UNDERSTANDING RESISTANCE AND TRANSCRIPTIONAL RESPONSES TO POTATO VIRUS Y INFECTION IN POTATO PLANTS

by

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ABSTRACT

The potato is one of the world's most important crops. Cultivation of potatoes occurs on every continent except Antarctica and in a wide variety of climates. Potatoes are susceptible to a multitude of pathogens that can decrease yield and market quality. Viruses are particularly problematic for potato growers, as most potato production involves the replanting of tubers grown the previous year. Because virus-infected potato plants can harbor virus in their tubers, these tubers can in turn be the source of infection in the next generation of plants. Strains of Potato virus Y are the most economically burdensome viruses for potato growers worldwide. In field settings, Potato virus Y is primarily transmitted to plant by aphids feeding on leaves, but PVY can also be transmitted mechanically through infected plant sap. The use of insecticides and the application of mineral oil to leaves can help limit aphid populations and prevent infection to an extent but are generally both less effective and more environmentally impactful than genetic antiviral resistance mechanisms. The incorporation of genes that provide durable resistance to Potato virus Y into commercial potatoes is a major focus of potato breeders. One form of resistance, called extreme resistance, is characterized by a lack of symptoms and little to no virus replication occurring at the site of infection, but the molecular mechanisms of this response are not well understood. A comprehensive analysis of the extreme resistance literature indicates that movement of the resistance protein from the cytoplasm to the nucleus of the cell directly after virus infection may be a key aspect of this immune response. The downstream, transcriptional aspects of the extreme resistance response are also not well understood. We analyzed the gene expression from a Potato virus Y-resistant potato variety, Payette Russet, and a commonly grown susceptible variety, Russet Burbank, at a series of time points after virus infection using RNA sequencing. Results of these analyses indicate that an immune response likely occurs in Payette Russet quickly after virus inoculation. These analyses also indicate that the virus-susceptible variety, Russet Burbank, exhibits changes in gene expression that are similar to other susceptible potato varieties during asymptomatic or tolerant infection. Furthering our understanding of the molecular mechanisms controlling resistance and severity of virus infections will help inform future breeding and genetic engineering efforts, which require detailed knowledge of the mechanisms of virus resistance.

CHAPTER ONE

OVERVIEW OF POTATO VIRUS Y AND POTATO PLANT ANTIVIRAL IMMUNITY

Potato Virus Y Infection Negatively Impacts Potato Production

The potato is a key component of global food security, with nearly 800 tons produced in 2017 [1]. Potatoes are susceptible to many pathogens that negatively impact both yield and tuber quality. Production of potatoes relies on vegetative growth from planted tubers and not the planting of true seeds, unlike many other major crops [2]. Tubers that are planted for the next year's crop, commonly referred to as "seed potatoes" can contain and seed the spread of pathogens to neighboring plants. One pathogen that is particularly economically problematic for seed potato production is Potato virus Y (PVY) [3]. PVY is a positive sense, single-stranded (+ss) RNA virus within the family *Potyviridae* that infects a variety of *Solanaceous* plants, including potato, tomato, eggplant, petunia, and pepper [4]. Potato virus Y (PVY) is a major problem for potato growers, particularly for seed potato growers. Seed potatoes that are grown to be sold to commercial growers are subject to strict pathogen abundance standards and routine testing for pathogens, with a particular emphasis on detecting and limiting PVY infections [2].

Like many other RNA viruses, PVY evolves quickly, as the virally encoded RNA-dependent RNA polymerase is relatively low fidelity and often jumps between different template RNA strands during replication, resulting in chimeric genomes composed of fragments from different strains [5]. The relative ease with which PVY strains and recombine to create new strains makes controlling PVY challenging, as the virus can evolve past host defenses quickly [3,6–8]. Recombinant PVY strains that include portions of PVY^N strain genomes and PVY^O

strain genomes (i.e., PVY^{NTN}, $PVY^{N:O}$, and $PVY^{N\text{-}Wilga}$) are currently the most abundant and most problematic for potato growers in the North America and Europe [4]. Many of the recombinant strains have evolved past resistance genes that were previously deployed to provide protection against PVY^{N} and PVY^{O} [9–12]. Potato plants that are infected with PVY can display a wide range of symptoms and the severity of the infection depends on the combination of the strain of PVY, the cultivar, or type, of potato plant that is infected, and the environmental conditions within which the infection is taking place [13].

Potato Antiviral Defense

Plants rely on nucleotide-binding, leucine-rich repeat proteins (NLRs) to recognize intracellular pathogens [14–16]. The NLR proteins act as sentinels within the cell, monitoring the cytoplasm for proteins that have been produced by pathogens. Plant NLRs are composed of three primary domains: an N-terminal coiled-coil domain; a central nucleotide-binding domain; and a C-terminal leucine-rich repeat domain [17]. Animal and plant NLR share structural and functional characteristics but are the result of convergent evolution [18]. Within plant genomes, NLRs are often among the most abundant gene species, as in many cases a single NLR can recognize a single plant pathogen [19].

After recognition and binding of a pathogenic protein, the NLR activates immune responses through mechanisms not well understood. Often, plant immune responses to viruses (and other pathogens) results in cell death local to the infected tissue, a reaction commonly referred to as the "hypersensitive response" (HR) [20]. Recent studies indicate that HR occurs after an influx of calcium ions into the cell and that this influx may be controlled by NLR oligomers forming pore-like structures in the cellular plasma membrane [21,22]. This localized

cell death act to stop the infection from progressing further, as viruses need intact and functioning host cells to replicate and spread [23]. Though HR is often treated as the tell-tale sign that an immune response to viruses has occurred, it is not the only type of plant immune response. Another type of resistance, termed 'extreme resistance,' does not result in cell death and allows for only minimal to no virus replication and spread [24–28]. The molecular mechanisms governing extreme resistance and how it relates to HR are not well understood [29–32]. Recently, Ry_{sto}, an NLR protein from the wild potato species, *Solanum stoloniferum*, has received considerable attention from potato breeders because of the quality of resistance to PVY that it confers [33–35]. Ry_{sto}-conferred extreme resistance provides symptomless resistance against all tested strains of PVY and Potato virus A, making it a considerable resource for potato growers worldwide. Beyond identifying the gene that codes for Ry_{sto}, we do not have a detailed understanding of the molecular mechanisms governing extreme resistance as conferred by Ry_{sto} or many of the other plant genes that confer extreme resistance.

Studies in potato indicate that extreme resistance may rely on NLR movement from the cytoplasm to the nucleus after pathogen recognition, but this hypothesis has only been tested for one gene, *Rx1*, that controls extreme resistance to Potato virus X [24,36–43]. The soybean NLR proteins, *Rsv1* and *Rsv3*, provide extreme resistance to various strains of Soybean mosaic virus [26,28,44–46]. Research on these proteins indicate that these NLR proteins rely on NLR-chaperone proteins (*Heat shock protein 90*, *SGT1*, *RAR1*) like NLRs that provide resistance through HR, as well as *Rx1* and Ry_{sto} [45,47,48]. Many of the NLR proteins that confer extreme resistance can also initiate immune responses that result in HR, indicating that the two seemingly disparate types of resistance may be intertwined [24]. Plant immune responses to viruses often

stimulate various chemical and biochemical pathways including reactive oxygen species production, systemic salicylic acid signaling, and phosphorylation cascades [49–51].

Beyond resistance by NLRs, plants rely heavily on the RNA interference (RNAi) pathway for both antiviral defense and host gene regulation [52–55]. RNAi relies on 20-24 nucleotide small RNAs, which once loaded into Argonaute proteins as a part of the RNAi induced silencing complex (RISC), can survey the cell for cognate RNA transcripts for slicing or translational repression [56–59]. Production of small RNAs relies on Dicer-like proteins, which recognize and cleave double-stranded RNAs (dsRNA) into small RNAs [60]. Plants encode non-coding RNAs that when expressed can form a “hairpin loop” secondary RNA structure [52]. These dsRNAs are recognized by Dicer-like and cleaved into microRNAs, which are a major component of plant transcriptional regulation. Similarly, RNA viruses, like PVY, must form a dsRNA intermediate during replication, which can be recognized by Dicer-like. The resulting virus derived small RNAs can then be used by RISC to target viral genomes and to limit infection [57].

Investigating Potato Host-Virus Interactions Using RNA Sequencing

Previous research studying the transcriptional responses to various strains of PVY have focused on resistance as conferred by HR and immune responses that occur very early during the infection process (i.e., less than one-week post-inoculation) [61,62]. To gain a better understanding of both the extreme resistance response and transcriptional responses to PVY in susceptible varieties as the infection progresses, we collected and sequenced RNA from the PVY-extreme resistant potato variety Payette Russet and the PVY-susceptible variety Russet Burbank from time points directly after inoculation (24 hours post inoculation) to systemic

infection (4 weeks post inoculation). Transcriptional and differential gene expression analyses indicate that transcriptional responses associated with the extreme resistance response occur very quickly after contact with PVY. Analyses of later time points post PVY infection, including systemic infection of virus, during the infection process in Russet Burbank indicate distinct responses occurring at 1 week and 4 weeks post-inoculation (wpi). Analyses of alternative splicing indicate that splice variants (or isoforms) of numerous genes, including poly(A) polymerase and a basic-leucine zipper (BZIP) transcription factor family protein, were more abundant in PVY-infected plants and may impact PYV infections. Small RNA sequencing was also performed in PVY-susceptible Russet Burbank at 1 wpi and 4 wpi to better understand the transcriptional landscape of small RNAs during PVY infection. Analyses of the small RNA responses revealed changes that are similar to those observed in other cultivars that undergo tolerant, largely asymptomatic PVY infections. Together these results further our understanding of both resistance and susceptibility to PVY and the genes identified may serve as the source of future studies into potato host-PVY interactions.

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CHAPTER TWO

EXTREME RESISTANCE TO VIRUSES IN POTATO AND SOYBEAN

Contributions of Authors and Co-authors

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Extreme Resistance to Viruses in Potato and Soybean

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Abstract

Plant pathogens, including viruses, negatively impact global crop production. Plants have evolved complex immune responses to pathogens. These responses are often controlled by nucleotide-binding leucine-rich repeat proteins (NLRs), which recognize intracellular, pathogen-derived proteins. Genetic resistance to plant viruses is often phenotypically characterized by programmed cell death at or near the infection site, a reaction termed the hypersensitive response. Although visualization of the hypersensitive response is often used as a hallmark of resistance, the molecular mechanisms leading to the hypersensitive response and associated cell death vary. Plants with extreme resistance to viruses rarely exhibit symptoms and have little to no detectable virus replication or spread beyond the infection site. Both extreme resistance and the hypersensitive response can be activated by the same NLR genes. In many cases, genes that normally provide an extreme resistance phenotype can be stimulated to cause a hypersensitive

response by experimentally increasing cellular levels of pathogen-derived elicitor protein(s). The molecular mechanisms of extreme resistance and its relationship to the hypersensitive response are largely uncharacterized. Studies on potato and soybean cultivars that are resistant to strains of Potato virus Y, Potato virus X, and Soybean mosaic virus indicate that abscisic acid-mediated signaling and NLR nuclear translocation are important for the extreme resistance response. Recent research also indicates that some of the same proteins are involved in both extreme resistance and the hypersensitive response. Herein, we review and synthesize published studies on extreme resistance in potato and soybean, and describe studies in additional species, including model plant species, to highlight future research avenues that may bridge the gaps in our knowledge of plant antiviral defense mechanisms.

Plant antiviral defense

The majority of plants are sessile, and thus there are strong selective pressures on the accurate, rapid sensing and response to pathogen or parasite infection (Jones and Dangl, 2006). Understanding these immune processes are of paramount importance to humans, as plants are the foundation of both the Earth's terrestrial ecosystems and the world economy. Of particular concern to humans are pathogens of crop species. Fungi, bacteria, and viruses are major threats and cause substantial crop losses. Controlling viral infection and spread in agricultural settings is challenging due to lack of chemical controls, rapid evolution of viruses, and transmission by insect vectors (Tian and Valkonen, 2013; Tromas et al., 2014). Genetic resistance (i.e., naturally evolved, breeder selected, and engineered) is a sustainable form of virus mitigation.

In plants, virus resistance is often controlled by dominantly inherited genes that encode nucleotide-binding leucine rich repeat proteins (NLRs) (Kourelis and Hoorn, 2018). Most plant NLR proteins are composed of three primary domains: an N-terminal coiled-coil, Toll/Interleukin-1 receptor, or divergent coiled-coil domain; a central nucleotide-binding domain; and a C-terminal leucine-rich repeat domain (Tamborski and Krasileva, 2020). These proteins function as intracellular immune receptors and their ability to elicit an immune response is controlled in part by nucleotide binding state. Specifically, inactive NLR proteins are bound to ADP, whereas recognition and binding of a pathogen effector protein allows for an NLR to transition to an active, ATP-bound state that is able to initiate an immune response (Takken et al., 2006; Wang et al., 2019a, 2019b).

Nod-like receptors in animals share structural and functional similarities with plant NLRs but are a result of convergent evolution (Urbach and Ausubel, 2017). Animal Nod-like receptor proteins oligomerize in the cytoplasm after pathogen detection, triggering the formation of plasma membrane pores, release of inflammatory cytokines, and cell death (Shi et al., 2017). The formation of NLR homo- and heterodimer protein complexes are often required for immune activation and downstream signaling (Wang et al., 2019b, 2019a) ‘Resistome’ structures formed by Toll/Interleukin-1 receptor NLRs hydrolyze cellular nicotaminide adenine dinucleoside (NAD), initiating immune and cell death responses (Ma et al., 2020; Martin et al., 2020) Resistomes formed by coiled-coil NLRs resemble bacterial pore-forming structures. How coiled-coil resistomes induces immunity in plants remains unknown, but t is possible that it forms a

membranous pore to induce cell death, similar to animal NLRs. (Adachi et al., 2019a, 2019b) . Plant NLR-mediated immune responses mounted against numerous pathogens often result in localized cell death – a reaction termed the hypersensitive response (HR). Plant immune responses that result in cell death can range in appearance from localized, microscopic lesions ("micro HR") to the death of the entire plant ("systemic HR") (Balint-Kurti, 2019). The hypersensitive response may exist as a point on a spectrum of plant physiological responses to pathogens (Bendahmane et al., 1999). A typical HR lies somewhere phenotypically between micro-HR and systemic HR, with cell death often occurring in the infection site and immediate vicinity.

Although commonly used as a sign of an active defense response, cell death is not necessarily a vital component of virus resistance mechanisms (Komatsu 2010,Bendahmane 1999) . What has been often categorized as another type of resistance, termed "extreme resistance", exists opposite systemic HR on the spectrum of plant antiviral immune responses. Extreme resistance is characterized by a lack of symptoms, and limited or lack of pathogen replication and pathogen spread (Bendahmane et al., 1999). Although the relationship between HR and extreme resistance is not well defined, in at least one example the same NLR gene can initiate both extreme resistance and HR, depending on the amount of the pathogen elicitor protein recognized in the cell (Bendahmane et al., 1999). Extreme resistance and HR may be phenotypic variations of the same response pathway gradient, or separate pathways that are sequentially activated. Plants can prime distal tissues for infection after pathogen recognition, resulting in a physiological state termed "systemic acquired resistance," which serves to limit future infections beyond the

infection site (Liu et al., 2010; Fu and Dong, 2013). Systemic acquired resistance can be initiated through both extreme resistance and HR, further indicating functional overlap between the two responses. Plant immune responses beyond the initial NLR-mediated pathogen recognition steps require further characterization. A mechanistic understanding of extreme resistance will further our understanding of plant immune responses to pathogens.

The incorporation of genes that control extreme resistance into favored crop cultivars is a focus of traditional and molecular breeding programs worldwide, particularly in crop species that are vulnerable to losses caused by virus infection. Research efforts have primarily focused on the identification and investigation of the genes and mechanisms that confer extreme resistance to viruses in potato (*Solanum tuberosum*) and soybean (*Glycine max*). There are examples of extreme resistance from other species as well, including *Arabidopsis thaliana*, an important model system. Notable genes conferring extreme resistance include *Rx1* (Resistance to Potato virus X 1) and Ry_{sto} (Resistance to Potato virus Y from *Solanum stoloniferum*) genes in potato, which provide resistance to certain strains of Potato virus X and Potato virus Y, and *Rsv* genes in soybean, which provide resistance to certain Soybean mosaic virus strains. This review summarizes existing knowledge of the molecular mechanisms of plant-evolved extreme resistance to viruses.

Extreme resistance to virus in potato

Research on *Rx1* in potato has illuminated the initial molecular steps of extreme resistance

The initial processes of extreme resistance are best-characterized in the potato *Rx1*-Potato virus X (PVX) system. Also referred to as *Rx*, *Rx1* is a coiled-coil NLR protein that confers extreme resistance to the *Potexvirus*, Potato virus X (PVX), which can cause yield losses of up to 20% and is a common problem for potato growers (Adams et al., 1985). The *Rx1* gene is located on chromosome XII and has been introgressed from the wild species of potato, *Solanum andigena*, into some commercial potato cultivars (e.g., cv. Cara, Atlantic) (Ritter et al., 1991; Bendahmane et al., 1997). The *Rx1* gene shares high sequence complementarity and is functionally redundant to another NLR, *Rx2*, which is found on potato chromosome V and was introgressed into potato from *Solanum acuale (Ritter et al., 1991; Bendahmane et al., 1997)*. Despite similarities between the two genes, *Rx1* has received a much greater research focus than *Rx2*.

Previous research on NLR protein function indicates that most, if not all, NLRs rely on chaperone proteins for stability and proper function (Kadota et al., 2010). A triad of highly conserved NLR chaperone proteins, SGT1 (SUPRESSOR OF G2 ALLELE OF SKP1), HSP90 (HEAT SHOCK PROTEIN 90), and RAR1 (REQUIRED FOR MLA12 RESISTANCE), are essential for disease resistance. Many studies involve the expression of *Rx1* in the genetically tractable model plant, *N. benthamiana*. Silencing of *NbSGT1* suppressed *Rx1*-mediated extreme resistance to PVX in *N. benthamiana (Nb)*, and indicated that the extreme resistance conferred by Rx1 relies on proteins similar to non-extreme resistance conferring NLRs (Botër et al., 2007) (**Figure 1**). Despite no known nuclear or cytoplasmic localization signals, Rx1 must be distributed in both the cytoplasm and nucleus for an extreme resistance immune response to

occur (Slootweg et al., 2010; Tameling et al., 2010). The Rx1 coiled-coil domain is necessary for accumulation in the nucleus, while the LRR domain is required for cytoplasmic localization (Slootweg et al., 2018). Binding between Rx1 and *N. benthamiana* RAN GTPase ACTIVATING PROTEIN (*Nb*RG2) results in the retention of Rx1 in the cytoplasm, which is required for their dual roles in pathogen sensing and gene activation (Rairdan and Moffett, 2006; Sacco et al., 2007; Tameling et al., 2010).

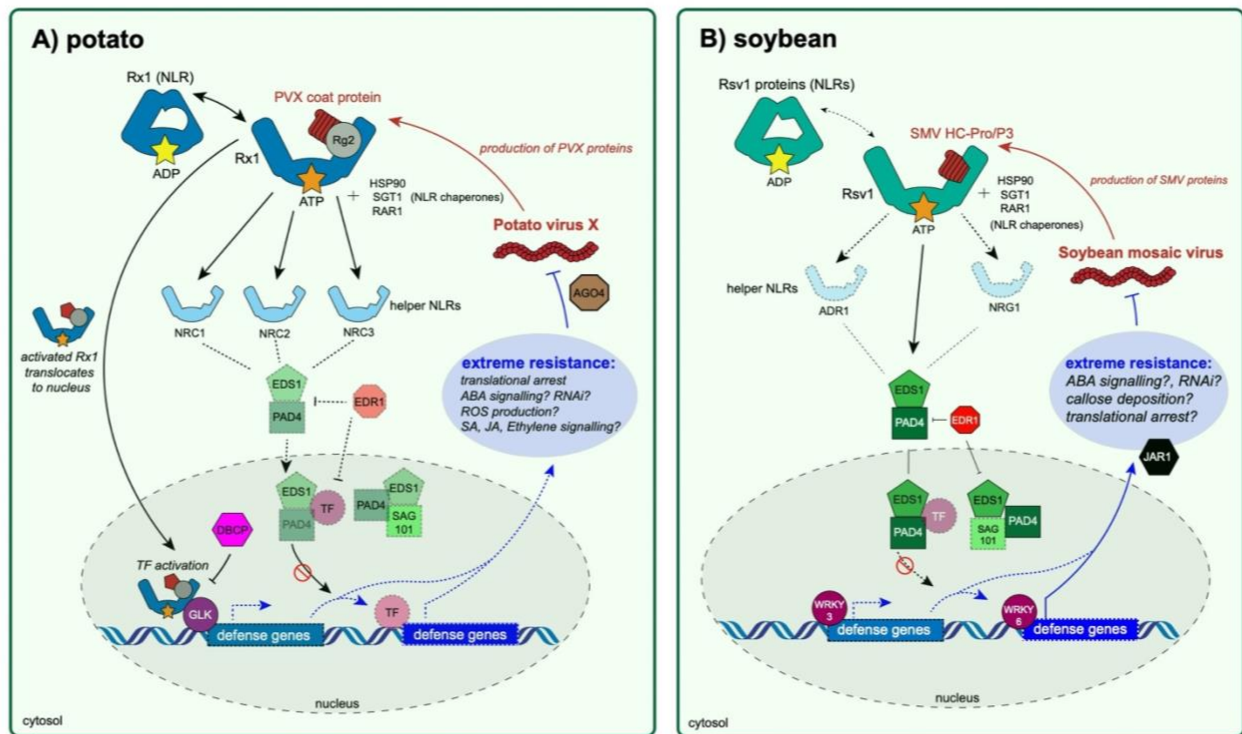

Figure 1: Extreme resistance to viruses in potato and soybean hosts.
The potato antiviral extreme resistance response to Potato virus X (PVX) is conferred by Rx1 in potato **(A)** and extreme resistance to soybean mosaic virus (SMV) is conferred by genes within the *Rsv1* locus in soybean **(B).** The first step in immune activation in either pathway relies on an NLR protein, Rx1 in potato **(A)** or an Rsv1 protein in soybean **(B),** directly or indirectly recognizing a pathogen-produced effector protein. The PVX coat protein is likely recognized after binding a RANGAP 2 (Rg2) protein 'guarded' by Rx1, although this relationship is yet to be experimentally validated. After pathogen recognition, the activated (ATP-bound) Rx1 then translocates to the nucleus, at which point it binds a GOLDEN 2-LIKE transcription factor

(GLK). The activity of Rx1-GLK complex is likely negatively regulated by a bromodomain-containing protein (DBCP). The Rx1-GLK complex binds DNA and may regulate expression of defense genes associated with the extreme resistance phenotype, although the genes that Rx1-GLK regulates are not known. The activated Rx1 protein relies on helper NLR proteins (NRC1, NRC2, NRC3) to transmit an immune response. Rx1-mediated extreme resistance causes translational arrest of the PVX genome to occur, possibly through involvement of *ARGONAUTE 4*. (B) The SMV HC-Pro or P3 protein is recognized directly or indirectly by an Rsv1 protein. Gene silencing of *ENHANCED DISEASE SUSCEPTIBLITY 1* (*EDS1*), *PHYTOALEXIN-DEFICIENT 4* (*PAD4*), *JASMONIC ACID-AMINO ACID SYNTHETASE* (*JAR1*), or *ENHANCED DISEASE RESISTANCE 1* (*EDR1*) abrogates the resistance response. Similarly, silencing of *WRKY3* and *WRKY6* disables resistance. In these diagrams, experimentally validated proteins involved in extreme resistance are highlighted, while proteins not validated but likely involved in extreme resistance responses are faded and have a dashed outline.

The function of *Nb*RG2 in the *Nb*RG2-Rx1 complex is not clear, although a few possible roles are apparent (Slootweg et al., 2010; Hao et al., 2013). RanGAP proteins, like *Nb*RG2, facilitate the GTPase activity of Ran GTPase proteins, which aid in the transport of protein complexes into and out of the nucleus (Dasso, 2002). There are no known transport activities of RanGAP proteins themselves and *Nb*RG2 hydrolasing activity is not required for a successful resistance response (Tameling and Baulcombe, 2007). Many NLR proteins have evolved to monitor host proteins that are vulnerable targets of pathogen effector proteins (Collier and Moffett, 2009). Slootweg *et al* 2010 suggest that *Nb*RG2 may act as a decoy target of the PVX coat protein, in which Rx1 would be “monitoring” *Nb*RG2 until bound by the PVX coat protein, after which Rx1 could initiate an immune response (Slootweg et al., 2010). This claim is buttressed by the findings that Gpa2, an NLR protein that shares a high amount of amino acid conservation with Rx1, guards *Nb*RG2 and initiates an immune response only when *Nb*RG2 is bound by effector proteins secreted by nematodes during infection (Sacco et al., 2009). Interactions between *Nb*RG2 and the PVX coat protein could produce a conformational change in *Nb*RG2, which could in turn allow Rx1 to reach an activated state (Hao et al., 2013). Many NLR proteins work

in pairs with other NLRs or helper NLRs to effectively transmit immune signals (Adachi et al., 2019a). A study by Wu *et al* 2017 found that silencing of three genes that encode helper NLR proteins, *NLR REQUIRED FOR CELL DEATH 1* (*NRC1*), *NRC2*, *NRC3*, disabled *Rx1*-conferred extreme resistance, but only if all three genes were silenced simultaneously (Wu et al., 2017). These results indicate levels of redundancy and possible robustness to interference by pathogens within plant immune signaling.

