Baby Leaf Lettuce Germplasm Enhancement: Developing Diverse Populations with Resistance to Bacterial Leaf Spot Caused by *Xanthomonas campestris* pv. *vitians*

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Additional index words. breeding, disease, disease resistance, distributive breeding, genetics, shelf-life, vegetable

Abstract. Baby leaf lettuce cultivars with resistance to bacterial leaf spot (BLS) caused by *Xanthomonas campestris* pv. *vitians* (Xcv) are needed to reduce crop losses. The objectives of this research were to assess the genetic diversity for BLS resistance in baby leaf lettuce cultivars and to select early generation populations of lettuce with BLS resistance. Greenhouse experiments using artificial Xcv inoculations were conducted to assess BLS resistance in 35 cultivars of 10 lettuce types used in baby leaf production and in F₂ through F_{3:4} progeny from ‘Batavia Reine des Glaces’ (BLS-resistant, green leaf color) × ‘Eruption’ (BLS-susceptible, red leaf color). Higher disease severity was identified in red leaf and red romaine cultivars compared with other types, indicating the need to target these types for resistance breeding. Selection for BLS resistance and red-colored leaves was therefore conducted among 486 F₂ plants, 38 F_{2:3} families, and two populations of F_{3:4} families from ‘Batavia Reine des Glaces’ × ‘Eruption’. Two populations were identified with uniform levels of BLS resistance equivalent to ‘Batavia Reine des Glaces’ and variable leaf morphology and color. These populations can be used by private and publicly employed lettuce breeders to select for diverse types of lettuce cultivars suitable for baby leaf production and with BLS resistance.

The popularity of commercially produced and packaged salads has increased in the United States (Glaser et al., 2001). Baby leaf

lettuce (*Lactuca sativa* L.) is the primary component of spring mix salads, which includes some mixture of a number of different leafy green vegetable species such as spinach (*Spinacia oleracea* L.), radicchio (*Cichorium intybus* L.), frisee (*Cichorium endivia* L.), arugula (*Eruca vesicaria* L.), tatsoi and mizuna (*Brassica rapa* L.), and mache [*Valerianella locusta* (L.) Laterr.]. Spring mix is ≈90% more valuable per ton than bulk iceberg or leaf lettuce and ≈4400 ha of production was worth over \$140 million to Monterey County, CA, in 2010 (Anonymous, 2010). Significant amounts are also grown in four other California counties, although the amount of this production is not known. In 2005, Yuma County in Arizona produced ≈2800 ha of spring mix. The crop is grown at extremely high densities, up to 7.4 million seeds per hectare (three million seeds per acre) on 203- to 213-cm wide (80 to 84 inches) raised beds with 24 to 32 seed lines per bed (Nolte, 2010; Smith et al., 2009). The crop is mechanically harvested when the first four true leaves are ≈5 to 13 cm (2 to 5 inches) long, typically 20 to 30 d after planting.

Approximately 15 to 20 different types of lettuce with unique combinations of leaf shape, margin serration, lobing, undulation, pliability, savoy, and color have been used for baby leaf lettuce production, although most commercially prepared salads contain less than 10 types of lettuce. Some cultivars of romaine, leaf, Batavia, and Latin-type lettuce are used for baby leaf and whole head production. However, it is increasingly common for cultivars of these types to be bred specifically for use in baby leaf production. Other specialty leaf-type lettuce such as oak leaf, lolla rossa, and tango are used nearly exclusively for baby leaf production in the United States. A limited number of characters is considered critical to the success of a cultivar intended for baby leaf production in California and Arizona. At a minimum, the cultivar should have an interesting leaf shape and color as a seedling and possess good post-packaging shelf life. Pest resistance is increasingly important, because the short time-frame from planting to harvest constrains pesticide use and considerable amounts of baby leaf production is organic.

The U.S. Department of Agriculture (USDA) lettuce breeding program in Salinas, CA, primarily operates as an enhancement breeding program, introgressing new economically valuable characters from unadapted germplasm into inbred lines of iceberg, romaine, or leaf lettuce. The inbred lines are dispersed to other private and public breeding programs for further selection, seed increase and sales, or crossing to develop new breeding populations. The diversity of types used in baby leaf production creates a challenge for public plant breeders attempting to enhance the cultivar gene pool with new traits such as pest resistance. Introgressing a new trait into a new inbred cultivar of each type is too resource-intensive to be feasible. In this case, a strategic approach is needed that balances the need to address the diverse types used in baby leaf production while ensuring that the types with the greatest susceptibility are prioritized. This could be accomplished through surveys of existing baby leaf cultivars of each type to determine which types have the greatest need for resistance. Additionally, breeders could select and release early-generation (F₂ to F₄) populations genetically fixed for disease resistance but with sufficient variability for leaf characters to encompass two or more lettuce types used in baby leaf production. External breeding programs could then select multiple lettuce types from a single resistant population. Development of these populations would rely on crossing parents that differ for resistance and leaf morphology.

The high planting densities of baby leaf lettuce exacerbate the severity of many lettuce pests, including leafminers (*Liriomyza langei*), downy mildew (*Bremia lactucae*), and bacterial leaf spot (BLS). Bacterial leaf spot of lettuce, caused by *Xanthomonas campestris* pv. *vitians* (Xcv), is an increasingly damaging disease in California during spring and fall production (Barak et al., 2001; Bull and Koike, 2005) and is a sporadic problem

Received for publication 13 Sept. 2013. Accepted for publication 11 Nov. 2013.

This research was supported by the California Leafy Greens Research Program and the California Department of Food and Agriculture. Partial funding for work by S. Gebben was provided by an NSF Science Talent Expansion Project (STEP) grant #0525444 Hartnell College, Salinas, CA. We thank Shamrock Seed Company and Synergene Seed & Technology for contributing baby leaf lettuce cultivars used in this research.

