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ORIGINAL PAPER

Simple sequence repeat markers linked to QTL for resistance to Watermelon mosaic virus in melon

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Abstract A population of recombinant inbred lines (RIL) derived from a cross between the *Watermelon mosaic virus* (WMV) resistant genotype TGR-1551 and the susceptible Spanish cultivar 'Bola de Oro' has been evaluated for WMV resistance in spring, fall and growth chamber conditions. The quantitative trait loci (QTL) analyses detected one major QTL (*wmv*) on linkage group (LG) XI close to the microsatellite marker CMN04_35. This QTL controls the resistance to WMV in the three environmental conditions evaluated. Other minor QTLs affecting the severity of viral symptoms were identified, but they were not detected in all the assayed environments. The screening of the marker CMN04_35 in an F_2 progeny, derived from the same cross, confirmed the effect of this QTL on the expression of WMV resistance also in early generations, which evidences the usefulness of this marker for a marker assisted selection program.

Introduction

Cucumis melo L. is one of the most important crops in tropical and subtropical countries. Similar to other cucurbits, this species is highly affected by viruses, most of them transmitted by insects. Among the viruses affecting melons, *Watermelon mosaic virus* (WMV) is one of the most important; it is widely distributed along the main melon production areas in Spain (Luis-Arteaga et al. 1998), California (Grafton-Cardwell et al. 1996), Brazil (Yuki et al. 2000), Turkey (Sevik and Arli-Sokmen 2003), and other countries (Abou-Jawdah et al. 2000; Ali et al. 2006; Ko et al. 2007). Recent reports indicate an increase in the importance of WMV in commercial melon crops (Desbiez et al. 2009).

WMV is a RNA virus with monopartite single-stranded genome and filamentous particle that is transmitted in melons by at least 38-aphid species in a non-persistent manner (Castle et al. 1992; Purcifull et al. 1984; Ward and Shukla 1991). Some of the symptoms of this virus are mosaic, leaf distortion, chlorosis and tip stunting, which results in poor fruit quality and low yields. The main damage is produced when the virus infects young plants, affecting seriously the production (Nameth et al. 1985). This damaging effect and the wide presence of the virus in extensive spring commercial fields, when aphid flights are abundant, make some control measures against this virus necessary.

Several approaches have been used to control the presence of WMV in melon crops. Cultural practices may reduce the infection levels in open fields (Hooks and Ferees 2006; Stapleton and Summers 2002), but these reductions are not enough to produce a profitable yield. The virus aphid transmission resistance gene (*Vat*) has been widely used by breeders to control WMV and other viruses transmitted by *Aphis gossypii* (Lecoq et al. 1979; Sauvion et al. 2005). However, Lombaert et al. (2009) reported the appearance of new *A. gossypii* biotypes that are able to overcome the resistance conferred by the gene

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Vat. Besides, this virus could be transmitted by other aphid species (Castle et al. 1992). This situation suggests that the most effective method to control WMV is the use of genetic resistance against the virus itself (Gómez et al. 2009).

Although Munger (1991) indicated the existence of tolerance to WMV in some exotic melon accessions, genetic resistance to WMV has only been described in two melon lines so far: PI 414723 and TGR-1551. The Indian PI 414723 (Gray et al. 1986) possesses a resistance controlled by a single dominant gene named *Wmr* which involves mild symptoms on the leaves at the beginning of the infection, followed by plant recovery (Gilbert et al. 1994). The resistance of the Zimbabwean genotype TGR-1551 controlled by one recessive gene together with other additional genetic factors (Díaz et al. 2003; Díaz-Pendón et al. 2005) shows restricted systemic virus accumulation. In this case, resistant plants could be asymptomatic or exhibit very mild virus symptoms. However, there are no commercial cultivars resistant to WMV, probably because the use of this exotic germplasm makes the introgression of the character difficult. On the other hand, the selection of virus resistant genotypes in a breeding program is a difficult task because viral symptoms take time to be expressed and susceptible plants could escape infection, a situation often affected by environmental conditions (Collard et al. 2005; Mohan et al. 1997; Shi et al. 2009). To avoid these problems, marker assisted selection (MAS) has been widely used to introduce virus resistance in main crops such as potato (Hämäläinen et al. 1997), soybean (Saghai Maroof et al. 2008; Shi et al. 2009), barley (Jefferies et al. 2003), rice (Sugiura et al. 2004) or tomato (Zamir et al. 1994). However, its application requires the previous identification of genetic markers tightly linked to the targeted genes. Regarding the resistance to WMV, neither mapping nor identification of markers linked to the resistance genes in melon has so far been described. Because the resistance found in TGR-1551 is recessive, which implies greater complexity in the introgression program, the use of molecular markers linked to it would make the selection of resistant genotypes much more effective.