Binding between Rx1 and PVX coat protein occurs in the cytoplasm (Bendahmane et al., 1995). Recognition of PVX coat protein by Rx1 and subsequent binding causes the release of *Nb*RG*2* from Rx1, allowing Rx1 to translocate to the nucleus through a yet unknown process, as there are no detectable nuclear localization signals within Rx1(Tameling and Baulcombe, 2007). However, nuclear localization signals are notoriously difficult to predict and can be hidden within secondary structure. Although many studies have focused on NLR functionality in the cytoplasm, there are other examples of NLR nuclear localization and function (Burch-Smith et al., 2007; Shen et al., 2007). The barley NLR, MLA, interferes with repression of defense genes by binding to WRKY transcription factors in the nucleus (Chang et al., 2013). Experiments in which nuclear exclusion signals or nuclear localization signals were added to an Rx1-GFP fusion protein indicated that the DNA binding capabilities of Rx1 are contingent upon Rx1 recognition of the PVX coat protein in the cytoplasm, which is followed by movement of Rx1 to the nucleus. Resistance, but not cell death responses were compromised in experiments in which Rx1 was localized predominantly to the nucleus or the cytoplasm (Bendahmane et al., 1999c; Knip et al., 2019; Richard et al., 2021). These results indicate that Rx1 must be activated (ATP-bound) in

order to successfully bind DNA and likely must be able to translocate from the cytoplasm to the nucleus in order to initiate the extreme resistance response. Activation of Rx1 and other NLRs occurs after binding effector proteins, in this case the PVX coat protein. *In vitro* binding assays indicate that the DNA binding activity of Rx1 is inhibited while in an inactivated state (ADP-bound), while the activated (ATP-bound) Rx1 can bind DNA (Fenyk et al., 2015). Further, recognition of the PVX coat protein likely results in a perturbed binding between the leucine-rich repeat (LRR) and ARC2 (Apaf-1, R proteins, and CED-4) domains of Rx1, a process which may play a role in initiation of resistance pathways. Rairdan and Moffett *et al* 2006 suggest that the LRR domain can repeatedly dissociate and reassociate with the ARC2 domain after recognition of the PVX coat protein, and that this iterative process may serve to amplify the resistance signal and could play a key role in the extreme resistance response (Rairdan and Moffett, 2006).

Upon entering the nucleus, the activated nucleotide-binding domain of *Rx1* allows for binding and melting of double-stranded DNA in a non-sequence specific manner, but with a higher affinity for DNA topologies similar to transcription start site bubbles (Finzi and Dunlap, 2010; Tang et al., 2011; Townsend et al., 2018). The DNA binding activities of Rx1 likely become sequence-specific when in complex with *Nb*GLK1 (a Golden 2-like transcription factor), although this remains to be definitively proven (Townsend et al., 2018). The activation state of Rx1 likely determines the DNA binding activity of *Nb*GLK1, as inactivated Rx1 in complex with *Nb*GLK1 does not bind DNA *in planta* (Townsend et al., 2018). Golden 2-like transcription factors preferentially bind DNA sequences with GLK-like motifs and are known to regulate the transcription of genes involved in abscisic acid (ABA) signaling (Ahmad et al., 2019). Although

the genes that *Nb*GLK1 regulates in response to PVX infection are not known, GLK-like transcription factors play a role in resistance to cucumber mosaic virus and fungal pathogens in *Arabidopsis* (Savitch et al., 2007; Murmu et al., 2014; Han et al., 2016).

There are likely other proteins that interact with Rx1 and *Nb*GLK1 in the nucleus. Sukarta *et al* 2020 recently described a direct interaction between Rx1 and a DNA-binding bromodomain containing protein, *Nb*DBCP (Sukarta et al., 2020). The precise role(s) of *Nb*DBCP remains unclear, though it likely acts as a repressor of *Rx1*-mediated resistance signaling. Silencing of *Nb*DBCP as well as co-expression of non-functional *Nb*DBCP decreased PVX coat protein accumulation during Potato virus X infection in plants expressing Rx1, indicating that *Nb*DBCP may negatively regulate extreme resistance responses. Binding of DNA by *Nb*DBCP occurs *in situ*, but not when co-expressed with Rx1 or during PVX infection. Size exclusion chromatography results indicate that Rx1, *Nb*DBCP, and *Nb*GLK1 may form a transient complex, however this idea remains theoretical and untested. These results conservatively indicate a negative regulatory role of *Nb*DBCP on *Rx1*-mediated extreme resistance, although its exact role(s) require more research (Sukarta et al., 2020).

Intriguingly, overexpression of *Nb*GLK1 in *N. benthamiana* confers immunity to PVX even in the absence of Rx1, and this immunity does not result in HR(Townsend et al., 2018). These results may signal that *Nb*GLK plays a role in controlling gene expression that is important for extreme resistance, but that role is likely independent or upstream of HR/cell death. Additionally, *Nb*DBCP overexpression, in the presence of Rx1 and during PVX infection, resulted in increased

cell death. Expression of a non-functional *Nb*DBCP resulted in decreased cell death, lending credence to the idea that 1) *Nb*DBCP negatively regulates the extreme resistance pathway and 2) extreme resistance and HR/programmed cell death are largely separate or sequentially activated pathways (Sukarta et al., 2020). Similar separation of cell death and resistance have been reported in *N. benthamiana* plants expressing the barley NLR, *MLA10*, with a nuclear localization tag (Bai et al., 2012). The *MLA10* gene provides resistance to the barley powdery mildew fungus, indicating that nuclear functions of NLRs may not be specific to virus resistance.

Extreme resistance conferred by *Rx1* does not limit viral spread through the phloem. Grafting experiments revealed that PVX moved from a susceptible rootstock through the phloem of a middle, resistant scion and into another upper, susceptible scion and caused infection (Bendahmane et al., 1999a). A reaction similar to *Rx1*-mediated extreme resistance can occur in protoplasts, while HR does not (Otsuki et al., 1972; BAULCOMBE et al., 1984; al, 1993b; Bendahmane et al., 1995). These results may indicate that intercellular signaling components or cell wall components may not be necessary for *Rx1*-conferred extreme resistance responses, whereas they are for HR responses. It is likely that the mechanisms controlling extreme resistance occur rapidly in the cell, as extreme resistance prevents viral replication and spread beyond the initial point of inoculation.

Overexpression of Rx1 in *N. benthamiana* results in HR, regardless of whether its elicitor, the PVX coat protein, is present or not. Transformation of *Rx1* under its native promoter into *N. benthamiana* and *N. tabacum* results in a typical, symptomless extreme resistance to PVX,

indicating that functionality and possible downstream interacting elements are conserved between species (Bendahmane et al., 1999b). Overexpression of PVX coat protein in *N. benthamiana* expressing *Rx1* under its native promoter results in HR. Bendahmane *et al* 1999 suggest that the continued production of the PVX coat protein after the initial recognition event and extreme resistance activation may signal to the cell that extreme resistance has been overcome and that further immune action may be warranted, hence the subsequent HR(Bendahmane et al., 1999a). This hypothesis is supported by the finding that extreme resistance conferred by *Rx1* is epistatic to HR, as plants expressing both *Rx1* and *N*, an NLR that provides resistance with an HR phenotype to Tobacco mosaic virus, were resistant to Tobacco mosaic virus infection but did not display HR when the virus was engineered to express both the PVX coat protein and the protein elicitor of *N* during infection.

The addition of a nuclear localization signal to *Nb*RG2 caused Rx1 to accumulate almost solely in the nucleus and prevented HR from occurring, even when auto-active *Rx1* mutants were overexpressed(Slootweg et al., 2010). Thus, it is likely that Rx1 must be located in the cytoplasm in order for HR and concurrent signaling to occur. This conclusion is congruent with cytosolic location of PVX replication and PVX coat protein detection by Rx1 (Slootweg et al., 2010).

The possible interconnectedness between HR and extreme resistance responses underscores the need for more sensitive resistance assays. To better understand extreme resistance, it is paramount that researchers try to replicate the native expression levels of *Rx1* (and other genes that confer extreme resistance) when experimenting outside of its native potato system. Slootweg *et al* 2010 expressed *Rx1* from a vector with a second start codon inserted upstream and out of

frame of the *Rx1* start codon. The resulting 'leaky' expression of Rx1 led to protein levels in *N. benthamiana* that were 5-10x lower than expression driven by a typical CaMV35S promoter, and a much more sensitive assay(Slootweg et al., 2010). Studies in *Arabidopsis thaliana* also indicate the expression level of NLR proteins may in part determine the phenotype of the resistance response. For example, resistance to the yellow strain of Cucumber mosaic virus is conferred by *RCY1*, a coiled-coil NLR, in *Arabidopsis* ecotype C24. Resistance to Cucumber mosaic virus (Y) via *RCY1* is normally accompanied by a hypersensitive response. Transgenic Arabidopsis lines that over-expressed *RCY1* at high levels (i.e., ~100x greater than its native promoter) exhibited the extreme resistance phenotype. Transgenic plants that expressed *RCY1* at moderately elevate levels (i.e., ~20x greater than native expression) exhibited enhanced resistance with very small areas of cell death ("micro-HR")(Sekine et al., 2008). The hypersensitive response was observed in transgenic lines with levels of *RCY1* expression similar to native expression. None of the *RCY1* transformed Arabidopsis lines became systemically infected with Cucumber mosaic virus (Y). These results indicate that *RCY1* expression levels, at least in part, govern virus-resistance phenotypes, possibly by determining the type of the subsequent immune response.

Other publications studying the NLR, HRT, which confers resistance to Turnip crinkle virus, have noted similar results (Cooley et al., 2000). However, levels of resistance protein expression are likely not the only factor governing immune responses. Overexpression of the Turnip crinkle virus coat protein, which is the binding target of HRT, resulted in severe HR, similar to the reaction that occurs after overexpression of the PVX coat protein plants expressing Rx1 (Cooley et al., 2000). Expression and activity levels of NLR proteins in plants are regulated in many ways

(e.g., transcriptionally, post-transcriptionally, post-translationally, etc.) (Borrelli et al., 2018). It is possible that increased expression of HRT or RCY1 is sufficient to overcome some negative regulation, resulting in faster immune responses.

The mechanistic details of Rx1 conferred resistance restricting PVX viral replication and spread are not yet known, however experiments indicate that the translational arrest of the PVX transcripts is likely a major component of these resistance processes (Richard et al., 2021) By employing an inducible effector protein expression system and nuclear- and cytoplasm-localized Rx1 expression, Richard *et al* 2021 demonstrate that Rx1-conferred extreme resistance likely relies on PVX transcript-specific translational arrest and that this response occurs within a few hours after infection (Meteignier et al., 2016; Richard et al., 2021). These data also demonstrate that nuclear- or cytoplasm-localized Rx1, expressed individually or together, results in HR or trailing necrosis (i.e., HR that trails viral spread throughout the plant) after 4 hours of the induction of PVX coat protein transcription, but does not induce extreme resistance. These results further support that upon recognition of the PVX coat protein in the cytoplasm, Rx1 must translocate to the nucleus in order to initiate the extreme resistance response. Translational arrest is a common host antiviral strategy (Machado et al., 2017) *Rx1*-expressing, PVX-infected potato protoplasts did not support replication of either Tobacco mosaic virus or Cauliflower mosaic virus, indicating that the Rx1-mediated antiviral response was a general antiviral response (al, 1993a; Bendahmane et al., 1995). Further, Rx1 may not be unique in this regard, as recognition of the resistance elicitor, Tobacco mosaic virus p50, by the tobacco NLR gene, *N*, can initiate an

immune response that prevents translation of PVX transcripts in *N. benthamiana*, but only in the presence of RNA containing the PVX coat protein coding sequence (Bhattacharjee et al., 2009).

These results collectively suggest that a conserved characteristic of viral RNAs, possibly secondary structure, may be specifically targeted by NLR-mediated translational inhibition responses and that this mechanism may play a key role in the extreme resistance response. Although the factor(s) governing translational arrest are not known, it is interesting to note that virus-induced gene silencing of *Argonaute 4 (AGO4)* disabled symptomless resistance responses, and in turn allowed systemic PVX infection in *N. benthamiana (Bhattacharjee et al., 2009)*. Similarly, in *N. benthamiana* plants in which the RNAi suppressor proteins from Beet western yellows virus and Turnip crinkle virus, P0 and P38, were expressed, the antiviral response was also disabled. The Turnip crinkle virus P0 protein targets and induces degradation of Argonaute proteins (Baumberger et al., 2007; Bortolamiol et al., 2007) The expression of other suppressors of RNAi, including potyviral HC-Pro, did not prevent an antiviral response from occurring, although HC-Pro disables RNAi through sequestration of virus-derived small RNAs, not through the degradation of Argonaute (Mallory et al., 2002). Another RNA virus, Tobacco rattle virus, expresses a suppressor of RNAi silencing protein, 16k, which binds AGO4 and *ago4* mutant *N. benthamiana* plants are more susceptible to infection (Ma et al., 2015; Fernández-Calvino et al., 2016).

Argonaute 4 is well known for its roles in transcriptional gene silencing and the RNA-directed DNA methylation pathway, as well as methylation-based antiviral defense against plant viruses

with DNA genomes (Zilberman et al., 2003; Li et al., 2006; Raja et al., 2008, 2014; Gao et al., 2010; Greenberg et al., 2011; Dowen et al., 2012; Wierzbicki et al., 2012; Ye et al., 2012) These well-characterized roles of AGO4 all occur in the nucleus. Interestingly, cytoplasm-localized AGO4 is necessary for resistance to the potexvirus virus, *Plantago asiatica* mosaic virus, in *Arabidopsis.* This resistance does not involve other protein components of the RNA-directed DNA methylation pathway (e.g., *DICER-LIKE 3*, *RNA POLYMERASE IV*, *RNA POLYMERASE 5*), indicating that AGO4 antiviral activity in this case is likely independent of its DNA methylation activity (Brosseau et al., 2016). The importance of AGO-4 non-methylation-based defense is not limited to antiviral responses, as silencing of AGO4 in *Arabidopsis* plants without functional RNA-directed DNA methylation pathways increased susceptibility to *Pseudomonas syringae (Agorio and Vera, 2007).*

Research on *Rx1*-conferred extreme resistance has illuminated the potential nuclear functions of Rx1 and laid a framework for future studies. In particular, gaining an understanding of the DNA sequences targeted by the Rx1-*Nb*GLK1 complex and the possible transcriptional changes that occur after recognition of the PVX coat protein will aid in the identification of other genes and mechanisms involved in *Rx1*-conferred extreme resistance. Future experiments employing RNA sequencing, chromatin-immunoprecipitation sequencing, and translatome analysis would increase understanding of the regulation of this defense system. Additional studies are required to determine the role(s) of bromodomain containing proteins, to identify the DNA sequences that are targeted by the Rx1-GLK complex, and if targeted gene(s) are responsible for the next stages of the *Rx1* conferred extreme resistance response. Further, studies that dissect the antiviral

translational repression response and possible antiviral roles of *AGO4* would provide a greater understanding of NLR-mediated immunity.

Genes conferring extreme resistance to Potato virus Y rely on conserved proteins that are also necessary for HR

The *Ry* genes in potato (e.g., Ry_{sto}, Ry_{fsto}, Ry_{adg}) provide resistance to particular strains of PVX and Potato virus Y (PVY). The Ry_{sto} gene (Resistance to PVY from *Solanum stoloniferum*), which conveys resistance to a broad spectrum of strains of PVY and Potato virus A (PVA) in potato and tobacco, is the only one of the genes controlling extreme resistance that has been isolated from the *Ry* loci (Cockerham, 1970; Barker, 1996; Grech-Baran et al., 2020). Global potato production is reliant on pathogen-free seed tubers, which are vulnerable to generational buildup and spread of pathogens, particularly viruses. Various Potato virus Y (PVY) strains (including PVY^{NTN} and $PVY^{N\text{-}Wi}$) are the most economically harmful viral pathogens involved in potato production and genetic resistance to PVY is a major focus of breeding programs (Karasev and Gray, 2013). Wild potato varieties and landraces are sources of PVY-specific NLR resistance genes that can be introgressed into commercial potato cultivars. Loci conferring extreme resistance to PVY have been mapped in *S. chacoense* (Ry_{chc}), *S. tuberosum* group Andigena (Ry_{adg}), and *S. stoloniferum* (Ry_{sto}) (Herrera et al., 2018). Grech-Baran *et al* employed resistance enrichment sequencing (RenSeq) to isolate the gene conferring Ry_{sto}-mediated extreme resistance from the commercial potato cultivar, Alicja(Grech-Baran et al., 2020). Introgressed from *S. stoloniferum,* $\mathrm{Ry}_{\mathrm{sto}}$ is a Toll-interleukin receptor (TIR) NLR protein, similar to other

potato virus resistance genes (e.g., *N*, *Pvr4*, *Y-1*, etc.). The broad-spectrum resistance conferred by Ry_{sto} lends it an attractive trait for breeders of Solanaceous plants. The Ry_{sto} gene is present in various commercial potato cultivars, including American cultivars Payette Russet and Castle Russet and European cultivars Alicj, White Lady, and Pirola. The $\mathrm{Ry}_{\mathrm{sto}}$ protein either directly or indirectly recognizes and binds the coat protein of PVY and PVA to elicit the extreme resistance response (Grech-Baran et al., 2020).

The Ry_{sto} gene has been cloned and expressed in PVY-susceptible Solanaceous plants. Challenge of transgenic plants expressing Ry_{sto} under its native promoter with PVY usually results in an extreme resistance response (i.e., no infection, no symptoms), but can cause veinal necrosis or HR in response to some isolates of $\mathrm{PVY}^{\mathrm{O}}$ (HINRICHS-BERGER et al., 1999). Co-expression of Ry_{sto} and the PVY coat protein under control of a CaMV35S promoter results in HR in potato and *N. benthamiana*. Expression of Ry_{sto} in tobacco and subsequent challenge with PVY produced some localized necrosis in inoculated leaves. Grech-Baran *et al* 2020 suggest that establishment of either extreme resistance or HR depends on at least three variables: expression level of the resistance gene; abundance of the cognate effector protein; and the genetic background of the host(Grech-Baran et al., 2020). Extreme resistance conferred by Ry_{sto} relies on at least two other genes for successful immune activation: the lipase-like *ENHANCED DISEASE SUSCEPTIBILTY* (*EDS1*) and the CC-NLR, *N REQUIREMENT GENE 1* (*NRG1*). These dependencies corroborate studies on other TIR-NLRs (Grech-Baran et al., 2020).

Similar to the Rx1-PVX system, the genes controlling *Ry$_{sto}$*-mediated extreme resistance downstream of virus recognition are not known, although some results have hinted at the involvement of proteins that interact with plasmodesmata. Beta-1,3-glucanase proteins aid in plant virus infection, likely by hydrolyzing callose and increasing the size exclusion limit of plasmodesmata, thus allowing for cell to cell spread of the virus (Iglesias and Meins, 2000). Callose is a polysaccharide that influences the size exclusion limit of the plasmodesmata and also serves as a deposition site for defense compounds (Zavaliev et al., 2011). Overexpression experiments of Beta-1,3-glucanase (class III) proteins were carried out in potato cultivars Santé (which displays extreme resistance to PVY) and Désirée (PVY-susceptible) within the context of PVY^{NTN} infection. Resistant Santé plants overexpressing *β-1,3-glucanase* exhibited modest, transient increases in PVY^{NTN} that dissipated within days of infection and the virus did not spread beyond the inoculated leaf. Susceptible Désirée plants that overexpressed *β-1,3-glucanase* may have exhibited slightly faster systemic infection of PVY^{NTN}, although a relatively small sample size precluded more definitive conclusions(Dobnik et al., 2013).

Callose deposition is also targeted by PVY during infection, as PVY-encoded Helper-component protease (HC-Pro) suppresses callose deposition during PVY^{O} infection through an unknown mechanism (Chowdhury et al., 2020). Callose deposition also occurs in cells surrounding HR activity during PVY infection and those cells can harbor viable PVY, thus further indicating that cell death is not the primary driver of resistance during HR and that callose deposition alone is not effective at arresting viral spread (Lukan et al., 2018). Cells undergoing HR/cell death processes may release signals to surrounding cells to initiate immune responses or that may act

as defense compounds themselves (Lamb and Dixon, 1997). These signals may include reactive oxygen species, which are a common component of plant immune responses (Qi et al., 2017). Whether or not reactive oxygen signaling acts a component of extreme resistance is not known, although there are examples of symptomless resistance to Tobacco mosaic virus in tobacco plants induced by application of reactive oxygen species (Kuenstler et al., 2016). The timing of foliar treatments was key to inducing resistance to Tobacco mosaic virus, as resistance did not occur in plants that were treated with reactive oxygen species three days after virus inoculation, but an HR-like reaction and cell death did.

The NLR resistance gene, *Ny-1*, also provides resistance to PVY, but the immune response is accompanied by HR. Extreme resistance conferred by Ry_{sto} is epistatic to *Ny-1*-mediated HR, as plants expressing both *Ny-1* and Ry_{sto} exhibit an extreme resistance phenotype but lack HR when challenged with a PVY^{NTN} isolate that is recognized by both Ry_{sto} and Ny-1 (Grech-Baran et al., 2020). For many TIR-NLR proteins, including *Ny-1*, resistance breaks down at high or low temperatures, while Ry_{sto} function is not limited by high ambient temperatures. Modulation of defense responses by temperature is likely controlled by NLR proteins, as point mutations in *NLR* genes can decrease nuclear accumulation of NLR proteins at higher temperatures and reduce NLR immune function (Zhu et al., 2010). At higher temperatures plants may preferentially activate pattern-triggered immunity, rather than NLR-dependent immunity (Cheng et al., 2013). Since NLR proteins confer resistance to plant pathogens that disable host pattern-triggered immune responses, a greater understanding of NLR thermosensitivity is needed as global temperatures continue to rise (Trebicki, 2020).

The expression levels of both the genes conferring extreme resistance and the viral elicitor protein seems to be a critical factor if HR or extreme resistance occurs in both Rx1-PVX and Ry_{sto}-PVY systems. The cellular distribution of Ry_{sto} both before and during resistance responses is not known but should be the focus of further research given the potential nuclear functions of Rx1. The advent and expanded use of resistance-enrichment sequencing (RenSeq), which allows for the expedited identification of NLR genes conferring specific resistance phenotypes, and CRISPR technologies, which allow for precise genome editing, should facilitate faster identification and breeding of resistance genes (Witek et al., 2016; Zhang et al., 2019). As extreme resistance is largely characterized by a lack of infection symptoms, there may be a pool of genes that confer extreme resistance to viruses that are yet to be discovered within landraces or wild Solanaceous species. For example, the *PVR4* gene, which encodes an NLR protein that originated in a landrace of hot pepper (*Capsicum annum*), confers extreme resistance to multiple potyviruses, including many PVY strains, Pepper mottle virus, and Pepper severe mosaic virus (Kim et al., 2015) The PVR4 protein recognizes the potyviral RNA-dependent RNA polymerase (NIb) to elicit the extreme resistance response (Kim et al., 2015). Global potato production relies on labor-intensive seed tuber certification programs to prevent pathogen accumulation, particularly viruses, with a large focus on PVY strains. Given that many NLR genes can be shuttled between *Solanaceous* species without a loss of function, transferring multiple, broad-spectrum NLR genes that target different potyviral components (i.e., Ry_{sto}, *PVR4*) to potato could provide durable and sustainable immunity to potyviruses (Rivero et al., 2012; Zhu et al., 2012; Jo et al., 2014).

Tobacco rattle virus-caused corky ringspot disease in potato is likely the result of an HR-like immune response

Potyviruses are not the only problematic viruses of potato production. Tobacco rattle virus (TRV), of the genus Tobravirus, causes necrosis in potato tubers, a disease commonly referred to as corky ringspot disease or "spraing." Corky ringspot disease can render tubers unmarketable. Tobacco rattle virus is vectored by various species of nematodes and can infect many different plant species. Although cultivars that display symptoms of TRV infection in tubers are often labeled as susceptible, the ringed, necrotic tuber tissue characteristic of corky ringspot disease are likely the result of HR in response to viral infection in the tuber(Xenophontos et al., 1998; Sahi et al., 2016). Some frequently grown cultivars are tolerant to TRV infection and can contain high levels of virus throughout the plant and yet remain largely asymptomatic, although these tolerant infections can have negative effects on yield and tuber size and also serve as inoculum sources(Dale et al., 2000; Brown et al., 2009). The potato cultivar, Saturna, displays an extreme resistance response in leaves when inoculated with the TRV isolate, PpK20, and no symptoms or detection of TRV in tubers. A yet undescribed protein in Saturna recognizes the TRV movement protein, 29K, to initiate an extreme resistance response (Ghazala and Varrelmann, 2007). Similar to potato cultivars that display extreme resistance to PVY and PVX, overexpression of the elicitor protein (29k, in this case) leads to HR in the infected leaf.