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throughout North America (Carisse et al., 2000; Pernezny et al., 1995; Sahin and Miller, 1997; Toussaint, 1999) and globally (Myung et al., 2010; Sahin, 2000; Wallis and Joubert, 1972). The pathogen causes small angular leaf spots, which are initially water-soaked and later become necrotic (brown to black) and papery. The leaf spots can coalesce, forming large necrotic regions (Bull and Koike, 2005). The symptoms reduce the quality and yield of lettuce and increase the potential for postharvest losses (Carisse et al., 2000). Lettuce crops grown for whole heads that experience severe disease levels may compel harvest crews to cull infected heads, leaving unmarketable plants in the field and only packing the less diseased or healthy plants. The dense plantings and mechanical harvest of baby leaf crops severely reduce the feasibility of culling infected leaves. Consequently, baby leaf crops with BLS may be abandoned resulting in loss of the entire crop. Initial symptoms on cultivars with red-colored leaves can be difficult to detect in the early stages of disease development but may develop further during post-harvest shipping. Therefore, cultivars with red-colored leaves, which are common in baby leaf crops, may contribute to increased post-harvest losses of spring mix crops.

Host resistance is an efficient and cost-effective tool to manage BLS of lettuce. Diverse sources of resistance are known including the Latin-type cultivar Little Gem and the Batavia-type cultivars Batavia Reine des Glaces (synonym Reine des Glaces), Iceberg, and La Brillante (Bull et al., 2007; Hayes et al., 2013). The USDA in Salinas, CA, developed and released seven resistant iceberg-type breeding lines from the cross ('Salad Crisp' × 'Iceberg') × 'Salinas 88' (Hayes et al., 2008). 'Little Gem' and its numerous derivatives are used in baby leaf production, whereas the remaining known sources of resistance are not suitable for baby leaf production. Additional germplasm with resistance to BLS that is suitable for baby leaf production is needed. In this article, we report on the genetic diversity for resistance to BLS in cultivars used for baby leaf production and the selection of early-generation baby leaf germplasm that are genetically fixed for resistance to BLS. The populations were further characterized for salad shelf life, resistance to leafminers, and resistance to downy mildew.

Materials and Methods

Bacterial leaf spot disease evaluation. All experiments to assess BLS resistance were conducted with greenhouse-grown seedlings in plug trays with plant populations of ≈ 2380 plants/m². The results from the greenhouse assay are closely correlated with results in field experiments (Bull et al., 2007; Hayes et al., 2008) and simulate the dense plant populations used in baby leaf production. Plug trays (22 cm wide and 65 cm long) with 31 rows of 11 cells that are 20 mm × 20 mm × 60 mm were filled with commercial plug-mix potting soil. The perimeter cells of plug trays

in all experiments were seeded with the susceptible iceberg cultivar Vista Verde, whereas the remaining 29 rows of nine cells were seeded with the experiment entries (experimental families, populations, parents, or control cultivars). Each entry was assigned to and seeded in plots, which was a single row of nine cells. After seeding, plug trays were placed in a growth chamber set at 10 °C with no light for 48 h to ensure consistent germination and then relocated to the greenhouse. After emergence the trays were thinned to a single plant per cell and plants were fertilized and watered as needed until the third true leaf had fully expanded at which time the seedlings were inoculated with *Xcv*. Seedlings were sprayed to runoff with the *Xcv* suspension and incubated under constant leaf wetness for 6 d. After a 24-h drying period, the plants were rated for disease severity (DS) using either a 0 to 3 rating scale (0 = no disease; 1 = less than 10 lesions of less than 3 mm in diameter; 2 = individual disease lesions greater than 3 mm in diameter or more than 10 lesions; 3 = large coalesced lesions) or an expanded 0 to 5 rating scale (0 = no disease; 1 = less than 10 lesions of less than 3 mm in diameter; 2 = individual disease lesions greater than 3 mm in diameter or more than 10 lesions; 3 = large coalesced lesions covering less than 20% of leaf area; 4 = lesions covering 20% to 50% of leaf area; 5 = lesions covering greater than 50% of leaf area).

Three *Xcv* strains (BS339, BS340, and BS347) were used as inoculum in all experiments except a single experiment evaluating F_{3,4} families where only strain BS347 was used. All of these strains were isolated from lettuce plants in California (Bull et al., 2007). The inoculum was prepared by spreading a single colony from each of the three strains across 100 × 15-mm petri dishes containing nutrient agar (Difco Laboratories, Detroit, MI) or yeast-peptone-glucose-agar consisting of 7 g yeast extract, 7 g Bacto Peptone (Difco Laboratories), 7 g glucose, and 15.0 g agar per liter, and incubated at 26 °C for 48 h to produce lawns. Plates were flooded with ≈ 15 mL of 0.01 M sterile phosphate buffer (pH 7.0) and the resulting suspensions were adjusted to $\approx 1 \times 10^8$ colony-forming units/mL (0.6 O.D. at 600 nm) using a Shimadzu (Kyoto, Japan) spectrophotometer model number UV-1601. The suspensions of each isolate were then mixed in equal proportions.

Lettuce populations and selection for BLS resistance. Lettuce is a diploid ($2n = 2x = 18$) autogamous species and cultivars are inbred homogenous lines. A population of 37 lettuce cultivars of 10 types was compiled to assess the genetic diversity for BLS resistance in currently available baby leaf cultivars. The cultivars were selected from the USDA, Salinas, CA, germplasm collection or were provided by participating seed companies. This population included as controls the susceptible iceberg cultivar Vista Verde and the resistant Latin-type cultivar Little Gem. This population was tested in two greenhouse experiments using a randomized complete block design (RCBD) with three replications.

