termed the rhizome tip and mature rhizome internode where it is known that the elongation zone/mature internode is largely a storage vessel for starches, proteins, and carbohydrates that aids in preparation for winter.

While the trends from fall to spring are similar across treatments and samples, it is not surprising that the D region shows increased differential expression levels in comparison to EL, especially in the spring, since active cell division and increased levels of transcription are linked. In addition, He et al. (2014) reported preferential expression of genes in either the apical tip (D) or the elongation zone (EL) when conducting transcriptomics on rhizomes of red rice (*Oryza longistaminata* A. Chev. & Roehrich) further supporting the theory that gene specialization and preferential timing of transcription is initiated in each cell type individually.

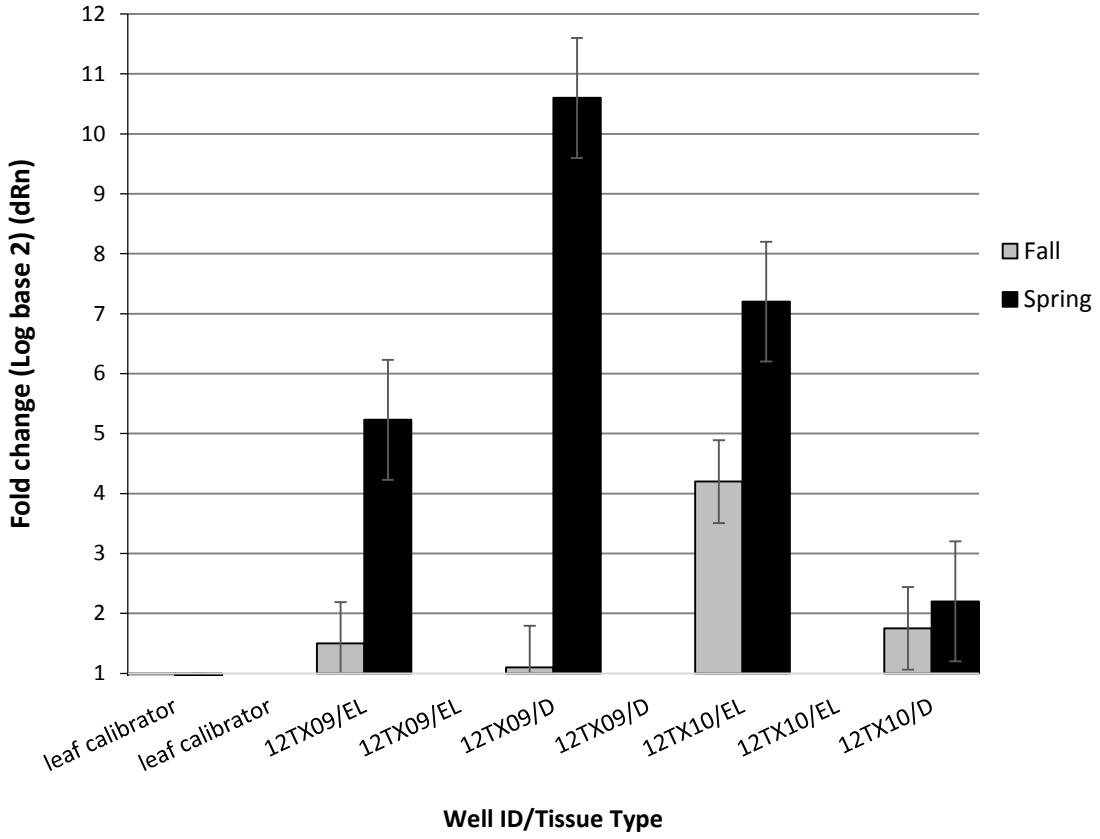


Figure 4. Relative expression of the AP2 gene in rhizomes of the 12TX09 and 12TX10 genotypes at Commerce, TX. Treatments shown here are the cell elongation (EL) and cell division (D) regions in fall as well as spring sampled rhizomes. Spring samples show differential expression from the control (leaf unstressed) in all treatments while only EL of 12TX10 shows differential expression in the fall. Error bars signify the standard error of the mean provided by the MxPro software.

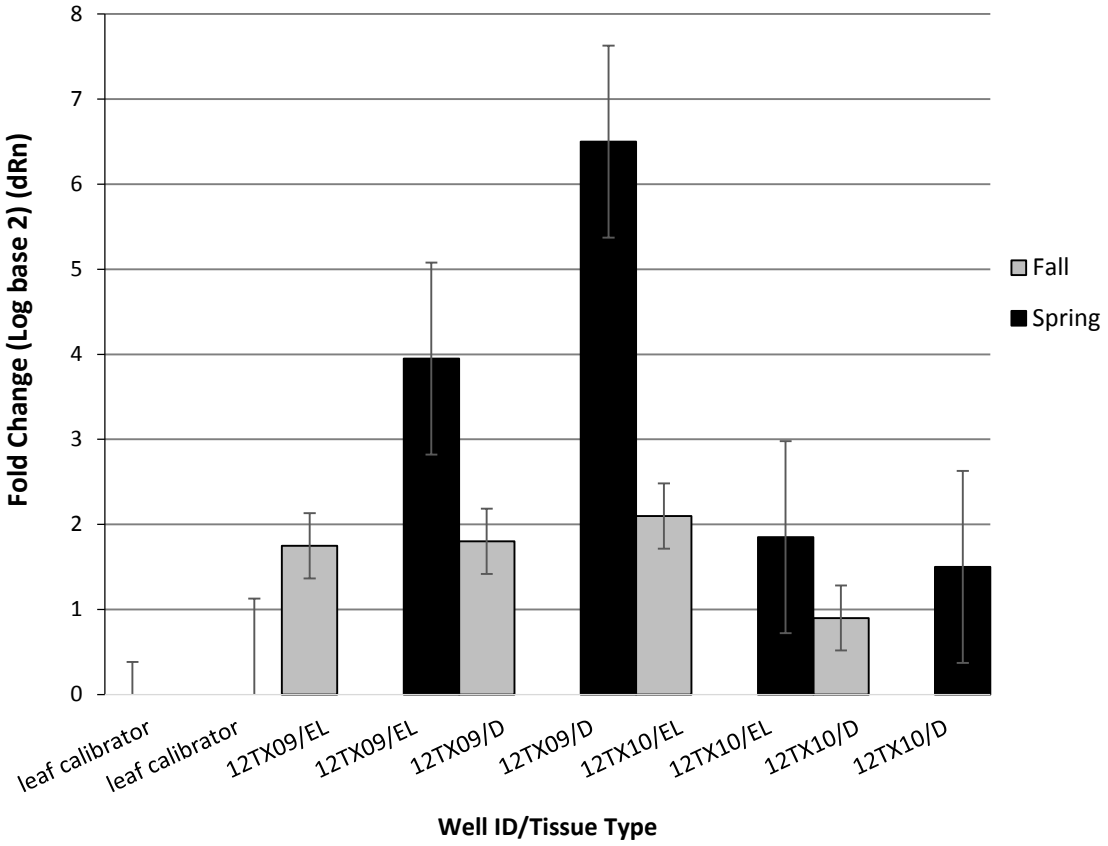


Figure 5. Relative expression of the RCI2 gene in rhizomes of the 12TX09 and 12TX10 genotypes at Commerce, TX. Treatments shown here are the cell elongation (EL) and cell division (D) regions as well as fall and spring samples rhizomes. Both 12TX09 regions show substantial differential expression in the spring while neither 12TX10 region showed differential expression from the control (leaf unstressed) in the spring. Error bars signify the standard error of the mean provided by the MxPro software.