Corky ringspot disease is a significant problem for growers in Europe, while TRV is a small but growing problem in the United States. Once TRV/corky ringspot disease occurs in a field it can be difficult to eliminate completely. Soil fumigation can reduce the presence of stubby root nematode vectors, but often does not completely eliminate TRV presence. Stubby root nematodes can feed on a wide variety of plants (i.e., potato, barley, corn, peas, brassicas, various common weeds, and others), often rendering crop rotations in infested fields non-effective at lowering vector pressure. Given that corky ringspot disease/spraing is caused by an HR-like immune response in tubers, then the largely symptomless immune response provided by extreme resistance would be a valuable asset to growers. Further, isolation of the gene(s) that confer extreme resistance to TRV in potato would be beneficial for breeding resistant cultivars.

Extreme resistance to viruses in soybean

The majority of the extreme resistance literature examines responses and pathways occurring after virus recognition in the soybean-Soybean mosaic virus pathosystem. Soybean mosaic virus (SMV) is a potyvirus that predominantly infects plants in the family *Fabaceae (Hajimorad et al., 2018)*. SMV is classified into seven strains, denoted G1-G7, with virulence in soybean generally increasing with strain number (e.g., isolates of strains G5-G7 are generally the most virulent in soybean cultivars) (Widyasari et al., 2020). Four dominant resistance loci, termed “Rsv”, (*Rsv1*, *Rsv3*, *Rsv4*, *Rsv5*) are effective against various strains and isolates of SMV and are located on soybean chromosomes 2, 13, and 14. The genes conferring resistance from some of these loci have been identified. China uses a separate system to designate SMV strains and resistance

genes, although many of the dominant resistance genes that have been identified by research teams in China (termed *Rsc* resistance genes) map to the same chromosomes and similar loci as Rsv resistance genes, although their relationships remain largely uncharacterized (WANG et al., 2017) (**Figure 1**).

The *Rsv1* and *Rsv5* loci are located within a complex region on the distal end of soybean chromosome 13 (Hayes et al., 2004). As *Rsv1* and *Rsv5* are tightly linked, they were once considered to be alleles of the same gene, however they are likely two separate NLR genes (Klepadlo et al., 2017). Because *Rsv1* and *Rsv5* are often inherited together, it is possible that some interpretations of *Rsv1*-mediated resistance are complicated by involvement of an undetected *Rsv5* allele (Klepadlo et al., 2017). Extreme resistance conferred by *Rsv5* prevents infection by SMV-G1 (Klepadlo et al., 2017). The mechanism of *Rsv5*-mediated resistance is unknown and the gene responsible has not been isolated or cloned. Resistance provided by *Rsv4* is unique in that it is not conferred via an NLR-type protein, as *Rsv4* encodes an RNAse-H family protein with the ability to degrade dsRNA. Interactions between the SMV protease 3 (P3) protein and Rsv4 promote dsRNA degradation and prevent viral replication (Maroof et al.; Hayes et al., 2000; Ishibashi et al., 2019). The ability of *Rsv4* to prevent infection declines with age, as mature plants are more susceptible to infection (Maroof et al.; Hayes et al., 2000). This unique form of resistance appears to be independent of extreme resistance and HR, although it is phenotypically similar to extreme resistance.

The resistance conferred by *Rsv3* to SMV may be mechanistically different from *Rsv1*-conferred extreme resistance (Zhang et al., 2009) Specifically, *Rsv3*-expressing soybean plants inoculated with SMV-G7 expressing beta-glucuronidase (GUS) exhibited small, isolated GUS expression foci at five days post inoculation, while *Rsv1*-plants inoculated with SMV-N expressing GUS did not (Zhang et al., 2009). *Rsv1* and *Rsv3*-expressing plants did not become systemically infected or exhibit cellular death; they were phenotypically indistinguishable from the mock-inoculated plants. Based on these results, Zhang *et al.* suggest that *Rsv1* and *Rsv3* provide resistance through functionally distinct immune responses. However, many publications describe *Rsv3*-conferred resistance as a form of extreme resistance despite the report by Zhang *et al.* 2009 (Seo et al., 2009, 2014; Khatabi et al., 2012; Ilut et al., 2016; Alazem et al., 2018). For consistency, herein *Rsv3*-conferred resistance is referred to as a form of extreme resistance. Recognizing that future studies may describe mechanistic differences between *Rsv3*-conferred and *Rsv1*-conferred resistance, as well as identify additional examples of NLR-conferred extreme resistance. Further experiments, including qPCR validation of limited SMV replication in *Rsv3*-expressing plants, as well as an extended time course comparison between *Rsv1*- and *Rsv3*-expressing plants directly after inoculation with various SMV strains expressing a reporter gene would likely produce more definitive conclusions.

Virus-induced gene silencing experiments have identified proteins involved in extreme resistance conferred by the Rsv1 locus

The *Rsv1* locus encodes multiple NLR genes, is highly complex, and is mapped to soybean chromosome 13 (Widyasari et al., 2020). The extreme resistance phenotype conferred by the *Rsv1* locus is contingent upon the strain of the infecting virus and the *Rsv1* allele present. There are 10 identified alleles of the *Rsv1* locus which are associated with strain-specific resistance to SMV (Klepadlo 2017). Various resistance phenotypes occur in plants containing the *Rsv1* locus that are infected with SMV strains G1-G7. The dominant *Rsv1* allele, which shares its name with the locus itself, confers extreme resistance to SMV strains G1-G6, but does not provide resistance to isolates of strain SMV-G7 and experimentally evolved SMV-G7d (Hajimorad et al., 2006).

Understanding of *Rsv1*-SMV interactions is limited because the gene(s) controlling *Rsv1*-conferred extreme resistance have not yet been identified. Gene silencing experiments concurrently targeting the expression of three non-Toll interleukin receptor NLR genes from the *Rsv1* allele of the *Rsv1* locus resulted in viral foci formation in resistant plants (cultivar L78-379), similar to that seen in susceptible plants (a near isogenic line of L78-379) (Zhang et al., 2012). It is likely that at least two of those three targeted genes is necessary for *Rsv1*-conferred extreme resistance (Wen et al., 2013). Individual silencing of each gene was not possible because of high sequence similarity between the three (Zhang et al., 2012). Extreme resistance conferred by the *Rsv1* locus may be dependent on host recognition of two viral proteins, as mutations in both the Helper-Component Protease (HC-Pro) and Protease 3 (P3) protein coding regions of the SMV genome are needed to break *Rsv1*-conferred extreme resistance (Wen et al., 2013)(Hajimorad et al., 2005, 2006, 2008; Zhang et al., 2009)

Studies that utilized recombinant inbred soybean lines derived from the *Rsv1*-allele containing PI969893 line have enhanced understanding of *Rsv1*-conferred extreme resistance. One recombinant inbred line, L800, contains one NLR gene from the *Rsv1* locus, denoted as *3gG2*. Another recombinant inbred line, L943, contains five NLRs from the *Rsv1* locus, but does not contain *3gG2*. Interestingly, both lines are resistant to SMV-N, but L943 recognizes the HC-Pro from SMV-N to induce resistance, while L800 recognizes P3 from SMV-N to induce resistance, suggesting that the *Rsv1* locus may contain at least two resistance genes that recognize separate SMV proteins to induce resistance (Wen et al., 2013). It is interesting that line L943, which contains five NLRs from the *Rsv1* locus, allowed limited virus replication, as a few viral foci were evident by GUS staining after inoculation with SMV-N expressing GUS, but virus did not spread beyond the inoculated leaf and the foci did not grow. In contrast, line L800, which only contains one NLR (3gG2) displayed no GUS expression when inoculated with SMV-N expressing GUS (Wen et al., 2013). These small GUS foci are similar to those seen when comparing *Rsv1* and *Rsv3* extreme resistance in Zhang *et al.* 2009 (Zhang et al., 2009). These results could indicate that separate mechanisms may inhibit viral replication and viral spread and that both may be induced by the extreme resistance response.

Mutations in the SMV-N HC-Pro and P3 coding regions, which are recognized by Rsv1 proteins, enabled virus replication in L943 and L800 soybean lines, but not in P196983, which contains the entire *Rsv1* locus. An additional mutation to SMV-N HC-Pro resulted in productive infections in L800, L943, and PI96983 soybean lines, further indicating that the *Rsv1* locus likely

contains multiple NLR or other genes that induce extreme resistance SMV. This idea is also supported by data indicating that SMV-N more easily evolves to evade variants that infect the single NLR (i.e., *Rsv1* 3gG2) containing L800 soybean line than the L943 soybean line that contains five NLRs from *Rsv1* or the full *Rsv1* locus-containing PI96983 line (Hajimorad et al., 2003; Wen et al., 2013).

The HC-Pro and P3 genes are next to each other in the potyviral genome and on the resulting polyprotein before self-cleavage. It is not known if the protein(s) from the *Rsv1* locus recognize the SMV polyprotein or enzymatically active HC-Pro or P3 (Hajimorad et al., 2008; Wen et al., 2013). In another example of extreme resistance outside of potato or soybean, the cowpea cultivar, Arlington, displays extreme resistance to Cowpea mosaic virus by recognizing the enzymatically active 24K-protease as it cleaves the polyproteins of the Cowpea mosaic virus' segmented genomes (Fan et al., 2011). It is also possible that closely related NLR genes from the *Rsv1* locus guard host proteins that are targeted by HC-Pro/P3 during the early stages of SMV infection (Hajimorad et al., 2008). It is also plausible that *Rsv1* contains multiple genes that confer variable levels of SMV resistance. For example, extreme resistance in the *3gG2* containing L800 soybean line could involve SMV-N P3 recognition, while other genes within the *Rsv1* locus could confer less effective resistance phenotypes (i.e., limited viral replication, but no spread) through SMV-N HC-Pro recognition. Further, line PI96983 could contain a yet unidentified NLR that recognizes a separate region of HC-Pro to induce extreme resistance.

Additional studies provide some indirect support that NLR proteins are likely involved in *Rsv1*-conferred extreme resistance to SMV, as silencing of genes that interact with NLR proteins resulted in increased virus load in resistant plants. Heat shock proteins often serve as molecular chaperones and *HEAT SHOCK PROTEIN 90* (*HSP90*) acts as a chaperone to NLR proteins in plants and animals (Kadota et al., 2010). Virus induced gene silencing (VIGS) of *HSP90* resulted in SMV infection foci in the leaves of resistant cultivars, resulting in a phenotype similar to SMV infection in a susceptible cultivar (Zhang et al., 2012). Two other genes, *RAR1* and *SGT1*, serve as co-chaperones to HSP90 to stabilize NLR proteins. Silencing of *RAR1* and *SGT1* in two independent publications provided conflicting results as to if either are involved in extreme resistance (Fu et al., 2009; Zhang et al., 2012). These differences are likely explained by both differences in experimental design and in that gene silencing assays rarely result in a complete loss of target gene expression.

Experimental VIGS was also used to target a suite of other defense-related genes, including soybean homologs of *ENHANCED DISEASE SUSCEPTIBILITY 1* (*EDS1*), *PHYTOALEXIN DEFICIENT 4* (*PAD4*), *ENHANCED DISEASE RESISTANCE 1* (*EDR1*), and *JASMONIC ACID-AMINO SYNTHETASE 1* (*JAR1*). Reducing expression of the aforementioned genes resulted in SMV infection foci in the inoculated leaves of extreme resistant soybean cultivar L78-379 (Zhang et al., 2012). These infections were phenotypically similar to infections in SMV-infected leaves of susceptible cultivars, thus indicating that the silenced genes are likely components of the extreme resistance defense response. The *EDS1* protein family includes *EDS1*, *PAD4*, and *SENESCENCE-ASSOCIATED GENE 101* (*SAG101*). Heterodimers between

EDS1/PAD4 or EDS1/PAD4/SAG101 are required for effector triggered immunity (ETI) in most seed plants. These heterodimers act downstream of pathogen recognition but upstream of transcription of defense genes. Recent research indicates that EDS1/PAD4 promote salicylic acid biosynthesis via the isochorismate pathway, but also control and preserve salicylic acid signaling through an alternative, parallel pathway. A third, separate salicylic acid signaling pathway relies on MAPK signaling (Cui et al., 2017). Salicylic acid is a primary signaling component of pattern-triggered and effector-triggered plant immune responses to many biotrophic pathogens and is therefore a primary target of plant pathogen interference. Downstream signaling and transcriptional reprogramming during ETI is controlled in part by the EDS1/PAD4 complex, which is in turn negatively regulated by the MAPKK kinase, *EDR1* (Neubauer 2020). Salicylic acid-mediated disease resistance is negatively regulated by *EDR1* and *Arabidopsis EDR1* mutants are sensitive to abscisic acid (ABA). Knocking down expression of *EDR1* would be expected to not have affected the resistant phenotype. Jasmonic acid signaling, which modulates defense responses to herbivory and often acts antagonistically to the salicylic acid pathway, is controlled in part by *JAR1* (Suza and Staswick, 2008).

Other genes targeted by VIGS within the context of *Rsv1*-conferred extreme resistance to SMV infection of soybean plants include WRKY transcription factors. WRKY proteins are among the largest transcription factors families in plants and largely regulate gene expression in response to abiotic and biotic stressors. A large scale VIGS study targeting 62 separate WRKY transcription factors revealed two genes, *GmWRKY30* and *GmWRKY6*, that compromised *Rsv1*-mediated resistance in soybeans that had been challenged with SMV (Zhang et al., 2012). Arabidopsis

WRKY6 is induced upon infection with a variety of viruses, which may suggest a conserved role for *WRKY6* within the context of virus infection across plant species (Whitham et al., 2003). *GmWRKY30* shares sequence similarity with Arabidopsis *WRKY3*, which is induced by pathogen infection and salicylic acid treatment in Arabidopsis (Liu et al., 2004; Lai et al., 2008; Pandey and Somssich, 2009). Further research is needed to understand which genes are regulated by *GmWRKY30* and *GmWRKY6* in response to viral infection and if similar antiviral roles are played by WRKY homologues in other plant species.

The factors controlling the relationship(s) between resistance, SMV strain, and *Rsv1* locus are not well understood and raise some interesting questions. Namely, why do some *Rsv1* alleles provide extreme resistance to particular SMV strains and others do not? For example, the *Rsv1* allele, *Rsv1-m*, provides extreme resistance to SMV strains G1, G4, and G5, but exhibits systemic necrosis when infected with G2, G3, G6, and G7 (Chen et al., 1991). The *Rsv1* allele confers resistance to SMV strains G1-G6, but systemic necrosis occurs in response to SMV-G7 infection. These differences may be attributed to allelic differences in elicitor binding/recognition efficiencies but may also be due to non-NLR host factors (Yuan et al., 2020) The cause(s) of systemic necrosis is not well understood but may be due to the delayed activation of the immune response/HR (Hajimorad and Hill, 2001; Hajimorad et al., 2003; Seo et al., 2009). In a study by Chen *et al.* 2017, *Rsv*-1-containing soybean plants in which *EUKARYOTIC TRANSLATION INITIATION FACTOR 5A* was silenced exhibited increased virus accumulation and less necrosis, indicating a possible role for *EUKARYOTIC TRANSLATION INITIATION FACTOR 5A* in defense responses (Chen et al., 2017).

These plants also exhibited lower expression of other defense genes and genes involved in ROS signaling. Genetic variation in the RNA interference machinery between species and cultivars can result in differential susceptibility to virus infection. For example, the *ARGONAUTE 2* (*AGO2*) gene in *A. thaliana* limits PVX infection, but *N. benthamiana AGO2* does not. Further, *AGO2* exhibits a high incidence of polymorphism between *A. thaliana* accessions, some of which affect antiviral activity. The *AGO2* sequences contain signatures of selective pressure, possibly due to co-evolution with viruses (Brosseau et al., 2020). Variation among non-NLR host factors may explain some of the differences observed between resistance phenotypes of different species or cultivars (i.e., plants that develop viral foci in inoculated leaves but not systemic infection) but more research is needed to understand the impact of these differences on antiviral defense in plants.

Future studies involving the cloning and testing of individual genes within the *Rsv1* locus and identification of possible plant protein binding partners and other host factors involved in antiviral defense could further our understanding of Rsv1-conferred extreme resistance. Isolation of genes within the *Rsv1* locus could also provide access to a suite of genes that confer varying levels of resistance, which may be experimentally valuable.

***Rsv3*-mediated extreme resistance response in soybean highlights importance of abscisic acid pathway**

The gene *GLYMA.14g204700* (referred to hereafter as simply '*Rsv3*'), which encodes a coiled-coil NLR, is likely the gene responsible for *Rsv3*-mediated extreme resistance in the L29 cultivar (Tran et al., 2018). The soybean cultivar, L29, exhibits extreme resistance to isolates of SMV strains G5, G6, G7, and G5H via the *Rsv3* locus but is susceptible to the SMV-G7H isolate. The cylindrical inclusion protein of SMV is indirectly recognized by Rsv3 to initiate an immune response. Virus strain SMV-G7H escapes *Rsv3*-mediated host detection via amino acid substitutions in the cylindrical inclusion protein (Seo et al., 2009).

Transcriptomic responses of the L29 soybean cultivar to SMV strains have been the focus of recent studies investigating the mechanism of extreme resistance to SMV (Seo et al., 2014; Alazem et al., 2018, 2019). Alazem *et al* 2018 postulate that the extreme resistance response may inhibit SMV replication and spread in three successive steps: 1) virus recognition by an NLR, resulting in rapid callose deposition at the plasmodesma of infected cells; 2) dsRNA detection, which induces viral genome destruction via RNAi; and 3) clearance of remaining viral proteins via the transient induction of autophagocytosis (Alazem et al., 2018). Seo *et al* 2014 compared the transcriptomic responses of L29 soybean plants when infected with either a virulent SMV strain (SMV-G7H) or an avirulent strain (SMV-G5H) at 8, 24, and 54 hours post infection (hpi) (Seo et al., 2014). Analysis of differential gene expression revealed that genes encoding Type 2C protein phosphatases were among the most differentially expressed in L29 soybean plants that exhibited extreme resistance to avirulent SMV infection. Type 2C protein phosphatases are a large class of serine/threonine phosphatases that are key regulators of the abscisic acid (ABA) signaling network in plants (Fuchs et al., 2013). Abscisic acid signaling plays key roles in

developmental regulation, stress responses, and likely defense responses (Flors et al., 2005; Fan et al., 2009; Alazem and Lin, 2017; Alazem et al., 2017; Xie et al., 2018; Pasin et al., 2020). L29 plants that overexpressed *PROTEIN PHOSPHATASE2C 3A* (*GmPP2C3a*) were not systemically infected when inoculated with virulent SMV, whereas all non-transgenic plants were systemically infected. Further analyses indicated that Type 2C protein phosphatases are likely regulators of the *Rsv3*-mediated extreme resistance response (Seo et al., 2014).

The aforementioned transcriptomic data was further analyzed in Alazem *et al* 2018 (Alazem et al., 2018). Genes involved in the ABA pathway exhibited increased expression at 8 and 24 hpi in plants infected with avirulent SMV, but expression of these genes was not increased by virulent SMV infection. The induction of genes involved in ABA signaling in plants infected with avirulent SMV dissipated by 54 hpi. Topical application of ABA to L29 soybean leaves 24 hours before infection with virulent SMV reduced virus accumulation by about 50% compared to non-treated plants, although virus replication was not completely eliminated. The specific mechanism(s) of ABA mediated virus reduction are an active area of research and are not well understood. It is likely that ABA signaling works in concert with other defense pathways including RNA interference and pattern-triggered immunity (PTI). Foliar ABA application of *N. benthamiana* plants induced the expression of genes involved in RNAi in response to Bamboo mosaic virus infection, specifically the argonaute protein encoding genes, *AGO2* and *AGO3* (Alazem et al., 2017). There are similar reports of increased expression of RNAi pathway components in Arabidopsis and soybean (Chen et al., 2013; Alazem et al., 2014; Alazem and Lin, 2017).

One effect of greater cellular ABA concentrations is increased callose deposition at plasmodesmata (Nishimura et al., 2003; Flors et al., 2005; Li et al., 2012). Abscisic acid negatively regulates β-1-3-glucanase expression, which encodes for proteins that break down callose tissue. The number of β-1-3-glucanase transcripts was reduced at all three time points in plants infected with avirulent SMV. Increased callose deposition at inoculation sites occurred in L29 plants infected with avirulent SMV and in L29 plants overexpressing *GmPP2C3a* and infected with virulent SMV. Similar increases in callose deposition were not observed in non-transgenic plants infected with virulent SMV. Foliar treatments of L29 leaves with the callose synthesis inhibitor, 2-deoxy-D-Glucose (DDG), resulted in increased avirulent SMV abundance in treated leaves. These experiments elegantly illustrate the importance and interplay of ABA signaling, GmPP2C3a expression, and callose deposition to the extreme resistance response as conferred by *Rsv3* in soybean.

Cellular concentrations of ABA increased dramatically in L29 plants that exhibited extreme resistance, but the abundance of another plant defense signaling molecule, salicylic acid (SA), did not change (Seo et al., 2014). The importance of SA signaling to antiviral defense in plants is well documented (Vleesschauwer et al., 2014). Increased SA accumulation occurred in L29 plants infected with virulent SMV but remained unchanged in plants infected with avirulent SMV, possibly suggesting that the antiviral role(s) of SA are not activated until after extreme resistance mechanisms have been broken. The ABA and SA pathways seem to interact in dueling antagonistic manner (i.e., high cellular ABA concentrations reduce SA biosynthesis and high SA

concentrations inhibit ABA signaling) (Nishimura et al., 2003; Zabala et al., 2009; Manohar et al., 2017). Interestingly, type 2C protein phosphatases (PP2Cs) bind both ABA and SA and may be important in modulating ABA and SA signaling (Manohar et al., 2017). ABA signaling is negatively regulated by Type 2C protein phosphatases which, in the absence of ABA, dephosphorylate ABA signaling kinases. Increased ABA concentration enhances binding between PP2Cs and PYR1-like regulatory elements, which inhibit PP2C dephosphorylation activity, resulting in the autophosphorylation of ABA signaling kinases and the expression of ABA responsive genes (Manohar et al., 2017).

Many SA-binding proteins have been documented. It is likely that a multitude of proteins are directly involved in regulating ABA and SA signaling. Staining leaves with DAB (3,3'-diaminobenzidine), which indicates reactive oxygen species presence, revealed that the *Rsv3*-mediated extreme resistance response does not rely on reactive oxygen species production. This result is contradictory to evidence indicating that reactive oxygen production is an important component of other plant immune responses, including extreme resistance. Therefore, it supports the idea that *Rsv3*-mediated extreme resistance differs mechanistically from *Rsv1*-mediated extreme resistance (Zhang 2009). Jasmonic acid (JA) is also an important defense signaling molecule during plant-pathogen interactions, particularly during interactions with herbivorous insects (Suza and Staswick, 2008). The relative importance of JA signaling during viral infection remains unclear. Genes involved in the JA pathway exhibited either no change or decreases in expression in reactions with avirulent SMV but saw increased expression during virulent SMV infection at all timepoints. These data suggest that the induction of JA signaling may be

important for establishing infection or may act as another layer of defense signaling. Another recent study by Alazem *et al.* illustrated that the effects of ABA treatment can be strain-dependent in cultivars lacking the *Rsv3* locus (Alazem et al., 2019). Topical treatment of leaves with ABA reduced the severity of virulent SMV infection but promoted avirulent SMV infection in an *Rsv3*-lacking soybean cultivar. The presence of *Rsv3* (along with other proteins involved in its network, potentially) is necessary to fully recapitulate the extreme resistance response. It is plausible that avirulent SMV (strain G5) has evolved to manipulate components of the ABA pathway for its own benefit, but more research is needed to understand these differences (Alazem et al., 2019).

