CALIFORNIA LEAFY GREENS RESEARCH PROGRAM
April 1, 2023 to March 31, 2024

GENETIC VARIATION IN LETTUCE

Richard W. Michelmore
The Genome Center and
The Department of Plant Sciences
University of California, Davis
rwmichelmore@ucdavis.edu

SUMMARY:

We continue to develop and apply novel methods for detecting, analyzing, and manipulating genetic variation in lettuce. This project aims to ensure that lettuce benefits from the application of genomic and biotechnological techniques and has four components: (i) transgene expression and genome editing in lettuce; (ii) genetic mapping, cloning, and characterization of disease resistance genes; (iii) developing genomic resources; and (iv) comparative genomics to identify candidate genes controlling horticultural traits. We identified the promoter of the lettuce ubiquitin gene as providing high levels of expression; we are currently confirming the stability of its expression over generations using the chimeric *RUBY* transgene as a visible reporter. We continue to make extensive use of high-throughput sequencing, marker technologies, and genome editing for gene identification. Version 11 of the reference genome assembly of cv. Salinas, which has nine telomere-to-telomere scaffolds and improved contiguity and sequence accuracy, was uploaded to GenBank. A telomere-to-telomere, ~gapless v14 genome assembly based on HiFi and nanopore long-reads is nearing completion. We are currently generating T2T genome assemblies for ~100 additional lines to develop a pangenome for lettuce and a catalog of resistance gene candidates. Genome sequences are being mined for candidate genes for traits, such as disease resistance and development. We are continuing programs to study corky root resistance, Verticillium wilt resistance, and salinity tolerance in lettuce. We continue to curate several databases that include genetic, molecular marker, phenotypic, and sequence data for lettuce. Key sequences have been uploaded to GenBank to make them widely accessible.

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PROJECT TITLE: **GENETIC VARIATION IN LETTUCE**

PRINCIPAL INVESTIGATOR: **Richard W. Michelmore**
The Genome Center and
The Department of Plant Sciences
University of California, Davis
rwmichelmore@ucdavis.edu

COOPERATING PERSONNEL: **Keri Cavanaugh, María José Truco, Dean Lavelle, Giulia Caliandro, Alex Kozik, Huaqin Xu, Maria Ferrer Ruiz, Beth Rowan,** UC Davis Genome Center and The Dept. of Plant Sciences, UC Davis.
Ivan Simko, Beiquan Mou, Steve Klosterman, UDSA-ARS, Salinas.
Michael Cahn, UC Cooperative Extension, Monterey Co.
Krishna Subbarao, Dept. of Plant Pathology, UC Davis.
Duke Pauli, School of Plant Sciences, U. Arizona.
German Sandoya, Horticultural Sciences Dept., U. Florida.
David Still, Dept. Plant Sciences, Cal Poly Pomona.

ABSTRACT:

We continue to develop and apply novel methods for detecting, analyzing, and manipulating genetic variation in lettuce. This project aims to ensure that lettuce benefits from the application of genomic and biotechnological techniques and has four components: (i) transgene expression and genome editing in lettuce; (ii) genetic mapping, cloning, and characterization of disease resistance genes; (iii) developing genomic resources; and (iv) comparative genomics to identify candidate genes controlling horticultural traits. We identified the promoter of the lettuce ubiquitin gene as providing high levels of expression; we are currently confirming the stability of its expression over generations using the chimeric *RUBY* transgene as a visible reporter. We continue to make extensive use of high-throughput sequencing, marker technologies, and genome editing for gene identification. Version 11 of the reference genome assembly of cv. Salinas, which has nine telomere-to-telomere scaffolds and improved contiguity and sequence accuracy, was uploaded to GenBank. A telomere-to-telomere, ~gapless v14 genome assembly based on HiFi and nanopore long-reads is nearing completion. We are currently generating T2T genome assemblies for ~150 additional lines to develop a pangenome for lettuce and a catalog of resistance gene candidates. Genome sequences are being mined for candidate genes for traits, such as disease resistance and development. We are continuing programs to study corky root resistance, Verticillium wilt resistance, and salinity tolerance in lettuce. We continue to curate several databases that include genetic, molecular marker, phenotypic, and sequence data for lettuce. Key sequences have been uploaded to GenBank to make them widely accessible.

