

2016

Identification of Genes Associated with Resistance to Brown Rust in Sugarcane and Prevalence of One Major Gene

Mavir Carolina Avellaneda Barbosa

Louisiana State University and Agricultural and Mechanical College, mcavellaneda@gmail.com

Follow this and additional works at: https://digitalcommons.lsu.edu/gradschool_dissertations



Part of the [Plant Sciences Commons](#)

Recommended Citation

Avellaneda Barbosa, Mavir Carolina, "Identification of Genes Associated with Resistance to Brown Rust in Sugarcane and Prevalence of One Major Gene" (2016). *LSU Doctoral Dissertations*. 3645.

https://digitalcommons.lsu.edu/gradschool_dissertations/3645

This Dissertation is brought to you for free and open access by the Graduate School at LSU Digital Commons. It has been accepted for inclusion in LSU Doctoral Dissertations by an authorized graduate school editor of LSU Digital Commons. For more information, please contact gradetd@lsu.edu.

IDENTIFICATION OF GENES ASSOCIATED WITH RESISTANCE TO
BROWN RUST IN SUGARCANE AND PREVALENCE OF ONE
MAJOR GENE

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Department of Plant Pathology and Crop Physiology

by
Mavir Carolina Avellaneda Barbosa
B.S. Pontificia Universidad Javeriana, 2002
M.S. Louisiana State University, 2014
May 2016

This dissertation is dedicated to my beloved son Nicolás.

ACKNOWLEDGMENTS

Thanks to God for granting me so many blessings and giving me the health, strength and discernment to pursue a research career.

I would like to sincerely and deeply thank Dr. Jeff Hoy for giving me the opportunity of pursuing graduate studies and accepting me as his student. His knowledge, guidance, supports and patience through the research project and course work has been fundamental for their completion. I would like to extend my gratitude to the members of my committee, Dr. Niranjan Baisakh, Dr. Lawrence Datnoff and Dr. Thomas Reagan. Their knowledge and advice have been very important for the success of the project. Without the economic support of the Louisiana State University Agricultural Center and the American Sugar Cane League, it never would have been possible to earn my degree.

I want to extend my gratitude to Carolyn Savario from the Sugarcane Lab for the support and friendship, Nathaniel Fickett who helped me with the WCSRG information, to my office mates from the Sugarcane Genetics Lab, especially to Andrés Felipe Gutiérrez for his companion during the MS and PhD process where we shared unforgettable moments for more than five years. Thank you, Andrés for always being there and bearing with my mood swings.

I must thank Dr. Chris Clark for being supportive and teaching me the basics of plant pathology. I need to thank my mentor, Dr. Jorge I. Victoria, who left a deep impression in my scientific career to follow in his footsteps by encouraging me to enjoy sugarcane pathology. I am thankful to all my roommates during the last five years: Dr. Tran Tran, Mrs. Deborah Xavier-Mis, Maryam Shahrtash, Kimberly Avellaneda, Cecilia Freitas, Rachel Sabat and Alejandra Jiménez who always found time to listen to me, hug me, and encourage me to follow my goals.

Also, thanks to my brother-in-law, Luis Alfaro, and friends, Dr. Sandra Galeano and Renesh Bedre, for the best coffee chats and memories in Baton Rouge. I would like to thank my friends that shared with me unforgettable moments but now are not near to me, Dr. Dina Gutiérrez, Dr. Margie Sánchez, Dr. Yamid Sanabria, Dr. Eduardo Sánchez and especially to my best friend Dr. Maria del Rocio Vega who despite the distance never let me lose my way, calmed my fears with a skype call, and had the time to answer a text immediately when I was in my worst moments. Thanks also to the Hoy's family for the hospitality and delicious Thanksgiving dinners that made me feel at home being away from my home. Thanks to fellow graduate students of the Plant Pathology and Crop Physiology Department and the friends from Zamorano Agricultural Society. To my friends from the Catholic Youth Pastoral Group from Christ the King: Jenny Moore, Alejandra Miranda, Erika Romero, Ghedy Matus, Mónica Tobón, Juan Rodríguez, César Escalante, Dr. Franklin Vacca, Carlos Escamilla, Ludwing Piña, Bro. César Muñoz, Bro. Renzo Alvarado, Fr. Eliécer Montañez Grimaldos M.C.M and auntie Rita Rangel - Thanks for your unconditional friendship, support and for being the best cheerleaders group.

I want to thank my entire "Barbosa family" for supporting me. I need to deeply thank my parents, Yolanda Barbosa and Silvio Avellaneda, who care for and educate my son Nicolás. Without their inexhaustible love, unconditional support, and dedication, it would be impossible to achieve my dreams. Thanks for letting me fly. I especially want to thank my sister, Kimberly Avellaneda, for always having time for me and for caring for my son as her own child. To my brother Silvio Avellaneda, thanks for always believing in me and reminding me not to forget my goals. Finally, I want to thank the most important person in my life, my son, who despite the distance, with every word, every look, and every smile fills me with energy, encouragement and strength to move forward. Thanks Nicolás for being the engine and joy of my life.

TABLE OF CONTENTS

ACKNOWLEDGMENTS	iii
LIST OF TABLES	vii
LIST OF FIGURES	viii
ABSTRACT.....	ix
CHAPTER 1: INTRODUCTION	1
1.1 OVERVIEW.....	1
1.2 SUGARCANE RESISTANCE TO BROWN RUST.....	4
1.3 SUGARCANE BROWN RUST MAPPING AND GENE EXPRESSION.....	6
1.4 REFERENCES CITED	12
CHAPTER 2: IDENTIFICATION OF GENES ASSOCIATED WITH RESISTANCE TO BROWN RUST IN SUGARCANE.....	18
2.1 INTRODUCTION.....	18
2.2 MATERIALS AND METHODS.....	21
2.2.1 Plant materials	21
2.2.2 Plant inoculation.....	21
2.2.3 Total RNA isolation.....	22
2.2.4 SSH library construction	22
2.2.5 Sequence processing and bioinformatics analysis.....	23
2.2.6. Expression analysis by semiquantitative RT-PCR.....	24
2.2.7 Mining of brown rust responsive ESTs for microsatellite markers.....	25
2.3 RESULTS	25
2.3.1 Identification of responsive genes in cultivar L 99-233.....	25
2.3.2 Expression analysis of brown rust responsive genes.....	30
2.3.3 Simple sequence repeat (SSR) markers derived from brown rust responsive genes....	33
2.4 DISCUSSION	34
2.5 REFERENCES CITED	37
CHAPTER 3: DISTRIBUTION AND PREVALENCE OF <i>Bru1</i> , A MAJOR BROWN RUST RESISTANCE GENE, IN THE SUGARCANE WORLD COLLECTION	42
3.1 INTRODUCTION.....	42
3.2 MATERIALS AND METHODS	46
3.2.1 Plant materials	46
3.2.2 Genomic DNA Isolation and PCR Genotyping.....	46
3.3.3 Genotype identifications.....	47
3.3 RESULTS.....	47
3.3.1 Classification of WCSRG Genotypes.....	47
3.3.2 Detection of <i>Bru1</i> in WCSRG.....	48
3.3.3 Geographical Distribution of <i>Bru1</i> in the World Collection of Sugarcane and Related Grasses.....	51

3.4 DISCUSSION	54
3.5 REFERENCES CITED	57
CHAPTER 4: CONCLUSIONS	62
VITA.....	64

LIST OF TABLES

Table 2.1 Sequences of primers used in reverse transcription PCR-mediated expression profiling.....	24
Table 2.2 Sequence statistics of the cDNAs isolated from suppressive subtraction library of L 99-233 in response to infection by <i>Puccinia melanocephala</i>	26
Table 3.1 Distribution and prevalence of <i>Bru1</i> molecular markers in the World Collection of Sugarcane and Related Grasses.....	50

LIST OF FIGURES

Figure 2.1 KEGG Pathway analysis (a) and gene ontology (b) of the brown rust resistance unigenes.....	26
Figure 2.2. Mapping of rust-responsive unigenes in sorghum genome.....	28
Figure 2.3 Temporal expression pattern of 11 selected rust-responsive unigenes in rust-resistant (L01-299, L99-233) and rust-susceptible (Ho95-988, L09-125) cultivars using semiquantitative RT-PCR. Sugarcane gene coding for elongation factor 1 α (<i>SoEF1α</i>) used as an internal control.....	30
Figure 2.4 Temporal expression pattern of 11 selected rust-responsive unigenes in rust-resistant (L 01-299, L 99-233) and rust-susceptible (Ho 95-988, L 09-125) cultivars using densitometry analysis of semiquantitative RT-PCR results. Sugarcane gene coding for elongation factor 1 α (<i>SoEF1α</i>) was used as an internal control.....	31
Figure 2.5 Distribution of simple sequence repeat (SSR) motifs in brown rust responsive genes in cultivar L 99-233.....	34
Figure 3.1 Genotypic classification of the World Collection of Sugarcane and Related Grasses.....	48
Figure 3.2 Representative gel images showing presence of <i>Bru1</i> detected by diagnostic PCR amplification products for R12H16 (570 bp product) and 9020-F4- <i>RsaI</i> (200 bp product) in <i>Saccharum</i> species or interspecific hybrid cultivars. Genotypes with positive <i>Bru1</i> detection provided by presence of either one or both markers are indicated by (+). <i>Saccharum spontaneum</i> : #1-7 represent ‘Unknown 2009:R433P78’, ‘IranSpont’, ‘SH3013’ (R12H16 marker only), ‘IND81161’, ‘US4625’, ‘SPONT 84089’, ‘WI 8711+2’. <i>Saccharum officinarum</i> : #1-6 represent ‘PMAG8428(221),’Puri’ Pundi’, IJ76316’, IJ76324’, IJ76319’. <i>Saccharum</i> hybrids: #1-5 represent ‘MEX856196’, ‘MOL6427’, ‘R570’, ‘B37161’, ‘Mesangen’ (R12H16 marker only). Other <i>Saccharum</i> species: #1-10 represent: ‘Merthi’(<i>S.sinense</i>), ‘Unknown’ (<i>S. sp</i>), ‘Nepal3’(<i>S.sinense</i>), ‘DB58661’(<i>S. sp</i>), ‘Mcilkrum’(<i>S.sinense</i>) (9020-F4 marker only), ‘Maneira’(<i>S.barberi</i>), ‘Kavangeri’(<i>S. officinarum</i>), ‘Merthizell’(<i>S.sinense</i>), ‘IK6340’ (<i>S. robustum</i>) (9020-F4 marker only), ‘IJ6480’(<i>S.robustum</i>).....	49
Figure 3.3 Geographical distribution of <i>Bru1</i> markers in World Collection of Sugarcane and Related Grasses.....	52

ABSTRACT

Development of resistant cultivars is the main control measure against sugarcane brown rust caused by *Puccinia melanocephala*. Durability is uncertain, since the pathogen possesses adaptive ability to overcome host plant resistance. A differential gene expression study utilizing suppressive subtraction hybridization was conducted to improve understanding of brown rust resistance mechanisms in sugarcane. The expression patterns of 11 unigenes representing biosynthetic pathways, defense-related genes, and signaling genes were analyzed in L 99-233, a cultivar exhibiting quantitative resistance, L 01-299, a resistant cultivar with the major resistance gene *Bru1*, and two susceptible cultivars, Ho 95-988 and L 09-125, at 24 h, 48 h, 72 h, and 1 week after inoculation with *P. melanocephala* using (semi)quantitative RT-PCR. All genes analyzed for their expression showed message accumulation upon infection in susceptible and resistant cultivars, but the maintenance of high amounts of mRNAs of the genes for a prolonged time period appeared to be the most important factor contributing to brown rust resistance. Differences in the time-course of gene expression were detected between L 01-299 and L 99-233 suggesting variable mechanisms for resistance between the cultivars. Molecular markers were used to screen the World Collection of Sugarcane and Related Grasses (WCSRG) for *Bru1* to determine its distribution and frequency in *Saccharum* species and related genera. A total of 1,282 clones were screened. *Bru1* was distributed across the *Saccharum* complex, but the frequency varied among species. *Bru1* was more prevalent in *S. robustum* clones (59.1%), whereas it occurred in low frequency and exhibited the highest level of variability in clones of *S. spontaneum* (18.8%). *Bru1* frequency was highest in the two secondary cultivated species, *S. barberi* (79.3%) and *S. sinense* (71.8%). The frequency of *Bru1* detection was 26.4% and 21.0% for *S. officinarum* and interspecific hybrid clones, respectively. The characterization of the

WCSRG for *Bru1* distribution and prevalence will complement efforts to characterize diversity in the *Saccharum* complex for the expected expanded use of marker-assisted selection in the future. Selection for quantitative resistance in combination with *Bru1* could allow breeding programs to develop sugarcane cultivars with effective and durable resistance against brown rust.

CHAPTER 1: INTRODUCTION

1.1 OVERVIEW

Sugarcane (inter-specific hybrids of *Saccharum* species) is an important crop in tropical and sub-tropical regions of the world. It was cultivated on 23.8 million hectares in more than 110 countries with an annual yield of 1.77 billion tons of cane in 2012 (FAOSTAT, 2012). Brazil produces the most sugarcane, with 670 million tons harvested in 2012, followed by India, China and Thailand (FAOSTAT, 2012). The United States produced 27.9 million tons (8th place) on approximately 350,000 hectares. Sugarcane occupies 5th place in the U.S. after maize, soybean, wheat and sugar beet. Sugarcane is commercially produced in Florida, Louisiana, Hawaii, and Texas.

Sugarcane is the most economically important field crop in Louisiana. It is grown on nearly 182,000 ha, with annual production averaging 14 million tons of cane and 1.4 million tons of sucrose. The total value to the farmers and processors is more than \$800 million with a total economic impact of \$2.2 billion. The sugarcane industry provides approximately 27,000 jobs (American Sugar Cane League, 2010). Louisiana produces about 20% of the total sugar produced in the U. S. (beet and cane combined).

Brown rust is caused by the fungus *Puccinia melanocephala* Syd. & P. Syd. It is an economically important disease in many sugarcane production regions (Ryan and Egan, 1989; Raid and Comstock, 2000). Brown rust was first reported in the continental U.S. in Florida (Dean *et al.*, 1979). The disease was then reported shortly thereafter in Louisiana (Koike, 1980).

Puccinia melanocephala belongs to the phylum Basidiomycota, class Pucciniomycetes, order Pucciniales, family Pucciniaceae and the genus *Puccinia* (Dixon *et al.*, 2010). Two species, *P. melanocephala* Syd. & P. Syd and *P. kuehnii* E. J. Butler, occur in the U.S. The characteristics

of the uredinial and telial stages can distinguish the two species that are phylogenetically distinct (Virtudazo *et al.*, 2001). *Puccinia melanocephala* has a simple life cycle with the urediniospore being the only known infectious spore.

The initial symptoms of brown rust are small, elongated, yellowish spots that are visible on both surfaces of the leaf. The spots develop into brown to orange-brown or red-brown lesions that range from 2 to 10 mm in length occasionally reaching 30 mm. The lesions are raised and are sometimes surrounded by a slight yellow halo (Raid and Comstock, 2000).

The lesions become pustular on the abaxial side of the leaf, and red-brown urediniospores are produced and released. Urediniospores have dense echinulation with darker brown and uniformly thick walls, and there are abundant capitate paraphyses in the uredinia. Spore production occurs 8-18 days after infection, depending on cultivar susceptibility and environmental conditions (Arya and Perello, 2010). Teliospores as well as urediniospores have been reported (Purdy *et al.*, 1983). Teliospores are occasionally found at the end of the season. Telia are dark brown to blackish and contain brown to dark brown teliospores with apically thickened walls (Virtudazo *et al.*, 2001). Basidiospores have been observed but do not initiate infection on sugarcane (Purdy *et al.*, 1983).

Once pustules rupture through the lower epidermis, urediniospores are exposed and passively released for aerial dispersal. Wind dispersal is the primary means of disease spread. The movement of diseased sugarcane stalks and contaminated equipment could also provide a means of spread.

On susceptible cultivars, numerous lesions coalesce resulting in premature leaf senescence and death. Plants of susceptible cultivars heavily infected with brown rust have a reddish-brown tinge that is visible outside the field (Comstock *et al.*, 1992). Infection by *P.*

melanocephala causes reduced growth of the plant due to reduced stalk height and weight, and stalk population may be reduced (Hoy and Hollier, 2009; Victoria *et al.*, 1984). Brown rust can reduce tonnage yield by 10-20 tons per hectare depending on the length of time brown rust is affecting the crop; however, fungicide applications can minimize losses (LSU AgCenter, 2010). Losses in total sucrose yield up to 22 % have been documented in Louisiana (Hoy and Hollier, 2009).

Puccinia urediniospores germinate then germ tubes typically form appressoria to enter through stomata (Sotomayor *et al.*, 1983). Appressoria form a penetration peg then a substomatal vesicle, infection hypha, haustorial mother cell, and haustorium that are produced in sequence to colonize the host (Sotomayor *et al.*, 1983).

Disease severity is determined primarily by an interaction of host genetic and physiological (plant age) factors and environmental conditions (primarily temperature and leaf wetness) with some effects from edaphic conditions. Spore germination may occur within a temperature range of 5-34 °C (Sotomayor *et al.*, 1983); however, the optimal temperatures for infection ranged between 17 and 27 °C (Barrera, 2010; Barrera *et al.*, 2012). Heavy rains can wash spores from leaves and the atmosphere (Comstock and Ferreira, 1986). Soil factors can also affect rust infection levels. Rust severity is higher for sugarcane growing on low pH soils and when soil moisture and levels of phosphorous and potassium in the soil are high (Anderson and Dean, 1986; Anderson *et al.*, 1990; Johnson *et al.*, 2007). Brown rust is more severe in younger plants between 2 and 6 months of age (Raid and Comstock, 2000).

Brown rust control is largely achieved through the use of resistant cultivars. However, fungicide programs to minimize losses have been developed with strobilurin fungicides providing the highest level of control (Hoy and Savario, 2007).

1.2 SUGARCANE RESISTANCE TO BROWN RUST

Brown rust is controlled primarily through the development and cultivation of resistant cultivars (Purdy *et al.*, 1983; Raid and Comstock, 2000). Selection for brown rust resistance results in the elimination of agronomically promising cultivars; however, breeding has provided control for the disease and reduced economic losses (Asnaghi *et al.*, 2001; Raid and Comstock, 2000).

The durability of resistance to brown rust is uncertain because *P. melanocephala* possesses the ability to adapt and overcome host plant resistance. This ability can cause a “boom and bust” cycle that results in periodic severe epidemics. The extent of cultivation of a resistant cultivar is one factor affecting resistance durability. Extensive cultivation of one cultivar can create a selection pressure on the pathogen population and increase the likelihood of a more rapid emergence of a genetic variant. Diversification of cultivars under cultivation may hold down the overall area-wide disease pressure and reduce the natural selection pressure leading to the emergence of new races.

