Breeding Science 71: 326–333 (2021)
doi: 10.1270/jsbbs.20141

Research Paper

Identification of quantitative trait loci for powdery mildew resistance in highly resistant cucumber (*Cucumis sativus* L.) using ddRAD-seq analysis

Koichiro Shimomura*[1)], Mitsuhiro Sugiyama[1)], Yoichi Kawazu[1)] and Yosuke Yoshioka[2)]

[1)] *Institute of Vegetable and Floriculture Science (NIVFS), National Agriculture and Food Research Organization (NARO)*, Kusawa 360, Ano, Tsu, Mie 514-2392, Japan

[2)] *Faculty of Life and Environmental Sciences, University of Tsukuba*, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8572, Japan

Powdery mildew, caused by *Podosphaera xanthii* (syn. *Sphaerotheca fuliginea* ex Fr. Poll.), is one of the most economically important foliar diseases in cucumber (*Cucumis sativus* L.). Cucumber parental line 'Kyuri Chukanbohon Nou 5 Go', developed from weedy cucumber line CS-PMR1, is highly resistant to powdery mildew and is promising breeding material. We performed quantitative trait locus (QTL) analysis using double-digest restriction-site-associated DNA sequencing (ddRAD-Seq) in a population from a cross between 'Kyuri Chukanbohon Nou 5 Go' and the Japanese native cultivar 'Kaga-aonaga-fushinari', which is susceptible to powdery mildew. The resistance of the population and its parents was evaluated using leaf disc assays and image analysis. We detected one major QTL on Chr. 5 that was effective at both 20°C and 25°C and one minor QTL on Chr. 1 effective at 20°C. We detected two additional QTLs in subpopulation: one on Chr. 3 effective at 20°C and one on Chr. 5 effective at both 20°C and 25°C in a position different from the major QTL. The resistance alleles at all four QTLs were contributed by 'Kyuri Chukanbohon Nou 5 Go'. The results of this study can be used to develop practical DNA markers tightly linked to genes for powdery mildew resistance.

Key Words: *Cucumis sativus*, *Podosphaera xanthii*, QTL analysis, ddRAD-Seq analysis.

Introduction

Powdery mildew is one of the most severe diseases in cucumber (*Cucumis sativus* L.) worldwide and is mainly caused by *Podosphaera xanthii* (syn. *Sphaerotheca fuliginea* ex Fr. Poll.). The fungus appears as white or grayish-white patches on the leaf surface, then it spreads throughout the leaves while feeding on the plant's nutrients, reducing photosynthesis and causing yellowing of leaves (Pérez-García *et al.* 2009). Despite the fact that this disease has been affecting cucumbers for many years, it is still not controlled sufficiently in cucumber production. Control of powdery mildew involves use of multiple fungicides applications. In greenhouse cultivation, environmental controls such as timing of the opening/closing of side curtains and vents are also used to control the spread of the disease. However, these methods have cost, labor, and materials, and they require training and experience with regard to optimal frequency and timing of fungicide sprays and effective use of environmental controls. Thus, breeding for powdery mildew resistance is one of the most desirable approaches for controlling this disease.

Much effort has been devoted to screening cucumber genetic resources and to genetic analysis of resistance to powdery mildew, and some resistant accessions have been identified such as BK2 (Zhang *et al.* 2015), CS-PMR1 (Fukino *et al.* 2013), IL52 (Zhang *et al.* 2018), S06 (Liu *et al.* 2008a, 2008b), WI 2757 (He *et al.* 2013), and PI197088 (Morishita *et al.* 2003, Sakata *et al.* 2006). Among them, CS-PMR1, a progeny line derived from repeated self-pollinations of PI 197088, possesses the highest level of resistance to powdery mildew, is stably resistant, and has been used for breeding and genetic analysis of resistance (Fukino *et al.* 2013, Morishita *et al.* 2003, Sakata *et al.* 2006). One of the cucumber parental lines used in this study, 'Kyuri Chukanbohon Nou 5 Go', was developed from a cross between CS-PMR1 and the other lines and has high powdery mildew resistance (Sakata *et al.* 2008). Although several cultivars are resistant to powdery mildew at 25°C (high temperature), but susceptible at 20°C (low temperature), 'Kyuri Chukanbohon Nou 5 Go' reported to have high resistance at high temperatures like CS-PMR1, and slightly higher resistance than CS-PMR1 at low temperature (Morishita *et al.* 2003, Sakata *et al.* 2008). The importance of low temperature resistance is due to the fact

Communicated by Katsunori Hatakeyama
Received November 4, 2020. Accepted February 8, 2021.
First Published Online in J-STAGE on June 25, 2021.
*Corresponding author (e-mail: shimomur@affrc.go.jp)

that one main cultivation period of cucumber is from winter to spring in Japan. Powdery mildew of cucumber has a serious problem in the greenhouse during the cultivation period. 'Kyuri Chukanbohon Nou 5 Go' is easier to use than CS-PMR1 as breeding material to develop new cultivars with powdery mildew resistance because the fruits of CS-PMR1 are soft and round, making them undesirable for certain markets, including Japan, whereas 'Kyuri Chukanbohon Nou 5 Go' has a longer, narrower fruit shape.

Recently, many QTLs for powdery mildew resistance have been detected in various populations derived from powdery mildew resistant materials, and several loci were estimated to be in the same or similar regions in different populations (Wang *et al.* 2020). In particular, a QTL on Chr. 5, *pm5.3* (some synonyms for the locus are used), has been detected in many populations, which suggests that this QTL is one of the most important loci for powdery mildew resistance (Chen *et al.* 2020, Fukino *et al.* 2013, He *et al.* 2013, Nie *et al.* 2015a, 2015b, Wang *et al.* 2018, 2020, Zhang *et al.* 2018).

Next-generation sequencing (NGS) has recently been introduced as a promising new platform for many genetic analyses. Correspondingly, methods for single nucleotide polymorphism (SNP) genotyping by NGS, such as genotyping by sequencing (GBS) and restriction-site-associated DNA sequencing (RAD-Seq), have become popular owing to their flexibility and relatively low cost (Davey *et al.* 2011). In cucumber, several investigations using NGS have been performed (Liang *et al.* 2016, Wang *et al.* 2018, Wei *et al.* 2014, 2016, Xu *et al.* 2015a, 2015b, Zhang *et al.* 2015, Zhu *et al.* 2016). For example, Wei *et al.* (2014) reported the first QTL analysis using the specific-length amplified fragment (SLAF) sequencing technique in cucumber. Zhang *et al.* (2015) identified six candidate genes associated with powdery mildew resistance using NGS and SLAF techniques.

