**Genotyping by sequencing for SNP discovery and genetic mapping of resistance to race 1 of
Fusarium oxysporum in watermelon**

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Abstract

Management of fusarium wilt caused by *Fusarium oxysporum* f. sp. *niveum* (*Fon*) (E.F. Sm.) W.C. Snyder & H.N. Han. in watermelon [*Citrullus lanatus* (Thunb.) Matsum. & Nakai] is largely dependent on cultivation of resistant cultivars. Application of marker-assisted selection (MAS) in conventional breeding programs can accelerate the release of new watermelon cultivars resistant to fusarium wilt. Towards developing tools for MAS, physical (1,024 SNPs) and genetic (389 SNPs) maps were developed in the current study for an F₂ population (n = 89; Calhoun Gray x Sugar Baby) segregating for resistance against *Fon* race 1 using the genotyping by sequencing platform. A modified tray-dip method was established for high-throughput phenotyping of the segregating F₃ population. A major quantitative trait locus (QTL) accounting for up to 38.4 % of the phenotypic variation in the F₃ population was identified on chromosome 1 on both the physical and genetic maps in a region previously associated with *Fon* race 1 resistance. This resistance locus was consistently detected over five different time points and three different phenotypic screens, showing the reliability of the screening method in discriminating susceptible and resistant genotypes. Eight resistance genes were found within the confidence interval of the identified QTL. SNPs close to this QTL may be exploited in MAS for fusarium wilt resistance in breeding programs. This study confirms the resistance locus on chromosome 1 and demonstrates the use of a physical map for QTL detection in watermelon. The SNPs reported here will be useful for future genetic studies in watermelon.

Keywords: fusarium wilt, race 1, watermelon, QTL, genetic mapping, SNP, tray-dip, phenotyping, GBS

1. Introduction

Watermelon [*Citrullus lanatus* (Thunb.) Matsum. & Nakai] is an important crop widely grown for its sweet flesh (Robinson and Decker-Walters 1997) and edible seeds (Achigan-dako et al. 2008; Edelstein and Nerson 2002). The United States has over 133,700 acres dedicated to watermelon production with an annual fresh market value of approximately half a billion dollars (United States Department of Agriculture 2014). The domestication of watermelon through selection for desirable traits has led to narrow genetic diversity in the current elite watermelon cultivars (Levi et al. 2001) and has resulted in the loss of alleles conferring resistance to important bacterial, viral and fungal diseases (Guo et al. 2013; Hawkins et al. 2001). Fusarium wilt caused by *Fusarium oxysporum* f. sp. *niveum* (*Fon*) (E.F. Sm.) W.C. Snyder & H.N. Hans is a production-limiting disease in watermelon growing regions of the world (Boyhan et al. 2001, 2003; Egel and Martyn 2007; Guner and Wehner 2004; Hawkins et al. 2001; Martyn and McLaughlin 1983; Zhou et al. 2010). Four *Fon* races (0, 1, 2 and 3) have been described in watermelon based on their aggressiveness or their ability to overcome specific resistance in a set of differential cultivars (Bruton 1998, Egel and Martyn 2007, Wehner 2008; Zhou et al. 2010).

The persistence of the pathogen in the soil and the evolution of new races make management of fusarium wilt difficult (Bernett 1936; Bruton 1998; Egel and Martyn 2007; Lin et al. 2009; Martyn and Netzer 1991; Yetisir et al. 2003). Current measures for managing the disease include avoiding infested fields, a 5-7 year crop rotation system, chemical and biological fumigation (Bruton 1998; Egel and Martyn 2007; Everts and Himmelstein 2015), the use of resistant root-stocks (Kuniyasu 1980) and growing watermelon genotypes resistant to the disease (Bruton 1998; Martyn 2014). Cultivating resistant cultivars is regarded as the best method for managing fusarium wilt (Bruton 1998; Hopkins et al. 1992; Lin et al. 2009; Lin et al. 2010;

