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ORIGINAL PAPER

Y. Sakata · N. Kubo · M. Morishita · E. Kitadani · M. Sugiyama · M. Hirai

QTL analysis of powdery mildew resistance in cucumber (*Cucumis sativus* L.)

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Abstract A population of F_7 recombinant inbred lines (RILs) was made from a cross between susceptible ('Santou') and resistant (PI197088-1) lines of cucumber in order to study powdery mildew resistance loci. Susceptibility to powdery mildew in the F_7 RIL individuals showed a continuous distribution from susceptible to resistant, suggesting that powdery mildew resistance is controlled by quantitative trait loci (QTLs). A QTL analysis identified two and three loci for powdery mildew resistance under 26 and 20°C conditions, respectively. One QTL was found in the same position under both temperature conditions. Therefore, it is more likely that one major QTL acts under both temperature conditions and that other QTLs are specific to the two temperature conditions. The above results suggest that the four QTLs are controlled in a different temperature manner, and that their combination played an important role in expressing a high level of resistance to powdery mildew in this cucumber population. Sequence-tagged site (STS) markers associated with each QTL were developed and would be useful for breeding a cucumber line with a high level of powdery mildew resistance.

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Y. Sakata and N. Kubo contributed equally to this work and are considered as first authors.

Y. Sakata · M. Sugiyama
Department of Fruit Vegetables, National Institute of Vegetables and Tea Science, Ano, Mie 514-2392, Japan

N. Kubo · M. Hirai (✉)
Graduate School of Agriculture, Kyoto Prefectural University,
Kyoto Prefectural Institute of Agricultural Biotechnology,
74 Oji, Kitainayazuma, Seika, Kyoto 619-0244, Japan
E-mail: mhirai@kab.seika.kyoto.jp
Tel.: +81-774-933526
Fax: +81-774-933528

M. Morishita
Department of Crop Breeding, National Agricultural Research Center for Hokkaido Region, Sapporo 062-0045, Japan

E. Kitadani
Department of Vegetable and Flower Research,
National Agricultural Research Center for
Kyushu Okinawa Region, Kurume 839-8503, Japan

Introduction

Powdery mildew caused by *Podosphaera xanthii* (formerly known as *Sphaerotheca fuliginea* Schlech ex Fr. Poll.) or *Golovinomyces cichoracearum* (formerly known as *Erysiphe cichoracearum* DC ex Mérat.) is one of the most serious diseases in cucumber (*Cucumis sativus* L.). These fungi belong to the same family (Erysiphaceae) and are often difficult to distinguish. To date, no race occurrence has been found for the above two fungi in cucumber, although this is not the case with the melon (Bardin et al. 1999). Although several commercial cultivars of cucumber show resistance to powdery mildew in field cultivation during summer, it is known that this resistance only acts under higher temperature conditions. The detailed mechanism of such temperature-dependent resistance remains unknown. As such, no resistant cultivar for greenhouse cultivation during the period from winter to spring exists to our knowledge. Several reports indicate the involvement of more than one gene for powdery mildew resistance in several cucumber accessions (see Pierce and Wehner 1990 for a review). Some linkages between the powdery mildew resistance locus and phenotypic markers have also been reported (Fanourakis and Simon 1987; Walters et al. 2001). However, no analysis has been undertaken on powdery mildew resistance genes using molecular markers. For this reason, no direct comparison between these genes has been made.

Previously, we developed a simple and improved method for evaluating powdery mildew resistance in cucumber. We found that accession PI197088-1,

originating from India, possesses the highest level of resistance among accessions of cucumber genetic resources (Morishita et al. 2002). PI197088-1 was segregated from the original accession PI197088, which was reported as a moderately resistant line (Zijlstra and Groot 1992). Because PI197088-1 is resistant to powdery mildew under both low (15–20°C) and high temperature conditions (< 26°C), it could be a good genetic material for breeding powdery mildew-resistant cucumbers. However, the detailed genetic nature of powdery mildew resistance is not yet well understood, although the involvement of recessive genes for this trait has been proposed (Morishita et al. 2003).

