FINE-MAPPING OF A MAJOR QUANTITATIVE TRAIT LOCUS (QTL) CONTROLLING FLOWERING TIME AND QTL MAPPING OF GUMMY STEM BLIGHT RESISTANCE IN WATERMELON

by

WINNIE RIZIKI ADU GIMODE

(Under the Direction of Cecilia McGregor)

ABSTRACT

Watermelon production is hindered by biotic and abiotic stresses that are intensified by long production cycles. Flowering time is a determining factor in early fruit production for growers, which results in reduced production costs and increased profitability. This study aimed to develop high-throughput molecular markers useful for selection of the early flowering trait to enable acceleration of cultivar development and improve diploid-triploid pairing in seedless watermelon breeding. Using QTL-seq and KASP assays, the flowering time locus was refined from a 5 Mb region to ~1.13 Mb region on chromosome 3. UGA3_10795402, UGA3_15424397 and NW0248748 were identified as markers appropriate for selection of flowering time depending on the genetic background. Diseases are another major challenge for growers in the Southeast due to the hot, humid weather that is conducive for plant disease development. Gummy stem blight caused by three *Stagonosporopsis* spp. is one of the most important diseases of watermelon and currently no resistant cultivars are available to growers. The three causal species exhibit differential sensitivity to fungicides, making disease management difficult. This work aimed to establish if the three species have similar host responses, as it remains unclear

whether differential host resistance is partially responsible for the lack of success in resistance breeding efforts. The effect of various *Stagonosporopsis* isolates on different *Citrullus* genotypes was evaluated and it was noted that isolate aggressiveness was not species dependent. In addition, some wild relatives of watermelon exhibited broad resistance to *Stagonosporopsis* isolates. Due to the lack of genetic resistance to GSB in commercial watermelon, we sought to identify chromosomal regions associated with this trait, with a future goal of resistance introgression into the commercial varieties. An interspecific population developed from a cross between a susceptible Crimson Sweet and resistant PI 482276 was used to map GSB resistance. Loci associated with GSB resistance were identified on chromosomes 3 (*ClGSB3.1*), 5 (*ClGSB5.1*) and 7 (*ClGSB7.1*) of watermelon, with the latter explaining up to 21% of the phenotypic variation. High throughput molecular markers including ClGSB5.1-1, ClGSB7.1-1 and ClGSB7.1-2 were developed for utility in MAS of GSB resistance in watermelon breeding.

INDEX WORDS: *Citrullus lanatus*, flowering time, *FT*, MAS, SNP, QTL-seq *Stagonosporopsis, caricae, citrulli, cucurbitacearum, Citrullus amarus,* gummy stem blight*, ClGSB7.1, ClCG07G013230*

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WINNIE RIZIKI ADU GIMODE

BSc, Kenyatta University, Kenya, 2010

MSc, Kenyatta University, Kenya, 2014

A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2020

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BLIGHT RESISTANCE IN WATERMELON

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WINNIE RIZIKI ADU GIMODE

Major Professor:	Cecilia McGregor
Committee:	Anish Malladi
	George Boyhan
	Marin Brewer
	Zenglu Li

Electronic Version Approved:

Ron Walcott
Interim Dean of the Graduate School
The University of Georgia
August 2020

DEDICATION

Dedicated to my beloved Davis Musia Gimode, for being a wonderful friend; for sharing interests, beliefs and hopes.

ACKNOWLEDGEMENTS

I am very grateful to Dr. McGregor. Thanks for offering me a chance to do this research, introducing me to a very interesting field of science. I admire your scientific enthusiasm and willingness to share your broad knowledge. Thanks for always finding time to answer my questions and for your honest and insightful comments. I am also very grateful to members of McGregor lab. Thanks for all your help with my projects, sharing knowledge, and creating a nice working atmosphere.

Special thanks to my parents Mr. and Mrs. Adu. Through the phases of life, you have been a constant encouragement and inspiration. Thank you for your support and faith in me. To my special siblings, much thanks for your love and support. I would not have made it this far without you.

To everyone else who made this work achievable, I am very grateful.

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CHAPTER 1

INTRODUCTION

Watermelon [*Citrullus lanatus* (Thunb.) Matsum. & Nakai] is a major vegetable crop cultivated in the warmer parts of the world for its sweet, juicy fruit. In some parts of the world, the seeds are a source of food as well (Zhang and Jiang 1990; Zhang 1996). Watermelon belongs to the xerophytic genus *Citrullus* Schrad. of the Cucurbitaceae family (Whitaker and Davis 1962; Robinson and Decker-Walters 1997) and flourishes on fertile sandy soils.

The *Citrullus* genus has been classified into seven distinct species (Chomicki and Renner 2015; Paris 2015; Renner et al. 2017). The cultivated species include *C. lanatus*: the sweet, dessert watermelon, its closest relative *C. mucosospermus*: bred for large edible (egusi) seed and *C. amarus*: cultivated in southern Africa and used in jam preparation or as a water source, for cooking and animal fodder. *C. amarus* is also known as preserving melon, citron, tsamma, or cow melon and has hard flesh that is often bitter or bland. The other sparingly cultivated species is *C. colocynthis* that is used for diverse medical purposes (Renner et al. 2017). The three additional species that are not cultivated include *C. ecirrhosus, C. rehmii*, and *C. naudinianus* (Jarret et al. 1997; Robinson and Decker-Walters 1997; Wasylikowa and van der Veen 2004; Dane and Liu 2007; McGregor 2012; Chomicki and Renner 2015; Paris 2015; Renner et al. 2017).

The geographic origin for cultivated watermelon is Africa, however, the exact location in Africa has been greatly debated. For a long time, it was widely accepted that

watermelon originated from southern Africa from wild *C. amarus* populations which grow throughout the region (Robinson and Decker-Walters 1997). A second hypothesis was that watermelon originated from somewhere in northern Africa. Wasylikowa and van der Veen (2004) reported the discovery of several 5000-year old seeds of wild watermelon in southwest Libya, proposing northern Africa as a center of domestication. *C. colocynthis*, which is found native in northern Africa, was also proposed to be a progenitor of cultivated watermelon. Some studies reported a close phylogenetic relationship between the two species (Singh 1978; Sain et al. 2002), however, hybrid plants obtained from crossing *C. lanatus* and *C. colocynthis* exhibit chromosome irregularities and reduced fertility (Whitaker and Davis 1962; Sain et al. 2002; Paris 2015), making *C. colocynthis* an unlikely progenitor. Western Africa was also proposed as a center of origin, with *C. mucosospermus* as the likely progenitor of sweet watermelon (Guo et al. 2013; Chomicki and Renner 2015; Paris 2015). In addition, a molecular phylogenetic study by Chomicki and Renner (2015) reported *C. mucosospermus* and *C. lanatus* to be sister species, which diverged 3.1 million years ago. More recently it was proposed that northeast Africa is the most likely center of origin for dessert watermelon, as wild germplasm historically classified as *C. lanatus* subsp. *cordophanus*, were found in Sudan and are more closely related to cultivated watermelon than any other *Citrullus* species (Renner et al. 2017).

Watermelon plants have a trailing growth habit with long vines and thin hairy stems having branched tendrils at each node (Wehner 2008). The flowers are staminate, pistillate or hermaphroditic, with monoecious plants most common but there are andromonoecious (male and hermaphroditic) and trimonoecious (male, female and hermaphroditic) types as well, especially the wild accessions (Rosa 1928; Porter 1933; Paris 2015). Male flowers

are produced first, with approximately one female flower produced for every seven male flowers (Wehner 2008), although this depends on the cultivar and the environmental conditions. Watermelon is predominantly outcrossing (Ferreira et al. 2000; Kumar et al. 2013) and pollination is mediated by honeybees and bumblebees that visit flowers to collect pollen and nectar.

Cultivated watermelon is naturally diploid ($2n = 2x = 22$), although tetraploids can be produced through chromosome doubling (Kihara 1951) by means of chemicals such as oryzalin. Moreover, triploid varieties which are developed from crossing tetraploids and diploids are used for seedless watermelon production (Kihara 1951; Andrus et al. 1971). The genome size of watermelon is 425 Mb (Arumuganathan and Earle 1991; Ren et al. 2012; Guo et al. 2013), which is relatively small compared with other crops such as wheat, tomato, cotton and corn.

Watermelon fruit is mainly comprised of water in addition to important nutritional compounds for example sugars, lycopene and amino acids, such as citrulline, arginine, and glutathione (Hayashi et al. 2005; Perkins-Veazie et al. 2006; Collins et al. 2007). Due to its sweet flesh, watermelon is enjoyed worldwide and is among the top five most consumed fresh fruits in the world (FAOSTAT 2018). China is the world's largest producer of watermelon, producing approximately 61% of the world total in 2018, while the United States ranks 8th worldwide, producing about 2% (FAOSTAT 2018). In 2019, over 106,000 acres of watermelon were grown in the U.S. (USDA-NASS 2019), producing approximately 1.8 million tons of fruit (FAOSTAT 2018). Most watermelon production in the U.S. occurs in Texas, Florida, Georgia, and California. Together, these states produce approximately 70% of all watermelon grown in the U.S. (USDA-NASS 2019). Georgia

watermelon production in 2019 was approximately 418,052 metric tons with an estimated market value of $ 101,216,000 (USDA-NASS 2019).

Production of seedless watermelon in the U.S. has increased to over 90% of the U.S. watermelon market in the recent past due to consumer demand. The seedless fruit is produced on triploid plants. However, these triploids do not produce sufficient amounts of viable pollen. Consequently, diploid pollen sources (pollenizers) must be interplanted with triploid cultivars to ensure fruit set (Maynard and Elmstrom 1992; Boyhan et al. 2000). Production of seedless watermelon is therefore reliant on synchronized flowering of diploid pollenizers and triploid watermelon cultivars (Dittmar et al. 2009, 2010; McGregor and Waters 2014). As such, flowering time is essential for successful fruit set in watermelon production. Moreover, flowering time is a determining factor in early fruit production for watermelon growers, which results in reduced production costs and increased profitability (Mohr 1986).

Watermelon production is hindered by diseases, which are intensified by long production times. Major diseases affecting watermelon in the southeastern U.S. include fusarium wilt (*Fusarium oxysporum* f. sp. *niveum; FON*), gummy stem blight (*Stagonosporopsis* spp.), bacterial fruit blotch (*Acidovorax citrulli*) and anthracnose (*Colletotrichum lagenarium*), among others (Kousik et al. 2016). Due to major yield losses that result from these diseases, a major goal in watermelon breeding and production is development of disease resistant cultivars. Varieties resistant to some pathogens e.g. fusarium wilt race 1 (Armstrong and Armstrong 1978; Correll 1991; Martyn and Netzer 1991; Zhou et al. 2010; Martyn 2014) and anthracnose (Wehner 2008) as well as sources of resistance for most of the other diseases affecting watermelon, including powdery

mildew and potyviruses, are available. However, gummy stem blight (GSB) remains a major problem, as commercial cultivars with high levels of genetic resistance to gummy stem blight in the field have not been developed. Management of GSB is complicated by the fact that it is caused by three different species of *Stagonosporopsis* (Stewart et al. 2015) with differential fungicide sensitivity (Brewer et al. 2015; Li et al. 2016; Li et al. 2019; Newark et al. 2019). Utilizing GSB-resistant cultivars would reduce yield losses, the high cost of disease control, and diminish hazards resulting from frequent fungicide application.

Traditional breeding methods are time-consuming and resource intensive, limiting the progress made. To accelerate watermelon breeding programs, adequate use of molecular tools such as marker assisted selection (MAS) would be beneficial. This requires a strong understanding of the genetics of the traits. In an effort to improve watermelon cultivar development, **this study aimed to (i) develop high-throughput markers for selection of flowering time (ii) determine the resistance potential in *Citrullus* genotypes infected with different *Stagonosporopsis* isolates and (iii) identify the genomic regions associated with resistance to gummy stem blight in watermelon**.

Watermelon breeding

Like most horticultural crops, it is important to incorporate many important traits into a single watermelon cultivar to meet the needs of the producers and consumers. Both seeded and seedless varieties of watermelon are bred, with the latter preferred in the US and some parts of Europe. Major breeding objectives for watermelon include desired fruit quality, high fruit yield, earliness, seedlessness and disease resistance.

Fruit quality

Watermelons are bred for both internal and external fruit quality. Internal fruit quality includes flesh color, sugar content and flesh texture. Breeders select for high sugar content, indicated by percentage of soluble solids (˚Brix) (Maynard 2001; Hashizume et al. 2003). Marketed watermelon fruit in the U.S. have a Brix value ranging from 10˚ to 14˚ (Maynard 2001). Firm and crisp flesh is usually more desirable than soft flesh. A wide range of flesh colors exist in watermelon, including scarlet red, red, yellow, canary yellow, salmon yellow, orange, and white (Henderson et al. 1998).

External qualities include fruit size and shape, and rind pattern. There are different market requirements for various groups of shippers and consumers with regard to shape and size. Fruit shape categories include round, oval, blocky, or elongate (Kumar 2009) while rind patterns vary from gray to striped and solid green or (Maynard 2001).

Yield

The average marketable yield for watermelon growers in the U.S. is approximately 19.8 metric tons/acre (USDA-NASS 2019). Yield is a quantitative trait, which is controlled by many genes with small effect. Yield components in watermelon include fruit count and fruit size (Kumar and Wehner 2011). Fruit size influences the fruit weight, and depending on size, watermelon fruit in the U.S. are classified into: icebox (<5.5 kg), small (5.5-8 kg), medium (8.1-11 kg), large (11.1-14.5 kg), and giant (>14.5 kg) (Maynard 2001).

Seedlessness

Due to consumer preference for seedless watermelon, there has been an increased production of triploid watermelon in the U.S. The seedless cultivars are developed by crossing a tetraploid plant (2n=4x=44) plant with a diploid plant (2n=2x=22). The

tetraploid plant is developed through artificial chromosomal doubling (Kihara 1951). The resulting triploid cultivar (2n=3x=33) is then pollinated using diploid varieties in the production field. Some seedless watermelon varieties include Fascination (Syngenta), Crunchy Red (Harris Morran), Warrior (Nunhems), Kingman (Sakata), Embasy (Bayer Cropscience), Captivation (Syngenta), Sweet Dawn (Syngenta), Summer Breeze (Seminis) and Joy Ride (Seminis).

Flowering time

Flowering marks the point at which a plant transitions from the vegetative growth phase to the reproductive phase (Levy and Dean 1998). Flowering in plants is regulated by the balance between different gene activities. Both inducers and repressors act to determine the timing of flowering. In *Arabidopsis* and most flowering plants, flowering locus T (*FT*) is one of the major regulators of flowering (Putterill 2001; Mouradov et al. 2002; Putterill et al. 2004). *FT* is believed to be produced in leaves and travels to the shoot apex to induce flowering. It belongs to the phosphatidylethanolamine binding protein family and interacts with the bZIP transcription factor flowering locus D (*FD*). *FD* then recruits *FT* to the promoter of florally expressed genes such as APETALA1 (*AP1*) and LEAFY (*LFY*), inducing the flowering process. Activity of *FT* has to be tightly controlled to ensure correct timing of flowering (Mouradov et al. 2002; Putterill et al. 2004).

The four major pathways that control floral transition include the photoperiodic, autonomous, vernalization, and hormonal (gibberellin) pathways (Mouradov et al. 2002; Simpson and Dean 2002; Poethig 2003; Putterill et al. 2004) (Fig. 1.1). The photoperiod and vernalization pathways respond to environmental signals such as light and temperature, whereas the autonomous and GA-dependent pathways monitor the endogenous

developmental state of the plant. These pathways can act independently to promote flowering, but converge on common downstream flowering pathway genes (Mouradov et al. 2002; Simpson and Dean 2002; Poethig 2003; Putterill et al. 2004). Photoperiodic pathway promotes flowering by day length. Photoreceptors initiate signals that interact with a circadian clock. The circadian clock affects expression of downstream genes that operate in the photoperiodic promotion pathway such as CONSTANS (*CO*) (Putterill et al. 1995). Under long days, GIGANTEA (*GI*) acts upstream of *CO* to increase its expression. Higher *CO* mRNA levels leads to the activation of *FT* and SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1 (*SOC1*) transcription, which ultimately leads to induction of flowering by increased expression of *AP1* and *LFY* (Mouradov et al. 2002). Floral repression through the photoperiod pathway is also needed to ensure a tight regulation of flowering time and TEMPRANILLO (*TEM1*) has been described as a repressor of *FT* (Castillejo and Pelaz 2008). Molecular and genetic analyses have shown that the balance between the activator *CO* and the repressor *TEM1* determines *FT* levels, and that *TEM1* downregulation marks the timing of flowering (Castillejo and Pelaz 2008).

Depending on the growth habit, some plants require a prolonged exposure to low temperatures for them to transition to the reproductive phase. These plants flower through the vernalization pathway, and the major gene involved is Flowering locus C (*FLC*) It encodes a MADS box transcription factor and high levels of its mRNA and protein expression leads to repression of flowering. Vernalization promotes flowering independently of *FLC,* as well as by *FLC* repression (Mouradov et al. 2002). The autonomous pathway is independent of photoperiod and vernalization pathways. It negatively regulates *FLC* expression leading to promotion of flowering. Some genes

involved in this pathway include *FCA/FY, FVE/FPA* and *LD*, which act upstream of *FLC* (Mouradov et al. 2002). Gibberellin, involved in the gibberellic acid (GA) pathway, is a hormone produced by plants that promotes flowering in *Arabidopsis*. High GA levels leads to activation of *LFY* expression (Wilson et al. 1992; Putterill et al. 1995; Blázquez et al. 2002).

Successful pollination and the subsequent development of seeds and fruits depend on plants producing flowers, and therefore flowering at the appropriate time is crucial for reproductive success (Zimmerman 1988; Jung and Müller 2009). Flowering time is an important trait in watermelon production as it determines fruit set, therefore defining the production time. In seedless watermelon production, where diploids are required as pollen sources for the triploid cultivars (Kihara 1951; Andrus et al. 1971), it is essential that the flowering time of the diploid pollenizers and triploids are synchronized for successful fruit production (McGregor and Waters 2014). Earliness, which is associated with flowering time in watermelon production is desirable because through early maturity, crops escape stresses such as diseases, and other abiotic stresses that intensify later in the season (Poland et al. 2009). Two aspects are associated with earliness in watermelon: time from transplant to flowering; and time from pollination to fruit maturity. Cultivars that produce flowers earlier in the season would therefore be ideal as they are likely to result in a shorter production time. This lowers the input costs for the grower and allows for escape from pathogens through earlier harvesting dates, before environmental conditions are favorable for high disease pressure (Maynard and Elmstrom 1992; Poland et al. 2009). Moreover, prices for watermelon are usually best at the beginning of the local season, meaning greater profits for the grower (Mohr 1986; Wehner 2008).

The breeding cycle in watermelon is determined by the time at which the first male and first female flowers are produced, and the interval between the opening of these flowers (Wehner 2008; McGregor et al. 2014). In watermelon, approximately one female flower is produced for every seven male flowers (Wehner 2008). Most wild accessions usually have a longer vegetative growth period and start flowering much later than cultivars and requiring more time for fruit maturity and ripening. Temperature and photoperiod are major factors that influence flowering time (Maheswaran et al. 2000) and in watermelon, flowering is promoted by high light intensity and high temperature (Wehner 2008).

To accelerate breeding, marker assisted selection (MAS) for the flowering time trait can be utilized in various crops. Quantitative trait loci (QTL) associated with flowering time have been identified in major crops such as maize, wheat, soybean and rice among others (Maheswaran et al. 2000; Yano et al. 2001; Thomson et al. 2003; Ducrocq et al. 2009; Yan et al. 2019), however, information on specific genes and mutations linked to the trait in crops remain unclear. Several genes interact to regulate the flowering process with *FT* playing a central role for most species (Kardailsky et al. 1999; Kobayashi et al. 1999; Ahn et al. 2006; Schwartz et al. 2009). Orthologs of *Arabidopsis FT* including *Hd3a* and *SFT* have been determined to promote flowering in rice and tomato, respectively (Lifschitz et al. 2006; Ordonez et al. 2010). In maize, the gene *dlfl* interacts with an *FT* ortholog to promote flowering while a favorable allele of the vernalization gene (*VRN3*) was found to promote flowering in barley and wheat (Ducrocq et al. 2009). FT orthologs have also been found to be part of the signaling system in cucurbits (Lin et al. 2007; Lu et al. 2014).

QTL associated with flowering time have been identified on watermelon chromosomes 2, 3 and 11 (McGregor et al. 2014). The locus that contributed to the highest

phenotypic variation (R^2=50%) for DMF (days to male flower) and DFF (days to female flower) was on chromosome 3 between 12-17Mb (Guo et al. 2013; McGregor et al. 2014). The QTL were identified in a Klondike Black Seeded (KBS; late flowering) x New Hampshire Midget (NHM; early flowering) recombinant inbred line (RIL) population. Among the 172 predicted genes in the major QTL were homologs of *FT* and *TEM1* (TEMPRANILLO) genes associated with flowering time in other species (Kojima et al. 2002; Lin et al. 2007; Castillejo and Pelaz 2008; Lu et al. 2014).

Development of markers that can be used by plant breeders for high-throughput genotyping to select for watermelon flowering time will speed up the development of new cultivars with desired flowering times.

Disease resistance

Selection for desirable fruit quality such as sweet taste, red flesh and appealing flesh color during domestication led to loss of some important traits such as disease resistance (Guo et al. 2013; Levi et al. 2017). Cultivated watermelon has a very narrow genetic base (Guo et al. 2013), which makes it vulnerable to several diseases. Some economically important diseases affecting watermelon include fusarium wilt, gummy stem blight, bacterial fruit blotch, anthracnose, powdery mildew and downy mildew.

Gummy stem blight

Gummy stem blight (GSB) is one of the most prevalent fungal diseases of watermelon and other cucurbits, that leads to major yield losses (Sherbakoff 1917; Chiu and Walker 1949; Sherf and MacNab 1986; Keinath 2011; Stewart et al. 2015). It is a major problem in all regions of the world where cucurbits are grown, which include tropical, subtropical and some temperate areas. The warm temperatures and high humidity

experienced in these regions provide a conducive environment for germination of the pathogen spores and disease development (Keinath 2011). Majority of the species in the Cucurbitaceae family, including watermelon (*Citrullus lanatus*), cucumber (*Cucumis sativus*), cantaloupe and muskmelon (*Cucumis melo*), squashes (*Cucurbita pepo*), and gourds (*Cucurbita* spp.) are affected by GSB (Chiu and Walker 1949; Keinath 2011). GSB was first observed on cucumber (*Cucumis sativus* L.) in 1891 by Fautrey and Roumeguere in France (Chiu and Walker 1949; Sherf and MacNab 1986). In the U.S., the disease was reported for the first time in 1917, affecting watermelon fruit from Florida (Sherbakoff 1917).

Previously, gummy stem blight of cucurbits was thought to be caused by a single pathogen, *Stagonosporopsis cucurbitacearum* (syn. *Didymella bryoniae*) (Aveskamp et al. 2010). GSB was later established to be caused by three species of the genus *Stagonosporopsis: S. cucurbitacearum, S. citrulli and S. caricae* (Stewart et al. 2015). The three species appear similar morphologically, but they differ genetically. Cucurbit hosts are affected by all three species, however, only one species, *S. caricae*, is pathogenic to papaya (Stewart et al. 2015). Variation in fungicide resistance among the different pathogen species has been observed (Brewer et al. 2015; Li et al. 2016; Li et al. 2019; Newark et al. 2019). Among the three, *S. citrulli* was reported as the most widely distributed species causing GSB in southeastern U.S. (Brewer et al. 2015; Stewart et al. 2015).

In warm and humid areas such as the southeastern U.S., the impact of GSB on watermelon production can be severe. In non-sprayed plots, yield losses in watermelon due to GSB averaged about 43% of the maximum yields (Keinath and Duthie 1998). Up to

100% yield losses due to GSB have been reported in Brazil in GSB research plots (Café-Filho et al. 2010). Yield losses incurred due to GSB can result from low fruit weight, or increased fruit rot, or sunburn on fruit due to excess foliage loss (Keinath and Duthie 1998; Keinath 2001).

Two spore stages are produced by the fungus, ascospores (sexual) and conidia (asexual), and the pathogen is self-compatible (Chiu and Walker 1949). On watermelon plants, symptoms develop on all above-ground parts (Maynard and Hopkins 1999; Brewer et al. 2015), and is evident as dark brown lesions on leaves, crown blight and stem cankers. A brown, gummy exudate is often produced on the surface of the lesions that occur on watermelon stems (Maynard and Hopkins 1999). Moreover, extensive defoliation, leaf and plant wilting, and fruit rot may occur in severe cases as well (Sherf and MacNab, 1986; Maynard and Hopkins, 1999).

The epidemiology of GSB is not clearly understood, but the fungus is most likely spread through infected seed (Brown et al. 1970; Lee et al. 1984), air-borne ascospores (Schenck 1968; Van Steekelenburg 1983), or transmitted through the soil (Bruton 1998; Keinath 2002, 2008). It is mostly introduced into greenhouses and fields through seed (Lee et al. 1984; Li et al. 2016) and transplant seedlings that are infected with the pathogen (Keinath 2011). The pathogen can also get established in weeds and plant debris from previously infected cucurbits (Lee et al. 1984; Keinath 2002, 2008, 2011). Ascospores serve as the primary inoculum while conidia, which arise from picnidia, can also be splash-dispersed and serve as secondary inoculum (Schenck 1968). For germination of the fungus and infection of plant tissue to occur, moisture and temperature are key factors. Infection is optimum at temperatures between 21°C – 26°C, and high moisture levels (above 90%

relative humidity), accompanied by leaf wetness (Van Steekelenburg 1984, 1985; Arny and Rowe 1991; Choi et al. 2010).

Management of GSB is mainly through a combination of cultural practices and chemical controls. Cultural practices include: early planting (to escape disease pressure that is highest later in the season); deep plowing (to promote the decay of crop debris that may carry inoculum from previous season); crop rotation; and irrigation practices that limit water splashing and prolonged leaf wetness (Keinath 2002; Stevenson et al. 2004; Keinath 2008). Due to limited effectiveness of cultural practices on their own, fungicides remain critical for successful management of GSB (Stevenson et al. 2004; Keinath 2012). Adequate control of GSB through fungicide applications can be challenging as well. For example, it is difficult to control GSB by spraying during high rainfall seasons when relative humidity remains high for long periods (Keinath 2000). Fungicides are also an additional expense to the farmers and their repeated use may have a negative impact on the environment, particularly if residues persist in the soil. Furthermore, differential sensitivity to fungicides among the three species causing GSB has been reported, posing a major challenge in management of GSB (Brewer et al. 2015; Li et al. 2016; Li et al. 2019; Newark et al. 2019). The best strategy for GSB management would be to utilize GSB-resistant cultivars, but currently commercial watermelon cultivars with high levels of genetic resistance to GSB have not been developed.

