Research

Mapping Quantitative Trait Loci for Lettuce Resistance to *Verticillium dahliae* Race 3, Plant Development, and Leaf Color Using an Ultra-High-Density Bin Map Constructed from F₂ Progeny

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Accepted for publication 6 March 2022.

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The mention of trade names or commercial products is solely for providing specific information and does not intend and imply recommendation or endorsement by the USDA.

Funding

This research was supported by grant numbers LGR-2020-09 and LGR-2021-13 from the California Leafy Greens Research Board to KVS. Funding for the project was also made possible by the U.S. Department of Agriculture's (USDA's) Agricultural Marketing Service through grant AM180100XXXXG003 to IS.

The author(s) declare no conflict of interest.

Abstract

Verticillium wilt is one of the most devastating soilborne diseases in lettuce, and the use of host resistance is the most optimal choice for its management. This study focused on identifying and mapping the genetic loci for resistance against *Verticillium dahliae* race 3 in a mapping population of 200 F_{2:3} families developed from a cross between moderately resistant red-leaf lettuce 'Sentry' and susceptible green-leaf lettuce 'La Brillante'. The population was genotyped using the tunable genotyping-by-sequencing (tGBS) approach. An ultra-high-density genetic linkage map containing 34,838 single nucleotide polymorphism markers grouped into 1,734 bins was constructed using F₂ progeny and a sliding window approach. Three quantitative trait loci (QTLs) for resistance to *V. dahliae* race 3 were located on linkage groups (LGs) LG 2 (*qVR3-2.1*) and LG 4 (*qVR3-4.1* and *qVR3-4.2*). Each of these QTLs explained up to ~10% of the total phenotypic variation for the trait. At each locus, the resistance alleles were derived from cultivar Sentry that is partially resistant to the pathogen. Additional loci resistant to the disease are expected in this population, and transgressive segregation indicates that some of those loci could originate from the susceptible cultivar La Brillante. In addition, two QTLs for plant development were identified on LG 2 (*qIPD-2.1*) and LG 7 (*qIPD-7.1*), although no relationship was detected between resistance in these genotypes and the rate of plant growth. A major effect of QTL for red leaf color was detected on LG 9 (*qRLC-9.1*). Candidate genes linked to some of the QTLs for *V. dahliae* race 3 resistance, plant development, and leaf color were identified. The QTLs for resistance identified in Sentry could diversify the resistance gene pool and provide an alternative tool to manage a newly emerged *V. dahliae* race 3.

Keywords: breeding, disease resistance, growth and development, inheritance, leaf color, lettuce, resistance loci, *Verticillium* wilt

Verticillium wilt, caused by the soilborne fungus *Verticillium dahliae*, is an economically significant disease of many high-value crops, including lettuce, tomato, artichoke, strawberry, and spinach in the Salinas and Pajaro Valleys of coastal Central California (Atallah et al. 2011; Subbarao et al. 2017) and many other hosts worldwide (Inderbitzin and Subbarao 2014; Klosterman et al. 2009; Ligoixigakis et al. 2002; Pegg and Brady 2002). Isolates of *V. dahliae* are classified as either race 1 or race 2 on tomato and lettuce (Fradin et al. 2009; Maruthachalam et al. 2010; Vallad et al. 2006). Most isolates recovered from affected lettuce fields in coastal California belong to race 1, with limited infestations of race 2 (Short et al. 2014). In 2017, however, race 3 on tomatoes was described in Japan (Usami et al. 2017). Based on the response of tomato rootstock cultivar ‘Aibou’ with resistance to both race 1 and race 2, Ingram et al. (2020) reclassified race 2 isolates from lettuce (VdLs17) and tomato (Le1811) as race 3, along with an additional isolate from tomato collected near Hollister, California, and eight isolates from North Carolina. Such reclassification contradicts the previously established race designation in lettuce based on the virulence patterns of isolates on lettuce differentials (Vallad et al. 2006).

Even though differential cultivar response-based race assignment in lettuce and tomato is the norm, the availability of molecular markers, including single nucleotide polymorphism (SNP) genetic markers (Short et al. 2014), has facilitated the identification of races among *V. dahliae* isolates from hosts in which either resistance or differential cultivars were unavailable. For example, isolates from artichoke, pepper, potato, spinach, and strawberry were classified as either race 1 or 2 using these markers (Short et al. 2014). Due to the unavailability of differential host cultivars to identify the presence of race 3 among *V. dahliae* populations from lettuce and other crops, we used molecular markers for avirulence genes developed from a comparative genomic analysis of three tomato isolates representing race 1 (JR2), race 2 (TO22), and race 3 (HoMCLT) (Wang et al. 2021). These avirulence genes from races 2 (Chavarro-Carrero et al. 2021) and 3 (VdAve3) were also functionally characterized to confirm that these were indeed avirulence genes. This race 3 marker was then used to classify isolates that had previously provided ambiguous results or to identify race 3 strains from newer collections of isolates (Wang et al. 2021). Kano and Usami (2019) elucidated that the race 3 isolate likely evolved from a race 1 divergence, although whole genome sequencing shows a distinct structural variation. One such isolate could not be classified as either race 1 or 2 previously, against which the race 1-resistant lettuce cultivars ‘Sentry’ and ‘La Brillante’ showed differential responses, and this was identified as race 3 using the race-specific marker and used in this study.

