2021-2022 CALIFORNIA LEAFY GREENS RESEARCH PROGRAM
ANNUAL REPORT

Project Title: Identification of Additional Viruses Contributing to Lettuce Dieback Disease

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Abstract

Lettuce dieback causes necrosis, stunting and death of lettuce plants throughout all western lettuce production regions in California and Arizona including the Salinas Valley and surrounding areas in Monterey, Santa Cruz, San Benito, San Luis Obispo, Santa Barbara, Fresno, Riverside and Imperial Counties, as well as the Yuma production region in Arizona. Losses resulting from lettuce dieback can range from a few plants to complete loss of crop. The disease was traditionally known to be caused by either of two viruses from the genus *Tombusvirus*; *Tomato bushy stunt virus* (TBSV) and *Moroccan pepper virus* (MPV; formerly known as Lettuce necrotic stunt virus), but in recent years these viruses have not been able to be detected from symptomatic plants. In order to identify additional causative agents associated with and responsible for causing lettuce dieback disease, lettuce samples were collected from several fields exhibiting symptoms of lettuce dieback disease. Symptomatic leaves were used to mechanically transmit any transmissible viruses to test plants. All lettuce plants, and plants to which the virus was passaged, were used for extraction of RNA, followed by RT-PCR for the presence of the tombusviruses, MPV and TBSV, as well as using degenerate primers for the detection of a broad range of related tombusviruses. Those lettuce samples testing negative for tombusviruses, but that were successful in mechanical transmission of a disease-causing agent (virus) to test plants were sent for high throughput sequencing, resulting in the identification of a previously unknown virus likely in the family *Phenuiviridae*. Primers were developed to the Phenui-like virus, now called Lettuce Dieback associated Virus (LDaV), and have shown a very close association between the presence of LDaV and lettuce dieback disease symptoms, lack of association with healthy lettuce, and preliminary testing indicates the virus is controlled by the *Tvr1* resistance gene. The genome of the virus has been sequenced and contains three RNAs. Mechanical transmission of LDaV is not very efficient; 38% efficiency to *Nicotiana benthamiana*, and exceptionally low efficiency to lettuce. This suggests the likelihood that LDaV is transmitted to lettuce by a biological vector, likely a soil-borne organism. Efforts through this project seek to begin work toward identification of a soil-borne vector, develop improved methods for transmission of the virus to test plants, and begin work toward serological detection methods that can be turned into field-level diagnostic tests.

Summary of previous accomplishments from this project relevant to this year's research:

Determination that Lettuce Dieback is actually caused by a newly identified virus previously unknown to science:

Lettuce samples were collected from several fields exhibiting symptoms of lettuce dieback disease. Symptomatic leaves were used to mechanically transmit virus to test plants (lettuce, *Nicotiana benthamiana* and others). Some plants, such as *N. benthamiana*, consistently developed a unique symptom regardless of whether the sample was obtained from the Salinas Valley or other regions, such as Yuma, AZ. All lettuce plants, and plants to which the virus was passaged, were used for extraction of RNA, followed by RT-PCR for the presence of the tombusviruses, MPV and TBSV, as well as using degenerate primers for the detection of a broad range of related tombusviruses. Those lettuce samples testing negative for tombusviruses, but that were successful in mechanical transmission of a disease-causing agent (virus) to test plants were saved for further evaluation. Specifically, the original lettuce RNA extracts and extracts of symptomatic *N. benthamiana* plants resulting from passaging virus from lettuce plants showing lettuce dieback-like symptoms were sent for high throughput sequencing (HTS) both through the lab of a colleague at Boyce Thompson Institute in Ithaca, NY, and at a private company (SeqMatic, Fremont, CA).

Sequencing of RNA from the field-grown lettuce plants and *N. benthamiana* plants to which virus was mechanically passaged, yielded sequences of multiple viruses, particularly with samples processed by SeqMatic. Many were typical of what one would expect from lettuce, whereas others were unknown, previously uncharacterized viruses, some of which were genetically divergent, but related to known viruses. However, high throughput sequencing (HTS) also identified sequences of a previously uncharacterized virus that was consistently associated with lettuce plants showing dieback-like symptoms. This novel virus was most closely related to a recently identified and poorly characterized virus from watermelon, a member of the family *Phenuiviridae*, order *Bunyavirales*, known as watermelon crinkle leaf associated virus, but shared only 31-36% identity with this virus (this is a very limited genetic relationship). Additionally, the new lettuce virus was found in older archived samples that had been previously associated with lettuce dieback disease or suspected of having lettuce dieback disease. Studies begun prior to the 2020 project year focused on complete sequencing of the genome of LDaV. This work continued during the past year and resulted in complete sequence of the LDaV genome.