GSB resistance breeding in watermelon began in the early 1960s, when PI 189225 was identified as the most resistant accession after evaluation of the USDA-ARS watermelon germplasm (Sowell and Pointer 1962). PI 271778, was later identified as an additional source of resistance (Sowell 1975). Crosses made between the two PI accessions

and elite watermelon cultivars led to the development of AU-Producer (Crimson Sweet × PI 189225) and AU-Jubilant (Jubilee × PI 271778) (Norton et al. 1986). These cultivars were however not resistant to GSB in the field (Song et al. 2002). Gusmini et al. (2005) identified additional accessions that displayed significant levels of resistance to gummy stem blight in both field and greenhouse conditions, which included PI 164248, PI 244019, PI 254744, PI 271771, PI 279461, PI 296332, PI 482276, PI 482379, PI 490383 and PI 526233. Identification of loci linked to GSB resistance will facilitate development of molecular markers that would increase the efficiency of introgression of resistance loci into commercial watermelon cultivars. To date, only one study has described QTL associated with resistance to GSB in watermelon, using PI 189225 as the resistance source (Ren et al. 2019). It is essential to determine whether additional resistant loci are present in other resistant accessions and to develop molecular markers that would allow for more efficient incorporation and potential pyramiding of GSB resistance into elite watermelon cultivars.

Genomic tools for watermelon breeding

Reference genome

Genomes of several economically important crops including maize, soybean, tomato, and rice (Goff et al. 2002; Schnable et al. 2009; Schmutz et al. 2010; The Tomato Genome Consortium 2012) have been assembled over the past twenty years. Currently, the genome sequences of two watermelon cultivars are publicly available: a Chinese elite watermelon inbred line; 97103 (Guo et al. 2013) (Guo et al. 2019) and Charleston Gray, an American watermelon cultivar (Wu et al. 2019).

The availability of the reference genome sequences of watermelon has significantly improved molecular breeding and expedited genetic studies of both qualitatively and

quantitatively inherited traits including fruit traits (Branham et al. 2017; Dou et al. 2018; Fall et al. 2019; Legendre et al. 2020), seed traits (Meru and McGregor 2013; Paudel et al. 2019a; Paudel et al. 2019b), flowering traits (Prothro et al. 2013; McGregor et al. 2014; Gimode et al. 2020), dwarfism trait (Dong et al. 2018) and disease resistance traits (Lambel et al. 2014; Ren et al. 2014; Meru and McGregor 2016a; Meru and McGregor 2016b; Branham et al. 2017; Branham et al. 2018; Branham et al. 2019a; Branham et al. 2019b; Ren et al. 2019).

Genotyping-by-sequencing (GBS)

GBS is an economical approach to finding single nucleotide markers (SNPs) for use in linkage and association mapping, and diversity studies (Elshire et al. 2011). SNPs are single base differences between haplotypes derived from specific DNA sequences linked to a gene or QTL which can be inexpensively assayed in a in a high-throughput manner (Rafalski 2002a; Rafalski 2002b; Collard and Mackill 2008). Due to their abundance, co-dominant inheritance, specificity and high-throughput scoring potential (Rafalski 2002a), SNPs are a powerful tool in QTL mapping and marker-assisted selection (Collard et al. 2005; Collard and Mackill 2008). They are easy to recognize and can be identified with several technologies. Functional SNPs are highly valuable for breeding because there is no recombination between the marker and the gene and they are frequently transferable between populations (Batley and Edwards 2007).

GBS allows for sequencing of DNA fragments tagged with short DNA sequences (barcodes) (Elshire et al. 2011). It utilizes restriction enzymes to target genomic subsets. It is relatively simple and highly multiplexed, facilitating population studies, germplasm characterization and breeding of diverse organisms at a low cost per sample (Elshire et al.

2011). GBS sometimes results in numerous missing data points due to low read coverage resulting from pooled samples, however, missing data can be included in analyses through imputation methods. In watermelon, GBS has been used to generate SNPs that were useful in linkage analysis (Lambel et al. 2014; Ren et al. 2015; Meru and McGregor 2016b; Branham et al. 2017; Branham et al. 2019a; Branham et al. 2019b) and diversity studies (Nimmakayala et al. 2014).

QTL mapping

Quantitative traits are characteristics that vary in degree and are controlled by many genes (Collard et al. 2005). Quantitative trait loci (QTL) are regions within genomes that are associated with a particular quantitative trait and the process of constructing linkage maps and conducting QTL analysis to identify genomic regions associated with traits is known as QTL mapping (Paterson et al. 1988; Mohan et al. 1997; Collard et al. 2005; Collard and Mackill 2008). QTL can only be detected for traits that segregate between the parents and segregating populations such as F_2, F_3 or backcross (BC) populations are often used for mapping. Recombinant inbred lines (RILs) which can be maintained and produced for a long time are however preferable because they allow replicated and repeated experiments and genotypes of the lines are fixed as homozygotes (Collard and Mackill 2008). In watermelon, QTL mapping has been applicable for economically important seed and fruit traits (Prothro et al. 2012a; Prothro et al. 2012b; Sandlin et al. 2012; Branham et al. 2017; Fall et al. 2019), flowering traits (Prothro et al. 2013; McGregor et al. 2014), as well as disease resistance traits (Lambel et al. 2014; Ren et al. 2015; Meru and McGregor 2016a; Meru and McGregor 2016b; Branham et al. 2019a; Branham et al. 2019b).

QTL-seq

QTL-seq is a relatively new genetic approach that employs next generation sequencing (NGS) technology for whole-genome resequencing of two DNA bulks of progeny from a mapping population, with extremely contrasting phenotypic trait values (Takagi et al. 2013). The two DNA bulks are prepared from an equal number of plants with the respective contrasting phenotypes, then whole genome sequencing is carried out, after which QTL identification is performed based on the SNP-index values (Takagi et al. 2013). QTL-seq has been found to be highly effective in identifying SNPs linked to quantitative traits of agronomic interest in different crops including rice, cucumber, chickpea, and peanut (Takagi et al. 2013; Lu et al. 2014; Das et al. 2015; Illa-Berenguer et al. 2015; Singh et al. 2016; Clevenger et al. 2018). In watermelon, it has been used to identify QTL and/or candidate genes associated with dwarfism (Dong et al. 2018), *FON* race 1 resistance (Branham et al. 2018; Fall et al. 2018), egusi trait (Paudel et al. 2019a), seed coat color (Paudel et al. 2019b), and flowering time (Gimode et al. 2020).

Marker assisted selection (MAS)

Marker assisted selection involves the use of DNA markers as a substitute for, or to aid in phenotypic selection (Collard and Mackill 2008). Markers tightly linked to the target loci are ideal and selection is based upon genotype rather than the phenotype (Collard et al. 2005). MAS is beneficial by overcoming the limitations associated with traditional selection strategies, since it is fast, nondestructive, and eliminates the environmental effect that influences estimation of most quantitative traits. Moreover, it can allow for evaluation of multiple traits simultaneously.

SNPs generated from various technologies can be used for MAS to improve efficiency of cultivar development and allow breeders to meet both consumer and grower demands. Utility of MAS requires identification of the loci controlling the trait of interest through methods such as QTL mapping or QTL-seq, validation of the loci, and SNP marker optimization for high-throughput genotyping.

Kompetitive Allele-Specific Assay (KASP)

KASP™ (LGC Genomics, Teddington, UK) is a genotyping technology useful for MAS, which allows for generation of fluorescent signals based on specific alleles present in a DNA sample (Semagn et al. 2014). KASPs includes the test DNA with the SNP of interest; two (FAM or VIC) allele-specific forward primers with unique tail sequences, one reverse primer and the KASP master mix containing the universal FRET cassette. Sample DNA is amplified with a thermal cycler using the allele-specific primers, leading to the separation of fluorometric dye and quencher when the FRET cassette primer is hybridized with DNA. If the genotype at a given SNP is homozygous, only one of the two possible fluorescent signals will be generated. If the genotype is heterozygous, a mixed fluorescent signal will be generated (He et al. 2014; Semagn et al. 2014). KASP assays are quick, easy, cheap, highly specific and can be used in a high throughput manner for genotyping (Semagn et al. 2014).

Research Approach and Objectives

This research focuses on two important aspects of watermelon production: flowering time and resistance to gummy stem blight.

Advancing marker assisted selection for flowering time.

Watermelon production is hindered by both biotic and abiotic stresses, which are intensified by long production cycles. Time to flowering is a determining factor in early fruit production for growers, which results in reduced production costs and increased profitability. Seedless watermelon production also requires synchronized flowering of female (triploid) and male (diploid) plants in the field. This study addresses these issues by studying the genetic control of flowering time. The objective was to develop high throughput molecular markers that can be used to select for the early flowering trait to enable acceleration of cultivar development. The focus was to refine a QTL associated with flowering time and identify candidate genes and markers linked to the trait. This was done by examining recombinants in a RIL mapping population as well as other $F_{2:3}$ segregating populations, developed from a cross between two watermelon cultivars with contrasting flowering time phenotypes. For validation, marker performance was also tested on a panel of watermelon cultivars with different genetic backgrounds. The results provide more insight into the regulation of flowering time in watermelon and will have implications in watermelon breeding programs. DNA assays that can be used by plant breeders to select for flowering time were developed.

Evaluate Citrullus germplasm based on new GSB pathogen classification.

A major challenge for growers in Georgia and the southeast is the hot and humid weather that is conducive for plant disease development, especially fungal diseases. For the sustainable production and marketing of watermelon, strategies to overcome constrains such as disease pressure need to be developed. Gummy stem blight is one of the most important watermelon diseases and currently no resistant cultivars are available to growers.

Moreover, management of GSB is further complicated by the fact that it is caused by three different species of *Stagonosporopsis* that exhibit differential sensitivity to fungicides. The important question that arises is: Do watermelon genotypes respond differently to the three species? To establish this, we investigated the effect of different *Stagonosporopsis* isolates on various *Citrullus* genotypes. With the isolates evaluated, we noted that isolate aggressiveness was not species-dependent, and some wild relatives of watermelon exhibited broad resistance to the *Stagonosporopsis* isolates, making them a good choice for GSB resistance breeding.

Develop markers for GSB resistance breeding.

Due to lack of genetic resistance in commercial watermelon cultivars, growers constantly suffer severe yield losses from GSB. Resistance has been found in wild relatives of watermelon, but introgression into elite germplasm has been unsuccessful. We therefore sought to identify chromosomal regions associated with resistance to GSB, with a future goal of resistance introgression into the commercial varieties. To accomplish this, we used an interspecific population developed from a cross between a susceptible Crimson Sweet and resistant PI 482276 to map GSB resistance. We then examined regions within the loci displaying significant association with the trait and identified some candidate genes. High throughput molecular markers linked to the trait were identified which will provide a useful tool for marker assisted selection of the loci in watermelon breeding programs. This will speed up the breeding process for resistant cultivars that will eventually reduce the need for weekly fungicide sprays, leading to reduced costs per acre for growers.

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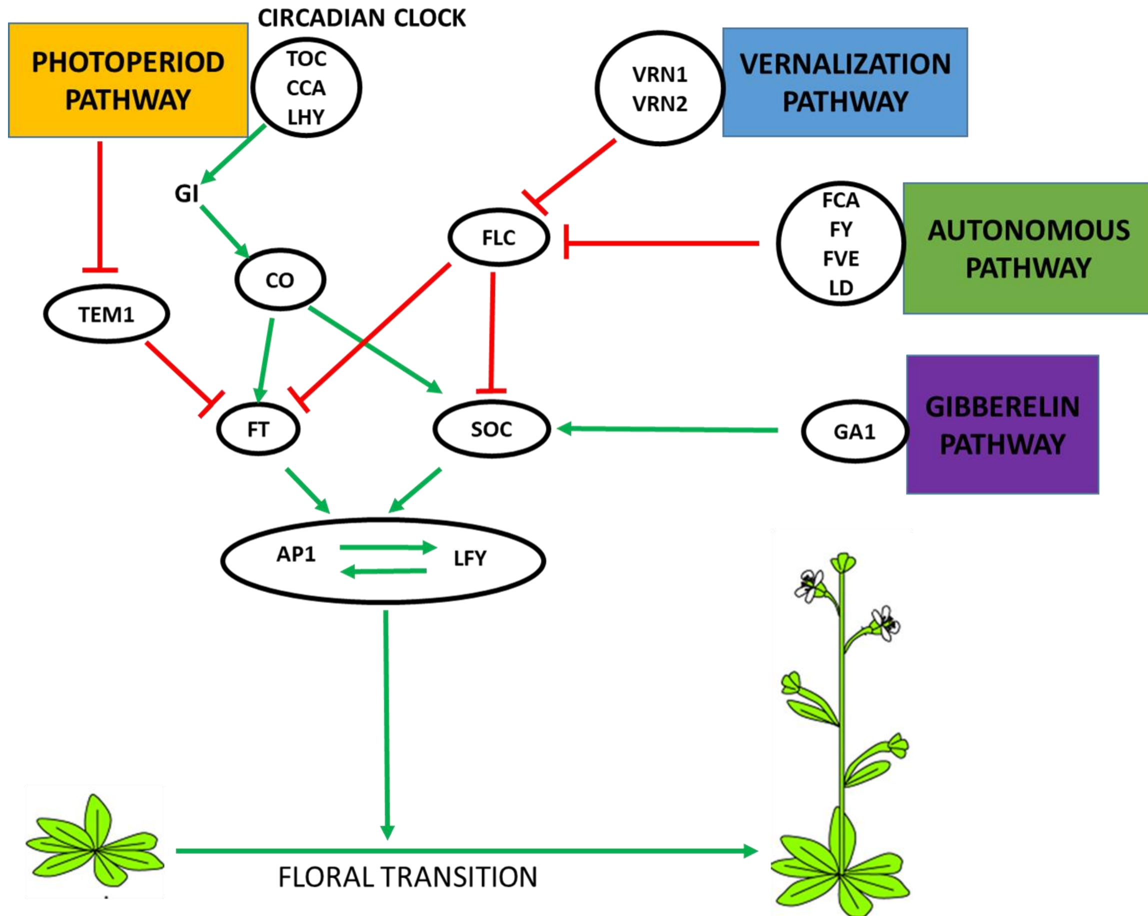

Fig 1.1: Pathways and major genes controlling flowering in *Arabidopsis*. Modified from Moradov et al. (2002); Putterill et al. (2004). Green arrows indicate up-regulation while red lines with bars indicate repression. Genes include: TOC (Timing of CAB 1), CCA (Circadian clock associated), LHY (Late elongated hypocotyl) VRN (Vernalization), GI (GIGANTEA), CO (CONSTANS), TEM1 (TEMPRANILLO) FT (Flowering Locus T), FLC (Flowering locus C), SOC (Suppressor of Overexpression of CO1), GA1 (Gibberellic acid 1), AP1 (APETALA 1), LFY (LEAFY).

CHAPTER 2

FINE-MAPPING OF A MAJOR QUANTITATIVE TRAIT LOCUS *Qdff3-1* CONTROLLING FLOWERING TIME IN WATERMELON

[1]Gimode W, Clevenger J, McGregor C (2020). Fine-mapping of a Major Quantitative Trait Locus *Qdff3-1* Controlling Flowering Time in Watermelon. Mol Breed 40 (1):1-12. Reprinted here with permission of the publisher

Abstract

Flowering time is crucial in watermelon (*Citrullus lanatus*) production as it determines time of fruit set. Early flowering is desirable because it enables crops to escape biotic and abiotic stresses that are intensified by long production cycles. Production of seedless watermelon is also reliant on synchronized flowering of diploid pollenizers and the triploid watermelon cultivars. Utilizing marker assisted selection (MAS) of flowering time in watermelon breeding would potentially aid in selection for early flowering, which would shorten the production time. A major quantitative trait locus *Qdff3-1* (12-17 Mb) associated with days to female flower (R^2=50%) was previously identified on chromosome 3 of watermelon. In this study, we validated the *Qdff3-1* locus using QTL-seq. To determine more precisely the interval of *Qdff3-1* and the candidate gene controlling flowering time, SNP markers were identified in the region and Kompetitive Allele Specific PCR (KASP) assays were developed for high throughput genotyping. Markers were tested for trait association on the mapping population, recombinant $F_{2:3}$ populations and a panel of differential cultivars. In the KBS × NHM genetic background the QTL was delineated to a 1.13 Mb region, flanked by markers UGA3_14537958 and NW0248748. This region includes the *FT* and a protein phosphatase 2C (*PP2C*) gene. Genotyping the regions of interest in a panel of genetically diverse cultivars suggest that genetic control of flowering time in watermelon is dependent on the genetic background. These results lay the foundation for a greater understanding of flowering mechanisms in watermelon and improved breeding strategies for this trait.

Keywords: *Citrullus lanatus*, flowering time, *FT*, MAS, SNP, *PP2C*, QTL-seq

Introduction

Flowering represents the transition from the vegetative to the reproductive growth phase of a plant (Levy and Dean 1998) and is essential for reproductive success (Zimmerman 1988). Flowering at the appropriate time is crucial because pollination and subsequent seed and fruit development depend on it (Jung and Müller 2009). In watermelon production, flowering is an important trait as it determines time of fruit set and therefore earliness of a cultivar. Earliness is desirable because it allows crops to escape diseases and other abiotic stresses that intensify later in the season (Poland et al. 2009). In addition, cultivars that produce flowers early in the season can result in shorter production times. Through the use of early flowering cultivars, grower input costs are significantly reduced and their profits increased through earlier harvesting dates (Mohr 1986).

Due to consumer demand for seedless fruit in the United States, there has been an increase in the production of triploid watermelon to approximately 90% of the U.S. watermelon market (USDA-NASS 2017). Diploid pollenizers are required for successful production of seedless watermelon because triploid plants used to produce the seedless fruit lack sufficient viable pollen (Maynard and Elmstrom 1992). Synchronized flowering time between the pollenizers and triploid cultivars is thus essential in seedless watermelon production (McGregor and Waters 2013).

The production cycle in watermelon is determined by the time at which the first male and first female flowers are produced, and the interval between the opening of these flowers (Wehner 2008; McGregor et al. 2014). Temperature and photoperiod are major factors that determine when a plant will flower and set seed (Maheswaran et al. 2000) and in watermelon, this is promoted by high light intensity and temperature (Wehner 2008).

Approximately one female flower is produced for every seven male flowers in watermelon (Wehner 2008), however, this depends on the cultivar and the environmental conditions.

Several genes have been found to control flowering in *Arabidopsis,* and pathways that control floral transition have been determined. These pathways include the photoperiodic, autonomous, vernalization, and hormonal (gibberellin) pathways (Mouradov et al. 2002; Simpson and Dean 2002; Poethig 2003; Putterill et al. 2004). In many species of flowering plants, flowering locus T (*FT*) appears to be one of the major genes that regulates flowering and it has been found to be part of the signaling system in cucurbits as well (Kardailsky et al. 1999; Ahn et al. 2006; Lin et al. 2007; Schwartz et al. 2009; Lu et al. 2014).

MAS for flowering time would be useful in breeding programs of several economically important crops. Quantitative trait loci (QTL) related to flowering time have been identified in various crops such as maize, wheat, soybean and rice (Maheswaran et al. 2000; Yano et al. 2001; Thomson et al. 2003; Yan et al. 2006; Ducrocq et al. 2009). A major gene, *Hd3a*, which is an ortholog of *Arabidopsis FT,* had a functional mutation associated with days to flowering in rice (Ordonez et al., 2010). In barley and wheat, a favorable allele of the vernalization gene (*VRN3*) was found to promote flowering, while in maize, the gene *dlfl* is hypothesized to interact with an *FT* ortholog to promote flowering (Ducrocq et al. 2009). In tomato, two QTL associated with days to flowering (DTF) were detected (Jimenez-Gomez et al. 2007; Sumugat et al. 2010) and an allele of the gene *SFT,* which is an ortholog of *FT* in *Arabidopsis*, promoted flowering (Lifschitz et al. 2006). Flowering time QTL have also been mapped in Brassica crops (Ferreira et al. 1995; Osborn et al. 1997; Bohuon et al. 1998; Robert et al. 1998; Rae et al. 1999) and genes orthologous

to *CONSTANS* (*CO*) in *Arabidopsis* were identified as candidate genes (Robert et al. 1998). In cucumber, *Csa1G651710*, which is an ortholog of the *FT* gene in *Arabidopsis*, was also identified as a possible candidate gene for early flowering (Lu et al. 2014). Although QTL associated with flowering time have been identified in most crops, information on specific genes and functional mutations associated with the trait remain elusive.

A major QTL associated with days to male flower [DMF (*Qdmf3-1*)] and days to female flower [DFF (*Qdff3-1*)] which contributed approximately 50% of the phenotypic variance in the Klondike Black Seeded (KBS; late flowering) × New Hampshire Midget (NHM; early flowering) recombinant inbred line (RIL) population (n=145), was previously identified on chromosome 3 of watermelon (McGregor et al. 2014). This QTL had a LOD-1.5 support interval stretching from 8.2 to 12 cM (≈12-17 Mb) (Guo et al. 2013; McGregor and Waters 2014) and a significant positive correlation between DMF and DFF (0.79, $P < 0.0001$) was observed in this population. One hundred and seventy two genes were predicted within this region (Guo et al, 2013), including *Cla009504*, an ortholog of the *FT* gene associated with flowering time in other species (Kojima et al. 2002; Lin et al. 2007; Lu et al. 2014), and *Cla000855*, an ortholog of the TEMPRANILLO (*TEM1*) gene in *A. thaliana* (Castillejo and Pelaz 2008). *TEM1* has been described as a transcription factor, regulated by the circadian clock which acts as a repressor of *FT* (Castillejo and Pelaz 2008).

Understanding the genetic control of flowering time in watermelon may reduce the production time by enabling selection for early flowering. It also has applications in seedless watermelon breeding as it could inform selection of the most suitable pollenizers for the triploid cultivars. This study specifically focused on DFF, since fruit set occurs on the female flower. Due to the high correlation between DMF and DFF however, association

between these two phenotypes would be expected. In this study we validated and refined the previously identified flowering time locus (*Qdff3-1*) using the QTL-seq method (Takagi et al. 2013), we developed KASP markers to span the region, used $F_{2:3}$ populations to further fine-map the region and tested the markers on a diverse cultivar panel.

Materials and methods

Plant materials

The F_6 KBS × NHM RIL mapping population used in the study was developed from a cross between KBS and NHM watermelon cultivars (Sandlin et al. 2012) with contrasting flowering time phenotypes (McGregor et al. 2014). The F_2 populations used for fine-mapping were obtained from selfing four different F_1 plants from the same cross.

DNA extraction and candidate gene sequencing

The two candidate genes identified within the previously identified *Qdff3-1* locus by Sandlin et al (2012), *Cla009504 (FT)* and *Cla000855* (*TEM1)* were sequenced (Eurofins Genomics LLC, Louisville, KY) in KBS and NHM. Genomic DNA was extracted from approximately 50 mg of young leaf tissue frozen in liquid nitrogen using the E.Z.N.A. Plant DNA kit (Omega Bio-Tek Inc., Norcross, GA) following the manufacturer instructions. The DNA was quantified using a Tecan NanoQuant Plate™ (Tecan, Group Ltd., Mannedorf, Switzerland) compatible with an Infinite M200pro reader (Tecan Group Ltd.). PCR amplifications were carried out in 20 µl containing 12.6 µl ddH_2O and 0.6 µl DMSO, and final concentrations of 1× Phusion HF Buffer, 0.2 mM dNTPs, 0.5 µM of forward and reverse primers (Supplementary Table 2.1), 0.4 U/µl Phusion Taq Polymerase (New England BioLabs Inc., Ipswich, MA) and 100 ng/µl genomic DNA. PCR conditions consisted of an initial incubation at 98°C for 30s, 35 cycles at 98°C for 5s, primer annealing

temperature for 20s, 72°C for 30s, followed by 1 cycle of 72°C for 10 min and then held at 4°C. Prior to sequencing, amplification of PCR products were confirmed by gel electrophoresis using 3 µl of the product on a 1.5% agarose gel. The PCR products were then purified using the E.Z.N.A. Cycle Pure kit (Omega Bio-Tek Inc.). Sanger sequencing was performed for two plants of each cultivar at Eurofins Genomics LLC. Sequences were quality trimmed then aligned to the 97103 watermelon genome (Guo et al. 2013) using Geneious 11.1.4 (http://www.geneious.com).

QTL-seq

To validate the location of the *Qdff3-1* locus and identify additional SNPs for fine-mapping, QTL-seq (Takagi et al. 2013) was performed.

I. Phenotyping

The KBS × NHM RIL population (n = 159) previously used to map flowering time (McGregor et al., 2014) was used for this study. During summer 2016, the population, parental and F_1 plants were transplanted at the Durham Horticulture Farm in Watkinsville, GA. Using a randomized complete block design (RCBD) with 5 blocks, one plant per RIL and 2 plants per parental cultivar/F_1 were planted per block. Plants were grown on plastic mulch with between-row spacing of 1.83 m and in-row spacing of 1.22 m. Data was collected three times a week, for days to anthesis for the first female flower (DFF).

II. Bulk construction and DNA extraction

RILs for each bulk were selected from the KBS × NHM population based on the average of the 2012 (McGregor et al., 2014) and 2016 flowering time data. The 2013 data described in McGregor et al. (2014) was not used due to the unusually high amount of rainfall in the period after transplanting during that year, which had an effect on flowering.

Two DNA bulks, ‘early’ (E-bulk: DFF=21 ± 0.8) and ‘late’ (L-bulk: DFF=32 ± 0.9), were constructed using DNA from 15 RILs each. DNA of the selected individuals was extracted and quantified as previously described for the sequenced samples. Equal amounts of DNA from the 15 respective individuals were bulked and shipped for library construction and 151 bp paired-end whole genome Illumina sequencing on the Illumina HiSeq X (Illumina, San Diego, CA) at the Hudson Alpha Genomic services laboratory (Huntsville, AL).

III. Analysis of short reads and SNP identification

FastQC (Andrews, 2010) was used to analyze the quality of the short reads obtained. The 97103 watermelon genome sequence (Guo et al. 2013) was indexed using BWA, after which reads were aligned using BWA MEM (Li et al. 2009). The reads were then converted from SAM to BAM format using SAMtools (Li et al. 2009), and the converted files were sorted and indexed. Genotype likelihoods were calculated using SAMtools and SNP calling was performed using BCFtools. The SNPs were then filtered with a minimum depth of 10 using a custom python script. A SNP-index value for each SNP was obtained by dividing the number of reads matching the reference by the total number of reads for that SNP. This was calculated separately for the ‘early’ (E) and ‘late’ (L) bulks. The ΔSNP-index was then calculated by subtracting the SNP-indices (E-bulk and L-bulk) of the bulks (Takagi et al. 2013). A threshold for $P < 0.01$ was calculated for the population, taking into account the population size, number of individuals in each bulk and read depth. To identify regions of the genome highly associated with flowering time, 1 Mb sliding window analysis with a 10 Kb step increment was applied.

