INVESTIGATING THE GENETIC BASIS OF AGRONOMICALLY IMPORTANT TRAITS IN SORGHUM

A Dissertation

by

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ABSTRACT

The genetic basis of three key agronomic traits *viz.* anthracnose resistance, tolerance to salinity and plant height, which affect sorghum grain, forage, and biomass production was investigated. Modern genomic tools like high-throughput genotyping, sequence-based marker technology and statistical software were used in conjunction with field or greenhouse based evaluation of recombinant inbred line (RIL) populations to identify the genomic regions and genes controlling these traits.

A total of 9 quantitative trait loci (QTL) that imparted variable levels of resistance to anthracnose, were identified from two different RIL populations. Of these QTL, three encoded stable resistance across all environments while two loci were environment specific. A brief examination of the candidate genes underlying one of the major resistance QTL revealed a block of genes that may play a role in host plant resistance. The molecular markers identified in this study serve as a tool for accelerated pyramiding of multiple anthracnose resistance loci into an elite sorghum genotype to provide effective and durable resistance. The analysis of a RIL population segregating for salt tolerance in greenhouse conditions consistently detected a QTL based on the percentage of total leaf area that was necrotic or damaged. Markers linked to the trait that could potentially be used for marker-assisted introgression of salt tolerance were identified. Of the genes observed on surveying the QTL, a cation/H^+ antiporter appears as a strong potential candidate conditioning salt tolerance to sorghum. The genomic locus controlling plant height in sorghum and harboring the dwarfing gene *Dw2* was

mapped at high resolution using individuals from a RIL population segregating for height. The locus was narrowed to ~0.1 Mbp and a candidate histone deacetylase gene and the molecular marker linked to this locus were identified. The functional validation of the candidate gene using *Agrobacterium*-mediated transformation was attempted but was not successful on account of the recalcitrance of sorghum for *in vitro* growth and transformation.

DEDICATION

Dedicated to my wonderful family; my parents Yuvaraj and Jayshree Patil for making me capable and giving me the best in life, my sisters Sheetal and Komal, brothers-in-law Rahul and Prateek for being my source of strength and motivation, my nephews Arav and Om for their unconditional love. Finally, thanks to my wife Kadambini for her patience and love.

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For the salt tolerance project, the initial germplasm and mapping population required was developed by MMR Genetics (LLC). A growth facility of Zia Tec (LLC) was utilized and phenotyping for salt tolerance was done by Dr. Marla Binzel. The statistical analysis of the phenotypic data was done in part by Dr. Mobashwer Alam.

For transformation of sorghum, technical assistance, growth chamber facilities, vectors pKANNIBAL, pCAM-Ubi-GUS, pART27 and *Agrobacterium tumefaciens* strain NTL4 harboring the disarmed Chry5 Ti plasmid designated pTiKPSF2 was

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NOMENCLATURE

AFLP	Amplified Fragment Length Polymorphism
BLUE	Best Linear Unbiased Estimators
BLUP	Best Linear Unbiased Predictors
BRs	Brassinosteroids
CaMV	Cauliflower Mosaic Virus
CDS	Coding DNA Sequence
CIM	Composite Interval Mapping
CS	College Station
DG	Digital Genotyping
GA	Georgia
HDAC	Histone Deacetylase Gene
HKT	High Affinity K^+ Transporter
hpRNA	Hairpin RNA
ICIM	Inclusive Composite Interval Mapping
IM	Interval Mapping
LOD	Logarithm of Odds
NB-LRR	Nucleotide Binding- Leucine Rich Repeat
NOS	Nopaline Synthase
OCS	Octopine Synthase
PCR	Polymerase Chain Reaction

PLD	Percent Leaf Damage
PVE	Phenotypic Variation Explained
PVPP	Polyvinylpolypyrrolidone
QTL	Quantitative Trait Locus
RAPD	Random Amplified Polymorphic DNA
REML	Restricted Maximum Likelihood
RIL	Recombinant Inbred Line
SNP	Single Nucleotide Polymorphism
SSR	Simple Sequence Repeat
UAV	Unmanned Aerial Vehicle

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CHAPTER I

INTRODUCTION

The world today faces challenges of increasing agricultural production and maintaining food security as the current world population of 7.2 billion is estimated to increase and reach 9.6 billion by 2050 (UN DESA, 2013). This will require world food production to rise by 70% and food production in the developing nations to double (FAO, 2009). This does not take into account the competing demand for grain for use in biofuel (ethanol) production, and this problem will be compounded further by the scarcity of natural resources like arable land and irrigation water and changing climate patterns. Meeting the increasing requirements of food, feed, fiber and fuel will necessitate bridging the gap between yield potential and actual yields of our agricultural crops in the face of these challenges. This will only be possible through better crop management practices and through genetic enhancement of our existing cultivars.

Sorghum [*Sorghum bicolor* (L.) Moench] is a major cereal crop grown around the world for food, fodder, fiber and fuel making it the fifth most important cereal crop worldwide (FAO, 2013). It serves as a staple food crop for millions of people, predominantly in the semi-arid tropics of Africa and Asia (Morris et al., 2013) where its grain is ground into flour as well as used for brewing. Stalks and leaves are used as cover crop and deferred animal feed as well as for hay and silage. Juicy sugar stems are used to make syrup and sugar and the sweet culm is often chewed by sustenance

farmers. The presence of directly fermentable sugars in stalks, which can be converted to biofuel, has brought sorghum into the list of dedicated bioenergy crops.

Sorghum is a C4 cereal with certain physiological and agronomic traits that make it an ideal crop for addressing the future demands placed on world food production. Compared to maize, sorghum is a crop that requires minimum input of nutrients and water and displays a wide range of adaptation and tolerance to various biotic and abiotic (e.g. high temperature, drought) stresses (Paterson, 2008). Being a C4 crop, sorghum is photosynthetically more efficient than rice and can produce 30% more dry matter per unit of water than a C3 crop (Shoemaker et al., 2010). Its complex biochemical and morphological specialization offers an advantage of enhanced carbon assimilation even at high temperatures. These characteristics make sorghum an ideal crop for subsistence as well as commercial farming.

The main target of temperate-zone commercial sorghum breeding programs has been grain yield and harvest index (Van der Weijde et al., 2013). However, since the 1980s, farmers increased the proportion of sorghum planted on marginal lands and their management practices were suboptimal for sorghum. As such, breeders responded in kind by developing cultivars with superior resistance to fungal diseases like downey mildew (Frederiksen, 1980), grain mold (Reddy et al., 2000), leaf blight, rust, anthracnose (Sharma et al., 2012), and insects like shoot fly (Jotwani, 1981; Van den Berg et al., 2005), stem borer (Sharma, 1997; Singh and Rana, 1989) and midge (Henzell et al., 1996), high and low growth temperatures (Burow et al., 2011; Nguyen et al., 2013;

Peacock, 1981; Singh, 1985), drought (Rosenow et al., 1983), poor soil fertility (Leiser et al., 2012) and soil salinity (Hassanein and Azab, 1993).

During the past several decades, vast amounts of information in genetics, genomics, biochemistry, molecular biology, and bioinformatics have been generated for sorghum, however there is still a need to bridge the gap that exists between the knowledge/tools that have been developed and their application in breeding programs. Sorghum scientists now face the challenge of determining how to take advantage of this knowledge to make crop improvement more efficient and enhance genetic gain. Thus, innovative approaches are being developed for animal and crop species to find the optimal breeding strategy to identify superior genotypes and increase the selection gains per unit time. The use of marker-based technology in applied plant breeding has continued to increase in the public and private sectors. Genome-wide marker coverage and associated costs have been the major limiting factors for many applied breeding programs until the recent advances in sequence based marker technology. Although genotyping is still a major expense, declining costs of high-throughput sequence-based marker systems has facilitated large-scale genotyping efforts in applied breeding programs, including those in sorghum. Breeders have now been equipped with powerful tools like next-generation sequencing technologies and improved genotyping/bioinformatics platforms for characterizing the genetic composition of their germplasm. It is anticipated that while selection based solely on marker effects will eventually displace phenotyping in specific generations, combining marker-based and

phenotypic-based selection will remain the norm for the foreseeable future in most public sorghum breeding programs.

The research detailed here addresses in part the long term goal of the TAMU sorghum research team through the use of modern genomic and biotechnological tools to elucidate the genetic basis and genes controlling key agronomic traits critical to sorghum germplasm improvement.

Specifically, this research focuses on the following objectives:

1. Map the genomic region conferring resistance to anthracnose using two mapping populations and develop molecular markers for use in marker-assisted breeding.
2. Elucidate the genetic basis of salt tolerance in sorghum through QTL analysis of a recombinant inbred line (RIL) population, and thereby identify molecular markers linked to the trait for introgression of salt tolerance into elite lines.
3. Map at high resolution the genomic locus harboring the dwarfing gene *Dw2* in sorghum and identify robust molecular markers linked to this locus.
4. Elucidate the genetic basis for dwarfism in sorghum by map-based cloning of *Dw2* and confirming the identity of the gene utilizing transgenic technology.

CHAPTER II

IDENTIFICATION OF QUANTITATIVE TRAIT LOCI ASSOCIATED WITH ANTHRACNOSE RESISTANCE IN SORGHUM

Introduction

Sorghum is a major cereal crop grown on nearly 42 million ha worldwide for food, fodder, fiber and fuel (http://faostat3.fao.org). Amongst the cereals, sorghum displays exceptional tolerance to heat and drought. However, a number of biotic and abiotic stresses are known to reduce sorghum yield potential (Wang et al., 2014b). Biotic stresses that impact sorghum production include fungal diseases (e.g., downy mildew, grain mold, leaf blight, and anthracnose) and parasitic insects (e.g., aphids, shoot fly, stem borer and midge).

One of the most severe diseases affecting sorghum grain yield and biomass production is anthracnose, with yield losses ranging from 50-70% having been reported in susceptible cultivars (Ali et al., 1987; Tesso et al., 2012). This disease is caused by the fungal pathogen *Colletotrichum sublineolum* and affects all above-ground portions of the plant. The first incidence of anthracnose in sorghum was reported from Togo, West Africa in 1902 (Sutton, 1980), and since then it has been observed in more than 65 countries across all continents except Antarctica (*Colletotrichum sublineolum* distribution map, 1988). The disease is most prevalent in hot, humid climates with frequent rain in both tropical and subtropical regions. The fungus most commonly infects leaves causing circular spots or elongated lesions with red, tan or blackish purple

margins and straw-colored centers which turn black during sporulation owing to the black colored asexual fruiting bodies, acervuli (Frederiksen and Odvody, 2000; Thakur and Mathur, 2000). The lesions may coalesce in susceptible genotypes resulting in senescence thereby restricting photosynthetic capacity. The fungus migrates from the lower leaves to infect the stalk, peduncle, and ultimately the grain. It invades the vascular tissue and disrupts the translocation of nutrients to the grain, reducing grain yield and quality.

Anthracnose infection begins when conidial spores from the acervuli are carried by the splashing action of raindrops or irrigation water (Bergstrom and Nicholson, 1999; Crouch and Beirn, 2009), and the spores adhere to the host surface with the aid of an extracellular matrix (Sugui et al., 1998). On susceptible genotypes, the conidia will germinate and undergo mitotic divisions to produce a germ tube with a globose appressorium that attaches itself to the host tissue and forms a penetration peg at the base. The fungus then penetrates the host cell through the cuticle by turgor pressure developed within the appressorium. To prevent this, the host forms papillae between the plasmalemma and host cell wall. The infection peg enlarges into a globose infection vesicle giving rise to filamentous primary hyphae which grow intracellularly and further colonize the adjacent cells. The secondary hyphae ramify throughout the epidermal, mesophyll and vascular tissue to begin the necrotic phase of infection (Crouch and Beirn, 2009; Münch et al., 2008; Wharton and Julian, 1996; Wharton et al., 2001). This is followed by formation of the acervuli containing setae and conidia bearing conidiophores and further spread of infection.

Agronomic practices can be used to lessen anthracnose infestation, but the nature of the pathogen and the presence of alternate hosts hamper their effectiveness. Conidial spores, the mycelium, sclerotia, microsclerotia, and hyphopodia can overwinter in decaying plant material and soil, which serve as a source of primary inoculum. The capability of *C. sublineolum* to survive on crop debris for nearly 18 months (Casela and Frederiksen, 1993) and in seed for up to 2.5 years (Crouch and Beirn, 2009) makes agronomic practices such as deep plowing of infected crop debris and crop rotation ineffective in controlling this disease. *C. sublineolum* infects most sorghum species and the fungal inoculum is capable of overwintering on the invasive weed Johnsongrass [*Sorghum halepense* (L.) Pers.] making the disease more persistent. The use of fungicides to control anthracnose can be partly effective (Gwary and Asala, 2006; Thomas et al., 1996), but fungicides prove to be uneconomical for most production fields while also posing a risk of pesticide residue on forage sorghums.

Of the control strategies for anthracnose, host plant resistance is widely regarded the most effective (Mehta et al., 2005; Rosenow and Frederiksen, 1982). However, the hypervariable nature of *C. sublineolum* makes understanding inheritance of anthracnose resistance in sorghum challenging and can impede the development of stable resistance. The different isolates (races) of *C. sublineolum* show varying pathogenicity in diverse environments, rendering isolate-specific resistant varieties potentially ineffective across locations (Pastor-Corrales, 1979; Reyes et al., 1969). Furthermore, with the evolution and introduction of new races over time and environments, the current arsenal of resistance genes may be rendered incompetent (Gorbet, 1987). Hence, a focus of applied

breeding programs is to identify multiple genetic resistance sources and then pyramid these genes into elite cultivars. Both dominant and recessive source of genetic resistance to anthracnose have been reported and resistance loci have been mapped with molecular techniques that include RAPDs (Boora et al., 1998; Singh et al., 2006), AFLPs (Perumal et al., 2009), SSRs (Klein et al., 2001; Murali Mohan et al., 2010), cDNA-AFLP transcript profiling in combination with virus-induced gene silencing (Biruma et al., 2012), and SNPs (Burrell et al., 2015; Upadhyaya et al., 2013).

The objective of the present research was to generate linkage maps identifying regions of the sorghum genome associated with anthracnose resistance loci that could be candidates for pyramiding multiple anthracnose resistance loci into an elite sorghum genotype. The ultimate goal is to provide effective and durable resistance against this fungal pathogen and to provide critical knowledge to eventually elucidate the molecular basis of resistance to this fungal disease.

Materials and methods

Mapping populations

Regular screening of sorghum germplasm in field environments across Texas and Georgia (USA) has led to the identification of a number of inbreds having a high level of resistance to anthracnose (Mehta, 2002). Amongst the various resistant lines identified, SC414-12E and SC155-14E displayed good agronomic fitness while possessing different genetic loci for anthracnose resistance (Mehta, 2002). To create bi-parental mapping populations to map anthracnose resistance loci, SC414-12E and SC155-14E were

crossed with the anthracnose susceptible inbred BTx623. The F_1 hybrids were selfed to create F_2 populations that were further advanced to $F_{4:5}$ recombinant inbred lines (RILs). For constructing linkage maps, ~100 $F_{4:5}$ recombinant inbred lines from each population were used and phenotypic evaluation was done in six environments: CS, Texas A&M University Research Farm, College Station, TX in 2011, 2012, 2013, 2015; and GA, University of Georgia College of Agricultural and Environmental Sciences campus, Tifton, GA in 2013 and 2015. The field design was a randomized complete block with two replications and each plot consisted of a 5.2 m long single row with 0.8 m row spacing. Standard agronomic practices for fertilization, irrigation and insect management were followed in both locations.

Inoculation of* Colletotrichum sublineolum *and phenotyping

All *C. sublineolum* isolates for field inoculations were collected from the Texas A&M AgriLife Research farms at Brazos Bottom, Burleson County, TX (supplied by L.K. Prom, USDA-ARS, College Station, TX). A mixture of *C. sublineolum* isolates was applied to categorize the response of the segregating populations to anthracnose in all CS experimental plots with isolates AMP 119, AMP 123, AMP 129, AMP 132, AMP 134, AMP 150, and AMP 159 being used in 2011 while 2012-2015 isolates consisted of FSP 2, FSP 5, FSP 7, FSP 35, FSP 36, FSP 44, FSP 50, and FSP 53. Inoculant was applied by dropping *C. sublineolum* colonized sorghum grains into the whorl of ~60 day-old sorghum plants (Erpelding and Prom, 2006). In CS environments, anthracnose disease perpetuation was dependent on seasonal rainfall and humidity. For GA environments, the sorghum mapping populations were naturally infected by local anthracnose isolates,

and the disease was perpetuated by the normal high humidity and rainfall supplemented with overhead sprinkler irrigation. To provide additional disease pressure, borders were planted with rows of susceptible genotype BTx623 in all field locations.

Anthracnose disease incidence ratings were recorded ~120 days after planting which corresponds to the late stages of grain fill to near physiological maturity. At this time, the characteristic symptoms of anthracnose infection were observed on the susceptible parent (Crouch and Beirn, 2009). Disease ratings on a visual basis were obtained for the whole plot as well as for leaf, stalk, and head of individual plants. A rating scale of 1 to 9 was used based on the area of the leaf covered with lesions (Thakur, 1995). The rating scale is as follows; 1 = no lesions, 2 = 1-5%, 3 = 6-10%, 4 = 11-20%, 5 =21-30%, 6 = 31-40%, 7 = 41-50%, 8 = 51-75%, and 9 = > 75% of the area covered with lesions. For disease rating of leaves, the foliage of plants was evaluated for anthracnose symptoms whereas the stalk rating was based on splitting the peduncle open and observing the spread of disease. Anthracnose incidence for panicles was rated by observing the infection on seeds and rachis of the panicle. For QTL analysis a whole plot reading was taken where each plot was assessed for foliar symptoms of anthracnose. In addition to anthracnose ratings, plant height was measured at physiological maturity from the ground to the panicle and days to anthesis was recorded when 50% of the plants in a plot were at mid-anthesis.

Statistical analysis

Data were analyzed with SAS version 9.4 (SAS Institute, 2014). Trait means, standard deviation, range, best linear unbiased estimators (BLUEs) and best linear unbiased predictors (BLUPs) were calculated. Analysis of variance for individual environments and combined environments was performed using PROC MIXED, which employs restricted maximum likelihood (REML) to estimate the variance components. The models used for analysis of individual and combined environments were $Y_{ij} = \mu + \mathrm{Re}\, p_i + Gen_j + \varepsilon_{ij}$ and $Y_{ijk} = \mu + Env_k + \mathrm{Re}\, p_i(Env_k) + Gen_j + Gen_j \times Env_k + \varepsilon_{ijk}$ respectively, where Y is the trait of interest, μ is the mean effect, Rep_i is the effect of the *i*th replicate, Gen_j is the effect of the *j*th genotype, Env_k is the effect of the *k*th environment, Rep_i (Env_k) is the effect of replicates nested within environments, Gen_j x Env_k is the genotype-by-environment interaction and ε_{ijk} is the error term. All the effects were treated as random and broad-sense Heritability (H^2) on an entry mean basis was estimated for individual and combined environments using the formulae $H^2 = \dfrac{\sigma_g^2}{\sigma_g^2 + \dfrac{\sigma_e^2}{r}}$ and $H^2 = \dfrac{\sigma_g^2}{\sigma_g^2 + \dfrac{\sigma_{gxe}^2}{l} + \dfrac{\sigma_e^2}{rl}}$ respectively, where σ^2_g is variance component for genotype, σ^2_{gxe} for genotype-by-environment, and σ^2_e for error; *r* is the number of replications; and *l* is the number of environments. To determine the relationship between the traits, the phenotypic correlations were calculated using PROC CORR. Correlation between anthracnose ratings of the whole plot (ANP), leaf (ANF),

stalk (ANS), and head (ANH) of plants as well as between plant height, days to anthesis and anthracnose disease ratings of the whole plot were calculated.

Genotyping, linkage mapping & QTL mapping

Digital Genotyping (DG), a high throughput method of genotyping-by-sequencing developed for C4 grasses, was employed for genotyping the parental lines and the 100 RILs of the two populations (Morishige et al., 2013). Plants were grown in Sunshine MVP growing media (Sun Gro Horticulture, Inc.) for 14 days in a greenhouse with temperatures varying from 24^0C (night) to 30^0C (day), under natural sunlight and sodium halide lights. Leaf tissue from 10 to 12 day-old seedlings of each genotype was collected and total genomic DNA was extracted using the FastPrep FP120 instrument (Bio 101 Savant, Inc.) along with the FastDNA Spin Kit (MP Biomedicals, Inc.) and quantified using a Qubit Fluorometer (Invitrogen, Inc.). 500 ng DNA of each line was digested with the methylation-sensitive enzyme *Fse*I (New England BioLabs, Inc.) and Illumina template library made as described by Burrell et al. (2015). Standard Illumina protocols were followed for cluster generation from the template and single-end sequencing was carried out on an Illumina GAIIx (Illumina, Inc.). The Illumina sequencing data obtained was processed through a number of custom Perl and python scripts, and processed Illumina reads from the parental lines were aligned to the sorghum reference genome [Sbicolor v.3.1, www.phytozome.jgi.doe.gov, Paterson et al., (2009)]. Using BLASTN, polymorphisms between parents were identified and further scored through the progeny using custom perl scripts as described (Morishige et al., 2013). A

file containing the SNP markers identified in the RILs was generated and used for genetic linkage map construction.

A genetic linkage map was created for both sorghum RIL populations using JoinMap V4.0 (Van Ooijen and Voorrips, 2006). SNP markers were excluded that were close to a flanking marker (<10,000 bp) or a neighboring SNP that showed identical segregation to a framework marker. Linkage groups were determined using the independence test LOD score and groups with a high LOD score (10) were selected for mapping. For each linkage group, the genetic distance between markers was calculated using Kosambi's mapping function.

BLUEs of anthracnose disease ratings, plant height, and days-to-anthesis for each RIL in each environment and BLUPs combined from all environments were used for QTL analysis. Single-marker analysis, interval mapping (IM) and composite interval mapping (CIM) were performed using WinQTL Cartographer V2.5 (Wang et al., 2012), and inclusive composite interval mapping (ICIM) was done in IciMapping V4.1 (Wang et al., 2016a). The CIM analysis was performed using standard stepwise regression model 6 with default settings, 1000 permutations to determine the LOD significance threshold, and a walk speed of 1.0 cM. Other parameters such as genomic positions, effects of significant QTL, and the phenotypic variation explained (PVE) were also calculated. For each significant QTL, the position of the peak marker and the flanking markers of the QTL interval (1-LOD interval window) were determined, and the annotated genes (sorghum genome, v.3.1) within the QTL 1-LOD interval were surveyed for a potential role in plant disease resistance.

