CALIFORNIA LEAFY GREENS RESEARCH PROGRAM

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PROJECT TITLE: **GENETIC VARIATION IN LETTUCE**

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OBJECTIVES:

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

1) Analysis of transgenes in lettuce and genome editing.

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

3) Development of a detailed genome assembly and identification of reliable, readily assayed markers linked to disease resistance genes.

4) Utilization of comparative genomics to identify candidate genes controlling horticultural traits and development of robust 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. Some 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 need. Introduction of genes into lettuce using *A. tumefaciens* is routine; however, transgenes that expressed well in some other plant species are expressed poorly after multiple generations in lettuce. We now use the Arabidopsis ubiquitin promoter in preference to the CaMV 35S promoter that is prone to silencing in lettuce. We continue to generate transgenics as components of other projects (see below); these provide additional data on transgene expression and stability.

Since 2012, a technology for precise genome editing based on the CRISPR/Cas9 system has been developed for use in plants and animals. This technology can be used to create gene knockouts, deletions, and replacements as well as for introduction of new genes and sequences. 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 (Table 1). Knock-outs of NCED4 resulting in high temperature germination as described in previous CLGRB reports has been published (Bertier *et al.* 2018. High-resolution analysis of the efficiency, heritability, and editing outcomes of CRISPR-Cas9-induced modifications of *NCED4* in lettuce (*Lactuca sativa*). *G3: Genes, Genomes, Genetics* **8**:1513-1521; https://doi.org/10.1534/g3.117.300396). We can now efficiently routinely knock out (mutate) genes in lettuce using *Agrobacterium*-mediated delivery of genes encoding Cas9 nuclease and polycistronic guide RNAs to explants and regenerating edited plants through tissue culture. However, it takes six to twelve months to complete the process of construct generation and regeneration of plants from tissue culture.

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 maintain a similar function. We are initially investigating genes potentially controlling traits such as nutrient content, leaf, flower, and root development, and flavor profile (Table 1).

Currently, we are testing a transgene-free approach based on transfection of ribonuclear proteins (CAS9 or Cpf1 RNPs) into protoplasts, as has been published by others (Woo *et al*., 2015. Nat. Biotechnol. 33:1162). We will use this system to transfect protoplasts with multiplexed gRNAs targeting NB-LRR genes that are *Dm* gene candidates. Regenerated tissue will be challenged with *B. lactucae* effectors using Agro-infiltration, or with isolates of *B. lactucae*.

Table 1. Genes in lettuce currently being studied using knock-outs.

Candidate Gene	Phenotype	Construct used (see 2018 report)	Status	Collaborators
NCED4	Germination thermosensitivity	v1	Completed	Kent Bradford
ERF1	Germination thermosensitivity	v2	Knockouts being tested	Kent Bradford
Vert1 (3 genes)	*Verticillium* wilt resistance (race 1)	v2, v4	Knockouts being tested	Steve Klosterman
Phytoene desaturase	Chlorophyll biosynthesis	v2	Completed	
XTH (6 genes)	Cell wall biosynthesis, post harvest characteristics	v3	Knockouts being tested	Annabelle Damerum, Gail Taylor
Cycloidea (3 genes)	Transcription factor involved in flower shape	v4	Knockouts being tested	
Cor (4 candidate genes)	Bacterial corky root resistance	v4	Underway	
Novel miRNA	Unknown	v4	Knockouts being tested	Suresh Pokhrel, Blake Meyers
Tipburn resistance	Physiological tissue breakdown	v4	Underway	
AAP	Nitrogen uptake	v4	Underway	
Lobe	Leaf shape	v4	Knockouts being tested	
FIGL1, FANCM, RECQ4A & *RECQ4B*	Recombination	v4	Underway	
Dm (multiple genes)	Downy mildew resistance	Protoplast RNP editing	Planned	David Tricoli
Tr	Herbicide (triforine) sensitivity	Protoplast RNP editing	Planned	Rong Tao, Hanhui Kang