KASP marker development and genotyping

DNA was extracted from leaf tissue of the RIL mapping population using a salt extraction method (King et al. 2014) with the following modifications: The tissue sample plates stored at -80˚C were placed in liquid nitrogen prior to using a TissueLyser II (QIAGEN, Hilden, Germany) for sample grinding. 500µl of the SDS/NaCl extraction buffer [40% (v/v) 5M NaCl and 60% (v/v) extraction buffer (200 mM Tris/HCl pH 7.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS)] was used and plates were centrifuged for 10 min at 3600 rpm.

KASP primers (Supplementary table 2) for SNPs identified through candidate gene sequencing and QTL-seq were designed and optimized using Primer3Plus (Untergasser et al, 2007). The KASP primers spanning the QTL region were designed for high throughput genotyping of the populations and cultivars and were named with the prefix 'UGA3_' followed by the physical position of the SNP on the chromosome in Mb. KASP primers for SNPs in the genetic map described by Sandlin et al. (2012) (NW0247945:12,804,860 Mb and NW0248748: 15,664,017 Mb) were not renamed. KASP assays were carried out in 4 µl volumes containing 1.94 µl of 2x low rox KASP master mix (LGC Genomics LLC, Teddington, UK), 0.06 µl primer mix with a final primer concentration of 0.81 µM, and 2 µl of 50-100 ng/µl genomic DNA. The PCR conditions used for the KASP assays consisted of an initial incubation at 95°C for 15 min, 10 cycles of touch down PCR with 20s at 95°C, 25s of primer annealing temperature + 9°C with 1°C decrease each cycle, and 15s of 72°C, followed by 35 cycles of 10s at 95°C, 1 min at primer annealing temp, and 15s at 72°C, then held at 4°C. KASP fluorescent end-point readings were measured using an Infinite M200Pro plate reader (Tecan Group Ltd.) and genotype calls were made using

KlusterCaller (LGC Genomics LLC). Flapjack version 1.18.06.29 (Milne et al. 2010) was used for haplotype visualization. To determine the marker/trait association, a one-way ANOVA followed by a Tukey-Kramer HSD test was used. R^2 values of each marker were tested using the Kruskal-Wallace test ($P = 0.05$) to determine the association of genotypes with flowering time in the mapping population and cultivar panel.

Fine-mapping the QTL interval for *Qdff3-1*

To determine more precisely the interval of *Qdff3-1* and the candidate gene(s) controlling the trait, recombinants were identified. From the F_6 KBS × NHM RIL population, one line (RIL-190) carrying a recombination in one of the major recombination points was selected. Three hundred and seventy-six seedlings from four KBS × NHM F_2 populations were genotyped using KASP markers from different haplotype blocks identified in the F_6 RIL population. Six recombinants (RIL-190 and five F_2 lines) were selected to contain heterozygous or homozygous genotypes at three different recombination points. The recombinant plants were selfed and the resulting seeds were sown in the greenhouse and the seedlings transplanted 5 weeks later at the UGA Durham Horticulture farm for flowering time evaluation in summer 2018. The populations, consisting of approximately 70 plants each and 7 plants each of parents and F_1s, were transplanted on June 7th, 2018, with between-row spacing of 1.83 m and in-row spacing of 1.22 m. The same parental and F_1 plants were used as controls for all populations. Phenotypic data was collected three times a week for DFF. Leaf tissue was collected from all plants of the six populations and DNA was extracted and KASP assays were performed to delineate the QTL region as previously described.

Validation using cultivars

A cultivar panel was compiled from watermelon of various pedigrees, consisting of both early and late flowering genotypes. The panel included: Sugar Baby (SB), NHM, Sugar Lump (SLMP), Strain II (SII), Mickylee (MICK), Golden Russian (GR), Charleston Gray (CG), Crimson Sweet (CS), AU-Producer (AUP), Calhoun Gray (CALG), Hopi Yellow (HOPI), Navajo Red (NAV), Sangria (SANG), KBS, Orangeglo (OG), Estrella (ES) and Allsweet (AS) (Supplementary Table 2.3).

In summer 2016 and 2017, 2 plants of each cultivar and the mapping population parents were transplanted per block in an RCBD with 5 blocks and 7 blocks, respectively. Data from the two years was averaged for analysis. All field experiments were done at the Durham Horticulture Farm in Watkinsville, GA. Similar to the RIL and recombinant populations, data was collected three times a week for DFF, after which leaf tissue was sampled for DNA extraction and genotyping as described earlier.

Results

SNP identification from candidate gene sequencing.

The *Cla000855* [12,382,520-12,383,470 Mb (*TEM1*)] gene (951 bp) consists of 1 exon, while the *Cla009504* [14,874,778-14,879,348 Mb (*FT*)] gene (4,571 bp) consists of 4 exons (Guo et al. 2013). No SNPs were identified in the exons of either of these genes (data not shown). For *FT*, 8 SNPs were found within the intron (*Cla009504*_14876335, 14876592, 14877025, 14877848, 14877931, 14878293, 14878858, and 14878904). Sequencing 1,944 bp upstream of *FT* also revealed 5 SNPs between KBS and NHM. These SNPs include *Cla009504*_14872838, 14872843, 14872958, 14873014 and 14873563. *FT* was also sequenced 965 bp downstream but no SNPs were identified between KBS and

NHM in this region. All the SNPs identified upstream and within the introns of *FT* had the same haplotype for all genotypes tested in this study. Only one SNP (*Cla009504*_14877931: KASP primer UGA3_14877931) is therefore shown to represent results from all *FT* SNPs (Supplementary fig 2.1).

QTL-seq

A total of 206,385,328 (99.32%) and 215,194,945 (99.42%) mapped reads were obtained through QTL-seq for the E and L bulks, respectively. On chromosome 3, 38,240 SNPs were identified between the two bulks and a significant QTL was detected between ≈10.7-16.2 Mb (Fig. 2.1a), which overlapped with the region previously identified as the major QTL using classical QTL mapping (≈12-17Mb) (McGregor et al. 2014). Several peaks were observed in this region, with 3 major peaks above the threshold signifying the possibility of more than one QTL/gene controlling the trait within the region. On further analysis of the significant regions, another potential candidate gene *Cla002795* (11,043,539-11,046,772 Mb) was identified at 11.04 Mb on chromosome 3 of watermelon. This gene is a phosphatidylinositol-4-phosphate 5-kinase (*PIP-kinase*), and is orthologous to a gene previously described to be involved in regulation of flowering time in *Arabidopsis* (Akhter et al. 2016) and rice (Ma et al. 2004). QTL-seq also identified numerous SNPs showing significant association with the flowering trait in this region. Twenty SNPs concentrated in the peak regions were selected for KASP assay development (Fig. 2.1b).

Marker-trait association in mapping population

KASP assays were designed for SNPs across the 10.7-16.2 Mb region (Supplementary Table 2) and tested on the KBS × NHM RIL population to determine which markers showed the highest association with the flowering phenotype. All markers

within the QTL region tested on the RIL displayed significant association with the flowering trait ($P < 0.0001$), with R^2 values ranging from 0.37 to 0.63 (Supplementary fig. 2.1). Based on the KASP assays, 22 recombinants out of the total 159 RILs were identified in the *Qdff3-1* region (Fig. 2.2a, Supplementary fig. 2.1). The recombinants were used to identify regions in the QTL that displayed the highest association with the phenotype. Similar marker performance was observed from the 11.05-16.34 Mb region for most markers. However, three RILs, RIL-146 and RIL-112, which were early flowering, and RIL-4, which was late flowering, indicated that the region after NW0248748 (15.66 Mb) was not associated with the trait. Better marker association was observed from 11.05-15.66 Mb flanked by markers UGA3_11046548 and NW0248748, where most of the earlier and later flowering recombinants had the NHM and KBS allele respectively. This region hosts both the *TEM1* and *FT* genes. The QTL was therefore narrowed down to ≈4.62 Mb by genotyping the KBS × NHM RIL population with additional markers. One of the recombinants, RIL-190, was selected for additional fine-mapping purposes because it was heterozygous in the 11.05-15.66 Mb region, which showed the highest association with the trait (Supplementary fig. 2.1).

<u>Fine mapping using recombinants</u>

In addition to RIL-190, 376 F_2 plants were genotyped to identify more recombinants to further refine the QTL. Five recombinants, KxNF$_2$-204, KxNF$_2$-205, KxNF$_2$-217, KxNF$_2$-218 and KxNF$_2$-222, were selected from the F_2 plants following genotyping. These five F_2 recombinants were heterozygous in different regions; from NW0247945 to UGA3_16339961, UGA3_10738714 to UGA3_14537958, UGA3_10738714 to UGA3_11046548, UGA3_11046548 to UGA3_15774973 and UGA3_11046548 to

UGA3_16339961, respectively (Fig. 2.2b). The RIL-190 (F_7) population (F_7RIL-190), which was heterozygous from UGA3_11046548 to UGA3_16339961, and the five $F_{2:3}$ populations were used for fine mapping. The continuous phenotypic distributions of DFF in the six recombinant families confirmed the quantitative nature of the trait (Fig. 2.3). The average DFF was 16.17 for the earliest flowering population (KxN3-205) and 27.22 for the latest flowering population (KxN3-217). F_7RIL-190, KxN3-204, KxN3-218 and KxN3-222 populations had average DFFs of 25.84, 21.13, 25.55 and 18.91, respectively.

Progeny testing of the six families showed that the QTL was located between UGA3_14537958 and UGA3_16339931. A significant difference between the NHM-type and KBS-type progeny was observed in the segregating regions of all populations except KxN3-205 and KxN3-217 (Table 1; Fig. 2.2b). These two populations, which were the earliest and latest flowering, respectively, were both homozygous between UGA3_14537958 and UGA3_16339931 markers. Based on results from the KBS × NHM RIL mapping population, with NW0248748 already determined as the right flanking marker, the QTL was further narrowed down to ≈1.13 Mb with results from the six populations (Fig. 2.2b). The region is flanked by markers UGA3_14537958 and NW0248748 and includes *FT* (*Cla009504*).

Cultivar panel

The majority of the cultivar panel had an intermediate to later flowering time, with few earlier flowering cultivars. Average DFF of the cultivar panel for 2016 and 2017 ranged from 16.4 to 36.8, with SB and AS being the earliest and latest flowering cultivars, respectively. SB, NHM, SLMP, SII and MICK were designated as ‘early’ cultivars (DFF=

16.4-24.6) while GR, CG, CS, AUP, CALG, HOPI, NAV, SANG, KBS, OG, ES and AS (DFF=30.2-36.8) were in the 'late' category (Supplementary fig. 2.2).

Marker performance was tested on the panel with different genetic backgrounds (Fig. 2.2c). Three markers (UGA3_10738714, UGA3_10795402, and UGA3_11016809) in the 10.7 to 11.04 Mb region were highly associated (R^2 = 0.80) with the phenotype (Supplementary fig. 2.2). This is in contrast to the KBS × NHM RIL population, where fine mapping excluded this region (Fig. 2.2), suggesting an additional region associated with control of flowering time in these diverse genetic backgrounds. The NW0248748 marker (15.66 Mb; R^2 = 0.60) gave the expected genotype for all cultivars except Golden Russian and Navajo. This marker was also the right flanking marker identified from the F_6 KBS × NHM RIL population recombinants, further validating the association of this region with DFF. Taking into account the cultivar panel, the 239 kb region between UGA3_15424397 and NW0248748 markers (Fig. 2.2c) displayed significant association with the trait. There are 15 genes (Supplementary Table 2.4) within this region, 7 with unknown function.

<u>Marker-trait association</u>

UGA3_15424397 and NW0248748 showed higher association in the mapping and recombinant populations compared to UGA3_10795402. However, in the cultivar panel UGA3_15424397 did not exhibit high association with the trait as all cultivars, except NHM, had the KBS genotype. The NW0248748 marker displayed better performance than UGA3_15424397 with all the earlier flowering cultivars having the NHM genotype while the later flowering cultivars had the KBS genotype, except Golden Russian and Navajo.

UGA3_10795402, unlike what was observed on the populations, exhibited significantly higher association with the trait on the cultivar panel (Fig. 2.4).

Discussion

Flowering time plays a key role in watermelon production as it is a major determinant of earliness and also a major factor in seedless watermelon production. Genetic control of flowering time is generally quantitative in nature and displays a continuous phenotypic variation. Several genes are involved in the regulation of this trait and flowering locus T (*FT*) has been described as one of the major genes that controls flowering in several species (Kardailsky et al. 1999; Kobayashi et al. 1999; Ahn et al. 2006; Lin et al. 2007; Lu et al. 2014). In this study, we used recombinants from a KBS × NHM RIL mapping population, KBS × NHM $F_{2:3}$ families and a cultivar panel of different watermelon genotypes to refine the *Qdff3-1* locus controlling flowering time in watermelon.

Previously, a major QTL (≈12-17 Mb) governing flowering time was identified on chromosome 3 of watermelon using conventional QTL mapping (McGregor et al., 2014). Among the 172 predicted genes within this region, *FT* and *TEM1* were previously proposed as candidate genes controlling flowering time in watermelon (McGregor, 2014). These two genes were sequenced in this study but no SNPs were identified in the exons of either of the genes. *FT*, which was a more obvious candidate gene, was also sequenced 1,944 bp upstream and 965 bp downstream. Since the QTL region described in (McGregor et al. 2014) was very large (≈5 Mb; 3.8 cM), we wanted to narrow down the region to identify a candidate gene and SNPs that could be useful for marker assisted selection. A significant QTL was observed on chromosome 3 of watermelon from 10.7-16.2 Mb, validating the previous results. QTL-seq has been found to be highly effective in identifying markers

linked to quantitative traits of agronomic interest in different crops including rice, cucumber, chickpea and peanut (Takagi et al. 2013; Lu et al. 2014; Das et al. 2015; Illa-Berenguer et al. 2015; Clevenger et al. 2018). More recently, it has been used to identify a dwarfism gene in watermelon (Dong et al. 2018). Several SNPs were identified in this region and were converted to KASP assays for high-throughput genotyping. The region was fine mapped using KBS × NHM populations developed from recombinants, which refined the region to a 1.13 Mb interval flanked by markers UGA3_14537958 and NW0248748. The *FT* gene (*Cla009504)* is located within this region.

Additional recombinants to further delineate the region were not available in the KBS × NHM genetic background. We turned our attention to historical recombination found in watermelon cultivars. All the cultivars in the cultivar panel, including early cultivars had the late (KBS) genotypes in the region spanning the *FT* gene (UGA3_14537968 to UGA3_15424397). *FT* transcription in *Arabidopsis* is controlled by distant (4.0 – 5.7 kb) *cis*-regulatory elements upstream of *FT* (Adrian et al. 2010). However, the KBS haplotype block in the cultivar panel stretches over ~2 Mb (UGA3_13418651 to UGA3_15424397). However, we only investigated SNPs and many other polymorphisms and modifications can affect gene function. Therefore, we cannot totally exclude the *FT* gene as playing a role in flowering time in these genetic backgrounds.

Marker NW0248748 had the expected allele for all but 2 of the cultivars. A possible explanation for our results is that the gene associated with DFF is found in the 239 kb between UGA3_15424397 and NW0248748. Recombination on either side of the gene, between the flanking markers would give the observed results. Despite several attempts,

we were unable to design additional KASP assays within this region to determine more accurately the recombination point. The 239 kb region contains 15 genes, including *Cla009546,* which is a protein phosphatase 2C (*PP2C)*. *PP2C* has been described as a positive regulator of flowering through regulation of the transcript levels of integrators and floral meristem identity genes, such as *Flowering locus C* (*FLC*)*, CO, Suppressor of overexpression of CONSTANS 1* (*SOC1*)*, LEAFY* (*LFY*)*, FT* and *Flowering Locus D* (*FD*) in *Arabidopsis* (Zhai et al. 2016) (Supplementary Table 2.3). This study proposes it as a major candidate gene that contributes to the genetic variation in watermelon flowering time. Further research quantifying gene expression of these candidate genes in diverse watermelon cultivars will be needed to confirm the roles of the different candidate genes in the control of flowering time in watermelon.

High associations (R^2 = 0.80) were observed for markers between UGA3_10738714 and UGA3_11016809 and DFF in the cultivar panel. This was contrary to observations in the KBS × NHM RIL and recombinant populations, where markers in this region displayed low association with the trait (0.37-0.45) and progeny tests found no significant difference in DFF between KBS and NHM genotypes. These results suggest that there is differential regulation of flowering in the diverse genetic backgrounds represented in the cultivar panel.

The most appropriate marker to use for marker assisted selection for flowering time in watermelon will depend on the genetic background used. UGA3_15424397 is effective for selection for DFF in the KBS × NHM background, but does not perform well in other backgrounds. NW0248748 may be useful to select for early flowering when SB (red flesh), SLMP (white flesh), SII (yellow flesh) or MICK (pink flesh) is used as a source of the early

allele, as long as the non-donor is not NAV or GR. UGA3_10795402 might be useful when NAV or GR are used, as long as MICK is not the early flowering donor. The utility of these markers for marker assisted selection in diverse backgrounds should be validated in segregating populations.

In conclusion, we refined the previously identified QTL region associated with DFF in KBS × NHM to a ≈1.13 Mb region of chromosome 3. A new candidate gene (*PP2C*) previously described to regulate flowering time genes in *Arabidopsis* was identified within this region. The results from diverse watermelon germplasm suggests that control of flowering time is dependent on genetic background. These findings provide more insight into the regulation of flowering time in watermelon and will have implications in watermelon breeding programs. The earlier harvesting days for the growers associated with the use of early flowering cultivars will reduce grower input costs and increase their profits.

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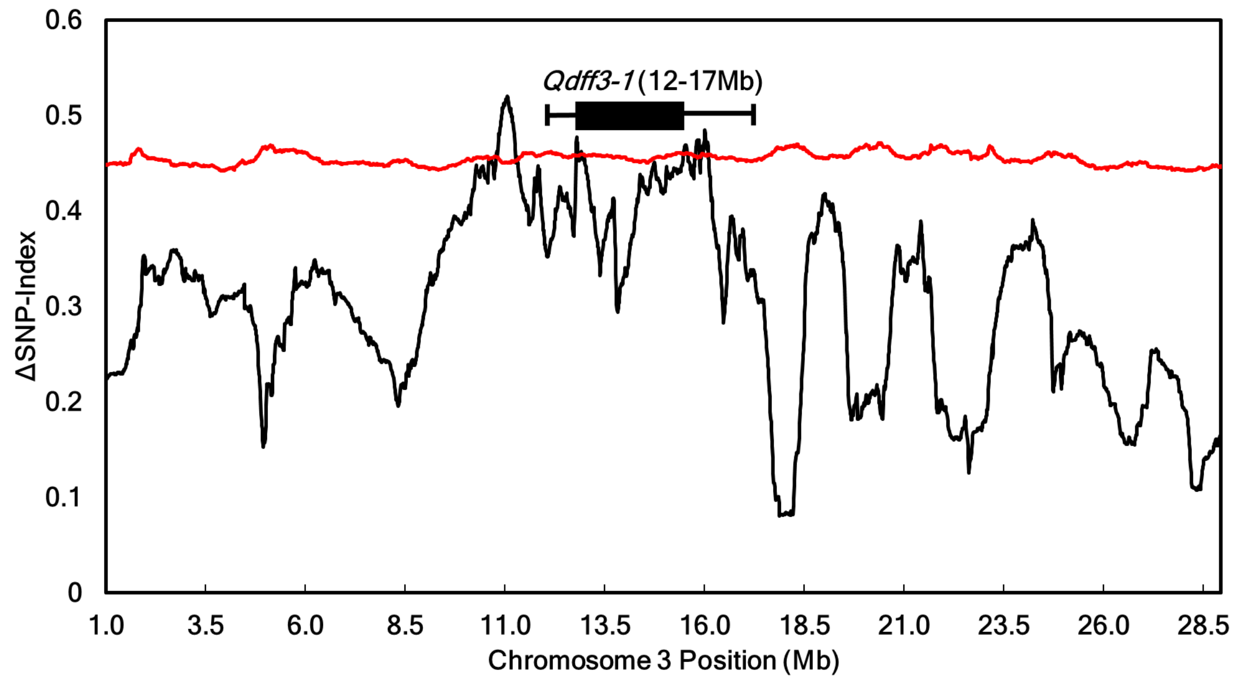
a



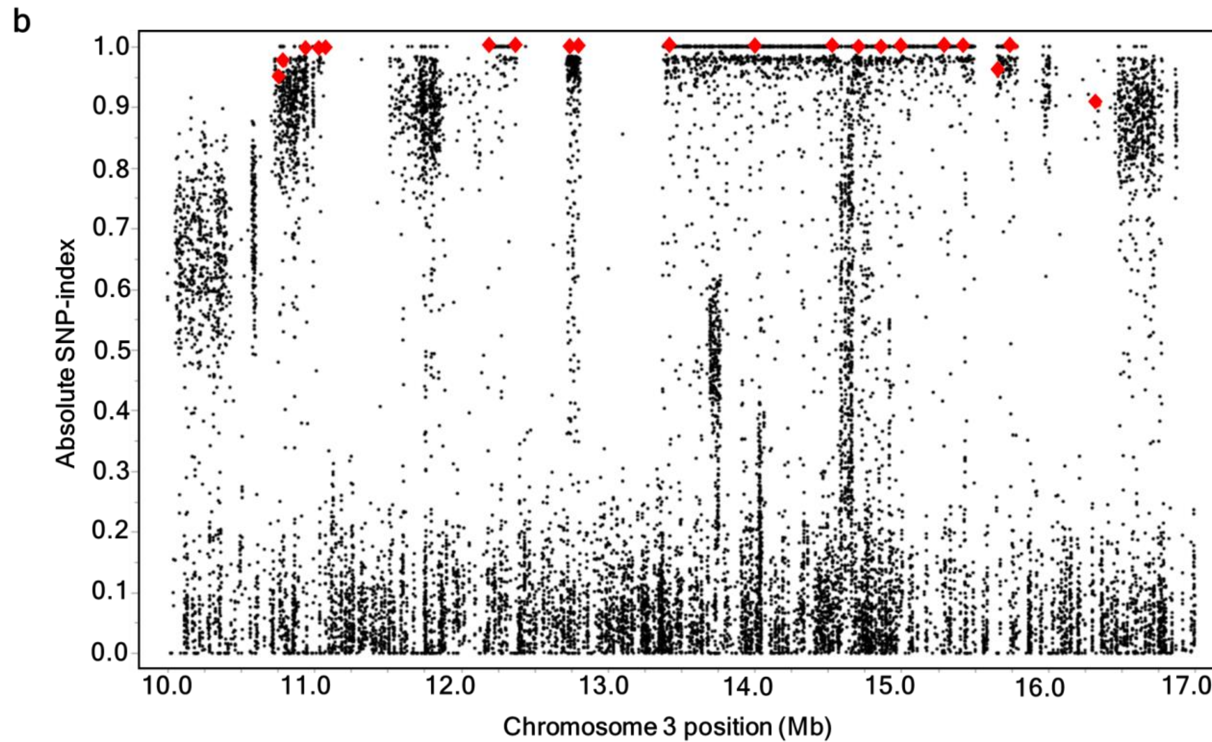


Fig 2.1: Validation of *Qdff3-1* locus (10.7-16.2Mb) on chromosome 3 using QTL-seq in the 'Klondike Black Seeded' (late) × 'New Hampshire Midget' (early) recombinant inbred line population. **(a)** ΔSNP-index graph plotted along with statistical confidence intervals under the null hypothesis of no QTL (P = 0.01) (red line). Box and whiskers above the peaks represent LOD-1 and LOD-1.5 support intervals respectively, of the previously mapped locus using classical QTL mapping (McGregor et al., 2014). **(b)** SNPs converted to KASP assays (red diamonds). Megabase (Mb) positions are based on the 97103 watermelon genome (Guo et al. 2013).

Markers
Chr3 (Mb)
UGA3_10738714
UGA3_10940115
UGA3_11046548
Phosphatidylinositol-4-phosphate 5-kinase (PIP-kinase)
Tempranillo (TEM1)
UGA3_12738922
NW0247945
UGA3_14537958
Flowering Locus T (FT)
UGA3_15424397
NW0248748
UGA3_15774973
UGA3_16339961
Line/Cultivar
Phenotype
a
NHM (Parent) E
112, 146 E
190 E
12 E
53 E
74 E
161 E
38, 83, 130 E
178 E
185 E
47, 54, 70, 186 L
63 L
33, 110, 195 L
173 L
4 L
KBS (Parent) L
b
F7RIL-190 0.0007
KxN3-204 <0.0001
KxN3-205 0.7741
KxN3-217 0.9815
KxN3-218 0.0013
KxN3-222 <0.0001
c
NHM E
SB E
SLMP E
SII E
MICK E
NAV L
HOPI L
GR L
SANG L
ES L
AS, AUP, CALG, CG, CS L
OG L
KBS L

Fig 2.2: Fine mapping of *Qdff3-1* locus using **(a)** recombinants from the KBS × NHM RIL mapping population, **(b)** populations used for fine mapping and **(c)** cultivar panel. The physical map with markers are shown at the top, while the black, white and grey bars denote the marker genotypes of KBS, NHM and heterozygous alleles, respectively. On the right is the phenotype of the specific line (E=early and L= late) and the significance level associated with progeny testing of the recombinant populations. On the left is the specific line or cultivar identification.

