

THE REGULATORY ROLE OF SMALL RNAS
IN IMMUNE RESPONSES OF SOLANUM
TUBEROSUM TO POTATO VIRUS Y
INFECTION

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Doctoral Dissertation
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VLOGA KRATKIH NEKODIRAJOČIH RNA PRI
IMUNSKEM ODGOVORU RASTLIN KROMPIRJA NA
OKUŽBO Z VIRUSOM KROMPIRJA Y

Doktorska disertacija

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To Davorin and my family

*“Success is not the key to happiness. Happiness is the key to success.
If you love what you are doing, you will be successful.”
(Albert Schweitzer)*

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Abstract

The increasing population of the world and its food demands require focused efforts for the improvement of crop plants to ensure sustainable food production. Small RNAs (sRNAs) are emerging as promising targets for genetic engineering to enhance crop yields by improving plant's performance under various environmental stress conditions, including more efficient response against pathogen attack. Potato virus Y (PVY) is one of the most important viruses affecting potato that causes substantial economic losses in worldwide potato production. We aimed at unraveling the roles of sRNAs in the complex immune signaling network controlling the establishment of tolerant (cv. Désirée and cv. Pentland Squire) and hypersensitive response (HR) of potato (cv. Rywal) to the virus. Employing high-throughput sequencing technology, we discovered more than 200 already annotated potato microRNAs (miRNAs), 199 novel potato miRNAs and more than 9000 potato phased interfering siRNAs (phasiRNAs). Expression patterns of these identified small RNAs (sRNAs) were characterized and compared between PVY-infected and healthy potato plants. In addition, this information was linked to expression profiles of their target transcripts to link sRNA level responses to physiological processes. Besides the already described regulation of *R* gene transcripts, we have discovered an interesting novel sRNAs-gibberellin regulatory circuit being activated in tolerant plants of cv. Désirée. Increased levels of potato miR167e and phasiRNA931 were reflected in decreased levels of transcripts involved in gibberellin (GA) biosynthesis. Moreover, decreased concentration of GA confirmed this regulation. We further showed that this regulation is salicylic acid (SA)-dependent as the miR167e and phasiRNA931 levels were diminished in SA depleted NahG-Désirée expressing severe disease symptoms. By comparing sRNA's responses to PVY infection between cv. Désirée and cv. Pentland Squire, we identified six commonly responding sRNAs. One of these potato sRNAs, miR390a, was shown to represent an additional regulatory potential of plants to downregulate GA signaling in order to promote immune responses against PVY. In resistant cv. Rywal, the levels of several sRNAs targeting immune receptor transcripts declined from lesions to surrounding tissue in response to PVY^{N-Wi} to modulate the immune responses. In addition, potato miR398c was shown to be important for ROS homeostasis during HR in cv. Rywal. We also optimized sRNA RT-qPCR protocol for high-quality sRNA expression studies from homogenized leaf material of potato, and showed that this protocol can be used as a validation tool for sRNA sequencing results. In addition, we also established a protocol for parallel extraction of both short sRNAs and long RNAs from low amounts of plant material (i.e. small tissue sections) as well as protocol for efficient DNA removal and RNA purification providing high-quality RNA/sRNAs suitable for RT-qPCR and sequencing of both sRNAs and mRNAs. Finally, we also optimized protocols for bioinformatics analysis of sRNA sequencing data using ShortStack tool. We showed that ShortStack pipeline allows faster, more stringent but reliable prediction for novel miRNA and *MIR* loci discovery.

Povzetek

Z naraščanjem števila svetovnega prebivalstva narašča tudi potreba po vedno večji in bolj učinkoviti pridelavi hrane, kar zahteva vedno bolj osredotočena prizadevanja po izboljšanju lastnosti agronomsko pomembnih rastlin. Male RNA molekule so se v zadnjem času pojavile kot obetavno orodje za uporabo v genskem inženiringu za izboljšanje donosa pridelka. Manipulacija specifičnih malih RNA lahko namreč izboljša odziv rastline na različne stresne pogoje v okolju, vključno z učinkovitejšim imunskim odzivom proti povzročiteljem bolezni. Virus krompirja Y (PVY) velja za enega izmed glavnih povzročiteljev bolezni na krompirju, ki povzroča velike gospodarske izgube v svetovni pridelavi krompirja. Naš cilj je bil razkriti regulacijo malih RNA in njihovo vlogo v kompleksni imunski signalizacijski mreži tolerantnih (cv. Désirée in cv. Pentland Squire) in odpornih (cv. Rywal) rastlin krompirja na okužbo s PVY. Z uporabo visokozmogljivega sekvenciranja smo odkrili več kot 200 anotiranih mikro RNA (miRNA) molekul, 199 novih miRNA molekul ter več kot 9000 faznih malih interferenčnih RNA (phasiRNA) molekul. S primerjavo profilov izražanja teh krompirjevih malih RNA med okuženimi in zdravimi vzorci in primerjavo profilov izražanja njihovih tarčnih transkriptov smo povezali odzive malih RNA s fiziološkimi procesi. Poleg že odkrite regulacije *R* genov smo pri tolerantnih rastlinah krompirja Désirée odkrili novo povezavo med malimi RNA in regulacijo giberelinske signalizacije. Po okužbi s PVY se je povečalo izražanje dveh krompirjevih malih RNA, miR167e in phasiRNA931, kar je vodilo v zmanjšanje količine transkriptov, ki kodirajo encime za sintezo giberelinov, ter zmanjšano koncentracijo samih giberelinov. Pokazali smo, da je regulacija odvisna od prisotnosti salicilne kisline. Regulatorni nivo miR167e in phasiRNA931 je bil namreč po okužbi z virusom nižji v transgenih rastlinah z zmanjšano sposobnostjo akumulacije salicilne kisline (NahG-Désirée). S primerjavo profilov izražanja malih RNA po okužbi s PVY med obema tolerantnima genotipoma krompirja smo odkrili šest skupnih podobno reguliranih malih RNA molekul. Za eno izmed njih, miR390a, smo pokazali, da njeno povečano izražanje predstavlja dodatno zanko za zmanjšanje giberelinske signalizacije, kar lahko vodi k boljšemu imunskemu odzivu. Pri odpornih rastlinah krompirja cv. Rywal smo našli številne prostorsko regulirane male RNA, katerih tarče so predvsem transkripti imunskih receptorjev. Prav tako smo za miR398c pokazali, da je pomembna za vzdrževanje homeostaze reaktivnih kisikovih zvrsti pri s preobčutljivostnim odzivom pogojeni odpornosti sorte Rywal na virus PVY. V okviru disertacije smo optimizirali protokol za analizo izražanja malih RNA v homogeniziranem rastlinskem materialu z uporabo metode RT-qPCR. Pokazali smo, da protokol deluje in da se lahko uporablja za validacijo sekvenčnih podatkov. Poleg tega smo vzpostavili protokol za hkratno izolacijo kratkih in dolgih RNA molekul iz majhnih koškov rastlinskega materiala, ter protokol za odstranjevanje DNA in čiščenje RNA. Protokola omogočata dobro kvaliteto in izplen ter se lahko uporabljajata za analize malih RNA in dolgih RNA molekul z uporabo metode RT-qPCR ali metode sekvenciranja. Optimizirali smo tudi bioinformatični protokol za napoved miRNA molekul in njihovih lokusov z uporabo orodja ShortStack. ShortStack omogoča strogo in zanesljivo napoved ter zmanjšuje potrebo po uporabi dodatnega orodja, s čimer lahko bistveno skrajšamo čas analiz.

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Abbreviations

ABA	... abscisic acid
AFB	... auxin F-box protein
AGO	... Argonaute protein
AOS	... allene oxide synthase
AP2/ERF	... APETALA2/ethylene responsive factor
ARF	... auxin response factor
Aux-IAA	... auxin/indole-3-acetic acid protein
Avr	... avirulence protein
BcTV	... beet curly top virus
BR	... brassinosteroids
CaMV	... cauliflower mosaic virus
cDNA	... complementary deoxyribonucleic acid
CDPK	... calcium/calmodulin-dependent kinases
CK	... cytokinin
CMV	... cucumber mosaic virus
COI1	... coronatine insensitive 1
Cox	... cytochrome oxidase
CP	... coat protein
Cq	... quantification cycle in quantitative polymerase chain reaction
DAMP	... damage-associated molecular pattern
DCL	... Dicer-like protein
DIECA	... diethyldithiocarbamic acid
DMT	... DNA methyltransferase
DNA	... deoxyribonucleic acid
dpi	... days post inoculation
dsRNA	... double-stranded RNA
EDTA	... ethylenediaminetetraacetic acid
ER	... extreme resistance
ET	... ethylene
ETI	... effector triggered immunity
ETS	... effector triggered susceptibility
GA	... gibberellin
gDNA	... genomic deoxyribonucleic acid
HD-ZIPIII	... Class III homeodomain-leucine zipper protein
hc-siRNA	... heterochromatic small interfering RNA
HR	... hypersensitive response
HST	... hasty protein
HYL1	... hyponastic leaves 1
JA	... jasmonic acid
LRR-RLK	... leucine rich repeat-receptor-like kinase
MAMP	... microbe-associated molecular pattern

MAPK	... mitogen-activated protein kinases
MeJA	... methyl jasmonate
miRNA	... micro RNA
MP	... movement protein
NBS-LRR	... nucleotide-binding site leucine-rich repeat receptors
NPR1	... nonexpresser of PR genes 1
NTC	... no template control
OPDA	... 12-oxophytodienoic acid
ORF	... open reading frame
PAMP	... pathogen-associated molecular pattern
PCD	... programmed cell death
PCR	... polymerase chain reaction
phasiRNA	... phased small interfering RNA
PLRV	... potato leafroll virus
PPR	... pentatricopeptide repeat protein
PR	... pathogenesis-related
PRR	... pattern recognition receptors
PTI	... pattern-triggered immunity
PTNRD	... potato tuber necrotic ringspot disease
PVS	... potato virus S
PVT	... potato virus T
PVY	... potato virus Y
PVX	... potato virus X
RLP	... receptor-like proteins
RDR	... RNA-dependent RNA polymerase
RIN	... RNA integrity number
RISC	... RNA-induced silencing complex
RT-qPCR	... reverse transcription quantitative PCR
<i>R gene</i>	... resistance gene
ROS	... reactive oxygen species
SA	... salicylic acid
SAR	... systemic acquired resistance
SE	... serrate protein
siRNA	... small interfering RNA
sRNA	... small RNA
TAS3	... TRANS-ACTING SIRNA GENE3
tasiRNA	... trans-acting small interfering RNA
TBSV	... tomato bushy stunt virus
TMV	... tobacco mosaic virus
TMVcg	... tobacco mosaic virus crucifier strain
TNV	... tobacco necrosis virus
ToMV	... tomato mosaic virus
ToRSV	... tomato ringspot virus
TYLCCNV	... tomato yellow leaf curl China virus
TYLCV	... tomato yellow leaf curl virus
SOD	... systemic acquired resistance
vsiRNA	... virus-derived small interfering RNAs
VSR	... viral suppressor of RNA silencing
WT	... wild-type

Chapter 1

Introduction

1.1 Plant Immune Response

Plants are under continuous biotic stress caused by different attackers (e.g., bacteria, fungi, viruses, oomycetes, insects and nematodes). To cope with their enemies, plants have evolved a multilayered immune system to recognize potential pathogens and activate an effective defense response. When pathogens overcome a passive layer of plant defense (i.e. wax layers, rigid cell wall, activity of antimicrobial enzymes), the first line of active plant immunity is triggered upon perception of conserved, pathogen-associated molecular patterns (PAMPs) by plant surface pattern recognition receptors (PRRs) such as receptor-like kinases (RLKs) or receptor-like proteins (RLPs) (reviewed by Jones and Dangl, 2006). As PAMPs are often also derived from non-pathogens, they are appropriately named microbe-associated molecular patterns (MAMPs) (Bigeard et al., 2015). Similar signals may arise from the plant itself because of the damage caused by microbes, in the form of what were originally called endogenous elicitors, which are now described as damage-associated molecular patterns (DAMPs). The recognition of MAMPs and DAMPs by the plant induces the pattern-triggered immunity (PTI; (Bigeard et al., 2015; Choi and Klessig, 2016)).

Within minutes after PAMP/DAMP recognition, signaling cascades trigger a set of different defense responses, including elevation of cytoplasmic Ca^{2+} levels, production of reactive oxygen species (ROS), nitric oxide, the activation of various calcium/calmodulin-dependent kinases (CDPKs) and mitogen-activated protein kinases (MAPK), stomatal closure and broad changes in gene regulation leading to production of various anti-microbial components, including induction of salicylic acid (SA) synthesis and signaling (Bigeard et al., 2015; Muthamilarasan and Prasad, 2013; Zipfel and Robatzek, 2010). Delayed response manifested by PTI also includes the deposition of callose near infection site and inhibition of growth processes (Boller and Felix, 2009). If PTI stops the pathogen attack, the outcome of the interaction is non-host resistance (Ruiz-Ferrer and Voinnet, 2009). However, many successful pathogens have evolved the ability to deliver effector proteins into the plant cells to suppress PTI and to promote their virulence. Plants that are not able to detect these effectors are susceptible to a pathogen, thereby establish effector-triggered susceptibility (ETS) (Peláez and Sanchez, 2013).

In turn, many plants have evolved resistance (*R*) genes that mediate intracellular specific recognition of effector proteins and initiate effector triggered immunity (ETI). The ETI signaling cascades often lead to hypersensitive response (HR) and programmed cell death (PCD) that locally counteracts pathogen multiplication and progression (reviewed by Zvereva and Pooggin, 2012). However, this is not a general rule because some MAMPs do induce a HR, whereas some resistance genes provide protection without a HR or PCD

(Boller and Felix, 2009). For instance, the tomato resistance protein Tm-1 relays resistance against tomato mosaic virus (ToMV) by inactivating the ToMV replicase protein without eliciting HR-associated cell death (Ishibashi et al., 2012). ETI also induces systemic acquired resistance (SAR) in tissue distal to the inoculation site, conferring broad spectrum resistance against biotrophic pathogens. SAR relies on the local accumulation of the plant hormone SA, which is regulated by small diffusible signals originating from the point of infection.

Most *R* genes in plants encode members of an extremely polymorphic superfamily of intracellular nucleotide-binding site leucine-rich repeat (NBS-LRR) receptors. Specific NBS-LRR proteins are activated by specific pathogen effectors. This can be via direct interaction, as receptor and ligand, or indirectly (Figure 1). In the first example of indirect recognition, the ETI is activated when pathogen effector binds to and modifies a plant target protein that is bound to and monitored (or guarded) by R protein. In the second indirect recognition example, ETI is initiated when R protein perceives modifications of a plant decoy protein that mimics a true effector target protein (Cui et al., 2015; Dangl et al., 2013). As the same plant target protein can sense multiple pathogen effectors and is guarded by only a single or few NBS-LRRs, the indirect effector recognition is likely to have evolved to enable plants with limited repertoire of NBS-LRRs (159 in Arabidopsis, 460 in rice, 755 in potato) to perceive sets of distinct pathogens (Cesari, 2018; Jupe et al., 2013). In addition, compared with direct NBS-LRR-ligand recognition, indirect recognition can provide more effective options for plant immunity as pathogen effectors have to lose or modify their function to escape indirect detection, which may impose greater fitness costs than simply avoiding NBS-LRR binding (Cesari, 2018).

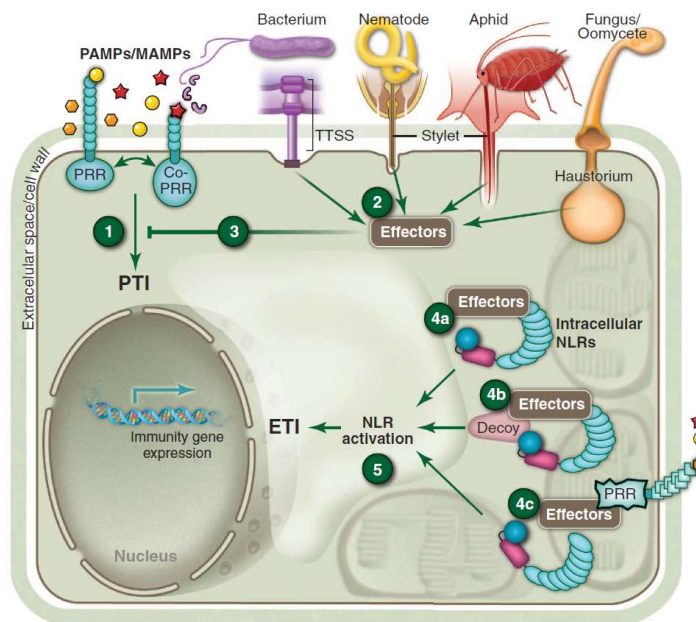


Figure 1: Schematic presentation of plant immune system. Pathogens express PAMPs/MAMPs. Plants perceive these via extracellular PRRs and initiate PTI (step 1). Pathogens deliver virulence effectors to the plant cell interior (step 2). These effectors have the ability to suppress PTI and facilitate virulence (step 3). Intracellular NBS-LRR receptors can sense effectors in three principal ways: first, by direct receptor ligand

interaction (step 4a); second, by sensing effector-mediated alteration in a decoy protein that structurally mimics an effector target, but has no other function in the plant cell (step 4b); and third, by sensing effector-mediated alteration of a host virulence target, like the cytosolic domain of a PRR (step 4c). Following recognition effector-triggered immunity (ETI) is triggered. Republished with permission of American Association for the Advancement of Science, from *Pivoting the Plant Immune System from Dissection to Deployment*, Dangl et al., 2013, Science, 10.1126/science.1236011, permission conveyed through Copyright Clearance Center, Inc.

Although the characteristics of immunity associated with PTI and ETI are different, many common responses such as oxidative burst, Ca^{2+} , downstream MAPK kinase cascade activation, hormonal changes, such as increased level of SA, and transcriptional reprogramming were found to be activated during both PTI and ETI, suggesting the overlap in the signaling machinery between PTI and ETI (Bigeard et al., 2015; Tsuda and Katagiri, 2010). However, ETI activated immune responses are faster and stronger in its amplitude than those in PTI. Molecular patterns that trigger PTI are conserved among pathogens and harmless microbes (Boller and Felix, 2009). If too vigorous immune responses during PTI would be rapidly activated after recognition, without previous discrimination among pathogenic and non-pathogenic microbes, the activation of immune response might be unnecessary in many cases, imposing a substantial cost on the fitness of plants. Therefore, PTI responses have likely been selected for weakness in early stages due to the low specificity in pathogen recognition. On the other hand, pathogen recognition by R protein is specific and thus rapid and strong ETI response is desirable to stop the pathogen proliferation (Tsuda and Katagiri, 2010).

1.1.1 Plant immune response against viruses

As obligate parasites with limited viral genome-encoded functions, plant viruses extensively use the host intracellular machinery for replication of their genomes, expression of viral genes and establishment of infection. As a consequence, they interact profoundly with the host during their biological cycle. Plant viruses are delivered into the cells by insect vectors or through opportunistic mechanical wounds. Once inside the cells, the viral particles, which consist of nucleic acids (RNA or DNA) encapsulated by the coat protein or capsid, are disassembled to release the viral genome and to initiate the infectious cycle, which includes expression and replication of the viral genome, cell to cell and long-distance movement of the viral particles and/or viral genome and vector-mediated transmission to new hosts (Calil and Fontes, 2017).

Plants have developed two major strategies to counteract virus infections: RNA silencing-based defenses and resistance (*R*) gene-mediated defenses. RNA silencing is an evolutionary conserved antiviral system in plants. The majority of plant viruses are positive-sense RNA viruses, with either single-stranded or double-stranded RNA genomes (Waterhouse et al., 2001). Within the cell viral single-stranded RNA gets converted into double-stranded RNA (dsRNA) replication intermediates by virus-encoded RNA-dependent RNA polymerases (RDRs). The dsRNAs are recognized by host ribonuclease III-like Dicer-like (DCL) proteins, predominantly DCL2 and DCL4, which cleave viral dsRNA structures and give rise to primary virus-derived small interfering RNAs (vsiRNAs). vsiRNAs are recruited into Argonaute (AGO) protein(s) forming RNA-induced

silencing complex (RISC) that targets and cleaves additional viral RNA molecules entering the plant cell (Figure 2) (Baulcombe, 2004). Cleaved viral RNA further serve as a template for host RDR1 or RDR6, to synthesize *de novo* dsRNAs that are processed by DCLs into secondary siRNAs which can silence even more viral RNA thus amplifying silencing effect (Zhang et al., 2017). In addition, a remarkable feature of RNA silencing, in case of plants, is the spread of the silencing signal from cell to cell through plasmodesmata and over long distance via phloem (Sharma et al., 2013) to confer nucleotide-sequence-specific resistance in distant tissues. As the signal contains fragments of the virus sequence, the recipient cells are thus ready to degrade viral RNAs containing these sequences before the virus arrives. However, if the virus moves ahead of the signal, it can generate an infection as soon as it enters the distant cells (Lu et al., 2008; Waterhouse et al., 2001).

To counter this host defense mechanism, viruses have evolved viral suppressors of RNA silencing (VSRs) (Figure 2) (Csorba et al., 2015). VSRs have been found in nearly all plant virus families (Moon and Park, 2016) and are important for replication, assembly and movement of viruses. These suppressors use different strategies to inhibit RNA silencing, either by binding to long or short viral-derived dsRNAs to prevent vsiRNAs from being loaded into AGO proteins, or they impede activity of AGOs or promote their degradation (Carbonell and Carrington, 2015; Ivanov et al., 2016; Moon and Park, 2016). Often, these silencing suppressors are recognized by *R* genes and function as effectors to trigger ETI responses (Figure 2). Many silencing suppressors can also inhibit endogenous sRNA-mediated regulation of mRNA turnover or mRNA translation, causing disruption of host transcript abundance. This results in developmental abnormalities and occurrence of disease symptoms usually observed in virus-infected plants (Pallas and Garcia, 2011). Also, vsiRNAs were found to have the ability to target host mRNAs leading to disease symptoms development. For instance, symptoms caused by CMV were shown to be a consequence of vsiRNA-directed silencing of chlorophyll biosynthetic gene *CHLI* (Pallas and Garcia, 2011; Wang et al., 2012).

In R-mediated defense response, plants employ NBS-LRRs proteins, which recognize viral effectors and activate ETI similar to that employed in non-viral infections. The viral effectors that are specifically detected by matching R proteins to activate ETI are termed avirulence (Avr) proteins. Pathogens containing *Avr* genes are avirulent to plants carrying the corresponding *R* genes and virulent to plants without the *R* genes. Due to the limitation of the coding capacity of viral genomes, virtually all virus proteins, such as replicase, movement proteins (MPs), coat proteins (CPs), can act as Avr determinants. As for non-viral pathogens, most plant antiviral *R* genes encode NB-LRR proteins that mediate resistance via specific (direct or indirect) recognition of a virus Avr factor (Gouveia et al., 2017).

The tobacco *N* gene, which confers resistance against tobacco mosaic virus (TMV) and encodes a TIR (Toll/interleukin receptor)-NB-LRR protein, was the first plant virus resistance *R* gene to be identified. The *N* resistance protein indirectly recognizes the helicase domain (the p50 effector) of TMV replicase via N receptor-interacting protein 1. Upon recognition, *N* protein undergoes conformational changes and moves to the nucleus where it interacts with transcriptional factors to modulate the expression of defense-related genes (Gouveia et al., 2017; Ueda et al., 2006). Interaction triggers HR response that induces establishment of necrotic local lesions which limit virus infection to the infection site (Mandadi and Scholthof, 2013; Ueda et al., 2006). Although necroses are observed concomitantly in HR-mediated resistance, the cell death can be uncoupled from resistance response (Künstler et al., 2016). Another example of viral resistance genes is the *Rx* gene in potato, which mediates extreme resistance (ER) against PVX. It encodes a well-characterized representative of the CC-NB-LRR class of R proteins, and is triggered by the viral CP.

Unlike other disease resistance responses, ER is not associated with HR at the site of infection but rather it is associated with an early arrest of viral accumulation in single cells (Künstler et al., 2016).

In contrast with resistant (also known as incompatible) plant virus-interaction, most susceptible (or compatible) virus infections either do not trigger HR or the HR is triggered but is inefficient in limiting the spread of the virus within the host plant (Baebler et al., 2014; Mandadi and Scholthof, 2013). However, a similar yet distinct form of necrosis, termed systemic necrosis, could be observed in susceptible interactions. For example, systemic necrosis was observed in *Nicotiana rustica* plants infected with TMV or in *Nicotiana benthamiana* plants with mixed infections of potexiviruses and potyviruses, including PVY. Both HR-associated necrosis and systemic necrosis involve PCD, trigger ROS production and alter expression of similar defense-related genes (Mandadi and Scholthof, 2013; Xu et al., 2012). Unlike HR-associated necrosis, systemic necrosis is manifested much later after infection and is primarily observed in upper non-inoculated tissue. In addition, the systemic necrosis results either from constitutive or uncontrolled cell death and is regarded as a lethal response that can kill the host plant, while HR response does not. Moreover, systemic necrosis does not prevent virus multiplication or its systemic movement, thereby resulting in susceptible interaction (Mandadi and Scholthof, 2013).

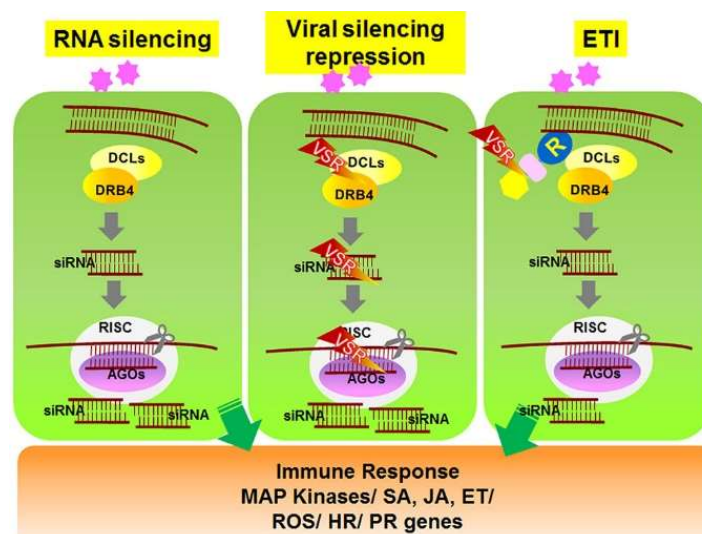


Figure 2: RNA silencing and R-mediated responses in plant cells. Upon amplification of viruses in plant cells, viral double-stranded RNAs (dsRNAs) activate RNA silencing mechanisms. Viral dsRNAs are processed into small RNA fragments (vsiRNAs). The vsiRNAs are recruited to RISC, which is associated with AGO protein. RISC complex then targets and degrades complementary viral RNA (left panel). Viruses express genes encoding silencing suppressor proteins (VSRs) that inhibit the RNA silencing mechanisms (center panel). In response, several R proteins recognize the VSRs and induce downstream ETI responses (right panel). Republished from Crosstalk in Viral Defense Signaling, Moon and Park, 2016, *Frontiers in Microbiology*, doi: <https://doi.org/10.3389/fmicb.2016.02068>, according to Creative Common Attribution ("CC BY") licence.

1.1.1.1 Plant immune response against potato virus Y

Potato virus Y (PVY) is considered to be one of the ten most important plant viruses of crops, because of its worldwide distribution and economic impact (Scholthof et al., 2011). PVY has a wide host range that includes many genera in the family *Solanaceae*, including important crop plants such as potato, pepper, tomato, and tobacco. Potato is the world's most important non-grain staple crop (Solomon-Blackburn and Barker, 2001). In potato, PVY is a yield-limiting pathogen that can cause 10-90 % yield loss depending on year, cultivar and location (Grech-Baran et al., 2018). The PVY may also cause post-harvest losses due to tuber necrosis and reduced storage quality (Piche et al., 2007). PVY is transmitted by sap-feeding aphid vector in a nonpersistent manner and vegetatively through propagated potato tubers, or mechanically by contact (Karasev and Gray, 2013).

PVY as for other members of the *Potyvirus* genus, have rod-shaped, flexuous particles (about 700 nm in length, 11–13 nm in diameter) encapsidating the viral RNA with multiple copies of a single coat protein (CP). The genome of PVY is a positive-sense single stranded RNA of approximately 9.7 kb. As with all potyviruses, the PVY genome has a poly(A) tail at the 3' end and a covalently linked VPg protein at the 5' terminus. The genome consists of two opening reading frames (ORFs) which encode 11 proteins. A single large ORF encodes a polyprotein cleaved into ten functional proteins P1, HC-Pro, P3, 6K1, CI, 6K2, VPg, NIa, NIb and CP, while a second small open reading frame called *pipo* (Pretty Interesting *Potyviriidae* ORF) encodes P3N-PIPO. P3N-PIPO is generated by either a ribosomal slippage creating a +2 frameshift within the P3 ORF or incorporating an additional nucleotide through slippage of the viral RNA-dependent RNA polymerase at a highly conserved G₁₋₂A₆₋₇ motif at the 5' end of PIPO sequence (Lacomme and Jacquot, 2017).

PVY is well-known for its propensity to evolve through accumulation of mutations and recombinations, where different strains of the virus exchange large (several hundred nt-long) segments of their genome (Green et al., 2018). Currently, fourteen strains of PVY have been defined, including five non-recombinant (PVY^O, PVY^{Eu-N}, PVY^{NA-N}, PVY^C, and PVY^{O-O5}) and nine recombinant strains (Green et al., 2018). Of them PVY^{NTN} and PVY^{N-Wi}, genetic recombinants between PVY^O and PVY^N, became the predominant strains in Europe and USA (Dupuis, 2017). Increased fitness appears to have enabled them to replace the non-recombinant variants in most potato growing areas of the world (Bellstedt et al., 2017).

In sensitive potato cultivars, PVY induces diverse disease symptoms such as leaf and vein necrosis, mosaic chloroses, yellowing and leaf drop, and necrotic ringspots on tubers referred to as potato tuber necrotic ringspot disease (PTNRD) (Baebler et al., 2009; Kogovšek and Ravnikar, 2013). Symptom appearance and severity of these diseases depend on genotypes of both, potato host plant and PVY pathogen (Figure 3). In addition, the response of a potato towards PVY infection can vary with various environmental conditions (temperature and light intensity) and various developmental and physiological growth stages of a plant (e.g., plant age) (Pompe-Novak and Lacomme, 2017). Several potato cultivars also exhibit a tolerant response in which virus replicates and spreads systemically, however, symptoms of the disease are mild or not visible at all (Baebler et al., 2011; Kogovšek and Ravnikar, 2013).

Resistance to PVY in potato may be conferred by two different types of resistance genes: ER conferred by *Ry* genes and HR conferred by *Ny* genes (Valkonen, 2015). In the case of ER, potato plants show no symptoms or very limited necrosis in the form of pinpoint lesions in some genotypes. Virus titres remain extremely low, below the limit of detection, due to either inhibition of virus multiplication in the infected cells or restricted cell-to-cell

movement of the virus (Pompe-Novak and Lacomme, 2017). The sources of the ER against PVY are the wild relatives of potato, and at least three single dominant *R* genes conferring ER to PVY have been identified. These are *Ry_{adg}* from *S. tuberosum* ssp. *andigena*, *Ry_{sto}* from *Solanum stoloniferum*, and *Ry_{chc}* from *Solanum chacoense*. Cultivated potatoes could obtain *Ry* genes only by classical breeding (e.g. Sante with introduced *Ry_{sto}* gene) (Grech-Baran et al., 2018; Karasev and Gray, 2013). Of them only *Ry_{sto}* resistance gene has been recently isolated and characterized (Grech-Baran et al., 2018). As breeding is an intensive and time-consuming process, successful isolation of *R* genes (i.e. determination of its gene sequence) offers an opportunity to speed up the resistance breeding process by introducing the required *R* gene to cultivars by genetic transformation (Valkonen, 2015).

In the case of HR, virus translocation from the initially infected leaf to other parts of the plant is prevented, although the virus replication and initial cell-to-cell movement are not blocked. Later, most of the infected cells die, which results in a localized necrotic lesion at the site of infection. For example, infection of cv. Rywal by PVY^{NTN} or PVY^{N-Wi} results in necrotic lesions on the inoculated leaves and inhibition of systemic virus movement, with no significant impact on yield (Szajko et al., 2008).

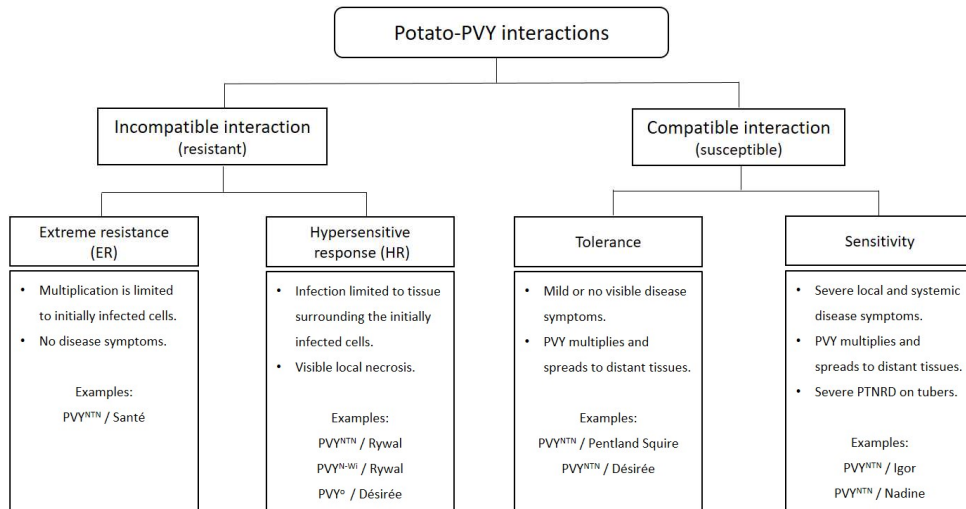


Figure 3: Outcomes of potato-PVY interaction. Outcomes depend on the host sensitivity and are manifested in different responses in terms of virus multiplication and disease symptoms development in different potato genotypes. Adapted from Potato virus Y: biodiversity, pathogenicity, epidemiology and management, First edition, Chapter 3: Molecular and Cellular Events During Infection of Potato by PVY, Pompe-Novak and Lacomme; Pages 21-42, 2017.

Until now, several *N* genes conferring HR in potato against PVY have been discovered, such as *Ny*, *Nc*, *Nz* and *Ny-1*, *Ny-2* (Szajko et al., 2014). However, none of them have been isolated so far. Unlike *R* genes, *N* genes are common in commercial potato cultivars and they are virus-strain specific (Karasev and Gray, 2013). For example, *Ny-1* can only trigger HR in potato plants infected with PVY^O, PVY^N, PVY^{N-Wi} and PVY^{NTN} (Szajko et al., 2008).

HR-conferred resistance mediated by *N* genes is also less durable compared to ER, as certain PVY strains can acquire several mutations/recombinations that help them overcome recognition by corresponding *N* genes (Valkonen, 2015). Another limitation of *N*-mediated resistance is the temperature sensitivity of some *N* genes. There are many examples where higher temperatures (usually above 28 °C) broke the resistance, which resulted in inefficient restriction of systemic movement of PVY (Baebler et al., 2014; Szajko et al., 2014).

1.1.2 Plant hormonal signaling pathways in immune response

The immune response is also regulated by plant hormones, which synergistically or antagonistically work in a complex network to regulate many aspects of plant growth, development and response to various environmental cues. This so-called hormone crosstalk provides the plant with a powerful capacity to finely regulate its immune response to the invader encountered and to utilize its resources in a cost-efficient manner (Pieterse et al., 2012).

Downstream of PTI or ETI activation, diverse plant hormones act as central players in triggering the plant immune signaling network (Pieterse et al., 2012). The essential roles of SA, ethylene (ET) and jasmonic acid (JA)-mediated signaling pathways in immune response to pathogens are well described. SA signaling is primarily induced by and involved in defense against biotrophic pathogens, whereas JA signaling is primarily induced by and involved in defense against insect herbivores and, in conjunction with ET, against necrotrophic pathogens. SA and JA signaling pathways are generally antagonistic to each other. For example, elevated SA signaling in response to biotrophic pathogens is often correlated with reduced JA signaling and decreased resistance to necrotrophic pathogens (Huot et al., 2014; Robert-Seilaniantz et al., 2011). Although valid for many plant-pathogen interactions, this traditional view is overly generalized, and accumulating evidence in both monocot and dicot systems suggest that reality is more complex (De Bruyne et al., 2014). Synergism between the SA and JA/ET signaling pathways has been reported. For example, components of the JA and ET signaling pathways work in combination with SA-mediated resistance response in defense of *Arabidopsis* against *P. syringae* and *H. parasitica* (Clarke et al., 2000). Moreover, a very recent study by our group revealed a previously unknown connection between ET and SA signaling in potato, namely that activation of the ET signaling module, through Ethylene Insensitive3 (EIN3), that induces the expression of *Nonexpresser of PR genes 1* (NPR1), an important regulator of SA signaling (Ramšak et al., 2018).

SA is a phenolic compound shown to affect many plant processes including growth, development, senescence, and stress responses and it is primarily recognized for its role in local defense induced against biotrophic and hemi-biotrophic pathogens. SA biosynthesis is triggered during PTI and ETI upon recognition of PAMPs or effectors of pathogens (Pieterse et al., 2012). Signaling downstream of SA is largely controlled by the regulatory protein NPR1, which upon activation by SA acts as a transcriptional coactivator of a large set of defense-related genes (Durrant and Dong, 2004; Moore et al., 2011). These *PR* (*pathogenesis-related*) genes are a diverse group, but several encode proteins with antimicrobial activity. Of the many *PR* genes identified, *PR1*, *PR2* and *PR5* have been shown to be induced by SA and have long been used as markers of SA signaling (Fu and Dong, 2013).

Once SA pathway is activated at the site of infection, a defense response is often triggered in distal parts of the plant to protect healthy tissues. This long-lasting and broad-

spectrum induced resistance is referred to as systemic acquired resistance (SAR) (Pieterse et al., 2012; Verma et al., 2016). The strongest evidence supporting SA's role as a critical local and systemic defense signal resulted from analyses of plants in which endogenous SA levels were altered. The first of these studies utilized transgenic tobacco or Arabidopsis plants expressing the bacterial *NahG* gene, encoding the SA-degrading enzyme salicylate hydroxylase. Following pathogen infection, these transgenic plants were unable to accumulate high SA levels, and they failed to develop SAR or express PR genes in the systemic leaves; instead, they displayed increased susceptibility to different virulent and avirulent bacterial and fungi pathogens (Vlot et al., 2009).

In addition, SA deficiency was shown to impair HR-conferred resistance to TMV or tomato ringspot virus (ToRSV) infection in tobacco, resulting in increased size of the lesions and inefficient restriction of systemic movement of the viruses (Jovel et al., 2011). In addition to HR-conferred resistance, SA was shown to control ER response. It was found that resistance response in tobacco to tomato bushy stunt virus (TBSV) requires intact SA signaling. Although TBSV did not accumulate in wild-type (WT) *Nicotiana tabacum* Xanthi, *NahG* lines of the same background were susceptible to infection even in the presence of the resistance gene (Alazem and Lin, 2014).

In compatible plant-pathogen interactions, overexpression of SA biosynthesis genes or application of SA or its analogues often improves plant defense to a certain degree by delaying the onset of viral infection, inhibit cell-to-cell and/or systemic movement and disease development (Baebler et al., 2011; Ji and Ding, 2001a). SA signaling may also enhance the efficiency of RNA silencing pathway in triggering immune responses against viruses by inducing *RDR1* expression levels (Carr et al., 2010; Yu et al., 2003), which is crucial for the maintenance of basal resistance to several RNA viruses, including PVY. The infection of *rdr1* mutant plants by TMV results in higher viral accumulation both in inoculated and in systemically infected leaves (Yu et al., 2003). The second line of evidence suggesting that SA and RNA silencing are connected came from experiments showing that certain viral silencing suppressor proteins also suppress SA-mediated defense, leading to replication and local movement of the virus (Ji and Ding, 2001a).

JA and its structurally related metabolites are lipid-derived compounds that are synthesized rapidly via the oxylipin biosynthesis pathway upon pathogen or insect attack. JA receptor (COI1) mutant plants are more susceptible to necrotrophic fungi, such as *Alternaria brassicicola* and *Botrytis cinerea*. Surprisingly, JA mutant plants exhibit an increased resistance to necrotroph *Fusarium oxysporum* (reviewed by Robert-Seilaniantz et al., 2011). Upon pest chewing and wounding during herbivory, JA synthesis is rapidly triggered, both locally at the injured site and systemically in undamaged leaves throughout the plants, to suppress or prevent the feeding, growth or reproduction of herbivores through activating expression of defense-related genes, production of toxic secondary chemicals and generation of volatile organic compounds which attract predators or parasitic enemies of pests (Yan and Xie, 2015).

JA may negatively affect host resistance to certain viruses such as TMV. Silencing of the JA receptor gene *COI1* or JA biosynthetic gene *AOS* in tobacco would enhance plant resistance to TMV accumulation. Moreover, exogenous application of JA conjugate methyl jasmonate (MeJA) increased TMV accumulation in inoculated leaves (Oka et al., 2013). An increasing body of evidence shows that some viruses may hijack JA signaling to promote plant susceptibility for either their own benefit or the benefit of insect vectors that transmit them (Yan and Xie, 2015). For instance, the C2 protein and its homologous protein L2 were identified as viral pathogenic factors of the geminivirus tomato yellow leaf curl virus (TYLCV) and beet curly top virus (BcTV) (Li et al., 2014), which interact with and interfere in the Arabidopsis CSN5 protein, resulting in attenuation of JA-mediated defenses required for conferring resistance against these viruses (reviewed by (Yan and Xie, 2015).

ET is a gaseous hormone that can act both positively and negatively on plant immunity. For instance, in *Arabidopsis*, ET potentiates SA-responsive *PR-1* expression, and in tobacco, it is essential for the onset of SAR. Conversely, the ET-responsive transcription factors EIN3 and EIL1 were implicated in global repression of PAMP-responsive genes in *Arabidopsis*, including the SA biosynthesis gene *ICS/SID2*, resulting in reduced accumulation of SA (reviewed by Pieterse et al., 2012). ET was shown to be involved in symptom development on cauliflower mosaic virus (CaMV) infection, systemic movement of tobacco mosaic virus crucifer strain (TMVcg) and the formation of necrotic lesions following infection by other viruses (e.g. TMV, tobacco necrosis virus (TNV)) (Alazem and Lin, 2014).

In addition, other hormones such as auxins, abscisic acid (ABA), cytokinins (CKs), gibberellins (GAs) and brassinosteroids (BRs) have recently emerged as crucial players in plant-pathogen interactions. ABA is an isoprenoid compound that functions as a regulator in development and adaptation to abiotic stress, in particular drought and salinity stress (Alazem and Lin, 2014; Denancé et al., 2013). ABA strongly antagonizes many hormone pathways, including the SA pathway, the ET pathway and the synergized dual ET/JA pathway (Broekaert et al., 2006). However, ABA seems to positively regulate JA biosynthesis and signaling during necrotrophic infection. ABA can positively regulate plant defense at the early stages of infection by the mediation of stomatal closure, or induction of callose deposition if the pathogen evades the first line of defense (Alazem and Lin, 2014).

GAs are tetracyclic diterpenoid plant hormones that act at all stages in the plant life cycle by promoting seed germination, stem elongation and modulate flowering. Through extensive physiological and molecular genetic studies of mutant plants impaired in GA perception, biosynthesis, or signaling, it is now clear that GAs are master regulators of plant growth, particularly in response to environmental conditions such as light, flooding, temperature and salt, and biotic stress (Gupta and Chakrabarty, 2013). GAs promote plant growth and development by inducing the degradation of DELLAs, a class of nuclear growth-repressing proteins. *Arabidopsis* mutants lacking four of the five DELLAs showed high levels of SA-dependent resistance when challenged with the hemibiotrophic pathogen *P. syringae*, and showed enhanced susceptibility against the necrotrophic fungus (Navarro et al., 2008). It has been contemplated that DELLAs modulate the balance between SA and JA/ET-mediated signaling pathways (De Bruyne et al., 2014; Navarro et al., 2008).

BRs are a unique class of polyhydroxylated steroidal phytohormones with important roles in regulating various physiological and developmental processes, including pollen tube elongation, seed germination, photomorphogenesis, flowering, and senescence. More than 50 brassinosteroids have been described, of which brassinolide and castasterone are the most active forms. Typically, BR-deficient and BR-insensitive mutants display dwarfism (reviewed by De Bruyne et al., 2014). BR can act both antagonistically and synergistically with PTI responses. In *Arabidopsis*, several independent studies confirmed that BR confers increased susceptibility to bacterial pathogens such as *Pseudomonas syringae*. Overexpression of *BRI1*, encoding a BR receptor favors infection by the pathogenic oomycete *Hyaloperonospora arabidopsidis*. On the other hand, in *N. tabacum*, BR treatments lead to reduced size of necrotic lesions caused by TMV (in the resistant cultivar Xanthi), and milder disease symptoms caused by powdery mildew (Lozano-Durán and Zipfel, 2015).

The role of CKs in biotic stress response has been demonstrated by several studies. Transgenic *Arabidopsis* plants having stabilized CK levels exhibited enhanced resistance against infection with hemibiotrophic pathogen *Verticillium longisporum* (Reusche et al., 2013). CKs are also known to crosstalk with SA signaling cascade to stimulate defense responses to biotrophs. CKs activate transcription factor ARR2 (*Arabidopsis* response regulator 2) which positively modulates SA signaling. Similarly, the synergistic interaction

between SA and CK, in an NPR1- and WRKY45-dependent manner, has been shown to increase rice resistance to the blast fungus *Magnaporthe oryzae* (Verma et al., 2016).

Auxins play a key role in plant growth and development. Many viral infections result in aberrant phenotypes such as stunting, leaf curl and loss of apical dominance, symptoms that resemble those of mutants with compromised biosynthesis and signaling of auxins. Elevated auxin signaling often correlates with increased susceptibility to pathogens and mainly antagonistically interact with SA signaling. The bacterial MAMP flg22, a peptide from flagellin protein was shown to increase the level of SA and suppressed the auxin signaling leading to increased resistance to the bacterium *Pseudomonas syringae* pv. *tomato* DC3000 and to the oomycete *H. arabidopsidis*. SA treatment stabilizes auxin repressor Aux-IAA (auxin/indole-3-acetic acid) proteins, probably indirectly through repression of the F-box auxin receptor TIR1, resulting in down-regulation of auxin-responsive genes (Fu and Wang, 2011). Reciprocally, activation of auxin signaling triggers the suppression of SA biosynthesis and SA signaling. The negative effect of auxins on regulation of pathogen resistance could also be indirect. Treatment of rice plants with auxin impaired the resistance to *Xanthomonas oryzae* pv. *oryzae* as a consequence of the activation of the biosynthesis of cell wall-associated expansins that caused cell wall loosening, which facilitates growth of the pathogen (Denancé et al., 2013).

While hormone crosstalk may provide the plant with a powerful regulatory potential to finely tune its defense (Ramšak et al., 2018), crosstalk is also a possible target for plant attackers to manipulate the immune signaling network for their own benefit (Pieterse et al., 2012). In recent years, numerous examples of plant pathogens that hijack specific hormone-regulated signaling pathways, e.g., by producing plant hormones, hormone mimics, or effectors that target hormone biosynthesis or signaling pathway components, have been described (Denancé et al., 2013; Pieterse et al., 2012; Robert-Seilaniantz et al., 2011). Even though some pathogens can also manipulate hormone signaling, the plant can attenuate this manipulation. Hence, the fine crosstalk between these pathways generates variable responses according to the challenging pathogen (Robert-Seilaniantz et al., 2011).