In the longer term, 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. Such stacks could contain multiple resistance genes effective against all known pathotypes of downy mildew and multiple viral, bacterial, fungal pathogens as well as insect pests and nematodes. This will enhance the durability of resistance by increasing the evolutionary hurdle that pathogens will have to overcome. Gene stacks could be expanded as more resistance genes become available and genes replaced when *Dm* genes are overcome by changes in the pathogens. A herbicide resistant gene (e.g. *ALS*) could be used as selectable marker for the gene stack. 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, need to be developed and genes for resistance to each disease need to be identified at the molecular level.

Resistance Gene Identification

We continue to map loci for resistance to downy mildew (DM), corky root, *Fusarium* and *Verticillium* wilts, onto the consensus genetic map and place them on the genome sequence. We are also continuing to develop molecular markers to assist the selection of resistance genes. Of the over 50 phenotypic resistance genes mapped in lettuce, most co-localize to one five major resistance clusters on chromosomes 1, 2, 3, 4, and 8 (MRCs 1, 2, 3, 4, and 8 respectively). The majority of these resistance phenotypes are linked to NB-LRR-encoding (NLR) genes as described in previous CLGRB reports that provide markers for these resistances.

Resistance to Downy Mildew

Numerous genes for resistance to DM have been introduced into cultivated lettuce from wild species (*L. serriola, L. saligna* and *L. virosa*) by repeated backcrossing (often six or more backcrosses) by public and commercial breeders. This has resulted in near-isogenic lines (NILs) that only differ for small chromosome regions that are potentially associated with resistance. Twelve advanced breeding lines previously released from the UC program show resistance to many, although not all, isolates of *B. lactucae* in California, including recent highly virulent isolates.

We have generated F_2 populations from crosses between these NILs and cv. Salinas in order to map the resistance to DM in the reference genome. Most populations segregated 3:1 for resistant and susceptible individuals, consistent with one dominant resistant gene determining resistance to downy mildew. F_2 progeny from these crosses were genotyped by sequencing and the resistance genes were mapped into the reference genome (Table 2, Fig. 1). New resistance genes from lines UC02202, UC02204, UC07105, UC07107, UC07108 were named as numbered *Dm* genes consistent with their prior resistance factor denominations described in Parra *et al*. (2016, *Euphytica* **210**:309. https://doi.org/10.1007/s10681-016-1687-1). New resistance genes from the lines UC12100, UC12101, UC12102 and UC12103 will be submitted to the International Bremia Evaluation Board (IBEB) for denomination.

Table 2. Advanced breeding lines with resistant donor accessions, *Dm* gene denominations and chromosomal locations. S.f.d. = to be submitted for denomination.

ID	Donor	*Dm* gene	*B. lactucae* isolate	Location
UC02202	*L. saligna* LJ85314	*Dm33*	1326	Chr1
UC02204	*L. virosa* LJ85289	*Dm35*	1326	Chr1
UC02206	*L serriola* W66331A	*Dm31*	1326	Chr1
UC07105	*L. sativa* PI491226	*Dm41*	1326	MRC4
UC07107	*L. saligna* PI491206	*Dm46*	1326	MRC1
UC07108	*L. saligna* PI491208	*Dm47*	1407	MRC2, Chr4
UC012100	*L. saligna* CGN9311	s.f.d.	1326, 1742	Chr1
UC012101	*L. saligna* CGN5318	s.f.d.	1326, 1691	MRC1, MRC2
UC012102	*L. saligna* CGN5282	s.f.d.	1326, 1742	MRC4
UC012103	*L. saligna* CGN5147	s.f.d.	1326	Chr1
UC012103	*L. saligna* CGN5147	s.f.d.	1691	MRC1

Figure 1: The genomic locations of new resistance genes on lettuce chromosomes.

