GENETIC MAPPING OF RESISTANCE TO FUSARIUM WILT AND SEED OIL TRAITS IN WATERMELON

by

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(Under the Direction of Cecilia McGregor)

ABSTRACT

Watermelon, (*Citrullus lanatus* var. *lanatus* (Thunb.) Matsum. & Nakai, a major horticultural crop in the world, is mainly grown for its flesh and seed consumption. Integration of marker assisted selection (MAS) in conventional breeding programs would accelerate the release of watermelon cultivars with improved nutrition and disease resistance. The current study aimed to elucidate the genetic factors associated with important seed nutrition traits and resistance to fusarium wilt caused by *Fusarium oxysporum* f. sp. *niveum* (E.F. Sm.) W.C. Snyder & H.N. Han. in watermelon. Bi-parental mapping populations segregating for seed traits (seed oil percentage, kernel percentage and seed size) and resistance to fusarium wilt (races 1 and 2) were used to identify genomic regions associated with these traits on the watermelon draft genome. Several quantitative trait loci and candidate genes associated with these traits were identified and are potential targets in MAS. To utilize the molecular tools developed in the current study in MAS, a seed based genotyping method that allows for efficient selection of target traits was developed.

INDEX WORDS: *Citrullus lanatus*, genotyping by sequencing, QTL, SNP, fatty acids, seed oil, egusi

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DEDICATION

I dedicate this dissertation to my late mother, Florence Meru, whose memories I cherish every day. I am grateful for the life lessons you taught me.

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CHAPTER 1

INTRODUCTION

Citrullus is an important genus of the Cucurbitaceae family (Robinson and Decker-Walters, 1997). Other important genera in this family include *Cucurbita* [pumpkin (*Cucurbita moschata*) and squash (*Cucurbita pepo*)] and *Cucumis* [melon (*Cucumis melo*) and cucumber (*Cucumis sativas*)] (Dane and Liu, 2007; Jeffrey, 1990). The *Citrullus* genus is composed of four species namely *C. lanatus* (Thunb.) Matsum. & Nakai, *C. colocynthis* (L.) Schrad., *C. ecirrhosus* (Cogn.) Chakrav., and *C. rehmii* De Winter. The origin of the four species can be traced back to Southern and Northern regions of Africa (Jeffrey, 1978; Jeffrey, 1990; Robinson and Decker-Walters, 1997). *C. lanatus* is further classified into *C. lanatus* var. *lanatus* and the *C. lanatus* var. *citroides* (Robinson and Decker-Walters, 1997).

China is the world's biggest producer of watermelon (Food and Agricultural Organization, 2013). The United States has over 133,700 acres dedicated to watermelon production with an annual fresh market value exceeding half a billion dollars (United States Department of Agriculture, National Agricultural Statistics Service, 2014). The crop is valued for its sweet-flesh and is a good source of vitamin A and C, minerals including potassium, iron and calcium, and high amounts of lycopene and citrulline (Ellul et al., 2007; Rimando and Perkins-Veazie, 2005). In addition, the seeds of watermelon provide a significant source of nutrition and income in several parts of the world (Achigan-Dako et al., 2008; Baboli and Kordi, 2010; Edelstein and Nerson, 2002).

Commercial production of watermelon in the US is primarily in Georgia, California, Texas, Florida and Arizona (United States Department of Agriculture, National Agricultural Statistics Service, 2014). Most of the area under watermelon cultivation in the US is dedicated to seedless, triploid hybrids. Breeding in watermelon is mainly by conventional methods (Wehner et al. 2008). Although effective, selection for traits by conventional methods is time consuming and resource intensive. Having markers linked to traits of interest can greatly accelerate conventional breeding and allow timely release of improved cultivars. However, application of marker-assisted selection (MAS) in watermelon breeding is not routine. The objective of the current study was to identify genomic regions associated with important seed nutrition traits and resistance to fusarium wilt (races 1 and 2) in watermelon using bi-parental mapping populations.

CHAPTER 2

LITERATURE REVIEW

Breeding in watermelon

Through domestication and deliberate plant breeding efforts, the late-maturing wild watermelon, characterized by small fruits with hard, bitter/ bland tasting white flesh, has been developed into the current elite watermelon cultivars through selection for a set of desirable traits (Wehner, 2008). In the United States, watermelon is either grown as diploid (open pollinated and F_1 hybrids) or triploid (seedless) cultivars with the latter dominating the market.

Yield

Yield is one of the most important traits in watermelon as it directly impacts the return on investment by growers. Typically, yield is measured in weight per acre of marketable size fruit (Wehner, 2008). In diploid watermelon production, yield is maximized by optimizing planting density with a higher density usually resulting in better yield per unit area (Duthie et al., 1999). In commercial triploid watermelon production, a certain proportion of the acreage is dedicated to diploid pollinizers that provide the viable pollen necessary for fruit set.

Fruit qualities

Fruit quality in watermelon is determined by a combination of different traits including sugar content, fruit size, flesh color, fruit shape, rind thickness and pattern. The sugar content of watermelon flesh is one of the most important traits in elite watermelon and is determined by the Brix value (°Brix) which is a measure of the total soluble solids (Hashizume et al., 2003; Sandlin et al., 2012; Wehner, 2008). Brix values are measured using a refractometer (Wehner, 2008). To

be marketable, watermelon must have a sugar content of at least 10% (Wehner, 2008) although recent watermelon cultivars have a sugar content reaching 14%. Desirable watermelon fruit size is dependent on consumer and shipper needs and is generally categorized into mini (<4.0 kg), icebox (4.0-5.5 kg), small (5.0-8.0 kg), medium (8.0-11.0 kg), large (11-14.5 kg) and giant (>14.5 kg) (Wehner, 2008). Unlike in the past where large watermelon fruits were valued by large-sized families, today's consumers prefer fruits that are smaller and easily handled or stored. For shipping needs, diploids within the range of 8-11 kg and triploids within the range of 6-8.5 kg are the desirable categories. In addition, rind thickness is important for shippers. Large fruits are required to have thick rinds to prevent cracking during shipping. Consumers have varying preferences for watermelon flesh color, which can be yellow (canary yellow and salmon yellow), red (scarlet red, coral red), white or orange (Ellul et al., 2007; Navot et al., 1990; Wehner, 2008). Breeders are also keen to satisfy the preference for specific fruit shape which may be oval, round, elongate, or blocky (Ellul et al., 2007; Wehner, 2008). The rind pattern of watermelon fruit also affects consumer preference and may be solid, gray or striped (Ellul et al., 2007; Wehner, 2008).

Seedlessness

The seedless trait in watermelon is characterized by fruits that contain undeveloped seeds that can be consumed along with watermelon flesh (Ellul et al., 2007). Following the production of the first watermelon seedless, triploids in Japan in 1951 (Kihara, 1951) and the adoption of chromosome doubling techniques for tetraploid induction (Andrus et al., 1971), the technology for seedless watermelon production has been widely adopted in the United States (Wehner, 2008). Triploid watermelons are produced by first developing tetraploid lines through chromosome doubling and crossing them with diploid cultivars (Andrus et al., 1971; Ellul et al.,

2007). Since tetraploid lines have reduced fertility (few seeds per fruit) and limited fruit production (fruit per plant), it takes a long time (> 10 years) to generate seed required to meet the commercial need for new triploids (Ellul et al., 2007). This is the main contributing factor to the high cost of triploid-hybrid seed which can be 5-10 times higher than that of seeded F_1 hybrid seed (Ellul et al., 2007).

Sex expression

Sex expression is an important trait in commercial production of watermelon F_1 hybrids (Prothro et al., 2013). Sex expression in watermelon is either andromonoecious, trimonoecious or monoecious (Prothro et al., 2013; Wehner, 2008). Monoecious sex expression is the most common type in commercial watermelon (Wehner, 2008). In the production of F_1 hybrids, the andromonoecious trait is undesirable since it necessitates emasculation of hermaphrodite flowers prior to hybridization (Prothro et al., 2013). The potential location of the andromonoecious trait (*a*) in watermelon has been narrowed to chromosome 3 (Prothro et al., 2013) on the draft genome (Guo et al., 2013). This QTL is a candidate in marker assisted selection (MAS) for sex expression in watermelon.

Seed traits

Watermelon breeders select for seed size to meet preferences by commercial growers and the consumers of edible seeds. Large seeds are preferred for direct sowing and for edible seeds (Prothro et al., 2012a; Zhang, 1996) while small seeds are preferred in the flesh of edible diploid-seeded cultivars (Prothro et al., 2012a). Seed size can be measured as seed weight, seed length or seed width. These traits are highly correlated (>91%) (Poole et al., 1941) leading to the use of seed length as a universal measure of seed size in watermelon (Poole et al., 1941; Prothro et al., 2012a). Watermelon seeds have been described as either small (6 mm), medium (10 mm) or

large (13 mm) (Poole et al., 1941). Genomic regions associated with seed size traits in watermelon have been identified in several watermelon populations of different genetic backgrounds (Prothro et al., 2012a). The QTL associated with seed weight, seed length and seed width were identified on chromosomes 2, 6 and 8, with the major QTL on chromosome 2 being stable across two independent populations (Prothro et al., 2012a; Zhang et al., 1995).

The seeds of watermelon provide a significant source of nutrition and income in several parts of the world including China (Zhang, 1996), Israel (Edelstein and Nerson, 2002), Iran (Baboli and Kordi, 2010) and Africa (Achigan-Dako et al., 2008; Al-Khalifa, 1996; El-Adawy and Taha, 2001). In particular, the egusi watermelon is the most popular watermelon type grown for edible seeds in Africa (Gusmini et al., 2004). The seeds of the egusi watermelon are large-sized with a unique fleshy pericarp and are nutritious with high levels of oils and proteins (Gusmini et al., 2004). Prothro et al. (2012b) determined that variation in seed oil percentage (SOP) between egusi and normal seed was mainly due to the *egusi* locus on chromosome (chr.) 6. However, the genetic cause of variation in SOP within seed types (egusi and normal) remained unclear. In other crops such as sunflower (*Helianthus annuus*) (Tang et al., 2006) and canola (*Brassica napus*) (Yan et al., 2009) kernel percentage (KP) and seed size have been implicated in determining SOP. In watermelon, Jarret and Levy (2012) reported KP to be highly correlated with SOP while Prothro et al. (2012b) mapped SOP on chr. in a region associated with seed size in independent populations, suggesting the role of the two traits in SOP.

Another important component of watermelon seed oil is its fatty acid profile. The type and ratio of fatty acids in a given vegetable oil determine its nutrition, flavor, shelf life, and its potential application (Brown et al., 1975). Watermelon seed oil with low saturated fatty acids and high unsaturated fatty acids is desirable as it reduces the risk of heart-related ailments

(Wassom et al., 2008). Watermelon has four primary fatty acids including palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1), and linoleic acid (18:2) (Al-Khalifa, 1996; Baboli and Kordi, 2010). The variation in linoleic acid (45.37% to 73%), oleic acid (7.89% to 33.95%), stearic acid (5.03% to 13.84%), palmitic acid (9.68% to 14.38%)] among the watermelon genebank accessions has been reported in a recent study by Jarret and Levy (2012). The unsaturated fatty acid profile (oleic and linoleic acids) in watermelon (81.3%) is similar to that of other cucurbits such as melon (80.1%) and cucumber (79.9%) (Al-Khalifa, 1996). Similarly, the levels of linoleic acid in watermelon (68.3%) and that in sunflower (68%) are comparable (Baboli et al., 2010). Although the fatty acid composition of watermelon seed is well documented, the underlying genetic basis has not yet been studied.

Disease resistance

Disease resistance is important in watermelon production since disease pressure can indirectly lead to reduction in yield. The domestication of watermelon through selection for suitable traits has led to a narrow genetic diversity in the current elite watermelon cultivars (Guo et al., 2013; Levi et al., 2001). Consequently, alleles conferring resistance to important fungal, bacterial and viral diseases have been lost (Guo et al., 2013; Hawkins et al., 2001), making elite watermelon susceptible to many diseases. Economically important pathogens affecting watermelon production include *Fusarium oxysporum* f. sp. *niveum* (fusarium wilt) (Martyn and McLaughlin, 1983; Martyn and Netzer, 1991), *Didymella bryoniae* (gummy stem blight) (Keinath, 2001), *Colletotrichum lagenarium* (anthracnose) (Suvanprakorn and Norton, 1980), *Sphaerotheca fuliginea* (powderly mildew) (Keinath and DuBose, 2004), *Pseudoperonospora cubensis* (downey mildew) (Palti and Cohen, 1980), *Verticillium dahlia* (verticillium wilt) (Bhat and Subbarao, 1999), *Erwinia* species (Bacterial rind necrosis) (Maynard and Hopkins, 1999),

Acidovorax avenae subsp. *citrulli* (fruit blotch) (Maynard and Hopkins, 1999), papaya ringspot virus (Fehr, 1993) and zucchini yellow mosaic virus (Boyhan et al., 1992).

Fusarium wilt

Fusarium wilt caused by *Fusarium oxysporum* f. sp. *niveum* (*Fon*) (E.F. Sm.) W.C. Snyder & H.N. Han. is a production-limiting disease in watermelon growing regions of the world (Boyhan et al., 2003; Egel and Martyn, 2007; Hawkins et al., 2001; Martyn and McLaughlin, 1983; Zhou et al., 2010). There are four races of *Fon* designated 0, 1, 2 and 3 based on their aggressiveness or the ability to overcome specific resistance in a set of differential cultivars (Bruton, 1998; Egel and Martyn, 2007; Wehner, 2008; Zhou et al., 2010). The disease was first described in 1894 by Smith in South Carolina and Georgia but has since spread to other states including Delaware, Florida, Oklahoma, Maryland and Texas (Bruton, 1998; Zhou and Everts, 2004; Zhou et al., 2010).

Pathogen biology and disease cycle

Fon has no known sexual stage and reproduces asexually through spores including microconidia, macroconidia and chlamydospores (Egel and Martyn, 2007; Leslie et al., 2006). Primary infection of watermelon root or hypocotyls starts with an infection-hyphae germinating from either the chlamydospores or macroconidia and can occur at any stage of plant growth (Egel and Martyn, 2007; Lin et al., 2010; Wehner, 2008). Using cell wall degrading enzymes and plant saponin-detoxifying enzymes, the fungal-hypae can penetrate the root-cortex by overcoming the primary plant resistance barriers such as the cell wall and constitutive phytoalexins (Bruton, 1998; Idnurm and Howlett, 2001; Inoue et al., 2002). Following penetration, the fungus grows within the xylem and produces microconidia that are translocated to the upper parts. Microconidia attach to the xylem walls and sporadically germinate to form

mycelia and other infection structures (Egel and Martyn, 2007; Wehner, 2008). The watermelon plant attempts to stop systemic spread of the pathogen by blocking the xylem vessels using special structures called tyloses that result from invaginations of the xylem parenchyma cells. Consequently, these structures restrict water flow thus causing wilting symptoms (Egel and Martyn, 2007; Lin et al., 2010). The speed at which these structures are formed determine, in part, the resistance level of a watermelon plant (Egel and Martyn, 2007). Other accompanying symptoms following wilting include yellowing and tissue necrosis that eventually lead to plant death (Bruton, 1998; Egel and Martyn, 2007; Wehner, 2008). Although the fungus is largely xylem-limited, profuse colonization of the plant surface is observed following plant death (Egel and Martyn, 2007). After plant death the fungus forms resting spores (chlamydospores) that remain viable in the soil for long periods of time.

Disease epidemiology

Infection by *Fon* is most favorable at temperatures of approximately 27 °C in light, sandy, slightly acidic soils with high organic matter (Boyhan et al., 2003; Bruton, 1998; Egel and Martyn, 2007; Wehner, 2008). Infection is retarded at temperatures above 33 °C (Bruton, 1998) often resulting in plants that are chlorotic and stunted but not wilted (Egel and Martyn, 2007). The propagules of the pathogen can be spread within and among cultivated fields through soil, water, plant debris, contaminated crops (transplants) and farm implements (Bruton, 1998; Egel and Martyn, 2007; Wehner, 2008). There are some reports that the pathogen can be seed-borne (Bruton, 1998; Egel and Martyn, 2007). Being a soil-borne pathogen, the ability of *Fon* to cause disease is influenced by the pathogen's interaction with the host and soil environment, which makes breeding for resistance difficult (Boyhan et al., 2003; Cohen et al., 2008; Geiger and

Heun, 1989; Martyn and McLaughlin, 1983). These interactions and the mechanisms influencing them are poorly understood (Boyhan et al., 2003).

Diagnosis and identification fusarium wilt

Routine diagnosis of fusarium wilt in watermelon and other cucurbits involves visual inspection of plants for symptoms (Egel and Martyn, 2007). It is therefore one of the most misdiagnosed diseases by farmers and plant pathologists (Bruton, 1998). This is in part because symptoms of fusarium wilt are commonly associated with other abiotic (e.g. drought stress) and biotic stress factors (e.g. verticillium wilt) in watermelon (Egel and Martyn, 2007). Reliable diagnosis of fusarium wilt is required for the commencement of an integrated disease management approach (Lin et al., 2010). Conventional *Fon* identification is based on fungal morphology and is not suitable in cases where other disease complexes are involved (Leslie et al., 2006; Lin et al., 2010). Molecular methods have been developed that discriminate *Fon* from other *Fusarium oxysporum* f. sp by Lin et al. (2010) and involve a PCR based on randomly amplified polymorphic DNA (RAPD) detection system. In addition, identification of specific races of *Fon* may be necessary in order to select suitable cultivars to grow in a field. The use of host differentials for *Fon* race identification is useful but laborious and time-consuming (Lin et al., 2010). An alternative to host differentials is the use of vegetative compatibility grouping (VCG) (Bruton, 1998; Hopkins et al., 1992; Zhou and Everts, 2007). Race 1 pathotype of *Fon* is grouped in the 0080 and 0081 VCG while race 2 is placed in the VCG 0082 (Bruton, 1998).

Management of fusarium wilt

Management of fusarium wilt is difficult because of the long-term survival of the pathogen in the soil and the evolution of new races (Bruton, 1998; Egel and Martyn, 2007; Lin et al., 2009; Martyn and Netzer, 1991; Yetisir et al., 2003). Evolution of *Fon* races was reported by

Hopkins et al. (1992) who documented the recovery of predominantly race 2 strain from wilted plants previously resistant to race 1 strain and less than 10% of the same race from wilted plants susceptible to race 1 after long periods of monocultures of watermelon in Florida, USA. Management options of fusarium wilt include the use of disease-free planting materials, avoidance of infested fields, 5-7 year crop rotation, chemical and biological fumigation (Bruton, 1998; Egel and Martyn, 2007; Hopkins et al., 1992), resistant root-stocks (Kuniyasu, 1980), solarization (Njoroge et al., 2008), suppressive soils (Biles and Martyn, 1989; Sun and Huang, 1985) and induced resistance (Biles and Martyn, 1989; Cohen, 2002). These methods are not singly effective and are expensive and environmentally harmful (Boyhan et al., 2003; Bruton, 1998; Egel and Martyn, 2007; Lin et al., 2010; Zhou et al., 2010). Cultivation of resistant cultivars is widely accepted as the best management method for fusarium wilt (Bruton, 1998; Hopkins et al., 1992; Lin et al., 2009; Lin et al., 2010; Martyn and McLaughlin, 1983; Martyn and Netzer, 1991; Zhou and Everts, 2004). To this end several disease screening experiments have been carried out to identify watermelon accessions with resistance to various *Fon* races. Consequently, many cultivars with resistance to *Fon* races 0 and 1 have been developed (Bruton, 1998; Wehner, 2008). The first fusarium wilt resistant cultivar, "Conqueror" was developed by Orton in 1913 by crossing the cultivar "Eden" with an African stock "Citron" (Bruton, 1998; Wehner, 2008). Since then, many other diploid cultivars with resistance to fusarium wilt race 1 have been bred (Wehner, 2008). Most commercially available triploid watermelon hybrids are susceptible to all races of *Fon* (Bruton, 1998; Zhou et al., 2010), although progress is being made towards incorporating race 1 resistance into these cultivars (Boyd, 2011).

Genetic resistance to fusarium wilt

The inheritance and nature of the genes involved in genetic resistance to *Fon* has been the topic of several studies (Guner and Wehner, 2004; Netzer and Weintall, 1980; Zhang and Rhodes, 1993). Resistance to *Fon* race 1 in the cultivar Calhoun Gray is thought to be controlled by a single dominant gene designated *Fo-1* with a few modifier genes (Guner and Wehner, 2004; Martyn and Netzer, 1991; Netzer and Weintall, 1980; Wehner, 2008; Zhang and Rhodes, 1993). Resistance to race 2, designated *Fo- 2* in PI 296341, is reportedly polygenic and controlled by at least a pair of recessive genes in epistasis with other minor genes (Hawkins et al., 2001; Martyn and Netzer, 1991; Wehner, 2008; Zhang and Rhodes, 1993).

Genetic mapping of resistance to fusarium wilt

Several studies have been carried out to identify genomic factors associated with resistance to *Fon* races 1 and 2. Xu et al. (1999) identified RAPD marker (OP01/700) linked (3 cM distance) to resistance to *Fon* race 1 in PI 296341-FW (var. *citroides*). This marker was cloned and sequenced by Xu et al. (2000) and converted into a sequence characterized amplified region (SCAR) marker. The SCAR marker was then used in discriminating between resistant and susceptible $F_{2:3}$ lines in a MAS program. Hawkins et al. (2001) constructed a genetic map for watermelon using F_2 and $F_{2:3}$ populations segregating for resistance to races 1 and 2 of *Fon*, also using PI 296341-FW as a resistant parent and New Hampshire Midget (*C. lanatus* var. *lanatus*) as the susceptible parent. This study reported "loose" linkage between RAPD markers and resistance loci to races 1 and race 2 at 40% and 33% recombination, respectively. In a recent study, Lin et al. (2009) designed a SCAR primer (G05-SCAR) derived from a RAPD marker (OP-G05) sequence from the resistant JSB (a mutant of Sugar Baby) (Chen et al., 2003) that was specific to watermelon lines resistant or tolerant to *Fon* race 1. Harris et al. (2009) used

degenerate primers to target R-genes that encode nucleotide binding site–leucine-rich repeat (NBS-LRR) proteins in watermelon. The three watermelon EST disease resistance homologs identified were mapped to different linkage groups but none mapped near the marker (OP01/700) linked to race 1 resistance by Xu et al. (1999). Most of these studies used populations with PI 296341-FW as resistant parent. As a result, segregation distortion of marker alleles in these populations led to low resolution maps and large unmapped regions (Levi et al., 2011). Breeders have used PI 296341-FW (var. *citroides*) to breed for resistance against race 2 of *Fon* in watermelon. However, the resulting cultivars have only been utilized as pollinizers (Boyd, 2011) due to undesirable traits such as small fruit size, undesirable flesh color and low sugar content. Wechter et al. (2012) recently identified several var. *citroides* accessions that were highly resistant to *Fon* race 2. The application of these accessions in breeding for fusarium wilt resistance may also lead to resistant cultivars with undesirable traits. Therefore there is need to identify new sources of resistance to *Fon* race 2 in var. *lanatus* that can be used for resistance breeding without the disadvantage of undesirable traits.

Recently, populations developed by crossing elite with elite watermelon cultivars have been used to identify markers linked to *Fon* race 1. Using bulk segregant analysis approach, Xu (2014) identified several SNP markers linked to *Fon* race 1 on chr. 1 using an $F_{2:3}$ population developed from a cross between Calhoun Gray (resistant) and Black Diamond (susceptible). In a separate study Lambel et al. (2014) mapped seven QTL associated with resistance to *Fon* race 1 using an $F_{2:3}$ population segregating for resistance. The main QTL identified in this study on chr. 1 is in proximity to several functionally annotated disease resistant gene homologs (Guo et al. 2013). Although progress has been made in identifying genomic regions associated with *Fon* race 1, no QTL have been successfully associated with resistance to *Fon* race 2.

Genomic tools for marker assisted selection in watermelon

MAS as a tool for conventional breeding allows breeders to select for desired traits without carrying out laborious and time consuming phenotypic assays (Kang et al., 1998). High throughput seed based genotyping systems for MAS have been developed for major crops such as maize (Gao et al., 2008) and allows for selection of suitable individuals prior to planting (Gao et al., 2008; Kang et al., 1998; von Post et al., 2003). The application of MAS in watermelon breeding lagged behind that of other cucurbit crops such as melon and cucumber due to lack of a high resolution genetic map (Ellul et al., 2007; Harris et al., 2009; Hawkins et al., 2001; Levi et al., 2001). Although maps based on interspecific and intersub-specific crosses have been developed with notably improved marker polymorphism, segregation distortion of some marker alleles has led to unmapped regions making it difficult for application in MAS (Ellul et al., 2007). However in the recent years, new maps providing better genome coverage have been developed. The recently constructed single nucleotide polymorphism (SNP) consensus map based on intra-specific and intersub-specific populations (Prothro et al., 2012a,b; Sandlin et al., 2012) provides a key tool for mapping genomic regions associated with agronomically important traits for watermelon. Ren et al. (2012) constructed a genetic map with a relatively high marker coverage consisting of 698 SSR, 219 insertion deletion (InDel) and 36 structure variation (SV) markers. In addition, several watermelon genetic maps have been used to develop an integrated genetic map that will allow comparative analysis of loci and traits mapped in different genetic backgrounds (Ren et al., 2014).

Following the availability of dense genetic maps for watermelon, scientists have identified genomic regions associated with several economically important traits such as fruit quality (size, shape, rind thickness, and sugar content) (Sandlin et al., 2012), seed size (Prothro et

al., 2012a), *egusi* seed phenotype and SOP (Prothro et al., 2012b), sex expression (Prothro et al., 2013), flowering time (McGregor et al., 2014) and resistance to *Fon* race 1 (Lambel et al., 2014). Further elucidation of these traits through functional characterization of the underlying genes will be facilitated by the recent availability of a watermelon draft genome sequence (Guo et al., 2013).

Advances in next-generation sequencing technologies have led to affordable genotyping platforms that allow plant breeders to study genome-wide allelic variation in populations in relation to specific traits of interest (Elshire et al., 2011; Lambel et al., 2014; Nimmakayala et al., 2014). Genotyping-by-sequencing (GBS) is a highly multiplexed next-generation sequencing technology that allows sequencing of DNA fragments tagged with short DNA sequences (Barcodes) (Elshire et al., 2011). These DNA fragments are typically generated by using appropriate restriction enzymes to digest whole genome of an organism to generate millions of sequences studied for allelic variation (Elshire et al., 2011). Recently, GBS has been used to generate thousands of SNPs for linkage analysis (Lambel et al., 2014) and diversity studies (Nimmakayala et al., 2014) in watermelon. Despite the ability of GBS to generate thousands of SNP markers, the low genetic diversity in cultivated watermelon limits the number of polymorphic markers available to study elite by elite populations. Using GBS, Lambel et al. (2014) were able to identify 266 SNPs in an elite by elite bi-parental watermelon mapping population in contrast to >30,000 and >130,000 SNPs in rice (*Oryza sativa*) (Spindel et al., 2013) and wheat (*Triticum aestivum*) (Rutkoski et al., 2013), respectively. Another potential application of GBS is in genome-wide association studies (GWAS), which requires dense marker coverage of the watermelon genome. Nimmakayala et al. (2014) used GBS to estimate genome-wide linkage disequilibrium decay in watermelon and identify selection sweeps resulting from

watermelon domestication. Such an approach will be useful in identifying genomic regions associated with important traits in watermelon through association mapping with a panel of diverse watermelon populations.

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CHAPTER 3

GENETIC MAPPING OF SEED TRAITS CORRELATED WITH SEED OIL PERCENTAGE IN WATERMELON[1]

[1] Meru, G. and C. McGregor. 2013. HortScience. 48:955-959.
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Abstract

Egusi watermelon (*Citrullus lanatus* (Thunb.) Matsum. & Nakai subsp. *mucosospermus* var. *egusi* (C. Jeffrey) Mansf. is known for its distinctive fleshy-pericarp seed phenotype and high seed oil percentage (SOP). The seed is part of the daily diet in West Africa where it is used in soups and stews or processed for cooking oil. Genetic mapping studies have revealed that most of the variation in SOP between egusi and normal, non-egusi seed is explained by the egusi (*eg*) locus, which is also associated with the unique seed phenotype. However, variation in SOP is also observed within egusi and normal seed types although the basis of this variation remains to be elucidated. A high correlation between kernel percentage (KP) and SOP has been observed in watermelon and other crops, and recent data also suggest association between seed size and SOP in watermelon. The aim of this study was to elucidate the relationship between SOP, KP and seed size traits in watermelon and to identify quantitative trait loci (QTL) associated with the latter traits to facilitate marker assisted selection (MAS) for traits correlated with SOP. KP showed a significant ($\alpha = 0.05$) positive correlation with SOP in both egusi and normal seed types while seed size traits showed significant negative correlations with SOP. QTL associated with KP and seed size traits in normal seed were co-localized with a previously mapped locus for SOP on linkage group (LG) 2 [(chromosome (chr.) 6)], but in egusi seed, a QTL explaining 33% of phenotypic variation in KP was localized on LG 7 (chr. 1). The results of this study show that SOP in watermelon is correlated with KP and seed size, but KP is associated with different loci in normal and egusi seed phenotypes.

Introduction

Watermelon is an important species of the genus *Citrullus* (Robinson and Decker-Walters, 1997), known mainly for its sweet, edible flesh. However, in Asia and West Africa, watermelon seeds are consumed in a wide variety of foods, including snacks and soups (Gusmini et al., 2004; Zhang, 1996). The seed of the egusi watermelon is high in oil, and is characteristically large in size with a fleshy pericarp. However the origin of this phenotype remains uncertain (Gusmini et al., 2004). The egusi seed is prepared for consumption by manually separating the seed coat from the kernel, after which it is eaten raw or ground into powder and added to soups and stews (Achigan-Dako et al., 2008). The seed may also be processed into cooking oil for subsistence use (Akoh and Nwosu, 1992) or utilized in traditional medicine as an ingredient in sedatives and anti-emetics (Achigan-Dako et al., 2008).

In a study comparing SOP of 475 *C. lanatus* genebank accessions, Jarret and Levy (2012) found that the average SOP for egusi seed was higher (n = 74; SOP = 35.6%) than normal, non-egusi seed from *C. lanatus* var. *lanatus* (n = 293; SOP = 23.2%) and *C. lanatus* var. *citroides* (n = 108; SOP = 22.6%). Prothro et al. (2012a) observed similar results in an F_2 mapping population and mapped the *eg* locus to LG 2 (chr. 6 of draft genome; Guo et al., 2013). This locus explained 83% of the phenotypic variation for SOP observed between the egusi and normal seed types. However, in both the Jarret and Levy (2012) and Prothro et al. (2012a) studies, variation in SOP was also observed within egusi (26 - 43%) and within normal (15 - 29%) seed types, suggesting additional genetic factors.

A positive correlation between SOP and KP has been reported in crops such as cotton (*Gossypium hirsutum* and *Gossypium barbadense*) (Song and Zhang, 2007), canola (*Brassica napus*) (Yan et al., 2009) and sunflower (*Helianthus annuus*) (Tang et al., 2006). In watermelon,

Jarret and Levy (2012) reported that KP was also highly correlated with SOP (R^2 = 0.83) and concluded that it was the thinner hulls of egusi seed, relative to normal seed that were responsible for the high SOP. In sunflower (Leon et al., 1995), canola (Yan et al., 2009) and cotton (Song and Zhang, 2007), SOP is a function of kernel oil percentage (KOP), hull components and KP and the QTL associated with these traits have been identified. However in sunflower breeding programs, emphasis is put on KP for improvement of SOP in hybrid cultivars (Leon et al., 1995).

The role of seed size in SOP has also been studied for several crop species. In sunflower (Tang et al., 2006), soybean (*Glycine max*) (Panthee et al., 2005) and safflower (*Carthamus tinctorius)* (Yermanos et al., 1967) a negative correlation between seed size and SOP was reported but a positive correlation was observed in jatropha (*Jatropha curcas)* (Kaushik et al., 2007). In watermelon, a seed size QTL (Prothro et al., 2012b) mapped in a similar chromosomal region as a QTL for SOP identified by Prothro et al. (2012a), pointing to a potential role for seed size in SOP.

The direct phenotypic selection for SOP and the indirect selection through correlated traits such as KP and seed size have proved to be viable options for increasing SOP in crops such as sunflower (Tang et al., 2006) and jatropha (Kaushik et al., 2007). However, phenotypic analysis of these traits can only be carried out after seed harvesting. Moreover, the phenotyping of traits such as KP and KOP is destructive. Therefore, the availability of molecular tools for marker assisted selection (MAS) for these traits in watermelon is imperative. Through MAS, selection for SOP can be carried out at the seedling stage, thus saving time. Therefore the aim of this study was to further elucidate the relationship between seed size traits, KP and SOP in

watermelon through phenotypic correlations and to identify genetic loci associated with the traits to facilitate MAS for higher SOP.

Materials and methods

Plant materials and genotyping

The F_2 mapping population used by Prothro et al. (2012a) to map loci controlling the egusi seed trait and SOP was used in the current study. Briefly, single nucleotide polymorphism (SNP) genotyping assays were performed on the parents and progeny of the mapping population using Illumina GoldenGate SNP arrays and BeadStudio software (Illumina, San Diego, CA, USA) as described in Sandlin et al. (2012). A genetic map was constructed that included 357 SNP markers spanning 14 LGs (11 chromosomes on the genome sequence) with an average gap of 4.2 cM between markers (Sandlin et al., 2012). One hundred F_2 individuals had the normal seed phenotype, while 42 had the egusi seed phenotype (Prothro et al., 2012a).

Phenotyping

Phenotyping was carried out on F_3 seeds derived from open pollinated F_2 individuals. The development of this population is described elsewhere (Prothro et al., 2012a). The KP (Strain II = 44.16 %, Egusi = 67.31 %) was measured as the weight of 15 manually dehulled seed as a percentage of the weight of the intact seed. Seed size was measured as the weight of 100 seeds (100SWT; Strain II = 4.98 g, Egusi = 12.95 g), the average seed length (SL; Strain II = 8.01 mm, Egusi = 15.01 mm) and seed width (SWD; Strain II = 5.30 mm, Egusi = 9.15 mm) of five seeds from each line.

Correlations and QTL detection

Pearson correlations of the phenotypic trait values were calculated using JMP version 9 (SAS Institute Inc., Cary, NC). The SOP values were previously determined by Prothro et al.

(2012a) using nuclear magnetic resonance [NMR (MiniSpec MQ20, NMR Analyzer, Bruker Optics Inc., Billerica, MA)].

The KP was arcsine square root transformed before QTL analysis since it was expressed as a proportion of intact seed weight (Wills et al., 2010). QTL were detected by composite interval mapping (CIM) performed with a 10 cM window in WinQTL Cartographer version 2.5 (Wang et al., 2011). The standard model (Model 6) with a walk speed of 1 cM was used for CIM analysis. Statistical significance of a QTL was determined by likelihood-odds (LOD) thresholds set by 1000 permutations ($\alpha = 0.05$) (Churchill and Doerge, 1994). All LGs and QTL were visualized using MapChart 2.2 (Voorrips, 2002).

Results and discussion

Phenotypic analysis of traits

When using the entire F_2 population (n = 142), positive correlations were observed between KP, seed size traits and SOP (Fig. 3.1). However, since we were interested in the factors affecting SOP within the two seed types, the data were split, and analyzed separately for the lines with egusi (n = 42) and normal seed (n = 100). Splitting the population this way eliminated the masking effect of the *eg* locus (R^2 = 83 %) already described by Prothro et al. (2012a).

KP ranged from 39.69 % to 58.24 % in normal seed and from 50.40 % to 75.85 % in egusi seed and followed a distribution (Fig. 3.2A) similar to that of SOP observed by Prothro et al. (2012a). The 100SWT for the normal seed type ranged from 4.48 to 16.70 g while values for the egusi seed type ranged from 9.00 to 16.16 g (Fig. 3.2B). Values for SL ranged from 8.47 to 15.65 mm in normal seed and from 12.08 to 16.04 mm in egusi seed (Fig. 3.2C) while SWD values ranged from 5.51 to 9.12 mm in normal seed and from 7.05 to 9.23 mm in egusi seed (Fig. 3.2D).

In both egusi and normal seed types, significant ($\alpha = 0.05$) positive correlations between KP and SOP were observed supporting the results of Jarret and Levy (2012) who also found a positive correlation ($R^2 = 0.83$) between KP and SOP in egusi watermelon accessions. Significant negative correlations were observed between seed size traits and SOP in both seed types (Table 3.1; Fig. 3.1). Tang et al. (2006) reported similar results in sunflower, where seed oil content was positively correlated with KP but negatively correlated with 100SWT, SL and SWD. These results are expected since breeding for improved seed oil content in sunflower has for many years focused on indirect selection for small seeds (Tang et al., 2006) and seeds with thinner hulls (Leon et al., 1995). Similar progress in breeding for increased SOP may be achieved for watermelon through the selection of small-sized seeds with thinner hulls in both egusi and normal seed types. Another way to increase watermelon seed oil content would be by increasing the total oil content per fruit which would be dependent on the seed yield per fruit. On the other hand, seed yield per fruit is a function of seed size and seed number (Nerson and Paris, 2000). Increased seed yield (kg/ha) in the edible seed watermelon types has been achieved by breeding for increased seed width (Zhang, 1996). Nerson and Paris (2000) found no relationship between fruit weight and seed weight in watermelon but noted that very small fruits (<500g) had smaller seeds in some cultivars. Therefore fruit size may not be an important factor in determining seed yield and hence not a crucial determinant of total fruit oil content.

KP was significantly negatively correlated with SWD and 100SWT in both seed types but only to SL in normal seed (Table 3.1) suggesting that larger seeds have a higher hull:kernel ratio than smaller seeds. As expected, there were significant positive correlations between the seed size traits (100SWT, SL and SWD) in this study for both seed types, confirming the results of Prothro et al. (2012b). The high correlation (0.89 - 0.92) between seed size traits (100SWT,

SL and SWD) in the normal seed type suggest that either of these traits may be used as a measure of seed size (Poole et al., 1941). KOP contributes to SOP and would have been a useful and more direct phenotype to measure for watermelon since seeds are usually dehulled before use. However, there was not enough seed available from each of the $F_{2:3}$ lines to measure KOP in the current study.