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

Resistance to *Fon* race 1 is widely described in Calhoun Gray and is thought to be controlled by a single dominant gene designated *Fo-1* with a few modifier genes (Guner and Wehner, 2004; Martyn and Netzer, 1991; Netzer and Weintall, 1980; Wehner 2008; Zhang and Rhodes, 1993). However, early efforts to identify loci associated with resistance to *Fon* race 1 primarily utilized the resistant PI 296341-FR (var. citroides). Using PI 296341-FR, Xu et al. (1999) identified a randomly amplified polymorphic DNA (RAPD) marker (OP01/700) weakly linked to *Fon* race 1 resistance at a 3 cM distance. This marker was cloned and sequenced by Xu et al. (2000) and converted into a sequence characterized amplified region (SCAR) marker for use in marker-assisted selection (MAS). Hawkins et al. (2001) attempted to identify quantitative trait loci (QTL) associated with resistance to *Fon* race 1 using F₂ and F₃ populations generated from a cross between PI 296341-FR and New Hampshire (susceptible). However, no useful marker-trait associations were found in this study. Harris et al. (2009) used degenerate primers to target resistant genes in PI 296341-FR encoding nucleotide binding site–leucine-rich repeat proteins and identified three expressed sequence tags (EST) disease resistance homologs. However, none of these EST markers mapped closely to RAPD marker (OP01/700) previously described by Xu et al. (1999) in PI 296341-FR. The inability to find useful marker-trait associations in PI 296341-FR in earlier studies was probably due to low genome coverage and high segregation distortion of marker alleles in this PI (Levi et al., 2011). The narrow genetic diversity among cultivated watermelon has previously hindered genetic mapping studies in elite by elite bi-parental populations due to low genetic diversity. However, the recent advancement in

next generation sequencing technology has provided an affordable platform for studying genome-wide variation in watermelon populations leading to the development of genetic maps of sufficient genome coverage (Cheng et al. 2016; Sandlin et al. 2012; Ren et al. 2012), including a consensus map based on 386 SNPs, 698 simple sequence repeats, 219 insertion-deletion and 36 structure variation markers (Ren et al. 2014). However, the marker-density in these maps is low compared to major crops such as maize (*Zea mays*) (1.15 million markers, Liu et al. 2015), soybean (*Glycine max*) (21,478 markers, Song et al. 2016), wheat (*Triticum aestivum*) (30,144 markers, Maccaferri et al. 2015) and rice (*Oryza sativa*) (30,984 markers, Spindel et al. 2013). Genotyping by sequencing (GBS) (Elshire et al. 2011) is a highly multiplexed next-generation sequencing technology that can generate thousands of markers in any plant species and involves sequencing of reduced genomic libraries followed by alignment of the generated reads to identify SNP variations (Barba et al. 2013; Elshire et al. 2011). GBS allows pooling of barcoded samples into a single sequencing lane thus reducing the cost of genotyping per data point (Elshire et al. 2011). Despite the ability of GBS to generate thousands of SNP markers, the low genetic diversity in cultivated watermelon limits the number of polymorphic markers available to study elite by elite populations. Using GBS, Lambel et al. (2014) identified only 266 usable SNPs in elite by elite F₂ population [HMw017 (resistant) x HMw013 (susceptible)] segregating for resistance against *Fon* race 1 and detected seven QTL associated with resistance, including a major QTL ($R^2 = 59.9\%$) on chromosome 1. This major QTL was independently confirmed by Ren et al. (2015) in a recombinant inbred population developed from a cross between PI 296341-FR and the cultivar 97103.

There is need to identify more SNPs among elite watermelon cultivars to allow the development of higher density genetic maps which would be useful in estimating the true

positions of QTLs underlying agronomically important traits in watermelon. Furthermore, future genome wide association studies (Zhao et al. 2011) and prediction of genomic breeding values in watermelon lines will require dense marker haplotype maps (Denis and Bouvet 2011).

Breeding for genetic resistance and developing molecular tools for MAS are dependent upon the ability to differentiate resistant and susceptible genotypes. These studies often require screening of large plant populations. However, as with many other crops (Barbedo 2014; Furbank and Tester 2011; Montes et al. 2007), advancement in high-throughput phenotyping technologies for robust trait evaluation in watermelon lags behind that of genotyping technologies for genomic applications. Several phenotyping methods have been developed for screening of fusarium wilt resistance in watermelon including the root-dip method, which is the most common in watermelon breeding and genetic studies (Dane et al. 1998; Freeman and Rodriguez 1993; Hawkins et al. 2001; Lambel et al. 2014; Martyn and McLaughlin 1983; Martyn and Netzer 1991; Ren et al. 2015; Yetisir et al 2003; Zhou and Everts 2004, 2007; Zhou et al. 2010). In this method, seedlings are uprooted at the first true leaf stage and the roots are washed off under running water, dipped in inoculum and transplanted into pots containing a substrate. Although reliable, this method is tedious and labor intensive, especially when applied to large populations. Several other phenotyping methods have been developed as alternatives to the root-dip method: pipetting, injection and the tray-dip method. The pipetting method delivers inoculum to the substrate surrounding the seedling (Gunter and Egel 2012; Kurt et al. 2008; Wechter et al. 2012; Zhou et al. 2010), while the injection method delivers the inoculum into the seedling stem using an insulin syringe (Boyhan et al. 2001, 2003). For the tray-dip method, seeds are sown in trays containing a substrate and placed inside a larger plastic flat containing the same substrate. At the first true leaf stage, the seedling tray is lifted off the larger flat and rinsed briefly