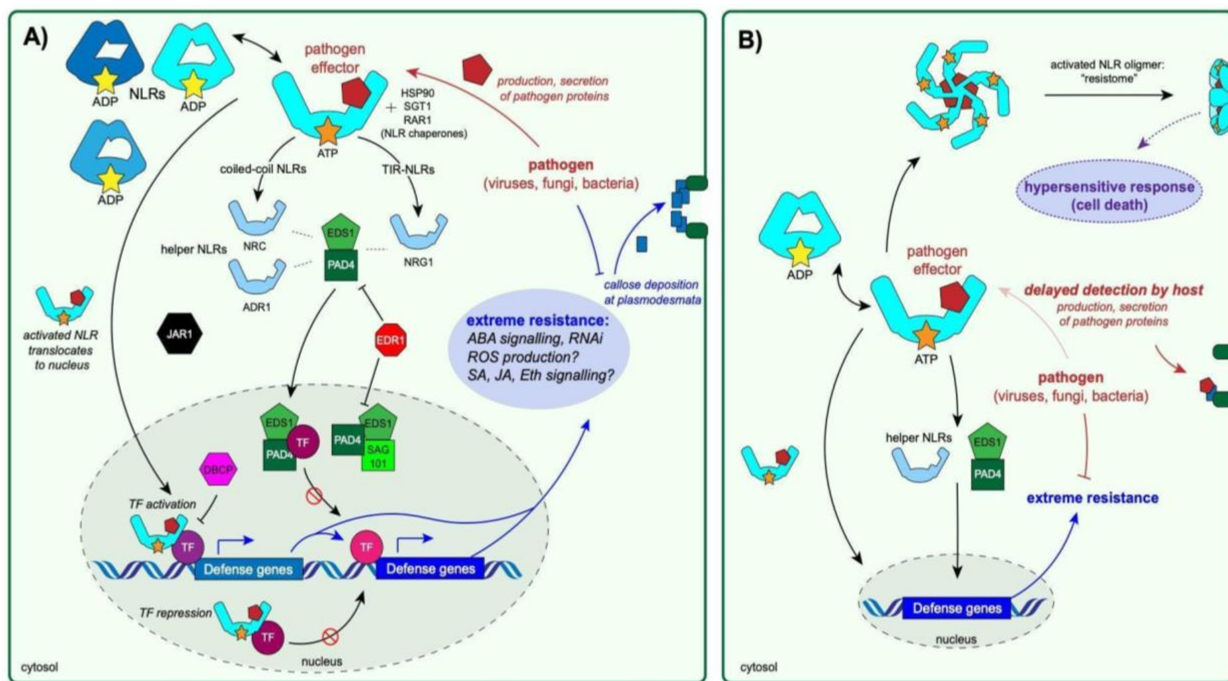

Figure 2: Diagrams of a hypothesized extreme resistance pathway and its possible relationship to the hypersensitive response.
Experimental evidence to date indicates that the proteins highlighted in these diagrams are important for antiviral defenses mechanisms, including extreme resistance **(A)** and the hypersensitive response **(B)**. These two pathways may share specific protein components and/or activation mechanisms, and therefore, may be thought of as a continuum of antiviral response rather than two distinct pathways. The conserved mechanisms of the extreme resistance response

remain largely unknown, although this review (and others(Kuenstler et al., 2016)) have noted some unifying themes. **(A)** The extreme resistance response depends on NLR activation and recognition of a pathogen-produced effector protein. The activated NLR may then translocate to the nucleus, where it could form a complex with transcription factor(s), resulting in either the activation of defense responses by binding to DNA and promoting transcription of defense genes or by preventing DNA binding of transcription factors that repress defense gene expression. Immune signaling through EDS1/PAD4/SAG101 proteins and resulting complexes likely also plays a role in initiating immune responses, but the mechanisms are not understood. Activation of extreme resistance defense pathways results in translational arrest, increased ABA signaling, which allows for callose deposition at plasmodesmata, increased expression of components of the RNA interference (RNAi) pathway, and likely other forms of hormonal signaling. **(B)** The relationship between extreme resistance and the hypersensitive response (HR) is not well understood. The timing of the pathogen recognition event by the host may play a role in determining the phenotype of the resulting resistance response. Delayed recognition of the pathogen could allow for more production or secretion of pathogen-derived proteins, many of which are involved in disabling host immune responses, including preventing callose deposition, which is a key aspect of extreme resistance. Eventual recognition of many pathogen proteins could lead to the oligomerization of activated NLR complexes and the formation of resistome pore-like structures in the cell wall, which may play a role in HR (Adachi et al., 2019b).

Summary

Understanding plant immune responses is a critical component to developing disease-resistant crops and limiting losses due to pathogens. Herein, we review the current literature of mechanisms to extreme resistance to viruses in the economically important plant species, potato and soybean. Although the mechanisms underlying extreme resistance are not well understood, there appear to be some possible unifying themes (as illustrated in **Figure 2a**):

1) Nuclear translocation of an activated NLR post-pathogen recognition may be an important component of extreme resistance: In particular, the NLR protein, Rx1, and its translocation to the nucleus following recognition of PVX coat protein. Further research on possible gene regulation by the nuclear-localized *Rx1-GLK1* complex after PVX detection could

identify other genes involved in extreme resistance. Additional research into whether activated Ry_{sto}, Rsv3, or proteins from individual genes isolated from the *Rsv1* locus translocate to the nucleus following pathogen recognition would provide insight as to if nuclear translocation of NLR proteins and gene regulation is a conserved aspect of the extreme resistance response.

2) **Key immune signaling components are shared between the hypersensitive response (HR) and extreme resistance responses:** This review outlines the current literature regarding extreme resistance to particular strains of PVY and SMV as conferred by the Ry_{sto} gene in potato and the *Rsv1* locus and *Rsv3* gene in soybean. Experiments that involved virus-induced gene silencing demonstrated that extreme resistance conferred by Ry_{sto}, *Rx1*, *Rsv1*, and other NLR genes and extreme resistance loci rely on many of the same proteins as resistance provided by HR (e.g., helper NLRs, *PAD4*, *EDS1*, etc.). Furthermore, many of the NLR proteins that confer extreme resistance can also elicit HR. Future experiments that determine if *Rsv3*-conferred extreme resistance relies on similar mechanisms will be vital to elucidating if extreme resistance across plant species can be attributed to a defined series of molecular events, or if plants have evolved multiple strategies that result in an extreme resistance phenotype.

3) **The interplay between hormone signaling, callose deposition, and translational arrest could form the basis of the extreme resistance response**: Recent studies of extreme resistance to SMV and PVY indicates that virus recognition promotes increases in ABA signaling, which results in increased callose deposition at plasmodesmata, thus preventing viral spread from cell to cell. Increased ABA concentrations in soybeans was associated with increased expression of

genes involved in RNAi, possibly resulting in targeted destruction of viral genomes (**Figure 2A**). It is noteworthy that virulent strains of PVY prevent callose deposition to promote infection. Further research into binding targets of extreme resistance-breaking virus strains will likely yield further insight into the mechanisms of extreme resistance. Likewise, virus-specific translational arrest appears to be an important component of preventing virus replication in extreme resistance conferred by *Rx1* (Bhattacharjee et al., 2009; Richard et al., 2021). The underlying mechanism(s) of translational arrest and the possible involvement of *Argonaute 4* are areas ripe for further research. Finally, genes involved in salicylic acid, ABA, and jasmonic acid signaling have all been implicated in the extreme resistance response. Determining the possible roles and interplay of these hormones during extreme resistance will provide for a better understanding of NLR-mediated virus defense.

There are many questions yet unanswered with regards to extreme resistance, particularly its relationship with HR. It seems that these seemingly distinct resistance phenotypes are connected and may represent ends of the plant immune response spectrum. Research indicates that the expression levels of both the NLR and the pathogen protein recognized by the NLR play roles in determining the phenotypic outcome of the interaction. The timing of immune activation could also be an important aspect in determining the resulting resistance phenotype. Delayed or inefficient recognition of pathogen infection may provide the pathogen with time to disrupt or disable early defense response (i.e., translational arrest, hormonal signaling, callose deposition, RNAi), thus triggering HR (**Figure 2B**), but this idea remains largely untested.

Disease resistance is a growing focus of crop breeding programs around the world. Given the mounting challenges to global agriculture posed by a changing climate and a burgeoning human population, a greater understanding of plant defense responses to viruses will be valuable assets to breeders and growers alike.

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CHAPTER THREE

TRANSCRIPTIONAL RESPONSES TO POTATO VIRUS Y INFECTION IN RESISTANT AND SUSCEPTIBLE POTATO CULTIVARS

Contributions of Authors and Co-authors

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Manuscript Information

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Transcriptional Responses to Potato virus Y Infection in Resistant and Susceptible Potato Cultivars

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Abstract

Potatoes are the world's most produced non-grain crops and an important food source for billions of people. Potatoes are susceptible to numerous pathogens that reduce yield, including Potato virus Y (PVY). Genetic resistance to PVY is a sustainable way to limit yield and quality losses due to PVY infection. Potato cultivars vary in their susceptibility to PVY and include susceptible varieties like Russet Burbank and resistant varieties, including Payette Russet. Although the loci and genes associated with PVY-resistance have been identified, many of the regulations and

mechanisms involved in virus resistance in potato have yet to be fully elucidated. To increase our understanding of PVY infection, host-resistance mechanisms, and transcriptional regulation of these processes, we utilized RNA sequencing to characterize the transcriptomes of two potato cultivars (i.e., PVY-resistant Payette Russet and susceptible Russet Burbank) at different stages post PVY-infection. Differential expression analyses indicate that transcriptional responses associated with the extreme resistance response occur very early after contact with PVY. Analyses of later time points post PVY infection, including systemic infection of virus, during the infection process in Russet Burbank indicate distinct responses occurring at 1 week and 4 weeks post-inoculation. Further small RNA sequencing revealed responses like those observed in other cultivars that undergo tolerant, largely asymptomatic PVY infections. Analyses of alternative splicing indicate that splice variants (or isoforms) of numerous genes, including poly(A) polymerase and a basic-leucine zipper (BZIP) transcription factor family protein, were more abundant in PVY-infected plants and may impact PVY infections. Together these results further our understanding of both resistance and susceptibility to PVY and the genes identified may serve as the source of future studies into potato host-PVY interactions.

1. Introduction

Potato (*Solanum tuberosum*) is the third most important food crop, in terms of worldwide production, with nearly 400 million metric tons produced per year (Devaux et al., 2014). Potato cultivation and consumption from 1990-2020 increased steadily in China, India, and sub-Saharan Africa. The global human population is projected to grow by 2 billion by the year 2050 with the most growth occurring in developing countries (Steinwand and Ronald, 2020). Balancing the

need to feed the world's population while preserving biodiversity will be a challenge. Planting crops that are more resistant to pathogens and abiotic stressors and, therefore, require fewer inputs is one way to minimize the impact of agriculture. Potatoes are reliably grown in diverse landscapes and environmental conditions, but abiotic and biotic stressors, including water stress, heat stress, and pathogens can negatively impact yield and tuber quality (Lal et al., 2017). Reliance upon clonal propagation renders potato production more vulnerable to vertical pathogen transmission and spread as compared to crops planted with true seed (Karasev and Gray, 2013a).

Strains of the Potyvirus, Potato virus Y (PVY), are the most damaging potato-infecting viruses worldwide. PVY infection can render tubers unmarketable and decrease yield. PVY is a positive sense, single-stranded RNA (+ssRNA) virus that infects many Solanaceous plant species, including the economically important vegetables potato, tomato, and pepper (Robaglia et al., 1989). PVY infections of potato plants and tubers arise from infected tubers, mechanical transmission in field settings, and non-circulative, stylet-borne aphid transmission (Ng and Falk, 2006; Fageria et al., 2014). Incidence of PVY infection in the field can increase greater than 10-fold from the start of the growing season to harvest and a yield reduction of 150 lbs/acre is estimated for every 1% increase in PVY prevalence (Nolte et al., 2004). Pathogen-free seed and genetic resistance to PVY are the most economical and environmentally sustainable ways to stem losses and spread of PVY.

Like many RNA viruses, genetic variation in PVY is generated via error-prone replication and recombination (Lauring et al., 2013). Recombination events between the once prevalent PVY^{O} and PVY^{N} strains has given rise to many recombinant strains, including PVY^{NTN} and $PVY^{N\text{-}Wi}$, which are now the most globally common and economically destructive (Karasev and Gray, 2013b). These recombinant PVY strains are more prevalent than the parent strains largely due to their ability to evade detection and replicate in potato cultivars with strain-specific resistance. The rise of destructive recombinant strains may be in part due to selection pressures imposed by resistant cultivars, as increased local prevalence of recombinant strains strongly correlates with increased propagation of cultivars with strain specific resistance (Funke et al., 2017).

Cultivar specific PVY-resistance is largely dependent on host protein recognition of a viral effector protein (Calil and Fontes, 2017). This response – termed effector triggered immunity (ETI) – is typified by build-up of reactive oxygen species (ROS) in infected and surrounding cells and the induction of defense related proteins (e.g., pathogenesis-related (PR) proteins). The hypersensitive response (HR) can be induced by ETI and results in programmed cell death within the infected and surrounding areas after ROS accumulation (Kourelis and Hoorn, 2018; Ross et al., 2021). HR prevents or limits the spread of pathogens that require living tissue to survive and replicate. ETI also results in the accumulation of the key immune signal, salicylic acid (SA), first in the infected area and then systemically, thereby priming non-infected distant tissues for possible pathogen invasion (Kumar, 2014). Planting of PVY-resistant potato cultivars limits growers' losses, but also results in the evolution and selection of virus strains that produce effector proteins with slightly altered recognition motifs. For example, the potato Ny_{tbr} gene

confers resistance to PVY^O by recognizing and binding two amino acid motifs (IGN and CCCT) on the PVY-encoded suppressor of RNA silencing, Helper-component protease (HC-Pro) (Tian and Valkonen, 2013). The host plant does not recognize PVY^N because the locations of the HC-Pro recognition motifs are reversed relative to PVY^O. Similarly, recombinant PVY-strains often have unrecognizable motifs and, in turn, escape host detection and successfully replicate and spread (Sztuba-Solińska et al., 2011; Tian and Valkonen, 2013).

Immune functions in all organisms must be finely regulated. In plants, many forms of pathogen resistance (e.g., ETI and HR) are controlled by a suite of nucleotide binding, leucine-rich repeat proteins (NLRs) (Kourelis and Hoorn, 2018). NLR proteins are responsible for detecting intracellular pathogens and initiating an appropriate immune response. NLR abundance and function are regulated at the transcriptional, post-transcriptional, and translational levels (Richard et al., 2018). The abundance of NLR transcripts increases after pathogen detection and may heighten detection and defense responses in surrounding cells (Lai and Eulgem, 2018). Slight changes of NLR transcript levels (e.g., less than two-fold) in the absence of pathogens can result in constitutively activated immune responses and growth defects in Arabidopsis (Stokes et al., 2002; Chang et al., 2013).

RNA interference (RNAi) mechanisms, including the small interfering RNA (siRNA) and microRNA (miRNA) pathways, are critical for antiviral defense and the regulation of gene expression in plants (Soosaar et al., 2005; Dunoyer et al., 2010). In brief, siRNAs are produced

by Dicer-like (DCL) proteins that cleave cytosolically located long double-stranded RNAs (dsRNAs), including the replicative intermediate form of +ssRNA viruses, into 21-24 nucleotide (nt) siRNA duplexes (Galun, 2005). An ARGONAUTE (AGO) protein in complex with other cofactors retains one strand of the siRNA duplex to form the RNAi induced silencing complex (RISC) (Vaucheret, 2008). The guide siRNAs facilitate binding of the RISC to cognate target RNA sequences, which are either sliced or translationally inhibited (Ding and Voinnet, 2007; Szittya and Burgyán, 2013; Fang and Qi, 2016).

MicroRNAs (miRNAs) primarily target host transcripts to regulate mRNA levels in the cell and are important in modulating potato gene expression, including genes involved in immune responses (Axtell and Bartel, 2005). MicroRNAs are 21-22 nt small RNAs produced from DCL-mediated cleavage of host-encoded dsRNA transcripts. The production of phased, small interfering RNAs (phasiRNAs) occurs when one or two miRNA-loaded RISC complexes bind to a single RNA transcript and cleave it (Zhai et al., 2011). RNA-dependent RNA polymerase 6 (RDR6) is recruited to the cleaved transcript which is used as a template for dsRNA synthesis. The long dsRNA is cleaved by DCL every 21-22 nucleotides, resulting in a characteristic 'phasing' pattern (Fei et al., 2013). The resulting phasiRNAs are incorporated into RISC where they target cognate sequences for silencing and effectively amplify the miRNA signal. MicroRNAs and phasiRNAs are key post-transcriptional regulators of NLR gene expression (Lai and Eulgem, 2018). For example, miR482 and miR2118 families in *Solanaceae* target conserved P-loop domains within NLR transcripts to initiate phasiRNA biogenesis, which results in reduced expression of target genes (Shivaprasad et al., 2012). The enzymatic activity of NLRs is

also regulated post-translationally. Proteins bound to ATP recognize and bind pathogen effectors and elicit an immune response whereas ADP-bound proteins do not (Tameling et al., 2002). It is hypothesized that NLRs likely exist in either an ATP or ADP-bound state in the cell (Wang et al., 2019). According to this model, the population of NLRs in a cell is constantly in equilibrium between these "On" and "Off" states, with the equilibrium shifted towards the ADP-bound, "Off" state until pathogen recognition and ligand binding (Wang et al., 2019).

To better understand potato antiviral defense mechanisms and identify genes and mechanisms that may prove useful in breeding resistant and/or tolerant cultivars, we investigated PVY-infection in two potato cultivars: Russet Burbank and Payette Russet. Russet Burbank is the most widely grown cultivar in the United States, due primarily to its uniform tuber production and favorable cooking qualities (Bethke et al., 2014). Russet Burbank is susceptible to many pathogens, including PVY. Resistance to PVY is usually virus strain dependent and immune activation in plants can present various visual symptoms (Funke et al., 2017; Ross et al., 2021). Extreme resistance is defined by a lack of symptoms and little to no detectable viral replication after inoculation. Payette Russet and other potato cultivars containing Ry_{sto}-mediated are resistant to most strains of PVY and Potato virus A (PVA) (Grech-Baran et al., 2020). Payette Russet has 'extreme resistance' governed by the NLR gene, Ry_{sto}, which has been introgressed from the wild potato species, *Solanum stoloniferum*, into commercial cultivars (Grech-Baran et al., 2020). The Ry_{sto} locus has been mapped to the distal arm of potato chromosome 12, which contains four clusters of resistance genes (Brigneti et al., 1997; Kondrák et al., 2020). An NLR protein encoded by the Ry_{sto} gene recognizes and binds to the coat protein encoded by strains of PVY

and PVA (Grech-Baran et al., 2020). The transcriptional responses following pathogen recognition by *Ry*$_{sto}$ that result in an extreme resistance phenotype have not been described.

We examined the transcriptional responses and small RNA profiles in the PVY-susceptible potato cultivar, Russet Burbank at multiple time points post-PVY infection. In parallel we examined the transcriptional response mounted by the PVY-resistant cultivar Payette Russet at 24-hours post inoculation, as we hypothesized that extreme resistance mechanisms likely occur soon after virus detection. Assessment of the initial antiviral responses in this cultivar facilitated further characterization of extreme resistance and comparison to the antiviral responses mounted by the PVY-susceptible Russet Burbank potato cultivar. We determined that PVY infection results in broad changes to the transcriptional and post-transcriptional landscape and although there is some overlap between the cultivars tested and time points observed, prominent differences were observed. Thousands of genes were differentially expressed in Payette Russet and these genes may be involved in the extreme resistance response. Similarly, PVY infection resulted in thousands of genes differentially expressed in Russet Burbank, some of which may be promising candidates for genetic engineering for resistance through loss of susceptibility. In addition, we characterized PVY-associated changes in small RNA profiles and alternative splicing which have not been previously described. Overall, this study improves our understanding of the transcriptional and post-transcriptional responses to PVY infection in potato and identifies genes that may be of interest to future studies.

2. Materials and Methods

2.1 Potato sources and growing conditions

Potato cultivars ‘Russet Burbank’ and ‘Payette Russet’ were propagated as pre-nuclear tissue culture plantlets using 4.44 g/L Murashige and Skoog medium with Gamborg’s vitamins (PhytoTechnology Laboratories, Shawnee Mission, KS). Plantlets were transplanted into 5″ pots containing Sunshine Mix 1 (Sungro Horticulture, Vancouver, Canada), and grown in a growth chamber at 22°C under a 16:8 day:night photoperiod. Watering was performed as needed and MiracleGro Shake 'n Feed All Purpose Plant food was applied biweekly. Plants were grown for four weeks in pots prior to mechanical inoculation.

2.2 PVY inoculum preparation and inoculation protocol

PYV strain Wilga ($PVY^{N\text{-}Wi}$) (NCBI Accession: HQ912863) was obtained from the Karasev Lab at the University of Idaho. $PVY^{N\text{-}Wi}$ stock was maintained in tobacco plants (*Nicotiana tabacum* ‘Samsun’), which were cultivated from seed with a 24 h photoperiod at 22°C in 10″ pots. Tobacco plants were grown for approximately four weeks prior to virus inoculation. Tobacco tissue that exhibited characteristic mosaic symptoms four to five weeks post-inoculation and was PVY-positive by Double antibody sandwich-ELISA was used to prepare inoculum. Virus

inoculum was prepared by placing 1 g infected tobacco leaf tissue in 10 mL inoculation buffer (0.05 M disodium phosphate, 0.05 M monopotassium phosphate, pH 7.5) in a 12 cm by 12 cm universal extraction bag (Bioreba, Reinach, Switzerland). Leaf tissue was homogenized in buffer using a tissue homogenizer (Bioreba, Reinach, Switzerland) mounted on a drill press (Craftsman, Hoffman Estates, IL). Carborundum powder was sprayed onto a targeted leaf and a cotton-tipped swab was used to gently abrade the leaf surface. PVY inoculum (200 uL) was applied to the leaf surface and spread across the abraded area with a pipette tip. Leaves were rinsed with water one hour after inoculations to remove any residual buffer. Mock-infected plants were treated with inoculation buffer only, with all other conditions held constant. Sampling of leaf tissue occurred at 24 hours post infection (hpi), and then at weekly intervals up until 4 wpi. Approximately 200 mg of leaf tissue was taken from the tip of the inoculated leaf at 24 hpi. Approximately 200 mg of leaf tissue was taken from upper, uninoculated leaves (the fourth leaf down from the apical meristem) at 1-4 wpi. Leaf tissue was stored in a -80°C freezer until analysis.

2.3 RNA isolation and cDNA synthesis

RNA was extracted from leaf tissue samples using TRIzol reagent (Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. Reverse transcription reactions (25 µl) were performed using 2 µg of total RNA and random hexamer primers (500 ng) (IDT, Coralville, IA) incubated with Maloney murine leukemia virus (M-MLV) reverse transcriptase (Promega, Madison, WI) for 1 h at 37 °C, according to the manufacturer's instructions.

2.4 Quantitative PCR (qPCR)

Quantitative PCR was performed in triplicate using a CFX Connect Real Time machine (BioRad, Hercules, CA). Each 20 µl reaction contained 2 µL cDNA, 1X ChoiceTaq Mastermix (Denville, Holliston, MA), 0.1 µM of each forward and reverse primer, 1X SYBR Green (Life Technologies, Carlsbad, CA), and 25 mM $MgCl_2$. To estimate the relative PVY abundance based on a standard curve, the corresponding segments of PVY were cloned into plasmids. Plasmid standards containing from 10^3 to 10^9 copies per reaction were used as templates for qPCR to generate standard curves. The accurate detection limit was 10^3 copies per reaction for the PVY^{N-Wi} qPCR primer set. The linear standard equations for the PVY^{N-Wi} qPCR primer set, generated by plotting the crossing point (Cp) versus the $\log_{10}$ of the initial plasmid copy number was as follows: PVY^{N-Wi} $Cp = -3.20x + 44.54$, $R^2 = 0.99$. Quantitative-PCR reactions containing no template served as negative controls. Melt point analyses, agarose gel electrophoresis, and sequencing of initial qPCR products were used to verify qPCR specificity (Ginzinger 2002).

The ΔΔC(t) method was used to calculate relative abundance of gene of interest (GOI) in individual leaf tissue samples because it was most accurate; the ΔΔC(t) method ensures that results are not skewed by inadvertent differences in RNA reverse-transcription efficiencies and starting cDNA template abundance. The ΔC(t) for each sample was calculated by subtracting the C(t) of the reference gene *Exosome-associated protein* from the GOI C(t). The potato gene encoding *Exosome-associated protein* was selected as an appropriate housekeeping gene for qPCR because analysis of the transcriptomic data presented herein confirmed that *Exosome-*

associated protein expression levels were similar in all sequenced libraries. The $\Delta\Delta C(t)$ was calculated by subtracting the average virus-infected $\Delta C(t)$ values from the ΔCt values for each treatment group. For host gene expression analyses and transcriptomic analysis validation, the percent gene expression for each gene of interest (GOI) was calculated using the following formula: $2^{-\Delta\Delta C(t)} \times 100 = \%$ gene expression, in which $\Delta C(t) = \text{GOI } C(t) - Ref\ C(t)$, and $\Delta\Delta C(t) = \text{sample } \Delta C(t) - \text{mock-infected control } \Delta C(t)$. Wilcoxon ranked-sum were used to identify statistical differences in host gene expression.

