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QTL Mapping of Downy Mildew Resistance in an Introgression Line Derived from Interspecific Hybridization Between Cucumber and *Cucumis hystrix*

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Abstract

Downy mildew (DM), caused by *Pseudoperonospora cubensis* (Berk. & M.A. Curtis) Rostovzev, is a worldwide major disease of cucumbers (*Cucumis sativus* L.). By screening 10 introgression lines (ILs) derived from interspecific hybridization between cucumber and the wild *Cucumis*, *C. hystrix*, through a whole plant assay, one introgression line (IL52) was identified with high DM-resistance. IL52 was further used as a resistant parent to make an F_2 population with 'changchunmici' (susceptible parent). The F_2 population (300 plants) was investigated for DM-yellowing, DM-necrosis and DM-resistance in the adult stage. A genetic map spanning 642.5 cM with 104 markers was constructed and used for QTL analysis from the population. Three QTL regions were identified on chromosome 5 and chromosome 6. By interval mapping analysis, two QTLs for DM-resistance were determined on chromosome 5 (DM_5.1 and DM_5.2), which explained 17.9% and 14.2% of the variation, respectively. QTLs for DM-yellowing were in the same regions as DM-resistance. For DM-necrosis, by interval mapping analysis, one QTL was determined on chromosome 5 (Necr_5.1) that explained 18.3% of the variation and one on chromosome 6 (Necr_6.1) that explained 13.9% of the variation. Our results indicated that the identification of molecular markers linked to the QTLs could be further applied for marker-assisted selection (MAS) of downy mildew resistance in cucumber.

Introduction

Downy mildew caused by *Pseudoperonospora cubensis* (Berk. & M.A. Curtis) Rostovzev is one of the most severe foliar disease of cucumber (*Cucumis sativus* L., 2n = 14) production worldwide. Yearly downy mildew epidemics threaten cucumber production in up to 80 countries, causing significant economic losses (Colucci et al. 2006; Olczak-Woltman et al. 2011). Among the different strategies used to control the disease, including cultural practices, intercropping, application of chemicals and sowing pathogen-free seeds, the development and deployment of resistant cultivars are generally considered the best approach for controlling downy mildew. However, the appearance of new physiological races resulted in loss of resistance in resistant cultivars: the dm-1 gene has not been effective since 2004 (Call et al. 2012). Thus, new DM-resistance genes are urgently required to ensure cucumber production.

The inheritance of cucumber resistance to downy mildew has been studied for the past 70 years (Criswell et al. 2008). However, the inheritance pattern is so complicated that, until now, there have been conflicting views as to whether the inheritance is monogenic or polygenic. Using different plant materials, it was reported that a single recessive gene (dm) controlled resistance in cucumbers (Van Vliet and Meysing 1974; Fanourakis and Simon 1987; Angelov 1994). On the other hand, three recessive resistance genes were first reported by Shimizu (1963) and were later repeated by McFerson (1978)

and Doruchowski and Łąkowska-Ryk (1992). Zhang et al. (2013) mapped five putative QTLs controlling downy mildew on chromosomes 1,5 and 6 using an F_2 population derived from the cross K8×K18. The different results regarding the inheritance of downy mildew resistance in cucumbers is likely due to different mechanisms used (Jamal-Ali et al. 2011). Van Vliet and Meysing (1974, 1976) used sporulation intensity, Fanourakis and Simon (1987) used the incidence of chlorotic and necrotic lesions on cotyledons and Doruchowski and Łąkowska-Ryk (1992) used necrotic lesions. Thus, different methods of identifying and evaluating resistance may show different inheritance patterns and should be thoroughly tested.

