2016

Development of Functional Markers for Resistance to Leaf Scald in Sugarcane

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DEVELOPMENT OF FUNCTIONAL MARKERS FOR RESISTANCE TO LEAF SCALD IN SUGARCANE

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 School of Plant, Environment and Soil Sciences

by
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B.S., Universidad del Valle, 2006
December 2016

This dissertation is dedicated to my mother Alba Myriam Viveros, who gave me the best gift that a parent can give to a son: love, values and education.

ACKNOWLEDGMENTS

I would like to thank my Major Professor, Dr. Niranjan Baisakh for giving me the opportunity to participate in his projects, the guidance and encouragement through the completion of this project.

I also want to thank Dr. Jeffrey Hoy for his support and the time that he spent with me in the field for the acquisition of new skills. I would like to extend my gratitude to the members of my committee Dr. Collins Kimbeng, Dr. Gerald Myers and Dr. Raymond Schneider for providing valuable advice in the design and revision of the experiment results.

I want to extend my acknowledgment to Carolyn Savario of the Sugarcane Disease Detection Lab, as well as, the members of the Sugarcane Genetics Lab, especially to my lab mate and unconditional friend Carolina Avellaneda for all the support in the US.

Finally, without the economic support of the United States Department of Agriculture (USDA) and the American Society of Sugar Cane Technologists, it never would have been possible to earn the degree.

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ABSTRACT

Leaf scald, caused by *Xanthomonas albilineans*, is a major sugarcane disease worldwide. The disease is managed primarily with resistant cultivars obtained through classical breeding; however, the erratic symptom expression hinders the reliability and reproducibility of the selection process. The development of molecular markers associated with incompatible/compatible reaction can overcome this limitation. Suppression subtractive hybridization (SSH) and quantitative trait locus (QTL) mapping were the strategies used to find leaf scald resistance-associated genes and molecular markers in sugarcane. SSH results showed that genes involved in signal perception and transduction, and DNA binding, were highly expressed in the resistant clone LCP 85-384 compared to the susceptible clone HoCP 89-846. Also, a higher proportion of overexpressed genes were located in the chloroplast in the resistant clone. Early accumulation and maintenance of high mRNA concentration was hypothesized as the determining factor for leaf scald resistance. A linkage map was constructed using 89 F_1 progeny of a cross between the cultivars LCP 85-384 (resistant) and L 99-226 (susceptible) using simple sequence repeat (SSR), leaf scald responsive genes-derived SSR and single nucleotide polymorphic (SNP) markers. Single marker analysis showed that the markers c3-579 (LOD = 3.7189; phenotypic variance explained (PVE = 17.56%), 1x71593 (LOD = 3.0453; PVE = 14.65%) and c1-586b (LOD = 3.013; PVE = 14.48%) were associated with leaf scald resistance. Interval mapping identified 15 QTLs associated with disease resistance that explained 2.5 to 18.6% of the phenotypic variance. Comparative genomic analysis with *Sorghum bicolor* identified genes previously associated with resistance or tolerance to biotic and abiotic stresses within and flanking the QTLs. The present study resulted in a strong platform for future functional validation of the genes to ascertain their role in leaf scald resistance and marker

validation in larger and diverse populations toward development of allele-specific markers for their use in breeding resistant sugarcane varieties.

CHAPTER 1: GENERAL INTRODUCTION

1.1 OVERVIEW

Sugarcane (*Saccharum* spp. hybrids) is a tropical grass in the *Poaceae* family which accounts for 70% of the raw sugar produced worldwide (Wei et al. 2006; Le Cunff et al. 2008; Andru et al. 2011; Aitken et al. 2014). Sugarcane is a perennial C4 plant with high photosynthetic efficiency able to partition carbon to sucrose in the stem, in contrast with other cultivated grasses that usually accumulate their reserve products within seeds. These characteristics have made it one of the most productive cultivated plants (Le Cunff et al. 2008). Recently, it has gained attention as an important source of renewable biofuel for ethanol production and electricity generation (Wei et al. 2006; Le Cunff et al. 2008). In addition, it is used as raw material for paper, plywood, industrial enzymes, and animal feed (Singh et al. 2013). In the United States, sugarcane is grown for sucrose in Florida, Louisiana, Texas, and Hawaii. Florida produces 48% of the total cane sugar in the United States (Baucum and Rice 2009), while Louisiana produces nearly 43% (NASS 2010).

Until the nineteenth century, sugarcane cultivars commonly grown were mostly clones of *Saccharum officinarum*, a species that accumulates high levels of sucrose in the stem but has poor disease resistance (D'Hont et al. 1996). *Saccharum spontaneum*, a related species, accumulates little sucrose and has thinner stalks and higher fiber content compared with *S. officinarum*; however, *S. spontaneum* is a polymorphic species with resistance or tolerance to different biotic and abiotic stresses, good ratooning ability, and adaptation to a wide range of habitats (Bull and Glasziou 1979; Aitken et al. 2014). Early in the twentieth century, hybridization attempts between *S. officinarum* (2n=8x=80; *x*=10) and *S. spontaneum* (2n=5x=40

to 2n=16x=128; *x*=8) and backcrossing with *S. officinarum* resulted in high sugar yields and disease resistance (Roach 1972). An analysis of parents used in breeding programs determined that only 20 *S. officinarum* and less than ten *S. spontaneum* clones had been involved in the breeding of the commercial cultivars available (Patade and Suprasanna 2008).

The breeding concept in sugarcane involves the combination of vigorous growth, ratooning ability, tolerance to abiotic stresses and disease resistance from *S. spontaneum* and high sucrose content from *S. officinarum*. Sugarcane cultivars are complex aneu-polyploids with chromosome numbers of 2n=100-120 (D'Hont et al. 1998; Aitken et al. 2014). In the development of modern cultivars, a phenomenon called female restitution was useful for faster recovery of high sucrose concentration in stem and other features associated with *S. officinarum*. In female restitution, when *S. officinarum* is used as female and *S. spontaneum* as male parent, the progeny will have a triploid chromosome number (2n + n = 100 to 130) (Sreenivasan et al. 1987); the female parent transmits 2n chromosomes, whereas the male parent (*S. spontaneum*) transmits the normal n chromosomes. The asymmetric transmission also occurs the first time that the hybrid is backcrossed to *S. officinarum* (Lu et al. 1994). The female restitution hastened the breeding process by decreasing the number of backcrosses needed to recover the features of *S. officinarum* in the hybrids (Sreenivasan et al. 1987). Consequently, modern cultivars contain approximately 80% of *S. officinarum* chromosomes, 10-15% *S. spontaneum* chromosomes and 5-10% recombinant chromosomes (D'Hont et al. 1996). This genome arrangement produces the coexistence of simplex and multiplex alleles and irregular chromosome numbers in various homo(eo)logous groups due to aneuploidy (Hoarau et al. 2001). The high ploidy levels, the aneuploidy and the cytogenetic complexity have made sugarcane a challenge for breeding, genetics and gene cloning (D'Hont and Glaszmann 2001; Rossi et al. 2003).

Pests and diseases are important problems that affect sugarcane productivity worldwide. Among the 120 diseases that have been described on sugarcane (Rott et al. 2000), leaf scald, caused by the bacterium *Xanthomonas albilineans* (Ashby) Dowson, is one of the major diseases worldwide (Rott et al. 1997; Wang et al. 1999; Rott and Davis 2000). The disease is characterized by chronic and acute symptoms varying in severity from a white, sharply defined longitudinal leaf stripe to death of shoots or entire plants (Ricaud & Ryan 1989; Rott et al. 1997; Wang et al. 1999). Latent infection can occur, making visual diagnosis problematic (Ricaud and Ryan 1989; Rott et al. 1997). Leaf scald causes high losses in tons of cane per hectare and reduction in the juice quality (Ricaud and Ryan 1989; Rott and Davis 2000). In addition, yield reductions have been associated with the acute form of the disease, in which whole fields planted with a susceptible variety could be destroyed in few months (Ricaud and Ryan 1989; Rott 1993).

Xanthomonas albilineans is a xylem-inhabiting gammaproteobacteria that belongs to the order Xanthomonadales (Janse 2005). It is a Gram-negative, aerobic, rod 0.25 - 0.3 µm by 0.6 - 10 µm with a single polar flagellum (Ricaud and Ryan 1989). The colonies are buff yellow and non-mucoid with optimal growth at 25°C; the bacteria grow slowly and appear after 4 – 6 days as circular honey-yellow colonies (Ricaud and Ryan 1989). *Xanthomonas albilineans* is an unusual bacterium because it apparently does not possess avirulence or pathogenicity genes that are typically found in phytopathogenic bacteria (Champoiseau et al. 2006).

Xanthomonas albilineans can cause three different phases of infection on sugarcane: chronic, acute and latent (Ricaud and Ryan 1989; Rott and Davis 2000; Saumtally and Dookun 2004). The chronic phase is characterized by symptoms that vary in severity, including white longitudinal streaks along leaf veins (pencil lines), leaf chlorosis, leaf necrosis progressing basipetally initially along pencil lines, abnormal development of side shoots exhibiting

symptoms, reddish discoloration of vascular bundles at node level, stunting, wilting, and death (Ricaud and Ryan 1989; Rott and Davis 2000; Birch 2001; Saumtally and Dookun 2004). Symptoms could be caused by xylem blockage and/or metabolic products (Birch 2001), while bleaching, chlorosis and necrosis are associated with changes in the cells caused by albicidin, a toxin produced by the pathogen. Albicidin is a phytotoxin that inhibits DNA replication and blocks plastid development (Hashimi et al. 2008). The acute phase occurs as a sudden wilting of plants resulting in death, with few or no symptom expression, and large areas of a field may be affected (Rott and Davis 2000; Saumtally and Dookun 2004). The acute phase was observed in a highly susceptible cultivar after a period of drought stress following rainy conditions (Ricaud and Ryan 1989). The latent phase occurs for unknown reasons (Rott and Davis 2000). Sometimes, the latency is observed in young shoots that emerge from infected setts and in ratoon crops. Symptomatic young shoots also can recover during stalk development (Ricaud and Ryan 1989). Detection of the disease is difficult when infection is latent, and this has resulted in worldwide spread of leaf scald during sugarcane germplasm exchanges (Daugrois et al. 2003).

Leaf scald is spread by the use of infected cuttings for planting and contaminated tools used at harvest (Ricaud and Ryan 1989; Rott and Davis 2000). Aerial transmission was reported in Guadeloupe, where the bacterium was exuded from the leaf hydathodes (Klett and Rott 1994). Hurricane conditions have also been associated with the pathogen spread (Ricaud and Ryan 1989; Hoy and Grisham 1994). In addition, maize and several weeds have been reported to be naturally infected by the bacterium (Rott and Davis 2000).

Breeding and selecting for host plant resistance has been the most important control measure for leaf scald. The use of hot water treatment and tissue culture techniques to produce healthy seed-cane, disinfection of cutting and harvest tools with bactericides, and quarantine

measures during germplasm exchanges are additional methods used to prevent and control the disease (Ricaud and Ryan 1989; Rott and Davis 2000). In Louisiana, leaf scald was reported for the first time in 1993 (Grisham et al. 1993). A survey found that leaf scald was widely distributed in the Louisiana industry and had the potential to cause severe symptoms and yield losses (Hoy and Grisham 1994). An indirect loss is caused by the elimination of promising clones in cultivar selection programs (Ricaud and Ryan 1989; Hoy and Grisham 1994). Up to 20% of sugarcane clones in the selection population are rejected annually due to susceptibility in Australia, even though crosses between susceptible parents are avoided (Birch 2001).

The development of resistant varieties is considered the best strategy to manage leaf scald in sugarcane. Screening trials to evaluate resistance are carried out in many countries, but assessment of cultivar reactions is difficult and time-consuming (Rott et al. 1997). Assessments generally are based on observation of symptom severity after artificial inoculation (Rott et al. 1997). However, the troublesome aspect of resistance evaluation is that symptom expression is affected by environmental conditions, and some sugarcane cultivars can tolerate the pathogen without exhibiting symptoms (Rott et al. 1997). The erratic symptom expression results in the failure to accurately detect susceptibility and thus multiple inoculations are needed. In addition, resistant genotypes can sometimes become systemically infected under severe inoculation conditions (Gutierrez et al. 2016). Under this scenario, the development of molecular markers was seen as a potential major breakthrough promising to overcome the limitations (Ruane and Sonnino 2007). The use of DNA markers for genetic analysis and manipulation of agronomic traits has become a useful tool in plant breeding (Zhang et al. 2004). The potential appears to be in accelerating the rate of gain from selection of desirable genotypes and in the manipulation of quantitative trait loci related with important economic traits of the crop (Zhang et al. 2004).

Marker-assisted selection (MAS) technique, which uses marker(s) linked to useful trait(s), is extensively used in improving yields and in breeding for resistance against pests and diseases in some crops (Manigbas and Villegas 2007).

The large genome (10 Gb), the absence of a genome sequence and the high complexity of the genome have hindered progress in genetic/genomic research and the application of genomic tools in sugarcane (Wang et al. 2010). Currently, all sugarcane genetic maps constructed are incomplete due to the large number of chromosomes and the limited sequence information available for developing markers (Wang et al. 2010). However, with the decreased cost of DNA sequencing technologies, the possibility of developing markers from sugarcane sequences is becoming less expensive than some years ago (Wang et al. 2010).

A high-density genetic map is a valuable tool to understand the genetic and genomic organization of sugarcane, a complex polyploid crop. Its autopolyploid nature with mostly random pairing plus inbreeding depression has limited the production of a more common experimental mapping population, such as double haploids or recombinant inbred lines (Aitken et al. 2014). Different features of the sugarcane genome, such as the coexistence of single and multi-dose alleles and the irregular number of chromosomes in the homo(eo)logy groups, have restricted genetic mapping (Aitken et al. 2014). Due to polyploidy, the development of a high-density genetic map for sugarcane requires more work than a diploid species (Singh et al. 2013).

The genetic maps developed to date for sugarcane cultivars, as well as for their ancestral species, are based on populations of full sib (F_1) individuals following a pseudo-test cross strategy and using only single-dose markers (Grattapaglia and Sederoff 1994). In a bi-parental population, a single-dose marker has either a single copy of an allele in one parent only

segregating 1:1 (presence:absence) or a single copy of the same allele in both parents segregating in a 3:1 ratio (presence:absence). In polyploids with irregular chromosome pairing, the loci showing either a 1:1 or a 3:1 segregation pattern are much more informative for genetic map construction than other markers showing more complex segregation patterns (Wu et al. 2002). Based on this method, partial genetic maps have been produced for *S. spontaneum* (da Silva et al. 1993; Ming et al. 1998), *S. officinarum* (Guimaraes et al. 1998; Aitken et al. 2006), interspecific hybrids (Daugrois et al. 1996, Alwala et al. 2008) and modern cultivars of sugarcane (Hourau et al. 2002; Andru et al. 2011; Singh et al. 2013; Aitken et al. 2014) using different types of molecular markers, including random amplified polymorphic DNA (RAPD), restriction fragment length polymorphisms (RFLP), amplified fragment length polymorphisms (AFLP), simple sequence repeats (SSR), sequence related amplified polymorphisms (TRAP), expressed sequence tag-SSR (EST-SSR) and diversity array technology (DArT) markers.

Despite the multiple problems associated with genetic mapping in sugarcane and other complex polyploids, the advent of a diverse array of molecular marker systems recently has increased efficiency in developing dense genetic linkage maps. Until a few years ago, the genetic maps for sugarcane had low genome coverage and limited information on genome organization due to the limited number of markers mapped (Aitken et al. 2014). The decrease in cost of DNA sequencing technologies will be useful to produce a higher number of DNA markers (Wang et al. 2010) and increase the saturation level of the molecular maps available, and the information can eventually be used in gene tagging, QTL mapping and map-based cloning (Le Cunff et al. 2008).

Multiple strategies can be used for the identification of the genes/alleles or QTL regions involved in resistance (or susceptibility) to leaf scald that will have great potential in breeding programs. Different methods have been reported for the identification and study of gene

expression in response to biotic or abiotic stresses in sugarcane. The suppression subtractive hybridization (SSH) is an efficient and widely used PCR-based method to obtain subtracted cDNA libraries and isolate differentially expressed genes (Bui et al. 2005). The procedure involves two successive tester-driver hybridization steps. The first induces a normalization of tester-specific molecules, allowing the subsequent cloning of rare, tester-specific transcripts. Because SSH can be initiated using PCR-amplified cDNAs, it seems particularly well-suited to tissues with low mRNA concentration (Bui et al. 2005). Furthermore, SSH does not require previous knowledge of gene sequences and can be suitable for species where only a small number of sequences are available in databases (Bui et al. 2005). SSH method has been used to identify genes related with cold tolerance (Khan et al. 2013), water deficit conditions (Almedia et al. 2013) and brown rust resistance in sugarcane (Oloriz et al. 2012).

For the identification of QTL regions associated with resistance to leaf scald, the development of a bi-parental population product of two contrasting parents in relation to the disease response will be a useful tool. Previously, a genetic map for the highly resistant cultivar LCP 85-384 was reported (Andru et al. 2011). In Louisiana, LCP 85-384 is considered as one of the most successful cultivars in the history of the sugar industry and generated significant monetary gains after its release in 1993 (Andru et al. 2011). The sugar yields of LCP 85-384 were 25% higher over the cultivars previously grown (Gravois and Bischoff 2008), and the cultivar occupied 91% of the Louisiana sugarcane acreage in 2004 because of its superior agronomic characters and resistance to various biotic and abiotic (post-freeze recovery) stresses, including leaf scald (Andru et al. 2011). LCP 85-384 is a BC_4 derivative of US 56-15-8 (*S. spontaneum*) from an introgression breeding effort that was initiated at the USDA-ARS Sugarcane Research Unit in 1972 (White et al. 2011). The introgression program was undertaken

to increase genetic variability, and LCP 85-384 retained many of the traits of its *S. spontaneum* ancestry, and its background is thought to be responsible for the 20% increase in sugar yields over varieties previously grown by Louisiana sugarcane farmers (White et al. 2011).

Although a genetic map is available for LCP 85-384 (Andru et al. 2011), a bi-parental population can be more useful in the development of molecular markers associated with leaf scald resistance due to the broad distribution of the disease response in the population. LCP 85-384 (female) was crossed with L 99-226 (male), a cultivar susceptible to the disease used as a parent in the Louisiana breeding program. Two different kinds of markers were selected for the map construction, SSRs and SNPs (obtained through next generation sequencing). With the use of SNPs markers and based on the sorghum homology with sugarcane, it is expected that the map obtained will show homologous regions with sorghum that can be used in a comparative analysis to elucidate the nature of the resistance to leaf scald in sugarcane and to fine resolve the QTLs associated with the resistance to the disease.

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CHAPTER 2: EXPRESSION PROFILE OF CANDIDATE GENES INVOLVED IN THE RESISTANCE RESPONSE OF SUGARCANE TO LEAF SCALD

2.1 INTRODUCTION

The widespread distribution of different diseases is one of the major problems adversely affecting sugarcane productivity. Leaf scald, caused by *Xanthomonas albilineans*, is a major sugarcane disease that occurs in most sugarcane producing countries (Rott et al. 1997; Wang et al. 1999; Rott & Davis 2000; Garces et al. 2014). The disease is characterized by chronic and acute phases varying in severity from a single, white to cream, sharply defined leaf stripe to death of shoots or entire plants (Ricaud & Ryan 1989; Rott et al. 1997; Wang et al. 1999; Croft et al. 2000). The disease can cause severe yield and juice quality reductions in susceptible cultivars (Ricaud & Ryan 1989; Hoy and Grisham 1994; Rott & Davis 2000), eliminate potential cultivars in the breeding program, and pose quarantine concerns for germplasm exchange (Garces et al. 2014).

Leaf scald is managed primarily with resistant cultivars developed through classical breeding (Wei et al. 2006). In classical breeding, the selection is carried out based on observable phenotypes of candidates clones and/or their relatives, but without knowing which genes are being selected (Ruane & Sonnino 2007). The development of molecular markers was considered as a major breakthrough promising to overcome the limitation with phenotypic evaluation (Ruane & Sonnino 2007). Marker-assisted selection (MAS) technique, which uses the marker(s) linked to useful trait(s), is extensively used in improving crop yields and in breeding for resistance against pests and diseases (Manigbas & Villegas 2007).

The high ploidy, interspecific origin, aneuploidy, and recombination among the chromosomes of the ancestral species make the sugarcane genome, possibly, the most complex among important crops (Grivet and Arruda 2001; D'Hont 2005; Wei et al. 2006). The complexity of the genome has hindered progress in the development and application of genetic/genomic tools in sugarcane breeding programs (Wang et al. 2010). However, with the decrease in the cost of DNA sequencing technologies, the development of sequence-based markers in sugarcane is now less expensive (Wang et al. 2010).

Identification of the genes/alleles involved in the resistance response to leaf scald will have potential for their application as markers in breeding programs. Several molecular approaches have been used to identify and study the expression profile of the genes in response to biotic and abiotic stresses in sugarcane. The suppression subtractive hybridization (SSH) method has been used to identify genes related to cold tolerance (Khan et al. 2013), water deficit conditions (Almedia et al. 2013), smut, mosaic and the stalk borer eldana (Butterfield et al. 2004), and brown rust resistance in sugarcane (Oloriz et al. 2012). In the present investigation, single resistant and susceptible clones were used to mine genes responsive to leaf scald with the long term objective to develop functional markers to facilitate breeding of cultivars resistant to the disease. In addition, the information obtained through the differential gene expression profiles in a resistant vis-à-vis susceptible clone will provide insight into the possible mechanisms involved in leaf scald resistance in sugarcane.

2.2 MATERIALS AND METHODS

2.2.1 Bacterial isolation and inoculation

Xanthomonas albilineans bacteria were isolated from the longitudinal section of a leaf with a pencil-line symptom. Tissue was surface-sterilized with NaOCl (0.5%) for 30 sec and rinsed with water. The leaf sections were cut in small pieces, and placed in a 1.5 ml tube containing 1 ml of sterile, distilled water. The tubes were incubated overnight at 4 °C, and a loop of bacterial suspension was transferred to semi-selective XAS (Wilbrinks) medium with benomyl, cycloheximide, novobiocin, cephalexin and kasugamycin (Davis et al. 1994) and incubated at 28 °C. After 5-8 days, single colonies were selected and streaked to obtain pure cultures on solid XAS medium without antibiotics. Pure cultures were incubated at 28 °C, and after 48 h, the bacterial suspension was diluted to obtain a concentration of 3.5×10^8 CFU/ml based on spectrometric absorbance (0.18 OD at 590 nm) (Garces et al. 2014). The bacterial suspension was used for inoculation of plants in the greenhouse.

The bacterial suspension was kept at 4 °C in the dark prior to inoculation. Approximately 2-month-old greenhouse grown plants of the clones LCP 85-384 (resistant to leaf scald) and HoCP 89-846 (susceptible to leaf scald) were inoculated by injection of the bacterial suspension near the apical meristem. Plants were inoculated with approximately 200 µl of bacterial suspension. Only two puncture wounds were done to the plant to minimize stress caused by mechanical wounding. For the non-inoculated controls, distilled water was injected near the meristem region to mimic the stress induced by mechanical wounding caused to the inoculated plants. Fifteen plants per clone were inoculated and four plants per clone were used as non-inoculated control for the subtraction library construction. Inoculations were done in the greenhouse at about sunset during the winter of 2012-2013. Leaf and meristem tissues of three

plants were harvested in liquid nitrogen from both clones 24 h, 48 h, 72 h, and 1 week after inoculation and stored at −80 °C for RNA isolation. The leaf and meristem tissues of non-inoculated control plants were collected at the same time points as the inoculated plants with one plant per time point. The inoculation method effectiveness was assessed at two weeks after inoculation, evaluating three inoculated plants per clone by visual evaluation and quantification of the bacterial population (Garces et al. 2014; Gutierrez et al. 2016).

2.2.2 RNA isolation and cDNA subtraction

Total RNA was isolated from leaf and meristem tissues using TRIzol (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. The quality and quantity of the RNA samples were evaluated with an agarose gel-based and a quantitative assay using a ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE), respectively. Equimolar concentrations of RNA samples of meristem and leaf tissues at different time points after inoculation were pooled, and 20 µg of RNA were used for cDNA synthesis. cDNA subtraction was performed using the PCR-select^{TM} cDNA subtraction kit (Clontech, Palo Alto, CA) following the manufacturer's instructions except that double-stranded cDNA was synthesized from 20 µg of control and inoculated total RNA using the Superscript^{TM} double-strand cDNA synthesis kit (Invitrogen, Carlsbad, CA). Double-stranded cDNA (5 µg) was digested with *Rsa*I (New England Biolabs, Ipswich, MA). 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 plants as the driver. Differentially expressed upregulated genes were amplified by primary PCR with 27 cycles of 94°C for 30 s, 66°C for 30 s and 72°C for 90 s. The primary PCR product was enriched by a secondary PCR with 12 cycles of 94°C for 30 s, 68°C for 30 s and 72°C for 90 s.

2.2.3 Cloning and sequencing of differentially expressed genes

The subtracted cDNAs were cloned into pGEM-T Easy Vector (Promega, Madison, WI) and transformed into *Escherichia coli* DH5α cells as described previously (Khan et al. 2013). Approximately 200 white colonies from the cDNA subtracted library of the resistant clone (LCP 85-384) and the susceptible clone (HoCP 89-846) were confirmed insert-positive with PCR using M13 forward and reverse primers. Plasmids extracted from the positive colonies were then single-pass sequenced using an ABI 3130xl sequencing platform.

2.2.4 Sequence processing and bioinformatics analysis

The vector sequences and the poly(A) tail were cleaned manually from the sequences. For the clean sequences, after excluding the exactly duplicated sequences, BLASTx- and BLASTn-based homology searches against NCBI non-redundant protein and nucleotide databases were performed (http://www.ncbi.nlm.nih.gov/) at e-value cut-off of 1e-06. Sequence match hits exceeding 50 nucleotides and more than 90% identity were considered significant. The sugarcane unigenes were mapped against the sorghum genome (http://www.plantgdb.org/SbGDB) using BLASTn at 1e-05 and 60% sequence identity. The syntenic regions were depicted in the sorghum genome map using the physical map location information retrieved from Phytozome v. 10.3 (https://phytozome.jgi.doe.gov/pz/#!info?alias=Org_Sbicolor). The maps, one per clone, were drawn using MapChart v. 2.3 (Voorrips 2002). Gene ontology (GO) IDs of the unigenes were retrieved from the Blast2GO output (Conesa et al. 2005). The GOSlim terms for biological process, molecular function, and cellular component associated with significant BLASTx hits were assigned to sugarcane unigenes using Blast2GO.

2.2.5 Transcript profiling of differentially expressed genes

The expression pattern of 17 differentially expressed sequence tags (ESTs), identified by cDNA subtraction, was validated by quantitative reverse transcription polymerase chain reaction (qRT-PCR) following the method described earlier (Khan et al. 2013). Gene-specific primers were designed using Primer3Plus software (Untergasser et al. 2012) (Table 2.1). All the primers were synthesized by Integrated DNA Technologies (IDT Inc, Coralville, IA). First-strand cDNA was synthesized from 1 µg of the total RNA isolated from leaf tissues of the control and inoculated plants of both clones (LCP 85-384 and HoCP 89-846) at 24 h, 48 h, 72 h, and 1 week using iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). Relative abundance of the genes was determined using qRT-PCR. The qRT-PCR was carried out in triplicate (three biological replications). The PCR was conducted in 20 µl final reaction volume containing 1 µl of 10x diluted first strand cDNA, 10 µl of SYBR Green Supermix (Bio-Rad, Hercules, CA) and 0.2 µM of each primer in a MyiQ real-time PCR detection system (Bio-Rad, Hercules, CA). A serial dilution of 100, 10, 1, 0.1 and 0.01 ng of 1st strand cDNA was used for all genes to generate a standard curve by plotting the Ct (threshold cycle) values against log (ng) of 1st strand cDNA to ensure that the PCR efficiencies for all transcripts were equal. The mRNA expression was normalized against the *S. officinarum* elongation factor (SoEF1; GenBank Acc. #EF581011) and calculated as the fold-change ratio in comparison to the control using the $2^{-\Delta\Delta Ct}$ method (Pfaffl 2001). The absolute fold-change values of the relative mRNA abundance were used for heat map analysis using gplots package in R v.3.1.3 (R Core Team, 2015) and Bar HeatMapper plus tool (http://bar.utoronto.ca/ntools/cgi-bin/ntools_heatmapper_plus.cgi).

2.2.6 Mining leaf scald disease responsive ESTs for microsatellite markers

The genes induced upon inoculation with *Xanthomonas albilineans* in LCP 85-384 and HoCP 89-846 were searched for the presence of simple sequence repeat (SSR) motifs using the GRAMENE SSR tool (Temnykh et al. 2001) with the criteria set to at least five repeats for dinucleotide motifs and at least three repeats for tri, tetra and penta nucleotide motifs. Primers flanking the SSR motifs were designed using Primer3Plus (Untergasser et al. 2012).

2.2.7 Genetic diversity of sugarcane cultivars using leaf scald responsive gene-derived SSR markers

Ninety six clones (Appendix 1) comprised of 93 sugarcane commercial hybrids and three *Saccharum spontaneum* clones were genotyped with 31 leaf scald responsive gene-derived SSR primers (Appendix 2). Fifty ng of genomic DNA, isolated using the potassium acetate protocol (Dellaporta et al. 1983), was used as the template in PCR reactions (final volume of 10 µl) containing 1X PCR buffer, 2.5 mM $MgCl_2$, 0.2 µM dNTP mix, 0.4 unit of *Taq* DNA polymerase (Promega, Madison, WI) and 0.75 µM of each primer. PCR products were resolved in 13 % polyacrylamide gels at 350 V for 4 h using 1X Tris-Glycine as running buffer in a HEGS electrophoresis apparatus (Nihon Eido, Tokyo, Japan). The gels were stained using ethidium bromide and visualized and documented in a Kodak GelLogic200 gel documentation system (Carestream, Rochester, NY).