Detection of QTL

Six QTL were detected on LG 2, 6 and 7 (chr. 6, 5 and 1 respectively) in normal and egusi seed phenotypes (Table 3.2 and Fig. 3.3). QTL for KP, 100SWT, SL and SWD in normal seed types were co-localized on LG 2 (chr. 6) and overlapped a QTL for SOP (R^2 = 35.70 %) identified for normal seed by Prothro et al. (2012a). A similar co-localization of QTL associated with SOP, KP and seed size traits has also been observed in sunflower (Bert et al., 2003; Leon et al., 1995; Tang et al., 2006). Co-location of QTL could indicate linkage of multiple QTL or pleiotropy (Song and Zhang, 2007) and fine mapping is required to distinguish between the two. The QTL for seed size detected on LG 2 (chr. 6) in normal seed is in the same region as QTL for seed size described by Prothro et al., (2012b), confirming that this region is associated with seed size in watermelon populations from diverse genetic backgrounds. A novel QTL (R^2 = 7.90 %) for SL was detected on LG 6 (chr. 5) in normal seed type (Fig. 3.3).

No genomic regions were associated with seed size in egusi seed phenotypes, perhaps due to the small sample size (n = 42). Population size is known to affect the ability to detect QTL, particularly those of minor effects. While studying resistance to stripe rust in barley using a double haploid population, Vales et al. (2005) demonstrated that in a small population (n = 50) only major QTL could be detected, while both the major and minor QTL could be detected when a large population size (n = > 300) was used. It is therefore not clear from our results whether

seed size in egusi seed is not associated with the QTL on LG 2 (chr. 6), or whether we are unable to detect the effect due to the small population size. A QTL for KP was detected on LG 7 (chr. 1) (R^2 = 33.80 %) in the egusi seed type. Once again, it is not clear whether the small population prevented detection of KP QTL on LG 2 (chr. 6), or whether that region does not play a role in KP in egusi seed.

The QTL detected for KP only accounted for a relatively small proportion of the observed phenotypic variation. QTL for KP explained only 22.30 % and 33.80 % of the observed phenotypic variation in normal and egusi seed, respectively. QTL for 100SWT, SL and SWD in normal seed explained 62.90 %, 60.80 % and 34.40 % of the observed phenotypic variation respectively. These results suggest additional genetic factors, not identified in the present study, may be contributing to the seed traits hence further research is required for elucidation.

This is the first report to establish the relationship between watermelon seed size and SOP and to identify QTL associated with KP in the normal and egusi seed types. Although selection for the egusi seed phenotype alone is a good indication of high SOP, the current results indicate that further enhancement of SOP in watermelon may be achieved through indirect selection of several phenotypically correlated seed traits as has been achieved for other crops. However caution is to be exercised when applying MAS for KP in the egusi and normal seed types as this trait may be associated with different loci in the two seed types.

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Table 3.1 Pearson correlations for seed oil percentage (SOP; Prothro et al., 2012a), kernel percentage (KP), 100 seed weight (100SWT), seed length (SL) and seed width (SWD) for individuals with (A) normal seed phenotype and (B) egusi seed phenotype in the Strain II (PI 279261) x Egusi (PI 560023) F_2 population.

(A)

	SOP	KP	100SWT	SL
KP	0.82*			
100SWT	-0.45*	-0.47*		
SL	-0.46*	-0.47*	0.90*	
SWD	-0.41*	-0.45*	0.89*	0.92*

(B)

	SOP	KP	100SWT	SL
KP	0.76*			
100SWT	-0.51*	-0.47*		
SL	-0.36*	-0.01	0.57*	
SWD	-0.36*	-0.32*	0.68*	0.64*

*Significant at $P < 0.05$.

Table 3.2 Chromosomal regions associated with kernel percentage (KP), 100 seed weight (100SWT), seed length (SL) and seed width (SWD) for individuals with the normal and egusi seed phenotype in the Strain II (PI 279261) x Egusi (PI 560023) F_2 population.

Trait	LG[z]	chr.[y]	Position (cM)	Closest marker[x]	Marker position on chr. (bp)[y]	LOD[w]	R^2 (%)[v]	Additive effect[u]	Dominance effect[u]	LOD-1 support interval (cM)	LOD-1 support interval (cM)
Normal seed											
KP	2	6	36.6	NW0250854	4,581,628-4,581,749	5.6	22.3	0.02	-0.01	11.8	48.6
100SWT	2	6	38.6	NW0250854	4,581,628-4,581,749	17.4	62.9	-3.73	-1.01	33.5	43.8
SL	2	6	33.6	NW0250854	4,581,628-4,581,749	23.1	60.8	-1.74	-0.34	29.6	42.1
	6	5	88.0	NW0248236	8,808,727-8,808,847	4.2	7.9	-0.80	-0.18	83.4	89.0
SWD	2	6	36.6	NW0250854	4,581,628-4,581,749	13.6	34.4	-0.88	-0.22	32.1	41.3
Egusi seed											
KP	7	1	102.21	NW0249392	20,067,660-20,067,779	5.83	33.8	1.25	-6.83	101.4	107.8

[z] Linkage group in Sandlin et al. (2012).

[y] Chromosome and marker position in the draft watermelon genome sequence (Guo et al., 2013; Ren et al., 2012)

[x] Marker sequence information available in Sandlin et al. (2012)

[w] Log_{10} likelihood ratio.

[v] Phenotypic variation explained.

[u] Negative values indicate that the effect is contributed by the allele from the egusi parent (PI 560023). For KP, the results are for the arcsine square-root transformed data.

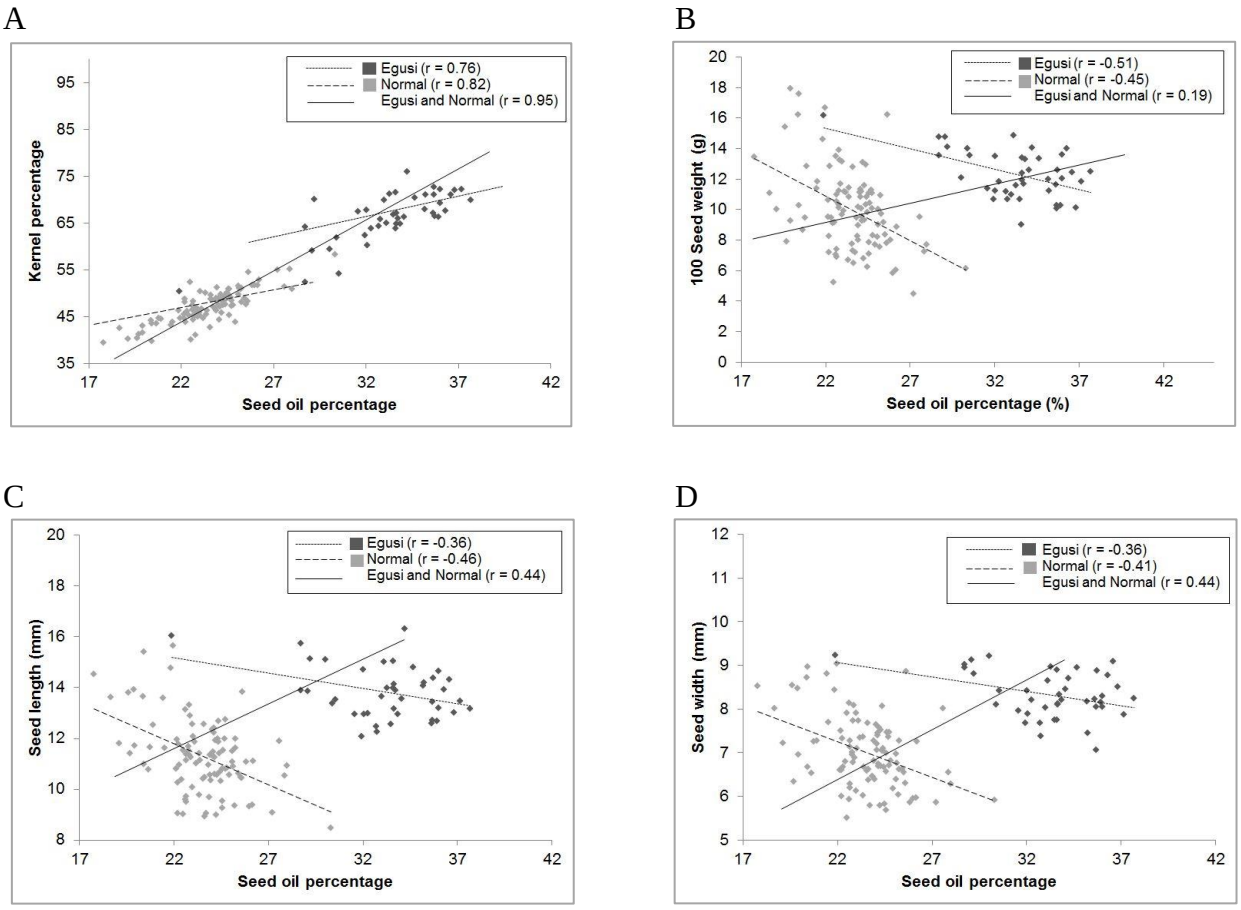
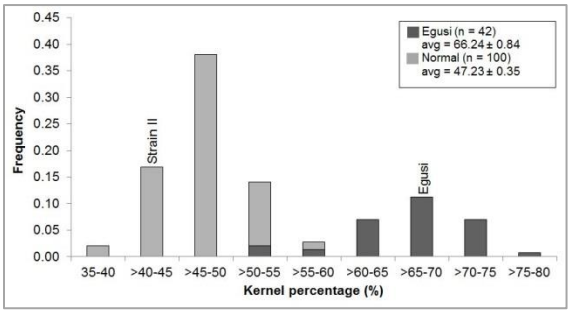
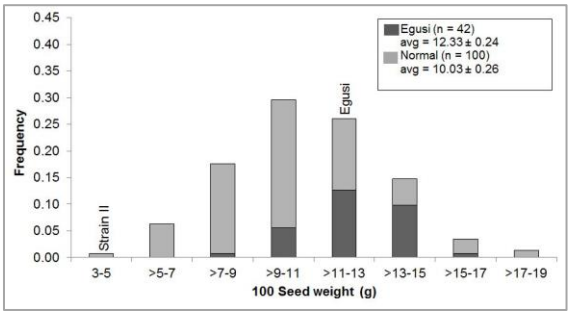


Fig. 3.1 Scatter plots and correlations for (A) kernel percentage, (B) 100 seed weight, (C) seed length, and (D) seed width in the normal and egusi seed types and the two seed types combined in the Strain II x Egusi F_2 population.

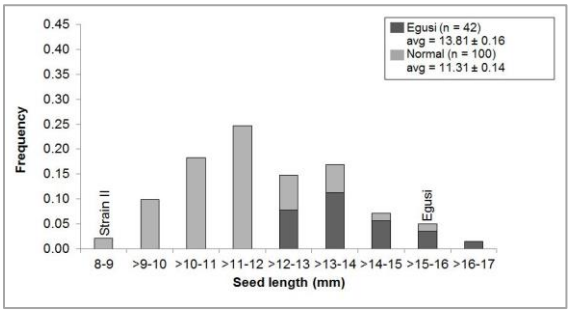
A



B



C



D

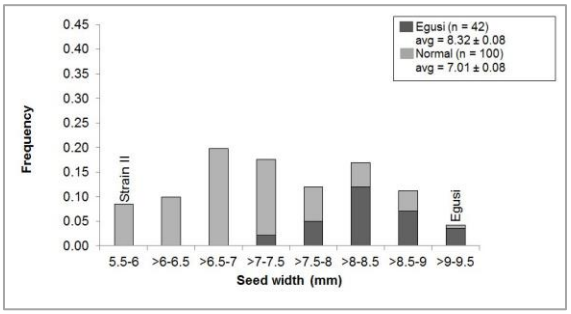


Fig. 3.2 Frequency distribution for (A) kernel percentage, (B) 100 seed weight, (C) seed length and (D) seed width in the Strain II x Egusi F_2 population as well as the parental phenotypes.

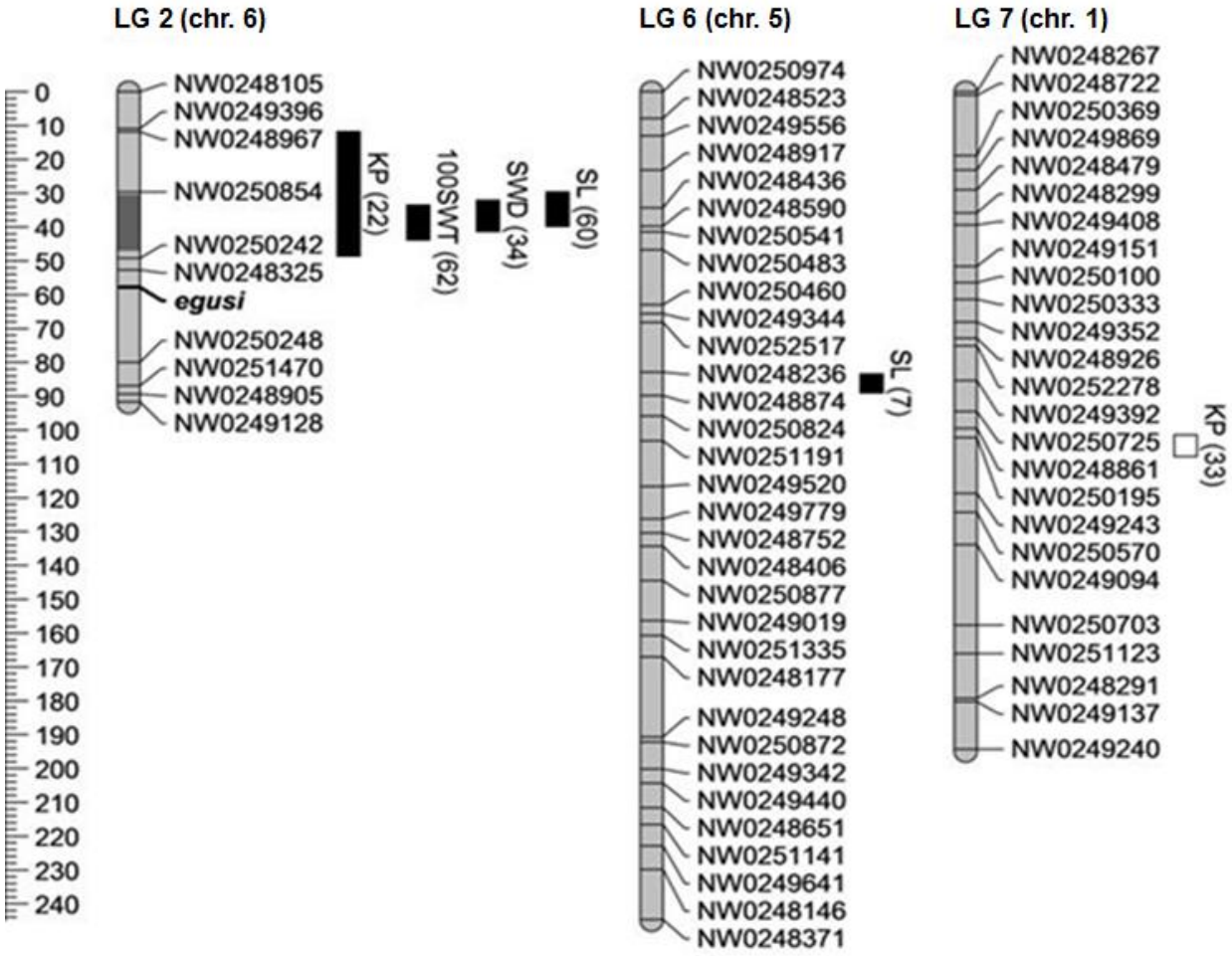


Fig. 3.3 Quantitative trait loci (length of the bar is equal to 1 LOD support interval) identified by composite interval mapping for kernel percentage (KP), 100 seed weight (100SWT), seed length (SL), and seed width (SWD) in the normal (black fill) and egusi (unfilled bars) seeds in the Strain II (PI 279261) x Egusi (PI 560023) mapping population. Values for the phenotypic variation (R^2) for the individual QTL are given in parenthesis. The location of the SOP QTL in normal seeds [shaded area on LG 2 (chr. 6)] and the egusi locus previously identified by Prothro et al. (2012a) on LG 2 (chr. 6) is indicated. Figure generated using MapChart version 2.2 (Voorrips, 2002).

CHAPTER 4

QUANTITATIVE TRAIT LOCI AND CANDIDATE GENES ASSOCIATED WITH FATTY ACID CONTENT OF WATERMELON SEED[1]

[1] Meru, G. and C. McGregor. 2014. J. Amer. Soc. Hort. Sci. 39:433-441.
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Abstract

Seed oil percentage (SOP) and fatty acid composition of watermelon (*Citrullus lanatus* (Thunb.) Matsum. & Nakai) seeds are important traits in Africa, the Middle-East and Asia where the seeds provide a significant source of nutrition and income. Oil yield from watermelon seed exceeds 50% (w/w) and is high in unsaturated fatty acids, and has a profile comparable to that of sunflower (*Helianthus annuus*) and soybean (*Glycine max*) oil. Due to novel non-food uses of plant-derived oils, there is an increasing need for more sources of vegetable oil. To improve the nutritive value of watermelon seed and position watermelon as a potential oil crop, it is critical to understand the genetic factors associated with SOP and fatty acid composition. Although the fatty acid composition of watermelon seed is well documented, the underlying genetic basis has not yet been studied. Therefore the current study aimed to elucidate the quality of watermelon seed oil and identify genomic regions and candidate genes associated with fatty acid composition. Seed from an F_2 population developed from a cross between an egusi type (PI 560023), known for its high SOP, and Strain II (PI 279261) was phenotyped for palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1) and linoleic acid (18:2). Significant (P<0.05) correlations were found between palmitic and oleic acid (0.24), palmitic and linoleic acid (-0.37), stearic and linoleic acid (-0.21) and oleic and linoleic acid (-0.92). A total of eight quantitative trait loci (QTL) were associated with fatty acid composition with a QTL for oleic and linoleic acid co-localizing on chromosome (chr.) 6. Eighty genes involved in fatty biosynthesis including those modulating the ratio of saturated and unsaturated fatty acids were identified from the functionally annotated genes on the watermelon draft genome. Several fatty acid biosynthesis genes were found within and in close proximity to the QTL identified in this study. A gene (*Cla013264*) homolog to fatty acid elongase (FAE) was found within the 1.5-likelihood-odds

(LOD) interval of the QTL for palmitic acid (R^2 = 7.6%) on chr. 2 while *Cla008157*, a homolog to omega-3-fatty acid desaturase and *Cla008263*, a homolog to FAE were identified within the 1.5-LOD interval of the QTL for palmitic acid (R^2 = 24.7%) on chr. 3. In addition, the QTL for palmitic acid on chr. 3 was located ~0.60 Mbp from *Cla002633*, a gene homolog to fatty acyl-[acyl carrier protein (ACP)] thioesterase B. A gene (*Cla009335*) homolog to ACP was found within the flanking markers of the QTL for oleic acid (R^2 = 17.9%) and linoleic acid (R^2 = 21.5%) on chr. 6 while *Cla010780*, a gene homolog to acyl-ACP desaturase was located within the QTL for stearic acid (R^2 = 10.2%) on chr. 7. On chr. 8, another gene (*Cla013862*) homolog to acyl-ACP desaturase was found within the 1.5-LOD interval of the QTL for oleic acid (R^2 = 13.5%). The genes identified in this study are possible candidates for the development of functional markers for application in marker-assisted selection for fatty acid composition in watermelon seed. To the best of our knowledge, this is the first study to elucidate the genetic control of the fatty acid composition of watermelon seed.

Introduction

Watermelon is an economically important crop of the Cucurbitaceae family, popular for its sweet edible flesh (Robinson and Decker-Walters, 1997). However, the seeds of watermelon provide a significant source of nutrition and income in other parts of the world including China (Zhang, 1996), Israel (Edelstein and Nerson, 2002), Iran (Baboli and Kordi, 2010) and Africa (Achigan-Dako et al., 2008; Al-Khalifa, 1996; El-Adawy and Taha, 2001).

Watermelon seeds are rich in oils (>50%) (Baboli and Kordi, 2010) and proteins (>27%) (Al-Khalifa, 1996; Baboli and Kordi, 2010; Loukou et al., 2007), thus playing a crucial role in supplementing the nutrients of the staple carbohydrate foods of the poor, who cannot afford animal-derived protein foods (Achu et al., 2005). The seed is manually dehulled to separate the kernels from the seed coat and eaten raw, roasted, made into soup or processed into cooking oil (Achigan-Dako et al., 2008; Al-Khalifa, 1996). In addition, edible seeds of other cucurbits such as melon (*Cucumeropsis mannii and Cucumis melo)* (Achigan-Dako et al., 2008; Loukou et al., 2007), squash (*Cucurbita pepo*) (Idouraine et al., 1996), pumpkin (*Cucurbita pepo* and *Cucurbita moschata*) (Al-Khalifa, 1996) and bottle gourd (*Lagenaria siceraria)* (Achigan-Dako et al., 2008; Achu et al., 2005) are also nutritious and form a part of the diet in these communities.

The egusi watermelon (*Citrullus lanatus* ssp. *mucosospermus* var. *egusi*), which is easily identifiable by its unique fleshy, thick seed pericarp (Gusmini et al., 2004), is popularly cultivated for its edible seeds in Africa. The egusi seed is nutritious with a high seed oil percentage (SOP) (Gusmini et al., 2004; Jarret and Levy, 2012; Prothro et al., 2012) and a high protein content (Gusmini et al., 2004). Whereas extensive research has been carried out towards improvement of the yield and quality of oil for the major oil crops such as soybean (*Glycine*

max), sunflower (*Helianthus annuus*), peanut (*Arachis hypogaea*), corn (*Zea mays)* and canola (*Brassica napus*), limited research has been done to improve these traits in cucurbit crops such as watermelon despite their agronomic and cultural importance (Loukou et al., 2007). The limited attention in research for cucurbits relative to staple crops has led to their underutilization and classification as orphan crops (Achigan-Dako et al., 2008; Baboli and Kordi, 2010; International Plant Genetic Resources Institute, 2002). Of the more than 500,000 known plant species, only 12 are currently exploited commercially to produce vegetable oil despite the increasing world demand (Baboli and Kordi, 2010; Mabaleha et al., 2007). Most of this demand is attributed to novel non-food uses including biofuel, oleochemicals, lubricants, pharmaceuticals, and cosmetics (Jarret and Levy, 2012; Panthee et al., 2006).

To improve the nutritive value of watermelon seed and establish watermelon as a potential oil crop, it is critical to understand the genetic factors associated with SOP and fatty acid composition. Prothro et al. (2012) elucidated the genetic factors associated with SOP in watermelon and identified four quantitative trait loci (QTL) associated with the trait. Among these, the egusi locus, which is also associated with the egusi seed phenotype, explained 83% of the phenotypic variation observed in SOP. Further studies have revealed that the high SOP in the egusi seed is due to a high kernel percentage (KP) (Jarret and Levy, 2012; Meru and McGregor, 2013) and that seed size plays a role in SOP in watermelon (Meru and McGregor, 2013).

The type and ratio of fatty acids in a given vegetable oil determine its nutrition, flavor, shelf life and its potential application (Brown et al., 1975; Panthee et al., 2006; Wassom et al., 2008; XinYou et al., 2011; Yang et al., 2010). Edible vegetable oils and seeds with low saturated fat content are desirable because they produce lower levels of low density lipoproteins that have been linked with arteriosclerosis and heart related ailments (Wassom et al., 2008). Therefore

plant breeders aim to reduce the levels of saturated fatty acids while increasing the levels of unsaturated fatty acids in oil crops. On the contrary, increased levels of saturated fats are desirable to decrease the cost and harmful effects associated with the processes of hydrogenation and trans-esterification in industries manufacturing margarine and similar products (Ascherio and Willett, 1997, Panthee et al., 2006).

The four primary fatty acids in watermelon seed oil are palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1) and linoleic acid (18:2) with linoleic acid being the most abundant (Al-Khalifa, 1996; Baboli and Kordi, 2010; El-Adawy and Taha, 2001; Giwa et al., 2010; Jarret and Levy, 2012; Loukou et al., 2007; Oluba et al., 2008). A study of fatty acid composition in watermelon genebank accessions by Jarret and Levy (2012) documented the range of different fatty acids in watermelon seeds [linoleic acid (45.37% to 73%); oleic acid (7.89% to 33.95%); stearic acid (5.03% to 13.84%); palmitic acid (9.68% to 14.38%)]. The fatty acid composition in watermelon seed is similar to that found in other cucurbit crops. For instance, Al-Khalifa et al. (1996) found similar levels of unsaturated fatty acids (oleic acid and linoleic acid) in watermelon (81.3%) and pumpkin [*Cucurbita pepo* (80.1%) and *Cucurbita moschata* (79.9%)]. In comparison to the major oil crops, Baboli and Kordi (2010) found similar levels of palmitic acid in watermelon seed (11.4%) and soybean (11.0%) and similar levels of linoleic acid in watermelon seed (68.3%) and sunflower (68.0%). However, watermelon seed was lower in oleic acid (13.3%; sunflower = 18.6%; soybean = 23.4%) but higher in stearic acid (7.0%; sunflower = 4.7%; soybean = 4.0%).

Although the fatty acid composition of watermelon seed oil is well documented, knowledge about the underlying genetic factors is lacking. The recent sequencing and functional annotation of the watermelon genome presents an opportunity for further research into the

molecular mechanisms underlying economically important traits including fatty acid composition (Guo et al., 2013). Similar tools have recently been made available for melon (Blanca et al., 2012) and cucumber (*Cucumis sativus*) (Huang et al., 2009) through the sequencing and functional annotation of their respective genomes. Previous studies aimed at understanding the regulation of stearoyl- acyl carrier protein (ACP) desaturation led to the isolation of full length cDNA clone of stearoyl-ACP protein desaturase in cucumber (Shanklin et al., 1991a, 1991b). However, no DNA markers have been developed for cucurbit crops for application in marker-assisted selection (MAS) for improved oil quality.

The association of DNA markers with genes regulating fatty acid composition has enabled the application of MAS to accelerate breeding for improved oil quality in canola (Hu et al., 2006), spring turnip rape (*Brassica rapa*) (Tanhuanpaa et al., 1998), sunflower (Hongtrakul et al., 1998; Perez-Vich et al., 2002), soybean (Cardinal et al., 2007; Fehr, 2007; Pham et al., 2010; Zhang et al., 2008) and peanut (Barkley et al., 2010, 2013; Chu et al., 2009). As a step towards developing such genomic tools for watermelon, the current study aimed to identify genetic loci and candidate genes associated with fatty acid composition in watermelon seed for future application in MAS for improved oil quality.

Materials and Methods

Plant material and genotyping

The F_2 population (n = 142) from a cross between Strain II of the Japanese cultivar Yamato-cream (PI 279261; normal seed type) and an egusi type from Nigeria (PI 560023) used previously (Meru and McGregor, 2013; Prothro et al., 2012) to map loci controlling the egusi seed trait, SOP and KP in watermelon was used in the present study. Single nucleotide polymorphism (SNP) assays were performed on the parents and progeny ($F_{2;}$ n = 142) of the

mapping population utilizing Illumina GoldenGate SNP arrays and BeadStudio software (Illumina, San Diego, CA) as described in Sandlin et al. (2012). A genetic map was developed that included 357 SNP markers spanning 14 linkage groups (LGs) (11 chromosomes on the genome, Guo et al., 2013) with an average gap of 4.2 cM between markers (Sandlin et al. (2012).

Fatty acid analysis

Phenotyping was carried out on seed derived from 142 open pollinated F_2 individuals (Prothro et al., 2012), the parental lines and F_1. The 15 seeds from each F_2 plant and parental line that were manually dehulled with a steel blade for the Meru and McGregor (2013) study were used for fatty acid analysis. The seed kernels from each sample were crushed with a mortar and pestle and 200 mg of the powder was weighed and transferred into 2.2-mL 96-well plates (Fisher Scientific, Pittsburgh, PA). Fatty acid methyl esters (FAMEs) were prepared using the standard method for analyses of fatty acid composition in fats and oils recommended by the American Oil Chemist's Society (Ce 1–62; American Oil Chemist's Society, 2009). To extract oil from the samples, 2 mL of hexane (Fisher Scientific) was added to each well followed by incubation at room temperature (25 °C) for 15 min. The supernatant (0.5 mL) from each sample was transferred to a new 96-well plate and evaporated to dryness with a stream of N_2 at 50 °C on a 96-well evaporator. To each well, 0.2 mL of ethyl ether (BDH, Poole, UK) was added to solubilize the lipids followed by the addition of 0.2 mL 0.1 M KOH and incubation at 50 °C for 10 min to convert the lipids into FAMEs. The methylation reactions were stopped by adding 0.2 mL of 0.15 M HCl. The samples were incubated at room temperature for 15 min, and an aliquot (0.5 mL) of the organic layer containing FAMEs was transferred to 2-mL auto-sampler vials (Fisher Scientific) for gas chromatography (GC) analysis. The FAMEs were separated by injecting 1 μL of sample onto GC column (DB-23) in a gas chromatograph (6890 Series;

Agilent, Wilmington, DE) equipped with an auto-sampler carousel. A standard (15A; Nu-Check Prep, Elysian, MN) with known concentrations for palmitic (16:0), stearic (18:0), oleic (18:1) and linoleic (18:2) acids was used to identify peaks. The profiles for the fatty acids were estimated from chromatograms using ChemStation Software (Agilent). Fatty acid extraction was carried out twice from different portions of the original ground sample and the average value for each line used in the data analysis.

Correlations, mean separation and QTL detection

Pearson correlations between the values of the phenotypic traits were calculated using JMP (version 11; SAS Institute, Cary, NC). The values for SOP for the population were previously determined by Prothro et al. (2012) while those of seed size (seed weight) were determined by Meru and McGregor (2013). The *student t-test* was performed in JMP to identify differences in the means of the various fatty acids in normal (n =100) and egusi (n = 42) seed sub-populations.

The fatty acid values were arcsine square root transformed before QTL analysis since they are expressed as a proportion of total fatty acids in the oil (Wills et al., 2010). Composite interval mapping (CIM) was used to detect QTL with a 5 cM window in WinQTL Cartographer version 2.5 (Wang et al., 2011a). The standard model (Model 6) with a walk speed of 1 cM was used for CIM analysis and the population type was designated as "RF3" (Wang et al., 2011a). Statistical significance of a QTL was determined by likelihood-odds (LOD) thresholds set by 1000 permutations ($\alpha = 0.05$) (Churchill and Doerge, 1994). QTL detected on the same LG were not considered different unless they were separated by at least 20 cM (Ravi et al., 2011). All LGs and QTL were visualized using MapChart 2.2 (Voorrips, 2002).

Fatty acid biosynthesis genes in watermelon

The genes involved in fatty acid biosynthesis in watermelon were determined by comparing annotated genes in the watermelon draft genome (Guo et al., 2013; International Cucurbits Genomics Initiative, 2012) with genes reported to be involved in fatty acid biosynthesis for other crops (Byfield et al., 2006; Cahoon et al., 1994; Cardinal et al., 2007; Kachroo et al., 2008; Li et al., 2010).

Candidate genes

Markers flanking the 1.5-LOD [~95% confidence interval (Silva et al., 2012)] interval for all the QTL were identified on the linkage map and their corresponding positions on the watermelon physical map (Guo et al., 2013) determined. The regions between the flanking markers were then inspected for genes in the fatty acid biosynthesis pathway.

Results and Discussion

Phenotypic analysis of traits

Linoleic acid was the predominant fatty acid detected. The levels of palmitic acid and linoleic acid in the Strain II parent (9.36% and 70.72%, respectively) were higher than in the egusi parent (8.50% and 61.15%, respectively) while those of stearic acid and oleic acid in the egusi parent (7.92% and 22.42%, respectively) were higher than in Strain II (5.59% and 14.33%, respectively) (Table 4.1). These results are similar to those reported by Jarret and Levy (2012) for egusi (palmitic, 9.68% to 12.82%; stearic, 8.63% to 13.84%; oleic, 8.14% to 17.26%; linoleic, 58.95% to 71.10%) and for the seeds of edible watermelon (palmitic, 9.68% to 14.38%; stearic, 5.03% to 11.52%; oleic, 7.89% to 25.67%; linoleic, 48.7% to 73%). In comparison to the major oil crops, the degree of unsaturation (oleic acid and linoleic acid) found in this study for

watermelon (Strain II = 85.1%; egusi = 83.6 %) was similar to that of soybean (84.4%) and sunflower (88.6%) (Baboli and Kordi, 2010).

Extraction of crude oil from watermelon seeds for subsistence use is common in West Africa (Achigan-Dako et al., 2008; Jarret and Levy, 2012; Oluba et al., 2008). However, this oil may have a low shelf life and less stability at high cooking temperature due to high linoleic acid content which is highly oxidative. High levels of linoleic acid content (57%) in soybean has led to breeding efforts to decrease the content of this fatty acid in favor of oleic acid, which is more stable at high cooking temperature (Baboli and Kordi, 2010; Lee et al., 2007). Linoleic acid is more oxidative due to the presence of two double bonds as opposed to oleic acid, which has a single double bond (Lee et al., 2007). For watermelon seed to be exploited commercially for the production of cooking oil, it is necessary to breed for reduced levels of linoleic acid and increased levels of oleic acid. From the study on watermelon genebank accessions by Jarret and Levy (2012), it is clear that natural variation exists in oleic acid (7.89% to 33.95%) and linoleic acid (45.37% to 73%) that can be exploited to produce cultivars with high oleic acid and lower linoleic acid. Adoption of watermelon as a minor oil crop would improve farmers' earnings while concurrently reducing the amount of solid waste resulting from the disposal of watermelon seeds (Baboli and Kordi, 2010; El-Adawy and Taha, 2001).

Within the population, palmitic acid ranged from 9.07% to 12.08%, stearic acid from 4.33% to 11.94%, oleic acid from 12.46% to 29.28% and linoleic acid from 50.65% to 70.28% (Table 4.1). Transgressive segregation was observed in one direction for palmitic acid and linoleic acid and in both directions for stearic acid and oleic acid whereby the progeny had trait values outside the range of the parents. Transgressive segregation is primarily associated with antagonistic additive effects (Rieseberg et al., 1999). Linoleic acid was found to be significantly

(P<0.05) negatively correlated with palmitic (-0.37), stearic (-0.21) and oleic (-0.92) fatty acids. However, palmitic acid was significantly positively correlated (0.24) with oleic acid (Table 4.2). Oyenuga and Fetuga (1975) found significant positive correlation between palmitic acid and oleic acid but negative correlation between palmitic acid and linoleic acid in watermelon. On the contrary, Jarret and Levy (2012) did not find significant correlations between these fatty acids in watermelon. In wheat (*Triticum aestivum*), Wang et al. (2011b) found linoleic acid to be significantly negatively correlated with palmitic, stearic, oleic and linolenic acids. Palmitic acid was the only trait significantly correlated with SOP (-0.22) (Table 4.2). This relationship may explain the observation in egusi watermelon whose seeds have high SOP (Gusmini et al., 2004; Meru and McGregor, 2013; Jarret and Levy, 2012; Prothro et al., 2012) but lower levels of palmitic acid relative to other watermelon types (Jarret and Levy, 2012). In the current study, significant differences (P<0.05) were found between palmitic acid levels in normal (low SOP) and egusi (high SOP) seeds further supporting this relationship. However, no unique QTL for palmitic acid were identified when mapping normal and egusi seed separately (data not shown). In canola, Zhao et al. (2008) found negative correlation between oil content and palmitic acid (-0.34) while in soybean, a negative correlation (-0.53) between linoleic acid and oil content has been documented (Li et al., 2011). No significant differences were found between egusi and normal seeds in the population for the levels of stearic acid, oleic acid and linoleic acid (data not shown). Seed weight was significantly positively correlated with oleic acid (0.27) but significantly negatively correlated with linoleic acid (-0.24). These results are similar to those obtained in soybean by Liu et al. (1995) who found a positive correlation between seed size and oleic acid but a negative correlation between seed size and linoleic acid.

Detection of QTL

Genetic analysis detected eight QTL on chr. 2, 3, 5, 6, 7 and 8, three each for palmitic acid and oleic acid and one each for stearic acid and linoleic acid (Fig. 4.1, Table 4.3). The QTL for oleic acid (R^2 = 17.9%) co-localized with a QTL for linoleic acid (R^2 = 21.5%) on chr. 6. Co-localization of the two traits was expected because of the high negative correlation between the traits (-0.92) (Table 4.2), which is consistent with the fact that the two fatty acids share a common pathway where desaturation of oleic acid by omega-6 fatty acid desaturase-2 (FAD2) yields linoleic acid in plants (Bachlava et al., 2009; Liu et al., 2011; Sharma et al., 2002; Yu et al., 2008). Co-localization of QTL for oleic acid and linoleic acid is common in crops including corn (Wassom et al., 2008; Yang et. al, 2010), wheat (Wang et al., 2011b) and soybean (Panthee et al., 2006).

Co-localization was also observed between the QTL for oleic acid and linoleic acid on chr. 6 and QTL for seed size (seed weight) in normal seed identified by Meru and McGregor (2013). These results might explain the significant correlation observed between seed size and the two fatty acids in the current study (Table 4.2). QTL for oleic acid were also detected on chr. 2 (R^2 = 10.7%) and chr. 8 (R^2 = 13.5%). QTL for palmitic acid were identified on chr. 2 (R^2 = 7.6%), chr. 3 (R^2 = 24.7%) and chr. 5 (R^2 = 12.7%) while a single QTL for stearic acid was identified on chr. 7 (R^2 = 10.2%) (Fig. 4.1, Table 4.3).

Fatty acid biosynthesis genes in watermelon

An examination of the functionally annotated genes in the watermelon draft genome (Guo et al., 2013) revealed eighty genes involved in fatty acid biosynthesis (Appendix 4.1). Of particular interest among the identified genes are those central in regulating the ratio of saturated (palmitic acid and stearic acid) and unsaturated (oleic acid and linoleic acid) fatty acids. This

ratio is important because it determines the potential use of vegetable oils (Brown et al., 1975; Panthee et al., 2006; Wassom et al., 2008; XinYou et al., 2011; Yang et al., 2010). The fatty acyl-ACP thioesterase-B (FATB) regulates the amount of saturated fatty acids by hydrolyzing the acyl group from saturated-ACP (16:0-ACP and 18:0-ACP) to release free saturated fatty acids (Barker et al., 2007; Bonaventure et al., 2003). In watermelon, five genes potentially encoding this enzyme were identified on several chromosomes. Similarly, five genes are predicted to encode FATB genes in peanut (Yin et al., 2013) and one each in arabidopsis (*Arabidopsis thaliana*) (Barker et al., 2007; Bonaventure et al., 2003) and Jatropha (*Jatropha curcas*) (Natarajan and Parani, 2011). In soybean, several low-palmitic acid cultivars have been developed by mutagenesis (ethyl methanesulfonate and N-nitroso-N-methyl urea and X-radiation) and molecular analysis has revealed mutation in various FATB genes (Cardinal et al., 2007).

