Molecular responses of *Nicotiana glutinosa* to infection to lettuce necrotic yellows virus

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A thesis submitted to
Auckland University of Technology
in complete fulfilment of the requirements for the degree of
Master of Philosophy

Faculty of Health and Environmental Sciences

School of Science

2024

Dedicating this thesis to my dear parents for their unwavering love, support, and inspiration.

Abstract

Lettuce necrotic yellows virus (LNYV), a cytorhabdovirus endemic to Australia and New Zealand, comprises two subgroups: subgroup I (SI) and subgroup II (SII). SI has seemingly become extinct in Australia, potentially outcompeted by SII. This research helps in understanding the host-virus interactions of the model species *N. glutinosa* with LNYV (Cytorhabdoviridae type species *Cytorhabdovirus lactucanecante*). This study investigates the molecular and metabolic responses of the model host to infection by each subgroup using one-step reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) and gas chromatography mass spectrometry (GCMS).

Gene expression analysis revealed that both LNYV subgroups induced increased accumulation of *AOX1a*, *NPR3*, and *RDR1*, with LNYV-SII causing weaker responses for *AOX1a* and *RDR1*. These results point to a salicylic acid-dependent response by the plant host to infection, implying that LNYV-SII may have evolved mechanisms to dampen the host's defensive response, potentially contributing to its competitive advantage over SI. In contrast, *RDR6* and *CPK3* transcript levels were unaffected by LNYV infection. To support these findings, reference genes *ACT*, *EF1α*, and *SAND* were validated for use in LNYV-infected *N. glutinosa*. *EF1α* was found to be the most stable, followed by *SAND* and *ACT*.

Metabolic profiling using GCMS demonstrated differential levels of amino acids and organic acids in LNYV-SI and LNYV-SII infected plants respectively compared to mock-inoculated plants. This increase in TCA cycle intermediates and activation of the TCA cycle pathway in SII-infected plants implies that these host plants may have low host resistance and hence require more energy to battle viral infection. Compared to SI, the host resistance to LNYV-SII seems to be limited. Higher viral loads are made possible by poor resistance, and this encourages quicker viral multiplication and more efficient vector transmission within plant populations. Common pathways affected by both subgroups included glyoxylate and dicarboxylate metabolism, the citrate cycle (TCA cycle), alanine, aspartate, and glutamate metabolism, and sulphur metabolism. However, SI infection uniquely impacted glutathione metabolism and the metabolism of glycine, histidine, serine, and threonine. Conversely, SII infection uniquely affected arginine biosynthesis and tyrosine metabolism.

These combined molecular and metabolomic analyses provide a greater understanding of LNYV infection in *N. glutinosa*, highlighting the distinct and overlapping impacts of the two LNYV subgroups. The findings suggest that LNYV-SII's ability to alter host defence responses and its unique metabolic impacts may explain its rapid spread and competitive displacement of LNYV-SI in Australia. This research represents a significant step towards understanding the molecular process underlying LNYV infection and offers insights into the mechanisms driving the dispersal and dominance of LNYV-SII.

Keywords: lettuce necrotic yellows virus, *N. glutinosa*, RT-qPCR, GCMS, host-virus interaction.

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Attestation of Authorship

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person (except where explicitly defined in the acknowledgements), nor material which to a substantial extent has been submitted for the award of any other degree or diploma of a university or other institution of higher learning.

30/07/2024

Signature Date

Co-authored works

Chapter 2 **Infection of *Nicotiana glutinosa* by lettuce necrotic yellows virus suggests a salicylic acid-dependent host response** *Submitted to the Plant Pathology Journal by Wiley (IF: 2.3)*	Shinde 80 % Valmonte-Cortes 10 % Lee 1 % Higgins 9 %
Chapter 3 **Lettuce necrotic yellows virus subgroup-specific metabolomic alterations in *Nicotiana glutinosa*** *In preparation for the Metabolomics Journal by Springer (IF: 3.5)*	Shinde 80 % Valmonte-Cortes 3 % Lee 2 % Chen 10 % Higgins 5 %

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Acknowledgements

I would like to express my deepest gratitude to all those who have contributed to the completion of this M. Phil thesis. Their unwavering support, guidance, and contributions have been invaluable throughout this journey.

First and foremost, I extend my heartfelt appreciation to my esteemed supervisory team, Professor Colleen Higgins, Dr. Gardette Valmonte-Cortes and Dr. Kevin Lee. Their insightful guidance, constructive feedback, and endless patience have been instrumental in shaping the direction of this research and refining its outcomes. Their knowledge and guidance have been crucial in assisting me in navigating through the challenges of the research process. I sincerely appreciate all the time and effort you have put into helping me over the past few years.

Furthermore, I would like to express deep appreciation to Professor Lindsey White for his kind support and giving me an opportunity to work with. I am also profoundly thankful to Auckland University of Technology for their financial support during difficult times. Thanks to the Australasian Plant Pathology Society, I was able to attend their conference and present my poster. I appreciate the AUT personnel and technicians for their help.

To the AUT Molecular Genetics Research Group, I would like to thank each one of you for all the fruitful discussions and enjoyable moments. Special thanks to Priya and Anthony, I am reminded of the significance of those initial interactions, as I reflect on my journey. Cheers, Tony and Lee for all the conference memories. To Elizabeth, Zoe, Natalia and Laura, I owe you so much for listening to me when I needed to vent, for your thoughtful feedback and words of encouragement.

My friends from AUT, Sadhvi, Mansi and Loretta your cheery nature and desire to help made the daunting task of settling in much simpler. Your commitment to ensuring that I felt at home from day one did not go unnoticed. Thank you, Sadhvi, Himanshi and Kamal, for looking after me and making sure my tummy is well-fed by all the yummy food. My gratitude goes to those kind people outside of AUT who offered encouragement and kept track of my progress by asking, "When are you finishing your Grad School?"

I am grateful to Karan and Aishwarya for being my sounding board, for applauding even the tiniest accomplishments, and for reminding me of the bigger picture when I was feeling overwhelmed. Finally, and most important of all, I am incredibly appreciative of my parents' constant support and inspiration. My academic successes are built on a foundation of their support, love, and sacrifices. The accomplishment would not have been achieved without your unending prayers, guidance, and blessings. Thank you for being my pillars of support, for acknowledging my efforts, and for being a source of comfort during my challenges. I love you both.

Chapter 1 General Introduction and Literature Review

1.1 Rationale and Significance of the study

In order to meet the growing demand for food worldwide without depleting natural resources, sustainable agriculture must address environmental issues and make use of plant-ecological and plant-microbe interactions to preserve resources for future generations. The spread of pathogens is anticipated to elevate in many areas of the world, bacteria, fungi, oomycetes, and viruses being the significant contributors. Of these, viruses alone cause almost 50% of the emerging infectious diseases documented in plants (Anderson et al. 2004; Aranda and Freitas-Astúa 2017). Understanding, regulating, and controlling plant damage caused by biotic stress agents will help develop sustainable agriculture. Studying gene expression and metabolic profiles of plants under stress can aid in resolving the signal transduction pathways that regulate stress tolerance in a natural environment (Sewelam et al. 2014). Several studies have been carried out so far to investigate the effect of single and combinatorial stresses to put together the puzzle of molecular interactions that control plant stress response (Atkinson 2012; Scandolera et al. 2024; Zandalinas et al. 2024).

In plants, stress may be defined as any modification in growth conditions that amend or distort its metabolic equilibrium among the plants' natural environment (Shulaev et al. 2008). Several cellular changes occur, such as biochemical, molecular, morphological, and physiological changes. The plant might come back to the first state, or a replacement physiological state if the stress is sustained or altered (Perez-Clemente et al. 2013). Several signalling cascade mechanisms, such as kinases, ion channels, reactive oxygen species (ROS) etc., are activated, and hormones such as abscisic acid, ethylene, jasmonic acid, salicylic acid, etc., accumulate once the abiotic or biotic stresses are sensed. Eventually, these result in changes in plant gene expression that helps prevent damage (Perez-Clemente et al. 2013; Kaur et al. 2022).

The identification of pathways involved in stress responses of plants is becoming more achievable due to the unfolding of several new "omics" based technologies. These approaches allow understanding of the factors influencing the growth and development of plants with and without stress, which can ultimately aid in understanding the interrelation between the metabolism of a plant and the stress caused due to biological entities or the environment. Changes in plant transcriptome, proteome, and metabolome result from gene expression changes mediated by plant responses to stress (Perez-Clemente et al. 2013; Gupta et al. 2023).

Although there have been numerous studies on plant responses to viruses, limited research is available on study of host responses to lettuce necrotic yellows virus (LNYV), or Cytorhabdoviruses in general; therefore, this study will increase understanding of how plants respond to this type of virus. Further, no study has reported specific host responses of *Nicotiana glutinosa* that respond to Cytorhabdoviruses; thus, this study will provide greater knowledge about this important, understudied model plant species. This research will focus on how this model plant responds to the two different subgroups of LNYV (Callaghan and Dietzgen 2005; Higgins et al. 2016) by analysing the impact of these subgroups on the transcript accumulation of known stress-responsive genes as well as metabolomic changes. Identifying plant host genes that respond to virus infection may provide important targets for breeding programmes developing virus resistant crops (Deng et al. 2020; Mores et al. 2021). This may lead to the development of novel breeding strategies for virus resistant plants, developing novel plant germplasm that may be of interest to farmers worldwide. Further, identifying virus responsive molecules in plants may provide tools for identification of infected plant material – an important tool for biosecurity agencies to protect local agriculture by identifying infected imported plant material rapidly (Delmiglio et al. 2023).

1.2 Research questions

The research questions addressed in this thesis are as follows.

1. Does the accumulation of specific host transcripts in *N. glutinosa* change in response to LNYV SI and SII infection?
2. What changes occur in the metabolite profile in *N. glutinosa* in response to LNYV SI and SII infection?

1.3 Thesis structure

The primary purpose of this study was to study plant-virus interaction under experimental conditions. This thesis is divided into four chapters, of which two constitute experimental chapters.

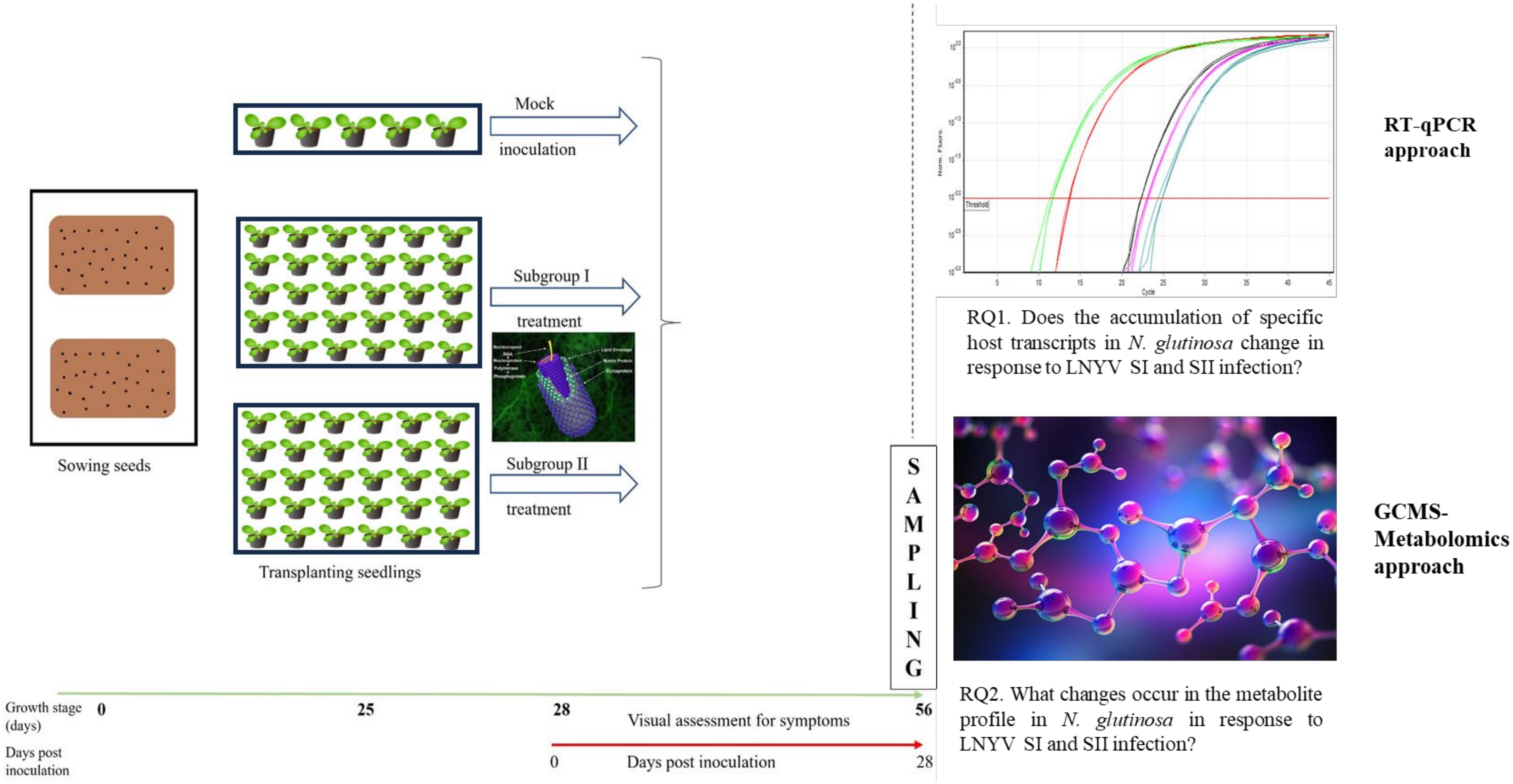
Chapter 1 contains a general introduction describing host responses to stress. It also highlights several features of LNYV, previous studies on LNYV in Australia and New Zealand, and the purpose of using a model host plant (*N. glutinosa*). It also reviews the literature on different methods used to study plant-virus interaction while identifying the knowledge gaps.

Chapter 2 describes the change in transcript accumulation of specific host genes in *N. glutinosa* in response to the LNYV subgroups. An RT-qPCR approach was used to measure the fold change in transcript accumulation. First, different algorithms were systematically evaluated to check the stability of reference genes [actin (*ACT*), elongation factor 1α (*EF1α*), and SAND family protein (*SAND*)] and, thereafter, relative expression of target genes [alternative oxidase 1a (*AOX1a*) (Wang et al. 2008), non-expressor of pathogenesis-related genes (*NPR3*) (Zhang et al. 2010), RNA-dependent RNA polymerase 1 (*RDR1*) (Liu et al. 2009) and RNA-dependent RNA polymerase 6 (*RDR6*) (Yang et al. 2011), calcium-dependent protein kinase 3 (*CPK3*)] was calculated.

Chapter 3 addresses the change in metabolite accumulation in *N. glutinosa* in response to infection by the LNYV subgroups. Untargeted metabolomics was used to examine the shift in primary metabolites such as amino acids and organic acids across various biosynthetic pathways. The functions of metabolites that differ under viral stress are portrayed.

Chapter 4 integrates the key findings and constitutes a general discussion. Finally, future perspectives and improvements for the research are discussed.

The graphical abstract for this thesis can be seen below in Figure 1



RQ1. Does the accumulation of specific host transcripts in *N. glutinosa* change in response to LNYV SI and SII infection?

RQ2. What changes occur in the metabolite profile in *N. glutinosa* in response to LNYV SI and SII infection?

Figure 1 Graphical abstract of the thesis structure; LNYV image taken from (Dietzgen et al. 2007).

1.4 An overview of lettuce necrotic yellows virus

There is a wide variety of pathogens in the *Rhabdoviridae* family that includes four subfamilies, and 56 genera (ICTV 2024). These viruses are known to infect both animals and plants, including birds, fish, reptiles, and mammals impacting public health, veterinary, and/or agricultural production. The subfamily *Betarhabdovirinae* includes six genera that includes *Betanucleorhabdovirus, Cytorhabdovirus, Dichorhavirus, Gammanucleorhabdovirus, Alphanucleorhabdovirus* and *Varicosavirus*. The locations of member virus replication and morphogenesis led to the establishment of two genera of unsegmented plant rhabdoviruses: *Cytorhabdovirus* and *Nucleorhabdovirus*. Cytorhabdoviruses comprise viruses with unsegmented genomes, exemplified by lettuce necrotic yellows cytorhabdovirus (the type species *Cytorhabdovirus lactucanecante)* and potato yellow dwarf nucleorhabdovirus (ICTV 2024). LNYV being the scope of this thesis is discussed in detail as below.

1.4.1 Host range and symptomology

LNYV was first identified in Victoria, Australia, in 1954 (Stubbs and Grogan 1963) and in New Zealand, near Blenheim in the South Island, in 1965 (Fry et al, 1973). It has been found that LNYV infects both monocotyledonous and dicotyledonous plants, both in field samples and in controlled experiments (Dietzgen et al. 2007). LNYV is known to infect a range of hosts belonging to *Alliaceae*, *Amaranthacae*, *Asteraceae*, *Chenopodiaceae*, *Compositae*, *Fabaceae*, *Leguminosa*, *Liliaceae*, and *Solanaceae* (Dietzgen et al. 2007). Species known to be infected by LNYV include lettuce (*Lactuca sativa*) and prickly lettuce (*L. serriola*), several species of sow thistle such *Sonchus oleraceus* and *S. hydrophilus*, as well as the native puha (*S. kirkii*) of New Zealand. Moreover, LNYV has been observed to invade lupin species such as *Lupinus albus* and *L. angustifolius*, garlic (*Allium sativum*), safflower (*Carthamus tinctorius*), chickpea (*Cicer arietinum*), peanut (*Arachis hypogaea*), common marigold (*Calendula officianlis*), jimsonweed (*Datura stramonium*), petunia (*Petunia hybrida*), spinach (*Spinacia oleracera*), globe amaranth (*Gomphrena globosa*), various tobacco species (*Nicotiana tabacum*, *N. benthamiana*, and *N.*

glutinosa), tomato (*Lycopersicon esculentum*), and false sow thistle (*Reichardia tingitiana*) (Fry et al. 1973; Dietzgen et al. 1989; Sward 1990; Dietzgen et al. 2007; Higgins et al. 2016).