1.1.3 Role of endogenous sRNAs in immune response

Plants are able to produce a variety of endogenous sRNAs that share many common features with the viral sRNAs (vsiRNAs) produced during virus infection. On the basis of their biogenesis and precursor structures, endogenous sRNAs are placed in two distinct groups: microRNAs (miRNAs) and siRNAs with functions at both transcriptional and post-transcriptional levels. Both miRNA and siRNA pathways have been best characterized in the model plant *Arabidopsis* (Katiyar-Agarwal and Jin, 2010).

miRNAs are non-coding RNA molecules ranging from 20 to 24 nucleotides in length. The biogenesis of miRNAs occurs in the nucleus where specific *MIR* genes are transcribed by RNA polymerase II into long primary transcripts (pri-miRNAs) which fold into hairpin-like structures (Figure 4A). These are typical transcripts capped at the 5' end and polyadenylated at the 3' end with several adenosines (Pantaleo et al., 2010). The pri-miRNAs are then cleaved by RNase III-like enzyme DCL1 in association with other plant proteins such as hyponastic leaves 1 (HYL1) and serrate (SE) into smaller stem-loop structures, called precursor miRNAs (pre-miRNAs). The pre-miRNAs are further cleaved by DCL1 to generate double-stranded miRNA:miRNA* duplex. These duplexes are protected against degradation by methylation at their 3' end by the methyltransferase HEN1 and further exported to the cytoplasm by HASTY (HST1), an exportin protein. Here, the duplexes are loaded into the RISC complex, containing AGO proteins. After

RISC loading, the miRNA:miRNA* duplexes are unwound primarily by the AGO1 protein and usually one strand of the duplex miRNA is directed to the exosome for degradation by sRNA degrading nucleases, while the other strand of the duplex named mature miRNA is inserted into the RISC to guide the post-transcriptional gene silencing through sequence-specific cleavage or translational repression of target mRNAs (Djami-Tchatchou et al., 2017; Ruiz-Ferrer and Voinnet, 2009). In addition to the control of targets at the post-transcriptional level, miRNAs can also regulate gene expression by inducing DNA and histone methylation (Wu et al., 2010). Besides the traditionally recognized mature miRNAs (i.e. miRNAs with the highest expression level), high abundance and ability to direct post-transcriptional gene silencing were also found for the miRNAs*, the complementary strand of mature miRNAs and isomiRs. The isomiRs are miRNA variants of variable lengths, produced by the same genomic loci as corresponding mature miRNA. The isomiRs are produced through individual or combined effects of: (1) Post-transcriptional enzymatic nucleotide additions, modifications, or removals that alter mature miRNA sequence during miRNA maturation; and, (2) imprecise or alternative cleavage of DCL1 during miRNA biogenesis resulting in different mature miRNA sequences with shifted start and/or end positions (Budak et al., 2015; Debat and Ducasse, 2014).

In contrast to miRNAs that are derived from imperfectly base-paired hairpin loop structures siRNAs are derived from perfectly paired double-stranded RNA (dsRNA) precursors. siRNAs are classified into at least two major classes heterochromatic siRNAs (hc-siRNAs) and phased siRNAs (phasiRNAs) (Axtell, 2013a). hc-siRNAs are predominantly 24-nt in length and are mainly derived from repetitive regions or transposons and regulate gene expression at transcriptional level. The hc-siRNAs associate with AGO4, AGO6 or AGO9 to target nascent transcripts transcribed by Pol V from the target loci. This targeting then recruits the DNA methyltransferases to induce *de novo* cytosine methylation at homologous DNA sites (Borges and Martienssen, 2015; Liu et al., 2018).

On the other hand phasiRNA, similarly as miRNA function mainly at the post-transcriptional level by either cleavage or translational suppression of target transcripts, although there is also evidence they can direct DNA methylation (Wu et al., 2012).

phasiRNAs can be produced from both coding and non-coding RNAs (the loci that produce them are all called *PHAS* loci) and their biogenesis relies on specific miRNA triggers. A subset of 22-nt miRNAs, with just a few exceptions, including highly conserved 21-nt miR390, is able to trigger the production of phasiRNAs from their cleavage mRNA products (Chen et al., 2010; Cuperus et al., 2010). The cleaved mRNA product serves as a substrate for RDR6, generating a dsRNA precursor, which is then cleaved sequentially in 21- (rarely also 24-nt) increments by DCL4 to produce the 21-nucleotide or 24-nucleotide phasiRNAs (Fei et al., 2015) (Figure 4B). Trans-acting siRNAs (tasiRNAs) are a special subclass of phasiRNAs generated from non-coding *PHAS* loci. The most known tasiRNAs are the set of trans-acting short interfering RNA-auxin response factors (tasiARFs) generated from *TRANS-ACTING SIRNA GENE3* (*TAS3*) (Allen et al., 2005; Axtell et al., 2006; Yoshikawa et al., 2005). The function of *TAS3*-derived tasiRNAs (tasiARFs) is to modulate transcript levels of auxin response factors (ARFs), known in Arabidopsis and other plants. In addition to non-coding *PHAS* loci, plant genomes also contain a large number of protein-coding *PHAS* loci. Genome-wide experimental and computational analysis has identified phasiRNAs from many gene families encoding NB-LRRs, pentatricopeptide repeat proteins (PPRs), MYBs, Ca²⁺-ATPase, and TIR1/AFB (auxin F-box protein) (Fei et al., 2015). Because phasiRNAs can function both in *cis*, targeting *PHAS* loci of origin, or in *trans*, targeting other related transcripts, a limited number of miRNA triggers can, by the production of phasiRNAs, dramatically amplify their

suppressive functions on the same or similar mRNA targets (Fei et al., 2013; Shivaprasad et al., 2012).

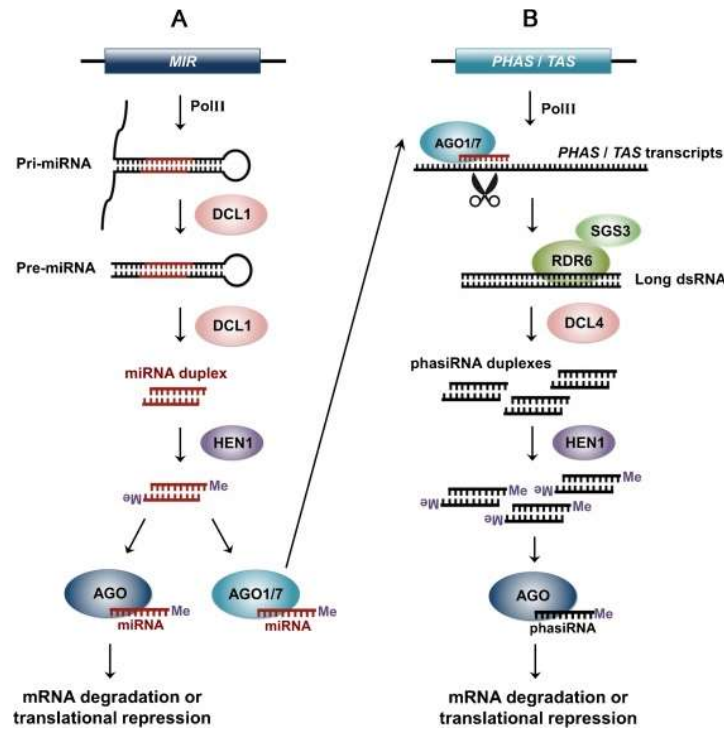


Figure 4: Biogenesis of miRNA and phasiRNA in plants. (A) miRNA biogenesis pathway. The primary transcripts of miRNAs (pri-miRNAs) are transcribed by RNA Polymerase II from endogenous *MIR* loci. pri-miRNAs are processed by the DCL1 complex in a two-step process; and the resulting double-stranded miRNA duplexes are subsequently stabilized by HEN1-mediated methylation. Usually, one miRNA strand is loaded into the AGO-containing RNA-induced silencing complex to suppress gene expression. (B) Some miRNA are able to trigger secondary siRNA (phasiRNA) production. These siRNAs are often in 21-nt (or 24-nt) regime, therefore, they are also called phased siRNAs or phasiRNAs. The transcripts of *PHAS* or *TAS* loci are cleaved by AGO/miRNA complex and used to synthesize long dsRNAs by RDR6 and SGS3 proteins. phasiRNAs are then generated from the long dsRNAs by DCL4 and subsequently loaded into AGO-containing RNA-induced silencing complex to induce target gene silencing. Republished with permission of Elsevier, from Small RNAs regulate plant responses to filamentous pathogens, Kuan et al., 2016, *Seminars in Cell & Developmental Biology*, doi: 10.1016/j.semcdb.2016.05.013, permission conveyed through Copyright Clearance Center, Inc.

In the past decades, plant miRNAs and phasiRNAs have been shown to be essential for numerous developmental processes, including growth and development of shoots, leaves, flowers, roots and seeds, among others. In addition, miRNAs and siRNAs are also involved in the plant responses to abiotic and biotic stresses, such as drought, temperature, salinity, nutrient deprivation, bacteria, virus and others (Yu and Zhang, 2017).

1.1.3.1 sRNA regulation in PTI

Arabidopsis miR393 was the first sRNA implicated in bacterial PTI. Transcription of miR393 (miR393-5p) was induced by the bacterial flagellin-derived PAMP peptide, flg22 to target mRNA encoding F-box auxin receptors, including TIR1, AFB2, and AFB3. This repression of auxin signaling was shown to correlate with enhanced disease resistance in plants, which was reflected in reduction of bacterial growth (Navarro et al., 2006). Enhanced miR393 accumulation was similarly found in Arabidopsis challenged with avirulent *P. syringae* pv. *tomato* DC3000 *hrcC*, which lacks a functional type-III secretion system required for virulence (Zhang et al., 2011). miR393 is conserved across plant species, as are its targets and target sites (Bai et al., 2017). Therefore, it is possible that its function in suppressing auxin signaling and enhancing PTI is also conserved. There is some support for this hypothesis of a conserved function; for example, in soybean, miR393 is upregulated upon infection by the oomycete pathogen *Phytophthora sojae* and miR393-knockdown plants displayed enhanced susceptibility to *P. sojae* (Wong et al., 2014). Interestingly, as a 22-nt miRNA, miR393 triggers phasiRNA biogenesis from the target transcripts of AFB2 and AFB3 in Arabidopsis, and these phasiRNAs together with miR393 repress transcript levels of TIR1, AFB2, and AFB3 (Si-Ammour et al., 2011). Consequently, via hormone crosstalk, both plant development and basal defense responses are regulated by miR393 and miR393-triggered phasiRNAs. Therefore, miR393-involved regulation of the auxin signaling pathway has crosstalk with immune responses at the level of PTI (Figure 5).

Furthermore, the level of complementary strand miR393-3p was previously reported to play a role in the control of plant disease resistance in Arabidopsis. miR393-3p targets the Membrin 12 mRNA encoding a Golgi-localized Synaptosome-Related (SNARE) protein, Membrin 12 (MEMB 12), whose down-regulation leads to increased exocytosis of antimicrobial pathogenesis-related PR1, which subsequently enhances plant resistance. Thus, miR393-5p and miR393-3p, two sRNAs generated from the same sRNA duplex have the ability to coordinately increase plant immunity (Huang et al., 2016).

miRNAs other than miR393 that suppress auxin signaling also play a role in plant immune responses. For example, overexpression of miR160a, a miRNA targeting *ARFs*, increases callose deposition in Arabidopsis treated with either flg22 or *P. syringae* (Li et al., 2010b) and, in rice, enhances plant immunity against *Magnaporthe oryzae* (Li et al., 2014). A recent study by Natarajan and coworkers (2018) demonstrated that miR160 plays an important role in modulating local defense and SAR responses in potato during *P. infestans* infection (Natarajan et al., 2018).

miR398, a miRNA targeting mRNAs of two copper superoxide dismutases (CSD1, CSD2), has different roles in plant disease resistance. Superoxide dismutases convert superoxide anion into hydrogen peroxide and therefore regulate ROS (Naya et al., 2014). During oxidative stress, expression miR398 is reduced to promote accumulation of *CSD1* and *CSD2*. In Arabidopsis, overexpression of miR398 decreased callose deposition upon flg22 treatment leading to increased susceptibility to *P. syringae* (Li et al., 2010b). In contrast, in rice, overexpression of miR398b yielded increased hydrogen peroxide production and inhibited fungal growth (Li et al., 2014). Another miRNA found to be involved in PTI in Arabidopsis is miR773. It targets the mRNA coding for the DNA methyltransferase 2 (DMT2). Transgenic plants overexpressing miR773 showed reduced *DMT2* mRNA levels, reduced callose deposition and enhanced proliferation and thus susceptibility to *P. syringae* strains, indicating that, like miR398, miR773 also negatively regulates PTI to *P. syringae* (Li et al., 2010c; Peláez and Sanchez, 2013).

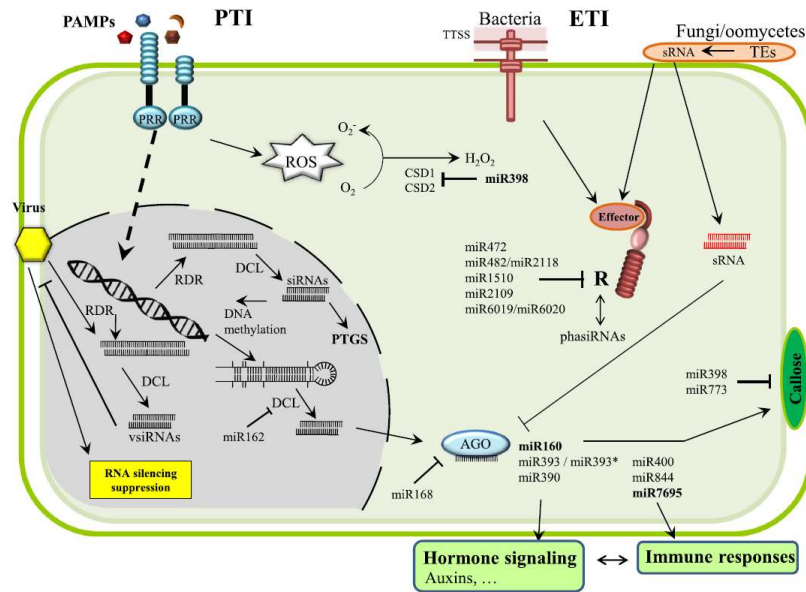


Figure 5: sRNAs involved in PTI and ETI. Most of the indicated miRNAs have been characterized in *Arabidopsis* and rice plants infected with bacterial or fungal pathogens. Dicer-like proteins (DCLs) process double-stranded RNAs or single-stranded RNAs with hairpin structures, giving rise to mature sRNAs which are loaded into Argonaute (AGO)-containing RNA-induced silencing complexes (RISCs) to induce gene silencing of their complementary targets. The RNA silencing-based antiviral defense involves the production of virus-derived sRNAs (vsiRNAs) by DCL activities that drive degradation of viral RNA in a sequence-specific manner. Two miRNAs, miR162 and miR168, are involved in the regulation of the miRNA pathway itself by guiding cleavage of DCL1 and AGO1 mRNAs, respectively. In rice, the involvement of miR160, miR398 and miR7695 in PTI responses was proposed. miR160 targets *Auxin Response Factors* (*ARFs*) whereas miR398 targets transcripts of *Cu/Zn superoxide dismutase* genes functioning in ROS scavenging. miR7695 targets an alternatively spliced transcript of the Natural resistance-associated macrophage protein 6 gene from rice. In the case of miR393, the two sRNAs of the duplex are functional during pathogen infection (miR393 targets TIR1, whereas miR393* regulates *MEM12*, encoding a SNARE protein that modulates exocytosis of antimicrobial PR1 proteins in *Arabidopsis*). Many miRNA families target mRNAs of NBS-LRRs and trigger the production of phasiRNAs to reinforce the silencing effect. *Arabidopsis* has no miR482 annotated, but its miR472 family members are closely related to miR482, and target *NBS-LRR* transcripts involved in ETI (Huang et al., 2016). Republished from *MicroRNAs in Rice Innate Immunity*, Baldrich and San Segundo, 2016, Rice, 10.1186/s12284-016-0078-5, according to Creative Commons Attribution 4.0 International License.

1.1.3.2 sRNA regulation in ETI

Various miRNAs were found to be directly involved in ETI responses in different plant species (Figure 5). Several miRNAs from miR482 family targeting *R* genes of NBS-LRR class were described in tomato (Shivaprasad et al., 2012). Members of miR482 family were predicted to target the conserved sequences encoding the P-loop motif of the NBS-LRR receptors, therefore they have the ability to suppress the expression of large numbers of

NBS-LRR defense genes. In the same study Shivaprasad and coworkers (2012) showed that upon the infection of virus or bacteria, the accumulation of miR482 is decreased to suppress the miR482-mediated silencing cascade, and subsequently increase the expression of defense-related mRNAs (Shivaprasad et al., 2012). They also showed that many *NBS-LRR* targeting miRNAs are capable of triggering the production of phased secondary phasiRNAs from their cleaved target mRNAs and at least one of these phasiRNAs targets other mRNAs of a defense-related protein. Zhai et al. (2011) described three additional abundant miRNA families miR2118, miR2109, miR472 and miR1510 that target diverse members of the NBS-LRR class of *R* genes in *Fabaceae* (Arikrit et al., 2014; Zhai et al., 2011). In another study, Li et al. (2012) identified two tobacco miRNAs nta-miR6019 and nta-miR6020 that guide cleavage of transcripts of the *N* gene that confers resistance to TMV. Overexpression of these two miRNAs in *N. benthamiana* attenuated *N* gene-mediated resistance to TMV. Interestingly, the 22-nt nta-miR6019 also triggers the production of phasiRNAs from the *N* transcripts (Li et al., 2011). In addition to the miRNAs that target several antibacterial or antiviral *R* genes, certain miRNAs were found to target *R* genes that confer resistance to fungal and oomycete pathogens. For example, miR6021, miR6022, and miR6023 target a tomato homolog of the *Cladosporium fulvum* resistance gene 9, which encodes a transmembrane protein and confers resistance to leaf mould fungus *C. fulvum* (Seo et al., 2013).

1.2 Methodologies for Discovery and Quantitative Profiling of sRNAs

sRNA studies are largely dependent on the availability of innovative strategies and methodological approaches for accurate detection of sRNA expression levels in specific tissues. Typically, sRNA profiling involves a series of steps that are highly sensitive to technical manipulations. Obtaining high quality, intact RNA is the first and often the most critical prerequisite in performing many fundamental molecular biology experiments, such as RT-qPCR and next generation sequencing analyses. Most RNA isolation procedures were developed and optimized to recover mRNAs while ignoring shorter RNA molecules. Unfortunately, these procedures can result in the loss of substantial amounts of short RNAs from samples. In recent years, novel RNA extraction methods were developed, allowing simultaneous isolation of both short sRNAs and long mRNAs. Although optimized for sRNAs recovering, these extraction methods may still suffer for differences in extraction efficiencies and for specific loss of some sRNAs, leading to incorrect sRNA expression estimation (Ach et al., 2008; Redshaw et al., 2013).

Until now, the method employing TRIzol-chloroform extraction followed by isopropanol precipitation developed by Chomczynski and Sacchi (1987) has still been the most widely used for RNA isolation for sRNA expression studies, including many potato sRNA studies (Kutnjak et al., 2015; Lakhota et al., 2014; Yang et al., 2015b), enabling efficient isolation of high-quality RNA as well as good retention of the short RNA population. However TRIzol-based extraction was shown unsuitable for the extraction of RNA from plant tissues that contain a high level of polyphenolics, polysaccharides, and fibrous materials (e.g., conifers and seeds) (Ding et al., 2008; Kalinowska et al., 2012). Nevertheless, several column-based methods have recently been adapted for short RNA isolation from plant tissues that claim to be faster and more efficient in providing high yields and purity of RNA (Kalinowska et al., 2012).

The isolation of high-quality RNA also depends greatly on the treatment and handling of the tissue prior to RNA extraction. Cellular ribonucleases act quickly and efficiently to

degrade RNA upon cell lysis; therefore, tissue frozen in liquid nitrogen and stored at no more than -70°C will provide the best possible material for further downstream sRNA analyses. Commercial reagents such as RNAlater® (Ambion) may increase storage stability without compromising the quality or quantity of the recovered RNA (Accerbi et al., 2010).

1.2.1 Reverse Transcription Quantitative-PCR-based Methods

Conventional technologies such as cloning, northern hybridization and microarray analysis were the first method employed for miRNA detection, however they are time-consuming, require relatively large amounts of RNA and are not sensitive enough to detect less-abundant miRNAs (Pritchard et al., 2012). In contrast, reverse transcription coupled with quantitative polymerase chain reaction (RT-qPCR) detection methods are very sensitive, specific, quantitative, fast and require small amounts of RNA for analysis; however, conventional qPCR methods require a template that is at least twice the length of either of the specific forward or reverse primers, each typically ~ 20 nt in length. Thus the target minimum length is ≥ 40 nt, making the small RNAs with ~ 22 nt in length too short to serve as templates (Kramer, 2012). In addition, unlike mRNAs, miRNAs lack a common sequence such as a poly(A) tail that can be used for selective enrichment or as a universal primer binding site for reverse transcription. Moreover, the mature miRNA sequence is present also in the primary transcript (pri-miRNA) and the precursor (pre-miRNA). It is therefore important to ensure that pri-miRNAs or pre-miRNAs do not provide templates in RT-qPCR amplification and do not contribute to the signal intensities detected. In addition, it was shown that miRNAs can differ by as little as a single nucleotide, making the ability to discriminate between forms with single nucleotide differences also important. Finally, many miRNAs have variants (so-called isomiRs) and measurement of different forms may be needed in some cases. Another challenge for profiling hundreds of miRNAs in parallel is that owing to their short length, variance in miRNA GC content leads to wide variance in melting temperatures (T_m) for annealing reactions, creating significant miRNA-specific biases (Pritchard et al., 2012).

Despite these challenges, two different RT-qPCR approaches were developed poly(A) RT-qPCR (Shi and Chiang, 2005) and stem-loop RT-qPCR method (Chen et al., 2005) (Figure 6). poly(A) RT-qPCR approach involves *in vitro* polyadenylation followed by oligo(dT)-mediated reverse transcription, and qPCR is subsequently performed using a miRNA-specific forward primer and a partial sequence in the oligo(dT) adapter as the reverse primer (Shi and Chiang, 2005). The stem-loop RT-qPCR employs miRNA specific RT primer for reverse transcription that is complementary to the 3' end of the miRNA. This primer contains a highly stable hairpin structure that unfolds upon denaturation, providing an elongated template. cDNA generated is then quantified using TaqMan qPCR assays with forward primer which is specific to miRNA sequence and includes extra 5' nts to optimize its melting temperature (T_m). The reverse primer is complementary to a sequence within the stem-loop RT primer. Stem-loop primers are more difficult to design but their structure reduces annealing of the primer to pre- and pri-miRNAs, therefore by increasing the specificity of the assay. Although linear primers are simpler to design, compared to stem-loop primers, linear primers may not discriminate between mature miRNA and their precursors (Benes and Castoldi, 2010). Consequently, the annealing and reverse transcription steps must be optimized to prevent any interaction between the primer and the pre- and pri-miRNAs. Stem-loop assay specificity is further enhanced by placement of the hydrolysis probe over miRNA sequence and the probe T_m is increased by

the addition of a minor groove binding (MGB) moiety to the probe (Chen et al., 2005; Kramer, 2012). Both these approaches were developed to assay the levels of mature miRNAs, however they can also be used to assay other small RNAs such as siRNA molecules. It was also shown that assays based on linear dT-primers often lacked specificity and require additional improvements such as incorporation of locked nucleic acid (LNA) nucleosides into primers (Adhikari et al., 2013; Varkonyi-Gasic et al., 2007).

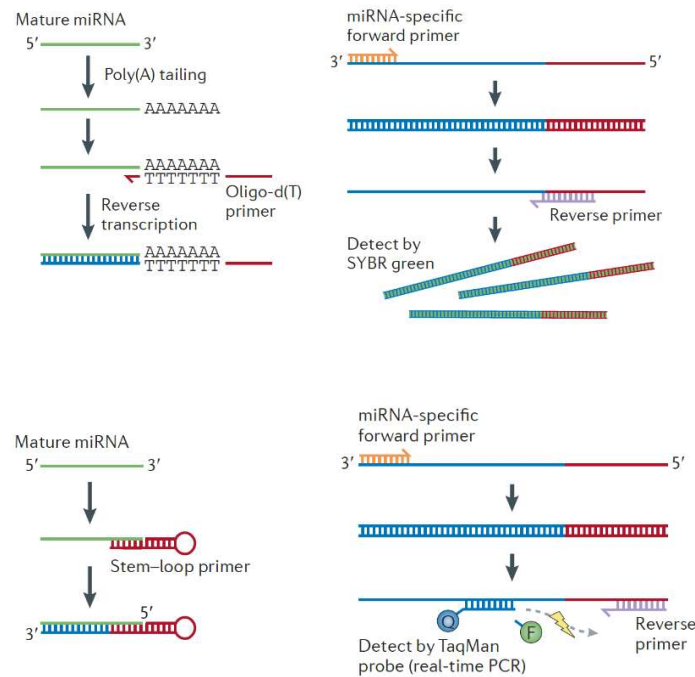


Figure 6: Two different RT-qPCR approaches for miRNA profiling. A) in poly(A) RT-qPCR miRNA is polyadenylated at 3' end and oligo-d(T) is used as a reverse transcription primer. A miRNA-specific forward primer and a reverse primer that anneals to the 3' portion of the miRNA sequence as well as to the poly(A) tail enable PCR amplification. The quantification is achieved through monitoring the fluorescence emission of the SYBR Green after its incorporation into double-stranded DNA during amplification. B) stem-loop RT-qPCR employs stem-loop primers specific to the 3' end of the miRNA for reverse transcription. The RT product is amplified using a miRNA specific forward primer and a reverse primer. As DNA polymerase proceeds along the template, the TaqMan probe is hydrolyzed and fluorescent dye molecule (F) is freed from the quencher (Q), resulting in fluorescence emission. Republished with permission of Springer Nature, from *MicroRNA profiling: approaches and considerations*, Pritchard, 2012, *Nature Reviews Genetics*, doi: 10.1038/nrg3198, permission conveyed through Copyright Clearance Center, Inc.

1.2.2 sRNA sequencing

The first NGS technology used to identify small RNAs was the 454 Life Sciences (later Roche) sequencing platform, allowing the sequencing of hundreds of thousands of templates simultaneously. First in plants and then in animals, 454 sequencing proved to be a useful tool to identify and monitor different sRNA populations. A few years later, in 2007, the

first publication to characterize miRNAs using the Illumina (then called Solexa) sequencing technology was published. The Illumina platform had better coverage than the 454 platform, producing millions of short reads. The decreased read length, when compared to 454, was offset by the large increase in the depth of coverage, and proved a great advantage in the discovery and profiling of complex short RNA populations. Since then, more publications have used Solexa for small RNA identification and profiling than any other platform (Armisen et al., 2012).

Typical RNA-sequencing experiment begins with the preparation of a sRNA cDNA library from the RNA sample of interest, followed by the “massively parallel” sequencing of millions of individual cDNA molecules from the library on a single run (Pritchard et al., 2012). First, sRNAs are size-selected on a denaturing polyacrylamide gel, cut out from the gel, and eluted. Then the adaptors are sequentially ligated to the 3′ and 5′-end of sRNAs. The ligated products are converted to cDNA and PCR amplified. Finally, libraries are gel-purified and subjected to sequencing (Figure 7). Deeply sequenced sRNA libraries allow discovery of various types of sRNAs (including novel and lowly abundant sRNAs), as well as their quantification based upon read-counts.

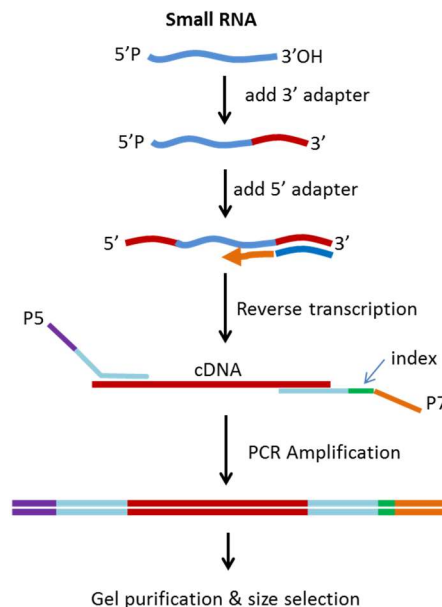


Figure 7: Schematic illustration of sRNA library preparation for Illumina platform. Isolated sRNAs are ligated to the 3′ preadenylated adapter and the 5′ adapter. The ligated products are converted to cDNA and PCR amplified. Finally, libraries are gel-purified and subjected to sequencing. Republished with permission of Novogene Corporation.

1.2.3 Computational tools for novel sRNA identification and categorization

Sequencing reads of a typical sRNA library represent a complex mixture of several types of molecules: miRNAs, siRNAs and a large number of “contaminating” tRNAs, rRNAs,

snRNAs and snoRNAs. To classify obtained short RNA reads and to select sRNAs of interest in subsequent analyses different bioinformatics (computational) analyses are usually employed. Known miRNA sequences can be readily identified by matching searches to annotated miRNA databases such as miRBase database ((Kozomara and Griffiths-Jones, 2014); <http://www.mirbase.org/>). A more difficult task is annotation of novel *bona fide* *MIR* loci and miRNAs. With the development of sRNA sequencing techniques, many miRNA prediction tools have emerged for the discovery of novel *MIR* loci and miRNAs in plants. Typically, the first step within prediction is mapping of short RNA reads on selected plant genome following identification of genomic regions with the potential to form hairpin secondary structures with structural features similar to known miRNA precursors (pre-miRNAs) (Axtell and Meyers, 2018; Meyers et al., 2008).

However, a typical genome contains thousands of regions with predicted secondary structures that resemble miRNA precursors, of which only a low percentage are likely to represent actual *MIR* loci. Usually, each of miRNA prediction tools follows some additional annotation criteria to predict high-confidence pre-miRNA, mature miRNA (highest expressed read) and corresponding miRNA*, such as limiting the precursor length to maximum of 300 nts, filtering based on folding free energy of predicted pre-miRNA precursor (< -0.2 kcal/mol/nt) and considering the strandedness of a candidate *MIR* locus (Axtell and Meyers, 2018). It has been demonstrated that *MIR* loci produce a discrete pattern of alignments to a single genomic strand, with a major species (the mature miRNA) separated by a short distance from a second, minor species miRNA* (Figure 8). By contrast, endogenous siRNAs originate from long dsRNA molecules and the precursor is processed such that numerous siRNAs accumulate from both strands of the dsRNA (Ambros et al., 2003). Another key property of sRNA genes is the distribution of sRNA sizes produced by the locus. For instance, in plants, *MIR* loci usually comprise 21 nt mature miRNA and miRNA* and their sequence variants (isomiRs) with distribution of sizes centered around 22 nt, rather than a discrete single length. Endogenous siRNAs are frequently 21 (phasiRNAs) or 24 nt long (hc-siRNAs) (Chen et al., 2010; Cuperus et al., 2010; Kasschau et al., 2007; Lu et al., 2006). On the other hand, sRNAs derived from fragmentation of abundant RNAs such as rRNA, tRNA, snRNA and snoRNA fragments, often have a wider size distribution in the 15–30 nt size range, which exceeds the size range normally associated with sRNA regulatory function (Axtell and Meyers, 2018; Wang et al., 2016).

The most prominent endogenous siRNA populations involved in PTGS are phasiRNAs. In contrast to miRNA-encoding *MIR* genes, identification of phasiRNA and *PHAS* loci requires a different computational prediction strategy. Based on sRNA sequences aligned to either genome or transcriptome reference, tools are searching for the pattern of phasing inside the mapped region. In general, these algorithms calculate the number of reads that are “in phase” against those that are “out of phase” in order to determine the probability that a particular locus is truly a *PHAS* locus (Chen et al., 2007; Gebert et al., 2017). Furthermore, one can also further classify phasiRNAs into ta-siRNA subgroup simply by comparing the mapping locations of the siRNA and its target transcript; if in trans, then siRNA can be further categorized in the ta-siRNA group (Morgado and Johannes, 2017).

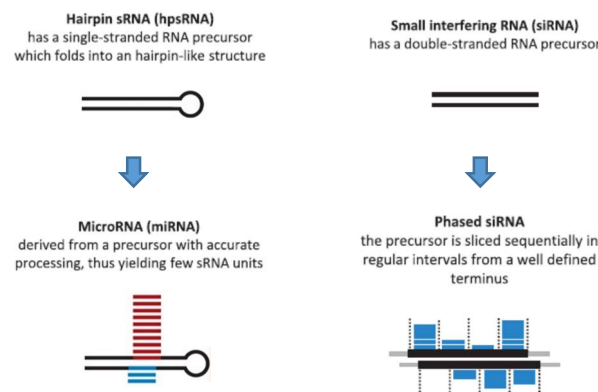


Figure 8: Difference in the prediction scheme for miRNAs and phasiRNAs. miRNA identification is primarily based on the prediction of the classical hairpin structure of a pre-miRNA yielding one highly abundant mature miRNA. phasiRNA identification relies on the detection of a phased pattern of mapped short reads. Republished with permission of Oxford University Press, from Computational tools for plant small RNA detection and categorization, Morgado and Johannes, 2017, Briefings in Bioinformatics, doi: 10.1093/bib/bbx136, and with permission of Annual Reviews, from Classification and Comparison of Small RNAs from Plants, Axtell, 2013, Annual Review of Plant Biology; doi: 10.1146/annurev-arplant-050312-120043, permission conveyed through Copyright Clearance Center, Inc.

1.2.4 Finding sRNA targets in plants

Once discovered, the identification of the target genes for any given sRNA is a key first step in determining its function. miRNAs and phasiRNAs direct post transcriptional regulation of gene expression by binding to the complementary site on the target mRNAs. The result of this binding is either immediate degradation or repression of translation. The degradation or blocking of translation depends on the degree of complementary relation between miRNA sequence and its target sequence. In plants, both experimental and computational techniques are used to identify target mRNAs (Djami-Tchatchou et al., 2017).

1.2.4.1 *In silico* prediction methods

In plants, bioinformatic predictions have been very successful in identifying sRNA targets. Plant miRNAs typically guide cleavage of target mRNAs. Cleavage of mRNA requires a high degree of miRNA:target base pairing, especially at the 5' and central positions of the duplex relative to the miRNA. In contrast, positions 2–13 are relatively mismatch-free and contain somewhat fewer G:U base pairs. In general, a scoring scheme that requires perfect or near perfect match within 2-13 region has been widely employed in published computational methods for predicting plant sRNA targets (Ding et al., 2012; Fahlgren and Carrington, 2010). The secondary structure of mRNA is also very important for predicting

exactly between the 10th and 11th nucleotide of complementarity relative to the small RNA 5' end. The resulting upstream fragment of the cleaved target rapidly degrades, while the downstream fragment is stable *in vivo* (Llave, 2002). The approach takes advantage of the free 5'-monophosphate and poly (A) tail present on the downstream 3' fragment after cleavage. Typically, degradome libraries are constructed by ligation of custom RNA adaptor containing a 3' MmeI site to a polyA-enriched fragments, followed by reverse transcription and second-strand synthesis (Figure 10). cDNA is then digested with the type restriction enzyme MmeI, which cleaves 20 nts 3' of the recognition site; the MmeI site is located in the 5'-adaptor, immediately 5' of the mRNA. Next, a 3' dsDNA adaptor with degenerate nts in the overhang region is ligated to the MmeI digestion products and the resulting material is PCR-amplified, gel-purified and subjected for sequencing (Ding et al., 2012; German et al., 2009).

After the sequencing, cleaved mRNA fragments are aligned back to the reference transcripts and used as evidence for sRNA mediated cleavage. Few degradome processing tools, such as CleaveLand, PAREsnip and SeqTar, have been developed to assist in identifying sRNA targets by combining the information of detected cleavage site on mRNA together with sRNA-mRNA complementarity and providing support for sRNA-target cleavage interaction (Ding et al., 2012). The limitation of Degradome-Seq is that it can detect only sRNA-mRNA target interactions that result in mRNA cleavage and can thus miss sRNA-target pairs when targets are lowly expressed or their 3' ends are highly unstable (Addo-Quaye et al., 2008).

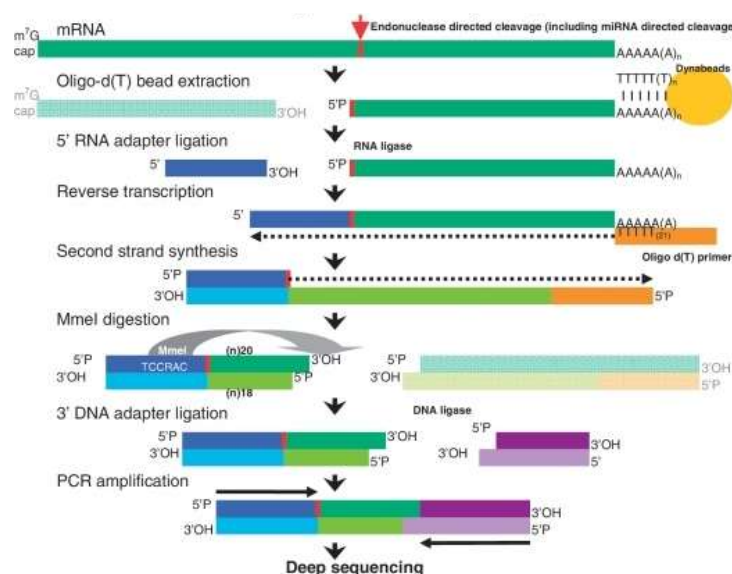


Figure 10: Schematic illustration of library preparation for Degradome-Seq. Procedure takes advantage of the free 5'-monophosphate remaining on the 3' fragment after sRNA-mediated cleavage, to which an RNA adaptor can be ligated without additional chemical modification following reverse transcription and PCR amplification prior to deep sequencing. Republished with permission of Oxford University Press, from Experimental strategies for microRNA target identification, Thomson et al., 2011, Nucleic Acids Research, doi: 10.1093/nar/gkr330, permission conveyed through Copyright Clearance Center, Inc.

1.3 Research Hypotheses

The main goal of this doctoral thesis was to unravel the roles of sRNAs regulatory level in different immune responses of potato to PVY infection, i.e. tolerant response and hypersensitive response (HR)-conferred resistance. In order to perform high-quality sRNA expression studies, we also aimed to establish the optimal workflows from sRNA isolation and purification, and for sRNAs quantification and sRNA sequencing data analysis.

The research objectives were:

- To optimize the sRNA isolation procedure that will be suitable for isolation of sRNAs from homogenized plant leaves as well as from small tissue section material.
- To optimize the RT-qPCR for sRNA expression studies that will allow validation of sRNA sequencing data.
- Employ sRNA sequencing coupled with optimized bioinformatics analyses for identification of novel potato miRNAs and siRNAs and sRNA quantification.
- Search for sRNA-mRNA target interactions to construct sRNA regulatory networks.
- Determine the differences between tolerant and resistant sRNA regulatory networks.
- Determine how lack of SA in susceptible plants can influence on the sRNA expression level and gene regulation.
- For the first time employ spatially resolved non-targeted approaches to get detailed insight into the expression and spatial distribution (gradients) of sRNAs and genes in response to PVY in HR-conferred resistance.

We have tested the following hypotheses:

- 1) Optimized sRNA analysis protocols will enable efficient and repeatable sRNA expression analyses from homogenized leaf material and small tissue section material.
- 2) Optimized protocols for bioinformatics analysis of sRNA sequencing data will enable prediction of novel sRNAs in potato and sRNA-target interactions.
- 3) sRNA regulatory network construction and integration of the sRNA and target gene expression profiles will reveal differences in regulatory circuits among different potato cultivars in response to PVY.

Chapter 2

Materials and Methods

2.1 Plant Material

2.1.1 Plant material source and growth conditions

Healthy potato (*Solanum tuberosum* L.) plants of four different cultivars (cv. Igor, cv. Désirée, cv. Pentland-Squire, cv. Rywal) and two transgenic lines expressing salicylic acid hydroxylase (*NahG*) gene in Désirée and Rywal background were used. Potato plants were obtained from tissue culture collection maintained at the National Institute of Biology (Department of Biotechnology and Systems Biology). Plants of cv. Désirée and NahG-Désirée were originally obtained from the Leibniz Institute of Plant Biochemistry, Halle, Germany, and plants of cv. Rywal, NahG-Rywal from the Institute of Plant Breeding and Acclimatisation–National Research Institute, Młochów, Poland.

Potato plants were grown in the stem node tissue culture as previously described by (Baebler et al., 2009). Two weeks after node segmentation, they were transferred to soil in a growth chamber and kept under controlled environmental conditions at 21 ± 2 °C in the light and 18 ± 1 °C in the dark, at a relative humidity of $75 \% \pm 2 \%$, with 70 – 90 mmol/m²/s² radiation and at 16 h day/ 8 h night cycle.

2.1.2 Plant material for the optimization of extraction procedures

To obtain uniform homogenized plant material for assessment of different total RNA extraction procedures for sRNA expression analyses, four bottom leaves of cv. Igor were collected from the same plant after 30 days of growth in the soil. The midvein was cut out, and both laminae of each leaf were pooled together, immediately grounded into fine powder in liquid nitrogen, aliquoted at approximately 50 mg per tube and stored frozen at -80 °C before total RNA extraction.

To obtain small tissue sections for comparison of two selected low-input RNA isolation protocols for extraction of both small and long RNAs, six leaf discs of the same size (sample dimensions: r=4 mm, surface: 50 mm², weight = 174 µg) were excised using cork borer from the leaf of a healthy plant of cv. Rywal and stored frozen at -80 °C before total RNA extraction.

In addition, an even smaller tissue section (e.g.; surface= 3 mm²) was cut by scalpel, immersed in RNAlater Stabilization Solution (Ambion) for RNA stabilization and stored

at 4 °C overnight (to allow the solution to thoroughly penetrate the tissue), next day the sample was moved to -80 °C for long-term storage. Tissue was used to test the performance of the selected RNA purification method.

2.1.3 Plant material for sRNA and gene expression after virus inoculation

After 4 weeks growth in soil, the potato plants were divided into two groups: virus-inoculated and mock-inoculated (control) groups. Inoculation procedure was carried out as described by (Baebler et al., 2011). In brief, three fully developed bottom leaves of each plant were dusted with carborundum (SiC) powder (Sigma) and gently rubbed with a sap prepared from homogenized either PVY^{NTN}- (isolate NIB-NTN, accession no. AJ585342) or PVY^{Wi}-infected plant material in 20 mM sodium phosphate buffer, supplemented with 0.025 g of diethyldithiocarbamic acid (DIECA), pH 7.6. The inoculum was left on the leaves for 10 min, then gently rinsed off with tap water. For the mock-inoculated plants, the same procedures were performed with sap from healthy potato plants. Samples of inoculated leaves were collected at 3 days post inoculation (dpi), and additionally from 4 dpi to 6 dpi for Pentland-Squire, using the second bottom inoculated leaf of three plants per genotype. The halves of the leaf laminae of each treated plant Désirée, NahG-Désirée and Pentland-Squire were excised and flash frozen in liquid nitrogen and stored at -80 °C prior to RT-qPCR and NGS analyses.

Leaf samples of cv. Désirée and NahG-Désirée were prepared and collected 3 dpi within the study of (Stare et al., 2015), for the study of gene expression response to PVY^{NTN} infection. Here, the same plant material sampled at 3 dpi was used to investigate the changes on sRNA expression level in response to PVY^{NTN}.

To study sRNA expression close to the viral entry in HR response of cv. Rywal and NahG-Rywal, small section of leaf tissue (region as small as 1 mm², containing only a few hundred cells) at the site of formed spot necrosis (lesions) and its surrounding tissue was sampled at the time when the lesions just became visible (3 dpi). From each second bottom inoculated leaf ~ 30 lesion samples (excluding lesion formed on veins) were pooled together. Similarly, ~ 30 sections of surrounding tissue of sampled lesions from the same leaf were pooled. To take the possible positional effect of the lesions into account, for mock-inoculated plants, narrow band of the tissue between veins from distal to proximal site of the leaf were excised. All small tissue section samples were stored in RNeasy Lysis Solution (Qiagen).

2.2 RNA Isolation

Prior to RNA extraction all plant tissue samples analyzed in this study, including those already grounded from Section 2.1.2, were subjected to mechanical homogenization using a Tissue Lyser (Qiagen) and stainless steel beads (Qiagen) for 3 min cycles at 30 Hz in a corresponding lysis buffer.

2.2.1 Optimization of sRNA extraction from homogenized and small tissue section potato leaf material

Five RNA isolation methods were compared for isolation of sRNA from leaf tissue samples. Because the assessment of different RNA isolation methods requires the use of a uniform source sample, all extraction methods were performed on the same homogenized leaf material (Section 2.1.2). Total RNA, including short RNA fraction, was extracted by three different techniques: phenol/guanidinium-based extraction using TRIzol Reagent (Invitrogen) followed by isopropanol precipitation, and two column-based techniques *mirVana* miRNA Isolation Kit (Ambion) and High Pure miRNA Isolation Kit (Roche) (see Figure 11 for more details). *mirVana* employs phenol:chloroform phase separation combined with silica column-based solid extraction method, while High Pure is a phenol-free method. In addition to total RNA extraction methods described above, we also followed two short RNA enrichment procedures with *mirVana* miRNA Isolation Kit and High Pure miRNA Isolation Kit, which enable the separation of short RNA molecules shorter than ~ 200 nt from the larger RNAs (Figure 11). All extraction protocols were performed in duplicates following manufacturer's instructions. MaXtract High Density tubes (Qiagen) were used with the TRIzol procedure for better organic and aqueous phase separation and to prevent carryover of contaminants. After elution, RNA samples were stored at -80 °C.

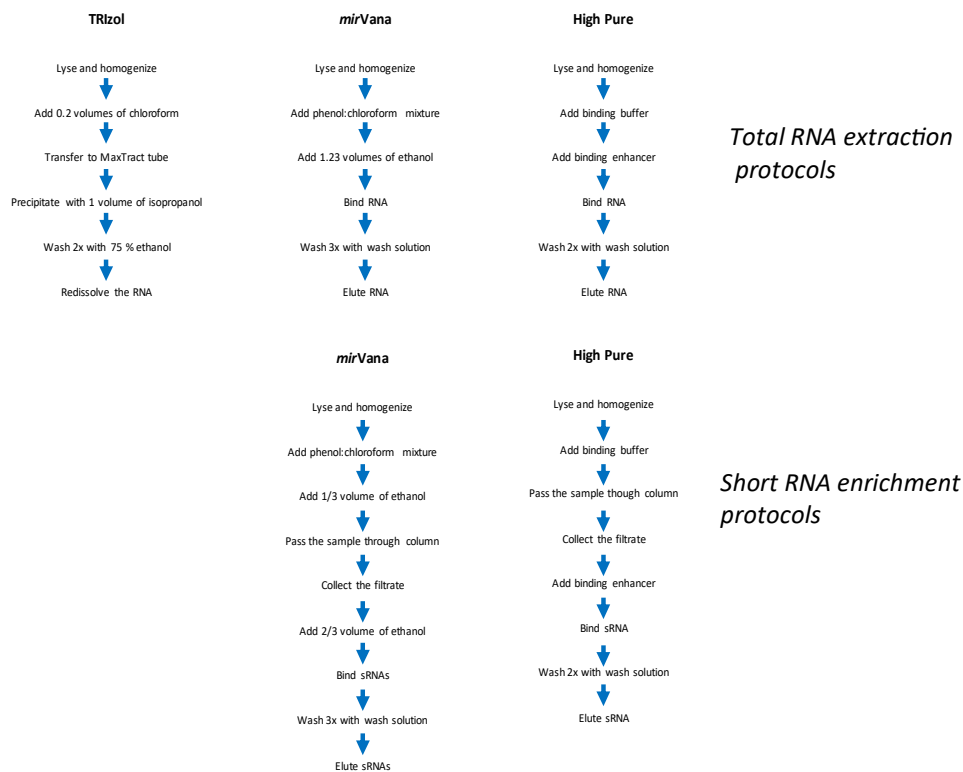


Figure 11: Flow charts of the different RNA isolation methods. RNA from potato leaves was isolated using the phenol-based method (TRIzol) and two column based technologies *mirVana* miRNA isolation kit and High Pure miRNA isolation kit following total RNA extraction or short RNA enrichment protocols.

For total RNA extraction from small tissue section material (Section 2.1.2), two different extraction procedures, designed for the extraction of RNA from low input starting material were tested: Direct-zol RNA MicroPrep Kit (Zymo Research, hereinafter referred to as Zymo) and Single Cell RNA Purification Kit (Norgen Biotek; hereinafter referred to as Norgen) (Figure 12). Within the Zymo protocol, TRIzol reagent was used for cell disruption prior to spin column RNA purification. Norgen's kit is also a column-based method originally employing phenol-free cell lysis Buffer RL, however, due to the manufacturer's suggestion the buffer was replaced by Norgen's phenol-free lysis buffer developed and optimized for processing of plant and fungi species. Both extraction protocols were performed in triplicates following the manufacturer's instructions with an additional modification step. For the elution step pre-heated nuclease-free water (80 °C) was applied to increase the RNA and sRNA yields. Eluted RNA was stored at -80 °C until further analysis.

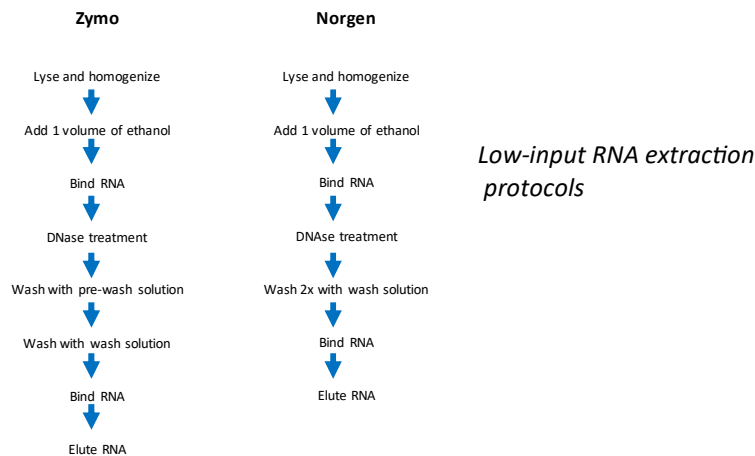


Figure 12: Flow chart over the low-input RNA extraction methods. RNA from small potato tissue sections was isolated using the Direct-zol RNA MicroPrep Kit (Zymo) and Single Cell RNA Purification Kit (Norgen).

