GENETIC CHARACTERIZATION OF RESISTANCE TO PHYTOPHTHORA CAPSICI AND MORPHOLOGICAL DIVERSITY IN CUCUMBER

By

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ABSTRACT

Cucumber (*Cucumis sativus* L.) is an economically important vegetable crop that is cultivated around the world. Pickling cucumber production in the United States is greatly impacted by Phytophthora fruit rot, caused by a soil-born oomycete pathogen, *Phytophthora capsici*. With no resistant variety available, genetic resources are needed to develop resistant varieties. The goal of this work was to identify quantitative trait loci (QTL) associated with resistance to Phytophthora fruit rot using multiple genomic approaches and populations. Young fruit resistance, which is observed during the state of rapid fruit growth prior to harvest, is a quantitative trait for which multiple QTL were identified. The largest effect QTL, *qPFR5.1*, located on chromosome 5 was fine mapped to a 1-Mb region. Genome-wide association studies (GWAS) and extreme-phenotype genome-wide association study (XP-GWAS) for young fruit resistance also were performed on a resequenced cucumber core collection representing > 96% of the genetic diversity of the USDA cucumber germplasm. Several SNPs overlapped with the QTL identified from QTL-seq analysis on biparental populations. In addition, novel SNPs associated with the resistance were identified from the germplasm. The resistant alleles were found mostly in accessions from India and South Asia, the center of origin for cucumber. The results from this work can be applied to future disease resistance studies and marker-assisted selection in breeding programs. When introgressing disease resistant traits, the resulting varieties also need to meet market standards, which are defined by morphological characteristics. Disease resistance and other valuable traits are often found in landraces or wild accessions that have fruit quality traits that are undesirable in commercial markets. To provide morphological and genetic information for the diversity of fruit traits in the cucumber core collection, external and internal fruit quality traits including fruit shape, skin color, netting, spine density, curvature, seed cavity size, flesh

thickness, hollowness, and flesh color, were measured in 2019-2022. The photographic and phenotypic data are deposited and publicly available on the Cucurbit Genome Database (GuGenDB, http://cucurbitgenomics.org/v2/). GWAS analyses were performed on all traits collected. The findings from the association analyses supported many previously identified QTL and candidate genes, suggested additional candidate genes, and provide genetic framework for morphological diversity of the core collection.

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CHAPTER 1

Literature Review

Cucumber production and domestication history

Domestication history

Cucumber has been widely cultivated around the world following domestication ~3000 years ago (Sebastian et al., 2010; Renner and Schaefer, 2016). The origin of cucumber is thought to be in India, China, Burma, Thailand, and the primary and secondary centers of diversity are in India and Southeast Asia, respectively ((McCreight et al., 2013; Naegele and Wehner, 2016). Following initial domestication, cucumber was introduced to China ~2,000 years ago, and was further introduced to East Asia (e.g., Japan) around ~100 CE (Lv et al., 2012). It was also dispersed westward to Europe and Africa through two routes: Persia into eastern and northern Europe by land in 6th or 7th century, and from Persia to western and southern Europe by sea in 10th century (Staub et al., 2008; Paris et al., 2012). The plant was later brought to the Americas by Columbus in 1490s.

Cucumber taxonomy

The cucumber species can be categorized into four botanical varieties: wild cucumber (*C. sativus* var. *hardwickii*), Sikkim cucumber (*C. sativus* var. *sikkimensis* Hook. F), semi-wild Xishuangbanna cucumber (XIS, *C. sativus* var. *xishuangbannanesis* Qi et Yuan), and cultivated cucumber (*C. sativus* var. *sativus*).

The wild cucumber, *C. sativus* var. *hardwickii* (Royle) Alef (HARD) is distributed widely in north-western Himalayan mountains. The plant is used as a laxative by native people (Deakin et al., 1971). The plant has strong branching habit, and produces a large number of small oval-shaped fruits (4-5 cm), which taste bitter and drop easily when mature (Deakin et al., 1971; Weng, 2021). The tiny seeds have strong dormancy, which require treatments such as removing seed coats to increase germination rates (Weston et al., 1992).

XIS cucumber is widely distributed in Xishuangbanna of southwest China and surrounding regions (Thailand, Laos, and Myanmar) and is mainly consumed by locals (Qi et al., 1983; Yang et al., 1991). The non-bitter fruit of XIS cucumber are large, mainly have five carpels, and produce seeds with strong dormancy (Qi et al., 1983; Renner, 2017; Weng, 2021). The distinct feature of XIS cucumber is orange flesh that is rich in β-carotene (Navazio and Simon, 2001; Bo et al., 2012). Genetic analysis showed that after domestication, XIS cucumber underwent diversification selection for unique traits (Bo et al., 2015). The inheritance and genetic mechanism of β-carotene accumulation was well studied and has been bred to increase nutrition level (Cuevas et al., 2010; Qi et al., 2013).

Sikkim cucumber is found in the Sikkim region of India and Nepal (Hooker, 1876). The plant produces large and bulky fruits (up to 38 cm in length and 12 cm in diameter) in brown color with netting, as well as a large centric hollow when mature. In addition to these distinct features, the Sikkim cucumber also have many lateral branches, later flowering and seeds with different degrees of dormancy (Wang et al., 2021b). Many accessions with similar morphological features also called Sikkim cucumber, also have been collected from Nepal, Bhutan and northern India (Sharma and Hore, 1996; Wang et al., 2021b; Yashiro et al., 2017).

Through natural and artificial selection, cucumber shows great differences compared to the wild progenitor. For example, cultivated cucumber has fewer lateral branches and bears fewer but larger fruits with fewer spines, thicker flesh, smaller seed cavity size, no intercentrum hollow, and loss of bitterness (Harlan, 1992; Walters et al., 1996; Abbo et al., 2014; Weng, 2021). The plants also have larger seeds without dormancy, which can facilitate the cultivation process. During the process of selection, cucumber underwent narrow bottlenecks which led to loss of diversity (Qi et al., 2013). Despite the overall trend of selection, the morphology of cucumber

varies depend on local environments and consumer preferences. As result, cultivated cucumber has been bred for specific traits and markets (Weng, 2021; Grumet et al., 2023).

Market classes of cucumbers

Consumer preferences and handling requirements for commercial production has deepened the distinct phenotypes between different market classes. Based on the use, whether consumed fresh or processed, and the different target markets (**Figure 1.1**), the types of cucumber can be categorized into several major market classes. In East Asia, long and slender cucumber are preferred and mostly consumed fresh. They can be further classified into North and South China types. The North China type, also called "Chinese Long", typically has long and tapered fruit covered with dense white spines, while the South China type has cylindrical fruit with green and white stripes and fewer black spines (Weng, 2021). North American and Europe fresh market cucumbers (slicing cucumbers) have smooth skin and intermediate fruit length (20-30 cm), while the processing cucumbers (pickling cucumbers) tend to have thicker and warty fruit surface, and are generally short in length (5-15 cm). Another major market type is parthenocarpic cucumber, typically produced in greenhouses, which includes two classes: long-fruited, about 30-40 cm long, and Beit Alpha (Mediterranean) cucumber, which has shorter fruit (12-15 cm) (Weng, 2021; Grumet et al., 2023).

Cucumber production in the U.S.

In the United States, the cucumber production primarily serves two different markets: slicing (fresh) and pickling (processing). Michigan is one of the leading pickling cucumber producing states with more than 171,000 tones harvested in 2015. In commercial pickling cucumber production in Michigan, cucumber plants are grown on the ground in high density to facilitate machine harvest (Michigan State University Extension,

http://msue.anr.msu.edu/news/pickling_cucumber_planting_density_affects_yield_and_dollar_value). Unlike hand-picked cucumbers, which allows multiple harvests to increase yield but requires higher labor cost and more field space, machine-harvested cucumbers are usually grown at higher density with 24-28 inches between row and 3-5 inches between plants within the row. Machine harvest is a once-over, large-scale, and destructive operation that usually harvests 1-1.5 fruit per plant on average. Pickling cucumbers are harvested when they are less than 5-1/2 inches in length or 1-7/8 inches in diameter (USDA Pickling Cucumbers Grades and Standards, https://www.ams.usda.gov/grades-standards/pickling-cucumbers-grades-and-standards). There are several diseases that can significantly impact the production of cucumber, such as bacterial wilt, powdery mildew, downy mildew, angular leaf spot, and Phytophthora fruit rot. Among these, the most severe diseases that impact cucumber production in Midwestern US , are downy mildew and Phytophthora fruit rot (Hausbeck and Lamour, 2004; Savory et al., 2011).

Genomic tools for cucumber

Reference genomes

Cucumber was the first sequenced cucurbit crop (Huang et al., 2009). Several characteristics make the plant suitable for facilitating genetic and genomic studies: it is diploid, has a low chromosome number (2n = 2x = 14), a relatively small genome size (~400 Mbp), low percentage of repetitive sequences, and a short life cycle. Currently, three draft genomes have been published from the cucumber research community: the North China type ‘Chinese Long’ inbred line 9930 (v3.0) (Li et al., 2019), European inbred line ‘B10’ (v3.0) (Osipowski et al., 2020), and the North American pickling type inbred line ‘Gy 14’ (v2.1) (Yu et al., 2023). All the reference genomes are available on the Cucurbit Genome Database (GuGenDB, http://cucurbitgenomics.org/v2/). With draft genomes and new genomic tools available, genetic

linkage maps have been generated from different populations targeting various traits of interests such as growth and flowering traits, yield, fruit shape and quality, and resistances to abiotic and biotic stresses. The constructed linkage maps, coupled with QTL identification, can ultimately boost marker-assisted breeding and map-based gene cloning.

Figure 1.1. Examples of cucumber market types. Left to right: Western fresh market (slicing); Beit Alpha/Mediterranean; parthenocarpic greenhouse; Western pickling; Chinese Long. (Figure from Grumet, Lin, et al., 2023).

The CucCAP project

The cucumber germplasm resources for breeding and genetic study are maintained in several collections worldwide including the USDA Agriculture Research Service National Plant Germplasm System (NPGS), Institute of Vegetables and Flowers at the Chinese Academy of Agricultural Sciences in China, and the Centre for Genetic Resources in the Netherlands. The NPGS stores 1,314 cucumber Plant Introduction (PI) accessions, which mainly are cultivars,

landraces, and varieties. The USDA-SCRI CucCAP project: Leveraging Applied Genomics to Increase Disease Resistance in Cucurbit Crops, aims to develop genomic tools for disease resistance for the crops in the Cucurbitaceae family (Grumet et al., 2020). To assess genetic diversity and relationships among accessions, the NPGS cucumber collection was sequenced using genotyping-by-sequencing (GBS) (Wang et al., 2018). Phylogenic analysis of the collection showed three distinct clades that reflect cucumber domestication history: India/South Asia, East Asia, and the rest of the world (Central/West Asia, Europe, Africa, and the Americas) (Lv et al., 2012; Qi et al., 2013; Wang et al., 2018). Within the major clades, there are subclades that showed regional divergence and morphology differences, for example, separation of accessions from eastern and western Türkiye, as well as fresh and pickling market classes in North American accessions (Wang et al., 2018; Grumet et al., 2020).

Based on the GBS sequence of the NPGS cucumber collection, a core collection of 388 accessions was established (Wang et al., 2018). The core collection captures > 95% of the allelic diversity in the NPGS cucumber collection and also includes historical cultivars with important agronomic traits and disease resistance (Wang et al., 2018). To reduce heterozygosity and heterogeneity within each accession, the accessions were self-pollinated for 2-3 generations. The self-pollinated core collection accessions were re-sequenced at 30-40× coverage and used to call SNPs as described by Yu et al. (2023). The SNP data is accessible on the Cucurbit Genomics Database website (CuGenDB) (http://cucurbitgenomics.org/v2/).

Resistance to Phytophthora fruit rot

Phytophthora blight, caused by the soil-borne oomycete pathogen *Phytophthora capsici* (Leon.), is one of the most serious concerns for cucurbit growers in the eastern and Midwestern United States. The pathogen can spread to other cucurbit growing areas through irrigation and

rainwater, and once present the oospores can linger in soil for many years. In very severe cases it can lead to 100% yield loss. Currently, there is no single effective management strategy to control the disease, preventative measures such as planting in clean fields and avoiding contaminated water sources are the most effective methods. However, once the field is infested, growers have no choice but to switch to other non-host crops. Since *P. capsici* can infect Cucurbitaceous, Fabeceous, and Solanaceous crops, farmers are left with a finite choice of crops.

Life cycle

The thick cell wall of *P. capsici* oospores protects it from desiccation and other extreme environmental conditions such as cold temperature. Under moist and warm conditions, oospores germinate and grow into hyphae, with lemon-shaped sporangia on the external plant surface (Lamour, 2013). Sporangia can release motile biflagellate zoospores in water, zoospores then germinate when in contact with the plant surface. When they perceive a suitable host surface, the hyphae grow out from zoospores and form appressoria, a specialized infection structure to breach the plant cuticle and cell walls. As a hemi-biotrophic pathogen, *P. capsici* first lives biotrophically in plant tissue. After penetrating the plant surface, some hyphae that grow within the plant tissue will form haustoria at the tip to acquire nutrients from host plants; often with no visible symptoms. In later infection stages, *P. capsici* kills host cells which results in necrosis and tissue collapse, followed by sporulation on the host surface which then can start a new infection cycle (Lamour et al., 2012).

Disease controls and prevention

In commercial cucumber production, fruits are located in contact with the ground under a canopy of leaves; the direct contact to soil increases the probability of *P. capsici* infection (Ando and Grumet, 2006). In addition to soil contact, the other major infection pathway is through

irrigation and rainwater, which help *P. capsici* to release mobile zoospores to spread within or between fields (Granke et al., 2012). In cucumber, *P. capsici* specifically infects fruits, especially young fruits, while leaves and vines remain healthy (Gevens et al., 2006). In addition, the latent period before obvious symptoms appear can result in serious economic loss, as infected fruits that lack symptoms can be harvested and later infect other fruits during storage.

There is no single management that can effectively control Phytophthora fruit rot, a combination of exclusion, cultural practices and chemical control strategies must be used to reduce epidemics of *P. capsici*. Among all the methods, avoiding the pathogen by planting in the fields that have no *P. capsici* infection history is the most crucial (Babadoost, 2005). Trellises, adding plant space, and cover crops are possible approaches to reduce disease level, however, these are not practical in large-scale commercial pickling cucumber production (Ando et al., 2009). The wide host range, genetic variance between isolates, and long-term survival of oospores increase the difficulty of disease management. Additionally, resistance to a commonly used fungicide, mefenoxam, has been reported in several states from different crops (Lamour and Hausbeck, 2000; Parra and Ristaino, 2001; Café-Filho and Ristaino, 2008; Davey et al., 2008; Dunn et al., 2010). Longevity of the oospores in soil also results in failure of non-host crop rotation although rotation can decelerate the buildup of disease pressure (Hausbeck and Lamour, 2004). A study conducted in Illinois showed that *P. capsici* can survive in soil for more than 36 months and still remain virulent (Babadoost and Pavon, 2013). Apart from the disease management strategies mentioned above, host resistance is the most desirable strategy for *P. capsici* management. Growing a resistant variety also can reduce the use of fungicides, which lead to lower cost and less potential environmental or health hazards. However, at this time there are no genetically resistant cucumber varieties available.

Inheritance and mechanisms and of host resistance to P. capsici

Efforts have been undertaken to introgress genes for resistance to *P. capsici* into commercial varieties or discover new resistance genes residing in related wild species for Solanaceous crops and some cucurbit crops. In watermelon, four accessions from *Citrullus lanatus* var. *lanatus* showed high resistance to fruit rot and one *C. colocynthis* and a *C. lanatus* var. *citroides* also exhibited resistance, but to a lower degree (Kousik et al., 2012). Broad resistance to Phytophthora fruit rot observed during post-harvest was identified in five watermelon germplasm lines (Kousik et al., 2022), and a breeding line, USVL531-MDR, resistant to both powdery mildew and Phytophthora fruit rot was recently released (Kousik et al., 2022, 2023). Screening of a collection of 308 melon (*Cucumis melo*) PIs and two commercial cultivars for *P. capsici* crown rot resistance identified three PIs that were highly resistant (Donahoo et al., 2013). Another screening for *P. capsici* resistance that was specifically focused on melon seedlings, identified two accessions that showed no symptoms in response to five *P. capsici* isolates from different hosts (Pontes et al., 2014). Several studies have identified multiple PIs in *Cucurbita moschata* and *Cucurbita pepo L.* (squash, pumpkin, and gourd) with crown rot resistance (Padley, Les D. et al., 2009; Krasnow et al., 2014; Michael et al., 2019; LaPlant et al., 2020). The sources identified could be used to expand available resistant germplasm and can be integrated to future breeding programs. QTL associated with *P. capsici* resistance have also been identified in multiple cucurbit crops. In melon, a QTL on chromosome 12 was identified, for which a candidate gene encoding a wall-associated receptor kinase (*WAK*) was proposed (Wang et al., 2020b). For squash, six QTL in *C. pepo* (Vogel et al., 2021) were identified using bulked segregant analysis (BSA) and four QTL were identified in C. *moschata* using BSA and QTL mapping approaches (Ramos et al., 2020; Michael et al., 2021).

In some crop species, organ-specific resistance is observed. In pepper, roots, stems, foliage, and fruit are all susceptible. In tomato, crown rot, leaf spot, and foliar blight are seen (Quesada-Ocampo and Hausbeck, 2010; Quesada-Ocampo et al., 2016). In cucumber, *P. capsici* mainly infects fruits, particularly young fruits, while leaves and vines remain largely unaffected (Gevens et al., 2006). In watermelon, similar to cucumber, fruit rot is more severe while foliar symptom is limited to water soaking, and in summer squash, both leaf and fruit are susceptible (Kousik et al., 2017; Quesada-Ocampo et al., 2023). Walker and Bosland, (1999) reported the organ specific expression of genes related to *P. capsici* resistance in pepper. They found that one independent dominant gene was essential for root rot resistance and another one for foliar blight resistance, with the presence of at least one dominant allele from the third gene. In addition to Phytophthora root rot and foliar blight, Sy et al. (2005) found an additional symptom, stem blight resistance, was controlled by one dominant gene. They concluded that there were three different dominant genes responsible for the resistance on each symptom (root rot, foliar blight and stem blight). Subsequent research conducted by Ogundiwin et al. (2005) supported these findings, however, instead of a single dominant gene, they identified QTLs from two recombinant inbred line (RIL) populations that had different effects on root and/or leaf symptoms.

P. capsici resistance in cucumber fruit

Age-related resistance

From the initial search for genetic resistance to *P. capsici* in cucumber, a population of 333 cucumber cultigens was tested (Gevens et al., 2006). However, instead of complete resistance, an age-related resistance (ARR) was observed that corresponds with fruit growth (Gevens et al., 2006). During the first few days after pollination, about 0-5 days-post-pollination (dpp), cucumber ovaries undergo cell division, which is then followed by a period of rapid cell

expansion, usually lasting until 12-16 dpp (Ando et al., 2012). Cucumber fruits are usually harvested immature, approximately 8-12 dpp, during mid to late exponential growth. Finally, fruit and seeds mature at ~30-35 dpp. Cucumber fruits are susceptible to *P. capsici* in the early fruit development stages, then gradually become resistant. The transition from susceptible to resistant begins at ~12 dpp, toward the end of exponential growth (Ando et al., 2009). ARR is also observed in other cucurbit crops such as watermelon and various squashes (Ando et al., 2009; Krasnow et al., 2017; Alzohairy et al., 2020, 2021).

Studies of cucumber fruit peels indicated that the fruit surface is important for ARR, and methanolic peel extracts of resistant-age peels suggested that biochemical components in the fruit peel may contribute to the ARR response (Ando et al., 2015). An untargeted metabolomics analysis showed a specific increase of terpenoid glycosides in resistant-age peels and transcriptomic analysis of the peel tissues identified enrichment of genes involved in terpene and flavonoid synthesis pathways (Mansfeld et al., 2017). Fruit exhibiting ARR also showed the ability to respond rapidly to the presence of *P. capsici* zoospores, as evidenced by a spike in defense response genes relative to susceptible-age fruit as early as 2 hours post inoculation and observable death of zoospores as early as 4 hour-post-inoculation (Mansfeld et al., 2020).

Young fruit resistance

Since ARR does not take effect until the end of exponential fruit growth, which is after the time for harvest, cucumber growers will still suffer yield loss due to *P. capsici* infection, even with the cultivar exhibit ARR. Given the need to protect young fruits, Colle et al. (2014) screened the full cucumber U.S. cucumber PI collection for young fruit resistance to *P. capsici*. Of 1,076 accessions screened, three were identified as potential sources of resistance to young fruit resistance. One accession, PI 109483 collected from Türkiye in 1935, showed stable resistance

over several generations of selfing. PI 109483 also has been found to be a potential source of resistance to belly rot caused by *Rhizoctonia solani* (Uchneat and Wehner, 1998). Morphologically, PI 109483 has dark green fruit that turns yellow when mature, the fruit shape is oblong with smooth skin and minor striping. A breeding line derived from PI 109483 was released as MSU 109483-53 (Grumet and Colle, 2017).

Objectives

The first objective of this thesis was to identify QTL associated with young fruit resistance to *P. capsici* and develop molecular markers to facilitate future introgression and breeding line development. A combination of genomic approaches, including QTL-seq, fine mapping, GWAS and XP-GWAS, and population types, including bi-parentally-derived populations and a diversity panel (the cucumber core collection) were utilized in this work.

Morphological diversity in the cucumber core collection

Fruit appearance is one of most important aspects that determines commercial value of cucumber. Key cucumber fruit quality traits include both external and internal features such as size and shape, flesh thickness, seed cavity size, and lack of internal hollowness; and skin features including color, spines and glossiness. The cucumber core collection exhibits extensive diversity in fruit traits including both desirable and undesirable features (**Figure 1.2**).

While a great deal of breeding research focuses on resistance to abiotic and biotic stresses to cope with existing and emerging challenges, the resulting breeding lines and varieties need to meet market requirements for fruit type. Many sources of disease resistance are found in wild or landrace accessions, which usually have more feral and undesirable morphology. For instance, a Sikkim type cucumber from India (PI 197087) was found to be resistant to downy mildew, powdery mildew, anthracnose and angular leaf spot (Wang et al., 2021b). However, the bulky,

netted, and large centric hollow fruit of Sikkim cucumber are highly unfavorable traits. To incorporate these resistant traits into commercial lines may require additional cycles of backcrossing to develop a morphologically acceptable variety. Selecting appropriate breeding materials can possibly achieve breeding goals with shorter breeding generations. Information about genetic regulation of the traits can facilitate these efforts. To this date, extensive research has been performed on bi-parental populations to identify QTL and candidate genes for various fruit quality traits, but few were performed on diversity panels.

Genetic control of cucumber fruit external traits

Fruit size and shape

Cucumber fruit size and shape related QTL have been well studied in numerous populations as summarized and reviewed in several papers (e.g., Weng 2015; Che and Zhang, 2019; Pan et al., 2020; Wang et al., 2020; Zhang et al., 2021a; Weng 2021;and Grumet et al. 2023). A set of 21 consensus fruit size and shape related QTL that were recurringly identified in different populations and studies was summarized by Weng et al. (2015).

Fruit shape is defined by the ratio between fruit length and diameter, which is one of the crucial attributes for market value. Many fruit shape candidate genes have been identified. The candidate gene for the QTL *FS1.2*, *CsSUN*, had a similar effect on fruit shape to its homolog gene *SUN* in tomato (Pan et al., 2017). A candidate gene for QTL *Fl4.1* encoding a homolog of an auxin efflux carrier family protein was downregulated in long-fruited lines (Xing et al., 2023). The gene *CRABS CLAW* (*CsCRC*) was found to be the candidate gene for QTL *FS5.2*, which resulted in longer fruit shape, potentially mediating auxin and gibberellic acid (GA) signaling pathways (Pan et al., 2022). The rare allele $CsCRC^{A}$ associated with short fruit only existed in round-fruited semi-wild Xishuangbanna cucumbers, while the alternative allele $CsCRC^{G}$ can be

found in both wild and cultivated cucumber (Che et al., 2023).