Genome sequencing of SC414-12E and SC155-14E

Genomic DNA for Illumina-based sequencing was extracted from leaf tissue of growth chamber-grown 14-day-old seedlings of SC414-12E and SC155-14E as detailed above for RIL genotyping. DNA library preparation was conducted by the Texas A&M AgriLife Genomics and Bioinformatics Service, followed by 100 bp paired-end sequencing in one lane on a HiSeq2000 (Illumina). Paired-end reads obtained by sequencing were uploaded to the CLC Genomics Workbench version 8.5.1 (CLC bio, Inc.) and duplicate reads were removed using the *Remove Duplicate Mapped Reads* feature. The remaining reads were trimmed and mapped to the BTx623 reference genome (Sbicolor v.3.1, www.phytozome.jgi.doe.gov, Paterson et al., 2009) using the *Trim Sequences* and *Map Reads to Reference* features, respectively. For read mapping, an alignment of at least 75% of length with more than 90% similarity was required. The mismatch cost was set to 2, and insertion and deletion costs were set to 3 and nonspecific read matches were mapped randomly. Variants were detected using the CLC Bio *Fixed Ploidy Variant Detection* feature with required variant probability set to 90%, neighborhood radius set to 5, minimum neighborhood quality set to 15 and minimum central quality of variant set to 20. The minimum coverage and count for a variant call were set to 15 and 3, respectively, and the variant had to be present in both forward and reverse reads. The annotated sorghum genome (v.3.1) downloaded from Phytozome v 11.0 was used to find coding variants in SC414-12E and SC155-14E compared to BTx623.

Results and discussion

Analysis of phenotypic data

Anthracnose disease incidence

To better understand the genetic basis of anthracnose resistance in sorghum, we evaluated two RIL populations from a cross of the elite sorghum inbred BTx623 that is susceptible to anthracnose with two anthracnose resistant adapted lines SC414-12E and SC155-14E. The sources of germplasm were selected not only due to their reported resistance to anthracnose (Mehta et al., 2005), but also because these improved 'converted' tropical accessions have been selected for agronomic fitness by sorghum improvement programs in the US. Thus, the genes or genomic regions of these resistant parents that harbor anthracnose resistance can be more readily utilized by breeding programs than sources of resistance that reside in genomes of more exotic (unadapted) germplasm.

In every environment, the mean anthracnose incidence of the RIL mapping populations was intermediate to their respective parents (Tables 1 and 2). The average disease incidence rating of susceptible (BTx623) and resistant (SC414-12E) parents varied among the environments, ranging from 4 to 8.8 and 1.25 to 3.5, respectively (Table 1). The second resistant parental genotype, SC155-14E, was highly resistant in all environments tested with an average disease incidence score of 1 (Table 2). The resistance classifications of these sorghum genotypes are in general agreement with previous studies (Mehta et al., 2005; Rooney et al., 2002). In the BTx623 × SC414-12E RIL population, the average anthracnose scores ranged from a low of 1.24 in the dry

environment of 2011-CS to 4.99 in 2013-GA where disease pressure was severe (Table 1). In the BTx623 × SC155-14E population, the average disease resistance rating for the RILs was consistently lower but the entries *per se* had a wider range of disease incidence ranging from highly susceptible (rating of 9) to highly resistant (rating of 1) (Table 2). The exception to these trends was the 2011-CS environment (ratings of 1-6), where the spread and development of disease were limited due to very dry conditions.

Transgressive segregation for anthracnose incidence was observed in both RIL populations; of particular interest were RILs displaying greater resistance than parent SC414-12E. This transgressive segregation may indicate that minor alleles for resistance are harbored in both BTx623 and SC414-12E and thus, favorable trait loci may be present in the susceptible parent (Young, 1996). Indeed, as is discussed later, minor QTL for resistance were located in this population, and the allele contributing resistance at one of these loci was derived from BTx623. Transgressive segregation was also observed in the population derived from resistant parent SC155-14E, but only for RILs that were slightly more susceptible than BTx623; no RIL was more resistant than SC155-14E, which was nearly disease free in the chosen environments (see Table 2).

Heritability estimates attributed a substantial portion of the phenotypic variation of anthracnose incidence to genetic effects. The broad sense heritability (H^2) estimates for anthracnose resistance were moderate to high, ranging from 0.36 to 0.86 in the BTx623 × SC414-12E population and from 0.73 to 0.94 for the BTx623 × SC155-14E population (Tables 1 and 2). These results are comparable to those reported previously

Table 1. Phenotypic trait values for parental inbreds BTx623 and SC414-12E and the derived 96 $F_{4:5}$ RILs evaluated in six environments and averaged across all environments. Values represent means with standard deviations (SD) shown in parentheses. Ranges for phenotypic values of the $F_{4:5}$ RILs along with broad sense heritability of each trait are shown.

Environment†	Trait	Mean (SD)			Range	Heritability
		BTx623	SC414-12E	RILs		
2011-CS	Anthracnose	NA‡	NA	1.24 (0.63)	1-5	0.36
2012-CS	Anthracnose	7.00 (0.00) [a]	2.00 (0.82) [b]	4.73 (1.80)	1-8	0.86
2013-CS	Anthracnose	7.75 (1.26) [a]	1.25 (0.50) [b]	3.43 (2.38)	1-9	0.79
2013-GA	Anthracnose	8.50 (0.58) [a]	2.67 (0.58) [b]	4.99 (2.52)	1-9	0.70
2015-CS	Anthracnose	6.50 (0.84) [a]	3.50 (1.05) [b]	4.65 (1.08)	1-7	0.69
2015-GA	Anthracnose	6.00 (1.10) [a]	2.83 (1.60) [b]	3.30 (1.87)	1-9	0.84
Combined	Anthracnose	7.00 (4.10)	2.60 (1.30)	3.72 (2.24)	1-9	0.82
2011-CS	Height	NA	NA	47.26 (8.07)	32-90	0.84
2012-CS	Height	47.75 (2.22) [a]	40.50 (6.35) [a]	49.66 (9.84)	31-74	0.90
2013-CS	Height	53.00 (3.56) [a]	45.50 (3.00) [b]	53.64 (8.74)	33-90	0.94
2013-GA	Height	NA	NA	NA	NA	NA
2015-CS	Height	52.50 (2.59) [a]	42.00 (2.10) [b]	48.47 (8.10)	24-68	0.93
2015-GA	Height	55.33 (3.72) [a]	47.33 (3.27) [b]	50.46 (10.23)	24-80	0.90
Combined	Height	52.50 (3.94)	44.00 (4.40)	49.90 (9.28)	24-90	0.93
2011-CS	Days to anthesis	NA	NA	69.58 (3.96)	60-81	0.85
2012-CS	Days to anthesis	73.00 (2.00) [a]	70.00 (0.00) [b]	70.04 (3.78)	60-84	0.71
2013-CS	Days to anthesis	81.75 (1.50) [a]	84.25 (2.87) [a]	83.77 (4.14)	64-93	0.70
2013-GA	Days to anthesis	NA	NA	NA	NA	NA
2015-CS	Days to anthesis	76.83 (2.93) [a]	81.50 (4.81) [a]	77.62 (5.74)	56-89	0.64
2015-GA	Days to anthesis	NA	NA	NA	NA	NA
Combined	Days to anthesis	77.14 (4.07)	79.00 (6.90)	75.25 (7.37)	56-93	0.82

† Defined as a combination of year and location; CS, College Station; GA, Georgia.

‡ Data not available.

Means followed by different alphabets [a] and [b] indicate significant differences of trait mean value between the two parents as calculated by Tukey's test.

Table 2. Phenotypic trait values for parental inbreds BTx623 and SC155-14E and the derived 100 $F_{4:5}$ RILs evaluated in six environments and averaged across all environments. Values represent means with standard deviations (SD) shown in parentheses. Ranges for phenotypic values of the $F_{4:5}$ RILs along with broad sense heritability of each trait are shown.

Environment†	Trait	Mean (SD)			Range	Heritability
		BTx623	SC155-14E	RILs		
2011-CS	Anthracnose	NA‡	NA	1.35 (0.78)	1-6	0.73
2012-CS	Anthracnose	7.00 (0.63) [a]	1.00 (0.00) [b]	2.68 (2.31)	1-8	0.94
2013-CS	Anthracnose	8.50 (0.53) [a]	1.00 (0.00) [b]	3.30 (2.84)	1-9	0.88
2013-GA	Anthracnose	8.88 (0.35) [a]	1.00 (0.00) [b]	2.91 (2.74)	1-9	0.90
2015-CS	Anthracnose	7.50 (0.58) [a]	1.00 (0.00) [b]	2.11 (1.63)	1-8	0.84
2015-GA	Anthracnose	4.00 (2.16)	NA	2.03 (1.70)	1-8	0.90
Combined	Anthracnose	7.57 (1.79)	1.00 (0.00)	2.39 (2.20)	1-9	0.90
2011-CS	Height	NA	NA	44.80 (6.88)	30-75	0.78
2012-CS	Height	50.83 (2.71) [a]	38.25 (3.86) [b]	49.85 (9.15)	31-75	0.87
2013-CS	Height	49.88 (2.90) [a]	36.25 (0.96) [b]	47.81 (8.38)	29-69	0.85
2013-GA	Height	NA	NA	NA	NA	NA
2015-CS	Height	55.75 (6.50) [a]	46.75 (9.18) [a]	50.28 (6.04)	36-62	0.79
2015-GA	Height	55.50 (4.73)	NA	52.06 (9.50)	28-76	0.81
Combined	Height	52.23 (4.57)	40.42 (7.06)	48.96 (8.45)	28-76	0.90
2011-CS	Days to anthesis	NA	NA	69.02 (4.25)	59-77	0.89
2012-CS	Days to anthesis	72.00 (2.19) [a]	74.00 (0.00) [a]	71.18 (3.57)	60-82	0.79
2013-CS	Days to anthesis	70.50 (1.41) [a]	71.50 (1.00) [a]	70.12 (4.16)	60-85	0.82
2013-GA	Days to anthesis	NA	NA	NA	NA	NA
2015-CS	Days to anthesis	72.25 (2.36) [a]	75.00 (1.15) [a]	74.57 (5.64)	56-92	0.82
2015-GA	Days to anthesis	NA	NA	NA	NA	NA
Combined	Days to anthesis	71.39 (1.97)	73.50 (1.73)	71.24 (4.93)	56-92	0.89

† Defined as a combination of year and location; CS, College Station; GA, Georgia.

‡ Data not available.

Means followed by different alphabets [a] and [b] indicate significant differences of trait mean value between the two parents as calculated by Tukey's test.

by Burrell et al. (2015). Based on these heritability estimates, selection for anthracnose resistance would likely result in significant genetic gains through the introgression of resistance from SC155-14E and SC414-12E into elite cultivars. It should be noted that the resistance present in SC155-14E is inherited as a recessive trait while resistance in SC414-12E is inherited as a dominant trait (Mehta et al., 2005). Despite the higher heritability estimates, introgression of a recessively inherited trait is more problematic in hybrid crops (including sorghum) as the trait must be introgressed into both parental lines that comprise the F_1 hybrid. Therefore, marker-assisted backcrossing will be of particular value in accelerating the introgression of this recessively inherited trait from SC155-14E into both male and female parental lines.

In both RIL populations, the analyses of variance for anthracnose in each environment, as well as the combined analysis across environments indicated significant phenotypic variation ($P < 0.001$ and $P < 0.05$) among the RILs. Additionally, the combined analysis of phenotypic data from all environments detected significant ($P < 0.001$) environmental effects and genotype-by-environment (G×E) interactions (Table 3). The different nature of the anthracnose reaction by specific genotypes in the various environments depends in part upon the races of *C. sublineolum* that are present in a given geographical location and to those environmental factors (e.g., humidity, rainfall) that influence the asexual reproduction and spread of the inoculum. Significant differences ($P < 0.001$ and $P < 0.01$) observed among the replicates for the 2013-CS environment in both populations suggested that the disease pressure varied amongst blocks in this environment. This block effect likely resulted from the unequal application

Table 3. Mean squares of ANOVA of anthracnose disease rating for the two RIL mapping populations in the six environments and combined across all environments.

BTx623 × SC414-12E	Mean squares					
Environment†	Genotype (G)	Replication	Error	Environment (E)	Replication(E)‡	G x E
2011-CS	0.49*	0.08	0.31			
2012-CS	5.70***	0.08	0.8			
2013-CS	8.91***	53.69 ***	1.87			
2013-GA	9.85***	0.02	2.93			
2015-CS	1.80***	0.34	0.56			
2015-GA	6.03***	2.32	0.97			
Combined	16.31***		1.24	380.50***	9.42***	3.28***
BTx623 × SC155-14E	**Mean squares**					
Environment†	**Genotype (G)**	**Replication**	**Error**	**Environment (E)**	**Replication(E)‡**	**G x E**
2011-CS	0.96***	0.41	0.26			
2012-CS	10.06***	1.62	0.60			
2013-CS	13.61***	24.94 **	1.65			
2013-GA	13.73***	0.26	1.40			
2015-CS	4.60***	0.05	0.72			
2015-GA	5.14***	0.53	0.53			
Combined	31.29***		0.85	95.72***	4.63***	3.23***

*, ** and *** are significant at the 0.05, 0.01 and 0.001 probability level, respectively.

† Defined as a combination of year and location; CS, College Station; GA, Georgia.

‡ Effect was nested within environment (E).

of inoculum due to a limited amount of inoculum remaining near the end of the inoculation process. The variation in disease incidence due to environmental and G×E effects was anticipated and thus, as many environments as possible were chosen for this study (along with different sources of resistance) to identify as many trait loci as possible for the eventual development of stable resistance to anthracnose (Rooney et al., 2002).

No significant correlations between anthracnose resistance and either plant height or days to anthesis were observed in the present populations (Table 4), which is unlike the results of Upadhyaya et al. (2013) who reported plant height and days to anthesis were negatively correlated with anthracnose severity. As plant height and days to anthesis are critical determinants of breeding value in sorghum, the lack of an association between anthracnose resistance in SC414-12E and SC155-14E indicates that utilization of these sources of anthracnose resistance will not be complicated by a negative association with plant height or flowering time. Similar to a previous report (Marley and Ajayi, 2002), a significant correlation between anthracnose ratings of the whole plot (ANP), leaf (ANF), stalk (ANS), and head (ANH) was observed in 2012-CS, 2013-CS, and 2013-GA (Table 5). Hence, the whole plot rating was considered to be a good predictive indicator of disease severity, and no further assessment of disease on stalks or heads was required in subsequent environments. This is noteworthy since scoring structures of individual plants (such as the stalks or heads) can be time-consuming, and is less amenable to rapid, high-throughput plant phenotyping by UAVs that are being presently utilized in many crop improvement programs including

Table 4. Pearson's correlation coefficients for height, days to anthesis and anthracnose of the two RIL mapping populations.

BTx623 × SC414-12E			
	Height	**Days to anthesis**	**Anthracnose**
Height	-	0.29***	-0.02
Days to anthesis		-	0.06
Anthracnose			-
BTx623 × SC155-14E			
	Height	**Days to anthesis**	**Anthracnose**
Height	-	0.13*	0.03
Days to anthesis		-	-0.09
Anthracnose			-

* and *** are significant at the 0.05 and 0.001 probability level, respectively.

Table 5. Pearson's correlation coefficients for anthracnose related traits of the two RIL mapping populations.

BTx623 × SC414-12E				
	ANP	**ANF**	**ANS**	**ANH**
ANP	-	0.84***	0.44***	0.80***
ANF		-	0.39***	0.89***
ANS			-	0.77***
ANH				-
BTx623 × SC155-14E				
	ANP	**ANF**	**ANS**	**ANH**
ANP	-	0.92***	0.73***	0.69***
ANF		-	0.61***	0.64***
ANS			-	0.79***
ANH				-

*** Significant at the 0.001 probability level.

ANP, anthracnose whole plot rating; ANF, anthracnose foliar rating; ANS, anthracnose stalk rating; ANH, anthracnose head rating.

sorghum. This will be of particular value in distant field locations where the use of a UAV to phenotype for anthracnose incidence could eventually be less costly and time-consuming.

Plant height and days to anthesis

While characterizing the genetics of anthracnose resistance in two sorghum RIL populations was the primary focus of this study, plant height and days to anthesis were analyzed to confirm consistency of the population both phenotypically and genetically. For most environments, mean values for the RIL populations were intermediate to the two parents of each population (Tables 1 and 2). As is commonly observed in populations derived from converted tropical sorghum accessions, transgressive segregation for plant height and days to anthesis was observed in RIL entries from both populations (Young, 1996). The relatively high heritability estimates for plant height (0.78-0.94) and days to anthesis (0.64-0.89) were expected as they are in agreement with previous reports for these traits in sorghum (Srinivas et al., 2009; Sukumaran et al., 2016; Zou et al., 2012).

Linkage maps

Digital genotyping of the BTx623 × SC414-12E and the BTx623 × SC155-14E populations was performed on an Illumina GAIIx. The unique sequences derived from the parents of each population were aligned to the BTx623 reference genome using BLASTN. The use of the methylation-sensitive enzyme *Fse*I reduced the representation of repetitive and pericentromeric heterochromatic regions allowing mostly unmethylated (gene-rich) regions to be sequenced. On aligning the reads of SC414-12E to the BTx623

reference genome, a total of 1420 single nucleotide polymorphism (SNP) markers were identified that were subsequently scored through the RILs. Of the 1420 SNPs, 425 SNPs were discarded for being redundant or having substantial missing data. The final linkage map contained a total of 857 unique SNPs spanning a length of 1732.6 cM that mapped to 12 linkage groups representing the 10 chromosomes of sorghum (Table 6). Two separate linkage groups spanned both chromosomes 3 and 6 in the final map. On average, one marker was present at 2 cM intervals across the linkage map.

For the BTx623 × SC155-14E population, 2061 SNPs were detected, of which 1105 were discarded for being redundant or having substantial missing data and 956 highly informative SNPs were mapped across 10 chromosomes covering a total map length of 1226.8 cM (Table 6). Markers on chromosome 6 mapped to two different linkage groups, resulting in a total of 11 linkage groups. The map was denser than that obtained for the BTx623 × SC414-12E RIL population with an average marker coverage of one marker per 1.2 cM.

With the high-quality sequenced genome of BTx623 being available (Sbicolor v.3.1, www.phytozome.jgi.doe.gov, Paterson et al., 2009), it was possible to ascertain how well each linkage map covered the sorghum genome. In general, all chromosomes were well covered by SNPs, although the gene-poor pericentromeric regions were under-represented with SNPs, which is expected based on the use of methylation-sensitive enzymes in Illumina template preparation (Morishige et al., 2013). This was observed for chromosomes 3 and 6 for the BTx623 × SC414-12E population and chromosome 6 for the BTx623 × SC155-12E population where the linkage groups could not be merged

Table 6. Linkage map marker coverage across the ten sorghum chromosomes generated for $F_{4:5}$ RIL mapping populations BTx623 × SC414-12E and BTx623 × SC155-14E.

BTx623 × SC414-12E											
Chromosome	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**Total**
Chromosome Length (cM)	199.7	149.3	125.5	207.1	170.5	119.7	199.2	194.3	197.6	169.7	1732.6
Number of Markers	131	109	107	92	71	61	66	57	76	87	857
Marker density† (cM)	1.52	1.37	1.17	2.25	2.40	1.96	3.02	3.41	2.60	1.95	2.02
Largest interval‡ (cM)	9.72	8.78	5.92	11.05	14.60	8.51	20.5	22.4	13.31	19.26	
Largest interval‡ (Mbp)	19.80	33.99	29.17	19.80	36.28	41.98	32.12	26.12	33.95	32.80	
BTx623 × SC155-14E											
Chromosome	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**Total**
Chromosome Length (cM)	183.2	163.7	129.1	137.8	114.8	101.7	103.3	113.5	71.2	108.2	1226.8
Number of Markers	166	114	131	109	82	96	67	60	42	89	956
Marker density† (cM)	1.10	1.44	0.99	1.26	1.40	1.06	1.54	1.89	1.70	1.22	1.28
Largest interval‡ (cM)	4.32	5.21	4.83	7.89	8.01	5.75	5.83	8.63	9.21	5.82	
Largest interval‡ (Mbp)	25.04	34.21	29.55	23.77	31.12	28.73	35.08	31.68	20.47	25.46	

† Defined as the distance between adjacent markers, measured in cM.

‡ Defined as the largest distance observed between adjacent markers in cM and million base pairs (Mbp).

due to low marker coverage around the centromere. The largest physical gaps between markers, which were found in the pericentromeric heterochromatic regions of the chromosome were ~42 Mbp and ~35 Mbp in the BTx623 × SC414-12E and BTx623 × SC155-12E populations, respectively.

Quantitative trait loci for anthracnose incidence, plant height, and days to anthesis

In total, 13 QTL in the BTx623 × SC414-12E RIL population and 16 QTL in the BTx623 × SC155-14E RIL population were detected for the three agronomic traits analyzed (Tables 7, 8, 9, 10). The threshold LOD score for a significant QTL was calculated as 3.3 for all traits and QTL in Tables 7 and 8 were significant in at least 3 environments for anthracnose and plant height, and two environments for days to maturity. Less consistent QTL, and those detected in fewer environments, are shown in Tables 9 and 10.

While linkage analysis of plant height was not a focus of this study, it did afford us the opportunity to assess the utility of these mapping populations and associated linkage maps to accurately locate regions of the sorghum genome that harbor important agronomic trait loci. QTL for height, one each on chromosomes 1, 7, and 9 were observed in the BTx623 × SC414-12E population (Table 7). The QTL on chromosome 1 (61.28-67.92 Mbp, LOD scores ranging from 3.39-7.54) accounted for 7 to 14% of the phenotypic variation. The QTL on chromosome 7 (8.72-57.49 Mbp, LOD scores ranging from 6.79-13.09), accounted for 20 to 35% of the PVE and was located nearly 3Mb away from the known *Dw3* gene. This QTL has been previously identified by Li et al. (2015) as qHT7.1 and found to be in repulsion linkage with the *Dw3* locus. The QTL on

chromosome 9 (52.82-58.14 Mbp, LOD scores ranging from 6.17-12.95) mapped to the position of the known height locus *Dw1* at ~57 Mb (Brown et al., 2008; Morris et al., 2013). BTx623 is recessive for the *dw1* locus whereas SC414-12E is dominant and hence expected to segregate in this population. A negative additive effect and phenotypic variation of 13 to 29% was attributed to this QTL. For the BTx623 × SC155-14E population (Table 8), a QTL for height was detected on chromosome 6 (LOD 4.88-9.54, PVE 14-27%) and was identified at a position between 41.92-44.83 Mbp, which corresponds to the known location of the *Dw2* locus (Brown et al., 2008; Li et al., 2015; Morris et al., 2013). BTx623 is dominant *Dw2* and SC155-14E recessive *dw2*, and thus segregation for this locus was expected in the RIL population. The quality and accuracy of the two linkage maps were thus assessed through the co-localization of QTL for plant height with known locations of dwarfing (*dw*) genes in the sorghum genome.

Table 7. Quantitative trait loci (QTL) detected for anthracnose disease incidence, plant height and days to anthesis in the $F_{4:5}$ RIL population of BTx623 × SC414-12E. QTL were predicted based on best linear unbiased estimators (BLUEs) for each environment.