OBJECTIVES:

To develop and apply new methods for detecting, analyzing, and manipulating variation in lettuce. We continue to pursue these objectives in four overlapping sub-projects:

1) Analysis of transgenes in lettuce and genome editing.

2) Molecular identification of genes for disease resistance and other horticultural traits.

3) Development of genomic resources.

4) Utilization of comparative genomics to identify candidate genes controlling horticultural traits and development of molecular markers for them.

In the first three projects, we are mostly emphasizing either novel forms of disease resistance or increasing the efficiency of selection for disease resistant genotypes. The fourth objective includes a wide range of horticultural traits. Several of these studies have been funded from federal grants and support from seed companies. All projects were initiated with CLGRP funds and application of the results to lettuce improvement is supported by CLGRP funds. All projects impact improvement of both crisphead and leafy types.

PROCEDURES AND RESULTS:

Transgene expression and genome editing in lettuce

Our studies on transgene expression remain a low priority, in part because there is not a major breeding objective that can only be addressed by transgenic lettuce and therefore commercial deployment of transgenes in lettuce is not a near-term goal or need. We continue to generate transgenics as components of other projects (see below); these provide additional data on transgene expression and stability.

Introduction of genes into lettuce using *A. tumefaciens* is routine. Last year we generated over ~1,000 transgenic plants for experimental purposes. We have continued to study the stability of gene expression driven by the ubiquitin promoter that we cloned from lettuce. So far expression has remained stable over multiple generations as evidenced by the expression of the RUBY reporter cassette in progeny.

Since 2012, technology for precise genome editing based on the CRISPR system has been developed for use in plants and animals. Gene knock-outs are currently much easier than sequence replacements or additions but the enabling technology is advancing rapidly. We continue to develop and apply genome editing technology for lettuce as part of our gene identification strategy. In addition to validating potential candidate genes for disease resistance, we are targeting a subset of the many genes that have been previously functionally characterized in other plant species, particularly Arabidopsis. We are using CRISPR-mediated gene knockouts to deduce whether the homologous genes in lettuce have a similar function. We are initially investigating genes potentially controlling traits such as nutrient content, leaf, flower, and root development.

We also have on-going experiments aimed at base editing (changing single bases in the DNA sequence). We have built on work of Sunchung Park & Bequian Mou who showed that a single nucleotide difference in the *LsCBF7* gene resulted no functional protein and was responsible for frost sensitivity in the majority of lettuce cultivars. We successfully made a single base change that should restore production of functional LsCBF7 protein. We have yet to demonstrate that this results in increased frost tolerance in the edited lines.

In the longer term, once allele replacement technology has been adequately developed, we will use genome editing of lettuce to create stacks of resistance genes containing several resistance and other genes at single chromosomal positions so that they will be inherited as single Mendelian loci in breeding programs. Gene stacking will greatly simplify breeding for disease resistance so

that breeders can focus on more complex traits such as water and nitrogen use efficiencies and nutritional quality. However, in order for this to happen, technology for inserting genes, preferably without tissue culture, needs to be developed and more genes for resistance to each disease need to be identified at the molecular level.

Molecular identification of genes for disease resistance and other horticultural traits

We previously mapped multiple genes for resistance to downy mildew (DM), corky root, and *Fusarium* and *Verticillium* wilts onto the consensus genetic map and placed them on the genome sequence. This provides molecular markers to assist the selection of resistance genes. Of the over 50 phenotypic resistance genes mapped in lettuce, most co-localize to one of five major resistance clusters (MRCs) on Chromosomes 1, 2, 3, 4, and 8. The majority of these resistance phenotypes are linked to NB-LRR-encoding (NLR) genes, as described in previous CLGRB reports, that provide markers for selecting for these resistances. Three populations have been phenotyped and sequenced to map new *Dm* genes during the past two years; identification and validation of candidate resistance genes is on-going.