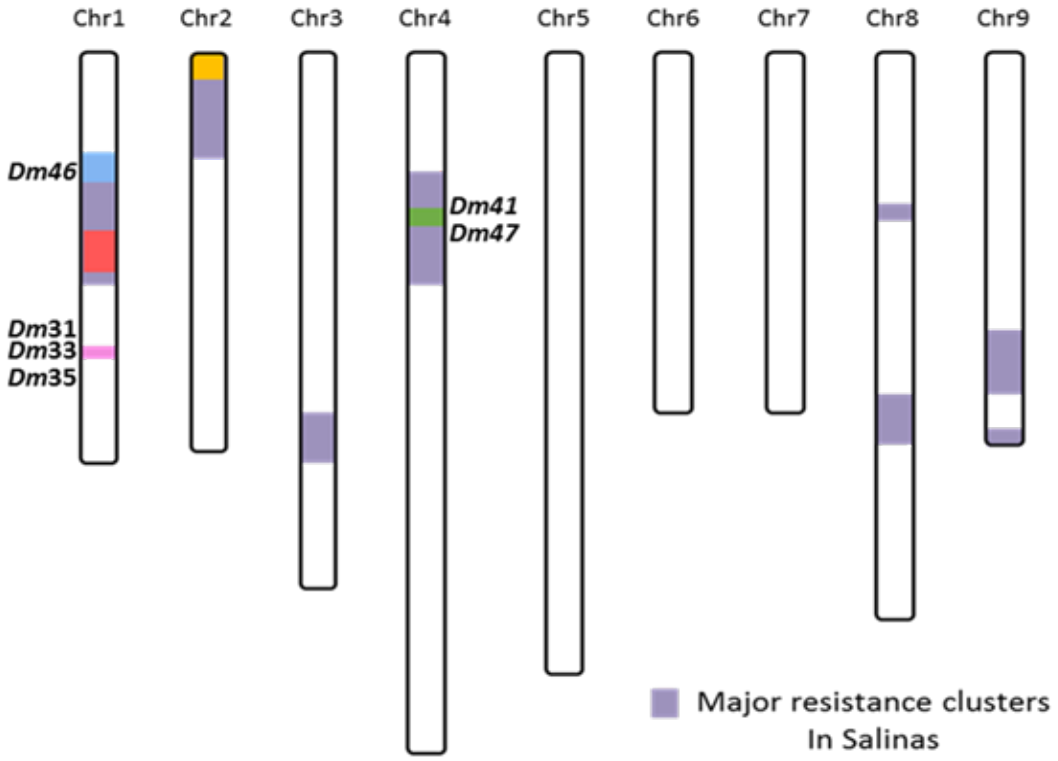

Table 3: Candidate genes for resistance to downy mildew obtained using k-mer association mapping.

gene	cultivar	isolate	candidate gene	location	type R gene
Dm 3	Dandie	1414	1 candidate	MRC2	NLR
	UC02203		1 candidate		
Dm 18	El Dorado	1452	1 candidate	MRC2	NLR
	Mariska		1 candidate		
Dm 45	UC12104	879	2 candidates	MRC1	NLR
Dm 36	Ninja	1326	1 candidate	MRC1	NLR
	FrSal-1		1 candidate		
	Discovery		1 candidate		
Dm 37	Frsal-1	1326	1 candidate	MRC1	NLR
	Discovery		1 candidate		
Dm 17	NunDm17	1926	4 candidates	MRC1	NLR
	LS102		4 candidates		
R33	UC02202	1326	1 candidate	Chr1	RLK
R35	UC02204	1326	1 candidate	Chr1	RLK
R31	UC02206	1326	6 candidates	Chr1	NLR
R41	UC07105	1326	1 candidate	MRC4	NLR
R46	UC07107	1326	5 candidates	MRC1	NLR
New	UC07108	1407	3 candidates	Chr4	NLR
New	UC12102	1326	2 candidates	MRC4	NLR
New	UC12103	1691	2 candidates	MRC1	NLR

