Molecular Mapping of High Resistance to Bacterial Leaf Spot in Lettuce PI 358001-1

Yunwen Wang, Huangjun Lu, and Jinguo Hu

First and second authors: Everglades Research and Education Center, University of Florida/Institute of Food and Agricultural Sciences, Belle Glade 33430; and third author: United States Department of Agriculture–Agricultural Research Service, Western Regional Plant Introduction Station, Washington State University, Pullman 99164.

Accepted for publication 12 May 2016.

ABSTRACT

Wang, Y., Lu, H., and Hu, J. 2016. Molecular mapping of high resistance to bacterial leaf spot in lettuce PI 358001-1. *Phytopathology* 106:1319-1325.

Lettuce (*Lactuca sativa* L.) is a diploid ($2n = 18$) with a genome size of 2,600 Mbp, and belongs to the family Compositae. Bacterial leaf spot (BLS), caused by *Xanthomonas campestris* pv. *vitians*, is a major disease of lettuce worldwide. Leaf lettuce PI 358001-1 has been characterized as an accession highly resistant to BLS and has white seed. In order to understand inheritance of the high resistance in this germplasm line, an F_3 population consisting of 163 families was developed from the cross PI 358001-1 $\times$ ‘Tall Guzmaine’ (a susceptible Romaine lettuce variety with black seed). The segregation ratio of reaction to disease by seedling inoculation with *X. campestris* pv. *vitians* L7 strain in the F_3 families was shown to be 32:82:48 homozygous resistant/heterozygous/homozygous susceptible, fitting to 1:2:1

($n = 162$, $\chi^2 = 3.19$, $P = 0.20$). The segregation ratio of seed color by checking F_2 plants was 122:41 black/white, fitting to 3:1 ($n = 163$, $\chi^2 = 0.002$, $P = 0.96$). The results indicated that both BLS resistance and seed color were inherited as a dominant gene mode. A genetic linkage map based on 124 randomly selected F_2 plants was developed to enable molecular mapping of the BLS resistance and the seed color trait. In total, 199 markers, comprising 176 amplified fragment length polymorphisms, 16 simple-sequence repeats, 5 resistant gene candidate markers, and 2 cleaved amplified polymorphic sequences (CAPS) markers were assigned to six linkage groups. The dominant resistance gene to BLS (*Xcvr*) was mapped on linkage group 2 and the gene locus *y* for seed color was identified on linkage group 5. Due to the nature of a single gene inheritance, the high-resistance gene should be readily transferred to adapted lettuce cultivars to battle against the devastating disease of lettuce.

Lettuce (*Lactuca sativa* L.) is a self-pollinated diploid ($2n = 18$) vegetable species with a genome size of 2,600 Mbp, and belongs to the family Compositae. Seven horticultural types, including crisphead, butterhead, romaine (cos), leaf, Latin, stem, and oilseed, are widely recognized in lettuce cultivars (Simko 2013). It is the most popular leafy vegetable in many countries, and the total world production was 24.9 million tons on 1.15 million ha in 2013 (FAOSTAT 2015). China is the largest production country for lettuce in the world, and produced approximately 36.9% of the total, followed by the United States (16.7%), Spain (3.9%), Italy (3.7%), and India (3.3%) (FAOSTAT 2015). From 10 years of data (2003 through 2013), the global harvested area of lettuce increased by 1.38% and total production increased by 1.59% annually worldwide (FAOSTAT 2015). In the United States, lettuce is one of the top 10 most valuable crops, with an annual economic value of over \$2 billion (USDA 2014). Head (crisphead and butterhead, 55.0% of the lettuce production), romaine (30.7%), and leaf (14.3%) are the major market types in this country (USDA 2014).

Bacterial leaf spot (BLS) is an economically important disease of lettuce. This disease is caused by *Xanthomonas campestris* pv. *vitians*. It was first reported in South Carolina and Virginia in the United States in 1918 (Brown 1918). Because there are no chemicals or cultural methods for control of the disease, use of host plant resistance is the only effective way to combat the disease. The majority of crisphead and romaine lettuce accessions tested thus far have been found to be susceptible (Lu and Raid 2013; Lu et al. 2014; Pernezny et al. 2000; Wang et al. 2015). However, two Latin

horticultural-type cultivars (‘Little Gem’ and ‘Pavane’), and a Batavia-type cultivar (‘La Brillante’) were shown to have resistance by being challenged with California *X. campestris* pv. *vitians* strains in California (Bull et al. 2007). A highly resistant leaf lettuce genotype (PI 358001-1) was identified against *X. campestris* pv. *vitians* strain L7 in Florida, and this genotype has much stronger BLS resistance than the well-known resistant Little Gem (Lu and Raid 2013; Lu et al. 2014; Wang et al. 2015). All of these resistant germplasms will provide desirable sources for development of resistant cultivars and investigation of the genetics of host resistance to BLS in lettuce.

Recent investigation reported that the resistance in Little Gem, La Brillante, and Pavane each followed a single dominant gene inheritance, and they may be the same gene or separate but closely linked genes (Hayes et al. 2014). The resistance gene in La Brillante (*Xar1*) was already mapped to lettuce linkage group (LG) 2 in the ‘Salinas 88’ $\times$ La Brillante population (Hayes et al. 2014). Using association mapping between a total of 179 lettuce accessions and 350 expressed-sequence tag (EST)-derived single-nucleotide polymorphism (SNP) markers, Lu et al. (2014) found that one SNP (QGB19C20.yg-1-OP5) on LG2 and two SNP (Contig 15389-1-OP1 and Contig 6039-19-OP1) on LG4 were associated with the BLS resistance in PI 358001-1. However, the inheritance mode and the chromosomal location of resistance in this lettuce genotype are as yet unknown.

