Investigating the role of miRNA160 and miRNA166 in defence response of potato

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By

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CERTIFICATE

Certified that the work incorporated in the thesis entitled, **"Investigating the role of miRA160 and miRA166 in defence response of potato"** submitted by **Mr. Harpreet Singh Kalsi** (Regn. No - 20132005) was carried out by the candidate, under my supervision. The work presented here or any part of it has not been included in any other thesis submitted previously for the award of any degree or diploma from any other University or Institution.

Date: 17th January, 2022.

Prof. A. K. Banerjee, IISER Pune,
(PhD Thesis Supervisor).

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DECLARATION

I declare that this written submission represents my ideas in my own words and where others' ideas have been included; I have adequately cited and referenced the original sources. I also declare that I have adhered to all principles of academic honesty and integrity and not misrepresented or fabricated or falsified any idea/data/fact/source in my submission. I understand that violation of the above will be cause for disciplinary action by the Institute and can also evoke penal action from the sources that have not been properly cited or from whom proper permission has not been taken when needed.

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Abbreviations

PAMP	Pathogen Associated Molecular Patterns
MAMP	Molecular Associated Molecular Patterns
PRR	Pattern Recognition Receptors
PTI	PAMP Triggered Immunity
R-protein	Resistance Protein
ETI	Effector Triggered Immunity
ETS	Effector Triggered Susceptibility
EF-Tu	Elongation Factor Tu
flg22	Bacterial PAMP, Flagellin 22
FLS2	Flagellin-Sensing 2
LPS	Lipopolysaccharide
PGN	Peptidoglycan
LRR-RLK	Leucine-Rich Repeat Rlk
BAK1	LRR-RLK Brassinosteroid Insensitive 1-Associated Kinase 1
BIK1	Botrytis-Induced Kinase 1
RLCK	A Receptor-Like Cytoplasmic Kinase
PBL1	Pbs1-Like 1
RBOHD	Respiratory Burst Oxidase Homolog D
MAPK	Mitogen-Activated Protein Kinases

MKK/MAP2K	MAPK Kinase
MAP3K/MEKK	MAPK Kinase Kinase
WRKY	W-Box Domain Containing Protein
ERF	Ethlyene Responsive Factor
TF	Transcription Factor
BSK1	BR Signalling Kinase 1
KAPP	Kinase-Associated Protein Phosphatase
BR	Brassinosteroid
BIR2	BAK1-Interacting Receptor Kinase 2
PUB	Plant U-Box E3 Ubiquitin Ligase Protein
T3SS	Type III Secretion System
Avr	Avirulent
SFD1/GLY1	Suppressor Of Fatty Acid Desaturase Deficiency 1
HopF2	Effector molecule
NOD-like receptor	Nucleotide-Binding Oligomerization Domain –Like Receptor
RPS2	Resistance To Pseudomonas Syringae 2
RPMI	RESISTANCE To PSEUDOMONAS SYRINGAE PV. MACULICOLA 1
PCD	Programmed Cell Death
HR	Hypersensitive Response
ALD1	AGD2-Like Defense Response Protein 1
AZI1	Aza Induced 1

RIN4	Rpm1-Interacting Protein 4
CDPKs	Calcium-Dependent Protein Kinases
SAR	Systemic Acquired Resistance
ROS	Reactive Oxygen Species
PP2A	Protein Phosphatase 2a
MeSA	Methyl Salicylate
SA	Salicylic Acid
Aza	Azelaic Acid
Pip	Pipicolic Acid
FMO1	Flavin-Dependent Monooxygenase 1
DA	Dehydroabietinal
DIR1	Defective In Induced Resistance 1
G3P	Glycerol-3-Phosphate
BSMT1	Benzoic Acid/Salicylic Acid Carboxyl Methyltransferase 1
NPR1	Nonexpressor Of Pathogenis Related Genes 1
SFD1	Suppressor Of Fatty Acid Desaturase Deficiency 1
JA	Jasmonic Acid
G3Pdh	Glycerol-3-Phosphate Dehydrogenase
SAMT	SA Carboxyl Methyltransferase
MeBA	Methyl Benzoic Acid
SABP2	Salicylic Acid-Binding Protein 2

TMV	Tobacco Mosaic Virus
PEX	Plant Exudate
K361M	Kinase-Impaired Version Of MEKK1
PAD4	Phytoalexin Deficient 4
EDS1	*Enhanced Disease Susceptible 1*
ET	Ethylene
TGA	Tgacg Sequence-Specific Binding Protein
DOF	DBA Binding With One Finger
cpr5	Constitutive Expressor Of PR5
acd6-1	Accelerated Cell Death
agd2	Aberrant Growth And Death
nahG	Salicylate hydroxylase gene
GRX480	NPR1-Dependent Glutaredoxin
BTH	Benzothiadiazole S-Methylester
ABA	Abscisic Acid
cev1	Constitutive Expression Of Vegetative Storage Protein 1
coi1	Coronatine-Insesetive 1
PR	Pathogenesis Related
(SCF)COI1	Skp1–Cullin–F-Box (SCF)COI1 Ubiquitin E3 Ligase Complex
JA-Ile	Jasmonoyl-L-Isoleucine
JAV1	J ASMONATE-ASSOCIATED VQ MOTIF GENE 1

JMT	Jasmonic Acid Carboxyl Methyltransferase
MeJA	Methyl Jasmonic Acid
OPDA	12-Oxophytodienoic Acid
OPR	12-Oxophytodienoic Acid Reductase
AOS	Allene Oxide Synthase
AOC	Allene Oxide Cyclase
PIF	Phytochrome interacting factor
IAA	Indole-3-Acetic Acid
InsP5	Inositol Pentakisphosphate
EAR	ERF-Associated Amphiphilic Repression
MYC2	JASMONATE INSENSITIVE 1
JAZ	Jasmonate ZIM-domain
TPRs	TPL-Related Proteins
EIN	Ethylene Insensitive
EIL1	Ethylene Insensitive3-Like1
ASA1	Anthranilate Synthase A1
YUCCA2	TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS
TOE	Target Of Eat
CMA	Coronamic Acid
CFA	Coronafacic Acid
miR	MicroRNA

DCL1	Dicer Like 1
HYL1	HYPONASTIC LEAVES 1
SE	Serrate
HEN1	HUA Enhancer 1
DDL	Dawdle
Pre-miR	Precursor miR
HST	HASTY
AGO1	Argonaute 1
RISC	RNA-INDUCED SILENCING COMPLEX
NBS	Nucleotide Binding Site
ARF	Auxin Response Factor
ARR15	Cytokinin Mediated Response Factor
LTH	Lijiangxin Tuan Hegu
R/FR light	Red/ Far red light
TIR1	TOLL LIKE RECEPTOR 1
P. infestans	*Phytophthora infestans*
A. solani	*Alternaria solani*
bHLH	Basic Helix loop helix

Synopsis

Investigating the role of miRNA160 and miRNA166 in defence response of potato

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Introduction

Plants are sessile and constantly battle with a plethora of pathogens. To combat these pathogens, plants use various methods to defend themselves. (Chisholm et al., 2006; Jones and Dangl, 2006). At the site of infection, plants recognize pathogen-associated molecular patterns (PAMPs) using pattern recognition receptors (PRRs) present on their membrane leading to the activation of PAMP-triggered immunity (PTI) (Chisholm et al., 2006). A few pathogens can evade this detection by releasing effector molecules that ultimately suppresses PTI response. In turn, plants have evolved resistance (R) proteins that can recognize effector molecules and activate effector-triggered immunity (ETI) (Jones and Dangl, 2006). PTI and ETI protect plants from local infection but they induce systemic defence in distal non-infected plant parts, resulting in a broad-spectrum, long-lasting resistance, known as systemic acquired resistance (SAR) (Dempsey and Klessig, 2012). Interestingly, plants relay the SAR response from infected tissue to systemic tissues via various SAR signals, majority of them have been reported to be mobile via phloem (Guedes et al., 1980; Tuzun and Kuc, 1985). Decades long research has led to the discovery of several SAR signals, and few of the well-studied ones include Methyl salicylate (MeSA) (Park et al., 2007), Azelaic acid (AzA) (Jung et al., 2009), Glycerol-3-Phosphate (G3P) (Chanda et al., 2011), dehydroabietinal (DA) (Chaturvedi et al., 2012) and pipecolic acid (Pip) (Návarová et al., 2012). The process of PTI, ETI and SAR has been found to be associated with salicylic acid (SA) and induction of defence related genes NON EXPRESSOR OF PATHOGENESIS-RELATED GENE 1 (NPR1), and PATHOGENESIS-RELATED (PR) family of genes (Cameron et al., 1999, Park et al. 2007).

Recent studies have reported the emerging role of microRNAs (miRs) in plants PTI and ETI responses (Seo et al., 2013). miRs are endogenous small ~21-24 nucleotide non-coding RNAs that are known to negatively regulate gene expression (Bartel and Bartel, 2003). Since their discovery, several miRs have been shown to play an important role in various plant processes such as seed germination,

root cap formation, and plant immunity to name a few (Wang et al., 2005; Kidner and Martienssen, 2005; Navarro et al., 2006; Liu et al., 2007; Jung and Park, 2007). The first report from Navarro et al., (2006), showed that treatment of Arabidopsis wild-type (WT) Columbia-0 seedlings with flg22 (a 22 amino acid N-terminal part of flagellin, that acts as a PAMP) leads to induction of miR393 and triggers PTI response in plants. The authors further reported that miR393 was involved in maintenance of antagonistic relationship between SA-mediated defence response and auxin-mediated growth (Navarro et al., 2006). Several other miRNAs, such as miR160, miR166, miR482, etc., have been shown to regulate PTI and ETI responses in plants (Li et al., 2010; Li et al., 2014; Salvador-Guirao-R et al., 2018; Wong et al., 2014). There is only one study that has described the importance of miR160 in both local and systemic defence responses (Natarajan et al., 2018). It was demonstrated that miR160 plays an essential role in regulating auxin-mediated signalling and SA-dependent defence response through *StARF10*, a target of miR160. But, authors did not attribute any specific defence related role to other miR160 targets, viz., *StARF16* and *StARF17.* Moreover, it was noticed that the miR160 deficient (knock-down) transgenic lines failed to elicit SAR response during *P*. *infestans* infection but the underlying mechanism still remains elusive. As miRs are important regulatory molecules, we hypothesised that there could be additional miRs, such as miR166, that may be involved in establishment of SAR as well. In potato, it has been observed that upon infection with *P. infestans* miR166 also accumulated similar to miR160 and is hypothesized to contribute towards plant defence response (Natarajan et al., 2018). Using potato–*Phytophthora infestans* interaction as a model system, we laid out a number of objectives to test our hypothesis.

Objectives:

1. Understanding the mechanistic link between miR160 target, StARF16 and defence response.
2. To investigate the compromised SAR response in miR160 knockdown lines.
3. Investigating the potential role of miR166 in the defence response of potato.

1. *StARF16*, a miR160 target, regulates *StNPR1* during plant-pathogen interactions

There are several studies that demonstrate the role of miR160 in the development process of plants (Wang et al., 2005; Liu et al., 2007; Hendelman et al., 2012; Damodharan et al., 2016). Moreover, miR160 role in defence response has also been elucidated in various plants species like Arabidopsis and rice (Li et al., 2010; Li et al., 2014). miR160 has been reported to target various Auxin Response Factors (ARFs) plants, namely ARF10, ARF16 and ARF17. Previous study from our lab (Natarajan et al., 2018),

have shown that StARF10 could directly bind to the promoter of *StGH3*.6, a homolog of *AtGH3*.5, and could contribute in maintaining the balance between free auxin and SA in the plant cells. We investigated whether *StARF16*, a miR160 target, could regulate defence response genes upon infection. We analysed the expression profile of miR160 and its targets during early infection with *P. infestans* (a-hemi biotroph) *and A. solani* (a necrotroph), and noticed that *StARF16* and *StNPR1* show a negative correlation with each other, suggesting either a direct or indirect regulation. As literature exhibits the role of miR160 during biotrophic and a necrotrophic pathogen infection, we used *P. infestans* (a-hemi biotroph) *and A. solani* (a necrotroph) to understand the differential response of miR160 upon infection. We found that miR160-*StARF10* shows a negative correlation, but interestingly, miR160-*StARF16* shows a positive correlation with each other. The positive correlation with miR160-*StARF16* displays a new unknown mechanism that regulates the biological processes in plants.

Using hormone treatment experiment, we observed that the negative correlation between *StARF16* and *StNPR1* was established upon treatment with defence hormones SA and JA, respectively. Furthermore, through yeast-1-hybrid (Y-IH), we proved that *StARF16* protein directly binds to the promoter of *StNPR1*. Additionally, our co-transfection assays and ChIP-qRT-PCR analyses (in planta) revealed that StARF16 binds to the promoter of *StNPR1* and negatively regulates its expression in potato. These results demonstrate that JA induced *StARF16* could bind to the promoter of StNPR1 and negatively regulate *StNPR1* expression.

2. MiR160 knockdown (KD) lines are deficient in G3P induced SAR response

miR160 has been depicted to contribute to plant's basal defence response in potato, Arabidopsis and rice (Li et al., 2010; Li et al., 2014; Natarajan et. al., 2018). It was only recently that miR160 was reported to be crucial in mounting SAR response in the plant. The miR160 knockdown (KD) lines were shown to exhibit compromised SAR upon infection with *P. infestans* (Natarajan et al., 2018). Authors hypothesised that miR160 KD lines must either be deficient in synthesizing or transport of SAR response but did not explain a mechanistic link for the same. In this study, we attempted to investigate the reasons for miR160 KD transgenic lines being SAR-deficient. To understand this, we treated WT potato plants with various SAR signals namely, SA, AzA, DA, G3P, and Arachidonic Acid (AA) and noticed that all these SAR signals successfully established SAR response. This result confirms that, the well known mobile SAR signals are functionally active in potato and prime plants for SAR.

In our further analysis, a miR160 KD transgenic line which was found to be deficient in the SAR response was able to mount a successful SAR response upon treatment with G3P. We tested a dose dependent response of G3P over miR160 KD lines and observed that there is a need of an optimal amount of G3P to mount a significant defence response in the miR166 KD lines. We then analysed various genes involved in the G3P dependent SAR response. Our study shows that genes like *StAZI1, StDIR1, and StALD1* were mis-regulated in the miR160 KD lines. Upon treatment with G3P in the miR160 KD lines, we noticed that the expression of these mis-regulated auxin related genes get partially rescued and could explain the basis for establishment of SAR response in miR160 KD lines. Moreover, the mis-regulation of auxin response in the miR160 KD lines was also partially rescued upon treatment with G3P suggesting a possible interaction of G3P and auxin signalling to mount a successful defence response. Future studies can only unfold the detailed mechanism in this regard. In this study, we establish that in potato, miR160 regulate G3P dependent SAR response upon *P. infestans* infection.

3. Investigating the role of miR166 in regulating the defence responses in potato plant upon treatment with *P. infestans.*

miR166 is well studied for its role in plant development process and abiotic stress responses in various plant species (Boialem et. al., 2008; Hawer et. al., 2004; Rubio-Somoza et. al., 2011; Ong et. al., 2012). Only recently, miR166 has been shown to contribute towards defence response in plant species like soybean and rice (Salvador-Guirao-R et al., 2018; Wong et al., 2014). In these reports, miR166 have been shown to contribute towards the PTI response and starts to accumulate as early as 15 minutes upon recognition of PAMP molecules from the pathogen. Similarly, a previous study from our lab reported the accumulation of miR166 during infection with *P. infestans* in WT potato plants. To understand the importance of miR166 in the potato plants during infection, we did an expression profile analysis of miR166 and its targets upon infection with *P. infestans*, and analysed the defence response of miR166 overexpression (OE) and KD lines in potato.

We found that upon *P. infestans* infection, miR166 accumulates as early as 3 hours post infection in both local and systemic leaves, indicating the role of miR166 in defence response. Upon further investigation, we noticed that miR166 is involved in the PTI response of the potato defence response, where it accumulates as early as 90 minutes post treatment with AA in WT potato plants. Along with miR166, various miR166 targets, viz. *StICU1, StICU2, and StREV* showed a negative correlation to the accumulation of miR166, whereas StPHV1 and StPHV2 failed to show a consistent negative correlation with the miR166 accumulation or decrease. We generated miR166 OE and KD lines

in WT *Solanum tuberosum L. cv. Désirée,* and did not observe any morphological changes, even though miR166 has been reported to play a role in leaf and root development earlier. We carried out SAR response in these transgenic lines and found that miR166 KD lines did not elicit SAR response, but miR166 OE lines exhibited SAR response. These results suggested that miR166 contributes to the establishment of SAR response in potato plants. To elucidate the regulatory mechanism of miR166 defence response in the transgenic lines, we generated transgenic lines overexpressing microRNA resistant *StICU1 (5'mStICU1).* During SAR assay, even the lines overexpressing *StICU1* failed to mount a SAR response in the plants. This result suggests that miR166 mediates potato defence response through StICU1. This study reveals the importance of miR166 in mounting a PTI response in the WT potato plants and its importance in eliciting SAR upon infection.

4. Summary

In this investigation, we showed that miR160 and miR166 both play crucial role in potato defence response. At the local site of infection, we noticed that expression of both miR160 and miR166 accumulated during early defence response. Our expression analysis upon infection with *P. infestans and A. solani* revealed that StARF16, a known target of miR160, shows a positive correlation with miR160 indicating a novel mechanism in regulating plant processes. We found that *StARF16* demonstrates negative correlation with StNPR1 upon both infection and hormone application. Moreover, we confirmed that *StARF16* directly binds to and negatively regulate the expression of *StNPR1,* a known defence gene. This study reports the importance of StARF16, a development related gene, in a regulation of defence response in potato through *StNPR1*. Further investigating the role of miR160 in establishment of SAR, we found that miR160 KD lines were deficient in G3P dependent SAR response. Upon exogenous application of G3P, miR160 KD lines were able to mount a successful SAR response in potato plants. This successful priming of miR160 KD lines was attributed to the partial attenuation of the G3P dependent SAR pathway and auxin signalling. In a follow up study, we observed that, another defence related microRNA, miR166 showed a dynamic expression profile and accumulated at early stages of infection with *P. infestan*s, suggesting its role in PTI response of potato. Further investigation revealed that miR166 also regulates the systemic response in potato plants. SAR assay showed that miR166 KD lines were deficient in establishment of SAR response and this response was regulated by *StICU1* in potato. This study emphasizes the role of miRNA160 and miRNA166 in regulation of local and systemic defence response in potato during plant-pathogen interactions.

Publications during PhD work

1. **Kalsi H**., Karkhanis A., Natarajan B., Bhide A., and Banerjee A. (2022). AUXIN RESPONSIVE FACTOR 16 (StARF16) regulates defence gene *StNPR1* upon infection with necrotrophic pathogen in potato. (Revision # 2 submitted to *Plant Molecular Biology* on 13th Jan, 2022).
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Chapter 1

Introduction

1.1. Background

Plants cannot run from the environmental challenges they encounter. These challenges include both biotic and abiotic stresses. Biotic stresses primarily include invasion by nefarious pathogens. Plants are attacked by pathogens, which can belong to any Kingdom, spanning from bacteria, viruses, fungi to animals. Pathogens use plants to obtain nutrition for growth and reproduction, thus resulting in damage to the host.

Plant pathogen interactions may result in substantial economic and food losses. In the past, these incidences have occurred. During 1840s in Ireland, late blight potato disease caused by *Phytophthora infestans* resulted in losing 1 million human lives due to food shortage. To reduce these economic and life losses, there is an utmost necessity for studying plant-pathogen interaction.

1.2. Types of plant pathogen

Pathogens are categorized into three classes, namely, biotroph, necrotroph and hemibiotroph, based on how they derive nutrients from the infected plant. Biotrophs hijacks the plant machinery to extract the nutrients from a live host like *Sphaerotheca pannosa,* a fungal pathogen. Necrotrophs, like *Ralstonia solanacearum,* a bacterial pathogen thrive in plants by killing the host and derive nutrients from dead tissue. Hemibiotrophs incorporate both biotrophic and necrotrophic lifestyles upon infection of plants. They utilize a biotrophic lifestyle during colonization and later shift to a necrotrophic lifestyle for reproduction, like *Phytophthora infestans*.

1.2.1. Biotroph

Biotrophs are pathogens that colonize and obtain nutrients from living plant cells without killing them. Biotrophs manipulate host plant molecular process to facilitate its colonization and proliferation in the plant cells. To keep the host alive, biotrophs usually induce plant growth by hijacking cell division or cell expansion processes. Biotroph-induced cell growth often results in visible phenotypic changes such as tumours and galls, as observed for the α-proteo-bacterium *Agrobacterium tumefaciens*. There are evidences that explain that plant

biotrophs (e.g., *Agrobacterium* and *Ustilago* spp.) manipulate plant's growth hormones such as auxin and cytokinin to induce cell expansion and division resulting in tumour/gall formation. Likewise, the *Xanthomonas campestris* PV. *vesicatoria* effector *AvrBs3* and the *Carlavirus Chrysanthemum virus B* protein P12 have been reported to induce expression of the plant growth-promoting bHLH (Basic Helix-Loop-Helix) transcription factor (TF) *UPA20,* which induces hypertrophy in leaf mesophyll cells. Thus biotrophic pathogens derive nutrition from plants through various biological processes, by regulating cell division and cell expansion of the host cells.

1.2.2. Necrotroph

Necrotrophs are the type of pathogens that extract nutrients from dead cells. Necrotrophs usually employ various chemicals that induce cell necrosis and cause leakage of nutrients, on which they feed. Necrotrophs are either classified as host-specific or as broad-host-range species. Host-specific necrotrophs are known to produce toxins that are host specific and are required for their pathogenicity and virulence. For example, HC-toxin produced by the fungal pathogen *Cochliobolus carbonum* which limits its infection to susceptible genotypes on which it causes the Northern corn leaf spot. Whereas the broad-host-pathogen produce plethora of toxins that can infect various species of plants and thus are broad range pathogens. The fungal pathogens *Botrytis cinerea*, *Alternaria brassicicola*, and *Sclerotinia sclerotiorum* and the bacterial pathogen *Erwinia carotovora* are well known broad-host-range necrotrophs that infect various plants. Broadly, the necrotrophic life cycle is a mode linked to death inducing virulence strategies, which modulates and counter the host immune responses upon infection. In summary, necrotrophs are pathogen specialized in inducing plant cell deaths to derive nutrient from the dying or the dead cells during the phase of infection.

1.2.3. Hemibiotroph

Hemibiotrops have a peculiar life cycle which is characterized by an initial phase of biotrophy followed by a secondary necrotrophy. This shift of mode of nutrition allows the pathogen to counter the host defence response during establishment of infection by killing the host tissues prior to their spread (e.g. some *Colletotrichum* species; Bailey et al., 1992). During colonization phase hemibiotrophs often develops specialized infection structures such as intracellular hyphae that are biotrophic in nature. Several fungi, e.g. *Cladosporium fulvum* (De

Wit, 1977), *Phytophthora infestans (Akino et al., 2014)* and *Pyrenopeziza brassicae* (Ashby, 1997), which are also often considered as hemibiotrophs, penetrate the leaf cuticle then spread via intercellular hyphae within the intracellular space of the host asymptomatically, before initiation of the destructive necrotrophic phase during which symptoms develop.

1.3. Plant Immune response

To fight invading pathogens, plants have evolved an innate immune system that can fight against invading pathogens. This plant innate response has two defence lines first, in the physical barrier of the plants, which include cuticle, cell wall, wax, and callose deposition. Successful pathogens can easily penetrate this first line of defence. The second line of defence comes in place, with local and systemic responses where the plant's response differs at the sites.

To recognize the invading pathogen, plants use the molecules present over the pathogen called the Pathogen Associated Molecular Patterns (PAMPs). These PAMPs are essential pathogen molecules present over the surface of the pathogens. These PAMPs are recognized through Pattern Recognition Receptors (PRR) present over the cell membrane of plants. This result in the initial mounting of the immune response via PAMP triggered Immunity (PTI). Some pathogens counter PTI by secreting effector proteins and molecules in plant cells, this makes the host susceptible to the pathogen and is called Effector Triggered Susceptibility (ETS). To counter the effector proteins and molecules secreted by the pathogen, plant uses Resistance protein (R-proteins) to launch an immune response called Effector-Triggered Immunity (ETI). This response has been well explained in Jones and Dangl, 2006 where the authors proposed a model in the plant immune response (Figure 1.1). During the pathogen attack, the plant recognizes PAMPs and initially mount PTI response but it is overcome by compatible pathogen by secreting effectors molecules and making the plant susceptible to the pathogen as the secreted effector molecules make the pathogen susceptible to the host. Resistant host plants can recognize the effectors present in cytoplasm through NB-LRR receptors and mount a successful ETI response, leading to a hypersensitive response, effectively delaying and suppressing the pathogen spread.

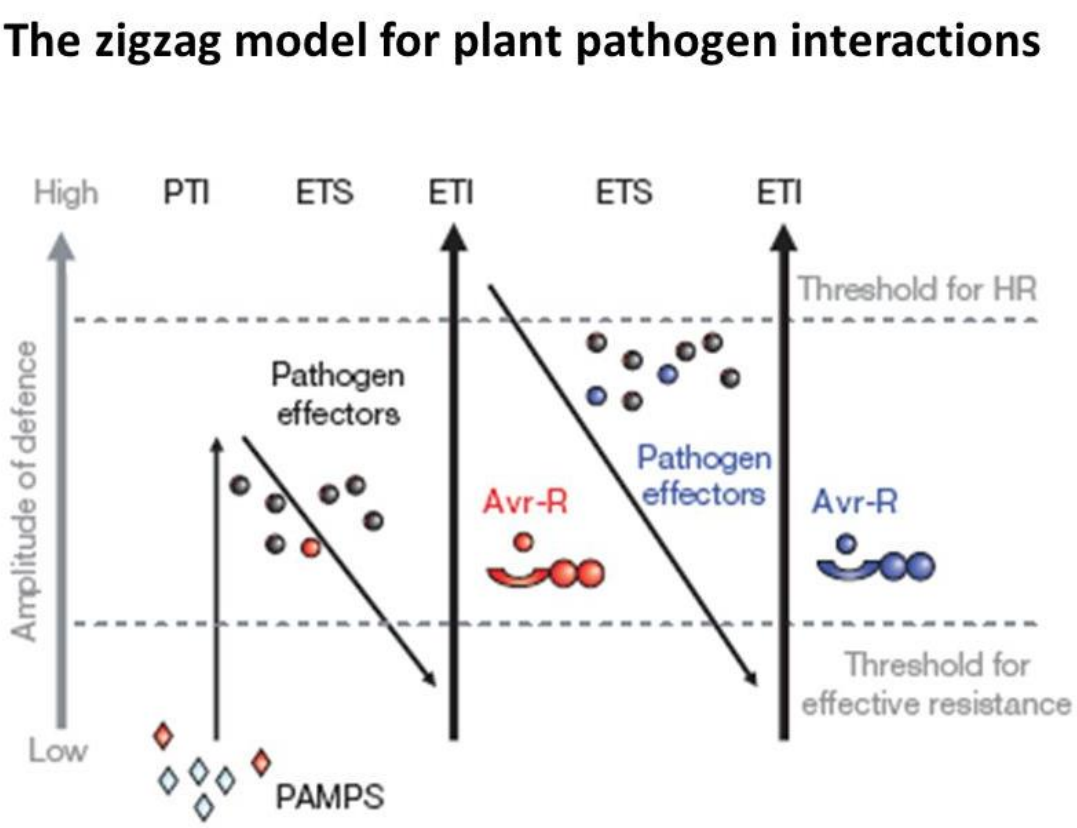


Figure 1.1 The zig zag model describing the PTI and ETI response in plant upon pathogen infection PAMP: pathogen-associated molecular pattern. PTI: PAMP-triggered immunity. ETS: effector-triggered susceptibility. ETI: effector-triggered immunity. (*Reproduced from Jones and Dangl, 2006; License is appended in Annexure I).*

1.3.1. Pattern-triggered immunity

Perception of PAMPs by PRRs present on the cell membrane activates the first line of defence response, termed as PTI. This response is responsible for evading attacks from non-adapted host pathogens (Bohm et al., 2014; Schwessinger and Ronald, 2012). The entire repertoire of PAMPs perceived by plants remains unknown, several crucial PAMPs, such as bacterial flagellin, lipopolysaccharide (LPS), peptidoglycan (PGN), elongation factor Tu (EF-Tu), and fungal chitin, have been well characterized and are known to elicit various defence responses in plant cells (Bohm et al., 2014; Boller and Felix, 2009; Schwessinger and Ronald, 2012). A well studied PAMP is 22-amino-acid peptide near the amino-terminus of flagellin, flg22 present over bacterial pathogen, is perceived by *Arabidopsis thaliana* PRR, FLAGELLIN-SENSING 2 (FLS2), a leucine-rich repeat RLK (LRR-RLK), which initiates immune signalling by

instantaneously heterodimerisation with another LRR-RLK BRASSINOSTEROID INSENSITIVE 1 - ASSOCIATED KINASE 1 (BAK1) (Chinchilla et al., 2007; Heese et al., 2007). FLS2 and BAK1 are in constitutive association with BOTRYTIS-INDUCED KINASE 1 (BIK1), a receptor-like cytoplasmic kinase (RLCK), and its homolog PBS1-LIKE 1 (PBL1). Upon perception of flgg22 BIK1, RLCK and PBL1 are rapidly phosphorylated and released from the receptor complex (Lu et al., 2010). BIK1 is responsible for early production of reactive oxygen species (ROS) after activation through various PAMPs/MAMPs by directly phosphorylating PM-resident NADPH oxidase RESPIRATORY BURST OXIDASE HOMOLOG D (RBOHD) (Kadota et al., 2014; Li et al., 2014). To fine tune the PTI response in plants FLS2 complex is negatively regulated via two plant U-box E3 ubiquitin ligases (PUB) 12 and 13. PUB 12 and 13 are recruited to the FLS2 complex upon flg22 perception via BAK1 complex, which directly ubiquitinate FLS2 and is marked for flg22-induced FLS2 degradation (Lu et al., 2011). To further tune the PTI response BAK1-INTERACTING RLK 2 (BIR2), an LRR-RLK, constitutively interacts with BAK1 and negatively regulates flg22-induced heterodimerisation of FLS2 and BAK1 (Halter et al., 2014) (Figure 1.2). It has been reported that, PROTEIN PHOSPHATASE 2A (PP2A) controls activation of the PRR complexes by modulating the phosphorylation of BAK1, thus providing further strict tuning of flg22 induced defence response (Segonzac et al., 2014). Moreover, rapid activation of mitogen-activated protein kinase (MAPK) cascades has been reported upon multiple MAMP perceptions (Meng and Zhang, 2013).