A cloned gene, *CsTRM4* (also named *CsTRM5, TONNEAU1 Recruiting Motif5*), which is a homolog of tomato *SlTRM5*, was identified for *FS2.1* QTL (Wu et al., 2018). Knocking out the expression of *CsTRM5* (CsaV3_2G013800) repressed cell expansion and resulted in shorter fruit length (Xie et al., 2023). Several *short fruit* mutants (*sf1*, *sf2*, *sf3*, and *sf4)* were cloned and

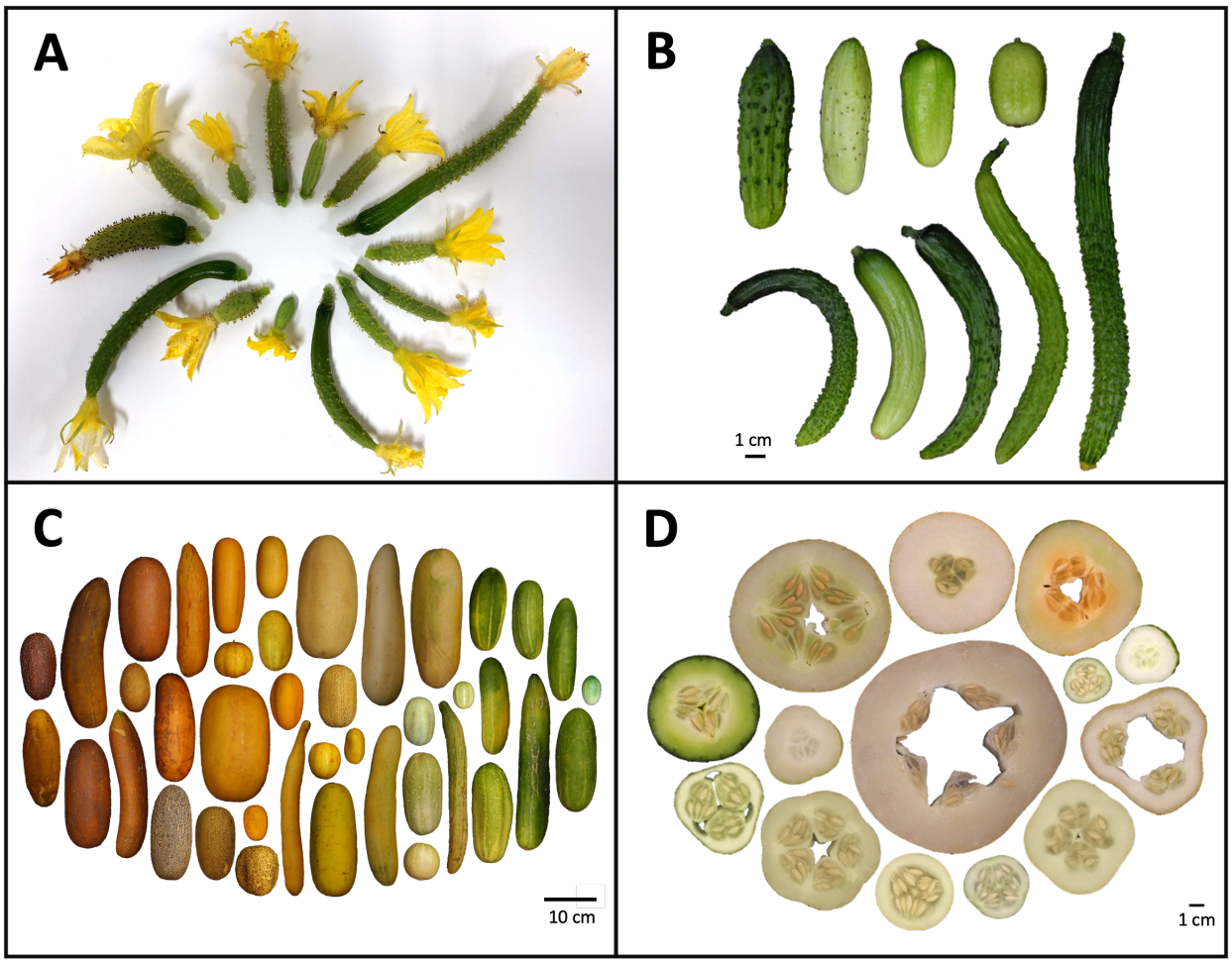

Figure 1.2. Examples of diversity in cucumber ovary and fruit morphology at different stages of development as observed in the CucCAP cucumber core collection. (A) Cucumber ovaries at anthesis or one day post-anthesis exhibit differences in size, shape, presence/absence/number of spines, spine color, warts, and ribs. (B) Variation in cucumber fruit development during early exponential growth 5–8 days post pollination (dpp). (C) Mature cucumber fruit vary in shape, size, color, surface texture, and netting. (D) Variation in internal properties at maturity (flesh thickness, seed cavity size, color, and hollows). (Figure from Grumet, Lin et al., 2023).

cucurbit-specific RING-type E3 ligase that regulates fruit elongation by mediating a key enzyme characterized from multiple mutagenesis populations. The *short fruit 1* (*sf1*) gene encodes a fourth short fruit mutant, *sf4*, encodes a homolog of an *O*-linked *N*-acetylglucosamine (*GlcNAc*) transferase (*OGT*), influences gene expression involved in cell division in *Arabidopsis* (Zhang et al., 2023). The CsFUL1^{A} allele of a *FRUITFULL* (*FUL*)–like MADS-box gene, which can be readily found in East Asia accessions with longer fruits, influences auxin accumulation and cell division and expansion (Zhao et al., 2019).

Curvature and Tapering

To integrate with modern large-scale commercial production, uniform and straight cucumber fruits are required to streamline packing and shipping. However, some accessions have higher tendency to produce curved or tapered fruit. Fruit curvature can be caused by an unbalanced distribution of auxin, where the convex side has higher auxin concentration than the concave size (Li et al., 2020). Applying auxin or overexpressing of *CsYUC10b*, a *YUCCA* involved in auxin biosynthesis, promoted auxin accumulation symmetrically on both sides of the fruits, and resulted in straight fruits (Li et al., 2020). A mutant exhibiting a tapered-shaped fruit phenotype (*mango fruit*, *mf*) that has underdeveloped seed cavity and carpel separation was caused by a mutant in a WUSCHEL-related homeobox1 (*CsWOX1*) gene (Niu et al., 2018).

Fruit skin color

Since cucumber fruits are consumed immature, fruits at this stage typically have different degrees of greenness, although some have yellow hue and even white color (**Figure 1.2B**). In mature fruit, however, there is a wide color variation ranging from white-green-yellow-orange-brown (**Figure 1.2C**). Chlorophyll and carotene are the main two players influencing fruit color (Egea et al., 2010). Several genes that cause light green skin color were identified through

mutagenesis, and were found to be involved in different processes affecting chloroplast number or chlorophyll biosynthesis. Disrupting the expression of a recessive gene *lgp*, which encodes a homolog of *ACCUMULATION AND REPLICATION OF CHLOROPLASTS 5* (*ARC5*), resulted in fewer but larger chloroplasts, probably due to interference with chloroplast division (Zhou et al., 2015b). Another gene, *CsYcf54* encoding an Ycf54-like protein localized in the chloroplast, was associated with a cyclase step in chlorophyll biosynthesis (Lun et al., 2016). Downregulating a transcription factor *MYB36* from the *CsMYB36* gene also led to decreased chlorophyll content in a yellow green peel mutant (*ygp*) (Hao et al., 2018). A major locus for pericarp color, *CsPC1* (*Pericarp color 1*), encoding a GATA transcription factor (*CsGATA1*) that had higher expression in light green pericarp, was implicated to be involved in chlorophyll biosynthesis (Huang et al., 2022). White peel color in immature fruit with lower chlorophyll content and fewer and smaller chloroplast was affected by a two-component response regulator-like *APRR2* (Liu et al., 2016; Jiao et al., 2017; Tang et al., 2018; Kishor et al., 2021a).

Spine density

Cucumber spines are multicellular non-glandular trichomes that consisted of an enlarged base and pointed stalk (Liu et al., 2022). Higher spine density is commonly found in North China type and Beit Alpha type cucumbers (Weng, 2021). The numerous spines (*ns*) locus located on chromosome 2 was identified by QTL mapping and GWAS analysis (Zhang et al., 2016; Xie et al., 2018; Liu et al., 2022). Overexpressing and knock out analyses suggested that *ns* was *Csa2G264590*, an auxin transporter (*CsAUX1*) that is specifically expressed in fruit peel (Xie et al., 2018; Liu et al., 2022). A non-functional ns haplotype which resulted in higher spine density was specifically selected in Eurasian cucumber accessions (Liu et al., 2022). Similarly, originating from mutagenesis in Chinese accessions with ultra-high fruit spine density phenotype

[e.g. *Csgl3* (*glabrous-3*) and *Tril* (*Trichome-less*) (Cui et al., 2016; Bo et al., 2019; Du et al., 2020)] were several allelic variations of an HD-Zip IV transcription factor essential for trichome formation in tomato (Yang et al., 2011). Spine initiation and differentiation is also associated with a WD-repeat homolog *CsTTG1* (*TRANSPARENT TESTA GLABRA1*), which positively regulated the density of fruit bloom trichomes and spines (Chen et al., 2016; Guo et al., 2020). *CsTTG1* also directly interacted with a class I homeodomain-leucine zipper gene, for which several mutants were identified including mict (*micro-trichome*)/*csgl1 (glabrous-1)*/*tbh* (*tiny branched hair*). Mutations of this gene led to tiny and stunted trichomes (Li et al., 2015; Zhao et al., 2015; Zhang et al., 2021b). Another factor, *CsMYB6*, a homolog of *MYB6* in Arabidopsis encoding a MIXTA-like MYB transcription factor, acts together with *CsTRY* , a homolog of *Arabidopsis TRIPTYCHON*, to negatively regulate and decrease trichome density (Yang et al., 2018).

Netting

Netted fruits are not often seen in commercial cucumber varieties, but can be observed in landraces or wild accessions, such as Sikkim type cucumber (Wang et al., 2021b; Weng, 2021). Microcracking on fruit surface occurs when cuticles on the outer cell walls fail to withstand the pressure of expansive growth (Knoche and Lang, 2017). These microcracks then may proceed to form fruit russeting, and netting (reticulation) (Petit et al., 2017; Zhang et al., 2022). Underneath the cracked surface is a layer of suberized wound-periderm in Sikkim type cucumber, which differs from cultivated cucumber, where the surface is composed of cutin and wax (Nomberg et al., 2022). A *Rs* (*Russet skin*) locus that conferred flaky skin that easily fell of the fruit was mapped to a 736 kb region on chromosome 1 (Wang et al., 2021b). Copy number variation of the *Rs* locus candidate gene, *CsSH1/WIN1* (*shine1/ WAX INDUCER1*), which is also associated with cuticle thickness (Rett-Cadman et al., 2019), was positively correlated with netting intensity

(Zhang et al., 2022). Another dominant locus, *H* (*Heavy netting*), was located chromosome 5, but is yet to be cloned (Miao et al., 2011; Zhou et al., 2015a; Wang et al., 2021b).

Genetic control of cucumber fruit internal traits

Cucumber produces fleshy, pepo fruit that consist of three parts: exocarp (or called epicarp), which is the outer layer; mesocarp, the fleshy and crisp middle layer; and the endocarp, the inner pulp layer around the seeds, which is also known as seed cavity. The proportion of fruit flesh and seed cavity size is of commercial value; in general, thicker flesh and smaller seed cavity size are preferred by consumers. In addition to flesh thickness and seed cavity size, other internal characteristics such as flesh color and hollowness are also important traits that impact commercial value of a variety or cultivar.

Carpel number

Most cucumber accessions have three carpels, although some accessions or varieties have five carpels, such as XIS cucumber and True Lemon type cucumber. Carpel number in cucumber is controlled by a major gene (*Cn*) located on chromosome 1, where three carpels are dominant to five carpels in True Lemon cucumber (Li et al., 2016). *Cn* gene is a homolog of *CLAVATA3* (*CsCLV3*), which is a component of the well-characterized *WUSCHEL (WUS)-CLAVATA3* (*CLV3*) pathway that regulates shoot apical meristem maintenance and floral organ development (Li et al., 2016; Somssich et al., 2016; Che et al., 2020). *CsCLV3* act as a negative regulator of carpel number and indirectly suppress *CsWUS* expression, while *CsWUS* acts as a promoter to increase variation in carpel number. Another gene, *CsFUL1A,* a *FRUITFULL-like MADS-box gene,* can bind to the promoter of *CsWUS* and induce expression, and *CsWUS* can further bind to the promoter of *CsCLV3* to activate its expression (Che et al., 2020). Additionally, auxin can also positively affect carpel number by interaction of *AUXIN RESPONSE FACTOR 14*

(*CsARF14*) with *CsWUS* (Che et al., 2020).

Flesh thickness

Flesh thickness represents the edible portion of a cucumber fruit. A QTL on chromosome 2 (*fft2.1*) was identified from an F_2 population crossed between a thick fruit flesh parent and a thin fruit flesh parent. From the QTL region of 0.19 Mb, a candidate gene *Csa2M058670.1* was identified, which had higher expression in thick flesh line (Xu et al., 2015). In addition to low expression level of *Csa2M058670.1*, a 4-bp deletion in the promotor region was found in all four thin flesh lines, suggesting the gene activity was associated with fruit flesh phenotypes (Xu et al., 2015). *Csa2M058670.1* encodes a homolog of a SET domain protein-lysine methyltransferase (PKMT) (Raunser et al., 2009) that is associated with cell division and growth in *Arabidopsis* (Horvath et al., 2003). *Csa2M058670* was specifically selected in a Xishuangbanna population, for which thinner fruit flesh is preferred (Lin et al., 2022). An additional six QTL were identified on chromosomes 1, 2, 3, and 6 from a RIL population derived from a North China type cucumber and a North European type cucumber (Yuan et al., 2008). Two bi-parental populations with different lines of Sikkim cucumber were also used to map various fruit quality QTL (Wang et al., 2021b). Two flesh thickness QTL, *fth3.1* and *fth5.1*, were identified on chromosomes 3 and 5, respectively, in both immature and mature fruits; *fth3.1* was associated with increased flesh thickness while *fth5.1* had the opposite effect.

Seed cavity size

Seed cavity size is highly correlated with fruit radial growth (Liu et al., 2020). Parthenocarpic fruits (i.e. without fertilization) that are widely grown for greenhouse production tend to have a greatly reduced seed cavity. Two QTL for seed cavity size at chromosomes 1 and 2 were found from a population derived from a cross between parthenocarpic and non-

parthenocarpic lines (Haaring and Huijbregts-doorduin, 2020). Yuan et al. (2008) identified seven QTL located on chromosomes 1, 2, 4, and 5 that explained 4-11.72% of phenotypic variation from the RIL population (S94 × S06). The QTL on chromosome 1 from both studies were located in similar location (Wang et al., 2020b). In a RIL population derived from a cultivated cucumber (CMCC) and an introgression line with *C. hystrix* background, two seed cavity size related QTL were identified on chromosome 5, and 6 (Wang et al., 2023b).

Hollowness

After double fertilization, a pistil comprised of 3-5 carpels develops into fruit and seeds of a cucumber. During the development, carpels fuse and the cell boundary between carpels become irregular and obscure (Zhou et al., 2022). Where carpels fail to fuse, the center becomes hollow. Hollowness is an undesirable trait that is not commonly seen in cultivated cucumber, especially in pickling cucumber, which may result in bloating effect during the brining process (Wilson and Baker, 1976). While environmental factors can affect hollowness formation, some cucumbers naturally form hollows, such as Sikkim type cucumber, where the size of hollowness was positively correlated with fruit diameter (Wang et al., 2021b). QTL mapping on populations with Sikkim cucumber background identified QTL on chromosome 1, 2,3, and 5, where *mfh1.1* and *mfh2.1* may increase the hollowness size while *mfh3.1* decreases hollowness (Wang et al., 2021b). In *Arabidopsis*, two bHLH transcription factors, *SPATULA* (*SPT*) and *ALCATRAZ* (*ALC*), regulate differentiation of the transmitting tract, which connects the stigma style and ovary (Heisler et al., 2001; Groszmann et al., 2008; Pabón-Mora et al., 2014). The double knockout mutant *Csspt Csalt* in cucumber causes a hollow center in cucumber fruit, and reduces fertility (Cheng et al., 2022). In the mutant, lignin accumulation in the transmitting tract promoted the separation of carpels and resulted in hollowness formation (Cheng et al., 2022). Another study

identified a candidate gene, *Csa1G630860* (*CsALMT2*), that encodes an aluminum-activated malate transporter protein *ALMT2* associated with fruit hollowness (Zhou et al., 2022). *CsALMT2* was highly expressed in ovules and non-hollow fruit material at all fruit development stages compared to hollow fruit material (Zhou et al., 2022). A homolog in watermelon *WMALMT-3* is involved in regulation of malic acid accumulation in vacuoles of pulp tissue during fruit development (Muhammad Jawad et al., 2020).

Flesh color

Typically, cucumber flesh color is white, with some ranging from green to yellow and orange. Green flesh can be found in *C. hardwickii*, and Xishuangbanna cucumber fruits are known for orange flesh (Qi et al., 1983; Renner, 2017). The yellow and orange are due to accumulation of carotene in mesocarp and endocarp, while green flesh results from chlorophyll accumulation (Li and Yuan, 2013). Orange flesh has been a breeding target due to the nutritional value of β-carotene content, which is a key precursor of vitamin A. Orange flesh is controlled by a single recessive gene, *ore* (Bo et al., 2012). A candidate gene was later identified for *ore*, *Csa3G183920*, which encodes B-carotene hydroxylase (*CasBCH1*) (Qi et al., 2013). Another orange flesh related gene, *CsOr*, was discovered from bi-parental populations from semi-white cucumber and an orange endocarp breeding line (Kishor et al., 2021b). The single recessive gene (CsaV3_6G040750) encodes a chaperone DnaJ protein (DnaJ) protein, which is associated with orange color in cauliflower and melon (Lu et al., 2006; Tzuri et al., 2015). A single recessive gene, *yf*, spanning 149 kb on chromosome 7 was identified from a yellow flesh and white flesh parents (Lu et al., 2015). Wang et al. (2023a) pinpointed another candidate gene, *yellow flesh 2* (*Csyf2*), that encodes an abscisic acid (ABA) 8'-hydroxylase. Downregulating *Csyf2* expression led to lower levels of lutein and higher β-cryptoxanthin contents without changing the β-carotene

content (Wang et al., 2023a). Lastly, green flesh was found to be controlled by two QTL, *qgf3.1* and *qgf5.1,* from a GWAS analysis (Bo et al., 2019).

Objectives

The second objective of this thesis was to morphologically characterize the cucumber core collection to provide publicly available photographic and phenotypic data that can be informative to breeders and researchers when choosing plant materials. I also sought to perform GWAS analyses to provide insights into the genetic basis of morphological diversity in the cucumber core collection.

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CHAPTER 2

Identification of QTL Associated with Resistance to Phytophthora Fruit Rot in Cucumber (*Cucumis sativus* L.)

Identification of QTL associated with resistance to Phytophthora fruit rot in cucumber (*Cucumis sativus* L.)

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All of the work presented and writing was performed by YC Lin with the exception of the age-related resistance QTL-seq and RNA-seq performed by B Mansfeld and M Colle. F Chen and Y Weng performed line development for the cucumber core population. X Tang and Z Fei performed bioinformatic analysis and SNP calling for the re-sequenced core population lines used for the GWAS and XP-GWAS analyses.

Abstract

Phytophthora fruit rot (PFR) caused by the soilborne oomycete pathogen, *Phytophthora capsici*, can cause severe yield loss in cucumber. With no resistant variety available, genetic resources are needed to develop resistant varieties. The goal of this work was to identify quantitative trait loci (QTL) associated with resistance to PFR using multiple genomic approaches and populations. Two types of resistances have been identified: age-related resistance (ARR) and young fruit resistance. ARR occurs at 12-16 days post pollination (dpp), coinciding with the end of exponential fruit growth. A major QTL for ARR was discovered on chromosome 3 and a candidate gene identified based on comparative transcriptomic analysis. Young fruit resistance, which is observed during the state of rapid fruit growth prior to commercial harvest, is a quantitative trait for which multiple QTL were identified. The largest effect QTL, *qPFR5.1*, located on chromosome 5 was fine mapped to a 1-Mb region. Genome-wide association studies (GWAS) and extreme-phenotype genome-wide association study (XP-GWAS) for young fruit resistance were also performed on a cucumber core collection representing > 96% of the genetic diversity of the USDA cucumber germplasm. Several SNPs overlapped with the QTL identified from QTL-seq analysis on biparental populations. In addition, novel SNPs associated with the resistance were identified from the germplasm. The resistant alleles were found mostly in accessions from India and South Asia, the center of diversity for cucumber. The results from this work can be applied to future disease resistance studies and marker-assisted selection in breeding programs.

Introduction

Cucumber production in the United States primarily serves two markets: slicing (fresh) and pickling (processing). Pickling cucumber production using primarily the once-over machine

harvesting system in the Midwestern U.S. is severely impacted by Phytophthora fruit rot caused by the soil-borne oomycete, *Phytophthora capsici* (Savory et al., 2011; Hausbeck and Lamour, 2004). The pathogen primarily infects cucumber fruits, especially young, rapidly growing fruits, while other plant parts such as leaves and vines remain intact (Gevens et al., 2006). The cucumber plants grown for processing are planted on bare ground in high density to facilitate machine harvest. The fruits, which are beneath the vegetative canopy, are in direct contact with the infested soil and the high density of foliage creates a moist environment ideal for the growth and spread of *P. capsici* (Ando and Grumet, 2006; Ando et al., 2007). The pathogen releases flagellate zoospores from sporangia that are mobile in water and can easily spread within or between fields through irrigation or rainwater (Granke et al., 2012). The symptoms start with water-soaked lesions and tissue collapse, followed by the growth of white mycelia and sporangia on the fruit surfaces (Quesada-Ocampo et al., 2023). Currently, growers rely on a combination of exclusion, cultural practices for management, and chemical control strategies to reduce outbreaks of *P. capsici* (Sanogo et al., 2022; Quesada-Ocampo et al., 2023). However, *P. capsici* can readily develop resistance to chemical controls and several commonly used fungicides such as metalaxyl and mefenoxam have been shown to be ineffective against the pathogen (Kousik et al., 2017; Quesada-Ocampo et al., 2023). Growing resistant varieties could reduce the use of fungicides, which should lead to lower cost and less potential environmental or health hazards. However, the complex nature and genetic variation in both host and pathogen hamper the development of resistant commercial cultivars. In cucumber, sources of resistance were discovered (Gevens et al., 2006; Colle et al., 2014), but are yet to be introgressed into commercial varieties.

The effort of searching for resistance to *P. capsici* initially led to the discovery of age-

related resistance (ARR) (Gevens et al., 2006; Ando et al., 2009). The fruits from accessions expressing ARR (ARR+) are susceptible at early fruit development stages, then gradually become resistant as fruits develop (Ando et al., 2015). The transition begins at ~12 days post pollination (dpp), toward the end of exponential fruit growth, and was demonstrated to be associated with the fruit peel (Ando et al., 2012, 2015). Preformed biochemical defenses and metabolites that are developmentally regulated were found to be associated with ARR, these include enzymes producing defense related compounds such as reactive oxygen species and terpenoid glycosides (Mansfeld, et al., 2017). Resistant-aged fruit also appear to be uniquely able to sense the presence of *P. capsi*ci zoospores, as evident by a spike in defense response genes as early as 2 hours post inoculation. This corresponds with observable death of zoospores early as in 4 hour-post-inoculation in fruits that exhibit ARR (Mansfeld et al., 2020).

While ARR is a largely effective form of resistance, cucumber fruits are usually harvested and consumed during mid- to late- exponential growth (approximately 8-12 dpp), prior to the transition to ARR. Hence, cucumber growers will still suffer yield loss due to *P. capsici* even if the cultivars exhibit ARR. Therefore, resistance expressed before the harvest age is desired to alleviate the yield loss caused by the pathogen. To search for young fruit resistance, Colle et al. (2014) surveyed the U.S. cucumber plant introduction (PI) by testing young cucumber fruits (~5-7 dpp). Three accessions with low disease scores were potential sources of young fruit resistance. One of them, PI 109483 from Türkiye, exhibited stable resistance in the following generations of selfing. The resulting S_6 progeny was released as a breeding line MSU 109483-53 (Grumet and Colle, 2017). The breeding line was later used for doubled haploid (DH) production, and the DH line ‘A4-3’, which shows delayed and reduced symptoms and slower rate of pathogen growth, was used to study young fruit resistance (Zhang et al., 2021).

The objectives of this work were to Identify genetic loci associated with both young fruit resistance and ARR and to develop molecular markers for future breeding efforts. Multiple genetic and genomic approaches, including bulk segregant analyses, fine mapping, transcriptome analyses and genome wide association studies identified several loci in association with the resistance traits. Association analyses identified several SNPs that overlapped with QTL identified from QTL-seq analysis as well as novel SNPs and potential sources of resistance.

Materials and Methods

Plant materials

Biparental mapping. The source of young fruit resistance was MSU109483-53 (Grumet and Colle, 2017), a breeding line obtained through a series of pure line selections from PI 109483, a landrace collected from Türkiye. Seed from MSU109483-53 was used for doubled haploid (DH) production via *in vivo*-induced parthenogenic embryo culture generously performed by Rijk Zwaan (De Lier, Netherlands). The DH line 'A4-3' was crossed with the susceptible parent, 'Gy14', an American type pickling cucumber, which has been broadly used in research and breeding programs and for which a high-quality reference genome is available (http://cucurbitgenomics.org/v2/). 'A4-3' also was crossed with an American fresh market cucumber, 'Poinsett 76', which exhibits ARR (Mansfeld et al., 2020), to provide a second population for QTL verification. To map ARR, 'Gy14' (ARR-) was crossed with 'Poinsett 76' (ARR+) and resultant F_1 seed was sent for DH production (Rijk Zwaan, De Lier, Netherlands).

Cucumber core collection. The cucumber core collection was selected based on genotyping-by-sequencing (GBS) data of United States National Plant Germplasm System (NPGS) collection (Wang et al., 2018). To reduce heterozygosity and heterogeneity within the accessions, individuals from each accession were self-pollinated for 2 or 3 generations. The self-pollinated

core collection lines were re-sequenced at 30-40× coverage and used to call SNPs as described by Yu et al. (2023) (http://cucurbitgenomics.org/v2/). The core collection lines were grown in the field from 2019-2022 with three plants per accession. The accessions that were tested are listed in **Supplementary Table 2.1**. The number of accessions planted each year varied depending on seed availability; each accession has 1-4 years of phenotypic data.

Growth conditions

Seeds for all experiments were sown in the greenhouse or growth room. Seedlings were transplanted to the greenhouse or field at the two true-leaf stage. No fungicide was applied after the onset of flowering in either the greenhouse or field to ensure that fungicide residue was not present on the fruit surface to interfere with phenotyping.

Greenhouse. Seedlings were transplanted to 1.5-gallon pots with Suremix Perlite soil medium and grown in the Michigan State University Plant Science Greenhouse Complex. The plants were fertigated twice a day (44 ppm nitrogen of Peters Professional 20-20-20 General Purpose; Scotts, Marysville, OH). LED lights were used to provide 16-hour photoperiod. Pest and disease management was based on general practice in the greenhouse using a combination of chemical and biological controls. For young fruit experiments, bumble bees (Koppert Biological Systems, Inc., Howell, MI) were introduced at week 4-5 for pollination. For ARR experiments, flowers were hand-pollinated; a single fruit was set per plant to prevent developmental effects of competition among fruits. All ARR experiments were grown in the greenhouse.

Field. Plants were grown at the Michigan State University Horticulture Teaching and Research Center (HTRC). Prior to transplanting, 300 lbs/acre of 19-19-19 fertilizer were applied to the field, and irrigation provided as needed throughout the season. To minimize contamination with other pathogens and avoid injury resulting from washing soil from the fruit, plants were grown on

raised black plastic mulch and trellised using T-posts and trellis netting. The space between plants was 0.45-0.6 m, depending on the field design each year. Pollination was facilitated by honeybees.

Screening for response to P. capsici

Fruit harvesting and handling. To provide uniform, high-inoculum pressure and optimal environmental conditions for disease development, fruits grown in both the greenhouse and field were harvested at the desired stage of development and brought into the laboratory for disease screening. For young fruit experiments, fruit were harvested at early exponential growth stage, 5-7 dpp (~7-10 cm long). Harvests were performed 2-3 times a week to provide 30-60 fruit/accession for the core collection, or 10-30 fruit/plant for biparental segregating populations. For ARR experiments, fruit were harvested at 16-18 dpp. Fruit from the field were rinsed with distilled water to remove soil and debris, sanitized by soaking in 1% bleach for one minute, and rinsed with distilled water thoroughly to remove bleach residue. Greenhouse fruit were rinsed with distilled water. Clean, dry fruits were placed in covered plastic trays lined with wet paper towel on the sides to maintain high humidity for pathogen growth as described by Gevens et al. (2006). Trays were incubated at 25-26 °C under constant light.