Environment†	Trait	Chromosome	Peak Position (cM)	Confidence interval‡ (cM)	Left Marker	Right Marker	LOD§	PVE (%)	Additive effect¶
2013-GA	Anthracnose	2	106.40	105.70-106.80	chr02_67.62	chr02_68.22	5.31	17	-0.93
2015-CS	Anthracnose	2	100.70	99.00-101.20	chr02_65.45	chr02_66.10	4.00	7	-0.27
2015-GA	Anthracnose	2	126.60	121.80-129.80	chr02_72.79	chr02_75.54	5.04	11	-0.61
Combined	Anthracnose	2	121.60	119.60-128.20	chr02_71.42	chr02_75.54	4.69	9	-0.28
2013-GA	Anthracnose	4	118.90	118.00-122.40	chr04_54.20	chr04_56.01	3.59	10	0.72
2015-GA	Anthracnose	4	112.20	102.90-116.20	chr04_53.28	chr04_54.10	5.53	14	0.81
Combined	Anthracnose	4	112.20	100.00-119.50	chr04_51.71	chr04_55.75	3.31	6	0.27
2012-CS	Anthracnose	5	108.20	108.20-120.20	chr05_64.02	chr05_66.05	8.74	25	0.90
2013-CS	Anthracnose	5	117.20	116.60-118.10	chr05_64.69	chr05_65.26	14.35	39	1.41
2015-CS	Anthracnose	5	119.90	116.60-124.80	chr05_64.69	chr05_66.98	12.41	31	0.55
Combined	Anthracnose	5	117.20	116.90-118.10	chr05_64.69	chr05_65.26	8.62	20	0.45
2012-CS	Height	1	116.40	115.60-123.10	chr01_64.44	chr01_67.92	7.54	14	3.67
2013-CS	Height	1	116.40	115.60-124.90	chr01_64.44	chr01_67.92	5.12	10	2.74
2015-CS	Height	1	104.80	99.50-110.20	chr01_61.28	chr01_64.09	3.39	7	2.12
2015-GA	Height	1	104.80	99.50-108.10	chr01_61.28	chr01_64.09	3.72	8	2.79
Combined	Height	1	118.40	116.00-123.10	chr01_64.87	chr01_67.92	7.93	13	2.72
2011-CS	Height	7	98.20	93.50-104.30	chr07_46.11	chr07_56.52	6.79	20	3.78
2012-CS	Height	7	93.00	91.80-93.20	chr07_08.72	chr07_46.11	11.00	24	5.75
2013-CS	Height	7	102.20	102.20-105.70	chr07_56.46	chr07_57.49	10.86	27	5.05
2015-CS	Height	7	102.20	102.20-105.30	chr07_56.46	chr07_57.49	10.62	31	4.91
2015-GA	Height	7	102.20	102.20-106.00	chr07_56.46	chr07_57.49	9.47	26	5.54

Table 7. Continued

Environment†	Trait	Chromosome	Peak Position (cM)	Confidence interval‡ (cM)	Left Marker	Right Marker	LOD§	PVE (%)	Additive effect¶
Combined	Height	7	102.2	101.80-105.30	chr07_55.34	chr07_57.49	13.09	35	4.67
2011-CS	Height	9	144.5	143.30-146.40	chr09_52.82	chr09_54.14	6.17	18	-3.8
2012-CS	Height	9	166.3	164.60-176.40	chr09_56.11	chr09_58.14	8.52	13	-4.51
2013-CS	Height	9	166.3	161.80-168.40	chr09_56.11	chr09_56.75	12.95	29	-5.35
2015-CS	Height	9	161.9	158.60-167.70	chr09_55.96	chr09_56.75	8.71	23	-4.37
2015-GA	Height	9	166.3	160.00-167.70	chr09_56.08	chr09_56.75	8.37	21	-5.06
Combined	Height	9	166.3	164.20-175.70	chr09_56.11	chr09_58.14	8.09	12	-3.28
2011-CS	Days to anthesis	9	186.9	185.50-189.00	chr09_57.42	chr09_58.36	6.35	17	-1.6
2012-CS	Days to anthesis	9	186.9	185.50-189.00	chr09_57.42	chr09_58.36	4.75	14	-1.24

† Defined as a combination of year and location; CS, College Station; GA, Georgia.
‡ Interval containing the peak marker ±1 LOD.
§ LOD, logarithm of odds.
¶ Positive values indicate contribution of BTx623 allele in increasing trait value, negative values indicate contribution of BTx623 allele in decreasing trait value or contribution of SC414-12E allele in increasing trait value.

Table 8. Quantitative trait loci (QTL) detected for anthracnose disease incidence, plant height and days to anthesis in the $F_{4:5}$ RIL population of BTx623 × SC155-14E. QTL were predicted based on best linear unbiased estimators (BLUEs) for each environment.

Environment†	Trait	Chromosome	Peak Position (cM)	Confidence interval‡ (cM)	Left Marker	Right Marker	LOD§	PVE (%)	Additive effect¶
2012-CS	Anthracnose	4	83.7	80.80-90.30	chr04_53.95	chr04_57.22	3.71	5	0.58
2013-GA	Anthracnose	4	92.2	91.60-93.30	chr04_57.23	chr04_57.74	6.24	16	1.10
2015-CS	Anthracnose	4	98.2	97.20-99.20	chr04_59.44	chr04_60.68	4.14	9	0.54
2015-GA	Anthracnose	4	98.2	95.70-99.20	chr04_58.61	chr04_60.68	3.71	9	0.51
Combined	Anthracnose	4	86.1	84.70-87.40	chr04_54.90	chr04_56.87	4.30	7	0.41
2011-CS	Anthracnose	9	0.9	0.20-2.00	chr09_00.74	chr09_01.28	5.80	19	0.31
2012-CS	Anthracnose	9	0.9	0.00-2.10	chr09_00.74	chr09_01.28	21.55	42	1.55
2013-CS	Anthracnose	9	1.6	0.00-1.70	chr09_00.74	chr09_01.28	27.65	62	2.16
2013-GA	Anthracnose	9	0.9	0.00-2.10	chr09_00.74	chr09_01.28	8.43	24	1.31
2015-CS	Anthracnose	9	0.9	0.40-4.80	chr09_00.74	chr09_01.59	8.85	23	0.74
2015-GA	Anthracnose	9	0.9	0.60-2.60	chr09_00.74	chr09_01.34	9.55	26	0.83
Combined	Anthracnose	9	0.9	0.60-1.80	chr09_00.74	chr09_01.28	17.37	40	0.97
2012-CS	Height	6	19.1	17.50-21.20	chr06_42.65	chr06_44.83	8.28	24	4.43
2013-CS	Height	6	18.3	15.88-20.10	chr06_42.53	chr06_44.83	4.88	14	2.91
2015-CS	Height	6	17.7	17.20-19.80	chr06_42.64	chr06_42.83	7.90	22	2.60
2015-GA	Height	6	17.5	16.10-20.60	chr06_42.53	chr06_44.83	6.36	19	4.11
Combined	Height	6	18.6	13.30-18.90	chr06_41.92	chr06_42.83	9.54	27	3.01
2011-CS	Days to anthesis	3	122.9	122.00-125.20	chr03_72.51	chr03_72.90	4.34	11	1.42
2012-CS	Days to anthesis	3	99.5	98.20-108.90	chr03_62.17	chr03_66.58	3.44	8	0.95
2013-CS	Days to anthesis	3	101.7	101.10-108.50	chr03_62.17	chr03_66.58	4.45	10	1.23
2015-CS	Days to anthesis	3	101.7	100.70-104.10	chr03_62.17	chr03_65.21	3.66	10	1.73
Combined	Days to anthesis	3	101.7	100.90-102.30	chr03_62.17	chr03_63.67	6.32	13	1.19

Table 8. Continued

Environment†	Trait	Chromosome	Peak Position (cM)	Confidence interval‡ (cM)	Left Marker	Right Marker	LOD§	PVE (%)	Additive effect¶
2011-CS	Days to anthesis	4	28.3	27.90-28.60	chr04_04.30	chr04_04.99	5.48	13	-1.51
2012-CS	Days to anthesis	4	28.3	27.90-28.90	chr04_04.30	chr04_04.99	6.87	17	-1.38
2013-CS	Days to anthesis	4	27.6	26.40-28.60	chr04_04.23	chr04_04.99	5.83	13	-1.44
Combined	Days to anthesis	4	28.3	27.70-28.60	chr04_04.30	chr04_04.99	6.2	13	-1.18

† Defined as a combination of year and location; CS, College Station; GA, Georgia.
‡ Interval containing the peak marker ±1 LOD.
§ LOD, logarithm of odds.
¶ Positive values indicate contribution of BTx623 allele in increasing trait value, negative values indicate contribution of BTx623 allele in decreasing trait value or contribution of SC155-14E allele in increasing trait value.

Table 9. Less consistent Quantitative trait loci (QTL) detected for anthracnose disease incidence, plant height and days to anthesis in the $F_{4:5}$ RIL population of BTx623 × SC414-12E. QTL were predicted based on best linear unbiased estimators (BLUEs) for each environment.

Environment†	Trait	Chromosome	Peak Position (cM)	Confidence interval‡ (cM)	Left Marker	Right Marker	LOD§	PVE (%)	Additive effect¶
2015-GA	Anthracnose	1	123.70	117.70-126.00	chr01_64.87	chr01_70.08	4.27	10	0.55
Combined	Anthracnose	1	100.40	94.50-103.40	chr01_61.28	chr01_62.91	3.98	7	0.24
2013-CS	Anthracnose	9	151.70	151.30-153.00	chr09_54.98	chr09_55.47	3.60	5	0.53
2015-CS	Anthracnose	9	162.90	157.20-167.70	chr09_55.47	chr09_56.75	6.64	14	0.40
2015-CS	Height	3	57.70	54.40-58.50	chr03_14.12	chr03_16.37	4.07	8	2.65
2012-CS	Days to anthesis	2	100.20	97.40-103.80	chr02_64.14	chr02_67.62	3.48	9	1.01
Combined	Days to anthesis	2	100.20	99.30-100.60	chr02_64.14	chr02_66.10	4.77	13	0.89
2011-CS	Days to anthesis	7	14.70	09.70-20.10	chr07_01.01	chr07_01.64	3.60	9	1.11
2011-CS	Days to anthesis	9	134.90	134.50-140.50	chr09_51.67	chr09_53.17	6.01	15	-1.57

† Defined as a combination of year and location; CS, College Station; GA, Georgia.

‡ Interval containing the peak marker ±1 LOD.

§ LOD, logarithm of odds.

¶ Positive values indicate contribution of BTx623 allele in increasing trait value, negative values indicate contribution of BTx623 allele in decreasing trait value or contribution of SC414-12E allele in increasing trait value.

Table 10. Less consistent Quantitative trait loci (QTL) detected for anthracnose disease incidence, plant height and days to anthesis in the $F_{4:5}$ RIL population of BTx623 × SC155-14E. QTL were predicted based on best linear unbiased estimators (BLUEs) for each environment.

Environment†	Trait	Chromosome	Peak Position (cM)	Confidence interval‡ (cM)	Left Marker	Right Marker	LOD§	PVE (%)	Additive effect¶
2013-GA	Anthracnose	1	52	50.80-56.60	chr01_13.07	chr01_14.40	3.78	8	-0.78
2012-CS	Anthracnose	8	61.6	61.20-64.00	chr08_54.57	chr08_55.85	4.44	6	0.61
2015-CS	Height	1	30.9	28.30-33.50	chr01_09.02	chr01_11.20	4.04	10	1.84
2015-CS	Height	1	175.9	175.40-177.10	chr01_79.04	chr01_79.51	3.82	8	1.67
Combined	Height	1	175.9	175.10-177.70	chr01_79.04	chr01_79.54	4.35	10	1.88
2012-CS	Height	2	97.8	96.00-99.20	chr02_65.04	chr02_65.72	4.73	12	-2.95
2011-CS	Height	3	42	38.30-42.70	chr03_07.42	chr03_07.78	3.36	10	2.31
2011-CS	Height	3	99.5	98.40-99.80	chr03_62.38	chr03_63.17	4.05	11	-2.23
2013-CS	Height	8	65.6	64.90-66.90	chr08_55.85	chr08_56.41	3.46	10	-2.40
2015-GA	Height	8	74.1	74.10-78.00	chr08_56.92	chr08_58.27	4.61	12	-3.08
Combined	Height	8	74.1	74.00-77.60	chr08_56.92	chr08_58.27	4.36	10	-1.83
2012-CS	Height	10	3.9	2.70-6.50	chr10_00.72	chr10_01.49	3.94	10	2.83
2013-CS	Days to anthesis	4	79.2	77.90-81.00	chr04_53.09	chr04_54.23	4.31	9	-1.19
2013-CS	Days to anthesis	10	61.0	59.80-63.30	chr10_48.93	chr10_52.28	3.77	8	1.16

† Defined as a combination of year and location; CS, College Station; GA, Georgia.

‡ Interval containing the peak marker ±1 LOD.

§ LOD, logarithm of odds.

¶ Positive values indicate contribution of BTx623 allele in increasing trait value, negative values indicate contribution of BTx623 allele in decreasing trait value or contribution of SC155-14E allele in increasing trait value.

In the BTx623 × SC414-12E population a QTL for days to anthesis with negative additive effect was located on chromosome 9 (57.42-58.36 Mbp) in two of the four environments (Table 7). This QTL co-localized with the known QTL, qFT9.1 (~46-74.61 Mbp) and SbFL9.1 (~58 Mbp) reported by Sukumaran et al. (2016) and Higgins et al. (2014), respectively. The LOD score and PVE values for this QTL ranged from 4.75 to 6.35 and 14 to 17%, respectively. For the BTx623 × SC155-14E population, two QTL for days to anthesis were recorded (Table 6). The QTL on chromosome 4 (4.23-4.99 Mbp, LOD scores ranging from 5.48-6.87), with a negative additive effect explained 13 to 17% phenotypic variation, and the QTL on chromosome 3 (62.17-72.9 Mbp, LOD scores ranging from 3.44-6.32) accounted for 8 to 13% phenotypic variation. The QTL for days to anthesis identified in previous studies on chromosome 3 at ~1.7 Mbp (El Mannai et al., 2012; Srinivas et al., 2009) did not co-localize with the flowering time locus in the present investigation. Both of these QTL found on chromosomes 3 and 4 are unique to this study and hence they may be considered as novel loci regulating flowering time in sorghum

Genetic studies have identified numerous resistant sources for anthracnose in sorghum (Marley and Ajayi, 2002; Mehta et al., 2005; Sharma et al., 2012; Singh, 2014), and two unique sources identified included converted lines SC414-12E and SC155-14E. The present QTL analyses of RIL populations derived from these resistance sources revealed a total of 9 unique QTL for anthracnose resistance, including 5 and 4 trait loci harbored by the genomes of SC414-12E and SC155-14E, respectively.

In the BTx623 × SC414-12E population, a QTL for anthracnose resistance on chromosome 2 was identified in three of the six environments tested (Table 7). This QTL (65.45-75.54 Mbp, LOD scores ranging from 4.00-5.31), explained 7 to 17% of the phenotypic variation and had a negative additive effect, implying the role of susceptible parent BTx623 in contributing a portion of the anthracnose resistance observed in the SC414-12E RIL population. This was supported by the observation that in some environments SC414-12E was moderately resistant (higher phenotypic ratings), and specific RILs had greater resistance than SC414-12E. Environment-specific QTL for anthracnose were detected on chromosomes 4 and 5. The QTL on chromosome 4 (51.71-56.01 Mbp, LOD scores ranging from 3.31-5.53), accounting for 6 to 14% of the phenotypic variation, was detected in the GA environments. Although this location-specific QTL was also detected in the same position in other environments (2013-CS and 2015-CS), the LOD score (1.83-2.28) was below the level to declare it significant in these other environments (data not shown). The QTL on chromosome 5 (64.02-66.98 Mbp, LOD scores ranging from 8.74-14.35) explained 25 to 39% of the phenotypic variance and was only detected in CS environments and the combined analysis, but not in GA environments. In 2011-CS this QTL was detected at the same position but with a lower LOD score of 2.8 (data not shown). As anthracnose susceptibility phenotype is influenced by plant genetics, the environment (rainfall, humidity), and disease pathotypes, it was not surprising that disease reactions differed across environments. In this study, a mixture of 7 to 8 known pathotype isolates was used for manual inoculation of disease in the CS environments whereas natural infestation with indigenous

pathotypes was used for disease pressure in all GA environments. In addition, overhead sprinkler irrigation was used to enhance the disease pressure in the GA environments while no supplemental overhead irrigation was employed in the CS environments. These different environmental and pathotype pressures allowed for the identification of environment-specific resistance loci along with loci conditioning resistance across environments. Identifying both classes of resistance QTL (environment-specific and general) will be critical to breeding for stable resistance across multiple environments especially if a majority of the breeding and selection is focused at a single location.

The high-quality genome sequence of sorghum facilitated a comparison of the physical location of anthracnose resistance loci identified in the present study with published QTL to identify novel and overlapping resistance loci. Various studies have identified the distal end of sorghum chromosome 5 as harboring various QTL for disease resistance. Recently, a greenhouse study of a PI 609251 x SC112-14 RIL population by Cuevas et al. (2014) reported an anthracnose resistance loci on chromosome 5 (63.68-65.66 Mbp) that overlapped with a resistance trait locus from the present study (64.02-66.98 Mbp). However, the source of resistance reported by Cuevas et al., (2014) was from an unrelated sorghum accession SC112-14, (working group zerazera) that is not in the same sorghum working group as SC414-12E (working group caudatum-kafir). Thus, it is inconclusive if the same chromosome 5 QTL for anthracnose resistance was identified in the two studies despite their overlapping genomic locations. Perumal et al. (2009) and Burrell et al. (2015) utilized resistant genotype SC748-5 to map an anthracnose resistance QTL to chromosome 5, but these QTL at ~70 Mbp were ~5 Mbp

away from the chromosome 5 QTL identified in SC414-12E in this study. Also the segregation of $F_{2:3}$ progeny derived from a cross between SC748-5 and SC414-12E indicates the presence of different sources of resistance in these genotypes (Mehta et al., 2005). Thus, it is conceivable that the same locus was not identified in the present study.

In the BTx623 × SC155-14E population, two QTL for anthracnose resistance were detected on chromosomes 4 and 9 (Table 8). The QTL on chromosome 9 (0.74-1.28 Mbp) was consistently found in all six environments and displayed the highest LOD score (27.65), the greatest additive genetic effect (2.16), and the highest PVE (62%) of all trait loci detected in the present study. This major anthracnose QTL is located at a different position than the two resistance-conferring loci *Cs*1A and *Cs*2A identified by Biruma et al. (2012), and represents an obvious target for marker-assisted introgression for stable anthracnose resistance across different environments. The QTL on chromosome 4 (53.95-60.68 Mbp, LOD scores ranging from 3.71-6.24) which accounted for 5 to 16% of the phenotypic variation, was found to overlap with the chromosome 4 QTL found in the BTx623 × SC414-12E population.

Identification of candidate genes underlying anthracnose QTL

Scanning the annotated reference sorghum genome (v.3.1) revealed 131 genes of known function, located within a 1-LOD interval of the anthracnose QTL found on chromosome 5 (~2.95 Mbp interval) in the BTx623 × SC414-12E population. Of these, ~36 genes were annotated as possibly being associated with plant disease resistance based on the present sorghum genome annotation. Genes within this QTL interval that may condition anthracnose resistance included the NB-ARC class of genes,

pathogenesis-related genes, genes involved in the synthesis and regulation of flavonoids, the hypersensitive response, cell death, cuticle development, and chitin and toxin catabolic processes. Therefore, an effective strategy for marker-assisted introgression may simply involve introgressing the distal end of chromosome 5 from resistant genotypes into more susceptible lines unless it is demonstrated that many resistance genes are linked in repulsion phase. In that event, further efforts are required to break up the linkage blocks harboring both resistant and susceptible genes for foliar disease resistance on chromosome 5.

For the anthracnose QTL found on chromosome 9 (~0.85 Mbp) in the BTx623 × SC155-14E population, the interval contained 72 annotated genes of which nearly 27 were annotated as having a biological role in disease resistance. Candidate genes within this QTL included those involved in programmed cell death, sesquiterpene biosynthesis, response to wounding, protein ubiquitination, xenobiotic stress response, oxidative stress response, flavonol biosynthesis, NB-ARC genes and chitinase biosynthesis.

To extend the investigation of candidate genes underlying the anthracnose resistance QTL, the genomes of resistant parental genotypes SC414-12E and SC155-14E were resequenced. Following removal of duplicate reads and read trimming, ~335.27 M paired-end sequences of inbred SC414-12E were generated and aligned to the BTx623 reference genome. Similarly, 249.99 M paired-end sequence reads were generated by resequencing the genome of resistant parent SC155-14E. Resequencing of the genomes of SC414-12E and SC155-14E led to the identification of ~1.65 M and 1.95 M sequence variants between BTx623 and SC414-12E and SC155-14E, respectively (data not

shown). The availability of sequence data that includes sequence polymorphisms between the susceptible and resistant parents afforded the opportunity to conduct a preliminary examination of sequence differences in potential candidate genes in the parental genotypes. The summation of sequence polymorphisms located within anthracnose QTL candidate genic regions is shown in Tables 11 and 12. In general, numerous nonsynonymous SNPs in candidate genes were observed as were SNPs in upstream regions that may harbor regulatory sequences. Additionally, sequence changes leading to insertion or/and deletion of amino acids, shift of the open reading frame, and premature termination of a protein due to a frameshift or introduction of a stop codon were observed. Some of these changes were in conserved protein domains (data not shown).

Candidate genes underlying the resistance trait locus on chromosome 9 were surveyed because this trait locus conferred resistance in all environments tested and also explained the greatest percentage of the resistant phenotype of any QTL detected. Four genes within the chromosome 9 trait locus belong to the NB-LRR gene family. Amino acid changes (ranging from 10-41 nonsynonymous substitutions) were detected in these genes, with Sobic.009G012900 having an amino acid substitution in the LRR domain. Many proteases and protease inhibitors (PIs) are associated with pathogen virulence and plant defense. Pathogens release proteases to digest host tissues, to counter which the host plant accumulates PIs. These PR plant proteins are known to have defensive capabilities against proteolytic enzymes produced by insects, fungi and bacteria during pathogenesis (Lorito et al., 1994; Turra and Lorito, 2011). Eight serine protease inhibitor

Table 11. Annotated sorghum genes with a role in plant disease resistance, under the 1 LOD interval of a major anthracnose QTL on sorghum chromosome 5. Mutations and amino acid changes in genes and coding DNA sequences (CDS) of parents BTx623 and SC414-12E were identified using resequencing data. Annotation file containing Arabidopsis and rice orthologs was downloaded from Joint Genome Institute's Genome Portal (http://phytozome.jgi.doe.gov).