Shifts from resistance to susceptibility have been reported in several cultivars in Florida, including CP 78-1247 (Raid, 1989), CP 79-1580 (Dean and Purdy, 1984), CP 74-2005 and CL 73-239 (Shine *et al.*, 2005). In Louisiana, the cultivar LCP 85-384 was ultimately grown on 91 % of the production area. In 2000, when the LCP 85-384 acreage had increased to over 40%, a severe epidemic of brown rust occurred in this cultivar that had previously been rated as a resistant (Hoy and Savario, 2007).

Resistant cultivars can be identified during selection, but resistance has not been durable on some cultivars, usually due to adaptation by the pathogen (Purdy *et al.*, 1983, Raid and Comstock, 2000). Previous studies evaluating variability in the pathogen population and

resistance responses in different cultivars demonstrated pathogenic variability related to host genotype. The reported shifts from resistance to susceptibility for cultivars in different regions (Dean and Purdy, 1984; Hoy and Grisham, 2005; Purdy *et al.*, 1983; Raid, 1989) were suggested to be due to pathogenic specialization. Differential cultivar reactions resulting from inoculation have been evaluated in four studies. It was concluded that specialization within the pathogen population to cultivar was not evident in Australia (Taylor, 1992), but studies in Florida (Shine *et al.*, 2005) and India (Srinivasan *et al.*, 1965) found differential reactions in cultivars inoculated with urediniospores collected from the same cultivars. In Louisiana, pathogenic specialization to cultivar was also detected (Avellaneda *et al.*, 2013; Hoy *et al.*, 2014). In addition, quantitative resistance was detected in one cultivar L 99-233 that could be very useful in on-going resistance research to ultimately improve breeding and selection for effective, durable resistance to brown rust. The lack of resistance durability is a very important aspect of brown rust epidemiology, worthy of study with the objective to improve the understanding of expression and basis for resistance in order to develop resistant cultivars with more durable resistance.

Sugarcane cultivars are complex interspecific aneuploids with chromosome numbers ranging from $2n=100-130$ (Sreenivassan *et al.*, 1987). Chu *et al.*, (1982) assumed that rust-susceptible genes of modern commercial cultivars are derived from *S. officinarum* clones which account for 85-90% of the genome of commercial cultivars, and it has been suggested that resistance was not likely to be determined by a single gene (D'Hont *et al.*, 1996). Tai *et al.*, (1981) observed marked transgressive segregation towards susceptibility in bi-parental crosses and selfed families and suggested that resistance to rust was partially dominant. Intermediate heritabilities for rust resistance were reported by Tai *et al.*, (1981) and Gonzales *et al.*, (1987). High narrow-sense and broad-sense heritability values of 0.84 and 0.73 determined by mid-

parent offspring regression were reported by Comstock *et al.*, (1992), and 0.84 and 0.78 heritability values were reported by Hogarth *et al.*, (1993). Daugrois *et al.*, (1996) attributed resistance to rust in the progeny of selfed cultivar R570 to a major resistance gene *Bru1* with dominant effect. A second major brown rust resistance gene, *Bru2* (Costet *et al.*, 2012; Raboin *et al.*, 2006), was then identified. *Bru1* was shown to provide resistance to all the rust isolates collected from several, diverse geographic regions (Asnaghi, 2000).

Brown rust resistance has been evaluated in sugarcane cultivars primarily by assessing natural infection severity. However, natural infection may not identify resistant cultivars due to variable environmental conditions and uneven inoculum exposure. Artificial inoculation exposes all plants under disease-favorable conditions to a high concentration of urediniospores.

Inoculation has been conducted under field conditions by introducing inoculum into the leaf whorl (Sood *et al.*, 2009). Inoculation under controlled conditions could provide information about resistance levels in potential parents or seedlings, and this approach has shown potential (Avellaneda *et al.*, 2013). The identification of clones resistant to brown rust without relying on natural infection could help in the breeding program to accurately characterize resistance in potential parents and determine appropriate crosses and thereby enhance the ability to produce new cultivars resistant to the disease.

1.3 SUGARCANE BROWN RUST MAPPING AND GENE EXPRESSION

Sugarcane probably has the most complex of all crop genomes due to its very high degree of polyploidy (as much as 12x) and interspecific nature (D'Hont, 2005). Cultivated sugarcane varieties are complex interspecific aneuploids with chromosome numbers ranging from $2n=100-130$ (Sreenivassan *et al.*, 1987). Modern sugarcane cultivars are derived from the combination of two polyploid species: *S. officinarum*, the domesticated sugar-producing species with $2n=8x=80$,

and *S. spontaneum*, a vigorous, grassy, wild species with $2n=5x=40$ to $2n=16x=128$ and many aneuploid forms (Sreenivasan *et al.*, 1987; D'Hont *et al.*, 1998). Both species are thought to have an autopolyploid origin (Sreenivasan and Ahloowalia, 1987; Grivet *et al.*, 1996).

Saccharum is a complex genus of mostly tall perennial bunch grasses that are highly polyploid. Morphology, chromosome number, and geographic distribution have traditionally been used for taxonomic classification, but ever since DNA markers became available, genomic data have been used to clarify the relationships among *Saccharum* species and accessions (Zhang *et al.*, 2014). The first inherited gene reported in sugarcane was for resistance to brown rust (Daugrois *et al.*, 1996). Rust resistance was generally considered to be a quantitatively inherited trait with high heritability (Hogarth *et al.*, 1993; Tai *et al.*, 1981). However, *Bru1* was identified in the French cultivar, R570 (Daugrois *et al.*, 1996) using a population consisting of selfed progeny. Brown rust resistance was observed to clearly and significantly segregate in a 3: 1 ratio, which is indicative of a single dominant resistance gene. *Bru1* was linked to a RFLP probe, CDSR29, which was initially not integrated in any linkage group (LG) in the R570 map. Subsequent additional mapping in R570 by Asnaghi (2000) indicated that *Bru1* was located on linkage group VII-1a in homology group (HG) VII (HGVII) of R570. Asnaghi refined the genetic map around *Bru1* on the basis of existing maize, rice and sorghum genetic maps. This approach revealed that the targeted region is orthologous to one end of sorghum consensus LG4, the end of the short arm of rice chromosome 2, and part of maize LG4 and LG5. It also enabled localization of *Bru1* at the end of one cosegregation group of the R570 HGVII (Le Cunff *et al.*, 2008). Further fine mapping of *Bru1* confirmed strong linkage disequilibrium due to the reduction in recombination in the *Bru1* region (Le Cunff *et al.*, 2008). This subsequently led to

the development of two PCR markers, R12H16 and 9020-F4, that can provide efficient molecular diagnosis for *Bru1* (Costet *et al.*, 2012).

A second major rust resistance gene was then identified by Raboin *et al.*, (2006) in MQ76–53 using a biparental cross between R570 and MQ76–53. The new rust resistance gene, *Bru2*, mapped to a linkage group in HGVIII, a different HG to the location of *Bru1*. Rust resistance has been analyzed in a third sugarcane population (McIntyre *et al.*, 2005), but in this population, rust resistance was quantitatively inherited with several QTLs identified that explained < 20 % of the phenotypic variation.

Bru1 as a source of resistance is of particular interest since it has been durable. *Bru1* resistance breakdown has not been detected despite intensive cultivation of R570 for >20 years in various regions of the world (Le Cunff *et al.*, 2008). Moreover, inoculation tests demonstrated that this gene provides resistance against diverse rust isolates collected in Africa and the Americas (Asnaghi *et al.*, 2001). In Colombia, 1,139 cultivars from local germplasm were screened, and 596 (48 %) contained *Bru1* and exhibited resistance to brown rust. In 412 (36 %) of these cultivars, both markers, R12H16 and 9020-F4, were detected. However, 5 (0.4 %) of the cultivars that contained *Bru1* exhibited susceptibility to brown rust (J. Victoria, personal communication).

The markers R12H16 and 9020-F4 were shown to be efficient in molecular diagnosis for *Bru1* (Costet *et al.*, 2012). *Bru1* was associated with resistance in 86 % of 194 clones from diverse locations (Costet *et al.*, 2012). Glynn *et al.*, (2013) detected a high frequency (42 %) of *Bru1* among Florida clones, while *Bru1* was detected in only 7 % of a limited collection of Louisiana clones. Racedo *et al.*, (2013) evaluated 129 clones in Argentina under natural field infection for resistance and subsequently screened for the presence or absence of the *Bru1* gene.

A total of 49 genotypes (38 %) were phenotyped as resistant to brown rust but only eight (16.3 %) harbored *Bru1*. To determine overall frequency of *Bru1* in the local sugarcane germplasm collection from Argentina, 190 additional genotypes were examined. Presence of *Bru1*, as determined by the diagnostic markers, was detected in only 7 % of the genotypes evaluated. In Guatemala, approximately one-third of the resistant clones contained *Bru1* (Molina *et al.*, 2013).

A marker-assisted screening of Louisiana sugarcane germplasm was performed with 506 clones, including 117 cultivars and elite breeding clones, 208 early generation progeny of crosses with wild/exotic germplasm, and 181 wild/exotic germplasm clones. Cultivars and advanced breeding clones showed a low frequency of detection with 5 out of 117 (4.3 %) testing positive for *Bru1*. In progeny from crosses involving wild/exotic germplasm, only 14 of 208 clones (6.7 %) tested *Bru1* positive. However, *Bru1* frequency was higher (28.7 %, 52 of 181 clones) in wild/exotic germplasm, which indicated that diverse genetic resources are available for *Bru1* introgression (Parco *et al.*, 2014). The low frequency (4.3 %) of *Bru1* found in the Louisiana commercial sugarcane breeding population concurred with the earlier report of Glynn *et al.*, (2013). The low frequency of *Bru1* in Louisiana commercial cultivars and elite clones may be related to the fact that a number of Louisiana sugarcane cultivars have become susceptible to brown rust while under cultivation (Parco *et al.*, 2014).

A high number of sugarcane expressed sequence tags (ESTs) have been generated from subtractive or cDNA libraries of plants subjected to different biotic or abiotic stresses (Sävenstrand *et al.*, 2000; Khan *et al.*, 2013). Oloriz *et al.*, (2012) described some aspects of the molecular basis of a brown rust resistant host reaction. This study utilized a complementary DNA (cDNA) subtraction method involving hybridization of cDNA from one population (tester) to excess of mRNA (cDNA) from another population (driver) and then separation of the

unhybridized fraction (target) from hybridized common sequences. It found that 88 % of 89 non-redundant sequences had similarity to functional genes. Genes involved in glycolysis, C4 carbon fixation and some transcription factors were identified during this study and 13 of these sequences were selected for transcript profiling in the resistant mutant and the susceptible donor clone. Differentially expressed genes can be studied using multiple methods, including representational difference analysis of cDNA (RDA) (Hubank and Schatz, 1994), serial analysis of gene expression (SAGE) (Yamamoto *et al.*, 2001), suppression subtractive hybridization (SSH) (Diatchenko *et al.*, 1996), cDNA microarray (Anahori and Vorst, 2002), cDNA-amplified fragment length polymorphism (AFLP) (Bachem *et al.*, 1998) and next generation sequencing such as RNA-Seq (Bedre *et al.*, 2015).

Suppression subtractive hybridization (SSH) is more effective than mRNA differential display and representational difference analysis. SSH is a technique that selectively amplifies cDNA fragments (differentially expressed) and also suppresses nontarget DNA amplification. SSH technique is simple and efficient for generating cDNAs highly enriched for differentially expressed genes of both high and low abundance. The high level of enrichment of rare transcripts has been achieved by the inclusion of a normalization step in the subtraction procedure. The normalization step equalizes the abundance of cDNA fragments within the target population, and the subtraction step excludes sequences that are common to the susceptible/resistant or uninfected/infected populations being compared. SSH has low false positive rates and is a less complex procedure. The technique is widely used to analyze genes related to plant disease resistance, abiotic stresses or genes expressed at different developmental stages (Diatchenko *et al.*, 1996).

The development and release of resistant cultivars is the best strategy to manage sugarcane brown rust. Multiple breeding programs in different parts of the world have inadvertently selected for *Bru1* because of the effective resistance it confers. Even though *Bru1* has exhibited durability, it is inadvisable to rely on one source of resistance against an adaptable pathogen. The availability of marker-assisted screening for *Bru1* will allow breeding programs to monitor the occurrence of *Bru1* during parent and offspring selection. In some cases, such as Louisiana and Argentina, monitoring will allow more utilization of *Bru1*. The ability to identify the presence of *Bru1* will allow the detection and incorporation of additional sources of resistance against brown rust and prevent over reliance on *Bru1*. The variable situation in *Bru1* frequency in different sugarcane industries around the world and on-going efforts to incorporate more genes for brown rust resistance from other *Saccharum* species and related genera suggest that the sugarcane world collection should be screened to identify the occurrence and distribution of *Bru1*. It would be of interest to determine the origin of *Bru1* in sugarcane germplasm.

Breeding efforts to develop effective and durable resistance to brown rust would be aided by a better understanding of other genes involved in the expression of resistance. The identification and expression analysis of transcripts differentially expressed in response to rust infection in cultivar L 99-233, a host cultivar without *Bru1* expressing quantitative resistance, could provide information about other possible mechanisms involved in brown rust resistance. This information could allow the development of additional marker-assisted screening tools to accurately and more broadly characterize resistance in potential parents, determine the most appropriate crosses, and thereby obtain a higher frequency of new cultivars with effective, durable resistant to the disease.

There were two objectives for this study. The first objective was to implement marker assisted screening of the sugarcane world collection for *Bru1* to determine its distribution and possible origin. The second objective was to perform a differential gene expression study of the resistance response to brown rust in L 99-233 using SSH technology to elucidate other mechanisms of resistance.

1.4 REFERENCES CITED

1. Aharoni, A. and Vorst, O. 2002. DNA microarrays for functional plant genomics. *Plant Molecular Biology* 48:99-118.
2. American Sugar Cane League. 2010. The Louisiana sugar industry. Retrieved September 6, 2010 from <http://www.amscl.org/SugarIndustry.pdf> Accessed 03/07/2012.
3. Anderson, D. L., and Dean, J. L. 1986. Relationship of rust severity and plant nutrients in sugarcane. *Phytopathology* 76:581-585.
4. Anderson, D. L., Raid, R. N., Ireby, M. S., and Henderson, L. J. 1990. Association of sugarcane rust severity with soil factors in Florida. *Plant Disease* 74:683-686.
5. Arya, A., and Perello, A. E. 2010. Management of Fungal Plant Pathogens. (Ed) CABI, page 221.
6. Asnaghi, C. 2000. Caractérisation d'un facteur génétique majeur de résistance à la rouille à chez la canne à sucre. Doctorat thesis. Université Paris-Sud.
7. Asnaghi, C., D'Hont, A., Glaszmann, J. C., and Rott, P. 2001. Resistance of sugarcane R 570 to *Puccinia melanocephala* isolates from different geographic locations. *Plant Disease* 85:282-286.
8. Avellaneda, M. C., Bombecini, J., and Hoy, J. W. 2013. Variability in *Puccinia melanocephala* pathogenicity and sugarcane cultivar resistance in Louisiana. *Phytopathology* 103(S2): 10.
9. Bachem, C. W. B., Oomen, R.J.F.J., and Visser, R.G.F. 1998. Transcript imaging with cDNA-AFLP: A step-by-step protocol. *Plant Molecular Biology Reporter* 16:157-173.
10. Barrera, W. A. 2010. Effects of environmental variables and crop growth on development of brown rust epidemics in sugarcane. M.S. Thesis. Louisiana State University Agricultural Center, Baton Rouge, 89 pp. Online publication. ETD-11182010-115435.

11. Barrera, W., Hoy, J. W., and Li, B. 2012. Temperature and leaf wetness effects on infection of sugarcane by *Puccinia melanocephala*. J. Phytopathol. 160:294-298.
12. Bedre, R., Rajasekaran, K., Mangu, V., Bhatnagar, D., and Baisakh, N. 2015. Genome-wide transcriptome analysis of cotton (*Gossypium hirsutum* L.) identifies candidate gene signatures in response to aflatoxin producing fungus *Aspergillus flavus*. PLOS ONE 10(9) DOI: 10.1371/journal.pone.0138025.
13. Chu, T. L., Serapion, J., and Rodriguez, J. L. 1982. Varietal reaction and heritance trends of susceptibility of sugarcane to rust (*Puccinia melanocephala* H. & P. Syd.). Journal of Agriculture of University of Puerto Rico 66:99-108.
14. Comstock, J. C., and Ferreira, S. A. 1986. Sugarcane rust: Factors affecting infection and symptom development. Proc. Int. Soc. Sugar Cane Technol. 19:402-410.
15. Comstock, J. C., Wu, K. K. and Schnell, R. J. 1992. Heritability of resistance to the sugarcane rust. Sugar Cane 6:7-10.
16. Costet, L., Le Cunff, L., Royaert, S., Raboin, L. M., Hervouet, C., Toubi, L., Telismart, H., Garsmeur, O., Rousselle, Y., Pauquet, J., Nibouche, S., Glaszmann, J. C., Hoarau, J. Y., and D'Hont, A. 2012. Haplotype structure around *Bru1* reveals a narrow genetic basis for brown rust resistance in modern sugarcane cultivars. Theor. Appl. Genet. 125:825-836.
17. Daugrois, J. H., Grivet, L., Roques, D., Hoarau, J. Y., Lombard, H., Glaszmann, J. C., and D'Hont, A. 1996. A putative rust resistance linked with a RFLP marker in sugar cane cultivar "R570". Theor. Appl. Genet. 92:1059-1064.
18. D' Hont, A. 2005. Unraveling the genome structure of polyploids using FISH and GISH:Examples of sugarcane and banana. Cytogenet. Genome Res. 109:27-33.
19. D'Hont, A., Grivet, L., Feldmann, P., Rao, S., Berding, N., and Glaszmann, J. C. 1996. Characterization of double genome structure of modern sugarcane cultivars (*Saccharum* spp.) by molecular cytogenetics. Mol. Gen. Genet. 250: 405-413.
20. D' Hont, A., Ison, D., Alix, K., Roux, C., and Glaszmann, J. C. 1998. Determination of basic chromosome numbers in the genus *Saccharum* by physical mapping of ribosomal RNA genes. Genome 41:221-225.
21. Dean, J. L., and Purdy, L. H. 1984. Races of the sugar cane rust fungus, *Puccinia melanocephala*, found in Florida. Sugar Cane 1:15-16.
22. Dean, J. L., Tai, P. Y. P., and Todd, E. H. 1979. Sugarcane rust in Florida. Sugar Journal 42(2):10.