Here, we report on the analysis of powdery mildew resistance derived from this accession. We made a population of F_7 recombinant inbred lines (RILs) from a cross between the cultivar 'Santou' and PI197088-1, and identified multiple quantitative trait loci (QTLs) for powdery mildew resistance. Of these QTLs, one locus was effective under both high and low temperatures. However, the other loci were effective only under either high or low temperature. This result suggests a temperature-dependent manner of gene expression for powdery mildew resistance, and that their combination plays an important role for expressing higher resistance to the powdery mildew pathogen. We have developed sequence-tagged site (STS) markers closely linked to the detected QTLs. The mechanism of powdery mildew resistance and the effectiveness of our STS markers are discussed.

Materials and methods

Plant material and DNA extraction

A population of 94 F_7 RILs was made from a cross between the cultivar 'Santou' and PI197088-1, using the single-seed-descent of F_1 plants after six selfings. PI197088-1 is highly resistant to the powdery mildew pathogen *P. xanthii* (Morishita et al. 2002), whereas 'Santou' was used as a susceptible parent. Genomic DNA was isolated from both young F_7 seedlings and the two parental lines with the Nucleon PhytoPure DNA extraction kit (Amersham Biosciences, Piscataway, NJ, USA), and was used for marker analysis.

Powdery mildew resistance test

Resistance to *P. xanthii* in the two parental lines and F_7 populations were tested as described previously (Morishita et al. 2002) with some modifications. Briefly, six seeds for each RIL were sown in soil on a plastic tray. When the cotyledons were fully expanded, they were inoculated with the pathogen by a spray of spore suspension (5×10^5 spores per ml). This was followed by incubation under 26 or 20°C condition with a 16 h photoperiod for 10 days. The inoculum of *P. xanthii* used in this test originated from a monospore culture maintained by infection of the melon susceptible cultivar 'Earl's Favourite Harukei 3'. The disease index (DI) was classified into the following ten categories based on visual infection of the leaf: 0 = no or almost no symptom; 1 = faint spot; 2–3 = thin mat of mildew; 4–5 = thick mat of mildew; 6–7 = very thick mat of mildew; 8–9 = whole leaf surface coated with mildew. The DI from the average value of three independent tests to each line was used for subsequent analysis since the triplicated mildew tests showed essentially similar scores and distribution patterns.

Marker analysis

An amplified fragment length polymorphic (AFLP) analysis was performed as described by Vos et al. (1995) with slight modification. Briefly, genomic DNA was digested with *Eco*RI and *Mse*I (Invitrogen, Carlsbad, CA, USA), and simultaneously ligated to *Eco*RI and *Mse*I adapters with T4 DNA ligase (Invitrogen) at 37°C for 2 h. After inactivation of enzymatic activity at 65°C for 20 min, pre-amplification was performed with *Eco*RI and *Mse*I pre-selective primers as follows: pre-incubation at 94°C for 2 min, followed by 20 cycles of denaturation at 94°C for 20 s, annealing at 56°C for 30 s, and extension at 72°C for 2 min. The final extension was performed at 72°C for 2 min and then at 60°C for 30 min. Sixty-four combinations of selective amplification primers (eight fluorescence-labeled *Eco*RI primers × eight nonlabeled *Mse*I primers) were used to screen polymorphic bands. The products of selective amplification were analyzed with CEQ2000XL sequencer (Beckman Coulter, Fullerton, CA, USA).

Simple sequence repeats (SSRs) were isolated from a *Sau*3AI-digested genomic library of 'Santou' as described previously (Suwabe et al. 2002) (Table 1). Previously reported SSRs and sequence characterized amplified regions (SCARs) of cucumber and melon were also tested (Katzir et al. 1996; Horejsi et al. 1999; Danin-Poleg et al. 2001; Fazio et al. 2002; Gonzalo et al. 2005). Randomly amplified polymorphic DNAs (RAPDs) (Welsh and McClelland 1990; Williams et al. 1990) were screened with 96 types of decamer primers (Operon Biotechnologies, Huntsville, AL, USA). PCR conditions and detection of SSRs and RAPD markers are described elsewhere (Suwabe et al. 2002; Hirai et al. 2004).