Plant hosts may react to LNYV infection symptomatically or asymptomatically. The impact of LNYV infection on lettuce varies depending on when the infection takes place in the plant's growth cycle (Fry et al. 1973; Dietzgen et al. 2007). When lettuce plants are infected early on, they display significant stunting Figure 2(a). Infections that occur prior to the formation of the lettuce's central heart result in necrosis of the innermost leaves, often extending to the point where the outer leaves turn bronze along their veins (Stubbs and Grogan 1963; Fry et al. 1973). These symptoms make the plant unsuitable for sale (Fry et al. 1973; Fletcher et al. 2005; Dietzgen et al. 2007; Fletcher et al. 2017). Conversely, infections that happen later in the plant's development cause the outer green leaves to lose their lustre and become faded. Bronzing and necrosis develop along the veins as the infection progresses. As time goes on, the older leaves become chlorotic or mottled and grow in a flattened and flaccid state Figure 2 (b). This typically results in the eventual death of the plant (Stubbs and Grogan 1963; Fry et al. 1973). Symptoms on *N. glutinosa* include systemic mild crinkling and necrosis vein clearing depending on the isolates as shown in Figure 3 (Stubbs and Grogan 1963; Fry et al. 1973; Dietzgen et al. 2007).

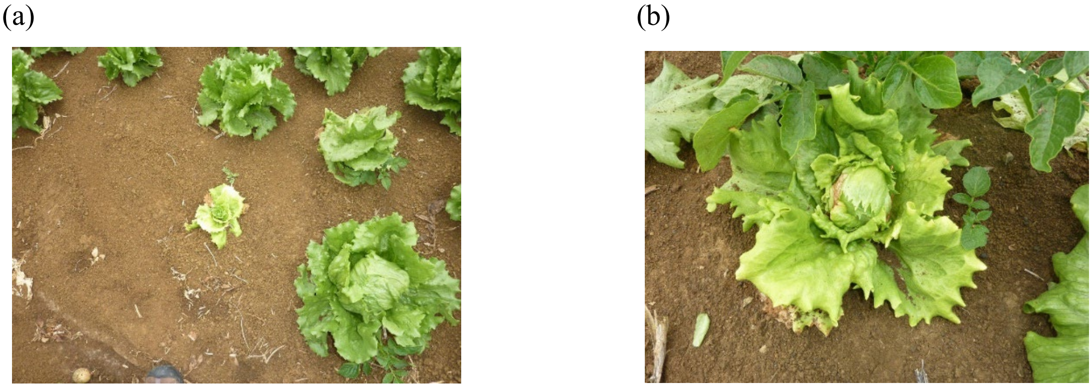


Figure 2 LNYV infected field lettuce showing range of symptoms (a) Early infection showing yellowing of leaves and stunted growth (b) late infection showing chlorotic outer leaves (Photos provided by Professor Colleen Higgins, Auckland University of Technology).

Figure 3 LNYV infected *N. glutinosa* showing systemic mild crinkling and necrosis vein clearing (taken by the author).

1.4.2 Epidemiology and transmission

In Australia, the epidemiology of LNYV is significantly influenced by its natural weed hosts, sow thistle being a prominent player (Randles and Carver 1971). Sow thistle is a perennial and widely distributed weed in Australia that tends to colonise barren or sparsely vegetated soils (Broster et al. 2022). Research from 1983 indicated that the incidence of LNYV in lettuce can be reduced up to 70% by eliminating adjacent sow thistle (Randles 1983; Dietzgen et al. 2007). It has been proposed that the surge in LNYV outbreaks in Australia during 1950s may have been triggered by the release of the myxomatosis virus, intended to control rabbit populations. This release led to an increase in sow thistle plants, as reduced grazing pressure from rabbits allowed them to thrive. This, in turn, created more reservoirs for LNYV, potentially contributing to the increased occurrence of the virus in lettuce (Randles 1983). Furthermore, a noticeable decline in disease incidence was observed as the distance from an external weed source was increased (Coutts et al. 2004). This study highlighted a pattern where LNYV-infected plants tend to cluster at the edge of a crop closest to the external weed source signifying the proximity to these reservoirs. In New Zealand, LNYV caused severe losses in lettuce crops during 1969 in Auckland, two years after it was first observed in the South Island (Fry et al. 1973). Growers of outdoor iceberg/crisp head lettuce cultivars in Mid Canterbury, Nelson, and the lower North Island reported losses. There was a high correlation between the plant virus lettuce necrotic yellows virus and crop losses resulting from necrosis symptoms on leaves (Fletcher et al. 2018; Fletcher et al. 2019).

LNYV is transmitted persistently by the aphids *Hyperomyzus lactucae* (L.) (Stubbs and Grogan 1963) and *H. carduellinus* (Theob.) (Randles and Carver 1971) in a circulative and propagative manner. More recently, the blackcurrant-lettuce aphid, *Nasonovia ribisnigri*, has also been identified as a vector. (Fletcher et al. 2019). LNYV is transmitted through at least two consecutive generations of *H. lactucae* via transovarial transmission, with roughly 20% of the progeny of an aphid capable of transmitting LNYV and 20% of these being able to transmit to the subsequent generation (Boakye and Randles 1974). The way vector interacts with the host species, *S. oleraceus* and lettuce, differs. The distribution of *S. oleraceus*, the reservoir for aphids, dictates the existence and abundance of *H. lactucae* as well as the size of the LNYV reservoir (Dietzgen et al. 2007). With *S. asper, S. arvensis*, and *Lactuca serriola* recently identified as reservoirs in New Zealand, sow thistle serves as the host for weed reservoirs (Dietzgen et al. 2020). In contrast, *H. lactucae* does not thrive on lettuce, aphids removed from a source plant would not settle and feed on lettuce unless they were starved for a few hours beforehand (Boakye and Randles 1974). The transmission of LNYV is illustrated as shown in Figure 4.

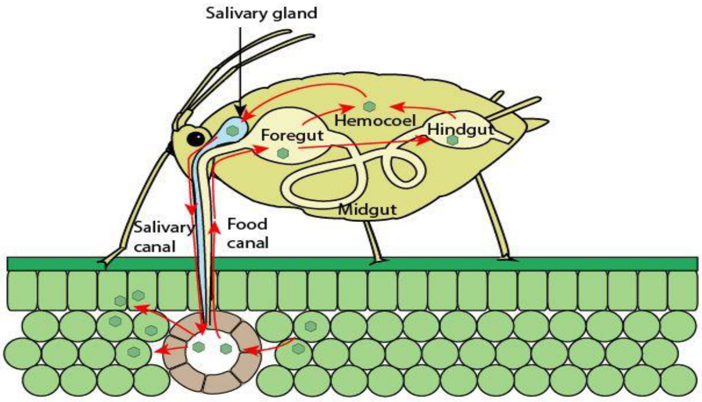


Figure 4 An illustration of how arthropods can spread viruses. Through the digestive tract, the virus enters the insect, grows, and moves to the salivary gland and canal, where the saliva distributes it. The red arrows represent the viral movement. Reproduced from (Hulo et al. 2011).

Briefly the virus acquisition and transmission occur when the aphid penetrates its stylet into the epidermal cells of leaves, injects saliva, and consumes the phloem sap all at the same time. Additionally, LNYV can be transmitted mechanically into *N. glutinosa* (Dietzgen et al. 2007; Higgins et al. 2016).

1.4.3 Virion morphology and genome structure

LNYV virion particles are bacilliform in shape measuring 360 nm x 52 nm in tissue sections Figure 5 (a) (Fry et al. 1973; Redinbaugh and Hogenhout 2005; Kuzmin et al. 2009). The particles are surrounded by a lipid envelope that has glycoprotein spikes and a nucleocapsid core that is enclosed in a hexagonal lattice Figure 5 (b) (Dietzgen et al. 2007). The matrix protein (M) forms a meshwork connecting the nucleocapsid to the glycoproteins (G). The viral nucleoprotein (N), negative-sense, single stranded RNA (12,807 nucleotides), phosphoprotein (P), and RNA dependent RNA polymerase (L) together constitute the nucleocapsid. The N protein is involved in genome encapsulation, regulation of viral replication and transcription, and association of the transcriptase enzyme (Wetzel et al. 1994). The P protein functions as the viral RNA silencing suppressor, while the L protein is involved in LNYV RNA replication and transcription (Dietzgen et al. 2007; Mann et al. 2016). The glycoprotein may be necessary for virion adhesion and insect host cell penetration (Gaedigk et al. 1986; Ammar et al. 2009); this hypothesis was supported by recent study (Prabowo et al. 2022). Figure 5 (c) shows the genome organisation of LNYV indicating its negative sense orientation, and the order of each open reading frame.

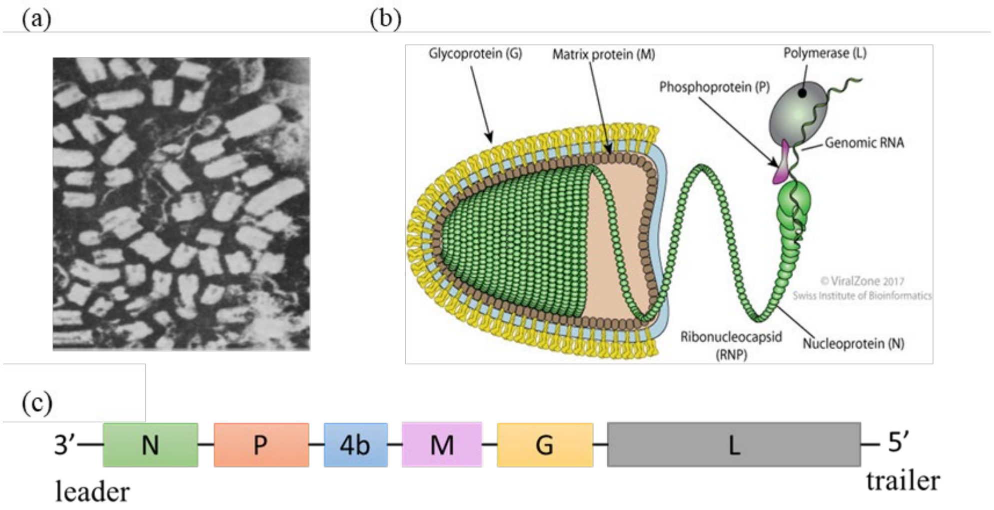


Figure 5 Illustration of LNYV (a) Electron micrograph of LNYV particles (Fry et al. 1973) (b) LNYV virion structure and proteins (Hulo et al. 2011) (c) LNYV genome organisation (image from https://viralzone.expasy.org/77

The average rhabdovirus coding capacity of this genome is 90.4% (Dietzgen et al. 2007). The N gene is 1530 nt and was the first gene to be fully sequenced (Wetzel T et al. 1994). The P gene is 1085 nt, 4b gene is 1046 nt, M gene is 631 nt, G gene is 1836 nt and L gene is 6332 in length (Dietzgen et al. 2007). The 5' UTRs range in length from 33 to 81 nucleotides, while the 3' UTRs vary between 45 and 150 nucleotides. The intergenic regions have specific lengths: six nucleotides for the region between the 3' leader and the N gene, seven between N and P, P and 4b, and 4b and M, eight between M and G and, finally, 31 nucleotides between G and L. The gene sizes (open reading frames and untranslated regions) encoded on the LNYV genome (3'-5') are listed in Table 1.

Table 1 Gene sizes (open reading frames and untranslated regions) encoded on the LNYV genome (3'-5')

Gene Regions	N	P (4a)	4b	M	G	L
gene (nt)	1530	1085	1046	631	1836	6332
5’ untranslated (nt)	78	81	38	55	33	78
Open reading frame (ORF) (amino acids)	459	300	302	177	551	2067
3’ untranslated (nt)	78	101	102	45	150	53

1.4.4 Phylogeny and subgroups

The LNYV population appears to be made up of two subgroups, SI and SII. A phylogenetic analysis comparing the complete N gene of eight different Australian LNYV isolates identified two distinct subgroups of LNYV (Callaghan and Dietzgen 2005). This study was further extended to include the N gene from New Zealand isolates (Higgins et al. 2016). Maximum Likelihood and BEAST analyses confirmed the existence of the two subgroups and demonstrated that they also exist in New Zealand. Further, SI appears to be slightly older than SII, with SII having dispersed much more rapidly than SI. Higgins et al (2016) suggested that SII outcompetes SI, possibly the result of greater vector transmission efficiency and/or greater replication efficiency in its host plant or insect vector. More sampling of LNYV isolates has been done in NZ in recent years, which supports the separation of SI and SII (Figure 6) (Ajithkumar 2018; Darling 2021). Although most of the studies have focussed on the N gene, a recent study reported analysis of glycoprotein sequences from NZ LNYV isolates in a phylogenetic study and confirmed that there are two distinct subgroups within the population of LNYV in New Zealand (Prabowo et al. 2022).

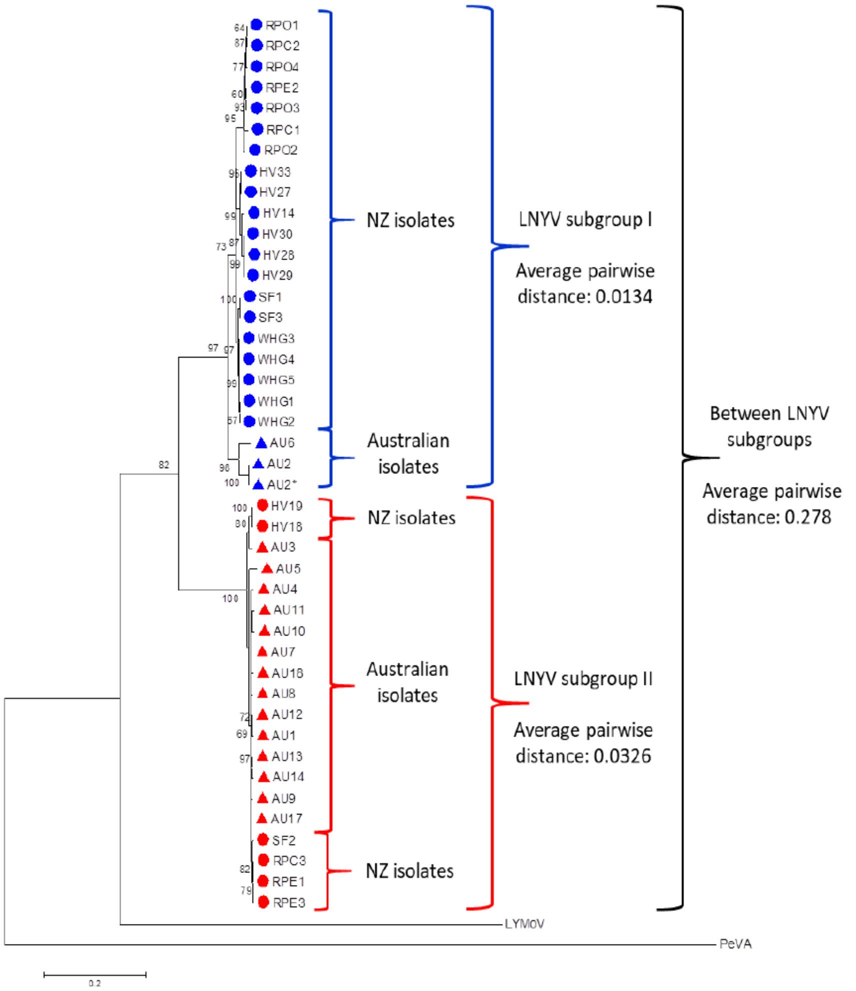


Figure 6 Phylogenetic analysis of LNYV N gene nucleotide sequences from isolates from Australia and NZ (Darling 2021)

1.5 *N. glutinosa* as a model host for the study of plant-virus interactions

The *Nicotiana* genus is relatively large and has been extensively utilised in the field of genetics research and plant breeding. Certain species within *Nicotiana* offer several advantages when it comes to research. These include phenotypic diversity, carrying out hybridisations, manipulating ploidy levels, high reproductive capacity, and a strong response to tissue culture techniques (Lewis 2011). Additionally, *Nicotiana* spp have served as valuable model systems for developing methods for genetically modifying plants and for investigating the functions of genes involving plant-pathogen interactions (Goodin et al. 2008). The initial reports of *N. glutinosa* and *N. tabacum* infected with TMV showed that the local necrotic lesions were an indication of plant virus infection (Holmes 1929, 1930). This ultimately opened up the prospects to determine viral load, virus isolation, study antiviral defences and using bioassays for quantification of viruses.

N. glutinosa has been commonly used as a plant host for LNYV studies to assess symptomology, to propagate and maintain LNYV, and to isolate the virus from naturally infected lettuce (Dietzgen et al. 1989). *N. glutinosa* was used in the foundation study of LNYV in order to determine its structure (Harrison and Crowley 1965). *N. glutinosa* was also used for cloning and sequencing the LNYV genome (Dietzgen et al. 2006). As it is rapidly infected by the virus and can serve as an inoculum for infecting other hosts, *N. glutinosa* has been recognised as a good differential host and indicator species for LNYV (Dietzgen et al. 2007; Higgins et al. 2016). While there have been reports of *N. glutinosa* responses to infection by other viruses (Wang et al. 2008; Liu et al. 2009; Zhang et al. 2010; Yang et al. 2011), there have been no research reported on the molecular responses of *N. glutinosa* to infection by LNYV, nor comparing responses to the LNYV subgroups. This study reports initial exploration of these molecular responses of *N. glutinosa*.

