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QTL mapping for resistance to *Cucurbit chlorotic yellows virus* in melon (*Cucumis melo* L.)

Yoichi Kawazu · Koichiro Shimomura · Shoichi Maeda · Yoichi Yamato · Shigenori Ueda · Shiori Okuda · Mitsuru Okuda · Mitsuhiro Sugiyama

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Abstract *Cucurbit chlorotic yellows virus* (CCYV), a member of the genus *Crinivirus*, is transmitted by the whitefly *Bemisia tabaci* and causes yellowing of leaves of cucurbit plants. The melon accession JP 138332 is resistant to CCYV. Here, we detected a single QTL conferring resistance to CCYV in melon. Two susceptible accessions ('Harukei No. 3' and 'AnMP-5') were crossed with JP 138332 to generate two mapping populations. Each F_2 population was evaluated for CCYV resistance in a greenhouse with the use of CCYV-infected *B. tabaci*, and genetic linkage maps were constructed using SSR and Indel markers. The map of the F_2 population derived from 'Harukei No. 3', with 12 linkage groups, comprises 171 markers, which span 1538 cM, with an average interval between markers of 9.7 cM. The map of the F_2 population derived from 'AnMP-5', also with 12 linkage groups, comprises 179 markers, which span 1419 cM, with an average interval of 8.5 cM. QTL analysis using each population detected one locus in the same region on chromosome 1, explaining 46.5–50% of the phenotypic variance. This is the first report of QTL mapping for CCYV resistance.

Keywords CCYV · *Crinivirus* · DNA marker · QTL · virus

Introduction

Cucurbit chlorotic yellows virus (CCYV), a member of the genus *Crinivirus* within the family *Closteroviridae*, was reported first in Japan (Gyoutoku et al. 2009) and later in many other countries, including China (Gu et al. 2011), Egypt (Amer 2015), Greece and Turkey

Y. Kawazu · K. Shimomura · M. Sugiyama (✉)
Institute of Vegetable and Floriculture Science, National Agriculture and Food Research Organization (NARO), 360 Kusawa, Ano, Tsu, Mie 514-2392, Japan
e-mail: sugimi@affrc.go.jp

S. Maeda · Y. Yamato
Kyushu Okinawa Agricultural Research Center, NARO, 1823-1 Miimachi, Kurume, Fukuoka 839-8503, Japan

S. Ueda
Hokkaido Agricultural Research Center, NARO, 9-4 Shinseiminami, Memuro, Kasai, Hokkaido 082-0081, Japan

S. Okuda
National Institute for Environmental Studies, 16-2 Onogawa, Tsukuba, Ibaraki 305-0053, Japan

M. Okuda
Central Region Agricultural Research Center, NARO, 2-1-18 Kannondai, Tsukuba, Ibaraki 305-8666, Japan

Present Address:
S. Okuda
Institute of Agrobiological Sciences, NARO, 1-2 Owashi, Tsukuba, Ibaraki 305-8634, Japan

(Orfanidou et al. 2014, 2017), Iran (Bananej et al. 2013), Lebanon (Abrahamian et al. 2012), Saudi Arabia (Al-Saleh et al. 2015), Sudan (Hamed et al. 2011), and Taiwan (Huang et al. 2010). CCYV is transmitted by the sweet potato whitefly, *Bemisia tabaci* (Gyoutoku et al. 2009). Both *B. tabaci* Middle East–Asia Minor 1 (B biotype) and Mediterranean (Q biotype) can transmit CCYV. CCYV infects many Cucurbitaceae species, including important crops such as melon (*Cucumis melo* L.), cucumber (*Cucumis sativus* L.), watermelon (*Citrullus lanatus* (Thunb.) Matsum. & Nakai), and squash (*Cucurbita pepo* L.) (Okuda et al. 2010). CCYV infection induces chlorotic spots and yellowing symptoms on leaves and causes a significant decrease in the sugar contents of fruits, reducing their market value. CCYV is currently controlled by cultural practices such as removing infected plants, using insect-proof net, and chemical treatment against its vector. One of the most effective methods of disease control is the use of resistant cultivars. Several reports describe sources of resistance to cucurbit-infecting criniviruses in melon, such as *Cucurbit yellow stunting disorder virus* (CYSDV) in TGR 1551 and TGR 1937 (López-Sesé and Gómez-Guillamón 2000), CYSDV and *Lettuce infectious yellows virus* (LIYV) in PI 313970 (McCreight 2000; McCreight and Wintermantel 2008), and *Beet pseudo-yellows virus* in Asian melon accessions (Esteva and Nuez 1992). But no melon cultivars have been reported to be resistant to CCYV. Okuda et al. (2013) evaluated 51 melon accessions for CCYV resistance and found that five showed no or only faint symptoms. One of them, JP 138332, showed a low level of CCYV RNA accumulation, while in the other four RNA accumulation was as high as susceptible commercial cultivar. Therefore, JP 138332 is a promising material for breeding for CCYV resistance, although there is no genetic information on its CCYV resistance.