The objective of this study was to conduct a QTL analysis of powdery mildew resistance using double-digest restriction-site-associated DNA sequencing (ddRAD-Seq) analysis and to detect loci associated with powdery mildew resistance. We investigated populations of F_2 and F_3 progenies derived from a cross between highly resistant and susceptible parental materials at both 20°C and 25°C. We evaluated the level of powdery mildew resistance by infected leaf discs, stained the spores and hyphae with aniline blue, and performing computer-aided image analysis, and then identified QTLs by using composite interval mapping (CIM). We discussed the positional relationships between the QTLs detected in this study and in past studies, which adds to our understanding of the inheritance of powdery mildew resistance.

Materials and Methods

Plant material and plant pathogen

A population of 189 F_2 progenies was derived from a cross between highly resistant line 'Kyuri Chukanbohon Nou 5 Go' (K5) and susceptible line 'Kaga-aonaga-fushinari' (KA). K5 is the progeny from crosses between CS-PMR1 and the other cultivars, 'Sharp 1' (Saitama Gensyu Ikuseikai Co., Saitama, Japan) and 'Rira' (Enza Zaden BV, Enkhuizen, the Netherlands) (Sakata *et al.* 2008). KA is a Japanese native cucumber that is susceptible to powdery mildew. The two parental cultivars and the F_3 individuals from each F_2 progeny plant ($F_{2:3}$) were used in leaf disc assays.

The *P. xanthii* used in this study originated from a single ascospore of pxB strain, as described by Fukino *et al.* (2008). Since *P. xanthii* is an obligate biotrophic pathogen, it requires live host tissue for reproduction, so it was maintained on leaves of a susceptible cultivar.

Leaf disc assay of inoculation test

$F_{2:3}$ progenies were sown in plastic seedling trays filled with vermiculite and grown in a closed-type artificial-light seedling production system (Mitsubishi Chemical Agri Dream Co., Ltd., Tokyo, Japan) with 16-h day length. The first true leaf was detached from 8 to 10 $F_{2:3}$ plants 2 weeks after sowing. Leaf discs of approximately 8-mm diameter were excised from the first leaf with a circular blade and placed onto 0.5% w/v agar medium containing 2% w/v mannitol and 1% w/v sucrose in 48-well plastic plates (Eppendorf Corporation, Hamburg, Germany) with the adaxial side up. Each open plastic plate was placed at the bottom of a cylindrical tower made of cardboard (10-cm diameter, 30-cm height), and the conidia of the powdery mildew fungus were blown on the top of the tower by using a glass pipette with a rubber bulb attached. The plates were then covered and incubated at 20°C or 25°C under a 16-h day length for 7 days. Hereafter, the assay at 20°C is referred to as LD20 and the assay at 25°C as LD25.

Aniline blue staining of spores and hyphae

The spores and hyphae of powdery mildew in the infected leaf discs were visualized using a method of Lu *et al.* (2011) and Schenk *et al.* (2014) with modifications. Leaf discs were fixed and destained in 1:3 acetic acid/ethanol solution until they turned yellow or white. The fixed leaf discs were washed in 50 mM phosphate buffer, pH 7.5, and stained in 0.01% aniline blue for two to three hours at room temperature. The discs were then transferred to glass slides with the adaxial side up, and images were obtained with a digital camera (EOS Kiss X4; Canon Inc., Tokyo, Japan) under a stereomicroscope (SZ2-ILST; Olympus Corporation, Tokyo, Japan).

Image analysis

Digital images representing a 2-mm^2 area containing disease symptoms were extracted from original images and analyzed using ImageJ software (Schneider *et al.* 2012). The digital images were converted into binary (black-and-white) images with a local thresholding method, Bernsen's algorithm (radius = 7.5) (Bernsen 1986). Then, the numbers

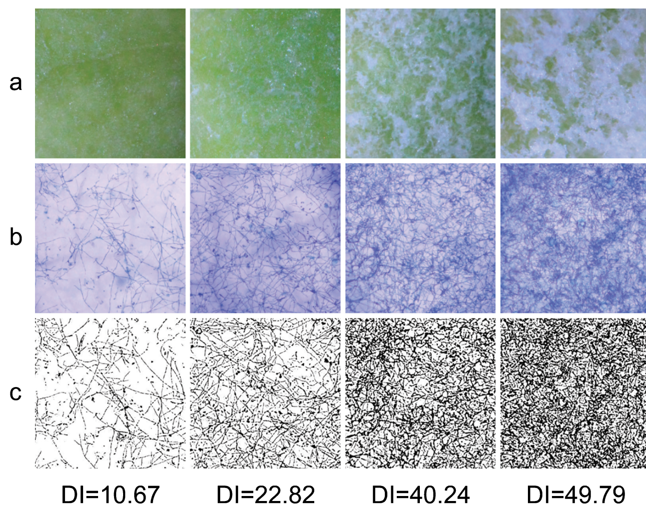

Fig. 1. Digital image of 2-mm^2 area of leaf disc and disease index (DI) calculated using image analysis. Within a column, each row represents the same leaf disc. (a) Spore and hyphae expansion in leaf disc. (b) Staining of spores and hyphae with aniline blue. (c) Stained image converted to black-and-white, and calculated DI.

of black and white pixels were counted separately and used to calculate the proportion of black pixels in each image. We used the percentage of black pixels (100% × the proportion of black pixels) as disease index (DI). Thus, a high DI indicates that the plant is susceptible to powdery mildew, and a low DI indicates resistance (**Fig. 1**). The average DI scores of 6 to 10 F_3 plants per F_2 were also calculated for the QTL analysis.

