Genetic Architecture of Downy Mildew (*Pseudoperonospora cubensis*) Resistance in Cucumber (*Cucumis sativus* L.)

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Abstract

Downy mildew (DM), caused by the obligate oomycete *Pseudoperonospora cubensis,* is one of the most devastating diseases of cucumber worldwide. For decades, cucumber production in the US was efficiently protected from DM damage with the deployment of *dm1* resistance gene derived from PI 197087. However, its resistance was overcome by new a strain in 2004 which has been devastating cucumber production ever since causing 40-100% loss for cucumber growers. At present, the control of DM relies mainly on fungicide sprays, which leads to a large amount of additional cost and threatens to long-term cucumber production. Fortunately, large-scale screening tests have identified several plant introduction (PI) lines with high resistances to the post-2004 DM strain(s) including PI 197085, PI 197088, PI 330628, and PI 605996. The genetic and molecular mechanisms of resistance to both pre-2004 and post-2004 DM strains in cucumber are not well understood. Therefore, the objectives of this thesis research were to understand the genetic architecture and molecular mechanisms of DM resistance conferred by WI7120 (PI 330628) and PI 197088 to the post-2004 strain, and that by the *dm1* gene in Gy14 for resistance to the pre-2004 DM strain.

QTL mapping of DM resistance in WI7120 was performed with 243 $F_{2:3}$ families derived from the cross between WI7120 and susceptible inbred line ‘9930’. Ninety-One F_2 plants were genotyped with 348 SSR and SNP markers for linkage map construction, while phenotypic data of DM inoculation responses were collected in four environments at three locations (the US, the Netherlands, and Italy) over two years (2013, 2014). Four QTL, *dm2.1*, *dm4.1*, *dm5.1* and *dm6.1* were consistently and reliably detected across at least three of the four environments, which together could explain 62-76% total phenotypic variances (R^2). Among them, *dm4.1* and *dm5.1* were major-effect QTL (R^2=15-30%) with only additive effects.

QTL mapping of DM resistance in PI 197088 was conducted with 148 $F_{7:8}$ recombinant inbred lines (RILs) developed from the cross between PI 197088 and the susceptible inbred line 'Coolgreen'. This population was genotyped with next-generation high-throughput sequencing (SLAF-Seq) that resulted in a high-resolution linkage map of 2,835 SNP and SSR marker loci. Responses to DM infection of the RILs were evaluated in five environments across three years at two locations. QTL analysis identified 11 QTL for DM resistance on six chromosomes with each explaining 2.0-31.0% total phenotypic variance including *dm1.1*, *dm2.1*, *dm2.2*, *dm3.1*, *dm3.2*, *dm4.1*, *dm5.1*, *dm5.2*, *dm5.3*, *dm6.1*, and *dm6.2*. Among them, *dm5.1*, *dm5.2* and *dm5.3* had the largest effects on DM resistance (R^2 = 19.7-31.0%). Based on 2.0-LOD support intervals, *dm4.1* and *dm5.1* from WI7120 were co-localized with *dm4.1* and *dm5.2* of PI 197088, respectively suggesting they may belong to the same genes. Interestingly, PI 197088 alleles of three QTL, *dm1.1*, *dm2.2* and *dm6.2*, conferred DM susceptibility, which may explain its relatively poor performance for DM resistance in the field at late plant growth stage.

PI 197088 is also highly resistant to the powdery mildew (PM) disease. QTL mapping for PM resistance in PI 197088 was conducted with phenotypic data collected from three replicated field trials. Four PM resistance QTL were identified including *pm1.1*, *pm2.1*, *pm5.1*, and *pm6.1* with *pm5.1* as the major-effect QTL (R^2=32.4%). Three PM and DM resistance QTL pairs, *dm2.1/pm2.1*, *dm5.3/pm5.1*, and *dm6.2/pm6.1* were co-localized at respective chromosome regions, which explained the close linkage of DM and PM resistances often found in cucumber.

Fine genetic mapping of *dm4.1* and *dm5.1* DM resistance QTL from WI7120 was performed to identify candidate genes for the two major-effect QTL. Near isogenic lines (NILs) were developed through marker-assisted backcrossing to Mendelize each QTL, and fine mapping was conducted in large, NIL-derived segregating populations, which were phenotyped in both field trials and screening tests in controlled environments. Starting from the 2.0 LOD interval with ~5Mb physical size, *dm4.1* and *dm5.1* were eventually delimited into 82 and 28 kb genomic DNA region, respectively. Genetic mapping and gene expression data indicated that *Csa4G416990* that encodes an F-box SKIP24 protein (SKP1-interacting partner 24), and *Csa5G471600* that encodes the PHOSPHATE1 (PHO1)-like protein, were two possible candidate genes for *dm4.1* and *dm5.1*, respectively.

QTL mapping for the downy mildew resistance gene *dm1* in two DM resistant lines Gy14 and WI 2757 was conducted with two RIL populations from the crosses of Gy14 × 9930 and WI2757 × True Lemon, respectively. High-density SNP-based genetic maps were developed for the two populations. QTL analysis with phenotypic data collected from multiple environments placed the *dm1* locus into a 3.4 Mb region in cucumber Chromosome 5. Fine-mapping in a large Gy14 × 9930 RIL population narrowed *dm1* down to 93.7-kb region. Twelve genes were predicted in this region. Among them, multiple lines of evidence suggested the cucumber *STAY-GREEN* gene (*CsSGR*, *Csa5G156180*) is the most possible candidate for *dm1*.

Finally, to utilize the DM resistance QTL identified in this study in cucumber breeding, molecular markers closely linked with *dm4.1* and *dm5.1* in WI7120 were developed and used in marker-assisted backcrossing to pyramid these two major-effect QTL into three elite but DM susceptible cucumber lines, Gy14, WI7204 and 9930, which belong to different market classes. QTL pyramiding was expedited with combined marker-assisted background and foreground selections as well as phenotypic selection. At BC_3F_2, these introgression lines were evaluated for DM resistance as well as horticultural performance in field trials. These lines will be accessed for their potential value for commercial release in additional tests.

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Chapter 1: General Introduction

Cucumber (*Cucumis sativus* L.)

Taxonomy, origin and production

The Cucurbitaceae family is composed of 126 genera and 989 accepted species including some economically important genera, such as *Cucumis* (cucumber and melon), *Cucurbita* (squash and pumpkin), *Citrullus* (watermelon), and *Luffa* (loofah) (The Plant List 2013, version 1.1). The genus *Cucumis* contains 52 annual and perennial species including melon (*C. melo*), cucumber (*C. sativus*) and its sister species *C. hystrix* (wild relative of cucumber) (Sebastian et al. 2010). In cucumber, four botanical varieties have been recognized including the cultivated cucumber *C. s.* var. *sativus*, the semi-wild Xishuangbanna cucumber, *C. s.* var. *xishuangbannanesis*, the Sikkim cucumber, *C. s.* var. *sikkimensis*, and the wild cucumber, *C. s.* var. *hardwickii* (Royle 1839; Hooker 1876; Qi and Yuan 1983).

It has been suggested that *C. sativus* var. *hardwickii* is the progenitor of the cultivated cucumber and is believed to originate from Himalayas and adjacent territories east of this mountainous belt (Zohary 2012; Yang et al. 2014). Fossil and literary evidence indicated that the wild cucumber was domesticated by the Indian people in the Indo-Gangetic plain at least 3,000 years ago (De Candolle 1886). More recently, from fingerprinting of 3,342 cucumber accessions collected worldwide, it was shown that independent migrations of cucumber have occurred from India to other parts of the world (Lv et al. 2012; Qi et al. 2013). According to historical records, cultivated cucumber was introduced through the Silk Road to China during Han Dynasty at 2nd century B.C., and westward through land and maritime routes to Europe around 5th to 13th century A.D. (Li 1969; Paris et al. 2012). In the 14th century, Portuguese explorers brought the cucumber plants to West Africa and Columbus is credited with bringing the cucumber to the Americas in 1494 (Robinson and Gecker-Walters 1997).

Cucumbers are now widely grown in the world with total harvest of more than two million hectares in 2015 after tomato, onion, and cabbage (FAOSTAT 2017). Cucumbers are mainly grown for culinary purposes including fresh consumption or processing by the pickling industry. According to the different preferences and consumption habits around the world, there are several important market types for cucumber production including North American processing type, North American slicing type, Northern China fresh market type, and European greenhouse type. In Europe and Asia, cucumber fruits are mainly produced in glass greenhouses or plastic tunnels, while in the U.S., most cucumbers are grown in open field adapting to the once-over machine harvest production system.

Draft genome assemblies

Among the 40 *Cucumis* species studied by Kirkbride (1993), *C. sativus* has the lowest chromosome number with 2*n*=14 chromosomes, while the rest have 2*n*=24 (such as melon, *C. melo*) and occasionally 2*n*=48 or 72 chromosomes. The estimated genome size of cucumber is relatively small as well with only 367Mb (Arumuganathan and Earle 1991).

Among cucurbits crops, cucumber is the first with a publicly released draft genome (Huang et al. 2009), followed by melon (Garcia-Mas et al. 2012) and watermelon (Guo et al. 2013). The first cucumber line used for sequencing is '9930', a northern China fresh market type. Huang et al. (2009) reported 243.5 Mbp sequences of the 9930 draft genome assembly (V1.0) which was the result of a combination of traditional Sanger and Solexa Next-generation sequencing technologies. By collaborating with Illumina GAII sequencing systems, Li et al. (2011) employed 5.23G additional sequencing data and improved the 9930 genome assembly to V2.0. In 9930 V2.0, a total of 23,248 protein-coding genes were predicted with evidence from RNA-seq data (http://www.icugi.org). Although the 9930 draft genome assembly V2.0 made a significant improvement, mis-assemblies in the draft genome still exist, which have been found in several linkage mapping studies (e.g., Yang et al. 2013; Wei et al. 2014; Zhou et al. 2015).

In addition to the 9930 genome assembly, the draft genomes of ‘Gy14’ (North American pickling type), ‘B10’ (European greenhouse type), and PI 183967 (*C. sativus var. hardwickii*, wild cucumber) were also developed (Wóycicki et al. 2011; Yang et al. 2012; Qi et al. 2013). The Gy14 genome was sequenced using the Roche/454 technology; the Gy14 assembly V1.0 composed of 193Mb and suggested 21,491 protein coding genes (http://www.phytozome.org). Unfortunately, both the 9930 V2.0 and Gy14 V1.0 assemblies covered only half the estimated cucumber genome size. Thus, a more complete, better annotated, higher quality genome assembly for cucumber is required, which will be beneficial to the cucurbits community in both forward and reverse genetic studies.

Molecular markers and genetic mapping

Early genetic studies in cucumber were hampered by the low level of polymorphism and lack of molecular markers due to the very narrow genetic base of cultivated cucumbers. For example, the earliest cucumber linkage maps were constructed with horticultural traits as markers such as downy mildew resistance (*dm*), powdery mildew resistance (*pm*), anthracnose resistance (*cla*), dull fruit skin color (*D*), and compact plant type (*cp*) (e.g., Fanourakis and Simon 1987). Few years later, few types of molecular markers became available in cucumber including RFLP (restriction fragment length polymorphism), RAPD (random amplified polymorphic DNA) and AFLP (amplified fragment length polymorphism) markers, which were applied to develop linkage map for quantitative trait loci (QTL) mapping and marker-assisted selection (MAS) (Kennard et al. 1994; Kennard and Havey 1995; Bradeen et at. 2001; Fazio et al. 2003). However, these early maps have relatively limited number of markers with low resolution, which made QTL mapping and map-based gene cloning difficult.

To date, the whole genome sequence offered a platform for large-scale molecular markers development. These markers would be further applied in both breeding and genetic research perspective including marker-assisted selection, map-based cloning, gene isolation, and genome-wide association studies.

Microsatellite, or simple sequence repeat (SSR) has been found abundant and distributed throughout the eukaryotic genome and was excellent in serving as molecular markers (Tautz and Renz, 1984). In the pre-genomics era, the development of SSR markers was difficult with only ~100 SSRs reported (Danin-Poleg et al. 2000; Fukino et al. 2008). However, based on the 9930 genome sequences, Ren et al. (2009) successfully developed over 2,000 SSR markers, which have been widely used in subsequent genetic studies in cucumber. Moreover, Cavagnaro et al. (2010) surveyed the whole genome of microsatellite sequences in the Gy14 assembly, and identified 112,073 SSRs. Among them, 83,000 SSRs were designed with forward and reverse primers for amplification. These large number of SSR markers has been applied to generate highly saturated linkage maps, which was proven to be useful in map-based cloning (e.g., Ren et al. 2009; Yang et al. 2013; Miao et al. 2016).

Single nucleotide polymorphism (SNP) and insertion/deletion polymorphism (INDEL) are other two types of DNA-level variations that exist widely in the genome and are ideal for use as molecular markers. With the increased use of new sequencing technologies, large-scale development of SNP/INDEL markers are becoming much easier, faster and cost-saving. One such approach for genome-wide SNP discovery is genotyping-by-sequencing (GBS), which uses restriction enzymes to reduce genome complexity and barcode-system to genotype multiple DNA samples (Elshire et al. 2011). Recently, Sun et al. (2013) developed an improved GBS approach with distinguishing characteristics in deeper sequencing, reduced sequencing costs, and dual barcode system for larger populations, and denoted it specific length amplified fragment sequencing (SLAF-seq). In cucumber, several genetic maps have been developed with SLAF-seq with each containing thousands of SNPs (Wei et al. 2014; Xu et al. 2015; Zhu et al. 2016).

The widespread use of new sequencing technologies makes it easy to develop almost unlimited number of molecular markers. However, this also brings the challenges for the marker ordering accuracy and linkage map construction, which is based on the recombination frequency that occur during meiosis as the basic foundation. For construction of a linkage group with *m* markers, the number of possible orders would be *m*!/2, which is impossible to compare all orders. Two aspects then are required to solve the ordering and calculation speed issues: optimization criteria and optimization algorithm.

During the development of a linkage map, most optimization criteria are functions of the recombination fraction between pairs of markers and the associated LOD score, except for the RECORD software (REcombination Counting and ORDering, Van Os et al. 2005). RECORD minimizes the total number of recombination events, which allows to order large numbers of markers rapidly and accurately (Ma et al. 2017). Some widely-used criteria based on the recombination fraction includes the maximum likelihood of an order (ML), the maximum sum of adjacent LOD scores (SALOD), the minimum sum of adjacent recombination fractions (SARF), and the likelihood through hidden Markov chains (LHMC) (Lender et al. 1987; Weeks and Lange 1987; Falk 1992).

A number of algorithms is also available for map construction. Wu et al. (2008) developed the MSTMAP approach based on a minimum spanning tree algorithm and claimed a more accurate marker order than RECORD in handling 10,000 to 100,000 markers. Rastas et al. (2013) proposed Lep-Map (Lepidoptera-Map) using a combination of multiple algorithms, which is optimized for some taxa without recombination on one sex and for ultradense genetic map with more than 40,000 SNPs. Liu et al. (2014) developed HighMap software using *k*-nearest neighbor algorithm with SARF criterion which also implemented with correcting genotyping errors and imputing missing genotypes. Among these methods, MSTMAP has been implemented in the R environment with the *R/ASMap* package, which also includes functions to analyze *R/qtl* objects. This package is user-friendly and convenient for the subsequent applications (for example, QTL mapping).

When building a map with large numbers of marker loci, it is necessary to choose from several algorithms and criteria, and the choice is not always simple. Since cucumber draft genome sequences are available, marker orders could be cross-validated with their physical positions. On the other hand, the high-density genetic map can also be used to detect assembly errors in the draft genome which are common for those assembled in early years. In addition, a reliable and saturated high-density genetic map can significantly improve the detection power of minor-effect QTL for target traits and reduce its logarithm of odds (LOD) score confidence interval. For example, using a high-density map consisting of 2,084 bins with unique recombination events, Zhou et al. (2015) was able to pinpoint the candidate genes for cucumber scab resistance locus in a 180-kb region.

QTL mapping

QTL mapping is commonly used to reveal the genetic regions responsible to certain phenotype variations. Below is a brief summary of traditional QTL mapping approaches, such as single marker analysis (SMA), simple interval mapping (SIM), composite interval mapping (CIM), and multiple-QTL mapping (MQM).

SMA is the simplest way to perform QTL mapping through marker regression. Each marker was grouped by their genotypes and compared with its phenotypic mean by ANOVA (*F*-test). SMA could be performed using basic statistics and does not require a complete linkage map which will be an advantage to test the significance of selective markers to candidate genes (e.g., examples in Chapter 3 and Chapter 4 of this study).

SIM models the correlation between phenotype and genotype by performing a likelihood ratio test at one-marker interval, while CIM combines SIM with linear regressions at each marker and using other markers as cofactors. CIM is nearly the most widely used approaches for QTL mapping since it was developed, which provided additional information and enhanced the detection of QTL with a less saturated linkage map in the past (Zeng et al. 1994). Zeng et al. (1999) stated that the use of tightly linked markers as cofactors could significantly reduce the statistical power in QTL detection. Thus, as the increasing utility of saturated SNP-based linkage map, CIM may suffer from this drawback.

The MQM approach uses the maximum likelihood (ML) or the restricted maximum likelihood (REML) algorithm, and employs a backward elimination strategy in QTL identification (Arends et al. 2010, 2014). As compared with CIM, MQM strengthens the identification of QTL in coupling and repulsion phases and reduces the chance of detection of 'ghost' QTL that is a non-existing QTL but can appear in-between two linked QTL. In general, MQM is a more advanced procedure that reduces both Type I and Type II errors and increases the accuracy of QTL location.

Cucumber Downy Mildew (*Pseudoperonospora cubensis*)

Taxonomy and morphology

The Pathogen *Pseudoperonospora cubensis* [(Berkeley & M. A. Curtis) Rostoyzev] is the causal agent of cucurbit downy mildew (DM), which is one of the most important foliar diseases of cucurbits worldwide. *Ps. cubensis* is a member of family Peronosporaceae in the order Peronosporales within the class Oomycetes.

Ps. cubensis, an obligate parasite, requires live host tissue to propagate and reproduce (Thines et al. 2009). *Ps. cubensis* forms large (20-40 x 14-25 µm in diameter) and lemon-shaped sporangia, which release 5 to 15 asexual and ovoid zoospores (10-13µm in diameter) under moisture environment for plant infection. The zoospores are biflagellate that allow them to swim through free moisture on the leaf surface to a stomate. Once a stomate is located, zoospores will penetrate the stomate via a germ tube (50-95 µm) and infect the host plant cell (Colucci and Holmes 2010). The mycelium grows within and between host cells and develops in mesophyll for 4 to 12 days before sporulation but not until there is sufficient moisture over the lesion (Gevens and Marks 2014). Then the sporangia are released to continue the disease cycle (Savory et al. 2011).

Population structure

Host specialization in *Ps. cubensis* was first studied in Cucurbitaceae by Doran (1932) and with two *Ps. cubensis* isolates by Hughes and Van Haltern (1952). Later, Thomas et al. (1987) studied eight isolates from US, Israel and Japan, and classified them into five pathotypes based on compatibility between the pathogen and six host taxa. Recent studies on the field populations of *Ps. cubensis* from different countries revealed a highly heterogeneous and dynamic nature of *Ps. cubensis* populations, which may be composed of many isolates, pathotypes or races with varying degree of pathogenicity or virulence (Lebeda and Urban 2007; Quesada-Ocampo et al. 2012; Lebeda et al. 2013; reviewed by Cohen et al. 2015). Molecular fingerprinting studies find that, while most *Ps. cubensis* isolate groups were present in all continents examined, the dominant ones were structured by geographic origin across continental scales (Quesada-Ocampo et al. 2012), which may result in differential inoculation responses on the same cucumber genotypes.

Epidemiology

Generally, as an obligate pathogen, *Ps. cubensis* could only survive and reproduce on living hosts. However, cucurbit plants are frost sensitive. Therefore, *Ps. cubensis* is not capable of overwintering in geographical areas that experience killing frosts. In the United States, cucumber host could only survive the winter in southern Florida, southern Texas, and along the Gulf of Mexico that have mild winters which allow the pathogen to survive during the winter season (Holmes et al. 2015). According to yearly epidemic trends detected in the US, cucumber DM originates every year from the southern states and moves north during the growing season (Holmes et al. 2015). It is believed that *Ps. cubensis* is dispersed through spring wind currents which carried the sporangia to the new host that could be several hundred miles far away (Doran 1932; Nusbaum 1944; Lebeda and Cohen 2010). Another source of primary inoculum of *Ps. cubensis* could be held by greenhouse production of cucurbits host in Canada or Northern states in the U.S. (Holmes et al. 2015).

In China, such epidemic trends from the southern to northern provinces have not been observed. It is believed that the oospores of *Ps. cubensis* initiated the local inoculum on cucumber in the northern area. The oospores of *Ps. cubensis* were reported in plant debris in the northern part of China but were hardly found in the southern area (Chen et al. 1959; Zhang et al. 2012).

The oospores are the sexual reproduction form of *Ps. cubensis* which may proceed with recombination among isolates and introduce new virulence that could overcome fungicides or resistant cultivars. This might be the causal reason for the appearance of new pathotypes of *Ps. cubensis* in Israel in 2002, Italy in 2003, and United States in 2004 (Cohen et al. 2003; Cappelli et al. 2003; Holmes et al. 2006).

Management and fungicide

The successful management of cucumber DM relies on the combination of utilizing resistant or tolerant cultivars, cultural practices, early disease detection, and timely application of fungicides. In the United State, a forecasting system for cucurbits DM appearance is available since 1998 which is called The Cucurbit Downy Mildew ipmPIPE (http://cdm.ipmpipe.org) (Ojiambo et al. 2011; Vankirk et al. 2012). The system could forecast and inform growers to effectively apply fungicides at the appropriate time before DM pressure reach the greatest point.

Ps. cubensis, as with many other oomycete pathogens, has been able to develop resistance to fungicides quickly. Many fungicides are no longer effective to *Ps. cubensis*, such as phenylamides, strobilurins and carbamates (Urban and Lebeda 2006). Thus, the different combination of multiple fungicides should be used when managing the disease, due to its ability for overcoming fungicides (Keinath et al. 2016).

Cucumber-Downy Mildew Interactions

For decades, cucumber production in the US was efficiently protected from DM damage with deployment of *dm1* derived PI 197087. However, in 2004, *dm1* locus was overcome by new strains. Currently, several new germplasms were identified conferring highly resistance to the post-2004 DM strains. The genetic basis for the breakdown and current resistance are still unknown. Thus, reviewing the cucumber-*Ps. cubensis* interaction during infection process may provide hints to search for candidate genes underlying the resistant or susceptible to DM in cucumber.

DM disease symptoms on susceptible cucumbers are characterized by the appearance of small and water-soaked lesions on the underside of leaves. Adaxial lesions are often angular and bound by leaf veins, eventually turning chlorotic and necrotic (Oerke et al. 2006). Every step of *Ps. cubensis* infection process could be against by host plants immune systems which might display a different level of DM resistance.

The initial stage of pathogen infection involves adhesion, penetration, and invasive growth within the host cell tissue. At this stage, elicitors are the molecules that stimulate a defense response in the host plant, most of which constitute pathogen-associated molecular patterns (PAMPs) (Henry et al. 2012). Elicitors are perceived as a microbial signature, likely through peripheral receptors by some plants, which require BAK1/SERK3 for their activity (Heese et al. 2007; Chaparro-Garcia et al. 2011). Several elicitors of defense responses have been extensively studied for oomycetes such as Cryptogein and Eicosapentaenoic acid in Arabidopsis, potato, and tobacco (Khatib et al. 2004; Kawaguchi et al. 2012; Henriquez et al. 2012; Wiesel et al. 2014). However, the elicitors of *Ps. cubensis* on cucumber have not yet been investigated and their receptors in cucumber are still unknown.

With the increasing immune responses triggered by pathogen's own elicitors, oomycetes must suppress it in order to sustain an intimate association with the host plant. Oomycetes are able to secrete effector proteins that can act in many different cellular compartments, and eventually alter the host plant’s physiological state to benefit colonization. Recent studies on *Ps. cubensis* genome have identified at least 271 candidate effector proteins with variable RXLR (Arg-X-Leu-Arg, where ‘X’ is any amino acid) translocation motifs, which is the major type of oomycete effectors that have been demonstrated to suppress basal host resistance (Bos et al. 2006; Fabro et al. 2011). The RXLR motif can be followed by an EER(Glu-Glu-Arg) motif, or similar motifs, such as QXLR (Gln-X-Leu-Arg) (Tian et al. 2011) and RXLQ (Arg-X-Leu-Gln) (Fabro et al. 2011), or it could be absent, such as in the case of ATR5 (Rehmany et al. 2005). Another important class of effectors is the Crinkler (CRN) family, for example, has a conserved LXLFLAK motif necessary for translocation into the host cytoplasm and subsequent import into plant nuclei where they elicit a rapid cell death response (Torto et al. 2003; Schornack et al. 2010). During the *Ps. cubensis* infection of cucumber leaves from 1 day post-inoculation (dpi) to 8dpi, at least 34 genes that belong the CRN family, were found expressed in at least one time point of infection or in isolated sporangia (Savory et al. 2012).

To counteract the secreted effectors from pathogens, host plants have evolved a second line of defense comprising resistance (R) proteins that recognize particular effectors. This recognition leads to effector-triggered immunity (ETI) (Jones and Dangl 2006; van der Hoorn and Kamoun 2008). In cucumber, several types of R genes have been investigated, with a total of 71 NBS-LRR type resistant gene homologs (RGHs), 178 LRR-RLP (receptor-like proteins) type resistant gene analogs (RGAs), and 56 LRR-RLK (receptor-like kinases) type RGAs (Wan et al. 2013; Yang et al. 2013; Wang et al. 2014). Some of the RGHs and RGAs were found clustered under few QTL identified from resistant cucumber cultivars. However, so far, no associations have been directly drawn out between R-genes and *Ps. cubensis* resistance in cucumber.

Research Objectives

The main objective of this thesis is to identify DM resistance QTL to both pre-2004 and post-2004 DM strains in multiple germplasm lines (Chapter 2, Chapter 3 and Chapter 5). By reviewing the QTL number, location, effect size, and dynamic magnitude underlying DM resistance in different DM resistance germplasm, we would be able to understand the genetic architecture of DM resistance for cucumber. This knowledge is important for efficient use of the resistance sources in cucumber breeding perspective. The second objective of this study is to fine-mapping the major-effect DM resistance QTL that are shared by different resistant sources (Chapter 4 and Chapter 5). Identification of the causal gene for DM resistances is the first step for understanding the genetic and molecular mechanisms of host resistance and the interactions between cucumber and *Ps. cubensis*. From a plant breeding perspective, exploration of the practical use of these resistance QTL is also critical. Thus, the third and last objective of this study is to develop a set of applicable molecular markers for marker-assisted selection in breeding for DM resistance and to pyramid those identified QTL into elite genetic backgrounds (Chapter 6). The development of such breeding lines with high DM resistance and other good horticultural quality traits would provide breeders with advanced material for commercial cultivar development.

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Chapter 2: QTL mapping for downy mildew resistance in cucumber inbred line WI7120 (PI 330628)

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Abstract

Downy mildew (DM) caused by the obligate oomycete *Pseudoperonospora cubensis* is the most devastating fungal disease of cucumber worldwide. The molecular mechanism of DM resistance in cucumber is poorly understood, and use of marker-assisted breeding for DM resistance is not widely available. Here we reported QTL mapping results for DM resistance with 243 $F_{2:3}$ families from the cross between DM-resistant inbred line WI7120 (PI 330628) and susceptible inbred line '9930'. A linkage map was developed with 348 SSR and SNP markers. Phenotyping of DM inoculation responses were conducted in four field trails in two years at three locations. Four QTL, *dm2.1*, *dm4.1*, *dm5.1* and *dm6.1* were consistently and reliably detected across at least three of the four environments, which together could explain 62-76% phenotypic variations (R^2). Among them, *dm4.1* and *dm5.1* were major-effect QTL (R^2=15-30%) with only additive effects; *dm2.1* (R^2=5-15%) and *dm6.1* (R^2=4-8%) had moderate and minor effects, respectively. Epistatic effects were detected for *dm2.1* and *dm6.1* with both *dm4.1* and *dm5.1*. One additional minor-effect QTL, *dm6.2* (R^2=3-5%) was only detectable with the chlorosis rating criterion. All alleles contributing to DM resistance were

from WI7120. This study revealed two novel QTL for DM resistance and the unique genetic architecture of DM resistance in WI7120 conferring high level resistance to prevailing DM populations in multiple countries. The effects of disease rating scales, rating time and criteria, population size in phenotyping DM resistance on the power of QTL detection, and the use of DM resistance in WI7120 in cucumber breeding were discussed.

Introduction

Downy mildew (DM) caused by the obligate biotrophic oomycete *Pseudoperonospora cubensis* [(Berkeley & M. A. Curtis) Rostoyzev] is an important foliage disease of all cucurbit crops worldwide. DM is especially devastating in cucumber. Disease symptoms on susceptible cucumbers are characterized by the appearance of small and water-soaked lesions on the underside of leaves. Adaxial lesions are often angular and bound by leaf veins, eventually turning chlorotic and necrotic (Oerke et al. 2006). Heavy sporulation can be observed within the lesions on the abaxial leaf surface. A general yellowing of affected leaves typically occurs as the lesions coalesce into one large lesion, eventually causing the leaf to wilt and die. Under favorable conditions, the disease can progress quite rapidly defoliating a cucumber field in a matter of days rendering limited flexibility of fungicide spray (Savory et al. 2011). *Ps. cubensis* spores spread rapidly by wind, splashing rain, and/or irrigation water. Disease management and prevention requires destruction of all plants from infected nurseries and disinfection of the facilities (Ojiambo et al. 2015). Seed transmission of *Ps. cubensis* has been found in cucurbits, and it was suspected that international trade of fruits or seeds of cucurbits might be associated with the recent global change in the population structure of *Ps. cubensis* (Cohen et al. 2014).

Among integrated pest management practices for disease control, deployment of resistant cultivars is clearly the most economic and environmentally sound method. The Unites States has a long history of breeding cucumber for DM resistance. Jenkins (1942) was the first to conduct systematic screening of DM resistance in cucumber who found that, among 12 varieties examined, Puerto Rico 37 (PR37) and a line from China (Chinese Long) had high resistance, and the progeny from the cross between them did not segregate for DM resistance. Jenkins (1946) then studied the inheritance of DM resistance in PR37 and found normal distribution of disease ratings in F_2 and F_3 families; he also found consistent performance of resistance at both seedling (in the greenhouse) and adult plant (in the field) stages. From the Chinese Long resistance source, Barnes et al. (1948) developed the cucumber cultivar 'Palmetto'; its resistance, however, was lost only two years after its release probably due to the appearance of new DM strain in the field (Epps and Barnes 1952). Following the defeat of resistance in Palmetto, Barnes and Epps (1954) identified a new source of resistance, PI 197087 from India. The resistance responses in PI 197087 were characterized by sparse pathogen sporulation, small necrotic lesions, tissue browning, and rapid cell death, indicative of the classical hypersensitive response (HR)-type resistance (Barnes and Epps 1954). Since then, DM resistance conferred by the recessive gene *dm-1* in PI 197087 has been widely used in commercial cultivar development in the US. Representative cultivars carrying *dm-1* include Polaris, Gy14, M21, Clinton, Chipper, and the Poinsett or Marketmore series (Wehner and Shetty 1997; Call et al. 2012a). The resistance conferred by *dm-1* was sufficient to prevent losses caused by DM without the use of fungicides for nearly 50 years until the emergence of a new DM strain in 2004 (Holmes et al. 2004; 2006). Fortunately, multi-year, multi-location, large scale screening tests identified several plant introduction (PI) lines with high resistance to the new DM strain including PI 197085, PI 197088, PI 330628, and PI 605996 (Call et al. 2012b).

In the post-2004 cucumber fields in the US, resistance tracing back to Puerto Rico 40 (PR40) provides only a slight advantage over lines that lack any resistance in their pedigree: cultivars containing the *dm-1* gene show moderate resistance whereas susceptible ones without any DM resistance gene become infected earlier in the season, and exhibit more severe damages than was observed previously (Holmes et al. 2004; Call 2012a, b). Cucumber lines possibly carrying both *dm-1* and PR40 DM resistances (for example, SC50 or PI 234517, WI2757, and M21) in general perform much better than *dm-1* only lines suggesting residual resistances of the two defeated genes (Call et al. 2012b; Kozik et al. 2013).

Meanwhile, from the progeny of a cross between Marketmore 97 and Ivory Queen with intermediate DM resistance, Holdsworth et al. (2014) selected a breeding line, MRNY264 with a high level of resistance to the new DM strain.

The inheritance of DM resistance in cucumber has been investigated in a number of studies through either classical quantitative genetic analysis or QTL mapping but with varying results. van Vliet and Meysing (1974) suggested a single recessive gene, *dm-1* was underlying the DM resistance in Poinsett, which was later located in cucumber chromosome 5 (Fanourakis and Simon, 1987; Kennard et al. 1994; Horejsi et al. 2000). Petrov et al. (2000) proposed that the resistance in WI2843 derived from PI 197087 was controlled by incomplete dominant genes. Genetic variance studies for Ames 2354 (a selection from SC50) revealed a small number of genes controlling DM resistance in this line and one of them seems to be allelic to *dm-1* (Kozik et al. 2013). Several QTL mapping studies identified five QTL in four cucumber chromosomes (Chr 1, 4, 5 and 6) for DM resistance in north China fresh market type (Chinese Long) cucumber lines ‘129’ (Ding et al. 2007), ‘S94’ (Bai et al. 2008) and ‘K8’ (Zhang et al. 2013). Pang et al. (2013) detected 3 QTL for DM resistance (1 on Chr6 and 2 on Chr5) that was believed to be from a *C. hystrix* introgression line IL52. Since the F_2 population used for mapping in Pang et al. (2013) was derived from a cross between IL52 (in the background of a North China type cucumber ‘Beijingjietou’) and a susceptible line ‘Changcunmici’, and the DM resistance in IL52 was comparable that in the moderately resistant cucumber line M21, it is not known whether the resistance was contributed by IL52 or ‘Beijingjietou’ or both.

Although the four PI lines, PI 197085, PI 197088, PI 330628, and PI 605996 all show high resistance against the post-2004 DM strain in the US, it is not known whether they share a similar genetic basis for DM resistance. This knowledge is important in efficient use of these resistance sources in cucumber breeding. Angelov (1982) suggested that DM resistance in PI 197088 is under the control of two recessive genes whereas that in Poinsett is due to one recessive gene. Yoshioka et al. (2014) conducted QTL mapping for DM resistance in the cucumber line CS-PMR1 (DM resistance from PI 197088) and identified 10 QTLs in 5 chromosomes (1, 3, 5, 6 and 7) including 3 QTL from the parental line Santou and the major-effect QTL contributed by PI 107088 being located in Chr5. More recently, Szczechura et al. (2015) identified three DM resistance QTL in Chr5 from PI 197085. In field screening tests, VandenLangenberg (2015) observed a significant number of susceptible offspring in F_2 populations derived from crosses among PI 197088, PI 330628, and PI 605996 suggesting possible genetic differences in these lines. VandenLangenberg (2015) also found that these PI lines responded differently to the DM disease over plant developmental stages, and suggested the preference of PI 330628 over PI 197088 due to its ability to maintain resistance for longer time. Therefore, the objective of the present study was to conduct QTL mapping to understand the genetic basis of DM resistance in WI7120 (PI 330628). Using F_3 families derived from two inbred lines, the DM resistant WI7120 and susceptible 9930, we collected phenotypic data from multiple environments in three countries over two years. QTL analysis identified QTL for DM resistance conferred by WI7120 in four cucumber chromosomes.

Materials and Methods

Plant materials

Two inbred lines, WI7120 and 9930 were used as the parental lines to develop an F_2 and F_2-derive F_3 populations for QTL mapping in the present study. WI7120 was the advanced self-pollinated inbred line from PI 330628, which was originally collected from Pakistan and is highly resistant to DM (Wehner and Shetty 1997; Call et al. 2012a). The North China type line 9930 was susceptible to DM. Ninety-One F_2 plants and $F_{2:3}$ families from 9930 × WI7120 mating were used for developing a linkage map and initial QTL mapping.

Phenotypic data collection and refined QTL mapping in target chromosomal regions were based on 243 $F_{2:3}$ families of the same population.

Phenotypic data collection and statistical analysis

Phenotypic data of inoculation responses of *Ps. cubensis* for 243 $F_{2:3}$ families were collected in four environments over two years (2013, 2014) in three countries, which were designated as US2013, US2014, NL2013 and IT2013, respectively. US2013 and US2014 were conducted at the Clinton Horticultural Crops Research Station of North Carolina State University at Raleigh, North Carolina in 2013 and 2014, respectively. NL2013 and IT2013 were performed in the field of Bayer Vegetable Seeds in the Netherlands, and in the greenhouses of Magnum Seeds in Fondi, Italy, respectively. The two parental lines and their F_1 were included in all screening tests. Some historical DM resistant cultivars carrying the PI 197087-derived *dm-1* resistance gene like Poinsett 87 and WI 2757, as well as the susceptible cultivar Straight 8 were also included as controls in the US2013 or US2014 trials.

The US2013 and US2014 experiments were a randomized complete block design with three replications. There were 5 plants per F_3 family per replication. Plots were exposed to natural epidemics encouraged by overhead irrigation during the growing season at least three times per week, or as needed. All entries were hand planted on June 27, 2013 and June 30, 2014. Disease severity of individual plants was evaluated weekly for three consecutive weeks based on percentage of symptomatic leaf area using disease rating scale 1 to 9, where 1 = 0-10%, 2 = 11-20%, 3 = 21-30%, 4 = 31-40%, 5 = 41-50%, 6 = 51-60%, 7 = 61-70%, 8 = 71-80%, 9 = 81-100% or dead (**Figure 2.1**). Since one of the major block effects was the genetic variation within F_3 families and there was no significant effect among replication in each environment, the family means from 15 plants of each family were used for statistical analysis and QTL mapping.

Field layout of the NL2013 experiment was similar to US2013 or US2014 but had 2 replications and 7 plants per family per replication (plot). The DM symptoms of each plot were measured twice with one week apart with three criteria: general impression (GI), necrotic lesion size (Nec), and chlorotic lesion size (Chl). Leaf chlorosis or yellowing was a symptom of the DM fungal infection and depends on both infection spread and on plant growth rate. Leaf necrosis was the appearance of necrotic (dead) spots on the leaf, either as a symptom of infection or as a hypersensitive response from the plant. The GI score was based on the general health and symptom expression in the plant (Pang et al. 2013). For each trait, the disease severity was rated on the plant breeder's 1-9 scale, where scale 1 = maximal disease symptoms and 9 = no visible symptom.

The IT2013 experiment was conducted in plastic tunnels with naturally infection. There were ten plants per F_3 family and no replications. To assure optimal conditions for DM symptom development, after transplanting, high humidity inside the tunnels was maintained by spraying with water twice a day when necessary. Rating of disease symptoms on each plant was based on a 1–5 scale where 1 = absence of symptoms and 5 = maximal disease symptoms.

Statistical analysis of phenotypic data was all performed in R (Version 3.1.1, http://www.r-project.org/). Means of disease score of each family were calculated by rating time, replication, trait and experiment and used for statistical analysis. Quantile-Quantile (Q-Q) plot was performed for each location to confirm the normal distribution of the phenotypic data. The ordinal rating data for each environment was standardized to standard score (z-score) for the same scale independently. Analysis of variance (ANOVA) was performed with the *R/lme4* package to estimate the genetic and environment effects with the following model: $R_{ij} = \mu + G_i + E_j + \varepsilon_{ij}$, where R=observed value for a given trait, μ = grand mean, G = genotype, E = environment and ε = residuals. Best linear unbiased predictors (BLUPs) were also extracted from the model and used for QTL analysis. Broad sense heritability estimates were calculated from variance components using $h^2 = \sigma_G^2 / (\sigma_G^2 + \sigma_\varepsilon^2)$, where σ_G^2 was the genotypic variance and σ_ε^2 was the residual variance. Spearman's rank correlation coefficient (r_s) among different environments was calculated before and after the scale standardization.

Marker development and linkage map construction

The linkage map was developed with both SSR and SNP markers. Cucumber SSR markers described by Ren et al. (2009), Cavagnaro et al. (2010), and Yang et al. (2012, 2013) were used for polymorphism screening between WI7120 and 9930. SNP genotyping was performed in the Bayer Vegetable Seeds, the Netherlands genotyping facility using the KBiosciences Competitive Allele-Specific PCR SNP genotyping system (KASPar). Polymorphic markers were used to genotype a subset of 91 F_2 plants. To refine QTL locations in four target regions, these markers mapped in 91 F_2 plants and 27 new SSRs were also applied to the larger population including all 243 F_2 plants. All markers were tested against the expected segregation ratio of 1:2:1 or 3:1 using Chi square tests ((χ^2, $P < 0.05$). Linkage analysis was carried out with JoinMap 4.0. Genetic map was developed with the regression mapping method and Kosambi mapping function. The physical locations of all mapped SSR markers in the Gy14 (V1.0) (Yang et al. 2012) and 9930 (V2.0) (Li et al 2011; http://www.icugi.org/) scaffold and draft genome assemblies were used to verify their genetic map positions.

DNA extraction, PCR amplification of molecular markers and gel electrophoreses was conducted as described in Li et al. (2013).

QTL analysis

QTL analysis was performed using the R/qtl package with the multiple-QTL model (MQM) (Broman et al. 2003; Arends et al. 2010). QTL detection started with “scanone” for preliminary QTL identification, and followed with “addqtl” and “addpair” to scan additional QTLs and QTL pairs. Then the function ‘refineqtl’ was used to refine the position of QTLs. The significance of each QTL interval was tested by a likelihood-ratio statistic (LOD). The LOD threshold for declaring significant QTLs was established separately for each trait and each environment using 1,000 permutations at significance threshold of 0.05. The refined significant QTLs were assessed for the additive and dominant effects, and percentage of phenotypic variations (R^2) explained. The support intervals for these QTLs were calculated using a 1.5 LOD drop interval. QTL naming conventions followed He et al. (2013) except each QTL was preceded with dm (for downy mildew).

Results

Phenotyping DM inoculation responses among $F_{2:3}$ families

Phenotypic data of DM inoculation responses among 243 $F_{2:3}$ families of 9930 × WI7120 were collected from four environments (US2013, US2014, NL2013, and IT2013) over two years (2013, 2014) in three countries. While disease rating in all experiments was based on a GI of foliage symptom development, two additional criteria, Nec and Chl were used in rating of DM disease symptoms in NL2013. The grand means and standard deviation (SD) of DM disease scores for the $F_{2:3}$ families, the two parental lines, F_1 and controls across the four environments are presented in **Table 2.1**. Frequency distribution of F_3 family means of the four experiments are illustrated in **Figure 2.2A-C**. In all experiments, the mean disease scores of the two parents (WI7120 and 9930) were located at the extreme ends of the largely normally distributed family means, and those for the F_1 and the F_3 families were close to midparent values suggesting the quantitative nature of DM resistance in WI7120. The normal distribution of residual phenotypic variations could also be reflected from QQ-plots of the four experiments (**Figure 2.3A**). As expected, the susceptible control Straight 8 remained highly susceptible as 9930 whereas the pre-2004 DM resistant Poinsett 76 and WI2757 showed intermediate resistance although the later performed a bit better (**Table 2.1**). These observations indicated satisfactory control of environmental factors in each experiment.

Different rating scales were employed in the three locations (1-9 in US2013, US2014; 9-1 in NL2013; 1-5 in IT2013). To compare environmental effects on DM resistance, the phenotypic data for each experiment were standardized using the z-score in which the IT2013 and NL2013 datasets were converted to the same scale as the US experiments. The frequency distribution of the standardized data from the four environments is shown in **Figure 2.2D**, which again was largely normal (**Figure 2.3B**). A one-way ANOVA was performed using the standardized data and the results are presented in **Table 2.2** which indicated no significant environmental effects on DM resistance. The broad-sense heritability estimate of mean disease scores (H^2) based on ANOVA was as high as 0.89 (**Table 2.2**) further indicating effective control of environments in these trials.

We calculated the Spearman's rank correlation coefficient (r_s) of different traits from the four environments using both the raw and standardized data. The results are provided in **Table 2.3**. The data among the four experiments were highly and significantly correlated with r_s ranging from 0.592 to 0.796 ($P < 0.001$) implying the consistence and reliability in DM ratings among environments. When the raw data were used in calculation of r_s, the mean disease scores of NL2013 (GI, Nec and Chl) were negatively correlated to those from other three experiments. This is reasonable because NL2013 experiment used a reversed rating scale (plants rated 1 were the most susceptible).

DM infection may result in chlorosis and necrosis of the plants. In NL2013, phenotypic data of DM inoculation responses were collected using three criteria: Chl, Nec, and Gl. No significant differences in mean disease scores were observed among the three criteria in both parents and segregating populations (**Table 2.1**). All three parameters were positively and highly correlated with each other and with those in US2013, US2014 and IT2013 (**Table 2.3**) suggesting that these symptoms may share similar underlying genetic mechanism in WI7120.

In US2013, US2014, and NL2013 trials, rating of disease symptoms was conducted multiple times with one week apart. The boxplots of mean disease scores at each time point are shown in **Figure 2.4A**. In US2013 and US2014, it was clear that with the progress of the disease, the overall mean disease score of the entire population shifted toward susceptibility with increasing variations within the population. On the other hand, this trend was not obvious in the NL2013 trial. The population dynamics had obvious consequences in QTL detection (see below).

To summarize, despite of the different environments, scoring scales and methods used in the four phenotyping experiments, data collected from these trials were highly correlated, consistent, and of good quality, which provided a solid foundation for subsequent QTL analysis.

Linkage map construction

Among 1,440 cucumber SSR markers screened, 441 (30.6%) were polymorphic between 9930 and WI7120, 271 of which were genotyped in 91 F_2 individuals. These plants were also subjected to KASPar SNP assay and 76 polymorphic SNPs were successfully mapped. On the resulting map, there was a large gap (~16 cM) at the top of Chr4. This $F_{2:3}$ population was segregating for mature fruit skin color and fruit spine color: WI7120 set netted brown fruits with black spines and 9930 had light yellow fruits with white spines. We phenotyped spine color in this population, and the underlying gene for black spine happened to be mapped in the 16-cM gap region. The location of the gene was consistent with previous work by Li et al. (2013) who identified a candidate gene for the black spine color gene *B* in the distal region of the short arm of cucumber chromosome 4. Therefore, it is reasonable to conclude that the black spine color in WI7120 was controlled by the same *B* locus as identified in Li et al. (2013).

The resulting genetic map is illustrated in **Figure 2.5** and the main statistics of the map are presented in **Table 2.4**. Detailed information of 348 mapped loci is provided in **Appendix 2.1**. While the majority of the marker loci fitted the expected 1:2:1 or 3:1 segregation ratio, 23 markers (~7%) showed distorted segregation (loci with asterisks in **Appendix 2.1**) which were mainly in two clusters in Chr5 and Chr7. In both clusters, the 9930 alleles showed preferred transmission over those of WI7120, which is consistent with observations in two previous studies (Miao et al. 2011; Rubinstein et al. 2015).

The genetic map covered 674.7 cM with 7 linkage groups (chromosomes) with an average interval of 2.0 cM (**Table 2.4**). According to the 9930 and Gy14 draft genome scaffolds associated with these markers, this map seemed to physically cover the majority of the cucumber genome. The marker orders were also highly consistent with their physical locations.

Detection of DM resistance QTLs

We conducted QTL analysis using the MQM approach with data for each location, time point and disease rating criterion. The LOD threshold to declare significance of QTL for each trait was determined with 1,000 permutations ($P = 0.05$), which varied from 3.4 to 3.6. We first investigated the effects of scoring time on the ability of QTL detection, and the results are shown in **Figure 2.4B**. We found that for US2013 and US2014, QTL on chromosomes 4 and

5 could be detected with data across all rating times whereas the additional QTL on chromosome 2 and 6 could only be detected in the later scorings (2nd and 3rd). On the other hand, both scoring times detected 3 QTL on chromosome 2, 4 and 5 for GI (general impression) and Nec (necrotic lesion size) in the NL2013 experiment (**Figure 2.4B**). This result was consistent with the phenotypic variations of mean disease scores at these time points. That is, larger variations within the population allowed detection of more QTL (**Figure 2.4A-B**). Since the last scoring time detected the most DM resistant QTL in all experiments, we used the datasets at this time point in subsequent discussions.

We examined the relationships of QTL detected with three different scoring criteria (GI, Nec, Chl) in NL2013 using MQM. The results are presented in **Table 2.5** and **Figure 2.6**. All three datasets detected QTL in Chr2, Chr4, and Chr5. The 1.5-LOD intervals and peak locations of QTL in Chr4 and Chr5 were largely overlapped suggesting they probably belonged to the same QTL in each chromosome although the LOD support value and the effects were somewhat different (**Table 2.5**). For the QTL in Chr2, the Nec QTL peak location was shifted from that for GI and Chl (**Figure 2.6**), which was probably a sampling error because its peak location was much closer to that of the other two QTL when a large population (243 $F_{2:3}$ families) was used in QTL analysis (see below). In chromosome 6, two QTL were detected, one with Chl (chlorotic lesion size) designated as *dm6.2-Chl*, which might be associated with chlorosis symptom development in response to DM infection. Another QTL, *dm6.1*, was detected with Nec, which had a LOD support score of 2.1 (**Table 2.5**) but was likely true since it was also detected with US2013, US2014 data and BLUPs (see below). These two QTL, *dm6.1* and *dm6.2-Chl* were clearly different because their peak locations in chromosome 6 were far away from each other (**Figure 2.6 and Table 2.5**). Overall, the results from NL2013 suggest that GI, Nec and Chl shared similar QTL in chromosomes 2, 4 and 5 whereas the QTL in Chr6 may be unique to responses to chlorosis.

Since GI represented integrated information of both necrotic and chlorotic responses to DM infection, the GI data from NL2013 were used for subsequent analysis with data from other environments.

QTL analysis was performed using the MQM approach with mean disease scores (raw data) from the four environments and BLUPs of the entire study. Details of each detected QTL including map location, LOD support value, percentages of total phenotypic variances explained (R^2), additive and dominant effects, and 1.5-LOD support interval are provided in **Table 2.5** and graphically presented in **Figure 2.7**. Note that the graph in **Figure 2.7** was generated with the *mqmscan* utility in R/qtl, thus the positions of QTLs in **Figure 2.7** might be somewhat different from data presented in **Table 2.5**. Comparing results among these experiments, it was clear that three DM resistance QTL, *dm2.1*, *dm4.1* and *dm5.1* located in chromosomes 2, 4, and 5, respectively, were consistently and reproducibly detected in all four environments and BLUPs. These QTL could explain 56.2-75.5% phenotypic variations. A fourth QTL, *dm6.1* in chromosome 6 could be detected in US2013, US2014, NL2013-Chl, as well as with BLUPs (**Table 2.5**).

Among the 4 DM resistant QTL, *dm4.1* on Chr4 had the largest effect accounting for 20.9-50.7% phenotypic variations followed by *dm5.1* (R^2 = 9.5-22.4%). The QTL on Chr2, *dm2.1* showed moderate effects with R^2 ranging from 5.4% in US2013 to 15.7% in IT2013. The fourth one, *dm6.1* in Chr6 was a minor-effect QTL that was detected in two environments and the BLUPs explained only 3.8-5.5% of the phenotypic variations (**Table 2.5**).

In US2013, US2014 and IT2013, all resistance contributing alleles were from WI7120 QTL which could be reflected from the negative additive effects of each QTL (reduction of disease score). Since the reverse DM rating scales were used in NL2013, the positive additive effects of all QTL indicated increase of the score (higher DM resistance). Dominance effects of all QTL were in general weak (**Table 2.5**).