Gene	Total mutations in		Amino acid changes	Arabidopsis/ Rice ortholog	Annotated Function	Biological process	Reference
	Gene	CDS					
Sobic.005G167400	42	33	9	AT3G07040	NB-ARC domain-containing disease resistance protein	defense response, plant-type hypersensitive response	(van Ooijen et al., 2008)
Sobic.005G167500	0	0	0	AT1G59620	Disease resistance protein (CC-NBS-LRR class) family	defense response	(Tan et al., 2007)
Sobic.005G167600	9	9	8	AT1G58410	Disease resistance protein (CC-NBS-LRR class) family	defense response, apoptosis	(Mace et al., 2014)
Sobic.005G167650	3	3	0	AT3G46730	NB-ARC domain-containing disease resistance protein	defense response, apoptosis	
Sobic.005G168500	15	2	0	AT3G54560	histone H2A 11	defense response to bacterium	(Ding and Wang, 2015)
Sobic.005G168600	4	4	1	AT5G28840	GDP-D-mannose 3\',5\'-epimerase	L-ascorbic acid biosynthetic process	(Taqi et al., 2011)
Sobic.005G168700	3	1	0	AT1G04220	3-ketoacyl-CoA synthase 2	response to osmotic stress, response to wounding	
Sobic.005G169000	0	0	0	AT1G19440	3-ketoacyl-CoA synthase 4	cuticle development	(Lee et al., 2009)
Sobic.005G169200	3	3	0	AT3G04720	pathogenesis-related 4	defense response to fungus	(Van Loon et al., 2006)
Sobic.005G169300	3	3	0	AT3G04720	pathogenesis-related 4	defense response to fungus	
Sobic.005G169400	0	0	0	AT3G04720	pathogenesis-related 4	defense response to fungus	
Sobic.005G172400	27	1	0	AT3G23400	Plastid-lipid associated protein PAP / fibrillin family protein	defense response to bacterium	(Singh and McNellis, 2011)
Sobic.005G172900	34	5	0	AT4G01070	UDP-Glycosyltransferase superfamily protein	flavonoid biosynthetic process	(Le Roy et al., 2016)
Sobic.005G173200	14	6	0	AT4G01070	UDP-Glycosyltransferase superfamily protein	flavonoid biosynthetic process	

Table 11. Continued

Gene	Total mutations in		Amino acid changes	Arabidopsis/ Rice ortholog	Annotated Function	Biological process	Reference
	Gene	CDS					
Sobic.005G175000	0	0	0	AT5G41210	glutathione S-transferase THETA 1	toxin catabolic process	(Shahrtash, 2013)
Sobic.005G175200	2	0	0	AT1G65790	receptor kinase 1	defense response	(Pastuglia et al., 2002)
Sobic.005G175500	218	218	0	AT4G05200	cysteine-rich RLK (RECEPTOR-like protein kinase) 25	defense response	(Chen et al., 2003)
Sobic.005G175600	7	4	0	AT4G23180	cysteine-rich RLK (RECEPTOR-like protein kinase) 10	defense response	
Sobic.005G176300	27	27	10	AT3G50950	HOPZ-ACTIVATED RESISTANCE 1	defense response	(Peele, 2015)
Sobic.005G176500	12	3	0	Os09g11790	DEFL14 - Defensin and Defensin-like DEFL family, expressed	defense response	(Stotz et al., 2009)
Sobic.005G177100	4	2	2	AT5G24090	chitinase A	response to wounding, chitin catabolic process	(Punja and Zhang, 1993)
Sobic.005G177400	2	1	0	AT5G24090	chitinase A	response to wounding, chitin catabolic process	
Sobic.005G177500	4	1	1	AT5G24090	chitinase A	response to wounding, chitin catabolic process	
Sobic.005G177600	1	1	0	AT5G24090	chitinase A	response to wounding, chitin catabolic process	
Sobic.005G179400	49	35	18	AT3G56860	UBP1-associated protein 2A	cell death, defense response	(Kim et al., 2008)
Sobic.005G181700	53	32	12	AT3G14470	NB-ARC domain-containing disease resistance protein	defense response	

Table 11. Continued

Gene	Total mutations in		Amino acid changes	Arabidopsis/ Rice ortholog	Annotated Function	Biological process	Reference
	Gene	CDS					
Sobic.005G181800	21	16	4	AT3G46730	NB-ARC domain-containing disease resistance protein	defense response	
Sobic.005G182100	5	5	4	AT3G07040	NB-ARC domain-containing disease resistance protein	defense response, plant-type hypersensitive response	
Sobic.005G182200	6	6	0	AT3G07040	NB-ARC domain-containing disease resistance protein	defense response, plant-type hypersensitive response	
Sobic.005G182800	21	21	2	AT3G46710	NB-ARC domain-containing disease resistance protein	defense response	
Sobic.005G182900	87	81	9	AT3G07040	NB-ARC domain-containing disease resistance protein	defense response, plant-type hypersensitive response	
Sobic.005G183000	191	190	10	AT1G58807	Disease resistance protein (CC-NBS-LRR class) family	defense response	
Sobic.005G183300	112	93	19	AT1G53350	Disease resistance protein (CC-NBS-LRR class) family	defense response	
Sobic.005G183400	64	64	11	AT3G14470	NB-ARC domain-containing disease resistance protein	defense response	
Sobic.005G183500	261	25	4	AT3G46530	NB-ARC domain-containing disease resistance protein	defense response, plant-type hypersensitive response	
Sobic.005G183700	22	22	2	AT5G48930	hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyl transferase	regulation of flavonoid biosynthetic process	(Miedes et al., 2015)

Table 12. Annotated sorghum genes with a role in plant disease resistance, under the 1 LOD interval of a major anthracnose QTL on sorghum chromosome 9. Mutations and amino acid changes in genes and coding DNA sequences (CDS) of parents BTx623 and SC155-14E were identified using resequencing data. Annotation file containing Arabidopsis orthologs was downloaded from Joint Genome Institute's Genome Portal (http://phytozome.jgi.doe.gov).

Gene	Total mutations in		Amino acid changes	Arabidopsis/ Rice ortholog	Annotated Function	Biological process	Reference
	Gene	CDS					
Sobic.009G008800	32	27	1	AT4G35350	xylem cysteine peptidase 1	programmed cell death , proteolysis	(Pogány et al., 2015)
Sobic.009G009000	14	14	1	AT5G23960	terpene synthase 21	sesquiterpene biosynthetic process	(Singh and Sharma, 2015)
Sobic.009G009200	156	153	6	AT5G23960	terpene synthase 21	sesquiterpene biosynthetic process	
Sobic.009G009300	33	33	0	AT5G23960	terpene synthase 21	sesquiterpene biosynthetic process	
Sobic.009G009400	1	1	0	AT2G38870	Serine protease inhibitor, potato inhibitor I-type family protein	defense response to fungus, response to wounding	(Turra and Lorito, 2011)
Sobic.009G009500	33	3	1	AT2G38870	Serine protease inhibitor, potato inhibitor I-type family protein	defense response to fungus, response to wounding	(Lorito et al., 1994)
Sobic.009G009600	30	30	1	AT5G43580	Serine protease inhibitor, potato inhibitor I-type family protein	defense response to fungus, response to wounding	
Sobic.009G009700	0	0	0	AT2G38870	Serine protease inhibitor, potato inhibitor I-type family protein	defense response to fungus, response to wounding	
Sobic.009G009800	0	0	0	AT2G38870	Serine protease inhibitor, potato inhibitor I-type family protein	defense response to fungus, response to wounding	

Table 12. Continued

Gene	Total mutations in		Amino acid changes	Arabidopsis/ Rice ortholog	Annotated Function	Biological process	Reference
	Gene	CDS					
Sobic.009G009900	1	1	0	AT2G38870	Serine protease inhibitor, potato inhibitor I-type family protein	defense response to fungus, response to wounding	
Sobic.009G010000	0	0	0	AT2G38870	Serine protease inhibitor, potato inhibitor I-type family protein	defense response to fungus, response to wounding	
Sobic.009G010150	13	13	0	AT2G38870	Serine protease inhibitor, potato inhibitor I-type family protein	defense response to fungus, response to wounding	
Sobic.009G010400	51	4	0	AT2G39720	RING-H2 finger C2A	protein ubiquitination	(Trujillo and Shirasu, 2010)
Sobic.009G010900	3	3	1	AT2G37660	NAD(P)-binding Rossmann-fold superfamily protein	defense response to bacterium	(Dehury et al., 2014)
Sobic.009G011000	0	0	0	AT1G07530	SCARECROW-like 14	xenobiotic stress response	(Fode et al., 2008)
Sobic.009G011200	22	5	2	AT2G37650	GRAS family transcription factor	xenobiotic stress response	
Sobic.009G011300	2	1	1	AT2G37650	GRAS family transcription factor	xenobiotic stress response	
Sobic.009G012100	31	11	1	AT3G12500	basic chitinase	defense response to fungus	(Punja and Zhang, 1993)
Sobic.009G012900	15	15	10	AT2G34930	disease resistance family protein / LRR family protein	disease resistance, defense response to fungus	(Shanmugam, 2005)
Sobic.009G013000	64	56	32	AT3G14470	NB-ARC domain-containing disease resistance protein	apoptosis, defense response	(Biruma et al., 2012)

Table 12. Continued

Gene	Total mutations in		Amino acid changes	Arabidopsis/ Rice ortholog	Annotated Function	Biological process	Reference
	Gene	CDS					
Sobic.009G013100	66	54	41	AT3G14470	NB-ARC domain-containing disease resistance protein	apoptosis, defense response	
Sobic.009G013300	65	49	41	AT3G14470	NB-ARC domain-containing disease resistance protein	apoptosis, defense response	
Sobic.009G013400	15	15	1	AT2G28900	outer plastid envelope protein 16-1	response to wounding	(Pollmann et al., 2007)
Sobic.009G013900	62	45	2	AT2G28840	XB3 ortholog 1 in Arabidopsis thaliana	ligase activity	(Wang et al., 2006b)
Sobic.009G016300	6	6	1	AT5G59820	C2H2-type zinc finger family protein	response to oxidative stress, response to wounding	(Sun et al., 2010)
Sobic.009G016600	6	0	0	AT3G46130	myb domain protein 48	flavonol biosynthesis,response to salicylic acid	(Park et al., 2008)
Sobic.009G016700	2	2	0	AT1G13440	glyceraldehyde-3-phosphate dehydrogenase C2	defense response to bacterium, response to oxidative stress	(Henry et al., 2015)

genes from Sobic.009G009400 to Sobic.009G010150 of the potato inhibitor I-type family were observed, and genes Sobic.009G009500 and Sobic.009G009600 had a single amino acid change. Genes Sobic.009G011000, Sobic.009G011200 and Sobic.009G011300 are SCARECROW-like 14 transcription factor orthologs, which are known to affect the transcription of stress responsive genes and involved in the detoxification of xenobiotics (Fode et al., 2008; Ramel et al., 2012). Two amino acid changes in Sobic.009G011200 and one in Sobic.009G011300 were observed. Studies have reported production of antimicrobial compounds as a mechanism of anthracnose resistance (Lo et al., 1999). Phytoalexins are toxic host metabolites produced in response to pathogen invasion or stress (Smith, 1996). Previous research (Snyder and Nicholson, 1990; Tenkouano et al., 1993) in sorghum has shown accumulation of reddish-brown flavonoid compounds at the site of fungal infection, and the pigments included 3-deoxyanthocyanidin flavonoids like luteolinidin, 5-methoxyluteolinidin, apigeninidin and a caffeic acid ester of arabinosyl 5-*O*-apigeninidin. Sobic.009G016600 is annotated as a putative transcription factor (MYB48) that functions to regulate flavonol biosynthesis. Sobic.009G009000, Sobic.009G009200 and Sobic.009G009300 are terpene synthase orthologs known to produce sesquiterpenes in Arabidopsis. As part of a plant defense mechanism, in some plant species, sesquiterpenes are produced to act as phytoalexins (Singh and Sharma, 2015).

In summary, this study provides much-needed information on regions of the sorghum genome that condition resistance to the fungal disease anthracnose. The location of trait loci that condition resistance across environments or resistance in a

specific environment will permit the use of marker-assisted introgression of resistance into elite cultivars with the aim of developing durable resistance to this critical disease. Finally, the brief survey conducted here of candidate genes underlying a major resistance QTL reveals that this genomic region encodes a wealth of genes that may play a role in host plant resistance, but also clearly demonstrates that identifying the resistance gene (or linked genes) underlying this trait locus will require linkage map refinement and validation of candidate genes, which is far beyond the scope of this study but this material will serve as a future resource to understand the molecular mechanisms conditioning anthracnose resistance in specific sorghum cultivars.

CHAPTER III

IDENTIFICATION OF QUANTITATIVE TRAIT LOCI CONDITIONING SALT TOLERANCE IN SORGHUM

Introduction

Soil salinity is one of the most severe abiotic stresses limiting the productivity of agricultural crops worldwide. Apart from natural causes of soil salinity (weathering of rocks, rising water tables, and wind/rain-mediated deposition of oceanic salts), irrigation and inappropriate land management are other major factors that increase soil salinity. More than 6% of the world's total landmass (i.e. 800 million hectares) is affected by salinity (www.fao.org), and nearly half of the total area of irrigated soils that contribute roughly one-third of global food production is affected by salinization.

Salinity stress affects crop growth and yield mainly by immediate osmotic effects or longer-term ion toxicity. Increase of salt concentration in soil decreases the water potential of soil water, which limits the uptake of water resulting in cellular dehydration. The ionic effect within the plant manifests itself over a period of time by passive accumulation of salt ions in plant cells thereby affecting photosynthetic, physiological and biochemical processes. The resulting phenotypes of salinity stress are manifested as premature senescence, chlorosis and necrosis of leaves. With salinization, agricultural profitability is adversely affected, and sustained production often requires increased inputs including increased planting densities, increased irrigation and fertilizer applications.

Remediation of saline soils has been implemented with some success, but only with substantial investment in resources. Removal of excess salts from the soil can be achieved through the application of low-ionic strength irrigation waters, but the availability of irrigation water for leaching is often a luxury, especially for arid and semi-arid regions. Soil amendments in the form of chemicals and fertilizers can also be used for reclamation of salt affected soils, but most of the methods of managing salt-affected soils are resource intensive and expensive. Therefore, sustainable agricultural production on saline soils will require the development and deployment of salt tolerant crops used in conjunction with management practices that minimize the salinization of soils.

Different crop species and crop cultivars can exhibit different levels of tolerance to salinity. In a review of published research on salt tolerance available from 1950-1975, (Maas and Hoffman, 1977) nearly 60 crops were classified based on their tolerance to salinity while (Munns and Tester, 2008) reported salt tolerance of cereals based on shoot dry matter accumulation of plants grown in a medium containing NaCl. Based on shoot dry matter accumulation, barley (*Hordeum vulgare*) was considered the most salt-tolerant, bread wheat (*Triticum aestivum*) moderately tolerant, and rice (*Oryza sativa*) was considered the least tolerant cereal. Salinity tolerance based on grain yield indicates that sorghum (*Sorghum bicolor*) is moderately tolerant to salt, but more salt tolerant than maize (*Zea mays*). Sorghum is also tolerant to heat and drought (Gong et al., 2005), and is a major cereal crop serving as a staple food predominantly in the arid and semi-arid regions of Africa and Asia. Identifying salt-tolerant sorghum genotypes and breeding for

enhanced salt tolerance could make sorghum an attractive crop for marginal agricultural lands that can experience severe abiotic stress including saline soils.

Crop improvement programs have attempted to develop salt-tolerant genotypes using traditional breeding and transgenic methods. Conventional breeding techniques have led to an improvement in salt tolerance of crops including barley, rice, maize, pearl millet (*Pennisetum glaucum*), alfalfa (*Medicago sativa*) and various forage grasses. By comparison, the commercial release of salt tolerant varieties is limited likely owing to the agronomic performance of saline tolerance genotypes in non-saline soils or to a limited market for salt-tolerant cultivars. Nevertheless, salt-tolerant cultivars have been commercialized for alfalfa and forage grasses (Dobrenz et al., 1983; Dobrenz, 1999), tomato (Rush and Epstein, 1981), soybean(Owen et al., 1994), rice (Mishra et al., 2003; Sankar et al., 2011; Singh et al., 2004), and wheat (Hollington, 1998; Munns et al., 2006).

In sorghum, genetic variation for salt tolerance in seedling and adult plants has been reported amongst cultivars, but little progress has been reported in breeding sorghum for salt tolerance (Krishnamurthy et al., 2007; Maiti et al., 1994). Various physiological and growth parameters have been used to screen sorghum germplasm for salt tolerance including seed germination, shoot growth, root growth, and biomass accumulation. Field trials of sorghum for grain production under saline conditions are limited, and further field-based studies are critical to translate the results obtained in laboratories and greenhouses to the agronomic conditions in which sorghum production normally occurs.

The transgenic approach to develop salt-tolerant plants has mainly focused on manipulating the expression of single genes involved in various mechanisms of salt tolerance. Overexpression of vacuolar and plasma membrane located ion transport proteins such as Na^+/H^+ antiporters conferred a level of salt tolerance in Arabidopsis, rice, wheat, tobacco, tomato, and alfalfa (Bao et al., 2009; Shi et al., 2003; Verma et al., 2007; Wu et al., 2004; Xue et al., 2004; Zhang et al., 2008; Zhang and Blumwald, 2001) . When salinity (NaCl treatment) was induced in growth chambers or glasshouses, transgenic lines displayed a level of salt tolerance based on biomass production, grain yield, germination rate, and/or root growth. Similar improvement in salt tolerance has also been observed in plants engineered for overproduction of different antioxidants, osmoprotectants, transcription factors, and heat-shock proteins. However, the commercial implementation of these promising transgenic approaches is hindered due to the lack of societal acceptance of genetically modified crops, especially for crops grown for grain production.

To elucidate the genetic basis of salt tolerance, various studies have identified quantitative trait loci (QTL) associated with tolerance. In tomato (*Lycopersicon esculentum*), a number of genomic regions affecting salt tolerance were identified (Foolad et al., 1998; Foolad, 1999; Foolad, 2004; Monforte et al., 1997), and a limited number of major QTL and several minor effect QTL controlled salt tolerance at each stage of tomato development. Studies on barley have also identified QTL conferring tolerance during germination and seedling development (Ellis et al., 1997; Mano and Takeda, 1997). Several of these QTL mapping efforts have culminated in the

identification of the genes underlying salt tolerance including sodium and potassium selective transporters in wheat (Ma et al., 2007; Xu et al., 2012) and rice (Bonilla et al., 2002; Flowers et al., 2000; Koyama et al., 2001). The *SKC1* locus in rice was mapped and the *SKC1* gene identified as a member of HKT-type transporter functioning as a sodium selective transporter (Ren et al., 2005). In sorghum, studies mapping loci conditioning salt tolerance are very limited. Wang et al. (2014a) identified six major QTL for salt tolerance in sorghum seedlings. However, since salt tolerance in seedlings may not be an indicator of tolerance during latter stages including grain filling (Ashraf and McNeilly, 1988; Foolad, 1999; Jones and Qualset, 1984), QTL mapping in non-juvenile vegetative and reproductive tissues are required to identify a suite of stage-specific and stage-independent resistance loci.

The objective of this study was to initiate a detailed genetic analysis of salt tolerance in sorghum by mapping trait loci conditioning a recombinant inbred line (RIL) population that segregates for salt tolerance. The research also aims to identify molecular markers tightly linked to the trait, to be used for marker-assisted introgression of salt tolerance into more susceptible sorghum cultivars.

Materials and methods

Plant growth and phenotyping

The initial germplasm and mapping population required for this research was developed by MMR Genetics (LLC), which has screened and developed a number of inbred lines having differential response in salinized soils. The mapping population

consists of 130 $F_{4:5}$ RILs derived (single-seed descent) from a cross between inbreds MMR338 and MMR31. MMR338 is characterized as a relatively salt-tolerant inbred whereas MMR31 is salt-sensitive (F.R. Miller, personal communication).

To phenotype for salt tolerance, a growth facility designed by Zia Tec (LLC) was utilized, which allowed for phenotyping under controlled environmental conditions including light, temperature and salinity levels. Salinity tolerance of sorghum was assessed in the vegetative stage after ~10 weeks of growth. Preliminary comparison with inbreds MMR381 and MMR31 indicated that the salt-tolerance phenotypes obtained in Zia Tec's growth facilities correlated well with field-based observations obtained by MMR Genetics (F.R. Miller, personal communication). To phenotype the RIL mapping population, plants were grown in Ray Leach Cone-tainers™ (SC-10 Super Cell, Stuwe & Sons, Inc) containing Sunshine VP potting mixture (Sun Gro Horticulture) and maintained at 28°C (day) and 21°C (night). 400-watt metal halide bulbs provided a day length of 12 hours. The Cone-tainers were placed in a rack, which was then placed in a large reservoir containing Peters Professional 10-30-20 nutrient solution diluted to a concentration of 150 ppm. The experiment was spatially designed using the biometrics software DiGGer (Coombes, 2009), which generated an experimental design consisting of 8 sites. Each site consisted of 8 trays with each tray comprised of 5 x 5 array of Cone-tainers. Once the fifth leaf emerged, the nutrient solution was supplemented with NaCl to a final concentration of 150 mM for a duration of 8 weeks. The nutrient solution was circulated on a daily basis and water was added twice a week to adjust the total volume, and electrical conductivity of the solution was

measured and stock NaCl added if needed. The nutrient/salt solution was replaced on a monthly basis.

To quantify the degree of salt tolerance of plants, digital images of the youngest fully emerged leaf and the next youngest leaf from each plant were obtained. Salt tolerance was based on the percentage of total leaf area that was necrotic or damaged, using the Assess 2.0 image analysis software (www.apsnet.org). Across the 8 sites, each RIL was phenotyped a minimum of 10 times while the parental inbreds (MMR338 and MMR31) were screened 64 times.

Statistical analysis

Data analysis was done using Genstat16.1 (Payne et al., 2011). Spatial analysis was conducted using REML mixed model to test the significance of fixed effects and estimate the variance components Fixed effects were tested considering Genotype, Site, Genotype X Site, Site X Tray, Tray X Row and Tray X Column as fixed effects and residual as random. Individual genotypic values and variance components were estimated using Genotype, Site, Genotype X Site and residuals as random effects and Site X Tray X row and Site X Tray X column as fixed effects. To determine the relationship between the traits, the phenotypic correlations were calculated using PROC CORR in SAS version 9.4. Correlations were calculated between percent leaf damage at leaf number 1 (PLD1), percent leaf damage at leaf number 2 (PLD2), and average value of PLD1 and PLD2 (PLD_AV).

Genotyping, linkage mapping & QTL analysis

A sequencing-based method developed for sorghum was employed for genotyping the parental inbreds and RIL population. The Illumina template library was prepared using the methyl-sensitive restriction enzyme *Fse*I and sequencing was done on the HiSeq2000 (Illumina). For library preparation, DNA was extracted from 14-day-old seedlings using the FastDNA Spin Kit (MP Biomedicals) and Illumina template was prepared as previously detailed in Chapter II. Standard Illumina protocols were followed for cluster generation from the template and single-end sequencing reads were obtained. Base calling was done using Illumina's Real Time Analysis (RTA) software and, the sequence text files were generated using GERALD in Illumina's CASAVA v1.7 software package. The sequencing data obtained was processed through a number of custom Perl and python scripts, and processed Illumina reads from the parental lines were aligned to the sorghum reference genome (Sbicolor v.3.1, www.phytozome.net, Paterson et al., 2009). Using BLASTN, polymorphisms between the parents were identified and further scored through the progeny as described in Chapter II. A file containing the SNP markers identified in the RILs was generated and used for genetic linkage map construction.