Here, we generated two mapping populations using JP 138332, evaluated F_2 plants for CCYV resistance, constructed genetic linkage maps, and performed QTL analysis for CCYV resistance. Our overall goal is to identify CCYV-resistance QTLs, to develop DNA markers closely associated with the resistance, and to use them for marker-assisted selection (MAS) in CCYV-resistance breeding.

Materials and methods

Plant materials

JP 138332 is a snapmelon (*Momordica* group) and a CCYV-resistant accession originating from India. 'Harukei No. 3' is an inbred cultivar of Earl's Favorite type. 'AnMP-5' is an inbred line of Earl's Favorite type with resistance to powdery mildew (*Podosphaera xanthii*) isolate pxA (Fukino et al. 2008), *Fusarium* wilt (*Fusarium oxysporum* f. sp. *melonis*; resistance gene *Fom-1*), and cotton-melon aphid (*Aphis gossypii*). 'Harukei No. 3' and 'AnMP-5' are both susceptible to CCYV. These 3 genotypes were used to develop F_1 and F_2 populations. We used 93 F_2 ('Harukei No. 3' × JP 138332) plants and 113 F_2 ('AnMP-5' × JP 138332) plants to test for association between marker genotypes and CCYV resistance.

Inoculation sources

To produce inoculation sources, we kept CCYV-infected cucumber plants and *B. tabaci* Mediterranean in a plant growth chamber at 28/23 °C under 16-h light/8-h dark conditions, with light provided by white fluorescent lamps at 60 μmol m^{-2} s^{-1}. Cucumber seedlings were periodically added or replaced to maintain CCYV infection and the *B. tabaci* population.

CCYV resistance tests

Seeds of F_2 ('Harukei No. 3' × JP 138332), F_1 ('Harukei No. 3' × JP 138332), F_1 (JP 138332 × 'Harukei No. 3'), and parental lines were sown on 1 September 2010. We evaluated 12 plants of each parental line and F_1 population, and 93 F_2 plants for CCYV resistance. Plants were inoculated with *B. tabaci* viruliferous for CCYV according to Okuda et al. (2013). They were kept in a cage with CCYV-infected cucumber plants and *B. tabaci* within a greenhouse from 8 to 13 September. The whiteflies were then killed with 50 ppm nitenpyram insecticide. The plants were then planted in single-row plots, 0.4 m apart in rows 1.2 m apart, in a greenhouse on 16 September. The level of resistance of each plant to CCYV was evaluated on 5 November on a disease index (DI) scale as follows: 0 = no symptoms; 1 = slight mottling; 2 = mild yellowing (leaf

yellowing less than 20% of leaf area); 3 = moderate yellowing (leaf yellowing 20% to 50% of leaf area); and 4 = severe yellowing (leaf yellowing over 50% of leaf area). DIs of the 6th to 15th true leaves were recorded, and the mean DI of each plant was used in QTL analysis.

Seeds of F_2 ('AnMP-5' × JP 138332), F_1 ('AnMP-5' × JP 138332), and parental lines were sown on 18 August 2014. We evaluated 12 plants of each parental line, 12 plants of the F_1 population, and 113 F_2 plants for CCYV resistance. Plants were inoculated with *B. tabaci* viruliferous for CCYV from 26 August to 3 September. The plants were planted in single-row plots, 0.35 m apart in rows 1.2 m apart, in a greenhouse on 4 September. The level of resistance of each plant to CCYV was evaluated on 5 November as above.

DNA analysis

Genomic DNA was isolated from young true leaves with a DNeasy 96 Plant Kit (Qiagen, Hilden, Germany). A total number of 326 SSR markers from previous reports (Danin-Poleg et al. 2001; Fazio et al. 2002; Fernandez-Silva et al. 2008; Fukino et al. 2007; Gonzalo et al. 2005; Ritschel et al. 2004) and provided by Syngenta (downloaded from the Cucurbit Genomics Database: http://cucurbitgenomics.org/) were used to select polymorphic SSR markers.

Additional polymorphic markers (Indel markers) were selected using genome sequence data. Whole genome resequencing, mapping to a reference genome, and Indel discovery were done by Macrogen Japan Corp. (Kyoto, Japan). All three parental accessions were sequenced on a HiSeq X Ten sequencer (Illumina, CA, USA), the reads were mapped to the DHL92 melon genome (http://cucurbitgenomics.org/), and Indels were found. PCR primers for detecting Indels were designed using the Primer3Plus software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi).