Pathogen inoculation and phenotyping. The *P. capsici* isolates, Bartley's 1, OP97, or NY-0644-RFP (Dunn et al., 2013), were cultured on V8 agar media as described in Gevens et al. (2006). After seven days, the plate was flooded with 6-7 mL sterile distilled water to stimulate zoospore production. The concentration of resuspended zoospore was measured using a Countess™ automated cell counter (Thermo Fisher Scientific, Waltham, MA). For young fruit experiments, zoospore suspensions of Bartley's 1 were diluted to 1×10^4 zoospore/mL. Two 30 µL droplets were applied to the surface of each fruit as described by Colle et al. (2014). Inoculated cucumber

fruits were photographed and scored at 5 days post inoculation (dpi) based on the disease rating scale shown in **Figure 2.1**. Ratings of 1-3 indicate mild symptoms limited the region of inoculation, 4-6 moderate to extensive water soaking, and 7-9 visible hyphal growth and sporulation. Symptoms were scored at both inoculated sites on each fruit; the score for an individual fruit was the mean of the two sites. The disease rating for a plant or line was the average of all fruit over all harvests for a given experiment. For the ARR experiments, OP97 or NY-0644-RFP zoospore suspensions were diluted to 1×10^5 zoospores/mL. Disease symptoms were monitored daily for 7-10 days and rated using either a 1-9 disease rating scale as above (F_2 experiments) or a 0-5 point disease score (0 – no symptoms, 5 – severe sporulation) (DH experiments). Fruits from the DH population were inoculated with 12 equally spaced 30 µL droplets. At 7 dpi, each fruit was assigned the rank of the most susceptible inoculation site.

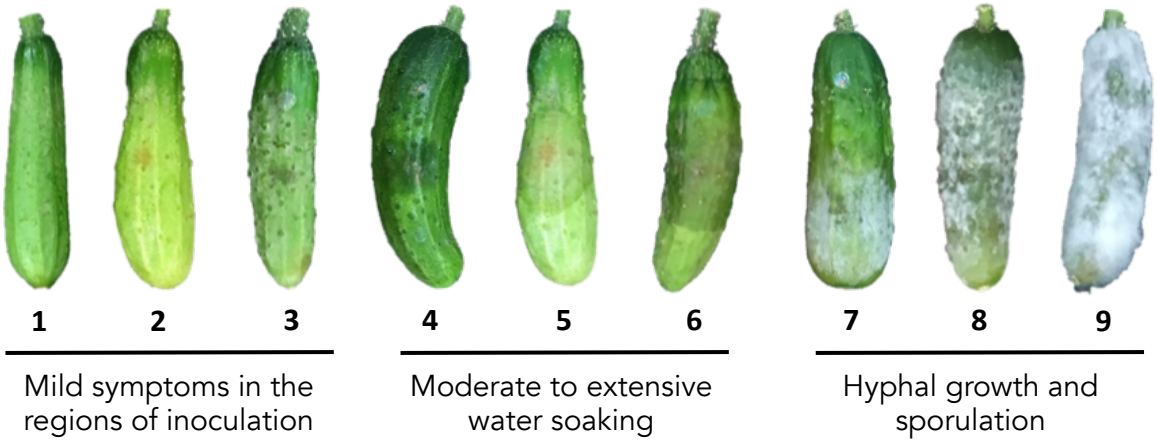


Figure 2.1. **Illustration of the 9-point disease scoring scale of *Phytophthora capsici* infection on cucumber fruit.** Ratings 1- 3: no or minor symptoms limited to inoculation sites; ratings 4-6: levels of water soaking and necrosis; ratings 7-9: different levels of hyphal growth and sporulation.

QTL-seq analysis

Young fruit resistance. An F_2 population (n=362) from the 'Gy14' × 'A4-3' was screened in 2018 in the field. Leaf tissue (~50mg) was collected from each seedling prior to transplanting to

the field, freeze-dried, and ground for DNA extraction. DNA was extracted from the parental lines and each plant selected for the bulks using the Kingfisher DNA extraction robot and Mag-Bind® Plant DNA DS 96 Kit (Omega Bio-tek, Norcross, GA) as described in Wang et al. (2018). The genomic DNA was quantified using PicoGreen (Invitrogen™, Waltham, MA). Equal amounts of DNA of the selected individuals for each bulk (n=19) were mixed for sequencing. Sequencing was performed at the Research Technology Support Facility at Michigan State University. Three libraries (resistant bulk, susceptible bulk, and 'A4-3') were prepared using Illumina TruSeq Nano DNA Library Preparation Kit and sequenced using paired-end sequencing of 150 bp on an Illumina HiSeq 4000 platform. After removing low-quality reads and sequencing adaptors using Trimmomatic v. 0.33 (Bolger, 2014), the reads from each bulk were aligned to the 'Gy14' reference genome v2.0 (http://cucurbitgenomics.org/v2/; Yu et al., 2023) using BWA-MEM (v0.7.8) (Li, 2013) with default parameters. Sequencing duplicates were marked using PicardTools (https://broadinstitute.github.io/picard/, v2.7.1) and the Genome Analysis Toolkit (GATK; v3.6) best practice pipeline was used for SNP calling (McKenna et al., 2010; DePristo et al., 2011; Van der Auwera et al., 2013). QTL-seq analysis (Takagi et al., 2013) was performed using the R package, QTLseqr (Mansfeld and Grumet, 2018). SNP filtering criteria in QTLseqr was set for "minimum depth ≥ 50, maximum depth ≤ 100, GQ ≥ 99, depth difference ≥ 20, minimum sample depth ≥ 20," which left 587,178 SNPs for further analysis. Delta SNP-index and G'-value (Magewene et al., 2012) were calculated using a sliding window size of 1Mb, where the 95% and 99% confidence intervals were calculated with 10,000 iteration in QTL-seq analysis, while filter method "deltaSNP" at the threshold of 0.1 in G' analysis.

ARR. Progeny of 79 DH lines (5 plants/line) along with both parents and F_1 seed were grown in the greenhouse in a replicated block design. In a separate experiment, 92 F_2 plants of the 'Gy14' ×

'Poinsett 76' cross were grown in the greenhouse; plants with the 15 highest and lowest disease scores were selected for the two bulks respectively. DNA extraction was as described above. After quantitation, all libraries were pooled in equimolar amounts and loaded on one lane of an Illumina HiSeq 2500 High Output flow cell (v2) and sequenced in a 2 ×150bp paired end format. Reads were cleaned and adaptor sequences were removed using Trimmomatic v. 0.33 (Bolger et al., 2014). QTL-seq analysis was performed as previously described with the following settings (F_2/DH): refAlleleFreq = 0.1/0.1, minTotalDepth = 20/30, maxTotalDepth = 50/100, depthDifference = 10/30, minGQ = 30/30, minSampleDepth = 10/15. A window size of 2 Mb was used for smoothing Δ(SNP-index) values.

Verifying and narrowing the genomic regions

Young fruit. A second F_2 population ('Gy14'× 'A4-3'; n=752) was used to verify the QTL regions and develop RIL and F_3 populations. Polymorphic SNPs flanking and within the QTL region were identified for KASP marker design and ordered from Sigma-Aldrich (St. Louis, MO). The 5 µL reaction mixture contained 2.5 µL of DNA (at 10 ng/µL), 2.5 µL of 2 × KASP Master Mix (LGC Biosearch Technologies, Hoddesdon, UK; 3CR Bioscience, Essex, UK), and 0.07 µL of primer mix. The cycling conditions were as follows: 94°C for 15 min followed by 10 touchdown cycles at 94°C for 20 s and 65°C for 60 s (decrease 0.6°C per cycle), then 38 amplification cycles of 94°C for 20 s and 55°C for 60 s, finally with 37°C for 10 s. Thermocycling and fluorescence readings were performed using a FX384 Real-Time thermal cycler (BioRad, Hercules, CA), where allele calls were determined using the CFX manager software (v.3.1). To narrow the genomic region, homozygous recombinant individuals (i.e., homozygous for the 'Gy14' allele at one and homozygous for the 'A4-3' allele at the other end) were selected and self-pollinated for 4-5 generations to develop a RIL population. Individuals

that were partially heterozygous (heterozygous at one end, and homozygous for the 'Gy14' or 'A4-3' allele at the other end) were selfed to produce F_3 families. KASP markers were designed at approximately every 0.5 Mb within the QTL region. The sequences of KASP markers and targeted SNP locations are in **Supplementary Table 2.2**. RIL families were grown in the field in 2020 with 30-60 fruits tested per line. In 2022, 99 homozygous recombinant individuals from 33 F_3 families were grown in the greenhouse. Cuttings were also taken from each plant and transplanted to the field. Fruit were harvested from both sets of plants and disease screening was performed as described above.

ARR. To verify the ARR QTL, KASP markers flanking the QTL were used to genotype 768 F_2 seedlings of 'Gy14' × 'Poinsett 76' as described above. Individuals homozygous for 'Gy14' or 'Poinsett76' alleles within the QTL region were self-pollinated to produce F_4 lines. Plants from 14 F_4 lines were grown in the greenhouse in a randomized complete block trial (5 plants/line). Phenotyping was performed as described above.

RNA-seq analysis

Sample collection and RNA extraction. Flowers of plants from the ARR parental lines ('Poinsett 76', 'Gy14') were hand pollinated so that 8 and 16 dpp fruit were harvested on the same day. Three fruit (biological replicates) were collected for each age and genotype. Uninoculated fruit peels were collected from 8 and 16 dpp fruit using a vegetable peeler and immediately frozen in liquid nitrogen. Samples were ground using a mortar and pestle in liquid nitrogen. RNA extraction was performed using the MagMAX Plant RNA Isolation Kit protocol (Thermo Fisher, Waltham, MA) as described in Mansfeld et al. (2020). Assessment of RNA concentration and quality was performed as described in Rett-Cadman et al. (2019). All samples had a minimum RNA quality score of 8.

TruSeq Library preparation and sequencing. Libraries were prepared at Michigan State University's Research Technology Support Facility, using the Illumina TruSeq Stranded mRNA Library Preparation Kit on a Sciclone G3 robot following manufacturer's recommendations. An additional cleanup with 0.8 × AmpureXP magnetic beads was performed after completion of library preparation. Quality control and quantification of completed libraries were performed using a combination of Qubit dsDNA HS and Advanced Analytical Fragment Analyzer High Sensitivity DNA assays. The libraries were divided into two pools of 15 libraries each. Pools were quantified using the Kapa Biosystems Illumina Library Quantification qPCR kit. Each pool was loaded onto one lane of an Illumina HiSeq 4000 flow cell and sequencing was performed in a 1 × 50 bp single read format using HiSeq 4000 SBS reagents. Base calling was done by Illumina Real Time Analysis (RTA) v2.7.7 and output of RTA was demultiplexed and converted to FastQ format with Illumina Bcl2fastq v2.19.1.

Differential expression analysis. Reads were cleaned, and adaptor sequences were removed using Trimmomatic v. 0.34 (Bolger et al., 2014) with the following settings: LEADING:3 TRAILING:3 SLIDINGWINDOW:4:15 MINLEN:35. Quality control was performed using FastQC (http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc). A cucumber transcriptome fasta file was made from the 'Chinese Long' (v2) (Huang et al., 2009; Li et al., 2011) genome using the gffread function from the cufflinks software package (Trapnell et al., 2010) and high-quality reads were then quasi-mapped to the transcriptome using Salmon v. 0.9.1 (Patro et al., 2017) with default settings. Read quantification data was imported into R using the tximport R package (Soneson et al., 2015) and differential expression analysis was performed using DEseq2 (Love et al., 2014) with log-fold-change-shrinkage. Age and genotype were combined into a single factor for differential expression analysis and contrasts between the four conditions ('Poinsett 76' 8 dpp,

'Poinsett 76' 16 dpp, 'Gy14' 8 dpp, 'Gy14' 16 dpp) were performed. Differentially expressed genes were called significant using an adjusted p-value (Benjamini-Hochberg adjustment; false discovery rate) of less than 5% and an expression change of greater than two-fold was used to define biological significance.

Association analysis

GWAS. The SNP data of the core collection was downloaded from CucGenDBv2 (Yu et al., 2023). SNPs were filtered using BCFtools (Danecek et al., 2021) and GATK (Van der Auwera et al., 2013) with the following criteria: bi-allelic, GQ scores >20, maximum read depth within one standard deviation of the mean read depth, minor allele frequency > 0.1, missing rate <20%, resulting in 1,168,270 SNPs for association analysis. Marker-trait association analyses were performed using best linear unbiased estimates (BLUEs). BLUEs were calculated using the R package lme4. Association analysis was performed using GAPIT 3.0 (Genome Association and Prediction Integrated Tool) with MLM, FarmCPU, BLINK, and MLMM models implemented within the software (Wang and Zhang, 2021). The significance threshold was calculated based on Bonferroni correction (p-value/N, N= number of SNPs used in the analysis), where the thresholds of adjusted p values of 0.05 and 0.01 corresponded to -log10(p) values of 7.368 and 8.06, respectively.

XP-GWAS. The 29 most resistant and 29 most susceptible accessions were selected based on 2019-2021 phenotypic data and grown in the field in a randomized complete block design with three blocks in 2022. A random bulk of 29 accessions was selected from the full core population (**Supplementary Table 2.1**). XP-GWAS analysis was performed as described in Yang et al. (2015). In brief, reference and alternative allele depths at each SNP site were extracted from the core resequencing data and calculated for each bulk. The input data was then computed using the

R package, XP-GWAS, with the depth filter set at 500, which ended with 3,444,143 SNPs for the analysis. The 5% false discovery rate (FDR) and the threshold of p = 0.05 with Bonferroni correction were calculated to detect significant SNPs.

Results

Identification of QTL for young fruit resistance

QTL-seq analysis

Screening for young fruit resistance to *P. capsici* was performed on an F_2 population (n=362) derived from the cross between the susceptible pickling cucumber breeding line, 'Gy14', and the doubled haploid line, 'A4-3', which shows reduced and delayed symptom development in response to inoculation (**Figure 2.2A**). Three harvests were performed with 10-20 young fruits (5-7 dpp; ~7-10 cm long) sampled from each F_2 plant. The normally distributed disease scores suggested that young fruit resistance is a quantitative trait controlled by multiple loci (**Figure 2.2B**). To verify phenotyping of plants to be selected for the resistant and susceptible bulk populations, the 30 highest and lowest scoring individuals from the first three harvests were harvested an additional time. Based on the four harvests, 19 plants which had consistent phenotypes were selected for the resistant and susceptible bulks, respectively (**Figure 2.2C**). Sequencing of the bulk populations generated ~77 million reads for each bulk with > 60× coverage of the cucumber genome. After processing and filtering, 558,625 SNPs were available for QTL-seq analysis, which identified QTL on chromosomes 1, 5, and 6 (**Figure 2.3** and **Table 2.1**). The most significant QTL was located on chromosome 5.

QTL validation and narrowing the genomic region of *qPFR5.1*

To validate the regions identified by QTL-seq, a second F_2 population (n=752) was genotyped using KASP markers flanking the QTL. Individuals homozygous for either susceptible

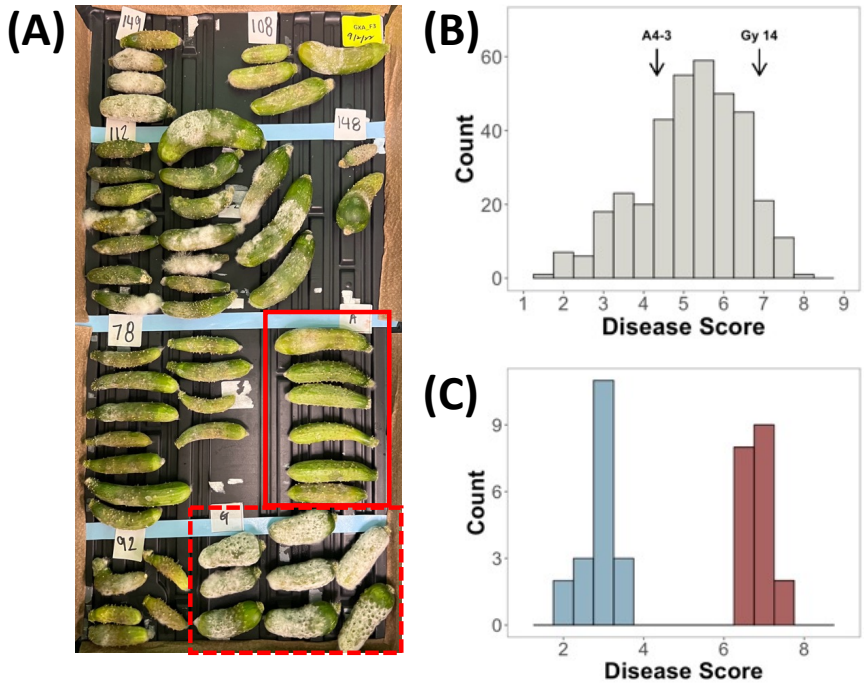
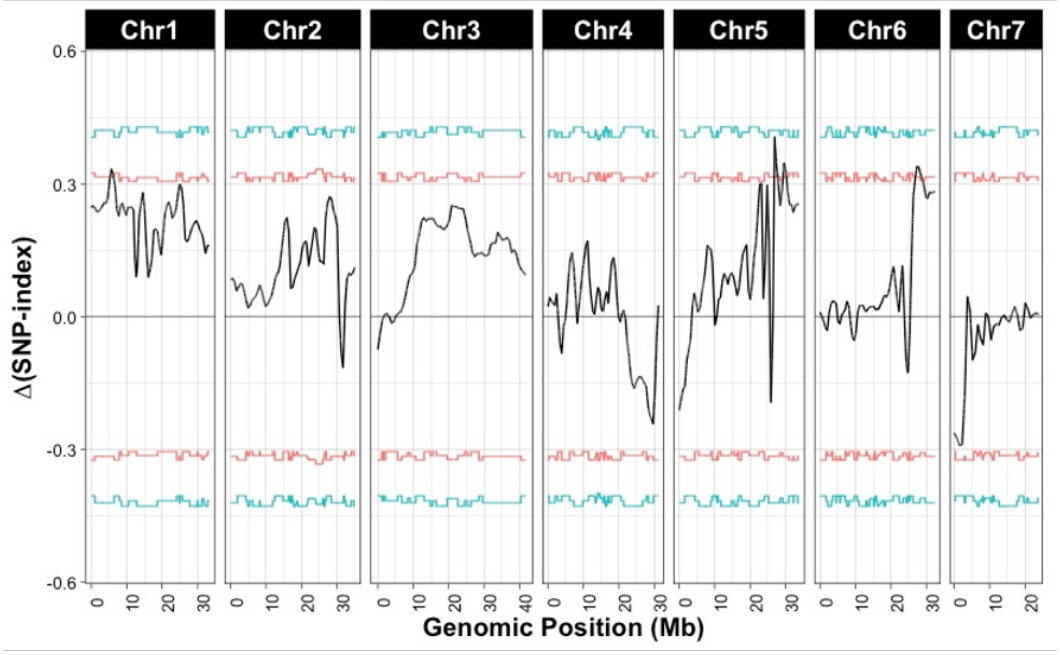


Figure 2.2. Screening of young fruit from the F_2 population of 'Gy14' × 'A4-3' for response to inoculation with *P. capsici*. Fruits were harvested at 5-7 days post pollination (dpp). **(A)** Example of disease screening; fruits were photographed at 5 days post inoculation (dpi). Red box, A4-3; Dashed box, 'Gy14'. **(B)** Disease score distribution of the F_2 population. Scores are the average of 10-20 fruit from each F_2 plant; fruit were scored at 5 dpi. **(C)** F_2 individuals selected for the resistant and susceptible bulks. Disease scores are the average of all four harvests.

'Gy14' (S) or resistant 'A4-3' I alleles on chromosome 5 and/or 6 were selected, providing four allelic combinations (chr5-chr6: Gy-Gy, Gy-A4-3, A4-3-Gy, and A4-3-A4-3). Presence of the 'A4-3' allele on chromosome 5 was associated with resistance with a frequency of 0.89 in the most resistant plants (disease score < 4) progressively dropping to 0.06 in the most susceptible plants (disease score > 7) (**Figure 2.4A**). Individuals with 'A4-3' alleles at chromosome 5 showed significantly lower disease score compared to those with 'Gy14' allele (**Figure 2.4B**). No significant allelic effect was observed for the QTL on chromosome 6 (**Figure 2.4B**). The allelic effect of the QTL on chromosome 1 was not verified.

The QTL on chromosome 5, named *qPFR5.1 (Phytophthora fruit rot 5.1)*, was tested in a



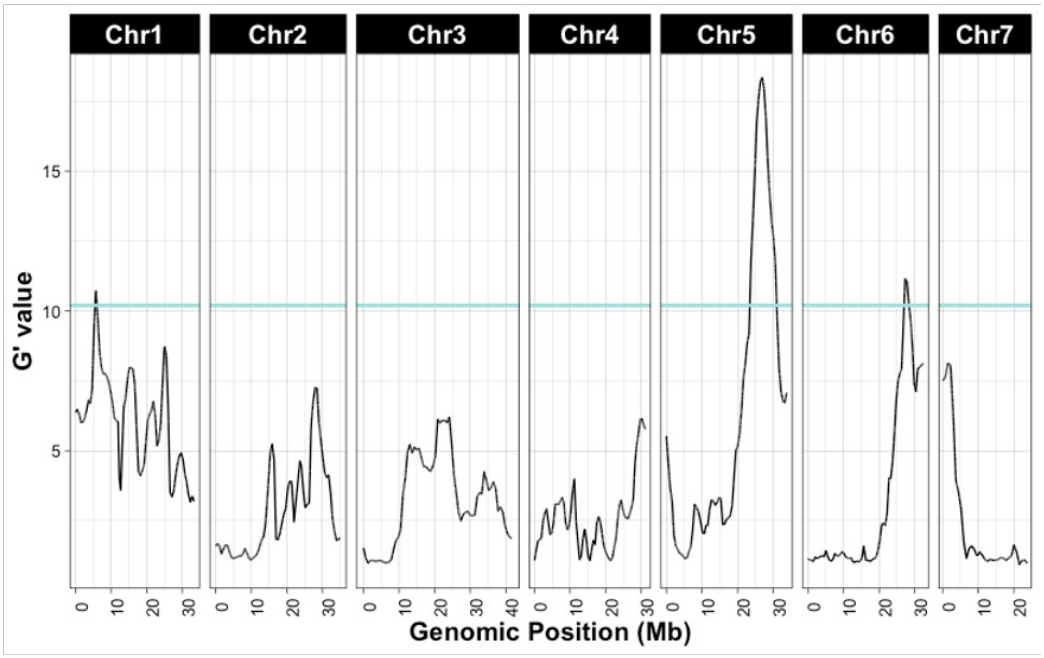


Figure 2.3. **QTL associated with young fruit resistance as identified by QTL-seq.** Δ(SNP-index) and G' were calculated with a window size of 1 Mb. Horizontal lines in Δ(SNP-index) represent confidence thresholds of 95% (red) and 99% (blue); for G' the threshold for false discovery is 0.01 (blue line). Allele frequencies for the resistant and susceptible bulks are provided in **Supplementary Figure 2.1**.

Table 2.1. QTL identified from QTL-seq in the 'Gy14' x 'A4-3' F_2 population.

	QTL-seq		G'	
Chromosome	**Location (Mb)[1]**	**Length**	**Location (Mb)**	**Length (Mb)**
1	5.35-6.27	0.92	5.53-6.00	0.47
5	26.87-27.71	0.84	23.40-30.94	7.53
5	29.24-30.19	0.95	-	-
6	26.92-28.95	2.03	27.04-28.38	1.34

[1]Genomic locations are according to Gy14 v. 2.1 (CuGenDB v.2; http://cucurbitgenomics.org/v2/)

second genetic background, 'Poinsett 76', a North American fresh market cucumber. F_2 individuals from 'Poinsett 76' × 'A4-3' (n=768) were genotyped with flanking markers for *qPFR5.1,* and individuals homozygous for either 'Poinsett 76' or 'A4-3' in that region were self-pollinated. Plants from the resulting 25 F_3 families were grown in the greenhouse and field and young fruit were harvested for inoculation. Consistent with the RIL population, the 'A4-3' allele showed strong association with resistance in the greenhouse and field, respectively), further confirming the effect of *qPFR5.1* (**Figure 2.4C**).

qPFR5.1 as identified by QTL-seq and G' spanned ~7Mb on chromosome 5. To facilitate fine mapping, we tested a recombinant inbred line (RIL) population and F_3 families selected for recombination within the QTL region. F_2 plants that were homozygous recombinant at *qPFR5.1* (*i.e.*, homozygous for the 'Gy14' allele at one end and homozygous for the 'A4-3' allele at the other end) were self-pollinated to the F_4 and F_5 generations; nine resulting RILs were grown in the field in 2020 with 30-60 fruits tested per line. Each plant was also genotyped with KASP markers

at approximately 0.5 Mb intervals within the QTL region (**Figure 2.5A**). Based on genotypic and phenotypic data, the region was narrowed to 3.22 Mb between markers M2 and M5 (25.17-28.39 Mb).

To further refine the QTL, an additional 768 F_2 plants were genotyped and 178 partially homozygous F_2 individuals (i.e., heterozygous at one end and homozygous for 'A4-3' or 'Gy14' alleles at the other end) were self-pollinated. Twelve F_3 families were selected for each of the four genotypic combinations (heterozygous-'Gy14', 'Gy14'-heterozygous, heterozygous-'A4-3', and 'A4-3'-heterozygous) and 16 individuals per family were genotyped. Of those, 99 homozygous recombinant individuals from 33 families were transplanted to the greenhouse. Cuttings were also taken from each plant and transplanted to the field for testing in a second environment. Additional KASP markers were designed between M2 and M5 (**Figure 2.5B**). The QTL identified from F_3 families was located between M53-M58 (26.09-27.13 Mb; 1.04 Mb) with a slight difference between the two seasons tested; M54-M58 (26.32-27.13 Mb) in the greenhouse, and M53-M41 (26.01-26.85 Mb) in the field.