1.6 Plant responses to viral stress

Plants are often exposed to short- term or long-term stress events reducing cell activity and minimising the growth. Biotic stresses in plants encompass challenges posed by bacteria, fungi, insects, protists, and viruses. Viral infections causing systemic or local symptoms further lead to pronounced effects on a plant's overall health including plant's morphology, physiology, photosynthetic activities, survival, and development. At molecular level, viruses have developed tactics for completing their life cycle in plant cell by manipulating host proteins. Microbe- or pathogen-associated molecular patterns (MAMPs or PAMPs) are molecules that are shared by a wide variety of microorganisms that are detected by plants. These are important components of microorganisms, therefore they can be found in a variety of forms, whether harmful (pathogens) or not (Jones and Dangl 2006; Newman et al. 2013). Plants possess unique receptors containing nucleotide-binding domains and leucine rich repeats (NLRs) known as pattern recognition receptors (PRRs) on their cell surfaces (Yuan et al. 2021). These receptors identify the MAMPs and initiate MAMP-triggered immunity (MTI), the plant's first defence mechanism (Newman et al. 2013). PTI often is associated with the influx of calcium ions and the generation of reactive oxygen species (ROS). In many instances the Ca^{2+} ion flux stimulates respiratory burst oxidase (RBOH), ensuring a continuous supply of ROS resulting in oxidative burst (Tsuda and Katagiri 2010). While these episodes of oxidative stress are frequently associated with cell death mediated by hypersensitive response (HR) in plants, the activation of intrinsic scavenging mechanisms can also generate distinct ROS for signal transduction (Tsuda and Katagiri 2010). Furthermore, HR induces local and systemic buildup of a diverse range of pathogenesis-related (PR) proteins. As the endogenous levels of phytohormones increase, the abundance of PR proteins contributes to systemic acquired resistance (SAR), providing protection against future attacks by a wide array of pathogens (Durrant and Dong 2004). Apart from ROS PAPM perception activates mitogen-activated protein kinases (MAPKs), which in turn phosphorylate target proteins, such as transcription factors and enzymes, and ultimately activate defense-related genes, the synthesis of camalexin, and the ROS-mediated hypersensitive response, which results in cell death, among other defence responses (Lee et al. 2018). A case study looking at barley yellow striate mosaic virus (BYSMV)- barley interaction has shown that the MAPK pathways react to insect-borne virus infections and negatively impact these viruses by directly phosphorylating their nucleotide proteins. (Ding et al. 2022). This suggest that activation of these pathways inhibits viral replication. Initially serving as a defensive mechanism that limits pathogen entry, PTI undergoes a decline in the immune response if the pathogen successfully overcomes this defense by producing specific toxins or effectors leading to a phenomenon known as effector-triggered susceptibility (ETS) (Bhar et al. 2022). In defense against the harmful impacts of pathotoxins, many plants possess the resistance gene (R gene), enabling the production of antitoxins to counteract these toxins (Kaur et al. 2021). ETI responses share similarities with PTI but exhibit a quicker and more robust reaction. Viral life cycle stages and plant immune response mechanisms are highlighted in the Figure 7 that illustrates the intricate interactions that occur between plant cells and different pathogens, such as bacteria, fungi, and viruses.

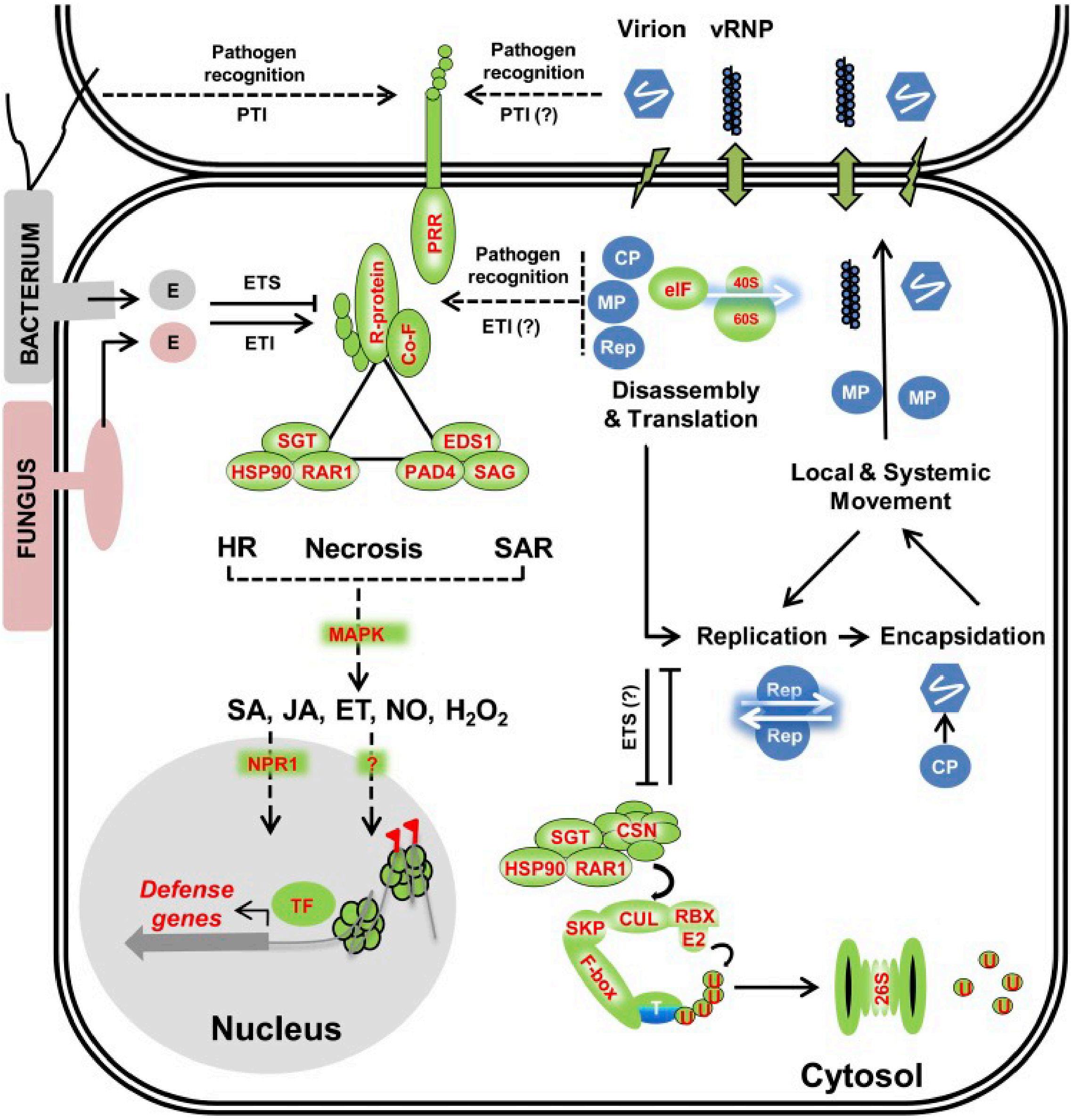


Figure 7 Plant responses to viral stress (adapted from Mandadi and Scholthof 2013)

The innate immunity of plants is remarkably complicated and adaptable in its ability to identify and respond to various pathogens including bacteria, fungi, or viruses (Pieterse and Van Loon 2007). Every plant cell has inherent defense mechanisms that can be constitutive and/or induced. The alterations in plant physiology upon virus infection can be categorised into four primary aspects of defence (Whitham et al. 2006) (Figure 8). The initial division involves the activation of defense and resistance genes triggered by a hypersensitive response HR. The second division includes stimulation of heat shock proteins (HSPs) in response to the accumulation of viral proteins. The third division comprises the activation of genes responsive to hormones, triggered by an imbalance of phytohormones (Verma et al. 2016). The last division encompasses the RNA interference (RNAi) pathway. These are discussed in detail below.

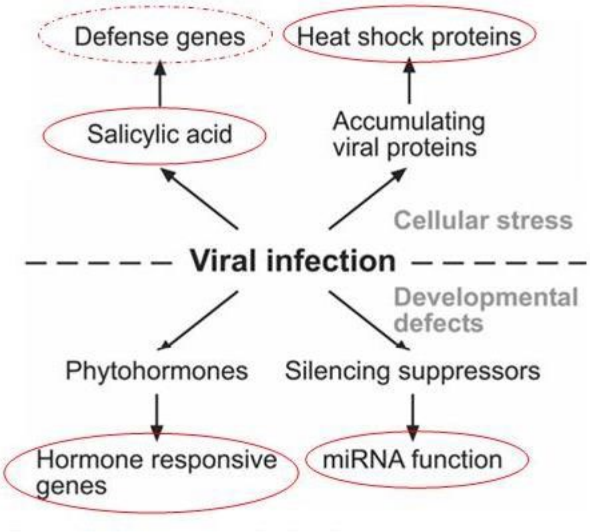


Figure 8 Host responses and altered gene expression associated with plant virus infections. (Whitham et al. 2006)

1.6.1 Cellular stress responses

These responses are mainly characterised by induction of defense genes (pathogenesis related PR genes and other genes associated with plant defence) and HSPs. Reactive oxygen species (ROS) and antioxidant enzymes play crucial roles in plant responses to biotic stress, including viral infections. Viral infections are known to trigger ROS bursts, which serve as a double-edged sword in plant physiology (Kim et al. 2022). On one hand, ROS act as signalling molecules, activating defense pathways such as the hypersensitive response (HR) and systemic acquired resistance (SAR), thereby limiting pathogen spread (Mittler et al. 2004). On the other hand, excessive ROS accumulation can lead to oxidative damage of cellular components, including lipids, proteins, and nucleic acids, exacerbating disease symptoms and promoting viral replication (Camini et al. 2017). The elevation of reactive oxygen species (ROS) and salicylic acid (SA) during HR does result in a systemic acquired resistance (SAR) response, diminishing the effectiveness of subsequent pathogenic attacks (Hernández et al. 2016). The expression levels of the PR-1 gene have proven to be a reliable and consistent molecular indicator for the activation of this pathway, making it a valuable tool in investigating various host-virus interactions (Chavez-Calvillo et al.

2016). There is a lack of literature on studies detailing the SAR response in plants infected by rhabdoviruses, indicating a potential avenue for research.

ROS is a characteristic feature and exists at a foundation level during routine cellular processes, being integral components of various signalling pathways and consistently generated through aerobic processes in organelles (Jiang et al. 2017). Under normal circumstances, cells have the capability to mitigate the impact of damage caused by ROS through the utilisation of antioxidant enzymes like superoxide dismutase (SOD) and glutathione S-transferases (GST) that convert hydrogen peroxide into oxygen and water (Afzal et al. 2014). While Increased levels of ROS are not solely attributed to viral infections, they have also been linked to various other biotic and abiotic stress factors (Khraiwesh et al. 2012). However, *Arabidopsis thaliana* and *N. benthamiana* in response to various viruses have induced levels of SOD and GST (Whitham et al. 2006).

External and internal stressors usually disturb cellular equilibrium, resulting in an instability of the cellular protein machinery and improper protein folding (Walter and Buchner 2002). To rectify this destabilisation, a set of chaperone proteins is mobilised, aiming either to facilitate the refolding of proteins or aid in directing abnormal proteins for ubiquitin-proteasome degradation (Marques et al. 2006). HSPs exhibit significantly increased induction levels in reaction to variety of stresses, encompassing environmental, physical, and chemical factors, as well as viral infections (Senthil et al. 2005; Liu et al. 2012). Numerous plant DNA and RNA viruses have been demonstrated to consistently trigger the expression of several HSPs such as HSP70, HSP83, HSP90, etc across various hosts including *A. thaliana*, *Cucurbita pepo*, *N. benthamiana*, *N. tabacum* and *Pisum sativum* (Whitham et al. 2003; Senthil et al. 2005; Whitham et al. 2006; Verchot 2012).

1.6.2 Developmental defect responses

Phytohormones, along with silencing suppressors, form an integral part of a complex network regulating developmental processes and defense pathways. The SAR pathway, that translates early signalling events induced by pathogens into activation of defense responses, relies on the synchronised activity of numerous phytohormones, extending beyond solely SA (Pieterse and Van Loon 2007; Verhage et al. 2010). Other phytohormones that help with fine tuning the plant-virus interactions include abscisic acid (ABA), ethylene (Et), jasmonic acid (JA), auxins, cytokinins, gibberellic acid (GA) and brassinosteroids (Alazem and Lin 2015).

RNA silencing, also known as RNA interference (RNAi) or post-transcriptional gene silencing (PTGS), involves small interfering RNAs (siRNAs) that guide sequence-specific cleavage of complementary target messenger RNA (mRNA) from a diverse pool of small RNAs (sRNAs) (Fang and Qi 2016). The microRNAs (miRNAs) in this RNAi pathway are believed to be disrupted by virus-encoded suppressors of RNAi (VSRs), leading to impaired plant development (Mengistu and Tenkegna 2021). Certain components of the RNAi machinery such as RNA dependent RNA polymerase (RDRs) are induced by viral infection (Schiebel et al. 1993) that are assessed using the RT-qPCR approach in Chapter 2. LNYV possesses a negative-sense RNA, thus, for its replication it must form a positive sense strand to act as the template for genome replication. These positive and negative sense strands form dsRNA that may act as a trigger of the host's RNAi pathway (Ali et al. 2020). These dsRNA intermediates would undergo processing by Dicer-like (DCL) enzymes, resulting in the generation of virus-derived small RNAs. These vsRNAs would then be cleaved by the RNA-induced silencing complex (RISC), ultimately leading to the silencing of the viral genome (Boualem et al. 2016; Silva-Martins et al. 2020). However, plant viruses including rhabdoviruses have evolved to impair the RNA silencing pathways by RNA silencing suppressors (RSSs). The phosphoprotein (P) protein of LNYV, identified as a weak RSS in plant but not insect cells, binds to RNA silencing machinery proteins (AGO1, AGO2, AGO4, RDR6 and SGS3) inhibiting RISC cleavage (Mann et al. 2015; Mann et al. 2016).

The host responses to a viral infection extensively studied so far are thus the disruption of the accumulation or functioning of host nucleic acids, proteins or above mentioned cellular and developmental effects. As there is little to no information available regarding the molecular responses of host plant to LNYV infection, this study was undertaken to lay the foundation of understanding the mechanism of LNYV-host interaction.

1.7 Approaches to studying plant responses to stress

In the realm of plant-virus interactions, so far, a multitude of responses have been uncovered, ranging from shifts in morphology and development to molecular level alterations (Wang 2015). Diverse technologies have emerged to delve into these responses, emphasising the importance of selecting the right method to gather and analyse data specific to the research question. Cutting edge "omics" technologies such as genomics, proteomics, and metabolomics are deciphering the genetic foundations of plant stress responses (Perez-Clemente et al. 2013; Desa et al. 2023; Hina et al. 2024). Under stress conditions, plants respond through significant alterations in gene expression, leading to modifications in the composition of the plant transcriptome, proteome, metabolome (Roy et al. 2023; Hina et al. 2024). The focus of this section is to explore use of RT-qPCR, and metabolomics approaches for understanding plant-LNYV interactions.

The following sub-sections provide a concise overview of methods available for quantifying gene expression in biological samples. Additionally, the strengths and limitations of the reported techniques are highlighted.

1.7.1 RT-qPCR

Reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR) is one of the most powerful methods for gene expression measurements in real-time (Vandesompele et al. 2002b; Artika et al. 2022). It is the most sensitive, reliable, efficient, and reproducible technique for the quantification of gene expression (Bustin et al. 2005; Nevone et al. 2023). It has been widely used to study mRNA transcript accumulation in host-virus interactions, including rhabdoviruses such as *Maize fine streak rhabdovirus* and *Maize Iranian mosaic virus* infecting maize (Chen et al. 2012; Hortamani et al. 2018). The assay is based on traditional PCR principles. In this process, an RNA template is converted to a DNA copy (cDNA), which is then used as the template in a PCR. These assays are customisable depending on the region of interest to be amplified. Unlike conventional PCR, qPCR utilises fluorescence at each amplification cycle to proportionally analyse the material present in a sample at a given time (Keer 2008; Venbrux et al. 2023).

RT-qPCR can be performed in one step or two steps assays. This approach has several benefits, including a reduction in experimental variation due to the fact that both reactions take place in the same environment and a reduction in the number of pipetting stages, which minimises the possibility of contamination (Smith and Osborn 2009). One step is also very reproducible and quicker than two step which makes it perfect for processing huge volumes of samples quickly. One step RT-qPCR can be carried out using a probe-based method such as Taqman probes or using DNA intercalating dyes such as SYBR green that bind to dsDNA (Gudnason et al. 2007). The latter is a widely used method due to its cost efficiency (Kokane et al. 2021). Identification of specific amplification is achieved by analysing the melting curve of PCR amplicons after amplification is complete.

Studies using RT-qPCR have been carried out in model host plants such as *Nicotiana* species, resulting in the establishment of reference genes that are stable for respective host plants infected with specific viruses (Mascia et al. 2010; Lilly et al. 2011; Zhang et al. 2013; Baek et al. 2017; Dong et al. 2022; Zhang et al. 2023). Currently, there is no set of defined reference genes for *N. glutinosa,* particularly for infected plants. Therefore, an RT-qPCR assay was designed to test a set of reference genes alongside the study of mRNA accumulation in the model host in response to LNYV infection. This is described in Chapter 2.