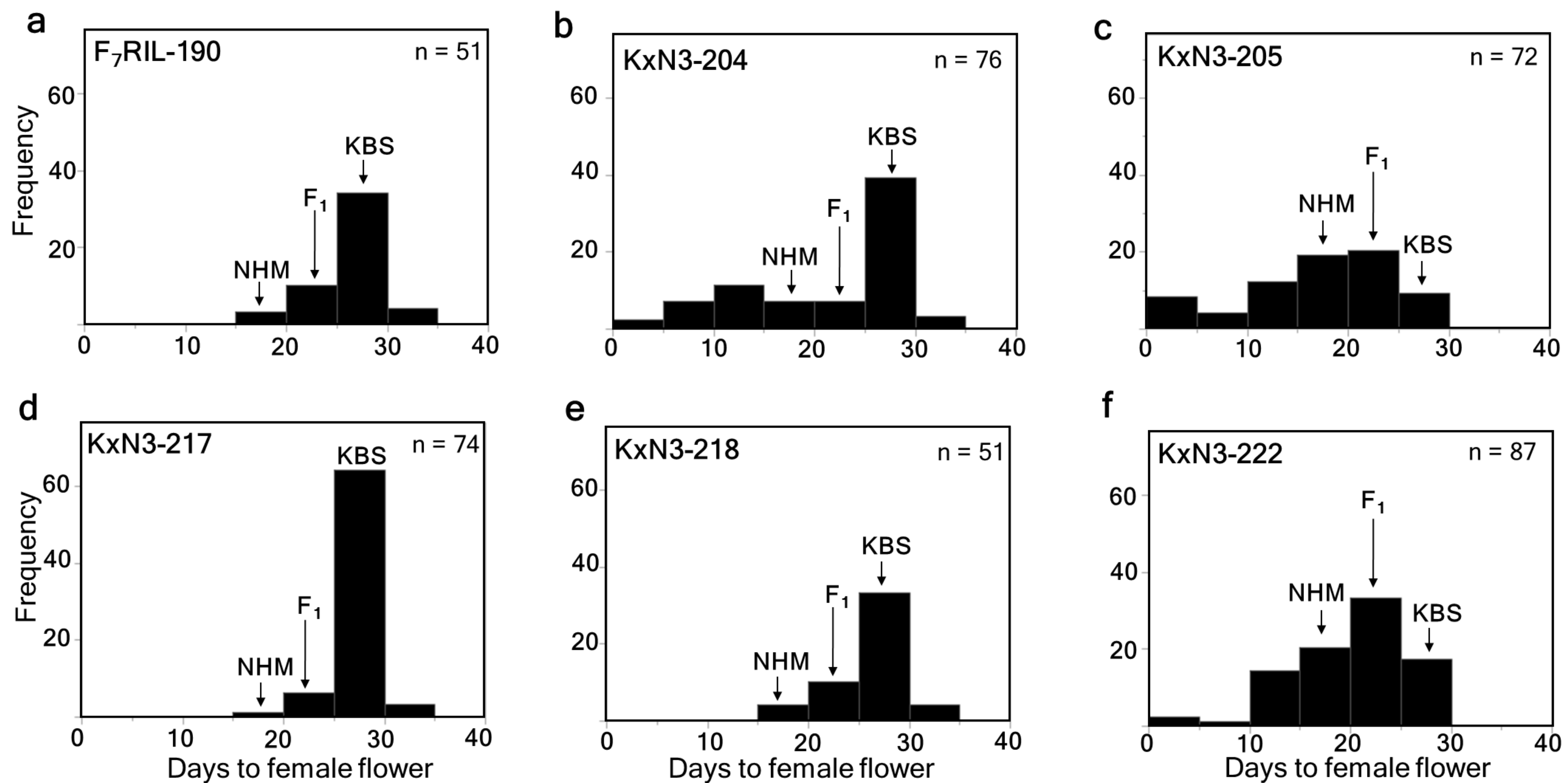
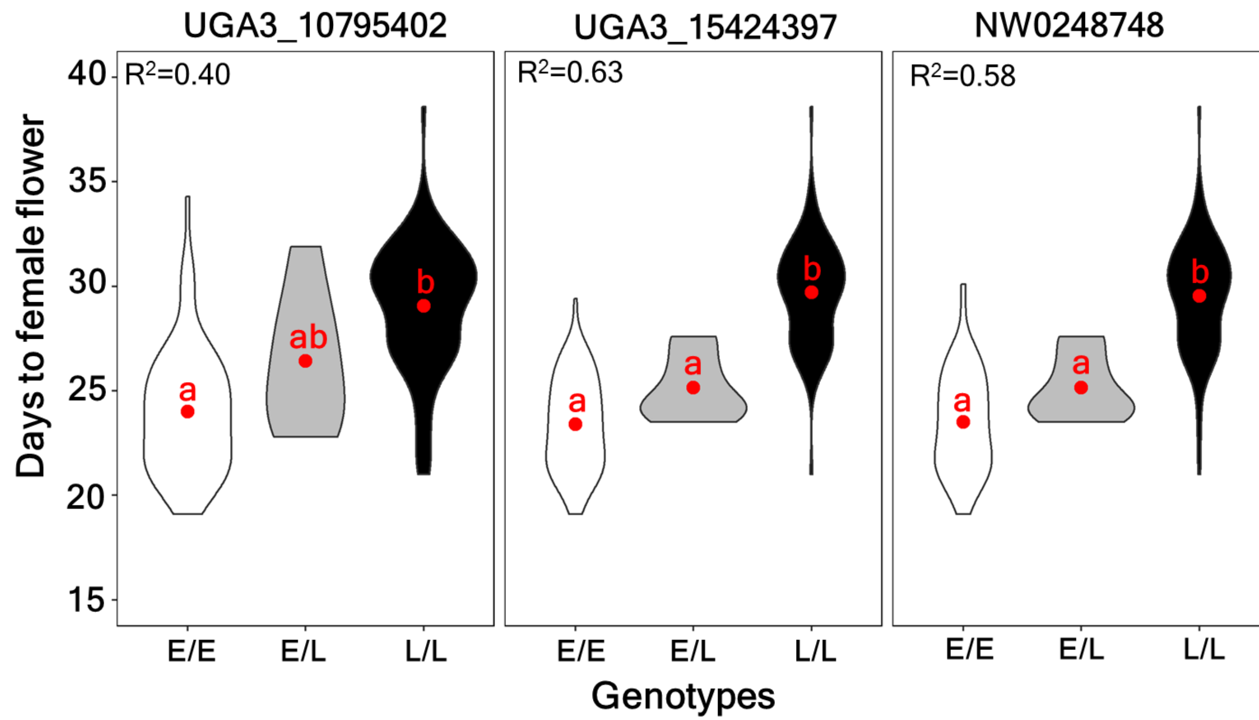


Fig 2.3: Frequency distribution of flowering time in six (5 $F_{2:3}$ and 1 F_7 RIL) watermelon populations. **(a)** F_7RIL-190, **(b)** KxN3-204, **(c)** KxN3-205, **(d)** KxN3-217, **(e)** KxN3-218, **(f)** KxN3-222. The average DFF of the different populations was 25.84, 21.13, 16.17, 27.22, 25.55 and 18.91 days, respectively.


a
UGA3_10795402
UGA3_15424397
NW0248748
R²=0.40
R²=0.63
R²=0.58
Days to female flower
40
35
30
25
20
15
a
ab
b
a
a
b
a
a
b
E/E
E/L
L/L
E/E
E/L
L/L
E/E
E/L
L/L
Genotypes

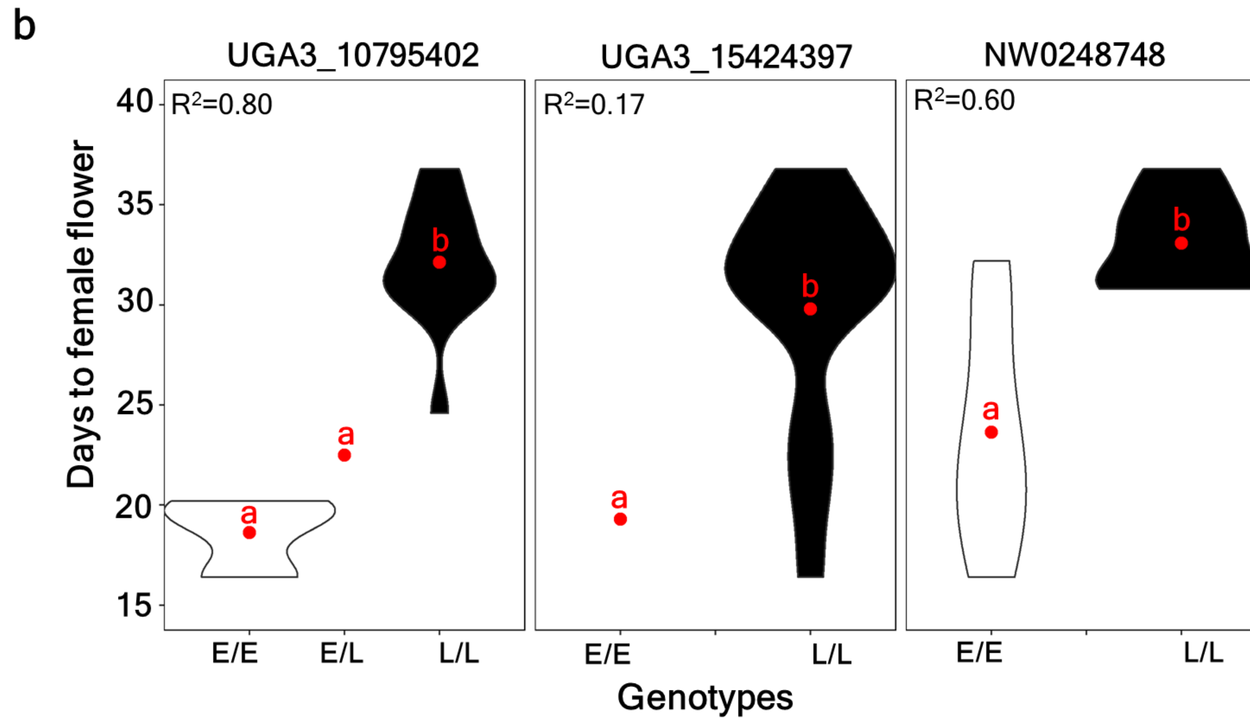


Fig 2.4: Performance of UGA3_10795402, UGA3_15424397 and NW0248748 on **(a)** RIL mapping population and **(b)** cultivar panel. Dots indicate mean and levels not connected by same letter are significantly different. E/E homozygous NHM (early) type allele, L/L homozygous KBS (late) type allele and E/L heterozygous.

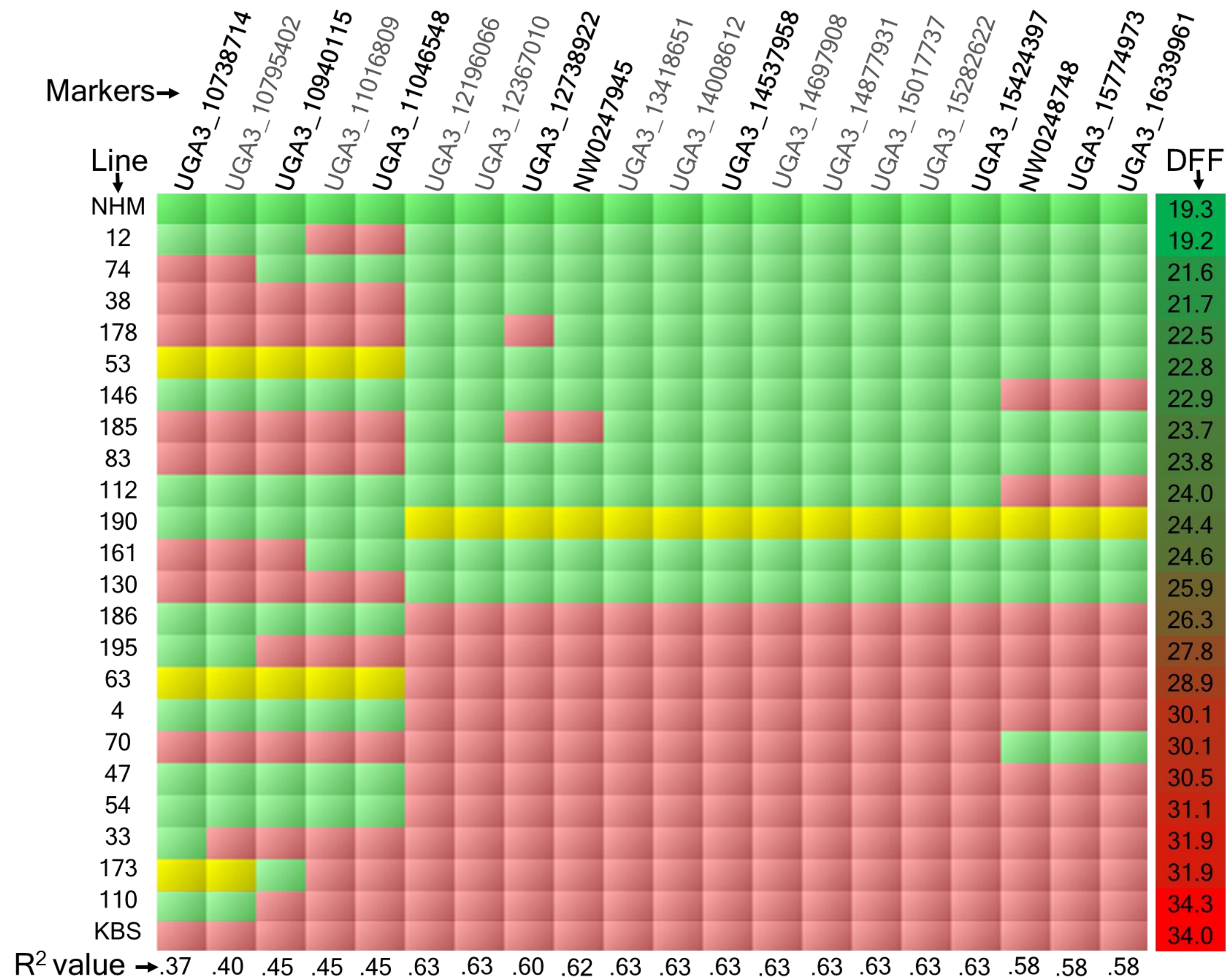
Table 2.1: Progeny test of recombinant plants

Parent	Progeny	Flowering time progeny test KBS[a]				NHM[b]				
		n	Mean μ	Variance σ^2	s.d σ	*n*	Mean μ	Variance σ^2	s.d σ	P- value
F_6RIL-190	F_7RIL-190	16	28.38	7.32	2.70	9	22.89	10.56	3.26	0.0007
KxN2-204	KxN3-204	23	28.17	13.51	3.68	14	11.57	61.80	7.86	<0.0001
KxN2-205	KxN3-205	14	16.07	53.61	7.32	19	14.32	65.78	8.11	0.7741
KxN2-217	KxN3-217	17	27.88	3.74	1.93	13	27.69	6.90	2.63	0.9815
KxN2-218	KxN3-218	16	28.38	3.98	2.00	12	23.17	18.56	4.30	0.0013
KxN2-222	KxN3-222	21	24.12	23.69	4.87	24	17.33	38.39	6.20	<0.0001

n = number of watermelon plants, *Mean* = average days to female flower, *s.d* standard deviation

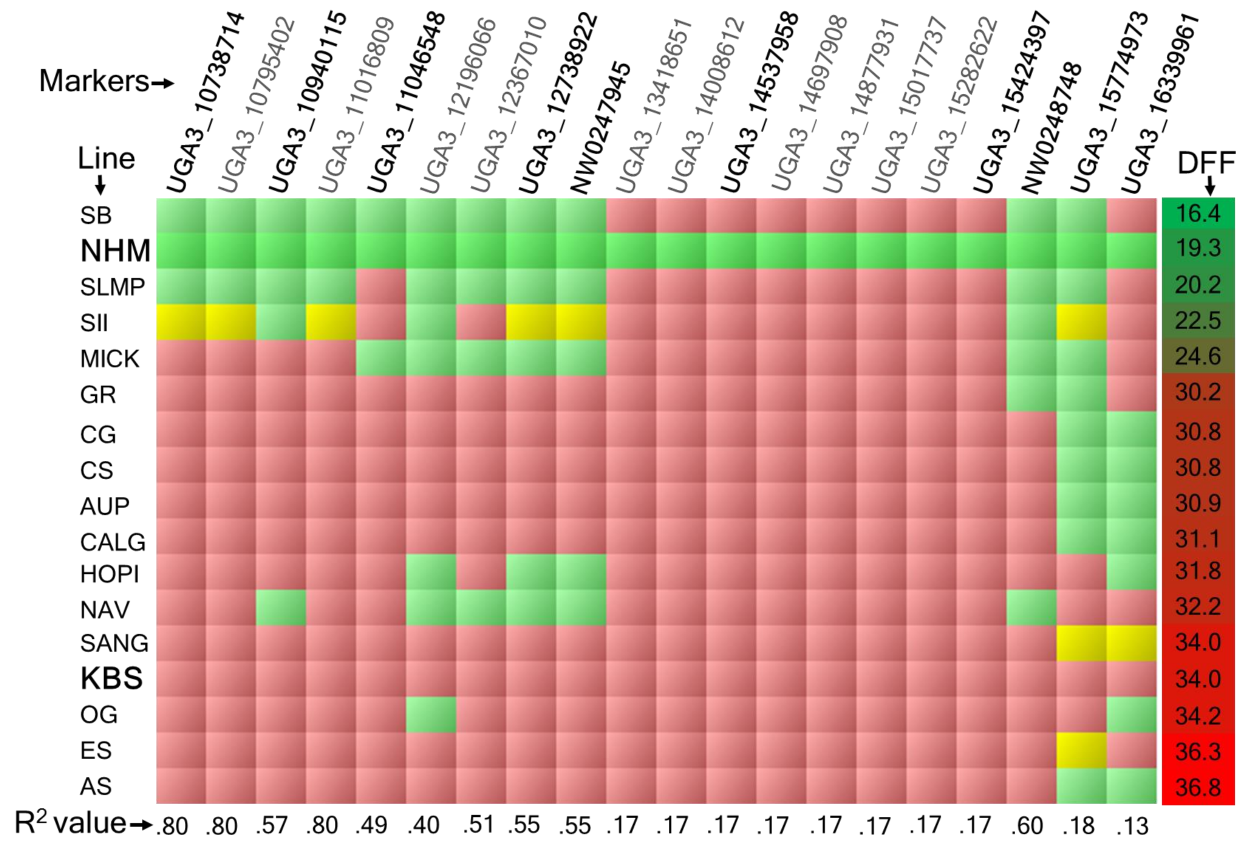
[a] Average DFF for KBS-type progeny

[b] Average DFF for NHM-type progeny



Markers
UGA3_10738714
UGA3_10795402
UGA3_10940115
UGA3_11016809
UGA3_11046548
UGA3_12196066
UGA3_12367010
UGA3_12738922
NW0247945
UGA3_13418651
UGA3_14008612
UGA3_14537958
UGA3_14697908
UGA3_14877931
UGA3_15017737
UGA3_15282622
UGA3_15424397
NW0248748
UGA3_15774973
UGA3_16339961
Line
DFF
NHM 19.3
12 19.2
74 21.6
38 21.7
178 22.5
53 22.8
146 22.9
185 23.7
83 23.8
112 24.0
190 24.4
161 24.6
130 25.9
186 26.3
195 27.8
63 28.9
4 30.1
70 30.1
47 30.5
54 31.1
33 31.9
173 31.9
110 34.3
KBS 34.0
R2 value .37 .40 .45 .45 .45 .63 .63 .60 .62 .63 .63 .63 .63 .63 .63 .63 .63 .58 .58 .58

Supplementary fig 2.1: Haplotype analysis of recombinants identified in the *Qdff3-1* locus from the KBS × NHM RIL mapping population. Genotype indicated in relation to the early flowering (NHM, green) and late flowering (KBS, red) parent alleles, and yellow blocks represent heterozygotes. The days to female flower (DFF) of the different lines are indicated on the right. Markers above the figure are ordered according to the physical map. Markers in bold are those also shown in Fig. 2 with R^2 values for each SNP at the bottom.



Supplementary fig 2.2: Haplotype analysis of the cultivar panel consisting of different watermelon genotypes. Green blocks represent NHM (early) type alleles, red blocks represent KBS (late) type alleles, and yellow blocks represent heterozygotes. The days to female

flower (DFF) of the different cultivars are indicated on the right. Markers above the figure are ordered according to the physical map. Markers in bold are those shown in Fig. 2 with R^2 values for each SNP at the bottom.

Supplementary Table 2.1: Sequencing primers for *Cla009504 (FT)* and *Cla000855 (TEM1)* genes

Name	F/R	bp position on gene	Sequence	Ta (˚C)
S1-U9504F	F	-2008	GATGCGTTGGTGTTATGTGC	64
S1-U9504R	R	-1076	GTTACCCCCTATCCCGAAAA	64
S2-U9504F	F	-1124	GGAATCGATGTCTGCTGTCTT	63
S2-U9504R	R	-273	TTTGAGGAAAAGTAGATGTGTGGA	63
S3-Cla009504F	F	-400	TGCAAATCAACGTAAGA	56
S3-Cla009504R	R	386	ATAGGATGTACGAAATTAGG	56
S4-Cla009504F	F	1	ATGCCGAGAGATCGTGA	61
S4-Cla009504R	R	666	TTTTACGCTTTACAAATCAAATCA	61
S5-Cla009504F	F	331	TTTTTATGTGAGTGAGAAGAATGAA	61
S5-Cla009504R	R	1293	TTGAAAAGATAGATCGATTATAGGAGA	61

S6-Cla009504F	F	796	AGATAGGTCAACCTCAATGTAGAAAA	64
S6-Cla009504R	R	1805	TGAAAAGATAGGTTAGCCTTTTACTGA	64
S7-Cla009504F	F	1599	TGCAATTATCTTCTCCCAAGTTT	63
S7-Cla009504R	R	2236	TGATCTATCAATTAGACAAGTTTCAGC	63
S8-Cla009504F	F	1924	TGAATAGACCTACATTTTCACTCCT	61
S8-Cla009504R	R	2742	GACACAACGGCCCTCACTAC	61
S9-Cla009504F	F	2297	GGGATTGAAGTGGAGGACAA	63
S9-Cla009504R	R	3270	TCAACTGAGTAGAAAACAGCAACC	63
S10-Cla009504F	F	3233	ATGTACGAGTAATAGGT	53
S10-Cla009504R	R	4592	TGGAGTGGGTTTAATTC	53
S11-D9504F	F	4537	CTGGTGGAAGGAGAAGAGTCC	63
S11-D9504R	R	5536	CTTACCTCACACGTTTCAGCA	63
S1-Cla000855F	F	-41	TCTCTTACACCAATTACCAAAATCC	63
S1-Cla000855R	R	972	ACAAGTCAGGTTGGGGAACTT	63

Supplementary Table 2.2: KASP™ assay primer sequences of SNPs in the *Qdff3-1* region on chromosome 3 of watermelon.

KASP Assay	Ta (˚C)	Position (bp)	Primer type	Primer sequence (5'-3')	Allele
UGA3_10738714	58	10,738,714	FAM	GAAGGTGACCAAGTTCATGCTCCCCATTATTTAACACATGCCTCA	NHM
			VIC	GAAGGTCGGAGTCAACGGATTCCCCATTATTTAACACATGCCTCG	KBS
			Reverse	TTGGTGCTCCAATATGGAGTAGGT	
UGA3_10940115	58	10,940,115	FAM	GAAGGTGACCAAGTTCATGCTGAGTCAATCGTAGGTTCGGGTTAGAAT	NHM
			VIC	GAAGGTCGGAGTCAACGGATTGAGTCAATCGTAGGTTCGGGTTAGAAC	KBS
			Reverse	TGCTTTAGATTAATGCTTGTTGATGA	
UGA3_10795402	58	10,795,402	FAM	GAAGGTGACCAAGTTCATGCTGTGCCTCAAACGTCCCTCAAA	NHM
			VIC	GAAGGTCGGAGTCAACGGATTGTGCCTCAAACGTCCCTCAAG	KBS
			Reverse	TCGAAGAATAAGTGTGTTTGGGTTC	
UGA3_11016809	58	11,016,809	FAM	GAAGGTGACCAAGTTCATGCTTTGGCCTAAGTATGACTAATCGAGTGA	NHM
			VIC	GAAGGTCGGAGTCAACGGATTTTGGCCTAAGTATGACTAATCGAGTGC	KBS
			Reverse	TGCCAAGTTGAGAAAGAGTGAAA	
UGA3_11046548	58	11,046,548	FAM	GAAGGTGACCAAGTTCATGCTGAAACAGAGTCAAGAGAAACAGTTGTG	NHM
			VIC	GAAGGTCGGAGTCAACGGATTGAAACAGAGTCAAGAGAAACAGTTGTC	KBS
			Reverse	AGGGTCACGCCCACTACCTT	
UGA3_12196066	58	12,196,066	FAM	GAAGGTGACCAAGTTCATGCTATGTTAATGCTGCACCTGCCATAG	NHM
			VIC	GAAGGTCGGAGTCAACGGATTATGTTAATGCTGCACCTGCCATAA	KBS
			Reverse	TCTGCTCCCTCAGCTCCTGT	

UGA3_12367010	58	12,367,010	FAM	GAAGGTGACCAAGTTCATGCTGTAGAGGGCGAATCGAGGAGA	NHM
			VIC	GAAGGTCGGAGTCAACGGATTGTAGAGGGCGAATCGAGGAGG	KBS
			Reverse	GCCGATTTTGAGAAATAGAAGAGGA	
UGA3_12738922	58	12,738,922	FAM	GAAGGTGACCAAGTTCATGCTCCTTAATCACCCTACCATGTGTTCACT	NHM
			VIC	GAAGGTCGGAGTCAACGGATTCCTTAATCACCCTACCATGTGTTCACC	KBS
			Reverse	TGTCTCGGAAGGAAAAACCAAA	
NW0247945	51	12,804,860	FAM	GAAGGTGACCAAGTTCATGCTTATTTGTGAGGATTCTTCCAA	NHM
			VIC	GAAGGTCGGAGTCAACGGATTTATTTGTGAGGATTCTTCCAG	KBS
			Reverse	GCCCTTTATAAACGTCAAGCA	
UGA3_13418651	58	13,418,651	FAM	GAAGGTGACCAAGTTCATGCTCGTCTATGGCACAACCTGAAGAA	KBS
			VIC	GAAGGTCGGAGTCAACGGATTCGTCTATGGCACAACCTGAAGAG	NHM
			Reverse	CGGCTGCCGATCAACATTA	
UGA3_14008612	58	14,008,612	FAM	GAAGGTGACCAAGTTCATGCTTTTGCCTGTGTGTCGTCCTG	KBS
			VIC	GAAGGTCGGAGTCAACGGATTTTTGCCTGTGTGTCGTCCTA	NHM
			Reverse	TCAGGGGTTCACGGTGTTTC	
UGA3_14537958	58	14,537,958	FAM	GAAGGTGACCAAGTTCATGCTTTTGTATATAAAGGCGTCTTAGCATTG	KBS
			VIC	GAAGGTCGGAGTCAACGGATTTTTGTATATAAAGGCGTCTTAGCATTA	NHM
			Reverse	TTTGAAGTTTTACCACAAAAGAGTCC	
UGA3_14697908	58	14,697,908	FAM	GAAGGTGACCAAGTTCATGCTGGGTTTGGTGGCAGAGGTC	KBS
			VIC	GAAGGTCGGAGTCAACGGATTGGGTTTGGTGGCAGAGGTT	NHM
			Reverse	CCTCTGCTCGACCAGCATTT	

UGA3_14877931	58	14,877,931	FAM	GAAGGTGACCAAGTTCATGCTGTGACCGGATCATAAAGACTAAATTAAAAT	KBS
			VIC	GAAGGTCGGAGTCAACGGATTGTGACCGGATCATAAAGACTAAATTAAAAC	NHM
			Reverse	CGTCGTTGTCTCTAAATTTCATGTG	
UGA3_15017737	58	15,017,737	FAM	GAAGGTGACCAAGTTCATGCTCGAAGTCAGACTACCTCACCGTCA	KBS
			VIC	GAAGGTCGGAGTCAACGGATTCGAAGTCAGACTACCTCACCGTCG	NHM
			Reverse	GACGACGACGGCCAATTAAA	
UGA3_15282622	58	15,282,622	FAM	GAAGGTGACCAAGTTCATGCTTGATTAAGTTGTTTTCACTCTCGAACA	KBS
			VIC	GAAGGTCGGAGTCAACGGATTTGATTAAGTTGTTTTCACTCTCGAACG	NHM
			Reverse	TCTCCAATATCTTTTGTAACTGCTCA	
UGA3_15424397	58	15,424,397	FAM	GAAGGTGACCAAGTTCATGCTCCAAGCTGCCCTAGCAACG	KBS
			VIC	GAAGGTCGGAGTCAACGGATTCCAAGCTGCCCTAGCAACA	NHM
			Reverse	CCTCTGTCGATGGGGTTTCA	
NW0248748	51	15,664,017	FAM	GAAGGTGACCAAGTTCATGCTGTGTTTTCTACATATCACAAACTCC	KBS
			VIC	GAAGGTCGGAGTCAACGGATTGTGTTTTCTACATATCACAAACTCT	NHM
			Reverse	TCTCTGGAGAGGACTTAGAGTGG	
UGA3_15774973	58	15,774,973	FAM	GAAGGTGACCAAGTTCATGCTTCAAGCTCGACTACTCAAGGGGTA	NHM
			VIC	GAAGGTCGGAGTCAACGGATTTCAAGCTCGACTACTCAAGGGGTG	KBS
			Reverse	CTAGTTTGGCAAGGGCATGG	
UGA3_16339961	58	16,339,961	FAM	GAAGGTGACCAAGTTCATGCTTCAGGTACGCCTTGTTGGACAT	KBS
			VIC	GAAGGTCGGAGTCAACGGATTTCAGGTACGCCTTGTTGGACAG	NHM
			Reverse	AAAAAGGAAAAAGTGGGAAGTATT	

Supplementary Table 2.3: Seed sources for cultivars used in the study

Cultivar	**Seed source**
Sugar Baby (SB)	Reimer Seeds
New Hampshire Midget (NHM)	USDA-ARS, Griffin, GA
Sugar Lump (SLMP)	Baker Creek Heirloom Seeds
Strain II (SII)	USDA-ARS, Griffin, GA
Mickylee (MICK)	Hollar Seeds
Golden Russian (GR)	Baker Creek Heirloom Seeds
Charleston Gray (CG)	Eden Brothers HeirloomSeeds

Crimson Sweet (CS)	Seedway Seeds
AU-Producer (AUP)	Hollar Seeds
Calhoun Gray (CALG)	USDA-ARS, Griffin, GA
Hopi Yellow (HOPI)	Baker Creek Heirloom Seeds
Navajo Red (NAV)	Baker Creek Heirloom Seeds
Sangria (SANG)	Seedway Seeds
Klondike Black Seeded (KBS)	USDA-ARS, Griffin, GA
Orangeglo (OG)	Seed Saver's Exchange Heirloom Seeds
Estrella (ES)	Seedway Seeds
Allsweet (AS)	Seedway Seeds

Supplementary Table 2.4: Genes within the 239 kb region.