The resistance against race 1 in lettuce is conferred by a single dominant *Verticillium resistance 1* (*Vr1*) locus initially identified on chromosomal linkage group (LG) 9 of the Batavia-type cultivar, La Brillante (Hayes et al. 2011b). The locus constitutes a complex cluster of four genes (*LsVe1*, *LsVe2*, *LsVe3*, and *LsVe4*), where only a single allele, *LsVe1L*, present in the resistant cultivar La Brillante confers wilt resistance (Inderbitzin et al. 2019). The resistance exerted by the *Vr1* locus is stable under the field conditions despite high pathogen pressures (Puri et al. 2017). Several heirloom lettuce cultivars and breeding lines also carry resistance to *V. dahliae* race 1 (Hayes et al. 2007; Vallad et al. 2006) and are currently being incorporated into commercial cultivars (Hayes et al. 2011a, 2018). In tomato, the *Ve* R-gene locus encodes two leucine-rich repeat receptor-like proteins, *Ve1* and *Ve2*, with *Ve1* being the gene conferring resistance to *V. dahliae* race 1 (Nazar et al. 2018). The *Ve* homologs are widely distributed among crops such as cotton (Chen et al. 2016), potato (Simko et al. 2004;

Song et al. 2017), wild eggplant, hop (Song et al. 2017), mint (Vining and Davis 2009), sunflower (Fick and Zimmer 1974), and others, and the gene is considered to be of ancient origin.

Unlike race 1 resistance, complete resistance in lettuce against race 2 is unavailable, but several germplasm accessions with partial resistance have been identified (Hayes et al. 2011a, 2018; Sandoya et al. 2017). The genetics of partial race 2 resistance is complex. Analyzing three mapping populations from crosses involving *Lactuca sativa* × *L. sativa* and *L. serriola* × *L. sativa*, Sandoya et al. (2021) identified a common quantitative trait locus (QTL), *qVERT6.1*, on LG 6 and other race 2 resistance QTLs in several LGs. In contrast, resistance against race 2 in tomato is conferred by only a single locus (Stamova 2005; Usami et al. 2017). Usami et al. (2017) identified two tomato rootstocks, Aibou and ‘Ganbarune-Karis’, effective against race 2 that serve as cultivars to differentiate race 2 (nonpathogenic on Aibou) from race 3 (pathogenic on Aibou) in tomato.

A strong association between flowering time and plant defense has been reported in various plant pathosystems, including vascular wilt pathogens (Dhar et al. 2019; Lyons et al. 2015b; Ogawara et al. 2003; Singh et al. 2014). QTLs conferring resistance to *Verticillium* spp. were mapped within the regions containing the flowering time gene in *Arabidopsis thaliana* (Haffner et al. 2010; Veronese et al. 2003). Plants respond to pathogens either by investing resources into immunity or by transitioning early into the reproductive phase, such as flowering and seed production, following a “growth versus defense conundrum” (Dhar et al. 2019; Huot et al. 2014; Lyons et al. 2015a). Lyons et al. (2015b) found a strong positive correlation between *A. thaliana* late flowering type and Fusarium wilt resistance. They further confirmed the upregulation of floral integrator genes or FLC, FT, and GI flowering loci following accelerated flowering in response to *F. oxysporum* infection. In contrast, the *A. thaliana* mutant *ndr1-1* that is highly susceptible to *Verticillium* spp. displays accelerated plant growth along with an early transition into flowering (Dhar et al. 2019, 2020a). Several hundred orthologs and paralogs of *Arabidopsis* flowering time genes are present in the lettuce genome, with 167 QTLs identified for bolting and flowering (Han et al. 2021). Thus, it is crucial to understand the association of these two traits in the lettuce-*V. dahliae* host pathosystem. A slight modification in harvesting time depending on disease incidence might be a strategy to rescue a crop from complete failure because the presence of *V. dahliae* in lettuce at the time of harvest does not alter the quality of harvested tissue during extended cold storage (Puri et al. 2019).

Anthocyanins are water-soluble, naturally occurring flavonoids that are responsible for color variation in leaves, flowers, and fruits in many plants, including lettuce (Su et al. 2020), and provide protection against biotic and abiotic stresses including photo-oxidative damage (Dhar et al. 2020a; Gould 2004; Steyn et al. 2002). Flavonoid glycosides and caffeic acid derivatives trigger reactive oxygen species during abiotic and biotic stresses in addition to directly absorbing UV radiation (Gill and Tuteja 2010; Winkel-Shirley 2002). An increased flavonoid production triggered by the PAL activity and cinnamic acid biosynthesis occurs as a resistance response in tomato against *V. dahliae* race 1 isolate Le1087 (Hu et al. 2019), and these were previously documented as defense factors and signaling molecules (Shirley 1996; Treutter 2006). Furthermore, the red-pigmented lettuce is enriched in anthocyanin, which provides significant health benefit to humans through its high antioxidant activity (Rice-Evans and Paganga 1997; Qin et al. 2018). Robinson et al. (1983) listed six genetic loci controlling anthocyanin accumulation and its inheritance in lettuce. Zhang et al. (2017), using genome-wide association mapping, found 12 QTLs that regulate the expression of

24 genes associated with flavonoid biosynthesis in lettuce. Thirty-four structural genes associated with the anthocyanin pathway in red-type lettuce were identified using transcriptome analysis (Zhang et al. 2016). More recently, Su et al. (2020) mapped, cloned, and characterized four genes (*RLI1*, *RLI2*, *rlI3*, and *rlI4*) promoting anthocyanin accumulation in lettuce. Considering the high antioxidant content and defense-related functions of many flavonoids in plant biology, we aimed to map these traits in the genetic background of Sentry to obtain high-quality lettuce varieties with increased resistance to *V. dahliae*.