Linkage and QTL analyses

Linkage analyses were performed with JoinMap 3.0 software (Van Ooijen and Voorrips 2001). Marker data were assigned to linkage groups (LGs) using a minimum logarithm of odds (LOD)-likelihood score of 4.0. The Kosambi map function (Kosambi 1944) was used

Table 1 Primer sequences of cucumber STS and SSR markers developed and mapped in this study

Marker	Core motif	Sense primer (5′–3′) Antisense primer (5′–3′)	Expected size (bp)
Cucumber STS			
EAACMCAC391-395STS		GAATTCAACCAAAAACCATAATCA ATATCAGGTCAAATCTATAATCCC	140[b]
EAAGMCAT280-282STS		AACACTCCTGCTTTAACAGCATC AATGTAATCGTCATTCAGCAGTGT	229[b]
EAAGMCTG171-179STS		GAATTCAAGGTTATTTTCTCATCA TTAACTGGCAAGCGTTCTTCTAAG	158[b]
EAAGMCAG154STS[a]		GAATTCAAGGGCAGTGGTGCAAC TTAACAGAGTCTCCTCACCCTGATTT	134[c]
EACAMCTG144-143STS		TTGAATAAGAATTCACATGCAT TAACTGGTGCTTTTGCATGTTCTG	129[b]
EAACMCTG116STS[a]		GAATTCAACTCATTCGAAAGTGTG TTAACTGTTGCTCATAAAAACTTC	95[b]
Cucumber SSR			
C31	$(GT)^{7}(GA)^{18}$	TTGATTTGAGTGTTTGAAATTGAG ATAGCTTCGTTGGCATTGACATT	201[c]
C35	$(AC)^{10}(TC)^{3}(AT)^{5}$ $N^{42}(TG)^{6}$	CCTCCTTCATCTCCCTTCGGTA CCATTTCTTATTATTGGAACCTCT	306[c]
C80	$(GT)^{7}(GA)^{18}$	TTGATTTGAGTGTTTGAAATTGAG ATAGCTTCGTTGGCATTGACATT	201[c]
C162	$(CT)^{13}$	CCTATGGCAACTTCGTCAGC TATCCTCCAATACAAAAACATACC	269[c]

[a]Dominant marker
[b]Size in PI197088-1
[c]Size in 'Santou'

to calculate the genetic distance between markers. An interval mapping analysis (Lander and Botstein 1989; Van Ooijen 1992) was conducted using the MapQTL 4.0 package (Van Ooijen et al. 2000) to detect QTLs. The region showing a LOD threshold value of 3.0 was treated as a putative QTL. In order to show the confidence intervals of map position for each QTL, one-LOD and two-LOD support intervals (Lander and Botstein 1989) were constructed, in which the LOD values are less than one and two from the maximum, respectively.

Cloning of AFLP fragments

The AFLP products were electrophoresed through a nondenatured 10% polyacrylamide gel, and visualized by silver staining. The band of expected size was cut from the gel, and the DNA was extracted as described by Metais et al. (2002). The isolated AFLP fragment was reamplified with selective amplification primers, cloned into a pCR-TOPO-XL vector (Invitrogen), and sequenced. The nucleotide sequences reported here have been deposited in the DDBJ/EMBL/GenBank databases under the accession nos. AB219081–AB219088. Primers were designed from the determined nucleotide sequences in order to amplify specific PCR products (Table 1). In case that nucleotide differences between 'Santou' and PI197088-1 were found at the border of the cloned fragment, a flanking sequence was obtained by TAIL-PCR (Liu and Whittier 1995).