Four genotypes (12TX09, 12TX10, 12TX18, and Merkeron) were analyzed in College Station, TX. Appendix B contains all of the charts for the genotypes analyzed at College Station for fall, spring, AP2, and RCI2. Spring samples across all genotypes had the greatest FC. The AP2 gene demonstrated a greater level of upregulation across all D

samples in the spring at College Station with 12TX09, 12TX10, 12TX18, and Merkeron having a differential expression FC of 13, 6, 12, and 11, respectively. While several other samples reached the minimum two-FC threshold and were considered as having differential expression from the control, most are not overly notable. Aside from the aforementioned group collected in the spring, the AP2 gene from the 12TX09 D region from the fall sampling and the RCI2 gene from the 12TX18 D region also from the fall sampling had a FC of 4 and 3.5, respectively. The strong expression levels of the D region in the spring match those results observed in the Commerce samples, and this is associated with a strong increase in transcriptional activity at the onset of spring, which coincides with spring regrowth of rhizome apical meristems. This also shows the importance of both genes, but especially AP2, in the initiation of signals for growth of quiescent rhizome meristems during critical times of deacclimation from the cold.

Identification of the AP2 and RCI2 genes in napiergrass and their subsequent upregulation during critical times of cold acclimation in the fall and deacclimation in the spring confirms that they probably participate in the cold-responsive gene regulatory pathway. The C-repeat-binding factors (*CBFs*) are upstream TFs from the AP2 family of genes, which have been shown to bind to the *cis*-element promoter region and trigger expression of genes associated with cold response (Thomashow, 1999). The results from this study confirm previous reports by He et al. (2014) regarding the importance of the AP2 family of genes in the cold response, and more specifically, as being enriched in the rhizome transcriptome. He et al. (2014) identified 199 unigenes that were highly-

enriched or specifically expressed in rhizome apical meristems of red rice, and 40% of these genes were associated with TFs, one of which is AP2. They have since been identified as playing a major role in the regulation of transcription of many biological processes as well as having an important function in several signal induction pathways including responses to hormones, biotic and abiotic stresses, salinity, drought, and cold (Rashid et al., 2012). In addition, the family of AP2 genes is quite diverse and involves in general growth and developmental processes such as regulating floral development, early identification of floral meristems, lateral and crown root development, and somatic embryogenesis (Rashid et al., 2012). It is likely the upregulation of AP2 in napiergrass' meristematic cells is associated with meristem development. While it is difficult to break the link between cold response and meristem development in this study, rhizome sampling and analysis of gene expression of AP2 during the winter could evidently link the two as AP2 expression is more than likely initiated later in late winter or early spring. Additional research is needed to determine if specialized AP2 genes regulate these processes or if they are acting in a pleiotropic manner. Zhu et al. (2007) suggested that cold stress triggers a multitude of transcriptional cascades because many cold responsive genes that are induced early encode TFs that may initiate genes that are induced after longer periods of exposure to cold. Zhu et al. (2007) also found only one TF that was down regulated during early cold stress indicating that cold responses in plants are initiated mainly by transcriptional activation rather than by repression of genes.

The discovery of crosstalk between cold-responsive and flowering regulatory pathways has been reported (Seo et al., 2009; Mawlong et al. 2015). Seo et al. (2009) discovered one feedback loop involving interactions between the transcriptional activator of floral development *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1* (*SOC1*), the upstream negative regulator of *SOC1*, *FLOWERING LOCUS C* (*FLC*), and cold responsive pathway regulators C-repeat/dehydration responsive element (CRT/DRE) binding factors (*CBFs*). In this example of crosstalk, the loop delays flowering through increasing *FLC* when a cold spell is transient in fall or early spring but suppresses the cold response when floral induction occurs through the repression of cold-inducible genes by *SOC1*. Seo et al. (2009) also suggested that *SOC1* negatively regulates the expression of the cold response genes through direct repression of *CBFs*. Linking this with AP2, Stockinger et al. (1997) found that *CBF1* of *Arabidopsis* encodes an AP2 domain-containing a transcriptional activator that binds to the CRT/DRE, which promotes the transcriptional response to cold temperatures. Ohme-Takagi and Shinshi (1995) determined that the AP2 domain of the CRT/DREs shares no significant resemblances in amino acid sequence or obvious similarities in structure with other known TFs or DNA binding motifs. According to Ohme-Takagi and Shinshi (1995), the domain seems to be a novel DNA-binding motif that only has been isolated in plant proteins. Implications of this include the likelihood that other AP2 domain proteins are simply one member of a larger superfamily of DNA-binding proteins that can identify other families of *cis*-acting regulatory elements. In addition, sequence variation

surrounding the principle CRT/DRE sequence would result in the mobilization of unique AP2 domain proteins, which are integrated into unique signal transduction pathways activated by different environmental, hormonal, and developmental cues (Ohme-Takagi and Shinshi, 1995). Mawlong et al. (2015) reported crosstalk between different developmental parameters and drought resistance when an AP2/ERF family transcription factor-encoding gene from rice was inserted into *Arabidopsis*. Increasing reports of this type of crosstalk is not surprising considering that the AP2/ERF family has been shown to perform several different functions towards conveying tolerance to a number of biotic and abiotic stresses as well as regulation of developmental processes, hormonal signal transduction, and metabolism (Ohme-Takagi and Shinshi, 1995; Aharoni et al., 2004; Broun et al., 2004; Yamaguchi-Shinozaki and Shinozaki, 2006; Century et al., 2008; Yin et al., 2010)