Gene ID	Position	Protein
Cla009532	15,437,774	Unknown Protein (AHRD V1)
Cla009533	15,438,424	Unknown Protein (AHRD V1)
Cla009534	15,438,915	Unknown Protein (AHRD V1)
Cla009535	15,455,829	Matrix metalloproteinase (AHRD V1- Q9LEL9_CUCSA)
Cla009536	15,471,588	Unknown Protein (AHRD V1)
Cla009537	15,512,429	Dicer-like protein (AHRD V1 - B9HHX6_POPTR)
Cla009538	15,518,604	Unknown Protein (AHRD V1)
Cla009539	15,529,511	Dicer-like protein (AHRD V1 - B9HHX6_POPTR)
Cla009540	15,547,732	Ribonuclease 3-like protein 3 (AHRD V1 - RTL3_ORYSJ)
Cla009541	15,597,443	Ycf60 protein (AHRD V1 - Q8DJZ7_THEEB)
Cla009542	15,600,235	Unknown Protein (AHRD V1)
Cla009543	15,606,094	2-phosphoglycolate phosphatase 1 (AHRD V1 - Q8L3U4_ARATH)
Cla009544	15,622,730	Unknown Protein (AHRD V1)

Cla009545	15,634,995	Serine carboxypeptidase-like 21 (AHRD V1 - F4J901_ARATH)
Cla009546*	15,661,333	Phosphatase 2C family protein (AHRD V1 - D7KZK8_ARALL)

* proposed candidate gene

CHAPTER 3

RESISTANCE RESPONSE OF *CITRULLUS* GENOTYPES TO *STAGONOSPOROPSIS* SPP. ISOLATES CAUSING GUMMY STEM BLIGHT

[1]Gimode Winnie, M. Lonnee, and C. McGregor. To be submitted to Cucurbit Genet Coop Rep

Abstract

Gummy stem blight (GSB) is a major disease of watermelon (*Citrullus lanatus*) that leads to severe yield losses. It is caused by three morphologically similar species of the genus *Stagonosporopsis: S. citrulli, S. caricae* and *S. cucurbitacearum*, which differ genetically and exhibit variation in fungicide resistance. In this preliminary study, we established that six isolates from the three *Stagonosporopsis* species displayed different host responses on a panel of 12 *Citrullus* genotypes with varying genetic backgrounds. The level of aggressiveness of the isolates was not species-dependent, with specific isolates within a species more aggressive than others. *S. citrulli* 12178A and *S. caricae* RG3 were the most aggressive isolates while the least aggressive isolate was *S. cucurbitacearum* GSB26. The *C. amarus* genotypes selected for this study displayed higher levels of resistance than the *C. lanatus* genotypes. To our knowledge, this is the first study to look at the effect of different *Stagonosporopsis* isolates on various *Citrullus* genotypes. Understanding the resistance potential in *Citrullus* genotypes when exposed to different *Stagonosporopsis* isolates will accelerate breeding efforts for resistant watermelon cultivars.

Keywords: *Stagonosporopsis, caricae, citrulli, cucurbitacearum,* watermelon*, amarus, lanatus*

Introduction

Gummy Stem Blight (GSB) is a major fungal disease affecting watermelon (*Citrullus lanatus*) and other cucurbits (Sherbakoff 1917; Chiu and Walker 1949; Sherf and MacNab 1986). The disease is also commonly known as black rot, when infection occurs on fruit of *Cucurbita* spp. (Chiu and Walker 1949; Maynard and Hopkins 1999). It is a serious problem for cucurbit growers, especially in tropical, subtropical and some temperate areas, where the warm and humid conditions are conducive for disease development (Robinson and Decker-Walters 1997). In the southeastern United States (US), GSB was identified as the second most important research priority in watermelon after fusarium wilt (Kousik et al. 2016).

GSB was previously thought to be caused by a single pathogen: *Stagonosporopsis cucurbitacearum* (syn. *Didymella bryoniae*). However, it was recently determined that GSB is caused by three species of the genus *Stagonosporopsis: S. cucurbitacearum, S. citrulli* and *S. caricae* (Stewart et al. 2015). Morphologically, the species appear similar, but they differ genetically. Among the three *Stagonosporopsis* species*, S. citrulli* was found to be the most widely distributed worldwide (Brewer et al. 2015; Stewart et al. 2015). The study by Stewart et al. (2015) established that most of the isolates obtained from different hosts in North and South America, Europe, north Africa, and Asia were *S. citrulli*. *S. caricae* isolates, some of which were obtained from *Carica papaya*, were found in samples from North and South America, Asia, and southeast Asia, while *S. cucurbitacearum* were specifically from temperate regions in North America, Europe, Asia, and New Zealand. Within the US, *S. citrulli* was the most abundant, especially in the southeast US, while *S. cucurbitacearum* isolates were more common in northeast US (Stewart et al. 2015).

Several studies have shown that the different pathogen species exhibit variation in fungicide sensitivity (Brewer et al. 2015; Li et al. 2016; Li et al. 2019; Newark et al. 2019). For example, tebuconazole resistance was reported in *S. caricae* isolates, whereas *S. citrulli* and *S. cucurbitacearum* isolates were shown to be sensitive to this fungicide (Li et al. 2016). A subsequent study reported sensitivity to boscalid and fluopyram among all *S. caricae* isolates but varied sensitivity among *S. citrulli* isolates to boscalid (Li et al. 2019). Resistance to thiophanate-methyl has also been detected among *S. citrulli* isolates from East China while most isolates from Florida remained sensitive to this fungicide (Newark et al. 2019). This differential sensitivity poses a major challenge in management of GSB, especially because current management efforts rely heavily on fungicide applications, since no commercial watermelon cultivars currently possess genetic resistance to the GSB in the field.

Cultivated watermelon has a very narrow genetic base as a result of domestication that led to loss of some traits while selecting for desirable fruit quality (Levi et al. 2017; Guo et al. 2019). Other *Citrullus* species have been used as a major source of disease resistance traits for various diseases in watermelon (Boyhan et al. 1994; Guner 2005; Thies and Levi 2007; Tetteh et al. 2010; Wechter et al. 2012; Levi et al. 2017). *Citrullus* germplasm resistant to GSB have been identified as early as 1962 (Sowell and Pointer 1962) and efforts to introgress this resistance into commercial cultivars has been attempted, though unsuccessful (Norton 1979; Norton et al. 1986; Norton et al. 1993; Sumner and Hall 1993; Norton et al. 1995; Song et al. 2002). Plant Introduction (PI) 189225 was initially identified as the most resistant accession evaluated from the USDA-ARS watermelon germplasm collection (Sowell and Pointer 1962). PI 271778, was later identified as an additional source of resistance (Sowell 1975; Norton 1979). Both PI 189225 and PI 271778 are wild accessions of *C. amarus,* a close relative of watermelon (Chomicki and Renner 2015; Renner et

al. 2017). Crosses between elite cultivars and resistant PIs were made to produce two lines: AU-Jubilant (Jubilee × PI 271778) and AU-Producer (Crimson Sweet × PI 189225) (Norton et al., 1986). However, these cultivars have not shown resistance to GSB in the field (Song et al. 2002).

Gusmini et al. (2005) identified a further ten PIs that displayed significant levels of resistance to GSB under both field and greenhouse conditions. These accessions consisted of genotypes from both *C. amarus* and *C. lanatus* species and included PI 164248, PI 244019, PI 254744, PI 271771, PI 279461, PI 296332, PI 482276, PI 482379, PI 490383 and PI 526233 (Gusmini et al. 2005). Despite all the resistant sources described, breeding efforts for GSB-resistant watermelon cultivars have been unsuccessful. With the discovery that GSB can be caused by three different *Stagonosporopsis* species, the question arises whether differential host resistance to the species might be partially responsible for the lack of success in resistance breeding efforts. To date, no studies have examined the effect of different *Stagonosporopsis* species on putative resistant *Citrullus* genotypes. It is vital to establish whether the three species have similar host responses. Understanding the level and breadth of resistance found in *Citrullus* genotypes will be essential in determining the appropriate sources of resistance to use in breeding efforts. The objective of this preliminary study was therefore to evaluate the level of resistance of 12 different *Citrullus* genotypes to six isolates from three different *Stagonosporopsis* species.

Materials and methods

Plant material

Seeds of 12 different *Citrullu*s genotypes that included both wild and elite genotypes were sown in the greenhouse in 48-cell seedling trays, approximately 2 weeks prior to screening. The genotypes consisted of cultivars and PIs belonging to *C. amarus, C. mucosospermus* and *C. lanatus*

species. They included: AU-Producer (AUP), Crimson Sweet (CS), Mickylee (MICK), Sugar Baby (SB), PI 189225, PI 244019, PI 279461, PI 482276, PI 482379, PI 549160, PI 560023 and PI 593359 (Table 3.1). The panel of genotypes used in this study were specifically chosen to represent a broad genetic background for watermelon. Moreover, five of the PI used in this study (PI 189225, PI 244019, PI 279461, PI 482276, PI 482379) were chosen because they had been previously described as resistant to GSB (Sowell and Pointer 1962; Gusmini et al. 2005)

The *C. lanatus* genotypes used in the study were from North America (CS, AUP, SB and MICK), Asia (Japan: PI 279461; China: PI 593359) and Africa (PI 549160). Many modern watermelon cultivars are related to CS, which is a parent of AUP (Norton et al. 1986; Wehner and Barrett 2002). SB, which is genetically distant from other North American cultivars, is an ancestral parent of MICK (Wehner and Barrett 2002). PI 549160 is a wild *C. lanatus* from northeast Africa, which is a center of domestication for watermelon (Renner et al. 2017). PI 560023 was the only *C. mucosospermus* (egusi) species used in this study. Egusi watermelon are utilized in West Africa for their edible seeds. The *C. amarus* species included PI 244019, PI 482379, PI 482276 and PI 189225 which are from South Africa, Zimbabwe, Zaire and Zimbabwe, respectively.

Fungal isolates

Six *Stagonosporopsis* isolates, provided by Marin Brewer (University of Georgia, Department of Pathology), were grown (16h/8h light/dark cycle) on potato dextrose agar (PDA) (Becton, Dickinson and Company, NJ, USA) for 2 weeks. Approximately 1 cm^2 agar plugs were then sub cultured on quarter-strength PDA (qPDA) where they were grown for another 2 weeks. The isolates included *S. citrulli:* 12178A and AcSq5, *S. cucurbitacearum:* RT2 and GSB26 and *S. caricae:* GA8007H and RG3 (Stewart et al., 2015; M. Brewer, *personal communication*) (Table 3.2).

Phenotyping

Three independent screens were performed in a growth chamber. During each screen, seven trays (6 isolates and 1 control) were sown, with four seeds of each genotype (12 genotypes total) per tray. On the day of inoculation, qPDA cultures were flooded with 10 ml of 0.1% tween20 and gently scraped with a sterile spatula to release spores. The inoculum was filtered through 2 layers of sterile cheese cloth and spore concentration was determined using a hemacytometer (Hausser Scientific, PA, USA). Spore concentrations were then adjusted to 5×10^5 spores/ml using 0.1% tween20 solution.

At the 2nd true leaf stage, seedling trays were placed in plastic tubs and each tray was sprayed with freshly made inoculum from one isolate using an airbrush sprayer (Master Airbrush Model E91) for 60 seconds. The control tray was sprayed with a mock inoculation consisting of 0.1% tween20 solution. The tubs were then sealed in a transparent, plastic bag to promote high relative humidity of approximately 95% which was measured using a data logger (Lascar Electronics UK). The tubs were placed in a growth chamber set to 26 °C day and 23 °C night with a 12h/12h light/dark cycle. On the 3rd day post-inoculation (dpi), the trays were removed from the tubs and disease severity data was collected 7dpi.

Disease symptoms were evaluated on a scale of 0 to 9 as described by (Lou et al. 2013), where 0 = no disease; 1 to 2 = mild trace of infection with less than 10% of leaves covered with lesions; 3 to 4 = 10 to 20% of leaves covered with lesions, 5 to 6 = 21 to 50 % of the leaves covered with small lesions; 7 to 8 = wilting plant and more than 50 % of the leaves covered with lesions; and 9 = dead plant. The scores of the four seedlings of each genotype were averaged before further analysis.

Data analysis

Statistical analyses were conducted using a fitted mixed linear model in R, whereby genotype, isolate and their interaction were the fixed effects while screen was treated as a random effect. Post hoc comparisons among groups after fitting the model were done using emmeans to obtain treatment values and significance levels after taking into account other terms in the model. Hierarchical cluster analysis using the averages was performed for both the isolates and the genotypes using JMP® Pro 14.1.

Results

No lesions were observed on mock inoculated plants in any of the screens. One of the isolates, *S. citrulli* AcSq5 had slightly lower spore concentration (4.34×10^5 spores/ml) in the first screen. In the subsequent screens, spores were not observed and therefore data from only one replication was included in the analysis for this isolate, which means conclusions cannot be drawn for this isolate. In the treated trays, similar trends were observed in the three screens with *S. citrulli* 12178A exhibiting higher aggressiveness than the other isolates, with most of the seedlings dead by 7dpi. Results of the ANOVA indicated a significant difference between the watermelon genotypes used ($P < 0.001$) as well as the isolates ($P < 0.001$), but no significant genotype × isolate interaction (Table 3.3).

S. citrulli 12178A and *S. caricae* RG3 were significantly the most aggressive of the isolates, followed by *S. cucurbitacearum* RT2 (Fig. 3.1). The least aggressive isolate was *S. cucurbitacearum* GSB26, however it was not significantly different from *S. caricae* GA8007H. Based on the hierarchical cluster analysis, the isolates formed two major clusters, with *S. citrulli* 12178A and *S. caricae* RG3 diverged from the four other isolates (Fig. 3.1).

The genotypes separated into two major clades in the hierarchical cluster analysis, with the *C. amarus* genotypes forming one clade and all the *C. lanatus* and the *C. mucosospermus* (PI 560023) genotypes in the other clade. AUP had the highest disease severity score overall (7.51) followed by SB (7.26), CS (7.09) and PI 279461 (6.63) (Fig. 3.1).

Discussion

Differences in aggressiveness among the isolates was detected, with *S. citrulli* 12178A and *S. caricae* RG3 significantly the most aggressive among the isolates. Based on the isolates selected for this study, results indicate that the level of aggressiveness was not species-dependent and that certain isolates within a species could be more aggressive than others. Unfortunately, the low sporulation of AcSq5 meant that only one replication for this isolate was included and therefore comparisons of *S. citrulli* with other species may not be meaningful.

The watermelon genotypes exhibited a wide distribution of resistance levels to the different isolates of *Stagonosporopsis* (Fig. 3.1) as would be expected from our choice of genotypes. Among the genotypes, PI 189225 (2.89) and PI 482276 (2.83) were generally more resistant than the other genotypes across isolates and they clustered together. These two lines had been previously described as resistant to GSB (Norton et al. 1993; Gusmini et al. 2005) and this study confirms their broad resistance to GSB isolates. The other two *C. amarus* lines, PI 482379 and PI 244019 also displayed intermediate resistance to most of the isolates, however the latter was more susceptible to *S. cucurbitacearum* RT2.

The most susceptible genotype was AUP with a disease severity score of 7.51. It is worth noting that AUP, which was formerly described as resistant to GSB (Norton et al. 1986) but demonstrated to be susceptible in the field (Song et al. 2002), only showed resistance to *S. cucurbitacearum* GSB26, the least severe of the isolates tested (Fig. 3.1). PI 279461 was among

the most resistant lines described by Gusmini et al. (2005) but displayed high disease severity in the present study. Similar to AUP, it was slightly more resistant to the least aggressive *S. cucurbitacearum* GSB26. It is tempting to speculate that an isolate similar to *S. cucurbitacearum* GSB26 was used in previous studies for phenotyping, but the current study does not allow us to determine that with any certainty. AUP however displayed very high susceptibility to all other isolates, confirming the susceptibility of this cultivar to GSB. The elite cultivars were generally susceptible to the various isolates (Fig. 3.1).

This study confirms that some *Stagonosporopsis* isolates are more aggressive than others, but with the isolates tested in this study, there is no pattern of aggressiveness within species. The two most aggressive isolates (12178A and RG3), which were *S. citrulli* and *S. caricae*, respectively, were originally obtained from *C. lanatus* hosts, therefore it could be argued that there could be some host specificity. However, RT2, which also displayed high aggressiveness, was obtained from *Cucurbita moschata,* while GA8007H which displayed lower aggressiveness was isolated from watermelon (Stewart et al., 2015).

Our results could explain the inconsistency that has been observed with GSB phenotyping in different research programs and why efforts to introgress GSB resistance into commercial cultivars have been complex and unsuccessful. It is possible that different *Stagonosporopsis* isolates with varying levels of aggressiveness are used for phenotyping, especially considering the pathogen in the screens is only referred to as *Didymella bryoniae*. It is also highly likely that a mixture of isolates exists in the field (Brewer et al., 2015). This further complicates the breeding process for GSB resistance. From the results of this study, it should be noted that phenotyping using a less aggressive isolate may confer resistance to the specific isolate, but when the genotype is challenged with a more aggressive isolate present in the field, it may not survive. Results from

Gusmini et al. (2017) also displayed large environmental effects associated with GSB, which would impact the severity of symptoms observed in the field.

It is still unknown whether the same resistant loci in *Citrullus* genotypes confer broad resistance against different *Stagonosporopsis* isolates. Knowledge of the effect of different *Stagonosporopsis* isolates on *Citrullus* genotypes may inform breeders on the appropriate resistance sources and pathogen isolates to utilize for breeding. These preliminary results can inform watermelon breeders in developing strategies for phenotyping and resistance loci deployment when breeding for GSB resistance. Future GSB resistance breeding efforts should pay more attention to selecting *Stagonosporopsis* isolates and species for phenotyping that will ensure broad resistance.

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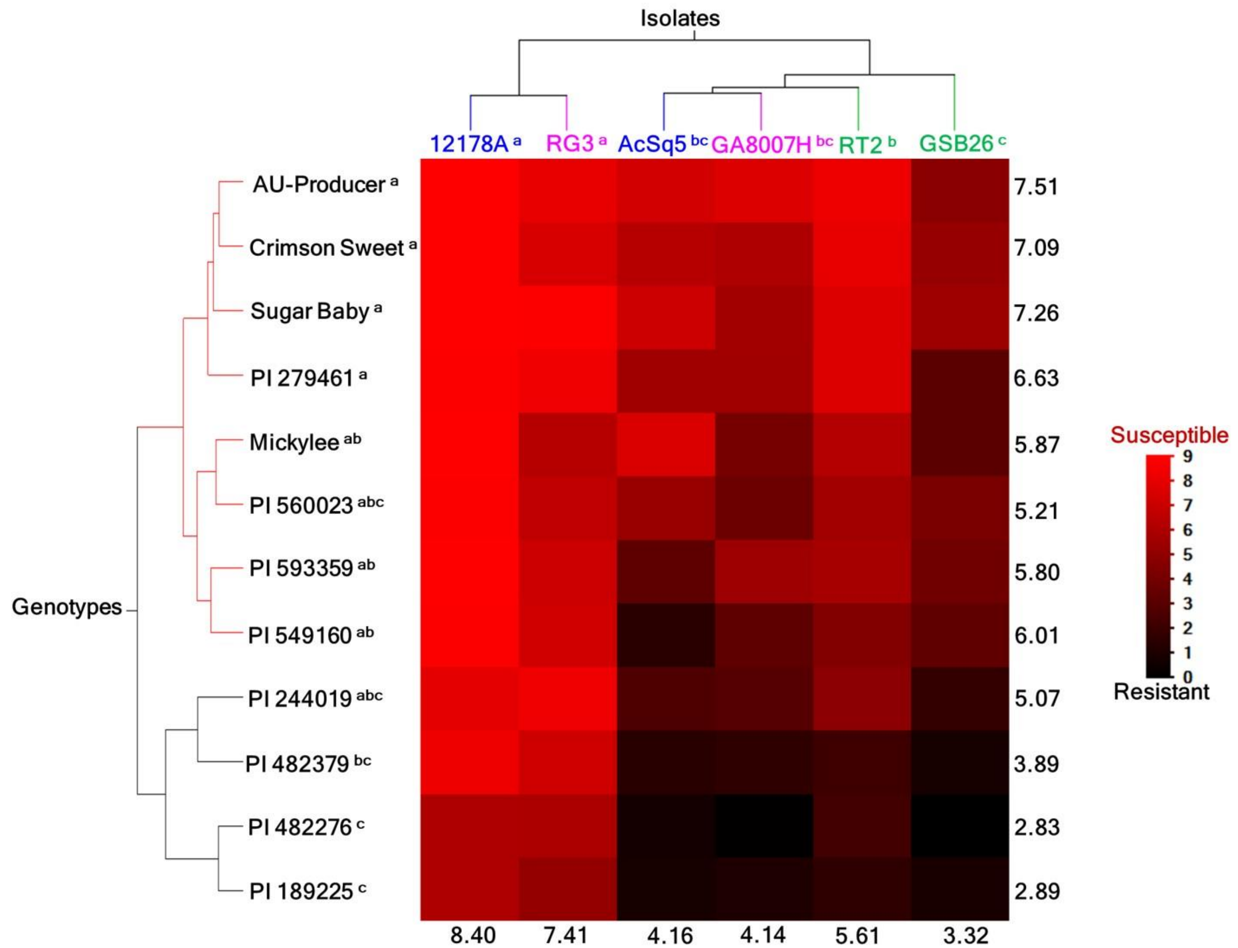

Fig 3.1: Heat map displaying the disease severity of different *Citrullus* genotypes (x-axis) against various *Stagonosporopsis* spp. isolates (y-axis). The isolates are *S. citrulli* (blue), *S. caricae* (pink) and *S. cucurbitacearum* (green). On the right and bottom are the mean severity scores for each genotype and isolate, respectively. Levels not connected by the same letter (superscript) are significantly different.