2.4 Transcriptome library preparation and sequencing

Prior to transcriptome library preparation, RNA from each sample was DNAse treated using an on-column DNase Treatment (Qiagen). The RNA quality was assessed using an Agilent 2200 Bioanalyzer (Santa Clara, CA, USA) and quantified with a Thermo Scientific NanoDrop 2000 Spectrophotometer (Waltham, MA, USA). The RNA was sent to Roy J. Carver Biotechnology Center at the University of Illinois at Urbana–Champaign for library preparation (Illumina TruSeq Stranded RNA Sample Prep kit). mRNA libraries were poly-A purified and sequenced on two 2x100 nucleotide lanes of the Illumina HiSeq 4000 which produced over 1.2 billion reads (15.8x coverage over the ~840 mB pair potato genome). Small RNA libraries were sequenced on one 1x50 nucleotide lane of the Illumina HiSeq 4000 which produced over 370 million reads. Sequence data was deposited into the NCBI Sequence Read Archive under submission number SUB10421751.

2.5 Differential gene expression analysis

FastQC and was used to remove low quality reads (<Q30). Illumina adapters were trimmed with Trimmomatic, and reads were aligned to the *S. tuberosum* genome assembly PGSC v6.1 with Hisat2; on average, ~85% of reads from each sample were mapped to the genome (**Supplementary Table – S1**). Alignments were assembled and count indices were made for each library via Stringtie (Love et al., 2014). Differential expression analysis of alignments was performed with the DESeq2 in R. Genes with strong evidence of differential expression between treatments (adj. p-value ≤ 0.05) were further analyzed with the gene and functional ontology program, g:Profiler (Raudvere et al., 2019). Small RNAs were aligned to the potato referenc e genome (PGSC v6.1) with Shortstack (Axtell 2015). PhasiRNAs were identified from small RNA clusters with phased scores > 30. MicroRNAs detailed in the text were identified as putative miRNAs by Shortstack and aligned using BLAST (Basic local alignment search tool) with experimentally verified miRNAs using Penn State's Small RNA database server, which facilitates searches against miRbase as well as small RNAs identified by Shortstack from hundreds of plant reference libraries (Altschul et al., 1990, Lunardon et al., 2020). Differential expression of small RNAs was done by taking the raw counts data from Shortstack and inputting those data into DESeq2.

2.6 BLAST search of potato transcript sequences against potato genome, wild potato species, and all organisms

The following Spuddb and NCBI NR sequence, annotation, accession and taxonomy sources and the DIAMOND executable were downloaded on September 14, 2021:

http://spuddb.uga.edu/data/DM_1-3_516_R44_potato.v6.1.working_models.func_anno.txt.gz,

http://spuddb.uga.edu/data/DM_1-3_516_R44_potato.v6.1.working_models.cdna.fa.gz,

ftp://ftp.ncbi.nih.gov/blast/db/FASTA/nr.gz,

ftp://ftp.ncbi.nlm.nih.gov/pub/taxonomy/accession2taxid/prot.accession2taxid.FULL.gz,

ftp://ftp.ncbi.nlm.nih.gov/pub/taxonomy/taxdmp.zip,

http://github.com/bbuchfink/diamond/releases/download/v2.0.11/diamond-linux64.tar.gz.

The DIAMOND database was built, and DIAMOND blastx was run on r5d.8xlarge and c5ad.24xlarge AWS EC2 instances respectively using the Amazon Linux 2 AMI as the machine image. Simplified versions of the commands building the DIAMOND database of NCBI's NR database and performing the DIAMOND blastx search comparing SpudDB gene models and NCBI's NR are as follows: "./diamond makedb --in nr.gz--taxonmap prot.accession2taxid.FULL.gz --taxonnodes ./taxdmp/nodes.dmp --taxonnames ./taxdmp/names.dmp --db nr.full"; "./diamond blastx –query DM_1-3_516_R44_potato.v6.1.working_models.cdna.fa --db nr.full --sensitive --iterate --evalue 0.001 --max-target-seqs 200 --max-hsps 1 --index-chunks 1 --block-size 7 --unal 1 --log --outfmt 6 qseqid sseqid evalue bitscore score salltitles -o diamond_results.tsv"

A custom script in R was used to extract the best (first) hit for each of *Solanum tuberosum*, potato breeding species, and 'any organisms' using regular expressions on the salltitles field. The potato breeding species used for these extractions were: *Solanum tuberosum*, *Solanum acaule*, *Solanum bulbocastanum*, *Solanum candolleanum*, *Solanum commersonii*, *Solanum demissum*, *Solanum maglia*, *Solanum microdontum*, *Solanum stoloniferum*, and *Solanum vernei*.

3. Results and Discussion

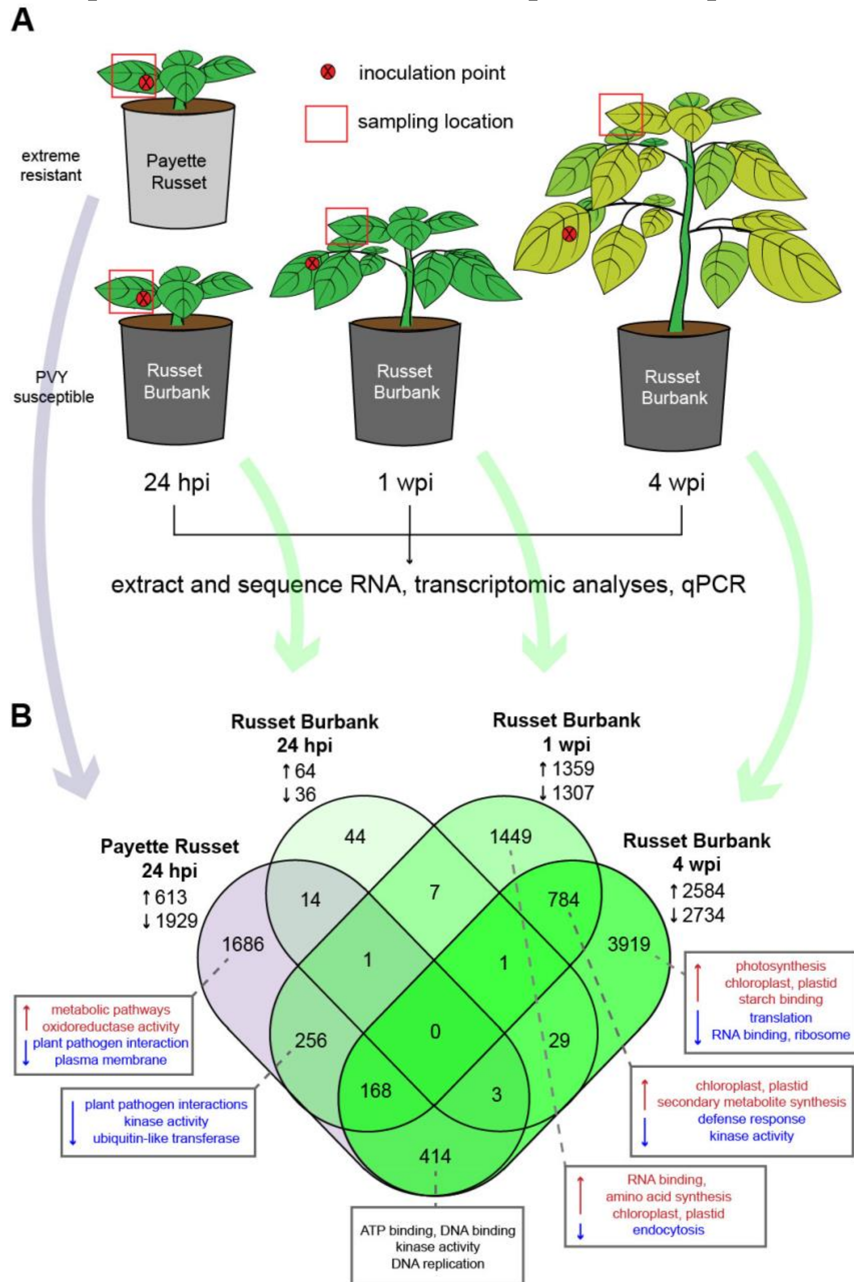
3.1. Transcriptome level assessment of Potato virus Y (PVY) infected potato cultivars

To characterize the transcriptional responses of PVY-resistant Payette Russet and PVY-susceptible Russet Burbank potato cultivars in response to PVY-infection, as well as to compare responses between cultivars, PVY-inoculated plants were sampled over a time course of infection and gene expression was examined by high throughput sequencing. For these studies, virus-free potato plantlets were propagated aseptically in tissue culture and subsequently transferred to potted soil in a growth chamber. For each of three biological replicates of the experiment, six plants were mechanically inoculated with PVY^{N-Wi} (NCBI Accession: HQ912863) or inoculation buffer (i.e., mock-infected) after four weeks of growth in soil (Boyd et al., 2018). Plants leaves were sampled at three time points after infection (24 hpi, 1 wpi, and 4 wpi), with the inoculated leaf being sampled at 24 hpi, and upper, uninoculated leaves sampled at the later time points. At each time point post infection leaf tissue samples were obtained from six plants per treatment (i.e., PVY- or mock-infected) and RNA was isolated and quantified (**Figure**

1A). Virus abundance in individual plants at each time point were quantified by quantitative PCR (qPCR) using RNA copy number (i.e., viral genomes and transcripts) as a proxy for virus abundance (**Supplemental File S1A**). Transcriptome sequencing libraries were produced with RNA obtained from a subset of individual plants in all treatment groups in biological replicate #1

(i.e., n = 3 plants per treatment group per timepoint) using the Illumina TruSeq Stranded mRNA Prep kit. High throughput sequencing was carried out using an Illumina HiSeq 4000.

Figure 1: Transcriptome level evaluation of the response of two potato cultivars indicates



that response to Potato virus Y-inoculation varies by cultivar and timepoint. (A) Schematic representation of the experiments performed to investigate potato antiviral immune responses. Potato plants were infected with PVY or mock-infected (n = 6 plants per treatment, per timepoint, per biological replicate). Leaf tissue samples were collected from the inoculation point

at 24 hours post infection (hpi) for both cultivars and at 1- and 4-weeks post infection (wpi) in PVY-susceptible Russet Burbank plants. RNA was extracted from leaf tissue samples and prepared for either qPCR or high throughput sequence analysis. qPCR was used to quantify PVY abundance in all plants and to validate differential gene expression results for a subset of genes in a subset of plants from all three biological replicates. (**B**) Potato transcriptional responses to PVY-infection are time and cultivar dependent. The Venn diagram illustrates the number of unique and shared differentially expressed genes (DEGs) in Payette Russet (blue) and Russet Burbank (shades of green) plants in response to PVY infection. No differentially expressed genes were shared between all timepoints and cultivars, though hundreds of differentially expressed genes were shared between Payette Russet at 24 hpi and Russet Burbank at 1 and 4 wpi. Full lists of differentially expressed genes and their corresponding fold changes are included in **Supplementary File S1**.

The resulting reads were aligned with HiSat2 and differential expression was assessed using Stringtie and DESeq2 (Love et al., 2014; Pertea et al., 2016) The reads were aligned to the potato DM version 6.1 genome (Pham et al., 2020). Transcript sequences from the DM version 6.1 working gene models were BLAST searched against potato, wild potato species commonly used for breeding purposes, and all organisms to provide a link between the potato genome online resources and the NCBI online resources (**Supplementary File S1**).

Transcriptome level analyses (RNAseq) identified thousands of potato genes that exhibited differential expression within the context of PVY infection, potato cultivar, and time point post infection (**Figure 1, Table 1, and Supplementary File S1**). Notable genes involved in plant immunity, including RNAi (i.e., *Dicer2-like, Dicer4-like, Argonaute1-like*), plant immune signaling (i.e., *NPR1-like, WRKY DNA-binding protein*), and other genes potentially involved in plant immunity (i.e., *Kunitz family trypsin and protease inhibitor protein*, *HSP70-interacting protein*) exhibited differential expression in at least one treatment group or time point (**Table 1**). For example, expression of the salicylic acid regulator, *NPR1-like*, was reduced PVY-infected

Payette Russet plants at 24 hpi (-1.04 log_2 fold change), and in PVY-infected Russet Burbank plants at 1 wpi (-1.07 log_2 fold change) and 4 wpi (-0.75 log_2 fold change), compared to the relevant mock-infected plants (**Table 1**). These results may indicate a decrease in salicylic acid signaling at these time points.

		Payette Russet	Russet Burbank		
gene name	**gene ID**	**24 hpi**	**24 hpi**	**1 wpi**	**4 wpi**
MLP-like protein	Soltu.DM.04G002870	-1.58	-0.90	-2.2	N
NPR1-like protein	Soltu.DM.07G014680	-1.04	N	-1.07	-0.75
Polynucleotidyl transferase, ribonuclease H-like superfamily protein	Soltu.DM.06G029420	-3.12	N	-1.70	-0.88
Leucine-rich repeat transmembrane protein kinase	Soltu.DM.02G014860	1.41	N	-1.30	-0.98
NAC (No Apical Meristem) domain transcriptional regulator superfamily protein	Soltu.DM.04G005970	-3.38	N	-1.75	-2.44
HSP70-interacting protein	Soltu.DM.03G032780	N	N	2.11	1.49
HSP70-interacting protein	Soltu.DM.03G032830	N	N	2.08	1.61
Argonaute1-like	Soltu.DM.03G019130	N	N	0.72	0.53
Dicer4-like	Soltu.DM.07G000040	N	N	-0.64	N
Dicer2-like	Soltu.DM.11G004150	N	N	N	0.94
TCP-1/cpn60 chaperonin family protein	Soltu.DM.04G011720	5.76	N	3.93	N
Calmodulin like	Soltu.DM.11G024590	-3.48	N	-2.38	N
WRKY DNA-binding protein	Soltu.DM.06G018840	-3.05	N	-1.05	-1.88
F-box family protein	Soltu.DM.11G022120	-3.92	N	-2.03	-2.44
Kunitz family trypsin and protease inhibitor protein	Soltu.DM.03G018580	N	N	2.36	2.12
Kunitz family trypsin and protease inhibitor protein	Soltu.DM.03G018450	N	N	2.34	1.44
Terpene synthase	Soltu.DM.10G000510	2.76	N	1.72	1.94
Terpene synthase	Soltu.DM.09G029760	1.37	3.69	N	N

Table 1. Select genes that exhibited notable changes in expression in PVY-infected potato plants relative to mock-inoculated plants.

Table highlights a small subset of DEGs identified by transcriptomic analysis; the relative expression of these genes of interest varies by time point (24 hours post inoculation, 1- and 4-weeks post inoculation) and potato cultivar (Payette Russet and Russet Burbank).

The expression of a subset of the differentially expressed genes (DEGs) that were identified by transcriptomic analysis for each time point (24 hours post inoculation, 1- and 4-weeks post

inoculation) and cultivar (PVY-resistant Payette Russet, PVY-susceptible Russet Burbank) was also assessed by qPCR of individual plant samples from all three biological replicates (n = 18 plant samples per treatment group) (**Figure 2, Supplemental Figures S1 and S2**). Statistical differences in gene expression between PVY-infected and mock-infected potato plants (n = 6 per biological replicate) were evaluated using Wilcoxon ranked sum test, *$p<0.05$. Specifically, qPCR assessment of the relative expression of *Dicer2-like*, *NPR1-like*, and *TCP* in PVY-inoculated Payette Russet leaves at 24 hpi relative level in mock-infected control plants, determined that their expression was similar, lower, and higher, respectively (**Figure 2A**). Evaluation of the expression of *Dicer2-like*, *MLP*, and *NPR-1* like in PVY-infected Russet Burbank plants relative to uninfected controls indicated that the only the expression of *MLP* was reduced by PVY-infection (**Figure 2B**). The qPCR gene expression results from upper, uninoculated leaves of PVY-infected Russet Burbank plants at 1 wpi compared to mock-infected controls indicate a reduced expression of *Dicer4-like* and *NPR1-like*, and a non-statistically significant increase the expression of a putative mitochondrial RNA helicase expression (**Figure 2C**). The qPCR gene expression results from upper, uninoculated leaf samples of PVY-infected Russet Burbank plants at 4 wpi compared to uninfected control plants indicated that the expression of *AGO1-like* and *Dicer2-like* was increased, whereas the *NPR1-like* expression was reduced (**Figure 2D**). All of the qPCR results mirrored the transcriptome level assessment results, except that the increased expression of RNA helicase in PVY-infected Russet Burbank plants was not statistically significant by qPCR whereas it was in the transcriptome data. The qPCR data validated the high throughput sequencing data and confirmed that the results were

robust, as they were similar in three independent biological replicates (**Supplemental Figures S1 and S2**).

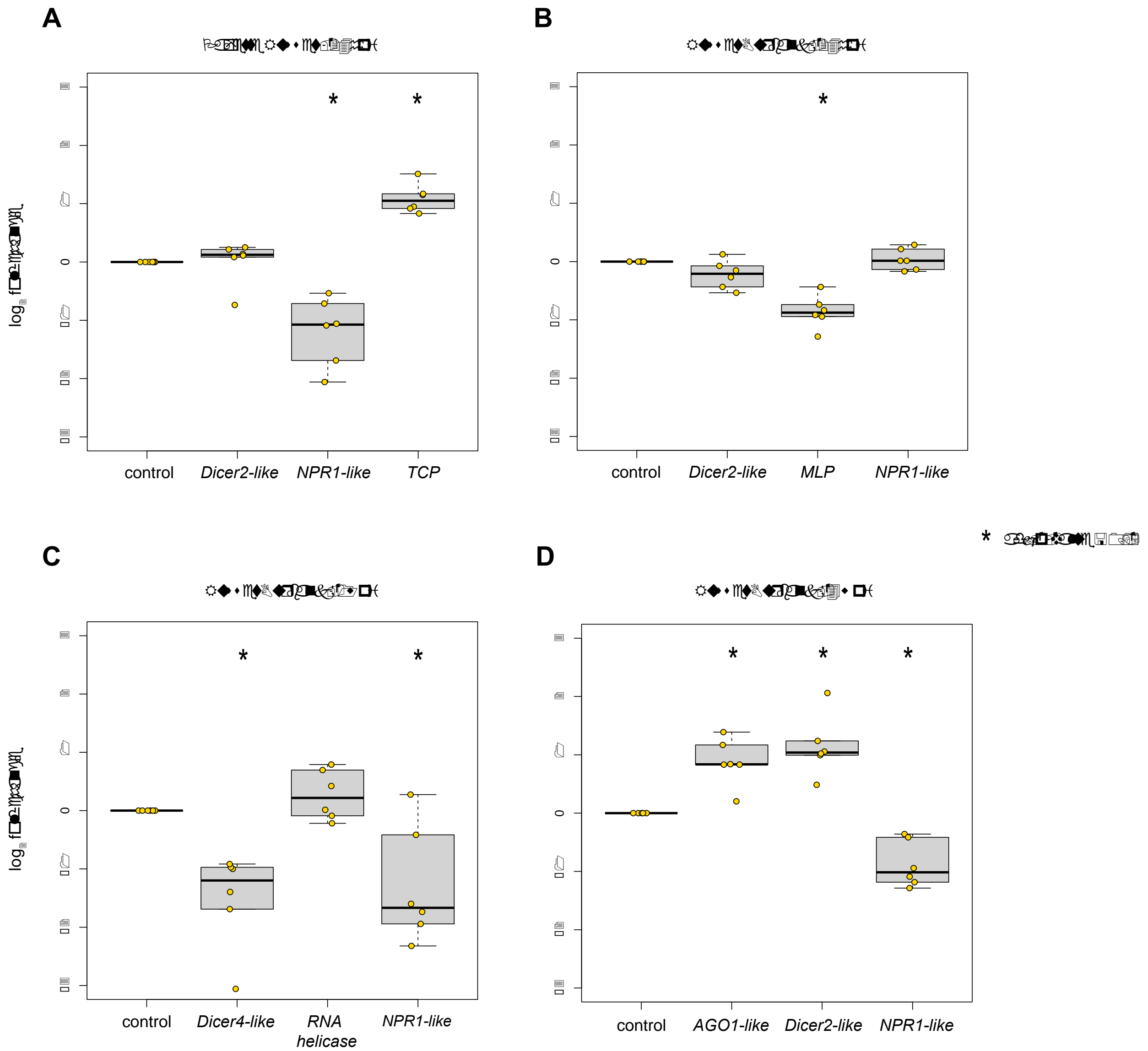


Figure 2: Potato transcriptome data supported by quantitative PCR analysis.

Quantitative PCR (qPCR) was used to validate the differential expression analysis results for a subset of genes for each time point (24 hours post inoculation, 1- and 4-weeks post inoculation) and cultivar (PVY-resistant Payette Russet, PVY-susceptible Russet Burbank) used in this study. (**A**) qPCR assessment of the relative expression of *Dicer2-like*, *NPR1-like*, and *TCP* in PVY-inoculated Payette Russet leaves at 24 hpi relative level in mock-infected control plants, determined that their expression was similar (0.04 log_2 fold change), lower (-1.11 log_2 fold change), and higher (1.10 log_2 fold change), respectively. **(B)** Evaluation of the expression of *Dicer2-like*, *MLP*, and *NPR1- like* in PVY-infected Russet Burbank plants at 24 hpi relative to uninfected controls indicated that the only the expression of *MLP* was reduced by PVY-infection (-0.84 log_2 fold change). **(C)** The qPCR gene expression results from upper, uninoculated leaves of PVY-infected Russet Burbank plants at 1 wpi compared to mock-infected controls indicate a reduced expression of *Dicer4-like* (-1.35 log_2 fold change) and *NPR1-like* (-0.98 log_2 fold change), and a non-statistically significant increase the expression of a *putative mitochondrial RNA helicase expression* (0.32 log_2 fold change). **(D)** The qPCR gene expression results from upper, uninoculated leaf samples of PVY-infected Russet Burbank plants at 4 wpi compared to uninfected control plants indicated that the expression of *AGO1-like* (0.92 log_2 fold change) and *Dicer2-like* (1.22 log_2 fold change) was increased, whereas the *NPR1-like* expression (-0.83 log_2 fold change) was reduced. Statistical differences in gene expression between mock-infected and PVY-infected potato plants (n = 6) were performed using Wilcoxon ranked sum test, *$p<0.05$. All of the qPCR results mirrored the transcriptome level assessment results, except that the increased expression of RNA helicase in PVY-infected Russet Burbank plants was not statistically significant by qPCR whereas it was in the transcriptome data. **Figure 2** includes a data from one representative biological replicate and the results from all three biological replicates are included (**Supplementary Figures S1 and S2**).

To evaluate the cellular pathways and mechanisms that are regulated in response to virus infection, genes that were differentially expressed in PVY-infected plants, compared to mock-infected plants, were further analyzed using gene ontology (GO) and cellular pathway enrichment analyses. Specifically, the GO program, g:Profiler, was used with the Phureja DM1-3 PGSC protein identification schema to characterize differentially expressed genes by pathway, molecular function, and biological process (Raudvere et al., 2019) (**Figure 1B**). Select GO terms and DEGs associated with particular comparisons are described in detail greater detail below.

3.1.1 Transcriptomic differences between PVY-resistant Payette Russet and susceptible Russet Burbank potato cultivars 24 hours post PVY-inoculation

The Payette Russet potato cultivar exhibits extreme resistance to PVY. Extreme resistance is characterized by little or no virus replication at the site of infection and a lack of visual symptoms of either infection or an immune response (i.e., hypersensitive response) (Bendahmane et al., 1999; Ross et al., 2021). We hypothesized that the PVY-limiting immune responses in Payette Russet would occur soon after virus inoculation. Therefore, to characterize these initial immune responses samples were obtained from the PVY-inoculated leaves of potato plants at 24 hours post infection (hpi). Virus levels in the PVY-infected leaves were near the limit of qPCR detection and accounted for less than or equal to 1% of the total reads in each of the 24 hpi sequencing libraries (**Supplemental File S1B**). Differential gene expression analysis of Payette Russet leaf samples at 24 hpi identified over 2,000 genes that were differentially expressed genes in PVY-inoculated plants as compared to mock-inoculated plants (adj. $p \leq 0.05$) (**Figure 1B, Supplemental File S1C**). The majority (1,929 genes) exhibited decreased expression while 613 exhibited increased expression (**Figure 1B, Supplemental File S1D**). GO analysis of the differentially expressed genes in Payette Russet at 24 hpi indicated that genes involved in phosphorylation pathways, the hypersensitive response, and plant-pathogen interactions were enriched among genes that exhibited decreases in expression (adj. $p \leq 0.05$) (**Supplemental File S1E**). Because this cultivar is resistant to PVY infection, it is likely that these DEGs contribute to initial events in potato antiviral defense. We identified differentially expressed genes with characterized functions in plant antiviral defense. For example, *NPR1-like*

(NONEXPRESSOR OF PATHOGENESIS-RELATED 1-LIKE), controls cellular salicylic acid concentrations, which is a key signaling molecular controlling responses to pathogen infection (Fan and Dong, 2002; Wu et al., 2012). RNA sequencing results indicate a decrease in transcription of *NPR1-like* (log_2 fold change = -1.04), and qPCR analyses confirmed this result (**Figure 1B, Supplemental Table 1, Supplemental Figures S1, S2**). Increased expression of *NPR1-like* results in greater cellular salicylic acid concentrations, so the observed decrease is possibly indicative of a waning immune response (Fitzgerald et al., 2004; Chern et al., 2005). In another example, pathogenesis-related proteins (PR proteins) are induced in leaf tissue following pathogen detection and can be used as immune response markers in plants (Breen et al., 2017). Seven different PR proteins exhibited decreased expression in PVY-treated Payette Russet leaves as compared to mock-infected plants, also possibly indicating that an immune response involved may have already occurred by 24 hpi (**Supplemental File 1D**). PR1 proteins, which are a part of a variety of defense responses in plants, contain a defense-related signaling domain and bind sterols, though their defense function(s) require further characterization (Lu et al., 2012). That the majority of the DEGs exhibited decreases in expression in Payette Russet at 24 hpi may indicate that 24 hpi represents the tail-end of the extreme resistance immune response in Payette Russet leaves, though without analysis of other, earlier time points this remains uncertain. Further research investigating when the transcriptional responses associated with extreme resistance to PVY begin and which genes are involved would enhance our understanding of this resistance response (Kogovšek et al., 2010; Goyer et al., 2016).