Thus, to evaluate whether a cucumber cultivar is resistant or not, molecular markers could be better than using disease rating of the phenotype. Random amplified polymorphism DNA (RAPD) was used by several researchers to identify molecular markers linked to cucumber resistance to downy mildew (Horejsi et al. 2000; Ding et al. 2007). Bai et al. (2008) mapped three putative QTLs controlling downy mildew on linkage group 1 and 6, using a recombinant inbred line (RIL) population derived from a cross between S94 (North China type) and S06 (European type). The number of markers was so limited that it could not facilitate QTL studies until the cucumber genome was sequenced, from which hundreds of simple sequence repeat (SSR) markers have now been developed (Ren et al. 2009). SSR markers are easy and inexpensive to use. Moreover, single-nucleotide polymorphisms (SNPs) have greater utility than other marker types for their higher genotyping efficiency, data quality, genome wide coverage, analytical simplicity and cost-effectiveness (Morin et al. 2004). So far, SNP markers within <5 cM from the downy mildew resistance gene have not been identified in cucumber.

Cucumber is a crop with a narrow genetic base. Thus, introgression of traits of interest from wild species into cultivars is a highly desired goal in cucumber improvement. *C. hystrix* Chakr. (2n = 24) has been identified as a novel source for downy mildew resistance. In 1995, interspecific hybrids between *C. sativus* and *C. hystrix* were first produced and *C. hytivus* (2n = 38) was obtained through chromosome doubling of the interspecific hybrid (Chen and Kirkbride 2000), followed by backcrossing to cucumber (Chen et al. 1997, 2003; Chen and Kirkbride 2000). The resulting progeny were selected and selfed for several generations to produce a series of introgression lines with 14 chromosomes. One of these derived introgression lines, IL52, was identified as highly resistant to downy mildew, which was confirmed in the USA, Europe and China. IL52 could become an important resistance resource to deal with new DM races.

In the present study, we used a SNP- and SSR-based genetic linkage map using IL52 as the resistant donor line. We identified regions of the genome with QTLs for resistance to downy mildew and identified molecular markers linked to the QTLs. The results from this study will facilitate genetic improvement of downy mildew resistance in cucumber breeding programmes.

Materials and Methods

Plant materials

Two cucumber cultivars and 10 ILs derived from backcrossing the *C. hytivus to cucumber 'beijingjietou' were used.*

An F_2 population was developed from a cross between the downy mildew resistant IL52 and downy mildew susceptible cucumber 'changchunmici'. Three hundred F_2 individuals were obtained by selfing the F_1 progeny. Some of the F_2 plants were self-pollinated to produce F_2-derived F_3 families.

Evaluation of downy mildew responses

Two cucumber cultivars and 10 ILs were tested for downy mildew resistance in summer 2009 of North Carolina State University, USA. The F_2 population was tested in August 2010 in the field at Nunhems in the Netherlands. Evaluation of downy mildew resistance comprises yellowing, leaf necrosis and other general health traits. Leaf yellowing is a symptom of the fungal infection and depends on both infection spread and on plant growth rate. Leaf necrosis is the appearance of necrotic (dead) spots on the leaf, either as a symptom of infection or as a hypersensitive response from the plant. The third trait phenotyped was DM-resistance, which consists of a score based on the general health and symptom expression in the plant. DM-yellowing explains a major part of the DM-resistance phenotype, while DM-necrosis is a smaller component that can be seen both in conjunction and separate from DM-yellowing and DM-resistance. Phenotypes were assigned a score between 1 and 9: Yellowing: 1 = no yellowing; 9 = maximal yellowing, DM-resistance: 1 = no disease symptoms; 9 = maximal disease symptoms and necrosis: 1 = no necrosis; 9 = maximal necrosis.

DNA extraction and molecular marker analyses

Genomic DNA was extracted from young leaves, as described by Murray and Thompson (1980). Total DNA was kept in sterile water and visualized after electrophoresis in 1% agarose gels with 1×TAE buffer stained with ethidium bromide.

The F_2 from the changchunmici×IL52 mapping population was genotyped with SNPs using the KBiosciences Competitive Allele-Specific PCR SNP genotyping system (KASPar). The details on the principle and procedure of the assay are available at http://www.kbioscience.co.uk/reagents/KASP_manual.pdf and http://www.kbioscience.co.uk/download/KASP.swf. SNP markers were first selected in eight F_2 individuals based on polymorphism and distribution in the genetic map.