A typical MAPK cascade comprises of three sequentially activated kinases starting with MAPK kinase kinase (MAP3K or MEKK), followed by a MAPK kinase (MAP2K, or MKK) and finally a MAPK, connecting upstream signals to downstream targets. All these MAPK convey the perception signal to regulatory molecules upon its own and target molecule by phosphorylation (Rodriguez et al., 2010). In *Arabidopsis*, two major MAPK signalling pathways have been identified to regulate plant defence response. MEKK-MKK4/MKK5-MPK3/MPK6 positively regulates the plant immune response whereas MEKK1-MKK1/MKK2-MPK4 act as a negative regulators in plant immunity (Meng and Zhang, 2013; Tena et al., 2011). WRKY (W-box containing Transcription factor) and ERF (Ethylene response factor) are the two major defence-related transcription factors studied in plants that are regulated via MAPKs. ERF 104 has been demonstrated to act as a substrate of MPK6 activated by flg22, and phosphorylation induces its release from MPK6 to 4 resulting in regulation of target gene expression (Bethke et al., 2009). Upon *Botrytis cinerea* infection, WRKY33 is reported to be phosphorylated by MPK3 and

MPK6 in vivo, promoting phytoalexin biosynthesis by inducing camalexin biosynthetic gene expression (Mao et al., 2011). In addition to these examples, ERF6 is also phosphorylated by MPK3 and MPK6 and plays a crucial role in plant defence against fungal pathogens (Meng and Zhang, 2013).

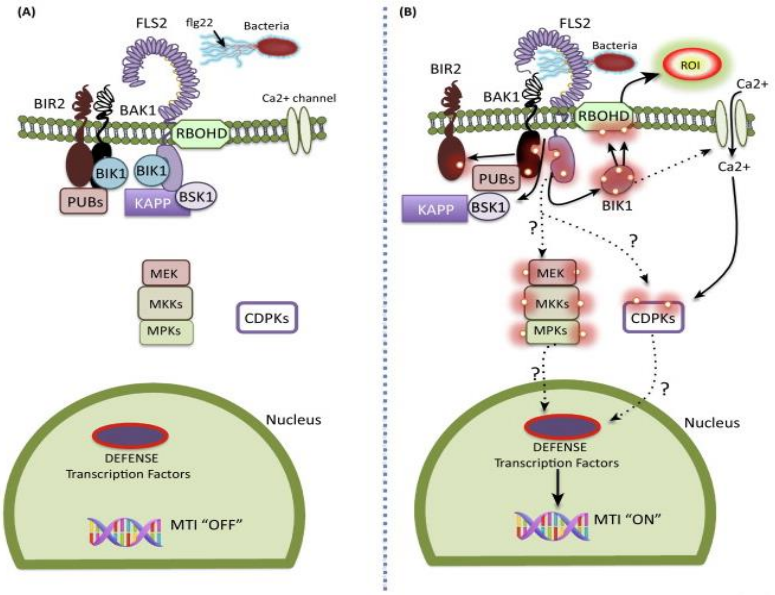


Figure 1.2 FLS2 mediated PTI response in Arabidopsis. A) In the absence of ligand, flg22, kinase-associated protein phosphatase (KAPP) inhibits the association of FLS2 with BIK1. 1, which is a member of the family of *AvrPphB* SUSCEPTIBLE1 (PBS1)-like proteins (PBLs). BIR2 sequesters BIK1 and regulates the interaction with FLS2 in the absence of flg22. (B) flg22 detection triggers formation of an FLS2–BAK1 hetero-oligomer. flg22 binding releases BAK1 from BIR2-BAK1 complex and causes dissociation of KAPP phosphatases. The FLS2–BAK1 oligomer undergoes a series of transphosphorylation events and phosphorylates BIK1. BIK1 then autophosphorylates and dissociates from the complex. BIK1 then targets NADPH oxidase RBOHD for phosphorylation, activating an extracellular superoxide burst and intracellular calcium level increases. MAPK cascade and calcium-dependent protein kinases (CDPKs) are activated by unknown mechanisms and contribute to the induction of flg22-responsive genes. Once flg22 signal have been transduced, BSK1 dissociates from the receptor complex, and BAK1 phosphorylates closely related PUBs, which in turn ubiquitylate FLS2 to regulate its presence at the cell membrane (*Reproduced form Belkhadir et al., 2014; License is appended in Annexure I)*.

1.3.2. Effector-triggered immunity

Host compatible pathogens secrete various virulence factors called, effectors, to establish successful infection by suppressing PTI response of the plant (Block et al., 2008; Dou and Zhou, 2012; Mudgett, 2005; Xin and He, 2013). It has been discovered that several pathogenic bacteria inject numerous effector molecules into host cells through Type III secretion system (T3SS). Many of these effector molecules are found to be important to interfere with host immune responses and physiology (Feng and Zhou, 2012). Multiple effectors, such as *AvrPto*, *AvrPtoB* and *HopF2*, have been reported to target BAK1, a PRR co-receptor, to dampen PTI response of the plant (Zhou et al., 2014). To recognize the effectors molecules secreted in the host cells, plants have evolved nucleotide-binding leucine-rich repeat (NLR) the intracellular receptors, called Resistance (R)-proteins. Upon recognizing these effector proteins or effector-mediated perturbations of host targets, plants elicit a second tier of defence responses, called ETI (Bonardi and Dangl, 2012; Gassmann and Bhattacharjee, 2012; Qi and Innes, 2013).

Plant's NLR proteins are structurally similar to mammalian nucleotide-binding oligomerization domain (NOD) - like receptors that recognize intracellular MAMPs to initiate immunity and inflammation in host cells (Maekawa et al., 2011). *AvrRpt2, a Pseudomonas syringae* effector, is recognized by Arabidopsis NLR protein RESISTANCE TO PSEUDOMONAS SYRINGAE 2 (RPS2), whereas *AvrRpm1* and *AvrB*, are recognized by RESISTANCE to PSEUDOMONAS SYRINGAE PV. MACULICOLA 1 (RPM1) to elicit an ETI response in plants. These ETI responses include transcriptional reprogramming and initiation of localized programmed cell death (PCD), termed hypersensitive response (HR) (Figure 1.3). Instead of direct NLR-effector interaction, RPS2 and RPM1 has been reported to perceive the perturbation of host protein RPM1-INTERACTING PROTEIN 4 (RIN4) when targeted by pathogen effectors to mount ETI defence response (Axtell and Staskawicz, 2003; Mackey et al., 2003). *HopF2* is a known effector molecule that suppresses RIPK-mediated activation of RIN4 by ADP-ribosylation of RIN4.

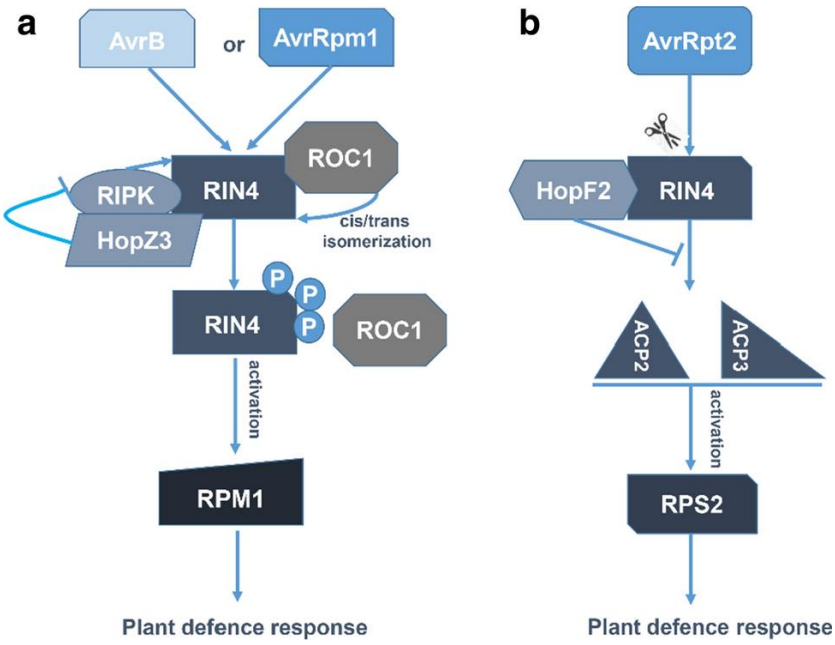


Figure 1.3 ETI response of *Arabidopsis* in response to AvrB, AvrRpm1 and AvrRpt2 controlled by RPS2 and RPM1. a *AvrB* or *AvrRpm1* suppresses PTI response by suppressing RIN4 and induces RIPK-mediated phosphorylation of RIN4, which, in turn, activates RPM1 and induces a defence response in plants. b *AvrRpt2* targets and cleaves RIN4, activates RPS2-mediated defence response; HopF2 targets RIN4 and induces the ADP-ribosylation of RIN4, and inhibits the activation of RPS2 (*Reproduced from Zhao et al., 2021; License is appended in Annexure I)*

1.3.3. Systemic Acquired Resistance

Upon recognizing a pathogen attack and mounting an immune response, whether PTI or ETI, plant relays a message to distal parts of the plant to build a prime state. During primed state, the plant is capable of fighting back recurring or new pathogen attacks for a long time. This type of broad and long time acquired resistance in plant is known as systemic acquired resistance (SAR). Under SAR response the plant responds faster and better to subsequent pathogen attack. It is a mechanism during which the plant's local defence response is used to induce a defence response at a distant non-infected site.

It is observed that during SAR response, the gene expression of defence-related genes related to Pathogenesis Related (PR) gene family are elevated at both local infection site and systemic non-infected site. These changes in gene expression of defence related genes at

infected and non-infected sites suggest a transfer of elevated resistance from the local infected site to distal systemic tissue. This suggests that some specific molecules are transported from the local site of infection to the distant site in systemic tissue to impart an elevated state of defence response. In 2007, Park *et al.* demonstrated that Methyl Salicylate (MeSA) is one such signal molecule that is transported to systemic site from local infected site to establish this immune response in the plants. Various other subsequent reports have established the role of Azelaic Acid (AzA), Pip, FMO1, DA, DIR1 and G3P as crucial regulators of SAR establishment at systemic tissue (Figure 1.4). These systemic signals role was discovered from mutants in Arabidopsis lacking the ability to establish SAR upon infection with an avirulent strain of *Pseudomonas syringae.* These crucial SAR molecules, genes and proteins altogether are essential for mounting a successful SAR response. But if the plant lacks the ability to generate or transport even one of these mobile signals to distal sites, it leaves the rest of the plant susceptible to future infection and SAR response is compromised.

1.3.3.1 Glycerol -3-Phosphate

Upon characterization of a SAR-defective mutant, sfd1/gly1 (suppressor of fatty acid desaturase deficiency 1), it was shown that it encodes for glycerol-3-phosphate dehydrogenase (G3Pdh) (Nandi et al., 2004). G3Pdh is a crucial protein that is responsible for generating Glycerol -3- Phosphate (G3P), the precursor for all glycerolipids essential for growth and defence response in plant (Chanda et al., 2008). Chanda et al. (2011) showed that G3P increases in local and systemic leaves during SAR, and the combined application of G3P and avirulent pathogen restored SAR in *gly1* mutant plants. However, transport of radio-labelled G3P from local to systemic leaves could not be observed, suggesting G3P itself may not be phloem-mobile, and its derivatives could be mobile. Further, these authors showed that G3P and DIR1 required each other for phloem translocation in *Arabidopsis* (Chanda et al., 2011). Further experimentations demonstrates that the G3P operates in a positive feedback mechanism with the lipid transport proteins (LTPs) DIR1 and AZI1, such that absence or lack of DIR1 or AZI1 impairs pathogen-induced G3P accumulation and lack of G3P results in reduced DIR1 and AZI1 transcripts (Yu et al., 2013). It has been shown that DIR1 and AZI1 complexes are unstable in the mutants lacking G3P and G3P is transported to systemic site only when these LTPs are present in the plants.

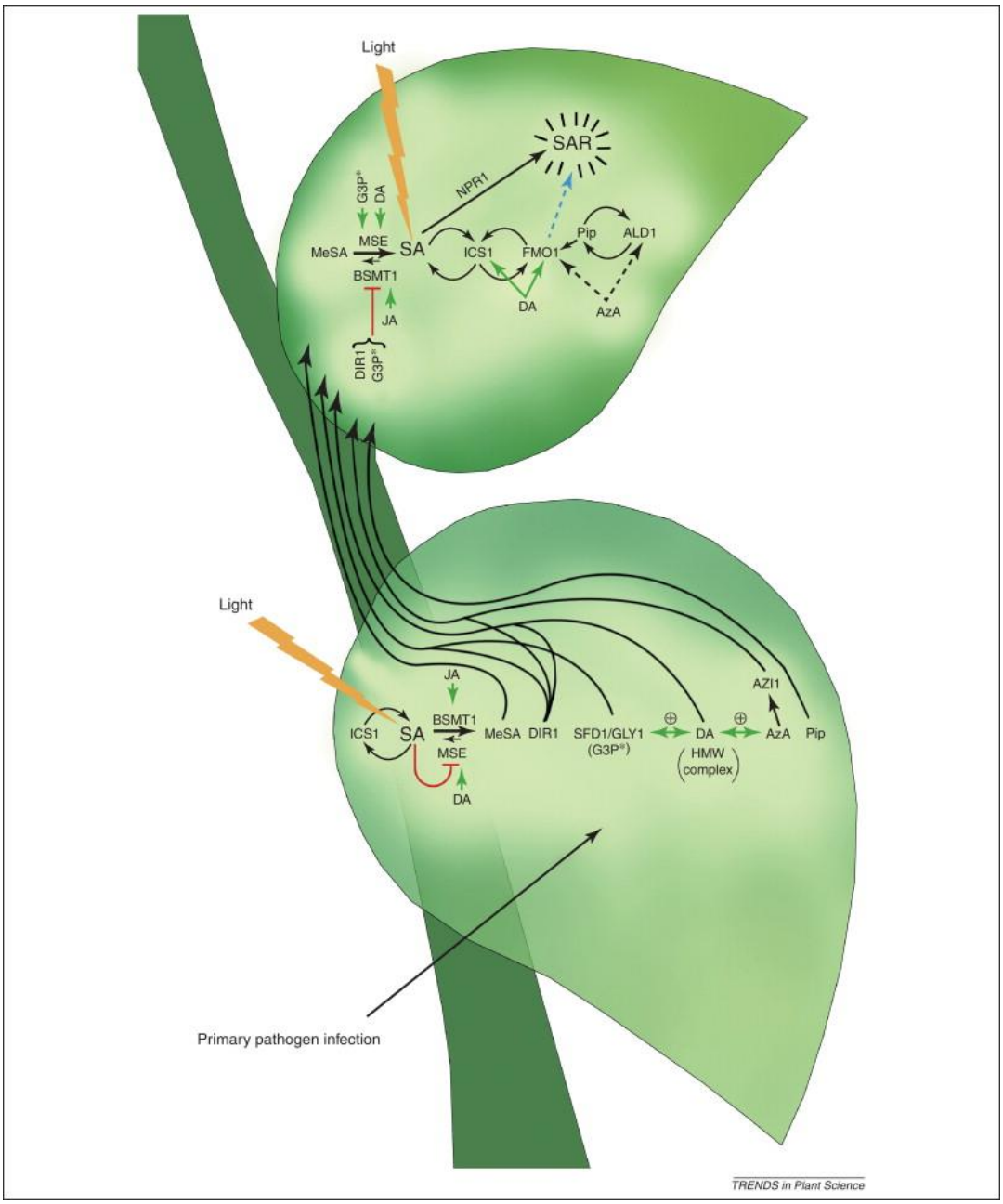


Figure 1.4 Diagrammatic representations of mobile SAR signals in the plants. Upon primary pathogen infection, plant produces various mobile SAR signals such as MeSA, DIR1, AzA, Pip, DA, and G3P that are transported as chemical molecules or in a complex form, from local infected site to systemic non-infected tissue. At systemic site, these molecules activate defence responsive genes ICS1, NPR1, FMO1, and ALD1 that leads to establishment of SAR. A brief description of different SAR signals is provided in text. (*Reproduced from Dempsey ad Klessig, 2012; License is appended in Annexure I)*

1.3.3.2. Methyl Salicylate

Methyl salicylate (MeSA) is a volatile ester that is induced upon infection and is absent during non-infected state of the plant. It has been shown that MeSA is synthesized from SA, catalytically triggered via SA carboxyl methyltransferase (SAMT), using the methyl donor *S*-adenosyl-l-Met and carboxylic acid-containing substrates (Park et al., 2007). Whereas, conversion of MeSA to SA has been demonstrated to be catalysed via SA methyl esterase SABP2 (salicylic acid-binding protein 2). Further investigation showed that SABP2-silenced tobacco plants have attenuated local resistance to tobacco mosaic virus (TMV), and were SAR-deficient. Upon infection, MeSA is found to increase in both primary infected and systemic tissue, regulated by SABP2 in the local infected tissue (Dempsey and Klessig 2013). It was also demonstrated that when *OsBSMT1, a homolog of SAMT,* overexpressors are infected with the fungal pathogen *Golovinomyces orontii*, or *Pseudomonas syringae* leads to reduced accumulation of SA, the inactive SA glycoside (SAG) and *PR1* compared to wild-type plants (Koo et al., 2007). Interestingly, *OsBSMT1* overexpressors triggered *PR1* induction in neighbouring wild-type plants, which was not dependent upon ICS1-derived SA but was dependent upon NPR1, a central positive regulator of systemic defence. This data suggests that MeSA alone is ineffective in inducing a defence response but can function as a volatile signalling molecule. Collectively, these data implicate MeSA as a mobile or volatile inducer of SAR.

1.3.3.3. Azelaic Acid

Azelaic acid (AzA), a fatty acid compound containing a nine carbon (C9) dicarboxylic acid. It was first identified by Jung et al. (2009) in the phloem exudate of *Arabidopsis* upon infection with avirulent pathogenic bacteria (Jung et al., 2009). Through radiolabelled experiments the authors were able to confirm the movement of AzA from local infected site to systemic site through phloem. Further, it was demonstrated that exogenous application of AzA induces local as well as systemic resistance. This systemic resistance was not induced by a direct increase of SA and PR1 levels in the systemic leaves, instead of by priming the leaves for enhanced resistance during the subsequent infection (Jung et al., 2009). It was also reported that AzA-mediated SAR induction requires AZI1 and DIR1 (Jung et al., 2009). The involvement of DIR1 in

AzA and MeSA signalling (Liu et al., 2011) suggests that different signals might be mediating the SAR process through other molecular players (Dempsey and Klessig, 2012).

1.4. SA in plants

SA has been well studied in the development of defence response in plants, but its role in plant growth has been studied very little in comparison to other plant hormones. Here, we describe the role of SA in both defence and development processes in plants.

1.4.1. SA in defence response

Significant progress has been made towards linking SA signalling and defence responses. Plants activate PTI response upon recognition of PAMPs. In *Arabidopsis*, recognition of the bacterial PAMP flagellin, flg22, activates MAPK module that leads to activation of basal defence response in plants. MEKK1 has recently been shown activate MAPK4 upon recognition of flg22 by plant defence response (Asai et al., 2002). MAPK4 has been reported to act as a negative regulator of SA signalling but is required to induce JA defence markers, representing a critical node mediating antagonism between SA/JA signalling (Petersen et al., 2000). mpk4 and mekk1 mutants have been shown to act exhibit severe dwarfism similar to mutants with constitutive activation of SA-dependent defences. This indicates the requirement of MEKK1 for suppression of SA signalling, in agreement with its proposed upstream signalling role in flg22-induced activation of MPK4 (Suarez-Rodriguez et al., 2007).

Moreover, it has been demonstrated that MKK1 can phosphorylate MPK4 *in vitro*, and following flg22 treatment, MKK1 activates MPK4. Additionally, *mkk1* mutants are found to be compromised in both flg22 activation of MPK4 (and MPK3 and MPK6) and resistance to virulent and avirulent strains of *P. syringae* (Meszaros et al., 2006). Downstream targets of MAPK4 have recently been discovered. Unexpectedly, genetic dissection of MAPK4 signalling revealed *PAD4/EDS1* mutations also act downstream of MAPK4 (Andreasson et al., 2005). PAD4 and EDS1 are proposed to improve JA/ET antagonism and partially rescue the dwarfism phenotype by activating SA and repressing JA/ET defences (Bodersen et al., 2006; Wiermer and Parker 2005).

Even though majority of studies depict SA signalling to be mediated by NPR1-dependent mechanisms, NPR1-independent mechanisms also contribute to plant defence response (Shah, 2003). Upon recognition of SA-induced redox changes NPR1 from cytosol gets converted to active monomers from disulfide-bound oligomers. NPR1 monomers then localize to nucleus and interact with the TGA (TGACG SEQUENCE-SPECIFIC BINDING PROTEIN) TFs leading to the expression of a several SA-dependent genes (Rochon et al., 2006). Even though, recruitment of NPR1 and TGA2 complex to the *PR1* promoter is autonomous and independent of SA. But, SA application stimulates the formation of a TGA2/NPR1 complex capable of inducing expression of *PR1* (Ndamukong et al., 2007). The TGA2 coactivator function of NPR1 is mediated by its BTB/POZ protein interaction domain and requires further Cys-oxidation of NPR1, consistent with the observation that the overexpression of NPR1 alone does not activate PR1 expression (Figure 1.5).

Recent reports emphasize that WRKY transcription factors have an extensive role to play in SA defence responses, downstream or alongside with NPR1, where they can act as both activators and repressors of SA- dependent transcription (Wang et al., 2006). Due to large numbers of WRKY TFs available in the plants, modulation of SA-mediated defence responses are incredibly complex in plant defence response (Eulgem and Somssich, 2007). For example, MAPK4 substrate MKS1 interacts with two WRKY TFs, WRKY25 and WRKY33. *wrky33* mutant have elevated *PR1* expression indicating a link between SA-mediated MAPK4 signalling. Along side, an independent study reported WRKY25 as a negative regulator of SA-mediated defence responses to *P. syringae (Zheng et al., 2007).* A short diagrammatic overview of how complex the SA dependent signalling becomes upon including WRKY TF is shown in Figure 1.5

1.4.2. SA in growth responses

Recently several studies have reported the role of SA in various plant development processes expanding from the seed germination to vegetative growth and plant senescence. We will briefly describe the role of SA and importance of NPR1 in plant growth. A detailed description of SA in plant growth and development has been reviewed in Vicente and Plasencia, (2011).

1.4.2.1. SA signalling pathway in *Arabidopsis* growth rate

Most of our current knowledge as how SA regulates growth is obtained by characterizing Arabidopsis mutants and generation of few transgenic plants that exhibit alteration in SA signalling. Arabidopsis mutant that shows constitutive expression of high levels of SA, such as *cpr5* (CONSTITUTIVE EXPRESSOR OF PR5; Bowling et al., 1997), *acd6-1* (ACCELERATED CELL DEATH; Rate et al., 1999), and *agd2* (ABERRANT GROWTH AND DEATH; Rate and Greenberg, 2001) shows severe decease growth in both roots and aerial parts of the plants. When SA is sequestered in Arabidopsis plants overexpressing NahG (salicylate hydroxylase), shows higher growth rate that exhibit increase in aerial shoots compared to WT plants (Abreu and Munné-Bosch, 2009; Du et al., 2009). Moreover, during low temperature stress it was found the plants with lower level of SA were able to grow faster than WT and this growth was attributed to increased levels of cytokinin and not to *amp1* (Xia et al., 2009). It has been reported that NahG plants that exhibit higher growth rate under low temperature stress is due to enhanced cell expansion rather than continuous cell division attributed to *amp1* mutants (Scott et al., 2004; Xia et al., 2009). Additionally, NahG transgenic plants have been shown to have higher levels of cyclin D3 (CYCD3; which drives the G1/S phase transition), suggesting that SA could negatively regulate its expression and thus leads to increased biomass in the plants (Xia et al., 2009). These results indicate towards a novel cross- talk existing between cytokinin, SA and brassinosteroids. Since, CYCD3 positive regulation has been attributed to cytokinin and brassinosteroids (Riou-Khamlichi et al., 1999; Hu et al., 2000).

Although these evidences suggest that SA negatively regulates the cell division in plants, it acts more as a regulator of plant growth. Depletion of SA using NahG transgene over *agd2* mutation background results in tumour-like growth in plants, representing that SA is required to carefully tune the plant growth. Similar effect was observed in acd6 NahG transgenic plants treated with the SA analogue benzothiadiazole S-methylester (Rate et al., 1999). Due to the complex nature of SA signalling and multiple receptors involved in these process the role of SA in plant growth is still in its rudimentary state.

1.4.2.2. NPR1 regulates plant growth and cell death

NPR1 is a crucial gene involved in the SA signalling and has been extensively studied for its role in defence response. Recently, reports are emerging that direct us towards the role of NPR1 in plant growth and senescence. Even though NPR1 is essential in SA mediated signalling but

NPR1 is not considered as a receptor of SA. There are five paralogues of NPR1 present in Arabidopsis genome, that exhibit partial redundancy to SA perception (Canet et al., 2010). A study demonstrated that upon mutation of *npr1* in acd6 background partially recues the *acd6* phenotype and contributes in a delayed response of cell death in these plants (Vanacker et al., 2001).

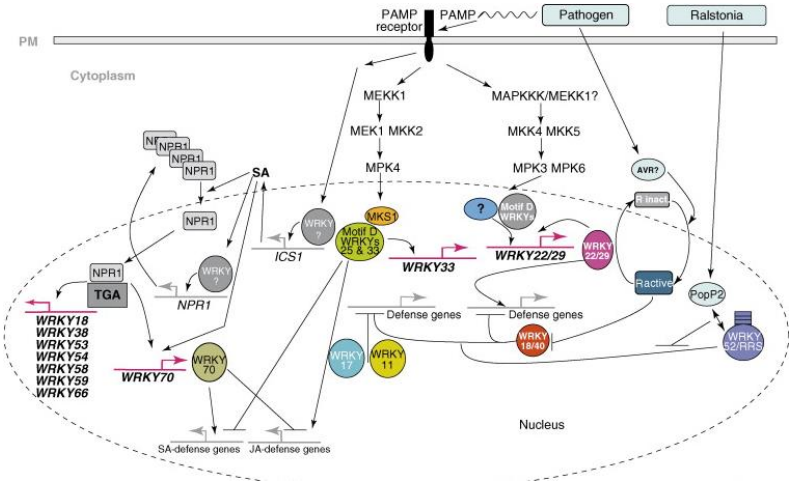


Figure 1.5 SA dependent defence response in plant. Plants trigger defence signalling upon recognition of PAMPs via PRRs present in the distinct cell membrane. These receptors then activate MAP kinase cascades. Defence responses are also initiated upon detection of effectors secreted by the pathogen within the host cell by major plant R proteins. A rapid alteration of gene expression ensues in the plants mediated by various TFs such as WRKY TFs. Upon pathogen-triggered SA signalling NPR1 is released from its oligomeric complexes into NPR1 monomers in the cytosol that are transported in to the nucleus. NPR1 monomers then binds with TGA TFs at various SA induced defence genes. A large set of WRKY genes both positively and negatively regulate downstream targets genes as indicated. (*Reproduced from Eulgem and Somssic., 2007; License is appended in Annexure I)*

Due to the involvement of SA in the plant growth it would be interesting to investigate whether SA dependent signalling could be involved in a cross-talk with other growth related phytohormones. These studies will expand our knowledge and provide us new insights towards how SA/NPR1 is involved in the cross-talk with JA, ABA, and ET pathways, in regulation of plant defence responses (Spoel et al., 2003; Yasuda et al., 2008; León-Reyes et al., 2009).

1.5. JA in Plants

Since the discovery of JA in plants, it has been vastly studied to understand its role in plant defence response upon pathogen attacks. But only recently its role in growth has started to emerge. The following section describes the role of JA in plant development and immunity.