The scoring of the gels and data analysis was done following the method described by Andru et al. (2012). Bands were scored for presence (1) and absence (0) in a binary matrix, which was subjected to genetic similarity (GS) and cluster analysis based on Dice's similarity coefficient using the unweighted pair group method with arithmetic means (UPGMA) in DendroUPGMA utility (http://genomes.urv.cat/UPGMA/), and the tree was drawn using

Dendroscope v. 3.5.7 (Huson & Scornavacca 2012). The principal coordinate analysis (PCoA) was performed using NTSYSpc v. 2.2 to validate the cluster distribution of the 96 clones.

2.3 RESULTS

2.3.1 Leaf scald response genes

A total of 384 white bacterial colonies representing the products of cDNA subtraction libraries (192 colonies of LCP 85-384 library and 192 colonies of HoCP 89-846 library) were sequenced. After cleaning and exclusion of rRNA and duplicated sequences, 158 sequences with lengths ranging from 90 to 1,039 bp (average length = 470 bp) were unigenes in the LCP 85-384 SSH library (Appendix 3) and 151 sequences with lengths ranging from 80 to 1079 bp (average length = 482 bp) were unigenes in the HoCP 89-846 SSH library (Appendix 4). The BLAST analysis showed that 20 unigenes of the LCP 85-384 SSH library and 29 unigenes of the HoCP 89-846 SSH library did not show significant similarity with the sequences in NCBI protein and nucleotide databases. These could represent sequences in the untranslated regions, non-coding RNAs or sequences specific to sugarcane (Khan et al. 2013). Confirmation of sugarcane sequence similarity to *Sorghum bicolor* was established by significant similarity of 140 unigenes of LCP 85-384 and 135 unigenes of the HoCP 89-846 SSH libraries. The unigenes were distributed over the 10 *S. bicolor* chromosomes and the Super 59 contig where chloroplast and mitochondrial sequences are located. The distribution of the unigenes on the *S. bicolor* genome was determined for LCP 85-384 (Figure 2.1) and for HoCP 89-846 (Figure 2.2).

2.3.2 Gene ontology (GO) analysis of LCP 85-384 SSH library

Analysis of 158 unigenes of the leaf scald resistant clone LCP 85-384 against the KEGG and NCBI databases revealed that 55 unigenes were involved in different metabolic pathways

(Figure 2.3a). The KEGG analysis showed that most of the pathways were involved in metabolism and degradation processes (Figure 2.3a). A majority of the ESTs were involved in purine metabolism followed by thiamine metabolism. In addition, ESTs were associated with lysine, benzoate, caprolactam, toluene, styrene, geraniol, valine-leucine-isoleucine and fatty acid degradation.

Gene ontology (GO) analysis of the ESTs showed that the GOslim terms for the biological process, molecular function and cellular component were assigned to 60.3, 22.4 and 17.4% of the ESTs, respectively (Figure 2.3b, Table 2.2). GO enrichment analysis indicated that most ESTs had hydrolase activity and ion binding under molecular function (Figure 2.3b) and an important number were represented in the plastid (Figure 2.1). In addition, a number of ESTs belonged to the transcription factor category involved in ion binding activity (Figure 2.3b). The ESTs represented in this category included the unigene 384-S76 that is related to nucleotide binding and homologous to *Zea mays* asf sf-2-like pre-mRNA splicing factor and 384-S82 that is associated with transduction system-phosphorelay signal and homologous to *Setaria italic* two component response regulatory protein. Other important biological processes represented in the GO analysis were the signaling and response to stimulus processes and the molecular function signal transduction (Figure 2.3b). The results obtained with KEGG pathway analysis and GOslim terms assignment represent the diversity of the expressed genes in a clone resistant to leaf scald after inoculation with the causal agent.

2.3.3 Gene ontology (GO) analysis of HoCP 89-846 SSH library

Analysis of 151 unigenes of HoCP 89-846 against the KEGG and NCBI databases revealed that 45 unigenes were involved in various metabolic pathways, and most of the

pathways were involved in metabolism (Figure 2.4a). Most of the ESTs were involved in purine metabolism (Figure 2.4a). In addition, ESTs were associated with amino sugar-nucleotide sugar, inositol phosphate, pyrimidine, seleno-compound and cysteine-methionine metabolism.

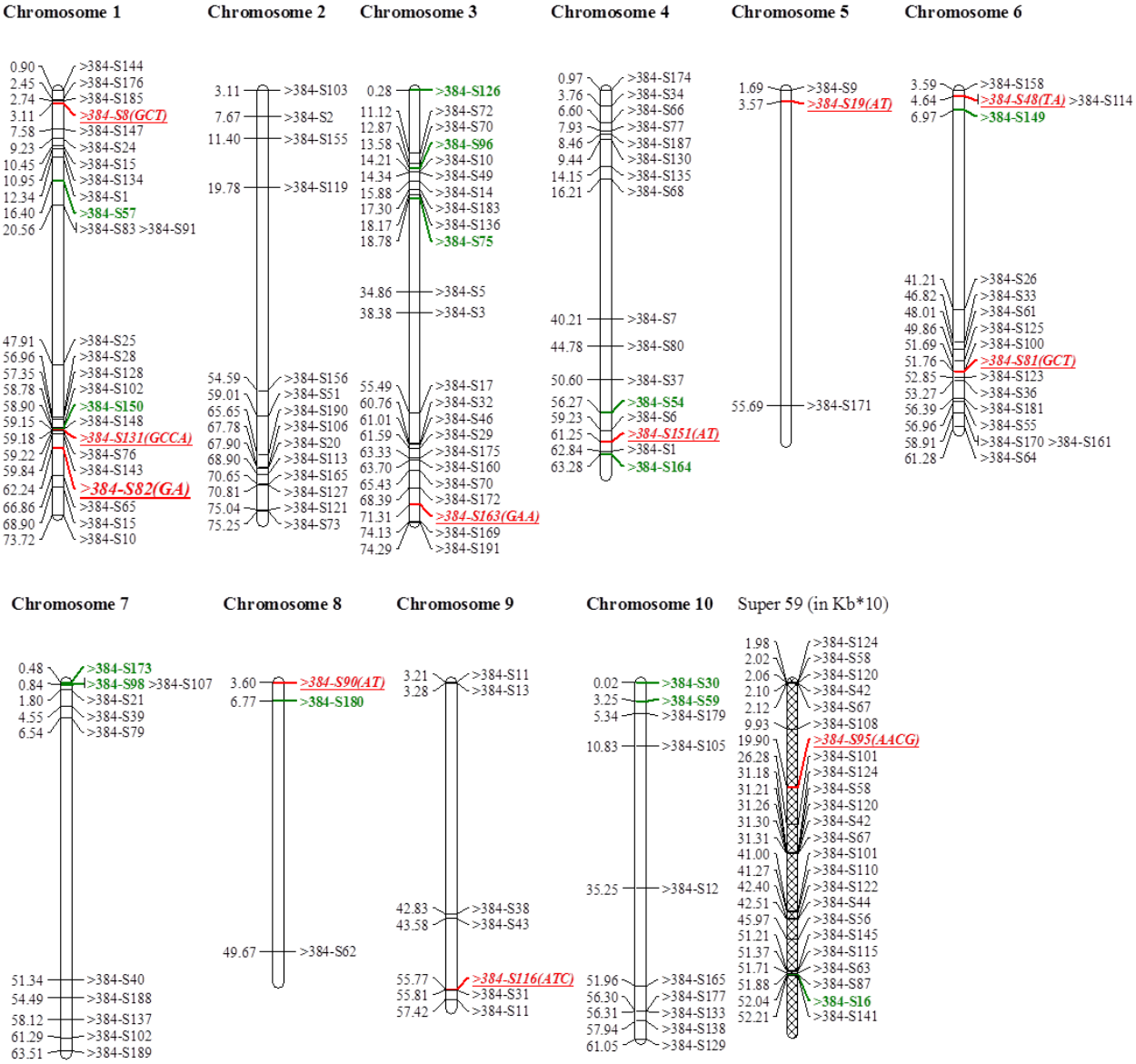


Figure 2.1 Sequence similarity of leaf scald resistant sugarcane clone LCP 85-384 against the *Sorghum bicolor* genome. Sequence IDs in red represent the *Xanthomonas albilineans*-responsive unigenes that were identified with SSR motifs. Sequence IDs in green represent the unigenes that were used in the qRT-PCR assays for the library validation and temporal expression pattern.

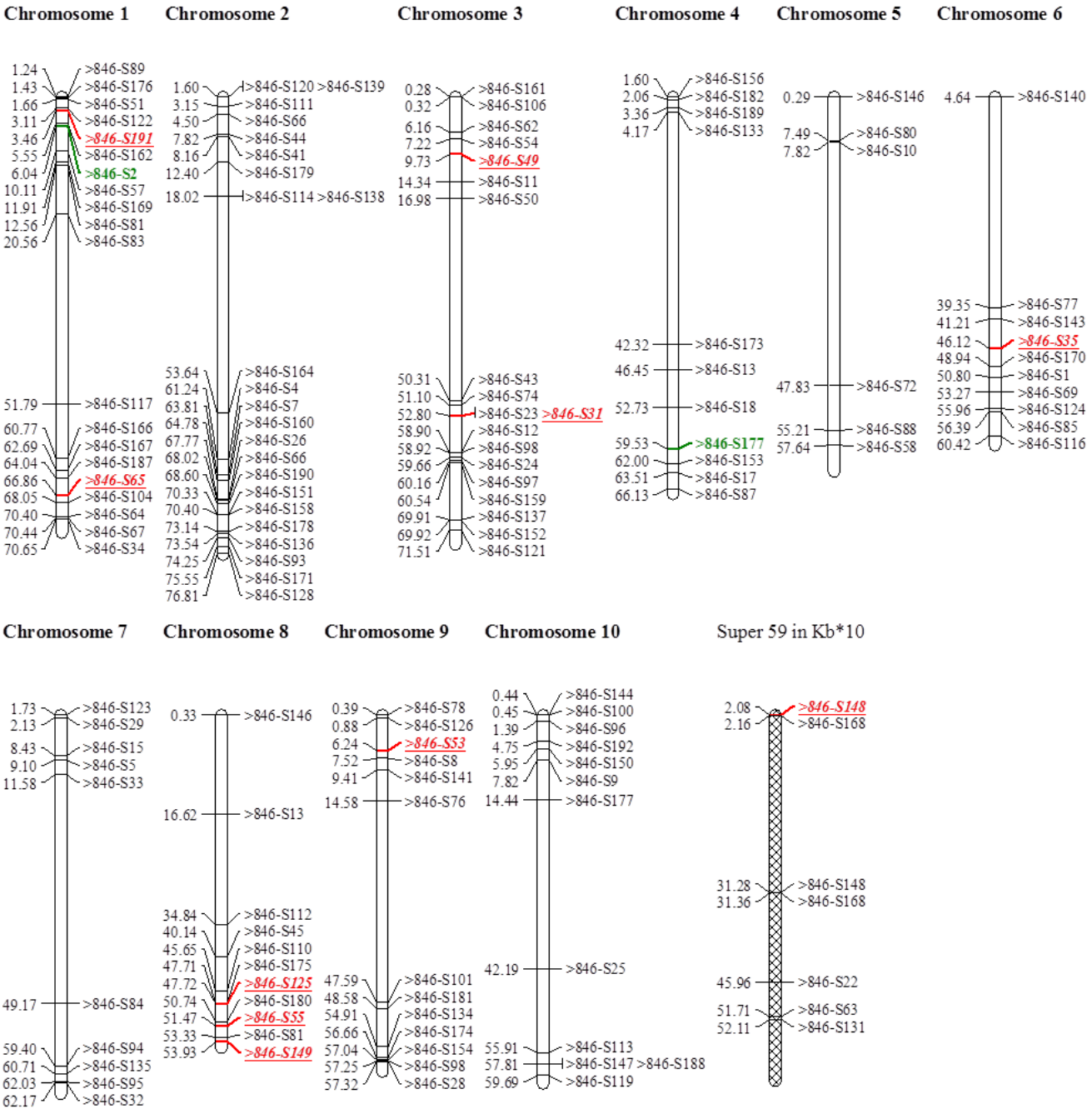


Figure 2.2 Sequence similarity of leaf scald susceptible sugarcane clone HoCP 89-846 against the *Sorghum bicolor* genome. Sequence IDs in red represent the *Xanthomonas albilineans*-responsive unigenes that were identified with SSR motifs. Sequence IDs in green represent the unigenes that were used in the qRT-PCR assays for the library validation and temporal expression pattern.

Table 2.1 Primer sequences used in real-time quantitative reverse transcription PCR-based expression profiling of differentially expressed genes in response to infection by the leaf scald pathogen in sugarcane.

Sequence ID	Primer name	Primer sequence (5' - 3')	PCR product size (bp)	BLASTn-based similarity to genes
846-S2	q846S2-F	GTTTGGTCATGTGGTGTAACCC	101	*Zea mays* serine/threonine-protein kinase SAPK8
	q846S2-R	ATGCGCTGAATTGTCTTGCG		
846-S177	q846S177-F	AGGACAACGCATCACTTTGC	87	Zinc finger protein CONSTANS-LIKE 11
	q846S177-R	TTATGGCCCGCCTTTTATGC		
	q846S180-R	ACGCGATTGTGCTCAAGATG		
384-S54	q384S54-F	ATGCTTTCGTTGGAGGCTTC	89	*Zea mays* adenosine kinase, putative
	q384S54-R	AACATTCGCGGCATAGCAAC		
384-S57	q384S57-F	ATCATGTTGTCGTCGGACCTC	93	*Zea mays* purple acid phosphatase precursor
	q384S57-R	AGGGCGGGGTCTTAAATTGG		
384-S96[a]	q384S96-F	TGCCACATGTTGAGTGCAAG	94	*Sorghum bicolor* CBL-interacting protein kinase 21
	q384S96-R	GGGGAACTCAAGCAACATCAAG		
384-S98	q384S98-F	AATCGCTTCAAGGCTTACGC	141	*Zea mays* adenylate kinase
	q384S98-R	ACTGCAGCATTTCCTTGCAC		
384-S59	q384S59-F	AATTCGGCACTCAACACTGC	119	*Zea mays* transcription elongation factor 1
	q384S59-R	AAACCATGGGGAAGAGGAAGTC		
384-S82	q384S82-F	TTGCTGTCACTGTCGTTCTC	128	*Zea mays* two-component response regulator-like
	q384S82-R	AACCTTTGGCAGCACGTTTG		
384-S134[a]	q384S134-F	AGTCCCTTTGGCAAATGCAC	110	*Zea mays* RING finger protein 5
	q384S134-R	AAGCAGCAGCATCTGGAAAC		
384-S75	q384S75-F	AACAATGGTGCCAACGAGTG	101	*Brachypodium distachyon* ABC transporter C family member 4-like
	q384S75-R	ATGCTGCTTGGCAATGTGAC		
384-S102[a]	q384S102-F	TTTCTGAGTGCTGTGCGATG	111	*Zea mays* nascent polypeptide-associated complex alpha subunit-like
	q384S102-R	AGCAAGCCAGCAAAGATGAC		
384-S113[a]	q384S113-F	AAGGTGTTCTCGCCACTGTAG	104	*Zea mays* autophagy-related protein 8 precursor
	q384S113-R	AAGAACACCTTGCCACCAAC		

(Table 2.1 Continued)

Sequence ID	Primer name	Primer sequence (5' - 3')	PCR product size (bp)	BLASTn-based similarity to genes
384-S116[a]	q384S116-F	TCGCAGCACAACACAAATCC	81	*Zea mays* histone deacetylase102
	q384S116-R	AGCAAAACAAGGCTGTGGTG		
384-S133[a]	q384S133-F	TCAGCAGGGATCTCCACAAAC	111	*Zea mays* FtsH6 protease
	q384S133-R	AAACAACAGGGAGGCCATTG		
384-S149	q384S149-F	ATGCTTTCTGCCAGTTCGTG	134	*Brachypodium distachyon* E3 ubiquitin-protein ligase
	q384S149-R	TGTCGCTCCAGTTGGATTTG		
384-S164	q384S164-F	TGGGCGTGACATTCAAAGTG	126	*Zea mays* glutaredoxin subgroup I
	q384S164-R	AGCTGTCCATTCGAAAGTGC		
384-S173	q384S173-F	ACAAAATGTGGCCGAGAAGC	78	*Zea mays* YT521-B-like family protein
	q384S173-R	TTGGCCTGGTTTTCAGAGAC		
384-S180	q384S180-F	ATAACGGAGCAGTAGCAGACG	107	*Saccharum* hybrid thioredoxin M
	q384S180-R	GCGCATATACACCAGCCATG		
384-S179[a]	q384S179-F	CGCAAGGAGTTTCTGGATGTC	141	*Zea mays* phospholipid hydroperoxide glutathione peroxidase 1
	q384S179-R	AGTGCTGGAGGATTTTTGGG		
384-S30	q384S30-F	TCTTCACCGACTTTGTTGCC	123	*Zea mays* bax inhibitor 1
	q384S30-R	TGTCCCCCAATTCATTCAGC		
384-S150	q384S150-F	ACTTGTGCTTCCGCTTTTGG	103	*Zea mays* transposon protein
	q384S150-R	TCCTTCAGCACTGTGTTCAC		
384-S22	q384S22-F	TCTGCTTCTAGTTCGACTTTCTG	73	*Saccharum arundinaceum* ATP synthase I subunit-like
	q384S22-R	AATTGCGTAGAGGGACCCTTG		
384-S126	q384S126-F	AAAGAGCGCATGATGAGGTG	81	*Sorghum bicolor* hypothetical protein
	q384S126-R	TGTCGACGATGTTGTGTCTG		
384-S74	q384S74-F	TGGTGGGTGAACAATCCAAC	97	Rice 25S ribosomal RNA
	q384S74-R	AAGCGTTCATAGCGACGTTG		

a. The primers designed for these sequences were not useful for the transcript profiling evaluation by qRT-PCR. Some of them either produced two or more bands of different size when RT-PCR products were visualized in agarose gels or did not produce amplification products.

Table 2.2 Sequence similarity and gene ontology (GO) of differentially expressed genes of the leaf scald resistant sugarcane clone LCP 85-384 in response to *Xanthomonas albilineans*.

Sequence ID	Chrom[a]	BLASTn-based similarity to genes	Accession number	GO terms
384-S1	1	*Zea mays* chaperone DNA J2	NM_001136845.1	Protein folding; Unfolded protein binding
384-S15	1	*Zea mays* ribulose-phosphate 3-epimerase	NM_001155113.1	Carbohydrate metabolic process; Isomerase activity
384-S57	1	*Zea mays* purple acid phosphatase precursor	EU970070.1	Phosphatase activity; Ion binding
384-S65	1	*Zea mays* 40S ribosomal protein S3a	NM_001155624.1	Structural constituent of ribosome; Translation
384-S76	1	*Sorghum bicolor* arginine/serine-rich splicing factor SR32 transcript I	KC425089.1	Molecular function
384-S82	1	*Zea mays* two-component response regulator-like PRR73	EU952116.1	Signal transduction
384-S134	1	*Zea mays* RING finger protein 5	EU976282.1	No data
384-S143	1	*Brachypodium distachyon* ribosome biogenesis protein BMS1 homolog	XM_003557930.1	Nucleus; GTPase activity; Ion binding
384-S147	1	*Zea mays* endo-1,4-beta-glucanase Cel1	NM_001158298.1	Carbohydrate metabolic process; hydrolase activity(glycosyl bonds)
384-S148	1	*Sorghum bicolor* hypothetical protein	XM_002467823.1	Structural constituent of ribosome; Translation
384-S185	1	*Sorghum bicolor* hypothetical protein	XM_002463589.1	Structural constituent of ribosome; Translation
384-S2	2	*Zea mays* cycloartenol-C-24-methyltransferase 1	EU961712.1	Biological process; Methyltransferase activity
384-S20	2	Sorghum bicolor hypothetical protein	XM_002462820.1	Molecular function
384-S73	2	*Zea mays* ferredoxin-dependent glutamate synthase1	NM_001112223.1	Oxidoreductase activity; Cellular aminoacid metabolic process; Biosynthetic process

(Table 2.2 Continued)

Sequence ID	Chrom[a]	BLASTn-based similarity to genes	Accession number	GO terms
384-S113	2	*Zea mays* clone 1692176 autophagy-related protein 8 precursor	EU958456.1	No data
384-S119	2	*Sorghum bicolor* hypothetical protein	XM_002459786.1	Signal transduction
384-S127	2	*Zea mays* eukaryotic translation initiation factor 2 gamma subunit	EU966655.1	GTPase activity; Ion binding
384-S155	2	*Zea mays* mitochondrial prohibitin complex protein 2 mitochondrial product	EU966008.1	Cellular component
384-S156	2	*Sorghum bicolor* hypothetical protein	XM_002462185.1	Carbohydrate metabolic process; Isomerase activity
384-S14	3	*Hemerocallis littorea* ribosomal protein S12 (rps12) gene, partial cds; ribosomal protein S7 (rps7) and NADH dehydrogenase subunit B (ndhB) genes	AY147480.1	Structural constituent of ribosome; Translation
384-S29	3	*Sorghum bicolor* hypothetical protein	XM_002458377.1	Structural constituent of ribosome; Translation
384-S32	3	*Sorghum bicolor* hypothetical protein	XM_002456173.1	Vesicle-mediated transport
384-S49	3	*Saccharum* hybrid putative ATP citrate lyase	JQ923438.1	Carbohydrate metabolic process; Transferase activity (acyl groups)
384-S70	3	*Zea mays* beta-5 tubulin	NM_001111988.1	Small molecule metabolic process; Cytoskeleton; GTPase activity; Ion binding; Nucleobase
384-S75	3	*Brachypodium distachyon* ABC transporter C family member 4-like	XM_003567625.1	ATPase activity; Transmembrane transport; Ion binding
384-S96	3	*Sorghum bicolor* CBL-interacting protein kinase 21	FJ901210.1	No data
384-S136	3	*Zea mays* full-length cDNA clone ZM_BFb0122D08	BT069004.1	Small molecule metabolic process; Oxidoreductase activity; lipid metabolic process

(Table 2.2 Continued)

Sequence ID	Chrom[a]	BLASTn-based similarity to genes	Accession number	GO terms
384-S163	3	*Zea mays* mitochondrial import receptor subunit TOM20, nuclear gene for mitochondrial product	EU958096.1	Protein targeting; Membrane organization; Mitochondrion
384-S172	3	*Saccharum* hybrid cytosolic fructose-1,6-bisphosphatase	X89006.1	Carbohydrate metabolic process; Phosphatase activity
384-S191	3	*Oryza sativa* Japonica Group cDNA Os01g0973400	NM_001052082.2	Ribosome biogenesis; Methyltransferase activity; Cellular nitrogen compound metabolic process
384-S34	4	*Zea mays* 60S ribosomal protein L37a	EU962243.1	Structural constituent of ribosome; Translation
384-S37	4	*Zea mays* alkaline/neutral invertase	EU955523.1	Hydrolase activity (acting on glycosyl bonds)
384-S54	4	*Zea mays* adenosine kinase 2	EU962200.1	Kinase activity
384-S66	4	*Zea mays* fumarylacetoacetase	EU959609.1	Cellular aminoacid metabolic process
384-S77	4	*Sorghum bicolor* hypothetical protein	XM_002451729.1	Biological process
384-S135	4	*Sorghum bicolor* hypothetical protein	XM_002451898.1	Oxidoreductase activity; lipid metabolic process
384-S164	4	*Zea mays* Grx_C3 - glutaredoxin subgroup I	NM_001156384.1	Oxidoreductase activity; Homeostasis process
384-S19	5	*Zea mays* uncharacterized LOC100272957 isoform X1	XP_008681407	Molecular function
384-S36	6	Sorghum bicolor hypothetical protein, mRNA	XM_002448219.1	Molecular function
384-S64	6	*Brachypodium distachyon* K(+) efflux antiporter 2, chloroplastic-like	XM_003579464.1	Transmembrane transport
384-S81	6	*Zea mays* PCO132326 mRNA sequence	AY108941.1	Structural constituent of ribosome; Translation

(Table 2.2 Continued)

Sequence ID	Chrom[a]	BLASTn-based similarity to genes	Accession number	GO terms
384-S100	6	*Brachypodium distachyon* probable inactive beta-glucosidase 14 transcript variant	XP_003581423.2	Carbohydrate metabolic process; hydrolase activity(on glycosyl bonds)
384-S149	6	*Zea mays* clone 285640 mRNA sequence	EU945789.1	Molecular function
384-S21	7	*Zea mays* 40S ribosomal protein S13	EU977066.1	Structural constituent of ribosome; Translation
384-S40	7	*Zea mays* HOTHEAD (LOC100281523)	NM_001154441.1	Oxidoreductase activity
384-S98	7	*Zea mays* adenylate kinase	EU955541.1	Ion binding; Kinase activity; Cellular nitrogen compound metabolic process
384-S173	7	*Zea mays* YT521-B-like family protein	EU957472.1	No data
384-S189	7	*Sorghum bicolor* hypothetical protein, mRNA	XM_002444775.1	Cellular component
384-S31	9	*Zea mays* eukaryotic translation initiation factor 3 subunit 5	EU960037.1	Translation process
384-S116	9	*Zea mays* histone deacetylase102	NM_001112161.1	No data
384-S12	10	*Zea mays* formate dehydrogenase 1	EU967680.1	Biological process; Molecular function
384-S30	10	*Zea mays* bax inhibitor 1	EU963304.1	No data
384-S105	10	*Zea mays* threonine endopeptidase	EU962752.1	No data
384-S129	10	*Zea mays* signal peptide peptidase-like 2B	EU961679.1	Peptidase activity
384-S133	10	*Zea mays* filamentation temperature-sensitive H 2A	EU257692.1	Ion binding; Peptidase activity
384-S165	10	*Saccharum* hybrid elongation factor 1 alpha	AF331850.1	Ion binding
384-S16	Super 59[b]	*Miscanthus sinensis* AtpF gene; chloroplast	HQ599894.1	Transmembrane transport; Cellular nitrogen compound metabolic process

(Table 2.2 Continued)

Sequence ID	Chrom[a]	BLASTn-based similarity to genes	Accession number	GO terms
384-S22	Super 59[b]	*Saccharum arundinaceum* ATP synthase I subunit-like	EU071786.1	Transmembrane transport; Cellular nitrogen compound metabolic process
384-S56	Super 59[b]	*Spodiopogon cotulifer* ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit; chloroplast	KC164343.1	Ion binding
384-S63	Super 59[b]	*Saccharum* hybrid chloroplast, complete genome Sequence	AE009947.2	Photosynthesis; Protein complex; Thylacoid
384-S87	Super 59[b]	*Zea mays* mitochondrion, complete genome	DQ490951.2	Transmembrane transport; Ion binding
384-S115	Super 59[b]	Maize chloroplast photosystem I ps1A1 and ps1A2 genes	M11203.1|MZECPPSI	Photosynthesis; Protein complex; Thylacoid
384-S141	Super 59[b]	Maize chloroplast phosphorylation coupling factor alpha subunit (atpA) and proteolipid subunit (atpH) genes	M27557.1|MZECPATPC	Transmembrane transport; Cellular nitrogen compound metabolic process

a. **Chrom,** Chromosome location in *Sorghum bicolor* (based on the homology of the sugarcane and sorghum sequences).
b. **Super 59**, is a contig with mainly the chloroplast and mitochondrial sequences.

Table 2.3 Sequence similarity and gene ontology (GO) of the differentially expressed genes of the leaf scald susceptible sugarcane clone HoCP 89-846 in response to *Xanthomonas albilineans*.

