California Leafy Greens Research Program
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Project Title: Development of management strategies for Bacterial Leaf Spot of Lettuce.

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ABSTRACT

Previously, using multilocus sequence analysis, we identified three major sequence types of *Xanthomonas campestris* pv. *vitians* (A, B, C, D, and E). In large part sequence types corresponded to pathogen-host reaction. We further divided the strains into additional sequence types using additional genes. B was further divided into B1 and B2. One strain from each group E and C became groups F and G, respectively, consisting of one unique strain. Screening of isolates from disease outbreaks in 2014 resulted in a single strain that represents a novel sequence type H. In addition to resistance to strains from groups A and B, we tentatively identified resistance to strains from group C. We used an understanding of the population structure of the pathogen to phenotype lettuce accessions to meet breeding objectives. Lastly, we finished the assembly on whole genome sequences and will move forward with the analysis in the coming year.

OBJECTIVES

Objective 1. Provide phenotypic data for breeding lines essential to the development of Bacterial Leaf Spot (BLS) resistant germplasm and the understanding of the genetics of BLS resistance.

Objective 2. Assemble genome sequences of *X. campestris* pv. *vitians* and related pathogens.

Objective 3. Identify avirulence and other pathogenicity related genes and sequences unique *X. campestris* pv. *vitians*.

Objective 4. To collect and monitor strains of *X. campestris* pv. *vitians* isolated from diseased lettuce throughout California for ability to overcome previously identified resistance in Little Gem.

METHODS

Resistance phenotyping:

Resistance to bacterial leaf spot is evaluated by two different methods. We screen for resistance using infiltration technique and use a spray inoculation technique to confirm resistance.

Depending on the experiment, accessions were planted in potting mix in 2 x 2 cm square cells in 11 x 15 cell flats or in four-inch pots. Flats or pots were incubated at 10°C for 2 days in the dark followed by incubation in the greenhouse. Lawns of *X. campestris* pv. *vitians* BS0347 and/or other strains were prepared and using a spectrophotometer adjusted to 0.600 OD at 600nm in phosphate-buffer (0.01 M pH 7.0). Leaves were marked and approximately 500 µl of the bacterial treatments or controls were infiltrated in to healthy leaf tissue. Buffer, BS0347, BS3127, or BS2861 are used as controls for various experiments. For each experiment there were at least two replications per treatment. Hypersensitive response is recorded for plant/pathogen combinations in which an incompatible reaction (cell collapse and death) occurred by 30 hours after infiltration. A compatible reaction was recorded for plant/pathogen combinations for which disease progressed and was visible only at 54 hour after infiltration.

Alternatively inocula were sprayed using a hand held spray bottle onto the leaves of three-week-old plants until run-off. Sterile phosphate buffer was used as a control. Plants were incubated in the greenhouse misting room (26°C, 100% RH) for a total of 21 days. Inoculated leaves were evaluated for disease severity and incidence 7, 14 and 21 days after the initial inoculation, using a rating scale modified from Bull et al., (2007). A rating of 0 was given for plants with no disease; 1, for plants with few lesions of < 3mm; 2, for plants with lesions > 3mm; 3, for plants with coalesced lesions; 4 for plants with many coalesced lesions < 30 of any leaf; 5, for plants with 50% or greater of any leaf diseased; 6, for plants with dead leaves.

Sequencing and genome assembly:

The genomes of 12 strains of *X. hortorum* including representative BLS pathogens were sent for sequencing using next generation sequencing technology. Specifically, DNA for 12 strains including multiple representatives from *X. campestris* pv. *vitians* genotypes (A, B1, B2, C) and pathotype strains of *X. hortorum* pv. *hederae*, *X. hortorum* pv. *taraxaci, X. hortorum* pv. *pelargonii* and a related pathogen from the composite radicchio were sequenced using Illumina HiSeq technology. A paired-end protocol with read length of 150 nt was used. *De novo* assembly was completed for all genomes sequenced using CLC Genomics Workbench (CLC Bio, Cambridge, Massachusetts).