The SSR primers used in this study were selected from the genetic map of the cucumber genome produced by Ren et al. (2009). The 20-μl PCR volume contained 10.1 μl H_2O, 1.6 μl dNTP (2.5 mM), 4 μl 5 × Phire reaction buffer, 2 μl 10 × LC green, 0.05 μl SSR forward primer (100 μM), 0.05 μl SSR reverse primer (100 μM) and 0.2 μl Phire DNA polymerase. 2 μl of DNA template was added to each reaction, which contained between 5 and 10 ng/μl DNA. PCR cycling started with an initial phase of 30 s at 98°C; then 40 cycles of 5 s at 98°C, 7 s at 60°C and a 30 s elongation step at 72°C; and one cycle of 72°C for 5 min. Genotyping was performed using a LightScanner high-resolution melting (HRM) system. The programme comprised: start temperature 60°C, end temperature 95°C and hold temperature 57°C.

Linkage map construction and QTL analysis

JoinMap4.0® software (Van Ooijen 2006) was used to generate the linkage map. Segregation distortion at each marker locus was tested against the expected 1 : 2 : 1 or 3 : 1 ratios for the F_2 population using a chi-squared test. The Kosambi mapping function (Kosambi 1944) was used to construct the map with the following JoinMap parameters: Rec = 0.4, logarithm of odds (LOD) = 3.0 and Jump = 5.

The genetic linkage map was used to identify markers associated with QTLs that have an effect on resistance to downy mildew, using the computer program MapQTL®5.0 (Van Ooijen 2004). To map downy mildew resistance, the level was evaluated as quantitative data, using classes 1–9 as numerical values. The analysis initially used interval mapping (IM) to identify putative QTLs. Markers located in the vicinity of the QTL were selected as the initial set of cofactors. The multiple-QTL model mapping (MQM) method was used to locate QTLs more precisely, using automatic cofactor selection. A permutation test was applied to each data set (1000 permutations) to determine the LOD thresholds. The integrated genetic linkage map was drawn using MapChart2.2 software (Voorrips 2002).

Results

Identification of ILs with downy mildew resistance

Ten ILs and two cucumber cultivars were tested for downy mildew resistance in summer 2009 in North Carolina State University. Cultivars NCSUM21 and WisSMR18 were used as the resistant and susceptible control, respectively (Table 1). Following three phenotypic identification methods: yellowing, leaf necrosis and general health traits, IL52 was regarded as highly resistant. In addition, IL 52 was then used as the resistant parent to make the F_2 population with 'changchunmici'.

Phenotypic variation of downy mildew resistance in the F_2 mapping population

Downy mildew resistance was scored in 293 individuals of the F_2 population. Nine phenotypic classes were identified, based on the level of infection. The symptoms of the different disease classifications of downy mildew leaves necrosis rating are shown on the left and downy mildew leaves rating are shown on the right (Fig. 1). The F_2 population showed a continuous distribution from resistance to susceptible, suggesting polygenic control of DM-resistance (Fig. 2). The dis-

Table 1 Estimate of disease rate in cultivar and introgression lines (ILs) in summer 2009

Code	DM	YEL	NEC
NCSU M21	4.3	2.0	3.8
IL52	4.7	2.0	4.3
IL08	4.8	6.0	2.0
IL16	4.9	3.0	5.0
IL06	5.1	3.0	3.0
IL15	5.3	2.0	3.0
IL02	5.3	6.0	5.0
IL07	5.5	4.0	5.0
IL03	5.6	6.3	4.3
IL09	6.1	6.5	5.5
WisSMR18	6.8	7.3	7.0
IL01	7.0	5.5	7.3

DM = DM-health, NEC = DM-necrosis, YEL = DM-yellowing. NCSUM21 was used as the resistant control, WisSMR18 as the susceptible control.

