Microsatellite markers linked to powdery mildew resistance locus in watermelon

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Abstract

Powdery mildew (*Podosphaera xanthii*) is one of the main foliar diseases that attack the watermelon (*Citrullus lanatus*) and other cucurbits species. The objective of this study was to identify microsatellite markers linked to the resistance locus to powdery mildew in a F_2 population of 'BRS Opara' (resistant) × 'Pérola' (susceptible). The parental plants, F_1 and F_2, were phenotyped for resistance or susceptibility to powdery mildew and 116 microsatellite markers were analyzed. Segregation data in the F_2 population demonstrated that resistance powdery mildew is controlled by a single dominant gene. The microsatellites MCPI_11, CYSTSIN and BVWS02441 showed linkage to the powdery mildew resistance gene at 2.6 cM distance. They were located on chromosome two of the watermelon genome. These markers can be used in the marker assisted selection in watermelon improvement programs and the identification of the chromosome region, in which these markers will facilitate future studies to identify the gene that confers resistance to powdery mildew in watermelon.

Keywords: Assisted selection; *Citrullus lanatus*; powdery mildew; resistance; SSR.
Abbreviations: MAS_Marker Assisted Selection, SSR_Simple Sequence Repeats, LOD_logarithm of the odds.

Introduction

The watermelon [*Citrullus lanatus* (Thunb.) Matsum & Nakai] is grown all over the world and one of the main cucurbits grown. Brazil is the fourth largest producer of watermelon with a production of 2,198,620 tons in year 2011 (Agrianual 2014). The majority of the cultivars used in Brazil are susceptible to diseases, such as powdery mildew, alternaria, viruses and anthracnose.

Powdery mildew (*Podosphaera xanthii*) is one of the main foliar diseases of the watermelon and of other cucurbits, cultivated or wild, in Brazil and in the world. In the United States and Israel, powdery mildew became a limiting problem for the cultivation of watermelon around the year 2000 (Davis et al., 2001; Cohen et al., 2000). However, in the Brazilian northeast, powdery mildew has already been cited as one of the main diseases that affected the watermelon crops since the late eighties (Souza et al., 1988). The fungus that causes powdery mildew in watermelon is *P. xanthii* (Castagne) Braun & Shishkoff [syn. *Sphaerotheca fuliginea auct. p.p.*] (Jahn et al., 2002), which is an obligatory parasite that shows physiological specialization. The symptoms appear all over the aerial part of the watermelon, but the leaves are affected the most. Initially, there is a white, dusty growth, which can be seen on the superior and inferior part of the leaves. It starts on the oldest leaves and on the oldest plants, in the fruiting stage, which contributes to the formation of fruits with a low content of soluble solids and of an inferior quality. Furthermore, the leaves that are much

attacked, dry out and the plant enters senescence earlier, reducing its productive period. It occurs in almost all areas where watermelon is cultivated (Terao et al., 2010). In Brazil, the occurrence of a specific race of powdery mildew that infects watermelon was reported. Studies carried out by Reis and Buso (2004) and Reis et al. (2005) confirmed that no fungal isolate of melon, pumpkin or cucumber powdery mildew was able to infect watermelon, and the obtained watermelon isolates were only capable of colonizing this species. The same was observed by Cohen et al. (2000) in Israel. Inheritance of resistance to powdery mildew, found in watermelon in Brazil, is of a monogenic dominant nature, with a segregation of three resistant and one susceptible gene (Borges et al., 2002). However, in the United States, two distinct powdery mildew races were identified, 1W and 2W (Davis et al., 2007; Tetteh et al., 2010), with monogenic and multigenic inheritance of resistance, respectively (Davis et al., 2002; Tetteh et al., 2013).

The genetic improvement programs generally aim to incorporate alleles for traits related to better fruit quality, yield increase or resistance to pests and diseases. Resistance to powdery mildew, apart from reducing the use of fungicides, makes a second crop of quality fruit viable because the plant does not suffer from the damage caused by the disease. The development of new watermelon cultivars with desirable agronomic traits and resistance to powdery mildew through classic improvement techniques is quite slow

and laborious, apart from undergoing environmental influence.