1.7.2 Metabolomics (with a focus on GCMS)

The metabolome is defined as a complete set of metabolites that can be detected in an organism, organ, tissue, or biological fluid whereas, metabolomics refers to techniques and methods used to study the metabolome (Duque et al. 2013; Mosa et al. 2017). One or a combination of several separation and analytical techniques are often employed to study the metabolome of a plant or given sample (Patel et al. 2021). Metabolomics tools such as gas chromatography mass spectrometry (GC-MS), liquid chromatography mass spectrometry (LC-MS), capillary electrophoresis mass spectrometry (CE-MS), fourier-transform ion cyclotron resonance mass spectrometry (FTICR-MS), matrix-assisted laser desorption/ionisation (MALDI), ion mobility

(IMS), and nuclear magnetic resonance (NMR) are routinely used for the qualitative and quantitative analyses of metabolites (Bianchi et al. 2018; Patel et al. 2021).

GCMS is an ideal technique for identifying and quantifying small metabolites including alcohols, amines, amino acids, fatty acids, organic acids, sterols, and sugars (Fiehn 2016). This is often achieved by using chemical derivatisations so that these metabolites are volatile enough for GC-MS. GCMS based metabolite profiling involves sampling and extraction, derivatisation followed by GCMS metabolite profiling and data analysis (Lisec et al. 2006). A targeted or untargeted metabolomics approach can be assigned to unravel the biochemical responses of plants to various stresses (Allwood et al. 2021). In targeted metabolomics, a single experimental platform and a particular extraction process are frequently sufficient because the focus is on particular group of metabolites. Whereas in untargeted metabolomics complete metabolome information can be obtained (Beale et al. 2018). Rather, a more comprehensive approach is required to account for the diverse range of metabolites that are present. A targeted approach is hypothesis-driven, analyses a predefined subset of metabolites, relies on known standards for comparison and provides absolute quantification. On other hand untargeted approach is hypothesis-generating, analyses a broad range of metabolites, uses large databases and libraries for comparison and provides relative quantification (Lelli et al. 2021). Therefore, an untargeted approach was used in current study which could provide us unbiased view of metabolome which can then be used for targeted and hypothesis-driven studies in order to quantify and validate specific metabolites. Plants produce a diverse range of biological and chemical compounds to manage various abiotic and biotic stresses. Hence, analysing the metabolites synthesised or accumulated by plants under stress conditions helps in understanding the regulating mechanisms. This connects the genetic makeup with the phenotype and investigates the changes in phenotype occurring under stress (Shaw et al. 2021). An untargeted approach was employed to study the metabolomic alterations in *N. glutinosa* on LNYV infection. This is described in Chapter 3.

To summarise, the combined approach of RT-qPCR and GCMS based metabolomics will help in better understanding of transcript accumulation of stress responsive genes in host plants and change in metabolomic profile respectively on infection to LNYV. This will also shed light on the dispersal patterns of two LNYV subgroups.

Chapter 2 Infection of *Nicotiana glutinosa* by lettuce necrotic yellows virus suggests a salicylic acid-dependent host response

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Abstract

Lettuce necrotic yellows virus (LNYV) is a cytorhabdovirus that appears to be endemic to Australia and New Zealand. The population of LNYV comprises two subgroups, SI and SII; SI appears to have become extinct in Australia, possibly outcompeted by SII. Previous work suggested that SII may have a more optimal interaction with its host than SI; thus, we investigated the response of the model host *Nicotiana glutinosa* to infection with each LNYV subgroup using one-step reverse transcriptase quantitative polymerase chain reaction (RT-qPCR). Here, we document the relative expression of the genes *AOX1a*, *NPR3*, *RDR1*, *RDR6* and *CPK3;* both LNYV subgroups caused increased accumulation of *AOX1a*, *NPR3*, *and RDR1* mRNAs*,* with weaker responses to LNYV-SII for *AOX1a and RDR1*. These responses suggest a salicylic acid-dependent defence by the plant host to infection, with LNYV-SII appearing able to dampen the host's fight against infection. *RDR6 and CPK3* transcript accumulation did not appear to be affected by LNYV infection. We also present validation of the reference genes *ACT*, *EF1α*, and *SAND* for use when studying LNYV-infected *N. glutinosa*. The order of decreasing stability was found to be *EF1α >SAND > ACT.* The impact of cytorhabdoviruses on the plant host at the molecular level are not well studied. Our findings represent an important step towards understanding the processes that result in infection, as well as deepening our understanding of the possible mechanisms for the rapid dispersal of LNYV-SII, particularly in Australia.

Keywords: RT-qPCR, reference genes, LNYV, gene expression, plant-virus interaction, *Nicotiana glutinosa*.

2.1 Introduction

Plant-virus infections cause changes in gene expression of a particular gene or set of genes in several ways. One common mechanism is through the activation of the plant’s defence response, involving the up or down-regulation of genes related to viral defence when the immune system detects viral RNAs and/or proteins, triggering this response (Wu et al. 2019; Ali et al. 2024). Viral infection can stimulate signalling pathways that control gene transcription. The salicylic acid (SA) signalling pathway, for instance, can be activated by viral infection (Singh et al. 2004; Spoel and Dong 2024), causing the overexpression of genes involved in pathogen defence via the regulation of transcription factors that govern the expression of several genes (Rivas-San Vicente and Plasencia 2011; Ding and Ding 2020). By interacting with the regulatory components of the gene itself, viral infection can also have a direct impact on how a particular gene expresses itself. For instance, viral proteins can bind to a gene's promoter region to either stimulate or repress gene transcription (Li et al. 2021). Viral infection can also influence host gene expression through suppression of the translation of target genes. This process is executed by small RNA molecules that are produced by the plant's RNA interference (RNAi) machinery, which is activated in response to viral infection (Mandadi and Scholthof 2013; Calil and Fontes 2017).

Plant viruses also influence the host’s physiological processes. For example, changes in carbohydrate, amino acid, and lipid profiles may cause metabolic shifts, the rate of photosynthesis may be reduced, and respiration rates may be modified (Radwan et al. 2007; Llave 2016; Souza et al. 2019). When a plant is infected by a virus, metabolic fluxes are activated, proteins are phosphorylated and dephosphorylated, and additional signalling molecules are produced. Hormone signalling, defence-responsive gene regulation, cell wall strengthening, phytoalexin accumulation, as well as several other physiological and molecular processes, are all promoted by this activation (Mishra et al. 2020).

LNYV belongs to the genus *Cytorhabdovirus* in the family Rhabdoviridae (Dietzgen et al. 2007), was first identified in *Lactuca sativa* (lettuce) in 1954 (Stubbs and Grogan 1963) and appears to be endemic to New Zealand and Australia. LNYV also infects several other monocot and dicot hosts (Dietzgen et al. 2007), with the model species *N. glutinosa* routinely used as an experimental host (Higgins et al. 2016). The virus is transmitted persistently by the aphids *Hyperomyzus lactucae* (Stubbs and Grogan, 1963)*, H. carduellinus (Randles and Carver 1971),* and *Nasonovia ribisnigri* (Fletcher et al, 2018), and mechanically by rub inoculation (Stubbs and Grogan 1963; Boakye and Randles 1974). LNYV is monopartite with a 12.8 kb single-stranded negative-sense RNA genome that encodes a nucleocapsid protein (N), phosphoprotein (P), movement protein (4b), matrix protein (M), glycoprotein (G), and polymerase (L), encased by a host-derived lipid bilayer envelope with G protein projections. Phylogenetic analysis of the N and G genes revealed that the virus population comprises two subgroups, I and II (Callaghan and Dietzgen 2005; Higgins et al. 2016; Prabowo et al. 2022). The dispersal of SII appears to have been more rapid in Australia than that of SI, leading to the apparent extinction of SI there (Callaghan and Dietzgen 2005), an event that has not happened in New Zealand (Higgins et al. 2016). It has been proposed that this rapid spread of SII may have been due to more effective replication/infection within either the plant or aphid host, or transmission by the aphid (Higgins et al. 2016; Prabowo et al. 2022).

Limited information is available on host responses to LNYV infection. RT-qPCR was used to analyse the impacts of SI and SII infection on *Nicotiana glutinosa* by comparing their effects on the expression of specific mRNAs. This method is widely used due to its high accuracy, reproducibility, sensitivity, and speed (Bustin et al. 2005; Peng et al. 2024). However, the challenge of RT-qPCR is selecting appropriate reference genes; that is, genes that are expressed stably irrespective of the tissue type or experimental treatment (Bustin et al. 2009). Accurate and reliable normalisation of mRNA expression data is only achievable if the expression of the reference genes used are adequately validated as stable to avoid false results (Vandesompele et al. 2002b; Guénin et al. 2009). Over the past decade, several tools such as geNorm (Vandesompele et al. 2002a), NormFinder (Andersen et al. 2004), BestKeeper (Pfaffl et al. 2004), and comparative delta Ct (Silver et al. 2006) have been used to identify stably expressed reference genes under different treatments. The candidate reference genes actin (*ACT*), elongation factor 1α (*EF1α*), and SAND family protein (*SAND*) were assessed for stable expression in *N. glutinosa* following infection by the LNYV subgroups.

Differences in host plant responses to the LNYV subgroups may explain the subgroups' different dispersal rates. The following target genes were chosen as they had been found previously to be virus-responsive in *N. glutinosa* (Table 2): alternative oxidase 1a (*AOX1a*) (Wang et al. 2008), non-expressor of pathogenesis-related genes (*NPR3*) (Zhang et al. 2010), RNA-dependent RNA polymerase 1 (*RDR1*) (Liu et al. 2009) and RNA-dependent RNA polymerase 6 (*RDR6*) (Yang et al. 2011). In addition, the calcium-dependent protein kinase 3 (*CPK3*) gene was included as it has been shown to respond to several abiotic and biotic responses (Valmonte et al. 2014). We hypothesised that LNYV-SI and -SII would have different impacts on the expression of these genes, such that host responses would be more likely to allow infection by LNYV-SII.

Table 2: Summary of findings for target genes from other studies in *N. glutinosa* except *CPK3*

Stress response gene	**Gene function**	**Stress**	**Technique used**	**Inference**	**Reference**
NgAOX1a (Alternative oxidase 1)	Involved in multi-signal transduction pathways and play a key role in defence response	Tobacco mosaic virus Potato virus X Potato virus Y	Northern blotting	Upregulated by each virus infections stresses	(Wang et al. 2008)
NgNPR3 (**Non expressor of pathogenesis related genes**)	Induces expression of downstream PR genes and plays a vital role in resistance to broad range of pathogens	Potato virus Y	Semi-quantitative RT-PCR	Upregulated due to biotic stress	(Zhang et al. 2010)
NgRDR1 (**RNA dependent RNA polymerase 1**)	RNA silencing, heterochromatin formation and natural gene regulation	Tobacco mosaic virus Potato virus X Potato virus Y Cucumber mosaic virus	Semi-quantitative RT-PCR	Upregulated by all viruses except potato virus X	(Liu et al. 2009)
NgRDR6 (RNA dependent RNA polymerase)	RNA silencing, heterochromatin formation and natural gene regulation	Tobacco mosaic virus Potato virus Y Cucumber mosaic virus	Semi-quantitative RT-PCR	Upregulated by cucumber mosaic virus infection, but no remarkable difference with infection by potato virus Y or tobacco mosaic virus.	(Yang et al. 2011)
AtCPK3 (Calcium-dependent protein kinase) (Studied in *Arabidopsis thaliana*)	Signal transduction	Cauliflower mosaic virus Tobacco mosaic virus Tomato spotted wilt virus Turnip mosaic virus Turnip yellow mosaic virus	RT-qPCR	Induced by all virus infections	(Valmonte 2016)

2.2 Methods

2.2.1 Plant growth and virus inoculation

N. glutinosa seeds provided by The New Zealand Institute for Plant and Food Research Limited (Rangahau Ahumāra Kai) were grown for four weeks on soil supplemented with Scott's Osmocote, after which seedlings were transferred to individual pots. Plant growth was carried out in a Conviron CMP5090 under 4 levels each of fluorescent and incandescent lighting to achieve 16/8 hr photoperiod at 20°C temperature with 80 % humidity. When they reached the 5 - 6 leaves stage, two sets of 30 plants were mechanically inoculated with LNYV-SI and LNYV-SII. A leaf from a previously infected plant was ground to a fine paste in the presence of 0.01 M phosphate buffer (pH 7), 0.1% (w/v) sodium sulphite (Na_2SO_3) and a dash of carborundum (600 mesh). This mixture was rubbed onto three apical young leaves, and after six hours, excess carborundum was removed by spraying with water. Five plants were mock inoculated with buffer only. After symptoms became evident at 26 dpi, three systemic, symptomatic leaves from virus-inoculated plants replicates were collected at 28 dpi and stored at -80℃ until further processing. For mock-inoculated plants, the youngest three systemic leaves were collected. However, one leaf from mock and all infected samples were tested for LNYV infection and transcript accumulation.

2.2.2 RNA extraction, quality assessment & detection for LNYV infection

Total RNA was extracted from 200-250 mg of leaf tissue using a Spectrum^{TM} Plant Total RNA kit (Sigma Aldrich) following the manufacturer's protocol with the following modifications. Elution was carried out twice with 25 ml of nuclease-free water, and the extracted RNA was stored at -80 °C until further use. One-step RT-PCR of the N gene using primers LNYV-440F (5'-TGACACAGATTCAGAACAACTC-3') and LNYV-1185R (5'-CGGACAATCCATCTCCACTA-3') was carried out to confirm LNYV infection. Mock and inoculated samples were tested using Superscript III RT-PCR System with Platinum Taq (Invitrogen) (Ajithkumar 2018).

2.2.3 Candidate reference genes, target genes and primer design

Reference genes that were most stable in other *Nicotiana* species under stress were selected for this study, namely *ACT*, *EF1α,* and *SAND* (Schmidt and Delaney 2010; Liu Deshui et al. 2012; Baek et al. 2017). Plant virus-responsive genes were selected based on previous reports on the same species, namely *AOX1a*, *NPR3*, *RDR1*, *RDR6*, and *CPK3.* Primers for each mRNA were designed using Geneious, version 11.0.2 (http://www.geneious.com/) and Integrated DNA Technologies (IDT) Oligoanalyzer 3.1 (https://sg.idtdna.com/calc/analyzer). Since the genome for *N. glutinosa* was unavailable, primers were designed by comparing the orthologous genes from the genomes of other *Nicotiana* species; *N. attenuata*, *N. benthamiana*, *N, sylvestris*, *N. tabacum,* and *N. tomentosiformis*. Sequence information was obtained from the website National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/) and compared to find highly conserved regions that flank an intron to ensure RNA was amplified rather than genomic DNA. Primer sequences are listed in Table 3.

Table 3: Primer sequences of candidate reference and target genes used in RT-qPCR analysis.

Gene Name	Primer Name	Primer Sequence (5'-3')	Tm (◦C)	GC (%)
Reference genes				
Actin	NgActin_F	ATG TAT GTC GCC ATT CAG GCT GTT CT	60.3	46.2
	NgActin_R	GAA CTG TGT GGC TGA CAC CAT CA	59.5	52.2
EF1α	NgEF1α_F	GAC TCC ACC ACT GGT GGT TTT GAA G	59.8	52.0
	NgEF1α_R	AAC CAG AGA TGG GGA CAA AGG GGA T	61.8	52.0
SAND	NgSAND_F	CCT GCT GGA CTT TGG CAT TTT ATG TAT CG	58.1	42.9
	NgSAND_R	CAT GCA TGG ATG CAT AAA GCT TCT GGT AAG	59.7	43.3
Target genes				
Alternative oxidase 1a	NgAOX1a_F	CCG CGA TGT CAA CCA CTT TGC AT	60.6	52.2
	NgAOX1a_R	GCT GGA GAG TCC TTC AGC TGT TG	59.7	56.5
Non-expressor of pathogenesis-related genes	NgNPR3_F	CAA GGA TGC AAG CCC TCT CGA AG	60.0	56.5
	NgNPR3_R	TCT GGA GTG CCC ATC TCA AGG AA	60.0	52.2
RNA-dependent RNA polymerase 1	NgRDR1_F	CGAGCACACAGTTGGATCATGATGTC	59.7	50.0
	NgRDR1_R	TGCACGGATCACTCATGGCCATATCA	61.9	50.0
RNA-dependent RNA polymerase 6	NgRDR6_F	CGGAAGTCAAACAGTTGGGTCGTCA	61.1	52.0
	NgRDR6_R	AGGCACTTCTCATCCAAAGCTCC	59.3	52.2
Calcium-dependent protein kinase 3	NgCPK3_F	AGC TAC AAT GCA CAT GAA TCG CAT GG	59.9	46.6
	NgCPK3_R	GTC TGT GTC AAC TTC AGC AAT GAT CTC	57.7	44.4

2.2.4 One-step RT-qPCR analysis

The transcript accumulation of the reference and target genes in *N. glutinosa* was measured by one-step RT-qPCR using a LightCycler® 96 instrument and LightCycler® EvoScript RNA SYBR® Green I Master kit (Roche Diagnostics, Ltd). The one-step RT-qPCR was carried out in a total reaction volume of 10 µl consisting of 2 µl of SYBR Green I master (5X), 5 µl of water, 0.25 µl of 10 µM of each forward and reverse primers and 2.5 µl of template RNA (15 µg/ml). Each sample was analysed with three technical replicates. The RT-qPCR parameters were an initial reverse transcription step at 60°C for 15 min, pre-incubation at 95°C for 10 min, followed by amplification of 40 cycles at 95°C for 10 s then 58°C for 30 s. This was followed by a melting curve analysis of 95°C for 10 s, 25°C for 60 s and 97°C for 1 s with continuous acquisition mode, followed by 40°C for 30 s.