Sequence ID	Chrom[a]	BLASTn-based similarity to genes	Accession number	GO names list
846-S2	1	*Zea mays* serine/threonine-protein kinase	EU960732.1	Cellular protein modification process; Ion binding; Kinase activity
846-S51	1	*Sorghum bicolor* hypothetical protein	XM_002466084.1	mRNA processing; Nucleoplasm
846-S57	1	*Pennisetum glaucum* chloroplast heat shock protein 70	EF495353.1	Response to stress
846-S64	1	*Zea mays* cell division cycle protein 48	JF915708.1	Ion binding
846-S162	1	*Brachypodium distachyon* probable methyltransferase PMT28-like	XM_003558577.1	Methyltransferase activity
846-S169	1	*Zea mays* RNA binding protein	EU968499.1	RNA binding; Cellular nitrogen compound metabolic process
846-S187	1	*Sorghum bicolor* hypothetical protein	XM_002465495.1	Cellular nitrogen compound metabolic process; biosynthetic process
846-S7	2	*Zea mays* clone 218683 endochitinase A2 precursor	EU959576.1	Catabolic process; Cell wall organization; Hydrolase activity
846-S41	2	*Zea mays* clone 211242 ADP-ribosylation factor 1	EU959162.1	Signal transduction; Ion binding; Intracellular
846-S44	2	*Phyllostachys edulis* cellulose synthase (CesA11)	HM068510.1	Carbohydrate metabolic process; transferase activity (glycosyl groups); Biosynthetic process
846-S111	2	*Zea mays* early responsive to dehydration protein (ERD4)	NM_001114650.1	Cellular component
846-S114	2	Sorghum bicolor hypothetical protein	XM_002459769.1	DNA binding; Nucleotidyl transferase activity
846-S120	2	*Zea mays* TPR domain containing protein	EU952451.1	Molecular function

(Table 2.3 Continued)

Sequence ID	Chrom[a]	BLASTn-based similarity to genes	Accession number	GO names list
846-S171	2	*Zea mays* elongation factor 1-beta	EU965401.1	Translation factor activity nucleic acid binding
846-S190	2	*Zea mays* clone 353620 protein phosphatase 2C isoform epsilon	EU970898.1	Molecular function
846-S11	3	*Saccharum* hybrid putative ATP citrate lyase	JQ923438.1	Lyase activity
846-S12	3	*Zea mays* clone RING-finger protein like	EU956797.1	No data
846-S43	3	*Brachypodium distachyon* probable protein phosphatase 2C 5-like	XM_003569137.1	Molecular function
846-S50	3	*Sesamum alatum* putative lipoamide dehydrogenase	AY873805.1	Oxidoreductase activity; Homeostatic process; Ion binding
846-S62	3	*Zea mays* triosephosphate isomerase, cytosolic	EU976695.1	Biological process; Isomerase activity
846-S97	3	*Zea mays* putative serine/threonine protein phosphatase superfamily protein isoform 1	NM_001174292.1	No data
846-S106	3	*Zea mays* OB-fold nucleic acid binding domain containing protein	NM_001153447.1	Nucleic acid binding
846-S16	4	*Zea mays* putative HLH DNA-binding domain superfamily protein	NM_001176472.1	DNA binding
846-S17	4	*Zea mays* cytochrome b561	EU962707.1	Cellular component
846-S133	4	*Zea mays* beta-1,3-galactosyltransferase sqv-2	NM_001155648.1	Carbohydrate metabolic process; transferase activity (glycosyl groups)
846-S177	4	*Zea mays* zinc finger protein CONSTANS-LIKE 11	EU972243.1	Ion binding
846-S10	5	*Brachypodium distachyon* FGFR1 oncogene partner-like, transcript variant 1	XM_003562678.1	Molecular function
846-S72	5	*Brachypodium distachyon* auxin response factor 23-like	XM_003575972.1	Nucleus; Cellular nitrogen compound metabolic process; Biosynthetic process

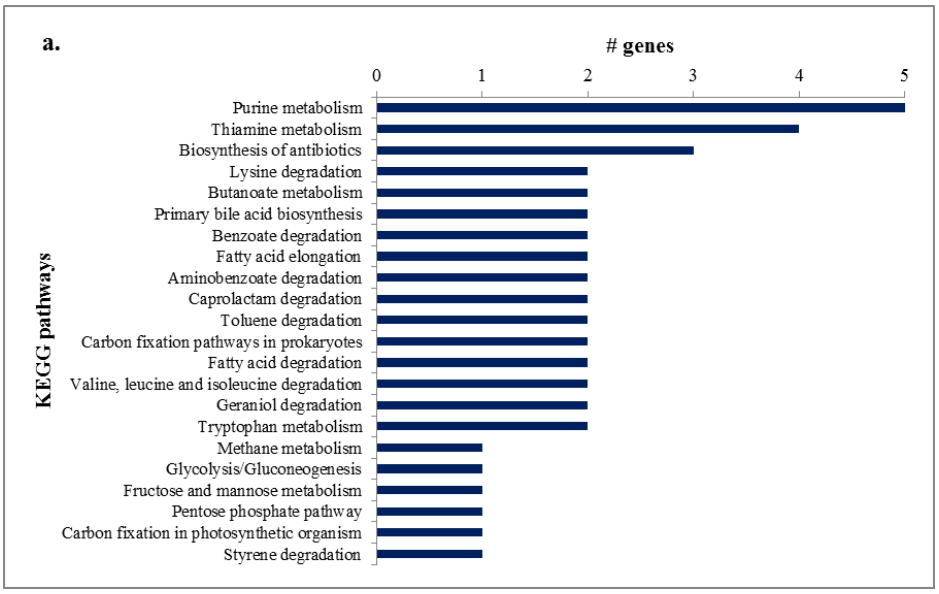
(Table 2.3 Continued)

Sequence ID	Chrom[a]	BLASTn-based similarity to genes	Accession number	GO names list
846-S88	5	*Sorghum bicolor* hypothetical protein	XM_002450985.1	Oxidoreductase activity; Ion binding
846-S1	6	*Zea mays* eukaryotic translation initiation factor isoform 4G-1-like	XM_008670990	Translation factor activity nucleic acid binding
846-S77	6	*Sorghum bicolor* hypothetical protein	XM_002446275.1	Biological process; Molecular function
846-S29	7	*Sorghum bicolor* hypothetical protein	XM_002444923.1	Molecular function
846-S32	7	*Zea mays* auxin response factor 1 (ARF1) gene	HM004516.1	No data
846-S13	8	*Sorghum bicolor* 5-methylcytosine DNA glycosylase (DME)	JF683319.1	DNA metabolic process; Response to stress
846-S45	8	*Sorghum bicolor* hypothetical protein	XM_002442115.1	Molecular function
846-S112	8	*Brachypodium distachyon* ABC transporter F family member 1-like	XM_003573173.1	ATPase activity; Ion binding
846-S149	8	*Zea mays* methionine synthase	AF439723.1	Ion binding; Methyltransferase activity; Cellular aminoacid metabolic process
846-S180	8	*Oryza minuta* bifunctional nuclease in basal defense response	DQ872164.1	Nuclease activity
846-S53	9	*Zea mays* auxin-binding protein ABP20 precursor	EU958158.1	No data
846-S98	9	*Zea mays* splicing factor U2af 38 kDa subunit	NM_001155027.1	Nucleus, RNA binding; Ion binding
846-S103	9	*Zea mays* UDP-sulfoquinovose synthase	NM_001155335.1	Molecular function
846-S126	9	*Sorghum bicolor* hypothetical protein	XM_002440391.1	Ion binding; lyase activity
846-S174	9	*Zea mays* clone 378431 ATP synthase beta chain	EU972246.1	Transmembrane transport; Protein complex
846-S9	10	*Sorghum bicolor* hypothetical protein	XM_002438041.1	Molecular function
846-S96	10	*Sorghum bicolor* hypothetical protein	XM_002436343.1	RNA binding; mRNA processing
846-S113	10	*Brachypodium distachyon* V-type proton ATPase catalytic subunit A-like	XM_003563289.1	Transmembrane transport

(Table 2.3 Continued)

Sequence ID	Chrom[a]	BLASTn-based similarity to genes	Accession number	GO names list
846-S147	10	*Sorghum bicolor* hypothetical protein	XM_002437437.1	Molecular function
846-S150	10	*Zea mays* 3-oxoacyl-synthase I	NM_001156167.1	Biological process; Molecular function
846-S188	10	*Glycine max* uncharacterized protein	XM_003539297.1	Molecular function
846-S80	No found	*Sorghum bicolor* tRNA-Met (tRNAfM) gene, complete sequence; and ATP synthase complex subunit 9 (atp9)	U61165.1	Small molecule metabolic process; transmembrane transport; cellular nitrogen compound metabolic process
846-S33	No found	*Brachypodium distachyon* pentatricopeptide repeat-containing protein At4g30825, chloroplastic-like	XM_003576487.1	Nucleus; Cell cycle
846-S22	Super 59[b]	*Spodiopogon cotulifer* ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	KC164343.1	Ion binding

a. **Chrom,** Chromosome location in *Sorghum bicolor* (based on the homology of the sugarcane and sorghum sequences).
b. **Super 59,** is a contig with mainly the chloroplast and mitochondrial sequences.



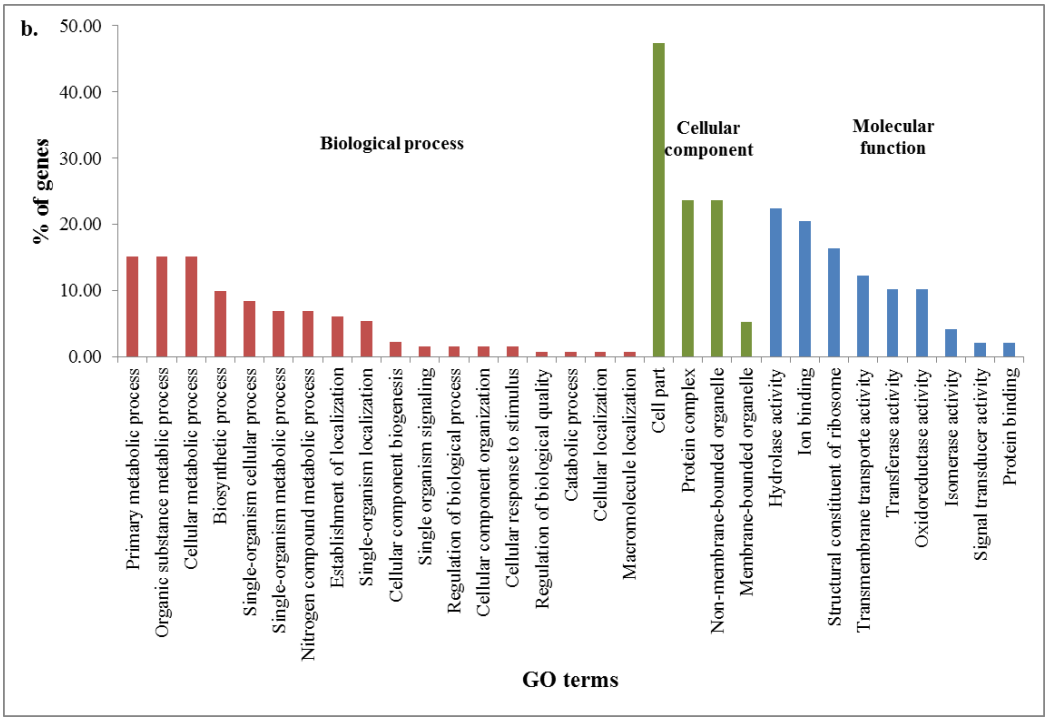


Figure 2.3 KEGG pathway mapping (a) and gene ontology (GO) analysis (b) of the overexpressed unigenes after the inoculation of *Xanthomonas albilineans* in leaf scald resistant sugarcane clone LCP 85-384.

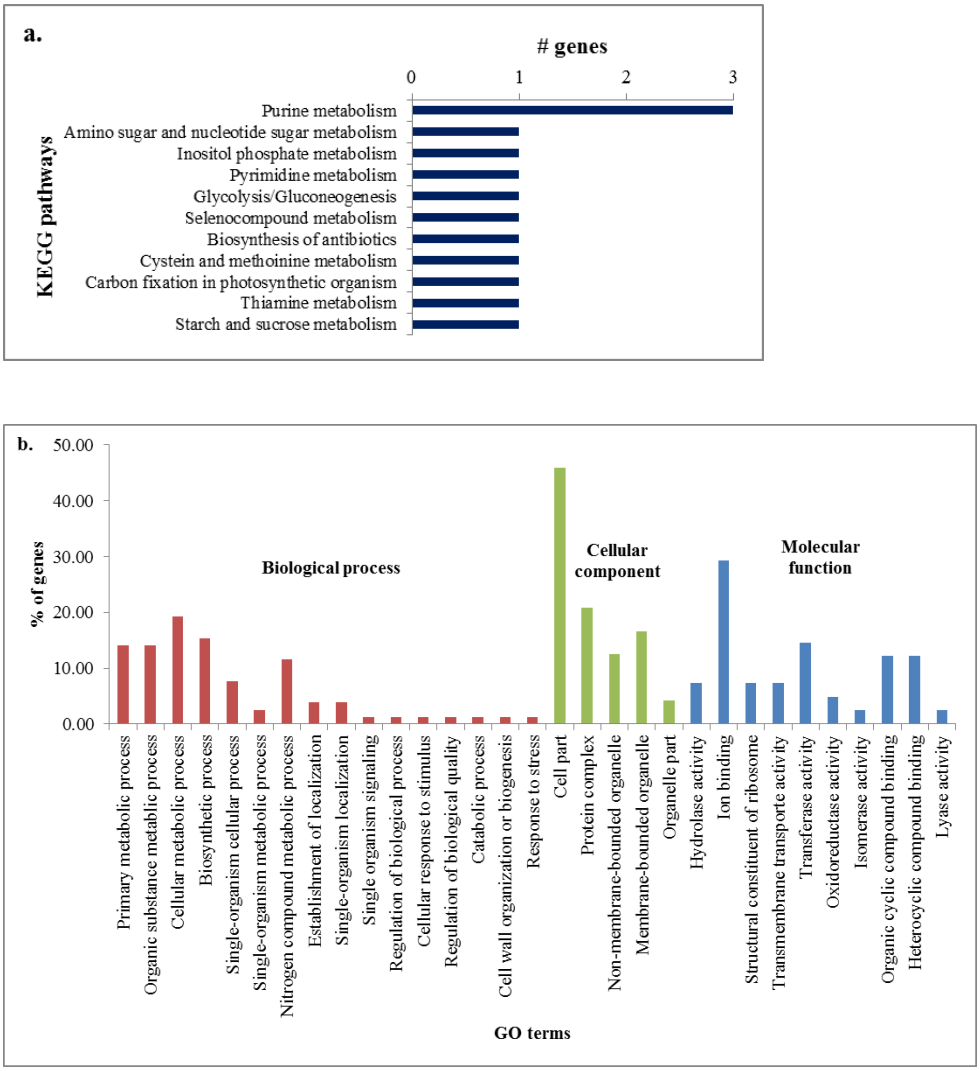


Figure 2.4 KEGG pathway mapping (a) and gene ontology (GO) analysis of the overespressed unigenes after the inoculation of *Xanthomonas albilineans* in leaf scald susceptible sugarcane clone HoCP 89-846.

Gene ontology (GO) analysis showed that the GOslim terms for biological process, molecular function and cellular component were assigned to 54.5, 28.7 and 16.8% of the ESTs, respectively (Figure 2.4b and Table 2.3) with most ESTs having ion binding and transferase activity under molecular function with a low number represented in the plastid (Figure 2.4b). A majority of the ESTs belonged to the transcription factor category involved in ion binding activity (Figure 2.4b). The response to stress and signal transduction, a process in which a signal

is conveyed to trigger a change in the activity or state of a cell, were important within the biological component. The result obtained with KEGG pathway analysis and GOslim terms assignment represent the diversity of the expressed genes in a clone susceptible to leaf scald after inoculation with the causal agent.

2.3.4 Temporal expression of *Xanthomonas albilineans*-responsive genes in resistant and susceptible clones under the pathogen stress

Expression pattern of 17 ESTs representing various biosynthetic pathways were analyzed at different time points in the leaf scald resistant clone LCP 85-384 and the susceptible clone HoCP 89-846 by qRT-PCR. Fifteen of these genes were overexpressed in LCP 85-384 and two were overexpressed in HoCP 89-846, based on the SSH libraries information. Different trends were observed in the expression pattern of the selected genes after the bacterial inoculation to the sugarcane plants. In general, the genes selected from the susceptible clone (HoCP 89-846) SSH library had different expression patterns than the genes selected from the resistant clone (LCP 85-384) SSH library (Figure 2.5). Although variation was observed in the expression of a few genes among the biological replicates, the resistant clone maintained a higher level of gene expression compared to the susceptible clone for genes selected from the resistant clone library and vice versa (Figure 2.5). Genes in the resistant clone responded early to the bacterial inoculation with higher accumulation of their transcripts within 24 or 48 h of stress under pathogen compared to the control. The time point for the highest expression of the genes was variable. For example, the highest expression of adenosine kinase and adenylate kinase was observed at 24 h, Thioredoxin M at 48 h, ATP synthase I subunit at 72 h and E3 ubiquitin protein ligase 1 week after bacterial inoculation (Figure 2.5). For the genes selected from the susceptible clone SSH library, the gene expression induction was observed between 24 to 48 h of

inoculation, with the highest expression at 72 h for the adenosine kinase (different isoform than the one overexpressed in the resistant clone) and 1 week after inoculation for serine/threonine protein ligase (Figure 2.5).

2.3.5 Leaf scald responsive unigenes-derived eSSR-based genetic diversity among sugarcane clones

Different SSR motifs (Figure 2.6a) and SSR motif types (Figure 2.6b) were found in the SSR-containing ESTs of the two sugarcane clones, with prevalence of tetranucleotide motifs (Figure 2.6b). Twenty-two and nine EST-derived SSR (eSSR) primers were designed using the EST sequence information of the LCP 85-384 and HoCP 89-846 SSH libraries, respectively.

Thirty-one eSSRs, derived from the leaf scald responsive unigenes, were tested in different sugarcane clones. Two of the eSSRs (eSSRS31 and eSSRR90) did not amplify and three (eSSRR82, eSSRR95, and eSSRR151A) were monomorphic among the 96 clones used for genotyping. The remaining 26 polymorphic eSSRs generated 120 alleles, ranging from 1 to 16 polymorphic bands per eSSR (Appendix 2). Genetic diversity among the clones was analyzed by UPGMA cluster and principal coordinate analyses (PCoA). The dendrogram obtained from the cluster analysis (Figure 2.7) revealed that a large genetic diversity existed among the commercial sugarcane hybrids and the *S. spontaneum* clones. However, hybrids displayed a narrower range of genetic diversity in comparison to the *S. spontaneum* clones. The phylogram showed a clear separation between the commercial hybrids and the *S. spontaneum* clones (SES234B, SES147B and US56-15-8 located at bottom of Figure 2.7). Three commercial clones (L 08-088, N 27 and L 94-426) were grouped into a small cluster, and the rest of commercial clones were grouped into two major clusters (Figure 2.7). The principal coordinate analysis (PCoA) supported the cluster analysis, where the ancestral *S. spontaneum* clones were clearly separated from the

cultivated sugarcane clones (Figure 2.8). Three coordinates cumulatively explained 51.7% of the total variation (coordinate 1, 2, 3 explaining 45.8%, 3.7% and 2.2% of the variation, respectively).

2.4 DISCUSSION

The complexity of the genome and the absence of a draft genome sequence in sugarcane pose difficulty in the identification of genes associated with traits of agronomic interest (Khan et al. 2013). Suppressive subtractive hybridization (SSH) library allowed identification of the genes differentially expressed in a leaf scald resistant clone (LCP 85-384) and a leaf scald susceptible clone (HoCP 89-846) after inoculation with the causal agent, *Xanthomonas albilineans*. SSH has been preferred over other popular low-scale RNA imaging techniques as cDNAs can be subtracted to identify genes regulated under a specific stress (Khan et al. 2013). The genes overexpressed with GO terms associated with the resistant and susceptible clones showed that genes involved in different biological processes, molecular functions and cellular components were differentially activated in the interaction between the pathogen and the plant. The results also suggested that extensive transcriptional and post-translational remodeling was associated with the plant's response to the pathogen.

2.4.1 Differences between resistant and susceptible clones related to chloroplast genome

A comparison of the genes induced between the susceptible and resistant clones showed differences associated with their response to the disease. An important characteristic of *Xanthomonas albilineans* is the production of an antibiotic phytotoxin called albicidin. Albicidin is a pathogenesis factor with an important role in systemic invasion of the pathogen in host plants (Hashimi et al. 2007). It is a potent DNA gyrase inhibitor that selectively blocks prokaryote

DNA replication and causes the characteristic chlorotic symptoms of leaf scald by blocking chloroplast development (Hashimi et al. 2007). Therefore, an important target to start the comparison among the pathogen-induced genes between two clones with differential disease reactions is the chloroplast. When the number of transcripts induced after *X. albilineans* inoculation were compared between the resistant and susceptible clones, the results showed that 16% of the induced genes were located in the chloroplast (based on the comparison with the *Sorghum bicolor* genome) in the resistant clone and only 5% in the susceptible clone (Figure 2.1 and Figure 2.2, respectively). It is noteworthy to mention that the mapped sorghum genome in the nuclear chromosomes is 654 Mb and the Super 59 contig (where the chloroplast genes were located) is only 620 Kb.

The genes in the chloroplast genome, induced after bacterial inoculation were mainly associated with transmembrane transport, cellular nitrogen compound metabolic process, ion binding and photosynthesis, based on the GO analysis (Table 2.1 and Table 2.2). The overrepresentation of chloroplastic genes in the resistant clone can be attributed to the role of albicidin, an antibiotic that affects the chloroplast development, during the initial steps of the bacterial infection. However, with the limited number of transcripts captured with small-scale sequencing of the SSH library, it was not possible to capture a gene associated with the action mechanism of the toxin, such as *gyrA* (a common target of the quinolones antibiotics) or genes involved in the re-ligation of the cleaved DNA intermediate products during the gyrase and topoisomerases activities (Hashimi et al. 2007).

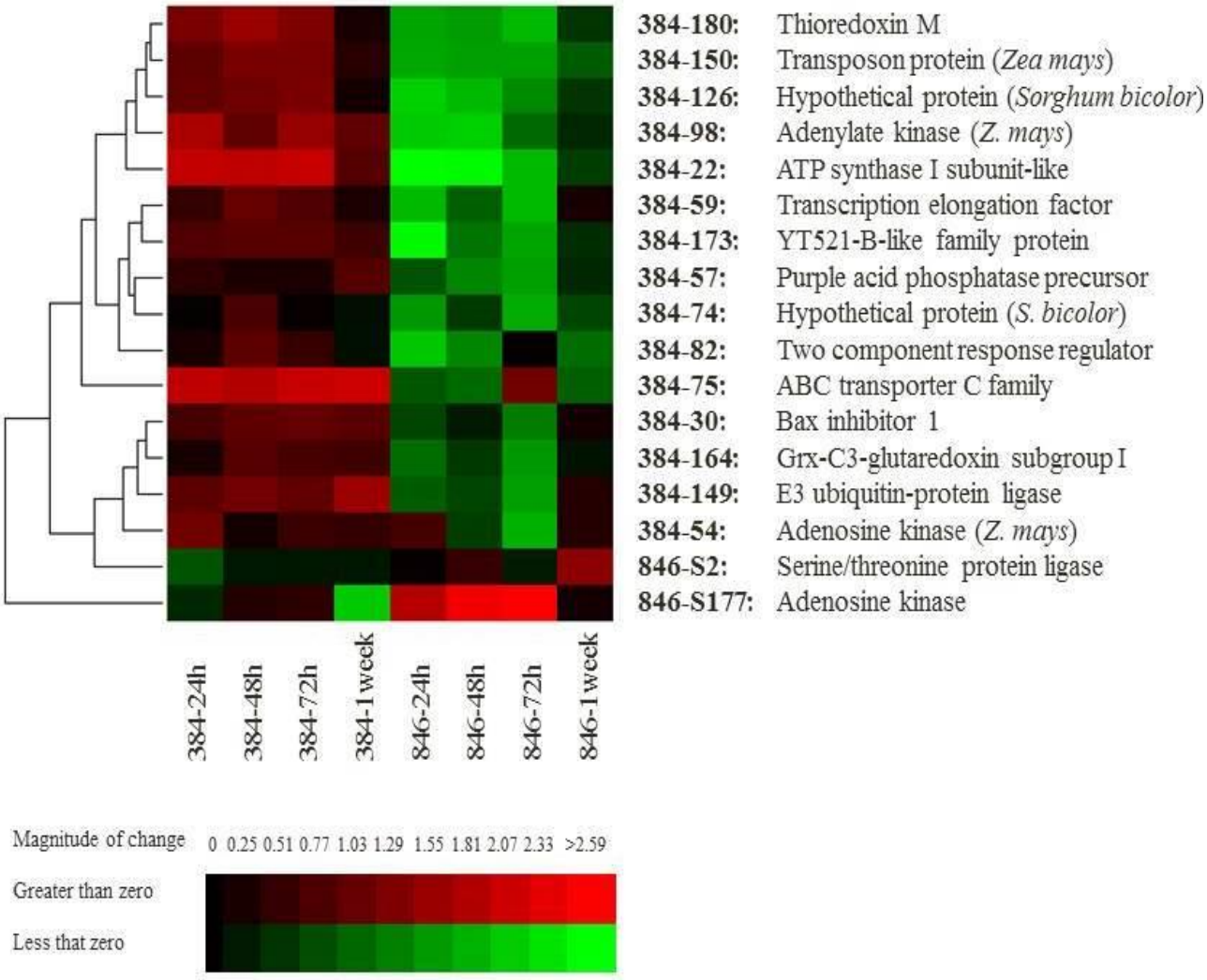


Figure 2.5 Heat map of the temporal expression pattern of 17 selected *Xanthomonas albilineans*-responsive unigenes of the resistant (LCP 85-384) and susceptible (HoCP 89-846) sugarcane clones. Total RNA was extracted from the leaf tissue of both clones collected at 24 h, 48 h, 72 h and 1 week after *X. albilineans* inoculation. qRT-PCR was performed using cDNA from control and inoculated plants. Elongation factor 1α (SoEF1α) was used as the reference gene. Fifteen unigenes were selected from the resistant clone (LCP 85-384) and two from the susceptible clone (HoCP 89-846), they are represented in each row and can be differentiated by the Sequence ID prefix (384 for LCP 85-384 and 846 for HoCP 89-846). In the heat map, each column represents a sample from the clone and time after inoculation: 384 for the resistant clone (LCP 85-384) and 846 for the susceptible clone (HoCP 89-846).

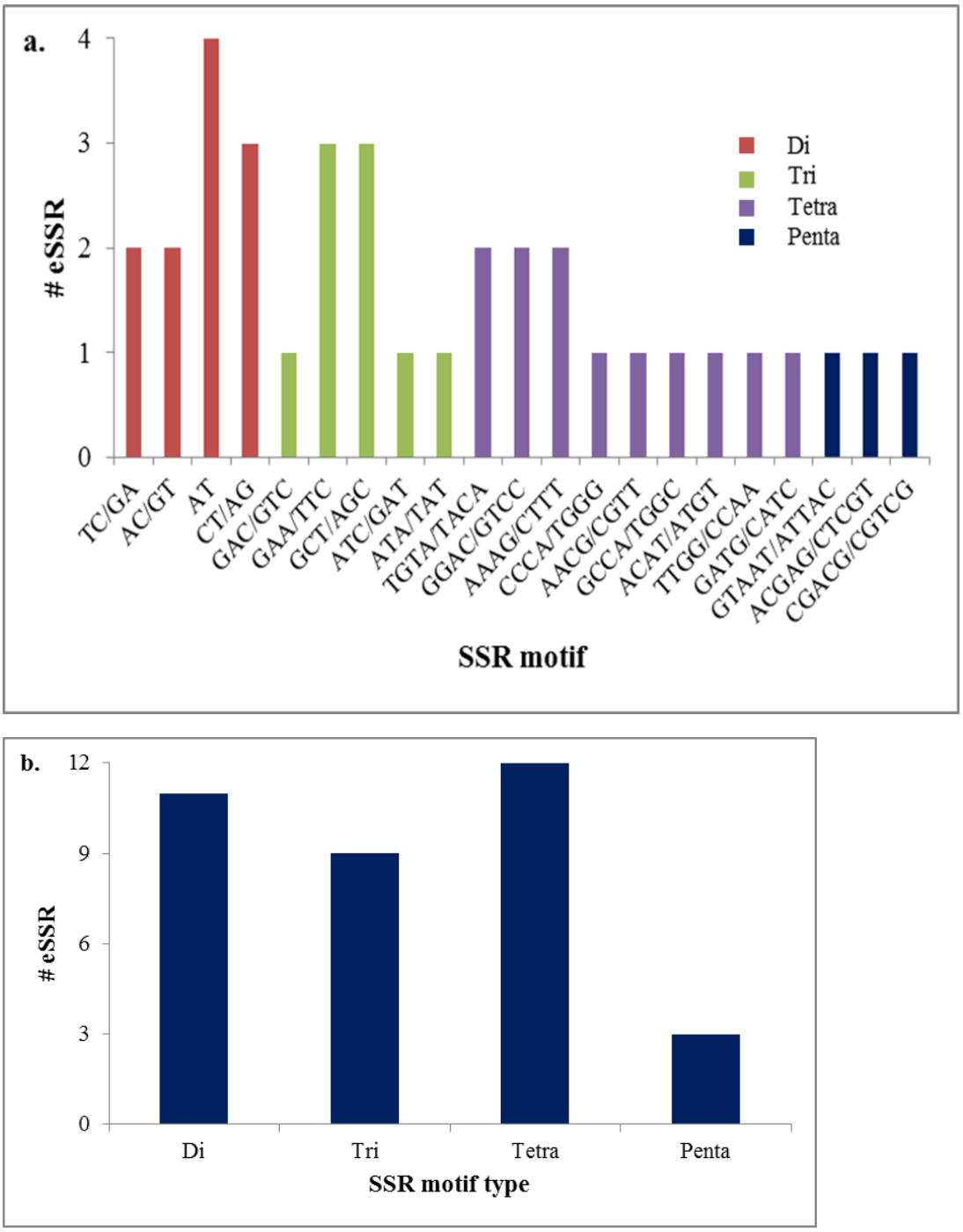


Figure 2.6 SSR motif distribution (**a**) and SSR type frequency (**b**) of the 31 eSSRs detected in the SSH libraries of the clones LCP84-384 (resistant to leaf scald) and HoCP 89-846 (susceptible to leaf scald) after inoculation with *Xanthomonas albilineans*.

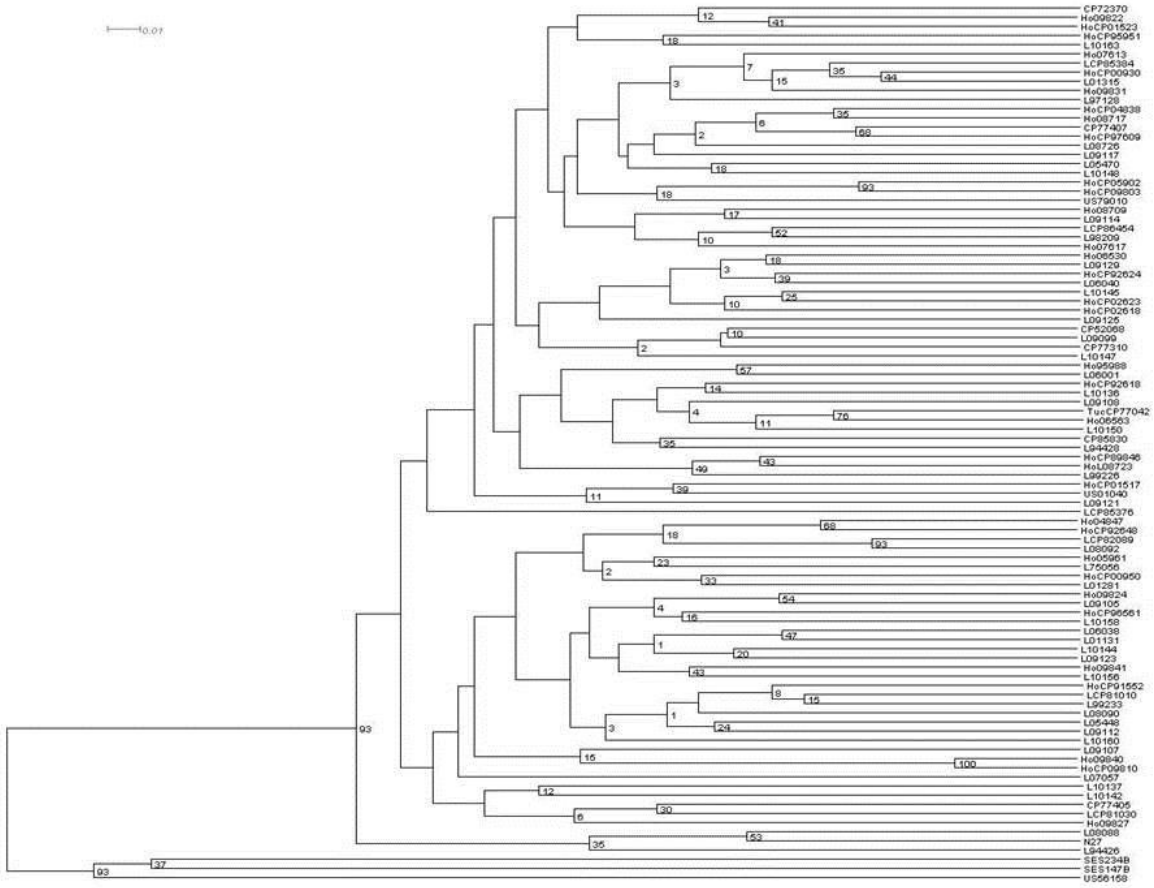


Figure 2.7 Phylogram inferred by the UPGMA method with Dice similarity coefficient showing the cluster pattern of the 93 sugarcane hybrids and three *Saccharum spontaneum* clones based on the allelic diversity at unigenes-derived simple sequence repeat loci (clone details in Appendix 2). Cophenetic correlation coefficient (CP) = 0.78.