2.2.2 RNA extraction from plant material used for studies of sRNA and gene expression studies after virus inoculation

Total RNA of mock- and PVY-inoculated samples of cv. Désirée and NahG-Désirée collected at 3 dpi was extracted from 100 mg of homogenized leaf tissue using TRIzol reagent (Invitrogen) and MaXtract High Density tubes (Qiagen) followed by isopropanol precipitation (as described in Section 2.2.1). Total RNA of mock- and PVY-inoculated samples of Pentland-Squire plants was extracted from 100 mg of homogenized leaf tissue using Direct-zol RNA MiniPrep kit (Zymo Research) and total RNA from a small section of leaf tissue of cv. Rywal and NahG-Rywal was extracted by Direct-zol RNA MicroPrep kit. In both Direct-zol kits, TRIzol reagent (Invitrogen) was used for cell disruption prior to spin column RNA extraction.

2.2.3 RNA purification

RNA extracted from small tissue sections (Section 2.1.2), including Rywal and NahG-Rywal samples (Section 2.1.3) was purified with RNA Clean & Concentrator kit protocol (Zymo Research). Protocol involves in-tube DNase I digestion (Zymo Research) followed by RNA binding to Zymo-Spin micro columns and repeated washing. In brief, to each RNA sample 5 μ l of DNase I and 5 μ l of DNase digestion buffer were added in a final volume of 50 μ l following the incubation at room temperature for 15 min. Subsequently, two volumes of RNA binding buffer and one volume of ethanol was added to each sample for RNA precipitation. The sample was transferred to Zymo-Spin micro column and centrifuged. By this step DNase I was removed and RNA bound to column was subsequently washed three times to get rid of contaminants, first with 400 μ l of RNA Prep buffer and twice with 400 μ l and 700 μ l of RNA Wash buffer. To remove traces of ethanol and washing buffers the columns were additionally centrifuged for 2 minutes with empty collection tubes. RNA was eluted using 16 μ l of pre-heated nuclease free water.

2.2.4 RNA quality and quantity control

RNA concentration and purity were determined using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific). The A_{260}/A_{230} and A_{260}/A_{280} ratios were used to assess the purity of the RNA samples. If the RNA is pure, we expect the A_{260}/A_{280} ratio of ~ 2.0 , if the ratio is appreciably lower, it is an indication of the presence of protein, phenol, or other contaminants that absorb strongly at or near 280 nm. The ratio of A_{260}/A_{230} is expected to be in the range of 2.0–2.2. A low A_{260}/A_{230} ratio may be an indication of the presence of carbohydrates, urea, EDTA, phenolate ions or guanidine residuals that absorb at or close to 230 nm.

The concentration and integrity of RNA was determined via an agarose gel electrophoresis or automated capillary electrophoresis using the Agilent RNA 6000 Nano or Pico Kits (Agilent Technologies) on the Agilent 2100 Bioanalyzer (Agilent Technologies) and according to manufacturer's protocols. The output of Bioanalyzer is presented as electropherograms and gel-like images, which allow visual inspection of the RNA quality much like an agarose gel but with higher sensitivity and higher resolution. Additionally, the Bioanalyzer calculates the RNA integrity number (RIN) ranging from 1 being the most degraded profile to 10 being the most intact using the Agilent 2100 Expert Software algorithm (Agilent Technologies).

The concentration and integrity of sRNAs were determined via an agarose gel electrophoresis or automated capillary electrophoresis using Agilent Small RNA Kit on the Agilent 2100 Bioanalyzer. For the short RNA fraction, the integrity of a sample was inferred from the resolution of peak areas in the electropherograms. Small RNA (from 0 to 200 nt) and miRNA (from 0 to 40 nt) concentrations as well as miRNA/small RNA ratio were calculated from electropherograms using Agilent 2100 Expert software (Agilent Technologies).

2.3 Reverse Transcription-Quantitative Polymerase Chain Reaction (RT-qPCR)

2.3.1 Viral RNA amount, gene and miRNA expression assays

PVY RNA was detected using a specific PVY-Uni RT-qPCR assay developed by Kogovšek et al. (2008). For *cox* and *PR1b* quantification predesigned assays (Baebler et al., 2011; Weller et al., 2000) were employed (Table 1).

Table 1: Primer and probe sequences used for PVY RNA quantification and gene expression analysis. Target, target gene ID, name of the assay, forward (F) and reverse (R) primer and probe (P) sequences together with the concentration used in qPCR mix. For probes, reporter and quencher dyes are also given. * For PVY RNA quantification coat protein (CP) nucleotide sequences of PVY were targeted with PVY-Uni assay (Kogovšek et al., 2008). Efficiencies of amplification for each assay were calculated as described previously in (Pirc et al., 2009).

Target	Target ID	Assay Name	Primer/probe sequences	Concentration (nM)	Efficiency of amplification (%)
<i>PVY CP</i>	AJ390300*	PVY-Uni	F: 5'-CATAGGAGAACTGAGATGCCAACT-3'	300	97.6
			R: 5'-TGGCGAGGTTCCATTTTCA-3'	300	
			P: 5'-FAM-TGATGAATGGGCTTATGGTTTGGTGCA-	150	
<i>cox</i>	PGSC0003DMG400002971; Sotub04g015050; X83206	COX	F: 5'-CGTCGCATTCCAGATTATCCA-3'	900	98.8
			R: 5'-CAACTACGGATATATAAGAGCCAAAACCTG-3'	900	
			P: 5'-FAM-TGCTTACGCTGGATGGAATGCCCT-TAMRA-3'	250	
<i>PR1b</i>	MICRO.5426.C4 PGSC0003DMG400002027, PGSC0003DMG400002028, PGSC0003DMG400002029	PR1b	F: 5'-GTATGAATAATTCCACGTACCATATGTTC-3'	300	93.4
			R: 5'-GTGGAAACAAGAAGATGCAATACTTAGT-3'	300	
			P: 5'-FAM-TGGTATAGTGGCTTACGTTGA-Zen Iowa BlackTM FQ-3'	250	

TaqMan MicroRNA Assays (Thermo Fisher Scientific) were ordered according to the sRNA-Seq sequence of the selected miRNAs (Table 2). Each assay contains one tube of stem-loop reverse transcription primer and one tube of TaqMan Assay (preformulated forward/reverse primer and MGB probe). All assays were tested for efficiency of amplification using standard dilution curve prior miRNA expression analyses (Table 2).

Table 2: RT-qPCR assays for selected miRNAs used for optimization of the sRNA analysis workflow and sRNA-Seq results validation. TaqMan MicroRNA Assays (Thermo Fisher Scientific), ordered according to the sRNA-Seq sequence of the selected miRNAs together with their IDs, mature miRNA sequence and efficiencies of amplification (calculated as described above).

Target miRNA	Assay name	Assay ID (Thermo Fisher Scientific)	Mature miRNA sequence (5'-3')	Efficiency of amplification (%)
stu-miR167a-5p.1	bna-miR167a	006933_mat	TGAAGCTGCCAGCATGATCTAA	98.3
stu-miR168	ath-miR168a	000351	TCGCTTGGTGCAGGTCGGGAA	96.5
stu-miR172a,b-e-3p	ath-miR172a	000359	AGAATCTTGATGATGCTGCAT	91.6
stu-miR390-5p	gma-miR390b	007109_mat	AAGCTCAGGAGGGATAGCACC	106.5
stu-miR398a-5p	stu-miR398a	469260_mat	GGGTGATTGAGAACATATG	91.6
stu-miR408b-5p	stu-miR408b	468849_mat	ACGGGACGAGACAGAGCATG	104.2
stu-miR4376-5p.1	sly-miR4376	473102_mat	ACGCAGGAGAGATGATGCTGGA	104.6
stu-miR6022-3p	stu-miR6022	475531_mat	TGGAAGGGAGAATATCCAGGA	100.7
stu-miR827-5p	stu-miR827	468282_mat	TTTGTGATGGTCATCTATTC	105.6

2.3.2 One-step RT-qPCR for studying the PVY^{NTN} multiplication dynamic

To follow the dynamic of PVY^{NTN} multiplication in the tolerant plants of Pentland-Squire, the amount of viral RNA was quantified in Pentland-Squire PVY-inoculated RNA samples collected from 3 to 6 dpi by using a specific PVY-Uni RT-qPCR assay developed by (Kogovšek et al., 2008). RNA samples were first treated with DNase I (Qiagen) (0.5 µl DNase per µg RNA) in a final volume of 10 µl and incubated at room temperature for 15 min. Reaction was stopped by adding 1 µl of 25 mM EDTA (pH 8.0) and heating to 65 °C for 10 min. Samples were then cooled at 4 °C to prevent RNA degradation. RT-qPCR reactions were performed on 2 µl of isolated DNase-treated RNA in a final reaction volume of 10 µl using the AgPath-ID One-Step RT-PCR Kit (Applied Biosystems) according to the manufacturer's recommendations and primer and probe concentrations as proposed by (Kogovšek et al., 2008) (Table 1). Two dilutions (1:10 and 1:100) of RNA were analyzed, each in duplicate. Reactions were carried out in 384-well reaction plates using the 7900HT Fast Real-Time PCR System (Applied Biosystems).

RT-qPCR conditions were as follows:

- a) 10 min at 48 °C (reverse transcription step);
- b) 10 min at 95 °C (polymerase activation step);
- c) 45 amplification cycles of:
 - i) 15 s at 95 °C (denaturation step);
 - ii) 60 s at 60 °C (extension step).

Baseline detection and the threshold value of the run were detected using the automatic settings in the SDS software (version 2.3, Applied Biosystems). Relative PVY RNA copy numbers were determined using a relative standard curve method. Relative copy number of PVY RNA was calculated for each sample and further normalized to the amount of

selected endogenous control cytochrome oxidase (*cox*) mRNA using quantGenius software (Baebler et al., 2017).

2.3.3 Two-step RT-qPCR for *cox* and *PR1b*

RNA quality and quantity of samples (Section 2.2.1) was also analyzed by estimating a cytochrome oxidase *cox* mRNA level. In addition to *cox* mRNA level in RNA obtained from small sections (Section 2.2.1), expression of *PR1b* gene was followed with the two-step RT-qPCR assay. RNA samples were DNase I (Qiagen) treated as described in Section 2.3.2 (homogenized plant material) and in Section 2.2.3 (small sections). DNase-treated RNA samples were then heated at 80 °C for 5 min to denature the RNA and then briefly cooled on ice. Subsequently, cDNA was synthesized using High-Capacity cDNA Reverse Transcription kit (Applied Biosystems) and random hexamers as primers, according to manufacturer's instructions. The reactions were incubated for 10 min at 25 °C and 120 min at 37 °C. Each RT product was 10- and 100-fold or 10- and 50-fold diluted prior to qPCR. Reactions were performed in duplicates and in a total volume of 10 µl, containing 8 µl of TaqMan Universal Master Mix II, with UNG (Applied Biosystems), assay with probe and primers for *cox* mRNA amplification designed by Weller et al. (2000) (Table 1) and 2 µl of diluted RT product. A 10-fold dilution series standard curve (10-times to 10000-times diluted) was prepared from a pool of RT product samples. As for tested samples, each dilution of the standard curve sample was analyzed in duplicates. We also included the control (RT-) to check for the presence of contaminating DNA in our samples.

cDNA synthesized RNA from small section samples was diluted 10- and 50-fold prior to qPCR. Reactions were performed in duplicates and in a total volume of 5 µl, containing Fast Start Universal Probe Master Mix (Roche), *cox* or *PR1b* assay (Table 1), and 2 µl of diluted RT product.

For amplification following general thermal cycling conditions were employed:

- a) 10 min at 95 °C (polymerase activation step);
- b) 45 amplification cycles of:
 - i) 15 s at 95 °C (denaturation step);
 - ii) 60 s at 60 °C (extension step).

Baseline detection and the threshold value of the run were detected using the automatic settings in the SDS software (version 2.3, Applied Biosystems). Relative *PR1b* abundance was determined using a relative standard curve method. Relative copy number of *PR1b* mRNA was calculated for each sample and further normalized to the amount of selected endogenous control cytochrome oxidase (*cox*) mRNA using quantGenius software (Baebler et al., 2017).

2.3.4 Two-step miRNA RT-qPCR for assessment of efficiency of sRNA extraction methods

Stem-loop miRNA RT-qPCR was conducted to evaluate the performance of the different extraction methods in terms of their efficiency in miRNA recovery and preservation of their

relative expression levels. RNA samples were DNase I (Qiagen) treated as described in Section 2.3.2. Samples were then cooled at 4 °C to prevent RNA degradation and half of each sample was used to prepare 10-fold dilutions. RT reactions were performed in duplicates using both the undiluted and diluted RNA solutions. miRNAs were reverse transcribed using either MultiScribe reverse transcriptase and TaqMan MicroRNA Reverse Transcription Kit (both Applied Biosystems), or SuperScript III First-Strand Synthesis System (Invitrogen) in combination with customized stem-loop Megaplex primer pool (Applied Biosystems) allowing simultaneous reverse transcription of many different miRNAs in one reaction. In brief, miRNA-specific primers were pooled and diluted in nuclease free water to obtain a final dilution of $0.05 \times$ each, ensuring minimal non-specific interactions between the different stem-loop primers (Mestdagh et al., 2008).

Three different reverse transcription protocols were tested:

1) TaqMan MicroRNA Reverse Transcription Kit reverse transcription reaction contained DNase-treated RNA, customized stem-loop Megaplex primer pool (in final concentration = $0.02 \times$), 100 mM dNTP, 150 units of MultiScribe Reverse Transcriptase (50 U/ μ l), $10\times$ RT buffer and RNase inhibitor (20 U/ μ l).

A final volume of 15 μ l was reverse-transcribed with the following conditions:

- a) 30 min at 16 °C
- b) 30 min at 42 °C
- c) 5 min at 85 °C

2) RT SuperScript III reactions contained stem-loop Megaplex primer pool (prepared as described above) in final concentration $0.02 \times$, 10 mM dNTP, $10\times$ RT buffer, 0.1 μ mole $MgCl_2$, 0.2 μ mole DTT, 40 units RNaseOUT, and 200 units SuperScript III RT enzyme were added to the reactions in a final volume of 20 μ l, according to manufacturer's instructions following the recommended thermal-cycling conditions:

- a) 50 min at 50 °C
- b) 5 min at 85 °C

3) In addition, to increase detection sensitivity of miRNAs (Varkonyi-Gasic et al., 2007)), pulsed RT reaction was also applied using the same SuperScript III protocol as described above, with modified thermal-cycling (pulsed) conditions as follows:

- a) 30 min at 16 °C
- b) followed by 60 cycles of:
 - i) 30 s at 30 °C
 - ii) 30 s at 42 °C
 - iii) 1 s at 50 °C
- c) 5 min at 85 °C

All SuperScript III RT reactions (protocols 2 and 3) were followed by an addition of *E. coli* RNase H (Thermo Fisher Scientific) and incubated at 37 °C for 20 min to remove RNA complementary to the cDNA. Additionally, no template control RT reactions were set to assess potential Megaplex primer pool background signal that could be a result of cross hybridization between different stem-loop primers.

Each RT product was 8- and 80-fold diluted prior to qPCR. Reactions were performed in duplicates and in a total volume of 10 μ l, containing 5 μ l of TaqMan Universal Master Mix II, with UNG (Applied Biosystems), 0.5 μ l of Taqman MicroRNA assay (Applied

Biosystems) and 4.5 µl of diluted RT product. To construct standard curve for each miRNA assay, 1 µl of each RT product was mixed in the same tube and diluted serially in 10-fold increments ranging from 1:8 to 1:80000 in nuclease free water. As for other samples, each dilution of the standard curve sample was analyzed in duplicates. In addition, no template qPCR control reactions were included by adding nuclease-free water instead of the cDNA template.

Thermal cycling conditions were as follows:

- a) 2 min at 50 °C
- b) 10 min at 95 °C
- c) followed by 45 cycles of:
 - i) 15 s at 95 °C
 - ii) 60 s at 60 °C

All PCR reactions were performed on the LightCycler® 480 (Roche). Raw C_q values were calculated with LightCycler 480 software (Roche), applying the Second Derivative Maximum method (Roche). Subsequently, relative quantification was done in quantGenius software, using relative standard curve method (Baebler et al., 2017). This way relative copy numbers of miRNAs were calculated for each sample and further normalized to the amount of endogenous control miRNA, miR167 (stu-miR167a-5p.1), which was determined to be the most robustly expressed in a sRNA sequencing dataset of potato plants that were uninfected or infected with a range of viruses (PVY, PVX, PVT (potato virus T), PVS (potato virus S) and PLRV (potato leafroll virus) (SRA accession no. SRP083247) (Križnik et al., 2017).

2.3.5 Two step miRNA RT-qPCR for sRNA expression studies

Total RNA (1 µg) of the PVY- and mock-inoculated samples of cv. Désirée and NahG-Désirée was DNase I (Qiagen) treated as described in Section 2.3.2 and reverse transcribed using SuperScript III First-Strand Synthesis System and stem-loop Megaplex primer pool following the previously optimized protocol 3 (see Section 2.3.4). All qPCR reactions were performed in duplicates in 10 µl reaction volume using two different dilutions of the cDNA samples (1:8 and 1:80). PCR amplification reactions and data analysis were performed as described in Section 2.3.4.

2.4 High Throughput RNA Sequencing

2.4.1 Library preparation and sequencing

sRNA libraries from three PVY- and three mock-inoculated cv. Désirée and NahG-Désirée DNase-treated total RNA samples collected at 3 dpi were constructed at SeqMatic LLC (California, USA) using the TailorMix miRNA Sample Preparation Kit (SeqMatic).

sRNA libraries of three PVY- and three mock-inoculated DNase-treated total RNA samples of cv. Pentland-Squire collected at 4 dpi, were generated at Omega Bioservices (Georgia, USA) using Bioservices NEXTflex Small RNA Seq Kit (Bioo Scientific).

mRNA and sRNA libraries of three PVY- and three mock-inoculated DNase-treated total RNA samples of cv. Rywal and NahG-Rywal collected at 3 dpi were generated at LC Sciences (Houston, USA) using TruSeq Stranded mRNA Library Preparation Kit and Illumina Truseq Small RNA Preparation kit (both Illumina).

The four degradome libraries (mock Désirée, PVY Désirée, mock NahG-Désirée, PVY NahG-Désirée) were constructed at LC sciences, from pooled RNA of the biological replicates, using internally developed and validated protocol (LC Sciences).

All libraries were sequenced on the Illumina HiSeq Sequencing System. For sRNA libraries 50 bp single-end or 100 bp single-end read sequencing (only for NEXTflex sRNA libraries), and for mRNA libraries 150 bp paired-end read sequencing was employed.

2.4.2 Tomato sRNA and mRNA sequencing data set

A dataset containing raw sRNA-Seq data from eight and raw RNA-Seq data from twelve samples of tomato yellow leaf curl virus (TYLCV)-infected and healthy tomato (*Solanum lycopersicum* cv. Moneymaker) plants sampled 2 or 7 dpi were obtained by the group of Eduardo Rodríguez Bejarano, University of Málaga.

In brief, tomato plants were grown in an insect-free growth chamber with a 16-h photoperiod at $250 \mu\text{mol} \cdot \text{s}^{-1} \cdot \text{m}^{-2}$ photosynthetically-active radiation (16/8 h, light/dark, 25 °C/20 °C) and 70 % relative humidity. Three-week-old tomato seedlings were infected with a clone of the isolate of TYLCV (Genbank Acc. No. AJ489258, (Morilla et al., 2005)) using *Agrobacterium tumefaciens* LBA4404. For the agroinfection, the axillary bud between the third and fourth tomato leaf were infiltrated. At 2 dpi and 7 dpi, the second most recently expanded leaves from the apex were harvested from both TYLCV-infected and virus-free *Agrobacterium*-inoculated (control) plants. For each infection method and time point, the leaf tissue from six different infected plants was pooled and used in downstream analysis. A total of three biological replicates were processed per condition and time point. Total RNA was extracted using TRIzol (Ambion) according to the manufacturer's protocol. For RNA-Seq three and for sRNA-Seq two biological replicates were set per condition and time point and used for sRNA library generation (TruSeq sRNA Kit, Illumina) or mRNA library generation (TruSeq Stranded mRNA Kit, Illumina). All libraries were sequenced on the Illumina HiSeq2000 instrument. For sRNA libraries 50 bp single-end and for mRNA libraries 75 bp paired-end read sequencing was employed.

2.4.3 Analysis of sequencing data

2.4.3.1 Preprocessing of sRNA and mRNA sequencing data

The raw sRNA and mRNA sequencing reads were first trimmed to remove adaptor sequences using the cutadapt tool (<https://cutadapt.readthedocs.io/en/stable/guide.html>) or CLC Genomics Workbench 8 (<https://www.qiagenbioinformatics.com/products/clc-genomics-workbench>) and further quality-assessed using the FastQC tool

(<https://www.bioinformatics.babraham.ac.uk/projects/fastqc>). To obtain high-quality sequences, reads were further trimmed by applying a quality score threshold of 30 for sRNA reads and 20 for mRNA reads using CLC Genomics Workbench 8. From sRNA sequencing dataset reads shorter than 18 nt and longer than 24 nt were discarded using CLC Genomics Workbench 8 or Filter Tool of the UEA sRNA Toolkit (Moxon et al., 2008).

2.4.3.2 Identification of known and novel miRNAs and *MIR* loci

To identify known potato miRNAs, the cleaned reads from sRNA sequencing samples of potato PVY-infected and mock sRNA-Seq samples were compared to potato miRNAs registered in miRBase database release 21 (Kozomara and Griffiths-Jones, 2014; <http://www.mirbase.org>), allowing no mismatches. Similarly, sRNA sequences from tomato sRNA-Seq samples which perfectly matched with miRNAs of tomato in the miRBase were acknowledged as known tomato miRNAs. miRNAs that had maximum of 3 nt difference in their 5' or 3' ends with respect to the annotated mature miRNAs deposited in miRBase were annotated as isomiRs. Additional isomiRs of known miRNAs were identified using computational pipeline isomiRID (Felipe et al., 2013).

To identify novel unannotated potato miRNAs and their loci of origin (i.e. *MIR* loci), filtered reads from cv. Désirée sRNA-Seq samples with 18-24 nt in length were submitted to miRcat tool of the UEA sRNA Toolkit using default parameters for plants and potato reference genome *Solanum tuberosum* Group Phureja DM1-3 Assembly Version 3 DM) (Xu et al., 2011; http://solanaceae.plantbiology.msu.edu/pgsc_download.shtml). An additional criterion we have imposed was that novel miRNAs should be present at least in two analyzed samples with more than five raw reads. To increase the confidence of predictions, novel miRNA candidates were verified with another plant miRNA prediction tool, miRPlant version 5 (An et al., 2014), using default parameters.

Prediction of novel unannotated tomato miRNAs and *MIR* loci from TLCY-infected and control tomato sRNA samples and search for additional novel potato miRNAs in sRNA-Seq samples of cv. Rywal was carried out using two recently emerged improved tools for plant miRNA prediction, miR-PREFeR and ShortStack (Axtell, 2013b; Lei and Sun, 2014). Both predictions were run with default parameters using either tomato reference genome Assembly version 2.5 (Tomato&Consortium, 2012; https://solgenomics.net/organism/Solanum_lycopersicum/genome) or potato genome (Xu et al., 2011), employing additional filtering of miRNAs having less than ten reads in at least two libraries.

Predicted miRNA sequences were additionally compared with tRNA, rRNA, snoRNA and snRNA sequences deposited in RNACentral database (Bateman et al., 2011; <https://rnacentral.org>) and only non-matching sequences were retained.

The output of all miRNA prediction tools may also contain the predictions of already annotated potato *MIR* loci, therefore in order to separate them from potential novel *MIR* candidates, all genome locations of predicted *MIR* loci were compared with already annotated potato or tomato *MIR* loci locations deposited in miRBase database (Griffiths-Jones et al., 2006; Kozomara and Griffiths-Jones, 2014; <http://www.mirbase.org>) using our internally developed script. Novel potato and tomato miRNAs were further classified into known or novel miRNA families by clustering their predicted pre-miRNA sequences with sequences of known plant pre-miRNAs from miRBase using CD-HIT-EST with an identity threshold of 0.8 (Huang et al., 2010; <http://weizhong-lab.ucsd.edu/cdhit-web-server/cgi-bin/index.cgi?cmd=cd-hit-est>). The sequences showing similarities with annotated pre-

miRNAs were grouped into corresponding known miRNA families, and sequences that did not show similarity with known plant miRNAs were classified as novel miRNA families.

For assessing the performance of three miRNA prediction tools, 1) miRCat tool of the UEA sRNA Toolkit, 2) miR-PREFeR and 3) ShortStack, preprocessed reads from two sRNA-Seq samples (one PVY-infected and one mock-inoculated) of cv. Rywal (3 dpi) were used as input for all three prediction pipelines. For all tools, the potato reference genome (Xu et al., 2011) was used for short read alignment. All tools were run using default parameter settings for plants. Since annotation of novel miRNAs and *MIR* loci requires support from at least two sRNA libraries (Axtell and Meyers, 2018), we imposed two additional criteria: novel *MIR* loci should be predicted in both analyzed samples and its corresponding mature miRNA should be represented with more than ten raw reads in both samples.

2.4.3.3 Prediction of phasiRNAs and phasiRNA-producing loci

Prediction of phasiRNAs and phasiRNA-producing loci (*PHAS* loci) from sRNA sequencing samples of cv. Désirée and NahG-Désirée was performed using ta-siRNA prediction tool of the UEA sRNA Toolkit (Chen et al., 2007; Moxon et al., 2008). Non-coding *PHAS* loci were detected by mapping short cleaned reads to the potato genome sequence (Xu et al., 2011), while coding *PHAS* loci were identified by mapping the reads to merged potato gene and unigene sequences StNIB_v1 (Ramšak et al., 2014), with no mismatches allowed. Analysis of phasing was performed in 21- and 24-nt intervals. To detect *PHAS* loci with a significant degree of phasing, very strict criteria were applied to avoid detection of false positives (phasing p-value < 0.0001; number of unique phasiRNAs detected at specific *PHAS* locus ≥ 4 , also to avoid detection of repeat-associated siRNAs). Additionally, the phasing p-values were corrected with strict Bonferroni correction and only loci with the p-value < 0.05 were considered as *PHAS* loci.

Prediction of novel tomato phasiRNAs and *PHAS* loci from TLCY-infected and control tomato sRNA samples and search of additional novel potato phasiRNAs and *PHAS* loci in sRNA samples of cv. Rywal was carried out using recently emerged phasiRNA prediction tool, unitas (Gebert et al., 2017). In contrast to ta-siRNA tool, unitas enables adjustment of a number of mismatches (< 2) during short read alignments to the reference.

This is a particularly useful feature because often there are differences between the sequences of analyzed genotype and reference transcriptome and genome sequences. Thus due to allowed mismatches, many more phasiRNAs and *PHAS* loci could be retrieved. Unitas also employs wider sliding window for phasiRNA prediction (1000 bp instead of ~ 300 bp in ta-siRNA tool) and reports only *PHAS* loci with at least five unique phasiRNAs and with phasing p-value < 0.05 (including Bonferroni correction) (Gebert et al., 2017).

2.4.3.4 sRNA and mRNA quantification and statistical analysis

Preprocessed reads from potato and tomato sRNA-seq samples were mapped to all identified known, novel miRNAs, isomiRs and phasiRNA sequences, and counted. sRNA counts were further subjected for differential expression analysis in R (R Development Core Team, 2011; version 3.2.2), using the limma package (Ritchie et al., 2015). In short, sRNA counts with a baseline expression level of at least two RPM (reads per million of mapped

reads) in at least three samples were TMM-normalized (edgeR package; Robinson et al., 2009) and analyzed using the voom function (Law et al., 2014). To identify differentially expressed sRNAs the empirical Bayes approach was used and the resultant p-values were adjusted using Benjamini and Hochberg's (FDR) method. Adjusted p-values below 0.05 were considered statistically significant.

Preprocessed reads from potato and tomato RNA-seq samples were mapped to the reference potato or tomato genome Assembly version 2.5 (Tomato&Consortium, 2012; https://solgenomics.net/organism/Solanum_lycopersicum/genome) or potato genome (Xu et al., 2011) with the RNA-Seq mapping algorithm (length fraction = 0.98; similarity fraction = 0.98) in CLC Genomics Workbench and counted. To determine significantly differentially expressed genes, mRNA counts mapped to annotated gene regions were further analyzed in R using the limma package and statistical analysis as described above.

2.4.3.5 Target prediction of sRNAs

2.4.3.5.1 In silico target prediction

In silico identification of potato transcripts targeted by identified sRNAs was carried out using the psRNATarget ((Dai and Zhao, 2011); <http://plantgrn.noble.org/psRNATarget/>) and StNIB_v1 sequences (Ramšak et al., 2014), following previously proposed stringent parameters (Zhang et al., 2013).

2.4.3.5.2 Degradome sequencing data analysis

For experimental validation of *in silico* predicted targets of identified sRNAs parallel analysis of RNA ends (PARE), i.e. degradome sequencing was employed. The degradome sequencing data were analyzed with CleaveLand4 ((Addo-Quaye et al., 2009); <http://sites.psu.edu/axtell/software/cleaveland4/>) using all our experimentally identified sRNAs and the StNIB_v1 transcript sequences allowing for maximum three mismatches. All identified degradation targets were classified into 5 categories as previously described (Addo-Quaye et al., 2009). Category '0' is defined as > 1 raw read at the position, with abundance at a position equal to the maximum on the transcript, and with only one maximum on the transcript. Category 'I' is described as > 1 raw read at the position, with abundance at the position equal to the maximum on the transcript, and more than one maximum position on the transcript. Category 'II' includes > 1 raw read at the position and abundance at the position less than the maximum but higher than the median for the transcript. Category 'III' comprised the transcripts with > 1 raw read at the position, and abundance at the position equal to or less than the median for the transcript. Category 'IV' comprised transcripts with one raw read at the cleavage position. Only categories with high confidence of cleavage (0, I, II, III) were considered for biological interpretation.

2.4.3.6 Comparison to transcriptome data, pathway analysis and regulatory network construction

In order to compare the expression of potato sRNAs in Désirée and NahG-Désirée with the expression of their target transcripts we used a microarray gene expression dataset

generated from the same samples ((Stare et al., 2015)); GEO accession no. GSE58593) and expression of sRNAs in Rywal and NahG-Rywal was compared with the expression of their targets determined by RNA-Seq in this study. All differentially expressed miRNAs and phasiRNAs were analyzed for functional overrepresentation in biological pathways of their targets with MapMan software ((Usadel et al., 2009)) using the ontology adapted for potato (Ramšak et al., 2014; Rotter et al., 2007). All sRNAs and their targets, obtained by *in silico* prediction and degradome sequencing were integrated with their expression data and used for the construction of regulatory networks in Cytoscape 3.4 (Shannon et al., 2003).

2.4.3.7 Identification of cis-regulatory elements in promoter regions of *MIR* genes

1000 nt sequences upstream of the predicted *MIR* gene hairpin sequences were extracted as putative miRNA promoter regions (Megraw and Hatzigeorgiou, 2010) and analyzed for *cis*-regulatory elements of plant transcription factors using position weight matrices and transcription binding sites in TRANSFAC (Matys et al., 2003); <http://www.biobase-international.com/product/transcription-factor-binding-sites>) and PlantCARE (Lescot et al., 2002) ; <http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>).

2.4.3.8 Gene Set Enrichment Analysis

To identify SA regulated genes in cv. Désirée and cv. Rywal the normalized expression values between mock NahG-Désirée vs. Désirée (calculated from the 3 dpi samples; data of Stare et al. (2015) and mock NahG-Rywal vs. Rywal samples were compared. Gene Set Enrichment Analysis algorithm (GSEA; (Subramanian et al., 2005) was run to perform analysis (false discovery rate (FDR) corrected $q \leq 0.01$) of expression profiles between both genotypes, using MapMan ontology as the source of the gene sets.

Chapter 3

Results

3.1 Optimization of Methodologies for sRNA Expression Analyses

3.1.1 Assessment of Total RNA Isolation Methods for sRNA Expression Analyses

Various RNA extraction methods have been developed for recovery of short RNAs. However, many studies revealed that different RNA isolation strategies vary in efficiency, potentially even in a sequence dependent manner, and could introduce bias in downstream sRNA profiling analyses such as RT-qPCR and sequencing. Since there is still no consensus as to which is the optimal RNA extraction method for sRNAs, we decided to compare three different total RNA isolation methods, 1) a phenol-based technique that relies on the use of organic solvents (TRIzol) followed by RNA precipitation, and two column-based techniques, 2) *mirVana* isolation kit and 3) High Pure kit. Additionally, we also tested short RNA enrichment protocols allowed by *mirVana* isolation and High Pure kits. Total RNA and short enrichment protocols were examined for their efficiency in sRNA isolation from potato homogenized leaf material to determine which would be the most suitable for future sRNA expression studies in potato.

sRNA recovery can be largely influenced not only by the extraction method used, but also by the amount of input material. By using equivalent starting amounts of leaf tissue we ensured comparable measurements among all tested RNA extraction protocols and at the same time ensured not to exceed the maximum amounts that may cause column saturation, which could hinder the optimal recovery of short RNA fraction during the column-based extractions (Brown et al., 2018; El-Khoury et al., 2016) of *mirVana* and High Pure kits. Additionally, including two replicates per isolation type allowed us to evaluate both the efficiency and variation in performance among all tested isolation protocols. Assessment of the quality and quantity of extracted RNA is not important only for gene expression analyses but also for reproducibility and accuracy in sRNA profiling analyses, therefore the RNA extractions were first evaluated in terms of purity, integrity and the yield of RNA (Figure 13).

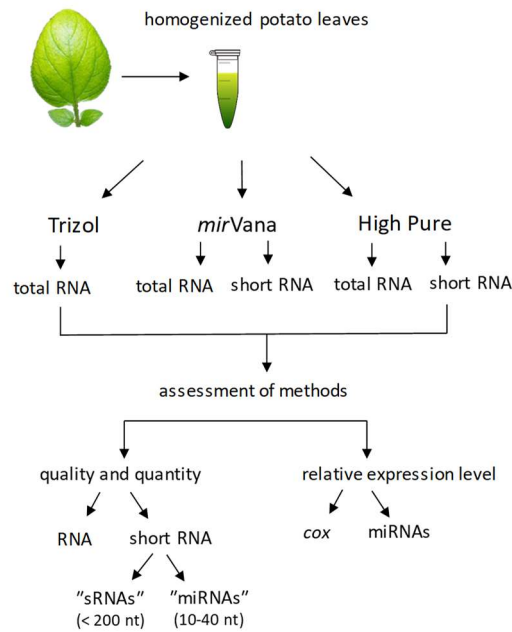


Figure 13: Workflow for assessment of total RNA and short RNA extraction methods for sRNA expression analyses from homogenized potato plant material.

3.1.1.1 TRizol and *mirVana* total RNA isolation protocols show similar yields and quality

The purity and the yield of extracted RNA were assessed from the absorbance measurements using NanoDrop spectrophotometer (Table 3). Both TRIZol and *mirVana* total RNA extraction protocols yielded highly pure RNA ($A_{260}/A_{280} > 2$; $A_{260}/A_{230} \sim 2$), indicating little or no contamination with proteins, phenols, polysaccharides, secondary metabolites etc. Also, RNA samples extracted following the High Pure total RNA protocol exhibited a lack of protein contamination, whereas A_{260}/A_{230} ratio was significantly lower, indicating the presence of contaminants absorbing at or near 230 nm. Since phenol-free lysis buffer was employed for the extraction, the low ratio could be related to components present in the lysis buffer or to carryover of carbohydrates or phenols originating from the plant tissue. We also observed lower A_{260}/A_{230} and A_{260}/A_{280} ratios in the short RNA enrichment samples, however we cannot state for sure whether the samples contained considerable amounts of contaminants or this is related to the low absorbance at 260 nm that cannot balance the measurement at 230 nm and at 280 nm or both (Table 3).

In terms of RNA yield, all three total RNA isolation protocols performed equally well, yielding on average 250 ng total RNA per mg of leaf tissue. As enrichment protocols retained only a short RNA fraction (< 200 nt), RNA yield in enrichment samples was expectedly lower in comparison to the yield observed using total RNA protocols, yielding approximately 30 ng of RNA per mg of tissue, with the exception of one *mirVana* enrichment sample, where the RNA yield was higher, comparable to the yields obtained using total RNA isolation protocols (Table 3).

Table 3: Performance of the different RNA extraction methods in terms of the quality and quantity of total RNA and short RNAs.

Extraction procedure	Absorbance ratios		RNA yield (ng/mg)	“sRNA” yield (ng/mg)	“miRNA” yield (ng/mg)	“sRNA” / RNA (%)	“miRNA” / RNA (%)	“miRNA” / “sRNA” (%)
	260/280	260/230						
TRIzol	2,02	1,99	221.7	50.0	1.0	22.5	0.4	2.0
	2	2,14	325.9	74.5	4.7	22.9	1.4	6.3
<i>mirVana</i>	2,15	1,99	275.3	45.4	2.7	16.5	1.0	6.0
	2,17	1,97	470.7	75.2	7.1	16.0	1.5	9.4
High Pure	2,02	1,42	150.4	19.6	2.9	13.0	1.9	14.8
	2,03	1,4	228.5	45.3	7.7	19.8	3.4	17.1
<i>mirVana</i> [E]	1,87	0,79	45.7	20.5	2.5	44.8	5.5	12.2
	1,98	0,68	201.3	38.5	1.9	19.1	1.0	5.0
High Pure [E]	1,84	0,7	23.3	15.8	3.2	67.8	13.9	20.5
	1,64	0,5	20.0	13.5	1.9	67.5	9.4	13.9

The quantity and quality of the RNA obtained were assessed spectrophotometrically at 230, 260 and 280 nm. The A_{260}/A_{280} ratio was used to detect the contamination with proteins and $A_{260}/230$ ratio was used to check for carbohydrate and phenol contamination. RNA yield indicates the amount of extracted total RNA in nanograms (ng) per milligram (mg) of sample fresh weight. “sRNA” (0-200 nt) and “miRNA” (10-40 nt) concentrations were determined by Bioanalyzer and were calculated per mg of sample fresh weight. “sRNA” / RNA ratio (%) - indicates the percentage of “sRNAs” in total RNA fraction. “miRNA” / RNA ratio (%) - indicates the percentage of “miRNAs” in total RNA fraction, and “miRNA” / “sRNA” ratio (%) - indicates the percentage of “miRNAs” in “sRNA” fraction. [E] – short RNA enrichment protocol.

The integrity of the RNA was determined by a capillary electrophoresis using Agilent Bioanalyzer with an Agilent RNA 6000 Nano Kit (Appendix, Supplementary Figure 1). The presence of two clearly resolved peaks of 18S and 25S rRNAs was used to evaluate the integrity of RNA samples. In TRIzol and *mirVana* total RNA samples some additional peaks were detected, probably corresponding to additional ribosomal RNAs. Because of these extra peaks, the RNA integrity number (RIN) was artificially underestimated. In spite of this effect, the RIN values in TRIzol and *mirVana* RNA samples were still quite high (RIN= 7-7.3), confirming the RNA with minimum degradation (Appendix, Supplementary Figure 1). Even though, for integrity evaluation of RNAs the presence of well resolved RNA peaks and not strictly the RIN value should be considered. By contrast, in High Pure total RNA samples, clear degradation effects were observed. One RNA sample showed only one peak close at 200 nt, corresponding to short RNA fraction, while in the other sample, start of degradation was indicated by less pronounced 18S and 25S rRNA peaks (Appendix, Supplementary Figure 1).

3.1.1.2 Total RNA isolation protocols yielded higher “sRNA” and “miRNA” amounts compared to short RNA enrichment protocols

To determine the quality of short RNA fraction (< 200 nt) and to analyze the differences among various isolation protocols in terms of the yield of sRNAs, total RNA samples were analyzed with the Agilent Bioanalyzer and Small RNA Kit (Figure 14 and Appendix, Supplementary Figure 2). If the integrity of short RNA is good, then electropherogram profile will be comprised of one transfer RNA (tRNA) peak at ~ 40-80 nt and multiple peaks between 80-200 nt, corresponding to other longer sRNAs (rRNAs, pri-miRNAs etc.).

All electropherograms show well resolved peaks, indicating good integrity of short RNA fraction, with the exception of one *mirVana* short RNA enrichment sample, where only one tRNA peak is retained (Figure 14C ; Appendix, Supplementary Figure 2). Comparing the tRNA peaks among electropherograms revealed that tRNA peak in *mirVana* short RNA enrichment sample is similar and as we did not detect an increase in the number of RNA fragments shorter than 40 nt, it seems unlikely that the *mirVana* sample suffered for degradation during preparation. However, it is more likely that sRNA species larger than 80 nt were lost during the isolation procedure.

To obtain the concentration of short RNA fraction, two distinct regions were defined arbitrarily on the resultant electropherograms. The “sRNA region”, ranging from 0 to 200 nt, and the “miRNA region”, ranging 10 to 40 nt (Appendix, Supplementary Figure 2). Results showed that all three total RNA isolation techniques were comparably efficient in recovering both “sRNAs” and “miRNAs”. Of note, the “miRNA” fraction discussed in this section does not comprise only miRNAs but all other sRNAs, ranging from 10 to 40 nt. By comparing the amounts of “sRNAs” and “miRNAs” with total RNAs, we observed that “sRNAs” accounted for approximately 20 % of the total RNA and “miRNAs” represent up to ~ 3 % of the total RNA (Table 3). Yield of sRNAs in short RNA enrichment samples was unexpectedly lower in comparison to the yields obtained by total RNA preparation methods. Similarly, when inspecting the efficiency of “miRNA” recovery among procedures, we observed that enrichment protocols did not prove to be more effective in gaining higher amounts of “miRNAs” compared to the total RNA protocols (Table 3).

In short RNA enrichment samples, “sRNAs” and “miRNAs” constitute up to ~ 70 % and ~ 14 % of total RNA fraction (Table 3), respectively, therefore it appears that these samples contain also significant amounts of RNAs longer than 200 nt. Among the short RNA enrichment samples, notable exception was again *mirVana* sample, showing lower percentages of “sRNA” and “miRNAs” present in total RNA, more resembling those obtained in RNA samples extracted by total RNA isolation protocols (Table 3). By running the short RNA enrichment samples on Bioanalyzer with an Agilent RNA 6000 Nano Kit, we indeed observed that in *mirVana* enrichment sample, substantial amounts of RNAs longer than 500 nt were detected, including major peaks of 18S and 25S rRNAs (> 1000 nt) observed in electropherogram profiles of total RNA isolation samples (Appendix, Supplementary Figure 1).

By comparing the amounts of “miRNA” in “sRNA” fraction, we detected that up to 20 % of “sRNA” fraction represented “miRNAs”. The highest percentages were obtained in RNA samples extracted with short RNA enrichment protocol of High Pure kit and in one short RNA enrichment sample of *mirVana*, whereas the percentage of “miRNAs” in “sRNA” fraction in other samples was significantly lower (Table 3). However, considering slightly lower intensity of peaks at ~ 150 nt (Figure 14D; Appendix, Supplementary Figure 2) and the poorer RNA integrity of RNA samples obtained by High Pure kit (Appendix, Supplementary Figure 1), and absence of short RNA peaks in *mirVana* enrichment sample, higher percentage of “miRNAs” detected in these samples may be a result of an ongoing degradation of RNAs into shorter RNA fragments.

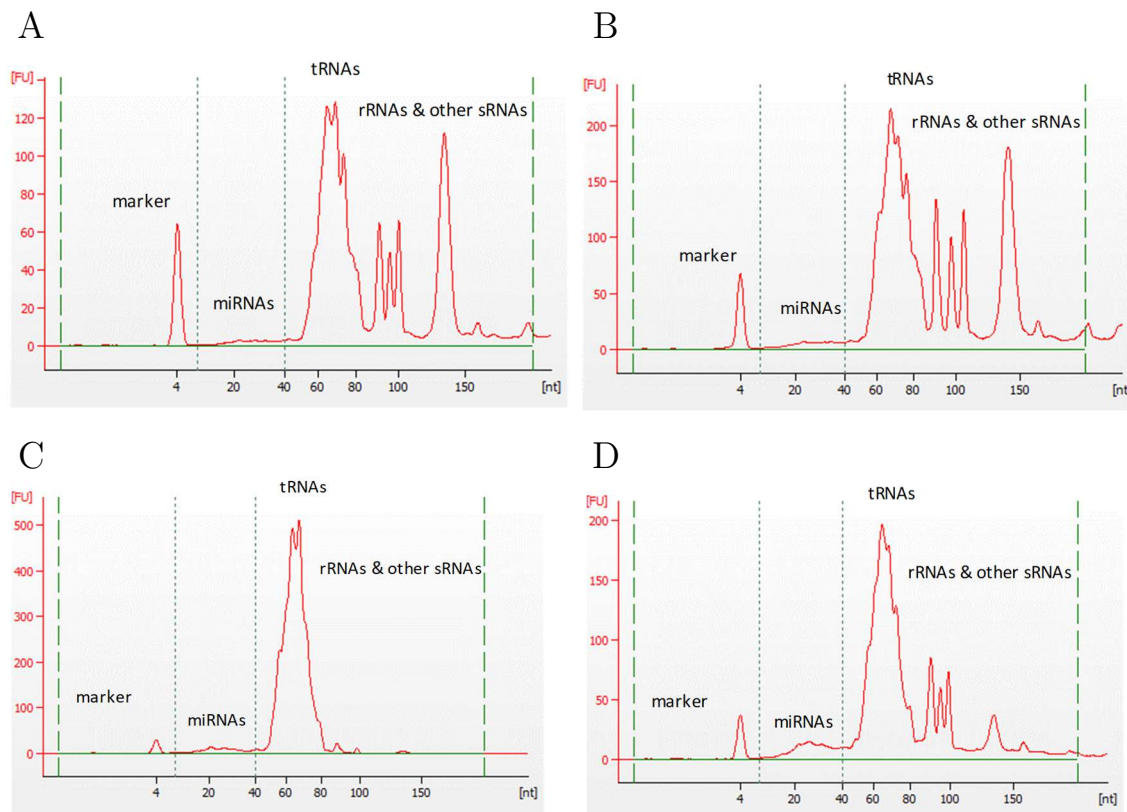


Figure 14: Examples of sRNA electropherograms showing the abundance and integrity of “sRNA” (0-200 nt) and “miRNA” (10-40 nt) fractions extracted by A) TRIzol, B) *mirVana*, C) *mirVana* short RNA enrichment protocol, D) High Pure short RNA enrichment protocol. RNA samples were analyzed with the Agilent Small RNA Kit on Agilent Bioanalyzer. The y axis represents fluorescence units (FU) and the x axis the length of small RNAs in nucleotides (nt). Electropherograms for other measurements can be found in Appendix, Supplementary Figure 2.

3.1.1.3 RNA isolation protocols are able to preserve ratios in expression of potato miRNAs

To evaluate whether the analyzed RNA extraction method could efficiently extract all miRNAs equally and preserve their expression level, the relative expression level of four potato miRNAs was measured by RT-qPCR. Relative expression levels of miR168, miR172 and miR390 were determined by normalization to the selected endogenous control miRNA, miR167 levels. Thus, miRNA relative expression level gave us the information how much quantity of specific miRNA is recovered by the isolation protocol with regard to the endogenous miR167 level. Meaning, if all three tested miRNAs and control miRNA were recovered with a similar efficiency, given the fact that identical tissue sample was used, then relative expression level of miR168, miR390 and miR172 should be similar, regardless of the extraction protocol used. By examining miR168, miR172 and miR390 relative expression levels among isolation protocols, we observed that *mirVana* total RNA samples

exhibit up to two-fold for miR168 and miR390 or even up to five-fold higher miR172 levels, compared to the levels observed in other RNA samples, in which the expression levels of all three miRNAs were close to one (Figure 15A-C).

Further, miRNA levels also differ considerably between both *mir*Vana isolation replicates (Figure 15C). By looking at amplification efficiencies (all close to 100 %; data not shown), we excluded the possibility that differences occurred due to inefficient PCR reactions.