Overall, the Verticillium wilt management in many crops or cropping systems has primarily relied on an integrated approach of cultural, biological, and chemical methods and host resistance (Atallah et al. 2011; Hayes et al. 2011a, 2011b; Puri et al. 2021; Sandoya et al. 2017; Subbarao et al. 2017). However, factors such as the broader host range and long-lived microsclerotia of *V. dahliae* in soil (Atallah et al. 2011), ineffective cultural practices (Inderbitzin et al. 2018), prohibition of methyl bromide with the unavailability of equally effective fumigant alternatives (Duniway 2002; Meadows 2013), and unavailability of resistant cultivars (Hayes et al. 2007, 2018; Sandoya et al. 2017, 2021) have stymied the successful management of this devastating disease.

The emergence of a new race has posed renewed challenges to lettuce breeding programs already saddled with efforts to identify and incorporate resistance against the preexisting races 1 and 2. It is therefore important to not only confirm the presence of race 3 among the *V. dahliae* populations from lettuce but also identify potential sources of resistance against this race and study the genetics of resistance. No genetic studies have been conducted thus far to elucidate resistance mechanisms against race 3 in tomato, lettuce, or other hosts.

The tunable genotyping-by-sequencing (tGBS) technology overcomes the high rate of genotypic errors and missing data that occur at the conventional genotyping-by-sequencing (GBS) approach, and for that reason, tGBS is one of the highly reliable technologies employed in the sequencing of heterozygous loci (Ott et al. 2017). The technology employs restriction enzymes to create overhangs in the opposite direction of DNA strands. A single-strand barcode adapter is ligated at each end, enabling the generation of high-quality GBS data with more than 97% accuracy during SNP calling (Ott et al. 2017). The technology is now widely used in many plant species, including vegetable and field crops such as lettuce (Simko et al. 2018), rice (Labroo et al. 2021), and corn (Li et al. 2021). Although the occurrence and distribution of race 3 in coastal California host systems is currently unknown, its emergence and eventual arrival in this area poses the most significant threat to lettuce production. Identifying new sources of resistance and their integration into the commercial cultivars will enable the preemptive management of this disease caused by *V. dahliae* race 3 when it eventually arrives. The current study was therefore conducted with a broader objective of identifying QTLs for resistance to *V. dahliae* race 3 using a biparental mapping population developed from a cross between cultivars Sentry and La Brillante and phenotyped in greenhouse conditions. Additionally, this study focused on identifying the QTLs controlling plant development and leaf variegation and explored the correlation among these traits to disease resistance in lettuce.

MATERIALS AND METHODS

Initial disease screening

Four *V. dahliae* isolates, namely VdLs16, the type isolate for race 1 in lettuce; VdLs17, the type isolate for race 2 from

lettuce; and newly classified race 3 isolates 303 from spinach and CB86.7.2 from potato, were tested on three lettuce differential cultivars, La Brillante, Salinas, and Sentry, and three plant introductions (PI 171674, PI 204707, and PI 226641) with partial resistance to VdLs17 (Hayes et al. 2011a). These tests indicated that the race 1-resistant cultivars, La Brillante and Sentry, had differential responses to isolates VdLs17, CB86.7.2, and 303. Cultivar Sentry, which is as susceptible as La Brillante to the race 2 isolate (VdLs17), showed consistent partial resistance to isolates CB86.7.2 and 303, whereas La Brillante was as susceptible to these two isolates as it was to VdLs17 (Fig. 1). We hypothesized that Sentry could be used as a differential cultivar to differentiate race 2 from race 3 and utilized to map the genetic factors governing partial resistance.

Population development and plant material

A biparental mapping population was developed from a cross between race 3 partially resistant cultivar Sentry and the susceptible cultivar La Brillante. The F₁ progeny was selfed to get F₂ families. About 450 F₂ seeds were randomly selected and used for seed increase to F_{2:3} using the single-seed descent method. Of the 450 F_{2:3} generated, 200 randomly selected lines were phenotyped against *V. dahliae* isolate 303 using replicated trials in the greenhouse.

Phenotyping of families and cultivars

The 200 F_{2:3} families and parents were evaluated for their response to *V. dahliae* isolate 303 and two phenotypic traits in three greenhouse experiments (E1, E2, and E3) composed of at least seven replications (plants) at the U.S. Agricultural Research Station in Salinas, California, in the spring season of 2020. Approximately 90 seeds (2 to 3 per cell) of each F_{2:3} family and the parental cultivars were sown in 128-well plug trays filled with sterilized Sunshine Mix number 4 (Sun Gro Horticulture, AB, Canada) and kept in the dark for 48 h to facilitate uniform seed germination. Seedlings were progressively thinned to retain a single robust seedling per cell. The isolate culturing, inoculum preparation, and so on, were done as previously described (Puri et al. 2017). Briefly, 100 µl of the cryopreserved isolate 303 suspensions at –80°C in 25% glycerol was transferred into full-strength PDA amended with 200 ppm of streptomycin and grown for a week. The conidia from the fully grown plate were further transferred into new PDA plates by streaking with an inoculation loop. The plates were then wrapped with parafilm and incubated at room temperature (~24°C) for 7 to 10 days. Inoculum was prepared by washing the cultures with sterilized ddH₂O, and the contents were filtered through a double-layered cheesecloth and used for seedling inoculation.