Diagnostics for corky root

Corky root has not been as severe or as problematic in coastal California during the past 30 years as it was prior to the 1990s. However, it is once again becoming a concern in Yuma and the Salinas Valley, but diagnostic tools are needed because the etiology of contemporary symptomatic roots is not clear. Using genome sequence information that we had previously generated for pathogenic and non-pathogenic corky root strains (see 2021-2022 variation report), we designed a diagnostic protocol for lettuce corky root with specific primer pairs for the chromosome of *Rhizorhapis suberifaciens* and the four plasmids of CA1, the pathogenic reference strain isolated in the 1980s (Tables 1, 2, and 3). These primer pairs amplify different sized products, so that they can be used in a multiplex reaction (Figure 1). The plasmid complement may vary between strains and it is not known what plasmids contemporary strains have. Therefore, the primary diagnostic should use the primer pairs for amplifying from the chromosome. We are interested in receiving feedback on results from the use of these primers on symptomatic roots, particularly if the results are positive.

Table 1. Primer information for *Rhizorhapis suberifaciens* CA1 strain.

Primer	Gene	PCR product	Ta (°C)	Prier sequence (5'- 3')
Rsub_CA1_Chr_F	Elongation factor Tu	800	54.2	GTGCCTGCTCTGGTGGTATA
Rsub_CA1_Chr_R			54.5	ATAGAGCTGACAACCCCTGC
Rsub_CA1_Chr_F_2	Elongation factor Tu	1,039	56	CCAACATCGACAAGGCTCCC
Rsub_CA1_Chr_R			54.5	ATAGAGCTGACAACCCCTGC
Rsub_CA1_p1_F	Type II toxin-antitoxin system YafQ family toxin	200	60	TGACATGGGCAAGCTGAAGG
Rsub_CA1_p1_F			62	TGCCGGCCCGAACAAAATA
Rsub_CA1_p2_F	Transposase	100	57	GGTGCAGATCAGCGAGCA
Rsub_CA1_p2_R			57	GCTTACCCTGATACCCGACG
Rsub_CA1_p3_F	Zeta toxin family protein	499	54.6	TATGAAACCCGCGTCTCTCC
Rsub_CA1_p3_R			54.8	TTATTGTGGGCTTCGGTCGT
Rsub_CA1_p4_F	Restriction endonuclease	300	58	ATGATGATCCCGCAGTCCTG
Rsub_CA1_p4_R			58	CAGGAAGTTCGCCCAGACTG

Table 2. PCR reaction.

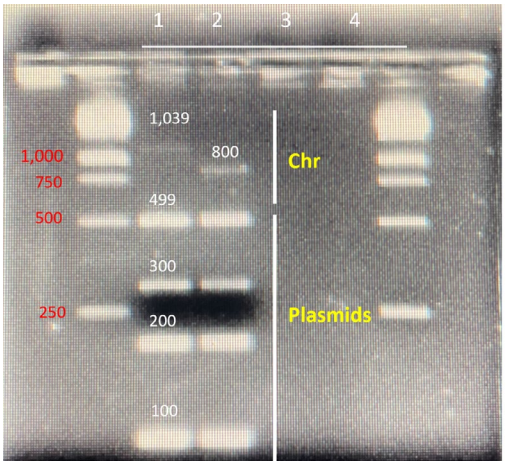
Type-it* Multiplex PCR Master Mix	10 µl
Primer F (10µM)	1 µl
Primer R (10µM)	1 µl
Bacterial DNA	1 µl of a 15 ng/µl DNA template
diH_2O	Add to 25 µl

* Qiagen, Valencia, CA, USA

Table 3. PCR conditions.

	Temperature	Time	Cycles
Initial Denaturation	94ºC	3 min	1
Denaturation	94ºC	30 s	30
Annealing	57ºC	30 s	
Extension	72ºC	1:30 min	
Final Extension	72ºC	5 min	1
Hold	4ºC	Hold	

Figure 1. The results of multiplex PCR performed on DNA of CA1, where the first and second wells display the amplicons from the chromosome and the four plasmids of CA1 after multiplex PCR. Lanes 1 and 2 differ in the primer pair employed to amplify from the chromosome. A more pronounced band was consistently obtained with the second primer pair for the chromosome. Note that the chromosomal band may not always amplify well in multiplex PCR. Therefore, it may be best to use just the primers to amplify from the chromosome for primary diagnosis.