Table 3.1: Seed sources for *Citrullus* genotypes used in this study

Genotype	Seed source	Species
AU-Producer (AUP)	Hollar Seeds	*C. lanatus*
Crimson Sweet (CS)	Seedway Seeds	*C. lanatus*
Sugar Baby (SB)	Reimer Seeds	*C. lanatus*
Mickylee (MICK)	Hollar Seeds	*C. lanatus*
PI 279461	USDA-ARS, Griffin, GA	*C. lanatus*
PI 593359	USDA-ARS, Griffin, GA	*C. lanatus*
PI 549160	USDA-ARS, Griffin, GA	*C. lanatus*
PI 560023	USDA-ARS, Griffin, GA	*C. mucosospermus*
PI 244019	USDA-ARS, Griffin, GA	*C. amarus*
PI 482379	USDA-ARS, Griffin, GA	*C. amarus*
PI 482276	USDA-ARS, Griffin, GA	*C. amarus*
PI 189225	USDA-ARS, Griffin, GA	*C. amarus*

Table 3.2: Sources of isolates used in this study (Stewart et al., 2015, M. Brewer, personal communication)

Isolate	Original host species	State of origin	***Stagonosporopsis* spp.**
12178A	*Citrullus lanatus* (watermelon)	Georgia	*S. citrulli*
AcSq5	*Cucurbita pepo* (acorn squash)	North Carolina	*S. citrulli*
RG3	*Citrullus lanatus* (watermelon)	California	*S. caricae*
GA8007H	*Citrullus lanatus* (watermelon)	Georgia	*S. caricae*
RT2	*Cucurbita moschata* (butternut squash)	Michigan	*S. cucurbitacearum*
GSB26	*Cucumis melo* (muskmelon)	New York	*S. cucurbitacearum*

Table 3.3: Analysis of variance for mean disease severity scores of 12 watermelon genotypes inoculated with six *Stagonosporopsis* species isolates

Source of variation	Sum Sq	Mean Sq	DF	F value	Pr (>F)
Genotype***	422.99	38.45	11	7.14	3.644×10^{-9}
Isolate***	671.30	134.26	5	24.92	$< 2.2 \times 10^{-16}$
Genotype × Isolate^{NS}	142.04	2.58	55	0.48	9.986×10^{-1}

CHAPTER 4

QTL MAPPING FOR GUMMY STEM BLIGHT RESISTANCE IN AN INTERSPECIFIC *CITRULLUS* POPULATION

[1]Gimode Winnie, K. Bao, Z. Fei, and C. McGregor. To be submitted to Theor Appl Genet

Abstract

Gummy stem blight (GSB), caused by three *Stagonosporopsis* spp., is a devastating fungal disease of watermelon (*Citrullus lanatus*) and other cucurbits that can lead to severe yield losses. Currently, commercial cultivars with genetic resistance to GSB in the field have not been developed. Utilizing GSB-resistant cultivars would reduce yield losses, decrease the high cost of disease control, and diminish hazards resulting from frequent fungicide application. The objective of this study was to identify quantitative trait loci (QTL) associated with GSB resistance in an $F_{2:3}$ interspecific *Citrullus* mapping population ($N = 178$), derived from a cross between Crimson Sweet (*C. lanatus*) and GSB-resistant PI 482276 (*C. amarus*). The population was phenotyped by inoculating seedlings with *Stagonosporopsis citrulli* 12178A in the greenhouse in two separate experiments, each with three replications. We identified three QTL (*ClGSB3.1*, *ClGSB5.1* and *ClGSB7.1*) associated with GSB resistance, explaining between 6.4 and 21.1% of the phenotypic variation. Locus *ClGSB7.1* accounted for the highest phenotypic variation and harbors twenty-two candidate genes associated with disease resistance. Among them is *ClCG07G013230*, encoding an Avr9/Cf-9 rapidly elicited disease resistance protein, which contains a non-synonymous point mutation in the DUF761 domain that was significantly associated with GSB resistance. High throughput markers were developed for selection of *ClGSB5.1* and *ClGSB7.1.* Our findings will facilitate the use of molecular markers for efficient introgression of the resistance loci and development of GSB-resistant watermelon cultivars.

Keywords: *Citrullus*, gummy stem blight*, Stagonosporopsis citrulli, ClGSB7.1, ClCG07G013230*

Introduction

Gummy stem blight (GSB) is a devastating fungal disease affecting cultivation of cucurbitaceous vegetable crops worldwide, leading to severe yield losses (Sherbakoff 1917; Chiu and Walker 1949; Sherf and MacNab 1986; Keinath 2011; Stewart et al. 2015). It has been reported to infect at least 12 genera and 23 species of Cucurbitaceae, including watermelon (*Citrullus lanatus*), cucumber (*Cucumis sativus*), cantaloupe and muskmelon (*Cucumis melo*), squashes (*Cucurbita pepo*), and gourds (*Cucurbita* spp.) (Keinath 2011). The occurrence of GSB is intensified by warm and humid environments that are conducive for germination of the spores and disease development (Keinath et al. 1995; Robinson and Decker-Walters 1997; Keinath 2011; Babu et al. 2015; Stewart et al. 2015). GSB was formerly thought to be caused by a single pathogen: *Didymella bryoniae* (syn. *Stagonosporopsis cucurbitacearum*) (Aveskamp et al. 2010), but it has since been established that the disease is caused by three *Stagonosporopsis* species: *S. cucurbitacearum* (syn. *Didymella bryoniae*), *S. citrulli*, and *S. caricae* (Stewart et al. 2015).

Current management of GSB in watermelon includes cultural practices and fungicide application. Due to the limited effectiveness of cultural practices on their own, fungicides remain critical for successful management of GSB (Stevenson et al. 2004; Keinath 2012). However, recent reports of differential fungicide resistance among the three causal *Stagonosporopsis* species presents a significant challenge to growers since the species cannot be differentiated based on symptoms (Brewer et al. 2015; Li et al. 2016; Li et al. 2019; Newark et al. 2019). In addition, fungicide applications greatly increase production costs and their repeated use may have a negative impact on the environment, particularly if residues persist in the soil. The best alternative would be to utilize GSB-resistant cultivars, but currently commercial watermelon cultivars with high levels of genetic resistance to GSB have not been developed.

Due to the narrow genetic base of cultivated watermelon following domestication (Guo et al. 2013; Levi et al. 2017), the *Citrullus amarus,* a wild relative of watermelon (*C. lanatus*) (Chomicki and Renner 2015; Renner et al. 2017) has been a major source of disease resistance alleles in watermelon breeding (Boyhan et al. 1994; Guner 2005; Thies and Levi 2007; Tetteh et al. 2010; McGregor 2012; Wechter et al. 2012; Levi et al. 2017; Branham et al. 2019a; Branham et al. 2019b). *Citrullus* germplasm sources with various levels of host resistance against GSB have been described (Sowell and Pointer 1962; Sowell 1975; Norton 1979; Gusmini et al. 2005). GSB resistance in *C. amarus* was described as early as 1962 in PI 189225 (Sowell and Pointer 1962) and later in PI 271778 (Sowell 1975; Norton 1979). Efforts to introgress resistance from these two sources into commercial cultivars was attempted, and led to the release of AU-Producer, AU-Jubilant, AU-Golden Producer and AU-Sweet Scarlet (Norton et al. 1986; Norton et al. 1993, 1995). However, these cultivars did not prove to be resistant in commercial production fields (Song et al. 2002). New sources of resistance that included accessions from both *C. amarus* and *C. lanatus* species were later described by Gusmini et al. (2005) and included PI 164248, PI 244019, PI 254744, PI 271771, PI 279461, PI 296332, PI 482379, PI 490383, PI 526233 and PI 482276. PI 482276 was found to be resistant to various isolates from all three *Stagonosporopsis* species (Gimode et al. unpublished).

Initial studies of GSB resistance in PI 189225 reported that resistance was mediated by a single gene, *db* (Norton 1979). However, later studies on PI 189225, PI 482283, and PI 526233 found that many genes with minor effects are most likely responsible for this trait (Gusmini et al. 2017; Hassan et al. 2019; Ren et al. 2019). Recently, a quantitative trait locus (QTL) underlying GSB resistance in PI 189225 was described on chromosome 8 of watermelon (Ren et al. 2019). This QTL explains ~32% of the phenotypic variance in the population. Identification of loci linked

to GSB resistance will facilitate development of molecular markers that would increase the efficiency of introgression of resistance loci into commercial watermelon cultivars.

The goal of the current study was to identify QTL associated with GSB resistance in an $F_{2:3}$ interspecific *Citrullus* population derived from a cross between Crimson Sweet and PI 482276 (Gusmini et al. 2005, Gimode et al. unpublished), and to develop high-throughput markers linked to the QTL to enable marker assisted selection for the trait.

Materials and methods

Plant material

The GSB-resistant PI 482276 (*C. amarus*) (Gusmini et al. 2005) was crossed with susceptible Crimson Sweet (*C. lanatus*) in the greenhouse to generate an interspecific F_1. A single F_1 plant was self-pollinated to produce an F_2 population. Individual F_2 plants were self-pollinated to produce 178 $F_{2:3}$ lines. Leaf material for parents, F_1 and each F_2 plant were collected and stored at -80°C prior to DNA extraction.

Inoculum preparation

A highly aggressive *Stagonosporopsis citrulli* isolate, 12178A (Gimode et al. unpublished), kindly provided by Marin Brewer (University of Georgia, Department of Pathology), was grown (16h/8h light/dark cycle) on potato dextrose agar (PDA) (Becton, Dickinson and Company, NJ, USA) for 2 weeks. Approximately 1 cm^2 agar plugs were then sub cultured on fresh PDA and grown for an additional 2 weeks. On the day of inoculation, PDA cultures were flooded with 10 ml of 0.1% tween20 and gently scraped with a microscope slide to release spores. The inoculum was filtered through 2 layers of sterile cheese cloth and spore

concentration was determined using a hemacytometer (Hausser Scientific, PA, USA). Spore concentrations were adjusted to 5×10^5 spores/ml using 0.1% tween20 solution.

Phenotyping

The seeds of the $F_{2:3}$ lines, F_1, and parental lines were grown in the greenhouse in 48-cell seedling trays under LED lights (Fluence Science, TX, USA) until the 3-4 leaf stage (approximately 3 weeks). Seedlings were inoculated by spraying with 5×10^5 spores/ml of freshly made *S. citrulli* inoculum until runoff. A randomized complete block design with three replicates was used, with four plants/genotype in each replicate (total of 12 F_3 plants/genotype in each experiment). Seedlings were placed in a humidity chamber in the greenhouse for 3 days (avg ~23.5 °C and ~96 % relative humidity) and then placed on a greenhouse bench and overhead watered as needed. Disease symptoms were scored 7 days post inoculation (dpi) for percentage of affected seedling using a 0-5 rating scale (0 = no symptoms, 1 = 1 to 20%, 2 = 21 to 40%, 3 = 41 to 60%, 4 = 61 to 80% and 5 = more than 80% of the seedling covered with lesions). Two independent experiments were performed from Dec 16, 2019 to Jan 13, 2020 and from Jan 24 to Feb 24, 2020, respectively.

Phenotypic data analysis

Analysis of variance (ANOVA) was performed using JMP® Pro 14.1 (SAS Institute Inc., Cary, NC) for the separate experiments with the effect of genotype and replication considered random. Further, an ANOVA of the combined experiments considered the random effects for genotype, replication, experiment and the interaction between genotype and experiment. BLUPs for each genotype, adjusted by the grand mean, was used for QTL mapping. Broad-sense heritability (H^2) was calculated from the ANOVA table as V_g / [V_g + $V_{g\times expt}$/expt + V_e/rep×expt],

where V_g = genotypic variance, $V_{g\times expt}$ = genotype by experiment variance, V_e = error (residual) variance, rep = number of replications, and expt = number of experiments (Holland et al. 2003). The distributions of disease severity within each experiment and in the joined data were tested for deviations from normality with Shapiro-Wilk tests (Shapiro and Wilk 1965). Correlation between experiments was assessed using pairwise Pearson correlations (*r*) calculated in JMP® Pro 14.1 (SAS Institute Inc., Cary, NC).

Genotyping, SNP analysis and map construction

For genotyping-by-sequencing (GBS) (Elshire et al. 2011), samples of the 178 F_2 individuals, four PI 482276, four Crimson Sweet and four F_1 individuals were freeze dried in two 96-well plates and shipped to Michigan State University for DNA extraction and quantification. Genomic DNA isolations and purifications were performed using the KingFisher flex (ThermoFisher Scientific Corporation, Waltham, MA) with Omega Mag-Bind kits (Omega Bio-Tek Inc., Norcross, GA). GBS was carried out at the Institute for Genomic Diversity (IGD), Cornell University, Ithaca, NY. Reads were aligned to the Charleston Gray watermelon genome (Wu et al. 2019) and SNPs were identified using TASSEL 5.0 GBS Discovery Pipeline (Glaubitz et al. 2014).

The identified SNPs were filtered for polymorphism between the mapping population parents, missing data rate in the population (no more than 5%), and segregation distortion ($P < 0.000001$). 1,525 SNPs were used for construction of the genetic map (Supplementary Table 4.1) using the regression mapping method in JoinMap 5.0 (Van Ooijen 2006) and distances between markers were calculated using the Kosambi mapping function (Kosambi 1943).

QTL mapping, candidate gene identification, and marker development

QTL detection for experiment 1, experiment 2 and the joined data was performed using composite interval mapping (CIM) (Zeng 1994) in WinQTLCart 2.5 (Wang 2007). Threshold values for all traits were calculated through permutation tests (1,000 permutations, $\alpha = 0.05$) (Churchill and Doerge 1994). CIM analysis was performed with a window size of 10 cM using the standard model (Model 6) with a walk speed of 1 cM and 5 marker cofactors determined by forward and backward regression.

The Charleston Gray (Wu et al. 2019) and the 97103 v2 (Guo et al. 2019) watermelon genomes were used to identify candidate genes within the 2-LOD interval of significant QTL. Syntenic regions associated with GSB resistance in other cucurbits were examined using the Synteny Viewer of the Cucurbit Genomics Database (Zheng et al. 2019) (http://cucurbitgenomics.org/). The genome sequence of PI 482276 (Guo et al. 2013) available at https://www.ncbi.nlm.nih.gov/sra/?term=SRP012850 was aligned to the Charleston Gray watermelon genome to identify polymorphisms in the candidate region. Pfam (https://pfam.xfam.org/) was used to determine protein domains likely to be associated with resistance.

KASP primers (Table 4.4) for SNPs closest to the QTL peaks were designed and optimized through Primer3Plus (Untergasser et al. 2007) and tested for polymorphisms with the two parents and the mapping population. The SNP of interest that was identified in the candidate gene region by aligning the genome resequencing reads of PI 482276 to the Charleston Gray reference genome was also developed into a KASP assay and the polymorphism confirmed in the parents and the population. All KASP assays were carried out in 4-µl volumes containing 1.94 µl of 2 × low rox

KASP master mix (LGC Genomics LLC, Teddington, UK), 0.06 µl primer mix with a final primer concentration of 0.81 µM, and 2 µl of 50–100 ng/µl genomic DNA. The PCR conditions used for the KASP assays consisted of an initial incubation at 95 °C for 15 min, 10 cycles of touchdown PCR with 20 s at 95 °C, 25 s of primer annealing temperature + 9 °C with 1 °C decrease each cycle, and 15 s of 72 °C, followed by 35 cycles of 10 s at 95 °C, 1 min at primer annealing temp, and 15 s at 72 °C, then held at 4 °C. KASP fluorescent end-point readings were measured using an Infinite M200Pro plate reader (Tecan Group Ltd.) and genotype calls were made using KlusterCaller (LGC Genomics LLC). Marker/trait association was analyzed using a one-way ANOVA followed by a Tukey-Kramer HSD test ($P = 0.05$) and R^2 values determined in JMP® Pro 14.1 (SAS Institute Inc., Cary, NC).

Results

Phenotypic data

The continuous phenotypic distributions of disease severity for the separate experiments as well as the joined data confirmed the quantitative nature of the trait (Fig. 4.1). All three distributions slightly deviated from a normal distribution according to the Shapiro-Wilk test for normality ($P = 0.005$, $P = 0.001$ and $P = 0.008$ for experiments 1, 2 and joined, respectively).

ANOVA showed significant effects for genotype ($P < 0.0001$) in individual (data not shown) and joined experiments (Table 4.1) but no significant effects were detected for the replication, experiment or interaction term of genotype × experiment in the joined analysis. The calculated variation between experiments accounted for only 0.7 % of the total variation in GSB resistance, while replication and interaction of genotype by experiment contributed to 4.2 % and 5.3 %, respectively (Table 4.1). A significant ($P < 0.0001$) positive correlation ($r = 0.57$) was

observed between the two experiments and the estimated broad sense heritability (H^2) of resistance to GSB was 72.6 % (Table 4.1).

GBS, SNP analysis and map construction

A total of 36,797 SNPs were obtained from the GBS analysis and 10,112 were found to be polymorphic between Crimson Sweet and PI 482276. After filtering, a genetic map was created containing 1,525 SNP markers with a 1.2 cM average distance between markers and a total length of 1,744 cM (Supplementary fig 4.1). Two regions on chromosome 8 and one on chromosome 5 had large gaps between markers: 25.58 cM and 21.71 cM on chromosome 8, and 18.77 cM on chromosome 5 (Supplementary fig 4.2).

QTL identification

GSB QTL were identified on chromosomes 3, 5 and 7. In experiment 1, QTL were identified on chromosomes 5 (*ClGSB5.1*: R^2=6.4%; 135.3-145.3 cM) and 7 (*ClGSB7.1*: R^2=15.4%; 114.3-116.3 cM) with maximum LOD scores of 4.4 and 6.5, respectively. In experiment 2, QTL were identified on chromosomes 3 (*ClGSB3.1*: R^2=14.1%; 76-79.1 cM) and 7 (*ClGSB7.1*: R^2=16%; 117.7-129 cM), with maximum LOD scores of 5.6 and 5.1, respectively. For the joined analysis, QTL with LOD scores of 5.9 and 8.6 were identified on chromosomes 5 (135.3-141.2 cM) and 7 (103.1-116.3 cM), explaining 10.2% and 21.1% of the phenotypic variance, respectively. These two QTL of the joined analysis both co-localized with the QTL for experiment 1 (Table 4.2 and Fig. 4.2). The closest SNP to the QTL peaks for *ClGSB3.1, ClGSB5.1* and *ClGSB7.1* were S03_12292063 (76.83 cM; experiment 2), S05_33279166 (139.22 cM; joined) and S07_30544246 (106.35 cM; joined), respectively.

Candidate gene identification

The total number of genes in the 2-LOD confidence interval for each QTL were: *ClGSB3.1:* 65; *ClGSB5.1:* 712; *ClGSB7.1:* 574 (data not shown). The GSB resistance loci and candidate genes identified in the present study were compared with those identified previously in cucurbit species (Lou et al. 2013; Liu et al. 2017; Zhang et al. 2017; Hassan et al. 2018; Hu et al. 2018; Ren et al. 2019).

ClGSB7.1 was responsible for the highest phenotypic variance and harbors several disease resistance-related genes. Among them are four nucleotide-binding site leucine-rich repeat (NBS-LRR) genes (*ClCG07G015790, ClCG07G015810, ClCG07G015870* and *ClCG07G015880*), and those encoding LRR containing proteins (*ClCG07G010720 ClCG07G012370, ClCG07G013540, ClCG07G014060, ClCG07G014730, ClCG07G015010, ClCG07G015800, ClCG07G015890*), receptor-like protein kinases (RLK), including LRR-RLKs (*ClCG07G010330, ClCG07G011290, ClCG07G011830, ClCG07G011880, ClCG07G012440, ClCG07G013510, ClCG07G014170, ClCG07G014760, ClCG07G015750*) and an Avr9/Cf-9 rapidly elicited disease resistance protein (*ClCG07G013230*) (Table 4.4).

Syntenic analysis revealed conserved synteny between *ClGSB7.1* and a locus in *Cucumis melo* (melon) chromosome 4 associated with GSB resistance (Hu et al., 2018) (Fig. 4.3). Eight candidate genes were reported in a 0.667 cM QTL region of chromosome 4 (Hu et al. 2018) and *MELO3C012987,* which displayed differential expression and sequence polymorphism between the resistant and susceptible melon lines, was determined as the most likely candidate gene associated with GSB resistance (Hu et al. 2018). In watermelon, *ClCG07G013230* is an ortholog of *MELO3C012987.*

The *ClCG07G013230* (29,622,088 - 29,622,708 Mb) gene is 621 base pairs (bp) long and contains no introns. Alignment of the PI 482276 sequence (Guo et al. 2013) to the Charleston Gray (parent of CS) genome (Wu et al. 2019) revealed four SNPs in the gene between the two genotypes. The four point mutations at bp positions 335 (C→A), 500 (T→G), 532 (G→C) and 598 (C→T) (Fig. 4.4) were all non-synonymous and the base substitutions cause an amino acid change from Alanine to Glutamic acid, Valine to Glycine, Alanine to Proline and Arginine to Tryptophan, respectively. A Pfam domain analysis indicated that *ClCG07G013230* harbors a DUF761 domain between 550 bp and 609 bp of the gene, which includes the Arginine to Tryptophan amino acid change. KASP assay confirmed the presence of the 598 (C→T) polymorphism between Crimson Sweet and PI 482276.

The candidate genes underlying *ClGSB5.1* include one NBS-LRR gene (*ClCG05G019540*), and those encoding F-box family proteins with LRR domains (*ClCG05G015740 ClCG05G015980, ClCG05G016910, ClCG05G020150*, *ClCG05G020210, ClCG05G020550*), RLKs (*ClCG05G014900, ClCG05G015780, ClCG05G017510, ClCG05G017520, ClCG05G018400, ClCG05G018970*) an enhanced disease resistance 2-like lipid binding protein (EDR2; *ClCG05G016060*) and a non-race specific disease resistance protein (*ClCG05G014750*). Among the 65 genes on *ClGSB3.1*, one of the genes is an LRR-RLK (*ClCG03G009870*) (Table 4.4).

Marker-trait association

High throughput KASP assays were developed for the SNPs closest to the peaks of *ClGSB5.1* and *ClGSB7.1* (Table 4.3). For all assays, disease severity was significantly lower for individuals homozygous for the resistant allele (R/R) than individuals homozygous for the susceptible allele (S/S) (Fig. 4.5). Assay ClGSB5.1-1 (S05_33279166; 139.22 cM) showed a

significant ($P = 0.019$) association with disease severity (RR = 2.9; SS = 3.3) and had an R^2 value of 4.5%. This marker had significant segregation distortion (P = 0.01), with the homozygous resistant genotype being underrepresented. Similar segregation distortion was observed for all the markers in this QTL region.

The closest marker to the QTL peak for *ClGSB7.1* (105.61 cM) was S07_30544246 (106.35 cM), and associated assay, ClGSB7.1-1, showed a significant difference ($P < 0.0001$) in disease severity between progeny homozygous for the PI 482276 (RR = 2.86) and the Crimson Sweet alleles (SS = 3.49) in the population ($R^2 = 14.2\%$).

ClGSB7.1-2, the KASP assay designed for the non-synonymous SNP in the DUF761 domain of *ClCG07G013230* gene, was polymorphic between the two parents (CS and PI 482276). This SNP mapped at 105.08 cM (data not shown), between S07_26211791 and S07_30544246 and displayed the highest R^2 value of 17.8% in the mapping population. Progeny with the 3 different ClGSB7.1-2 genotypes were significantly different from each other [($P < 0.0001$) (Fig. 4.5)] and the mean disease severity scores for progeny with the RR and SS genotypes were 2.79 and 3.57, respectively.

Discussion

The phenotypic distribution in the $F_{2:3}$ population from the Crimson Sweet × PI 482276 cross was continuous, confirming the quantitative nature of GSB resistance in this population. Transgressive segregation was observed in the direction of susceptibility. The correlation between the two experiments performed was moderate ($r = 0.57$). Variability in evaluations for GSB resistance in cucurbits has been observed in previous studies. A study by Wehner and Shetty (2000) reported low correlation between GSB ratings in cucumber, while Zhang et al. (1997) reported significantly high correlations ($r = 0.50 – 0.92$) among greenhouse experiments and inoculated

field trials. Gusmini et al. (2005) reported a low correlation (r = 0.10 – 0.36) in the evaluation for GSB resistance in watermelon. More recently, Ren et al. (2019) reported a significantly high correlation (r = 0.92) for GSB disease incidence in watermelon seedlings between two greenhouse experiments. We calculated a relatively high (72.6 %) broad sense heritability, with no significant interaction observed between the genotype × experiment. Previous QTL studies on GSB resistance (Lou et al. 2013; Liu et al. 2017; Zhang et al. 2017; Ren et al. 2019) did not partition the overall variance into genetic versus environmental components so it was not possible to compare the heritability estimates with other findings. Due to the non-significant experiment, and genotype-by-experiment interaction, the joined analysis data was considered most informative as it incorporated data from 24 plants/genotype, which gives a more accurate estimate of the $F_{2:3}$ family means.

Different modes of inheritance have been proposed for GSB resistance in cucurbits, including monogenic recessive (Norton 1979; Frantz and Jahn 2004; Hassan et al. 2018), monogenic dominant (Zuniga et al. 1999; Frantz and Jahn 2004; Wolukau et al. 2007; Hu et al. 2018), and polygenic (Lou et al. 2013; Gusmini et al. 2017; Liu et al. 2017; Zhang et al. 2017; Hassan et al. 2019; Ren et al. 2019) inheritance patterns. A QTL associated with GSB resistance in watermelon was recently mapped on chromosome 8 (*Qgsb8.1*) using PI 189225 as the resistance source (Ren et al. 2019). We identified three QTL significantly associated with GSB resistance on chromosomes 3, 5 and 7 of watermelon in an interspecific Crimson Sweet × PI 482276 cross, which represent a novel source of resistance to GSB. It is worth noting that the location of *Qgsb8.1* described by Ren et al. (2019) is within the 21.71 cM (10.2 Mbp) gap on chromosome 8 on our genetic map. Due to low marker density in this region, we cannot determine the potential association of the region with GSB resistance in the present study. The large genetic distances

between markers in this location may be due differences in chromosome structure and distorted segregation that often occurs in interspecific crosses. Sandlin et al. (2012) reported a 33.04 cM gap between markers in the ZWRM50 (*C. lanatus*) × PI 244019 (*C. amarus*) map. Another possibility for lack of detection of *Qgsb8.1* in our population could be the utilization of different resistance sources and pathogen isolates in the two studies. Ren et al. (2019) used PI 189225 and a *S. cucurbitacearum* isolate while we used PI 482276 and a *S. citrulli* isolate. It is still unclear whether the resistance loci provide resistance across different *Stagonosporopsis* species.

ClGSB7.1 was stable across the two experiments while *ClGSB5.1* and *ClGSB3.1* were dependent on the environment. *ClGSB7.1* appears to have the greatest potential for introgression into cultivated watermelon since it not only explained the highest proportion of variation in GSB resistance (21%) but was also stable in the two experiments and the joined analysis. None of the progeny in our study was as resistant as the PI 482276 parent (Fig. 4.1), however selecting for *ClGSB7.1* provides an intermediate level of resistance (disease severity = 2.79). Further research is needed to determine the effectiveness of this level of resistance under field conditions. Efforts to breed for resistance to GSB in watermelon began in the 1970s (Norton 1979; Norton et al. 1986) but to date, no commercial cultivars with field-level resistance have been developed. It is likely that this is at least partially due to the complex genetic control of GSB resistance by separate loci with different effects. The identification of several resistance QTL from different resistance sources would allow for pyramiding multiple resistance alleles into cultivated watermelon.