Association analysis of resistance to P. capsici

GWAS of the core collection

The re-sequenced cucumber core collection, consisting of 388 accessions along with several breeding lines with important agronomic traits, represents >96% of genetic diversity in the U.S. NPGS (Wang et al., 2018; Yu et al., 2023). The collection was planted in the field and fruits were tested from 2019 to 2021. The number of accessions grown each year varied depending on seed availability. Phenotypic data was collected from 370 accessions with 1-3 years of disease scores per accession. The normally distributed disease score of the core collection further affirmed that young fruit resistance is a quantitative trait (**Figure 2.6A**); the correlation between

years ranged from 0.48-0.80.

GWAS analysis was performed using BLUE values calculated from disease scores from 2019-2021 with one single-locus model (MLM) and three multi-locus models (FarmCPU, BLINK and MLMM) (**Figure 2.6B, Table 2.2**). A total of 11 SNPs were identified from the different models: seven in FarmCPU, five in BLINK, one in MLMM, and five in MLM. The phenotype variance explained (PVE) of the SNPs ranged from 0.38-24.49%. Several significant SNPs were identified in at least two models. S1_21117743 (A/G) was significant in BLINK and MLM models with PVE of 9.01% and 7.74%, respectively. S2_10226744 (A/G) was detected in FarmCPU, MLMM, and MLM models, and a closely located SNP 27.69 kb upstream was detected in the BLINK model. This was the only significant SNP identified in the MLMM model with a PVE of 24.49%. Another SNP, S3_37752706 (C/T), was detected in FarmCPU and BLINK models.

Phenotypes were significantly different between accessions carrying homozygous reference vs. alternate alleles for all significant SNPs except S3_18480786 (**Supplementary Figure 2.2**). Of the nine SNPs, five alternate alleles led to increased resistance (lower disease scores). Of those, only two of the alternate alleles were present in 'A4-3' (SNP S1_21117743 and S3_37752706), suggesting that the other alleles identified by GWAS may provide additional sources of resistance. When the alternate alleles associated with lower disease scores were rare in the core collection (< 10%, i.e., < 38 accessions), the majority of accessions (64%-81%) carrying the alternate allele originated from the India/South Asia region (e.g., S1_21117743, S5_23563699, and S6_29175300) (**Table 2.3**). Conversely, four of the five SNPs associated with increased resistance (S1_21117743, S4_8024257, S5_23563699, and S6_29175300) were very uncommon in the East Asian accessions (0-3%). For S2_10226744, where the rare alternate allele

was associated with increased susceptibility, 77% of the accessions were from East Asia. When the alternate alleles occurred frequently in the germplasm (>50%) (e.g., S3_37752706 and S7_3391182), the origins were widely distributed across regions.

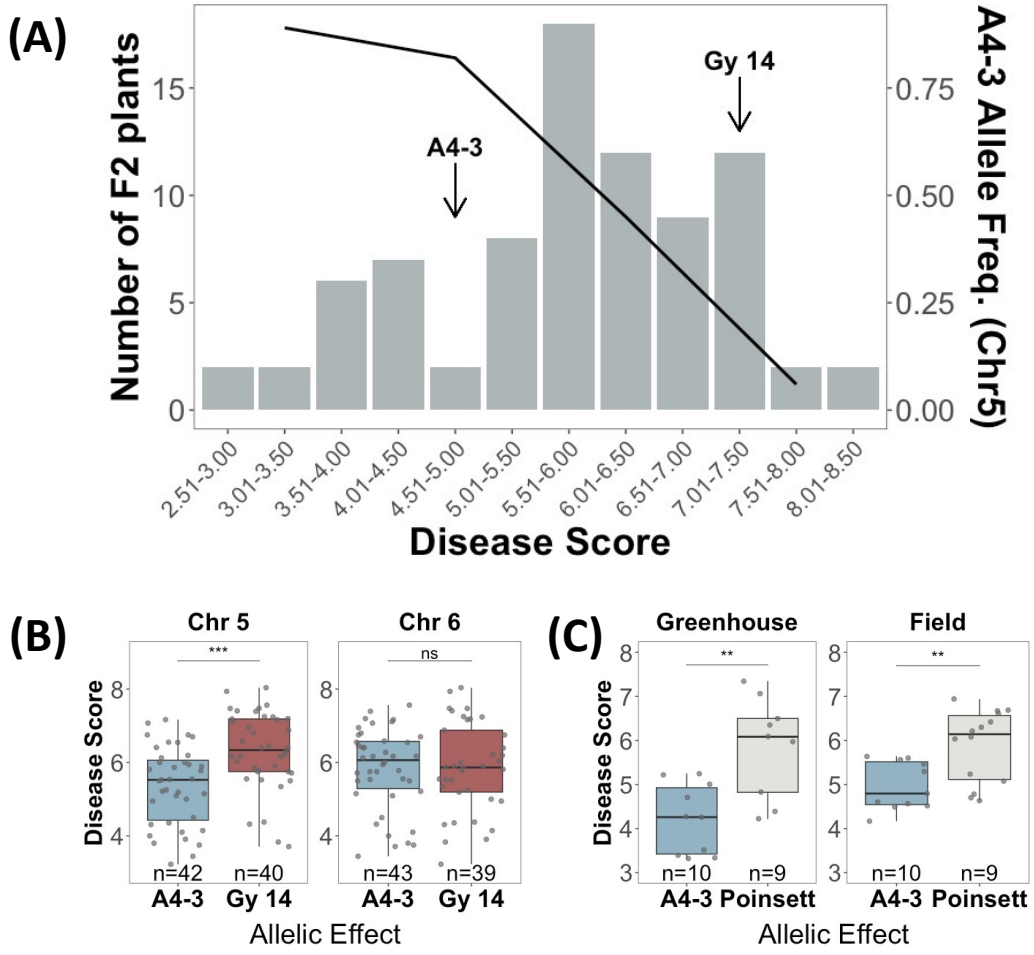


Figure 2.4. Verification of the allelic effect of QTL for young fruit resistance identified by QTL-seq analysis. (A) Disease distribution at 5 days post inoculation for the selected F_2 plants ('Gy 14' × 'A4-3') homozygous for the 'Gy14' or 'DH4-3' alleles (n=82) (bar graph), and the frequency of the resistant 'A4-3' allele on chromosome 5 (black line). Each F_2 value is the mean of 10-30 fruit/plant. **(B)** Disease scores of F_2 individuals of 'Gy14' (pickling cucumber) × 'A4-3' possessing either the 'A4-3' or 'Gy14' allele at *qPFR5.1*. **(C)** F_3 families of 'Poinsett 76' (fresh market cucumber) × 'A4-3' possessing either the 'A4-3' or 'Poinsett' allele at *qPFR5.1*. Each point is the mean of >20 fruits/family from the greenhouse and >50 fruits/family from the field.

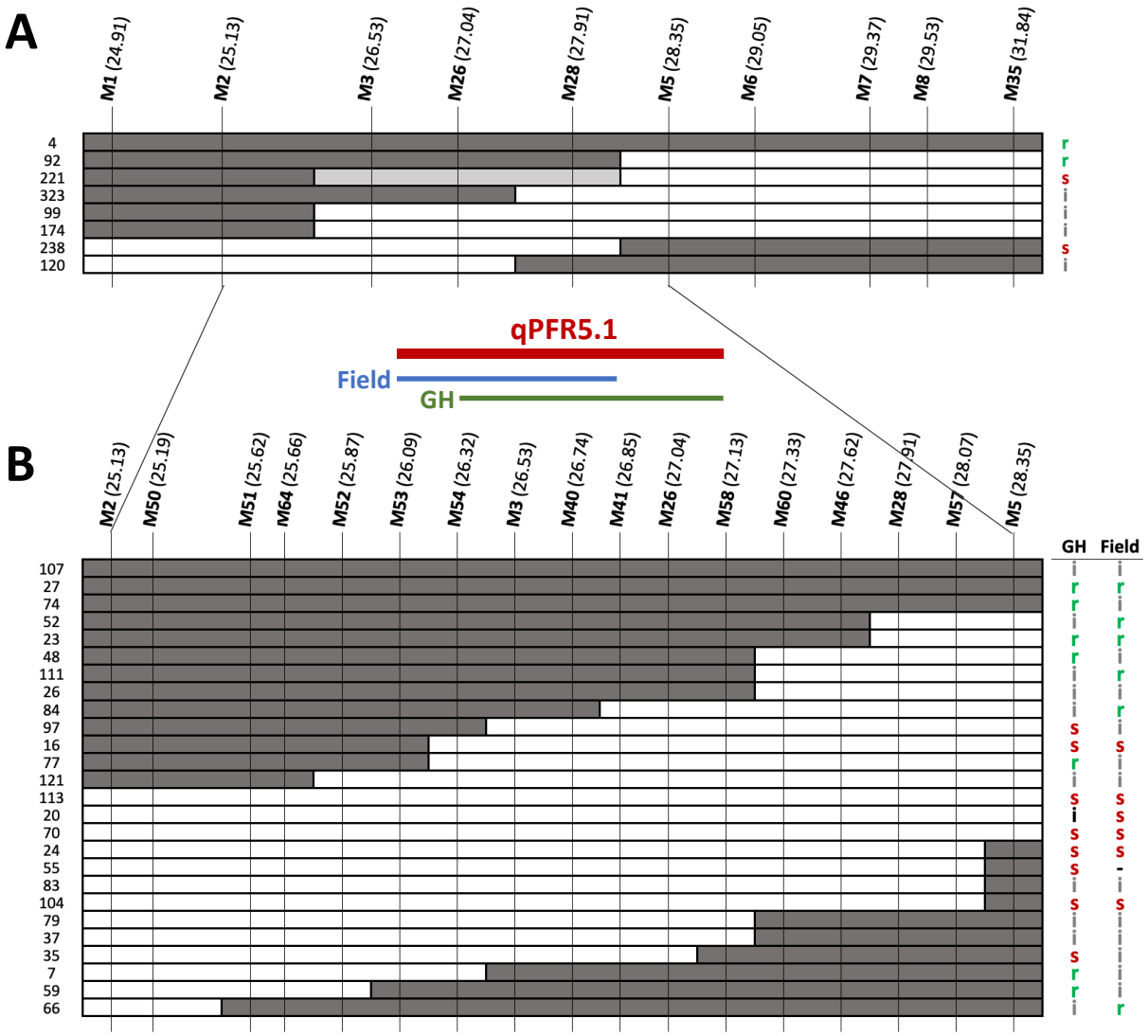


Figure 2.5. **Fine mapping of *qPFR5.1* in (A) RIL population and (B) F_3 families.** Dark, white, grey bars represent 'A4-3', 'Gy14', and heterozygous alleles, respectively. Letters on the right indicate phenotypes of each line/family: r, resistant (score < 4.5); s, susceptible (score > 6.0); I, intermediate (scores 4.5 – 6.0). Genomic locations shown in parentheses for each KASP marker (Mb) as per Gy14 v. 2.1 (CuGenDB v.2; http://cucurbitgenomics.org/v2/).

Table 2.2. Significant SNPs identified in multiple GWAS models (FarmCPU, Blink, MLMM, and MLM) for young fruit resistance in the cucumber core collection.

SNP	Chr	Position (bp) [1]	p-value / PVE (percent phenotypic variation explained) (%)			
			FarmCPU	BLINK	MLMM	MLM
S1_21117743	1	21,117,743	-	5.15E-13 / 9.01	-	1.73E-8 / 7.74
S2_10199046	2	10,199,046	-	8.31E-10 / 3.63	-	-
S2_10226744	2	10,226,744	3.85E-5 / 3.60	-	9.76E-10 / 24.49	5.95E-9 / 1.44
S3_18480786	3	18,480,786	1.06E-8 / 0.38	-	-	-
S3_37752706	3	37,752,706	3.37E-11 / 1.53	4.49E-10 / 2.09	-	-
S4_8024257	4	8,024,257	-	2.64E-9 / 2.25	-	-
S5_23563699	5	23,563,699	1.73E-8 / 3.23	-	-	-
S6_29086459	6	29,086,459	2.70E-8 / 2.03	-	-	-
S6_29175300	6	29,175,300	3.31E-11 / 5.48	-	-	-
S7_3391182	7	3,391,182	9.52E-9 / 0.79	-	-	-
S7_17739386	7	17,739,386	-	6.04E-9 / 1.35	-	-

[1]Genomic locations are according to Gy14 v. 2.1 (CuGenDB v.2; http://cucurbitgenomics.org/v2/)

Table 2.3. Geographical origin of accessions carrying the alternate alleles for the significant SNPs for young fruit resistance to *P. capsici* as identified by GWAS.

SNP	Effect[b]	Region of origin[a]								
		Africa	Europe	East Asia	Central/ West Asia	India/ South Asia	North America	Turkey	Other	Total
S1_2111743	↓	-	-	-	2	9	1	2	-	14
S3_37752706	↓	7	28	82	13	24	13	30	-	197
S4_8024257	↓	4	24	1	11	18	4	32	2	96
S5_23563699	↓	-	1	1	1	26	3	-	-	32
S6_29175300	↓	-	2	1	-	27	4	-	-	34
S3_18480786	-	1	2	23	2	5	1	-	-	34
S2_10226744	↑	1	2	23	-	2	2	-	-	30
S7_3391182	↑	5	43	79	30	47	37	32	2	275
S7_17739386	↑	1	17	17	7	15	10	17	-	84

[a] The regions are as defined in Wang et al. (2018).

[b] The effect of alternative alleles compared to reference alleles in disease score. ↓ - decreased disease score (more resistant); ↑ - increased disease score (more susceptible)

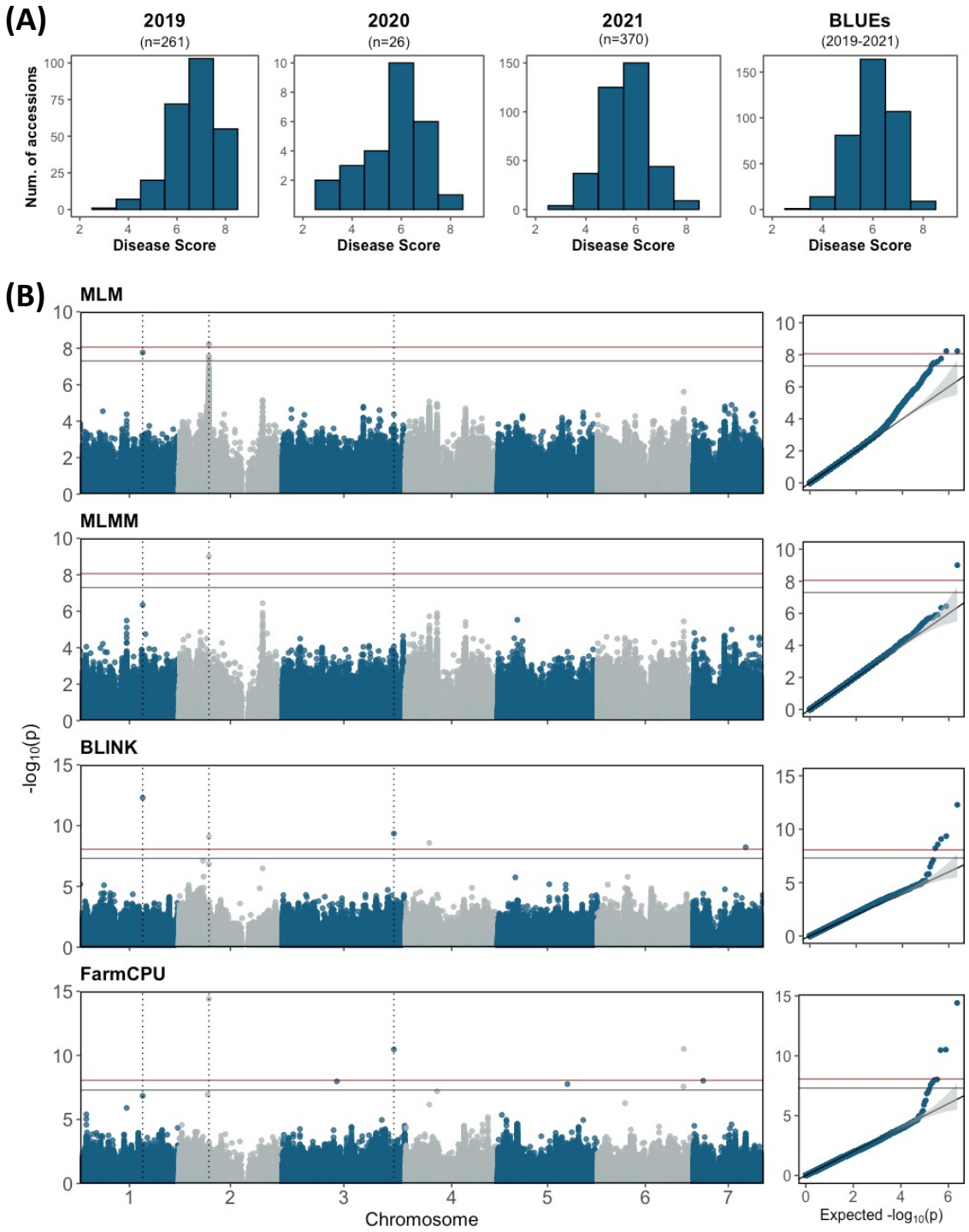


Figure 2.6. (A) Disease score distribution for young fruit resistance to *Phytophthora capsici* from the cucumber core collection and BLUE distribution of combined data 2019-2021. The score for each accession is the mean of 30-50 fruits. (B) Manhattan plots and quantile–quantile plots of the genome-wide association study analyses for young fruit resistance in the cucumber core population. The horizontal blue and red lines represent significance thresholds of Bonferroni -corrected *P* values of 0.05 and 0.01, respectively. The dotted vertical lines show the locations of SNPs that were significant in at least two models.

XP-GWAS of the extreme phenotype bulks

Precise phenotyping is crucial to identify QTL associated with traits of interest, especially for quantitative traits composed of multiple small effect QTL. However, depending on the trait, phenotyping can be expensive, especially when screening a diversity panel with many lines. To determine reproducibility of QTL identified from GWAS and increase replication and accuracy of phenotyping, we used an XP-GWAS approach (Yang et al., 2015). The accessions with extreme resistant or susceptible phenotypes (29 accessions in each bulk) were retested for additional phenotyping in 2022.

The disease score distributions of the resistant and susceptible bulks showed clear differences in multiple years (**Figure 2.7A**) and was reproduced in the replicated trial in 2022 (**Figure 2.7B**), verifying accuracy of the bulk selection for XP-GWAS analysis. Correlations for the selected resistant and susceptible bulks among 2019, 2021, 2022 were 0.755-0.912. SNP data from the selected accessions were combined via in-silico bulking as described in methods. XP-GWAS analysis identified 165 significant SNPs (5% FDR threshold) distributed across the seven chromosomes. The 39 significant SNPs based on the Bonferroni corrected p=0.05 threshold were located on chromosomes 1 and 5 (**Figure 2.7C, Supplementary Table 2.3**). The XP-GWAS SNP identified on chromosome 5 overlapped with the QTL identified by QTL-seq.

Identification of QTL for age-related resistance

QTL-seq analysis

Screening for ARR was performed on two populations: an F_2 population from 'Gy14' (ARR-) (ARR+) (**Figure 2.8A**); and DH lines produced from F_1 seed of 'Gy14' × 'Poinsett 76' (**Figure 2.8B**). Plants were grown in the greenhouse and a single, hand-pollinated fruit per plant was harvested at 16-18 dpp. The 15 most resistant and susceptible F_2 individuals were selected

for QTL-seq analysis (mean disease ratings of 1.32 and 7.88 for resistant and susceptible bulks, respectively; 1-9 scale). Disease scores for fruit from the 79 DH lines (3-5 fruit/line) showed high within-line variability for lines showing intermediate susceptibility; fruit from the most resistant and susceptible lines responded consistently with a mean rating of 0 and 4.8, respectively (scale 0-5) (**Supplementary Figure 2.3A**). Seed from the 15 most resistant and susceptible DH lines were regrown and fruit were phenotyped in a second screen (**Supplementary Figure 2.3B**). The eight lines with the most consistent resistant and susceptible disease ratings in both screens were selected.

DNA from the 15 most resistant and susceptible F_2 individuals and the 8 most resistant and susceptible DH lines were pooled for QTL-seq bulk segregant analysis. A total of 72,699 and 92,607 filtered SNPs were used in the F_2 and DH analysis, respectively. Both analyses identified a major locus associated with resistance on chromosome 3, *qPARR3.1* (*Phytophthora ARR 3.1)* located at 34.62-38.07 and 31.08-41.68Mb, respectively (**Figure 2.8A, B**). To verify the QTL, KASP markers flanking the QTL on chromosome 3 were used to genotype 768 F_2 seedlings and individuals homozygous for 'Gy14' or 'Poinsett 76' within the QTL region were self-pollinated to produce F_4 lines. Phenotyping of fruit from a replicated trial (5 plants/F_4 family) verified a strong effect of the QTL (**Figure 2.8D**).

Identification of genes of interest within the linked locus

Transcriptome analysis of the parental lines at 8 and 16 dpp was used to identify genes of interest within the region of *qPARR3.1*. ARR results from developmental changes that occur prior to inoculation, either as a result of production of preformed resistance mechanisms, or a change in capacity to rapidly respond to infection (Mansfeld et al., 2017, 2022). Therefore, we sought to identify developmental changes in gene expression that are unique to cultigens that

become resistant vs. those that remain susceptible. Genes were considered of interest if they showed differential expression with age in 'Poinsett 76' *and* were also differentially expressed in 'Poinsett 76' vs. 'Gy14' at 16dpp. Of the 1,240 annotated genes in this region (CL9930v2; CuGenDB), only four genes were uniquely upregulated in resistant 'Poinsett 76' (i.e., up in 'Poinsett 76' fruit peels at 16 dpp vs. 'Poinsett' 76 at 8 dpp, and in 'Poinsett 76' at 16 dpp vs. 'Gy14' at 16dpp). Thirteen genes were uniquely downregulated in resistant peels (down in 'Poinsett 76' fruit peels at 16 dpp vs. 'Poinsett 76' at 8 dpp, and in 'Poinsett 76' at 16 dpp vs. 'Gy14' at 16dpp) (**Figure 2.9A; Supplementary Table 2.4**).

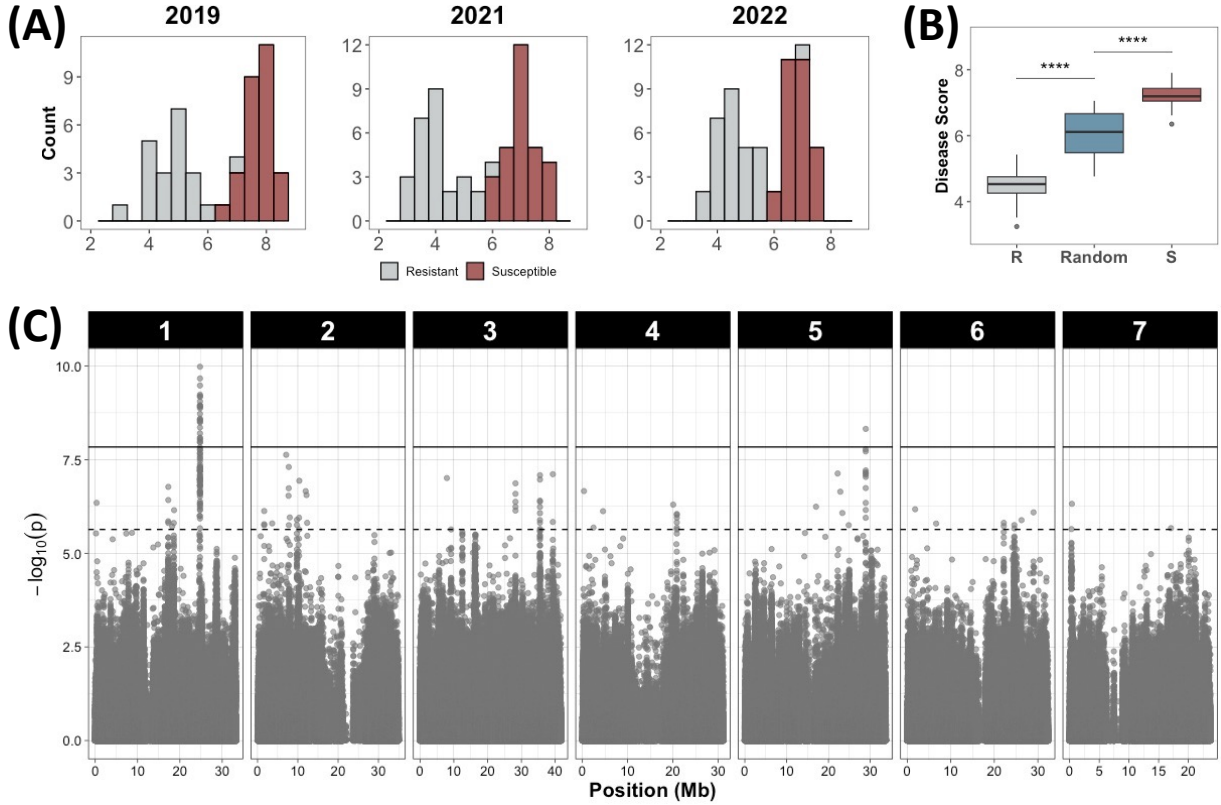


Figure 2.7. **Disease score distribution and Manhattan plot of the XP-GWAS analysis to identify SNPs associated with young fruit resistance.** (A) Disease score distribution of the resistant and susceptible bulks in different years. (B) Disease score values of the resistant I, susceptible (S), and random bulks (**** indicates P<0.0001, Wilcoxon test). (C) Manhattan plot of the XP-GWAS analysis. The dashed line indicates the 5% FDR threshold; the solid line indicates significance threshold of Bonferroni-corrected *P* value of 0.05.