Well	Primer	PCR product
1	Rsub_CA1IF_multiplex_F	1,039; 499; 300; 200; 100
	Rsub_CA1IF_multiplex_R	
2	Rsub_CA1IF_multiplex_F	800; 499; 300; 200; 100
	Rsub_CA1IF_multiplex_R	
3	Rsub_CA1IF_multiplex_F	Negative Control
	Rsub_CA1IF_multiplex_R	
4	Rsub_CA1IF_multiplex_F	Negative Control
	Rsub_CA1IF_multiplex_R	

We are interested in receiving symptomatic corky root samples to establish by sequencing whether the current disease is caused by *Rhizorhapis suberifaciens,* and if it is, how similar the contemporary strain is to strains that caused the earlier outbreaks. We are particularly interested in symptomatic roots that have given a positive result with the diagnostic assay described above. Anyone interested in submitting symptomatic

root samples should contact Maria Ferrer (mferrerruiz@ucdavis.edu) or Stephanie Slinski, (sslinski@arizona.edu) for samples from Yuma.

Important caveats:

- These PCR primers were initially developed for laboratory experiments of plasmid stability. They may not be optimal for diagnostics of field samples. Better primers for diagnostics may be released later.
- Figure 1 and these reaction conditions were developed with DNA extracted from a culture of the CA1 strain of *Rhizohapis suberifaciens*. They may need to be modified for assays of naturally infected roots. We will work on this when we have such samples.
- As in Fig.1, amplification of the genomic fragments is weaker than those of the plasmid-derived fragments. Therefore, initial diagnostics should be done with just the primer pairs for the genomic fragments.
- The plasmid composition of contemporary strain(s) may be very different from CA1. Therefore, if a symptomatic root is positive with one or more of the genomic primer pairs but negative with the plasmid primer pairs, it should still be considered potentially positive for corky root.
- We are still working on determining the sensitivity and specificity of this assay for diagnostics. We are therefore very interested in receiving samples of positive symptomatic roots. These will be subjected to whole genome shotgun sequencing to determine the microbes present. Only by sequencing multiple samples of symptomatic and healthy roots will we be able to determine specificity for *R. suberifaciens* in the rhizobiome of lettuce roots.

Resistance to *Verticillium dahliae*

We are dissecting the *Vr1* locus using *Agrobacterium*-mediated transient expression of the Ave1 effector of *V. dahliae*. This method that is faster than inoculating with the fungal strain (see 2022-2023 variation report). We are currently screening reactions of T_3 progeny from five transgenic knock-out lines and 17 transgenic complementation lines to the Ave1 effector. This should confirm which genes are necessary and sufficient for resistance to race 1.

In addition to Ave1, we have cloned genes encoding the Ave2, VdR2e, and VdR3e effectors of *V. dahliae* into an expression vector driven by the 35S promoter. The *Ave2* effector gene, located in a race 2-specific region, triggers *V2*-mediated resistance in tomato (Chavarro-Carrero *et al*., 2021. Environmental Microbiology, 23:1941–1958), while the *VdR2e* and *VdR3e* genes encode secreted proteins from race 2 and race 3, respectively (Wang et al., 2021. Microbiology Spectrum, 9:e01118-21). These four effector genes can be used to screen for resistance to *V. dahliae* races 1, 2, and 3.

Remote sensing

In collaboration with Dr. Duke Pauli (University of Arizona), we conducted another field trial at the Maricopa Agricultural Center, AZ in an attempt to study water use efficiency in lettuce. The Maricopa Agricultural Center hosts the largest analytical field robot in the world. The high-throughput phenotyping field-scanning robot has a gantry that moves on rails while imaging the crops growing below with a diverse array of cameras and sensors, including scanners for hyperspectral and thermal imaging, 3D laser, and chlorophyll fluorescence. This is the fourth trial conducted at the Maricopa Agricultural Center to understand the genetic basis controlling agronomic and stress-adaptive traits, to monitor growth and development, and to quantify water deficit stress and the impacts on photosynthetic efficiency as well as to develop analytical tools for remote sensing. However, once again the wet spring in Arizona meant that there was no drought stress in the plots that were meant to be water deprived. Therefore, we focused on the well-watered plots.