Association of LDaV with Lettuce Dieback Symptoms:

Lettuce with dieback-like symptoms was collected from symptomatic fields for the first three years of the project to determine how tightly the presence of LDaV is linked to the presence of disease symptoms, and whether or not the virus can be found in lettuce from fields that do not exhibit lettuce dieback symptoms. Nucleic acid (RNA) was extracted from field-collected lettuce leaf samples, and tested for the presence of LDaV using LDaV-specific primers developed through this project, as well as primers for detection of TBSV and MPV, the two viruses

previously known to cause lettuce dieback, using RT-PCR (Wintermantel and Hladky, 2013; Wintermantel and Bachinsky, 2014). Results of field sampling have consistently found LDaV to be associated closely with lettuce dieback symptoms in the field. Plants with typical symptoms of lettuce dieback are nearly universally infected with LDaV, whereas lettuce that did not exhibit lettuce dieback symptoms were consistently found to be free of LDaV, as were those that exhibited symptoms of other diseases but not symptoms of lettuce dieback (0 positives/22 tested). This strongly supports that LDaV is a causative agent of lettuce dieback disease.

Sequence of the LDaV Genome:

LDaV is not closely related to any known viruses, sharing only approximately 31-36% sequence identity with the recently sequenced virus from watermelon, watermelon crinkle leaf associated virus (Xin et al., 2017). Deep sequencing yielded near full-length sequences of three virus genomic RNAs. We used traditional RT-PCR on the original RNA used for deep sequencing and primers that bind to sequenced regions of the virus to obtain what we believe is the complete sequence of the genome of the newly identified lettuce virus. Genome ends were completed using the traditional 5' and 3' RACE techniques (a method frequently used to determine sequences of the ends of virus RNAs). To date we have three fully sequenced virus RNAs, have identified the proteins encoded by these RNAs, and believe this to be the complete genome of the virus (**Fig. 1**). The availability of the sequence of the LDaV genome (*Objective 2*) is facilitating comparisons to other isolates of LDaV as well as other viruses, and may provide insight into the biological nature of this virus including whether or not it has a biological vector that transmits it.

Studies on improving experimental Mechanical transmission of LDaV and further laboratory validation that LDaV is controlled by the Tvr1 resistance gene:

In an effort to improve efficiency and experimental transmission of LDaV to lettuce by mechanical inoculation, LDaV positive lettuce plants were used for mechanical inoculation of *Nicotiana benthamiana* plants. It is already clear from previous research that LDaV is not readily mechanically transmissible. Transmission to *N. benthamiana* was much more successful than transmission to lettuce, although rates of transmission to *N. benthamiana* were also low (38% in recent experiments), whereas transmission to lettuce by mechanical transmission, although shown previously, is not reliable, and it remains possible that the previous transmission of LDaV from *N. benthamiana* to lettuce, although confirmed, could have had a contribution by a biological vector as yet to be determined. As noted above, viruses in the family, *Phenuiviridae,* are not readily transmitted mechanically, which supports the likelihood that a biological vector contributes to transmission in the field.

In order to clarify infectivity of lettuce we also attempted to mechanically inoculate several varieties of lettuce varying for the presence of the either the dominant *Tvr1* resistance allele that protects plants from developing lettuce dieback symptoms or the susceptible *tvr1* allele (Table 1). Seedling lettuce plants with two true leaves were inoculated mechanically with sap from symptomatic *N. benthamiana* plants that had been confirmed to be infected with LDaV.

N. benthamiana plants with four true leaves were inoculated as controls (Fig. 2). Results were inconclusive, as mechanical transmission to lettuce was unsuccessful. Results of these mechanical inoculation experiments demonstrated clearly that LDaV is not easily transmitted in this manner, and strongly supports the likelihood that LDaV has a biological vector.