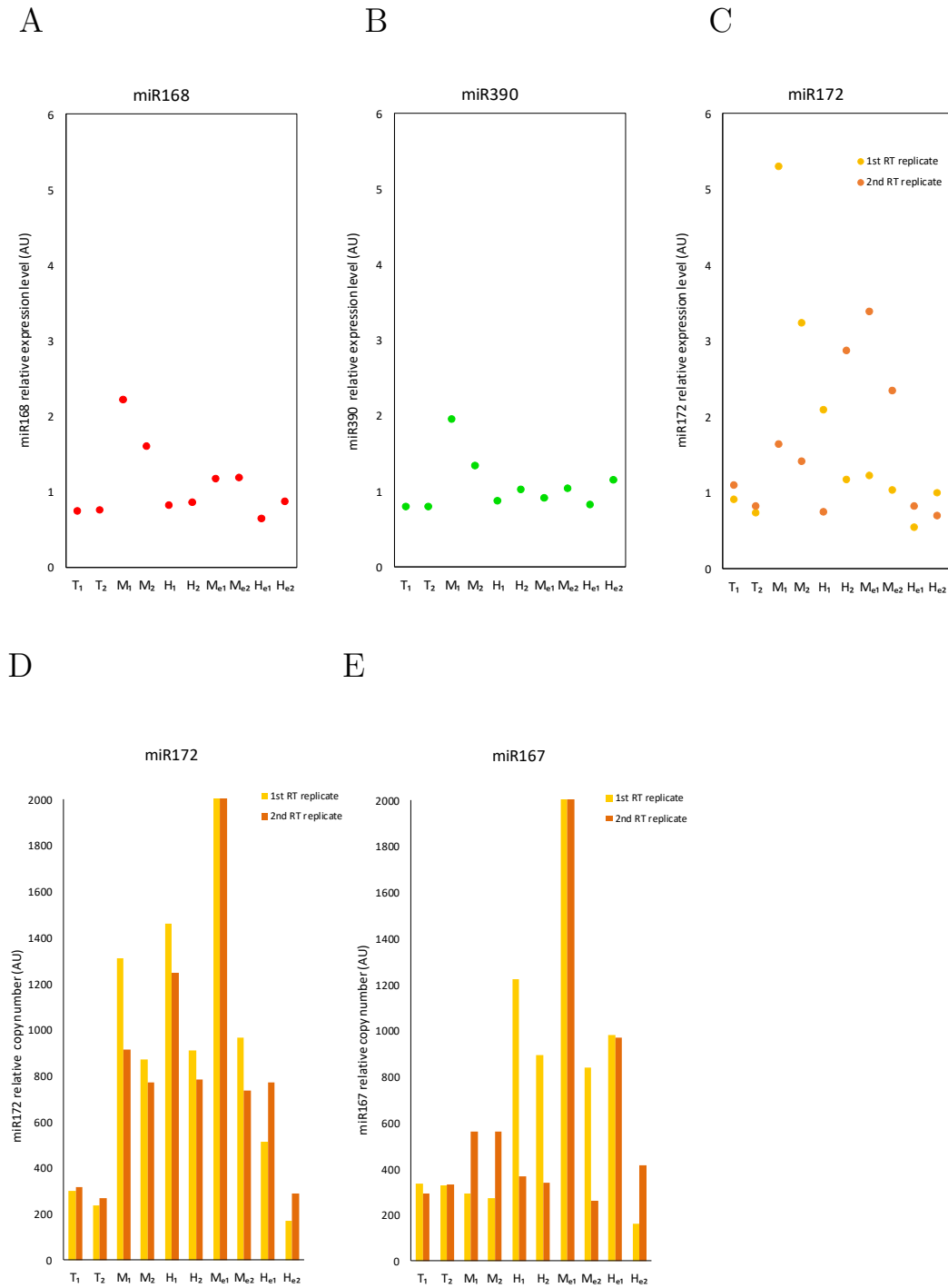


Figure 15: Effect of different RNA extraction protocols on relative expression levels of three tested miRNAs. Relative expression levels of A) miR168, B) miR390 and C) miR172. The miRNA expression levels were quantified by RT-qPCR and further normalized to control miR167 expression level. Each extraction protocol was tested in duplicates. For determination of the miR172 relative expression level and to test the repeatability of RT, two duplicate reverse transcription (RT) reactions for miR172 and miR167 were performed. D) miR172 and E) miR167 relative copy numbers as determined quantGenius from both duplicate RT reactions. AU – absorbance units; T – TRIzol; M – *mirVana*; H – High Pure; e – short enrichment protocol; 1, 2 – two technical replicates of the same procedure; miR168 – stu-miR168; miR390 – stu-miR390-5p; miR172 – miR172 a,b-e-3p, miR167 – stu-miR167a-5p.1.

For miR172 and control miR167, duplicate RT reactions were carried out. As shown in Figure 15C, relative expression levels of miR172 between RT duplicate reactions vary considerably not only between both *mirVana* total RNA isolation samples but also among High Pure total RNA and *mirVana* short RNA enrichment samples. By examining the copy numbers of miR172 and miR167 between the duplicate RT reactions (Figure 15DE), we found that observed differences in relative expression levels of miR172 are mainly attributed to a less efficient reverse transcription of miR167 (Figure 15E).

Thus, results suggested that the observed differences in miRNA expression levels emerged due to variable efficiency of RT reactions, rather than the bias introduced during the RNA extraction. It is possible that MultiScribe reverse transcriptase RT suffered from repeatability problems due to too concentrated RNA applied, which smothered the RT reactions, or there was too much co-isolated carryover inhibitors present in RNA solution that interfere with conversion to cDNA. To increase the efficiency, we carried out the additional RT reaction using diluted (10-fold) RNA samples. 10-fold dilutions shifted the Cq values towards the limit of quantification, making quantification/comparisons between miR172 and miR167 expression levels impossible.

We thus decided to perform new RT reactions on the same undiluted RNA samples using SuperScript III enzyme, which has been proved as more sensitive and efficient than MultiScribe employed in our previous protocol. SuperScript III RT reactions were first performed using recommended thermal cycling conditions for SuperScript III action (i.e. at 50 °C). Both, high repeatability of RT reactions and similar miR172 relative expression levels were observed in all samples, including *mirVana* total RNA isolation samples, further confirming that the previously observed differences in miRNA relative expression levels were attributed to differences in RT reactions and not differences in extractions (Figure 16A). As the input concentration of the RNA in RT reactions was similar, and amounts of RNA used in the RT step were well within the manufacturers' specifications, it is less likely that MultiScribe RT reactions were saturated due to too high concentrations of RNA used in RT.

Since the Cq values obtained using SuperScript III reactions were higher than expected, even though the similar RNA concentrations were used for RT, we decided to additionally perform pulsed RT reaction using the same SuperScript III enzyme, but with employing three different annealing temperatures with cycling pulses (pulsed RT). By using the same undiluted RNA samples, we observed that the Cq values of miR172 and miR167 were substantially lower in all samples, indicating improved sensitivity and efficiency of pulsed RT reactions to non-pulsed (Figure 16C). In addition to good concordance between RT duplicate reactions, miR172 relative expression levels were similar in all samples,

confirming the previous results obtained by employing non-pulsed SuperScript III RT protocol were the result of inefficient reverse transcription (Figure 16B).

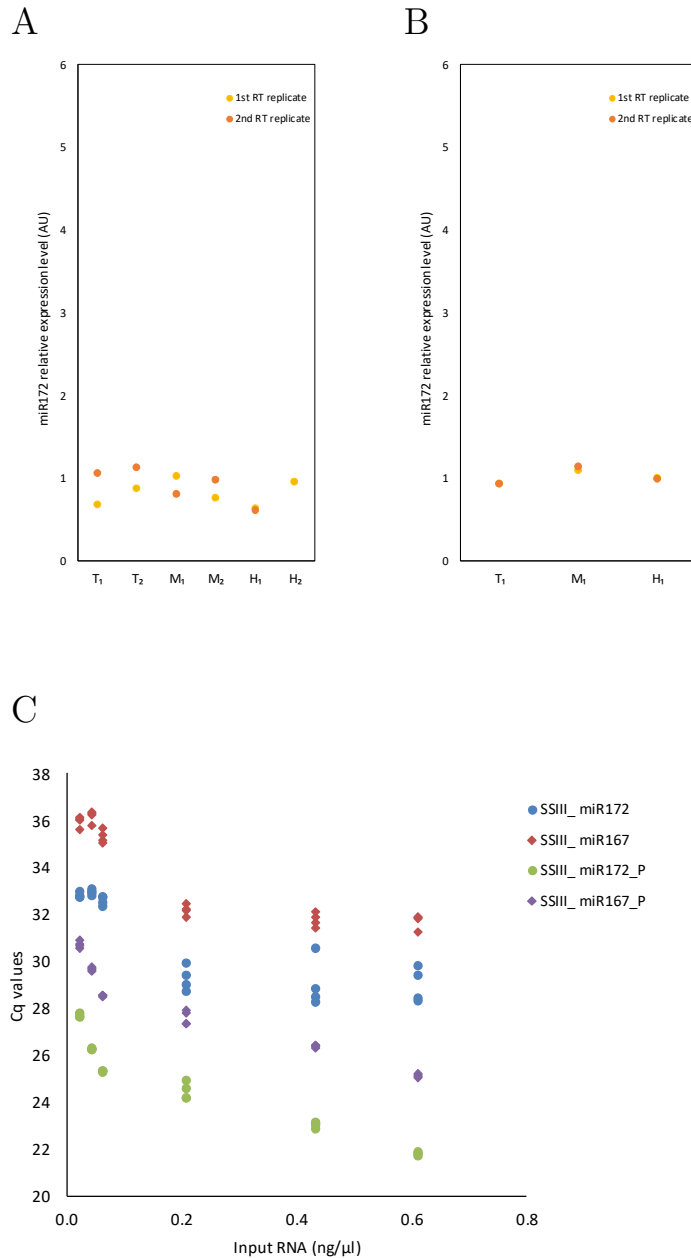


Figure 16: Effect of different RNA isolation protocols on relative expression level of miR172 normalized to the expression of miR167 as determined with RT-qPCR by using either A) SuperScript III RT system or B) SuperScript III RT system employing pulsed RT reaction. Each extraction protocol was tested in duplicates. For determination of the miR172 relative expression level, two duplicate RT reactions for miR172 and miR167 were performed. C) Cq values for miR172 and miR167 obtained by SuperScript III and SuperScript III pulsed RT reactions. AU – absorbance units; T – TRIzol; M – *mirVana*; H – High Pure, miR172 – miR172 a,b-e-3p; 1,2 – two technical replicates of the same extraction procedure; SSIII – SuperScript III; P-pulsed; miR172 – miR172 a,b-e-3p, miR167 – *stu-miR167a-5p.1*.

3.1.2 Protocol for simultaneous RNA and sRNA isolation from small amounts of starting plant material

To establish the RNA extraction protocol that will gain enough RNA for both long transcript and sRNA expression studies from very small amounts of leaf tissue (e.g. to perform spatial analysis of responses following PVY infection), we chose to compare two recently developed column-based kits, Direct-zol micro kit (hereinafter referred to as Zymo) and Single cell purification kit (hereinafter referred to as Norgen). The first employs the use of TRIzol for cell disruption, while the second uses phenol-free lysis buffer. Both protocols are fast and easy and enable the use of very small elution volumes $> 8 \mu\text{l}$ and reportedly allow purification of both long and short RNAs ($> 17 \text{ nt}$). For a comparison of the two extraction kits, RNA was isolated from equal amounts of potato leaf tissue (leaf discs), in triplicates. Kits were assessed in terms of the total RNA yield, purity of samples and sRNA recovery (Figure 17).

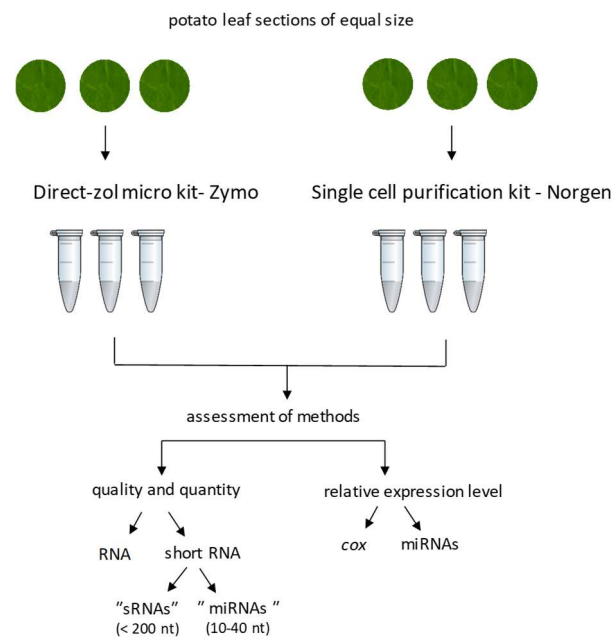


Figure 17: Workflow for assessment of RNA extraction methods for mRNA and sRNA expression analyses from small amounts of plant material.

In terms of the total RNA yield, Zymo kit performed slightly better than Norgen, yielding approximately 50 ng per μg of plant tissue (Table 4). However, in RNA samples extracted by Zymo kit, purity assessment ratios, A_{280}/A_{260} and A_{260}/A_{230} were lower than expected, indicating the presence of contaminants that absorb at or close to 280 nm and 230 nm. On the other hand, even though the Norgen lysis buffer induced extensive foaming during extraction, it appears that this did not affect either the total RNA yield or purity of RNA solution. Both kits proved to be equally efficient yielding similar "sRNA" and "miRNA" quantities and good integrity of short RNA fractions (Appendix Supplementary Figure 3). "sRNAs" represented approximately 27 % of total RNAs, indicating good

recovery of short RNA fraction (Table 4). In both kits, “miRNAs” were estimated to represent $\sim 18\%$ of the “sRNA” fraction, which was substantially higher than obtained by total RNA extraction protocols described in Section 3.1.1.

Table 4: Performance of the low-input RNA extraction methods in terms of the quality and quantity of RNA and short RNAs.

Extraction procedure	Absorbance ratios		RNA (ng/ μ L)	RNA yield (ng/ μ g)	“sRNA” yield (ng/ μ g)	“miRNA” yield (ng/ μ g)	“sRNA”/RNA (%)	“miRNA”/RNA (%)	“miRNA”/“sRNA” (%)
	260/280	260/230							
Zymo	1.97	1.44	57.63	3.31	ND	ND	ND	ND	ND
	1.76	1.05	44.59	2.56	0.68	0.12	26.6	4.7	17.7
	1.96	1.47	53.34	3.07	ND	ND	ND	ND	ND
Norgen	2.08	2.01	21.63	1.24	ND	ND	ND	ND	ND
	2.04	1.96	34.21	1.97	0.53	0.09	27.2	5.00	18.3
	1.98	1.89	35.36	2.03	ND	ND	ND	ND	ND

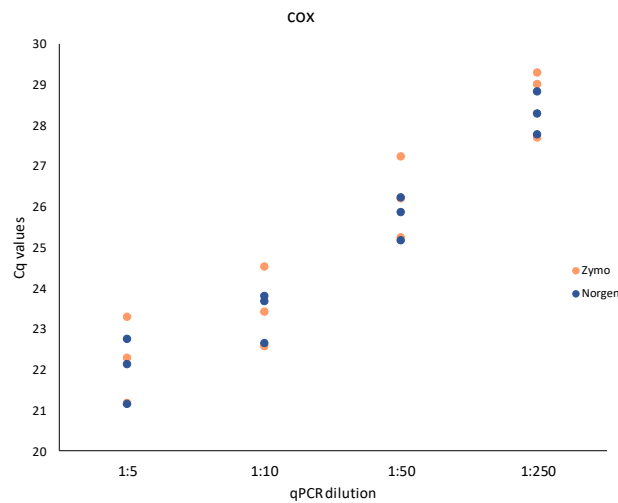
The quantity and quality of the RNA of three separate isolations per method (Zymo, Norgen) were assessed spectrophotometrically at 230, 260 and 280 nm. The A_{260}/A_{280} ratio was used to detect the contamination with proteins and A_{260}/A_{230} ratio was used to check for carbohydrate and phenol contamination. Total RNA concentration (ng/ μ L) as determined by spectrophotometric measurements. RNA yields indicate the amount of extracted RNA in nanograms (ng) per micrograms (μ g) of sample fresh weight. “sRNA” (0-200 nt) and “miRNA” (10-40 nt) concentrations were determined by capillary electrophoresis system. “sRNA” and “miRNA” yields were calculated per μ g of sample fresh weight. “sRNA” / RNA ratio (%) - indicates the percentage of “sRNAs” in total RNA fraction. “miRNA”/RNA ratio (%) - indicates the percentage of “miRNAs” in total RNA fraction, and “miRNA”/“sRNA” ratio (%) - indicates the percentage of “miRNAs” in “sRNA” fraction. ND – not determined.

Total RNA quantity and quality were also examined by quantification of *cox* mRNA level by RT-qPCR, assuming equal RT and qPCR efficiencies. In all RNA samples, the obtained Cq values were very similar among samples, with a maximum of $\sim$ one cycle difference between RNA samples of Zymo and Norgen kit (Figure 18A). Surprisingly, one of the RNA samples extracted by Zymo kit exhibits the lowest *cox* mRNA level, despite the fact that assessed RNA concentration of this sample (i.e. 44 ng/ μ L, Table 4) was higher compared to all RNA samples extracted with Norgen’s kit. By looking at low A_{280}/A_{260} and A_{260}/A_{230} ratios determined in this particular sample, the apparent increase in concentration most probably resulted from a contamination by residual phenol, which led to an erroneous overestimation of RNA quantity.

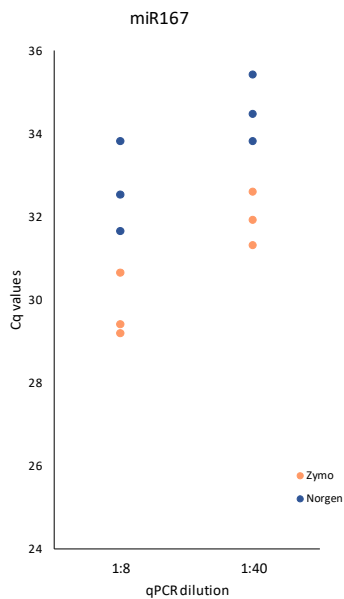
To further examine if any of the extraction protocols used may affect miRNA profiling results, the miRNA RT-qPCR was performed to compare the expression levels of three selected miRNAs. We chose to look at the level of miR172, with a low GC content, at miRNA with high expression in potato leaves miR6022, and control (reference) miRNA, miR167. When we compared the Cq values obtained by RT-qPCR, we found that the RNA samples extracted by Norgen’s kit yielded significantly higher Cq values, suggesting a lower recovery of miRNAs (Figure 18B-D). The levels of miR172, miR6022 and miR167 in RNA samples extracted with Zymo kit were 2-200-fold higher, even though comparable input RNA amounts were used. This was also observed with *cox* mRNA quantification results. Since the measured Cq values for miR172 and miR167 in RNA samples obtained by Norgen’s kit were close to the limit of quantification, relative quantification was not possible. However, the relative expression level of miR172 and miR6022 observed in RNA samples extracted with Zymo kit were in good agreement among the extraction replicates, except for one RNA sample, where the relative expression level of miR172 was three-fold higher than expected, even though the SuperScript III pulsed RT reaction was employed

(Figure 19). The amplification efficiency of PCR reactions was close to 100 %, therefore we assumed that the difference occurred due to suboptimal efficiency of RT, most probably due to the presence of higher amounts of inhibitors co-extracted with RNA (low A_{280}/A_{260} and A_{260}/A_{230} ratios, Table 4). Whereas Zymo kit showed overall superiority in isolating sRNAs and mRNAs from small tissue sections it also showed that in some cases it may yield RNA with lower purity which could affect miRNA profiling results. Therefore, for the samples showing lower ratios than expected, additional purification would be recommended.

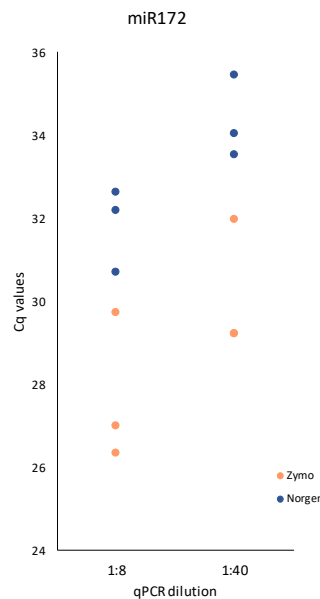
A



B



C



D

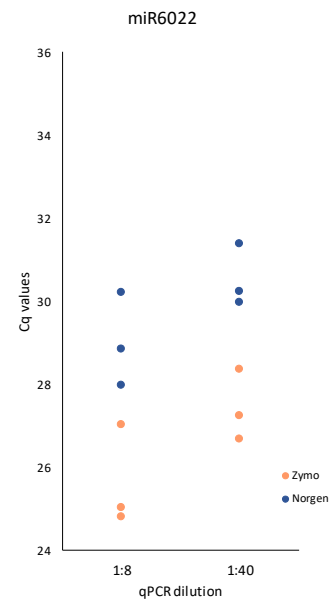


Figure 18: Assessment of mRNA and miRNA recovery of low-input RNA extraction methods. A) *cox* mRNA and B) miR167 C) miR172 and D) miR6022 recovery of Zymo (Direct-zol micro kit) and Norgen (Single cell purification kit) extraction kits as

evaluated by the Cq values obtained by RT-qPCR. Cq values of *cox* mRNA indicate that similar amounts of RNA were used for RT-qPCR. RNA samples obtained with Norgen kit are showing higher Cq values for three selected miRNAs, indicating poorer performance of the kit in miRNA recovery compared to Zymo kit. Blue and orange dots represent triplicate isolations per extraction method for 8- and 40-fold dilutions used in qPCR reactions. miR167 – stu-miR167a-5p.1, miR172 – miR172 a,b-e-3p, miR6022 – stu-miR6022-3p.

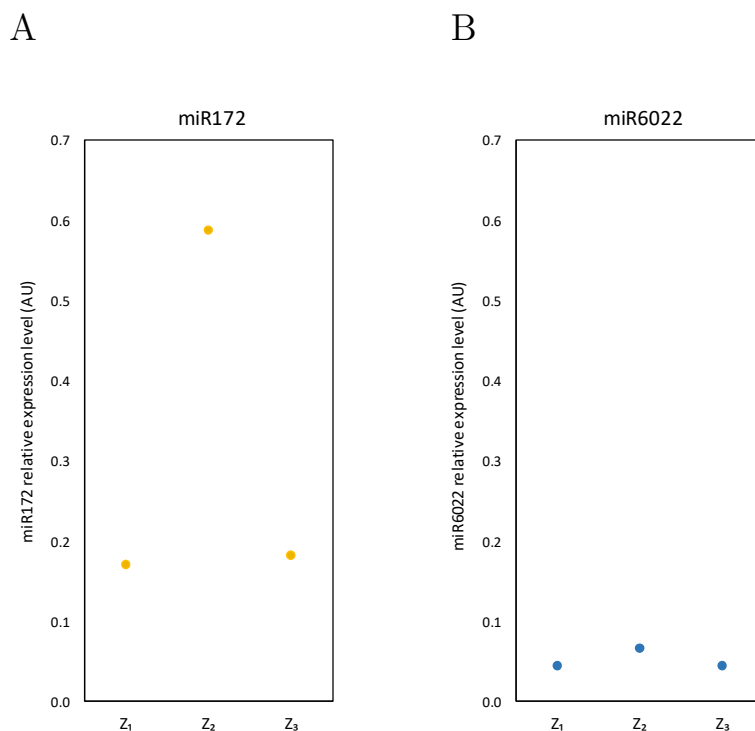


Figure 19: Effect of Zymo extraction protocol on relative expression levels of miRNAs. Expression levels of A) miR172 and B) miR6022 were quantified by RT-qPCR and further normalized to miR167 expression level. Extractions were performed in triplicates. AU – absorbance units, Z – Zymo (Direct-zol micro kit); 1,2,3 – three replicates of the extraction procedure. miR167 – stu-miR167a-5p.1, miR172 – miR172 a,b-e-3p.

3.1.3 Optimization of DNA removal from RNA samples

Previously, our lab has been employing DNA digestion protocol, in which DNase I treatment is followed by addition of EDTA and heating step for DNase I inactivation. As EDTA was discovered to interfere with several ligation steps during library generation and sequencing (LC Sciences), it was usually removed by an additional cleaning step. Nevertheless, the protocol is time-consuming and the heating step employed within the protocol may have a more pronounced effect on RNA samples of very low concentrations. We were thus looking for a faster RNA purification protocol, enabling DNase treatment and purification of short and long RNAs, while excluding EDTA and heating steps. We tested the performance of recently developed Clean RNA Clean & Concentrator kit (Zymo Research). The procedure involves the DNase I treatment followed by RNA binding to the

column and repeated washing steps to remove impurities, enabling the purification of both short (> 17 nt) and long RNAs. We aimed to evaluate the suitability of the protocol for samples with limiting amounts of RNAs, therefore very small pieces of potato leaf tissue were used (Figure 20).

First, RNA was extracted using Direct-zol micro kit and checked for the quantity and integrity. The same RNA was used to analyze the efficiency of RNA Clean & Concentrator kit (hereinafter referred to as DNase-cleaning protocol) and subsequently to compare its performance with our currently used DNase I digestion protocol (hereinafter referred to as DNase-EDTA protocol). Because the columns for RNA binding used within Direct-zol micro kit were identical to those employed during the DNase-cleaning protocol, we assumed that they are efficient in binding and preserving expression levels of short RNAs (i.e. miRNAs) as demonstrated in Section 3.1.2. In addition, we aimed to analyze whether the columns were efficient in preserving the levels of mRNAs. To test this, DNase-cleaning protocol was carried out in duplicates using the same amount of input RNA. At the same time, two duplicate reactions were set on the identical RNA sample employing current DNase-EDTA protocol (Figure 20).

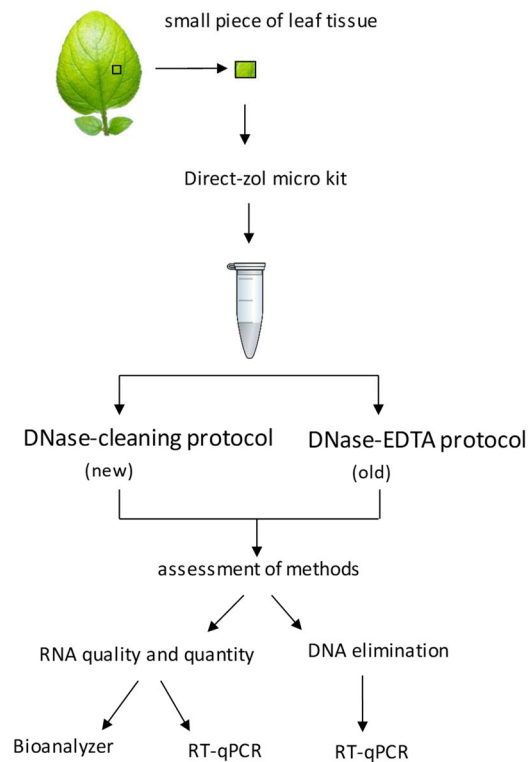


Figure 20: Workflow for assessment of the efficiency of RNA purification protocol for RNA samples obtained from very small pieces of leaf tissue.

DNase-treated and cleaned RNA samples were firstly examined for the quantity and integrity using capillary electrophoresis using Agilent Bioanalyzer with an Agilent RNA 6000 Pico Kit. The resulting electropherograms showed good quantity and integrity of RNA. When compared to the electropherogram of RNA sample prior treatment we observed good preservation of the integrity of both short and long RNAs in both samples. Both RNA samples subjected to DNase-EDTA protocol also showed good integrity of RNAs > 1000 nt, including well preserved 18S and 25S RNAs, however instead of three peaks at ~ 100-150 nt, samples displayed two peaks, one at 120 nt and one with a lower absorbance at 160 nt, indicating the loss of some short RNAs (Appendix, Supplementary Figure 4).

DNase-treated and cleaned RNA samples were further subjected to RT-qPCR for quantification of the *PR1b* and *cox* mRNAs levels. By examining the relative expression level of *PR1b* (normalized to *cox*) between two independent replicate reactions per each protocol, we observed that both DNase-cleaning and DNase-EDTA protocols were equally efficient in preserving both *PR1b* and *cox* mRNA expression levels, implying good repeatability of both protocols (Figure 21A).

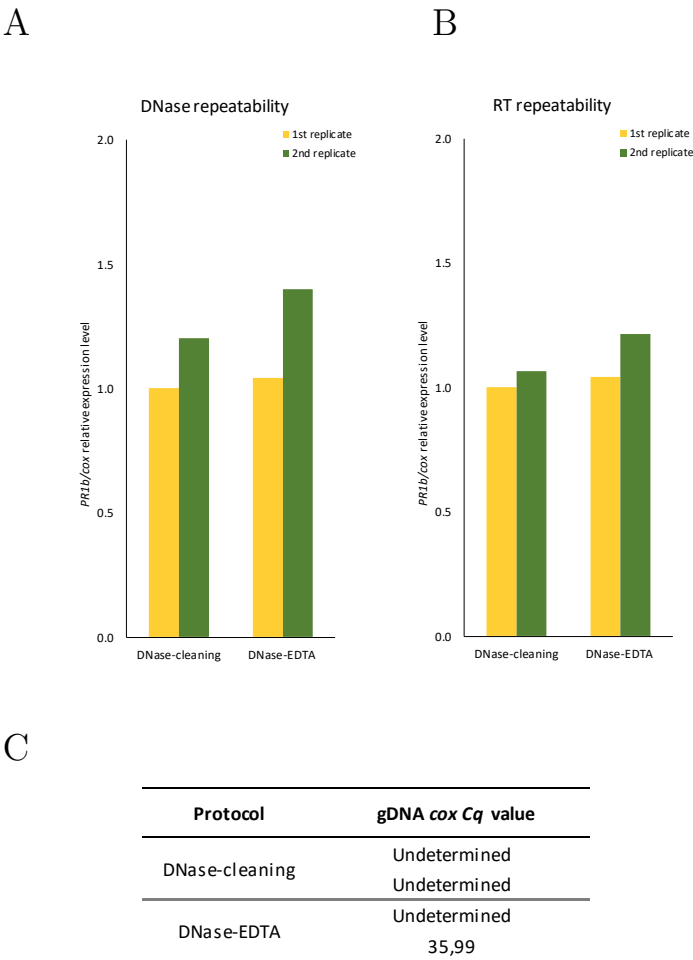


Figure 21: Repeatability of RNA quantification following DNA removal by two protocols, DNase-cleaning and DNase-EDTA protocols. Evaluation was performed with either two independent A) DNase reactions or two independent B) reverse transcription (RT) reactions. Repeatability was assessed by preservation of ratios in the expression level of

PR1b mRNA and *cox* mRNA. C) Efficiency of DNAs removal from RNA samples. Genomic DNA was quantified by RT-qPCR with *cox* primers and probes (Table 1). Undetermined – under the level of detection ($C_q > 45$).

Furthermore, by quantifying *PR1b* and *cox* mRNA levels in two replicate RT reactions (Figure 21B), we aimed to indirectly evaluate the purity of the processed samples (spectrophotometric assessment was not possible because of too low RNA amounts) by monitoring of putative inhibitory effects through the efficiencies of RT and PCR reactions. Results of RT duplicates reactions show similar *PR1b* and *cox* mRNAs levels in both DNase-cleaning and DNase-EDTA RNA samples, indicating that RNA is pure enough not to interfere with RT-qPCR. Also, EDTA in RNA samples subjected to DNase-EDTA protocol did not inhibit reverse transcriptase efficiency (Figure 21B). Further, to check the efficiency of DNA removal performed within DNase-cleaning protocol and DNase-EDTA protocol, *cox* DNA level was screened in duplicate reactions via qPCR without RT. Results showed that DNase-cleaning protocol yielded pure RNA free of DNA contamination, while in one RNA sample subjected to DNase-EDTA protocol, some residuals of DNA could still be detected (Figure 21C). Altogether, these data demonstrate that DNase-cleaning protocol could be used as a protocol of choice for purification of RNA samples for sequencing.

3.1.4 Comparison of plant miRNA prediction tool

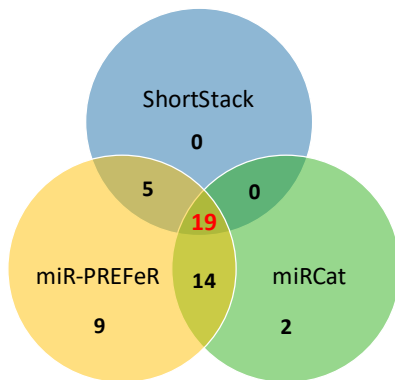
With the development of sRNA sequencing techniques, several miRNA prediction tools have emerged for the discovery of novel *MIR* loci and miRNAs in plants. The evaluation of their performance is however lacking. Thus, we decided to do a comparative analysis of the performance of three plant miRNA prediction tools, two standalone tools ShortStack (Axtell, 2013b) and miR-PREFeR (Lei and Sun, 2014) and miRCat, a tool from the UEA sRNA workbench (Moxon et al., 2008). sRNA-Seq data of two potato (cv. Rywal) samples were used to assess the sensitivity and accuracy of their prediction of novel *MIR* loci and miRNAs.

3.1.4.1 miRNA prediction tools vary significantly in the number of predicted *MIR* loci

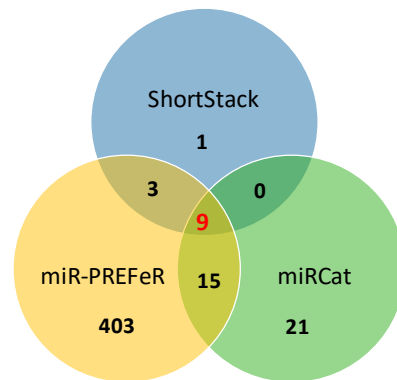
In total miR-PREFeR predicted 447 *MIR* loci, whereas predicted number of *MIR* loci by miRCat and ShortStack was more than five times lower, being 80 and 37, respectively (Figure 22AB and Figure 23). For 224 annotated potato *MIR* loci found in miRBase (Kozomara and Griffiths-Jones, 2014), miR-PREFeR successfully identified 47 of them, while the miRCat and ShortStack identified 35 and 24 of them, respectively. 28 *MIR* loci were predicted by all three tools, of them 19 were known and 9 were novel potato *MIR* loci (Figure 22AB). Although the number of ShortStack predictions was the lowest, almost all predicted *MIR* loci were confirmed by at least one of the other tools (Figure 22AB). On the other hand, more than 400 novel *MIR* loci were detected only by miR-PREFeR (Figure 22B).

We further examined the number of known *MIR* loci that were not predicted (false negatives) by the tested tools, even though their corresponding miRNAs were detected in sRNA-Seq samples (having > 10 raw reads in both analyzed sRNA-Seq samples; see Section 2.4.3 for used parameters within predictions). miR-PREFeR was unable to predict 6 of 36 known *MIR* loci, while miRCat and ShortStack predictions missed 19 and 16 known *MIR* loci (Figure 22C).

A known potato *MIR* loci



B novel potato *MIR* loci



C not predicted known potato *MIR* loci

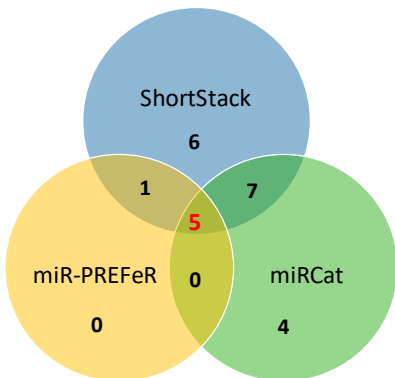


Figure 22: Venn diagrams of the numbers of unique and common predicted A) known (annotated), B) novel *MIR* loci and C) not predicted known (annotated) *MIR* loci (false negatives) among different miRNA prediction tools.

A

[illegible]

B

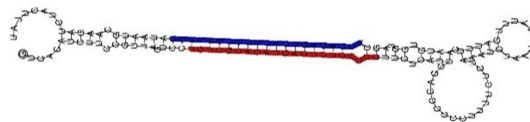


Figure 23: Example of predicted *MIR* loci, given as coordinates on potato genome (Xu et al., 2011) and pre-miRNA precursor sequence in ShortStack tool. A) The location on the genome and the sequence of a precursor pre-miRNA is given at the top. Below the sequence base-paired secondary structure is predicted in dot-bracket notation followed by derived mature miRNA and miRNA* sequences. The additional sequences mapped to the same *MIR* loci represent the mature miRNA variants (isomiRs). The numbers on the right represent lengths and abundance of reads. l – length, a – number of reads for mapping sequence. B) The fold of the pre-miRNAs into a hairpin with miRNA and miRNA* sequences on 5' and 3' arms visualized by RNA/Folding Annotation tool from the UEA sRNA workbench (Moxon et al., 2008).

3.1.4.2 ShortStack is more efficient in eliminating non-*MIR* like loci

As the higher number of predicted *MIR* loci may also indicate higher probability of false positive predictions, we further aimed to evaluate the extent of false positive results by inspecting the folding free energy of predicted hairpin structure of pre-miRNA, which should be < -0.2 kcal/mol/nt (Axtell and Meyers, 2018; Kozomara and Griffiths-Jones, 2014; Meyers et al., 2008). If the folding energy of predicted pre-miRNA precursor was higher, then the predicted loci was not considered as true *MIR* loci. In the false positive set, we additionally included the *MIR* loci, whose corresponding mature miRNA sequence matched the sequences of other non-coding RNAs (tRNA, rRNA, snoRNA, snRNA) in *Viridiplantae* (<https://rnacentral.org/>) (Bateman et al., 2011). Among tested tools, ShortStack was found to be the most specific with no false positives detected (considering both our false positive sets), while ~ 35 % of miRCat and ~ 26 % of miR-PREFeR predictions were found to be non-*MIR* like loci (Figure 24).

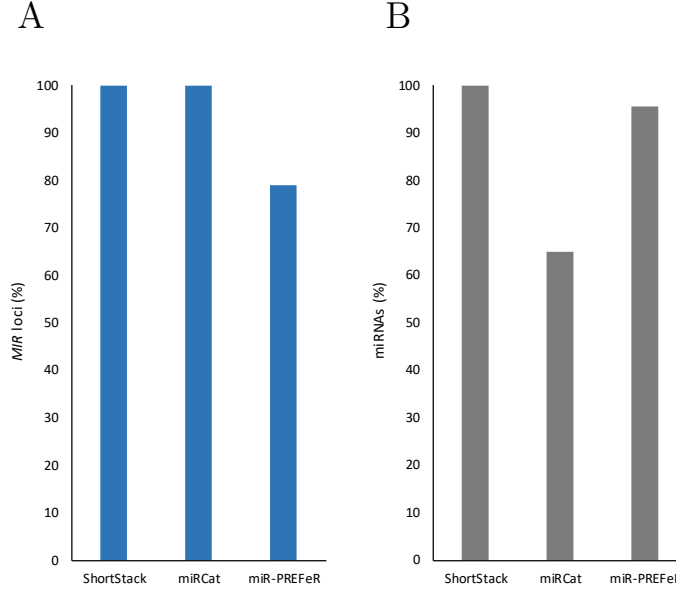


Figure 24: Performance of predictions tools in discriminating the *MIR* loci and miRNAs from non-*MIR* loci and non-miRNAs. A) Percentage of predicted *MIR* loci that have a folding free energy of < -0.2 kcal/mol/nucleotide. B) Percentage of mature miRNAs from predicted *MIR* loci that did not map to the other ncRNA *Viridiplantae* sequences (tRNA, rRNA, snRNA or snoRNA).

3.1.4.3 Prediction tools vary in the prediction of pre-miRNA sequence length

We further examined whether the tools were concordant in predicting identical mature miRNA from the same predicted *MIR* loci. By looking at the miRNA sequences from commonly predicted *MIR* loci, we found that all three tools predicted the identical miRNA sequence from the same *MIR* loci (Appendix, Supplementary Table 1).

The differences in prediction algorithms were, however, in the length and sequences of predicted pre-miRNAs. Among pre-miRNAs predicted by at least two tools, only one pre-miRNA sequence was found to be identical (Appendix, Supplementary Table 1). By comparing the sequences and their folding free energies, between 28 *MIR* loci, predicted by all three tools, we observed that, on average, ShortStack predicted longer *MIR* loci with up to 50 nt difference in length and with a less negative folding free energy of predicted hairpin structure, while miRCat on average predicted the shortest *MIR* loci with more negative folding free energy of predicted hairpin precursor (Appendix, Supplementary Table 2).

3.2 sRNA in Potato-PVY Interaction

3.2.1 Known and Novel endogenous sRNAs identified in potato leaves

We have sequenced 36 sRNA libraries from both control and PVY-infected leaves of cv. Désirée, NahG-Désirée, cv. Pentland-Squire, cv. Rywal, NahG-Rywal and identified 262 unique miRNAs belonging to 105 plant miRNA families. 209 of miRNA sequences matched to previously described potato miRNAs (miRBase version 22) and 53 of them correspond to miRNAs found in other plant species. Additionally, 45 known miRNA length variants (i.e. isomiRs) were identified (Figure 25, Appendix, Supplementary Dataset 1). By using *in silico* miRNA prediction, 199 unique novel miRNA sequences coded by 160 novel *MIR* loci were also detected (Figure 25, Appendix, Supplementary Dataset 1 and 2). Based on the similarity of their predicted precursor sequences with other annotated plant precursor sequences, novel miRNA sequences were assigned to 19 known and 141 novel miRNA families (Figure 25, Appendix, Supplementary Dataset 2).

When assessing the miRNA regulatory network, the amplification of silencing through phasiRNA biogenesis was also considered. In total, 769 *PHAS* loci were predicted, coding for more than 9000 phasiRNAs (Figure 25, Appendix, Supplementary Dataset 3 and 4). 540 *PHAS* loci were located on protein-coding regions of genes, with the majority encoding NBS-LRRs (nucleotide binding site-leucine rich repeat proteins) and LRR-RLKs (leucine rich repeat-receptor-like kinases) (Appendix, Supplementary Dataset 3). Interestingly, several novel *PHAS* loci were discovered in coding regions of genes associated with stress signaling, such as superoxide dismutase, calcium-transporting ATPase and auxin signaling F-box proteins. Moreover, we have also confirmed that *StAGO1* is a *PHAS* locus, as previously described in Arabidopsis (Mallory and Vaucheret 2009) (Appendix, Supplementary Dataset 3). Moreover, we also searched for miRNAs with the ability to trigger phasiRNA production and similarly to previous reports (Li et al., 2011; Shivaprasad et al., 2012), we observed that the vast majority of predicted miRNA triggers belong to the conserved miR482 family as well as to three *Solanaceae*-specific miR6023, miR6024 and miR6027 families (Appendix, Supplementary Dataset 5).

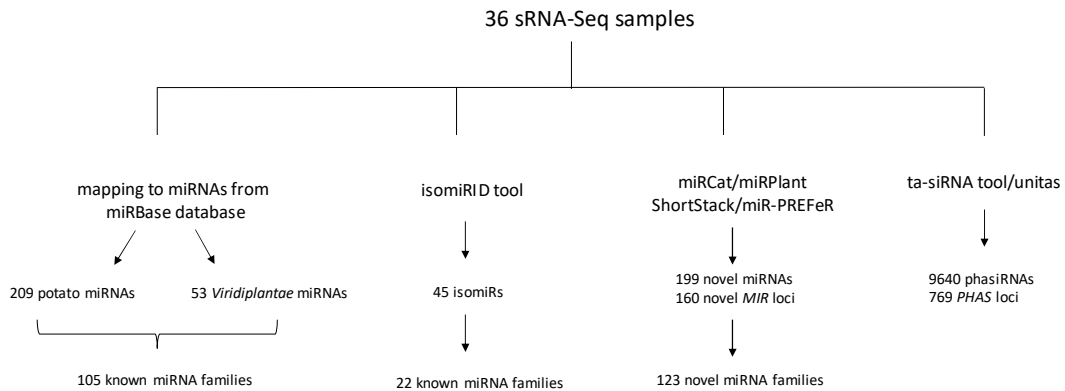


Figure 25: Numbers of known and novel miRNAs, isomiRs and phasiRNAs identified from 36 analyzed sRNA-Seq samples.

3.2.2 Identification and validation of targets of endogenous miRNAs and phasiRNAs

To study the importance of post-transcriptional regulation relevant to our research system, we performed searching of miRNAs and phasiRNA target transcript by employing the *in silico* and Degradome-Seq approach.

Most of the determined targets of miRNAs and phasiRNAs were found to encode transcription factors belonging to AP2/ERF, bHLH, MYB and GRAS family proteins known to participate in plant development and/or stress response, genes encoding putative immune receptors (NBS-LRRs, LRR-RLKs) as well as genes involved in biosynthesis and signaling of different phytohormones (Appendix, Supplementary Dataset 6 and 7).

Several miRNA-mRNA pairs conserved across plant species, such as miR156-*SPL11*, miR160-*ARF10*, miR160-*ARF17*, miR172-*AP2*, miR396-*GRF5* or miR398-*CSD* (Curaba et al., 2014) have been confirmed using both *in silico* prediction and Degradome-Seq. Additionally, by connecting miRNAs with phasiRNAs and their targets, potato sRNA regulatory network was constructed, which revealed also many novel connections in the plant immune signaling (see example in Appendix, Supplementary Figure 5, Supplementary Dataset 6 and 7).

Our data confirmed the well-defined miR393-mediated cleavage of transcripts encoding members of TIR/AFB gene family, receptors implicated in the control of auxin signaling (Figure 26) (Si-Ammour et al., 2011). We also discovered that these transcripts were targets of several *TIR1*-derived phasiRNAs (phasiTIRs) (Figure 26, Appendix, Supplementary Dataset 6 and 7). Moreover, our *in silico* analysis predicted that the stu-miR393- and phasiTIR-network is targeting downstream auxin-modulated transcription factor StARF1 and other phytohormone signaling pathways, such as transcripts involved in ethylene signaling, in jasmonate signaling and in brassinosteroid biosynthesis (Figure 26, Appendix, Supplementary Dataset 6 and 7). Two of these alternative phasiTIR targets, *StAP2* and *StOPR1*, were also confirmed by Degradome-Seq (Appendix, Supplementary Dataset 7).

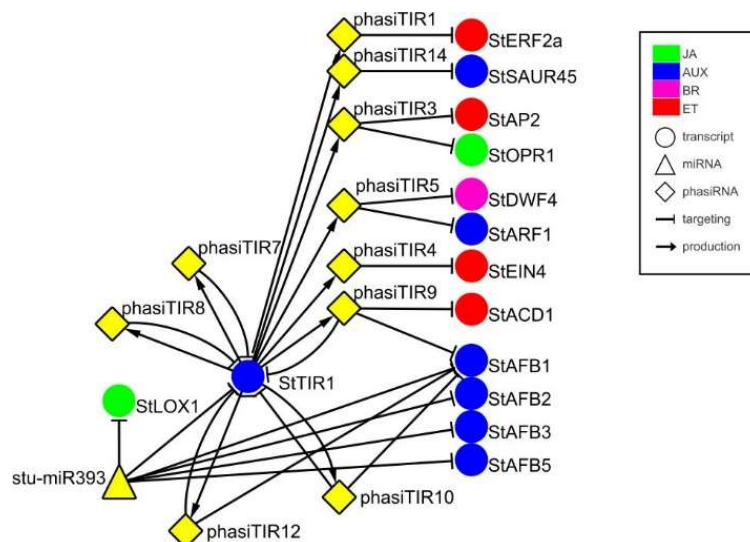


Figure 26: miR393-mediated cleavage of *StTIR1* leads to the production of phasiTIRs that are predicted to target diverse phytohormone signaling components. Targets of

phasiTIRs were predicted *in silico*, cleavage of two of them, *StAP2* and *StOPR1*, was also confirmed by degradome sequencing (PARE). Node shapes represent classes of sRNAs (triangle – miRNA; diamond – phasiRNA) or transcripts (circle). Node colors indicate components related to different hormone signaling pathways: green – jasmonic acid (JA); blue – auxin (AUX); magenta – brassinosteroid (BR); red – ethylene (ET). Arrows connect sRNAs and targets (blunt-end arrow) or *PHAS* loci and producing phasiRNAs (regular arrow). Node *stu-miR393* represents miR393-5p and miR393-5p.1 and node *StAFB1* represents *StAFB1.1*, *StAFB1.2*, and *StAFB1.3*. For details of the target transcripts/genes see Appendix, Supplementary Table 3. *StTIR1* – Transport inhibitor response 1, *StLOX1* – Lipoxygenase 1, *StERF2a* – Ethylene responsive transcription factor 2a, *StSAUR45* – Small auxin upregulated RNA 45, *StAP2* – APETALA2, *StOPR1* – 12-oxophytodienoate (OPDA) reductase, *StDWF4* – Dwarf4, *StARF1* – Auxin response factor 1, *StEIN4* – Ethylene insensitive 4, *StACD1* – 1-aminocyclopropane-1-carboxylic acid deaminase 1, *StAFB1/2/3/5* – Auxin F-box 1/2/3/5. Republished from Salicylic Acid Perturbs sRNA-Gibberellin Regulatory Network in Immune Response of Potato to Potato virus Y Infection, Križnik et al., 2017, *Frontiers in Plant Science*, doi: 10.3389/fpls.2017.02192, according to Creative Common Attribution ("CC BY") licence.