RCI2 has been confirmed to play a critical role in the cold response pathway. It has been repeatedly shown to be induced by low temperatures to confer cold-tolerance, and eight RCI2 genes have been cloned from *Arabidopsis* (A-H) (Capel et al., 1997; Medina et al., 2007; Sivankalyani et al., 2015). Even though RCI2 is not correlated to a TF superfamily, it has been shown to be induced by multiple abiotic stress responses including drought, salt, and cold (Misuya et al., 2005; Gimeno et al., 2009; Long et al., 2015). Drought-induced genes in mandarin, *Citrus reticulata* Blanco, leaves and roots were isolated using microarrays, and qRT-PCR that showed a high homology to RCI2 hydrophobic membrane proteins from *Arabidopsis*. This verifies that RCI2 is not organ

specific and is induced upon exposure to drought, salt, and cold in different plant tissues and plant species (Gimeno et al., 2009). The RCI2 proteins in plants have high homology to the yeast plasma membrane proteolipid protein, PMP3, which functions in decreasing the electrical potential of cell membranes and reducing the buildup of cations in the cell (Navarre and Goffeau, 2000). RCI2 mutants were inserted into *Arabidopsis* by Mitsuya et al. (2005), and they observed the hyperaccumulation of Na^{2+} in the knockout mutants. Conversely, overexpression of RCI2 in the same experiment lead to a reduction in Na^{2+} taken up by the plants and increased their tolerance to salt. In a similar experiment as Gimeno et al. (2009), several orthologs showing high homology to RCI2 from *Arabidopsis* were isolated and characterized in *Medicago sativa* L. and *Medicago truncatula* Gaertn. further confirming that these proteins are ubiquitous across plant species. Similar reports linking RCI2 and salt tolerance have been reported for rice, barley, and wheatgrass (Morsy et al., 2005; Goddard et al., 1993; Galvez et al., 1993). These findings are promising for the future of cold, salt, and drought tolerance in crop plants, and they also confirm the role of RCI2 in abiotic stress responses. While the transcript levels of RCI2 were inconsistent for the different genotypes, cell types, and seasons in this experiment, it is clear the RCI family of proteins play an important role in how these plants respond to cold.

Conclusions

Several studies have attempted to correlate fluctuations of metabolic and biochemical adaptation to responses to cold-tolerance. However, these focused on rapid, short-term responses to low-temperature exposure in controlled conditions while neglecting or disregarding the prolonged response provided by *in situ* analysis. This latter type of approach is validated because previous reports are lacking in functional annotation when BLAST searches are done on genes specifically expressed from the rhizome transcriptome (Jang et al., 2006). The finding of AP2 and RCI2 in napiergrass is promising for the future cold adaptation of the species because it validates the regulatory mechanisms for cold-tolerance, as well as drought and salt, both of which already exist in napiergrass. In conjunction with next-generation biotechnology, genomics, transcriptomics, metabolomics, proteomics, and traditional breeding techniques, progress can be made regarding cold-tolerance and other major abiotic stresses that utilize genes from the AP2 superfamily as well as the RCI proteins. At the very least, these findings can more precisely elucidate the signal transduction, transcriptional machinery, and protein-protein interactions regulating tolerance to cold leading towards improved overwintering in valuable tropical and temperate perennial species including napiergrass.

CHAPTER IV

CONCLUSIONS

Napiergrass is a valuable forage grass with high nutritive value for ruminant livestock and potential as a bioenergy crop. It produces large amounts of biomass, and expanding its range of adaptation can greatly increase its production area and importance. This research has shown that the northern fringe for napiergrass can be expanded with phenotypic selection. Additionally, the species may be more winter hardy than previously expected due to Merkeron, the experimental control, demonstrating equivalent winter hardiness to most of the selections. An orthologous AP2 gene from *S. bicolor* present in napiergrass proved to be important in overwintering, and at a minimum, is important in proper timing of spring growth initiation. In addition, the hydrophobic membrane protein, RCI2, showed some degree of importance in overwintering likely by protecting cellular membranes. Further research is needed to elucidate how multiple genetic pathways interact with environmental, hormonal, and developmental cues leading to the materialization of cold-tolerance. Attempts to incorporate genes associated with cold-tolerance via wide hybridization with wild *Pennisetum* species was unsuccessful. All genotypes had pollen tubes reaching the ovary and micropyle, except for accession 433, which was the only pollinator that displayed a negative pollen-pistil interaction with PEPU09TX01. Analysis of post-fertilization activities in napiergrass ovaries should reveal the barriers in napiergrass x buffelgrass and napiergrass x oriental fountaingrass hybrids. The production of interspecific hybrids

between napiergrass and members of the tertiary gene pool to incorporate genes associated with cold-tolerance is difficult, tedious, and not practical from the implications of this study. Selecting and improving elite individuals within napiergrass and including them in a breeding program to increase the overall tolerance to cold are most feasible and have the greatest likelihood of success.

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APPENDIX A

Table 8. Pollen germination and tube growth of napiergrass (PEPU09TX01) when self-pollinated.

Time after pollination (hours)	Number of pistils observed	Number of pollen grains observed†	Pollen germination (%)‡	Number of tubes to:			
				stigma†	style†	ovary†	micropyle†
				----------No.----------			
1	15	578 ±5.27	53.20 ±11.88	128 ±1.79	0 ±0	0 ±0	0 ±0
2	13	798 ±7.78	57.04 ±9.49	137 ± 2.76	0 ±0	0 ±0	0 ±0
3	14	881 ±9.01	50.53 ±11.28	157 ±3.38	0 ±0	0 ±0	0 ±0
4	15	1140 ±5.65	54.76 ±8.65	223 ±2.87	0 ±0	0 ±0	0 ±0
6	14	937 ±4.89	59.65 ±9.81	168 ±4.14	6 ±0.47	0 ±0	0 ±0
9	15	1207 ±6.31	61.90 ±13.70	212 ±4.82	7 ±1.11	0 ±0	0 ±0
12	14	753 ±4.09	47.44 ±12.69	141 ±6.09	11 ±1.02	3 ±0.11	0 ±0
24	13	916 ±7.11	50.98 ±10.54	179 ±3.41	16 ± 2.12	10 ±0.21	7 ±0.13
Overall	113	7210	54.44	1345	40	13	7

† Sum ± standard error of the mean
‡ Mean ± standard error of the mean

Table 9. Pollen germination and tube growth after pollinations between napiergrass (PEPU09TX01) and pearl millet (PEGL09TX04).

† Sum ± standard error of the mean

‡ Mean ± standard error of the mean

Time after pollination (hours)	Number of pistils observed	Number of pollen grains observed†	Pollen germination (%)‡	Number of tubes to:			
				stigma†	style†	ovary†	micropyle†
				----------No.----------			
1	23	1749 ±14.68	84.73 ±14.35	812 ±8.52	55 ±0.76	22 ±0.41	1 ±0.04
2	19	1502 ±12.44	81.82 ±11.27	735 ±7.42	98 ±0.93	76 ±0.81	18 ±0.26
Overall	42	3251	83.28	1547	153	98	19

Table 10. Pollen germination and tube growth after pollinations between napiergrass (PEPU09TX01) and napiergrass (Merkeron).