Russet Burbank is susceptible to most PVY strains, including $PVY^{N\text{-}Wi}$, and thus we wanted to compare the transcriptional changes between extreme resistant Payette Russet and Russet Burbank. Ninety-nine genes were differentially expressed at 24 hpi in PVY-infected Russet Burbank plants as compared to mock-infected plants (adj. $p \leq 0.05$) (**Supplemental File S1F, G**). The majority of the differentially expressed genes (DEGs) (64 out of 99 exhibited decreased expression while 36 genes exhibited increased expression in PVY-infected plants (**Figure 1B, Supplemental File S1G**). Gene ontology analysis indicated that PVY-infected Russet Burbank plants exhibited enrichment for DEGs involved in transmembrane transport, photosynthesis (photosystem I, II), and metabolism at 24 hpi (adj. $p \leq 0.05$). Among the DEGs with increased transcription, terpene synthase activity, ion homeostasis, and transmembrane transporter activity were enriched. Among the DEGs with decreased transcription, kinase activity, chlorophyll binding, and photosynthesis activities were enriched (**Supplemental File S1H**).

Like Payette Russet, the genes that exhibited the greatest increases in expression at 24 hpi included genes involved in transmembrane transport and terpene synthesis. Among the most statistically supported genes were a cation exchanger (Soltu.DM.09G008230, $\log_2$ fold change = 2.31), a cyclic nucleotide-gated cation channel protein (Soltu.DM.10G002160, $\log_2$ fold change = 1.90), and a Calcium-binding EF-hand family protein (Soltu.DM.10G027990, $\log_2$ fold change = -1.20) (**Supplemental File SG)**. These three genes are all implicated in ion exchange, particularly calcium ion exchange, which may be a key component of the hypersensitive response (Chen 2021, Bi 2021). Similarly, three genes encoding terpene synthases (Soltu.DM.09G029760, Soltu.DM.09G029770, Soltu.DM.09G029880) exhibited increased

expression at 24 hpi in Russet Burbank and Payette Russet (**Supplemental File S1I**). Terpenes are volatile organic compounds produced and released by plants to combat herbivory and infection (Tholl, 2006; Singh and Sharma, 2015). Expression of terpene synthesis-related genes is in part controlled by cellular jasmonic acid concentrations (Memelink et al., 2001). The jasmonic acid pathway can act antagonistically to the salicylic acid pathway, which contributes to antiviral defense (Niki et al., 1998; Shim et al., 2013). That the same terpene synthase genes exhibited increased expression in both resistant Payette Russet and susceptible Russet Burbank at 24 hpi may indicate that terpene synthesis may be conserved early response to PVY infection.

3.1.2 Upper, uninoculated Russet Burbank leaves exhibit different transcriptional responses before and after systemic infection

The potato cultivar Russet Burbank is consistently one of the most widely grown potato cultivars in North America due to its consistent tuber shape and cooking qualities (Bethke et al., 2014). Russet Burbank is also very susceptible to most strains of PVY. Characterization of the transcriptional responses to PVY in Russet Burbank will provide a better understanding of the effects of PVY infection on Russet Burbank, as well as identify genes that may facilitate PVY replication and spread. Thus, the transcriptional response to PVY was further assessed over a time course of infection, including the previously discussed 24 hpi timepoint and additional timepoints (i.e., 1- and 4-weeks post infection (wpi). Systemic infection of PVY takes approximately four weeks to develop in Russet Burbank plants. For these additional timepoints, PVY samples were obtained from leaves distal to the inoculation site, since the effects of PVY

infection on distal, yet uninfected regions of potato plants is not well understood, as many previous sequencing efforts have focused on the early responses (i.e., 6 hpi to 5 days post inoculation) of inoculated leaves (Kogovšek et al., 2010; Goyer et al., 2016).

At 1 wpi, PVY-infected Russet Burbank plants did not show any visible symptoms of virus infection at 1 wpi and virus levels were below the detection limit of qPCR (i.e., < 1,000 RNA copies per 40 ng RNA) (**Supplemental File S1B**). Over 2,500 genes were differentially expressed in upper, non-inoculated leaves as compared to control plants. A similar number of genes that exhibited either increased or decreased expression (1,359 up, 1,307 down) (**Figure 1B, Supplemental File S1J, K**). Gene ontology analysis of the genes that exhibiting decreased expression indicated that this gene list was enrichment in genes involved in the Calvin pathway, non-coding RNA metabolism, and hydrolase activity (**Figure 1B, Supplementary File S1L**). GO analysis also indicated that DEGs with increased expression were involved in processes related to chloroplast and plastid organization and function. Plant viruses have a wide range of effects on photosynthesis and the photosynthetic machinery of plants (Kogovšek et al., 2010; Stare et al., 2015; Bhattacharyya and Chakraborty, 2017). Many plant viruses, including potyviruses like PVY, use chloroplast membranes as replication structures (Bhattacharyya and Chakraborty, 2017). Processes including protein modification and phosphorylation, ubiquitination, and plant-pathogen interactions were enriched among DEGs with decreased transcript abundance in PVY-infected plants at 1 wpi (**Figure 1B**). These results indicate that by 1 wpi, virus infection is associated with reduced expression of defense-related genes before virions spread to distal portions of the plant, thus possibly facilitating viral spread.

Systemic infection of PVY in Russet Burbank results in the widespread accumulation of viral RNA genomes and proteins in leaf tissue throughout the plant. At 4 wpi, the inoculated Russet Burbank plants were systemically infected with PVY (**Supplemental File S1A)**. Analysis of the sequencing libraries at 4 wpi indicate that ~30% of the reads are viral in origin **(Supplemental File S1B).** Differential gene expression analysis indicated that slightly more differentially expressed genes exhibited decreased expression (2,734) than increased expression (2,584) in upper, non-inoculated leaf samples from PVY-infected Russet Burbank (**Figure 1B, Supplemental File S1M, N**) (adj. p-value < 0.05). Gene ontology analysis indicated that processes including photosynthesis, oxidation-reduction, and small molecule metabolism were enriched among genes that showed increased expression in PVY-infected plants, whereas biological processes including ribosomal structure, translation, plant-pathogen interactions, and cellular signaling were enriched among transcripts that decreased in expression (**Supplemental File S1O**). Virus infection affects nearly every aspect of the plant cell. Infection by plant viruses often results in chlorosis or mosaic patterning in infected leaves. These symptoms are indicative of damage to chloroplasts or the photosynthetic machinery. Importantly, Russet Burbank plants in this study were largely asymptomatic and did not display any chlorosis or strong mosaic symptoms. As PVY reached high levels of systemic infection without obvious phenotypic effects on the plants, this infection could be categorized as a tolerant infection (Křižnik, 2017; Prigigallo et al., 2019; Křižnik et al., 2020). A decrease in the expression of defense genes in conjunction with an increase in expression of genes involved in photosynthesis and chloroplast functioning may contribute to the establishment of tolerant PVY infection in Russet Burbank.

3.2 Differences and similarities in differentially expressed genes between potato cultivars and time points reveal patterns of infection and possible antiviral genes

Differentially expressed genes between timepoints and cultivars were analyzed to determine which of the genes were shared between timepoints or cultivars (**Figure 1B**, **Supplemental File 1P-W**). Genes that are differentially expressed in two or more treatments or between timepoints may indicate that those genes could be involved in potato antiviral defense or in roles that benefit the virus. Gene ontology analysis of common differentially expressed genes was also conducted to ascertain patterns of types of genes or pathways that may be involved in multiple timepoints.

3.2.1 DEGs shared between PVY-infected Payette Russet and Russet Burbank may indicate antiviral roles

Genes that were differentially expressed in both PVY-inoculated Payette Russet and Russet Burbank may be involved in antiviral immunity. The resistance response in Payette Russet likely happens early after recognition of the virus, while Russet Burbank does not mount a successful resistance response but may still engage in some defensive measures as the infection progresses. Payette Russet samples from 24 hpi and Russet Burbank samples from 1 wpi shared 425 differentially expressed genes, of which 256 were shared exclusively between the two groups (**Figure 1B, Supplemental File S1P)**. Among these shared genes, there was gene ontology enrichment for protein serine/threonine kinase activities, plant pathogen interactions, and

ubiquitin-protein transferase activity (**Supplemental File S1Q**). Enrichment within these treatment groups was exclusive to genes that exhibited decreased expression. For example, the salicylic acid concentration-controlling gene, *NPR1*-like, decreased in expression in Payette Russet at 24 hpi and in Russet Burbank at 1 wpi and 4 wpi (**Figure 2, Supplemental Table 1, Supplemental File S1L**). Although genes involved in defense responses and plant pathogen interactions were enriched for both Payette Russet at 24 hpi and Russet Burbank at 1 wpi, that these enrichments occurred only among genes exhibiting decreases in expression may indicate that the stress of PVY infection at inoculated leaves is already having strong effects on distal, not yet infected regions of the susceptible Russet Burbank plants. Leaf tissue in which transcription of defense genes is decreased may be more prone to PVY infection as the virus spreads systemically.

Gene ontology analysis of differentially expressed genes that increased in expression exclusively in Russet Burbank at 1 wpi identified enrichment of chloroplast and plastid-related genes, as well as genes involved in RNA binding and amino acid synthesis (**Figure 2, Supplemental File S1L**). Many potyviruses, including PVY, use chloroplast membranes as replication complexes (Wei et al., 2009; Li et al., 2016). Increased transcription of genes involved in chloroplast/plastid organization could be due to an increased need for both chloroplast membranes as well as an increased demand to produce amino acids for chloroplast production and for future translation of viral proteins. Chloroplasts are devoid of RNAi activity and therefore serve as a prime location for viral replication and translation activity (Bhattacharyya and Chakraborty, 2017).

More than 500 genes were differentially expressed in both Payette Russet (24 hpi) and Russet Burbank plants at 4 wpi, of which 414 were exclusively shared between these two groups. The exclusively shared gene list was enriched for genes involved in molecular functions including ATP binding, kinase activity, sequence-specific DNA binding, and phosphotransferase activity. Biological processes associated with DNA replication and break repair and the mini-chromosome maintenance complex, which is also involved in DNA replication, were also enriched (**Supplemental File S1S**).

3.2.2 DEGs shared between Russet Burbank time points may be important to successfully establishing PVY infection or involved in antiviral defense

Viruses are adept at manipulating cellular conditions to promote their own replication and spread (Laliberté and Sanfaçon, 2010; Laliberté and Zheng, 2014; Bhattacharyya and Chakraborty, 2017; Medina-Puche and Lozano-Duran, 2019; Aguilar et al., 2020). Genetic engineering to limit interactions between viral proteins and the host proteins required for viral replication can also be a sustainable form of antiviral resistance (Paven 2009). Genes that were differentially expressed in Russet Burbank at both 1 wpi and 4 wpi may be important for establishing PVY infection or antiviral defense and could serve as targets for future PVY resistance editing experiments. A total of 953 differentially expressed genes were shared between Russet Burbank at the 1 wpi and 4 wpi timepoints (**Supplemental File S1T**). Among the shared genes that exhibited increased expression, there was strong enrichment for the cellular components, plastid and chloroplast, and secondary metabolite biosynthesis, as well as enrichment for the carotenoid biosynthesis and

carbon metabolism pathways (Kanehisa and Goto, 2000) (**Supplemental File S1U**). Enrichment among shared genes that exhibited decreased expression included the defense response and kinase activity. These results indicate that an overall decrease in transcription of defense response genes and genes involved in kinase activity/phosphorylation cascades may be an important aspect of establishing PVY infection in Russet Burbank.

Beyond gene ontology, patterns of expression among individual genes or gene families among and between treatment groups provides additional information regarding the PVY infection process in Russet Burbank potato plants. Four genes encoding Heat shock protein 70-interacting proteins differentially increased in expression in Russet Burbank at both 1 wpi and 4 wpi (**Supplemental File S1T**). Heat shock protein 70 (HSP70) serves important pro-viral roles during plant virus infections. For example, successful infection by Tomato yellow leaf curl virus depends on interactions between tomato HSP70 and the viral coat protein (Gorovits et al., 2013). The assembly of the replicase complex of Red clover necrotic mosaic virus also depends on interactions with HSP70 and HSP90 (Mine et al., 2012). Interestingly, HSP70 is also required for successful deployment of plant defense responses in *Nicotiana benthamiana* (Kanzaki et al., 2003). The specific roles of HSP70-interacting are not well understood but could play pivotal pro- or antiviral roles in during PVY infection. Five genes encoding Kunitz family trypsin and protease inhibitor proteins also exhibited greater expression in PVY-infected Russet Burbank at 1 wpi and 4 wpi (**Supplemental File S1T**). Kunitz family trypsin and protease inhibitor proteins negatively regulate programmed cell death and the hypersensitive response in Arabidopsis in response to fungal and bacterial pathogens (Li et al., 2008). As the Russet Burbank plants remain

mostly asymptomatic even when systemically infected with PVY, it is possible that these Kunitz family trypsin and protease inhibitor also negatively regulate defense responses to viral pathogens, though more research is needed to test this hypothesis.

3.3 Expression and type of small RNA varies by time point and cultivar

Small RNAs are employed by plants in myriad ways and influence nearly every aspect of plant biology (Borges and Martienssen, 2015). The length of the small RNA in plants can, in part, dictate function. Non-coding RNAs with secondary stem loop structure can be processed by the RNAi machinery into microRNAs (miRNAs), which are often 21 or 22-nt in length. Argonaute-bound miRNAs bind complementary mRNA transcripts and regulate gene expression by either transcript cleavage or translational inhibition of the target mRNA (Millar, 2020). MicroRNAs and other small RNAs functioning within the RNAi pathway in plants are generally involved in negative regulation of gene expression, particularly transcriptional and post-transcriptional gene silencing. Transcriptional regulation by DNA methylation is in part controlled by the targeting of complementary long, non-coding RNAs by Argonaute proteins bound to 24-nucleotide small RNAs, which are often the most common length of small RNA found in flowering plants (Daxinger et al., 2009; Gao et al., 2010).

While ~ 40% of the small RNAs in systemically PVY-infected Russet Burbank plants at 4 wpi aligned to the PVY genome, the focus of this analysis was primarily on 21-24 nucleotides small

RNAs that aligned within gene regions of the potato genome (**Supplemental File S2A**). Identification of abundant clusters of 21-24 nt small RNAs with Shortstack are likely to be biologically relevant through the RNAi pathway (Lunardon et al., 2020). Though many small RNAs, particularly 24-nucleotide length, align outside of gene regions and can impact transcription of genes by binding to promoter regions or repress transcription of transposable elements (Lunardon et al., 2020). he majority of the differentially expressed small RNAs that aligned to genes in Russet Burbank were made up of either 21 or 24-nucleotide small RNAs, which is typical of flowering plants (**Figure 3, Supplemental File S2B, D**) (Lunardon 2020). Interestingly, the genes aligned to differentially expressed small RNAs were dominated by 24-nucleotide small RNAs at 1 wpi and by 21-nucleotide small RNAs at 4 wpi and the abundance of almost all these small RNAs increased (**Supplemental File S2B, D**). At 1 wpi the genes that aligned to 24-nucleotide small RNAs that decreased in abundance were enriched for transporter activity, carbohydrate metabolism, and cell wall/cell periphery (**Supplemental File S2C**). Many of the 21-nucleotide small RNAs differentially expressed at 1 wpi aligned to NLR resistance genes, which are typically subject to negative regulation by small RNAs. Further, about half of those genes were also aligned with 21-nucleotide small RNAs at 4 wpi (**Figure 3**). Among the 21-nucleotide differentially expressed clusters at 1 wpi there was enrichment for ADP binding and defense responses.

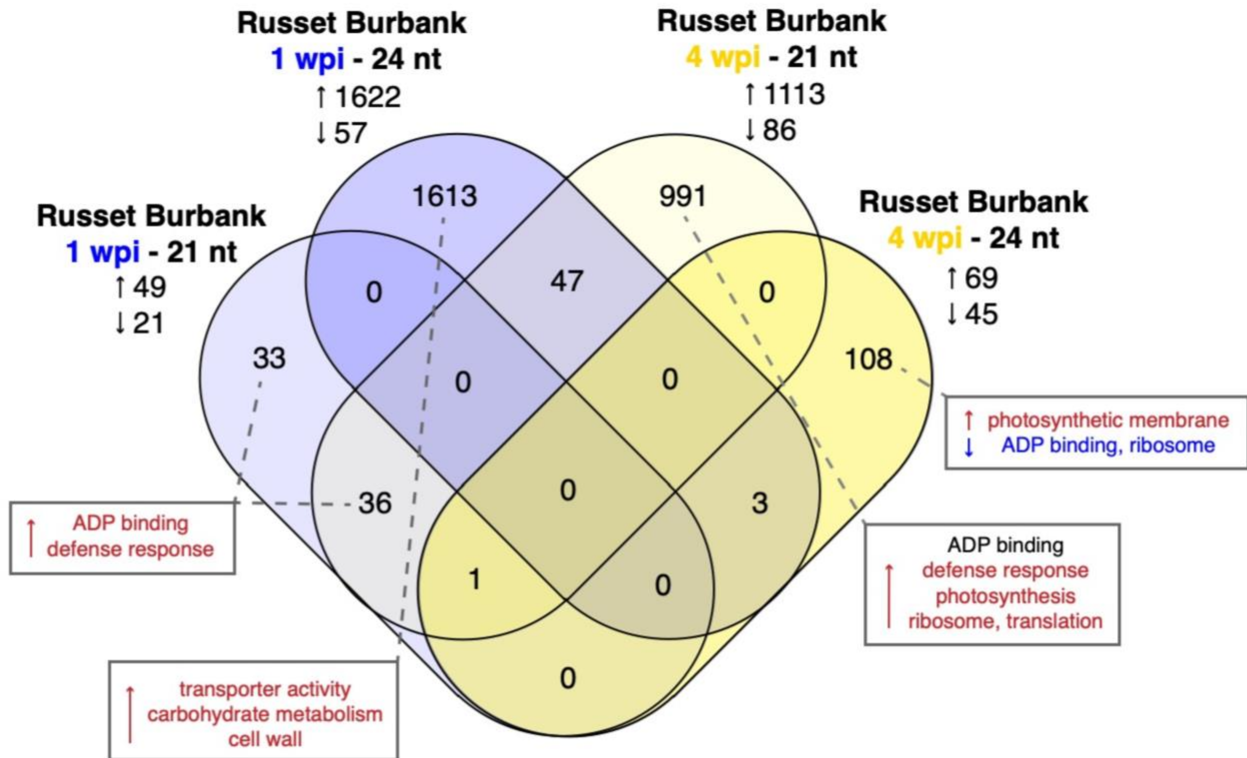


Figure 3: Potato small RNA transcriptional response to PVY-infection is time and length dependent. A Venn diagram depicts unique and shared differentially expressed small RNAs in Russet Burbank at 1 wpi (blue) for 21- and 24-nucleotide small RNAs and Russet Burbank at 4 wpi (yellow) for 21- and 24-nucleotide small RNAs response to PVY infection. No differentially expressed small RNAs were shared between all time points and cultivars. Over half of the 21-nucleotide small RNAs that increased in abundance at 1 wpi were also differentially expressed at 4 wpi. Most of the differentially expressed small RNAs at 1 wpi with 24 nucleotides longs, while most of those differentially expressed at 4 wpi were 21 nucleotides long, with many fewer 24-nucelotide small RNAs at that time point. Gene ontology results indicate that defense response and ADP binding were enriched among all differentially expressed 21-nucleotide small RNAs, while enrichment for photosynthesis, ribosome and translation were enriched for 21-nucleotide small RNAs at 4 wpi only. Transporter activity, carbohydrate metabolism, and the cell wall were enriched among 24-nucleotide small RNAs at 1 wpi, with the photosynthetic membrane, ADP binding, and the ribosome were enriched among 24-nucleotide small RNAs at 4 wpi. Full lists of differentially expressed genes and their corresponding fold changes are reported in **Supplementary File S2**.

The small RNAs in Russet Burbank plants with systemic PVY infections (4 wpi) aligned to numerous host genes (**Supplemental File S2D**). Specifically, 21-nucleotide small RNAs aligned with over 1,000 genes and the 24-nucleotide RNAs aligned to over 100 genes. Within the genes that were aligned to 21-nucleotide small RNAs there was GO enrichment for ADP binding among small RNAs that both exhibited increased and decreased in abundance, as well as enrichment for defense responses, photosynthesis, ribosomes, and translation among the small RNAs that increased in expression (**Supplemental File S2E**). Enrichment analysis of the 24-nucleotide small RNA clusters revealed that the photosynthetic membrane was enriched among clusters that increased in expression. Conversely, ADP binding and ribosome were enriched among those clusters that displayed decreases in expression.

In some instances, the observed increased abundance in small RNAs that aligned to potato genes inversely corresponded to changes in mRNA abundance. For example, Russet Burbank genes that exhibited decreased expression at 1 wpi and 4 wpi included a higher number of genes involved in defense responses than would be expected by chance (**Supplemental File S1C, E**). Correspondingly, the abundance of small RNAs targeting defense genes was greater at 1 wpi and 4 wpi. Intuitively these results make sense, as increases in small RNAs would in theory result in decreases in target gene transcript. However, this pattern was not observed in all the results, as enrichment for ribosomal processes/translation existed among both mRNA and small RNAs that decreased in expression/abundance.

The roles of small RNAs in regulating defense responses and other physiological traits are well established, but the regulation of ribosomal proteins is much less well understood. Plant small RNAs target transcripts from NLR genes and tightly regulate the expression of plant immune responses (Deng et al., 2018b, 2018a; Lai and Eulgem, 2018). We analyzed the composition of genes that were aligned with small RNAs in PVY-infected Russet Burbank plants. At 1 wpi, 35.2% (25/71) of the differentially expression 21-nucleotide small RNAs aligned with NLR-type disease resistance genes (**Supplemental File S2B**). Similarly, of the differentially expressed 21-nucleotide small RNAs at 4 wpi, 21.8% (240/1099) aligned to annotated disease resistance genes (**Supplemental File S2D**). All 21-nucleotide small RNA clusters aligned to disease resistance genes exhibited increased expression at 4 wpi in PVY-infected plants. The potato genome encodes 786 NLR-type disease resistance genes, meaning that ~32% of the total disease resistance genes are increasingly targeted by 21-nt small RNAs during systemic PVY infection at 4 wpi (Jupe et al., 2013; Pham et al., 2020). This likely indicates that small RNAs and RNAi mediated gene regulation are important in limiting the antiviral responses mounted by Russet Burbank potato plants against PVY.

There were distinct differences in the small RNA composition in Russet Burbank plants at 1 week post PVY infection compared to 4 wpi, when the plants were systemically infected. These differences were striking, as the 1 wpi time point was dominated by differences in 24-nucleotide small RNA abundance and 4 wpi is dominated by differences in the 21-nucleotide small RNAs (**Figure 3**). DNA-methylation as conferred in part by the 24-nucleotide small RNA pathway often targets transposable elements to prevent expression (Diezma-Navas et al., 2019). It is

possible that PVY infection also induces transposable element release and that this causes the rise increase in 24-nucleotide small RNAs observed in this study.

3.3.1. The miRNA profiles of PVY-infected Russet Burbank plants mirrors mRNA results and is similar to PVY-tolerant potato cultivars

MicroRNAs are not well annotated within the potato genome and many miRNA identification software are prone to high rates of false positive annotations (Shahid and Axtell, 2014). Shortstack is a conservative predictor of putative miRNAs by design. Because of the conservative nature of Shortstack and the relative lack of miRNA annotation in the potato genome, we decided to only analyze the abundance of small RNAs that were identified as miRNAs by Shortstack and were also similar in sequence to annotated plant miRNAs.