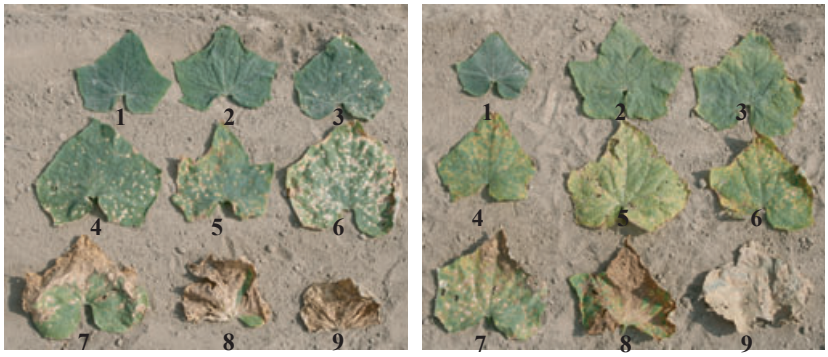

Fig. 1 The symptom of different disease classification on downy mildew leaves necrosis scoring (left) and downy mildew leaves scoring (right). Number under each leaf corresponds to the scoring (1–9).

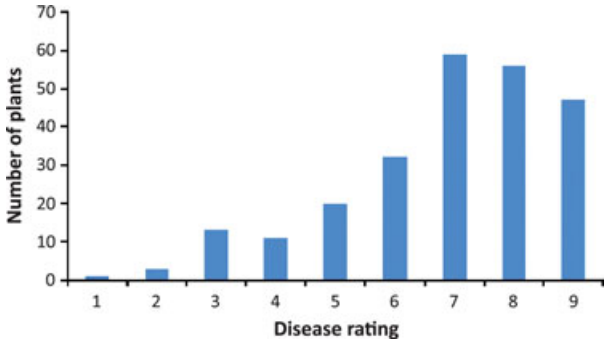

Fig. 2 Phenotypic distribution of DM-resistance in the F_2 mapping population derived from the cross between changchunmici (susceptible) × IL52 (resistance) 1 = no disease symptoms; 9 = maximal disease symptoms.

ease rating of the phenotype was confirmed for resistance to downy mildew from the F_3 families obtained by selfing each F_2 plant.

Linkage map construction

Construction of the genetic linkage map from the set of 300 F_2 plants developed from a cross between 'changchunmici' and IL52, comprising 100 SNP markers and four SSR markers. The SNP markers were selected from 1656 SNP, according to the SNP map from Nunhems, the Netherlands. The SSR markers were genotyped by high-resolution melting analysis, which divided them into three curves. The different coloured curves represent different genotypes (Fig. 3).

The map comprised seven chromosomes and spanned a genetic distance of 642.5 cM, with an average distance of 6.2 cM among markers (Fig. 4). The size of the chromosomes and the number of markers assigned to the different chromosomes varied between 61.6 cM for chromosome 7 (nine loci) and 105.2 cM for chromosome 3 (20 loci). The number of markers in these chromosomes ranged from nine (chr7) to 21 (chr5), with an average of 15 markers per chromosome. A detailed description of this map is provided in Table 2.

QTL mapping of downy mildew resistance

The genetic map was used to perform QTL analysis for yellowing, necrosis and general health traits for

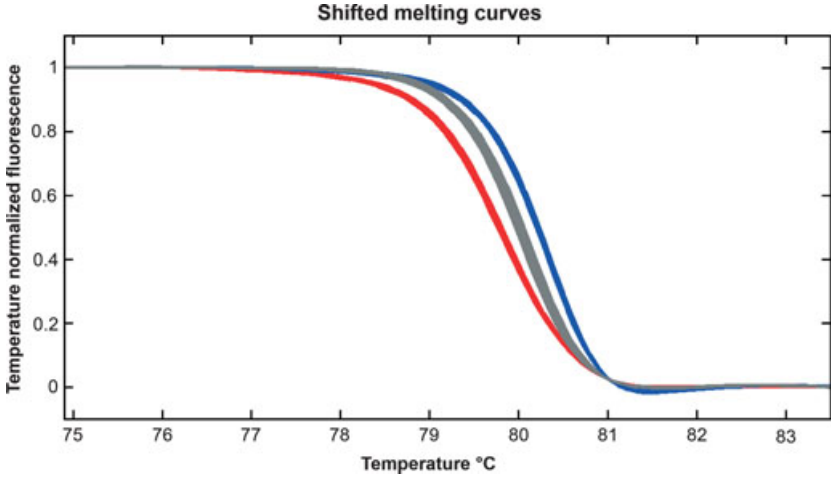

Fig. 3 One SSR marker was genotyped in part of F_2 population by high-resolution melting (HRM) analysis.