1.5.1. JA in defence response

During plant immune response, JA has been implicated as a critical regulator that play important role in plant defence response (Pieterse et al., 2012). Several studies have been carried out that shows the importance of coronatine-insensitive1 (*coi1*) mutants as a core protein responsible for JA mediated signalling in plants. COI1 has been reported to function as part of the JA receptor that interacts with the Skp1/Cullin to form the Skp1–Cullin–F-box (SCF)COI1 ubiquitin E3 ligase complex. This SCF/COI1 complex is formed upon perception of elevated levels of JA in the system which then interacts with JAZ repressor proteins in the presence of jasmonoyl-L-isoleucine (JA-Ile) (the active form of JA). After marking JAZ proteins for degradation, several JA responsive TFs then regulate JA-responsive genes (Thines et al. 2007). Through *coi1* mutant, importance of COI1 in JA signalling has been demonstrated by reduction in expression of several PR family members (Feys et al. 1994, Xie et al. 1998, Devoto et al. 2005). It has been observed that *coi1* mutants are susceptible to necrotrophic pathogens, thus suggesting the importance of JA in plant defence response against necrotrophic pathogens. Similarly, *jar1*-1 a mutant defective in JA-Ile synthesis demonstrated reduced expression of JA-regulated genes and is highly susceptible to a soil fungus, *Pythium irregulare* (Staswick et al. 1998, Staswick et al. 2002). In contrast, *cev1* (CONSTITUTIVE EXPRESSION OF VEGETATIVE STORAGE PROTEIN 1), a JA-hypersensitive mutant, is found to be resistant to *Erysiphe cichoracearum* due to constitutive production of JA and ethylene (Ellis and Turner, 2001). These results thus suggest that JA–COI1 signalling positively contribute towards plant immunity in *Arabidopsis*.

Recently, Hu et al. (2013) reported that JAV1 (JA-ASSOCIATED VQ MOTIF GENE 1) negative regulates JA-mediated immunity against both insect attack and pathogen infection. The JA–COI1 signalling module, has been shown to mark JAV1 for degradation via the 26S proteasome upon activation, to trigger the expression of defensive genes and impart resistance to *Spodoptera exigua*, a herbivore, and *Botrytis cinerea*, a necrotrophic fungus. During uninfected conditions, JAV1 interact with several TFs, such as WRKY, to turn off their active

functions. After pest or pathogen invasion, degradation of JAV1 activates various downstream regulators, leading to the positive regulation of the defence signal cascades. This negative regulation is required by plants to fine-tune their JA-responsive defence reactions to balance growth and defence response during pathogen attack.

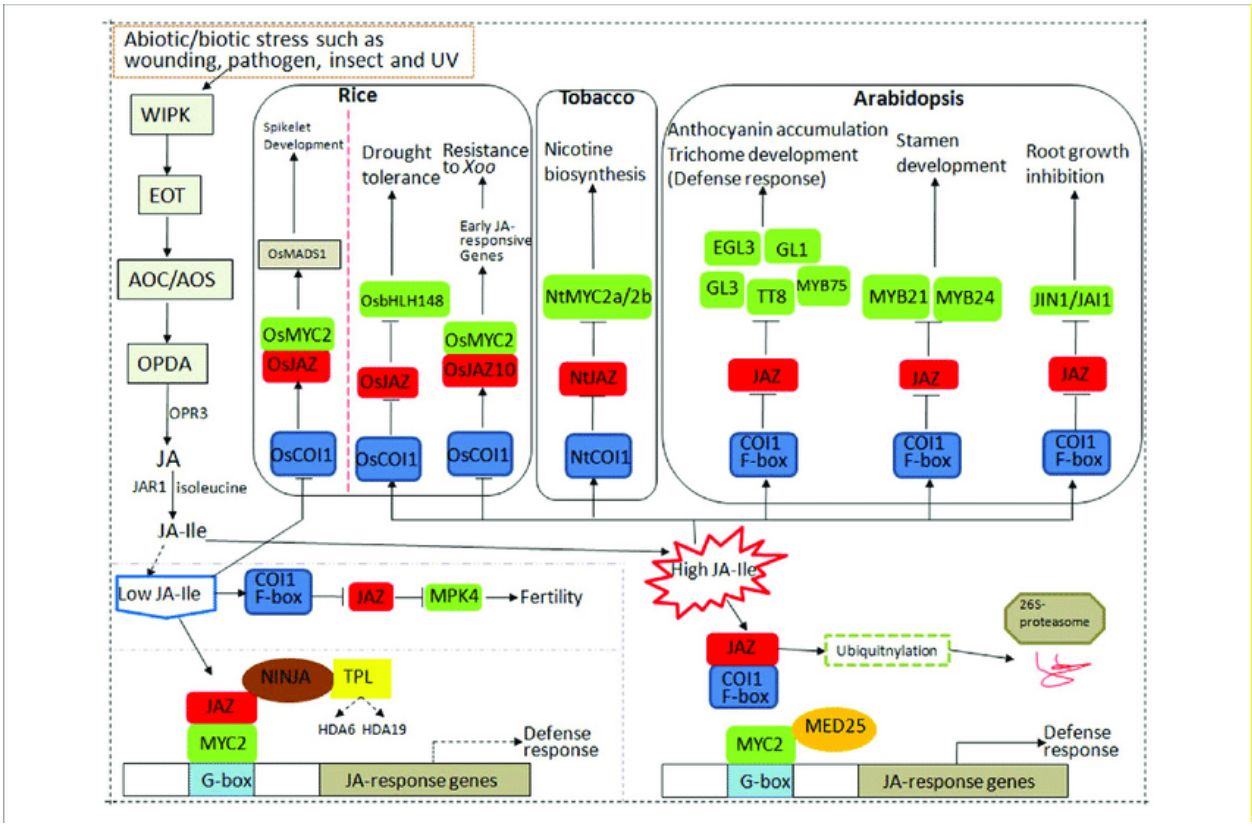


Figure 1.6 Jasmonic acid signalling in plant defence and growth response. CORONATINE INSENSITIVE 1 (COI1) protein, JASMONATE ZIM DOMAIN PROTEIN (JAZ), and MYC constitute the core of JA signalling. Under control conditions, JA-isoleucine (Ile) is very low plants. Several JAZ repressors bind to MYC2 to inhibit its transcriptional activation on downstream genes. Upon Infection JA levels are largely activated, which are then perceived by JA receptor COI1. After that SKP1/CULLIN/F-box (SCF) COI1 binds to JAZs for ubiquitination and mark the proteins for degradation through the 26S proteasome pathway, resulting in the release of the downstream TFs that activates MYCs and the activation of JA responses *(Reproduced from Yang et al., 2019, Frontiers in Plant Science; under the Creative Commons Attribution License (CC BY))*

Other than JA-Ile several JA derivatives are also known to contribute towards plant defence response. *Arabidopsis opr3* mutants that lack 12-oxophytodienoic acid reductase (OPR)

activity, fails to convert 12-oxophytodienoic acid (OPDA) to OPC8:0, effectively stopping the JA biosynthesis process (Stintzi and Browse 2000). Interestingly *opr3* mutant have been found to impart increased resistance to the necrotrophic fungus *Alternaria brassicicola,* which JA-insensitive plants such as the coi1-1 and jar1-1 mutants fails to establish. These results suggest that OPDA may act in JA-independent manner to impart defence against certain pathogen species. Methyl jasmonate (MeJA), an airborne jasmonate, similar to MeSA, can also induce defence-related genes, in plants (Manners et al. 1998). Conversion of JA to MeJA is regulated by S-adenosyl-L-methionine: jasmonic acid carboxyl methyltransferase (JMT) (Seo et al. 2001). Transgenic plants overexpressing JMT gene exhibit the higher accumulation of MeJA but not JA, and constitutive expression of JA-responsive genes VSP and PDF1.2. It has been attributed that plants with higher amount of MeJA could defend themselves against *B. cinerea*, suggesting that MeJA acts as an active molecule in plant defence response (Seo et al. 2001). Thorpe and his co-workers reported that the MeJA is a mobile molecule and is transported through both xylem and phloem pathways in tobacco. The authors also demonstrated that the transport of MeJA to systemic site is regulated by sugar transport machinery available in the vascular structures (Thorpe et al. 2007). Taken together, these findings indicate that several JA derivatives are produced by plants and contribute to the diversity of plant defence actions.

It has been shown that rice possesses a COI1 family, unlike *Arabidopsis*, three COI1 homologs have been predicted and functionally characterized in rice. Upon introduction of rice COI1 homologs, *OsCOI1a* and *OsCOI1b* in *Arabidopsis coi1-1* mutant, JA insensitivity was fully complemented in both the cases (Lee et al. 2013). Moreover, rice *OsJAZ8* is known to interact with *OsCOI1b* (referred to as *OsCOI1H* in the original paper) in a COR-dependent manner. The overexpression of *OsJAZ8ΔC*, which is defective in the C-terminal region responsible for the binding to *COI1s* (defined as a Jas motif, a 20 amino acid length of the conserved domain in the JAZ family), caused an insensitivity to JA. It increased susceptibility to bacterial blight *Xanthomonas oryzae pv. oryzae* (Yamada et al. 2012). These findings reveal that the COI1–JAZ signalling system, which is conserved in Arabidopsis and rice, is the central machinery for JA-mediated plant defences against pathogens.

1.5.2. JA in Growth

In the last decade, a numerous studies have focused upon understanding the role of JA in plant growth and development. A large number of studies have established that JA and several of its derivatives are involved in a number of plant development processes, such as primary root growth, leaf senescence, and reproduction (Wasternack and Hause 2007; Kim et al. 2015) (Figure 1.7). In this section, we summarize importance of JA in few of these plant development processes.

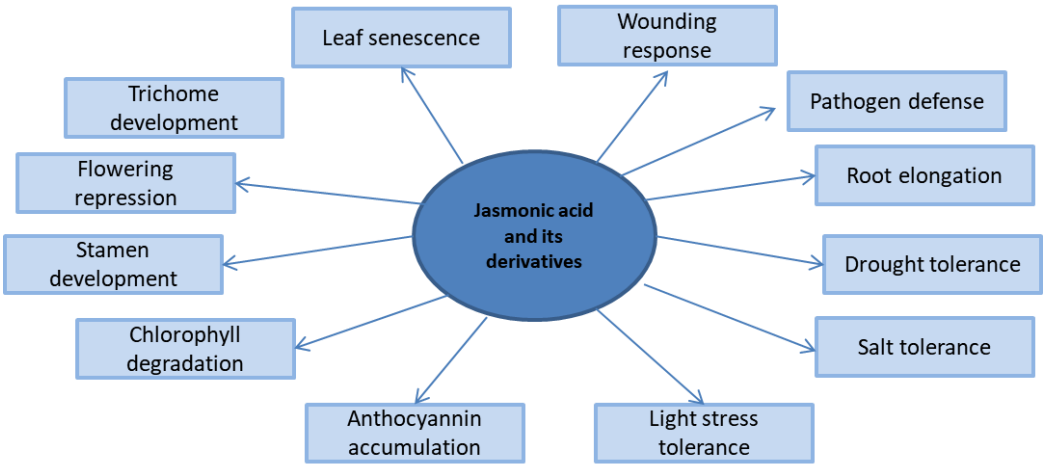


Figure 1.7 Diverse plant processes regulated by JA in the plant.

1.5.2.1. Effects on seed germination

Several phytohormones, such as ABA, IAA, and JA are essential role player during seed germination (Xiao et al. 2018). It has been shown that both ABA and JA inhibit seed germination, but the molecular mechanism behind this process is still unclear (Tang et al. 2020). In *Arabidopsis*, JA signalling is controlled in a COI1-independent manner (Dave et al., 2011). In *Triticum aestivum* plants, cold-stimulated germination of seeds resulted in an increase in the endogenous level of JA after upregulation of JA biosynthesis-related gene, and JA promotes cold-stimulated germination (*Xu et al. 2016; Avramova 2017*). Recently, *SAPK10-bZIP72-AOC'* pathway was identified in rice, here it was shown that ABA stimulates JA biosynthesis to synergistically inhibit the germination of rice seeds (Wang et al., 2020). *SAPK10* molecule promotes its binding to the G-box cis-element of AOC promoter by phosphorylation of *bZIP72* TF to enhance AOC transcription in the presence of elevated concentrations of JA (Wang et al. 2020).

1.5.2.2. Inhibition of root growth

Several reports have shown that JA acts as a suppressor of root maturation in plants. *coi1* mutants are found to be insensitive to JA mediated suppression of primary root formation (Yan et al., 2009). These results has been substantiated via application of coronatine-O-methyl oxime, a competitive JA antagonist, suppresses the inhibitory outcome of coronatine on primitive root growth (Monte et al. 2014). JAZ proteins that are known to suppress the JA response in plants are found to be essential role players in regulating primary root formation, where combined mutations in *JAZ7, JAZ8, JAZ10,* and *JAZ13* resulted in inhibition of root formation in plants due to activation of JA signalling (Thireault et al. 2015; Thatcher et al. 2016). In *Arabidopsis*, *bHLH* type transcription factors (MYC2 and its homologs *MYC3/4/5*) has been shown to interact with JAZ proteins (Qi et al., 2015). MYC2/3/4 are involved in the inhibition of primary root apex; which is regulated by JA (Gasperini et al., 2015). Moreover, MYC2 inhibits *PLETHORA genes* (*PLT1 and PLT2*) expression to minimize the activity of meristematic root cells and suppress primary root growth (Chen et al., 2011).

JA mediated primary root growth inhibition and root hair formation has been attributed to interaction of JAZ proteins with *ETHYLENE INSENSITIVE (EIN) 3* and EIN3-LIKE1 (EIL1) TFs, a part of ethylene signalling (Zhu et al., 2011). In *Arabidopsis*, JA is also known to stimulate the lateral root formation by upregulating ERF109 expression, which in turn stimulates ANTHRANILATE SYNTHASE A1 (ASA1) and YUCCA2 expression to regulate auxin biosynthesis in plants (Cai et al., 2014). In contrast, via *COI1-MYC2/3/4* signalling JA negatively regulates adventitious root formation in plants (Gutierrez et al., 2012).

1.5.2.3. Delay of flowering

In *Arabidopsis*, JA has been shown to contribute to vegetative-reproductive maturation transition. *COI1–JAZ* interactions inhibits flowering, where *coi1* plants, exhibits an early flowering response. Moreover, *TARGET OF EAT* TFs (*TOE1 and TOE2*), *APETALA2/ERF* domain TFs are known to repress plant flowering upon interacting with JA-induced JAZ proteins, by inactivating the transcription of *FLOWERING LOCUS T* (FLT). On the other hand, overexpression of *TOE1* and/or *TOE2* inhibits *coi1* leading to early flowering phenotype (Zhai et al. 2015).

1.5.2.4. Inhibition of hypocotyl growth

JA has been shown to inhibit hypocotyl elongation under light stress conditions such as far-red and blue wavelengths, regulated through *COI1* (Chen et al., 2013). Studies have shown that JA-deficient mutant *jar1* or *coi1* mutants have longer hypocotyls compared to WT plants when they were grown in the dark or under low R/FR light ratio far-red lights conditions (Chen et al., 2013; Robson et al. 2010). Similar observation was noted for the *myc2*/*jin1* mutant grown under low R/FR light ratio (Robson et al. 2010), but these plants also show a shorter hypocotyl under blue light as well (Yadav et al. 2005). Differential hypocotyl growth under different light conditions is attributed to bHLH type TF MYC2, that is known to be induced upon JA accumulation.

1.5.2.5. Stamen development in *Arabidopsis*

In *Arabidopsis*, many male-sterile plants were found to be JA-deficient mutants. For example, Arabidopsis mutants such as *coi1, lox3 lox4, aos, opr3; fad3 fad7 fad8*, *defective in anther dehiscence1 (dad1), JAZ1Δ3A* and *JAZ10.4* were found to exhibit incomplete stamen development (Song et al. 2013). However, upon exogenous JA application stamen development was rescued only in plants deficient in JA biosynthesis but not in JA signalling mutants (Jewell and Browse 2016). Moreover, in *coi1* background, expression of *COI1* in a tissue specific manner can retrieve anther dehiscence, filament elongation, as well as pollen maturation (Jewell et al. 2016). *JA* regulated TFs such as *MYB21*, *MYB24*, and *MYB57* interact directly with JAZs and their double mutants *myb21-myb24* exhibit delayed anther dehiscence, non-viable pollen grains and short filaments (Song et al. 2011). It has been reported that *MYB21* and *MYB24* physically binds with *MYC2, MYC3, MYC4,* and *MYC5* to control stamen development. Interestingly, overexpression of *MYC5* and *MYC3* in *coi1-1* plants can restore stamen maturation and productivity (Qi et al., 2015).

1.6. SA-JA antagonism

Several plant hormones are utilized in mounting an effective defence response in plants. SA and JA are well recognized as major defence hormones in plants (Browse 2009, Vlot et al. 2009). As it has ben established that SA defence response is catered for infection against biotrophic pathogens and JA against necrotrophic pathogens, a so called hormone cross-talk

between these too major defence related hormones. The first study indicating SA-JA cross talk was reported in tomato, where it was demonstrated that SA and its acetylated form MeSA could suppress of the JA-dependent wound response (Doherty et al. 1988, Peña-Cortés et al. 1993). Since the establishment of SA-JA antagonism, in tomato several studies have reported that same in several other plant species (Spoel et al. 2003, Van Wees et al. 1999). This interplay between SA and JA is required for optimum induction of the immune response against virulent *Pseudomonas syringae*, that is known to stimulate both the SA and JA pathways (Spoel et al. 2003). Thus, SA-JA cross talk becomes essential for selecting a defence response based upon the type of pathogen attacking the plant. Due to prioritizing of one of the defence response trade-offs between SA-dependent resistance to biotrophs and JA-dependent defence against necrotrophs have been reported repeatedly (Bostock 2005, Kunkel & Brooks 2002, Verhage et al. 2010). For example, induction of the SA pathway by avirulent *P. syringae* suppresses JA signaling and rendered infected Arabidopsis leaves more susceptible to the necrotrophic fungus *Alternaria brassicicola* (Spoel et al. 2007). Similarly in cabbage, prior activation of SA pathway through biotrophic pathogen H*yaloperonospora arabidopsidis*, suppresses JA-mediated defence response activated by caterpillar herbivore Pieris rapae (Koornneef et al. 2008).

The underlying mechanism how these hormones regulate each other could only be understood through studying the gene expression during pathogen attacks. As described above, SA seems to dominate the regulation of JA during infection. Thus, interference of SA in suppression of JA responses could be attributed to suppression of JA transcription machinery upon SA-induction. There are several transcriptional co-activators, such as NPR1, and WRKY, that are involved in SA-mediated suppression of JA-responsive genes (Figure 1.8). These molecules can act as suppressor of JA-mediated defence response. However, precise mechanism through which these transcriptional co-activators regulate JA response pathway still remain unclear.

NPR1 has been implicated as a central role player of SA mediated defence response to regulate several defence genes. It has been demonstrated that NPR1 nuclear localization is essential for SA-induced defence response, but interestingly SA-mediated suppression of JA pathway seems to be independent of NPR1 transport. It was shown that cytosolic NPR1 seems to be sufficient for SA-mediated suppression of JA pathway (Spoel et al., 2003). But, we should not neglect the presence of nuclear NPR1 that could potentially regulate several SA-responsive

TFs contributing to indirect suppression of JA pathway. Although the mechanism of how NPR1 suppresses JA-pathway remains unclear, few studies have demonstrated the role of WRKY TFs contribution in this suppression. One such example includes WRKY62 as a central role player in SA-JA crosstalk (Mao et al. 2007). WRKY62 has been reported to be induced by both SA and JA in wild-type Arabidopsis. SA mediated induction of WRKY62 is regulated by NPR1 as mutant of *npr1* failed to induce WRKY62 expression. Mutant *wrky62* plants exhibit higher expression of several JA-responsive genes, whereas overexpression of WRKY62 suppresses the JA pathway. These finding suggests that SA- and NPR1-inducible negatively regulate JA pathway through WRKY62 (Mao et al., 2007).

On the basis of studies carried out, it seems there have been significant information available in how SA regulates JA pathway, but very limited information is available regarding the JA mediated regulation of SA pathway during pathogen infecton.

1.7. Plant microRNAs

MicroRNAs (*miR*s) are endogenous small ~21-22 nucleotide (nt) non-coding RNAs that act as negative regulators of gene expression (Bartel and Bartel, 2003; Dugas and Bartel, 2004). RNA Pol II transcribes these non-coding genes and generates primary miR transcripts (Katiyar-Agarwal and Jin, 2010). The primary miR transcript then forms a stem-loop structure that is the recognized and processed by the Dicer-like protein complex (*DCL1-HYL1-SE*) along with the DAWDLE (*DDL*) to produce precursor miR (pre-miR) (Yu et al., 2008). The DCL1-HYL1 complex further processes the pre-miR to generate stable 21-22 nt *miR*:*miR* $*$ duplex, that is later methylated at the 3'-ends by *HUA ENHANCER 1 (HEN1)* (Yu et al., 2005). These methylated miRs are transported to the cytoplasm by an exportin homolog, HASTY (HST) (Ruiz-Ferrer and Voinnet, 2009). The mature miR is further incorporated into the RNA-induced silencing complex (RISC) containing *Argonaute1 (AGO1)* protein. The RISC is recruited to the target gene based on sequence complementarity of miR and the target mRNA (Vazquez et al., 2004). *AGO1* then represses gene expression by initially cleaving and then degrading the target mRNA or repressing its translation (Figure 1.9). Since their discovery, plant miRs have been found to play crucial role in plant development processes such as embryo development, seed germination, reproduction and immunity (Wang et al., 2005; Kidner and Martienssen, 2005; Jones-Rhoades

et al., 2006; Navarro et al., 2006; Liu et al., 2007; Jung and Park, 2007; Nogueira et al., 2009; Borges and Martienssen, 2015; Li and Zhang, 2016).

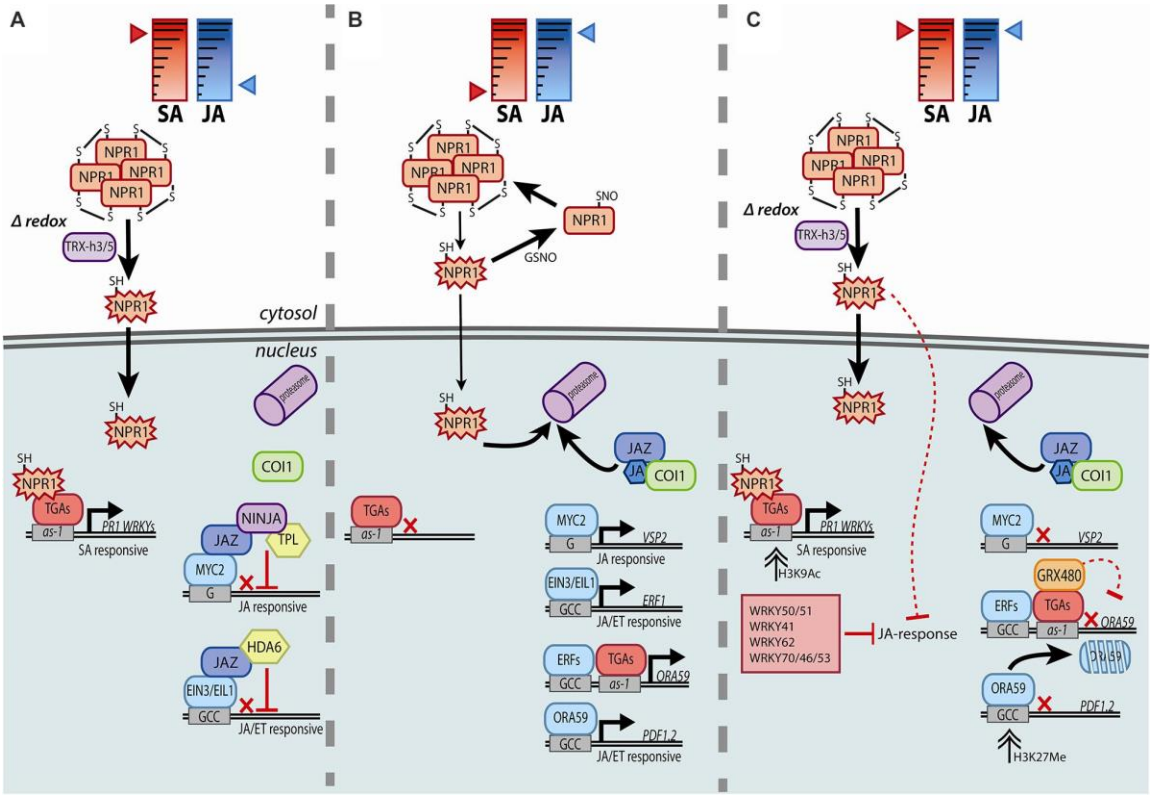


Figure 1.8 A model explaining the molecular mechanism involved in transcription regulation upon treatment with SA (A), JA (B) and JA-SA antagonism (C). SA activates NPR1 by breaking its oligomeric complex to monomers, which then gets transported to nucleus and triggers gene expression. JA-responsive genes are suppressed by intact JAZ repressors in the absence of JA. In the presence of JA, COI1 forms a complex with JAZ and JA and is marked for degradation. Later MYC or ERF TFs activate JA-responsive genes, in absence of SA. When both SA and JA are activated pathways leads to antagonism of JA-responsive gene repression by SA. Solid lines indicate established activities and dashed lines hypothesized activities, where black arrows specify activation and red blocks suppression. Red crosses indicate that gene transcription is hampered. (*Reproduced form Caarls et al., 2015, Frontiers in Plant Sciences; under the Creative Commons Attribution License (CC BY)*)

1.7.1. miR in defence response

MicroRNAs have been shown to play a crucial role in plant defence against various types of pathogens infecting plants. Plants utilize miRs to mount a quick response to the invading pathogens. Under non-infective conditions, miRs involve in the defence response either are themselves repressed or suppresses plant defence machinery but upon infection these *miR*s can swiftly turn on the defence response by regulating various genes involved in defence response. *miR*s have been shown to act both as a positive and negative regulator of defence. In *Arabidopsis,* Navarro et al. (2006) have shown that upon elicitation with flg22 (a PAMP) from *Psuedomonas syringae*, *miR393* accumulates and inhibits F-box auxin receptors in plants and mount a successful PTI response. These authors showed that miR393 targets TIR1, the auxin receptor that de-represses the inhibition of ARFs. As miR393 targets TIR1, it was proposed that miR393 is involved in the growth-defence trade-off response and mediates PTI induction. Under non-infective conditions, miR393 levels are low; as a result, *TIR1* levels are high. These increased *TIR1* levels inhibit the *AUX*/*IAA* repressors by marking them for degradation. Thus, ARFs are free to carry out the transcription of auxin-responsive genes and facilitate the plant's growth (Navarro et al., 2006; Ruiz-Ferrer and Voinnet, 2009; Sunkar et al., 2012).

After the discovery of miR393's role in PTI, some miRs are shown to target R-genes, which are involved in ETI response of the plant (Zhai et al., 2011; Li et al., 2012). These miRs are known to act as signal amplifiers upon detection of pathogen attack, by generation of siRNAs (Shivaprasad et al., 2012). For example, tomato miRs, miR482 and miR2118, under non infective conditions negatively regulates the presence of R-genes in cell but upon infection, miR82 levels decrease and in turn accumulates R-genes in the host cells. It has been observed that miR482-mediated silencing of R-genes is actively suppressed by a viral and bacterial infection (Shivaprasad et al., 2012), suggesting a counter-counter-defence strategy adopted by pathogens. Similar to the above studies, multiple reports further unveiled the role of miRs in various plant-pathogen interactions. A brief overview of various miRs involved in the defence response is summarized in Table 1.1.