Seed color was among the traits that were first analyzed for inheritance in lettuce. Two major genes (*W* and *Y*) were identified to control the seed color in lettuce in early studies (Durst 1929; Thompson 1943). Later, three gene loci for brown seed (*br*), yellow seed (*y*), and white seed (*w*) were reported (Waycott et al. 1999). Of the three loci, *w* was mapped on lettuce LG7 (Chen 2005; Kwon et al. 2013; Truco et al. 2007; Simko 2013; Simko et al. 2013) and *y* was mapped on LG2 (Chen 2005; Waycott et al. 1999). Although the locus *br* was assigned to an LG, it has never been assigned to any chromosome of lettuce (Waycott et al. 1999).

Corresponding authors: H. Lu; E-mail address: hjlu@ufl.edu and Y. Wang; E-mail address: ywwang@ufl.edu

<http://dx.doi.org/10.1094/PHYTO-09-15-0238-R>
© 2016 The American Phytopathological Society

Due to the economic importance of BLS in lettuce production worldwide, it is highly desirable to understand the genetics of the high resistance present in PI 358001-1. The primary objective of this study was to investigate inheritance of BLS resistance in PI 358001-1 and to map the resistance with molecular markers. Additionally, it is also interesting to know of any new chromosomal location of a gene responsible for seed color in lettuce.

MATERIALS AND METHODS

Plant materials. An F₂ segregating population with 163 plants was generated from a cross between *L. sativa* accession PI 358001-1, a leaf lettuce resistant to BLS and having white seed, and *L. sativa* ‘Tall Guzman’, a romaine lettuce susceptible to BLS and having black seed. F₃ families were derived by self-pollination from each of the 163 F₂ plants, and seed color (black versus white) was recorded when seed were harvested from the F₂ plants. Except for one F₃ family without enough plants, 162 F₃ families were tested for resistance to BLS. Then, 124 F₂ individuals were randomly chosen for genotyping.

Genomic DNA extraction. Healthy leaf tissues were collected from individual F₂ plants at the 7-week stage and immediately stored in a freezer at –80°C. Genomic DNA was extracted from frozen leaves using the cetyltrimethylammonium bromide method of Doyle and Doyle (1990), with minor modifications as described by Liu and Wu (2012). The DNA quantity and quality were measured by an ND1000 spectrophotometer (NanoDrop Products, Wilmington, DE) and 1.0% agarose gel electrophoresis, respectively.

Amplified fragment length polymorphism marker assay. A final concentration of the genomic DNA was diluted to 125 ng µl^{–1}. The amplified fragment length polymorphism (AFLP) procedure was performed using the IRDye Fluorescent AFLP Kit (LI-COR

Biosciences, Lincoln, NE) following the manufacturer’s instructions. In total, 64 three-nucleotide selective primer combinations of restriction enzyme *EcoRI/MseI* were employed (Table 1). AFLP fragments were detected using 6.5% polyacrylamide gels with a LI-COR 4300 DNA Analyzer (LI-COR Biosciences). The size of a clear AFLP marker was visually scored using Saga software (version 3.3; LI-COR Biosciences). AFLP markers were designated according to Standard List for AFLP Primer Nomenclature (KeyGene, Wageningen, The Netherlands) followed by the marker size.

Simple-sequence repeat marker assay. Genomic DNA concentration was diluted to 75 ng µl^{–1}. In all, 45 simple-sequence repeat (SSR) primer pairs, including 33 genomic SSR and 12 EST-SSR which have been anchored on the LG of lettuce, were obtained from Rauscher and Simko (2013) and Simko (2009), respectively. These SSR primer pairs were first tested for polymorphism on the two parent lines. Only 12 SSR primer pairs (10 genomic SSR and 2 EST-SSR) with unique polymorphic alleles were amplified on the F₂ mapping population (Table 2). Amplification of SSR was carried out on a Biosystems 2720 thermal cycler (Applied Biosystems, Foster City, CA) in 10.5-µl volumes containing the following components: 1.5 µl of template DNA at 75 ng/µl, 1.34 µl each of forward and reverse primers at 1.0 µM, 0.2 µl each of dNTP at 10 mM, 1.6 µl of 10× polymerase chain reaction (PCR) buffer, 0.2 µl of IR-M13 forward primer at 1.0 µM, 0.05 µl of *Taq* enzyme at 50 U/µl, and 4.27 µl of nuclease-free water. Cycling parameters were 95°C for 5 min; followed by 14 cycles of 94°C for 20 s, 58°C for 1 min, and 72°C for 30 s; an additional 28 cycles of 94°C for 20 s, 55°C for 1 min, and 72°C for 30 s; and a final extension of 72°C for 10 min. A 5.0-µl Blue Stop Solution (95% formamide, 25 mM EDTA, and 2% bromophenol blue) was added to each PCR tube (total volume = 15.5 µl), mixed thoroughly, spun down, and denatured for 3 min at 94°C in the thermocycler. Detection of SSR amplification products was the same as AFLP analysis.

TABLE 1. Primer combinations for amplified fragment length polymorphism analysis are shown with the number of markers fitting to 3:1 segregation ratio and the total number of polymorphic markers in parenthesis

Primer	M47 CAA	M48 CAC	M49 CAG	M50 CAT	M59 CTA	M60 CTC	M61 CTG	M62 CTT	Total
E32 AAC	0(0)	8(9)	4(4)	0(0)	6(8)	2(4)	4(5)	3(3)	27(33)
E33 AAG	0(0)	0(1)	4(7)	2(2)	1(2)	5(5)	2(2)	3(3)	17(22)
E35 ACA	3(4)	2(2)	5(8)	0(0)	8(9)	1(1)	7(8)	2(4)	28(36)
E36 ACC	1(1)	4(6)	3(5)	2(3)	1(1)	2(2)	4(5)	4(4)	21(27)
E37 ACG	1(1)	3(3)	4(9)	2(2)	2(3)	7(7)	1(1)	0(0)	20(26)
E38 ACT	0(0)	1(1)	4(5)	2(2)	2(3)	4(4)	1(2)	3(3)	17(20)
E40 AGC	0(0)	1(1)	2(3)	2(2)	5(5)	0(1)	3(4)	3(3)	16(19)
E41 AGG	4(6)	0(0)	7(8)	1(1)	5(5)	2(2)	9(9)	2(3)	30(34)
Total	9(12)	19(23)	33(49)	11(12)	30(36)	23(26)	31(36)	20(23)	176(217)