In order to identify the resistance genes present in the advanced breeding lines and distinguish these genes from resistance genes previously described in other cultivars, we have used long fragment sequencing (PacBio) for the characterization of the resistance gene repertoires present in each breeding line and the different lettuce cultivars, carrying known *Dm* genes. Resistance gene enrichment sequencing (RenSeq) allowed the identification of numerous NBS-LRR sequences from 48 lettuce genotypes, including the UC advanced breeding lines. This data in combination with phenotypic data from these lines was used for k-mer association analysis, which allowed the identification of several candidate genes for known and new resistances against *B. lactucae* (Table 2). Resistance genes will be validated using CRISPR-mediated knock-outs of the candidate gene.

Genetic Analysis

We have continued to construct detailed genetic maps using genotyping by sequencing on populations segregating for a variety of traits in collaboration with other researchers (See Table 2, 2016-2017 CLGRB report). Last year, we genotyped and analyzed a single population developed by German Sandoya at University of Florida that segregated for resistance to bacterial leaf spot. We have complemented this research on bacterial leaf spot (BLS) by analyzing two other populations RIL populations segregating for bacterial leaf spot resistance: Reine des Glaces (RG) x Eruption and RG x Delsay (in collaboration with Ivan Simko and Ryan Hayes, USDA Salinas and Brigitte Maisoneuve, INRA France). We identified a major QTL for resistance in LG2 in both populations; additional minor QTLs were also detected in other linkage groups (Figure 2). The major QTL collocates with *Xar1* and *Xcvr* genes for BLS resistance that had previously been identified in two other populations.