Isolation of *X. campestris* pv. *vitians* isolated from diseased lettuce:

Despite the drought conditions, bacterial leaf spot was observed in central coastal California. Lesions from symptomatic leaves from several plants from each outbreak were surface-sterilized with 0.5 % sodium hypochlorite for 1 min followed by rinsing in sterile distilled water three

times. Small (3 X 3 mm) sections of tissue were excised aseptically from leaf spot margins and macerated in 40 µl of sterile distilled water. The resulting suspensions or a dilution were streaked onto nutrient agar and incubated at 24 to 26°C. After 3 to 5 days, single colonies were purified and stored. Multilocus sequence analysis using published protochols (Young et al., 2008) was used to identify the pathogens to their respective sequence groups.

RESULTS AND DISCUSSION:

Pathogen diversity and disease resistance: Previously, using multilocus sequence analysis we identified three major sequence types of the pathogen A, B, C, D, and E and in large part sequence types corresponded to pathogen-host reaction. Recently we further divided the strains into additional sequence types using additional genes. B was further divided into B1 and B2. One strain from each group E and C became groups F and G, respectively, consisting of one unique strain. Thus, additional diversity was detected. Simultaneously, additional diversity was recognized in phage sensitivity. In a related project not funded by CLGRB, it was shown that bacteriophage isolated from grower's fields infested with BLS could infect and kill strains from sequence type B. A survey of all strains demonstrated that the phage could kill strains from sequence types B, D, and E but not C or A. However, not all group B strains were lysed (52 were lysed and 31 were not lysed). Of those that were not lysed 1/3 belonged to group B1 while 2/3 belonged to group B2. These findings of greater diversity than previously thought impact our strategies for breeding and sequencing. We need to determine if resistance has already been identified for groups F and G, in addition to determining if there are differences among the two group B strains.

So far we have identified resistance to groups A, B, D and E. We are currently testing all strains in subgroups B1 and B2 as well as group F and G. We continue to assay resistance in populations as the breeding effort moves forward. For example, to obtain red baby leaf F2 plants from RH08-0111 x Merlot were screened for resistance to BS0347 (sequence type B). F3 families are being evaluated for HR this summer and resistance in spray inoculation experiments will be conducted this fall. In a separate effort, breeding for resistance to sequence type A strains is moving forward. F2 families will be screened for resistance to BS127 this summer.

In order to identify HR based resistance to strains from sequence type C, we screened a wide variety of germplasm to a single strain BS2861 (sequence type C) using methods that we successful in identifying resistance to sequence type A. This year we evaluated 495 lines for HR to group C. Recently we identified three lines that may be resistant to BS2861. We are simultaneously screening additional germplasm and growing these potentially resistant lines for further testing with all strains in sequence type C and other strains.

The goal of objective 4 is to determine if new strains or strains for which we do not have resistance are becoming more prevalent in California. Although drought conditions in reduced the incidence of bacterial leaf spot, we obtained 47 isolates from samples from King City, Salinas, and Watsonville in 2014. Additionally, we have received additional strains from global outbreaks to monitor what sequence types might eventually make it to California (data not shown). The sequences of three genes, *rpoD, fyuA, gyrB,* and *gap1* are needed to differentiate among sequence types. These genes were sequenced and comparison of concatenated sequences of these genes to those of representatives of each sequence type is being conducted. Of the strains evaluated, the majority of the strains had sequence type B1, which is common in the Salinas Valley. Approximately, 43% of all strains evaluated have this sequence type. A new sequence type (H) was identified from strain BS3675 isolated from lettuce grown in King City (Figure 1). We are currently testing this strain to determine if any of the cultivars previously identified as having complete immunity to groups B and A also have immunity to this strain.