Biotechnological techniques, such as molecular markers permit selecting individuals based on their genotype, independently from biotic or abiotic factors at any stage of plant development. The identification of molecular markers linked to phenotypic traits, such as powdery mildew, permits a better understanding of inherited characteristics between the progenies and the reduction of the number of individuals to be examined posteriorly in the field (Bhering et al., 2009). In this way, marker assisted selection (MAS) tends to speed up improvement programs because the selection can still be done in the initial development stages of the plant, which currently, is of fundamental importance for the development process of new cultivars.

The microsatellite markers, also denominated SSR (Simple Sequence Repeats), consist of units of about one to six nucleotides repeated in tandem (Oliveira et al., 2006; Caixeta et al., 2009). They have been developed for various cultivated plant species and are rapidly substituting other types of markers in various types of genetic studies, mainly due to its reproducibility, technical simplicity, the small quantity of required DNA, the low cost, great resolving power and high levels of polymorphism (Caixeta et al., 2009).

Ren et al. (2012) published a genetic map of the watermelon, with genetic linkage groups highly saturated with SSR markers. Guo et al. (2013) published the genome of the watermelon, which together with the work of Ren et al. (2012), will facilitate marker assisted selection and identification of genes that are responsible for desirable agronomic traits.

The objective of this study was to identify microsatellite markers linked to the resistance locus to powdery mildew in a F_2 population of 'BRS Opara' × 'Pérola' to assist improvement programs for watermelon.

Results

True F_1 identified by microsatellite markers

The used method of controlled hand-pollination was efficient to obtain the F_1 and F_2 populations from the cross of the cultivars BRS Opara × Pérola, free of contamination. The five microsatellite markers (MCPI_04, MCPI_05, MCPI_11, MCPI_16 and MCPI_26) that used to confirm hybridization, were able of discriminating between the F_1 population and parents. The individuals of the F_1 population showed alleles from both the female parent (BRS Opara) and the male parent (Pérola).

Powdery mildew resistance is conditioned by a dominant allele

When phenotyping the F_1 and F_2 populations for resistance to powdery mildew, we found that parents were pure lines for resistance to powdery mildew, as 100% of the examined plants of 'BRS Opara' were found to be resistant and 100% of the 'Pérola' plants were susceptible. In the F_1 population, 100% of the plants were resistant and in the F_2 population 158 (79%) of the plants were resistant and 42 (21%) were susceptible (Table 1). The values observed for resistance and segregation to powdery mildew in the F_2 population for in the field indicate that the resistance to the disease is governed by a dominant segregation gene with a ratio of 3:1.

Three microsatellite are closely linked to powdery mildew genes

The screening was carried out with 116 microsatellite markers developed for watermelon. Twelve primer pairs with polymorphism among the parents (Table 2) were screened. Among these, only three markers showed the expected allelic pattern, with the presence of alleles in resistant individuals and absence in susceptible individuals (Fig. 1). The three markers were: MCPI_11 developed by Joobeur et al. (2006), the CYSTSIN developed by Guerra-Sánz (2002) and BVWS02441 developed by Ren et al (2012). These three markers were selected to genotype the F_2 populations of 200 individuals, and only nine recombinant individuals were found by the quoted markers. The analyses of linkage done in JoinMap 2.0 showed that the microsatellites MCPI_11, CYSTSIN and the BVWS02441 are linked to powdery mildew resistance gene in watermelon at a distance of 2.6 cM with LODs 'score' of 31.42. The blast search for the sequences of the three primer pairs and its PCR products was done in the watermelon genome, available at http://www.icugi.org. It showed that the gene related to resistance to powdery mildew is located on chromosome two. In addition, these three markers are very close together with the following approximate distances: (1) MCPI_11 to BVWS02441 57.44 kb (2) BVWS02441 to CYSTSIN 33.82 kb, and (3) MCPI_11 to CYSTSIN 91.26 kb. The analysis of the microsatellite markers MCPI_11, CYSTSIN and BVWS02441 in 17 commercial watermelon cultivars showed that the cultivar 'BRS Opara' (resistant to powdery mildew) has an allele that is different from all the other analyzed cultivars (susceptible to powdery mildew) in relation to the three analyzed markers (Fig 2).

Discussion

The microsatellite markers MCPI_04, MCPI_05, MCPI_11, MCPI_16 and MCPI_26, were used to confirm hybridization between the cultivars, BRS Opara and Pérola. They can be used as an identification tool in paternity analyses of watermelon hybrids for these cultivars. In many cases, when it is not possible to confirm hybridization, differentiating a hybrid from its parents, or even confirming its paternity, based only on morphological descriptors, molecular markers, such as microsatellite markers, is an alternative.