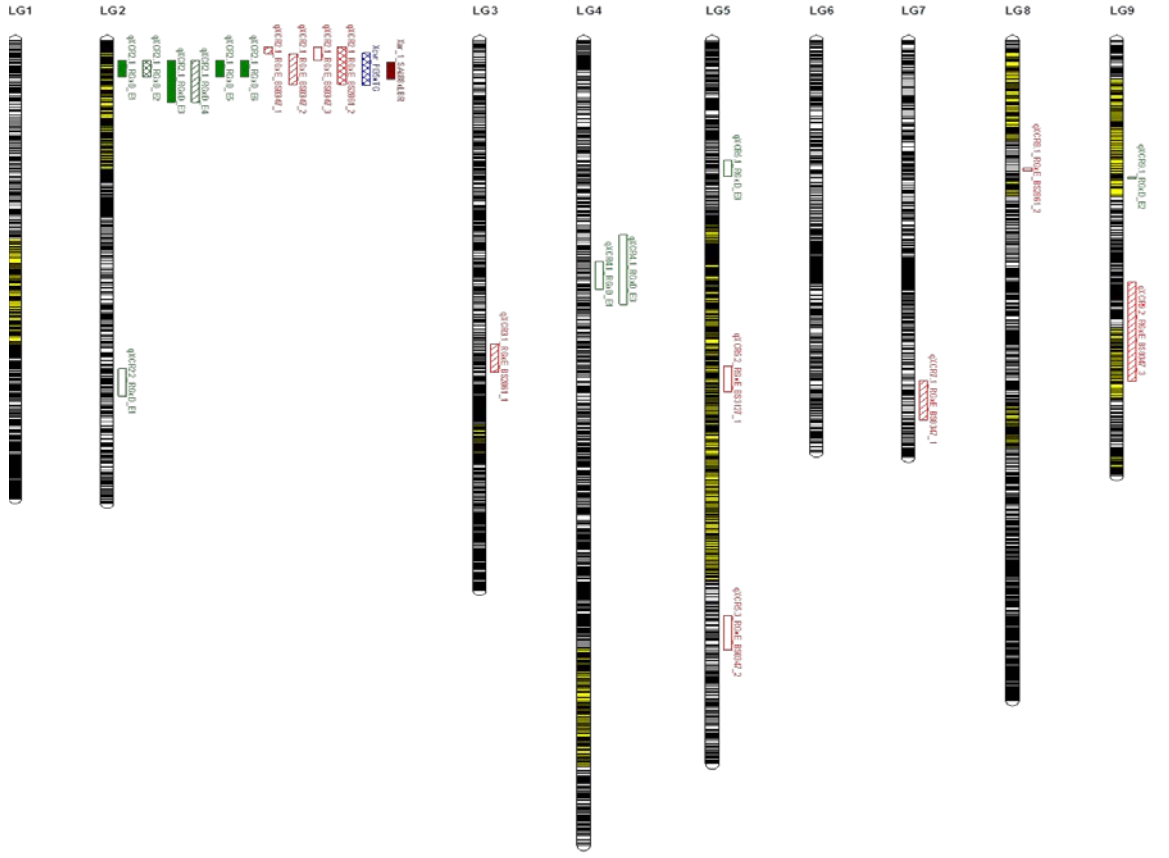

Figure 2: Quantitative trait loci (QTLs) identified in the RIL populations Reine des Glaces (RG) × Eruption (red QTL bars) and RG × Delsay (green QTL bars). Depending on the percentage of phenotypic variation (R^2) explained by a given QTL, QTL bars are empty (R^2< 10%), hatched (10%< R^2<20%), cross-hatched (20%<R^2<50%), or solid (R^2> 50%). Genetic maps of these two populations and maps of the populations: Salinas 88 × La Brillante and PI 358001-1 × Tall Guzmaine from which the loci *Xar1* (maroon bar) and *Xcvr* (blue bar), respectively, were previously identified (Hayes et al. 2014; Wang et al. 2016), were aligned to the lettuce genome (Reyes-Chin_Wo *et al*., 2017). Note that LG is used to abbreviate linkage group. Yellow segments on the linkage groups correspond to the major resistance clusters (MRCs) in lettuce genome (Christopoulou *et al*., 2015).

Genetic Analysis of Tipburn Resistance

A QTL for resistance to tip burn had been previously identified in linkage group 5 in a RIL population from a cross between cvs. Emperor and El Dorado (Jenni *et al*., 2013. *Theor. Appl. Genet*. 126:3065-3079). Subsequently, we fine mapped this QTL by phenotyping lines selected as being recombinant using codominant molecular markers flanking and within the QTL region and phenotyping those recombinant lines in field experiments in different environments (see previous CLGRB reports for more information). These recombinant lines were sequenced and analysis of chromosomal recombination points reduced the genomic interval associated with tipburn resistance to a region containing 21 candidate genes. RNAseq studies of expression level differences in the recombinant lines, further reduced the number of candidate genes. We are currently generating CRISPR-mediated knock-out lines for three candidate genes to test whether they affect tipburn.

In collaboration with I. Simko, R. Hayes USDA and S. Jenni, Canada, seven recombinant inbred line (RIL) populations had been assessed over the past 15 years for tipburn in multiple environments and years (Table 3). Several other morphological traits were also assessed including core height, head firmness, head closure, leaf crinkliness, plant fresh weight, and leaf savoy. These populations were genotyped by sequencing and analyzed to elucidate the genetic architecture of resistance to tipburn and to identify QTL for tipburn resistance.

Table 3. RIL populations used to study tipburn and identified QTLs for resistance.

RIL population	# Trials	QTLs for tipburn
Iceberg x Saladin	2	-
F_1 (Valmaine x Salinas 88) x Salinas	5	LG2, LG7
Salinas 88 x La Brillante	4	**LG1**, LG4, **LG5**, LG9
Emperor x El Dorado	1	**LG5**, LG7
Salinas x Calicel	2	**LG1**, LG2, LG3, **LG5**, LG7, LG8
Grand Rapids x Iceberg	2	LG2, LG3, LG4, **LG5**, LG7, LG9
Salinas x *L.serriola* UC96US23	3	LG3, **LG5**, LG7

Analysis of these seven populations revealed that the genetic architecture for tipburn in lettuce consists of major, intermediate, and minor QTL scattered throughout the lettuce genome (See Fig. 4, 2017-2018 CLGRB report). The significance of these QTL varied from population to population, possibly because either causal genes were not segregating in some populations, or that they sometimes had small undetected effects, or because they were influenced by the

environment. This study has been published: Macias-González *et al*. 2019. Genetic architecture of tipburn resistance in lettuce. *Theoretical and Applied Genetics* doi: 10.1007/s00122-019-03349-6).