In addition to the genes located in the Super 59 contig, other genes located in the nuclear chromosomes were related with functions in the chloroplast. *X. albilineans*-induced *Saccharum officinarum* chloroplastic envelope membrane protein (384-S125) and *Zea mays* endo-1,4-beta-glucanase (384-S147) in the resistant clone SSH library, and *Brachypodium distachyon* pentatricopeptide repeat-containing protein chloroplastic-like (846-S33) and *Saccharum* hybrid ferredoxin-NADP reductase (846-S100) in the susceptible clone SSH library were encoded by nuclear genes with possible role in the chloroplast.

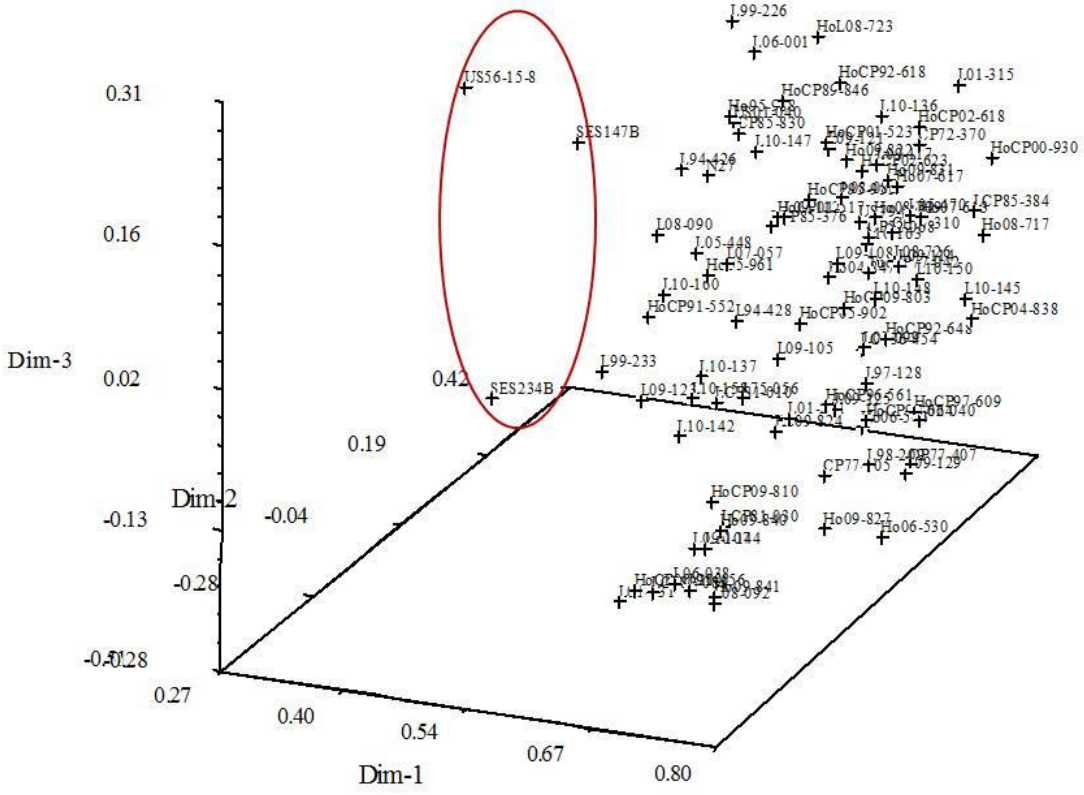


Figure 2.8 Principal coordinate analysis showing clustering of 96 *Saccharum* clones discriminated by the leaf scald responsive gene-derived eSSRs markers. Inside the red oval are the three delimited *Saccharum spontaneum* clones.

2.4.2 Signal transduction and DNA related genes

An important step in the plant's defense response to biotic stress is pathogen perception and signal transduction (Oloriz et al. 2012). In the resistant clone SSH library, transcripts involved in signaling and the gene regulation (transcription factors) were identified (Table 2.2). Based on the similarity with the *Sorghum bicolor* arginine/serine-rich splicing factor SR32 (384-S76) was induced, that protein plays a key role during pre-mRNA processing that leads to alternative splicing by influencing the selection of splice sites through their relative abundance and phosphorylation state (Rauch et al. 2013). Alternative spliced isoforms of several SR

proteins in plants are tissue-specific and/or developmentally regulated in response to environmental stress, such as *X. albilineans* infection (Rauch et al. 2013). Other genes induced in the resistant clone were similar to (1) two-component response regulator-like PRR73 (384-S82) that controls photoperiodic flowering response (a component of the circadian clock), (2) eukaryotic translation initiation factor 2 gamma subunit (384-S127) that catalyzes the first regulated step of protein synthesis initiation, promoting the binding of the initiator tRNA to 40S ribosomal subunits to activate the translation complex, and (3) filamentation temperature-sensitive H 2A (FtsH2A; 384-S133), a protease that is implicated in stress response in plant (Yue et al. 2010).

In the susceptible clone, the genes overexpressed were similar to (1) stress associated chloroplastic heat shock protein 70 (846-S57), a nuclear gene for chloroplast product, (2) splicing factor U2af 38 kDa subunit (846-S98) involved in the splicing of the RNA, and (3) serine/threonine-protein kinase SAPK8 (846-S2), which is known to be activated under hyperosmotic stress or under the abscisic acid (ABA) signal. Another important gene that was activated in the susceptible clone was the serine/threonine protein phosphatase superfamily protein isoform 1 (846-S97), which was previously reported from a SSH library to be associated with brown rust resistance in sugarcane (Oloriz et al. 2012). This protein has been associated with multiple regulatory proteins, which dephosphorylates a number of biological targets, and it is essential for cell division, glycogen metabolism and protein synthesis.

2.4.3 Marker development

The SSH method provides a tool for discovering genes that are differentially expressed during *X. albilineans* – sugarcane interactions. The information of the sequences isolated from

the SSH library was used for the development of molecular markers for future use. EST-derived microsatellite markers (eSSRs) have become markers of choice for the sugarcane scientific community for their ease of use, inexpensive development, and amenability to high throughput operation (Khan et al. 2013). The eSSRs derived from the sugarcane leaf scald responsive genes also had high sequence similarity with sorghum, so these can be used as anchor markers for comparative mapping and could prove useful for marker-assisted selection (Khan et al. 2013). In total, 31 eSSRs were detected and the polymorphism of these markers was tested in a population with 96 *Saccharum* clones (93 commercial clones and three *S. spontaneum* accessions).

In the present study, 84% of the eSSRs were observed to be polymorphic, which is similar to the values that were reported in earlier studies for sugarcane SSR markers (Aitken et al. 2005; Khan et al. 2013). *S. spontaneum* showed the most diversity, which is in agreement with the previous reports of eSSR markers designed from cold-responsive genes (Khan et al. 2013) and TRAP markers (Andru et al. 2012). On the other hand, genetic similarity value was higher among sugarcane hybrids, which validated that only a few parental clones were involved in the development of the foundation clones through nobilization in breeding programs (Alwala et al. 2006). The eSSR markers will be useful to assess genetic diversity and to discriminate between different species of the *Saccharum* complex, and could be used with gSSR and other marker systems for mapping in sugarcane.

In summary, genes involved in diverse biological/cellular/molecular mechanisms were identified through SSH library in sugarcane in response to *X. albilineans* infection. The study also allowed comparison of the difference in the response to the bacterial inoculation between a resistant and a susceptible clone. Genes involved in signal transduction and post-translational modifications were isolated. The difference in the gene expression between both clones was

evident, where a higher percentage of transcripts located in the chloroplast were identified in the resistant clone. The difference in the transcription profile was corroborated using qRT-PCR of selected genes from both libraries. The expression profile showed that the genes overexpressed in one clone were not overexpressed in the other clone in the early hours after the pathogen inoculation. An early accumulation and maintenance of high mRNA concentration in LCP 85-384 could be the determining factor for its leaf scald resistance. A more comprehensive genome-wide comparative transcriptome profiling through next generation sequencing will lead to precise understanding of the *X. albilineans* and sugarcane genes interaction on a global scale. This will also lead to the large-scale identification of candidate resistance gene-networks, which will help in devising strategies to identify target genes for their genetic manipulation to develop leaf scald resistant sugarcane varieties.

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CHAPTER 3: IDENTIFICATION OF GENOMIC REGIONS CONTROLLING LEAF SCALD RESISTANCE IN SUGARCANE USING A BI-PARENTAL MAPPING POPULATION DENSELY ENRICHED WITH SNP MARKERS

3.1 INTRODUCTION

Sugarcane (*Saccharum* spp. hybrids) is a tropical C4 member of the Poaceae family, which accounts for 70% of the raw sugar produced worldwide (Wei et al. 2006; Le Cunff et al. 2008; Andru et al. 2011; Aitken et al. 2014). Cultivated sugarcane is derived from inter-specific hybridizations between two polyploid species *Saccharum officinarum* and *S. spontaneum* with different basic chromosome numbers (Aitken et al. 2014). The hybridization involved the combination of vigorous growth, tolerance to abiotic stresses and disease resistance from *S. spontaneum* with the high sucrose content from *S. officinarum*. In the development of modern cultivars, the initial hybrids were backcrossed with *S. officinarum* to recover high sucrose content. Thus, the modern cultivars are complex aneu-polyploids with chromosome numbers of 2n=100-120 (D'Hont et al. 1998; Aitken et al. 2014), and they contain approximately 80% of *S. officinarum,* 10-15% of *S. spontaneum* and 5-10% recombinant chromosomes (D'Hont et al. 1996). The high ploidy level, the aneuploidy and the cytogenetic complexity have made sugarcane a challenge for breeding, genetics and gene cloning (D'Hont and Glaszmann 2001; Rossi et al. 2003).

Diseases are one of the most important problems that affect sugarcane productivity (Rott et al. 2000). Leaf scald, caused by *Xanthomonas albilineans* (Ashby) Dowson, is one of the major diseases worldwide (Rott et al. 1997; Wang et al. 1999; Rott and Davis 2000). The disease is characterized by chronic and acute phases varying in severity from a white, sharply defined longitudinal leaf stripe to death of shoots or entire plants (Ricaud and Ryan 1989; Rott et al.

1997; Wang et al. 1999). Latent infection can occur, making visual diagnosis problematic (Ricaud and Ryan 1989; Rott et al. 1997). Leaf scald causes high losses in tons of cane per hectare and reduction in juice quality (Ricaud and Ryan 1989; Rott and Davis 2000). The use of hot water treatment and tissue culture to produce healthy seed-cane, disinfection of cutting and harvesting tools with bactericides, and quarantine measures during germplasm exchanges are methods used to prevent and control the disease (Ricaud and Ryan 1989; Rott and Davis 2000).

The development of resistant varieties is considered the best strategy to manage leaf scald in sugarcane. The troublesome aspect of resistance evaluation is that symptom expression is affected by environmental conditions, and some sugarcane cultivars can tolerate the pathogen without exhibiting symptoms (Rott et al. 1997). The erratic symptom expression results in the failure to accurately detect susceptibility and thus multiple inoculations are needed. In addition, inoculation can result in systemic infection of resistant clones (Gutierrez et al. 2016). Under this scenario, the development of molecular markers was considered as a major breakthrough promising to overcome the limitations with phenotypic evaluation (Ruane and Sonnino 2007). The use of DNA markers for genetic analysis and manipulation of agronomic traits has become a useful tool in plant breeding (Zhang et al. 2004). Marker-assisted selection (MAS) technique, which uses marker(s) linked to useful trait(s), is extensively used in improving crop yields and in breeding for resistance against pests and diseases (Manigbas and Villegas 2007).

The large (10 Gb) and complex genome, the absence of a reference genome draft, the coexistence of single and multi-dose alleles, and the irregular number of chromosomes in the homo(eo)logy groups have hindered the progress in the development and application of genetic/genomic tools in sugarcane (Wang et al. 2010). Currently, all sugarcane genetic maps constructed appear incomplete due to the large number of chromosomes and the limited

sequence information available for marker development (Wang et al. 2010). However, with the decrease in the cost of DNA sequencing technologies, it will be possible to produce a higher number of DNA markers (Wang et al. 2010) that will help saturate the available molecular maps, and the information can be used in gene tagging, QTL mapping and map-based cloning (Le Cunff et al. 2008).

The genetic maps developed for sugarcane cultivars, as well as for their ancestral species, are based on populations of full sib (F_1) individuals following a pseudo-test cross strategy using only single dose markers (Grattapaglia and Sederoff 1994). In a bi-parental population, a single dose marker has either a single copy of an allele in one parent segregating in 1:1 (presence: absence) or a single copy of the same allele in both parents segregating in 3:1 (presence: absence). Based on this method, partial genetic maps have been produced for *S. spontaneum* (Da Silva et al. 1993; Ming et al. 1998), *S. officinarum* (Guimaraes et al. 1998; Aitken et al. 2006), interspecific hybrids (Daugrois et al. 1996), and modern cultivars of sugarcane (Hoarau et al. 2001; Andru et al. 2011; Singh et al. 2013; Aitken et al. 2014).

Genetic tools for sugarcane have only recently become adequate to quantify the effect of many genomic regions on a trait (Aljanabi et al. 2007). Earlier studies in sugarcane genetics have reported the association of DNA markers with disease resistance, for example, brown rust (Daugrois et al. 1996) and yellow spot (Aljanabi et al. 2007). For brown rust resistance, the studies conducted by Daugrois et al. (1996) were confirmed in a larger population (Asnaghi et al. 2004) that led to the development of two molecular markers linked to the QTL region associated with brown rust resistance (*Bru*1) (Le Cunff et al. 2008). *Bru*1 provides an example that marker-assisted selection is feasible in sugarcane, and the use of *Sorghum bicolor* genome information is

an important tool in the map saturation process and the identification of possible gene candidates in the QTL regions (Le Cunff et al. 2008).

For the identification of QTLs associated with resistance to leaf scald, a bi-parental population was developed from the cross between two parents with contrasting disease response-resistant cultivar LCP 85-384 (female) and susceptible cultivar L 99-226 (male). Two different kinds of markers were selected for the map construction, SSRs (including those from the leaf scald responsive ESTs) and SNPs (obtained through genotyping by sequencing). With the use of SNP markers and the synteny between sorghum and sugarcane, a comparative genomic analysis was conducted to elucidate the nature of the resistance to leaf scald and pinpoint regions associated with disease resistance.

3.2 MATERIALS AND METHODS

3.2.1 Plant materials

High heterozygosity of the sugarcane clones makes it possible to use a F_1 population as the mapping population (F_1 populations in sugarcane are considered as pseudo F_2 populations). The progeny derived from the cross between a leaf scald resistant clone LCP 85-384 (female) and a susceptible clone L 99-226 (male) was used to develop a linkage map. LCP 85-384 cultivar was selected from the progeny of a cross between CP 77-310 and CP 77-407 (Milligan et al. 1994). The cultivar L 99-226 was selected from the progeny of a cross between HoCP 89-846 and LCP 81-30 (Bischoff et al. 2009). The seedling progeny of the mapping population was germinated in the greenhouse. The seedlings were transplanted to seedling trays after three weeks and the survivor clones in the process went to the field. A random sample of 186 individuals was taken from the population and used in the linkage mapping study. The

population was maintained as clones in field plots. The parents and grandparents were also included to track the origin of markers segregating in the population.

3.2.2 Leaf scald susceptibility evaluation

The population was evaluated in two growing seasons (summer 2014 and 2015) in field trials planted at the LSU AgCenter Sugar Research Station, Saint Gabriel, LA. Phenotypic evaluations were performed in plant cane crops. Bacteria isolation and quantification, and plant inoculation were performed following the protocols previously described (Garces et al. 2014). For inoculation, a bacterial suspension with a concentration of 3.5×10^8 CFU/µL (0.18 OD at 590 nm) was kept at 4°C in the dark prior to inoculation. Plants were inoculated using the decapitation method by placing the bacterial suspension with a sprayer on the surface of the shoot cut above the apical meristem with scissors dipped in the inoculum suspension (Koike 1965). The inoculations were performed at sunset on approximately 15-20 plants per clone. In the summer of 2014, inoculation was performed on June 12. Two inoculations were performed in 2015, in different sugarcane plantings, the first inoculation was performed on May 29 and the second was performed on June 9.

Each trial evaluated 188 different clones (186 F_1 clones and parents). The trials followed a completely randomized layout where each clone represented a single plot of 2.1 - 2.4 m long. Disease severity was evaluated on plant cane according to the type of symptoms observed 8 weeks after inoculation in intact leaves that emerged after the inoculation in 6 to 14 stalks. Visual symptoms were assessed for systemically infected leaves and rated using a 1 to 9 scale where 1-3 was considered to be resistant, 4-6 as moderately susceptible, and 7-9 as highly susceptible. The assessment was performed using the TVD -2 (Top Visible Dewlap) leaf, and

disease severity was evaluated for each clone using the formula: DS = [(1 × NS) + (3 × PL) +(5 × ML) + (7 × N) + (9 × D)]/ T, where NS = number of stalks without symptoms; PL= number of stalks with the TVD -2 leaf exhibiting one or two narrow, white, pencil-line streaks; ML = number of stalks with more than two pencil-line streaks in leaves; N = number of stalks with leaf necrosis or bleaching; D = number of dead stalks or stalks with side shooting; and T = total number of stalks per clone.

In addition to visual symptom evaluation, the bacterial populations were quantified at 8 weeks after inoculation in the TVD -2 leaves using three composite samples (each sample consisted of three leaves of different stalks) per clone. The quantification was performed using a TaqMan qPCR using the protocol as previously described (Garces et al. 2014; Gutierrez et al. 2016).

3.2.3 Phenotypic data analysis

For the QTL analysis, two different sets of phenotypic data were collected: visual rating and bacterial population titer at 8 weeks after inoculation. Both data sets were transformed in order to meet the normal distribution requirement of the analysis. The visual ratings (scale 1-9) were transformed using the Box-Cox transformation with λ values of -1.2 (2014 data), -0.2 (first set of 2015) and 0.1 (second set 2015) using the formula $(y^{\lambda} - 1)/\lambda$ (if $\lambda \neq 0$). The Box-Cox coefficients (λ) were obtained using SAS software v. 9.3 (SAS Institute Inc., Cary, NC). The data after transformation were evaluated using the Shapiro and Wilk test for normality in SAS software v. 9.3 (SAS Institute Inc., Cary, NC). For the bacterial population titer (scale 0 to 10^9) a LOG_{10} transformation was used. Although the transformed data did not meet the normality requirement, the histogram shapes and the Box-Cox normality plots showed a better shape as

compared with the non-transformed data. Using the transformed data, the VARCOMP procedure of SAS software v. 9.3 (SAS Institute Inc., Cary, NC) was used to calculate the broad-sense heritability of the visual evaluation of the leaf scald symptoms.

3.2.4 DNA extraction and SSR genotyping

Young leaf tissue was collected on ice from the clones growing in field plots and stored at -80°C until DNA extraction. Genomic DNA was isolated using the potassium acetate protocol (Dellaporta et al. 1983). DNA concentrations were estimated using Nanodrop 1000 spectrophotometer (Nanodrop, Bethesda, MD) at 260 nm wavelength and the quality was checked using the 260 nm / 280 nm ratio information. DNA samples with values lower than 1.8 were cleaned using an ammonium acetate/ethanol DNA purification protocol (Crouse and Amorese 1987), and the DNA was stored at -20°C until further use. A total of 121 SSR primers from the Sugarcane Microsatellite Consortium (Cordeiro et al. 2000; Pan 2006) and 31 eSSRs developed from the leaf scald suppressive subtractive hybridization cDNA library (described in Chapter II) were used in this study. Fifty ng of genomic DNA was used as the template in PCR reactions in a final volume of 10 µl containing 1X PCR buffer, 2.5 mM $MgCl_2$, 0.2 µM dNTP mix, 0.4 unit of *Taq* DNA polymerase (Promega, Madison, WI) and 0.75 µM of each primer. PCR amplification reactions were conducted on C1000 Touch Thermal Cycler equipped with 384 well block (Bio-Rad, Hercules, CA) with a thermal profile of initial denaturation of 95°C for 5 min, 35 cycles of 95°C for 15 sec, 58°C for 15 sec and 72°C for 1 min, and a final extension of 72°C for 10 min. PCR products were resolved in 13% polyacrylamide gels and run at 350 V for 4 h using 1X Tris-Glycine as running buffer in a HEGS electrophoresis apparatus (Nihon Eido, Tokyo, Japan). The gels were stained using ethidium bromide and visualized and documented in a Kodak GelLogic200 gel documentation system (Carestream, Rochester, NY).

The SSRs and eSSRs amplified fragments were manually scored as '1' for presence and '0' for absence (Andru et al. 2011).

3.2.5 Genotyping by sequencing and SNP markers development

The DNA samples of parents, grandparents and 89 F_1 clones selected based on the disease symptom evaluation in 2014 (36 resistant, 28 moderate resistant, 16 moderate susceptible and 9 susceptible clones; the samples in each disease reaction groups were represented in similar proportions in the original population of 186 progeny) were used for genotyping by sequencing. The presence of inhibitors in the DNA samples was tested indirectly through an enzymatic restriction using 20 U of *Eco*RI (NEB, Ipswich, MA) per 125 ng of DNA and incubating for 3 hours at 37°C. Five hundred ng of DNA (20µL at 25 ng of DNA) of each clone was used for library preparation as per Elshire et al. (2011). Briefly, DNA of each clone was restricted by *ApeKI* enzyme and ligated with adapters for barcoding. Barcoded DNA from parents, grandparents, and 89 progeny were pooled and 96-plex sequenced in a single flow cell on a Illumina HiSeq2500 platform at the Institute of Biotechnology of Cornell University, BRC Genomics Facility (Ithaca, NY). In the absence of the sugarcane reference genome, *Sorghum bicolor* genome, because of its microsynteny with sugarcane (Wang et al. 2010), was used for SNP calling using the Tassel GBS pipeline. After the filtering out the SNP markers with more than 10% of missing data and/or without parent information, a χ^2 test was performed to select the bi-allelic SNPs that segregated in a single dose (SD) manner.

3.2.6 Segregation analysis

Mono- and polymorphic fragments were produced by all the marker systems. In sugarcane, several segregation ratios are possible in the F_1 population. With the assumptions of

polysomic inheritance and absence of segregation distortion, single dose (SD) markers are present only once in the genome and they are expected to segregate in 1:1 (present in one parental genome) and 3:1 (bi-parental single dose). Double dose (DD) markers are present twice in one parental genome, either in an 11:3 ratio (for x = 8) or in a 7:2 ratio (for x = 10) (Da Silva et al. 1993). Each marker was tested against expected segregation ratio using a χ^2 test (df = 1) at 5% error level (type I) for SD or bi-parental SD segregation ratios.

3.2.7 Linkage map construction

Mapping of the SD markers onto linkage groups was done using OneMap v. 2.0-4 package of the R software v.3.1.3 (Margarido et al. 2007). The SSR and eSSR markers were mapped as a dominant marker (presence versus absence). The linkage map construction was performed in two steps following method suggested for genetic mapping in polyploid species (Wu et al. 1992). Only SD markers were used to build the framework map with LOD (Log_{10} of odds) score threshold of 4.0 and a recombination fraction value of 0.40. Genetic distances between markers were computed using the Kosambi mapping function. Linkage groups with significant QTLs with high LOD scores and percentage of phenotypic variance explained (PVE) were selected for saturation. In the saturation process, the markers that were previously discarded but flanking the QTL regions (based on the genome information of the *Sorghum bicolor*) were selected with a less stringent selection (Bonferroni correction was applied in the χ^2 test) for integration into the map. The graphic representation of the linkage groups was performed using the software MapChart v.2.3 (Voorrips 2002) and/or Windows QTL Cartographer Software v.2.5 (Wang et al. 2012).

3.2.8 QTL mapping

QTL mapping was carried out using single marker analysis (SMA), interval mapping (IM) and composite interval mapping (CIM). QTL analysis was performed on the transformed phenotypic data from the three field trials over two crop years, using the Windows QTL Cartographer Software v.2.5 (Wang et al. 2012) and QTL ICIM Mapping Software v.4.1(Wang et al. 2016). To confirm the location of the QTLs, CIM was undertaken with all default settings in Windows QTL Cartographer Software v.2.5 (Wang et al. 2012). A permutation (1,000 iterations) based LOD threshold of 2.5 and a 5% PVE threshold were used as the criteria to declare a QTL significant (Churchill and Doerge 1994).

3.2.9 Search of candidate genes based on sorghum information

Based on the microsynteny between sugarcane and sorghum genomes (Wang et al. 2010), and the information of the sugarcane SNP markers, the location of the single markers and composite interval QTLs were ascertained in the sorghum genome that facilitated the search for the genes flanking the QTL regions. Genes, located within 40-kb surrounding the QTL regions and previously reported to be associated with biotic or abiotic stress responses were compiled as candidate genes associated with the resistance response to leaf scald. These genes will be characterized in subsequent studies.

3.3 RESULTS

3.3.1 Leaf scald screening of the F_1 progeny in the field

Leaf scald reaction of the F_1 population was evaluated 8 weeks after artificial inoculation on plant cane in three different trials (one in 2014 and two in 2015). Two different methods were used for the disease evaluation: the first method was the visual symptom rating (scale 1-9) and

the second was the bacterial population titer in leaf tissue (scale 0-10^9). In both cases, the phenotypic distributions were not normal and skewed to the left. The left skewed distribution was due to the high number of resistant clones present in the F_1 population. Different strategies were used to obtain the normal distribution requirement for ANOVA-based broad sense heritability (H^2) calculation.

For the visual symptom evaluation, the use of the Box-Cox transformation showed low to intermediate correlation among the three field trials (Table 3.1). In contrast, the correlation among the different trials evaluated with the average of the visual symptom evaluation was high (Table 3.1). For H^2 calculation, the transformed data appeared to be normally distributed (Shapiro-Wilk test; p-value = 0.4157, W = 0.9943). In addition, the skewness value was near to zero (0.0860), a good indicator of the transformation effect in the elimination of the left skewness. The heritability in broad sense of the leaf scald reaction (H^2=0.2757), based on the symptom expression, showed a low to medium genetic variance component and a high effect of the environment on the leaf scald symptom expression.

Table 3.1 Pearson correlation among different measures of leaf scald reaction in the field of the progeny of the bi-parental F_1 population of LCP 85-384 x L 99-226.

	Visual symptom rating				**Bacterial population titer**			
Trials	**2014**	**2015a**	**2015b**	**Average**	**2014**	**2015a**	**2015b**	**Average**
2014	1	0.3486	0.2558	0.6665	1	0.2911	0.0012	0.7163
		(0.0009)a	(0.0162)	(<.0001)		(0.0059)	(0.9911)	(<.0001)
2015a		1	0.3865	0.7808		1	0.1461	0.6719
			(0.0002)	(<.0001)			(0.1696)	(<.0001)
2015b			1	0.7133			1	0.5828
				(<.0001)				(<.0001)

a. Values in the parenthesis represent p-values for Pearson correlation.

For the bacterial population titer measured through qPCR, the use of LOG_{10} transformation showed low correlation among the three field trials (Table 3.1). However, the

correlation among the trials with the average of the bacterial population was medium to high (Table 3.1). The heritability calculation was not possible because the data after transformation did not show a near-normal distribution (Shapiro-Wilk test; p-value <0.0001, W = 0.9667). In addition, the skewness value was not close to zero (0.2339), an indicator that the data transformation could not eliminate the left skewness. Other kinds of data transformations were also tested, but the results failed to normalize the data (data not shown). The low to medium correlation among the data sets with both methods of disease reaction evaluation led to the use of all the data sets in the QTL analysis. The QTLs reported in this study (described later in this chapter) were found with at least two of the three field evaluations. The medium (bacterial population titer) to high (visual symptom rating) correlations of the average data with the trials allowed using the average information for the initial QTL mapping with both methods.

3.3.2 SSRs, eSSRs and SNP markers

A total of 332 unambiguous alleles were obtained with genotyping of the F_1 progeny using 121 polymorphic SSR markers. Genotyping using 31 polymorphic eSSR markers resulted in 24 alleles. Of these, 202 SSR (60.8%) and 20 eSSR (83.3%) alleles that segregated as SD markers by χ^2 test were included for linkage mapping. From the genotyping by sequencing of 95 individuals (89 F_1 individuals plus parents and grandparents), a total of 27,260 SNP markers were called using *Sorghum bicolor* genome as the reference. A cleaning process was implemented to select only the bi-allelic markers that are present in the parent(s) and grandparent (s), with less than 10% of missing data and no duplicated information. A total of 5,835 selected markers were tested for allelic dosage using a $\chi 2$ test, and 1,726 (29.6%) were SD markers that were used along with the SD SSR and eSSR markers for linkage mapping.