Double-digest restriction-site-associated DNA sequencing (ddRAD-Seq)

Genomic DNA was extracted from 189 F_2 individuals using the DNeasy Plant Kit 96 (Qiagen, Hilden, Germany) and DNA samples were diluted to a final concentration of 20 ng/μL. Library construction and sequencing analysis of the population and the parents were performed as described by Shirasawa *et al.* (2016) with minor modification. The ddRAD-Seq libraries were constructed with a combination of two restriction enzymes, *Pst*I and *Msp*I (Thermo Fisher Scientific, Waltham, MA, USA). Digested DNA was ligated to adapters using T4 DNA ligase (Takara Bio Inc., Shiga, Japan) and purified using Agencourt AMPure XP (Beckman Coulter, Brea, CA, USA) to eliminate short (<300 bp) DNA fragments. Purified DNA was amplified by PCR with indexed primers. Amplified DNA fragments were purified using the QIAquick PCR Purification Kit (Qiagen), and 300–1000 bp fragments were fractionated by 2.0% agarose gel (Kanto Chemical Co., Inc., Tokyo, Japan) electrophoresis and isolated using a QIAquick Gel Extraction Kit (Qiagen). These libraries were sequenced on a HiSeq X sequencer (Illumina Inc., San Diego, USA) in 150-bp paired-end reads. Nucleotide sequence data were deposited in the DDBJ Sequenced Read Archive under the accession numbers DRA011481.

NGS data acquisition and SNP calling

After removal of low-quality reads, the sequencing data were trimmed and filtered in FaQCs v. 2.08 software (Lo and Chain 2014). Then the data were mapped to the cucumber (‘Chinese Long’ v3) reference sequence (Li *et al.* 2019) in the Cucurbit Genomics Database of the International Cucurbit Genomics Initiative (http://www.icugi.org) with BWA-MEM v. 0.7.17 software (https://arxiv.org/abs/1303.3997). The resultant sequence alignment/map (SAM) format files were converted to binary sequence alignment/map (BAM) format files. SNP calling and genotyping were performed with the mpileup tool in SAMtools v. 0.1.15 and BCFtools v. 1.9 software (Li 2011). SNPs with >8 reads in >80% of progenies were used for analysis.

Simple sequence repeat (SSR) markers

SSR markers from previous studies (Cavagnaro *et al.* 2010, Fukino *et al.* 2008, Ren *et al.* 2009) were used for detection of polymorphism. PCR amplification was carried out using a post-labeling method for multiplexed genotyping analysis with a bar-coded split tag (BStag) as described by Shimizu and Yano (2011) with minor modifications. PCR was performed in a 10-μL reaction mixture that consisted of 1–5 ng of genomic DNA, 0.5 pmol of both the tagged forward primer and the fluorescently labeled BStag primer, 2 pmol of reverse primer, and 2× QIAGEN Multiplex PCR Master Mix (Qiagen). DNA was amplified in a Mastercycler Pro 384 (Eppendorf Corporation) under the following thermal protocol: 95°C for 5 min; 33 cycles of 95°C for 20 s, 55°C for 1 min 30 s, 72°C for 30 s; an additional three cycles of 95°C for 20 s, 49°C for 1 min 30 s, 72°C for 30 s; and 68°C for 10 min. The sizes of the amplified fragments were estimated on an automated DNA fragment analyzer (model 3730xl, Applied Biosystems, Foster City, CA, USA) with a GeneScan-500LIZ size standard (Applied Biosystems). Fragment length was determined in GeneMapper software (Applied Biosystems).

Linkage map construction and QTL analysis

Genetic linkage maps of 189 $F_{2:3}$ segregation population and 48 $F_{2:3}$ subpopulation were constructed in AntMap v. 1.2 (Iwata and Ninomiya 2006) from the SNP markers obtained by ddRAD-Seq analysis. The SNP markers were selected by removing low-quality loci (>5 progenies with missing values at the locus). In addition, we used 45 SSR markers. QTL analysis for DI in the LD20 and LD25 assay was performed by composite interval mapping (CIM) in Windows QTL Cartographer v. 2.5 software (Wang *et al.* 2007) with the following parameter settings: model 6, forward and backward stepwise regression model, 5 maximum background marker loci, window size 10, and 1 cM walking speed along chromosomes. The LOD thresholds for QTL detection in both mapping methods were determined by 1000 permutations.

Results

The continuous distribution of the average F_2 DI scores from each F_3 individuals in the LD20 (20°C) and LD25 (25°C) assays suggested polygenic control of powdery mildew resistance (**Fig. 2a**). However, the presence of a bimodal distribution, especially in the LD25 assay, indicated that a small number of genes might control the resistance in this population. As expected, the highly resistant parent K5 had low DI scores (12.12 ± 8.04 for LD20 and 6.92 ± 4.64 for LD25), and the susceptible parent KA had high scores (42.38 ± 7.35 for LD20 and 41.83 ± 8.21 for LD25). The DI scores between LD20 and LD25 were strongly correlated (Pearson's product-moment correlation coefficients (r) was 0.92*). The average DI scores of $F_{2:3}$ progenies were 33.06 and 30.13 and the median scores were 35.12 and 32.83 in the LD20 and LD25 assays, respectively (**Fig. 2b**). Although K5 was reported to have temperature-independent resistance (Sakata *et al.* 2008), our results showed that the DI score of K5 in the LD20 assay was higher than that in the LD25 assay.

ddRAD-Seq analysis using 189 F_2 individuals returned an average of 1.3 million high-quality reads per sample, and an average Q30 score of 90.15 (Q30 is a quality score specified by Illumina, indicating 99.9% confidence), and a total of 420 out of 2137 SNP sites were informative. The 420 SNPs were combined with 45 SSR markers, giving a total of 465 markers used for constructing linkage maps (**Fig. 3**). The constructed map had 7 linkage groups covering 630.84 cM, with an average distance between interval of 1.36 cM. The 7 linkage groups corresponded to the cucumber 'Chinese Long' v3 reference genome structure (Li *et al.* 2019). However, several long intervals (>10 cM) had no markers on Chr. 2, Chr. 6, and Chr. 7.