Refinement of QTL locations

Our above-described initial QTL analysis with 91 $F_{2:3}$ families identified four DM resistant QTL in WI7120 including two major-effect (*dm4.1* and *dm5.1*), one moderate-effect (*dm2.1*) and two minor-effect (*dm6.1* and *dm6.2-Chl*) QTL (**Table 2.5**). While the 1.5-LOD interval and peak locations of *dm4.1* and *dm5.1* were relatively consistent across different environments, those for *dm2.1* and *dm6.1* showed more variations (**Figure 2.7**), which may be due to the small population size and their relatively smaller contributions to overall phenotypic variations. Thus, we expanded the population size to include all 243 $F_{2:3}$ families that we phenotyped in four environments aiming to refine the QTL locations on the genetic map. We also added more markers in the four target QTL regions. Information on the resulting genetic map (99 loci with 27 new SSR or indel markers) is provided in **Appendix 2.2**. LOD profiles of *dm2.1*, *dm4.1*, *dm5.1* and *dm6.1* based on data of four environments and BLUPs are presented in **Figure 2.8**; detailed information for each QTL is summarized in **Table 2.6**.

The increased population size (from 91 to 243) allowed detection of new QTL. First, *dm6.1* was identified with the NL2013-GI data which failed to detect in the 91-F_3 population (**Table 2.5**). Second, the *dm6.2-Chl* locus was supported by IT2013 dataset which did not identify *dm6.1* though. This was probably due to the fact that the scoring of disease symptoms in IT2013 relied more on chlorotic lesion sizes. This large population size not only confirmed the four QTL (*dm2.1*, *dm4.1*, *dm5.1* and *dm6.1*), but also significantly improved the precision of the location of each QTL. The peak location and 1.5-LOD support interval of each QTL from each environment as well as BLUPs were highly consistent. The QTL detected with BLUPs had the highest LOD support (**Figure 2.8**) suggesting BLUP is more powerful in detecting the small effects QTL in this population. The size of the 1.5 LOD-interval was also significantly narrowed down as compared with that based on the small population size (**Figure 2.8**). For example, the physical size of the 1.5-LOD interval for the major-effect QTL *dm4.1* in Chr4 was reduced from 4.2 Mbp (**Table 2.5**) to 1.2 Mbp in the 9930 V2.0 draft genome assembly that was delimited by two flanking markers UW083868 and UW004293 (**Appendix 2.2**).

We investigated possible interactions among these QTL. Line interaction plots of *dm2.1* (CHXSSR00134 at 37.6 cM), *dm4.1* (UW059163 at 22.9 cM), *dm5.1* (SSR15321 at 51.6 cM), and *dm6.1* (SSR18651 at 46.9 cM) (**Appendix 2.2**) are illustrated in **Figure 2.9**. Typical parallels across all genotypes were observed indicating that there was no strong epistatic interaction between two major-effect QTL *dm4.1* and *dm5.1*(**Figure 2.9B**). However, it seems that both *dm2.1* and *dm6.1* had epistatic interactions with either *dm4.1* or *dm5.1* and with each other (**Figure 2.9A, C-F**). The dominant effect of *dm2.1* in either *dm4.1* or *dm5.1* was evident from the fact that the mean disease score of the heterozygotes (ab) was the same as the homozygotes (bb) in both interaction plot (**Figure 2.9A and C**).

Discussion

Rating scales and times for phenotyping DM inoculation responses in cucumber: more is better?

In this study, we performed QTL analysis using phenotypic data collected in four environments (US2013, US2014, IT2013 and NL2013) and identified 4 DM resistant QTL in 4 chromosomes (*dm2.1*, *dm4.1*, *dm5.1* and *dm6.1*). Despite of the fact that the four experiments were different in experimental design, rating criteria and scales, environmental conditions, and population dynamics of the DM pathogen (see below for discussion), all 4 QTL were consistently identified in at least 3 of the 4 environments with high LOD support

(**Figure 2.8**, **Appendix 2.2** and **Table 2.6**) suggesting these DM resistant QTL are authentic and the phenotypic data are of high quality. Many factors affect the QTL detection power, some of which, such as multiple-location and multi-year trials, or large segregating population sizes for data collection, are well known from the statistical point of view. Our work also revealed some other important factors such as disease rating scale, scoring time or summary statistics that may affect the detection power for DM resistant QTL in cucumber.

In QTL mapping studies for DM resistance in cucumber, the rating scales of disease severity varied from 0-5 (Zhang et al. 2013), 1-9 (Kozik et al. 2013), 9-1 (Pang et al. 2013), and 0-9 (Call et al. 2012a) (from most resistant to most susceptible). It is a challenge to compare the data from different studies. In the present study, three different rating scales were used including 1-9 in US2013 and US2014 (**Figure 2.1**), 9-1 in NL2013, and 1-5 in IT2013, which provided a good opportunity to assess the efficiency of different rating scales on QTL detection. Since the phenotypic data showed largely normal distribution in all 4 environments (**Figure 2.2**, **Figure 2.3**), we were able to standardize the ordinal rating data for each environment using the same scale as US2013 and US2014, which made it possible to evaluate the inheritance of DM resistance in WI 7120 across multiple environments and extract BLUPs from linear models.

The different ordinal rating scales seemed to contribute to some extends of the varying power for detection of DM resistance QTL. As compared with other three experiments using 9 rating scales, IT2013 data (with 5 rating scales) failed to detect *dm6.1* in both small and large F_3 populations, and the LOD support score for QTL detected with this dataset was relatively lower (**Table 2.5 and Table 2.6**). Since there were only five categories (1, 2, 3, 4, and 5), each interval includes a larger range of disease severities, which cause the deficiency of additivity and reduce the power in QTL detection (Xie et al. 2012). Increasing the categories in ordinal rating allows addition of more distinguishable units, thus reduce bias of the class midpoints and residual variances. In general, the closer the scale of collected data is to a ratio scale with normal distribution, the more powerful methods are available for analysis (Hartung and Piepho 2007; Poland and Nelson 2011). Therefore, for phenotyping of DM inoculation responses, a 9-scale rating system may be preferable to the 5-scale method. However, considering other factors (phenotyping environment, inoculation method, pathogenicity of DM pathogen inoculum etc.) may also influence the QTL detection power in the IT2013 experiment, more rigorous experiments may be needed to make a solid conclusion. Nevertheless, while the 9-scale rating system can increase the QTL detection power, it also offers advantage for comparison with data deposited in public databases such as the USDA Germplasm Resources Information Network (GRIN) (Postman et al. 2010).

Natural inoculation of the DM pathogen was used in US2013, US2014 and NL2013 trials, and phenotypic data were collected from the adult plants. The performance of the plants was the interaction of intrinsic genetics and environmental factors. In the field, the distribution of DM inoculum was not uniform across the plots, which depends largely on the direction of the airflow (wind). This was evident from the heatmaps of disease scores in the field (data not shown) over time. Therefore, to reveal the true QTL underlying host resistance, it is important to conduct the scoring multiple times especially for evaluating adult plants. Indeed, in US2013 and US2014 trials, among the three datasets collected at three time points, the last dataset could detect the most DM resistant QTL, whereas no additional QTL information was obtained in the second scoring time in NL2013 (**Figure 2.4**; **Table 2.5**) suggesting that the third time of US2013 and US2014 or either time of NL2013 was the preferred time points for phenotypic data collection.

In reality, it is difficult to predict the best time for scoring. Due to the associated cost in time and labor, the number of times for scoring is also limited. Often, DM resistant QTL detected in different environments were not overlapped (e.g., Zhang et al. 2013; Yoshioka et al. 2014), or the QTL detected were in the same region but with different peak locations, large LOD-support interval or insignificant LOD support (e.g., **Figure 2.7**). In the present study, we found that use of BLUPs was able to help mitigate the effects of growth stages or scoring time on disease scores and increase QTL detection power (**Figure 2.8**). As compared to using the grand means to estimate the true value, BLUP allows information to be borrowed across trials, depending on the size of the genetic correlations, which can improve the prediction at each particular trial, especially when within site replication is low (Gilmour et al. 2009). This was especially true in the large population (243 $F_{2:3}$ families) (**Figure 2.8**). In a small population, the environment variations could be overestimated and the BLUP would deviate too much to the "true value" while the estimation of the environment and population variances is more accurate in a larger population. This can explain why the BLUP-based DM resistance QTL had the strongest LOD support and were highly consistent in location and 1.5-LOD interval with another QTL (**Figure 2.8**).

Chlorosis, necrosis and general impression: different criteria under the same mechanisms?

Cucumber DM is a foliar disease. Its symptoms are easily recognizable by the formation of chlorotic on leaf surface, which become necrotic with the progress of the disease (Oerke 2006; Savory et al. 2010). The speed of necrosis depends on the environment condition which occurs more quickly in hot, dry weather (Cohen and Rotem 1971). Criswell et al. (2008) and Call et al. (2012b) found that Chl and Nec of DM symptoms are highly correlated and suggested that they are likely the same trait. Pang et al. (2013) used Chl (yellowing), Nec as well as GI to evaluate DM inoculation responses, and identified two QTL for both Chl (YL-5.1, YL-5.2) and GI (DM-5.1 and DM-5.2); YL-5.1 and DM-5.1, as well as YL-5.2 and DM-5.2 were, respectively co-localized in chromosome 5 suggesting the two traits may be under the control of the same genetic mechanism(s). Of the two Nec QTL identified, one (Nec-5.1) was co-localized with YL-5.1/GI-5.1, and the other was mapped in chromosome 6 (Nec-6.1) (Pang et al. 2013) which seemed to be consistent with the *dm6.1* location in the present study (**Figure 2.8**). In this study, we found that QTL detected by Chl, Nec and GI in NL2013 were co-localized in chromosomes 2, 4, 5, and 6. These locations were also consistent with corresponding QTL detected with datasets from other three environments (**Figure 2.8**, **Table 2.6**). On the other hand, the minor-effect QTL *dm6.2-Chl* detected by NL2013-Chl data was mapped in a different location from *dm6.1* in chromosome 6 that was also detected with IT2013 data in the 243-F_3 population (**Figure 2.6**, **Figure 2.8**), which may be uniquely associated with chlorosis development upon DM pathogen infection. Despite of this, since the two studies (Pang et al. 2013; this study) employed very different sets of plant materials, these results suggest that DM resistance for chlorosis, necrosis or general impression in cucumber may share the common mechanism(s). Therefore, it is probably not necessary to use all the three criteria in future QTL mapping studies for DM resistance.

WI7120 (PI 330628): a genetically unique source of resistance for downy mildew pathogen?

In this study, the alleles of the 4 DM resistant QTL contributing to increased disease resistance were all from WI7120 (**Table 2.6**; **Figure 2.8**). We compared QTL mapping results from the present study and early studies, and the approximate locations of previously identified QTL are aligned on the genetic map developed herein, which is shown in **Figure 2.5**. This map only contain QTLs detected from PI lines that were reported to confer high resistance to the post-2004 strain in the US (Call et al. 2012a, b) including PI 197085 (Szczechura et al. 2015), PI 197088 (Yoshioka et al. 2014), and PI 330628 (this study). QTL mapping studies with lines from North China cucumber types were also included (Ding et al 2007; Bai et al. 2008; Zhang et al. 2013).

DM resistant QTL have been detected in all seven cucumber chromosomes. Yoshioka et al. (2014) detected two QTL in Chr3 (from CS-PMR1), one in Chr7 (from Santou). Chr2 harbored the moderate-effect QTL *dm2.1* detected only in the present study. Using 77 $F_{2:3}$ families and the BSA (bulked segregant analysis) approach, Ding et al. (2007) identified a single recessive locus that was responsible for the DM resistance in a north China type cucumber line '129', which seemed to be close to *dm4.1*, the major-effect QTL identified in WI7120 from our study (**Figure.2.5**). Bai et al. (2008), Zhang et al. (2013) and Yoshioka et al. (2014) all detected a DM resistance QTL at the distal end of the short arm of chromosome 1 with the resistance from north China or Japanese cucumber lines 'S94', 'K8' and 'Santou', respectively. Since Santou also had north China cucumber in its pedigree (Yoshioka et al. 2014), it seems that the north China fresh market cucumber carries the DM resistance QTL in chromosome 1 (**Figure 2.5**), which was not detected in any PI lines of India or Pakistan origin (PI 197085, PI 197088 or PI 330628). On the other hand, the minor-effect QTL *dm6.1* detected in the present study shared the similar location with a minor-effect QTL detected in the north China type cucumber line K8 by Zhang et al. (2013).

Cucumber chromosome 5 seems to harbor major-effect QTL for DM resistance from different sources (**Figure 2.5**) including *dm-1*, the single recessive DM resistance locus originated from PI 197087 which was loosely linked with the dull fruit skin gene *D* (van Vliet and Meysing 1974; Fanourakis and Simon 1987; Kennard et al. 1994; Horejsi et al. 2000). Zhang et al. (2013), Yoshioka et al. (2014) and Szczechura et al. (2015) each identified 3 QTL in chromosome 5 for DM resistance in K8, PI 197088 and PI 197085, respectively although the exact locations of these QTL in each line and their relationships are not well characterized. Bai et al. (2008) identified one QTL from S94 in chromosome 5 which was in the distal end of the short arm of Chr5 whereas the only QTL, *dm5.1* we identified in WI7120 seemed to correspond to the QTL region detected by Zhang et al. (2013) and Yoshioka et al. (2014) (**Figure 2.5**). These results suggest that, although PI 197085, PI 197088 and PI 330628 were all highly resistant to the post-2004 DM pathogen strain in the US cucumber field, the underlying genetic mechanisms may not be the same. Regardless, our study revealed the unique genetic architecture of DM resistance in WI7120. The three QTL, *dm2.1*, *dm4.1* and *dm5.1* together could explain up to 75% phenotypic variations with both *dm4.1* and *dm5.1* major-effect QTL showing additive effects (**Table 2.6**). Such information is important for efficient use of this resistance source in cucumber breeding for DM resistance.

DM resistance in WI7120 versus Ps. cubensis races: one-fit-all?

The population of the DM pathogen *Ps. cubensis* in the field is highly heterogeneous and dynamic, which may consist of many isolates, pathotypes or races with varying degree of pathogenicity or virulence (Lebeda and Urban 2007; Quesada-Ocampo et al. 2012; Lebeda et al. 2013; reviewed by Cohen et al. 2015). Molecular fingerprinting studies have revealed that, while most *Ps. cubensis* isolate groups were present in all continents examined, the dominant ones were structured by geographic origin across continental scales (Quesada-Ocampo et al. 2012), which may result in differential inoculation responses on the same cucumber genotypes. For example, the *Ps. cubensis* population structure in the post-2004 cucumber field in the US was different from that in Europe; cucumber cultivars carrying *dm-1* from PI 197087 have only moderate resistance in post-2004 fields in the US (Call et al. 2012a, b), which however still performs quite well in European countries (e.g., Lebeda 1999; Kozik et al. 2013). The pathogen population structure may change over time and the virulence of *Ps. cubensis* population in the field may also shift (Quesada-Ocampo et al. 2012; Lebeda et al.

2013) thus rendering host resistance ineffective. This may be the reason for the defeat of host resistance genes in cucumber to the DM pathogen in the US in 1950's and early 2000's (Epps and Barnes 1952; Holmes 2004, 2006).

In this study, the responses of the WI7120 × 9930 F_3 mapping population to natural inoculation of the DM pathogen in US2013, US2014 and NL2013 were highly consistent which could be reflected from the significantly high and positive correlations of mean disease scores across these trials (**Table 2.3**). QTL analysis with data from these environments all supported the presence of four DM resistance QTL in WI7120 (**Figure 2.8**; **Table 2.4**). It is known that the population structures of *Ps. cubensis* in these fields are different (Quesada-Ocampo et al. 2012). Such results may suggest that host resistance in WI7120 is highly effective to current dominant isolate groups in the fields of the three countries (Italy, the Netherlands and the US), which is consistent with screening results in which PI 197088 and PI 330628 showed high resistance to DM inoculation in different countries in Asia, Europe and the US (Call et al. 2012b; Chen and Cohen 2013). However, this does not mean that WI7120 is resistant to all isolates in the field pathogen populations. Innark et al. (2014) conducted field screening of cucumbers for DM resistance in Thailand and found that cucumber accessions from East Asia or Southeast Asia (e.g., PI 489752 from China) exhibited better DM resistance than those from India or surrounding areas; for example, PI 330628 from Pakistan only had moderate resistance. Chen and Cohen (2013) found that PI 330628 (WI7120) was highly resistant against multiple isolates (A, B and C) of *Ps. cubensis* under laboratory, greenhouse and field conditions in Israel. However, the F_1 and F_2 plants of PI 330628 × SMR18 (susceptible) showed differential inoculation responses to the three isolates. These observations imply that there are isolates in the field could overcome different resistant genes in current resistance sources such as WI7120, which may potentially evolve to more virulent races through isolates mating and become the prevailing races in the future

(Cohen 2015). Therefore, further investigations are needed to understand the genetic and molecular mechanisms of DM resistance in WI7120, which is important for efficient deployment of this resistance especially in the context of population dynamics of the DM pathogen in the fields.

Author contributions

YHW performed the research and conducted data analysis. YQW designed this experiment, participated in data analysis. YHW, KV and TCW collected field data for US2013 and US2014 field trials. PAG and JS collected data for NL2013 experiment. XYZ and KO conducted IT2013 experiment. YHW and YQW wrote the manuscript with input from other coauthors. All authors reviewed and approved this submission.

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Conflict of interest statement

The authors declare no conflict of interest.

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Table 2.1 Mean and standard deviation (SD) of downy mildew disease scores (raw data) of the $F_{2:3}$ families, the two parental lines, their F_1 and controls across four environments.

Environments [a]	**9930 (P1)**	**WI7120 (P2)**	**F_1**	**$F_{2:3}$** [b]	**Poinsett 76**	**Straight 8**	**WI2757**
US2013	8.3±0.6	3.1±0.8	5.6±0.5	5.2±1.0 (2-9)	5.5±0.8	7.4±0.8	3.5±0.8
US2014	9.0±0.0	2.6±0.6	5.7±0.9	5.5±1.3 (2-9)	n/a [c]	n/a	n/a
NL2013-GI[d]	2.7±0.8	7.6±0.6	6.5±0.7	5.4±1.1 (2-8)	n/a	2.0±0.0	4.5±0.7
NL2013-Nec[d]	2.3±0.6	8.0±0.0	5.0±0.0	5.1±1.4 (2-8)	n/a	1.0±0.0	4.0±0.8
NL2013-Chl[d]	2.3±0.6	7.0±0.0	5.0±0.0	4.4±1.3 (1-8)	n/a	2.5±0.6	5.0±0.0
IT2013	5.0±0.0	1.3±0.5	4.0±0.0	3.0±0.8 (1-5)	n/a	n/a	3.3±0.5

Note:

[a] US2013 and US2014 were conducted at the Clinton Horticultural Crops Research Station of North Carolina State University at Raleigh, North Carolina in 2013 and 2014, respectively. NL2013 and IT2013 were performed in the field of Bayer Vegetable Seeds in the Netherlands, and in the greenhouses of Magnum Seeds in Fondi, Italy, respectively.

[b] Grand family means of disease scores. Range of disease scores in parentheses.

[c] Lines were not included in the trial and the data were not available.

[d] F-test revealed that there are no significant differences among three criteria (GI, Nec, and Chl) in NL2013 for both parents and segregating population.

Table 2.2 Analysis of variance (ANOVA) of F_3 family DM disease scores (standardized data) across four environments and estimate of broad-sense heritability.

Source of variations	**df**	**Mean Square**	**F value**	**P value**
Genotype (G)	242	2.92	9.35	<2e-16
Environment (E)	3	0.32	1.01	0.386
Residuals	669	0.31		
Heritability (H^2)	0.89±0.07			

Table 2.3 Spearman's rank correlation coefficients (r_s) of DM disease scores of F_3 families from different experiments with raw data (above the diagonal) and standardized data (below the diagonal).

	US2013	**US2014**	**NL2013-GI**	**NL2013-Nec**	**NL2013-Chl**	**IT2013**
US2013		0.796**	-0.599**	-0.706**	-0.527**	0.704**
US2014	0.796**		-0.563**	-0.684**	-0.537**	0.713**
NL2013-GI	0.599**	0.563**		0.776**	0.513**	-0.587**
NL2013-Nec	0.706**	0.681**	0.776**		0.486**	-0.596**
NL2013-Chl	0.527**	0.537**	0.513**	0.486**		-0.556**
IT2013	0.712**	0.712**	0.587**	0.592**	0.556**	

Note: ** $P < 0.01$

Table 2.4 Statistics of the linkage map developed with 348 markers and 91 F_2 plants from 9930 × WI7120 cross.

Linkage group (chr)	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**Sum**
# Mapped loci	53	50	52	48	55	55	35	348
Map Length (cM)	99.8	116.4	111.3	86.8	81.9	109.5	68.9	674.7
Average Map Interval (cM)	1.9	2.4	2.2	1.8	1.5	2.0	2.0	2.0
Maximum map interval (cM)	8.1	12.5	8.4	9.3	6.4	12.9	8.3	12.9

Table 2.5 Downy mildew resistance QTL detected in four environments with 91 $F_{2:3}$ families based on mean disease scores (raw data) or BLUP by the MQM model.

Environments	**QTL**	**Chr**	**Significant Loci**	**Peak (cM)**	**LOD value**	**1.5 LOD support interval**				**Additive Effects**	**Dominance effects**	**R^2 (%)**	**Total variations explained (%)**
						Left marker	**Left Position**	**Right marker**	**Right position**				
US2013	*dm2.1*	2	SSR13105	38.32	5.45	SSR398134	24.9	UW230458	47.0	-0.33	0	5.37	75.3
	dm4.1	4	SSR13864	57.39	18.87	SSR05125	49.6	SSR10368	59.8	-1.1	0	50.68	
	dm5.1	5	SSR20165	32.27	6.84	SSR03082	24.7	SSR15321	44.8	-0.46	0	9.46	
	dm6.1	6	SSR02768	97.24	3.6	SSR01012	17.2	SSR05946	105.8	-0.28	0.28	3.8	
US2014	*dm2.1*	2	SSR13105	38.32	5.3	UWSTS0384	28.1	UW230458	47.0	-0.52	0	8.6	72.2
	dm4.1	4	UW083868	53.54	11.2	SSR07130	48.5	UW083868	53.5	-1.08	0	27.2	
	dm5.1	5	SSR15321	44.85	9.9	UW053068	37.3	UW001847	51.8	-0.99	0.05	21.1	
	dm6.1	6	mCU13568	86.31	3.4	SSR07248	42.5	SSR05946	105.8	-0.46	0	5.5	
NL2013-GI	*dm2.1*	2	mCU18017	68.00	3.6	SSR00219	55.4	SSR16135	76.7	0.33	0.36	6.9	56.5
	dm4.1	4	mCU11245	52.00	6.8	SSR05415	46.1	SSR10368	59.8	0.63	0	15.2	
	dm5.1	5	mCU1293	35.25	11.0	SSR20165	32.3	SSR32717	42.0	0.90	0	27.9	
NL2013-Nec	*dm2.1*	2	UW084818	23.00	3.8	SSR00204	16.9	CHXSSR00134	36.1	0.65	0	10.1	65.7
	dm4.1	4	UW083868	53.54	13.5	SSR07130	48.5	SSR10368	59.8	1.14	0	27.4	
	dm5.1	5	SSR15321	44.85	12.6	UW053068	37.3	UW001423	46.8	0.99	0.20	22.4	
	*dm6.1**	6	SSR12510	87.40	2.1	SSR15245	19.2	mCU18950_k	109.5				

NL2013-Chl	*dm2.1*	2	SSR00219	58.00	7.2	UW023284	48.4	SSR13275	71.6	0.51	0.35	12.1	72.3
	dm4.1	4	mCU11245	52.00	12.4	SSR05415	46.1	SSR13864	57.4	0.84	0	21.5	
	dm5.1	5	SSR32717	40.00	10.4	SSR19178	36.1	mCU261_k	44.7	0.83	0	21.0	
	dm6.2-Chl	6	UW083983	64.00	5.1	SSR15067	51.1	mCU126_k	72.4	0.50	0	8.9	
IT2013	*dm2.1*	2	CHXSSR00134	36.15	5.6	UWSTS0384	28.1	UW023284	48.4	-0.31	0	15.7	61.8
	dm4.1	4	SSR17911	56.83	7.0	SSR05125	49.6	SSR10368	59.8	-0.43	0.02	20.9	
	dm5.1	5	SSR32717	41.98	4.4	UW043165	34.5	UW001847	51.8	-0.35	0	11.5	
BLUP	*dm2.1*	2	UW230458	46.97	4.1	CHXSSR00134	36.1	SSR12083	60.6	-0.29	0	8.8	56.2
	dm4.1	4	UW083868	53.54	11.9	SSR05125	49.6	UW083868	53.5	-0.66	0	30.5	
	dm5.1	5	SSR10348	22.88	7.4	SSR14517	20.3	SSR19178	36.1	-0.46	0.11	16.1	
	dm6.1	6	SSR18651	99.07	3.4	SSR12510	87.4	mCU18950_k	109.5	-0.2	0	5.9	

Note: **dm6.1* emerged under environment NL2013-Nec but is below the threshold, we listed it here as a comparison but not included for explained variation calculation.

Table 2.6 Consensus DM resistance QTL in WI7120 detected across four environments.

QTL	**Chr**	**Associated Marker**	**Peak (cM)**	**LOD Score**	**1.5 LOD support interval[a]**		**Additive effects[b]**	**Dominance effects**	**Phenotypic variation (%)**	**Environments and BLUPs Detecting the QTL with 243 F3 families**
					Left marker	**Right marker**				
dm2.1	2	CHXSSR00134	37.6	2.7-8.1	UW084818 (27.6)	UW230458 (48.3)	0.2-0.5	0	2.5-7.3	US2013, US2014, NL2013 (Chl, GI, Nec), IT2013, BLUP
dm4.1	4	UW059163	22.9	22.0-49.5	UW083868 (19.7)	UW004293 (24.7)	0.6-1.2	0-0.1	20.3-40.6	US2013, US2014, NL2013 (Chl, GI, Nec), IT2013, BLUP
dm5.1	5	SSR15321	51.6	10.8-28.5	UW002465 (41.9)	UW001370 (54.0)	0.4-0.9	0-0.1	8.5-20.8	US2013, US2014, NL2013 (Chl, GI, Nec), IT2013, BLUP
dm6.1	6	SSR18651	46.9	4.6-8.0	SSR01148 (39.1)	SSR05946 (52.0)	0.2-0.5	0	3.1-3.7	US2013, US2014, NL2013 (GI), BLUP
dm6.2-Chl	6	UW000765	13.5	2.8-4.6	SSR13996 (3.0)	SSR17818 (22.7)	0.2-0.5	0	2.9-4.5	NL2013 (Chl), IT2013

Note:

[a] Number in parentheses is map position (cM) of the corresponding marker.

[b] Absolute value (additive effects are negative for all except NL2013 due to inversed.

Figure 2.1 Disease rating scales and corresponding symptoms used phenotyping DM inoculation responses in US2013 and US2014 field experiments.

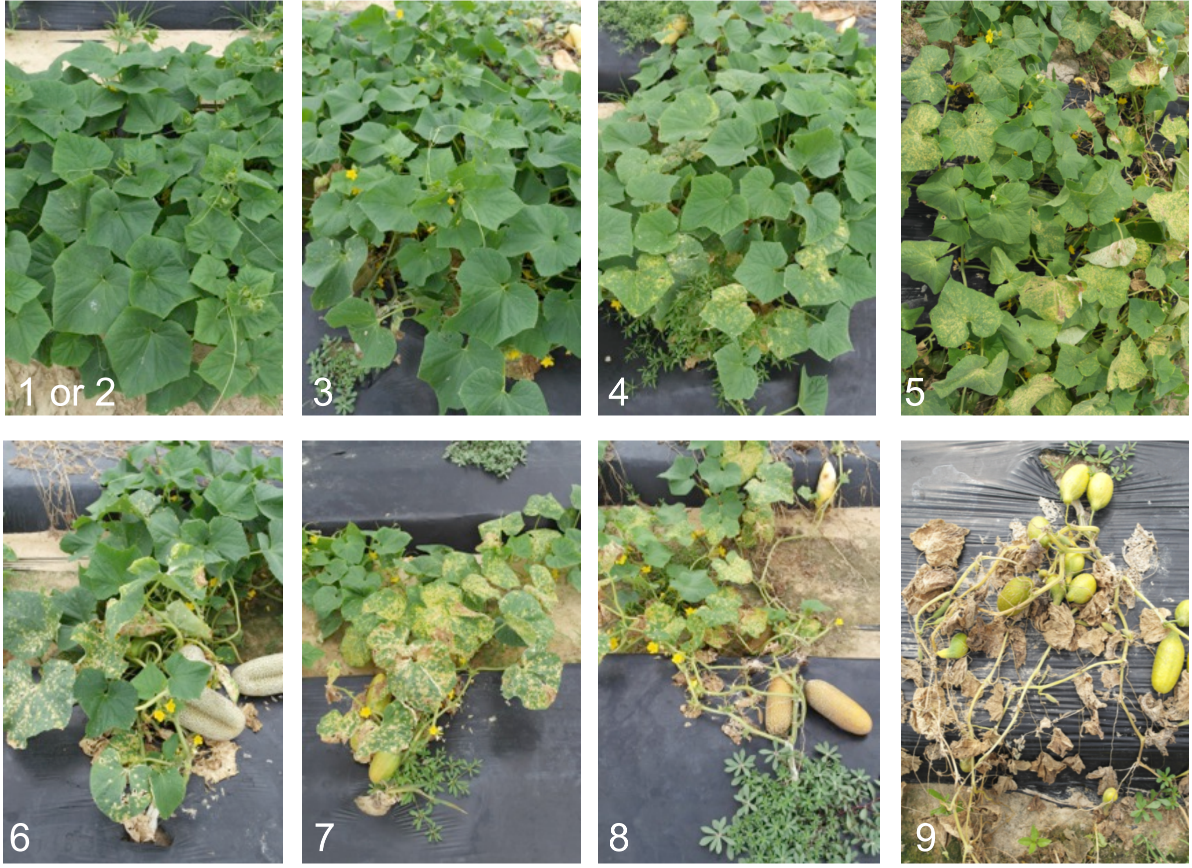


Figure 2.2 Frequency distribution of F_3 family means of downy mildew disease scores in US2013 and US2014 (A), NL2013 (B, GI dataset), IT2013 (C), and standardized data of all four environments. Note that the rating scale for IT2013 was 1–5, and 1–9 for other three experiments

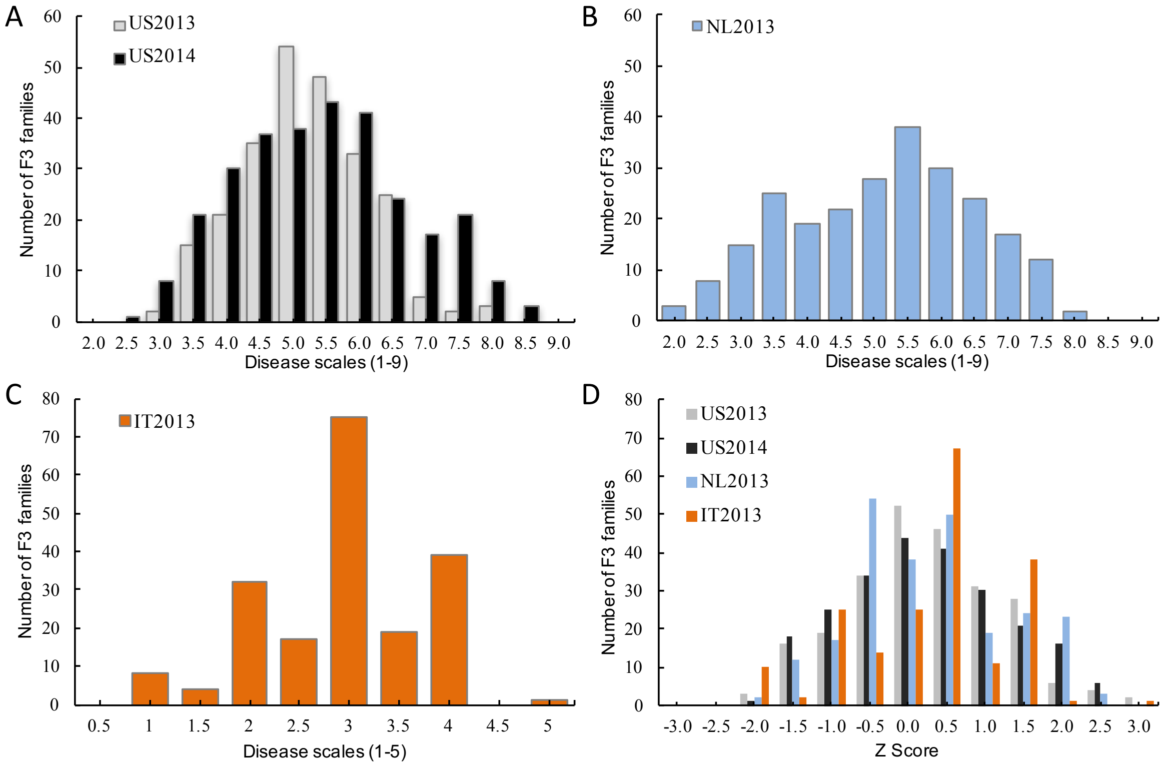


Figure 2.3 Q-Q plots on raw data (A) and standardized data (B) from four environments.

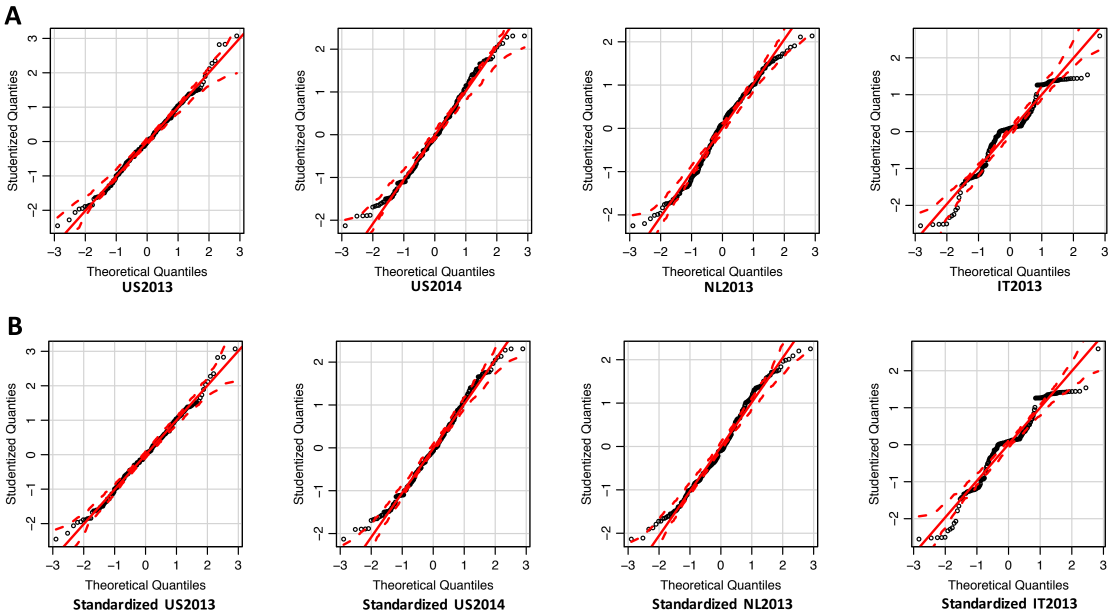


Figure 2.4 Boxplot of mean disease scores of 91 $F_{2:3}$ families at different rating stages in four environments (A) and chromosomal of locations of DM resistance QTLs detected at each stage. (B). The bottom, top lines of each box and the horizontal line inside the box represents 25th percentile, 75th percentile and the median of family means, respectively. The whiskers are the minimum and maximum observed values. The white circle is the mild outlier for single data point. Three ratings were conducted for US2013 (A1 to A3) and US2014 (B1 to B3). Plants were rated twice for NL2013-GI (C1, C2), and NL2013-Nec (D1 and D2). The chlorosis of NL2013 (Chl) was only scored once (E2). In B, the numbers are chromosomes where the DM resistance QTL were detected at each time point of disease scoring. One QTL was detected on each chromosome.

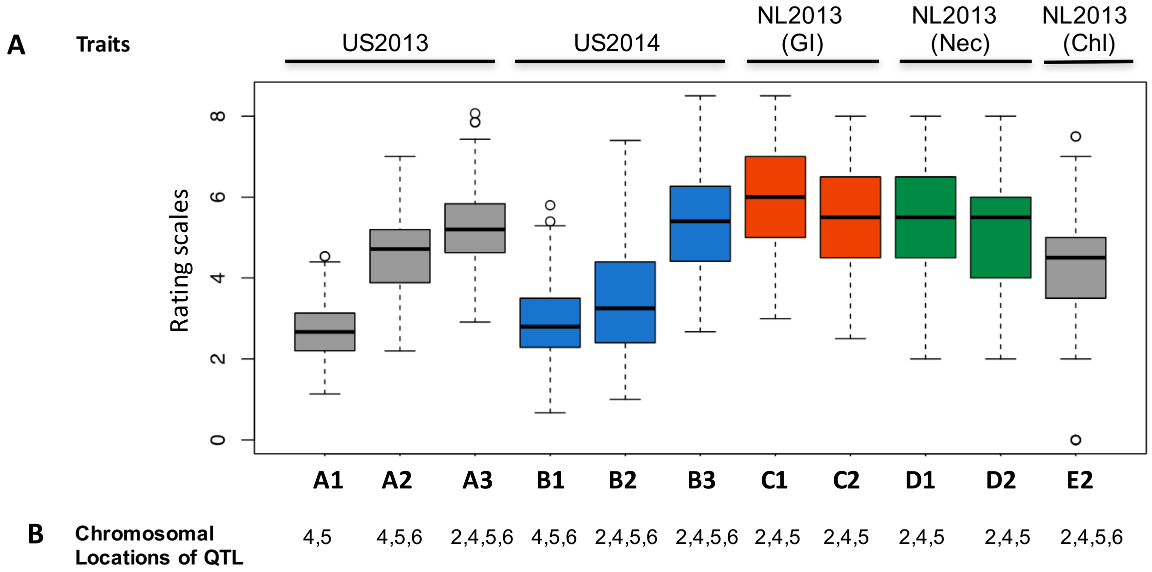


Figure 2.5 Linkage map developed with 348 marker loci and locations of DM resistance QTL from previous and present studies. DM resistance QTL detected in the present study are based on mean disease scores from 243 $F_{2:3}$ families of the 9930 × WI7120 cross; the 1.5-LOD support interval of each QTL detected from this study is represented by a vertical bar. The green dot within each bar indicates the approximate peak location. QTL locations from other studies were based on common markers, or inferred from *in silico* PCR or BLASTn using primer sequences of QTL-associated molecular markers, and are approximations.

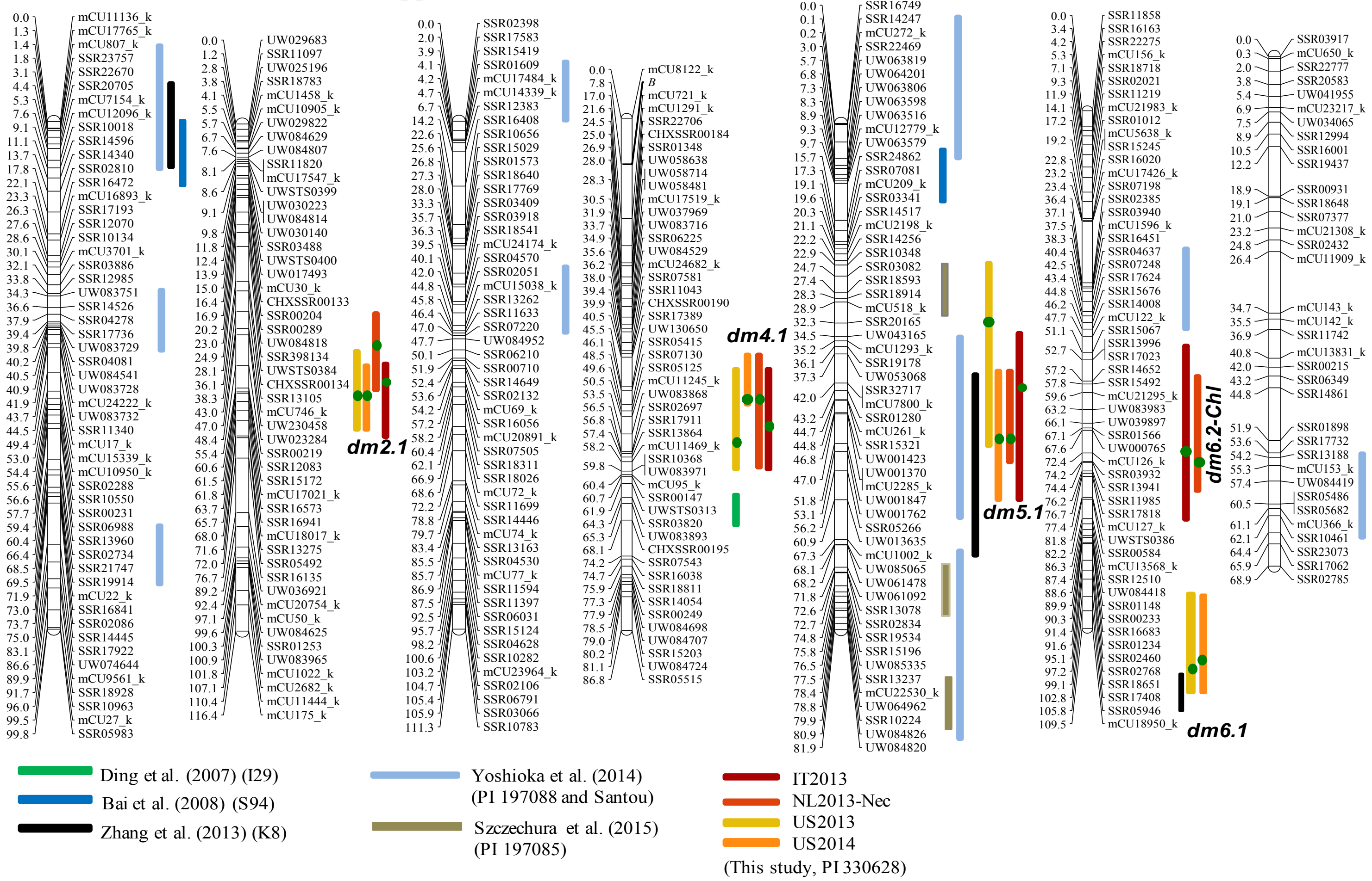


Figure 2.6 Whole genome view of QTL locations for DM resistance based on mean disease scores of three rating criteria (GI, Nec and Chl) from 91 $F_{2:3}$ families in NL2013 with the MQM model. The horizontal line represents LOD threshold obtained with 1,000 permutation tests (*P* = 0.05).

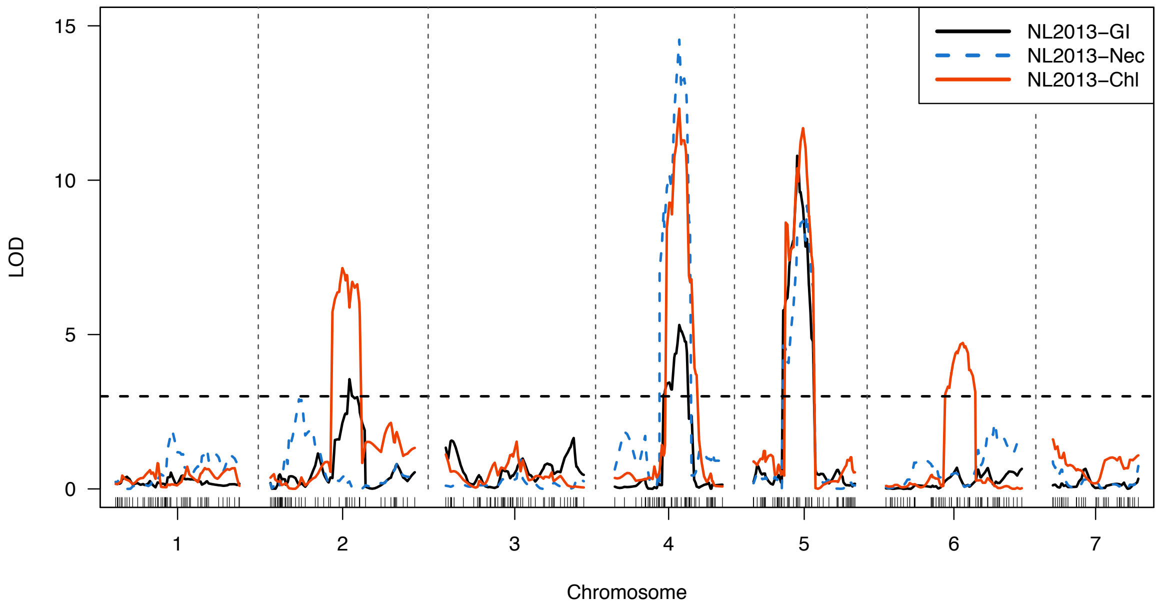


Figure 2.7 Whole genome view of QTL locations for DM resistance detected across four environments (US2013, US2014, IT2013 and NL2013) with data from 91 F_3 families by the MQM model. The GI data were used for NL2013 experiment. The horizontal line represents LOD threshold obtained with 1,000 permutation tests (*P* = 0.05).

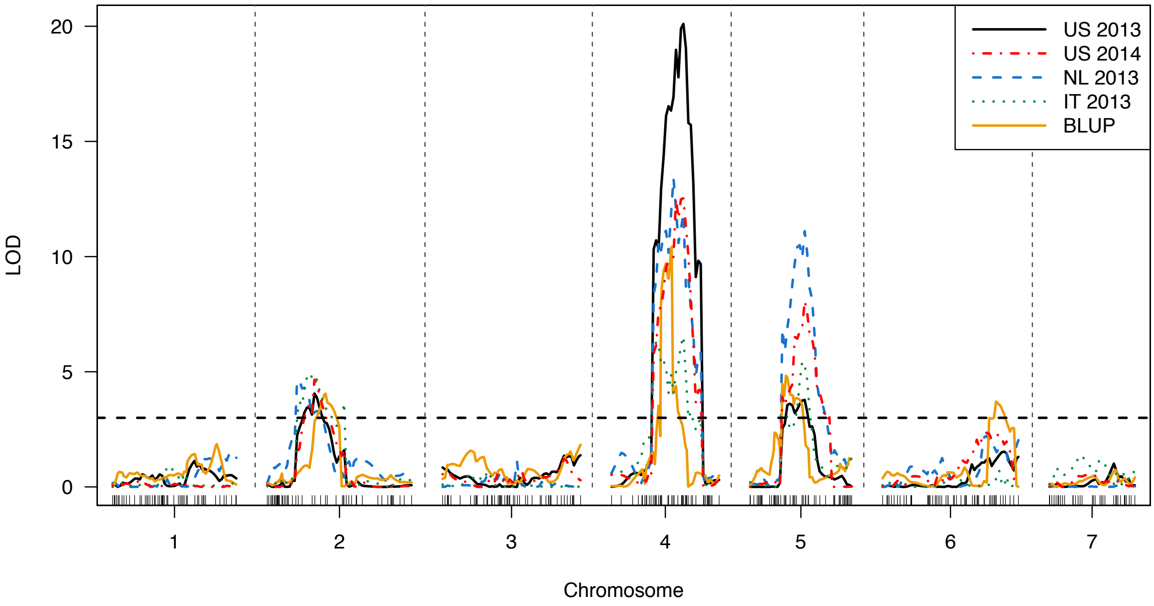


Figure 2.8 LOD profiles of downy mildew resistance QTLs in cucumber chromosomes 2, 4, 5, and 6 detected with the MQM model using mean disease scores and BLUPs of 243 $F_{2:3}$ families of the 9930 × WI7120 cross.

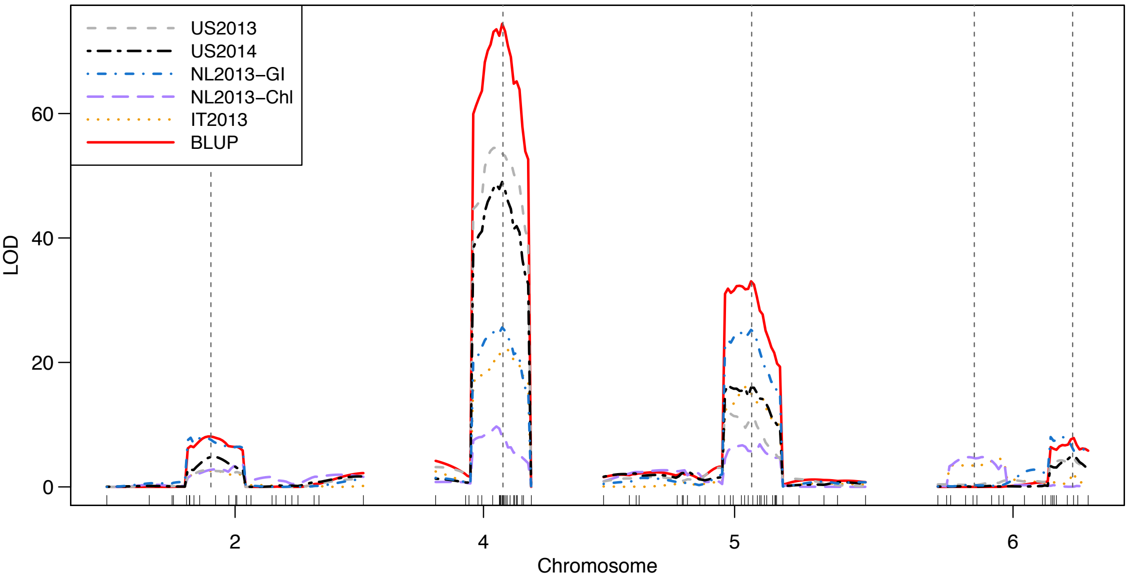
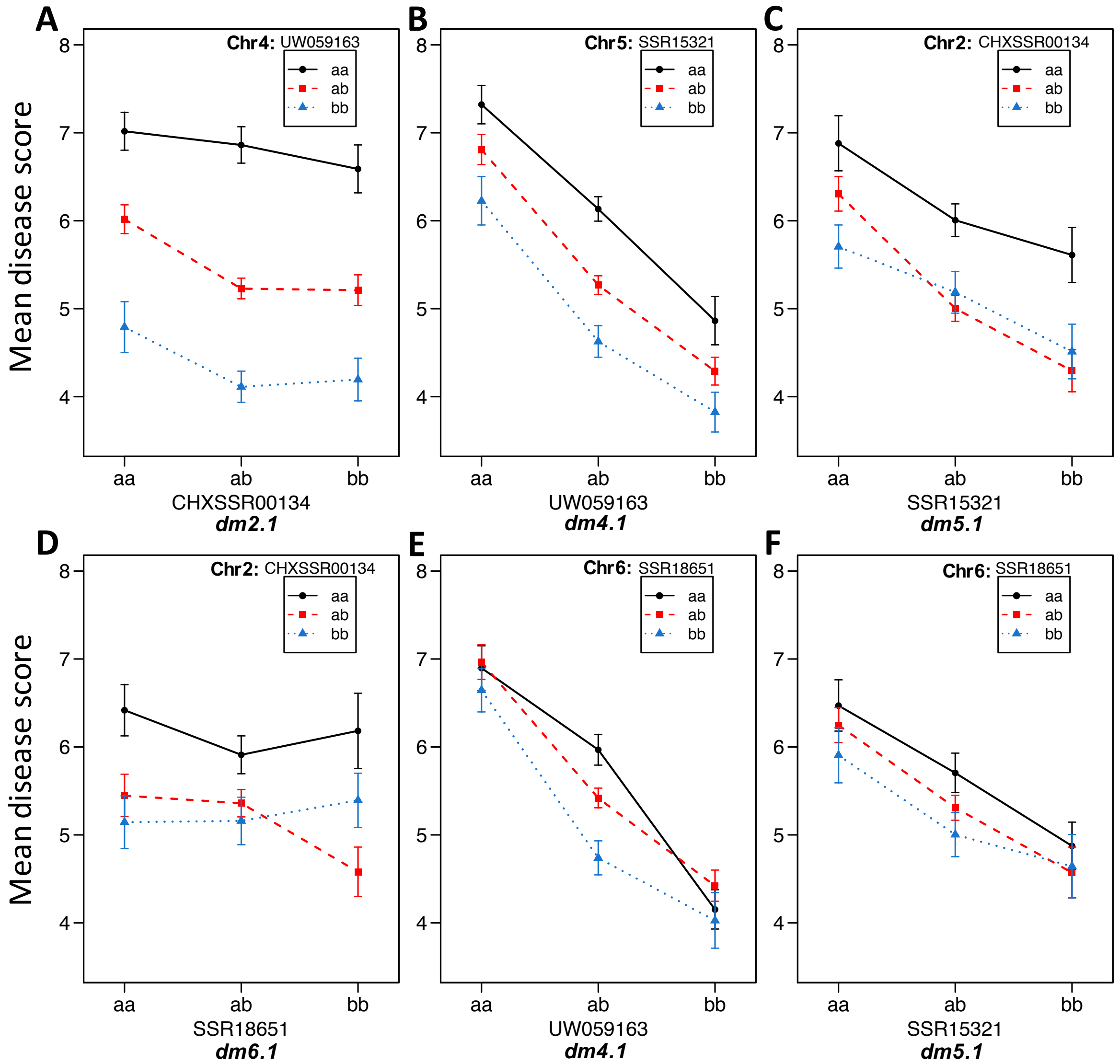


Figure 2.9 Effect plots showing interactions among loci near *dm2.1* (CHXSSR00134, 37.6 cM), *dm4.1* ((UW059163, 22.9 cM), *dm5.1* (SSR15321, 51.6 cM), and *dm6.1* (SSR18651, 46.9 cM) on DM resistance in 243 $F_{2:3}$ families from 9930 × WI 7120. Line interaction plots demonstrate that *dm2.1* has a completely dominant effect on DM resistance and strong epistatic interactions with *dm4.1* and *dm5.1*. There are no significant interactions between *dm4.1* and *dm5.1*. Note that "a" is allele from susceptible parent line 9930, while "b" is allele from resistant parent WI7120.