Time after pollination (hours)	Number of pistils observed	Number of pollen grains observed†	Pollen germination (%)‡	Number of tubes to:			
				stigma†	style†	ovary†	micropyle†
				----------No.----------			
1	14	875 ±4.00	79.44 ±2.96	269 ±1.54	0 ±0	0 ±0	0 ±0
2	13	1048 ±4.14	84.54 ±3.24	429 ±2.27	4 ±0.17	3 ±0.16	0 ±0
3	15	1072 ±2.57	73.32 ±2.44	412 ±1.50	16 ±0.22	4 ±0.11	0 ±0
4	15	1757 ±11.04	67.73 ±5.86	665 ±3.05	33 ±0.36	22 ±0.28	7 ±0.16
Overall	57	4752	76.26	1775	53	29	7

† Sum ± standard error of the mean
‡ Mean ± standard error of the mean

Table 11. Pollen germination and tube growth after pollinations between napiergrass (PEPU09TX01) x buffelgrass (Common).

Time after pollination (hours)	Number of pistils observed	Number of pollen grains observed†	Pollen germination (%)‡	Number of tubes to:			
				stigma†	style†	ovary†	micropyle†
				----------No.----------			
1	17	1410 ±9.65	58.09 ±5.85	288 ±2.56	2 ±0.07	1 ±0.06	0 ±0
2	15	1496 ±15.21	50.53 ±11.28	334 ±4.74	33 ±0.52	24 ±0.34	8 ±0.19
3	13	1214 ±14.38	68.62 ±11.10	351 ±4.99	47 ±0.98	39 ±0.85	17 ±0.41
4	16	1599 ±14.01	63.98 ±10.42	510 ±5.92	96 ±0.85	57 ±0.54	22 ±0.33
Overall	61	5719	60.31	1483	178	121	47

† Sum ± standard error of the mean
‡ Mean ± standard error of the mean

Table 12. Pollen germination and tube growth after pollinations between napiergrass (PEPU09TX01) and buffelgrass (Frio).

Time after pollination (hours)	Number of pistils observed	Number of pollen grains observed†	Pollen germination (%)‡	Number of tubes to:			
				stigma†	style†	ovary†	micropyle†
				----------No.----------			
1	21	696 ±4.30	57.04 ±2.49	155 ±1.18	0 ±0	0 ±0	0 ±0
2	22	980 ±5.18	63.67 ±3.11	276 ±1.45	0 ±0	0 ±0	0 ±0
3	27	1046 ±3.97	68.36 ±2.95	286 ±1.01	12 ±0.12	5 ±0.07	0 ±0
4	21	698 ±3.29	64.18 ±2.19	201 ±1.02	26 ±0.22	14 ±0.16	2 ±0.06
6	14	312 ±2.11	83.44 ±1.57	136 ±0.79	50 ±0.46	39 ±0.43	18 ±0.24
Overall	105	3732	67.34	1054	88	28	20

† Sum ± standard error of the mean
‡ Mean ± standard error of the mean

Table 13. Pollen germination and tube growth after pollinations between napiergrass (PEPU09TX01) and oriental fountaingrass (PEOR 09TX01).

Time after pollination (hours)	Number of pistils observed	Number of pollen grains observed†	Pollen germination (%)‡	Number of tubes into:			
				stigma†	style†	ovary†	micropyle†
				----------No.----------			
1	13	702 ±5.23	77.35 ±4.71	179 ±2.45	0 ±0	0 ±0	0 ±0
2	20	1132 ±4.36	72.17 ±4.08	470 ±2.14	5 ±0.12	0 ±0	0 ±0
3	17	1528 ±8.42	75.33 ±6.66	512 ±2.71	43 ±0.39	17 ±0.26	9 ±0.13
4	17	1478 ±6.50	83.40 ±4.94	637 ±3.08	80 ±0.54	68 ±0.53	19 ±0.32
Overall	67	4840	77.06	1798	128	85	28

† Sum ± standard error of the mean
‡ Mean ± standard error of the mean

Table 14. Pollen germination and tube growth after pollinations between napiergrass (PEPU09TX01) and oriental fountaingrass (PI 269961).

Time after pollination (hours)	Number of pistils observed	Number of pollen grains observed†	Pollen germination (%)‡	Number of tubes into:			
				stigma†	style†	ovary†	micropyle†
				----------No.----------			
1	21	1769 ±12.60	78.01 ±10.72	338 ±2.53	0 ±0	0 ±0	0 ±0
2	27	3379 ±21.72	79.43 ±18.14	1228 ±7.95	4 ±0.09	0 ±0	0 ±0
3	21	1586 ±10.66	78.59 ±9.20	642 ±5.12	20 ±0.23	10 ±0.13	1 ±0.05
4	15	2514 ±12.05	81.30 ±10.74	782 ±5.64	26 ±0.36	8 ±0.16	1 ±0.06
6	14	767 ±6.96	68.10 ±4.83	240 ±2.20	51 ±0.78	37 ±0.64	20 ±0.36
Overall	98	10015	77.09	3230	101	55	22

† Sum ± standard error of the mean
‡ Mean ± standard error of the mean

Table 15. Pollen germination and tube growth after pollinations between napiergrass (PEPU09TX01) x oriental fountaingrass (PI 314994).

Time after pollination (hours)	Number of pistils observed	Number of pollen grains observed†	Pollen germination (%)‡	Number of tubes into:			
				stigma†	style†	ovary†	micropyle†
				----------No.----------			
1	15	2107 ±14.99	78.03 ±13.09	440 ±3.43	0 ±0	0 ±0	0 ±0
2	15	2458 ±11.81	75.22 ±9.96	927 ±5.65	9 ±0.21	0 ±0	0 ±0
3	14	2465 ±22.31	73.51 ±14.67	888 ±12.21	15 ±0.26	8 ±0.20	0 ±0
4	15	1676 ±13.98	69.51 ±11.0	565 ±5.95	19 ±0.27	9 ±0.18	0 ±0
6	15	1416 ±11.54	74.93 ±7.36	591 ±4.60	43 ±0.52	21 ±0.34	9 ±0.18
Overall	74	10122	74.24	3411	86	38	9

† Sum ± standard error of the mean
‡ Mean ± standard error of the mean

Table 16. Pollen germination and tube growth after pollinations between napiergrass (PEPU09TX01) and oriental fountaingrass (PI 271595).