The 2021–2022 trial consisted of 272 diverse lines grown in two different water treatments, with three repetitions per treatment for a total of 1,632 plots (see 2021- 2022 CLGRB report). The 2022-2023 trial had partially the same layout of diverse cultivars and the addition of a RIL population. The 2023 – 2024 trial had a reduced diversity panel of 200 cultivars plus two RIL populations that segregated for root architecture: Salinas x *L. serriola* (already grown in the previous year) and Armenian *L. serriola* x PI 251246 (Fig. 2). For these two populations, we excavated the roots of three plants per plot per rep for a total of nine samples per family. We also collected nine root samples from a subset of the diversity panel of 48 cultivars representing different lettuce types. We took images from all the roots and the analysis for root architecture is now underway.

Figure 2. The trial at the University of Arizona Maricopa Agricultural Center about to be harvested in March 2024. The Lemnatec Field Scanalyzer is positioned at the end of the plot; the rails for the gantry are visible on either side.

We harvested one head of lettuce for each plot and measured fresh and dry weight and stem length. These measurements will be used to ground-truth the growth measurements made with remote sensors on the gantry. The remote sensor measurements of all plants in each plot will be used for GWAS of horticultural traits. We have finished whole genome sequencing of all of the lines for which sequence data was not already available. We aligned the sequences the v11 reference genome of Salinas and called sequence variation (SNPs) for each line. These will be used to identify genomic regions associated with phenotypic variation.

Genetics of salt tolerance in wild *Lactuca* species

We continue to investigate the genetic basis of salt tolerance as the foundation for breeding lettuce cultivars with increased salt tolerance. As described in last year's report, we initiated a genetic analysis of lettuce root system architecture (RSA) in response to salinity stress using an existing RIL population derived from Armenian 999 x PI 251246. One hundred and twenty RILs were evaluated under similar conditions for nine days to identify QTLs for RSA changes due to salinity stress. Seedlings were grown on plates of ½ MS media with or without 100 mM NaCl for seven days. Photographs were taken at multiple time points and images were analyzed using SmartRoot software to quantify root lengths. In initial screens of the parents, PI 251246 exhibited a significant decrease in primary root length (PRL), total lateral root length (TLRL), total root length (TRL), and lateral root number (LRN) ($p < 1.0^{-3}$) compared to Armenian 999, which did not exhibit significant differences in the same RSA traits. Additionally, Armenian 999 exhibited little lateral root production compared to PI 251246. There was a significant genotype x treatment effect ($p < 1.0^{-2}$) for all RSA traits. Images of the RIL population were taken at 0, 3, 5, 7, and 9 days after exposure to 100 mM NaCl. The area under the curve was calculated for each RSA trait. RSA traits measured for the QTL analysis include PRL, TRL, TLRL, and LRN as well as apical zone (AZ), branching zone (BZ), branching density of primary root (BDP), and branching density of branching zone (BDB) as shown in Figure 3.