The significant loci detected in this study all harbor potential candidate genes including those encoding NBS-LRRs, LRR domains, RLKs, an Avr9/Cf-9 protein, an EDR2 protein and a non-race specific disease resistance protein, which are all associated with plant defense against pathogens. One of these candidate genes, *ClCG07G013230,* is an ortholog of *MELO3C012987,*

an Avr9/Cf-9 rapidly elicited disease resistance protein, that was proposed as the single dominant GSB-resistance gene in melon (Hu et al. 2018). Moreover, a SNP within the DUF761 domain of this gene was found to be significantly associated with the trait in resistant and susceptible *C. melo* lines (Hu et al. 2018). The four SNPs found in the *ClCG07G013230* gene in *ClGSB7.1* all led to a change in amino acid. ClGSB7.1-2 KASP assay for the C → T SNP in the DUF761 domain which is associated with disease resistance (Zhang et al. 2019), was polymorphic between Crimson Sweet and PI 482276, confirming what was observed from the genome alignments. This SNP displayed significant association with GSB resistance in the mapping population. We therefore propose *ClCG07G013230* as a candidate gene for resistance to GSB in watermelon. Further analysis of this gene through expression studies in GSB-resistant and -susceptible watermelon lines will be required to confirm its role. The utility of the KASP assays described in this study needs to be validated in other genetic backgrounds to confirm their usefulness in marker assisted selection (MAS) for GSB resistance in watermelon breeding.

One of the major drawbacks in the quest to breed for GSB-resistant cultivars has been the labor-intensive phenotyping process and inconsistencies observed with phenotyping results (Wehner and Shetty 2000; Gusmini et al. 2005; Wehner 2008). Application of molecular breeding tools such as marker-assisted selection would greatly improve GSB-resistant cultivar development by minimizing the labor-intensive and time-consuming steps in the breeding process. We have developed high throughput KASP assays for MAS that will allow for more efficient incorporation of GSB resistance into elite watermelon cultivars.

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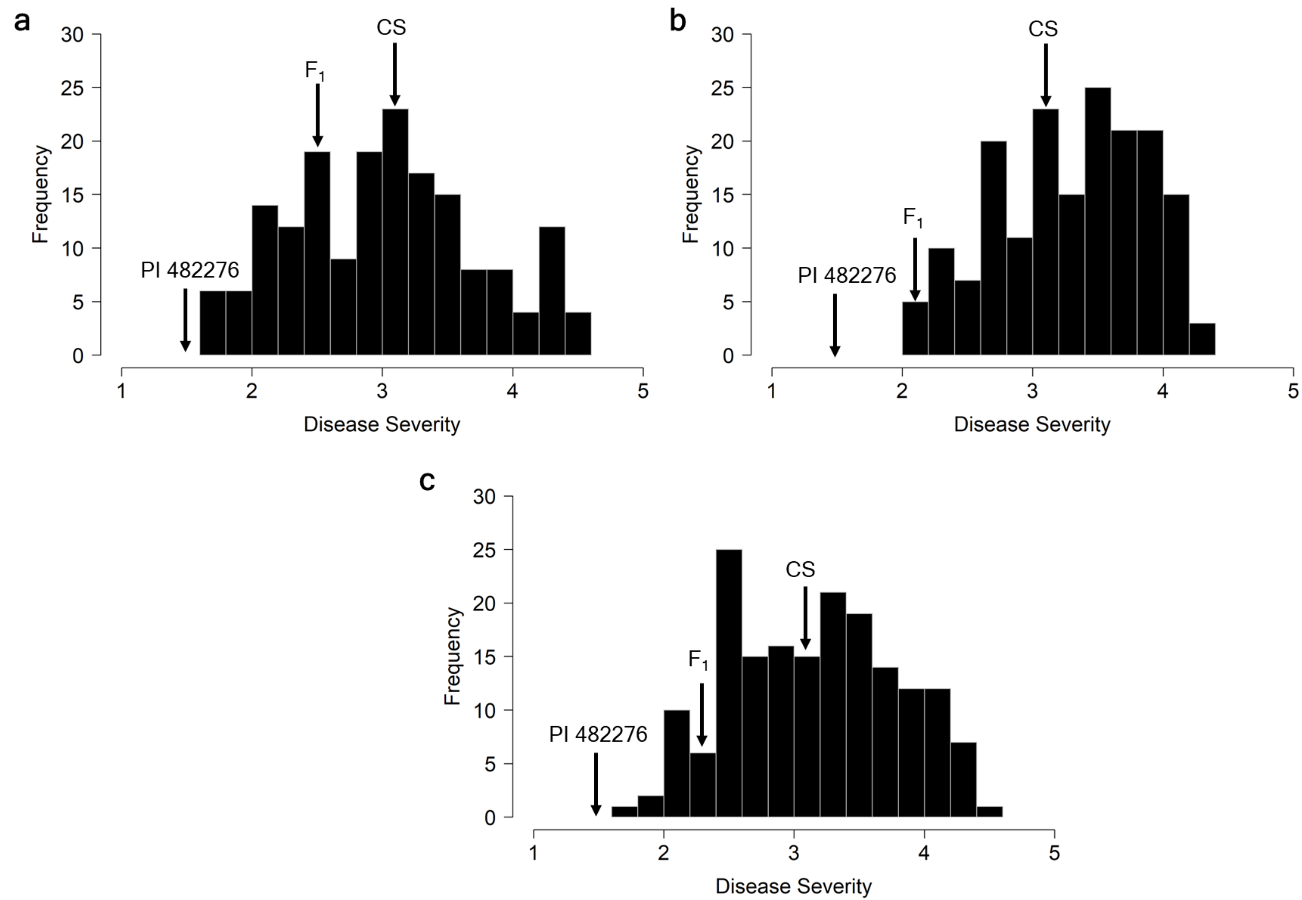

Fig 4.1: Frequency distribution for disease severity scores at 7 days post-inoculation for **(a)** experiment 1, **(b)** experiment 2 and (**c**) joined data in the Crimson Sweet (CS) × PI 482276 $F_{2:3}$ watermelon population (*N*=178) inoculated with *Stagonosporopsis citrulli*.

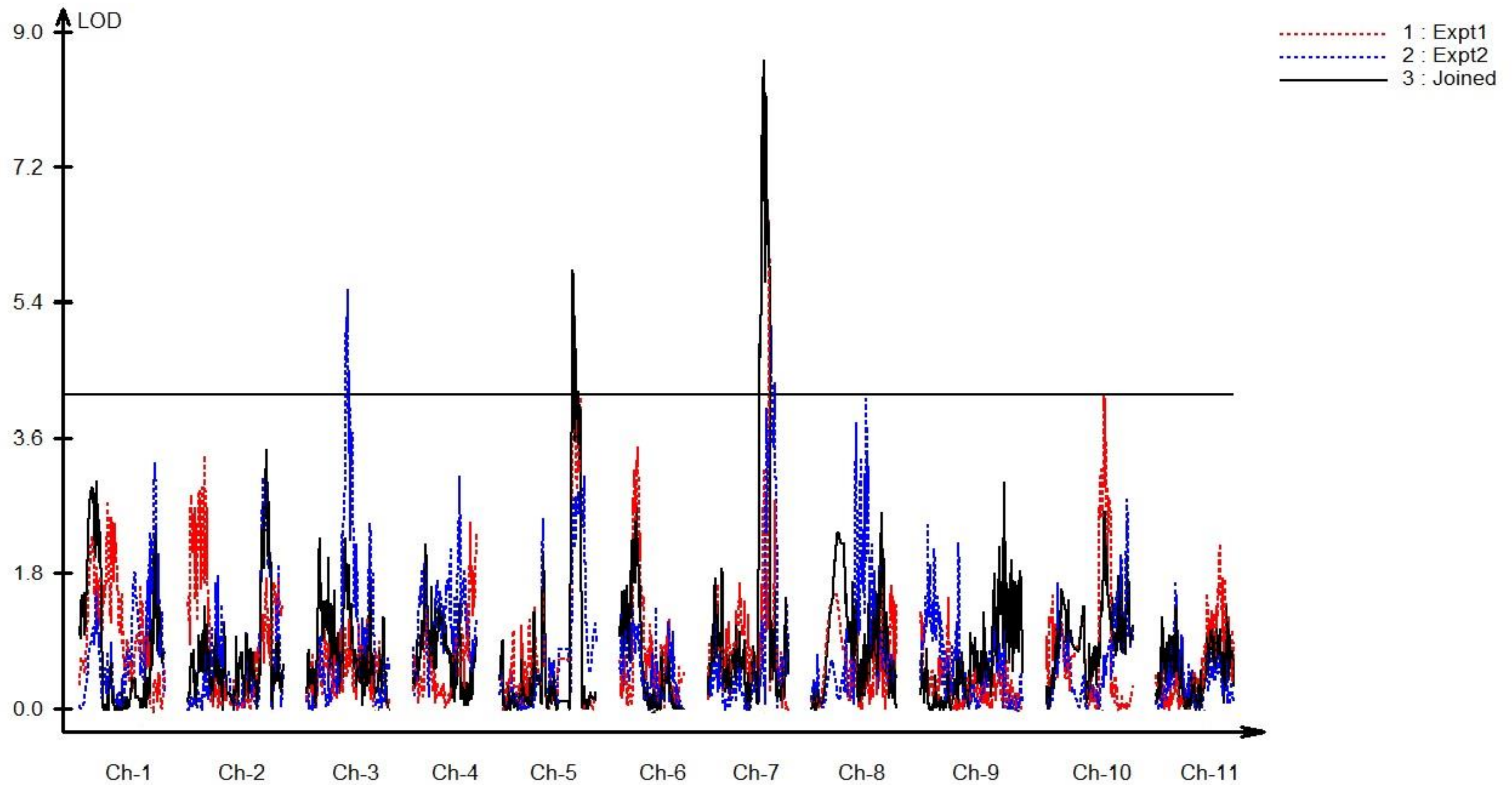

Fig 4.2: QTL associated with GSB resistance in the Crimson Sweet (CS) × PI 482276 $F_{2:3}$ watermelon population (*N*=178) in experiment 1, experiment 2 and joined data 7 days after inoculation with *Stagonosporopsis citrulli*.

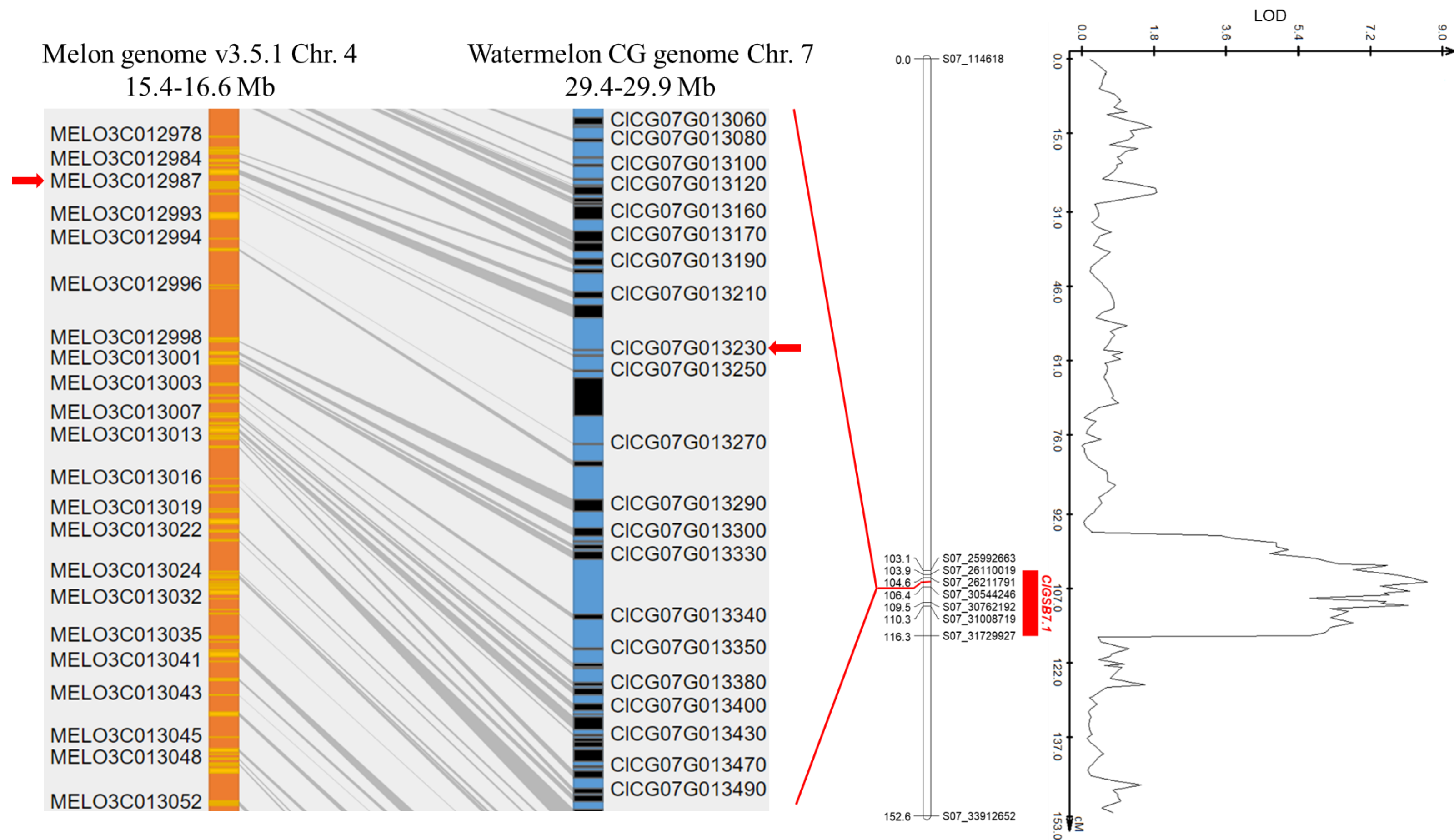

Fig 4.3: Syntenic analysis of the GSB resistance region in *Cucumis melo* (Hu et al. 2018) with the QTL region on *Citrullus lanatus* chromosome 7. Orange and blue represent *Cucumis melo* and *Citrullus lanatus* chromosome 4 and 7, respectively. The disease resistance melon gene *MELO3C012987* is an ortholog of watermelon gene *ClCG07G013230*.

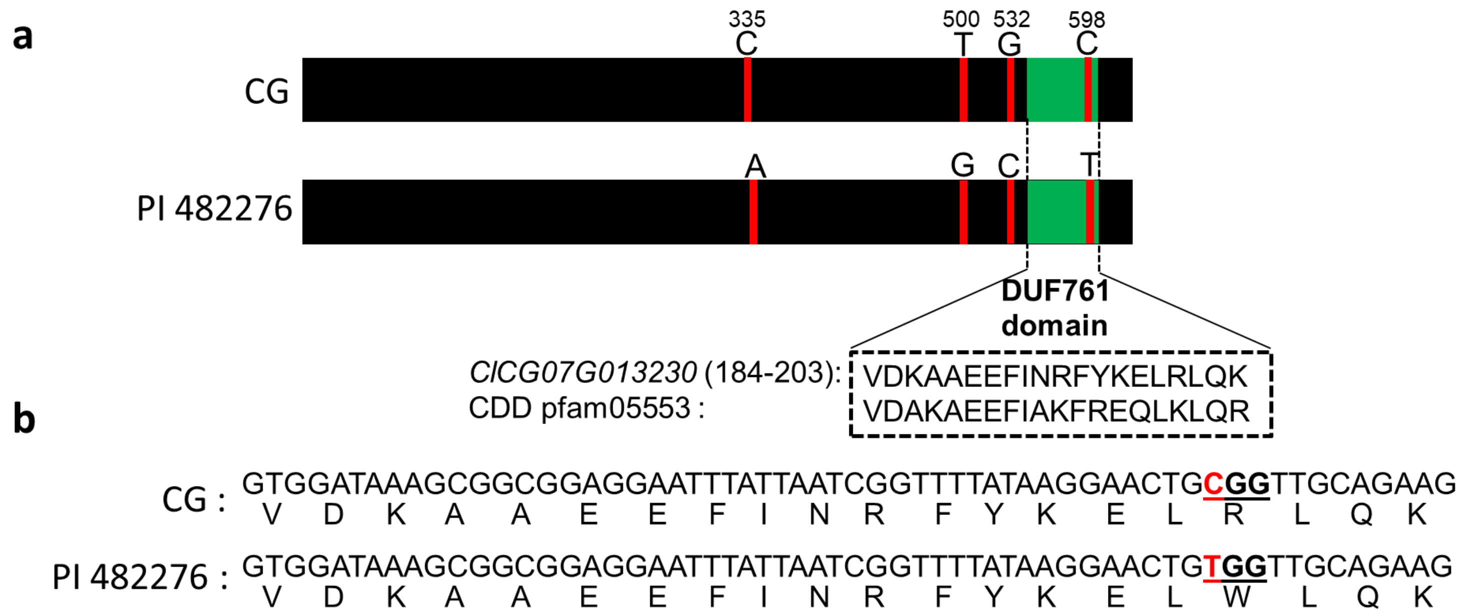

Fig 4.4: Alignment of the ClCG07G013230 gene between the Charleston Gray (CG) reference genome and PI 482276. **a** SNPs and their bp positions on the gene. **b** The DUF761 domain with a non-synonymous substitution from C to T that changes the amino acid from Arginine to Tryptophan.

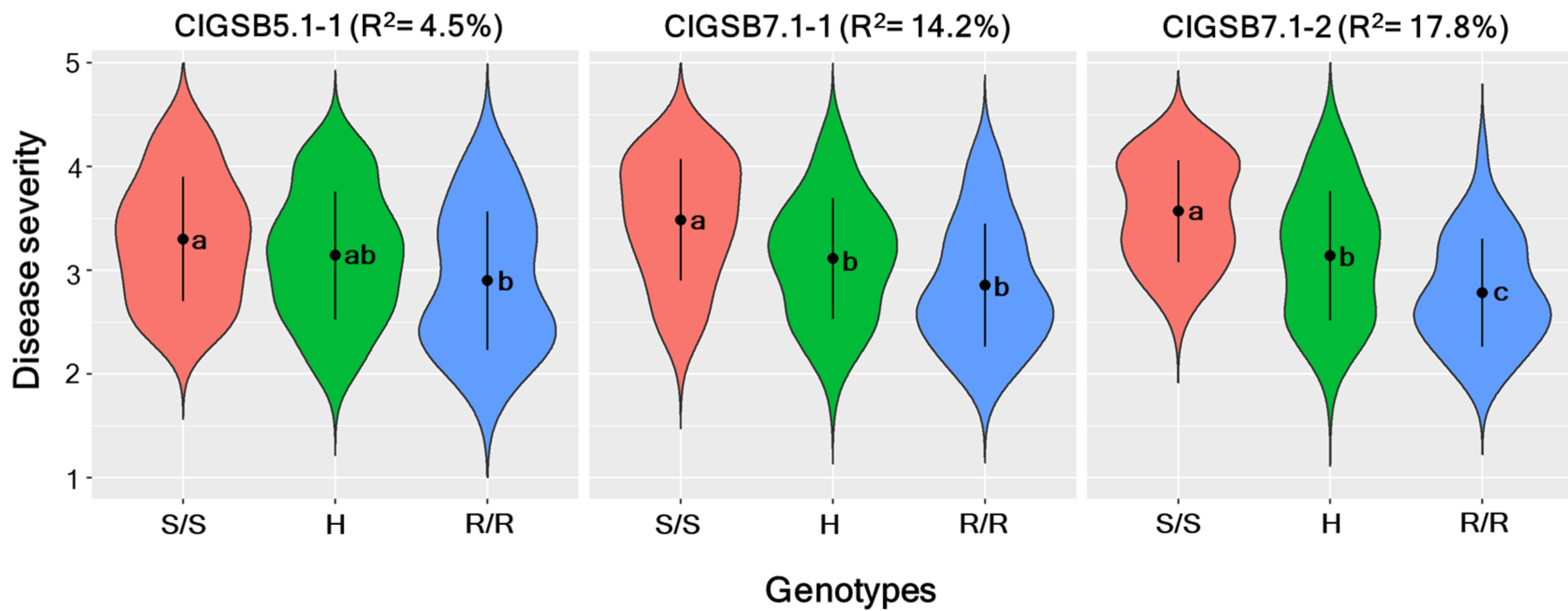

Fig 4.5: Performance of KASP assays in the Crimson Sweet (CS) × PI 482276 $F_{2:3}$ mapping population. Dots indicate mean and levels not connected by the same letter are significantly different, where S represents the susceptible (CS) allele, H represents the heterozygote and R represents the resistant (PI 482276) allele.

Table 4.1: ANOVA table for variance components and broad sense heritability (H^2) of gummy stem blight resistance in the Crimson Sweet × PI 482276 $F_{2:3}$ watermelon population after inoculation with *Stagonosporopsis citrulli*

Variance component	**Variance**	**Standard Error**	**$\%V_P$**[a]
Genotype***	54.7	8.4	32.4
Experiment[NS]	1.1	5.5	0.7
Genotype × experiment[NS]	8.9	4.7	5.3
Replicate within experiment[NS]	7.1	5.4	4.2
Residual	97.1	5.2	57.5
H^2	72.6		

[a] Percent of total phenotypic variance

***Significant: $P < 0.0001$

[NS]: Not significant

Table 4.2: Quantitative trait loci (QTL) associated with resistance to *Stagonosporopsis citrulli* and the corresponding 2-LOD support interval for separate and joined data for disease severity observed at 7 d post-inoculation from two screens of the Crimson Sweet × PI 482276 $F_{2:3}$ watermelon population.

Trait	QTL name	Chromosome	Peak (cM)	LOD[a]	Additive[b]	Dominance[c]	2- LOD interval (cM)[d]	Right flanking marker (Mb)	Left flanking marker (Mb)	R^2(%)[e]
Expt 1	*ClGSB5.1*	5	143.41	4.4	0.2851	0.0385	135.3-145.3	24,766,876	33,886,603	6.4
Expt 1	*ClGSB7.1*	7	114.71	6.5	0.3655	-0.0876	114.3-116.3	31,234,486	31,729,927	15.4
Expt 2	*ClGSB3.1*	3	76.81	5.6	-0.1394	0.3044	76.0-79.1	12,183,997	16,245,609	14.1
Expt 2	*ClGSB7.1*	7	119.11	5.1	0.2496	-0.169	117.7-129.0	31,760,991	32,678,198	16.0
Joined	*ClGSB5.1*	5	137.71	5.9	0.3354	0.0918	135.3-147.1	24,766,876	33,964,634	10.2
Joined	*ClGSB7.1*	7	105.61	8.6	0.3517	-0.0996	103.1-116.3	25,992,663	31,729,927	21.1

[a] Logarithm of odds ratios at the position of the peak

[b] Additive effect of QTL

[c] Dominance effect of QTL

[d] The QTL interval on genetic map

[e] Percent of phenotypic variance explained by the QTL

Table 4.3: KASP™ assay primer sequences of SNPs associated with GSB resistance

KASP Assay	Ta (˚C)	SNP	Primer type	Primer sequence (5'-3')	Allele
ClGSB5.1-1	57	S05_33279166	FAM	GAAGGTGACCAAGTTCATGCTGACGAGGATGGTATGTTCAAATTCT	CS
			VIC	GAAGGTCGGAGTCAACGGATTGACGAGGATGGTATGTTCAAATTCG	PI 482276
			Reverse	GAACGAAGCAACCGCAATTC	
ClGSB7.1-1	58	S07_30544246	FAM	GAAGGTGACCAAGTTCATGCTCGGAGTCCGAAAGGATCTTCA	CS
			VIC	GAAGGTCGGAGTCAACGGATTCGGAGTCCGAAAGGATCTTCG	PI 482276
			Reverse	TTCCATGGCCTCTTCTGCAT	
ClGSB7.1-2	57.5	*ClCG07G013230;* 598 (C→T)	FAM	GAAGGTGACCAAGTTCATGCTTCCGCCGTCTTCTGCAAC	CS
			VIC	GAAGGTCGGAGTCAACGGATTTCCGCCGTCTTCTGCAAT	PI 482276
			Reverse	CCGATGCTGGTTAGGCAGTT	

Table 4.4: GSB resistance candidate genes within the QTL regions

Gene ID	Chromosome	Start (Mb)	End (Mb)	Strand	Annotation
ClCG03G009870	CG_Chr03	15757768	15780203	-	Leucine-rich repeat receptor-like protein kinase family protein
ClCG05G014750	CG_Chr05	25408792	25409433	+	Non-race specific disease resistance protein 1-like protein b
ClCG05G014900	CG_Chr05	25899733	25902609	-	Receptor-like kinase
ClCG05G015740	CG_Chr05	27630305	27637763	+	F-box/LRR-repeat protein
ClCG05G015780	CG_Chr05	27688890	27697011	+	Leucine-rich receptor-like protein kinase family protein
ClCG05G015980	CG_Chr05	27976417	27977658	-	F-box family protein with LRR domain
ClCG05G016060	CG_Chr05	28188299	28189177	+	Enhanced disease resistance 2-like lipid binding protein
ClCG05G016910	CG_Chr05	29218923	29222502	-	F-box protein with LRR domain
ClCG05G017510	CG_Chr05	29814511	29817269	+	Receptor-like kinase
ClCG05G017520	CG_Chr05	29822221	29835381	+	Receptor-like protein kinase
ClCG05G018400	CG_Chr05	30659730	30660297	+	LRR receptor-like protein kinase
ClCG05G018970	CG_Chr05	31252009	31256889	+	Leucine-rich repeat receptor-like protein kinase family protein
ClCG05G019540	CG_Chr05	31779884	31783097	-	NBS-LRR resistance protein

ClCG05G020150	CG_Chr05	32367048	32368349	-	Leucine-rich repeat (LRR) family protein
ClCG05G020210	CG_Chr05	32407294	32410880	-	F-box protein with LRR domain
ClCG05G020550	CG_Chr05	32625712	32632871	+	Leucine-rich repeat (LRR) family protein
ClCG07G010330	CG_Chr07	26049905	26056663	+	Receptor-like protein kinase
ClCG07G010720	CG_Chr07	26595881	26598780	-	Leucine-rich repeat (LRR) family protein
ClCG07G011290	CG_Chr07	27323834	27337194	+	Leucine-rich repeat receptor kinase
ClCG07G011830	CG_Chr07	28050919	28055264	-	Leucine-rich repeat receptor-like protein kinase PEPR2
ClCG07G011880	CG_Chr07	28178597	28180263	-	Receptor-like protein kinase
ClCG07G012370	CG_Chr07	28782357	28783289	-	Leucine-rich repeat/extensin 1
ClCG07G012440	CG_Chr07	28834828	28836168	-	Leucine-rich repeat receptor-like protein kinase
ClCG07G013230	CG_Chr07	29622088	29622708	-	Disease resistance gene
ClCG07G013510	CG_Chr07	29929867	29948050	+	Leucine-rich repeat protein kinase family protein
ClCG07G013540	CG_Chr07	29977725	29980571	+	F-box/RNI-like superfamily protein with LRR domain
ClCG07G014060	CG_Chr07	30591416	30591940	+	Leucine-rich repeat/extensin 2
ClCG07G014170	CG_Chr07	30634407	30655058	-	Leucine-rich repeat receptor-like protein kinase family protein