A total of 20 miRNAs that met our requirements were identified in the sequencing libraries generated from Russet Burbank plants at 1 wpi (**Supplemental File S2H**). Of those 20 miRNAs, 19 were more abundant in PVY-infected plants compared to non-infected plants. Two miRNAs that were much more abundant in PVY-infected plants most closely resembled miR7992 and miR6149, both of which exhibit complementarity to regions of the PVY RNA genome and could in theory target PVY (Iqbal 2016). MiR162 was one of the most abundant miRNAs in PVY-infected plants and regulates the expression of *DICER1-LIKE*, which is a key component of the plant miRNA pathway in Arabidopsis (Kidner 2005). Mir482, which targets NLR transcripts, was the most abundant miRNA at 1 wpi. At 4 wpi we identified 25 miRNAs that fit our criteria (**Supplemental File S2I**). Of those miRNAs, 24 were more abundant in PVY-infected plants

compared to non-infected Russet Burbank plants. The most abundant miRNA belonged to a mir167 miRNA family, which target transcripts involved in plant growth regulation (Kinoshita et al., 2012). Multiple miRNAs annotated as species of miR408 were more abundant in PVY-infected plants at 4 wpi. The greater abundance of miR408 may result in attenuated immune responses, as similar results have been described in sweet potato (Kinoshita et al., 2012; Kuo et al., 2019).

Understanding tolerance to virus infection has recently received increased scientific attention, as tolerant plants are less likely to put strong evolutionary pressures on viruses to evolve mechanisms or structures that render resistance genes and immune responses ineffective (Prigigallo et al., 2019; Pagán and García-Arenal, 2020). However, tolerant plants can harbor very high viral loads and thus can serve as viral reservoirs in fields, potentially seeding outbreaks to neighboring, more susceptible plants and allowing for mixed infections and possible recombination events between different viral strains. Recent research indicated that upregulation of key miRNAs in a tolerant potato cultivar, Desiree, may assist in establishing a tolerant infection to PVY^{NTN} (Křižnik, 2017). Our results corroborate some of these findings, as miR167, miR391, miR171, miR164, and miR390 were all differentially increased in expression at either 1 wpi or 4 wpi in $PVY^{N\text{-}Wi}$-infected Russet Burbank plants (**Supplemental File S2H, I**). These miRNAs have been implicated in facilitating tolerance and mutualism in host plants. That we found similar results in a tolerant infection with a different potato cultivar (i.e., Russet Burbank) and different PVY strain (i.e., $PVY^{N\text{-}Wi}$), further strengthens previous findings (Křižnik, 2017). Symptoms of PVY infection can vary by environmental conditions and while we observed

tolerant-like infections in Russet Burbank plants grown from sterile tissue culture in growth chambers, this does not necessarily mean that these results would replicate in field conditions.

3.3.2. Analysis of phasiRNAs in PVY-infected potato plants reveals enrichment of NLR resistance gene targets and similarities between timepoints

In plants, AGO proteins loaded with distinct 22-nucleotide small RNAs can trigger phased cleavage of a target RNA by dicer-like proteins, ultimately resulting in the production of many small RNAs (phased siRNAs, or phasiRNAs) from a single transcript. The resulting phasiRNAs can be employed to target other transcripts produced from the same gene or locus, or other transcripts with similar target regions (Liu et al., 2020). PhasiRNA production results in distinctive alignment signatures that can be computationally identified by programs such as Shortstack (Axtell, 2013; Coruh et al., 2014; Shahid and Axtell, 2014). We identified putative phasiRNAs within the small RNA alignments of Russet Burbank at 1 wpi and 4 wpi using Shortstack to gain a better understanding of how phasiRNA are regulated in response to PVY infection in susceptible plants. Analysis of phasiRNAs in PVY-infected Russet Burbank plants at 1 wpi resulted in 52 phasiRNA loci from gene regions, with 37 (71%) of those loci composed of 21-nucleotide phasiRNAs and the remaining 15 (29%) phasiRNA loci composed of 24-nucleotide phasiRNAs (**Figure 4A, B, Supplemental File S2J**). Shortstack analysis of the PVY-infected Russet Burbank plants at 4 wpi resulted in 104 phasiRNA loci, which were composed of

a mixture of 21-nucleotide (92%) and 24-nucleotide (8%) small RNA clusters (**Supplemental File S2K**).

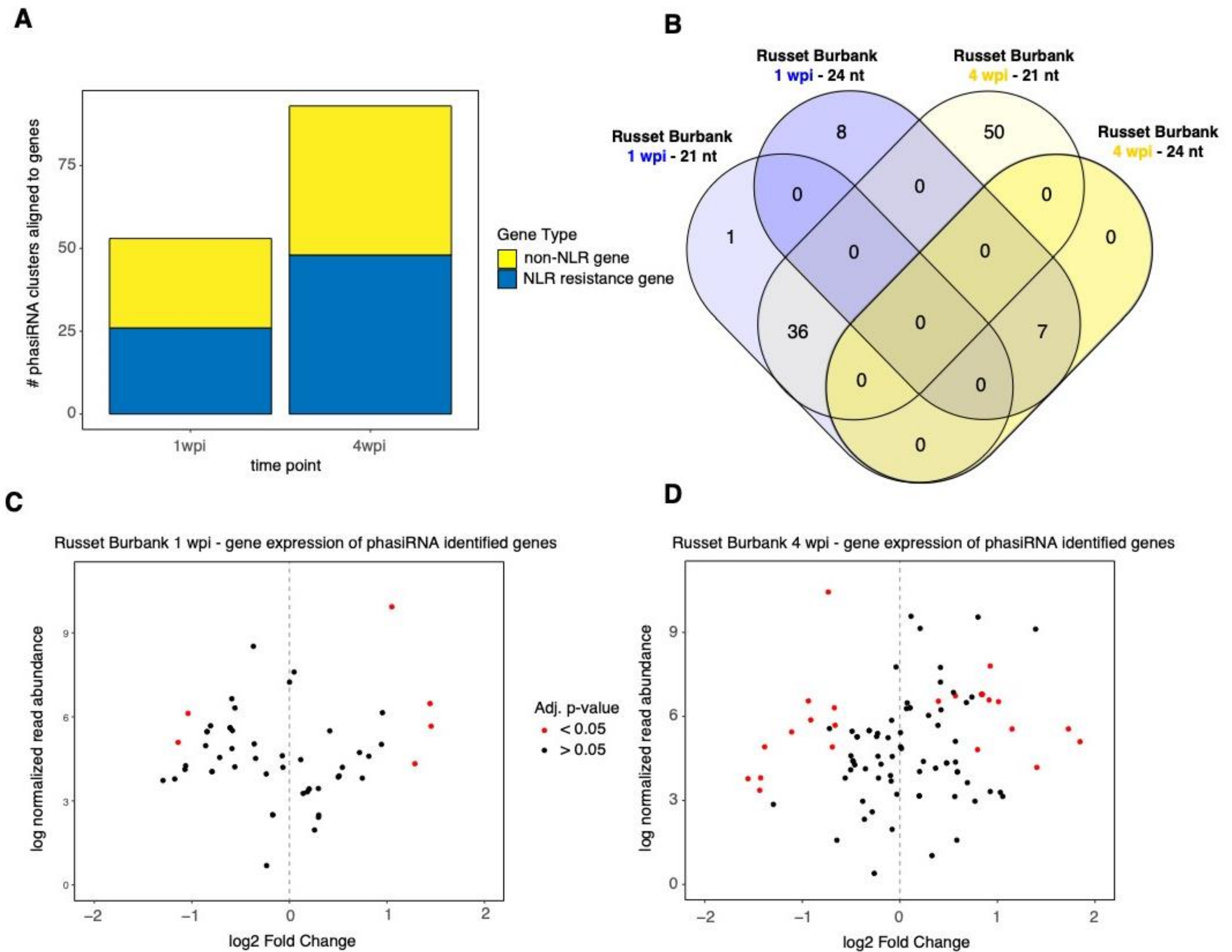


Figure 4: Phased short-interfering RNAs target predominantly NLR proteins in Russet Burbank and are not correlated with expression patterns of mRNAs.
(A) PhasiRNAs separated by time point and gene type. Approximately half (25/52 at 1 wpi, 48/93 at 4wpi) of the phasiRNAs aligned to annotated NLR resistance genes at each time point. (**B**) Similar phasiRNAs are shared between timepoints and among small RNA lengths. All but one of the 21-nucleotide phasiRNAs identified at 1 wpi was also identified at 4 wpi. Similarly, all seven 24-nucleotide phasiRNAs at 4 wpi were also identified as phasiRNAs at 1 wpi. (**C, D**) Normalized expression of genes targeted by phasiRNAs colored by state of differential expression. Genes targeted by phasiRNAs do not exhibit a correlation with either increased or

decreased expression. Full lists of differentially expressed phasiRNAs and their corresponding fold changes are reported in **Supplementary File S2.**

PhasiRNA production aids in the regulation of defense-related genes (Liu et al., 2020). The 21-nucleotide phasiRNAs aligned to predominantly NLR genes and other defense-related genes at both time points in PVY-infected plants, while the 24-nucleotide phasiRNAs did not align to any NLR genes but did align to transcripts from a protein kinase superfamily protein (Soltu.DM.07G005020) and a Kunitz family trypsin and protease inhibitor protein (Soltu.DM.03G023530). Nearly half (26/52) of the phasiRNA loci at 1 wpi aligned to NLR-type resistance genes (**Figure 4B**). Nearly twice as many phasiRNA alignment loci were detected at 4 wpi (93) in Russet Burbank than at 1 wpi. The types of genes that phasiRNAs at 4 wpi aligned to were similar in type and proportion as at 1 wpi, as slightly more than half (48/93) aligned to NLR-type resistance genes. The non-NLR resistance genes that contained phasiRNA alignments consisted of other defense-related genes, including *Dicer2-like* (Soltu.DM.11G004150), and a Kunitz family trypsin and protease inhibitor (Soltu.DM.03G023530), both of which are involved in antiviral defense or defense responses in plants against other pathogens (Deleris et al., 2006). These results indicate that while NLR-type resistance genes are the primary target of phasiRNAs, they are not the only type of defense-related genes that are targeted by phasiRNA in potato in response to PVY infection.

We earlier described that the transcriptional responses in Payette Russet and Russet Burbank exhibited some similarities between timepoints, but that the majority of the differentially

expressed genes were distinct to that cultivar and time point. We hypothesized that phasiRNA targets may also segregate by timepoint in Russet Burbank at 1 wpi and 4 wpi. All but one of the genes aligned with 21-nucleotide phasiRNAs was also targeted by phasiRNAs at 4 wpi, while 12 phasiRNA loci were unique to 4 wpi. All seven of the 24-nucelotide phasiRNA loci detected at 4 wpi were also targeted by 24-nucleotide phasiRNAs at 1 wpi, while the remaining eight, 24-nucleotide phasiRNA loci were unique to 1 wpi (**Figure 4B**). These results indicate that phasiRNAs do not necessarily match the transcriptional patterns as observed in the mRNA data, but that phasiRNA may target similar genes at different time points during PVY infection of Russet Burbank.

Post-transcriptional regulation by phasiRNAs can result in decreases of target transcripts (Liu 2020). In this study, detected phasiRNA presence did not exhibit strong correlation with changes in abundance among affiliated genes (**Figure 4C, D**). Many of the genes which had phasiRNA alignments were not differentially expressed or exhibited differential expression in either a positive or negative direction at both 1 wpi and 4 wpi (**Figure 4C, D)**. These results indicate that detection of phasiRNAs via sequencing alignments does not necessarily correlate with reduced abundance of target mRNAs.

The phasiRNAs identified in this study, and their corresponding genes may be involved in either promoting or reducing PVY infections in Russet Burbank plants. In this study, we identified a poly(A) binding protein (Soltu.DM.01G000470) transcript that was less abundant in PVY-

infected plants, compared to uninfected controls, that was the target of a phasiRNA cluster that was more abundant in PVY-infected plants. Poly(A) binding proteins are necessary for replication of other potyviruses, but their involvement in in PVY replication has not yet been described (Wang et al., 2000). Our transcriptome and small RNA analysis suggest that poly(A) binding protein may be important for PVY infection. Genes that produce proteins required for PVY replication and spread could be targets for gene editing techniques to alter viral protein-host protein interaction sites and thus may be useful in the development of PVY-resistant plants (Pavan et al., 2009).

3.4. Alternative splicing analysis reveals genes outside of differential expression analysis with potential impacts on PVY infection

Most transcripts in eukaryotes can be alternatively spliced (Graveley, 2001; Wang and Brendel, 2006). Alternative splicing allows for a single transcript to produce a diversity of protein structures and functions while also providing another avenue to regulate gene expression (Graveley, 2001; Black, 2003; Chen and Manley, 2009). Patterns of alternative splicing can be influenced both by tissue and cell type but can also be a component of a response to environmental stimuli, including regulation of defense responses to invading pathogens. Alternative splicing is a relatively understudied area of host responses to plant viruses. To date there have been no studies published detailing the alternative splicing landscape in host plants following PVY infection. Therefore, we used the R package, IsoformSwitchAnalyzeR, to

identify and analyze alternative splicing patterns following PVY infection in both Payette Russet and Russet Burbank potato cultivars (Vitting-Seerup and Sandelin, 2019). Alternative splicing analysis of the transcriptomic libraries indicated that hundreds of genes had transcripts that were alternatively spliced in response to PVY infection in both cultivars (adj. p-value < 0.05) (**Supplemental File S3A-D**).

Within Payette Russet samples at 24 hpi, 49 genes exhibited differential splicing patterns in PVY-inoculated leaves as compared to mock-inoculated plants (**Figure 5, Supplemental File S3A**). The transcripts that exhibited the greatest statistical strength of differential splicing belonged to an ARM repeat superfamily protein (Soltu.DM.05G013700), a potassium (K+) uptake permease (Soltu.DM.02G003540), a protein of unknown function containing a DUF538 domain (Soltu.DM.12G024860), and a glycine-rich RNA-binding protein (Soltu.DM.02G030950). Gene ontology analysis of the differentially spliced genes determined that there was enrichment for genes involved in glucose-6-phosphate 1-epimerase activity (**Supplemental File S3E**). PVY-infected Russet Burbank leaves exhibited 24, 203, and 81 alternatively spliced genes at 24 hpi and 1 and 4 wpi as compared to mock-inoculated plants (**Figure 5, Supplemental File S3B-D**). Gene ontology analysis of differentially spliced genes at 1 wpi revealed enrichment of genes involved in mRNA binding/processing, chloroplast function, and the citrate cycle (**Supplemental File S3F**). Differentially spliced genes associated with chloroplasts, organelles, and the splicesome were enriched at 4 wpi (**Supplemental File S3G**). A total of 18 genes were differentially spliced in PVY-infected Russet Burbank at both 1 wpi and 4

wpi and gene ontology enrichment revealed enrichment for oxidoreductase and terpene metabolism activity (**Supplemental File S3H**).

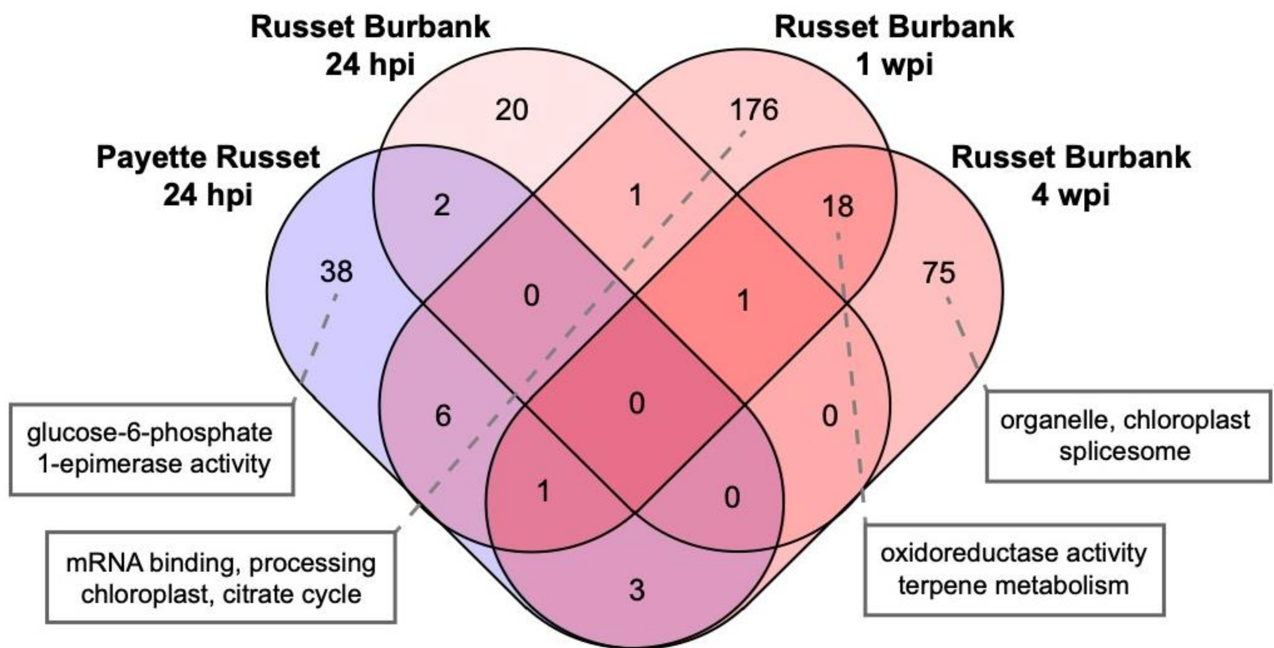


Figure 5: Alternative splicing analysis in Payette Russet and Russet Burbank potato cultivars in response to PVY infection.

Hundreds of genes differentially spliced genes were identified in this study, some of which shared splicing patterns between timepoints. Gene ontology analysis of the spliced genes revealed glucose-6-phosphate enrichment among differentially spliced genes in Payette Russet at 24 hpi. The highest number of differential splicing occurred at 1 wpi in Russet Burbank, with over 200 genes that were likely differentially spliced and ontology enrichment for mRNA binding/processing, chloroplast, and the citrate cycle.

At 1 wpi, the transcripts that exhibited the most statistical strength of differential splicing belonged to genes encoding a Polynucleotidyl transferase, ribonuclease H-like superfamily protein (Soltu.DM.04G031630) and a basic-leucine (bZIP) transcription factor family protein (Soltu.DM.08G019590) (**Supplemental File S3C**). The Polynucleotidyl transferase, ribonuclease H-like superfamily protein may also be annotated as a Werner Syndrome-like exonuclease (WEX). Some WEX proteins act as cofactors to Argonaute proteins in Arabidopsis and are critical to RISC function. These proteins degrade uridylated 5' single-stranded RNAs cleaved by Argonaute proteins, thus allowing for RISC to target other transcripts (Wang et al., 2015). Isoform 2 of ribonuclease H-like superfamily protein is truncated at the C-terminal end and would theoretically be missing components of catalytic domains necessary for proper functioning of RISC. RNA interference is a primary antiviral defense pathway in plants, therefore dysfunctional RISCs would render a plant more susceptible to viral infection. That the alternative splicing of this transcript occurs at 1 wpi in leaves that are yet to be infected suggests that PVY may be able to alter the state of uninfected cells to facilitate incoming infection. Plants also rely extensively on the intercellular trafficking of virus derived small RNAs from infected tissue to distal cells to 'prime' those cells for impending virus infection. Interference with RISC function in distal, yet to be infected tissues could prevent priming of uninfected cells. The alternative splicing of this Polynucleotidyl transferase, ribonuclease H-like superfamily gene will require further investigation to understand its impact during PVY infection.

One gene which exhibited differential splicing at 4 wpi in Russet Burbank was *syntaxin of plants* (Soltu.DM.10G025490), homologous to the Arabidopsis syntaxin of plants 121 (SYP121).

SYP121 proteins are critical component of SNARE complexes, which control bulk transport and vesicle traffic in the cell (**Figure 6B**) (Lipka et al., 2007). Syntaxin proteins are also involved in various defense pathways in Arabidopsis (Zhang et al., 2008). Isoform 1 was less abundant in PVY-infected plants, while isoform 2 levels were higher. Isoform 2 differs from isoform 1 in that it contains an N-terminal Nuclear factor YA domain and an intrinsically disordered domain with a binding region. Nuclear factor Y binding domains in plants are generally individually encoded in different gene families and oligomerize in response to various stressors to form a transcription factor complex recognizing the CCAAT box in target gene promoters. It is possible that differential splicing of *SYNTAXIN OF PLANTS* in potato to include the Nuclear factor YA domain results in virus-specific regulation of defense responses, but further research is necessary to gain a better understanding.

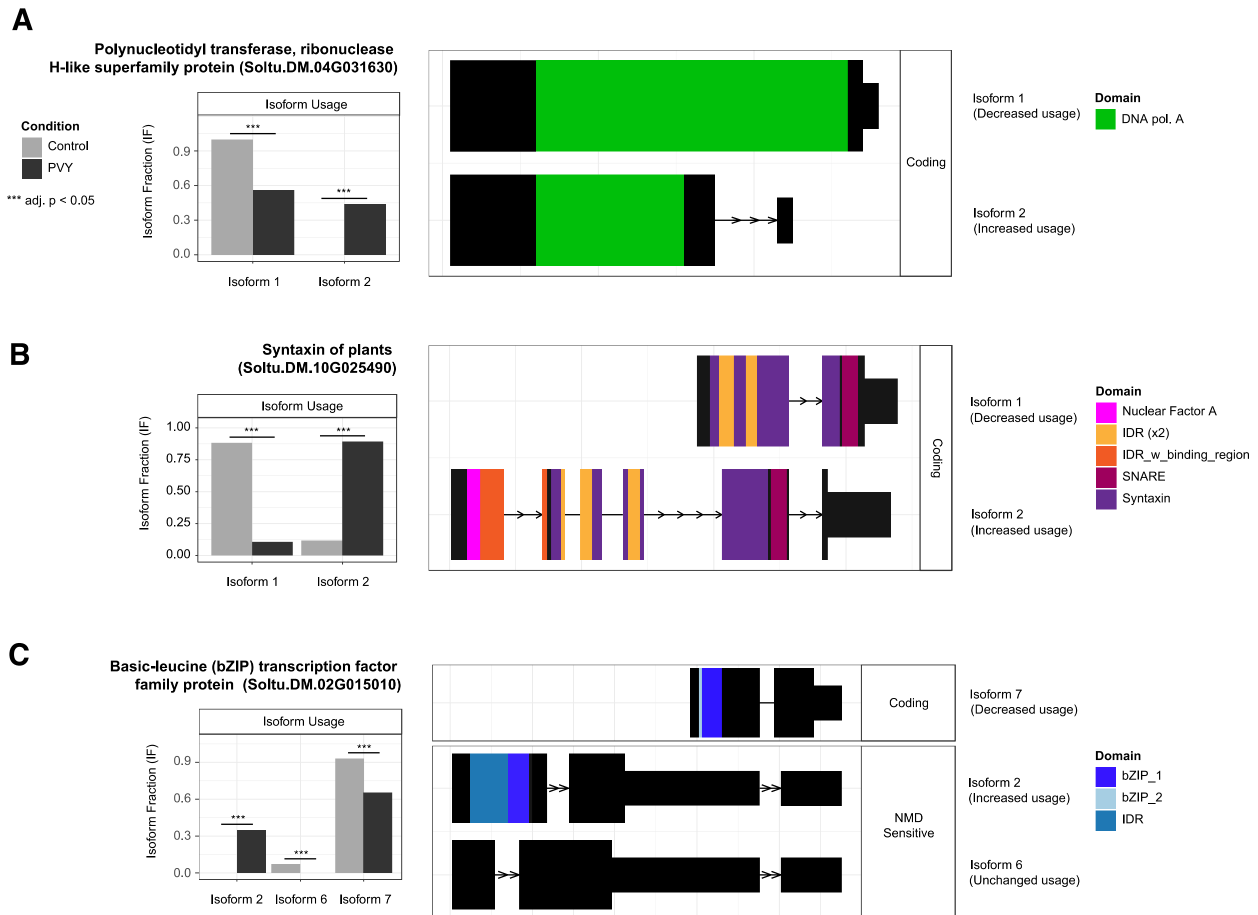


Figure 6: Alternative splicing profiles of select genes in PVY-infected Russet Burbank plants.