Time after pollination (hours)	Number of pistils observed	Number of pollen grains observed†	Pollen germination (%)‡	Number of tubes into:			
				stigma†	style†	ovary†	micropyle†
				----------No.----------			
1	15	861 ±3.73	59.35 ±3.89	223 ±2.79	0 ±0	0 ±0	0 ±0
2	14	618 ±5.06	63.13 ±3.08	181 ±1.84	0 ±0	0 ±0	0 ±0
3	14	722 ±8.99	45.15 ±2.87	109 ±0.96	3 ±0.11	0 ±0	0 ±0
4	15	603 ±4.38	61.49 ±2.16	165 ±1.23	18 ±0.25	5 ±0.12	1 ±0.06
6	13	662 ±7.24	68.19 ±4.69	207 ±2.45	29 ±0.36	21 ±0.37	7 ±0.23
Overall	71	3466	59.46	885	50	26	8

† Sum ± standard error of the mean

‡ Mean ± standard error of the mean

Table 17. Pollen germination and tube growth after pollinations between napiergrass (PEPU09TX01) and oriental fountaingrass (PI 271596).

Time after pollination (hours)	Number of pistils observed	Number of pollen grains observed†	Pollen germination (%)‡	Number of tubes into:			
				stigma†	style†	ovary†	micropyle†
				----------No.----------			
1	15	881 ±6.44	63.77 ±4.98	212 ±2.21	0 ±0	0 ±0	0 ±0
2	14	473 ±5.06	71.88 ±1.98	115 ±0.85	0 ±0	0 ±0	0 ±0
3	15	749 ±8.99	53.52 ±1.51	232 ±1.20	0 ±0	0 ±0	0 ±0
4	13	468 ±4.38	70.94 ±3.01	181 ±1.40	12 ±0.25	0 ±0	0 ±0
6	14	898 ±7.24	59.69 ±2.72	269 ±1.40	62 ±0.54	25 ±0.34	6 ±0.13
Overall	71	3469	63.96	1009	74	25	6

† Sum ± standard error of the mean
‡ Mean ± standard error of the mean

Table 18. Pollen germination and tube growth after pollinations between napiergrass (PEPU09TX01) and oriental fountaingrass (PI 315867).

Time after pollination (hours)	Number of pistils observed	Number of pollen grains observed †	Pollen germination (%)‡	Number of tubes into:			
				stigma†	style†	ovary†	micropyle†
				----------No.----------			
1	19	723 ±4.90	53.18 ±1.8	80 ±0.68	0 ±0	0 ±0	0 ±0
2	16	789 ±6.68	52.74 ±3.45	156 ±1.33	7 ±0.22	0 ±0	0 ±0
3	18	863 ±7.44	58.85 ±3.47	222 ±1.57	13 ±0.19	3 ±0.09	0 ±0
4	17	869 ±7.02	59.03 ±4.13	252 ±2.13	51 ±0.53	36 ±0.45	15 ±0.20
Overall	70	3244	56.45	710	71	39	15

† Sum ± standard error of the mean

‡ Mean ± standard error of the mean

Table 19. Pollen germination and tube growth after pollinations between napiergrass (PEPU09TX01) and oriental fountaingrass (Cowboy).

Time after pollination (hours)	Number of pistils observed	Number of pollen grains observed†	Pollen germination (%)‡	Number of tubes into:			
				stigma†	style†	ovary†	micropyle†
				----------No.----------			
1	14	716 ±5.17	55.63 ±2.41	140 ±1.31	0 ±0	0 ±0	0 ±0
2	14	1131 ±8.54	65.52 ±8.54	301 ±2.03	0 ±0	0 ±0	0 ±0
3	18	869 ±4.48	59.72 ±2.53	209 ±1.20	4 ±0.13	0 ±0	0 ±0
4	15	2570 ±13.55	67.32 ±10.89	704 ±5.02	12 ±0.21	5 ±0.18	2 ±0.09
6	15	1155 ±7.23	72.68 ±4.17	324 ±2.48	35 ±0.41	22 ±0.39	8 ±0.19
Overall	76	6441	64.17	1678	51	27	10

† Sum ± standard error of the mean
‡ Mean ± standard error of the mean

Table 20. Pollen germination and tube growth after pollinations between napiergrass (PEPU09TX01) and oriental fountaingrass (433).

Time after pollination (hours)	Number of pistils observed	Number of pollen grains observed†	Pollen germination (%)‡	Number of tubes into:			
				stigma†	style†	ovary†	micropyle†
				----------No.----------			
1	15	362 ±4.78	47.12 ±2.76	42 ±0.67	0 ±0	0 ±0	0 ±0
2	11	286 ±6.11	53.55 ±4.91	31 ±0.88	0 ±0	0 ±0	0 ±0
3	13	332 ±4.21	61.21 ±6.26	46 ±0.56	0 ±0	0 ±0	0 ±0
4	15	329 ±7.76	49.87 ±4.99	62 ±0.33	0 ±0	0 ±0	0 ±0
6	11	348 ±5.19	51.33 ±2.23	39 ±0.24	0 ±0	0 ±0	0 ±0
9	14	531 ±4.33	56.28 ±3.60	58 ±0.91	0 ±0	0 ±0	0 ±0
12	14	353 ±4.02	43.36 ±3.09	67 ±0.83	0 ±0	0 ±0	0 ±0
24	13	95 ±2.47	46.68 ±1.79	44 ±0.21	0 ±0	0 ±0	0 ±0
Overall	106	2636	51.18	389	0	0	0