Appendix 2.1 Information of 348 SSR, SNP or morphological markers placed on the 9930 × WI7120 linkage map.

Appendix 2.1 is available in the following website as Table S2:
https://static-content.springer.com/esm/art%3A10.1007%2Fs00122-016-2719-x/MediaObjects/122_2016_2719_MOESM1_ESM.pdf

Appendix 2.2 Downy mildew resistance QTL detected in four environments with 91 F2:3 families based on mean disease scores (raw data) or BLUP by the MQM model. Appendix 2.2 is available in the following website as Table S3: https://static-content.springer.com/esm/art%3A10.1007%2Fs00122-016-2719-x/MediaObjects/122_2016_2719_MOESM1_ESM.pdf

Chapter 3: QTL mapping of downy and powdery mildew resistances in PI 197088 cucumber with genotyping-by-sequencing in RIL population

Abstract

The downy mildew (DM) and powdery mildew (PM) are the two most economically important foliar diseases of all cucurbits worldwide. PI 197088, an accession collected from India exhibits high level resistances to both pathogens. Here, this study reported the QTL mapping results for DM and PM resistances with 148 $F_{7:8}$ recombinant inbred lines (RILs) from a cross between PI 197088 and susceptible inbred line 'Coolgreen'. The phenotypic data on responses to natural infection of DM and PM pathogens were collected in multi-year and multi-location replicated field trials. A high-density genetic map with 2,780 SNPs from genotyping-by-sequencing and 55 SSR markers was developed using this RIL population. QTL analysis identified 11 QTL for DM resistance on six chromosomes with each explaining 2.0-31.0% of the phenotypic variance. Among them, three conferred susceptibility, which may explain the relatively poor performance of PI 197088 in DM resistance at late growth stage. Four QTL for PM resistance were detected with each contributing 1.8-32.4% of phenotypic variance. Three of the four PM QTL were co-localized with DM QTL which was consistent with the observation that PM and DM resistances in cucumber is often closely linked. Joint analysis of linkage mapping and transcriptome data revealed 139 differentially expressed genes in five QTL intervals including *dm3.2*, *dm4.1*, *dm5.1*, *dm5.2*, and *dm5.3*, which provided a good start point for identification of candidate genes for these QTL.

Introduction

The cucurbit downy mildew (DM) is caused by the obligate oomycete *Pseudoperonospora cubensis* (Berk. & Curt.) Rostovzev, while the powdery mildew (PM) is caused mainly by the fungus *Podosphaera xanthii* (Fr.) Braun and Shishkoff (syn. *Podosphaera fusca*). They are the two most economically important foliar diseases of all cucurbits worldwide. The symptoms of DM on susceptible cucumbers are characterized by the water-soaked lesions limited by leaf veins that turn chlorotic and necrotic with brown/black sporulation on the abaxial leaf surface, eventually causing the death of entire plants (Oerke et al. 2006). The PM fungi do not usually kill the plants. PM appears as white or grayish-white patches on the plant leaves, petioles, stems, and fruits, sometimes causing chlorosis due to decreased photosynthesis (Sitterly 1972; Pérez-García et al. 2009).

Early breeding efforts identified many cucumber lines with both DM and PM resistances, such as ‘Puerto Rico 37’ and the Indian accession PI 197087 (Roque and Adsuar 1939; Smith 1948; Epps and Barnes 1952; Kooistra 1968). van Vliet and Meysing (1974, 1977) found that the DM and PM resistance in the cultivar ‘Poinsett’ (derived from PI 197087) was often closely linked. However, Fanourakis and Simon (1987) failed to draw the association between DM and PM resistances in other PI 197087-derived cucumber lines such as WI2757 and Gy2.

For decades, PI 197088, an accession collected from a region of India close to where PI 197087 originated, has consistently exhibited a high level of resistance to PM (Clark 1975; Zijlstra and Groot 1992; Block and Reitsma 2005; Nie et al. 2015a). El Jack and Munger (1983) observed partial dominance of PM resistance in PI 197088 at the early growing stage. Morishita et al. (2003) suggested the PM resistance in PI 197088-5, a selection of PI 197088, is due to two genes, one recessive and another incompletely dominant. In PI 197088-1, Sakata et al. (2006) identified four temperature-dependent quantitative trait loci (QTL) for PM resistance on chromosome 1, 5, 6, and 7. With a detached-leaf assay, Fukino et al. (2013) detected seven

QTL on chromosomes 1 to 6 in CS-PMR1 (also derived from PI 197088). More recently, Berg et al. (2015) and Nie et al. (2015a) identified a candidate gene for the major-effect PM resistance QTL *pm5.1* (syn. *pm-h*), and found that the resistance is due to loss of function of the susceptibility gene *CsMLO1* (or Cs*MLO8*) on chromosome 5. Nie et al. (2015b) further found that a 1-bp insertion in this candidate gene in PI 197088 resulted in a premature stop codon and PM resistance.

As compared with PM resistance, a complete picture of the genetic basis of DM resistance in PI 197088 is still lacking. Large scale screening tests identified PI 197088 with high level resistance to prevailing field DM strains in the US (Call et al. 2012a, b). Caldwell et al. (2011) and Shetty et al. (2014) reported three DM resistance QTL in PI 197088, which were located on chromosomes 2, 4 and 5, respectively. Yoshioka et al. (2014) identified 10 QTL on chromosomes 1, 3, 5, 6, and 7 from the cross between CS-PMR1 (derived from PI 197088) and Santou. Several studies also identified DM resistance QTL in other cucumber lines. Szczechura et al. (2015) identified three DM resistance QTL on chromosome 5 from PI 197085. Wang et al. (2016) reported five QTL in WI7120 (PI 330628) on chromosomes 2, 4, 5, and 6, and revealed the dynamics of DM resistance through disease progress. In field screening tests, VandenLangenberg (2015) observed the segregation of DM resistance in F_2 populations developed from crosses among PI 197088, PI 330628, and PI 605996 which may suggest different genetic basis of DM resistance among these accessions. VandenLangenberg and Wehner (2016) also observed different responses to DM natural infection among several accessions over plant development stages indicating the dynamics of active QTL during the process of DM resistance.

In the PI 197088-derived breeding line CS_PMR1, seven QTL for DM and PM resistances were found to be co-localized (*dm1.2/pm1.1, dm1.3/pm1.2, dm3.1/pm3.1, dm5.1/pm5.1, dm5.2/pm5.2, and dm5.3/pm5.3, dm6.1/pm6.1*) (Fukino et al. 2013; Yoshioka et al. 2014).

DM/PM resistances in these studies were evaluated with single DM/PM strain by leaf-disc assays. It is not known if the host plants will perform the same way when infected with mixed DM/PM strains under field conditions. Moreover, due to the low resolution of genetic maps developed in previous studies, the precise locations of mapped DM and PM QTL are not known, which prevents fine mapping and cloning of the major-effect QTL.

A robust and high-density genetic map is useful for many marker-based applications such as fine mapping of genes/QTL, as well as improvement of the genome assembly. With the availability of cucumber draft genomes (e.g., Huang et al. 2009; Yang et al. 2012) and use of next-generation sequencing technologies, several high-density linkage maps in cucumber have been developed (e.g., Yang et al. 2012; Wei et al. 2014; Xu et al. 2015; Zhou et al. 2015; Rubinstein et al. 2015; Zhu et al. 2016). These maps can significantly improve the detection power of minor-effect QTL for target traits and reduce the confidence LOD interval of the QTL. For example, using a high-density map consisting of 2,084 bins with unique recombination events, Zhou et al. (2015) were able to pinpoint the candidate genes for the cucumber scab resistance locus in a 180-kb region.

The main objective of this study is to conduct QTL mapping and reveal the genetic architecture of DM and PM resistances in PI 197088. An $F_{7:8}$ RIL population from the cross between PI 197088 and Coolgreen was phenotyped for DM and PM resistances under field conditions in multiple years. A high-resolution linkage map with saturated SNP and SSR markers was developed from the same cross and used for QTL analysis in this study.

Materials and Methods

Plant materials

A RIL population derived from the cross between PI 197088 and Coolgreen was used to identify QTL for DM and PM resistances. PI 197088, a cucumber accession collected from

India, is highly resistant to DM and PM diseases. Coolgreen, a cucumber variety that developed by Seminis Vegetable Seeds Inc. (formerly Asgrow Seed Co.) is highly susceptible to both DM and PM. The PI 197088 × Coolgreen population with 169 $F_{6:7}$ RILs was developed by the Horticultural Field Laboratories of North Carolina State University (VandenLangenberg 2015). The whole population was evaluated for DM resistance in field trials. A subset of 148 RILs was self-pollinated to create the $F_{7:8}$ RILs at the Walnut Street Greenhouse (WSGH) at the University of Wisconsin-Madison, and was used for PM resistance studies and SSR/SNP genotyping.

Phenotypic data collection

Field evaluation of responses to natural infection

For DM phenotypic data collection, in the summer field seasons of 2013, 2014 and 2015, 169 RILs and the two parental lines were directly seeded in test plots at the Horticultural Crops Research Stations of North Carolina State University in Clinton and Castle Hayne, North Carolina, which were designated as CL2013, CL2014, CL2015, CH2013, and CH2014, respectively. All entries were planted on 27 June 2013, 30 June 2014, and 30 June 2015 at Clinton, and 20 June 2013 and 26 June 2014 at Castle Hayne and the plants were grown with recommended horticultural practices (VandenLangenberg 2015).

All experiments used a randomized complete block design (RCBD) with two replications per location per year. There were 15 plants per replication. In each trial, disease severity was assessed three times with one week apart starting at 41 days after planting (dap) (1st rating). The rating scale was based on percentage of symptomatic leaf area (per plot) with a scale from 0 to 9, where 0=no damage, 1=1-10%, 2=11-20%, 3=21-30%, 4=31-40%, 5=41-50%, 6=51-60%, 7=61-70%, 8=71-80%, 9=81-100% (or dead) (Jenkins and Wehner 1983).

Powdery mildew field evaluation

Phenotypic data of PM resistance among 148 $F_{7:8}$ RILs of PI 197088 × Coolgreen were collected from field trials in 2014, 2015 and 2016 at the University of Wisconsin Hancock Agricultural Research Station (HARS) at Hancock, Wisconsin, which were designated as WI2014, WI2015 and WI2016, respectively. Fertilizer was incorporated before planting with 126 kg/ha of ESN-44-0-0 Slow Release Nitrogen (Agrium Inc.). For each growing season, the experimental design was conducted with RCBD consisting of two replications with six plants per replication per line. Plots were exposed to natural epidemics of PM. Disease severity of each plot was evaluated ~80 days after planting when two parental lines showed clearly distinctive symptoms on PM infection. Disease evaluation was based on sporulation level on leaves using disease rating scales 1 to 9, where 1 = 0-10% surface area covered with PM spores, 2 = 11-20%, 3 = 21-30%, 4 = 31-40%, 5 = 41-50%, 6 = 51-60%, 7 = 61-70%, 8 = 71-80%, 9 = 81-100% (He et al. 2013).

Statistical analysis

Analysis of variance (ANOVA) was performed with the *R/lme4* package to estimate the genetic and environmental effects (Bates et al. 2014). DM resistance trait was fitted in a linear mixed-effects model (LMM) with location and year effects for each rating time, while PM disease score was fitted in an LMM with year effects only. The best linear unbiased predictors (BLUPs) were also extracted from the models and used for further QTL analysis.

Heritability estimates were calculated from variance components from mixed-effects model. Since the dominant variance and covariance of additive and dominant effects are equal to zero in the $F_{6:7}$ and $F_{7:8}$ RIL populations, the heritability was considered as narrow sense estimate (Cockerham 1983).

Genotyping

Bulked Segregant Analysis for DM resistance with microsatellite markers

Two bulks of extreme phenotypes (highly resistant and highly susceptible) were used for bulked segregant analysis (BSA), based on the DM rating scores from CL2013. Equal quantities of DNA were pooled from eight susceptible and eight resistant $F_{6:7}$ RIL plants to give two bulks. SSR markers described in Ren et al. (2009), Cavagnaro et al. (2010), and Yang et al. (2012) were used to screen two parental lines (PI 197088 and Coolgreen); polymorphic ones were then applied to the two bulks. Polymorphic markers between bulks were used for further single marker analysis. Moreover, these markers and addition neighboring polymorphic markers between parents were used to genotype 148 RILs; the genotypic data were integrated with SNP markers for genetic map development.

Specific Length Amplified Fragment (SLAF) Sequencing (SLAF-Seq)

The 148 $F_{7:8}$ RILs were genotyped with next-generation sequencing-based SLAF-Seq, which was an improved genotyping by sequencing (GBS) technology with distinguishing characteristics in deeper sequencing, reduced sequencing costs, and dual barcode system for large populations (Sun et al. 2013). Leaf samples of all RILs and two parental lines were freeze-dried and shipped to the Beijing Agriculture and Forestry Academy of Sciences for SLAF-seq. The SLAF-Seq library was constructed following Wei et al. (2014) and sequenced with an Illumina HiSeq 2500 sequencer (Illumina, San Diego, CA, USA), which was performed at the Beijing Biomarker Technologies Corporation (http://www.biomarker.com.cn).

Raw SLAF-Seq reads were demultiplexed based on barcodes, and low-quality reads (quality score <20) were filtered out. Reads of 100 base pair from the same samples were mapped onto the 9930 cucumber draft genome sequence (http://www.icugi.org, version 2.0) using SOAPdenovo2 software (Luo et al. 2012). All sequences aligned to the same position were defined as a SLAF locus. SLAF loci that contained fewer than three SNPs (single nucleotide polymorphism) with an average of 3× depths of coverage were kept for genetic map construction.

Linkage map construction with SNP and SSR markers

The physical order of all SNP markers on the 9930 draft genome assembly was examined with paired recombination fraction (RF) by LOD score for a test of RF=0.5 using the function *est.rf* in *R/qtl* (Broman et al. 2003). On the RF-LOD plot, off-position and unlinked markers on each linkage group could be easily inspected manually. Next, the function *mstmap* in *R/ASMap* was used to re-order and adjust the marker positions, which was based on the minimum-spanning-tree (MST) algorithm and is efficient and fast for handling dense maps (Wu et al. 2008; Taylor and Butler 2015). After correcting errors in marker order, the RF between each marker pair was re-examined. On the RF plot, the correctness of the order of markers could be judged with the following criteria: nearby markers are clearly associated, and distant markers showed no association. To integrate the SSR with SNP markers, all SSR markers were assigned to unknown chromosomes first, and the function *tryallpositions* in *R/qtl* was then used to try all possible positions for each marker by keeping all other markers fixed. Finally, the genetic distances were re-estimated using the function *est.map* in *R/qtl* which was based on the likelihood through hidden Markov chains algorithm (LHMC or Lander-Green algorithm. Lander et al. 1987). The resulting map with both SNP and SSR markers was then used for further analysis. Kosambi mapping function was applied during genetic map distance calculation.

For genetic and physical map integration, the SLAF reads of mapped SNPs were used to search for homologous sequences in the Gy14 V1.0 draft genome assembly using BLAST+ (Camacho et al. 2009) with a cut-off E-value <1.0E-10.

QTL analysis

Single marker analysis

The single marker analysis (SMA) was conducted for polymorphic SSR markers between the two bulks by performing ANOVA to test if variation among genotypic classes was significant, and to estimate additive effects for each marker ($P<0.05$).

QTL analysis with SNP-based high-density genetic map

QTL analysis for PM and DM resistances was performed in the *R/qtl-MQM* package (Arends et al. 2010, 2014). Both simple interval mapping (SIM) and composite interval mapping (CIM) methods performed in R/qtl revealed that there were multiple QTL on one chromosome. Often a non-existing QTL may appear in between two linked QTL termed ‘ghost’ QTL. To reduce the chance in detecting ghost QTL, the multiple-QTL mapping (MQM) method was employed which also strengthen the identification of QTL in coupling and repulsion phases. The function *mqmaugmentdata* was first used for filling in missing genotypes, and the cofactors were determined by function *scanone* and initial *mqmscan* with a forward stepwise approach. Then the function *mqmscan* was employed again for unsupervised backward elimination to identify significant QTL. The significance of each QTL was tested by the function *mqmpermutation* for each trait using 1,000 permutations at a significance threshold of 0.05. These QTL and all interactions were further investigated in *R/lme4* by fitting linear models to test their statistical significance. All significant QTL and QTL interactions were kept for fitting a full model to evaluate the additive effects and percentage of phenotypic variance (R^2) explained (Jamann et al. 2015). The support intervals for these QTL were calculated using a 1.5 LOD drop interval.

Gene ontology (GO) analysis

Candidate genes were predicted from the 1.5 LOD interval of each QTL based on the 9930 draft genome assembly V2.0. All candidate genes within the target QTL interval were filtered based on data from transcriptome profiling obtained with RNA-Seq by Burkhardt and Day (2016). Those differentially expressed genes (DEGs) in PI 197088 for at least one time point post inoculation (1day post inoculation/dpi, 2dpi, 3dpi, 4dpi, and 6dpi) were classified by singular enrichment analysis tool in agriGO (http://bioinfo.cau.edu.cn/agriGO/).

Results

Performance of mildew resistances in the RIL population

Phenotypic variations of responses to downy mildew infection in RIL population

Phenotypic data on DM inoculation responses of the 169 PI 197088 × Coolgreen RILs were collected in three years (2013, 2014 and 2015) at the North Carolina State University Clinton (CL) and Carl Haynes (CH) Research Stations. In general, with the progress of the disease development in the field, the performance of the entire population shifted toward susceptibility with increased variations of disease scores within the population across all five environments (CL2013, CL2014, CL2015, CH2013, and CH2014). The observed ratings followed normal distribution that covered a large range of scores at each environment and at each rating time (**Table 3.1; Figure 3.1**).

From the 1^{st} rating to the 3^{rd} rating, the average correlations of disease scores among environments increased from 0.50 to 0.69 (P<0.01), implying the accrued genotypic effects in late stages of DM development (**Figure 3.2**). Thus, the increased (narrow sense) heritability estimates were observed from 0.83 to 0.88 across the three rating times in all environments

(**Table 3.2**). Although the heritability was as high as 0.88, the year effects on the phenotype variance were significant. The interaction effects of genotype-by-location and genotype-by-year were also significant at all ratings, although the three-way genotype-by-location-by-year interaction became less significant from the 1st to the 3rd ratings (**Table 3.2**). Therefore, QTL analysis was performed with data from each environment and BLUPs to minimize the environmental effect. QTL mapping was also conducted for the three ratings to study the dynamic resistance to DM disease in PI 197088.

Phenotypic variations of PM resistance and its correlation with DM resistance

Data for responses to natural infection of the RIL population were collected for three years (2014 to 2016) at the University of Wisconsin Hancock Agricultural Research Station for 148 RILs. As shown in **Figure 3.2**, disease scores for the PM among the three years were highly and significantly correlated (r_s = 0.62-0.80) with non-normal distribution. The disease score of F_1 plants was similar to that of the susceptible parent Coolgreen indicating the recessive nature of PM resistance in PI 197088 (**Table 3.3**). The interaction effects between genotype and year were detected, and the heritability was as high as 0.90 (**Table 3.4**).

The Spearman's rank correlation coefficients between DM and PM resistances of three rating times ranged from 0.61 to 0.66 (P<0.01) (**Figure 3.3**) suggesting possible common genetic basis of resistances against the two pathogens in PI 197088.

Genotyping and linkage map construction

Bulked Segregant Analysis and SMA identified significant marker-DM resistance associations

Among 1,536 SSR markers screened between PI 197088 and Coolgreen, 422 were polymorphic (27.47%). Of the 422 markers, 312 evenly distributed across the cucumber genomes were applied to the resistant and susceptible bulks, and 19 were polymorphic between bulks. The 19 markers were distributed in five chromosomes (Chr1, 3, 4, 5, and 6). Single marker analysis revealed 16 out of 19 markers that were significantly associated with DM resistance (**Table 3.5**) suggesting at least six QTL were underlying DM resistance in PI 197088.

Analysis of SLAF markers

SLAF-seq of 148 RIL plants generated 12.83 Gb data containing 110,421,590 pair-end reads, of which 87.67% and 92.99% bases had quality scores higher than 30 (Q30) or Q20, respectively. Among 58,886 high quality SLAFs identified, 11,620 (19.73%) were polymorphic between the two parental lines. The following criteria were used to filter the SLAF markers: 1) sequence depth in both parents > 10× coverage; 2) marker missing data rate <10% in RILs; 3) no duplicated (co-segregating) markers among 148 RIL plants (bin mapping); and 4) a stringent threshold was used for distinguishing genotypic errors and segregation distortion at 0.5% level cutoff with a Bonferroni correction [$-\log10(P)>6.2$]. Finally, 2,780 SNP markers were kept for genetic map construction (**Table 3.6**).

Segregation distortion (SD) analysis

Each codominant SNP marker was tested for significant deviation from the expected Mendelian segregation ratio with X^2 tests and the *P*-value for each marker was extracted (calculated in R). Since cucumber is diploid with seven pairs of chromosomes, at least 14 independent genomic regions are expected. A threshold of at least 0.05/14 ~0.004 ($-\log10(P)\approx2.40$) with Bonferroni correction would be needed to obtain a genome-wide error rate of alpha=0.05. Thus, a region with the SNP genotype frequencies ($-\log10(P)$) larger than 2.40 and at least five tightly linked distorted SNPs bin was considered in distorted region. The SD patterns in the RIL population were visualized by plotting the $-\log10(P)$ value of each marker along the seven chromosomes (**Figure 3.4)**. Marker order was based on their physical positions in the 9930 draft genome assembly V2.0.

Five large SD regions were identified in the PI 197088 × Coolgreen RIL population which were located on chromosomes 1, 2, 3, 5, and 6 with all in favor of Coolgreen alleles.

Genetic map construction and evaluation

Marker ordering with large number of markers is challenging for linkage map construction, which is critical for accurate QTL mapping. To obtain a reliable marker order, firstly, the 2, 780 SNP obtained from SLAF-Seq were ordered by their physical positions based on the 9930 draft genome assembly V2.0. The recombination fraction (RF) of each pair of the SNP markers was then examined. As shown in the RF-plot (**Figure 3.5A**), the majority of the markers was in the right order for linkage map construction, but several from chromosomes 3, 6, and 7 were misplaced.

To optimize the off-position markers, SNPs were re-ordered in *R/ASMap* and generated a new RF-plot which displayed a good order of markers with nearby markers clearly associated, and no distant markers showed any association (**Figure 3.5B**). The genetic distances were estimated and the linkage map was constructed according to the new marker order (**Appendix 3.1**).

To examine the quality and explore the utility of the genetic map, 19 SSR markers from the SMA and 36 neighboring SSR markers were chosen to genotype the 148 RILs, and the genotypic data were integrated with 2,780 SNP markers to construct a new genetic map. The new RF-plot also displayed a good order (**Figure 3.5C**). Importantly, all 55 SSR markers that embedded in the nearly 3000 SNP markers seemed to be located at their correct physical positions indicating a good marker ordering of the genetic map. Moreover, some SSR markers were able to fill large gaps left on the SNP-only map thus improving the quality of genetic map

(**Appendix 3.1**). Overall, the final genetic map spanned 873 cM with an average marker interval of 0.3 cM (**Table 3.7**).

The high-density map allowed detection of mis-assemblies in the 9930 cucumber draft genome

During linkage map construction, few off-position markers were identified from the RF-plot that created from the physical position of the 9930 draft genome assembly. Since bin map was used in this study, the bin size of these disorder markers was investigated. The corresponding sequences were extracted from the 9930 draft genome assembly V2.0 and were aligned with Gy14 assembly V1.0 (Yang et al. 2012). As showed in **Appendix 3.2**, four bins in 9930 draft genome assembly were mis-assembled and showed 99% identity to a different chromosome in Gy14 assembly.

A whole genome alignment was further conducted among the high-density linkage map, the 9930 draft genome assembly V2.0, and the Gy14 assembly V1.0. As shown in **Figure 3.6**, in addition to the four mis-assembled intra-chromosomal regions, the alignment also revealed two inter-chromosomal regions on chromosome 5 and 7 in 9930 draft genome assembly V2.0. A group of markers showed cross over at the top of chromosome 5 and 7. The collinearity between genetic and their physical position**,** and the detailed mis-assembled regions were listed in **Appendix 3.1**.

QTL analysis of mildew resistances in PI 197088

The RIL means and BLUPs of DM and PM disease scores from all experiments were used for QTL analysis with the MQM approach. A global view of all QTL detected across the seven chromosomes is provided in **Appendix 3.3**. Details of each identified QTL including peak location, LOD value, 1.5-LOD support interval, additive effect, and percentages of total phenotypic variances explained (R^2) are provided in **Table 3.8** and **Appendix 3.4**. Slight shifts of the peak position were observed across rating times and environments for DM and PM resistances, but the 1.5 support LOD intervals of these QTL were highly consistent. Therefore, this study mainly reported the results of QTL analysis from BLUPs which were more accurate because the BLUPs had incorporated the environmental effects.

QTL for downy mildew resistance

Eleven QTL were detected across three rating times which were located on six chromosomes with each QTL explaining 1.98-30.97% of the phenotypic variance (**Table 3.8**).

A careful examination of QTL and their effects over three rating times allowed to reveal the dynamic effects of the QTL on disease development. The QTL *dm5.1*, *dm5.2*, and *dm5.3* are major-effect QTL (R^2 = 19.68-30.97%) with negative additive effects suggesting these QTL contribute to DM resistance (reduced disease scores). The three QTL were detected at all three rating times of all environments indicating their important roles in conferring DM defense at entire plant growth stage (**Figure 3.7**). Epistatic interaction between *dm5.2* and *dm5.3* was observed at 1st and 2nd rating times, which explained 2.56% of the phenotypic variance (**Table 3.8**). The phenotypic variance explaining by *dm3.2* decreased from 13.36, 10.03, to 5.26% at three rating times (early to late), respectively, whereas *dm2.1* and *dm4.1* showed increased effects (larger phenotypic variance explained) in later rating. Interestingly, the alleles from PI 197088 at *dm1.1*, *dm2.2*, and *dm6.2* loci all exhibited positive additive effects suggesting that they all conferred susceptibility to DM infection. These three susceptible QTL explained relatively small proportion of the phenotypic variance and were detected only at the 2nd and 3rd rating time.

The magnitudes of additive effects of the 11 QTL that were estimated by the peak markers across five environments are plotted in **Figure 3.8**. The effects of all alleles at location CH dropped dramatically from 2013 to 2014, but showed stable at location CL with only slight difference from 2013 to 2015. This could be reflected by population distribution as showed in **Figure 3.1**, CH2013 had the highest and CH2014 had the lowest population mean, while CL2013, CL2014, and CL2015 had similar population mean across five environments. The possible reason may be due to the variation of DM epidemics at CH, which was close to the ocean with strong sea breezes that are known to promote disperse of DM spores through wind currents.

QTL analysis of powdery mildew resistance in PI 197088

Using BLUPs from three environments (WI2014, WI2015 and WI2016), four QTL, *pm1.1*, *pm2.1*, *pm5.1*, and *pm6.1*, were identified, which explained 1.82-32.38% of the phenotypic variance (**Table 3.8, Figure 3.9**). The major-effect QTL, *pm5.1*, was peaked at 115.0 cM flanked by the SNP marker Marker5_24647715 (114.30 cM, 24.65Mb of chromosome 5) and SSR15196 (115.10 cM, 25.03Mb of chromosome 5). Within this region, a candidate gene for PM resistance, *CsMLO1* (*CsMLO8*) was previously identified (at 24.83Mb) (Nie et al. 2015a, b; Berg et al. 2015). Nie et al. (2015b) found that in PI 197088, *CsMLO1* has a 1-bp insertion resulted in a premature stop codon and PM resistance. Very likely, *pm5.1* detected in the present study was the same as *CsMLO1* identified in the previous study. This result exemplified the importance of a high quality and high-density genetic map (of course, high quality phenotypic data) in improving the power of QTL detection.

The other three QTL, *pm1.1*, *pm2.1*, and *pm6.1*, had relatively small effects on contributing PM resistance with each explaining 1.82-5.56% of the phenotypic variance. Among them, *pm6.1* had positive additive effects conferring PM susceptibility in PI 197088.

Co-localization of DM and PM resistance QTL

By a quick look at the map locations of the DM and PM QTL detected in the present study, it was clear that three DM and PM QTL pairs had overlapped 1.5-LOD intervals, which included *dm2.1/pm2.1*, *dm5.3/pm5.1*, and *dm6.2/pm6.1* (**Table 3.8; Figure 3.10**). However, the peak locations of *dm2.1* and *dm5.3* were obviously different from that of *pm2.1* and *pm5.1*, respectively, suggesting they may be different but linked loci conferring resistances to the two pathogens. On the other hand, *dm6.2* and *pm6.1* shared the same peak location and both contributed to disease development (positive additive effects) which may imply both QTL share the same genetic base. The co-localization of DM and PM QTL also explained the observed high correlation between DM and PM phenotypes.

Identification of candidate genes for DM resistance

The gene expression level is one of the major regulations to determine the cell function induced by internal or external signals (O'Connor and Adams 2010). Upon pathogen infection, the expression of many genes including the causal resistance gene per se is up- or down-regulated to contribute to host defense responses. Therefore, examining the transcriptome level of PI 197088 after DM inoculation could provide new insights to DM resistance pathway. Burkhardt and Day (2016) studied the transcriptome dynamics during a time course of DM infection on PI 197088 and susceptible line 'Vlaspik' through RNA-Seq using the 9930 draft genome assembly V2.0 as the reference. Thus, investigating the expression level of the genes within QTL intervals may help identification of possible causal genes.

Five QTL (*dm3.2*, *dm4.1*, *dm5.1*, *dm5.2*, and *dm5.3*) were reproducibly detected across all environments under three rating times with each explained more than 5% of the phenotypic variance suggesting they play important roles in contributing DM resistance throughput the plant growth. Thus, genes located within the 1.5 LOD intervals of the five DM resistance QTL were extracted for differentially expressed genes (DEGs) analysis from the 9930 draft genome assembly V2.0, since the transcriptome data were analyzed and annotated using 9930 assembly V2.0. One difficulty is that the QTL *dm5.1* was mapped in a mis-assembled region on chromosome 5 in the 9930 draft genome assembly. To obtain the accurate number of genes underlying *dm5.1*, all SNP markers within 1.5 LOD interval was anchored to the Gy14 assembly V1.0 to estimate candidate genome size. Then, the whole sequence of this region was aligned back to the 9930 draft genome. Finally, all corresponding genes were collected from the fragmented 9930 sequence. In total, 1,220 annotated genes in the five QTL regions were selected for additional analysis.

Among the 1,220 genes, 558 showed differential expression in PI 197088 in at least one time point post inoculation (1dpi, 2dpi, 3dpi, 4dpi, and 6dpi) (**Table 3.9**). As shown in **Figure 3.11**, the cluster analysis and heat map revealed the separation of DEGs between PI 197088 and Vlaspik under DM infection. However, the DEGs at 1 dpi in PI 197088 showed closer correlation with those in Vlaspik, indicating a large number of DEGs were induced by DM inoculation in both PI 197088 and Vlaspik. Furthermore, 263 of the 558 DEGs persisted from 2dpi to 6dpi in PI 197088, while 139 DEGs in PI 197088 were non-DEGs or opposite-DEGs with those in susceptible line Vlaspik (**Table 3.9**). This result dramatically reduced the candidate gene number for further study by filtering out more than 80% of total genes within these QTL intervals.

To investigate the gene functions, functional GO analysis was performed which revealed that most of the 263 DEGs in PI 197088 were related to catalyst activity (GO:0003824) (**Figure 3.12**). The most commonly enriched secondary terms of genes were related to hydrolase activity (GO:0016787), nucleotide binding (GO:0000166), transferase activity (GO:0016740), and ion binding (GO:0043167). Cellular component GO analysis indicated that most of the genes were located at the membrane (GO:0016020) and intracellular (GO:0005622). Many of these DEGs were directly or indirectly related to some known plant disease defense pathways, including several typical R genes, such as receptor-like protein/kinase, WRKY transcription factors, F-box protein, and E3 ubiquitin-protein.

Discussion

SD regions, high-density map, and improvement of genome assembly

Segregation distortion (SD) is a widespread phenomenon in plants and animals, in which the frequencies of segregating alleles skew from the expected Mendelian ratios (Lyttle, 1991). In cucumber, SD has been observed in a number of studies, but the genetic mechanism is still unclear. Ren et al. (2009) reasoned the SD to the stronger viability of ‘wild’ alleles from *C. s. hardwickii* PI 183967 than the ‘domesticated’ ones from Gy14. Rubinstein et al. (2015) suggested that the preference of one parent over the other would be the possible reason with examples for the preferential in male alleles. In this study, five SD regions were identified in the PI 197088 × Coolgreen RIL population with all alleles in favor of Coolgreen. The reason is unknown. More systematic studies would be required to address the reasons from the nuclear factor, cytoplasm factors, or gametophytic selection in causing SD in cucumber.

In many studies, distorted markers were removed during linkage map construction for the reason that SD may introduce errors in map distance estimation and marker order, and thus affect QTL mapping results (e.g., Lorieux et al. 1995). But more recent studies indicated that SD has little or no effect on mapping accuracy and even improves QTL mapping in some cases (Hackett and Broadfoot 2003; Xu 2008; Zhang et al. 2010; Bartholome et al. 2015). In this study, two of the five SD regions on chromosomes 1 and 5 spanned a large proportion of the genome. If we discarded these markers in SD, the map lengths of the two chromosomes would be much shorter. More importantly, three QTL (*dm1.1*, *dm2.1*, and *dm3.1*) were mapped in the SD regions; their map locations would otherwise be inaccurate without these markers. This piece of information is also important from a marker-assisted breeding perspective. If the gene for a target trait is located within a SD region, the trait will also show SD (e.g., Pan et al. 2017). If the favorable allele in the SD region contributes to the desirable trait, the selection for this target trait would be easier. On the other hand, if the contributing allele is unfavorable in SD, molecular marker assisted selection (MAS) would be an efficient way to increase the frequency of the favorable allele. Therefore, among the three QTL, using MAS to select alleles of *dm2.1* and *dm3.1* that conferred DM resistance from PI 197088 may accelerate the breeding process.

The high-density genetic map developed in this study helped identify mis-assemblies in the 9930 draft genome. In the 9930 draft genome assembly (V2.0), two inter-chromosomal mis-assembled regions on chromosome 5 and 7 have been reported in several studies (Sun et al. 2013; Wei et al. 2014; Zhou et al. 2015; Rubinstein et al. 2015), which were confirmed in the present work (**Figure 3.6**). In addition, using this high-density genetic map, four small mis-assembled sequences ranged from 7.8 kb to 281 kb were also identified in the 9930 draft genome assembly V2.0, which provided additional evidence to improve the 9930 draft genome assembly in the future (**Figure 3.6; Appendix 3.2**).

Co-localization of DM and PM resistances

PI 197088 possesses resistance to multiple diseases such as DM, PM, angular leaf spot (caused by *Pseudomonas syringae* pv. *lachrymans*), and target leaf spot (caused by *Corynespora cassiicola*) (Staub et al. 2002). Understanding the genetic mechanism underlying PI 197088 for a broad-spectrum disease resistance, would allow us to better use this germplasm to develop cucumbers with multiple-disease resistances. In this study, QTL mapping identified three overlapped QTL between DM and PM phenotypes (*dm2.1/pm2.1*, *dm5.3/pm5.1* and *dm6.2/pm6.1*) which can explain the significant correlation between DM and PM phenotypes.

The increased correlation from 0.61 to 0.66 ($P< 0.01$) between PM and DM from 1st to 3rd rating times could account for the increased genetic effects of *dm2.1* and *dm6.2* at later stage.

Co-localization of DM and PM resistance QTL has been reported in the cucumber line PI 197087, and is common in other crops such as melon, tomato and Arabidopsis (Van Vliet and Meysing 1977; Perchepied et al. 2005; Van Damme et al. 2009; Fukino et al. 2013; Huibers et al. 2013; Yoshioka et al. 2014; Gao et al. 2015). A notable example is the Arabidopsis *dmr1* (*downy mildew resistant 1*) gene, which encodes a homoserine kinase that mediates resistance to both DM (*Hyaloperonospora arabidopsidis*) and PM (*Oidium neolycopersici*) diseases (Van Damme et al. 2009; Huibers et al. 2013). In cucumber, *CsMLO1* has been shown to be the underlying the *pm5.1* major-effect QTL, which was co-localized with *dm5.3* but with different peak locations. Further investigation is needed to see if *CsMLO1* also plays any roles in DM resistance in PI 197088.

DM resistance QTL in PI 197088

Several previous studies have identified DM resistance QTL in PI 197088. Caldwell et al. (2011) and Shetty et al. (2014) detected three DM resistance QTL on chromosome 2, 4, and 5 from PI 197088, but the confidence intervals of these QTL spanned nearly two thirds of the respective chromosomes. Using single isolate and a detached-leaf assay and the CS-PMR1 × Santou RIL population, Yoshioka et al. (2014) conducted QTL mapping of DM resistance in CS-PMR1 (derived from PI 197088) and detected 10, in which 7 were contributed by CS-PMR1 and 3 by Santou. However, of the 10 QTL detected by Yoshioka et al. (2014), only four (*dm3.1*, *dm5.1*, *dm5.2*, and *dm5.3*) showed consistent chromosome locations with the QTL detected by the present study (based on 1.5 LOD interval of each QTL). In addition, the major-effect QTL identified by Yoshioka et al. (2014) was *dm1.1* and contributed by moderate resistant line Santou. In our study, the three QTL in chromosome 5 conferred the major effects in DM resistance with each contributed 19.68-30.97% of the phenotypic variance.

Several reasons may contribute to the discrepancies of results among these studies. Yoshioka et al. (2014) used a single isolate of the DM pathogen to inoculate the plants with the detached leaf assay whereas the present study used natural inoculation with field strains. The population of the DM pathogen *Ps. cubensis* in the field is a mixture with many isolates that varied in the degree of pathogenicity or virulence (Lebeda and Urban 2007; Quesada-Ocampo et al. 2012; Lebeda et al. 2013; reviewed by Cohen et al. 2015). The four common QTL (*dm3.1*, *dm5.1*, *dm5.2*, *dm5.3*) detected by both studies may confer resistance to DM pathogen strains at both locations. It is not known if the different inoculation methods used in the two studies may affect the outcome of QTL mapping. In addition, the high-density genetic map developed in the present study increased the power of QTL detection especially for QTL with minor effects.

Three QTL conferred susceptibility to DM in PI 197088

From field tests with multiple DM resistant lines, VandenLangenberg and Wehner (2016) reported that PI 197088 appears to develop disease symptoms faster than other resistant PI accessions such as PI 330628 and PI 605996 (**Figure 3.13**, redrawn from VandenLangenberg and Wehner, 2016). They suggested that PI 330828 (WI7120) and PI 605996 are preferable to PI 197088 since the latter hardly holds the DM resistance at later growth stages.

In the present study, phenotypic data of the RIL population to DM inoculation responses were scored at 41, 48 and 55 days after planting (dap). QTL analysis identified 3 QTL, *dm1.1*, *dm2.2*, and *dm6.2* that had positive additive effects. That is, these QTL conferred susceptibility to DM infection in PI 197088. The two QTL, *dm2.2* and *dm6.2,* were mapped at later rating times (48 and 55 dap) and explained increasing phenotypic variance. This result may explain the observation that PI 197088 did not hold its resistance after 42 dap and showed similar resistance level as Ashley and Poinsett 76 (**Figure 3.13**). These susceptible QTL may be responsible for the reduced resistance in PI 197088 at later plant growth stages. Previous QTL mapping study in PI 330628 revealed 4 QTL conferring DMR without any susceptible QTL (Wang et al. 2016). It might be able to explain the fact that WI7120 (PI 330628) has fewer DM resistant QTL than PI 197088 but performed similar resistance level at early growing stage and can maintain a higher level of resistance than PI 197088 for weeks past flowering stage.

The potential of PI 197088 in cucumber breeding for DM resistance

Among over 1200 accessions examined, PI 197088 was the top performer for DM resistance in multi-year and multi-location screening tests (Call et al. 2012a, b). PI 197088 DM resistance is being used widely in both public and commercial breeding programs. The present study offers some new perspectives on the use of PI 197088 resistance source in cucumber breeding with both pros and cons. First, two pairs of PM and DM resistance QTL (*dm2.1/pm2.1* and *dm5.3/pm5.1*) were co-localized; especially, both *dm5.3* (R^2 = 31%) and *pm5.1* (R^2=32%) were major-effect QTL with large positive effects on disease resistance (**Table 3.8**), which makes it more convenient to develop cucumber lines with resistance to both pathogen. Second, three DM susceptible alleles in PI 197088 (*dm1.1*, *dm2.2*, *dm6.2*) were identified at second and third ratings which may explain why PI 197088 developed disease symptoms faster at later growth stages as compared to PI 330628 (WI7120) and PI 605996 (VandenLangenberg and Wehner, 2016). Thus, to enhance the utility of PI 197088, the use of molecular markers in tracking these alleles would be necessary to minimize their negative effects on DM resistance from PI 197088. Third, two QTL (*dm2.1* and *dm3.1*) conferring DM resistance in PI 197088 were mapped in SD regions, which are all in favor of alleles from susceptible parental line Coolgreen. Using a traditional breeding scheme, these DM resistance alleles would have lower-than-expected frequency in the progeny, which may even get lost under stringent selection. Thus, a large breeding population would be needed to ensure the selection of PI 197088 resistance alleles. However, the use of molecular marker to track these alleles should be efficient to increase their frequency during selection.

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Table 3.1 Mean and standard deviation of downy mildew disease scores of the 169 $F_{6:7}$ RIL population (PI 197088 × Coolgreen), the two parental lines, their F_1 and controls across all environments and rating time.

Environment	**Rating time**	**PI197088**	**Coolgreen**	**F_1**	**Gy14**	**Poinsett76**	**Ashley**	**Polaris**
CL2013	**1**	0.44±0.52	3.85±0.89	3.22±0.66	3.40±0.54	1.42±0.78	3.25±0.70	2.00±0.00
	2	0.88±0.33	6.28±0.48	3.44±0.52	4.60±1.14	2.42±1.13	4.62±0.74	4.25±0.95
	3	1.00±0.50	7.42±0.53	5.11±0.78	5.40±0.54	4.14±0.37	5.00±0.53	5.50±0.57
CH2013	**1**	1.20±0.42	5.33±0.81	3.50±0.53	3.00±0.00	2.00±0.53	2.25±0.50	3.20±0.91
	2	2.30±3.56	7.66±0.51	5.62±0.74	4.75±0.70	2.37±0.51	3.50±0.57	4.60±0.84
	3	1.70±0.48	8.00±0.00	4.87±0.35	7.12±1.12	3.12±0.64	5.50±0.57	6.10±0.87
CL2014	**1**	0.11±0.33	4.22±0.66	3.60±0.51	n.a	1.70±0.82	3.90±0.73	2.00±0.66
	2	0.11±0.33	6.11±0.60	3.60±0.51	n.a	3.10±0.31	3.60±0.51	3.30±0.48
	3	1.11±0.33	6.22±0.66	5.10±0.73	n.a	2.40±0.69	3.70±0.94	3.90±0.73
CH2014	**1**	0.44±0.52	6.62±0.74	4.28±0.75	n.a	1.80±0.63	3.60±0.51	3.30±0.82
	2	1.00±0.00	7.62±0.91	4.85±0.89	n.a	2.30±0.48	4.30±0.48	3.70±0.67
	3	1.00±0.00	7.12±0.99	5.00±0.57	n.a	3.90±0.73	3.80±0.78	4.00±0.00

Note: n.a: not available.

Table 3.2 Analysis of variance, year means, variance component estimates, and heritability (h^2) for DM disease scores in the169 $F_{6:7}$ RILs of PI 197088 × Coolgreen.

Source of variation	**df**	**Mean Square** **Rating 1**	**Rating 2**	**Rating 3**
Genotype (G)	168	7.48**	12.73**	13.16**
Location (L)	1	0.74	2.39	0.27
Year (Y)	2	21.36**	31.73**	20.25**
G × L	168	1.34**	1.35**	1.2**
G × Y	266	1.37**	1.71**	1.75**
G × L × Y	131	1.05**	1.21*	0.74
L × Y	1	20.54**	34.21**	25.61**
Block (L Y)	11	0.083	0.04	0.05
Residual	1482*	1.014	0.97	0.87
Heritability (h^2)		0.83±0.20	0.88±0.24	0.88±0.15

Note * The degree of freedom for residual in Rating 1 is 1482, in Rating 2 is 1506, and in Rating 3 is 1512; **with a significant level at $P<0.01$.

Table 3.3 Mean and standard deviation of downy mildew disease scores of the 148 $F_{7:8}$ RILs of PI 197088 × Coolgreen cross, the two parental lines, their F_1 and controls across all environments and rating time.

Environment	**PI197088**	**Coolgreen**	**F_1**	**Poinsett76**	**Ashley**
WI2014	1.0±0.0	8.0±0.0	7.0±0.0	1.5±0.7	5.0±0.0
WI2015	1.0±0.0	9.0±0.0	7.5±0.7	2.0±1.4	5.5±0.7
WI2016	1.0±0.0	9.0±0.0	7.0±0.7	n.a	n.a

Note: n.a: not available.

Table 3.4 Analysis of variance, year means, variance component estimates, and heritability (h^2) for powdery mildew disease scores of 148 $F_{7:8}$ PI 197088 × Coolgreen RILs.

Source of variation	**df**	**Mean Square**
Genotype (G)	147	22.77**
Year (Y)	2	14.92
G × Y	280	2.31**
Block (Y)	4	0.129
Residual	708	1.302
Heritability (h^2)		0.90±0.12

Note: **with a significant level at $P<0.01$.

Table 3.5. Single marker analysis for association of polymorphic markers between bulks and BULPs of DM disease scores.

Nr.	**Marker Name**	**Chr**	**9930 assembly v2.0 Position**	**Additive effect**	**Adjusted R^2**	***Pr(>F)***
1	SSR15108	1	1488524	ns	ns	0.72
2	SSR14596	1	3951558	ns	ns	0.32
3	SSR07505	3	23920626	-0.48	0.04	0.008
4	SSR02106	3	36931487	-0.51	0.04	0.006
5	SSR07581	4	10875872	-0.61	0.07	0
6	SSR17911	4	14823864	-0.88	0.15	0
7	SSR04649	4	14904229	-0.87	0.14	0
8	SSR18870	4	15126951	-0.78	0.11	0
9	SSR20165	5	12594760	-1.09	0.24	0
10	SSR18729	5	13005885	-1.03	0.21	0
11	SSR01280	5	16669445	-1.28	0.32	0
12	SSR07531	5	17208433	-1.23	0.3	0
13	SSR15321	5	17443753	-1.25	0.31	0
14	SSR03529	5	21686805	-1.24	0.29	0
15	SSR06521	5	22155275	-1.07	0.22	0
16	SSR15196	5	25021262	-1.31	0.33	0
17	SSR18489	5	25662340	-1.23	0.29	0
18	SSR03940	6	9245205	-0.46	0.04	0.01
19	SSR14934	6	23187094	ns	ns	0.58

Note: ns: non-significant at $P<0.05$ level.

Table 3.6 SLAF marker on each chromosome using the 9930 draft genome assembly v2.0 as reference and the anchored scaffolds statistics in both 9930 assembly v2.0 and Gy14 assembly v1.0.

Chromome	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**Sum**
Length of Chromosome (9930 v2.0)	29,149,675	23,174,626	39,782,674	23,425,844	28,023,477	29,076,228	19,226,500	
SLAF Number	8914	6996	12182	7196	8598	8982	6018	58886
Polymorphic SLAF Marker #	1606	1518	2428	1435	1696	1861	1076	11620
Polymorphism Ratio	18.02%	21.70%	19.93%	19.94%	19.73%	20.72%	17.88%	19.73%
High-quality Mapped SNPs on SLAF	331	376	546	368	481	372	316	2780
Total Scaffold Number (9930 v2.0)	50	36	58	61	38	35	29	307
Anchored Scaffold Number	38	27	40	32	32	23	25	217
Ratio of Anchored/Total	0.76	0.75	0.69	0.52	0.84	0.66	0.86	0.71
Total Scaffold Number (Gy14 v1.2)	90	43	73	99	82	77	65	529
Anchored Scaffold Number	60	28	36	55	62	34	40	315
Ratio of Anchored/Total	0.67	0.65	0.49	0.56	0.76	0.44	0.62	0.60

Table 3.7 Statistics of the linkage map developed with 2,780 SLAF and 55 SSR markers for 148 $F_{7:8}$ PI 197088 × Coolgreen RILs.

Linkage Group (Chr)	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**Sum**
#SNP Marker	331	376	546	368	481	362	316	2780
#SSR Marker	5	0	11	10	22	7	0	55
Map Length (cM)	110.3	137.3	150.7	108.1	128.1	114.9	83	853.3
Average Map Interval (cM)	0.3	0.4	0.3	0.3	0.2	0.3	0.3	0.3
Maximum Map Interval (cM)	4.4	8.9	4.9	7.5	3.2	3.0	2.7	8.9

Table 3.8 QTL for DM and PM resistances detected with BLUPs of mean disease scores in 148 $F_{7:8}$ PI 197088 × Coolgreen RILs.