23. Diatchenko, L., Lau, Y. F. C., Campbell, A. P., Chenchik, A., Moqadam, F., Huang, B., Lukyanov, S., Lukyanov, K., Gurskaya, N., Sverdlov, E. D. and Sievert, P. D. 1996. Suppression subtractive hybridization: A method for generating differentially regulated or tissue-specific cDNA probes and libraries. *Proc. Natl. Acad. Sci.* 93:6025-6030.
24. Dixon, L. J., Castlebury, L. A., Aime, M. C., Glynn, N. C., and Comstock, J. C. 2010. Phylogenetic relationships of sugarcane rust fungi. *Mycol. Prog.* 9:459–468.
25. FAOSTAT. 2012. Food and Agriculture Organization of the United Nations (FAO). 2012. Statistical reports. Sugarcane crop. Accessed 01/28/2014, from <http://faostat3.fao.org/faostat-gateway/go/to/browse/Q/QC/E>.
26. Glynn, N. C., Laborde, C., Davidson, R. W., Irely, M. S., Glaz, B., D' Hont, A., and Comstock, J. C. 2013. Utilization of a major brown rust resistance gene in sugarcane breeding. *Mol. Breed.* 31:323-331.
27. Gonzales, R.V., Manzano, C. A., Ordosgoitti, F. A., and Salazar, P. 1987. Genética de la reacción de la Caña de Azúcar (*Saccharum spp*) a *Puccinia melanocephala*, causante de la roya. *Agronomía Tropical* 37: 99-116.
28. Grivet, L., D' Hont, A., Roques, D., Feldmann, P., Lanaudd, C., and Glaszmann, J. C. 1996. RFLP mapping in cultivated sugarcane *Saccharum spp.*: Genome organization in a highly polyploid and aneuploid interspecific hybrid. *Genetics* 142:987-1000.
29. Hogarth, D. M., Ryan, C. C., and Taylor, P. W. J. 1993. Quantitative inheritance of rust resistance in sugarcane. *Field Crops Res.* 34: 187-193.
30. Hoy, J. W., Avellaneda, M. C. and Bombecini, J. 2014. Variability in *Puccinia melanocephala* pathogenicity and resistance in sugarcane cultivars. *Plant Dis.* 98: 1728-1732.
31. Hoy, J. W., and Grisham, M. P. 2005. Impact of rust on LCP 85-384. *Sugar Bull.* 845(1):12-13.
32. Hoy, J. W., and Hollier, C. A. 2009. Effect of brown rust on yield of sugarcane in Louisiana. *Plant Dis.* 93:1171-1174.
33. Hoy, J. W., and Savario, C. F. 2007. Can fungicides provide an alternative control measure for brown rust? *J. Amer. Soc. Sugar Cane Technol.* 27:60.
34. Hubank, M. and Schatz, D. G. 1994. Identifying differences in mRNA expression by representational difference analysis of cDNA. *Nucleic Acids Res.* 22:5640-5648.
35. Johnson, R. M., Grisham, M. P., and Richard, E. P., Jr. 2007. Relationship between sugarcane rust severity and soil properties in Louisiana. *Phytopathology* 97:748-755.

36. Khan, N., Bedre, R., Parco, A., Bernaola, L., Hale, A., Kimbeng, C., Pontif, M., and Baisakh, N. 2013. Identification of cold responsive genes in sugar/energycane for their use in genetic diversity analysis and future functional marker development. *Plant Sci.* 211:122-131.
37. Koike, H. 1980. Rust of sugarcane in Louisiana: A first report. *Plant Dis.* 64:226.
38. Le Cunff, L., Garsmeur, O., Raboin, L. M., Pauquet, J., Telismart, H., Selvi, A., Grivet, L., Philippe, R., Begum, D., Deu, M., Costet, L., Wing, R., Glaszmann, J.C., and D'Hont, A. 2008. Diploid/polyploid syntenic shuttle mapping and haplotype specific chromosome walking toward a rust resistance gene (*Bru1*) in highly polyploid sugarcane (2n=12x~115). *Genetics* 180:649-660.
39. Louisiana State University Agricultural Center (LSU AgCenter). 2010. Best management practices for minimizing the impact of brown rust in sugarcane. Accessed 5/20/2014, http://www.lsuagcenter.com/mcms/webtools/printable.aspx?url=/en/crops_livestock/crop/s/sugarcane/pathology/Best-Management-Practices-for-Minimizing-the-Impact-of-Brown-Rust-in-Sugarcane.
40. McIntyre, C. L., Casu, R. E., Drenth, J., Knight, D., Whan, V. A., Croft, B. J., Jordan, D. R., and Manners, J. M. 2005. Resistance gene analogues in sugarcane and sorghum and their association with quantitative trait loci for rust resistance. *Genome* 48:391-400.
41. Molina, L., Queme, J. L., and Rosales, F. 2013. Comparative analysis between phenotype and *Bru1* marker for incidence to brown rust in sugarcane. *Proc. Int. Soc. of Sugar Cane Technol.* 28:738-743.
42. Oloriz, M. I., Gil, V., Rojas, L., Portal, O., Izquierdo, Y., Jiménez, E., and Hofte, M. 2012. Sugarcane genes differentially expressed in response to *Puccinia melanocephala* infection: Identification and transcript profiling. *Plant Cell Rep.* 31:955-969.
43. Parco, A. S., Avellaneda, M. C., Hale, A. H., Hoy, J. W., Pontif, M. J., Gravois, K. A., and Baisakh, N. 2014. Frequency and distribution of the brown rust resistance gene *Bru1* and implications for the Louisiana sugarcane breeding programme. *Plant Breed.* 133:654-659.
44. Purdy, L. H., Liu, L. J., and Dean, J. L. 1983. Sugarcane rust, a newly important disease. *Plant Dis.* 67:1292-1296.
45. Raboin L-M., Pauquet J., Butterfield M., D'Hont A., and Glaszmann, J-C. 2006. Analysis of genome-wide linkage disequilibrium in the highly polyploid sugarcane. *Theor. Appl. Genet.* 116:701-714.
46. Racedo, J., Perera, M. F., Bertani, R., Funes, C., Gonzales, V., Cuenya, M. I., D'Hont, A., Welin, B., and Castagnaro, A. P. 2013. *Bru1* gene and potential alternative sources of resistance to sugarcane brown rust disease. *Euphytica* 191:429-436.

47. Raid, R. N. 1989. Physiological specialization in sugarcane rust (*Puccinia melanocephala*) in Florida. *Plant Dis.* 73:183.
48. Raid, R. N., and Comstock, J. C. 2000. Common rust. Pages 85-89 in: *A Guide to Sugarcane Diseases*. P. Rott, R. A. Bailey, J. C. Comstock, B. J. Croft., A. S. Saumtally, eds. CIRAD/ISSCT Montpellier, France.
49. Raid, R. N., and Comstock, J. C. 2006. Sugarcane rust disease. University of Florida, Florida Sugarcane Handbook.
50. Ryan, C. C., and Egan, B. T. 1989. Rust. Pages 189-210, in: *Diseases of Sugarcane. Major Diseases*. C. Ricaud, B. T. Egan, A. G. Gillaspie, Jr., and C. G. Hughes, eds. Elsevier, Amsterdam.
51. Sandoval, I., Picornell, V., Chaves, R., and Ramos, N. 1983. *Puccinia melanocephala* H&P. Syd.: Biological and ecological aspects. *Proc. Int. Soc. Sugar Cane Technol.* 18:845-853.
52. Sävenstrand, H., Brosche, M., Ängelagen, M., and Strid, Å. 2000. Molecular markers for ozone stress isolated by suppression subtractive hybridization: Specificity of gene expression and identification of a novel stress-regulated gene. *Plant Cell Environ.* 23:689-700.
53. Shine, J. M., Jr., Comstock, J. C., and Dean, J. L. 2005. Comparison of five isolates of sugarcane brown rust and differential reaction of six sugarcane clones. *Sugar Cane Int.* 23(5):24-29.
54. Sood, S. G., Comstock, J. C., and Glynn, N. C. 2009. Leaf whorl inoculation method for screening sugarcane rust resistance. *Plant Dis.* 93:1335-1340.
55. Sotomayor, I. A., Purdy, L. H., and Trese, A. T. 1983. Infection of sugarcane leaves by *Puccinia melanocephala*. *Phytopathology* 73:695-699.
56. Sreenivasan, T. V., Ahloowalia, B. S., and Heinz, D. J. 1987. Cytogenetics. Pages 211-235 in *Sugarcane improvement through breeding*. D. J. Heinz ed. Elsevier, Amsterdam.
57. Srinivasan, K. V., and Muthaiyan, M. C. 1965. A note on physiological races of *Puccinia erianthi* Padw. & Khan affecting sugarcane varieties. *Proc. Int. Soc. Sugar Cane Technol.* 12:1126-1128.
58. Tai, P. Y. P., Miller, J. D., and Dean, J. L. 1981. Inheritance of resistance to rust in sugarcane. *Field Crops Res.* 4:261-268.
59. Taylor, P. W. J. 1992. Evidence for the existence of a single race of common rust caused by *Puccinia melanocephala*, in Australian sugar cane cultivars. *Aust. J. Agric. Res.* 43:443-450.

60. Victoria, J. I., Ochoa B. O., y Gómez, J. F. 1984. La roya de la caña en el valle del Cauca: Diseminación y efecto en la producción. Memorias del I Congreso de la Sociedad Colombiana de Técnicos de la Caña de Azúcar, Tecnicaña. 1:209-218.
61. Virtudazo, E. V., Nojima, H., and Kakishima, M. 2001. Taxonomy of *Puccinia* species causing rust diseases on sugarcane. Mycosci. 42:167-175.
62. Yamamoto, M., Wakatsuki, T., Hada, A., and Ryo, A. 2001. Use of serial analysis of gene expression (SAGE) technology. J. Immunol. Meth. 250:45-66.
63. Zhang, J., Zhou, M., Walsh, Z., Zhu, L., Chen, Y., and Ming, R. 2014. Sugarcane genetics and genomics. Pages 623-643 in: Sugarcane: Physiology, Biochemistry, and Functional Biology, First Edition. P. H. Moore and F. C. Botha eds. John Wiley & Sons, Inc. Ames, Iowa, USA.

CHAPTER 2: IDENTIFICATION OF GENES ASSOCIATED WITH RESISTANCE TO BROWN RUST IN SUGARCANE

2.1 INTRODUCTION

Brown rust, caused by *Puccinia melanocephala* H. & P. Syd., is an important disease of sugarcane (inter-specific hybrids of *Saccharum* L.) worldwide. Brown rust is controlled primarily through the development and cultivation of resistant cultivars (Purdy *et al.*, 1983; Raid and Comstock, 2000). However, cultivar shifts from resistance to susceptibility while under commercial cultivation have been repeatedly reported (Dean and Purdy, 1984; Hoy and Grisham, 2005; Purdy *et al.*, 1983; Raid, 1989). The lack of durability in resistance is the most important aspect of the disease that warrants an investigation to improve understanding of the expression and genetic basis of resistance in order to develop resistant cultivars with effective and durable resistance. Natural infection severity has been the means of assessing rust resistance in sugarcane cultivars (Asnaghi *et al.*, 2004; Raid and Comstock, 2000). Although natural infection is useful in assessing resistance, it is not always efficient in identifying resistant cultivars due to variable environmental conditions and uneven inoculum exposure.

Resistance to brown rust is a quantitatively inherited trait with high heritability (Hogarth *et al.*, 1993; Tai *et al.*, 1981). The genetics of sugarcane is complex. Modern sugarcane cultivars are derived from the combination of two polyploid species: *S. officinarum*, the domesticated sugar-producing species with $2n=8x=80$, and *S. spontaneum*, a vigorous, grassy, wild species with $2n=5x=40$ to $2n=16x=128$ and many aneuploid forms (Sreenivasan and Ahloowalia, 1987; D'Hont *et al.*, 1998). As a result, modern cultivars have a complex aneuploid and polyploid genome consisting of 100-130 chromosomes with a total size of about 10 Gbp (D'Hont 2005). Brown rust susceptibility genes of modern commercial cultivars may be transmitted from *S.*

officinarum which accounts for 80-90% of the genome of interspecific hybrid clones (Chu *et al.*, 1982).

A major gene for brown rust resistance, *Bru1*, was identified in a self-population of sugarcane cultivar R570 (Daugrois *et al.*, 1996), and subsequently, a second brown rust resistance gene, *Bru2*, was reported (Raboin *et al.*, 2006). The resistance conferred by *Bru1* has exhibited durability under extensive cultivation of R570 and in diverse cultivars in different regions (Asngahi *et al.*, 2004; Glynn *et al.*, 2013). However, the mechanism of resistance associated with *Bru1* is unknown. Molecular markers for *Bru1* were developed (Costet *et al.*, 2012) that enabled monitoring of the frequency of the gene in breeding programs and detection of brown rust resistance in the absence of *Bru1* (Glynn *et al.*, 2013; Molina *et al.*, 2013; Parco *et al.*, 2014; Racedo *et al.*, 2013). Brown rust resistance that was quantitatively expressed in the absence of *Bru1* was detected in a cultivar L 99-233 in Louisiana (Hoy *et al.*, 2014).

To understand the mechanisms and molecular regulation of brown rust resistance, the relevant subsets of differentially expressed genes of interest need to be identified, cloned, and studied in detail. A number of expressed sequence tags (ESTs) have been described in sugarcane libraries from different metabolic pathways involved in responses to different biotic or abiotic stresses (Sävenstrand *et al.*, 2000; Khan *et al.*, 2013, Park *et al.*, 2015). In a study of the interaction of *P. melanocephala* with sugarcane, 88% of 89 non-redundant sequences had similarity to functional genes, and 13 of these genes, involved in glycolysis, C4 carbon fixation and some transcription factors, showed differential transcript profiles between a resistant mutant and the susceptible donor clone (Oloriz *et al.*, 2012). Resistance gene analogues also have been shown to be associated with brown rust resistance in sugarcane (Glynn *et al.*, 2008; McIntyre *et al.*, 2005).

Differentially expressed genes can be studied using multiple methods, including representational difference analysis of cDNA (RDA) (Hubank and Schatz, 1994), serial analysis of gene expression (SAGE) (Yamamoto *et al.*, 2010), suppression subtractive hybridization (SSH) (Diatchenko *et al.*, 1996), cDNA microarray analysis (Anahori and Vorst, 2002), cDNA-amplified fragment length polymorphism (AFLP) (Bachem *et al.*, 1998), and high-throughput method using next generation sequencing technology, such as RNA-Seq (Bedre *et al.*, 2015).

SSH is simple and efficient for generating cDNAs highly enriched for differentially expressed genes of both high and low abundance. The high level of enrichment of rare transcripts is achieved by the inclusion of a normalization step in the subtraction procedure. SSH is based on a suppression PCR effect and combines normalization and subtraction in a single procedure. The normalization step equalizes the abundance of cDNA fragments within the target population, and the subtraction step excludes sequences that are common to the susceptible/resistant or uninfected/infected populations being compared. SSH has low false positive rates and is a less complex procedure.

The identification of transcripts differentially expressed in response to infection by *P. melanocephala* and semi-quantitative reverse transcriptase PCR (RT-PCR) analysis in cultivar L 99-233, a host cultivar without *Bru1* expressing quantitative resistance, could provide information about other possible mechanisms involved in brown rust resistance. This information could allow the development of tools to breed and select sugarcane cultivars with effective and durable resistance to brown rust. Therefore, a differential gene expression study of the resistance response to brown rust in L 99-233 was performed using SSH technology to identify genes associated and elucidate mechanisms of resistance.

2.2 MATERIALS AND METHODS

2.2.1 Plant materials

Plants of two sugarcane cultivars resistant to brown rust, L 01-299 and L 99-233, and two susceptible cultivars Ho 95-988 and L 09-125 were produced vegetatively from single-node cuttings in the greenhouse. L 01-299 was the only commercial cultivar in which *Bru1* was detected (Parco *et al.*, 2014), and L 99-233 was demonstrated to exhibit quantitative resistance to brown rust (Hoy *et al.*, 2014). Twelve plants of each cultivar were grown in 3.8 L pots with a 1:1 mixture of silt loam soil and sand and had approximately four fully emerged leaves at the time of inoculation.

2.2.2 Plant inoculation

Urediniospores of *P. melanocephala* were collected by vacuum from the abaxial surface of multiple naturally infected leaves in naturally infected commercial fields of cultivar Ho 95-988 and stored at -80 °C. Ten plants of each cultivar were inoculated with a urediniospore concentration of 1×10^6 / ml determined with haemocytometer and suspended in a solution of 0.1 % Tween 20. The inoculum was applied to both sides of two fully emerged leaves per plant with a paint brush until a film of moisture was visible (Barrera *et al.*, 2012). Spore germination rate was determined at the time of each inoculation by plating on water agar and microscopic observation 24 hours after inoculation. Inoculated plants were placed inside a plastic sheeting moist chamber for 15 h at a temperature of 23 ± 1 °C. Additional distilled water was sprayed to the leaves with an atomizer and cool mist generators were used to create a humid atmosphere and maintain constant leaf wetness for the entire 15 hours. After the infection period, plants were placed on shelves under 12 h artificial lighting at 23 ± 1 °C for 2 weeks.

2.2.3 Total RNA isolation

Two leaves per plant were collected 24, 48, 72 h and 1 week after inoculation and immediately frozen in liquid nitrogen. Leaf tissues collected from non-inoculated plants served as a control. Leaf tissue (100 mg) was ground in a mortar and pestle with liquid nitrogen, using 1 ml of Trizol (Invitrogen, Carlsbad, CA), vortex mixed, centrifuged at 13,000 rpm for 10 min at 4 °C, precipitated with one volume of isopropanol at -70°C for at least 1 h and centrifuged again at 13,000 rpm for 10 min at 4 °C. Pellet of RNA was washed with 75% ethanol, air-dried and resuspended with 50 µl of RNase-free water. Genomic DNA was removed by digestion with DNase I (Qiagen, Valencia, CA). Qualitative and quantitative assays of RNA were performed by both gel electrophoresis and ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE).

2.2.4 SSH library construction

Control RNA (33 µg) and RNA pooled over four different time points (15 µg each) of inoculated cultivar L 99-233 were used for cDNA synthesis. cDNA subtraction was performed using the PCR-selectTM cDNA subtraction kit (Clontech, Palo Alto, CA) following manufacturer instructions, except that the double-stranded cDNA was synthesized from total RNA instead of mRNA using the SuperscriptTM double-stranded cDNA synthesis kit (Invitrogen, Carlsbad, CA). Double-stranded cDNA was digested with *RsaI*. The cDNA from the inoculated plants was ligated with two different adaptors and used as the tester. Two rounds of forward subtractions were performed using cDNA from non-inoculated control plants as the driver. Differentially expressed, up-regulated genes were amplified using the Advantage 2 PCR Kit (Clontech, Palo Alto, CA). Products of subtraction were cloned into the pGEM-T Easy Vector System (Promega, WI, USA) and transformed into *Escherichia coli* DH5α competent cells following the method

described by Ramanarao *et al.*, (2011) and plated onto Luria Bertani (LB) agar containing 100 mg L⁻¹ ampicillin, 1 mM isopropyl β-D-thiogalactopyranoside, and 80 mg L⁻¹ 5-bromo-4chloro-3indolyl β-D-galactopyranoside. Following incubation at 37 °C overnight, positive white colonies were picked and arrayed into 96-well microplates and then cultured in LB containing 100 mg L⁻¹ ampicillin. The resultant subtractive cDNA library was stored at -80 °C with 15% glycerol. Three-hundred sixty-three colonies were randomly selected for PCR analysis to confirm the presence of inserts prior to sequencing.