3.3.3 Linkage map construction

A total of 1,948 SD (SSR, eSSR, and SNP) markers were obtained from the genotyping of the 89 F_1 progeny of the cross between LCP 85-384 x L 99-226. One thousand seven hundred and twenty six (88.6%) of the SD markers were SNP markers generated by the genotyping by sequencing strategy. A simplex framework map was built using 1,146 SD markers, which were assigned to 205 linkage groups (LGs) with the genome coverage of 19,230 cM (Appendix 5, Appendix 6). Of the 205 linkage groups, 95 LGs were assigned to LCP 85-384 (31 LGs with bi-parental 3:1 SD markers exclusively) with a genome length of 2,793 cM by 272 SD markers, and109 LGs were assigned to L 99-226 (31 LGs with bi-parental SD markers exclusively) with a genome length of 4,121 cM by 348 markers (Appendix 5). Also, 32 LGs characterized with SD markers from both parents were obtained with a genome length of 12,880 cM by 593 SD markers (Appendix 5). The length of the LGs varied from 0.0001 cM (LG-90) to 5,217 cM (LG-3) with an average of 93.80 cM per LG and an average distance of 16.78 cM between two adjacent markers. The number of mapped markers per LG varied from 2 to 163 (Appendix 5).

3.3.4 Initial QTL mapping

The phenotypic data of leaf scald reaction obtained through visual symptom evaluation and bacterial population titer were considered as quantitative traits for QTL mapping. QTL mapping was performed using three different strategies: single marker analysis (SMA), interval mapping (IM) and composite interval mapping (CIM). In all the strategies, a putative QTL was called positive when the LOD score (Log_{10} of odds) was higher than 2.5 and the percentage of the phenotypic variance explained (PVE) was higher than 5%. Table 3.2 (for SMA) and Table 3.3 (for IM and CIM) summarize the QTLs found in the initial QTL mapping.

Table 3.2 Summary of the single marker analysis (SMA) for the detection of single dose (SD) markers associated with the leaf scald resistance in LCP 85-384 x L 99-226 F_1 population.

Trait[a]	LG[b]	Position	Marker	LOD[c]	PVE (%)[d]	Add[e]	Dom[f]
Visual	20	0.00	c1_586b	2.5964	12.61	-0.1881	-0.2601
Visual	6	110.88	c3_579	2.4837	11.99	-0.1287	-0.2753
Bacteria	6	110.88	c3_579	3.8923	18.31	-0.2755	-0.8111

a. Trait, Two different methods were used for the disease assessment in the F_1 population used for QTL analysis. "Visual" refers to visual symptom evaluation and "Bacteria" refers to bacterial population titer measured through qPCR.
b. LG, Linkage group.
c. LOD, Logarithm -base 10- of odds score (threshold=2.5, to call a SMA QTL positive).
d. PVE (%), Percentage of phenotypic variance explained by the marker.
e. Add, Estimated additive effect of QTL (of the marker).
f. Dom, Estimated dominant effect of QTL (of the marker).

Table 3.3 Summary of interval mapping and composite interval mapping QTL analysis for the detection of regions associated with the leaf scald resistance in the LCP 85-384 x L 99-226 F_1 population.

Trait[a]	LG[b]	Pos[c]	Left Marker	Right Marker	LOD[d]	PVE (%)[e]	Add[f]	Dom[g]	Left CI[h]	Right CI[h]
Interval Mapping										
Visual	21	291.5	6_4830d	c6_548b	4.9011	11.68	0.1224	-0.4763	288.25	298.25
Visual	35	109.5	10_192a	10_165	4.9808	9.07	0.0393	0.3276	101.25	109.75
Visual	42	8.5	c10_38	10_321a	2.627	10.62	0.0296	0.3997	0	16.75
Visual	44	54.5	5_1527g	5_1527e	8.2299	19.03	-0.7295	-0.2655	53.75	61
Bacteria	3	74.5	CA1172c	1_7232	4.5392	5.27	1.7829	-1.7525	62.75	82.75
Bacteria	3	2151	1_1515d	1_1745a	4.5424	7.85	-1.3817	-1.7751	2143.75	2159.75
Bacteria	6	109.5	2_7637b	c3_579	6.7471	12.56	-0.3441	-1.0454	105.75	110.5
Bacteria	130	86.5	c10_19b	6_6359a	4.5663	10.20	-1.0768	-1.5135	82.25	90.25
Bacteria	167	3.5	3_5544	c4_659a	5.1195	8.88	1.4266	-0.9752	0	12.25
Composite Interval Mapping										
Visual	2	763	3_6381	2_7699a	3.3943	10.61	-0.5841	-0.0355	752.75	766.25
Visual	3	1860.5	CA1602b	SR8-1	2.7549	8.10	0.0749	-0.7322	1824.25	1872.25
Visual	3	3169.5	ci1_713	c1_525a	2.5067	8.14	-0.0608	-0.7263	3156.75	3177.75
Visual	44	54.5	5_1527g	5_1527e	3.9824	2.78	-0.3285	0.0994	47.75	61
Visual	119	321	c6_540a	6_5843a	4.8421	7.84	0.1064	0.7942	312.25	328.25

a. Trait, Two different methods were used for the disease assessment in the F_1 population used for QTL analysis. "Visual" refers to visual symptom evaluation and "Bacteria" refers to bacterial population titer measured through qPCR.
b. LG, Linkage group
c. Pos, The scanning position in cM on the linkage group.
d. LOD, Logarithm -base 10- of odds score (threshold=2.5, to call an IM QTL positive).
e. PVE (%), Percentage of the phenotypic variation explained by QTL at the current scanning position.
f. Add, Estimated additive effect of QTL at the current scanning position.
g. Dom, Estimated dominance effect of QTL at the current scanning position.
h. Left CI and Right CI, Confidence intervals calculated by one-LOD drop from the estimated QTL position.

3.3.5 Saturation of QTL regions

After the initial QTL mapping, nine LGs (3, 6, 20, 21, 35, 42, 44, 130, and 167) showed the presence of QTL regions with high PVE % and high LOD scores. These QTL regions were saturated with the SNPs that were previously not included in the linkage mapping but were found flanking the QTL regions based on the *Sorghum bicolor* genome information. For the integration of the additional SNPs, the Bonferroni correction was applied in the χ^2 test of these markers. Also, the markers that could not be mapped to the *S. bicolor* genome and were located in super contigs were analyzed with the Bonferroni correction for their integration into the map. The Bonferroni correction was not applied previously, during the construction of the framework linkage map, due to the addition of markers with different dosage in the analysis (especially double dose markers). That strategy was followed for the small population size used in the present study for linkage mapping.

The nine LGs with QTLs selected for saturation initially covered a genome length of 6,123 cM with 217 SD markers. The saturation process resulted in 16 LGs that covered a genome length of 15,570 cM with 657 markers (Table 3.4, Figure 3.1), taking into account the LGs with previously mapped SD markers and/or LGs with QTLs.

3.3.6 Final QTL mapping

A second round of QTL analysis was performed after the saturation process using SMA (Table 3.5), IM and CIM (Table 3.6). The results showed the location of new markers by SMA analysis and the QTLs flanked with markers added in the saturation process. However, some of the QTLs, such as the QTL region located in the LG 44 (IM, Table 3.6) and LG 35 (CIM, Table 3.6) could not be fine resolved after the saturation attempt.

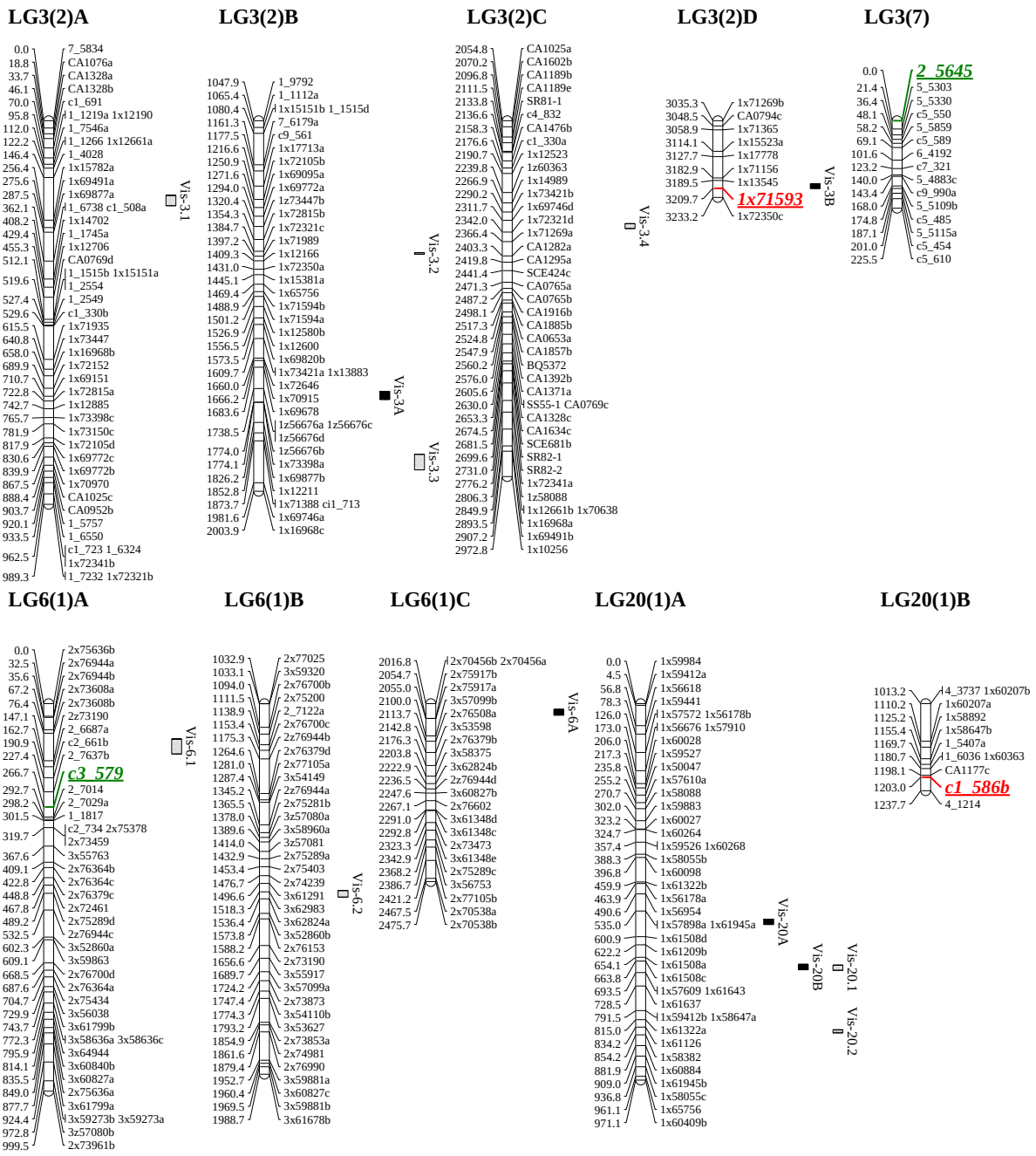
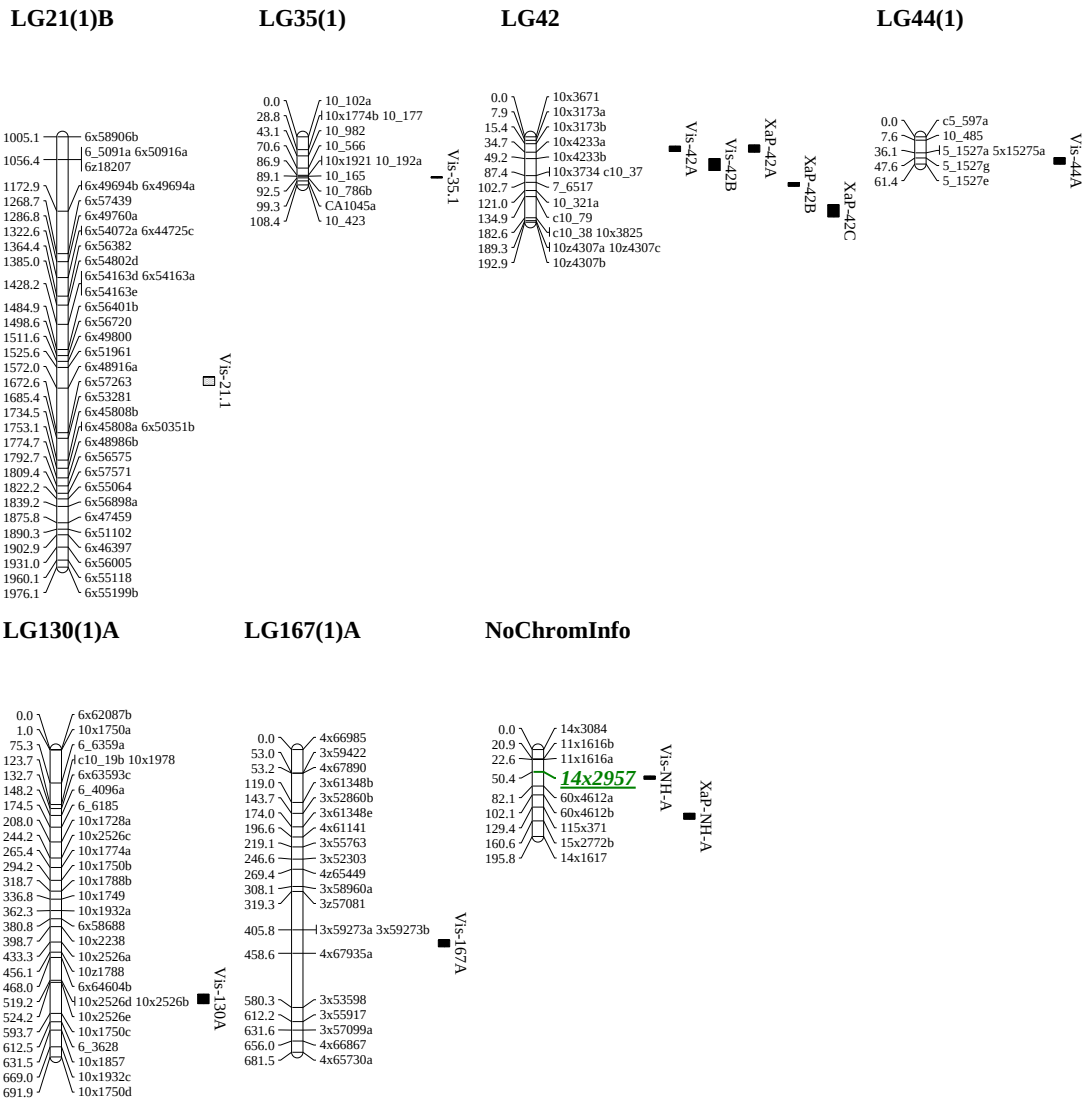


Figure 3.1 Genetic map of sugarcane cultivars LCP 85-384 and L 99-226 after the saturation process of the QTL regions detected in the initial screening. Bonferroni correction was used in the χ^2 test for the detection of single dose markers flanking the QTL regions. For the detection of markers and regions associated with leaf scald resistance were used three different QTL strategies. Markers associated with leaf scald resistance using the visual evaluation as phenotypic trait (red) and the bacterial populations (green) were detected using single marker analysis. Multiple regions were found using interval mapping (solid black bars on the right side of each linkage group (LG) and composite interval mapping (hatched bars on the right side of each LG) QTL analysis. The QTL names show if they were detected using the visual evaluation (Vis) or the bacterial population (Xa) data.



(Figure 3.1 Continued)

3.3.7 Candidate genes in QTL regions

Based on the synteny between *Sorghum bicolor* and sugarcane, the genes located within and neighboring the QTL regions were identified in the *S. bicolor* genome. The selection of the regions was based on the distance between the flanking markers. The QTL regions that could not be selected were because of the prohibitive distance between the flanking markers or the location of the flanking markers on different chromosomes. The genes that were identified within and

around the QTLs located in LG 3, 6, and 44 belonged to transcription factors, DNA binding proteins, helicases, transcription activators, pathogen induced compatible 1 (PIC; Sb03g034810.1) and genes associated with resistance to *Pseudomonas syringae* pv. maculicola (Sb05g008340.1 and Sb05g008350.1, chromosome 5 of *S. bicolor*) (Table 3.7). In addition, neighboring mRNAs and ESTs of the markers detected in the single marker analysis and the markers common in different QTLs included genes associated with nucleic acid binding, helicases, exonucleases, pathogen induced 1 (PI1), pathogen-infected compatible 1 (PIC1) and a cDNA overexpressed after salicylic acid-treatment in seedlings (Sb01g035130.1) (Table 3.8). The identification of ESTs such as pathogen induced 1 (PI1) and pathogen-infected compatible 1 (PIC1) that are known to be associated with plant-pathogen interaction suggested that the QTL analysis and the use of the microsynteny between *S. bicolor* and *Saccharum* spp could be a valuable tool in sugarcane research.

3.4 DISCUSSION

Erratic symptom expression, the association between environment and symptomatology, the possibility that some sugarcane cultivars can tolerate the pathogen without exhibiting symptoms (Rott et al. 1997), and occasional systemic infection of inoculated resistant clones (Gutierrez et al. 2016) have made leaf scald resistance evaluation a difficult task. The evaluation based on symptom expression has been the standard method for assessment of the disease response in different sugarcane clones; however, the problems associated with leaf scald evaluation suggested the need for new methods for the disease evaluation. A qPCR method for quantification of bacterial populations titer was described previously that showed good correlation with the visual symptom evaluation (Garces et al. 2014; Gutierrez et al. 2016). Both methods were used in the present study for the evaluation of the disease response in a F_1

population progeny of the cross between a leaf scald resistant parent (LCP 85-384) and a susceptible parent (L 99-226). The population phenotype distribution was skewed for broad sense heritability (H^2) calculation (based on ANOVA test), and hence the data were transformed to obtain normal distribution.

The results showed that the bacterial population data, after different transformation strategies, did not meet the normal distribution requirement for the ANOVA test. This could be due to the qPCR design that had a detection limit of 3.5×10^3 CFU/µL (Garces et al. 2014), and it is possible that the bacterial populations at 8 weeks after inoculation in different resistant clones were below the threshold level of detection. So, the qPCR may have failed to detect the small differences in the bacterial titer present in highly resistant clones, which resulted in the skewed data that could not achieve normal distribution even after the data transformation. In contrast, the visual symptom evaluation data could differentiate the resistant clones after data transformation (Box-Cox transformation), and the normality requirement was met for the broad sense heritability calculation by ANOVA. Using the transformed data of the visual symptom evaluation, the broad sense heritability for leaf scald resistance obtained in this study (H^2=0.2757) was similar to other sugarcane diseases, such as red rot (h^2=0.19 to 0.31; Yin et al. 1996) and smut (h^2=0.41 ± 0.08 for plant cane; Chao et al. 1990). The low to moderate H^2 value obtained in the present study was due to the erratic symptom expression of the disease, latency, and the influence of the environment on symptom expression.

Linkage mapping in sugarcane requires a large number of progeny and markers in comparison with diploid plants (Andru et al. 2011). In the present study, the genotyping by sequencing method by next generation sequencing produced a large number of markers that allowed the use of a small population for constructing linkage maps, which were comparable

with previously reported sugarcane linkage maps (Hoarau et al. 2001; Andru et al. 2011). The low number of markers decreases the reliability of estimating useful genetic distances between the markers (Andru et al. 2011). This problem was circumvented by using the *Sorghum bicolor* genome information and the synteny between *S. bicolor* and *Saccharum* spp. (Wang et al. 2010, Aitken et al. 2014), which resulted in the development of a reliable and informative linkage map. The exclusive use of SD markers for the construction of the framework linkage map, followed by the saturation process with SD and DD dose markers (added after Bonferroni correction), ensured high reliability in estimating genetic distances (Andru et al. 2011).

Table 3.4 Linkage groups obtained after the saturation of the QTL regions.

LG[a] final	Length (cM)	Markers final[b]	Markers added[c]	3:1 (both parents)	1:1 (LCP 85-384)	1:1 (L99-226)	1:1:1 :1	Chrom in sorghum[d]
3.1	302.40	25	5	12	6	7	0	1
3.2	3233.17	136	71	50	44	41	1	1, 7, 9
3.4	246.69	21	5	2	10	9	0	1
3.7	225.47	15	0	9	4	2	0	5, 2, 6, 7
3.8	140.07	11	0	2	4	5	0	3
3.11	82.37	6	0	2	1	3	0	7
6.1	2475.71	99	90	45	34	19	1	2, 3
20.1	1237.67	53	47	17	18	18	0	1, 4
21.1	3071.46	120	112	43	31	46	0	6, 2
21.2	12.83	2	0	1	0	1	0	3
35.1	108.35	11	2	0	0	11	0	10
42.1	192.89	15	10	0	0	0	0	10, 7
44.1	61.40	6	1	1	5	0	0	5, 10
130.1	1215.14	37	31	16	8	12	0	6, 10
167.2	2768.89	91	90	37	29	23	2	3, 4
NM[e]	195.82	9	9	6	2	0	1	ND[f]
Total	15570.34	657	473	243	196	197	5	All except 8

a. LG, Linkage group. The name of the linkage group was based on the original LG prior ro the saturation and a consecutive number.
b. Markers final, Number of markers present in the linkage group.
c. Markers added, Markers added in the saturation process after the Bonferroni correction in the χ^2 test
d. Chrom in sorghum, Based on the SNP information (*Sorghum bicolor* genome was used for the SNP calling), the linkage groups were discriminated using the SNPs contained in each LG.
e. NM, Not mapped on the *Sorghum bicolor* genome
f. ND, No data. No SNPs markers on the LG, so it was not possible to assign a chromosome in sorghum to the LG.

Table 3.5 Summary of the single marker analysis after the saturation process for the detection of single dose markers associated with leaf scald resistance in the LCP 85-384 x L 99-226 F_1 population.

Trait[a]	LG[b]	Position	Marker	LOD[c]	PVE (%)[d]	Add[e]	Dom[f]
Visual	3.2	3209.69	1x71593	3.0453	14.65	-0.0738	-0.3497
Bacteria	3.7	0	2_5645	2.0375	10.03	-0.0682	0.6505
Bacteria	6.1	266.72	c3_579	3.7189	17.56	-0.5928	-0.4387
Visual	20.1	1203.01	c1_586b	3.013	14.48	-0.0342	-0.3921
Bacteria	NM	50.44	14x2957	2.8566	13.80	-1.303	-1.1466

a. Trait, Two different methods were used for the disease assessment in the F_1 population used for QTL analysis. "Visual" refers to visual symptom evaluation and "Bacteria" refers to bacterial populations measured through qPCR.
b. LG, Linkage group
c. LOD, Logarithm -base 10- of odds score.
d. PVE (%), Percentage of phenotypic variance explained by the marker.
e. Add, Estimated additive effect of QTL of the marker.
f. Dom, Estimated dominant effect of QTL of the marker.

In linkage map construction, the LOD scores and the recombination frequency threshold generally determine the number of LGs present in the map (Andru et al. 2011). In sugarcane, LOD scores ≥3.0 and recombination frequency values between 0.25 and 0.45 have been commonly used (Da Silva et al. 1993; Grivet et al. 1996; Alwala et al. 2008; Andru et al. 2011), although the maximum detectable recombination generally depends on the size of the mapping population (Andru et al. 2011). In the present study, a maximum recombination frequency value of 0.40 and LOD score values ≥ 4.0 were used to avoid false linkages. The high number of unlinked markers and short LGs with less than four markers per LG resulted in unsaturation in the final linkage map, which could be due to the small population used (Andru et al. 2011). Similarly, the long distance between some markers and the presence of long LGs (LG 3, for example), despite the use of LOD scores and recombination thresholds similar to previously reported linkage map studies (Da Silva et al. 1993; Grivet et al. 1996; Alwala et al. 2008; Andru et al. 2011), was possibly due to the small population size. However, the use of *S. bicolor* genomic information extrapolated with the markers was important to overcome these limitations, and the comparative analysis in the QTL mapping showed important regions that can be associated with leaf scald resistance in sugarcane.

Table 3.6 Summary of the interval mapping and composite interval mapping QTL analysis after the saturation process for the detection of regions associated with the leaf scald resistance in the LCP 85-384 x L 99-226 F_1 population.

Trait[a]	LG[b]	Pos[c]	Left Marker	Right Marker	LOD[d]	PVE (%)[e]	Add[f]	Dom[g]	Left CI[h]	Right CI[h]
Interval Mapping										
Visual	3.2	1755	1z56676d	1z56676b	2.5677	11.58	-0.2179	0.385	1745.75	1766.75
Visual	3.2	3204.5	1x13545	1x71593	3.4202	13.29	-0.0886	-0.4915	3198.25	3209.75
Visual	6.1	2039.5	2x70456a	2x75917b	3.5764	6.65	-0.111	0.7975	2031.75	2046.25
Visual	20.1	562.4	1x61945a	1x61508d	3.6602	10.86	-0.5282	-0.0348	555.661	569.458
Visual	20.1	679.7	1x61508c	1x57609	3.6713	8.00	-0.1009	0.6745	672.433	686.529
Visual	42.1	26.6	10x3173b	10x4233a	3.2177	6.27	-0.44	0.13	21.45	32.9501
Visual	42.1	66.3	10x4233b	10x3734	2.5377	7.59	0.1137	0.7485	49.7498	76.0494
Bacteria	42.1	31.5	10x3173b	10x4233a	2.7955	2.46	-1.8086	-1.4754	18.85	34.35
Bacteria	42.1	106.3	7_6517	10_321a	3.1217	3.19	-1.4305	-1.4772	103.349	111.349
Bacteria	42.1	168.5	c10_79	c10_38	4.85	4.21	1.4692	-1.1141	153.35	180.352
Visual	44.1	54.8	5_1527g	5_1527e	3.9372	18.62	0.2784	0.117	47.5498	61.2996
Visual	130.1	558.5	10x2526e	10x1750c	2.8359	5.24	-0.5821	-0.0551	550.75	571.75
Visual	167.2	436.3	3x59273b	4x67935a	3.3905	5.90	0.24	0.8485	428.667	443.668
Visual	NM	65.9	14x2957	60x4612a	2.8708	11.27	-0.134	-0.7936	60.0497	66.4496
Bacteria	NM	58.2	14x2957	60x4612a	5.9117	4.44	-1.3874	-1.5255	51.8498	75.7494
Composite Interval Mapping										
Visual	3.2	210	1_4028	1x15782a	3.1258	7.80	-0.5752	-0.0396	192.25	219.25
Visual	3.2	1390	1x72321c	1x71989	2.6335	6.40	-0.5455	0.0035	1388.25	1392.25
Visual	3.2	1929	ci1_713	1x69746a	2.8246	7.36	-0.5552	-0.0196	1908.25	1948.25
Visual	3.2	2327	1x69746d	1x72321d	2.5526	6.01	-0.1079	-0.7307	2320.25	2332.75
Visual	6.1	107.5	2x73608b	2z73190	2.5936	9.78	-0.1109	0.7879	91.25	129.75
Visual	6.1	1527.5	3x62983	3x62824a	2.5716	7.34	-0.0611	0.6357	1514.75	1531.75
Visual	6.1	2039.5	2x70456a	2x75917b	3.5764	9.22	-0.111	0.7975	2031.75	2046.25
Visual	20.1	681.9	1x61508c	1x57609	2.8295	10.91	-0.0084	0.7048	674.332	687.929
Visual	20.1	845.1	1x61126	1x58382	2.5681	11.77	-0.1559	-0.6542	840.992	849.19
Visual	21.1	1558.2	6x51961	6x48916a	3.0903	8.70	-0.0063	-0.7202	1546.92	1565.91
Visual	35.1	90.5	10-165	10-786b	2.6475	12.58	-0.4651	-0.1224	90.4492	92.4492

a. Trait, Two different methods were used for the disease assessment in the F_1 population used for QTL analysis. "Visual" refers to visual symptom evaluation and "Bacteria" refers to bacterial populations measured through qPCR.
b. LG, Linkage group
c. Pos, The scanning position in cM on the Linkage Group.
d. LOD, Logarithm -base 10- of odds score (threshold=2.5, to call an IM QTL positive).
e. PVE (%), Percentage of the phenotypic variation explained by QTL at the current scanning position.
f. Add, Estimated additive effect of QTL at the current scanning position.
g. Dom, Estimated dominance effect of QTL at the current scanning position.
h. Left CI and Right CI: Confidence interval calculated by one-LOD drop from the estimated QTL position.

After the QTL analysis, multiple genomic regions and markers were identified to be associated with leaf scald resistance in the present study. The saturation process, focused on QTL regions controlling leaf scald response, allowed to reduce the gap between the markers flanking

some of the QTLs. Also, the LG (NM; 195.82 cM) that was formed after saturation with nine markers, which did not map to *S. bicolor* genome and were located in SuperContigs, contained one marker and two QTLs associated with leaf scald resistance (Table 3.5 and Table 3.6).

Comparative analysis with *S. bicolor* genome was performed for the markers and the QTLs with a narrow distance between the flanking markers. The analysis showed that some of those QTLs contain or are surrounded by ESTs and mRNAs previously known to be responsive to other diseases and/or sequences associated with transcriptional activation. The location of genes previously associated with disease response near to the QTLs supports the robustness of the methodologies used for the disease evaluation, and linkage and QTL mapping. In addition, the *S. bicolor* genomic information accounted to each SNP marker helped to overcome the limitations associated with the small population used in the mapping process and the high environmental influence in the symptom expression of the disease.

The neighboring ESTs and genes identified in this study are valuable resources for subsequent analysis of allelic polymorphism and gene expression that can enhance our knowledge of the nature of leaf scald resistance in sugarcane. The QTL markers such as c3_579 (LG 6, 17.56% PVE for bacteria population), 1x71593 (LG 3, 14.65% PVE for visual symptom evaluation) and c1_586b (LG 20, 14.48% PVE of visual disease evaluation), and QTLs flanked by 5_1527g and 5_1527e (LG 44, 18.62 % PVE for visual symptom evaluation) will serve as the starting point for subsequent analysis because of the high value of PVE and the information on the neighboring ESTs and mRNAs that are associated with disease resistance. Validation of the markers identified in this study needs to be conducted on larger population and diverse germplasm and allele-specific markers need to be developed for their use in breeding programs.