Trait	Rating time	QTLs	Chr	Associated marker	Peak Position (cM)	Peak Lod Score	1.5 Lod Left Marker	1.5 Lod Right Marker	*P-value*	Additive effects	Phenotypic Variation (%)	Total Phenotypic Variation (%)
DM	1st, 2nd, 3rd	*dm1.1*	1	Marker1_16905088	79	1.91	Marker1_15701257	Marker1_26471365	0.00542	0.52	4.89	73.52
	1st, 2nd, 3rd	*dm2.1*	2	Marker2_8521946	34	2.65	Marker2_7976333	Marker2_10788428	0.00133	-0.56	6.28	
	2nd, 3rd	*dm2.2*	2	Marker2_20890971	116	2.00	Marker2_19519994	Marker2_21497402	0.0518	0.33	1.98	
	3rd	*dm3.1*	3	Marker3_9882197	37	2.12	SSR16264	SSR13312	0.0304	-0.39	2.73	
	1st, 2nd, 3rd	*dm3.2*	3	Marker3_37674467	147	2.28	Marker3_36702815	Marker3_39669543	0.00339	-0.49	5.26	
	1st, 2nd, 3rd	*dm4.1*	4	SSR17911	72	13.37	Marker4_14377388	Marker4_16172943	0	-0.77	13.7	
	1st, 2nd, 3rd	*dm5.1*	5	Marker5_64722	40	4.72	Marker5_11387337	Marker5_850061	0	-0.97	19.68	
	1st, 2nd, 3rd	*dm5.2*	5	Marker5_16754619	67	6.20	Marker5_16446482	Marker5_18140438	0	-1.05	27.76	
	1st, 2nd, 3rd	*dm5.3*	5	Marker5_25566302	119	11.92	Marker5_24908668	Marker5_26224508	0	-1.14	30.97	
	1st, 2nd, 3rd	*dm6.1*	6	Marker6_5079443	20	2.12	Marker6_3684277	Marker6_5856618	0.00362	-0.51	5.33	
	3rd	*dm6.2*	6	Marker6_26132968	97	1.81	Marker6_24230045	Marker6_27973837	0.0191	0.41	3.29	
	1st, 2nd, 3rd	*dm2.2:dm3.1*							0.00817	-0.22	4.45	
	1st, 2nd	*dm5.2:dm5.3*							0.00742	-0.17	2.56	

Trait	Rating time	QTLs	Chr	Associated marker	Peak Position (cM)	Peak Lod Score	1.5 Lod Left Marker	1.5 Lod Right Marker	*P-value*	Additive effects	Phenotypic Variation (%)	Total Phenotypic Variation (%)
PM	n.a*	*pm1.1*	1	Marker1_25833145	101.3	3.82	Marker1_24814391	Marker1_28110025	0	-0.55	5.56	63.04
	n.a	*pm2.1*	2	Marker2_12541521	49.15	5.12	Marker2_8520308	Marker2_12912775	0.00458	-0.3	2.14	
	n.a	*pm5.1*	5	SSR15196	115	30.47	Marker5_24647715	Marker5_26224508	0	-1.16	32.38	
	n.a	*pm6.1*	6	Marker6_26132968	117.56	5.45	Marker6_23875073	Marker6_26499531	0.00892	0.29	1.82	

*n.a: not applied.

Table 3.9 Differentially expressed genes (DEGs) within 2.0-LOD support intervals of five DM resistance QTL using filtering criteria from the transcriptome data of Burkhardt and Day (2016).

Filter Step	***dm3.2***	***dm4.1***	***dm5.1***	***dm5.2***	***dm5.3***	**Sum**
Step0: Total genes within 5 QTL intervals	277	209	134	210	390	1220
Step1: DEGs in PI197088 for at least one time point	136	82	51	109	180	558
Step2: DEGs in PI197088 from 2dpi to 6dpi	64	34	25	50	90	263
Step3: Non-DEGs, opposite-DEGs, different expression level genes in Vlaspik	35	16	9	27	52	139

Figure 3.1 Phenotypic distribution of mean DM disease scores among 169 PI 197088 × Coolgreen RILs presented with violin plot and boxplot for three rating times at all five environments.

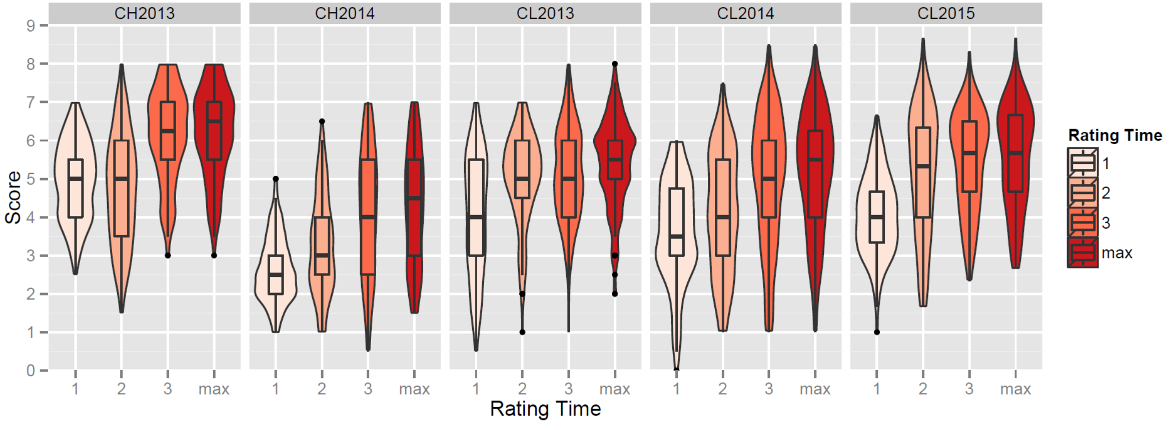
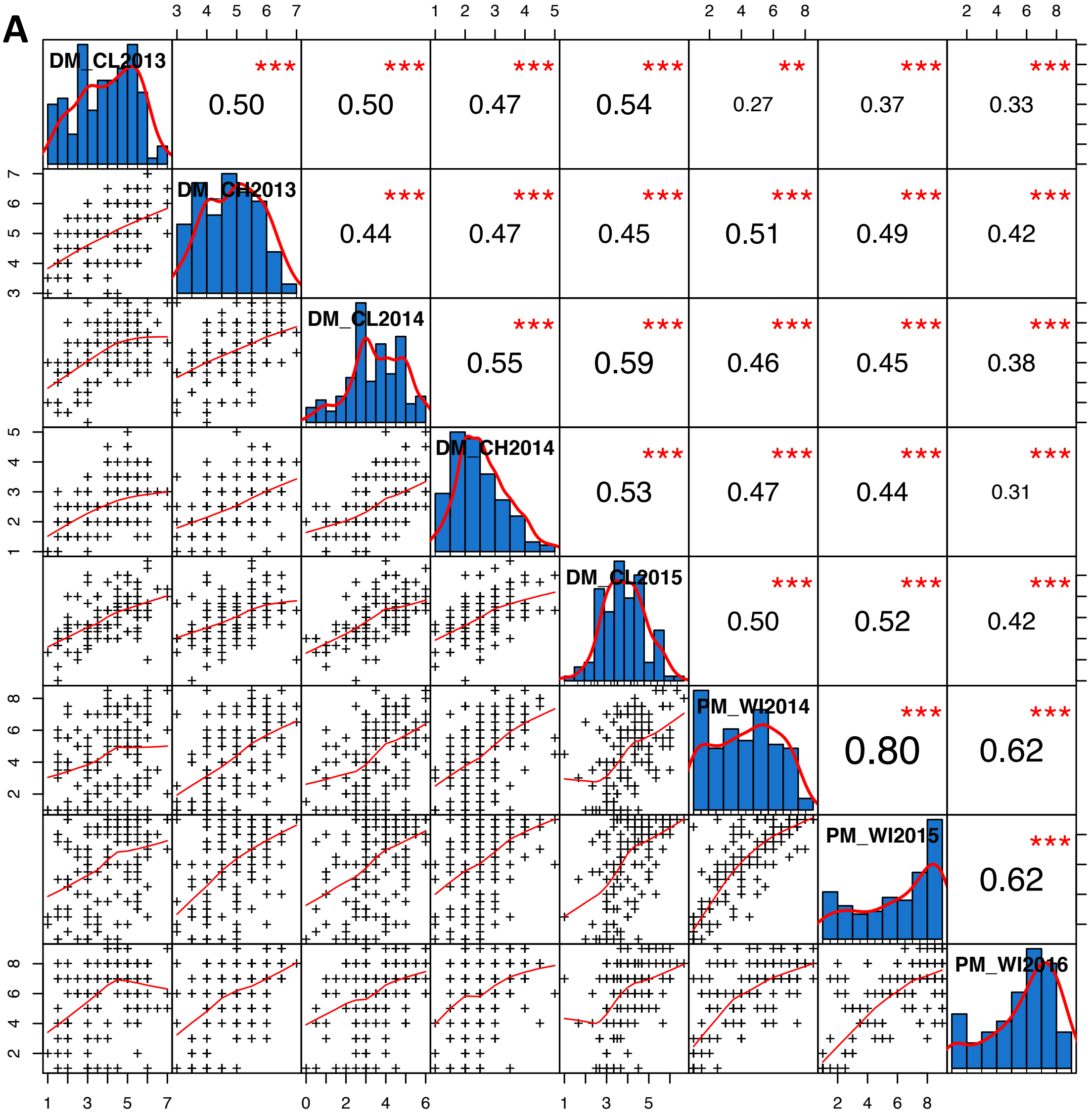
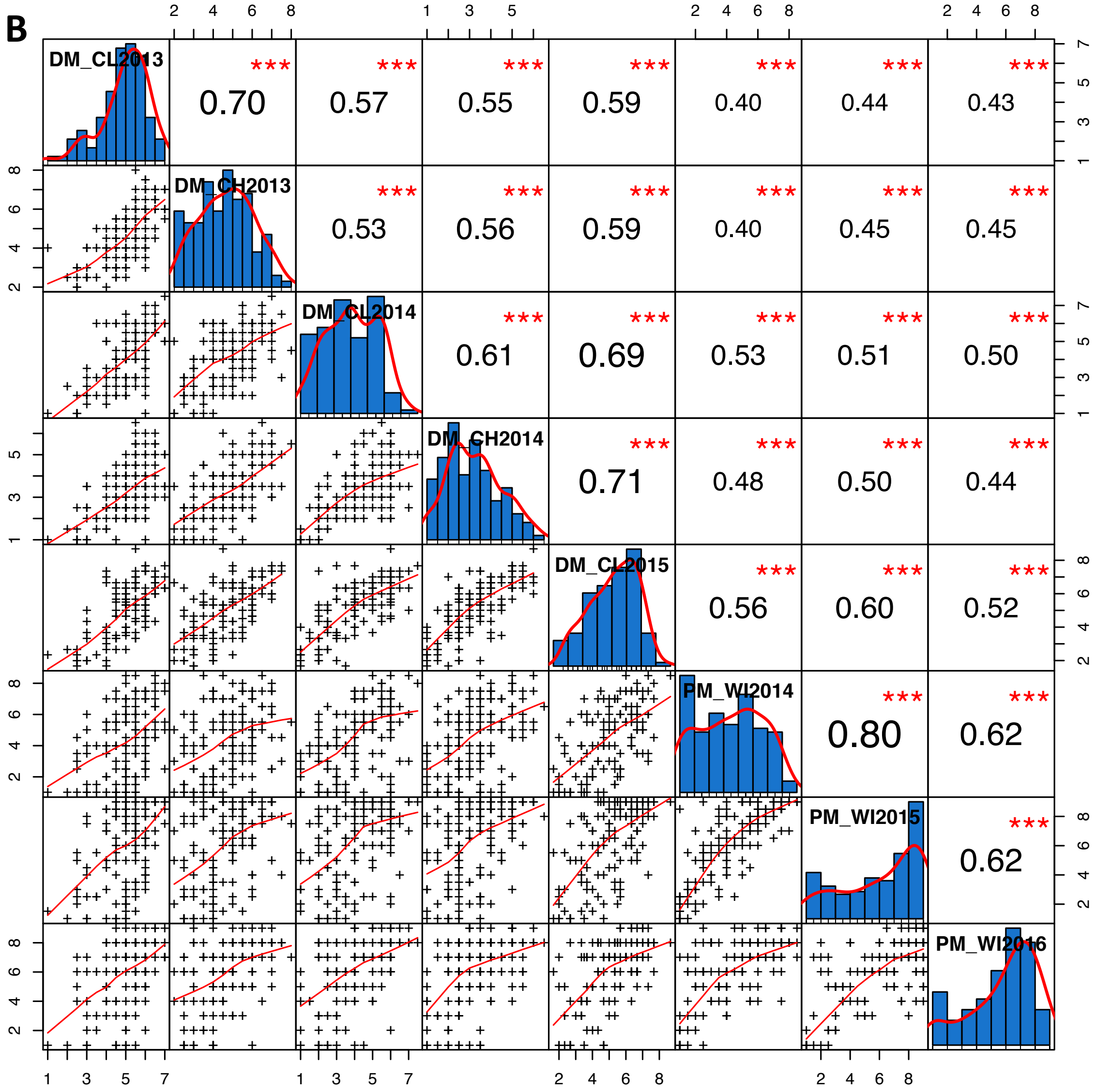


Figure 3.2 The phenotypic analysis of DM disease scores at first (A), second (B) and third (C) rating among different environments. The plots on the diagonal show the phenotypic distribution of DM and PM scores at each environment. The values above the diagonal show the pairwise Spearman's rank correlation coefficients, and the plots below diagonal are scatter plot of compared phenotypes. *, *P< 0.05*; **, *P<0.01*; ***, *P<0.001*.




B
DM_CL2013
DM_CH2013
DM_CL2014
DM_CH2014
DM_CL2015
PM_WI2014
PM_WI2015
PM_WI2016
*** 0.70
*** 0.57
*** 0.55
*** 0.59
*** 0.40
*** 0.44
*** 0.43
*** 0.53
*** 0.56
*** 0.59
*** 0.40
*** 0.45
*** 0.45
*** 0.61
*** 0.69
*** 0.53
*** 0.51
*** 0.50
*** 0.71
*** 0.48
*** 0.50
*** 0.44
*** 0.56
*** 0.60
*** 0.52
*** 0.80
*** 0.62
*** 0.62

C

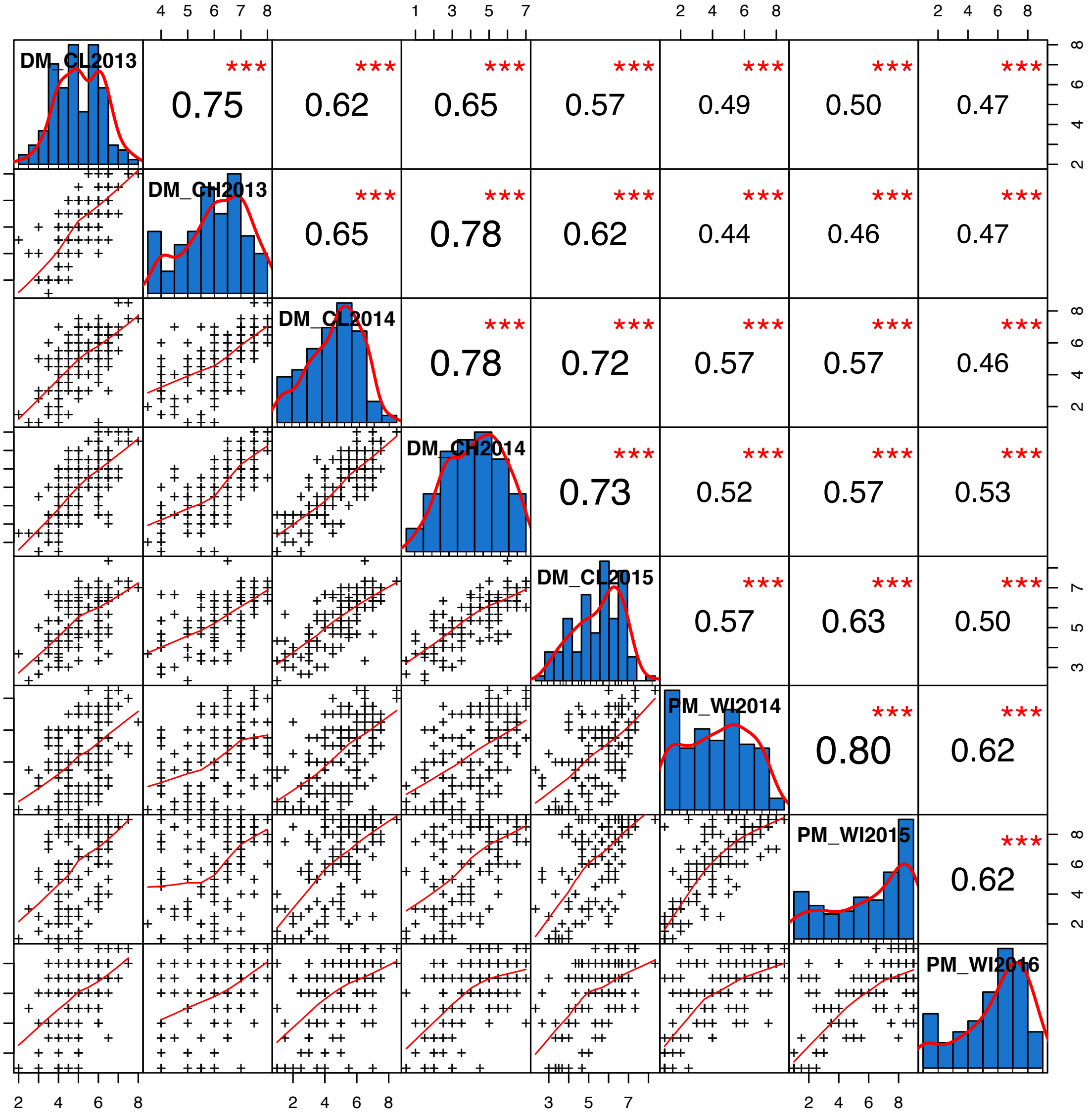

DM_CL2013
DM_CH2013
DM_CL2014
DM_CH2014
DM_CL2015
PM_WI2014
PM_WI2015
PM_WI2016
*** 0.75
*** 0.62
*** 0.65
*** 0.57
*** 0.49
*** 0.50
*** 0.47
*** 0.65
*** 0.78
*** 0.62
*** 0.44
*** 0.46
*** 0.47
*** 0.78
*** 0.72
*** 0.57
*** 0.57
*** 0.46
*** 0.73
*** 0.52
*** 0.57
*** 0.53
*** 0.57
*** 0.63
*** 0.50
*** 0.80
*** 0.62
*** 0.62
4 5 6 7 8
1 3 5 7
2 4 6 8
2 4 6 8
2 4 6 8
2 4 6 8
3 5 7
2 4 6 8

Figure 3.3 The phenotypic analysis of BLUPS of DM and PM disease scores among rating times. The plots on the diagonal show the phenotypic distribution of DM and PM scores at each rating time. The values above the diagonal show the pairwise Spearman's rank correlation coefficients, and the plots below diagonal are scatter plot of compared phenotypes. *, $P < 0.05$; **, $P<0.01$; ***, $P<0.001$.

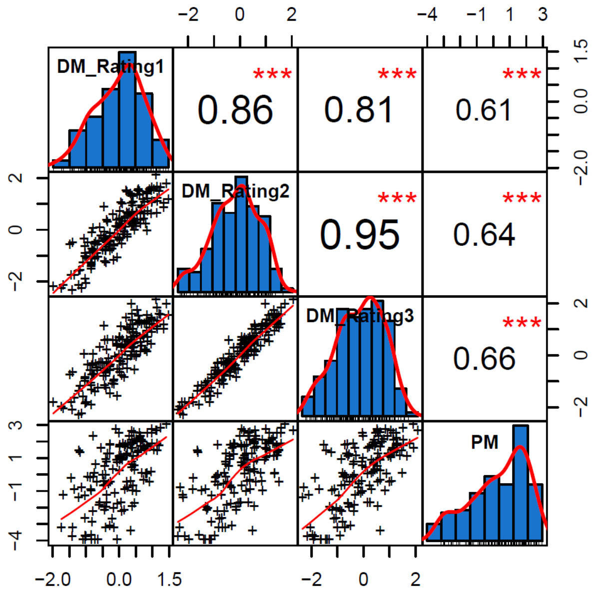


Figure 3.4 Chromosomal distribution of SNPs with segregation distortion. Seven chromosomes were separated by blue and orange colors. Black horizontal line is the threshold for determining segregation distortion at *P* < 0.05 with Bonferroni correction for genome wide error (–log10(*P*)=2.40). The x-axis corresponds to the SNP index with the order physical position in the 9930 draft genome assembly v2.0, and the y-axis corresponds to the –log10(*P*) value. The SNPs with –log10(*P*) > 6.2 were trimmed off as genotyping error.

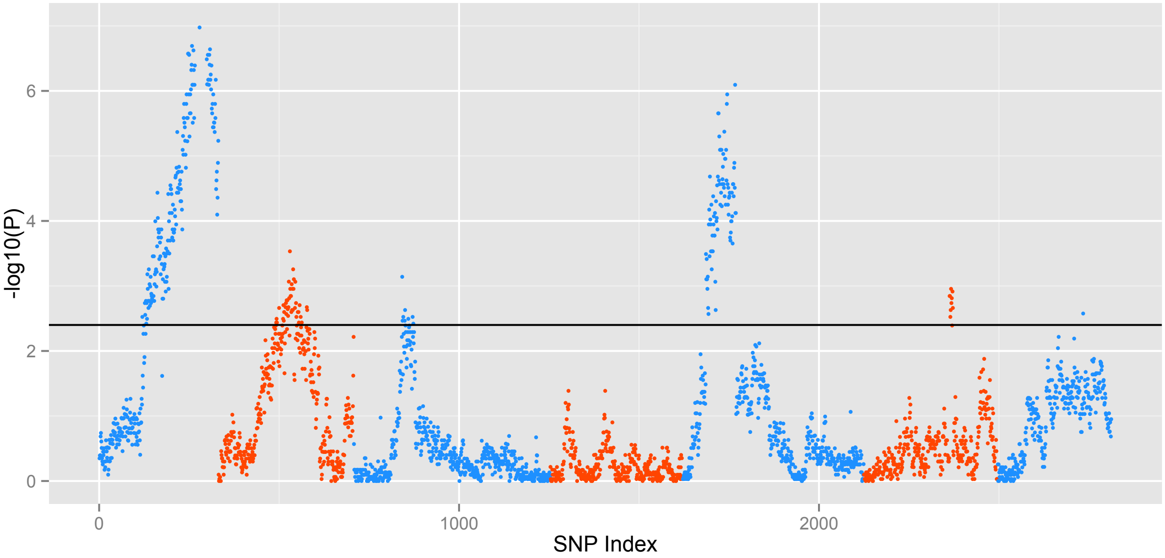


Figure 3.5 Pairwise recombination fractions (upper left diagonal) and LOD scores (lower right diagonal) for all markers. A) SNP markers were ordered by their physical positions in the 9930 draft genome assembly v2.0. Disordered markers showed closer linkage with other chromosomes; B) SNP marker order generated by *R/ASMap* displayed a good quality of the marker ordering; C) Integrated map wth SSR and SNP markers reordered by *R/ASMap*.

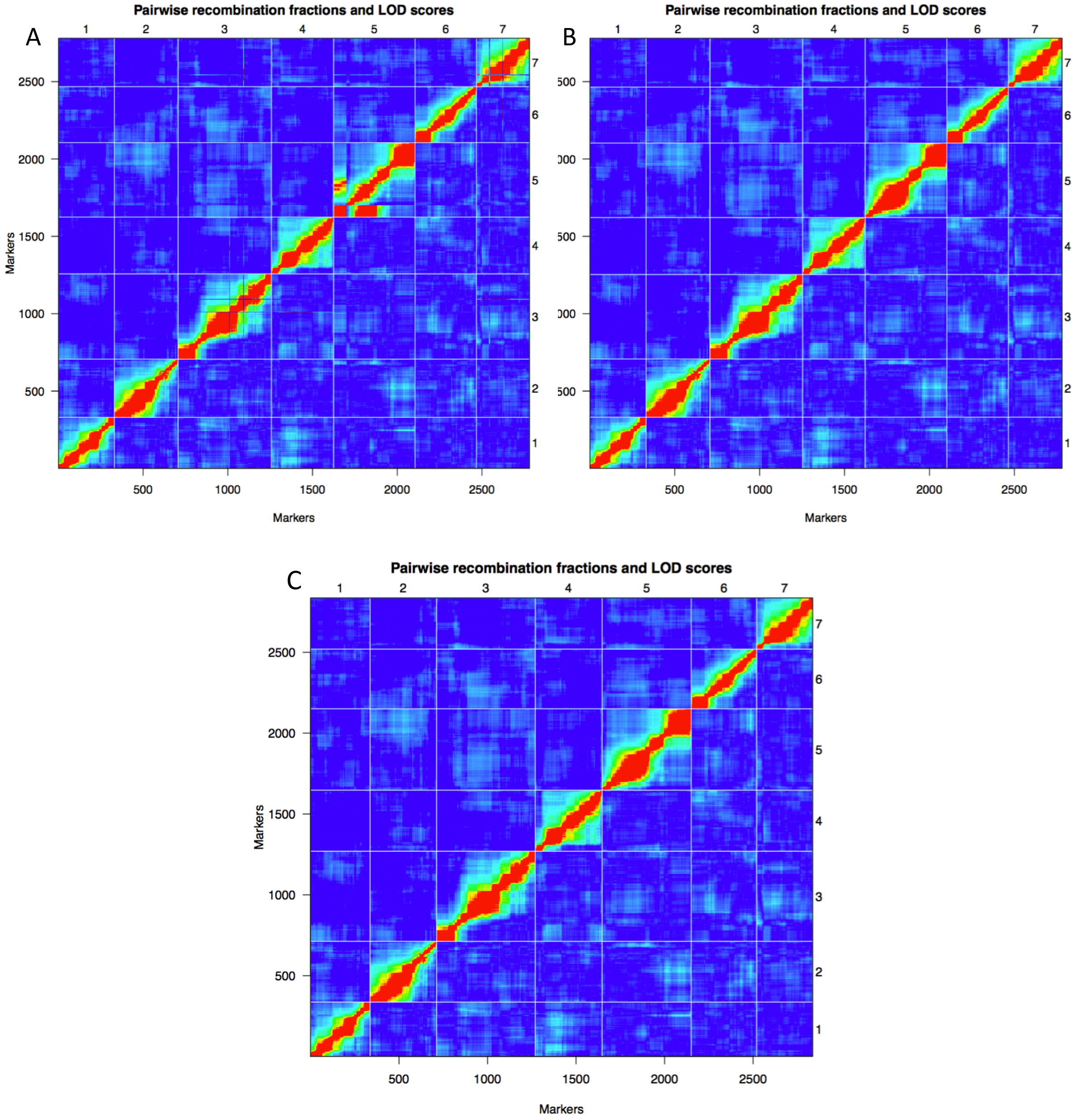


Figure 3.6 Comparison map between genetic and physical positions of the 9930 draft genome assembly v2.0 and the Gy14 assembly v1.0 in seven chromosomes. In y-axis, one unit equals to 1 Mb of physical length or 5 cM linkage map length. Blue dashed line connects mis-assembly markers and their physical locations.

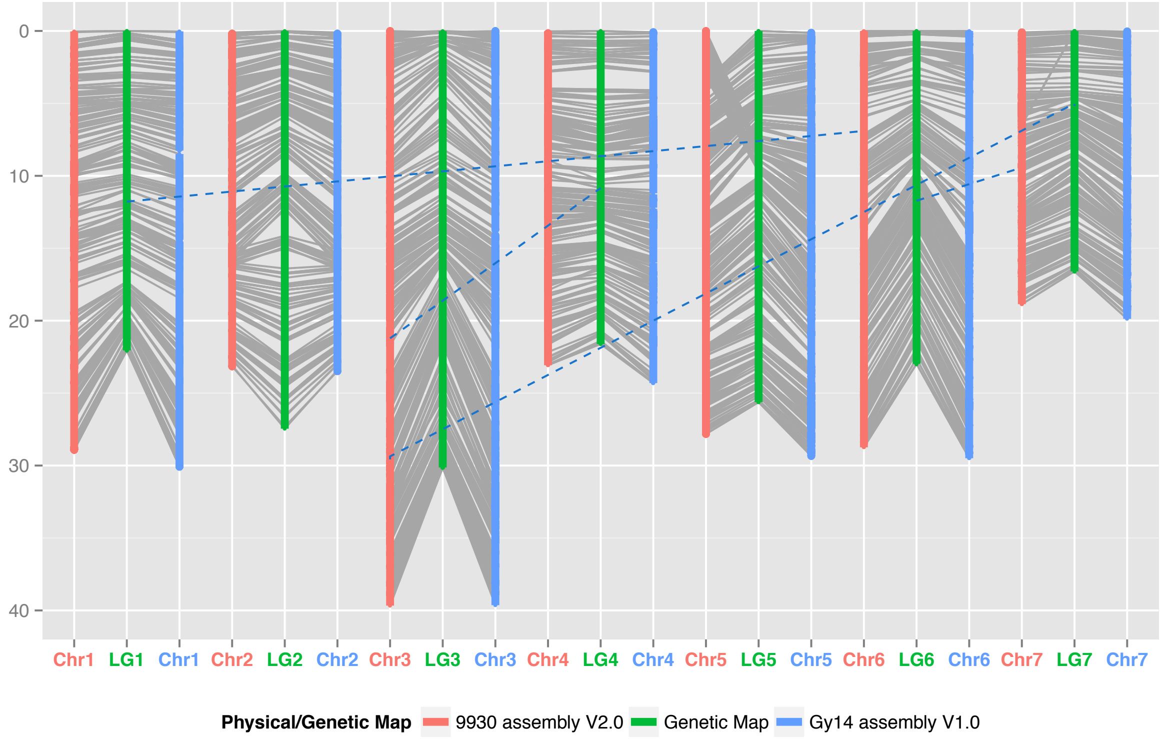


Figure 3.7 Downy mildew resistance QTL on chromosome 5 detected with BLUPs. A) LOD score profile of QTL on chromosome 5 detected with MQM with BLUPs of disease scores from three rating times. The horizontal dashed line represents LOD threshold value for alpha=0.05 after 1000 permutations. B) Genotypic effects on DM disease scores at the peak location of *dm5.1* (Marker5_64722, left), *dm5.2* (Marker5_16754619, middle), and *dm5.3* (Marker5_25566302, right). Red, green, and blue lines represent the first, second, and third ratings, respectively. '*a*' and '*b*' are alleles from PI 197088 and Coolgreen, respectively. Error bars are +/- 1 standard error (SE).

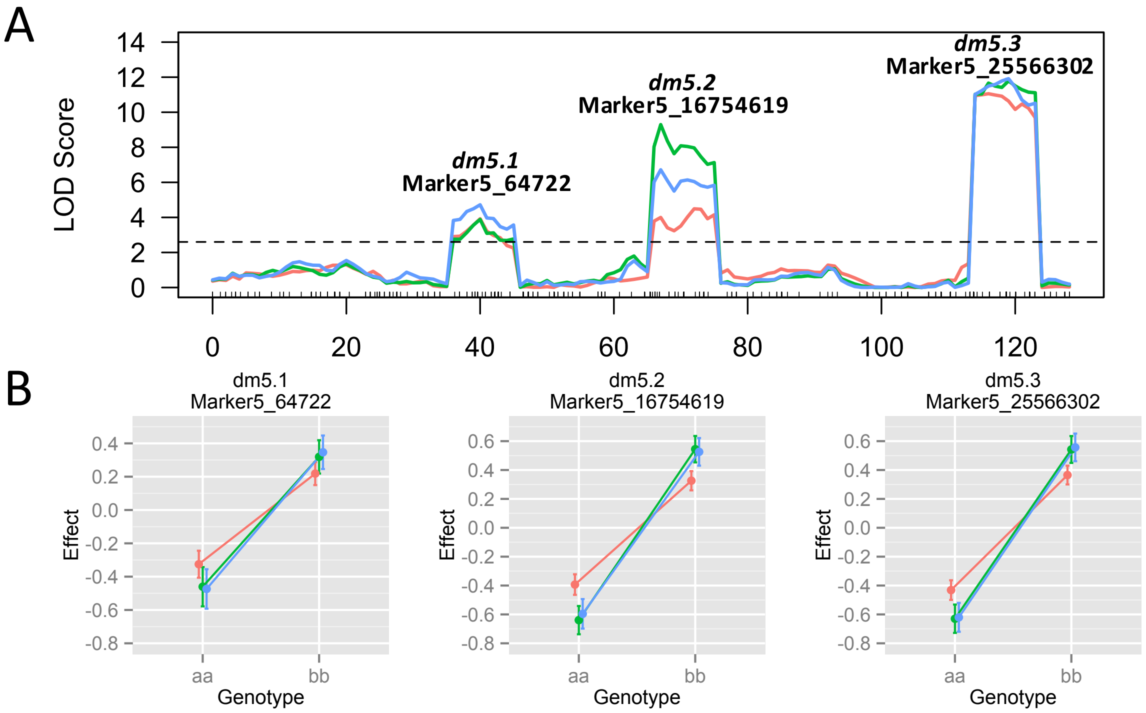


Figure 3.8 The dynamics of QTL effects across multiple environments (CH2013, CH2014, CL2013, CL2014, and CL2015) on DM resistance at the third rating in PI197088 × Coolgreen RIL population. The QTL names and their peak markers are listed as the title of each plot. '*a*' and '*b*' are alleles from PI 197088 and Coolgreen, respectively. Error bars are +/- 1 standard error (SE).

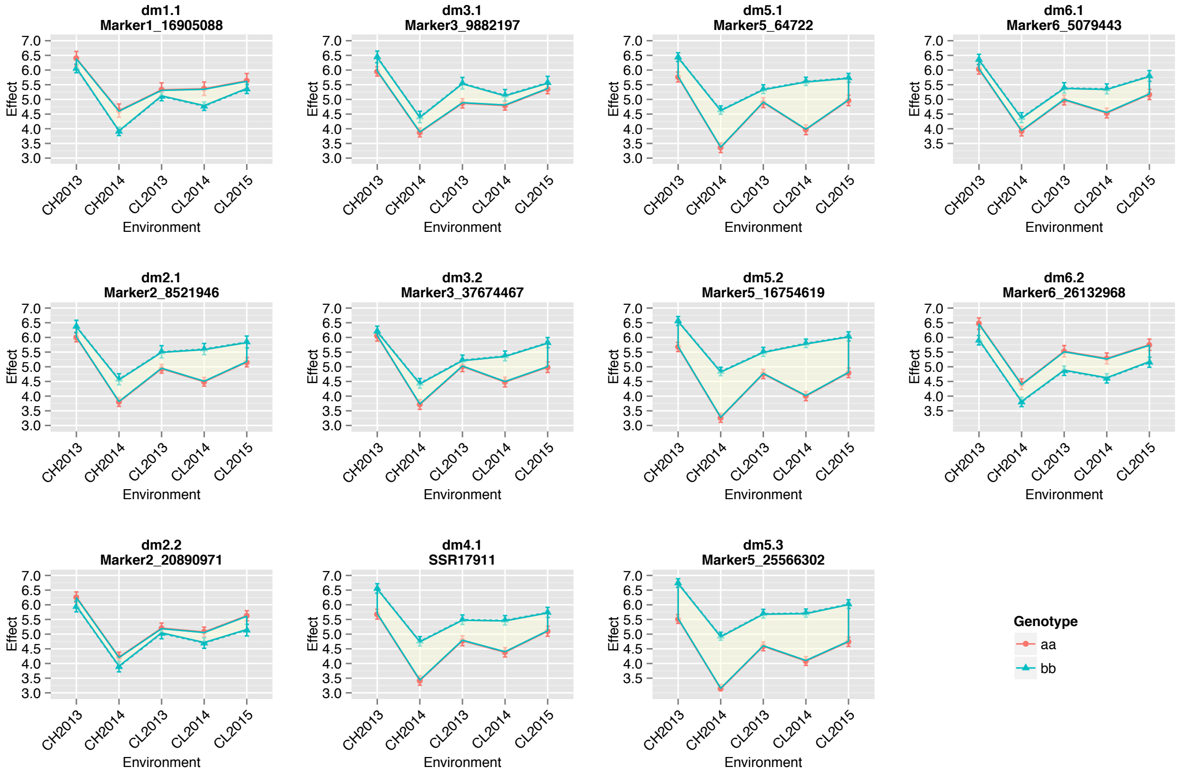


Figure 3.9 Whole genome view of QTL for powdery mildew resistance detected with 148 PI 197088 × Coolgreen RILs with BLUPs from three environments (WI2014, WI2015 and WI2016).

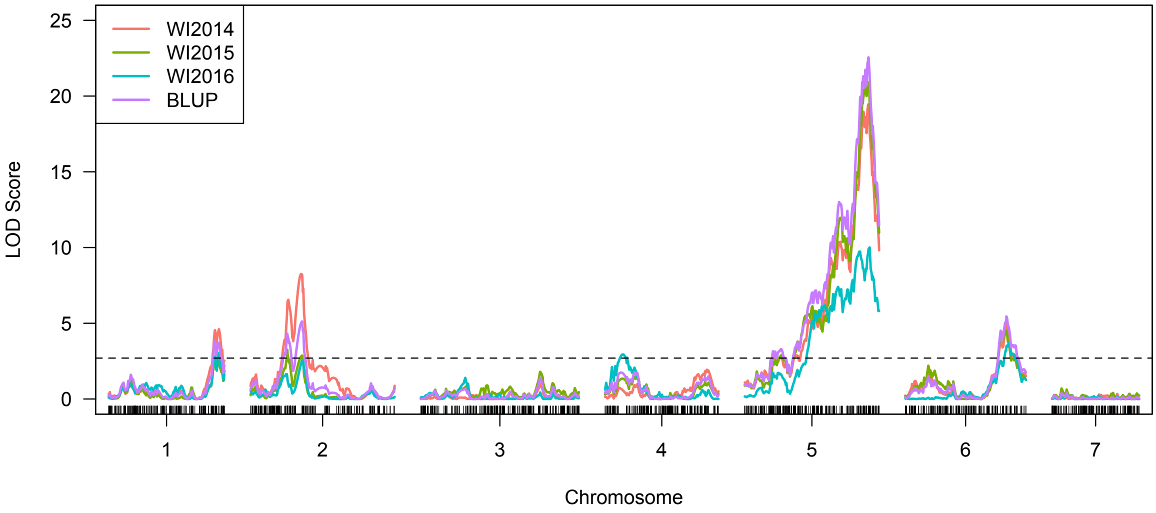


Figure 3.10 Venn diagram showing co-localized QTL for powdery mildew and downy mildew resistances detected in the PI 197088 × Coolgreen RIL population.

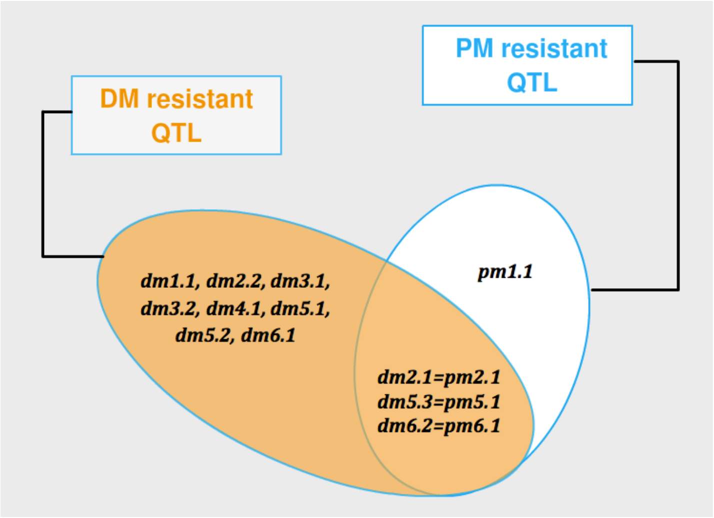


Figure 3.11 Heat map expression levels of 558 differentially expressed genes (DEGs) in PI 197088 at different time points after inoculation. The raw data was extracted from Burkhardt and Day (2016). The heat map reveals a closer relationship between PI 197088-1dpi and Vlaspik-1dpi. dpi: day post inoculation; PI088 stands for PI 197088; Vlas stands for Vlaspik.

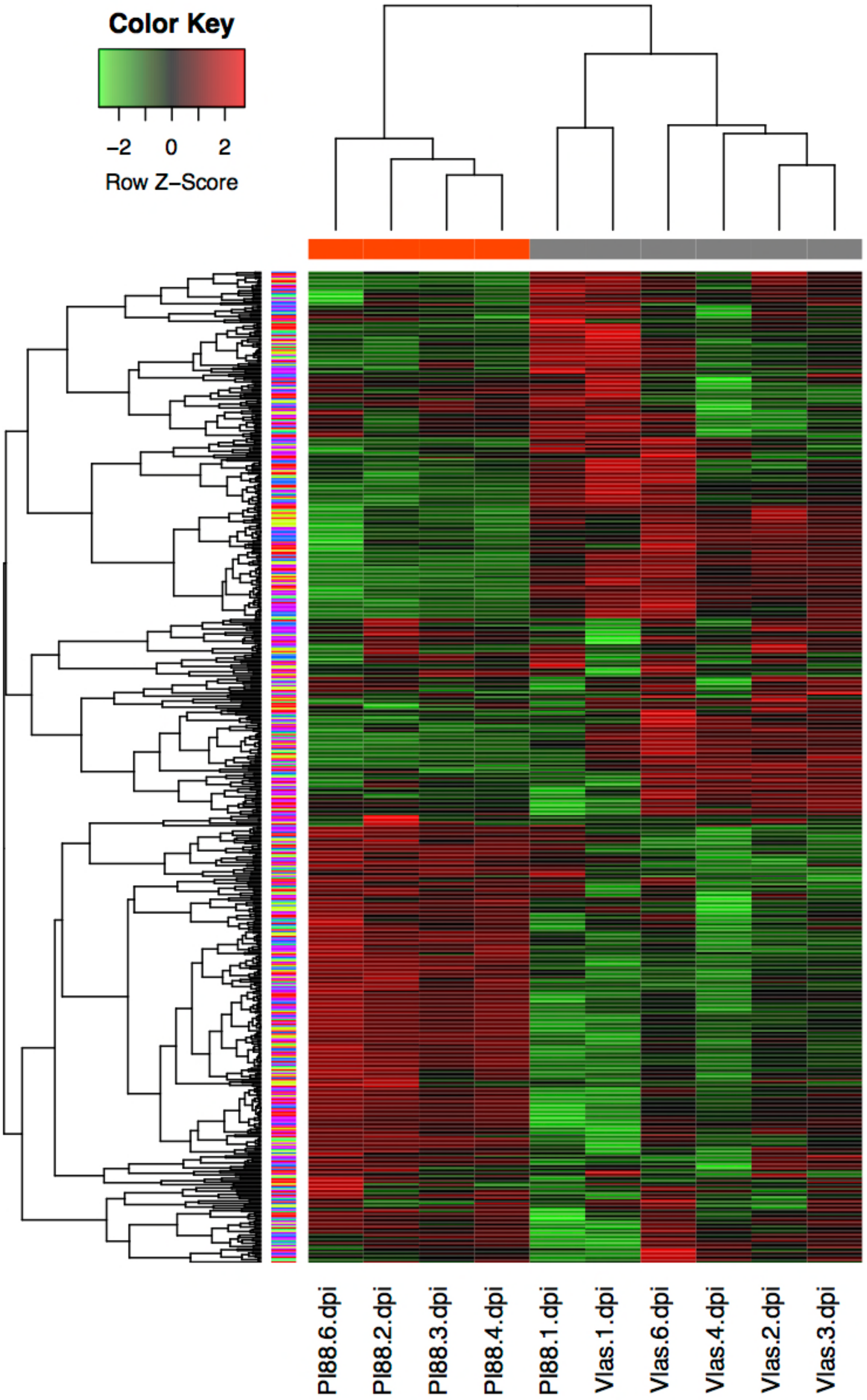


Figure 3.12 GO term analysis for 558 DEGs, which suggest that 40, 20 and 20% of these differentially expressed genes are involved in the primary metabolic process, catalyze activity and are located on the cell membrane, respectively.

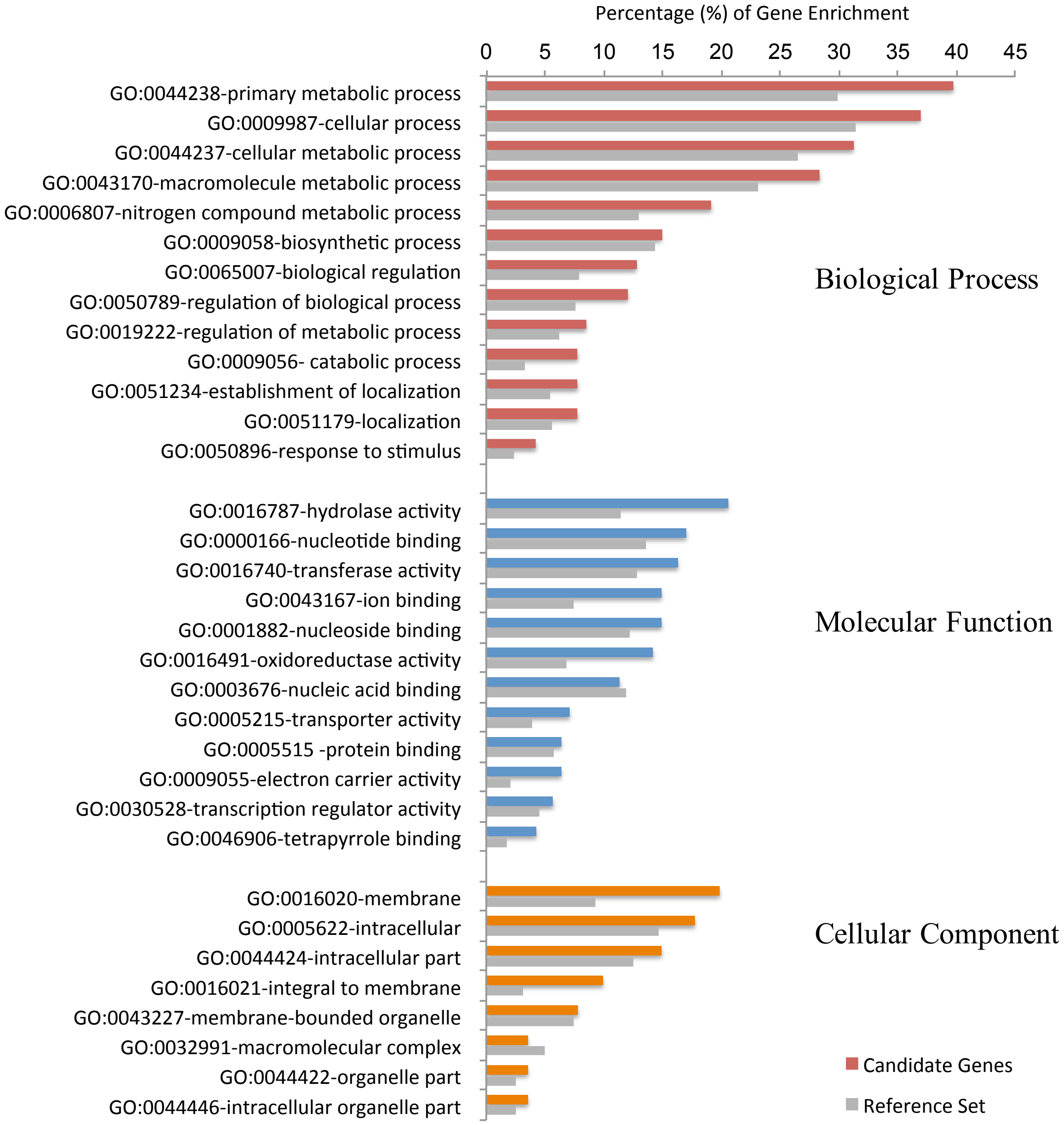
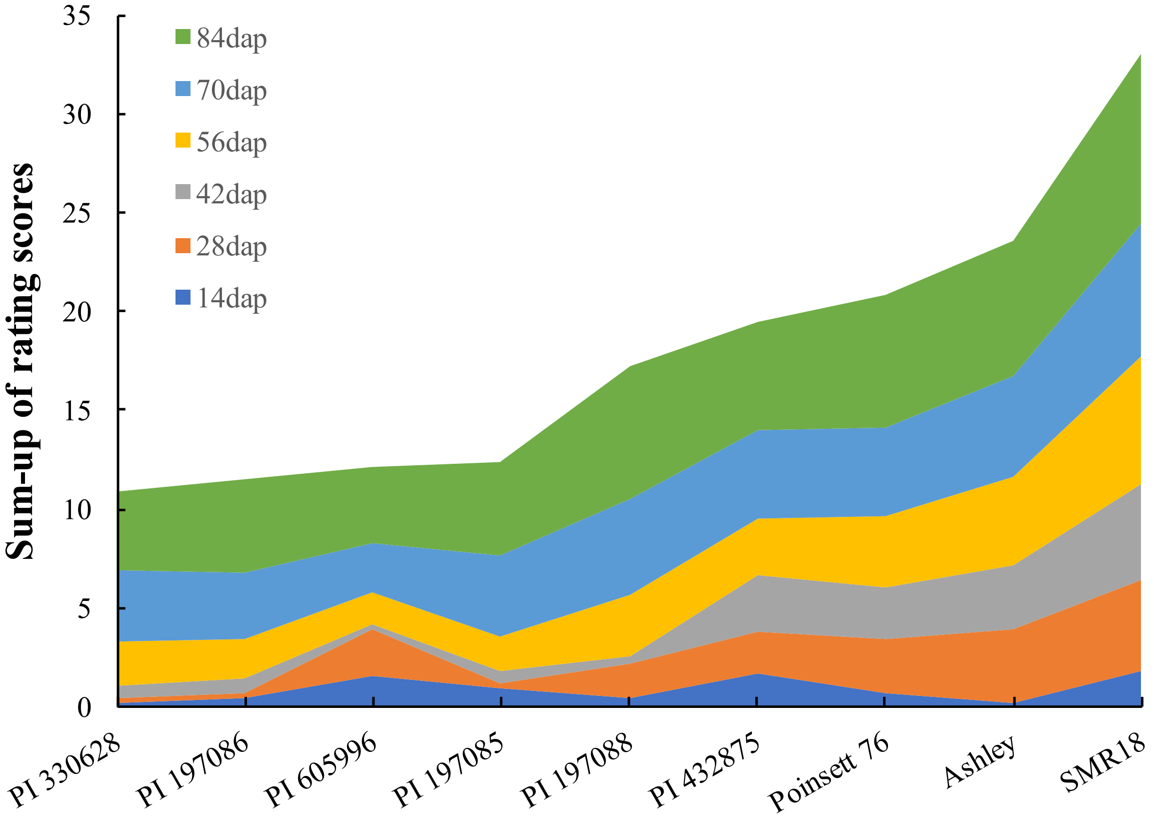


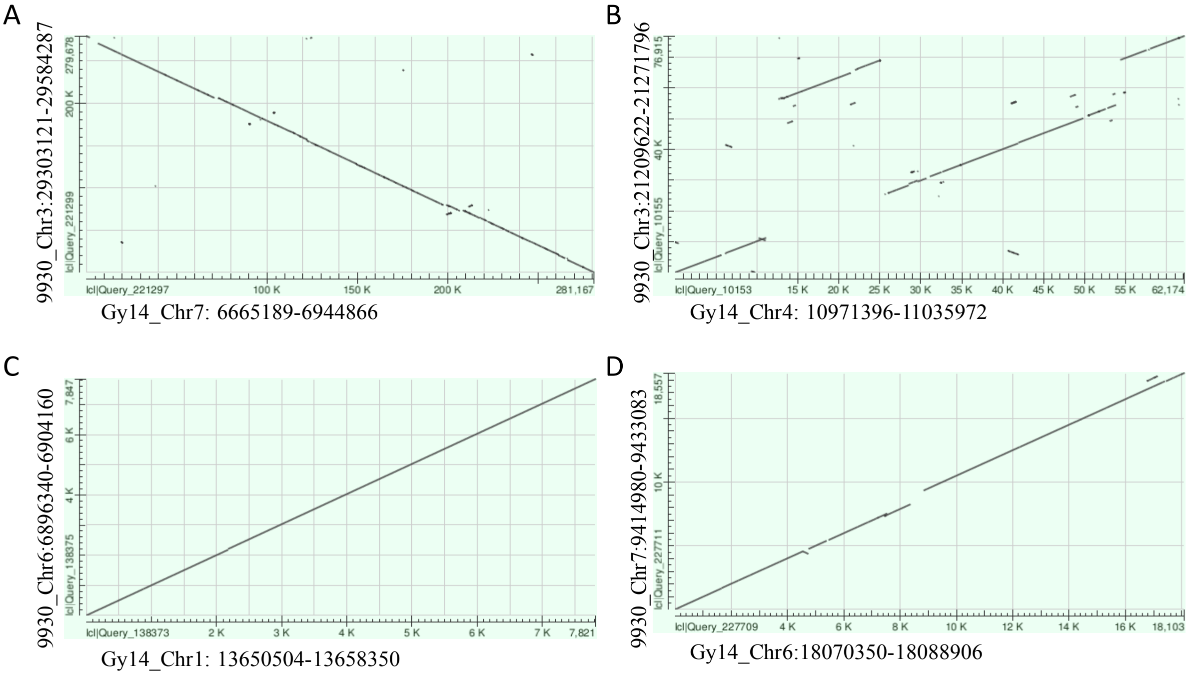
Figure 3.13 Sum-up of the rating scores from 14 day after planting (dap) to 84 dap of resistant accession and susceptible lines (Modified from VandenLangenberg and Wehner 2016).