3.2.3 Several PVY-derived siRNAs trigger degradation of host transcripts

The primary plant defense mechanism against invading viruses is RNA silencing involving the production of vsiRNAs. The population of vsiRNAs detected in the infected samples cv. Désirée and NahG-Désirée consisted of more than 46 000 unique sequences of 20-24 nt in length (Appendix, Supplementary Dataset 8). In order to take into account the unlikely possibility that PVY^{NTN} produces its own miRNAs, we first ran the miRNA prediction pipeline on vsiRNAs and the viral genome. However, we found no sequence that would fulfil the criteria for a viral miRNA. Subsequently, we searched for potential host transcripts targeted by vsiRNAs in our experimentally validated target degradation dataset (Appendix, Supplementary Dataset 9). We found that vsiRNAs are indeed able to target multiple potato transcripts, among them mRNAs coding for immune receptor proteins, various transcription factors and proteins involved in hormonal signaling pathways (Appendix, Supplementary Dataset 9). For example, several vsiRNAs were detected with the confirmed ability to guide the cleavage of transcripts encoding Heat shock proteins and RLKs implicated in stress signaling responses, as well as GRAS transcription factors, ARFs and Auxin induced-like proteins involved in regulation of growth and developmental processes (Figure 27).

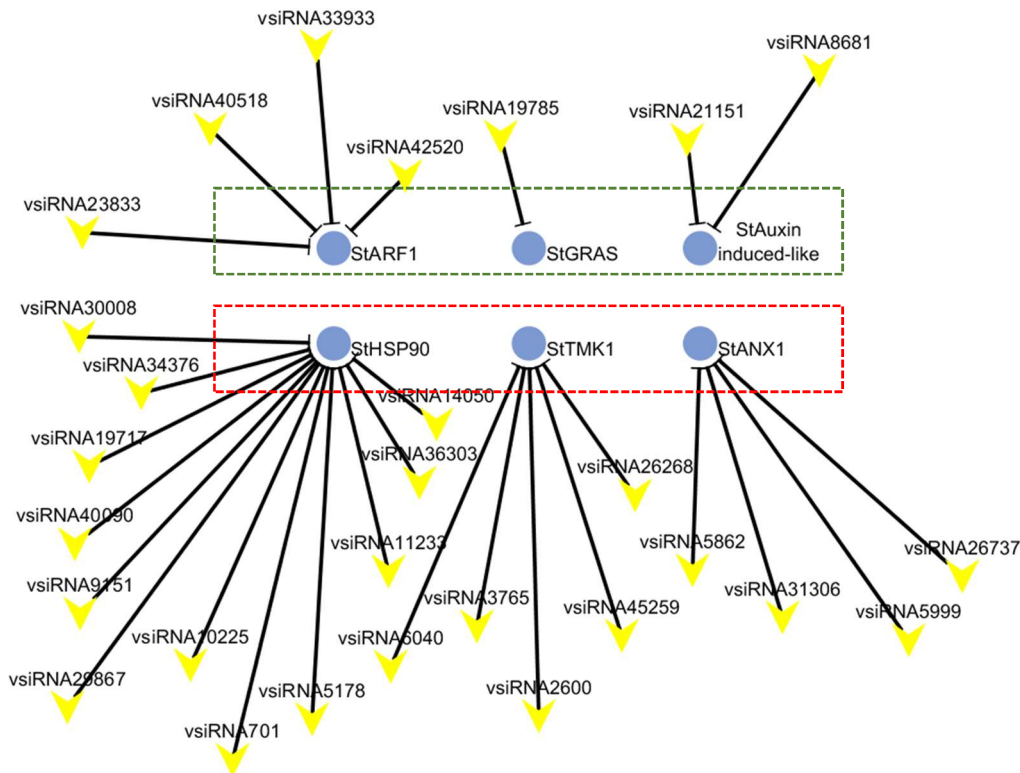


Figure 27: vsiRNAs guide the cleavage of host transcripts involved in immune, growth and developmental signaling. Network shows a set of vsiRNAs and their targets, identified by degradome sequencing. Only a subset of targets with reduced expression level following PVY^{NTN} infection in the tolerant plants of Désirée are shown (FDR-adjusted p-value < 0.05; Stare et al. 2015). Node shapes represent different components: arrowhead – vsiRNA, circle – target transcripts. Green rectangle indicates growth and development related transcripts, red rectangle indicates targets involved in immune signaling. StHSP90 – Heat shock protein 90, StTMK1 – Leucine rich receptor like kinase TMK1, StANX1 – Leucine rich receptor like kinase ANX1, StARF1 – Auxin response factor 1, StGRAS – transcription factor GRAS, StAuxin induced-like – Auxin induced-like protein F25G13.70. For details of the target transcripts/genes see Appendix, Supplementary Table 3. Republished from Salicylic Acid Perturbs sRNA-Gibberellin Regulatory Network in Immune Response of Potato to Potato virus Y Infection, Křižník et al., 2017, Frontiers in Plant Science, doi: 10.3389/fpls.2017.02192, according to Creative Common Attribution ("CC BY") licence.

3.2.4 sRNAs involved in tolerant response of potato cv. Désirée to PVY

3.2.4.1 PVY infection caused an increase in the levels of sRNAs in cv. Désirée

To identify miRNAs and phasiRNAs involved in modulation of tolerant response against PVY^{NTN}, the expression profiles of miRNAs and phasiRNAs were compared between PVY^{NTN}-infected versus mock-inoculated leaves of tolerant cv. Désirée in early stages of

virus infection (3 dpi; (Baebler et al., 2011; Stare et al., 2015)). In total, 61 unique miRNAs were found to be significantly differentially expressed, and virus infection predominantly caused an increase in miRNAs levels (Appendix, Supplementary Dataset 1). Additionally, we identified 36 phasiRNAs as differentially regulated 3 dpi, mainly originating from non-coding *PHAS* and *NBS-LRR* loci (Appendix, Supplementary Dataset 4). To validate the obtained sRNA-Seq results, abundance of six differentially expressed miRNAs was analyzed by our optimized stem-loop RT-qPCR protocol. As shown in Appendix, Supplementary Figure 6, all sRNA-Seq differential expression results were confirmed, except in cases where concentrations of miRNAs were below the limit of the quantification.

High number of differentially expressed miRNAs and phasiRNAs were detected to regulate transcript coding for transcription factors, immune receptors and proteins involved in hormonal signaling or secondary metabolism (Figure 28A, Appendix, Supplementary Dataset 6 and 7). The regulation mediated by certain sRNA can be greatly expanded to many processes as the same sRNA often target more than one target transcripts. However, when looking at the level of gene expression of target transcripts measured at 3 dpi (data published in (Stare et al., 2015) we observed that changes in their expression following PVY infection were less pronounced compared to changes of sRNAs (Figure 28B, Appendix, Supplementary Dataset 6 and 7). This could be attributed either to the sRNA's fine-tuned regulation on the level of translational repression of target mRNAs and/or to the limitation of microarray measurements. For example, in a case when cleavage of transcripts occurs outside the range of the microarray probe, the decrease in mRNA level using microarray approach cannot be detected.

A sRNAs

B target mRNAs

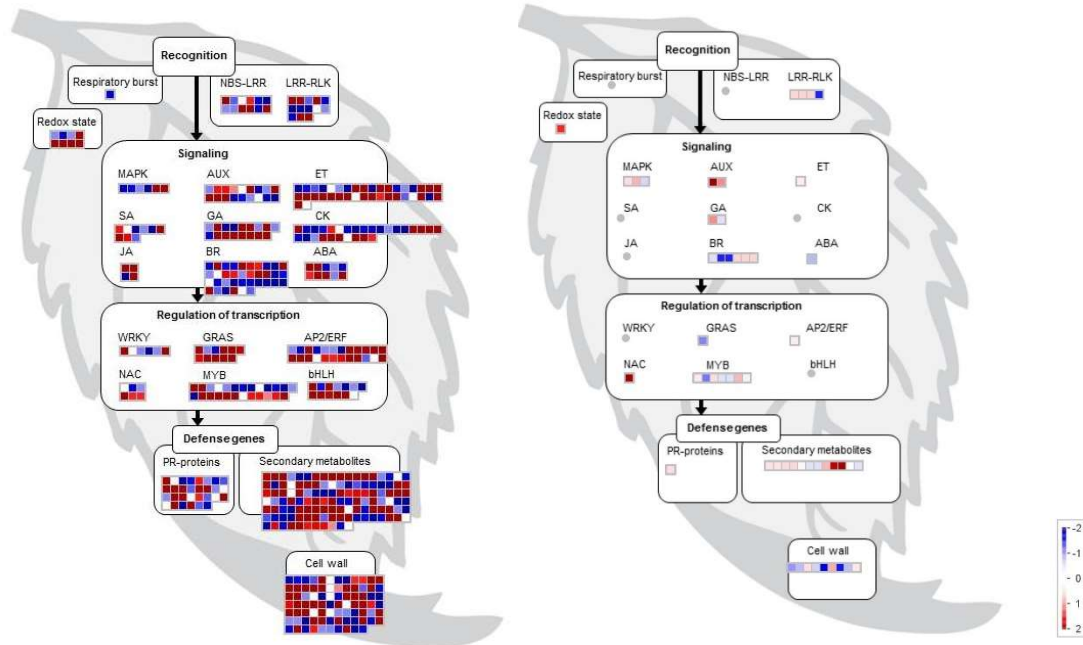


Figure 28: sRNAs are targeting immune and hormonal signaling components in *Désirée* at the onset of PVY^{NTN} multiplication. A) Visualization of differentially expressed miRNAs/phasiRNAs in PVY^{NTN}-infected *Désirée* at 3 dpi according to the function of their predicted mRNA targets and B) Visualization of the expression changes on the level of target mRNAs. Each square represents log₂ ratios of either (A) sRNA or (B) mRNA expression between PVY^{NTN}- and mock-inoculated plants (red – upregulated; blue – downregulated). MapMan ontology bins: respiratory burst (20.1.1), redox state (21.6), MAPK (30.6), SA (17.8), JA (17.7), AUX (17.2), GA (17.6), BR (17.3), ET (17.5), CK (17.4), ABA (17.1), WRKY (27.3.32), NAC (27.3.27), GRAS (27.3.21), MYB (27.3.25), AP2/ERF (27.3.3), bHLH (27.3.6), PR-proteins (20.1.7), secondary metabolites (16), cell wall (10). The NBS-LRR and LRR-RLK bins were custom constructed for this study, based on their harboring domains (obtained from PFAM database; (Finn et al., 2016)). These bins represent differentially expressed miRNAs/phasiRNAs targeting *NBS-LRRs* or *LRR-RLKs*. NBS-LRR – nucleotide binding site-leucine-rich repeat protein, LRR-RLK – leucine-rich repeat receptor-like kinase, MAPK – mitogen activated protein kinase, SA – salicylic acid, JA – jasmonic acid, AUX – auxin, GA – gibberellin, BR – brassinosteroid, ET – ethylene, CK – cytokinin, ABA – abscisic acid, PR – pathogenesis-related. Republished from Salicylic Acid Perturbs sRNA-Gibberellin Regulatory Network in Immune Response of Potato to Potato virus Y Infection, Křižník et al., 2017, *Frontiers in Plant Science*, doi: 10.3389/fpls.2017.02192, according to Creative Common Attribution ("CC BY") licence.

3.2.4.2 sRNA regulatory network response in tolerant interaction resembles the one in mutualistic symbiosis

Further analysis of the differentially expressed miRNAs and phasiRNAs together with the levels of the target transcripts revealed the presence of many known, as well as novel regulatory cascades involving NBS-LRRs. Several *NBS-LRRs* were predicted to be targeted by miR482, miR6024 and miR6027 family members. Moreover, *NBS-LRRs* are regulated also by phasiRNAs, where most phasiRNAs have multiple *NBS-LRR* targets (Figure 29, Appendix, Supplementary Figure 7, Dataset 6 and 7) due to the shared conserved P-loop or Walker A motif (Shivaprasad et al., 2012). In all the previous studies, miR482 family members were downregulated following pathogen infection (Ouyang et al., 2014; Shivaprasad et al., 2012; Yang et al., 2015a). In our study, however, the one regulated member of the miR482 family (stu-miR482e) targeting *NBS-LRR* transcripts was upregulated following the PVY^{NTN} infection (Appendix, Supplementary Dataset 1), similarly as observed in the establishment of mutualistic symbiosis in soybean roots (Li et al., 2010). Moreover, several miRNAs that were upregulated in response to PVY^{NTN} in cv. Désirée, such as miR160, miR164, miR167, miR169, miR171, miR319, miR390 and miR393 have also been reported to regulate nodulation and arbuscular mycorrhizal symbiosis in different legume species (Appendix, Supplementary Dataset 1; (Devers et al., 2011; Lelandais-Brière et al., 2009; Mao et al., 2013; Nizampatnam et al., 2015; Yan et al., 2015)). In addition to NBS-LRR proteins, LRR-RLKs are also important mediators of immune as well as important triggers of mutualistic symbiosis signaling cascades (Antolin-Llovera et al., 2014; Hohmann et al., 2017). We have predicted a novel miRNA-LRR-RLKs interaction in which miR6022 levels decrease in response to PVY^{NTN} infection in cv. Désirée, which is further linked to upregulation of its predicted target genes encoding LRR-RLKs (Figure 29, Appendix, Supplementary Dataset 6).

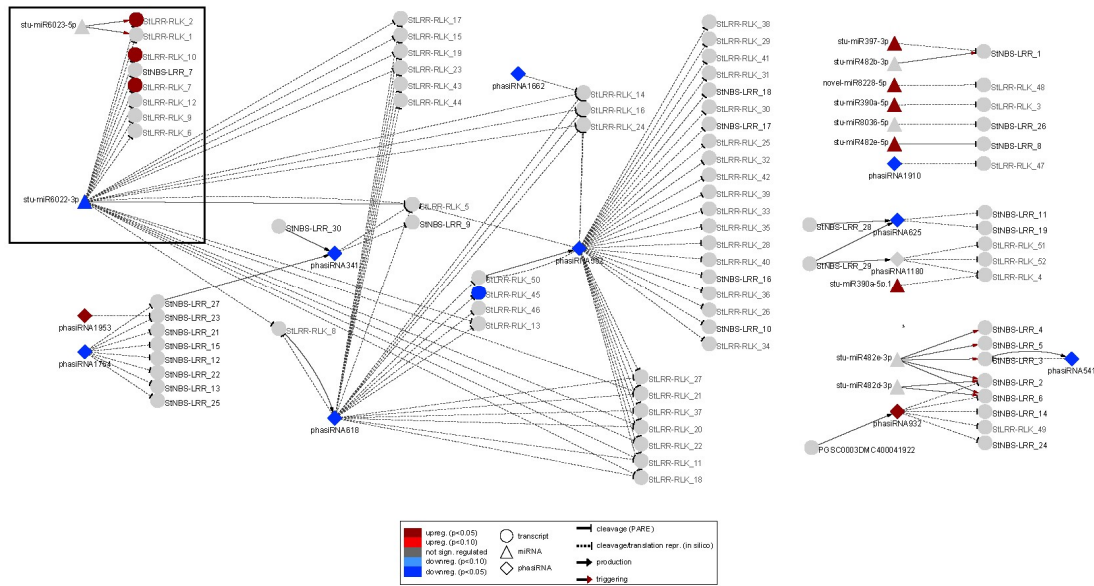


Figure 29: Regulatory network of sRNAs and *R* genes in cv. Désirée. *NBS-LRRs* and *LRR-RLKs* targets of differentially expressed sRNAs in Désirée plants at the onset of viral replication. Node shapes represent different components: triangle – miRNA;

diamond – phasiRNA; circle – transcript and rectangle – metabolite. Node colors indicate expression changes: red – upregulated; blue – downregulated; and grey – no significant difference in expression. Blocked solid arrow – cleavage observed by Degradome-Seq, blocked dashed-line arrow – *in silico* predicted cleavage, dashed-line arrow with circle – *in silico* predicted translational repression. Black solid line with red arrow connects miRNA trigger and their *PHAS* loci. Black solid line with black arrow connects *PHAS* locus and its producing phasiRNAs. Black rectangle indicates miR6022-LRR-RLKs interaction. For more details of interactions between sRNA-target see Appendix, Supplementary Dataset 6 and 7.

3.2.4.3 sRNA-mediated downregulation of gibberellin biosynthesis genes is reflected in lower level of gibberellin (GA3)

Interestingly, we found that GA biosynthesis and downstream signaling are targeted by a sRNA-mediated regulatory network and that the changes in sRNA levels following PVY^{NTN} infection are reflected also in the changes of their target transcripts levels (Figure 30A, Table 5, Appendix, Supplementary Dataset 6). GA20-oxidase (GA20ox) and GA3-oxidase (GA3ox) are enzymes that catalyze the last steps in the formation of bioactive GAs (Yamaguchi, 2008). In Désirée plants miR167e was predicted to cause translational repression of the *StGA20ox* transcript (Figure 30A, Appendix, Supplementary Dataset 6). An additional layer of GA biosynthesis regulation is represented by the increased production of phasiRNA931, which promotes cleavage of the *StGA3ox* transcript (Figure 30A, Table 5, Appendix, Supplementary Dataset 6). The transcriptomics results support these interactions as the targeted transcripts are significantly downregulated in Désirée upon PVY^{NTN} infection (Figure 30A, Table 5, Appendix, Supplementary Dataset 6). Additionally, vsRNAs were found to target transcripts encoding two enzymes involved in GA biosynthesis StGA1 and StGA20ox (Figure 30A, Appendix, Supplementary Dataset 9). One also has to note that all of the miRNAs/phasiRNAs discovered to be involved in sRNA-GA biosynthesis regulation have so far not been identified in Arabidopsis and among them only miR167e was also discovered in tomato (Griffiths-Jones et al., 2006; Zhang et al., 2014). Moreover, downstream GA signaling was also found to be targeted by sRNAs in potato – PVY interaction on multiple levels. Four miR319 family members, close relatives of the Arabidopsis miR159 family, were predicted to cleave the transcript encoding StMYB33, an orthologue of GAMYB transcription factor involved in GA signal transduction (Millar and Gubler, 2005). Moreover, phasiRNA931 can cleave a potato orthologue of a DELLA protein, a GA-signaling repressor (Figure 30A, Appendix, Supplementary Dataset 6).

Such interconnectedness between plant defense-related miRNA/phasiRNA network and GA biosynthesis/signaling has not been identified previously and may represent a link between defensive and developmental signaling. Thus, we decided to functionally evaluate these results by measuring the concentrations of a set of plant hormones. Hormone concentration measurements were performed using gas chromatography coupled with mass spectrometry method by the group of Prof. Dr. Stephan Pollmann at the Centre for Plant Biotechnology and Genomics UPM – INIA (Křižník et al., 2017).

As predicted by the sRNA-target transcript analyses, we detected a reduced level of GA3 in PVY^{NTN} infected Désirée plants (Figure 30B). The levels of SA, jasmonic acid (JA), the JA precursor 12-oxophytodienoic acid (OPDA), abscisic acid (ABA) and indole-3-acetic

acid (IAA) remained unchanged at 3 dpi (Figure 30B, Appendix, Supplementary Dataset 10). To inspect if GA deficiency has any impact on plant growth, plant height was monitored till 21 dpi. However, no differences were observed between all studied groups of plants.

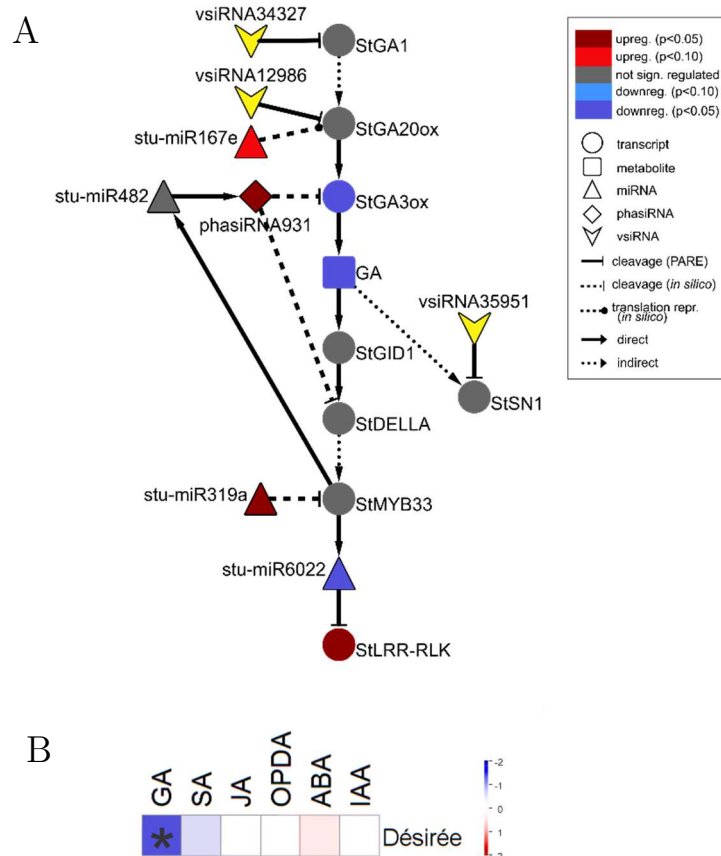


Figure 30: sRNAs are targeting gibberellin (GA) signaling components in Désirée at the onset of PVY^{NTN} multiplication. (A) Network of differentially expressed endogenous sRNAs and vsiRNAs targeting mRNAs of GA biosynthesis and signaling pathways in Désirée 3 days post PVY^{NTN} inoculation. Node shapes represent classes of sRNAs (triangle – miRNA; diamond – phasiRNA; arrowhead – vsiRNA), transcripts (circle) or metabolites (rectangle). Statistical significances of expression differences (FDR-adjusted p-values) and direction of expression change are represented by the node colors (see the legend). Arrows indicate the type of interaction (solid-line normal arrow – direct interaction; dashed-line normal arrow – indirect interaction; blunt-end solid arrow – cleavage observed by Degradome-Seq, blunt-end dashed-line arrow – *in silico* predicted cleavage (or translational repression as proposed by Rogers and Chen (2013), dashed-line oval arrow – *in silico* predicted translational repression). Node stu-miR319a represents stu-miR319a-3p and stu-miR319a-3p.2. Node StGA20ox represents StGA20ox, StGA20ox1, StGA20ox3 and StGA20ox4; stu-miR167e – stu-miR167e-3p; stu-miR482 – miR482f-3p, miR482c-3p; stu-miR6022 – miR6022-3p; StGA1 – GA

REQUIRING 1 (ent-copalyl diphosphate synthase); StGA20ox – GA20-oxidase; StGA3ox – GA3-oxidase; StGID1 – GA receptor GA INSENSITIVE DWARF1C hydrolase; StSN1 – Snakin-1; StDELLA – DELLA protein; StLRR-RLK – leucine-rich repeat receptor-like protein kinase. B) Changes in concentrations of a set of plants hormones in cv. Désirée 3 days after PVY^{NTN} infection. sRNA-mediated repression of GA biosynthesis was confirmed by reduced GA3 levels in PVY-infected Désirée plants. Colors present as log₂ ratios of mean concentrations between PVY^{NTN}- and mock-inoculated plants (n=4; red – increased, blue – decreased level). * indicates statistically significant values (ANOVA; FDR-adjusted p-value < 0.05). SA – salicylic acid, JA – jasmonic acid, OPDA – 12-oxophytodienoic acid, ABA – abscisic acid, IAA – indole-3-acetic acid. Republished from Salicylic Acid Perturbs sRNA-Gibberellin Regulatory Network in Immune Response of Potato to Potato virus Y Infection, Křižník et al., 2017, Frontiers in Plant Science, doi: 10.3389/fpls.2017.02192, according to Creative Common Attribution ("CC BY") licence.

Table 5: Endogenous sRNAs and vsiRNAs targeting mRNAs of GA biosynthesis and signaling pathways in cv. Désirée 3 days post PVY^{NTN} inoculation.

sRNA	log ₂ FC	target	log ₂ FC
stu-miR167e	0.7 [†]	<i>StGA20ox</i>	-0.1
stu-miR319a	2.1**	<i>StMYB33</i>	0.1
stu-miR319a.2	2.1**	<i>StMYB33</i>	0.1
stu-miR482c	0.4	<i>PHAS</i> [#]	NA
stu-miR482f	0.0	<i>PHAS</i> [#]	NA
stu-miR6022	-1.3**	<i>StLRR-RLK</i>	0.7*
phasiRNA931	4.2**	<i>StGA3ox</i>	-0.5*
		<i>StDELLA</i>	-0.5
vsiRNA12986	NA	<i>StGA20ox</i>	-0.1
vsiRNA34327	NA	<i>StGA1</i>	-0.2
vsiRNA35951	NA	<i>StSN1</i>	-0.1

For each sRNA and its target the expression levels are given together with statistical significances of expression differences (FDR-adjusted p-value). ** – p < 0.01; * – p < 0.05; [†] – p < 0.1; NA – not available. [#]*PHAS* – phasiRNA-producing locus *PGSC0003DMC400041922*. Republished from Salicylic Acid Perturbs sRNA-Gibberellin Regulatory Network in Immune Response of Potato to Potato virus Y Infection, Křižník et al., 2017, Frontiers in Plant Science, doi: 10.3389/fpls.2017.02192, according to Creative Common Attribution ("CC BY") licence.

3.2.4.4 *In silico* prediction showed interconnection between miRNA regulatory network and transcriptional regulation

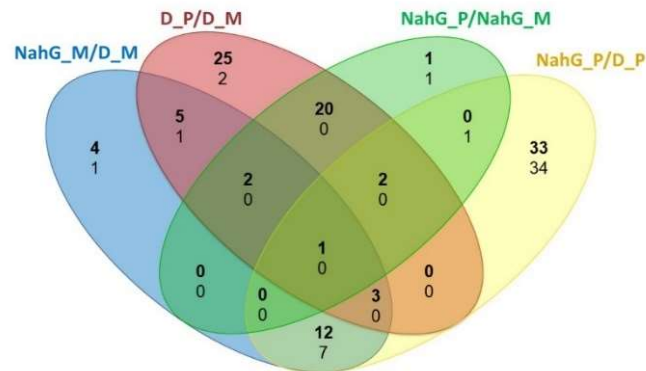
Given the critical role of miRNAs in gene regulation, cis-regulatory elements of differentially expressed *MIR* genes involved in *R*-gene regulation, GA and auxin signaling, *MIR6022*, *MIR319a* and *MIR167e*, *MIR160a* and *MIR390a* were investigated together with *MIR482f* and *MIR482c* genes encoding miRNA triggers of phasiRNA931 production.

Interestingly, GAMYB binding sites were detected in the promoters of the *MIR6022* and *MIR482f* genes (Appendix, Supplementary Dataset 11), while *MIR160a* contain P-box, gibberellin-responsive regulatory element. Moreover, *MIR6022*, *MIR482f*, *MIR160a* and *MIR390a* genes harbor WRKY8/28/48 binding sites, while *MIR319a* and *MIR482c* harbor a general WRKY binding W-box regulatory element. Additionally, cis-acting elements involved in SA and JA responsiveness were identified in the promoter of the *MIR482f* gene (Appendix, Supplementary Dataset 11). cis-acting element involved in SA responsiveness was also found in *MIR160a* promoter. The promoter of *MIR167e* is only partially assembled in the current version of the potato genome; thus, the promoter analysis was performed only for the first 80 nt upstream of the predicted hairpin precursor sequence. However, no defense-related regulatory elements were detected in promoter region of *MIR167e* (Appendix, Supplementary Dataset 11).

3.2.4.5 SA depletion attenuates sRNA response following PVY^{NTN} infection

As the link between repression of GA signaling and disease symptoms severity was already established in Arabidopsis (Du et al., 2014) we have further investigated the activity of discovered sRNA-GA circuit in susceptible potato-PVY^{NTN} interaction. We have shown previously that SA depletion breaks the equilibrium between disease and tolerance. In NahG-Désirée plants, the pronounced disease symptoms appeared both on the inoculated and systemic leaves (Baebler et al., 2011). Furthermore, the viral multiplication was detected one day earlier than in non-transgenic Désirée plants (at 4 dpi), while the final concentrations of the virus were not significantly higher (Baebler et al., 2011). Here, we performed the analysis of sRNA response as well as the measurements of hormonal concentrations in the interaction of this susceptible genotype with the virus. Interestingly, we found that the overall response of sRNAs was attenuated in NahG-Désirée at 3 dpi. In NahG-Désirée only 28 miRNAs were differentially expressed, with the majority showing a lower degree of induction than in Désirée plants (Figure 31 and 32A, Appendix, Supplementary Dataset 1). Attenuated response on sRNA level was also reflected on the level of the target transcripts, showing no remarkable change in regulation (Figure 32B, Appendix, Supplementary Dataset 6 and 7).

A miRNAs



B phasiRNAs

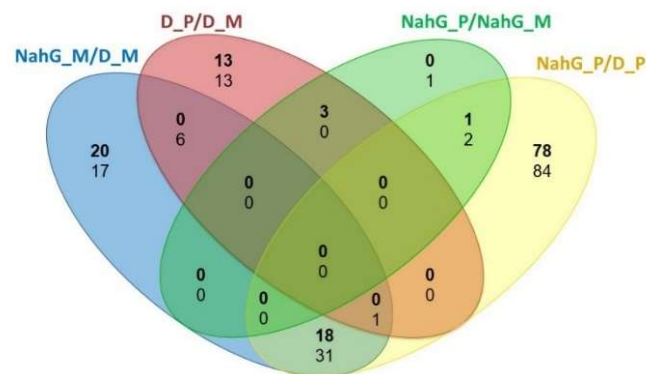


Figure 31: Numbers of unique and common differentially expressed miRNAs and phasiRNAs 3 days post PVY^{NTN} inoculation in comparison to SA-deficient and non-transgenic Désirée plants. Venn diagrams show the number of differentially expressed (FDR-adjusted p-value < 0.05) (A) miRNAs and (B) phasiRNAs in mock- or PVY^{NTN}-inoculated potato leaves of cv. Désirée and NahG-Désirée. Upregulated miRNAs/phasiRNAs are shown in bold and downregulated in normal text. D – Désirée, NahG – NahG-Désirée, M – mock, P – PVY^{NTN}. Republished from Salicylic Acid Perturbs sRNA-Gibberellin Regulatory Network in Immune Response of Potato to Potato virus Y Infection, Křižník et al., 2017, Frontiers in Plant Science, doi: 10.3389/fpls.2017.02192, according to Creative Common Attribution ("CC BY") licence.

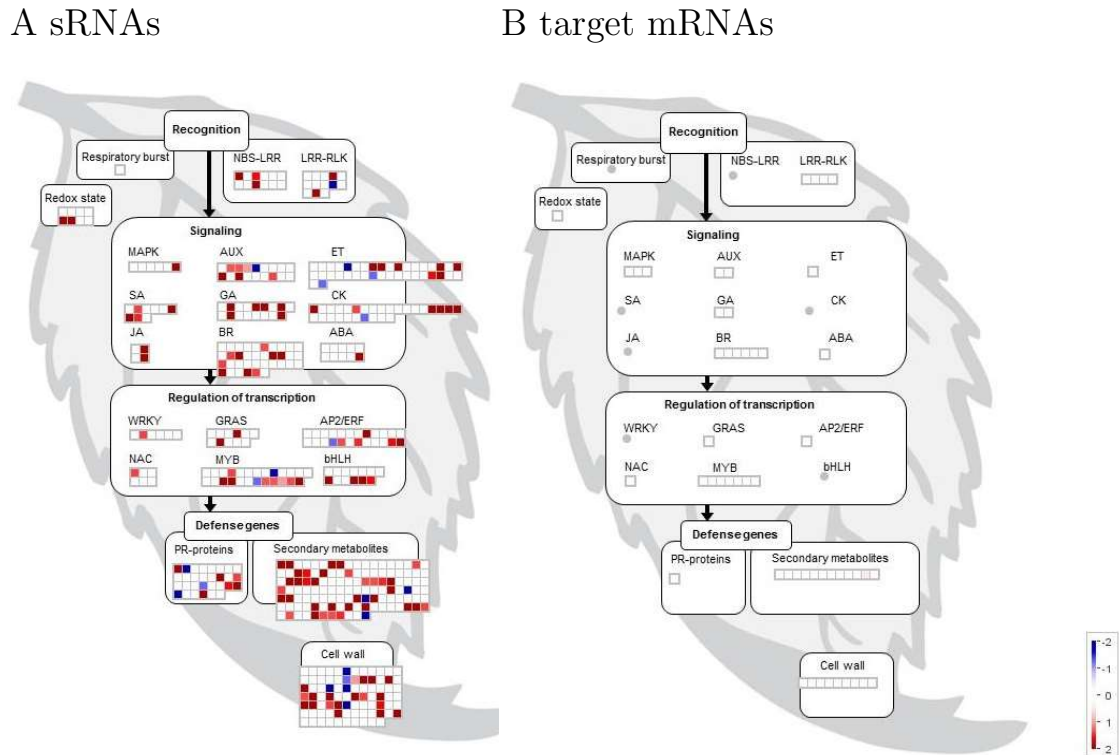


Figure 32: sRNA response is attenuated in susceptible SA depleted plants following PVY^{NTN} infection. (A) Visualization of differentially expressed miRNAs/phasiRNAs in PVY^{NTN}-infected NahG-Désirée according to the function of their mRNA targets. (B) Visualization of the expression changes on the level of target mRNAs. Each square represents log₂ ratios of either sRNA (A) or mRNA (B) expression between PVY^{NTN}- and mock-inoculated plants (red – upregulated; blue – downregulated). For abbreviations and other details of the scheme, see the caption of Figure 28. Republished from Salicylic Acid Perturbs sRNA-Gibberellin Regulatory Network in Immune Response of Potato to Potato virus Y Infection, Křižník et al., 2017, *Frontiers in Plant Science*, doi: 10.3389/fpls.2017.02192, according to Creative Common Attribution ("CC BY") licence.

Inspecting specifically the discovered link between sRNAs regulation, GA signaling and immune signaling in the sRNA and transcriptional datasets we observed that the responses of miR167e and miR319a were diminished in NahG-Désirée plants (Figure 33A, Table 6, Appendix, Supplementary Dataset 1). Interestingly, although the phasiRNA931 was also upregulated in NahG-Désirée plants, albeit to a lower extent, this was not translated into downregulation at the target transcript level (Figure 33A, Table 6, Appendix, Supplementary Dataset 6). Adding to the significance of this finding, in NahG-Désirée plants, the level of bioactive GA was not significantly different in infected leaves (Figure 33B). Furthermore, the relieved silencing of *LRR-RLKs* by miR6022 that is predicted to modulate the immune response and which is also linked with GAMYBs was absent in NahG-Désirée plants (Figure 33A, Table 6, Appendix, Supplementary Figure 6). We also inspected sRNA-mediated responses which resembled responses in mutualistic symbioses in tolerant plants of Désirée and found that miR482e was also upregulated in NahG plants,

while regulation of miR160, miR164, miR167, miR169, miR171, miR319, miR390a and miR393 was diminished (Appendix, Supplementary Dataset 1).

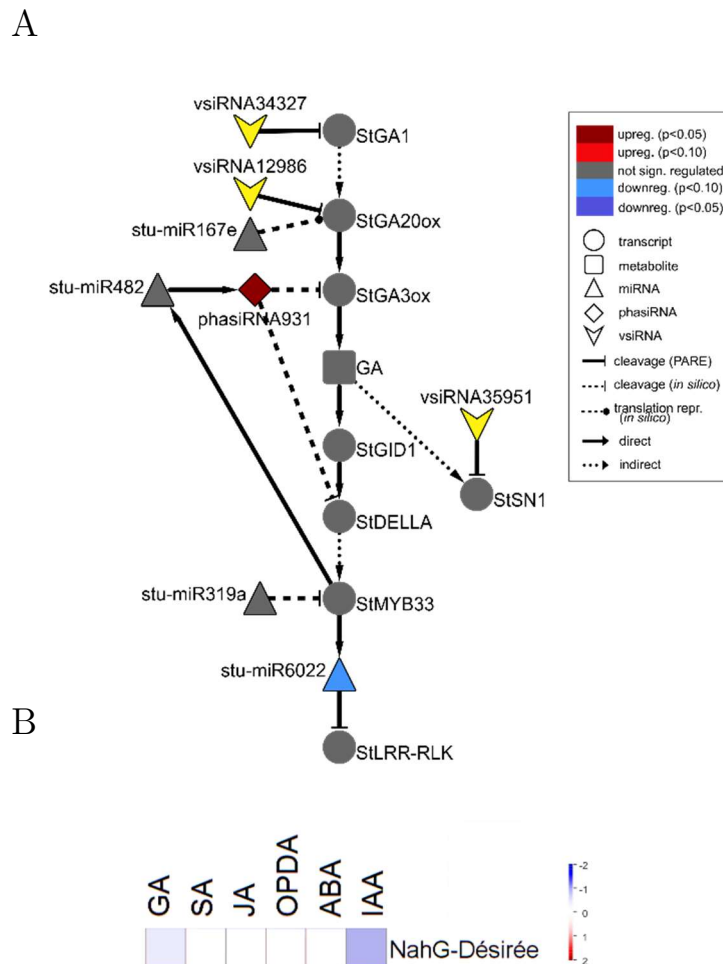


Figure 33: sRNAs targeting gibberellin signaling components are attenuated in NahG-Désirée at the onset of PVY^{NTN} multiplication. A) Network of endogenous sRNAs and vsiRNAs targeting mRNAs of GA biosynthesis and signaling pathways in NahG-Désirée 3 days post PVY^{NTN} inoculation. B) Concentrations of a set of plant hormones in NahG-Désirée 3 days after PVY^{NTN} infection. The levels of all analyzed hormones remained unchanged in NahG plants following the PVY^{NTN} infection. Colors present as log₂ ratios of mean concentrations between PVY^{NTN}- and mock-inoculated plants (n=4; red – increased, blue – decreased level). * indicates statistically significant values (ANOVA; FDR-adjusted p-value < 0.05). For abbreviations and other details of the scheme, see the caption of Figure 30. Republished from Salicylic Acid Perturbs sRNA-Gibberellin Regulatory Network in Immune Response of Potato to Potato virus Y Infection, Křižník et al., 2017, *Frontiers in Plant Science*, doi: 10.3389/fpls.2017.02192, according to Creative Common Attribution ("CC BY") licence.

Table 6: Endogenous sRNAs and vsiRNAs targeting mRNAs of GA biosynthesis and signaling pathways in NahG-Désirée 3 days post PVY^{NTN} inoculation.

sRNA	log ₂ FC	target	log ₂ FC
stu-miR167e	-0.4	<i>StGA20ox</i>	-0.3
stu-miR319a	0.4	<i>StMYB33</i>	-0.9
stu-miR319a.2	0.3	<i>StMYB33</i>	-0.9
stu-miR482c	-0.4	<i>PHAS#</i>	NA
stu-miR482f	-0.2	<i>PHAS#</i>	NA
stu-miR6022	-0.9 [†]	<i>StLRR-RLK</i>	-0.2
phasiRNA931	3.2*	<i>StGA3ox</i>	-0.1
		<i>StDELLA</i>	-0.1
vsiRNA12986	NA	<i>StGA20ox</i>	-0.3
vsiRNA34327	NA	<i>StGA1</i>	-0.1
vsiNA35951	NA	<i>StSN1</i>	-0.1

For each sRNA and its target the expression levels are given together with statistical significances of expression differences (FDR-adjusted p-value). ** – $p < 0.01$; * – $p < 0.05$; [†] – $p < 0.1$; NA – not available. #*PHAS* – phasiRNA-producing locus *PGSC0003DMC400041922*. Republished from Salicylic Acid Perturbs sRNA-Gibberellin Regulatory Network in Immune Response of Potato to Potato virus Y Infection, Křižník et al., 2017, Frontiers in Plant Science, doi: 10.3389/fpls.2017.02192, according to Creative Common Attribution ("CC BY") licence.

3.2.4.6 *In silico* prediction of cis-regulatory elements of *MIR* genes reveals interconnection between miRNA regulatory network and transcriptional regulation

To evaluate the direct effect of the SA deficiency in NahG plants, independently of the viral infection, we also compared the sRNA expression profiles in mock-inoculated leaves of non-transgenic and SA-depleted Désirée (Figure 31, Appendix, Supplementary Dataset 1). We found 36 miRNAs regulated by SA. It seems that SA in the normal growing conditions generally cause the reduction in the levels of miRNAs as the majority (27) of miRNAs were detected at significantly higher levels in NahG-Désirée plants (Appendix, Supplementary Dataset 1). When we similarly compared the expression profiles of the transcripts in NahG-Désirée to those in Désirée, we noticed that the most strongly induced by SA signaling are notably different WRKY transcription factors (Table 7, Appendix Supplementary Dataset 12 and Dataset 13), among them orthologues of Arabidopsis WRKY70, which was already shown to be positively regulated by SA (Li et al., 2004).

As *MIR160a*, *MIR319a*, *MIR390a*, *MIR482c*, *MIR482f* promoters harbor WRKY transcription factors binding sites (Appendix, Supplementary Dataset 11), we discovered a potential direct link between SA signaling and miRNA regulatory network in potato. This link was experimentally confirmed by differential response of miRNAs (originating from these loci), to PVY infection in SA-depleted versus non-transgenic genotype. The miR160a, miR319a, miR482c levels were found to be positively regulated by SA (Appendix, Supplementary Dataset 1). miR160a and miR319a were induced following PVY infection in Désirée, while their level in NahG-Désirée was not altered, supporting the hypothesis that they require WRKY transcription factor for this response. On the other hand, the

levels of miR390a and miR482f were negatively regulated by SA, indicating more complex regulation indicated also by prediction of other response elements in their promoter regions.

Table 7: SA-dependent transcriptional responses of potato leaves in cv. Désirée.

Functional group (BIN ID)	No. of genes in BIN	NahG-Désirée vs. Désirée
WRKY transcription factor family (27.3.32)	215	-
Biotic stress related regulation of transcription (20.1.5)	140	-
Pathogenesis-related proteins PR-4,8,11 (chitinases and chitin binding proteins) (20.1.7.3)	46	-
Photosynthesis LHC-II (1.1.1.1)	87	-
Glycolysis PEPC (4.3.15)	28	-
Degradation of histidine (13.2.7)	25	-

Gene set enrichment of differences in expression profile was performed comparing mock-inoculated samples of both genotypes and comparing PVY-infected samples of both genotypes using MapMan ontology. Only the gene groups (BINs) significantly enriched (FDR-adjusted p-value < 0.01) in both comparisons (regulated only by SA and not by the virus) are listed. The full list of enriched BINs is given in Appendix, Supplementary Dataset S13. “-“ downregulation of genes assigned to particular BIN was observed in SA deficient plants. No. of genes in BIN – number of all genes assigned to the BIN. Republished from Salicylic Acid Perturbs sRNA-Gibberellin Regulatory Network in Immune Response of Potato to Potato virus Y Infection, Křižník et al., 2017, *Frontiers in Plant Science*, doi: 10.3389/fpls.2017.02192, according to Creative Common Attribution ("CC BY") licence.

3.2.5 sRNAs involved in tolerant response of potato cv. Pentland-Squire to PVY

3.2.5.1 PVY^{NTN} multiplication in Pentland-Squire was detected one day later than in Désirée

In order to examine whether the sRNA-mediated regulation observed in tolerant plants of cv. Désirée could be regarded as a common tolerant response to PVY infection, we decided to study response at sRNA level using additional tolerant potato cultivar Pentland-Squire. As for Désirée, we were interested in response in early stages of infection at the onset of viral multiplication. Since PVY multiplication dynamic can differ among different potato cultivars, we first examined PVY accumulation in Pentland-Squire over time from 3 dpi to 6 dpi. Using this approach we aimed at identifying physiologically similar time-point as used in Désirée-PVY study, corresponding to two days prior to the first detection of increased viral multiplication. In the PVY-inoculated leaves of Pentland-Squire, PVY RNA was already detected at 3 dpi and 4 dpi (Figure 34). However, this was most probably due to remnants of PVY in residual inoculum that was not efficiently washed from the leaf surface. The drop of relative PVY RNA concentration from 4 dpi to 5 dpi (Figure 34) supports this, as the PVY RNA on the leaf surface gets degraded (Baebler et al., 2011; Pompe-Novak et al., 2018). From 5 dpi to 6 dpi the detected amount of PVY RNA clearly increased, indicating active multiplication of PVY inside the infected cells takes place. In contrast to Désirée in which the PVY multiplication increased from 4 dpi to 5 dpi, in PVY-

infected Pentland-Squire, a one day delay of the onset of multiplication was observed. PVY and mock-inoculated Pentland plants were also monitored for plant height and disease symptoms until 6 dpi. No difference in plant height was observed between virus-infected and healthy plants and expectedly, PVY^{NTN}-infected plants did not develop any disease symptoms.

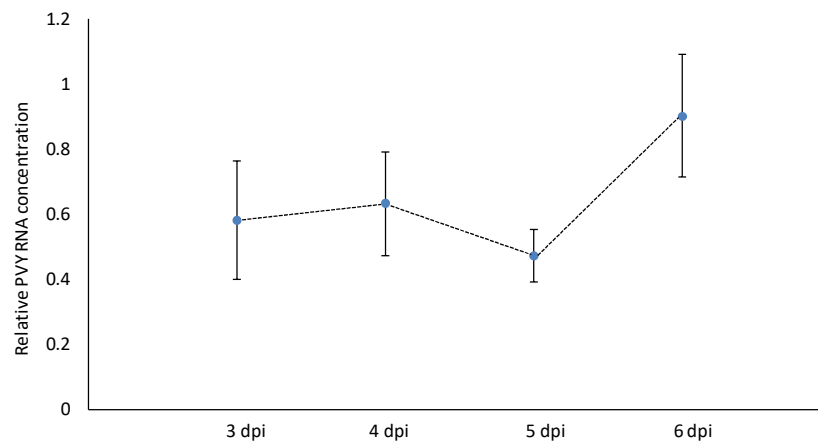


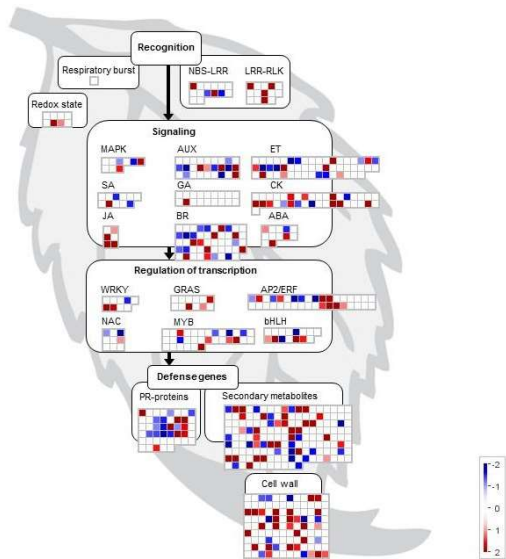
Figure 34: The relative amount of PVY RNA measured by RT-qPCR in the inoculated leaves of cv. Pentland-Squire from 3 dpi to 6 dpi. Error bars represent the standard error ($n = 3$).

3.2.5.2 Modulation of miRNA and phasiRNA levels by PVY infection in tolerant Pentland-Squire plants

Based on PVY^{NTN} multiplication results we chose to sequence six sRNA libraries from three mock-inoculated (control) and three PVY^{NTN}-infected samples collected at 4 dpi.

In total, 57 different sRNAs were found to be significantly differentially expressed (DE) following PVY infection in cv. Pentland-Squire. Similarly as for Désirée, infection by PVY caused mainly increases in levels of most miRNAs and phasiRNAs, as abundance of 45 sRNAs was increased and level of only 12 sRNA decreased (Appendix, Supplementary Dataset 1 and 4). The number of differentially expressed miRNAs and phasiRNAs showed that Pentland's response to PVY infection resembles more the response observed in Désirée than in NahG-Désirée (Figure 28A, Figure 32A and Figure 35A). However when inspecting the individual differentially expressed miRNAs and phasiRNAs in both virus-infected tolerant potato genotypes we observed a relatively low number of commonly responding sRNAs (Appendix, Supplementary Dataset 1). Six miRNAs and one phasiRNAs were found differentially expressed in both Désirée and Pentland-Squire plants to PVY infection, whereas their expression was not altered in NahG-Désirée plants (Figure 35B, Appendix, Supplementary Dataset 1).