TABLE 2. Information for 10 genomic simple-sequence repeat (SSR) and 6 expressed sequence tag SSR used for linkage mapping^a

Marker	Repeat motif	Forward (5'–3')	Reverse (5'–3')	Fragment size (bp)	Ta (°C) ^b
LSSA06-2	(TTTAT) ₃	GCA AGA ATG GTC CCT GTT GT	TGG GTG GGT GTG ATA ATA AGG	235	59
LSSA07	(CCA) ₅	AAG ATA CAC ACA ACC ACC CTT G	GGG CAT TTT GCA AAC AGA A	167	59
LSSA12	(GT) ₁₁	TCG AAA ATT TGG AGA GAG TTT CTT	ACA AGG CCC AAT CCT TTT CT	271	61
LSSA14	(AG) ₁₈	TGA GTT GTA GAG CAA CGA GTG C	CAT GGA AGG TCA TAA GTC CCC	217	60
LSSA28-1	(GA) ₂₈	TTA ATC TCT CTC CTC CTT CAG C	ATC CCC ATT GTC CTC CC	151	55
LSSB31	(TC) ₁₄	TTG GTT TGA CCC CTG TTT TC	TCA CAC CCA AGC TGT TGC TA	307	59
LSSB37	(TC) ₂₈	GGT AGG GCA GTC AAG CAA GA	AAT GAT GAT TTT GCC CTT GG	271	59
LSSB40	(CT) ₂₀	TGG TTT TGG TCC CTG TGA TT	GGT CCG ATA TGT GGT GCT CT	227	61
LSSB51	(TC) ₂₀	TCA TTG CGT TTT TGG TC	AAA GGA AGA AGA AGG AGG	218	52
LSSB55-3	(AGAGT) ₃	TTA ATT ATA TCC AAA AGA C	CGT ACT GAC GCC CTA CAG GT	83	57
SML-001	(CATGAT) ₆	CCA TGG ATC CTG TGT GAA GA	CAC CAT GTT CCA CTT CCA CTT	196–220	...
SML-003	(GTTTT) ₅	CGG GCT GGT TTT GAT TTT TA	TGT CAA ATC GTC ACG TGG TT	110–120	...
BOMZ-ssr	(CA) ₈	ATA GTA CAG ATT GTT GGT GG	AGA TAT AAC TCC ATT TTC CC	276	50
BOKW-ssr	(CAT) ₅	GAT TCA ATG AAA GTG ATT GT	ATC AAG AGT ATG TTT GCC TA	279	50
BGTM-ssr	(GA) ₅	ACA ACT CTA TAA CAA CCG AAC TCC	AAA AAG TCT CTC CAA CAT AAA ACG	322	58
AVMP-ssr	(AT) ₅	GAT AGG CAA GAA AGT GGA TAA AGA	AAT ATA TGC TGC AAA ACA GGT ATG	154	58

^a Markers 1 to 12 were from Rauscher and Simko (2013) and 13 to 16 from van Leeuwen et al. (2010).

^b Ta = annealing temperature.

Cleaved amplified polymorphic sequences (CAPS) marker assay. Two CAPS markers were developed from two SNP (QGB19C20.yg-1-OP5 and Contig 15389-1-OP1) that had association with BLS resistance in PI 358001-1 (Lu et al. 2014) (Table 3). Two restriction enzymes, *Taq*I and *Bsp*HI, were identified for designing CAPS markers for QGB19C20.yg-1-OP5 and Contig 15389-1-OP1 (Kwon et al. 2012), respectively, using NEBcutter V2.0 (New England Biolabs Inc., Ipswich, MA). The enzymes each had only one cutter in the recognition site located on the polymorphic position of SNP. PCR primers were designed by online NCBI/Primer-BLAST (National Center for Biotechnology Information, U.S. National Library of Medicine, Bethesda, MD). The PCR amplification solutions of two CAPS markers were the same as those of SSR markers, and a touchdown PCR procedure was used as follows: an initial denaturation at 95°C for 5 min; followed by 5 cycles of 95°C for 45 s, 68°C for 5 min, reduced by 2°C per cycle, and 72°C for 1 min; denaturation at 95°C for 45 s, 58°C for 2 min, and 72°C for 1 min; 95°C for 45 s; 56°C for 2 min and 72°C for 45 s; then 10 cycles of 95°C for 45 s, 54°C for 2 min, and 72°C for 1 min; an additional 23 cycles of 95°C for 45 s, 58°C for 2 min, and 72°C for 1 min; and a final extension of 72°C for 10 min.

For digestion of the PCR products, reaction mixtures containing 2.0 µl of PCR product, 2.0 µl of 10× NEBuffer, and 10 U of enzyme (0.5 µl of *Taq*I or 1.0 µl of *Bsp*HI) in a total volume of 20 µl were incubated for 14 h at 65°C (*Taq*I) and 37°C (*Bsp*HI), respectively. Gel visualization of CAP markers was the same as AFLP analysis.

Saturation mapping of the BLS resistance gene region. Based on the initial LG developed by mapping previous AFLP, CAPS, and SSR markers, we identified 96 additional SSR from the mapped sequences which were displayed at 0 to 40 centimorgans (cM) of LG2 on lettuce Affymetrix GeneChip (van Leeuwen et al. 2010). Search parameters selected for SSR Locator V.1 software (da Maia et al. 2008) included 5-di-, tri-, tetra-, 4-penta-, hexa-, 3-hepta-, octa-, nona-, and decanucleotide repeats. All other parameters for SSR searching were set to program defaults. The parameters of the primer design with SSR Locator V.1 software were as follows: the amplicon size was set from 140 to 350 bp; GC clamp was set 0; the primer size was set from 18 to 22 bp, with optimum size of 20 bp; the content of G/C was set from 45 to 50; both start and end points of a region scanned were set as automatic; and the end stability was set as 250. SSR primers were synthesized by Sigma-Aldrich (The Woodlands, TX). An M13-tagged sequence (CACGACGTTGTAAACGAC) was added to the 5' end of each forward primer. In addition, 8 resistant gene candidate (*RGC*)-derived markers which have been anchored on lettuce LG2 were obtained from McHale et al. (2009). PCR conditions and product visualization were the same as used in SSR analysis.

