Project Title: Understanding the molecular mechanisms of viral infection in lettuce leaves in support of the USDA lettuce breeding program
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Abstract

The incidence of INSV on lettuce in the Salinas Valley has increased dramatically since 2019, leading to significant economic losses and lettuce shortages across the country. An improved understanding of the immune response in lettuce and rapid solutions are necessary. Gene expression studies or transcriptomics studies are hypothesis-independent methods of developing testable hypotheses about the immune responses of plants, and have the potential of significantly improving our understanding of the mechanisms involved in disease resistance. Genes can be "turned on" or "turned off" in different parts of the plant or under different circumstances (including insect defense or disease incidence), and by understanding how the plant "turns on" or "turns off" genes in different situations allows us to develop hypotheses that may indicate resistance mechanisms.

In this study, we sequenced the transcriptome of lettuce plants from the cultivars 'Cavalry,' 'Eruption,' and 'Ruben's Red,' which Richardson et al. (2024) have found to have differential responses to thrips and INSV. We found evidence that 'Cavalry' up-regulated or "turned on" genes involved in repairing damaged leaf tissue, and that salicylic acid may be the plant hormone responsible for the defense response to thrips in 'Eruption.' In 'Ruben's Red,' we identified a putative disease resistance protein, which may be involved in detecting thrip feeding and initiating the defense response in the plant. We also found evidence that part of that defense response may involve the hypersensitive response, which leads to localized cell death surrounding the site of damage. These data provide valuable testable hypotheses that can be investigated further to elucidate the mechanisms of the defense response in lettuce. Understanding gene expression in lettuce under thrip and INSV pressure may suggest mechanisms of control of the insect vector and/or the virus. It has the potential of helping to identify resistance genes for marker-assisted selection, and can provide support for candidate resistance genes that are identified via future mapping studies. At minimum, it improves our understanding of molecular mechanisms involved in the immune response in lettuce.

Objectives

- ***Objective 1:*** To sequence the transcriptome of lettuce leaves under thrip and INSV pressure in order to develop testable hypotheses for the molecular mechanisms involved in biotic stress response

- ***Deliverable:*** Quantitative data on genes expressed ("turned on") in lettuce cultivars that are resistant and susceptible to thrips and/or INSV under control, thrip, and viruliferous thrip pressure along a time series. These data will indicate broadscale gene transcriptional reprogramming in the plant immune system localized in the leaves in response to these treatments. At minimum, this is important information for understanding the lettuce immune system. These data may suggest control measures or markers for marker-assisted selection of resistant cultivars.

Procedures

We grew 12 plants of the cultivars 'Cavalry,' 'Eruption,' and 'Ruben's Red' in 4-inch pots in an insect-free growth room for 6 weeks, at approximately the 4th true leaf stage. These cultivars were chosen for their differential response to INSV and thrips as described by Richardson et al. (2024).

Four plants of each cultivar were put into two thrip-proof cages. Each plant was covered with a plexiglass tube with a thrip-proof net at the top to completely enclose each plant. A Whatman filter paper (approximately 4-inch diameter), was cut and placed around the base of each plant at the soil to discourage thrips from burrowing into the soil. In one of the two thrip-proof cages, 10 thrips were released onto each plant covered with a plexiglass tube. In the second thrip-proof cage, the plants were not exposed to thrips, but still covered with the plexiglass tubes; these plants were the controls. The plants were left for 48 hours. After 48 hours, the thrip-proof cages were opened, and approximately 500 mg of leaf tissue was harvested from the second and third youngest leaf from each plant and flash frozen immediately in liquid nitrogen. Plants were stored in a -80 degree freezer until shipped to Novogene Corporation, Inc. (Sacramento, CA) for tissue processing and RNA sequencing.

RNA-seq is a method of isolating and sequencing the RNA that is expressed at a particular point in time. The data returned is a snapshot of the proteins that the plant will use to respond to the experimental stimulus, and provides researchers with testable hypotheses about the molecular mechanisms plants use to respond to the experimental stimulus. The primary limitation of this method is that it provides only a snapshot. The transcriptome data returned constitute the plant's response at the moment of collection. If that moment of collection is poorly planned by the researcher, then important clues to the plant's response are lost. There is some amount of trial and error in deciding the most appropriate time of collection. We chose to collect samples 48 hours after exposure to thrips based on evidence that thrips will likely feed during that time. However, not all thrips likely fed at the same time, and the putative low-palatability of some cultivars might delay thrip feeding in that cultivar. The methods for inoculating lettuce with viruliferous thrips have proven difficult, and virus transfer is limited under controlled conditions. At this point, we have data only on lettuce exposed to non-viruliferous thrips, and intend to repeat the experiment in the future with viruliferous thrips when the method is optimized. The response of lettuce to non-viruliferous thrips is a critical part of the understanding of this system and the plant's defense response system, and the conditions of the experiment can be repeated to produce comparable datasets.

Statistics: Gene expression data are relative. Differences in gene expression among samples is identified by comparison among samples, such that samples exposed to thrips are compared to samples exposed to no thrips. Among the samples, we identify differentially expressed genes, which are genes that have significantly different expression levels among the

two treatments. If the two treatments induce the same gene expression patterns, there are no (or few) differentially expressed genes. Differentially expressed genes were identified as having a B-H adjusted p value < 0.05, and a log2fold change > |2|.