with water. The roots growing through the cells' drainage holes are trimmed to a uniform length of 2.5-3 cm and the entire tray is dipped into a shallow flat containing 250-300 mL of inoculum for 10-20 minutes. The tray is then placed back to the larger flat containing the substrate (Martyn and Netzer 1991; Zhou et al. 2010). Although effective, these methods are also resource intensive for large-scale screening of fusarium wilt resistance.

The aim of the current study was to confirm the genetic locus associated with resistance to *Fon* race 1 and identify SNPs for future genetic studies among elite watermelon cultivars. In addition, we validated a high throughput phenotyping method for large-scale screening of watermelon seedlings in breeding and genetic studies using a modified tray-dip method.

2. Materials and methods

2.1 Plant materials

A cross between Calhoun Gray (resistant) and Sugar Baby (susceptible) cultivars was made in the greenhouse and a single F₁ plant was selfed to yield F₂ (n = 89) seeds. Each individual F₂ plant was selfed to generate F₃ families that were phenotyped for resistance to *Fon* race 1 in the greenhouse. Charleston Gray, which is described as having intermediate resistance to *Fon* race 1, was also included in the study as a control.

2.2 Fungal inoculum preparation

Race 1 of *Fon* [(B05-07), provided by Anthony Keinath, Clemson University], was grown (14h/10h dark cycle) on quarter-strength potato dextrose agar (Becton, Dickinson and Company, NJ, USA) for 12 days. After that time, 1 cm² agar plugs were transferred into 250 mL erlenmeyer flasks containing 100 mL potato dextrose broth (Becton, Dickinson and Company). The fungal cultures were grown (14h/10h light/dark cycles) on a Mini-Orbital shaker (Henry

Troemner, NJ, USA) at 200 rpm for 10 days, at which point the inoculum was filtered through four layers of sterile cheese cloth. The microconidial concentration in the inoculum suspension was determined using a hemacytometer (Hausser Scientific, PA, USA) and adjusted to 1×10^6 mL⁻¹ using sterile water.

2.3 Phenotyping

Phenotyping was carried out using a modified tray-dip method as follows: seeds from Sugar Baby, Calhoun Gray, Charleston Gray, the F₁, and each of the F₃ (n = 89) lines were sown in the greenhouse in plastic trays (53.3 x 27.9 x 5.1 cm, Sun Gro Horticulture, MA, USA) with 48-cell (5.98 x 3.68 x 4.69 cm) tray inserts filled with steam-pasteurized sand: peat: vermiculite (4: 1: 1) amended with Osmocote 14N-4.2P-11.6K. Unlike the original tray-dip method, the plastic trays were not placed inside larger plastic flats containing substrate. For each cultivar/line, there were a total of four replications per experiment in a randomized complete block design. Each replication consisted of four subsamples. One of these was designated as a control for each cultivar/line and was sown in separate trays. At the first true leaf stage, the cells with the seedlings were transferred to webbed trays (53.7 x 26.9 x 6.28 cm, Sun Gro Horticulture) and placed in plastic tubs (67.3 x 40.6 x 16.8 cm, Sterilite Corporation, MA, USA) containing 7 liters of inoculum for 15 min. In this step, rinsing of trays and trimming of roots to a uniform length prior to tray dipping was not carried out as required in the original tray-dip method. During this period, the plastic tubs remained undisturbed to avoid inoculum from running over the top of the soil in the trays. Seedlings that were not at the first true leaf stage were uprooted prior to inoculation. Control seedlings were treated as above, but were placed into plastic tubs containing sterile water. A plastic tub containing 7 liters of inoculum was sufficient for inoculating four seedling trays (192 seedlings). The inoculated seedlings were then

transferred into hole-less trays (53.3 x 27.9 x 5.1 cm, Sun Gro Horticulture) and placed on the greenhouse bench.