Table 3.7 Expressed sequence tags (ESTs) and/or mRNAs within and neighboring the QTL regions controlling leaf scald resistance in sugarcane.

LG[a]	Left Marker				Right Marker				Sb-JGI mRNA[e]	
	Marker	Type[b]	Ch[c]	Position[d]	Marker	Type[b]	Ch[c]	Position[d]	Locus[f]	Description[g]
6.1	2x73608b	D1.13	2	73608702	2z73190	D2.18	2	73190930	Sb02g039195.1	DNA binding / DNA-directed RNA polymerase/ nucleic acid binding / transcription regulator/ zinc ion binding
									Sb02g039290.1	Zinc finger (FYVE type) family protein
									Sb02g039300.1	DNA binding / transcription factor
6.1	3x62983	D1.13	3	62983640	3x62824a	B3.7	3	62824456	Sb03g034670.1	Transcription factor
									95005061	Pathogen-induced: compatible
									Sb03g034780.1	Ethylene responsive element binding factor 1; DNA binding / transcription activator/ transcription factor
									Sb03g034810.1	Vascular plant one zinc finger protein; transcription activator (Sb-EST 18067043 PIC1)
3.2	1x72321c	D2.18	1	72321199	1x71989	D2.15	1	71989743	Sb01g049020.1	Agamous-like 20; transcription factor
									Sb01g049070.1	DEAD/DEAH box helicase, putative (RH10) (Sb-EST 18067525 PIC1)
									Sb01g049110.1	ATP-dependent helicase/ double-stranded RNA binding / protein binding / ribonuclease III (Sb-EST 18061110 PIC1)
									Sb01g049180.1	(TOM THREE HOMOLOG 1); virion binding (Sb-EST 9852932 PI1)
									Sb01g049260.1	Pentatricopeptide (PPR) repeat-containing protein
									18065578	Pathogen-infected compatible 1 (PIC1)

(Table 3.7. Continued)

LG[a]	Left Marker				Right Marker				Sb-JGI mRNA[e]	
	Marker	Type[b]	Ch[c]	Position[d]	Marker	Type[b]	Ch[c]	Position[d]	Locus[f]	Description[g]
44.1	5_1527g	D1.10	5	15275658	5_1527e	D1.10	5	15275602	Sb05g008340.1	RESISTANCE TO P. SYRINGAE PV MACULICOLA 1; nucleotide binding / protein binding (Sb-EST 9849959 PI1)
									Sb05g008350.1	RESISTANCE TO P. SYRINGAE PV MACULICOLA 1; nucleotide binding / protein binding (Sb-EST 9308565 PI1)

a. LG, Linkage group (after the saturation of QTL regions)
b. Type, Marker type according to Wu et al. 2002 notation.
c. Ch, Chromosome in *Sorghum bicolor* genome
d. Position, Position (bp) in the chromosome of *S. bicolor*.
e. Sb-JGI mRNA, *Sorghum bicolor*-Joint Genome Institute mRNA
f. Locus, Locus of the mRNA reported. Number without Sb prefix corresponds to the Sequence ID of an EST
g. Description, Description of the mRNA or EST sequence. Some mRNAs have in parenthesis the Sequence ID and description of the EST located in the same position.

Table 3.8 Expressed sequence tags (ESTs) and mRNAs flanking the markers associated with leaf scald resistance in sugarcane and identified by the single marker analysis.

Marker	Type[a]	Chrom[b]	Position[c]	Sb-JGI mRNA[d]	
				Locus[e]	Description[f]
c3_579	C.8	3	57957141	18069453	Pathogen-infected compatible 1 (PIC1)
1x71593	D1.13	1	71593863	Sb01g048630.1	(glucan synthase-like 12); 1,3-beta-glucan synthase/ transferase, transferring glycosyl groups (Sb-EST 18064092 PIC1)
2_5645	B3.7	2	56458139	Sb02g023060.1	Nucleic acid binding / protein binding / zinc ion binding
c1_586b	C.8	1	58647793	Sb01g035130.1	Beta-adaptin, putative (Sb-EST 31332215)
1x69746d	D1.10	1	69746895	Sb01g046585.1	3-5 exonuclease domain-containing protein / helicase and RNase D C-terminal domain-containing protein / HRDC domain-containing protein (Sb-EST 9851334 PI1)
1x72321d	D2.15	1	72321229	Sb01g049310.1	Acetyl-CoA C-acetyltransferase/ hydroxymethylglutaryl-CoA synthase (Sb-EST 9306869 PI1)
2x70456a	B3.7	2	70456040	Sb02g036040.1	S-locus lectin protein kinase family protein (Sb-EST 18062857 PIC1)
1x61945a	D1.10	1	61945200	Sb01g038410.1	Protein phosphatase 2C, putative / PP2C, putative (Sb-EST 18063952 PIC1)
10x4233b	D2.15	10	4233034	Sb10g004760.1	Leucine-rich repeat transmembrane protein kinase

a. Type, Marker type according to the Wu et al. 2002 notation.
b. Chrom, Chromosome in *Sorghum bicolor* genome
c. Position, Position (bp) in the chromosome of *S. bicolor*.
d. Sb-JGI mRNA, *Sorghum bicolor*-Joint Genome Institute mRNA
e. Locus: Locus of the mRNA reported. Number without Sb prefix corresponds to Sequence ID of an EST
f. Description, Description of the mRNA or EST sequence. Some mRNAs have in parenthesis the Sequence ID and description of the EST located in the same position

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CHAPTER 4: GENERAL CONCLUSIONS AND PROSPECTS FOR FUTURE STUDIES

4.1 GENERAL CONCLUSIONS

- The present study identified genes involved in diverse biological/cellular/molecular mechanisms in sugarcane in response to *Xanthomonas albilineans* infection through a suppression subtractive (SSH) library, which allowed comparison of the difference in response between a resistant clone and a susceptible clone to the disease. Genes involved in signal transduction and post-translational modifications were over-represented.
- Differences in the transcription profile of leaf scald responsive genes between resistant and susceptible clones were observed.
- A higher percentage of genes located in the chloroplast genome and/or related with chloroplast function were identified in the cDNA-SSH library of the resistant cultivar LCP 85-384 as compared with the cDNA-SSH library of the susceptible cultivar HoCP 89-846 after the inoculation with *X. albilineans*.
- The expression profile showed that the genes overexpressed in one clone were not overexpressed in the other clone during the early hours after pathogen inoculation. The early accumulation and maintenance of high mRNA concentration in LCP 85-384 could be the determining factor for its leaf scald resistance.
- The eSSR markers, designed from the transcript sequence information of cDNA-SSH libraries, were able to differentiate sugarcane clones from *Saccharum spontaneum* accessions. The eSSR markers can be used to assess genetic diversity and discriminate among different species of the *Saccharum* complex, and for genetic mapping in sugarcane.

- The qPCR failed to differentiate clones that were highly resistant to leaf scald in the F_1 progeny of a bi-parental cross between a highly resistant clone (LCP 85-384) and a susceptible clone (L 99-226). That negatively affected the ability to calculate the broad sense heritability (H^2) of the bacterial population titer data of the progeny, even after data transformation. In contrast, the visual evaluation data could differentiate the resistant clones and after the data transformation the normal distribution requirement was met for H^2 calculation. The broad sense heritability ($H^2 = 0.2757$) obtained for leaf scald resistance was similar to other sugarcane diseases, such as red rot or smut. The low to moderate H^2 value can be attributed to the disease characteristics, such as the erratic symptom expression and latency.
- The genetic analysis identified multiple markers and QTL regions associated with leaf scald resistance. The saturation process to fine resolve the QTLs controlling leaf scald response reduced the gap between the flanking markers in most cases.
- Comparative analysis using the synteny between *Saccharum* spp. and *Sorghum bicolor* showed that some of the QTL regions contain or are flanked by ESTs and mRNAs previously known to be associated with the response to other diseases in *S. bicolor* and/or sequences associated with transcriptional activation.

4.2 PROSPECTS FOR FUTURE RESEARCH

The expression profile analysis using cDNA-SSH libraries was the first step towards identifying differentially expressing genes in response to leaf scald pathogen. Considering the limitation of low transcript coverage by SSH, genome-wide co-expression networks need to be established through next generation sequencing of the transcriptome of resistant as well as susceptible clones. Validation of the networks through protein-protein interaction and transgenic

overexpression/knock down will provide a comprehensive understanding of the resistance response of sugarcane against the leaf scald pathogen, which ultimately will provide better clues to devising strategies to breed leaf scald resistant sugarcane varieties.

The ESTs and mRNAs within and neighboring the QTL regions are important outcomes of the present study and will serve as a powerful foundation for subsequent analysis of polymorphism and gene expression studies that can further elucidate the nature of the resistance to leaf scald in sugarcane. The QTL markers, such as c3_579 (LG 6, 17.56% of PVE of bacterial population titer), 1x71593 (LG 3, 14.65% PVE of visual disease symptom evaluation), c1_586b (LG 20, 14.48% PVE of visual disease symptom evaluation) and the QTL flanked by 5_1527g and 5_1527e (LG 44, 18.62 % PVE visual disease evaluation) need to be pursued in further fine mapping analysis because the high value of phenotypic variance explained by these regions. The markers identified through single marker analysis and closely linked to QTL through (composite) interval mapping need to be validated in a larger and diverse population and allele-specific markers need to be developed for their use in breeding. The ESTs and mRNAs within and neighboring the QTLs need to be functionally validated to ascertain their role in leaf scald resistance.

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APPENDIX 1. List of the *Saccharum* spp. clones used in the genetic diversity evaluation using the leaf scald responsive genes-derived simple sequence repeat markers (eSSRs).

Order	Clone ID	Species
1	CP72-370	*Saccharum* hydrid
2	CP52-068	*Saccharum* hydrid
3	CP77-310	*Saccharum* hydrid
4	L10-147	*Saccharum* hydrid
5	L05-448	*Saccharum* hydrid
6	L09-112	*Saccharum* hydrid
7	Ho09-822	*Saccharum* hydrid
8	HoCP01-523	*Saccharum* hydrid
9	Ho95-988	*Saccharum* hydrid
10	L06-001	*Saccharum* hydrid
11	HoCP95-951	*Saccharum* hydrid
12	HoCP92-618	*Saccharum* hydrid
13	L10-136	*Saccharum* hydrid
14	L09-108	*Saccharum* hydrid
15	CP85-830	*Saccharum* hydrid
16	L94-428	*Saccharum* hydrid
17	LCP85-376	*Saccharum* hydrid
18	HoCP 89-846	*Saccharum* hydrid
19	L99-226	*Saccharum* hydrid
20	HoL08-723	*Saccharum* hydrid
21	Ho04-847	*Saccharum* hydrid
22	HoCP92-648	*Saccharum* hydrid
23	LCP82-089	*Saccharum* hydrid
24	L08-092	*Saccharum* hydrid
25	LCP86-454	*Saccharum* hydrid
26	L98-209	*Saccharum* hydrid
27	Ho07-617	*Saccharum* hydrid
28	Ho05-961	*Saccharum* hydrid
29	L75-056	*Saccharum* hydrid
30	HoCP00-950	*Saccharum* hydrid
31	L01-281	*Saccharum* hydrid
32	L09-099	*Saccharum* hydrid
33	L09-107	*Saccharum* hydrid
34	Ho09-840	*Saccharum* hydrid
35	HoCP09-810	*Saccharum* hydrid
36	Ho08-709	*Saccharum* hydrid
37	L09-114	*Saccharum* hydrid
38	L08-088	*Saccharum* hydrid
39	N27	*Saccharum* hydrid
40	HoCP96-561	*Saccharum* hydrid

(APPENDIX 1: Continued)

Order	Clone ID	Species
41	L07-057	*Saccharum* hydrid
42	Ho09-824	*Saccharum* hydrid
43	L09-105	*Saccharum* hydrid
44	L06-038	*Saccharum* hydrid
45	L01-131	*Saccharum* hydrid
46	L99-233	*Saccharum* hydrid
47	LCP81-010	*Saccharum* hydrid
48	L10-158	*Saccharum* hydrid
49	L10-144	*Saccharum* hydrid
50	L09-123	*Saccharum* hydrid
51	Ho09-841	*Saccharum* hydrid
52	L10-156	*Saccharum* hydrid
53	L10-160	*Saccharum* hydrid
54	HoCP91-552	*Saccharum* hydrid
55	L08-090	*Saccharum* hydrid
56	L10-137	*Saccharum* hydrid
57	Ho06-530	*Saccharum* hydrid
58	L09-129	*Saccharum* hydrid
59	HoCP92-624	*Saccharum* hydrid
60	L06-040	*Saccharum* hydrid
61	L10-145	*Saccharum* hydrid
62	HoCP02-623	*Saccharum* hydrid
63	HoCP02-618	*Saccharum* hydrid
64	TucCP77-042	*Saccharum* hydrid
65	Ho06-563	*Saccharum* hydrid
66	L10-150	*Saccharum* hydrid
67	L09-125	*Saccharum* hydrid
68	Ho07-613	*Saccharum* hydrid
69	LCP 85-384	*Saccharum* hydrid
70	HoCP00-930	*Saccharum* hydrid
71	L01-315	*Saccharum* hydrid
72	Ho09-831	*Saccharum* hydrid
73	L97-128	*Saccharum* hydrid
74	HoCP04-838	*Saccharum* hydrid
75	Ho08-717	*Saccharum* hydrid
76	CP77-407	*Saccharum* hydrid
77	HoCP97-609	*Saccharum* hydrid
78	L10-163	*Saccharum* hydrid
79	L05-470	*Saccharum* hydrid
80	L10-148	*Saccharum* hydrid
81	L08-726	*Saccharum* hydrid
82	L09-117	*Saccharum* hydrid
83	L10-142	*Saccharum* hydrid
84	CP77-405	*Saccharum* hydrid

(APPENDIX 1: Continued)

Order	Clone ID	Species
85	LCP81-030	*Saccharum* hydrid
86	Ho09-827	*Saccharum* hydrid
87	HoCP01-517	*Saccharum* hydrid
88	US01-040	*Saccharum* hydrid
89	L09-121	*Saccharum* hydrid
90	HoCP05-902	*Saccharum* hydrid
91	HoCP09-803	*Saccharum* hydrid
92	US79-010	*Saccharum* hydrid
93	L94-426	*Saccharum* hydrid
94	SES234B	*Saccharum spontaneum*
95	SES147B	*Saccharum spontaneum*
96	US56-15-8	*Saccharum spontaneum*

APPENDIX 2. EST-SSRs primers sequences developed from the SSH libraries information and used for the genetic diversity evaluation of *Saccharum* spp.

Seq ID[a]	SSR motif	Reps[b]	SSR name	Poly/total[c]	eSSR primer name	Primer sequence (5' - 3')
846-S31	TC	5x	SSRS31	No amp	SSRS31-F	CCGCGAAAAGTGCTAAGACG
					SSRS31-R	ACCTTGTTTTCTGGGTGGCA
846-S35	CT	5x	SSRS35	6 / 9	SSRS35-F	CGTGTTTCTGTGTTGTGCCC
					SSRS35-R	ACAATAGTTCAGTAGCAGAGTATGA
846-S49	TGTA	3x	SSRS49	13 / 16	SSRS49-F	GAATAACAGCAGCCAAGCAA
					SSRS49-R	ACCATTTCATCTTGGTTTCTAC
846-S53	GAC	4x	SSRS53	6 / 10	SSRS53-F	CATGCAGCAGACACACGTTC
					SSRS53-R	GGTCACCTTCTTGGACGACG
846-S55	GGAC	3x	SSRS55	5 / 9	SSRS55-F	CCATGGCCTTGGAAGAAAT
	AAAG	3x			SSRS55-R	TTAGAGGGAGGAGCAGGGAC
846-S65	CCCA	3x	SSRS65	3 / 17	SSRS65-F	GACCAGCACCAAACCCTGAT
					SSRS65-R	CGGCAACTGCGATTCCAAC
846-S71	AC	6x	SSRS71	2 / 4	SSRS71-F	AGCACAGGTTGGTTCACAAGA
					SSRS71-R	TTCTCCCTTCCGCACAAAGT
846-S125	GTAAT	3x	SSRS125	7 / 9	SSRS125-F	GCTCGGGGTTGGTCTGATTT
					SSRS125-R	TGCATTACAAGCACAAAGGCA
846-S148	GAA	8x	SSRS148	2 / 10	SSRS148-F	CGCGAGCGGTACTGAAAAGA
					SSRS148-R	GTGGACGAGCAAGGCAGTAA
846-S149	GCT	4x	SSRS149	1 / 3	SSRS149-F	TCCCCGTGTGACCAATGAAG
					SSRS149-R	TGCTGGGCATCCAATCTAGC
846-S191	TTC	4x	SSRS191	8 / 12	SSRS191-F	GGCGACCCAAATTGAAGGAC
					SSRS191-R	GCCTCCACGATCACCAAGAA
846-S128	ACGAG	3x	SSRS128A	16 / 20	SSRS128A-F	ATACATGACCACGAGTAGCCC
	CGACG	3x			SSRS128A-R	CCCTACTACTGAACGGACGG
846-S128	TGTA	3x	SSRS128B	9 / 17	SSRS128B-F	GCCCTACTACTGAACGGACG
					SSRS128B-R	TGACCACGAGTAGCCCTTTG
846-S132	ACAT	4x	SSRS132A	5 / 10	SSRS132A-F	CTCCCATAAGTATATGTATCGCAAC
					SSRS132A-R	CATGCATGAAGGAGACGTGC

(APPENDIX 2. Continued)

Seq ID[a]	SSR motif	Reps[b]	SSR name	Poly/total[c]	eSSR primer name	Primer sequence (5' - 3')
846-S55	ATA	7x	SSRS55A	2 / 4	SSRS55A-F	CTGCTGCTGGTGGGGAAAATA
					SSRS55A-R	AGTCAGTCGGTCAGTGGATG
846-S55	GGAC	3x	SSRS55B	6 / 11	SSRS55B-F	TAGCTTCTTCTGCTGCTGCT
	AAAG	3x			SSRS55B-R	ATTGGATTGGGTGGGAGGGA
846-S55	TTGG	3x	SSRS55C	6 / 12	SSRS55C-F	AGCTAGGTGCTCCCGAATTT
	GATG	3x			SSRS55C-R	CCATCCATGGCGCAAAAGAG
846-S191	TTC	4x	SSRS191A	1 / 2	SSRS191A-F	CTACCCGGAGAAGGTCAACG
					SSRS191A-R	GTCCTTCAATTTGGGTCGCC
384-S8	GCT	4x	SSRR8	1 / 2	SSRR8-F	GAGTGGTGATGTTGGGCGTA
					SSRR8-R	TGCCAGCCACCTCAAGTATC
384-S19	AT	6x	SSRR19	3 / 4	SSRR19-F	CATTGGCAGTGCTTCAGAGC
					SSRR19-R	GCGTTTTGCTCGAGGTTCAG
384-S48	CA	5x	SSRR48	3 / 6	SSRR48-F	ACACCCGCATACATGAGCA
					SSRR48-R	TGGTAACAGGAGGAGCAGCA
384-S81	GCT	4x	SSRR81	2 / 3	SSRR81-F	CCAGAAGAGGAGAGCATCGC
					SSRR81-R	CGAGACATCGAAGCATAGAGGA
384-S82	GA	7x	SSRR82	0 / 2	SSRR82-F	CCACCATCTGTGAACTCCCT
					SSRR82-R	TGCATCAGCTCCAACCTCAT
384-S90	AT	9x	SSRR90	No amp	SSRR90-F	TGGCATGGTGTAATTGAT
					SSRR90-R	GAACCAATTATATATTCGT
384-S95	AACG	3x	SSRR95	0 / 1	SSRR95-F	AGGATAAGGTAGCGGCGAGA
					SSRR95-R	ACGAACGAGTTGAACAATGAAGA
384-S116	ATC	4x	SSRR116	2 / 6	SSRR116-F	CCTGCCTCTGGCTTCTTAGG
					SSRR116-R	GCTGATGGGAAGGAGCAGAA
384-S131	GCCA	3x	SSRR131	1 / 2	SSRR131-F	AATGGTTGGAGGCAGTTGGG
					SSRR131-R	CCACTCCACCTGAACCATCAA
384-S151	AT	5x	SSRR151	5 / 8	SSRR151-F	CTGCTCGACTTGCGGACATA
					SSRR151-R	GGTTTTCCGAGCATGAGTGC
384-S163	GAA	4x	SSRR163	3 / 5	SSRR163-F	GACACAAGCATCCTCAGCCT
					SSRR163-R	GGCATTGATCTTGCCAACCC
384-S82	CT	6x	SSRR82A	2 / 5	SSRR82A-F	GATTTTGAGCGTCGCAGAGC

(APPENDIX 2. Continued)

Seq ID[a]	SSR motif	Reps[b]	SSR name	Poly/total[c]	eSSR primer name	Primer sequence (5' - 3')
					SSRR82A-R	AAGCGCTACCCATTAGCCTG
384-S151	AT	5x	SSRR151A	0 / 3	SSRR151A-F	CTCCCAAGGATGTTGCTGCT
					SSRR151A-R	TTCCCACCTTGTTTCCTTGAA

a. **Seq ID,** Name used in the different analysis performed for the sequence with the SSR motif

b. **Reps,** Number of repetitions of the SSR motif in the sequence.

c. **Poly/total**, Ratio between the number of polymorphic alleles (poly) and the total number of bands (total) observed in the population.

APPENDIX 3. Functional annotation of differentially expressed genes in the leaf scald resistant sugarcane clone LCP 85-384 in response to *Xanthomonas albilineans* infection.

Seq ID	BLASTn-based similarity to genes	Accession number
384-S1	*Zea mays* chaperone DNA J2	NM_001136845.1
384-S2	*Zea mays* cycloartenol-C-24-methyltransferase 1 Mrna	EU961712.1
384-S3	*Sorghum bicolor* cultivar BTx623 chloroplast, complete genome	EF115542.1
384-S4	No significant similarity found.	
384-S5	No significant similarity found	
384-S6	*Phyllostachys edulis* cDNA clone: bphyem210n24, full insert sequence	FP093091.1
384-S7	*Sorghum bicolor* hypothetical protein	XM_002451994.1
384-S8	*Brachypodium distachyon* magnesium-chelatase subunit chlD, chloroplastic-like (LOC100824575)	XM_003563892.1
384-S9	*Sorghum bicolor* hypothetical protein	XM_002450134.1
384-S10	*Eleusine coracana* 16S ribosomal RNA gene; plastid	HQ183502.1
384-S11	*Zea mays* aspartic proteinase oryzasin-1 (LOC100284362)	NM_001157257.1
384-S12	*Zea mays* formate dehydrogenase 1	EU967680.1
384-S13	No significant similarity found.	
384-S14	*Hemerocallis littorea* ribosomal protein S12 (rps12) gene, partial cds; ribosomal protein S7 (rps7) and NADH dehydrogenase subunit B (ndhB) genes, complete cds; and tRNA-Leu (trnL) gene; chloroplast genes for chloroplast products	AY147480.1
384-S15	*Zea mays* ribulose-phosphate 3-epimerase	NM_001155113.1
384-S16	*Miscanthus sinensis* voucher YDK2009819 AtpF gene; atpF-atpH intergenic spacer; and AtpH gene; chloroplast	HQ599894.1
384-S17	*Zea mays* uncharacterized LOC100382178	NM_001174938.1
384-S19	No significant similarity found.	
384-S20	*Sorghum bicolor* hypothetical protein, mRNA Sequence	XM_002462820.1
384-S21	*Zea mays* 40S ribosomal protein S13	EU977066.1
384-S22	*Saccharum arundinaceum* ATP synthase I subunit-like	EU071786.1
384-S24	*Zea mays* hypothetical protein (LOC100273166)	NM_001147612.1
384-S25	*Medicago truncatula* Kinase-START (MTR_5g027200)	XM_003612587.1
384-S26	No significant similarity found.	
384-S27	No significant similarity found.	
384-S28	*Zea mays* mRNA sequence	EU976030.1
384-S29	*Zea mays* 40S ribosomal protein S24	EU961447.1
384-S30	*Zea mays* bax inhibitor 1	EU963304.1
384-S31	*Zea mays* eukaryotic translation initiation factor 3 subunit 5	EU960037.1
384-S32	*Zea mays* full-length cDNA clone ZM_BFb0234H04	BT067202.1

(APPENDIX 3. Continued)

Seq ID	BLASTn-based similarity to genes	Accession number
384-S33	*Zea mays* clone 316348	EU968184.1
384-S34	*Zea mays* 60S ribosomal protein L37a	EU962243.1
384-S36	*Zea mays* hypothetical protein (LOC100382076)	NM_001174839.1
384-S37	*Zea mays* alkaline/neutral invertase	EU955523.1
384-S38	*Zea mays* LOC100284086 (cl44852_1b)	NM_001156984.1
384-S39	*Zea mays* glycerol 3-phosphate permease (LOC100281074)	NM_001153993.1
384-S40	*Zea mays* protein HOTHEAD (LOC100281523)	NM_001154441.1
384-S42	*Saccharum* hybrid chloroplast, complete genome	AE009947.2
384-S43	No significant similarity found.	
384-S44	*Sorghum bicolor* chloroplast, complete genome	EF115542.1
384-S45	*Sorghum bicolor* hypothetical protein (SORBIDRAFT_1138s002030)	XM_002488920.1
384-S46	*Zea mays* PCO081749	AY105545.1
384-S48	No significant similarity found.	
384-S49	*Saccharum* hybrid cultivar GT28 putative ATP citrate lyase	JQ923438.1
384-S51	*Sorghum bicolor* hypothetical protein	XM_002462332.1
384-S54	*Zea mays* adenosine kinase, putative	AJ012281.1
384-S55	*Zea mays* coproporphyrinogen III oxidase (cpx2)	NM_001195846.1
384-S56	*Sorghum halepense* voucher HCCN-PJ008548-PB260 ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene; chloroplast	KC164342.1
384-S57	*Zea mays* purple acid phosphatase precursor	EU970070.1
384-S58	*Panicum virgatum* cultivar Kanlow chloroplast, complete genome	HQ731441.1
384-S59	*Zea mays* transcription elongation factor 1	EU973809.1
384-S61	*Zea mays* histone H3	EU959087.1
384-S62	*Zea mays* diacylglycerol kinase 1	EF088691.1
384-S63	*Saccharum* hybrid cultivar NCo 310 chloroplast DNA, complete genome Sequence	AP006714.1
384-S64	*Brachypodium distachyon* K(+) efflux antiporter 2, chloroplastic-like (LOC100824553)	XM_003579464.1
384-S65	*Zea mays* 40S ribosomal protein S3a (LOC100282717)	NM_001155624.1
384-S66	*Zea mays* fumarylacetoacetase	EU959609.1
384-S67	*Saccharum* hybrid chloroplast, complete genome	AE009947.2
384-S68	*Saccharum* hybrid O-methyltransferase-like protein gene, promoter region	GU062719.1
384-S70	*Zea mays* beta-5 tubulin (TUBB5)	NM_001111988.1
384-S72	*Zea mays* LOC100276317 (pco137802(98))	NM_001150133.1
384-S73	*Zea mays* ferredoxin-dependent glutamate synthase1 (fgs1)	NM_001112223.1
384-S75	*Brachypodium distachyon* ABC transporter C family member 4-like (LOC100831839)	XM_003567625.1
384-S76	*Sorghum bicolor* arginine/serine-rich splicing factor SR32 transcript I (SR32), SR32I allele, alternatively spliced	KC425089.1
384-S77	*Zea mays* clone Contig452	BT016619.1

(APPENDIX 3. Continued)

Seq ID	BLASTn-based similarity to genes	Accession number
384-S78	*Saccharum* hybrid cultivar 23S ribosomal RNA gene, partial sequence; tRNA-Ala (trnA) gene; hypothetical protein gene, complete cds; ACR pseudogene, complete sequence; tRNA-Ile (trnI) gene, partial sequence; and ORF72 pseudogene, partial sequence; chloroplast genes for chloroplast products	AY082604.1
384-S79	*Zea mays* LOC100277035 (pco133003)	NM_001150706.1
384-S80	No significant similarity found.	
384-S81	*Sorghum bicolor* hypothetical protein	XM_002448139.1
384-S82	*Zea mays* two-component response regulator-like PRR73	EU952116.1
384-S83	*Sorghum bicolor* hypothetical protein	XM_002467035.1
384-S85	No significant similarity found.	
384-S86	No significant similarity found.	
384-S87	Maize mitochondrial ATP-alpha gene encoding F1-ATPase alpha subunit	M16222.1|MZEMTATP
384-S90	*Sorghum bicolor* hypothetical protein	XM_002441800.1
384-S91	No significant similarity found.	
384-S96	*Sorghum bicolor* CBL-interacting protein kinase 21	FJ901210.1
384-S97	No significant similarity found.	
384-S98	*Zea mays* adenylate kinase	EU955541.1
384-S99	No significant similarity found.	
384-S100	No significant similarity found.	
384-S101	*Saccharum officinarum* chloroplast DNA, trnK intron region	AB732019.1
384-S102	*Zea mays* nascent polypeptide-associated complex alpha subunit-like protein mRNA	EU961468.1
384-S103	*Zea mays* PCO087457	AY104465.1
384-S104	No significant similarity found.	
384-S105	*Zea mays* threonine endopeptidase	EU962752.1
384-S106	No significant similarity found.	
384-S107	No significant similarity found.	
384-S108	Maize chloroplast genes for ribosomal proteins L14, S8 and L16 partial	X06734.1L
384-S110	Saccharum hybrid cultivar SP-80-3280 chloroplast, complete genome	AE009947.2
384-S111	No significant similarity found.	
384-S113	*Zea mays* autophagy-related protein 8 precursor	EU958456.1
384-S114	No significant similarity found.	
384-S115	Maize chloroplast photosystem I ps1A1 and ps1A2 genes	M11203.1|MZECPPSI
384-S116	*Zea mays* histone deacetylase102 (hdt102)	NM_001112161.1
384-S118	No significant similarity found.	
384-S119	*Zea mays* histidine-containing phosphotransfer protein 1	EU963994.1
384-S120	*Panicum virgatum* cultivar Kanlow chloroplast, complete genome	HQ731441.1