Table 1,1 List of various miRs involve in regulating defence response in plants. (*adapted and modified from Huang et al., 2016; License is appended in Annexure I)*

miRNA	Host	Pathogen	Target genes	Expression of gene upon infection	Roles in plant-pathogen interaction	References
miR159	*Arabidopsis*	*P. syringae*	MYB33, MYB65, and MYC101	Up	Regulate gibberellin (GA) and ABA signalling pathways.	Zhang et al. (2011)
miR160	*Arabidopsis*	*P. syringae*	ARF10, ARF16, and ARF17	Up	Increase PAMP-induced callose deposition.	Li et al. (2010)
miR160	*M. esculenta*	*C. gloeosporioides*	ARF10	Up	Regulate plant auxin and enhance plant defense responses.	Pinweha et al. (2015)
miR160	*O. sativa*	*M. oryzae*	ARF16 and a B3 DNA-binding domain-containing protein	Up	Over-expression of miR160 increases the accumulation of hydrogen peroxide and defense-related genes and attenuates fungal growth.	Li et al. (2014)
miR160	*Solanum tuberosuum*	*oomycete P. infestans*	ARF10 and ARF16	Up	Altering miR160 levels leads to susceptibility in miR160 OE and KD lines. miR160 KD lines are found to be SAR-deficient.	Natarajan et al., (2018)
miR167	*Arabidopsis*	*P. syringae*	ARF8, ARF6	Up	Regulate auxin signalling pathway and enhance plant defense response.	Fahlgren et al. (2007); Zhang et al. (2011)
miR168	*O. sativa*	*RSV and RDV*	AGO1	-	Infection induces accumulation of AGO18 which sequesters miR168. AGO1 expression is then	Wu et al. (2015)

					rescued, resulting in enhanced plant defense.	
miR390	*Arabidopsis*	*P. syringae*	TAS3	Down	Trigger the accumulation of ta-siRNAs that regulate the expression of *ARF3* and *ARF4*, genes involved in auxin signalling.	Zhang et al. (2011)
miR393	*Arabidopsis*	*P. syringae*	TIR1, AFB2, and AFB3	Up	Regulate auxin signalling and enhance plant defense response.	Navarro et al. (2006); Fahlgren et al. (2007)
miR393	*M. esculenta*	*Colletotrichum gloeosporioides*	TIR1	Up	Regulate auxin signalling and enhance plant defense response.	Pinweha et al. (2015)
miR393b*	*Arabidopsis and Nicotiana benthamiana*	*P. syringae*	MEMB12	Up	Increase the secretion of antimicrobial pathogenesis-related protein PR1.	Zhang et al. (2011)
miR396a-5p	*Solanaceae*	*Oomycete P. infestans*	GRF	Down	Over-expression of miR396a-5p decreases plant resistance to *P. nicotianae*.	Chen et al. (2015)
miR398	*Arabidopsis*	*P. syringae*	COX5b.1, CSD1 and CSD2	Down	Negatively regulate callose deposition and is involved in the suppression of auxin signalling and detoxification of ROS.	Jagadeeswaran et al. (2009); Li et al. (2010)

miR398	*Hordeum vulgare L.*	*Blumeria graminis f. sp. hordei*	SOD1	-	Mla and Rom repress miR398-mediated SOD1 expression to change the HR response to fungus.	Kerchev et al. (2013)
miR398	*O. sativa*	*M. oryzae*	SOD2	Up	Over-expression of miR398 increases the accumulation of hydrogen peroxide and defense-related genes and decreases fungal growth.	Li et al. (2014)
miR399	Citrus	Ca. L. asiaticus	PHO2	Up	Contribute to HLB symptoms and phosphorus homeostasis and signalling.	Zhao et al. (2013)
miR408	*Arabidopsis*	*P. syringae*	Copper protein plantacyanin, laccase copper protein and copper ion binding protein genes (predicted targets)	Up/Down	-	Zhang et al. (2011)
miR408	*Wheat*	*Puccinia striiformis f. sp. tritici*	TaCLP1, a type of plantacyanin protein	Up/Down	Negatively regulate wheat resistance to stripe rust.	Feng et al. (2013)
miR472	*Arabidopsis*	*P. syringae*	CC-NBS-LRR	-	Over-expression of miR472 decreases plant resistance to bacteria.	Boccara et al. (2014)
miR482	*S. lycopersicu*	*es TCV, CMV and TRV*	NBS-LRR	Down	Virus and bacteria infection down-regulates the expression of miR482 and induces the	Shivaprasad et al. (2012)

	m				expression of R protein.	
miR482	*G. raimondii*	*V. dahlia*	NBS-LRR	Down	Fungal pathogen infection down-regulates the expression of miR482 and induces the expression of R protein.	Zhu et al. (2013)
miR482	*S. lycopersicum*	*F. oxysporum*	*Solyc08g075630, Solyc08g076000*	Down	Fungus infection down-regulates the accumulation of miR482 to increase the expression of NB domain genes.	Ouyang et al. (2014)
miR773	*Arabidopsis*	*P. syringae*	*MET2*	Down	Negatively regulate callose deposition and disease resistance to bacteria.	Li et al. (2010)
miR825	*Arabidopsis*	*P. syringae*	Remorin, zinc finger homeobox family, frataxin-related	Up	-	Fahlgren et al. (2007)
miR1507	*M. truncatula*	-	NBS-LRR	-	-	Zhai et al. (2011)
miR1885	*Brassica napus*	*TuMV*	TIR–NBS–LRR	Up	Repress ETI	Wroblewski et al. (2007)
miR2109	*Medicago*	-	NBS-LRR	-	-	Zhai et al. (2011)
miR2118	*Medicago*	-	NBS-LRR	-	-	Zhai et al. (2011)

miR2118	*S. lycopersicum*	*es TCV, CMV and TRV*	NBS-LRR	Down	Virus and bacteria infection down-regulates the expression of miR482 and induces the expression of R protein.	Shivaprasad et al. (2012)
miR5300	*S. lycopersicum*	*F. oxysporum*	*Solyc05g008650, tm-2*	Down	Fungus infection down-regulates the accumulation of miR5300 to increase the expression of NB domain genes.	Ouyang et al. (2014)
miR6019/ miR6020	*N. tabacum*	*TMV*	TIR-NBS-LRR	-	Over-expression of miR6019 and miR6020 attenuates N-gene mediated resistance to viruses.	Li et al. (2012)
miR7695	*O. sativa*	*M. oryzae*	OsNramp6	-	Over-expression of miR7695 enhances plant defense resistance.	Campo et al. (2013)
miR9863	*Hordeum vulgare L.*	*Blumeria graminis f. sp. hordei*	Mla1	-	Over-expression of miR9863 reduces fungal resistance and cell-death signalling.	Liu et al. (2014)

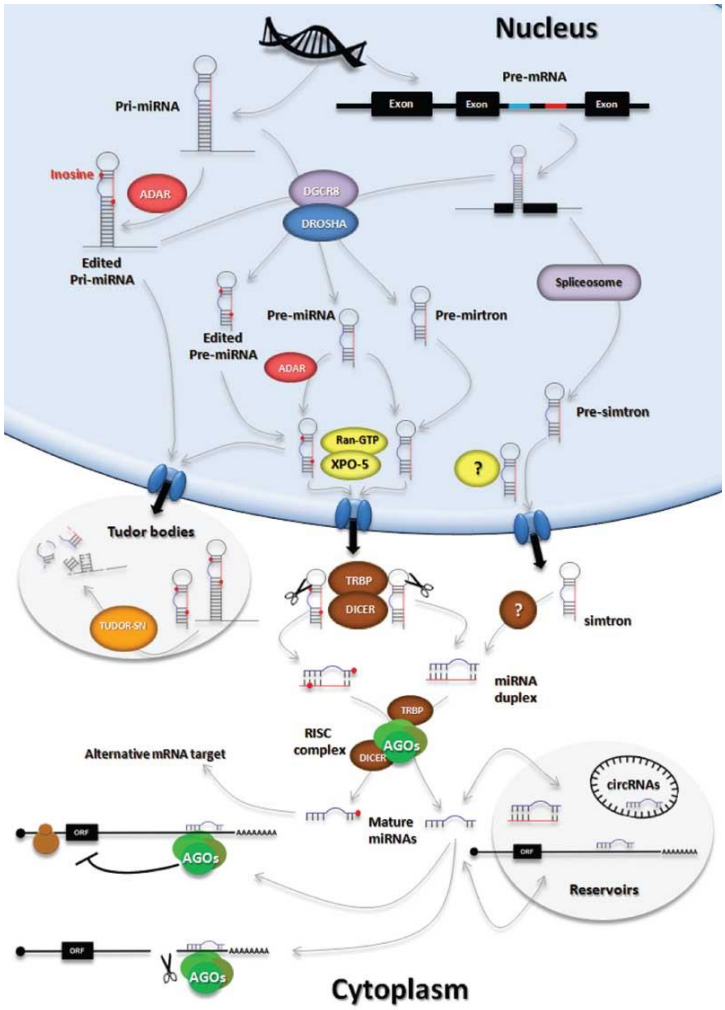


Figure 1.9 A model describing *miR* biogenesis in plants. Primary miR transcripts are generated by transcription of noncoding genes using RNA Pol II. DCL1-HYL1-SE protein complex bids to the stem loop structure of primary *miR*s and process them into pre-miR. Pre-miRs is then further processed by DCL1-HYL1 complex to generate 21-22 nt miRs. The miR-miR * duplex is methylated at 3' ends by HEN1 and transported into cytoplasm by HST. Mature miR is then incorporated into RISC containing AGO1 protein. The RISC is then recruited to the target gene on the basis of sequence complementarity with incorporated *miR* leading to gene repression by either mRNA degradation or translational arrest. (*Reproduced from Lopez et al., 2013, BioMolecular Concepts; under the Creative Commons Attribution License (CC BY))*

1.7.2. The role of miR160 and miR166 in defence

miR160 has been shown to be crucial in various development process in plants (Wang et al., 2005; Mallory et al., 2005; Liu et al., 2007; Gutierrez et al., 2012; Hendelman et al., 2012; Liu et al., 2013; Turner et al., 2013; Huang et al., 2016; Damodharan et al., 2016). Moreover, the role of miR160 in defence response has also been elucidated in several plants species like Arabidopsis and rice (Li et al., 2010; Li et al., 2014). miR160 has been reported to target various Auxin Response Factors (ARFs) plants, namely ARF10, ARF16 and ARF17. Previous study from our lab has demonstrated the role of miR160 target StARF10 and its potential role in regulating StGH3.6, a homolog of AtGH3.5 in potato (Natarajan et .al., 2018). *AtGH3*.5 has been implicated to play a role in maintenance of balance between free Auxin and SA in plants. Unfortunately, we do not know how miR160 regulates the development of SAR (Natarajan et al., 2018).

Similar to *miR*160, the function of *miR*166 has been reported in plant development, which targets homeodomain-leucine zipper class III (HD-ZIP IIII) family transcription factor, such as REVOLUTA, PHABULOSA and PHABOLUTA, to specify the fate of the shoot apical meristem (Zhu et al. 2011; Li et al. 2019). Recently, miR166 function has been established in plant immunity in rice. Studies have reported differential expression of miR166 members in response to infection with *M. oryzae.* For example, *miR*166 was found to be down-regulated following treatment with *M. oryzae* elicitors in susceptible rice varieties (Campo et al., 2013). In another rice variety found to be moderately resistant, expression of both miR166k-3p and miR166j-3b was upregulated following *M. oryzae* infection (Zhang et al. 2018). In a *dcl1* mutant, expression of miR166j-5 was observed to be down-regulated, whereas expression of miR166a/b/c/d-3p/f was upregulated upon infection with *M. oryzae* (Zhang et al. 2018). However, a recent study in the rice shows that *miR*166k-166h polycistron leads to co-expression of *miR*166k and *miR*166h upon infection with *M. oryzae* (Baldrich et al. 2016). Up-regulation of *miR*166k and *miR*166h from an activation mutant *miR*166k-*166* h exhibits an increased resistance to rice blast disease, associated with activation of the ET-signalling pathway and high marked up-regulation of defence-related genes. In the same study, authors demonstrate that *miR*166k-5p suppresses the expression of two *OsEIN2* genes (Salvador-Guirao et al. 2018). Other than in rice, miR166 was found in the cross-kingdom RNAi, where pathogens utilize sRNAs to help in successful

establishment of infection. It has been reported that cotton (*Gossypium hirsutum*) and Arabidopsis roots accumulates miR166 upon infection with *V. dahliae* (Zhang et al., 2016). Even in soybean, miR166 was observed to be upregulated upon infection with *P. sojae* (Wong et al., 2014). In the same study, they have shown that miR166 expression increases upon treatment with heat-inactivated *P. sojae*. In contrast, miR166 is shown to be down-regulated in response to *C. graminicola (Balmer et al., 2017).* These studies depict the importance of miR166 in defence response against variety of pathogens in plants.

1.8. *Phytophthora* and potato defence response

Phytophthora-induced plant disease is known as blight diseases that causes wilting, damping-off, chlorosis, root rot, and the rotting of other organs. Among the many *Phytophthora* species identified, *Phytophthora infestans* (Montague) discovered by Aton deBary is infamous as the causal agent of the Irish potato famine in the 1840s (Fry, 2008). *P. infestans* follows a hemi-biotrophic form of life style and is considered to be a very complex pathogen that carries a plethora of effector genes to successfully infect its host (Haas et al., 2009). Due to the importance of potato as cash crop, various attempts to transfer R-genes from wild relatives of potato to the cultivated varieties by conventional crossing techniques (Kumar et al., 2006; Sliwka et al., 2010). However, due to high evolving nature of *P. infestans* combined with large number of effector genes in its genome, renders the resistance gained through these strategies were ineffective.

Though there are various studies available regarding identification of R-genes in potato but the knowledge of SAR response in potato is still in its initial phase. There is only one report that describes the role of miR160 in defence response during potato-Phytophthora interactions, where authors have demonstrated the importance of miR160 in developing local and systemic defence responses in potato (Natarajan et al., 2018). This was also the first study that reported the involvement of miR, miR160, in establishment of SAR response in potato pivoted over the effective response of SA dependent SAR. Other studies have also reported the indispensable role of SA in potato defence response against *P. infestans*, Potato virus X and Potato virus Y (Halim et al., 2007, 2009; Sánchez et al., 2010; Baebler et al., 2014). Alongside, Manosalva et al., (2010) shows that similar to tobacco and Arabidopsis, MeSA is a mobile signal in potato and StMES1 (otholog of SABP2), is involved in the conversion of SA to MeSA in the local leaves

treated with AA (Manosalva et al., 2010). These few reports undoubtedly, represent the need of further studies to understand the role of miRs and process of SAR response in potato-*Phytophthora* interactions.

1.9. Hypothesis

Numerous reports have demonstrated that miRs could regulate PTI and ETI responses in plants. Recently, we have shown that miR160 is essential for plant's local and systemic defence response (Natarajan et al., 2018). It was found that miR160 plays a crucial role in maintaining auxin-mediated growth signalling and SA-dependent defence responses through *StARF10*, a target of miR160. But, the potential defence related role if any, for other miR160 targets, viz., *StARF16* and *StARF17* could not have been studied. Additionally, it was noticed that the miR160 deficient transgenic lines (miR160 KD) failed to elicit SAR response during *P*. *infestans* infection but the underlying mechanism remained unclear. As miRs are important regulatory molecules, we hypothesised that there could be additional miRs, such as miR166, that may be involved in establishment of SAR as well. In potato, it has been observed that upon infection with *P. infestans* miR166 also accumulated similar to miR160 and is hypothesized to contribute towards plant defence response (Natarajan et al., 2018). Using potato–*Phytophthora infestans* interaction as a model system, we laid out a number of objectives to test our hypotheses.

1. Understanding the mechanistic link between miR160 target, StARF16 and defence response.
2. To investigate the compromised SAR response in miR160 knockdown lines.
3. Investigating the potential role of miR166 in the defence response of potato.

Chapter 2

MiRNA160 target, *StARF16*, regulates defence gene *StNPR1* during plant pathogen interactions

2.1. Introduction

2.1.1. Role of miR160 in plant development and immunity

Since the discovery of miRNA's role in defence response, many miRNAs have shown to be associated with plant immunity (Navarro et al., 2006, Natarajan et al., 2018). One such miRNA, miR160, has been studied widely in both dicots and monocots for its role in development and defence responses (Ben-Gera et al., 2016; Li et al., 2010; Yu et al., 2017; Armenta-Medina et al., 2017). MiR160 has been known to target three Auxin Response Factors (ARF10, ARF16, and ARF17) that have well implicated roles in the control of root, flower, leaf and shoot development in various plants (Liu et al., 2007; Qiao et al., 2012). Studies have also established that ARF17 binds to the *GH3*.5 promoter in *Arabidopsis* and, regulates plant development (Mallory et al., 2005). It is also demonstrated that GH3.5 regulates free SA and auxin available in plants by adenylating these hormones; hence playing a dual role in regulating plant immunity and development (Park et al., 2007; Ostrowski and Jakubowska, 2013; Zhang et al., 2008). Both are energy intensive processes that enable the plants to maintain balance between development and defence. Recently, Natarajan et al., 2018 demonstrated a potential role of StARF10 in the regulation of defence response in potato. It was established that *StGH3*.6, a homolog of *AtGH3.5,* could govern the plant's defence response which is a target for ARF10 in potato (Natarajan et al., 2018). Another gene, *PP2A-B'γ* has been demonstrated to regulate defence response against a necrotrophic pathogen, *Botrytis cinerea,* and leaf senescence in *Arabidopsis* by regulating genes involved in the SA pathway (Durian et al., 2020).

2.1.2. Role of ARFs in plant development

There are 23 predicted ARFs in *Arabidopsis* and each ARF gene is expressed in a dynamic and differential pattern during development, and various genetic studies have shown that individual ARFs control distinct developmental processes (Rademacher et al., 2012). ARF proteins contain three domains: a N-terminal DNA-binding domain, a middle region containing transcriptional activation or repression domain, and a C-terminal protein-protein interaction domain (Guilfoyle and Hagen, 2007; Di et al., 2015).–During auxin perception and signaling processes, ARF binds to a consensus motif of TGTCTC, called the ARF binding site, which exists in the promoters of many auxin responsive genes. Expression of *ARF10*, *ARF16*, and *ARF17* (targets of miRNA160) shows that they are the crucial role players during shoot, leaf, carpel

and root cap development (Gepstein, 2004; Wojcikowska and Gaj, 2017; Nizampatnam et al., 2015; Liu et al., 2016; Ye et al., 2016) and have been suggested to act as repressive transcriptional factors (Hendelman et al., 2012). Despite ARFs significant role in plant development, their role in defence response is still not clearly established.

This study was carried out with following objectives

1. Expression analysis of the miR160 targets during infection with hemi-biotrophic and necrotrophic pathogen.

2. To predict the potential defence response genes regulated by miR160 targets.

3. To understand the interactions between defence response genes and miR160 target.

2.2. Material and methods

2.2.1. Plant and pathogen material and growth conditions

All the potato plants used in this study were *Solanum tuberosum* L. cv. Désirée. The plants were grown in a greenhouse under 25 °C with a 16-hr light / 8-hr dark photoperiods for five weeks before conducting detached leaf and pathogen assay experiments against different pathogens. Potato plants were grown in tissue culture on Murashige and Skoog (MS) basal medium with 2 % sucrose and 0.2 % phytagel at pH 5.8 for two weeks before transferring to soil. The soil plants were transferred to growth chambers before carrying out pathogen assays. The oomycete pathogen *Phytophthora infestans* strain A2 was maintained at 18 °C in pea agar media whereas, the fungal pathogen *Alternaria solani* was maintained at 28 °C in potato agar media. The *A. solani* was confirmed using As-Tub-FP and AS-Tub-RP primers from RNA of *A. solani* conidia.

2.2.2. Infection assay and growth conditions for *Phytophthora infestans* and *Alternaria solani*

For all the infection experiments including *P. infestans*, sporangia were used at a concentration of 1 x 10^6 sporangia/ml and treated plants were incubated at 18 °C and 90 % humidity (Natarajan et al., 2018). Time course experiments in potato plants were performed by inoculating 1 x 10^4 spores on the abaxial side of the 4th and 5th leaf, counted from the apical meristem. Simultaneously, control plants were treated with sterile water. For infection experiment including *A. solani*, sporangia were used at a concentration of 1 x 10^6sporangia/ml and treated plants were incubated at 28 °C and 90 % humidity. Similar to *P. infestans* infection, 4th and 5th leaf from the top of the plant was inoculated with 1 x 10^4 spores on the abaxial side of the leaf. Samples treated with both pathogens and controls were harvested at 3, 6, 9, and 12 hours post infection.

2.2.3. Hormone treatment

For in-vitro hormone treatment, 5-weeks old leaves were detached and incubated in MS media, pH 5.8 with or without 0.25 mM SA, 1 µM IAA and 100 µM JA. Leaf samples were then incubated under 16-hr light / 8-hr dark photoperiods and harvested at 24, 48 and 72 hrs after treatment. Before the hormone treatment experiment, leaves were washed three times with distilled water containing 50 mg/L Ampicillin. For in vivo hormone treatment, 5-week old plants

were treated with or without 0.25 mM SA, and 100 µM JA. Leaf samples were then incubated under 16-hr light / 8-hr dark photoperiods, and harvested at 24, 48 and 72 hours post after treatment. At the selected time-point, leaves were detached and then flash frozen in liquid nitrogen and stored at -80°C for further analysis.

2.2.4. Generation of Plasmid Constructs

The *StNPR1* promoter (~3.3 kbp) was PCR amplified from the genomic DNA of *Solanum tuberosum* L. cv. Desiree, using forward and reverse primers with HindIII and BamHI restriction sites (Supplementary Table S1), and cloned into pBI221 vector, and the sequence was verified. The *StARF16* and *StWRKY6* CDS sequence was PCR amplified from the cDNA of *Solanum tuberosum* L. cv. Desiree, using forward and reverse primers with BamHI and SacI sites, and cloned into pBI221 vector and the sequence was verified. For generating *5'mStARF16* construct, we sub-cloned the native *StARF16* in pGEM-T-Easy vector (Promega) and then amplified the whole plasmid construct using *5'mStARF16* (FP-RP) primers, and transferred the miRNA resistant *StARF16* to pBI221 vector and sequence was verified. For promoter deletion construct, we removed two ARF binding sites present within close proximity of each other for every deletion construct, and each of these four constructs of length 2.7kb, 2.5kb, 1.5kb and 600bp were cloned in pBI221 vector using specific primers mentioned in Table2.2.

2.2.5. Total RNA extraction, cDNA preparation and quantitative real-time PCR (qRT-PCR) analysis

Total RNA from *A. solani* and *P. infestants* treated plants was extracted using RNAIso-Plus (Takara), following the manufacturer's protocol. The concentration of RNA was determined on Nanodrop by measuring A260. Two micrograms of total RNA was reverse transcribed into cDNA using Promega MMLV–RT with oligo(dT) primers and these cDNAs were used as templates in quantitative Real time – PCR (qRT-PCR). For analysis of *StNPR1, StARF16, StARF10, SARF17, StWRKY6 and StMYC2* were selected. For normalization, *SteIF3e* gene was used. qRT-PCRs were carried out using SYBR green mix (Takara). The reaction conditions for *StARF16, StARF10, SARF17, StWRKY6 and StMYC2* were 95°C for 2min, 40 cycles of 95°C 15 sec and 60°C 20 sec and for *StNPR1* were 95°C for 2min, followed by 40 cycles of 95°C 15 sec, 50°C 15 sec and 68°C 20 sec. Melting curve analysis was included in all the programs to check the PCR specificity and the data was analyzed using 2^{-ddCt} method (Livak and Schmittgen, 2001).

2.2.6. Quantitative real-time PCR (qRT-PCR) analysis of miR160

To analyze miR160 levels upon *P. infestans* infection, total RNA was isolated from local and systemic leaves harvested at selected time points by RNAIso-Plus. Two microgram (2µg) of total RNA was used for reverse transcription reaction using miRNA stem-loop (STP) primers. All the quantitative RT-PCR (qRT-PCR) reactions were setup using the SYBR Green Mix (TAKARA) in Bio-Rad CFX96 system. For miRNAs, qRT-PCR was carried out using miRNA specific forward and universal reverse primers, and reactions were carried out at 95°C for 5 min followed by 40 cycles of 95°C for 5 sec, 60°C for 10 sec, and 68°C for 8 sec. For normalization, U6 was used and qRT-PCR was performed using same miRNA, cDNA and gene-specific primers (U6-FP and U6-RP) with PCR conditions as 95°C for 5 min followed by 40 cycles of 95°C for 5 sec, 60°C for 10 sec, and 68°C for 8 sec. Melting curve analysis was included in the program to check PCR specificity and data was analyzed by using 2^{-ddCt} method (Livak and Schmittgen, 2001).

2.2.7. Yeast one-hybrid assay

The protocol used is similar to as mentioned in Natarajan et al., 2018. Briefly, the coding sequences of *StARF10*, and *StARF16*, and the promoter sequences ~3.3kb upstream of *StNPR1* and ~3.0kb upstream of *AtGH3.5* were cloned into Gateway destination vectors using gateway cloning technology (Thermo Fisher Scientific). StNPR1 and AtGH3.5 were used as baits in the assay, which were cloned into destination vector pMW#2 through the donor vector pDONRP4-P1r. This bait vector was transferred to the yeast strain, YM4271, and selected over SD -His (Synthetic dropout without histidine) medium. StARF10 and StARF16 were used as prey in the assay, which were cloned into destination vector pDEST-2µ-Gal4-AD via the donor vector pDONR221. Both prey vectors were transferred to the yeast strain, Yα1867 and selected over SD -Trp (Synthetic dropout without tryptophan) medium. To understand interaction between the bait and prey, the yeast clones containing prey and bait were mated and selected over SD -His, -Trp medium. The interaction was substantiated by growing mated yeast clones on SD -His, -Trp medium supplemented with increasing concentrations of 3-amino-1,2,4-triazole (3-AT). After 3 days of sustained growth of the mated colonies on the selection media, images were taken and data was recorded.

2.2.8. Tobacco Protoplast Isolation

Fully expanded leaves from 3 to 4 weeks old tobacco plants were excised and placed in K3 basal media (Kao and Michayluk, 1975) supplemented with 0.4 M sucrose, 0.25 % (w/v) cellulases (EMD Millipore Corp) and 0.05 % (w/v) macerases (Calbiochem), and incubated overnight at 28°C. After incubation, the liberated protoplasts were filtered through 1 µm cell strainer and centrifuged for 10 min at 150 g. Protoplasts were collected from the bottleneck area and washed once in K3 media with 0.4 M sucrose, and suspended in K3 media containing 0.4 M glucose to a final concentration of 4×10^6 protoplasts per milliliter (Chen et al., 2004).

2.2.9. Potato Protoplast Isolation

The protocol for isolation and purification of potato protoplasts was adapted from Foulger and Jones, 1985 and Shepard and Totten, 1977; and minor modifications were made. From the top, the second to fourth green leaves were excised from the soil grown *Solanum tuberosum* L.cv. Desiree plants and surface sterilized as described in Shepard et al (1997). The leaves were then placed in medium A (Shepard and |Totten, 1977) supplemented with 5% (w/v) celite and gently abrased on both sides using a soft nylon brush, until their color turned light green. The leaves were cut into 1.5-2 cm square pieces, and the cut pieces from two leaves were transferred to a petri plate containing 25ml medium A, 2% (w/v) PVP, 0.25% (w/v) cellulases (EMD Millipore Corp.), 0.05%(w/v) macerases (Calbiochem) and 10mM MES, pH was adjusted to 5.6. Following vacuum infiltration for 10 minutes, the petri plates were incubated at 24°C for 20hrs, under dark conditions for complete/ efficient digestion. After incubation, protoplasts were released from the digested leaves by gently shaking the petri-plates. The released protoplasts were sieved through a 1µm cell strainer and the filtrate was centrifuged at 150g for 10mins at RT. The viable protoplasts suspended on the top meniscus, were collected in a fresh falcon and washed twice with medium A (centrifugation at 150g for 5mins at RT). After purification, the protoplast density was adjusted to 1×10^6 cells/ml using medium B (Shepard and Totten, 1977) supplemented with 0.35M mannitol and 2% glucose.

2.2.10. Electroporation and transient protein analysis

For each transfection analysis, 1400 µl of tobacco protoplasts and 700 µl of potato protoplasts (prepared as described above) were mixed with 30 µl of 2 M KCl and plasmid DNA in an electroporation cuvette with 0.4 cm electrode gap. The plasmid DNA was a mixture of 10

µg of the reporter construct, 5 µg of the 35SCaMV:LUC construct as internal control, and a different combination of 30 µg of each effector plasmid. After electroporation of tobacco protoplasts (voltage= 170V, capacitance = 125 µF); and potato protoplasts (voltage = 275V and capacitance = 1000µF) using Gene Pulser Transfection Apparatus; Bio-Rad, Hercules, CA, USA 4.0 ml of Murashige and Skoog (1962) basal media was added to tobacco protoplasts, and 5 ml of medium B with 0.35M glucose and 0.35M mannitol to potato protoplasts. The protoplasts were incubated in the dark at room temperature for 40–48 hours before conducting GUS and LUC activity assays. Transfections were performed three times for each effector combinations (Mahajan et al. 2012, Shepard and Totten, 1977).

Luciferase assays were performed by adding 100 µl of LUC substrate (Promega, Madison, WI, USA) into 20 µl of extract and measuring the emitted photons for 15 sec in a TD-20 luminometer. Fluorometric GUS assays were performed as described by Jefferson (Jefferson et al. 1987). A fluorescence multiwell plate reader, Varioscan, was used to measure GUS activity at 365 nm (excitation) and 455 nm (emission). Each sample was measured three times for both LUC and GUS activities. Relative GUS–LUC activity was calculated by dividing the ratio of GUS activity to LUC activity from different effectors, with the ratio from reporter plasmid alone. Relative activities calculated from three transfection replications were presented as a mean ± SE.

2.2.11. Western Blot

After 48 hours of incubation, 1.5ml of harvested protoplasts were crushed in 150 µl of Laemmli Buffer and specific proteins were detected by western blotting using Anti-6xHis M2 monoclonal antibody (Invitrogen, Cat. No. 37-2900) or FLAG antibody (Sigma, Cat No.F1804) at a dilution of 1:1000 followed by anti-mouse IgG horseradish peroxidase (Sigma-Aldrich A9044) at a dilution of 1:5000 as a secondary antibody. Western blots were developed using Clarity™ Western ECL Substrate (Bio-Rad, Cat. No. 1705060), under chemiluminescence settings.

2.2.12. ChIP-qRT-PCR Analysis

The potato protoplasts were collected after 40-48 hours of incubation post transfection. The cells were pelleted by centrifugation at 1500 g for 2 min at RT. Then the samples were treated with 37 % formaldehyde to crosslink the proteins to the DNA. After crosslinking, ChIP (Chromatin Immunoprecipitation) was performed using the reagents and protocol provided in

a universal plant ChIP-Seq kit (Cat. No. C01010152; Diagenode), as per the manufacturer's instructions. The sheared chromatin was immunoprecipitated using the DiaMag protein A-coated magnetic beads (Diagenode) and 2 µg of either anti-His or anti-IgG antibody (Cat. No. C15410206; Diagenode), is added in each reaction. Finally, eluted DNA was used for subsequent qPCR analysis with gene-specific primers.

