2020-2021 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 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.

Summary of previous accomplishments from this project relevant to this year's research:
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*, *N. clevelandii*, pepper, *Datura stramonium*, *Chenopodium quinoa*, 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.

We continue to evaluate field-grown lettuce 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 (see Objective 1). This is an ongoing effort although sampling of fields is occurring at a much lower rate now than early in the project. Results of additional field sampling continue to consistently find 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.

Objectives

Objective 1. Continue to collect lettuce samples with lettuce dieback-like symptoms and evaluate them for the presence of the newly identified Lettuce Dieback associated Virus (LDaV), as well as TBSV and MPV by RT-PCR.
Deliverables: This will clarify the relationship between presence of LDaV and symptoms of lettuce dieback disease, as well as determine how frequently TBSV and/or MPV occur together with LDaV in association with disease. Overall, this contributes to knowing if LDaV is the primary causative agent of lettuce dieback.

Objective 2. Complete sequencing of the genome of LDaV to determine its taxonomic relationship to known viruses and gain insight into its possible biological properties.
Deliverables: Knowing the genome and sequence of LDaV will allow us to determine what other viruses are most closely related to LDaV, which will provide insight into the biology of the virus including possible vectors that may be involved in transmission.

Objective 3. Purify LDaV and conduct experiments to infect resistant (*Tvr1* gene) and susceptible lettuce cultivars with the purified virus to determine if this newly identified virus is responsible for causing lettuce dieback disease and if it is controlled by the *Tvr1* gene.
Deliverables: This will conclusively demonstrate whether LDaV causes lettuce dieback symptoms on lettuce, and if the virus is controlled by the *Tvr1* resistance gene.

Objective 4. Begin efforts to determine if a biological vector may be involved in transmission of LDaV to lettuce.
Deliverables: This will begin the process of determining if there is a biological organism that transmits LDaV to lettuce.

Procedures

Objective 1. Continue to collect lettuce samples with lettuce dieback-like symptoms and evaluate them for the presence of the newly identified lettuce dieback associated virus (LDaV), as well as TBSV and MPV by RT-PCR.

DNA primers for RT-PCR were developed against a replication associated region of LDaV during the fall of 2018 and have been used to test RNA extracts from symptomatic lettuce for the presence of LDaV.

Lettuce fields with symptoms resembling lettuce dieback were collected by Dr. Wintermantel's lab with assistance from industry and extension representatives for identification of symptomatic fields. Nucleic acid (RNA) was extracted from field-collected lettuce leaf samples, and tested for the presence of LDaV using the aforementioned LDaV primers, as well as for TBSV and MPV, the two viruses previously known to cause lettuce dieback, using RT-PCR (Wintermantel and Hladky, 2013; Wintermantel and Bachinsky, 2014). Samples that tested

positive for LDaV and negative for the two tombusviruses were selectively increased for further studies on transmission to lettuce. Samples were mechanically passaged to the indicator host plant, *Nicotiana benthamiana*, to determine if an infectious agent was present in the symptomatic lettuce plant using standard methods for mechanical transmission and whether the symptoms on *N. benthamiana* matched those induced by LDaV, or of the tombusvirues, TBSV and MPV. Infection was confirmed in inoculated test plants by RT-PCR.

Objective 2. Complete sequencing of the genome of LDaV to determine its taxonomic relationship to known viruses and gain insight into its possible biological properties.

This objective was completed by the time this project was approved for funding (see 2019 Annual Report).

Deep sequencing studies conducted through this project during the 2018-19 project year identified a multipartite RNA virus and yielded near full-length sequences of three virus genomic RNAs. The virus is not closely related to any known viruses, with its closest relative being a virus identified from watermelon, watermelon crinkle leaf associated virus, which shares only approximately 31-36% sequence identity with LDaV (Xin et al., 2017). We used traditional RT-PCR on the original RNA that had been 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. The genome sequence has been deposited in GenBank and will be released publicly upon publication of a manuscript in preparation.

Objective 3. *Purify the new virus and conduct experiments to infect resistant (Tvr1 gene) and susceptible lettuce cultivars with the purified virus to determine if this uncharacterized virus is responsible for causing lettuce dieback disease.*

LDaV infected *N. benthamiana* leaves showing stunting, yellowing and necrosis symptoms were ground in sodium phosphate buffer containing 100 mM sodium sulfite and rub-inoculated to young two-true leaf lettuce plants and to additional 4 leaf stage *N. benthamiana* plants. Development of infection in lettuce and *N. benthamiana* test plants was observed over four weeks followed by RT-PCR for confirmation of infection of test plants.