2.2.5 Sequence processing and bioinformatics analysis

The insert cDNAs of the selected clones were sequenced using T7 sequencing primer at the Washington State University high-throughput sequencing facilities using BigDye™ terminator kit on an ABI 3730xl genetic analyzer (Applied Biosystems, Foster city, CA). After removal of vector sequences, adapters, and low quality sequences, sequence similarity searches were performed against NCBI non-redundant DNA and protein database using BLASTN and BLASTX (www.ncbi.nlm.nih.gov/BLAST/) tools, respectively, with default parameters. Sequence assembly was performed using web tool CAP3 (Huang and Madan, 1999) to identify contigs and singlets. Functional classification of the unigenes with query coverage of more than 50% or E-value cut-off at 1-05 was carried out according to the MIPS functional categories (<http://mips.gsf.de/proj/thal/db/index.html>). The top scoring hits were annotated for Gene Ontology (GO) terms according to the putative function obtained from BLASTX and using Blast2GO (Conesa *et al.*, 2005).

Sequences of 217 unigenes were blasted against *Sorghum bicolor* genome sequences using the Blast interface of Phytozome v11.0 resource to obtain the coordinates of their location in the genome. The coordinates were used as input in MapChart software to generate the

graphical linkage map of sorghum chromosomes showing the brown rust responsive unigenes.

2.2.6. Expression analysis by semiquantitative RT-PCR

Eleven unigenes from the subtractive library were selected to study their expression in a time-course experiment for incompatible (resistant cultivars L 99-233 and L 01-299) and compatible (susceptible cultivars Ho 95-988 and L 09-125) reactions. Primers for semiquantitative RT-PCR of selected genes were designed using Primer3 software (Table 2.1). All primers were synthesized by Integrated DNA Technologies (IDT Inc, Coralville, IA). Products of PCR were

Table 2.1 Sequences of primers used in reverse transcription PCR-mediated expression profiling

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	Band size (bp)
<i>Ran-A1</i>	AAGCCTCCGGAAGTTACCAT	GGCACAACTCCTGCTCAAGT	156
<i>CP</i>	TATGCAGGGGTTCCAATGTT	ACATCTTTGCTGCCTTCGAT	206
<i>Bi1</i>	CGTGCTGATGTTCTTCGTGT	GATGGCACAGCACACTCCTA	161
<i>Trxh1</i>	TTCTTTGCCAGCTCATCCTT	CGCACCATAGCTCCAATCTT	194
<i>H2A</i>	CAGGCAAGCGTTACACAGAA	GCACATTCACAAGTCCCTGA	169
<i>FtsH6</i>	GAGGACATTGATTCGGCAGT	GCAGGGATCTCCACAAACTC	176
<i>Ubiquitin conjugating enzyme</i>	TGTTGCTGCTGTTCTTGGTC	CAGACACCGCCTTGGTAGAT	180
<i>Cks</i>	CGTTGCTGTGAGGCACTAAA	TGTCGAATGCATCATCTGGT	181
<i>ALAT 2</i>	GGGCTACTCCAGGATGTGAA	ACCCACAGAAATTCCGTTCC	196
<i>V-ATPase B1</i>	TGCACTTCGTGAGGTTTCTG	AAGGTCAGGAGTTGGGTGTG	190
<i>Nucleic binding protein</i>	AGCAGCGCTTGAAAGAGAAG	TGGGATGACCGTATGACAGA	176
<i>Actin</i>	CTGGAATGGTCAAGGCTGGT	TCCTTCTGTCCCATCCCTACC	112

analyzed by electrophoresis in 2% agarose gels, stained with ethidium bromide and visualized in a KODAK GelLogic200 documentation system (KODAK, New Haven, CT).

2.2.7 Mining of brown rust responsive ESTs for microsatellite markers

Three-hundred sixty-three brown rust responsive ESTs were searched for the presence of simple sequence repeat (SSR) motifs using the Gramene web resource SSRIT (Temnykh *et al.*, 2001). Criteria for SSR identification were set to the presence of at least five repeats for dinucleotide motifs and three repeats for tri, tetra and penta nucleotide motifs.

2.3 RESULTS

2.3.1 Identification of responsive genes in cultivar L 99-233

A total of 363 ESTs from the L 99-233 subtractive library were sequenced. Cleaning the vector sequence contamination and removal of duplicate sequences produced 357 non-redundant ESTs longer than 50 bp, which were used for downstream bioinformatics analysis. Sequence assembly resulted in 75 contigs and 142 singlets, thereby 217 unigenes with a size ranging between 142 bp to 1235 bp and an average length of 444 bp (Table 2.2).

All 217 unigenes matched to sequences in the sugarcane gene index (SoGI, release 3.0) at a cut off of $1e-05$ and 60% similarity. BLAST analysis with NCBI non-redundant nucleotide and protein database, sorghum gene index (SbGI, release 9.0) and SwissProt database assigned putative functions to 205 out of 217 unigenes. Unigenes without a hit may represent sequences in the untranslated regions, non-coding RNAs or sugarcane-specific sequences.

Forty-five of the putative rust responsive unigenes were involved in various metabolic pathways with abundance of amino acid metabolism as revealed from their search against the KEGG database. Thirteen unigenes were involved in purine metabolism followed by 11 in

thiamine metabolism and six in lipopolysaccharide metabolism (Figure 2.1, a.). Gene ontology analysis using Blast2GO classified the unigenes into biological process (39.7%), molecular

Table 2.2 Sequence statistics of the cDNAs isolated from suppressive subtraction library of L 99-233 in response to infection by *Puccinia melanocephala*.

Parameter	Number
Total number of sequences	363
Number of sequences (> 50 bp) after vecscreen cleaning	358
Number of duplicate sequence	1
Total unique sequences (> 50 bp) used for assembly	357
Total unigenes obtained	217
Total contigs	75
Total Singlets	142
Maximum length of unigenes (bp)	1235
Minimum length of unigenes (bp)	99
Mean length of unigenes (bp)	444.26 (~444)
Number of sequences annotated	205

function (36.5%), and cellular component (23.7%) categories. GO enrichment analysis indicated that the majority of the unigenes under biological process were involved in metabolic processes with cellular metabolic process as the most enriched (Figure 2.1, b.). Similarly intracellular part along with intracellular and membrane-bound organelle dominated the cellular component. On the other hand molecular function of most of the unigenes was characterized by their ion binding function followed by hydrolase activity (Figure 2.1, b.).

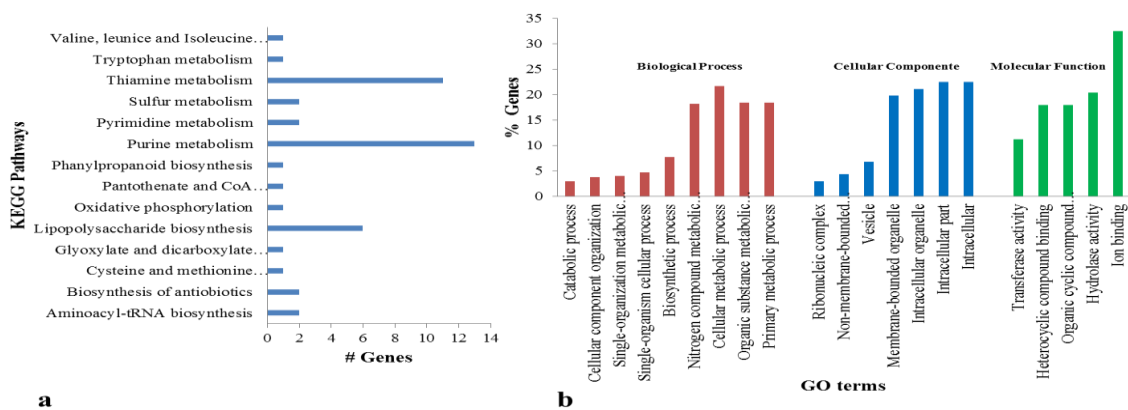


Figure 2.1 KEGG pathway analysis (a) and gene ontology (b) of the brown rust responsive unigenes

The 217 unigenes were distributed irregularly across the 10 chromosomes of Sorghum (Figure 2.2). Majority of the unigenes were localized in chromosomes 1, 3, 6, and 7. Unigenes selected for gene expression analysis were highlighted with red color while unigenes containing SSR motifs were highlighted in green.

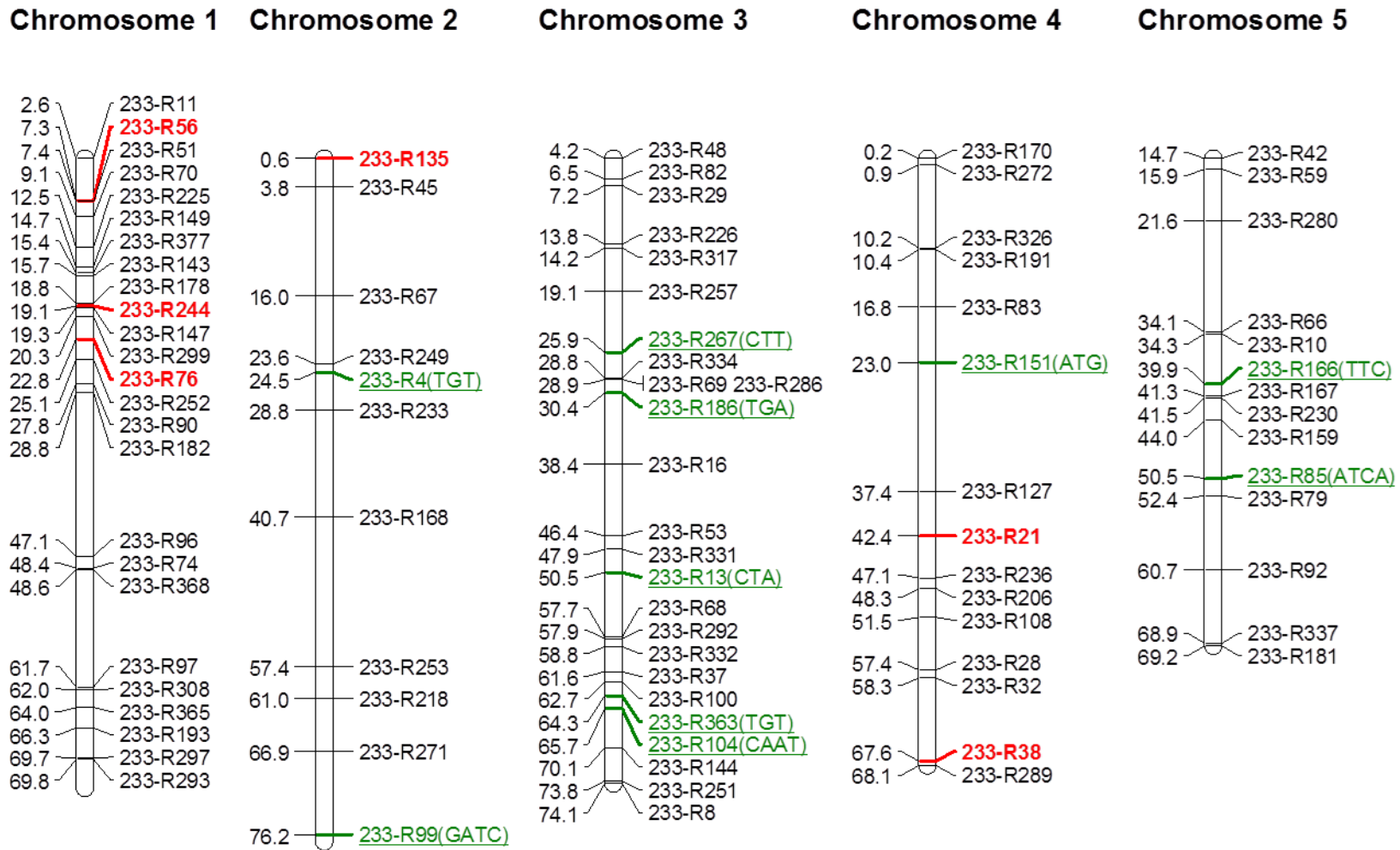
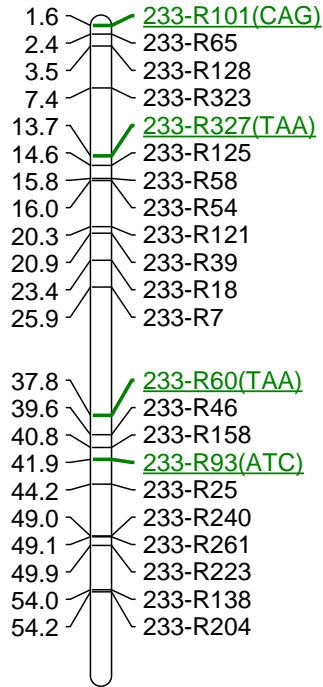


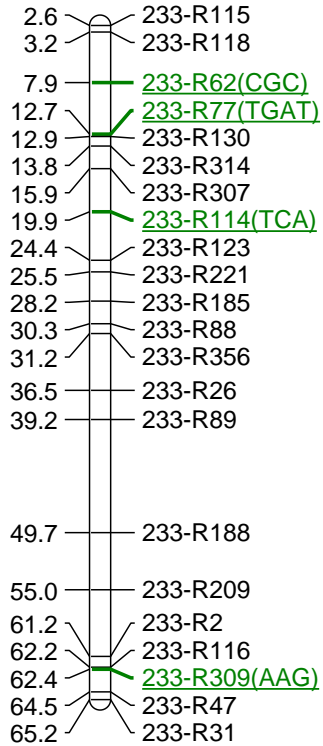
Figure 2.2. Mapping of rust-responsive unigenes on sorghum genome.

(Figure 2.2. continued)

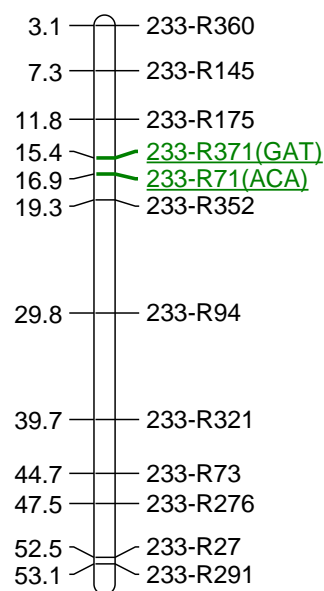
Chromosome 6



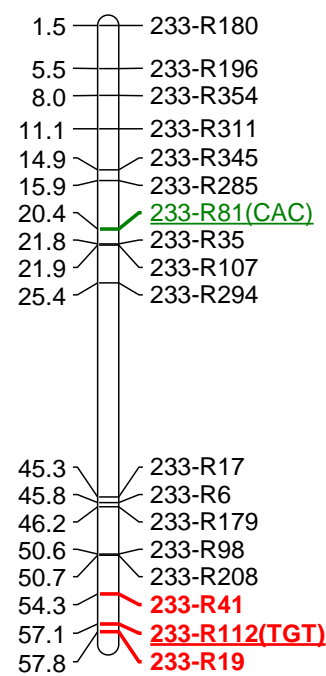
Chromosome 7



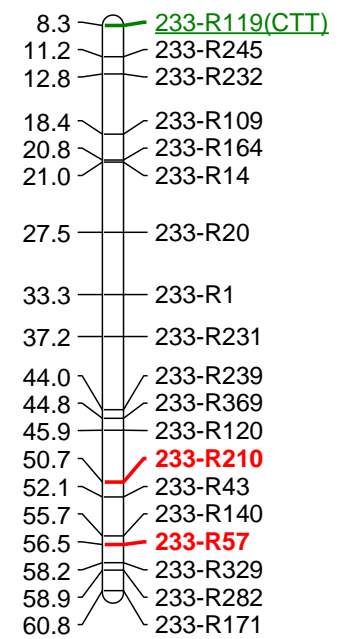
Chromosome 8



Chromosome 9



Chromosome 10



2.3.2 Expression analysis of brown rust responsive genes

The expression patterns of 11 unigenes representing biosynthetic pathways and defense-related and signaling genes were analyzed in the two resistant cultivars L 99-233 and L 01-299 and the two susceptible cultivars Ho 95-988 and L 09-125 at different time points of the *P. melanocephala*-sugarcane interaction using semiquantitative RT-PCR (Figures 2.3 and 2.4). Interestingly, almost all genes in both resistant and susceptible cultivars showed message accumulation 24 h after infection by *P. melanocephala*. Variable expression patterns were observed among and within resistant and susceptible cultivars in controls and at later times after infection.

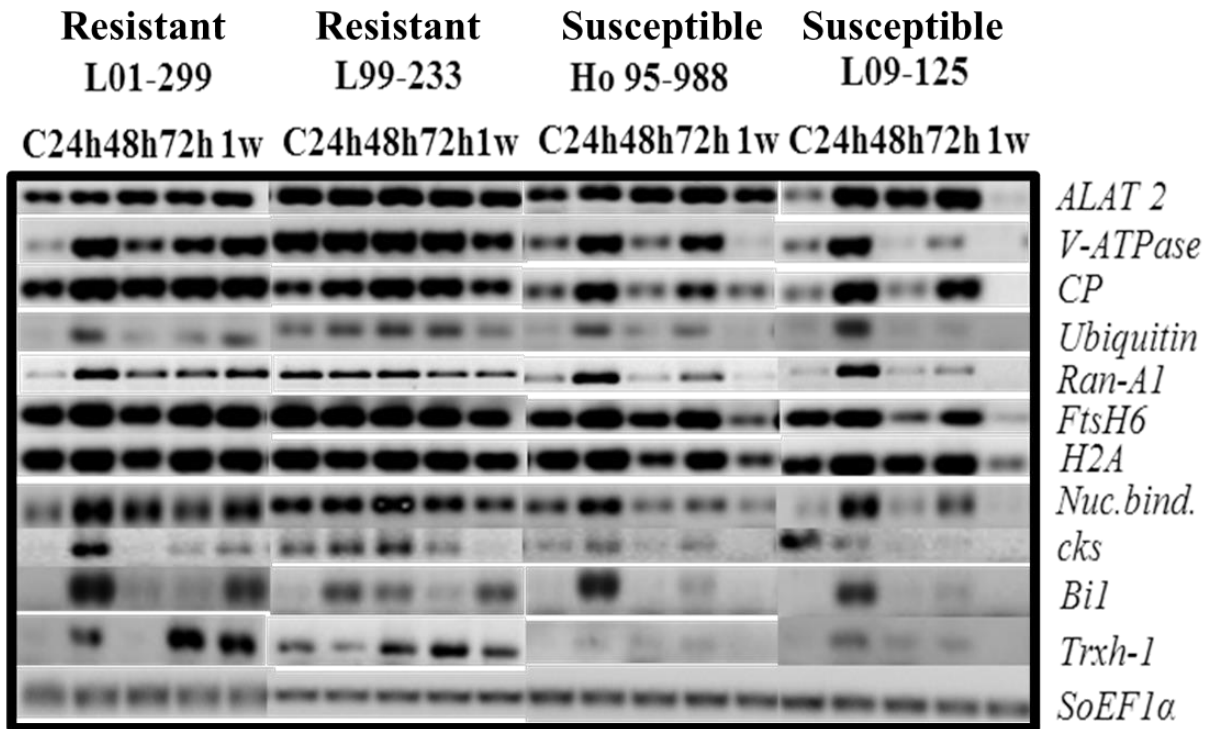


Figure 2.3 Temporal expression pattern of 11 selected rust-responsive unigenes in rust-resistant (L 01-299, L 99-233) and rust-susceptible (Ho 95-988, L 09-125) cultivars using semiquantitative RT-PCR. Sugarcane gene coding for elongation factor 1 α (SoEF1 α) was used as an internal control.