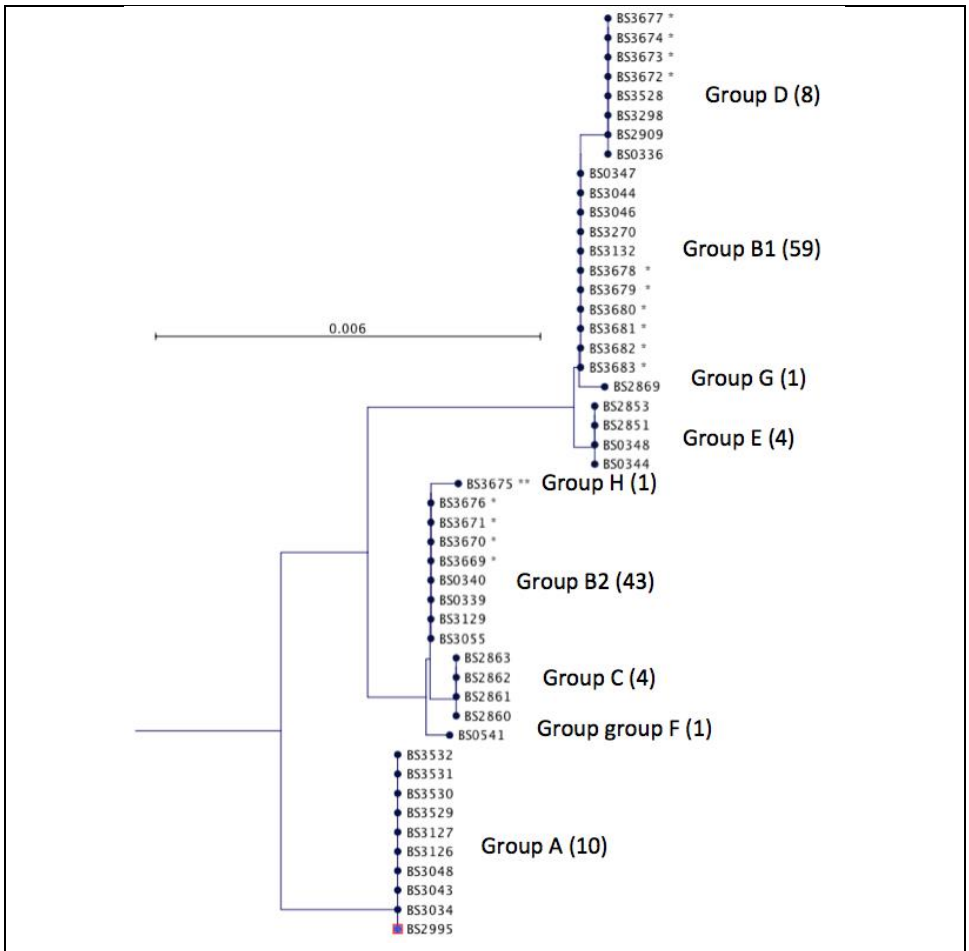

Figure 1. *Xanthomonas campestris* pv. *vitians* sequence types. Representatives of the 47 isolates characterized are presented previously defined sequence types of *Xanthomonas campestris* pv. *vitians*. Representative new strains are labeled with an asterisk. BS3675 isolated from lettuce in King City represents a new sequence type that has not previously been observed. It is the only isolate of this type observed

For most of the outbreaks single sequence types were isolated from all plants sampled. However, strains from the outbreak near King City had four different sequence types including the novel type (H). We have been concerned about the distribution of group C strains because we had not previously identified complete resistance to this group. None of the 2014 strains were group C strains. It is interesting that this novel group of strains were also from the King City area. Ideally we would like to receive more samples from this area.

Our whole genome sequencing objectives are moving forward. Of the 12 strains sent for sequencing, the results from 10 strains are useable. Sequencing was not successful for two important strains from group B (BS0347 and BS3046). These strains will be sent for sequencing again. We are currently debating if a subset of strains and additional strains from group F and G should also be sequenced. Assemblies of 10 strains with representatives from groups A, B, and C have been completed and analysis with these can move forward.

We had several additional accomplishments in this funding year due to the support of the CLGRB. Due to previous and ongoing support of the CLGRP we received recent funding from CDFA SCBGP (2014-2017) grant **Titled:** *Developing Lettuce Cultivars with Resistance to Bacterial Leaf Spot and Tospoviruses* (Simko, PI). We also Published two papers from previous CLGRB funding (Bull et al., 2015; 2014)

References:

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