One large QTL peak was detected at the same region of Chr. 5 in both the LD20 and LD25 assays, and another small QTL peak was detected on Chr. 1 only in the LD20 assay (**Fig. 4a**, **Table 1**). QTLs detected by CIM in the LD20 and LD25 assays were significant at LOD thresholds of 3.7 and 3.5, respectively. The LOD scores of these QTLs were 48.77 (Chr. 5 QTL, LD20 assay), 69.72 (Chr. 5 QTL, LD25 assay), and 3.78 (Chr. 1 QTL, LD20 assay). The scores for phenotypic variation (R^2) were 0.37 (Chr. 5 QTL, LD20 assay), 0.43 (Chr. 5 QTL, LD25 assay), and 0.02 (Chr. 1 QTL, LD20 assay). We named the detected QTLs according to the names of previously reported loci at the same estimated positions (Wang *et al.* 2020). The QTL on Chr. 5, at the position of *pm5.3*, had an extremely large effect; to exclude its effect, we performed additional QTL analysis using a subpopulations of 48 $F_{2:3}$ progenies that were homozygous for the K5 (resistant parent) genotype at the peak position of *pm5.3*. The average F_2 DI scores from each F_3 individuals were also continuously distributed in the subpopulation (**Supplemental Fig. 1**). The linkage map constructed using this subgroup also had 7 linkage groups, consisting of 276 markers and 630.18 cM in total (**Supplemental Fig. 2**). Three small-effect QTLs were detected on Chr. 1, Chr. 3, and Chr. 5. However, the QTL on Chr. 5 was not at the same position as *pm5.3*, and the QTLs on Chr. 1 and Chr. 3 were detected only in the LD20 assay (**Fig. 4b**, **Supplemental Table 1**). The QTLs on Chr. 1, Chr. 3, and Chr. 5 in the subpopulation analysis were also estimated to correspond to three previously detected loci: *pm1.1*, *pm3.2*, and *pm5.1* (Wang *et al.* 2020). To examine the effects of these three loci in more detail, we investigated the

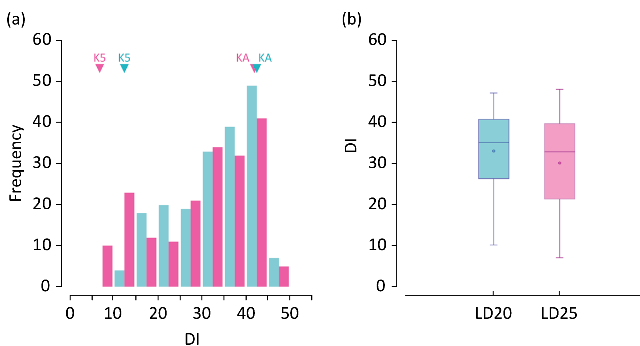

Fig. 2. Differences in disease index (DI) between the LD20 and LD25 assays. (a) Frequency distribution of powdery mildew resistance in average DI scores of the $F_{2:3}$ individuals derived from 'Kyuri Chukanbohon Nou 5 Go' (K5) × 'Kaga-aonaga-fushinari' (KA) cross. (b) Box plot of DI. The *horizontal line* in each box indicates the median; *dots* indicate averages. *Blue* indicates LD20; *pink* indicates LD25.

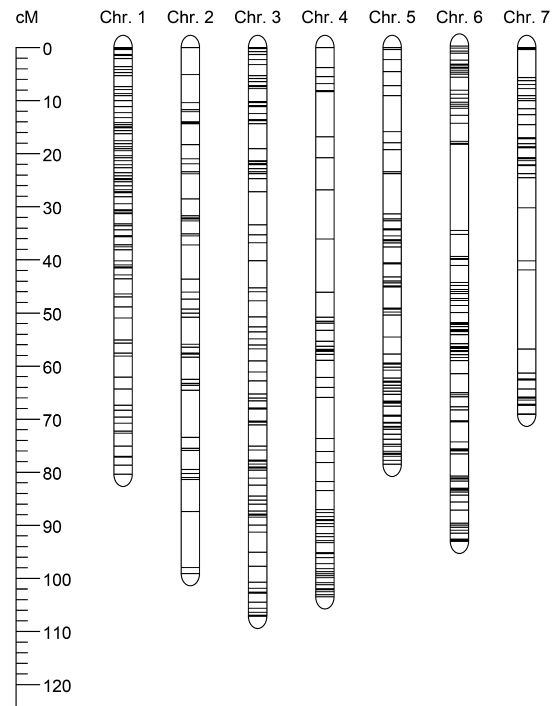

Fig. 3. Linkage map constructed from cross of 'Kyuri Chukanbohon Nou 5 Go' (K5) × 'Kaga-aonaga-fushinari' (KA). Linkage groups were assigned to chromosomes by using the cucumber reference genome ('Chinese Long' v3). *Black lines* indicate loci.

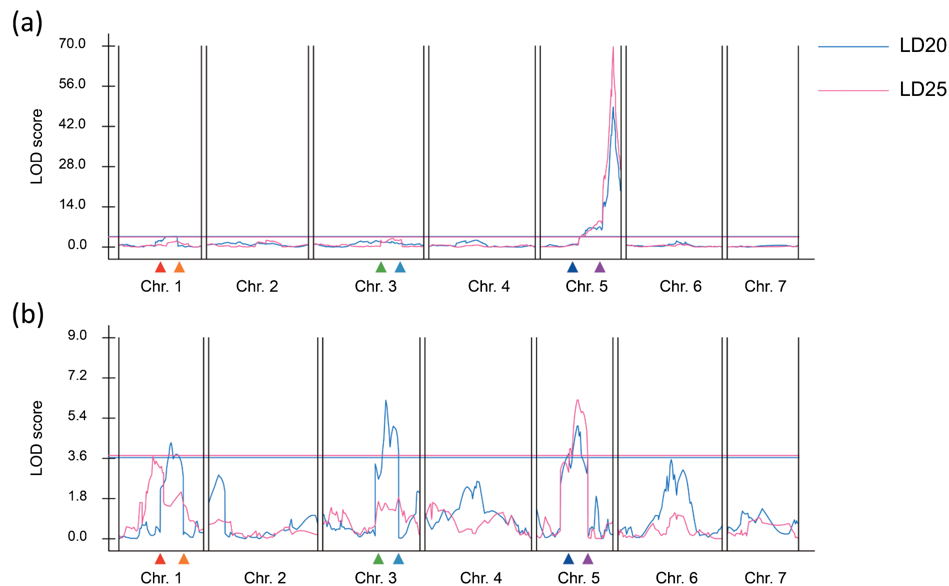

Fig. 4. Positions and LOD scores of QTLs associated with powdery mildew resistance in LD20 and LD25 assays. (a) QTL analysis performed using full $F_{2:3}$ population. (b) QTL analysis performed using subpopulation of $F_{2:3}$ population homozygous for the K5 (resistant parent) genotype at the position of *pm5.3*. *Arrowheads of the same color* indicate the same locus.