Plants were evaluated for symptom severity on a scale of 0 to 5 with a score of 0 representing asymptomatic plants, a score of 1 for plants showing initial wilting on one leaf, a score of 2 for plants showing continued wilting in more than one leaf, a score of 3 for plants with all the leaves wilted, a score of 4 for plants with all leaves wilted and stems collapsing, and a score of 5 for dead plants (Fig. 1). Disease severity data was collected at 11, 14, 17, 20, 23, and 26 days after inoculation (DAI) and the area under disease progress curve (AUDPC) was determined by the trapezoidal integration method (Shaner and Finney 1977). The first disease screening experiment was carried out in August 2013, and was repeated in November 2013 and February 2014. The AUDPC values for the parents, the F₁ and Charleston Gray were analyzed using the PROC GLM procedure of SAS (SAS Institute Inc., Cary, NC) and the means separated using Fisher's protected least significant difference test (Ott and Longnecker 2001). Pearson correlations for the DAI and AUDPC values in the population were calculated using JMP Version 9 (SAS).

Fig. 1 The 0-5 rating scale used in the experiment where (A) indicates a score of 0 for asymptomatic plants, (B) a score of 1 for plants showing initial wilting on one leaf, (C) a score of 2 for plants showing wilting in more than one leaf, (D) a score of 3 for plants with all the leaves wilted, (E) a score of 4 for plants with all leaves wilted and stems collapsing and (F) a score of 5 for dead plants. (color: online only)

2.4 Genotyping by sequencing, SNP analysis and map construction

Leaf material from the parents, the F_1 and each of the 89 F_2 plants was used for DNA extraction using the E.N.Z.A 96-well format kit (Omega Bio-tek, GA, USA) according to the manufacturer's instructions. The concentration and quality of the DNA was determined by

measurement of absorbance (Infinite M200 PRO, Tecan Group Ltd., ZH, CH) and by agarose gel electrophoresis. Genotyping of the parents, F₁ and F₂ population was carried out using GBS (Elshire et al. 2011) at Cornell University's Institute for Genomic Diversity. Briefly, the DNA was digested using *ApeKI* restriction enzyme and fragment alignment and SNP calling was performed using the GBS reference pipeline in TASSEL version 3.0.160. SNP calls were generated in HapMap format and filtered in Microsoft Office Excel for missing data, polymorphism between the mapping population parents and segregation distortion ($P < 0.0001$). Based on these parameters, two SNP data sets were created, (i) one consisting of 1,024 SNPs that included markers with up to 70% missing data points in the F₂ individuals, and (ii) a subset of 389 SNPs with no more than 10% missing data. A physical map was created by placing the 1,024 SNPs (Supplementary Table 1) on their respective physical locations on the reference genome (Guo et al. 2013). The data set consisting of 389 SNPs was used to construct the linkage map in JoinMap version 4.1 (Van Ooijen 2006) using regression mapping. Independence LOD and maximum likelihood algorithm were used for grouping and ordering of markers respectively (Van Ooijen 2006).

216 Table 1 Single nucleotide polymorphisms obtained in the physical map (Guo et al. 2013) and linkage map in the Sugar Baby and
 217 Calhoun Gray F₂ population.

Physical map			Genetic map		
Chromosome	Number of markers	Length (Mb)	Linkage group	Number of markers	Length (cM)
1	75	33.6	1	33	365.5
2	105	34.3	2	49	450.7
3	86	28.9	3	36	409.8
4	48	23.7	4	8	86.2
5	108	33.2	5	44	537.3
6	90	26.9	6	23	305.0
7	102	31.3	7	27	315.3
8	74	26.1	8	27	269.3
9	138	34.9	9	65	545.4
10	116	27.3	10	50	414.6
11	82	26.7	11A	8	40.2
			11B	19	215.9
Total	1,024	326.9	Total	389	3,955.2

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2.5 QTL detection

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

3. Results

3.1 Phenotypic analysis

Disease severity was higher in the experiments conducted in the months of August and November 2013 than the experiment conducted in February 2014 (Fig. 2-5). In all the experiments, the severity of disease symptoms increased over time and was highest at 26 DAI (Fig. 3-5). Disease severity of the susceptible parent (Sugar Baby) and intermediate resistant control (Charleston Gray) was observed to be significantly higher ($P < 0.05$) across the experiments than that of the resistant parent (Calhoun Gray), which showed minimal symptoms (Fig. 2A-C). The F_1 plants were generally intermediate between the two parents (Fig. 2A-C). Segregation for resistance was observed in the F_3 population in all three experiments (Fig. 3-5). Significant positive correlations ($\alpha = 0.05$) were observed between all time points as well as AUDPC measured in the three experiments (data not shown).