A population for selection of BLS-resistant baby leaf germplasm was developed by crossing the cultivars Batavia Reine des Glaces and Eruption using the method of Ryder and Johnson (1974) to produce F₁ seed. 'Eruption' was used as the pollen parent. The F₂, F_{2,3}, and F_{3,4} generations were produced by growing plants in the greenhouse and allowing each plant to naturally self-pollinate. Seed from each plant was kept separate unless otherwise noted. The parent 'Batavia Reine des Glaces' (PI634668, <<http://www.ars-grin.gov/cgi-bin/npgs/acc/display.pl?1594749>>; CGN05864, <<http://applicaties.wageningenur.nl/applications/cgngenis/AccessionDetails.aspx?ID=uh22ve45&acnumber=CGN05864>>) is a heirloom cultivar with crisp, highly serrate or frilled, medium green leaves. 'Batavia Reine des Glaces' possess shelf life of packaged salad that is commercially acceptable (Simko et al., 2012). 'Eruption' (PI 613577, <<http://www.ars-grin.gov/cgi-bin/npgs/acc/display.pl?1600187>>) is a dark red-colored cultivar developed by the seed company Enza Zaden B.V. for use in commercial baby leaf production. 'Eruption' carries the *short leaf 1* (*sll*) gene, which confers a diminutive stature to 'Eruption' that is architecturally similar to many Latin-type cultivars (Hayes et al., 2011a). Progeny from intertype crosses with 'Eruption' that do not inherit *sll* can have a romaine-type architecture. The shelf life of packaged salad prepared from 'Eruption' is inferior to many commercial cultivars (Simko et al., 2012), but the cultivar has nonetheless been used in commercially prepared spring mix salads.

The F₂ and F_{2,3} generations of 'Batavia Reine des Glaces' × 'Eruption' were selected for BLS resistance equivalent to 'Batavia Reine des Glaces' and for red-colored leaves. The F₂ progenies were grown and tested with the parent cultivars in a single experiment; no data were collected on F₂ seedlings or parents. Seedlings selected from this experiment were grown to produce F_{2,3} seeds. The resulting F_{2,3} families were evaluated in three experiments using a RCBD with four blocks. Control cultivars (Little Gem, La Brillante, and Vista Verde) and parent cultivars were included in these experiments. After the first experiment, only the most desirable F_{2,3} families were retained for testing in the subsequent two experiments as was a single susceptible family for use as an additional susceptible control. From this series of experiments, two F_{2,3} families were selected for further inbreeding. Plants were randomly selected from within these F_{2,3} families and allowed to self-pollinate to generated two populations comprised of 38 and 72 F_{3,4} seed lots. The F_{3,4} families were evaluated for BLS resistance in an experiment using an augmented randomized complete block design (ARCB) with 22 blocks and eight plots per block. The control cultivar Vista Verde and the parent cultivars were replicated once in each block, whereas F_{3,4} families were unreplicated and randomly distributed to the remaining plots.

Lettuce salad shelf life, leafminer and downy mildew assessments in field experiments. Two field experiments were conducted using

two F_{2,3} families from 'Batavia Reine des Glaces' × 'Eruption' to assess shelf life of salad cut lettuce and susceptibility to leafminers (*Liriomyza langei*) and downy mildew (*Bremia lactucae*) in 2012. The first field experiment was planted on 26 June as a RCBD with three blocks at the USDA Spence research farm near Salinas, CA. The cultivars Parris Island Cos (green romaine used in commercial baby leaf production and good salad shelf life control), Annapolis (red romaine used in commercial baby leaf production), the parent cultivars Batavia Reine des Glaces and Eruption, and two F_{2,3} families were evaluated for salad shelf life. The lettuce was harvested on 3 Oct. and processed into salad on 4 Oct. Shelf life was evaluated once per week for 4 weeks. 'Batavia Reine des Glaces', 'Eruption', and the F_{2,3} families were also evaluated for leafminer damage on 29 July and 16 Sept. The fourth or fifth fully expanded leaf was sampled from 25 to 30 plants of each family grown in the USDA Spence research farm experiment. An image of the leaves from each family was taken using

a Canon PowerShot SX110 IS digital camera (Tokyo, Japan).

The second field experiment was planted on 26 July at the USDA, U.S. Agricultural Research Station in Salinas, CA, and used a completely randomized design (CRD) with two replications. 'Parris Island Cos', 'Triple Threat' (green romaine and poor salad shelf life control), 'Annapolis', 'Batavia Reine des Glaces', 'Eruption', and two F_{2,3} families were harvested on 17 Sept. and processed into salad on 18 Sept. Shelf life was evaluated once per week for 5 weeks. These same cultivars, plus the green leaf cultivar Grand Rapids, were evaluated for leafminer damage on 28 Aug. and 26 Sept. and for downy mildew damage 17 Sept., 24 Sept., and 1 Oct.

Damage from leafminers and downy mildew was the result of natural infestations. Leafminer stings/cm² on a 20-cm² leaf area with highest sting density and the number of mines per plant were counted on two randomly selected plants per replication. Downy mildew severity of each plot was assessed using a scale of 0 for no disease to 5 for

severe disease on leaves (Simko et al., 2012). Salad shelf life was evaluated by harvesting fully grown mature heads and processing and packaging the salad according to the methods of Hayes and Liu (2008). One head of lettuce was used to make one bag of salad cut lettuce that consisted of 170 g of 2.5-cm² lettuce pieces in transparent 22.8 × 15-cm transparent bags. The film to make the bags was 63.5-μm-thick polyethylene coextruded film with an oxygen transmission rating of 0.94 nmol·s⁻¹·m⁻²·Pa⁻¹ as determined by the manufacturer (Printpack, Atlanta, GA). Before sealing the bags closed, the packaged was triple-flushed with N₂ to prevent discoloration of the cut surfaces. Decay of cut lettuce was visually evaluated on a 0 through 10 scale that corresponds to the estimated percentage of decayed tissue divided by 10.