The number of genes potentially encoding acyl-ACP desaturases in watermelon (12) was higher than that in peanut (8) (Yin et al., 2013) and Jatropha (4) (Natarajan and Parani, 2011). Acyl-ACP desaturases are soluble enzymes that introduce double bonds into acyl-ACPs in oxygen-dependent reactions to form monounsaturated fatty acids hence modulate the ratio of saturated and unsaturated fatty acids (Guy et al., 2011). For example, the stearoyl-ACP protein desaturase (SADP) introduces a double bond at C_9 in stearic acid (18:0) to form oleic acid (18:1). In soybean, mutations in the SADP have been sought to increase the levels of stearic acid (and reduce the levels of oleic acid) in soybean oil to decrease the cost of hydrogenation (Byfield et al., 2006). Although stearic acid is a saturated fatty acid, it does not increase cholesterol levels in human and thus does not pose a health risk in contrast to palmitic acid (Ruddle et al., 2013). Most of the high stearic acid soybean germplasm lines have been developed using mutagenesis

targeting SADP (Ruddle et al., 2013) although a natural mutation in the gene has also been reported in one cultivar (Byfield et al., 2006; Ruddle et al., 2013).

Increased levels of oleic acid and concomitant reduction in levels of linoleic acid have been achieved in oil crops by targeting FAD2 that converts oleic acid into linoleic acid by inserting a double bond at C_{12} (Heppart et al., 1996; Yu et al., 2008). High oleic acid peanut cultivars have been obtained both through radiation induced mutagenesis and exploitation of natural mutations in FAD2 (Yu et al., 2008). Four genes potentially encoding FAD2 were identified in watermelon on chr. 2, 6 and 11, a number lower than that of FAD2 in peanut (13) (Yin et al., 2013).

Candidate genes

Cla013264 (E-value: 2E-200) and *Cla008263* (E-value: 2E-195), homologs to 3-ketoacyl-CoA synthase in *Populus trichocarpa* (Appendix 4.1), were found within the 1.5-LOD interval of the QTL for palmitic acid on chr. 2 (R^2 = 7.6%) and chr. 3 (R^2 = 24.7%) respectively. 3-ketoacyl-CoA synthase is a subunit of fatty acid elongase (FAE) complex that condenses acyl-CoA with malonyl-CoA as the first step in the synthesis of very long fatty acids (Bach et al., 2008, 2010; Barret et al., 1998). The association of this QTL with variation in palmitic acid may be explained by the use of palmitic acid to form palmitoyl-CoA which is used as a substrate by 3-ketoacyl-CoA synthase (Bach et al., 2008, 2010). In canola, FAE is associated with variation in erucic acid (22: 1) and is targeted in breeding for low erucic acid (cooking oil) and high erucic acid (industrial applications) canola cultivars (Barret et al., 1998; Fourmann et al., 1998).

In addition, a gene (*Cla008157*) homolog (E-value: 1E-136) of omega-3 desaturase (FAD3) in *Betula pendula* (Appendix 4.1) was found within the flanking markers of the QTL for palmitic acid on chr. 3. FAD3 catalyzes the addition of a double bond to linoleic acid to yield

linolenic acid and is a central target for breeders in reducing levels of linolenic acid in soybean oil (Anai et al., 2005). Interestingly, *Cla002633* a homolog (E-value: 4E-61) of the FATB gene in *Ricinus communis* (Appendix 4.1) is just outside (~0.60 Mbp from NW0250413) the 1.5-LOD interval of the palmitic acid QTL on chr. 3. The FATB hydrolyzes the acyl group from palmitoyl-ACP to release free palmitic acid and ACP (Bonaventure et al., 2003; Cardinal et al., 2007). A natural deletion in FATB in soybean resulted in reduced palmitic acid levels (Cardinal et al., 2007). Similarly, reduction in the levels of palmitic acid have been reported in chemical and induced mutants that resulted in perturbation of FATB isoforms in soybean (Cardinal et al., 2007).

Cla009335 homolog (E-value: 1E-26) of ACP in *Fragaria vesca* (Appendix 4.1) was found within the flanking markers of the QTL for oleic acid and linoleic acid on chr. 6. ACPs are acidic proteins involved in de novo fatty acid synthesis, acyl chain modification and chain-length termination during fatty acid biosynthesis (Li et al., 2010).

Cla010780 homolog (E-value: 6E-46) of SADP gene in *Picea glauca* and *Cla013862* a homolog (E-value: 3E-173) to SADP gene in *R. communis* were located within the flanking markers of QTL for stearic acid on chr. 7 and oleic acid on chr. 8 respectively. SADP encode enzymes that catalyze the conversion of stearic acid to oleic acid (Barker et al., 2007; Byfield et al., 2006) and may explain why the gene is associated with the QTL for stearic acid and oleic acid in watermelon seed. In soybean, silencing of SADP resulted in a 3.6- fold reduction in oleic acid levels and a six-fold increase in stearic acid levels when compared to the wild type (Kachroo et al., 2008) while as in *Thunbergia alata,* overexpression of the gene led to accumulation of oleic acid (Cahoon et al., 1994). Physiologically, altering the function of SADP in plants results in changes in membrane integrity and fluidity which in turn affects the ability to

acclimatize to cold or activate the salicylic-dependent pathogen defense pathway (Kachroo et al., 2008).

The genes identified in this study are possible candidate genes for the development of functional markers for application in MAS for improved oil quality in watermelon seed. The main QTL for palmitic acid on chr. 3 (R^2 = 24.7%) and that for oleic acid (R^2 = 17.9%) and linoleic acid (R^2 = 21.5%) on chr. 6. present possible targets in MAS for manipulating the levels of saturated and unsaturated fatty acids in watermelon seed. In the major oil crops, the key genes in the fatty acid biosynthesis pathway have been cloned and well characterized (Barkley et al., 2013; Byfield et al., 2006; Cardinal et al., 2007). Availability of these genomic tools in these crops has led to the development of functional markers and identification of markers tightly linked to key fatty acid biosynthesis genes. Allele-specific primers for the FAD2 gene have been developed for canola (Hu et al., 2006), spring turnip rape (Tanhuanpaa et al., 1998), sunflower (Hongtrakul et al., 1998; Perez-Vich et al., 2002), soybean (Pham et al., 2010) and peanut (Barkley et al., 2010, 2013; Chu et al., 2009). Similarly, candidate genes for SADP (Pham et al., 2010; Zhang et al, 2008) and FATB (Cardinal et al., 2007; Fehr, 2007) have been tagged with molecular markers in soybean.

In breeding for oil quality in watermelon seed, targeting genes encoding FATB, SADP and FAD2 would be logical since these enzymes are central in determining the fatty acid composition in oil crops. Given the variation in fatty acid composition reported for watermelon genebank accessions by Jarret and Levy (2012), conventional breeding can be used to develop watermelon cultivars of superior oil quality by selecting combinations of the naturally occurring alleles in the gene pool.

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Table 4.1 Fatty acid composition (percentage by weight of watermelon seed oil) of palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1) and linoleic acid (18:2) in Strain II (PI 279261), Egusi (PI 560023), F_1 and the F_2 population (n = 142) derived from a cross between Strain II and Egusi.

Fatty Acid	Strain II	Egusi	F_1	Population (F_2)				
				mean	SD	range	min	max
Palmitic	9.36	8.50	10.95	10.57	0.58	3.01	9.07	12.08
Stearic	5.59	7.92	8.08	8.37	1.22	7.61	4.33	11.94
Oleic	14.33	22.42	18.50	18.8	3.03	16.81	12.46	29.28
Linoleic	70.72	61.15	62.48	62.12	3.25	19.63	50.65	70.28

Table 4.2 Pearson correlations for fatty acids, seed oil percentage (SOP; Prothro et al., 2012) and seed weight (SWT) (Meru and McGregor, 2013) in the Strain II (PI 279261) x Egusi (PI 560023) F_2 watermelon population (n = 142).

Trait	SOP	Seed size (SWT)	Palmitic acid	Stearic acid	Oleic acid
Palmitic acid	-0.22*	0.01			
Stearic acid	0.11	0.02	-0.01		
Oleic acid	0.12	0.27*	0.24*	-0.15	
Linoleic acid	-0.12	-0.24*	-0.37*	-0.21*	-0.92*

*Significant at $P<0.05$.

Table 4.3 Chromosomal (chr.) positions (Mbp) and the corresponding 1.5-likelihood-odds (LOD) support interval associated with fatty acid composition in the Strain II (PI 279261) x Egusi (PI 560023) F_2 watermelon population.

Fatty acid	LG[z]	Position (cM)	chr.[y]	LOD[x]	R^2 (%)[w]	Additive effect[v]	Dominance effect[v]	LOD-1.5 support interval (cM)	Flanking marker[u]	Position on chr. (Mbp)[y]	LOD-1.5 support interval (cM)	Flanking marker[u]	Position on chr. (Mbp)[y]
Palmitic	9B	106.74	2	3.67	7.55	0.20	-0.44	98.08	NW0248254	29.63	112.74	NW0248056	31.13
Palmitic	11B	44.96	3	10.70	24.73	0.67	0.35	38.53	NW0250413	3.36	59.39	NW0251825	0.33
Palmitic	6	188.02	5	4.94	12.67	-0.45	-0.12	173.02	NW0248177	26.20	199.21	NW0249342	29.07
Stearic	8	7.99	7	3.85	10.17	-0.85	-0.82	2.68	NW0250663	29.41	12.45	NW0250095	30.82
Oleic	9B	3.01	2	5.13	10.67	-1.65	0.04	0.00	NW0250803	15.40	5.27	NW0249789	19.15
Oleic	2	36.59	6	6.43	17.86	-1.69	-2.06	23.89	NW0248967	3.77	44.59	NW0250242	6.44
Oleic	4	17.57	8	6.46	13.48	1.78	-0.63	12.30	NW0249450	17.39	35.37	NW0249252	14.33
Linoleic	2	36.59	6	5.88	21.46	1.59	2.09	13.89	NW0248967	3.77	43.59	NW0250242	6.44

[z] Linkage group in Sandlin et al. (2012).

[y] Chromosome and position in the draft watermelon genome sequence (Guo et al., 2013; Ren et al., 2012).

[x] Log_{10} likelihood ratio.

[w] Phenotypic variation explained.

[v] Negative values indicate that the effect is contributed by the allele from the egusi parent (PI 560023).

[u] Marker sequence information available in Sandlin et al. (2012)

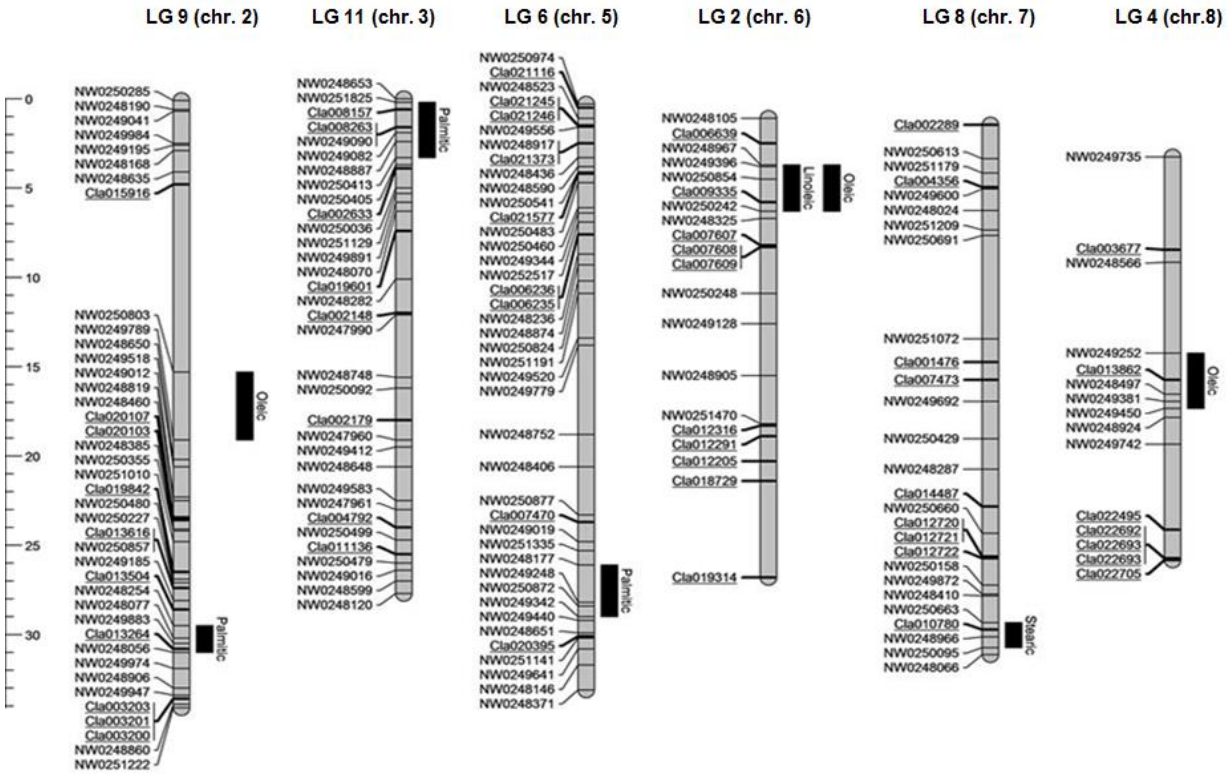

Fig. 4.1 Quantitative trait loci [length of the bar represents the region between the markers flanking the 1.5-likelihood-odds (LOD) support interval] identified by composite interval mapping for the content of palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1) and linoleic acid (18:2) in watermelon seed in the Strain II (PI 279261) x Egusi (PI 560023) watermelon population. The genes involved in the fatty acid biosynthesis pathway are underlined. Figure generated using MapChart version 2.2 (Voorrips, 2002).

Appendix 4.1 Chromosomal (chr.) placement of genes encoding enzymes involved in fatty acid biosynthesis in watermelon and their homology (E-value) to corresponding genes in other plant species (Guo et al., 2013; International Cucurbits Genomics Initiative, 2012).

Unigene[z]	chr.[y]	Symbol	Enzyme	Homology[x]		
				Accession No.	E-value	Species
Cla015916	2	FAE	3-ketoacyl-CoA synthase	NP_179223	5E-57	*Arabidopsis thaliana*
Cla013264	2	FAE	3-ketoacyl-CoA synthase	XP_002313455	2E-200	*Populus trichocarpa*
Cla020107	2	FAE	3-ketoacyl-CoA synthase	XP_002315256	1E-155	*Populus trichocarpa*
Cla020103	2	FAE	3-ketoacyl-CoA synthase	XP_002327205	5E-167	*Populus trichocarpa*
Cla008263	3	FAE	3-ketoacyl-CoA synthase	XP_002313455	2E-195	*Populus trichocarpa*
Cla007470	5	FAE	3-ketoacyl-CoA synthase	XP_002309451	4E-168	*Populus trichocarpa*
Cla020395	5	FAE	3-ketoacyl-CoA synthase	ABA01490	9E-267	*Gossypium hirsutum*
Cla021116	5	FAE	3-ketoacyl-CoA synthase	ABX10440	1E-210	*Gossypium hirsutum*
Cla021577	5	FAE	3-ketoacyl-CoA synthase	XP_002312562	5E-226	*Populus trichocarpa*
Cla006236	5	FAE	3-ketoacyl-CoA synthase	BT004205	1E-118	*Arabidopsis thaliana*
Cla006235	5	FAE	3-ketoacyl-CoA synthase	BT004205	4E-123	*Arabidopsis thaliana*
Cla012291	6	FAE	3-ketoacyl-CoA synthase	ACY78677	3E-257	*Pistacia chinensis*
Cla002289	7	FAE	3-ketoacyl-CoA synthase	XP_002309451	2E-82	*Populus trichocarpa*

Cla022495	8	FAE	3-ketoacyl-CoA synthase	ACQ41892	3E-256	*Camellia oleifera*
Cla017158	10	FAE	3-ketoacyl-CoA synthase	XP_002298171	2E-173	*Populus trichocarpa*
Cla012095	4	fabG	3-oxoacyl-(Acyl-carrier-protein) reductase	Q9X248	1E-18	*Thermotoga maritima*
Cla022705	8	fabG	3-oxoacyl-(Acyl-carrier-protein) reductase	P28643	3E-116	*Cuphea lanceolata*
Cla003677	8	fabG	3-oxoacyl-(Acyl-carrier-protein) reductase	XM_003608029	7E-07	*Medicago truncatula*
Cla017175	10	fabG	3-oxoacyl-(Acyl-carrier-protein) reductase	Q93X67	1E-13	*Brassica napus*
Cla017174	10	fabG	3-oxoacyl-(Acyl-carrier-protein) reductase	P51831	4E-15	*Bacillus subtilis*
Cla016982	10	fabG	3-oxoacyl-(Acyl-carrier-protein) reductase	Q9X248	1E-18	*Thermotoga maritima*
Cla001918	11	fabG	3-oxoacyl-(Acyl-carrier-protein) reductase	AF324985	1E-12	*Arabidopsis thaliana*
Cla013616	2	ACCase	Acetyl-CoA carboxylase	ACN85391	3E-55	*Suaeda salsa*
Cla002148	3	ACCase	Acetyl-CoA carboxylase	AAA75528	0.0	*Glycine max*
Cla001454	4	ACCase	Acetyl-CoA carboxylase	AY142630	5E-88	*Arabidopsis thaliana*
Cla001455	4	ACCase	Acetyl-CoA carboxylase	ACR61637	9E-163	*Jatropha curcas*
Cla018129	4	ACCase	Acetyl-CoA carboxylase	FJ441419	3E-246	*Arabidopsis thaliana*
Cla021246	5	ACCase	Acetyl-CoA carboxylase	FJ441419	4E-145	*Arabidopsis thaliana*
Cla021245	5	ACCase	Acetyl-CoA carboxylase	FJ441419	3E-26	*Arabidopsis thaliana*
Cla009059	1	ACP	Acyl carrier protein	P53665	2E-16	*Arabidopsis thaliana*
Cla019842	2	ACP	Acyl carrier protein	CAA04768	4E-31	*Fragaria vesca*
Cla019842	2	ACP	Acyl carrier protein	CAA04768	4E-31	*Fragaria vesca*
Cla019842	2	ACP	Acyl carrier protein	CAA04768	4E-31	*Fragaria vesca*
Cla002179	3	ACP	Acyl carrier protein	ABP38063	6E-46	*Jatropha curcas*
Cla002179	3	ACP	Acyl carrier protein	ABP38063	6E-46	*Jatropha curcas*

Cla021373	5	ACP	Acyl carrier protein	ACJ07135	2E-39	*Arachis hypogaea*
Cla009335	6	ACP	Acyl carrier protein	CAA04768	1E-26	*Fragaria vesca*
Cla019314	6	ACP	Acyl carrier protein	P93092	5E-29	*Casuarina glauca*
Cla015524	9	ACP	Acyl carrier protein	P93092	1E-19	*Casuarina glauca*
Cla012205	6	DESA1	Acyl-[ACP] desaturase	XP_002517641	3E-61	*Ricinus communis*
Cla010780	7	DESA1	Acyl-[ACP] desaturase	AAM12238	6E-46	*Picea glauca*
Cla012720	7	DESA1	Acyl-[ACP] desaturase	ADC80920	2E-157	*Vernicia fordii*
Cla012721	7	DESA1	Acyl-[ACP] desaturase	D3YLK2	9E-61	*Vernicia fordii*
Cla012722	7	DESA1	Acyl-[ACP] desaturase	ADC80920	2E-50	*Vernicia fordii*
Cla022693	8	DESA1	Acyl-[ACP] desaturase	XP_002531889	1E-189	*Ricinus communis*
Cla013862	8	DESA1	Acyl-[ACP] desaturase	XP_002526163	3E-173	*Ricinus communis*
Cla022692	8	DESA1	Acyl-[ACP] desaturase	XP_002531889	6e-172	*Ricinus communis*
Cla022693	8	DESA1	Acyl-[ACP] desaturase	XP_002531889	1E-189	*Ricinus communis*
Cla014825	9	DESA1	Acyl-[ACP] desaturase	ADC80920	1E-59	*Vernicia fordii*
Cla000627	11	DESA1	Acyl-[ACP] desaturase	ACG59946	6E-193	*Ricinus communis*
Cla016938	11	DESA1	Acyl-[ACP] desaturase	ADC80920	3E-14	*Vernicia fordii*
Cla013504	2	fabF	Beta 3-keto-acyl-ACP synthase II	NP_178533	9E-179	*Arabidopsis thaliana*
Cla011136	3	fabF	Beta 3-keto-acyl-ACP synthase II	ADK23940	8E-207	*Gossypium hirsutum*
Cla019601	3	fabF	Beta 3-keto-acyl-ACP synthase II	ACJ07141	2E-212	*Arachis hypogaea*
Cla011561	1	fabH	Beta 3-keto-acyl-ACP synthase III	XP_002529789	4E-172	*Ricinus communis*
Cla007607	6	CPA-FAS	Cyclopropane fatty acid synthase	ABG37642	2E-141	*Populus trichocarpa*
Cla007608	6	CPA-FAS	Cyclopropane fatty acid synthase	AAT74602	4E-20	*Gossypium hirsutum*

Cla007609	6	CPA-FAS	Cyclopropane fatty acid synthase	NM_113256	0.0	*Arabidopsis thaliana*
Cla007473	7	CPA-FAS	Cyclopropane fatty acid synthase	Q3L7F1	2E-57	*Gossypium hirsutum*
Cla001981	9	CPA-FAS	Cyclopropane fatty acid synthase	AAT74602	3E-16	*Gossypium hirsutum*
Cla001982	9	CPA-FAS	Cyclopropane fatty acid synthase	ABG37642	6E-12	*Populus trichocarpa*
Cla003203	2	fabI	Enoyl-[ACP] reductase	ABB83365	3E-89	*Malus domestica*
Cla004792	3	fabI	Enoyl-[ACP] reductase	ABB83365	1E-92	*Malus domestica*
Cla014934	9	FATA	Fatty acyl-ACP thioesterase A	AAB51523	2E-144	*Garcinia mangostana*
Cla003904	1	FATB	Fatty acyl-ACP thioesterase A	XP_002526311	5E-150	*Ricinus communis*
Cla002633	3	FATB	Fatty acyl-ACP thioesterase A	XP_002511148	4E-61	*Ricinus communis*
Cla012316	6	FATB	Fatty acyl-ACP thioesterase A	XP_002515564	1E-183	*Ricinus communis*
Cla015415	9	FATB	Fatty acyl-ACP thioesterase A	ABI20760	6E-21	*Glycine max*
Cla016747	11	FATB	Fatty acyl-ACP thioesterase A	ABC47311	6E-171	*Populus tomentosa*
Cla014487	7	fabZ	Hydroxymyristoyl ACP dehydratase	BT098415	1E-22	*Glycine max*
Cla008157	3	FAD3	Omega 3 fatty acid desaturase	AAN17504	1E-136	*Betula pendula*
Cla006639	6	FAD3	Omega 3 fatty acid desaturase	XP_002511936	5E-218	*Ricinus communis*
Cla004356	7	FAD3	Omega 3 fatty acid desaturase	ACE80931	1E-250	*Cucumis sativus*
Cla003201	2	FAD2	Omega-6 fatty acid desaturase	AAO37752	5E-194	*Trichosanthes kirilowii*
Cla003200	2	FAD2	Omega-6 fatty acid desaturase	AAT72296	8E-146	*Nicotiana tabacum*
Cla018729	6	FAD2	Omega-6 fatty acid desaturase	ABU96742	6E-206	*Jatropha curcas*
Cla017539	10	FAD2	Omega-6 fatty acid desaturase	AAS19533	4E-213	*Cucurbita pepo*
Cla003659	1	PPT	Palmitoyl-protein thioesterase	AK317387	1E-82	*Arabidopsis thaliana*
Cla003660	1	PPT	Palmitoyl-protein thioesterase	ACG35638	7E-115	*Zea mays*

Cla005617	10	PPT	Palmitoyl-protein thioesterase	BT020483	6E-81	*Arabidopsis thaliana*

[z] Unigene assigned in the draft watermelon genome (Guo et al., 2013; Ren et al., 2012)

[y] Chromosome in the draft watermelon genome sequence (Guo et al., 2013; Ren et al., 2012).

[x] Homology to sequences in Genbank, Swiss-Prot, TrEMBL and TAIR (International Cucurbits Genomics Initiative, 2012).

CHAPTER 5

THE EFFECT OF SOIL TYPE ON DISEASE SEVERITY OF *FUSARIUM OXYSPORUM* FSP. *NIVEUM* (RACE 1) IN WATERMELON[1]

[1] Meru, G. and C. McGregor. To be submitted to Plant Dis

Abstract

Fusarium wilt of watermelon (*Citrullus lanatus* var. *lanatus* (Thunb.) Matsum. & Nakai caused by *Fusarium oxysporum* f. sp. *niveum* (*Fon*) (E.F. Sm.) W.C. Snyder & H.N. Han. is a major disease, causing huge economic losses in watermelon producing areas of the world. Breeding for genetic resistance is the most effective management strategy for the disease and depends on the ability to discriminate resistant and susceptible genotypes. The complex interactions between *Fon* and the soil environment are poorly understood and require breeders to use a reliable soil media in disease screening assays to ensure reliable results. Although several soil media have been described for use with fusarium species in cucurbits, their effects on disease severity have not been evaluated for watermelon. The current study compared disease severity in watermelon infected with *Fon* race 1 in sand-peat (1:1), sand-perlite (1:1), sand-peat-vermiculite (4:1:1), peat-perlite (1:1) and Fafard 3B soil media using Calhoun Gray (resistant), Sunsugar (resistant), All Sweet (moderately resistant), Sugar Baby (susceptible) and Charleston Gray (susceptible) cultivars. Disease severity appeared highest in sand-peat and peat-perlite soil media but plants exhibited poor growth in these media, possibly confounding results. It was not possible to discriminate resistant from susceptible cultivars in sand-peat, peat-perlite and sand-perlite (no significant difference in disease severity, $\alpha = 0.05$). However in sand-peat-vermiculite and Fafard 3B, significant differences in disease severity were observed between the resistant and susceptible cultivars. In this study, suitable soil media for use in disease screening assay with *Fon* race 1 were identified.

Introduction

Watermelon (*Citrullus lanatus* var. *lanatus*) is an economically important crop of the Cucurbitaceae family popular for its sweet edible flesh (Robinson and Decker-Walters, 1997). Watermelon production is severely limited by a vascular disease caused by *Fusarium oxysporum* f. sp. *niveum* (*Fon*) (E.F. Sm.) W.C. Snyder & H.N. Han. (Boyhan et al., 2003; Egel and Martyn, 2007; Martyn and McLaughlin, 1983; Zhou et al., 2010). There are four races of *Fon* designated 0, 1, 2 and 3 based on their aggressiveness or the ability to overcome specific resistance in a set of differential cultivars (Bruton, 1998; Egel and Martyn, 2007; Wehner, 2008; Zhou et al., 2010).

Management of fusarium wilt is difficult because of the long-term survival of the pathogen's chlamydospores in the soil and the evolution of new races (Bruton, 1998; Egel and Martyn, 2007; Lin et al., 2009). Examples of management options for the disease include use of disease-free planting materials, crop rotation, chemical and biological fumigation (Bruton, 1998; Egel and Martyn, 2007; Hopkins et al., 1992), resistant root-stocks (Kuniyasu, 1980), solarization (Njoroge et al., 2008), suppressive soils (Biles and Martyn, 1989; Sun and Huang, 1985) and induced resistance (Biles and Martyn, 1989; Cohen, 2002). The ecological risk of the chemical fumigants has led to the phasing out of methyl-bromide (Montreal Protocol and the Clean Air Act, 1998) leaving even fewer options for managing fusarium wilt. Watermelon growers prefer resistant cultivars as the primary management method for fusarium wilt (Bruton, 1998; Hopkins et al., 1992; Lin et al., 2009; Lin et al., 2010; Martyn and McLaughlin, 1983; Martyn and Netzer, 1991; Zhou and Everts, 2004).

Breeding for resistant cultivars depends on the ability to discriminate resistant and susceptible genotypes which can be difficult due to complex interactions between *Fon*, the host and the environment (Boyhan et al., 2003; Scholthof, 2006). Important host factors contributing

to the development of fusarium wilt include the age and inherent susceptibility of the plant to *Fon* (Boyhan et al., 2003; Bruton, 1998; Cohen et al., 2008; Egel and Martyn, 2007; Geiger and Heun, 1989; Zhou and Everts, 2007) while pathogen factors include the virulence of the race and inoculum density. Martyn and McLaughlin (1983) reported that high inoculum density elicited higher disease severity than lower inoculum density in watermelon. Other important environmental factors include temperature, light, moisture levels and soil type (Cohen et al., 2008; Scholthof, 2006).

The mechanisms and interactions between soil-borne fusarium species and the soil environment are poorly understood (Boyhan et al., 2003) making the choice of soil medium an important factor in obtaining reliable results in disease screening experiments. Depending on the plant species, certain soil media will suppress severity, while others increase severity of soil-borne pathogens. Compost and other soils have been shown to suppress the development of soil-borne diseases even in presence of pathogens (Cohen et al., 2008; Larkin et al., 1996). Cohen et al. (2008) reported that peat-based soil media induced susceptibility of melons to *Fusarium oxysporum* f. sp. *melonis*. On the contrary, peat-based media has been shown to suppress disease development for some soil-borne pathogens such as *Alternaria brassicicola*, *Leptosphaeria maculans*, *Rhizoctonia solani* and *Fusarium oxysporum* f. sp. *lycopersici* (Cohen et al., 2008). It is therefore imperative to determine a suitable pathogen-soil media combination for use in screening for specific soil-borne pathogens. In cucurbit breeding programs, soil media used in disease screening with fusarium species include sand-peat-vermiculite (Martyn and Netzer, 1991), vermiculite-peat moss (Zhou and Everts, 2004), commercial potting mix such as Metromix (Boyhan et al., 2003), Metromix-sand-vermiculite (Wechter et al., 2012), sand-soil (Lambel et al., 2014), peat-perlite, sand-peat and sand-perlite (Cohen et al., 2008).

Currently, no information is available on the effect of different soil media on disease severity of *Fon* in watermelon. Therefore the objective of the current study was to evaluate the effect of different soil media on disease severity of *Fon* in watermelon.

Materials and methods

Plant materials and soil media

Five watermelon cultivars with known disease reactions to *Fon* race 1 [Sugar Baby (susceptible; Reimer Seed Company, Mount Holly, NC, USA), Charleston Gray (susceptible, Reimer Seed Company), All Sweet (moderately resistant; SeedWay, NY, USA), SunSugar (resistant; SeedWay), and Calhoun Gray (resistant; SeedWay)] (Bruton, 1998; Elmstrom and Hopkins, 1981; Martyn and Netzer, 1991; Netzer and Weintall, 1980; Wehner, 2008; Zhou and Everts, 2003; Zhou and Everts, 2004) were used in this study. Four of the soil media used in the study were obtained locally and mixed in specific ratios [sand-peat (1:1), sand-perlite (1:1), sand-peat-vermiculite (4:1:1), and peat-perlite (1:1)]. In addition, a commercial soil mix, Fafard 3B (Sun Gro Horticulture, MA, USA), was included.

Inoculum preparation

Fon race 1 [(B05-07), provided by Anthony Keinath, Clemson University], was grown (14h/10h dark cycle) on quarter-strength potato dextrose agar (Becton, Dickinson and Company, NJ, USA) for 12 days and 1 cm^2 agar plugs transferred into 250 mL erlenmeyer flasks containing 100 mL potato dextrose broth (Becton, Dickinson and Company). The fungal cultures were grown (14h/10h dark cycles) on a Mini-Orbital shaker (Henry Troemner, NJ, USA) at 200 rpm for 10 days and the inoculum was filtered through four layers of sterile cheese cloth. The microconidial concentration in the inoculum suspension was determined using a hemacytometer (Hausser Scientific, PA, USA) and adjusted to 1 x 10^{6} mL^{-1} using sterile water.

Greenhouse evaluations

Seeds were sown in 20.32 cm pots (Sun Gro Horticulture, MA, USA) filled with steam-pasteurized soil media described above that were amended with Osmocote Classic Osmocote 14N-4.2P-11.6K. Plants were inoculated at cotyledonary stage by pouring 40 mL of inoculum around each seedling using a beaker. For each soil medium, four seeds of each cultivar were sown per replication with a total of three replications in a randomized complete block design. Mock inoculations were carried out as described above but using sterile potato dextrose broth diluted (1:10) with sterile water. The pots were transferred into hole-less trays (53.3 x 27.9 x 5.1 cM, Sun Gro Horticulture, MA, USA) to contain run-off. The experiment was carried out in Mar. 2014 and repeated in June of the same year. The temperature in the greenhouse was maintained at 27 ± 3 °C.

Data collection and analysis

Plants were evaluated for symptom severity on a scale of 0 to 5 with a score of 0 representing asymptomatic plants, score of 1 for plants showing initial wilting on one leaf, score of 2 for plants showing continued wilting in more than one leaf, score of 3 for plants with all the leaves wilted, score of 4 for plants with all leaves wilted and stem collapsing, and a score of 5 for dead plants (Fig. 5.1). Disease severity data were collected at 4, 7, 10, 13, 16, 19 and 27 days after inoculation (DAI) and the area under disease progress curve (AUDPC) determined by the trapezoidal integration method (Shaner and Finney, 1977). To determine the vigor of plants growing in the soil media, dry weight of the mock inoculated controls at 27 DAI was determined by oven-drying plants at 55 ± 5 °C for four days. The data for AUDPC and dry weight was analyzed using PROC GLM procedure of SAS (SAS Institute Inc., 1999) and mean separation determined using the Tukey's significance difference test (Ott and Longnecker, 2001).

Results and discussion

No significant differences ($\alpha = 0.05$) in disease severity were observed between the two experiments (Mar. and June, 2014; data not shown). Therefore data collected in the two experiments was averaged and used in data analysis. Poor plant vigor in all cultivars was observed in peat-perlite and sand-peat soil media (Fig. 5.2 and Table 5.1). Fusarium wilt severity was also high in these soil media and it was not possible to discriminate resistant and susceptible cultivars (Table 5.2). Poor plant vigor reduces the ability of plants to resist diseases (Cohen et al., 2008; Egel and Martyn, 2007; Geiger and Heun, 1989; Zhou and Everts, 2007) and might explain the high disease severity observed in the resistant cultivars in peat-perlite and sand-peat soil media. In melon, Cohen et al. (2008) reported that seedlings grown in peat based media developed more severe disease symptoms than those grown in media lacking peat. However, the growth vigor of the seedlings in these soil media was not reported.

Plant vigor in sand-perlite was significantly different from that in peat-perlite and sand-peat but not significantly different from that in Fafard 3B and sand-peat-vermiculite (Table 5.1). However, no significant difference ($\alpha = 0.05$) in disease severity was observed among the cultivars phenotyped in this soil medium (Table 5.2). Similarly in melon, Cohen et al. (2008) could not discriminate resistant and susceptible cultivars using sand-perlite. In Fafard 3B and sand-peat-vermiculite, high disease severity was observed in Sugar Baby (susceptible) and Charleston Gray (susceptible). However no symptoms were observed in All Sweet (moderately resistant), Sunsugar (resistant) and Calhoun Gray (resistant) (Table 5.2). Conflicting levels of resistance have been reported for the All Sweet cultivar with some authors describing it as resistant (Gunter and Egel, 2012) while others moderately resistant (Zhou and Everts, 2003).

This study established Fafard 3B and sand-peat-vermiculite as the best soil media for discrimination of resistant and susceptible cultivars with *Fon* race 1 pathogen. Although sand-peat-vermiculite is routinely used in disease screening experiments with *Fon* (Martyn and Netzer, 1991), Fafard 3B can be used as a suitable commercial-mix alternative.

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Table 5.1 The mean dry weight of mock inoculated watermelon plants at 27 days after inoculation in peat-perlite (1:1), sand-peat (1:1), sand-perlite (1:1), Fafard 3B, and sand-peat-vermiculite (4:1:1).

Soil medium	Dry weight (g)
Peat-perlite	1.67[d]
Sand-peat	2.16[d]
Sand-perlite	3.48[ab]
Fafard 3B	6.90[a]
Sand-peat-vermiculite	4.94[ab]

[z] Means separated by the same letter are not significantly different.

Table 5.2 The mean disease severity (area under disease progress curve) in Sugar Baby (susceptible), Charleston Gray (susceptible), All Sweet (moderately resistant), SunSugar (resistant) and Calhoun Gray (resistant) infected with *Fusarium oxysporum* f. sp. *niveum* in peat-perlite (1:1), sand-peat (1:1), sand-perlite (1:1), Fafard 3B, and sand-peat-vermiculite (4:1:1).

	Area under disease progress curve				
Cultivar	Peat-perlite	Sand-peat	Sand-perlite	Fafard 3B	Sand-peat-vermiculite
Sugar Baby	1581.05[a]	1121.40[a]	303.00[a]	622.61[a]	586.96[a]
Charleston Gray	1422.38[ab]	1028.00[a]	276.84[a]	474.55[a]	375.63[b]
All Sweet	1342.50[ab]	860.50[ab]	234.76[a]	0[b]	0[b]
SunSugar	1171.36[ab]	625.90[bc]	175.00[a]	0[b]	0[b]
Calhoun Gray	771.58[c]	470.90[c]	168.64[a]	0[b]	0[b]

[z] Means separated by the same letter within columns are not significantly different.