Objectives

Objective 1. *Antiserum will be developed for serological detection of Lettuce dieback associated virus (LDaV).* This will produce antiserum that can be used for ELISA and potentially development of immunostrips for rapid detection of the virus.

Objective 2. *Infectious clones of LDaV to will be developed to allow for easier transmission of the virus to lettuce and other host plants.* These clones should greatly improve efficiency of virus transmission for evaluation of lettuce for resistance and for studies on virus host range or other biological factors.

Objective 3. *Begin efforts to determine if a biological vector may be involved in transmission of LDaV to lettuce.* These studies will begin to evaluate whether a soil-borne vector may be responsible for transmission of LDaV in lettuce fields.

Procedures

Objective 1. *Antiserum will be developed for serological detection of Lettuce dieback associated virus (LDaV).*

RNA3 of LDaV encodes a 282 amino acid nucleocapsid protein, which is believed to make up the protective shell of the virus based on sequence similarities. The gene encoding the LDaV nucleocapsid protein will be cloned into a protein expression vector using standard molecular biology methods. This clone will be used to express the nucleocapsid protein *in vitro*. The expressed protein will be purified using standard methods for protein purification from expression vectors and sent to a commercial antibody production facility and used for antibody development. The purified antibody, provided by the commercial company, will be evaluated for performance as both direct and indirect ELISA and through western blotting. Assuming antibodies perform well in these validation tests, we plan to seek partnership with a company to allow commercialization of the antibody to facilitate availability to the public and development of immunostrips for rapid virus detection from field samples.

Objective 2. *Infectious clones of LDaV to will be developed to allow for easier transmission of the virus to lettuce and other host plants.*

We are preparing to develop an agro-inoculation system for easier inoculation of plants during greenhouse evaluation of germplasm for LDaV resistance and for routine study of the host range and other biological aspects of LDaV. The LDaV genome is composed of three viral genomic RNAs (RNA1, 7, 060 nts; RNA2, 1,453 nts; RNA3, 1,461 nts). Each of the three RNAs will

be cloned separately into an *Agrobacterium tumefaciens* vector using standard molecular biology methods and verified by sequencing to confirm proper insertion and that clones are correct and complete. Once confirmed, we will transform Agrobacterium cells with each construtct, then grow each clone separately in an incubator using standard methods. We will then inoculate plants using a method known as agroinoculation. Agroinoculation delivers virus directly into cells more efficiently than traditional mechanical (leaf-rub) inoculation and should greatly improve efficiency of establishing infections. Infections will be confirmed using LDaV-specific RT-PCR methods already available in the lab.

Objective 3. *Begin efforts to determine if a biological vector may be involved in transmission of LDaV to lettuce.*

Based on genetic relationships to other viruses in the family *Phenuiviridae* (order Bunyavirales), it is suspected that LDaV may have a biological vector that transmits it. This vector would likely be associated with wet, poorly drained soils; common in areas where lettuce dieback disease is generally observed. Although it is probable that a biological vector exists, this likely is not a typical insect vector, because the disease is limited to specific field conditions and does not fit the pattern of a disease transmitted by an airborne insect such as thrips, aphids or whiteflies. A number of soil-borne organisms are currently being considered as possible vectors, and this year will be only the beginning of the search for a vector.

Specific soil-borne organisms (insects, fungi, and fungus-like organisms which are intentionally not listed here) will be established in cultures for use in evaluation of disease transmission. Once cultures of these organisms are established, these will be used to determine if the potential vector organisms can acquire LDaV from diseased lettuce (confirmation by RT-PCR). Subsequently we will see if LDaV can be transmitted to new susceptible lettuce when the potential vector organisms are introduced into soil around healthy lettuce seedlings following a period on which they are given access to feed on LDaV-infected lettuce.

Results

Note: This project was significantly delayed during the 2021-2022 project year due to two unexpected vacancies in the virology research program. Both the molecular biology and biological sciences tech positions were vacant during most of the project year as the molecular biology technician shifted full-time to the entomology project and the biological sciences technician passed away during summer 2021. While we are moving forward, progress was severely delayed and is only now beginning to pick up with the hiring of the new biological sciences technician in June 2022. Some progress was made by a laboratory assistant who has begun work on Objective 3 to determine a vector for transmission of LDaV and is also conducting studies to determine host range of LDaV. Additionally, a postdoc predominantly focused on a different project has been instrumental in initial studies to establish isolates in live culture, and to prepare virus sources for antiserum development under Objective 1.