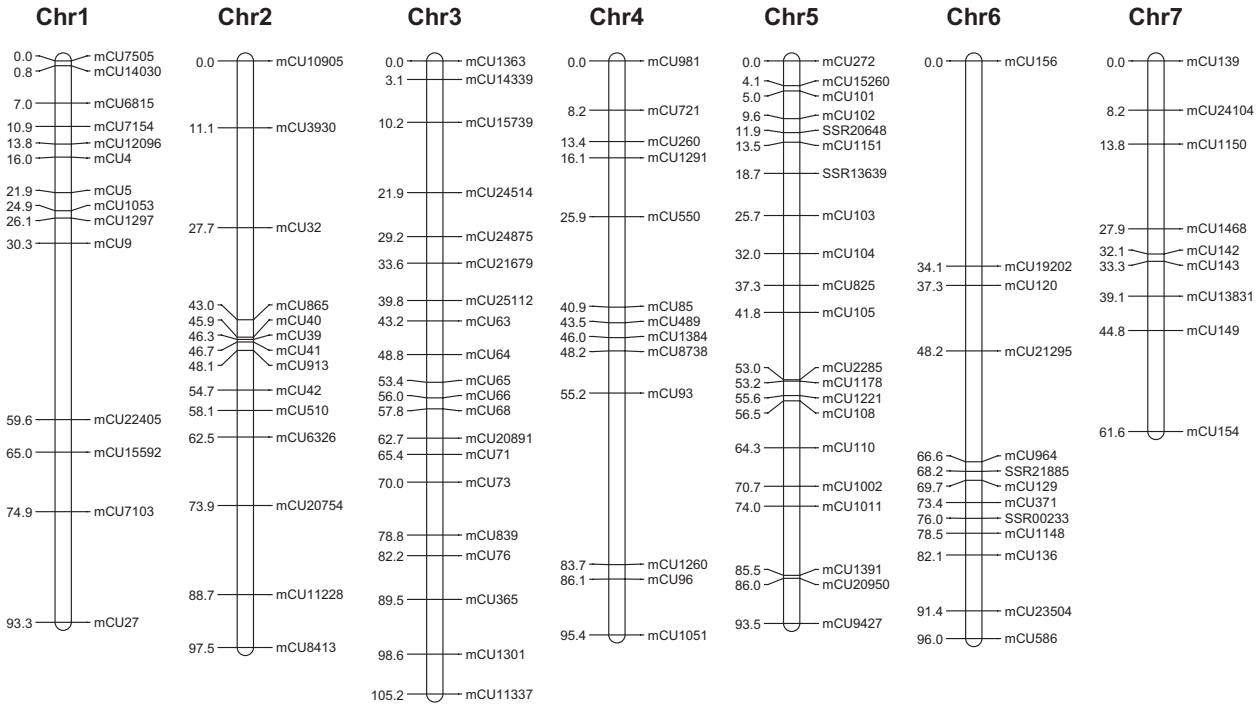

Fig. 4 Genetic linkage map based on the F_2 population derived from the cross changchunmici×IL52. The marker name and map distances in centi-Morgans (cM) are indicated on the *right* and *left* side of the chromosome.