BLS resistance evaluation. Tests of resistance to BLS were conducted according to Lu and Raid (2013) and Wang et al. (2015).

The tests were repeated twice. Seed of each F₃ family were randomly sown in a single row with 10 rows in a flat tray (30 by 50 cm) filled with Fafard growing mix (Sun Gro Horticulture, Agawam, MA). In every other tray, the resistant parent and the susceptible parent were added as two controls. Eight plants of each family were grown on a row. *X. campestris* pv. *vitians* strain 7, the most virulent *X. campestris* pv. *vitians* isolate recovered from the Everglades Agricultural Area in Florida, was used for inoculation. The first replicate was sown on 14 October 2014 and seedlings were inoculated on 21 November 2014. The second replicate was sown on 27 October 2014 and seedlings were inoculated on 26 November 2014. All procedures for *X. campestris* pv. *vitians* inoculum preparation, inoculation, and severity rating were carried out as described by Lu and Raid (2013) and Wang et al. (2015). Thus, in total, 16 plants were tested for each F₃ family. The disease severity of a plant was evaluated using the 0-to-4 rating system described by Lu and Raid (2013), where 0 = no symptoms; 1 = 1 to 10 lesions, with each diameter less than 3 mm; 2 = more than 10 lesions, with each diameter less than 3 mm; 3 = lesions with diameters larger than 3 mm; and 4 = coalesced lesions. The reaction to disease for a single plant was identified as a resistant type with a score of 0 or 1 for disease severity, and as a susceptible type with a score of higher than 1.

Seed color investigation. The seed color of PI 358001-1 was white and Tall Guzmame was black. Black color has been shown to be dominant to white (Durst 1929; Thompson 1943). The seed color of each F₃ family was observed visually as homozygous recessive white and dominant black.

Linkage analysis. Segregation ratios for all markers, BLS resistance type, and seed color were analyzed using a χ^2 test. Codominant markers, including SSR and CAPS markers, and the BLS reaction types were tested against the 1:2:1 ratio. Dominant markers (AFLP) and seed color trait were tested against the 3:1 ratio. Markers were classified into the distorted type if they were significant at a level of $P < 0.05$. A genetic linkage map with molecular markers and two phenotypic traits were constructed using MapMaker/EXP version 3.0b (Lander et al. 1987; Lincoln et al. 1993; Sharma and Kaur 2014). Markers were assigned to LG by increasing the logarithm of odds (LOD) value for grouping in steps of 0.5 LOD units. Determination of LG was based on the stability of groups over different LOD values and consequent grouping of SSR and CAPS anchor markers into independent LG. On the LOD value of 4.0, an additional step was performed with increasing LOD 0.1 unit to split up a large LG at a threshold LOD value of 4.1 because the anchored markers indicated that they belonged to two different LG. Marker orders were determined using the procedure of mapping a slightly larger group described by Lincoln et al. (1993). Kosambi's mapping function was used to calculate map distance in centimorgans. No order was forced during the linkage analysis. A visual map was created using MapChart 2.2 (Voorrips 2002).

TABLE 3. Information for two CAP markers used for linkage mapping

Marker	Contig in LSGermOPA ^a	Primer sequence (5'-3')	Restriction enzyme	Fragment size (bp)	Ta (°C) ^b
QGB19C20	QGB19C20.yg-1-OP5	F-TAT CAA ACG TAA AGT TGG AGC TTG T R-CCA AAC ACC TTG TGC ATT GCT	<i>Taq</i> I incubated at 65°C	90	59.24/60.20
Cntg15389	Contig 15389-1-OP1	F-TGA GTG ATA AAATGG AAG AAA CCC C R-TGC TTT ATC ATT TCT CTC CAA AGG T	<i>Bsp</i> HI incubated at 37°C	90	59.29/58.75

^a Kwon et al. 2012.

^b Ta = annealing temperature.

TABLE 4. Segregation of reactions to bacterial leaf spot disease (BLS) inoculated by *Xanthomonas campestris* pv. *vitians* strain L7 in 162 lettuce F₃ families derived from *Lactuca sativa* acc. PI 358001-1 × *L. sativa* Tall Guzmame

Reaction	Number of F ₃ families	Homozygous resistant	Heterozygous	Homozygous susceptible	P of χ^2 for 1:2:1 ratio
Observed	162	32	82	48	0.20
Expected	162	40.5	81	40.5	...

RESULTS

BLS disease. The parent PI 358001-1 was used as resistant check and parent Tall Guzmaine as susceptible check for every two trays, with a total of 18 rows planted per replicate. The BLS reaction testing was repeated twice. All plants of PI 358001-1 were identified as resistant, whereas all plants of Tall Guzmaine were susceptible. It assured that successful inoculations were applied in phenotyping. Among 162 F₃ families, 32 were homozygous resistant, 82 were heterozygous, and 48 homozygous susceptible, fitting to a 1:2:1 segregation ratio ($\chi^2 = 3.19$, $P = 0.20$) (Table 4). The F₂ population was previously tested against the L7 strain and the segregation ratio was 3:1 resistant/susceptible (data not shown). Thus, a dominant gene inheritance was confirmed.

Seed color. The seed color of Tall Guzmaine was black and that of PI 358001-1 was white. Of 163 F₃ families, the seed color of 122 families was black and the remaining 41 families had white seed. This also fit to a single dominant gene segregation ratio of 3:1 ($\chi^2 = 0.002$, $P = 0.96$) (Table 5).