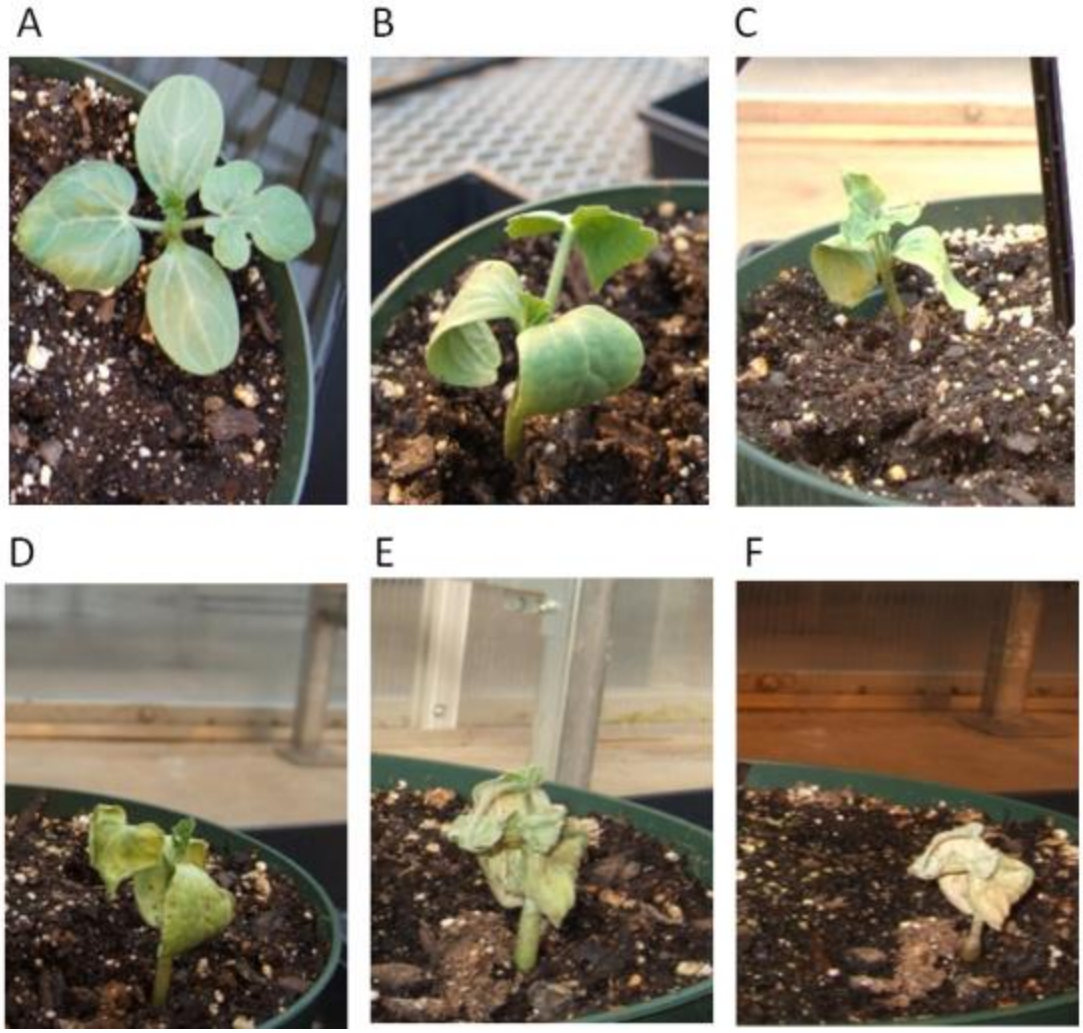

Fig. 5.1 Rating scale for *Fusarium oxyxporum* f. sp. *niveum* showing different severity scores. (A) A score of 0 representing an asymptomatic plant, (B) score of 1 for a plant showing initial wilting on one leaf, (C) score of 2 for a plant showing continued wilting in more than one leaf, (D) score of 3 for a plant with all the leaves wilted, (E) score of 4 for a plant with all leaves wilted and stem collapsing, and (F) score of 5 for a dead plant.

Fig. 5.2 Mock inoculated watermelon plants at 27 days after inoculation grown in (A) peat-perlite (1:1), (B) sand-peat (1:1), (C) sand-perlite (1:1), (D) Sand-peat-vermiculite (4:1:1), and (E) Fafard 3B.

CHAPTER 6

GENETIC MAPPING OF QUANTITATIVE TRAIT LOCI (QTL) ASSOCIATED WITH RESISTANCE TO RACE 1 OF *FUSARIUM OXYSPORUM* F. SP. *NIVEUM* IN WATERMELON[1]

[1] Meru, G. and C. McGregor. To be submitted to Mol. Breeding

Abstract

Watermelon (*Citrullus lanatus* (Thunb.) Matsum. & Nakai) is an important vegetable crop widely grown for its sweet flesh and edible seeds. Production of watermelon is severely limited by vascular disease caused by *Fusarium oxysporum* f. sp. *niveum* (*Fon*) (E.F. Sm.) W.C. Snyder & H.N. Han. Markers linked to fusarium wilt resistance would greatly enhance conventional breeding through marker assisted selection (MAS). In the current study, genotyping by sequencing (GBS) was used for discovery of single nucleotide polymorphisms (SNPs) in an F_2 population (n = 89) developed from a cross between Calhoun Gray (resistant) and Sugar Baby (susceptible). Individual F_2 plants were selfed to generate F_3 families that were phenotyped for resistance against *Fon* race 1. A major QTL accounting for 44% of the phenotypic variation observed in the F_3 population was identified on chromosome (chr.) 1 in a region previously associated with *Fon* race 1 resistance in elite by elite populations. Sequence analysis of putative disease resistant genes (*Cla004916*, *Cla004959*, *Cla0011391*, and *Cla0011463*) close to the QTL revealed three SNPs between Sugar Baby and Calhoun Gray on *Cla0011463*. This study confirms the region on chr. 1 to be associated with resistance to *Fon* race 1 in diverse genetic backgrounds. Markers in proximity to this QTL are candidates for the development of tools for MAS in watermelon breeding programs.

Introduction

Watermelon (*Citrullus lanatus* (Thunb.) Matsum. & Nakai is an important crop widely grown for its sweet flesh (Robinson and Decker-Walters, 1997) and edible seeds (Achigan-Dako et al., 2008; Baboli and Kordi, 2010; Edelstein and Nerson, 2002). The United States has over 133,700 acres dedicated to watermelon production with an annual fresh market value of approximately half a billion dollars (United States Department of Agriculture, National Agricultural Statistics Service, 2014).

The domestication of watermelon through selection for suitable traits has led to narrow genetic diversity in the current elite watermelon cultivars (Levi et al., 2001) and resulted in the loss of alleles conferring resistance to important fungal, bacterial and viral diseases (Guo et al., 2013; Hawkins et al., 2001). Fusarium wilt caused by *Fusarium oxysporum* f. sp. *niveum* (*Fon*) (E.F. Sm.) W.C. Snyder & H.N. Han. is a production-limiting disease in watermelon growing regions of the world (Boyhan et al., 2003; Egel and Martyn, 2007; Hawkins et al., 2001; Martyn and McLaughlin, 1983; Zhou et al., 2010). Four *Fon* races (0,1, 2 and 3) have been described in watermelon based on their aggressiveness or the ability to overcome specific resistance in a set of differential cultivars (Bruton, 1998; Egel and Martyn, 2007; Wehner, 2008; Zhou et al., 2010).

The persistence of the pathogen in the soil and the evolution of new races make management of fusarium wilt difficult (Bruton, 1998; Egel and Martyn, 2007; Lin et al., 2009; Martyn and Netzer, 1991; Yetisir et al., 2003). Current management measures include growing watermelon genotypes resistant to the disease (Bruton, 1998), avoidance of infested fields, 5-7 year crop rotation system, chemical and biological fumigation (Bruton, 1998; Egel and Martyn, 2007) and the use of resistant root-stocks (Kuniyasu, 1980). Cultivation of resistant cultivars is regarded as the best method for combating fusarium wilt (Bruton, 1998; Hopkins et al., 1992;

Lin et al., 2009; Lin et al., 2010; Martyn and McLaughlin, 1983; Martyn and Netzer, 1991; Zhou and Everts, 2004) and to this end many cultivars resistant to *Fon* races 0 and 1 and a few to race 2 have been developed through breeding programs (Bruton, 1998; Lambel et al., 2014; Wehner, 2008).

Resistance to *Fon* race 1 in Calhoun Gray is thought to be controlled by a single dominant gene designated *Fo-1* with a few modifier genes (Guner and Wehner, 2004; Martyn and Netzer, 1991; Netzer and Weintall, 1980; Wehner, 2008; Zhang and Rhodes, 1993). Several studies have been carried out to identify genomic factors associated with resistance to *Fon* race 1 with the aim of developing molecular tools for marker-assisted selection (MAS). Xu et al. (1999) identified a randomly amplified polymorphic DNA (RAPD) marker (OP01/700) linked (3 cM distance) to resistance to *Fon* race 1 in PI 296341-FR. This marker was cloned and sequenced by Xu et al. (2000) and converted into a sequence characterized amplified region (SCAR) marker for use in MAS. Hawkins et al. (2001) attempted to identify quantitative trait loci (QTL) associated with resistance to fusarium wilt race 1 using F_2 and F_3 populations generated from a cross between PI 296341-FR (resistant to race 1) and New Hampshire (susceptible). However, no useful linkages were found between the RAPD markers tested and resistance to *Fon* race 1. Lin et al. (2009) designed a SCAR primer (G05-SCAR) derived from the sequence of a RAPD marker (OP-G05) in JSB cultivar (Chen et al., 2003) that was specific to watermelon lines resistant or tolerant to *Fon* race 1. Harris et al. (2009) used degenerate primers to target R-genes that encode nucleotide binding site–leucine-rich repeat (NBS-LRR) proteins in watermelon and identified three watermelon expressed sequence tags (EST) disease resistance homologs. However, none of these EST markers mapped closely to RAPD marker (OP01/700) previously linked to race 1 resistance by Xu et al. (1999). The efforts to map loci associated with resistance

to *Fon* race 1 have mainly utilized PI 296341-FR, a var. citroides, as the source of resistance thus leading to high segregation distortion of marker alleles and low resolution maps (Levi et al., 2011).

Recently, watermelon populations developed from elite by elite crosses have been used to identify markers linked to resistance against *Fon* race 1. Using an F_3 population developed from a cross between Calhoun Gray (resistant) and Black Diamond (susceptible), Xu (2014) used bulk segregant analysis to identify several SNP markers linked to resistance against *Fon* race 1 on chromosome (chr.) 1 of the draft genome sequence (Guo et al., 2013). In an independent study, Lambel et al. (2014) used an F_3 population derived from a cross between HMw017 (resistant) and HMw013 (susceptible) to map seven QTL associated with resistance to *Fon* race 1 that included a major QTL (Fo-1.1) on chr. 1. Several putative disease resistance genes (*Cla004916, Cla004959*, *Cla0011391,* and *Cla0011463*) (Guo et al., 2013) were found close to Fo-1.1 QTL.

Recent advances in next-generation sequencing technologies have led to affordable genotyping platforms that permit plant breeders to study genome-wide allelic variation in populations in relation to traits of interest (Elshire et al., 2011; Lambel et al., 2014; Nimmakayala et al., 2014). Genotyping by sequencing (GBS) allows pooling of hundreds of barcoded samples into a single sequencing lane (Elshire et al., 2011) thus reducing the cost of genotyping per data point. The GBS method involves sequencing of reduced genomic libraries followed by alignment of the generated reads to identify SNP variations (Barba et al., 2013; Elshire et al., 2011). Recently, a GBS protocol for watermelon was optimized (Elshire et al., 2011; Lambel et al., 2014) and has been used in genetic mapping (Lambel et al., 2014) and diversity studies (Nimmakayala et al., 2014).

The aim of the current study was to identify genomic regions associated with resistance to *Fon* race 1, characterize resistance genes in the region, and develop molecular tools for MAS in watermelon.

Materials and methods

Plant material and DNA extraction

A cross between Calhoun Gray (resistant) and Sugar Baby (susceptible) cultivars was made in the greenhouse and a single F_1 plant was selfed to yield F_2 seeds. Each individual F_2 plant was selfed to generate F_3 families that were phenotyped for *Fon* race 1 resistance in the greenhouse. Leaf material from the parents, the F_1 and each of 89 F_2 plants, were used for DNA extraction using the E.N.Z.A 96-well format kit (Omega Bio-tek, GA, USA) according to the manufacturer's instructions. The concentration and quality of the DNA was determined by measurement of absorbance (Infinite M200 PRO, Tecan Group Ltd., ZH, CH) and by agarose gel electrophoresis. To ensure that the quality of the extracted DNA was sufficient for digestion with restriction enzymes, samples of the DNA were digested using EcoRI (New England Biolabs, MA, USA).

Genotyping by sequencing, SNP analysis and map construction

Genotyping of the parents, F_1 and F_2 was carried out using GBS (Elshire et al., 2011) at the Institute for Genomic Diversity (IGD), Cornell University. At IGD, the DNA from the parents, F_1 and 89 F_2 plants were digested using *ApeK*I restriction enzyme and fragment alignment and SNP calling were performed using the GBS reference pipeline in TASSEL version 3.0.160. The generated SNP calls in HapMap format provided by IGD were filtered in Microsoft Office Excel for missing data, polymorphism between the mapping population parents and segregation distortion ($P<0.0001$).

Based on these filtering parameters, two SNP data sets were created (Appendix 6.1), (i) one consisting of 1,024 polymorphic SNPs that included markers with up to 70% missing data points in the F_2 individuals, and (ii) a subset of 389 polymorphic SNPs with no more than 10% missing data. A physical map was created by placing the 1,024 SNPs on the reference genome (Guo et al., 2013). The data set consisting of 389 SNPs was used to construct the linkage map in JoinMap version 4.1 (Van Ooijen, 2006) using regression mapping and Kosambi map units. The physical and linkage maps were visualized using MapChart 2.2 (Voorrips, 2002).

Fungal inoculum preparation

Race 1 of *Fon* [(B05-07), provided by Anthony Keinath, Clemson University], was grown (14h/10h dark cycle) on quarter-strength potato dextrose agar (Becton, Dickinson and Company, NJ, USA) for 12 days and 1 cm^2 agar plugs transferred into 250 mL erlenmeyer flasks containing 100 mL potato dextrose broth (Becton, Dickinson and Company). The fungal cultures were grown (14h/10h dark cycles) on a Mini-Orbital shaker (Henry Troemner, NJ, USA) at 200 rpm for 10 days and the inoculum filtered through four layers of sterile cheese cloth. The microconidial concentration in the inoculum suspension was determined using a hemacytometer (Hausser Scientific, PA, USA) and adjusted to 1 x $10^{6.}mL^{-1}$ using sterile water.

Phenotyping

Seeds of Sugar Baby, Charleston Gray (susceptible control), the F_1, and F_3 lines as well as Calhoun Gray and SunSugar (resistant control) were sown in the greenhouse in cells (5.98 x 3.68 x 4.69 cM) filled with steam-pasteurized sand:peat:vermiculite (4:1:1) amended with Osmocote 14N-4.2P-11.6K. At the first true leaf stage, the cells with the seedlings were immersed in the inoculum contained in plastic tubs for 15 minutes and transferred into hole-less trays (53.3 x 27.9 x 5.1 cM, Sun Gro Horticulture, MA, USA). For each disease screening

experiment, four seeds of each cultivar/line were sown per replication with a total of three replications in a randomized complete block design. Control seedlings were immersed into plastic tubs containing sterile water. Plants were evaluated for symptom severity on a scale of 0 to 5 with a score of 0 representing asymptomatic plants, a score of 1 for plants showing initial wilting on one leaf, a score of 2 for plants showing continued wilting in more than one leaf, a score of 3 for plants with all the leaves wilted, a score of 4 for plants with all leaves wilted and stems collapsing, and a score of 5 for dead plants. Disease severity data was collected at 11, 14, 17, 20, 23, and 26 days after inoculation (DAI) and the area under disease progress curve (AUDPC) determined by the trapezoidal integration method (Shaner and Finney, 1977). The first disease screening experiment was carried out in Aug. 2013 and repeated in Nov. 2013 and Feb. 2014. The average values for all the measured traits (AUDPC and DAI) across the three experiments were determined. Pearson correlations for the AUDPC, DAI and their means were calculated using JMP Version 9 (SAS Institute Inc., Cary, NC).

QTL detection

QTL detection using markers on the genetic and physical map was performed by composite interval mapping (CIM) with a 5-cM window in WinQTL Cartographer Version 2.5 (Wang et al., 2011). A walk speed of 1 cM was used in the standard model (Model 6) for CIM analysis and the statistical significance of a QTL determined by likelihood-odds thresholds set by 1000 permutations ($\alpha = 0.05$) (Churchill and Doerge, 1994). The QTL was visualized using MapChart 2.2 (Voorrips, 2002).

Cloning and sequence analysis of candidate resistance genes

Four disease resistance genes (*Cla004916, Cla004959, Cla0011391,* and *Cla0011463*) (Guo et al., 2013) close to the detected QTL on chr. 1 were cloned and sequenced in Calhoun

Gray and Sugar Baby for polymorphism detection. As a control, these genes were also cloned and sequenced in one Calhoun Gray and Sugar Baby plant that were not parents of the mapping population. For each gene, primers (Table 6.1) were designed within the 300 bp flanking region and used to amplify the homologs in each of the cultivars. Each PCR amplification reaction was performed in a 20 uL reaction volume with 1x Phusion high fidelity (HF) buffer (New England Biolabs), 0.2 Mm of each dNTP (New England Biolabs), 0.5 µM of each primer, 40 ng of genomic DNA and 0.5 U of Phusion HF Taq DNA polymerase (New England Biolabs). For PCR, a single cycle of initial denaturation was done at 98 °C for 30 sec. followed by 35 cycles at 98 °C for 5 sec., annealing temperature (52 - 57 °C; Table 6.1) for 20 sec. and 72 °C for 90 sec. Final extension was performed in a single cycle at 72 °C for 10 min. Amplicons of the expected size (Table 6.1) were excised from an agarose gel and purified using E.Z.N.A gel-extraction kit (Omega Bio-tek) and ligated into a pGEM-T easy vector (Promega Corporation, WI, USA). Subsequently, the vector was transformed into JM109-*Escherichia coli* competent cells (Promega Corporation) and the plasmid DNA was isolated using E.Z.N.A plasmid isolation kit (Omega Bio-tek). Sequencing of the cloned gene inserts was carried out by Eurofins Genomics (AL, USA). Sequence assembly and alignment to reference genes (Guo et al., 2013) was carried out in Sequencher 5.0 (Gene Codes Corporation, MI, USA).

Results and discussion

Genotyping by sequencing, SNP analysis and map construction

In this study, GBS analysis generated a total of 933,662 tags among which 761,835 (81.6%) aligned to unique positions, 51,216 (5.5%) aligned to multiple positions while 120,611 (12.9%) could not be aligned to the watermelon reference genome (Guo et al., 2013). The number of tags generated in this study with *ApeK*I restriction enzyme exceeded those generated

(527,844 tags) with the same enzyme in an F_2 population made from a cross between elite watermelon lines (Lambel et al., 2014). Analysis of the tags aligning to unique positions revealed 20,889 HapMap SNP calls that were filtered according to missing data points, polymorphism between mapping population parents and segregation distortion to generate 1,024 and 389 SNP markers for the physical and linkage map, respectively (Appendix 6.1, Table 6.2 and Fig. 6.1).

The SNP markers generated in this study had a large amount of missing data, which was expected with the GBS method due to low coverage of reads that results from the pooling of bar-coded samples in a single sequencing lane (Barba et al., 2013). A genetic map was constructed that contained 389 SNP markers spanning 12 linkage groups with a total length of 3,955.2 cM and an average marker interval of 10.1 cM (Table 6.2). The map length observed in this study is larger than that obtained in previous watermelon mapping studies (Hashzume et al., 2003; Hawkins et al., 2001; Levi et al., 2002, 2006, 2011; Ren et al., 2012, 2014; Sandlin et al., 2012). Massive elongation of the genetic maps often occurs when working with SNP data generated through the GBS method due to high levels of genotyping errors that tend to over-estimate the number of double cross-over events (Barba et al., 2013; Spindel et al., 2013). In rice (*Oryza sativa*), a genetic map constructed from SNP markers generated through GBS was found to be 130 times larger than the consensus genetic map for the crop (Spindel et al., 2013). In the current study, the genetic map was approximately 5 times longer than the integrated genetic map (798 cM) for watermelon (Ren et al., 2014). To address high levels of missing data and genotyping errors in GBS method, several imputation and error correction algorithms have been developed respectively. Imputation algorithms utilize available marker information to predict missing genotype values while error correction algorithms test for the accuracy of recombination break points in the population (Barba et al., 2013; Rutkoski et al., 2013, Spindel et al., 2013). Examples

of imputation methods include mean imputation, k-nearest neighbor's imputation, singular value decomposition imputation, expectation maximization imputation, and random forest regression imputation (Rutkoski et al., 2013). In rice, Spindel et al. (2013) used random forest imputation and a post-imputation genotyping error correction algorithm in Python (PLUMAGE 2.0) to correct for missing data and genotyping errors respectively. These interventions resulted in a saturated genetic map of comparable size to the consensus genetic map. In grapevine (*Vitis vinifera*), Barba et al. (2013) corrected for genotyping errors in GBS data by discarding individuals and markers exhibiting high proportion of cross over events in R/QTL software (Broman et al., 2003). In the current study, implementation of random forest imputation and error correction in R/QTL did not improve the length of the genetic map (data not shown).

For the construction of the physical map, SNP markers with up to 70% missing data were allowed to ensure good coverage of the genome. This allowed for coverage in regions not represented on the genetic map (Fig. 6.1).

Phenotypic analysis

Wilting of the susceptible parent (Sugar Baby) was observed in all the disease screening experiments while the resistant parent (Calhoun Gray) showed minimal symptoms (Fig. 6.2A-6.2C). As expected, severe wilting was observed in the susceptible control (Charleston Gray) (AUDPC for screen 1 = 798.0, screen 2 = 444.5, and screen 3 = 368.6), but the resistant control (SunSugar) showed minimal symptoms [AUDPC for screen 1 = 38.0, screen 2 = 130.0, and screen 3 = 7.5). The F_1 showed moderate susceptibility to fusarium wilt in two of the experiments (Oct. and Nov. 2013) (Fig. 6.2A and B) but minimal symptoms in the experiment conducted in Feb. 2014 (Fig. 6.2C). Segregation for resistance to fusarium wilt was observed in the F_3 population (Fig. 6.2A-D). Lambel et al. (2014) reported continuous segregation pattern for

resistance to *Fon* race 1 in an F_3 population but observed no symptoms in the 18 phenotyped F_1 plants. The difference in disease severity in the F_1 between the two studies might have resulted from the variation in the inoculation method and soil type used. In the current study, a tray dip method was used for inoculation in sand: peat: vermiculite soil medium while Lambel et al. (2014) used the root dip and sand: soil for the inoculation method and soil medium respectively. The method of inoculation and soil media are known to influence severity of fusarium wilt in cucurbits (Boyhan et al., 2001; Cohen et al., 2008; Meru and McGregor, unpublished). Significant positive correlations ($\alpha = 0.05$) were observed between all the time points measured in the three experiments (Table 6.3). The relationship between the data taken at different time points indicate that plant breeders can efficiently select for *Fon* race 1 resistance in the greenhouse within the first two weeks after inoculation instead of waiting longer to make decisions.

QTL detection

QTL mapping using markers on either the physical or genetic map revealed the same number of QTL (Table 6.4 and 6.5) for the time points and AUDPC measured. In addition, the position, the LOD and the phenotypic variation explained by each QTL were comparable between the two maps (Table 6.4 and 6.5). A major QTL associated with resistance *Fon* race 1 was identified on chr. 1 (Fig. 6.3, Table 6.4 and 6.5). This QTL was designated *Qfon1* and explained up to 47.5% of the phenotypic variation observed in the F_3 population. It was possible to detect *Qfon1* with data collected at 14, 17, 20, 23 and 26 DAI as well AUDPC across the three experiments. For 11 DAI, this QTL was detected in experiment 1 and 2 but not 3. This is not surprising because the disease severity in experiment 3 at 11 DAI was lower than in experiment 1 and 2 (data not shown). It was possible to detect *Qfon1* at 11, 14, 17, 20, 23 and 26 DAI as

well AUDPC with the average trait data, averaged across the three experiments (Fig. 6.3, Table 6.4 and Table 6.5). Lambel et al. (2014) identified a major QTL for *Fon* race 1 in a region close to the identified QTL using data collected at 4 weeks after inoculation. Several putative resistant genes (*Cla004916, Cla004959, Cla011391* and *Cla011463*) (Guo et al., 2013) were found in proximity to the genomic region underlying this QTL making them candidates for functional analysis in the elucidation of the mechanism for disease resistance associated with the identified QTL. A QTL was identified on chr. 10 in experiment 2 (20, 23 and 26 DAI, and ADUPC) but not in experiment 1 and 3 (Table 6.4.and 6.5). The inability to detect this QTL across the three experiments is not surprising because of the small number (n = 89) of lines in the segregating population and potential variation in greenhouse micro-environment. Although greenhouse experiments have a controlled environment, variations in temperature are common and may influence expression of phenotypes controlled by QTLs of moderate effects.

Lambel et al. (2014) identified 7 QTL associated with resistance to *Fon* race 1 in watermelon. Although we confirmed the main QTL on chr. 1, we did not detect the other seven QTL. This is perhaps due to the difference in population size, method of inoculation and the use of parents of different genetic background in the two studies.

Cloning and sequencing analysis of candidate resistance genes

Sequencing analysis of *Cla004916, Cla004959* and *Cla0011391* (Guo et al., 2013) genes in Calhoun Gray and Sugar Baby revealed no polymorphisms between the two parents. However three point mutations (SNPs) were identified in *Cla0011463*, two in a non-coding region at position 2,677,348 and 2,679,644 bp (Fig. 6.4A and B) and one in the coding sequence (CDS) of the gene at position 2,679,498 (Fig. 6.4C). The SNP marker at position 2,679,498 bp corresponded to the SNP marker UGA_1_2679498 generated using GBS. The point mutation in

the CDS at position 2,679,498 bp is synonymous and does not lead to an amino acid change (Fig. 6.4C and D). The codon in which the mutation lies encodes Leucine, an amino acid with several possible codon usages. In Sugar Baby and Calhoun Gray, the mutation results in a TTG and TTA codon respectively, both of which encode Leucine (Fig. 6.4D).

This study confirms the QTL on chr. 1 to be associated with resistance to *Fon* race 1 in watermelon of diverse genetic background and is a viable candidate for MAS. However, it is difficult to determine the precise location of the identified QTL in the current study due to a missing chromosomal portion (approximately 2 Mb) at the edge of chr.1 on both the genetic and physical maps. To increase the mapping resolution of this QTL, markers in the missing region of the chromosome ought to be added. Sandlin et al. (2012) identified four SNP markers (NWO252073, NW0251293, NW0250418, NW0250430) in the region missing from chr. 1. These markers together with a SNP marker UGA_1_7268 identified by Meru and McGregor (unpublished) in a different var. *lanatus* by var. *lanatus* population may provide useful information on the precise position of the QTL. Other markers close to this region include UGA_1_2169311, UGA_1_2299218, UGA_1_2679498, UGA_1_3288352, NW0248267 and NW0248722). For a marker to be useful in MAS, it must be inherited (functional or tightly linked) with the trait of interest. The marker (S1_67050) described by Lambel et al. (2014) as inheriting with resistance to *Fon* race 1 in their population was found to be monomorphic between Sugar Baby (susceptible) and Calhoun Gray (resistant) (data not shown). Therefore its usefulness is limited to the specific population in which it was developed. Moreover, the SNP markers described by Xu (2014) to be linked to *Fon* race 1 resistance have been patented and would be costly to use in pubic breeding programs. Therefore, there is an urgent need to develop

publicly available markers for adoption in MAS. Markers found in proximity to *Fon* race 1 QTL (*Qfon1*) in the current study should be investigated for linkage with *Fon* race 1 resistance.

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Table 6.1 The size, position and target primers for the four putative resistance genes close to the QTL associated with resistance to *Fon* race 1 on chromosome 1 (Guo et al., 2013).

Gene	Size (bp)	Start position (bp)	End position (bp)	Forward primer	Reverse Primer	Annealing temperature (°C)
Cla004916	2,409	424,758	427,166	TGAATTCGATCATCCAGTTTCG	GCAACCAGCAATTGAAGCAA	57
Cla004959	1,223	829,911	831,133	CCAATCATCGCCATCTCCTT	CACAACCTCACAAGCCGCTAC	57
Cla011391	2,573	1,862,928	1,865,500	TTGGCAAAATACTAAATATGAGAGG	AGACAGAAGCCCTTGCAGTT	52
Cla011463	3,444	2,676,548	2,679,991	TCTAGGATTGAACATGTACACGGAAT	GGAATTCGGTTTTGGCTTCC	57

Table 6.2 The number of single nucleotide polymorphisms (SNPs) obtained per chromosome and linkage group in the physical map (Guo et al., 2013) and linkage map respectively in the Sugar Baby and Calhoun Gray F_2 population.

Chromosome	Physical map		Genetic map		
	Number of markers	Length (Mb)	Linkage group	Number of markers	Length (cM)
1	75	33.6	1	33	365.5
2	105	34.3	2	49	450.7
3	86	28.9	3	36	409.8
4	48	23.7	4	8	86.2
5	108	33.2	5	44	537.3
6	90	26.9	6	23	305.0
7	102	31.3	7	27	315.3
8	74	26.1	8	27	269.3
9	138	34.9	9	65	545.4
10	116	27.3	10	50	414.6
11	82	26.7	11A	8	40.2
			11B	19	215.9
Total	1024	326.9	Total	389	3955.2

Table 6.3 Pearson correlations for area under disease progress curve and days after inoculation for experiment 1, 2, 3 and the mean in the Sugar Baby x Calhoun F_3 population. All values are significant ($\alpha = 0.05$).

Trait (Experiment)	11(1)	14(1)	17(1)	20(1)	23(1)	26(1)	AUDPC (1)	11(2)	14(2)	17(2)	20(2)	23(2)	26(2)	AUDPC (2)	11(3)	14(3)	17(3)	20(3)	23(3)	26(3)	AUDPC (3)	11DAI (mean)	14DAI (mean)	17DAI (mean)	20DAI (mean)	23DAI (mean)	26DAI (mean)	AUDPC (mean)
11(1)	1.00																											
14(1)	0.87	1.00																										
17(1)	0.78	0.94	1.00																									
20(1)	0.74	0.92	0.98	1.00																								
23(1)	0.70	0.87	0.94	0.98	1.00																							
26(1)	0.66	0.83	0.91	0.95	0.98	1.00																						
AUDPC(1)	0.78	0.94	0.98	0.99	0.98	0.96	1.00																					
11(2)	0.34	0.47	0.45	0.46	0.43	0.41	0.45	1.00																				
14(2)	0.41	0.50	0.50	0.53	0.52	0.51	0.53	0.90	1.00																			
17(2)	0.44	0.54	0.57	0.60	0.60	0.60	0.60	0.81	0.94	1.00																		
20(2)	0.43	0.54	0.58	0.61	0.61	0.61	0.61	0.80	0.93	0.99	1.00																	
23(2)	0.42	0.54	0.58	0.60	0.62	0.61	0.61	0.73	0.86	0.93	0.95	1.00																
26(2)	0.44	0.55	0.60	0.62	0.64	0.63	0.62	0.65	0.78	0.85	0.88	0.96	1.00															
AUDPC(2)	0.44	0.55	0.58	0.61	0.61	0.61	0.61	0.83	0.95	0.99	0.99	0.97	0.91	1.00														
11(3)	0.29	0.39	0.47	0.51	0.54	0.55	0.51	0.32	0.42	0.51	0.52	0.56	0.55	0.52	1.00													
14(3)	0.32	0.43	0.51	0.55	0.58	0.59	0.55	0.32	0.44	0.53	0.54	0.58	0.57	0.55	0.98	1.00												
17(3)	0.34	0.45	0.53	0.57	0.60	0.60	0.57	0.32	0.45	0.54	0.55	0.59	0.58	0.55	0.96	0.99	1.00											
20(3)	0.36	0.47	0.55	0.59	0.62	0.62	0.59	0.35	0.46	0.55	0.57	0.60	0.59	0.57	0.94	0.98	0.99	1.00										
23(3)	0.43	0.54	0.63	0.65	0.67	0.68	0.65	0.32	0.45	0.54	0.56	0.60	0.60	0.56	0.90	0.94	0.96	0.96	1.00									
26(3)	0.47	0.58	0.66	0.68	0.70	0.70	0.68	0.33	0.46	0.55	0.57	0.62	0.62	0.58	0.84	0.88	0.91	0.92	0.96	1.00								
AUDPC(3)	0.40	0.51	0.60	0.63	0.65	0.66	0.62	0.34	0.46	0.55	0.57	0.61	0.61	0.57	0.94	0.97	0.99	0.99	0.99	0.96	1.00							
11DAI(mean)	0.77	0.80	0.76	0.76	0.73	0.70	0.78	0.82	0.83	0.81	0.80	0.77	0.73	0.82	0.58	0.59	0.60	0.61	0.62	0.64	0.63	1.00						
14DAI(mean)	0.68	0.81	0.81	0.82	0.80	0.78	0.83	0.78	0.88	0.88	0.88	0.85	0.81	0.90	0.64	0.67	0.68	0.69	0.71	0.72	0.71	0.95	1.00					
17DAI(mean)	0.63	0.78	0.84	0.85	0.85	0.83	0.85	0.69	0.81	0.89	0.90	0.87	0.84	0.90	0.70	0.74	0.75	0.77	0.78	0.79	0.78	0.88	0.96	1.00				
20DAI(mean)	0.61	0.76	0.83	0.87	0.87	0.85	0.86	0.66	0.79	0.87	0.88	0.87	0.84	0.88	0.73	0.77	0.78	0.80	0.81	0.81	0.81	0.87	0.95	0.99	1.00			
23DAI(mean)	0.60	0.75	0.83	0.86	0.88	0.87	0.86	0.58	0.71	0.81	0.83	0.86	0.85	0.84	0.75	0.79	0.81	0.82	0.86	0.86	0.85	0.82	0.91	0.96	0.98	1.00		
26DAI(mean)	0.60	0.75	0.83	0.86	0.89	0.89	0.87	0.52	0.66	0.76	0.78	0.82	0.85	0.79	0.74	0.78	0.80	0.81	0.86	0.89	0.85	0.79	0.88	0.94	0.95	0.99	1.00	
AUDPC(mean)	0.64	0.79	0.84	0.87	0.87	0.86	0.87	0.66	0.79	0.86	0.87	0.87	0.85	0.88	0.73	0.77	0.78	0.80	0.82	0.83	0.82	0.88	0.96	0.99	1.00	0.99	0.97	1.00

Table 6.4 Chromosomal (chr.) (Guo et al., 2013) positions (cM) of the QTL associated with resistance to *Fon* race 1 on the genetic map and the corresponding 1-likelihood-odds (LOD) support interval in the Sugar Baby x Calhoun F_3 watermelon population.

Trait	Exp[z]	chr.	Position (cM)[y]	LOD[x]	R^2 (%)[w]	Additive effect[v]	Dominance effect[v]	LOD-1 support interval (cM)	LOD-1 support interval (cM)
11 DAI	1	1	3.01	5.13	20.66	0.32	-0.16	0	11.8
14 DAI	1	1	0.01	7.12	27.94	0.62	-0.19	0	5.3
17 DAI	1	1	1.01	5.52	21.35	1.03	-0.29	0	4.2
20 DAI	1	1	0.01	6.01	21.00	1.22	-0.26	0	4.0
23 DAI	1	1	0.01	9.55	34.93	1.19	-0.21	0	3.0
26 DAI	1	1	1.01	8.10	30.35	1.14	-0.21	0	5.2
AUDPC	1	1	0.01	6.52	22.72	319.42	-69.08	0	3.7
11 DAI	2	1	0.01	6.55	21.68	0.38	-0.09	0	4.7
14 DAI	2	1	1.01	7.85	30.73	0.82	-0.25	0	5.7
17 DAI	2	1	0.01	10.07	37.92	1.20	-0.35	0	5.1
20 DAI	2	1	1.01	9.03	37.19	1.29	-0.26	0	5.6
23 DAI	2	1	0.01	9.71	33.47	1.09	-0.68	0	5.1
26 DAI	2	1	0.01	9.69	31.62	1.04	-0.32	0	3.5
AUDPC	2	1	0.01	10.28	38.40	320.42	-59.94	0	4.5
20 DAI	2	10	0.01	3.69	11.10	0.41	0.86	0	3.9
23 DAI	2	10	0.01	3.41	8.63	0.32	0.72	0	16.1
26 DAI	2	10	0.01	4.02	10.46	0.42	0.64	0	11.4
AUDPC	2	10	0.01	3.70	10.20	59.86	213.64	0	3.9
14 DAI	3	1	0.01	2.92	12.97	0.23	0.12	0	7.2
17 DAI	3	1	0.01	3.10	11.52	0.29	0.20	0	12.8
20 DAI	3	1	0.01	3.32	13.52	0.49	0.11	0	5.0
23DAI	3	1	0.01	4.29	19.18	0.78	-0.15	0	4.3
26 DAI	3	1	0.01	6.62	24.10	0.97	-0.42	0	5.5

AUDPC	3	1	0.01	4.68	20.53	157.75	-51.42	0	3.7
11 DAI	Mean	1	0.01	12.53	47.47	0.35	-0.11	0	2.7
14 DAI	Mean	1	0.01	12.13	41.86	0.59	-0.11	0	2.7
17 DAI	Mean	1	0.01	11.08	41.42	0.81	-0.17	0	3.4
20 DAI	Mean	1	0.01	10.32	39.36	0.92	-0.19	0	3.4
23 DAI	Mean	1	0.01	10.22	36.56	0.99	-0.30	0	2.9
26 DAI	Mean	1	0.01	11.14	37.46	1.06	-0.30	0	2.6
AUDPC	Mean	1	0.01	11.78	44.39	249.25	-50.45	0	2.8

[z] Experiment in which data for QTL mapping was derived.

[y] Chromosome on the watermelon draft genome (Guo et al., 2013).

[x] Log_{10} likelihood ratio.

[w] Phenotypic variation explained.

[v] Negative values indicate that the effect is contributed by the allele from Calhoun Gray.

Table 6.5 Chromosomal (chr.) (Guo et al., 2013) positions (Mb) of the QTL associated with resistance to *Fon* race 1 on the physical map and the corresponding 1-likelihood-odds (LOD) support interval in the Sugar Baby x Calhoun F_3 watermelon population.