2.2.5 Gene expression stability of reference genes and relative quantification of target genes

LinRegPCR 11.1 was used to perform baseline correction, log transformation, and determine PCR efficiency (Ruijter et al. 2009). The stability of each reference gene was evaluated using the algorithms geNorm (Vandesompele et al. 2002a), NormFinder (Andersen et al. 2004), BestKeeper (Pfaffl et al. 2004), and the comparative delta Cq method (Silver et al. 2006) through the RefFinder platform (Xie et al. 2012; Wieczorek et al. 2013). The transcript accumulation levels for the target genes *AOX1a*, *NPR3*, *RDR1, RDR6,* and *CPK3* were normalised against the two most stable reference genes. Finally, the relative expression levels of target genes were quantified using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001; Huang et al. 2024). Follow-up statistical analysis, including t-tests, was conducted to assess the significance of observed differences in expression levels between experimental conditions, with significance levels set at $p < 0.05$.

2.3 Results

2.3.1 Symptoms and confirmation of LNYV infection

No correlation was observed between appearance of symptoms and LNYV subgroup at day 28 following mechanical inoculation of *N. glutinosa* indicating no correlation between the type and severity of symptoms. Mild symptoms such as mosaic patterns and curling of leaves became evident after day 26 post inoculation but did not differentiate the viral subgroups. Illustrative examples of mock inoculated, SI infected, and SII infected plants are shown in Figure 9. While this was unreplicated, inoculation with LNYV-SII did, however, result in a higher infection rate of 26 % compared to 15 % for LNYV-SI (n= 30 plants), supporting the ability of LNV-SII to infect its host more efficiently.

Figure 9: Representative *N. glutinosa* plants 28 dpi a) Mock-inoculated b) LNYV-SI infected c) LNYV-SII infected.

2.3.2 Primer specificity and amplification efficiency

The primers designed for each target gene and each reference gene gave rise to the expected single products as indicated by the melt curve analyses, confirming amplification specificity (Figure 10). All the tested genes showed an amplification efficiency of 1.85- 2.23, suggesting an amplification effectiveness of at least 90% (Ruijter et al. 2009).

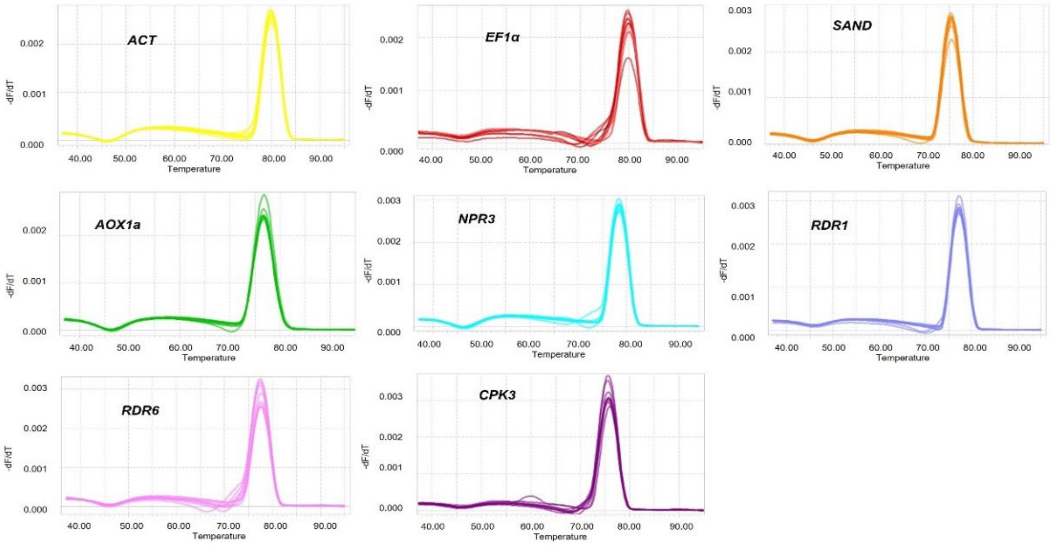

Figure 10: Melting curve analysis of three reference genes (*ACT, EF1α* and *SAND*) and five target genes (*AOX1a, NPR3, RDR1, RDR6* and *CPK3*) across all the samples of *N. glutinosa*.

2.3.3 Identification of stable reference genes

The variability in the transcript accumulation of the reference genes and target genes across all infected samples is shown in Figure 11. The average Ct values ranged from 21.86 (*AOX1a*) to 28.16 (*SAND*). The mean Ct values of candidate reference genes were 26.67 for *ACT*, 24.53 for *EF1α*, and 28.16 for *SAND*.

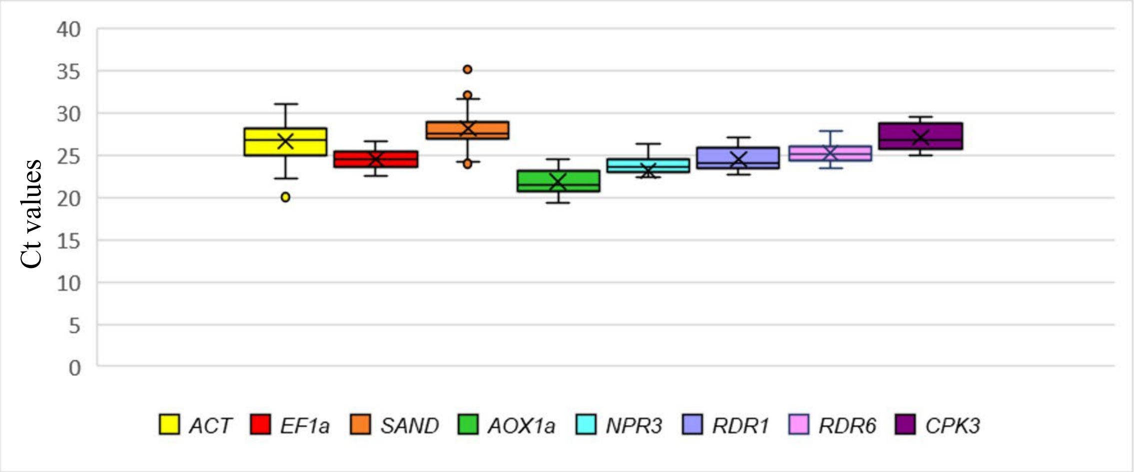

Figure 11: Cycle threshold values (Ct) of three reference genes *(ACT, EF1α, and SAND)* and five target genes *(AOX1a*, *NPR3*, *RDR1*, *RDR6, and CPK3)* across all infected samples.

A range of statistical algorithms was used to rank the stability of the chosen reference genes (Table 4). RefFinder, which incorporates algorithms such as geNorm, Normfinder, BestKeeper, and the delta Ct method, calculated the geometric mean for each gene to get a composite index ranking. The stability value of *EF1α* was 1.00, 1.682 for *SAND,* and 3.00 for *ACT*. From this, *EF1α* showed the most stable expression followed by *SAND*, while *ACT* showed the least stable expression.

Table 4: RefFinder data for comprehensive ranking of the reference genes in *N. glutinosa*

Ranking Order (Better--Good--Average)			
Method	**1 (Better)**	**2 (Good)**	**3 (Average)**
Delta C	*EF1α*	*SAND*	*ACT*
BestKeeper	*EF1α*	*SAND*	*ACT*
Normfinder	*EF1α*	*SAND*	*ACT*
Genorm	*EF1α* | *SAND*		*ACT*
Recommended comprehensive ranking	*EF1α*	*SAND*	*ACT*

2.3.4 Stress-responsive genes were upregulated during LNYV-infection in *N. glutinosa*

The target genes showed varying responses to LNYV infection, and different responses to LNYV-SI as compared to LNYV-SII (Figure 12). The expression levels of *AOX1a*, *NPR3, and RDR1* were found to increase in LNYV-infected *N. glutinosa* for both subgroups post symptom development. While marked differences (relative fold change) were observed in the responses of these genes to infection by the different LNYV subgroups, the only one that showed statistical significance was *AOX1a* when compared to mock vs SI to mock vs SII. This gene showed a 3.8-fold change in response to LNYV-SI, while there was a 2.5-fold change in response to LNYV-SII infection (p value=0.03) The *RDR6* and *CPK3* genes did not appear to respond to LNYV infection.

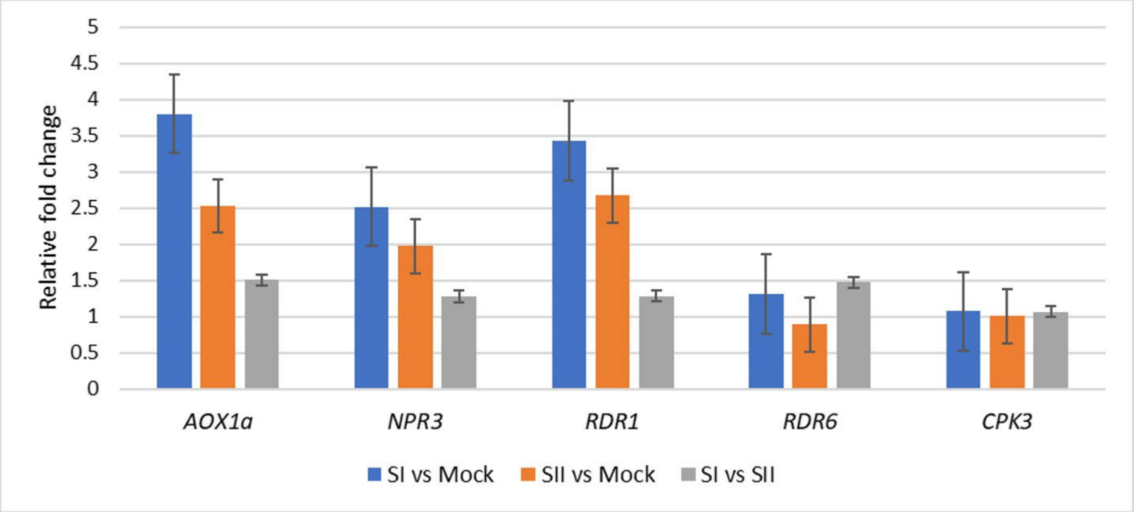

Figure 12: Relative expression of target genes (*AOX1a*, *NPR3*, *RDR1*, *RDR6* and *CPK3*) in mock, subgroup I infected and subgroup II infected samples. The error bars represent the standard error (*AOX1a* is significant; p value=0.03 using t-test)

2.4 Discussion

RT-qPCR analyses of the accumulation of specific mRNAs (Table *2*) were carried out to further understand the defence processes in response to infection of the model plant host *N. glutinosa* by the cytorhabdovirus LNYV. The virus population is made up of two subgroups (Callaghan & Dietzgen, 2005, Higgins et al, 2016, Prabowo et al, 2022), and it was of interest to determine if these subgroups have different impacts on the host at the molecular level to explain their differences in infection and dispersal rates (Higgins et al, 2016).

Increased accumulation of *AOX1a,* and possible increased accumulation of *RDR1* and *NPR3* mRNAs in response to LNYV suggests the host's response is induced by salicylic acid. *RDR1* leads to increased viral RNA degradation via the RNAi pathway as the host plant attempts to fight off infection (Jin et al. 2022) and has been shown to increase following infection by several viruses (Liu Y et al. 2009; Anjanappa et al. 2018; Leibman et al. 2022). This appears to be a salicylic acid (SA) - induced response (Ordog et al. 2002; Anjanappa et al. 2018; Liu et al. 2020). A previous study suggested that the LNYV viral RNAi silencing suppressor (VSR), the P protein, delays systemic silencing and may interfere with a step after siRNA biogenesis such as interfering with AGO protein(s), DNA methylation or amplification of silencing (Mann et al. 2015). The increased accumulation of *RDR1* in LNYV-infected plants suggests that the amplification of silencing may be affected. Increase in *AOX* has also been observed in virus infected plants (Chivasa and Carr 1998; Király et al. 2008; Wang et al. 2008; Fu et al. 2010; Liao et al. 2012; Liao et al. 2020). SA also appears to inhibit the electron transport chain within the mitochondria, which would be expected to lead to an increase in ROS (Poór et al. 2019); however, an increase in *AOX* expression appears to dampen this process. NPR3 is a negative regulator of defence genes that is responsive to SA binding (Raskin 1992; Kuai et al. 2015; Ding et al. 2018). Pathogen infection is known to suppress NPR3 protein degradation (Ding et al. 2018; Ding and Ding 2020) and LNYV infection of *N. glutinosa* also appears to increase *NPR3* mRNA accumulation. This was also observed by Zhang et al (2010) in *N. glutinosa* plants infected with *Rhizoctonia solani, Phytophthora parasitica*, *Alternaria alternata*, *Pseudomonas solanacearum*, and potato virus Y (PVY). Like *NPR1*, *NPR3* is an SA receptor, but activates a parallel signalling pathway to repress the expression of defence and immunity-related genes (Ding et al. 2018; Liu et al. 2020; Spoel and Dong 2024). Taken together, the increased accumulation of mRNA for these three genes suggests that LNYV infection upregulates an SA-dependent defence response.

The accumulation of *AOX1a* and possibly *RDR1* were lower in response to LNYV-SII than SI, with this being statistically significant for *AOX1a*. It is possible that SII is able to weaken the host's response to infection and that this may be what leads to its higher infection and dispersal rates (Higgins et al. 2016). In plants infected by CMV, the VSR, 2b, led to a reduced induction of RNAi and the *AOX* gene, as well as SA-induced resistance to viral replication (Singh et al. 2004). The LNYV VSR, the P protein, may play a similar role. The reduced accumulation of *RDR1* in SII-infected plants suggests reduced amplification of silencing, and lower levels of *RDR1* would lead to increases in infective viral genomes while lower accumulation of *AOX1a* would result in higher ROS levels, perhaps making the host more vulnerable to infection through reduced defensive signalling (Singh et al. 2004; Mukherjee et al. 2024).

No differences were observed in accumulation of *RDR6,* and *CPK3* mRNA following infection by either subgroup of LNYV. In addition to the antiviral RNAi pathway, *RDR6* is involved in RNAi pathways related to plant development (Jin et al. 2022). LNYV infection had no impact on *RDR6* mRNA accumulation, perhaps because it is not as vital to the infection process for this virus. While the calcium sensor *CPK3* has been shown to be virus responsive in other systems (Perraki et al. 2017; Valmonte-Cortes et al. 2022), it was not responsive in *N. glutinosa* at the mRNA level to infection by LNYV. *CPK3* is a multifunctional signalling protein that acts as an upstream element in the transcriptional activation of the plant defensin gene *PDF*1.2, which plays a key role in jasmonate-dependent defense responses (Kanchiswamy et al. 2010). This might imply that the jasmonate pathway is not a major factor in the recognition and establishment of LNYV infection in *N. glutinosa*.

This is the first reporting of suitable reference genes and their use with RT-qPCR to study transcript accumulation in *N. glutinosa* infected with LNYV and its subgroups. Due to the differing calculation philosophies of the various algorithms evaluating the expression stability of reference genes (Vandesompele et al. 2002b), we used RefFinder to generate a thorough assessment of gene expression stability since it takes into account four statistical techniques, namely geNorm, NormFinder, BestKeeper, and Delta-Ct (Xie et al. 2012). Our findings showed that *EF1α* was the most stable reference gene, similar to the finding of others studying virus - infected plants such as TMV-infected *N. tabacum* (Baek et al. 2017), tomato and *A. thaliana* infected with TMV, cauliflower mosaic virus (CaMV) turnip mosaic virus (TuMV), tomato spotted wilt virus (TSWV), or turnip yellow mosaic virus (TYMV), (Lilly et al. 2011; Wieczorek et al. 2013).This differed from studies of tomato infected with tomato yellow leaf curl virus, CMV, potato virus Y, tomato mosaic virus, and TSWV (Mascia et al. 2010). The second most stable reference gene, *SAND*, was also found to be suitable for *Arabidopsis* infected with TMV, CaMV, TuMV, or TYMV (Lilly et al. 2011) and sorghum infected with *Brome mosaic virus* (BMV) (Zhang et al, 2013). *ACT* was found to be the least stable of all reference genes in our study similar to these studies (Nicot et al. 2005; Gutierrez et al. 2008; Thellin et al. 2009) in contrast to others (Wieczorek et al. 2013; Baek et al. 2017). The contradictory nature of publications describing reference genes highlights the importance to test and validate a set of reference genes in a given species under similar experimental conditions as there is no universal reference gene suitable for all situations (Gutierrez et al. 2008).

This study presents evidence supporting a salicylic acid- induced response to LNYV infection of *N glutinosa*. Of particular interest, host responses to LNYV-SII appear to be weaker than to SI. Higgins et al (2016) hypothesised that LNYV-SII is able to replicate more effectively in either the insect or plant host. This study suggests that this subgroup may be able to do this via ensuring the plant host does not respond too vigorously to infection. This study presents evidence of defence gene expression in LNYV-infected *N. glutinosa* specific transcript accumulation at 28 dpi - a time course study would be of interest to determine when these changes take place following inoculation. Including transcript levels of LYNV would have provided valuable insights into the comparative viral load between SI and SII. Such data could have clarified whether SI exhibited a higher or lower titre than SII, offering a deeper understanding of the infection dynamics and potential differences in host-virus interactions. Current work is underway to assess the holistic response by *N. glutinosa* to infection with each LNYV subgroup using transcriptomics approaches. This study has also validated reference genes for RT-qPCR analysis of LNYV infected *N. glutinosa*. This study is an important advance in our understanding of the infection process of plant hosts by cytorhabdoviruses.