Analysis of baby leaf cultivar data. Bacterial leaf spot DS data on individual plants within plots was averaged into a single plot mean for use in subsequent analysis. The arithmetic DS means of the two experiments were significantly correlated ($r = 0.45$,

Table 1. Bacterial leaf spot disease severity in 36 lettuce cultivars used in baby leaf production and the iceberg cultivar Vista Verde evaluated in two replicated greenhouse experiments inoculated with *Xanthomonas campestris* pv. *vitians* strains BS339, BS340, and BS347.

Cultivar	Market type	Number of replications ^a	Bacterial leaf spot disease severity ^b					
			Median	Minimum	Maximum	Estimate	Relative treatment effect ^c	
							99% CI	
							Lower	Upper
Vista Verde	Iceberg, susceptible control	6	2.1	0.8	2.9	0.8	0.37	0.91
Little Gem	Latin, resistant control	6	0.6	0.3	1.0	0.2	0.10	0.38
Brigade	Latin	6	0.9	0.4	1.5	0.3	0.15	0.61
Evergreen	Green romaine	6	0.7	0.2	1.2	0.2	0.10	0.50
Green Acres	Green romaine	6	1.1	0.1	1.6	0.4	0.23	0.68
Lexington	Green romaine	6	0.8	0.5	1.0	0.3	0.16	0.38
Mutiny	Green romaine	6	0.5	0.0	1.0	0.1	0.05	0.37
Skyway	Green romaine	4	0.9	0.0	2.0	0.4	0.08	0.81
Lolla Verde	Lolla verde	6	1.1	0.6	1.4	0.4	0.25	0.61
Barrage	Lolla rossa	6	1.8	1.0	1.8	0.7	0.43	0.83
Fortress	Lolla rossa	6	1.3	0.7	2.0	0.6	0.34	0.82
Lolla Rossa	Lolla rossa	6	0.8	0.2	1.6	0.3	0.11	0.67
Rampage	Lolla rossa	6	1.1	1.0	2.8	0.6	0.34	0.84
Green Miles	Green leaf	5	1.1	0.4	1.7	0.4	0.19	0.72
Green Miles Elite	Green leaf	5	1.4	0.5	1.6	0.5	0.25	0.77
Revenge	Green leaf	6	1.8	0.5	2.5	0.7	0.28	0.91
Sergeant	Green leaf	6	1.1	0.0	2.0	0.4	0.18	0.43
Beijing Green	Green oak leaf	5	1.1	0.9	2.4	0.6	0.25	0.87
General	Green oak leaf	5	0.5	0.1	1.1	0.2	0.06	0.53
Stardom	Green oak leaf	6	1.2	0.8	2.0	0.6	0.35	0.77
Crimson	Red oak leaf	5	0.8	0.6	1.2	0.3	0.14	0.51
Red Tamale	Red oak leaf	6	1.4	0.4	2.1	0.6	0.30	0.82
SSC 1980	Red oak leaf	6	1.0	0.0	2.1	0.4	0.16	0.77
Shanghai Red	Red oak leaf	5	1.1	0.3	1.8	0.5	0.21	0.80
Stealth	Red oak leaf	6	0.8	0.4	1.6	0.3	0.13	0.58
Cardinale	Red leaf	6	2.2	1.3	2.7	0.9	0.64	0.96
Cavalry	Red leaf	6	1.1	0.7	2.0	0.5	0.24	0.75
Eruption	Red leaf	6	1.4	0.6	2.0	0.6	0.30	0.80
Picante Red	Red leaf	6	1.3	0.6	2.0	0.5	0.23	0.81
Red Hot	Red leaf	6	1.5	0.8	1.9	0.6	0.40	0.79
Rouge De Grenoblouse	Red leaf	6	1.6	1.0	1.9	0.7	0.47	0.82
SSC 2633	Red leaf	6	1.3	0.4	2.1	0.5	0.24	0.81
Annapolis	Red romaine	6	1.6	1.0	2.8	0.7	0.41	0.87
Flagship	Red romaine	6	1.1	0.3	2.1	0.5	0.20	0.77
Red Wonder	Red romaine	6	1.6	0.9	2.4	0.7	0.42	0.87
Red Zin	Red romaine	6	1.0	0.8	2.7	0.5	0.23	0.75
Rouge d'Hiver	Red romaine	6	1.9	1.1	2.4	0.8	0.54	0.91

^aTotal number of replicates in two experiments. Each experiment had no more than three replications per cultivar.

^bDisease severity (DS) was evaluated on the top three leaves (0 = no disease; 1 = less than 10 lesions of less than 3 mm in diameter; 2 = individual disease lesions greater than 3 mm in diameter or more than 10 lesions; 3 = large coalesced lesions).

^cThe relative treatment effect and 99% confidence intervals (CI) were calculated from analysis of the rank values of the disease severity data.

$P = 0.0047$) and the cultivar $\times$ experiment interaction in the final analysis was not significant ($P = 0.1$). Therefore, the data from the two experiments were combined into a single analysis. The treatments (cultivars) exhibited heterogeneous variances and the errors were not normally distributed. Therefore, these data were analyzed using analysis of variance-type statistics of ranked data using the PROC Mixed procedure in SAS Version 9.3 (SAS Institute, Cary, NC) and the LD_CI macro to generate relative treatment effects (RTE) for each treatment and 99% confidence intervals for detection of statistical differences between treatments (Brunner et al., 2002; Shah and Madden, 2004). Significant differences between RTE were declared when the 99% confidence intervals for two cultivars did not overlap. The median and maximum DS was calculated for each cultivar.