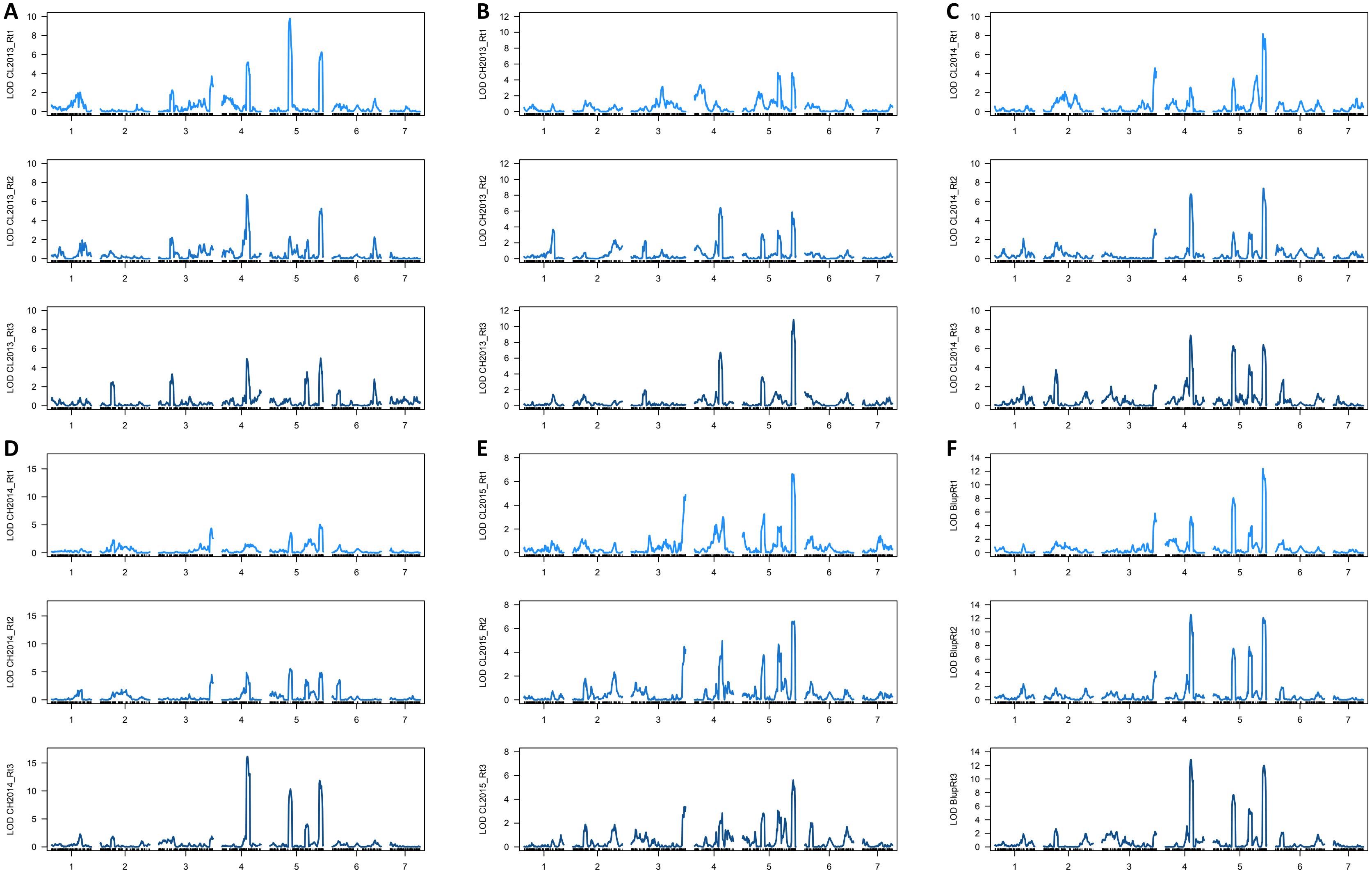
Appendix 3.1 Information of SSR and SNP markers placed on the PI197088 × Coolgreen linkage map, includes linkage position before and after integrated SSR markers, SNP nucleotide among 9930, PI 197088 and Coolgreen, physical position on the 9930 draft genome assembly v2.0 and the Gy14 assembly v1.0.
The Appendix 3.1 is uploaded at the following link:
http://cranberry.vcru.wisc.edu/owncloud/index.php/s/3W8uWGmnyjtlOO2

Appendix 3.2 The corresponding mis-assembly regions in 9930 assembly v2.0 identified by mis-placed markers in linkage group, and the alignment dot-matrix between the 9930 draft genome assembly v2.0 and the Gy14 assembly v1.0.

Marker Name	LG	9930 v2.0 Chr	Bin Size (9930 v2.0)		Gy14 v1.0 Chr#	Bin Size (Gy14 v1.0)		Identity	Dot Matrix
			Left Pos	Right Pos		Left Pos	Right Pos		
Marker3_29366233	7	3	29303121	29584287	7	6665189	6944866	99%	A
Marker3_29466582	7	3			7				
Marker3_29498560	7	3			7				
Marker3_29575926	7	3			7				
Marker3_21214463	4	3	21209622	21271796	4	10971396	11035972	99%	B
Marker6_6901436	1	6	6896340	6904160	1	13650504	13658350	100%	C
Marker7_9428392	6	7	9414980	9433083	6	18070350	18088906	99%	D



Appendix 3.3 Genome wide QTL mapping for three rating times using BLUP (F) and RIL means from CL2013 (A), CH2013 (B), CL2014 (C), CH2014 (D), and CL2015 (E).



Appendix 3.4 DM and PM resistance QTL detected across all environments and rating time in the PI 197088 × Coolgreen RIL population.
Appendix 3.4 is uploaded at the following link:
http://cranberry.vcru.wisc.edu/owncloud/index.php/s/qnY3M4M9A8bSOjX

Chapter 4: Fine mapping and characterization of candidate genes of two major-effect QTL *dm4.1* and *dm5.1* for downy mildew resistance in WI7120 (PI 330628)

Abstract

Downy mildew (DM), caused by the obligate oomycete *Pseudoperonospora cubensis*, is the most devastating foliar disease of cucumber worldwide. However, the molecular mechanisms of DM resistance in cucumber is still unknown. Two major-effect quantitative trait loci (QTL), *dm4.1* and *dm5.1*, for DM resistance have been identified from the resistant inbred line WI7120 (PI 330628). Here, this study reported fine mapping of *dm4.1* and *dm5.1* using mapping populations derived from near isogenic lines (NIL) for each QTL. Segregating populations for each Mendelized QTL were phenotyped for DM inoculation responses in field trials at multiple locations in 2015 and 2016, which allowed to narrow down each QTL into a 2 Mb region. Refinement of map location of each QTL was conducted by identifying recombinants in large NIL-derived segregating populations. Progeny tests of recombinants to DM artificial inoculation response were performed in the growth chamber with controlled environment. Finally, *dm4.1* and *dm5.1* were located to ~82 kb and ~28kb genomic regions, respectively. Integrated genetic mapping and transcriptome analysis identified two possible candidate genes for *dm4.1* and *dm5.1*, which encodes an F-box SKIP24 protein (SKP1-interacting partner 24) and a PHOSPHATE1 (PHO1)-like protein, respectively.

Introduction

The downy mildew (DM) is the common name describing the symptoms caused by a group of highly specialized obligate oomycetes of vascular plants. The pathogens causing the DM disease belong to the Peronosporaceae family that contains more than 600 species in 17 genera (Thines et al. 2009). A number of host resistance genes to DM have been cloned in Arabidopsis and Lettuce. In Arabidopsis, the resistance to DM is controlled by *RPP* genes (*__r__esistance to __P__eronospora __p__arasitica*, formerly *Hyaloperonospora arabidopsis*) including *RPP1*, *RPP2*, *RPP3*, *RPP5*, *RPP7*, *RPP8*, and *RPP13*, which all belong to the NB-LRR (nucleotide-binding leucine-rich repeat) type resistance genes (Botella et al. 1998; Cooley et al. 2000; Bittner-Eddy et al. 2000; Van Der Biezen et al. 2002; Eulgem et al. 2004; Noël et al. 2007; Yi and Richards 2008). Other DM resistance genes isolated in Arabidopsis include *IOS1*, *DMR1* and *DMR6*. *IOS1* (*Impaired oomycete susceptibility 1*) is a receptor-like protein kinase (RLK) type R gene (Hok et al. 2011). *DMR1* encodes a homoserine kinase, which is a key enzyme in primary amino acid metabolism (van Damme et al. 2009). *DMR6* was discovered through ethl methane sulfonate (EMS) mutagenesis, which encodes a defense-associated 2-oxoglutarate (2OG)-Fe(II) oxygenase (Zeilmaker et al. 2015). In Lettuce, DM caused by the pathogens *Bremia lactucae* Regel, has been characterized gene-for-gene plant-pathogen interactions for more than 25 genes. Among them, *Dm3* is the only one that has been cloned, which is a NB-LRR type gene (Shen et al. 2002; Christopoulou et al. 2015).

In cucumber, DM is caused by the oomycete *Pseudoperonospora cubensis*. Several quantitative trait loci (QTL) mapping studies have identified a number of QTL for DM resistance in different sources of resistant materials, but none has been cloned (Zhang et al. 2013; Yoshioka et al. 2014; Wang et al. 2016; Win et al. 2017; Chapter 3). The molecular mechanisms of DM resistance in cucumber remain unknown. Several studies tried to associate DM QTL with known resistance genes through the candidate gene approach. For example, the cucumber genome has approximately 71 NB-LRR resistant genes analogs (RGAs), most of which are distributed in 11 clusters (Yang et al. 2013; Wan et al. 2013). In the study of resistant line K8, Zhang et al. (2013) conducted QTL mapping for its DM resistance and found ten predicted NBS-type RGAs in the *dm5* major-effect QTL region, but none in the regions where *dm1.1* and *dm6.1* QTL reside. Wang et al. (2014) conducted genome-wide analysis of the LRR-receptor-like protein (RLP) and LRR-receptor-like kinase (LRR-RLK) type genes in the cucumber genome and found that some DM resistance QTL were co-localized with these types of R genes. Schouten et al. (2014) identified one or two the homologs of Arabidopsis *DMR1* and *DMR6* in cucumber and found that *CsDMR6-2* co-localized with QTL *dm5.1* of PI 197088. These studies have provided useful information in understanding the molecular mechanisms of DM resistance in cucumber. They also suggested possibly different mechanism underlying cucumber DM resistance as compared with Arabidopsis. It is also clear that understanding the genetic and molecular mechanisms of DM resistance in cucumber is important to clone the genes or QTL, which has not been available so far. In our previous study of the WI7120 (PI 330628) cucumber inbred line, we identified two major-effect DM resistance QTL (*dm4.1* and *dm5.1*) that could explain more than 50% of the phenotypic variance (Wang et al. 2016). Although varied in the magnitude of QTL effects, these two QTL seem to be consistent in map locations with several other DM QTL detected in the resistant lines like K8, PI 197088, PI 197085, and TH118FLM (Caldwell et al. 2011; Zhang et al. 2013; Szczechura et al. 2016; Win et al. 2017; Chapter 3). In all cases, both QTL do not have obvious recessive and epistasis effects, which is advantageous in fine mapping of the two QTL and their use in cucumber breeding.

Map-based cloning of simply inherited genes or QTL has been a routine in model species such as Arabidopsis and rice, but it is still a non-trivial task in vegetable crops like cucumber. A successful map-based cloning approach requires development of large segregating populations with informative recombinants in the candidate gene region. To avoid the interference by other QTL, the majority of the genome except for the target donor fragment should be substituted with recurrent parent (Remington et al. 2001). In reality, map-based cloning of disease resistance QTL has proven extremely difficult for several reasons: 1) relative small effects on phenotype contributed by each QTL, 2) variations in disease severity across different environments, and 3) lack of uniformity in the evaluation of disease symptoms (Flint and Mott 2001; Salvi and Tuberosa 2005; Drinkwater and Gould 2012). Thus, it is not surprising that, in cucumber, only one disease resistance QTL, *pm5.1* or *pm-h* has been cloned which belongs to the MLO type gene conferring powdery mildew resistance (Nie et al. 2015). The objective of this study was to fine map the *dm4.1* and *dm5.1* major-effect QTL from WI7120. Near isogenic lines (NILs) for each QTL were developed in the genetic background of the 9930 susceptible line. Segregating populations were then developed for fine mapping and identification of the candidate genes.

Materials and Methods

Plant materials

A marker-assisted backcrossing (BC) strategy was employed to develop NILs for the two QTL. The F_1 from WI7120 × 9930 cross was backcrossed with the recurrent parent 9930 to produce BC_1F_1 to initiate NIL development following the strategy showed in **Figure 4.1**. At each BC generation, the progeny were genotyped by SSR markers for background selection of 9930 alleles and foreground selection of target WI7120 DM resistance QTL alleles. The $BC_3F_{2:3}$ population was produced by three rounds of backcrossing and followed by self-fertilization for two generations (**Figure 4.1**). The initial $BC_3F_{2:3}$ family was employed as the fine mapping population which was phenotyped for DM inoculation responses in field screening tests. Informative recombinant-derived $BC_3F_{2:3}$ families and homozygous $BC_3F_{3:4}$

families (from BC_3F_3 individuals) were evaluated for artificial inoculation response of the DM pathogen in growth chamber tests.

Greenhouse conditions and plant maintenance

Cucumber seeds were grown for 2-3 weeks in 96-well (8×12) plastic trays for genotyping to identify informative plants that carried 9930 background and target QTL. Selected seedlings were transferred to 9-inch plastic pots for backcrossing or self-pollination. Fruits were harvested at 35 to 40 day post pollination for seed extraction. Seeds were treated with 1% hydrogen peroxide (H_2O_2) for 3h before planting to ensure the uniform germination. All crosses were performed at the University of Wisconsin-Madison Walnut Street Greenhouses (WSGH) or the greenhouses at the USDA Dairy Forage Research Center (DFRC) facility at Madison, WI.

DNA extraction and PCR

Genomic DNA was isolated from the cotyledon of one-week old seedlings. Tissue samples were first lyophilized in freeze-dryer and then grounded into fine powder with a high-throughput homogenize (OPS Diagnostics, Lebanon, NJ). Genomic DNA was extracted using CTAB method and purified with phenol/chloroform/isoamyl alcohol (Murray and Thompson, 1980). The DNA concentration of all samples was determined using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Tech, Wilmington, DE) and adjusted to a concentration of 50 ng μL^{-1}. PCR amplification with molecular markers and gel electrophoreses of PCR products followed Li et al. (2011).

Screening tests for downy mildew resistance in segregating populations

Field trials

Phenotypic data of segregating population to DM inoculation responses were collected for two $BC_3F_{2:3}$ populations (*dm4.1* and *dm5.1*) in four environments across three countries (US, Italy, and the Netherlands) of two years (2015 and 2016), which were designated as US2015, IT2015, NL2015, and NL2016, respectively. The US2015 experiment was conducted at the Horticultural Crops Research Station of North Carolina State University in Clinton, North Carolina. IT2015 was performed in the plastic tunnels of Magnum Seeds in Capratica, Italy (now Harris Moran Clause Seed Company). NL2015 and NL2016 were conducted in the fields of Bayer Vegetable Seeds in the Netherlands in 2015 and 2016, respectively.

The US2015 experiment was a randomized complete block design (RCBD) with three replications with each $BC_3F_{2:3}$ family containing 10 plants per replication. Plots were exposed to natural epidemics encouraged by overhead irrigation during the growing season at least three times per week. Disease severity of individual plants was evaluated weekly for three consecutive weeks based on the general impression (GI) of symptomatic leaf area using disease rating scale 1 to 9, where 1= 0-10% of symptoms area and 9= 81-100% symptoms area or dead.

The IT2015 experiment was conducted in the plastic tunnels with 12 to 15 plants per $BC_3F_{2:3}$ family without replications. Rating of disease symptoms on each plant from artificial inoculation was based on the scale from 0 to 9, where 0=absence of symptoms and 9=95% more disease symptoms with criterion GI.

The field layout of the NL2015 and NL2016 experiments was similar to that in US2015 but had two replications. There were 15 plants per family per replication (plot). The DM symptoms were measured twice with one week apart based on entire plot performance. NL2015 only used the criterion GI to evaluate DM resistance, while NL2016 evaluated the plants more carefully with four criteria including GI, necrotic lesion size (Nec), chlorotic lesion size or yellowing (Yel), and severity of sporulation (Spor). For each criterion, the disease severity was rated on the plant breeder's 1 to 9 scale with 1=maximal disease symptoms and 9=no visible symptoms.

Downy mildew screening tests in growth chambers

More accurate evaluation of DM inoculation responses of NILs carrying individual QTL was achieved in the growth chambers under controlled environmental conditions. Seeds were pre-germinated in Petri-dishes followed by planting in 4×12 (or 6×12) plastic trays (one seed per well) filled with PRO-MIX HP BIOFUNGICIDE + MYCORRHIZAE Growing Medium (Premier Tech Ltd. Québec, Canada). The DM pathogen (*Ps. cubensis*) was collected from the field, which was kindly provided by Dr. Alyson Thorton of Harris Moran Clause Seed Company (Sun Prairie, WI) and was cultured and maintained on DM susceptible Straight 8 and 9930 cucumbers. Seedlings were grown at 27/25°C (day/night) temperatures with 12 hours of light, and then were inoculated at 1-true-leaf stage by misting the adaxial side of leaves with the sporangial solution in a concentration of 1×10^5 spore mL^{-1} with ~50 mL per tray. The inoculated seedlings were incubated in dark for 48 h at 20°C and 100% relative humidity (RH). After incubation, plants were kept in the growth chamber with 24/18°C (day/night), 12 hours of light and 100% RH to promote symptom development. Disease assessments were made 5-7 days after inoculation with a qualitative scoring system in which each plant was rated as susceptible (S, 60% or more of the symptom area), intermedium resistance (I, 30-60% of the symptom area), or resistant (R, less than 30% of symptom area).

Marker development in candidate QTL regions

The DM resistance donor parent WI7120 was re-sequenced with the Illumina Hi-Seq 2000 platform (Illumina Inc., San Diego, CA). The resulting short reads were mapped to the 9930 draft reference genome (V2.0) using the BWA-Samtools programs for single nucleotide polymorphism (SNP) calling and insertion–deletion (INDEL) identifications (Li and Durbin 2009; Li et al. 2009). The BWA-mem default values were used for reads alignment. Only high quality reads were considered for SNP/INDEL calling. The SNP/INDEL positions within the aligned reads were identified by the *mpileup* function in SAMtools utilities (v.1.1). To ensure high-quality, reliable alignment of the reads, the varFilter module in *vcfutils* was implemented with the following quality criteria: 1) SNP with a minimum mapping quality (-Q) of 30; 2) maximum read depth equal to mean read depth + 3 standard deviation of read depth; 3) minimum read depth of 3; and 4) SNP must not be within 10 bp of a gap. The polymorphic SNP and INDEL were used for marker-assisted selection (MAS) during NIL development and the evaluation of SNP distribution in QTL candidate regions.

SNP genotyping

The DNA samples of BC_3F_2 individuals were adjusted to 20 ng μL^{-1}. SNP genotyping was performed with the KASPar (KBioscience Ltd., Hoddesdon, UK) assay, which was performed in Magnum Seeds. SNP genotypic data were used to select recombinants among BC_3F_2 plants to advance to BC_3F_3. In primer design for the KASPar assay, 50 bp upstream and 50bp downstream flanking sequences around the SNP position were selected. On the basis of the fluorescence obtained, alleles were called and viewed graphically as a scatter plot using the SNPViewer (LGC Limited, Middlesex, UK).

Statistical analysis

The single marker analysis (SMA) was used to verify the association of markers and the phenotype of each line ($P<0.05$) through ANOVA. P-value of each marker was extracted and converted with $-\log10(P)$ for comparison of significance.

Results

Marker-assisted backcrossing for NIL development of dm4.1 and dm5.1 QTL

Our previous QTL mapping for DM resistance in the inbred line WI7120 revealed two major-effect QTL, *dm4.1* and *dm5.1*, which together explained more than 50% of the phenotypic variance (Wang et al. 2016). The initial candidate regions of *dm4.1* and *dm5.1* were selected according to the 2.0 LOD interval; both spanned ~5 Mb on chromosomes 4 and 5, respectively. For fine mapping of the two QTL, NILs were first developed to Mendelize each QTL with marker-assisted selection (MAS) in which WI7120 was the donor of DM resistance and the DM susceptible 9930 was the recipient and recurrent parent.

For NIL development for *dm4.1*, 122 SSR markers evenly distributed across the seven cucumber chromosomes (~5 cM apart) were employed for background selection of the 9930 alleles, and five SSR markers located within the 2.0-LOD interval of *dm4.1* were selected to track donor fragment. During the NIL development for *dm5.1*, the SSR markers used in MAS were largely same as those for *dm4.1* except for those in the target region. All the markers used for MAS were chosen from Wang et al. (2016) which are presented in **Table 4.1**. At each generation (BC_2, BC_3), 48 to 96 seedlings were genotyped. Seedlings carrying target QTL region but with maximum 9930 background were kept for backcrossing with 9930 to advance to the next generation. At BC_3F_1 generation, all background markers from 9930 were fixed (homozygous) except for the regions harboring the *dm4.1* or *dm5.1* QTL, which were heterozygous carrying marker alleles from both WI7120 (resistant) and 9930 (susceptible). Therefore, the BC_3F_1 plants were self-pollinated to create a segregation population (BC_3F_2) at the two target regions. The detailed schemes of NIL development are shown in **Figure 4.1**.

For *dm4.1*, 650 BC_3F_2 plants were genotyped with 11 SNPs covering the 2.0 LOD interval QTL region. Among them, 157 showed recombination in the target region. Owing to the limited greenhouse space, for each type of recombination, only one or two plants were kept for self-pollination to obtain $BC_3F_{2:3}$ families for phenotyping. Finally, a NIL population consisting of 79 $BC_3F_{2:3}$ families were obtained, which was designated as dm4.1-NIL.

For *dm5.1*, 200 BC_3F_2 plants were genotyped by six SSR markers, which identified 33 BC_3F_2 recombinant individuals. All of them were self-pollinated to obtain $BC_3F_{2:3}$ families and employed as a NIL population for *dm5.1* fine mapping (designated as dm5.1-NIL).

Fine genetic mapping of dm4.1 and dm5.1 QTL

Fine mapping of *dm4.1* and *dm5.1* was conducted with two segregating populations, dm4.1-NIL and dm5.1-NIL consisting of 79 and 33 $BC_3F_{2:3}$ families, respectively. To narrow down the candidate gene region, additional SNP or INDEL markers were developed for screening recombinants in the two target regions. At the same time, phenotypic data for DM inoculation responses were collected from five environments (US2015, IT2015, NL2015, and NL2016). ANOVA/SMA were performed to test the significance of each marker and phenotypic value.

Fine mapping of *dm4.1*

The dm4.1-NIL population was genotyped with 63 SSR/INDEL and 10 SNP markers. Detailed information of these markers is presented in **Appendix 4.1**. Single marker analysis was conducted using phenotypic data from different rating times and rating criteria (GI, Yel, Nec, and Spor). The results among environments were highly consistent: all peaks fell into a 2 Mbp region in Chromosome 4 (**Figure 4.2**). To further confirm the region obtained from SMA, homozygous recombinants within this region (14.34 to 16.28 Mb in 9930 V2.0) were self-pollinated to create $BC_3F_{3:4}$ families. In total, homozygous recombinants of 14 haplotypes for *dm4.1* were obtained. These lines were carefully evaluated for DM inoculation responses twice in the growth chambers (two replications, 24 plants per replication for each test). The accurate phenotyping and genotyping allowed to delimit the *dm4.1* locus into a ~322kb region (**Figure 4.3A and Figure 4.4**).

To further narrow down the candidate gene region for *dm4.1*, two flanking markers UW057915 and UW004293, were used to genotype 768 BC_3F_2 plants which resulted in 13 recombinants within the 322kb region. Twelve additional INDEL markers were then developed to further resolve the breakpoints of these recombinants (**Table 4.4**), which were self-fertilized to obtain $BC_3F_{2:3}$ families for DM resistance assessment in growth chamber tests. For each $BC_3F_{2:3}$ families, 30 to 40 seedlings were evaluated. These efforts finally placed the *dm4.1* locus into a genomic region of 82 kb (**Figure 4.3B and Figure 4.4**).

Fine mapping of *dm5.1*

The dm5.1-NIL population was genotyped with 57 SSR or INDEL markers. The detailed physical information of each marker is listed in **Appendix 4.1**. Unlike the QTL *dm4.1*, *dm5.1* explained relatively smaller phenotypic variance, which made accurate field evaluation much more challenging. It turned out that only the NL2016 experiment was able to draw the association between markers and phenotypes in SMA. Consistent with this, the DM inoculation responses in NL2016 were assessed with more detailed criteria rather than just based on general impression (GI). These criteria included necrotic lesion size (Nec), chlorotic lesion size or yellowing (Yel), and severity of sporulation (Spor). As shown in **Figure 4.5**, the significantly associated markers were located in a 2Mbp region on Chromosome 5. To refine this region defined by SMA, homozygous recombinants within this region (15.75 to 18.32 Mbp) were self-pollinated to create $BC_3F_{3:4}$ families. Sixteen homozygous recombinants in this region were identified. Association of phenotypic data and haplotypes among these recombinants confirmed that the *dm5.1* locus was located in a region of ~628 kb in length (**Figure 4.6A and Figure 4.7**).

Two flanking markers of the *dm5.1* locus, CsDm5-044 and UW058914, were used to genotype 768 BC_3F_2 plants, from which 15 recombinants between the two markers were identified. Fifteen new INDEL markers were developed in this interval for identification of new recombinants (**Table 4.4**). The 15 recombinants were self-fertilized to obtain $BC_3F_{2:3}$ families for DM resistance screening in the growth chamber tests. For each $BC_3F_{2:3}$ family, 30 to 40 seedlings were evaluated. Eventually, the *dm5.1* QTL was narrowed down to approximately 28,323 bp region (**Figure 4.6B and Figure 4.7**).

Candidate genes for dm4.1 and dm5.1

Fine genetic mapping placed *dm4.1* into an 82 kb region, in which 13 genes were predicted (http://www.icugi.org). Detailed information of the 13 genes is presented in **Table 4.2.** Sequence alignment of WI7120 re-sequence reads against the 9930 draft genome identified 59 SNPs and INDELs between the two parental lines in this 82kb region. Among the 59 polymorphisms, 56 were located in intergenic regions (**Appendix 4.2**). Of the three remaining SNPs, one is synonymous mutation in *Csa4G418580*. The only gene, *Csa4G416990*, was predicted to encode an F-box SKIP24 protein with two amino acid substitutions in the coding region, which was very likely to be the candidate gene for *dm4.1*.

Within the 28,323 bp candidate region for *dm5.1*, three genes were predicted in the 9930 draft genome assembly v2.0 and two in the Gy14 draft genome assembly (V1.0) (**Table 4.3**). Among the three genes in 9930 v2.0, *Csa5G471100* is predicted as unknown protein and is not predicted by Gy14 v1.0. A homolog search using its predicted coding DNA sequence (CDS) in other species could not find any similar sequences suggesting a pseudogene. Sixteen SNPs between WI7120 and 9930 were identified in this region (**Appendix 4.2**). One synonymous SNP was found in the *Csa5G471090* coding region and one SNP presented in its 3’ UTR. Moreover, five SNPs and INDELs presented within 1.5 kb upstream on

Csa5G471600; one SNP in coding region led to a Phenylalanine (F) to Leucine (L) amino acid substitution; nine SNPs or INDELs were found within 1.0 kb downstream region of this gene. *Csa5G471600* is a homolog of PHO-1 like of Arabidopsis that encodes a phosphate transporter, and is a candidate for *dm5.1*.

Expression dynamics of dm4.1 and dm5.1 candidate genes

Our QTL mapping studies (Wang et al. 2016; Chapter 3) suggest that WI7120 and PI 197088 may share the same QTL *dm4.1* and *dm5.1* (*dm5.2* in PI 197088) although their contributions to the observed phenotypic variance are somewhat different. In Chapter 3, we investigated the expression levels of 1,220 genes that harbors in the 1.5 LOD interval of five major QTL, and finalized 139 most possible candidate genes. Importantly, the two candidate genes (*Csa4G416990* and *Csa5G471600*) identified in this study were among the 139 differential expressed genes. It was found that *Csa4G416990* (for *dm4.1*) was down-regulated from 1dpi (days post inoculation) to 6dpi, whereas *Csa5G471600* (for *dm5.1*) was dramatically and continuously up-regulated during this time period (**Figure 4.8**). Nevertheless, additional evidence is still needed to establish their candidacy for *dm4.1* and *dm5.1*.

Discussion

Fine mapping of QTL with near isogenic lines

QTL mapping is often followed by refinement of QTL position towards the goal of QTL cloning, which is challenging in quantitative genetics (Rockman 2008). One approach to QTL cloning is the development of near-isogenic lines (NILs) (Monforte and Tanksley 2000). Each line in a NIL population carries a different size of donor parent fragment, while the entire population carries introgressions spanning the target candidate region with the majority of the genome being substituted by the recurrent parents (Eshed and Zamir 1995;

Remington et al. 2001). However, the generation of NIL population is a laborious and time consuming process, while the phenotyping of NIL population for disease resistance traits is even more challenging due to the reduced genetic effect with single QTL.

In the present study, the MAS approach was employed with background selection for the recurrent parent and foreground selection of target QTL region from the donor parent. Evenly distributed markers with ~5 cM apart across the whole genome were used for background selection. With this strategy, it took less than two years (April 2013 to November 2014) to fix all background markers in the NIL populations with target region in segregation. Initial screening with SSR markers was time consuming, but much fewer markers are needed at later generations. In the future, high-throughput SNP-genotyping would be a more efficient alternative for MAS.

The QTL *dm4.1* and *dm5.1* were chosen for NIL development because they together explained more than 50% of the phenotypic variance, and they both showed only additive effects without epistasis effects. Thus, each QTL could still perform a decent degree of resistance in the susceptible genetic background (9930). This is advantageous for phenotyping and fine mapping of their locations. Nevertheless, the successful cloning of candidate genes for the two QTL could also be attributed to reliable and accurate disease evaluation of individual NILs in the present study. NIL populations were phenotyped in both field trials and growth chamber tests. In field trials, DM resistance was evaluated in four environments with multi-rating times and criteria in order to reduce environmental effects. In growth chamber test, the seedlings were evaluated with several replications and repeated at least twice to guarantee a reliable outcome. To summarize, the present study illustrated the importance of using MAS in accelerating development of NIL population, the choice of target QTL, and accurate phenotyping in QTL cloning.

Characterization of candidate genes underlying dm4.1 and dm5.1

By phenotyping large NIL-derived populations, *dm4.1* and *dm5.1* were respectively delimited into 82-kb interval which harbored 13 annotated genes, and a 28-kb region harboring three predicted genes. In the *dm4.1* region, most polymorphic SNPs between WI7120 and 9930 were intergenic, and *Csa4G416990* was the only one with non- synonymous mutations resulting in two amino acid substitutions in its CDS. Similarly, in the *dm5.1* region, among the 16 SNPs between two parents, the only SNP in CDS was located in *Csa5G471600* which was predicted to cause to an F to L amino acid substitution and a number of SNPs were found to present at 1.5kb up and down stream of its CDS. In addition, from transcriptome profiling, among the 139-DEG, upon DM infection (Chapter 3; Burkhardt and Day 2016), these two candidate genes (*Csa4G416990* and *Csa5G471600*) showed consistent expression dynamics at different time points after inoculation. These data support that *Csa4G416990* and *Csa5G471600* were the two most possible candidates for *dm4.1* and *dm5.1*, respectively.

The candidate gene for *dm4.1*, *Csa4G416990*, encodes an F-box SKIP24 protein (SKP1-interacting partner 24). *SKIP24*, a component of SCF (Skip-cullin-F-box) E3 ubiquitin ligase complexes, acts in proteasomal degradation process through the recognition and ubiquitination of specific protein substrates in response to various stimuli including pathogen attacks (Skaar et al. 2013; Reiner et al. 2016). Microbial effector proteins could target the host SCF E3 Ub-ligases complexes through F-box protein, which subvert the host protease to promote disease (Duplan et al. 2014). With the proposed function of *Csa4G416990*, further studies in clarifying the binding site are needed to draw the association of nonsynonymous SNP and allelic effects. Further efforts will be required to keep delimiting the causal region into a smaller size and to determine the gene eventually.

The candidate gen for *dm5.1*, *Csa5G471600*, encodes PHOSPHATE1 (PHO1)-like protein, which shows highest homology with the Rcm1 receptor in mouse and human for the xenotropic and polytropic murine leukemia retroviruses (XPR). Rcm1 receptor is a protein involved in the signal transduction pathway (Hamburger et al. 2002). *PHO1* genes play critical roles in plant growth and development, well known for inorganic phosphate (Pi) sensing and transport (Bowling 1981; Clarkson 1993). Some *PHO1* genes in Arabidopsis, such as *AtPHO1;H10,* was reported to be induced by numerous abiotic and biotic stresses, including local wounding, salinity, osmotic, and pathogens (Ribot et al. 2008a; Eshraghi et al. 2014). The accumulation of *AtPHO1;H10* expression against pathogen attacks involves the ABA-mediated signaling pathway. Two cis-acting ABA responsive elements and one CE3 coupling element were identified within 1 kb of promoter sequence of *AtPHO1;H10*, which could be recognized by members of the bZIP (basic-domain Leu zipper) transcription factor including proteins that involved the ABA-mediated plant defense (Ribot et al. 2008b).

While the present study identified two most possible candidate genes for *dm4.1* and *dm5.1* QTL, clearly, additional evidence is needed to confirm this preliminary observation. The bottom line is, within the two candidate regions, no known typical R genes such as NB-LRR type, LRR-RLP and LRR-RLK were annotated. Thus, the two DM resistance QTL cloned herein may represent some novel resistance mechanisms for DM resistance in cucumber.

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Table 4.1 Molecular markers used for foreground and background selections for development of near isogenic lines for major-effect DM resistance QTL *dm4.1* and *dm5.1* from WI7120 in 9930 genetic background.

NIL Type	**Chr No.**	**Marker (Foreground Selection)**	**Maker (Background Selection)**
dm4.1/dm5.1	Chr1	n.a*	SSR22670**, SSR20705, SSR10018, SSR14340, SSR02810, SSR16472, SSR17193, SSR03886, SSR14526, UW083728, SSR11340, SSR06988, SSR13960, SSR02734, SSR19914, SSR02086, SSR14445, SSR17922, SSR18928, SSR10963, SSR05983
dm4.1/dm5.1	Chr2	n.a	UW029683, SSR18783, UW084807, SSR03488, SSR00204, UW084818, UWSTS0384, CHXSSR00134, SSR13105, UW230458, UW023284, SSR00219, SSR12083, SSR16941, SSR13275, SSR16135, UW036921, UW084625, UW083965
dm4.1/dm5.1	Chr3	n.a	SSR02398, SSR01609, SSR12383, SSR16408, SSR10656, SSR15029, SSR03918, SSR04570, SSR13262, SSR06210, SSR00710, SSR07505, SSR18026, SSR11699, SSR14446, SSR04530, SSR11594, SSR06031, SSR04628, SSR10282, SSR03066, SSR10783
dm4.1	Chr4	UW130650, SSR05125, UW083868, SSR00147, UW083893,	SSR22706, UW058481, UW084529, CHXSSR00190, CHXSSR00195, SSR07543, UW084698, UW084724, SSR05515
dm5.1	Chr4	n.a	SSR22706, UW058481, UW084529, CHXSSR00190, UW130650, SSR05125, UW083868, SSR00147, UW083893, CHXSSR00195, SSR07543, UW084698, UW084724, SSR05515
dm4.1	Chr5	n.a	SSR16749, UW063819, UW063579, SSR24862, SSR14517, SSR10348, SSR18914, SSR20165, SSR19178, SSR32717, UW001423, UW001847, SSR05266, UW085065, SSR13078, SSR15196, UW084826
dm5.1	Chr5	SSR18914, SSR20165, SSR19178, SSR32717, UW001423	SSR16749, UW063819, UW063579, SSR24862, SSR14517, UW001847, SSR10348, SSR05266, UW085065, SSR13078, SSR15196, UW084826
dm4.1/dm5.1	Chr6	n.a	SSR22275, SSR18718, SSR02021, SSR01012, SSR16020, SSR02385, SSR04644, SSR15676, SSR15067, SSR14652, UW083983, UW000765, SSR03932, SSR17818, UWSTS0386, SSR12510, SSR01234, SSR02768, SSR17408, SSR05946
dm4.1/dm5.1	Chr6	n.a	SSR03917, UW041955, SSR16001, SSR19437, SSR18648, SSR02432, SSR11742, SSR06349, SSR14861, SSR01898, UW084419, SSR10461, SSR17062, SSR02785

Note: *n.a: not applied; **The detailed information of each marker was listed in Appendix 2.1, including physical position, genetic position, and forward and reverse primers.

Table 4.2 List of 13 annotated genes and their predicted functions in an 82-kb region harboring major-effect DM resistance QTL *dm4.1* Annotated genes are based on the 9930 draft genome assembly v2.0.

GeneID	**Annotation**
Csa4G416980	MYB transcription factor; contains IPR009057 (Homeodomain-like)
Csa4G416990	F-box protein SKIP24; contains IPR001810 (F-box domain, cyclin-like)
Csa4G417490	Zinc import ATP-binding protein ZnuC; contains IPR027417 (P-loop containing nucleoside triphosphate hydrolase)
Csa4G417500	Presenilin family protein; contains IPR006639 (Peptidase A22, presenilin signal peptide)
Csa4G417510	Protein grpE, putative; contains IPR000740 (GrpE nucleotide exchange factor)
Csa4G417520	ELMO domain-containing protein A; contains IPR006816 (Engulfment/cell motility, ELMO)
Csa4G418520	Unknown protein; contains IPR005516 (Remorin, C-terminal)
Csa4G418530	Unknown protein
Csa4G418540	Chaperone DnaK; contains IPR013126 (Heat shock protein 70 family)
Csa4G418550	Chaperone DnaK; contains IPR013126 (Heat shock protein 70 family)
Csa4G418560	KRI1-like protein; contains IPR018034 (KRR1 interacting protein 1), IPR024626 (Kri1-like, C-terminal)
Csa4G418570	Pentatricopeptide repeat-containing protein; contains IPR002885 (Pentatricopeptide repeat), IPR011990 (Tetratricopeptide-like helical)
Csa4G418580	RING finger protein; contains IPR013083 (Zinc finger, RING/FYVE/PHD-type)

Table 4.3 List of annotated genes and their predicted functions in a 28-kb region harboring major-effect DM resistance QTL *dm5.1*. Annotated genes are based on the 9930 draft genome assembly v2.0 and Gy14 assembly v1.0.

GeneID (9930 v2.0)	**GeneID (Gy14 v1.0)**	**Annotation**
Csa5G471090	Cucas.033870	5'->3' exoribonuclease, putative; contains IPR027073 (5'-3' exoribonuclease)
Csa5G471100		Unknown protein
Csa5G471600	Cucas.033880	Xenotropic and polytropic retrovirus receptor-like protein; contains IPR004331 (SPX, N-terminal), IPR004342 (EXS, C-terminal)

Table 4.4 Primer sequences and their physical positions in the 9930 draft genome assembly v2.0 for molecular markers used in fine mapping of *dm4.1* and *dm5.1* major-effect QTL.

QTL	**Marker Name**	**9930 V2.0 Position**	**Forward Primer**	**Reverse Primer**
dm4.1	UW057915	15375026	CGAGGAAGATTTAGAAAGTTTGATG	TCGATGACAAGAGAACAACGA
	CsDm4-046	15417455	TTTGGCACAGAGTTGCAAGA	TCATCCACCAACTTCCACTG
	CsDm4-066	15421000	GAGGGAGTTGGTCACTCCAA	AAAAGGCATTATGAGCCTGA
	SSR13864	15481204	CTCCCTCCTTTCTCCTGCTT	AAACTTGGAAGCCCTCATCC
	CsDm4-047	15514106	TTATTTTCACGATCCCCAAT	CGCTCTAACCTCCAAAACTG
	CsDm4-072	15532410	TTGCAAGAGCTTTGATTGGA	GGTGAGGCGTCGGTAATAGA
	CsDm4-071	15543570	AAGAGTCATTTCACCTTTGCT	CAGTTAATCTCGGATCGTTG
	CsDm4-070	15599350	AAAGCACATCGAAGATTTGTA	CAGCTTGTAGAGCCAAGTCA
	CsDm4-005	15614161	TGAACATAATTAACACCAAAATTCAA	TGGAAGAGAAGAAATCACAAAAA
	CsDm4-006	15637832	TTGATCCATGCAAACTGCTT	CCCAGACTCCAGGGACTATCT
	UW057881	15657140	GAGGGGAAAGAGGCTTGATT	TTTATCGGGGGATTTCACCT
	CsDm4-068	15661140	AAAATCCCAACAATTTACATGC	AAATCCCAATGTTAAATAGAGCA
	CsDm4-074	15684941	GTGCACCCCATGAGAAGAAA	ACGAGCAAGTTCACATGACG
	CsDm4-069	15718850	GTGGTCTTGTGGGACAATTT	AACCTAACCTACGCGTCAAC
	UW004293	15743586	AGAAACCAACAAACGCCATC	CTGCTTCTGGACCTGTGGTT
dm5.1	CsDm5-044	16261450	GTTGCCCCATTAAGTGATTG	TTCAAAAGAGCTTTGGCATT
	CsDm5-063	16292130	GTGGGGGTGTTTGTATTGGA	TGTGAATAAACAGCGCAACG
	CsDm5-064	16323200	AAACTCGTCGGGAAACATGA	TTCTCGACGCATATGACACC
	CsDm5-065	16350620	GTTGCTATTGGAAGGGGAAA	TCGATTGATGCTCAACCCTA
	CsDm5-066	16372490	ATGGGAAGAGAAGGGGAGAA	TGGTTAAGAGGAGAGTGCAACA
	CsDm5-067	16399450	GTAGGGCCAAACAAACCTCA	CAGTACTGCGACAGGACACC
	CsDm5-049	16468212	TTGCAACAGAACCCTAAACC	CATTTGATTTGCAAGCACAC
	CsDm5-068	16508850	AAAACATGATTTGGGATGCTG	TCAAGCTAATGGTGGAAGTGG
	CsDm5-050	16569111	AAAGGACACTTGTAAATTCAGC	TACACCCGAAATGTCCTCTT
	CsDm5-082	16569620	ATGCATTCATCACACACACG	TTTGCACCATAAACGACACA
	CsDm5-086	16649667	GGATGGGCAATAAACCTATGA	CACACATTTTGTATTTCGAGCA
	SSR01280	16669445	TGAGTGTGAACAGCCACTGA	AAATCCAATTTGATGTCAAAAACA
	CsDm5-080	16678081	ACTTCTTTCATCCCCTCTCAA	CCCCAAATTTGAATTGTAACG
	CsDm5-073	16699200	CAAGTTGATTTTGGGAAGATTTG	CATCAAGTTTACAAACTGAGGGTTT

QTL	Marker Name	9930 V2.0 Position	Forward Primer	Reverse Primer
dm5.1	CsDm5-074	16726700	TTCAAAGTGTAGAAGCATACGTTTT	TATTGGCCTGAACGAATGTG
	CsDm5-075	16754790	ACTTTCGTGGCTTTGGAAGA	CACACAACAACTTCCATTAAACAA
	CsDm5-076	16775960	GCCATCACAAAAATGTTGGAA	CAAAGTCAATGTGAAGTTTGTCA
	CsDm5-077	16795360	GAAAACAAGCATGAACTAACTGGA	CATCAAAAGGAAACCCTTCG
	CsDm5-078	16816020	TCAAGCTTTAACATACCTTTGCTT	CAAACTTGTTTCTCCATTCAAAA
	UW058914	16889680	TTGTTATACCACATATTGTTGATGGA	AAGCATGAGGGGGAgACATA

Figure 4.1 Schemes for marker-assisted backcrossing to develop near isogenic lines (NILs) for *dm4.1* and *dm5.1* DM resistance QTL with WI7120 of resistance source as the donor and susceptible 9930 as the recipient parents.

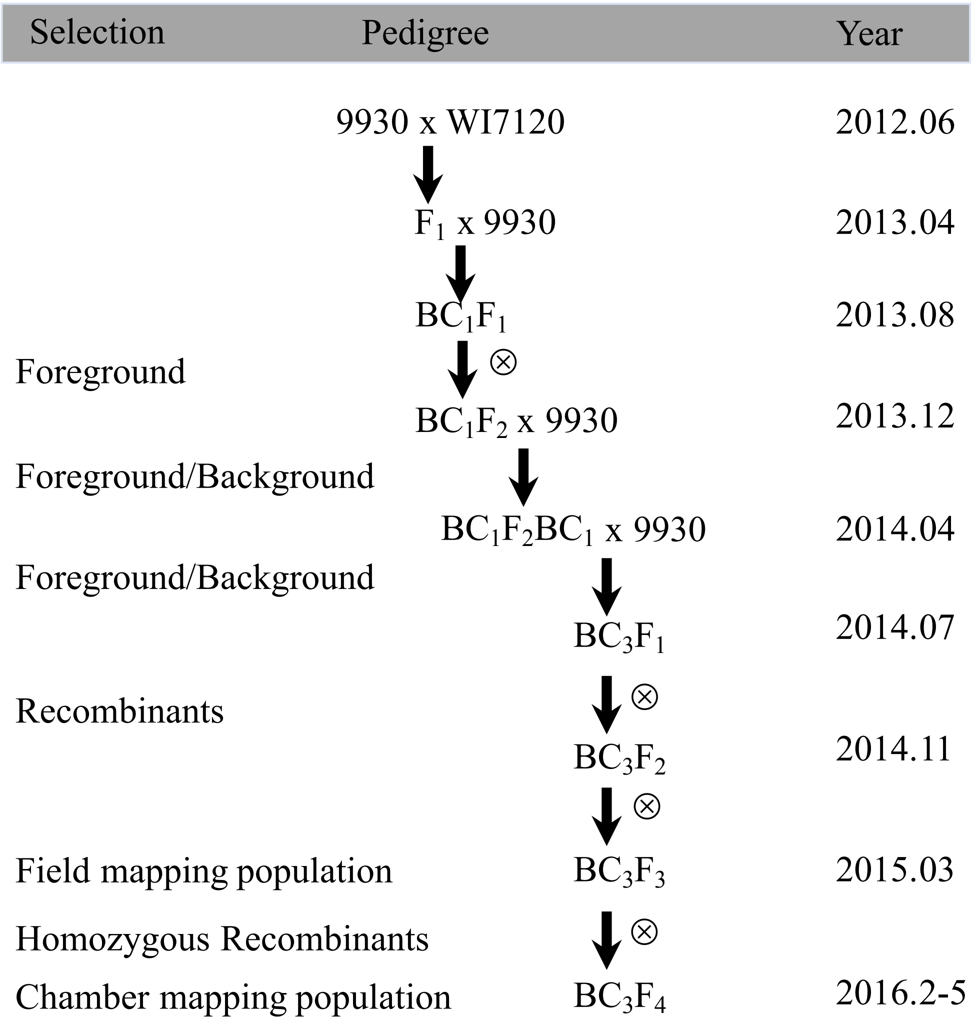


Figure 4.2 QTL fine mapping of *dm4.1* using 79 NILs population. A) Preliminary 2.0 LOD-interval of QTL *dm4.1* (Modified from Wang et al 2016). B) Single marker analysis for the dm4.1-NIL population using 88 SSR/INDEL/SNP markers. Y-axis is according to markers' physical position (Mbp) in the 9930 draft genome assembly v2.0, X-axis is –log10(*P*) value.

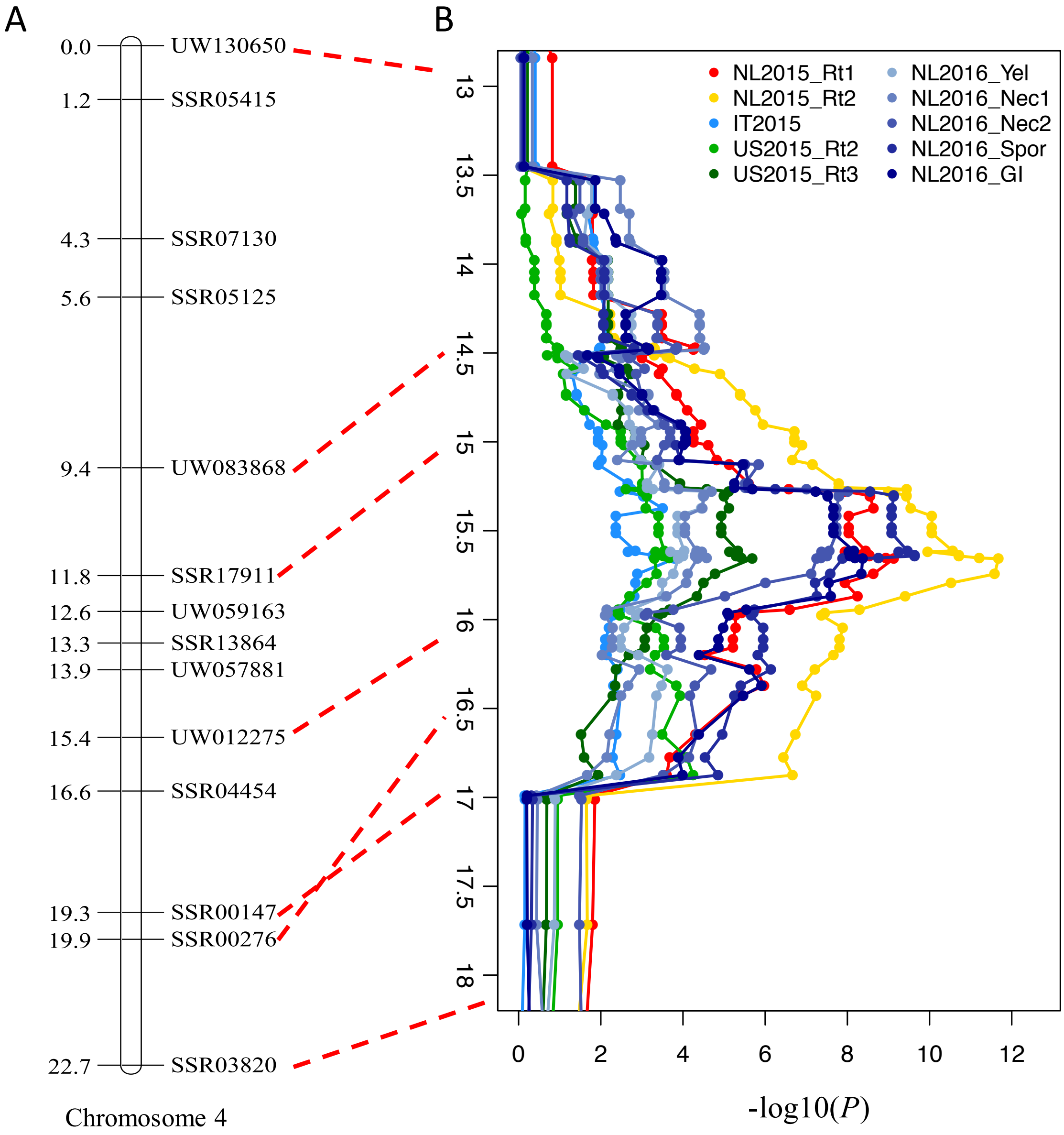


Figure 4.3 QTL fine mapping of *dm4.1* under growth chamber evaluations. A) Fine mapping of *dm4.1* with selective homozygous $BC_3F_{3:4}$ families, which delimited the *dm4.1* into approximately 322kb region. B) Fine mapping of *dm4.1* with recombinants selected from a large $BC_3F_{2:3}$ families, which further delimited the *dm4.1* into 82kb region. Darkly shaded rectangles, the homozygous genotype with alleles derived from WI7120; open rectangles, the homozygous 9930 genotype; gray shaded rectangles, heterozygous genotype. Phenotype Class: R=resistance, S=susceptible, Seg=segregating of phenotypes.