Chapter 3 Lettuce necrotic yellows virus subgroup-specific metabolomic alterations in *Nicotiana glutinosa*

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Abstract

Lettuce necrotic yellows virus (LNYV) is a cytorhabdovirus that appears to be endemic to Australia and New Zealand. The population of LNYV comprises two subgroups, SI and SII; SI appears to have become extinct in Australia, possibly outcompeted by SII. In the current study, we investigated the metabolic alterations of *Nicotiana glutinosa* in response to infection by LNYV subgroups. Metabolome analysis of primary metabolites using gas chromatography-mass spectrometry showed differential levels of amino acids and organic acids in SI-infected and SII-infected plants compared to mock-inoculated plants. Pathways that showed changes in response to LNYV infection and were found to be common between SI and SII against mock included glyoxylate and dicarboxylate metabolism, citrate cycle (TCA cycle), alanine, aspartate and glutamate metabolism and sulfur metabolism. Pathways affected by SI infection only included glutathione metabolism, glycine, serine and threonine metabolism and histidine metabolism whereas pathways affected by SII infection only included arginine biosynthesis and tyrosine metabolism. Our study provides valuable information regarding plant metabolomic signatures that characterise LNYV infection as well as subgroup-specific infection and provides insights on how the spread of LNYV-SII is more rapid than SI.

Keywords: Metabolomics, GCMS, LNYV, plant-virus interaction, derivatisation

3.1 Introduction

The economic effect of plant viruses in agriculture and associated yield losses are estimated to be more than US$30 billion per year (Rao and Reddy 2020; Maina and Jones 2023). With 8,600 tonnes produced yearly, lettuce (*Lactuca sativa* L.) is one of the top ten vegetables cultivated and consumed in New Zealand (HortNZ 2017). Crop catastrophes in New Zealand's lettuce industry, with losses as high as 50%, have been ascribed to viral infection (Fletcher et al. 2005). Specifically, losses due to necrosis symptoms on leaves have been strongly linked to the virus lettuce necrotic yellows virus (LNYV) (Fry et al. 1973).

LNYV was first reported in *Lactuca sativa* (lettuce) in 1954; *Cytorhabdovirus* is the type species of the genus *Cytorhabdovirus*, family *Rhabdoviridae*, and order *Mononegavirales* (Stubbs and Grogan 1963; Walker et al. 2022). LNYV is transmitted to plants via aphids; the blackcurrant-sowthistle aphid *Hyperomyzus lactucae* is the primary transmission vector (Stubbs and Grogan 1963; Fry et al. 1973; Dietzgen et al. 2007). LNYV appears to be endemic to Australia and New Zealand (NZ); the LNYV population is divided into two subgroups, SI and SII (Callaghan and Dietzgen 2005), with SI apparently extinct in Australia (Higgins et al. 2016). The origin of LNYV is still not fully understood. It is possible that LNYV could have transitioned between native and weed hosts in either Australia or New Zealand before infecting lettuce. Alternatively, there might have been at least two separate incursions of LNYV, with the first one possibly happening when European agriculture began in the region (Higgins et al. 2016). The disappearance of subgroup I in Australia could have been due to less efficient spreading compared to subgroup II, possibly due to less-than-ideal interactions with plant and/or insect hosts. The introduction of subgroup II to New Zealand seems to be a more recent occurrence, hence both subgroups are still present (Higgins et al. 2016; Prabowo et al. 2022).

A plant's physiological status is often directly reflected by metabolites that constitute the end products of a plant's response to environmental factors, such as biotic and abiotic stress. Understanding metabolic profiles under different conditions may support the development of new plant varieties with desirable characters (Fernie and Schauer 2009; Sharma et al. 2021; Oh et al.

2023; Haq et al. 2024). In plant metabolomics research, identification and quantification of metabolites involved in primary metabolic pathways, such as sugars, sugar alcohols, amino acids, organic acids, and polyamines, are regularly analysed (López-Gresa et al. 2012; Mandal et al. 2012; Sade et al. 2014; More et al. 2022; Chaowongdee et al. 2023). Analytical techniques such as gas chromatography-mass spectrometry (GCMS), nuclear magnetic resonance spectroscopy (NMR), liquid chromatography-mass spectrometry (LCMS) and capillary electrophoresis (CE) are commonly used for plant metabolomics research (Dunn and Ellis 2005; Choi et al. 2006; Lisec et al. 2006; Manickam et al. 2023; Serag et al. 2023). Several studies in recent years have demonstrated the utility of metabolomics in studying plant-pathogen interactions (Castro-Moretti et al. 2020; Maia et al. 2023; Kumari et al. 2024). Different metabolic profiles following plant-pathogen interaction is a sign of altered metabolic pathways. Finding metabolites with different abundances in control and infected samples may reveal the signalling and defence mechanisms underlying plant-pathogen interactions.

Understanding host responses to LNYV infection remains limited. To gain insights, a metabolite profile analysis was conducted on LNYV-SI and -SII infected *N. glutinosa* leaves to determine levels of various metabolites after the onset of viral infection compared to mock-inoculated plants. *N. glutinosa* was used as it is a model differential host and indicator species for LNYV used for biological indexing, maintenance, propagation, and LNYV purification (Higgins et al. 2016). Metabolomic profiling is thus a powerful tool for understanding how plant viruses like LNYV interact with their host plants by comparing infected and control plants. This analysis reveals key insights into biochemical pathways involved in the infection process, shedding light on how cytorhabdoviruses impact critical aspects of plant biology including defence responses, energy utilisation and nutrient dynamics. By examining the metabolic profiles associated with each subgroup, we hypothesise that LNYV-SI and SII induce distinct changes in host plant metabolite profile, with a potential advantage for LNYV-SII spread.

3.2 Methods

3.2.1 Plant growth, virus inoculation and confirmation of LNYV infection

***N. glutinosa* seeds provided by The Institute** for Plant and Food Research (Auckland) were grown for four weeks on soil supplemented with Scott's Osmocote after which seedlings were transferred to individual pots. Plant growth was carried out in a Conviron CMP5090 under 4 levels each of fluorescent and incandescent lighting to achieve 16/8 hr photoperiod at 20°C temperature with 80 % humidity. Plants were allowed to acclimatise for 2 to 3 days until they reached the 5 to 6 leaves stage. LNYV-SI and LNYV-SII were mechanically inoculated onto 30 plants each. A leaf from a previously infected plant was ground to a fine paste in the presence of 0.01M phosphate buffer (pH 7), 0.1% (w/v) sodium sulphite (Na_2SO_3) and a dash of carborundum (600 mesh). This mixture was rubbed onto three apical young leaves and excess carborundum removed by spraying with water after six hours. Five plants were mock inoculated with buffer only. Twenty-eight days post-inoculation, three systemic, symptomatic leaves from three virus infected plants were collected and stored at -80℃ until further processing. For mock-inoculated plants, the youngest three systemic leaves were collected. The RNA was extracted from leaves of mock-inoculated plants and LNYV inoculated plants showing visual symptoms of LNYV infection (Ajithkumar 2018). LNYV infection was confirmed using one step RT-PCR; mock and inoculated samples were tested using Superscript III RT-PCR System with Platinum Taq (Invitrogen) (Ajithkumar 2018). Leaf material of mock-inoculated, SI-infected, and SII-infected *N. glutinosa* was frozen in liquid nitrogen and stored at -80°C until further extraction. Three biological replicates of each group were used for the subsequent metabolomic analysis.

3.2.2 Extraction and sample preparation

Leaf material (10-20 mg) frozen in liquid nitrogen was weighed in a 2 ml silanised glass vial, powdered, and spiked with 20 µl of 10 mM d-4 alanine as an internal standard (Ravi et al. 2020). Each spiked sample was freeze-dried overnight and then resuspended in 500 µl cold (4°C) 50 % v/v methanol solution by vortexing for 30 sec. The samples were centrifuged at 1200 rcf (Hermle centrifuge Labortechnik GmbH, model Z-216 MK) for 5 min, and each supernatant was collected into another 2 ml silanised vial. The extraction step was repeated using 500 µl cold (4°C) 80 % v/v methanol solution, and the supernatant was pooled with the previous supernatant. The samples were freeze-dried and stored at -20°C until methylchloro formate (MCF) derivatization, which was performed according to Villas-Bôas et al. (Villas-Bôas et al. 2003). The solids, after the freeze-drying process, were redissolved in 200 µl of 1 M sodium hydroxide (NaOH) and mixed with 34 µl of pyridine and 167 µl of methanol. The alkylation reaction was initiated by adding 20 µl of MCF, vortexing for 30 s, followed by another 20 µl of MCF and vortexing again for 30 s. For the separation of the MCF derivatives, 400 µl of chloroform was added before shaking vigorously for 10 s, followed by addition of 400 µl of 50 mM sodium bicarbonate (NaHCO3) with further vortexing for another 10 s. The upper aqueous layer was removed, and a small amount of anhydrous sodium sulphate added to the chloroform phase to absorb residual moisture. The dried chloroform phase was then transferred into a GC glass micro-insert seated in a 2 ml GC vial and subjected to GC-MS analysis. Solutions of known standards were treated similarly; blank samples were also prepared containing all the components except the leaf material.

3.2.3 Gas Chromatography Mass Spectrometry

The MCF-derivatised samples were analysed using the Agilent 7890B GC equipped with a 5977B mass spectrometer detector (MSD) with an Electron Impact ionisation source. For the MCF-derivatised samples, the separation was achieved by the Agilent DB-1701 column (30m x 0.25mm x 0.25 µm). The inlet temperature was set at 290°C, and sample volumes of 1 µl were injected in split mode with a split ratio of 20:1. For the MCF-derivatised samples, the initial column temperature was held at 45 °C for 2 minutes, ramped at 9 °C/min to 180 °C and held for 5 minutes, then ramped at 40 °C/min to 220 °C and held for 5 minutes, then ramped at 40 °C/min to 240 °C and held for 11.5 minutes, then ramped 40 °C/min to 280 °C and held for 2 minutes. MSD transfer line was held at 250 °C, the ion source at 250 °C and the quad at 150 °C. The data was acquired in 29 minutes with 2 minutes solvent delay in the mass range of 38-650 Da.

3.2.4 Data processing and analysis

Preprocessing of the spectra obtained from the GC was performed using the Automated Mass Spectral Deconvolution and Identification System (AMDIS V2.66). The National Institute of Standards and Technology (NIST14) library and in-house MCF library were used to identify metabolites of MCF-derivatized samples, by comparing the mass-to-charge ratios and abundance of each compound detected against a standard mass chromatogram. Identification was carried out using an in-house MS library parallel to NIST, with a minimum matching percentage of 80 %, which is standard based on the metabolite's MS spectrum and retention time (Stein 1999). Records were evaluated for quality and manually checked for the presence of contaminants, and any anomalous records were removed. To account for potential technical variations (e.g., variable metabolite recoveries) and sample-specific biomass, metabolite peak intensities within each dataset were normalised against the relevant internal standard. Metaboanalyst 5.0 (https://www.metaboanalyst.ca/) was used to perform statistical analysis on metabolite data; data were log-transformed and mean-centred for multivariate analyses to reduce heteroscedasticity and provide a Gaussian distribution of metabolite abundances (Pang et al. 2021). Based on the underlying structure of the data, unsupervised principal component analysis (PCA) was used to identify the natural groupings of all leaf samples (Nguyen et al. 2019). PLS-DA (supervised partial least squares discriminant analysis) was used to distinguish all groups (mock inoculated, SI-infected, and SII-infected) and perform a classification and regression model (Nguyen et al. 2019). The key classifiers' variable importance in projection (VIP) scores were used to identify them. All metabolites with VIP score values greater than 0.7 were deemed important for distinguishing between infected and control groups. To provide an intuitive visualisation of expression patterns and between-sample variability, the top metabolites were subjected to a combined heatmap and hierarchical cluster analysis (Euclidian distance; Wards criterion). Pathway analysis methods -- Quantitative Enrichment Analysis (QEA) (Xia and Wishart 2010) and Network Topology Analysis (NTA) (Nikiforova and Willmitzer 2007) -were used to investigate functional relationships among annotated metabolites and highlight differential regulation within these at the pathway level.

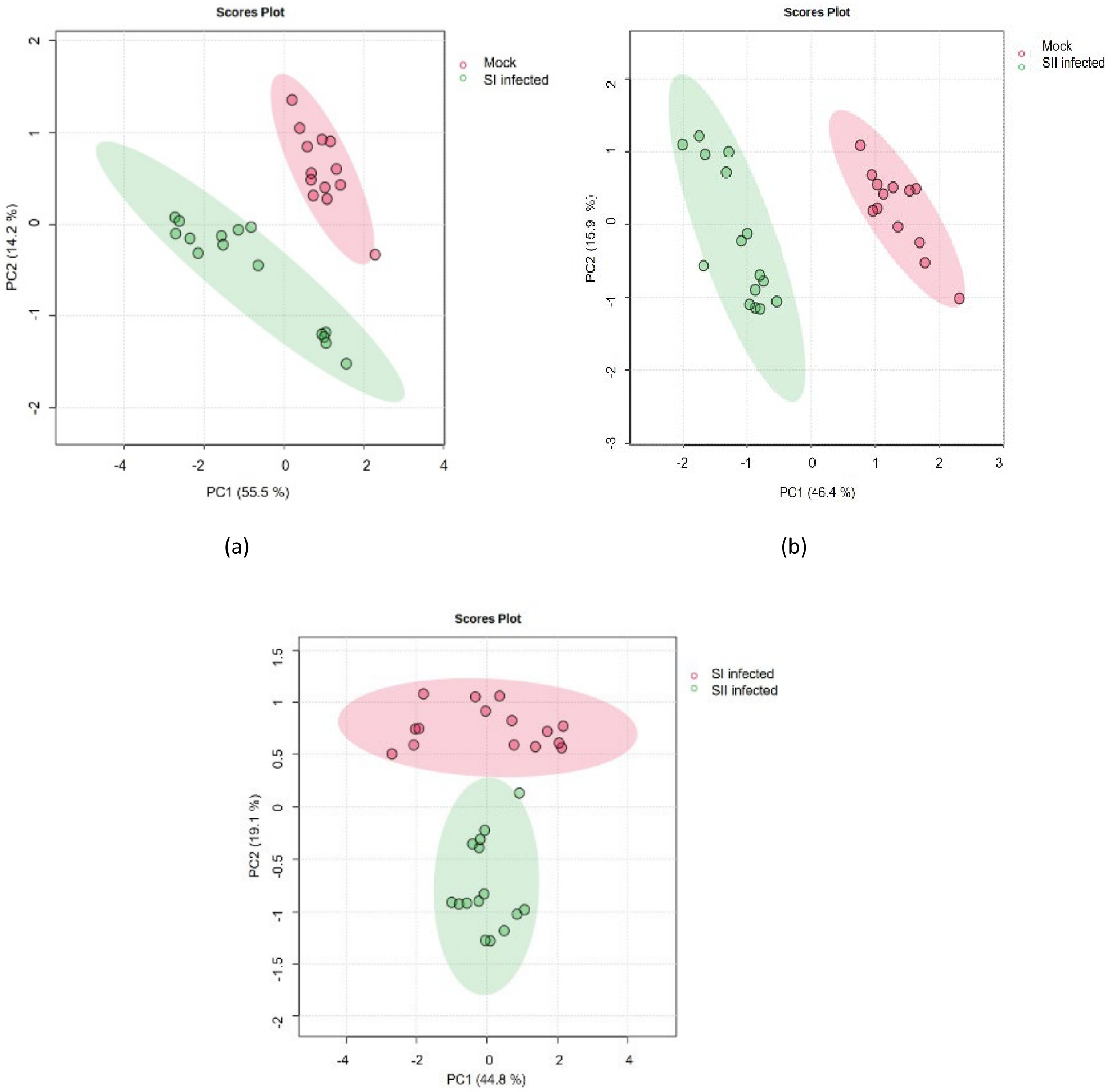
3.3 Results

3.3.1 Symptoms and confirmation of LNYV infection

The mock inoculated leaves of *N. glutinosa* did not show any alterations morphologically. LNYV-infected plants exhibited mild symptoms such as mosaic patterns and crinkling of leaves, which started to appear at 26 dpi; however, there was no marked difference between the symptoms of the two subgroups. Inoculating the plants with LNYV-SII led to a higher infection rate of 26% as confirmed by RT-PCR of systemic leaves, whereas the infection rate for LNYV-SI was 15% (n=30 plants for each subgroup). This supports LNYV-SII being more effective at infecting its host (Higgins et al 2016).

3.3.2 Multivariate analysis of the metabolite profile

Among the various multivariate analysis methods available, PCA was employed to reduce the dimensionality of the data and visualise the relationships among the mock inoculated and LNYV infected samples (Figure 13). The PCA plots showed distinct clustering of mock, SI-infected, and SII-infected samples, indicating differential metabolite compositions between these groups. Virus-infected plants demonstrated variance from mock-inoculated plants (Figure 13 a and b), and, importantly, SI-infected plants demonstrated variance from SII-infected plants (Figure 13 c). These data indicate that the host is impacted by LNYV at the metabolite level, and that the host responds differently to each subgroup of LNYV.