Analysis of 'Batavia Reine des Glaces' $\times$ 'Eruption' families. Greenhouse and field experiments involving 'Batavia Reine des Glaces' $\times$ 'Eruption' families, control cultivars, and parents in RCBD or CRD were analyzed in Proc Mixed of SAS Version 9.3 using the methods recommended by Littell et al. (2006). The data from each experiment were analyzed separately. Entry (family, cultivar, or parent) was treated as a fixed effect. The downy mildew data from the three assessment dates were averaged into a single value for analysis, whereas leafminer and salad shelf life assessment dates were analyzed separately. Salad shelf life data were transformed to the square root scale before analysis to improve normality; means were back-transformed for reporting on tables. Individual plant data of leafminer damage and BLS DS within plots were averaged into a single value before analysis in Proc Mixed. Bacterial leaf spot DS from the greenhouse experiment with $F_{3,4}$ families using the ARCBD was first analyzed in Proc Mixed of SAS to compare the two populations of 'Batavia Reine des Glaces' $\times$ 'Eruption' $F_{3,4}$ families to control cultivars and parents. Entry (population, cultivar, or parent) was treated as a fixed effect, whereas each $F_{3,4}$ family was treated as a replicate of each population. The data were then analyzed to compare the DS of cultivars to the DS of each individual $F_{3,4}$ family in Proc Mixed using the methods of Wolfinger et al. (1997). For all analyses, least square means of populations, families, or cultivars were outputted from SAS and compared using the Tukey-Kramer multiple comparison procedure. To determine if families or populations contained genetic variation for BLS resistance or salad shelf life, the variance within $F_{2,3}$ families or within populations of $F_{3,4}$ families was calculated and compared using F -tests to an estimate of environmental variance. The environmental variance was estimated by calculating the variance between seedlings within each cultivar or between plots of each cultivar. This variance for each cultivar was then pooled to arrive at a single estimate of environmental variance. Family or population

variances greater than this were attributed to the presence of genetic variation.

Results and Discussion

Genetic variation for BLS resistance in baby leaf lettuce cultivars. Genetic variation for BLS resistance was detected within a population of 37 lettuce cultivars using a

greenhouse evaluation method. The greenhouse evaluation method used in this research is indicative of results from field experiments, as Bull et al. (2007) used the same greenhouse method and reported a correlation of 0.77 between the disease levels observed in field and greenhouse experiments. The median DS of the susceptible control 'Vista Verde' was 2.1, whereas the

Table 2. Bacterial leaf spot (BLS) disease severity of $F_{2,3}$ families from the cross 'Batavia Reine des Glaces' $\times$ 'Eruption'; the resistant cultivars Batavia Reine des Glaces, La Brillante, and Little Gem; and the susceptible cultivars Eruption and Vista Verde in two replicated greenhouse experiments inoculated with *Xanthomonas campestris* pv. *vitiensis* strains BS339, BS340, and BS347.

Cultivar name or family number	Greenhouse Expt. 1			Greenhouse Expt. 2		
	Number of plants ^x	BLS disease severity ^z		Number of plants	BLS disease severity ^y	
		Mean	Variance between plants		Mean	Variance between plants
Batavia Reine des Glaces	32	1.3 fgh ^w	1.0	36	2.6 de	1.3
Eruption	31	2.9 a	1.9	36	3.7 ab	2.9
La Brillante	31	0.7 h	0.8	36	1.8 fg	0.7
Little Gem	34	0.8 gh	0.2	36	1.2 g	0.8
RH11-1906	35	2.0 cdef	0.3	36	2.9 cd	2.0
RH11-1907	31	2.3 abc	1.3	36	2.8 ce	2.3
RH11-1909	31	2.0 cde	1.3	36	2.9 ce	2.0
RH11-1912	36	2.6 abc	0.1	36	3.1 bcd	2.6
RH11-1922	34	1.6 def	0.6	36	2.5 def	1.6
RH11-1927	33	1.4 eg	0.6	36	2.2 ef	1.4
RH11-1931 ^v	28	2.7 ab	2.3 ^t	36	3.3 ac	2.7
RH11-1933	21	2.1 bd	1.5 ^t	36	3.1 acd	2.1
RH11-1936	33	2.6 abc	0.8	36	3.1 acd	2.6
Vista Verde	34	2.9 a	1.1	36	3.8 a	2.9
Environmental variance ^u			0.98			1.75

^zDisease severity was evaluated on the top three leaves where 0 = no disease; 1 = less than 10 lesions of less than 3 mm in diameter; 2 = individual disease lesions greater than 3 mm in diameter or more than 10 lesions; 3 = large coalesced lesions.

^yDisease severity was evaluated as 0 = no disease; 1 = less than 10 lesions less than 3 mm in size; 2 = one lesion greater than 3 mm or more than 10 lesions; 3 = coalesced lesions covering less than 20% of leaf area; 4 = lesions covering 20% to 50% of leaf area; 5 = lesions covering greater than 50% of leaf area.

^xTotal number of plants tested from four reps as a randomized complete block design with up to nine plants per replication.

^wMeans with different letters are significantly different at $P < 0.05$. Significant differences were determined using the Tukey-Kramer multiple comparison procedure.

^v $F_{2,3}$ family selected for susceptibility and retested as a control.

^uThe environmental variance was calculated as the variance among seedlings pooled across cultivars.

^tVariance of $F_{2,3}$ family significantly greater than the environmental variance at $P < 0.05$ using an F -test.

Table 3. Bacterial leaf spot disease severity of two populations of $F_{3,4}$ families from 'Batavia Reine des Glaces' $\times$ 'Eruption', the resistant cultivar Batavia Reine des Glaces, and the susceptible cultivars Eruption and Vista Verde in a greenhouse experiment inoculated with *Xanthomonas campestris* pv. *vitiensis* strain BS347.