† Sum ± standard error of the mean
‡ Mean ± standard error of the mean

APPENDIX B

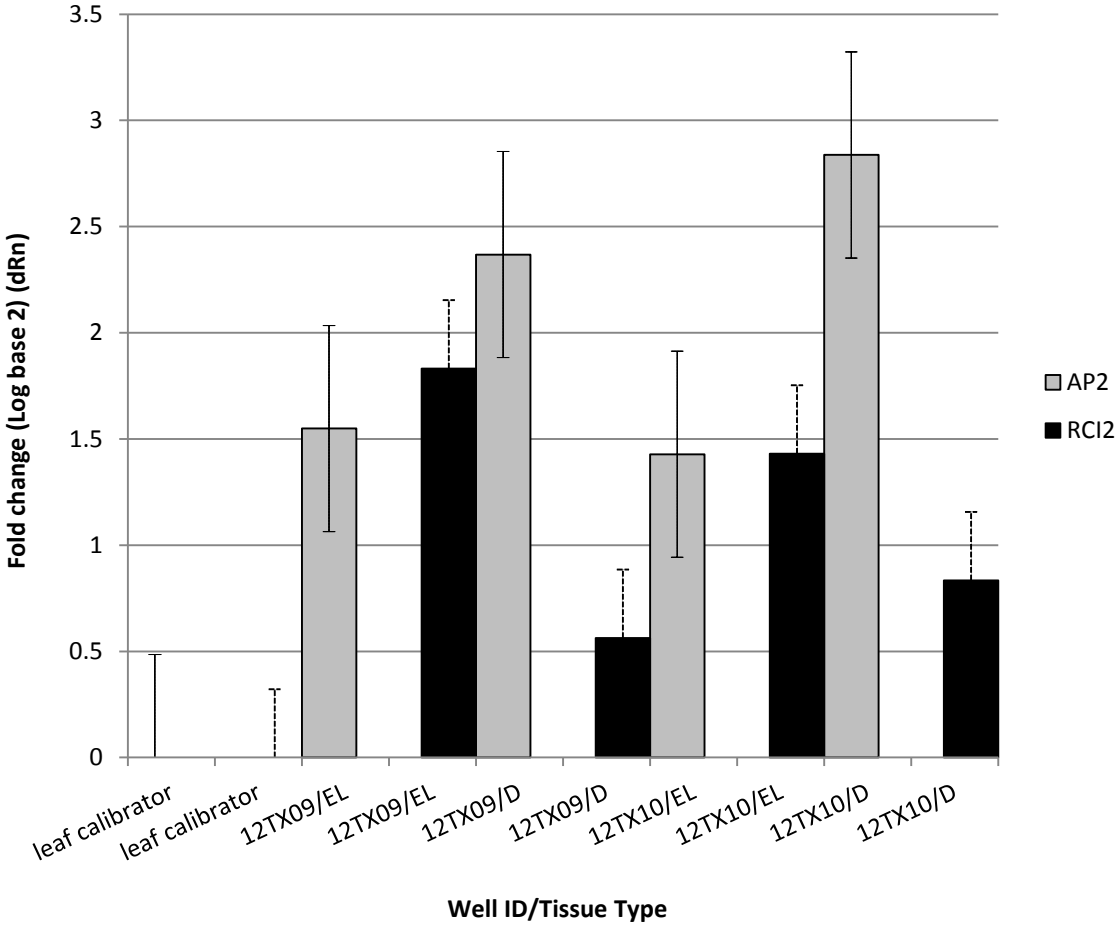

Figure 6. Relative expression of the AP2 and RCI2 genes in rhizomes of the 12TX09 and 12TX10 genotypes at Commerce, TX, in the fall of 2013. Treatments shown here are the cell elongation (EL) and cell division (D) regions for each genotype.

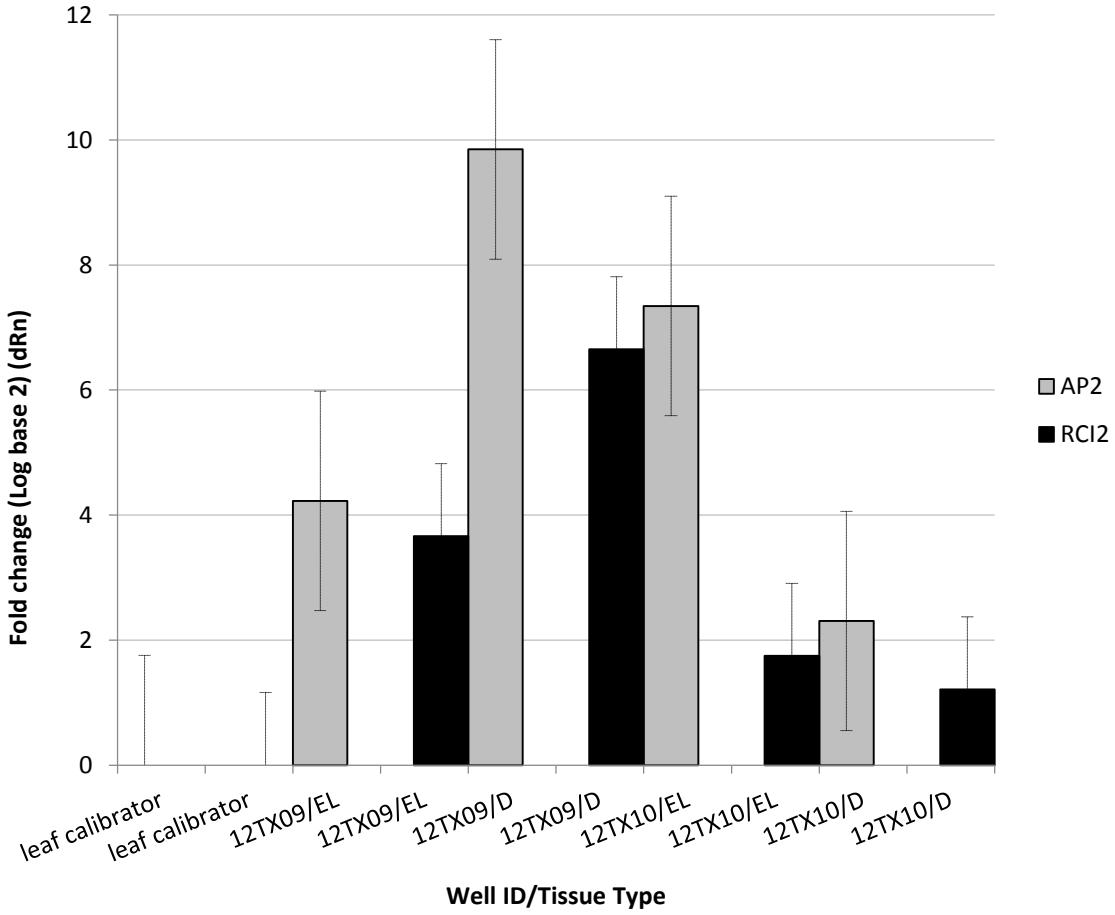

Figure 7. Relative expression of the AP2 and RCI2 genes in rhizomes of the 12TX09 and 12TX10 at Commerce, TX, in the spring of 2014. Treatments shown here are the cell elongation (EL) and cell division (D) regions for each genotype.