Molecular linkage map. In total, 240 polymorphic markers, including 217 AFLP, 16 SSR, 5 *RGC*, and 2 CAPS, were detected in the mapping population. On average, 3.39 polymorphisms were detected for each AFLP primer combination. Of the first 33 tested genomic SSR markers, 10 (30.3%) were polymorphic, whereas only 2 polymorphic markers were identified from 12 EST-SSR markers, with a polymorphic rate of 16.7%. During the saturation mapping, 96 additional SSR designed from mapped sequences in the 0- to 40-cM region of LG2 on lettuce Affymetrix GeneChip (van Leeuwen et al. 2010) were screened in this study, and only 4 SSR (4.2%) were mapped. However, five of eight *RGC* markers were polymorphic and mapped. Of 217 AFLP, 176 (81.1%) fit to a 3:1 segregation ratio for a dominant marker at $P < 0.05$, whereas all SSR and CAPS markers fit to a 1:2:1 ratio for a codominant marker at $P < 0.05$. Of these markers that fit the expected ratio, 179 markers were grouped to 11 LG, and another 20 markers consisting of 19 AFLP and 1 SSR did not group with other markers. For the 11 LG, 6 were assigned to lettuce chromosomal LG based on the anchor markers on the reference maps (McHale et al. 2009; Rauscher and Simko 2013) or association analysis (Lu et al. 2014), and the remaining LG were not assigned, including an LG with 25 AFLP markers and 4 small LG with a few AFLP markers (data not shown) (Table 6). The LG spanned 1,707.3 cM.

In total, 23 anchor markers, including 15 SSR, 5 *RGC*, 2 CAPS, and 1 AFLP were mapped to six LG (Table 6). LG8 and LG9 were

each anchored by one SSR marker. LG5 had two anchored SSR markers and LG6 had three anchored SSR markers. LG4 was anchored by one SSR and one CAPS. LG2 was anchored by two SSR (LSSA07 and LSSB37), one CAPS marker (QGB19C20), and one AFLP marker (E41M59_274) during initial map construction (Fig. 1). Because the gene for resistance to BLS was found to locate on LG2 (see below), nine more markers with known chromosomal locations were then added to determine the chromosomal region harboring the resistance gene.

Loci for BLS and seed color. The resistance to BLS in PI 358001-1 was controlled by a dominant gene, *Xcvr*, that was mapped to LG2 (Fig. 1). *Xcvr* was found to locate at one end of LG2, with the AFLP marker E32M48_528 being 13.5 cM from the gene. The closest anchor marker (LR0089) mapped at 17.1 cM from *Xcvr*. Although the CAPS marker derived from QGB19C20.yg-1-OP5 was also mapped to LG2, the genetic distance between this marker and *Xcvr* was 256.6 cM (Fig. 1). The gene for seed color was mapped to LG5 and was flanked by AFLP markers E33M50_360 and E35M61_322 at an interval of 30.2 cM (Fig. 1).

DISCUSSION

Although a number of molecular markers have been applied to lettuce genetic analysis, AFLP, SSR, and SNP markers are commonly used for construction of a framework linkage map and mapping of genes or quantitative trait loci (QTL) in lettuce. The average AFLP polymorphic rate of 3.39 in this study complied with the previous report that the polymorphism was relatively low in lettuce (van Treuren 2001) but was far less than that detected in the F₂ mapping population developed from an interspecific cross (Jeuken et al. 2001; Syed et al. 2006). Jeuken et al. (2001) observed a rate of 81% for polymorphism between *L. saligna* CGN 11341 and *L. sativa* Norden. Because only a few AFLP primer combinations were shared with other linkage maps and the polymorphism level was low (Jeuken et al. 2001; Syed et al. 2006), limited AFLP markers were identified as anchor markers between this linkage map and consensus maps. The polymorphic rate for SSR markers in the present study was also lower than that scored from the F₂ mapping population developed from an interspecific cross (Rauscher and Simko 2013). The results indicated that the genetic analysis for an intraspecific mapping population demands testing more molecular markers than for an interspecific one. From these 16 SSR markers, only 1 genomic marker, LSSA06 was not mapped to any LG, and 1 genomic marker, LSSA07 was mapped to LG2 instead of LG6 in the consensus map (Rauscher and Simko 2013). LSSA06 and LSSA07 amplified multiple alleles. This may be the reason for disagreement with consensus map. Although an LG with 25 AFLP markers was not assigned with a chromosomal LG due to lack of anchor markers, there were two lettuce LG (LG1 and LG3) which were not identified in this study. The genomic locations of the two target traits (i.e., BLS resistance and seed color) were identified by mapped markers. In particular, the location of the BLS resistance

TABLE 5. Segregation of seed color in 163 lettuce F₃ families derived from *Lactuca sativa* acc. PI 358001-1 × *L. sativa* Tall Guzmaine

Reaction	Number of F ₃ families	Black	White	<i>P</i> of χ^2 for 3:1 ratio
Observed	163	122	41	0.96
Expected	163	122.25	40.75	...

TABLE 6. Information of linkage groups (LG) of the *Lactuca sativa* acc. PI 358001-1 × *L. sativa* Tall Guzmaine linkage map^a

LG	Number of AFLP markers	Number of SSR markers	Number of <i>RGC</i>	Number of CAP markers	Total number of markers	Length of LG (cM)	Number of anchor markers
LG2	25	6	5	1	37 ^b	302.2	13
LG4	12	1	0	1	14	185.4	2
LG5	19	2	0	0	21 ^c	236.0	2
LG6	8	3	0	0	11	120.9	3
LG8	32	1	0	0	33	286.7	1
LG9	28	1	0	0	29	364.0	1
Unknown	25	0	0	0	25	212.1	0
Total	149	14	5	2	170	1,707.3	23

^a Abbreviations: AFLP = amplified fragment length polymorphism, SSR = simple-sequence repeat, *RGC* = resistant gene candidate, and cM = centimorgans.

^b A single dominant locus for gene resistant to bacterial leaf spot disease was on LG2.

^c A single dominant locus for black seed color was on LG5.

gene on LG2 was determined by three anchor markers, including one AFLP marker (E41M59-274), one SSR marker (LSSB37), and one CAP marker (QGB19C20), and was then confirmed by adding nine more anchor markers to the region of the resistance gene.