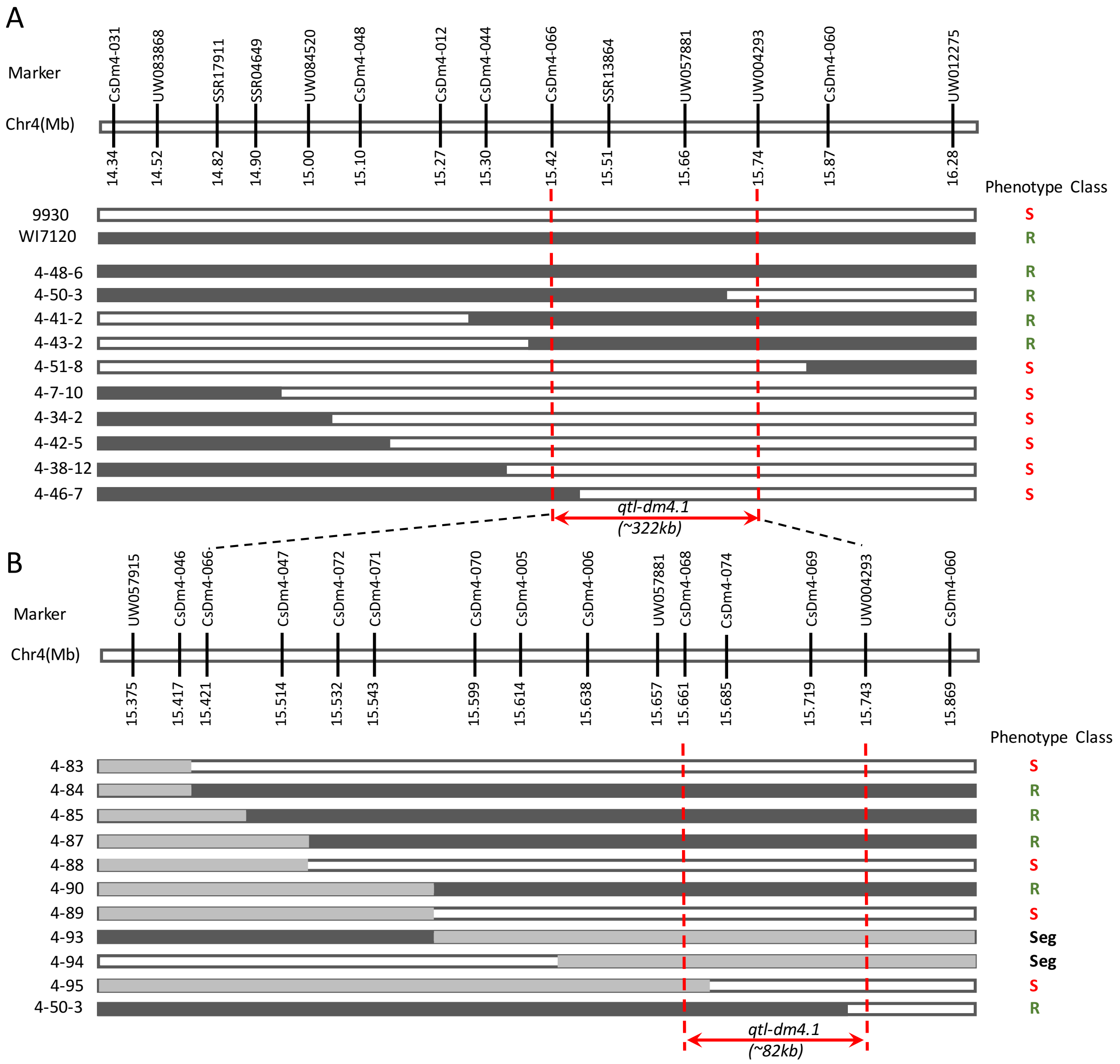


Figure 4.4 Performance of different plants carrying varying size of WI7120 donor DNA fragments in the *dm4.1* QTL region upon DM pathogen inoculation in growth chamber tests.

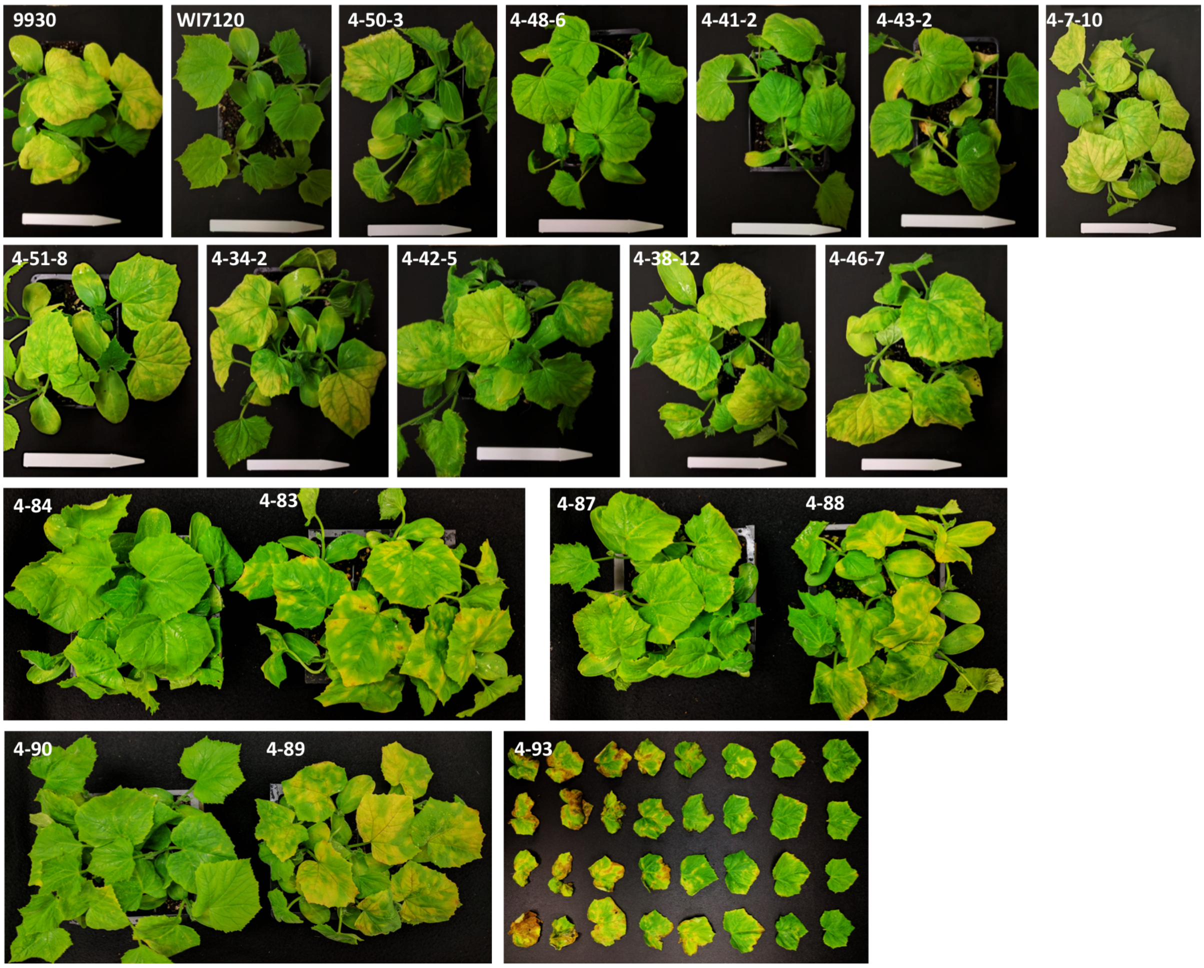


Figure 4.5 QTL fine mapping of *dm5.1* using 33 NILs population. A) Preliminary 2.0 LOD-interval of QTL *dm5.1* (Modified from Wang et al 2016). B) Single marker analysis for the *dm5.1*-NIL population using 57 SSR/INDEL markers. Y-axis is according to physical position (Mbp) of each marker in the 9930 draft genome assembly v2.0; X-axis is –log10(*P*) value.

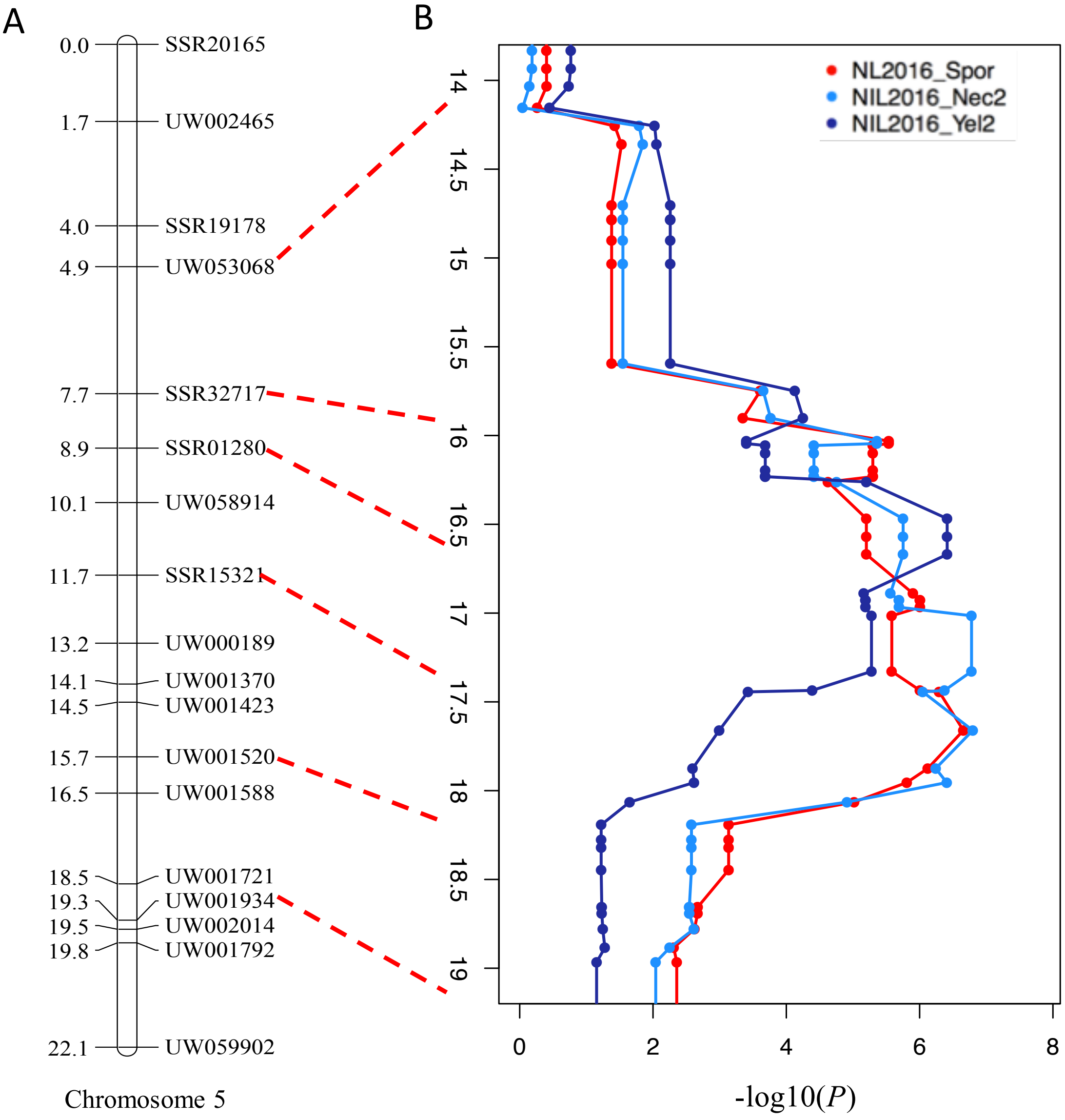


Figure.4.6 QTL fine mapping of *dm5.1* with growth chamber tests. A) Fine mapping of *dm5.1* with selective homozygous $BC_3F_{3:4}$ families, which delimited the *dm4.1* into approximately 628kb region. B) Fine mapping of *dm5.1* with recombinants selected from a large $BC_3F_{2:3}$ families, which further delimited the *dm5.1* into 28kb region. Darkly shaded rectangles, the homozygous genotype with alleles derived from WI7120; open rectangles, the homozygous 9930 genotype; gray shaded rectangles, heterozygous genotype. Phenotype Class: R=resistance, S=susceptible, Seg=segregating of phenotypes.

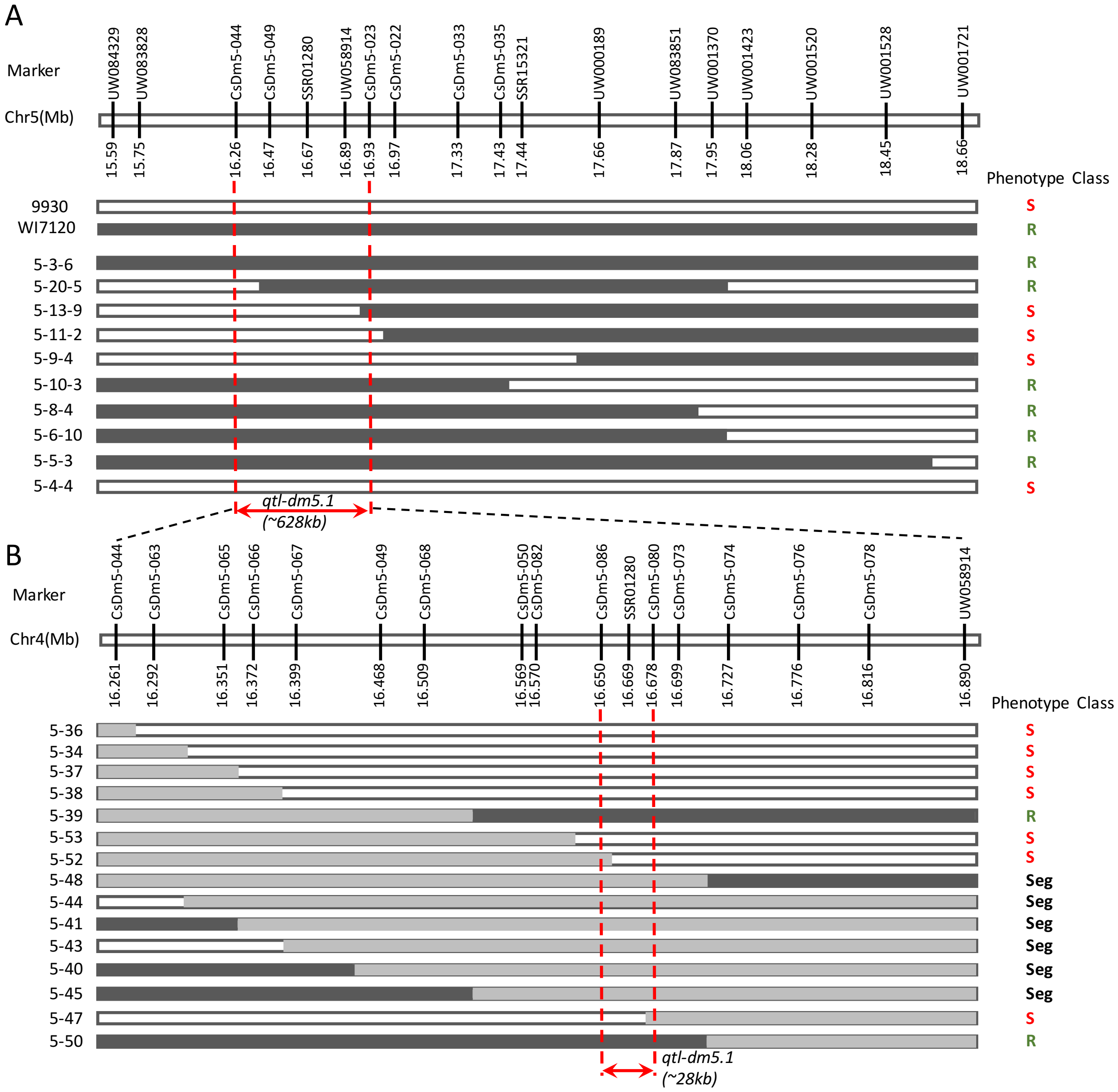


Figure 4.7 Performance of different plants carrying varying size of WI7120 donor DNA fragments in the *dm5.1* QTL region upon DM pathogen inoculation in growth chamber tests.

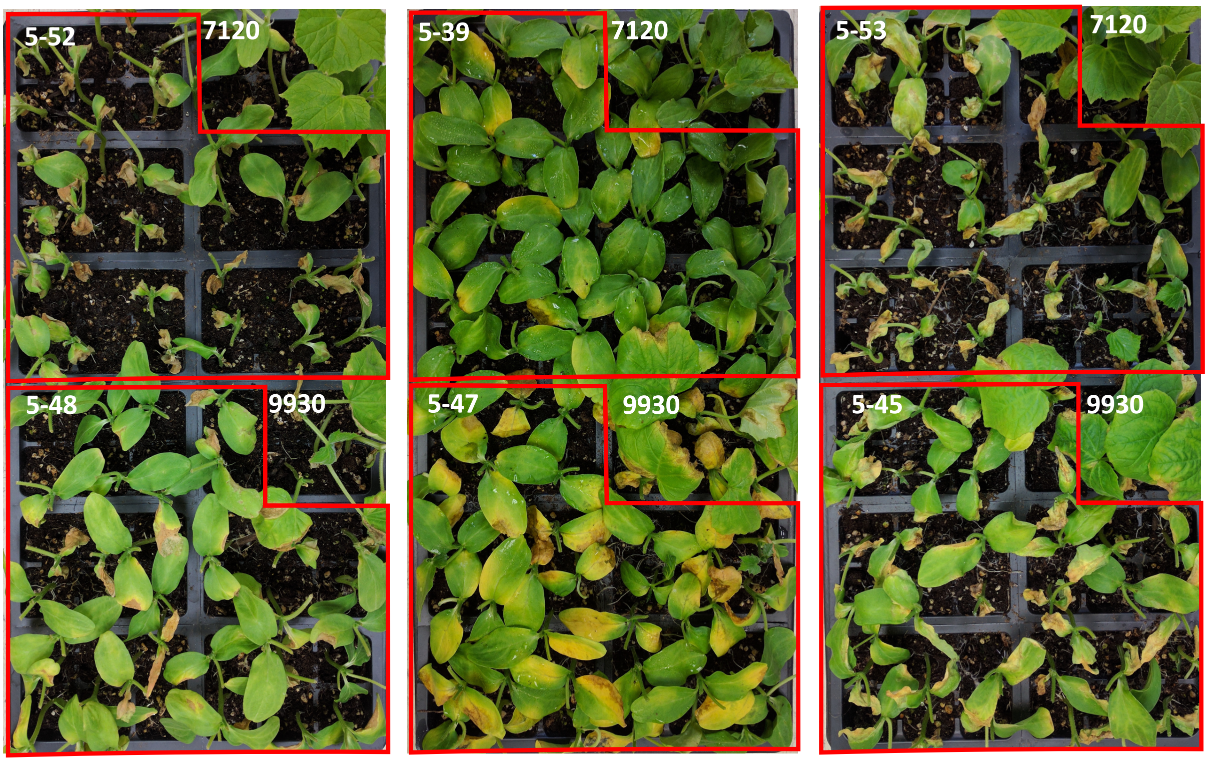
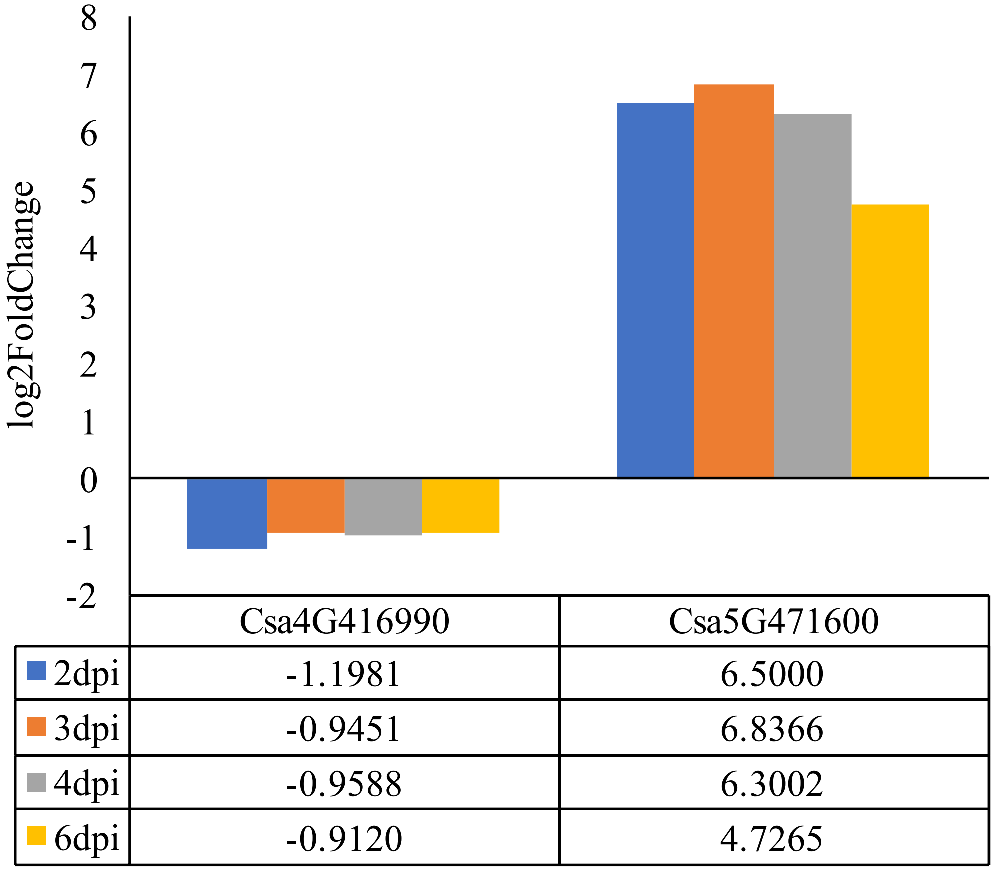


Figure 4.8 The Log2FoldChange of *Csa4G416990* and *Csa5G471600* at 2 day post inoculation (dpi) to 6dpi compared to Mock in PI 197088. Data was extracted and redrawn from Burkhardt and Day (2016).



	Csa4G416990	Csa5G471600
🟦 2dpi	-1.1981	6.5000
🟧 3dpi	-0.9451	6.8366
⬜ 4dpi	-0.9588	6.3002
🟨 6dpi	-0.9120	4.7265

Appendix 4.1 SSR/INDEL markers used for single marker analysis in dm4.1-NIL and dm5.1-NIL populations.
Appendix 4.1 is uploaded with the following link:
http://cranberry.vcru.wisc.edu/owncloud/index.php/s/DP73qb0J2d6seaf

Appdenix.4.2 The filtered information of SNP calling between 9930 and WI7120 in vcf format.

Chr	9930 V2.0 Pos	REF[a]	ALT[b]	QUAL	INFO[c]	FORMAT[d]	WI7120.bam[e]
4	15661046	G	A	999	DP=70	GT:PL:DP:GQ	1/1:239,15,0:5:15
4	15661543	A	C	999	DP=60	GT:PL:DP:GQ	1/1:255,30,0:10:30
4	15661598	G	T	999	DP=80	GT:PL:DP:GQ	1/1:255,24,0:8:24
4	15661764	AATA	AA	999	INDEL	GT:PL:DP:GQ	1/1:255,27,0:9:27
4	15661983	A	G	999	DP=66	GT:PL:DP:GQ	1/1:255,21,0:7:21
4	15661989	T	C	999	DP=63	GT:PL:DP:GQ	1/1:255,21,0:7:21
4	15662229	G	T	999	DP=78	GT:PL:DP:GQ	1/1:255,18,0:17:18
4	15662561	G	A	999	DP=97	GT:PL:DP:GQ	1/1:255,39,0:13:39
4	15662568	T	A	999	DP=98	GT:PL:DP:GQ	1/1:255,39,0:13:39
4	15662575	T	G	999	DP=91	GT:PL:DP:GQ	1/1:255,51,0:17:51
4	15662716	C	G	999	DP=56	GT:PL:DP:GQ	1/1:246,15,0:5:15
4	15663386	ATTTTT	ATTTT	999	INDEL	GT:PL:DP:GQ	1/1:90,33,0:11:33
4	15663578	T	A	999	DP=60	GT:PL:DP:GQ	1/1:158,12,0:4:12
4	15663705	GTTAAATT	GT	999	INDEL	GT:PL:DP:GQ	1/1:255,24,0:8:24
4	15663735	T	C	999	DP=73	GT:PL:DP:GQ	1/1:255,33,0:11:33
4	15663791	T	A	999	DP=79	GT:PL:DP:GQ	1/1:255,42,0:14:42
4	15663829	A	T	999	DP=80	GT:PL:DP:GQ	1/1:255,39,0:13:39
4	15663880	ATTTT	ATTT	999	INDEL	GT:PL:DP:GQ	1/1:171,33,0:11:33
4	15671517	A	C	999	DP=64	GT:PL:DP:GQ	1/1:255,15,0:5:15
4	15671703	A	T	999	DP=66	GT:PL:DP:GQ	1/1:255,30,0:10:30
4	15672216	T	C	999	DP=66	GT:PL:DP:GQ	1/1:255,42,0:14:42
4	15672591	T	G	999	DP=43	GT:PL:DP:GQ	1/1:246,15,0:5:15
4	15672687	A	G	999	DP=44	GT:PL:DP:GQ	1/1:255,24,0:8:24
4	15672866	C	A	999	DP=66	GT:PL:DP:GQ	1/1:255,30,0:10:30
4	15672907	GAAAA	GAAAAA	999	INDEL	GT:PL:DP:GQ	1/1:88,12,0:4:12
4	15673123	G	A	999	DP=67	GT:PL:DP:GQ	1/1:255,42,0:14:42
4	15673198	C	T	999	DP=64	GT:PL:DP:GQ	1/1:231,15,0:5:15
4	15673483	T	A	999	DP=62	GT:PL:DP:GQ	1/1:255,33,0:11:33
4	15673498	C	T	999	DP=68	GT:PL:DP:GQ	1/1:255,42,0:14:42
4	15673522	A	G	999	DP=73	GT:PL:DP:GQ	1/1:255,33,0:11:33
4	15674989	ATTT	ATT	999	INDEL	GT:PL:DP:GQ	1/1:157,18,0:6:18
4	15677605	A	G	999	DP=70	GT:PL:DP:GQ	1/1:255,36,0:12:39
4	15677607	C	T	999	DP=84	GT:PL:DP:GQ	1/1:255,36,0:12:39
4	15693218	G	A	999	DP=71	GT:PL:DP:GQ	1/1:255,36,0:12:36
4	15694011	C	T	999	DP=84	GT:PL:DP:GQ	1/1:255,45,0:15:45
4	15710691	A	C	999	DP=52	GT:PL:DP:GQ	1/1:255,39,0:13:42
4	15727651	T	C	999	DP=64	GT:PL:DP:GQ	1/1:255,25,0:12:25
4	15731848	G	A	999	DP=107	GT:PL:DP:GQ	1/1:255,39,0:13:42
4	15737967	A	C	999	DP=76	GT:PL:DP:GQ	1/1:255,6,0:13:9
4	15738066	G	A	999	DP=53	GT:PL:DP:GQ	1/1:255,24,0:8:24
4	15738164	G	A	999	DP=80	GT:PL:DP:GQ	1/1:255,24,0:8:24
4	15738683	C	T	999	DP=72	GT:PL:DP:GQ	1/1:255,57,0:19:57
4	15740254	G	T	999	DP=53	GT:PL:DP:GQ	1/1:255,27,0:9:30
4	15740925	A	G	999	DP=50	GT:PL:DP:GQ	1/1:255,27,0:9:27
4	15741046	A	T	999	DP=60	GT:PL:DP:GQ	1/1:255,18,0:6:18

Chr	**9930 V2.0 Pos**	**REF**	**ALT**	**QUAL**	**INFO**	**FORMAT**	**WI7120.bam**
4	15741220	T	A	999	DP=69	GT:PL:DP:GQ	1/1:255,30,0:10:30
4	15741221	T	A	999	DP=70	GT:PL:DP:GQ	1/1:255,30,0:10:30
4	15742107	G	A	999	DP=69	GT:PL:DP:GQ	1/1:255,24,0:8:24
4	15742504	G	A	999	DP=62	GT:PL:DP:GQ	1/1:255,27,0:9:27
4	15742507	G	A	999	DP=68	GT:PL:DP:GQ	1/1:255,27,0:9:27
4	15742527	T	A	999	DP=75	GT:PL:DP:GQ	1/1:255,30,0:10:30
4	15742559	C	A	999	DP=70	GT:PL:DP:GQ	1/1:255,33,0:11:33
4	15743058	T	C	999	DP=65	GT:PL:DP:GQ	1/1:255,27,0:9:27
4	15743230	TTTTCATAAATGTTTCATAAAT	TTTTCATAAAT	999	INDEL	GT:PL:DP:GQ	1/1:255,24,0:8:24
4	15743289	A	G	999	DP=87	GT:PL:DP:GQ	1/1:255,24,0:8:24
4	15743301	A	T	999	DP=76	GT:PL:DP:GQ	1/1:255,30,0:10:30
4	15743739	T	A	999	DP=71	GT:PL:DP:GQ	1/1:255,24,0:8:24
4	15743761	TAAC	TAACAAC	999	INDEL	GT:PL:DP:GQ	1/1:255,21,0:7:21
4	15743809	AC	A	999	INDEL	GT:PL:DP:GQ	1/1:100,15,0:5:15
5	16650274	C	T	999	DP=70	GT:PL:DP:GQ	1/1:255,27,0:9:27
5	16661793	C	T	999	DP=68	GT:PL:DP:GQ	1/1:255,69,0:23:66
5	16671662	C	G	999	DP=76	GT:PL:DP:GQ	1/1:255,33,0:11:33
5	16671728	G	A	999	DP=94	GT:PL:DP:GQ	1/1:255,39,0:13:39
5	16671869	ATAG	A	999	INDEL	GT:PL:DP:GQ	1/1:255,54,0:18:54
5	16672015	A	G	999	DP=65	GT:PL:DP:GQ	1/1:255,36,0:12:36
5	16672141	T	G	999	DP=92	GT:PL:DP:GQ	1/1:255,24,0:8:24
5	16674615	C	G	999	DP=85	GT:PL:DP:GQ	1/1:255,39,0:13:39
5	16677753	GAAAAAAAAA	GAAAAAAAAAAAAA	999	INDEL	GT:PL:DP:GQ	1/1:192,15,0:5:15
5	16678059	C	T	999	DP=77	GT:PL:DP:GQ	1/1:255,30,0:10:30
5	16678078	G	A	999	DP=75	GT:PL:DP:GQ	1/1:255,33,0:11:33
5	16678182	C	T	999	DP=70	GT:PL:DP:GQ	1/1:255,36,0:12:36
5	16678229	TTCT	TT	999	INDEL	GT:PL:DP:GQ	1/1:255,33,0:11:33
5	16678263	C	T	999	DP=85	GT:PL:DP:GQ	1/1:255,39,0:13:39
5	16678307	G	T	999	DP=87	GT:PL:DP:GQ	1/1:255,39,0:13:39
5	16678486	T	C	999	DP=69	GT:PL:DP:GQ	1/1:255,30,3:10:27
5	16678576	CTTTTTGTATTTGGTTT	CTTT	999	INDEL	GT:PL:DP:GQ	1/1:255,24,0:8:24

Note:

[a] REF referred to reference allele of the 9930 draft genome assembly v2.0;

[b] ALT referred to alternative allele in WI7120;

[c] DP is the raw read depth during SNP calling;

[d] GT: Genotype, PL: List of Phred-scaled genotype likelihoods, DP: high-quality bases, GQ: genotype quality;

[e] The score of each term in FORMAT column and is separated by colon ":".

Chapter 5: *STAY-GREEN (CsSGR)* is a putative candidate gene for the *dm1* locus conferring downy mildew resistance in the field for over 50 years of US cucumber production

Abstract

The downy mildew (DM) resistance in PI 197087 is controlled by a single recessive gene denoted as *dm1*. This gene was used to develop many important DM resistant cultivars that efficiently controlled DM disease for over 50 years of the United States cucumber production until 2004 when a new strain emerged. Currently, the *dm1* locus is still effective in other countries but exhibits moderate resistance in US cucumber fields. However, the causal gene underlying *dm1* locus and its molecular mechanism of DM resistance are still poorly understood. This study reports fine mapping results for *dm1* from two resistant cucumber lines Gy14 and WI2757, both resistance could be traced back to PI 197087. The *dm1* locus was eventually delimited into a 93.7kb region in chromosome 5, harboring 12 candidate genes. Among them, *Csa5G156180 (CsSGR)* that encodes a senescence-inducible chloroplast *STAY-GREEN* protein, is the putative candidate gene. A SNP in the third exon resulted in a Glutamine (Q) to Arginine (R) amino acid substitution in Gy14 and WI2757. The phylogenetic tree with 131 cucumber accessions revealed the Indian origin of *dm1* which was preserved during breeding practices in modern times.

Introduction

Downy mildew (DM), one of the most destructive foliar diseases of cucurbits, is caused by the oomycete *Pseudoperonospora cubensis* [(Berkeley & M. A. Curtis) Rostoyzev] (Palti and Cohen 1980). *Ps. cubensis*, first described by Berkeley and Curtis (1868) in Cuba, caused DM disease and significant yield losses in cucumber in early 1900s (Kurosawa 1927; Jardine 1938). Breeding of DM resistance in cucumber was initiated in Puerto Rico Agricultural Station in the 1930s by systematic screening for over 150 varieties (Roque and Adsuar 1939). One of the varieties, which was introduced from China, showed high resistance to DM. This variety latter named as ‘Chinese Long’ was used for breeding Puerto Rico (PR) 37, PR39 and PR40 (Roque and Adsuar 1939). Barnes developed the very first DM resistant American slicing cucumber ‘Palmetto’ from the cross between PR40 and 'Cubit' in 1948. Its resistance, however, was lost only two years after its release probably due to the appearance of new DM strains in the field (Epps and Barnes 1952).

Barnes and Epps (1954) described a new type of resistance to DM in the Indian accession PI 197087. The symptoms of PI 197087 to DM infection was characterized by small irregularly shaped, light brown lesions, with a slight water-soaked appearance, becoming necrotic without passing through a yellowing stage and resembling a classic hypersensitive response (HR). PI 197087 was promising for cucumber breeding because it also showed tolerance or resistance to several other cucumber diseases, including anthracnose (caused by *Colletorichum lagenarium*), powdery mildew (mainly caused by *Podosphaera xanthii*), scab (caused by *Cladosponum cucumerinum*), and mosaic (caused by *Cucumber mosaic virus*), but less so to aphids and mites (Barnes and Epps 1952; Leppik 1968). PI 197087 was extensively used by Dr. Carol Barnes at Clemson University who developed a series of multiple-disease resistant cultivars, such as ‘Polaris’, ‘Poinsett’, ‘Pixie’, ‘Chipper’, ‘Sumter’, and ‘Gy1-A’, which were released between 1961 and 1973 (Palti and Cohen 1980; Wenner and Shetty

1997). DM resistance in most modern varieties released in the US could be traced to PI 197087 including the very widely used US processing cucumber inbred line Gy14 and the multi-disease resistant germplasm line WI 2757 (Peterson et al. 1982). With the deployment of the DM resistance gene *dm1* from PI 197087, DM was only a minor problem on cucumber from 1961 to 2003. St. Amand and Wehner (1991) reported only an average 2.9% yield loss per year caused by DM from 1982 to 1988. However, in 2004, *dm1* was overcome by a new DM strain that has devastated cucumber production in US with 40-100% yield loss (Colucci et al. 2006). Nevertheless, the *dm1* gene is still effective in European and Asian countries for cucumber production and offers only moderate resistance to DM infection in US cucumber fields.

Since the 1970s, a number of studies have investigated the inheritance of DM resistance in the PI 197087-derived cultivars through classical qualitative genetic analysis. van Viet and Meysing (1974) reported the recessive nature of DM resistant in cultivar ‘Poinsett’ (derived from PI 197087) denoted it as *dm*. In 1977, van Viet and Meysing further emphasized the closely-link between *dm* and powdery mildew resistance (*pm*). Since then, *dm* or *dm1* were unified and referred to the recessive DM resistance gene from PI 197087 in subsequent studies. Fanourakis and Simon (1987) discovered the association of *dm1* and anthracnose resistance (*cla*) in WI2757, while *pm* was correlated with few morphology traits, such as dull fruit skin (*D*), uniform skin color (*u*) and tuberculate fruits (*Tu*), which has been mapped to the end of chromosome 5 in later studies. Kennard et al. (1994) used restriction fragment length polymorphism (RFLP) markers and mapped the *dm1* locus from Gy14 between markers CsC588/H3 or CsC230/E1 and CsC593/D1 in Gy14. The CsC588/H3 sequence (GenBank: CF542159.1) aligned to ~6.5Mb of Chromosome 5 in the 9930 draft genome assembly V2.0 (Huang et al. 2009). Horejsi et al. (2000) identified the *dm1* locus from WI 1983G (derived from Gy14) flanked by random amplified polymorphic DNA (RAPD) markers $AS5_{800}$, $BC526_{1000}$, and $G14_{800}$ using 55 F_3 families. $AS5_{800}$ and $BC526_{1000}$ sequences were aligned to Chromosome 5 at 8.2Mb and 6.9Mb in the 9930 draft genome assembly V2.0, respectively. Bradeen et al. (2001) developed a cucumber consensus map, on which *dm1* was linked with RFLP markers C7 and AJ18 which were located between 4.5Mb and 9.2Mb of Chromosome 5 in the 9930 draft genome assembly V2.0.

After *dm1* was defeated in 2004, little work has been done on *dm1*. The molecular mechanism why *dm1* provide 50-year resistance and the reasons why it was defeated are still unknown. Since *dm1* still provides moderate resistance to the new DM strains and performs well in other countries, answering these questions may still be of significance for efficient use of host resistance against the DM. Furthermore, the rapid progress in the development of genetic and genomic resources in cucumber also provide an excellent opportunity to re-investigate the causal gene underlying *dm1* locus. Therefore, the objective of this study was to conduct fine mapping of the *dm1* gene. Two resistance lines carrying *dm1*, Gy14 and WI2757, were crossed with highly susceptible line 9930 and True Lemon, respectively, to develop two recombinant inbred line (RIL) populations. The two RILs were genotyped by large numbers of SNP markers and phenotyped in multiple environments.

Materials and Methods

Plant materials

Gy14 and WI2757 are two inbred lines with high resistance to pre-2004 DM strains but moderate resistance to current DM strains (Call et al. 2012). Gy14 was an advanced selection from the cross between Gy3 and Wis SMR18. Gy3 was developed from PI 197087, PI 196289 and PI 220860 (Peterson et al. 1975; Chung et al. 2003). WI 2757 has a complicated pedigree consisting of many varieties, including ‘Expo’ (Netherlands), PI 220860 (Korea; source of gynoecy), PI 197087 (India; DM and anthracnose resistance source), PI 212233

(Japan; powdery mildew resistance source) (Peterson et al. 1982). In both pedigrees of Gy14 and WI2757, PI 197087 is the major DM resistance source that carried *dm1* locus.

Gy14 was crossed with 9930 (North China fresh market type) and used to create an $F_{7:8}$ RIL population (hereinafter referred to as G9RIL) consisting 129 lines (Yang et al. 2012; Weng et al. 2015). Additional 375 G9RIL $F_{7:8}$ lines were used for fine-mapping of *dm1*. WI2757 was crossed with True Lemon to develop 87 $F_{5:6}$ RILs through single seed descent from the F_2 population described by He et al. (2013) (hereinafter referred to as WTRIL). Both 9930 and True Lemon were highly susceptible to DM.

Downy mildew resistance screening

Field Screening

For a preliminary QTL mapping, the phenotypes of G9RIL and WTRIL to DM resistance were collected in two locations for three years. The details of all experiments are summarized in **Table 5.1**. Experimental location included the Horticultural Crops Research Station of North Carolina State University in Clinton, North Carolina (NC) and Bayer Vegetable Seeds, the Netherlands (NL). The phenotypes of G9RIL were collected in NC2013, NC2014, and NL2014, while those of WTRIL were collected in NL2014 and NC2015.

The NC experiments were carried out with randomized complete block designs with two replications. There were 5 to 10 plants per line per replication. Plots were exposed to natural epidemics encouraged by overhead irrigation during the growing season at least three times per week, or as needed. Disease severity of each plot was evaluated for two or three consecutive weeks. In NC2013, DM symptoms of the G9RIL population were measured using two rating criteria: general impression (GI) and chlorotic lesion size or yellowing leaf area (Yel). In NC2014 for G9RIL and NC2015 for WTRIL, only the criterion GI was used. For each criterion, the disease severity was based on percentage of symptomatic leaf area on rating scale of 1 to 9, where 1 = 0–10%, 2 = 11–20%, 3 = 21–30%, 4 = 31–40%, 5 = 41–50%, 6 = 51–60%, 7 = 61–70%, 8 = 71–80%, 9 = 81–100%, or dead.

Field layout of NL2014 for G9RIL experiments was similar to NC with two replications and seven plants per family per replication. The DM symptoms of each plot were recorded twice with one week apart with two criteria: GI and Yel. For each criterion, the disease severity was rated on the plant breeder's 1 to 9 scale, where scale 1 = maximal disease symptoms and 9 = no visible symptom.

At fine-mapping stage, the recombinants identified from G9RIL population were tested for DM inoculation responses in NC2015 and NL2016 experiments using the same experimental design and rating criterion GI as described above. Two addition environments were employed in 2015 for phenotyping including the Florida experimental field of Seminis Vegetable Seed Inc. (FL2015) and plastic tunnels of Magnum Seeds in Fondi, Italy (IT2015). Both experiments were conducted with natural infection of 20 plants per RIL without replications. Individual plants were scored with GI, and family means were calculated from averaging data from 20 plants.

DM screening tests in controlled environments

The RILs was screened for DM inoculation responses under controlled environment conditions at two locations. The first was the experiment for WTRIL in 2014 in a walk-in climate control room at Bayer Vegetable Seeds in the Netherlands (NL2014). Each RIL had two replications, and there were 2 to 3 plants per RIL per replication. Plants were artificially inoculated at two to three true-leaf stage with sporangial suspension at a concentration of 1×10^5 spore mL^{-1} **(Figure 5.1)**. The DM symptoms were evaluated with two criteria: Yel and leaf collapsing (Col). Each plant was measured twice with one week apart with the same rating scale in field experiment of NL.

The second experiment was conducted in the growth chambers at the University of Wisconsin-Madison, WI. The seedlings growth condition and DM isolates maintenance were the same as described in Chapter 4. The artificial inoculation was conducted at the first true leaf by misting the adaxial side of leaves with the sporangial solution in a reduced concentration of 5 × 10^4 spore/mL with ~50 mL per tray. The inoculated seedlings were incubated in dark for 48 h at 20°C and 100% relative humidity (RH). After incubation, plants were grown at condition of 24/18°C (day/night) with 12 hours of light in the growth chamber, in which 100% RH was maintained to promote DM symptom development. Leaf samples of these materials were collected and used for RNA extraction and real-time PCR experiments.

Genotyping and linkage map development

Linkage map construction of G9RIL population

An ultra-high-density genetic map was developed with 129 Gy14 × 9930 $F_{7:8}$ G9RILs, which contains 11,156 SNP loci spanning 600 cM in seven linkage groups (Rubinstein et al. 2015). From this map, a subset of 458 SNPs evenly distributed across seven chromosomes were selected to develop a new map for QTL analysis of RIL data in this study. More details of the SNP-based RIL map for G9RIL could be found in Weng et al. (2015).

Linkage map construction of WTRIL population

The leaf samples of 87 $F_{5:6}$ WTRILs and two parents (WI2757 and True Lemon) were freeze-dried and shipped to the Beijing Agriculture and Forestry Academy of Sciences for SLAF-seq (Specific Length Amplified Fragment sequencing). SLAF library construction, genotyping procedure, SNP filter criteria and SNP calling method were the same as the described in Chapter 3.

The *R/ASMap* package was used to establish marker order (Wu et al. 2008; Taylor and Butler 2015), and the function *est.map* in *R/qtl* package was used to estimate the genetic distance for linkage map construction (Broman et al. 2003). Kosambi mapping function was applied during genetic map distance calculation.

Statistical Analysis

Statistical analysis of phenotypic data was all performed in R (Version 3.1.1, http://www.r-project.org/). Mean disease score of each family was calculated from replications under different rating times, criteria and environments for statistical analysis. The original ordinal rating data for NL environment were subtracted by 9 to convert to same scale with other environments. Analysis of variance (ANOVA) was performed with the *R/lme4* package (Gilmour et al. 2009) to estimate the genetic effects, environmental effects and heritability for G9RIL. Heritability estimates are considered narrow sense estimates because the dominance variance and covariance of additive and dominance effects are equal to zero (Cockerham 1983). The Spearman's rank correlation coefficient (r_s) among different environments was calculated for each rating time and rating criterion.

QTL Analysis

QTL analysis was performed using the *R/qtl* package with the multiple-QTL model (MQM) (Broman et al. 2003). QTL detection started with the function *scanone* for preliminary QTL identification, and followed with *addqtl* and *addpair* to scan additional QTL and QTL pairs, respectively. The function *refineqtl* was followed up to refine the position of QTLs. The significance of each QTL interval was tested by a likelihood-ratio statistic (LOD). The LOD threshold for declaring significant QTL was established separately for each rating criterion and each environment using 1000 permutations ($P<0.05$). The refined significant QTL were assessed for the additive effects and percentage of phenotypic variations (R^2) explained. The support intervals for these QTL were calculated using a 1.5 LOD drop interval. QTL naming conventions followed He et al. (2013) except that each QTL was preceded with *dm* (for downy mildew).

Fine mapping of candidate QTL

To determine the physical position of candidate QTL, the sequences of flanking markers of 1.5 LOD interval were anchored to the 9930 draft genome assembly V2.0 and Gy14 genome assembly V1.0 using BLAST+ (Camacho et al. 2009) with a cut-off E-value <1.0E-10. The candidate sequence of 9930 and Gy14 were then aligned to discover new SNPs, INDELs, or SSR markers for genotyping of in the large G9RIL population and identifying new recombinants.

RNA extraction and qRT–PCR

The inoculated true leaves were collected at 0, 3, 5, and 7 day post inoculation (dpi). Total RNA was extracted using GeneJET Plant RNA Purification Mini Kit (Thermo Scientific). DNase I (Ambion) was applied to remove the trace amounts of remain DNA. The first-strand cDNA was synthesized using RevertAid^{TM} First Strand cDNA Synthesis Kit and analyzed by quantitative PCR with the SYBR Green PCR master mix (Applied Biosystems Inc., USA) on a iCycler iQ^{TM} Real-Time System (Bio-Rad). The RNA expression level of *CsSGR* was examined using the following primers: forward: 5’-CATGGAATGGTTCCTATGGCG-3’, and reverse: 5’-TGTGTGAGCGTATAAGTCCTTGG-3’. The cucumber actin gene (*Csa2G301530*) was used to normalized the transcripts level of *CsSGR* with the following primer pairs: forward: 5’-ATTGTTCTCAGTGGTGGTTCTAC-3’, and reverse: 5’-CCTTTGAGATCCACATCTGCT-3’. The relative gene expression levels were determined by $2^{\Delta\Delta CT}$. The qRT-PCR program was conducted with the following procedure: 95°C for 30 seconds, 40 cycles of 95°C for 10s, 58°C for 20s, and 72°C for 30s, followed with a melting curve analysis started at 72°C with 0.5 degree increments for every 5s to 95°C.

Allelic diversity and in silico bulk segregant analysis (BSA) of dm1 locus

Whole-genome resequencing data of 131 cucumber lines were available, 115 of which were available from the NCBI database (Qi et al. 2013) and 16 were re-sequenced with the Illumina Hi-seq 2000 platform. To understand the origin and evolution of the *dm1* locus, the nucleotide variations in *dm1* locus were explored in the 131 cucumber lines (listed in **Appendix 5.1**). Illumina sequence reads of 16 lines were aligned with GS Reference Mapper (V2.8) software using the 9930 draft genome assembly V2.0 as the reference for SNP calling. These SNPs were further integrated with the SNPs of 115 lines which were downloaded from CuGenDB (http://www.icugi.org/). Among them, six cucumber lines are known to carry the *dm1* locus and six lines do not; they were selected for in silico bulked segregant analysis (BSA) to detect consensus SNPs between the two bulks, which were considered as putative causal SNPs and used for further analysis (Li et al. 2016). Moreover, a set of SNPs in the regions upstream and downstream of the *dm1* SNP position were selected for hierarchical clustering by basic R function *hclust* with ‘complete’ linkage method.

Phylogenetic analysis of CsSGR protein sequences

The phylogenetic relationship of cucumber CsSGR protein and other plant SGR/SGRL family proteins were investigated. The accession numbers of SGRs and SGRLs in the GenBank used in this study are as followers: AtSGR1 (*Arabidopsis thaliana*), At4G22920.1; AtSGR2, At4G11910.1; AtSGRL, At1G44000.1; CaSGR (pepper, *Capsicum annuum*), NP_001311847.1 ; CsSGRL, XP_016569624.1 ; ClaSGR(watermelon, *Citrullus lanatus*), Cla011503; CmSGR (*Cucumis melo*), XP_008437189.1; CmSGRL, XP_016899407.1; CsSGR(*Cucumis sativas*), Csa5G156180.1; CsSGRL, Csa6G349840.1; GmSGR1 (soybean, *Glycine max*), GLYMA11G027400; GmSGR2, GLYMA01G214600; HvSGR (Barley, *Hordeum vulgare*), AAW82955.1; NtSGR(tobacco, *Nicotiana tabacum*), XP_016501400.1; NtSGRL, XP_016437764.1; SlSGR(tomato, *Solamum lycopersicum*), NP_001234723.1; SlSGRL, XP_004237702.1; StSGR (potato, *Solanum tuberosum*), XP_006358848.1; StSGRL, XP_006365914.1; OsSGR (*Oryza sativa*), Os09g36200; OsSGRL, Os04g59610; ZmSGR1 (*Zea mays*), ZEAMMB73_024579; ZmSGR2, ZEAMMB73_905872. These sequences were aligned and clustered by MEGA 7.0 software (http://www.megasoftware.net/) using nearest neighbor-joining parameters with 1000 bootstrap replications.

Results

Phenotypic characterization of DM resistance in G9RIL population

Phenotypic data of DM inoculation responses among 129 G9RILs were collected from three environments with two criteria GI and Yel, which were designated as NC2013-Yel, NC2013-GI, NC2014-GI, NL2014-Yel, and NL2014-GI, respectively. The grand means and standard deviation of DM disease scores for the two parental lines and their F_1 across the three environments are presented in **Table 5.2**. The probability densities of the phenotypes at each rating time are illustrated by violin plot and boxplot in **Figure 5.2**. Frequency distribution and the Spearman’s rank correlations of the three experiments for each rating time and criterion in G9RIL are presented in **Figure 5.3.**

In NC2013 and NL2014 trials, rating of disease symptoms was conducted two to three times with one week apart. As shown in **Figure 5.3**, the correlation among environments decreased as the rating time increased. This was consistent with the fact that *dm1* locus showed moderate resistant to current DM strains and lost its durable resistance at later plant stage, which is also reflected in **Figure 5.2**.

As compared with the first rating time in all environments, the two rating criteria GI and Yel were highly and significantly correlated with r_s ranging from 0.45 to 0.73 ($P < 0.001$) suggesting that symptom of yellowing (Yel) may be the major component of a general disease symptom (GI) in Gy14. When the raw data were used in calculation of r_s, the mean disease scores of NL2014 (GI and Yel) were negatively correlated to those from NC2013 and NC2014. This is reasonable because NL2013 experiment used a reversed rating scale (plants rated 1 were the most susceptible).

Reverse rating scales were employed between two locations (1-9 in NC2013, NC2014; 9-1 in NL2014). To compare environmental effects on DM resistance, the phenotypic data from NL2014 datasets were subtracted by 9 to convert to the same scale as the NC experiments. A one-way ANOVA using the criteria GI and 1st rating time was performed and the results are presented in **Table 5.3**, which indicated no significant genotype-by-environment effects on DM resistance. The heritability estimate of mean disease scores (h^2) based on ANOVA was only 0.57 (**Table 5.3**).