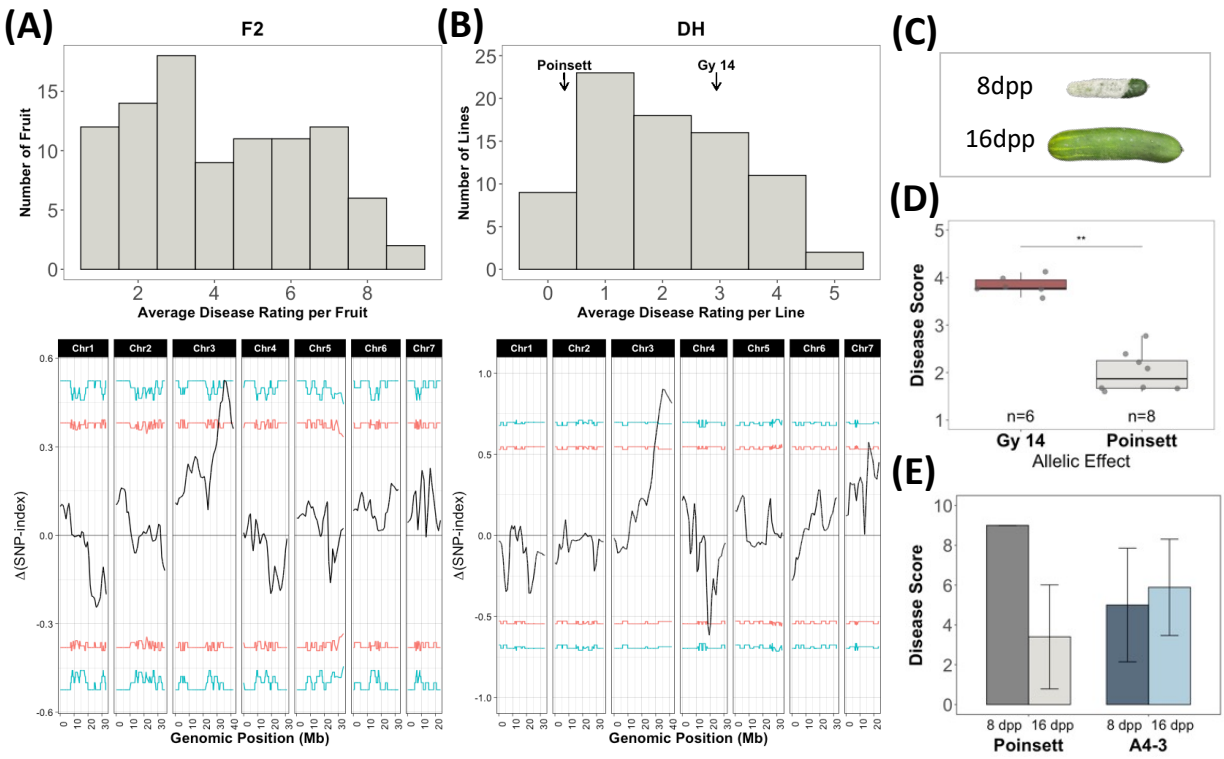


Figure 2.8. QTL-seq analysis for age-related resistance (ARR) to Phytophthora fruit rot using segregating populations derived from 'Gy14' (ARR-) × 'Poinsett 76' (ARR+). (A) Top: Disease rating distributions for F_2 individuals (n=95). All fruit were harvested at 16-18 dpp. Disease rating on a 1-9 scale. Fruit were scored at 10 dpi. The 15 most resistant and susceptible individuals were selected for QTL-seq. Bottom: QTL-seq analysis. Δ(SNP-index) was calculated with a window size of 2Mb. Horizontal lines represent confidence thresholds of 95% (red) and 99% (blue). **(B)** Top: Disease rating distributions for doubled haploid lines (n=79, 3-5 fruit/line). Disease ranking on 0-5 scale. Fruit were scored at 7 dpi. The 8 most resistant and susceptible lines were selected for QTL-seq. Bottom: QTL-seq analysis. Δ(SNP-index) was calculated with a window size of 2Mb. Allele frequencies for the resistant and susceptible bulks are provided in Sup. Fig. 1. **(C)** Phenotype of 'Poinsett 76' fruit at 8 and 16 dpp photographed at 7 days post inoculation (dpi). **(D)** Mean disease ratings of F4 families (5 plants/F_4 family) homozygous for the 'Gy14' or 'Poinsett 76' allele within the QTL on chromosome 3. **I** Disease ratings for 'Poinsett 76' and 'A4-3' fruit harvested at 8 and 16 dpp and scored at 7 dpi. Each value is the mean of 8-14 fruit.

To further examine genes potentially contributing to ARR, we screened our previously published transcriptome data from developing 'Vlaspik' fruit (ARR+) compared to 'Gy 14' fruit (ARR-) (Mansfeld et al., 2017) to identify genes uniquely up- or downregulated in both ARR+ cultivars at 16 dpp. Of the 17 genes identified above, one upregulated gene and three downregulated genes had similar expression patterns in 'Poinsett 76' and 'Vlaspik' (**Figure 2.9B**). The up-regulated gene, *Csa3G872720 (CsGy3G041010 in Gy14 v.2.1),* showed consistently significantly higher expression in resistant 'Poinsett 76' and 'Vlaspik' at 16 dpp. Expression in 16 dpp 'Poinsett 76' fruit was >2.5-fold higher compared to 8 dpp susceptible 'Poinsett 76' fruit and susceptible 'Gy 14' 16 dpp fruit. Similarly, expression in resistant 'Vlaspik' fruit was greater than 4-fold higher when compared to susceptible 'Gy14' 16 dpp fruit, and 1.8-fold higher when compared to susceptible 'Vlaspik' 8 dpp fruit. In contrast, while the downregulated genes had statistically lower values in resistant fruit in both genotype comparisons, the patterns observed were not obviously consistent with our model of ARR; i.e., distinctly different expression levels in 16 dpp resistant fruit compared to the susceptible samples.

CsGy3G041010 located at 38,365,049-38,373,217 bp (Gy14 v. 2.1), is annotated to encode a putative RING-type E3 ubiquitin transferase, a U-box domain and WD40 repeat containing protein. Sequence comparisons from 2 kb upstream until the end of its 3'UTR in 'Gy 14' (ARR-), 'Poinsett 76' (ARR+) and 'Vlaspik' (ARR+) identified 15 SNPs and 5 INDELs (**Supplementary Table 2.5**) that differed between 'Gy14' and 'Poinsett 76'. Four of the variants were within 2kb upstream of the transcription start site; eight were in introns, one of which was close to a splice site; three were in the 3'UTR. Six SNPs were in exons, five of which cause non-synonymous amino acid changes. 'Vlaspik', which is a commercial F_1 hybrid, was heterozygous at all variants. In contrast to 'Poinsett 76' which shows a strong drop in disease score for 16 dpp

vs. 8 dpp fruit, the disease rating of 'A4-3' remained essentially constant at 8 dpp vs. 16 dpp indicating the distinct nature of ARR and young fruit resistance in these genotypes (**Figure 2.8E**). The *CsGy3G041010* allele in 'A4-3' matches that of 'Gy14'.

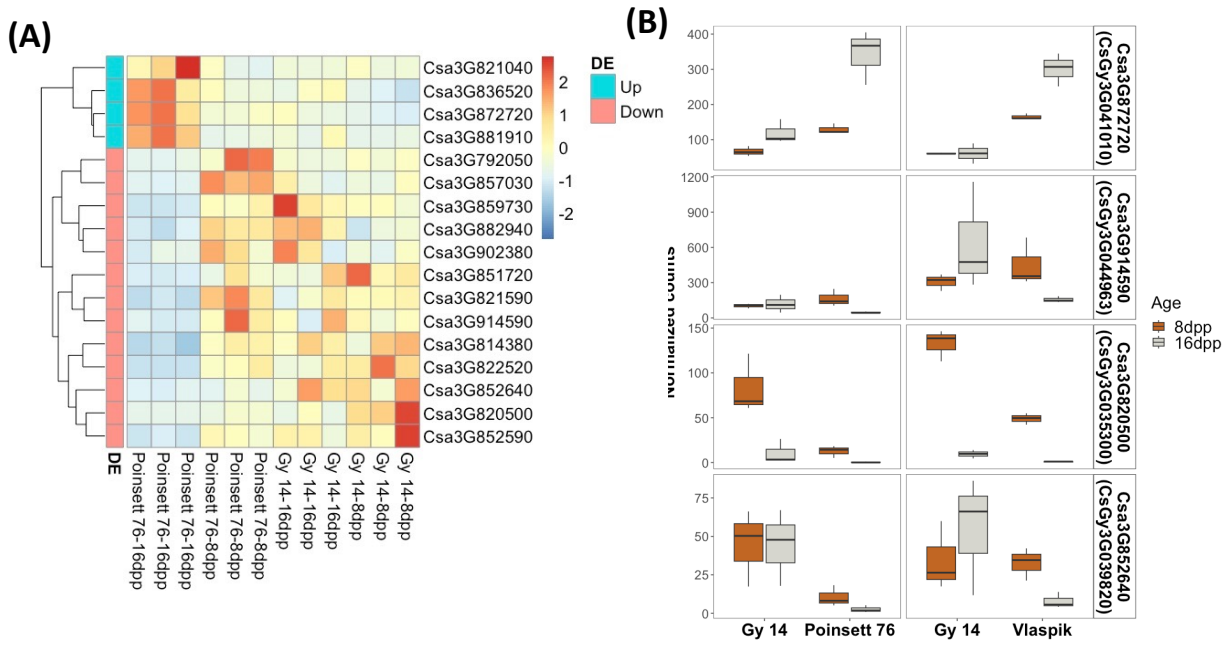


Figure 2.9. Identification of genes uniquely expressed in resistant fruit. (**A**) Heatmap of genes within the locus identified as uniquely differentially expressed in resistant-aged (16 dpp) 'Poinsett 76' fruit. Row clustering was based on Euclidean distances. Heatmaps are scaled by row and indicate deviation relative to mean expression across all samples. Gene names are according to Chinese Long v.2 (CuGenDB; http://cucurbitgenomics.org/). (Read count data and corresponding Gy14v.2.1 gene names are provided in Supplemental Table 4.) (**B**) Genes uniquely up- or down-regulated in transcriptome comparisons with two ARR+ genotypes ('Poinsett 76' and 'Vlaspik'). Boxplots show the distribution and median (dark line) of normalized read counts from two experiments. 'Gy 14' is susceptible at both ages (8 and 16 dpp); 'Poinsett 76' and 'Vlaspik' are susceptible at 8 dpp and become resistant at 16 dpp. Three biological replicates of each age-genotype combination were used in each experiment.

Discussion

Employing multiple strategies to detect QTL for resistance to Phytophthora fruit rot

The types of populations most frequently used to identify QTL associated with traits of interest are segregating biparental populations and genetically variable natural populations. In this work, both types of populations were used to search for QTL associated with young fruit

resistance to Phytophthora fruit rot in cucumber. QTL-seq, a bulk segregant analysis (BSA) approach performed on progeny from biparental populations that express extreme phenotypes, provides a quick and specific way to detect QTL. Furthermore, using different population structures such as F_2, RIL, and DH can provide additional power for QTL detection. QTL-seq was performed on both young fruit resistance and ARR. The analyses discovered three QTL associated with the young fruit resistance and one for ARR. For young fruit resistance, the QTL were located on chromosomes 1, 5, and 6; the strongest effect was from the QTL on chromosome 5, *qPFR5.1*. For ARR, which appears to have a major gene component, a QTL at the end of chromosome 3 was identified. The ability to identify QTL and the size of the genomic region identified is limited to the genetic variation between the two parents and is influenced by population structure and size (Mackay and Caligari, 2000; Li and Xu, 2021). The lengths of QTL from QTL-seq can be large, for example, *qPFR5.1* was ~7 Mb and *qPARR3.1* was ~10 Mb. To narrow the QTL for young fruit resistance, screening of additional RIL and F_3 populations that were enriched for recombination within the region refined *qPFR5.1* to ~1 Mb.

The second approach, use of a diversity panel such as the cucumber core collection, provides the opportunity to identify additional QTL associated with the resistances in a germplasm with higher genotypic diversity. The main drawbacks of using a diversity panel include higher expenses to phenotype and genotype the large population size, and the difficulty to detect rare alleles associated with the traits (Alqudah, et al., 2020; Uffelmann et al., 2021). In addition to traditional GWAS, where the whole collection is phenotyped and genotyped, a second method of association analysis, extreme-phenotype genome-wide association study (XP-GWAS) can be used to reduce experimental costs by reducing the number of entries to be included (Yang et al., 2015). XP-GWAS is typically used to reduce sequencing costs and has been applied to

several crops with different target traits. Some recent examples include rice (Xiao et al., 2017), apple (Kumar et al., 2022), switchgrass (Poudel et al., 2021), sugar beet (Ries et al., 2016), and wheatgrass (Crain et al., 2023). In this case, however, as sequence data was already available for the full collection (Yu et al., 2023), we were able to use XP-GWAS to reduce phenotyping costs associated with additional replications. By focusing on the lines with extreme phenotypes, the rare alleles associated with resistance can be enriched within the bulk and are more likely to be detected (Yang et al., 2015; Zou et al., 2016). Although performing XP-GWAS still requires known phenotypes of each line, once candidates for the extremes are identified, the subsequent phenotyping expense for replication can be reduced. In this research a preliminary screening of the complete cucumber core collection was performed from 2019-2021. To verify the phenotypes, selected accessions were grown in the following year with additional replications to increase the accuracy of phenotyping.

It should be noted that when performing association analysis, the same data sets analyzed using different programs can give somewhat different results due to the default assumptions written within each software. As a result, peak SNPs may be offset by several Mb. For example, the significant SNP on chromosome 3 detected in rMVP (Ying et al., 2021), another widely used R-based GWAS software, was about 3 Mb away from the significant SNP detected on chromosome 3 in GAPIT3.0 (34,803,363 vs. 37,752,706, respectively). Therefore, replication and comparison among tools and models are recommended to avoid false positives (Chanock et al., 2007). Similarly, different experiments with standard QTL or QTL-seq analyses can give somewhat different estimates of QTL location (e.g., powdery mildew and downy mildew resistance QTL (Wang et al., 2020). In other cases, QTL may be somewhat complex, composed of more than one contributing factor as was observed for cucumber downy mildew (Berg et al.,

2020), possibly contributing to different assessments of QTL location. These observations can have implications for consideration of appropriate regions for introgression of disease resistance QTL.

Using multiple approaches, several SNPs significantly associated with resistance to Phytophthora fruit rot were identified in closely-located positions, giving greater confidence to their contributions **(Figure 2.10)**. In our results, regions were identified by more than one approach on chromosomes 1, 2, 3, 5, 6, and 7. The QTL peaks detected on chromosome 1 were located at 21.17 Mb by GWAS and 24.75 Mb by XP-GWAS. The QTL on chromosomes 5 and 6 were consistently identified by QTL-seq, GWAS and XP-GWAS methods (on chromosome 5 all were located within 7 Mb, and on chromosome 6 all were within 3 Mb). On chromosome 3, the peak SNP was located at 37.75 Mb in GWAS and at 39.29 Mb in XP-GWAS. Both fall within the QTL region previously identified from QTL-seq analysis for ARR including the candidate gene *CsGy3G041010* located at 38.36 Mb. In most cases, the QTL identified for Phytophthora fruit rot also coincided with previously identified QTL for other diseases.

The Identification of multiple QTL for young fruit resistance Is consistent with quantitative traits consisting of multiple small effect QTL. Studies identifying QTL for resistance to *P. capsici* in other species also have indicated polygenic architecture, including several examples in pepper (*Capsicum annum*) and squash (*Cucurbita pepo* and *Cucurbita moschata)* (Barchenger et al., 2018; Ramos et al., 2020; Vogel et al., 2021). The signal on chromosome 5 was stronger in QTL-seq and XP-GWAS compared to GWAS, possibly due to the use of the resistant line ‘A4-3’ in the bi-parental QTL-seq analysis, and the enrichment of rare alleles in the resistant bulk for XP-GWAS analysis.

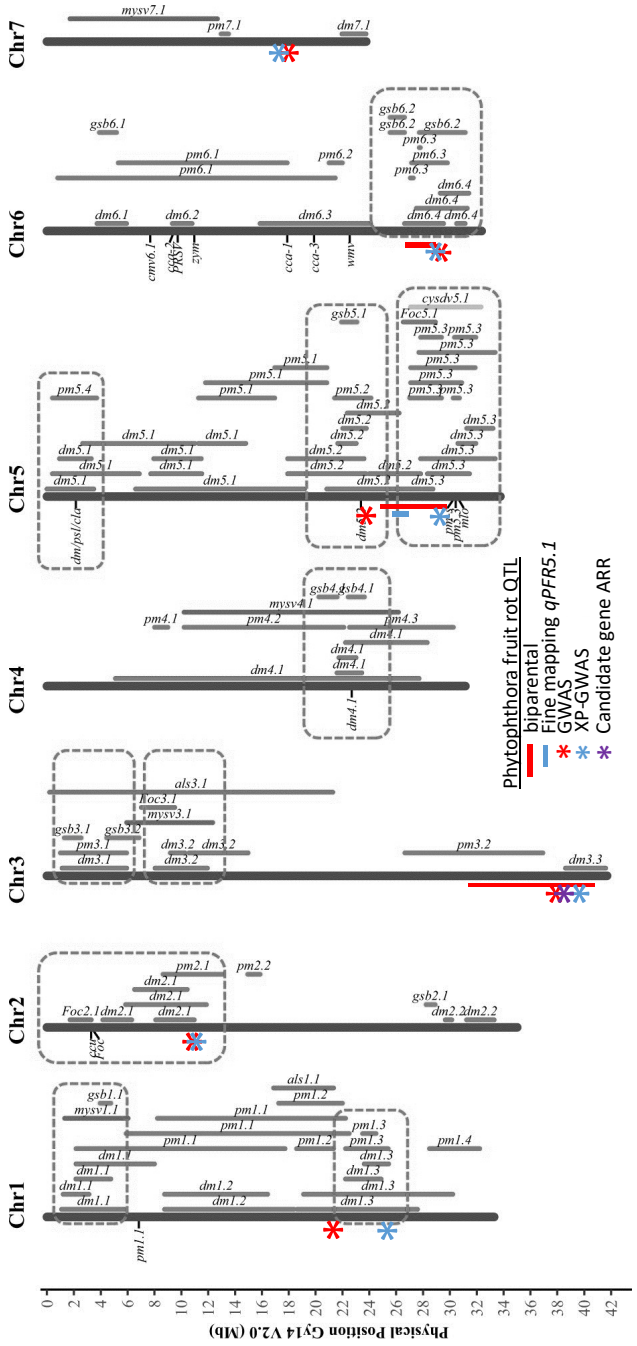


Figure 2.10. **Chromosomal locations of QTL identified for Phythophthora fruit rot of cucumber in relation to prior QTL identified for resistances to other cucumber diseases.** The indicated PFR QTL were identified from multiple analyses: red bar – biparental QTL-seq; blue bar – fine mapping of biparental populations; red asterisk GWAS of cucumber core collection; blue asterisk XP-GWAS; purple asterisk – candidate gene identified by RNAseq analyses. Figure is adapted from Wang et al., 2020 *Horticulture Research* under Creative Commons license http://creativecommons.org/licenses/by/4.0/. Abbreviations: DM, downy mildew; PM, powdery mildew; ALS, angular leaf spot; Foc, fusarium wilt; GSB, gummy stem blight; MYSV, melon yellow spot virus; CYSDV, cucurbit yellow stunting disorder virus.

Although the SNPs identified from the association analyses in this study were screened for young fruit resistance, *qPARR3*.1 from QTL-seq analysis was also detected by GWAS and XP-GWAS analyses. This may result from closely located genes within the *qPARR3*.1 region that confer young fruit resistance. It may also be possible that for some accessions the ARR-associated QTL may function earlier during fruit development and contribute to young fruit resistance. A BLAST search (Swiss-Prot database) of the protein sequence for the candidate gene for ARR on chromosome 3 reveals that this gene is a homologue of the *LIN* gene found in *Medicago truncatula* (E = 0e0, Identity = 58.38%, Positives = 72.86%). In *M. truncatula* LIN functions in early rhizobial symbiotic nodule formation and mutation of LIN leads to a suppression of nodule development (Kiss et al., 2009). *LIN* was shown to not be required for nodule organogenesis, however *LIN* expression was associated with rhizobial root infection (Kiss et al., 2009). Interestingly, while other rhizobial-symbiosis mutants were more resistant to infection, the *lin-2* mutant was shown to be extremely susceptible to the pathogen *Phytophthora palmivora* (Rey et al., 2015). It has been hypothesized that the protein modulates defense responses by means of its U-box domain and the ubiquitination of target proteins (Kiss et al., 2009).

In addition to the QTL identified by QTL-seq in biparental populations, novel SNPs were also discovered by association analyses, providing potential additional resources for future breeding efforts. Multiple alternative alleles of the significant SNPs leading to stronger resistance were found in accessions originating from India and South Asia, the primary and secondary centers of origins of cucumber (Lv et al., 2012; McCreight et al., 2013). During the process of subsequent domestication and dissemination, cucumber germplasm diverged between East Asia vs. Eurasia and the West (Europe, Africa, North America) (Qi et al., 2013; Wang et al., 2018). This divergence, which is evident in fruit morphology, is also reflected in genetic composition,

showing differentiation of cucumber into three major phylogenetic clades: India/South Asia, East Asia, and the West (Qi et al., 2013; Wang et al., 2018; Grumet et al., 2021). Consistent with the resistance-associated alleles identified in this study, prior screening indicated that the accessions that were resistant to Phytophthora fruit rot were mainly from India, especially North and Central India (Colle et al., 2014; Grumet et al., 2020). Although there were also resistant accessions that originated from East Asia, alleles associated with higher susceptibility were more frequently traced to East Asian accessions.

QTL hotspots for disease resistance

Numerous studies have been conducted to identify QTL of various disease resistances in cucumber as summarized in a recent review by Wang et al. (2020). Collectively these studies implied the presence of disease resistance gene/QTL hot spots on several chromosomes. Interestingly, most of the QTL and significant SNPs identified here for Phytophthora fruit rot also co-localized with the hot spots including resistances to downy mildew, powdery mildew, fusarium wilt, and gummy stem blight (**Figure 2.10**). The aggregation of these QTL suggests that these genomic regions play an important role in disease resistance to fungal and oomycete pathogens. Oomycete and fungi are two evolutionary distinct groups; however, they share similar strategies in terms of infection, e.g., specialized infection structures such as appressoria, infection hyphae and haustoria, and secreting cell-wall-degrading enzymes to facilitate cell wall penetration (Latijnhouwers et al., 2003). Though the mechanisms of resistances to these pathogens remain unknown, similar or the same defense-associated genes might be activated in response to infection and result in clusters of disease resistance QTL.

In addition to *Phythophora* the other major disease leading to severe loss of pickling cucumber production in midwestern United States is downy mildew caused by

Pseudoperonospora cubensis (Savory et al., 2011; Hausbeck and Lamour, 2004). Toward the end of chromosome 5 there are two disease resistance QTL clusters including resistances to powdery mildew and downy mildew pathogens. *qPFR5.1* for young fruit resistance identified in this work is located in one of these clusters and is adjacent to two downy mildew resistance QTL, *dm5.2* and *dm5.3* (Wang et al., 2016; Tan et al., 2022). The narrowed QTL identified by fine mapping allowed us to distinguish the boundaries between these three QTL. With distinct borders between the QTL, molecular markers targeting specific QTL can be designed for marker assisted selection, and to develop breeding lines that can confer multiple diseases by pyramiding resistance QTL from different genetic backgrounds.

Conclusions

Resistance to *Phytophthora capsici* is an agronomically important trait in cucumber but currently no resistant commercial varieties are available due to the limited research and intricate genetic natures of both the pathogen and the host. A combination of approaches of QTL-seq and associated analyses were used to identify QTL for resistances to Phytophthora fruit rot in cucumber. Multiple QTL were identified for young fruit resistance. The largest effect QTL, *qPFR5.1*, was located on chromosome 5, and narrowed to approximately 1 Mb. A major effect QTL for ARR, *qPARR3.1*, was found at the end of chromosome 3, and a candidate gene identified from comparative transcriptomic analyses of cucumber peels. Additional SNPs associated with resistance were discovered from GWAS and XP-GWAS analyses of the USDA cucumber core collection. The close vicinity of the QTL and SNPs identified from multiple analyses strengthened the credibility of these findings. Several of the findings also corresponded with previously identified disease resistant hot spots in cucumber. Collectively, the results of this work can provide useful information for future studies to understand mechanisms of resistance to *P.*

capsici in cucumber and breed for varieties with resistance to Phytophthora fruit rot.

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APPENDIX A: Supplementary Materials

Supplementary Tables (provided in a separate Excel file)

Sup. Table 2.1. List of cucumber core collection accessions tested for young fruit resistance to Phytophthora capsici in GWAS and XP-GWAS analyses.

Sup. Table 2.2. KASP primer sequences and the location and ref/alt alleles of the targeted SNPs for fine mapping *qPFR5.1*.

Sup. Table 2.3. Significant SNPs detected in XP-GWAS analysis. Threshold indicates significance of the SNPs.

Sup. Table 2.4. Genes within the *qPARR3.1* locus identified as differentially expressed uniquely in resistant-aged `Poinsett 76' fruit.

Sup. Table 2.5. Polymorphisms detected starting from 2 kb upstream of *Csa3G872720* until the end of its 3'UTR in four genotypes: 'Chinese Long 9930' (ARR-, the reference genome), 'Gy 14' (ARR-), 'Poinsett 76' (ARR+) and 'Vlaspik' (ARR+).

Supplementary Figures

Figure A2.1. SNP-indexes in QTL-seq analyses for young fruit resistance and age-related resistance (ARR) in cucumber.

Figure A2.2. Allelic effect of significant SNPs associated with young fruit resistance identified from GWAS analysis.

Figure A2.3. Disease distribution and individual selection in ARR DH population.