A genetic map was created for the RIL populations using JoinMap V4.0 (Van Ooijen and Voorrips, 2006). The SNP markers, which were close to each other (<10,000 bp) or showed identical segregation among progeny, were excluded since they did not improve map resolution. The linkage groups were determined using the independence test LOD score and groups with a LOD score of at least 10 were selected for mapping.

For each linkage group, the genetic distance between markers was calculated using Kosambi's mapping function.

For QTL analysis, BLUP estimates of salt tolerance for leaf PLD1 were obtained for each spatial site along with BLUP estimates for phenotypes averaged across the 8 spatial sites. The data was subjected to single-marker analysis, interval mapping (IM), and composite interval mapping (CIM) using the software WinQTL Cartographer V2.5 (Wang et al., 2006a; Wang et al., 2012). For the CIM analysis, a standard stepwise regression model 6 with default settings, a walk speed of 1.0 cM and 1000 permutations to determine the LOD significance threshold were applied. The genomic position of the significant QTL, effects of significant QTL, and the phenotypic variation explained (PVE) were calculated. For the significant QTL detected, the position of the peak marker and the flanking markers of the QTL interval (1-LOD interval window) were determined. The QTL interval was scanned in the annotated sorghum genome (v.3.1) for identifying potential candidate genes underlying the salt tolerance QTL.

Results and discussion

Analysis of phenotypic data

A summary of the statistical analysis of phenotypic data of RILs and parental inbreds MMR31 and MMR338 is presented in Table 13. In agreement with field-based classification of MMR31 as salt sensitive and MMR338 as salt tolerant, salt-induced damage of leaf 1 and 2 (PLD1 and PLD2) of inbred MMR31 was significantly higher than that of MMR338. The percentage of leaf damage for MMR338 ranged from 3.84%

to 14.64% for leaf 1 and 1.42% to 8.12% for leaf 2. By comparison, leaf damage of inbred MMR31 ranged from 55.23% to 88.33% for leaf 1 and 35.97% to 76.14% for leaf 2. Trait mean values for the RIL population were intermediate to the two parental inbreds with salt-induced damage ranging from 23.64% to 33.84% and 10.90% to 23.76% for PLD1 and PLD2, respectively. Transgressive segregation for salt-induced leaf damage was observed with select RILs that showed no apparent leaf necrosis and RILs with complete necrosis. This transgressive segregation in salt-induced damage amongst the RILs indicates that genes/alleles conferring salt tolerance may reside in both parental inbreds. Of particular interest were the RILs that showed no apparent damage from the salt treatment and did not appear stunted or quiescent in response to the stress. The apparent salt-tolerant RILs may represent germplasm that can be utilized by sorghum improvement programs to introgress salt tolerance into elite inbreds. However, further field-based evaluation of select salt-tolerant RILs in salinized soils are necessary to determine the utility of this germplasm in efforts to breed salt tolerant sorghum hybrids.

Table 13. Phenotypic trait values for parental inbreds MMR338 and MMR31 and the derived 130 $F_{4:5}$ RILs evaluated at 8 sites. Values represent means with standard deviations (SD) shown in parentheses. Ranges for phenotypic values of the $F_{4:5}$ RILs are shown.

Site[†]	Trait	Mean (SD)			Range
		MMR338	MMR31	RILs	
1	PLD1	4.69 (5.05) [a]	69.60 (26.18) [b]	26.65 (29.18)	0-100
2	PLD1	3.84 (3.72) [a]	64.13 (22.25) [b]	28.28 (27.53)	0.49-100
3	PLD1	4.90 (3.34) [a]	65.49 (15.83) [b]	31.57 (27.62)	1.92-100
4	PLD1	6.65 (4.44) [a]	60.54 (19.97) [b]	31.82 (30.11)	0.35-100
5	PLD1	9.96 (7.39) [a]	61.85 (23.93) [b]	23.97 (25.93)	0.34-98.75
6	PLD1	5.96 (8.11) [a]	55.23 (23.06) [b]	23.64 (25.88)	0.01-100
7	PLD1	9.63 (5.14) [a]	73.28 (18.05) [b]	30.69 (28.22)	1.23-100
8	PLD1	14.64 (3.99) [a]	88.33 (15.74) [b]	33.84 (27.85)	0.68-100
1	PLD2	1.42 (1.38) [a]	55.87 (31.63) [b]	15.01 (25.05)	0.01-100
2	PLD2	2.41 (2.08) [a]	45.77 (23.44) [b]	11.05 (20.28)	0.01-100
3	PLD2	5.91 (3.63) [a]	35.97 (11.53) [b]	16.07 (20.50)	0.43-100
4	PLD2	5.88 (4.56) [a]	44.21 (22.52) [b]	23.76 (29.39)	0.10-100
5	PLD2	4.50 (3.42) [a]	47.99 (32.16) [b]	10.90 (19.76)	0.01 -89.94
6	PLD2	2.46 (2.87) [a]	41.81 (24.60) [b]	11.12 (20.01)	0.01-100
7	PLD2	6.64 (4.41) [a]	58.41 (28.53) [b]	15.14 (24.24)	0.15-100
8	PLD2	8.12 (9.25) [a]	76.14 (21.34) [b]	15.17 (23.75)	0.03-100

[†] Each site was comprised of 8 trays with each tray having 5 rows and 5 columns.
Means followed by different alphabets [a] and [b] indicate significant differences of the trait mean value between the two parents as calculated by Tukey's test.
PLD1, percent of leaf damage at leaf number 1; PLD2, percent of leaf damage at leaf number 2.

Table 14. Wald test for PLD1, PLD2 and PLD_AV for the RIL mapping population combined across all sites.

	Wald-statistic				
Trait	**Genotype**	**Genotype x Site**	**Tray**	**Tray x Row**	**Tray x Column**
PLD1	16284.25***	973.08	15.61*	37.74	53.85**
PLD2	4127.02***	1011.72	6.19	43.87	21.65
PLD_AV	10227.08***	965.68	9.82	44.43	30.52

*, **, *** Significant at the 0.05, 0.01 and 0.001 probability level, respectively.
PLD1, percent of leaf damage at leaf number 1; PLD2, percent of leaf damage at leaf number 2; PLD_AV, average value of PLD1 and PLD2.

Wald test indicated significant genotypic variation ($P < 0.001$) among sites for all traits, significant variation for Tray ($P < 0.05$) and Tray x Column ($P < 0.01$) for trait PLD1 and non-significance of Genotype X Site interaction was also observed (Table 14). This variation can be largely attributed to variation in the micro-environment (e.g., air movement, temperature) that existed in the Zia Tec growth facilities. These sources of variation were anticipated based on previous studies in this growth facility, and were accounted for using the spatial design generated by DiGGer software.

Table 15. Pearson's correlation coefficients for PLD1, PLD2 and PLD_AV as measured at 8 sites and averaged across all sites.

Site	Trait	PLD1	PLD2	PLD_AV
1	PLD1		0.88***	0.97***
2	PLD1		0.76***	0.96***
3	PLD1		0.87***	0.98***
4	PLD1		0.76***	0.94***
5	PLD1		0.88***	0.98***
6	PLD1		0.89***	0.98***
7	PLD1		0.84***	0.96***
8	PLD1		0.81***	0.96***
Average of all sites	PLD1		0.82***	0.96***
1	PLD2			0.97***
2	PLD2			0.92***
3	PLD2			0.96***
4	PLD2			0.94***
5	PLD2			0.96***
6	PLD2			0.96***
7	PLD2			0.95***
8	PLD2			0.94***
Average of all sites	PLD2			0.95***

*** Significant at the 0.001 probability level.

PLD1, percent of leaf damage at leaf number 1; PLD2, percent of leaf damage at leaf number 2; PLD_AV, average value of PLD1 and PLD2.

Pearson correlation coefficients calculated between percent leaf damage at leaf 1 (PLD1), percent leaf damage at leaf 2 (PLD2), and average value of PLD1 and PLD2 indicated a significant ($P < 0.001$) positive correlation between these phenotypic determinations (Table 15). The strong positive correlation between PLD1 and newly emerging leaves indicates that the estimates of damage are consistent regardless of which of these immature leaves were phenotyped. Based on this correlation of salt damage between the leaves phenotyped, PLD1 was considered a good predictive indicator of salt tolerance and hence used for QTL analysis.

Linkage map

On aligning the reads of parental inbreds MMR31 and MMR338 to the reference genome of BTx623, a total of 3,096 single nucleotide polymorphism (SNP) markers were identified that were subsequently scored through the RILs. Of these, 579 SNPs were discarded for having substantial missing data. The final linkage map for QTL mapping contained 1083 unique SNPs spanning a length of 1250.56 cM that mapped to 10 linkage groups representing the 10 chromosomes of sorghum (Table 16). On average, one marker was present at 1 cM intervals across the linkage map. Except for the pericentromeric heterochromatic regions, which had the largest gaps between markers, the SNPs were well distributed across all chromosomes. This was anticipated as the Illumina template was prepared using a methylation-sensitive enzyme to reduce the representation of gene-poor heterochromatic regions.

Table 16. Linkage map marker coverage across the ten sorghum chromosomes generated for $F_{4:5}$ MMR338×MMR31 RIL mapping population.

MMR338 × MMR31											
Chromosome	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**Total**
Chromosome Length (cM)	154.84	143.05	170.77	79.07	111.69	105	137.96	107.01	115.74	125.43	1250.56
Number of Markers	175	133	158	68	66	92	95	79	93	124	1083
Marker density† (cM)	0.88	1.08	1.08	1.16	1.69	1.14	1.45	1.35	1.24	1.01	1.15
Largest interval‡ (cM)	6.01	5.62	10.14	10.17	7.27	8.42	5.44	7.87	4.46	6.83	
Largest interval‡ (Mbp)	14.78	31.16	29.16	3.84	26.09	27.75	22.22	16.99	24.08	21.86	

† Defined as the distance between adjacent markers, measured in cM.

‡ Defined as the largest distance observed between adjacent markers in cM and million base pairs (Mbp).

Quantitative trait loci and candidate genes for salt tolerance

As calculated by the permutation test, the threshold score for declaring a QTL significant was 2.5. A statistically significant QTL was consistently observed on chromosome 7 in all the sites tested (Table 17). This QTL located between 58.29 and 59.84 Mbp (59.51-61.06 Mbp v.3.1) had a LOD score ranging from 5.1 to 5.3 and explained 12.2 to 12.8% of the phenotypic variation. The negative additive effect observed implies the presence of alleles in the MMR338 parent, which contribute to salt tolerance by decreasing the percent leaf damage. Markers chr7_58.29 and chr7_59.84, linked to the trait and delimiting the ±1LOD interval were identified as left and right markers to be used for marker-assisted introgression of salt tolerance into more susceptible sorghum cultivars.

While only 1 QTL was above the threshold value for significance in the present mapping study, at least 6 QTL were within 1.2 LOD score of the threshold LOD score established by permutation analysis. These include QTL on chromosome 5 (65.6-84.0 cM), chromosome 6 (81.1-89.1 cM), chromosome 8 (75.7-81.4 cM), chromosome 9 (61.3-66.5 cM and 89.1-110.2 cM), and chromosome 10 (96.8-103.6 cM). The LOD score for these QTL ranged from 1.3 to 1.9 and the additive values had a range of -0.2 to 0.2. This lack of power of the present QTL analysis is likely due to the variation that existed in the micro-environmental conditions in which the phenotypes were obtained along with the inherent morphological variation that existed amongst the RILs. The analysis of variance clearly indicated that a significant portion of the observed phenotypic variance could be explained by spatial effects and despite our effort to

account for these differences, it is likely that the lack of uniformity in the growth environment and our ability to account for this source of variance precluded the power of this QTL analysis. In addition, significant morphological variation amongst the RILs existed for traits including leaf architecture, leaf size, and plant height (data not shown). These differences could hinder our ability to accurately assess the extent of salt damage, especially when the plant structure (leaves) being phenotyped varied markedly in morphology. The parental inbreds of this population were chosen based on salt tolerance rather than uniformity of growth or leaf architecture, and future efforts to map salt tolerance using these parental lines may require selection during the development of RILs for morphological uniformity. Alternatively, creating a backcross mapping population rather than using RILs may alleviate a significant amount of the morphological variation that confounded the ability to accurately and precisely phenotype salt damage and map trait loci.

A preliminary survey of the annotated genes within a one-LOD interval spanning the chromosome 7 salt tolerance trait loci was conducted to identify potential candidate genes underlying the QTL. Scanning the annotated reference sorghum genome (v.3.1) revealed 147 genes of known function, located within a 1-LOD interval of the QTL (~1.55 Mbp interval) and of these genes, ~45 candidates with a potential role in different mechanisms of salt tolerance were identified (Table 18). This includes genes involved in signal transduction, ion transport, lipid catabolism, oxidation-reduction process, cell wall modification, calcium ion binding, response to salt stress, flavonoid biosynthesis, wax

Table 17. Quantitative trait loci (QTL) detected for salt tolerance in the $F_{4:5}$ RIL population of MMR338 × MMR31. QTL were predicted based on best linear unbiased predictors (BLUPs) of PLD1 for 8 sites and the average values of PLD1 across 8 sites. Phenotypic variation explained (PVE) and additive genetic effects are shown for each significant QTL in each environment.

Site[†]	Trait	Chromosome	Peak Position (cM)	Confidence interval[‡] (cM)	Left Marker	Right Marker	LOD[§]	PVE (%)	Additive effect[¶]
1	PLD1	7	114.5	109.0-117.3	chr7_58.29	chr7_59.84	5.2	12.8	-10.10
2	PLD1	7	114.5	108.9-117.3	chr7_58.29	chr7_59.84	5.1	12.6	-9.70
3	PLD1	7	114.3	109.0-117.3	chr7_58.29	chr7_59.84	5.2	12.8	-10.00
4	PLD1	7	114.6	109.0-117.3	chr7_58.29	chr7_59.84	5.2	12.8	-10.10
5	PLD1	7	114.3	109.0-117.3	chr7_58.29	chr7_59.84	5.2	12.3	-9.90
6	PLD1	7	114.5	109.0-117.3	chr7_58.29	chr7_59.84	5.1	12.7	-9.90
7	PLD1	7	114.3	108.9-117.3	chr7_58.29	chr7_59.84	5.1	12.2	-9.50
8	PLD1	7	114.5	109.0-117.3	chr7_58.29	chr7_59.84	5.3	12.6	-9.70
Average	PLD1	7	114.6	109.0-117.3	chr7_58.29	chr7_59.84	5.2	12.8	-9.60

[†] Each site comprised of 8 trays with each tray having 5 rows and 5 columns.

[‡] Interval containing the peak marker ±1 LOD.

[§] LOD, logarithm of odds.

[¶] Negative values indicate contribution of MMR31 allele in increasing trait value.
PLD1, percent of leaf damage at leaf number 1.

Table 18. Annotated sorghum genes with a potential role in salinity tolerance, under the 1 LOD interval of the QTL on sorghum chromosome 7. Annotation file containing Arabidopsis orthologs was downloaded from Joint Genome Institute's Genome Portal (http://phytozome.jgi.doe.gov).

Gene	Arabidopsis ortholog	Annotated Function	Biological process	Reference
Sobic.007G160600	AT1G12680	phosphoenolpyruvate carboxylase-related kinase 2	intracellular signal transduction	(Monreal et al., 2013)
Sobic.007G160700	AT5G65380	MATE efflux family protein	antiporter/transporter activity	(Nimmy et al., 2015)
Sobic.007G160900	AT1G52190	Major facilitator superfamily protein	low-affinity nitrate transport	(Henderson et al., 2014)
Sobic.007G161500	AT2G05620	proton gradient regulation 5	electron carrier activity	(Shahid et al., 2016)
Sobic.007G162200	AT5G33370	GDSL-like Lipase/Acylhydrolase superfamily protein	lipid catabolic process	(Naranjo et al., 2006)
Sobic.007G162300	AT4G28730	Glutaredoxin family protein	cell redox homeostasis,	(Gong et al., 2005)
Sobic.007G162400	AT4G25750	ABC-2 type transporter family protein	transmembrane transport	(Li et al., 2011)
Sobic.007G163200	AT4G28720	Flavin-binding monooxygenase family protein	auxin biosynthesis, oxidation-reduction	(Cheol Park et al., 2013)
Sobic.007G163700	AT2G36930	zinc finger (C2H2 type) family protein	nucleic acid binidng	(Ma et al., 2016)
Sobic.007G163800	AT2G36910	ATP binding cassette subfamily B1	transmembrane transport	
Sobic.007G164000	AT5G60790	ABC transporter family protein	transmembrane transport	
Sobic.007G164101	AT3G28880	Ankyrin repeat family protein		(Sakamoto et al., 2008)
Sobic.007G164300	AT4G34870	rotamase cyclophilin 5	signal transduction	(Ruan et al., 2011)
Sobic.007G164400	AT3G54700	phosphate transporter 1;7	phosphate ion transport	(Cubero et al., 2009)
Sobic.007G165500	AT4G00350	MATE efflux family protein	antiporter/transporter activity	
Sobic.007G165600	AT1G63770	Peptidase M1 family protein	proteolysis, response to cadmium ion	(Zhou et al., 2011)
Sobic.007G165701	AT5G23950	Calcium-dependent lipid-binding (CaLB domain) family protein		(De Silva et al., 2011)
Sobic.007G166000	AT5G22890	C2H2 and C2HC zinc fingers superfamily protein	nucleic acid binding	(Mukhopadhyay et al., 2004)
Sobic.007G166200	AT5G58330	lactate/malate dehydrogenase family protein	oxidation-reduction process	(Wang et al., 2016b)
Sobic.007G166300	AT5G58330	lactate/malate dehydrogenase family protein	oxidation-reduction process	
Sobic.007G166500	AT3G55500	expansin A16	plant-type cell wall modification	(Chen et al., 2016)
Sobic.007G166600	AT2G28190	copper/zinc superoxide dismutase 2	cellular response to salt stress	(Jing et al., 2015)

Table 18. Continued

Gene	Arabidopsis ortholog	Annotated Function	Biological process	Reference
Sobic.007G167000	AT3G03430	Calcium-binding EF-hand family protein	calcium ion binding	(Liu and Zhu, 1998)
Sobic.007G167100	AT5G03560	Tetratricopeptide repeat (TPR)-like superfamily protein	cation symporter activity	(Rosado et al., 2006)
Sobic.007G167400	AT2G36690	2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein	biosynthetic process, oxidation-reduction	(Colebrook et al., 2014)
Sobic.007G167500	AT3G45750	Nucleotidyltransferase family protein	transferase activity	(Yu et al., 2016)
Sobic.007G167800	AT3G23940	dehydratase family	response to salt stress	(Zhang et al., 2015)
Sobic.007G168000	AT1G19670	chlorophyllase 1	chlorophyll catabolic process	(Ambede et al., 2012)
Sobic.007G168300	AT3G11540	Tetratricopeptide repeat (TPR)-like superfamily protein	cation symporter activity	
Sobic.007G168800	AT5G41220	glutathione S-transferase THETA 3	response to toxic substance	(Sharma et al., 2014)
Sobic.007G169000	AT3G44380	Late embryogenesis abundant (LEA) hydroxyproline-rich glycoprotein family	response to desiccation	(Tabaei-Aghdaei et al., 2000)
Sobic.007G169100	AT5G03880	Thioredoxin family protein	cell redox homeostasis	(Zhang et al., 2011)
Sobic.007G169200	AT5G04170	Calcium-binding EF-hand family protein	calcium ion binding	
Sobic.007G169900	AT4G33790	Jojoba acyl CoA reductase-related male sterility protein	wax biosynthetic process	(Wang et al., 2016c)
Sobic.007G170100	AT5G22500	fatty acid reductase 1	response to salt stress	
Sobic.007G170400	AT5G13930	Chalcone and stilbene synthase family protein	flavonoid biosynthesis	(Lijuan et al., 2015)
Sobic.007G170500	AT4G35160	O-methyltransferase family protein	melatonin biosynthesis	(Liang et al., 2015)
Sobic.007G170700	AT5G22500	fatty acid reductase 1	response to salt stress	
Sobic.007G170800	AT5G22500	fatty acid reductase 1	response to salt stress	
Sobic.007G171000	AT3G52880	monodehydroascorbate reductase 1	hydrogen peroxide catabolism	(Kavitha et al., 2010)
Sobic.007G171950	AT3G48330	protein-l-isoaspartate methyltransferase 1	response to salt stress	(Oge et al., 2008)
Sobic.007G174600	AT5G53460	NADH-dependent glutamate synthase 1	L-glutamate biosynthesis	(Dluzniewska et al., 2007)
Sobic.007G175800	AT1G79400	cation/H+ exchanger 2	cation transport	(Sze et al., 2004)
Sobic.007G176000	AT5G38030	MATE efflux family protein	antiporter/transporter activity	
Sobic.007G176100	AT5G38030	MATE efflux family protein	antiporter/transporter activity	

biosynthesis, and hydrogen peroxide catabolism among others. The most prominent candidate gene closest to the peak Sobic.007G175800, encodes a cation/H^+ antiporter.

Plants have developed three main mechanisms towards salinity stress, which include osmotic stress tolerance, Na+ exclusion and tolerance of accumulated Na+ in the tissue. The exclusion mechanism entails reducing the ionic stress on plants by preventing the accumulation of toxic concentrations of Na^+ in leaves (Pardo et al., 2006) whereas the tolerance mechanism involves compartmentalization of Na^+ into particular cell types and organelles of the shoot to avoid toxic build up within the cytoplasm. Exclusion of salt ions into the apoplast is mediated through a number of plasma membrane located ion channels like Na^+/H^+ antiporters whereas vacuolar Na^+ compartmentalization is conferred by tonoplast Na^+/H^+ antiporters such as those belonging to the Na^+/H^+ exchanger family in Arabidopsis (Carillo et al., 2011). The exclusion and compartmentalization of ions involves up and down regulation of genes expressing various ion channels and transporters (Apse et al., 1999; Davenport et al., 2005). The QTL detected here harbors genes encoding various transporters such as MATE efflux family protein, PGR5, ABC transporter family protein, phosphate transporter 1;7, TPR-like superfamily protein, and cation/H+ exchanger 2, which play an important role in regulating ion balance. In addition to genes encoding ion transporters, genes implicated with a role in oxidation-reduction process and antioxidant production were present within the trait locus, which included glutaredoxin family protein, flavin-binding monooxygenase family protein, lactate/malate dehydrogenase family protein, copper/zinc superoxide dismutase 2, glutathione S-transferase THETA 3, thioredoxin

family protein, chalcone and stilbene synthase family protein and monodehydroascorbate reductase 1. Plants affected by salinity stress are at a high risk of oxidative damage due to formation of free radicals and require mechanisms to maintain redox homeostasis (Gong et al., 2005), and each of above mentioned genes may play a role in these mechanisms. Nevertheless, despite the wealth of potential candidates with this region of the genome, further fine mapping studies with greater power and precision are necessary to eventually identify the salt-tolerance gene underlying this chromosome 7 QTL.