Resistance to *Verticillium* race 1

We characterized sequence diversity at the locus conferring the resistance to *V. dahliae* race 1 in lettuce that had previously been mapped to a single dominant *Verticillium resistance 1* (*Vr1*) locus in linkage group 9 in collaboration with Krishna Subbarao and Ivan Simko. This locus contains a cluster of several genes with sequence similarity to the tomato *Ve* genes. Genome sequencing and PCR screening was used to characterize *Ve*-like genes in 152 accessions of lettuce segregating for resistance to *V. dahliae* race 1. A single allele that was present in all resistant accessions and absent in all susceptible accessions was identified. A PCR assay was developed as a molecular marker for resistance based on sequence polymorphisms between resistant and susceptible alleles. This has been accepted for publication: Inderbitzin, P. *et al*. *BMC Plant Biology* where details can be found.

Genetics of Salt Tolerance in wild *Lactuca* species

We have initiated an investigation of the genetic basis of salt tolerance in order to breed lettuce cultivars with increased salt tolerance. In the past year, we have screened wild *Lactuca* germplasm for salinity tolerance. Data analysis is underway; at the phenotypic level there appears to be clear variation between accessions in response to varying levels of soil salinity. Accessions in this screen will be selected as parents for mapping populations to identify QTLs and candidate genes for salinity tolerance, as well as to characterize physiological responses to salinity.

Genome and Transcriptome Sequencing

The v8 of the reference genome of lettuce is now publically available at https://lgr.genomecenter.ucdavis.edu/ as well as several public databases such as GenBank, CoGe, and Phytozome and is being widely used. The v9.0 version of the lettuce genome has nine chromosomal scaffolds and is being prepared for release. This has improved contiguity and better resolution of each genetic bin of scaffolds that are now assembled into chromosome-scale genomic scaffolds.

In collaboration, with Ivan Simko, Eric Schranz (Wageningen Agricultural University) and others ~250 lettuce cultivars and wild accessions have been sequenced and SNPs identified relative to the reference genome that are being made available through our website (see below).

In collaboration with academic groups working on different aspects of lettuce biology, we are continuing to conduct RNAseq profiling experiments to provide an atlas of genes expressed in lettuce at different developmental stages, under different abiotic stresses, and during resistance and susceptibility to diverse diseases. We now have over 800 tracks of RNAseq data from multiple diverse experiments. These data are being made available through our website.

Databases

We continue to curate several publicly accessible databases for lettuce. The Compositdb contains several searchable databases for lettuce (accessible through http://compositdb.ucdavis.edu/) and is the main portal for distributing information generated by the Compositae Genome Project (CGP). Chiplett provides access to data from the Affymetrix lettuce Genechip project as well as the ultra-dense genetic map (http://chiplett.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 as genetic chromosomal pseudomolecules. Lettcv (http://compositdb.ucdavis.edu/database/lettcv2/display/) archives extensive genetic, passport and performance data on lettuce cultivars. The CGP database (http://compgenomics.ucdavis.edu/compositae_index.php) contains extensive sequence and related information as well as links to lettuce genetic maps (lettuce genetic map viewer) and marker information. Morphodb (http://compgenomics.ucdavis.edu/morphodb_index.php) is an archive of and provides access to phenotypic information on *Lactuca* species; this database utilizes standard ontologies to facilitate searches across databases. The lettuce v7 genome assembly is publically available at https://lgr.genomecenter.ucdavis.edu/. These databases are being modified to facilitate access to marker information for breeding purposes from disease-centric, breeder-oriented perspectives. 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).