The objective of this work was to perform a quantitative trait loci (QTL) analysis to better depict the genetic architecture of the WMV resistance from TGR-1551 and to identify molecular markers useful for a MAS program directed to introgress this resistance into commercial cultivars. To get this objective, a population (F_7) of recombinant inbred lines (RIL) derived from a cross between TGR-1551 and the highly susceptible Spanish cultivar 'Bola de Oro' has been used.

Materials and methods

Plant material

A RIL population was developed by the Single Seed Descent (SSD) method. A single F_1 plant derived from a cross between the Zimbabwean genotype TGR-1551 (WMV resistant) and the Spanish cultivar 'Bola de Oro' (WMV susceptible) was used to generate F_2 individuals, which were self-pollinated until F_7 generation.

This RIL population, together with the parental genotypes and their F_1, has been evaluated for WMV resistance using four plants per genotype in three different environments: spring-glasshouse (25–14°C), fall-glasshouse (30–18°C) and growth chamber (25–20°C, 16 h light/8 h dark). The number of RIL evaluated was 58, 77 and 66 in spring, fall, and growth chamber, respectively. In all of the experiments, melon plants were grown in plastic pots (12 cm Ø) filled with soil-substrate composed of peat (60%), litonite (10%) and compost (30%).

In addition, in order to validate the markers linked to the resistance, an F_2 population of 200 individuals was derived from the same cross and evaluated for WMV resistance in the spring-glasshouse (25–14°C) conditions.

Evaluation of WMV resistance

Virus inoculation and evaluations were made following the method described by Díaz-Pendón et al. (2005). Mechanical inoculations were performed on the first leaf and cotyledons of plants at one-to-two leaf stage. The virus isolate M116-WMV, used in the experiments, was kindly provided by Dr. Moriones (Virology Department, IHSM-La Mayora, CSIC). Young leaves of WMV infected zucchini squash ('Diamante' F_1) showing clear symptoms of infection were used as the inoculum source.

Symptoms were rated according to an integer scale ranging from 0 to 5, where 0 = no symptoms; 1 = apical leaf with dispersed vein clearing or mottle and no mosaic; 2 = mild mosaic only in the apical leaf; 3 = mild mosaic and mild leaf distortion in the three youngest leaves; 4 = mosaic and leaf distortion in the three or four youngest leaves; and 5 = severe mosaic and leaf distortion in the five to six youngest leaves. WMV symptoms in melon plants started to appear 2 weeks after virus inoculation. The symptom score recorded at twenty-one days after inoculation was considered the phenotypic value of each plant. The presence of virus in plants was confirmed following Díaz et al. (2003) using double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) (Clark and Adams 1977) and a commercial polyclonal antiserum for WMV (Loewe Biochemica GmbH, Otterfing,

Germany). The mean symptom scores recorded on each RIL genotype and environment was used for different analyses. Because the distribution of genotypes did not follow a normal distribution, the effect of the different factors on the symptom score was analyzed using a Generalized Lineal Model (GLM), with symptom score as the dependent variable, and RIL genotype, plants replicate (inside each RIL genotype) and evaluation, as the predictors. Heritability for each evaluation was calculated following Perchepied and Pitrat (2004) using $h^2 = \sigma_G^2/\left(\sigma_G^2 + \left(\sigma_e^2/n\right)\right)$, where σ_G^2 is the genotypic variance, σ_e^2 is the environmental variance and n is the number of evaluated plants per RIL. All statistical tests were performed using SPSS for Windows v.17.0 (SPSS for Windows, Rel. 11.0.1. 2001. Chicago: SPSS Inc).