The results obtained from phenotyping the F_1 and F_2 populations and from its parents for resistance to powdery mildew in watermelon, validate the Mendelian segregation of 3:1 (resistant: susceptible) with dominance of a gene that confers resistance to the disease. In this way, it can be substantiated that the inheritance of resistance, in the used source, is monogenic and dominant. These results are in agreement with the results reported by Borges et al. (2002). However, in South Korea, Kim et al. (2013) reported a source of resistance to powdery mildew, race 1 W, as monogenic and partially dominant. On the other hand, in the United States, Davis et al. (2002) confirmed that resistance to race 1 W is multigenic. It is not possible to confirm whether these are different races, if there is some kind of speciation of this race that depends on the locality, or for being sources of different resistance. They behave in a different way in relation to the type of inheritance of resistance to the pathogen. Apart from being limited, the available data suggest that the geographical

Table 1. Number of analyzed plants for powdery mildew for resistance and susceptibility in BRS Opara, Pérola cultivars and population F_1 and chi-square test in F_2 of watermelon population.

Resistance or susceptibility to powdery mildew			
Genotypes	Number of analyzed plants	Resistant plants	Susceptible plants
BRS Opara	30	30 (100%)	0
Pérola	30	0	30 (100%)
F_1(BRS Opara × Pérola)	100	100 (100%)	0
F_2	200	158(79%)[NS]	42(21%)[NS]

[NS] Non-significant at 5% probability by the Chi-square test.
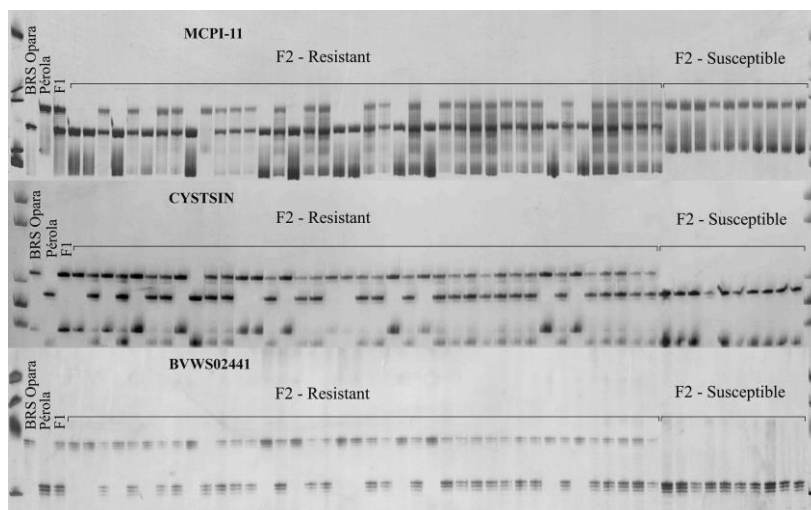

Fig 1. Allelic pattern of the cultivar BRS Opara (resistant), of cv. Pérola (susceptible), F_1 and of 50 individuals of the F_2 population (40 resistant × 10 susceptible) using three microsatellite markers (MCPI_11, CYSTSIN and BVWS02441) associated with powdery mildew in watermelon.

distribution of races is complex and mutable over time (Mohamed et al., 1995). However, the best region to select sources of resistance for some plant disease and to develop programs for genetic improvement with the objective of transferring resistance to cultivars of interest, are those regions where the host grows naturally or where it is grown for centuries, which permits only the survival of hosts that are resistant to the pathogen.

With genotyping the F_2 population for resistance to powdery mildew using microsatellite markers, the results showed three SSRs markers (MCPI_11, CYSTSIN and BVWS02441) capable of identifying the presence of alleles in resistant plants and absent in susceptible plants. These microsatellite markers identified only nine recombinant individuals in a population of 200 individuals. The genetic basis of linkage mapping is the genetic recombination, resulting from crossing over between homologous during meiosis. Genetic recombination is measured by recombination fraction, which is the ratio of recombinant gametes to total gametes. The closer together on the same chromosome are two loci, the less recombination between them; the further apart, the more recombination (Liu, 1997). The analyses of linkage done in JoinMap 2.0 showed that the microsatellites MCPI_11, CYSTSIN and the BVWS02441 are linked to powdery mildew resistance gene in watermelon at a distance of 2.6 cM with LODs 'score' of 31.42. This means that these markers are very close to the allele that confers the resistance to powdery mildew in watermelon. Therefore, they are suitable for being used in the marker assisted selection for resistance to powdery mildew and considerably accelerate the genetic improvement programs of watermelons of various research institutions that are trying to achieve resistance to powdery mildew.