Table 2 Distribution of molecular markers among seven chromosomes established on a genetic linkage map using an F_2 population derived from the cross changchunmici×IL52

Chromosome	Number of markers	Average interval (cM)	Chromosome size (cM)
1	14	6.7	93.3
2	14	7.0	97.5
3	20	5.3	105.2
4	13	7.3	95.4
5	21	4.4	93.5
6	13	7.4	96.0
7	9	6.8	61.6

downy mildew. After a first round of IM, markers with the highest LOD value were used as cofactors for MQM in the MapQTL®5.0 software. Both IM and MQM approaches detected the same QTLs. IM analysis for DM-resistance identified two QTLs on chromosome 5 (DM_5.1 and DM_5.2). DM_5.1 and DM_5.2 were supported by LOD scores of 9.66 and 7.48 and explained 17.9 and 14.2% of the variation, respectively. Two QTLs for DM-yellowing were located in the same regions as DM-resistance, showing LOD scores (15.04, 7.25) and explaining 26.8% and 14% of the variation, respectively. For necrosis, one QTL was determined on chromosome 5 (Necr_5.1) that explained 18.3% of the variation and one on chromosome 6 (Necr_6.1) that explained 13.9% of the variation (Fig. 5). A permutation test showed that the LOD threshold was 3.4 to obtain 90% confidence of detecting a putative QTL. Biometrical characteristics of the QTLs are presented in Table 3.

Discussion

Cucumis hystrix Chakr. (2n = 24, HH) is a wild *Cucumis* species having valuable traits with considerable potential for cucumber improvement. One IL was identified as highly resistant downy mildew among 10 introgression lines. This paper is the first report of QTL mapping of downy mildew resistance in a cucumber introgression line derived from a cross with *C. hystrix*. Three QTL regions were found in the introgression line IL52, which could be used for pyramiding of downy mildew resistance to provide durable protection.

Two different patterns of inheritance have been reported for resistance to downy mildew in cucumber: qualitative resistance and quantitative resistance. This may be explained by certain factors, for example, the different identification and evaluation methods used in previous studies. In this study, three disease identification methods were used to evaluate the F_2

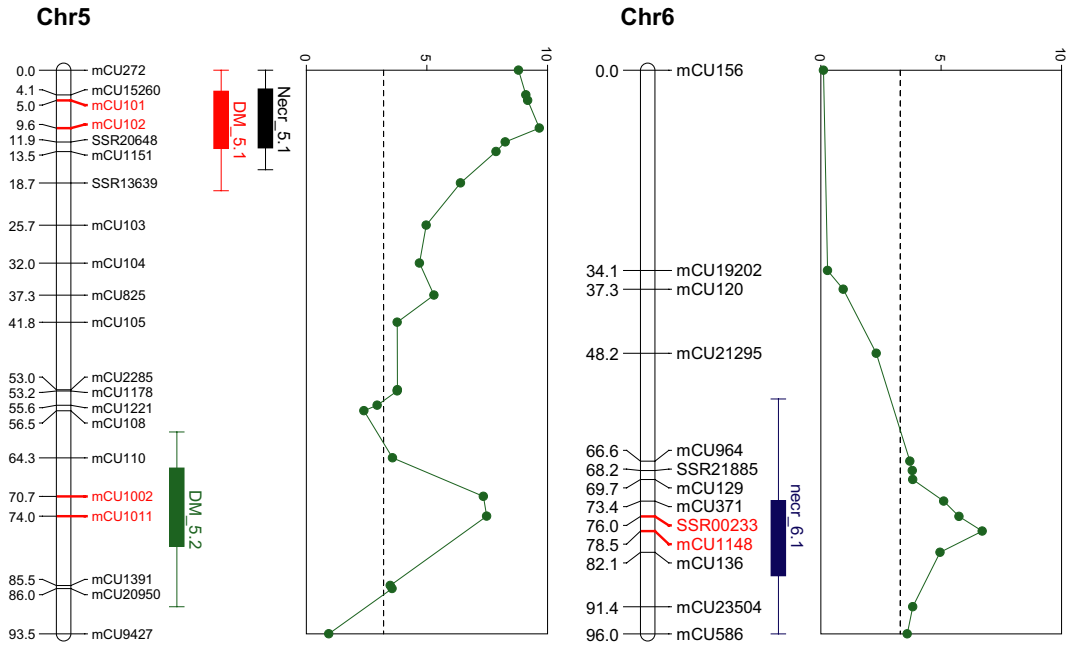

Fig. 5 Genetic map of chromosome 5 and 6 with QTL intervals and graphs. Genetic map: red marker = two markers with highest linkage. QTL intervals: Bar = 2-LOD interval, line = 4 LOD interval. QTL graph: dotted line = $P_{0.95}$ 1000 permutation genome wide confidence interval. Datapoints = LOD value per marker.