Fourteen days after seedling emergence, the first inoculation with a suspension of 1×10^7 conidia/ml of *V. dahliae* isolate 303 was performed by nearly saturating the soil (~3 ml inoculum/well) in each cell. Two additional inoculations to ensure successful infection were done at 7 and 14 days after the first inoculation. Thirty-day-old inoculated and uninoculated seedlings were then transplanted into 500-ml Styrofoam cups filled with a 2:1 vol/vol mixture of pasteurized potting mix and sand. One week after transplanting, the fourth and final inoculation was performed by pouring 10 ml of conidial suspension of 1×10^7 conidia/ml per cup. The seedlings from each family were arranged in a randomized complete block design, replicated at least seven times with seven single plants per replication ($202 \times 7 = 1,414$ total cups per experiment and three experiments). The control plants for each parent were treated with sterile distilled water

at the same time as the inoculations and placed on a separate bench. Plants were grown for approximately 10 weeks until 70% of plants showed the first flower and were evaluated for disease symptoms. For disease evaluation, plants were uprooted, washed, and the taproot cut longitudinally to assess disease severity based on the extent of vascular discoloration along with the above-ground symptoms using the previously developed disease rating scale of 0 (symptomless) to 5 (most severe symptoms) (Hayes et al. 2011b).

In addition, at the time of disease rating, the plant development (index of plant development, IPD) stages were classified as pre-bolting (at rosette stage), bolting, or flowering. The number of plants with red or green leaf color was also recorded.

Genotyping of families and cultivars

At the 2- to 3-leaf stage, foliar tissue was collected from 259 F₂ plants, both parents (Sentry and La Brillante), as well as from the cultivar Salinas that was previously used for the construction of lettuce reference genome and from an F₁ plant (Sentry × La Brillante). The leaf tissue was immediately lyophilized and submitted for DNA extraction and genotyping (Data2Bio, Ames, IA, <http://www.data2bio.com>) using the tGBS approach (Ott et al. 2017). Genotyping was performed on a single sample from each F₂ plant, five samples from the F₁ plant, and eight samples each from cultivars Sentry, La Brillante, and Salinas. Identification of SNPs and their physical position was based on the lettuce genome sequence of cultivar Salinas, version 8 (Reyes-Chin-Wo et al. 2017).

Bioinformatic analyses

Genotyping was performed with a modified tGBS protocol using digestion restriction enzyme *Bsp*1286I (Ott et al. 2017). Low-quality reads with a *Phred* quality score below 15 of a total of 40 (63% error rate) (Ewing et al. 1998; Ewing and Green 1998) were removed before alignment. The read quality was further assessed in a two-step process, where the initial reads were scanned at each

end, and nucleotides with quality values lower than the threshold were removed. Next, the remaining nucleotides were scanned using an overlapping window of 10 bp, and all sequences below the average quality value threshold were trimmed using software LUCY 4.3 (Chou and Holmes 2001; Li and Chou 2004). The trimmed reads were then aligned to the reference genome using the Genomic Short-read Nucleotide Alignment Program (Wu and Nacu 2010). All high confident reads were filtered and used for subsequent analyses if mapped uniquely (e.g., ≤2 mismatches every 36 bp and fewer than five bases for every 75 bp as tails).

Bin map construction and genetic mapping

The original dataset of 132,508 tGBS SNPs was first filtered using the following criteria for each SNP: Genotype data were available for more than 50% of samples, more than 87% of cultivar Salinas samples were homozygous and identical to the reference genome, more than 87% of cultivar Sentry samples were homozygous and identical, more than 87% of cultivar La Brillante samples were homozygous and identical, alleles of homozygous samples from one parent were different from alleles of homozygous samples of the other parent, and more than 80% of F₁ samples were heterozygous. The filtered set of 34,838 SNPs was used to construct a genetic bin map employing the sliding window approach (Huang et al. 2009). First, the detected SNPs were ordered according to their physical position in the reference genome. Consecutive SNPs were examined in a sliding window of 15 SNPs and a step size of a single SNP. As the window glided along the chromosome, recombination breakpoints were determined by the junction of two different genotypes, thus grouping consecutive markers into bins (Huang et al. 2009). A total of 1,741 identified bins of the physical map were subsequently used as markers to construct a genetic linkage map using MST_{MAP} software (Wu et al. 2008), Kosambi mapping function, and LOD score of 10. Seven bins not assigned to any of the nine LGs were eliminated from further analyses, leaving 1,734 bin markers in the genetic linkage map. The locations of bin markers on the physical and

FIGURE 1 Symptoms of tap root discoloration on cultivars La Brillante (susceptible) and Sentry (partially resistant) inoculated with *Verticillium dahliae* race 3 isolate 303 (A) and the relative marginal effects, median disease score (the numbers within the bars scored on a 0-to-5 scale), and confidence intervals (shown as error bars) associated with disease severity (B).

genetic linkage maps were compared using Pearson (r), Spearman (ρ), and Kendall (τ) correlation coefficients implemented in JMP Pro 15.0.0 (SAS Institute, Cary, NC).

QTL analysis

QTL mapping was performed with QGene v. 4.4.0 software (Joehanes and Nelson 2008) using the composite interval mapping approach with an automated, forward selection of cofactors. The threshold for significant QTLs was set at the genome-wide $\alpha = 0.05$ and determined through permutations with 1,000 iterations. The threshold for suggestive QTLs was set at $\alpha = 0.1$. To minimize a type I error (false positive), suggestive QTLs were reported only if no significant QTL was detected for the particular experiment-trait combination.