Table 1. QTLs for powdery mildew resistance by using composite interval mapping in an $F_{2:3}$ population derived from cross of 'Kyuri Chukanbohon Nou 5 Go' (K5) × 'Kaga-aonaga-fushinari' (KA)

Assay	Chr.	QTL[a]	Position (cM)	LOD peak	Contributing parent	Estimated range within 'Chinese Long' v3 genome	R^2
LD20	1	*pm1.1*	55.7	3.78	K5	23796093–25266488	0.02
	5	*pm5.3*	70.8	48.77	K5	18320307–31743090	0.37
LD25	5	*pm5.3*	70.8	69.72	K5	18320307–31743090	0.43

[a] QTL names were assigned according to the names of corresponding loci (reviewed by Wang *et al.* 2020).

differences in powdery mildew resistance among three subpopulations: one homozygous for the K5 genotype at *pm5.3*, one homozygous for the KA (susceptible parent) genotype at *pm5.3*, and one heterozygous (**Table 2**). All three loci (*pm1.1*, *pm3.2*, and *pm5.1*) in the LD20 assay and only *pm5.1* in the LD25 assay showed significant differences among genotypes in the subpopulation homozygous for the K5 genotype at *pm5.3*. Furthermore, there were also significant differences among genotypes at *pm5.1* in the subpopulation heterozygous at *pm5.3* in both the LD20 and LD25 assays.

Discussion

In this study, as expected, K5 was highly resistant to powdery mildew in both the LD20 and LD25 assays, whereas KA was susceptible in both assays. Several cultivars and lines have been reported to be resistant to the disease at 25°C but susceptible at 20°C (Morishita *et al.* 2003). Sakata *et al.* (2008) reported that K5 is highly resistant to powdery mildew at both temperatures. However, in our results, the DI score of K5 was slightly higher in the LD20 assay than in the LD25 assay, indicating that the resistance level of K5 was affected by temperature to some extent (**Fig. 2**). The different results of the two studies might be explained by the differences in evaluation methods. Although disease severity is frequently evaluated by visual inspection and field evaluation, our method quantified stained spores and hyphae on the leaf disc using digital microscopic images. Therefore, we could evaluate subtle differences in disease severity that are difficult to detect through visual inspection (**Fig. 1**).

In the disease severity assays, the distribution of the average F_2 DI scores from each F_3 individuals was continuous but was bimodal, especially in the LD25 assay. This result suggested that powdery mildew resistance is controlled by a few genes, as reported previously (Sakata *et al.* 2008). When all 189 $F_{2:3}$ progenies were included in the analysis, only two QTLs were detected, one on Chr. 1 and one on Chr. 5 (**Fig. 4a**, **Table 1**). The QTL on Chr. 5, which had a major effect on resistance, was detected in both the LD20 and LD25 assays. Several other QTLs with a large effect on resistance to powdery mildew have been reported in the same chromosomal region as our large-effect QTL on Chr. 5; these QTLs are referred to as *pm5.3* (some synonyms for the locus are used) (Berg *et al.* 2015, Fukino

Table 2. Differences in powdery mildew resistance among three subpopulations classified by *pm5.3* genotype in regions of three QTLs

pm5.3 genotypes of subgroup		LD20			LD25		
		*pm1.1**	*pm3.2**	*pm5.1**	*pm1.1*	*pm3.2*	*pm5.1**
K5 homozygous (48)	K5 homozygous	17.46 ± 3.84 (11)	17.23 ± 3.75 (14)	18.69 ± 4.11 (30)	11.10 ± 3.34 (11)	11.63 ± 3.02 (14)	11.53 ± 2.31 (30)
	KA homozygous	21.97 ± 4.77 (15)	23.57 ± 3.38 (11)	26.39 ± 2.97 (2)	14.43 ± 3.95 (15)	14.49 ± 4.15 (11)	19.26 ± 1.22 (2)
	Heterozygous	20.32 ± 3.68 (22)	20.35 ± 3.89 (23)	22.20 ± 3.39 (16)	13.76 ± 3.17 (22)	13.87 ± 3.47 (23)	16.04 ± 3.43 (16)

pm5.3 genotypes of subgroup		LD20			LD25		
		pm1.1	*pm3.2*	*pm5.1*	*pm1.1*	*pm3.2**	*pm5.1*
KA homozygous (56)	K5 homozygous	40.96 ± 2.77 (19)	41.23 ± 3.41 (11)	40.68 ± 4.13 (3)	41.51 ± 2.84 (19)	38.92 ± 2.47 (11)	39.96 ± 2.62 (3)
	KA homozygous	42.14 ± 3.17 (12)	41.59 ± 2.00 (15)	40.78 ± 3.60 (34)	41.63 ± 2.42 (12)	43.54 ± 1.66 (15)	41.25 ± 3.22 (34)
	Heterozygous	40.75 ± 3.45 (25)	40.84 ± 3.58 (30)	41.79 ± 2.05 (19)	41.08 ± 3.47 (25)	41.14 ± 3.02 (30)	41.73 ± 2.76 (19)

pm5.3 genotypes of subgroup		LD20			LD25		
		pm1.1	*pm3.2*	*pm5.1**	*pm1.1*	*pm3.2*	*pm5.1**
Heterozygous (85)	K5 homozygous	33.63 ± 5.38 (14)	33.93 ± 5.57 (17)	32.91 ± 4.04 (20)	31.90 ± 5.32 (14)	30.47 ± 4.65 (17)	30.80 ± 5.42 (20)
	KA homozygous	35.85 ± 4.02 (19)	35.31 ± 3.18 (13)	37.27 ± 3.57 (17)	33.28 ± 5.17 (19)	34.86 ± 4.40 (13)	34.77 ± 3.76 (17)
	Heterozygous	35.11 ± 4.70 (52)	35.31 ± 4.70 (55)	35.12 ± 4.94 (48)	31.91 ± 4.95 (52)	32.13 ± 5.11 (55)	31.90 ± 5.03 (48)

Numbers in parentheses indicate the number of F_2 individuals.
* Significant differences at the 0.05 probability level among the three genotypes by one-way ANOVA.