A



B

sRNA	Target description	Process description (MapMan BIN)	log ₂ FC	
			Désirée	Pentland
stu-miR160a-3p	Cathepsin B*	29.5.3 protein.degradation.cysteine protease	1.9	3.1
stu-miR390a-5p	Ubiquitin ligase protein COP1*	29.5.11.4.6 protein.degradation.ubiquitin.E3.DCX 30.11 signalling.light 33.99 development.unsigned	2.1	2.0
stu-miR399a-o-5p.1	F-box family protein*	protein.degradation.ubiquitin.E3.SCF.FBOX	3.1	6.0
stu-miR167c-3p	Purple acid phosphatase	26.13 misc.acid and other phosphatases	2.0	1.8
stu-miR530-5p.1	Zinc knuckle (CCHC-type) family protein	27.3.12 RNA.regulation of transcription.C3H zinc finger family	3.7	1.1
stu-miR530-5p.1	AP2 transcription factor	27.3.3 RNA.regulation of transcription.AP2/ERF, APETALA2/ethylene-responsive element binding protein family	3.7	1.1
phasIRNA850	NAD(P)H-quinone oxidoreductase*	1.1.2.2 PS.II light reaction.photosystem I.PSI polypeptide subunits 1.1.6 PS.II light reaction.NADH DH	2.8	5.4

Figure 35: sRNA response in Pentland-Squire following PVY^{NTN} infection at 4 dpi. A) Visualization of differentially expressed miRNAs/phasiRNAs in PVY^{NTN}-infected Pentland-Squire according to the function of their targets. Each square represents log₂ ratios of expression between PVY^{NTN}- and mock-inoculated plants (red – upregulated; blue – downregulated). B) six commonly differentially expressed miRNAs in both tolerant genotypes of potato following PVY^{NTN} inoculation. For each sRNA, its expression levels (log₂FC; FDR-adjusted p-value < 0.05) detected in both genotypes are given together with their target mRNA, full description and description of the process in which they are involved.* denotes targets identified by the degradome sequencing approach.

Two miRNAs, miR160 and miR390 associated with regulation of processes in the arbuscular mycorrhizal symbiosis (Hobecker et al., 2017), were found upregulated both in Désirée and Pentland-Squire after PVY infection (Figure 35B). In previous plant studies, both miR160 and miR390 were found to directly or indirectly target transcripts coding for Auxin Response Factors (ARFs). miR160 targets *ARF10*, *ARF16*, and *ARF17*, and miR390 directs cleavage of non-coding *TAS3* transcripts, leading to the production of *TAS3*-tasiRNAs that negatively regulate *ARF2*, *ARF3* and *ARF4* mRNAs (Allen et al., 2005). The miR390-TAS3-ARF module is predicted to be conserved in several plant species, including in potato, and plays an important role in plant growth and development, including leaf morphology, developmental timing and patterning, and lateral root growth (Xia et al., 2017).

Our Degradome-Seq results confirmed stu-miR160a-5p mediated cleavage of *StARF10* and additionally identified that stu-miR160a-3p, a complementary strand of stu-miR160a, mediates the cleavage of *CathepsinB*, involved in the induction of programmed cell death (Figure 36A, Appendix, Supplementary Dataset 7) (Birch, 2009). stu-miR160-5p targeting *StARF10* showed a significant increase in abundance in infected plants of Pentland-Squire and a slight increase in Désirée (Appendix, Supplementary Dataset 1), while the level of *StARF10* in inoculated leaves of Désirée was not altered (Appendix, Supplementary Dataset 1; Stare et al., 2015). Interestingly, when looking at *StARF10* expression in upper leaves of PVY-infected plants of Désirée (Stare et al., 2015; data unpublished), we observed significant downregulation of *StARF10* ($\log_2FC = -0.88$), while the level was not changed in NahG-Désirée genotype. Similarly, stu-miR160a-3p was found upregulated in both tolerant genotypes but the level of *CathepsinB* in inoculated and upper non-inoculated leaves remained unchanged (Figure 36A).

Recently, Xia et al. (2017) predicted miR390a-*TAS3-ARF* module in potato. In our study, using *in silico* phasiRNA prediction analysis we were unable to identify *TAS3* locus and *TAS3*-producing tasiRNAs. To address the possibility that potato *TAS3* and *TAS3*-producing tasiRNAs were not detected within our phasiRNA prediction analysis because they displayed poor phasing pattern, we manually checked and counted sequences matching to *TAS3*-producing tasiRNAs in our sRNA-seq samples. We observed that the numbers of sequences matching *TAS3*-producing tasiRNAs were either very low (< 10 raw counts/sample) or/and their abundance level was not significantly different between infected and healthy Désirée or Pentland-Squire plants. The transcriptomic data also showed that the mRNA levels of *ARF2*, *ARF3* and *ARF4* in Désirée were not altered following PVY^{NTN} infection (Stare et al., 2015). Thus, it seems that the miR390a-*TAS3-ARF* module is not actively involved in the regulation of potato tolerance to PVY^{NTN}.

Notably, however, we discovered an additional link between the miRNA regulation and GA signaling. stu-miR390a-5p, was found to cleave *Constitutive Photomorphogenic 1* (*StCOP1*) transcript, a negative regulator of light signaling (Figure 36B, Appendix, Supplementary Dataset 7). COP1 was shown to positively regulate expression of PIF4 (PHYTOCHROME INTERACTING FACTOR 4) (Gangappa and Kumar, 2018), a basic-helix-loop-helix (bHLH) transcription factor which promotes growth and development and negatively regulates immune responses (Gangappa and Kumar, 2018). Upregulation of stu-miR390a-5p was not reflected in the decreased level of *StCOP1* in our data. However, the level of *StPIF4* was significantly downregulated in PVY-inoculated leaves of Désirée (Stare et al., 2015). *PIF4* is also positively regulated by GA, thus reduced GA level observed in tolerant cv. Désirée can cause a reduction in PIF4 level. Notably, we revealed two different miRNA regulatory pathways, one regulated by stu-miR390a and the second by stu-miR167e/phasiRNA931, which are activated in the tolerant responses upon PVY infection and converge at the level of PIF4 (Figure 36B).

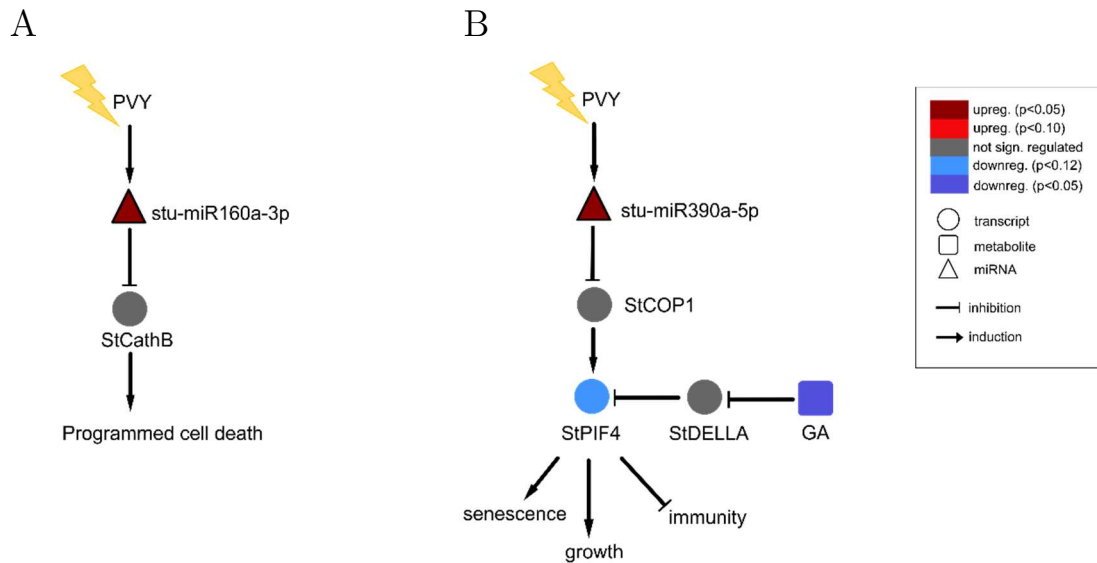


Figure 36: miR160a and miR390a are upregulated in cv. Désirée and cv. Pentland-Squire following PVY^{NTN} infection. Networks of two potato miRNAs, A) *stu-miR160a-3p* and B) *stu-miR390a-5p* targeting *cathepsin B* (*StCathB*) and *Constitutive Photomorphogenic 1* (*StCOP1*) transcripts. Both target cleavage regulations were experimentally validated using Degradome-Seq. Both *stu-miR390a*-mediated negative regulation of *StCOP1* and decrease in GA level observed in cv. Désirée may reduce PHYTOCHROME INTERACTING FACTOR 4 (*StPIF4*) levels in response to virus infection to promote immune responses. Node shape represents miRNAs (triangle), transcripts (circle) or metabolites (rectangle). Statistical significances of expression differences (FDR-adjusted p-values) and direction of expression change are represented by the node colors (see the legend). Arrows indicate the type of interaction (solid-line normal arrow – induction; blunt-end solid arrow – inhibition).

3.2.6 sRNAs involved in HR-conferred resistance in cv. Rywal

3.2.6.1 Spatially regulated sRNAs involved in HR in cv. Rywal targets immune receptors

Mechanisms blocking PVY multiplication and spread in HR-conferred resistance in cv. Rywal are occurring close to the point of viral entry and seem to be highly spatially regulated. To study the role of sRNA in HR-conferred resistance response of cv. Rywal, we decided to preserve the spatial information of immune response by sampling a very small section of leaf tissue, containing only a few hundred cells at the site of formed spot necrosis (lesions) on the leaf and its surrounding tissue at the time of first lesion appearance (3 dpi) (Figure 37). Altogether nine sRNA libraries for both lesions and surrounding tissue from three PVY^{N-Wi}-infected plants and control plants of cv. Rywal were sequenced and analyzed. To compare the expression of sRNAs with the expression of their target transcripts we used gene expression results generated from the same samples by RNA-Seq.

In total, 84 sRNAs were found to be significantly differentially expressed following PVY^{N-Wi} infection in the lesion samples, and 34 sRNAs were differentially expressed in the surrounding tissue of cv. Rywal compared to mock-inoculated samples. Virus infection predominantly caused an increase in sRNAs levels in both analyzed tissue areas (Appendix, Supplementary Dataset 1 and 4). By comparing the sRNA levels detected in lesion samples to their levels in surrounding tissue, 9 miRNAs and 24 phasiRNAs sRNA showing spatial distribution pattern were identified (Appendix, Supplementary Dataset 1 and 4). The majority of them displayed decreased abundance from lesion outwards. Of them, miRNAs belonging to miR482, miR6024 and miR6025 families as well as several phasiRNAs targeting transcripts encoding *NBS-LRRs*, *LRR-RLKs* and polyphenol oxidases (laccases), involved in cell wall formation and ROS generation (Appendix, Supplementary Dataset 6 and 7).

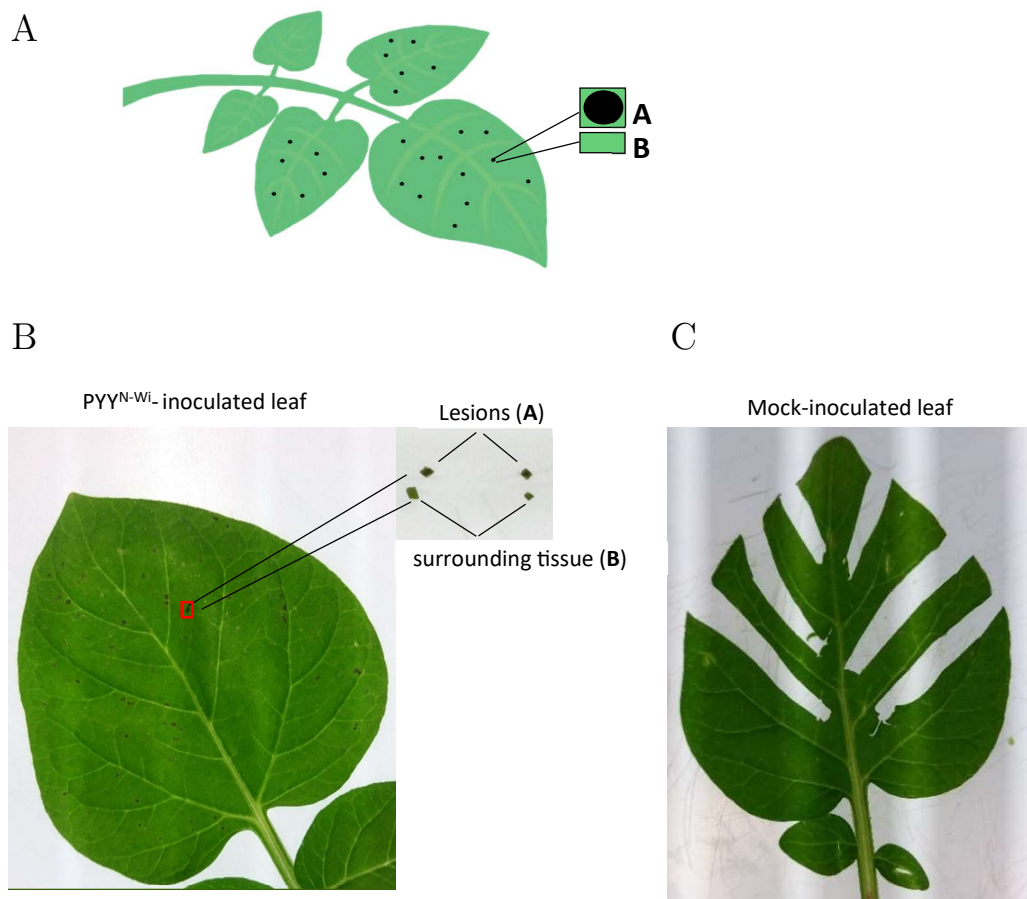
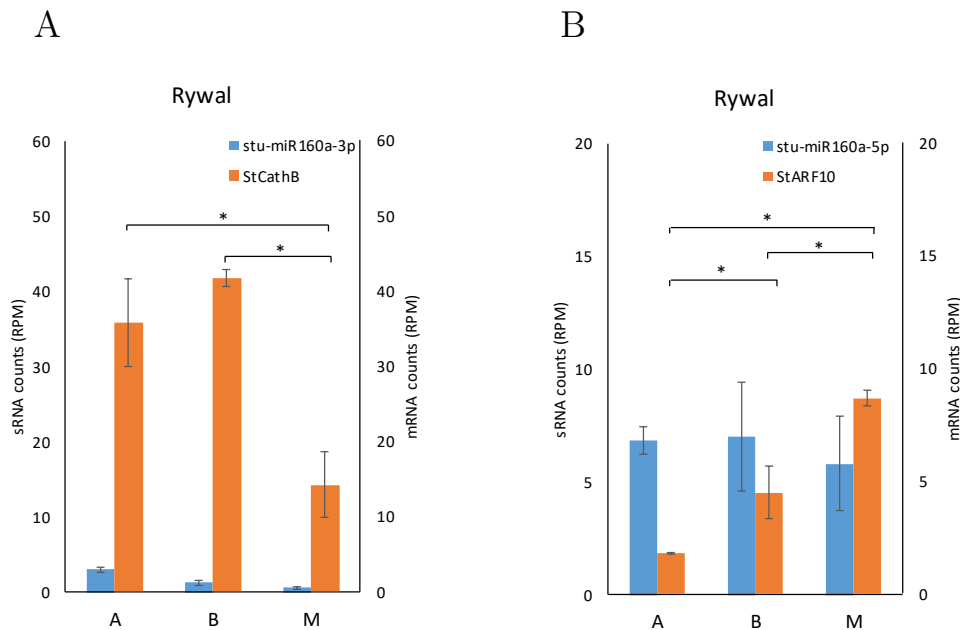


Figure 37: Sampling scheme (A) and “real” sampling (B) of small sections from PVY^{N-Wi}-inoculated leaves of Rywal and NahG-Rywal at 3 dpi. From each second bottom inoculated leaf per individual plant ($n=3$) ~ 30 lesion samples (piece A; excluding lesion formed on veins) were pooled together. Similarly, ~ 30 sections of surrounding tissue (piece B) of sampled lesions from the same leaf were pooled. (C) For mock-inoculated plants, narrow band of the tissue between veins from distal to proximal site of the leaf were excised.

3.2.6.2 Newly discovered phasiRNA3874-StCAT module is activated in HR

As cell death is usually accompanied with HR, we further inspect the activity of miR160a-*StCathB* module in response to PVY^{N-Wi} in cv. Rywal. Increased level of *StCathB* was detected both at the site of early visible lesion as well as in its surrounding tissue, whereas the level of miR160a-3p was not altered (Figure 38A). Furthermore, the level of complementary strand miR160a-5p targeting *StARF10* was also examined. The level of *StARF10* was significantly reduced in the lesion area compared to the levels observed in control plants, and miR160a-5p displayed slight but not significant increase (Figure 38B, Appendix, Supplementary Dataset 1).

In addition to cell death, production of ROS is another characteristic feature connected with HR (Coll et al., 2011). Enzymatic ROS-scavenging mechanisms in plants include superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and glutathione peroxidase (GPX). SOD, acting as the first line of defense against ROS, converts superoxide to less toxic hydrogen peroxide (Guan et al., 2013). Neither miR398 expression nor the level of its target potato *copper/zinc SOD* (*StCSD*) changed upon PVY^{N-Wi} infection in cv. Rywal (Figure 38C). While the miR398-*CSD* module is well documented and is regarded as conserved among many plant species (Naya et al., 2014), the link between the sRNA regulation of transcripts encoding catalases which act downstream of superoxide dismutases to further detoxify the hydrogen peroxide into oxygen and water, has so far not been discovered. We found phasiRNA3874 originating from *NBS-LRR* transcripts (Appendix, Supplementary Dataset 3 and 4) promoting the cleavage of mRNA encoding potato catalase transcripts (*StCAT*) (Appendix, Supplementary Dataset 7). Notably, phasiRNA3874 was significantly upregulated in lesions of Rywal, while the level of target *StCAT* was downregulated (Figure 38D).



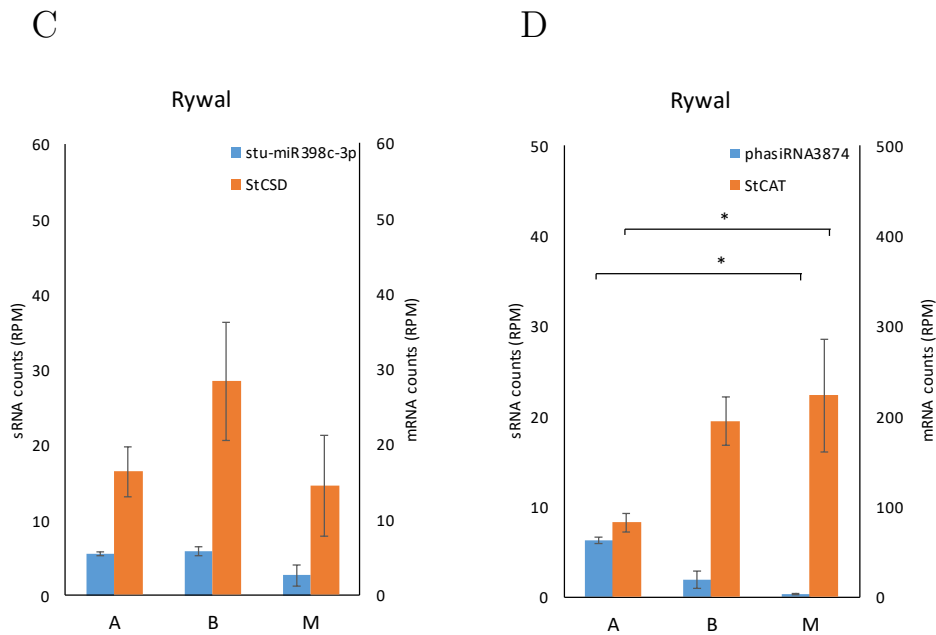


Figure 38: Expression of sRNAs and their targets in cv. Rywal at 3 dpi following PVY^{N-Wi} infection. Expression patterns A) stu-miR160a-3p, B) stu-miR160a-5p, C) stu-miR398c-3p, D) phasiRNA3874 and corresponding target transcripts in lesions (A), surrounding tissue (B) and in mock plants (M). Error bars represent the standard error (n = 3). * represents statistically significant changes in expression level (FDR adjusted p-value < 0.05).

3.2.6.3 Response on sRNA level in resistant and susceptible SA depleted Rywal plants is highly similar

We further examined the effect of SA depletion on the sRNA level and their regulation in the lesions and in the surrounding tissue of the lesions in transgenic NahG-Rywal plants, which react to PVY^{N-Wi} with HR and develop lesions at the site of the infection, similar to Rywal. However, these plants due to the lack of SA cannot restrict the virus spread and prevent disease symptoms (Baebler et al., 2014). To get insight into the spatial regulation of sRNAs in this susceptible potato genotype and to compare the response to that observed in cv. Rywal, nine sRNA and mRNA libraries from both lesions and surrounding tissue samples of three PVY^{N-Wi}-infected and three control plants of NahG-Rywal were sequenced and analyzed.

In total, 60 differentially expressed sRNAs were detected in lesion samples, and 34 in surrounding tissue of NahG-Rywal plants at 3 dpi following PVY^{N-Wi} infection (Figure 39, Appendix, Supplementary Dataset 1 and Dataset 4). In contrast to diminished response observed in NahG-Désirée (Figure 32A), the sRNA response in NahG-Rywal to PVY^{N-Wi} infection at the site of the formed lesion as well as in the surrounding tissue was very similar to that observed in cv. Rywal. A large number of sRNAs was also found differentially expressed and the majority of them were commonly regulated in both

developed lesions and their surrounding tissue in both genotypes (Figure 39, Appendix, Supplementary Dataset 1).

A lesions

B surrounding tissue

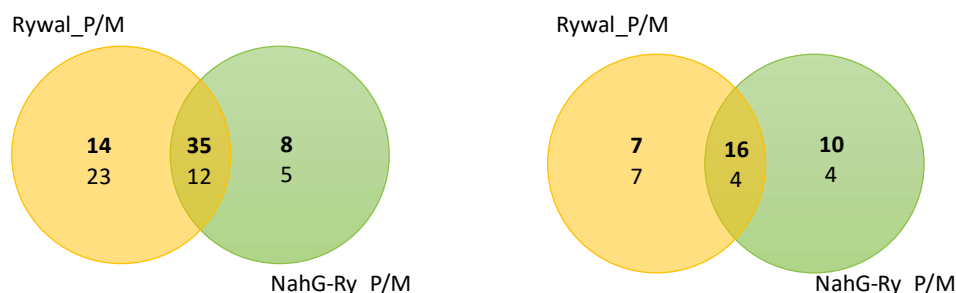


Figure 39: Numbers of unique and common differentially expressed miRNAs and phasiRNAs 3 days post PVY^{N-Wi} inoculation in lesion (A) or surrounding tissue section samples (B) in cv. Rywal and NahG-Rywal. Upregulated sRNAs are shown in bold and downregulated in normal text. NahG-Ry – NahG-Rywal; P – PVY^{N-Wi}; M – mock.

Similarly as observed in Rywal, miR160a-5p was not significantly altered, while the level of target *StARF10* was significantly downregulated both in lesion and surrounding tissue in response to PVY^{N-Wi}. Furthermore, target of miR160a-3p the *StCathB* was also found upregulated both at the site of the lesions as well as in the cells of surrounding tissue (Figure 40AB). Moreover, expression of phasiRNA3874 increased, which was coupled with reduced level *StCAT*, similarly as observed in cv. Rywal (Figure 40D). Contrary to cv. Rywal, miR398c was found upregulated, both at the site of the lesion as well as in the surrounding tissue sections of NahG-Rywal (Figure 40C). Consistently with miR398c upregulation, the level of target *StCSD*, albeit non-significantly, decreased in response to PVY^{N-Wi} in NahG-Rywal ($\log_2FC = -0.8$, FDR adjusted p-value = 0.15) (Figure 40C).

To evaluate the direct effect of the SA deficiency in NahG plants, independently of the viral infection, we also compared the sRNA expression profiles in mock-inoculated leaves of non-transgenic and SA-depleted Rywal. Only two sRNAs were found positively regulated by SA (Appendix, Supplementary Dataset 1 and 4). When we similarly compared the expression profiles of the transcripts in both healthy NahG-Rywal and Rywal plants and checked for significantly enriched or depleted groups of genes under SA, nine genes were found regulated by SA (Appendix, Supplementary Dataset 12). Consequently, due to such limited number of genes, process enrichment of genes differentially regulated by SA in cv. Rywal was not possible. Of nine differentially regulated genes, eight were positively regulated by SA. SA-induced genes encoding a PR protein (endochitinase), a lipoxygenase involved in JA biosynthesis and a subtilisin-like protease (subtilases) involved in activating downstream immune signaling processes (Figueiredo et al., 2014), were also found regulated by SA in cv. Désirée (Appendix, Supplementary Dataset 12). PR proteins, lipoxygenase and subtilases have already been shown to be induced by SA in other plant systems (Figueiredo et al., 2014; Khalili et al., 2009; Zhang et al., 2010).

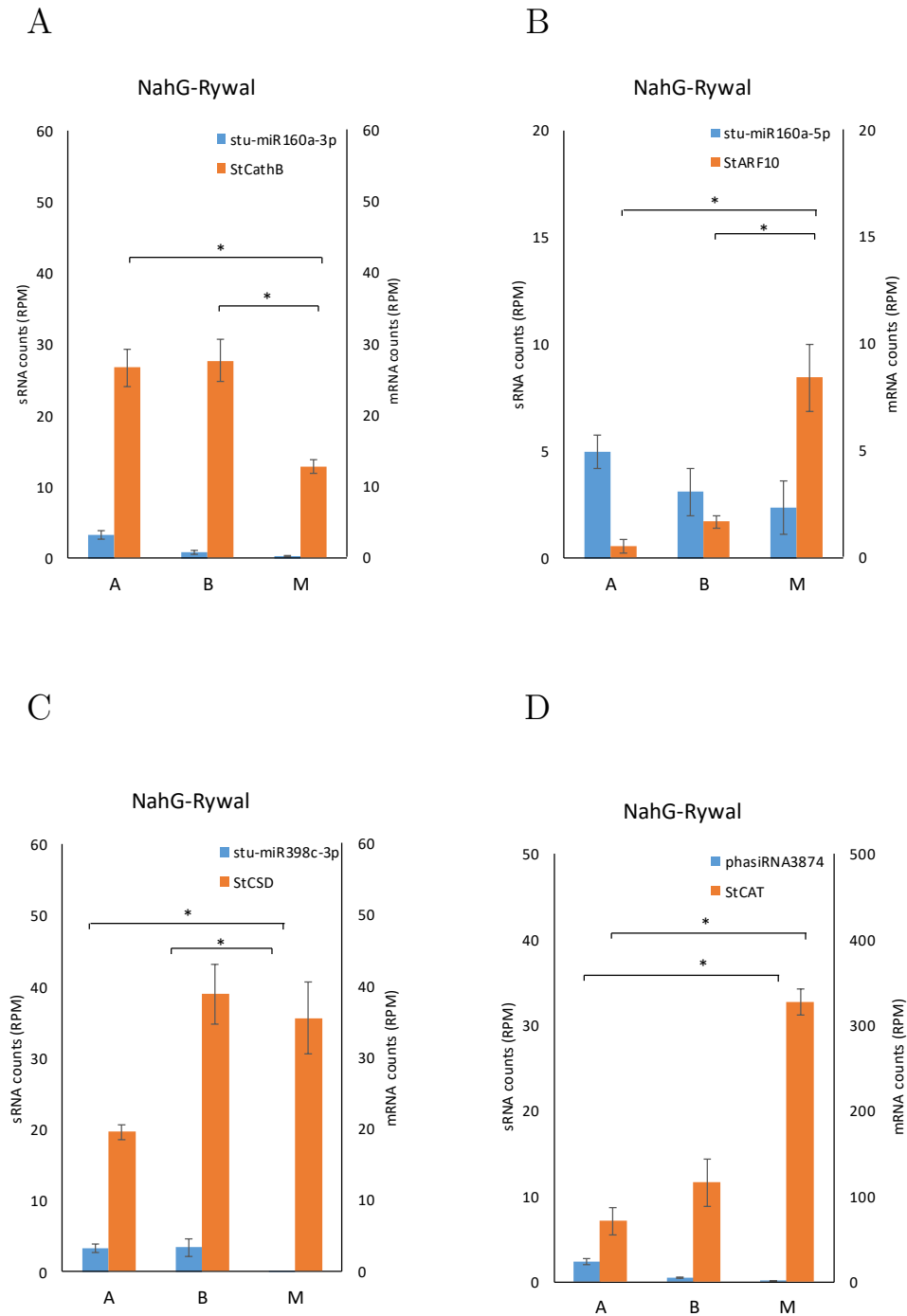


Figure 40: Expression of sRNAs and their targets in NahG-Rywal at 3 dpi following PVY^{N-Wi} infection. Expression patterns A) stu-miR160a-3p, B) stu-miR160a-5p, C) stu-miR398c-3p, D) phasiRNA3874 and corresponding target transcripts in lesions (A), surrounding tissue (B) and in mock plants (M). Error bars represent the standard error (n = 3). * represents statistically significant changes in expression level (FDR adjusted p-value < 0.05).

3.2.7 Only a few sRNAs are differentially regulated in susceptible response of cv. Moneymaker to TYLCV infection

To determine how comparable is the sRNA-mediated regulation observed in compatible, sensitive response (NahG-Désirée and NahG-Rywal) of potato against PVY NahG-Désirée and NahG-Rywal in other plant-virus pathosystems, we studied the response on sRNA level in compatible TYLCV-tomato interaction at 2 dpi (before viral multiplication) and 7 dpi (at the start of viral multiplication).

TYLCV-infected plants exhibited diminished response on sRNA level at both analyzed time points. At 2 dpi, only two tomato miRNAs from miR7981 family were found upregulated. Five days later, when disease symptoms appeared and virus multiplication was detected, the levels of 15 additional sRNAs was significantly changed (Appendix, Supplementary Dataset 13). The majority of them were again miRNAs from miR7981 family. These miRNAs were found to cleave various transcripts, such as mRNAs encoding oxidoreductase, LRR-RLKs or NAC transcription factors (Appendix, Supplementary Dataset 13 and Dataset 14). Of them only upregulation of one miRNA variant from miR7981 family was reflected in its decreased target transcript level coding for Mob1-like proteins (sly-MOB1-like), involved in plant cell proliferation and cytokinesis (Figure 41, Appendix, Supplementary Dataset 14) (Citterio et al., 2006). Moreover, at 7 dpi, two upregulated phasiRNAs, sly-phasiRNA1043 and sly-phasiRNA9598, were detected, targeting tomato *LRR-RLKs* or *NBS-LRRs*. However, their upregulation did not affect the level of their target transcript (Figure 41, Appendix, Supplementary Dataset 13).

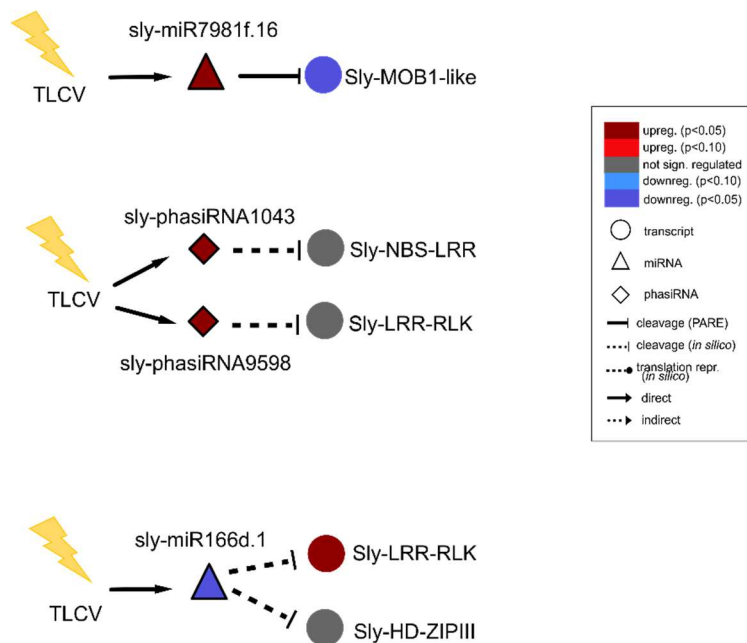


Figure 41: Differentially expressed endogenous tomato sRNAs are targeting mRNAs of immune receptor genes and genes involved in development processes in cv. Moneymaker 7 days post TYLCV inoculation. Node shape represents classes of sRNAs (triangle – miRNA;

diamond – phasiRNA), transcripts (circle). Statistical significances of expression differences (FDR-adjusted p-values) and the direction of expression change are represented by the node colors (see the legend). Arrows indicate the type of interaction (solid-line normal arrow – direct interaction; dashed-line normal arrow – indirect interaction; blunt-end solid arrow – cleavage observed by Degradome-Seq, blunt-end dashed-line arrow – *in silico* predicted cleavage, dashed-line oval arrow – *in silico* predicted translational repression). Sly-MOB1-like – Mps one binder kinase activator-like 1A, Sly-NBS-LRR – nucleotide binding site-leucine-rich repeat protein; Sly-LRR-RLK – leucine-rich repeat receptor-like protein kinase; Sly-HD-ZIPIII – Class III homeodomain-leucine zipper.

Moreover, TLCV infection in tomato plants caused downregulation of an isomiR from miR166 family (sly-miR166d.1) at 7 dpi, targeting *LRR-RLK* and *Class III homeodomain-leucine zipper* (*HD-ZIPIII*) transcripts. Consistently with downregulation of tomato sly-miR166d.7, the level of target *LRR-RLK* transcripts increased significantly, whereas the level of *HD-ZIPIII* remained unchanged (Figure 41, Appendix, Supplementary Dataset 13). We further inspected whether the miR166-mediated regulation of *LRR-RLK* and *HD-ZIPIII* was similarly activated in response of potato to PVY infection. In potato, several members of the miR166 family able to target orthologues of tomato *HD-ZIPIII* transcripts (but not *LRR-RLK*) were found. However, miR166 levels in both susceptible SA potato genotypes were not altered upon PVY infection (Appendix, Supplementary Dataset 6). On the other hand, the levels of *HD-ZIPIII* transcripts were significantly downregulated in both Rywal and NahG-Rywal in response to PVY^{N-Wi}.

Chapter 4

Discussion

4.1 Methodologies for High-Quality sRNA Expression Studies

4.1.1 Optimization of sRNA analysis protocols

As there is still no consensus on a gold standard isolation method and studies comparing different isolation protocols for sRNAs are lacking and only performed on animal samples, we decided to evaluate different RNA isolation methods for their efficiency, reproducibility and overall suitability for sRNA expression studies. In particular we chose to evaluate a) a phenol-based technique that relies on the use of organic solvents (TRIzol), phase separation using chloroform and recovery of RNA by precipitation method, b) *mirVana* isolation kit which utilizes the phenol and chloroform for RNA separation and columns for RNA adsorption and c) High Pure kit which is the column-based technique that uses a phenol-free lysis buffer and a column for RNA recovery. Both column-based techniques allow two different protocols, total RNA or short RNA enrichment protocols. The first is designed to recover both short and long RNAs, whereas the other one retains only short RNAs up to 200 nt in length and eliminates the larger ones. Besides comparing the performance of three different total RNA preparation methods, we also chose to follow short RNA enrichment protocols in order to examine whether the yields of short RNAs (< 200 nt) could be significantly higher to the yields obtained from total RNA extraction procedures.

4.1.1.1 Measured miRNA expression level was affected most by suboptimal setting of reverse transcription reaction rather than RNA extraction method

The performance of the methods was not assessed only in terms of the highest efficiency of sRNA recovery, but also through the quality of extracted RNA. The quality and quantity of extracted RNA are not important only for gene expression analyses but also for reproducibility and accuracy in sRNA profiling. Plant RNA preparations may contain traces of soluble and/or insoluble contaminants such as phenol, polysaccharides, secondary metabolites that have been carried over through the purification steps. Such contaminants are shown to inhibit/interfere with enzymatic reactions during RT-qPCR or library generation and even sequencing (Bustin and Nolan, 2004; Schrader et al., 2012). Further, the integrity of RNA is also not negligible. The RNA extraction method should provide

RNA with high integrity, because the short fragments produced can mask or dilute the presence of sRNAs that are of particular interest within the study. This could pose a problem, especially during sRNA sequencing where the short degradation fragments will compete with plant's miRNAs, siRNAs, which could lead both to reduced number of “target” sRNA sequences and to underestimation of their levels.

We showed that in terms of the RNA yield, all tested total RNA protocols provided comparable RNA quantities (Table 3). Both TRIzol and *mirVana* also yielded highly intact and pure RNA, while in High Pure samples, the purity and integrity were found to be compromised (Appendix, Supplementary Figure 1). The High Pure protocol was also less efficient in removing contaminants that absorb at or near 230 nm and RNA samples suffer for RNA degradation (Table 3). Considering that the RNA yields were comparable with those observed in RNA samples of TRIzol and *mirVana*, the difference encountered in RNA stability with High Pure might be due to contamination of samples with RNases after extraction or due to reduced stability of RNA in the applied elution buffer. Several studies demonstrated that TRIzol-based protocol often resulted in high organic and phenolic contamination (Ach et al., 2008; El-Khoury et al., 2016). With this problems in mind, we employed Phase Lock Gel tube (MaXtract High Density tubes) within the TRIzol procedure to gain RNA samples of better purity. Similarly to the results of El-Khoury et al. (2016) and Yockteng et al. (2013), we also showed that employing gel tubes within the TRIzol procedure may greatly facilitate separation of the organic and aqueous phases and helped us to obtain very good yields and purity of the RNA samples.

Due to their length, sRNAs, including miRNAs, seem to be more stable and exhibit fewer recognition sites for nucleases, so they might be less affected by the overall degradation compared to longer RNAs (Aryani and Denecke, 2015)). Even though, the degradation of short RNA fragments may have the same effect as degradation of longer RNAs as described above. Therefore, the integrity of short RNA fraction was also examined. All RNA samples extracted following total RNA protocols showed good integrity of short RNA fraction and comparable yields of both “sRNAs” and “miRNAs” (Table 3, Appendix, Supplementary Figure 2). By comparing the percentage of “miRNAs” detected in “sRNA” fraction, we examined which method would gain the highest enrichment. The RNA samples extracted by High Pure kit showed a significantly higher percentage to those in RNA samples obtained by TRIzol or *mirVana* total RNA protocols (Table 3). However, considering lower integrity of High Pure RNA samples, the significant rise in “miRNA” amount observed could also be due to the formation of short RNA fragments caused by ongoing degradation, leading to overestimation of “miRNAs” in these samples. Hence, the miRNA and sRNA quantification using small RNA chip might be problematic and unreliable when the integrity of RNA or short RNA is low. Nevertheless, even when the integrity of the samples would be high, these measurements could only give us a rough assessment of extraction efficiency of short RNA species (i.e. miRNAs, sRNAs) recovery. For sRNA quantification studies, however, it is of particular importance that the extraction method is efficient in preserving original sRNA (miRNA) levels.

Several reports have emerged indicating differences in extraction efficiency depending on the method used for RNA isolation. The paper of Kim et al. (2011) was retracted as they found that their conclusions about differences in miRNA expression from cells grown at different confluencies (high density versus low density) and when they were detached from the culture dish (adherent versus suspension) were actually accounted for by differences in RNA extraction efficiency using the TRIzol method (Kim et al., 2012). They found that miRNAs with low GC content were lost during extraction, rather than being degraded in the cells as they had initially published. Moreover, El-Khoury et al. (2016) found differences in miRNA recovery from cells, plasma and urine/plasma-derived exosomes when comparing TRIzol method with two column-based kits such as miRNeasy

and miRCURY extraction kits. The authors found that also a column-based method such as miRCURY can affect the sRNA quantification results, due to poor miRNA recovery. To examine whether any of our tested RNA isolation protocols would have an impact on sRNA quantification results, the expression of three selected miRNAs was analyzed by RT-qPCR. Since all protocols were tested on identical tissue sample, the variability in miRNA levels should be preserved in all RNA preparations.

Contrary to the results obtained by Kim et al. (2012), our miRNA quantification results showed good concordance in relative expression levels of miRNAs among all different RNA extraction protocols, indicating efficient and non-biased recovery of selected potato miRNAs, regardless of the content of GC within miRNA sequence (Figure 15). Additionally we have observed that relative expression results of miRNAs were affected by the method used for reverse transcription reaction (Figure 15). MultiScribe reverse transcriptase RT reaction showed poor repeatability for some samples leading to significant differences in miRNA relative expression levels that could be misinterpreted if the duplicate RT were not included (Figure 15C). It was already shown that different reverse transcriptases may have a different efficiency of converting RNA into cDNA and different tolerance to inhibitors (Arezi et al., 2010; Baranauskas and Paliksa, 2012). The usual way to improve efficiency is to dilute the RNA sample and thus also the effect of inhibitors. However, in our case, this did not solve the problem, because the Cq values were shifted towards the limit of quantification. As SuperScript III reportedly has higher internal stability, increased cDNA synthesis capability and shows overall better performance than wild-type MMLV and MMLV RNase H-minus enzymes (Baranauskas and Paliksa, 2012), this prompted us to perform additional RT reactions employing SuperScript III RT system instead of MultiScribe reverse transcriptase. Despite different buffer formulation, SuperScript III RT reactions contained a similar amount (Units/ μ L) of enzyme and the same concentrations of custom stem-loop Megaplex primer pool (see Materials and Methods). Our result showed improved repeatability of SuperScript III and negated previously observed differences in miRNA expression level (Figure 16). Our results also corroborate a previous study showing that Superscript III is the best performing enzyme in terms of reproducibility and sensitivity, including for low copy RNA samples. Additionally, Tang and coworkers (2006) showed that employing a pulsed RT reaction increases RT efficiency and reduces non-specific interactions between stem-loop primers for different miRNAs (Tang et al., 2006). Even though the optimal temperature for SuperScript III quantitative synthesis of full-length cDNA is 50–55 °C, our results are in agreement to the results of Tang and coworkers, confirming that by applying pulsed cycling conditions, using three different primer annealing temperatures, sensitivity and efficiency of reverse transcription could be even more improved.

4.1.1.2 Short RNA enrichment protocols performed worse than total RNA extraction protocols

When comparing the performance of total RNA extraction protocols to short RNA enrichment protocols, we observed that short RNA enrichment protocols did not prove to be more efficient in gaining higher yields of “sRNAs” or “miRNAs”, compared to the total RNA protocols (Table 3). Although, expectedly, short RNA enrichment protocols displayed higher levels of “sRNAs” and “miRNAs” relative to total RNAs than observed in non-enriched RNA samples, they still contain significant amounts of larger RNAs, up to 30 % in RNA samples of High Pure or even up to 55 % in RNA sample of *mirVana* (Table 3).

The performance of *mirVana* short enrichment protocol has already been studied by Redshaw and coworkers on the human brain samples (Redshaw et al., 2013). They discovered that enrichment procedure did not have an equal effect on all measured miRNAs, and that observed miRNA yield ranged between 12%-35%, leading to significant changes in relative miRNA levels compared with the non-enriched protocols. It was therefore assumed, that loss of short RNA is connected to their binding to longer RNAs and their concurrent elimination within the protocol (Brown et al., 2018; El-Khoury et al., 2016; Redshaw et al., 2013). In our study, however, by measuring the relative expression level of three selected potato miRNAs, we did not observe any bias in extraction efficiencies among short RNA enrichment protocols (Figure 15). On the other hand, short RNA enrichment protocols displayed other issues in performance. *mirVana* short RNA enrichment protocol suffered for the elimination of sRNAs > 80 nt or retention of “undesired” longer RNAs with up to 4000 nt, indicating poor repeatability of the protocol (Appendix, Supplementary Figure 1 and 2). There was also an indication of the presence of contaminants in RNA short enrichment samples of both *mirVana* and High Pure kits (Table 3). Overall, our data suggest that the TRIzol and *mirVana* total RNA protocols outperformed High Pure total RNA protocol and that it is still more advisable to rely on the use of total RNA extraction protocols rather than short RNA enrichment protocol since they can provide better quality and yields of sRNAs. As both long and short RNA fractions are retained, the gene and sRNA expression studies could be performed on the same RNA sample, and there would also be a lower probability that short RNAs will be lost during the procedure.

4.1.2 Optimized protocols for combined transcriptomics and sRNAomics analyses in small tissue sections

When studying spatially regulated responses in plants, sampling of very small pieces of leaf tissue, containing up to 100 hundred cells, is required. The main challenge is to recover enough RNA material from such a small section that it is amenable for further downstream analyses. We sought to find the method that will allow efficient extraction of both short and long RNAs and produce sufficient RNA and sRNA yields for subsequent sRNA and mRNA library generation and sequencing. Moreover, since it is well known that RNA can be easily degraded, the method should provide short handling time to reduce the possibility of RNA degradation.

Column-based extraction protocols developed for homogenized plant material cannot be easily applied for small tissue samples, due to a wider diameter of columns and requirements for higher elution volumes (> 30 µl). On the other hand, TRIzol extraction protocol was shown to be less efficient than column-based kits for RNA and miRNA extraction from a small number of human epithelial cells (El-Khoury et al., 2016). Moreover, as described above, miRNAs with low GC content were shown to be selectively lost during TRIzol extraction from a small number of human breast cancer cells (Kim et al., 2012). To our knowledge there is just one study published on comparing the different phenol-based RNA extraction methods for small quantities of plant tissue, however even though they claimed to work on small amounts of tissue, the amounts of plant material they used (~ 100 mg) was comparable to the amount we used for RNA extraction from homogenized leaf samples. We, therefore, decided to test two recently developed column-based kits, Direct-zol micro kit (Zymo) and Single cell purification kit (Norgen).

4.1.2.1 Zymo kit is preferable over Norgen for RNA and sRNA/miRNAs extraction from small tissue sections

Comparing the results from both extraction methods, we observed slightly better RNA, “sRNA” and “miRNA” yields obtained by Zymo kit (Table 4). RNA samples isolated using the Zymo kit also performed, in general, better, resulting in lower Cq values of all three analyzed miRNAs than RNA samples deriving from the Norgen isolation procedure, showing that Zymo kit had higher efficiency in miRNA recovery (Figure 18). These results are similar to results from Mäki et al., (2017), who already demonstrated that Direct-zol RNA MicroPrep kit gained the highest RNA and sRNA yields from phytoplankton cells compared to the similar low-input method. By examining the relative expression level of selected miRNAs, we additionally demonstrated that the method also efficiently preserved original miRNA levels (Figure 19). Even though the TRIzol reagent was used for cell disruption prior RNA column adsorption, the relative expression level of miRNA with low GC content (i.e. miR172) was not affected as was highlighted previously in the study by Kim and coworkers (Kim et al., 2012). Hence, the loss of miRNAs is unlikely related to the inefficiency of TRIzol reagent itself, but rather to downstream procedures that follow, such as poor chloroform extraction or RNA precipitation or losses during washing of the pellet. Concerning the Zymo kit protocol, the only problem we observed was low purity of RNA samples (Table 4). Contaminants can induce overestimation of RNA quantity and more importantly, they can interfere with downstream applications. This is especially true for reverse transcription of miRNAs, as we already demonstrated in one of the previous sections. Even though we replaced the MultiScribe enzyme for SuperScript III and applied pulsed RT reaction to optimize the efficiency of reverse transcription, we observed that it is still possible that higher levels of contaminants in RNA sample may reduce the reverse transcription efficiency and affect the miRNA quantification results (Figure 19A). Overall, we identified the Zymo kit as a better choice than the Norgen kit for extraction of RNA from low-input samples, despite the higher probability to obtain less optimal purity of the RNA samples. However, to obtain reliable sRNA RT-qPCR or sRNA-Seq quantification results, it is highly advisable to clean low purity RNA samples prior further downstream analyses.

4.1.2.2 RNA Clean & Concentrator kit preserves RNA integrity and does not affect miRNA and mRNA levels

We tested the performance of recently developed RNA Clean & Concentrator kit for purification of RNA samples. We demonstrated that EDTA free DNase treatment protocol is highly repeatable, and importantly, the columns employed are efficient in binding and preserving both miRNAs and mRNA levels (Figure 19 and Figure 21). In comparison to currently used DNase-EDTA protocol, DNase-cleaning protocol tested here performed better in elimination of genomic DNA and did not affect the integrity of RNA samples (Figure 21C, Appendix, Supplementary Figure 4). On the other hand, RNA samples processed by DNase-EDTA protocols showed reduction in integrity of short RNAs, while the integrity of longer RNAs was preserved (Appendix, Supplementary Figure 4). It has been known that RNA is hydrolyzed in the presence of divalent cations and the addition of chelating agent EDTA before heat denaturation of the DNase enzyme can help overcome the RNA degradation (Malek et al., 2000). Based on preserved integrity of longer RNAs

(Appendix, Supplementary Figure 4) and confirmed repeatability of RT-qPCR reaction of *Pr1b* and *cox* it seems that EDTA might be more beneficial for stabilization of larger RNAs, while shorter RNAs become more vulnerable. However, further analyses will be needed to evaluate the effect of DNase-EDTA protocol on sRNA expression analyses to exclude the possibility of effects on short RNA integrity and putative inhibitory effect of EDTA on miRNA reverse transcription.

4.1.3 Establishment of optimized protocol for novel *MIR* loci and miRNA discovery

Several computational tools have been developed to complement experimental approaches to identify and validate novel miRNAs and miRNA-producing loci from high throughput sequencing data (Huang et al., 2011). These tools may employ different mapping algorithms for short RNA reads, different algorithm used for predicting the secondary structures and may also differ in number and stringency of integrated criteria for miRNA annotation (Axtell and Meyers, 2018).