Objective 1. Efforts to develop antiserum against LDaV are still in early stages. (This will take some time as we need purified virus or virus coat protein cloned and expressed from an expression vector for development of antiserum. In order to optimize our chance of success we plan to use both approaches, but as noted above progress has been slow as staff with the appropriate skills for these efforts were unexpectedly not available for most of the project year. Progress is picking up as one of the positions is now filled and the other is in process of replacement. We anticipate cloning of the virus coat protein gene into an expression vector by this fall (2022) and are hopeful for additional support through another grant that will allow a postdoc to be hired who can focus specifically on this project.

Objective 2. This objective was unable to be addressed as it requires a large time commitment from a molecular biologist. This aspect of the project is on hold temporarily as it is the most complex of the three objectives and was not feasible due to the staff shortages. This will be addressed if the grant mentioned above is funded.

Objective 3. With the hiring of a new biological science technician, we are beginning work toward isolating specific soil-borne fungi that are suspected of being possible vectors of LDaV. Soils containing some potential vectors are available, while others are being collected and organisms present in the soils characterized. These will be used to obtain pure isolates of the organisms, and once these isolates are determined to be virus free, studies will move forward to evaluate their ability to acquire and transmit LDaV to lettuce.

Supplemental. Determining the host range of LDaV: Due to staff limitations impacting other aspects of the research, we focused on elements that were feasible with existing staff, including studies to determine alternate hosts of LDaV in addition to lettuce (*Lactuca sativa*) and *Nicotiana benthamiana*.

Prior to this year only lettuce and the tobacco relative and research plant, *Nicotiana benthamiana*, were known to be susceptible to systemic (whole plant) infection by LDaV. As we prepare for more extensive studies including development of clones and characterization of vector organisms it is helpful to know what other hosts can be infected by LDaV, as well as what plants might be sources of the virus from which it can infest soil and subsequently infect lettuce plants. To evaluate potential host plants we conducted mechanical (rub inoculation) experiments in which LDaV propagated in *N. benthamiana* plants was inoculated to seedlings of each of several potential host plant species. It should be noted that we have already determined that mechanical transmission of LDaV is very inefficient, and this could impact results. Nevertheless, it is the most effective means to determine alternate hosts. In these experiments at least 10 plants of each test host species were inoculated with LDaV by grinding infected *N. benthamiana* tissue (*N. benthamiana* was infected by passage of virus from infected lettuce) in phosphate buffer, pH7.4 and a small amount of sterile diatomomaceous earth (Celite) as an abrasive. Ground sap was gently rubbed on the upper surface of test plants by gloved hand. Symptoms were observed weekly for 5 weeks. Any positive symptoms were confirmed by RT-PCR. In these experiments only jimsonweed (*Datura stramonium*) developed infections, and these were in the form of local lesions produced on inoculated leaves (**Table 1**).

Plants did not become infected systemically. No other plants developed infections. Experiments are continuing.

Table 1. Number of plants of each type with local and systemic infection by LDaV of total number tested.

Plant Type	Local lesions[1]	Systemic infection[2]
Tomato (Oregon Cherry)	0/10	0/10
Tomato (Moneymaker)	0/11	0/11
Tomato (Patio #3)	0/10	0/10
Ground cherry (*Physalis wrightii*)	0/10	0/10
Tomatillo (*Physalis ixocarpa*)	0/10	0/10
Nicotiana clevelandii	0/10	0/10
Jimsonweed (*Datura stramonium*)	10/10[3]	0/10
Devil's trumpet *(Datura metel)*	0/10	0/10
Eggplant (*Solanum melongena*)	In progress	In progress
Pepper (Jalapeno)	In progress	In progress

[1] Local lesions refer to chlorotic or necrotic spots on inoculated leaves

[2] Systemic infection refers to ability of the virus to infect non-inoculated parts of a plant.