Results

Segregation of powdery mildew resistance

The extent of resistance or susceptibility to powdery mildew was tested in F_7 RILs as well as their parental lines based on the visual inspection of the leaf. In 'Santou', the DI was 2.19 and 4.94 under 26 and 20°C conditions, respectively. However, PI197088-1 was immune (DI = 0.00 under both temperature conditions), as determined in our earlier report (Morishita et al. 2002). In the F_1 plant, the DI was 5.48 and 6.89 under 26 and 20°C conditions, respectively, confirming the involvement of recessive gene in this trait. The DI in the F_7 population showed a continuous distribution from resistant to susceptible phenotypes, without showing any typical segregation pattern (Fig. 1). This was irrespective of the two temperature conditions. When compared with the 26°C condition, the DI at 20°C increased in many F_7 lines. These results demonstrate that powdery mildew resistance in this accession is a quantitative trait, and is probably controlled by more than one gene.

QTL analysis of powdery mildew resistance

A linkage map was constructed with 154 molecular markers that mainly consisted of AFLPs but included 40 SSRs (27 from cucumber and 13 from melon), five SCARs, and three RAPDs. This map resulted in nine LGs spanning 533.3 cM (data not shown). Among these

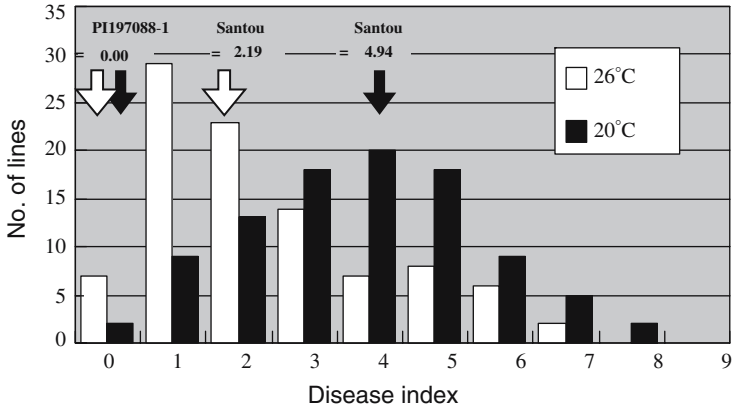

Fig. 1 Frequency distribution of disease incidence in the parental lines and F_7 populations. The disease index (*DI*) and number of lines are shown on the *x* and *y axes*, respectively. DI scores under 26 and 20°C conditions are represented by *open* and *filled bars*, respectively. DI scores of the parental lines 'Santou' and PI197088-1 are indicated by *white* and *black arrows* for 26 and 20°C conditions, respectively

LGs, a QTL analysis indicated that four genomic regions are involved in powdery mildew resistance. Two QTLs were detected on LGs I and II under the 26°C condition (Fig. 2, open bars). The QTL on LG II had the largest effect and explained 49.6% of the phenotypic variation of the powdery mildew resistance observed in the RILs (Table 2). The combination of the two QTLs on LGs I and II explained 72.4% of the observed phenotypic variation.

In contrast, three QTLs were found on LGs II, III, and IV under the 20°C condition (Fig. 2, filled bars). The combination of the three QTLs explained 56.9% of the observed phenotypic variation (Table 2). Among them, the QTL showing the largest effect on LG II seems to be the same locus as that detected under the 26°C condition because their peaks of LOD scores lay in the same position (Fig. 2, open and filled triangles).

It is interesting to note that the QTLs on LGs I and IV gave negative additive effects (Table 2), suggesting that the loci are derived from the 'Santou' allele. This assumption is also supported by the fact that 'Santou' showed slight resistance to powdery mildew as shown above (DI = 2.19 at 26°C and DI = 4.94 at 20°C). LG I corresponds to the LG 1 reported by Fazio et al. (2003) because of the map position of one SCAR (CSL18-3-SCAR) and two SSR markers (CSWCT16 and CSWGCA01). However, we failed to detect any apparent correspondence for the LGs II, III, and IV because of a paucity of anchor markers. Increasing the number of the anchor markers using the reciprocal mapping of common markers among researchers such as ourselves might provide clues for solving the origin of these QTLs.