This study was successful in elucidating the genetic basis of salt tolerance in sorghum through QTL analysis of a recombinant inbred line (RIL) population and the subsequent examination of candidate genes within the limits of this salt-tolerance trait loci. The research also identified molecular markers linked to the trait, to be used for marker-assisted introgression of salt tolerance into more susceptible sorghum cultivars. Furthermore, the QTL identified is replete with genes known to have a role in different mechanisms of salt tolerance. However, identification of the salt tolerance gene(s) from the candidates will require additional fine mapping efforts in conjunction with analyses of expression studies, mutagenesis, cDNA library analysis, and plant transformation.

CHAPTER IV

HIGH RESOLUTION MAPPING *Dw2* DWARFISM LOCUS IN SORGHUM

Introduction

Sorghum accessions in the world collection range in plant height from 1-to-4 meters (Quinby and Karper, 1953), with the tall accessions preferred for subsistence agriculture where sorghum stover can be used for livestock grazing or as a building material. In temperate-zone production agriculture, tall varieties with higher biomass are suitable as forage and more recently for cellulosic-based biofuel production (Salas Fernandez et al., 2009). By contrast, tall plants are undesirable for mechanized (combine) grain harvesting and more susceptible to lodging, thus a major effort was initiated in the 1940s to breed sorghum in the United States for dwarfism (Quinby, 1974). In conjunction with this, Quinby and colleagues (Ayyangar et al., 1937; Karper, 1932; Sieglinger, 1932) initiated efforts to identify the number of genes controlling dwarfism while plant physiologists labored to understand the physiological parameters controlling this trait. This pioneering genetic work initiated by Quinby and colleagues has been carried forth to the present day by sorghum researchers that investigate gene regulation of key agronomic traits including dwarfism (Childs et al., 1997; Multani et al., 2003; Murphy et al., 2014; Murphy et al., 2011).

Investigations have described the diverse biochemical and molecular regulation of plant height in different species. Gibberellic acid is a plant hormone known to stimulate stem elongation by promoting cell division and elongation (Metraux, 1987),

and mutations in the gibberellin biosynthetic pathway have resulted in dwarf phenotypes which respond to external GA application with stem elongation. This has been observed in dwarf mutants of maize (*Zea mays* L.), rice (*Oryza sativa* L.) and pea (*Pisum sativum* L.) (Phinney, 1984). However, mutations in the signal transduction pathway can block the utilization of GA leading to GA-insensitive dwarf phenotypes as reported in wheat (*Triticum aestivum* L.), rye (*Secale cereale* L.), maize and rice (Milach and Federizzi, 2001). Brassinosteroids (BR) are another family of hormones that affect plant height by promoting cell elongation. Dwarf phenotypes have been observed in tomato (*Solanum lycopersicum* L.), *Arabidopsis* and pea mutants defective in BR biosynthesis and signaling pathway (Salas Fernandez et al., 2009). A number of studies have indicated that type-B phytochrome acts by altering GA biosynthesis or by modifying the response to GA (Lopez-Juez et al., 1995; Weller et al., 1994). Overexpression of phytochrome A and phytochrome B genes has been shown to cause dwarfness in tomato, tobacco (*Nicotiana tabacum* L.) and *Arabidopsis* plants (Milach and Federizzi, 2001). Finally, down regulation of histone deacetylase, a transcriptional regulator in plants, has shown to affect various developmental functions and plant height in *Arabidopsis* (Tian and Chen, 2001).

Several dwarfing genes in crop species have been identified and studied so far, and several are critical to crop improvement programs as dwarfism genes can have a positive pleiotropic effect on grain yield. The dwarfing genes *Rht1* and *Rht2* of wheat (Börner et al., 1997; Konzak, 1987) and *sd1* of rice (Cho et al., 1994) have been substantially used throughout the world in developing semi-dwarf varieties. One of the

first dwarfing genes to be cloned was *gai* of *Arabidopsis thaliana* (Peng et al., 1994), which encodes a mutant protein (GA signal transduction component) that causes reduction in GA response and thereby a dwarf phenotype. In rice a semi-dwarf gene, *sd-1* encoding gibberellin 20-oxidase, a key enzyme of the gibberellin biosynthesis pathway was isolated by positional cloning (Monna et al., 2002).

In sorghum four independent loci *Dw1, Dw2, Dw3* and *Dw4* have been characterized to affect plant height by primarily influencing stem internode length (Quinby and Karper, 1953). Tallness is partially dominant to shortness (Hadley, 1957) and five height classes are recognized (0-4 dwarf phenotypes) that refer to the number of dwarfism loci (*Dw1-Dw4*) that are homozygous recessive. Usually three of the *Dw* loci (termed "3-dwarf") are homozygous recessive to temperate zone commercial grain hybrids whereas dual purpose (grain and forage) are often 2-dwarfs. Of the four dwarfing loci in sorghum, *Dw3* located on chromosome 7 has been identified as encoding a P-glycoprotein auxin efflux carrier orthologous to *PGP1* in *Arabidopsis* (Multani et al., 2003). The mutant allele *dw3* has been used in sorghum grain hybrids, but the recessive allele is unstable and reverts back to the dominant (tall) allele with a frequency of 0.1-0.5%. This reversion at the *Dw3* locus is often evident in production fields by the presence of "tall mutations" in otherwise uniformly short fields of sorghum. In addition, decreased sorghum grain yields through reduced shoot biomass and grain size have been associated with the recessive *dw3* allele (George-Jaeggli et al., 2011). The *Dw1* locus has been proposed to be on chromosome 9 where a major height gene *Sb.Ht9.1* has also been reported (Brown et al., 2008; Pereira and Lee, 1995). The *Dw4*

locus has so far not been mapped conclusively to a chromosome, but the gene is proposed to be linked with a bloom locus in sorghum (Madhusudhana and Patil, 2013). Recent association mapping studies have suggested the potential physical position of *Dw4* at ~6.6 Mbp on chromosome 6 (Morris et al., 2013).

The *Dw2* dwarfism locus was reported by Quinby and Karper (1945) to be linked to the maturity locus, *Ma1*, and both of these loci are critical to sorghum's adaptation to temperate-zone agriculture (Lin et al., 1995). Utilizing both AFLP and SSR genetic markers, *Dw2* was previously mapped (Klein et al., 2008; Lin et al., 1995) to the distal end of chromosome 6, delimited by the AFLP markers *Xtxa2124* (42.57 Mb) and *Xtxa3407* (44.46 Mb). Recent association mapping studies have resolved the location of the *Dw2* locus to ~100kb around 42.2Mb of chromosome 6 (Morris et al., 2013). While examination of the *Dw2* locus based on the mapping efforts of Klein et al., (2008) revealed ~53 genes. While several of these genes including cytochrome P540, alpha amylase, and histone deacetylase represent viable candidates for encoding *Dw2*, however, identification of the gene underlying the *Dw2* locus has not been experimentally verified.

The objective of the present research was to map at high resolution the sorghum dwarfing gene *Dw2* to permit the identification of candidate genes. The research aims to fine map the *Dw2* gene using residual heterozygous lines from an F_2 population and to confirm the identity of the gene utilizing transgenic technology.

Materials and methods

High resolution mapping of the Dw2 locus

The parents of this mapping population were BTx3197 (*dw1Dw2dw3dw4*) and BTx616 (*dw1dw2dw3dw4*) (Miller et al., 1999; Quinby, 1974). An F_2 mapping population from a cross of BTx3197 and BTx616 was created by F.R. Miller (Professor Emeritus, Texas A&M University), and this F_2 population was utilized by Klein et al. (2008) to delimit the *Dw2* locus to an ~1 Mbp region of sorghum chromosome 6. To further resolve the *Dw2* locus, F_2 plants with residual heterozygosity within the limits of the *Dw2* locus were identified by genotyping the entire F_2 mapping population (202 individuals) with SSR markers flanking (and within) the *Dw2* locus. Those F_2 individuals heterozygous for the *Dw2* locus-delimiting SSR markers were selected for further selfing to potentially generate additional recombinant events within the locus. This process of selfing, genotyping, and phenotyping plants with residual heterozygosity was repeated for all F_3 plants that were still heterozygous for the *Dw2* locus-delimiting markers, and additional recombinant (crossover) events within the locus were identified. This process was repeated until all individuals within the mapping population were homozygous at the *Dw2* locus (i.e. no further crossover events were possible), and thus no further refinement of the locus was possible with this mapping population.

Genotyping and phenotyping

The SSR markers required for genotyping were identified by analyzing the sequence spanning the *Dw2* locus with the simple sequence repeat identification tool, SSRIT (http://archive.gramene.org/db/markers/ssrtool). Potential SSRs covering the

Dw2 locus were chosen and primers designed (Primer3 v.0.4.0) for PCR amplification (Table 19). DNA was extracted from seedling leaf tissue using the FastDNA Spin Kit (MP Biomedicals) and PCR was performed using DNA template, 5X Green GoTaq® Reaction Buffer, 5U/µl GoTaq® DNA Polymerase, 25mM $MgCl_2$, 2.5mM dNTPs, 2.5 pmol forward and reverse primers and water to volume. Depending on the annealing temperatures of the primers, either a single annealing temperature of 55°C was used or a touchdown protocol was used. The two PCR cycling parameters were as follows: STS-55, initial denaturation at 95°C for 2 min, followed by 25 cycles of 95°C for 45 s, 55°C for 45 s, and 72°C for 1 min, and a final synthesis step at 72°C for 20 min; or TD-65, initial denaturation at 95°C for 2 min, followed by 11 cycles of 95°C for 45 s, 65°C for 45 s, and 72°C for 45 s, 20 cycles of 95°C for 45 s, 58°C for 45 s, and 72°C for 30 s and a final synthesis step at 72°C for 30 min. The parents of the mapping population were screened for polymorphism through size variation on a 3130xl Genetic Analyzer (Applied Biosystems), and alleles called with GeneMapper Software v4.0 (Applied Biosystems). Those SSRs exhibiting different allele sizes between BTx3197 and BTx616 were used to screen the selected progeny as detailed above.

Phenotypic data for height was collected from the plants grown in a greenhouse or the field after physiological maturity. For greenhouse phenotyping, plants were grown in Sunshine MVP soil (Sun Gro Horticulture) with a temperature range of 24°C (night) to 30°C (day). A day length of 12 hours was provided using sodium halide lights and natural sunlight.

Table 19. Primer sequences for amplifying simple sequence repeat (SSR) markers covering the *Dw2* locus. Maker names, genomic positions, type and number of repeats along with predicted size of the amplification product and the annealing temperatures of primers are shown.

Marker name	Marker Position	Type of SSR(s)[†]	Sequence of forward primer (5'to3')	Sequence of reverse primer (5' to 3')	Product Size[‡] BTx3197/ BTx616	Annealing temperature ^{O}C
txp434	42653043	(TCG)8	CGAGGTCCAGGAGTACACG	CGGCCTCCATGAGGAGTAAT	155/147	55
txi56[§]	-	INDEL	AGGACAAGGTGGAGTTCACG	ATGTCAGCATGTGCAGTTGG	219/214	55
txp741	42690137	(CCT)8	TCTCGATCTGCTCCTCCTTG	GGCAGCTTGCTTGTGCTATAA	292/290	55
txp742	42691036	(AGC)5	ACTTTTTCGCGTCCATTGTT	GGTTTATTGACGCCTTGCTC	228/227	55
txp743	42717697	(CCGC)6 + (CG)5	GTACGAGAGATGGCGTCCTT	ACACGTTGTCTGCGTCTGC	385/378	65
txp737	42722260	(CAA)13	GCTCATCTGACACAGCCTTTC	TAGCGTACCCCAACTTGCTT	172/190	55
txp738	42778077	(CCG)5	CAACTCGATGCAGAGTGTCC	GAGCAGCGAAAATCCAAGTC	190/184	55
txp739	42800383	(AC)12	CTGCACTGCATCCCTTTCTT	CACATGAGTCTTGCCGATTC	247/243	55
txp547	42807635	(AAT)12	GAGAGAGAGCGCGATGAGAC	ATCCATCGCAAACCGATAAA	203/190	55
txp690	42824911	(CGG)9	CAATGATTCCGAACCAGGAT	AACCACACTAGCCCCTTCCT	170/165	55
txp691	42840525	(GC)10	CCAACCTTAGCCAAATCGAG	CACCCACTCGAAAGCTTCAT	179/407	55
txp535	43208978	(AT)7	TTGAAATTTATTGCATCCTAA	AAAGAACTCTGATAAATACTTCC	117/120	55
txp559	43634595	(TCC)7	TAAGCAAGTCGTCACCCGTCA	GGCATGGCATACCCGAACA	360/357	55

[†] Number of repeats; a '+' indicates the SSRs are separated by more than five bases.

[‡] Amplification product size of genotypes BTx3197 and BTx616.

[§] Insertion/deletion (INDEL) marker.

For field analyses, plants were grown at the Texas A&M University Research Farm in College Station, TX (USA).Plants were grown in a randomized complete block design with two replications. Plots consisted of a single 5.2 m long row with row spacing of 0.8 m. Production practices standard for sorghum fertilization, irrigation and pest management were employed. Total plant height and other parameters including height of the first, second, and third internodes below the peduncle (IN-1, IN-2, IN-3), base to first node below the peduncle, peduncle length and panicle length were measured. The phenotypic data was then subjected to one way analysis of variance to compare the means of parents and progeny and differentiate them into two height classes.

Candidate gene identification and functional validation

Following refinement of the *Dw2* locus using residual heterozygous individuals, the annotated genes within the fine-mapped region were surveyed (http://www.phytozome.net), and all genes of known or suspected biological function in controlling height were regarded as potential candidate gene. For verification of the function of a candidate gene, two approaches were considered; complementation of a recessive *dw2* cultivar with the dominant *Dw2* gene, or down-regulation of the expression of the candidate gene (gene silencing) in a dominant *Dw2* cultivar using RNA interference (RNAi).

Construct design for gene silencing

For *Dw2* gene silencing, a construct encoding a hairpin RNA (hpRNA) consisting of an inverted repeat separated by a spacer intron was prepared. Total RNA was extracted from the leaves of 35-day-old seedlings of sorghum genotype RTx430

(*Dw2*) using the miRNeasy Mini Kit (QIAGEN). From the total RNA, first-strand cDNA was synthesized with the SuperScript® III First-Strand Synthesis System for RT-PCR (Invitrogen). For generating the inverted repeats, ~300 bp cDNA fragment from the candidate gene was amplified by two separate PCR reactions that added restriction sites to the ends of the PCR product. The primer sets 1 and 2 (Table 20) incorporated the restriction sites *Xba*I, *Hind*III and *Xho*I, *Kpn*I, respectively flanking the PCR product. The standard PCR cycling protocol detailed above (55^{o}C annealing temperature) was used for PCR amplification and PCR products were purified using the QIAquick PCR Purification Kit (QIAGEN). Purified PCR products were then cloned into a pCR®II-TOPO® TA vector using the TA Cloning® Kit (Invitrogen) to form two silencing constructs; SIL-1 (restriction sites *Xba*I and *Hind*III) and SIL-2 (restriction sites *Xho*I and *Kpn*I). The constructs were then transformed into One Shot® TOP10F' Chemically Competent *E. coli* cells and transformants analyzed. Transformants were grown in LB medium and plasmid DNA was extracted using the Zyppy™ Plasmid Miniprep Kit (ZYMO RESEARCH). Recombinant pCR®II plasmids were screened by DNA sequencing employing the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) along with M13 forward and reverse primers. Inserts with the correct sequence (e.g., absence of PCR-induced sequencing errors) were sequentially subcloned into the pKANNIBAL vector (Figure 1) in sense and antisense orientation, flanking an intron sequence of the vector. To subclone into pKANNIBAL, SIL-1 and pKANNIBAL were digested with *Xba*I and *Hind*III followed by gel extraction, purification and finally

Table 20. Primer sequences for amplifying and sequencing DNA in silencing and over-expression experiments.

	Primer Sequence (5'-3')
	Silencing
Primer Set 1	TCTAGAGAGTTTGCGGTCCATACAGC
	AAGCTTTCACAATCACTTTGACCTGCT
Primer Set 2	CTCGAGGAGTTTGCGGTCCATACAGC
	GGTACCTCACAATCACTTTGACCTGCT
Primer Set 4	TTATTAACTTCTAAATGGATTGAC
	GGCGGTAAGGATCTGAGCTA
Primer Set 5	CGCACAATCCCACTATCCTT
	CTTCGTCTTACACATCACTTGTCA
M13 Forward	GTAAAACGACGGCCAG
M13 Reverse	CAGGAAACAGCTATGAC
	Primer Sequence (5'-3')
	Over-expression
Primer Set 3	AGATCT AGCG ATGCTGGAGAAAGAC
	GAGCTCCAGATTAGCGTGCGC
Primer Set 6	GACATTGATGTCCATCATGG
	GCAAGTGAATTAACCAGATA
	ATTTGAACAGTAAGACCTATC
Primer Set 7	GCAGCATCTATTCATATGCTCTAAC
	TTTAGCCCTGCCTTCATACG
	TGCTTAACGTAATTCAACAGA
T3	ATTAACCCTCACTAAAGGGA
T7	TAATACGACTCACTATAGGG

ligation of the digested SIL-1 insert (~300 bp) with vector pKANNIBAL using T4 DNA ligase (Promega).

This construct was then transformed into competent cells and transformants were analyzed and sequenced as described above. Upon identification of clones containing the pKANNIBAL vector and SIL-1 insert without mutations, the above process was repeated with restriction enzymes *Xho*I and *Kpn*I to incorporate the SIL-2 insert into pKANNIBAL_SIL-1. Transformants bearing the construct pKANNIBAL_SIL-1_SIL-2 (Figure 2), were then sequenced with primer sets 4 and 5 (Table 20) to confirm the correct clone. Finally, the fragment from pKANNIBAL_SIL-1_SIL-2, containing the CaMV35S promoter, OCS terminator and the hpRNA was excised using *Not*I and further subcloned into a binary vector, pART27 (Figure 3) by digestion and ligation. The new construct pART27_ SIL-1_SIL-2 (Figure 4) thus formed, was used for *Agrobacterium*-mediated transformation of the genotype RTx430 (*Dw2*).

Construct design for gene complementation

For gene complementation, the coding DNA sequence (CDS) of the candidate gene was PCR amplified from cDNA. Primer set 3 (see Table 20) that added restriction sites *Bgl*II, and *Sac*I, at the ends of the CDS was used for amplification. The PCR cycle TD-65 was performed using cDNA template, 5X Q5 Reaction Buffer, 0.02 U/µl Q5 High-Fidelity DNA Polymerase, 2.5mM dNTPs, 10 pmol target-specific primers, and water to volume. The PCR product was loaded on a 0.7% agarose gel containing 0.2M guanosine and run using 1x TBE buffer. The target CDS fragment was excised from the gel using QIAquick Gel Extraction Kit (QIAGEN) and purified. A single 3'adenosine

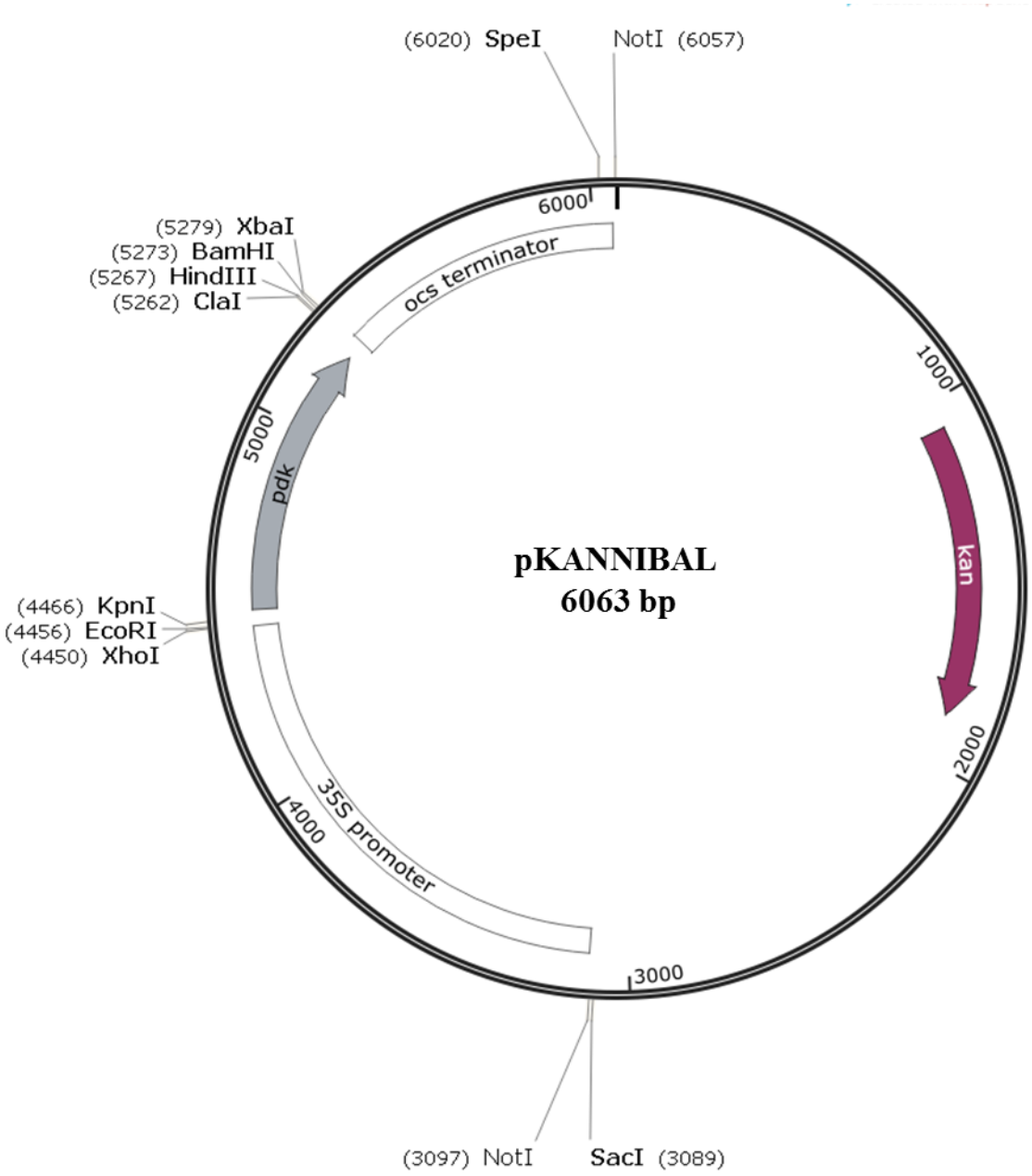

Figure 1. Map of the pKANNIBAL construct with CaMV35S promoter, pdk gene intron, and OCS terminator.