A χ^2 test was performed to check the goodness-of-fit of monogenic resistance. For this analysis, RILs with symptom score <2 were considered resistant, while RILs with ≥ 2 were considered susceptible. Mild mosaic in apical leaf was a clear signal of viral infection and ELISA test confirmed the virus presence in the plant.

Extraction of genomic DNA and marker analysis

Bud tips of plants were stored at -70°C until DNA extraction. Liquid nitrogen was added to quickly freeze-dry the sample before grinding. Plant DNAzol Reagent (Invitrogen, Germany) was used for genomic DNA extraction. The concentration and quality of extracted DNA in samples was determined by reading at 230, 260 and 280 nm using Nanodrop Spectrophotometer ND-100 (Nanodrop Technologies, Delaware, USA). DNA was diluted to get a working dilution of 10 ng/µl.

For the molecular evaluation of the RIL population, we used mainly simple sequence repeat (SSR) markers described previously. PCR amplifications and nomenclature assignment were carried out following the indications suggested by the different authors. They were named as CM- and CS- (Daning-Poleg et al. 2001), CSW- (Fazio et al. 2002), CMBR (Ritschel et al. 2004), CM-N (Gonzalo et al. 2005), CMN (Fukino et al. 2007, http://cse.naro.affrc.go.jp/nbk/List_CMN.xls), CM (Kong et al. 2007), and ECM, GCM and 5A6U marker (Fernández-Silva et al. 2008).

Random Amplification of Polymorphic DNA (RAPD) analyses were performed using the primers OPC09, OPC17, and OPE01 (Operon Technologies, Alameda, CA). PCR amplification and DNA electrophoresis were carried out according to López-Sesé et al. (2002). Each marker was designated by the name of the RAPD primer used for its amplification followed by its molecular size in base pairs (e.g., OPC09-750).

In addition, a set of SCAR and SSR markers previously reported as being linked to several resistance genes were mapped: SV01, SV06 (Oumouloud et al. 2008), AM, FM (Wang et al. 2000), SSR138, SSR154, SSR178 (Joobeur et al. 2004) for resistance to fusarium wilt (*Fom-1* and *Fom-2*); MarkerD and MarkerE (Dogimont et al. 2007) for the *Vat* locus; and 24L19D (van Leeuwen et al. 2005) for the melon resistance gene homologue MRGH18. The codominant PCR-based markers PM1-SCAR, PM2-CAPS, PM3-CAPS and PM4-dCAPS, linked to powdery mildew resistance of TGR-1551 described by Yuste-Lisbona et al. (2011) were also screened in the mapping population.

Linkage map and QTL analyses

Linkage analyses were performed using JoinMap® 4.0 software (van Ooijen 2006). Marker data were assigned to linkage groups using a minimum logarithm of odds (LOD) score of 4.0 and a recombination frequency value of 0.3. The Kosambi map function (Kosambi 1944) was used to calculate the genetic distance between markers. The linkage groups were designed according to Périn et al. (2002).

QTL analyses were conducted separately for the three RIL evaluations using the different methods provided by the software MapQTL® 5.0 (van Ooijen 2004). With the Interval Mapping method (IM), the map location, the LOD score, and the phenotypic effect (expressed as the percentage of phenotypic variance explained) of potential QTLs were estimated. This information was applied to extend the QTL search through a Multiple QTL Model (MQM) using the closest marker to each QTL detected by IM method as cofactors. Using the set of cofactors, the map location, LOD score, and percentage of phenotypic variance explained were estimated. A permutation test (10,000 cycles) was used to determine the LOD threshold score at which the QTL was considered to be present in a particular genomic region with a confidence interval of 99%. Finally, molecular markers with potential significant effect on the expression of the WMV resistance ($P \leq 0.05$) were identified with the Kruskal–Wallis method (KW), a non-parametric test equivalent to the one-way ANOVA.