The mode of inheritance was estimated from mean values detected at bins linked to the QTLs. The d/a ratio was calculated from the values of dominance (d) and additivity (a), where $d = \mu_{sl} - (\mu_{ss} + \mu_{ll})/2$, $a = (\mu_{ss} - \mu_{ll})/2$, and μ_{ss} , μ_{ll} , and μ_{sl} are the mean values for progeny homozygous for alleles originating from cultivar Sentry or cultivar La Brillante or heterozygous.

Statistical analyses

The phenotypic data of selected 200 $F_{2:3}$ families, parents, and controls from all three experiments (E1, E2, and E3) were analyzed. All phenotypic data were analyzed in JMP Genomics v. 8.1 (SAS Institute), if not mentioned otherwise. The traits analyzed included resistance to *V. dahliae* race 3 (VR3), plant development (IPD), and red leaf color (RLC). Individual experiments and the overall mean of the three experiments (EM) were statistically analyzed. The IPD was calculated from percentages of plants at bolting (BOL %) and flowering (FLO %) stages as follows: $IPD = (BOL \% + 2 \times FLO \%)/200$. The index ranged from 0, when all plants were at the rosette stage (pre-bolting), to 1, when all plants were at the flowering stage. As the parental cultivars differ in leaf color (Sentry is red-colored, whereas La Brillante is green-colored), $F_{2:3}$ families were segregated for this trait. The presence of red leaf color on leaves was recorded and converted to 0 (green color) and 1 (red color) scale and used for downstream analysis.

The statistical analysis of phenotypic variation in disease severity was achieved by analysis of variance (ANOVA) fitting in the linear model (lm) using the 'Stats' Package in R (R Core Team 2020). The relative marginal effects of disease severity in each parent for the initial screening were analyzed using the LD_CI macro at 95% confidence intervals (CIs) using SAS v. 9.4 (SAS Institute) (Shah and Madden 2004). The broad-sense heritability (H^2) of phenotypic data was calculated using the mixed model equations ($mmer$) function of the 'sommer' package in R using the formula $H^2 = \sigma_g^2 / (\sigma_g^2 + \sigma_e^2/n)$, where σ_g^2 is genetic variance, σ_e^2 is residual variance, and n is the number of replications. The Pearson correlation coefficient (r) was used to evaluate the linear relationship between traits or experiments. Basic mathematical calculations were performed in Microsoft Excel v. 16.52 (Microsoft, Redmond, WA), and statistical analyses were executed using JMP Pro v. 15.0.0 (SAS Institute).

RESULTS

Phenotypic variation to Verticillium wilt

In preliminary disease screening, both parents (i.e., Sentry and La Brillante) showed significantly different responses to *V. dahliae* isolate 303 ($P < 0.05$), with median disease severity

ratings of 1 and 3.5, respectively (Fig. 1). All three greenhouse experiments and the overall mean showed a near normal distribution of $F_{2:3}$ families to their responses to *V. dahliae* race 3, with means of 2.50 in E1 (range 0.67 to 4.71), 2.51 in E2 (range 0.25 to 4.33), 2.27 in E3 (range 0.14 to 4.14), and 2.42 in EM (range 0.97 to 4.10) (Fig. 2). The disease severity ratings for cultivar Sentry were lower than for La Brillante: 0.40 versus 1.88 in E1, 1.63 versus 3.67 in E2, 1.88 versus 3.14 in E3, and 1.30 versus 2.89 in EM. These data indicate transgressive segregation of resistance in this population because the progeny had both a greater resistance than the partially resistant parent and greater susceptibility than the susceptible parent. The disease severity data from all three experiments were combined following the test of normality (Shapiro-Wilk test, $W = 0.997$, $p = 0.42$). The ANOVA of disease severity on combined data indicated a significant variance among the $F_{2:3}$ families ($P < 0.001$). The broad-sense heritability

FIGURE 2

The frequency distribution of disease severity (DS) scores on 200 $F_{2:3}$ families to *Verticillium dahliae* race 3 isolate 303 in the greenhouse. The families were derived from a cross between race 3 partially resistant cultivar Sentry and the susceptible cultivar La Brillante. Disease scoring was done based on vascular root discoloration of tap root and foliar symptoms typical to Verticillium wilt on a 0-to-5 scale, where 0 = no vascular discoloration; 1 = 1 to 25% of the vascular tissue exhibiting discoloration; 2 = 26 to 50%; 3 = 51 to 75%; 4 = 76 to 100% discoloration and no foliar symptoms; and 5 = 100% discoloration and the presence of foliar symptoms (Hayes et al. 2011b). Average DS of 0 was classified as resistant, whereas scores up to 2 were classified as moderately resistant. The E1 (top), E2 (middle), and E3 (bottom) represent the three independent experiments with experimental means of 2.50, 2.51, and 2.27, respectively. The EM is the overall mean (2.42) across three experiments.

(H^2) was 0.618 with a standard error of 0.047, demonstrating a strong genetic effect on the disease-resistance trait.

Phenotypic variation in plant development and leaf color variation

The $F_{2:3}$ progenies showed significant variation in plant development and red leaf color compared with the parents. The mean IPD score for Sentry and La Brillante was 0.40 and 0.19, respectively, whereas the mean score for plants from $F_{2:3}$ families was 0.38. The cultivar La Brillante had green leaf color, whereas Sentry had a red color. Approximately 63 families were only green colored, 52 were only red colored, and 85 comprised a mix of green- and red-colored plants.