2.2.13. Accession number

Table 2.1 Accession numbers

Name	Accession	Source
StPR1	AY050221	NCBI
StNPR1	XM_006357647	NCBI
StARF16	PGSC0003DMT400062489	PGSC
StARF10	PGSC0003DMT400020874	PGSC
U6	*X60506*	NCBI
AtG3.5	*AT4G27260*	TAIR
WRKY6	PGSC0003DMT400038852	PGSC
MYC2	*XM_006352794.2*	NCBI

2.2.14. Primer details

Table 2.2 Primer details

miRNA160 qPCR	
miR160-STP	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGGCATA
miR160-FP	TGGAGTTTGCCTGGCTCCCTG
U6-STP	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTTGGAC
Univ-miR-RP	AGTGCAGGGTCCGAGGT
miRNA160 target qRT-PCR	
StARF10-FP	GTCCAGCAGTCCTTTCTGTTGTTT
StARF10-RP	GCTGCAACACGCTGGAAACTT

StARF16-FP	GGCAACCCCCTCAGGTCTAG
StARF16-RP	TGCAACTTTTCGCTACGGTGGA
StARF17-FP	TAGCTTCCCTATGACAGGGTTG
StARF17-RP	ACAACCCAAGGTTATTGCT
qRT-PCR of Defence-related Genes	
StPR1-FP	GTACCAACCAATGTGCAAGCG
StPR1-RP	TGTCCGACCCAGTTTCCAAC
StMYC2-FP	TGCACGTCTAGGTCTAATTCCAT
StMYC2-RP	AGGCAAGATTGTAGACGTGGATA
StPDF1.2-FP	CAGTAATATTTGTGACCCCATGGTT
StPDF1.2-RP	TTGGAAAAAGAGTGACAAGTGGAAT
StNPR1-FP	AAGAGGCTCACTAGGCTT
StNPR1-RP	GCTTCATACGCAAATCATCG
StWRKY6-FP	CTCTGGCCTCCAATCGTCTCAAG
StWRKY6-RP	AGTGAAGTGATGGCTGCTGCT
Auxin Responsive genes	
StARP-FP	TGCCAACCTATGAAGATAAAGATG
StARP-RP	GCTTCTGATCCTTTCATTATGCG
IAA3-FP	TTAGCATGGATGGAGCACCT
IAA3-RP	CACCAATGGGATGCTTGAAC
Y1-H Analysis	
ARF10_Y1H_FP	AAAAAGCAGGCTTCATGAAGGAGGTTTTGGAGAAGT
ARF10_Y1H_RP	CAAGAAAGCTGGGTTCTATGCAAAGATGCTAAGAGG
ARF16_Y1H_FP	AAAAAAGCAGGCTTCACCATGGAGGTGGTGGAAGAG
ARF16_Y1H_RP	CAAGAAAGCTGGGTTCTAGACAACGTTGAGGATTGG
ATTB1_Y1H_FP	GGGGACAAGTTTGTACAAAAAAGCAGGCT
ATTB2_Y1H_RP	GGGGACCACTTTGTACAAGAAAGCTGGGT
Prom-NPR1_Y1H_F	TATAGAAAAGTTGTCAGATATACCTACTTGGAGGAAAGAA
Prom-NPR1_Y1H_R	TTTGTACAAACTTGCTACAGCATACGTTCTGTATGTTTAT
Prom-AtGH3.5_Y1H_F	TATAGAAAAGTTGTCTTTTAAATTAACTAAGTTCGATAAACTGTG

Prom-AtGH3.5_Y1H_R	TTTGTACAAACTTGCGGTTTAAGAGAAAGAGAGAAGTC
p53_Y1H_F	TATAGAAAAGTTGTCTACCAGGCATGCCTAGCA
p53_Y1H_R	TTTGTACAAACTTGCATACAGAGCACATGCCTC
ATTB4_FP	GGGGACAACTTTGTATAGAAAAGTTGTC
ATTB1_RP	GGGGACTGCTTTTTTGTACAAACTTGC
Protoplast construct	
promNPR1pbi121_FP	TTTTTTAAGCTTAGATATACCTACTTGGAGGAAAGAA
promNPR1(-1&2)qF	TTTTTAAGCTTAGCAAACTTCAAATTCACCCTACT
promNPR1(-1to4)qF	TTTTTAAGCTTGTGGAAGTGATGGAAAATGATGCT
promNPR1(-1to6)qF	TTTTTAAGCTTGCGAGTTGGCAAGTGAAG
promNPR1(-1to8)qF	TTTTTAAGCTTGGAGTATTAATTGTTCCTACAAACTT
promNPR1RP	TTTTTGGATCCTACAGCATACGTTCTGTATGTTTAT
StARF16-FP	AAAAAAGGATCCATGGAGGTGGTGGAAGAG
StARF16-RP	AAAAAAGAGCTCTCAATGATGATGATGATGATGATGATGGACAAC GTTGAGGATTGGCAG
St5'mARF16-FP	CCGTCTGACAACAGTACTGCACCCACACCGGAAGTCAAGCCTGTT CAATTTGGAGTACC
St5'mARF16-RP	GGTACTCCAAATTGAACAGGCTTGACTTCCGGTGTGGGTGCAGTA CTGTTGTCAGACGG
StWRKY6-FP	AA TCTAGAATGGCCAAAGGAAGTGGA
StWRKY6-RP	AAAGAGCTCTCACTTGTCGTCATCGTCTTTGTAGTCATCAATTTGC TGGAAAACTC
ChIP-qPCR Analysis	
promNPR1frag1_2_qF	TGGGAACCAAGGTTTGAACT
promNPR1frag1_2_qR	TTGTACACCTCTACTTCTGAAGATT
promNPR1frag3_qF	AGCAAACTTCAAATTCACCCTACT
promNPR1frag3_RP	CTACCTTTGTTAGTACAAGGGACA
promNPR1frag4_qF	ACCAAATGCATGATGATGTAGCA
promNPR1frag4_qR	CCCAGTGATGATGAGATTAGGTTCA
promNPR1frag5_qR	GTGGAAGTGATGGAAAATGATGCT
promNPR1frag5_qF	CCTCTCTTTTACTTGTTTCCTCA

promNPR1frag6_qF	AGAGAAGTAAGCGGAGACACA
promNPR1frag6_qR	TGCACTAGAGAGTTTGAAGCTAA
promNPR1frag7_qF	GCGAGTTGGCAAGTGAAG
promNPR1frag7_qR	TTTATTAATAATTGCCTACCCTAC
promNPR1frag8_qR	TAAAAATGAACTAAAATATACGT
promNPR1 frag8_qF	TGTCGAAACGTCAGATAAAA
Reference gene	
ElF3e-FP	GGAGCACAGGAGAAGATGAAGGAG
ElF3e-RP	CGTTGGTGAATGCGGCAGTAGG
GAPDH-FP	GAAGGACTGGAGAGGTGGA
GAPDH-RP	GACAACAGAAACATCAGCAGT
Pathogen Detection	
PINF	CTCGCTACAATAGGAGGGTC
ITS5	GGAAGTAAAAGTCGTAACAAGG
As-Tub-FP	ACGACATCTGCATGAGGACCCTC
As-Tub-RP	AACCATGTTGACGGCCAACTTCCTC

2.3. Results

2.3.1. Establishing the hemi-biotroph and necrotoph pathogens.

To decipher the role of miR160's target role in potato defence response, we selected *Solanum tuberosum* L. cv. Desiree as plant model, and *P. infestans* as hemi-biotroph, and *A. solani* as the necrotrophic pathogen model, respectively. *P. infestans* strain A2, was obtained from Central Potato Research Institute (CPRI, Shimla, India) which was maintained on pea agar media throughout the study (Figure 2.1) The *P. infestans* strain was previous confirmed by amplifying Internal Transcribed Spacer 2 (ITS2) (Natarjan et. al., 2018).

A. solani was obtained from Thapar University, (Patiala, India), and maintained over potato dextrose agar (Figure 2.1). We confirmed the *A. solani* by amplifying tubulin and using As tub-FP and As-tub-RP (Figure 2.1). To understand the potato defence response against the necrotrophic pathogen, we performed *in planta* infection studies (Figure 2.2). In the infected leaves generation of *A. solani,* hyphae were seen as early as 24 hours post infection, as indicated by Trypan Blue staining (Figure 2.2). We also standardized the amount of spores required for infection and found that 10^4 conidia application was selected to observe the presence of necrotrophic lesions on the plant (Figure 2.1).

2.3.2. StARF16 is differentially regulated upon infection with hemi-biotrophic and necrotrophic pathogen

To understand the involvement of miR160 and its targets in defence response, expression analysis was performed in leaves of potato upon infection with *P. infestans*, (a hemi-biotrophic pathogen) and *A. solani*, (a necrotrophic pathogen), independently. Five weeks old soil grown *Solanum tuberosum* L. cv. Désirée plants were infected with equivalent amount of pathogen (1 x 10^4 spores) and total RNA was isolated from the infected tissues at different time points. We performed quantitative Real Time–PCR (qRT-PCR) analysis, which revealed significant increase of miR160 levels at 12 and 9 hours post infection (hpi) upon treatment with *P. infestans* and *A. solani,* respectively (Figure 2.3 A and D). We evaluated expression levels of all three miR160 targets, *StARF10* (Figure 2.3 C and F), *StARF16* (Figure 2.3 B and E) and *StARF17* (Figure 2.4) upon infection and found that S*tARF16 and StARF17* expression profiles are not typical to that of miRNA and its target. *StARF10* exhibited negative correlation with its regulating miRNA, miR160 (Figure 2.3 C and F) upon infection with either *P. infestans* or *A. solani*. However,

expression profile of *StARF16* altered as the infection progressed, but failed to establish a negative correlation with its proposed regulator miR160 (Figure 2.3 B). *StARF16* levels increased at 6 and 12 hpi in the leaves infected with *P. infestans,* whereas it constantly accumulated until 9 hpi with *A. solani* (Figure 2.3 B and E). However, *StARF17* did not significantly change in the tissues infected with either *P*. *infestans* or *A*. *solani* infection (Figure 2.4). Although, *StARF10* and *StARF16* have been shown to be the targets of miR160, only *StARF10* showed a strong negative correlation with the miR160.

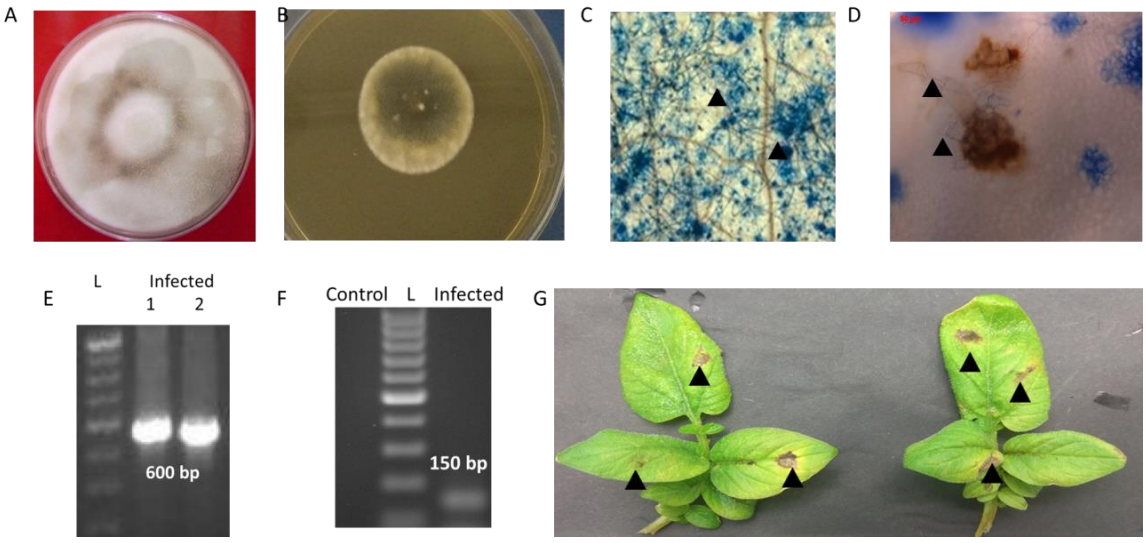


Figure 2.1 Maintenance and *In planta* infection of *P. infestans and A. solani in WT plants*. Culture of *P. infestans (A) and A. solani* (B) maintained in pea agar and potato dextrose agar, respectively. (C) Infection of *P. infestans* in WT potato plants after Trypan blue staining 94 hpi. (D) Conidia of *A. solani g*enerating hyphae in the plants 24 hpi. (E) Confirmation of *P. infestans (E) and A.solani (F)* using As-Tub-FP and As-Tub-RP (~150bp). The infection progression of *A. solani* in WT potato plants under infection with 10^6 spores (G). Black arrow in C and D shows hyphae presence in WT potato plants infected the *P. infestans* and *A. solani*, respectively. Black arrow in G represents necrotrophic lesions. (*P. infestans* data A, and C has been reproduced from Natarajan PhD Thesis with permission, E is adapted from Natarajan et. al., 2018)

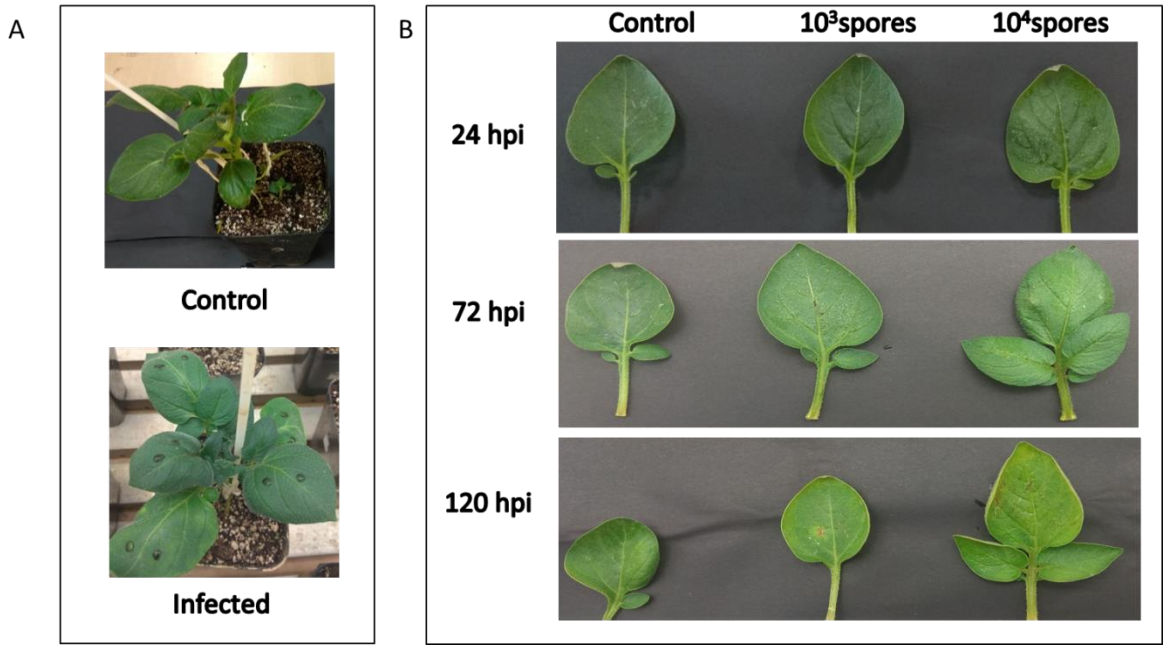


Figure 2.2 *In planta* infection experiment. (A) Control and infected plants kept in the growth chamber with 28°C. The concentration of the conidia was adjusted to 10^6 conidia /ml and 10^3, and 10^4 conidia are then applied on soil grown plants and then samples were collected at 24, 72, and 120 hpi the disease progression (B). We observed formation of necrotrophic lesions with 10^4 conidia.

We observed a reduced expression of *StARF10* in infected leaves compared with mock-treated plants in both necrotrophic and hemi-biotrophic infection. However, in plants treated with necrotrophic pathogen, we noticed a steady increase of *StARF16* expression over the infection duration of 6 to 12 hpi and it accumulates only during interactions with *A. solani* and not during biotrophic phase of *P. infestans*. These results suggest that miR160 targets attenuate the plant defence response, in response to the pathogen invading the plant. A previous study (Natarajan et al., 2018) has demonstrated that miR160 modulate plant's response, upon infection, by regulating essential defence genes. Thus, we analyzed the transcript levels of genes involved in the defence responses during the hemi-biotrophic and necrotrophic pathogen infection.

2.3.3. Both hemi-biotrophic and necrotrophic pathogen induces differential defence response during infection in potato

We performed expression analysis for crucial defence genes *StNPR1, StPR1, and StWRKY6,* involved in biotrophic defence response, and genes involved in necrotrophic defence response,

StMYC2, in the infected leaves of WT plants. The expression of *StNPR1, StPR1 and StWRKY6* steadily increased post treatment with *P. infestans* across all time points (Figure 2.5 A, B, and C), whereas the expression of these genes decreased upon treatment with *A. solani* at 9 and 12 hpi (Figure 2.5 E, F and G). *StMYC2* expression did not change upon treatment with *P. infestans* but upon treatment with *A. solani,* the levels of *StMYC2* significantly increased only at 12 hpi (Figure 2.5 D and H). All these genes showed ~~a~~ differential expression response upon infection with *P. infestans* and *A. solani*. We noticed that the expression of defence genes upon infection with *P. infestans*, *StNPR1* and *StPR1* (Figure 2.5 A, B, E and F) increased, and the levels of *StARF16* (Figure 2.3 C) were downregulated. In contrast, upon infection with *A. solani*, *StARF16* showed an opposite response (Figure 2.3 B, 2.5 E and F).

During hemi-biotrophic infection, increase in the levels of *StNPR1, StPR1* and *StWRKY6* suggests that SA dependent pathway gets activated to mount a defence response. However, the increase in *StMYC2* expression, suggests the activation of JA dependent pathway, upon necrotrophic pathogen infection.

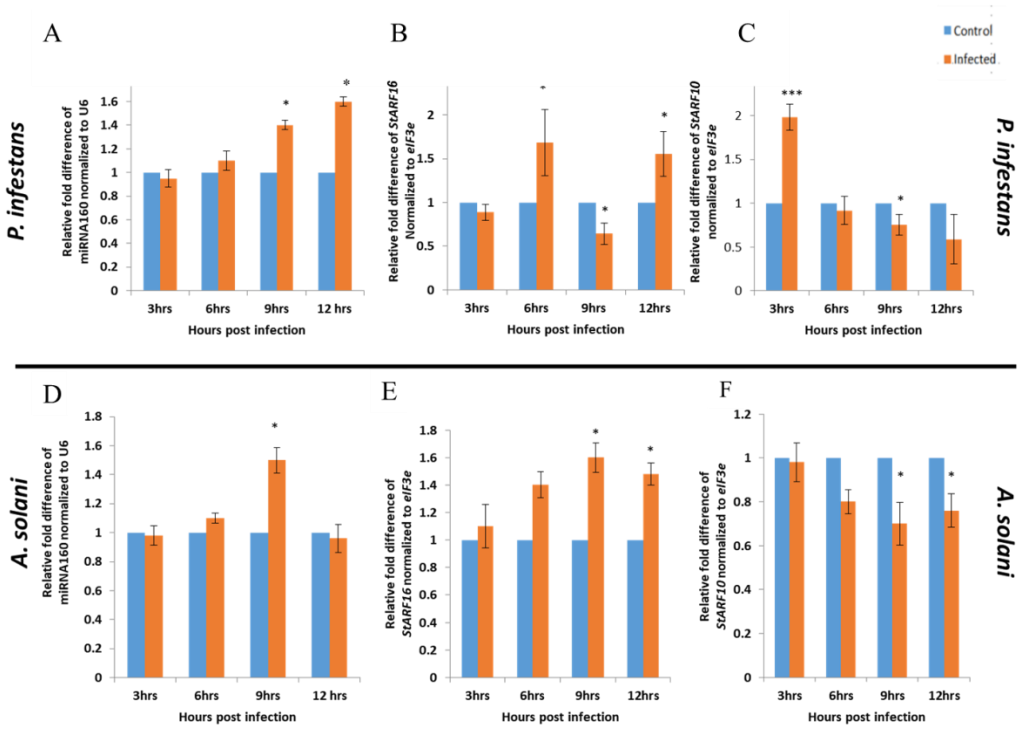


Figure 2.3. Expression profile of miR160, *StARF16*, and *StARF10* during early infection with P. infestans and *A. solani*. Quantitative RT-PCR, upon infection with *P. infestans,* miR160 (A), *StARF16* (B), and *StARF10* (C), targets of miR160. Quantitative RT-PCR, upon infection with *A. solan,i* miR160 (D), *StARF16* (E), and *StARF10* (F), targets of miR160. Leaves were treated with 10^4 spores of *P. infestans* and *A. solani*. Total RNA was extracted from control and pathogen-treated leaves. Gene expression is relative to high expressing reference gene *eIF3e* for leaves. Asterisks indicate significant difference by t-test from the WT (*P<0.05; **P<0.01, ***P<0.001). Data are means ± standard deviation (SD) for four independent biological replications.

2.3.4. Expression levels of *StARF16* and *StNPR1* are perturbed by exogenous application of SA and JA

After analyzing the effect of different pathogens on the expression profile of miR160, its target and defence genes, we further elucidated the defence response upon treatment of SA and JA phytohormones. We carried out qRT-PCR analysis for different time points, 24, 48, and 72 hours post treatment (hpt) for *in vivo* and *in vitro* conditions to analyze the effect of hormones on *StARF16 and StNPR1* expression in plants (Figure 2.7, and 2.6 A). At 24 hpt, expression of *StARF16*, did not show any significant changes either SA or JA treatment. At 48 hpt however, *StARF16* expression levels increases upon JA treatment, while decreases upon SA treatment, respectively (Figure 2.7). While at 72 hpt, the level of *StARF16* reverts back to control levels in both *in vitro* and *in vivo* conditions (Figure 2.7 A, B and C) during both SA and JA treatment. We have also analyzed effect of exogenous auxin on transcript levels of key auxin responsive genes, and observe that *StARF16* transcript level decreases (Figure 2.6). But *StIAA3* levels increase whereas *StARP* levels decrease at 24 and 48 hpt, respectively (Figure 2.6 B). Further, transcript levels of *StWRKY6* and *StNPR1* were elevated upon treatment with 0.25 mM SA, even at 72 hpt (Figure 2.7 A and C). We noticed a different trend for JA responsive genes, *MYC2* and *PDF1.2*. The levels increased upon treatment at 48 hpt, whereas at 72 hpt, their levels were comparable to that of control leaves treated with or without 100 µM JA (Figure 2.7 B and C). The decreased *StNPR1* level and increased *StARF16* expression, during JA treatment, showed that these genes have a negative correlation and suggest a potential regulation of *StNPR1* through StARF16 upon infection or hormone treatment.

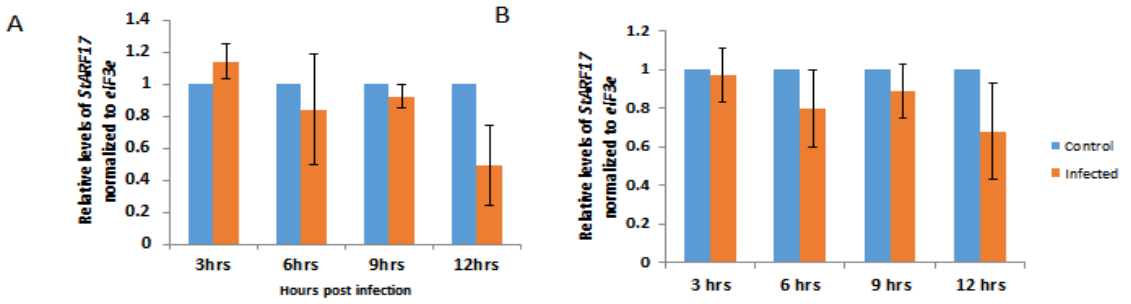


Figure 2.4 Expression of *StARF17* in Potato plants infected with (a) *P. infestans* and (b) *A. solani*. The expression of *StARF17* does not show a dynamic relation as *StARF16* and *StARF10,* upon infection with either *P. infestans* or *A. solani*. As the levels of *StARF17* levels do not change as infection progression, the gene was not included in the study. Gene expression is relative to high expression reference gene *elf3e* for leaves. The sample do not show any significant difference as tested using t-test from the WT potato leaves infected with pathogen. Data are means ± standard deviation (SD) for four independent biological replications.

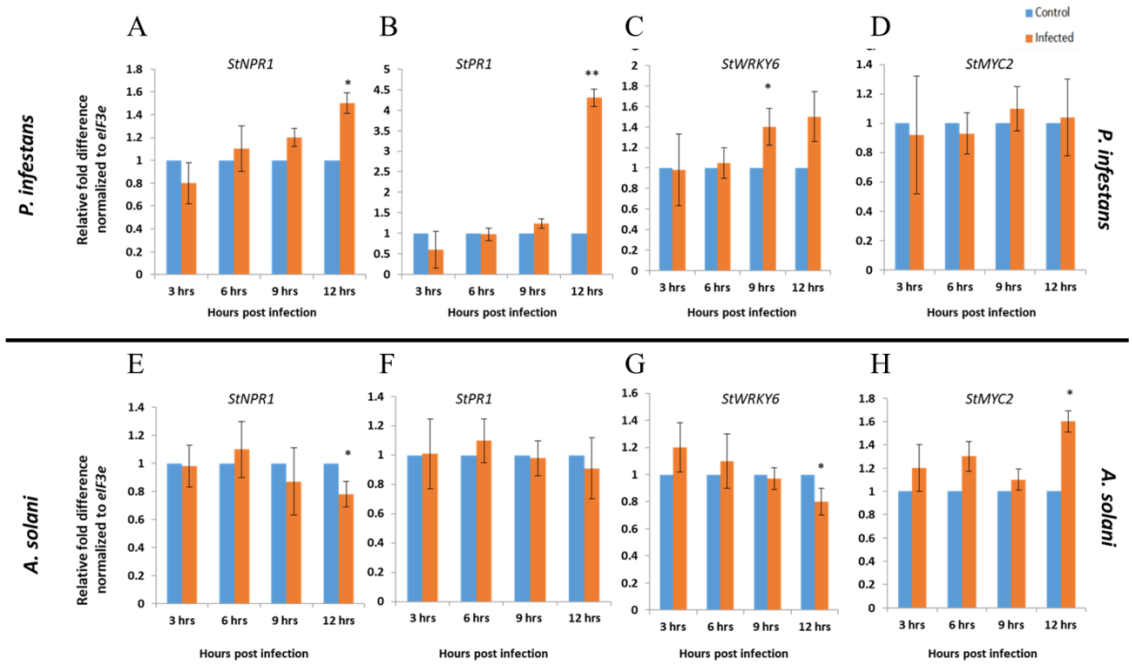


Figure 2.5 Activation of key defence response gene upon infection with *P. infestans* and *A. solani*. (A-D) Quantitative RT-PCR analysis of *NPR1* (A), *PR1* (B), *WRKY6* (C) and *MYC2* (D) upon infection with *P. infestans*. (E-H) Quantitative RT-PCR analysis of *NPR1* (E), *PR1* (F), *WRKY6* (G)

and *MYC2* (H) upon infection with *A. solani*. Leaves were treated with 10^4 spores of *P. infestans* and *A. solani*. Total RNA was extracted from control and pathogen-treated leaves. Gene expression is relative to high expression reference gene *eIF3e* for leaves. Asterisks indicate significant difference by t-test from the WT (*P<0.05; **P<0.01). Data are means ± standard deviation (SD) for four independent biological replications.

2.3.5. StARF16 binds to the *StNPR1* promoter

Previous studies have demonstrated how important JA–SA antagonistic cross-talk is to mount an effective defence response in plants against different pathogen (Spoel et al., 2007; Spoel et al., 2003). In this study, we attempted to understand whether JA can cross-talk with SA pathway via StARF16. To determine whether StARF16 is indeed regulating defence response, we analyzed if StARF16 directly binds to the promoter and regulate *StNPR1*. We analyzed the promoter of *StNPR1,* an ortholog of NPR1 in potato, and found that there are many putative ARF binding sites (viz. GGGACA, ACAGAG, TGTCCC, and GAGACA) on the promoter sequence (sequence are provided in Annexure I). Our VISTA analysis for NPR1 promoter of *S. tuberosum* with various plant species belonging to *Brassicaceae* and *Solanaceae*, shows that the promoter sequences of *Solanaceae* family members were more similar than those of *Brassicaceae* family (Figure 2.8). Notably, *Solanaceae* family members such as tomato, tobacco and potato showed high nucleotide similarity in the promoter of *NPR1* (Figure 2.8) indicating potential regulation of *NPR1* through ARF proteins.