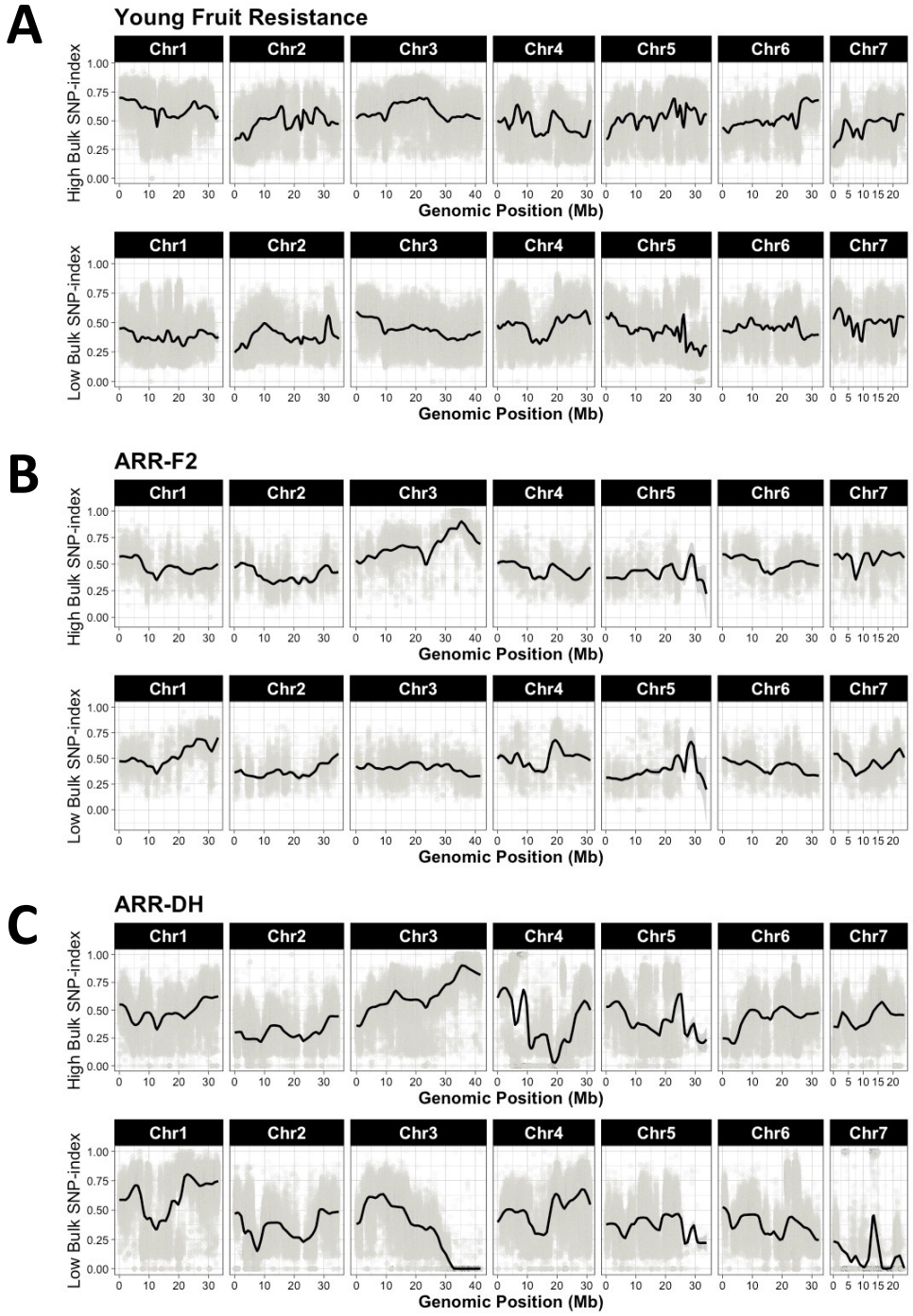


Figure A2.1. SNP-indexes in QTL-seq analyses for young fruit resistance and age-related resistance (ARR) in cucumber. (A) Young fruit resistance (B) ARR in F_2 population, (C) ARR in doubled haploid (DH) population.

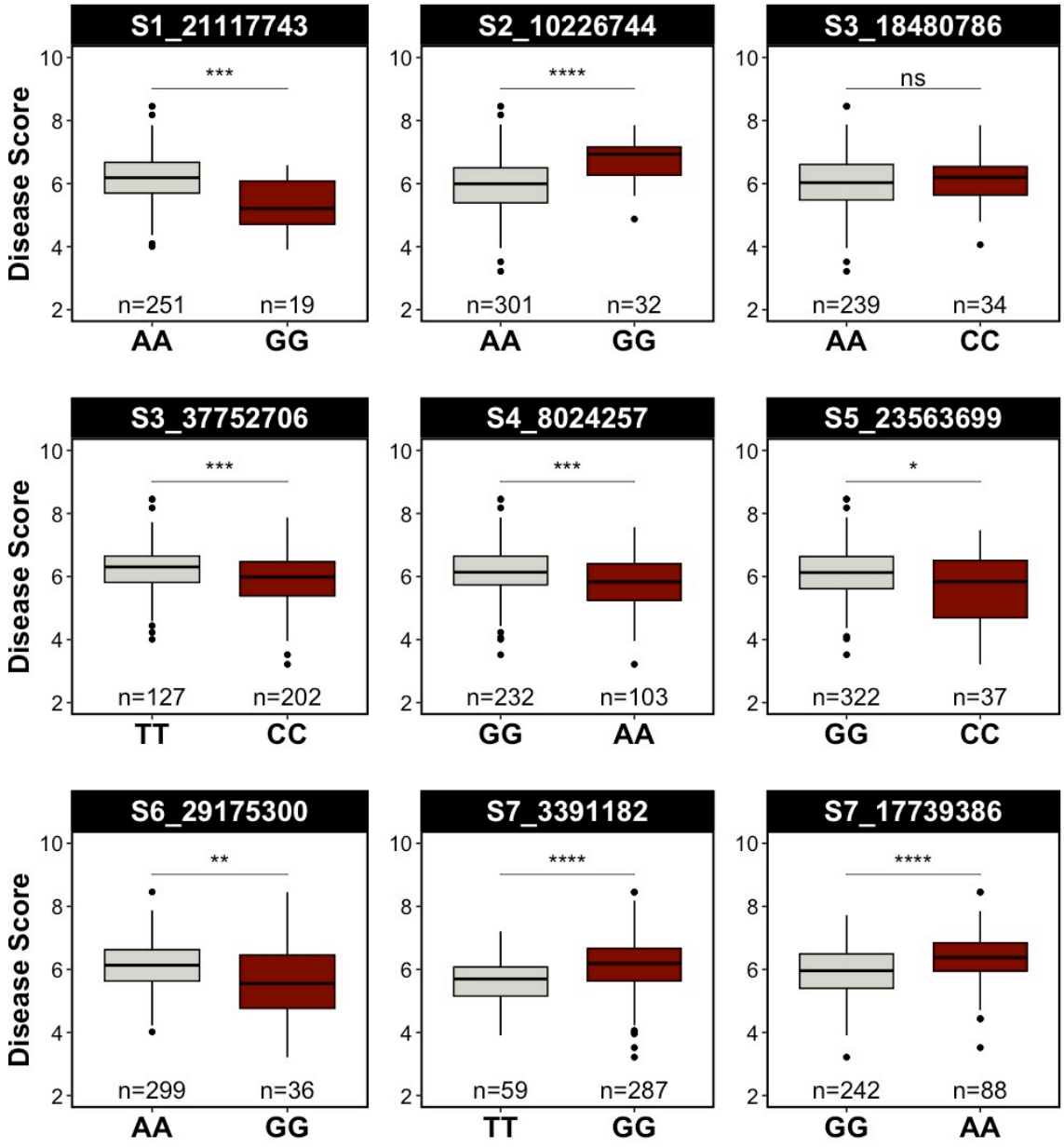


Figure A2.2. Allelic effect of significant SNPs associated with young fruit resistance identified from GWAS analysis.

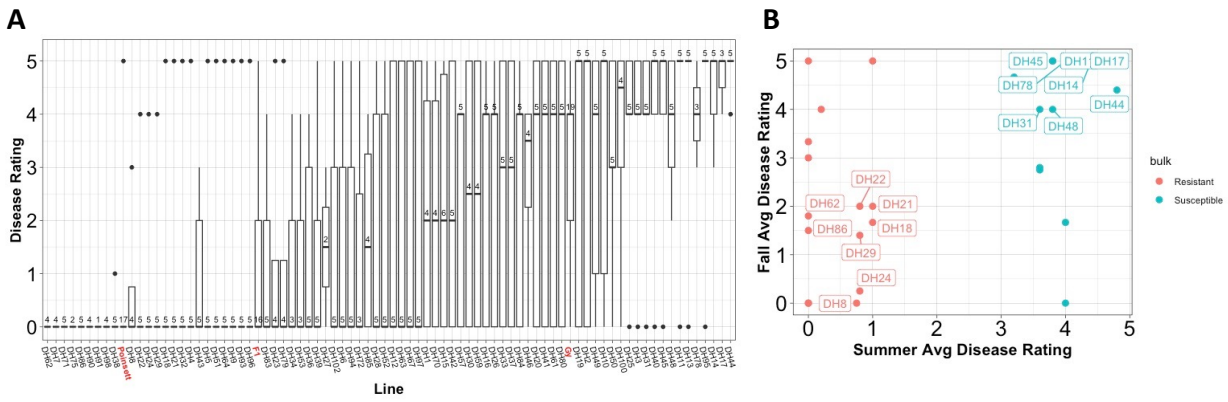


Figure A2.3. **Disease distribution and individual selection in ARR DH population.** (A) Disease rating distributions within each doubled haploid line. Lines are sorted by mean disease rating. Dark lines indicate the median disease rating for each line. Numbers above the median indicate the number of fruit tested for that line. In red are the parental lines and the F_1. (B) These lines were then regrown and phenotyped in a second verification screen in the fall of 2018. The y axis represents the mean line disease rating scores from the first screen (Summer) and the x axis represents the score in the second screen (Fall). Lines labeled showed consistent phenotypes and were selected for bulk segregant analysis.

CHAPTER 3

Phenotypic and Genetic Characterization of Morphological Diversity in the Cucumber Core Collection

Introduction

Cucumber (*Cucumis sativus* L.), which is primarily produced for consumption of immature fruit, is one of the most highly cultivated vegetable crops worldwide. *C. sativus* has undergone a long history of domestication. Its origin is thought to be in India, China, Burma, Thailand, with primary and secondary centers of diversity in India and Southeast Asia, respectively (McCreight et al., 2013; Naegele and Wehner, 2016). Following initial domestication, cucumber was introduced to China ~2,000 years ago, and was further introduced to East Asia (e.g., Japan) around ~100 CE (Lv et al., 2012). It was also dispersed westward to Europe and Africa through two routes: Persia into eastern and northern Europe by land in 6th or 7th century, and from Persia to western and southern Europe by sea in 10th century (Staub et al., 2008; Paris et al., 2012). The plant was later brought to the Americas by Columbus in 1490s.

Through extensive natural and artificial selection, modern cultivated cucumber shows great differences compared to its wild progenitor. For example, cultivated cucumber has fewer lateral branches and bears fewer but larger fruits (Harlan, 1992). Many have thicker flesh with smaller seed cavity size and no intercentrum hollow, as well as no bitterness (Abbo et al., 2014; Weng, 2021). The plants also have larger seeds without dormancy, which can facilitate the cultivation process. Despite these general features, cucumber fruit morphology varies geographically, reflecting selection based on local environments, consumer preference, and target market classes (Weng, 2021; Grumet et al., 2023). The fruits at immature stage are usually crisp with underdeveloped seeds for ease of consumption. The major types of cucumber can be categorized based on region and usage (fresh or processing). In East Asia, long and slender cucumber are preferred and mostly consumed fresh. They can be further classified into North and South China types. The North China type, also called “Chinese Long”, typically has long and

tapered fruit covered with dense white spines, while the South China type has cylindrical fruit with green and white stripes and fewer black spines (Weng, 2021). North American and Europe fresh market cucumbers (slicing cucumbers) have smooth skin and intermediate fruit length (20-30 cm), while the processing cucumbers (pickling cucumbers) tend to have thicker and warty fruit surface, and are generally short in length (5-15 cm). Another major market type is parthenocarpic cucumber, typically produced in greenhouses, which includes two classes: long-fruited, about 30-40 cm long, and Beit Apha (Mediterranean) cucumber, which has shorter fruit (12-15 cm) (Weng, 2021; Grumet et al., 2023).

Breeders seeking to develop improved cultivars with crucial traits such as resistances to abiotic and biotic stresses to cope with existing and emerging challenges, must also meet market standards for fruit quality. These traits include, but are not limited to, fruit size and shape, skin and flesh color, spine density, as well as internal traits such as seed cavity size, flesh thickness and hollowness. All of these features are crucial when considering the commercial value of a variety. Identification of QTL or genes associated these desirable fruit quality traits can facilitate breeding efforts to introgress novel resistance or production traits into new cultivars.

Recent years have seen a rapid increase in genomic tools available for cucumber. The USDA-SCRI CucCAP project: Leveraging Applied Genomics to Increase Disease Resistance in Cucurbit Crops, has been developing publicly available genetic, genomic , and bioinformatic tools for the crops in the *Cucurbitaceae* family (Grumet et al., 2020). One of the tools developed was a cucumber core collection derived from the USDA Agriculture Research Service National Plant Germplasm System cucumber collection (NPGS; https://www.ars-grin.gov/Collections#plant-germplasm) (Wang et al., 2018). The NPGS cucumber collection comprised of 1,314 cultivars, landraces, and varieties was sequenced using genotyping-by-sequencing (GBS). The resulting

core collection consists of 388 accessions, which capture > 96% of the allelic diversity in the NPGS cucumber collection, and also includes historical cultivars with important agronomic traits and disease resistance (Wang et al., 2018). To reduce heterozygosity and heterogeneity, the accessions were self-pollinated for 2 or 3 generations. The self-pollinated core collection accessions were re-sequenced at 30-40× coverage and used to call SNPs as described by Yu et al., (2023). The SNP data are accessible on Cucurbit Genomics Database website (CuGenDB, http://cucurbitgenomics.org/v2/).

In this work, we sought to provide phenotypic and genotypic information about key external and internal fruit quality traits present in the cucumber core collection. The resulting photographic and quantitative data have been deposited in the publicly accessible CuGenDB, and were used to perform Genome-wide association analysis (GWAS) analyses to identify QTL associated with the fruit quality traits. With known morphological features and genetic characterization of the accessions in the core collection, breeders can select suitable materials that fit breeding goals, and further devise efficient and effective strategies to introduce desirable traits while maintaining fruit quality standards for the market.

Materials and Methods

Plant materials and growth conditions

The cucumber core collection was selected based on genotyping-by-sequencing (GBS) data of United States National Plant Germplasm System (NPGS) collection (Wang et al., 2018). To reduce heterozygosity and heterogeneity within the accessions, individuals from each accession were self-pollinated for 2 or 3 generations. The self-pollinated core collection lines were re-sequenced at 30-40× coverage and used to call SNPs as described by Yu et al. (2023). The core collection lines were grown in the field from 2019-2022. The accessions that were tested

are listed in **Supplementary Table 3.1**.

The collection was grown at the field of Michigan State University Horticulture Teaching and Research Center (HTRC) in 2019-2022 with three plants per accession. Prior to transplanting, 300 lbs/acre of 19-19-19 fertilizer were applied to the field, and irrigation provided as needed throughout the season. The plants were grown on raised black plastic mulch with 0.45-0.6 m between plants, depending on the field design each year. The number of accessions planted each year varied depending on seed availability. Each accession has 1-4 years of phenotypic data for mature fruit and in most cases 1 year of data for young fruit (2021 or 2022).

Young fruits were harvested at about 5-7 days-post-pollination (dpp), and mature fruits were 30-40 dpp. A total of 10-12 fruits were collected from each accession, arranged by size, and six representative fruits were selected to photograph. The fruits were rinsed with distilled water to remove soil and debris, and then air dried. The fruits were placed a black cloth with a ruler and a color correction card for photograph. Data were collected using Fiji software (*Fiji Is Just ImageJ*, Schindelin et al., 2012). Multiple morphological traits were measured from the photographed fruits as described below.

Fruit trait measurements

Fruit length, diameter, fruit shape index, carpel number, seed cavity, flesh thickness, hollowness, curvature, and tapering

The fruit length was the distance between the blossom and stem ends; when fruits curved, length measurement followed the shape of the fruit (**Figure 3.1A**). Diameter was measured from a cross section at the mid-section of the fruit (**Figure 3.1B**). Fruit shape index was the ratio of fruit length/diameter (L/D ratio). Seed cavity size (sc), flesh thickness (ft) and hollowness (h) were measured as shown in **Figure 3.1B**.

Curvature and tapering were measured in young fruits. The curvature measurement was adapted from (Clement et al., 2013), which was the ratio of fruit diameter measured at the center of the fruit (d) and the distance from the center of the fruit to a line connecting the two ends of the fruit (d') (**Figure 3.1C).** Higher values indicate greater curvature. Multiple curves were measured if a fruit had more than one. Taper was the ratio of the fruit width at the first (q1) and third (q3) quarters of a fruit, with higher values indicating more tapering (**Figure 3.1D**).

Skin and flesh color, netting, and spine density

Each photo was color corrected using Chart White Balance plugin in ImageJ (https://imagejdocu.list.lu/plugin/color/chart_white_balance/start). RGB color values were measured at top, middle and bottom sections for mature fruits and the first and third quarters sections for young fruits. The netting was scored for each fruit based on the scale shown in **Figure 3.1E**, where the scale describes the fruit skin from smooth, to lightly and deeply netted surface. Spine density was measured on young fruits using ImageJ. The total number of spines on each fruit were counted using the cell counter plugin (https://imagej.nih.gov/ij/plugins/cell-counter.html) and divided by the area of the fruit. The area of the fruit was obtained by thresholding color to capture the shape of each fruit.

Genome-wide association analysis

The SNP data of the core collection was downloaded from CuGenDBv2 (Yu et al., 2023). SNPs were filtered using BCFtools (Danecek et al., 2021) and GATK (Van der Auwera et al., 2013) with the following criteria: bi-allelic, GQ scores >20, maximum read depth within one standard deviation of the mean read depth, minor allele frequency >0.1, missing rate <20%, resulting in 1,179,473 SNPs for association analysis. Marker-trait association analyses were performed using best linear unbiased estimates (BLUEs) calculated from multi-year data. BLUEs

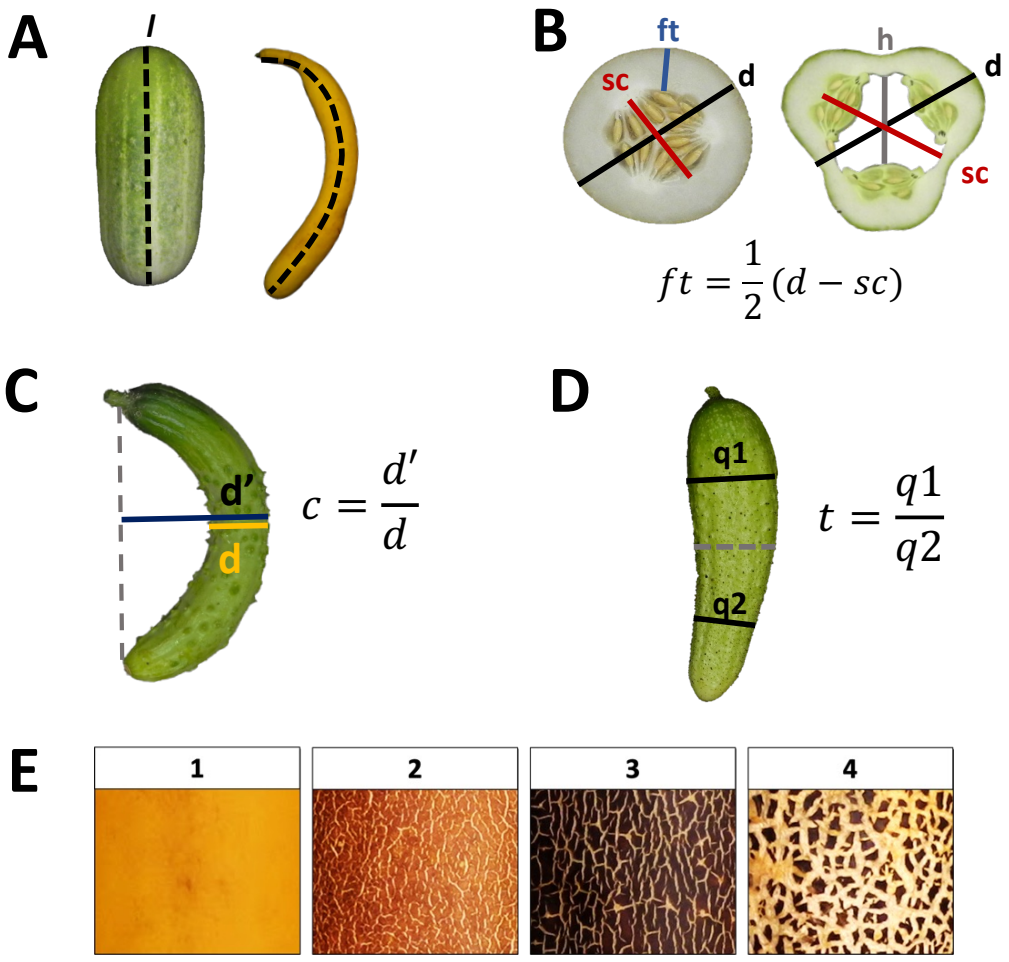


Figure 3.1. Illustration of cucumber fruit trait measurement. (A) fruit length (l); (B) internal traits: diameter (d, black lines), seed cavity (sc, red lines), hollowness (h, gray line) and flesh thickness (ft, blue line); (C) curvature; (D) tapering; (E) fruit netting rating scale.

were calculated using the R package lme4 (Bates et al., 2015). Association analysis was performed using GAPIT 3.0 (Genome Association and Prediction Integrated Tool) with MLM, FarmCPU, BLINK, and MLMM models implemented within the software (Wang and Zhang, 2021). The significance threshold was calculated based on Bonferroni correction (p-value/N, where N was the number of SNPs used in the analysis), where the thresholds of adjusted p-values of 0.05 and 0.01 corresponded to -log10(p) values of 7.37 and 8.06, respectively.

Results

Phenotypic data in the Cucurbit Genomics Database (CuGenDB)

Representative mature and young fruits were harvested from 388 accessions during 2019-2022. The fruits were photographed and phenotyped for a range of fruit quality traits. Examples showing diversity for various traits are shown in **Figure 3.2**. The list of accessions evaluated, region of origin and BLUE values for each measured trait are listed in **Supplementary Table 3.1**. The photographic and associated phenotypic data collected in each year have been deposited at Cucurbit Genomics Database (CuGenDB, http://cucurbitgenomics.org/v2/), where the resequenced genome data of the core collection is also publicly available.

Phenotypic diversity in the core collection

Profuse diversity in morphological external and internal traits in mature and young fruits was observed in the cucumber core collection (**Figure 3.2** and **Figure 3.3**). Most of the traits were normally distributed, such as mature fruit length, diameter, seed cavity size, flesh thickness, and tapering, suggesting that there are a wide range of diversity of these traits in the core collection. In contrast, several traits showed less variation. Only a few accessions had visibly noticeable hollowness at the center of the fruits. Most of the accessions had straight fruit, with low spine density and a smooth, non-netted fruit surface (**Figure 3.3**). Fruit shape related traits and internal traits were highly reproducible between years (R = 0.63-0.98) (**Supplementary Table 3.2**). There was a larger range of correlation in color traits among years (R = 0.11-0.89), likely due to variation in maturity at the time of harvest.

Fruit skin color in young fruits primarily varied for degree of greenness, but later exhibited a wide range of colors from dark brown, orange, green, to yellow as the fruit matured (**Figure 3.4A** and **Figure 3.4B**). In order to provide quantitative measurements of color to

facilitate GWAS analysis, RGB values were determined for color traits. When dissecting color into RGB descriptive values, low R/G values in young fruits depicted dark green; fruit become light green with increasing R/G/B values (**Figure 3.4A**). In mature fruits, less variation was observed in B value compared to R and G values, and low R/G values were associated with darker fruit surface. Mature fruit with high B values tended to be orange/yellow while those with low B values remained green (**Figure 3.4B**). Fruit flesh color was predominantly cream and white color, with a few showing green or yellow tint (**Figure 3.4C**). B values in flesh were substantially higher than in the peel.

Correlation between traits

Correlations among the traits are shown in **Figure 3.5**. Fruit shape-related traits such as young and mature L/D ratios, mature fruit length, and curvature were highly correlated to each other (r = 0.83-0.93), and moderately negatively correlated with diameter and seed cavity size (r = -0.59-0.72). The longer the fruit, the higher the L/D ratio and greater frequency of curvature. For fruit internal traits, diameter was positively correlated with seed cavity size, flesh thickness and hollowness (r = 0.56-0.79). Young fruit R and G values were negatively correlated with fruit shape traits such as L/D ratios, mature fruit length and curvature (r = 0.42-0.50), and positively correlated with seed cavity size and hollowness (r = 0.34-0.44), indicating that fruits with high L/D, small seed cavity size and small hollowness tend to have dark green young fruits. Netting is a notable trait that largely exists in wild accessions and landraces, which tend to have larger seed cavity size and hollowness, and was negatively correlated with fruit shape traits including mature fruit length, young and mature L/D ratios and curvature (**Figure 3.5**).