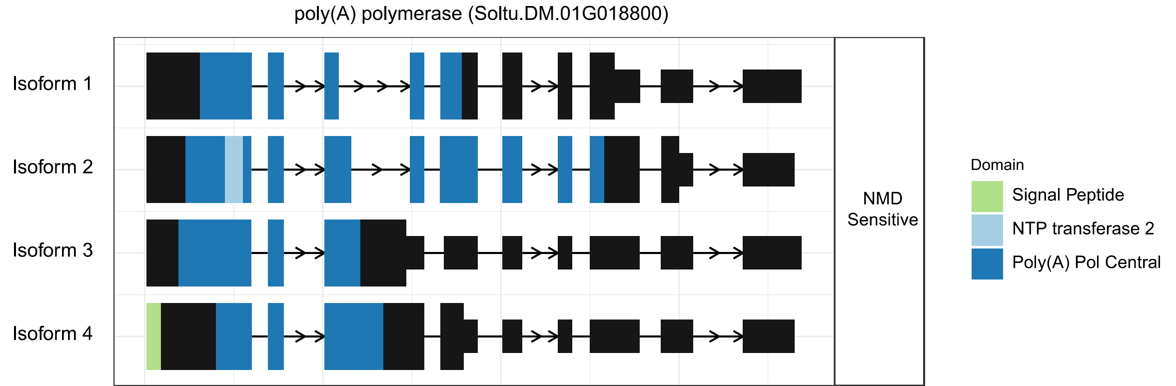
(**A**) Isoform 2 of the gene Polynucleotidyl transferase, ribonuclease H-like superfamily protein (Soltu.DM.04G031630) exhibited increased usage in PVY-infected Russet Burbank plants at 1 wpi, resulting in a higher proportion of transcripts encoding a truncated C-terminal end, which may be important for an effective RNA interference response. (**B**) Isoform 2 of the gene *Syntaxin of plants* (Soltu.DM.10G025490) exhibits increased usage relative to isoform 1 in PVY-infected Russet Burbank plants at 4 wpi. (**C**) Isoform 2 of the gene *Basic-leucine (bZIP) transcription factor family protein* (Soltu.DM.02G015010), which contains an intrinsically disordered domain, exhibits increased usage at 4 wpi in PVY-infected Russet Burbank plants.

Consistent patterns of alternative splicing across timepoints in PVY-infected plants may be indicative of specific splicing patterns that are important for PVY infection. The basic-leucine

zipper (BZIP) transcription factor family protein (Soltu.DM.08G019590) exhibits sequence similarity to Arabidopsis BZIP17, which regulates salt stress responses in Arabidopsis (Liu et al., 2007). The transcript Isoform 2 was more abundant in PVY-infected Russet Burbank plants at 1 wpi and 4 wpi, while isoform 7 was less prevalent at both timepoints (**Figure 6C**). The two transcripts differ in that isoform 2 has an N-terminal intrinsically disordered domain and is missing a second basic leucine zipper domain that is present in isoform 7. The functional ramifications of this alternative splicing pattern are not understood, but often intrinsically disordered regions within transcription factors are important to facilitating interactions with DNA and gene regulation (Minezaki et al., 2006; Das et al., 2011, 2012). It is possible that the potato BZIP protein regulates immune genes in response to PVY infection, but further studies are needed to investigate possible defense roles or pro-viral roles. Transcripts from other potentially interesting genes were differentially spliced at both 1 wpi and 4 wpi in Russet Burbank (silencing defective, Soltu.DM.07G024420; RNA-binding (RRM/RBD/RNP motifs family protein, Soltu.DM.01G005860) and may be promising targets of future research on potato-PVY interactions as well.

The only gene that was differentially spliced in Russet Burbank at all three timepoints was poly(A) polymerase (Soltu.DM.01G018800) (**Figure 7**). Poly(A) polymerases enhance transcript stability and translational efficiency by adenylating the 3’ ends of pre-mRNAs (Li et al., 1996). The potato genome encodes multiple poly(A) polymerases and one of them (i.e., Soltu.DM.01G018800) has four different isoforms. All four isoforms were expressed at 24 hpi, 1 wpi, and 4 wpi (**Figure 7**). Isoform 2 contains an NTP transferase 2 domain, which is not found

in the other three isoforms, while isoform 4 is the only isoform to contain a signal peptide. Poly(A) polymerase was differentially expressed at 4 wpi, but not at 24 hpi or 1 wpi. The increase in expression was likely due to increased expression of isoform 3. All four isoforms are sensitive to nonsense mediated decay, indicating that it is possible that some or all of the transcripts described may not be translated into functional proteins, as the NMD pathway does not automatically degrade all transcripts with pre-mature stop codons (Chang et al., 2007). The analysis of alternatively spliced transcripts adds another dimension of information to our growing understanding of the interactions between potato cultivars and PVY. Although additional studies are required, the genes and isoforms identified in this study may play key roles in host responses to PVY infection.


A
poly(A) polymerase (Soltu.DM.01G018800)
Isoform 1
Isoform 2
Isoform 3
Isoform 4
NMD Sensitive
Domain
Signal Peptide
NTP transferase 2
Poly(A) Pol Central

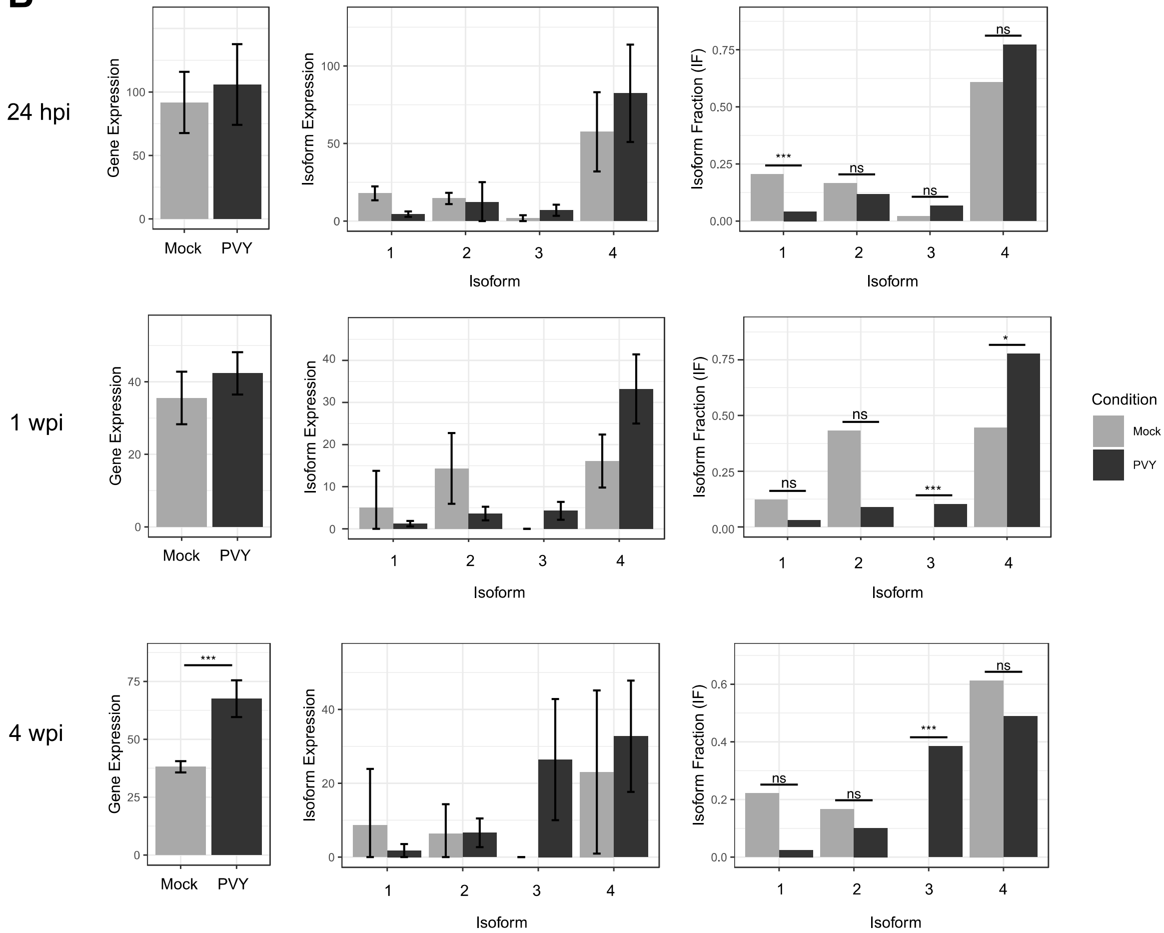

B
24 hpi
1 wpi
4 wpi
Gene Expression
Isoform Expression
Isoform Fraction (IF)
Mock
PVY
Isoform
Condition

Figure 7: Poly(A) polymerase exhibits alternative splicing at 24 hpi, 1 wpi, and 4 wpi in PVY-infected Russet Burbank plants.
Transcript isoforms of the potato gene, poly(A) polymerase (Soltu.DM.01G018800) exhibit similar differential splicing patterns in all sampled timepoints of Russet Burbank during PVY infection. (**A**) A splicing graph depicting the four different isoforms of poly(A) polymerase expressed in Russet Burbank. Isoform 2 contains and NTP transferase 2 domain and isoform 4 contains a signal peptide domain. (**B**) Gene expression, isoform expression, and isoform usage graphs for poly(A) polymerase at 24 hpi, 1 wpi, and 4 wpi in Russet Burbank plants. All four isoforms were expressed at 24 hpi, 1 wpi and 4 wpi. Isoform 2 contains an NTP transferase 2 domain. Poly(A) polymerase is differentially expressed at 4 wpi, but not at 24 hpi or 1 wpi. This increase in expression is likely due to an increase of expression in isoform 3, which also exhibits a significant increase in isoform usage at 4 wpi.

4. Conclusions

In this study we employed a variety of transcriptomic analysis tools to gain a better understanding of both extreme resistance to PVY in the potato cultivar Payette Russet and the antiviral responses mounted by the PVY-susceptible cultivar, Russet Burbank. We identified thousands of differentially expressed genes in Payette Russet at 24 hours post infection. Gene ontology analysis indicated that defense responses were enriched among genes that decreased in expression, while metabolic processes were enriched among genes that increased in expression. That the majority of genes, many of which are involved in defense responses, decreased in expression at 24 hpi may indicate that the transcriptional responses to PVY begin earlier than 24 hpi and require further investigation. We also identified thousands of differentially expressed genes in the PVY-susceptible cultivar, Russet Burbank at later time points (1 wpi, 4 wpi) of infection. Gene functions largely segregated by time point and suggest that PVY has a large influence on distal, yet uninfected regions of the plant relatively early in infection. Analyses of small RNAs from the same time points indicate similarities to tolerant infections in other potato cultivars, further solidifying the results of other studies (Križnik, 2017; Križnik et al., 2020;

Pagán and García-Arenal, 2020). Tolerant plants may serve as viral reservoirs that may produce recombinant viruses and/or outbreaks and therefore tolerance to PVY infection is not a useful trait for potato breeders. However, understanding the mechanisms of tolerance could aid in the engineering of beneficial or mutualistic relationships to improve crop yield and/or decrease nutrient input uses in potato and other important crop species (Arif 2020, Trivedi 2017). Analyses of alternative splicing responses to PVY and identification of possible impactful genes that are not transcriptionally regulated in response to virus infection provide fodder for future studies. A greater understanding of plant transcriptional responses to provide more information for breeders to create disease resistant plants.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

Author contributions are as follows: Conceptualization, B.T.R, N.Z., and M.L.F., writing – original draft preparation B.T.R, M.L.F, writing – review and editing B.T.R, N.Z., and M.L.F, visualization B.T.R and M.L.F, supervision N.Z and M.L.F., funding acquisition N.Z. and M.L.F. All authors have read and agreed to the published version of the manuscript.

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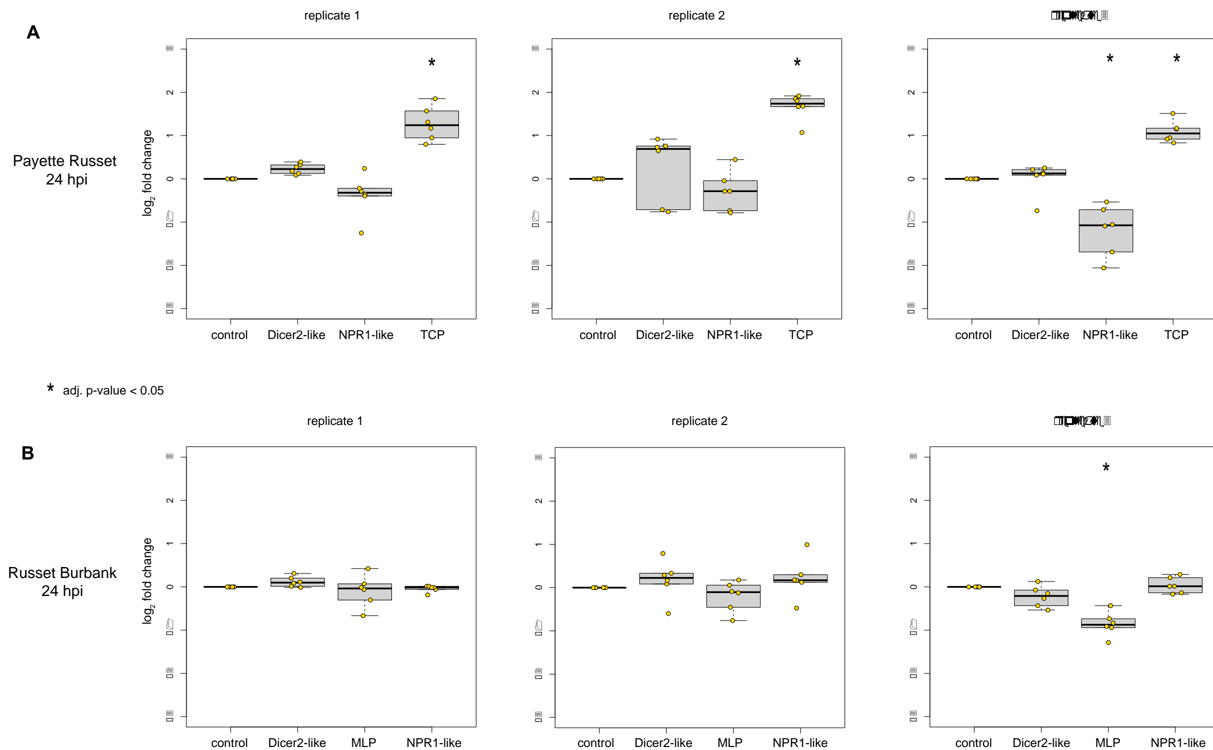
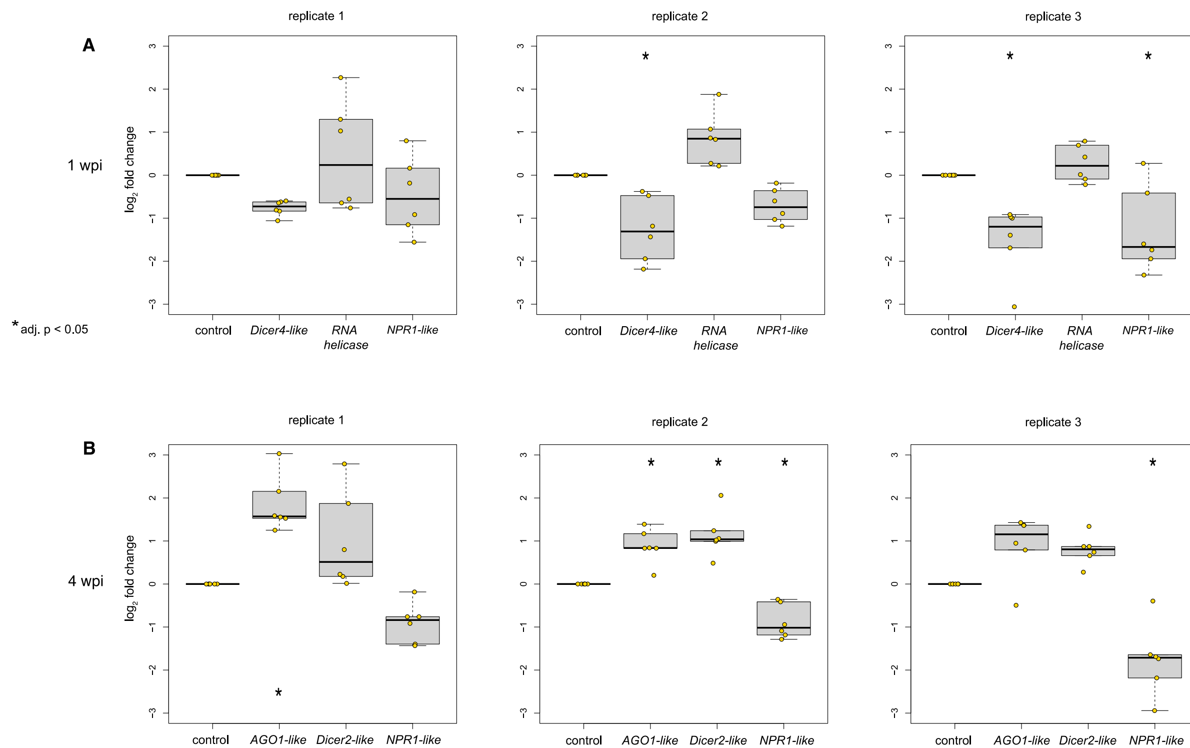


Supplemental Figure 1. qPCR validation of select genes in Payette Russet and Russet Burbank plants at 24 hpi.

(**A**) qPCR gene expression results from three biological replicates in PVY-inoculated Payette Russet plants support transcriptome data. Specifically, *TCP* exhibited increased in expression in all three biological replicates(1.32, 1.69, 1.11 $\log_2$ fold change), *NPR1-like* expression was decreased in biological replicate 3 (-1.11 $\log_2$ fold change) and, although not supported by statistical analysis, trended lower in in biological replicates 1 (-0.31 $\log_2$ fold change) and 2 (-0.22 $\log_2$ fold change), whereas *Dicer2-like* expression levels were similar to levels in mock-infected control plants (0.22, 0.42, 0.04 $\log_2$ fold change) **B**) PVY-inoculated Russet Burbank plant exhibited lower *MLP* expression in biological replicate 3 (-0.84 $\log_2$ fold change), while no changes in gene expression were observed for *MLP* (-0.06, -0.17 $\log_2$ fold change) in the other two replicates, *NPR1-like* (-0.03, 0.27, 0.05 $\log_2$ fold change), or *Dicer2-like* (0.12, 0.23, -0.21 $\log_2$ fold change) in any of the replicates. The RNA sequencing results suggested a decrease in expression of *MLP* (-1.58 $\log_2$ fold change), but no changes for either *NPR1-like* or *Dicer2-like*. Statistical differences in gene expression between mock-infected and PVY-infected potato plants (n = 6 per treatment group per biological replicate) were performed using Wilcoxon ranked sum test, *p<0.05.



Supplemental Figure 2. qPCR validation of select genes in Payette Russet and Russet Burbank plants at 1 and 4 wpi.

A) qPCR gene expression results from three biological replicates of PVY-infected Russet Burbank plants at 1 wpi. *Dicer4-like* exhibits decreases in expression in replicates 2 (-1.10 $\log_2$ fold change) and 3 (-1.35 $\log_2$ fold change), and a decreasing trend in replicate 1 (-0.75 $\log_2$ fold change), corroborating the transcriptomic analysis data. *NPR1-like* also exhibited a decrease in

expression in replicate 3 (-0.98 log_2 fold change) and downward trends in replicates 1 (-0.24 log_2 fold change) and 2 (-0.65 log_2 fold change), also matching the transcriptomic data. The *putative mitochondrial RNA helicase* increased in expression in the transcriptomic data, but was not found to increase by qPCR, though the replicates did exhibit a slight increasing trend of expression (0.90, 0.97, 0.32 log_2 fold change). **B)** qPCR gene expression results from three biological replicates of PVY-infected Russet Burbank plants at 4 wpi. *NPR1-like* exhibited a decrease in expression in replicates 2 (-0.82 log_2 fold change) and 3 (-1.56 log_2 fold change) and downward trends in replicates 1 (-0.84 log_2 fold change), matching the transcriptomic analysis data. *AGO1-like* increased in expression in replicates 1 (1.99 log_2 fold change) and 2 (0.92 log_2 fold change), while *Dicer2-like* increased in replicate 2 (1.23 log_2 fold change) only, but trended up in replicates 1 (1.38 log_2 fold change) and 3 (0.83 log_2 fold change). Both gene displayed increases in expression in the transcriptomic data. Statistical differences in gene expression between mock-infected and PVY-infected potato plants (n = 6) were performed using Wilcoxon ranked sum test, $^*p<0.05$.

CHAPTER FOUR

GENERAL CONCLUSIONS AND DISCUSSION

Potato Transcriptional Responses to Potato virus Y

Here, studies at the transcriptional level were carried out to further elucidate potato (*Solanum tuberosum*) host-Potato virus Y (PVY) interactions in both resistant and susceptible potato varieties. PVY is a economic burden for potato growers worldwide, as infection can result in yield losses, decreases in tuber quality, and in the case of seed potato growers, a potential loss of certification. Resistance to PVY is focus of breeding programs worldwide and thus gaining a better understanding of the mechanisms controlling resistance is of benefit, as genetic resistance to PVY is a sustainable way to limit yield and quality losses due to PVY infection. Although the loci and genes associated with PVY-resistance have been identified, many of the regulations and mechanisms involved in virus resistance in potato have yet to be fully elucidated.

To increase our understanding of PVY infection, host-resistance mechanisms, and transcriptional regulation of these processes, we utilized RNA sequencing to characterize the transcriptomes of two potato cultivars (i.e., PVY-resistant Payette Russet and susceptible Russet Burbank) at different stages post PVY-infection. Differential expression analyses indicate that transcriptional responses associated with the extreme resistance response occur very early after contact with PVY. We identified thousands of differentially expressed genes in Payette Russet at 24 hours post infection. Gene ontology analysis indicated that defense responses were enriched among genes that decreased in expression, while metabolic processes were enriched among genes that increased in expression. That most genes and many involved in defense responses

decreased in expression at 24 hpi may indicate that the transcriptional responses to PVY begin earlier than 24 hpi and require further investigation. We also identified thousands of differentially expressed genes in the PVY-susceptible cultivar, Russet Burbank at later time points (1 wpi, 4 wpi) of infection. Gene functions largely segregated by time point and suggest that PVY has a large influence on distal, yet uninfected regions of the plant relatively early in infection. Analyses of small RNAs from the same time points indicate similarities to tolerant infections in other potato cultivars, further solidifying the results of other studies (Križnik, 2017; Križnik et al., 2020; Pagán and García-Arenal, 2020).

Tolerance to PVY infection is generally not a goal of breeders, as tolerant plants can serve as virus reservoirs and can lead to more severe PVY outbreaks, as well as provide genetic fodder for virus evolution past resistant plants. However, research into tolerance is necessary for understanding the genetic basis of mutualisms and for the potential engineering of beneficial plant-microbe relationships to improve crop yield and/or decrease nutrient input uses (Arif 2020, Trivedi 2017). Analyses of alternative splicing responses to PVY and identification of possible impactful genes that are not transcriptionally regulated in response to virus infection provide the basis for future studies. Analyses of alternative splicing indicate that splice variants (or isoforms) of numerous genes, including poly(A) polymerase and a basic-leucine zipper (BZIP) transcription factor family protein, were more abundant in PVY-infected plants and may impact PYV infections. A greater understanding of plant transcriptional responses to provide more information for breeders to create disease resistant plants.

Extreme resistance to plant viruses remains an under-researched area of plant immunity. The review provided in Chapter 2 is the first comprehensive review of the extreme resistance

literature and will provide the basis for future studies into understanding the underlying molecular mechanisms of extreme resistance. Some of the main questions regarding extreme resistance revolve around if there are conserved mechanisms that define the response across species, or if similar resistance phenotypes have arisen as a result of convergent evolution. A few key areas of future research stand out, particularly further investigation into the movement of NLRs that confer extreme resistance after pathogen recognition. As *Rx1* in potato is only able to provide extreme resistance after binding the PVX coat protein in the cytoplasm and then moving to the nucleus, experimenting with other extreme resistance-providing NLRs (i.e., Ry_{sto}, *Rsv1*) and if they need to also translocate to the nucleus after virus recognition will aid in determining if NLR nuclear translocation is a conserved aspect of the extreme resistance response. Further, more research into the relationship between extreme resistance and callose deposition at the plasmodesmata outside of the soybean *Rsv3* system will also delineate if callose deposition is a conserved aspect of extreme resistance, or if *Rsv3* is mechanistically distinct from other forms of extreme resistance.

Beyond mechanistic studies into extreme resistance, there are questions of how common extreme resistance is outside of the few crops or model species systems from which it has been identified. As extreme resistance is challenging to differentiate phenotypically from non-host resistance and usually requires the identification of a resistance locus or NLR to verify, answering this question will be challenging outside of species for which detailed breeding programs and genomic tools have been developed. However, it stands to reason that if future studies are able to delineate particular, conserved mechanisms that define extreme resistance, it

is very possible that extreme resistance is a much more common phenomenon among plant NLRs than was once realized.

These studies describe the transcriptional responses to Potato virus Y in resistant and susceptible potato varieties and provide a review and avenues of future work on mechanisms of extreme resistance. The results of these studies can be used to identify genes that may be involved in antiviral immunity and to further our understanding of extreme resistance. Research based off these studies may in the future help alleviate economic harm caused by plant viruses, particularly PVY. Further, as humanity transitions into an era of molecular biology in which the editing of genes and engineering of biological pathways becomes more commonplace, we are also beginning to experience the harsher and more volatile effects of global climate change. To apply the powerful new tools of molecular biology quickly, safely and effectively, basic research into systems of which we do not fully understand but nevertheless rely on for sustenance is important.

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