3.2 Genotyping by sequencing, SNP analysis and QTL mapping

GBS analysis generated a total of 933,662 tags, of which 761,835 (81.6%) aligned to unique positions, 51,216 (5.5%) aligned to multiple positions and 120,611 (12.9%) could not be aligned to the watermelon reference genome (Guo et al. 2013). Analysis of the tags which aligned to unique positions revealed 20,889 HapMap SNP calls, which were then filtered

Fig. 2 Disease severity of Calhoun Gray (CALG), Charleston Gray (CG), F₁ and Sugar Baby (SB) in (A) experiment 1 (August 2013), (B) experiment 2 (November 2013) and (C) experiment 3 (February 2014). The numbers to the right side of the figure indicate the calculated area under disease progress curve for each cultivar and the F₁. Values followed by the same letters are not significantly different using Fisher's protected least significant difference test.

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Fig. 3 Frequency distribution for disease severity in the F₃ population at (A) 11, (B) 14, (C) 17, (D) 20, (E) 23 and (F) 26 DAI, as well as (G) the area under disease progress curve (AUDPC) for experiment 1 (August 2013). The disease severity scores for Calhoun Gray (CALG), Sugar Baby (SB) as well as the F₁ are indicated.

Fig. 4 Frequency distribution for disease severity in the F₃ population at (A) 11, (B) 14, (C) 17, (D) 20, (E) 23 and (F) 26 DAI, as well as (G) the area under disease progress curve (AUDPC) for experiment 2 (November 2013). The disease severity scores for Calhoun Gray (CALG), Sugar Baby (SB) as well as the F₁ are indicated.

Fig. 5 Frequency distribution for disease severity in the F₃ population at (A) 11, (B) 14, (C) 17, (D) 20, (E) 23 and (F) 26 DAI, as well as (G) the area under disease progress curve (AUDPC) for experiment 3 (February 2014). The disease severity scores for Calhoun Gray (CALG), Sugar Baby (SB) as well as the F₁ are indicated.

according to missing data points, polymorphism between mapping population parents and segregation distortion to generate 1,024 SNP markers for the physical map (Table 1 and Supplementary Table 1) and 389 SNPs for the genetic map (Table 1 and Supplementary Table 2). The genetic map spanned 12 linkage groups with a total length of 3,955.2 cM and an average marker interval of 10.1 cM (Table 1). A major QTL associated that explained up to 34.3% and 38.40% of the phenotypic variation for resistance to *Fon* race 1 observed in F₃ population was detected on chromosome 1 on the physical and genetic maps, respectively (Table 2 and 3). This QTL was consistently detected using disease severity data collected at 14, 17, 20, 23 and 26 DAI as well as the AUDPC from the three experiments.

4. Discussion

4.1 Phenotypic analysis

Significant differences in AUDPC were observed between Calhoun Gray, Charleston Gray and Sugar Baby cultivars for the first two experiments (August and November 2013; Fig 2A-B). These results are in agreement with numerous studies that have established resistance to *Fon* race 1 in Calhoun Gray, intermediate resistance in Charleston Gray and susceptibility in Sugar Baby (Martyn and McLaughlin 1983; Martyn and Netzer 1991; Netzer and Weintall 1980; Wehner 2008; Zhang and Rhodes 1993). In the third experiment (February 2014), the difference in disease severity observed between Charleston Gray and Sugar Baby was not statistically significant (Fig. 2C). The inability to differentiate between these two cultivars has been previously reported (Zhou and Everts 2004), and may be due to the overall low disease severity in experiment three.

The AUDPC of the F₁ plants was significantly different from Calhoun Gray (Fig. 2A-C), contradicting the theory that resistance to *Fon* race 1 in Calhoun Gray is controlled by a single

303 Table 2 Chromosomal (chr.) (Guo et al. 2013) positions (Mb) of the QTL associated with resistance to *Fon* race 1 on the physical map
 304 and the corresponding 1-likelihood-odds (LOD) support interval in the Sugar Baby x Calhoun F₃ watermelon population for
 305 experiment 1 (August 2013), 2 (November 2013) and 3 (February 2014).