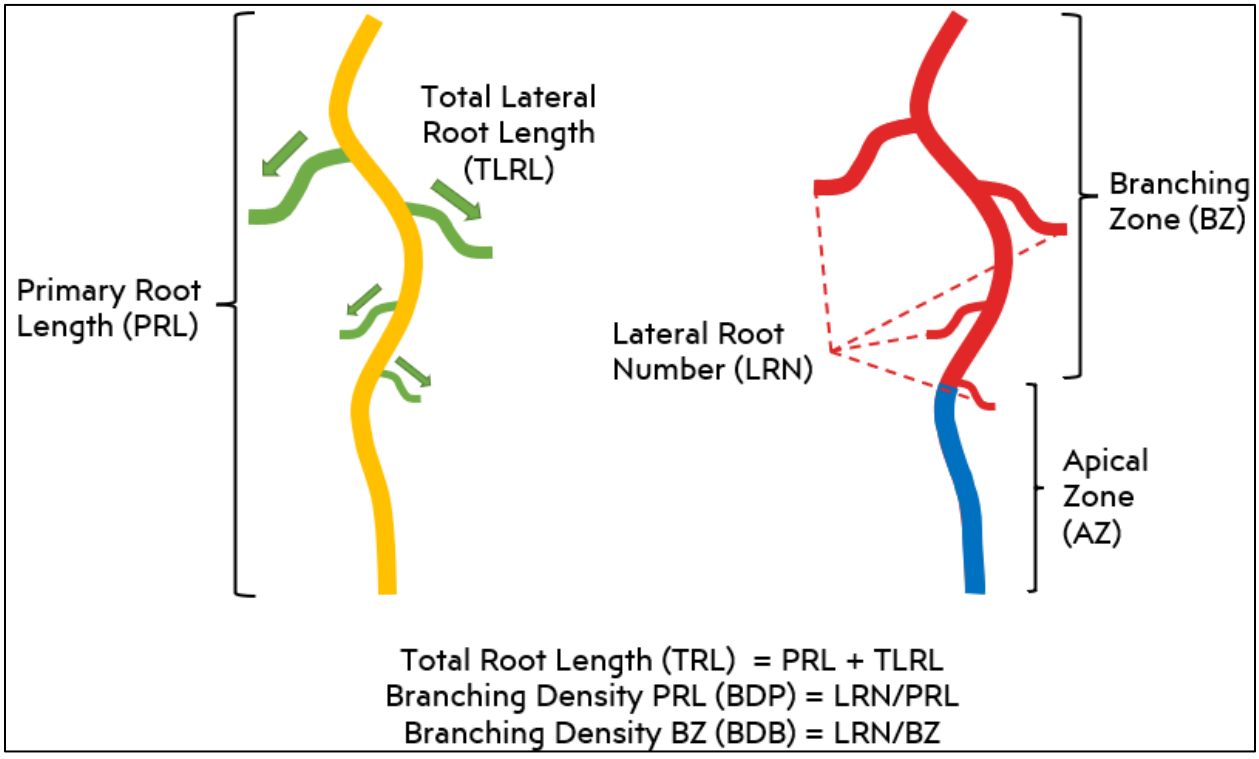


Figure 3. Diagram of the RSA traits measured for the QTL study of the Armenian 999 x PI 251246 RIL population.

Linkage analysis was achieved using composite interval mapping in Rstudio. A genetic map of the Armenian 999 x PI 251246 RIL population with 2,581 SNP markers was used. The genetic map was generated using imputed genotypes based on SNPs compared to the PI251246 v14 reference genome using GBS markers. RSA traits were mapped separately in 0 and 100 mM NaCl conditions. A total of 22 QTLs were identified among chromosomes 4, 5, 8, and 9 across both treatments (Figure 4). Multiple QTLs overlapped to form four major QTL (>15% PVE) clusters and one minor QTL (<15% PVE) cluster in addition to the singular minor QTL qBZ8 on chromosome 8 (Fig. 4). The LOD scores of QTLs ranged from 3.9 to 13.55 and PVE ranged from 9.42% to 34.55%. Chromosome 4 QTLs were identified in 0 mM NaCl, chromosome 5 QTL clusters were identified in 100 mM NaCl, and most QTLs on chromosome 9 were identified in 100 mM NaCl with the exceptions of qTRL9.1 and qBZ9.1. The QTL clusters containing QTLs for TLRL were largely associated with lateral root development meanwhile QTL clusters containing QTLs for PRL were largely associated with primary root length.