By comparing three different plant miRNA prediction tools we showed that the number of predicted *MIR* loci varies substantially depending on the tool used, from 37 to more than 400 *MIR* loci from the same sRNA sequencing data set (Figure 22). miR-PREFeR was recognized as the most sensitive with the ability to predict the highest number of annotated potato *MIR* loci, however the number of potential novel *MIR* loci was more than 400. The higher number of *MIR* loci is always questionable, since many studies have reported the problems with some tools in terms of high false positive rate (An et al., 2014; Axtell and Meyers, 2018; Lei and Sun, 2014). By examining folding free energy of predicted hairpin structure, which is one of the main criteria for accurate annotation, and with comparing corresponding mature miRNA sequences with other ncRNA sequences, we tried to evaluate, at least to some extent, the ability of the tools in discriminating *bona fide* *MIR* loci from non-*MIR* like loci.

Of three tested tools presented here, ShortStack predicted the lowest number of *MIR* loci, however, it was more efficient in eliminating non-*MIR* like loci and non-miRNAs (Figure 24). On the other hand, more than 25 % of miR-PREFeR and 35 % of miRCat predictions were recognized as false positives, which further poses the question of the reliability in predictions for other *MIR* loci (Figure 24). In our previous study (Križnik et al., 2017), we were aware of the problems of false positives, thus we tried to increase the fidelity of miRNA/*MIR* loci prediction by considering only those miRNA/*MIR* loci predicted by two miRNA prediction tools. This approach might be efficient, however it is time consuming. When we compare the numbers of commonly predicted *MIR* loci, we found that except in one case, all ShortStack's predicted *MIR* loci were confirmed also by at least one other tested miRNA prediction tool (Figure 22AB), indicating that they can be confidently annotated as *MIR* loci. These results additionally showed that ShortStack pipeline is highly stringent, and even though some *MIR* loci and miRNAs can be missed, the ones that are predicted can indeed be regarded as high confidence miRNAs/*MIR* loci. Notably, this specific feature attributed to ShortStack's predictions can also minimize the need for confirmation with another tool, thereby reducing the time for analyses.

We also showed that even though all three tools are highly concordant in predicting the identical mature miRNA sequence from the same predicted *MIR* loci (Appendix, Supplementary Table 1), the predictions of length and the energy of pre-miRNA hairpin precursor vary considerably (Appendix, Supplementary Table 2). However, all predictions

follow the current miRNA annotation criteria that limit the length of pre-miRNA precursor to 300 nt and folding free energy of hairpin structure (Axtell and Meyers, 2018). Thus, based on these criteria all predicted pre-miRNA sequences are equally likely. The length of pre-miRNA precursor is not relevant for sRNA quantification studies. But for other studies, when the length and sequence of pre-miRNAs needs to be resolved, sequencing technology to capture longer sequences, such as Illumina 300 bp paired-end read sequencing or PacBio instead of bioinformatic prediction, should be followed.

4.2 Role of sRNAs in Potato-PVY Interaction

Up to now, studies on potato-PVY interaction have been focused on phenotypisation and in detection of changes in plant transcriptome, proteome and metabolome, especially related to plant hormonal signaling (Baebler et al. 2011; Stare et al. 2015). Of hormones, salicylic acid (SA) was found to be the crucial component for attenuation of disease symptoms in tolerant plants of cv. Désirée (Baebler et al. 2011) and in the orchestration of the events restricting PVY spread in resistant cv. Rywal. However, the understanding of the mechanisms that underlie tolerance or resistance response, such as HR to PVY, is still incomplete.

Advancement in molecular biology demonstrated the importance of sRNAs in regulating essential plant metabolic processes at the post-transcriptional level including plant immune responses. These riboregulators can therefore serve as potential molecules that can be manipulated to help the plant to cope with its pathogens and thus improve plant productivity. However, none of the studies performed so far investigated the regulatory network of sRNAs in different potato cultivars infected with a PVY. Within the study we aimed to gain insight into regulatory mechanisms governed by sRNAs and elucidate potato sRNA's role in establishment of a tolerant response and hypersensitive response of potato to PVY infection.

Employing high-throughput sequencing technology, we characterized and compared the expression patterns of known and newly discovered sRNAs between PVY-infected and healthy potato plants. In addition, this information was linked with expression profiles of their target transcripts identified by *in silico* and Degradome-Seq, and used for sRNA regulatory networks construction, which confirmed many known, but also revealed several novel regulatory circuits associated with immunity regulation and hormone signaling.

4.2.1 Differentially expressed miRNAs regulate various immune receptor genes in tolerant response of cv. Désirée

When plants are exposed to pathogens, NBS-LRRs and LRR-RLKs are the key players in sensing and transducing stress signals (Antolin-Llovera et al., 2014; Jones and Dangl, 2006). Viral suppressors of silencing can release the tight control of *R*-gene silencing by sRNAs and activate immune responses in plants (Li et al., 2011; Shivaprasad et al., 2012). Our study investigated regulatory processes occurring early after infection, before virus concentration significantly increased thus the effects we detected were not caused by extensive HC-Pro or any other viral protein accumulation. Even so, we have detected diverse regulation of *NBS-LRRs* and *LRR-RLKs* and their targeting miRNAs as expected

according to their specialized roles (Figure 29). In our study, *StLRR-RLKs* were found to be upregulated in response to PVY^{NTN} in cv. Désirée through reduced activity of miR6022 (Figure 29 and 30A) even before quantity of viral RNA significantly increased. However, this regulation was diminished in susceptible SA depleted plants, as well as in tolerant Pentland-Squire plants, suggesting that the regulation is specific within Désirée-PVY^{NTN} interaction.

A recent study by Yang et al. (2015) confirmed that miR482e overexpression decreases NBS-LRR gene expression, which enhances the sensitivity of potato to *Verticillium dahliae* infection (Yang et al., 2015b). In our system, the miR482e miRNA was found upregulated in response to PVY in both tolerant as well as in susceptible NahG-Désirée genotypes attenuating the response of *NBS-LRR* transcripts. Upregulation of miR482e was similarly observed in soybean nodulation (Li et al., 2010a). In this case defense responses should be limited in the presence of rhizobia to facilitate infection to establish beneficial microbial interactions. Results thus suggest that miR482e overexpression in plants most probably reduces immune response in infected plant which can contribute to successful infection and unrestricted multiplication of the virus observed in all three analyzed genotypes (Désirée, Pentland, NahG-Désirée). Moreover, several miRNAs that were upregulated in response to PVY^{NTN} in cv. Désirée have also been reported to regulate nodulation in soybean. Of them several miRNAs that have also been linked to auxin signaling (miR160, miR164, miR167 and miR393, miR390) (Bazin et al., 2012).

4.2.2 sRNAs are involved in crosstalk between SA and auxin signaling

Auxin is a well-known promotor of plant growth, which provides carbon and nitrogen sources for biotrophic pathogens and increases their virulence and susceptibility by suppressing SA-mediated defense responses. Until now, many miRNAs were confirmed to regulate auxin signaling in potato (Lin et al., 2015; Navarro et al., 2006). A complex miR393/TIR/phasiTIR auxin signaling network was identified in this study, showing that same miRNA can, by triggering the production of phasiRNAs, affect different hormonal signaling pathways (Figure 26). However, none of sRNAs involved in this signaling network was differentially expressed after PVY infection, showing that miR393-TIR module was not activated in response to PVY at 3 dpi in Désirée or at 4 dpi in Pentland-Squire.

On the other hand, miR160a-5p repressing ARFs, involved in downstream auxin signaling was found upregulated upon PVY infection in both Désirée and Pentland-Squire. miR160 is known to play a vital role in plant growth and development, however its role in defense was discovered only recently. Overexpression of miR160 in rice was shown to induce increased basal resistance against rice blast (*M. oryzae*) (Li et al., 2014). Moreover, a recent study by Natarajan and coworkers showed that miR160 is involved in eliciting local defense and SAR in systemic leaves of potato plants (cv. Désirée) challenged either with oomycete pathogen *Phytophthora infestans* or bacterial pathogen *Ralstonia solanacearum*. The miR160 knockdown lines displayed reduced levels of SA in both local and systemic leaves, suggesting that miR160a is able to positively regulate SA accumulation in potato by some as yet unknown mechanism (Natarajan et al., 2018). Their results further show the involvement of miR160a-ARF10 module in antagonistic crosstalk between SA and auxin signaling. The similar mechanism therefore could exist in Désirée and Pentland-Squire plants, in which increased level of miR160a-5p would promote SA signaling pathway. Even though *STARF10* expression was not found altered in infected leaves of cv. Désirée, significant decrease of its level was detected in upper non-inoculated leaves cv. Désirée. It

thus may be speculated that reduced level of *StARF10* is the result of stronger miR160-mediated regulation in the upper leaves. Since infected leaves of Désirée exhibit a less pronounced increase in miR160a-5p level compared to the levels observed in Pentland-Squire (Appendix, Supplementary Dataset 1), it could also be possible that miR160a-5p acts as a systemic signal that moves into distant tissue to induce SAR. The movement of miR160a has not been explored yet, however it was shown that several miRNAs such as miR166, miR172, miR399 are capable of moving long distances (Kasai et al., 2010; Marín-González and Suárez-López, 2012; Pant et al., 2008). However, further analyses will be needed to confirm this hypothesis. Increased level of complementary strand miR160a-3p, on the other hand might be important for the modulation of cathepsinB to prevent PCD (Figure 35B).

4.2.3 Linking viral defense and developmental signaling: sRNA – gibberellins circuit identified in potato

Novel link between sRNA regulatory network and GA signaling was discovered. Biotic stress was previously shown to repress GA signaling pathways. Additionally, it was shown that modulation of bioactive GA levels can affect disease resistance, which is also reflected in symptoms development. We discovered that GA biosynthesis and signaling are post-transcriptionally regulated via multiple miRNAs, phasiRNAs as well as vsiRNAs in potato leaves following infection with PVY^{NTN} in cv. Désirée. The effect of this regulatory circuit was confirmed by reduced bioactive GA level in the tolerant Désirée plants (Figure 30). This reduction was however not reflected in decreased plant growth and was thus most probably transient and localized in nature (Karasov et al., 2017). In other plant species, GA biosynthesis was shown to be indirectly targeted by miRNAs regulating the activity of the corresponding transcription factors (miR319-TCP14-GA2ox/GA20ox; miR393-GRF2-GA3ox/GA20ox) (Curaba et al., 2014), while direct interactions were to our knowledge not yet reported. Also of note, the sRNAs regulating GA biosynthesis identified here (miR482f/phasiRNA931 and miR167e) were not identified in Arabidopsis and seem to be *Solanaceae* specific.

Moreover, downstream GA signaling components seem to be affected by the sRNA regulatory network as well. Potato orthologue of DELLA protein was detected as a target of phasiRNA931, its regulation did, however, not affect the level of transcript (Figure 30). It is possible that phasiRNA downregulates its protein production which is not observable on the level of transcript accumulation or regulation via its promotor overshadows the effects of sRNAs. On the other hand the measured decrease in GA concentration (Figure 30B) leads to slower degradation of DELLA protein (Fonouni-Farde et al., 2016). Decrease of GA coupled with increase of DELLA protein concentrations was shown to trigger components of rhizobial and mycorrhizal signaling, showing yet another similarity between tolerant response of potato to viral infection and response of plants in mutualistic symbiosis. Further downstream StMYB33, an orthologue of Arabidopsis GA-induced MYB-like proteins (GAMYBs) MYB33, is regulated by miR319a, a close relative of miR159 (Palatnik et al., 2007). The two Arabidopsis GAMYBs were previously shown to be regulated by miR159 (Allen et al., 2005). Lower activity of GAMYB caused by decreased levels of GA and potential translational repression of DELLA and GAMYB itself by sRNAs could be crucial in the regulation of tolerant-like response against PVY^{NTN}, since these GAMYBs have already been linked to generation of disease symptoms following infection by cucumber mosaic virus (CMV) in Arabidopsis (Du et al., 2014) and *N. benthamiana*.

plants (Liao et al., 2015). With the discovery of GAMYB binding sites in the *MIR482f* and *MIR6022* promoters, we predicted the circuit in sRNA-GA signaling and additionally a link between GA signaling and *R* gene expression. Effect of sRNAs on GA signaling in tolerant plants of Désirée was confirmed also by decreased abundance of miR6022 (Figure 30A).

Importantly, an additional link between miR390a and GA signaling was discovered. miR390a was found to promote the cleavage of transcript of *StCOP1*, an E3 ubiquitin ligase that has been studied extensively in the photomorphogenesis and light-related processes in Arabidopsis. COP1 plays a prominent role in drought stress tolerance in Arabidopsis. Additionally, in a recent study, COP1 was recognized as a negative regulator of immune responses in Arabidopsis plants towards bacterial pathogen *P. syringae* pv. *tomato* DC3000 (Jazi et al., 2018). COP1 modulates immunity through regulation of the expression of PIF4 (Gangappa and Kumar, 2018). Consistently, *pif4* mutant plants displayed downregulation of growth-related genes and upregulation of defense genes and enhanced resistance to *P. syringae* pv. *tomato* DC3000 (Gangappa et al., 2017). PIF4 is also positively regulated by GA, thus both the reduced levels of GA as well as increased levels of miR390 could synergistically work to diminish the levels of PIF4 to prioritize defense over growth in tolerant interactions.

4.2.4 The role of SA in modulation of defense versus development trade-offs

Baebler and coworkers have previously shown that depletion of SA in NahG-Désirée plants leads to the development of necrotic lesions 5 days post PVY^{NTN} infection (Baebler et al., 2011). In this study we have additionally shown that response of sRNA regulatory networks controlling immune receptors and hormonal signaling is strongly attenuated in the NahG-transgenic plants in the early stage of viral infection (3 dpi; Figure 32 and 33). It has been reported previously that SA and RNA silencing-mediated defenses cooperate to limit virus spread (Alamillo et al., 2006; Ji and Ding, 2001b), which was corroborated by faster virus multiplication in the NahG-Désirée plants observed in our previous studies (Baebler et al., 2011; Stare et al., 2015). The molecular mechanisms of this synergism have, however, remained elusive. SA has been shown to induce *RDR1* expression, which is crucial for the maintenance of basal resistance to several RNA viruses (Carr et al., 2010; Yu et al., 2003). In potato depletion of SA however does not affect expression of *RDR1* (Appendix, Supplementary Dataset 12), indicating this crosstalk is not effective in this species. Here, we have additionally predicted SA-directed transcriptional regulation of *MIR482f*, the miRNA linking the GA signaling circuit and *R* gene expression (Figure 30A, Appendix, Supplementary Dataset 11), which could be an additional link between SA signaling and symptoms development in potato-PVY interaction. Regulation of miR482f expression by SA was indeed experimentally confirmed in comparison between non-transgenic and NahG-transgenic plants (Appendix, Supplementary Dataset 1).

4.2.5 Potato miR398 is important for ROS homeostasis during HR

HR is induced by certain plant pathogens and involves PCD to restrict the spread of pathogens from the infection site. In many cases, HR is manifested in the development of

necrotic lesions. In potato cv. Rywal, HR is mediated by *Ny-1* resistance gene and is manifested as the formation of lesions on inoculated leaves 3 dpi (Szajko et al., 2008). Similarly, in SA depleted transgenic plants the lesions started to be visible at 3 dpi, but the lack of SA seems to compromise HR, as lesions continuously expanded into large necrotic lesions covering the majority of the leaf and the virus spreads systemically in the plant (Baebler et al., 2014; Lukan et al., 2018).

Caspases, a family of cysteine proteases emerged as the key orchestrators of cell death process in animals. Study by Gilroy and coworkers (2007) on *Nicotiana benthamiana*-*Phytophthora infestans* pathosystem demonstrated direct role of cathepsin B in promoting PCD during HR in plants (Gilroy et al., 2007). Upregulation of *StCathB* detected in lesion samples as well as in surrounding tissue of Rywal and NahG-Rywal (Figure 38A and 40A) clearly shows its involvement in establishment of PCD during PVY^{N-Wi} infection. Both lesions and surrounding tissue of Rywal and NahG-Rywal also displayed a decreased level of *StARF10*, involved in auxin signaling (Figure 38B and 40B). miRNAs that regulate *StCathB* and *StARF10* were however not differentially regulated (Figure 38AB and 38AB), suggesting that some other, additional mechanisms exist that are faster and stronger compared to the regulation exerted by sRNAs. Also ROS can contribute to suppression of auxin signaling as previously demonstrated by (Oa et al., 2011).

In contrast to differences in Désirée and NahG-Désirée, response of Rywal and NahG-Rywal to PVY^{N-Wi} infection on the sRNA level was highly similar, which is somehow not surprising, as in both genotypes cell death responses are triggered at the infection site. However, some dissimilarities in response were observed that could be linked to symptoms progression or inability to restrict virus movement in susceptible SA depleted plants.

As already discussed, many miRNAs and phasiRNAs are able to regulate the *NBS-LRRs* or *LRR-RLKs*. The levels of several sRNAs targeting immune receptor transcripts were found to decline from lesions to surrounding tissue in response to PVY^{N-Wi} in cv. Rywal (Appendix, Supplementary Dataset 1). This indicates that sRNA-mediated silencing in the cells of lesion surrounding area is relieved to promote activity of immune receptors and might be important for resistance, as such pattern was absent in susceptible plants.

Another characteristic feature of HR is the rapid and intense production of ROS. ROS, which include the superoxide anion radical, hydroxyl radical, and hydrogen peroxide are potentially harmful to the cell and cause oxidative damage to proteins, DNA and lipids. Plants have evolved complicated mechanisms to regulate the delicate balance between ROS production and scavenging through ROS detoxification system. Several ROS-scavenging enzymes play a positive role in scavenging ROS and protecting the cells against oxidative stress. miRNA belonging to miR398 family was shown to negatively regulate *copper/zinc superoxide dismutases* (*CSDs*) which detoxify superoxide radicals in response to various environmental stress including biotic stress (Leng et al., 2017).

Our results showed that PVY^{N-Wi} infection in Rywal did not alter miR398c expression level or target transcript *StCSD* levels, whereas in NahG-Rywal the expression of miR398c was significantly increased, coupled with reduced accumulation of *StCSD* level (Figure 38C and 40C). The decrease in *CSD* level may cause an increase in the level of superoxide radicals and lead to increased oxidative damages (Leng et al., 2017). It was shown previously that downregulation of miR398 by oxidative stresses could be advantageous as it leads to the accumulation of CSDs, which in turn increased the plant's tolerance to a variety of stresses (Sunkar, 2006).

Moreover, the link between miR398 and disease symptoms has also been reported. miR398 overexpression was shown to promote susceptibility to *S. sclerotiorum* infection in *N. benthamiana* plants and cause an increased size of lesions compared to wild-type plants (Naya et al., 2014). These results are in agreement with the expanded lesions observed in NahG-Rywal plants. It might be thus possible that in Rywal plants homeostasis between

miR398c and CSD levels after PVY^{N-Wi} infection is better maintained to balance the amount of superoxide radicals and hydrogen peroxide, while the lack of SA impairs ROS-scavenging capability leading to progression of uncontrolled cell death reflected in larger lesions. Furthermore, miR398 upregulation in NahG-Rywal might also interfere with the amount of callose. The overexpression of miR398 causing decrease in callose deposition was shown to enhance bacterial proliferation and increase susceptibility to both virulent and nonpathogenic strains of *P. syringae* (Li et al., 2010c). Callose is in an early defense strategy employed by plants to limiting the spread of the virus as well (Li et al., 2012). It is believed that the callose deposition change the size exclusion limit of plasmodesmata and thus restrict the movement of virus between cells. Our spatial transcriptomic data showed that transcripts encoding two Beta-1 3-glucanases (BGLUs) BGLU (Sotub10g021570) and BGLU 3 (Sotub01g009310); Appendix, Supplementary Table 3), a callose turnover enzymes were significantly upregulated in response to PVY^{N-Wi} in both analyzed genotypes (*BGLU* $\log_2FC = 9.3$ (lesions) and 8.2 (surrounding tissue) in Rywal; *BGLU* $\log_2FC = 10.5$ (lesions) and 10.2 (surrounding tissue) in NahG-Rywal; *BGLU3* $\log_2FC = 9.6$ (lesions) and 7.1 (surrounding tissue) in Rywal; *BGLU3* $\log_2FC = 11.8$ (lesions) and 10.6 (surrounding tissue) in NahG-Rywal). As the induction level of both *BGLU* and *BGLU3* was higher in NahG-Rywal both in lesions as well as in surrounding cells compared to those in Rywal, these results might suggest stronger tendency toward reduction of callose amount on plasmodesmata. In Rywal plants callose deposits were detected in the lesion areas at 3 dpi (Baebler et al., 2014; Lukan et al., 2018), however none of the studies so far investigated the callose deposition in NahG-Rywal in response to PVY^{N-Wi}. Thus, further studies will be needed to determine whether the miR398-mediated regulatory network negatively affects callose deposition in NahG-Rywal and to additionally determine if the miR398 regulation is responsible for impaired restriction of the movement of the virus.

4.2.6 Regulation of sRNAs in susceptible TYLCV-tomato interaction does not resemble sRNA's response in sensitive potato-PVY interaction

Tomato yellow leaf curl disease caused by TYLCV is one of the most devastating viral diseases affecting tomato crops in tropical, subtropical and temperate regions of the world (Díaz-Pendón et al., 2010). The symptoms of infected tomato plants are marginal chlorosis and reduced size of the apical young leaves, upward curling or cupping of leaf margins and chlorosis in older leaves, and drastic reduction in plant height because of the shortened internodes. The plant appears bushy, and floral abortion often causes total yield loss. TYLCV is a single-stranded DNA virus, which belongs to the genus *Begomovirus* (family *Geminiviridae*) and is transmitted in a circulative, persistent manner by the whitefly *Bemisia tabaci* (Navas-Castillo et al., 2011). Even though the PVY and TYLCV differ in their genome (the first one being RNA and the second one DNA virus), pathology and mode of transmission, our aim was to examine whether the response on sRNA level observed in the early stages of virus infection in susceptible cv. Moneymaker shares similarity to those observed in susceptible potato-PVY interaction (e.g. NahG-Désirée or NahG-Rywal).

We found that response on sRNA level in cv. Moneymaker upon TYLCV infection was diminished, even more than observed in NahG-Désirée-PVY^{NTN} interaction (Appendix, Supplementary Dataset 13 and 14). A very low number of differentially expressed sRNAs

at 2 dpi and 7 dpi indicates that sRNA regulation may not have such a profound role in response to TYLCV infection at early stages of viral infection as observed in potato-PVY interaction. The differentially expressed sRNAs found in tomato upon virus infection were also not regulated in sensitive potato genotypes and vice versa, therefore we were unable to determine sRNAs that could be linked to sensitivity to both viruses. It might be that the differences in response are related to the type of plant-pathogen interactions or to difference in aggressiveness of PVY and TYLCV. Moreover, NahG-Désirée and NahG-Rywal may not represent “real” sensitive-PVY interaction, as they possess genetic background of tolerant and resistant potato genotypes. Perhaps higher similarity in sRNA’s response would be observed if sensitive potato cultivar such as cv. Igor or Nadine would be additionally analyzed.

Interestingly we found the link between tomato miR166 isomiR and *LRR-RLK* and *HD-ZIPIII*. Even though the upregulation of miR166 isomiR did not reduce the levels of *HD-ZIPIII*. This modulation could be important for disease symptoms development in cv. Moneymaker, as Yang and coworkers (2008) studying tomato yellow leaf curl China virus (TYLCCNV) showed that virus interacts with ASYMMETRIC LEAVES 1 (AS1) tomato protein which downregulates the miR166 levels and increases levels of *HD-ZIPIII* that cause alterations in leaf development resulting in the manifestation of disease symptoms (Yang et al., 2008).

Chapter 5

Conclusions

In this work, we demonstrated that TRIzol and *mirVana* total RNA extraction protocols are more suitable for RNA isolation compared to High Pure kit or short RNA enrichment protocols, as they provide the highest quality and yield of RNA, including sRNAs. We additionally showed that sRNA expression results were significantly affected by suboptimal efficiency of MultiScribe reverse transcriptase rather than RNA extraction method. By replacing MultiScribe enzyme for SuperScript III reverse transcriptase and employing pulsed cycling condition, both efficiency and repeatability of RT reaction were improved gaining more reliable sRNA expression results. Optimized RT-qPCR protocol was also shown to have a high concordance with sRNA-Seq results and thus can be employed as a validation tool of sRNA sequencing data in the future sRNA studies.

For isolation of both sRNAs and longer RNAs from a small amount of plant material, Direct-zol RNA MicroPrep (Zymo) extraction protocol was demonstrated as a preferable choice, providing high and non-biased sRNA recovery. The sRNA and total RNA quantities were also large enough for the use in sequencing or RT-qPCR analyses. Of note, both sRNAs and mRNAs are extracted together, which also reduces the time and costs if separate extractions for sRNAs and mRNAs would be performed. In some cases lower purity of the samples was observed. To reduce the possibility that contaminants present in RNA solution will interfere with downstream analyses, we also established fast and efficient RNA purification protocol (DNase-cleaning) that should be followed to clean low purity RNA samples. Besides efficient removal of contaminants we demonstrated that protocol is especially suitable for DNA removal from RNA samples prior to sequencing as it does not involve EDTA and heating step for DNase inactivation, which may negatively impact the integrity of low input RNA samples or interfere with ligation steps during library preparations and sequencing. Importantly, we showed that DNase-cleaning protocol is efficient in eliminating DNA and in providing pure RNA, efficient in binding of sRNAs and long RNAs and preserving their integrity as well as their expression level. Direct-zol RNA MicroPrep (Zymo) extraction protocol coupled with DNase-cleaning protocol also enabled us to obtain high-quality sRNA and RNAs to study the spatially regulated responses of sRNAs and their target genes in hypersensitive response of cv. Rywal to PVY infection.

We also established an optimized protocol for novel *MIR* loci and miRNA discovery using ShortStack prediction pipeline which was shown to be highly stringent. Even though pipeline can miss some of the true *MIR* loci and miRNAs, predictions can be regarded as high confidence miRNAs/*MIR* loci.

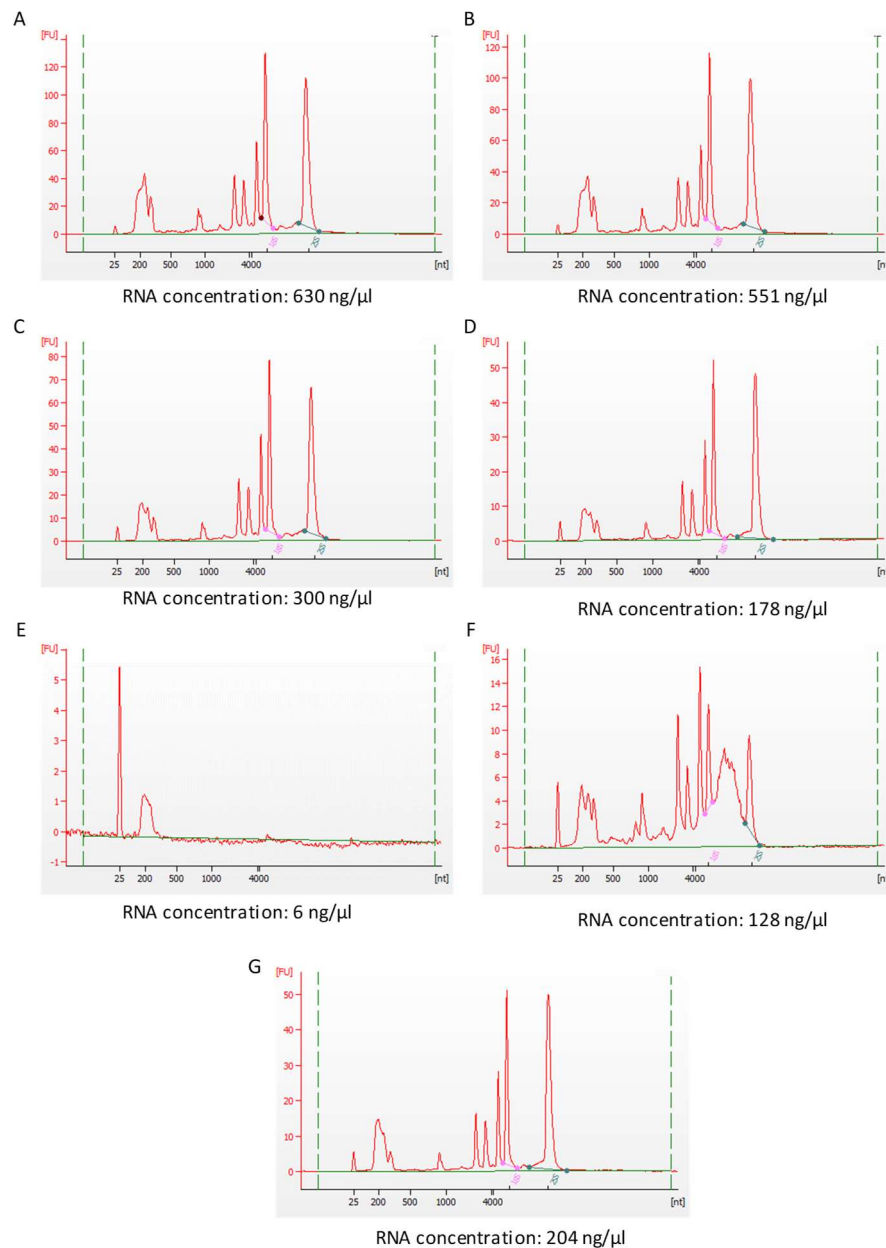
By employing high-throughput sequencing technology coupled with bioinformatics analyses, we identified many known and novel potato miRNAs, and phasiRNAs together with their producing loci. By comparing responses between tolerant and resistant plants with sensitive SA depleted counterpart plants (NahG) we showed that sRNA's response

differed depending on the type of interaction. Certain sRNAs were demonstrated to be important for modulation of plant tolerant response, while others were found to be involved in hypersensitive resistance response. By connecting sRNAs and their targets identified by *in silico* and Degradome-Seq approach, we also discovered many novel connections between sRNA regulatory network and hormonal (GA and auxin) signaling pathways (tolerant interaction) as well as components involved in ROS signaling (resistant interaction), which increased our understanding of sRNA-mediated regulation of plant immune responses in different potato cultivars. The sRNAs linked to tolerance (i.e. miR160a, miR167e, phasiRNA931, miR319a, miR390a) and/or resistance response (i.e. miR398c) identified within this study, can be further utilized as a valuable tool to manipulate target gene expression in susceptible genotypes to improve crop immunity, impair pathogen virulence, and increase disease resistance of potato to PVY.

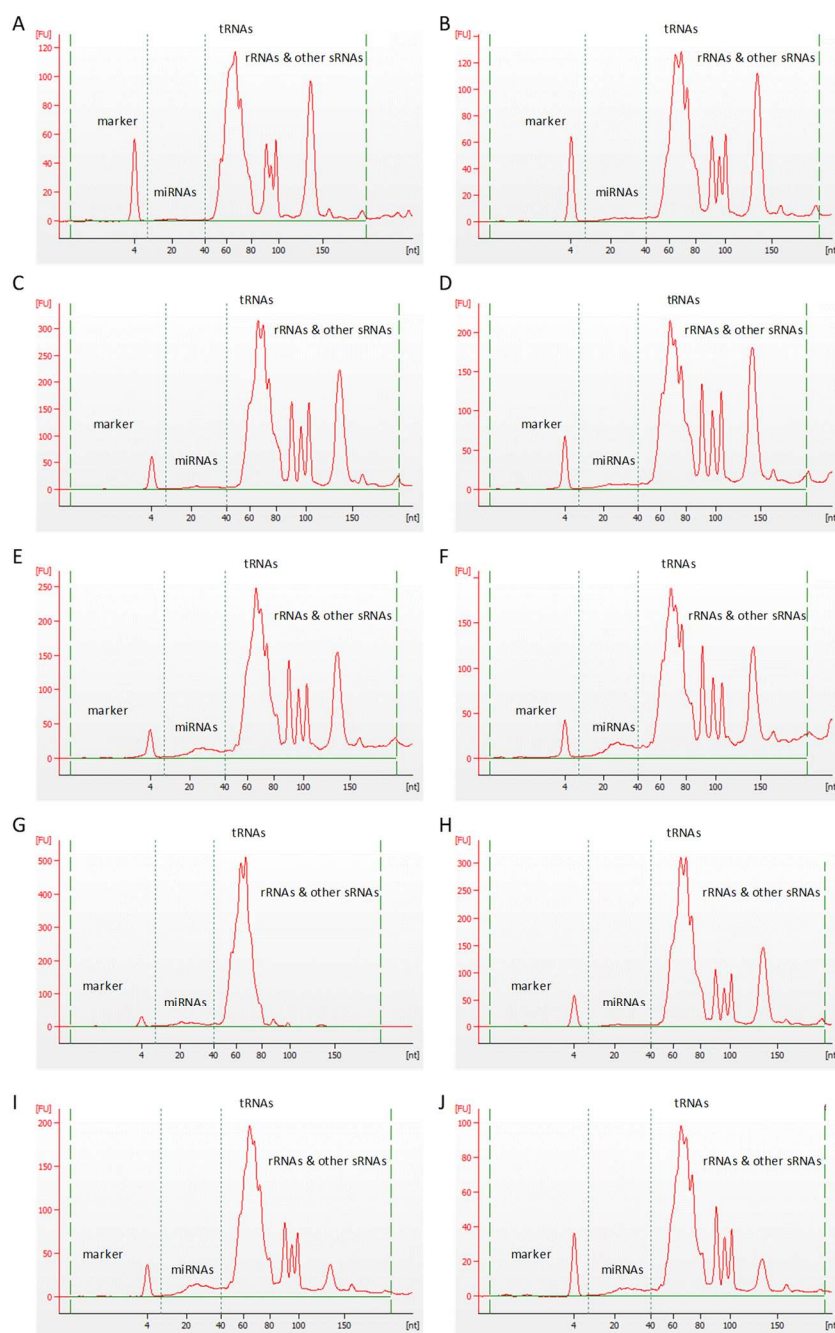
Appendix A

Supplementary Material

A.1 Supplementary Material for Section 3.1

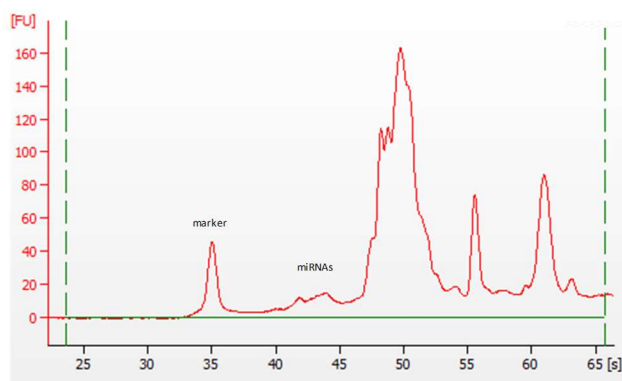


Supplementary Figure 1: Concentration and integrity of RNA extracted from homogenized potato plant material. Electropherograms showing the integrity of total RNA extracted by total RNA extraction protocols: A) and B) Trizol; C) and D) *mirVana* kit; E) and F) High Pure kit, or G) *mirVana* kit employing short RNA enrichment protocols. RNA samples were analyzed with the Agilent RNA 6000 Nano Kit on Agilent Bioanalyzer. The y-axis represents fluorescence units (FU) and the x-axis length of RNAs in nucleotides (nt); 18S – 18S rRNAs; 25S – 25S rRNAs.

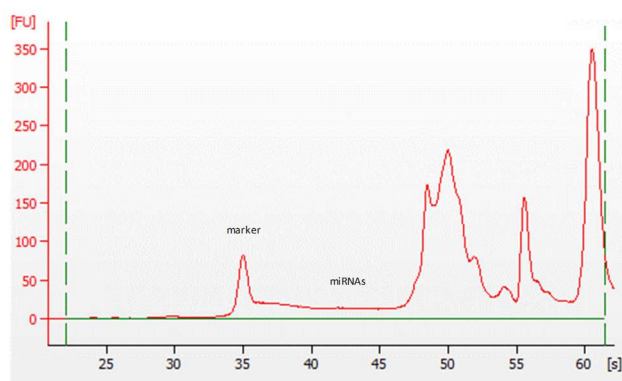


Supplementary Figure 2: Quantity and integrity of short RNAs extracted from homogenized potato plant material. Electropherograms showing the abundance and integrity of small RNA (0-200 nt) and miRNA (10-40 nt) fractions extracted by Trizol (A and B), *mirVana* (C and D) and High Pure (E and F) total RNA and *mirVana* (G and H) and High Pure (I and J) short RNA enrichment protocols. RNA samples were analyzed with the Agilent Small RNA Kit on Agilent Bioanalyzer. The y axis represents fluorescence units (FU) and the x axis length of small RNAs in nucleotides (nt).

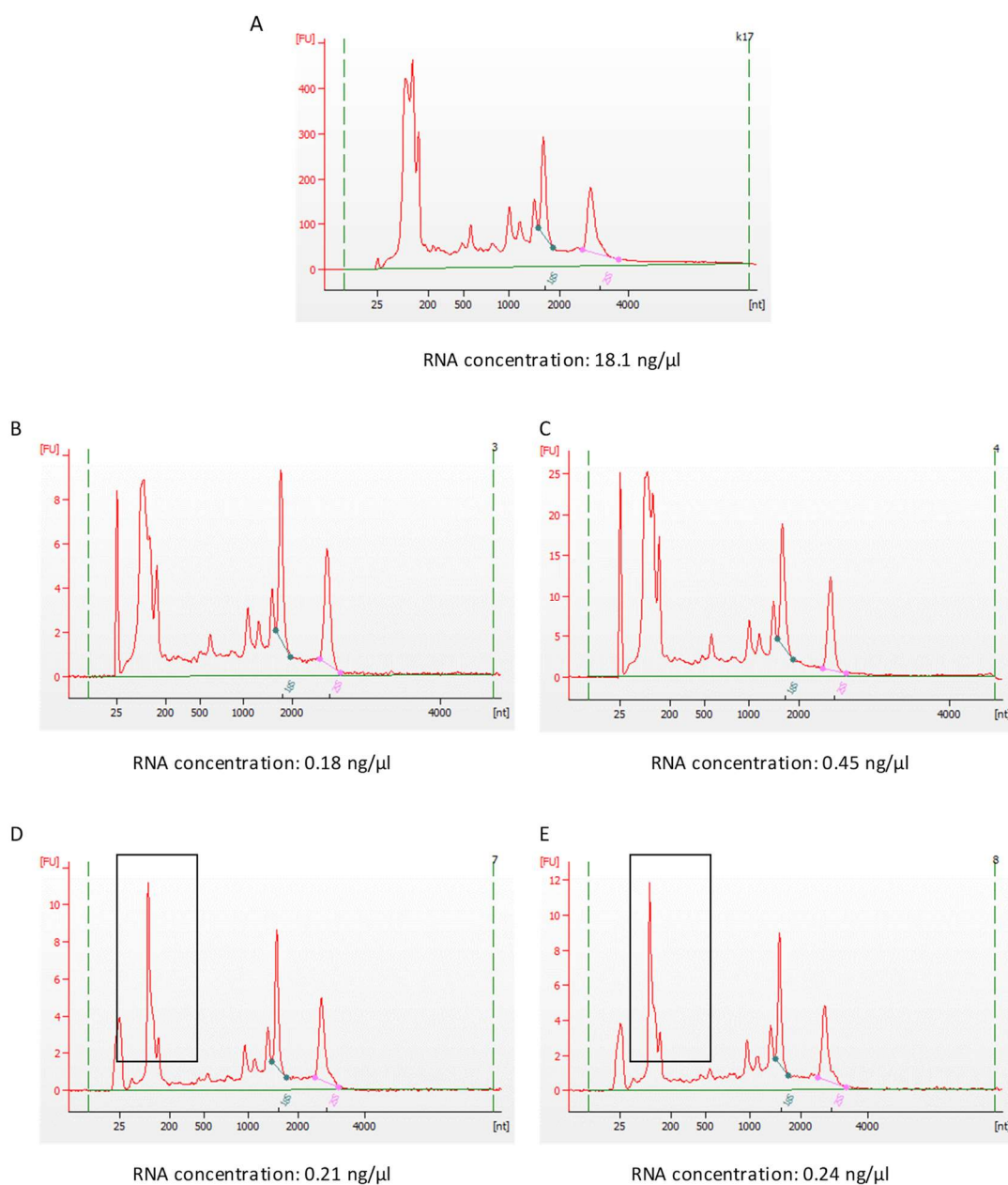
A



B



Supplementary Figure 3: Quantity and integrity of short RNAs obtained from small amount of plant material. Electropherograms showing the abundance and integrity of small RNA (0-200 nt) and miRNA (10-40 nt) fractions extracted by A) Direct-Zol micro kit (Zymo) and B) Single Cell RNA Purification Kit (Norgen). RNA samples were analyzed with the Agilent Small RNA Kit on Agilent Bioanalyzer. The y axis represents fluorescence units (FU) and the x axis the length of small RNAs in nucleotides (nt).



Supplementary Figure 4: Concentration and integrity of RNA prior and after RNA purification. A) Electropherogram showing the concentration and integrity of total RNA extracted by Direct-Zol micro kit and analyzed with the Agilent RNA 6000 Nano Kit on Agilent Bioanalyzer. B) and C) concentration and integrity of the same RNA sample after DNase-cleaning protocol (Zymo Research). D) and E) Concentration and integrity of the same RNA sample after DNase-EDTA protocol. All DNase-treated RNA samples were analyzed with the Agilent RNA 6000 Pico Kit on Agilent Bioanalyzer. The y axis represents fluorescence units (FU) and the x axis the length of RNAs in nucleotides (nt); 18S – 18S rRNAs; 25S – 25S rRNAs. Black squares indicate loss of short RNAs.

Supplementary Table 1: Pre-miRNA and miRNA sequences among three different miRNA prediction tools. The comparison was made on 28 *MIR* loci that were predicted by all three tools. pre-miRNAs and miRNA sequences are given for each prediction. Identical pre-miRNA sequences are highlighted in red.