(APPENDIX 3. Continued)

Seq ID	BLASTn-based similarity to genes	Accession number
384-S121	*Zea mays* speckle-type POZ protein	EU966879.1
384-S122	Maize chloroplast ribosomal protein S12 gene, exons 2 and 3, and ribosomal protein S7 gene	M17841.1|MZECPRPS2
384-S123	*Solanum lycopersicum* BMS1 homolog, ribosome assembly protein (yeast) (BMS1)	XM_004230065.1
384-S124	*Saccharum* hybrid cultivar SP-80-3280 chloroplast, complete genome	AE009947.2
384-S125	*Saccharum officinarum* chloroplast gene for envelope membrane protein, partial cds, strain: Badila, clone: OBa-59kb	AP007033.1
384-S126	*Sorghum bicolor* hypothetical protein	XM_002454822.1
384-S127	*Zea mays* eukaryotic translation initiation factor 2 gamma subunit	EU966655.1
384-S128	*Sorghum bicolor* hypothetical protein	XM_002467739.1
384-S129	*Zea mays* signal peptide peptidase-like 2B	EU961679.1
384-S130	PREDICTED: *Brachypodium distachyon* citrate synthase, glyoxysomal-like (LOC100838601)	XM_003571799.1
384-S131	*Medicago truncatula* Katanin p60 ATPase-containing subunit A-like protein (MTR_1g088750)	XM_003591508.1
384-S133	*Zea mays* FtsH6 - Zea mays FtsH protease	EU961437.1
384-S134	*Zea mays* RING finger protein 5	EU976282.1
384-S135	*Zea mays* peroxisomal fatty acid beta-oxidation multifunctional protein	EU957569.1
384-S136	*Zea mays* glyoxysomal fatty acid beta-oxidation multifunctional protein MFP-a (LOC100285945)	NM_001158834.1
384-S137	*Zea mays* clone 7838 mRNA sequence	DQ244464.1
384-S138	*Sorghum bicolor* hypothetical protein	XM_002438859.1
384-S141	Maize chloroplast phosphorylation coupling factor alpha subunit (atpA) and proteolipid subunit (atpH) genes	M27557.1| MZECPATPC
384-S143	*Brachypodium distachyon* ribosome biogenesis protein BMS1 homolog (LOC100828930)	XM_003557930.1
384-S144	No significant similarity found.	
384-S145	*Triticum aestivum* chloroplast DNA, 21.1-kb fragment bearing RNA polymerase subunit (rpo) genes	AB027572.1
384-S147	*Zea mays* endo-1,4-beta-glucanase Cel1 (LOC100285405)	NM_001158298.1
384-S148	*Zea mays* clone 280872 40S ribosomal protein S21	EU964691.1
384-S149	*Brachypodium distachyon* E3 ubiquitin-protein ligase UPL3-like	XM_003581528.1
384-S150	*Zea mays* transposon protein (LOC100281653)	NM_001154572.1
384-S151	*Sorghum bicolor* hypothetical protein	XM_002454407.1
384-S152	No significant similarity found.	
384-S154	*Saccharum* hybrid cultivar NCo 310 chloroplast DNA, complete genome	AP006714.1

(APPENDIX 3. Continued)

Seq ID	BLASTn-based similarity to genes	Accession number
384-S155	*Zea mays* mitochondrial prohibitin complex protein 2 mRNA; nuclear gene for mitochondrial product	EU966008.1
384-S156	*Sorghum bicolor* hypothetical protein	XM_002462185.1
384-S158	*Sorghum bicolor* hypothetical protein	XM_002446073.1
384-S160	No significant similarity found.	
384-S161	No significant similarity found.	
384-S163	*Zea mays* mitochondrial import receptor subunit TOM20 mRNA; nuclear gene for mitochondrial product	EU958096.1
384-S164	*Zea mays* Grx_C3 - glutaredoxin subgroup I (LOC100283484)	NM_001156384.1
384-S165	Saccharum hybrid elongation factor 1 alpha	AF331849.1
384-S169	*Zea mays* hypothetical protein	EU964240.1
384-S170	*Zea mays* PCO071732	AY104995.1
384-S171	No significant similarity found.	
384-S172	*Saccharum* hybrid cultivar H65-7052 mRNA for cytosolic fructose-1,6-bisphosphatase	X89006.1
384-S173	*Zea mays* YT521-B-like family protein	EU957472.1
384-S174	*Saccharum officinarum* clone Y71-374-T2 hypothetical protein-like	EU048802.1
384-S175	*Zea mays* cupin, RmlC-type	EU960133.1
384-S176	*Sorghum bicolor* hypothetical protein	XM_002463574.1
384-S177	*Zea mays* hypothetical protein	EU972131.1
384-S178	No significant similarity found.	
384-S179	*Zea mays* phospholipid hydroperoxide glutathione peroxidase 1	EU973175.1
384-S180	*Saccharum* hybrid cultivar GT28 thioredoxin M-type	JN591763.1
384-S181	No significant similarity found.	
384-S183	*Sorghum bicolor* hypothetical protein	XM_002455550.1
384-S184	No significant similarity found.	
384-S185	*Zea mays* 40S ribosomal protein S23	EU952753.1
384-S186	No significant similarity found.	
384-S187	*Sorghum bicolor* hypothetical protein	XM_002451754.1
384-S188	*Saccharum* hybrid 14-3-3-like protein	AY222859.1
384-S189	*Sorghum bicolor* hypothetical protein	XM_002444775.1
384-S190	*Sorghum bicolor* hypothetical protein	XM_002460519.1
384-S191	*Oryza sativa* Japonica Group Os01g0973400	NM_001052082.2
384-S192	No significant similarity found.	

APPENDIX 4. Functional annotation of differentially expressed genes of the leaf scald susceptible sugarcane clone HoCP 89-846 in response to *Xanthomonas albilineans* infection.

Seq ID	BLASTn-based similarity to genes	Accession number
846-S1	*Sorghum bicolor* hypothetical protein	XM_002448079.1
846-S2	*Zea mays* serine/threonine-protein kinase SAPK8	EU960732.1
846-S4	*Zea mays* uncharacterized LOC100382709	NM_001175432.1
846-S5	*Brachypodium distachyon* 26S proteasome non-ATPase regulatory subunit 1-like	XM_003571499.1
846-S6	No significant similarity found.	
846-S7	*Zea mays* endochitinase A2 precursor	EU959576.1
846-S8	*Sorghum bicolor* hypothetical protein	XM_002439346.1
846-S9	*Sorghum bicolor* hypothetical protein	XM_002438041.1
846-S10	*Brachypodium distachyon* FGFR1 oncogene partner-like, transcript variant 1	XM_003562678.1
846-S11	*Saccharum* hybrid cultivar GT28 putative ATP citrate lyase	JQ923438.1
846-S12	*Zea mays* RING-finger protein like	EU956797.1
846-S13	*Sorghum bicolor* 5-methylcytosine DNA glycosylase (DME)	JF683319.1
846-S14	No significant similarity found.	
846-S15	*Sorghum bicolor* hypothetical protein	XM_002443978.1
846-S16	*Zea mays* putative HLH DNA-binding domain superfamily protein	NM_001176472.1
846-S17	*Zea mays* cytochrome b561	EU962707.1
846-S18	*Zea mays* calmodulin-binding protein mRNA, 3'end	L01496.1| MZECMBPA
846-S19	No significant similarity found.	
846-S21	*Oryza sativa* Japonica Group genomic DNA, chromosome 9, fosmid clone:OSJNOa063K24, Range 1: 9219 to 9635	AP009051.1
846-S22	*Spodiopogon cotulifer* ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit gene, chloroplast	KC164343.1
846-S23	*Sorghum bicolor* hypothetical protein	XM_002455808.1
846-S24	*Brachypodium distachyon* UPF0667 protein C1orf55 homolog	XM_003569521.1
846-S25	*Sorghum bicolor* hypothetical protein	XM_002438399.1
846-S26	*Zea mays* serine carboxypeptidase-like precursor	EU974114.1
846-S28	*Zea mays* hypothetical protein	EU960328.1
846-S29	*Sorghum bicolor* hypothetical protein	XM_002444923.1
846-S31	No significant similarity found.	
846-S32	*Zea mays* auxin response factor 1 (ARF1) gene	HM004516.1
846-S33	*Brachypodium distachyon* pentatricopeptide repeat-containing protein At4g30825, chloroplastic-like	XM_003576487.1
846-S34	*Zea mays* 60S ribosomal protein L23	EU976422.1
846-S35	*Zea mays* protein disulfide isomerase11	NM_001112333.1

(APPENDIX 4. Continued)

Seq ID	BLASTn-based similarity to genes	Accession number
846-S37	No significant similarity found.	
846-S38	No significant similarity found.	
846-S40	*Zea mays* full-length cDNA clone ZM_BFc0120O22	BT065006.1
846-S41	*Zea mays* ADP-ribosylation factor 1	EU959162.1
846-S43	*Brachypodium distachyon* probable protein phosphatase 2C 5-like (LOC100825920)	XM_003569137.1
846-S44	*Phyllostachys edulis* cellulose synthase (CesA11)	HM068510.1
846-S45	*Sorghum bicolor* hypothetical protein	XM_002442115.1
846-S46	No significant similarity found.	
846-S49	No significant similarity found.	
846-S50	*Sesamum alatum* putative lipoamide dehydrogenase	AY873805.1
846-S51	*Zea mays* LOC100285191 (IDP798)	NM_001158085.1
846-S52	No significant similarity found.	
846-S53	*Zea mays* auxin-binding protein ABP20 precursor	EU958158.1
846-S54	*Zea mays* clone 261099 mRNA sequence	EU945354.1
846-S55	*Zea mays* clone 284960 mRNA sequence	EU945765.1
846-S57	*Pennisetum glaucum* chloroplast heat shock protein 70 mRNA; nuclear gene for chloroplast product	EF495353.1
846-S58	*Sorghum bicolor* hypothetical protein	XM_002449811.1
846-S61	No significant similarity found.	
846-S62	*Zea mays* triosephosphate isomerase, cytosolic	EU976695.1
846-S63	*Saccharum* hybrid cultivar SP-80-3280 chloroplast, complete genome	AE009947.2
846-S64	*Zea mays* cell division cycle protein 48	JF915708.1
846-S65	No significant similarity found.	
846-S66	*Zea mays* 60S ribosomal protein L15	EU966076.1
846-S68	No significant similarity found.	
846-S69	No significant similarity found.	
846-S71	No significant similarity found.	
846-S72	PREDICTED: *Brachypodium distachyon* auxin response factor 23-like (LOC100823699)	XM_003575972.1
846-S74	*Zea mays* mRNA for legumain-like protease (see2b gene)	AJ131719.1
846-S76	*Zea mays* aquaporin TIP4.1	EU974338.1
846-S77	*Sorghum bicolor* hypothetical protein	XM_002446275.1
846-S78	*Saccharum* hybrid TFIIA small subunity	GU120202.1
846-S80	*Sorghum bicolor* tRNA-Met (tRNAfM) gene, complete sequence; and ATP synthase complex subunit 9 (atp9) gene; mitochondrial genes for mitochondrial products	U61165.1
846-S81	*Zea mays* pyruvate dehydrogenase E1 component subunit beta	NM_001157001.1
846-S82	No significant similarity found.	
846-S83	*Sorghum bicolor* hypothetical protein	XM_002467035.1
846-S84	*Zea mays* clone 259857 mRNA sequence	EU945336.1
846-S85	*Zea mays* maltose excess protein 1-like	NM_001155986.1
846-S87	*Sorghum bicolor* hypothetical protein	XM_002454688.1

(APPENDIX 4. Continued)

Seq ID	BLASTn-based similarity to genes	Accession number
846-S88	*Sorghum bicolor* hypothetical protein	XM_002450985.1
846-S89	*Sorghum bicolor* hypothetical protein	XM_002466061.1
846-S91	No significant similarity found.	
846-S92	No significant similarity found.	
846-S93	No significant similarity found.	
846-S94	*Zea mays* low molecular weight protein-tyrosine-phosphatase slr0328	EU959344.1
846-S95	*Saccharum officinarum* clone SCCCRZ1001G11, complete sequence	AY596608.1
846-S96	*Sorghum bicolor* hypothetical protein	XM_002436343.1
846-S97	*Zea mays* putative serine/threonine protein phosphatase superfamily protein isoform 1	NM_001174292.1
846-S98	*Zea mays* splicing factor U2af 38 kDa subunit	NM_001155027.1
846-S100	*Saccharum* hybrid ferredoxin-NADP reductase	JN591761.1
846-S101	*Sorghum bicolor* hypothetical protein	XM_002439683.1
846-S103	*Zea mays* UDP-sulfoquinovose synthase	NM_001155335.1
846-S104	*Zea mays* hypothetical protein (LOC100274071)	NM_001148450.1
846-S106	*Zea mays* OB-fold nucleic acid binding domain containing protein (LOC100280528)	NM_001153447.1
846-S108	No significant similarity found.	
846-S110	*Zea mays* clone 228969 tryptophanyl-tRNA synthetase	EU960838.1
846-S111	*Zea mays* early responsive to dehydration protein (ERD4)	NM_001114650.1
846-S112	*Brachypodium distachyon* ABC transporter F family member 1-like (LOC100840133)	XM_003573173.1
846-S113	*Brachypodium distachyon* V-type proton ATPase catalytic subunit A-like (LOC100842214)	XM_003563289.1
846-S114	*Sorghum bicolor* hypothetical protein	XM_002459769.1
846-S116	No significant similarity found.	
846-S117	*Zea mays* branched-chain-amino-acid aminotransferase	EU962032.1
846-S119	*Zea mays* cytosolic glyceroldehyde-3-phosphate dehydrogenase GAPC2	NM_001112230.2
846-S120	*Zea mays* clone 1281211 TPR domain containing protein	EU952451.1
846-S121	*Zea mays* histidine kinase2	NM_001111396.1
846-S122	*Brachypodium distachyon* magnesium-chelatase subunit chlD, chloroplastic-like (LOC100824575)	XM_003563892.1
846-S123	*Zea mays* 60S acidic ribosomal protein P1	EU967302.1
846-S124	*Sorghum bicolor* hypothetical protein	XM_002448382.1
846-S125	*Sorghum bicolor* hypothetical protein	XM_002442267.1
846-S126	*Sorghum bicolor* hypothetical protein	XM_002440391.1
846-S128	No significant similarity found.	
846-S130	No significant similarity found.	
846-S131	*Saccharum officinarum* chloroplast gene for RNA polymerase betaII subunit,	AP006961.1
846-S132	No significant similarity found.	

(APPENDIX 4. Continued)

Seq ID	BLASTn-based similarity to genes	Accession number
846-S133	*Zea mays* beta-1,3-galactosyltransferase sqv-2	NM_001155648.1
846-S134	*Oryza sativa* (indica cultivar-group) small GTP binding protein (Rab7)	AY226827.1
846-S135	*Zea mays* putative 14-3-3 protein	AY744160.1
846-S136	*Zea mays* 40S ribosomal protein S6	EU959790.1
846-S137	*Zea mays* IM30 protein-like protein (LOC100191375)	NM_001136809.1
846-S138	No significant similarity found.	
846-S140	No significant similarity found.	
846-S141	*Sorghum bicolor* hypothetical protein	XM_002440725.1
846-S143	No significant similarity found.	
846-S144	*Zea mays* 60S ribosomal protein L9	EU961109.1
846-S146	*Sorghum bicolor* hypothetical protein	XM_002441621.1
846-S147	*Sorghum bicolor* hypothetical protein	XM_002437437.1
846-S149	*Zea mays* methionine synthase	AF439723.1
846-S150	*Zea mays* 3-oxoacyl-synthase I (LOC100283265)	NM_001156167.1
846-S151	*Sorghum bicolor* hypothetical protein	XM_002460800.1
846-S154	*Sorghum bicolor* hypothetical protein	XM_002440194.1
846-S155	*Sorghum bicolor* hypothetical protein (SORBIDRAFT_1138s002030)	XM_002488920.1
846-S156	*Zea mays* LOC100281725 (si486102h02)	NM_001154645.1
846-S158	*Zea mays* nucleoside transporter	EU970960.1
846-S159	No significant similarity found.	
846-S160	No significant similarity found.	
846-S161	*Z. mays* zmcpt mRNA triose phosphate/phosphate translocator	Z26595.1
846-S162	*Brachypodium distachyon* probable methyltransferase PMT28-like (LOC100822061)	XM_003558577.1
846-S164	*Sorghum bicolor* hypothetical protein	XM_002462163.1
846-S166	*Sorghum bicolor* hypothetical protein	XM_002467922.1
846-S167	*Zea mays* DNA binding protein (LOC100284393)	NM_001157288.1
846-S169	*Zea mays* RNA binding protein	EU968499.1
846-S170	*Triticum* sp. mRNA for DP protein	AJ271917.1
846-S171	*Zea mays* elongation factor 1-beta	EU965401.1
846-S173	*Sorghum bicolor* hypothetical protein	XM_002453762.1
846-S174	*Zea mays* ATP synthase beta chain	EU972246.1
846-S175	*Sorghum bicolor* hypothetical protein	XM_002442266.1
846-S176	No significant similarity found.	
846-S177	*Zea mays* zinc finger protein CONSTANS-LIKE 11	EU972243.1
846-S178	*Zea mays* elongation factor 1-delta 1 (LOC100283380)	NM_001156281.1
846-S179	No significant similarity found.	
846-S180	*Oryza minuta* bifunctional nuclease in basal defense response	DQ872164.1
846-S181	*Sorghum bicolor* hypothetical protein	XM_002441026.1
846-S182	*Zea mays* phosphatidate cytidylyltransferase	EU969653.1
846-S184	No significant similarity found.	
846-S187	*Sorghum bicolor* hypothetical protein	XM_002465495.1

(APPENDIX 4. Continued)

Seq ID	BLASTn-based similarity to genes	Accession number
846-S188	*Glycine max* uncharacterized protein LOC100780983	XM_003539297.1
846-S189	*Sorghum bicolor* hypothetical protein	XM_002451497.1
846-S190	*Zea mays* protein phosphatase 2C isoform epsilon	EU970898.1
846-S191	No significant similarity found.	
846-S192	*Sorghum bicolor* isolate SVP1 vaculor H+-pyrophosphatase	HM143921.1

APPENDIX 5. Summary of the LCP 85-384 x L 99-226 (F1 population) linkage map analysis.

LG[a]	Length (cM)	SD[b] Markers	3:1 (both parents)	1:1 (LCP 85-384)	1:1 (L99-226)	Chrom in sorghum[c]
1	88.18	7	0	7	0	1
2	1233.93	60	18	21	21	All
3	5217.03	163	60	54	49	1, 3, 7, 5, 6, 9, 4, 2
4	109.78	7	4	1	2	1, 2
5	132.65	6	1	5	0	1
6	110.88	9	3	4	2	2, 1
7	17.18	3	0	0	3	1, 7
8	4.51	2	0	0	2	1
9	6.78	2	0	0	2	1
10	248.20	9	7	1	1	2, 1, 5, 9
11	24.08	3	0	3	0	1
12	913.35	39	12	20	7	5, 7, 10, 2, 4, 8, 3, 1
13	27.10	2	0	2	0	1
14	24.45	2	0	0	2	1, 7
15	27.10	2	0	2	0	1
16	3.65	2	1	1	0	1
17	58.66	4	0	4	0	10, 1
18	104.07	6	1	5	0	1
19	129.50	7	2	4	1	1
20	73.70	6	2	3	1	1, 4
21	318.66	12	7	0	5	6, 3, 1, 2
22	153.87	8	5	0	3	10, 1, 6, 3, 4, 7
23	28.60	2	0	0	2	1
24	24.55	3	1	1	1	1, 5
25	47.04	3	0	3	0	1
26	123.32	7	4	0	3	1, 2, 3, 8, 10
27	37.07	3	2	1	0	1
28	58.81	4	3	1	0	3, 1, 2
29	518.62	20	4	2	14	10, 1, 9, 2, 7
30	13.24	2	0	2	0	1
31	346.08	23	6	12	5	8, 9, 3, 7, 1
32	11.97	3	1	0	2	1
33	50.29	3	0	0	3	1, 2
34	166.19	7	0	0	7	5, 1
35	128.91	9	0	0	9	10
36	20.38	2	0	2	0	10
37	9.19	2	0	0	2	10
38	11.33	2	1	0	1	10, 9
39	8.11	3	2	0	1	10
40	12.37	2	1	0	1	1, 4

(APPENDIX 5. Continued)

LG[a]	Length (cM)	SD[b] Markers	3:1 (both parents)	1:1 (LCP 85-384)	1:1 (L99-226)	Chrom in sorghum[c]
41	340.94	16	5	10	1	10, 1, 5, 6, 3
42	80.09	5	4	0	1	10, 7
43	26.08	2	0	0	2	10
44	61.40	5	1	4	0	5, 10
45	0.00	2	1	0	1	10
46	37.29	5	4	1	0	10
47	84.27	4	0	0	4	10, 6
48	12.45	2	0	0	2	10
49	29.71	3	0	0	3	10
50	169.53	10	2	5	3	6, 10
51	72.27	6	1	5	0	6, 10
52	833.14	60	28	21	11	2, 8, 7, 5, 4, 6
53	131.26	10	2	4	4	8, 2, 1
54	3.76	2	0	2	0	2
55	16.75	2	1	1	0	2, 8
56	57.96	4	1	0	3	2
57	76.31	5	1	4	0	2, 8
58	32.74	3	2	1	0	2, 1
59	0.00	2	1	1	0	2
60	61.84	5	2	0	3	2
61	27.85	2	1	0	1	2, 7
62	100.05	6	4	1	1	2, 6
63	140.79	10	3	5	2	2
64	431.55	18	2	1	15	2, 3, 5, 7, 4
65	6.86	2	0	2	0	2
66	15.05	2	0	2	0	2
67	0.00	2	1	1	0	2
68	18.40	3	2	1	0	3, 2
69	29.64	3	1	1	1	2, 4
70	104.86	6	2	3	1	2, 3
71	30.16	2	0	2	0	2
72	11.48	2	1	0	1	2
73	110.99	5	0	0	5	2
74	61.09	4	0	0	4	2
75	161.77	11	6	4	1	2, 8, 5
76	47.12	4	3	0	1	2
77	33.16	3	1	1	1	2
78	0.00	2	1	1	0	2
79	2.25	2	0	0	2	2
80	0.00	2	1	1	0	2
81	24.39	4	3	0	1	3
82	164.00	11	5	3	3	3
83	116.09	9	2	2	5	3, 1

(APPENDIX 5. Continued)

LG[a]	Length (cM)	SD[b] Markers	3:1 (both parents)	1:1 (LCP 85-384)	1:1 (L99-226)	Chrom in sorghum[c]
84	11.85	2	1	0	1	3
85	73.52	5	3	0	2	3, 1
86	13.97	2	1	1	0	3, 6
87	75.57	6	2	3	1	3, 4
88	40.68	3	0	0	3	3
89	27.47	2	0	0	2	3
90	0.00	2	0	2	0	3
91	40.33	3	1	2	0	3, 4
92	23.38	2	2	0	0	3
93	131.09	6	1	0	5	3
94	7.30	2	1	1	0	3
95	52.40	4	1	2	1	3
96	40.63	5	0	5	0	3
97	12.77	2	0	0	2	3
98	44.50	4	0	4	0	3
99	64.11	3	1	0	2	9, 1
100	0.00	2	0	2	0	3
101	23.41	2	0	0	2	3, 7
102	31.28	3	1	2	0	4
103	10.88	2	0	0	2	4
104	29.54	4	1	0	3	4
105	0.00	2	1	0	1	4
106	170.16	10	6	2	2	4
107	184.50	15	4	9	2	4, 7
108	55.57	4	1	0	3	4
109	221.84	12	4	0	8	4
110	138.20	8	1	0	7	4, 2
111	96.49	7	4	2	1	4
112	91.48	9	7	0	2	4
113	92.74	5	0	0	5	4
114	30.16	2	0	2	0	4, 7
115	52.98	5	0	0	5	4
116	2.33	2	0	0	2	4, 5
117	117.83	8	3	5	0	4
118	33.91	4	3	0	1	4, 5
119	498.52	26	11	9	6	6, 5, 4
120	50.74	4	1	0	3	4, 7
121	7.06	2	1	1	0	4, 7
122	30.82	3	0	3	0	5
123	90.10	5	1	4	0	5, 6
124	0.00	2	1	1	0	5
125	8.38	2	1	0	1	4, 5
126	4.54	2	1	0	1	5

(APPENDIX 5. Continued)

LG[a]	Length (cM)	SD[b] Markers	3:1 (both parents)	1:1 (LCP 85-384)	1:1 (L99-226)	Chrom in sorghum[c]
127	42.12	3	1	2	0	5
128	0.00	2	1	0	1	5
129	2.38	2	0	0	2	5
130	92.54	5	1	1	3	6, 10
131	32.47	3	2	1	0	6, 1
132	27.54	3	2	0	1	1, 6
133	13.78	3	2	0	1	6
134	28.60	2	0	2	0	6
135	30.59	2	0	2	0	6
136	6.89	3	2	1	0	6
137	26.94	3	1	1	1	6, 1
138	5.81	3	2	1	0	7
139	70.82	5	3	0	2	7, 2
140	116.54	6	0	6	0	7
141	0.00	2	1	1	0	7
142	10.49	2	1	1	0	7, 3
143	1.14	2	0	0	2	7
144	9.16	3	1	0	2	7
145	17.79	2	2	0	0	7, 1
146	15.22	2	1	0	1	7
147	2.57	2	2	0	0	7
148	7.58	2	1	1	0	8
149	0.00	2	1	0	1	8, 5
150	33.49	5	0	0	5	8
151	94.65	5	0	5	0	9
152	47.76	3	1	2	0	9, 2
153	18.82	2	0	2	0	9
154	12.62	2	0	2	0	9
155	31.86	3	1	2	0	9
156	36.88	3	0	3	0	9
157	70.34	3	0	0	3	2, 6
158	12.53	2	1	0	1	9
159	31.77	2	0	0	2	9
160	51.91	6	3	3	0	9
161	20.56	3	1	0	2	9
162	37.50	4	3	0	1	9, 4
163	95.87	5	2	0	3	9, 8
164	17.50	2	2	0	0	1
165	10.54	2	2	0	0	1
166	53.96	4	3	1	0	1, 3
167	39.48	3	1	0	2	3, 4, 7
168	33.75	3	3	0	0	1
169	20.97	2	2	0	0	1

(APPENDIX 5. Continued)

LG[a]	Length (cM)	SD[b] Markers	3:1 (both parents)	1:1 (LCP 85-384)	1:1 (L99-226)	Chrom in sorghum[c]
170	5.21	2	1	0	1	1
171	25.80	2	2	0	0	1, 3
172	6.68	3	3	0	0	9, 10
173	39.42	3	1	2	0	6
174	18.96	2	2	0	0	2
175	8.10	2	2	0	0	2
176	23.71	2	2	0	0	2
177	21.83	3	3	0	0	5, 7, 9
178	13.77	2	2	0	0	3
179	1.21	2	2	0	0	3
180	19.25	2	2	0	0	3, 7
181	20.00	2	2	0	0	3
182	47.33	3	3	0	0	3
183	1.31	2	2	0	0	3
184	16.53	2	2	0	0	4
185	13.96	2	2	0	0	6, 9
186	45.59	3	3	0	0	6
187	3.66	2	2	0	0	7
188	22.43	2	2	0	0	8
189	53.79	5	4	0	1	8, 2
190	43.45	3	3	0	0	8
191	23.69	3	3	0	0	9
192	5.35	2	2	0	0	9
193	4.00	2	2	0	0	9
194	4.96	2	2	0	0	9
195	19.06	2	2	0	0	9
196	28.28	2	0	0	2	ND[d]
197	12.49	2	1	0	1	2
198	42.09	3	1	2	0	ND[d]
199	18.27	2	0	2	0	ND[d]
200	24.24	2	0	0	2	ND[d]
201	29.00	2	0	0	2	ND[d]
202	25.70	2	2	0	0	5
203	20.35	2	1	0	1	ND[d]
204	33.60	3	1	2	0	9
205	11.43	2	0	0	2	ND[d]
Total	**19230.01**	**1146**	**426**	**361**	**359**	**All**

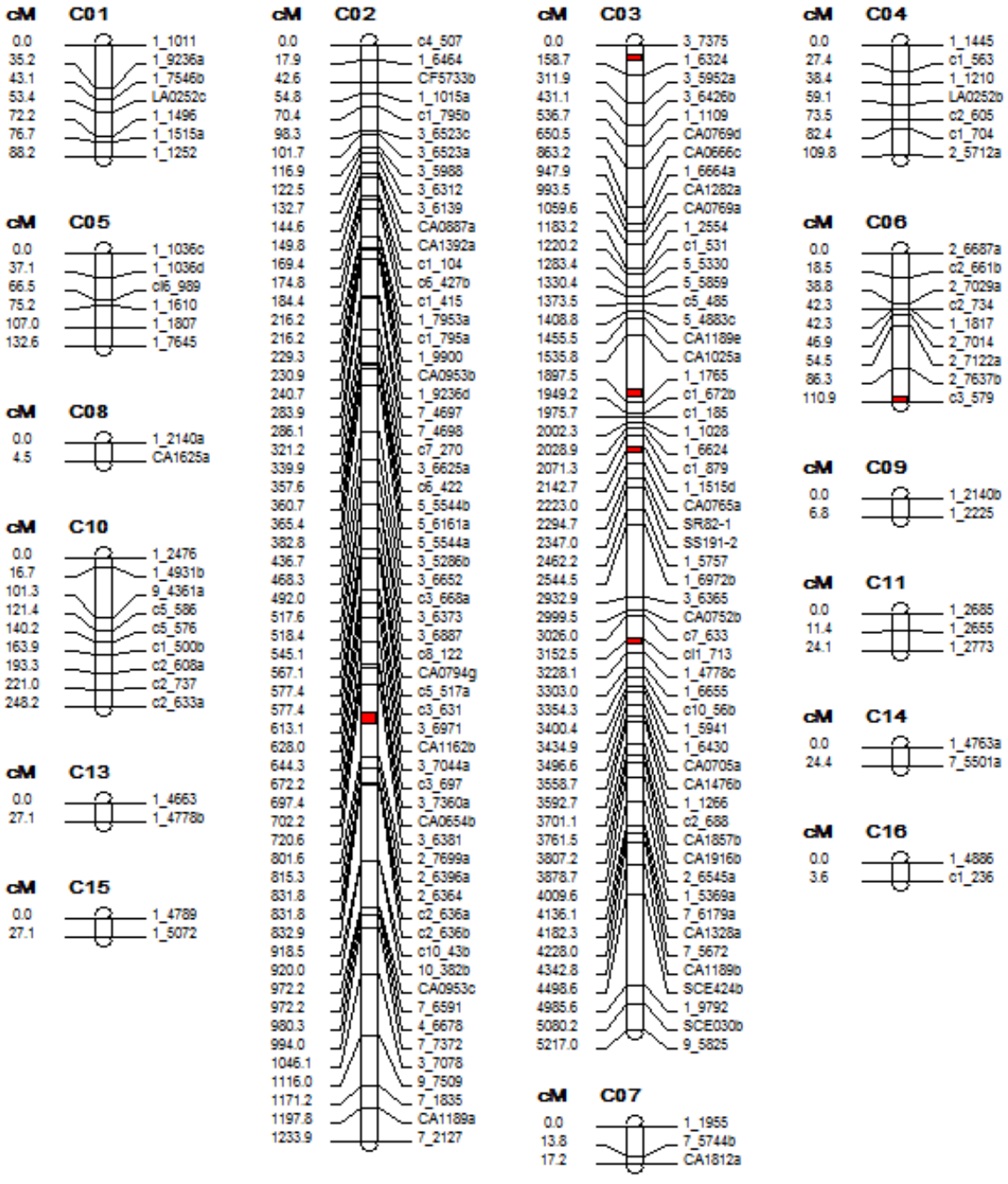
a. LG, Linkage group.