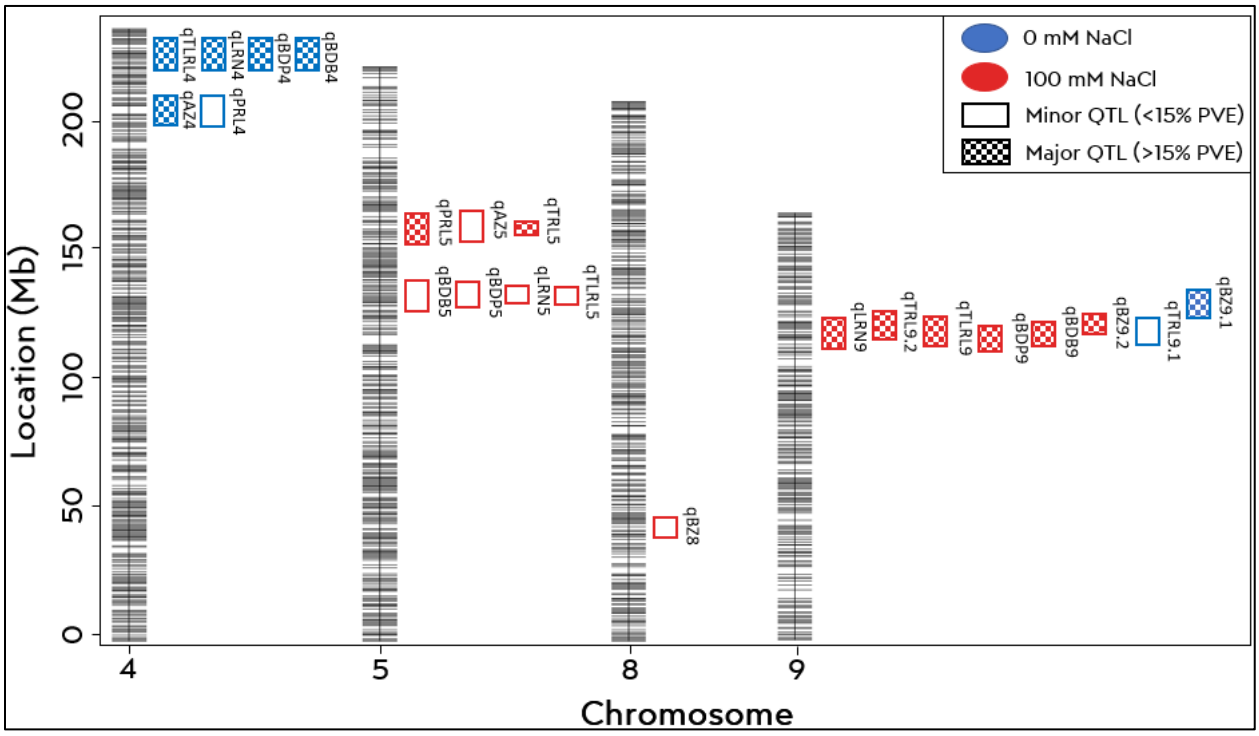
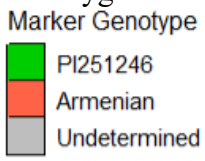


Figure 4. Diagram of minor and major QTLs identified for all RSA traits in 0 mM and 100 mM NaCl separately.

RILs containing recombination events within the QTLs in Fig. 4 were identified using combined marker information from the SNP-based genetic map used in the QTL study as well as a k-mer-based genetic map generated using AFLAP. A subset of 42 recombinant RILs were evaluated phenotypically using the same methodology as the initial QTL study to fine map the major QTL clusters with a focus on the chromosome 9 QTL cluster, which had 29 recombinants in the 170-182 Mb interval (Fig. 5). The interval of the QTL cluster on chromosome 9 was reduced from 12 to 4 Mb by fine mapping based on the distribution of phenotypic averages for

RSA traits compared to genotypes across the interval. Most recombinants with the PI251246 genotype had greater TRL and TLRL between 174 Mb and 178 Mb while most recombinants with the Armenian genotype had little to no lateral root development. Undetermined genotypes at marker positions were due to the lack of GBS data or the 1 Mb window likely being heterozygous.