PCR was performed using a post-labeling method with bar-coded split tags (BStags) (Shimizu and Yano 2011). BStags were labeled with fluorescent dyes (Applied Biosystems): CTAGTATCAGGACTAC-6-FAM, CTAGTATTAGGACGCC-NED, CTAGTATTA-GGA-CCCG-PET, and CTAGTATTAGGACAGG-VIC. An unlabeled BStag was attached at the 5′ end of the forward primer. PCR was carried out in a 5-μL reaction mix containing 1–3 ng of genomic DNA, 2.5 μL of 2 × Qiagen Multiplex PCR Master Mix, 0.05 μM labeled BStag, 0.05 μM forward primer, and 0.2 μM reverse primer. Reaction conditions were as follows: 95 °C for 5 min; 33 cycles of 95 °C for 20 s, 55 °C for 90 s, and 72 °C for 30 s; 3 cycles of 95 °C for 20 s, 49 °C for 90 s, and 72 °C for 30 s; and 72 °C for 5 min. The sizes of the amplified fragments were estimated by an automated DNA analyzer (model 3730xl, Applied Biosystems, Foster City, CA, USA) against a GeneScan-500LIZ size standard (Applied Biosystems). Fragment length was determined with GeneMapper software (Applied Biosystems).

Linkage map construction and QTL analysis

F_2 populations and DNA markers selected as described above were used to construct linkage maps in MAPMAKER/EXP 3.0 software (Lander et al. 1987). Marker data with a logarithm of odds (LOD) likelihood score of ≥ 4.0 were assigned to linkage groups (LGs). The Kosambi map function (Kosambi 1943) was used to calculate the genetic distance between markers. QTL analysis was performed using composite interval mapping in QTL Cartographer v. 2.5 software (Wang et al. 2012). Putative QTLs were estimated from the calculated LOD threshold score after 1000 permutation tests.

Results

Evaluation of resistance to CCYV

We evaluated 'Harukei No. 3', JP 138332, and their F_1 and F_2 for CCYV resistance in 2010. All JP 138332 plants were symptomless (Fig. 1a). 'Harukei No. 3' is a susceptible cultivar, but only 7 out of 12 plants showed symptoms (Fig. 1b), indicating unsuccessful inoculation of some plants in this test. Ten out of 12 F_1 ('Harukei No. 3' × JP 138332) and 5 out of 12 F_1 (JP 138332 × 'Harukei No. 3') showed symptoms (Fig. 1c). When averages of the disease severity index (DI) were calculated from the results of symptomatic plants, the mean DI values of 'Harukei No. 3', F_1 ('Harukei No. 3' × JP 138332), and F_1 (JP 138332 × 'Harukei No. 3') were 2.5 ± 0.20, 2.8 ± 0.21, and 3.4 ± 0.05, respectively. The F_1 scores were greater than that of 'Harukei No. 3', suggesting a recessive phenotype for CCYV resistance. Of the 93 F_2

plants, 54 symptomatic plants had average DI scores of 2.51–4.00, greater than that of symptomatic 'Harukei No. 3' plants, another 25 plants had average DI scores of 0.01–2.50, and the other 14 plants were symptomless, either resistant or not infected (Fig. 2).

We evaluated 'AnMP-5' (DI = 3.6 ± 0.40), JP 138332 (DI = 0.0 ± 0.00), and their F_1 (DI = 4.0 ± 0.07) and F_2 for CCYV resistance in 2014. The mean DI score of the F_1 was almost the same as that of 'AnMP-5', indicating a recessive nature of CCYV resistance. Of the 113 F_2 plants, 70 highly symptomatic plants had average DI scores of 3.51–4.00, another 30 plants had mild symptoms with average DI scores of 0.51–3.50, and the other 13 plants had little or no symptoms with average DI scores of 0.00–0.50 (Fig. 3).

Construction of linkage maps

To select DNA markers which are polymorphic between JP 138332 and 'Harukei No. 3' or between JP 138332 and 'AnMP-5', melon and cucumber SSR markers of previous reports were used (see Materials and methods). One-hundred thirty-two SSR markers which are polymorphic between JP 138332 and 'Harukei No. 3' and 131 SSR markers which are polymorphic between JP 138332 and 'AnMP-5' were selected for linkage map construction. The 'Harukei No. 3' × JP 138332 linkage map had 15 linkage groups (LGs), which spanned 1294 cM, with an average interval of 11.1 cM. The 'AnMP-5' × JP 138332 linkage map had 14 LGs, which spanned 1181 cM, with an average interval of 10.1 cM. The number of LGs of each linkage map was more than the chromosome number of melon (n = 12).