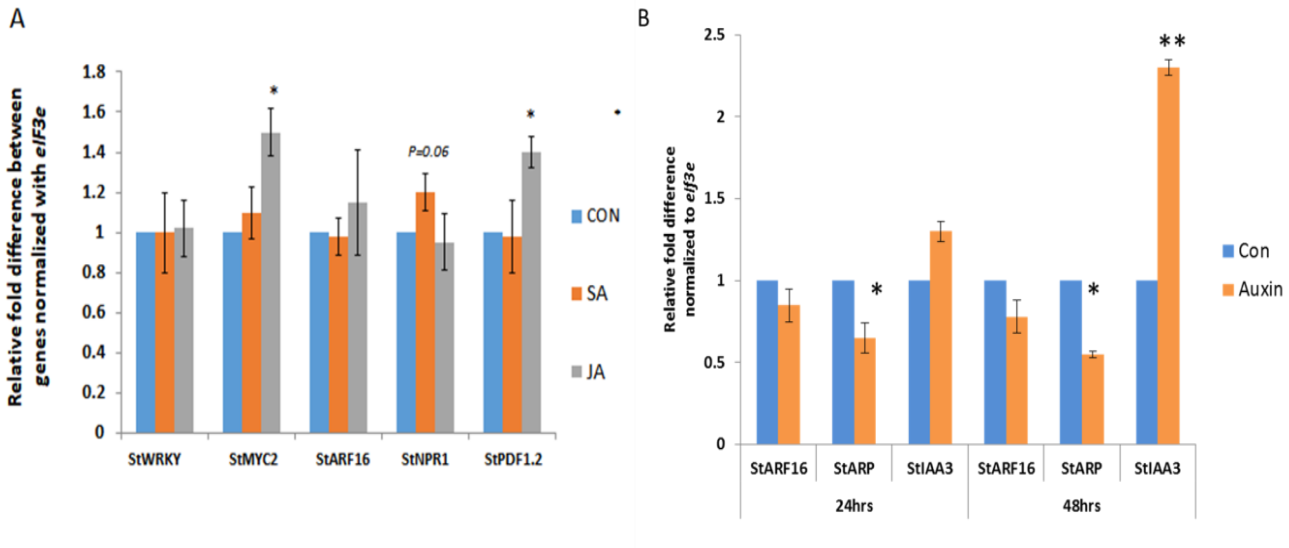


Figure 2.6 Treatment of JA and SA regulates *StARF16* and *StNPR1* expression in potato at 24 hrs. (A) Quantitative RT-PCR analysis of *StWRKY*, *StARF16*, *StNPR1*, *StMYC2*, and *StPDF1.2* with or without treatment of 0.5mM SA and 100µM JA at 24hrs over detached leaves of WT potato. At 24 hpt, there was no significant change in *StARF16* expression levels. (B) qRT-PCR analysis of *StARF16* and auxin responsive genes, *StIAA3* and *StARP* upon treatment with auxin. Gene expression is relative to high expression reference gene *elf3e* for leaves. Asterisks indicate significant difference by t-test from the WT treated by SA or JA on the same day (*P<0.05). Data are means ± standard deviation (SD) for three independent biological replications.

To understand whether StARF16 can bind to putative ARF binding sites in *StNPR1* promoter (Figure 2.9 A), we performed a Yeast 1-hybrid (Y1H) assay using proteins of two miR160 targets, *StARF1* and *StARF16,* and the promoter of potato *StNPR1* (Figure 2.9 B). The promoter sequence of *Arabidopsis AtGH3.5* was used as a positive control. For Y1H assays, the interaction of *StARF10 and StARF16* proteins, with the ~3.3 kb promoter of *StNPR1* and the ~3.0 kb promoter of *AtGH3.5* was explored (Figure 2.9). Mated yeast colonies containing *StARF16* with the promoter of either *StNPR1* or *AtGH3.5*, grew robustly on the selection media (SD -His -Trp) with increasing concentrations of 3-AT. However, mated yeast colonies containing *StARF10* with the promoter of *StNPR1* did not grow over increasing concentrations of 3-AT. This suggested that only StARF16 could bind to the promoter sequence of *StNPR1*, whereas StARF10 could not bind to the same sequence (Figure 2.9 C). These results and expression analysis data suggested that StARF16 could possibly affect *StNPR1* expression,

thereby modulating the cross-talk between JA-and SA-mediated defence response during the pathogen attack.

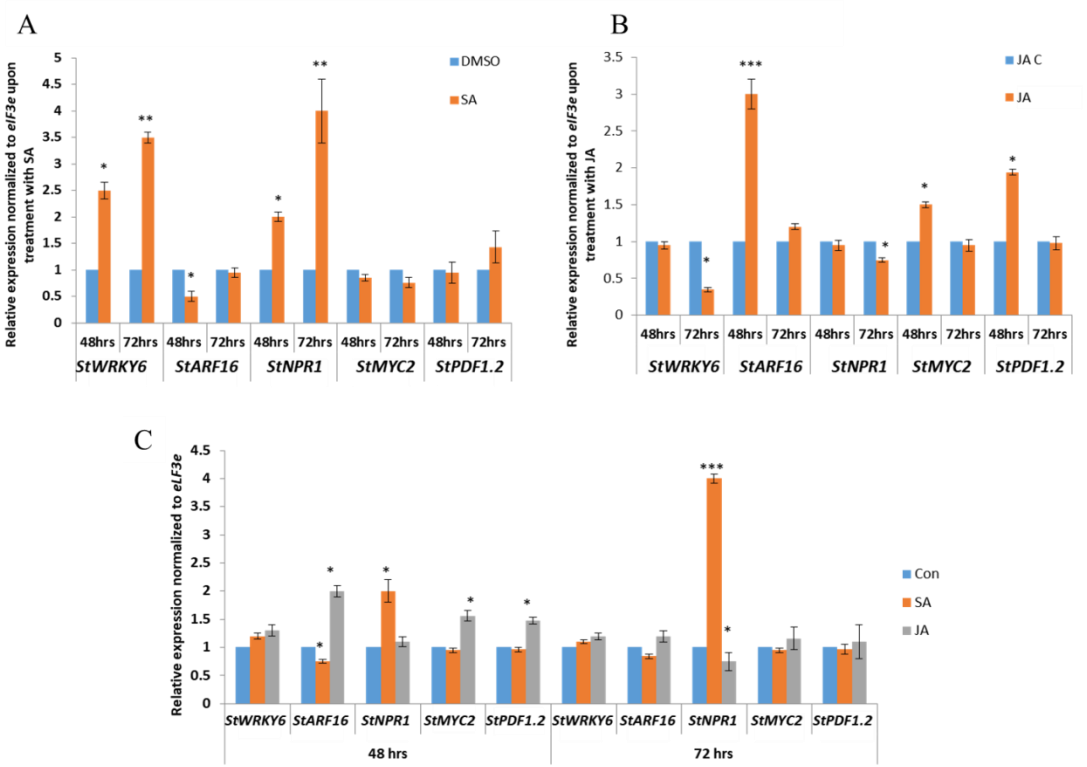


Figure 2.7 Treatment of JA and SA regulates *StARF16* and *StNPR1* expression in potato. (A-B) Quantitative RT-PCR analysis of *StWRKY6*, *StARF16*, *StNPR1*, *StMYC2*, and *StPDF1*.2 with or without treatment of 0.5 mM SA (A) and 100 µM JA (B) at 48 and 72 hrs over detached leaves of WT potato. (C) Quantitative RT-PCR analysis of *StWRKY6*, *StARF16*, *StNPR1*, *StMYC2*, and *StPDF1.2* with or without treatment of 0.5 mM SA and 100 µM JA (C) at 48 and 72hrs in soil grown WT potato. Gene expression is relative to high expression reference gene *eIF3e* for leaves. Asterisks indicate significant difference by t-test from the WT treated by SA or JA on the same day (*P<0.05; **P<0.01, ***P<0.001). Data are means ± standard deviation (SD) for three independent biological replications.

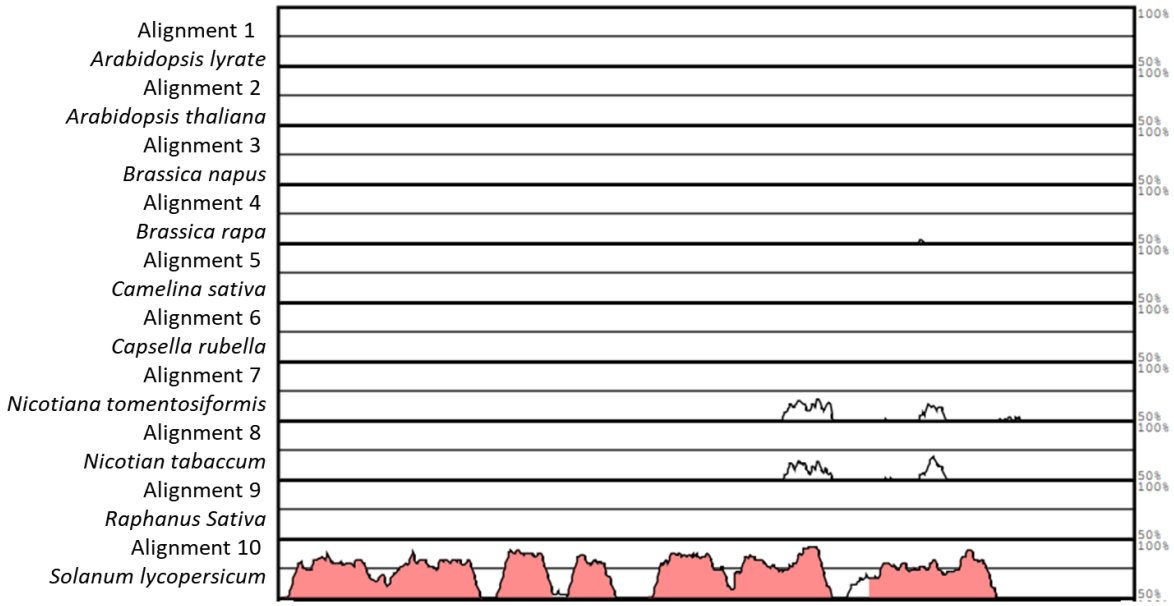


Figure 2.8. VISTA analysis of *NPR1* promoter of potato, with promoter of *NPR1* promoter, in plants from *Brassicaceae* and *Solanaceae*. We observed no conserved region in *Brassicaceae* and *Solanaceae* plants. Interestingly we observed six conserved regions in *NPR1* promoters of potato and tomato. Both members of *Solanaceae* family have high conserved regions in *NPR1* promoter. A 3kb upstream region of *NPR1* from potato, Arabidopsis and tomato was selected for VISTA analysis. VISTA analysis was carried out with a base 50% to show peaks and minimum 70% similarity depicted in red color, with a word length of 100bp. Ll the sequences used for VISTA analysis are provided in Annexure II.

2.3.6. StARF16 represses *StNPR1* expression

To validate the effect of StARF16 binding to *StNPR1* promoter, we co-transfected the tobacco and potato protoplasts with plasmids, constitutively expressing *StARF16*, and with promoter of *StNPR1,* respectively. The β-Glucuronidase(GUS) was used as a marker gene, which was driven by the promoter of *StNPR1.* Multiple constructs were designed to understand the mechanistic relation between ARF16 and *NPR1*, represented diagrammatically in Figure 2.10 A. WRKY6 was used as a control, which is known to positively regulate the expression of *StNPR1* during the SA dependent senescence response in *Arabidopsis* (Chai et al. , 2014). *In silico* sequence analysis revealed that tobacco miR160 is also able to target and cleave *StARF16* transcripts. Hence, to get an efficient protein expression of *StARF16* in tobacco protoplasts, miR160 resistant form of *StARF16, 5'mStARF16,* was used. The GUS-LUC assays were carried out in the co-transfected

protoplasts. Our initial co-transfection experiments with either 35SCaMV:*StARF16* or 35SCaMV:*St5'mARF16*, and prom*StNPR1*:GUS, resulted in no significant change in the GUS/LUC activity (Figure 2.10). We speculated that the activation of SA dependent pathway in tobacco protoplast during protoplast isolation could be the reason for non-significant GUS/LUC activity. To validate this, we analyzed the expression of tobacco *NPR1* in the protoplasts and found to be highly expressed (Figure 2.11 A). In order to attenuate this tobacco defence response, the protoplasts were treated with JA. We noted that 100 µM of JA treatment showed effective suppression of SA-mediated defence response (Figure 2.12 B). Further, co-transfection assay were performed using tobacco and potato protoplasts pretreated with 100 µM of JA, yielded comparable results (Figure 2.13 and 2.14).

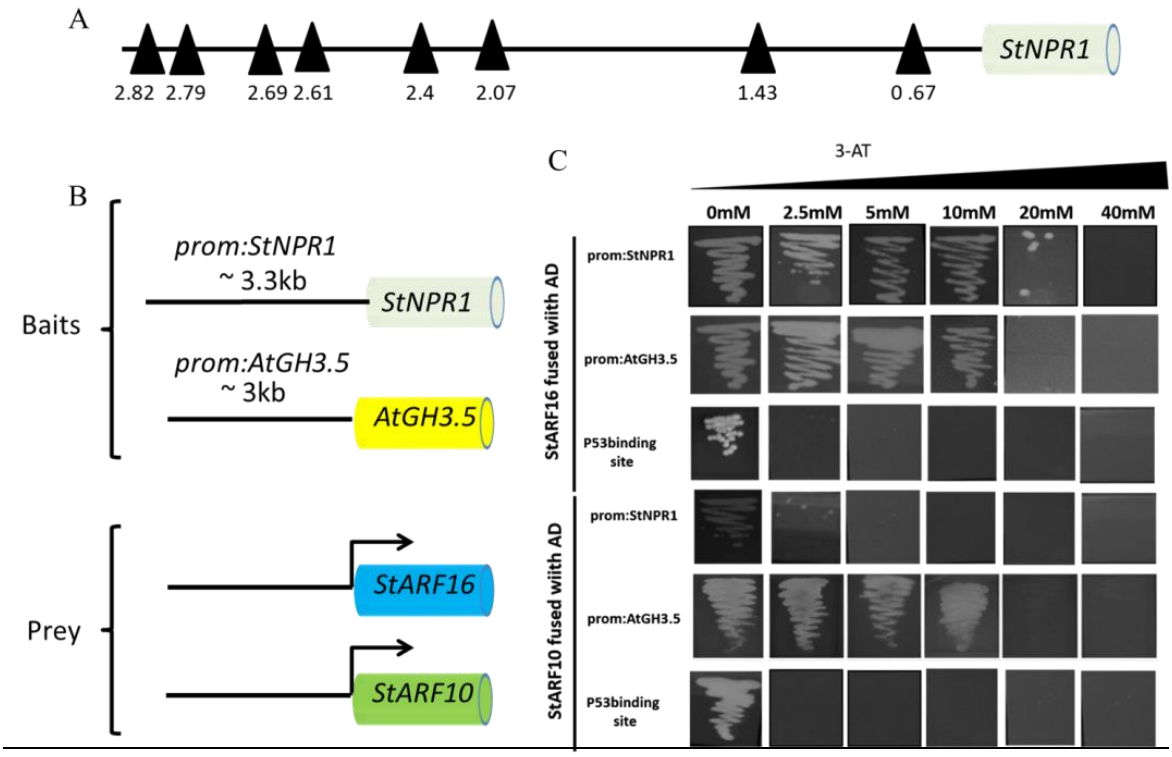


Figure 2.9 StARF16 binds to promoter of *StNPR1* and *AtGH3.5*. (A) Promoter of *StNPR1* showing putative ARF binding sites at 670, 1437, 2070, 2400, 2618, 2694, 2790 and 2824 bp upstream to the transcription start site (TSS). (B) A diagrammatic representation of baits and prey used in yeast one-hybrid assay. We have taken a 3.3kb region upstream to *StNPR1* and 3 kb region upstream to AtGH3.5 TSS for preparation of baits and the full-length coding

sequences of *StARF10* and *StARF16* was taken as prey. (C) Yeast strains containing StARF16 prey protein and *prom:StNPR1* bait grew in media containing up to 20 mM 3-amino-1,2,4-triazole (3-AT); strains containing StARF16 prey and prom:AtGH3.5 bait grew in media containing up to 10 mM 3-AT. The growth of yeast strains indicates the binding of StARF16 to both *StNPR1* and *AtGH3.5* promoters, whereas StARF10 do not bind with *prom:StNPR1*. The p53 binding site was used as a negative control; inhibition of yeast growth at all the concentrations of 3-AT suggests no interaction between the p53 binding site and StARF16 protein. AD is activation domain.

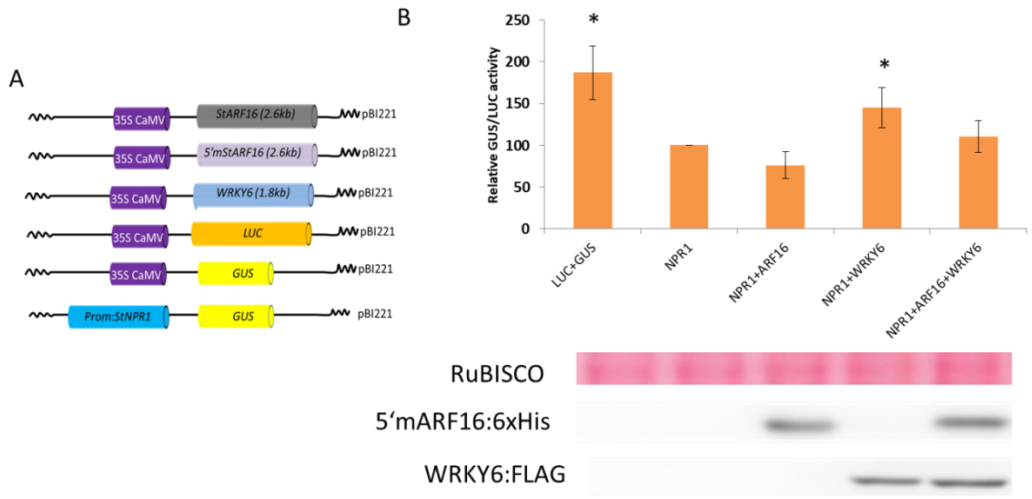


Figure 2.10 GUS activity of prom:*StNPR1* as measured in tobacco co-transfection without pretreatment with Jasmonic acid. (A) Diagrammatic representation of different constructs used in protoplast co-transfection assay. (B) Background activity of GUS driven by prom:*StNPR1* remain constant even upon co-transfection of the samples together with *5'mStARF16*. Interestingly, under the same background we were able to see a better activation of *StNPR1* promoter upon providing StWRKY6 in the transfection system. GUS activity was measured by 4-methylumbelliferyl-β-D-glucuronide (MUG) fluorescence assay. A relative value for GUS expression was obtained by dividing the GUS activity by the specific luciferase activity. Asterisks indicate significant difference by t-test from the promStNPR1:GUS (*P<0.05). Data are means ± standard deviation (SD) for three independent biological replications from 2 independent experiments.

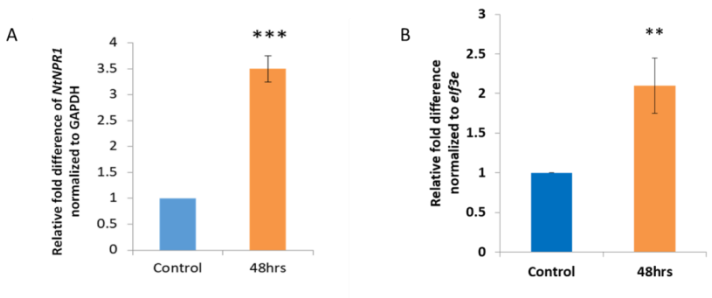


Figure 2.11 Activation of *NtNPR1,* upon treatment with macerase and cellulases, in tobacco protoplasts. *NtNPR1* levels of tobacco increase upon treatment with the combination of cellulases and macerase relative to beginning of the experiment in tobacco (A) and in potato (B). Gene expression is relative to high expression reference gene *GAPDH* for leaves. Asterisks indicate significant difference by t-test from the WT treated by JA in a time dependent study (***P<0.001). Data are means ± standard deviation (SD) for three independent biological replications.

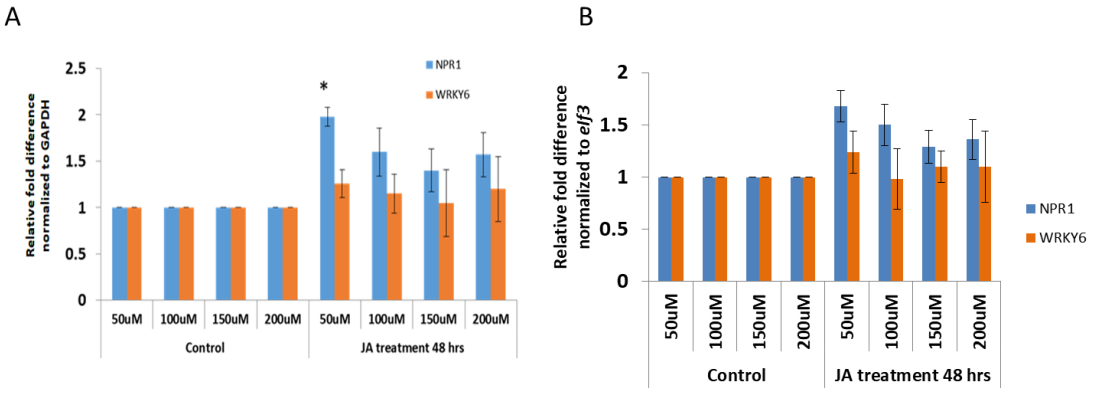


Figure 2.12 Treatment with JA suppresses SA dependent activation of *NtNPR1* in tobacco protoplast in tobacco (A) and in potato (B). To suppress the activation of *NtNPR1* in the tobacco protoplast we supplied various concentration of JA, and observed that 100µM JA treatment is successful to keep the levels of *NtNPR1* similar to control level. Gene expression is relative to high expression reference gene *GAPDH* for leaves. Asterisks indicate significant difference by t-test from the WT treated by JA in a time dependent study (*P<0.05). Data are means ± standard deviation (SD) for three independent biological replications.

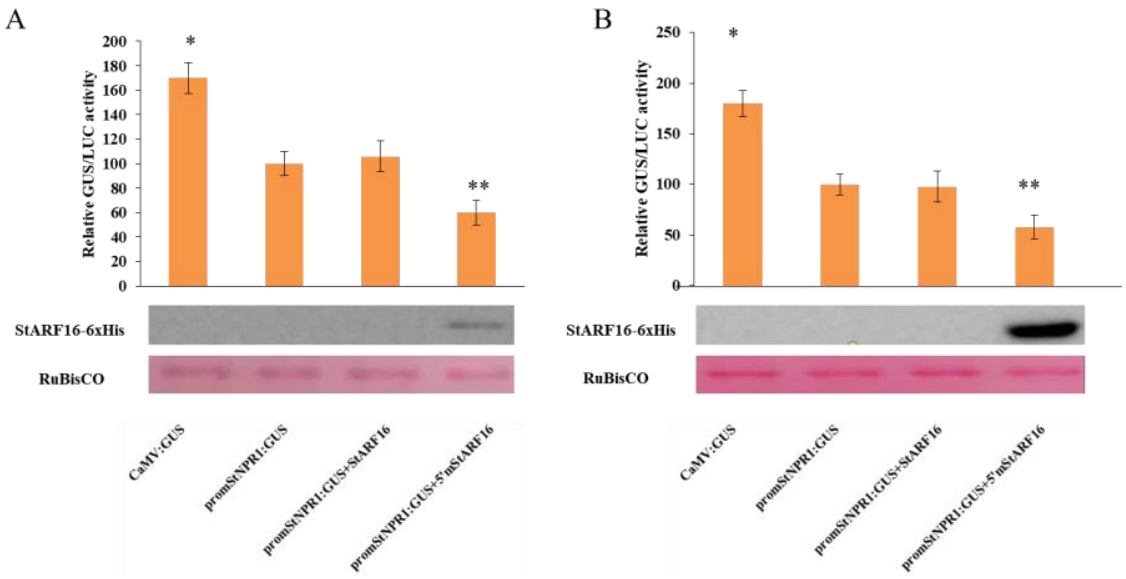


Figure 2.13 StARF16 regulates *StNPR1* expression in potato. StNPR1:GUS activity in the tobacco protoplast (A) And potato protoplast (B) pretreated with 100 µM JA. 35SCaMV:GUS in pBI221 has been used as a positive control for the experiment. RuBisCO was used as western loading control. GUS activity was measured by 4-methylumbelliferyl-β-D-glucuronide (MUG) fluorescence assay. A relative value for GUS expression was obtained by dividing the GUS activity by the specific luciferase activity. Asterisks indicate significant difference by t-test from the *promStNPR1*:GUS (*P<0.05; **P<0.01). Data are means ± standard deviation (SD) for three independent biological replications from 3 independent experiments.

The JA treated tobacco and potato protoplasts were co-transfected with either 35SCaMV:*StARF16*or 35SCaMV:*St5'mARF16*, and the *promStNPR1*:GUS and relative GUS-LUC activity was measured for each combination. Upon transfection with 35SCaMV:*St5'mARF16*, the *promStNPR1*:GUS reporter activity decreased suggesting that *StARF16* could repress *StNPR1 (*Figure 2.14 B and C*)*. However, when co-transfected with *35SCaMV*:*StARF16*, it did not result in any change in the reporter gene activity. We observed that in tobacco protoplasts, StWRKY6 can positively regulate the expression of *StNPR1* (Figure 2.10). As native form of *StARF16* can be cleaved by miR160, western blot analysis was employed to confirm the expression of His-tagged *StARF16* in the samples (Figure 2.13 B and C). The translation of StARF16 in both tobacco and potato protoplasts was found to be negligible when 35SCaMV:*StARF16* construct was used whereas, higher levels of StARF16 accumulated when 35SCaMV:*5'mStARF16* construct was employed in co-transfection. (Figure 2.13 B and C).

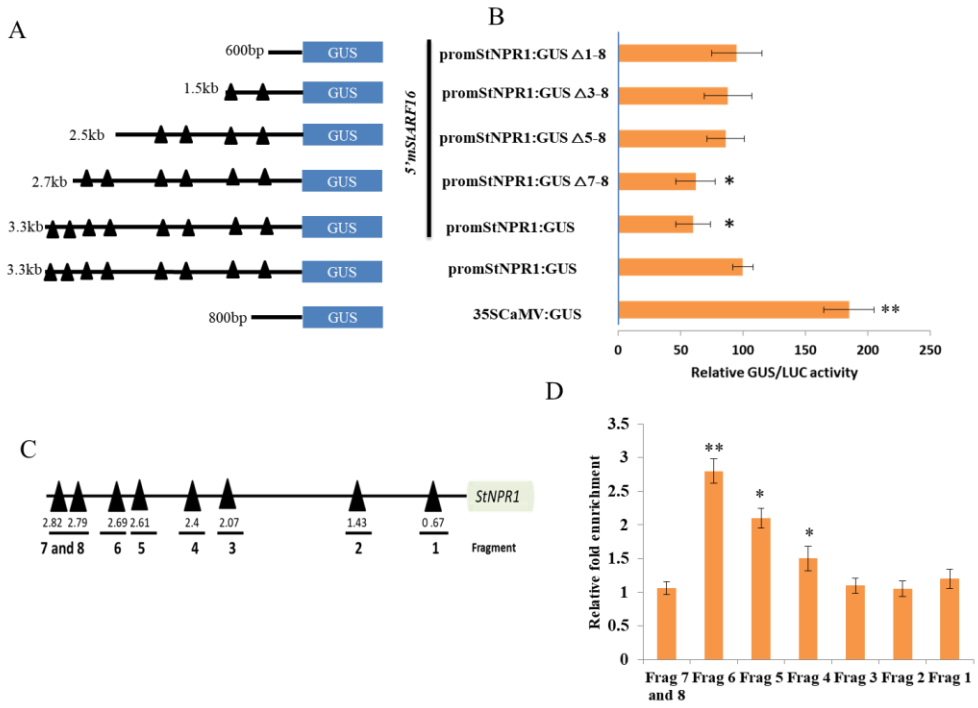


Figure 2.14 Tandem ARF binding sites are necessary for an effective suppression of *StNPR1*. (A) Schematic diagram showing different constructs used in the potato protoplast transfection using deletion constructs. The luciferase gene in pBI221 under the control of the 35SCaMV promoter was included as an internal control. Two putative ARF binding sites have been removed from each deletion construct. (B) StARF16 represses prom*StNPR1*:GUS activity in the potato protoplasts pretreated with 100 µM JA. 35SCaMV:GUS in pBI221 has been used as a positive control for the experiment. Removal of four ARF binding sites from the *promStNPR1*, viz., Δ5-8 and Δ7-8, showed repression of *promStNPR1*:GUS upon addition of 5'mStARF16. Significant repression was lost upon deletion of 5th to 6th ARF binding sites. Filled triangles represent positions of putative ARF binding sites on the promoter of *StNPR1* (C) Schematic diagram of different fragments that are analyzed using ChIP-qRTPCR. (D) Relative enrichment levels of StARF16 over different fragments, i.e. Frag 1 to 6, using specific primers corresponding to the promoter regions of *StNPR1*. GUS activity was measured by 4-methylumbelliferyl-β-D-glucuronide (MUG) fluorescence assay. A relative value for GUS expression was obtained by dividing the GUS activity by the specific luciferase activity. Asterisks indicate significant difference by t-test from the *promStNPR1*:GUS (*P<0.05; **P<0.01). Data are means ± standard

deviation (SD) for three independent biological replications. For (F) Data are means ± standard deviation (SD) for two independent biological replications

Our sequence analysis for *StNPR1* promoter revealed that there are altogether eight putative ARF binding sites in *StNPR1* promoter similar to *AtGH3*.6 promoter (The complete sequence is provided in Annexure II). To validate the importance and necessity ARF binding sites present in the promoter of *StNPR1*, we generated several truncated constructs lacking ARF binding sites (Figure 2.14 A). The reporter gene activity showed significant decrease when the potato protoplasts were co-transfected, with Δ7-8 and Δ5-8 truncated promoter sequences, along with, 35SCaMV:5'm*StARF16* construct. This attenuation suggests that StARF16 could regulate *StNPR1* expression only if a minimum of 6 ARF binding sites are present over the promoter (Figure 2.14 A and B western blot for ARF16 expression is shown in Figure 2.15 C and D). The ARF binding sites present in the range of 2.6 kb region upstream to *NPR1* CDS if deleted, results in total loss of *StNPR1* regulation by StARF16(Figure 2.14 B). We obtained similar results in tobacco protoplast where deletion of 6 ARF binding site showed significant decrease of reporter gene activity (Figure 2.15 B). To further confirm the regulation by ARF16 and to assess the enrichment of StARF16 on promoter of *StNPR1* chromatin immunoprecipitation (ChIP) - qRTPCR analysis was performed of the transfected potato and tobacco protoplasts. The tobacco protoplast system we observed that ARF16 could bind to the exogenously provided *StNPR1* promoter and is specifically binds to 5^{th} and 6^{th} putative ARF binding site. Meanwhile for, the potato protoplasts, we transfected 35SCaMV:*St5'mARF16:6xHis* to confirm the binding of *StARF16* to the promStNPR1 present in the native chromatin of the potato protoplasts. A ChIP -qRTPCR analysis revealed that *StARF16* was significantly enriched over the 4^{th}, 5^{th}, and 6^{th} ARF binding positions on the *StNPR1* promoter (Figure 2.14 D and 2.15C Fig. S8D and 7D; western blot for ARF16 expression is shown in Figure 2.15 E). These sites correspond to the sequences position 2.4 kb, 2.61 kb, and 2.69 kb upstream to *StNPR1* gene, respectively (Figure 2.14 C and D). Our results confirm the repression of *StNPR1* by JA mediated induction of *StARF16*, through the putative ARF binding sites present upstream in its promoter in potato.