Cultivar name or population number	Number of replications	Number of $F_{3,4}$ families	Bacterial leaf spot disease severity ^z		Percent $F_{3,4}$ families significantly better than Eruption ^y
			Mean	Variance between $F_{3,4}$ families or cultivar replicates	
Batavia Reine des Glaces	22		1.2 a ^x	0.08	
Eruption	22		3.0 b	0.08	
RH11-1922		38	1.2 a	0.05	100
RH11-1927		72	1.2 a	0.08	94
Vista Verde	22		3.0 b	0.06	
Environmental variance ^w				0.07	

^zDisease severity was evaluated as 0 = no disease; 1 = less than 10 lesions less than 3 mm in size; 2 = one lesion greater than 3 mm or more than 10 lesions; 3 = coalesced lesions covering less than 20% of leaf area; 4 = lesions covering 20% to 50% of leaf area; 5 = lesions covering greater than 50% of leaf area.

^yMean disease severity significantly lower than Eruption at $P < 0.05$ determined using the Tukey-Kramer multiple comparison procedure.

^xMeans with different letters are significantly different at $P < 0.05$ using the Tukey-Kramer multiple comparison procedure.

^wEnvironmental variance was calculated as the variance of replicated plots pooled across cultivars.

resistant control 'Little Gem' had a median DS of 0.6 (Table 1). The RTE of 'Little Gem' (0.2) and 'Vista Verde' (0.8) had 99% confidence intervals that slightly overlap ('Little Gem': 0.10 to 0.38; 'Vista Verde': 0.37 to 0.91), indicating no significant difference between 'Little Gem' and 'Vista Verde' in these experiments. 'Little Gem' and 'Vista Verde' were previously reported to possess different levels of BLS resistance (Bull et al., 2007), and the inability to statistically separate the DS of 'Little Gem' and 'Vista Verde' in these experiments was likely the result of disease escapes occurring in the susceptible cultivar. The occurrence of disease escapes was apparent in the large difference between minimum (least diseased replicate) and maximum (most diseased replicate) DS within susceptible cultivars compared with resistant cultivars. For example, the minimum DS for 'Vista Verde' was 0.8, whereas the maximum DS was 2.9. In contrast, the minimum and maximum DS of 'Little Gem' was 0.3 and 1.0, respectively. Significant differences among other cultivars were detected. The lowest DS was observed in the green romaine cultivar Mutiny (median DS = 0.5 and RTE = 0.1). The DS observed in 'Mutiny' was significantly lower than the 'Barrage', 'Cardinale', 'Red Hot', 'Rouge De Grenoblouse', 'Annapolis', 'Red Wonder', and 'Rouge d'Hiver'. All of these susceptible cultivars except 'Barrage' are either red leaf or red romaine-type cultivars. Although the remaining red leaf or red romaine cultivars were not significantly different from 'Mutiny', all red leaf and red romaine cultivars had a median DS of 1 or greater and maximum DS of 1.9 or greater. These results indicated that breeding BLS-resistant red leaf and red romaine germplasm was a higher priority than breeding resistant cultivars of other lettuce types.

Selection for BLS resistance in 'Batavia Reine des Glaces' × 'Eruption'. From a population of 486 'Batavia Reine des Glaces' × 'Eruption' F₂ seedlings, 38 were selected for low disease severity and red color. The subsequent experiment evaluating the resulting 38 F_{2,3} families, cultivars, and parents identified genetic differences for BLS DS. 'Little Gem' (DS mean = 0) and 'La Brillante' (DS mean = 0.1) had significantly lower DS compared with 'Batavia Reine des Glaces' (DS mean = 0.8) and 'Eruption' (DS mean = 1.6). 'Batavia Reine des Glaces' had less disease than 'Eruption', which approached significance ($P = 0.0937$). 'Batavia Reine des Glaces' and 'Eruption' both had significantly less disease than 'Vista Verde' (DS mean = 3.0). The DS means of F_{2,3} families ranged from 0.7 to 2.1. Thirteen F_{2,3} families had significantly greater DS than 'Batavia Reine des Glaces'. Only one F_{2,3} family (RH11-1927) had significantly lower DS than 'Eruption'. Regardless, eight F_{2,3} families that were uniform or segregating for red-colored leaves and having numerically lower DS than 'Eruption' were selected for additional testing. One additional family (RH11-1931) was selected for retesting as an additional susceptible control. The remaining 29 families were discarded.

Nine selected F_{2,3} families, their parents as well as 'Little Gem', 'La Brillante' (resistant controls), and 'Vista Verde' (susceptible control) demonstrated genetic differences for BLS resistance in two independent experiments (Table 2). In both experiments, 'Batavia Reine des Glaces' had significantly less disease than 'Eruption' and 'Vista Verde'. 'Little Gem' and 'La Brillante' had significantly less disease than 'Eruption' and 'Vista Verde' in greenhouse Expt. 1 and significantly less disease than 'Batavia Reine des Glaces', 'Eruption', and 'Vista Verde' in greenhouse Expt. 2. Among the F_{2,3} families, RH11-1931 (susceptible F_{2,3} family) had the greatest amount of disease in both experiments. Three F_{2,3} families (RH11-1906, RH11-1922, and

RH11-1927) had DS means that were significantly less than 'Eruption', not significantly different from 'Batavia Reine des Glaces', and with a variance between seedlings not significantly different from the between seedling environmental variance. Therefore, these families were potentially genetically fixed for BLS resistance alleles. Among these three families, RH11-1922 and RH11-1927 segregated for plants with dark red- and green-colored leaves. These families were selected for additional testing, whereas the remaining F_{2,3} families were discarded.