To expand genome coverage of each map, additional polymorphic markers were searched using genome sequence data of three accessions JP 138332, 'Harukei No. 3' and 'AnMP-5'. Indel markers were selected in regions not covered by the first linkage maps. A total of 171 and 179 markers were used to construct linkage maps from F_2 ('Harukei No. 3' × JP 138332) and F_2 ('AnMP-5' × JP 138332) populations, respectively. The former contained 12 LGs, which spanned 1538 cM, with an average interval between markers of 9.7 cM (Fig. 4). The latter also contained 12 LGs, which spanned 1419 cM, with an average interval of 8.5 cM (Fig. 5).

QTL analysis for resistance to CCYV

QTL analysis was performed using the data from F_2 ('Harukei No. 3' × JP 138332) population. As the 14 F_2 plants that showed no symptoms could not be distinguished between resistant or uninfected, only genotype data of those 14 plants were used. QTL analysis detected one major locus on chromosome 1 (Chr. 1), which explained 46.5% of the phenotypic variance, with a maximum LOD of 12.9 (Fig. 4, Table 1).

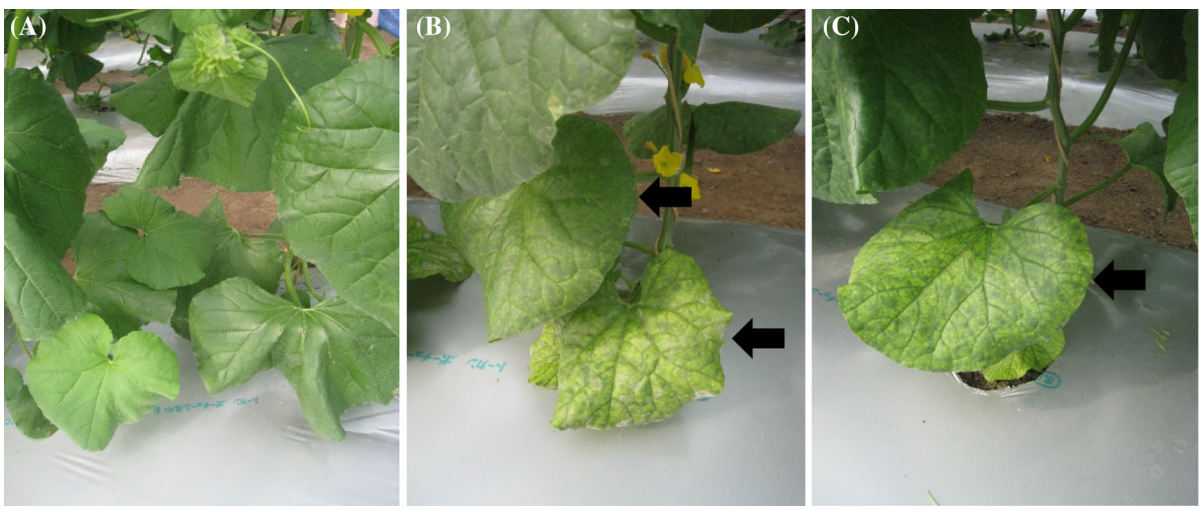

Fig. 1 Plants of **a** JP 138332 (no symptoms), **b** 'Harukei No. 3' (yellowed), and **c** F_1 ('Harukei No. 3' × JP 138332) (yellowed) 70 days after inoculation with CCYV. Leaves of 'Harukei No. 3' and F_1 ('Harukei No. 3' × JP 138332) plants show yellowing (*arrows* in **b** and **c**)