Trait	Exp ^v	chr. ^w	Position (Mb) ^x	LOD ^y	R ² (%) ^z	Additive effect	Dominance effect	LOD-1 support interval (Mb)	LOD-1 support interval (Mb)
14 DAI	1	1	0.01	7.75	28.21	0.61	-0.17	0	1.6
17 DAI	1	1	0.01	7.88	28.19	0.79	-0.11	0	2.1
20 DAI	1	1	0.01	7.20	29.20	0.94	-0.33	0	2.3
23 DAI	1	1	0.14	6.49	24.92	1.13	-0.38	0	1.3
26 DAI	1	1	0.01	8.68	27.36	1.09	-0.22	0	1.1
AUDPC	1	1	0.01	10.45	33.56	257.14	-89.29	0	1.1
14 DAI	2	1	0.01	8.25	30.59	0.87	-0.10	0	0.5
17 DAI	2	1	0.01	8.53	32.21	1.17	-0.24	0	0.3
20 DAI	2	1	0.01	8.79	33.65	1.22	-0.35	0	0.5
23 DAI	2	1	0.01	9.48	30.80	1.03	-0.74	0	1.8
26 DAI	2	1	0.01	8.43	28.29	1.00	-0.30	0	0.3
AUDPC	2	1	0.01	8.59	34.29	311.20	-79.88	0	0.3
14 DAI	3	1	0.01	3.14	12.70	0.25	-0.07	0	0.4
17 DAI	3	1	0.01	3.08	13.03	0.25	0.31	0	7.8
20 DAI	3	1	0.01	3.33	12.08	0.45	-0.11	0	0.3
23DAI	3	1	0.01	4.73	18.66	0.76	-0.16	0	0.4
26 DAI	3	1	0.01	6.08	23.35	0.99	-0.35	0	0.3
AUDPC	3	1	0.01	4.53	17.75	145.45	-40.85	0	0.4

306 ^v Experiment from which data for QTL mapping was derived.

307 ^w Chromosome on the watermelon draft genome (Guo et al. 2013).

308 ^x The position of the QTL on the chromosome (Guo et al. 2013).

309 ^y Log₁₀ likelihood ratio.

310 ^z Phenotypic variation explained.

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Table 3 Linkage group positions (cM) of the QTL associated with resistance to *Fon* race 1 on the genetic map and the corresponding 1-likelihood-odds (LOD) support interval in the Sugar Baby x Calhoun F₃ watermelon population.

Trait	Exp ^v	LG ^w	Position (Mb) ^x	LOD ^y	R ² (%) ^z	Additive effect	Dominance effect	LOD-1 support interval (Mb)	LOD-1 support interval (Mb)
14 DAI	1	1	0.01	7.12	27.94	0.62	-0.19	0	5.3
17 DAI	1	1	1.01	5.52	21.35	1.03	-0.29	0	4.2
20 DAI	1	1	0.01	6.01	21.00	1.22	-0.26	0	4.0
23 DAI	1	1	0.01	9.55	34.93	1.19	-0.21	0	3.0
26 DAI	1	1	1.01	8.10	30.35	1.14	-0.21	0	5.2
AUDPC	1	1	0.01	6.52	22.72	319.42	-69.08	0	3.7
14 DAI	2	1	1.01	7.85	30.73	0.82	-0.25	0	5.7
17 DAI	2	1	0.01	10.07	37.92	1.20	-0.35	0	5.1
20 DAI	2	1	1.01	9.03	37.19	1.29	-0.26	0	5.6
23 DAI	2	1	0.01	9.71	33.47	1.09	-0.68	0	5.1
26 DAI	2	1	0.01	9.69	31.62	1.04	-0.32	0	3.5
AUDPC	2	1	0.01	10.28	38.40	320.42	-59.94	0	4.5
14 DAI	3	1	0.01	2.92	12.97	0.23	0.12	0	7.2
17 DAI	3	1	0.01	3.10	11.52	0.29	0.20	0	12.8
20 DAI	3	1	0.01	3.32	13.52	0.49	0.11	0	5.0
23DAI	3	1	0.01	4.29	19.18	0.78	-0.15	0	4.3
26 DAI	3	1	0.01	6.62	24.10	0.97	-0.42	0	5.5
AUDPC	3	1	0.01	4.68	20.53	157.75	-51.42	0	3.7

^v Experiment in which data for QTL mapping was derived.

^w Linkage group 1 on the linkage map corresponds to chromosome 1 on the watermelon draft genome (Guo et al. 2013).

^x Log₁₀ likelihood ratio.

318 ^y Phenotypic variation explained.

319 ^z Negative values indicate that the effect is contributed by the allele from Calhoun Gray.