Correlation among various traits

A significant but relatively weak correlation was observed among experiments in the reaction of families to *V. dahliae* E1 to E2 ($r = 0.346$, $P < 0.0001$), E1 to E3 ($r = 0.400$, $P < 0.0001$), and E2 to E3 ($r = 0.332$, $P < 0.0001$) (Fig. 3). A stronger and highly significant correlation existed among experiments for both IPD (r from 0.555 to 0.629, $P < 0.0001$) and RLC (r from 0.463 to 0.545, $P < 0.0001$). However, no significant correlation between disease severity and IPD (r from -0.073 to 0.048 , all $P > 0.325$) or red leaf variegation (r from -0.121 to 0.135 , all $P > 0.071$) was observed, indicating that Verticillium wilt resistance is not substantially associated with either plant development or the leaf color variation in lettuce as assessed at the time of resistance evaluation.

FIGURE 3

Correlations between Verticillium wilt disease severity (VR3), plant development (IPD), and red leaf color (RLC) evaluated in three experiments (E1, E2, and E3). Graphs below the diagonal line show the linear correlation trends between traits and the 95% confidence interval for the trend line. The color of circles in the graphs above the diagonal line indicates the value of Pearson correlation coefficients (scale is shown on the right side). Circle size indicates P values, with a larger circle size meaning a more significant correlation. Only nine correlation coefficients between three experiments within each phenotypic trait were significant ($P < 0.0001$), all other correlation coefficients were not significant at $P = 0.05$.

Development of genetic maps

Following the quality control analysis of the initial set of 132,508 tGBS SNPs, a total of 34,838 filtered SNPs were used to construct the genetic linkage map and to map QTLs. The filtered SNPs were reduced to 1,734 bin markers that were positioned across nine chromosomal LGs (Fig. 4). The total genetic distance covered by the bin-based map was 1,883.5 cM, with the length of individual LGs ranging from 92.0 cM (LG 6) to 304.2 cM (LG 4). The highest number of bin markers were positioned on LG 8 (296 bins), whereas lowest number of bin markers were on LG 6 (79 bins).

The locations of bins on the genetic and physical (reference genome of ‘Salinas V8’) maps showed a very strong correlation for all LGs ($r = 0.963$ to 0.996 , $\rho = 0.987$ to 0.999 , $\tau = 0.925$ to 0.986 , all $P < 0.0001$). From all LGs, the order of markers, as indicated by two nonparametric rank tests (ρ , τ), was relatively least congruent on LG 9 (Fig. 4). Detailed inspection of this LG revealed a weak negative correlation in the order of about

20 markers located in the centromeric region ($\rho = -0.244$, $\tau = -0.207$, both nonsignificant with $P > 0.14$) (Fig. 4).

QTLs for resistance to *V. dahliae* race 3

Composite interval mapping using a total of 1,734 SNP-based bin markers and phenotypic datasets detected three QTLs significantly associated with resistance to *V. dahliae* race 3 on two LGs (LG 2 and LG 4). The first QTL, *qVR3-2.1*, was detected in E2 (at a suggestive level) and EM experiments and explained 6.3 to 10.5% of the total phenotypic variation. The resistance allele originated from cultivar Sentry, and the dominance-to-additivity ratio (d/a) test indicated additive to partially dominant resistance effect. The additional two significant QTLs for resistance were detected on LG 4, *qVR3-4.1* in E1 and *qVR3-4.2* in E3, separated by approximately 50 cM. At both QTLs, the allele associated with resistance originated from the cultivar Sentry. Each of these QTLs explained almost 10% of the total phenotypic variation. There is a possibility, though, that these two loci represent a

FIGURE 4

Relationship between the positions of bin markers on the physical map (horizontal axis) and the molecular linkage map (vertical axis). The bin position on the physical map is based on the location of the bin's first single nucleotide polymorphism (SNP) marker at the lettuce reference genome of cultivar Salinas, version 8 (Reyes-Chin-Wo et al. 2017). The best linear fit between two maps and its 95% confidence interval is indicated by the blue lines and the light blue shaded areas, respectively. Values for Pearson (r), Spearman (ρ), and Kendall (τ) correlation coefficients are shown for each chromosome, as well as the number of bins in each linkage group and the linkage group length. All correlation coefficients were significant at $P < 0.0001$. The whole linkage map is 1,883.5 cM long, comprising 1,734 bins.

single QTL but are detected in different chromosomal regions due to a relatively high variability in the phenotypic trait.

QTLs for plant development

Two QTLs significantly associated with plant development were detected on LG 2 and LG 7 (Table 1). The first QTL, *qIPD-2.1*, was detected in E1, E3, and EM, and the second QTL, *qIPD-7.1*, was detected in E1, E2, E3, and EM. The QTL *qIPD-2.1* explained 11.8 to 25.3% of the phenotypic variation, whereas *qIPD-7.1* explained 15.6 to 20.5% of the variation. Alleles for more rapid plant development, as indicated by the percentage of bolting and flowering plants, originated from both parents, cultivar La Brillante at *qIPD-2.1* and cultivar Sentry at *qIPD-7.1*.

QTLs for red leaf color variation

Only a single QTL was identified that was associated with red leaf color in all experiments (Table 1). A QTL, *qRLC-9.1*, significantly associated with red leaf color was positioned on LG 9, which explained 29.0 to 45.8% of the total phenotypic variation of the trait, and alleles for red color originated from red-colored cultivar Sentry.