et al. 2013, He *et al.* 2013, Nie *et al.* 2015a, 2015b, Sakata *et al.* 2006, Wang *et al.* 2018, Zhang *et al.* 2018). The *pm5.3* locus in several lines harbors a powdery mildew resistance gene, *CsMLO1*, which encodes a barley and Arabidopsis *MLO* homolog (Berg *et al.* 2015, Nie *et al.* 2015a, 2015b). Other reports identified a GATA transcription factor gene and a cyclin-like gene as candidate genes for powdery mildew resistance in the *pm5.3* locus in IL52 and in BK2, respectively (Zhang *et al.* 2015, 2018). Our QTL on Chr. 5, the most effective in this study, is suggested to correspond to *pm5.3* because the LOD peak was closely linked to the genome position of *CsMLO1*, according to the cucumber reference genome (Li *et al.* 2019). On the other hand, the small-effect QTL detected on Chr. 1 only in the LD20 assay was estimated to correspond to a previously reported QTL, *pm1.1* (Fukino *et al.* 2013, Wang *et al.* 2020, Xu *et al.* 2016). K5 was developed from crossing between CS-PMR1 and other lines, so it is expected that several loci for resistance to powdery mildew would have been inherited from CS-PMR1. In fact, *pm1.1* and *pm5.3* were detected in both CS-PMR1 and K5 (Fukino *et al.* 2013). Sakata *et al.* (2008) reported that K5 had slightly higher resistance to powdery mildew than CS-PMR1 at 20°C, suggesting that there are other important genetic components conferring powdery mildew resistance in K5.

To search for these other loci, we performed additional QTL analysis using a subpopulation of $F_{2:3}$ progenies homozygous for the K5 genotype at the position of *pm5.3*. Although the effects detected were not very large, a total of three QTLs were detected (**Fig. 4b**, **Supplemental Table 1**). These loci appear to correspond to *pm1.1* on Chr. 1, *pm3.2* on Chr. 3, and *pm5.1* on Chr. 5, which is at a different position than *pm5.3* (Fukino *et al.* 2013, He *et al.* 2013, Liu *et al.* 2008b, Wang *et al.* 2020, Xu *et al.* 2016). When marker genotype and powdery mildew resistance were examined among three subpopulations with differing alleles at the position of *pm5.3* (K5 homozygous, KA homozygous, and heterozygous), these other three loci appeared to play an important role in powdery mildew resistance to some extent (**Table 2**). QTLs corresponding to *pm1.1* and *pm3.2* showed significant differences in resistance only in the LD20 assay; therefore, these two QTLs are considered to control temperature-dependent resistance. On the other hand, *pm5.1* was more effective than the other two QTLs because differences were detected in two *pm5.3* subpopulations, K5 homozygous and heterozygous, at both temperatures. The most resistant progeny had K5 genotypes at all four loci (*pm1.1*, *pm3.2*, *pm5.1*, and *pm5.3*), and progenies with K5 genotypes at both *pm5.1* and *pm5.3* tended to be more resistant than those with only *pm5.3* (**Supplemental Table 2**). On the other hand, progenies that had the KA genotype at *pm5.3* were susceptible even when *pm5.1* and the other loci had K5 genotypes (**Supplemental Table 2**). These results suggested that, although *pm5.3* was necessary for resistance, the three other QTLs were also important, especially *pm5.1*. Furthermore, the difference in resistance between K5 and CS-PMR1 can also be explained by the presence of resistance alleles at *pm3.2* and *pm5.1*, neither of which is present in CS-PMR1.

K5 has been used as a powerful breeding material for producing cucumber resistant to powdery mildew. To develop new varieties with desired traits introgressed from genetic resources, breeders perform repeated backcrossing and selection for many years. However, it is likely that unfavorable linkages between disease resistance and other important agronomic traits will adversely affect the efficiency of breeding during gene accumulation for disease resistance. The most desirable breeding program for powdery mildew

resistance using K5 would seem to require accumulation of the K5 alleles for *pm1.1*, *pm3.2*, *pm5.1*, and most importantly *pm5.3*.

Besides powdery mildew, many other diseases have caused serious problems in cucumber cultivation, and resistance to them has been investigated. Resistance to downy mildew, caused by *Pseudoperonospora cubensis* (Berk. & Curt.) Rostov, has been well studied genetically by several groups. For example, Yoshioka *et al.* (2014) identified a total of 14 QTLs using recombinant inbred lines (RILs) derived from CS-PMR1, and reported that the most effective QTLs were detected on Chr. 1 (*dm1.1*) and Chr. 5 (*dm5.1* and *dm5.3*). Wang *et al.* (2018) identified a total of 11 QTLs using RILs derived from PI197088, and the most effective QTLs were on Chr. 5 (*dm5.1*, *dm5.2*, and *dm5.3*). In particular, *dm5.3* is closely linked to *pm5.3* (Wang *et al.* 2018, 2020, Yoshioka *et al.* 2014). QTLs for many important characteristics of cucumber fruit, such as length, diameter, length-to-diameter ratio (L/D ratio), were detected on Chr. 1, Chr. 5, and Chr. 6 (Bo *et al.* 2015, Gao *et al.* 2020, Shimomura *et al.* 2017, Wang *et al.* 2020, Weng *et al.* 2015, Yuan *et al.* 2008). Therefore, development of DNA markers tightly linked to important resistance genes for powdery mildew is an urgent requirement for efficiently breeding new cultivars combining genes for resistance to multiple diseases and important agronomic traits. This study makes it possible to develop practical DNA markers for selection of powdery mildew resistance loci and contributes to the accumulation of information about powdery mildew resistance.

Author Contribution Statement

KS conducted assays, analyzed data, performed genetic experiments, and drafted the manuscript. KS, MS and YK were involved in the $F_{2:3}$ population. YY developed method of image analysis. All authors discussed and guided all steps of the experiments, and contributed to the preparation of the final version of the manuscript.

Acknowledgments

We thank Dr. Yoshiteru Sakata and Dr. Nobuko Fukino of National Agricultural Research Organization, and Dr. Yuichiro Iida of Setsunan University, for their useful suggestions. We also thank Dr. Motoki Shimizu of Iwate Biotechnology Research Center for advice on NGS data analysis, and Dr. Kenta Shirasawa of Kazusa DNA Research Institute for instruction on ddRAD-Seq analysis. Finally, we thank F. Hori, S. Masuji, D. Yamashita, Y. Wazaki, M. Shindo, M. Wakabayashi, Y. Taki, A. Suzuki, C. Yamada, A. Tsunoda, and M. Sano of the Institute of Vegetable and Floriculture Science, NARO, for technical assistance.