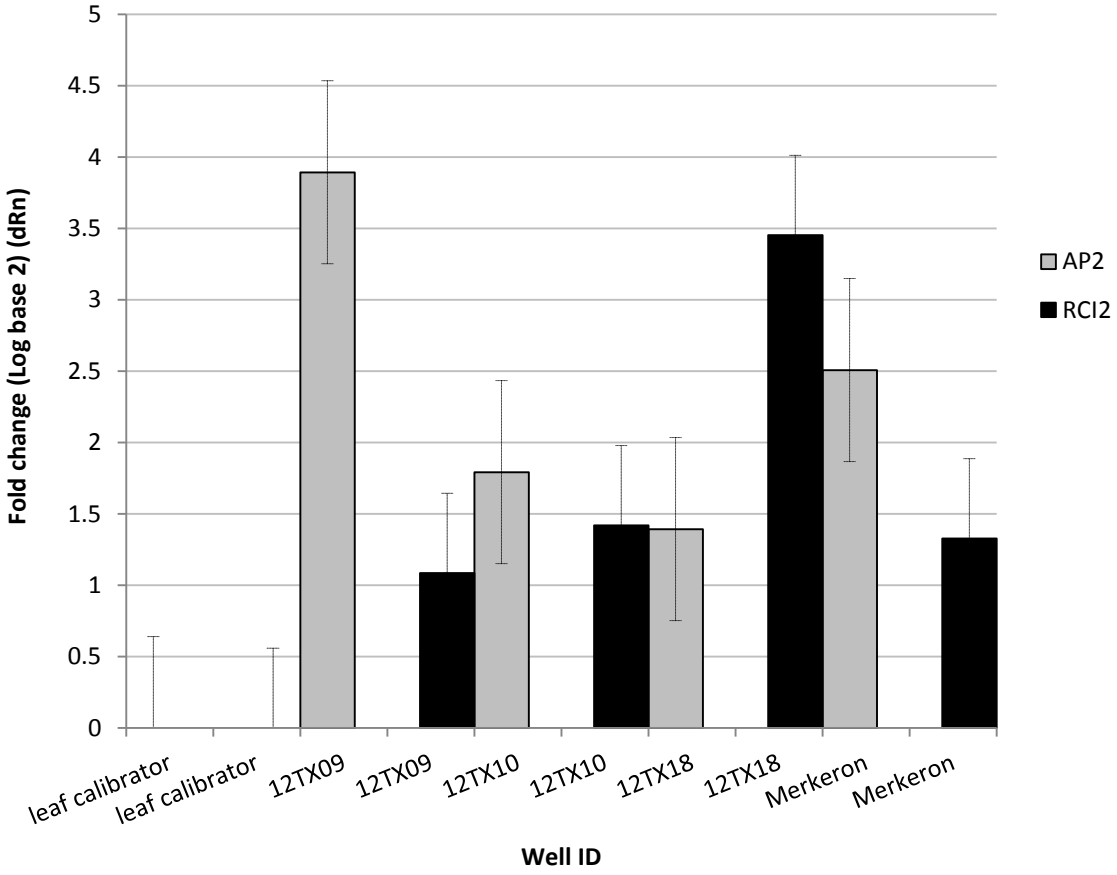

Figure 8. Relative expression of the AP2 and RCI2 genes in the region of cell division for 12TX09, 12TX10, 12TX18, and Merkeron at College Station, TX, in the fall of 2013.

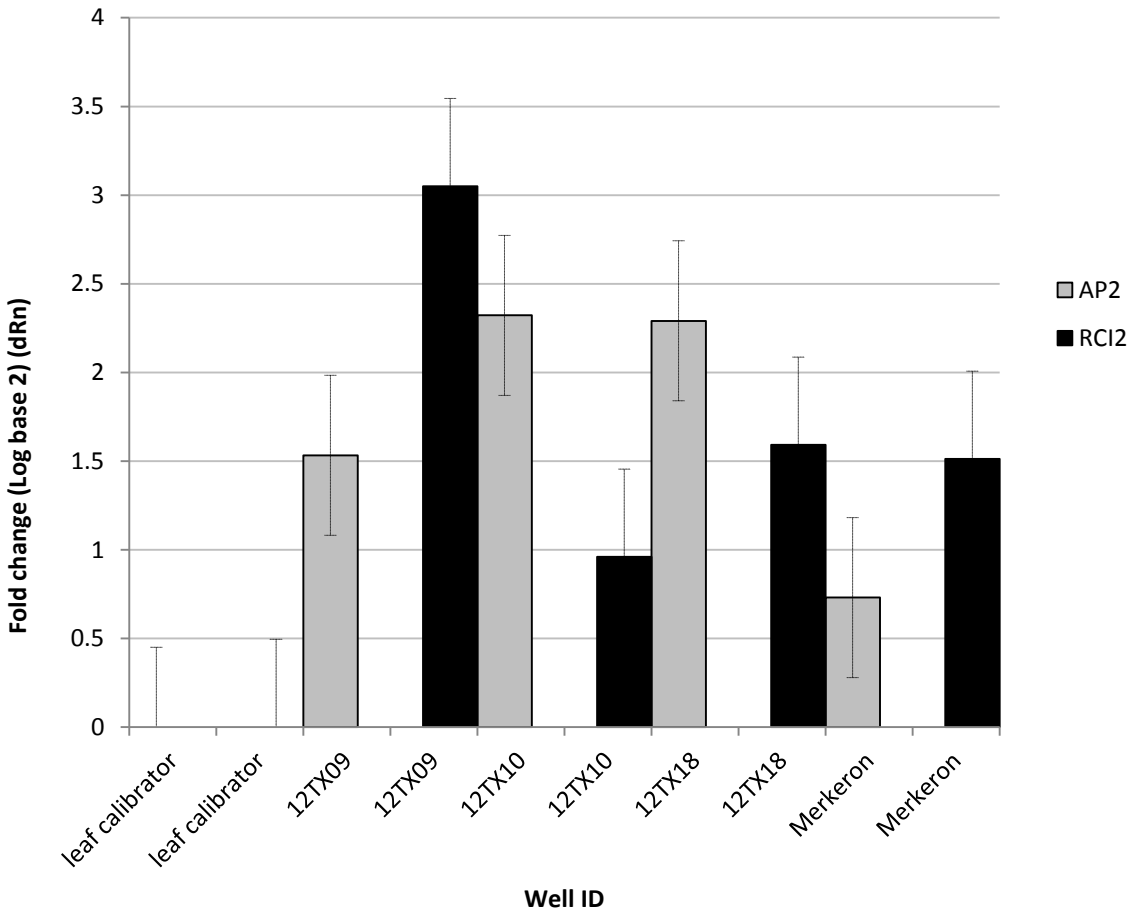

Figure 9. Relative expression of the AP2 and RCI2 genes in the region of cell elongation for 12TX09, 12TX10, 12TX18, and Merkeron at College Station, TX, in the fall of 2013.