DISCUSSION

Resistance in lettuce to different races of *V. dahliae* is either governed by a single dominant gene (*Vr1*) as in race 1 (Hayes et al. 2011a) or is a function of complex genetic traits governed by a few highly environment-dependent QTLs (e.g., *qVERT6.1*) as in race 2 (Sandoya et al. 2021). Besides lettuce, in many other hosts, including tomato, cotton, and sunflower, resistance to race 1 is governed by a single dominant locus (Fick and Zimmer 1974; Hayes et al. 2011a; Mert et al. 2005; Schaible et al. 1951). The resistance against race 2 is host dependent and governed by a single gene in tomato (Stamova 2005; Usami et al. 2017), whereas 12 QTLs governing resistance against race 2 have been reported in lettuce (Sandoya et al. 2021). The complex genetic

makeup of *V. dahliae* races from lettuce and tomato (Chavarro-Carrero et al. 2021; Wang et al. 2021), and the unavailability of differential host cultivars to identify races among *V. dahliae* populations poses greater complexity for lettuce-breeding programs. The *V. dahliae* isolate 303 used in this experiment carries avirulence gene characteristic for race 3 (*VdAve3*) and is structurally different from many originally classified race 2 isolates, including VdLs17 (Wang et al. 2021). This study, thus, will provide a foundation for genetic studies to elucidate resistance mechanisms against *V. dahliae* race 3 in lettuce.

Using 259 (Sentry × La Brillante) F₂ progeny, 132,508 tGBS SNPs, and a sliding window approach, we constructed an ultra-high-density genetic linkage map comprising 1,734 bins with a total length of 1,883.5 cM. This map is similar in length to the ultra-high-density linkage map (1,585 cM) constructed using the interspecific mapping population (*L. sativa* cultivar Salinas × *L. serriola* accession US96UC23) of 213 F₇ recombinant inbred lines (RILs) (Truco et al. 2013).

Phenotyping of 200 randomly selected F_{2:3} families revealed three QTLs for resistance to *V. dahliae* race 3. Each of these QTLs explained up to ~10% of the total phenotypic variation for the trait. The resistance alleles for all three QTLs originated from cultivar Sentry that is partially resistant to *V. dahliae* race 3. The *qVR3-4.2* is positioned in the same chromosomal region as *qVERT4.2* for resistance to *V. dahliae* race 2 (Sandoya et al. 2021). Notably, this QTL is located in the major resistance cluster (MRC4) that contains several candidate genes involved in disease resistance (Christopoulou et al. 2015). The *qVR3-2.1* seemed to be separated from MRC2 located on chromosome 2 (Christopoulou et al. 2015). However, sequence annotation of this chromosomal region revealed at least two candidate resistance genes (PADRE protein upregulated after infection by *Sclerotinia sclerotiorum*, AT3G03280.1, and NB-ARC domain-containing disease-resistance protein AT3G14470.1) (Didelon et al. 2020; Nandety et al. 2013) within 200 kb of *qVR3-2.1*.

The resistance alleles from *qVR3-2.1* and *qVR3-4.2* showed an additive effect. When La Brillante alleles in a homozygous state were present at both loci, the average disease score (in EM)

TABLE 1

Quantitative trait loci (QTLs) identified for resistance to *V. dahliae* race 3 (VR3), plant development, and red leaf color in the Sentry × La Brillante mapping population

Trait	QTL	LG	Position (cM) ^a	Position (physical) ^b	Peak LOD	R ² (%)	<i>d/a</i> ^c	Origin ^d	Experiment ^e
VR3	<i>qVR3-2.1</i>	2	213.7	154,445,004	4.6	10.5	0.59	Sen	EM
	<i>qVR3-2.1</i>	2	215.6	156,118,795	2.7 ^f	6.3	0.67	Sen	E2
	<i>qVR3-4.1</i>	4	198.7	281,126,519	4.3	9.8	0.56	Sen	E1
	<i>qVR3-4.2</i>	4	249.5	322,393,238	4.4	9.9	-0.38	Sen	E3
IPD	<i>qIPD-2.1</i>	2	2.1	14,743,078	11.8	25.3	-0.28	LB	E1
	<i>qIPD-2.1</i>	2	3.1	1,134,532	9.6	20.1	-0.56	LB	EM
	<i>qIPD-2.1</i>	2	4.2	1,134,532	5.2	11.8	-0.91	LB	E3
	<i>qIPD-7.1</i>	7	1.1	3,239,861	9.8	20.5	-0.37	Sen	EM
	<i>qIPD-7.1</i>	7	1.9	3,239,861	8.1	17.7	-0.35	Sen	E3
	<i>qIPD-7.1</i>	7	2.1	2,775,830	6.6	15.6	-0.56	Sen	E2
	<i>qIPD-7.1</i>	7	6.8	3,700,693	6.9	15.7	-0.08	Sen	E1
	<i>qRLC-9.1</i>	9	108.8	149,474,495	17.4	33.5	0.05	Sen	E3
RLC	<i>qRLC-9.1</i>	9	108.8	149,474,495	26.2	45.8	0.24	Sen	EM
	<i>qRLC-9.1</i>	9	114.4	149,944,505	13.1	29.0	0.36	Sen	E2
	<i>qRLC-9.1</i>	9	117.1	147,291,926	12.9	36.8	0.52	Sen	E1

^a Position of nearest bin on the molecular linkage map.