Table 3 Biometrical parameters of QTLs identified showing linkage groups, position of QTLs, LOD scores, phenotypic variation explained and the most significant marker associated with disease resistance phenotypes

QTL	Chromosome	Position (cM)	Marker	LOD	%Explained
YEL-5.1	5	0–25.7	mCU102	15.04	26.8
YEL-5.2	5	64.3–85.5	mCU1002	7.25	14
DM-5.1	5	0–25.7	mCU102	9.66	17.9
DM-5.2	5	64.3–85.5	mCU1011	7.48	14.2
Nec-5.1	5	0–25.7	mCU102	9.11	18.3
Nec-6.1	6	69.7–91.4	mCU1148	6.71	13.9

The markers are those of highest LOD scores and variation explained of the respective QTL. DM = DM-health, Nec = DM-necrosis, YEL = DM-yellowing, Position = map position in cM, LOD = log. Likelihood of marker-trait linkage.

population: yellowing, necrosis and resistance. This may have made the results more reliable and accurate. An investigation by Oerke et al. (2006) showed that as infection progresses, the chlorotic with expand and may become necrotic. Other researchers showed the occurrence of necrosis and chlorosis was highly correlated, indicating that these two phenotypes may be a manifestation of the same genetic mechanism (Criswell et al. 2008). In the present study, one QTL was just determined on chromosome 5 in the same region for yellowing and necrosis and for DM-resistance. This may be caused by a hypersensitive response from the plant, because necrosis is highly linked to DM-resistance. One QTL was found on chromosome 6 only for necrosis, but this QTL is neither linked to DM-resistance nor yellowing, so perhaps simply causes necrosis.

The whole cucumber genome was sequenced in 2009, from which hundreds of SSR markers have since been developed. Using these markers, several genes and QTLs have been mapped (Zhang et al. 2010; Li et al. 2011; Miao et al. 2011). In the present study, four SSR markers were mapped in the genetic map successfully (Fig. 2). Two markers are located on chromosome 5 and two on chromosome 6. The four SSR markers were genotyped by HRM analysis rather than the conventional method. In the previous study, electrophoresis through a 6% polyacrylamide gel was used. However, HRM is a rapid procedure for characterization of sequence differences immediately following PCR amplification. It has been used to successfully genotype single-nucleotide polymorphisms (SNPs) by Michael et al. 2004. In this paper, we adapted this system to successfully genotype single sequence repeats.

In general, quantitative resistance, which is under polygenic control, is more durable than resistance conferred by a single dominant gene (Kelly and Vallejo 2006). However, many factors affect whether a QTL is detected or not, because the LOD score of QTL

mapping may be affected by population size, distribution of markers associated to a putative QTL, QTL effects and error variance (Li et al. 2008). Previously, five QTLs for resistance to downy mildew were mapped to chromosomes 1, 5 and 6 using the F_2 and $F_{2:3}$ families derived from the cross K8 × K18 (Zhang et al. 2013). In this study, three putative QTLs were detected on chromosomes 5 and 6 in the introgression line 52. Nevertheless, comparative analysis of the QTLs detected in the two studies may not be successful because of two factors. One is the different populations: the resistance parent is a cucumber introgression line that was originally produced from a cross with *C. hystrix*, which is different from t K8. The other factor is the lack of common molecular markers. Therefore, more SSRs should be tested to integrate the SNP map and the SSR map. Although our results could not be compared with previous results, one QTL in the region of DM_5.2 was identified using another F_2 population from the same parents ('changchunmici' and IL52). The population was screened under greenhouse conditions and genotyped by SNP markers. The QTL is in almost the same position and explains a similar amount of observed variation (Zhou 2010).

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