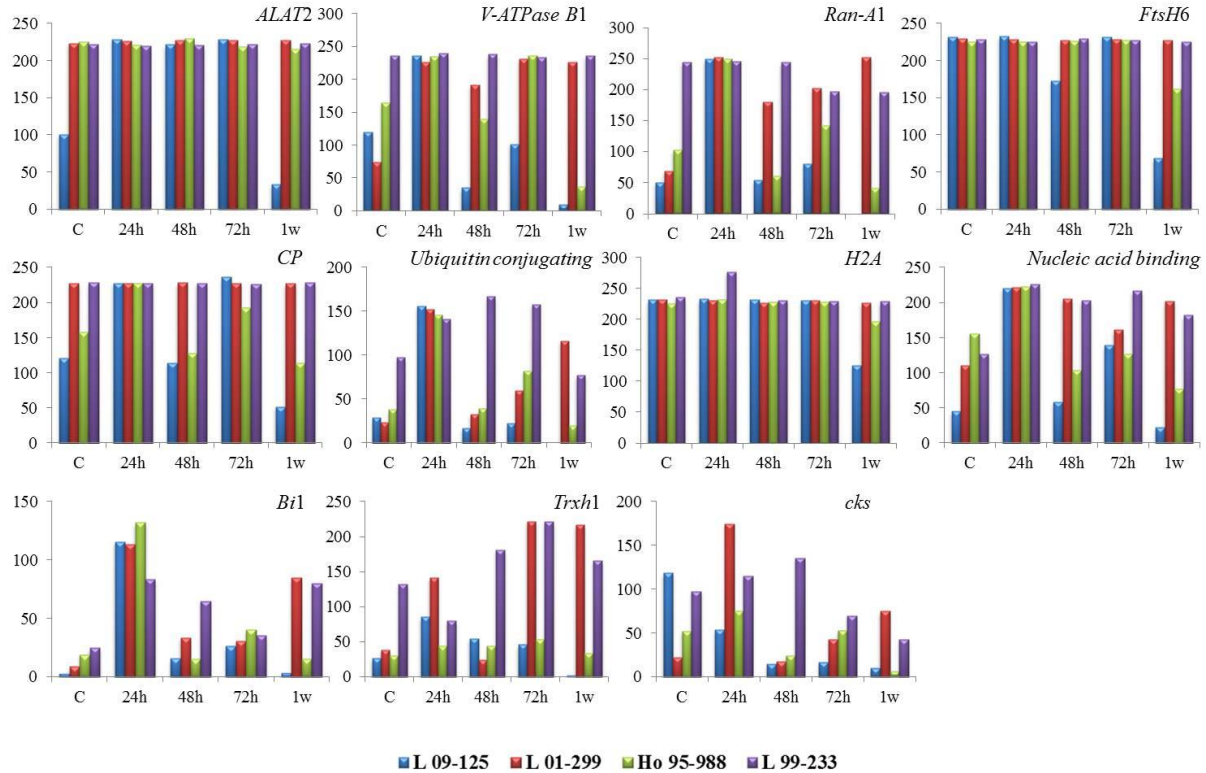


Figure 2.4 Temporal expression pattern of 11 selected rust-responsive unigenes in rust-resistant (L 01-299, L 99-233) and rust-susceptible (Ho 95-988, L 09-125) cultivars using densitometry analysis of semiquantitative RT-PCR results. Sugarcane gene coding for elongation factor 1 α (*SoEF1 α*) was used as an internal control.

2.3.2.1 Genes involved in primary metabolism and transport

The gene *ALAT2* coding for an enzyme that catalyzes the reversible transfer of an amino group from glutamate to pyruvate to form 2-oxoglutarate and alanine in the nitrogen metabolism was expressed in non-inoculated control plants of both resistant and susceptible cultivars, and expression was detected following infection until 72 h (Figure 2.4). The gene was down-regulated by 1 week in the susceptible cultivar Ho 95-988. The unigenes encoding vacuolar ATP synthase B1 subunit (V-ATPase B1) had a roller coaster expression pattern in both susceptible cultivars where the expression was induced after 24 h of infection, down-regulated at 48 h, up-regulated at 72 h, then finally repressed at 1 week (Figure 2.4). The gene showed significant up-regulation at all time points in resistant cultivar L 01-299. In the second resistant cultivar, L 99-

233, basal expression was higher than for all other cultivars, the expression was maintained until 1 week.

2.3.2.2 Genes coding for proteases

The genes encoding proteases, cysteine proteinase (CP) and a putative small ubiquitin-conjugating enzyme (Ubiquitin), showed differential temporal expression in susceptible and resistant cultivars. Cysteine proteinase expression was almost identical to the V-ATPase B1 gene in the susceptible cultivars with alternate up- then down-regulation after infection with the fungus, whereas both resistant cultivars showed consistent high expression (Figure 2.4).

Ubiquitin conjugating gene expression was low in the control plants of all the cultivars, except the resistant cultivar L 99-233. Its mRNA accumulation was then up-regulated at 24 h for all cultivars, and 48 and 72 h for L 99-233. Ubiquitin was still being expressed at 1 week in both the resistant cultivars.

2.3.2.3 Genes involved in binding activities

Genes involved in binding activities were studied during the *P. melanocephala*-sugarcane interaction through the expression of four genes encoding GTP binding nuclear protein (*Ran-A1*), ATP binding protein (*FtsH6*), Histone 2A protein (*H2A*), and nucleic acid binding protein. *Ran-A1* showed a strong up-regulation at all time points in the resistant cultivar L01-299, whereas it was expressed basally and continuously after infection in L 99-233 (Figure 2.4). Its expression in the susceptible cultivars was up-regulated at 24 h. Genes *H2A* and *FtsH6* showed generally similar patterns of expression in the resistant and susceptible cultivars (Figure 2.4). The genes were expressed basally and at all time points until 1 week after infection when the susceptible cultivars began to exhibit some down-regulation. Nucleic acid binding protein was up-regulated in all cultivars at 24 h (Figure 2.4). Expression was then down-regulated in the

susceptible cultivars with evidence of repression at 1 week, while expression remained high for the resistant cultivars.

2.3.2.4 Gene involved in signal transduction

The expression of a unigene CMP-KDO synthetase (*cks*) involved in signal transduction was strongly up-regulated at 24 h in L 01-299, and expression continued in L 01-299 at 1 week (Figure 2.4). Expression was repressed in susceptible cultivar L 09-125 at all time points and in Ho 95-988 at 1 week. In L 99-233, *cks* expression was similar to the control at 24 and down-regulated at 72 h and 1 week.

2.3.2.5 Defense-related genes

Expression analysis of sugarcane defense-related genes *Bi1* (brown plant hopper induced) and *Trxh1* (thioredoxin cytosolic) showed contrasting expression patterns between the resistant and susceptible cultivars (Figure 2.4). *Bi1* was strongly up-regulated in all cultivars at 24 h. Expression was down-regulated thereafter in the susceptible cultivars, but expression was detected at 1 week in both resistant cultivars. *Trxh-1* expression was low or not evident in the susceptible cultivars, whereas it was expressed at 72 h and 1 week in both resistant cultivars. *Trxh-1* was basally expressed in L 99-233 but not in L 01-299.

2.3.3 Simple sequence repeat (SSR) markers derived from brown rust responsive genes

Mining of the 217 rust responsive genes identified 118 unigenes to contain SSR motifs. Of these, 77 unigenes had SSRs with single motif repeats (perfect SSRs), and 41 were complex SSRs with two or more SSR motifs separated by ≤ 100 bp. SSRs with dinucleotide repeats were the highest (136) in number followed by tri- (67), and tetra-nucleotide (12) motifs (Figure 2.5). Among the dinucleotide motifs, AT/AT type was the most abundant followed by TG/CA and CA/GT. Among the trinucleotide motifs, AAG/CTT was the highest followed by AGC/GCT.

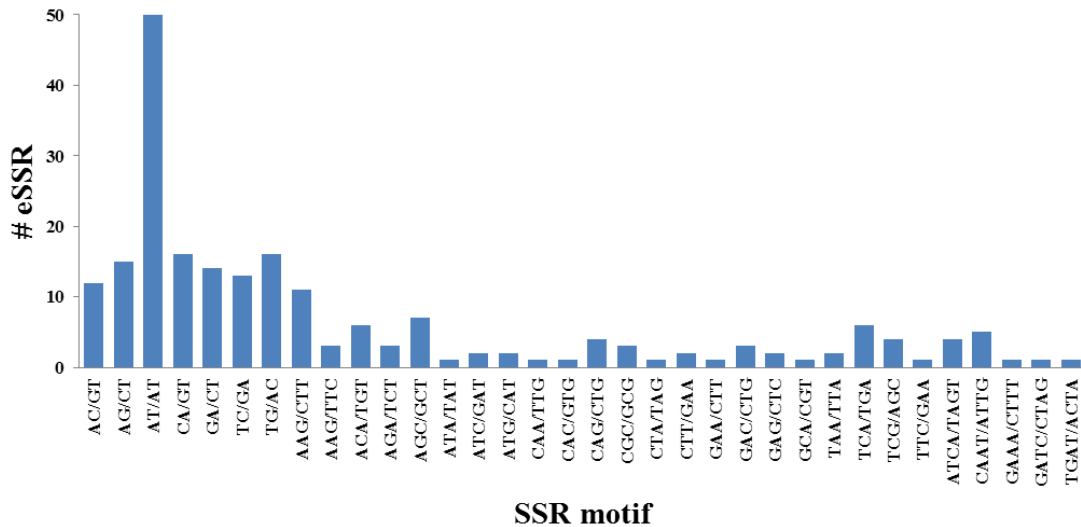


Figure 2.5 Distribution of simple sequence repeat (SSR) motifs in brown rust responsive genes in cultivar L 99-233

2.4 DISCUSSION

Host plant resistance to a disease is the result of a cascade of genes involved in signal perception and transduction, oxidative bursts, activation of defense related genes and transcriptional regulation. Identification of differentially expressed genes can provide vital information towards the understanding of molecular and biochemical bases of defense mechanisms of plants in response to a pathogen (Valculescu *et al.*, 2005). Knowledge on the molecular backgrounds of an interaction between sugarcane and the brown rust pathogen is limited. Two studies evaluated differential gene expression in resistant clones generated from a brown rust susceptible cultivar, B4362, by somaclonal variation (Carmona *et al.*, 2004) and chemical mutagenesis (Oloriz *et al.*, 2012). Genes associated with resistance processes were identified in somaclonal variants by cDNA-AFLP (Carmona *et al.*, 2004) and by SSH in a mutant showing a post-haustorial hypersensitive response (Oloriz *et al.*, 2012). In the present study, a low-scale transcriptome analysis of a cultivar, L 99-233, expressing quantitative

resistance against *P. melanocephala* (Hoy *et al.*, 2014), was performed through SSH as a first step towards identifying resistance-associated genes in this particular cultivar.

The results showed that genes associated with primary metabolism, protease activity, nucleotide binding, defense responses, as well as signal transduction, were differentially expressed in response to *P. melanocephala*, indicating a complex interplay of an array of genes in sugarcane in response to pathogen infection. All genes analyzed for their expression showed that their messages accumulated upon infection in both susceptible and resistant cultivars, but the maintenance of high expression of the genes for a prolonged time period appeared to be the contributing factor for resistance to brown rust.

Continuous high expression of genes involved in primary metabolism (ALAT2) and proton pump (V-ATPase B1) was observed in the resistant cultivars L 01-299 and L 99-233 but not the two susceptible cultivars Ho 95-988 and L 09-125. ALAT2 functions in breaking down alanine to pyruvate during recovery from hypoxia and in response to both biotic and abiotic stresses in several species (Kendziorek *et al.*, 2012, Miyashita *et al.*, 2007). Similarly, V-ATPase B1 acts in energizing transport of ions and metabolites, and its mRNA and protein content were altered in response to environmental stress (Ratajczak, 2000). Maintenance of primary metabolic activity by over-expression of these genes could be involved in the response of the resistant cultivars to prevent infection at 1 week, by which time the fungus would have invaded cells through haustoria in a susceptible reaction.

Cysteine proteinases (CP) are proteolytic enzymes that have been shown to be induced in response to stresses, including wounding (Linthorst *et al.*, 1993), and in programmed cell death (Solomon *et al.*, 1999; Xu and Chye, 1999). Ubiquitin-conjugating enzymes, also known as E2 enzymes, perform the second step in the ubiquitination reaction that targets a protein for

degradation via the proteasome (Wang *et al.*, 2014). Otubian-like cysteine proteases are a component of ubiquitin-proteasome that precisely cleaves proteins at the ubiquitin-protein bond (Balakirev *et al.*, 2003). However, the expression of CP and Ubi did not follow the same pattern among the cultivars. While Ubi was up-regulated in all cultivars 24 h after inoculation, its expression was reduced in the susceptible cultivars subsequently but remained at a higher level in the resistant cultivars at 1 week after inoculation. In a previous study, expression of the *Sumo1* gene involved in ubiquitinylation, showed strong up-regulation between 3 days post-inoculation until 7 days in the compatible reaction of a brown rust susceptible mutant (Oloriz *et al.*, 2012). Contrastingly, strong basal and post-infection expression of CP in the resistant cultivars may be related to the incompatible reaction with the fungus.

The CMP-KDO synthetase (*cks*) gene involved in signal transduction activates 3-Deoxy-D-manno-2-octulosonic acid KDO, which is an important component of the rhamnogalacturonan II- Boron (B-RG-II) complex that is essential for the cell wall integrity of rapidly growing tissues (Kobayashi *et al.*, 2011). Significant induction of the gene 24 h after inoculation in resistant cultivars and its maintenance at 1 week could possibly explain the lack of successful colonization.

GTP binding protein Ras-related nuclear protein (*Ran-A1*) is a small GTP-binding protein that has been shown to be involved in the regulation of growth and metabolism of maize plants infected with *Maize rough dwarf virus* (Li *et al.*, 2011). *Ran-A1* has been implicated in abiotic stress responses in plants, but very little is known about its role in biotic stress response. Again, the gene over-expression and/or induction and subsequent maintenance for a prolonged period post-infection may be involved in the prevention of disease development in the resistant cultivars.

The defense related cytosolic gene thioredoxin (*Trxh1*) is involved in the redox regulation of cytosolic enzymes (Hara *et al.*, 2006). A thioredoxin-like 1 (TRX) transcript was also reported in the SSH library produced from the *P. melanocephala* - mutant sugarcane interaction (Oloriz *et al.*, 2012). This gene appears to be an important component of the defense system of the resistant cultivars, especially L 01-299 based on its level of expression at 72 h and 1 week after inoculation. In L 99-233, *Trxh1* was basally expressed and showed moderate levels of expression until 1 week.

The present study provided first-hand information about changes in the expression profile of a selected set of genes in L 99-233, a sugarcane cultivar exhibiting quantitative resistance in response to infection by *P. melanocephala*. However, a more comprehensive genome-wide transcriptome analysis through RNA-Seq will identify the complex co-expression networks and will provide better clues to the quantitative resistance reaction of the cultivar in response to fungal infection. These genes would have a great potential in identifying resistance-associated genes that will be useful to mine for SNPs/indels for QTL and association mapping to identify functional markers associated with brown rust resistance. Furthermore, gene-derived SSRs underlying the QTL region can directly be used as functional markers. These novel genes could be combined with the *Bru1* gene in the breeding program to develop sugarcane cultivars with more durable and effective resistance against brown rust.

2.5 REFERENCES CITED

1. Aharoni, A. and Vorst, O. 2002. DNA microarrays for functional plant genomics. *Plant Mol Biol* 48:99-118.
2. Asnaghi, C., Roques, D., Ruffel, S., Kaye, C., Hoarau, J. Y., Telismart, H., Girard, J.C., Raboin, L. M., Risterucci, A. M., Grivet, L. and D'Hont, A. 2004. Targeted mapping of a sugarcane rust resistance gene (*Bru1*) using bulked segregant analysis and AFLP markers. *Theor Appl Genet* 108:759-764.

3. Bachem, C. W. B, Oomen, R. J. F. J. and Visser, R.G.F. 1998. Transcript imaging with cDNA-AFLP: A step-by-step protocol. *Plant Mol Biol Rep* 16:157-173.
4. Balakirev, M. Y., Tcherniuk, S. O., Jaquinod, M. and Chroboczek, J. 2003. Otubains: a new family of cysteine proteases in the ubiquitin pathway. *EMBO* 4:517-22.
5. Barrera, W., Hoy, J. W. and Li, B. 2012. Temperature and leaf wetness effects on infection of sugarcane by *Puccinia melanocephala*. *J Phytopathol* 160:294-298.
6. Carmona, E., Vargas, D., Borroto, C. J., Lopez, J., Fernandez, A. I., Arencibia, A. and Borrás, O. 2004. cDNA-AFLP analysis of differential gene expression during sugarcane –*Puccinia melanocephala* interaction. *Plant Breed* 123:499–501.
7. Chu, T. L., Serapion, J. and Rodríguez, J. L. 1982. Varietal reaction and heritance trends of susceptibility of sugarcane to rust (*Puccinia melanocephala* H. & P. Syd.). *Journal of Agriculture of University of Puerto Rico* 66:99-108.
8. Conesa, A., Götz, S., García-Gómez, J. M., Terol, J., Talón, M. and Robles, M. 2005 Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. *Bioinformatics* 21:3674-3676.
9. Costet, L., Le Cunff, L., Royaert, S., Raboin, L. M., Hervouet, C., Toubi, L., Telismart, H., Garsmeur, O., Rousselle, Y., Pauquet, J., Nibouche, S., Glaszmann, J. C., Hoarau, J. Y. and D'Hont, A. 2012. Haplotype structure around *Bru1* reveals a narrow genetic basis for brown rust resistance in modern sugarcane cultivars. *Theor Appl Genet* 125:825-836.
10. D'Hont, A. 1996. A putative major gene for rust resistance linked with a RFLP marker in sugarcane cultivar 'R570'. *Theor Appl Genet* 92:1059-1064.
11. D'Hont, A. 2005. Unravelling the genome structure of polyploids using FISH and GISH; examples of sugarcane and banana. *Cytogenet Genome Res* 109:27-33.
12. D' Hont, A., Ison, D., Alix, K., Roux, C. and Glaszmann, J. C. 1998. Determination of basic chromosome numbers in the genus *Saccharum* by physical mapping of ribosomal RNA genes. *Genome* 41:221-225.
13. Daugrois, J. H., Grivet, L., Roques, D., Hoarau, J. Y., Lombard, H., Glaszmann, J. C., and D'Hont, A. 1996. A putative rust resistance linked with a RFLP marker in sugar cane cultivar "R570". *Theor. Appl. Genet.* 92:1059-1064.
14. Dean, J. L. and Purdy, L. H. 1984. Races of the sugar cane rust fungus, *Puccinia melanocephala*, found in Florida. *Sugar Cane* 1:15-16.
15. Glynn, N.C., Comstock, J. C., Sood, S. and Chaparro, J. X. 2007. Isolation of nucleotide binding site-leucine rich repeat and kinase resistance gene analogues from sugarcane (*Saccharum* spp). *Pest Manag Sci* 64:48-56.