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dominant gene designated *Fo-1* (Netzer and Weintall 1980). Generally, the F₁ and Calhoun Gray had similar disease severity rating early in the experiment, but the F₁ showed more severe symptoms as the experiment progressed (Figs 3, 4 and 5). The difference in disease severity observed in the F₁ in this study and previously published work might have resulted from a variation in the inoculation method and soil type used. In the current study, a modified-tray dip method was used for inoculation in sand: peat: vermiculite medium while in most studies, the root dip method and various combinations of sand, soil, peat and vermiculite are used (Dane et al. 1998; Freeman and Rodriguez 1993; Hawkins et al. 2001; Lambel et al. 2014; Martyn and McLaughlin 1983; Martyn and Netzer 1991; Yetisir et al 2003; Zhou and Everts 2004, 2007; Zhou et al. 2010). These results show that researchers should carefully consider the time (DAI) of data collection and the method of inoculation used when drawing conclusions from genetic studies of *Fon*.

Previous studies in watermelon have reported similar disease severity using the root-dip, pipette and tray-dip methods (Martyn and Netzer 1991; Zhou et al. 2010). However no studies have compared the time, cost, and labor requirements between these techniques. In melon (*Cucumis melo*), a study revealed that the root-dip method took four times longer to complete than the pipette method and eight times longer than the tray-dip method (Latin and Snell 1986). The modified tray-dip method presented here saves time when applied to large-scale screening experiments since it eliminates rinsing of trays and cutting of seedling roots to a uniform length. In addition, elimination of the substrate requirement in the larger flats may reduce the cost of phenotyping.

4.2 Genotyping by sequencing and SNP analysis

The number of tags (933,662) generated in this study with *ApeKI* restriction enzyme exceeded those generated (527,844 tags) with the same enzyme in an elite by elite F₂ watermelon population described by Lambel et al. (2014). Due to the low genetic diversity among cultivated watermelon (Levi et al. 2001), the number of usable SNPs (1,024) generated in the current study was small compared to that reported for other crops such as rice (>30,000) (Spindel et al. 2013) and wheat (>130,000) (Rutkoski et al. 2013) using GBS. The SNPs generated through GBS had a large number of missing data points. This is expected with the GBS method due to low coverage of reads that results from the pooling of bar-coded samples in a single sequencing lane (Barba et al. 2013). In the current study, 389 SNPs with no more than 10% missing data were used for construction of the genetic map so as to reduce marker order errors (Hackett and Broadfoot 2003). In grapevine (*Vitis vinifera*), Barba et al. (2013) allowed up to 10% missing data in GBS-derived SNPs for genetic map construction. The genetic map obtained in this study was significantly longer (3,955.2 cM) than that obtained in previous watermelon mapping studies (Hashzume et al., 2003; Hawkins et al., 2001; Levi et al., 2002, 2006, 2011; Ren et al., 2012, 2014; Sandlin et al., 2012). Massive elongation of the genetic maps often occurs when working with SNP data generated through GBS due to high levels of genotyping errors that tend to over-estimate the number of double cross-over events (Barba et al. 2013; Spindel et al. 2013). In rice, a genetic map constructed from SNP markers generated through GBS was found to be 130 times larger than the consensus genetic map for the crop (Spindel et al. 2013). In the current study, the genetic map was approximately 5 times longer than the integrated genetic map (798 cM) for watermelon (Ren et al. 2014). To address high levels of missing data and genotyping errors in GBS method, several imputation and error correction algorithms have been developed

respectively. Imputation algorithms utilize available marker information to predict missing genotype values while error correction algorithms test for the accuracy of recombination break points in the population (Barba et al. 2013; Rutkoski et al. 2013, Spindel et al. 2013). Examples of imputation methods include mean imputation, k-nearest neighbor's imputation, singular value decomposition imputation, expectation maximization imputation, and random forest regression imputation (Rutkoski et al. 2013). In rice, Spindel et al. (2013) used random forest imputation and a post-imputation genotyping error correction algorithm in Python (PLUMAGE 2.0) to correct for missing data and genotyping errors respectively. In grapevine, Barba et al. (2013) corrected for genotyping errors in GBS data by discarding individuals and markers exhibiting high proportion of cross over events in R/QTL software (Broman et al. 2003). In the current study, implementation of random forest imputation and error correction in R/QTL did not improve the length of the genetic map (data not shown).

Physical mapping using 1,024 SNP markers with up to 70% missing data was used to improve coverage in regions not represented on the genetic map and to determine the physical position of the QTL.