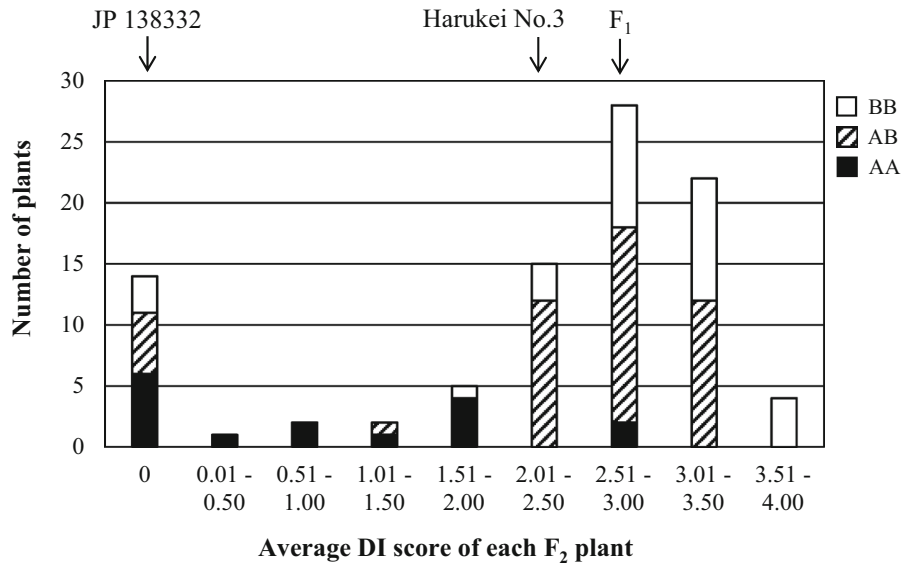

Fig. 2 Frequency distribution of average disease index (DI) scores and genotype at marker ECM 230 of F_2 ('Harukei No. 3' × JP 138332) plants inoculated with CCYV (mean of the 6th to 15th true leaves per plant). AA, homozygous for JP 138332 allele; AB, heterozygous; BB, homozygous for 'Harukei No. 3' allele. DI scores were recorded as follows: 0 = no symptoms; 1 = slight mottling; 2 = mild yellowing (leaf yellowing less than 20% of leaf area); 3 = moderate yellowing (leaf yellowing 20% to 50% of leaf area); and 4 = severe yellowing (leaf yellowing over 50% of leaf area). Arrows indicate the mean DI scores of JP 138332, 'Harukei No. 3', and F_1 ('Harukei No. 3' × JP 138332) and F_1 (JP 138332 × 'Harukei No. 3')

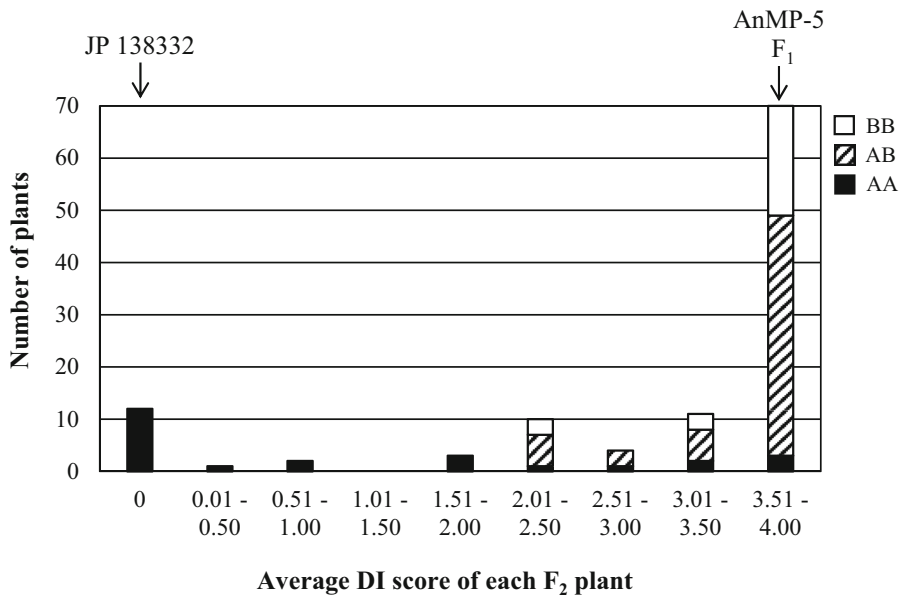

Fig. 3 Frequency distribution of average disease index (DI) scores and genotype at marker ECM 230 of F_2 ('AnMP-5' × JP 138332) plants inoculated with CCYV (mean of the 6th to 15th true leaves per plant). AA, homozygous for JP 138332 allele; AB, heterozygous; BB, homozygous for 'AnMP-5' allele. Arrows indicate the mean DI scores of JP 138332, 'AnMP-5', and F_1 ('AnMP-5' × JP 138332)

QTL analysis using the data from F_2 ('AnMP-5' × JP 138332) population detected one major locus on Chr. 1, which explained 50.0% of the phenotypic variance, with a maximum LOD of 25.5 (Fig. 5, Table 1). This locus was located in almost the same region as that detected in the other population.