^b Position of the nearest bin on the physical map. The bin position was based the position of the bin's first single nucleotide polymorphism marker on the lettuce genome sequence of cultivar Salinas, version 8 (Reyes-Chin-Wo et al. 2017).

^c *d/a* is the dominance-to-additivity ratio that indicates the mode of inheritance. The value of 1 indicates that alleles from cultivar Sentry are dominant, the value of 0 indicates that alleles have an additive effect, and the value of -1 indicates that alleles cultivar La Brillante are dominant.

^d Origin of the alleles associated with higher disease resistance (VR3), more advanced developmental stage, or red leaf color. LB = alleles originating from cultivar La Brillante; Sen = alleles originating from cultivar Sentry.

^e Three experiments (E1, E2, E3) and the mean of the three experiments (EM).

^f Suggestive QTL at genome-wide $\alpha \leq 0.1$.

was 3.06, whereas when alleles originating from cultivar Sentry were homozygous at the two loci, the average disease score was only 1.82. For comparison, the average disease score (in EM) of cultivar La Brillante was 2.89, whereas that for cultivar Sentry was 1.30. Together with observed transgressive segregation in F_{2:3} families, these data indicate that more alleles for resistance to *V. dahliae* race 3 were present in this population, some of which might originate from the susceptible cultivar La Brillante. Although cultivar La Brillante is more susceptible to race 3 than cultivar Sentry is, it harbors *Vr1*, the single dominant gene of major effect for resistance to *V. dahliae* race 1 located at LG 9 (Hayes et al. 2011b; Inderbitzin et al. 2019). However, additional loci might exist in this cultivar that provide a minor effect for resistance to other races of *V. dahliae* besides race 1.

Understanding of Verticillium wilt severity-related traits such as plant height, development, bolting, and flowering are important for detecting the disease-resistance dependency and complexity of those traits. Unlike a strong positive correlation of *A. thaliana* late-flowering type to *F. oxysporum* wilt resistance (Lyons et al. 2015b), no relationship was observed between the plant development, flowering, and disease-resistance phenotype in the present study. Sandoya et al. (2021) also reported a different plant-pathogen interaction from the one observed here in *V. dahliae* race 2-lettuce interaction, where infected plants became more symptomatic at the flowering stage. However, this agrees with the previous finding of negative correlations between bolting and lettuce drop within and between environments (Hayes et al. 2010; Mamo et al. 2019). Thus, such a relationship observed in the current study was likely either because of the absence of an association between the genomic loci conferring plant development and disease resistance or because of microclimatic variations or a combination of both.

Although cultivars Sentry and La Brillante had an approximately similar rate of development, two significant alleles were detected to be associated with IPD, *qIPD-2.1* and *qIPD-7.1*. Considering a substantial transgressive segregation for IPD in this population, it is not surprising that alleles for more rapid development originated both from cultivar La Brillante (*qIPD-2.1*) and cultivar Sentry (*qIPD-7.1*). More than 160 QTLs were previously reported to be associated with plant development (Han et al. 2021; Simko et al. 2021), including those for bolting and flowering located in the same region of LG 7 (*qFLT7.5*, *sqBLT7.1*) and LG 2 (*qBFR2.1*) as the QTLs detected in this study (Rosental et al. 2021; Sandoya et al. 2021). These two genomic regions harbor major clusters of genes reported to be linked with lettuce bolting and flowering (Han et al. 2021). QTL *qIPD-2.1* is colocalized with *qBFR2.1*, the major locus for bolting/flowering identified in another mapping population and tightly linked with a lettuce homolog of the *Flowering Locus T* (*LsFT*) from *Arabidopsis* (Rosental et al. 2021).

Leaves of cultivar Sentry are red colored, whereas leaves of cultivar La Brillante have a light green color. The red color of lettuce leaves is caused by anthocyanins (Simko 2020). At least six candidate genes responsible for the variation of anthocyanin in lettuce leaves were previously described (Zhang et al. 2017). The major QTL detected in this study (*qRLC-9.1*) on LG 9 is colocalized with *R9* for anthocyanin production originating from cultivar 'Eruption' (Mamo et al. 2019). Considering the similarity in the chromosomal locations, this QTL might be associated with the candidate gene for leaf color, *ANS* (anthocyanidin synthase) (Zhang et al. 2017). The high phenotypic variation governed by QTL (*qRLC-9.1*) indicated that red leaf color in this population is likely controlled by a major gene.

In conclusion, cultivar Sentry contains loci for resistance to *V. dahliae* race 3 and thus can be used to develop lettuce-breeding

lines with partial resistance against this pathogen. Sandoya et al. (2021) identified a major QTL at LG 6 (*qVERT6.1*) against the race 2 isolate VdLs17 in the genetic background of *L. sativa* and *L. serriola*. Thus, the QTLs identified in the Sentry background will diversify the resistance gene pool for the overall race 2 and race 3 breeding programs and open up the possibility of gene pyramiding. Markers linked to the resistance QTLs described in this study can aid in the introgression of the resistance loci and alleles into desirable genotypes using marker-assisted selection. Additional genetic and functional studies are needed, however, to identify the genes that confer phenotypes with partial resistance to *V. dahliae* race 3.

ACKNOWLEDGMENTS

The authors thank Rosa Marchebout, Lorraine Landeros, Denise Soto, and Bullo Erena Mamo for assistance in different phases of this research. Special thanks to Jose Orozco for developing the F₁ cross.

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