Trait	Exp[z]	chr.[y]	Position (Mb)	LOD[x]	R^2 (%)[w]	Additive effect[v]	Dominance effect[v]	LOD-1 support interval (Mb)	LOD-1 support interval (Mb)
11 DAI	1	1	0.14	6.45	25.23	0.33	-0.20	0	0.6
14 DAI	1	1	0.01	7.75	28.21	0.61	-0.17	0	1.6
17 DAI	1	1	0.01	7.88	28.19	0.79	-0.11	0	2.1
20 DAI	1	1	0.01	7.20	29.20	0.94	-0.33	0	2.3
23 DAI	1	1	0.14	6.49	24.92	1.13	-0.38	0	1.3
26 DAI	1	1	0.01	8.68	27.36	1.09	-0.22	0	1.1
AUDPC	1	1	0.01	10.45	33.56	257.14	-89.29	0	1.1
11 DAI	2	1	0.01	6.11	20.31	0.37	-0.08	0	0.4
14 DAI	2	1	0.01	8.25	30.59	0.87	-0.10	0	0.5
17 DAI	2	1	0.01	8.53	32.21	1.17	-0.24	0	0.3
20 DAI	2	1	0.01	8.79	33.65	1.22	-0.35	0	0.5
23 DAI	2	1	0.01	9.48	30.80	1.03	-0.74	0	1.8
26 DAI	2	1	0.01	8.43	28.29	1.00	-0.30	0	0.3
AUDPC	2	1	0.01	8.59	34.29	311.20	-79.88	0	0.3
20 DAI	2	10	2.42	6.33	18.53	0.25	1.26	0	2.7
23 DAI	2	10	1.30	3.20	8.89	0.18	1.02	0	2.8
26 DAI	2	10	1.30	5.30	13.36	0.42	0.79	0	1.8
AUDPC	2	10	2.42	6.57	19.41	59.27	329.88	0	2.6
14 DAI	3	1	0.01	3.14	12.70	0.25	-0.07	0	0.4
17 DAI	3	1	0.01	3.08	13.03	0.25	0.31	7	7.8
20 DAI	3	1	0.01	3.33	12.08	0.45	-0.11	0	0.3
23DAI	3	1	0.01	4.73	18.66	0.76	-0.16	0	0.4
26 DAI	3	1	0.01	6.08	23.35	0.99	-0.35	0	0.3

AUDPC	3	1	0.01	4.53	17.75	145.45	-40.85	0	0.4
11 DAI	Mean	1	0.01	7.49	33.11	0.26	-0.09	0	0.1
14 DAI	Mean	1	0.01	11.73	40.53	0.59	-0.10	0	0.8
17 DAI	Mean	1	0.01	10.56	32.50	0.72	-0.21	0	1.0
20 DAI	Mean	1	0.01	10.00	31.25	0.82	-0.24	0	1.0
23 DAI	Mean	1	0.01	10.37	39.42	1.05	-0.28	0	0.2
26 DAI	Mean	1	0.01	9.58	30.96	0.96	-0.37	0	1.1
AUDPC	Mean	1	0.01	11.04	41.40	241.11	-65.38	0	0.2

[z] Experiment in which data for QTL mapping was derived.

[y] Chromosome on the watermelon draft genome (Guo et al., 2013).

[x] The position of the QTL on the chromosome (Guo et al., 2013; Ren et al., 2012).

[w] Log_{10} likelihood ratio.

[v] Phenotypic variation explained.

[v] Negative values indicate that the effect is contributed by the allele from Calhoun Gray.

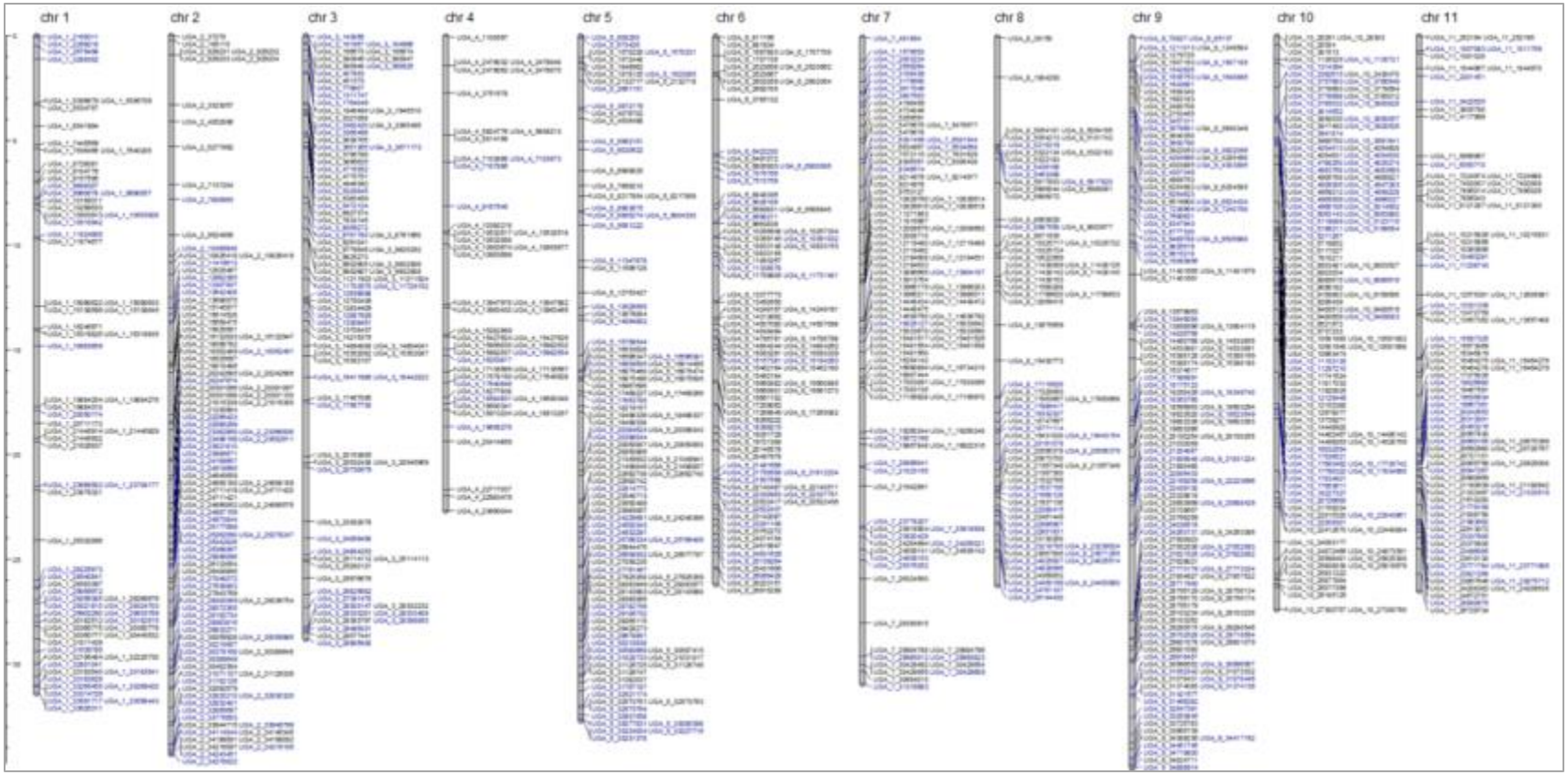

Fig. 6.1 The watermelon physical map consisting of 1,024 single nucleotide polymorphisms (SNPs) and spanning 11 chromosomes of the watermelon draft genome sequence (Guo et al., 2013). Loci in blue font represent markers on the genetic map. Figure generated using MapChart Version 2.2 (Voorrips, 2002).

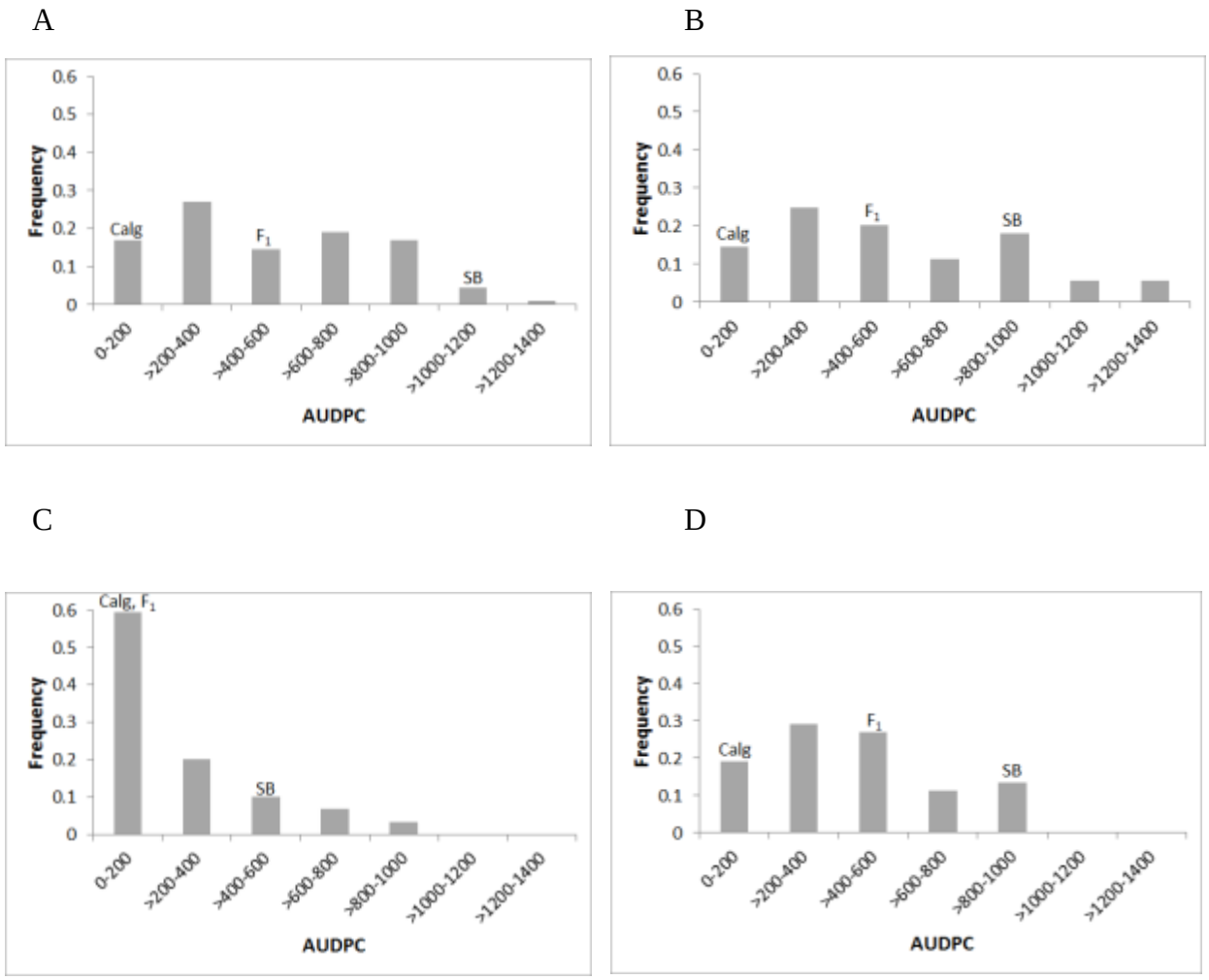

Fig. 6.2 Frequency distribution for the area under disease progress curve (AUDPC) for *Fon* race 1 phenotyping in the greenhouse in (A) Aug. 2013 (B) Nov. 2013, (C) Feb. 2014, and (D) mean AUDPC for the three experiments in the Calhoun Gray x Sugar Baby, F_3 population as well as the parental and F_1 genotypes.

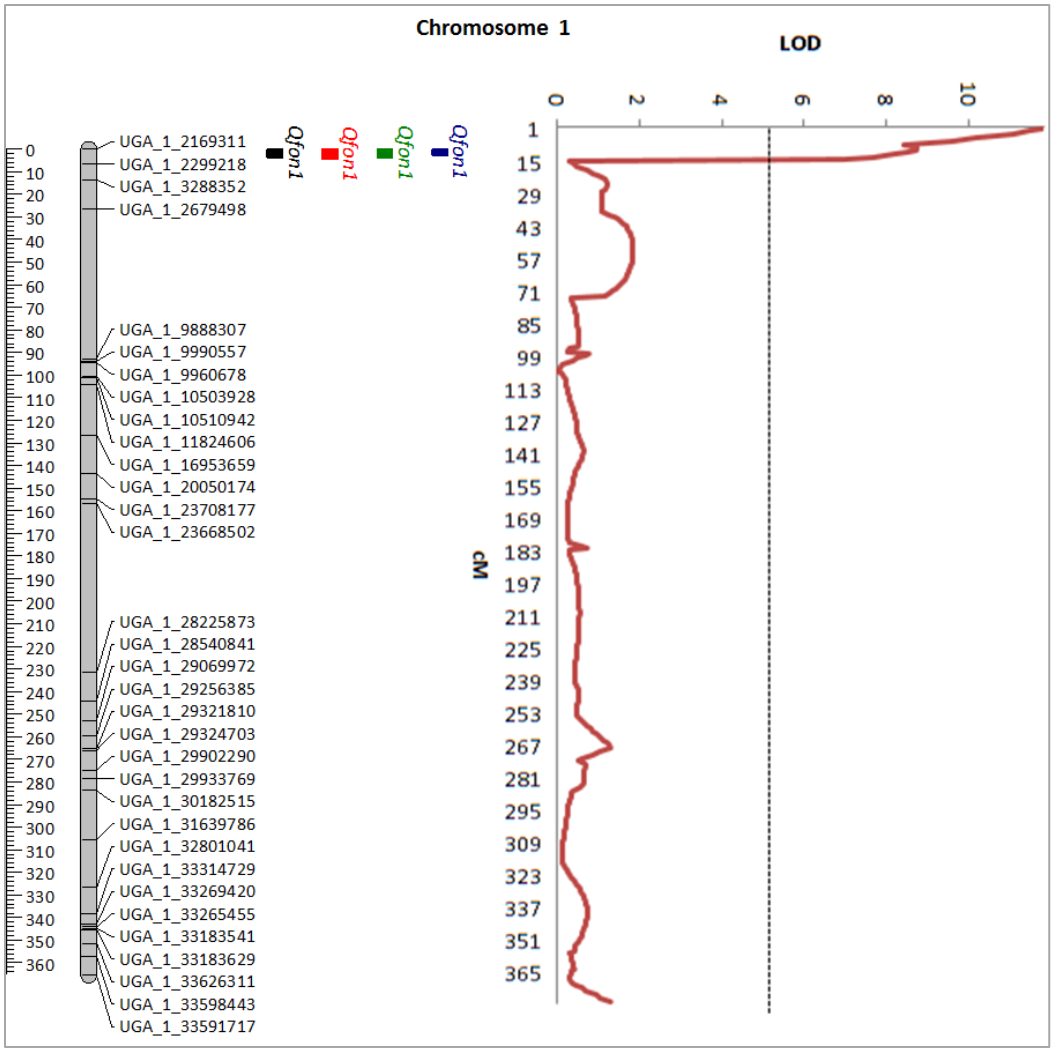

Fig. 6.3 Quantitative trait locus associated with resistance to race 1 of *Fusarium oxysporum* f. sp. *niveum* on chromosome 1 [length of the bar is equal to 1-LOD support interval] identified by composite interval mapping in the Calhoun Gray x Sugar Baby F_2 population on the genetic map. The black, red, green and blue bars represent QTL mapped using AUDPC values from experiment 1, 2, 3 and their mean respectively. The Log_{10} likelihood ratio (LOD) values for this QTL are shown next to the QTL. Cutoff value for declaring significance (LOD = 5.2) is indicated by the dotted line. Figure generated using MapChart Version 2.2 (Voorrips, 2002) and Microsoft Office Excel.

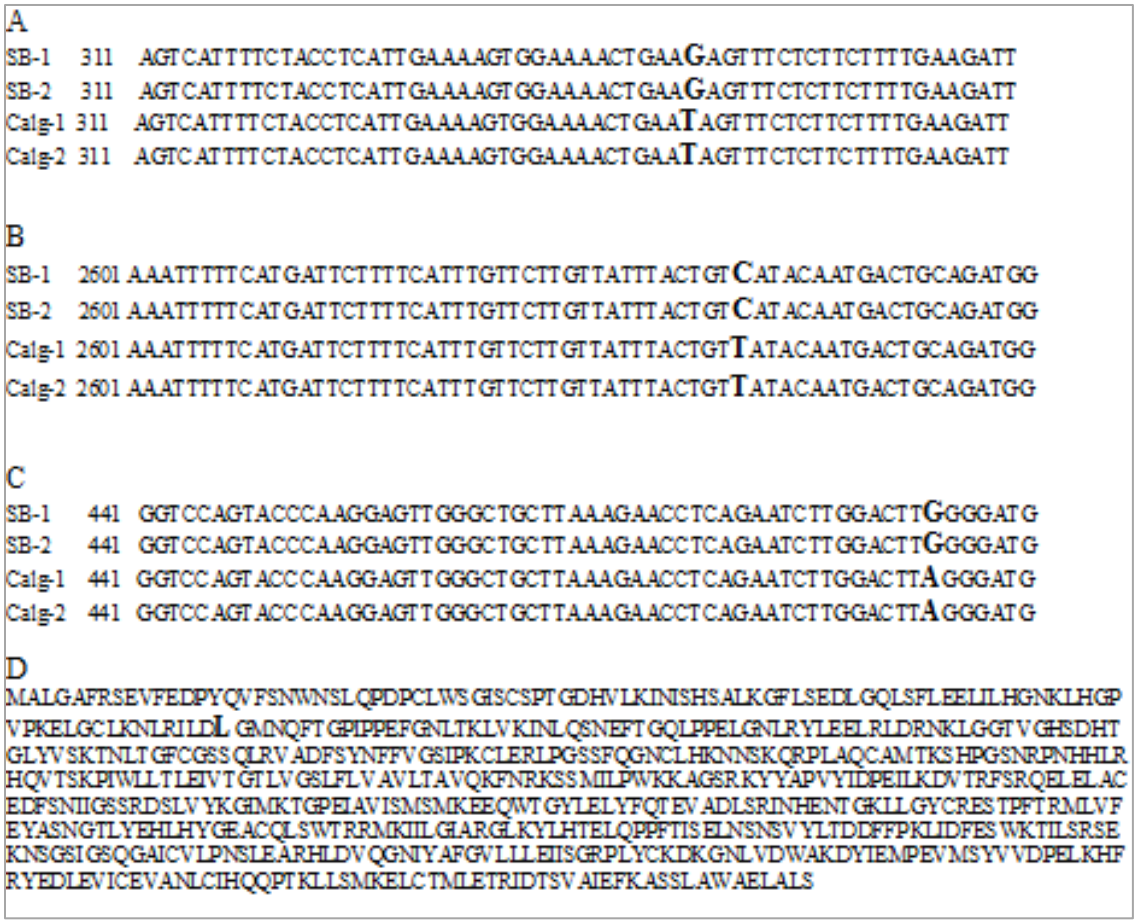

Fig. 6.4 Polymorphisms identified between Sugar Baby (SB) and Calhoun Gray (Calg) in *Cla011463* gene. (A) Mutations at position 2,679,644 bp and (B) 2,677,348 bp are in the intron region while, (C) mutation at position 2,679,498 is in an exon. (D) The amino acid sequence encoded by *Cla011463* (Guo et al., 2013). The 96th amino acid is Leucine (bolded) and is coded by TTG and TTA codons in SB and Calg respectively.

Appendix 6.1 The name, chromosomal position (Guo et al., 2013) and genotype of Sugar Baby (SB) and Calhoun Gray (Calg) for the 1,084 single nucleotide polymorphisms discovered by genotyping by sequencing in the SB x Calg F_2 population.

			Genotype	
Locus name	**Chromosome**	**Position (bp)**	**SB**	**Calg**
UGA_1_2169311	1	2,169,311	C	T
UGA_1_2299218	1	2,299,218	A	G
UGA_1_2679498	1	2,679,498	C	T
UGA_1_3288352	1	3,288,352	A	G
UGA_1_5356679	1	5,356,679	G	C
UGA_1_5356709	1	5,356,709	T	C
UGA_1_5534757	1	5,534,757	C	A
UGA_1_6541994	1	6,541,994	A	G
UGA_1_7445569	1	7,445,569	T	C
UGA_1_7636466	1	7,636,466	C	G
UGA_1_7640205	1	7,640,205	A	G
UGA_1_8728081	1	8,728,081	G	A
UGA_1_9104776	1	9,104,776	A	C
UGA_1_9157595	1	9,157,595	T	A
UGA_1_9888307	1	9,888,307	A	G
UGA_1_9960678	1	9,960,678	T	C
UGA_1_9990557	1	9,990,557	A	G
UGA_1_10189311	1	10,189,311	A	G
UGA_1_10299533	1	10,299,533	T	A
UGA_1_10503913	1	10,503,913	G	A
UGA_1_10503928	1	10,503,928	A	G
UGA_1_10510942	1	10,510,942	C	A
UGA_1_11824606	1	11,824,606	G	A
UGA_1_11874677	1	11,874,677	G	A
UGA_1_15090622	1	15,090,622	A	G
UGA_1_15090633	1	15,090,633	T	A
UGA_1_15138599	1	15,138,599	C	A
UGA_1_15138645	1	15,138,645	A	G
UGA_1_16246971	1	16,246,971	G	T
UGA_1_16318826	1	16,318,826	G	A
UGA_1_16318835	1	16,318,835	C	T
UGA_1_16953659	1	16,953,659	T	C
UGA_1_19884254	1	19,884,254	A	T

UGA_1_19884276	1	19,884,276	C	A
UGA_1_19884310	1	19,884,310	G	A
UGA_1_20050174	1	20,050,174	G	A
UGA_1_20711172	1	20,711,172	C	G
UGA_1_21445914	1	21,445,914	A	G
UGA_1_21445929	1	21,445,929	C	T
UGA_1_21445932	1	21,445,932	G	C
UGA_1_21626837	1	21,626,837	G	A
UGA_1_23668502	1	23,668,502	C	T
UGA_1_23708177	1	23,708,177	A	G
UGA_1_23878321	1	23,878,321	T	C
UGA_1_26332096	1	26,332,096	T	G
UGA_1_28225873	1	28,225,873	A	G
UGA_1_28540841	1	28,540,841	C	G
UGA_1_28583397	1	28,583,397	C	A
UGA_1_29069972	1	29,069,972	G	A
UGA_1_29256385	1	29,256,385	A	G
UGA_1_29268676	1	29,268,676	C	G
UGA_1_29321810	1	29,321,810	G	A
UGA_1_29324703	1	29,324,703	C	G
UGA_1_29902290	1	29,902,290	A	C
UGA_1_29933769	1	29,933,769	T	C
UGA_1_30182512	1	30,182,512	C	A
UGA_1_30182515	1	30,182,515	A	G
UGA_1_30360775	1	30,360,775	A	C
UGA_1_30360776	1	30,360,776	G	T
UGA_1_30360777	1	30,360,777	A	C
UGA_1_30445532	1	30,445,532	G	A
UGA_1_31011439	1	31,011,439	T	A
UGA_1_31639786	1	31,639,786	T	C
UGA_1_32195484	1	32,195,484	A	T
UGA_1_32225730	1	32,225,730	C	T
UGA_1_32801041	1	32,801,041	G	C
UGA_1_33183540	1	33,183,540	C	T
UGA_1_33183541	1	33,183,541	C	T
UGA_1_33183629	1	33,183,629	G	C
UGA_1_33265455	1	33,265,455	A	T
UGA_1_33269420	1	33,269,420	C	G
UGA_1_33314729	1	33,314,729	G	A
UGA_1_33591717	1	33,591,717	G	T
UGA_1_33598443	1	33,598,443	G	A

UGA_1_33626311	1	33,626,311	A	C
UGA_2_37270	2	37,270	C	T
UGA_2_165115	2	165,115	T	C
UGA_2_929231	2	929,231	A	T
UGA_2_929232	2	929,232	A	T
UGA_2_929233	2	929,233	G	T
UGA_2_929234	2	929,234	G	T
UGA_2_3323057	2	3,323,057	A	G
UGA_2_4052896	2	4,052,896	G	A
UGA_2_5277992	2	5,277,992	T	G
UGA_2_7137204	2	7,137,204	A	C
UGA_2_7808963	2	7,808,963	A	C
UGA_2_9524956	2	9,524,956	G	A
UGA_2_10489848	2	10,489,848	G	C
UGA_2_10826418	2	10,826,418	G	C
UGA_2_10826419	2	10,826,419	G	T
UGA_2_11419613	2	11,419,613	T	C
UGA_2_12525467	2	12,525,467	T	C
UGA_2_12982385	2	12,982,385	G	C
UGA_2_13307937	2	13,307,937	G	C
UGA_2_13642408	2	13,642,408	G	A
UGA_2_13698975	2	13,698,975	G	T
UGA_2_15145977	2	15,145,977	C	T
UGA_2_15614528	2	15,614,528	C	T
UGA_2_15658475	2	15,658,475	A	T
UGA_2_15826561	2	15,826,561	A	G
UGA_2_16132933	2	16,132,933	A	G
UGA_2_16132947	2	16,132,947	A	T
UGA_2_18098782	2	18,098,782	G	T
UGA_2_18352489	2	18,352,489	G	A
UGA_2_18352491	2	18,352,491	T	A
UGA_2_18525697	2	18,525,697	T	C
UGA_2_18810486	2	18,810,486	T	C
UGA_2_20242685	2	20,242,685	T	G
UGA_2_20242686	2	20,242,686	T	A
UGA_2_20247674	2	20,247,674	A	C
UGA_2_20301096	2	20,301,096	C	A
UGA_2_20301097	2	20,301,097	T	G
UGA_2_20301098	2	20,301,098	T	A
UGA_2_20301100	2	20,301,100	G	C
UGA_2_21015339	2	21,015,339	A	G

UGA_2_21015350	2	21,015,350	A	G
UGA_2_21230904	2	21,230,904	A	T
UGA_2_22295423	2	22,295,423	G	C
UGA_2_22586299	2	22,586,299	T	A
UGA_2_23362868	2	23,362,868	A	G
UGA_2_23366936	2	23,366,936	C	T
UGA_2_23498199	2	23,498,199	C	A
UGA_2_23502611	2	23,502,611	A	G
UGA_2_23521810	2	23,521,810	G	A
UGA_2_23906671	2	23,906,671	C	T
UGA_2_24169657	2	24,169,657	C	T
UGA_2_24518665	2	24,518,665	T	C
UGA_2_24645659	2	24,645,659	G	T
UGA_2_24658188	2	24,658,188	A	C
UGA_2_24658189	2	24,658,189	T	A
UGA_2_24711419	2	24,711,419	C	A
UGA_2_24711420	2	24,711,420	T	G
UGA_2_24711421	2	24,711,421	G	A
UGA_2_24866852	2	24,866,852	G	T
UGA_2_24886576	2	24,886,576	A	T
UGA_2_24887159	2	24,887,159	T	C
UGA_2_24973844	2	24,973,844	G	A
UGA_2_25177699	2	25,177,699	A	G
UGA_2_25262090	2	25,262,090	C	A
UGA_2_25278247	2	25,278,247	A	T
UGA_2_25342826	2	25,342,826	G	A
UGA_2_25368067	2	25,368,067	A	C
UGA_2_25896096	2	25,896,096	T	C
UGA_2_26133554	2	26,133,554	A	T
UGA_2_26406866	2	26,406,866	A	T
UGA_2_27048072	2	27,048,072	G	A
UGA_2_27638883	2	27,638,883	A	T
UGA_2_27943769	2	27,943,769	G	A
UGA_2_28000069	2	28,000,069	A	G
UGA_2_28036754	2	28,036,754	G	T
UGA_2_28072355	2	28,072,355	T	C
UGA_2_28192734	2	28,192,734	T	C
UGA_2_28983816	2	28,983,816	A	G
UGA_2_29832271	2	29,832,271	G	T
UGA_2_30059928	2	30,059,928	G	A
UGA_2_30059986	2	30,059,986	G	A

UGA_2_30215557	2	30,215,557	A	G
UGA_2_30379168	2	30,379,168	A	C
UGA_2_30389648	2	30,389,648	T	A
UGA_2_30389649	2	30,389,649	T	A
UGA_2_30482394	2	30,482,394	C	T
UGA_2_31071157	2	31,071,157	T	C
UGA_2_31129336	2	31,129,336	G	A
UGA_2_31152126	2	31,152,126	A	G
UGA_2_32092579	2	32,092,579	C	T
UGA_2_32825210	2	32,825,210	T	A
UGA_2_32830326	2	32,830,326	G	C
UGA_2_32832401	2	32,832,401	C	T
UGA_2_32865697	2	32,865,697	G	C
UGA_2_33775653	2	33,775,653	A	T
UGA_2_33944715	2	33,944,715	G	C
UGA_2_33949799	2	33,949,799	C	G
UGA_2_34114044	2	34,114,044	T	G
UGA_2_34146346	2	34,146,346	T	G
UGA_2_34199091	2	34,199,091	A	C
UGA_2_34199092	2	34,199,092	G	C
UGA_2_34216097	2	34,216,097	A	C
UGA_2_34216106	2	34,216,106	C	T
UGA_2_34243451	2	34,243,451	A	G
UGA_2_34278822	2	34,278,822	C	T
UGA_3_143056	3	143,056	G	A
UGA_3_161957	3	161,957	T	C
UGA_3_184966	3	184,966	C	G
UGA_3_195673	3	195,673	C	A
UGA_3_195674	3	195,674	T	G
UGA_3_380946	3	380,946	G	T
UGA_3_380947	3	380,947	A	C
UGA_3_380948	3	380,948	G	T
UGA_3_390925	3	390,925	G	A
UGA_3_407853	3	407,853	C	G
UGA_3_451373	3	451,373	G	A
UGA_3_779807	3	779,807	G	A
UGA_3_1311747	3	1,311,747	A	G
UGA_3_1764049	3	1,764,049	A	C
UGA_3_1946494	3	1,946,494	A	G
UGA_3_1946510	3	1,946,510	A	G
UGA_3_3321088	3	3,321,088	A	G

UGA_3_3362420	3	3,362,420	C	T
UGA_3_3365495	3	3,365,495	C	T
UGA_3_3365496	3	3,365,496	C	T
UGA_3_3638765	3	3,638,765	C	A
UGA_3_3651365	3	3,651,365	A	G
UGA_3_3671173	3	3,671,173	A	T
UGA_3_3756786	3	3,756,786	T	C
UGA_3_3895625	3	3,895,625	A	C
UGA_3_4716352	3	4,716,352	C	G
UGA_3_4775751	3	4,775,751	C	T
UGA_3_4946392	3	4,946,392	G	T
UGA_3_5220845	3	5,220,845	C	T
UGA_3_5385459	3	5,385,459	A	G
UGA_3_5472124	3	5,472,124	T	A
UGA_3_6927374	3	6,927,374	T	A
UGA_3_7833145	3	7,833,145	C	T
UGA_3_8059272	3	8,059,272	G	A
UGA_3_8761780	3	8,761,780	G	T
UGA_3_8761960	3	8,761,960	T	G
UGA_3_9291041	3	9,291,041	A	T
UGA_3_9779546	3	9,779,546	T	C
UGA_3_9825252	3	9,825,252	C	A
UGA_3_9825273	3	9,825,273	T	C
UGA_3_9932905	3	9,932,905	T	A
UGA_3_9932906	3	9,932,906	C	T
UGA_3_9932907	3	9,932,907	A	C
UGA_3_9932908	3	9,932,908	G	T
UGA_3_11211923	3	11,211,923	A	T
UGA_3_11211924	3	11,211,924	A	G
UGA_3_11703570	3	11,703,570	C	G
UGA_3_11729152	3	11,729,152	G	T
UGA_3_12639098	3	12,639,098	G	C
UGA_3_12700436	3	12,700,436	T	C
UGA_3_12834409	3	12,834,409	G	A
UGA_3_12887929	3	12,887,929	T	C
UGA_3_13309451	3	13,309,451	T	C
UGA_3_13708437	3	13,708,437	C	T
UGA_3_14215375	3	14,215,375	T	G
UGA_3_14894008	3	14,894,008	C	T
UGA_3_14894041	3	14,894,041	T	C
UGA_3_15352082	3	15,352,082	G	T

UGA_3_15352087	3	15,352,087	T	A
UGA_3_15352107	3	15,352,107	A	G
UGA_3_16411686	3	16,411,686	C	T
UGA_3_16442023	3	16,442,023	T	C
UGA_3_17467095	3	17,467,095	T	A
UGA_3_17587730	3	17,587,730	C	T
UGA_3_20153805	3	20,153,805	T	C
UGA_3_20532438	3	20,532,438	A	T
UGA_3_20546969	3	20,546,969	G	T
UGA_3_20730679	3	20,730,679	T	C
UGA_3_23302078	3	23,302,078	C	T
UGA_3_24069458	3	24,069,458	A	G
UGA_3_24884253	3	24,884,253	A	C
UGA_3_25114112	3	25,114,112	A	C
UGA_3_25114113	3	25,114,113	G	A
UGA_3_25200121	3	25,200,121	T	C
UGA_3_25976676	3	25,976,676	G	C
UGA_3_26825682	3	26,825,682	C	T
UGA_3_27391470	3	27,391,470	C	T
UGA_3_28303147	3	28,303,147	T	A
UGA_3_28332232	3	28,332,232	G	C
UGA_3_28333201	3	28,333,201	T	A
UGA_3_28333408	3	28,333,408	G	A
UGA_3_28383797	3	28,383,797	T	C
UGA_3_28398893	3	28,398,893	T	C
UGA_3_28489331	3	28,489,331	T	C
UGA_3_28577441	3	28,577,441	T	A
UGA_3_28905930	3	28,905,930	T	A
UGA_4_1100597	4	1,100,597	C	T
UGA_4_2479032	4	2,479,032	A	G
UGA_4_2479048	4	2,479,048	C	G
UGA_4_2479062	4	2,479,062	T	C
UGA_4_2479075	4	2,479,075	G	A
UGA_4_3751578	4	3,751,578	G	A
UGA_4_5804776	4	5,804,776	C	T
UGA_4_5809213	4	5,809,213	A	G
UGA_4_5814168	4	5,814,168	G	A
UGA_4_7103868	4	7,103,868	T	C
UGA_4_7103873	4	7,103,873	C	G
UGA_4_7107895	4	7,107,895	G	A
UGA_4_9167545	4	9,167,545	T	A

UGA_4_10380218	4	10,380,218	T	C
UGA_4_10532517	4	10,532,517	A	T
UGA_4_10532518	4	10,532,518	T	A
UGA_4_10532558	4	10,532,558	G	A
UGA_4_10953674	4	10,953,674	A	G
UGA_4_10953677	4	10,953,677	A	G
UGA_4_10953699	4	10,953,699	A	G
UGA_4_13947978	4	13,947,978	G	A
UGA_4_13947982	4	13,947,982	C	T
UGA_4_13963430	4	13,963,430	G	A
UGA_4_13963465	4	13,963,465	C	T
UGA_4_15282569	4	15,282,569	C	T
UGA_4_15427624	4	15,427,624	C	A
UGA_4_15427625	4	15,427,625	C	G
UGA_4_15959035	4	15,959,035	T	G
UGA_4_15982532	4	15,982,532	C	T
UGA_4_15982537	4	15,982,537	T	G
UGA_4_15982554	4	15,982,554	T	A
UGA_4_16253817	4	16,253,817	G	A
UGA_4_17135566	4	17,135,566	A	C
UGA_4_17135567	4	17,135,567	G	T
UGA_4_17578150	4	17,578,150	A	G
UGA_4_17640628	4	17,640,628	A	C
UGA_4_17640646	4	17,640,646	C	T
UGA_4_18277838	4	18,277,838	T	G
UGA_4_18584831	4	18,584,831	T	G
UGA_4_18600340	4	18,600,340	A	C
UGA_4_18600341	4	18,600,341	G	A
UGA_4_18913234	4	18,913,234	C	T
UGA_4_18913257	4	18,913,257	T	G
UGA_4_19658276	4	19,658,276	A	T
UGA_4_20414859	4	20,414,859	T	C
UGA_4_22717037	4	22,717,037	T	C
UGA_4_22983479	4	22,983,479	G	T
UGA_4_23660044	4	23,660,044	A	G
UGA_5_608200	5	608,200	T	G
UGA_5_673426	5	673,426	A	G
UGA_5_1570228	5	1,570,228	T	A
UGA_5_1570231	5	1,570,231	A	C
UGA_5_1572448	5	1,572,448	G	A
UGA_5_1848962	5	1,848,962	A	C

UGA_5_1878125	5	1,878,125	T	G
UGA_5_1923085	5	1,923,085	G	A
UGA_5_2132717	5	2,132,717	C	A
UGA_5_2132718	5	2,132,718	T	G
UGA_5_2661151	5	2,661,151	T	A
UGA_5_3972179	5	3,972,179	G	A
UGA_5_4076702	5	4,076,702	T	C
UGA_5_4535498	5	4,535,498	T	C
UGA_5_5562151	5	5,562,151	G	A
UGA_5_6020622	5	6,020,622	C	T
UGA_5_6989025	5	6,989,025	T	C
UGA_5_7669210	5	7,669,210	C	T
UGA_5_8217854	5	8,217,854	T	C
UGA_5_8217856	5	8,217,856	G	A
UGA_5_8903675	5	8,903,675	G	A
UGA_5_8955274	5	8,955,274	G	T
UGA_5_9004330	5	9,004,330	G	T
UGA_5_9581222	5	9,581,222	G	A
UGA_5_11347878	5	11,347,878	A	G
UGA_5_11596128	5	11,596,128	C	A
UGA_5_12753427	5	12,753,427	G	A
UGA_5_13526555	5	13,526,555	T	C
UGA_5_13878884	5	13,878,884	T	C
UGA_5_14094882	5	14,094,882	G	T
UGA_5_15758544	5	15,758,544	G	A
UGA_5_16533628	5	16,533,628	A	T
UGA_5_16596347	5	16,596,347	A	C
UGA_5_16596381	5	16,596,381	G	A
UGA_5_16809505	5	16,809,505	G	A
UGA_5_16814495	5	16,814,495	G	C
UGA_5_16875460	5	16,875,460	T	A
UGA_5_16875474	5	16,875,474	A	T
UGA_5_16875496	5	16,875,496	T	C
UGA_5_16875505	5	16,875,505	G	T
UGA_5_16967695	5	16,967,695	T	C
UGA_5_17468227	5	17,468,227	A	G
UGA_5_17468266	5	17,468,266	T	C
UGA_5_17653785	5	17,653,785	A	C
UGA_5_18219157	5	18,219,157	T	G
UGA_5_18498336	5	18,498,336	C	T
UGA_5_18498337	5	18,498,337	A	C

UGA_5_18498338	5	18,498,338	G	T
UGA_5_20384624	5	20,384,624	C	T
UGA_5_20398043	5	20,398,043	A	G
UGA_5_20398044	5	20,398,044	G	A
UGA_5_20850887	5	20,850,887	C	T
UGA_5_20850893	5	20,850,893	C	A
UGA_5_20850906	5	20,850,906	A	C
UGA_5_21349932	5	21,349,932	G	A
UGA_5_21349941	5	21,349,941	T	G
UGA_5_21498849	5	21,498,849	A	G
UGA_5_21498857	5	21,498,857	A	G
UGA_5_22692738	5	22,692,738	A	T
UGA_5_22692740	5	22,692,740	T	A
UGA_5_22692742	5	22,692,742	A	C
UGA_5_22814773	5	22,814,773	C	G
UGA_5_23548713	5	23,548,713	T	C
UGA_5_23550489	5	23,550,489	A	T
UGA_5_23985067	5	23,985,067	A	G
UGA_5_24229581	5	24,229,581	C	T
UGA_5_24248399	5	24,248,399	G	A
UGA_5_24500343	5	24,500,343	T	C
UGA_5_24932291	5	24,932,291	C	G
UGA_5_25766334	5	25,766,334	A	G
UGA_5_25766406	5	25,766,406	G	T
UGA_5_25944476	5	25,944,476	C	A
UGA_5_26658932	5	26,658,932	A	G
UGA_5_26677797	5	26,677,797	A	C
UGA_5_27058235	5	27,058,235	C	G
UGA_5_27791467	5	27,791,467	C	T
UGA_5_27925358	5	27,925,358	T	A
UGA_5_27925359	5	27,925,359	A	C
UGA_5_28083949	5	28,083,949	A	C
UGA_5_28083977	5	28,083,977	C	A
UGA_5_28143983	5	28,143,983	A	T
UGA_5_28143988	5	28,143,988	A	T
UGA_5_28589366	5	28,589,366	G	A
UGA_5_28782756	5	28,782,756	T	C
UGA_5_29106732	5	29,106,732	T	C
UGA_5_29265115	5	29,265,115	T	C
UGA_5_29428273	5	29,428,273	A	C
UGA_5_29678861	5	29,678,861	T	C