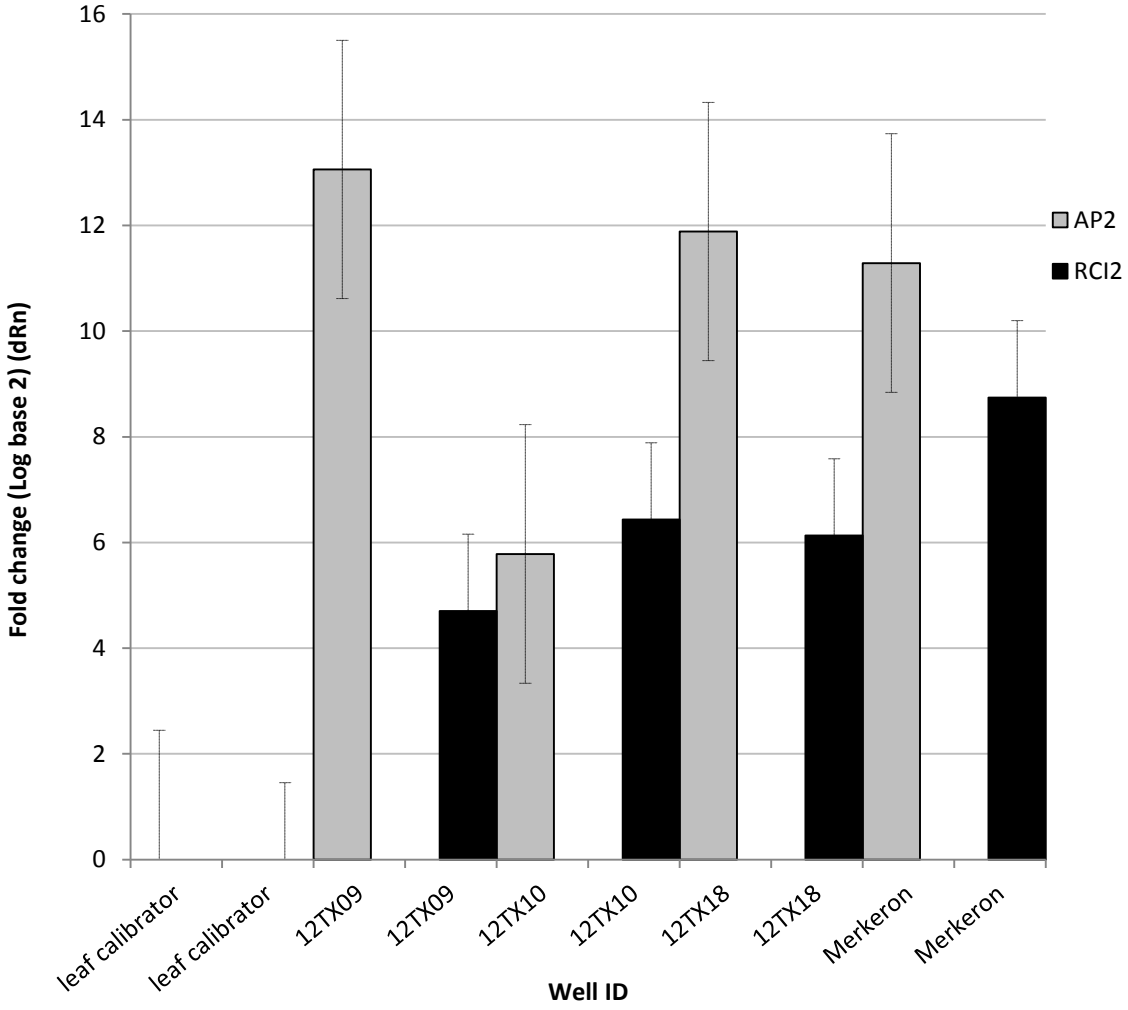

Figure 10. Relative expression of the AP2 and RCI2 genes in the region of cell division for 12TX09, 12TX10, 12TX18, and Merkeron at College Station, TX, in the spring of 2014.

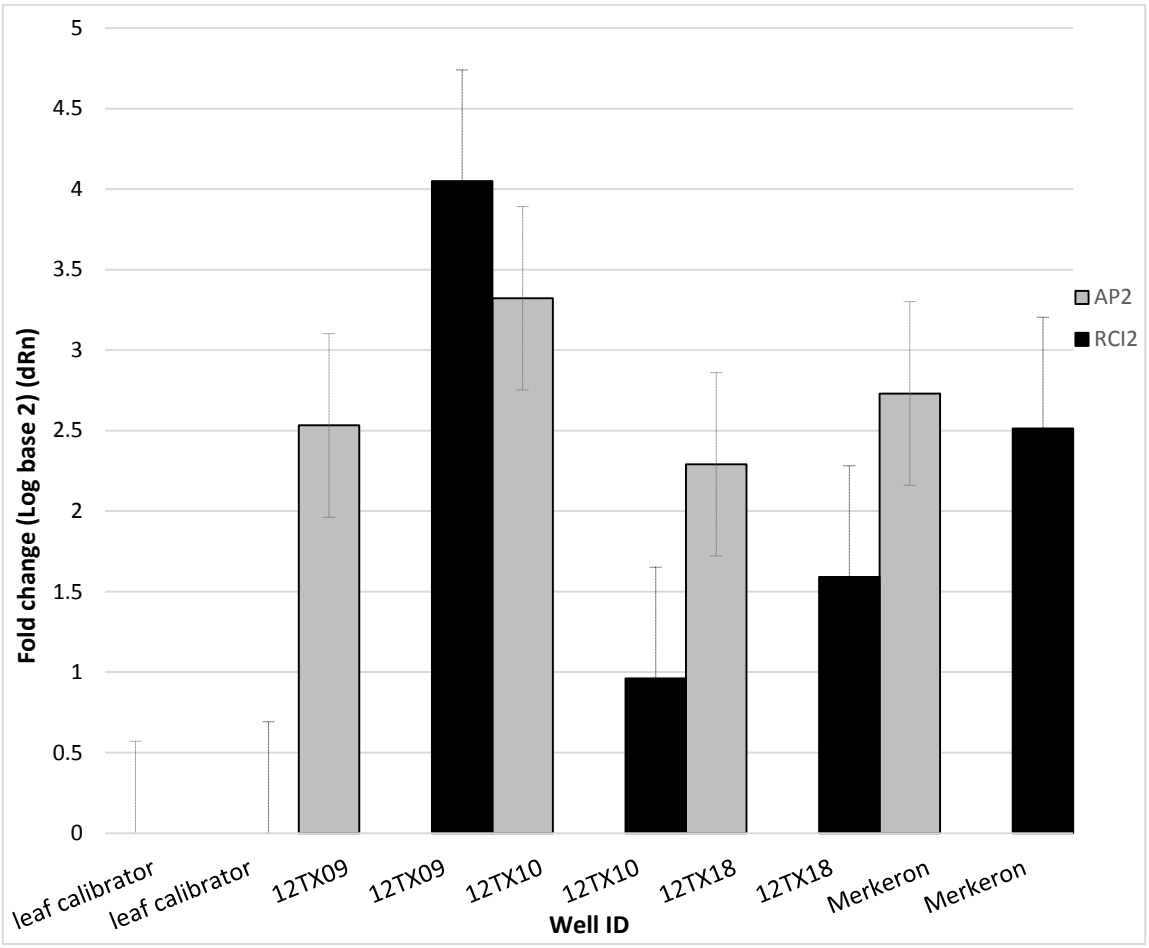

Figure 11. Relative expression of the AP2 and RCI2 genes in the region of cell elongation for 12TX09, 12TX10, 12TX18, and Merkeron at College Station, TX, in the spring of 2014.