Conversion of AFLP bands to STS markers

The AFLP bands associated with the QTLs were cloned for conversion to STS markers (Fig. 2, asterisks). Six AFLP fragments were successful for cloning. A database search analysis revealed that two kinds of AFLP fragments, EACAMCTG144-143 and EAAGMCAT280-282, are homologous to part of the bacterial artificial chromosome (BAC) end sequence of *Lotus corniculatus* (accession no. AG237935) and the TAT-binding protein TBP1 homolog from several plant species, respectively. The other four kinds of fragments did not show significant homology to any known sequence deposited in the databases. Of the six cloned fragments, we were able to convert four markers to codominant STS markers, termed EAACMCAC391-395STS, EAAGMCAT280-282STS, EAAGMCTG171-179STS, and EACAMCT-G144-143STS (Table 1 and Fig. 3). The other two markers (EAAGMCAG154STS and EAACMCT-G116STS) resulted in dominant markers specific to the 'Santou' and PI197088-1 alleles, respectively (Table 1). Of these, EAACMCAC391-395STS and EAACMCT-G116STS were located on the peak of the LOD score on LGs II and IV, respectively (Fig. 2).

Discussion

There have been several studies on the construction of cucumber linkage maps using molecular markers (e.g., Kennard et al. 1994; Danin-Poleg et al. 2000; Park et al. 2000; Bradeen et al. 2001; Fazio et al. 2003). Although some disease resistance loci are mapped on these molecular linkage maps, no such approach has been reported for powdery mildew resistance. In the present study, we made a linkage map in order to identify QTLs for powdery mildew resistance using RILs derived from a cross between 'Santou' and PI197088-1. The present map did not result in seven LGs expected from the chromosome number of cucumber, and the total map length was slightly shorter than that from the estimated genome size (750–1000 cM) (Staub and Meglic 1993). However, we infer that our map covers most of the cucumber genome because the mean marker interval of our map (3.5 cM) is comparable to that of the previous maps, and because no ungrouped marker remained during the present map construction. We successfully localized tens of sequence-specific markers such as SSRs and SCARs on this map. Unfortunately, they did not merge well with other cucumber genetic maps, except for the LG I (Fig. 2), because of paucity of common anchor markers (data not shown). Although the main objective