Results

Evaluation of WMV resistance

All the inoculated plants of 'Bola de Oro' were susceptible to WMV and showed severe mosaic and leaf distortion (symptom score 5). The TGR-1551 plants showed a resistant response, without any symptoms in most of the cases (symptom score 0), although a dispersed vein clearing in the apical leaf could be observed in a few plants

(symptom score 1). Plants of the F_1 were susceptible, showing mosaic and leaf distortion (symptom score 4), which confirmed the recessive inheritance of the character (Díaz-Pendón et al. 2005).

According to symptom score averages (Fig. 1), there was a high proportion of resistant RILs showing no symptoms in any of the evaluations (spring: 46.5%; fall: 45.7%; growth chamber: 40.9%), and <10% of RILs scored a symptom score of 1. The percentages of susceptible plants observed in all evaluations ranged between 5 and 20% for each score (Fig. 1). The mean symptom score for each evaluation was similar, with values of 1.96, 1.82 and 1.92 for spring, fall and growth chamber, respectively.

The results of the GLM analysis (Table 1) indicated that the RIL genotype was the only factor responsible for the virus symptom variation ($P = 0.001$). Different plant replicates from the same RIL showed the same symptom score in the different environmental conditions evaluated ($P \geq 0.05$). Heritability values were also high in all the environments, reaching the values of 0.88, 0.88 and 0.82 in spring, fall and growth chamber, respectively.

In all of the evaluations, the observed resistant: susceptible segregation ratios confirmed the recessive monogenic inheritance hypothesis (spring: $\chi^2 = 0.27$, $P = 0.60$; fall: $\chi^2 = 0.06$, $P = 0.81$; growth chamber: $\chi^2 = 0.00$, $P = 1$).

Linkage map and QTL analyses

A total of 114 RIL genotypes were used as mapping population. The linkage map generated included 204 loci (181 SSR, 7 RAPD, 16 SCAR/CAPS/dCAPS) distributed across 24 linkage groups (LG). Based on the collinearity of SSR shared with other genetic maps, these 24 LG could be assigned as LG I–XII, representing the 12 chromosomes of the melon genome. This map covers a genetic distance of 830.61 cM, with an average of 4.01 cM/marker.

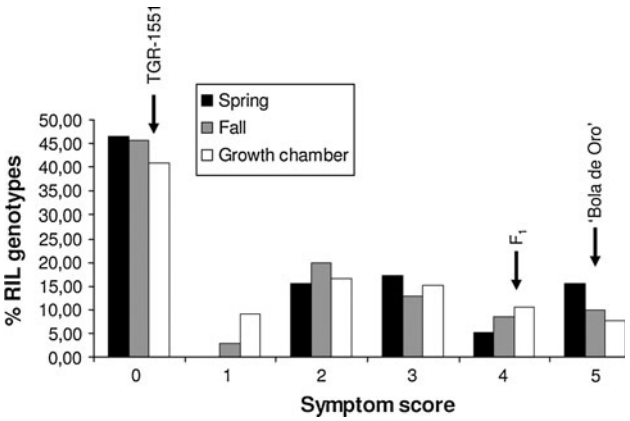

Fig. 1 Percentage of RILs showing WMV symptoms 21 days after their inoculation in the three environmental conditions assayed. Symptom score ranged from 0 = no symptoms to 5 = severe mosaic and leaf distortion, considering score <2 as resistant and >2 as susceptible plants. Symptom scores of parental lines and *F1* are indicated by *black arrows*