At the present time, a number of genetic linkage maps have been published in lettuce (Atkinson et al. 2013; Hayashi et al. 2008; Jeuken et al. 2001; Kesseli et al. 1994; Landry et al. 1987; Syed et al. 2006; Truco et al. 2007, 2013; Waycott et al. 1999) and a total of 14 map versions are accessible online at <http://cgpdb.ucdavis.edu/GeneticMapView/display/>. Genomic positions of disease resistance genes and QTL, including Verticillium wilt, turnip mosaic virus, downy mildew, powdery mildew, big vein, Fusarium wilt, and anthracnose, have been mapped on the genetic linkage map of lettuce (Simko 2013). The lettuce chromosome 2 has a resistance-gene-rich region that harbors at least nine downy mildew resistance genes (McHale et al. 2009; Simko et al. 2015; Wroblewski et al. 2007), one locus for resistance to root aphid (Ellis et al. 1994), and the BLS resistance gene *Xar1* in La Brillante (Hayes et al. 2014). The BLS resistance gene *Xcvr* in PI 358001-1 was also mapped to this region in the present study. It would be interesting to know the relationship between *Xar1* and *Xcvr*. The BOMS SNP marker and the AFLP marker E35/M49-189 flanked the *Xar1* gene at a genetic distance of 15.8 cM in the Salinas 88 × La Brillante population (Hayes et al. 2014). These two markers were 8.2 cM apart from each other in the 'Salinas' × 'UC96US23' population that has no resistance to BLS (McHale et al. 2009). Neither of these two markers was polymorphic in this mapping population. However, we were able to map the *RGC* markers LR0089, LR0035, LK0036, and LR0029 and the BOMZ-ssr marker to LG2 (Fig. 1). These five markers were very close to one another, with a cumulative distance of 0.4 cM, and were approximately 3.4 to 4.0 cM proximal to the AFLP E32M45_528, the closest marker to the resistance gene. The four *RGC* markers were also mapped to LG2, with a cumulative distance of 3.8 cM by McHale et al. (2009) and 1.3 cM by Hayes et al. (2014). Although the map orders of the *RGC* markers were slightly different in these three

studies, it was conclusive that the markers were tightly linked to each other and were close to one end of LG2. In the study of Hayes et al. (2014), the marker BOMS cosegregated with LR0029 in the Salinas × UC96US23 population and *Xar1* was 1.2 cM proximal to BOMS in the Salinas 88 × La Brillante population. In the present study, all markers were mapped proximally to the gene *Xcvr*. The closest marker, E32M48_528, was 13.5 cM from *Xcvr* and the *RGC* markers were at least 17.1 cM from *Xcvr*. These comparisons indicate that *Xcvr* and *Xar1* are different genes for resistance to BLS.

The SNP marker QGB19C20 from LG2 was associated with disease severity in both the Q general linear model and Q + K mixed linear model in a previous study (Lu et al. 2014). The R^2 value was 0.09 with a P value of 0.0002 in the Q general linear model, and the R^2 was 0.04 with a P value of 0.02 in the Q + K mixed linear model. As expected, the CAPS marker derived from QGB19C20 by using *Taq*I to digest the SNP site was mapped on LG2 in this study. However, the CAPS marker QGB19C20 was mapped at a significant distance (256.6 cM) from *Xcvr*. The disagreement between these two studies was likely due to a false positive in association analysis because false positives are often observed in association studies (Atwell et al. 2010; Lu et al. 2015).

BLS and downy mildew are two major diseases of lettuce. These diseases can occur in the field at the same time or sequentially in the growing season in main production areas of the United States. It is desirable to develop resistant cultivars that combine both BLS resistance and downy mildew resistance. Over 30 *Dm* genes have been identified in lettuce, and the resistance-gene-rich region of LG2 has been known to harbor 9 such genes in different genetic backgrounds, including *Dm1*, *Dm2*, *Dm3*, *Dm6*, *Dm14*, *Dm15*, *Dm16*, *Dm18*, and *Dm50* (Simko et al. 2015). The BLS resistance gene *Xar1* was mapped to this region in a previous study (Hayes et al. 2014). Our study showed that *Xcvr* is also located in the same region. Because this is a resistance gene hotspot, lettuce breeders should monitor it to know what resistance genes they have in their breeding programs.

Fig. 1. Location of a dominant gene (*Xcvr*) resistant to bacterial leaf spot disease on linkage group 2 (LG2) and a dominant gene (*y*) for black seed color on LG5. Genetic distances in Kosambi centimorgans are shown at the left of each LG. Asterisked markers are anchored markers.

The seed color of lettuce is widely classified as white, black (brown), and yellow types. Classic genetic investigation found that two major genes, *W* and *Y*, conferred one black phenotype (*W_Y_*), one yellow phenotype (*wwY_*), and two white phenotypes (*W_yy* and *wwyy*) (Durst 1929; Lindqvist 1960; Thompson 1943). Using the RYDER population, three seed color trait loci, including white seed (*w*), brown seed (*br*), and yellow seed (*y*) were assigned to three different LG with random amplified polymorphic DNA markers, and white seed was considered to have an epistatic effect to brown seed (Waycott et al. 1999). Based on the lettuce integrated map from the RYDER population with six other populations, white seed (*w*) and yellow seed (*y*) were mapped to lettuce LG7 and LG2, respectively (Chen 2005; Truco et al. 2007). However, the brown seed (*br*) trait was never assigned to any chromosomal LG. Now, there are more investigations reporting that seed coat color trait was mapped on lettuce LG7 (Kwon et al. 2013; Simko 2013); however, no evidence confirmed the *y* locus on LG2. In our study, Tall Guzmaine was homozygous for black seed, and its genotype should be *WWYY*, whereas PI 358001-1 was homozygous for white seed, and there was no yellow seed progeny among the *F*₃ families; therefore, its genotype should be *WWyy*. The black seed color trait that has been mapped to LG5 in this study appears to be contributed by the *y* locus, not *w*. The single gene (*Y*), jointly with *W* to confer dominant seed color trait of black to white, was inherited from Tall Guzmaine.