(a)

(b)

(c)

Figure 13 Multivariate analysis of metabolite profiles in mock-inoculated, SI-infected, and SII-infected leaf samples of *N. glutinosa* (a) M vs SI (b) M vs SII (c) SI vs SII

The relative abundance of metabolites in each group was visualised by generating a heatmap. A combined heat map and hierarchical cluster analysis (HCA) revealed distinct groupings based on sample class membership using the top metabolites ranked by their t-test and p-values. Each of the three heatmap columns represents the $\log_2$-fold change of relative peak intensity from one of the treatments: Mock, SI-infected, or SII-infected (Figure 14). The levels of the majority of amino acids were elevated in LNYV-SI-infected samples compared to mock-inoculated and LNYV-SII-infected samples. Among the 15 identified amino acids, threonine, lysine, asparagine, proline, alanine, glutamine, valine, leucine, aspartic acid, isoleucine, phenylalanine, and glycine exhibited higher levels in SI-infected plants in comparison to both mock and SII-infected plants. Conversely, SII-infected plants demonstrated reduced levels of glutamic acid and serine, while tyrosine was elevated. In contrast, the levels of the organic acids; succinic acid, citric acid, fumaric acid, quinic acid, malic acid, citramalic acid, hexanoic acid, octanoic acid, palmitic acid, and stearic acid, were increased in SII-infected samples compared to both mock and SI-infected samples.

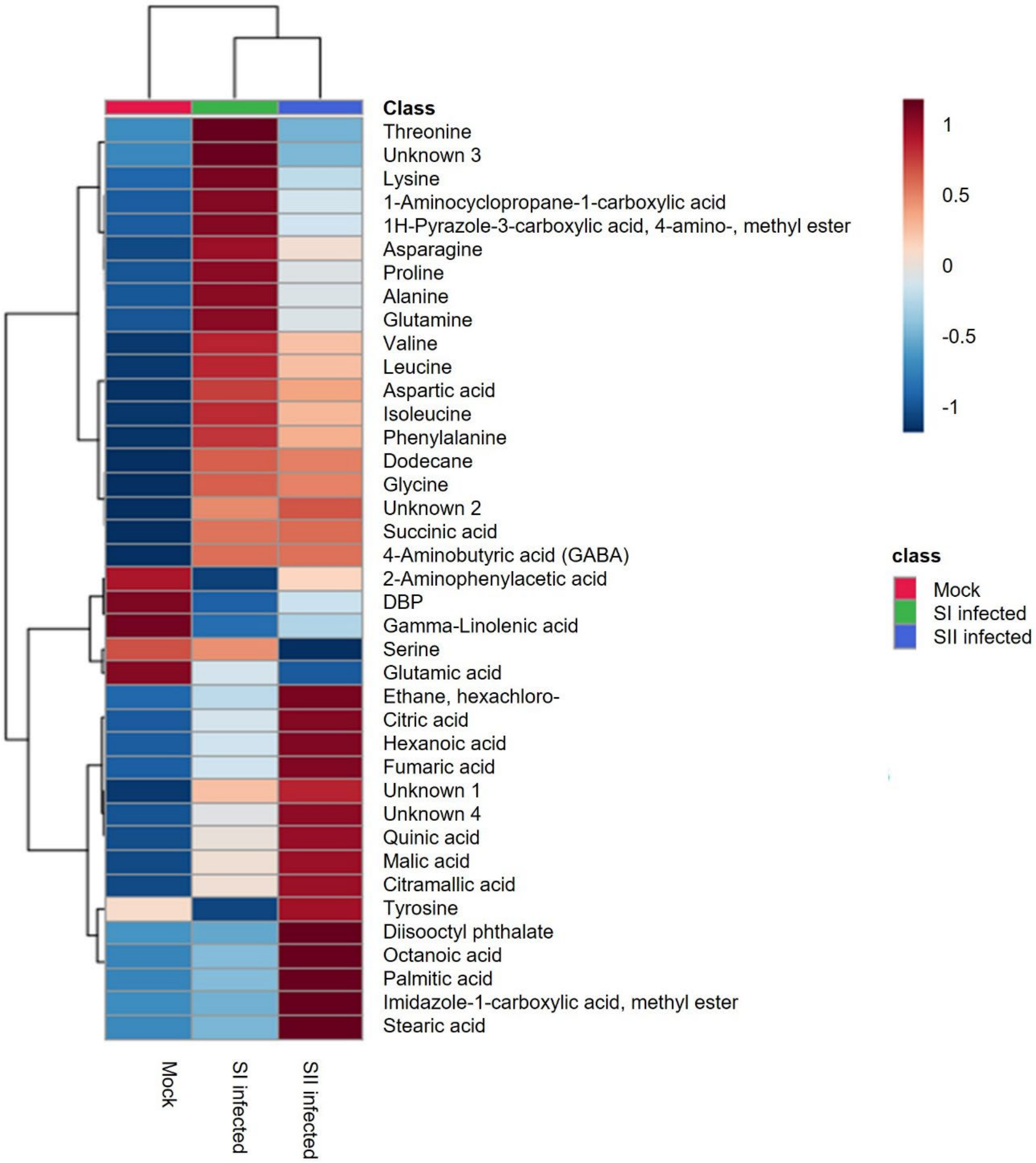
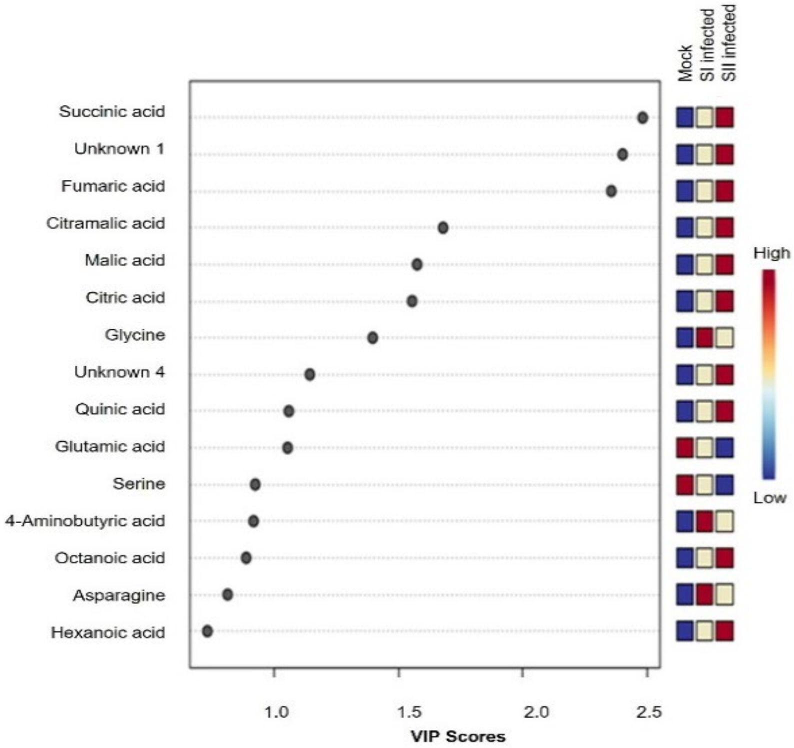


Figure 14 Effects of LNYV infection on *N. glutinosa* leaf metabolome via heatmap and hierarchical cluster analysis (Euclidean distance; Ward's criterion) of the top metabolites ranked by their t-test and p-values. Columns represent samples (red = mock inoculated; green = SI-infected and blue = SII-infected), and rows represent metabolites. The blue/red colour scale represents standardised (log-transformed and mean-centred) abundance data, where red are higher values and blue are lower values.

With VIP scores > 0.7, metabolites such as succinic acid, fumaric acid, citramalic acid, malic acid, citric acid, glycine, quinic acid, glutamic acid, serine, and asparagine were identified as strong sample discriminators between mock inoculated, SI, and SII-infected samples (Figure 15a). In contrast, metabolites that discriminated SI- infected from SII-infected samples are shown in

Figure 15b and include fumaric acid, citric acid, asparagine, citramalic acid, malic acid, serine, histidine, glycine, alanine, and threonine.

(a)



(b)

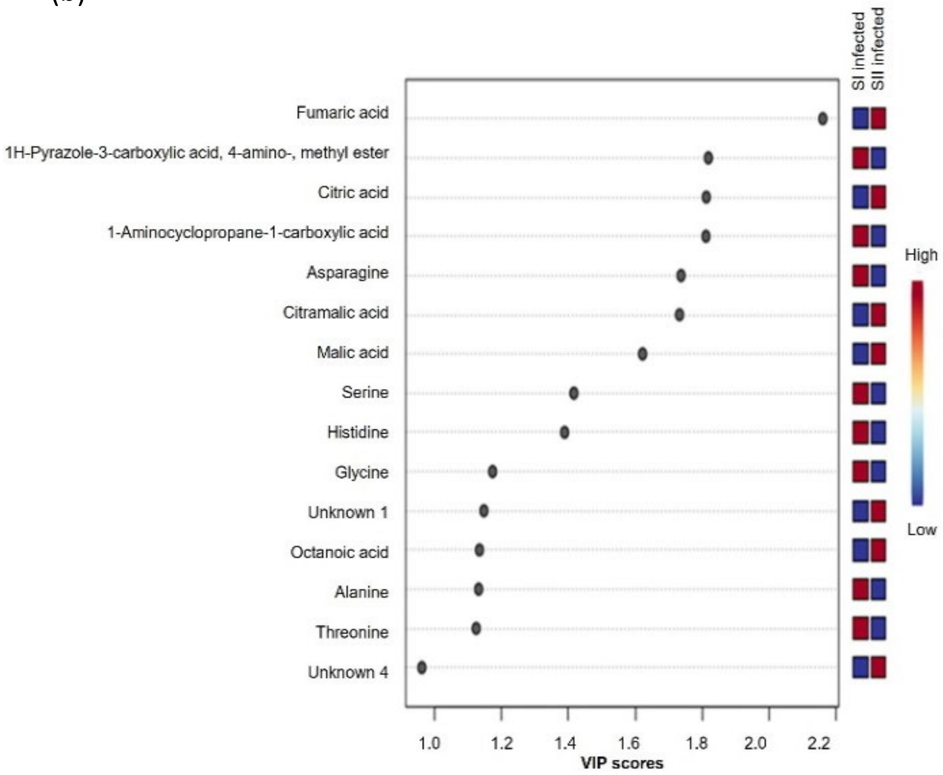
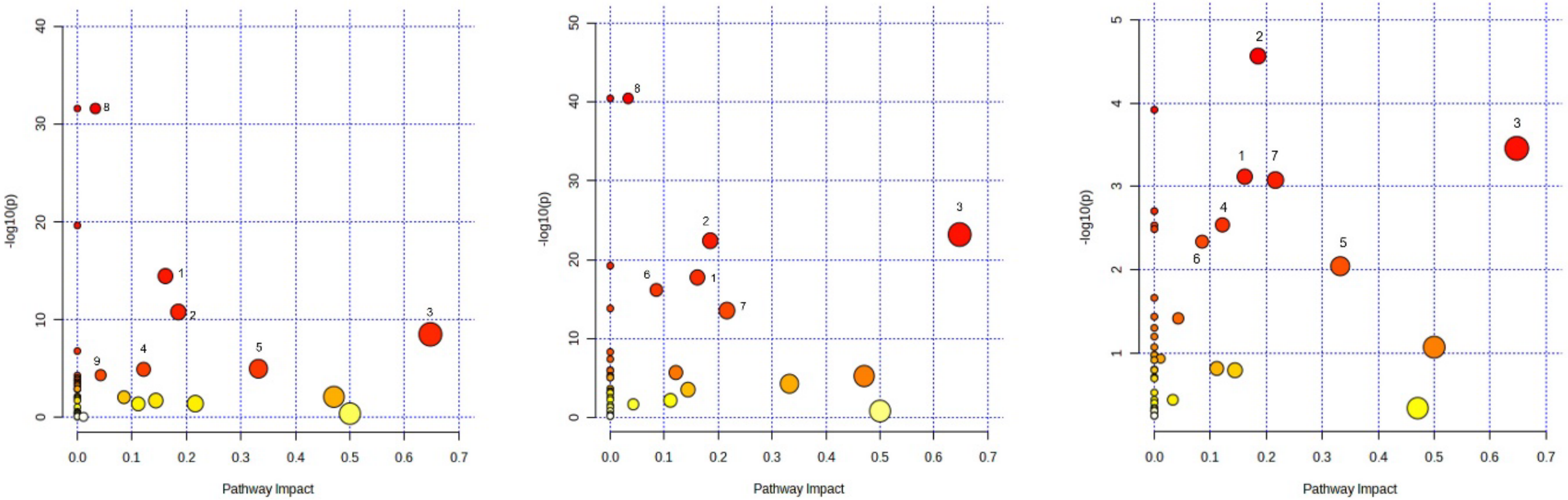


Figure 15 Variable importance in projection (VIP) scores generated from partial least squares discriminant analysis (PLS-DA). The coloured boxes on the right indicate the relative concentrations of the corresponding metabolites. Top contributors based on the VIP scores for (a) mock vs SI infected and SII infected (b) SI vs SII infected.

3.3.3 Metabolic pathways altered by LNYV infection

Topology-based pathway analysis was conducted to reveal the most relevant pathways associated with the LNYV infection (Figure 16 a, b, and c). The p-values and the pathway impact scores of comparisons between the mock vs SI, mock vs SII, and SI vs SII are shown in Table 5. The pathways that contained metabolites with simultaneous QEA p-values < 0.05 and NTA Pathway Impact (PI) values greater than 0.01 were considered as potential primary target pathways of interest relating to the treatment (LNYV infection, SI vs SII). Based on this, biochemical pathways with evidence of differential regulation upon LNYV infection, and upon infection by the different LNYV subgroups, were identified. The greatest impact by SI infection appeared to be on amino acid metabolic pathways, while SII infection appeared to impact pathways involving organic acids when compared to mock inoculated plants and when compared to each other.



(a) (b) (c)

Figure 16 Topology-based pathway analysis shows metabolic networks in *N. glutinosa* on LNYV infection. The most impacted metabolic pathways are designated by the volume and the colour of the circles (yellow = least relevant; red = most relevant; small circles = low pathway impact scores, large circles = high pathway impact scores) according to their statistical relevance and pathway impact (PI) values resulting from Quantitative Enrichment Analysis (QEA) and Network Topology Analysis (NTA), respectively. (a) Mock vs SI (b) Mock vs SII (c) SI vs SII.

Table 5: Significant metabolite pathways with $p < 0.05$ across the three different treatments (mock, SI infected, and SII infected). ✓ indicates metabolic pathways that were altered whereas x indicates pathways that were not altered across three different treatments.

Sr. no.	Pathway name	Total Compounds	Hits	Metabolite	p value	FDR	Pathway Impact Score	Mock vs SI	Mock vs SII	SI vs SII
1	Glyoxylate and dicarboxylate metabolism	29	5	Citrate, Glycine, L-Glutamate, L-Glutamine, Succinate	3.40E-15	3.66E-14	0.16159	ü	ü	ü
2	Citrate cycle (TCA cycle)	20	3	Succinate, Citrate, Fumarate	1.60E-11	1.38E-10	0.18531	ü	ü	ü
3	Alanine, aspartate, and glutamate metabolism	22	7	L-Aspartate; L-Asparagine, L-Alanine, L-Glutamate, L-Glutamine, Fumarate, Succinate	3.05E-09	2.19E-08	0.64748	ü	ü	ü
4	Glutathione metabolism	26	2	Glycine, L-Glutamate	1.16E-05	5.56E-05	0.1216	ü	x	ü
5	Glycine, serine, and threonine metabolism	33	3	Glycine, L-Aspartate, L-Threonine	1.00E-05	5.40E-05	0.33218	ü	x	ü
6	Arginine biosynthesis	18	4	L-Glutamate, L-Aspartate, Fumarate, L-Glutamine	6.83E-17	3.71E-16	0.08544	x	ü	ü
7	Tyrosine metabolism	16	2	L-Tyrosine, Fumarate	2.76E-14	1.17E-13	0.21622	x	ü	ü
8	Sulphur metabolism	15	1	Succinate	2.55E-32	5.49E-31	0.03315	ü	ü	x
9	Histidine metabolism	15	1	L-Histidine	4.60E-05	0.000196	0.04264	ü	x	x

3.4 Discussion

This study highlights the first identification and characterisation of metabolomic changes in *N. glutinosa* in response to LNYV. Further, the metabolomic profiling allows exploration of the proposition that the different dispersal rates of the LNYV subgroups is due to different interactions with the host (Higgins et al. 2016). GCMS profiling was carried out after the onset of LNVY symptoms (28dpi) and showed differential responses of LNYV-infected plants compared to mock inoculated plants. Further, plants also showed differential responses to infection by the LNYV subgroups SI and SII. This study can form the basis to further understanding the interaction between plants and cytorhabdoviruses.

Common responses between mock and infected samples (both SI and SII) indicate that several metabolites, including threonine, lysine, asparagine, proline, alanine, glutamine, valine, leucine, aspartic acid, isoleucine, phenylalanine, glycine, succinic acid, and GABA, are increased in infected samples compared to mock. Conversely, metabolites such as serine, glutamic acid, citric acid, hexanoic acid, quinic acid, tyrosine, palmitic acid, and stearic acid are decreased in infected samples relative to the mock. When comparing differential responses between SI and SII infections, the SII-infected samples show increased levels of serine, glutamic acid, citric acid, hexanoic acid, quinic acid, tyrosine, and imidazole-1-carboxylic acid, while levels of threonine, lysine, asparagine, proline, alanine, glutamine, valine, leucine, aspartic acid, isoleucine, phenylalanine, glycine, succinic acid, GABA, and several other metabolites are reduced in SII compared to SI. This trend suggests that SI and SII infections induce distinct metabolic adjustments, reflecting differences in their physiological impact on the host.

Previous studies focusing on *N. glutinosa* revealed significant alterations in amino acid levels subsequent to LNYV-SI infection, particularly post-symptom development (Randles and Coleman 1972). These authors found a reduction in the proportion of ribosomes occurring as polysomes in infected leaves at the onset of symptoms compared to healthy leaves, and that this continued to decline with a net loss of ribosomes. They suggested that symptom development was associated with a reduced rate of protein synthesis and/or an increase in protein breakdown. A decrease in the proportion of ribosomes present as polysomes, combined with an overall decrease in the total ribosome count, signifies a reduction in the cell's active protein synthesis. This reduction in protein synthesis can lead to the accumulation of amino acids within the cell. This accumulation occurs because the cell still undergoes its routine protein breakdown processes, but the slower rate of protein synthesis means that the amino acids aren't being utilized as quickly to construct new proteins.