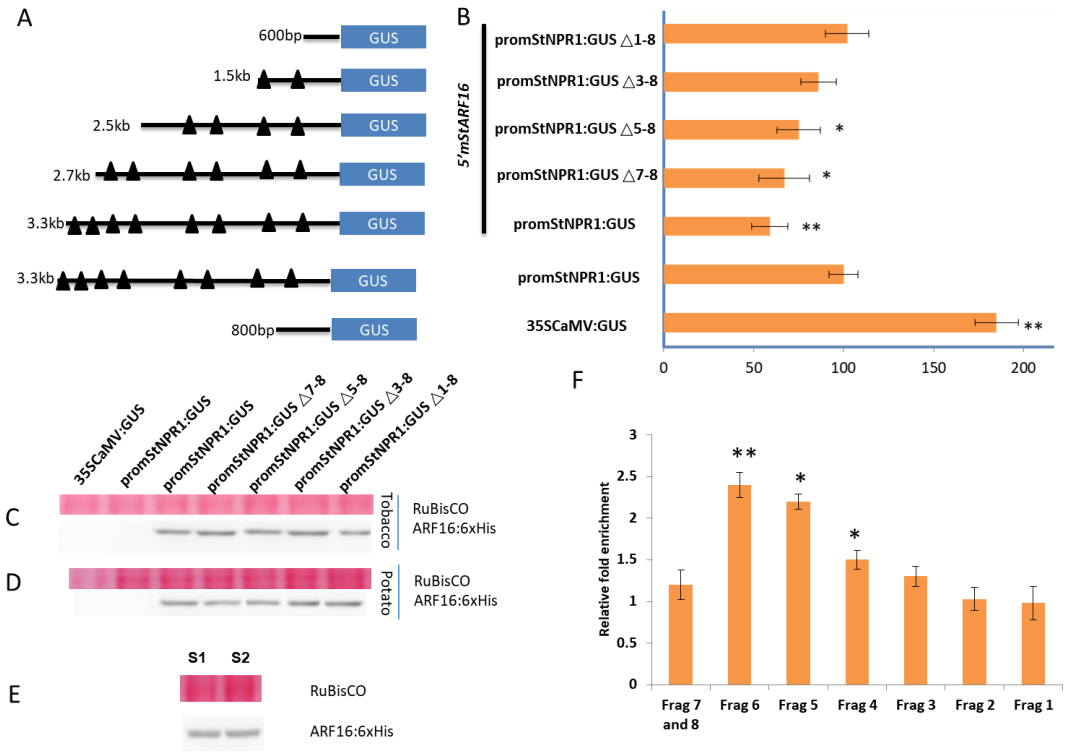


Figure 2.15 Tandem ARF binding sites are necessary for an effective suppression of *StNPR1* *in tobacco.* (A) Schematic diagram showing different constructs used in the tobacco protoplast transfection using deletion constructs. The luciferase gene in pBI221 under the control of the 35S CaMV promoter was included as an internal control. Two putative ARF binding sites has been removed from each deletion construct. (B) StARF16 represses *StNPR1:GUS* activity in the tobacco protoplast pretreated with 100 µM JA. CaMV:GUS in pBI221 has been used as a positive control for the experiment. Removal of up to four ARF binding sites from the *promStNPR1* Δ5-8, and Δ7-8 showed repression of *promStNPR1*:GUS upon addition of 5'mStARF16. Significant repression was lost upon deletion of existing six and eight ARF binding sites. Filled triangles represent positions of putative ARF binding sites on the promoter of StNPR1. (C and D) Western blot shows equal expression of ARF16 during transfection in tobacco (C)and potato (D). (E) Western blot analysis shows the expression of StARF16 :6xhis in potato protoplasts transfected with 35SCaMV*:5'mStARF16,* for ChIP qRT-PCR in potato protoplast. *(*F) Relative enrichment levels of ARF16 over different fragments, i.e. Frag 1 to 8, using specific primers corresponding to the promoter regions of StNPR1. RuBISCO was used as loading control. GUS activity was measured by 4-methylumbelliferyl-β-D-glucuronide (MUG) fluorescence assay. A relative value for GUS expression was obtained by dividing the GUS

activity by the specific luciferase activity. Asterisks indicate significant difference by t-test from the promStNPR1:GUS (*P<0.05; **P<0.01). Data are means ± standard deviation (SD) for three independent biological replications. For (F) Data are means ± standard deviation (SD) for two independent biological replications

2.4. Discussion

Plants utilize two major phytohormones, SA and JA to regulate defense response against various invading pathogens. To mount an effective immune response against a specific pathogen, SA and JA both have an antagonistic relationship with each other (Spoel et al., 2003; Spoel et al., 2007; Leon-Reyes et al., 2009; Spoel et al., 2009). Previous studies in Arabidopsis have demonstrated the role of NPR1 in modulating JA response (Spoel et al., 2003). However, there are very few studies that explain the role of JA in regulating SA pathway via PR1 (Laurie-Berry et al., 2006). Recently, miR160 has been demonstrated to contribute to the defense response against P. infestans by regulating SA pathway. A previous study from our laboratory has suggested that StARF10, a target of miR160, could potentially regulate GH3.6, a homolog of GH3.5 of Arabidopsis, and maintains the balance between development and defense responses by regulating SA and auxin upon infection with P. infestans (Natarajan et al., 2018). Here, we investigated whether StARF16, another target of miR160, could contribute to regulating the defense response in potato. By using various approaches, such as sequence analysis, expression profiling, infection analysis, and protoplast co-transfection assays, we showed that JA-mediated StARF16 is crucial for regulating the expression of SA pathway upon infection in potato.

2.4.1. Defence response during hemi-biotrophic and necrotrophic interactions

MiR160 has been demonstrated to be differentially expressed in infected and systemic leaves of various plants upon infection with either a biotrophic or a necrotrophic pathogen (Xin et al., 2010; Lang et al., 2011; Natarajan et al., 2018). It has been shown to positively regulate defence response in Jujube (Ma et al., 2020) upon infection with biotrophic pathogen. However, in case of necrotrophic pathogen, miR160 has been shown to be downregulated upon infection with *B. cinerea* in tomato (Jin and Wu, 2015). Our findings show contrasting expression profile for miR160 upon infection with *P. infestans* and *A. solani* (Figure 2.3. A and D). MiR160 and its target, *StARF10*, exhibited a negative correlation with each other, but *StARF16* did not show such correlation during early infection stages (Figure 2.3). Lack of negative correlation between miR160 and *StARF16* has been reported during the regulation of leaf curvature in potato (Natarajan and Banerjee, 2020). In tomato also, miR160 and *StARF16* do not show negative correlation, and has been shown to be crucial in ovary patterning, floral organ abscission and lamina outgrowth (Damodharan et al., 2016). These evidences indicate

that even when miRNA and its target do not show negative correlation, they are intertwined to regulate important development and defence responses through an unknown mechanism.

Previously, we have reported the involvement of miR160 in regulation of SA pathway to mount a successful local and systemic defence response during potato-*Phytophthora* interactions (Natarajan et al., 2018). It was established that SA accumulates during the early phase of infection which corresponds to the hemibiotrophic phase of *P. infestans*. During *A. solani* infection, genes such as *MYC2* and *PDF1*.2 (Ellis and Turner, 2001) are activated to regulate JA mediated defence response in the plants. Studies conducted in tomato describe that upon infection with *A. Solani,* levels of *NPR1* peaks at 3 days post infection (Dey et al., 2019; Shinde et al., 2018). In potato, the *PR1* levels accumulation has been reported at 2 days post treatment (Natarajan et al., 2018). In this study, we observed that the levels of SA dependent genes (*StNPR1, StPR1,* and *StWRKY6*) increased upon *P. Infestans* infection (Figure 2.5 A, B and C) but, decreased upon infection with *A. solani* (Figure 2.5 E, F and G) during early phase of infection (12 hpi). In *Arabidopsis*, expression of *NPR1*, *PR1*, *WRKY6* and *MYC2* have been reported to elevate after 24 hpi with *Psuedomonas syringae and Colletotrichum orbiculare,* respectively (Rogers and Ausubel, 1997; Fernandez-Calvo et al., 2011). A study in tomato reports accumulation of *MYC2* transcripts upon treatment with *B. cinerea* at 48 hpi (Du et al., 2017). However, we found that upon infection with *A. solani, StMYC2* levels were upregulated at 12 hpi (Figure 2.5 H), but no change in expression was observed during *P. infestans* infection (Figure 2.5 D). Previous study illustrates the importance of SA-JA balance to successfully mount an early defence response against *A. solani* in potato (Brouwer et al., 2020). Upon infection progression, JA-mediated defence response becomes evident in regulating plant's immune response against *A. solani* infection.

2.4.2. JA and SA regulates *StARF16*

ARF16 has been reported to contain a transcriptional repressor domain (Hendelman et al., 2012), and its negative correlation with *StNPR1* prompted us to investigate whether it directly regulates *StNPR1* and contributes in defence response (Figure 2.3 E and 2.5 E). In *Arabidopsis,* ARF17 dependent regulation of JA-Ile has been shown to be crucial in root cap formation, but its effect on JA is not discussed (Gutierrez et al., 2012). Hormone treatment experiment revealed that JA and SA could independently regulate *StARF16* expression profile in *in vivo and in vitro* conditions (Figure 2.5). *StARF16* levels were found to be increased at 72 hpt of JA

application while it decreased at 48 hpt on SA application (Figure 2.5). SA has been shown to induce the expression of *NPR1* in a dose dependent manner in various plant species (Horvath and Chua, 1996; Dong, 2004; Uquillas et al., 2004). We found similar *StNPR1* induction in potato leaves treated with SA (Figure 2.5 A and C). However, *StNPR1* expression decreased at 72 hpt of JA application (Figure 2.5 B and C). In rice, *WRKY6* has been shown to regulate *PR10a* in a SA dependent manner upon infection with *Xanthomonas oryzae* pv. *oryzae* (Choi et al., 2015). A similar study in *Arabidopsis* shows that *WRKY6* is a SA inducible gene that positively regulate expression of *NPR1 via* MAPK4 (Chai et al., 2014). We found that, like *Arabidopsis* and rice, *StWRKY6* is a SA inducible gene in potato, where gene accumulation initiated at 48 hpt and continued until 72 hpt (Figure 3A). *MYC2* and *PDF1.2* has been shown to accumulate after 24 hpt with JA (Fernandez-Calvo et al., 2011). Consistently, we also noted their accumulation in potato leaves started at 24 hpt, peaked at 48 hpt, and returned to control levels at 72 hpt (Figure 2.5 B). An increased level of *StARF16* before the suppression of *StNPR1* upon treatment with JA, suggests that *StARF16* plays a potential role in regulating defence response during infection with a biotrophic or a necrotrophic pathogen in potato.

2.4.3. StARF16 regulates *StNPR1,* by binding to its promoter and suppresses downstream SA mediated defence pathway

Previous studies in *Arabidopsis* have demonstrated the significance of NPR1 in modulation of various JA-responsive genes like *LOX2*, *VSP*, and *PDF1.2* (Spoel et al., 2003; Leon-Reyes et al., 2009). Simultaneous infection with both biotrophic and necrotrophic pathogens results in an increased susceptibility to the latter. This indicates SA mediated suppression of JA defence response in *Arabidopsis* (Spoelstra et al., 2007; Spoel et al., 2003). Suppression of JA mediated defence response also appears to be exerted by cytoplasmic NPR1, which upon SA induction limits JA dependent signaling (Spoel et al., 2003; Ndamukong et al., 2007; Yuan et al., 2007). Upon activation of JA responsive gene *COI1 (CORONATE INSENSITIVE 1)*, the JA pathway is known to suppress the expression of *PR1*, but the nature of regulation remains unclear (Laurie-Berry et al., 2006).

Our study reports a new regulatory mechanism related to the JA mediated modulation of SA induced defence response in potato. We noted that the expression profiles of *StARF16* and *StNPR1* exhibited a negative correlation during early stages of infection and also during hormonal treatment experiments (Figure 2.3 B- 2.5 A and 2.3 E-2.5 E), indicating that JA could

potentially regulate SA pathway. These results led us to hypothesize that JA mediated induction of StARF16 could regulate *StNPR1* expression. Sequence analysis of *StNPR1* promoter revealed the presence of multiple ARF binding sites (Figure 2.8). VISTA analysis (Frazer et al., 2004) also unraveled presence of a family specific feature in the promoter of *StNPR1* in *Solanaceae*. We observed sequence similarity in the promoter of *NPR1* between tomato, tobacco and potato (Figure 2.8), but no such similarity was found within the members of *Brassicaceae* (Figure 2.8). We hypothesize that the regulation of *StNPR1 via* StARF16 could be a family specific phenomenon, but it requires further investigations. Through Y1H, we confirmed that the potato StARF16 can directly interact with the promoter of *StNPR1* (Figure 2.9 C). Further, by sequential deletion of the ARF binding motifs in *StNPR1* promoter, we demonstrate that the StARF16 could suppress the expression of *StNPR1*, and requires a minimum of six ARF binding sites for its efficient functioning (Figure 2.14 B and D, Figure 2.15 B and D). Also similar to the findings in *Arabidopsis* (Chai et al., 2014), we observe that StWRKY6 could positively regulate *StNPR1* (Figure 2.10). The presence of ARF binding site at ~2.6 kb upstream of TSS is required for effective regulation of the *StNPR1* expression. Our ChIP –qRT-PCR analysis shows StARF16 enrichment over 4th, 5th and 6th ARF binding motifs, present upstream to the StNPR1 CDS (Figure 2.14D). Hence, the presence of ARF binding sites upstream to *StNPR1* is crucial for effective regulation of gene expression. Similar to previous reports (Damodharan et.al., 2016; Natarajan and Banerjee, 2020), an upregulation of miR160 (Figure 2.3 D) during *A. solani* infection could be playing a crucial role in regulating defence response through its target *StARF16,* even if they fail to show negative correlation with each other.

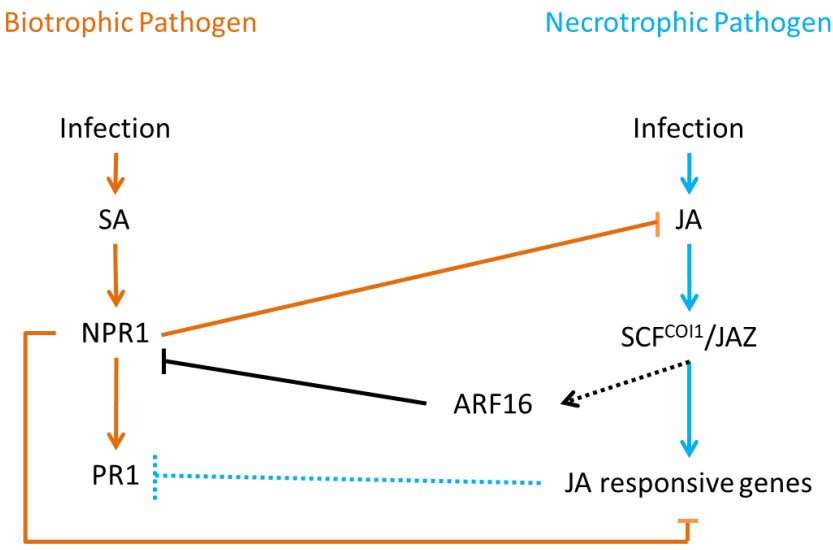


Figure 2.16. A model describing the role of StARF16 in plant pathogen interaction to regulate SA-JA cross talk. During biotrophic pathogen interaction, SA antagonizes with JA by suppressing JA accumulation and JA responsive genes. Whereas, upon infection with necrotrophic pathogen, JA mediate the expression of *StARF16* in the plants. In turn, StARF16 suppress the expression of *StNPR1* and regulate the SA pathway. Dotted line indicates putative interactions only.

Taken together, our study demonstrates a new regulation of SA pathway by JA *via StNPR1* in potato during necrotrophic pathogen infection. It provides new insights about the role of JA-induced *StARF16*, a development related gene, to play a crucial role in regulating SA pathway through *StNPR1* in potato. Our findings suggest that StARF16 could contribute to JA-SA antagonistic relationship during early defence response (Figure 2.16). We speculate that the miR160-*StARF16-StNPR1* module functions differentially upon infection either with a biotrophic or a necrotrophic pathogen. Our investigation has attributed a novel role to StARF16 during establishment and regulation of defence response upon plant–pathogen interactions.

Kalsi H., Karkhanis A., Natarajan B., Bhide A., and Banerjee A. (2022). *AUXIN RESPONSIVE FACTOR 16 (StARF16) regulates defence gene StNPR1 upon infection with necrotrophic pathogen in potato. Plant Molecular Biology DOI:10.1007/s11103-022-01261-0.*

Chapter 3

MiRNA160 knockdown (KD) lines are deficient in G3P induced SAR response

3.1. Introduction

Plants utilize various chemicals to induce systemic response upon pathogen infection. Studies have shown the use of different chemicals viz., SA, methylated form of SA-MeSA, AzA, DA, pipecolic acid, and G3P, to elicit SAR response in the plants. These chemicals were discovered via treating mutants of plants that lack or were compromised in the synthesis of these vital SAR signals. It has been shown that few select chemicals are transported from the locally infected site to the systemic site, and are responsible for mounting an effective SAR response for future infection.

3.1.1. Branches of SAR inducers

SAR is a complex phenomenon that involves various chemicals and proteins, and these molecules can be categorized in one of the two main branches that comprise the SAR pathway. One branch involves SA dependent on NPR1, and the other involves the free radicals NO and ROS that regulate AzA and G3P (Wang et al., 2014; Wendehenne et al., 2014; El-Shetehy et al., 2015). Upon infection, plants synthesis and accumulate SA that leads to synthesis of MeSA, biologically active form of MeSA required for SAR establishment (Shulaev et al., 1997;Koo et al., 2007). At the infected site SA is converted to mesa through SA methyltransferases (SAMT/BSMT), and SAMT is required for the phloem accumulation of MeSA. Upon translocation to the distal tissue MeSA is converted back to SA via MeSA esterase activity of the SA binding protein (SABP) 2 (Kumar and klessig et al., 2003; Chen et al., 2003). In presence of SA, NPR1 octamer disscociates an forms NPR1 mpnonmers that are transported to nucleus for activation of SA mediated defence genes. NPR1 in nucleus interacts with TGA proteins and activate defence pathway in plants (Tada et al., 2008; Mou et al., 2003). Pip and DA are known to induce SA accumulation, suggesting activation of the SA branch of SAR response but not with the application of G3P and AzA (Chaturvedi et al., 2012; Návarová et al., 2012). The presence of two SAR branches is supported by the fact that SA cannot restore SAR in mutants defective in NO, ROS, or G3P biosynthesis, while NO/ROS cannot confer SAR on mutants defective in SA synthesis or signaling. These results suggest the independent establishment of SAR response in plants.

Additionally, studies including inhibitors of NO synthesis or NO scavengers show attenuation of SA-induced SAR in tobacco (Song and Goodman, 2001). It has been reported

that NO-ROS-AzA-G3P branch confers SAR response in a dose dependent manner, unlike that of SA dependent pathway (Wang et al., 2014). Free radicals, like NO, have been reported to operate similar to =animal systems, where plants produce opposing effects depending on their optimal concentration (Delledonne et al., 1998; Besson-Bard et al., 2008; Wink et al., 2011). Free radicals participate in SAR by oxidation of carbon 18 unsaturated fatty acids (FAs) to di-carboxylic acid, AzA. It has been demonstrated that AzA application cannot confer SAR on mutants deficient in G3P synthesis, indicating its function that is upstream of G3P. Exogenous AzA application induces the expression of the G3P synthesizing *GLY1* and *GLI1* genes, which encode G3P dehydrogenase and glycerol kinase, respectively. Upon exogenous application og G3P and WT plant exudate, even *gly1/gli1* plants are capable of producing SAR response (Chana et al., 2011). G3P operates in a positive feedback mechanism with the lipid transport proteins (LTPs), DIR1 and AZI1 such that the absence or lack of DIR1 or AZI1 impairs pathogen-induced G3P accumulation and lack of G3P results in reduced *DIR1* and *AZI1* transcripts (Yu et al., 2013). DIR1 and AZI1 are known to form homo- and heteromers suggesting that a complex of these proteins is required for the establishment of SAR. It has been shown that these complexes are unstable in the mutants lacking G3P, and it is transported to the systemic site only when these LTPs are present in the plants. However, direct interaction between G3P and DIR1 has not yet been confirmed, raising the possibility that G3P may be derivatized and then transported to distal tissues. Radiolabelled feeding experiments showed that G3P is indeed converted to an unidentified derivative that can translocate from infected to distal tissues in a DIR1-dependent manner (Chanda et al., 2011). Along with these studies, it has been shown that mutation in *sid2*, a gene involved in biosynthesis of SA in Arabidopsis, compromises G3P induced SAR, suggesting a basal amount of SA is required for G3P to confer SAR (Chanda et al., 2011).

3.1.2. Objectives

In our previous study (Natarajan et al., 2018) , we establish that miR160 has a crucial role in regulating the defence genes responsible for SA-mediated defence response in potato, during *P. infestans* infection. It was shown that, upon infection with *P. infestans,* the plants show high accumulation of miR160 during early defence response. As the infection progresses, the SA-mediated defence response is activated. Upon generating miR160 knockdown (KD) transgenic lines, eTM160, it was observed that the plants were compromised in developing an

effective defence response, in both local and systemic sites. Upon treatment with Arachidonic acid, a known PAMP and SAR elicitor in potato, the transgenic line plants showed deficiency in accumulation of SA and MeSA. Moreover, various SA-mediated genes were found to be mis-regulated upon AA treatment in eTM160 lines. Further, grafting experiments suggested that eTM160 transgenic lines were compromised in either synthesizing or in transporting SAR signals. The study did not explain why miR160 KD lines were compromised in SAR. There are various SAR signals, such as G3P, SA, AzA, etc., are required to produce SAR, and their necessity to establish the SAR response has been well reported (Dempsey and Klessig, 2013). In this study, we subjected miR160 KD lines to several SAR signals to analyse the SAR response of miR160 KD transgenic plants. Upon successful establishment of SAR response, we attempted to dissect the downstream signalling components that could be involved in regulating SAR response in miR160 KD lines.

To further understand the nature of SAR response in the plants and what causes miR160 KD lines to show compromised SAR, we followed three approaches in this study:

1. Exogenous application of SAR elicitors to mount SAR response in potato WT plants
2. To rescue the compromised SAR response in miR160 KD lines of potato.
3. investigating the regulation of successful SAR defence response in miR160 KD lines

3.2. Material and methods

3.2.1. Plant and pathogen material

The wild-type (WT) and transgenic potato (*Solanum tuberosum* L. cv. Désirée) plants were maintained in *in vitro* at 25°C under long day conditions (LD, 16 hours light: 8 hours dark) in tissue culture. After two weeks, the plants were transferred to soil and maintained at 22°C under LD conditions in the greenhouse for three weeks. In the fourth week, plants were transferred to the environmental plant growth chambers (Percival Scientific) for experimentations. Oomycete pathogen, *Phytophthora infestans* was maintained on a pea agar at 18°C in petri-dish. Prior to infection the fungal hyphae were scrapped and transferred to sterile water Petri-dish and incubated at 4°C to induce the release of viable zoospores. Sporangia concentration was then adjusted to $1x10^6$ per ml, and plants were infected with $1x10^4$ spores on the abaxial side of the leaves.

3.2.2. Chemical treatment assay

To find out the deficiency of SAR signal in the miR160 KD transgenic lines, four-week old soil grown WT and eTM160-26 KD lines were treated with 100 µM G3P (Chanda et al., 2010), 5 µM Arachidonic acid (AA) (Mansalova et al., 2010), 0.25 mM SA (Jendoubi et al. 2017), 100µM AzA and 10 pM DA (Chaturvedi et al 2012) on to 5^{th} and 6^{th} leaves from the top to trigger SAR response. Treated and untreated plants were kept in separate growth chambers at 18°C. Four days post treatment, a systemic infection with $1x10^6$ C.F.U. per ml (OD_{600} = 0.1) of *R. solanacearum* was introduced to 2^{nd} and 3^{rd} mature leaves from the top, and incubated at 28°C. Post five days of secondary treatment, one cm^2 leaf piece was cut from infected leaves. The collected leaf pieces were crushed in sterile water, and plated on nutrient agar post serial dilution for bacterial count.

3.2.3. Systemic Acquired Resistance Assay

Four weeks old potato (WT, miR160 OE and miR160 KD) plants were given a primary infection with *P. infestans,* followed by secondary infection with *Ralstonia solanacearum.* For primary infection, $1x10^4$ spores of *P. infestans* were swabbed on the abaxial side of the 5^{th} and 6^{th} mature leaf from the apex, and plants were incubated at 18°C for four days. On fifth day, 2^{nd} and 3^{rd} mature leaves from the top were infiltrated with $1x10^6$ C.F.U. per ml (OD_{600} = 0.1) of

R. solanacearum and Incubation was carried out in growth chamber, at 28°C for secondary infection. After five days of secondary infection, one square centimeter leaf piece was removed from *R. solanacearum* infected leaf and crushed in sterile water. The sample was serially diluted and plated on nutrient agar medium and bacterial count was recorded.

3.2.4. RNA isolation and cDNA preparation

Total RNA was isolated from the infected and control leaf samples of WT, miR160 OE and miR160 KD potato plants using RNAIsoPlus (TAKARA) reagent as per manufacturer's protocol. cDNA was prepared using two micrograms (2 µg) of total RNA, oligo(dT) primers and MMLV Reverse Transcriptase (Promega) by following manufacturer's instructions.

3.2.5. Quantitative real-time (RT-PCR) analysis

For analysis of defence pathway genes, *StNPR1, StAZI1, StALD1, StDIR1, and StPR1* were selected; whereas *StTIR1, StYUCCA1 and StARF16* were selected for the analysis of auxin pathway genes. For normalization, *SteIF3e* gene was used. qRT-PCRs were carried out using SYBR green mix (Takara). The reaction conditions for genes *StTIR1, StYUCCA1, SteIF3e, StAZI1, StALD1, StDIR1, and StPR1* were 95°C for 2 min, 40 cycles of 95°C for 15 sec and 60°C for 20 sec and for *StNPR1* conditions were 95°C for 2 min, followed by 40 cycles of 95°C for 15 sec, 50°C for 15 sec and 68°C for 20 sec. Melting curve analysis was included in all the programs to check the PCR specificity and the data was analyzed using 2^{-ddCt} method (Livak and Schmittgen, 2001).