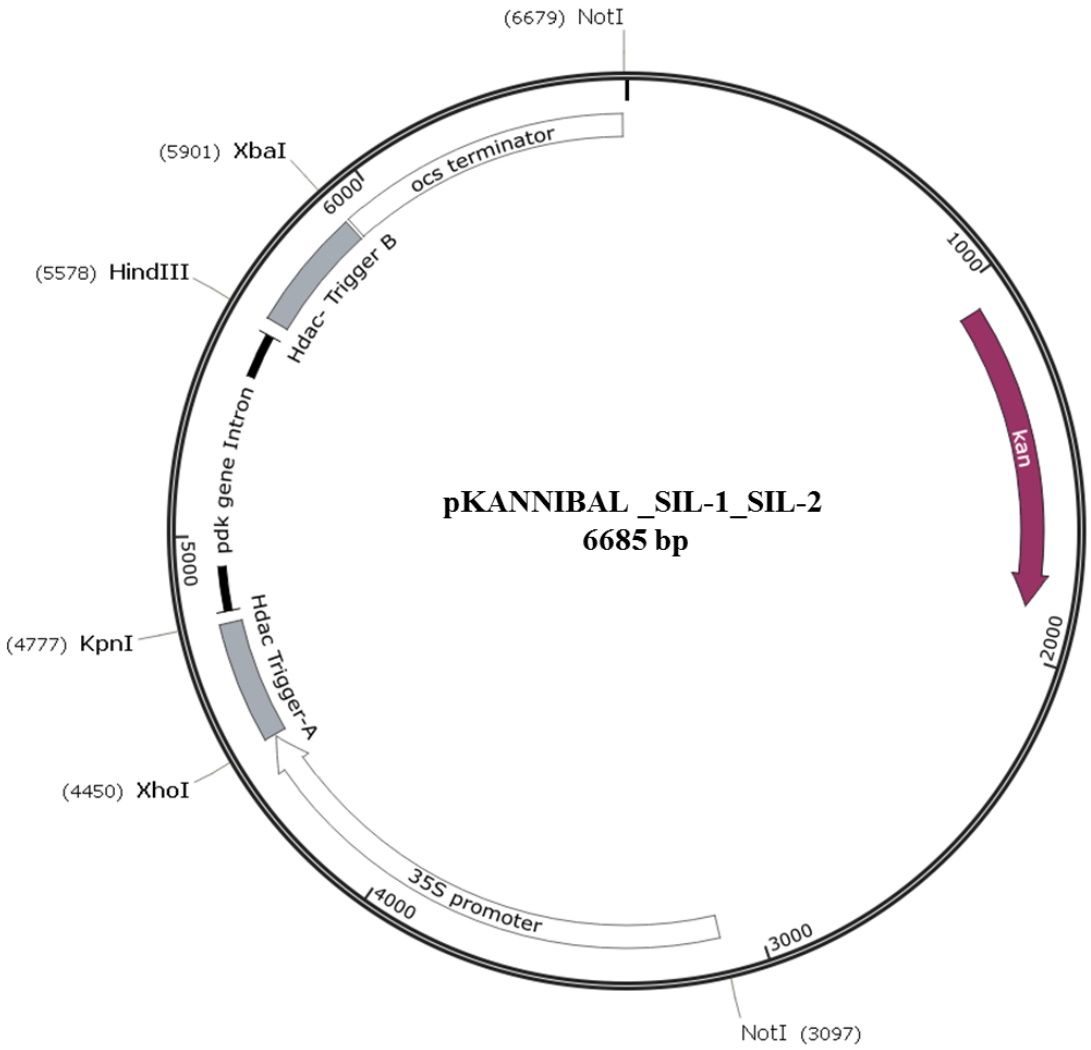

Figure 2. Map of the pKANNIBAL_SIL-1_SIL-2 construct with CaMV35S promoter, OCS terminator, inverted repeats Hdac-Trigger-A and Hdac-Trigger-B separated by the spacer pdk intron.

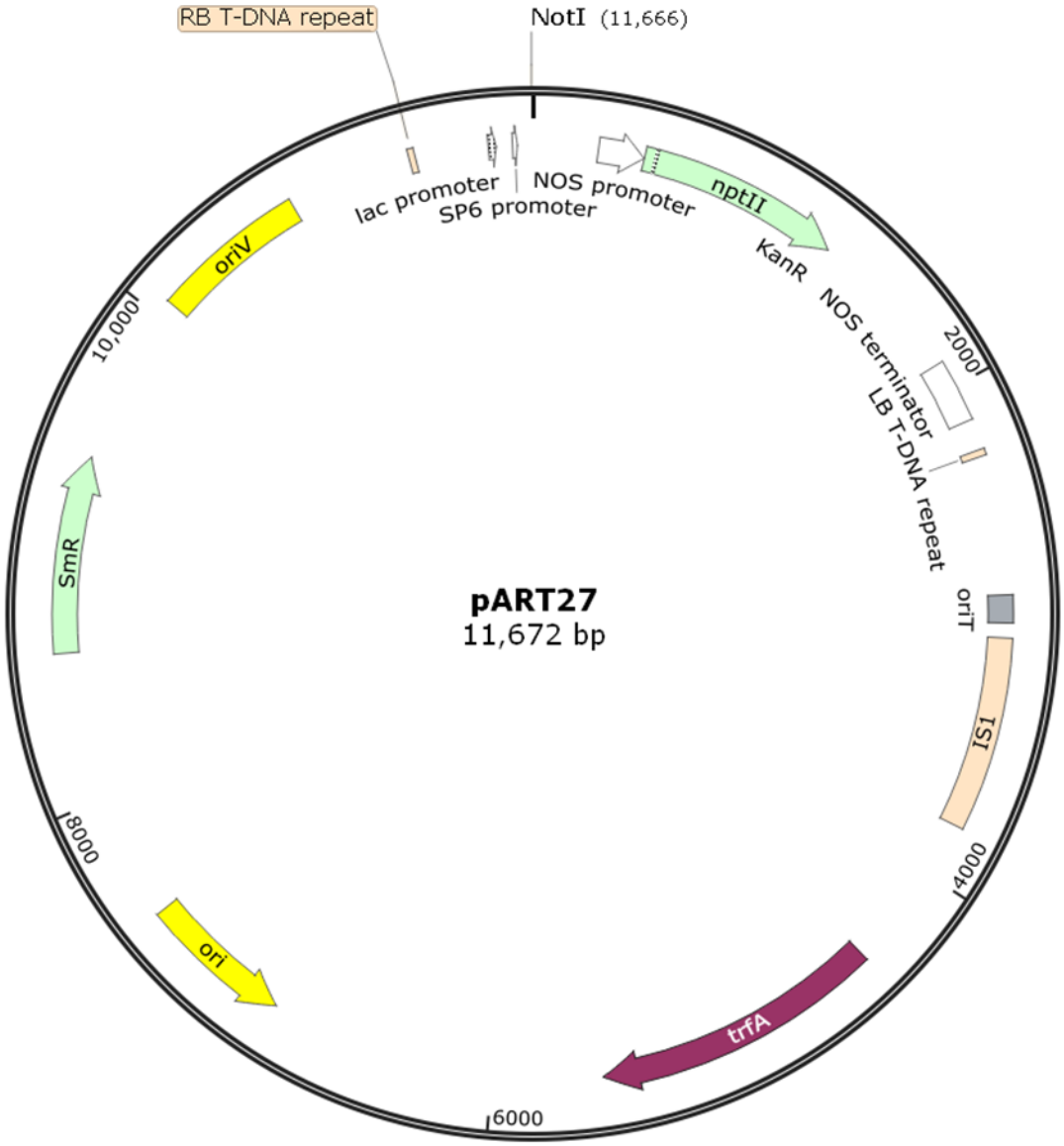

Figure 3. Map of the pART27 construct with *npt*II plant selection gene.

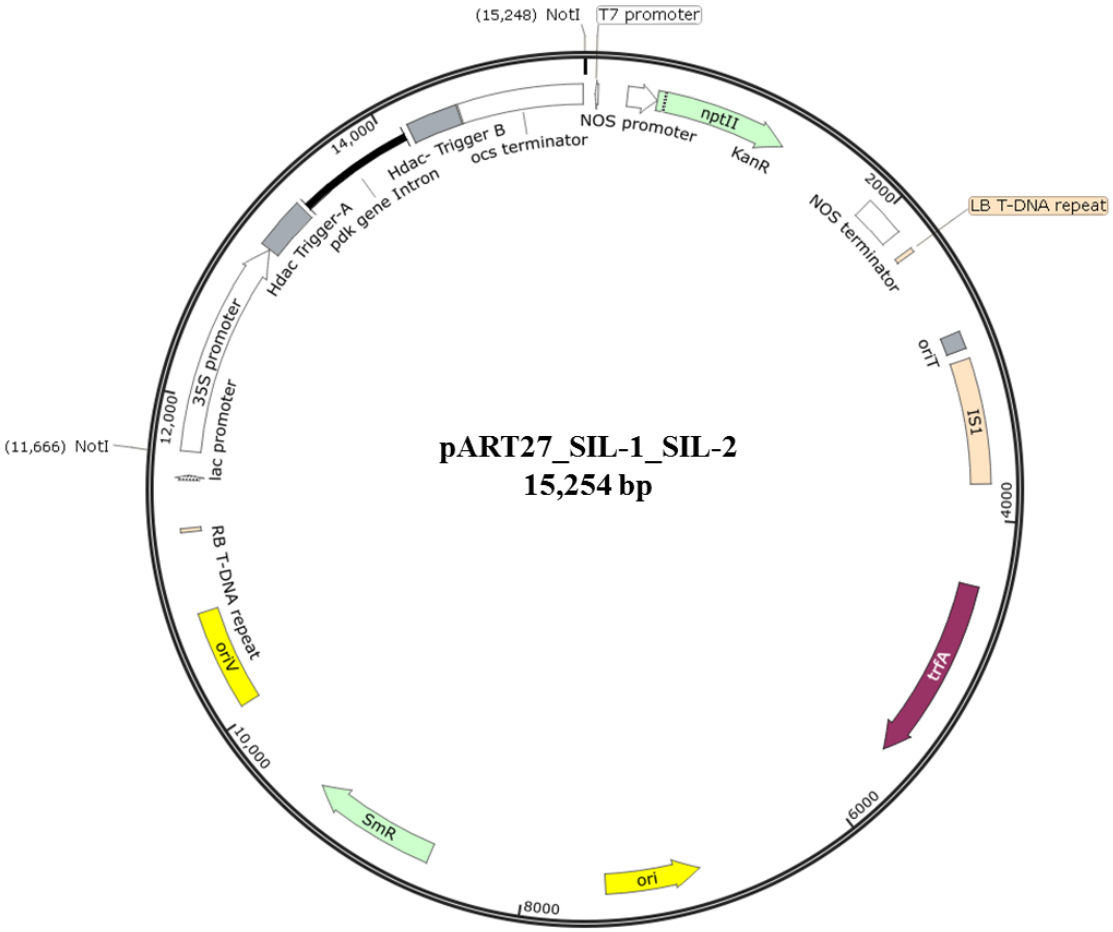

Figure 4. Map of the pART27_SIL-1_SIL-2 construct with *Not*I fragment digested from pKANNIBAL_SIL-1_SIL-2 construct.

residue was added by incubating the PCR fragment with 1mM dATP and GoTaq Flexi DNA Polymerase in 5X GoTaq Reaction Buffer and 25mM $MgCl_2$ at 70 ^{o}C for 15-30 mins. The A-tailed fragment was then cloned into a pCR™4-TOPO® TA vector using the TOPO® TA Cloning® Kit (Invitrogen). Transformation of competent cells, plasmid DNA extraction and inserts sequenced with primer set 6 (Table 20) and T3/ T7 primers, and the clones containing the target insert were subcloned into a pCAMBIA-based vector. For subcloning into the pCAMBIA-based vector, clones were digested with *Bgl*II and *Sac*I, and the vector pCAM-Ubi-GUS (Figure 5) was digested with *BamH*I and *Sac*I. The digestion reactions were run on an agarose gel and the digested fragments were gel extracted as detailed above, and the purified insert was ligated to vector pCAM-Ubi-GUS using T4 DNA ligase (Promega). This construct was then transformed into One Shot® TOP10F' chemically competent *E. coli* cells and transformants were analyzed and sequenced with primer sets 6 and 7 (Table 20) as described earlier. This new construct, termed pCAM_OE (Figure 6), harbored the entire CDS of the target gene plus a maize ubiquitin promoter and NOS terminator, and this construct was used for *Agrobacterium*-mediated transformation of the sorghum genotype P898012 (*dw2/dw2).* All the primers used for amplifying and sequencing DNA fragments of the designed constructs are shown in Table 20.

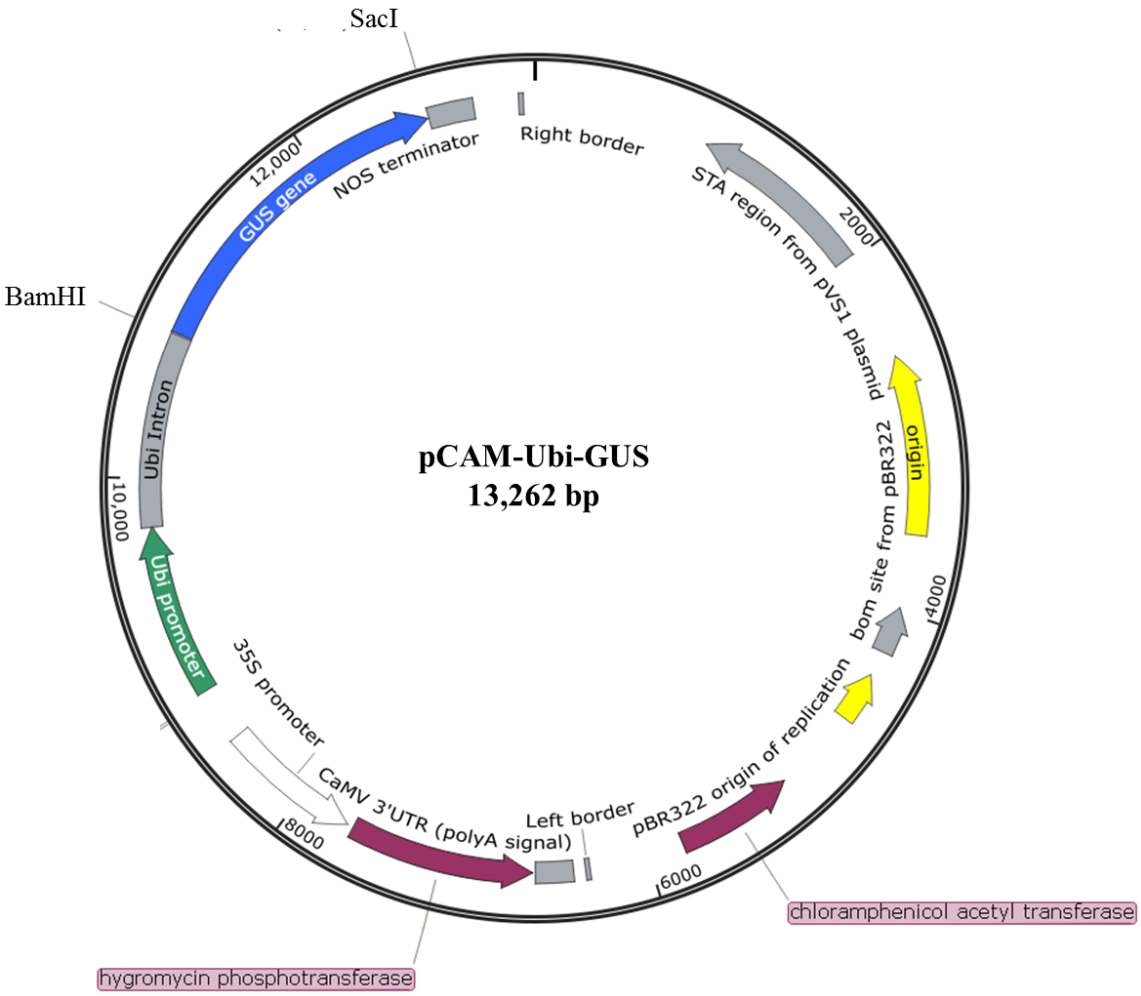

Figure 5. Map of the pCAM-Ubi-GUS construct with ubiquitin promoter, ubiquitin intron, GUS gene and NOS terminator.

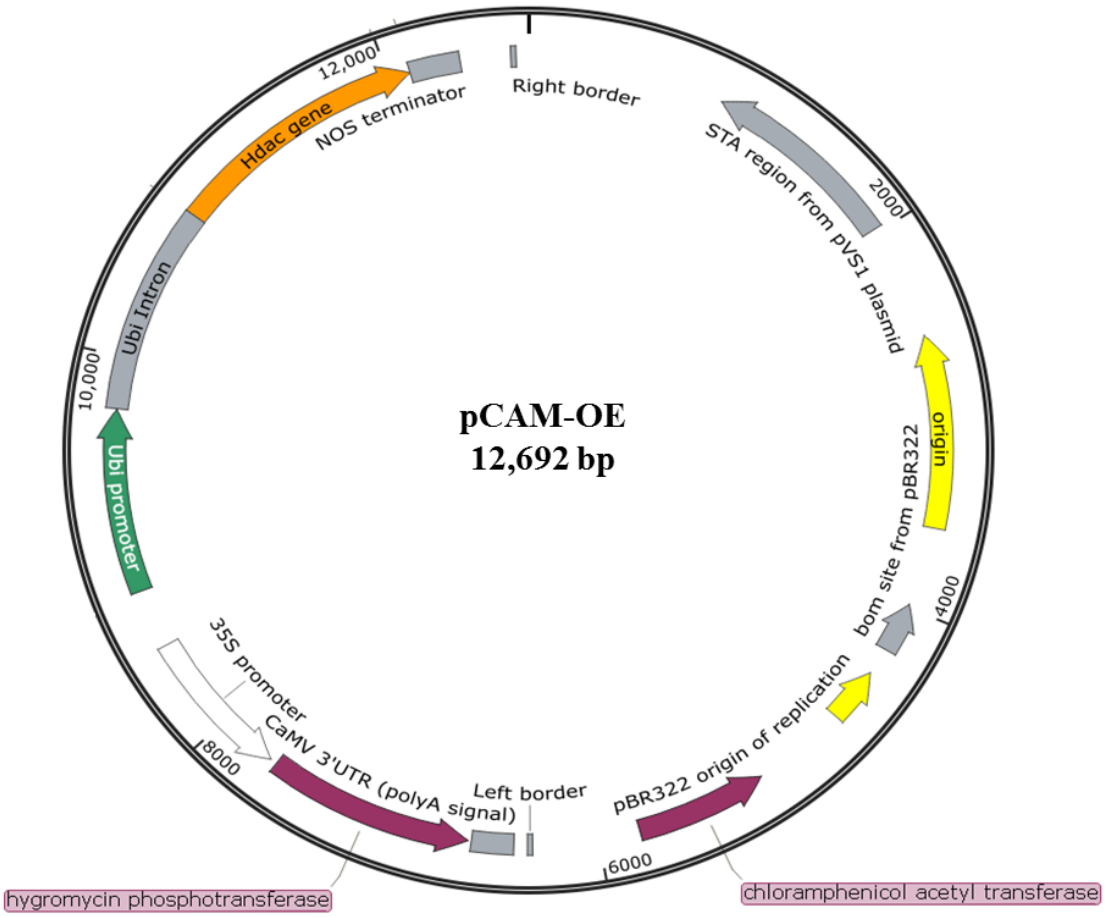

Figure 6. Map of the pCAM_OE construct with *hpt*II plant selection gene. GUS gene of the pCAM-Ubi-GUS construct is replaced by the histone deacetylase CDS.

Agrobacterium-*mediated transformation*

The binary vector constructs, pART27_SIL-1_SIL-2 (for silencing) and pCAM_OE (for over-expression) were mobilized by electroporation into *Agrobacterium tumefaciens* strain NTL4 harboring the disarmed Chry5 Ti plasmid designated pTiKPSF2 (Palanichelvam et al., 2000). Genotypes P898012 (*dw2/dw2*) and RTx430 (*Dw2/Dw2*) were grown under greenhouse conditions and in field plots in College Station, TX. Before flowering the panicles were covered with pollination bags, and immature seeds were harvested within 12-15 days of flowering. Seeds were sterilized by soaking in 70% ethanol for 1 minute, followed by a rinse with sterilized water, and soaked in 30% commercial bleach while shaking for 20 mins at 200 rpm. Seeds were then rinsed three times with sterilized water and placed in an open petri plate to air dry. Immature embryos, 1-1.5 mm in length were excised aseptically under a microscope and placed with their scutellum side up on a stack of 4 sterile Whatman filter papers saturated with 4.2 ml M11 media in a petri plate (100 x 15 mm) and incubated overnight in the dark at 28^{o}C.

The preparation of *Agrobacterium* using appropriate antibiotics followed by induction with acetosyringone was done in accordance with the method described by Howe et al. (2006). The inoculum was kept on ice until ready for use. The excised embryos were moved to a sterile 1.5 ml tube containing the M11 medium. Excess medium was removed, leaving just enough to cover the embryos, and the tubes were incubated in a controlled water bath at 43^{o}C for 3 min followed by cooling at 25^{o}C (Gurel et al., 2009). The embryos were then washed 3 times with modified PHI-T co-

culture medium (mPHI-T) (Howe et al., 2006) supplemented with 40 mg/l acetosyringone and 200 mg/l L-Cysteine, and infected with 1 ml of pre-induced *Agrobacterium* culture for 10 min (after which the bacterial culture was removed). The embryos were placed with scutellum side up on a stack of 4 sterile Whatman filter papers saturated with 4.2 ml of mPHI-T medium in a 100 x 25 mm Petri plate. The embryos were co-cultured in the dark at 25^{o}C for 5-6 days, after which they were moved to resting medium DBC3 (Wu et al., 2014) for 7-10 days at 28^{o}C in the dark. On the 14th day from infection, the growing coleoptiles of the embryos were removed and the embryos were moved to selection medium DBC3 supplemented with appropriate antibiotics for selection (30 mg/L geneticin for RTx430 embryos and 20mg/L hygromycin for P898012 embryos) and cultured for 2 weeks at 28^{o}C in the dark. The embryos were transferred to fresh selection medium every 2-3 weeks for a total period of 6-9 weeks. The surviving calli were transferred to pre-regeneration medium containing antibiotics for selection in tall plates for 1 week under light at 28^{o}C, and then to regeneration medium with the antibiotic concentration reduced to half under the same conditions until shoots regenerated. Developing shoots were moved to jars containing rooting medium with full strength antibiotics for selection. Shoots with well-developed roots were transferred to Sunshine LP5 soil medium for hardening on bench top under room light. The composition of media used in this study are presented in Table 21. Since immature embryo explants of P898012 produce substantial amount of phenolic compounds, 10g/l polyvinylpolypyrrolidone (PVPP) was added to all media during transformation of this genotype.