[3] LDaV infection confirmed by RT-PCR

Discussion

We continue to evaluate field-grown lettuce to determine how tightly the presence of LDaV is linked to the presence of disease symptoms. Results of additional field sampling have continued to consistently find LDaV to be associated closely with lettuce dieback symptoms in the field, and we have not found LDaV in symptomless lettuce plants or lettuce plants exhibiting symptoms of other pathogens. Plants with typical symptoms of lettuce dieback are nearly universally infected with LDaV (based on studies in the Wintermantel Lab at USDA-ARS and the Koike Lab at Tri-Cal), whereas lettuce that did not exhibit lettuce dieback symptoms were consistently found to be free of LDaV, as were those that exhibited symptoms of other diseases but not symptoms of lettuce dieback (0 positives/22 tested). This strongly supports that LDaV is a causative agent of lettuce dieback disease. As noted in previous years, this is a much stronger correlation between infection and disease symptoms than had been seen with either tombusvirus, TBSV or MPV. This is not to suggest that the tombusviruses do not actually cause lettuce dieback disease, but their correlation with the disease is not as strong as current data suggest for LDaV. It is possible that either of the two tombusviruses or the unrelated LDaV could cause lettuce dieback disease, although it is not common to find two unrelated viruses that can cause the same disease symptoms (but it does happen sometimes). Alternatively, it is possible that LDaV has always been the only virus responsible for causing lettuce dieback, but we were unaware of its presence until it was identified in 2018, and perhaps the tombusviruses opportunistically infect plants already infected with LDaV when they are present (although they

have not been common in recent years). This latter possibility would explain why infection by the two tombusviruses has never been 100 percent correlated with disease incidence.

Research toward confirming that LDaV causes lettuce dieback disease through inoculation of lettuce with LDaV passaged from lettuce to *N. benthamiana*, then back to new lettuce plants was not successful. Symptomatic lettuce positive by RT-PCR for LDaV and negative for MPV and TBSV was routinely passaged to *N. benthamiana* to obtain infection where infection rates are generally low to moderate (38% in recent studies). Infected *N. benthamiana* plants develop a yellow, stunted and bushy appearance with some leaf necrosis and plants showing these symptoms were confirmed to be infected using RT-PCR. LDaV infected *N. benthamiana* leaves ground in sodium phosphate buffer and rub-inoculated to young lettuce plants has resulted in infection of romaine lettuce and reproduction of lettuce dieback symptoms, followed by confirmation by RT-PCR in two plants to date, but this is a very low rate of transmission and suggests that mechanical transmission is not the primary means by which LDaV infects lettuce. Rather it suggests that a biological vector may be more likely to transmit the disease. Another important point is that LDaV infected lettuce or *N. benthamiana* tissue that has been frozen loses its infectivity. Freezing tissue for short periods does not eliminate transmission, but long-term storage at – 80 C for four months or more generally results in loss of transmission even to *N. benthamiana*.

This project made rapid progress toward characterization of a new virus, Lettuce dieback associated virus (LDaV) that is likely responsible for causing lettuce dieback disease, during the first three years of this project. However, progress has been hampered by staff vacancies during the past year. These positions are being replaced, and we will continue to advance work on this project through internal USDA support for this research and external grants, even though the CLGRP funding for this project is coming to a close. Lettuce dieback has caused losses for lettuce production, particularly in fields with high soil-moisture since the 1990s, and probably longer under a different name, brown blight (Jagger, 1940). Although tombusvirus infection has been believed to cause the disease since 2001 (Obermeier et al., 2001), there have been numerous cases in which no tombusviruses were found in diseased lettuce. The newly identified virus, LDaV, which is not closely related to any other known viruses and likely will be classified in its own genus, appears to be nearly universally correlated with the presence of lettuce dieback symptoms, but absent from healthy lettuce or lettuce with symptoms of other diseases. Characterization of the LDaV genome and development of primers for detection of LDaV show a tight correlation demonstrating that LDaV is likely the primary cause of lettuce dieback disease. Although low efficiency of mechanical transmission makes a reliable assay for greenhouse screening of plants for LDaV challenging, we hope that additional research will lead to clones that can more efficiently deliver the virus to plants during controlled inoculations, the development of rapid serological diagnostic tools, and identification of an organism capable of transmitting LDaV to lettuce plants. If we can achieve this it will greatly improve the efficiency of breeding for resistance and will also allow studies to continue to select for resistance against LDaV and lettuce dieback, based on *Tvr1*, the lettuce dieback resistance gene (Grube et al., 2005; Simko et al. 2009, 2010).

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