UGA_5_30210838	5	30,210,838	G	A
UGA_5_30580669	5	30,580,669	C	T
UGA_5_30587415	5	30,587,415	T	C
UGA_5_31028733	5	31,028,733	A	C
UGA_5_31031017	5	31,031,017	C	T
UGA_5_31126725	5	31,126,725	C	T
UGA_5_31126746	5	31,126,746	T	C
UGA_5_31126747	5	31,126,747	A	T
UGA_5_31382037	5	31,382,037	T	G
UGA_5_31767187	5	31,767,187	A	C
UGA_5_32521174	5	32,521,174	C	T
UGA_5_32573761	5	32,573,761	T	G
UGA_5_32573763	5	32,573,763	C	A
UGA_5_32573764	5	32,573,764	T	C
UGA_5_32931558	5	32,931,558	G	A
UGA_5_33077831	5	33,077,831	A	G
UGA_5_33085398	5	33,085,398	C	T
UGA_5_33224004	5	33,224,004	C	T
UGA_5_33227716	5	33,227,716	C	T
UGA_5_33231378	5	33,231,378	C	T
UGA_6_811166	6	811,166	T	C
UGA_6_881934	6	881,934	A	G
UGA_6_1697883	6	1,697,883	G	C
UGA_6_1707705	6	1,707,705	G	C
UGA_6_1707735	6	1,707,735	T	C
UGA_6_2523658	6	2,523,658	G	A
UGA_6_2523662	6	2,523,662	A	G
UGA_6_2523667	6	2,523,667	C	G
UGA_6_2602059	6	2,602,059	A	T
UGA_6_2602064	6	2,602,064	A	T
UGA_6_2692705	6	2,692,705	T	C
UGA_6_3785132	6	3,785,132	G	A
UGA_6_6422230	6	6,422,230	A	C
UGA_6_6491072	6	6,491,072	G	A
UGA_6_6800933	6	6,800,933	G	A
UGA_6_6800935	6	6,800,935	G	C
UGA_6_7076766	6	7,076,766	G	A
UGA_6_7513759	6	7,513,759	A	G
UGA_6_8848336	6	8,848,336	G	A
UGA_6_9026106	6	9,026,106	A	C
UGA_6_9589831	6	9,589,831	A	G

UGA_6_9589845	6	9,589,845	A	G
UGA_6_9590211	6	9,590,211	A	C
UGA_6_9663839	6	9,663,839	G	A
UGA_6_10256938	6	10,256,938	T	C
UGA_6_10257334	6	10,257,334	G	A
UGA_6_10359145	6	10,359,145	A	T
UGA_6_10391032	6	10,391,032	T	C
UGA_6_10833148	6	10,833,148	A	G
UGA_6_10833153	6	10,833,153	A	G
UGA_6_10833155	6	10,833,155	G	A
UGA_6_11063257	6	11,063,257	T	C
UGA_6_11338078	6	11,338,078	T	G
UGA_6_11709805	6	11,709,805	A	G
UGA_6_11731401	6	11,731,401	C	G
UGA_6_13377773	6	13,377,773	A	G
UGA_6_13450550	6	13,450,550	C	A
UGA_6_14249167	6	14,249,167	C	T
UGA_6_14249191	6	14,249,191	A	G
UGA_6_14313662	6	14,313,662	C	T
UGA_6_14507080	6	14,507,080	T	C
UGA_6_14507098	6	14,507,098	T	C
UGA_6_14549494	6	14,549,494	T	C
UGA_6_14795781	6	14,795,781	A	G
UGA_6_14795798	6	14,795,798	G	A
UGA_6_14804244	6	14,804,244	G	A
UGA_6_14804262	6	14,804,262	T	C
UGA_6_15093291	6	15,093,291	T	C
UGA_6_15093339	6	15,093,339	G	C
UGA_6_15157381	6	15,157,381	T	A
UGA_6_15194263	6	15,194,263	G	T
UGA_6_15462164	6	15,462,164	T	C
UGA_6_15462180	6	15,462,180	A	G
UGA_6_15462184	6	15,462,184	T	C
UGA_6_15660882	6	15,660,882	G	A
UGA_6_15660885	6	15,660,885	G	A
UGA_6_15660931	6	15,660,931	C	A
UGA_6_15661073	6	15,661,073	C	T
UGA_6_15661102	6	15,661,102	C	G
UGA_6_17209062	6	17,209,062	G	A
UGA_6_17266849	6	17,266,849	C	T
UGA_6_17269382	6	17,269,382	C	T

UGA_6_18266222	6	18,266,222	C	A
UGA_6_18399215	6	18,399,215	A	G
UGA_6_19351729	6	19,351,729	C	T
UGA_6_19721308	6	19,721,308	G	T
UGA_6_20146578	6	20,146,578	T	C
UGA_6_20497978	6	20,497,978	T	C
UGA_6_21481686	6	21,481,686	A	G
UGA_6_21750508	6	21,750,508	T	G
UGA_6_21812234	6	21,812,234	A	T
UGA_6_21937598	6	21,937,598	A	C
UGA_6_22140487	6	22,140,487	C	T
UGA_6_22140511	6	22,140,511	A	C
UGA_6_22330953	6	22,330,953	C	T
UGA_6_22337751	6	22,337,751	A	G
UGA_6_22522417	6	22,522,417	G	C
UGA_6_22522436	6	22,522,436	A	G
UGA_6_22522437	6	22,522,437	A	C
UGA_6_23142087	6	23,142,087	A	C
UGA_6_23251106	6	23,251,106	A	G
UGA_6_23762272	6	23,762,272	T	C
UGA_6_24074154	6	24,074,154	G	A
UGA_6_24515647	6	24,515,647	A	T
UGA_6_24831629	6	24,831,629	T	C
UGA_6_25139264	6	25,139,264	T	G
UGA_6_25431606	6	25,431,606	G	A
UGA_6_26099428	6	26,099,428	T	A
UGA_6_26223151	6	26,223,151	T	G
UGA_6_26915258	6	26,915,258	A	C
UGA_7_481894	7	481,894	A	T
UGA_7_1570653	7	1,570,653	A	G
UGA_7_2003224	7	2,003,224	C	T
UGA_7_2559264	7	2,559,264	A	T
UGA_7_3106439	7	3,106,439	C	T
UGA_7_3176660	7	3,176,660	G	C
UGA_7_3617046	7	3,617,046	A	G
UGA_7_3807603	7	3,807,603	G	A
UGA_7_4196459	7	4,196,459	C	T
UGA_7_4734046	7	4,734,046	A	T
UGA_7_5256591	7	5,256,591	G	A
UGA_7_5476676	7	5,476,676	A	T
UGA_7_5476677	7	5,476,677	A	C

UGA_7_5476678	7	5,476,678	G	T
UGA_7_6301459	7	6,301,459	C	T
UGA_7_6301544	7	6,301,544	G	A
UGA_7_6534957	7	6,534,957	C	T
UGA_7_6534958	7	6,534,958	A	G
UGA_7_7572115	7	7,572,115	A	C
UGA_7_7631629	7	7,631,629	A	C
UGA_7_8306351	7	8,306,351	C	A
UGA_7_8306438	7	8,306,438	G	A
UGA_7_8348614	7	8,348,614	G	C
UGA_7_9214976	7	9,214,976	C	A
UGA_7_9214977	7	9,214,977	T	G
UGA_7_9214978	7	9,214,978	T	A
UGA_7_9763127	7	9,763,127	T	C
UGA_7_10829760	7	10,829,760	C	G
UGA_7_10836614	7	10,836,614	C	G
UGA_7_10836616	7	10,836,616	C	T
UGA_7_10836618	7	10,836,618	A	G
UGA_7_11271883	7	11,271,883	G	A
UGA_7_11816967	7	11,816,967	T	A
UGA_7_12008676	7	12,008,676	A	G
UGA_7_12008692	7	12,008,692	T	C
UGA_7_12008710	7	12,008,710	A	C
UGA_7_12119480	7	12,119,480	A	G
UGA_7_12119488	7	12,119,488	T	G
UGA_7_12119492	7	12,119,492	C	T
UGA_7_12194608	7	12,194,608	G	A
UGA_7_12194631	7	12,194,631	A	G
UGA_7_12194633	7	12,194,633	T	C
UGA_7_13896665	7	13,896,665	T	A
UGA_7_13904107	7	13,904,107	A	T
UGA_7_13913764	7	13,913,764	T	C
UGA_7_13966179	7	13,966,179	C	T
UGA_7_13966203	7	13,966,203	C	G
UGA_7_13966211	7	13,966,211	A	T
UGA_7_13986011	7	13,986,011	A	G
UGA_7_14448434	7	14,448,434	A	G
UGA_7_14448472	7	14,448,472	C	G
UGA_7_14448475	7	14,448,475	T	C
UGA_7_14636760	7	14,636,760	G	A
UGA_7_14636792	7	14,636,792	A	G

UGA_7_15025127	7	15,025,127	A	G
UGA_7_15033642	7	15,033,642	A	G
UGA_7_15033670	7	15,033,670	A	G
UGA_7_15033690	7	15,033,690	T	C
UGA_7_15481517	7	15,481,517	T	C
UGA_7_15481526	7	15,481,526	A	G
UGA_7_15481554	7	15,481,554	C	A
UGA_7_15481558	7	15,481,558	G	A
UGA_7_15481560	7	15,481,560	G	C
UGA_7_16254142	7	16,254,142	G	C
UGA_7_16690894	7	16,690,894	A	T
UGA_7_16734210	7	16,734,210	T	A
UGA_7_16887444	7	16,887,444	C	G
UGA_7_17003091	7	17,003,091	G	A
UGA_7_17003096	7	17,003,096	G	A
UGA_7_17003135	7	17,003,135	T	C
UGA_7_17156928	7	17,156,928	G	A
UGA_7_17156970	7	17,156,970	T	C
UGA_7_19260244	7	19,260,244	G	A
UGA_7_19260248	7	19,260,248	C	T
UGA_7_19572166	7	19,572,166	A	T
UGA_7_19867848	7	19,867,848	A	C
UGA_7_19922316	7	19,922,316	G	A
UGA_7_20866041	7	20,866,041	C	G
UGA_7_21023155	7	21,023,155	A	G
UGA_7_21942891	7	21,942,891	G	T
UGA_7_23778207	7	23,778,207	A	C
UGA_7_23918904	7	23,918,904	T	C
UGA_7_23918938	7	23,918,938	C	T
UGA_7_23920429	7	23,920,429	G	A
UGA_7_24264994	7	24,264,994	A	C
UGA_7_24265021	7	24,265,021	T	G
UGA_7_24939141	7	24,939,141	G	A
UGA_7_24939142	7	24,939,142	G	A
UGA_7_24939153	7	24,939,153	A	T
UGA_7_25376252	7	25,376,252	G	A
UGA_7_26324593	7	26,324,593	A	G
UGA_7_28393915	7	28,393,915	T	A
UGA_7_29984788	7	29,984,788	A	T
UGA_7_29984796	7	29,984,796	A	G
UGA_7_29985012	7	29,985,012	A	T

UGA_7_29993823	7	29,993,823	G	A
UGA_7_30429492	7	30,429,492	A	G
UGA_7_30429554	7	30,429,554	A	T
UGA_7_30429555	7	30,429,555	T	A
UGA_7_30429609	7	30,429,609	G	A
UGA_7_30684015	7	30,684,015	C	T
UGA_7_31316683	7	31,316,683	C	T
UGA_8_38150	8	38,150	C	T
UGA_8_1884293	8	1,884,293	C	T
UGA_8_5054181	8	5,054,181	T	A
UGA_8_5054195	8	5,054,195	C	T
UGA_8_5054213	8	5,054,213	T	C
UGA_8_5101742	8	5,101,742	A	G
UGA_8_5215319	8	5,215,319	T	C
UGA_8_5322134	8	5,322,134	G	A
UGA_8_5322182	8	5,322,182	A	G
UGA_8_5322183	8	5,322,183	C	T
UGA_8_5436168	8	5,436,168	A	G
UGA_8_5463398	8	5,463,398	T	C
UGA_8_5817803	8	5,817,803	A	G
UGA_8_5817820	8	5,817,820	T	C
UGA_8_6906044	8	6,906,044	A	G
UGA_8_6906061	8	6,906,061	A	G
UGA_8_6906072	8	6,906,072	C	T
UGA_8_8663629	8	8,663,629	T	C
UGA_8_8987558	8	8,987,558	C	T
UGA_8_9003677	8	9,003,677	C	T
UGA_8_9871935	8	9,871,935	T	G
UGA_8_10325717	8	10,325,717	A	T
UGA_8_10325722	8	10,325,722	G	T
UGA_8_10325724	8	10,325,724	T	A
UGA_8_10522569	8	10,522,569	T	G
UGA_8_11438089	8	11,438,089	A	G
UGA_8_11438126	8	11,438,126	A	T
UGA_8_11438142	8	11,438,142	A	C
UGA_8_11438145	8	11,438,145	T	C
UGA_8_11438153	8	11,438,153	G	A
UGA_8_11590288	8	11,590,288	C	T
UGA_8_11799620	8	11,799,620	T	C
UGA_8_11799633	8	11,799,633	C	T
UGA_8_12059415	8	12,059,415	G	A

UGA_8_13675659	8	13,675,659	T	C
UGA_8_15430773	8	15,430,773	A	C
UGA_8_17119520	8	17,119,520	G	A
UGA_8_17220893	8	17,220,893	T	C
UGA_8_17863667	8	17,863,667	A	T
UGA_8_17863668	8	17,863,668	A	T
UGA_8_17886417	8	17,886,417	C	T
UGA_8_18332327	8	18,332,327	T	C
UGA_8_18747697	8	18,747,697	T	G
UGA_8_18771114	8	18,771,114	T	C
UGA_8_19431830	8	19,431,830	T	C
UGA_8_19443154	8	19,443,154	G	T
UGA_8_20161078	8	20,161,078	G	T
UGA_8_20595378	8	20,595,378	C	G
UGA_8_20595379	8	20,595,379	T	C
UGA_8_20873700	8	20,873,700	T	A
UGA_8_21367348	8	21,367,348	A	C
UGA_8_21367349	8	21,367,349	G	T
UGA_8_21367350	8	21,367,350	A	T
UGA_8_21522766	8	21,522,766	A	G
UGA_8_21637156	8	21,637,156	G	A
UGA_8_21660128	8	21,660,128	C	T
UGA_8_21937136	8	21,937,136	G	C
UGA_8_22288415	8	22,288,415	C	T
UGA_8_22461449	8	22,461,449	T	A
UGA_8_22586957	8	22,586,957	A	G
UGA_8_22801831	8	22,801,831	G	A
UGA_8_23130260	8	23,130,260	G	A
UGA_8_23218785	8	23,218,785	A	C
UGA_8_23239524	8	23,239,524	T	C
UGA_8_23667506	8	23,667,506	C	T
UGA_8_23671265	8	23,671,265	T	C
UGA_8_24025007	8	24,025,007	G	A
UGA_8_24025514	8	24,025,514	G	T
UGA_8_24028066	8	24,028,066	C	T
UGA_8_24369052	8	24,369,052	T	A
UGA_8_24450199	8	24,450,199	C	G
UGA_8_24453980	8	24,453,980	C	T
UGA_8_24761107	8	24,761,107	A	G
UGA_8_26144430	8	26,144,430	T	A
UGA_9_70827	9	70,827	T	G

UGA_9_85137	9	85,137	A	T
UGA_9_1211013	9	1,211,013	C	T
UGA_9_1249584	9	1,249,584	C	T
UGA_9_1279725	9	1,279,725	A	G
UGA_9_1507163	9	1,507,163	T	G
UGA_9_1507185	9	1,507,185	G	A
UGA_9_1542925	9	1,542,925	G	A
UGA_9_1635753	9	1,635,753	G	C
UGA_9_1640886	9	1,640,886	C	T
UGA_9_1643601	9	1,643,601	T	G
UGA_9_1658340	9	1,658,340	C	A
UGA_9_1983183	9	1,983,183	G	C
UGA_9_2480708	9	2,480,708	A	T
UGA_9_2753455	9	2,753,455	T	C
UGA_9_3457311	9	3,457,311	G	A
UGA_9_3579501	9	3,579,501	C	T
UGA_9_3640349	9	3,640,349	G	T
UGA_9_3640350	9	3,640,350	A	C
UGA_9_3650790	9	3,650,790	T	C
UGA_9_3922053	9	3,922,053	G	C
UGA_9_3922056	9	3,922,056	C	T
UGA_9_4284995	9	4,284,995	A	T
UGA_9_4285490	9	4,285,490	C	A
UGA_9_4303885	9	4,303,885	T	C
UGA_9_4303886	9	4,303,886	T	C
UGA_9_4307349	9	4,307,349	T	C
UGA_9_4909753	9	4,909,753	G	A
UGA_9_6254582	9	6,254,582	A	G
UGA_9_6254585	9	6,254,585	G	A
UGA_9_6254623	9	6,254,623	C	T
UGA_9_6519900	9	6,519,900	G	A
UGA_9_6524434	9	6,524,434	C	T
UGA_9_7238964	9	7,238,964	G	C
UGA_9_7240788	9	7,240,788	C	T
UGA_9_7690621	9	7,690,621	C	T
UGA_9_8347943	9	8,347,943	T	G
UGA_9_8777330	9	8,777,330	G	T
UGA_9_9489788	9	9,489,788	A	G
UGA_9_9529988	9	9,529,988	G	A
UGA_9_9826519	9	9,826,519	C	A
UGA_9_9915218	9	9,915,218	C	G

UGA_9_10093895	9	10,093,895	T	A
UGA_9_11401665	9	11,401,665	G	A
UGA_9_11401679	9	11,401,679	A	T
UGA_9_11401691	9	11,401,691	G	A
UGA_9_13679863	9	13,679,863	G	A
UGA_9_13845093	9	13,845,093	C	T
UGA_9_13858596	9	13,858,596	G	A
UGA_9_13904119	9	13,904,119	A	C
UGA_9_14026768	9	14,026,768	G	A
UGA_9_14480768	9	14,480,768	A	T
UGA_9_14533855	9	14,533,855	G	T
UGA_9_14533881	9	14,533,881	A	G
UGA_9_14533891	9	14,533,891	A	G
UGA_9_15388128	9	15,388,128	G	A
UGA_9_15388169	9	15,388,169	A	G
UGA_9_15388174	9	15,388,174	A	C
UGA_9_15388180	9	15,388,180	A	G
UGA_9_16374617	9	16,374,617	C	A
UGA_9_17395891	9	17,395,891	C	T
UGA_9_18175123	9	18,175,123	G	T
UGA_9_18258426	9	18,258,426	C	T
UGA_9_18349740	9	18,349,740	T	C
UGA_9_18363760	9	18,363,760	G	A
UGA_9_18595863	9	18,595,863	T	C
UGA_9_18603294	9	18,603,294	C	T
UGA_9_19523520	9	19,523,520	C	T
UGA_9_19523549	9	19,523,549	A	T
UGA_9_19603336	9	19,603,336	T	C
UGA_9_19603363	9	19,603,363	A	G
UGA_9_19603366	9	19,603,366	T	C
UGA_9_20100254	9	20,100,254	C	T
UGA_9_20100255	9	20,100,255	G	A
UGA_9_21032089	9	21,032,089	C	T
UGA_9_21204087	9	21,204,087	C	T
UGA_9_21806848	9	21,806,848	G	A
UGA_9_21831224	9	21,831,224	A	G
UGA_9_21880460	9	21,880,460	C	T
UGA_9_22006433	9	22,006,433	A	G
UGA_9_22169258	9	22,169,258	A	G
UGA_9_22223696	9	22,223,696	A	C
UGA_9_22439130	9	22,439,130	G	A

UGA_9_23329818	9	23,329,818	A	G
UGA_9_23683999	9	23,683,999	T	C
UGA_9_23688429	9	23,688,429	T	C
UGA_9_23729657	9	23,729,657	C	G
UGA_9_23766259	9	23,766,259	T	C
UGA_9_24036516	9	24,036,516	C	A
UGA_9_24263131	9	24,263,131	T	C
UGA_9_24263386	9	24,263,386	G	A
UGA_9_27500923	9	27,500,923	A	G
UGA_9_27552536	9	27,552,536	A	T
UGA_9_27552593	9	27,552,593	A	G
UGA_9_27621525	9	27,621,525	A	G
UGA_9_27622062	9	27,622,062	C	G
UGA_9_27629021	9	27,629,021	G	A
UGA_9_27773179	9	27,773,179	C	A
UGA_9_27773334	9	27,773,334	T	C
UGA_9_27854927	9	27,854,927	A	G
UGA_9_27857522	9	27,857,522	T	C
UGA_9_28711940	9	28,711,940	T	G
UGA_9_28756129	9	28,756,129	G	T
UGA_9_28756134	9	28,756,134	C	T
UGA_9_28756170	9	28,756,170	C	T
UGA_9_28756174	9	28,756,174	C	T
UGA_9_28756179	9	28,756,179	C	T
UGA_9_29103234	9	29,103,234	T	G
UGA_9_29103235	9	29,103,235	T	G
UGA_9_29103262	9	29,103,262	G	T
UGA_9_29280515	9	29,280,515	G	T
UGA_9_29280546	9	29,280,546	C	A
UGA_9_29702528	9	29,702,528	G	A
UGA_9_29718594	9	29,718,594	G	C
UGA_9_29801078	9	29,801,078	T	G
UGA_9_29801079	9	29,801,079	C	T
UGA_9_29801080	9	29,801,080	T	G
UGA_9_29916457	9	29,916,457	T	C
UGA_9_30996552	9	30,996,552	C	T
UGA_9_30996567	9	30,996,567	T	C
UGA_9_31052342	9	31,052,342	G	C
UGA_9_31073332	9	31,073,332	T	G
UGA_9_31078431	9	31,078,431	G	A
UGA_9_31078446	9	31,078,446	A	G

UGA_9_31374085	9	31,374,085	T	C
UGA_9_31374136	9	31,374,136	G	T
UGA_9_31421577	9	31,421,577	A	C
UGA_9_31466282	9	31,466,282	A	T
UGA_9_32547391	9	32,547,391	T	C
UGA_9_33263845	9	33,263,845	C	T
UGA_9_33725783	9	33,725,783	A	G
UGA_9_33965739	9	33,965,739	T	C
UGA_9_34388036	9	34,388,036	A	T
UGA_9_34417192	9	34,417,192	T	C
UGA_9_34461746	9	34,461,746	T	G
UGA_9_34719800	9	34,719,800	T	C
UGA_9_34824711	9	34,824,711	T	C
UGA_9_34888914	9	34,888,914	T	G
UGA_10_28291	10	28,291	G	A
UGA_10_28303	10	28,303	T	A
UGA_10_28304	10	28,304	C	A
UGA_10_381513	10	381,513	G	A
UGA_10_1135525	10	1,135,525	C	T
UGA_10_1135721	10	1,135,721	A	G
UGA_10_1314394	10	1,314,394	A	G
UGA_10_2392615	10	2,392,615	G	A
UGA_10_2438470	10	2,438,470	A	G
UGA_10_3757683	10	3,757,683	C	T
UGA_10_3766549	10	3,766,549	T	C
UGA_10_3778693	10	3,778,693	T	C
UGA_10_3778694	10	3,778,694	G	A
UGA_10_3778698	10	3,778,698	G	A
UGA_10_3785012	10	3,785,012	G	C
UGA_10_3785032	10	3,785,032	A	G
UGA_10_3800828	10	3,800,828	C	A
UGA_10_3814552	10	3,814,552	T	C
UGA_10_3850030	10	3,850,030	A	G
UGA_10_3850057	10	3,850,057	T	C
UGA_10_3917400	10	3,917,400	A	G
UGA_10_3928526	10	3,928,526	A	G
UGA_10_3941814	10	3,941,814	C	T
UGA_10_3989700	10	3,989,700	T	G
UGA_10_3991841	10	3,991,841	C	T
UGA_10_4054513	10	4,054,513	C	T
UGA_10_4054608	10	4,054,608	T	G

UGA_10_4054631	10	4,054,631	T	C
UGA_10_4054635	10	4,054,635	A	G
UGA_10_4788250	10	4,788,250	G	C
UGA_10_4825274	10	4,825,274	A	G
UGA_10_4883768	10	4,883,768	A	G
UGA_10_4883993	10	4,883,993	C	T
UGA_10_4906788	10	4,906,788	A	C
UGA_10_4906821	10	4,906,821	G	A
UGA_10_4929305	10	4,929,305	T	C
UGA_10_4947283	10	4,947,283	T	C
UGA_10_4959212	10	4,959,212	G	T
UGA_10_4959228	10	4,959,228	C	T
UGA_10_4965559	10	4,965,559	T	C
UGA_10_4966037	10	4,966,037	A	G
UGA_10_4966106	10	4,966,106	T	C
UGA_10_5014652	10	5,014,652	T	A
UGA_10_5063143	10	5,063,143	A	T
UGA_10_5063900	10	5,063,900	A	C
UGA_10_5119089	10	5,119,089	C	A
UGA_10_5123716	10	5,123,716	A	T
UGA_10_5199311	10	5,199,311	A	C
UGA_10_5199554	10	5,199,554	G	A
UGA_10_5211267	10	5,211,267	C	T
UGA_10_5719852	10	5,719,852	A	G
UGA_10_6117027	10	6,117,027	G	A
UGA_10_7915211	10	7,915,211	T	C
UGA_10_8803481	10	8,803,481	C	T
UGA_10_8803527	10	8,803,527	G	A
UGA_10_8803534	10	8,803,534	A	C
UGA_10_9086615	10	9,086,615	A	G
UGA_10_9086616	10	9,086,616	G	A
UGA_10_9099182	10	9,099,182	G	A
UGA_10_9159583	10	9,159,583	G	T
UGA_10_9159595	10	9,159,595	A	G
UGA_10_9295439	10	9,295,439	G	C
UGA_10_9400612	10	9,400,612	G	C
UGA_10_9400619	10	9,400,619	T	C
UGA_10_9400659	10	9,400,659	C	T
UGA_10_9409003	10	9,409,003	C	T
UGA_10_9521972	10	9,521,972	G	A
UGA_10_9737233	10	9,737,233	A	G

UGA_10_10581880	10	10,581,880	T	C
UGA_10_10581882	10	10,581,882	A	G
UGA_10_10581948	10	10,581,948	T	C
UGA_10_10581998	10	10,581,998	T	C
UGA_10_10963474	10	10,963,474	A	T
UGA_10_11153135	10	11,153,135	G	C
UGA_10_11297210	10	11,297,210	G	A
UGA_10_11741624	10	11,741,624	G	A
UGA_10_11817033	10	11,817,033	T	C
UGA_10_11920825	10	11,920,825	G	A
UGA_10_12129449	10	12,129,449	A	C
UGA_10_12152388	10	12,152,388	A	G
UGA_10_12978077	10	12,978,077	T	C
UGA_10_13739271	10	13,739,271	G	C
UGA_10_14448920	10	14,448,920	C	G
UGA_10_14462467	10	14,462,467	A	G
UGA_10_14495142	10	14,495,142	W	T
UGA_10_14499263	10	14,499,263	T	C
UGA_10_14528759	10	14,528,759	G	T
UGA_10_16032554	10	16,032,554	A	T
UGA_10_17536521	10	17,536,521	C	T
UGA_10_17693492	10	17,693,492	C	T
UGA_10_17735742	10	17,735,742	C	G
UGA_10_17790322	10	17,790,322	A	C
UGA_10_17834560	10	17,834,560	C	A
UGA_10_17834921	10	17,834,921	A	G
UGA_10_17953611	10	17,953,611	A	T
UGA_10_18227327	10	18,227,327	A	G
UGA_10_20726600	10	20,726,600	C	T
UGA_10_21709234	10	21,709,234	T	G
UGA_10_22015320	10	22,015,320	T	C
UGA_10_22043961	10	22,043,961	G	T
UGA_10_22209301	10	22,209,301	T	A
UGA_10_22412670	10	22,412,670	A	G
UGA_10_22440884	10	22,440,884	T	C
UGA_10_24093177	10	24,093,177	G	T
UGA_10_24872466	10	24,872,466	C	T
UGA_10_24873391	10	24,873,391	T	G
UGA_10_25598481	10	25,598,481	G	A
UGA_10_25625308	10	25,625,308	A	G
UGA_10_25808038	10	25,808,038	T	A

UGA_10_25816979	10	25,816,979	A	G
UGA_10_25831222	10	25,831,222	T	A
UGA_10_25877864	10	25,877,864	A	G
UGA_10_26077399	10	26,077,399	C	A
UGA_10_26185125	10	26,185,125	T	A
UGA_10_27300757	10	27,300,757	C	T
UGA_10_27300760	10	27,300,760	A	C
UGA_11_252194	11	252,194	A	T
UGA_11_252195	11	252,195	C	T
UGA_11_1007083	11	1,007,083	T	C
UGA_11_1011759	11	1,011,759	G	A
UGA_11_1081028	11	1,081,028	C	T
UGA_11_1844967	11	1,844,967	C	A
UGA_11_1844970	11	1,844,970	A	G
UGA_11_2201451	11	2,201,451	A	G
UGA_11_3422525	11	3,422,525	T	G
UGA_11_3805788	11	3,805,788	A	G
UGA_11_4117999	11	4,117,999	G	A
UGA_11_5966967	11	5,966,967	C	A
UGA_11_6393710	11	6,393,710	A	G
UGA_11_7224974	11	7,224,974	T	C
UGA_11_7224988	11	7,224,988	T	C
UGA_11_7402007	11	7,402,007	A	G
UGA_11_7402008	11	7,402,008	A	G
UGA_11_7895014	11	7,895,014	C	T
UGA_11_7895026	11	7,895,026	G	A
UGA_11_7895043	11	7,895,043	T	C
UGA_11_8121287	11	8,121,287	G	A
UGA_11_8121300	11	8,121,300	C	T
UGA_11_10215826	11	10,215,826	A	G
UGA_11_10215831	11	10,215,831	T	C
UGA_11_10215856	11	10,215,856	T	C
UGA_11_10363656	11	10,363,656	T	C
UGA_11_10493281	11	10,493,281	A	G
UGA_11_11238743	11	11,238,743	T	G
UGA_11_12575381	11	12,575,381	A	G
UGA_11_12605981	11	12,605,981	A	G
UGA_11_13331339	11	13,331,339	G	A
UGA_11_13472759	11	13,472,759	C	T
UGA_11_13557352	11	13,557,352	T	C
UGA_11_13557408	11	13,557,408	T	C

UGA_11_15587325	11	15,587,325	C	T
UGA_11_15673069	11	15,673,069	C	A
UGA_11_16345819	11	16,345,819	G	C
UGA_11_16464275	11	16,464,275	A	C
UGA_11_16464276	11	16,464,276	G	C
UGA_11_16464278	11	16,464,278	T	G
UGA_11_16464279	11	16,464,279	C	G
UGA_11_17375035	11	17,375,035	C	T
UGA_11_19026680	11	19,026,680	C	A
UGA_11_19467591	11	19,467,591	T	C
UGA_11_19635634	11	19,635,634	C	A
UGA_11_19667391	11	19,667,391	G	A
UGA_11_20242652	11	20,242,652	A	G
UGA_11_20312001	11	20,312,001	G	C
UGA_11_20463216	11	20,463,216	C	G
UGA_11_20567830	11	20,567,830	C	G
UGA_11_20660165	11	20,660,165	T	C
UGA_11_20670399	11	20,670,399	G	A
UGA_11_20692980	11	20,692,980	T	A
UGA_11_20720767	11	20,720,767	T	C
UGA_11_20721101	11	20,721,101	T	C
UGA_11_20865799	11	20,865,799	G	T
UGA_11_20925008	11	20,925,008	T	A
UGA_11_20947267	11	20,947,267	G	C
UGA_11_20960669	11	20,960,669	A	G
UGA_11_21180639	11	21,180,639	A	G
UGA_11_21180642	11	21,180,642	C	A
UGA_11_21333497	11	21,333,497	C	T
UGA_11_21333519	11	21,333,519	G	A
UGA_11_21610200	11	21,610,200	A	T
UGA_11_21779189	11	21,779,189	C	G
UGA_11_21938758	11	21,938,758	A	G
UGA_11_21983692	11	21,983,692	A	T
UGA_11_22613072	11	22,613,072	A	T
UGA_11_23337605	11	23,337,605	A	G
UGA_11_23375935	11	23,375,935	A	G
UGA_11_23496085	11	23,496,085	A	G
UGA_11_23610130	11	23,610,130	A	G
UGA_11_23771784	11	23,771,784	C	A
UGA_11_23771906	11	23,771,906	T	C
UGA_11_23821268	11	23,821,268	A	G

UGA_11_23867646	11	23,867,646	T	C
UGA_11_23875712	11	23,875,712	A	G
UGA_11_24208362	11	24,208,362	T	C
UGA_11_24209535	11	24,209,535	A	G
UGA_11_24672751	11	24,672,751	A	T
UGA_11_25850675	11	25,850,675	A	T
UGA_11_26729734	11	26,729,734	G	T

CHAPTER 7

GENETIC MAPPING OF QTL ASSOCIATED WITH RESISTANCE TO RACE 2 OF *FUSARIUM OXYSPORUM* F. SP. *NIVEUM* IN WATERMELON[1]

[1] Meru, G. and C. McGregor. To be submitted to J. Amer. Soc. Hort. Sci.

Abstract

Fusarium wilt of watermelon (*Citrullus lanatus* (Thunb.) Matsum. & Nakai caused by *Fusarium oxysporum* f. sp. *niveum* (*Fon*) [(E.F. Sm.) W.C. Snyder & H.N. Han.] is a devastating soil-borne disease limiting watermelon production across the world. Although many cultivars have been bred for resistance to *Fon* race 0 and 1, no edible watermelon cultivars resistant to *Fon* race 2 are currently available. Marker assisted selection (MAS) can be exploited as a tool in conventional breeding to facilitate the release of cultivars resistant to *Fon* race 2. However, to date no molecular markers linked to *Fon* race 2 resistance have been identified, thus impeding the application of MAS in improving resistance against the disease. In the current study, the genotyping by sequencing (GBS) platform was used to generate single nucleotide polymorphisms (SNPs) in an F_2 population (n = 178) developed from a cross between WS147 (resistant) and Charleston Gray (susceptible). An F_3 population was developed by selfing individual F_2 plants and phenotyped for resistance against *Fon* race 2. Five hundred and one single nucleotide polymorphisms (SNPs) were placed on the physical map and used in the mapping of quantitative trait loci (QTL). An intermediate QTL associated with resistance to *Fon* race 2 was identified on chromosome (chr.) 11 and accounted for 17% of the phenotypic variation observed in the population. This study is a first step towards using MAS in the improvement of resistance to *Fon* race 2 in watermelon.

Introduction

Watermelon (*Citrullus lanatus* (Thunb.) Matsum. & Nakai is a widely cultivated crop of the Cucurbitaceae family popular for its sweet flesh and edible seeds (Achigan-Dako et al., 2008; Baboli and Kordi, 2010; Edelstein and Nerson, 2002; Robinson and Decker-Walters, 1997). The intense selection for suitable traits in watermelon has led to low genetic diversity in the cultivated watermelon leading to narrow genetic diversity and consequently susceptibility to important pests and diseases (Guo et al., 2013; Hawkins et al., 2001; Levi et al., 2001). *Fusarium oxysporum* f. sp. *niveum* (*Fon*) (E.F. Sm.) W.C. Snyder & H.N. Han. is a soil-borne pathogen that causes vascular wilting in watermelon often resulting in yield reduction and crop failure in watermelon growing regions of the world (Boyhan et al., 2003; Egel and Martyn, 2007; Hawkins et al., 2001; Martyn and McLaughlin, 1983; Zhou et al., 2010). Four *Fon* races have been described (0, 1, 2 and 3) based on their aggressiveness or the ability to overcome specific resistance in a set of differential cultivars (Bruton, 1998; Egel and Martyn, 2007; Wehner, 2008; Zhou et al., 2010).

The ability of the pathogen's chlamydospores to persist in the soil for a long time combined with the rapid evolution of its races has rendered the management of fusarium wilt difficult (Bruton, 1998; Egel and Martyn, 2007; Lin et al., 2009; Martyn and Netzer, 1991; Yetisir et al., 2003). Currently employed management options for the disease include the use of resistant cultivars (Bruton, 1998), avoidance of infested fields, crop rotation, chemical and biological fumigation (Bruton, 1998; Egel and Martyn, 2007) and the use of resistant root-stocks (Kuniyasu, 1980).

The use of resistant cultivars is the most preferred management method for fusarium wilt (Bruton, 1998; Hopkins et al., 1992; Lin et al., 2009; Lin et al., 2010; Martyn and McLaughlin,

1983; Martyn and Netzer, 1991; Zhou and Everts, 2004). Breeding of cultivars resistant to *Fon* race 1 has been successful due to availability of well described resistant sources and the simplicity of the trait. *Fon* race 1 is controlled by a single dominant gene designated *Fo-1* with a few modifier genes (Guner and Wehner, 2004; Martyn and Netzer, 1991; Netzer and Weintall, 1980; Wehner, 2008; Zhang and Rhodes, 1993). In contrast, resistance to *Fon* race 2 designated *Fo- 2* in PI 296341-FR (the best described source) is thought to be polygenic and controlled by at least a pair of recessive genes in epistasis with other minor genes (Hawkins et al., 2001; Martyn and Netzer, 1991; Wehner, 2008; Zhang and Rhodes, 1993). To date only a few sources of resistance to *Fon* race 2 in *C. lanatus* var *citroides* have been described (Martyn and Netzer, 1991; Wechter et al., 2012). Due to linkage drag, attempts to incorporate *Fon* race 2 resistance from PI 296341-FR (var. *citroides*) into edible watermelon cultivars have resulted in resistant cultivars with poor edible qualities which have been used primarily as pollinizers (Boyd, 2011). Recently, several additional var. *citroides* accessions resistant to *Fon* race 2 have been identified (Wechter et al., 2012). However, the incorporation of the identified resistance into edible watermelon (var. *lanatus*) from var. *citroides* may also lead to cultivars with undesirable traits. It is highly desirable to identify a source of *Fon* race 2 resistance in var. *lanatus* accessions since it is likely that incorporation into edible watermelon cultivars would be easier.