Computations were partially performed on the NIG supercomputer at ROIS National Institute of Genetics.

Literature Cited

Berg, J.A., M. Appiano, M. Santillán Martínez, F.W.K. Hermans, W.H. Vriezen, R.G.F. Visser, Y. Bai and H.J. Schouten (2015) A transposable element insertion in the susceptibility gene *CsaMLO8* results in hypocotyl resistance to powdery mildew in cucumber. BMC Plant Biol. 15: 243.

Bernsen, J. (1986) Dynamic thresholding of grey-level images. Proc. of the 8th Int. Conf. on Pattern Recognition, pp. 1251–1255.

Bo, K., Z. Ma, J. Chen and Y. Weng (2015) Molecular mapping reveals structural rearrangements and quantitative trait loci underlying traits with local adaptation in semi-wild Xishuangbanna cucumber (*Cucumis sativus* L. *var. xishuangbannanesis* Qi et Yuan). Theor. Appl. Genet. 128: 25–39.

Cavagnaro, P., D. Senalik, L. Yang, P. Simon, T. Harkins, C. Kodira, S. Huang and Y. Weng (2010) Genome-wide characterization of simple sequence repeats in cucumber (*Cucumis sativus* L.). BMC Genomics 11: 569.

Chen, Q., G. Yu, X. Wang, X. Meng and C. Lv (2020) Genetics and resistance mechanism of the cucumber (*Cucumis sativus* L.) against powdery mildew. J. Plant Growth Regul. 40: 147–153.

Davey, J.W., P.A. Hohenlohe, P.D. Etter, J.Q. Boone, J.M. Catchen and M.L. Blaxter (2011) Genome-wide genetic marker discovery and genotyping using next-generation sequencing. Nat. Rev. Genet. 12: 499–510.

Fukino, N., T. Ohara, A.J. Monforte, M. Sugiyama, Y. Sakata, M. Kunihisa and S. Matsumoto (2008) Identification of QTLs for resistance to powdery mildew and SSR markers diagnostic for powdery mildew resistance genes in melon (*Cucumis melo* L.). Theor. Appl. Genet. 118: 165–175.

Fukino, N., Y. Yoshioka, M. Sugiyama, Y. Sakata and S. Matsumoto (2013) Identification and validation of powdery mildew (*Podosphaera xanthii*)-resistant loci in recombinant inbred lines of cucumber (*Cucumis sativus* L.). Mol. Breed. 32: 267–277.

Gao, Z., H. Zhang, C. Cao, J. Han, H. Li and Z. Ren (2020) QTL mapping for cucumber fruit size and shape with populations from long and round fruited inbred lines. Hortic. Plant J. 6: 132–144.

He, X., Y. Li, S. Pandey, B.S. Yandell, M. Pathak and Y. Weng (2013) QTL mapping of powdery mildew resistance in WI 2757 cucumber (*Cucumis sativus* L.). Theor. Appl. Genet. 126: 2149–2161.

Iwata, H. and S. Ninomiya (2006) AntMap: constructing genetic linkage maps using an ant colony optimization algorithm. Breed. Sci. 56: 371–377.

Li, H. (2011) A statistical framework for SNP calling, mutation discovery, association mapping and population genetical parameter estimation from sequencing data. Bioinformatics 27: 2987–2993.

Li, Q., H. Li, W. Huang, Y. Xu, Q. Zhou, S. Wang, J. Ruan, S. Huang and Z. Zhang (2019) A chromosome-scale genome assembly of cucumber (*Cucumis sativus* L.). GigaScience 8: 1–10.

Liang, D., M. Chen, X. Qi, Q. Xu, F. Zhou and X. Chen (2016) QTL mapping by SLAF-seq and expression analysis of candidate genes for aphid resistance in cucumber. Front. Plant Sci. 7: 1000.

Liu, L., R. Cai, X. Yuan, H. He and J. Pan (2008a) QTL molecular marker location of powdery mildew resistance in cucumber (*Cucumis sativus* L.). Sci. China Life Sci. 51: 1003–1008.

Liu, L., X. Yuan, R. Cai, J. Pan, H. He, L. Yuan, Y. Guan and L. Zhu (2008b) Quantitative trait loci for resistance to powdery mildew in cucumber under seedling spray inoculation and leaf disc infection. J. Phytopathol. 156: 691–697.

Lo, C. and P.S. Chain (2014) Rapid evaluation and quality control of

next generation sequencing data with FaQCs. BMC Bioinformatics 15: 366.

Lu, Y., S. Chanroj, L. Zulkifli, M.A. Johnson, N. Uozumi, A. Cheung and H. Sze (2011) Pollen tubes lacking a pair of K^+ transporters fail to target ovules in *Arabidopsis*. Plant Cell 23: 81–93.

Morishita, M., K. Sugiyama, T. Saito and Y. Sakata (2003) Powdery mildew resistance in cucumber. Jpn. Agric. Res. Q. 37: 7–14.

Nie, J., H. He, J. Peng, X. Yang, B. Bie, J. Zhao, Y. Wang, L. Si, J.S. Pan and R. Cai (2015a) Identification and fine mapping of *pm5.1*: a recessive gene for powdery mildew resistance in cucumber (*Cucumis sativus* L.). Mol. Breed. 35: 7.

Nie, J., Y. Wang, H. He, C. Guo, W. Zhu, J. Pan, D. Li, H. Lian, J. Pan and R. Cai (2015b) Loss-of-function mutations in *CsMLO1* confer durable powdery mildew resistance in cucumber (*Cucumis sativus* L.). Front. Plant Sci. 6: 1155.

Pérez-García, A., D. Romero, D. Fernández-Ortuño, F. López-Ruiz, A. De Vicente and J.A. Torés (2009) The powdery mildew fungus *Podosphaera fusca* (synonym *Podosphaera xanthii*), a constant threat to cucurbits. Mol. Plant Pathol. 10: 153–160.

Ren, Y., Z. Zhang, J. Liu, J.E. Staub, Y. Han, Z. Cheng, X. Li, J. Lu, H. Miao, H. Kang *et al.* (2009) An integrated genetic and cytogenetic map of the cucumber genome. PLoS ONE 4: e5795.