Molecular markers, when associated with phenotypic characteristics, such as resistance to powdery mildew, can speed up the improvement programs. Alpuerto et al. (2009) estimated that, with the use of marker assisted selection in rice improvement programs for tolerance to salinity and phosphor deficiency, there was a reduction of 3 to 6 years for the development of a new cultivar in comparison to conventional improvement.

The results of the validation of the microsatellite markers MCPI_11, CYSTSIN and BVWS02441 reinforce the use of these markers in assisted selection aiming to achieve resistance to powdery mildew. Once these markers were analyzed in 17 commercial watermelon cultivars (Fig 2), the cultivar BRS Opara (resistant to powdery mildew) showed an allelic pattern, distinct from all the other examined cultivars, which are susceptible to powdery mildew. Few studies have been found in the literature that report markers associated with phenotypic characteristics in watermelon. Kim et al. (2013) analyzed 5493 RAPD primers and identified one marker (OP-R483) linked to resistance to powdery mildew, race 1 W, at a distance of 3.6 cM. Subsequently, these authors cloned the amplicon that was found with this marker and converted to SCAR1-F and SCAR1-R, which corresponded to the locus OP-S483. The sequences obtained with OP-S483 and OP-R483 showed five single nucleotide polymorphisms (SNP), which were used to develop the CAPs marker.

The comparison of the data from this study and the data from the study by Kim et al. (2013) is difficult because different sources of resistance are involved and the authors of the latter study did not provide the primer sequence (OP-R483). Therefore it is not possible to verify whether the microsatellite markers in their study and those that were found in this study are in the same chromosome region.

Considering other economically important crops, assisted

Table 2. Name and primer sequence of the polymorphic primers among the cultivars BRS Opara and Pérola.

Primer Name	Primer sequence (5'-3')
MCPI_04[a]	AGCAAATGCATGGGGAAAAC/TGTTGAATGGAGGCTTTGAG
MCPI_05 [a]	ATTTCTGGCCCCAGTGTAAG/GAACAACGCAACCACGTATG
MCPI_11 [a]	GAGCAGGGGAGAAGGAAAAC/CCAGTAGCTTTTTCCGATGC
MCPI_16 [a]	TGCTCAATCCACCCTTTCTC/AAAAACAGCAACTCTCCCATC
MCPI_26 [a]	CAGAGGAACGAGAGGGAGTG/GGGGAGCCCATATTTTAACC
CYSTSIN [b]	ATTTTCTTGCTTCAAATGGA/ATAAGCAAAAGCATCGAAAG
EST00680 [b]	CCTTATCTCAACTCTTTTCGGA/AGGATTGGGCTTGATTGTTG
BVWS00241[c]	CAGAGGAACGAGAGGGAGTG/GGGGAGCCCATATTTTAACC
BVWS2447 [c]	CAGAGGAACGAGAGGGAGTG/GGGGAGCCCATATTTTAACC
BVWS01050 [c]	GAATTTGCTTCAGCCTTTGG/TTCATTTCTAGGTTGGTTTTAAGATTT
BVWS02384 [c]	TCCAAGTGGCTTGCTCTTTT/TCTCAACCTCAAATTCCGAGA
BVWS01358 [c]	CCCTATTGCCTATTTTTCTCAA/AAATTTGTGCTCTTCGTGGG