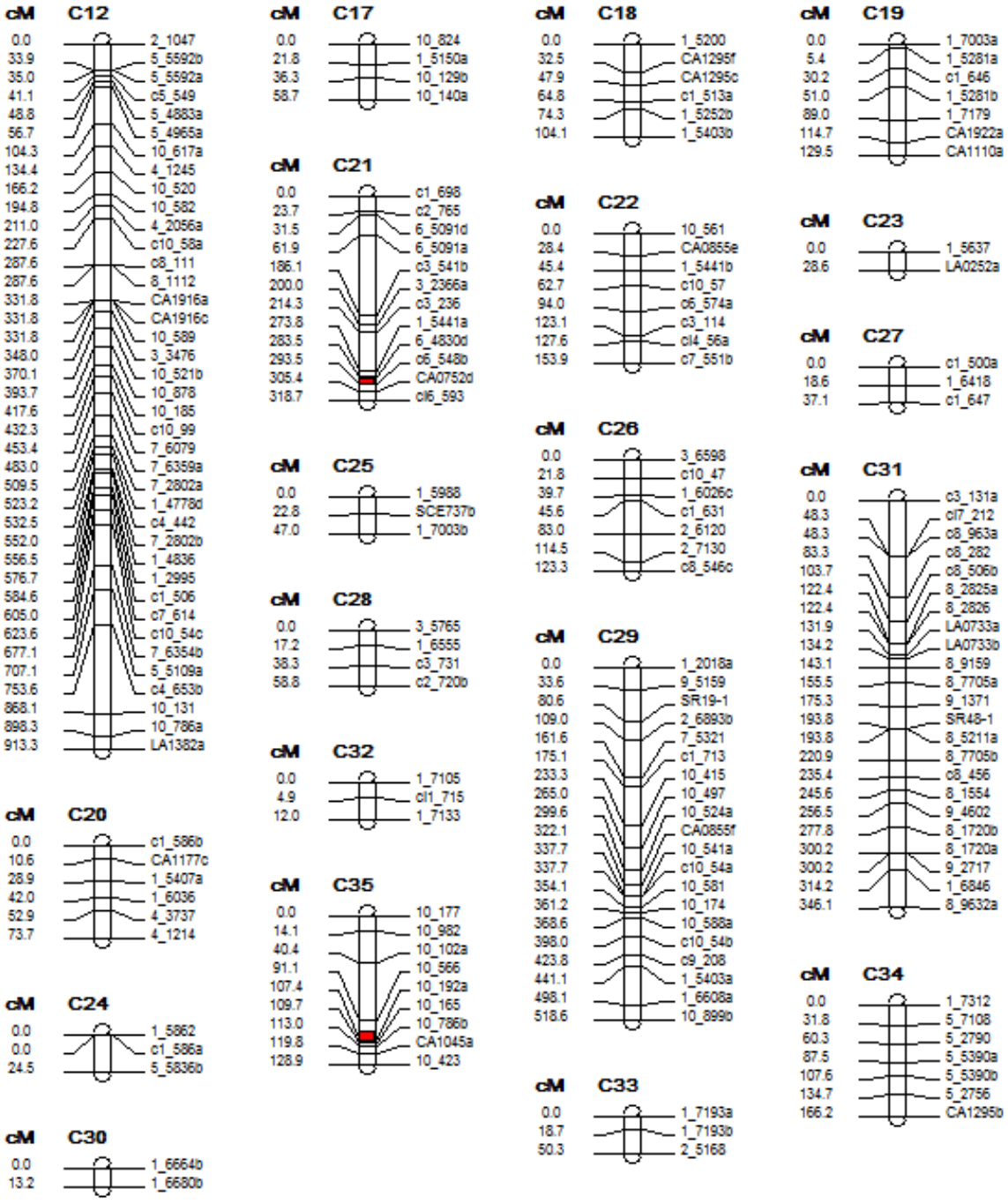
b. SD, Single dose

c. Chrom in sorghum, Based on the SNP information (*Sorghum bicolor* genome was used for SNP calling). The linkage groups were discriminated using the SNPs contained in each LG.

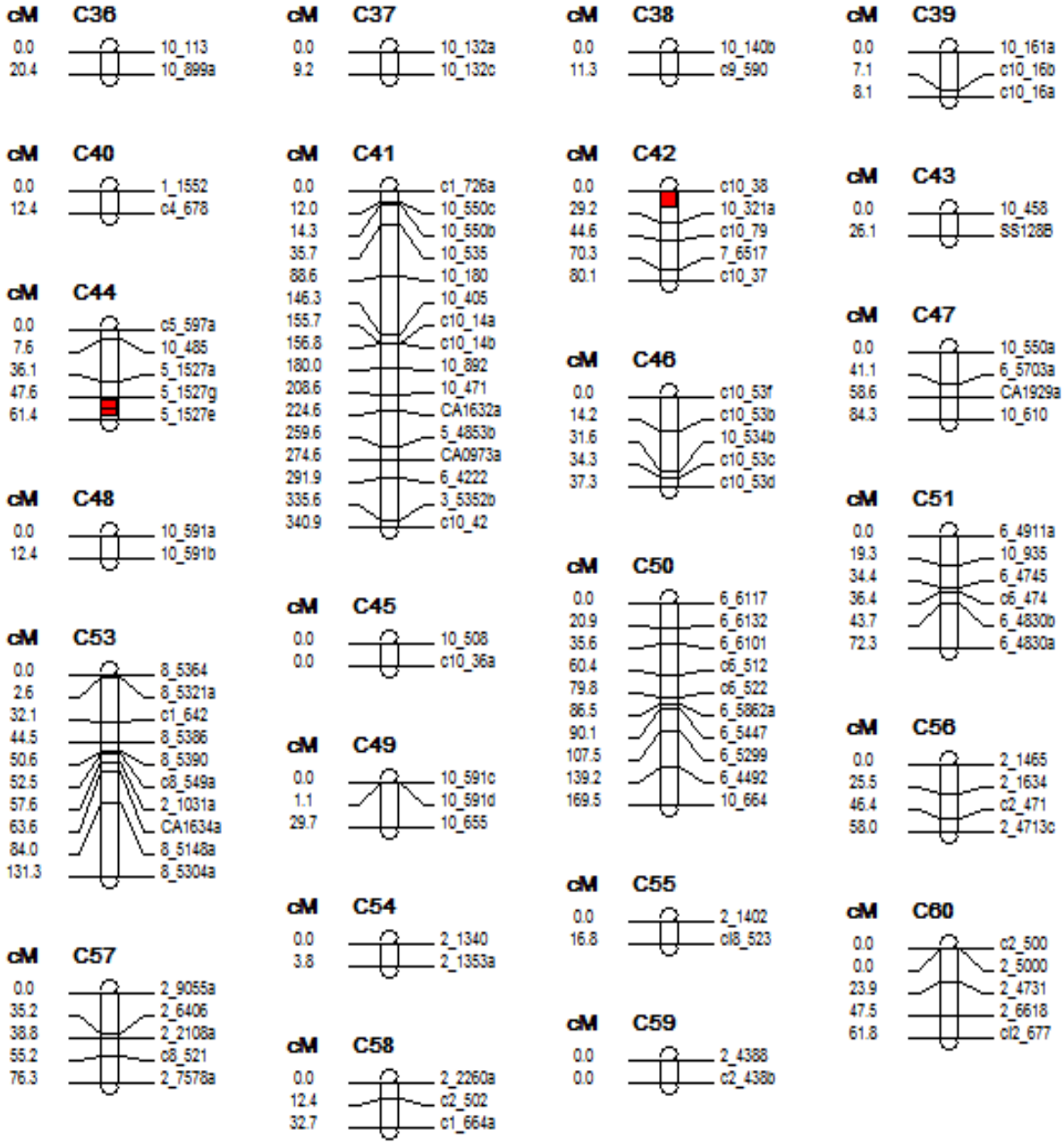
d. ND, No data. SNPs markers in the LG could not be assigned to a chromosome in sorghum.

APPENDIX 6. Genetic linkage map[a] of the cross between LCP 85-384 and L 99-226 sugarcane cultivars based upon genotype data of 89 F_1 progeny using SSR, eSSR and SNP markers.

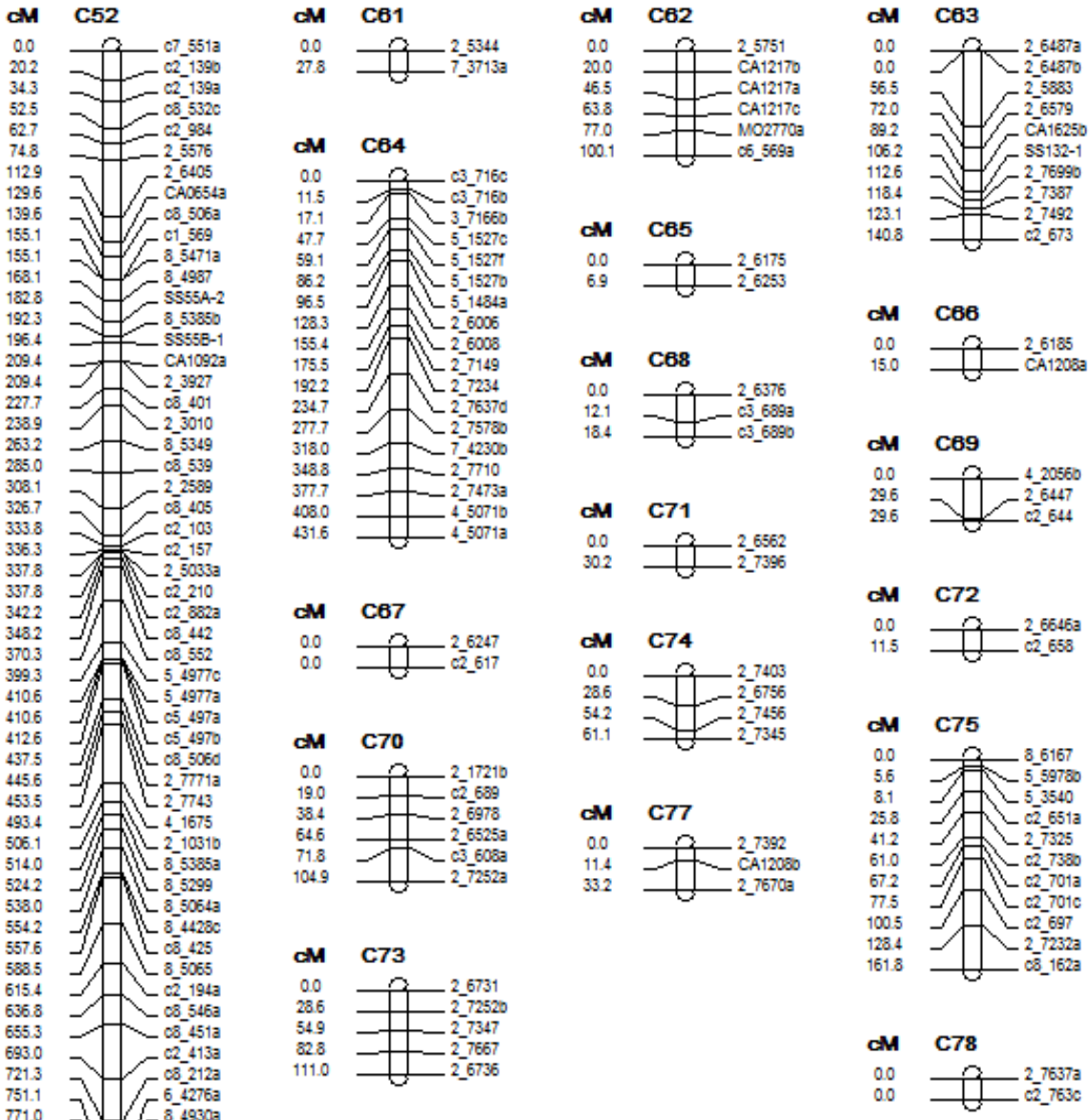




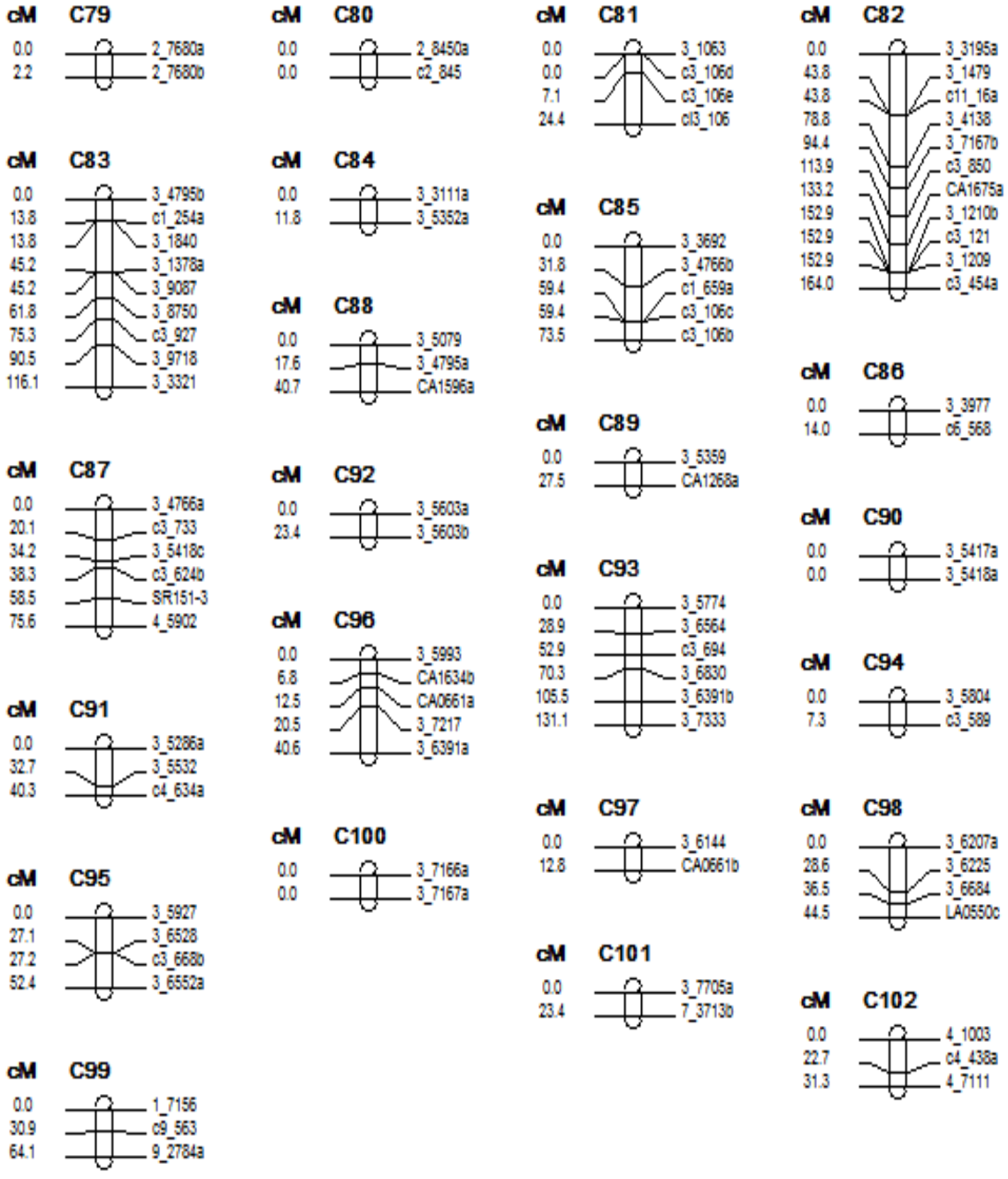
(APPENDIX 6. Continued)



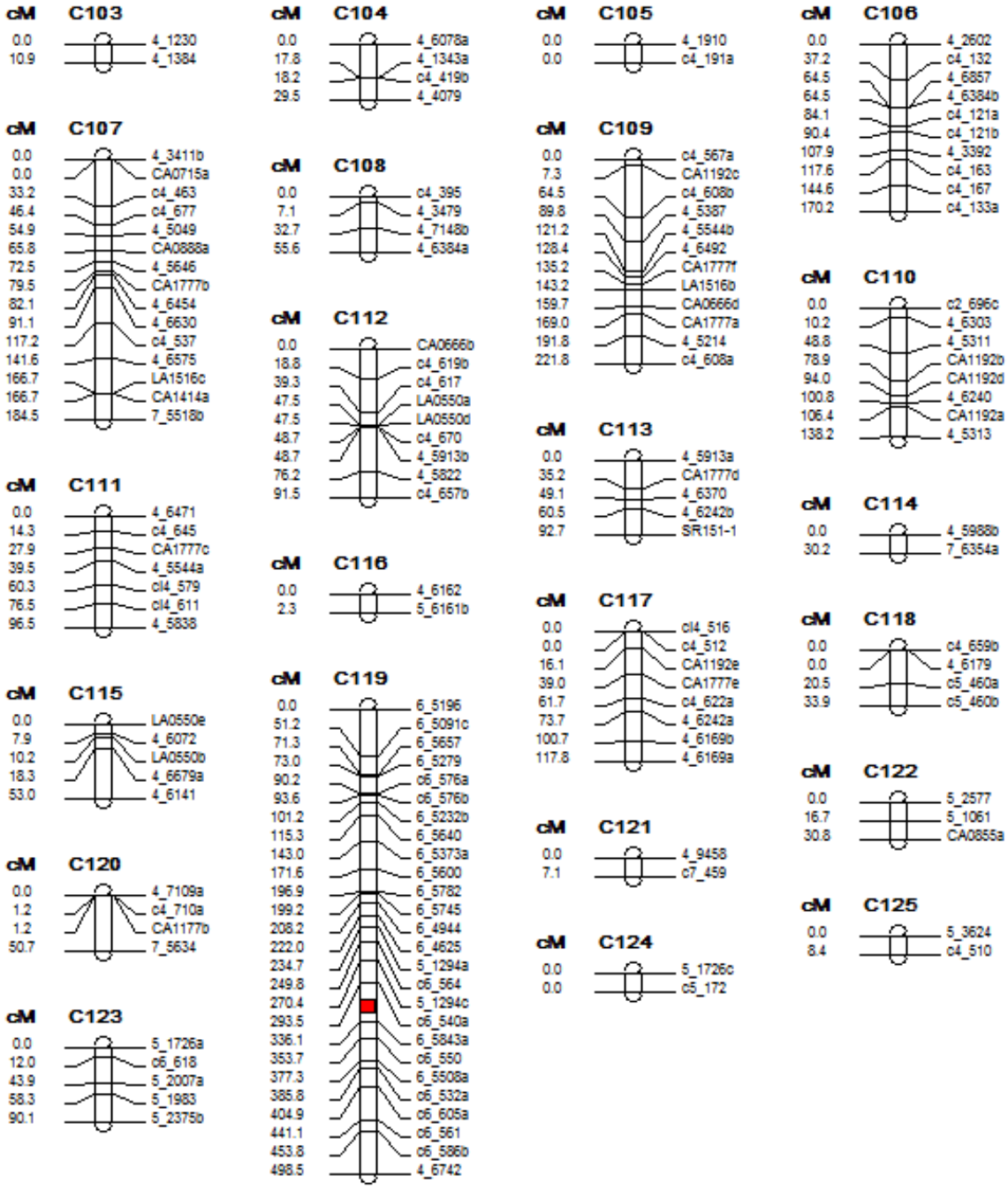
(APPENDIX 6. Continued)



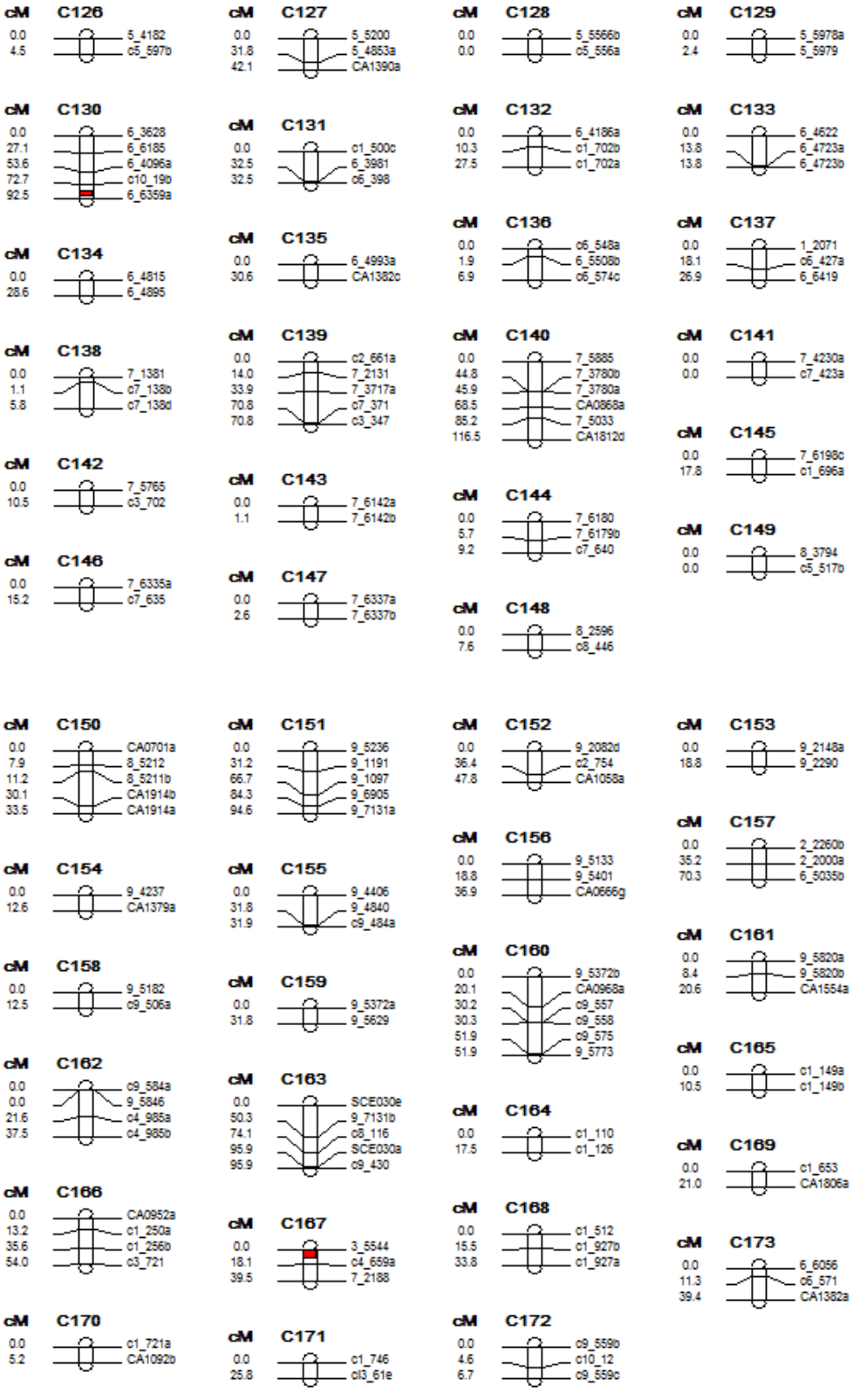
(APPENDIX 6. Continued)



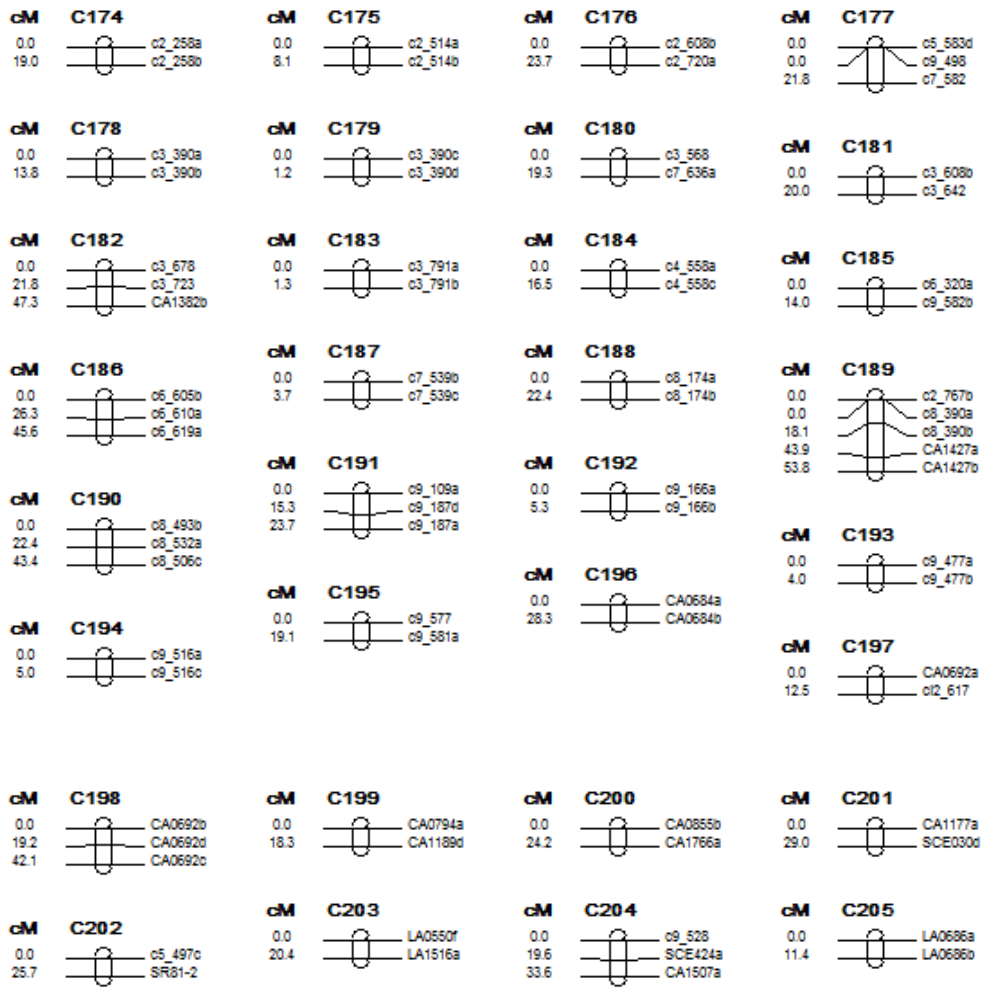
(APPENDIX 6. Continued)



(APPENDIX 6. Continued)



(APPENDIX 6. Continued)



a. The map was constructed using a LOD score >4.0 and a recombination frequency of 0.4. A total of 1,146 single dose markers were assigned onto 205 linkage groups (LGs). The Kosambi map distances (cM) and the marker names are indicated on the left and right sides, respectively, of each LG. The fourteen red color areas represent QTL regions associated with leaf scald resistance (see Table 3 for more information on the QTLs).

VITA

Andres Felipe Gutierrez was born in Cali, Colombia, in 1984. In 2000, he started his bachelor studies in Biology with emphasis in genetics in the Universidad del Valle in Cali, Colombia. After his graduation, he was a young investigator in CIDEIM (International Center of Training and Medical Research) sponsored by COLCIENCIAS (Administrative Department of Science, Technlogy and Innovation of Colombia). In CIDEIM, he worked on projects associated with drug resistance surveillance and molecular characterization of the resistance in *Plasmodium falciparum*, causal agent of malaria. In 2008, he started to work with sugarcane in Cenicana (Colombian Sugarcane Research Center) as a research assistant. In Cenicana, he worked on projects to develop molecular markers associated with resistance to *Sugarcane Yellow Leaf Virus* and the molecular characterization of *Puccinia melanocephala*, the causal agent of brown rust in sugarcane. In 2011, he was accepted in Louisiana State University for the Ph.D. program under the supervision of Dr. Niranjan Baisakh. In 2014 received his Master of Science degree of the Department of Plant Pathology and Crop Physiology under the guidance of Dr. Jeffrey Hoy. He is a candidate to receive the Doctoral of Philosophy degree in the School of Plant, Environment and Soil Sciences (SPESS) in December, 2016.