Results and Discussion

Some work has already been compiled on these cultivars (Richardson et al. 2024). Richardson et al. looked a palatability of these cultivars, among other cultivars, to thrips. Thrips were given a choice of a total of 13 cultivars over the course of 2 weeks, and thrips feeding damage was scored on a scale of 0-5 in three 2.5 cm discs selected from outer, middle, and inner leaves. 'Cavalry' plants had an average feeding score of 1, that is, they exhibited an average of 1-5 feeding scars per plant. 'Eruption' plants had an average feeding score of 1.5, and 'Ruben's Red' plants had an average feeding score of 2, equal to an average 6-10 feeding scars per plant. The highest feeding scores of 3 were found in 'Beacon' and 'Pacific.' This suggests that 'Cavalry' appears to be less palatable to thrips than 'Ruben's Red,' and 'Eruption' has intermediate palatability. All three cultivars had similarly high total polyphenol levels, when grown under low biotic and abiotic stress conditions. Total polyphenol content (TPC) is an inducible biosynthesis pathway, and the growing conditions under which they were tested was intended to describe baseline TPC only. Under inoculation tests, 'Cavalry' had lower INSV symptom scores when exposed to viruliferous thrips, however 'Ruben's Red' had lower INSV symptom scores when INSV was transferred mechanically using an abrasive rather than thrips. These data suggest that both resistance to the vector thrips and to the virus play a role in INSV resistance. 'Cavalry' has a high TPC and appears to be less palatable to thrips, and therefore may resist INSV by resisting the vector. 'Ruben's Red' may have better resistance to the virus, however they may have more susceptibility to thrips. This relationship is still unclear, and experiments are on-going.

This RNA-seq analysis produced over 0.5 TB of sequencing data. The analysis presented here is by necessity limited to the most significant results.

The five most significantly differentially expressed genes among 'Cavalry' plants exposed to thrips and not exposed to thrips included the following:

Protodermal factor 1	Increased with thrips	Involved in cell membrane of growing tips of the plants
Glycine-rich cell wall structural protein	Increased with thrips	Involved in cell wall
ABC transporter	Decreased with thrips	Transmembrane protein, involved in the cell membrane
Cytochrome P450	Decreased with thrips	Involved in detoxification of xenobiotics, integrating cell signaling, and biosynthesis of secondary metabolites
Root allergen	Decreased with thrips	Induced by the plant defense response system

We see an increase in expression of two genes involved in the cell membrane and the cell wall, which could be the plant's response to the damage caused by thrip feeding. This is a

testable hypothesis that will be investigated in the future. It is interesting that two potential defense response genes, the cytochrome P450 and root allergen (detected in leaf tissue) were decreased under thrips pressure. This could be the result of an elicitor produced by the thrip that actively suppresses the plant immune system, but this is a testable hypothesis that will be investigated in the future.

The five most significantly differentially expressed genes among 'Eruption' plants exposed to thrips and not exposed to thrips included the following:

Auxin-related gene (ARGOS)	Increased with thrips	Regulated directly by the plant hormone auxin, and changes cell growth and plant organ growth
Unknown gene	Increased with thrips	Unknown
EIN3-binding F-box protein 2	Increased with thrips	Repress the plant hormone ethylene and promote growth
F-box protein PP2-B10	Increased with thrips	Located in the phloem, binds carbohydrates, expression is induced by the plant hormone salicylic acid
LOB domain-containing protein 41	Increased with thrips	Transcription factors involved in plant development

Again, we see genes that are related to growth, which may be up-regulated by the plant to repair tissue damage caused by the thrip feeding. The gene for F-box protein PP2-B10 is induced by salicylic acid, which is a plant hormone commonly associated with insect resistance. This suggests that salicylic acid is the hormone regulating the defense response of lettuce to thrips, and is a testable hypothesis that can be investigated in the future. The unknown gene is highly differentially expressed, and more work is necessary to characterize this gene and its role in plant defense.

The five most significantly differentially expressed genes among 'Ruben's Red' plants exposed to thrips and not exposed to thrips included the following:

Uncharacterized long non-coding RNA	Decreased with thrips	Involved in gene regulation for growth and stress response, but specific information is lacking
Uncharacterized protein	Decreased with thrips	Unknown
Putative disease resistance protein	Increased with thrips	Disease resistance proteins are usually attached to the plant cell wall or membrane that recognize something that the insect produces in order to start the cascade response of the plant defense system
E3 ubiquitin-protein ligase	Increased with thrips	Part of plant defense response system

Uncharacterized long non-coding RNA	Decreased with thrips	Involved in gene regulation for growth and stress response, but specific information is lacking

In the other cultivars, we identified genes that were tangentially associated with the defense response system. In 'Ruben's Red,' we have identified genes that are more directly related to the defense system, a putative disease resistance protein. There is increased expression of a putative disease resistance protein encoding gene that could potentially code for a protein that recognizes an unidentified elicitor the insect produces as a result of feeding, and there is a downstream gene that may have been upregulated in response to the putative disease resistance protein as part of the plant defense system and the onset of the hypersensitive response, which causes cell death immediately surrounding the wound area to prevent spread of the insect or disease. Interestingly, there were also two long non-coding RNAs that were significantly down-regulated in response to thrip pressure. It is not clear what role these long non-coding RNAs play generally in the plant defense system, or specifically in this system. The roles of the resistance protein and the E3 ubiquitin-protein ligase are potentially important parts of the plant defense system against thrips, and constitute testable hypotheses that can be investigated further.

We have described several testable hypotheses from these RNA-seq data to more fully understand the defense response system of lettuce to thrips. Further work is currently in process to repeat this experiment with viruliferous thrips and to compare the response to understand if viral infection changes the plant's response. Further work may be necessary to optimize the timing of collections in order to test additional hypotheses from these data.

References

Richardson, K. L., Nayak, S., Hasegawa, D. K., and Eriksen, R. L. 2024. Evaluation of lettuce germplasm for resistance to impatiens necrotic spot virus. Euphytica 220:33. https://doi.org/10.1007/s10681-023-03285-z.