Identification of Additional Viruses Contributing to Lettuce Dieback Disease

California Leafy Greens Research Program 2018-19 Annual Report

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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) (Obermeier et al, 2001; Wintermantel and Hladky, 2013), 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 saved for further evaluation. 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. Two previously uncharacterized viruses were found consistently associated with lettuce plants showing dieback-like symptoms; one associated with the Phenuiviridae that has been consistently associated with diseased lettuce, and another associated with the Soymoviridae, which appears to have simply been common during the growing season but not directly associated with dieback, based on subsequent analysis. Primers were developed to the Phenui-like virus, now called Lettuce dieback associated virus (LDaV), and studies are in progress to confirm association with disease and lack of association with healthy lettuce.

Objectives

Objective 1. Identify lettuce samples with lettuce dieback-like symptoms that test negative for TBSV and MPV by RT-PCR, but positive for a virus-like agent through mechanical inoculation of test plants.

Objective 2. Conduct RNA-seq analysis and compare to lettuce genome sequences and virus-like sequences to identify virus sequences present in nucleic acid extracts from diseased plants.

Objective 3. Develop primers for RT-PCR detection of newly identified targets and evaluate against additional field isolates.

Procedures

Objective 1. *Identify lettuce samples with lettuce dieback-like symptoms that test negative for TBSV and MPV by RT-PCR, but positive for a virus-like agent through mechanical inoculation of test plants.*

Lettuce fields with symptoms resembling lettuce dieback were collected by Dr. Wintermantel's lab through coordination with industry and extension representatives for identification of symptomatic fields. Each sample was mechanically passaged to indicator host plants to determine if an infectious agent was present in the lettuce plant using standard methods for mechanical transmission of tombusviruses. Additionally, nucleic acid (RNA) was extracted from lettuce leaf samples, and tested for the presence of TBSV and MPV, the two viruses currently known to cause lettuce dieback, using RT-PCR (Wintermantel and Hladky, 2013; Wintermantel and Bachinsky, 2014). Samples were identified that tested negative for both tombusviruses, and that also were able to be passaged to test plants, demonstrating the presence of a transmissible virus-like agent.

Objective 2. *Conduct RNA-seq analysis and compare to lettuce genome sequences and virus-like sequences to identify virus sequences present in nucleic acid extracts from diseased plants.*

Lettuce samples identified in Objective 1 as testing negative for both tombusviruses and other common viruses in the region, but inducing virus-like symptoms on indicator plants, were evaluated further using high-throughput sequencing. RNA was extracted from original symptomatic lettuce tissue using TRIzol (Invitrogen, Inc.) and the Direct-zol RNA MiniPrep kit (Zymo Research Corporation, USA) following the manufacturers'instructions. RNA-seq libraries were constructed using the paired-end method (Zhong et al., 2011), and sequenced on a Hi-Seq 2500 (Illumina, Inc.). RNA-seq data was analyzed and compared against the genome of *Lactuca sativa* for identification of non-*Lactuca* sequences and for the presence of sequences related to but distinct from those of MPV and TBSV. Results were also evaluated using either or both of two pipelines currently available for the detection of plant virus-like sequences from transcriptome data using the applications, Virus Detect and VirFind (Li et al., 2012; Di Bello et al., 2015; Ho and Tzanetakis, 2014; Ho et al., 2016). Unfortunately, results did not yield any pathogens that showed promise as a causative agent, so a second round of analysis was conducted as well. Material was prepared in the same manner and sent to a private sequencing laboratory, SeqMatic, for library preparation and high throughput sequencing. This time, sequence analysis identified several virus-like sequences, and two were consistently associated with all but one symptomatic lettuce and *Nicotiana benthamiana* test plants to which sap was used for mechanical inoculation.

Objective 3. *Develop primers for RT-PCR detection of newly identified targets and evaluate against additional field isolates.*

With the identification of two viruses showing a high degree of association with lettuce dieback based on high-throughput sequencing, DNA primers for RT-PCR were designed to sequences of each of the two newly identified viruses. These were used to test against original samples of lettuce dieback from the freezer, dating as far back as 1999 to establish a correlation between presence or absence of these viruses with lettuce that exhibited dieback-like symptoms.

Results and Discussion

Lettuce samples were collected from several fields exhibiting symptoms of lettuce dieback disease (**Fig. 1**). 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 (**Fig. 2**) 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 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. Two previously uncharacterized viruses were found consistently associated with lettuce plants showing dieback-like symptoms. A total of 5/6 samples contained a virus associated with the genus *Soymovirus*. This is likely an insect-transmitted virus. Additionally, 5/6 samples also consistently contained a virus associated with the family *Phenuiviridae*, order *Bunyavirales*. This unknown virus shared only limited homology 31-36% with a recently identified and poorly characterized virus from watermelon. It is very distantly related to the virus causing lettuce big vein disease, *Mirafiori lettuce big vein virus*, and therefore might have a soil-borne organism as a vector. The putative Soymovirus was not found in archived samples prior to 2018, suggesting it just happened to be prevalent in 2018. Furthermore, we would not anticipate an insect-transmitted virus to cause lettuce dieback disease. However, the putative soil-borne virus from the *Phenuiviridae* was found in older archived samples and did appear to be consistently associated with the disease.

Figure 1. Symptoms of lettuce dieback disease on romaine lettuce in a Salinas Valley lettuce field.

Figure 2. Symptoms on *Nicotiana benthamiana* after mechanical inoculation with sap from lettuce exhibiting lettuce dieback disease symptoms. Lettuce plants were confirmed by RT-PCR to be free of infection by TBSV or MPV, yet another infectious agent was passaged.

DNA primers for RT-PCR were developed against a replication associated region of the newly identified virus during Fall 2018. These were based on the information obtained from the high throughput sequencing. These primers are now being used to determine the prevalence of this possible causative agent, now called Lettuce dieback associated virus (LDaV) in both archived

lettuce samples, frozen archived RNA extracts, and new samples collected from fields. To date, we have identified positives in most recent samples confirmed to have lettuce dieback and in many freezer samples, including several over ten years old (**Table 1**). Older frozen tissue may have been of reduced quality, therefore we are less concerned with negatives in older freezer samples, as we do not know how stable LDaV may be in frozen tissue. Additionally, some samples in the freezer, although suspected of being lettuce dieback samples, may have been the result of other causes. Therefore, we are quite optimistic based on the numbers of positives and that this may be the missing causative agent.

Table 1. RT-PCR evaluation of a 957 nucleotide section of the genome of the newly identified virus, LDaV, from frozen archived lettuce dieback samples to identify association between LDaV, tombusviruses, and lettuce dieback disease

YEAR	# PLANTS TESTED	% TOMBUS	% LDaV
PRE 2011*	17	100	29
2011	9	100	78
2012	12	100	67
2013	4	0	75
2014	24	17	58
2015	11	33	91
2016	6	0	67
2017	17	0	65
2018	4	0	75
TOTAL	108		

*ALL RNA EXTRACTS WERE CONFIRMED TO BE VIABLE BY RT-PCR RE-AMPLIFCATION OF THE TOMBUSVIRUS

We are continuing 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. To date the new virus has only been found in association with dieback symptoms, and the limited number of healthy lettuce samples tested to date did not contain LDaV.

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