ACKNOWLEDGMENTS

We thank P. McCord, M. Kanaan, and K. McCorkle at the United States Department of Agriculture (USDA) Agricultural Research Service U.S. Sugar Cane Field Station for providing facilities for DNA quality measurement and sample loading; J. Todd for computer software assistance; and A. Carroll, Ph.D. student R. Kandel, and internship undergraduate student G. Faroutine for lab and greenhouse assistance. This work was supported by grants from Florida Specialty Crop Block Grant Program, USDA grant number 00114165, and the CRIS project number FLA-BGL-005105 to H. Lu.

LITERATURE CITED

- Atkinson, L. D., McHale, L. K., Truco, M. J., Hilton, H. W., Lynn, J., Schut, J. W., Micheltmore, R. W., Hand, P., and Pink, D. A. 2013. An intra-specific linkage map of lettuce (*Lactuca sativa*) and genetic analysis of postharvest discoloration traits. *Theor. Appl. Genet.* 126:2737-2752.
- Atwell, S., Huang, Y. S., Vilhjálmsson, B. J., Willems, G., Horton, M., Li, Y., Meng, D., Platt, A., Tarone, A. M., Hu, T. T., Jiang, R., Mulyati, N. W., Zhang, X., Amer, M. A., Baxter, I., Brachi, B., Chory, J., Dean, C., Debieu, M., de Meaux, J., Ecker, J. R., Faure, N., Kniskern, J. M., Jones, J. D. G., Michael, T., Nemri, A., Roux, F., Salt, D. E., Tang, C., Todesco, M., Traw, M. B., Weigel, D., Marjoram, P., Borevitz, J. O., Bergelson, J., and Nordborg, M. 2010. Genome-wide association study of 107 phenotypes in *Arabidopsis thaliana* inbred lines. *Nature* 465:627-31.
- Brown, Z. A. 1918. Some bacterial diseases of lettuce. *J. Agric. Res.* 13: 367-388.
- Bull, C. T., Hayes, R., Madden, L. V., Koike, S. T., and Ryder, E. 2007. Genetic diversity of lettuce for resistance to bacterial leaf spot caused by *Xanthomonas campestris* pv. *vitians*. Online publication. *Plant Health Prog.* doi:10.1094/PHP-2007-0917-02-RS
- Chen, F. 2005. Lettuce Genetic Map Viewer Setup. Online publication. University of California, Davis. http://cgpgdb.ucdavis.edu/database/genome_viewer/viewer/daMaia/L.C./Palmieri/D.A./deSouza/V.Q./Kopp/M.M./deCarvalho/F.I.F./anddeOliveira/A.C.2008.SSRLocator:ToolforSimpleSequenceRepeatDiscoveryIntegratedwithPrimerDesignandPCRSimulation.Int.J.PlantGenomics2008:Article412696. doi:10.1155/2008/412696
- Doyle, J. J., and Doyle, J. K. 1990. Isolation of plant DNA from fresh tissue. *Focus* 12:13-15.
- Durst, C. E. 1929. Inheritance in lettuce. *Science* 69:553-554.
- Ellis, P. R., Pink, D. A. C., and Ramsey, A. D. 1994. Inheritance of resistance to lettuce root aphid in the lettuce cultivars 'Avoncrisp' and 'Lakeland'. *Ann. Appl. Biol.* 124:141-151.
- FAOSTAT. 2015. FAOSTAT Domains. Lettuce and chicory. Online publication. <http://faostat3.fao.org/browse/Q/QC/E>
- Hayashi, E., Aoyama, N., and Still, D. W. 2008. Quantitative trait loci associated with lettuce seed germination under different temperature and light environments. *Genome* 51:928-947.
- Hayes, R. J., Trent, M. A., Truco, M. J., Antonise, R., Micheltmore, R. W., and Bull, C. T. 2014. The inheritance of resistance to bacterial leaf spot of lettuce caused by *Xanthomonas campestris* pv. *vitians* in three lettuce cultivars. *Hortic. Res.* 1:Article 14066. doi:10.1038/hortres.2014.66
- Jeuken, M., Van Wijk, R., Peleman, J., and Lindhout, P. 2001. An integrated interspecific AFLP map of lettuce (*Lactuca*) based on two *L. sativa* × *L. saligna* *F*₂ populations. *Theor. Appl. Genet.* 103:638-647.
- Kesseli, R. V., Paran, I., and Micheltmore, R. W. 1994. Analysis of a detailed genetic linkage map of *Lactuca sativa* (lettuce) constructed from RFLP and RAPD markers. *Genetics* 136:1435-1446.
- Kwon, S., Simko, I., Hellier, B., Mou, B., and Hu, J. 2013. Genome-wide association of 10 horticultural traits with expressed sequence tag-derived SNP markers in a collection of lettuce lines. *Crop J.* 1:25-33.
- Kwon, S. J., Truco, M. J., and Hu, J. 2012. LSGermOPA, a custom OPA of 384 EST-derived SNPs for high-throughput lettuce (*Lactuca sativa* L.) germplasm fingerprinting. *Mol. Breed.* 29:887-901.
- Lander, E. S., Green, P., Abrahamson, J., Barlow, A., Daly, M. J., Lincoln, S. E., and Newberg, L. A. 1987. MAPMAKER: An interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. *Genomics* 1:174-181.
- Landry, B. S., Kesseli, R. V., Farrara, B., and Micheltmore, R. W. 1987. A genetic map of lettuce (*Lactuca sativa* L.) with restriction fragment length polymorphism, isozyme, disease resistance and morphological markers. *Genetics* 116:331-337.
- Lincoln, S. E., Daly, M. J., and Lander, E. S. 1993. Constructing genetic linkage maps with MAPMAKER/EXP Version 3.0: A tutorial and reference manual. A Whitehead Institute for Biomedical Research Technical Report, 3rd ed. McGill University, Montreal, Quebec, Canada.
- Lindqvist, K. 1960. Inheritance studies in lettuce. *Hereditas* 46:387-470.
- Liu, L. L., and Wu, Y. 2012. Identification of a selfing compatible genotype and mode of inheritance in switchgrass. *BioEnergy Res.* 5:662-668.
- Lu, H., Hu, J., and Kwon, S. J. 2014. Association analysis of bacterial leaf spot resistance and SNP markers derived from expressed sequence tags (ESTs) in lettuce (*Lactuca sativa* L.). *Mol. Breed.* 34:997-1006.
- Lu, H., and Raid, R. N. 2013. A novel screening method for evaluation of lettuce germplasm for bacterial leaf spot resistance. *HortScience* 48: 171-174.
- Lu, Q., Zhang, M., Niu, X., Wang, S., Xu, Q., Feng, Y., Wang, C., Deng, H., Yuan, X., Yu, H., Wang, Y., and Wei, X. 2015. Genetic variation and association mapping for 12 agronomic traits in *indica* rice. *BMC Genomics* 16:1067.
- McHale, L. K., Truco, M. J., Kozik, A., Wroblewski, T., Ochoa, O. E., Lahre, K. A., Knapp, S. J., and Micheltmore, R. W. 2009. The genomic architecture of disease resistance in lettuce. *Theor. Appl. Genet.* 118:565-580.
- Pernezny, K., Raid, R. N., and Collins, J. 2000. Cultivar resistance and seed treatment for management of bacterial leaf spot of lettuce. EREC Research Report EV-2000-2, University of Florida/IFAS, Belle Glade, FL.
- Rauscher, G., and Simko, I. 2013. Development of genomic SSR markers for fingerprinting lettuce (*Lactuca sativa* L.) cultivars and mapping genes. *BMC Plant Biol.* 13:11.
- Sharma, N., and Kaur, R. 2014. MAPMAKER/EXP Version 3.0 b: Though used since time immemorial still difficult for beginners to start with map construction for the genetic studies. *J. Transl. Proteomics Res.* 1: 17-27.
- Simko, I. 2009. Development of EST-SSR markers for the study of population structure in lettuce (*Lactuca sativa* L.). *J. Hered.* 100:256-262.
- Simko, I. 2013. Marker-assisted selection for disease resistance in lettuce. Pages 267-289 in: *Translational Genomics for Crop Breeding*, Vol. 1: Biotic Stress. R. K. Varshney and R. Tuberosa, eds. John Wiley & Sons Ltd., Chichester, UK.
- Simko, I., Atallah, A. J., Ochoa, O. E., Antonise, R., Galeano, C. H., Truco, M. J., and Micheltmore, R. W. 2013. Identification of QTLs conferring resistance to downy mildew in legacy cultivars of lettuce. *Sci. Rep.* 3:Article 2875. doi:10.1038/srep02875
- Simko, I., Ochoa, O. E., Pel, M. A., Tsuchida, C., Forcada, C. F., Hayes, R. J., Truco, M. J., Antonise, R., Galeano, C. H., and Micheltmore, R. W. 2015. Resistance to downy mildew in lettuce 'La Brillante' is conferred by *Dm50* gene and multiple QTL. *Phytopathology* 105:1220-1228.
- Syed, N. H., Sørensen, A. P., Antonise, R., van der Wiel, C., van der Linden, C. G., van 't Westende, W., Hooftman, D. A. P., den Nijs, H. C. M., and Flavell, A. J. 2006. A detailed linkage map of lettuce based on SSAP, AFLP and NBS markers. *Theor. Appl. Genet.* 112:517-527.
- Thompson, R. C. 1943. Inheritance of seed color in *Lactuca sativa*. *J. Agric. Res.* 66:441-446.
- Truco, M. J., Antonise, R., Lavelle, D., Ochoa, O., Kozik, A., Witsenboer, H., Fort, S. B., Jeuken, M. J. W., Kesseli, R. V., Lindhout, P., Micheltmore, R. W., and Pelem, J. 2007. A high-density, integrated genetic linkage map of lettuce (*Lactuca* spp.). *Theor. Appl. Genet.* 115:735-746.