To assess whether the marker genotype linked to the major QTL could be used to select for CCYV resistance, we examined the relationship between it

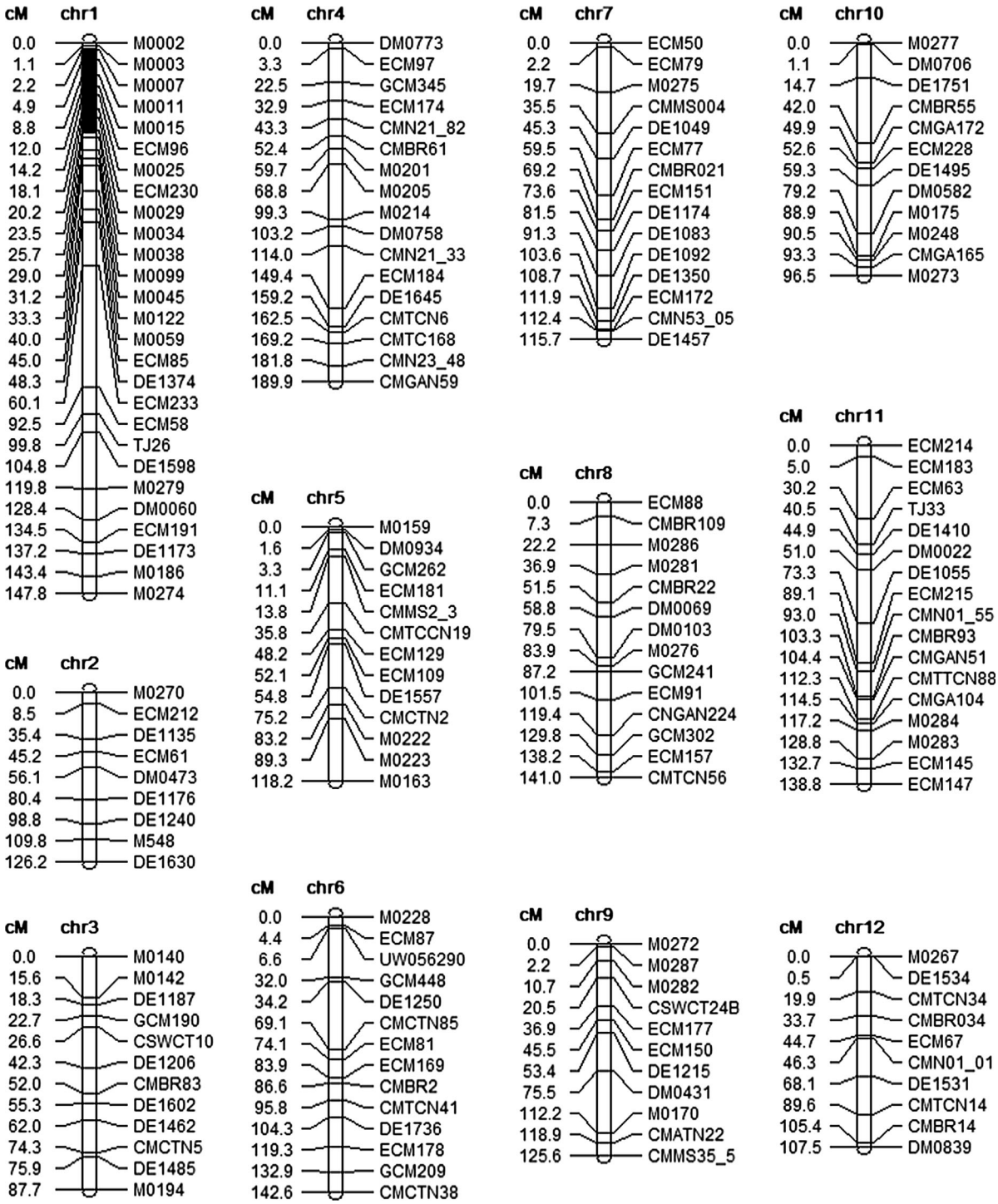

Fig. 4 Linkage map for F_2 ('Harukei No. 3' × JP 138332) population. Black segment indicates significant QTL for CCYV resistance

and CCYV resistance in F_2 progeny (Figs. 2, 3, Table 2). The SSR marker ECM230, which is located near the maximum LOD positions in both F_2 populations, was used for the examination. The mean DI