The three methods applied for the QTL detection in every evaluation allowed the identification of one robust QTL on LG XI. The IM analysis placed the putative resistance gene between the markers ECM215 and CMN04_35, at approximately 3 cM away from the last one, with LODs of 6, 16 and 8 and percentages of explained variance of 45, 76 and 47%, for spring, fall and growth chamber, respectively. As expected, the percentage of explained variance was slightly lower (39, 58 and 44% respectively) when associated to the adjacent marker, CMN04_35. The amplicon sizes for ECM215 marker were 260 and 265 bp, and for CMN04_35 marker were 240 and 230 bp in TGR-1551 and Bola de Oro, respectively (Table 2). MQM analysis using this marker as cofactor confirmed the existence of one QTL in this region. This major QTL was named *wmv* (Fig. 2). The other markers associated with resistances, used in this report, did not cosegregate with our major QTL.

The same marker, CMN04_35, was highly significant with the KW method ($P < 0.0001$) and flanking markers showed a gradient in the statistic test towards it. This evidences the occurrence of a QTL in this region being CMN04_35 the closest marker. Other regions along the genome were also involved in the resistance expression; they reached lower levels of significance and they were dependent on environmental conditions. At least one marker was highly significant ($P < 0.01$) in the linkage groups IVa (CMN04_37B), IVb (CMN06_25), Va (ECM203), VIIIb (ECM130), and XIIb (5A6U) in at least one evaluation. When the average score of the three evaluations was used in the analysis, only two regions, LG IVa (marker ECM53) and LG XIIb (5A6U), remained significant ($P < 0.01$).

In general, mean symptom scores of RILs with TGR-1551 alleles in those markers were lower than symptom average scores of RILs with 'Bola de Oro' alleles. The score reduction produced by TGR1551 allele was about 2.5–3 degrees for CMN04_35 marker but only 1 degree for ECM53 and 5A6U markers (Table 3). In the last cases, this reduction was still detected when the CMN04_35 effect was subtracted, but to a lesser extent. A combined effect of two or more markers is difficult to quantify because of the unbalanced size of the samples obtained (Table 3).

The linkage between the CMN04_35 marker and WMV resistance was confirmed in an F_2 progeny. Homozygous plants for the TGR-1551 allele displayed a symptom score average of 0.27, whereas homozygous plants for the 'Bola de Oro' allele and heterozygous plants exhibited symptom score averages of 2.75 and 2.61, respectively.

Table 1 General Linear Model analysis for WMV resistance in a RIL population (F_7) derived from a cross between TGR-1551 x 'Bola de Oro'. The environmental conditions assayed were spring-glasshouse, fall-glasshouse and growth chamber

Source of variation	Chi-square of Wald	*df*	*P*
Environment	2.999	2	0.223
Plant replicates	38.403	215	1.000
RIL	133.195	84	0.001

df degree of freedom

Table 2 Description of the molecular markers close to the *wmv* QTL detected on linkage group XI

Marker	Sequence of forward and reverse primers (5′–3′)	Approximate amplicon size (bp)	Reference
ECM215	TGGACATTCATATTCAGGCTTC	TGR-1551: 260	(Fernández-Silva et al. 2008)
	CTCCTTCGATAATGCAAGCAC	'Bola de Oro': 275	
CMN04_35	TGAATTTTGCTCCCCAAATC	TGR-1551: 240	(Fukino et al. 2007)
	GGGGAATTTGGCATTTTCTT	'Bola de Oro': 230	