Objective 4. 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 in the field. 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. Several soil-borne organisms are currently being considered as possible vectors, and this objective will begin to test each of the candidate vectors for their potential to transmit LDaV.

Although the plan was originally to address identification of a vector, two factors complicated this goal during 2020. The departure of Alejandro Del Pozo, collaborating extension entomologist, and restrictions on staff time during Covid-19. Therefore, this objective has been delayed until the 2021 project year.

Results

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.

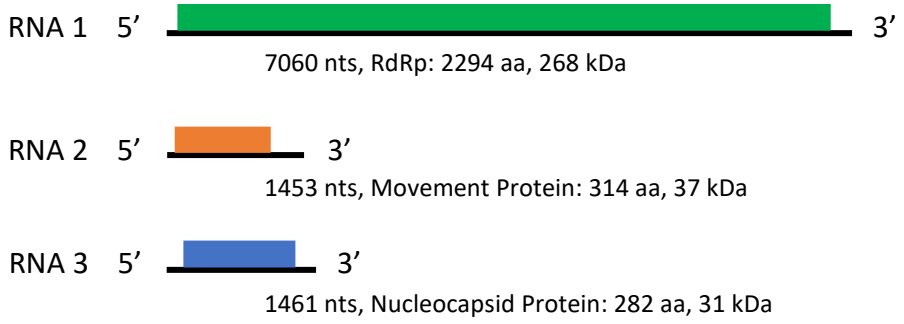

Figure 1. Genome Organization of Lettuce Dieback associated Virus

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

Samples were collected from fields as requested by industry cooperators, although the number of samples provided has been lower in 2020 and 2021 because many are being screened by companies using primers developed against LDaV through this project. Of 19 lettuce samples exhibiting potential symptoms of lettuce dieback disease, 15 tested positive for LDaV, none were infected with MPV or TBSV, and of these, nine were tested for the presence of INSV and TSWV, but were also negative for those unrelated viruses. The latter were tested because of the prevalence of INSV in the Salinas Valley during 2020.

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. Future studies covering Objective 4 should address this possibility. Furthermore, results suggest that if we plan to improve efficiency of transmission during experimentation or in plant breeding, it may be necessary to develop clones of each of the LDaV RNAs for use in agroinoculation, a much more efficient delivery system than traditional mechanical inoculation.

Table 1. Numbers of lettuce plants by variety inoculated with LDaV in each of three experiments to evaluate performance of the Tvr1 gene LDaV[b]

GENOTYPE	10/14/20	11/17/20	2/24/21
SM09A (*Tvr1* [b])	12	6	10
Triple Threat (*Tvr1*)	10	10	10
Grand Rapids (*Tvr1*)	5	-	10
La Brillante (*Tvr1*)	2	10	10
Little gem (*tvr1* [b])	1	-	8
Iceberg (*tvr1*)	11	8	10
Darkland (*tvr1*)	12	5	10
Valmaine (*tvr1*)	6	11	10
N. benthamiana	4	7	2

[a] Plants were left in the dark for 24 hours before inoculation to improve transmission efficiency.
[b] *Tvr1* = resistant, *tvr1* = susceptible to developing disease symptoms

Fig. 2. Inoculation of lettuce (upper panel) and *N. benthamiana* (lower panel) to test mechanical transmission and performance of the *Tvr1* gene against LDaV.

Identification of a possible minor mechanically transmissible virus in lettuce:

In a separate experiment involving three different sources of fresh inoculum in which lettuce with LDaV infections from the field were used to mechanically inoculate *N. benthamiana* plants which were subsequently confirmed to be infected with LDaV and used for inoculation of lettuce. As in previous mechanical transmission studies, the plants were left in the dark for 24 hours prior to inoculation to improve mechanical transmission efficiency. Surprisingly, symptoms were observed on plants four days post-inoculation in sample 21-03. Most of the inoculated lettuce plants showed symptoms on the inoculated leaves and this was highly unusual for transmission of LDaV based on previous studies. RT-PCR amplification from RNA extracts of lettuce plants showed nonspecific bands (bands that were the wrong size for RT-PCR amplification of LDaV). The PCR product was sequenced, and indicated the presence of a

tobamovirus related to turnip vein clearing virus. Further testing may be necessary to confirm the identity of the contaminating virus, but it was clearly not LDaV.

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 has made rapid progress toward characterization of a new virus, Lettuce dieback associated virus (LDaV) that is likely responsible for causing lettuce dieback disease. This disease 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 are facilitating rapid accumulation of data that show a tight correlation suggesting 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. 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 based on *Tvr1*, the lettuce dieback resistance gene (Grube et al., 2005; Simko et al. 2009, 2010), against LDaV.

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