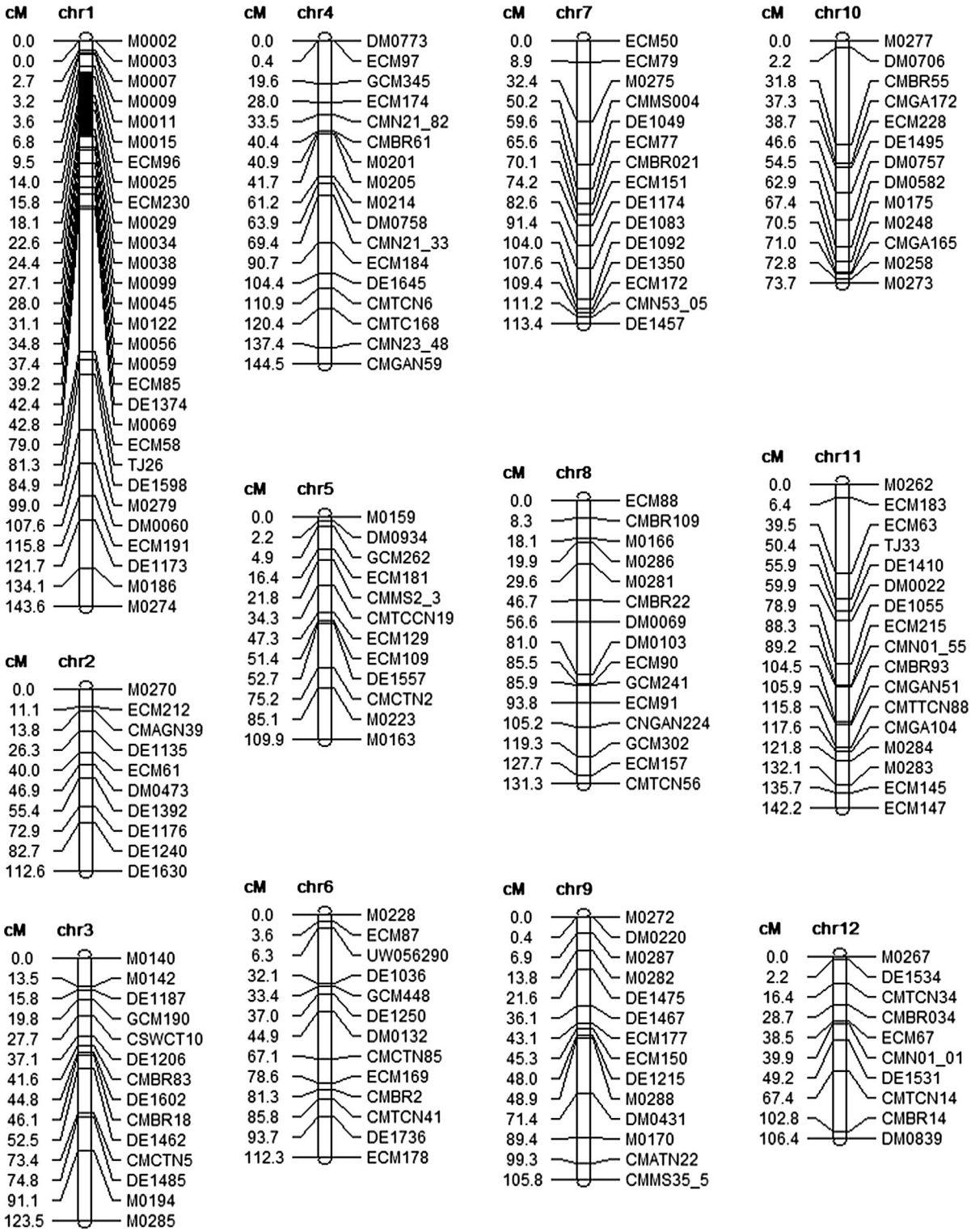

Fig. 5 Linkage map for F_2 ('AnMP-5' × JP 138332) population. Black segment indicates significant QTL for CCYV resistance

scores of F_2 progeny homozygous for the alleles derived from the resistant parent (genotype AA) were 1.6 and 1.2, respectively. These scores were significantly ($P < 0.05$) lower than those of genotypes AB (3.0 and 3.6) and BB (2.7 and 3.6). This result shows that the SSR marker linked to the major QTL can be used to select for resistance to CCYV.

Discussion

We report that CCYV resistance of JP 138332 is recessive, and that a major locus for the resistance is located on Chr. 1. This is the first report of QTL analysis for resistance to CCYV in melon. Genetic resistance to other criniviruses in melon has been reported. A single dominant gene controls LIYV resistance in PI 313970 (McCreight 2000). A single recessive gene controls CYSDV resistance in PI 313970 (McCreight and Wintermantel 2011). Ríus et al. (2016) identified two major QTLs for resistance to CYSDV in both LG V of TGR 1551, and McCreight et al. (2017) reported the resistance to be recessive. Marco et al. (2003) reported that mechanism of resistance to CYSDV in TGR 1551 may involve a restriction of the virus movement in the vascular system of the plants and/or prevention of high levels of virus accumulation. JP 138332 shows high resistance to CCYV and the resistance to be recessive. Okuda et al. (2013) reported that JP 138332 had a lower level of CCYV accumulation in the inoculated and upper leaves at 3 and 6 weeks after inoculation. CCYV resistance in JP 138332 might be associated with inhibition of virus multiplication. However, the mechanisms of resistance to CCYV in JP 138332 are unclear.