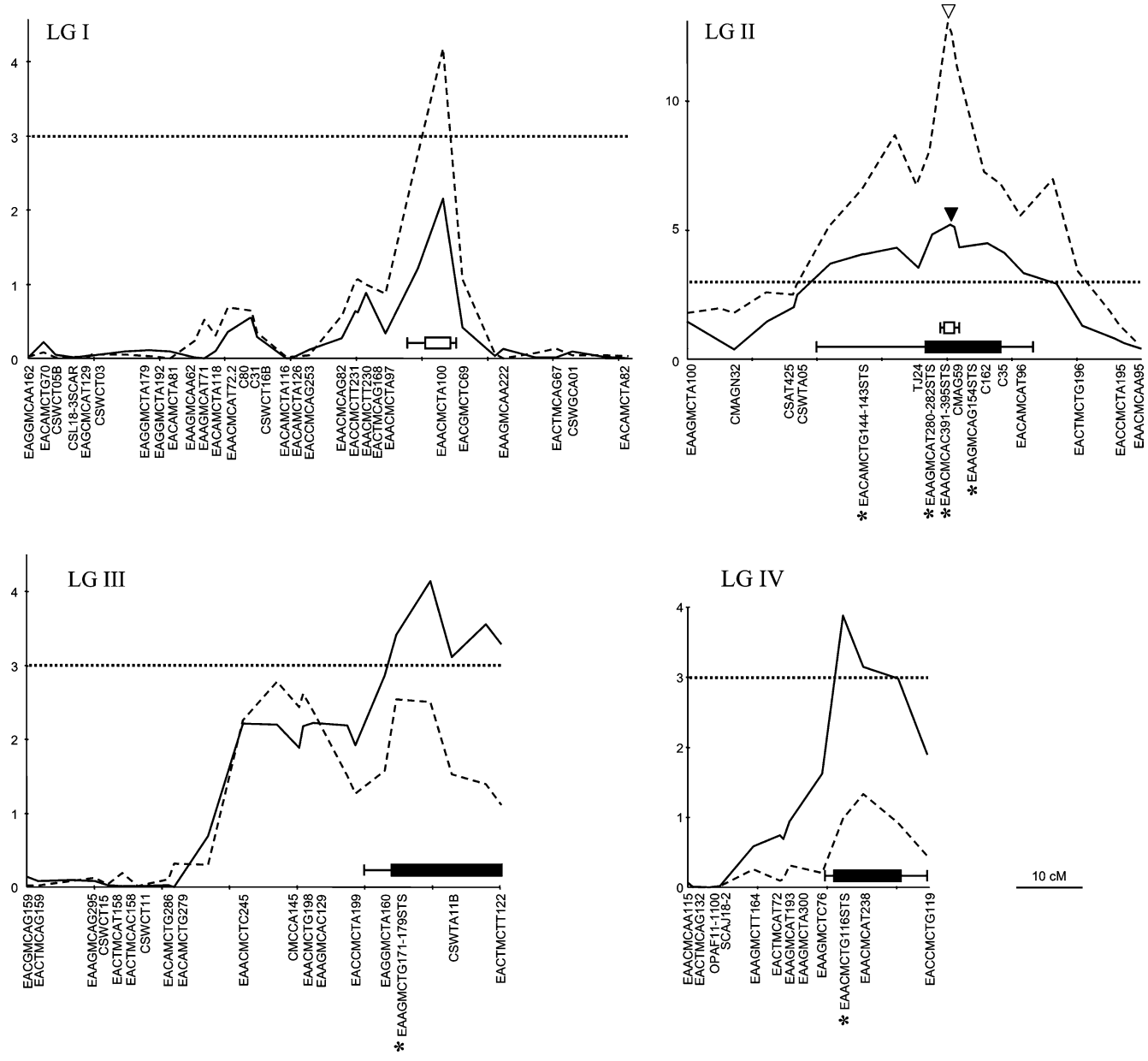

Fig. 2 Positions of QTLs for powdery mildew resistance in the linkage map derived from the F_7 population of 'Santou' × PI197088-1. Only linkage groups (*LGs*) holding the QTLs are shown. The map positions of each marker are given in cM on the *x*-axis. STS markers developed in this study are marked by *asterisks* (*). AFLP and RAPD markers are indicated by *E_M_* and *OP_*, respectively. Others are SSR and SCAR markers of cucumber and melon (this study; Katzir et al. 1996; Horejsi et al. 1999; Danin-Poleg et al. 2001; Fazio et al. 2002; Gonzalo et al. 2005). LOD scores are shown on the *y*-axis. The LOD threshold of 3.0 is indicated by a *horizontal dotted line*. The QTL-likelihood profiles under 26 and 20°C conditions are indicated by *broken* and *solid lines*, respectively. One-LOD support intervals under the 26 and 20°C conditions are shown by *open* and *filled bars*, respectively. Two-LOD support intervals that bound the QTL with ~95% confidence (Van Ooijen 1992) are shown by *outer bars*. For the QTL on LG II, the peaks of LOD score under 26 and 20°C conditions are indicated by *open* and *filled triangles*, respectively

of this study was to detect QTLs for powdery mildew resistance, and development of STS markers closely linked to this trait, improvement of the present map will be more informative for future genetic studies in cucumber.