Accessions with long fruit shape and high L/D ratios predominantly originated from East Asia, where long, thin fruits are preferred (**Figure 3.6A**). Highly curved fruits were also mostly

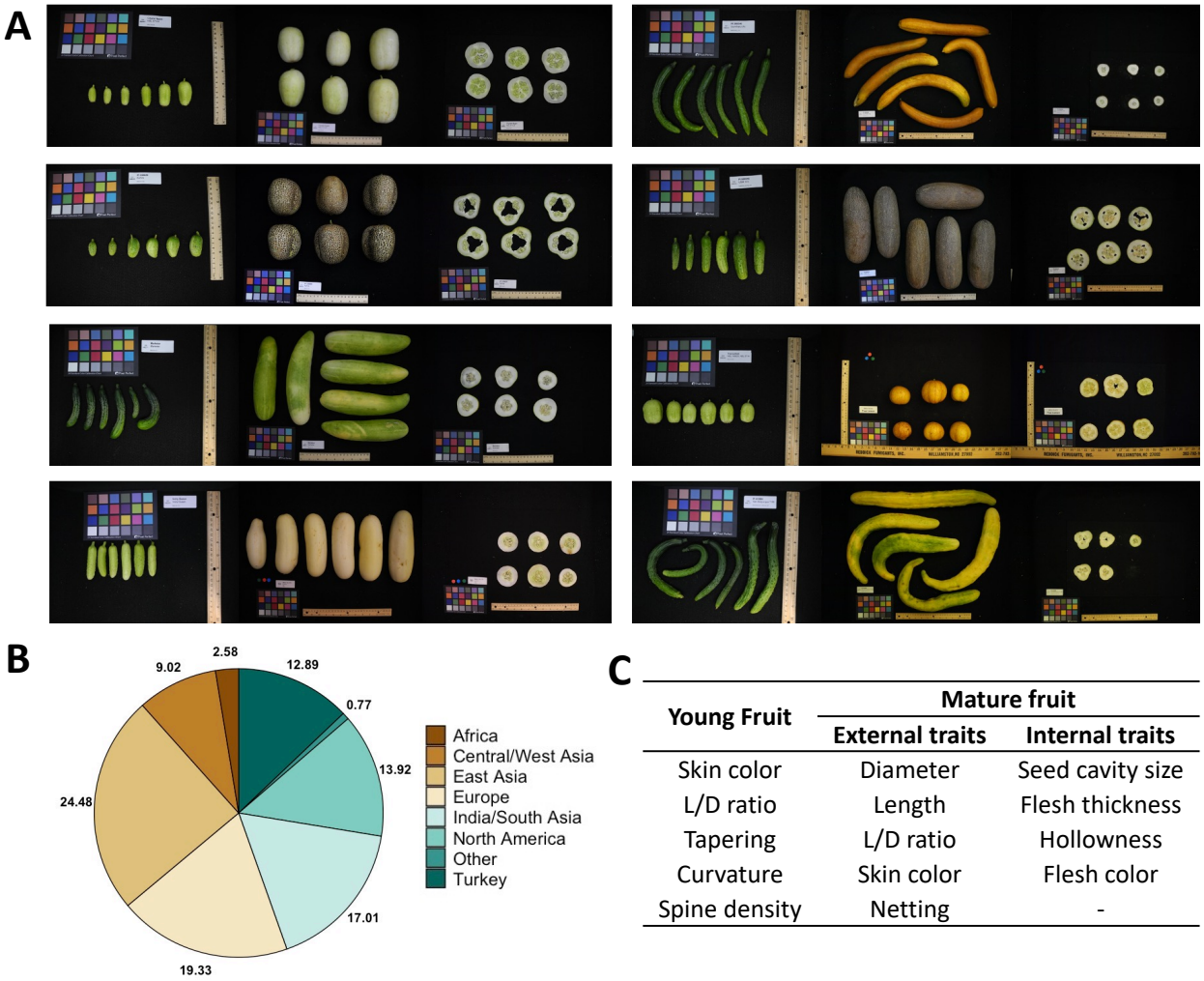
found in East Asia accessions. These accessions were more likely to have dark green young fruit (lowest R/G values) and turn yellow when mature (high R/G) (**Figure 3.6B**). While the majority

of the accessions had fruit with small seed cavity size and smooth skin surface, accessions from India and South Asia, which is the center of origin of cucumber, had extensive variation for these traits, for example, larger seed cavity size and hollowness, and deeply netted fruit surface. These accessions tended to have brown color mature fruits (mid R/G and low B values), and a few have flesh color with yellow tone (high R/G and low B values) (**Figure 3.6B**). Accessions from Central/West Asia and Africa also showed moderate variation for netting and hollowness.

GWAS on morphological external and internal fruit traits

GWAS was performed on the external and internal traits measured in mature and young fruits in the core collection using four models, FarmCPU, BLINK, MLMM, and MLM (**Supplementary Table 3.3**). The Manhattan and QQ plots of FarmCPU model are shown in **Figure 3.7**. The chromosomal locations of significant SNPs identified from the GWAS FarmCPU model, and the SNPs that have >10% PVE (phenotypic variance explained) are illustrated in **Figure 3.8**. Though the SNPs identified from the GWAS analysis were widely distributed across all chromosomes, QTL for some of the traits were closely clustered (**Figure 3.8**). For example, on chromosome 1 at ~10 Mb, SNPs for several highly correlated fruit size and shape traits, including mature fruit length, young fruit L/D, carpel number, and seed cavity size, were closely located. Several external fruit traits also mapped to same region on chromosome 1, such as netting, spine density, young fruit color R/G values.

Several QTL identified by GWAS fell within the region of previously identified fruit quality QTL and candidate genes (**Figure 3.8**). The cluster of genes located at ~10 Mb on chromosome 1 fell within the consensus QTL for fruit size (FS), and fruit shape index (FSI),



C

Young Fruit	Mature fruit	
	External traits	Internal traits
Skin color	Diameter	Seed cavity size
L/D ratio	Length	Flesh thickness
Tapering	L/D ratio	Hollowness
Curvature	Skin color	Flesh color
Spine density	Netting	-

Figure 3.2. Phenotyping fruit quality traits of the cucumber core collection. (A) Examples of photographic records of young fruit, mature fruit, and cross sections of the cucumber core collection. Graphic and phenotypic data for the full collection can be accessed at Cucurbit Genomics Database (CuGenDB) (http://cucurbitgenomics.org/v2/). (B) Geographic distribution of accessions in the core collection. (C) Phenotypic traits collected from young and mature fruits in the cucumber core collection.

CsFS1.1 and *CsFSI1.1*, (Pan et al., 2020). *CsFS1.1* was found to contribute to both length and diameter growth (Weng et al., 2015). On chromosome 2, significant SNPs for fruit diameter, mature fruit L/D, carpel number, and tapering were in the range of *CsFS2.1* and *CsFSI2.1* at ~10 Mb. *CsFS2.1* was found to contribute to fruit radial growth leading to round fruit shape of landrace WI7239 (Pan et al., 2017b). At ~5 Mb on chromosome 3, significant SNPs for flesh

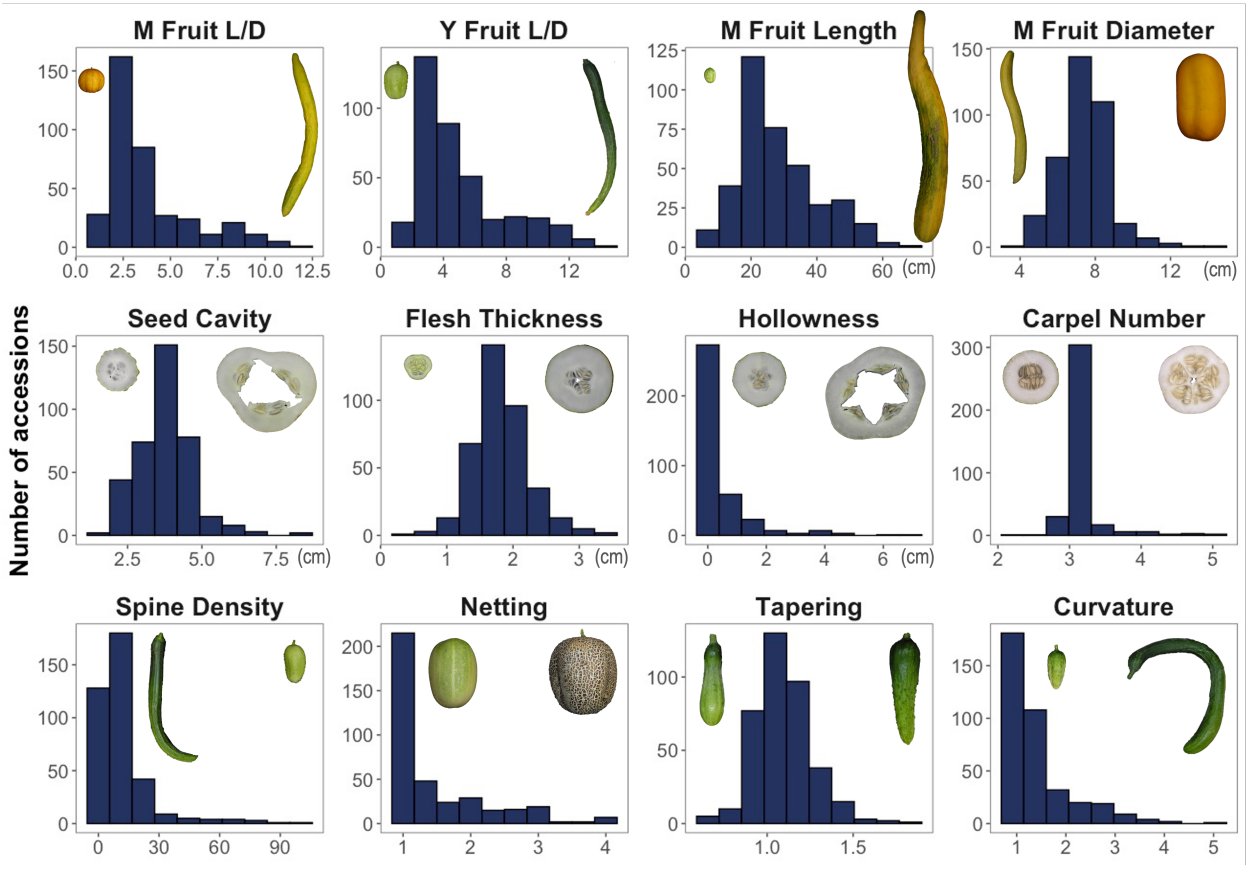


Figure 3.3. **Phenotypic distribution of mature and young fruits traits as observed in the cucumber core collection.** Phenotypes were measured as described in methods. Values of each trait were the BLUEs calculated from 1-4 years of data measured for each trait. M – mature fruit; Y – young fruit. Photos of each fruit within each plot are to scale.

thickness and carpel number were located at *fth3.1* (Yuan et al., 2008), while at the end of chromosome 3, several fruit size and shape related SNPs were also identified in the region of *CsFS3.2*, which was associated with fruit elongation (Weng et al., 2015; Pan et al., 2020). A QTL on chromosome 5 identified for spine density overlapped with a previously identified QTL for wart density, *fws5.1*. Though spine density was measured in this study, spine and wart densities are closely related traits (Shimomura et al., 2017). At beginning of chromosome 6, significant SNPs for seed cavity, mature L/D, carpel number and tapering were colocalized with *CsFSI6.1* (Pan et al., 2020). Consistent with an adjacent pair of fruit shape QTL previously identified at the end of chromosome 6 (*CsFS6.1* and *CsFS6.2*) (Pan et al., 2020), two QTL for mature fruit length

were also identified by GWAS in these locations (**Supplementary Table 3.3**). Lastly, a significant SNP for diameter was within the region of *CsFS7.2*, which was the consensus QTL combining multiple fruit shape QTL from Shimomura et al. (2017) (Pan et al., 2020). Although not a fruit shape and size trait, significant SNPs for netting were also located in the SNP clusters on chromosomes 1, 6, and 7, possibly due to the correlation of netting with other internal traits

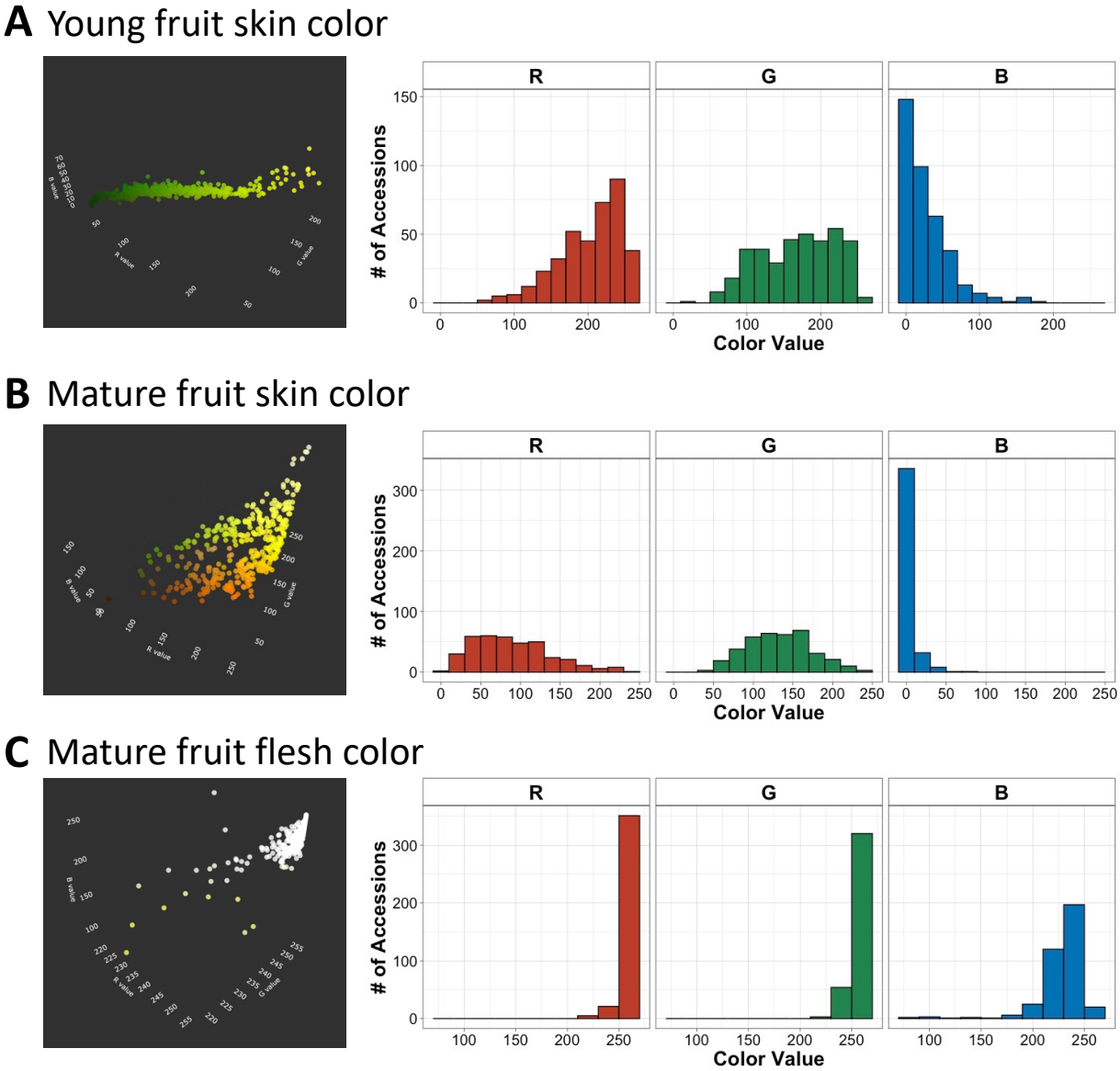


Figure 3.4. **Color traits of the cucumber core collection and the distribution of RGB values measured for each trait.** (A) young fruit skin color; (B) mature fruit skin color; (C) mature fruit flesh color.

and fruit shape traits. Although not detected by FarmCPU, SNPs located in the region of *scs1.2*, a QTL associated with seed cavity size (Yuan et al., 2008), was identified on chromosome 1 in the BLINK model (**Supplementary Table 3.3**).

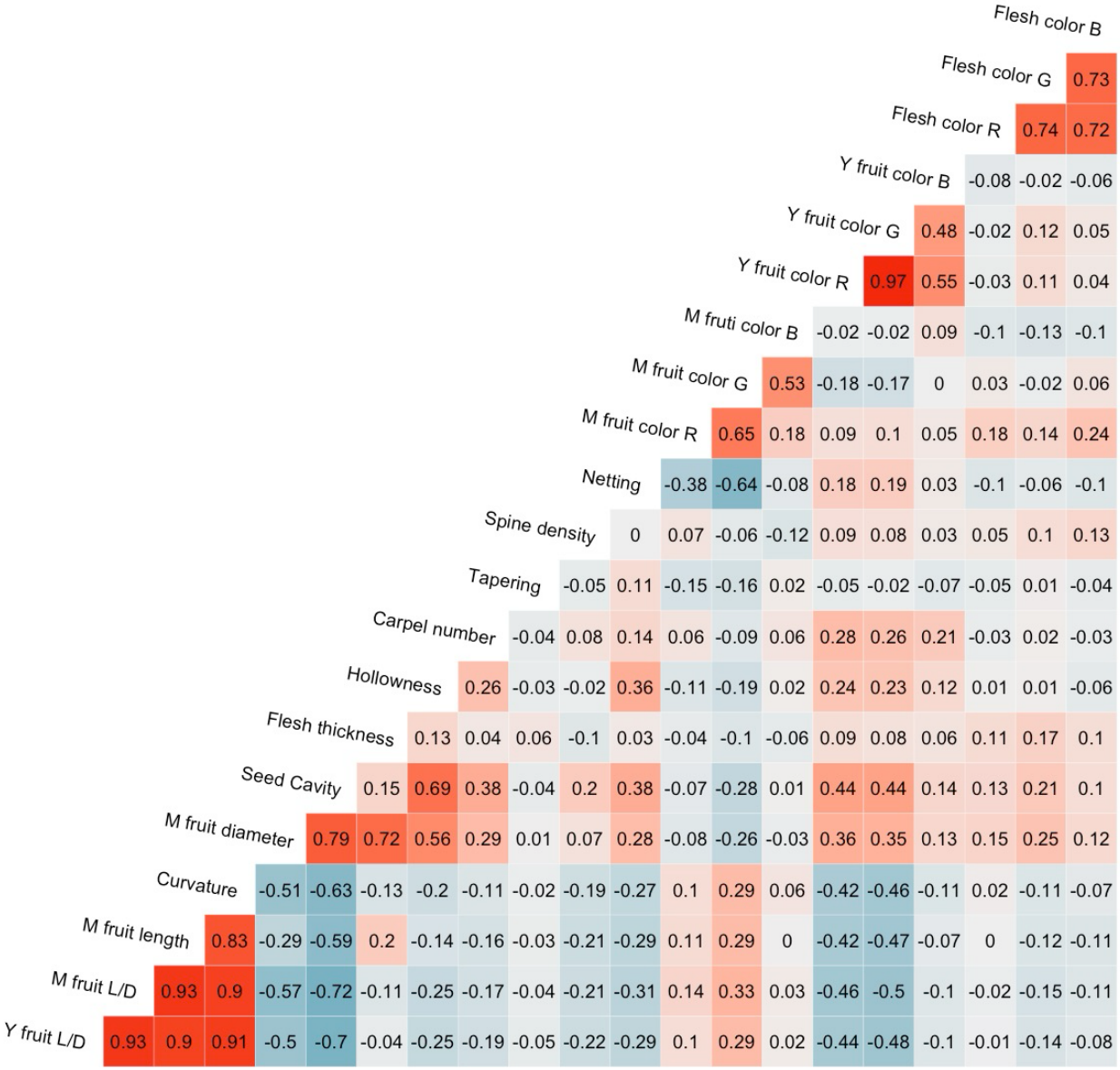


Figure 3.5. **Correlation (r) matrix of the external and internal traits for young and mature fruits in the cucumber core collection.** M – mature fruit; Y – young fruit.

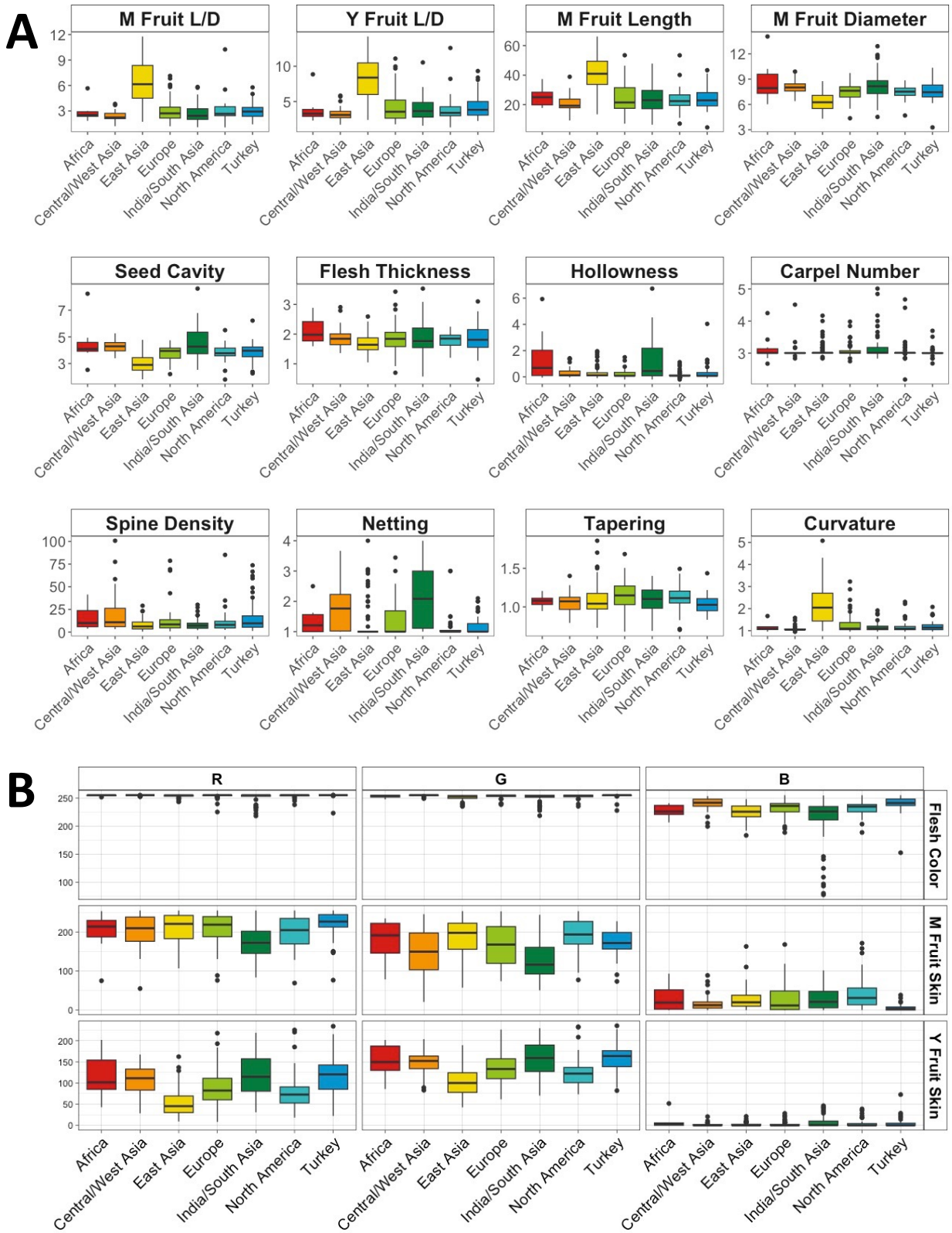


Figure 3.6. **Distribution of the fruit traits in the core collection based on originated region of origin**. (A) fruit external and internal traits; (B) color traits. M – mature; Y – young.

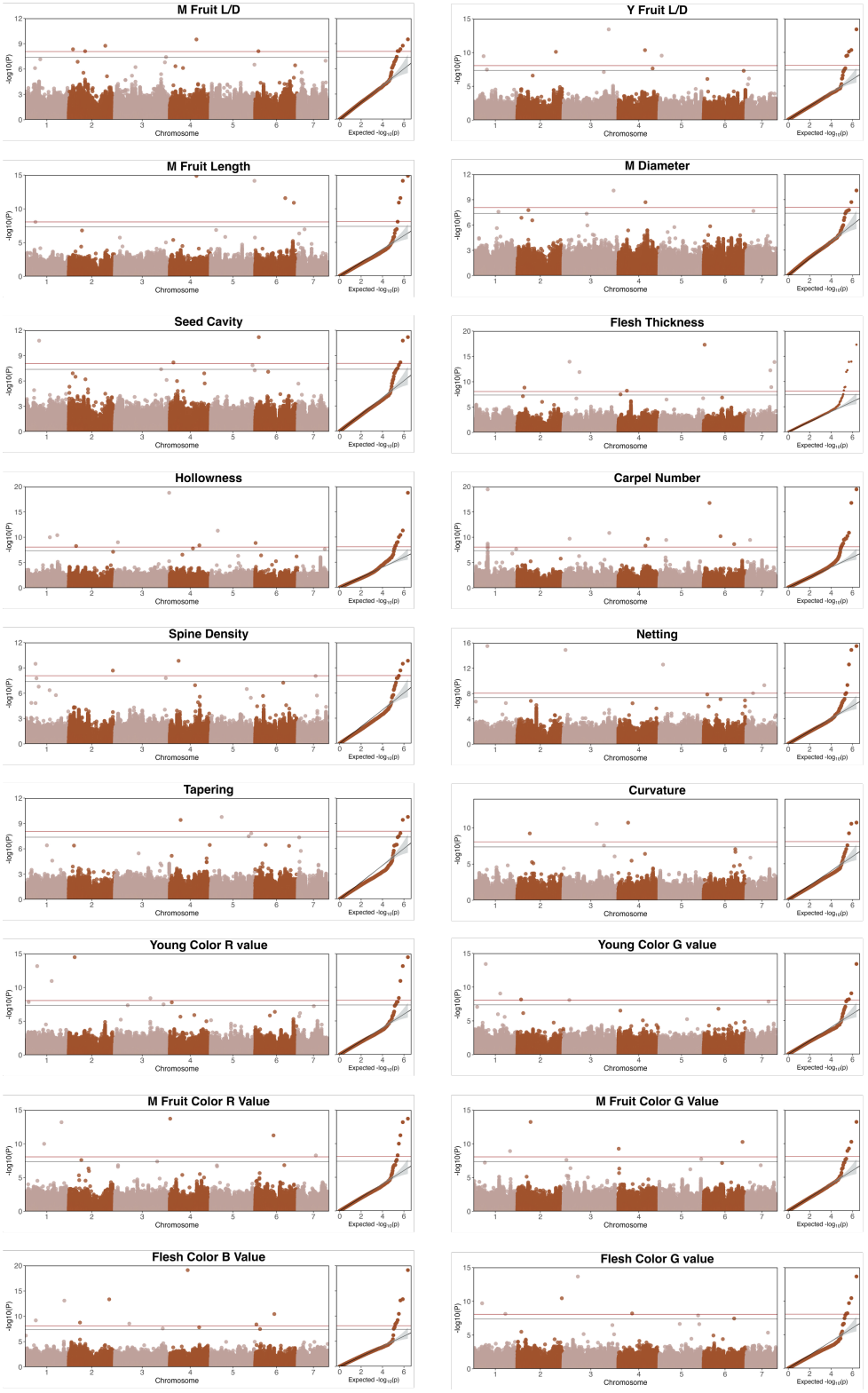


Figure 3.7. **Manhattan and quantile-quantile (QQ) plot of GWAS analysis of the morphological traits using FarmCPU model in cucumber core collection.** The gray and red horizontal lines represent p-value of 0.05 and 0.01, respectively.