Plants rely on multiple mechanisms to defend themselves against diseases and pests. Resistance genes are involved in the defense, and include two types: dominant resistance genes (*R*-genes) and recessive alleles of genes for resistance. *R*-genes encode proteins with conserved motifs such as nucleotide-binding site (NBS), leucine-rich-repeat (LRR), coiled-coil (CC), and Toll/interleukin-1 receptor (TIR) domains (Sekhwal et al. 2015). A major class of resistance genes encodes proteins with NBS and LRR domains, and has been reported in melon. *Fom-2* confers resistance to *Fusarium oxysporum* f. sp. *melonis* (*Fom*) races 0 and 1 (McHale et al. 2006). *Fom-1* confers resistance to races 0 and 2 (Brotman et al. 2013). *Prv* confers resistance to *Papaya ringspot virus* (Brotman et al. 2013). All three of these genes encode NBS-LRR proteins. *Vat* confers resistance to infestation by *A. gossypii* and to *A. gossypii*-mediated viruses, and encodes a CC-NBS-LRR protein (Dogimont et al. 2014). All of these genes are dominant. The genome sequence of melon (Garcia-Mas et al. 2012) includes 81 putative disease resistance genes which encode proteins with NBS, LRR, or TIR domains, but none of those genes are localized near the locus that we detected for CCYV resistance, consistent with the result that CCYV resistance is recessive.

Most recessive resistance genes isolated to date are the eukaryotic translation initiation factors (eIF) 4E and eIF4G and their isoforms ("eIF4Es") (Hashimoto et al. 2016). eIF4Es-mediated resistance has been reported in melon as well. The recessive *nsv* confers resistance to *Melon necrotic spot virus*, and encodes an eIF4E (Nieto et al. 2006). We searched for (putative) eIF4E and eIF4G in the DHL92 melon reference genome in the Cucurbit Genomics Database but did not find any at the locus for CCYV resistance that we

Table 1 QTL for CCYV resistance in F_2 plants

Flanking markers	Position (cM)	Maximum LOD score	MaximumLOD position (cM)	Additive effect[a]	R^2 (%)
F_2 ('Harukei No. 3' × JP 138332)					
M0007–M0038	2.2–24.5	12.9	15.2	− 0.90	46.5
F_2 ('AnMP-5' × JP 138332)					
M0015–M0038	8.8–24.4	25.5	15.8	− 1.15	50.0

[a]Additive main effect of the parent contributing the higher-value allele; negative values indicate that higher-value alleles come from JP 138332

Table 2 Relationship between marker genotype near major QTL and average disease severity index (DI) scores in CCYV-inoculated F_2 plants

Cultivar and populations	No. of plants	Genotype[a] at marker ECM230	Average DI of 6th to 15th leaves[b]
JP 138332	12	AA	0.0
'Harukei No. 3'	12	BB	2.5
F_1 ('Harukei No. 3' × JP 138332)	12	AB	2.8
F_2 ('Harukei No. 3' × JP 138332)	10	AA	1.6[a]
	28	AB	3.0[b]
	41	BB	2.7[b]
JP 138332	12	AA	0.0
'AnMP-5'	12	BB	3.6
F_1 ('AnMP-5' × JP 138332)	12	AB	4.0
F_2 ('AnMP-5' × JP 138332)	25	AA	1.2[a]
	27	AB	3.6[b]
	61	BB	3.6[b]

[a]AA, homozygous for JP 138332 allele; AB, heterozygous; BB, homozygous for 'Harukei No. 3' or 'AnMP-5' allele

[b]Values followed by the same letter are not significantly different at the 5% level by Tukey–Kramer HSD test

detected (data not shown). The recently identified recessive *cmv1*, which confers resistance to *Cucumber mosaic virus* subgroup II strains in melon accession PI 161375 (Giner et al. 2017), encodes vacuolar protein sorting 41 (VPS41) protein, which is involved in membrane trafficking to the vacuole as part of the homotypic fusion and vacuole protein sorting (HOPS) complex. We searched for VPS proteins in the melon reference genome and found that one gene (*MELO3C018764*, encoding vacuolar protein sorting–associated protein 18-like) has been mapped on Chr. 1, although not near the QTL for CCYV resistance.

The plants of susceptible cultivars show symptoms on upper leaves about 30 days after inoculation with CCYV, therefore, it is difficult to select the resistant plants at young seedling stage by inoculation assay in CCYV resistant breeding. MAS enables us to select resistant plants at young seedling stage and to develop resistant cultivars more rapidly. In this study, we demonstrated that SSR marker linked to the major QTL for CCYV resistance is effective for selection of CCYV-resistant plants. Further studies are needed to identify CCYV resistance genes. Our results will accelerate gene identification and the development of CCYV-resistant cultivars through the use of marker-assisted selection.

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Compliance with ethical standards

Conflict of interest The authors of this study declare that there is no conflict of interest.

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