ClCG07G014730	CG_Chr07	31128770	31132515	-	Protein kinase superfamily protein with LRR domain
ClCG07G014760	CG_Chr07	31191612	31205212	-	Receptor-like protein kinase
ClCG07G015010	CG_Chr07	31441825	31446423	+	Leucine-rich repeat-containing protein
ClCG07G015750	CG_Chr07	32238769	32243750	-	Leucine-rich receptor-like protein kinase family protein
ClCG07G015790	CG_Chr07	32277725	32280985	+	CC-NBS-LRR resistance protein
ClCG07G015800	CG_Chr07	32281502	32281940	-	Fom-2 protein with LRR domain
ClCG07G015810	CG_Chr07	32283089	32286325	-	NBS-LRR resistance protein
ClCG07G015870	CG_Chr07	32330744	32331177	+	CC-NBS-LRR resistance protein
ClCG07G015880	CG_Chr07	32331618	32332439	+	NBS-LRR resistance protein
ClCG07G015890	CG_Chr07	32332552	32333123	+	Fom-2 protein with LRR domain

'1' '2' '3' '4' '5' '6'

'7'

cM	Marker
0.0	S07_114618
2.5	S07_478623
4.1	S07_781631
5.9	S07_948517
7.0	S07_902478
8.5	S07_1386615
9.7	S07_1385352
10.7	S07_1182896
11.2	S07_1169639
13.8	S07_1498420
14.5	S07_1499575
16.3	S07_1903735
17.3	S07_2229338
18.1	S07_2040391
18.6	S07_1890511
19.8	S07_2281975
20.9	S07_2631775
21.3	S07_2631817
21.8	S07_2543518
24.1	S07_2802508
25.0	S07_2844006
26.0	S07_2998440
27.2	S07_3012167
27.8	S07_2975555
29.2	S07_3051636
31.7	S07_3634400
33.0	S07_3743400
33.8	S07_3802433
34.9	S07_3983630
35.7	S07_4046830
37.6	S07_4095620
39.0	S07_4404449
42.0	S07_4966068
43.0	S07_5226312
45.1	S07_5495746
50.2	S07_6685973
52.2	S07_6925969
53.8	S07_6956264
54.3	S07_7008156
55.0	S07_7126866
55.8	S07_7048563
56.3	S07_7554107
58.7	S07_7819729
59.2	S07_8051578
59.8	S07_8070675
60.6	S07_8083586
61.3	S07_8758628
64.0	S07_10325158
65.8	S07_10838805
67.1	S07_11287353
68.4	S07_12838438
69.5	S07_15184634
70.4	S07_15185734
72.5	S07_20508996
73.1	S07_19890161
75.6	S07_21268922
76.7	S07_21856691
77.6	S07_21856721
78.1	S07_21856649
78.6	S07_21477218
79.4	S07_21963413
80.5	S07_22340580
83.1	S07_22954087
85.2	S07_23450771
86.0	S07_23699591
88.5	S07_24369641
89.7	S07_24346708
90.8	S07_24564246
91.5	S07_24734340
94.2	S07_25476916
95.5	S07_25164505
96.1	S07_25144057
96.7	S07_25476979
97.6	S07_25467536
98.4	S07_25332089
99.1	S07_25643144
99.8	S07_25641595
101.9	S07_25873360
102.1	S07_25921729
102.4	S07_25815428
103.1	S07_25992663
103.9	S07_26110019
104.6	S07_26211791
106.4	S07_30544246
107.4	S07_30000088
107.8	S07_29532637
108.2	S07_30017298
108.5	S07_27342596
108.9	S07_26953736
109.5	S07_30762192
109.8	S07_26642510
110.3	S07_31008719
110.9	S07_30977359
111.4	S07_31032881
112.8	S07_31234486
114.7	S07_31318805
116.3	S07_31729927
116.6	S07_31689639
117.7	S07_31760991
119.1	S07_31846633
120.1	S07_31959383
120.6	S07_31990061
121.2	S07_32053019
122.1	S07_32226762
122.4	S07_32226714
122.7	S07_32226644
125.0	S07_32329293
126.3	S07_32328806
126.9	S07_32347957
129.0	S07_32528184
130.3	S07_32678198
130.8	S07_32672663
131.8	S07_32725463
132.8	S07_32819043
133.4	S07_32874690
134.3	S07_32967438
135.3	S07_33148105
136.3	S07_33007331
137.1	S07_32997140
140.2	S07_33409510
140.9	S07_33463519
143.5	S07_33664903
144.6	S07_33664946
146.5	S07_33723919
147.5	S07_33735659
148.7	S07_33753660
149.9	S07_33809769
151.1	S07_33809755
152.6	S07_33912652

'8'

cM	Marker
0.0	S08_891496
4.5	S08_2304945
6.0	S08_2614411
7.3	S08_1542629
8.3	S08_1137218
8.9	S08_1775969
9.9	S08_1830092
10.9	S08_374481
12.5	S08_3814195
38.1	S08_6123544
59.8	S08_16365681
62.1	S08_16896195
63.4	S08_15902759
64.2	S08_15900602
64.9	S08_16365671
67.3	S08_17658671
71.7	S08_19512270
73.7	S08_19012567
74.7	S08_19322464
75.2	S08_19012494
75.9	S08_19294210
76.3	S08_19065378
76.8	S08_19483946
77.9	S08_19656084
78.6	S08_19799827
79.2	S08_19896065
79.6	S08_19759346
81.9	S08_20309688
82.2	S08_20377789
82.8	S08_20600543
83.2	S08_20641978
84.5	S08_20686849
84.7	S08_20764738
85.1	S08_20764684
86.6	S08_20991021
87.3	S08_21048058
87.6	S08_21046164
88.5	S08_21129366
89.7	S08_21477283
90.1	S08_21495604
92.1	S08_21859022
92.5	S08_21791191
93.6	S08_21891016
94.1	S08_21920091
94.4	S08_22290243
95.2	S08_22290350
96.5	S08_22681897
97.6	S08_22891031
98.2	S08_22780017
98.3	S08_22811416
99.2	S08_22922383
99.8	S08_23155327
100.3	S08_23122805
100.5	S08_23208415
101.0	S08_23208496
101.3	S08_23251062
101.7	S08_23250961
102.5	S08_23337365
103.0	S08_23314142
103.3	S08_23422415
104.0	S08_23641400
104.2	S08_23634955
104.6	S08_23810581
105.1	S08_23849450
105.9	S08_23971089
106.7	S08_24010123
107.7	S08_24356534
108.0	S08_24329028
108.4	S08_24323695
108.7	S08_24323771
108.9	S08_24539926
109.2	S08_24326959
109.9	S08_24476105
110.8	S08_24951363
111.5	S08_24790437
112.3	S08_25209693
112.8	S08_25184898
113.9	S08_25321103
114.2	S08_25316393
114.5	S08_25317244
115.4	S08_25333744
116.7	S08_25796629
117.3	S08_25785941
118.0	S08_26034368
118.8	S08_25664624
119.5	S08_26266446
120.8	S08_26310720
121.8	S08_26382749
123.2	S08_26378591
124.3	S08_26605471
125.3	S08_26318121
127.0	S08_26718714
127.3	S08_26828656
128.2	S08_27081050
129.5	S08_27222079
131.5	S08_27365613
133.7	S08_27893282
134.0	S08_27863599
134.5	S08_27946402
135.1	S08_27864236
137.5	S08_28347741
138.7	S08_28097055
142.1	S08_28402519
143.7	S08_28437478
144.6	S08_28493097
145.6	S08_28690922
147.0	S08_29053645
147.5	S08_29012183
148.6	S08_29260783
149.2	S08_29186598
149.6	S08_29222135
150.3	S08_29462144
150.8	S08_29552007
151.1	S08_29399757
151.9	S08_29462203
152.7	S08_29685345
153.6	S08_29751971
155.0	S08_29348630
157.9	S08_29589752
160.7	S08_29967336

'9'

cM	Marker
0.0	S09_135300
2.5	S09_167815
3.6	S09_117704
4.2	S09_76827
4.9	S09_160736
7.0	S09_799304
7.9	S09_487337
8.8	S09_725110
9.3	S09_799373
10.2	S09_1040267
10.5	S09_1094344
11.3	S09_1143200
13.2	S09_1225000
13.7	S09_1242453
14.5	S09_1299055
15.5	S09_1713453
16.4	S09_1878320
17.4	S09_2016256
17.8	S09_1968620
18.0	S09_1934068
18.7	S09_2035545
20.6	S09_2273872
22.8	S09_2495642
23.1	S09_2492476
24.2	S09_2616632
25.3	S09_3016851
26.8	S09_3157510
27.8	S09_3205559
28.3	S09_3211098
29.1	S09_3440214
29.6	S09_3414151
31.3	S09_3615247
31.6	S09_3640468
33.3	S09_3806885
34.1	S09_3800096
34.8	S09_3822771
36.0	S09_3868891
36.3	S09_3922129
37.4	S09_4040204
37.8	S09_4122330
38.1	S09_4033653
38.6	S09_4140024
40.3	S09_4335628
42.9	S09_4560830
43.5	S09_4490655
44.1	S09_4582195
44.7	S09_4595486
45.5	S09_4652787
47.0	S09_4847054
47.6	S09_4821309
47.9	S09_4744334
48.3	S09_4785444
50.5	S09_5443023
51.1	S09_5442909
52.1	S09_5576288
52.5	S09_5442971
52.8	S09_5488083
53.0	S09_5367785
53.3	S09_5439372
53.6	S09_5348674
55.8	S09_5958781
56.5	S09_6073192
56.8	S09_6166627
57.4	S09_6202440
57.8	S09_6072675
59.5	S09_6484477
59.9	S09_6681425
61.2	S09_6916655
61.6	S09_6862420
62.0	S09_6825173
62.4	S09_6865187
63.7	S09_7075145
65.8	S09_7193610
66.1	S09_7250185
66.3	S09_7358125
68.0	S09_7508243
68.4	S09_7504526
69.0	S09_7607455
69.4	S09_7646445
70.1	S09_7733042
71.9	S09_8268380
72.4	S09_8259351
72.9	S09_8313744
73.3	S09_8317550
73.9	S09_8456715
76.2	S09_8642707
78.1	S09_9008440
78.5	S09_9160374
79.4	S09_9318976
80.3	S09_9388501
80.6	S09_9461245
81.0	S09_9480336
81.8	S09_9787109
83.5	S09_10121070
84.1	S09_10005510
85.0	S09_9892748
85.6	S09_10253289
87.2	S09_10362767
88.3	S09_10473869
89.5	S09_10453844
92.7	S09_11246907
93.6	S09_11243196
94.4	S09_11280540
95.2	S09_11280408
96.4	S09_11607089
100.3	S09_12066191
101.9	S09_12714182
103.6	S09_12850984
104.6	S09_13372184
106.0	S09_14339813
106.6	S09_14167674
107.5	S09_14751894
108.5	S09_14923673
109.0	S09_14923485
109.8	S09_15724956
111.8	S09_17165277
114.4	S09_19071222
114.8	S09_18583365
115.4	S09_19864605
115.6	S09_18976177
116.4	S09_20244160
116.8	S09_20018490
117.8	S09_24436281
118.4	S09_22207859
118.8	S09_21259081
119.3	S09_21231925
119.8	S09_27770382
120.5	S09_28198786
120.9	S09_30502384
121.3	S09_26926788
121.9	S09_32352262
123.7	S09_33602703
126.6	S09_34429846
127.4	S09_34635937
127.7	S09_34664440
128.1	S09_34652309
128.7	S09_34764461
129.8	S09_34656997
131.1	S09_34930260
132.3	S09_35197670
133.8	S09_35338061
135.3	S09_35503839
136.5	S09_35528124
137.5	S09_35647119
140.0	S09_35909627
140.5	S09_36001651
142.4	S09_36235333
142.8	S09_36116204
143.4	S09_36200541
145.7	S09_36430760
147.6	S09_36552623
148.0	S09_36513514
148.6	S09_36625232
149.3	S09_36565158
149.7	S09_36633384
150.5	S09_36702871
152.3	S09_36895364
152.9	S09_37016294
154.2	S09_37052042
156.0	S09_37249244
158.5	S09_37574337
159.1	S09_37616951
159.5	S09_37545856
160.0	S09_37480213
160.4	S09_37459729
161.0	S09_37617460
164.8	S09_38019237
165.5	S09_38187452
166.9	S09_38367018
167.5	S09_38397365
167.7	S09_38321918
168.0	S09_38367102
169.5	S09_38363115
170.8	S09_38529765
171.8	S09_38481745
173.1	S09_38412556
175.6	S09_38821630
177.6	S09_39193544
179.0	S09_39314970
180.3	S09_39431656
181.0	S09_39480908
181.6	S09_39496468
183.1	S09_39588431
184.2	S09_39766028
185.7	S09_40005982
186.8	S09_39998123
189.5	S09_39430299
194.2	S09_39813582

'10'

cM	Marker
0.0	S10_204351
1.0	S10_133425
1.6	S10_111540
3.1	S10_308221
4.6	S10_534427
5.4	S10_595763
6.8	S10_613739
8.8	S10_784616
10.0	S10_1056896
10.5	S10_1019810
11.3	S10_1224747
12.5	S10_1512695
13.4	S10_1450240
14.0	S10_1468106
14.9	S10_1673324
15.5	S10_1671767
16.8	S10_1777086
17.7	S10_1880000
19.6	S10_2119181
19.9	S10_2352665
20.4	S10_2376136
20.8	S10_2269982
21.4	S10_2376205
22.3	S10_2674243
23.1	S10_2674087
24.8	S10_3178368
25.3	S10_3191048
26.9	S10_3510059
28.0	S10_3899815
29.1	S10_3940398
36.5	S10_5309435
38.1	S10_5245006
38.8	S10_5350341
40.0	S10_5509046
41.7	S10_5508985
43.1	S10_6080893
44.2	S10_6243284
45.8	S10_6408610
53.6	S10_7303770
70.9	S10_22080227
79.1	S10_23337437
81.2	S10_24007453
82.8	S10_23422542
84.1	S10_24007767
85.2	S10_24244389
86.2	S10_24622163
87.1	S10_24563112
87.9	S10_24647547
88.3	S10_24657719
88.7	S10_24647472
89.9	S10_24750075
90.2	S10_24938565
92.5	S10_25863388
93.5	S10_25336247
94.1	S10_25752965
94.6	S10_25880984
95.2	S10_28620698
95.6	S10_25465315
96.0	S10_26863330
96.3	S10_28428017
96.6	S10_26269380
98.8	S10_30231301
99.4	S10_30347076
99.6	S10_30219654
99.9	S10_30347121
100.2	S10_30404314
100.5	S10_30357436
101.7	S10_30483044
103.4	S10_30507990
104.6	S10_30656353
105.8	S10_30934478
107.2	S10_31152945
108.2	S10_31289340
108.6	S10_31243061
109.0	S10_31267086
109.4	S10_31379787
109.9	S10_31361960
110.3	S10_31469413
110.9	S10_31648831
111.1	S10_31648788
111.4	S10_31743616
112.0	S10_31648735
112.5	S10_31893203
112.7	S10_31901827
114.6	S10_32136579
116.0	S10_32211885
116.6	S10_32393132
121.0	S10_32631590
121.7	S10_33117493
122.3	S10_33112542
123.5	S10_33394000
123.8	S10_33404890
125.2	S10_33524426
125.8	S10_33571313
127.0	S10_33770904
129.4	S10_34308855
130.2	S10_34308766
130.4	S10_34268285
130.9	S10_34293617
131.2	S10_34277080
132.8	S10_34495218
133.3	S10_34471722
133.5	S10_34485922
134.6	S10_34627895
136.2	S10_34886360
137.9	S10_35036210
138.5	S10_35144853
139.0	S10_35048989
139.5	S10_35139277
139.7	S10_35136116
140.3	S10_35194164
140.6	S10_35194035
142.0	S10_35451061
142.5	S10_35369929
143.0	S10_35451098
143.7	S10_35663980
145.4	S10_35710713
146.1	S10_35724708
147.4	S10_35973346
148.6	S10_36164604
149.9	S10_36184055
151.4	S10_36259334
151.7	S10_36259490
152.2	S10_36353804
153.9	S10_36350125
155.0	S10_36580094
155.9	S10_36747606
156.6	S10_36809618
157.4	S10_36849637
159.8	S10_36848241
161.5	S10_37058691
164.1	S10_37011453

'11'

cM	Marker
0.0	S11_88847
1.2	S11_163588
2.0	S11_90087
3.0	S11_262130
3.9	S11_254031
5.8	S11_489613
6.5	S11_630440
8.8	S11_1129070
9.8	S11_1163740
11.2	S11_887170
11.7	S11_1021098
12.3	S11_1016041
13.4	S11_1244604
14.7	S11_1394765
16.1	S11_1602041
16.7	S11_1679232
17.7	S11_1734489
18.8	S11_1808421
19.2	S11_1808389
20.0	S11_1816864
20.7	S11_1853038
22.8	S11_1998955
23.8	S11_2082387
24.7	S11_3173487
25.1	S11_2300909
26.4	S11_3913119
27.0	S11_3912510
27.7	S11_3758288
28.0	S11_3855014
28.5	S11_3837831
30.1	S11_4210398
30.7	S11_4265609
31.5	S11_4287459
32.4	S11_4443676
33.3	S11_4672109
35.0	S11_5181009
36.5	S11_5101978
37.0	S11_5195429
37.4	S11_5004921
37.7	S11_5102271
38.3	S11_5101910
39.1	S11_5337593
39.4	S11_5472818
39.9	S11_5447965
41.8	S11_5986107
42.1	S11_5766745
42.6	S11_5787974
42.8	S11_5787983
43.2	S11_5874957
44.4	S11_6087149
45.3	S11_6122077
47.0	S11_6388840
47.5	S11_6381052
48.5	S11_6436131
49.2	S11_6546124
51.0	S11_6791124
52.5	S11_6949869
52.8	S11_6993203
53.4	S11_7085798
53.8	S11_7094879
54.1	S11_7085882
55.1	S11_7183021
55.5	S11_7267063
55.9	S11_7251683
56.3	S11_7264666
56.9	S11_7372089
57.2	S11_7378084
58.8	S11_7893326
59.2	S11_7893218
60.5	S11_10343233
60.7	S11_11453177
61.0	S11_11729317
61.2	S11_15911841
61.5	S11_10570876
61.7	S11_16300375
62.0	S11_16300434
62.7	S11_18665757
63.5	S11_19134246
64.5	S11_19463544
64.7	S11_20026688
64.9	S11_19689706
65.2	S11_20022284
65.6	S11_20025533
67.7	S11_21449408
68.1	S11_21355709
68.4	S11_21098444
68.9	S11_22045469
70.5	S11_22376167
71.3	S11_22731081
71.6	S11_22598843
72.0	S11_22631986
72.6	S11_22731138
73.1	S11_22989055
73.8	S11_23308519
74.9	S11_24069347
75.9	S11_24338835
77.2	S11_24495920
79.0	S11_25297877
79.3	S11_25109573
79.8	S11_25297821
81.2	S11_25492831
81.5	S11_25506652
82.0	S11_25614905
82.4	S11_25506582
82.8	S11_25557227
83.1	S11_25557275
83.6	S11_25556270
84.2	S11_25714967
85.0	S11_25567453
85.5	S11_25987882
86.3	S11_26062881
86.9	S11_26009234
89.4	S11_26294364
89.8	S11_26428091
91.2	S11_26228111
93.6	S11_26719867
94.4	S11_26668405
95.7	S11_26769307
97.3	S11_26859447
98.2	S11_26971311
98.8	S11_27259778
101.1	S11_27625919
101.9	S11_27703757
103.5	S11_27815927
104.0	S11_27825586
104.6	S11_27978919
106.5	S11_28166945
107.8	S11_28339888
108.4	S11_28335602
109.9	S11_28558103
110.9	S11_28611127
111.7	S11_28614154
113.5	S11_28935001
114.3	S11_29022015
114.7	S11_29030534
115.6	S11_29159661
117.9	S11_29450017
119.7	S11_29737667
120.1	S11_29737768
120.9	S11_29907373
124.6	S11_30463801
124.7	S11_30356940
124.8	S11_30176070
125.1	S11_30242579
126.2	S11_30497695
126.9	S11_30550710
127.8	S11_30363120
129.5	S11_30684249
129.9	S11_30721039
132.9	S11_31364163
134.4	S11_31644353
134.9	S11_31559918
135.6	S11_31642145
136.5	S11_31528134
138.1	S11_31940844
138.4	S11_31968462
139.1	S11_31969059
140.1	S11_32075914
141.5	S11_32246640
144.4	S11_32014475
146.7	S11_32246550

Supplementary fig 4.3: CS (*Citrullus lanatus*) × PI 482276 (*Citrullus amarus*) genetic linkage map with 1,525 SNPs. The genetic positions (cM) of SNPs are labeled on the left of each chromosome and the SNP ID [named by chromosome and physical position of the Charleston Gray draft genome (Wu et al. 2019)] on the right.

CHAPTER 5

CONCLUSION

Watermelon is one of the most consumed fresh fruits, widely grown in the warmer parts of the world (FAOSTAT 2018). Both biotic and abiotic stresses hinder watermelon production, and these are intensified by long production cycles (Poland et al. 2009). For the sustainable production and marketing of watermelon, strategies to overcome these stresses need to be developed and this requires a strong understanding of the genetic processes involved. In an effort to improve watermelon cultivar development, this research focused on determining the genetic basis of flowering time and resistance to gummy stem blight disease, with the aim of developing molecular markers for selection of these traits. Utilization of molecular tools such as marker assisted selection for these traits would be beneficial and increase efficiency in watermelon breeding programs.

Time to flowering is an important trait in watermelon breeding as it is one of the determinants of earliness (Zimmerman 1988; Jung and Müller 2009). Growers would prefer earlier varieties as they are likely to result in a shorter production time which would enable their crops escape stresses such as diseases, and other abiotic stresses that intensify later in the season (Poland et al. 2009). Earliness is advantageous as it would result in reduced production costs and increased profitability for the growers (Mohr 1986). Flowering time is also a key trait in production of seedless watermelon, which are predominantly cultivated in the U.S. due to consumer demand. These seedless varieties are produced on triploids, which are interplanted with diploids pollen sources in the field to

ensure fruit set (Maynard and Elmstrom 1992; Boyhan et al. 2000). Synchronized flowering time between the triploids and diploid is essential (Dittmar et al. 2009, 2010; McGregor and Waters 2014) and as such, an understanding of the genetic basis of flowering is key. Utilizing MAS of flowering time in watermelon breeding would potentially aid in selection for early flowering, which would shorten the production time and may also improve diploid-triploid pairing in seedless watermelon breeding.

Flowering time is a quantitative trait that involves many genes. In this study, we validated *Qdff3-1* (McGregor et al. 2014) using QTL-seq on a RIL mapping population developed from a cross between KBS and NHM. The locus harbors three candidate genes associated with flowering time: phosphatidylinositol-4-phosphate 5-kinase (*PIP-kinase*), TEMPRANILLO (*TEM1*) and flowering locus T (*FT*). Twenty Kompetitive Allele Specific PCR (KASP™) assays were developed using the SNPs identified from QTL-seq to narrow down the region using recombinants in the RIL and five $F_{2:3}$ populations from the same parents for further fine-mapping. A panel of watermelon cultivars with different genetic backgrounds were used for marker validation. The QTL was narrowed down to a ≈1.13 Mb region of chromosome 3 and markers were identified for MAS. Results indicated that there is differential regulation of flowering in diverse genetic backgrounds of watermelon cultivars and the most appropriate marker to use for MAS will depend on the genetic background. UGA3_15424397 is effective for selection for flowering time in the KBS × NHM background but does not perform well in other backgrounds. NW0248748 may be useful to select for early flowering when SB (red flesh), SLMP (white flesh), SII (yellow flesh) or MICK (pink flesh) is used as a source of the early allele, as long as the non-donor is not NAV or GR. UGA3_10795402 might be useful when NAV or GR are

used, as long as MICK is not the early flowering donor. These findings provide more insight into the regulation of flowering time in watermelon and will have implications in watermelon breeding programs.

Watermelon production is hindered by diseases which lead to severe losses for growers. Gummy stem blight caused by three *Stagonosporopsis* spp. (Brewer et al. 2015) is one of the most important diseases of watermelon and was identified as the second most important research priority in watermelon after fusarium wilt in the southeastern US. (Kousik et al. 2016). The hot and humid weather in the southeast is conducive for germination of the pathogen spores and disease development (Keinath 2011). Management of GSB is mainly through fungicide applications since no commercial watermelon cultivars currently possess genetic resistance to GSB in the field. The three species exhibit differential sensitivity to fungicides (Brewer et al. 2015; Li et al. 2016; Li et al. 2019; Newark et al. 2019), making management of the disease even more difficult. Resistance sources, especially from wild relatives of watermelon, have been described, however, breeding efforts for GSB-resistant watermelon cultivars have been unsuccessful. This work aimed to establish if the three species have similar host responses, as it remains unclear whether differential host resistance is partially responsible for the lack of success in resistance breeding efforts. 12 different *Citrullus* genotypes were evaluated for resistance by inoculating seedlings with six isolates from the three *Stagonosporopsis* species. It was noted that isolate aggressiveness was not species-dependent, and some wild relatives of watermelon exhibited broad resistance to the *Stagonosporopsis* isolates. The preliminary results from this section of the work can inform watermelon breeders in developing

strategies for phenotyping and resistance loci deployment when breeding for GSB resistance.

Due to the narrow genetic base of cultivated watermelon following domestication (Guo et al. 2013; Levi et al. 2017), the *Citrullus amarus*, a wild relative of watermelon (Chomicki and Renner 2015; Renner et al. 2017) has been a major source of disease resistance alleles in watermelon breeding. Introgression of this resistance into elite germplasm has however been unsuccessful despite several breeding efforts (Norton et al. 1986; Song et al. 2002). This lack of genetic GSB resistance in commercial watermelon cultivars has resulted in severe yield losses for growers. Our aim was therefore to identify chromosomal regions associated with resistance to GSB, with a future goal of resistance introgression into the commercial varieties. An interspecific population developed from a cross between a susceptible Crimson Sweet and resistant PI 482276 was used to map GSB resistance. Loci associated with GSB resistance were identified on chromosomes 3 (*ClGSB3.1*), 5 (*ClGSB5.1*) and 7 (*ClGSB7.1*) of watermelon, with the latter explaining up to 21% of the phenotypic variation. A candidate gene (*ClCG07G013230*) in the peak of *ClGSB7.1* containing a non-synonymous C → T SNP in the DUF761 domain was also identified. Regions displaying the highest association with the trait were examined and high throughput molecular markers linked to the trait were developed. ClGSB5.1-1, ClGSB7.1-1 and ClGSB7.1-2 KASP assays explained 4.5%, 14.2% and 17.8% of the phenotypic variation, respectively, in the CS × PI482276 mapping population. Our results provide the basis for marker assisted selection for GSB resistance in watermelon.

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