[a]Primers developed by Joobeur et al. (2006); [b]Primers developed by Guerra-Sánz (2002); [c]Primers developed by Ren et al. (2012)
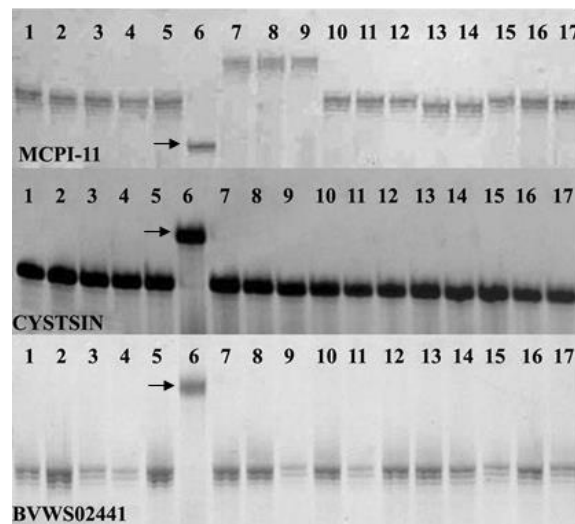

Fig 2. Allelic pattern for three microsatellite loci CYSTSIN, MCPI_11 E BVWS02441, linked with resistance to powdery mildew, examined in 17 watermelon cultivars. Cultivars: 1 = Minipool, 2 = Pérola, 3 = Smile, 4 = BRS Soleil, 5 = BRS Kuarah, 6 = BRS Opara, 7 = Crimson Sweet, 8 = Crimson Select, 9 = Nova Crimson, 10 = Top Gun, 11 = Omaru Yamato, 12 = Sugar Baby, 13 = Fairfax, 14 = Emperor Charleston Bonanza, 15 = Charleston Gray, 16 = Peacock, 17 = Sunshade. Only BRS Opara cultivar is resistant to powdery mildew.

selection by molecular markers has been well used in improvement programs, such as, accelerated development of quality protein maize hybrid through marker-assisted introgression of opaque-2 allele (Gupta et al., 2013).

The great advances have been made in molecular studies in watermelon, especially by providing the genome of this crop (Guo et al., 2013). These authors made it possible to identify the chromosome that suitable microsatellite markers MCPI_11, CYSTSIN and BVWS02441can be located on.

Materials and Methods

Plant materials

A number of crosses were carried out by means of controlled hand-pollination (CHP), as described by Dias et al. (2001), among two watermelon cultivars, contrasting for resistance to powdery mildew: 'BRS Opara' (resistant) and 'Pérola' (susceptible), to obtain population F_1. Samples of young leaves were collected from each parent and stored in freezer at -80 °C for extraction of DNA. After harvesting of hybrid fruits from the cross, the seeds were washed and put to dry. Subsequently, these seeds were sown in polystyrene trays and

kept in a greenhouse for 12 days. When the seedlings started to develop, leaf samples were collected from F_1 individuals for extraction of DNA and for confirmation of hybridization by PCR, using microsatellite markers.

The seedlings from the F_1 population were transplanted to an experimental field and self-fertilization was carried out during the flowering stage by means of CHP to obtain population F_2. They were examined for resistance to powdery mildew. After harvesting the fruits, the seeds were washed and put to dry. Subsequently, a final field experiment was carried out to examine the resistance to powdery mildew by using 10 plants of each parent, 100 plants of the F_1 population and 200 plants of the F_2 population. When the seedlings started to develop, samples of young leaves were collected from the F_2 population and conserved in a freezer at -80 °C for extraction of DNA.

Extraction of DNA and microsatellite analysis of the F_1s

DNA extraction of the leaves was done by using the 2x CTAB protocol according to Doyle and Doyle (1990), modified to: 7.500 and 10.000 rpm at the first and second centrifugation, respectively; 2-mercaptoethanol at 2% and

incubation at 60 °C for 30 min for all the samples. The DNA was resuspended in a Tris-EDTA buffer and treated with RNAse to remove co-isolated RNA. Quantification and integrity analysis of the DNA was performed in agarose gel at 0.8%, followed by storage of the samples in a freezer at -20 °C.

Five microsatellite markers, developed for watermelon by Joobeur et al. (2006), were used (MCPI_04, MCPI_05, MCPI_11, MCPI_16 and MCPI_26) for inclusion of paternity and confirmation of the hybrids. These SSRs were selected from the work by Gama et al. (2013) because they showed PCR products of different sizes for these cultivars. The PCR reaction was done by following the protocol of Joobeur et al. (2004) with some modifications for a final volume of 12 μL, containing 50 ng of DNA, 5 μM of each 'primer', 0.1 mM of dNTP, 1.5 mM of $MgCl_2$, 1x PCR buffer and 0.75 units of the Taq DNA polymerase enzyme. Programming of the thermal cycler for amplification consisted of: a) an initial cycle of 94 °C for 2 min, followed by 30 cycles at 94 °C for 15s, at 56 °C for 30 s and at 72 ºC for 2 min and a final cycle at 72 ºC for 30 min.