Table 21. Media formulations for transformation of sorghum.

Media	Medium formulation
M11	MS salts 4.3 g/l; sucrose 30 g/l; myo-inositol 100mg/l; M11 vitamin stock 1 ml/l; 2,4-D 2 mg/l; KH_2PO_4 1.03 g/l; asparagine 900 mg/l; proline 2 g/l; pH 5.7
mPHI-T	MS salts 2.16 g/l; NPT stock 1 ml/l; sucrose 20 g/l; myo-inositol 100mg/l; casein hydrolysate 1 g/l; 2,4-D 2 mg/l; glucose 10 g/l; MES 500 mg/l; proline 2 g/l; acetosyringone 40 mg/l; L-Cysteine 200 mg/l; pH 5.2
DBC3	MS salts 4.3 g/l; myo-inositol 250 mg/l; casein hydrolysate 1.0 g/l; thiamine HCL 1.0 mg/l; 2,4-D 1.0 mg/l; maltose 30 g/l; proline 0.69 g/l; cupric sulfate 1.22 mg/l; BAP 0.5 mg/l, phytagel 3.5 g/l, pH 5.8; carbenicillin 200 mg/l; cefotaxime 100 mg/l
Pre-regeneration	MS salts 4.3 g/l; myo-inositol 100 mg/l; modified B5 vitamin stock 10 ml/l; sucrose 30 g/l; kinetin 0.25 mg/l; phytagel 2 g/l ; pH 5.7; carbenicillin 200 mg/l; cefotaxime 100 mg/l; IAA 0.5 mg/l
Regeneration	MS salts 4.3 g/l; myo-inositol 100 mg/l; modified B5 vitamin stock 10 ml/l; sucrose 30 g/l; kinetin 0.5 mg/l; phytagel 2 g/l ; pH 5.7; carbenicillin 200 mg/l; cefotaxime 100 mg/l; IAA 1 mg/l
Rooting	MS salts 2.15 g/l; myo-inositol 100 mg/l; modified B5 vitamin stock 10 ml/l; sucrose 20 g/l; NAA 0.5mg/l; IBA 0.5 mg/l; phytagel 2 g/l; pH 5.7
M11 vitamin stock	Nicotinic acid 13 mg/10 ml; thiamine HCl 10 mg/10 ml; pyridoxine HCl 10 mg/10 ml;
NPT stock	Nicotinic acid 5 mg/10 ml; thiamine HCl 10 mg/10 ml; pyridoxine HCl 5 mg/10 ml;
Modified B5 vitamin stock	Nicotinic acid 1 mg/l; thiamine HCl 10 mg/l; pyridoxine HCl 1 mg/l; glycine 7.7 mg/l; niacinamide 1.3 mg/l

MS salts are as described by Murashige and Skoog (1962).

Results and discussion

Analysis of phenotypic data and refinement of Dw2 locus

The results of field-based phenotyping of internode length and plant height is presented in Table 22. The mean values of the parent BTx3197 (*dw1**Dw2**dw3dw4*) for all measurements recorded of internode length were significantly greater than for BTx616 (*dw1**dw2**dw3dw4*). The height (from base of plant to tip of panicle) of BTx3197 ranged from 74.5-105.5 cm while the height of BTx616 ranged from 40.7-59.0 cm. The dwarfing genes *Dw1*, *Dw2*, *Dw3* and *Dw4* have been characterized as brachytic, primarily affecting plant height by influencing stem internode length. The lack of a significant effect of recessive *dw2* on peduncle and panicle length in part explains why sorghum breeders utilize this dwarfing gene in breeding grain hybrids. The lack of a strong positive relationship of the recessive *dw2* allele and panicle length are reflected in the correlation coefficient values for these traits (Table 23). Finally, a one way analysis of variance indicated significant differences ($P < 0.001$) in the parental genotypes for plant height, and the genotypes were classified into two height groups, which is consistent with BTx3197 and BTx616 differing at only dwarfing locus *dw2*.

With an aim to map the *Dw2* locus at higher resolution, SSR and INDEL markers (Table 19) spanning the locus as delimited by Klein et al. (2008) were examined to identify additional recombinant events. The *Dw2* locus reported by Klein et al. (2008) was resolved to ~1 Mbp of chromosome 6, and was flanked by SSR markers *Txp434* (position 42.65 Mbp) and *Txp559* (position 43.63 Mbp). Of the original 202 F_2 progeny from the cross of BTx3197 and BTx616, 12 were determined to be heterozygous for

genetic markers flanking the region and thus, were useful for additional *Dw2* locus resolution. A total of 16 SSR sequences within the locus were identified, and of these 12 (75%) were determined to be polymorphic and informative (Table 24). In combination with F_2 progeny showing residual heterozygosity, these new markers identified additional crossover events within the locus, which effectively reduced the size of the locus by 0.8 Mbp. Ultimately, 6 of the 12 (50%) F_2 progeny with residual heterozygosity showed recombinant events within the *dw2* locus, and the refined locus spans 0.1 Mbp and 11 genes (see Table 25 and discussion below). Given the estimated local recombination rate of 150 kb/cM within the *Dw2* locus, to resolve the locus to a similar extent by increasing the original mapping population size would have required an estimated 92 additional F_2 individuals (Dinka et al., 2007). Thus, using F_2 individuals with residual heterozygosity was an effective strategy for increasing the resolution of the *Dw2* locus without the additional time and labor associated with increasing the original mapping population size. The use of progeny with residual heterozygosity has been shown to be an effective locus refinement strategy, as observed for the flowering time loci *FT1* and *FT2* in soybean (Watanabe et al., 2011; Yamanaka et al., 2005), semidwarfism (Tong et al., 2016) and grain length loci (Shao et al., 2010) in rice.

Table 22. Phenotypic mean values for height of different parts of plants of parental inbreds BTx3197 and BTx616. Values represent means with standard deviations (SD) shown in parentheses.

Height	BTx3197	BTx616
IN-1	11.86 (2.47) [a]	3.83 (1.02) [b]
IN-2	7.63 (2.29) [a]	2.32 (0.42) [b]
IN-3	7.33 (2.41) [a]	2.26 (0.53) [b]
Base to IN-1	49.93 (7.39) [a]	14.93 (1.81) [b]
Peduncle+Panicle	41.63 (5.57) [a]	36.43 (5.74) [a]
Total	91.57 (9.28) [a]	51.36 (5.06) [b]
Range	74.50-105.50	40.7-59.0

IN-1, IN-2, IN-3 are first, second and third internodes below the peduncle.
Means followed by different alphabets [a] and [b] indicate significant differences of the trait mean value between the two parents as calculated by Tukey's test.

Table 23. Pearson's correlation coefficients for height of different parts of plants of parental inbreds and progeny.

	IN-1	IN-2	IN-3	Base to IN-1	Peduncle+Panicle	Total
IN-1	1	0.74***	0.64***	0.86***	0.29***	0.82***
IN-2		1	0.85***	0.89***	0.48***	0.90***
IN-3			1	0.83***	0.58***	0.88***
Base to IN-1				1	0.37***	0.95***
Peduncle+Panicle					1	0.63***
Total						1

*** Significant at the 0.001 probability level.
IN-1, IN-2, IN-3 are first, second and third internodes below the peduncle.

Table 24. Genotype scores of parents BTx3197 and BTx616 and the derived progenies for SSR markers covering the *Dw_2* locus. The scores 1 and 3 indicate alleles contributed by BTx3197 and BTx616, respectively. Phenotypic data and height group of parents and progenies are shown.

Genotype	Marker name													Average Total Ht[†] (cm)	Average Base to IN-1 Ht[‡] (cm)	Height group[§]
	txp 434	txi 56	txp 741	txp 742	txp 743	txp 737	txp 738	txp 739	txp 547	txp 690	txp 691	txp 535	txp 559			
BTx3197	1	1	1	1	1	1	1	1	1	1	1	1	1	91.57	49.93	Tall (Dw2)
BTx616	3	3	3	3	3	3	3	3	3	3	3	3	3	51.36	14.93	Short (dw2)
5468-18-02-04_A02	3	3	3	3	3	3	3	3	3	3	1	-	-	56.41	22.80	Short (dw2)
5468-18-02-04_E05	3	3	3	3	3	3	3	3	3	3	1	-	1	55.07	20.60	Short (dw2)
5468-18-02-04_F04	3	3	3	3	3	3	3	3	3	3	1	-	1	58.37	21.70	Short (dw2)
5468-18-02-04_H06	3	3	3	3	3	3	3	3	3	3	1	-	1	56.95	21.30	Short (dw2)
5471-02-06-03-A9	1	3	3	3	3	3	3	3	3	3	-	-	3	56.68	21.55	Short (dw2)
5471-02-06-03-D7	1	3	3	3	3	3	3	3	3	3	-	-	3	57.72	21.60	Short (dw2)
5471-09-B6	-	1	3	3	3	3	3	3	3	3	3	-	-	52.92	21.60	Short (dw2)
5471-02-04-05-07_F11	3	3	3	3	3	1	1	1	1	1	1	-	-	77.00	41.20	Tall (Dw2)
5471-02-04-05-07_B09	3	3	3	3	3	1	1	1	1	1	1	-	-	77.90	41.30	Tall (Dw2)
5484-20-07-08-03_H11	1	1	1	1	1	1	1	1	1	3	3	-	3	73.70	34.75	Tall (Dw2)
5484-20-07-08-03_C07	1	1	1	1	1	1	1	1	1	3	3	-	3	78.90	39.70	Tall (Dw2)
5471-02-13-_G04	3	1	1	1	1	1	1	1	1	1	1	-	1	88.14	46.41	Tall (Dw2)

[†] Defined as average height measured from ground to the top of panicle.

[‡] Defined as average height measured from ground to the topmost node below peduncle.

[§] Height groups short and tall are defined on the basis of one-way analysis of variance of mean height of genotypes.
Crossover events between markers are shown in box.

Candidate gene identification and validation

The refined locus of *Dw2* is delimited by SSR markers *Txp743* and *Txp690* and spanned 0.1 Mbp (SBi-06 42.71-42.82 Mbp). Examining the annotated reference sorghum genome (v.3.0) revealed 11 genes of known function located within the fine mapped *Dw2* locus (Table 25). Annotated genes within the *Dw2* locus included a 1,4-alpha-glucan-branching enzyme (Sobic.006G066800), PPR repeat containing protein (Sobic.006G067000), putative F-box domain proteins (Sobic.006G0067150, Sobic.006G0067300), Serine/Threonine protein phosphatase family proteins (Sobic.006G066900, Sobic.006G067400, Sobic.006G067500), histone deacetylase (Sobic.006G067600), ACG kinase (Sobic.006G067700), and ribosomal inactivating protein (Sobic.006G067100). Examining the proposed function of each of these annotated genes provided a good indication of the most likely candidate for the *Dw2* dwarfism gene. While several of these annotated genes could be involved in dwarfism, Sobic.006G067600, which encodes a protein histone deacetylase, was regarded as the strongest candidate. Histone deacetylases (HDACs) are known to play an essential role in eukaryotic gene regulation via transcriptional gene silencing, and present studies indicate that HDACs play a key role in regulating plant growth. Down-regulation of histone deacetylase in plants has been shown to regulate growth-related processes including plant height in *Arabidopsis* (Tian and Chen, 2001), reduced peduncle elongation, and altered morphology of leaves and stem in rice (Hu et al., 2009; Jang et al., 2003). The remaining 10 genes within the *Dw2* locus had a series of cellular functions that potentially could be involved in dwarfism, but none were considered as

likely candidates compared to the HDAC encoded by Sobic.006G067600. Excluding HDAC, the annotated function of genes in the *Dw2* locus included the following: 1,4-alpha-glucan-branching enzyme that has hydrolase activity and is involved in the starch biosynthetic process; PPR domain containing proteins, which are a large family of RNA-binding proteins primarily involved in gene expression in plant organelles; ribosomal-inactivating proteins (RiPs), which are catalytic toxins produced by some plants and bacteria; F-box domain proteins that form one of the largest multigene superfamilies and control many important biological functions including mediating ubiquitination of proteins, signal transduction and cell cycle regulation; serine/threonine protein phosphatase family proteins that are known to play a role in stress signaling pathways; ACG kinase that is involved in protein phosphorylation and intracellular signal transduction; and 60S acidic ribosomal protein that is a structural component of ribosome with a function in translational elongation.

Despite the known function of HDACs in plant growth processes, validation of the identity of the *Dw2* candidate gene was necessary. Two approaches were chosen for functional validation; RNAi-mediated gene silencing of candidate gene Sobic. 006G067600 in a wild-type (*Dw2*) sorghum genotype, and over-expression of the candidate gene in a dwarf (*dw2*) sorghum genotype. To overexpress the wild-type allele, cDNA was prepared from RNA of BTx3197 (*Dw2*) at a stage of plant development where internode elongation was apparent (~30 days post germination). A full-length cDNA of 1293 nucleotides comprising the HDAC gene was synthesized and the accuracy of the clone was confirmed to assure no mutations were introduced during

Table 25. Annotated sorghum genes within the fine mapped *Dw2* locus. Genomic positions of the genes and markers are shown. Annotation file containing Rice orthologs was downloaded from Joint Genome Institute's Genome Portal (Sorghum bicolor v1.0, http://phytozome.jgi.doe.gov).

Gene/Marker start (bp)	Gene/Marker end (bp)	Gene name	Marker name	Rice ortholog	Annotated function
42707963	42717710	Sobic.006G066800	*Txp743*†	LOC_Os02g32660	1,4-alpha-glucan-branching enzyme
42718980	42722791	Sobic.006G066900	*Txp737*†	LOC_Os04g33470	Ser/Thr protein phosphatase family protein
42723881	42725688	Sobic.006G067000		LOC_Os03g11690	PPR repeat containing protein
42753303	42756717	Sobic.006G067100		LOC_Os02g05590	ribosome inactivating protein
42758806	42759413	Sobic.006G067150		LOC_Os06g49530	OsFBX206 - F-box domain containing protein
42769007	42770832	Sobic.006G067300		LOC_Os03g46510	OsFBX103 - F-box domain containing protein
42774078	42778987	Sobic.006G067400	*Txp738*†	LOC_Os04g33470	Ser/Thr protein phosphatase family protein
42781244	42785442	Sobic.006G067500		LOC_Os04g33470	Ser/Thr protein phosphatase family protein
42785485	42802516	Sobic.006G067600	*Txp739*†	LOC_Os04g33480	histone deacetylase
42803037	42807520	Sobic.006G067700		LOC_Os12g29580	AGC_PVPK_like_kin82y.19
			Txp547		
			Txp690		
42823157	42825066	Sobic.006G067800		LOC_Os02g32760	60S acidic ribosomal protein

† SSR markers located within the genes.

cDNA preparation. Following the series of cloning events as detailed in the methods section, pCAM_OE, which contained a maize ubiquitin promoter, NOS terminator and *hpt*II plant selection gene (encoding hygromycin resistance) and the entire HDAC CDS was used for complementation of the recessive *dw2* allele of the genotype P898012. For silencing the HDAC gene, a 300 bp fragment of the coding region was targeted using a self-complementary single-stranded hpRNA consisting of an inverted repeat and separated by a spacer intron. This region of Sobic. 006G067600 was chosen based on its low sequence similarity to other genic sequences thereby reducing the possibility of cross-silencing of non-target genes. On sequencing the RNAi construct, a single base change (G→A) was observed in the ~300 bp gene insert. However, the single base change should not affect the formation of the hairpin and thus, should not preclude RNAi. Following a series of cloning events, the derived construct pART27_SIL-1_SIL-2 (Figure 2) was used for RNAi-mediated gene silencing of candidate gene Sobic. 006G067600 in sorghum genotype RTx430 (*Dw2*).

Sorghum genotypes RTx430 (*Dw2*) and P898012 (*dw2*) were chosen for RNAi-meditated gene silencing and complementation of the *dw2* allele because of their known allelic state at the *Dw2* locus and their suitability for agrobacterium-mediated transformation (Do and Zhang, 2015).

Genotype P898012 posed unique tissue regeneration challenges due to the high tannin (phenolics) of this sorghum line. Phenolics exuded by immature embryos of P898012 negatively impacted callus tissue growth and differentiation during the resting phase and plant regeneration from callus (Gao et al., 2005; Zhao et al., 2000). In

addition, phenolics are toxic to *Agrobacterium* cells (Nguyen et al., 2007), and each of these factors prevented successful transformation of genotype P898012. While ~2,350 embryos were infected with *Agrobacterium* for transformation, none of them survived beyond the plantlet pre-regeneration stage. The use of PVPP in all media to bind phenolics met with limited success and did not drastically improve the rate of survival of the growing calli. Towards the end of this investigation, it was determined that infecting the embryos with *Agrobacterium* on the same day of embryo excision without pre-culture on M11 medium resulted in less injury of the embryos and thus, reduced phenolic synthesis. While this modification limited callus formation, it may facilitate the successful recovery of T1 plants allowing for complementation of the HDAC gene in genotype P898012.

For the genotype RTx430, ~2,750 embryos were used for transformation, of which ~800 embryos were lost to bacterial or fungal infections during different stages of the process. A number of remaining embryos formed calli and underwent selection in the presence of hygromycin. Based on the ability to grow on media containing the selectable marker, ~27 putative transgenic calli regenerated into plants and formed roots. Following transplanting to potting mixture, the acclimatization process of the environmentally-sensitive T1 plantlets was done within the laboratory by maintaining the plantlets under high humidities and low light intensities. Despite the effort to reduce the shock of transplanting T1 plantlets, none of the plantlets survived the acclimatization process. Examination of the T1 plantlets revealed that their failure to form new roots after transferring to soil is a probable reason for plant death during hardening.

Based on the inability of the aforementioned investigation to validate the identity of the *Dw2* gene, additional studies were recently initiated, but will not reach fruition prior to the completion of this dissertation. Nevertheless, a collaboration was established with a crop transformation laboratory at the University of Nebraska-Lincoln (Thomas and Wendy Clemente) that has a very high success rate of sorghum transformation and plant regeneration. Constructs for complementation and RNAi-mediated gene silencing of *Dw*2 candidate gene HDAC are in the process of being used to generate T1 plants of genotypes RTx430 and P898014. It is my assertion that these studies will provide conclusive evidence to determine if the correct *Dw2* candidate gene has been identified.

This study was successful in improving the resolution of the *Dw2* locus using a strategy that involved further characterization of individuals with residual heterozygosity across the dwarfism locus. Without increasing the original F_2 population size, the *Dw2* locus of ~1 Mbp was refined to a 0.1 Mbp of chromosome SBi-06 by discovering additional recombinant events (i.e. crossovers) that occurred in F_2 plants that were still segregating for markers spanning the locus. The resulting *Dw2* locus encoded a limited number (11) of annotated genes with the best candidate being a histone deacetylase, a family of genes known to play a key role in regulating plant growth. The two validation approaches, gene complementation and RNAi-mediated gene silencing, were conducted, but the lack of success of *Agrobacterium*-mediated sorghum transformation precluded the ability to confirm the identity of *Dw2* as a member of the histone deacetylase gene family. The recalcitrance of sorghum for *in vitro* growth makes transformation

challenging, and success in sorghum transformation by teams at Texas A&M University will require acquiring expertise from laboratories that routinely transform this crop.

CHAPTER V

CONCLUSION

With a primary focus on improving grain sorghum germplasm, molecular genetic analyses were conducted on key agronomic traits that included anthracnose resistance, tolerance to salinity, and plant dwarfism. To elucidate those regions of the sorghum genome conditioning anthracnose resistance, two RIL populations from a cross of anthracnose-susceptible inbred BTx623 with two anthracnose resistant sorghum genotypes SC414-12E and SC155-14E were evaluated in multiple environments. Inbred SC414-12E displayed a moderate level of resistance across all locations while inbred SC155-14E had a high level of stable resistance in all environments tested. QTL analysis revealed a total of 9 trait loci for anthracnose resistance with three of these disease resistance loci (one in SC414-12E and two in SC155-14E) consistently detected across all environments. In addition these mapping efforts revealed environment-specific QTL, which likely arise from the different pathogen pressures that exist. One disease resistance locus was detected with BTx623 contributing the resistance allele implying a role of susceptible parent BTx623 in anthracnose resistance amongst the RILs. A preliminary investigation of the anthracnose resistance trait loci revealed a series of annotated genes with a role in disease resistance. Many of these genes have sequence variation in the coding regions, but further studies are necessary to identify causative genes and functional mutations conferring anthracnose resistance. Nevertheless, this

study provides the necessary information to introgress or pyramid anthracnose resistance QTL into elite cultivars through a marker-assisted backcrossing strategy.

A greenhouse study to elucidate the genomic region conditioning salt tolerance in sorghum identified one QTL conditioning salt tolerance in a RIL population created from a cross of sorghum inbreds MMR338 and MMR31. By quantifying the extent of leaf damage in plants exposed to saline growth conditions, it was determined that inbred MMR338 displayed ~80 to 95% less leaf damage than MMR31, and MMR338 was contributing the resistance allele for the one QTL detected. In addition, the RIL population showed transgressive segregation for salt tolerance, which suggests that both sorghum inbreds harbor genes that confer salt tolerance in the RILs. Despite efforts to statistically account for the spatial variation that existed in the microenvironment across the greenhouse, it is apparent that growth conditions varied markedly and likely was responsible for the low power of this mapping study. A brief examination of the annotated genes spanning the one significant QTL revealed a number of genes with a role in different mechanisms of salt tolerance, with the most prominent being a gene encoding a cation/H^+ antiporter. However, to identify the salt tolerance genes, studies are necessary in which growth conditions are closely monitored and adjusted to increase the accuracy and power to identify salt tolerance trait loci.

The dwarfing gene *Dw2* was fine-mapped using a strategy that involved further characterization of individuals with residual heterozygosity across the genomic region encoding *Dw2*. Without increasing the original F_2 population size, the *Dw2* locus of ~1 Mbp was refined to a 0.1 Mbp region of chromosome SBi-06 by discovering additional

recombinant events (i.e. crossovers) that occurred in F_2 plants that were not fixed for markers spanning the locus. The resulting *Dw2* locus encoded a limited number (11) of annotated genes with the best candidate being a histone deacetylase, a family of genes known to play a key role in regulating plant growth. The two approaches (gene complementation and RNAi-mediated gene silencing) were conducted for gene validation, but the lack of success of *Agrobacterium*-mediated sorghum transformation precluded the ability to confirm the identity of *Dw2* as a member of the histone deacetylase gene family. Nevertheless, the histone deacetylase gene that resides in the refined *Dw2* trait locus represents a strong candidate for controlling dwarfism in sorghum, and further refinement of sorghum transformation will eventually permit a determination of the identity of the *Dw2* gene. For sorghum to become a model genetic system used in identifying key agronomic genes, plant transformation must become routine for researchers even if transformation is limited to a few select sorghum genotypes.

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