Thirty-eight F_{3,4} families randomly selected from RH11-1922 and 72 F_{3,4} families randomly selected from RH11-1927 demonstrated BLS resistance equivalent to 'Batavia

Table 4. Leafminer stings/cm² and number of leafminer mines per plant at two assessment dates in two replicated field experiments and downy mildew disease severity in a single replicated field experiment for 'Batavia Reine des Glaces' × 'Eruption' F_{2,3} families RH11-1922 and RH11-1927 and six lettuce cultivars.

Cultivar or family	Spence farm field experiment			Salinas field experiment			Downy mildew severity ^z
	Leafminer damage			Leafminer damage			
	29 July			28 Aug.			
	Stings/cm ²	Number of mines	16 Sept. Stings/cm ²	Stings/cm ²	Number of mines	26 Sept. Stings/cm ²	
RH11-1922	13.1 b ^y	0.8 ab	9.7 a	7.8 bc	2.8 a	7.1 bd	2.5
RH11-1927	17.3 a	1.5 a	10.8 a	13.7 ab	2.0 ab	8.9 bc	2.7
Batavia Reine des Glaces	17.8 a	1.5 a	11.6 a	16.5 a	2.8 a	12.1 a	2.0
Eruption	5.7 c	0.5 b	5.9 b	9.6 b	2.0 ab	10.9 ab	2.4
Annapolis				1.9 c	0.5 b	3.8 d	2.5
Grand Rapids				2.5 c	2.8 a	4.9 cd	1.8
Triple Threat				9.6 b	2.5 a	12.0 a	3.1
Parris Island Cos				10.00 b	2.0 a	13.1 a	2.5

²Downy mildew severity was assessed on 17 Sept., 24 Sept., and 1 Oct. using a scale of 0 for no disease to 5 for severe disease and then averaged into a single value. Downy mildew means not significantly different.

^yMeans with different letters are significantly different at $P < 0.05$ using the Tukey-Kramer multiple comparison procedure.

Table 5. Mean and variance of salad cut lettuce decay of 'Batavia Reine des Glaces' × 'Eruption' F_{2,3} families RH11-1922 and RH11-1927 and five cultivars in two replicated field experiments.

Cultivar or family	Salad shelf life decay ^z						
	Number of bags	Week 3		Week 4		Week 5	
		Mean	Var ^y	Mean	Var	Mean	Var
Salinas field experiment							
RH11-1922	28	0.4 c ^x	0.9	1.7 c	1.9	5.0 b	4.8
RH11-1927	26	0.4 c	1.0	0.8 c	5.2 ^v	3.0 c	7.9 ^v
Parris Island Cos	20	0.4 c	0.5	0.9 c	1.4	3.5 bc	3.6
Batavia Reine des Glaces	20	0.4 c	0.6	1.7 c	2.1	3.6 bc	2.5
Annapolis	21	0.7 c	0.6	4.2 b	7.3	7.7 a	5.6
Eruption	22	1.6 b	3.3	4.7 b	4.0	8.2 a	4.2
Triple Threat	19	8.5 a	2.0	10.0 a	0.0	10.0 a	0.0
Environmental variance ^w			1.5		3.1		3.3
Spence farm field experiment							
RH11-1922	31	0.7 b	0.8	2.4 cb	7.4		
RH11-1927	29	2.2 a	3.1	4.1 ab	8.7		
Parris Island Cos	18	0.4 b	2.9	1.5 c	6.5		
Batavia Reine des Glaces	43	0.8 b	2.6	2.4 c	5.2		
Annapolis	30	0.2 b	1.3	2.7 cb	13.9		
Eruption	32	2.1 a	3.0	5.5 a	6.0		
Environmental variance ^w			2.4		7.7		

²Decay scored weekly after processing as 0 = 0% decay to 10 = 100% decay. Decay data were transformed to the square root scale before analysis to improve normality. Means were back-transformed for reporting on tables.

^yVar = Variance between bags of salad within a cultivar or line. One head of field-grown lettuce was used to make one bag of salad.

^xMeans with different letters are significantly different at $P < 0.05$ using the Tukey-Kramer multiple comparison procedure.

^wEnvironmental variance was calculated as the variance of replicated plots pooled across cultivars.

^yVariance of RH11-1927 significantly greater than the environmental variance at $P < 0.05$.

Reine des Glaces', 'Batavia Reine des Glaces' and the $F_{3:4}$ populations RH11-1922 and RH11-1927 all had DS means of 1.2, which was significantly less than 'Eruption' and 'Vista Verde' (Table 3). Analysis of DS of $F_{3:4}$ families within the RH11-1922 and RH11-1927 populations indicated that these populations do not segregate for BLS resistance. The variance between $F_{3:4}$ families was not significantly different from the environmental variance. Additionally, 100% of RH11-1922 $F_{3:4}$ families and 94% of RH11-1927 $F_{3:4}$ families had significantly lower DS than 'Eruption'.

Salad shelf life and susceptibility to leafminer and downy mildew. The $F_{2:3}$ generation of RH11-1922, RH11-1927, and 'Batavia Reine des Glaces' was similar for the number of stings/cm² and the number of mines per plant in the Spence Farm experiment, all generally having greater leafminer damage than the 'Eruption' (Table 4). In the Salinas field experiments, RH11-1922, RH11-1927, 'Batavia Reine des Glaces', 'Triple Threat', 'Parris Island Cos', and 'Eruption' were similar for the number of stings/cm² and the number of mines per plant, although some significant differences for mean stings/cm² were detected. 'Annapolis' had significantly fewer mines compared with all other cultivars or families, whereas 'Annapolis' and 'Grand Rapids' had significantly fewer stings/cm² compared with all other cultivars and $F_{2:3}$ RH11-1927. Downy mildew severity ranged from 1.8 ('Grand Rapids') to 3.1 ('Triple Threat'), although the differences between these cultivars were not significant. RH11-1922, RH11-1927, 'Batavia Reine des Glaces', and 'Eruption' appear to have similar levels of susceptibility to downy mildew.