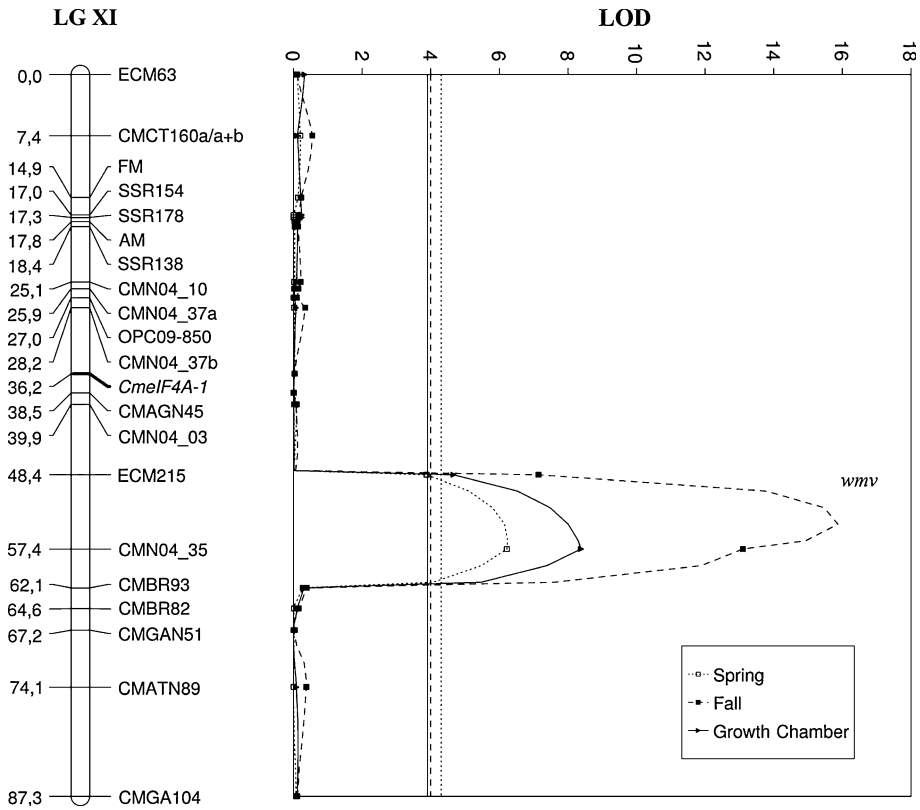

Fig. 2 A multiple QTL model mapping analysis showed the location of the wmv QTL for resistance to WMV on linkage group XI of the melon genome. The SSR marker CMN04_35 was used as a cofactor. Map distances (cM) were estimated using Kosambi's function

Discussion

According to the results obtained by the GLM analysis, differences between genotypes explained most of the WMV resistance variation expressed in our RIL population ($P = 0.001$), with an irrelevant contribution from experimental factors such as RIL replicates or environmental conditions. These results agree with the high heritabilities of the character found in the three evaluations, even considering the small size of the RIL population.

Both the segregation analyses and the QTL analyses carried out confirm the monogenic and recessive control of the WMV resistance in TGR-1551, as suggested before (Díaz-Pendón et al. 2005). On the other hand, the results of the QTL analyses evidenced the existence of a major QTL, called *wmv*, placed at LG XI (CMAGN45-CMBR93

Table 3 Mean WMV symptom scores associated with (a) each single marker and (b) two marker combinations in a RIL population derived from a cross between TGR-1551 and 'Bola de Oro'

(a) Single marker allelic effect			
Alleles	**CMN04_35**	**ECM53**	**5A6U**
TGR	0.50 (41)	1.24 (44)	1.17 (37)
BO	3.00 (42)	2.22 (37)	2.21 (48)
(b) Combined allelic effects			
Allelic combinations			
CMN04_35	**ECM53**	**Symptoms score**	
TGR	TGR	0.63 (30)	
TGR	BO	0.17 (10)	
BO	TGR	2.74 (13)	
BO	BO	2.96 (26)	
CMN04_35	**5A6U**		
TGR	TGR	0.34 (22)	
TGR	BO	0.74 (18)	
BO	TGR	2.56 (14)	
BO	BO	3.14 (26)	

The number of RILs for each allelic combination is shown in brackets. 0 = no symptoms to 5 = severe mosaic and leaf distortion