Previous attempts to identify genomic regions associated with resistance to *Fon* race 2 from *citroides* have been unsuccessful. Hawkins et al. (2001) attempted to identify quantitative trait loci (QTL) associated with resistance to *Fon* race 2 using F_2 and F_3 populations generated from a cross between PI 296341-FR (resistant) and New Hampshire (susceptible). However, no useful linkages were found between the markers tested and resistance to *Fon* race 2. Harris et al. (2009) used degenerate primers to target R-genes that encode nucleotide binding site–leucine-

rich repeat (NBS-LRR) proteins in watermelon and identified three watermelon expressed sequence tags (EST) disease resistance homologs. However, none of these EST markers were linked to disease resistance to *Fon* race 2. The efforts to map QTL associated with resistance to *Fon* race 2 have utilized PI 296341-FR (var. *citroides*) as the resistant parent in a cross with a susceptible var. *lanatus* cultivar. This has led to high segregation distortion of marker alleles and resulted in genetic maps of low resolution (Levi et al., 2011) making identification of QTL difficult.

Using next-generation sequencing (NGS) technologies, plant breeders can study genotypic profiles of populations to identify variations associated with a trait of interest (Elshire et al., 2011; Lambel et al., 2014; Nimmakayala et al., 2014). Highly multiplexed NGS technologies allow pooling of barcoded samples into a single sequencing lane (Elshire et al., 2011) thus reducing the cost of genotyping per data point. Genotyping by sequencing (GBS) is a NGS technology that involves sequencing of reduced genomic libraries followed by alignment of reads for SNP discovery (Barba et al., 2013; Elshire et al., 2011). Recently, a GBS protocol for watermelon was optimized (Elshire et al., 2011; Lambel et al., 2014) and has been used in genetic mapping of resistance to *Fon* race 1 (Lambel et al., 2014) and diversity studies (Nimmakayala et al., 2014). The current study aimed at utilizing GBS for SNP discovery and identification of QTL associated with resistance to *Fon* race 2 in watermelon.

Materials and methods

Plant material and DNA extraction

A cross was made in the greenhouse between WS147 (resistant, Boyhan et al., 2003) and Charleston Gray (susceptible). A single F_1 plant was selfed to yield 178 F_2 plants that were further selfed to generate F_3 families. DNA was extracted from leaves of the parents, the F_1 and

each of the F_2 plants using the E.N.Z.A 96-well format kit (Omega Bio-tek, GA, USA) according to the manufacturer's instructions. The concentration and quality of the DNA was determined by absorbance measurements (Infinite M200 PRO, Tecan Group Ltd., ZH, CH) and by agarose gel electrophoresis. To ensure that the quality of the extracted DNA was sufficient for digestion with restriction enzymes, samples of the DNA were digested using EcoRI (New England Biolabs, MA, USA).

Genotyping by sequencing, SNP analysis and map construction

Genotyping of the parents, F_1 and F_2 was carried out using GBS (Elshire et al., 2011) at the Institute for Genomic Diversity (IGD), Cornell University. The DNA was digested using *ApeK*I restriction enzyme and fragment alignment and SNP calling performed using the GBS reference pipeline in TASSEL version 3.0.160. The generated SNP calls in HapMap format provided by IGD were filtered in Microsoft Office Excel for missing data (with up to 70% missing data), polymorphism between the mapping population parents and segregation distortion ($P<0.00001$). Based on this criterion, a physical map (Guo et al., 2013) comprising 501 SNPs was developed (Appendix 7.1) and visualized using MapChart 2.2 (Voorrips, 2002).

Fungal inoculum preparation

Fon race 2 [(04-04122), kindly provided by Bennie Bruton, United State Department of Agriculture- Agricultural Research Service], was grown (14h/10h dark cycle) on quarter-strength potato dextrose agar (Becton, Dickinson and Company, NJ, USA) for 12 days and 1 cm^2 agar plugs transferred into 250 mL erlenmeyer flasks containing 100 mL potato dextrose broth (Becton, Dickinson and Company). The fungal cultures were grown (14 h/10h dark cycles) on a Mini-Orbital shaker (Henry Troemner, NJ, USA) at 200 rpm for 10 days and the inoculum was filtered through four layers of sterile cheese cloth. The microconidial concentration was

determined using a hemacytometer (Hausser Scientific, PA, USA) and adjusted accordingly using sterile water.

Phenotyping

Disease screening experiments were performed using either a spore concentration of 1 x 10^{6} mL^{-1} (field and greenhouse experiment 1) or 5 x 10^{5} mL^{-1} (greenhouse experiments 2 and 3). Inoculation in the field was carried out at the University of Georgia Horticulture Research farm in Watkinsville, GA in Apr. 2013 by pouring 40 mL of inoculum (1 x 10^{6} mL^{-1}) onto 3-week old watermelon transplants consisting of Charleston Gray (susceptible parent), WS147 (resistant parent), the F_1 and F_3 lines, as well as Sugar Baby (susceptible control) and SP4, SP6, USLV 246, 252 and 335 (resistant controls). There were four transplants of each cultivar/line per replication with a total of three replications in a randomized complete block design. Sugar Baby transplants were used as un-inoculated controls. In the greenhouse, three disease screening experiments were carried out in Sep. 2013 (1 x 10^{6} mL^{-1}), and Apr. and July 2014 (5 x 10^{5} mL^{-1}). Watermelon seedlings (as described above) were sown in cells (5.98 x 3.68 x 4.69 cM) filled with steam-pasteurized sand:peat:vermiculite (4:1:1) amended with Osmocote 14N-4.2P-11.6K. At the first true leaf stage the cell trays with seedlings were immersed in spore suspension contained in plastic tubs for 15 minutes. For each disease screening experiment, four seeds of each cultivar/line were sown per replication with a total of three replications in a randomized complete block design. Controls were immersed into plastic tubs containing sterile water.

Plants in both the greenhouse and the field were evaluated for symptom severity on a scale of 0 to 5 with a score of 0 representing asymptomatic plants, a score of 1 for plants showing initial wilting on one leaf, a score of 2 for plants showing continued wilting in more than one leaf, a score of 3 for plants with all the leaves wilted, a score of 4 for plants with all

leaves wilted and stem collapsing, and a score of 5 for dead plants. Disease severity data was collected at 11, 14, 17, 20, 23, and 26 days after inoculation (DAI). Pearson correlations for DAI were calculated using JMP Version 9 (SAS Institute Inc., Cary, NC).

QTL detection

The detection of QTL was performed using composite interval mapping (CIM) with a 5-cM window in WinQTL Cartographer Version 2.5 (Wang et al., 2011) and a walk speed of 1 cM. The standard model (Model 6) was used for CIM analysis and the statistical significance of a QTL determined by likelihood-odds thresholds set by 1000 permutations ($\alpha = 0.05$) (Churchill and Doerge, 1994). The QTL was visualized using MapChart 2.2 (Voorrips, 2002).

Results and discussion

Genotyping by sequencing, SNP analysis and map construction

In this study, GBS analysis generated a total of 1,963,049 tags of which 1,604,031 (81.7%) aligned to unique positions, 85,763 (4.4%) aligned to multiple positions while 273,255 (13.9%) could not be aligned to the watermelon reference genome (Guo et al., 2013). The number of tags generated in this study with *ApeK*I restriction enzyme exceed those generated by Lambel et al. (2014) (527,844 tags) and Meru and McGregor (unpublished) (933,662 tags) with the same enzyme in F_2 populations made from elite x elite crosses. Analysis of the tags (1,604,031) aligning to unique positions revealed 23,286 HapMap SNP calls, a number similar to that (20,889) reported by Meru and McGregor (unpublished) in a F_2 population generated from a cross between Calhoun Gray x Sugar Baby. Of the 23,286 SNPs, 30.78% (7,165) were found to be polymorphic between WS147 and Charleston Gray. The number of polymorphic SNPs in this study is higher than that (28.66%; 5,986) found between Calhoun Gray and Sugar Baby (Meru and McGregor, unpublished). The difference in the number of polymorphic SNPs between the

two studies is expected because WS147 x Charleston Gray represents a wider cross than Calhoun Gray x Sugar Baby cross.

The 23,286 HapMap SNP calls were filtered according to missing data points, polymorphism between mapping population parents and segregation distortion. Polymorphic SNPs with up to 70% missing data and segregation distortion ($P<0.00001$) were allowed on the physical map to ensure a good coverage of the genome. As expected with GBS method, large amounts of missing data were observed due to low coverage of reads resulting from pooling of samples in a single sequencing lane (Barba et al., 2013). Following the described criterion, 501 SNPs were mapped on the physical map (Guo et al., 2013) (Appendix 7.1, Table 7.1 and Fig.7.1) and utilized in QTL mapping.

Phenotypic analysis

Following heavy rainfall, flooding occurred in the field during the first two weeks after inoculation. Wilting symptoms were observed in both the inoculated and non-inoculated transplants suggesting physiological effects of flooding. In the field experiment, the resistant parent (Fig. 7.2E) and two of the resistant controls showed a similar or higher level of disease severity (disease severity at 26 DAI for SP4 = 3.5 and SP6 = 2.2) when compared to the susceptible parent (Fig. 7.2E) and susceptible control (disease severity at 26 DAI for Sugar Baby = 2.3). However, three of the resistant controls (USLV 246, 252 and 335) did not show any symptoms at 26 DAI in the field (disease severity = 0). The correlations between data taken at different DAI were not significant among the various experiments but were significant within the experiments (Table 7.1).

Segregation for resistance to *Fon* race 2 was observed in all the three greenhouse experiments. In the first experiment (Sep. 2013) inoculated at a high inoculum concentration (1 x

10^{6} mL^{-1}), disease progressed rapidly and resulted in disease in most genotypes in the population (Fig. 7.2A) and resistant controls [disease severity at 26 DAI for SP4 = 2.6, SP6 = 4.8, USLV 246 = 4.8, USLV 252 = 3.6, and USLV 335 = 3.3)]. To ensure discrimination of resistant and susceptible genotypes, subsequent screening assays (Apr. and July 2014) were carried out at a reduced inoculum concentration (5 x 10^{5} mL^{-1}). As expected, reduction in the inoculum concentration lowered the disease severity in the population for the two experiments (Fig. 7.2C-D) and the resistant controls [disease severity for Apr. and July 2014 experiments at 26 DAI for SP4 (1.2 and 2.0), SP6 (2.4 and 2.6), USLV 246 (3.3 and 2.2), USLV 252 (1.8 and 2.3), and USLV 335 (2.2 and 2.8)]. The inoculum concentration of a pathogen is known to play an important role in disease epidemiology and has been shown to affect the severity symptoms of fusarium wilt in watermelon (Martyn and McLaughlin, 1983).

QTL detection

Genetic mapping revealed an intermediate QTL associated with resistance to *Fon* race 2 on chr. 11 (Table 7.3 and Fig. 7.3) of the watermelon draft genome sequence (Guo et al., 2013). This QTL (designated *Qfon11*) was detected in the three greenhouse experiments at different DAI, but not in the field experiment perhaps due to effects of flooding (Fig. 7.2E). For the experiment inoculated at a high inoculum concentration (Sep. 2013; 1 x 10^{6} mL^{-1}), the QTL was detected early (11 DAI, Fig. 7.2B) but not late in the experiment when most of the plants had succumbed to wilting. Conversely, for the two experiments inoculated at a low inoculum concentration (Apr. and July 2014; 5 x 10^{5} mL^{-1}) this QTL was detected later (23 and 26 DAI) in the experiment. In addition it was also possible to detect this QTL using the data averaged over the three experiments (Table 7.3 and Fig. 7.3). The phenotypic variation (R^2) explained by the identified QTL ranged from 9.39 to 16.89 (Table 7.2).

Three receptor-like kinase pathogen-related genes (*Cla016557*, *Cla016650* and *Cla016744*) (Guo et al., 2013) were found within the 1.5-LOD of the identified QTL (Fig. 7.3). Receptor-like kinase genes play an important role in pathogen defense by acting as sentinels in both broad-spectrum and race-specific plant responses (Goff and Ramonell, 2007). *Cla016557*, *Cla016650 and Cla016744* are candidate genes for investigation of the molecular mechanisms underlying resistance to *Fon* race 2 in watermelon.

The QTL identified in the current study accounts only for a small percentage of phenotypic variation observed in the population. This suggests that additional genetic factors not identified in the current study are involved in resistance against *Fon* race 2 in WS147 and that environmental effect could play a role in the expression of *Fon* race 2 resistance. Therefore further studies are necessary to identify additional genetic factors involved in resistance against *Fon* race 2 in WS147.

WS147 as a source of resistance to Fon race 2

Wild sources of ressitance to *Fon* race 2 such as PI 296341-FR (var. *citroides*) have many undesirable fruit quality traits. Therefore breeding for resistance with these accessions has been limited to inedible watermelon pollinizers. The resistant accession used in the current study (WS147; var. *lanatus*) has fruit qualities similar to those in edible watermelon including red flesh and sweet flesh (Brix value of >8%) and will povide a suitable source of resistance in breeding programs.

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Table 7.1 Pearson correlations for days after inoculation for experiment 1, 2, 3 and the field in the WS147 x Charleston Gray F_3 population.

Trait (Experiment)	11(1)	14(1)	17(1)	20(1)	23(1)	26(1)	11(2)	14(2)	17(2)	20(2)	23(2)	26(2)	11(3)	14(3)	17(3)	20(3)	23(3)	26(3)	11(field)	14(field)	17(field)	20(field)	23(field)	26(field)
11(1)	1.00																							
14(1)	0.83	1.00																						
17(1)	0.76	0.96	1.00																					
20(1)	0.69	0.87	0.93	1.00																				
23(1)	0.64	0.82	0.88	0.96	1.00																			
26(1)	0.56	0.75	0.81	0.91	0.95	1.00																		
11(2)	-0.08	-0.09	-0.06	-0.03	-0.05	-0.04	1.00																	
14(2)	0.10	0.12	0.11	0.11	0.12	0.10	0.27	1.00																
17(2)	0.05	0.03	0.04	0.07	0.01	0.01	0.60	0.28	1.00															
20(2)	0.04	0.05	0.07	0.10	0.05	0.04	0.63	0.27	0.91	1.00														
23(2)	0.05	0.01	0.01	0.04	0.01	-0.02	0.42	0.25	0.69	0.77	1.00													
26(2)	0.03	0.00	0.00	0.04	0.01	-0.01	0.43	0.26	0.69	0.79	0.98	1.00												
11(3)	0.18	0.15	0.11	0.07	0.08	0.03	-0.09	0.00	0.01	0.02	0.08	0.07	1.00											
14(3)	0.21	0.21	0.17	0.17	0.17	0.13	-0.11	-0.02	0.00	0.01	0.09	0.08	0.84	1.00										
17(3)	0.32	0.34	0.32	0.33	0.33	0.31	0.01	0.13	0.14	0.13	0.16	0.16	0.53	0.71	1.00									
20(3)	0.34	0.37	0.35	0.37	0.36	0.35	0.02	0.14	0.16	0.14	0.18	0.17	0.49	0.68	0.99	1.00								
23(3)	0.35	0.39	0.37	0.38	0.38	0.36	0.03	0.14	0.17	0.15	0.18	0.18	0.47	0.65	0.97	0.99	1.00							
26(3)	0.36	0.40	0.38	0.39	0.39	0.37	0.04	0.14	0.18	0.16	0.19	0.18	0.45	0.63	0.95	0.99	1.00	1.00						
11(field)	0.07	0.07	0.05	0.04	0.08	0.08	0.03	0.21	0.07	0.05	0.01	0.05	-0.08	0.01	0.09	0.10	0.09	0.09	1.00					
14(field)	0.05	0.03	0.03	0.03	0.06	0.07	-0.01	0.19	0.07	0.04	0.01	0.04	0.00	0.05	0.08	0.08	0.07	0.05	0.94	1.00				
17(field)	-0.01	-0.02	-0.01	0.01	0.05	0.06	0.03	0.20	0.09	0.05	0.02	0.04	0.03	0.09	0.11	0.11	0.09	0.08	0.93	0.98	1.00			
20(field)	-0.03	-0.04	-0.03	0.00	0.05	0.07	0.02	0.19	0.06	0.03	-0.01	0.02	0.05	0.10	0.11	0.11	0.10	0.09	0.90	0.96	0.99	1.00		
23(field)	-0.03	-0.02	-0.01	0.02	0.07	0.08	0.00	0.18	0.03	0.03	0.00	0.03	0.01	0.07	0.11	0.12	0.11	0.11	0.88	0.92	0.95	0.96	1.00	
26(field)	-0.03	-0.02	-0.01	0.03	0.07	0.09	-0.01	0.18	0.02	0.02	-0.01	0.02	0.00	0.07	0.11	0.11	0.11	0.10	0.87	0.92	0.95	0.96	1.00	1.00

Table 7.2 The length (Mb) of each chromosome and the corresponding number of single nucleotide polymorphisms (SNPs) identified on the physical map in the F_2 population developed from a cross between Charleston Gray (CG) and WS147.

Chromosome	Physical map	
	Number of markers	Length (Mb)
1	40	34.05
2	43	30.55
3	27	27.93
4	23	23.66
5	59	33.31
6	64	26.94
7	51	29.57
8	45	25.09
9	58	34.89
10	44	28.15
11	47	26.77
Total	501	320.98

Table 7.3 Position (Mb) of the QTL associated with resistance to *Fon* race 2 on chromosome 11 and the corresponding 1-likelihood-odds (LOD) support interval for experiment 1 [11 days after inoculation (DAI)], experiment 2 (26 DAI), experiment 3 (26 DAI), and their mean [(experiment 1 (11DAI), experiment 2 (26 DAI), and experiment 3 (26 DAI)] in the Charleston Gray x WS147 F_3 population.

Trait	Exp[z]	Position (Mb)[y]	LOD[x]	R^2 (%)[w]	Additive effect[v]	Dominance effect[v]	LOD-1 support interval (Mb)	LOD-1 support interval (Mb)
11 DAI	1	22.88	5.57	9.39	-0.23	-0.06	22.7	24.8
26 DAI	2	22.77	7.59	12.33	-0.24	0.10	22.5	22.8
26 DAI	3	22.82	4.91	9.89	-0.34	0.39	22.6	23.9
Mean	Mean	22.77	10.81	16.84	-0.29	0.09	22.5	23.0

[z] Experiment in which data for QTL mapping was derived.

[y] The position of the QTL on the chromosome (Guo et al., 2013; Ren et al., 2012).

[x] Log_{10} likelihood ratio.

[w] Phenotypic variation explained.

[v] Negative values indicate that the effect is contributed by the allele from the resistant parent (WS147).

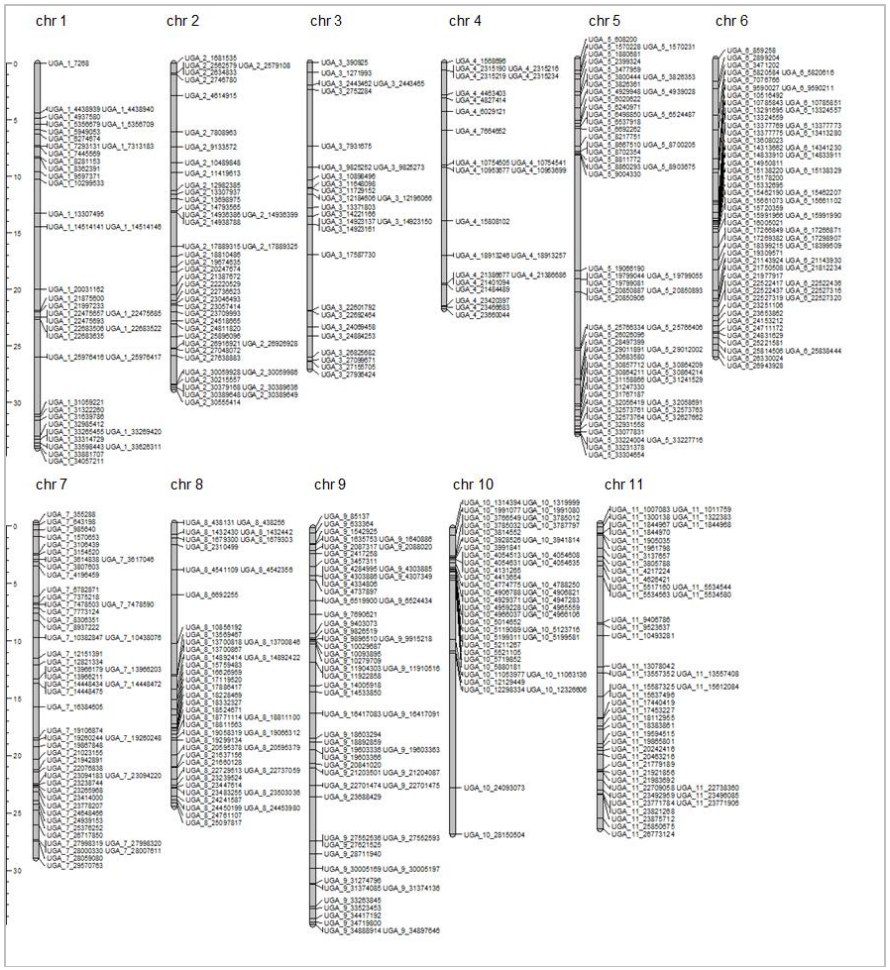
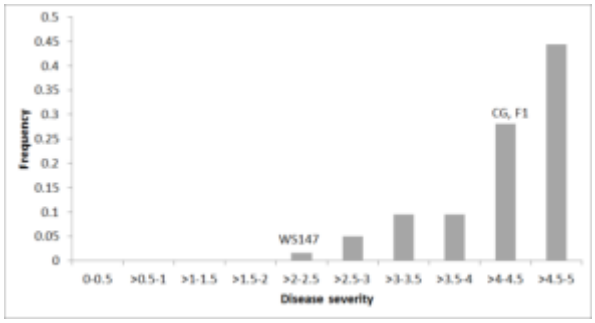
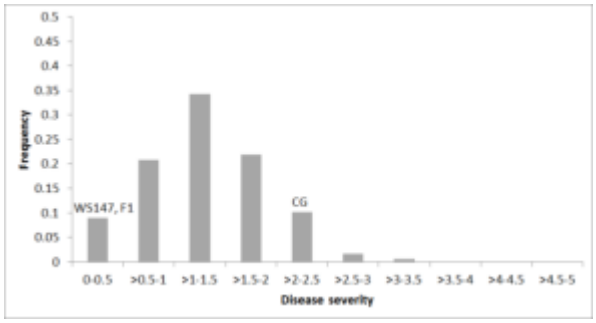


Fig. 7.1 The physical map developed from 501 single nucleotide polymorphisms (SNPs) in the Charleston Gray (CG) and WS147 F_2 population. Figure generated using MapChart Version 2.2 (Voorrips, 2002).

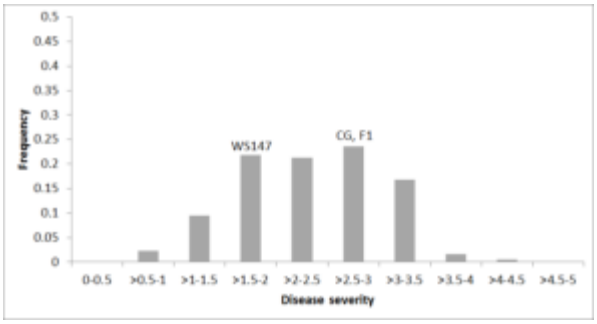
A



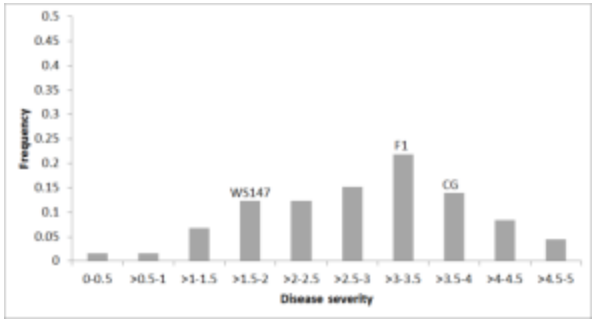
B



C



D



E

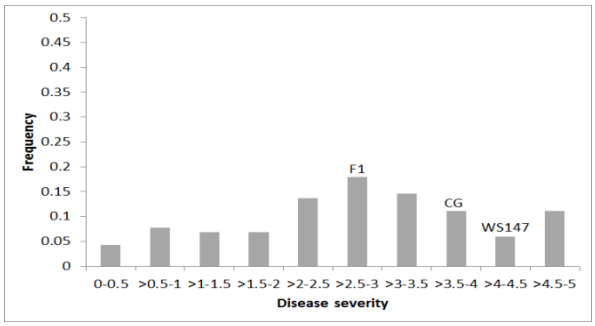


Fig. 7.2 Frequency distribution for disease severity for (A) experiment 1 (26 DAI) (B) experiment 1 (11 DAI), (C) experiment 2 (26 DAI), (D) experiment 3 (26 DAI), and (E) field experiment (26DAI) in the Charleston Gray (CG) x WS147 F_3 population.

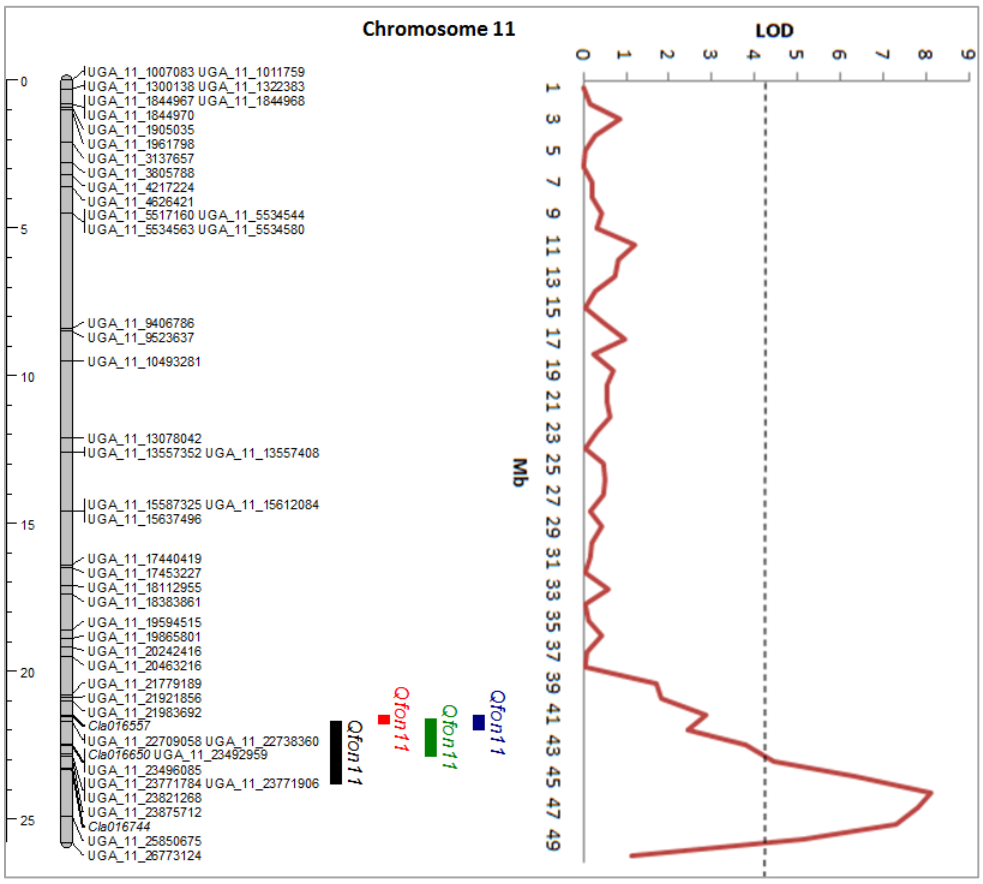


Fig. 7.3 Quantitative trait loci associated with resistance to *Fon* race 2 on chromosome 11 [length of the bar is equal to 1-LOD support interval] identified by composite interval mapping in the Charleston Gray x WS147 F_3 population. The black, red, green and blue bars correspond to the QTL identified in experiment 1 (11 DAI), experiment 2 (26 DAI), experiment 3 (26 DAI), and the mean DAI [(experiment 1 (11DAI), experiment 2 (26 DAI), and experiment 3 (26 DAI)]. The three receptor-like kinase genes (*Cla016557, Cla016650* and *Cla016744*) located within the 1.5-LOD support interval of the identified QTL are italicized. The Log_{10} likelihood ratio (LOD) values for the QTL (mean for experiment 1, 2, 3) are shown next to the QTL. Cutoff value for declaring significance (LOD = 4.1) is indicated by the dotted line. Figure generated using MapChart Version 2.2 (Voorrips, 2002) and Microsoft Office Excel.

Appendix 7.1 The name, chromosomal position (Guo et al., 2013) and genotype of Charleston Gray (CG) and WS147 for the 501 single nucleotide polymorphisms discovered by genotyping by sequencing in the CG x WS147 F_2 population.

Locus name	Chromosome	Position (bp)	Genotype	
			CG	WS147
UGA_1_7268	1	7,268	T	A
UGA_1_4438939	1	4,438,939	A	T
UGA_1_4438940	1	4,438,940	A	T
UGA_1_4937580	1	4,937,580	C	T
UGA_1_5356679	1	5,356,679	C	G
UGA_1_5356709	1	5,356,709	C	T
UGA_1_5949053	1	5,949,053	G	A
UGA_1_6274674	1	6,274,674	T	A
UGA_1_7293131	1	7,293,131	T	G
UGA_1_7313183	1	7,313,183	T	C
UGA_1_7445569	1	7,445,569	C	T
UGA_1_8281153	1	8,281,153	A	T
UGA_1_8362391	1	8,362,391	G	A
UGA_1_9597371	1	9,597,371	A	G
UGA_1_10299533	1	10,299,533	T	A
UGA_1_13307495	1	13,307,495	T	C
UGA_1_14514141	1	14,514,141	A	G
UGA_1_14514146	1	14,514,146	T	C
UGA_1_20031162	1	20,031,162	C	T
UGA_1_21875600	1	21,875,600	A	G
UGA_1_21997233	1	21,997,233	A	G
UGA_1_22475657	1	22,475,657	T	C
UGA_1_22475685	1	22,475,685	G	A
UGA_1_22475693	1	22,475,693	C	A
UGA_1_22683506	1	22,683,506	T	C
UGA_1_22683522	1	22,683,522	A	T
UGA_1_22683635	1	22,683,635	G	A
UGA_1_25976416	1	25,976,416	G	A
UGA_1_25976417	1	25,976,417	A	C
UGA_1_31059221	1	31,059,221	C	T
UGA_1_31322260	1	31,322,260	T	A
UGA_1_31639786	1	31,639,786	T	C
UGA_1_32985412	1	32,985,412	G	A
UGA_1_33265455	1	33,265,455	T	A
UGA_1_33269420	1	33,269,420	G	C
UGA_1_33314729	1	33,314,729	A	G
UGA_1_33598443	1	33,598,443	A	G
UGA_1_33626311	1	33,626,311	C	A
UGA_1_33881707	1	33,881,707	T	C
UGA_1_34057211	1	34,057,211	T	G
UGA_2_1681535	2	1,681,535	T	C

UGA_2_2562579	2	2,562,579	G	A
UGA_2_2579108	2	2,579,108	T	C
UGA_2_2634833	2	2,634,833	C	T
UGA_2_2746780	2	2,746,780	G	A
UGA_2_4614915	2	4,614,915	G	A
UGA_2_7808963	2	7,808,963	A	C
UGA_2_9133572	2	9,133,572	A	G
UGA_2_10489848	2	10,489,848	C	G
UGA_2_11419613	2	11,419,613	C	T
UGA_2_12982385	2	12,982,385	C	G
UGA_2_13307937	2	13,307,937	C	G
UGA_2_13698975	2	13,698,975	G	T
UGA_2_14793565	2	14,793,565	G	A
UGA_2_14936386	2	14,936,386	C	T
UGA_2_14936399	2	14,936,399	A	T
UGA_2_14938788	2	14,938,788	C	T
UGA_2_17889315	2	17,889,315	A	C
UGA_2_17889325	2	17,889,325	T	G
UGA_2_18810486	2	18,810,486	T	C
UGA_2_19674635	2	19,674,635	A	G
UGA_2_20247674	2	20,247,674	C	A
UGA_2_21387672	2	21,387,672	A	G
UGA_2_22220529	2	22,220,529	T	C
UGA_2_22736623	2	22,736,623	N	C
UGA_2_23046493	2	23,046,493	T	A
UGA_2_23057414	2	23,057,414	G	A
UGA_2_23709993	2	23,709,993	A	C
UGA_2_24518665	2	24,518,665	C	T
UGA_2_24811820	2	24,811,820	C	T
UGA_2_25896096	2	25,896,096	C	T
UGA_2_26916921	2	26,916,921	T	C
UGA_2_26926928	2	26,926,928	C	G
UGA_2_27048072	2	27,048,072	G	A
UGA_2_27638883	2	27,638,883	T	A
UGA_2_30059928	2	30,059,928	A	G
UGA_2_30059986	2	30,059,986	A	G
UGA_2_30215557	2	30,215,557	G	A
UGA_2_30379168	2	30,379,168	C	A
UGA_2_30389636	2	30,389,636	A	T
UGA_2_30389648	2	30,389,648	A	T
UGA_2_30389649	2	30,389,649	A	T
UGA_2_30555414	2	30,555,414	A	G
UGA_3_390925	3	390,925	G	A
UGA_3_1271993	3	1,271,993	G	A
UGA_3_2443462	3	2,443,462	G	A
UGA_3_2443465	3	2,443,465	G	A
UGA_3_2752284	3	2,752,284	T	C
UGA_3_7931675	3	7,931,675	C	T
UGA_3_9825252	3	9,825,252	C	A
UGA_3_9825273	3	9,825,273	C	T

UGA_3_10898496	3	10,898,496	A	G
UGA_3_11648098	3	11,648,098	C	G
UGA_3_11729152	3	11,729,152	T	G
UGA_3_12184606	3	12,184,606	T	A
UGA_3_12196066	3	12,196,066	A	G
UGA_3_13371803	3	13,371,803	A	G
UGA_3_14221166	3	14,221,166	C	T
UGA_3_14923137	3	14,923,137	T	G
UGA_3_14923150	3	14,923,150	G	A
UGA_3_14923161	3	14,923,161	G	A
UGA_3_17587730	3	17,587,730	T	C
UGA_3_22601792	3	22,601,792	A	G
UGA_3_22692464	3	22,692,464	C	G
UGA_3_24069458	3	24,069,458	G	A
UGA_3_24884253	3	24,884,253	C	A
UGA_3_26825682	3	26,825,682	T	C
UGA_3_27099671	3	27,099,671	A	C
UGA_3_27155705	3	27,155,705	A	C
UGA_3_27936424	3	27,936,424	C	T
UGA_4_1568696	4	1,568,696	A	G
UGA_4_2315190	4	2,315,190	T	A
UGA_4_2315216	4	2,315,216	T	G
UGA_4_2315219	4	2,315,219	A	G
UGA_4_2315234	4	2,315,234	G	A
UGA_4_4463403	4	4,463,403	N	N
UGA_4_4827414	4	4,827,414	C	A
UGA_4_6029121	4	6,029,121	A	C
UGA_4_7664652	4	7,664,652	G	A
UGA_4_10754505	4	10,754,505	T	C
UGA_4_10754541	4	10,754,541	G	A
UGA_4_10953677	4	10,953,677	A	G
UGA_4_10953699	4	10,953,699	A	G
UGA_4_15808102	4	15,808,102	A	G
UGA_4_18913246	4	18,913,246	T	C
UGA_4_18913257	4	18,913,257	T	G
UGA_4_21386677	4	21,386,677	C	T
UGA_4_21386686	4	21,386,686	A	G
UGA_4_21401094	4	21,401,094	A	N
UGA_4_21484489	4	21,484,489	G	T
UGA_4_23420397	4	23,420,397	G	A
UGA_4_23466683	4	23,466,683	G	A
UGA_4_23660044	4	23,660,044	A	G
UGA_5_608200	5	608,200	G	T
UGA_5_1570228	5	1,570,228	A	T
UGA_5_1570231	5	1,570,231	C	A
UGA_5_1880681	5	1,880,681	T	A
UGA_5_2399324	5	2,399,324	C	A
UGA_5_3477959	5	3,477,959	A	C
UGA_5_3800444	5	3,800,444	T	C
UGA_5_3826353	5	3,826,353	C	T

UGA_5_3826361	5	3,826,361	T	C
UGA_5_4929948	5	4,929,948	A	G
UGA_5_4939028	5	4,939,028	T	C
UGA_5_6020622	5	6,020,622	T	C
UGA_5_6240971	5	6,240,971	G	C
UGA_5_6498850	5	6,498,850	T	C
UGA_5_6524487	5	6,524,487	A	G
UGA_5_6537918	5	6,537,918	A	G
UGA_5_6692262	5	6,692,262	T	C
UGA_5_8217751	5	8,217,751	T	C
UGA_5_8667510	5	8,667,510	A	T
UGA_5_8700205	5	8,700,205	T	C
UGA_5_8702354	5	8,702,354	A	C
UGA_5_8811772	5	8,811,772	A	G
UGA_5_8860293	5	8,860,293	G	A
UGA_5_8903675	5	8,903,675	A	G
UGA_5_9004330	5	9,004,330	T	N
UGA_5_19066190	5	19,066,190	G	A
UGA_5_19799044	5	19,799,044	G	A
UGA_5_19799055	5	19,799,055	C	T
UGA_5_19799081	5	19,799,081	C	T
UGA_5_20850887	5	20,850,887	C	T
UGA_5_20850893	5	20,850,893	C	A
UGA_5_20850906	5	20,850,906	A	C
UGA_5_25766334	5	25,766,334	G	A
UGA_5_25766406	5	25,766,406	T	G
UGA_5_26026096	5	26,026,096	A	G
UGA_5_28497399	5	28,497,399	T	A
UGA_5_29011891	5	29,011,891	T	G
UGA_5_29012002	5	29,012,002	A	T
UGA_5_30683580	5	30,683,580	A	C
UGA_5_30857712	5	30,857,712	T	C
UGA_5_30864209	5	30,864,209	C	T
UGA_5_30864211	5	30,864,211	G	C
UGA_5_30864214	5	30,864,214	C	A
UGA_5_31158866	5	31,158,866	G	T
UGA_5_31241529	5	31,241,529	T	A
UGA_5_31247330	5	31,247,330	A	G
UGA_5_31767187	5	31,767,187	C	A
UGA_5_32056419	5	32,056,419	C	A
UGA_5_32058691	5	32,058,691	C	T
UGA_5_32573761	5	32,573,761	T	G
UGA_5_32573763	5	32,573,763	C	A
UGA_5_32573764	5	32,573,764	T	C
UGA_5_32627662	5	32,627,662	C	T
UGA_5_32931558	5	32,931,558	G	A
UGA_5_33077831	5	33,077,831	G	A
UGA_5_33224004	5	33,224,004	T	C
UGA_5_33227716	5	33,227,716	T	C
UGA_5_33231378	5	33,231,378	T	C