Marker	9_170	9_171	9_172	9_173	9_174	9_175	9_176	9_177	9_178	9_179	9_179.78	9_180	9_181	9_182
Chr	9	9	9	9	9	9	9	9	9	9	9	9	9	9
Map	SNP	SNP	SNP	SNP	SNP	SNP	SNP	SNP	SNP	SNP	AFLAP	SNP	SNP	SNP
Mb	169	170	171	172	173	174	175	176	177	178	178.78	179	180	181
4	A	A	-	B	B	B	B	B	B	B	B	B	B	B
54	A	A	A	A	-	-	B	B	B	B	B	B	B	B
226	A	A	A	A	A	B	B	B	B	B	B	B	B	B
64	A	A	A	A	A	-	B	B	B	B	B	B	B	B
83	A	A	A	A	A	-	B	B	B	B	B	B	B	B
76	A	A	A	A	A	A	-	B	B	B	B	B	B	B
147	A	A	A	A	A	A	-	B	B	B	B	B	B	B
107	A	A	A	A	A	A	A	A	A	-	B	B	B	B
141	A	A	A	A	A	A	A	A	A	-	B	B	B	B
34	A	A	A	A	A	A	A	A	A	-	B	B	B	B
55	A	A	A	A	A	A	A	A	A	A	A	B	B	B
111	A	A	A	A	A	A	A	A	A	A	A	A	-	B
79	A	A	A	A	A	A	A	A	A	A	A	A	-	B
172	A	A	A	A	A	A	A	A	A	A	A	A	A	A
128	A	A	A	A	A	A	A	A	A	A	A	A	A	A
168	-	A	A	A	A	A	A	A	A	A	A	A	A	A
62	B	B	A	A	A	A	A	A	A	A	A	A	A	A
228	B	B	B	B	B	-	A	A	A	A	A	A	A	A
237	B	B	B	B	B	-	A	A	A	A	A	A	A	A
113	B	B	B	B	B	B	A	A	B	B	B	B	B	B
186	B	B	B	B	B	B	B	B	-	A	A	A	A	A
42	B	B	B	B	B	B	B	B	-	A	A	A	A	A
212	B	B	B	B	B	B	B	B	B	A	A	A	A	A
174	B	B	B	B	B	B	B	B	B	A	A	A	A	A
46	B	B	B	B	B	B	B	B	B	-	A	A	A	A
108	B	B	B	B	B	B	B	B	B	-	A	A	A	A
116	B	B	B	B	B	B	B	B	B	-	A	A	A	A
71	B	B	B	B	B	B	B	B	B	B	B	-	A	A
5	B	B	B	B	A	A	-	-	-	B	B	B	B	B

Figure 5. Genotype table of the RILs recombinant within the major QTL cluster on chromosome 9 in order of recombination events. Green A markers represent PI251246, Red B markers represent Armenian 999 haplotypes, and gray dashes represent undetermined consensus genotype of 1 Mb windows.

Development of genomic resources

We have continued to refine the reference genome of lettuce to provide the foundation for understanding the genetic architecture of traits in *Lactuca* spp. In December 2022, we uploaded the v11 sequence of cv. Salinas to GenBank to make it widely available. GenBank then generated a Refseq annotation. After many years of effort and constantly improving sequencing technologies, we are finalizing the complete, telomere-to-telomere (T2T), completely gapless assembly for the reference cultivar cv. Salinas. The final v15 assembly is based on both PacBio HiFi and Oxford Nanopore long reads and Bionano data and has a total genome size of nearly 2.6

Gb. This will be released to GenBank and made publicly available as soon as it is checked in the coming weeks.

It is now possible to rapidly and inexpensively generate T2T, near gapless, assemblies of any lettuce genotype. In April 2022, the UC Davis Genome Center was one of the first places to get a PacBio Revio DNA sequencer that efficiently generates large amounts of HiFi reads. We are currently using this to generate telomere-to-telomere assemblies of ~100 wild and cultivated *Lactuca* spp. that exhibit a broad diversity of disease resistance and developmental phenotypes. We have so far assembled over 20 genotypes. These data will provide an extensive catalog of resistance genes that will provide the foundation for pyramiding resistance genes using marker-assisted selection.

In order to generate a pan-genome that captures the genetic diversity within *Lactuca spp*., we have sequenced several diverse wild and cultivated lettuce lines using PacBio HiFi / Oxford Nanopore long-read technologies and Illumina short-read sequences. Currently high-quality reference assemblies of *L. sativa* cvs. Salinas, Ninja, *La Brillante*, PI251246, VIAE, and *L. serriola* accessions Armenian and US96UC23 are assembled. D*e novo* annotation is currently underway to reveal differences in gene content, particularly with regard to their resistance gene repertoires.

Databases

We continue to curate several publicly accessible databases for lettuce accessible through http://michelmorelab.ucdavis.edu. The G2G site (http://scri.ucdavis.edu/) provides access to information generated as part of the Next-Generation Lettuce Breeding: Genes to Growers (G2G) and CLGRP-funded projects. Our GBrowse genome viewer (http://gviewer.gc.ucdavis.edu/cgi-bin/gbrowse/lettucePublic/) provides access to the ultra-dense map of genetic chromosomal pseudomolecules. The majority of the sequencing data is uploaded to GenBank to facilitate broad access and avoid legacy issues. The Bremia Database displays virulence phenotypes, mating type, and fungicide sensitivity for Californian isolates of *B. lactucae* characterized from 2001 to the present (http://bremia.ucdavis.edu/bremia_database.php).