TGR TGR-1551 allele; *BO* Bola de Oro allele

interval). This region was significantly associated with WMV resistance in all three of the QTL analyses considered across the different environments assayed. The IM analysis placed the putative resistance gene between the markers ECM215 and CMN04_35, with CMN04_35, approximately 3 cM away, the closest marker to the gene. RILs carrying the TGR-1551 allele for this marker caused, on average, a reduction of symptom scores of 2.5–3 degrees, discriminating resistant RILs (average score 0.5) from susceptible RILs (average score of approximately 3). In the F_2 population, the presence of the TGR-1551 allele of CMN04_35 in the homozygous state also distinguished resistant (average score of 0.27) from susceptible individuals (average score of 2.75 for homozygous 'Bola de Oro' allele and 2.61 for heterozygous). These results prove the high potential of this marker for the selection of WMV resistant genotypes.

However, *wmv* QTL does not explain the entire phenotypic variance observed. The symptom graduation observed in the expression of the WMV resistance points to the presence of other factors affecting the character (Díaz-Pendón et al. 2005) that should have a smaller effect than *wmv* QTL because it was more difficult to identify them, especially in our RIL population. According to theory, a RIL population is an efficient and powerful tool for QTL detection because of their increased homozygosity and homogeneity, resulting in increased additive genetic variance and heritability estimates. In addition, the high recombination level improves the separation of linked QTLs. However, the same homogeneity makes that those QTLs exhibiting significant dominant effects cannot be detected and therefore might have failed to become significant. On the other hand, melon consanguinity (or endogamic depression) hampers the development of large RIL populations reducing the power of the QTL analysis. The significant regions found with KW test are candidates to harbour these minor factors, which do not determine the resistance/susceptibility of the trait but can enhance it through a reduction of the symptoms (Table 3). In addition, QTL analyses over a larger RIL population and a single-cross family derived from the same cross could confirm this point and help to depict the genetic architecture of the WMV resistance from TGR-1551, denoting the different genomic regions, as well as the genomic and environmental interactions involved.

The WMV resistance provided by TGR-1551 is a recessive resistance. Fraser (1990) suggested that the recessive resistance could be due to the occurrence in the host plant of a loss or mutation of some components required for the virus to complete its life cycle. One of these components, with a great effect on potyvirus resistance, is the eukaryotic translation factor 4E (eIF4E) or its isoform (eIF(iso)4E) (Robaglia and Caranta 2006). Similar factors have been found along the melon genome (Essafi et al. 2009) and one of them, *CmelF4A-1*, is present in the same linkage group as the *wmv* QTL, but away from the significant region (Fig 2), indicating that *CmeIF4A-1* is not involved in the WMV resistance of TGR-1551. Nevertheless, we cannot discard the possibility that the resistance described here might be encoded by another translation initiation factor gene. Initiatives on sequencing and functional genomics of melon currently in progress (MELONOMICS: http://www.gen-es.org) will provide an excellent tool for identifying the translation initiation factors associated with the different resistance to pathogens and pests affecting this species.

This is the first report in which a major QTL and a molecular marker linked to WMV resistance in melon have been described. Although several virus resistance genes have been mapped or cloned, i.e. *Papaya ring spot virus*

(*Prv*; Brotman et al. 2005), *Zucchine yellow mosaic virus* (*Zym*, Daning-Poleg et al. 2002), *Melon necrotic spot virus* (*nsv*, Morales et al. 2005; Nieto et al. 2006) and *Cucumber mosaic virus* (*cmv1*, Essafi et al. 2009), WMV had not been considered until now.

The closest marker to the WMV gene resistance, CMN04_35 has been revealed as very useful marker for resistance selection in early (F_2) and later generations (RIL). Its codominant nature enables the distinction of heterozygous from homozygous genotypes which is essential to handle a recessive resistance.

The information generated in this work has provided the tools needed to set a MAS program to introgress this resistance into melon commercial cultivars. In addition, the location of the main factor controlling the WMV resistance in the melon genetic map establishes a route by which future research can lead to the isolation and characterization of the gene(s) underlying this major QTL. The cloning of this QTL will help to enhance our understanding of the mechanisms involved in the expression of this resistance.

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