16. Glynn, N. C., Laborde, C., Davidson, R. W., Irey, M. S., Glaz, B., D' Hont, A. and Comstock, J. C. 2013. Utilization of a major brown rust resistance gene in sugarcane breeding. *Mol Breed* 31:323-331.
17. Hara, S., Motohashi, K., Arisaka, F., Romano, P. G., Hosoya-Matsuda, N., Kikuchi, N., Fusada, N. and Hisabori, T. 2006. Thioredoxin-h1 reduces and reactivates the oxidized cytosolic malate dehydrogenase dimer in higher plants. *J Biol Chem* 281:32065-32071.
18. Hogarth, D., Ryan, C. and Taylor, P. 1993. Quantitative inheritance of rust resistance in sugarcane. *Field Crops Res* 34:187-193.
19. Hoy, J. W., Avellaneda, M. C. and Bombecini, J. 2014. Variability in *Puccinia melanocephala* pathogenicity and resistance in sugarcane cultivars. *Plant Dis.* 98:1728-1732.
20. Hoy, J. W. and Grisham, M. P. 2005. Impact of rust on LCP 85-384. *Sugar Bull* 845:12-13.
21. Huang, X. and Madan, A. 1999. CAP3: A DNA sequence assembly program. *Genome Res* 9:868-877.
22. Hubank, M. and Schatz, D. G. 1994. Identifying differences in mRNA expression by representational difference analysis of cDNA. *Nucleic Acids Res* 22:5640-5648.
23. Kendziorek, M., Paszkowski, A. and Zagdanska, B. 2012. Differential regulation of alanine aminotransferase homologues by abiotic stresses in wheat (*Triticum aestivum* L.) seedlings. *Plant Cell Rep* 31:1105–1117.
24. Khan, N. A., Bedre, R., Parco, A., Bernaola, L., Hale, A., Kimbeng, C., Pontif, M. and Baisakh, N. 2013. Identification of cold-responsive genes in energycane for their use in genetic diversity analysis and future functional marker development. *Plant Sci* 211:122-131.
25. Kobayashi, M., Kouzu, N., Inami, A., Toyooka, K., Konishi, Y., Matsuoka, K. and Matoh, T. 2011. Characterization of Arabidopsis CTP: 3-deoxy-D-manno-2-octulosonate cytidyltransferase (CMP-KDO synthetase), the enzyme that activates KDO during rhamnolacturonan II biosynthesis. *Plant Cell Physiol* 52:1832-1843.
26. Li, K., Xu, C. and Zhang, J. 2011. Proteome profile of maize (*Zea Mays* L.) leaf tissue at the flowering stage after long-term adjustment to rice black-streaked dwarf virus infection. *Gene* 485:106–113.
27. Linthorst, H. J. M., Vanderdoes, C., Brederode, F. T. and Bol, J. F. 1993. Circadian expression and induction by wounding of tobacco genes for cysteine proteinase. *Plant Mol Biol* 21: 685–694.

28. McIntyre, C. L., Casu, R. E., Drenth, J., Knight, D., Whan, V. A., Croft, B. J., Jordan, D. R. and Manners, J. M. 2005. Resistance gene analogues in sugarcane and sorghum and their association with quantitative trait loci for rust resistance. *Genome* 48:391-400.
29. Miyashita, Y., Dolferus, R., Ismind, K. P. and Good, A.G. 2007. Alanine aminotransferase catalyses the breakdown of alanine after hypoxia in *Arabidopsis thaliana*. *Plant J* 49:1108–1121.
30. Molina, L., Queme, J. L. and Rosales, F. 2013. Comparative analysis between phenotype and *Bru1* marker for incidence to brown rust in sugarcane. *Proc Int Soc of Sugar Cane Technol* 28:738-743.
31. Oloriz, M.I., Gil, V., Rojas, L., Portal, O., Izquierdo, Y., Jiménez, E. and Hofte, M. 2012. Sugarcane genes differentially expressed in response to *Puccinia melanocephala* infection: Identification and transcript profiling. *Plant Cell Rep* 31:955-969.
32. Parco, A. S., Avellaneda, M. C., Hale, A. H., Hoy, J. W., Kimbeng, C. A., Pontif, M. J., Gravois, K. A. and Baisakh, N. 2014. Frequency and distribution of the brown rust resistance gene *Bru1* and implications for the Louisiana sugarcane breeding programme. *Plant Breed* 133: 654-659.
33. Park, J. W., Thiago, R., Benatti, T. R., Marconi, T., Yu, Q., Solis-Gracia, N., Mora, V. and da Silva, J. A. 2015. Cold responsive gene expression profiling of sugarcane and *Saccharum spontaneum* with functional analysis of a cold inducible *Saccharum* homolog of NOD26- like intrinsic protein to salt and water stress. *PLoS ONE* 10(5): e0125810. doi:10.1371/journal.pone.0125810
34. Purdy, L. H., Liu, L.J. and Dean, J.L. 1983. Sugarcane rust, a newly important disease. *Plant Dis* 67:1292–1296.
35. Racedo, J., Perera, M.F., Bertani, R., Funes, C., Gonzales, V., Cuenya, M. I., D'Hont, A., Welin, B. and Castagnaro, A. P. 2013. *Bru1* gene and potential alternative sources of resistance to sugarcane brown rust disease. *Euphytica* 191:429-436.
36. Raid, R. N. 1989. Physiological specialization in sugarcane rust (*Puccinia melanocephala*) in Florida. *Plant Dis* 73:183.
37. Raid, R. N. and Comstock, J.C. 2000. Common rust. Pages 85-89 in: *A Guide to Sugarcane Diseases*. P. Rott, R. A. Bailey, J. C. Comstock, B. J. and Croft., A. S. Saumtally, eds. CIRAD / ISSCT Montpellier, France.
38. Ramanarao, V. M., Weindorf, D., Breitenbeck, G. and Baisakh, N. 2011. Differential expression of the transcripts of *Spartina alterniflora* Loisel. (smooth cordgrass) induced in response to petroleum hydrocarbon. *Mol Biotechnol* 51:18-26.

39. Ratajczak, R. 2000. Structure, function and regulation of the plant vacuolar H translocating ATPase. *Biochimica et Biophysica Acta* 1465:17-36.
40. Sävenstrand, H., Brosche, M., Ängelsten, M. and Strid, Å. 2000. Molecular markers for ozone stress isolated by suppression subtractive hybridization: Specificity of gene expression and identification of a novel stress-regulated gene. *Plant Cell Environ* 23:689-700.
41. Solomon, M., Belenghi, B., Delledonne, M., Menachem, E. and Levine, A. 1999. The involvement of cysteine proteases and protease inhibitor genes in the regulation of programmed cell death in plants. *The Plant Cell* 11:431-444.
42. Sreenivasan, T. V., Ahloowalia, B. S. and Heinz, D. J. 1987. Cytogenetics. Pages 211-235 in *Sugarcane improvement through breeding*. D. J. Heinz ed. Elsevier, Amsterdam.
43. Tai, P. Y. P., Miller, J. D. and Dean, J. L. 1981. Inheritance of resistance to rust in sugarcane. *Field Crops Res* 4:261-268.
44. Temnykh, S., Lukashova, A., Cortinhour, S., De Clerck, G., Lipovich, L. and McCouch, S. 2001. Computational and experimental analysis of microsatellites in rice (*Oryza sativa* L.): frequency, length variation, transposon associations, and genetic marker potential. *Genome Res* 11:1414-1452.
45. Velculescu, V. E., Zhang, L., Vogelstein, B. and Kinzler, K. W. 1995. Serial analysis of gene expression. *Science* 270:484-487.
46. Wang, Y., Wang, W., Cai, J., Zhang, Y., Qin, G. and Tian, S. 2014. Tomato nuclear proteome reveals the involvement of specific E2 ubiquitin-conjugating enzymes in fruit ripening. *Genome Biol* 15:548.
47. Xu, X. F. and Chye, L. M. 1999. Expression of cysteine proteinase during developmental events associated with programmed cell death in brinjal. *The Plant Journal* 17:321-328.
48. Yamamoto, M., Wakatsuki, T., Hada, A. and Ryo, A. 2001. Use of serial analysis of gene expression (SAGE) technology. *J Immunol Meth* 250:45-66.

CHAPTER 3: DISTRIBUTION AND PREVALENCE OF *Bru1*, A MAJOR BROWN RUST RESISTANCE GENE, IN THE SUGARCANE WORLD COLLECTION

3.1 INTRODUCTION

Sugarcane is a perennial bunch grass that belongs to the genus *Saccharum* with a high degree of polyploidy and interspecific origin (Daniels and Roach 1987, Lu *et al.*, 1994, D'Hont *et al.*, 1998, Grivet *et al.*, 2004). The *Saccharum* genus belongs to the *Poaceae* family and *Andropogoneae* tribe, which grows in tropical and subtropical regions and also includes other grasses in related genera, including two crop plants maize and sorghum (D'Hont *et al.*, 1998). Until the end of the 19th century, most sugarcane cultivars were the “noble canes” provided by *Saccharum officinarum* (2n=80) with *S. sinense* (2n=81-124) and *S. barberi* (2n=111-120) as taxonomic secondary species derived after natural hybridizations between *S. officinarum* and *S. spontaneum* (D'Hont *et al.*, 1996). Modern sugarcane cultivars are inter-specific hybrids of the domesticated species *S. officinarum* (2n=8x=80, x=10), which is characterized by low fiber and high sucrose content, and the wild species *S. spontaneum* (2n=5x-16x=40-128, x=8), which is high in fiber and low in sucrose content but resistant to biotic and abiotic stresses (D'Hont *et al.*, 1996, D'Hont *et al.*, 1998, Piperidis and D'Hont 2001). Modern sugarcane cultivars have a complex aneu-polyploid genome consisting of 100-130 chromosomes with a total size of about 10 Gbp (D'Hont 2005) and estimated genome composition of 70-80% from *S. officinarum* and 10-20% from *S. spontaneum* (D'Hont *et al.*, 1996, Piperidis and D'Hont 2001).

The *Saccharum* genus contains five major species, including two wild species, *S. spontaneum* and *S. robustum* (thought to be the ancestor of *S. officinarum*), and three cultivated species, *S. officinarum*, *S. barberi*, and *S. sinense* (Daniels and Roach 1987, Lu *et al.*, 1994, D'Hont *et al.*, 1998, Grivet *et al.*, 2004). A controversial report suggested the existence of only

two *Saccharum* species: *S. officinarum* and *S. spontaneum* (Irvine 1999). The *Saccharum* genus together with related genera, including *Erianthus*, *Miscanthus*, *Narenga*, and *Schlerostachya*, were referred to as the “*Saccharum* complex” (Cai *et al.*, 2005, Selvi *et al.*, 2006). Two duplicated *Saccharum* complex germplasm collections known collectively as the “World Collection of Sugarcane and Related Grasses” (WCSRGR) are currently being conserved. One WCSRGR is maintained in Coimbatore in India and the other in Miami, Florida in the United States (Alexander and Viswanathan 1995, Comstock *et al.*, 1995). The WCSRGR contains significant genetic diversity and valuable alleles for numerous morphological traits, biomass yield, resistance to biotic and abiotic stresses, and other quality traits (Nayak *et al.*, 2014).

Brown rust caused by *Puccinia melanocephala* H. & P. Syd is an economically important disease in many regions where sugarcane is grown (Raid and Comstock 2000). Brown rust symptoms consist of reddish-brown lesions on the leaves, and severe infections can cause leaf necrosis and premature death of even young leaves (Raid and Comstock 2000). Brown rust can cause reductions in stalk weight and number adversely affecting yield in susceptible cultivars (Comstock *et al.*, 1992, Hoy and Hollier 2009). Disease development can be affected by weather conditions, plant growth stage, plant nutrition, and soil characteristics (Anderson and Dean 1986, Anderson *et al.*, 1991, Raid and Comstock 2000, Barrera *et al.*, 2013), as well as genetic interactions between the host and pathogen.

The development and cultivation of resistant cultivars has been the primary means of disease control. Unfortunately, brown rust resistance durability is uncertain, since the pathogen, possesses adaptive ability to overcome host plant resistance. Shifts from resistance to susceptibility have been reported for cultivars in different regions (Purdy *et al.*, 1983, Dean and Purdy 1984, Hoy 2005, Raid 1989). Three studies have shown differential sugarcane cultivar

reactions resulting from inoculations with different pathogen isolates, indicating specialization in *P. melanocephala* to host genotype (Srinivasan and Muthaiyan 1965, Shine *et al.*, 2005, Hoy *et al.*, 2014).

Brown rust resistance has been reported to be a quantitatively inherited trait with high heritability (Tai *et al.*, 1981, Hogarth *et al.*, 1993). However, molecular genetic tools identified a major gene, *Bru1*, for brown rust resistance in a selfed population of sugarcane cultivar R570 from the Reunion breeding program (Daugrois *et al.*, 1996, Asnaghi *et al.*, 2004). Resistance was observed to segregate in a 3: 1 ratio indicative of a single dominant resistance gene. This gene was linked to a RFLP probe, CDSR29, which was initially not integrated in any linkage group in a R570 map. Subsequent additional mapping in R570 (Asnaghi *et al.*, 2004) indicated that *Bru1* was located on the linkage group VII–1a in homology group VII (HGVII) of R570. Results of mapping of R570 revealed in an atypical RFLP profile the presence of one band cosegregating with *Bru1* suggesting that it might originate from *S. spontaneum* (Le Cunff *et al.*, 2008). Raboin *et al.*, (2006) identified a second major brown rust resistance gene nonorthologous to the *Bru1* of R570 in MQ76-53, an old Australian cultivar lacking *Bru1*, which came from a cross between an interspecific cultivar (Trojan) and a *S. spontaneum* clone (SES528).

Bru1 as a source of resistance to brown rust is of particular interest since it has been durable. *Bru1* resistance breakdown was not been detected despite intensive cultivation of R570 for more than 20 years in different regions of the world (Le Cunff *et al.*, 2008). Moreover, inoculation tests revealed *Bru1* provided resistance against diverse rust isolates in Africa and the Americas (Asnaghi *et al.*, 2004).

Strong linkage disequilibrium was detected in the *Bru1* region of the genome, and 22 molecular markers used in a worldwide sample of 380 cultivars showed that the presence of

Bru1 was associated with brown rust resistance in 86% of resistant cultivars (Costet *et al.*, 2012). Two markers, R12H16 and 9020-F4, were strongly linked to *Bru1* and only found in resistant genotypes, and these markers subsequently enabled molecular diagnosis and marker assisted selection for *Bru1*. Glynn *et al.*, (2013) found that 27% of 1072 clones carried the *Bru1* gene when Canal Point Florida sugarcane germplasm was screened. In the same study, *Bru1* was detected in 7% of Louisiana clones and 59% of Florida clones that were resistant to brown rust. Recently, two studies performed in Argentina and Guatemala showed that 49 of 129 (38%) and 26 of 80 (32%) clones showed the presence of *Bru1*, respectively (Molina *et al.*, 2013, Racedo *et al.*, 2013). A comprehensive marker- assisted screening of Louisiana sugarcane germplasm was performed with 506 clones, including 117 cultivars and elite breeding clones, 208 early generation progeny of crosses with wild/exotic germplasm, and 181 wild/exotic germplasm clones (Parco *et al.*, 2014). Cultivars and advanced breeding clones showed a low frequency of detection with 5 out of 117 (4%) testing positive for *Bru1*. In progeny from crosses involving wild/exotic germplasm, only 14 of 208 clones (7%) tested *Bru1* positive. However, *Bru1* frequency was higher (29%, 52 of 181 clones) in wild/exotic germplasm, which indicated that diverse genetic resources were available for *Bru1* introgression.

The variable situation in *Bru1* frequency in different sugarcane industries around the world and increasing efforts to incorporate other sources of brown rust resistance and genes for additional traits from other *Saccharum* species and related genera suggest that the WCSRG should be screened to identify the distribution, prevalence, and existence of any variability for *Bru1*. It also would be of interest to determine the origin of *Bru1* in the *Saccharum* complex. The objective of this study was therefore to determine the distribution and prevalence of *Bru1* in the

WCSRG to assist in the development of the most effective strategies for breeding programs to breed for brown rust resistance along with additional gene introgression from wild species.

3.2 MATERIALS AND METHODS

3.2.1 Plant materials

All sugarcane and related genotypes in the World Collection of Sugarcane and Related Grasses (WCSRG) that is part of the United States National Plant Germplasm System (NPGS) were included in the study. All genotypes were clonally maintained in the field or pots at the United States Department of Agriculture-Agricultural Research Service Subtropical Horticulture Research Station at Miami, Florida. A total of 1,282 clones from the WCSRG were collected as leaf pieces sampled for DNA extraction.

3.2.2 Genomic DNA Isolation and PCR Genotyping

Total genomic DNA was extracted from approximately 100 mg of leaf tissue using CTAB miniprep methodology (Doyle and Doyle 1990). The quantity and quality of DNA was determined using a ND-100 spectrophotometer (Nanodrop Technologies Inc, Wilmington, DE, USA). PCR reactions were performed with 100 ng of total DNA with *Bru1*-specific markers, R12H16 and 9020-F4 (Costet *et al.*, 2012), following the method described earlier (Parco *et al.*, 2014). PCR reactions were carried out in a total volume of 20 µl containing 100 ng template DNA, 0.4 µM of each primer; 0.4 mM of each dNTP, 2.5 mM MgCl₂, and 0.5 units Taq Polymerase with 1X PCR buffer. The primer sequences used were: R12H16 Fw – CTACGATGAAACTACACCCTTCTC, R12H16 Rv – CTTCTGTAAGCGTGACCTATGGTC; 9020-F4 Fw – TACATAATTTTAGTGGCACTCAGC, 9020-F4 Rv - ACCATAATTCAATTCTGCAGGTAC. Thermocycling was performed as follows: 4 min denaturation at 94 °C followed by 35 cycles of 94 °C for 30 s, 55.5 °C for 45 s, 72 °C for 72 s and

final elongation for 8 min at 72 °C. Ten microliters of PCR product amplified with 9020-F4 primers were digested overnight at 37 °C with *RsaI* in a total volume of 20 µl. Restriction fragments were resolved on 3% agarose gels in 1X TAE buffer and stained with ethidium bromide for visualization and documentation in a KODAK Gel Logic 200 Imaging system (Kodak, New Haven, CT). Presence of *Bru1* was indicated by presence of an amplification product of 570 bp with the R12H16 marker or a 200 bp fragment produced after *RsaI* digestion of 9020-F4 marker amplicon.