Our study documents elevated levels of glutamine and aspartic acid in SI-infected plants, which aligns with the ninhydrin positive material in SI infected leaves described by Randles and Coleman (1972). Other amino acids such as glutamic acid, asparagine, alanine, proline, threonine, valine, methionine and 4-amino butyric acid were downregulated in this ninhydrin positive material, a trend not observed in our current study. Several factors could contribute to this disparity, including the isolate of virus being used for infection and subsequent time required for successful infection. The 1972 study examined the amino acid profile at 5 dpi whereas our study focused on the metabolomic profile at 28 dpi which was 2 days after symptom development as the strain was milder than the strain used by them. Compared to the ninhydrin approach, GC-MS offers extremely high sensitivity and can identify amino acids at much lower quantities. It is especially helpful for metabolomics research and trace-level analysis, which require the detection of low quantities of amino acids (Xu et al. 2020). GC-MS is renowned for its high sensitivity and specificity, allowing the detection of subtle metabolic changes that might have been overlooked with older, less advanced techniques. Its precise quantification and ability to identify metabolites based on spectral matching enable robust analysis, even at low concentrations. However, its metabolite coverage is limited to volatile or derivatizable compounds, which may exclude some classes of metabolites, necessitating complementary techniques for a more comprehensive metabolic profile.

Most of the metabolites identified following LNYV infection were classified as organic acids and amino acids. Such changes in primary metabolism after infection are thought to support viral replication and host defence mechanisms, which in turn influences the course of the infection (susceptibility or resistance) (Manasseh et al. 2023). Aromatic amino acids, including phenylalanine and tyrosine, where found to be elevated in SI and SII-infected plants, respectively. These amino acids play a pivotal role as precursors for the synthesis of numerous secondary metabolites, encompassing alkaloids, coumarins, flavonoids, glucosinolates, phenolic acids, and more (Tzin and Galili 2010). These secondary metabolites can be involved in plant defence responses against pathogens. Some amino acids or their metabolic byproduct accumulation results in resistance responses against pathogens that may or may not be dependent on SA- and ROS-mediated defence mechanisms (Rojas et al. 2014). This suggests a complex interplay of various metabolic pathways in plant defence responses against pathogens. In cases of viral infections, plants react by altering the synthesis of amino acids that are necessary for effective plant defence responses as well as for virus proliferation (Kogovsek et al. 2016). Several other studies in systems such as *Amaranthus hypochondriacus* L., *Arabidopsis thaliana*, *Solanum lycopersicum*, and Rossa di Tropea' onion infected with ageratum enation virus, tobacco rattle virus, tomato yellow leaf curl virus, and onion yellow dwarf virus, respectively, have documented the effects of viral stress on the levels of amino acids and TCA cycle intermediates (Srivastava et al. 2012; Fernández-Calvino et al. 2014; Sade et al. 2014; Taglienti et al. 2020). However, there is no literature available demonstrating the changes in metabolite profile in plants infected by cytorhabdoviruses.

Decreased levels of amino acids might indicate their breakdown to precursors or intermediates of the TCA cycle and their subsequent utilisation to meet the host's energy demands (Hildebrandt et al. 2015). Despite the increase in tyrosine levels, most of the amino acids were downregulated and TCA cycle intermediates were upregulated in SII-infected plants when compared to mock inoculated and SI-infected plants. The TCA cycle is widely recognised not only for its role in oxidising respiratory substrates to produce ATP but also for providing carbon structures to support anabolic processes (Araujo et al. 2012). Additionally, it plays an important role in the interaction between carbon and nitrogen as well as contributing to responses to biotic stress (Zhang et al. 2018). The substantial need to acquire carbon in response to infection will likely involve shuttling amino acids into energy generating pathways such as the TCA cycle (Bolton 2009). Carbon is necessary for plants to produce energy, maintenance of cellular structures, and synthesis of defence compounds (Caretto et al. 2015). Since energy production is lowered, the respiration rate increases to provide resources for defence responses and/or pathogen multiplication (Kogovsek et al. 2016). In the current study the levels of organic acids such as fumaric acid, succinic acid, malic acid, and citric acid were elevated in SII-infected plants compared to SI-infected & mock inoculated plants. Elevated activity of anaplerotic enzymes is thought to be essential for restoring the intermediate compounds in the tricarboxylic acid (TCA) cycle, thereby furnishing the essential components required for the synthesis of viral proteins and the generation of energy (Jiang and Zhou 2023). Organic acids such as malic, citric, succinic, and fumaric were found to be altered in a previous report on the metabolic profile of tomato in response to infection by tomato mosaic virus (López-Gresa et al. 2012). The TCA cycle intermediates were also seen to accumulate in *A. hypochondriacus* L. infected with ageratum enation virus (AEV) and the upper leaves of tobacco infected with tobacco mosaic virus (TMV) (Bazzini et al. 2011; Srivastava et al. 2012). In contrast, no significant differences in the accumulation of TCA cycle intermediates were observed on infection by tobacco rattle virus (Fernández-Calvino et al. 2014). This apparent disparity could be attributed to the TCA cycle's rapid flux of metabolic intermediates and reactions. This revving up of TCA cycle intermediates and activation of the TCA cycle pathway in SII-infected plants suggest that these host plants may have limited host resistance and thus require more energy to combat viral infection. The host resistance to LNYV-SII appeared to be more limited than to SI; the elevated TCA cycle pathway may be an indicator of this. Poor resistance permits greater viral loads, which promote faster viral proliferation and more effective vector transmission within plant populations Thus further testing of viral loads could help us investigate the spread and replication of LNYV.

The observation that SI-infected plants produce increased amounts of amino acids whereas, SII-infected plants exhibit enhanced synthesis of intermediates in the tricarboxylic acid (TCA) cycle shows the variety of responses that plants use to combat viral threats. In particular, the increased production of amino acids may be a source for defence-related mechanisms, such as improved defence protein synthesis or secondary metabolites that support pathogen resistance (Hildebrandt et al. 2015; Kumar et al. 2023). The rise of TCA cycle intermediates, on the other hand, points to a potential shift towards greater energy generation, possibly in response to the increased energy requirements that may facilitate viral replication and spread within the host plant (Nagy and Lin 2020). The connection between disease resistance and metabolic reprogramming highlights the delicate balance that plants must preserve to allocate resources effectively in the face of viral infections. Insights into the tactics used by plants to fend off viral assaults and improve their overall resilience can be gained from further study of the molecular mechanisms behind these metabolic alterations.

While this study detected only a specific group of metabolites, exploring the alteration in the metabolite set before and after the onset of LNYV infection and symptom development could provide intriguing insights for future investigations. A more comprehensive metabolite profiling would facilitate a complete characterization of the events occurring after plant infection by either of the subgroups providing clear evidence of host responses would be more likely to allow infection by LNYV-SII. A targeted approach can be used in future studies to quantify the metabolites detected in the current study. Amino acids and organic acids could be thus used as potential biomarkers for LNYV infection. To further explore the differences between SI and SII infections, specific experiments such as quantifying viral loads across infection stages using qRT-PCR or high-throughput sequencing could provide a deeper understanding of infection dynamics. These experiments would help confirm whether the observed metabolic changes correlate with viral titre variations. Additionally, investigating metabolic reprogramming during earlier infection stages, using techniques like GC-MS or LC-MS, could reveal initial host responses and potential tipping points in infection progression.

Chapter 4 General Discussion

4.1 Insights from the study

The overall objective of this thesis was to assess the LNYV-responsive biochemical and molecular mechanisms that play an important role during systemic infection of *Nicotiana glutinosa* 28 days post inoculation. This work focussed on the important metabolic pathways such as those involved in defence mechanism, amino acid metabolism, TCA cycle, and plant hormonal signal transduction affected by LNYV subgroup infection. This chapter discusses the main findings of this thesis and presents future recommendations.

When initiating an infection, viruses are anticipated to exert an influence on numerous pathways, thereby altering the gene expression within the host plant (Perez-Clemente et al. 2013; Zeier 2013; Suzuki et al. 2014; Zhang et al. 2023). Common responses to viral infection have been observed across various plant hosts (Senthil et al. 2005; Whitham et al. 2006; Berger et al. 2007; Anzano et al. 2021), categorised into cellular responses and developmental responses. Transcriptomic studies from microarray assays have previously been conducted on *Solanaceae* plant hosts to determine genes that are significantly up or down regulated in response to viral infection (Chen et al. 2013). Although extensive data were acquired in these investigations regarding the varying expression of genes in distinct host plants, it is essential to carry out specific plant host-virus studies to validate whether similar patterns emerge in the context of LNYV infection in *N. glutinosa.*

Chapter 2 presents evidence supporting a salicylic acid- induced response to LNYV infection of *N glutinosa*. Of particular interest, host responses to LNYV-SII appeared to be weaker than to SI. Higgins et al (2016) hypothesised that LNYV-SII is able to replicate more effectively in either the insect or plant host. This study suggests that this subgroup may be able to do this via ensuring the plant host does not respond too vigorously to infection. This study presents evidence of specific transcript accumulation at 28 dpi - a time course study would be important to determine when these changes take place following inoculation. This study has also validated reference genes for RT-qPCR analysis of LNYV infected *N. glutinosa*. This study is an important advance in our understanding of the infection process of plant hosts by cytorhabdoviruses. Current work is underway to assess the holistic response by *N. glutinosa* to infection with each LNYV subgroup using transcriptomics approaches.

Chapter 3 shows that SI-infected plants produce increased amounts of amino acids whereas SII-infected plants exhibit enhanced synthesis of intermediates in the tricarboxylic acid (TCA) cycle. This shows the variety of responses that plants use to combat viral threats, and that these responses can vary depending on the specific viral strain. In particular, the increased production of amino acids may be a source for defence-related mechanisms, such as improved defence protein synthesis or secondary metabolites that support pathogen resistance (Hildebrandt et al. 2015; Kumar et al. 2023). The rise of TCA cycle intermediates, on the other hand, points to a potential shift towards greater energy generation, possibly in response to the increased energy requirements that may facilitate viral replication and spread within the host plant (Nagy and Lin 2020). The host resistance to LNYV-SII appeared to be more limited than to SI. Poor resistance permits greater viral loads, which promote faster viral proliferation and more effective vector transmission within plant populations; thus, promoting efficient dispersal. The connection between disease resistance and metabolic reprogramming highlights the delicate balance that plants must preserve to allocate resources effectively in the face of viral infections. Insights into the tactics used by plants to fend off viral assaults and improve their overall resilience can be gained from further study of the molecular mechanisms behind these metabolic alterations.

In plants, defence responses to pathogen invasion are coordinated by a complex interplay of signalling channels and metabolic activities. The accumulation of NPR3, a key player in the regulation of the salicylic acid pathway, is an essential part of this defence mechanism (Raskin 1992; Zhang et al. 2010; Liu et al. 2020). The metabolic shifts taking place during an immune response are further highlighted by the role of amino acids, especially phenylalanine. PAL enzyme, which is a plant defence enzyme catalysing biosynthesis of phenylalanine, facilitates the transition between primary and secondary metabolism (Pappi et al. 2021). Isochorismate synthase

(ICS) and phenylalanine ammonia-lyase (PAL) genes are essential for synthesising SA and signal transduction processes for activating the hypersensitive response and generating system-acquired resistance (SAR) (Métraux et al. 1990; Ding et al. 2018; Zhang and Li 2019). Thus, the elevated levels of *NPR3* in LNYV infected *N. glutinosa* (Chapter 2 findings) ultimately leads to elevated levels of amino acids, especially phenylalanine (Chapter 3 findings).

The induction of SA pathway and the roles of *NPR3*, *AOX1a* and *RDR1* highlight metabolic reprogramming that plants undergo during LNYV infection. Enhanced amino acid metabolism during LNYV infection supports accumulation of aa that could be further used for synthesis of defence related metabolites. The intermediates of the TCA cycle that are used up in anaplerotic reactions restore the supply of precursors needed for the production of secondary metabolites and amino acids. Hence, amino acids and TCA cycle intermediates could be potential biomarkers for SI & SII infection, respectively.

4.2 Future recommendations

It is important to note that the gene expression changes observed in this study are specific to the model plant N. glutinosa and may not be applicable to L. sativa. However, replicating this study using L. sativa, a significant commercial host for the virus, is challenging due to the difficulty of mechanical infection. Additional genes could be identified to help develop a more comprehensive understanding of the gene regulatory networks that LNYV affects during infection, and how these networks differ between subgroups. By uncovering this information, we may gain insights into how genetic differences between subgroups reveal and provide a deeper understanding of the mechanisms of cytorhabdoviruses and, more broadly, rhabdoviruses.

Studying the response of LNYV infection in the environment rather than controlled conditions would help in better understanding disease development in natural conditions. With the projected global climate changes, plants will need to endure heat waves, droughts, and pathogens simultaneously or consecutively. Heat and drought trigger opposing physiological responses, while pathogens might benefit from climate changes depending on their nature. Numerous studies, such as those by Zhang and Sonnewald (2017), have aimed to identify stress-specific markers or universal commonalities among different stresses. Traditionally, this has been done by comparing various single stress treatments. However, this method has proven challenging, as most studies comparing individual and combined stress conditions have found that each stress type results in distinct transcriptional changes. Although these changes at the individual gene level are highly variable and stress-specific, central metabolic and signaling responses tend to be common, often resulting in overall reduced plant growth. The challenge lies in understanding how specific transcriptional changes are linked to stress adaptations and in identifying key regulatory hubs that control these interactions.

Plant health and resistance to viral infections can be significantly improved by studying the rapidly expanding topic of epigenetics in plant-virus interactions. Techniques such as bisulfite sequencing, chromatin immunoprecipitation, CRISPR/dCas9 epigenetic editing, epigenome-wide association studies (EWAS), etc can be used to enhance knowledge of the regulation of plant host methylation in response to viral infection. Additionally, transcriptomics, metabolomics (LCMS) and proteomics can be used to identify secondary metabolites and host proteins in plants affected by LNYV infection. Application of enzyme [defence related enzymes such as peroxidases, polyphenol oxidases, superoxide dismutase (SOD), catalase (CAT)] and hormonal (measurement of SA and JA) based assays can help in elucidating defence mechanisms against LNYV, identifying biomarkers for resistance or LNYV infection.

There is no recent survey available on LNYV infection it would be interesting to do a current survey of LNYV infection both in Australia and New Zealand. A new survey would provide the latest information on the prevalence and distribution of LNYV infections, which is crucial for current disease management strategies. This would enhance our understanding of the virus’s epidemiology, including its spread and evolution, which is necessary for long-term control and prevention measures of LNYV. It would also help us gain insights into how LNYV affects crops in different climates and ecosystems in Australia and New Zealand that could reveal important regional variation in the virus’s impact and spread.

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Appendix

Table 6 Average Ct, ΔCt and ΔΔCt values between reference genes and genes of interest under different experimental conditions

	Average Ct			*EF1a* as reference gene						*SAND* as reference gene					
	Mock	SI	SII	Mock ΔCt	SI ΔCt	SII ΔCt	SI/Mock ΔΔCt	SII/Mock ΔΔCt	SI/SII ΔΔCt	Mock ΔCt	SI ΔCt	SII ΔCt	SI/Mock ΔΔCt	SII/Mock ΔΔCt	SI/SII ΔΔCt
AOX1a	22.04	21.13	22.23	-1.96	-3.64	-2.57	-1.67	-0.60	-1.075	-4.93	-6.85	-6.77	-1.91	-1.83	-0.08
NPR3	23.87	23.42	24.02	-0.14	-1.36	-0.78	-1.21	-0.64	-0.57	-3.11	-4.56	-4.98	-1.45	-1.87	0.41
RDR1	24.88	23.98	24.86	0.86	-0.80	0.05	-1.67	-0.80	-0.86	-2.09	-4.00	-4.13	-1.90	-2.03	0.12
RDR6	24.64	25.09	26.20	0.62	0.30	1.39	-0.32	0.76	-1.08	-2.34	-2.89	-2.80	-0.55	-0.46	-0.09
CPK3	26.38	27.17	27.77	2.36	2.38	2.96	0.02	0.60	-0.57	-0.60	-0.81	-1.23	-0.21	-0.62	0.41
EF1a	24.01	24.78	24.80												
SAND	26.98	27.99	29.00												

Table 7 Averaged ΔΔCt values of genes of interest under different experimental conditions against *EF1α* and *SAND* reference genes in mock and LNYV subgroup I and II infected *N. glutinosa*.

	Average SI/Mock ΔΔCt	**Average SII/Mock** ΔΔCt	**Average SI/SII** ΔΔCt
AOX1a	-1.79	-1.21	-0.57
NPR3	-1.33	-1.25	-0.07
RDR1	-1.79	-1.42	-0.36
RDR6	-0.43	0.15	-0.59
CPK3	-0.09	-0.01	-0.08

Table 8 $2^{-\Delta\Delta Ct}$ values for each of the three genes of interest against *EF1α* and *SAND* reference genes in mock and LNYV subgroup I and II infected *N. glutinosa*

$2^{-\Delta\Delta Ct}$	SI vs Mock	SII vs Mock	SI vs SII
AOX1a	3.47	2.32	1.49
NPR3	2.52	2.38	1.05
RDR1	3.46	2.68	1.28
RDR6	1.35	0.89	1.50
CPK3	1.06	1.00	1.05