4.3 QTL detection

A major QTL (designated *Qfon1*) was detected on chromosome 1 of watermelon (Table 2 and 3) using the physical and genetic maps in the same genomic region (0 to 2.3 Mbp) as *Qfon1.1*, recently identified in two studies (Lambel et al. 2014; Ren et al. 2015). Variations in LOD, R^2 values, additive effects, dominance effects, and locus intervals were observed across time points and experiments, but the position of the identified QTL was consistent from 14 DAI for all experiments, despite the lower symptom severity in experiment 3. Thus data needs to be collected only at a single DAI, as long as it is at least 14 DAI.

It is difficult to determine the precise location of the identified QTL in the current study due to a missing portion (approximately 2 Mb) at the end of chromosome1. To increase the mapping resolution of this QTL, markers in the missing region of the chromosome ought to be added. Sandlin et al. (2012) identified four SNPs (NW0252073, NW0251293, NW0250418, NW0250430) in the region missing from chromosome 1. Addition of these markers to the maps may provide useful information on the precise position of the QTL. Further analysis on these SNPs will reveal their usefulness in MAS for fusarium wilt resistance in breeding programs. Three receptor-kinase genes (*Cla004916*, *Cla011391* and *Cla011463*), one lipoxxygenase gene (*Cla004959*), one glucan endo-1,3-bglucosidase precursors (*Cla004990*) and three acidic chitinase genes (*Cla004914*, *Cla004920* and *Cla004921*) (Guo et al. 2013) were found within the confidence interval for the identified QTL. These resistance genes are candidates for functional analysis and elucidation of the disease resistance mechanism associated with the identified QTL.

The current study did not detect the other minor QTLs identified on chromosome 1, 3, 4, 9, 10 by Lambel et al. (2014). This is perhaps due to differences in population size, method of inoculation and parental genetic background between the two studies. These minor QTLs were also not detected in a recent *Fon* race 1 genetic mapping study using PI-296341-FR (Ren et al. 2015).

To the best of our knowledge, this is the first study in watermelon to demonstrate the feasibility of using the physical map (marker order on the reference genome) for determining the physical position of a QTL of interest. In wheat, physical mapping has been used to determine the physical positions of genes and QTLs especially in areas of unknown or uneven recombination rates (Dilbirligi et al. 2006; Erayman et al. 2004). Furthermore, physical mapping allows for comparative and syntenic analysis among genotypes in a chromosomal region of

interest (Dilbirligi et al. 2006). Although it was possible to detect the QTL using the physical map in the current study, there were some discrepancies in marker order between the physical and genetic maps (Supplementary Table 1 and 2). Marker order differences between physical and genetic maps may be due to differences in marker order between the sequenced reference genome and the genotypes being mapped. However, differences may also result from sequencing/ genotyping errors as has been reported in barley (*Hordeum vulgare*) (Mascher et al. 2013) and wheat (Dilbirligi et al. 2006).

The detection of *Qfon1* using data collected at 14, 17, 20, 23, 26 DAI as well as the AUDPC values from all three experiments emphasizes the consistency of the modified-tray dip method and its ability to identify QTLs early in experimentation, which can save on breeding resources (time, greenhouse space etc.). However given the observation that QTLs at early time points have lower LOD (Table 2 and 3), it is probably prudent to select a later day (e.g. 23 DAI) if data will only be collected at a single time point. In future, the modified-tray dip method could be combined with precision phenotyping (Barbedo 2014; Cobb et al. 2013; Fiorani and Schurr 2013; Furbank and Tester, 2011; Junker et al. 2015; Montes et al. 2007) using image sensors for more accurate and objective disease severity ratings (Mahlein 2015). However, the growth habit of watermelon presents real challenges for imaging systems since it is difficult to keep individual plants in seedling trays or small pots separated as they grow trailing vines. The ability to collect data earlier (14 DAI), while plants are still relatively contained, would be very advantageous for imaging systems.

5. Conclusions

This study confirms the QTL on chromosome 1 to be associated with resistance to *Fon* race 1 in watermelon populations of diverse genetic backgrounds (Lambel et al. 2014; Ren et al.

2015) and is a viable candidate for MAS. Further analysis of the candidate resistant genes in this region will help elucidate the molecular mechanisms underlying the function of the identified QTL. The SNPs generated through GBS in the current study will be useful in future genetic studies in watermelon since only a limited number of SNPs are currently available for elite germplasm. The modified tray-dip method developed in the current study provides an important stride towards high-throughput precision phenotyping in watermelon. The relative simplicity and ease with which large scale screenings can be carried out should make this method an attractive alternative to the current, more labor intensive root-dip method.

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