3.3.3 Genotype identifications

Genotype names from the WCSRG were defined on a curator's name system available from the USDA Germplasm Resources Information Network (USDA-GRIN) as part of the NPGS. Accession numbers and descriptors of each genotype are maintained at the National Germplasm Repository in Miami, Florida.

3.3 RESULTS

3.3.1 Classification of WCSRG Genotypes

A total of 1,282 genotypes from the WCSRG were screened for the presence of *Bru1* gene using two linked markers. The species *S. spontaneum* and *S. officinarum* and *Saccharum* interspecific hybrids comprised the major portion of the collection with 40.6, 19.2, and 13.2% of the genotypes, respectively (Figure 3.1). *Saccharum robustum*, *S. sinense*, and *S. barberi* comprised 5.1, 3.0, and 2.2% of the genotypes. Other *Saccharum* species, including *S. arundinaceum*, *S. bengalense*, *S. brevibarbe*, *S. edule*, *S. kanashiroi*, *S. procerum*, *S. ravennae* and *S. rufipilum* represented 12.6% of the genotypes screened, while genotypes belonging to other genera such as *Coix*, *Erianthus*, *Imperata* and *Miscanthus* comprised 3.2%. Genotypes without classification comprised 10.9% of the collection.

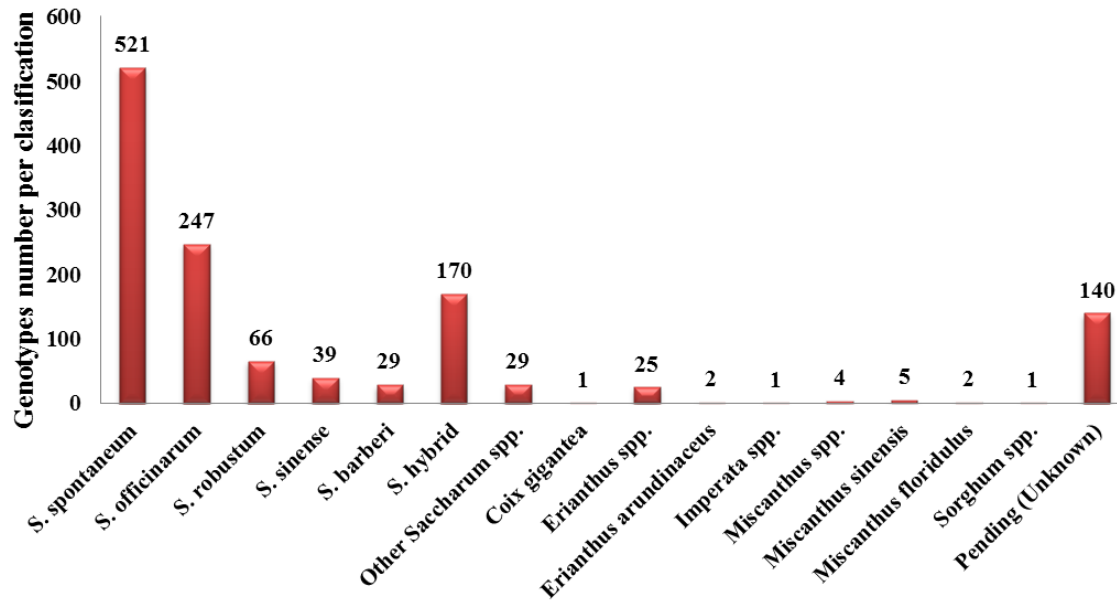


Figure 3.1 Genotypic classification of the World Collection of Sugarcane and Related Grasses

3.3.2 Detection of *Bru1* in WCSRG

The presence of *Bru1* was indicated by the detection of amplification products for the R12H16 (570bp) and/or 9020-F4-*RsaI* (200bp) markers (Figure 3.2). A total of 280 (21.8%) of the 1,282 genotypes in the WCSRG tested positive for *Bru1* as indicated by the detection of one or both markers (Table 3.1). The R12H16 marker was detected alone in 72 (25.7%) of the 280 *Bru1* positive genotypes, while marker 9020-F4-*RsaI* was detected alone in 70 (25%) of the 280 *Bru1* positive genotypes (Table 3.1). Both molecular markers, R12H16 and 9020-F4-*RsaI*, associated with *Bru1* were detected in 138 (49.3%) of 280 *Bru1* positive genotypes (Table 3.1). The frequency of *Bru1* detection for single markers alone or both markers varied among *Saccharum* species (Table 3.1). The proportion of genotypes with *Bru1* was highest for *S. barberi* (79.3%), *S. sinense* (71.8%), and *S. robustum* (33.3%). Interspecific hybrids and *Saccharum officinarum* genotypes had similar lower percentages of *Bru1* (26.4% and 21.0%, respectively), and *S. spontaneum* genotypes had the lowest percentage with *Bru1* (13.2%).

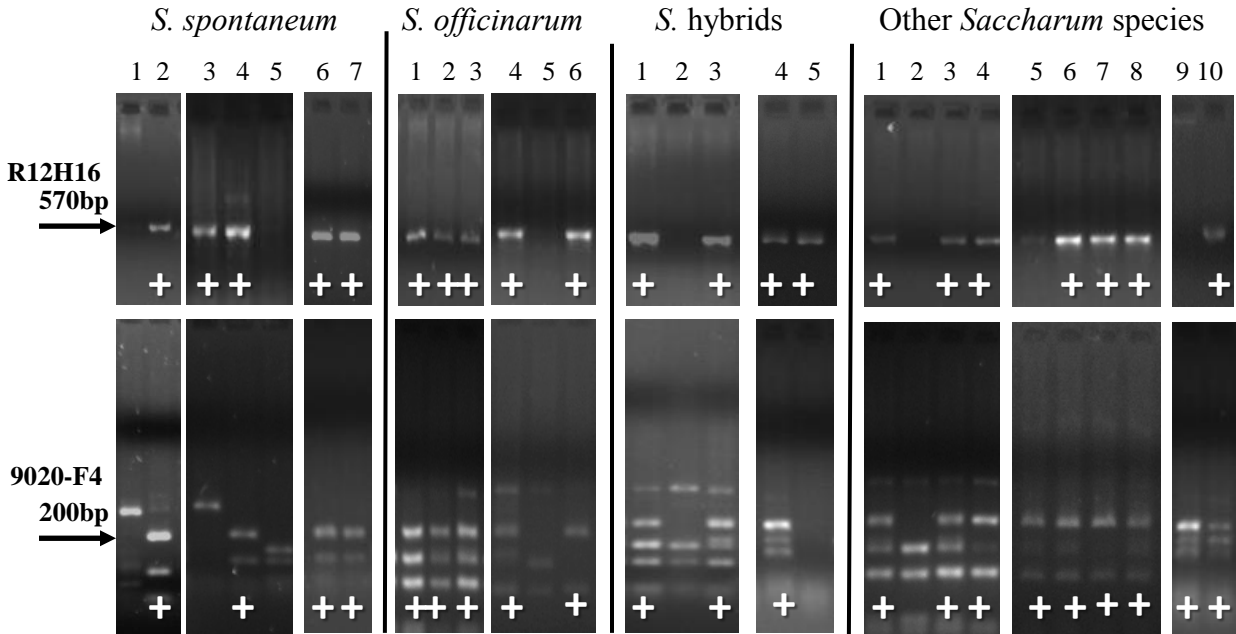


Figure 3.2 Representative gel images showing presence of *Bru1* detected by diagnostic PCR amplification products for R12H16 (570 bp product) and 9020-F4-*RsaI* (200 bp product) in *Saccharum* species or interspecific hybrid cultivars. Genotypes with positive *Bru1* detection provided by presence of either one or both markers are indicated by (+). *Saccharum spontaneum*: #1-7 represent ‘Unknown 2009:R433P78’, ‘IranSpont’, ‘SH3013’ (R12H16 marker only), ‘IND81161’, ‘US4625’, ‘SPONT 84089’, ‘WI 8711+2’. *Saccharum officinarum*: #1-6 represent PMAG8428(221),Puri’ Pundi’, IJ76316’,IJ76324’,IJ76319’. *Saccharum* hybrids: #1-5 represent ‘MEX856196’, ‘MOL6427’, ‘R570’, B37161’, ‘Mesangen’ (R12H16 marker only). Other *Saccharum* species: #1-10 represent: ‘Merthi’(*S.sinense*), ‘Unknown’ (*S. sp*), ‘Nepal3’(*S.sinense*), ‘DB58661’(*S. sp*), ‘Mcilkrum’(*S.sinense*) (9020-F4 marker only), ‘Maneira’(*S.barberi*), ‘Kavangeri’(*S. officinarum*), ‘Merthizell’(*S.sinense*), ‘IK6340’ (*S. robustum*) (9020-F4 marker only), ‘IJ6480’ (*S. robustum*).

The unknowns that probably include additional hybrids had 22.1% of the genotypes with *Bru1*, other *Saccharum* species 10.3%, and the other genera, such as *Erianthus* spp. and *Miscanthus sinensis*, had a low percentage of genotypes (14%, and 2.4%, respectively), that contained *Bru1*.

The frequencies of genotypes with both markers or only a single marker also varied across *Saccharum* species (Table 3.1). *Saccharum sinense* (71.4%), *S. officinarum* (65.4%), the

interspecific hybrids (62.2%), and the unknowns (64.5%) had the highest percentages of genotypes with both markers. *Saccharum robustum* and *S. sinense* had intermediate percentages of genotypes with both markers (59.1% and 43.5%, respectively). *Saccharum spontaneum* genotypes had the lowest frequency with only 18.8 % containing both markers. None of the genotypes from other genera and other *Saccharum* species that were *Bru1* positive had both markers.

Table 3.1 Distribution and prevalence of *Bru1* molecular markers in the World Collection of Sugarcane and Related Grasses

	Number of genotypes with positive detection of <i>Bru1</i> based on the presence of single or both markers			Total genotypes in the collection <i>Bru1</i> + ^d	Total genotypes in the collection
	Genotypes with only R12H16 ^c	Genotypes with only 9020-F4 ^c	Genotypes with both markers ^c		
<i>Saccharum spontaneum</i>	23 (33.3%)	33 (47.8%)	13 (18.8%)	69 (13.2%)	521
<i>Saccharum officinarum</i>	13 (25.0%)	5 (9.6%)	34 (65.4%)	52 (21.1%)	247
<i>Saccharum</i> hybrids	8 (17.7%)	9 (20.0%)	28 (62.2%)	45 (26.5%)	170
<i>Saccharum robustum</i>	5 (22.7%)	4 (18.1%)	13 (59.1%)	22 (33.3%)	66
<i>Saccharum sinense</i>	3 (10.7%)	5 (17.8%)	20 (71.4%)	28 (71.8%)	39
<i>Saccharum barberi</i>	12 (52.1%)	1 (4.3%)	10 (43.5%)	23 (79.3%)	29
Other <i>Saccharum</i> species ^a	1 (33.3%)	2 (66.7%)	0 (0%)	3 (10.3%)	29
Other Genus ^b	2 (28.6%)	5 (71.4%)	0 (0%)	7 (17.1%)	41
Pending (Unknown)	5 (16.1%)	6 (19.3%)	20(64.5%)	31 (22.1%)	140
Total	72 (25.7%)	70 (25%)	138 (49.3%)	280 (21.8%)	280/1,282

^a Other *Saccharum* species that included *Bru1* positive genotypes: *Saccharum arundinaceum* (2) and *S.edule* (1).

^b Other genera that included *Bru1* positive genotypes: *Erianthus* spp. (6) and *Miscanthus sinensis* (1).

^c Number of genotypes *Bru1* positive with percentage of the total number of genotypes *Bru1* positive for that taxonomic group in parentheses.

^d Total genotypes *Bru1* positive for each taxonomic group with percentage of the total number of genotypes of that group in collection in parentheses.

3.3.3 Geographical Distribution of *Bru1* in the World Collection of Sugarcane and Related Grasses

The genotypes screened represent clonal accessions obtained from 55 locations in 48 countries. Countries in Southeast Asia contributed the highest proportions of genotypes to the WCSRG compared with the rest of the world. India was the source of the greatest number of clones with 226 genotypes followed by Indonesia and Papua New Guinea with 174 and 133, respectively (Figure 3.3). The majority of the genotypes in the collection were obtained from breeding programs and associated germplasm collections located around the world at locations where sugarcane does not occur naturally. The geographic origins of these genotypes within the natural range of *Saccharum* are uncertain.

The frequency of *Bru1* positive genotypes within species varied by geographic location from which the clones were obtained for some *Saccharum* species (Figure 3.3). The frequencies of *Bru1* positive genotypes of *S. officinarum* were 18.1% (17 of 94) for clones with a known origin of Papua New Guinea compared to 36.7% (11 of 30) for clones with a known Indonesian origin. Across the entire collection, 21.1% (52 of 247) of *S. officinarum* genotypes were positive for *Bru1*. The same pattern was evident for the frequency of *Bru1* positive genotypes for the ancestral species, *S. robustum*, with a higher frequency of detection for clones known to originate from Indonesia (31.6%, 6 of 19) compared to genotypes known to originate from Papua New Guinea (21.4%, 6 of 28). *Saccharum spontaneum* which occurs over a wider geographic range with more variability in climatic conditions exhibited some variation in *Bru1* frequency. The frequencies of *Bru1* were 15.4% (24 of 156) for genotypes from India, 14.8% (12 of 81) for genotypes from Indonesia, 9.6% (8 of 83) from the Philippines, 11.1% (1 of 9) from Sri Lanka, 11.4% (4 of 35) from Taiwan, and 16.7% (3 of 18) from Thailand, with an overall collection.

Genotypes distribution and detection of <i>Bru1</i>																				
Location	<i>Saccharum spontaneum</i>		<i>Saccharum officinarum</i>		<i>Saccharum hybrid</i>		<i>Saccharum robustum</i>		<i>Saccharum sinense</i>		<i>Saccharum barberi</i>		Other <i>Saccharum</i> species		Other genera		Pending (Unknow)		Total genotypes	
	<i>Bru1</i> marker presence																			
	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+		-
Afganisthan	1																			1
Argentina	1		2		1															4
Australia	1		1		2													2		6
Bangladesh	2				5	3														10
Barbados	15	4	2		6	2							1							30
Brazil				1	1					1										3
Canal Point					18	9							1					1	1	30
China	6			1					4	6										17
Costa Rica										1										1
Cuba			3	3	2					1										9
Dominican Republic					2															2
Fiji			3	1	3	2		1												10
Guadeloupe			3																	3
Guam	3	1																		4
Guatemala					5															5
Guyana						1						1								2
Hawaii			26	7	9	4	1													47
Houma					2											2				4
India	131	24	9	1	5	4			5	11	5	20	9				1	1		226
Indonesia	69	12	19	11	22	4	13	6	1	1	1	1	8	3	3					174
Iran	14	3																		17
Japan	4					1				1										6
Kenya	3																	5	1	9
LSU				1		3												1	1	6
Malaysia	5		11																	16
Mauritius	1		3	1	4											1				10
Mexico						1													1	2

Figure 3.3 Geographical distribution of *Bru1* markers in World Collection of Sugarcane and Related Grasses

(Figure 3.3 continued)

Location	Genotypes distribution and detection of <i>Brul</i>																		Total genotypes	
	<i>Saccharum spontaneum</i>		<i>Saccharum officinarum</i>		<i>Saccharum hybrid</i>		<i>Saccharum robustum</i>		<i>Saccharum sinense</i>		<i>Saccharum barberi</i>		Other <i>Saccharum</i> species		Other genera		Pending (Unknow)			
	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+		
Myanmar	1		1	1	1															4
Nepal										1				1						2
New Caledonia			2	1																3
Nicaragua			1																	1
Pakistan	14	2	1			2												3		22
Panama		1																		1
Papua New Guinea	3	1	77	17	4	1	22	6					1		1					133
Philippines	75	8	7	2	1															93
Puerto Rico			2		2													1		5
Reunion					1	2														3
Saudi Arabia	4																			4
South Africa			3		10	1				3										17
Spain				1																1
Sri Lanka	8	1																		9
Surinam			1																	1
Taiwan	31	4	2		3	5							1							46
Tanzania										1										1
Texas					2															2
Thailand	15	3	1		1	1									1		6			28
Timor														1						1
Turkistan	1																			1
Uganda	4																			4
United States	2				2								1		6		4			15
Unknown	38	5	11	4	8	2	8	8	1	1			1		20	6	84	28		225
Vanuatu			2					1												3
Vietnam													2							2
Virgin Islands			1																	1
Total genotypes	452	69	195	52	125	45	44	22	11	28	6	23	26	3	34	7	109	31		1282

frequency of 13.2% (69 of 521). The accessions of species with the highest frequencies of *Bru1*, *S. sinense* and *S. barberi*, came primarily from the countries where they originated with 68.7% (6 of 10) *Bru1* positive from China with 84.8% (28 of 39) positive overall for *S. sinense* and 80.0% (20 of 25) *Bru1* positive from India with 79.3% (23 of 29) positive overall for *S. barberi*.

3.4 DISCUSSION

Molecular markers could provide a valuable tool for the evaluation of sugarcane germplasm to assist breeding programs in identifying the best genotypes to maximize genetic gain in crossing for traits, such as disease resistance. The discovery of *Bru1* (Daugrois et al., 1996) and development of linked markers for its detection (Costet *et al.*, 2012) have led to the first successful application of marker-assisted selection in sugarcane. The availability of the markers has allowed the characterization of germplasm collections associated with breeding programs in different regions to determine the frequency of occurrence of *Bru1* and its association with resistance to brown rust (Asnaghi *et al.*, 2004, Costet *et al.*, 2012, Glynn *et al.*, 2013, Molina *et al.*, 2013, Racedo *et al.*, 2013, Parco *et al.*, 2014). The present study determined the distribution and prevalence of *Bru1* in the World Collection of Sugarcane and Related Grasses (WCSRGR) maintained in Miami, Florida. *Bru1* is distributed throughout the *Saccharum* complex represented in the collection, but its prevalence varied among species and genera. The proportion of genotypes containing *Bru1* varied widely within the different *Saccharum* species. *Bru1* occurs in both of the wild species, *S. spontaneum* and *S. robustum* (Daniels and Roach 1987, Lu *et al.*, 1994; Grivet *et al.*, 2004). *Bru1* is more prevalent in *S. robustum* clones, whereas it occurs in low frequency and exhibits the highest level of variability in clones of *S. spontaneum*, the *Saccharum* species with the highest levels of genetic diversity and phenotypic variability and widest geographic distribution.