Sakata, Y., N. Kubo, M. Morishita, E. Kitadani, M. Sugiyama and M. Hirai (2006) QTL analysis of powdery mildew resistance in cucumber (*Cucumis sativus* L.). Theor. Appl. Genet. 112: 243–250.

Sakata, Y., M. Morishita, E. Kitadani, M. Sugiyama, T. Ohara, K. Sugiyama and A. Kojima (2008) Development of a powdery mildew resistant cucumber (*Cucumis sativus* L.), 'Kyuri Chukanbohon Nou 5 Go'. Hort. Res. (Japan) 7: 173–179 (in Japanese with English summary).

Schenk, S.T., C. Hernández-Reyes, B. Samans, E. Stein, C. Neumann, M. Schikora, M. Reichelt, A. Mithöfer, A. Becker, K. Kogel *et al.* (2014) *N*-acyl-homoserine lactone primes plants for cell wall reinforcement and induces resistance to bacterial pathogens via the salicylic acid/oxylipin pathway. Plant Cell 26: 2708–2723.

Schneider, C.A., W.S. Rasband and K.W. Eliceiri (2012) NIH Image to ImageJ: 25 years of image analysis. Nat. Methods 9: 671–675.

Shimizu, T. and K. Yano (2011) A post-labeling method for multiplexed and multicolored genotyping analysis of SSR, indel and SNP markers in single tube with bar-coded split tag (BStag). BMC Res. Notes 4: 161.

Shimomura, K., N. Fukino, M. Sugiyama, Y. Kawazu, Y. Sakata and Y. Yoshioka (2017) Quantitative trait locus analysis of cucumber fruit morphological traits based on image analysis. Euphytica 213: 138.

Shirasawa, K., H. Hirakawa and S. Isobe (2016) Analytical workflow of double-digest restriction site-associated DNA sequencing based on empirical and in silico optimization in tomato. DNA Res. 23: 145–153.

Wang, S., C. Basten and Z. Zeng (2007) Windows QTL Cartographer 2.5. Department of Statistics, North Carolina State University, Raleigh. https://brcwebportal.cos.ncsu.edu/qtlcart/WQTLCart.htm.

Wang, Y., K. VandenLangenberg, C. Wen, T.C. Wehner and Y. Weng (2018) QTL mapping of downy and powdery mildew resistances in PI 197088 cucumber with genotyping-by-sequencing in RIL population. Theor. Appl. Genet. 131: 597–611.

Wang, Y., K. Bo, X. Gu, J. Pan, Y. Li, J. Chen, C. Wen, Z. Ren, H. Ren, X. Chen *et al.* (2020) Molecularly tagged genes and quantitative trait loci in cucumber with recommendations for QTL nomenclature. Hortic. Res. 7: 3.

Wei, Q., Y. Wang, X. Qin, Y. Zhang, Z. Zhang, J. Wang, J. Li, Q. Lou and J. Chen (2014) An SNP-based saturated genetic map and QTL analysis of fruit-related traits in cucumber using specific-length amplified fragment (SLAF) sequencing. BMC Genomics 15: 1158.

Wei, Q., W. Fu, Y. Wang, X. Qin, J. Wang, J. Li, Q. Lou and J. Chen (2016) Rapid identification of fruit length loci in cucumber (*Cucumis sativus* L.) using next-generation sequencing (NGS)-based QTL analysis. Sci. Rep. 6: 27496.

Weng, Y., M. Colle, Y. Wang, L. Yang, M. Rubinstein, A. Sherman, R. Ophir and R. Grumet (2015) QTL mapping in multiple populations and development stages reveals dynamic quantitative trait loci for fruit size in cucumbers of different market classes. Theor. Appl. Genet. 128: 1747–1763.

Xu, X., R. Xu, B. Zhu, T. Yu, W. Qu, L. Lu, Q. Xu, X. Qi and X. Chen (2015a) A high-density genetic map of cucumber derived from specific length amplified fragment sequencing (SLAF-seq). Front. Plant Sci. 5: 768.

Xu, X., L. Lu, B. Zhu, Q. Xu, X. Qi and X. Chen (2015b) QTL mapping of cucumber fruit flesh thickness by SLAF-seq. Sci. Rep. 5: 15829.

Xu, X., T. Yu, R. Xu, Y. Shi, X. Lin, Q. Xu, X. Qi, Y. Weng and X. Chen (2016) Fine mapping of a dominantly inherited powdery mildew resistance major-effect QTL, *Pm1.1*, in cucumber identifies a 41.1 kb region containing two tandemly arrayed cysteine-rich receptor-like protein kinase genes. Theor. Appl. Genet. 129: 507–516.

Yoshioka, Y., Y. Sakata, M. Sugiyama and N. Fukino (2014) Identification of quantitative trait loci for downy mildew resistance in cucumber (*Cucumis sativus* L.). Euphytica 198: 265–276.

Yuan, X.J., J.S. Pan, R. Cai, Y. Guan, L.Z. Liu, W.W. Zhang, Z. Li, H.L. He, C. Zhang, L.T. Si *et al.* (2008) Genetic mapping and QTL analysis of fruit and flower related traits in cucumber (*Cucumis sativus* L.) using recombinant inbred lines. Euphytica 164: 473–491.

Zhang, K., X. Wang, W. Zhu, X. Qin, J. Xu, C. Cheng, Q. Lou, J. Li and J. Chen (2018) Complete resistance to powdery mildew and partial resistance to downy mildew in a *Cucumis hystrix* introgression line of cucumber were controlled by a co-localized locus. Theor. Appl. Genet. 131: 2229–2243.

Zhang, P., Y. Zhu, L. Wang, L. Chen and S. Zhou (2015) Mining candidate genes associated with powdery mildew resistance in cucumber via super-BSA by specific length amplified fragment (SLAF) sequencing. BMC Genomics 16: 1058.

Zhu, W., L. Huang, L. Chen, J. Yang, J.N. Wu, M. Qu, D. Yao, C. Guo, H. Lian, H. He *et al.* (2016) A high-density genetic linkage map for cucumber (*Cucumis sativus* L.): based on specific length amplified fragment (SLAF) sequencing and QTL analysis of fruit traits in cucumber. Front. Plant Sci. 7: 437.