Six μL of formamide denaturation buffer 98% (EDTA pH 8.0 10mM, 1 mg/mL of Xylene Cyanol and 1 mg/mL of Bromophenol blue) was added to the solution from the PCR reaction, followed by complete denaturation at 94 °C for 5 min in a thermal cycler and put on ice until applying in polyacrylamide gel. Two and half μL of the denatured PCR reaction was applied to the 6% polyacrylamide gel. The electrophoresis carried out for approximately 3 h with a constant power of 45 W. The gels were stained with silver nitrate according to the procedure described by Creste et al. (2001). It was observed if the hybrids exhibited alleles from both the female parent (BRS Opara) and the male parent (Pérola).

Phenotyping for resistance to powdery mildew of the F_1, F_2 and parents

After the flowering period, powdery mildew inoculation was carried out by using a suspension of sterile water and powdery mildew from watermelons in a concentration of 10^6 conidia/ml, adding a spreader sticker ("Tween 80"). The plants that showed 0-30 colonies of powdery mildew were considered resistant and those that showed more than 31 colonies were considered susceptible.

Genotyping of the F_2 population for resistance to powdery mildew, using microsatellite markers

Initially, a screening was done with 116 microsatellite markers developed for watermelon (Jarret et al., 1996; Guerra-Sánz, 2002; Joobeur et al., 2006, Ren et al., 2012). In order to select the primers that show different sizes of the PCR product among the parents, they were used to analyze five resistant and five susceptible individuals from the F_2 population to identify alleles that were present in the resistant individuals and absent in the susceptible individuals or vice versa. Subsequently, the primers that were capable to verify this characteristic, were used to genotype the 200 individuals of the F_2 population. The methodology that was used for DNA extraction, PCR reaction using microsatellite markers and polyacrylamide gels was the same as was described previously for the F_1 topic DNA extraction and microsatellite analyses.

Statistical analysis and identification of the chromosome region of resistance to powdery mildew

The data were annotated taking 'b' for the dominant ('AA') and the heterozygote ('Aa') and 'd' for the recessive ('aa') microsatellite genotypes. The data that were obtained by the microsatellite markers and by visual examination of resistance/susceptibility to powdery mildew were submitted to the chi-square test at 0.05 for verification of the Mendelian of expected segregation for a trait monogenic and dominant in a F_2 population of 3:1. The linkage analyses were done by using the program JoinMap version 2.0 (Stam and Van Ooijen, 1995) and the values obtained by the recombination frequencies were converted into maps of genetic distance (centiMorgan) by using the Kosambi function (Kosambi, 1944).

A blast search was done for primer sequence and its PCR products, linked to resistance to powdery mildew, in the watermelon genome available at: http://www.icugi.org (Guo et al., 2013), to determine in which chromosomes these primers are located.

Validation of microsatellite markers linked to resistance to powdery mildew

In the current study, three microsatellite markers linked to resistance to powdery mildew were selected out of 116 markers. They were analyzed in 17 commercial cultivars of 'Minipool', 'Pérola', 'Smile', 'BRS Soleil', 'BRS Kuarah', 'BRS Opara', 'Crimson Sweet', 'Crimson Select', 'Nova Crimson', 'Top gun', 'Omaru Yamato', 'Sugar Baby', 'Fairfax', 'Emperor Charleston Bonanza', 'Charleston Gray', 'Peacock' and 'Sunshade' watermelons. Among these, only the cultivar 'BRS Opara' is resistant to powdery mildew and all the other analyzed cultivars were susceptible. The resistant and susceptible genotypes ['BRS Opara' (resistant) × 'Pérola' (susceptible)] then used to create the F_1 and F_2 populations.

Conclusions

The microsatellite markers MCPI_11, CYSTSIN and BVWS02441, are closely linked with resistance to powdery mildew genes and may offer support for watermelon improvement programs with resistance to this disease and other desirable agronomic traits. The identification of the chromosome region, in which these markers are located, will facilitate future studies to identify the gene that confers resistance to powdery mildew in watermelon.

Acknowledgments

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