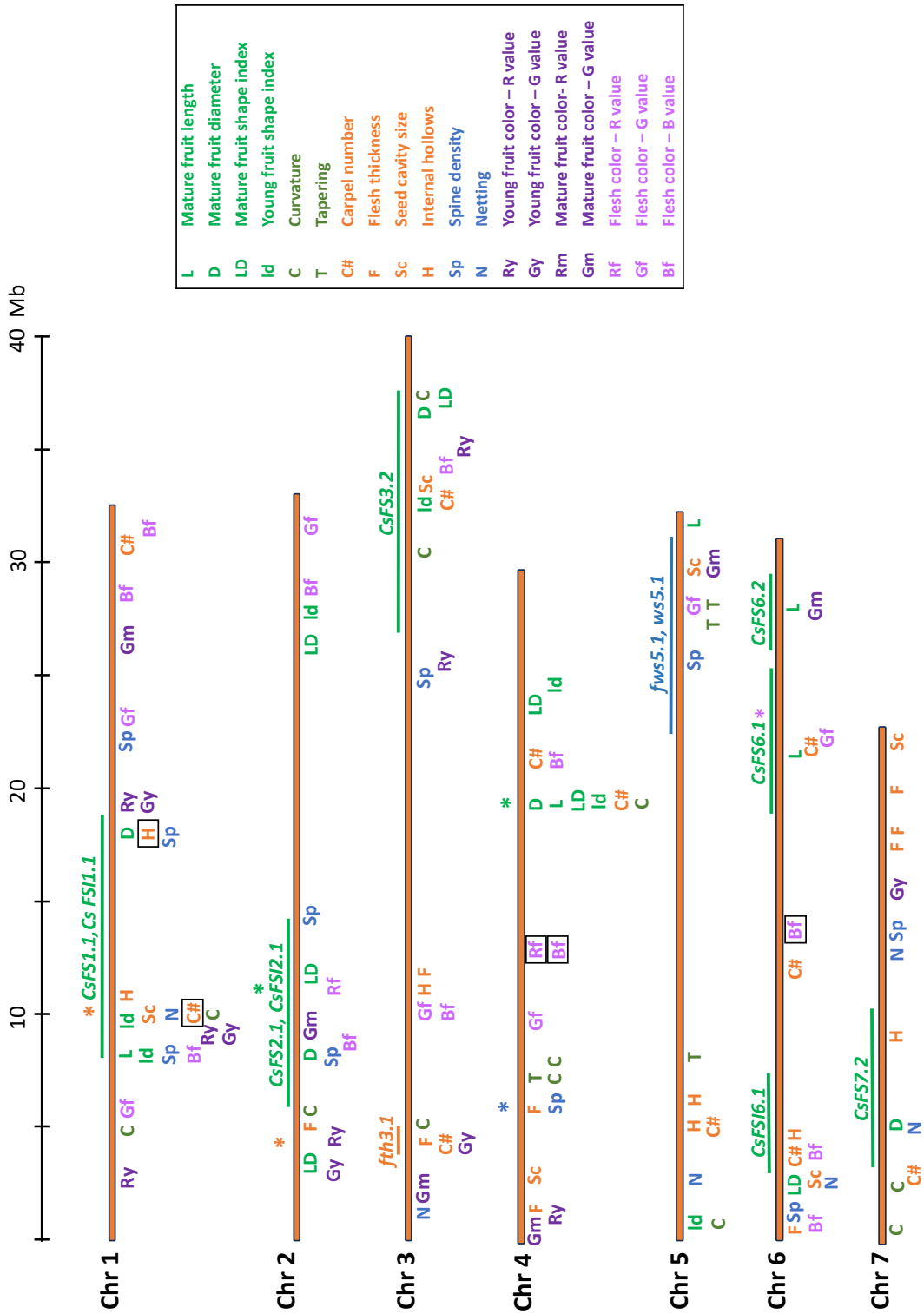


Figure 3.8. Chromosomal location of the significant SNPs identified in GWAS analysis using FarmCPU model for the morphological traits measured in the cucumber core collection. SNPs with >10% PVE (phenotypic variance explained) are boxed. The asterisks indicate candidate genes identified from prior studies. QTL nomenclature follows Wang et al. (2020b).

Table 3.1. Previously identified QTL or genes located within 1 Mb of the significant SNPs identified from the GWAS analysis using FarmCPU model.

SNP	Chr	Pos (bp)	P-value	PVE (%)	QTL/Candidate gene	Gene	Predicted function	Ref[a]
Mature L/D								
S2_12445336	2	12,445,336	7.68E-09	3.10	*CsTRM4/CsTRM5*	CsGy2G011350	TON1 Recruiting Motif homolog, LONGIFOLIA1	1
S4_20273647	4	20,273,647	3.09E-10	5.98	*Fl4.1*	CsGy4G016160	auxin efflux carrier family protein, PIN-like 6	2
Young L/D								
S4_20280932	4	20,280,932	4.39E-11	4.60	*Fl4.1*	CsGy4G016160	auxin efflux carrier family protein, PIN-like 6	2
Mature length								
S4_20284337	4	20,284,337	1.29E-15	10.57	*Fl4.1*	CsGy4G016160	auxin efflux carrier family protein, PIN-like 6	2
Flesh thickness								
S2_5438072	2	5,438,072	1.45E-09	1.65	*fth2.1*	CsGy2G006100	SET domain protein-lysine methyltransferase	3
Carpel Number								
S1_10811977	1	10,811,977	1.76E-09	14.22	*CsCLV3*	CsGy1G014910	*CLAVATA3*	4
S1_10811982	1	10,811,982	3.46E-20	20.03	*CsCLV3*	CsGy1G014910	*CLAVATA3*	
Spine density								
S4_6717620	4	6,717,620	4.34E-09	2.69	*CsTTG1*	CsGy4G008590	WD repeat protein (*TRANSPARENT TESTA GLABRA1*)	5
Flesh Col - G								
S6_23122636	6	23,122,636	3.53E-08	1.83	*CsOr*	CsGy6G025020	DnaJ Zn finger protein	6

[a] **References**: 1- Wu et al., 2018, (Xie et al., 2023); 2- (Xing et al., 2023); 3- (Xu et al., 2015); 4- Li et al., 2016; 5- Chen et al., 2016; 6- Kishor et al., 2021

Relationship of GWAS identified QTL to prior identified QTL and candidate genes

Several significant SNPs identified by GWAS were also in close vicinity to prior identified candidate genes (**Table 3.1** and **Figure 3.8**). *CsCLV3* was located in close proximity to two significant SNPs for carpel number at 10.8 Mb on chromosome 1 accounting for 14% and 20% of the PVE (**Supplementary Table 3.3**). *CsCLV3* is the homolog of *Arabidopsis* gene *CLAVATA3* (Li et al., 2016), a regulatory protein involving in controlling cell division and differentiation in shoot and floral meristem (Clark et al., 1995; Sablowski, 2011). The expression of *CsCLV3* was significantly lower in True Lemon, a variety with five carpels, compared to WI2757 with three carpels, possibly due to mutation in *CsCLV3* in True Lemon (Li et al., 2016). A significant SNP for flesh thickness was located near a candidate gene for the flesh thickness QTL *fth2.1*, which encodes a SET domain protein-lysine methyltransferase (PKMT, CsGy2G006100). A 4-bp deletion in the promoter region of the PKMT gene resulted in thinner flesh (Xu et al., 2015). A candidate gene for fruit shape on chromosome 2, *CsTRM4* (also named *CsTRM5, TONNEAU1 Recruiting Motif5*), which is a homolog of tomato *SlTRM5*, was identified for *FS2.1* QTL (Wu et al., 2018), and was closely associated with a significant SNP for fruit shape (L/D). Knocking out the expression of *CsTRM5* (CsaV3_2G013800) repressed cell expansion and resulted in shorter fruit length (Xie et al., 2023). For spine density, a significant SNP on chromosome 4 is close to a candidate gene *CsTTG1* (TRANSPARENT TESTA GLABRA1, CsGy4G008590), a TTG1-like homolog in cucumber which encodes a WD repeat protein. Mutation of *TTG1* in Arabidopsis hampers trichome development (Koornneef, 1981; Walker et al., 1999). Upregulating the *CsTTG1* gene increased the density of trichomes and spine in cucumber fruit (Chen et al., 2016). A tightly clustered set of significant SNPs on chromosome 4 at ~ 20 Mb was associated with multiple highly correlated fruit shape traits such as fruit length,

diameter, and young and mature L/D ratios, including a SNP for fruit length explaining 11% of the variance. An annotated gene (CsGy4G016160) encoding an auxin efflux carrier family protein (PIN-LIKES 6 protein) was located within the QTL region and identified as a candidate gene for fruit length (Xing et al., 2023). The expression of the gene was negatively correlated with fruit length. For flesh color, the *CsOr* locus (*Orange*, CsGy6G025020) encoding a DnaJ Zn finger protein was close to a significant SNP for flesh color G value on chromosome 6. The *CsOr* locus was identified from CS-B line which had orange flesh due to higher β-carotene content in endocarp (Kishor et al., 2021).

Potential novel genes identified by GWAS analysis

We also sought to identify potentially novel genes that may influence fruit traits. We searched for annotated genes expressed in fruit (according to RNA-seq data accessed in CuGenDBv2) that were located within, or within 2 kb upstream, of the GWAS-identified significant SNPs . Of the 41 SNPs that fell within or near fruit-expressed genes, 17 were located in introns (**Supplementary Table 3.4**). Five affected amino acids in exons, but only one caused a non-conservative amino acid substitution, glutamine to histidine in an uncharacterized protein. Of the remaining SNPS, two each were within 5' or 3' untranslated regions and 15 within 2kb upstream of the transcriptional start, potentially affecting gene expression.

Of particular interest was a set of significant SNPs that were identified for mature fruit length and young and mature fruit L/D ratios located within the coding region of a homolog of SCAR (suppressor of cAMP receptor) protein (CsGy4G015630) on chromosome 4 at 20.27 Mb. The homolog of SCAR protein in *Arabidopsis thaliana* is a part of the well-annotated actin nucleating promoting complex, WAVE (SCAR/WAVE/WASP [Wiskott–Aldrich syndrome protein] family verprolin homologous protein). SCAR/WAVE serves as an activator of the

cytoskeleton-associated actin-related protein complex (ARP2/3) (Deeks and Hussey, 2005; Yanagisawa et al., 2013). The role of cytoskeleton involved in cell division and cell morphology and organ shape, could potentially influence fruit shape development (Li and Staiger, 2018; Livanos and Müller, 2019). However, neither of the identified significant SNPs caused a non-synonymous amino acid change; one was located in an intron and the second caused a conservative amino acid change from phenylalanine to leucine.

Two additional significant SNPs were located upstream of genes associated with cell division factors: one for fruit diameter located 1623 bp upstream of an actin-associated gene on chromosome 1, a homolog of Villin-4 (Miears et al., 2018), and one on chromosome 3 for young fruit shape located 41bp upstream of a homolog of a gamma-tubulin complex associated protein, AUGMIN subunit 1 (Hotta et al., 2012). Also of potential interest was an additional SNP for young fruit shape on chromosome 5 located in the 5'UTR of a homolog of a brassinosteroid-signaling kinase, BSK-1. Brassinosteroids have been demonstrated to be important for early fruit growth (e.g., Fu et al., 2008).

A second pair of SNPs identified by GWAS for mature fruit color R and G values were located close to a gene encoding a DJ-1 homolog D protein (CsGy4G000550) at 0.32 Mb on chromosome 4. One SNP was 1.2 kb upstream and the second one was in the 3' UTR of the gene, potentially influencing gene expression. The DJ-1 protein family has been shown to be essential in chloroplast development in *Arabidopsis thaliana* and poplar (*Populus trichocarpa*) (Lin et al., 2011; Wang et al., 2023). Disrupting or knockout the expression of DJ-1 protein resulted in albino phenotype with malformed chloroplasts (Lin et al., 2011; Wang et al., 2023b).

Discussion

Morphological features are crucial when considering breeding for new varieties. New

sources of disease resistance and other valuable traits are often found in landraces or wild accessions, many of which have fruit quality traits that are undesirable in commercial markets, for example, large seed cavity size and internal hollowness. However, the fruit quality information of these accessions is often not available. If an accession possesses a target trait, i.e. disease resistance, but distinct fruit quality traits from the market, it might take extra effort and resources to introgress the traits and reach acceptable fruit quality. Breeders may prefer to search for alternate source materials to pursue breeding goals. Here, the phenotypic data of the core collection can provide valuable morphological information for researchers and breeders to acquaint them with the plant materials. The 388 accessions in the core collection contain not only diverse morphological features, as well as numerous disease resistances and key agronomic traits. With available genotypic and phenotypic resources for the core collection, breeders can utilize the resources to formulate efficient strategies and develop new varieties.

Morphological diversity in the core collection

In this work, morphological external and internal traits were documented and characterized (**Figure 3.1**). Most of the traits exhibit a wide range of diversity in the core collection, except for a few that were less variable, including hollowness, carpel number, spine density, netting and curvature (**Figure 3.2**). Though many are landraces and wild cultivars, the phenotypes reflect the results of long history of domestication (Wang, 2015; Grumet et al., 2023). During the process of domestication (~3,500 years), most cultivated cucumber nowadays is phenotypically distinct from the wild ancestor, in addition to fruit traits such as increased flesh thickness, smaller seed cavity size, no internal hollowness, and smoother skin surface (Qi et al., 2013; Weng, 2021). Some distinct phenotypes can also be found in specific regions, showing the geographical and cultural selection preference of the crop (Wang, 2015; Grumet et al., 2023). A

noticeable feature was the fruit shape of the accessions originating from East Asia, where these accessions predominantly bear long and slender fruit with the average L/D ratios of 8.15 and 6.55 for young and mature fruit, respectively (**Figure 3.6**). The tendency of curving fruit was also higher in the fruits from this region, probably due to greater capacity for long, slender fruit to curve.

Other evident differences in morphological features were observed in the accessions from India and South Asia. The accessions from these regions tended to have larger seed cavity size and hollowness, as well as more heavily netted fruits. These traits are less favorable for consumption, and were mostly eliminated during domestication. The fact that India and South Asia are the center of origins of cucumber suggests that these accessions preserve the diversity of morphology. Additionally, it is known that other than white, yellow and green flesh color, orange flesh cucumbers were found in subtropical Xishuangbanna of southwest China and surrounding regions (Qi et al., 1983). However, these traits were not observed in the core collection.

Association among identified fruit trait QTL

Several SNPs linked to different fruit shape QTL were tightly clustered with each other and also located close to, or within, prior QTL or annotated genes (**Figure 3.8**). A set of 21 consensus fruit size and shape index QTL identified from multiple populations has been reported and summarized (Pan et al., 2020; Wang et al., 2020; Weng, 2021). Among them, some were reported to have pleiotropic effects where a single gene or protein influenced multiple traits. For example, two fruit shape QTL, *CsFS1.4* and *CsFS2.3*, contribute to both fruit radial growth and elongation in the U.S. processing cucumber (Sheng et al., 2020). It is also possible that some traits could be polygenic and controlled by multiple closely linked QTL or genes through additive or epistatic effects. In addition, several prior identified QTL spanned relatively large genomic

regions which might include multiple components that can affect phenotypes. For instance, the fruit shape QTL on chromosome 3, *CsFS3.2*, was associated with fruit elongation but not diameter growth (Weng et al., 2015). However, GWAS analysis identified several significant SNP s for mature fruit diameter and young and mature fruit L/D ratios that spanned a 4 Mb distance within this QTL region, indicating that the SNPs associated with these traits might be associated with distinct genes. Further dissecting the relationship between genomic region and the traits could help in pinpointing candidate genes.

In some cases, different QTL were identified in different stages of fruit development. For instance, SNPs of young fruit L/D were found in the cluster on chromosome 1, but not mature fruit L/D, while SNPs for mature fruit L/D were found on chromosomes 2 and 6, but not young fruit L/D, suggesting that these QTL may be active in different stages of fruit length and diameter growth (**Figure 3.8**). Detailed analyses have shown that different stages of development are associated with peak longitudinal versus lateral expansion and that differences in both cell number and cell shape contribute to differences in fruit shape (Colle et al., 2017). Peak L/D ratios typically occur early in development and timing varies with genotype (Colle et al. 2017; Pan et al., 2020). A prior study of cucumber fruit growth identified several QTL that were active at different stages of ovary and fruit development (Weng et al., 2015). Adjacent QTL located near the end of chromosome 6, *CsFS6.1* and *CsFS6.2*, both affect length and diameter growth, but *CsFS6.1* was detected during fruit maturation, while *CsFS6.2* was active during development from ovary to immature fruit (Weng et al., 2015). Our GWAS analysis also identified two adjacent, but distinct, QTL on chromosome 6 corresponding with the locations of *CsFS6.1* and *CsFS6.2*. Understanding the dynamic of QTL function in different development stages may also be helpful for identifying candidate genes.

From SNPs to candidate genes

Two SNPs on chromosome 4 (S4_20280932 and S4_20284337) located within the gene CsGy4G015630 were identified in multiple fruit shape related traits including mature fruit diameter and length, young fruit L/D ratio, and curvature, hinting that the gene associated with these SNPs could play a crucial role in fruit development. CsGy4G015630 encodes a homolog of an actin-associated SCAR protein that potentially affects cell division and fruit shape. Prior work had identified a different candidate gene within the same QTL *Fl4.1,* CsGy4G016160, encoding a homolog of an auxin efflux carrier family protein, PIN-LIKES 6 protein (Xing et al., 2023). Auxin has been demonstrated to play an important role in cucumber fruit growth, influencing features such as fruit length, neck length, and curvature (e.g., Li et al., 2020; Sharif et al., 2022; Wang et al., 2022). While the *PIN-LIKES 6* gene was close to the SNPs identified in this study (~0.6Mb away), no significant SNPs were detected within the *PIN-LIKES 6* gene by GWAS analysis by any of the models tested. Examination of the gene expression data provided by Xing et al. (2023) for the 52 genes identified within the interval of the QTL *Fl4.1* showed that the *SCAR* gene exhibited a similar expression pattern to the auxin efflux carrier family protein gene. Both were significantly downregulated in longer fruit compared to shorter fruit, suggesting that both are credible candidate genes.

Two of the SNPs identified for fruit skin color fell within a gene encoding a homolog of a DJ-1 protein, which is involved in chloroplast development. Not surprisingly, numerous prior studies have demonstrated the importance of chloroplasts in fruit skin color, where higher chloroplast content led to darker skin color (e.g., Zhou et al., 2015; Liu et al., 2016; Tang et al., 2018; Wang et al., 2020a).

While GWAS has been widely used to identify QTL for various traits, there is not a clear

standard for determining causal genes from GWAS analysis and the criteria have been determined case by case. Most of the SNPs identified by GWAS are in non-coding and intergenic regions (Farh et al., 2015), with difficulty to link to a casual gene. It was reported that the casual genes can be located up to 2 Mb away from the SNP, and the closest genes were not necessarily the target genes (Brodie et al., 2016). Many factors can affect the precision of GWAS analysis, such as accuracy and distribution of phenotypic data, population size and structure, allele frequency of traits of interest, and linkage disequilibrium (LD) or haplotype blocks (Alqudah et al., 2020). Therefore, many GWAS analyses to date use a combination of criteria, for example, physical distance, LD and minor allele frequency, to determine casual variants. In this work, we searched for candidate genes by two computational approaches: 1) QTL and genes that were previously identified from literature and located within 1 Mb from the SNPs; and 2) inspected the genes that were located within 2 kb upstream of the significant SNPs. We also took advantage of extensively curated RNA-seq data available in CuGenDB (http://cucurbitgenomics.org/v2/) to identify those that are expressed in cucumber fruits (**Supplementary Table 3.4**). While these approaches resulted in a more condensed list of candidate genes, ultimately, it will be necessary to directly verify the function and expression of the candidate genes.

Using RGB values in phenotyping color-related traits

Fruit skin and flesh color in this study were measured using calibrated RGB values (**Figure 3.4**). The use of RGB values can provide advantages relative to categorical color rating scales or analysis of pigment content, for example, providing digitally accessible image data, quantitative measurement compared to visual rating, and reduced cost relative to biochemical analyses of chlorophyll or carotenoid content (Jiao et al., 2017; Hao et al., 2018; Kishor et al., 2021; Sun et al., 2023). RGB values have been widely used in various high-throughput

phenotyping research such as surveying vegetation or monitoring plant growth (Chen et al., 2020; Sánchez-Sastre et al., 2020; Han et al., 2021).

More intense color both in young and mature fruits was associated with low R and G values, , likely representing increased chloroplast (and subsequent chromoplast) content. Similarly, flesh tissue ,which was predominantly white and lacking chloroplasts, had higher R and G values. Mature fruits with lower G values tended to be brown or orange, instead of green; while in flesh color, lower G values corresponded to increased yellow color, suggesting those might have higher carotenoid content. Multiple models have been proposed to scrutinize the relationship between RGB values and traits of interest (Zakaluk and Sri Ranjan, 2006; Beamish et al., 2018; Alves et al., 2022; Olivoto, 2022). Consistent with our observations, research tracking seasonal vegetation changes in low-Arctic tundra found that G value highly reflects chlorophyll content, while R-based index reflected chlorophyll to carotenoid ratio moderately (Beamish et al., 2018).

Conclusion

This work provided morphological characterization of fruit quality traits in the cucumber core collection. The multi-year phenotypic and photographic data have been deposited the in CuGenDB website. In addition, GWAS analysis was performed for the morphological traits measured and provided a refined list of candidate genes that could be useful for future research. In many cases, QTL identified from this study overlapped with prior QTL mapping studies, providing confidence in the analyses and further support for QTL and candidate genes in the literature. Notably, most of the morphological QTL identified in cucumber to date, are from QTL mapping using bi-parental populations. Only a few have been performed using diversity panels for a limited number of traits: green fruit flesh (Bo et al., 2019b), spine density (Bo et al., 2019a), pericarp color (Huang et al., 2022b), and fruit skin waxiness (Huang et al., 2022a). Bi-parental

populations derived from two parents with extreme phenotypes provide a quick and specific method to explore QTL underlying a trait. However, the genetic variation is limited to the two parents and the size of QTL is affected by population structure and size (Mackay and Caligari, 2000; Li and Xu, 2022). A diversity panel composed of a set unrelated individuals can provide a greater range of diversity, but requires higher expense of genotyping and phenotyping, as well as lower ability to detect rare alleles (Alqudah et al., 2020; Uffelmann et al., 2021). In this work we were able to take advantage of the established cucumber core collection and the associated resequencing data providing the necessary genetic data needed for genomic analysis. The collected fruit quality phenotypic documentation and photographic data will provide an additional valuable resources for breeding research.

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APPENDIX B: Supplementary Materials

Supplementary Tables (provided in a separate Excel file)

Sup. Table 3.1. The list of accessions n the core collection and the morphological phenotypic data measured in 2019-2022.

Sup. Table 2.2. Correlation between 2019-2022 of mature fruit external and internal traits.

Sup. Table 2.3. Previously identified QTL or candidate genes located within 1 Mb of the significant SNPs identified from the GWAS analysis in all models (FarmCPU, BLINK, MLMM, MLM) for morphological traits measured.

Sup. Table 2.4. Cucumber fruit trait SNPs identified by GWAS (FarmCPU) within or near (<2kb upstream) fruit-expressed annotated genes (Gy v.2.1; CuGenDB v.2).

CHAPTER 4

Conclusion and Future Directions

Phytophthora fruit rot caused by the oomycete pathogen, *Phytophthora capsici*, is a severe disease that causes loss for pickling cucumber growers. The intricate genetic natures of both the pathogen and the host hampers the development of resistant cucumber varieties. With the recent development of advanced genetic and genomics tools, we were able to use multiple genomic approaches and different types of populations to understand the genetics behind the resistance in cucumber and to develop molecular markers and genomic information for future breeding efforts for resistance to *P. capsici*.

We studied young fruit resistance, which is observed during rapid fruit growth prior to harvest. First, we utilized a doubled haploid resistant parent derived from the resistant breeding line (MSU 109483-53) and developed bi-parental populations for QTL-seq analysis. The results confirmed that the young fruit resistance is quantitative trait, with the largest effect QTL, *qPFR5.1*, located on chromosome 5. The effect of *qPFR5.1* was additionally verified in a fresh market cucumber variety. Then we fine mapped the region to ~1 Mb with KASP markers from two F_3 populations in two seasons and environments. The QTL position and the sequence of flanking makers are available for future breeding work to introgress the QTL into existing breeding materials or varieties. It is also important to pay heed to the fact that this is a quantitative trait. Though *qPFR5.1* had the largest effect, additive or epistatic interactions with other QTL should be considered. Two other QTL on chromosomes 1 and 6 were also identified from QTL-seq analysis, however, were not pursued due to little phenotypic effect or failure to develop markers around the QTL region. Understanding the genetic mechanisms of these QTL may shed light on a more comprehensive view of young fruit resistance.

The cucumber core collection provides a wide range of diversity that is useful to mine for valuable traits existing in natural populations. With multiple years of data collected, we performed

two types of association analysis: GWAS and XP-GWAS, to search of SNPs that are linked to young fruit resistance. Several SNPs were overlapped or located close to the QTL identified from the QTL-seq study, as well as QTL that were previously identified for resistances to other diseases, such as downy mildew, powdery mildew, and gummy stem blight. These findings were consistent with previously observed hot spots for disease resistance on multiple chromosomes. Additionally, several accessions consistently had lower disease ratings with minor symptom development and can be used as additional sources of *P. capsici* resistance. Interestingly, the three most resistant accessions originate from different regions (Bhutan, China, and the United States) and have distinct fruit morphology, suggesting that the resistance to *P. capsici* may be preserved selectively in some accessions around the world but are yet to be discovered. The SNPs reported in this work can be informative when choosing materials from the core collection as materials to breed for resistances. Collectively, the results of this work can provide useful information for future studies to understand mechanisms of resistance to *P. capsici* in cucumber and breed for varieties with resistance to Phytophthora fruit rot.

In addition to resistance to *P. capsici*, we also characterized morphological diversity in the cucumber core collection. The importance of cucumber fruit quality traits is greatly reflected in its commercial value. As numerous abiotic and biotic challenges arise, breeders and researchers often seek novel sources of valuable traits in diverse germplasm. However, many sources of traits of interest reside in materials with feral phenotypes which are undesirable in today's market. Therefore, we assessed multiple crucial fruit quality traits in the cucumber core collection. The photographic documentation and quantitative data for these traits are available on the CuGenDB website. This publicly accessible information provides a quick glimpse of the diverse phenotypes in the core collection, and breeders can scheme breeding strategies accordingly. Selecting

appropriate breeding materials can possibly achieve breeding goals with shorter breeding generations.

We also performed GWAS analyses on the morphological trait data collected in 2019-2022. We identified and summarized the significant SNPs detected for each trait, and proposed a refined list of candidate genes that could be useful for future research. In many cases, QTL identified from this study overlapped with prior QTL mapping studies, providing confidence in the analyses and further support for QTL and candidate genes in the literature. We also identified potential novel genes important for these traits. The results from this work provide a broad view of the diversity of fruit quality traits in the core collection. The SNPs identified and the candidate genes proposed can be further functionally tested for more in depth understanding of the genetic mechanisms of these traits.