Powdery mildew resistance genes have been studied in a number of plant species such as cereals, legumes, Solanaceae, and Rosaceae. In particular, extensive analyses have been concentrated on barley, wheat, and *Arabidopsis*. To date, a number of resistance alleles have been found in barley, in which qualitative and quantitative resistance loci have been identified (Backes et al. 2003). This observation suggests the presence of multiple mechanisms for powdery mildew resistance (Hückelhoven and Kogel 1998). In fact, resistant alleles in *Mla*, some of which have been isolated using map-based cloning (Halterman et al. 2001; Zhou et al. 2001), would mediate programmed cell death, the so-called hyper-

Table 2 QTL analysis of powdery mildew resistance in cucumber

Condition	Marker[a]	LG	Position[b]	LOD	R^2 [c]	Add[d]
26°C	EAACMCTA100	I	63.3	4.18	22.8	−1.048
26°C	EAACMCAC391-395STS	II	40.1	12.99	49.6	1.258
20°C	EAACMCAC391-395STS	II	40.1	5.22	23.2	0.868
20°C	EAAGMCTG171-179STS	III	54.7	3.42	15.6	0.693
20°C	EAACMCTG116STS	IV	22.3	3.88	18.1	−0.752

[a]The marker on the peak or in the vicinity of the peak of the LOD score
[b]Position of the marker in the linkage group (*LG*) in cM
[c]Percentage of phenotypic variation explained
[d]Additive effect of QTLs of the PI197088-1 allele

sensitive response (HR). In contrast, the *Mlg* gene seems to mediate the arrest of fungal development in papillae. Unlike such well-characterized, race-specific, dominant-acting resistance genes, broad-spectrum resistance to powdery mildew is conferred by a recessive loss-of-function *mlo* gene (Büschges et al. 1997). Little is presently understood about recessive resistance genes because of the few cases of gene cloning studied (see Chu et al. 2004 and references therein). In cucumber, many of the powdery mildew resistance genes, including those in this study, are controlled by recessive genes. The detailed mechanism of these recessive genes is unknown, although our previous investigation showed tiny HR-like spots on the leaf surface of the resistant line PI197088-1 after inoculation of the pathogen (Morishita et al. 2003). Severe decrease in the number of haustorium and retardation of hyphal growth was also observed on the leaf surface of PI197088-1. Therefore, the powdery mildew resistance of PI197088-1 may involve retardation of hyphal growth and development after the penetration of epidermal cells by the pathogen. In addition, we found chlorosis-like spots on the leaf surface of some F_7 lines (data not shown). However, we failed to identify any apparent correlation between powdery mildew resistance and leaf chlorosis, unlike an earlier report (Zijlstra et al. 1995). This is probably because of the