Phenotypic characterization of DM resistance in WTRIL population

The DM symptoms among 87 WTRILs were collected from two environments: NL2014 and NC2015. While disease rating in NC2015 was based on a GI of foliage symptom development, NL2014, conducted in a climate control room, used two different criteria Yel and Col. The probability densities of the phenotypes are illustrated by violin plot and boxplot in **Figure 5.4**. The grand means and standard deviation of DM disease scores for the two parental lines and their F_1 across the three environments are presented in **Table 5.2**.

Frequency distribution and Spearman rank correlations of the three experiments for each rating time and criteria in WTRIL were listed in **Figure 5.5.**

As showed in **Figure 5.1** and **Table 5.2**, the F_1 plants were as susceptible as the parent True Lemon, which suggested the recessive nature of DM resistance in WI2757. The frequency distribution in NC2015 showed continuous and largely normal distribution with high correlation among each rating time. This suggested that WI2757 was able to hold moderate DM resistance in later plant growth stage. Moreover, unlike G9RIL, the correlation among rating criteria of WTRIL showed only low to moderate scale with r_s ranging from 0.23 to 0.49 ($P < 0.001$). These observations suggested that, in addition to *dm1*, other DM resistance QTL may exist in WTRIL. When the raw data were used in calculation of r_s, the mean disease scores of NL2014 (Yel and Col) were negatively correlated to those from NC2015, since reversed rating scale was applied in the two environments.

Linkage map construction for the WTRIL population

The WTRIL population with 87 RILs was genotyped by SLAF-seq. In total, 6.48 Gb data containing 40,504,760 pair-end reads were generated using the Illumina Genome Analyzer. Among them, 80.13 and of 96.32% reads were of high quality with quality scores of at least 30 (Q30) and Q20 threshold, respectively. Among the detected 50,256 high quality SLAF tags, 6,491 (12.92%) were polymorphic between the two parents. After filtering with criteria described in Chapter 3, 1,618 SNP markers were obtained for genetic map construction. The resulting genetic map spanned 1,187.6 cM in 7 linkage groups (chromosomes) with an average marker interval of 0.7 cM (**Table 5.4**, **Appendix 5.2**).

Detection of DM resistance QTL in G9RIL population

The QTL analysis was performed using the MQM approach with data from each location, rating time and rating criterion. The LOD threshold to declare significance of QTL for each trait was determined with 1,000 permutations ($P<0.05$). As presented in **Figure 5.6**, each location and rating criterion detected only one QTL at the top of chromosome 5 at the same position. By using criterion GI, QTL was detected only with the data from the 1st rating, which was disappeared as the disease developed in the later ratings (2nd and 3rd rating). Moreover, QTL detected with the rating criterion Yel showed higher LOD score and larger additive effects than those detected with GI in both NC2013 and NL2014 suggesting that Yel may be a more accurate parameter in rating the DM symptoms conferred by *dm1* locus.

Detailed information of detected QTL including map location, correlated peak maker, 1.5 LOD interval, additive effect, and percentages of phenotypic variance is provided in **Table 5.5**. Comparing results among these experiments, it was clear that the *dm1* locus was located in an interval delimited by SNP markers SNP.74621 and SNP.111257, which were physically located between 4.07 and 6.31Mb of Chromosome 5 in the 9930 draft genome assembly V2.0.

Detection of DM resistance QTL in WTRIL population

QTL analysis in the WTRIL population was conducted with MQM and data from NC2015 with GI criterion at three rating times and NL2014 with Yel and Col criteria. As shown in **Figure 5.7**, two QTL were consistently identified across all environments. Detailed information of all detected QTL including map location, correlated peak maker, 1.5 LOD interval, additive effect, and percentages of phenotypic variance are provided in **Table 5.5**.

The QTL detected by NL2014-Yel and NC2015-GI of 1st rating was defined by SNP Marker5_3374580 and Marker5_6536439, which was largely overlapped with the QTL, *dm1*, detected in the G9RIL population. Another QTL detected by NL2014-Col and NC2015-GI of 2nd and 3rd rating was delimited by its 1.5-LOD interval from 15.37Mb to 17.38Mb in Chromosome 5. This interval was overlapped with the QTL *dm5.1* identified from 9930 × WI7120 population (Wang et al. 2016) or *dm5.2* in PI 197088 × Coolgreen population (Chapter 3).

To summarize, QTL mapping identified one QTL for *dm1* in both G9RIL and WTRIL populations located on Chromosome 5 between 3.37Mb to 6.54Mb. In WI2757, there was a second QTL, which was consistent with *dm5.1* detected in WI7120 and *dm5.2* in PI 197088 (Wang et al. 2016; Chapter 3).

Fine mapping of dm1 in a larger RIL population

To further narrow down the region of *dm1*, recombinants were identified in a large G9RIL population containing 375 $F_{7:8}$ RILs. Using two flanking SSR markers UW063935 and SSR00398 defined the 1.5-LOD interval of *dm1*, 18 recombinants were identified. Additional nine polymorphic SSR and SNP markers within the QTL region were developed and used to genotype these recombinants. Information of all markers used is provided in **Table 5.6.**

These 18 recombinants were tested for DM inoculation responses in the field trials for four environments including NC2015, FL2015, IT2015, and NL2016. The mean disease scores and standard deviation (SD) of each line were calculated across four environments to infer its phenotype. As shown in **Figure 5.8**, the *dm1* locus was eventually delimited to a 93.7kb region, which harbored 12 annotated genes.

Variants of candidate genes sequences in natural cucumber populations

Twelve genes were predicted (from *Csa5G156160* to *Csa5G157260*) within the 93.7kb target region according to the 9930 draft genome assembly V2.0. We examined allelic diversity of the candidate gene region in 12 cucumber lines with and without the *dm1* gene. The six lines carrying the *dm1* gene included Gy14, WI2757, Gy8, H19, G421, and 2A; those without *dm1* included 9930, Straight 8, Coolgreen, Wis SMR18, WI7120, and PI 197088. The last two were DM resistant but do not carry the *dm1* locus (Wang et al. 2016; Chapter 3). Using the 9930 draft genome assembly V2.0 as the reference, 50-75 SNPs or INDELs were detected between any of the 11 cucumber lines and 9930 within this 93.7kb region. However, complete consistency between the marker genotype and DM resistance phenotype was found only at one SNP locus, which was located in the coding region of *Csa5G156180*. Distribution of all SNPs in the 12 genes is presented in **Table 5.7**.

STAY-GREEN (CsSGR) is a putative candidate gene for the dm1 locus

The above evidence suggested that *Csa5G156180,* is a possible candidate gene for *dm1*, which encodes a senescence-inducible chloroplast *STAY-GREEN* protein and designated as *CsSGR* hereinafter. *CsSGR* was predicted to contain 4 exons and its coding region was 771bp encoding a putative protein with 256 amino acids (aa) residues. At No. 323 nucleotide of CDS in *CsSGR*, there is a transition from A (9930) to G (Gy14), which causes No.108 residue coding protein substitution from Glutamine (Q, 9930) to Arginine (R, Gy14). The DNA and protein sequences of *CsSGR* in the 12 cucumber lines were compared in **Figure 5.9**.

To further confirm the function of *CsSGR* in DM resistance, the transcript expression level was examined in DM resistant line Gy14 and WI2757, and susceptible line 9930 at 0, 3, 5, and 7 days post inoculation (dpi). As shown in **Figure 5.10**, the expression level of *CsSGR* in 9930 was significantly increased at 7dpi as compared with Gy14 and WI2757, when the different symptoms were clearly distinct between 9930 (yellowing leaves) and Gy14 or WI2757 (necrotic spots but staying green).

The CsSGR protein sequence is highly conserved across dicot and monocot plants

In various plant species, *STAY-GREEN* family contains only two or three SGR homologs, which could be classified into two subfamilies: the SGR and SGR-like (SGRL) subfamily in higher plants (Sakuraba et al. 2015). The cucumber genome has one *CsSGR* (*Csa5G156180*) and one *CsSGRL* (*Csa6G349840*) in SGR family. To understand the structural and functional relationship among *CsSGR* in cucumber and its homologs in other plant species, a phylogenic tree was constructed using amino acid sequences from cucumber *CsSGR* and *CsSGRL*, and 22 other *STAY-GREEN* proteins in dicot and monocot plant species **(Figure 5.11A**). The domain structure of SGR homologs in Arabidopsis, rice, pepper, tomato, melon, and cucumber lines 9930 and Gy14 were examined as well, which revealed that the Q to R amino acid substitution was located at the conserved SGR domain (**Figure 5.11B**). The protein structure of the SGR domain encoded by *CsSGR* was therefore predicted by RaptorX (http://raptorx.uchicago.edu/). The predicted 3D model of the SGR domain with highlighted substituted amino acid residue revealed that the Q to R aa substitution caused shorten β-fold sheet that connected to the α-helix at the SGR domain, which might affect its functions (**Figure 5.12**).

The dm1 allele is of Indian cucumber origin

Since PI 197087 was collected from India, to ascertain the Indian origin of the *dm1*, the SNP distribution at the *dm1* locus among 131 cucumber accessions with resequencing data was examined, with 115 lines obtained from Qi et al. (2013) and 16 lines were re-sequenced with the Illumina Hi-Seq 2000 platform. Among them, 59 were cultivated cucumbers (*C. sativus* var. *sativus*, CSS hereinfater) from various geographic regions in Asia and Europe were assigned as Eurasian1 to Eurasian59, respectively. Thirteen and 19 belonged to the wild cucumber (*C. s.* var. *hardwickii*. CSH1 to CSH13) and semi-wild Xishuangbanna cucumbers (*C. s.* var. *xishuangbannanesis*. XSBN1 to XSBN19), respectively. For convenience, the CG# of each line by Qi et al. (2013) was kept, and those Indian originated cucumbers and American cultivars were listed with their common names and PI accession names. One hundred and eighty-six SNPs in a 20 kb vicinity of the *dm1* locus were used for a phylogenetic tree construction for the 131 accessions, which is illustrated in **Figure 5.13**. It is very likely the *dm1* allele is a single occasional mutation in PI 197087 which is originated from India, and was preserved during breeding practice in modern times.

Discussion

Identification of dm1 locus

Barnes and Epps (1954) described a new type of DM resistance in PI 197087 originated from India, which has been extensively used for DM resistance breeding for over 50 years in US cucumber production. The causal gene in PI 197087 is a recessive gene and named as *dm1* conferring highly resistant to pre-2004 DM strains and moderately resistant to current strains.

The major distinct feature of *dm1* resistance was characterized by the appearance of necrotic lesion without passing through a yellowing stage. To carefully examine the *dm1* resistance, three criteria Yel, Col and GI were used to differentiate the symptoms to DM infection in this study. GI refers to general impression which scored the symptoms of both Yel and Col under DM infection. Yel and Col only evaluated yellowing and collapsing symptoms, respectively. In both G9RIL and WTRIL populations, the QTL detected using Yel explained larger additive effects and phenotypic variance compared to GI, suggesting Yel was the major susceptible symptom under DM infection. Therefore, the QTL that mapped at the top of chromosome 5 ranging from 3.3Mb to 6.5Mb in 9930 assembly v2.0 was considered as the *dm1* locus. The other QTL detected in the WTRIL populations with Col data may be inherited from other DM resistance source(s).

Fine mapping narrowed the *dm1* locus down to a 93.7kb region which harbored 12 predicted genes. By examining the SNP variants within this region using an in silico BSA strategy, the gene *Csa5G156180* (STAYGREEN, Cs*SGR*) seemed to be the best candidate gene of *dm1*. Yoshioka et al. (2014) suggested that the *dm5.1* identified in PI 197088 could be the candidate QTL for *dm1* gene. However, after carefully examining the physical locations of *dm5.1* in PI 197088 and *dm1* in the present study, the two QTL were at least ~3Mb apart and there was no overlap in their 1.5 LOD interval.

Characterization of CsSGR as a putative candidate gene of dm1

In several plant species, it was found that mutations in the *SGR* genes induced the stay-green phenotype (Park et al. 2007; Ren et al. 2007). Protein to protein interaction studies revealed that SGR physically interacts with light-harvesting complex of PSII (LHCII) and other six chlorophyll degradation enzymes to trigger chlorophyll and chlorophyll-binding protein degradation during senescence and embryo degreening (Hörtensteiner 2009; Mecey et al. 2011; Sakuraba et al. 2012; Delmas et al. 2013). Recently, *SGR* gene was also found to encode the magnesium-dechelatase (Shimoda et al. 2016). The *SGR* expression can be induced by pathogen infection which can serve as a signal transduction component in the defense network (Mecey et al. 2011). However, the detailed functions of the *SGR* gene in disease resistance are still unknown.

SGR family is conservative with only few SGR genes and SGR-like genes homologs in various plants (Delmas et al. 2013; **Figure 5.11**). From all annotated gene in 9930 and Gy14 assembly, only one SGR (*Csa5G156180*) and one SGR-like (*Csa6G349840*) gene were identified. In this study, using in silico BSA between *dm1*-present and absent bulks, only one

SNP was obtained which caused a Q to R amino acid substitution in *CsSGR*. A 3D model was predicted to evaluate the structure change resulting from amino acid substitution, which showed a shorten beta-fold sheet in SGR domain. The changes of protein structure could be one of the reason for the modification of gene function during DM infection. Furthermore, the phylogenetic tree with 131 cucumber accessions revealed the India origin of *dm1* locus which was preserved during breeding practice in modern times. Further studies are needed to identify the possible binding site in *CsSGR* that altered its function, including but not limited to gene knock out, gene silencing, or editing the single mutated nucleotide in the *CsSGR* gene.

Additional DM resistance QTL in WI2757

Besides *dm1*, another QTL *dm5.1* in WI 2757 was also identified, which corresponded to necrotic symptom only. WI 2757 has a complicated pedigree, it is difficult to verify its resistance sources (Peterson et al. 1982). Compared the 1.5 LOD interval of *dm5.1* with some other QTL that were mapped at the similar position, it is found that *dm5.1* of WI2757 shared a large interval with QTL *dm5.1* in WI7120 and *dm5.2* in PI 197088 (Wang et al. 2016; Chapter 3). In Chapter 4, *Csa5G471600* has been identified to be the putative candidate gene for *dm5.1* in WI7120. Further work is needed to verify if *Csa5G471600* is also the causal gene underlying *dm5.1* in WI2757.

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Table 5.1 Details of environments used for evaluation f DM inoculation responses in G9RIL and WTRIL populations for QTL mapping of the *dm1* resistance locus.

Population	**Environments**[a]	**Trial**	**Criteria**[b]	**Experimental design**[c]
G9RIL ($129\ F_{7:8}$)	NC2013	Field	Yel, GI	RCBD, two reps, 5-10 plants per line per rep, rating by plot
	NC2014	Field	GI	RCBD, two reps, 5-10 plants per line per rep, rating by plot
	NL2014	Field	Yel, GI	RCBD, two reps, 7 plants per line per rep, rating by plot
WTRIL ($87\ F_{5:6}$)	NL2014	Greenhouse	Yel, Col	Two rep, 2-3 plants per line per rep, rating by individuals
	NC2015	Field	GI	RCBD, two replications 5-10 plants per line per rep, rating by plot
Selected Recombinants (18 lines)	FL2015	Field	GI	No rep. 20 plants per line, rating by individuals
	IT2015	Plastic Tunnel	GI	No rep. 20 plants per line, rating by individuals
	NC2015	Field	GI	RCBD, three reps, 10-15 plants per line per rep, rating by plot
	NL2016	Field	Yel	No rep, 10-15 plants per line, rating by plot

Note:

[a]: NC=Horticultural Crops Research Station of North Carolina State University in Clinton, North Carolina; NL=Bayer Vegetable Seeds in the Netherlands; FL=Florida experimental field of Seminis Vegetable Seed Inc.; IT= plastic tunnels of Magnum Seeds in Fondi, Italy.

[b]: Yel=Yellowing or Chlorosis symptoms; Col=Collapsing or Necrosis symptoms; GI=General Impression or General judgement of all the symptoms.

[c]: RCBD=Randomized Complete Block Design; rep=replication.

Table 5.2 Phenotypic means and standard deviation of DM disease scores at the first rating time with different criteria in parental lines, their F_1, and G9RILs and WTRILs from individual experiments.

Pop	**Lines**	**NC2013-Yel***	**NC2013-GI**	**NC2014-GI**	**NL2014-Yel**	**NL2014-GI**
G9RIL	Gy14	1.00±0.00	4.50±0.50	3.00±0.00	8.50±0.50	7.00±1.00
	F_1	6.50±2.50	6.00±0.00	6.00±0.00	7.00±0.00	5.00±0.00
	9930	9.00±0.00	6.00±0.00	6.50±0.50	6.00±0.00	6.00±0.00
	RILs	4.75±2.72	5.26±1.08	4.60±0.82	6.14±1.20	5.82±1.82
Pop	**Lines**	**NL2014-Yel**	**NL2014-Col**	**NC2015-GI**		
WTRIL	WI2757	7.22±0.78	8.94±0.23	3.67±0.47		
	F_1	4.44±0.68	2.67±1.37	6.67±0.47		
	True Lemon	4.00±0	1.56±1.00	5.67±0.47		
	RILs	6.18±1.53	5.27±2.60	5.93±1.12		

Note: *NC=Horticultural Crops Research Station of North Carolina State University in Clinton, North Carolina; NL=Bayer Vegetable Seeds in the Netherlands; Yel=Yellowing or Chlorosis symptoms; Col=Collapsing or Necrosis symptoms; GI=General Impression or General judgement of all the symptoms.

Table 5.3 Analysis of variance (ANOVA) and estimation of heritability (*h^2*) for DM disease scores using GI (general impression) data of the G9RIL population.

Source of variation	**df**	**Mean Square**
Genotype (G)	150	3.85**
Environment(E)	2	10.39**
G × E	270	1.24
Block (E)	6	1.01
Residual	800	0.97
Heritability (h^2)		0.57±0.27

Note: **with a significant level at $P<0.01$.

Table 5.4 Main statistics of a linkage map developed with 1618 SNP marker loci for 87 WTRIL population.

Linkage Group (Chr)	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**Sum**
Number of SNP Marker	358	168	254	179	309	129	221	1618
Map Length (cM)	199	176.6	204.6	175.1	131.7	156	144.8	1187.9
Average Map Interval (cM)	0.6	1.1	0.8	1	0.4	1.2	0.7	0.7
Maximum Map Interval (cM)	11.3	13.6	9.8	14.2	8.1	12.8	6.4	14.2

Table 5.5 Information of QTL for DM resistance identified from Gy14 × 9930 RIL (G9RIL) and WI2757 × True Lemon RIL (WTRIL) populations in different environments with different rating criteria.

Pop	Environment	Rating Time	QTL	Chr	Associated marker	Peak Position (cM)	Peak Lod Score	9930 v2.0 Pos	1.5LOD Left Marker	1.5LOD Right Marker	Additive effects	Phenotypic Variation (%)
G9RIL	NC2013-Yel	1	*dm1*	5	SNP.176869	12.0	22.63	5040372	SNP.74621	SNP.111257	-1.84	44.35
	NC2013-Yel	2	*dm1*	5	SNP.176869	12.0	16.23	5040372	SNP.74621	SNP.111257	-1.44	33.48
	NC2013-GI	1	*dm1*	5	SNP.176869	11.3	10.62	5040372	SNP.74621	SNP.111257	-0.56	26.02
	NC2013-GI	2	-	-	-	-	-	-	-	-	-	-
	NC2014-GI	1	*dm1*	5	SNP.176869	11.3	7.29	5040372	SNP.74621	SNP.73809	-0.39	20.35
	NC2014-GI	2	-	-	-	-	-	-	-	-	-	-
	NC2014-GI	3	-	-	-	-	-	-	-	-	-	-
	NL2014-Yel	1	*dm1*	5	SNP.111257	14.0	18.03	6306083	SNP.74621	SNP.146681	0.89	38.76
	NL2014-Yel	2	*dm1*	5	SNP.176869	12.0	18.5	5040372	SNP.74621	SNP.111545	0.77	39.49
	NL2015-GI	1	*dm1*	5	SNP.176869	12.0	6.45	5040372	SNP.74621	SNP.146681	0.56	16.37
	NL2015-GI	2	-	-	-	-	-	-	-	-	-	-
	NL2015-GI	3	-	-	-	-	-	-	-	-	-	-
WTRIL	NL2014-Yel	1	*dm1*	5	Marker5_4972558	3	20.2	4972558	Marker5_4735130	Marker5_6432618	1.24	64.24
	NL2014-Col	1	*dm5.2*	5	Marker5_16720220	79.5	14.1	16720220	Marker5_16104657	Marker5_17384531	1.66	39.19
	NC2015-GI	1	*dm1*	5	Marker5_5154518	3.08	3.46	5154518	Marker5_3374580	Marker5_6536439	-0.39	9.59
	NC2015-GI	1	*dm5.2*	5	Marker5_16720220	80.1	3.51	16720220	Marker5_15371270	Marker5_17384531	-0.44	12.28
	NC2015_GI	2	*dm5.2*	5	Marker5_16720220	81	4.12	16720220	Marker5_15371270	Marker5_17384531	-0.47	15.79
	NC2015-GI	3	*dm5.2*	5	Marker5_16720220	81	4.31	16720220	Marker5_15371270	Marker5_17384531	-0.47	15.96

Table 5.6 The primer sequences and physical positions in the 9930 draft genome assembly v2.0 of SSR and SNP markers used in fine mapping of *dm1* locus.

Marker Name	**Marker Type**	**9930 v2.0 Position**	**Forward Primer (5' to 3')**	**Reverse Primer (5' to 3')**	**Enzyme**
SSR01243	SSR	4790046	AATCCTCAAACCCTCCAACC	TGCAGAAGGAAAACAATGGA	
UW063935	SSR	5158139	CGAACTTTTGCTCGATAGCAC	TTTTGATTTGCATGTTATCATTTTT	
SNP10	SNP	5399858	CTTGTCTTGATAGGCATCCATAT	TGACCTTCAAGCACTTCTGC	*Nde* I
UW063781	SSR	5437900	ACGTCACAAGACACAGCAGC	ATACTCTGCCCCTCAAGCCT	
SNP08	SNP	5463839	TTTTCTGGAATGGGTGTTAAA	CATTCTCCTACCACTTCATCCGCT	*Bsr* BI
SNP07	SNP	5469491	AGGGCATCCAACCTACCATT	ATGCCGGCATAAATTAATAACTT	*Dde* I
SNP05	SNP	5531753	CAAAATCACTTTTATTTTCTTGTCAAA	GGGAGCCATGTGAATAACACATA	*Nde* I
Ar92-21	SSR	5701690	GAACCCACGTGCAGGTTATT	TCTTCGTCCCATGAATCTATCC	
UW085228	SSR	5935183	TGTGCATGCTTCCTTAGCAC	TTGGCCTTTGGGTAACATTT	
UW063352	SSR	6191263	GATCCGACGTCCGAAAATAA	TCCGCCTCTTGACACTCTTT	
SSR00398	SSR	6616839	ATTCAAACCCCGTTTAACCC	AGTGAAAATGGCGGAAACTG	

Table 5.7 Annotated genes and predicted functions in the *dm1* locus within candidate 93.7kb region based on 9930 draft genome assembly V2.0.

Gene ID	**SNP Nr.**	**aa substitution**	**Annotation**
Csa5G156160	0	n.a*	Protein FAM63A; contains IPR007518 (Protein of unknown function DUF544)
Csa5G156170	0	n.a	MADS box transcription factor; contains IPR002487 (Transcription factor, K-box)
Csa5G156180	A to G	Q to R	Senescence-inducible chloroplast stay-green protein; contains IPR024438 (Staygreen protein)
Csa5G156190	0	n.a	Guanine nucleotide-binding protein subunit beta-like protein; contains IPR015943 (WD40/YVTN repeat-like-containing domain)
Csa5G156200	0	n.a	AT3g08600/F17O14_7; contains IPR010605 (Protein of unknown function DUF1191)
Csa5G156210	0	n.a	Expansin-A7; contains IPR007118 (Expansin/Lol pI), IPR014733 (Barwin-like endoglucanase)
Csa5G156220	0	n.a	Putative transcription factor; contains IPR011598 (Myc-type, basic helix-loop-helix (bHLH) domain)
Csa5G157220	0	n.a	Transcription factor bHLH36; contains IPR011598 (Myc-type, basic helix-loop-helix (bHLH) domain)
Csa5G157230	0	n.a	Transcription factor bHLH36; contains IPR011598 (Myc-type, basic helix-loop-helix (bHLH) domain)
Csa5G157240	0	n.a	Subtilisin-like serine protease; contains IPR015500 (Peptidase S8, subtilisin-related)
Csa5G157250	0	n.a	Putative NUDIX hydrolase family protein; contains IPR015797 (NUDIX hydrolase domain-like)
Csa5G157260	0	n.a	Cytochrome P450; contains IPR001128 (Cytochrome P450)

Note: *n.a: not applied.

Figure 5.1 Performance of DM resistance in WI2757, True Lemon and their F$_1$ upon inoculation of DM pathogen in a climate control room in NL2014 experiment. A) DM symptoms of one week after DM infection on the first three leaves. B) DM symptoms of two weeks after DM infection on the first three leaves. Images curtesy of Dr. Jos Suelmann, Bayer Vegetable Seeds, Inc.

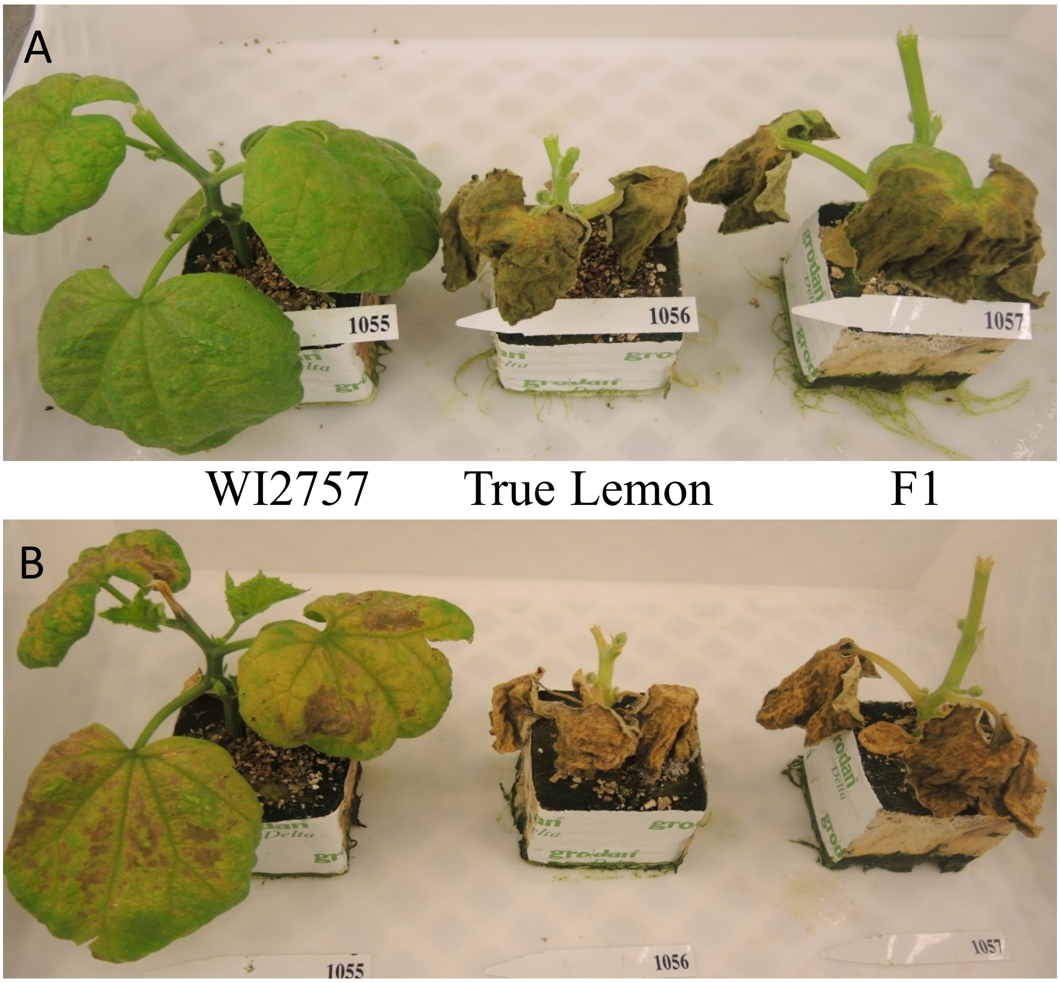


Figure 5.2 Phenotypic distribution of DM resistance in the Gy14 × 9930 RIL (G9RIL) population presented with violin plot and boxplot with mean disease scores from three rating times of different criteria in three environments.

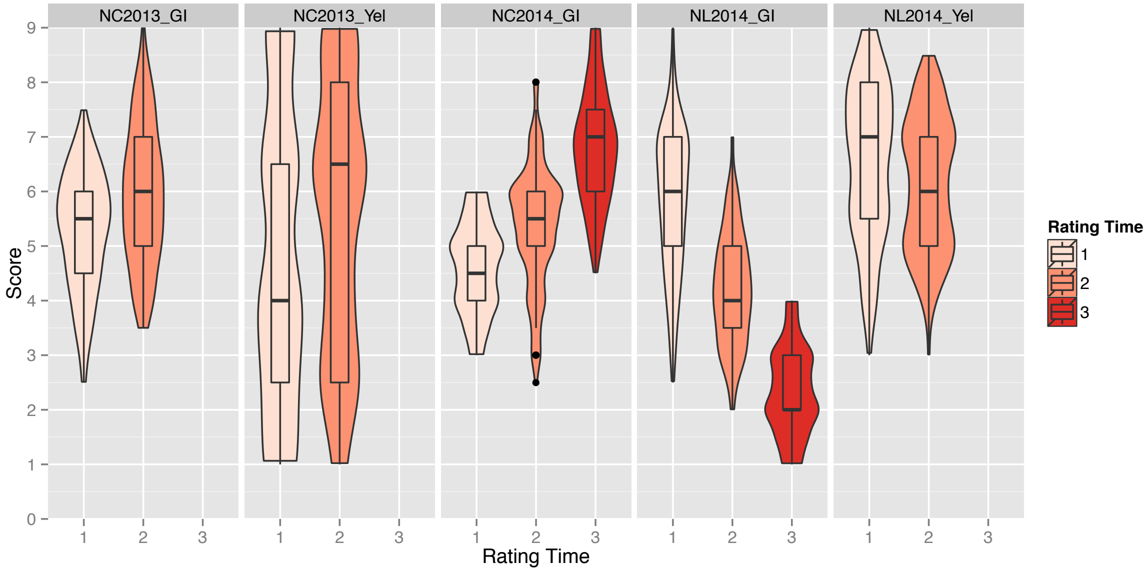


Figure 5.3 The phenotypic analysis of DM disease scores of Gy14 × 9930 RIL (G9RIL) population among different environments, rating criteria and rating times. The plots on the diagonal show the phenotypic distribution of DM scores. The values above the diagonal show the pairwise Spearman's rank correlation coefficients, and the plots below diagonal are scatter plot of compared phenotypes. *, *P< 0.05*; **, *P<0.01*; ***, *P<0.001*.

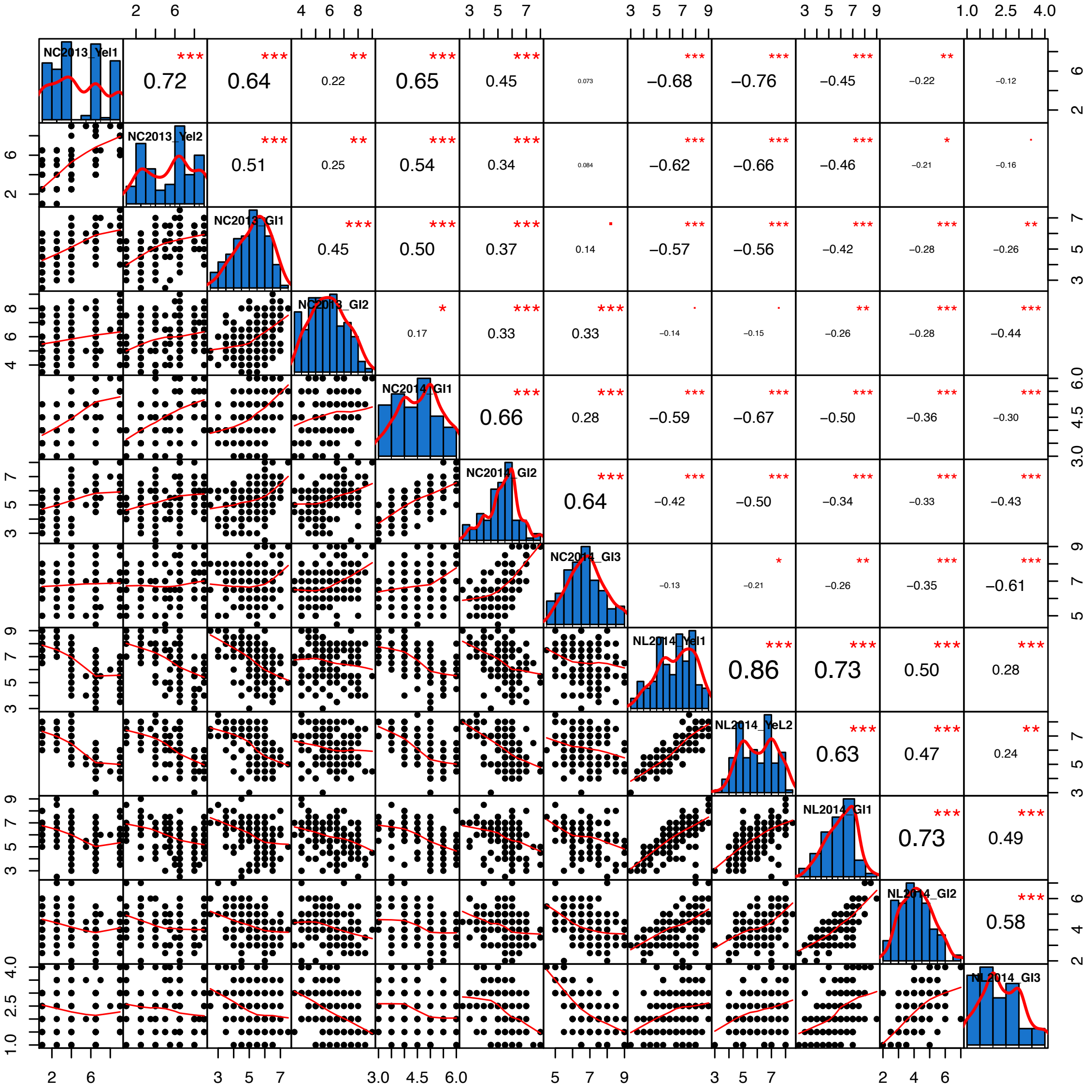


Figure 5.4 Violin plot and boxplot of mean disease scores from three environments upon DM inoculation in the WI2757 × True Lemon RIL (WTRIL) population.

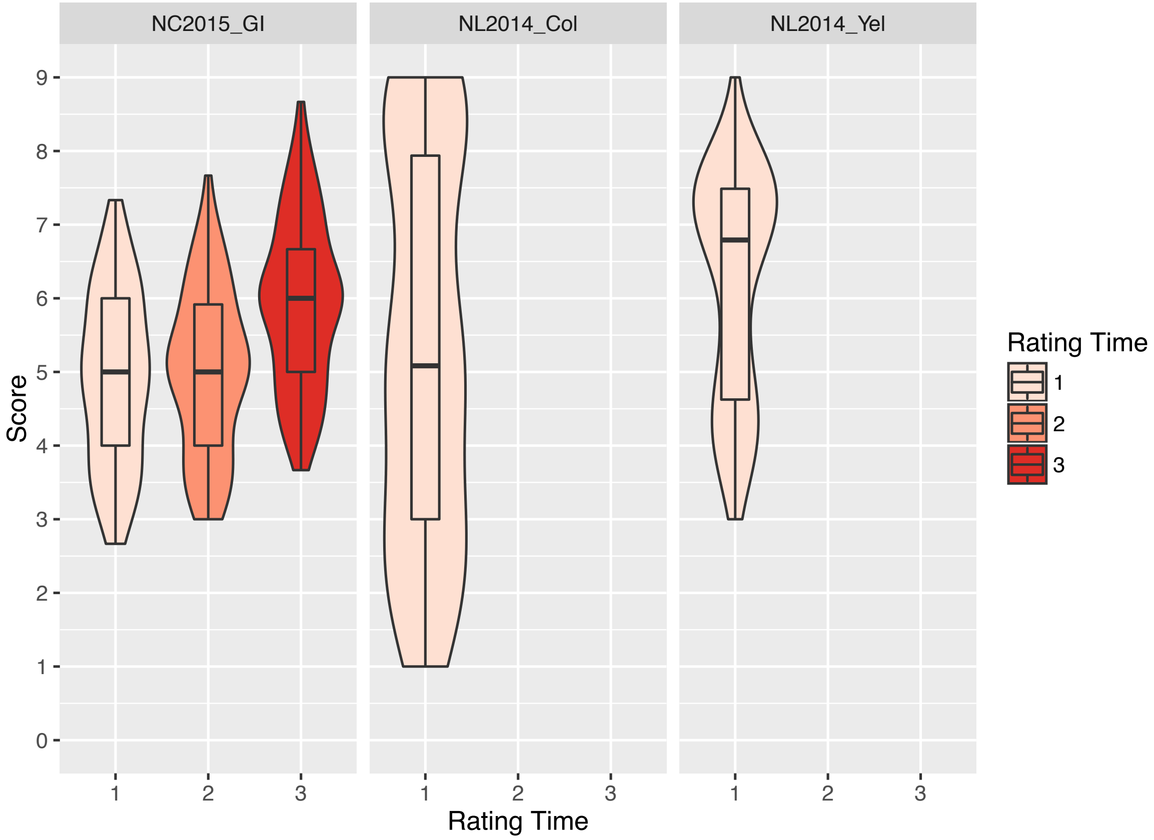


Figure 5.5 The phenotypic analysis of DM disease scores of WI2757 × True Lemon RIL (WTRIL) population among different environments, rating criteria and rating times. The plots on the diagonal show the phenotypic distribution of DM scores. The values above the diagonal show the pairwise Spearman's rank correlation coefficients, and the plots below diagonal are scatter plot of compared phenotypes. *, *P< 0.05*; **, *P<0.01*; ***, *P<0.001*.

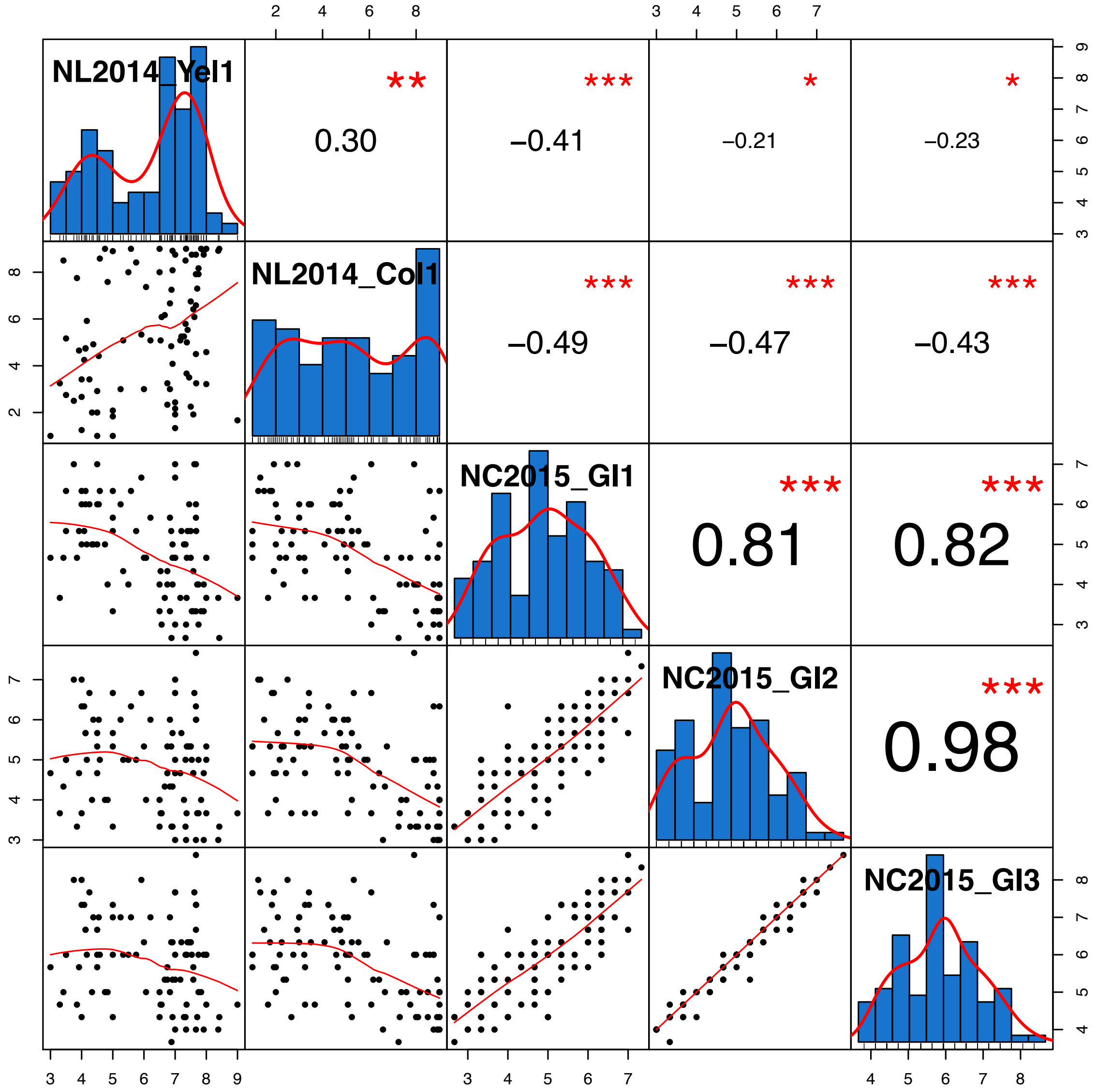


Figure 5.6 LOD profiles of DM resistance QTL on chromosome 5 detected with the G9RIL population from five environments with different rating rimes and criteria. The X-axis is the genetic map distance in cM of chromosome 5; the Y-axis is the LOD support score. YEL=yellowing symptoms with 1 and 2 represent the first rating and second rating, respectively; GI=General Impression.

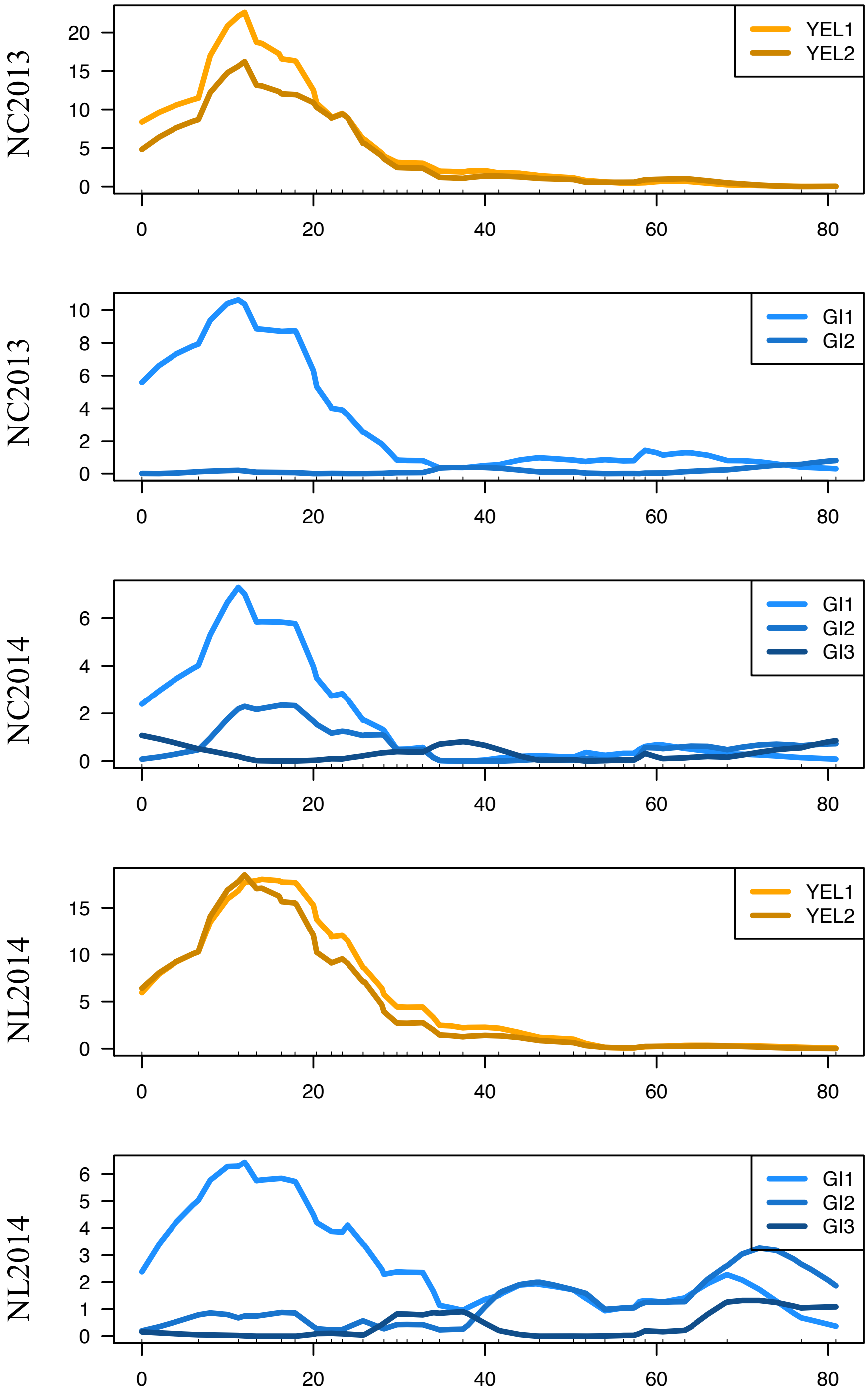


Figure 5.7 LOD profiles of DM resistance QTL on chromosome 5 detected with the WTRIL population from five environments with different rating rimes and criteria. The X-axis is the genetic map distance in cM of chromosome 5; the Y-axis is the LOD support score. YEL1=symptoms yellowing at the first rating; Col1=symptoms collapsing at the first rating time; GI=General Impression with 1 to 3 representing the first to third rating time, respectively.

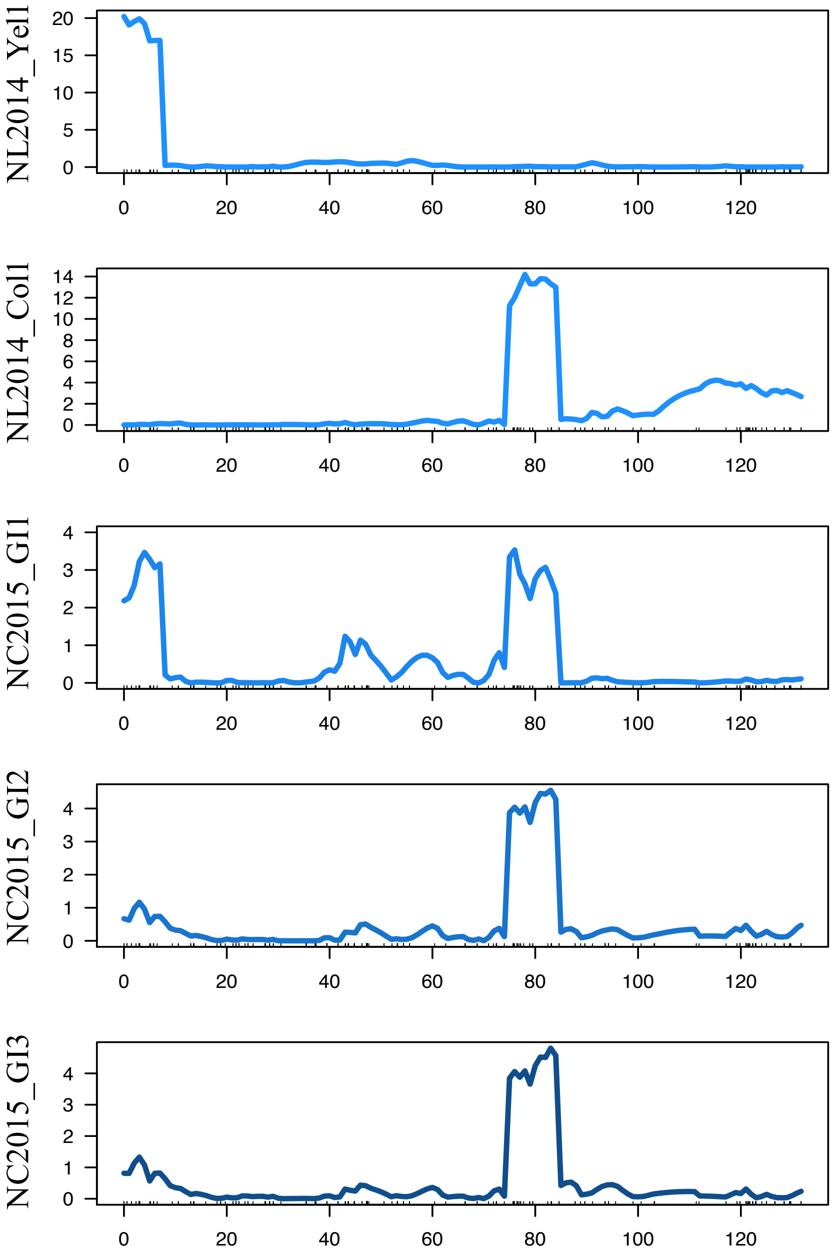


Figure 5.8 Fine mapping of *dm1* with 375 G9RIL population. Dark and white rectangle regions carry homozygous Gy14 and 9930 alleles, respectively.