The frequency of *Bru1* was lower in the *S. officinarum* clones in the collection than in *S. robustum* and, as might be expected, similar to the frequency in the interspecific hybrids. The prevalence of *Bru1* was highest in the two secondary cultivated species, *S. barberi* and *S. sinense*, which are derived from interbreeding among *S. officinarum*, *S. spontaneum*, and *S. robustum* (Lu *et al.*, 1994, Grivet *et al.*, 2004) and *Bru1* was present in other related genera as well.

A genetic explanation for the wide distribution of *Bru1* with varying frequencies in the different *Saccharum* species and related genera is not clear. The results suggest that the *Bru1* present in *S. officinarum* and the interspecific hybrids may have come from *S. robustum* rather than *S. spontaneum*. Although, some evidence suggests *S. robustum* evolved from *S. spontaneum* in association with some other genera. The explanation for the high frequencies of *Bru1* in both *S. barberi* and *S. sinense* also is unclear. These two species might provide good sources for brown rust resistance along with other genes for introgression into commercial sugarcane clones.

Variability in the occurrence of *Bru1* as indicated by the presence of only one of the two molecular markers was detected to varying degrees in different *Saccharum* species. Variability was highest in *S. spontaneum* for which a majority of the clones amplified only a single marker. However, *Bru1* was detected by a single marker in eight of 14 *Saccharum* species while in other genera *Bru1* was detected only by a single marker. The implications of this variability are uncertain. This result suggested that a possible weak (less strong) linkage disequilibrium exists between the two diagnostic markers (Costet *et al.*, 2012). Another possibility is that variations in the sequences around *Bru1* among the genotypes that may lead to changes in the priming and/or restriction sites (Parco *et al.*, 2014).

Many sugarcane breeding programs are attempting to introgress additional genes from other wild *Saccharum* species, especially *S. spontaneum*, and other related genera to widen the narrow genetic base of commercial sugarcane cultivars (Jannoo *et al.*, 1999). Louisiana has had one of the most active efforts in this area (Dunckelman and Breaux 1972, Dunckelman 1979). The characterization of the WCSRG for *Bru1* distribution and prevalence will complement efforts to characterize diversity in the *Saccharum* complex for the expected expanded use of marker-assisted selection in the future. The high level of genetic variability in *S. spontaneum* is a proven valuable asset in sugarcane breeding, providing the first interspecific hybrids that allowed the establishment of modern industries worldwide (Daniels and Roach 1987, Lu *et al.*, 1994, Grivet *et al.*, 2004). The more recent effort in Louisiana was successful in improving sucrose content and resistance to mosaic caused by *Sorghum mosaic virus* (Dunckelman and Breaux 1972, Dunckelman 1979). However, in the absence of another major disease, smut, a *S. spontaneum* clone, US 56-15-8, was chosen to be utilized in the breeding effort that subsequently turned out to be highly susceptible to the disease. The later incursion of smut (Koike *et al.*, 1981) then resulted in extensive losses of promising clones in the breeding program due to smut susceptibility causing serious indirect losses to the industry (J. Hoy, unpublished). The characterization by molecular markers of *S. spontaneum* clones and other potential gene introgression sources for brown rust resistance in the absence of the pathogen or under conditions of low disease pressure may prevent unanticipated disease susceptibility problems from limiting future success in other breeding endeavors.

Interspecific hybrid populations under recurrent selection for resistance to brown rust based on natural infection ratings unknowingly increased the frequency of *Bru1* (Asnaghi *et al.*, 2004, Glynn *et al.*, 2013), and it was suggested that this has resulted in a potentially risky

dependence on *Bru1*, for disease resistance worldwide (Costet *et al.*, 2012, Glynn *et al.*, 2013). The prevalence of *Bru1* as indicated by the two molecular markers, is much lower in two more isolated, related breeding populations under more subtropical conditions in Louisiana (Parco *et al.*, 2014) and Argentina (Racedo *et al.*, 2013). This difference has resulted in different breeding strategies utilizing marker-assisted selection for *Bru1*. There is a common interest in using the absence of *Bru1* in clones exhibiting brown rust resistance to suggest possible alternative sources of resistance. Programs with high frequency of *Bru1* are then attempting to reduce over reliance, while programs with low frequency of *Bru1* occurrence are using marker-assisted selection to make bi-parental crosses that will increase the frequency of this source of demonstrated effective and durable resistance gene with other sources of resistance. One Louisiana genotype positive for *Bru1* was rated susceptible to brown rust (Parco *et al.*, 2014), and there have been unpublished reports of susceptibility in *Bru1* positive clones in other sugarcane production areas. In Colombia, five brown rust susceptible cultivars showed the presence of *Bru1* (J. Victoria, personal communication). Sugarcane breeding programs are now attempting to use the ability to monitor *Bru1* to breed and select for cultivars with the brown rust resistance it confers in combination with other genes for resistance to obtain effective, durable resistance to this important disease.

3.5 REFERENCES CITED

1. Alexander, K. and Viswanathan, R. 1995. Conservation of sugarcane germplasm in India given the occurrence of new viral diseases. In: B. J. Croft, C. M. Piggin, E. S. Wallis, D.M. Hogarth (eds), Sugarcane Germplasm Conservation and Exchange, 19-21. ACIAR Proceedings, Canberra.
2. Anderson, D. L. and Dean, J. L. 1986. Relationship of rust severity and plant nutrients in sugarcane. *Phytopathology* 76: 581-585.
3. Anderson, D. L., Henderson, L. J., Raid, R. N. and Ireby, M. S. 1991. Sugarcane rust severity and leaf nutrient status. *Sugar Cane* 3: 5-10.

4. Asnaghi, C., Roques, D., Ruffel, S., Kaye, C., Hoarau, J. Y., Telismart, H., Girard, J.C., Raboin, L. M., Risterucci, A. M., Grivet, L. and D'Hont, A. 2004. Targeted mapping of a sugarcane rust resistance gene (*Bru1*) using bulked segregant analysis and AFLP markers. *Theor. Appl. Genet.* 108: 759-764.
5. Barrera, W. A., Hoy, J. W. and Li, B. 2013. Effects of temperature and moisture variables on brown rust epidemics in sugarcane. *J. Phytopathol.* 161: 98-106.
6. Cai, Q., Aitken, K. S., Fan, Y. H., Piperidis, G., Jackson, P. and McIntyre, C. L. 2005. A preliminary assessment of the genetic relationship between *Erianthus rockii* and the "*Saccharum* complex" using microsatellite (SSR) and AFLP markers. *Plant Sci.* 169: 976-984.
7. Comstock, J. C., Wu, K. K. and Schnell, R. J. 1992. Heritability of resistance to the sugarcane rust. *Sugar Cane* 6:7-10.
8. Comstock, J., Schnell, R. and Miller, J. 1995. Current status of the world sugarcane germplasm collection in Florida. In: B. J. Croft, C. M. Piggins, E. S. Wallis, D. M. Hogarth (eds), *Sugarcane Germplasm Conservation and Exchange*, 17-18. ACIAR Proceedings, Canberra.
9. Costet, L., Le Cunff, L., Royaert, S., Raboin, L. M., Hervouet, C., Toubi, L., Telismart, H., Garsmeur, O., Rousselle, Y., Pauquet, J., Nibouche, S., Glaszmann, J. C., Hoarau, J. Y. and D'Hont, A. 2012. Haplotype structure around *Bru1* reveals a narrow genetic basis for brown rust resistance in modern sugarcane cultivars. *Theor. Appl. Genet.* 125:825-836.
10. Daniels, J. and Roach, B. T. 1987. Taxonomy and evolution. In: D. J. Heinz (eds), *Sugarcane Improvement Through Breeding*, 7-84. Elsevier, Amsterdam.
11. Daugrois, J. H., Grivet, L., Roques, D., Hoarau, J. Y., Lombard, H., Glaszmann, J. C. and D'Hont, A. 1996. A putative major gene for rust resistance linked with a RFLP marker in sugarcane cultivar 'R570'. *Theor. Appl. Genet.* 92: 1059-1064.
12. Dean, J. L. and Purdy, L. H. 1984. Races of the Sugar Cane Rust Fungus, *Puccinia melanocephala*, found in Florida. *Sugar Cane* 1: 15-16.
13. D'Hont, A. 2005. Unravelling the genome structure of polyploids using FISH and GISH; examples of sugarcane and banana. *Cytogenet. Genome. Res.* 109:27-33.
14. D'Hont, A., Grivet, L., Feldmann, P., Rao, S., Berding, N. and Glaszmann, J. C. 1996. Characterization of double genome structure of modern sugarcane cultivars (*Saccharum* spp.) by molecular cytogenetics. *Mol. Gen. Genet.* 250: 405-413.
15. D'Hont, A., Ison, D., Alix, K., Roux, C. and Glaszmann, J. C. 1998. Determination of basic chromosome numbers in the genus *Saccharum* by physical mapping of ribosomal RNA genes. *Genome* 41:221-225.

16. Doyle, J. J. and Doyle, J. L. 1990. A rapid total DNA preparation procedure for fresh plant tissue. *Focus* 12:13-15.
17. Dunckelman, P. 1979. Advancement of new basic sugarcane breeding lines. *Sugar Bull.* 57: 16-19.
18. Dunckelman, P. and Breaux, R. D. 1972. Breeding sugarcane varieties for Louisiana with new germplasm. *Proc. Int. Soc. Sugar Cane Technol.* 14:233-239.
19. Glynn, N. C., Laborde, C., Davidson, R. W., Ireby, M. S., Glaz, B., D' Hont, A. and Comstock, J. C. 2013. Utilization of a major brown rust resistance gene in sugarcane breeding. *Mol. Breed.* 31: 323-331.
20. Grivet, L., Daniels, C., Glaszmann, J. C., and D'Hont, A. 2004. A review of recent molecular genetics evidence for sugarcane evolution and domestication. *Ethnobotany Res. Appl.* 2:9-17.
21. Hogarth, D., Ryan, C. and Taylor, P. 1993. Quantitative inheritance of rust resistance in sugarcane. *Field Crops Res.* 34:187-193.
22. Hoy, J. W. 2005. Impact of rust on LCP 85-384. *Sugar Bull.* 84: 12-13.
23. Hoy, J. W., Avellaneda, M. C. and Bombecini, J. 2014. Variability in *Puccinia melanocephala* pathogenicity and resistance in sugarcane cultivars. *Plant Dis.* 98:1728-1732.
24. Hoy, J. W. and Hollier, C. A. 2009. Effect of brown rust on yield of sugarcane in Louisiana. *Plant Dis.* 93: 1171-1174.
25. Irvine, J. E. 1999. *Saccharum* species as horticultural classes. *Theor. Appl. Genet.* 98:186-194.
26. Jannoo, N., Grivet, L., Dookun, A. and D'Hont, A. 1999. Linkage disequilibrium among modern sugarcane cultivars. *Theor. Appl. Genet.* 99:1053-1060.
27. Koike, H., Fontenot, D., Damann, K. and Schulb, R. 1981. Smut of sugarcane in Louisiana. *Plant Dis.* 65: 1018.
28. Le Cunff, L., Garsmeur, O., Raboin, L. M., Pauquet, J., Telismart, H., Selvi, A., Grivet, L., Philippe, R., Begum, D., Deu, M., Costet, L., Wing, R., Glaszmann, J. C. and D'Hont, A. 2008. Diploid/polyploid syntenic shuttle mapping and haplotype specific chromosome walking toward a rust resistance gene (*Bru1*) in highly polyploid sugarcane (2n=12x~115). *Genetics* 180: 649-660.
29. Lu, Y. H., D'Hont, A., Walker, D. I. T., Rao, P. S., Feldman, P. F. and Glaszmann, J. C. 1994. Relationships among ancestral species of sugarcane revealed with RFLP probes using single copy maize nuclear probes. *Euphytica* 78: 7-18.

30. Molina, L., Queme, J. L. and Rosales, F. 2013. Comparative analysis between phenotype and *Bru1* marker for incidence to brown rust in sugarcane. Proc. Int. Soc. of Sugar Cane Technol. 28:738-743.
31. Nayak, S. N., Song, J., Villa, A., Pathak, B., Ayala-Silva, T., Yang, X., Todd, J., Glynn, N. C., Kuhn, D. N., Glaz, B., Gilbert, R. A., Comstock, J. C. and Wang, J. 2014. Promoting utilization of *Saccharum* spp. genetic resources through genetic diversity analysis and core collection construction. PLoS ONE 9, e110856.doi:10.1371/journal.pone.0110856.
32. Parco, A. S., Avellaneda, M. C., Hale, A. H., Hoy, J. W., Kimbeng, C. A., Pontif, M. J., Gravois, K. A. and Baisakh, N. 2014. Frequency and distribution of the brown rust resistance gene *Bru1* and implications for the Louisiana sugarcane breeding programme. Plant Breed. 133:654-659.
33. Piperidis, G. and D'Hont, A. 2001. Chromosome composition analysis of various *Saccharum* interspecific hybrids by genomic in situ hybridization (GISH). Proc. Int. Soc. Sugar Cane Technol. 2:565-566.
34. Purdy, L.H., Liu, L. J. and Dean, J. L. 1983. Sugarcane rust, a newly important disease. Plant Dis. 67:1292-1296.
35. Raboin, L. M., Oliveira, K. M., Le Cunff, L., Telismart, H., Roques, D., Butterfield, M., Hoarau, J. Y. and D'Hont, A. 2006. Genetic mapping in sugarcane, a high polyploid, using bi-parental progeny: identification of a gene controlling stalk colour and a new rust resistance gene. Theor. Appl. Genet. 112: 1382-1391.
36. Racedo, J., Perera, M. F., Bertani, R., Funes, C., Gonzales, V., Cuenya, M. I., D'Hont, A., Welin, B. and Castagnaro, A. P. 2013. *Bru1* gene and potential alternative sources of resistance to sugarcane brown rust disease. Euphytica 191:429-436.
37. Raid, R. N. 1989. Physiological specialization in sugarcane rust (*Puccinia melanocephala*) in Florida. Plant Dis. 73:183.
38. Raid, R. N. and Comstock, J. C. 2000. Common rust. In: P. Rott, R. A. Bailey, J. C. Comstock, B. J. Croft, and A. S. Saumtally (eds), A Guide to Sugarcane Diseases, 85-89. CIRAD and ISSCT, Montpellier.
39. Selvi, A., Nair, N., Noyer, J., Singh, N., Balsaundaram, N., Bansal, K. C., Koundal, K. R. and Mohapathra, T. 2006. AFLP analysis of the phenetic organization and genetic diversity in the sugarcane complex, *Saccharum* and *Erianthus*. Genet. Resour. Crop. Ev. 53:831-84.
40. Shine, J. M. Jr., Comstock, J. C. and Dean, J. L. 2005: Comparison of five isolates of sugarcane rust and differential reaction on six sugarcane clones. Proc. Int. Soc. Sugar Cane Technol. 25: 638-647.

41. Srinivasan, K. V. and Muthaiyan, M. C. 1965. A note on physiological races in *Puccinia erianthi* Padu & Khan affecting sugarcane varieties. Proc. Int. Soc. Sugar Cane Technol. 12:1126- 1128.
42. Tai, P. Y. P., Miller, J. D. and Dean, J. L. 1981. Inheritance of resistance to rust in sugarcane. Field Crops Res. 4: 261-268.

CHAPTER 4: CONCLUSIONS

- A transcriptome analysis of L 99-233, a sugarcane cultivar with quantitative resistance to brown rust, using suppression subtractive hybridization found that genes associated with primary metabolism, protease activity, nucleotide binding, defense responses, as well as signal transduction, were expressed in sugarcane in response to infection by *P. melanocephala*.
- All genes analyzed for their expression showed message accumulation upon infection in both susceptible and resistant cultivars, but the maintenance of high amounts of mRNAs of the genes for a prolonged time period appeared to be the most important factor contributing to brown rust resistance.
- Differences in the time-course of expression were detected for some genes between L 01-299 (containing *Bru1*) and L 99-233 (lacking *Bru1*) suggesting differences in the mechanisms for brown rust resistance between the cultivars.
- Comprehensive genome-wide transcriptome analysis through RNA-Seq is needed to identify complex co-expression networks and provide a more complete explanation of the quantitative resistance reaction of L 99-233.
- Identification of novel genes could be useful to mine for SNPs/indels for QTL and association mapping to identify functional markers associated with brown rust resistance. These markers in combination with those for the *Bru1* gene will allow breeding programs to develop sugarcane cultivars with more durable and effective resistance against brown rust.
- The frequency of the major brown rust resistance gene, *Bru1*, varies among species in the *Saccharum* complex. *Bru1* occurs in both of the wild species, *S. spontaneum* and *S.*

robustum but the frequency of detection was much lower in *S. spontaneum*. The prevalence of *Bru1* was high in the two secondary cultivated species, *S. barberi* and *S. sinense*, which are derived from interbreeding among *S. officinarum*, *S. spontaneum*, and *S. robustum*.

- The characterization by molecular markers of *S. spontaneum* clones and other potential gene introgression sources for *Bru1* associated brown rust resistance in the absence of the pathogen or under conditions of low disease pressure may prevent unanticipated disease susceptibility problems from limiting success in other breeding endeavors.
- The characterization of the WCSRG for *Bru1* distribution and prevalence will complement efforts to characterize diversity in the *Saccharum* complex for the expected expanded use of molecular marker-assisted selection in the future.

VITA

Mavir Carolina Avellaneda Barbosa was born in Bogotá, Colombia in 1981. In 1997, she started her bachelor studies in Industrial Microbiology in the Pontificia Universidad Javeriana in Bogotá, Colombia. She began her research career as an intern student in the Colombian Sugarcane Research Center-CENICANA under the mentoring of Dr. Jorge Victoria, her project of research during her internship was supported with the National Phytopathological Award from the Colombian Plant Pathology Association ASCOLFI. After her graduation in 2002, she was a junior researcher in CENICANA sponsored by COLCIENCIAS (Administrative Department of Science, Technology and Innovation of Colombia). During seven years, she was involved in different projects related with sugarcane viral diseases.

In January 2011, she joined Dr. Jeff Hoy's lab and started to work on the screening for brown rust using controlled conditions methods. During her time in graduate school she was awarded with LSU Graduate School Travel Award, LACA (Louisiana Agricultural Consultant Association) and ASSCT (American Society of Sugarcane Technologist) fellowships. Mavir Carolina served as treasurer of the PPCP-Graduate Student Association from June 2013 to April 2015. In 2014 she was invited to join the Omicron Delta Kappa Honor Society and received her Master of Science degree in Plant Health in August, 2014. She is a candidate to receive the Doctoral of Philosophy degree in Plant Health in May, 2016.