RH11-1922, RH11-1927, the parents, and control cultivars did not exhibit significant differences for salad decay in the first 2 weeks of storage in either field experiment (data not shown). At Weeks 3, 4, and 5, the parents and control cultivars decayed at a rate consistent with previously published experiments (Hayes and Liu, 2008; Simko et al., 2012) (Table 5). The families RH11-1922 and RH11-1927 decayed at a similar rate as 'Batavia Reine des Glaces' and 'Parris Island Cos' in the Salinas experiment. In the Spence farm experiment, RH11-1922 decayed at a faster rate (higher decay means) than 'Batavia Reine des Glaces' and 'Parris Island Cos'. RH11-1922 and RH11-1927 demonstrated improved shelf life compared with 'Eruption', although the difference between RH11-1927 and 'Eruption' was not significant at Week 4 in the Spence farm experiment. RH11-1922 and RH11-1927 were significantly better than 'Annapolis' at Weeks 4 and 5 in the Salinas experiment but not significantly different from 'Annapolis' in the Spence farm experiment at Week 4. All families or cultivars had superior shelf life compared with 'Triple Threat' in the Salinas experiment; 'Triple Threat' is a known poor shelf life control (Hayes and Liu, 2008; Simko et al., 2012). The variance between bags within RH11-1927 was significantly greater than

the environmental variance in the Salinas experiment. Selection for slow decay within RH11-1927 may result in genetic improvement of salad shelf life.

Morphological description and availability of germplasm. RH11-1922 and RH11-1927 are romaine-type populations that segregate for leaf color, savoy, shape, undulation, and margin serration (Fig. 1). RH11-1922 segregates for black and white seed color. When grown to whole head maturity, the plants are generally romaine type with open tops (rather than closed-top hearting-type romaine). Variability for days to flower and seed set as well as resistance or susceptibility to other diseases or physiological defects not discussed in this report is unknown. Equal aliquots of the remaining seed of all $F_{3:4}$ families from RH11-1922 and RH11-1927 were massed together to produce populations RH12-3370 and RH12-3371 for distribution. Limited samples of seed are available for distribution to all interested parties for the development and commercialization of new cultivars. Requests for seed should be sent to the corresponding author. It is requested that appropriate recognition be made if these breeding lines contribute to research or the development of new germplasm, breeding lines, or cultivars.

Conclusion

Resistance to BLS in lettuce is available in diverse lettuce cultivars encompassing most lettuce types (Table 1; Bull et al., 2007), which is advantageous for breeding commercially viable lettuce cultivars. Intra-type crosses (romaine $\times$ romaine, iceberg $\times$ iceberg) often result in higher frequencies of

progeny that are in the same market type as their parents. Because this typically translates to higher frequencies of desirable plants, smaller populations are needed for successful breeding. In this research we have used the opposite approach to conduct enhancement breeding and developed a population from an intertype cross possessing extensive segregation for leaf morphology. As a result, multiple market types of lettuce may be selected from this population. These populations (RH12-3370 and RH12-3371) were released into the public domain with no intellectual property protection. It is intended that these populations will be used by diverse institutions to select baby leaf lettuce cultivars with BLS resistance. These populations were characterized for salad shelf life, leafminer resistance, and downy mildew resistance. Both populations appear to have industry-acceptable salad shelf life. The populations are susceptible to downy mildew and leafminer, which is not surprising because 'Batavia Reine des Glaces' and 'Eruption' do not appear to have economically useful levels of resistance to these pests. Crossing selected plants from RH12-3370 and RH12-3371 to leafminer or downy mildew-resistant parents will be needed to breed cultivars with resistance to multiple pests.

The breeding scheme applied in this research uses early generation testing, which is difficult to apply to characters that are determined by numerous genes or have low trait heritability (Fehr, 1991). Consequently, the application of this breeding approach to other economically important characters of baby leaf lettuce is highly dependent on the inheritance of the targeted traits. The inheritance

Fig. 1. Leaves of $F_{2:3}$ families RH11-1922 (A) and RH11-1927 (B) grown in a field experiment at the Spence farm near Salinas, CA. RH11-1922 and RH11-1927 are from the cross 'Batavia Reine des Glaces' $\times$ 'Eruption'. The experiment was planted on 26 June; pictures were taken when plants had four to five fully expanded leaves. Each leaf is from a different plant.

of salad cut lettuce shelf life can be simple (Hayes et al., 2011b) or complex (Zhang et al., 2007). The inheritance of BLS resistance is known only in 'La Brillante', in which resistance is conferred by a single dominant gene (Hayes et al., 2013). Bacterial leaf spot DS evaluations in the 'Batavia Reine des Glaces' × 'Eruption' cross were conducted on families with prior selection for resistance. Regardless, intermediate levels of resistance were observed suggesting that BLS DS in randomly selected progeny could be quantitatively distributed. Greenhouse BLS testing of seedlings, which facilitates the evaluation of large progeny populations, may have contributed to the success of this breeding effort. By testing large populations, the likelihood of selecting progeny possessing all the necessary genes for resistance increases.

We confirmed high-level resistance in the Latin-type cultivar Little Gem and the Batavia cultivar La Brillante to California isolates of *Xanthomonas campestris* pv. *vitians*. The level of resistance in 'Little Gem' and 'La Brillante' appears to be greater than in 'Batavia Reine des Glaces'. Deployment of new cultivars with resistance derived from 'Little Gem' or 'La Brillante' may result in greater levels of BLS control.

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