<i>MIR</i> loci	pre-miRNA sequence			mature miRNA sequence		
	miR-PREFeR	miRCat	ShortStack	miR-PREFeR	miRCat	ShortStack
1	ATGGGAAGTGGAGA AACAACACTTGCTAA AAGAAGTTCATCAGT GATTCTCTCTCTTT TGGAGTTTTTTTAGC AAGAGTTGTTTTCCC TCTTCTCAA	TGGGAAGTGGAGAA ACAACACTTGCTAAA AGAAGTTCATCAGTG ATTCTCTCTTCTTT GGAGTTTTTTTAGCA AGAGTTGTTTTCCCT CTTCTCA	AAATTGGTAATGGGAA GTGGAGAAACAACACT TGCTAAAAGAAGTTCA TCAGTGATTCTCTCTT CTTTTGGAGTTTTTTT AGCAAGAGTTGTTTTC CCTCTTCTCAATCCG TCTCTC	TTTAGCAAGAGT TGTTTTCCC	TTTAGCAAGAGT TGTTTTCCC	TTTAGCAAGAGT TGTTTTCCC
2	GGAGAGAGCTTTTGT ATTGCGGGGAATGTT GTCTGGCTCGAGGAA TCAAATTCTTTATTA CTCATAGATCTGTAT ATTGTTTATAGATCT ATAATGTTTTTGACG TCGTCGGACCAGGCT TCATTCCCCACAATG AATATTGCTCTCTCA	GAGAGCTTTTGTATT GCGGGGAATGTTGT CTGGCTCGAGGAATC AAATTCTTTATTACT CATAGATCTGTATAT TGTTTATAGATCTAT AATGTTTTTGACGTC GTCGGACCAGGCTTC ATTCCCCACAATGAA TATTGCTCTC	AGAGAGCTTTTGTATT GCGGGGAATGTTGTCT GGCTCGAGGAATCAAA TTCTTTATTACTCATA GATCTGTATATTGTTT ATAGATCTATAATGTT TTTGACGTCGTCGGAC CAGGCTTCATTCCCCA CAATGAATATTGCTCT CTC	TCGGACCAGGCTT CATTCCCC	TCGGACCAGGCTT CATTCCCC	TCGGACCAGGCTT CATTCCCC
3	TGGGTTAGAACACTG GAGGCAGCGGTTTCAT CGATCTGTTCCCTGA AAAGCGATAAATATA GCAACAGGAAGCGA	GGGTTAGAACACTG GAGGCAGCGGTTCA TCGATCTGTTCCCTG AAAAGCGATAAATAT AGCAACAGGAAGCG	TTTTGTTGGGTTAGAA CACTGGAGGCAGCGGT TCATCGATCTGTTCCC TGAAAAGCGATAAATA TAGCAACAGGAAGCGA	TCGATAAACCTCTG CATCCAG	TCGATAAACCTCTG CATCCAG	TCGATAAACCTCTG CATCCAG

	TCGATAAACCTCTGC ATCCAGTGCTTAACCG	ATCGATAAACCTCTG CATCCAGTGCTTAACCC	TCGATAAACCTCTGCA TCCAGTGCTTAACCCG CCTCACATT			
4	TCTATGGGAATTGGT GGATTGGAAAGCTTT TATTTTCTTCTGATT AGCTTTCCAATTCCA CCCATTCCCTATGGT	CTATGGGAATTGGT GGATTGGAAAGCTTT TATTTTCTTCTGATT AGCTTTCCAATTCCA CCCATTCCCTATGG	GAGTTTTTCGAAAATC TATGGGAATTGGTGGA TTGGAAAGCTTTTATT TTCTTCTGATTAGCTT TCCAATTCCACCCATT CCTATGGTTTTTCGGT ATCCTCTC	TTTCCAATTCCACC CATTCCTA	TTTCCAATTCCACC CATTCCTA	TTTCCAATTCCACC CATTCCTA
5	CAGAGGAGGAGAAAA GAAGAGGGAAAAAT GGAGTGGGTGATAT TGTTGACAGAAGATA GAGAGCACATATGAT GAAATGCTGCTGCTG CTAAATCTGTCTGGA GAGTCCAACAATAAA TAAAAGCATTTCACT TCATTTGTGCTCTCT ATGCTTCCGTCATCC CTTTCACCCACTTTT CCTTCTATTTTCTC TTCCTCTC	TGACAGAAGATAGA GAGCACATATGATGA AATGCTGCTGCTGCT AAATCTGTCTGGAGA GTCCAACAATAAATA AAAGCATTTCACTTC ATTTGTGCTCTCTAT GCTTCCGTCA	AAATGGAGTGGGTGAT ATTGTTGACAGAAGAT AGAGAGCACATATGAT GAAATGCTGCTGCTGC TAAATCTGTCTGGAGA GTCCAACAATAAATA AAGCATTTCACTTCAT TTGTGCTCTCTATGCT TCCGTCATCCCTTTCA CCCACCTTTTCCTTC	TTGACAGAAGATA GAGAGCAC	TTGACAGAAGATA GAGAGCAC	TTGACAGAAGATA GAGAGCAC
6	AGTGGAGCTCCTTGA AGTCCAACAGAAGAT CTAACAGGTTAGATT GAGCTGCTGACCTAT GGATTCCCTCAGCCCT ATCTATTTATGATTT AACATATAGATAGCT TTGTGGGTTTGCATA	TAGTTGTTATGGGA GTGGAGCTCCTTGAA GTCCAACAGAAGATC TAACAGGTTAGATTG AGCTGCTGACCTATG GATTCCCTCAGCCCTA TCTATTTATGATTTA ACATATAGATAGCTT	TATTAGTTGTTATGGG AGTGGAGCTCCTTGAA GTCCAACAGAAGATCT AACAGGTTAGATTGAG CTGCTGACCTATGGAT TCCTCAGCCCTATCTA TTTATGATTTAACATA TAGATAGCTTTGTGGG	TTTGGATTGAAGG GAGCTCTA	TTTGGATTGAAGG GAGCTCTA	TTTGGATTGAAGG GAGCTCTA

	TGTCAGGAGCTTTAT TTTACCTTTTGTG ATCATTTTTTGGATT GAAGGGAGCTCTACA	TGTGGGTTTGCATAT GTCAGGAGCTTTATT TTACCTTTTGTGTTGA TCATTTTTTGGATTG AAGGGAGCTCTACAT TCGTATCTCCTTGCT A	TTTGCATATGTCAGGA GCTTTATTTTACCTTT TGTTTGATCATTTTTT GGATTGAAGGGAGCTC TACATTCGTATCTCCT TGCTAG			
7	TATGGGTGAAGGAG AGGTTATTGGAGTG GGAGGAATCGAAAG ATATAAGGATTTATT TATTTTTTCAATTG ATTGGAAAAATAGAA AAAAAAGAATTATG TCTTTCCGATGCCTC CCATTCCCATGGCTT CCGTTCACTTCTCCC ATT	GTGAAGGAGAGGTT ATTGGAGTGGGAGG AATCGAAAGATATAA GGATTATTTATTTT TTCAATTTGATTGGA AAAAATAGAAAAAAA AGAATTATGTCCTTC CGATGCCTCCCATT CCATGGCTTCCGTT AC	AAGGAGAGGTTATTGG AGTGGGAGGAATCGAA AGATATAAGGATTTAT TTATTTTTTCAATTG ATTGGAAAAATAGAAA AAAAAAGAATTATGTC TTTCCGATGCCTCCCA TTCCCATGGCTTCCGT TCAC	TATGTCTTTCCGAT GCCTCCCA	TATGTCTTTCCGAT GCCTCCCA	TATGTCTTTCCGAT GCCTCCCA
8	CTTGCTAGTGCAGCA CCATCAAGATTCACA TAGAAAATATGGACT ATGAAATGAAATATG CCCAATTTTTGAATA CATGAGAATCTTGAT GATGCTGCATTGGCA AA	TTGCTAGTGCAGCAC CATCAAGATTCACAT AGAAAATATGGACTA TGAAATGAAATATGC CCAATTTTTGAATAC ATGAGAATCTTGATG ATGCTGCATTGGCAA	TACAGTTGTTGCTTGC TAGTGCAGCACCATCA AGATTCACATAGAAAA TATGGACTATGAAATG AAATATGCCCAATTTT TGAATACATGAGAATC TTGATGATGCTGCATT GGCAAATTGATGACTT GAT	AGAATCTTGATGA TGCTGCAT	AGAATCTTGATGA TGCTGCAT	AGAATCTTGATGA TGCTGCAT
9	TGTTGAGGAGAATG TCGTCTGGTTCGAGA TCTTTCATAAGAAGG TAACATGCAATGAGA TGATTTTCGGACCAGG	GTTGAGGAGAATGT CGTCTGGTTCGAGAT CTTTCATAAGAAGGT AACATGCAATGAGAT	GACATGTAATATTGTT GAGGAGAATGTCGTCT GGTTCGAGATCTTTCA TAAGAAGGTAACATGC AATGAGATGATTTTCGG	TCGGACCAGGCTT CATTCCCC	TCGGACCAGGCTT CATTCCCC	TCGGACCAGGCTT CATTCCCC

	CTTCATTCCCCTCAA CT	GATTTCGGACCAGGC TTCATTCCCCTCAAC	ACCAGGCTTCATTCCC CTCAACTCTGATCCAA CAAGT			
10	AGGGAGCTTTTGGA GTGGGTGGCATGGT AAGATATCATATTTT GTTTTTCATAAAAAA TGGAATTGAAATCTT ACCGATTCCCCCAT TCCAATGGCTTCCA	GGGAGCTTTTGAG TGGGTGGCATGGTA AGATATCATATTTTG TTTTTCATAAAAAAT GGAATTGAAATCTTA CCGATTCCCCCAT CCAATGGCTTCC	GAATGTTTCAGGGAGC TTTTGGAGTGGGTGGC ATGGTAAGATATCATA TTTTGTTTTTCATAAA AAATGGAATTGAAATC TTACCGATTCCCCCA TTCCAATGGCTTCCAG TCATTTCTCC	TTACCGATTCCCCC CATTCCAA	TTACCGATTCCCCC CATTCCAA	TTACCGATTCCCCC CATTCCAA
11	TGGAGTGGGTGGTG TGGTAAGATTTTCATA TTTATTTTCCATAAA AATTGAGATCTTGCC AATACCGCCCATTC T	GGAGGTGTTGGAGT GGGTGGTGTGGTAA GATTTCATATTTATT TTCCATAAAAAATTGA GATCTTGCCAATAACC GCCCATTCCCTATGGC TTCC	GTGTTTCGGGGAGGTG TTGGAGTGGGTGGTGT GGTAAGATTTTCATATT TATTTTCCATAAAAAAT TGAGATCTTGCCAATA CCGCCCATTCCTATGG CTTCCGATCATTCT	TCTTGCCAATACCG CCCATTCC	TCTTGCCAATACCG CCCATTCC	TCTTGCCAATACCG CCCATTCC
12	ACACTGGAGGCAGC GGTTCATCGATCTGT TCCCTGAAAGCTGAA TATATATGCAAAAAT ACAGCAAAAGGAACC GATCGATAAACCTCT GCATCCAGTGC	GGTTGAAGACACTG GAGGCAGCGGTTCA TCGATCTGTTCCCTG AAAGCTGAATATATA TGCAAAAAATACAGCA AAAGGAACCGATCGA TAAACCTCTGCATCC AGTGCTTAACC	TTTAGTTGGTTGAAGA CACTGGAGGCAGCGGT TCATCGATCTGTTCCC TGAAAGCTGAATATAT ATGCAAAAATACAGCA AAAGGAACCGATCGAT AAACCTCTGCATCCAG TGCTTAACCCCTGCTA ATTG	TCGATAAACCTCTG CATCCAG	TCGATAAACCTCTG CATCCAG	TCGATAAACCTCTG CATCCAG
13	CAGTCGTTGTTTGCG TATGTAGCATAATCA AGATTACATGAAAA GTTGCAAATTTGTTA TATAATTGATGAAAT	AGTCGTTGTTTGCGT ATGTAGCATAATCAA GATTCACATGAAAAG TTGCAAATTTGTTAT ATAATTGATGAAATT	TACAGTCGTTGTTTGC GTATGTAGCATAATCA AGATTACATGAAAAG TTGCAAATTTGTTATA TAATTGATGAAATTAA	AGAATCTTGATGA TGCTGCAT	AGAATCTTGATGA TGCTGCAT	AGAATCTTGATGA TGCTGCAT

	TAATGGCTAGCTATT TGAAATTCATGAGAA TCTTGATGATGCTGC ATCAGCAATAAATGA CTA	AATGGCTAGCTATTT GAAATTCATGAGAAT CTTGATGATGCTGCA TCAGCAATAAATGAC T	TGGCTAGCTATTTGAA ATTCATGAGAATCTTG ATGATGCTGCATCAGC AATAAATGACTATTGC			
14	ATGGTCCTTTTTGTT TTTTTCCACAGCTTT CTTGAAGTGCATCTA ATTACAATTTCAATT CACAAAATGAATAAT TGATTCATATTGTTG TTGCGGTTCAATAAG GCTGTGGGAAGATA CAGATAGGGTCAA	TGGTCCTTTTTGTTT TTTTTCCACAGCTTTC TTGAAGTGCATCTAA TTACAATTTCAATTC ACAAAATGAATAATT GATTCATATTGTTGT TGCGGTTCAATAAGG CTGTGGGAAGATACA GATAGGGTCA	AAATGGTCCTTTTTGT TTTTTCCACAGCTTT CTTGAAGTGCATCTAA TTACAATTTCAATTCA CAAAATGAATAATTGA TTCATATTGTTGTGTC GGTTCAATAAGGCTGT GGGAAGATACAGATAG GGTCAAACAG	TTCCACAGCTTTCT TGAAGT	TTCCACAGCTTTCT TGAAGT	TTCCACAGCTTTCT TGAAGT
15	AGTCATGCTTTTCCA CAGCTTTCTTGAAGT TCTTCTTGCTAAATT TTGATCTCTAAATTG ATAATTTTGAGATGA AATTTTGAAGCTAT GAAAGTCCAAGAAA GCTGTGGGAAAAGA TGGCA	TTTTCCACAGCTTTC TTGAAGTCTTCTTG CTAAATTTTGATCTC TAAATTGATAATTTT GAGATGAAATTTTTG AAGCTATGAAAGTCC AAGAAAGCTGTGGG AAA	ACTAAGTCCTAGTCAT GCTTTTCCACAGCTTT CTTGAAGTCTTCTTG CTAAATTTTGATCTCT AAATTGATAATTTTGA GATGAAATTTTGAAG CTATGAAAGTCCAAGA AAGCTGTGGGAAAAGA TGGCAAGCTTTGGACT T	TTCCACAGCTTTCT TGAAGTT	TTCCACAGCTTTCT TGAAGTT	TTCCACAGCTTTCT TGAAGTT
16	TTAGGAAGATATATA TAGATATTGATGTGG CTCAATCTGAAGACA TGGCTAAATATGTAA TTAGCCATGTGATTT TGGATTGAGCCGCGT CAATATCTCTCTTT CCTAT	AGATATTGATGTGGC TCAATCTGAAGACAT GGCTAAATATGTAAT TAGCCATGTGATTTT GGATTGAGCCGCGTC AATATCT	ACGTTTAGGAAGATAT ATATAGATATTGATGT GGCTCAATCTGAAGAC ATGGCTAAATATGTAA TTAGCCATGTGATTTT GGATTGAGCCGCGTCA ATATCTCTCTCTTCCT ATTTCCAGTTAC	TTGAGCCGCGTCA ATATCTCT	TTGAGCCGCGTCA ATATCTCT	TTGAGCCGCGTCA ATATCTCT

17	CGAATCGCTCCGACG CCTTTGATGGTCACA TCTTCCGTTAGTCCT GTCGCAGATGACTTT CGCCCTTCTCATTC AGCCCCCTTTCTCT AAATCTAATTAATTT GATTTCAATGTGGTA GGACGAGAGTCATCT GTGACAGGATAATGC AAGATCTAGTTATCA AAGGCTTATTGGGGT CGTTCT	GTCCTGTCGCAGATG ACTTTCGCCCTTCTC ATTCCAGCCCCCTTT TCTCTAAATCTAATT AATTTGATTTCAATG TGGTAGGACGAGAG TCATCTGTGACAGGA T	GTCACATCTTCCGTTA GTCCTGTCGCAGATGA CTTTCGCCCTTCTCAT TCCAGCCCCCTTTCT CTAAATCTAATTAATT TGATTTCAATGTGGTA GGACGAGAGTCATCTG TGACAGGATAATGCAA GATCTAGTTAT	ACGAGAGTCATCT GTGACAGG	ACGAGAGTCATCT GTGACAGG	ACGAGAGTCATCT GTGACAGG
18	TCGCTCCGATGCCTT TGATGTTACATCTT CTGGTAGTCCTGTCG CAGATGACTTTCTCC CTTCTCATTACAGCCC CTTCTCTAAATCTA ATTAATTTGATTTCA ATGTGGTAGGACGA GAGTCATCTGTGACA GGATAATGGAAGAT CTAGTTATCAAAGGC TTATTGGGGCGT	GTCCTGTCGCAGATG ACTTCTCCCTTCTC ATTCAGCCCCCTTCT CTAAATCTAATTAAT TTGATTTCAATGTGG TAGGACGAGAGTCAT CTGTGACAGGAT	TTCACATCTTCTGGTA GTCCTGTCGCAGATGA CTTCTCCCTTCTCAT TCAGCCCCCTTCTCTA AATCTAATTAATTTGA TTTCAATGTGGTAGGA CGAGAGTCATCTGTGA CAGGATAATGGAAGAT CTAGTTAT	ACGAGAGTCATCT GTGACAGG	ACGAGAGTCATCT GTGACAGG	ACGAGAGTCATCT GTGACAGG
19	AAGTGAAGCTGCCAG CATGATCTAAACTTG GCCACACTTATAAAG AAAAAAATGGGTG ACTAAGGTTAGGTCA TGCTCGGACAGCCTC ACTC	AGTGAAGCTGCCAGC ATGATCTAAACTTGG CCACACTTATAAAGA AAAAAAATGGGTGA CTAAGGTTAGGTCAT GCTCGGACAGCCTCA CT	AAGGTCTAGAGTGGA CAAGTGAAGCTGCCAG CATGATCTAAACTTGG CCACACTTATAAAGAA AAAAAATGGGTGACTA AGGTTAGGTCATGCTC GGACAGCCTCACTCCT	TGAAGCTGCCAGC ATGATCTA	TGAAGCTGCCAGC ATGATCTA	TGAAGCTGCCAGC ATGATCTA

			TCCTTTCTTGATGAAA A			
20	TCGTGAACCACTAGC AGTTGAAGCTGCCAG CATGATCTAAACTTT TCTATTGCTCTGTAC GAGGAAAGATCAGA TCATGTGGCAGCATC ACCTGTTGATGGTTT CGCGG	TCGTGAACCACTAGC AGTTGAAGCTGCCAG CATGATCTAAACTTT TCTATTGCTCTGTAC GAGGAAAGATCAGA TCATGTGGCAGCATC ACCTGTTGATGGTTT CGCGG	GATCGTGAACCACTAG CAGTTGAAGCTGCCAG CATGATCTAAACTTTT CTATTGCTCTGTACGA GGAAAGATCAGATCAT GTGGCAGCATCACCTG TTGATGGTTTCGCGGA AT	TGAAGCTGCCAGC ATGATCTA	TGAAGCTGCCAGC ATGATCTA	TGAAGCTGCCAGC ATGATCTA
21	AGTTGAAGCTGCCAG CATGATCTAAACTTA CCTACAAATTTATAT TGAGGCAAGATCAG ATCATGTGGTTGCTT CACCC	GAAGCTGCCAGCATG ATCTAAACTTACCTA CAAATTTATATTGAG GCAAGATCAGATCAT GTGGTTGCTTC	TAGCGTATACCACTAT CAGTTGAAGCTGCCAG CATGATCTAAACTTAC CTACAAATTTATATTG AGGCAAGATCAGATCA TGTGGTTGCTTCACCC GTTAGTTCGTACTACG AAA	TGAAGCTGCCAGC ATGATCTA	TGAAGCTGCCAGC ATGATCTA	TGAAGCTGCCAGC ATGATCTA
22	TTTTACATGGTATGA GAACCAGATTCATCC CCGTTTGAGCTTTTCG GTTCTCGCTCAAACG GGGATGAACCTGGTT CTGATACCATGTAA C	TTACATGGTATGAGA ACCAGATTCATCCCC GTTTGAGCTTTTCGGT TCTCGCTCAAACGGG GATGAACCTGGTTCT GATACCATGTAA	CAAACTTAATTTTAC ATGGTATGAGAACCAG ATTCATCCCCGTTTGA GCTTTCGGTTCTCGCT CAAACGGGGATGAACC TGGTTCTGATACCATG TAAACCTAAGTGCGAA G	TATGAGAACCAGA TTCATCCC	TATGAGAACCAGA TTCATCCC	TATGAGAACCAGA TTCATCCC
23	TCTATAAAGCTCAGG AGGGATAGCACCATG AATACATCTTAATAA CTATCTATAGCGCTA	GGTATAGAGAATCTA TAAAGCTCAGGAGG GATAGCACCATGAAT ACATCTTAATAACTA TCTATAGCGCTATCC	ATTGGTATAGAGAATC TATAAAGCTCAGGAGG GATAGCACCATGAATA CATCTTAATAACTATC TATAGCGCTATCCATC	AAGCTCAGGAGGG ATAGCACC	AAGCTCAGGAGGG ATAGCACC	AAGCTCAGGAGGG ATAGCACC

	TCCATCTTGAGTTTT ATGGC	ATCTTGAGTTTTATG GCTTCTTCATGCT	TTGAGTTTTATGGCTT CTTCATGCTCGCTT			
24	TGTTATACAGAGAAA CCGCTGTCGAGTCTG ATAAAAAAATTTGGT CTTCTCATGCTCCAC AGCGTTTCTCTGTAT AACC	GTTATACAGAGAAAC CGCTGTCGAGTCTGA TAAAAAAATTTGGTC TTCTCATGCTCCACA GCGTTTCTCTGTATA AC	TGTTGGTCCTTACCAT TCTGTTATACAGAGAA ACCGCTGTCGAGTCTG ATAAAAAAATTTGGTC TTCTCATGCTCCACAG CGTTTCTCTGTATAAC CAAATGTTTTAGGGTC ATTAT	ACAGCGTTTCTCTG TATAACC	ACAGCGTTTCTCTG TATAACC	ACAGCGTTTCTCTG TATAACC
25	TGTATGTCAAAGATT GCAACACACCTGAAT CGTCAAATATTTTTT AATATATATTTGTTG ATTCAGGTTTGTATG CAAACATGACATAT T	GTATGTCAAAGATTG CAACACACCTGAATC GTCAAATATTTTTTA ATATATATTTGTTGA TTCAGGTTTGTATGC AAACTATGACATAT	TAGCCTTTGTATGTCA AAGATTGCAACACACC TGAATCGTCAAATATT TTTTAATATATATTTG TTGATTCAGGTTTGTA TGCAAACATGACATA TTTTAAAGGAT	TTGCAACACACCTG AATCGTC	TTGCAACACACCTG AATCGTC	TTGCAACACACCTG AATCGTC
26	ATGGCTCTTTTTGTA TTTTTCCACAGCTTT CTTGAAGTGCATATA TAATTATTCGATGAA GAGAGTTAAATTGTT GCGGTTCAATAAAGC TGTGGGAAGATATAT ATAGGGTCAA	TGGCTCTTTTTGTAT TTTTCCACAGCTTTC TTGAAGTGCATATAT AATTATTCGATGAAG AGAGTTAAATTGTTG CGGTTCAATAAAGCT GTGGGAAGATATAT ATAGGGTCA	AAATGGCTCTTTTTGT ATTTTTCCACAGCTTT CTTGAAGTGCATATAT AATTATTCGATGAAGA GAGTTAAATTGTTGCG GTTCAATAAAGCTGTG GGAAGATATATATAGG GTCAAGAAT	TTCCACAGCTTTCT TGAAGT	TTCCACAGCTTTCT TGAAGT	TTCCACAGCTTTCT TGAAGT
27	AGTTGATTTCTCGGG TGCTTACTCAACTAA TAGTTATTGTTTAAAG AAAATCAATAATATT GGAAGCAAGGAGAA TGGTGACTTTCAGGA TGATAACTATTGGTT	GTTGATTTCTCGGGT GCTTACTCAACTAAT AGTTATTGTTTAAAGA AAATCAATAATATTG GAAGCAAGGAGAAT GGTGACTTTCAGGAT GATAACTATTGGTTG	CTGAGATTTAGTTGAT TTCTCGGGTGCTTACT CAACTAATAGTTATTG TTTAAGAAAATCAATA ATATTGGAAGCAAGGA GAATGGTGACTTTCAG GATGATAACTATTGGT	TTGGTTGAGTGAG CATCTAAG	TTGGTTGAGTGAG CATCTAAG	TTGGTTGAGTGAG CATCTAAG

	GAGTGAGCATCTAAG AAATCGACG	AGTGAGCATCTAAGA AATCGAC	TGAGTGAGCATCTAAG AAATCGACGTGATTCT GAGA			
28	AATAGGGGCAACTTG AGATCACATGTTGTC ATTTTTCTTTTTAG TTTGATGATTTGTAT CTCAACATGTGTTCT CAGGTCGCCCCTGTC	GGTGAAAGTATTCAA TAGGGGCAACTTGA GATCACATGTTGTCA TTTTTCTTTTTTAGT TTGATGATTTGTATC TCAACATGTGTTCTC AGGTCGCCCCTGTCG AATTATTTTTCACC	ATTGGTGAAAGTATTC AATAGGGGCAACTTGA GATCACATGTTGTCAT TTTTCTTTTTTAGTTT GATGATTTGTATCTCA ACATGTGTTCTCAGGT CGCCCCTGTCGAATTA TTTTTCACCTAA	GGGGCAACTTGAG ATCACATG	GGGGCAACTTGAG ATCACATG	GGGGCAACTTGAG ATCACATG

Supplementary Table 2: Lengths and folding free energy values of predicted pre-miRNA precursors among three different miRNA prediction tools. The comparison was made on 28 *MIR* loci that were predicted by all three tools. pre-miRNAs length and folding free energy values are given for each predicted *MIR* loci.

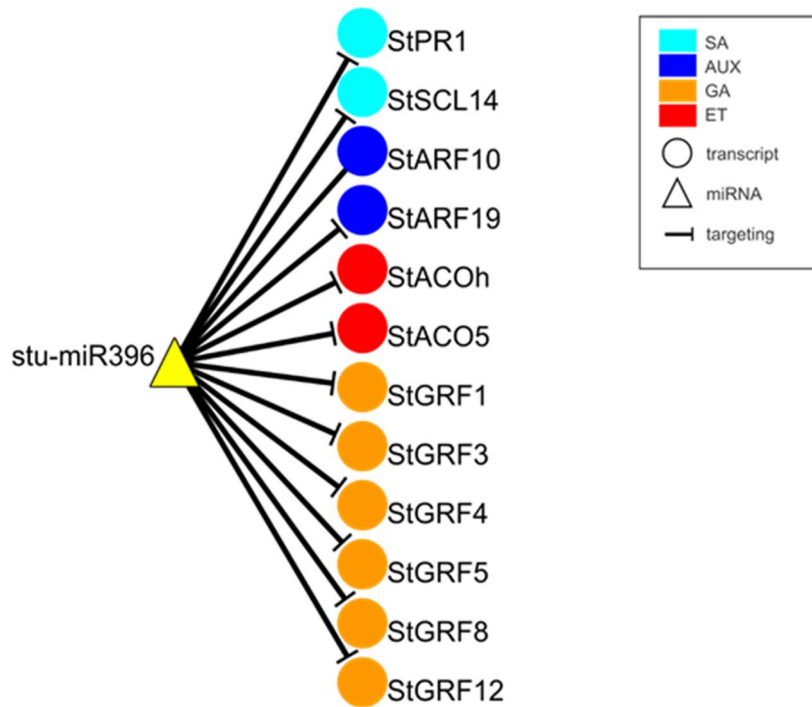
MIR loci	pre-miRNA length (nt)			folding free energy (kcal/mol/nt)		
	miR-PREFeR	miRCat	ShortStack	miR-PREFeR	miRCat	ShortStack
1	98	96	118	-0.43	-0.45	-0.37
2	150	144	147	-0.45	-0.43	-0.43
3	106	104	121	-0.48	-0.48	-0.42
4	74	72	104	-0.51	-0.52	-0.45
5	200	114	158	-0.48	-0.42	-0.41
6	180	210	214	-0.44	-0.40	-0.39
7	150	135	132	-0.44	-0.45	-0.40
8	107	105	131	-0.40	-0.64	-0.35
9	91	89	117	-0.50	-0.50	-0.39
10	102	100	122	-0.49	-0.50	-0.41
11	75	92	111	-0.56	-0.54	-0.47
12	100	114	132	-0.45	-0.44	-0.40
13	138	136	144	-0.37	-0.37	-0.35
14	132	130	138	-0.39	-0.41	-0.37
15	123	107	145	-0.39	-0.39	-0.36
16	110	82	124	-0.55	-0.63	-0.48
17	186	105	139	-0.41	-0.42	-0.36
18	175	102	136	-0.44	-0.42	-0.36
19	93	91	129	-0.46	-0.39	-0.42
20	109	109	114	-0.49	-0.49	-0.47
21	79	71	115	-0.38	-0.39	-0.33
22	91	87	113	-0.69	-0.71	-0.59
23	80	102	110	-0.39	-0.40	-0.36
24	79	77	117	-0.44	-0.45	-0.37
25	91	89	107	-0.42	-0.43	-0.39
26	115	113	121	-0.47	-0.47	-0.44
27	128	126	148	-0.45	-0.47	-0.44
28	90	118	124	-0.47	-0.47	-0.44

A.2 Supplementary Material for Section 3.2

Supplementary Table 3: List of genes referred to in the manuscript together with their descriptions and corresponding IDs. Gene symbols and potato gene names, their full descriptions and corresponding potato StNIB_v1 identifiers (IDs; Ramšak et al., 2014), together with potato IDs of paralogous genes (potato gene and unigene sequences StNIB_v1), used in the manuscript, are shown. Potato genes were named according to the *Arabidopsis thaliana* orthologs, obtained from GoMapMan (www.gomapman.org; Ramšak et al., 2014).

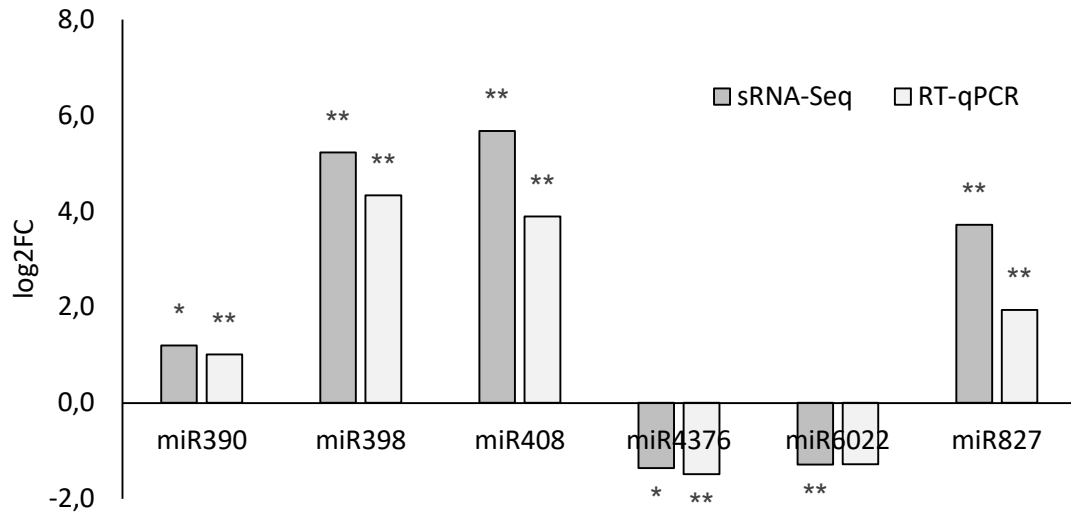
Gene symbol	Potato gene name	StNIB_v1 ID	Full description	IDs of all paralogs	<i>Arabidopsis thaliana</i> ortholog ID
ACD1	StACD1	Sotub03g022940	1-aminocyclopropane-1-carboxylic acid deaminase 1	Sotub03g022940	AT1G48420
ACO5	StACO5	Sotub02g024300	1-aminocyclopropane-1-carboxylate oxidase 5	MICRO.2939.C2; MICRO.2939.C3; TC197510; TC213446	AT1G05010
ACOh	StACOh	Sotub09g026280	1-aminocyclopropane-1-carboxylate oxidase homolog	Sotub09g026280; PGSC0003DMC400030140	AT1G03400; AT1G03410
AFB	StAFB1.1	Sotub06g008630	Auxin F-box protein 1	Sotub06g008630; TC197735; MICRO.9052.C2; MICRO.9052.C1; PGSC0003DMC400009476	AT1G12820; AT2G39940; AT3G26810; AT3G62980; AT4G03190; AT4G24390; AT5G49980
AFB	StAFB1.2	Sotub06g008900	Auxin F-box protein 1	Sotub06g008900; Sotub06g008780; PGSC0003DMC400036051; MICRO.13137.C1; TC219660; TC215657; Sotub06g008800	AT1G12820; AT2G39940; AT3G26810; AT3G62980; AT4G03190; AT4G24390; AT5G49980
AFB	StAFB1.3	Sotub06g034990	Auxin F-box protein 1	Sotub06g034990; PGSC0003DMC400041556	AT1G12820; AT3G26810; AT3G62980; AT4G03190
AFB	StAFB2	Sotub06g008730	Auxin signaling F-box 2	Sotub06g008730; PGSC0003DMC400038062; PGSC0003DMC400038063	AT1G12820; AT3G26810; AT3G62980; AT4G03190
AFB	StAFB3	Sotub06g008920	Auxin signaling F-box 3	PGSC0003DMC400064891; MICRO.16535.C1; TC215350; EGO13617; SDBN005N07u.scf	AT1G12820; AT2G39940; AT3G26810; AT3G62980; AT4G03190; AT4G24390; AT5G49980
AFB	StAFB5	Sotub02g022180	Auxin F-box protein 5	Sotub02g022180; PGSC0003DMC400038842; MICRO.15100.C1; TC196340	AT1G12820; AT3G26810; AT3G62980; AT4G03190
ANX1	StANX1	Sotub09g014230	Leucine-rich repeat receptor-like kinase	Sotub09g014230	AT3G51550
AP2	StAP2	Sotub10g024610	APETALA2	Sotub10g024610; PGSC0003DMC400021291; Sotub04g013820; PGSC0003DMC400021292; PGSC0003DMC400021293; PGSC0003DMC400019509; MICRO.5083.C3; TC217078; PGSC0003DMC400019512; Sotub02g031630; PGSC0003DMC400019514; PGSC0003DMC400007140; MICRO.5083.C1; PGSC0003DMC400019513; TC208874; TC211656; CK249226; MICRO.7449.C1	AT4G36920
ARF1	StARF1.1	Sotub01g040050	Auxin response Factor 1	Sotub01g040050	AT1G59750
ARF1	StARF1.2	Sotub01g038660	Auxin response Factor 1	Sotub01g038660	AT3G62290
ARF2	StARF2	Sotub03g031730	Auxin response factor 2	Sotub03g031730	AT5G62000
ARF10	StARF10	Sotub11g026490	Auxin response factor 10	Sotub11g014110; PGSC0003DMC400048339	AT2G28350; AT4G30080
ARF19	StARF19	Sotub07g008830	Auxin response factor 19	Sotub05g021800; PGSC0003DMC400024204	AT1G19220; AT5G20730
BGLU	StBGLU	Sotub10g021570	Beta-1 3-glucanase	Sotub10g021570	AT3G57240
BGLU3	StBGLU3	Sotub01g009310	Beta-1 3-glucanase 3	Sotub01g009310	AT3G57240; AT3G57260
CAT	StCAT	Sotub02g026140	Catalase	Sotub02g026140	AT4G35090
CathB	StCathB	Sotub02g015280	CathepsinB	Sotub02g015280; TC199372	AT1G02300; AT1G02305; AT4G01610
CSD	StCSD	Sotub11g024220	Superoxide dismutase	Sotub11g024220	AT2G28190
DELLA	StDELLA	Sotub02g037540	DELLA protein	Sotub11g008520; PGSC0003DMC400027527	AT1G14920; AT2G01570; AT3G03450
DWF4	StDWF4	Sotub02g028500	Dwarf4	Sotub02g028500	AT3G50660

EIN4	StEIN4	Sotub11g005240	Ethylene insensitive 4	Sotub11g005240	AT3G04580
ERF2a	StERF2a	Sotub01g009690	Ethylene responsive transcription factor 2a	PGSC0003DMG400046911; Sotub01g009590.1.1; Sotub01g009600.1.1; Sotub01g009610.1.1; Sotub01g009640.1.1; Sotub01g009660.1.1; Sotub01g009670.1.1; Sotub01g009690.1.1	AT1G03800; AT1G04370
F25G13.70	StF25G13.70	Sotub05g018740	Auxin induced-like protein	Sotub05g018740	AT3G25290; AT4G12980; AT4G17280; AT5G35735; AT5G47530
GA1	StGA1	Sotub06g034690	GA requiring 1	Sotub06g034690	AT4G02780
GA20ox	StGA20ox	Sotub10g011620	Gibberellin 20-oxidase	Sotub10g011620; PGSC0003DMC400061378	AT4G25420AT5G51810
GA20ox	StGA20ox1	Sotub03g007160	Gibberellin 20-oxidase-1	Sotub03g007160; Sotub06g009210; PGSC0003DMC400041949	AT4G25420
GA20ox	StGA20ox3	Sotub11g029030	Gibberellin 20-oxidase 3	Sotub11g029030	AT4G25420AT5G51810
GA20ox	StGA20ox4	Sotub01g031210	Gibberellin 20-oxidase 4	Sotub01g031210; MICRO.15941.C1; PGSC0003DMC400000034; PGSC0003DMC400000035	AT4G25420AT5G51810
GA3ox	StGA3ox	Sotub06g023360	Gibberellin 3-oxidase	Sotub06g023360; PGSC0003DMC400028884	AT1G15550; AT1G80340; AT4G21890
GRF1	StGRF1.1	Sotub02g030550	Growth-regulating factor 1	BF186907; MICRO.328.C1; Sotub04g030450; PGSC0003DMC400008794; PGSC0003DMC400037092; Sotub02g030550; TC211003	AT2G22840; AT4G37740
GRF1	StGRF1.2	Sotub07g014780	Growth-regulating factor 1	Sotub07g014780; MICRO.3833.C1; TC217359; PGSC0003DMC400023044	AT2G06200
GRF3	StGRF3	Sotub08g008530	Growth-regulating factor 3	Sotub08g008530; cSTB21A15TH; PGSC0003DMC400032221; Sotub08g021020; PGSC0003DMC400053791; PGSC0003DMC400032220; PGSC0003DMC400032219; PGSC0003DMC400032217	AT2G06200; AT2G22840; AT2G36400; AT2G45480; AT3G13960; AT3G52910; AT4G24150; AT4G37740; AT5G53660
GRF4	StGRF4	Sotub03g016800	Growth-regulating factor 4	Sotub03g016800; PGSC0003DMC400005813	AT5G53660
GRF5	StGRF5	Sotub12g028430	Growth-regulating factor 5	MICRO.4557.C1; PGSC0003DMC400051174	AT2G06200; AT2G22840; AT2G36400; AT2G45480; AT3G13960; AT3G52910; AT4G24150; AT4G37740; AT5G53660
GRF5	StGRF5	Sotub12g028430	Growth-regulating factor 5	MICRO.4557.C1; PGSC0003DMC400051174	AT2G06200; AT2G22840; AT2G36400; AT2G45480; AT3G13960; AT3G52910; AT4G24150; AT4G37740; AT5G53660
GRF8	StGRF8	Sotub08g027110	Growth-regulating factor 8	Sotub08g027110; TC219670; MICRO.1264.C1; PGSC0003DMC400021643	AT4G24150
GRF12	StGRF12	Sotub01g030660	Growth-regulating factor 12	Sotub01g030660; PGSC0003DMC400022619	AT2G45480
HD-ZIPIII	StHD-ZIPIII	Sotub12g022220 Sotub11g026520	Class III homeodomain-leucine zipper	Sotub12g022220, Sotub11g026520	AT1G52150, AT5G60690
HSP90	StHSP90	Sotub12g013560	Heat shock protein 90	Sotub12g013560	AT5G52640; AT5G56000; AT5G56010; AT5G56030
IAA16	StIAA16	Sotub01g034780	Indoleacetic acid-induced protein 16	Sotub01g034780	AT3G04730
IAR3	StIAR3	Sotub12g009860	IAA-amino acid hydrolase IAR3	Sotub12g009860	AT1G51760; AT1G51780
ILR1	StILR1	Sotub06g018160	IAA-amino acid hydrolase ILR1	Sotub06g018160	AT3G02875
LOX1	StLOX1	Sotub01g036960	Lipoxygenase 1	Sotub01g036960	AT1G55020
LRR-RLK	StLRR-RLK	Sotub01g006120	Leucine-rich repeat receptor-like kinase	Sotub01g006120; Sotub01g006170; Sotub01g017420;	AT1G71390; AT1G71400
MYB33	StMYB33	Sotub06g030530	MYB domain protein 33	Sotub06g030530	AT5G06100
OPR1	StOPR1	Sotub11g017720	12-oxophytodienoate (OPDA) reductase	Sotub11g017720	AT1G76680; AT1G76690
PR1	StPR1.1	Sotub01g043930	Pathogenesis-related protein 1	Sotub09g006110	AT1G50060
PR1	StPR1.2	Sotub01g043880	Pathogenesis-related protein 1	TC224536	AT2G14580; AT2G14610
SAUR45	StSAUR45	Sotub10g025790	Small auxin upregulated RNA 45	Sotub10g025790	AT2G36210
SCL14	StSCL14	Sotub04g010480	Scarecrow-like 14	Sotub04g010480; PGSC0003DMC400011269	AT1G07520; AT1G07530; AT2G29060; AT2G37650
SN1	StSN1	Sotub04g031180	Snakin-1	Sotub04g031180	AT2G14900
SPL11	StSPL11	Sotub05g016440	Squamosa promoter-binding-like protein 11	Sotub05g016440	AT5G43270
TIR1	StTIR1	Sotub09g022650	Transport inhibitor response 1	Sotub09g022650; PGSC0003DMC400020096; POADF41TV; PGSC0003DMC400020095; MICRO.1332.C1; TC213418	AT3G62980
TMK1	StTMK1	Sotub01g046210	Receptor-like serine/threonine-protein kinase	Sotub01g046210	AT2G01820; AT1G66150

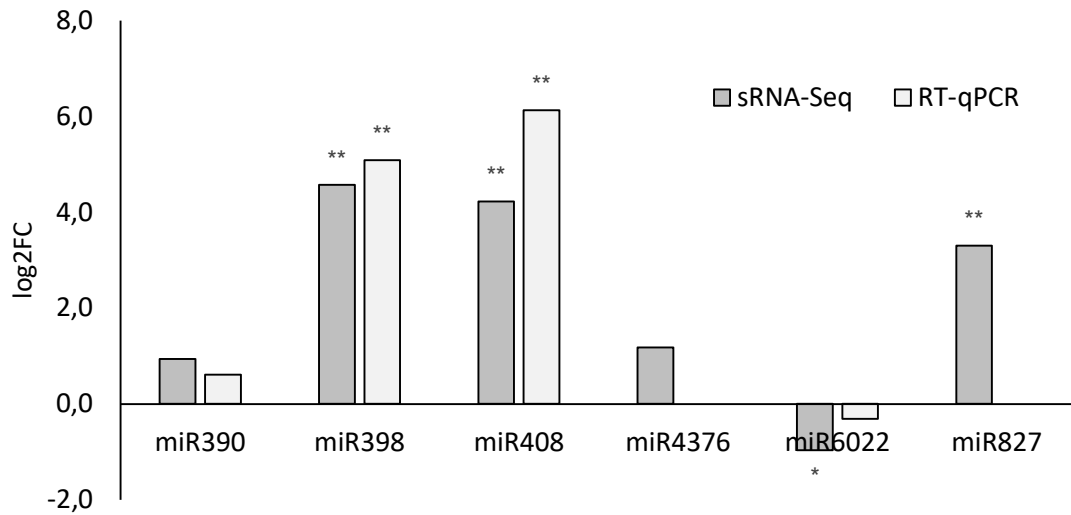


Supplementary Figure 5: miR396-auxin signaling network. miR396 targets as detected by *in silico* prediction and degradome sequencing. miR396 exhibited diverse target repertoire from already well known conserved targets such as *GRFs*, to transcripts coding for GRAS protein SCARECROW-like 14 (StSCL14), ACOs involved in the ethylene biosynthesis, ARFs involved in auxin signaling and even pathogenesis-related protein 1 (PR1). Thus, besides their important developmental role, miR396 family is also shown to be involved in the control of phytohormone- and defense-related genes. Similarly to the study of Hewezi et al. (2012), in which many miR396-GRF-regulated genes were discovered to be involved in phytohormone signaling pathways. Node shapes represent components: triangle – miRNA; circle – transcripts, while node colors indicate different signaling pathways-related components: turquoise – salicylic acid (SA); blue – auxin (AUX); orange – gibberellin (GA); red – ethylene (ET). Arrows connect miRNA and its targets. Node stu-miR396 represents four miR396 family members stu-miR396-5p, stu-miR396a,b-5p.1, stu-miR396a,b-5p and stu-miR396,a,b-5p.1, node StPR1 represents StPR1.1 and StPR1.2 and node StGRF1 represents StGRF1.1 and StGRF1.2. Gene name symbols are explained in Supplementary Table 3.

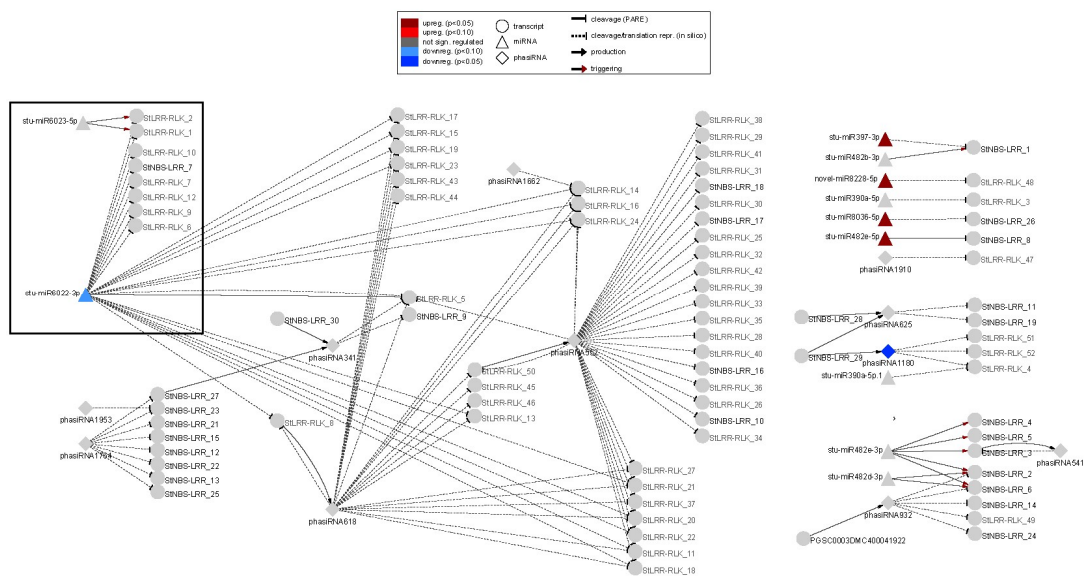
A Désirée



B NahG-Désirée



Supplementary Figure 6: Validation of sRNA-Seq results by stem-loop RT-qPCR. The $\log_2$ ratios of expression of six miRNA in PVY^{NTN}-infected samples versus mock inoculated samples of potato cv. (A) Désirée or (B) NahG-Désirée as determined by sRNA-Seq (dark grey) or RT-qPCR (light grey). The expressions of miR827 and miR4376 in NahG-Désirée samples were under the limit of quantification. Asterisks indicate statistically significant changes (**p-value < 0.05; *p-value < 0.1). miR390 – stu-miR390-5p; miR398 – stu-miR398a-5p; miR408 – stu-miR408b-5p; miR4376 – stu-miR4376-5p.1; miR6022 – stu-miR6022-3p; miR827 – stu-miR827-5p.



Supplementary Figure 7: Regulatory network of sRNAs and *R* genes in NahG-Désirée. *NBS-LRRs* and *LRR-RPKs* targets of differentially expressed sRNAs in NahG-Désirée plants at the onset of viral replication. Node shapes represent different components: triangle – miRNA; diamond – phasiRNA; circle – transcript and rectangle – metabolite. Node colors indicate expression changes: red – upregulated; blue – downregulated; and grey – no significant difference in expression. Blocked solid arrow – cleavage observed by degradome-sequencing, blocked dashed-line arrow – *in silico* predicted cleavage, dashed-line arrow with circle – *in silico* predicted translational repression. Black solid line with red arrow connects miRNA trigger and their *PHAS* loci. Black solid line with black arrow connects *PHAS* locus and its producing phasiRNAs. Black rectangle indicates miR6022-LRR-RLKs interaction. For more details of interactions between sRNA-target see Appendix Supplementary Dataset 6 and 7.

A.3 Supplementary Datasets

Supplementary Datasets are deposited at the following address:

<https://oblak.nib.si/index.php/s/4nODJgWGtsUkrqZ>

Supplementary Dataset 1: Known and novel miRNAs identified in PVY- and mock-inoculated samples of five analyzed potato cultivars together with their expression levels.

Supplementary Dataset 2: Novel *MIR* loci and miRNAs discovered in potato.

Supplementary Dataset 3: Potato *PHAS* loci identified by genome and transcriptome-wide phasing analysis.

Supplementary Dataset 4: phasiRNAs expression profiling.

Supplementary Dataset 5: Predicted 22-nt miRNA triggers of phasiRNA production.

Supplementary Dataset 6: Predicted targets of endogenous potato sRNAs by *in silico* approach.

Supplementary Dataset 7: Experimentally validated targets of endogenous potato sRNAs by Degradome-Seq.

Supplementary Dataset 8: PVY-derived siRNAs.

Supplementary Dataset S9: Targets of PVY-derived siRNAs identified by Degradome-Seq in potato.

Supplementary Dataset 10: Phytohormone concentrations at 3 dpi in the PVY^{NTN}-infected and mock inoculated samples of Désirée and NahG-Désirée potato plants.

Supplementary Dataset 11: Regulatory elements in the miRNA promoter regions of potato.

Supplementary Dataset S12: SA-dependent gene expression changes in cv. Desiree and cv. Rywal.

Supplementary Dataset 13: Differentially expressed sRNAs and/or their differentially expressed *in silico* predicted targets in tomato cv. Moneymaker in response to TYLCV infection at 2 dpi and 7 dpi.

Supplementary Dataset 14: Differentially expressed sRNAs and/or they differentially expressed experimentally validated targets in tomato cv. Moneymaker in response to TYLCV infection at 2 dpi and 7 dpi.

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Publications Related to the Thesis

Journal Articles

Križnik, M., Petek, M., Dobnik, D., Ramšak, Ž., Baebler, Š., Pollmann, S., Kreuze, J., Gruden, K. (2017). Salicylic Acid Perturbs sRNA-Gibberellin Regulatory Network in Immune Response of Potato to Potato virus Y Infection, *Front. Plant Sci.* 8:2192.
doi: 10.3389/fpls.2017.02192

Stare, T., Ramšak, Ž., Križnik, M., Gruden, K. (2019). Multi-omics analysis of potato response to potato virus Y infection, *Scientific Data* (accepted for publication).

Pompe-Novak, M., Križnik, M., and Gruden, K. (2018). The Titre of the Virus in the Inoculum Affects the Titre of the Viral RNA in the Host Plant and the Occurrence of the Disease Symptoms, *Acta chimica slovenica*, 65:1–5.
doi:10.17344/acsi.2019.4585

Biography

Maja Križnik was born on April 4, 1989 in Celje, Slovenia. She started studying Biology at the Biotechnical Faculty, University of Ljubljana, Slovenia, in 2008. During the 3rd year of the study, she was involved in the project work within the subject Physiology of Plants where she was investigating the effects of light intensity and temperature on photosynthesis of common duckweed (*Lemna minor* L.), under the supervision of Asst. Prof. Matevž Likar (Biotechnical Faculty, University of Ljubljana). The experimental work resulted in a scientific publication entitled “*Vpliv jakosti svetlobe in temperature na fotosintezno aktivnost male vodne leče*” (co-authored by Buh S., Dovč P., Fegeš A., Fortič A., Gojkovič M., Jurček A., Novak L., Žnidarič M.). Due to her interest in molecular biology, more specifically in plant-pathogen interactions, in 2010, she joined Prof. Dr. Kristina Gruden’s research group at the Department of Biotechnology and Systems Biology of the National Institute of Biology, where she started studying the correlation between the concentration of the virus in the inoculum used for potato infection and induction of disease symptom development in inoculated plants, under the supervision of Asst. Prof. Maruša Pompe Novak and Prof. Dr. Kristina Gruden. The result was a scientific publication entitled “*The titre of the virus in the inoculum affects the titre of the virus in the host plant and the occurrence of the disease symptoms*” (co-authored by Pompe-Novak M. and Gruden K.). In 2011, she finished her Bachelor's degree in Biology and continued working in Prof. Dr. Kristina Gruden’s research group. In 2014, she graduated with a Master's degree in Microbiology with the diploma thesis entitled “*Vloga malih RNA (miRNA) pri odgovoru rastlin krompirja (Solanum tuberosum L.) na okužbo s krompirjevim virusom Y*”, under the supervision of Asst. Prof. Blagajana Herzog Velikonja (Biotechnical Faculty, University of Ljubljana) and Prof. Dr. Kristina Gruden (National Institute of Biology). Part of her experimental work resulted in a scientific publication “*Fluorescently tagged Potato virus Y : a versatile tool for functional analysis of plant-virus interactions*” (co-authored by Rupar, M., Faurez F., Tribodet M., Gutiérrez-Aguirre I., Delaunay A., Glais L., Dobnik, D., Gruden K., Jacquot E., Ravnikar M.). In 2014, she started working as a young researcher at the Department of Biotechnology and Systems Biology, under the supervision of Prof. Dr. Kristina Gruden. In the same year she enrolled in the third-level study program Nanosciences and Nanotechnologies at the Jožef Stefan International Postgraduate School, Ljubljana, Slovenia. Her research is focused on investigation of interaction between *Potato virus Y* (PVY) and potato, in particular in revealing the roles of RNA silencing-based immune responses, mediated by sRNA-regulated pathways. Her main focus of research is to establish the optimal workflows for wet and dry lab sRNA experiments and to identify key players in sRNA-regulatory networks that modulates different outcomes of the plant-virus interactions (e.g. susceptibility, tolerance, resistance). In 2015, based on her PhD research results, she published her first first-author publication entitled “*Salicylic Acid Perturbs sRNA-Gibberellin Regulatory Network in Immune Response of Potato to Potato virus Y Infection*” (co-authored by Petek M., Dobnik D., Ramšak Ž., Baebler Š., Pollmann S., Kreuze J., Žel J., Gruden K.). Throughout her study she attended several scientific meetings to extend the knowledge of her research topics and to present her work to an international scientific community. She actively participated in several workshops on the topic of omics data integration, bioinformatics and systems biology. Moreover, Maja actively participated in events aimed at educating the public, such as Fascination of Plants Day, Researchers' Night and Znanstival.