UGA_5_33304654	5	33,304,654	A	T
UGA_6_859258	6	859,258	C	A
UGA_6_2899204	6	2,899,204	C	G
UGA_6_3471202	6	3,471,202	G	A
UGA_6_5820584	6	5,820,584	G	A
UGA_6_5820616	6	5,820,616	C	A
UGA_6_7076766	6	7,076,766	A	G
UGA_6_9590027	6	9,590,027	C	A
UGA_6_9590211	6	9,590,211	C	A
UGA_6_10516492	6	10,516,492	T	A
UGA_6_10785843	6	10,785,843	C	T
UGA_6_10785851	6	10,785,851	C	T
UGA_6_13291695	6	13,291,695	A	G
UGA_6_13324557	6	13,324,557	C	T
UGA_6_13324559	6	13,324,559	A	G
UGA_6_13377769	6	13,377,769	G	A
UGA_6_13377773	6	13,377,773	G	A
UGA_6_13377775	6	13,377,775	A	G
UGA_6_13413280	6	13,413,280	C	T
UGA_6_13608023	6	13,608,023	C	T
UGA_6_14313662	6	14,313,662	C	T
UGA_6_14341230	6	14,341,230	G	A
UGA_6_14833910	6	14,833,910	C	A
UGA_6_14833911	6	14,833,911	G	T
UGA_6_14950811	6	14,950,811	T	C
UGA_6_15138220	6	15,138,220	G	N
UGA_6_15138329	6	15,138,329	A	C
UGA_6_15178200	6	15,178,200	A	T
UGA_6_15332695	6	15,332,695	T	C
UGA_6_15462190	6	15,462,190	A	T
UGA_6_15462207	6	15,462,207	G	A
UGA_6_15661073	6	15,661,073	C	T
UGA_6_15661102	6	15,661,102	C	G
UGA_6_15720359	6	15,720,359	C	T
UGA_6_15991966	6	15,991,966	C	G
UGA_6_15991990	6	15,991,990	A	G
UGA_6_16005021	6	16,005,021	G	A
UGA_6_17266849	6	17,266,849	T	C
UGA_6_17266871	6	17,266,871	T	A
UGA_6_17269382	6	17,269,382	C	T
UGA_6_17298907	6	17,298,907	A	G
UGA_6_18399215	6	18,399,215	G	A
UGA_6_18399509	6	18,399,509	G	A
UGA_6_19309571	6	19,309,571	T	C
UGA_6_21143924	6	21,143,924	G	A
UGA_6_21143930	6	21,143,930	G	A
UGA_6_21750508	6	21,750,508	G	T
UGA_6_21812234	6	21,812,234	T	A
UGA_6_21977917	6	21,977,917	A	C
UGA_6_22522417	6	22,522,417	C	G

UGA_6_22522436	6	22,522,436	G	A
UGA_6_22522437	6	22,522,437	C	A
UGA_6_22527316	6	22,527,316	A	T
UGA_6_22527319	6	22,527,319	T	A
UGA_6_22527320	6	22,527,320	C	A
UGA_6_23251106	6	23,251,106	G	A
UGA_6_23653862	6	23,653,862	T	G
UGA_6_24153212	6	24,153,212	A	C
UGA_6_24711172	6	24,711,172	C	T
UGA_6_24831629	6	24,831,629	C	T
UGA_6_25221581	6	25,221,581	T	A
UGA_6_25814506	6	25,814,506	A	T
UGA_6_25838444	6	25,838,444	C	T
UGA_6_26330024	6	26,330,024	A	G
UGA_6_26943928	6	26,943,928	G	N
UGA_7_355288	7	355,288	C	A
UGA_7_643198	7	643,198	G	A
UGA_7_985640	7	985,640	G	C
UGA_7_1570653	7	1,570,653	A	G
UGA_7_3106439	7	3,106,439	C	T
UGA_7_3154520	7	3,154,520	G	A
UGA_7_3614838	7	3,614,838	C	T
UGA_7_3617046	7	3,617,046	A	G
UGA_7_3807603	7	3,807,603	G	A
UGA_7_4196459	7	4,196,459	T	C
UGA_7_6782871	7	6,782,871	A	G
UGA_7_7375218	7	7,375,218	G	T
UGA_7_7478503	7	7,478,503	T	C
UGA_7_7478590	7	7,478,590	A	T
UGA_7_7773124	7	7,773,124	A	G
UGA_7_8306351	7	8,306,351	A	C
UGA_7_8937222	7	8,937,222	A	G
UGA_7_10382847	7	10,382,847	T	C
UGA_7_10438076	7	10,438,076	C	T
UGA_7_12151391	7	12,151,391	T	G
UGA_7_12821334	7	12,821,334	C	G
UGA_7_13966179	7	13,966,179	T	C
UGA_7_13966203	7	13,966,203	G	C
UGA_7_13966211	7	13,966,211	T	A
UGA_7_14448434	7	14,448,434	G	A
UGA_7_14448472	7	14,448,472	G	C
UGA_7_14448475	7	14,448,475	C	T
UGA_7_16384605	7	16,384,605	G	A
UGA_7_19106874	7	19,106,874	G	A
UGA_7_19260244	7	19,260,244	A	G
UGA_7_19260248	7	19,260,248	T	C
UGA_7_19867848	7	19,867,848	C	A
UGA_7_21023155	7	21,023,155	G	A
UGA_7_21942891	7	21,942,891	T	G
UGA_7_22076838	7	22,076,838	G	A

UGA_7_23094183	7	23,094,183	G	A
UGA_7_23094220	7	23,094,220	T	C
UGA_7_23238744	7	23,238,744	N	A
UGA_7_23265968	7	23,265,968	T	C
UGA_7_23414000	7	23,414,000	A	C
UGA_7_23778207	7	23,778,207	A	C
UGA_7_24648466	7	24,648,466	A	G
UGA_7_24939153	7	24,939,153	A	T
UGA_7_25376252	7	25,376,252	A	G
UGA_7_26717850	7	26,717,850	A	G
UGA_7_27998319	7	27,998,319	A	T
UGA_7_27998320	7	27,998,320	G	A
UGA_7_28000330	7	28,000,330	G	A
UGA_7_28007611	7	28,007,611	T	G
UGA_7_28059080	7	28,059,080	A	C
UGA_7_29570763	7	29,570,763	C	A
UGA_8_438131	8	438,131	G	A
UGA_8_438256	8	438,256	G	T
UGA_8_1432430	8	1,432,430	T	C
UGA_8_1432442	8	1,432,442	C	T
UGA_8_1679300	8	1,679,300	T	C
UGA_8_1679303	8	1,679,303	A	G
UGA_8_2310499	8	2,310,499	A	G
UGA_8_4541109	8	4,541,109	A	C
UGA_8_4542356	8	4,542,356	C	T
UGA_8_6692255	8	6,692,255	C	T
UGA_8_10856192	8	10,856,192	T	A
UGA_8_13569467	8	13,569,467	G	C
UGA_8_13700818	8	13,700,818	A	G
UGA_8_13700846	8	13,700,846	A	G
UGA_8_13700867	8	13,700,867	C	T
UGA_8_14892414	8	14,892,414	G	A
UGA_8_14892422	8	14,892,422	G	A
UGA_8_15759483	8	15,759,483	A	G
UGA_8_16626959	8	16,626,959	C	T
UGA_8_17119520	8	17,119,520	A	G
UGA_8_17886417	8	17,886,417	T	C
UGA_8_18228469	8	18,228,469	G	A
UGA_8_18332327	8	18,332,327	C	T
UGA_8_18524671	8	18,524,671	G	A
UGA_8_18771114	8	18,771,114	C	T
UGA_8_18811100	8	18,811,100	T	C
UGA_8_18811563	8	18,811,563	T	C
UGA_8_19058319	8	19,058,319	C	G
UGA_8_19066312	8	19,066,312	C	T
UGA_8_19299134	8	19,299,134	T	C
UGA_8_20595378	8	20,595,378	G	C
UGA_8_20595379	8	20,595,379	C	T
UGA_8_21637156	8	21,637,156	A	G
UGA_8_21660128	8	21,660,128	T	C

UGA_8_22729513	8	22,729,513	A	T
UGA_8_22737059	8	22,737,059	A	G
UGA_8_23239524	8	23,239,524	C	T
UGA_8_23447614	8	23,447,614	G	C
UGA_8_23483255	8	23,483,255	C	T
UGA_8_23503036	8	23,503,036	C	T
UGA_8_24241587	8	24,241,587	G	A
UGA_8_24450199	8	24,450,199	C	G
UGA_8_24453980	8	24,453,980	C	T
UGA_8_24761107	8	24,761,107	A	G
UGA_8_25097817	8	25,097,817	A	G
UGA_9_85137	9	85,137	T	A
UGA_9_633364	9	633,364	C	T
UGA_9_1542925	9	1,542,925	A	G
UGA_9_1635753	9	1,635,753	C	G
UGA_9_1640886	9	1,640,886	T	C
UGA_9_2087317	9	2,087,317	T	C
UGA_9_2088020	9	2,088,020	T	C
UGA_9_2417258	9	2,417,258	A	T
UGA_9_3457311	9	3,457,311	A	G
UGA_9_4284995	9	4,284,995	T	A
UGA_9_4303885	9	4,303,885	C	T
UGA_9_4303886	9	4,303,886	C	T
UGA_9_4307349	9	4,307,349	C	T
UGA_9_4334806	9	4,334,806	G	A
UGA_9_4737897	9	4,737,897	A	G
UGA_9_6519900	9	6,519,900	A	G
UGA_9_6524434	9	6,524,434	T	C
UGA_9_7690621	9	7,690,621	T	C
UGA_9_9403073	9	9,403,073	G	A
UGA_9_9826519	9	9,826,519	A	C
UGA_9_9896510	9	9,896,510	T	G
UGA_9_9915218	9	9,915,218	G	C
UGA_9_10029687	9	10,029,687	A	T
UGA_9_10093895	9	10,093,895	A	T
UGA_9_10279709	9	10,279,709	A	G
UGA_9_11904303	9	11,904,303	C	G
UGA_9_11910516	9	11,910,516	A	G
UGA_9_11922858	9	11,922,858	A	G
UGA_9_14005918	9	14,005,918	T	A
UGA_9_14533850	9	14,533,850	T	C
UGA_9_16417083	9	16,417,083	C	T
UGA_9_16417091	9	16,417,091	C	T
UGA_9_18603294	9	18,603,294	T	C
UGA_9_18892859	9	18,892,859	G	A
UGA_9_19603336	9	19,603,336	C	T
UGA_9_19603363	9	19,603,363	G	A
UGA_9_19603366	9	19,603,366	C	T
UGA_9_20841020	9	20,841,020	A	G
UGA_9_21203501	9	21,203,501	A	G

UGA_9_21204087	9	21,204,087	T	C
UGA_9_22701474	9	22,701,474	T	A
UGA_9_22701475	9	22,701,475	T	A
UGA_9_23688429	9	23,688,429	T	C
UGA_9_27552536	9	27,552,536	T	A
UGA_9_27552593	9	27,552,593	G	A
UGA_9_27621525	9	27,621,525	G	A
UGA_9_28711940	9	28,711,940	G	T
UGA_9_30005169	9	30,005,169	G	A
UGA_9_30005197	9	30,005,197	T	G
UGA_9_31274796	9	31,274,796	G	T
UGA_9_31374085	9	31,374,085	C	T
UGA_9_31374136	9	31,374,136	T	G
UGA_9_33263845	9	33,263,845	T	C
UGA_9_33523453	9	33,523,453	C	T
UGA_9_34417192	9	34,417,192	C	T
UGA_9_34719800	9	34,719,800	C	T
UGA_9_34888914	9	34,888,914	G	T
UGA_9_34897646	9	34,897,646	G	T
UGA_10_1314394	10	1,314,394	G	A
UGA_10_1319999	10	1,319,999	A	C
UGA_10_1991077	10	1,991,077	A	T
UGA_10_1991080	10	1,991,080	A	T
UGA_10_3766549	10	3,766,549	C	T
UGA_10_3785012	10	3,785,012	C	G
UGA_10_3785032	10	3,785,032	G	A
UGA_10_3787797	10	3,787,797	T	C
UGA_10_3814552	10	3,814,552	C	T
UGA_10_3928526	10	3,928,526	G	A
UGA_10_3941814	10	3,941,814	T	C
UGA_10_3991841	10	3,991,841	T	C
UGA_10_4054513	10	4,054,513	T	C
UGA_10_4054608	10	4,054,608	G	T
UGA_10_4054631	10	4,054,631	C	T
UGA_10_4054635	10	4,054,635	G	A
UGA_10_4131265	10	4,131,265	G	A
UGA_10_4413654	10	4,413,654	G	T
UGA_10_4774775	10	4,774,775	T	G
UGA_10_4788250	10	4,788,250	C	G
UGA_10_4906788	10	4,906,788	C	A
UGA_10_4906821	10	4,906,821	A	G
UGA_10_4929371	10	4,929,371	C	T
UGA_10_4947283	10	4,947,283	C	T
UGA_10_4959228	10	4,959,228	T	C
UGA_10_4965559	10	4,965,559	C	T
UGA_10_4966037	10	4,966,037	G	A
UGA_10_4966106	10	4,966,106	C	T
UGA_10_5014652	10	5,014,652	A	T
UGA_10_5119089	10	5,119,089	A	C
UGA_10_5123716	10	5,123,716	T	A

UGA_10_5199311	10	5,199,311	C	A
UGA_10_5199581	10	5,199,581	T	A
UGA_10_5211267	10	5,211,267	T	C
UGA_10_5521105	10	5,521,105	C	T
UGA_10_5719852	10	5,719,852	A	G
UGA_10_5880181	10	5,880,181	C	A
UGA_10_11053977	10	11,053,977	A	G
UGA_10_11063136	10	11,063,136	A	T
UGA_10_12129449	10	12,129,449	C	A
UGA_10_12298334	10	12,298,334	G	A
UGA_10_12326606	10	12,326,606	C	T
UGA_10_24093073	10	24,093,073	A	G
UGA_10_28150504	10	28,150,504	C	A
UGA_11_1007083	11	1,007,083	C	T
UGA_11_1011759	11	1,011,759	A	G
UGA_11_1300138	11	1,300,138	A	G
UGA_11_1322383	11	1,322,383	A	G
UGA_11_1844967	11	1,844,967	C	A
UGA_11_1844968	11	1,844,968	G	T
UGA_11_1844970	11	1,844,970	G	A
UGA_11_1905035	11	1,905,035	C	T
UGA_11_1961798	11	1,961,798	G	A
UGA_11_3137657	11	3,137,657	G	A
UGA_11_3805788	11	3,805,788	G	A
UGA_11_4217224	11	4,217,224	G	C
UGA_11_4626421	11	4,626,421	T	A
UGA_11_5517160	11	5,517,160	T	C
UGA_11_5534544	11	5,534,544	G	A
UGA_11_5534563	11	5,534,563	A	G
UGA_11_5534580	11	5,534,580	G	A
UGA_11_9406786	11	9,406,786	A	G
UGA_11_9523637	11	9,523,637	C	T
UGA_11_10493281	11	10,493,281	G	A
UGA_11_13078042	11	13,078,042	T	G
UGA_11_13557352	11	13,557,352	T	C
UGA_11_13557408	11	13,557,408	T	C
UGA_11_15587325	11	15,587,325	T	C
UGA_11_15612084	11	15,612,084	C	T
UGA_11_15637496	11	15,637,496	T	G
UGA_11_17440419	11	17,440,419	C	T
UGA_11_17453227	11	17,453,227	T	C
UGA_11_18112955	11	18,112,955	G	A
UGA_11_18383861	11	18,383,861	A	G
UGA_11_19594515	11	19,594,515	T	C
UGA_11_19865801	11	19,865,801	C	G
UGA_11_20242416	11	20,242,416	T	A
UGA_11_20463216	11	20,463,216	G	C
UGA_11_21779189	11	21,779,189	G	C
UGA_11_21921856	11	21,921,856	G	C
UGA_11_21983692	11	21,983,692	T	A

UGA_11_22709058	11	22,709,058	G	C
UGA_11_22738360	11	22,738,360	C	A
UGA_11_23492959	11	23,492,959	C	T
UGA_11_23496085	11	23,496,085	G	A
UGA_11_23771784	11	23,771,784	A	C
UGA_11_23771906	11	23,771,906	C	T
UGA_11_23821268	11	23,821,268	G	A
UGA_11_23875712	11	23,875,712	G	A
UGA_11_25850675	11	25,850,675	T	A
UGA_11_26773124	11	26,773,124	A	G

CHAPTER 8

A NON-DESTRUCTIVE GENOTYPING SYSTEM FROM A SINGLE SEED FOR MARKER ASSISTED SELECTION IN WATERMELON[1]

[1] Meru, G., D. McDowell, V. Waters, A. Seibel1, J. Davis, and C. McGregor. 2013. Genet. Mol. Res. 12:702-709.

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Abstract

Genomic tools for watermelon breeding are becoming increasingly available. A high throughput genotyping system would facilitate the use of DNA markers in marker-assisted selection. DNA extraction from leaf material requires prior seed germination and is often time-consuming and cost prohibitive. In an effort to develop a more efficient system, watermelon seeds of several genotypes and various seed sizes were sampled by removing ⅓ or ½ sections from the distal ends for DNA extraction, while germinating the remaining proximal parts of the seed. Removing ⅓ of the seed from the distal end had no effect on seed germination percentage or seedling vigor. Different DNA extraction protocols were tested to identify a method that could yield DNA of sufficient quality for amplification by polymerase chain reaction (PCR). A sodium dodecyl sulphate (SDS) extraction protocol with 1% (w/v) polyvinylpyrrolidone (PVP) yielded DNA that could be amplified with microsatellite primers and was free of pericarp contamination. In this study, an efficient, non-destructive genotyping protocol for watermelon seed was developed.

Introduction

The application of DNA markers to aid the selection of important traits in plant breeding is routine for many crops (Gao et al., 2008; von Post et al., 2003). Leaf-based genotyping systems for marker-assisted selection (MAS) are resource-intensive, requiring prior germination of seeds and the collection and storage of leaf samples in expensive, ultra-low-temperature freezers (Gao et al., 2008; Kang et al., 1998). Seed-based genotyping systems provide an appealing alternative that saves resources and allows breeders to select suitable individuals prior to planting (Gao et al., 2008; von Post et al., 2003). This method has been adopted for several crops, including barley (von Post et al., 2003), maize (Gao et al., 2008), soy bean (Kamiya and Kiguchi 2003), wheat (Abd-Elsalam et al., 2011), and rice (Kang et al., 1998). To be useful, a non-destructive sampling technique that does not affect the germination potential of remnant seed embryos must be developed and the DNA extracted from the seed must be of sufficient quantity and quality to allow genotypic analysis. DNA extractions from seed can be problematic and DNA of low quality and quantity is observed frequently (Abd-Elsalam et al., 2011). Moreover, the extracted DNA must be devoid of pericarp contamination, which can lead to genotyping errors (Gao et al., 2008). Here we describe a non-destructive seed based genotyping system for watermelon.

Materials and methods

Plant materials, seed sampling, and germination test

Three watermelon genotypes [Sugar Baby, Charleston Gray, and Florida Giant (Reimer seed company, NC, USA)] were chosen due to their difference in seed size. Seed samples for DNA extraction were obtained by removing ⅓ or ½ of the distal portions with a steel blade (Fig. 8.1). The remaining proximal portions were germinated in cells (5.98 × 3.68 × 4.69 cM) filled

with Fafard 3B soil amended with Osmocote (14N–4.2P–11.6K) in the greenhouse (14 h light/10 h dark, 22-32°C). Germination data and individual plant height (indicator of vigor) were measured 15 days after planting (DAP). The experiment was carried out in July, 2011 and repeated in August and December of the same year with 24 seeds for each treatment-cultivar combination in each experiment.

DNA extraction and simple sequence repeat (SSR) analysis

Cut seeds were placed in individual tubes in a 96-tube plate (Greentree Scientific, NY, USA), immersed in liquid nitrogen for 5 min, then ground using 5-mm beads (Qiagen Inc, CA, USA) in a Tissuelyser II (Qiagen) for 5 min at 30 Hz/s. Four DNA extraction buffers were tested using 2 different protocols.

Protocol 1 (modified from McGregor et al., 2000; Gao et al., 2008): samples were incubated in 450 µL 1% Cetyl trimethylammonium bromide (CTAB), 1% Sarcosyl (SAR), or 1% sodium dodecyl sulphate (SDS) in a water bath at 65°C for 30 min. Each buffer also included 100 mM Tris-HCl (pH 7.5), 700 mM NaCl, 50 mM EDTA (pH 8), and 0.2% v/v β-mercaptoethanol, which was added just before incubation. An equal volume of chloroform-isoamyl alcohol (24:1) was added and samples centrifuged at 2,465 *g* for 10 min at room temperature. The supernatant (300 µL) was transferred to clean tubes and 0.6 × volume cold isopropanol added. DNA was allowed to precipitate for 30 min at -20 °C and collected by centrifugation (2,465 *g*) for 10 min at room temperature. The pellets were washed twice with cold 70% ethanol and allowed to air dry before suspension in 50 µL Tris-EDTA (TE).

Protocol 2 (PVP+SDS) (Modified from: Abd-Elsalam et al., 2011): Ground samples were incubated in 450 µL 0.5% SDS, 1% PVP, 200 mM Tris-HCl (pH 7.5), 250 mM NaCl, 25 mM EDTA (pH 8), and 0.2% v/v β-mercaptoethanol in a water bath at 65°C for 30 min. NaOAc (0.3×

volume, 3 M) was added to each tube, then centrifuged (2,465 *g*) for 10 min. The supernatant was transferred to a clean tube and an equal volume of cold isopropanol added. DNA was allowed to precipitate for 10 min at room temperature and collected by centrifugation (2,465 *g*) for 10 min at room temperature. The DNA pellets were washed and suspended as previously described.

DNA concentration and quality were determined on a NanoDrop 8000 (Thermo Scientific, USA) and by agarose gel electrophoresis. Amplification of all samples was initially tested using internal transcribed spacer (ITS) primers (Blattner, 1999). An SSR marker (MCPI-13) (Joobeur et al., 2006) was used to determine the presence of pericarp contamination by amplifying DNA obtained from seed (SDS + PVP) and from the corresponding leaf samples of a segregating population of 30 F_2 plants. The amplicons were separated an ABI 3730 capillary sequencer (Applied Biosystems, MA, USA) at the Georgia Genomics Facility and the output was analyzed using Genemapper 4.0 (Applied Biosystems).

Statistical Analysis

Data were analyzed using the PROC GLM procedure of SAS (SAS Institute, 1999) and the means of the tested variables separated using the Tukey's honest significance test (Ott and Longnecker, 2001).

Results and discussion

Germination percentage and plant vigor

In order to adopt a seed-based genotyping system for watermelon, a non-destructive sampling method that generates viable embryos must be developed. In the current study, no significant differences ($\alpha = 0.05$) in germination percentage were observed between the ⅔ proximal ends and uncut controls at 15 DAP for all the cultivars tested (Table 8.1). However,

there was a significant difference in germination percentage between samples in which ½ of the distal ends were removed for DNA extraction and uncut controls for the Sugar Baby cultivar (Table 8.1), which had the smallest seed. Similarly, seedling heights at 15 DAP did not differ significantly between the ⅔ proximal ends and uncut controls for any of the cultivars tested (Table 8.1). However, removing ½ the distal ends for DNA extraction from Sugar Baby seeds significantly affected the plant height in comparison to uncut controls. The germination percentage of the cut seed and the vigor of the resulting seedling depend on embryo integrity and availability of nutrition after sampling, respectively (Gao et al., 2008). For watermelon cultivars with small seeds such as Sugar Baby, sampling large portions of the seed for DNA extraction should be avoided as this depletes energy reserves required for germination and growth. In a similar study in maize, Gao et al. (2008) reported reduced germination rates in genotypes whose seeds had a large proportion of the endosperm sampled for DNA extraction. In the current experiment, removing the ⅓ distal portion of the seed for DNA extraction did not influence the germination and vigor of watermelon seedlings.

DNA quality, quantity, and genotypic analysis

Based on the results for germination and seedling vigor, ⅓ distal ends were chosen for DNA extraction. DNA was obtained with all the four buffers tested, regardless of protocol (Fig. 8.2). The SDS + PVP buffer yielded significantly higher amounts of DNA than the other buffers (Table 8.2). DNA yields per single ⅓ distal portion ranged from 1.7-20.6 µg [Charleston Gray (9.96-15.95 µg), Florida Giant (5.75-20.6 µg), and Sugar Baby (1.7-4.8 µg)] and is sufficient for analysis of 170-2000 PCR reactions, which are sufficient for most MAS applications (Gao et al., 2008).

The mean absorbance at 260/280 nm (A260:280) ranged from 0.88 for CTAB buffer to 1.92 for SDS + PVP while that at 260/230 nm ranged from 0.34 for CTAB buffer to 0.6 for 0.5% SDS buffer (Table 8.2). Through PCR, it was shown that only the DNA obtained from the SDS + PVP buffer was of sufficient quality (A260:280 = 1.92) to amplify consistently with ITS primers (Fig. 8.3) (Thermo Scientific, 2008). DNA obtained with other buffers had A260:280 values of 0.88-1.51, indicating protein contamination (Thermo Scientific, 2008). Contaminants such as proteins, polysaccharides and polyphenols can be co-purified with DNA and are known to inhibit PCR (Gao et al., 2008; Thermo Scientific, 2008). Levels of proteins and polysaccharides in watermelon seed can exceed 45 % w/v (Al-Khalifa, 1996; Baboli et al., 2010) and could result in DNA with PCR inhibitors. PVP is often used to obtain good quality DNA from samples with high levels of polyphenols and polysaccharides as it removes these compounds during extraction (Li et al., 2007; Schween et al., 2002). The inclusion of PVP in the extraction buffer might be useful to ensure yield of good quality DNA from watermelon seed as indicated by the results from this study.

To investigate possible retention of maternal tissue from the pericarp in the extracted DNA, a polymorphic SSR marker was used to detect genotyping errors in a segregating F_2 population. Pairwise comparison of cotyledon genotypes and corresponding leaf genotypes showed no contamination with maternal alleles in the embryos (Table 8.3). Pericarp contamination may be detected if the cotyledon genotype is heterozygous while the leaf genotype is homozygous (Gao et al., 2008).

In addition to its application for MAS, this method will be a suitable alternative to leaf-based genotyping for other watermelon crop studies such as genetic diversity studies, DNA fingerprinting, and tests for varietal purity.

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Table 8.1 Germination percentages and plant height at 15 days after planting for uncut seeds (control) and remnant two thirds or halves for three different watermelon cultivars.

Treatments (Proximal ends)	Cultivars					
	Charleston Gray		Florida Giant		Sugar Baby	
	Germination (%)	Height (cm)	Germination (%)	Height (cm)	Germination (%)	Height (cm)
Uncut control	87.5	6.8	94.4	6.5	79.1	4.9
Two third	91.6	6.7	98.6	7.1	83.3	4.2
Half	87.5	6.2	93.0	6.3	61.1*	3.4*

*Significant at $P < 0.05$.

Table 8.2 Properties of DNA extracted using four different buffers. All DNA samples were dissolved in 50 µl TE.

Buffers	DNA concentration ng/µl	A260: 280	A:260/230
CTAB	170.1	0.88*	0.34*
SAR	184.4	1.38	0.49
SDS	121.3	1.51	0.60
SDS + PVP	261.9*	1.92	0.53

*Significant at $P < 0.05$.

Table 8.3 Alleles amplified in a segregating F_2 population (subset) with MCPI-13 SSR marker (Joobeur et al., 2006) in DNA extracted from leaf and seed tissue. Results visualized with Genemapper 4.0 (Applied Biosystems).

SSR Alleles	
Leaf	Seed
Parent 1	
222/222	222/222
Parent 2	
210/210	210/210
F_1	
210/222	210/222
F_2 (subset)	
210/210	210/210
222/222	222/222
222/222	222/222
210/210	210/210
222/222	222/222
210/210	210/210
222/222	222/222
210/210	210/210
222/222	222/222

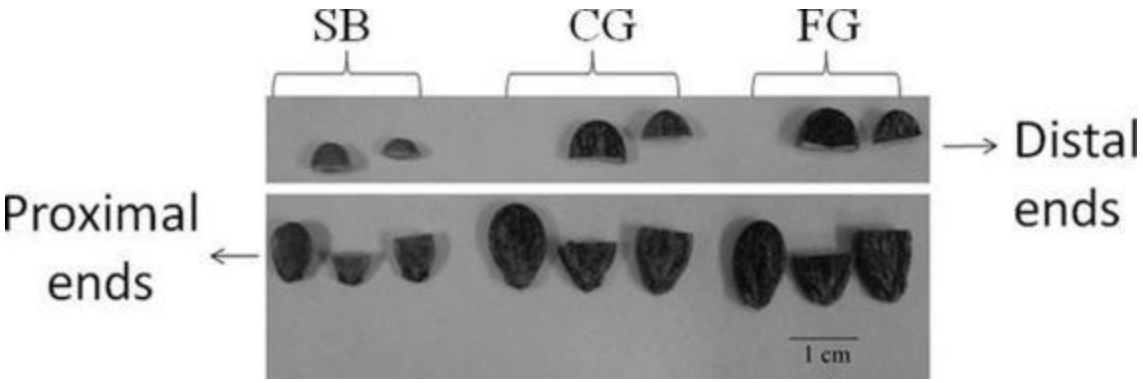

Fig. 8.1 Uncut and cut seeds from watermelon seeds of Charleston Gray (CG), Florida Giant (FG) and Sugar Baby (SB) indicating the size of proximal and distal parts.

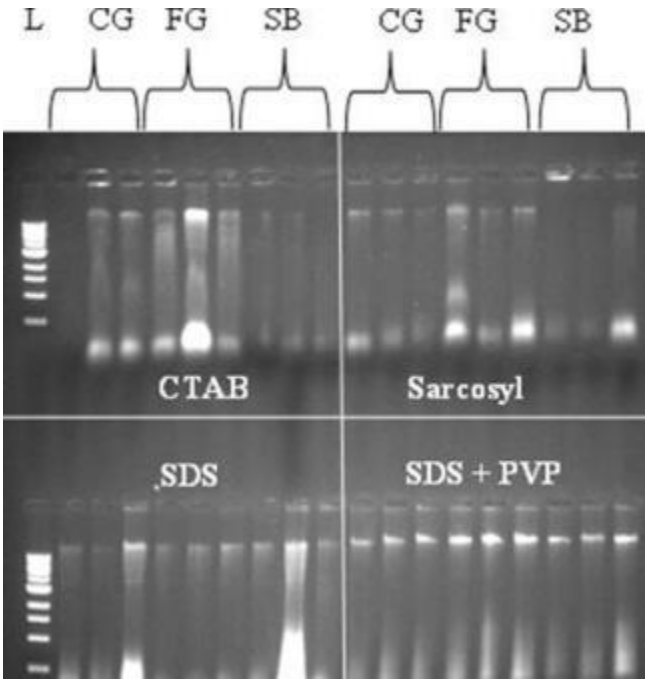

Fig. 8.2 Electrophoresis on 0.8 % agarose gel of DNA extracted from watermelon seeds of Charleston Gray (CG), Florida Giant (FG) and Sugar Baby (SB) using four different extraction buffers. "L" denotes the 1 kb DNA size marker (Promega, CA, USA).

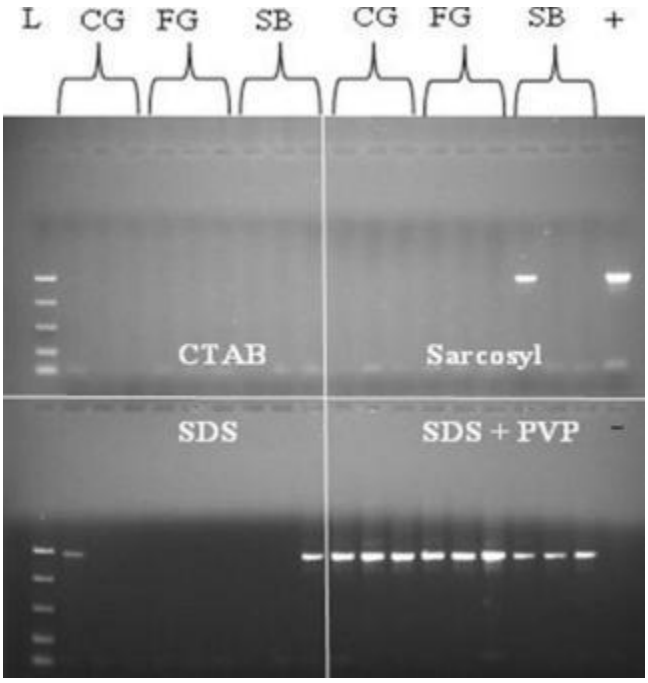

Fig. 8.3 PCR amplification of DNA extracted using four different extraction buffers from watermelon seeds of Charleston Gray (CG), Florida Giant (FG) and Sugar Baby (SB) using ITS primers (Blattner, 1999). The amplicons are separated on a 1.5 % agarose gel. “L” denotes a 100 bp DNA size marker (Promega).

CHAPTER 9

CONCLUSION

It is estimated that the world food production will need to increase by 60% to feed an increased world population by 2050 and that crop production will account for more than 80% of this production (FAO, 2012). To meet this demand, plant breeders (for both agronomic and horticultural crops) must use efficient ways to increase yield while protecting it from abiotic and biotic stresses. To meet this objective, it is critical to dissect the components of phenotypic variance (genetic, environmental, and genetic x environment interaction) associated with economically important traits.

In watermelon, progress in breeding for key traits such as yield, fruit quality and disease resistance has been accomplished primarily through conventional breeding (Ellul et al., 2007; Wehner et al., 2008). In the past, inheritance studies were used in an attempt to determine the nature and number of genetic factors associated with important traits. Such information allowed plant breeders to make decisions on the type of population and number of individuals therein to use in their breeding programs. However, it was not until recently that scientists have been able to estimate the locations of these genetic factors in the watermelon genome. This information is indispensable in the development of markers for application in MAS. Although application of MAS in watermelon breeding programs is not routine, several studies have identified quantitative trait loci (QTL) associated with economically important traits in watermelon (Prothro et al., 2012a; Prothro et al., 2012b, McGregor et al., 2014, Prothro et al., 2013; Sandlin

et al., 2013). The current study aimed to identify genetic factors associated with seed nutrition traits and resistance to fusarium wilt (races 1 and 2) in watermelon.

In this study, the relationship between seed oil percentage (SOP) and seed traits was determined. Seed size (seed weight, seed length and seed width) was negatively correlated to SOP in both egusi and normal seed while kernel percentage (KP) was positively correlated with SOP in the two seed types. This implies that selection for increased SOP in watermelon may be achieved by selecting for small seeds with thinner hulls. The genomic regions associated with seed size traits and kernel percentage in watermelon were identified on chromosomes (chrs.) 1, 5, and 6.

The fatty acid composition of watermelon seed oil will determine the potential application of the oil for industrial and culinary applications. This study determined the quality of watermelon seed oil by quantifying palmitic, stearic, oleic and linoleic acids in the seed. Linoleic acid was predominant of the four fatty acids in the seed. Eight QTL associated with variation in these fatty acids were mapped on chrs. 2, 3, 5, 6, 7 and 8. The major QTL for oleic and linoleic acid co-localized on chr. 6 suggesting pleiotropic effects/linkage. This QTL overlapped the QTL for SOP, KP and seed size in normal seed suggesting the role of this region in controlling multiple seed traits in watermelon. Eighty genes involved in fatty acid biosynthesis pathway in watermelon were identified from the functionally annotated genome.

A study on the effect of soil type on disease severity symptoms of fusarium wilt in watermelon revealed peat-perlite and sand-peat as unsuitable media for screening resistance against race 1 of *Fusarium oxysporum* f. sp. *niveum* (*Fon*) due to inability to support healthy seedling growth. On the other hand, sand-perlite could not discriminate resistant and susceptible watermelon cultivars. However, normal seedling growth was observed in Fafard 3 B and sand-

peat-vermiculite and it was also possible to discriminate resistant and susceptible cultivars in these two soil types.

Genotyping by sequencing (GBS) was exploited in the current study for SNP discovery in an elite x elite F_2 population. This population was developed from a cross between Calhoun Gray and Sugar Baby and segregated for resistance against *Fon* race 1. In this population, 1,024 and 389 SNP markers were mapped on the physical map and genetic map respectively. An evaluation of the F_3 population revealed segregation for resistance to *Fon* race 1. A major QTL (*Qfon1*) was identified on chr. 1 and explained 44% of the phenotypic variation. Several resistance R candidate genes (*Cla004916, Cla004959, Cla011391*, and *Cla011463*) close to this QTL were cloned and sequenced. The sequence analysis of *Cla004916, Cla004959* and *Cla011391* revealed no polymorphisms between Sugar Baby and Calhoun Gray. However in *Cla011463* three SNP mutations were identified at position 2,677,348 bp, 2,679,498 bp and 2,679,644 bp on chr. 1. Markers in proximity to *Qfon1* are candidates for development of MAS for disease resistance.

Another F_2 population developed from a cross between var. *lanatus* and var. *lanatus* (WS147 and Charleston Gray) was genotyped by GBS to further saturate the watermelon physical map. A physical map consisting of five hundred and one SNP markers was developed from this study and used to map a QTL (of moderate effect) associated with race 2 resistance in watermelon. This study forms an important first step in the development of molecular tools for MAS against *Fon* race 2 resistance in watermelon.

To facilitate the application of the molecular tools developed in this study in MAS, a seed based genotyping method was developed for watermelon. This system relies upon the ability to sample a portion of a seed for DNA extraction while maintaining the germination viability of the

remaining seed. Sampling 1/3 of the seed for DNA extraction did not affect plant germination and vigor of seedlings while sodium dodecyl sulphate buffer with 1% (w/v) polyvinylpyrrolidone yielding high quality and quantity DNA. The DNA obtained was free of pericarp contamination.

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