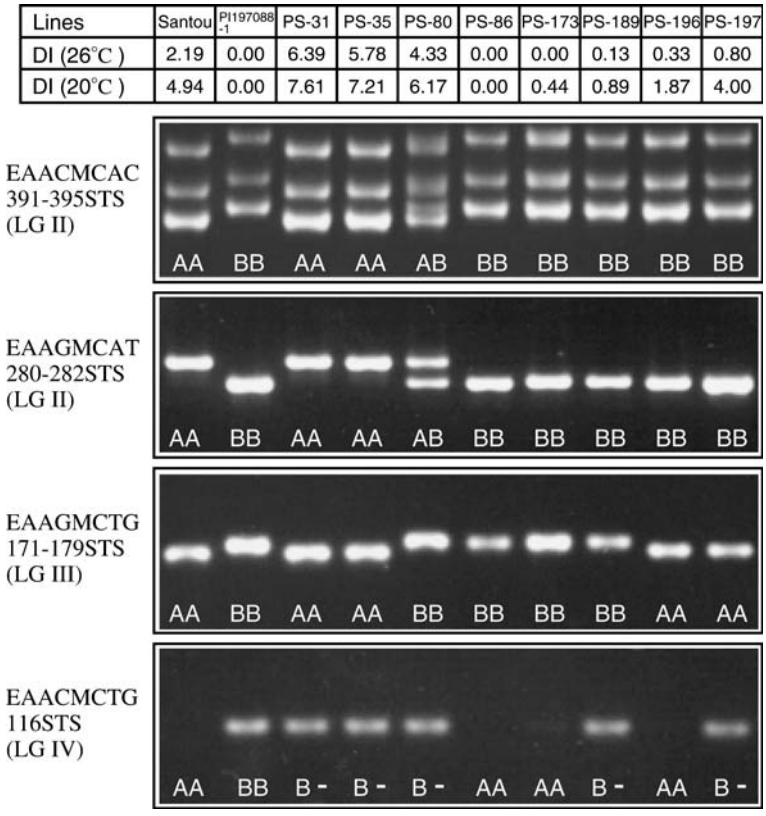

Fig. 3 The band patterns of STS markers linked to powdery mildew resistance QTLs developed in this study. Examples of four STS markers for several lines are shown. Names of lines and disease indexes (*DIs*) under 26 and 20°C conditions are indicated above the *columns*. Marker names and their linkage groups (*LGs*) are shown at *left*. Genotypes are indicated within the *column*, where 'Santou' and PI197088-1 alleles are represented as "*A*" and "*B*", respectively. In the case of EAACMCTG116STS, the genotypes in lines PS-31, PS-35, PS-80, PS-189, and PS-197 are indicated as "*B*–" because one allele is unknown because of a dominant marker

differences in the plant materials used and the nature of leaf chlorosis, which is greatly affected by environmental conditions.

We found four QTLs in this study. Of these, two loci were derived from PI197088-1, and the other two were derived from 'Santou'. Our results also suggest that these QTLs are controlled differently. Although the locus on LG II seems to be active under both high and low temperatures, the relative effect may decrease under low temperature since RILs having only this QTL decreased their resistance under low temperature (e.g., Fig. 3, the line 197). A QTL on LG I was effective under high temperature and lost its activity under low temperature. However, the relative effects of QTLs on LGs III and IV increased under low temperature. The above results suggest that the QTLs act in a temperature-dependent manner. Temperature-dependent resistance has been reported in many cases in other crops, but the detailed molecular mechanism is unclear. Upon cloning of barley *Mla* and *mlo* genes (Büschges et al. 1997; Halterman et al. 2001; Zhou et al. 2001), no temperature-dependent cascade has been reported. The loci we found could provide examples for studies on temperature-dependent resistance in plant pathology.

The QTL analysis under the high temperature condition showed that the locus on LG II is most effective. This QTL is derived from the PI197088-1 allele. Thus, resistance of the present resistant parent may often look monogenic. Indeed, our previous results suggest that resistance is controlled by a single recessive gene under the 26°C condition (Morishita et al. 2003). On the other hand, the present study provides evidence that the combination of the multiple loci could play an important role in expressing the high level of resistance to powdery mildew under low temperature. Our preliminary segregation analysis suggested that two genes confer powdery mildew resistance in PI197088-1 under low temperature (Morishita et al. 2003). The result obtained in the present study is in good agreement with the above assumption because two QTLs derived from PI197088-1 alleles were detected (Table 2). However, the RILs with these two resistance QTLs were not as immune as the parent except for one RIL (Fig. 3, the line 86). We took a region with a LOD < 3 as a QTL in the present study, but cannot rule out the possibility that additional QTL(s) with minor effects could play a role in powdery mildew resistance under low temperature. Further studies may be necessary to clarify this issue. In any case, because of the presence of more than one resistant locus, breeding of cucumber cultivars resistant under lower temperature seems to be difficult without suitable genetic markers. The QTLs we identified here were assigned by STS markers, most of which are codominant and located at the top of the LOD scores in each QTL. Therefore, they would be useful for marker-assisted selection to breed a cucumber line with a high level of powdery mildew resistance. By using these STS markers, a program for breeding cucumber varieties with a high level of powdery mildew resistance is being undertaken.

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