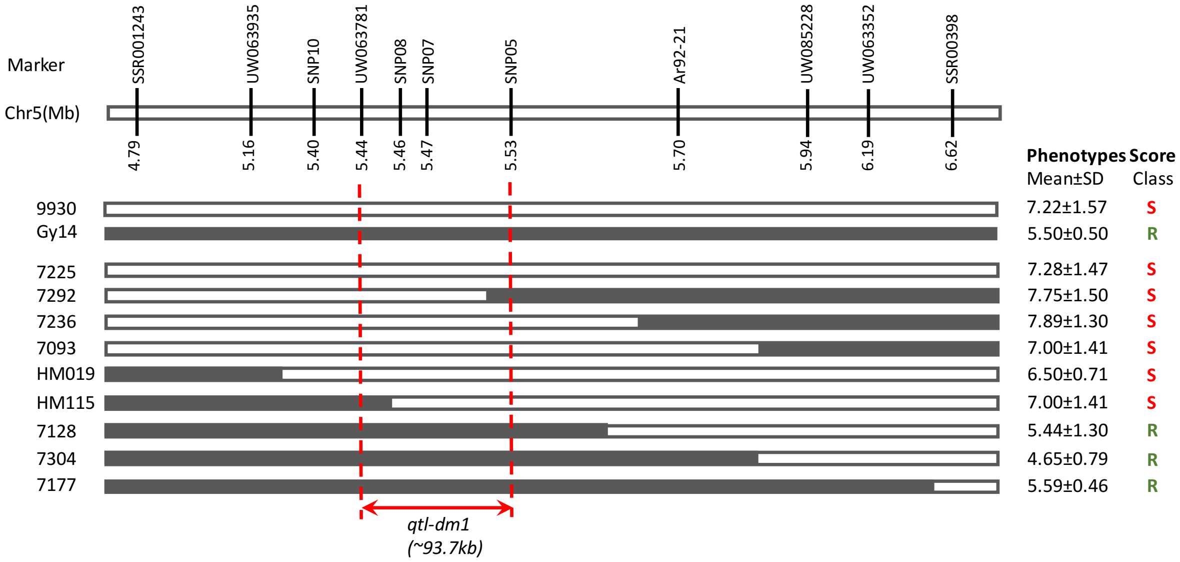
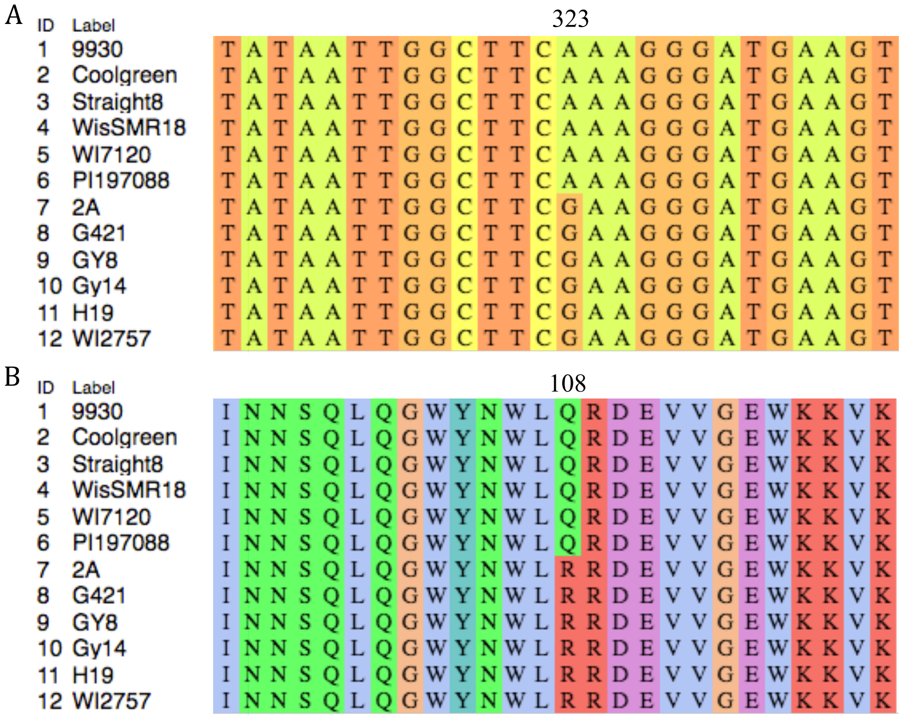


Figure 5.9 Alignment of genomic DNA (A) and deduced amino acid (B) sequences of the *dm1* candidate gene (*Csa5G156180*) from 12 cucumber lines. Among these lines, five carry the resistance allele (2A, G421, Gy8, Gy14, H19, and WI2757) and five carry the susceptible allele (9930, Coolgreen, Straight 8, Wis SMR18, WI7120, and PI 197088,).



A

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ID	Label	
1	9930	T A T A A T T G G C T T C A A A G G G A T G A A G T
2	Coolgreen	T A T A A T T G G C T T C A A A G G G A T G A A G T
3	Straight8	T A T A A T T G G C T T C A A A G G G A T G A A G T
4	WisSMR18	T A T A A T T G G C T T C A A A G G G A T G A A G T
5	WI7120	T A T A A T T G G C T T C A A A G G G A T G A A G T
6	PI197088	T A T A A T T G G C T T C A A A G G G A T G A A G T
7	2A	T A T A A T T G G C T T C G A A G G G A T G A A G T
8	G421	T A T A A T T G G C T T C G A A G G G A T G A A G T
9	GY8	T A T A A T T G G C T T C G A A G G G A T G A A G T
10	Gy14	T A T A A T T G G C T T C G A A G G G A T G A A G T
11	H19	T A T A A T T G G C T T C G A A G G G A T G A A G T
12	WI2757	T A T A A T T G G C T T C G A A G G G A T G A A G T

B

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ID	Label	
1	9930	I N N S Q L Q G W Y N W L Q R D E V V G E W K K V K
2	Coolgreen	I N N S Q L Q G W Y N W L Q R D E V V G E W K K V K
3	Straight8	I N N S Q L Q G W Y N W L Q R D E V V G E W K K V K
4	WisSMR18	I N N S Q L Q G W Y N W L Q R D E V V G E W K K V K
5	WI7120	I N N S Q L Q G W Y N W L Q R D E V V G E W K K V K
6	PI197088	I N N S Q L Q G W Y N W L Q R D E V V G E W K K V K
7	2A	I N N S Q L Q G W Y N W L R R D E V V G E W K K V K
8	G421	I N N S Q L Q G W Y N W L R R D E V V G E W K K V K
9	GY8	I N N S Q L Q G W Y N W L R R D E V V G E W K K V K
10	Gy14	I N N S Q L Q G W Y N W L R R D E V V G E W K K V K
11	H19	I N N S Q L Q G W Y N W L R R D E V V G E W K K V K
12	WI2757	I N N S Q L Q G W Y N W L R R D E V V G E W K K V K

Figure 5.10 Expression level of *CsSGR* candidate genes in Gy14, WI2757 and 9930 at different days post inoculation (dpi) (A) and the progress of symptoms on the first true leaves in the three cucumber lines (B). ** indicated significantly different expression *CsSGR* at $P <$ 0.01.

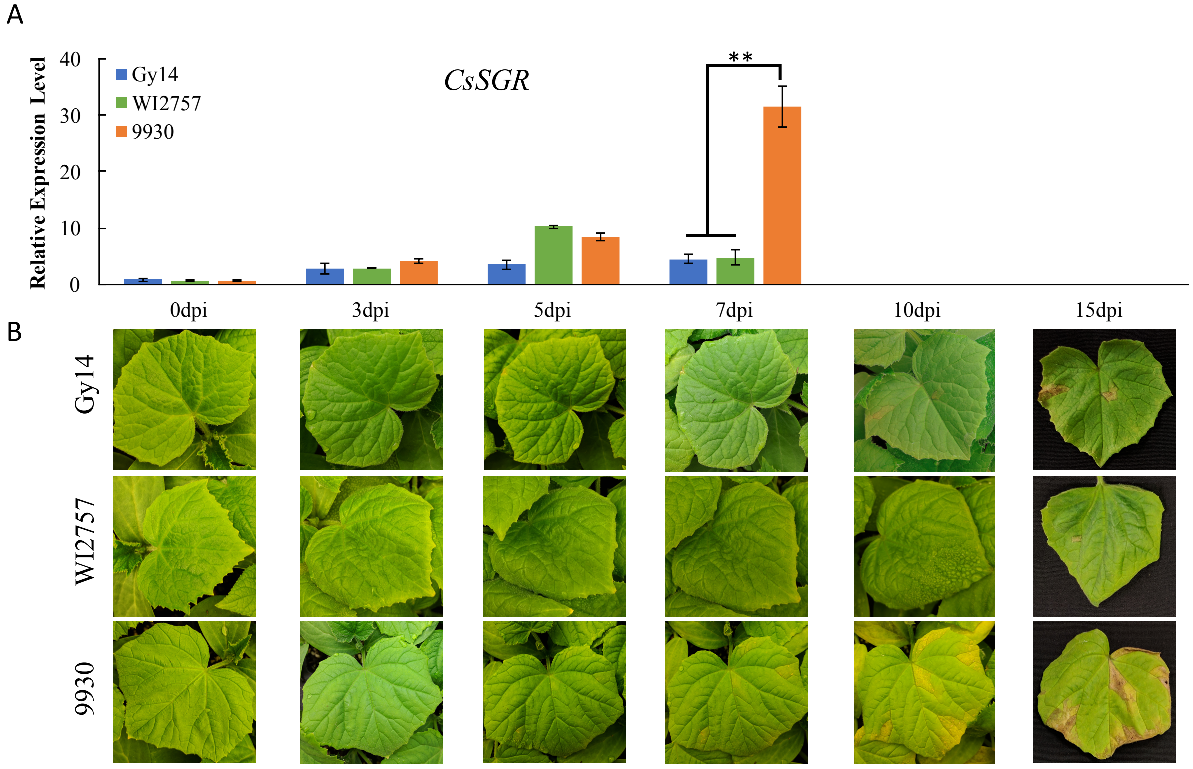


Figure.5.11 Phylogenetic relationships (A) and domain structure (B) of cucumber *CsSGR* with homologs in other plants species. Numbers at branch points represent bootstrap values of 1000 replicate trees. Blue line: Chloroplast transit peptides; Red line: Conserved SGR domain; Green line: variable C-terminal region; Red tangle: mutation aa in Gy14.

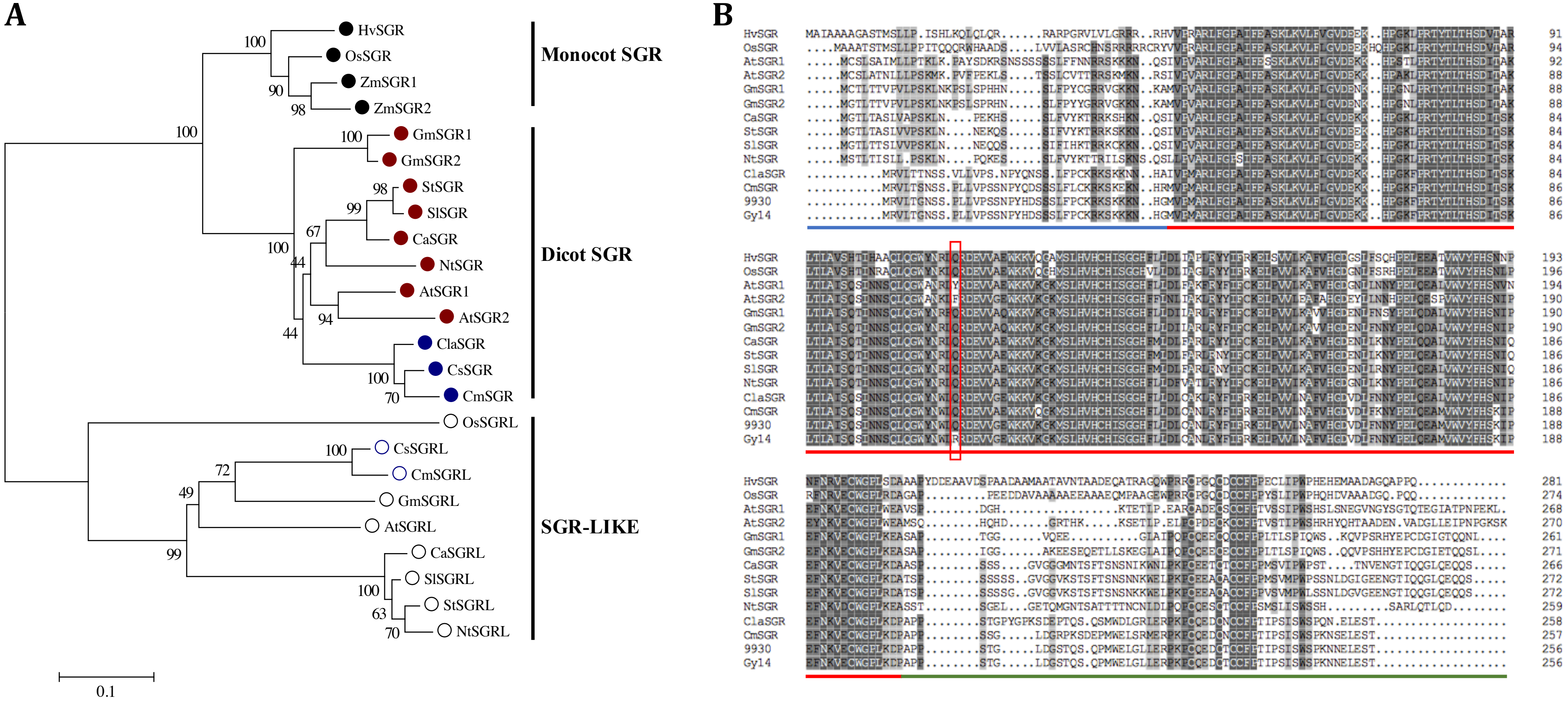


Figure 5.12 The predicted 3D model of SGR domain of *CsSGR* protein in 9930 and Gy14. The one amino acid substitution: Glutamine (Q) to Arginine (R) caused shorten β-fold sheet that connected to the α-helix which might influenced its proper function.

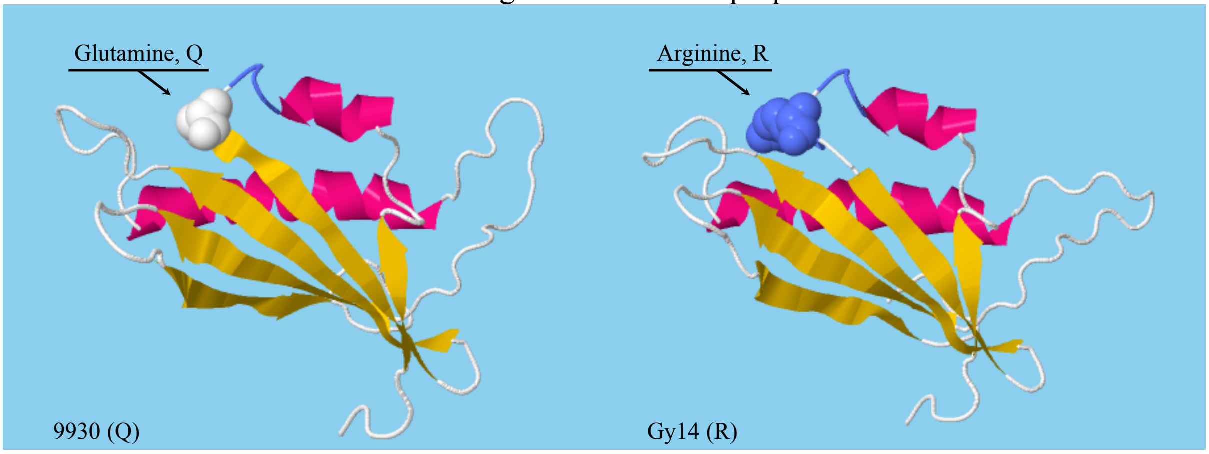
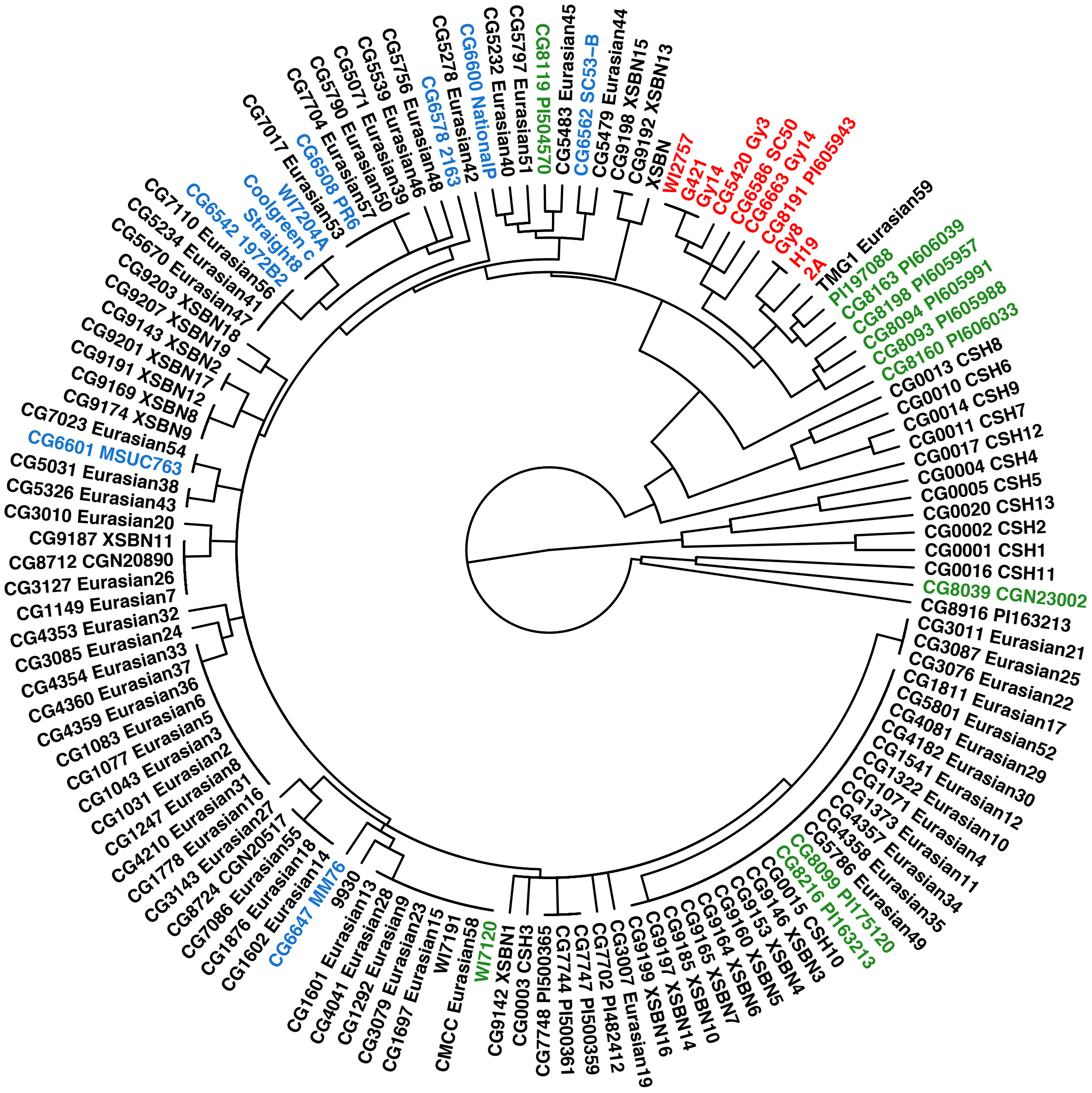


Figure 5.13 Dendrogram of 131 cucumber accessions from three cucumber taxa based on 186 SNPs within the 20-kb region of the *dm1* candidate gene (*CsGSR*) locus. XIS = semi-wild cucumber, *C. sativus* var. *xishuangbannanesis*; CSH = wild cucumber, *C. sativus* var. *hardwickii*. All others belong to cultivated cucumber, CSS (*C. sativus* var. *sativus*) from Asia and Europe (Eurasian). Accessions in red color were known to carry the *dm1* DM resistance gene; those in blue were developed in US but do not carry the *dm1* gene; accessions in green were of India origin.



Appendix 5.1 Cucumber materials used in the present study for phylogenetic analysis at *dm1* locus.
Appendix 5.1 is uploaded with the following link:
http://cranberry.vcru.wisc.edu/owncloud/index.php/s/n7BQMyGygF0FOLo

Appendix 5.2 The 1,618 SNP markers information in the linkage map of WTRIL, includes genetic map position and physical position in the 9930 draft genome assembly V2.0. Appendix 5.2 is uploaded with the following link: http://cranberry.vcru.wisc.edu/owncloud/index.php/s/ODNO5obORjW7hd6

Chapter 6: Marker-assisted pyramiding of downy mildew resistance QTL *dm4.1* and *dm5.1* into elite cucumber genetic backgrounds

Abstract

Cucurbit downy mildew (DM) is a serious threat to cucumber production in both greenhouse and field production around the world. In the United States, the genetic resistance that used for more than half century in most commercial cultivars was overcome by a new DM strain in 2004. Thus, new resistance cucumbers are in demands for sustainable cucumber production. Traditional breeding was laborious and less effective for developing cucumber lines with DM resistance under the control of quantitative trait loci (QTL). In the present study, marker-assisted backcrossing method was applied to pyramid DM resistance QTL into elite genetic backgrounds. Two major-effect QTL for DM resistance, *dm4.1* and *dm5.1* identified in WI7120, explained more than 50% of the phenotypic variance. They were successfully transferred and pyramided into Gy14, WI7204 and 9930 backgrounds, which represented three major cucumber market classes. DM resistance in backcross progeny was evaluated in growth chamber tests and the horticultural traits were assessed in replicated field trials.

Introduction

Downy mildew (DM) causes rapid foliage death on cucumber and is one of the major threats in greenhouse and open-field cucumber production in the United States. The effective protection of cucumber from *dm1* locus for more than 50 years was overcome by the new DM strain emerged in 2004. Currently, the management of cucumber DM relies mainly on timely use of fungicides. For the absence of fungicide application, cucumber yield loss could be up to 40 to 100%, leading to substantial economic losses (Holmes et al. 2006; Colucci 2008; Savory et al. 2011). *Pseudoperonospora cubensis* (*Ps. cubensis*) is the causal agent of DM on cucurbit crops and it is known to develop resistance to fungicides very rapidly (Holmes et al. 2015). Hausbeck (2014) reported that commonly sprayed fungicides are no longer working well in Michigan cucumber production from the last several years. Furthermore, Call et al. (2012) found no commercial cultivars with DM resistance that was comparable to the pre-2004 level. Therefore, the development and deployment of durable and high-level host resistance is the most efficient, economical and environmentally friendly approach to protect the cucumber against DM.

Gene pyramiding for a specific trait, aimed at accumulating multiple desirable genes into a single genetic background, is a widely-employed method for host disease resistance breeding. It is an important strategy to prevent or delay the breakdown of resistance because the probability of simultaneous pathogen mutations for virulence to defeat two or more effective genes is lower than for a single one (Mundt 1990). In some cereal and vegetable crops, gene pyramiding has been applied in transferring multiple genes for qualitative and quantitative disease resistance, such as bacterial blight and blast resistances in rice (Hittalmani et al. 2000; Fukuoka et al. 2015), powdery mildew resistance in wheat (Liu et al. 2000), as well as root-knot nematode resistance in pepper (Djian-Caporalino et al. 2014). However, the conventional gene introgression or R-gene pyramiding for disease resistance breeding is a slow and uncertain process, especially when more than one locus confers resistance against diseases. In cucumber, the development of cucumbers with high level of DM resistance is still a time-consuming process, which may take more than five years (Holdsworth et al. 2014). Marker-assisted selection (MAS) is an effective and efficient approach that offers rapid and precise transfer of the target genes even at very early stage during gene pyramiding. Therefore, to accelerate the breeding process, MAS was integrated in gene pyramiding to develop high level of DM resistance cucumber in the present study.

Several recent studies identified at least 13 QTL conferring DM resistance on seven chromosomes from different sources including PI 197088, PI 197085, PI 330628, and K8 (Zhang et al. 2013; Yoshioka et al. 2014; Szczechura et al. 2015; Wang et al. 2016; Chapter 3). Among the DM resistant sources, WI7120 (PI 330628) and PI 197088 are the two main sources of DM resistance against the post-2004 DM strains in the US. They shared at least four QTL (*dm2.1*, *dm4.1*, *dm5.1/dm5.2*, and *dm6.2*) but with different magnitudes of effects on DM resistance (**Table 6.1;** Wang et al. 2016; Chapter 3). Although PI 197088 is being used widely in commercial breeding programs, its resistance does not hold at late development stage in the field due probably to the three DM susceptible QTL (VandenLangenberg and Wehner 2016; Chapter 3). In contrast, WI7120 has the ability to hold long-term DM resistance even in late growth stage, which is a preferable source of DM resistance (VandenLangenberg and Wehner 2016). Therefore, WI7120 would be a preferable source of DM resistance over PI 197088 for cucumber breeding. Further, the QTL *dm 4.1* and *dm5.1* from WI7120 also present in several other resistant sources, and each of them has been mapped to a relatively small region (Chapter 4), which may improve the accuracy of MAS in QTL pyramiding. The main objective of this study was to pyramid the two DM resistance QTL *dm4.1* and *dm5.1* from WI 7120 (PI 330628), through MAS in three elite commercial inbred lines Gy14, WI7204, and 9930, which belong to the US pickling, beit alpha, and the north China fresh market cucumber, respectively.

Materials and Methods

Plant materials

Gy14, WI 7204 and 9930 are elite cucumber lines that representing three major cucumber market types: the North American pickling type cucumber, the beit alpha greenhouse cucumber (Mediterranean type, or mini cucumber), and the Northern China fresh market type cucumber (Chinese Long), respectively. They have excellent horticultural traits for the target markets, but are moderately resistance (Gy14) or susceptible (9930 and WI7204) to the infection by the post-2004 DM strain. These three lines were used as the recipients and recurrent parents. The cucumber landrace WI7120 (PI 330628) with high level DM resistance to the post-2004 field strain was used as the donor parent. The two QTL *dm4.1* and *dm5.1* from WI7120 were the targets for pyramiding into Gy14, WI 7204, and 9930 backgrounds. Both MAS and morphology trait selection were conducted during gene pyramiding. The schemes are outlined in **Figure 6.1**.

Greenhouse conditions and plant maintenance

Plants of all relevant materials were grown at the University of Wisconsin Walnut Street Research Greenhouses (WSGH) located at Madison, WI. Plants were grown in Classic 1000 plastic pots ($20.6 \times 25 \times 23.2$ cm^3) with PRO-MIX HP BIOFUNGICIDE + MYCORRHIZAE Growing Medium (Premier Tech Ltd, Québec, Canada) at 25-27°C day/20-22°C night air temperatures and supplemental lighting. Plants were fertilized with 380 ppm of 20N-10P-20K Peters Professional fertilizer twice per week. Cucumber fruits were harvested 35 to 40 days after pollination. Cucumber seeds were either stored at least 4 weeks after harvest or pre-treated with 1% of hydrogen peroxide (H_2O_2) for 3 hours before planting to break the dormancy and ensure uniform germination.

Development of molecular markers for MAS

Cucumber SSR and INDEL markers described by Ren et al. (2009), Cavagnaro et al. (2010), Yang et al. (2012, 2013), and Chapter 4 were used for polymorphism screening between the donor parent (WI 7120) and the recurrent parents (Gy14 or WI 7204). Polymorphic markers were aligned to the 9930 draft genome assembly V2.0 (Huang et al. 2009) for their physical positions. Certain polymorphic markers which covered the 2.0 LOD interval of candidate QTL region were selected and used for further MAS. The molecular markers for breeding lines with 9930 background were directly selected from Chapter 4 within the candidate region for each QTL.

DNA extraction and PCR

Genomic DNA was isolated from the cotyledon of one-week old seedlings. Tissue samples were lyophilized in freeze-dryer and ground into fine powder with a high-throughput homogenizer (OPS Diagnostics, Lebanon, NJ). Genomic DNA was extracted using the cetyltrimethyl ammonium bromide (CTAB) method (Murray and Thompson 1980) and purified with phenol/chloroform/isoamyl alcohol. The DNA concentration was determined using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Tech, Wilmington, DE) and adjusted to a concentration of 50 ng μL^{-1}.

All polymerase chain reactions (PCR) were performed using an Applied Biosystems 2720 thermal cycler (Applied Biosystems, Foster City, CA). Each PCR was performed in a 10 µL-volume reaction that consisted of 1 µL of diluted DNA (50 ng μL^{-1}), 1 µL of 10 × DreamTaq Buffer (Thermo Fisher Scientific Inc., Waltham, MA), 1 µL of 2.5µM forward and reverse primers, 0.2 μL of 10 μM dNTPs (Bioline USA Inc, Taunton, MA), 0.05 μL of DreamTaq polymerase (5U μL^{-1}) (Thermo Fisher Scientific Inc., Waltham, MA), and 5.7 μL of ddH_2O. A modified touchdown PCR program described in Weng et al. (2005) was utilized for all primer sets as follows: 3 minutes initial denaturation at 94°C; six cycles of 20 seconds at 94°C for denaturation, 20s at 68°C for annealing, 20s at 72°C for extension, with the annealing temperature being reduced by 2°C per cycle; eight cycles of 20s at 94°C, 20s at 58°C, 20s at 72°C, with the annealing temperature being reduced by 1°C per cycle; a 22 cycles of 20s at 94°C, 20s at 50°C, 20s at 72°C; a final extension at 72°C for 5 min.

PCR based marker visualization

Denaturing polyacrylamide-gel electrophoresis (PAGE) was used to separate PCR amplicons. A 5.7% denaturing polyacrylamide gel was prepared from 300 mL of 28.6% acrylamide stock solution (500 g acrylamide and 17.2 g bis-acrylamide for a final volume of 1.75 L) and 200mL of 5×Tris-Borate-EDTA buffer stock solution for a final volume of 1,000 mL. The 106-well combs were applied during gel solidifying (Jingke Weiye Lab Equipment Co. Ltd, Beijing, China). Only 1.2-1.5 μL of PCR products were loaded and run at 170V for 1.5 hours.

After electrophoresis, the gels were visualized with silver staining. Briefly, the gel was detached from the glass plate and stained in the staining solution (0.1% (w/v) $AgNO_3$) for 12 min. After one min rinse in ddH_2O, the gel was transferred into the developing solution [15% (w/v) Na_2CO_3, 0.15% (v/v) of 37% formaldehyde] for 5 to 10 min. The reaction was stopped by rinsing in regular water two to three times. Banding patterns were scored manually and digital photographs were produced for permanent record.

Evaluation of resistance to Ps. cubensis and other horticultural traits

The cucumber seedlings from BC_2 and later generations in Gy14 and WI7204 backgrounds were evaluated for DM resistance in growth chambers in Horticulture Department in Madison, which is as same as described in Chapter 4.

Horticultural traits were evaluated in field trials performed at the University of Wisconsin Hancock Agricultural Research Station in 2016. The following plant materials were included: recurrent parents Gy14, WI 7204 and 9930; donor parent WI7120; BC_2 breeding lines. The experiments were conducted with two replications of twenty plants per replication. Plants were grown on plastic beds with 2.5-foot spacing. Fertilizers were incorporated before planting with 126 kg ha^{-1} of ESN-44-0-0 Slow Release Nitrogen (Agrium Inc. Marston, MO).

Results

Polymorphic marker development

From QTL mapping in the WI7120 × 9930 population, the 2.0-LOD interval for both *dm4.1* and *dm5.1* were estimated to be 5 Mb in the 9930 draft genome (Wang et al. 2016). Two hundred and sixty-four SSR/INDEL markers spanning both regions were selected for polymorphism screening among Gy14, WI7204 and WI7120. Eventually, 7 and 16 SSR/INDEL markers located respectively at the *dm4.1* and *dm5.1* loci were identified, which were polymorphic between both recipient/donor pairs (Gy14/WI7120 and WI7204/WI7120) (**Table 6.2**). In Chapter 4, QTL *dm4.1* and *dm5.1* were narrowed to a region of less than 500 kb. Thus, those polymorphic INDELs developed between WI7120 and 9930 were also tested in each pair of Gy14/WI7120 and WI7204/WI7120. Additional 4 and 3 INDELs were found closely linked with *dm4.1* and *dm5.1*, respectively. These markers were all applied for MAS during cucumber DM resistant breeding while using Gy14 or WI7204 as recurrent parents.

Pyramiding DM resistance QTL through MAS in Gy14

Gy14 is a gynoecious inbred line with excellent horticultural characteristics, which is a representative of the US pickling cucumber market type. Gy14 carries *dm1* gene derived from PI 197087 which exhibits moderate resistance to prevailing DM strains in the field. The addition of *dm4.1* and *dm5.1* from WI7120 to Gy14 may allow the Gy14 cucumber to achieve higher resistance level against the DM. The F_1 plant of Gy14 × WI7120 was selfed to generate the F_2 generation. The pedigree of breeding scheme is shown in **Figure 6.1A**. Two pairs of SSR markers (SSR04649/CsDm4-008 for *dm4.1* and UW053068/SSR15321 for *dm5.1*) were selected to screen for the donor fragments of WI7120 in 24 F_2 plants. As a result, 5 plants carrying WI7120 alleles at both loci were selected for backcrossing with Gy14 to obtain BC_1 seeds. The growth habits, fruit and seed characteristics of F_2 plants were compared with those of Gy14, and the ones sharing the most characteristics with Gy14 were selected to advance to next generation. Twelve BC_1 plants were grown in the growth chamber for DM resistance test and tracked with the *dm4.1* and *dm5.1* by the same set of markers. Among them, three seedlings showed moderate resistance to DM and carried both *dm4.1* and *dm5.1* alleles from WI7120, which were kept for backcrossing with Gy14 to obtain BC_2 seeds. One of the three BC_1 plants had the following traits: gynoecious sex expression, white spine color, intermediate fruit size, and smooth fruit skin, which was very similar to Gy14. Thus, the BC_2 seeds from this BC_1 plant were kept for genotyping and backcrossed with Gy14 again to obtain BC_3 seeds. At this generation, more closely linked markers were developed in fine-mapping of *dm4.1* and *dm5.1* (Chpater 4). Therefore, a new set of INDEL markers flanking ~200kb of *dm4.1* (CsDm4-066 and CsDm4-072) and *dm5.1* (CsDm5-049 and CsDm5-023) were chosen to genotype 22 BC_2 plants for MAS. As shown in **Figure 6.2**, plants No. 2, 12 and 20 carried WI7120 alleles at both *dm4.1* and *dm5.1* loci, while plants

No. 5 and No. 8 carried only *dm4.1* and *dm5.1*, respectively. These seedlings were transplanted in WSGH for backcrossing and self-pollination to generate BC_3F_1 and BC_2F_2.

Pyramiding DM resistance through MAS in WI7204

The F_1 plant of WI7204 × WI7120 was crossed with PI 197088 to generate a three-way cross F_1. Two pairs of SSR markers (SSR04649/CsDm4-008 for *dm4.1*, UW053068/SSR15321 for *dm5.1*) were selected to examine the presence of the donor fragments of WI7120 in 48 three-way cross F_1 plants. The selection scheme is shown in **Figure 6.1B**. As a result, five plants carrying WI7120 alleles at both loci were selected for backcrossing with WI7204 to obtain BC_1 seeds. The plant growth habit, fruit and seed characters of the 3-way cross F_1 plants were compared with those of WI7204. Plants sharing the more characteristics with WI7204 were selected to advance to the next generation. Twelve BC_1 plants were tested in the growth chamber for DM resistance and at the same time genotyped with markers for *dm4.1* and *dm5.1* using the same set of SSR markers. Among them, three seedlings showed moderate resistance to DM and carried both *dm4.1* and *dm5.1* alleles from WI7120, which were transplanted in WSGH for backcrossing with WI7204 to obtain BC_2 seeds. One of the three BC_1 plants exhibited gynoecious sex expression with dark-green immature fruit skin color, set long, smooth and glossy, white-spined fruits that was very similar to WI7204. Thus, the seeds from this plant were used to advance to next generation. Again, at his generation, due to progress made in fine mapping of the *dm4.1* and *dm5.1* QTL (Chapter 4), a new set of more closely linked INDEL markers in ~200kb regions of *dm4.1* (CsDm4-047 and CsDm4-072) and *dm5.1* (CsDm5-049 and CsDm5-074) were used to genotype 21 BC_2 plants for MAS. As shown in **Figure 6.3**, the individuals No. 4 carried alleles of two QTL from WI7120 was resistant to DM, while No. 2 did not carry any WI7120 alleles in the two regions was highly susceptible to DM under growth chamber tests. Finally, plants No. 4, 17 and 20 that carried WI7120 alleles of both QTL, and plants No. 11 and No. 1 carried only *dm4.1* and *dm5.1*, respectively, were transplanted for backcrossing and self-pollination to generate BC_3F_1 and BC_2F_2.

Pyramiding DM resistance through MAS in 9930

Two near-isogenic lines (NILs) were developed for *dm4.1* and *dm5.1* in the 9930 background (Chapter 4). Growth chamber tests and field evaluations revealed that the DM resistance conferred by each single QTL was incomplete or moderate. Thus, pyramiding both QTL into one genetic background will enhance the DM resistance in 9930. The NIL plants homozygous for either *dm4.1* or *dm5.1* were selected from BC_3F_3 families and crossed with each other to obtain F_1 that carried both resistant loci. The F_1 plants were self-pollinated to create segregating F_2 generation. Two sets of INDEL markers that closely-linked with *dm4.1* (CsDm4-068 and CsDm4-072) and *dm5.1* (CsDm5-049 and CsDm5-074) (Chapter 4) were used to genotype 70 F_2 plants. Among them, four F_2 plants fixed for both QTL (homozygous) were self-pollinated to obtain homozygous F_3 seeds for further field evaluation (**Figure 6.1C**).

Horticultural trait evaluation

For each Gy14 and WI7204 backcrossing line, 40 BC_2 seeds were planted with two replications in the University of Wisconsin Hancock Agricultural Research Station in the summer of 2016. These plants were evaluated for several important horticultural traits and compared with their recurrent parents. No obvious late flowering time and poor plant growth vigor was observed. As shown in **Figure 6.4A-B**, the fruits of BC_2 plants were very similar to those of recurrent parents (Gy14 and WI7204) from 0 to 15 day post flowering (dpf) at the typical commercial harvest date. It is worthy to note that no obvious linkage drag of the introgressed chromatins was observed among these introgression lines.

The plants morphology, fruits shape and fruit-set habits from F_2 plants in 9930 background were also assessed at 15 and 30 day post pollination (dpp) in WSGH during seed increase. As shown in **Figure 6.4C**, the F_2 fruits were nearly identical to their recurrent parent 9930.

Discussion

In traditional cucumber breeding practice, the development of high level of DM resistant cucumber breeding lines was a time-consuming process, which usually takes more than five years (Holdsworth et al. 2014). To improve the selection efficiency, in the present study, we practiced both MAS and phenotypic selection during marker-assisted pyramiding of *dm4.1* and *dm5.1* in three genetic backgrounds. It allowed very fast recovery of the genetic background of the recurrent parent and at the same time transfer the target QTL. As shown in **Figure 6.3** and **Figure 6.4**, in BC_2 generation, the plants showed moderate resistance to DM in a heterozygous state, and their fruits already resembled to their recurrent parents. During the developing process, the selection was carried out at the seedling stage with molecular markers, which was goal-oriented and allowed to save growing space. Furthermore, only a small number of seedlings was genotyped and even few were advanced to next generation. It demonstrated the efficiency of MAS, which does not require a large pool of plants for selection as compared to conventional breeding.

More importantly, as the progress made in fine-mapping the QTL *dm4.1* and *dm5.1*, more closely linked molecular markers were identified and used for MAS in the later generations (as described in Chapter 4). In both cases of Gy14 and WI7204, the target donor fragments were still presented when using the new set of markers which proved that using further-away molecular markers in MAS was effective as well. This work demonstrated that when very closely linked markers to the target gene/QTL are available, markers defining the 2.0 LOD interval are still efficient for marker-assisted selection.

In the present study, we obtained the BC_2F_2 and BC_3F_1 breeding lines with Gy14 and WI7204 background. Further work is still needed to identify the homozygous plants with fixed *dm4.1* and *dm5.1* alleles from WI7120, and to evaluate their potential value for commercial uses. The assessment includes but not limited to DM resistance and yield-related traits such as flowering time, fruit numbers, fruit weight, and fruit length/width ratio.

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Table 6.1 Comparison of DM resistant QTL mapped in WI7120 and PI 197088.

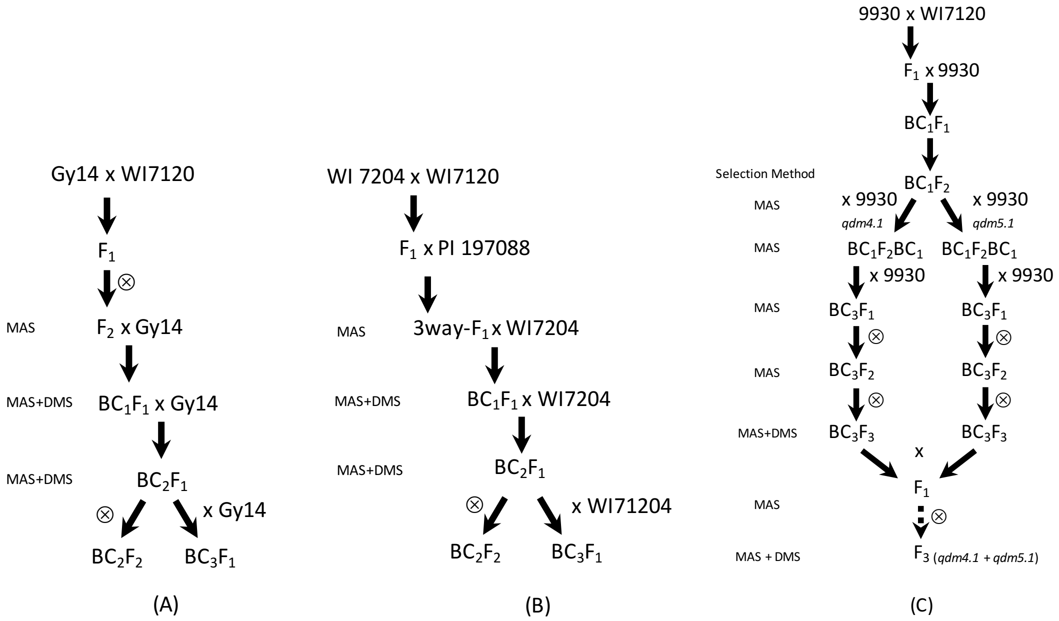
Lines	**WI7120**		**PI197088**	
QTL name	**Peak Maker**	**1.5Lod Interval (Mb)**	**Peak Maker**	**1.5Lod Interval (Mb)**
dm1.1	n.a*	n.a	Marker1_16905088	15.70-26.47
dm2.1	CHXSSR00134	5.77-11.67**	Marker2_8521946	7.98-10.79
dm2.2	n.a	n.a	Marker2_20890971	19.52-21.50
dm3.1	n.a	n.a	Marker3_9882197	9.15-1024
dm3.2	n.a	n.a	Marker3_37674467	36.70-39.67
dm4.1	UW059163	14.52-15.72	SSR17911	14.38-16.17
dm5.1	n.a	n.a	Marker5_64722	8.50-11.39
dm5.2	SSR15321	13.47-17.95	Marker5_16754619	16.44-18.14
dm5.3	n.a	n.a	Marker5_25566302	24.91-26.22
dm6.1	n.a	n.a	Marker6_5079443	3.68-5.86
dm6.2-chl	UW000765	14.99-21.03	n.a	n.a
dm6.2	SSR18651	25.95-28.09	Marker6_26132968	24.23-27.97

Note: *n.a: not applied; **Physical position range in the 9930 draft genome assembly v2.0.

Table 6.2 List of polymorphic markers among WI7120, Gy14 and WI 7204 that are located within 2.0 LOD intervals of DM resistance QTL *dm4.1* and *dm5.1*, and were used in marker-assisted pyramiding.

QTL	**Marker Name**	**9930 v2.0 Chr**	**9930 v2.0 position**	**Left Primer**	**Right Primer**
dm4.1	SSR05125	4	13529802	TGGCTCCTTCACATTGTTGT	TGGGAAAAAGGGTATGGAAA
dm4.1	UW085410	4	14562700	CAAACATGGGTTTGGCAAG	CATTATTGGAGAGGTGATAATTGG
dm4.1	UW084598	4	14874712	TACCTCCATGCTCCATCACA	TGGTGAAGTTAAAGGGTAAATCG
dm4.1	SSR04649	4	14904229	TTTGAAATTGATGACATCCCA	ACATGGAGGAAGACAGGCAC
dm4.1	UW084014	4	15057482	TGTGGACCCACAACTTTtAT	TGGTTTTTGACCAATTTACC
dm4.1	CsDm4-066	4	15421000	GAGGGAGTTGGTCACTCCAA	AAAAGGCATTATGAGCCTGA
dm4.1	CsDm4-047	4	15514106	TTATTTTCACGATCCCCAAT	CGCTCTAACCTCCAAAACTG
dm4.1	CsDm4-072	4	15532410	TTGCAAGAGCTTTGATTGGA	GGTGAGGCGTCGGTAATAGA
dm4.1	CsDm4-007	4	15641896	AGTTGGACCTTCTTGCCAAA	AAAGCAAAACAGCACAACCA
dm4.1	CsDm4-008	4	15653384	AAAGATGCTGGTAGTGGTGGA	CTTGGGTTAAGTGGGTGCTT
dm4.1	CsDm4-069	4	15718850	GTGGTCTTGTGGGACAATTT	AACCTAACCTACGCGTCAAC
dm5.1	UW002466	5	13463483	TGGAACCCAGAATCTAGCCTT	GCCTACAATGACACACCAACC
dm5.1	UW002465	5	13468929	TCAATGAAGTGGAAGTGACACA	CAGCAGGCCCTCTTTAGTTG
dm5.1	UW053011	5	13885458	ACGTAATTTTGGCTTGCACc	AACAAGAAACGGCCAAAATG
dm5.1	UW053068	5	14033768	GTCCTCTCTTCTCCAGTCGC	TAGGGGaaAACGGATTTCAA
dm5.1	SSR13420	5	14077578	GGGTTTGGGTTGGTTTAGGT	CTTTCATCACACCCTCCTCC
dm5.1	UW085348	5	14625312	GGATGGCCAACATAATAGTCC	AAATATTTGATGTGAGGTTGAGG
dm5.1	SSR23132	5	15530725	GCCCTATCACTTCTTTGTTTTCA	CAAGAAATGGGGTGGAAGAA
dm5.1	UW083828	5	15749559	AAGAATGATTGTAGTGCTGGTATG	CGAAGGGGTTTTTCAACTCTT
dm5.1	UW084561	5	16100650	CATTTGTTGCAAGTGAAGCTG	AAGACTCTACACACCAAACACA
dm5.1	CsDm5-049	5	16468212	TTGCAACAGAACCCTAAACC	CATTTGATTTGCAAGCACAC
dm5.1	CsDM5-074	5	16726700	TTCAAAGTGTAGAAGCATACGTTTT	TATTGGCCTGAACGAATGTG
dm5.1	UW058914	5	16889680	TTGTTATACCACATATTGTTGATGGA	AAGCATGAGGGGGAgACATA
dm5.1	CsDm5-023	5	16927127	GGTGGAGCAGAGATGGAGAC	CGTCAATTTGCGATTTCTTA
dm5.1	UW058982	5	17062353	AACGACACACACCAAACCAA	TCATCTCCCTTACTCCCATCA
dm5.1	UW084654	5	17359033	GAAAACAGATTGATTGGTATCATTG	CAAAGCAAGAAGTTTGGAGGT
dm5.1	SSR15321	5	17443753	TCAATGTAGGTAGAGCACCACG	TCCAATTGCTTGACCAATGA
dm5.1	UW000189	5	17660713	CCTTGAAAATTATTCGGCCA	TGACAAGCTCGATTCAAACGTA
dm5.1	UW083851	5	17875135	CGGAGGCTTTGATTTTGGTA	CAAACACCCCCAAATTCATC
dm5.1	UW001370	5	17954417	GCACAACAACAATCCATCCA	TTGGAAATTGAGCCAAGACC

Figure 6.1 Schemes for marker-assisted pyramiding of dm4.1 and *dm5.1* QTL in Gy14 (A), WI7204 (B), and 9930 (C) backgrounds. MAS: Marker Assisted Selection. DMS: Downy Mildew Resistance Selection.



(A) (B) (C)

Figure 6.2 Marker assistant selection (MAS) for 22 individuals from BC_2F_1 families with Gy14 background by flanking markers of *dm4.1* (CsDm4-066 and CsDm4-072) and *dm5.1* (CsDm5-049 and CsDm5-023). Plants carrying *dm4.1* and *dm5.1* DM resistance alleles from WI7120 are highlighted with red rectangle. Plants carrying single QTL DM resistance allele are highlighted with green rectangle.

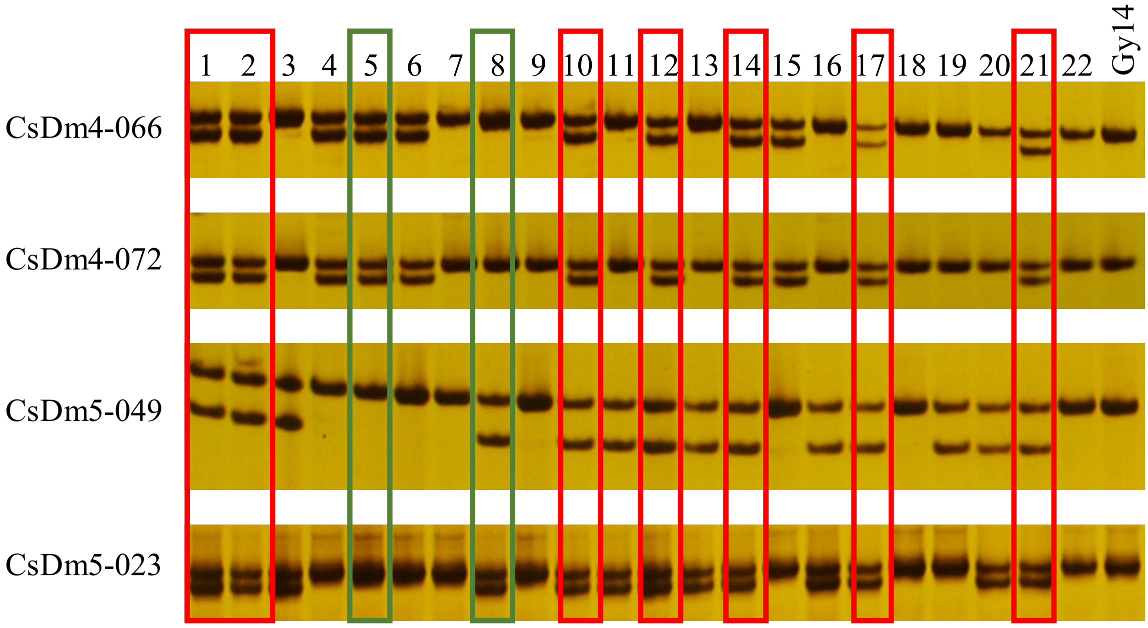


Figure 6.3 Marker assistant selection (MAS) for 21 individuals from BC_2F_1 families with WI7204 background. A) Gel images of each individual that was genotyped with flanking markers of *dm4.1* (CsDm4-072 and CsDm4-047) and *dm5.1* (CsDm5-049 and CsDm5-074). Plants carrying *dm4.1* and *dm5.1* DM resistance alleles from WI7120 are highlighted with red rectangle. Plants carrying single QTL DM resistance allele are highlighted with green rectangle. B) and C) The phenotypes of DM resistant individual No.4 and susceptible plant No.2.

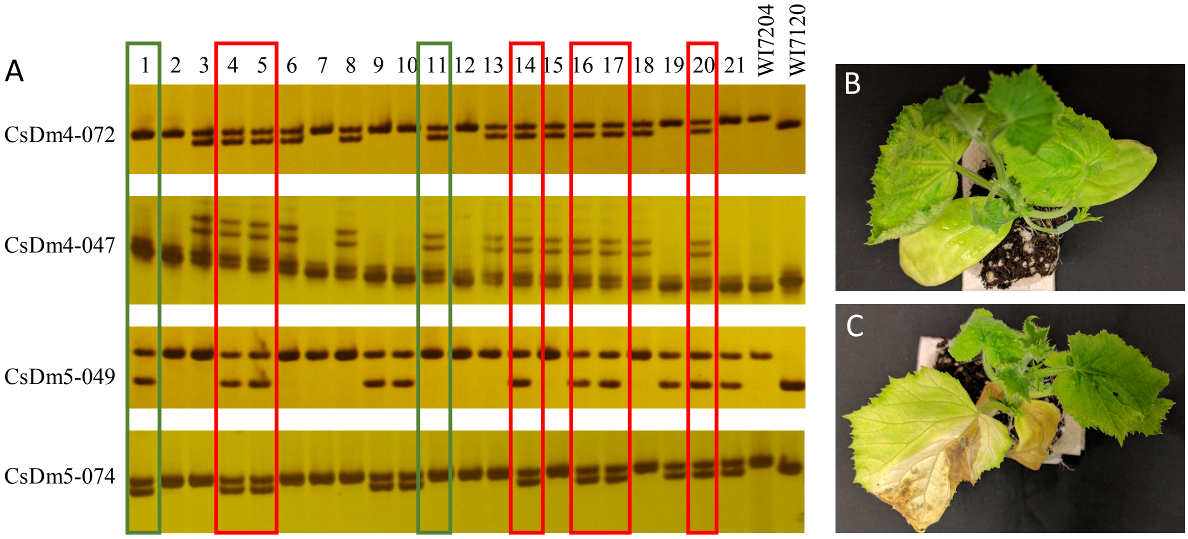


Figure 6.4 Fruit trait assessment of Wisconsin DM resistant breeding lines. A) Fruits collected from 0 to 15 day post flowering in BC_2F_1 generation with Gy14 background; B) Fruits collected from 0day post flowering to 15d in BC_2F_1 generation with WI7204 background; C) Fruits picture at 15 and 30 day post pollination(dpp) of 9930 and F_2 plants with 9930 background. A and B were collected from University of Wisconsin Hancock Agricultural Research Station; C was taken in Walnut Street Research Greenhouses.