- Truco, M. J., Ashrafi, H., Kozik, A., van Leeuwen, H., Bowers, J., Wo, S. R. C., Stoffel, K., Xu, H., Hill, T., Van Deynze, A., and Michelmore, R. W. 2013. An ultra-high-density, transcript-based, genetic map of lettuce. *G3* (Bethesda) 3:617-631.
- USDA. 2014. USDA-National Agricultural Statistics Service Homepage. <https://www.nass.usda.gov>
- van Leeuwen, H., Ashrafi, H., and Reyes Chin-Wo, S. 2010. Lettuce SFP Chip Project. Online publication. University of California, Davis. <http://chiplett.ucdavis.edu/index.php>
- van Treuren, R. 2001. Efficiency of reduced primer selectivity and bulked DNA analysis for the rapid detection of AFLP polymorphisms in a range of crop species. *Euphytica* 117:27-37.
- Voorrips, R. E. 2002. MapChart: Software for the graphical presentation of linkage maps and QTLs. *J. Hered.* 93:77-78.
- Wang, Y., Lu, H., Raid, R. N., Nuessly, G. S., and Faroutine, G. 2015. Diverse responses of lettuce cultivars and germplasm lines to infections of three isolates of *Xanthomonas campestris* pv. *vitians*. *HortScience* 50:650-655.
- Waycott, W., Fort, S. B., Ryder, E. J., and Michelmore, R. W. 1999. Mapping morphological genes relative to molecular markers in lettuce (*Lactuca sativa* L.). *Heredity* 82:245-251.
- Wroblewski, T., Piskurewicz, U., Tomczak, A., Ochoa, O., and Michelmore, R. W. 2007. Silencing of the major family of NBS-LRR-encoding genes in lettuce results in the loss of multiple resistance specificities. *Plant J.* 51: 803-818.