3.2.6. Accession numbers

Following are the accession numbers of transcripts analyzed in this chapter:

Table 3.1 Accession numbers

Name	Accession	Source
StPR1	AY050221	NCBI
StNPR1	XM_006357647	NCBI
StARF16	PGSC0003DMT400062489	PGSC
StYUCCA1	PGSC0003DMT400067103	PGSC
StTIR1	PGSC0003DMT400029517	PGSC

StDIR1	PGSC0003DMT400029446	PGSC
StAZI1	PGSC0003DMT400065895	PGSC
StEIF3E	PGSC0003DMT400086547	PGSC
StALD1	PGSC0003DMT400059031	PGSC

3.2.7. Primer Sequences

Table 3.2 List of primers

Primer Name	Sequence (bp; 5'- 3')
ElF3e qFP	GGAGCACAGGAGAAGATGAAGGAG
ElF3e qRP	CGTTGGTGAATGCGGCAGTAGG
YUCCA1_FP	AAATTAGGTCTCCGGCGA
YUCCA1_RP	TTTCCTTCACACCTGGCAT
TIR1_FP	AACCCTGAGCTTGGCAAGC
TIR1_RP	GGCCTTGCTCCGTCAAGGTT
NPR1_FP	AAGAGGCTCACTAGGCTT
NPR1_RP	GCTTCATACGCAAATCATCG
PR1_FP	GTACCAACCAATGTGCAAGCG
PR1_RP	TGTCCGACCCAGTTTCCAAC
DIR1_FP	GAGTGCCTGGTTCATTTTATAC
DIR1_RP	GGACTTGTACTTGCCATGTAA
AZI1_FP	CATCATTGGTGAGACCAAGCTC
AZI1_RP	TGGTATGCCTTTGTCCTCAGT
ARF16_FP	GGCAACCCCCTCAGGTCTAG
ARF16_RP	GCATCAACTTGTTGGGAAGCGG
StALD1_FP	GTGCAGCCCTGAGAATGATT
StALD1_RP	GCACAAGTAAATGGTTCAAT

3.3. Results

3.3.1. SAR elicitors trigger SAR response in WT plants

Diverse molecules have been reported to play crucial roles in SAR establishment for *Arabidopsis* and tobacco, however, SAR responses have not been well studied in potato. Previous studies suggest that potato plants could have a more complex and different regulation of SAR response (Mansalova et al., 2010; Yu et al., 1997). Thus to confirm, whether the known SAR elicitors can mount a successful SAR response in potato as well, we treated WT plants with *P. infestans*, SA, AA, AzA, G3P and DA, and monitored their SAR response. After the primary treatment of WT plants with either water (Control) or a SAR elicitor (Aza, G3P, DA, AA, SA and, *P. infestans*) plants were given a secondary infection at distal tissue site with *R. solanacearum* after 3 days of post treatment. We observed that WT potato plants treated with SAR elicitors were able to successfully mount a SAR response (Figure 3.1). The most robust response was observed in plants treated with AA and SA. These results confirm the fact that the known SAR elicitors identified in *Arabidopsis and* tobacco, can mount SAR response in potato as well.

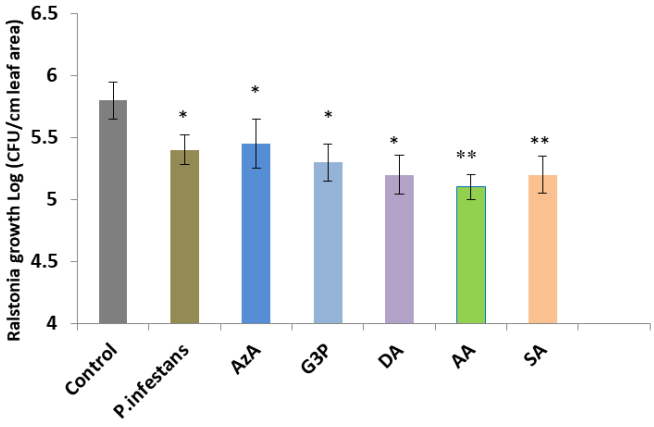


Figure 3.1 Establishment of SAR upon treatment with SAR elicitor. SAR response establishment in WT potato plants upon treatment with *P. infestans* and various SAR elicitors, viz, SA, AA, AzA, DA, and G3P. Primary leaves were treated with a SAR elicitor and, at 72 hpt distal leaves were inoculated with R. *solanacearum* and, leaf discs were collected after 5 days post inoculation. Data represents mean ± SD of at least three biological replicates having three technical

replicates each. Statistical analysis was carried out with Student's t-test. Asterisks indicate values that were significantly different from the control plants, $P < 0.05$ (*) and $P < 0.01$ (**).

Previously published results show us a differential regulation of SA and AA inducible SAR response in potato (Manosalva et al., 2010; Yu et al., 1997). We treated the WT potato plants with 0.25mM and 0.5mM of SA, and 0.05mM, 0.25mM and 0.5mM of AA and, analyzed dose dependent SAR response in the treated plants. We noticed that there exists a correlation between the SAR response and the dose of the SAR elicitor given (Figure 3.2). Even though all the concentration confers SAR in potato plants but higher doses of both SA and AA yielded a robust response.

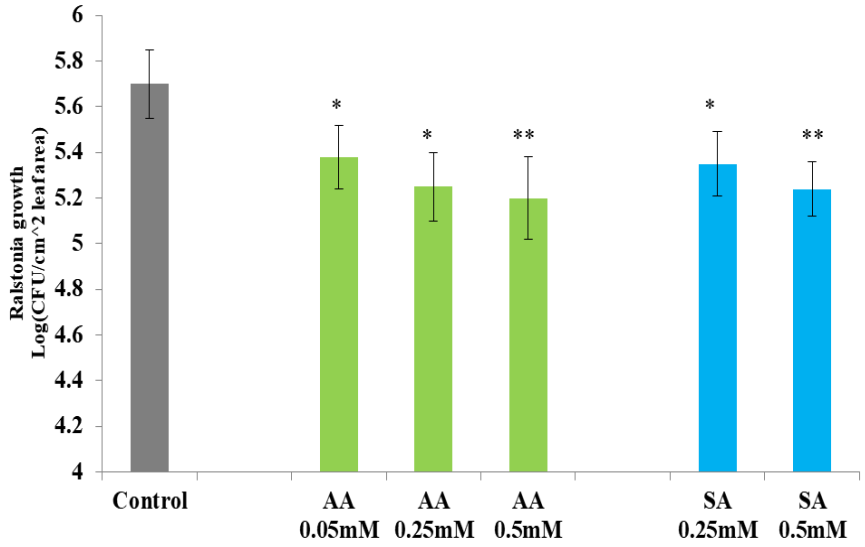


Figure 3.2 Dose dependent response of SAR in WT potato plants. WT plants were treated with concentrations of 0.05mM to 0.5mM AA and 0.25mM and 0.5mM of SA. The primary leaves were treated with SAR elicitors and 3 days post treatment distal leaves were inoculated with *R. solanacearum* and incubated at 28°C for 5 days post infection before CFU count. Data represents mean ± SD of at least three biological replicates having three technical replicates each. Statistical analysis was carried out with Student's t-test. Asterisks indicate values that were significantly different from the control plants, $P < 0.05$ (*) and $P < 0.01$ (**).

3.3.2. miR160 KD lines are deficient in SAR mobile signal

In our previous study, we carried out the SAR response assay to investigate the role of miR160 in systemic defence response. Upon treatment of miR160 transgenic lines with *P. infestans* as a primary pathogen, and *R. solanacearum* as a secondary pathogen in the distal site, we observed that miR160 KD lines were deficient in SAR response, whereas miR160 OE lines were able to mount a successful and robust SAR response (Figure 3.3) (Natarajan et al., 2018). This result suggested that miR160 KD lines are deficient in either synthesis or transport of SAR mobile signals. We tested this hypothesis using grafting experiment, wherein we used miR160 KD line and WT plants to generate homografts and heterografts (Figure 3.4). We found that WT/WT homografts exhibited robust SAR development while miR160 KD homografts (eTM160-26/eTM160-26) failed to exhibit SAR response (Figure 3.4). This confirmed that grafting process have not affected SAR response but interestingly, none of the heterografts exhibited the SAR response as well, suggesting that knock down of miR160 in potato renders both local and systemic leaves defective in SAR response. To rescue this deficiency in SAR response, we treated the miR160 KD lines with various SAR elicitors that we established to mount SAR response in potato.

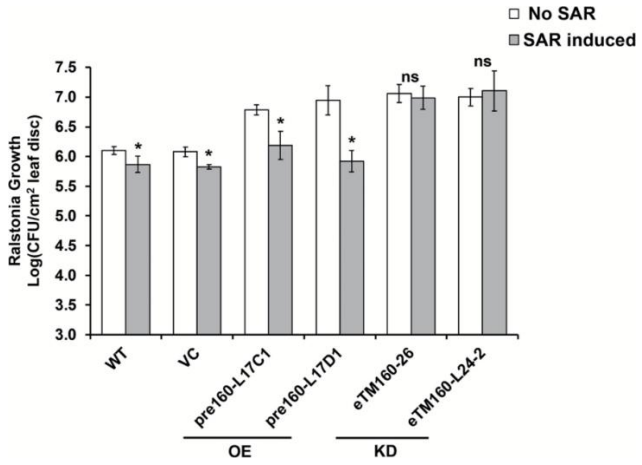


Figure 3.3 SAR response of miR160 KD and OE lines of potato. miR160 KD lines failed to establish SAR response, whereas miR160 OE lines were able to mount a successful SAR response, similar to WT and VC plants. No SAR represent plants treated with sterile water and SAR induced were treated with *P. infestans*. Data represents mean ± SD of at least three biological replicates having three technical replicates each. Statistical analysis was carried out with Student's t-test. Asterisks indicate values that were significantly different from the control

plants, P < 0.05 (*). 'ns' indicates difference is non-significant. (*Reproduced* from our previously published data Natarajan et al., 2018, *under the Creative Commons Attribution License (CC BY)*. Authors Natarajan and Harpreet contributed equally).

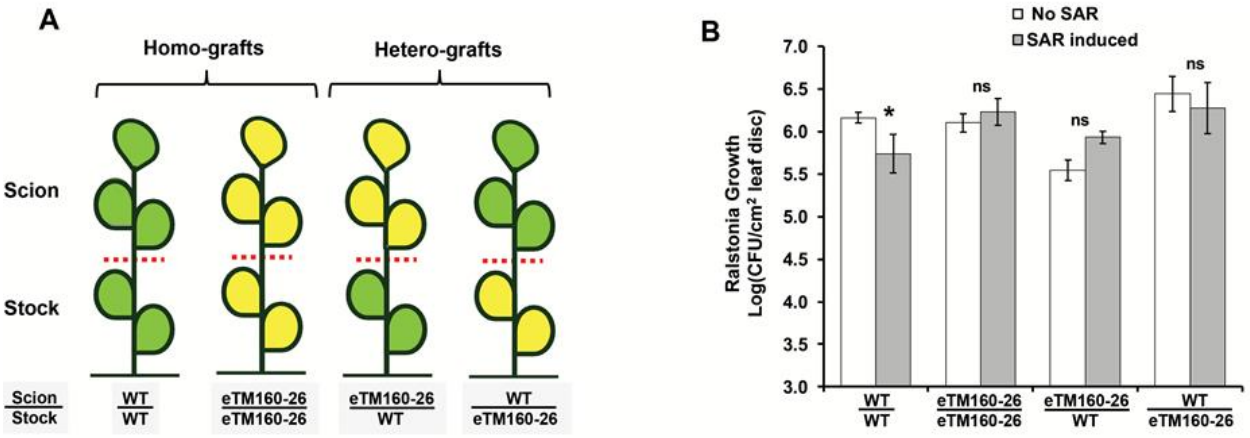


Figure 3.4 miR160 KD lines are deficient in SAR signal synthesis or transport. (A) Diagrammatic representation of grafts developed using WT plants and the miR160 KD line eTM160-26: homografts (WT/WT and eTM160-26/eTM160-26) and heterografts (WT/eTM160-26 and eTM160-26/WT). (B) SAR responses of the homografts and heterografts. All SAR data represent the mean ± SD of at least three biological replicates with three technical replicates each. 'No SAR' represents primary treatment with sterile water and 'SAR induced' represents primary treatment with *P. infestans.* Statistical analysis was carried out with Student's t-test. Asterisks indicate values that were significantly different from the control plants, P < 0.05 (*). 'ns' indicates difference is non-significant. (Adapted from previously published data Natarajan et. al., 2018, *under the Creative Commons Attribution License (CC BY)*. Authors Natarajan and Harpreet contributed equally to the figure)

Before treating miR160 KD lines with SAR elicitors, we treated miR160 KD and OE lines with *P. infestans* as a primary pathogen and *R. solanacearum* as a secondary pathogen at a distal site, and found that miR160 KD lines are compromised in establishment of SAR (Figure 3.5 A). We treated the miR160 KD lines with the SAR elicitor similar to WT, and found that only G3P mounted a successful SAR response in these plants (Figure 3.5 B). Different concentration of G3P were given to the miR160 KD lines to obtain a physiological relevant concentration of G3P, and thus,100 µM G3P was required to establish a robust SAR in miR160 KD lines (Figure 3.6).

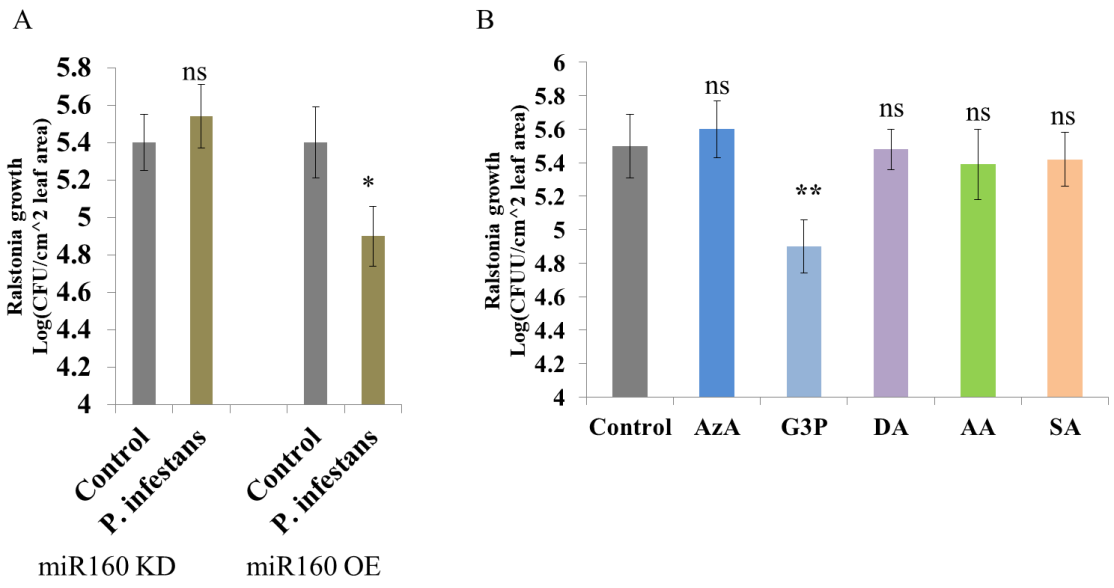


Figure 3.5 miR160 KD lines establish SAR upon treatment with G3P. (A) miR160 KD lines do not shows SAR response upon infection with *P. infestans* for four days at 18°C, followed by a secondary infection on distal site with R. *solanacearum* incubated at 28°C for five days. miR160 OE lines shows a robust SAR response. (B) Treatment of miR160 KD lines with SAR elicitor, viz, AA, SA, AzA, DA and G3P as the primary treatment and then a secondary inoculation with *R. solanacearum,* incubated at 28°C for five days before calculating bacterial titer in the plants. Data represents mean ± SD of at least three biological replicates having three technical replicates each. Statistical analysis was carried out with Student's t-test. Asterisks indicate values that were significantly different from the control plants, P<0.05 (*). 'ns' indicates difference is non-significant.

3.3.3. Activation of ROS-NO-AzA-G3P pathway

Upon treatment of miR160 KD lines with G3P, we observed that these transgenic lines were deficient in G3P response. Thus, we explored how the ROS-NO-AzA-G3P pathway is activated in the potato plants. We analyzed genes involved in this pathway in WT plants treated with G3P at 48 hpt and 72 hpt (Figure 3.7). We found that G3P treatment down- regulates *StARF16* expression and leads to accumulation of *NPR1, PR1, AZI1, ALD1, and WRKY6* at both local (Figure 3.7 A and B) and systemic sites (Figure 3.7 C and D). This suggests that WT potato plants are capable of activating ROS-NO-AzA-G3P pathway upon treatment with G3P.

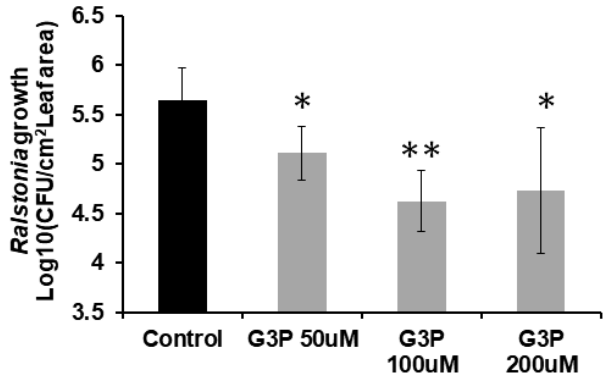


Figure 3.6 Dose responses of miR160 KD lines upon treatment with G3P. SAR response in miR160 KD lies upon treatment with 50µM, 100µM and 200µM concentrations of G3P at the local leaves. The distal leaves were inoculated with R. *solanacearum* at 72 hours post treatment with primary leaves. Data represents mean ± SD of at least three biological replicates having three technical replicates each. Statistical analysis was carried out with Student's t-test. Asterisks indicate values that were significantly different from the control plants, $P < 0.05$ (*) and $P < 0.01$ (**).

Natarajan et al., (2018) have also reported that miR166 KD line showed mis-regulation of auxin biosynthesis gene, *StYUCCA1*, and auxin signaling gene, *StTIR1* (Figure 3.8) (Natarajan et. al., 2018). Further, we analyzed three auxin responsive genes in our study upon treatment with G3P, namely *StARF16, StYUCCA1* and *StTIR1*, which were shown to be attenuated during *P. infestans* infection in potato. We found that, upon G3P treatment, *StYUCCA1, StARF16 and StTIR1* levels decreased (Figure 3.9).

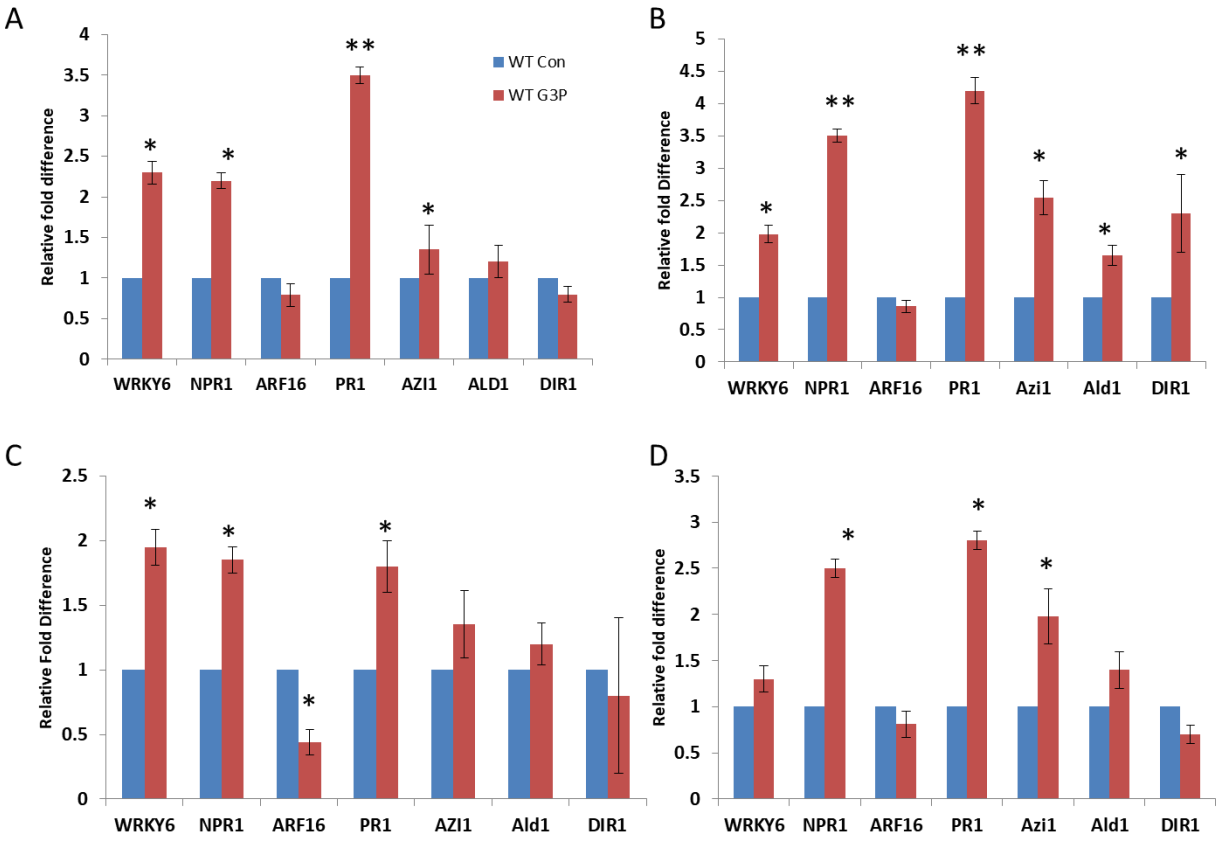


Figure 3.7 Induction of ROS-NO-AzA-G3P pathway upon treatment with G3P in WT plants. qRT-PCR analysis shows G3P induces NPR1 dependent pathway in WT where expression of *StNPR1* and *StPR1* was induced upon G3P treatment at 48 (A) and 72 hpt (B) at local site (A and B) and systemic site (C and D). We also observed increase of genes downstream of G3P, namely AZI1 and ALD1, and they accumulate at 48 hpt local site (A), 72 hpt at local sites (B) and Systemic site (D). Data represents mean ± SD of at least three biological replicates having three technical replicates each. Statistical analysis was carried out with Student's t-test. Asterisks indicate values that were significantly different from the control plants, $P < 0.01$ (**) $P < 0.05$ (*).

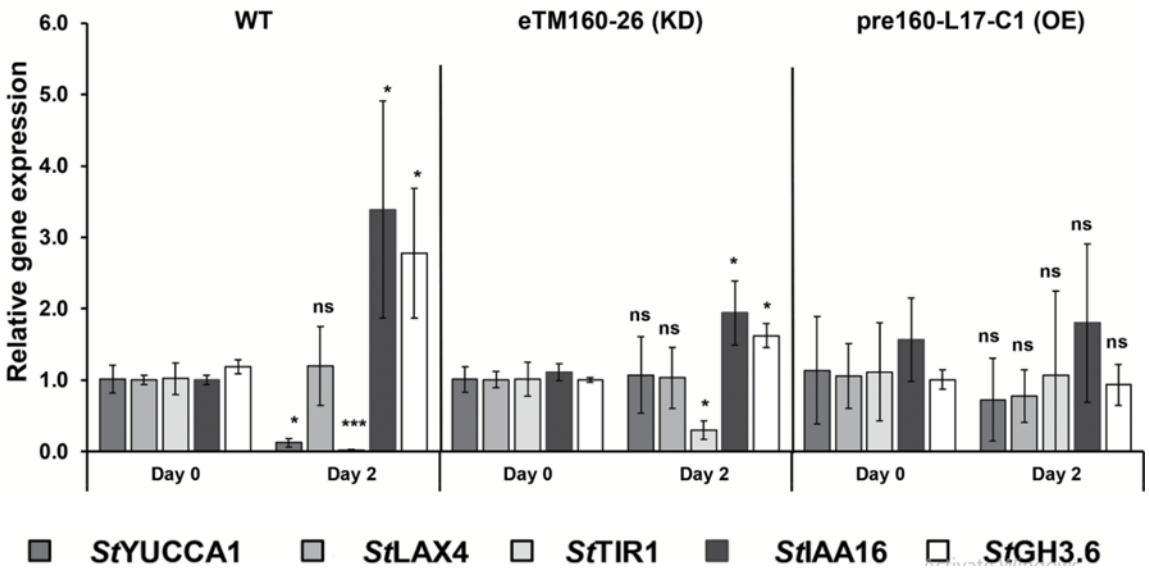
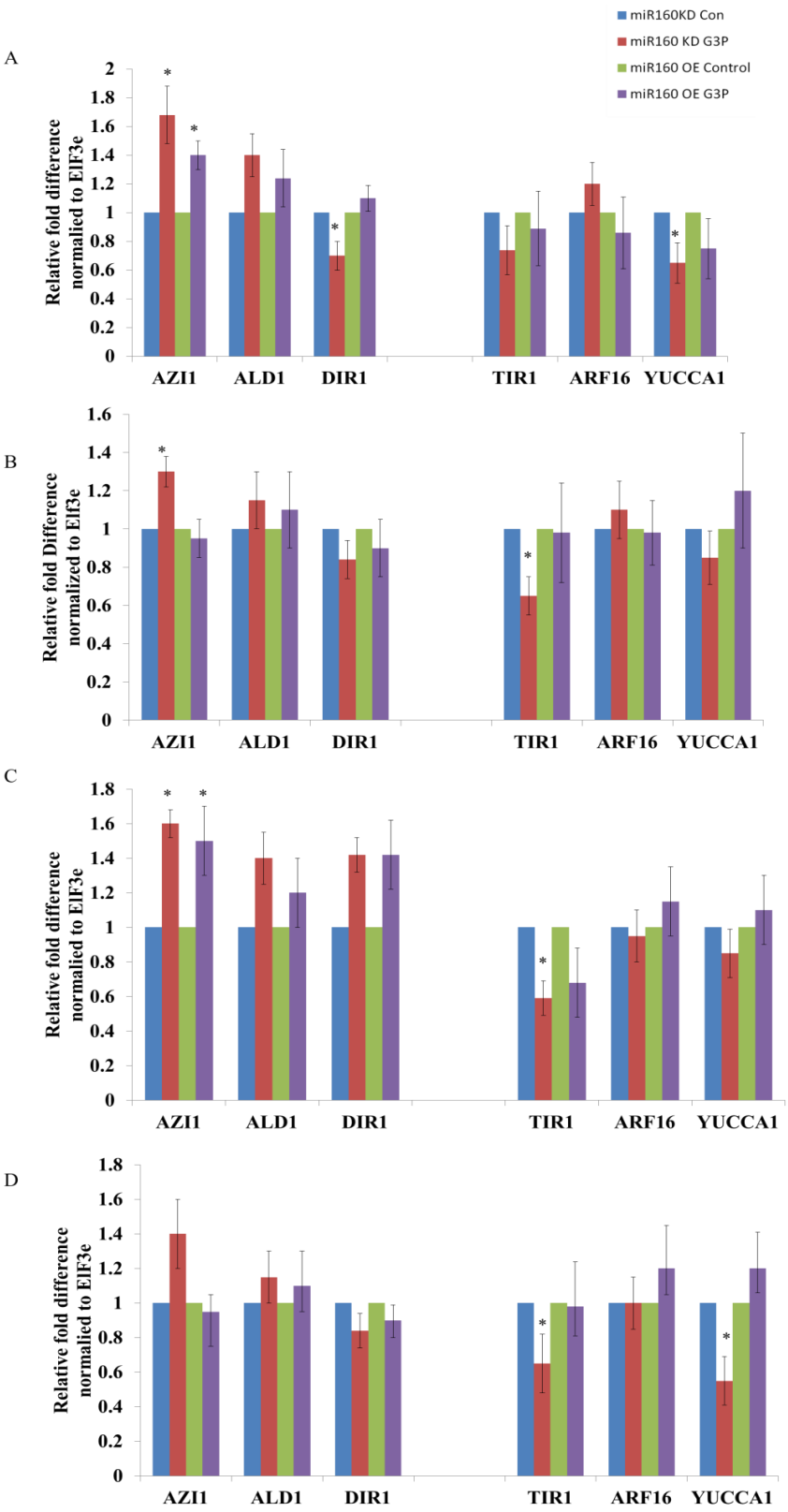


Figure 3.8 Mis-regulation of auxin pathway genes upon infection with *P. infestans*. Expression profile of auxin pathway genes, *StYUCCA1, StLAX4, StTIR1, StIAA16, and StGH3.6,* in WT, miR160 OE and KD plants, upon infection with *P. infestans*. Data represent mean ± SD of at least three biological replicates having three technical replicates each. Statistical analysis was carried out with Student's t-test. Asterisks indicate values that were significantly different from the control plants, P< 0.01 (**) P < 0.05 (*). (Adapted from previously published data Natarajan et al., 2018 *under the Creative Commons Attribution License (CC BY)*. Natarajan and Harpreet contributed equally).


A
miR160KD Con
miR160 KD G3P
miR160 OE Control
miR160 OE G3P
Relative fold difference normalied to EIF3e
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
AZI1
ALD1
DIR1
TIR1
ARF16
YUCCA1
B
Relative fold Difference normalized to EIf3e
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
AZI1
ALD1
DIR1
TIR1
ARF16
YUCCA1
C
Relative fold difference normalied to EIF3e
1.8
1.6
1.4
1.2
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0.6
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0.2
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AZI1
ALD1
DIR1
TIR1
ARF16
YUCCA1
D
Relative fold difference normalied to EIF3e
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
AZI1
ALD1
DIR1
TIR1
ARF16
YUCCA1

Figure 3.9 Dysregulation of ROS-NO-AzA-G3P and Auxin pathway in miR160 KD and OE lines. qRT-PCR analysis shows dysregulation of various genes involved in ROS-NO-AzA-G3P pathway and Auxin genes, in miR160 KD and OE plants at 48 hours (A and C) and 72 hours (B and D) for local site (A-B) and systemic site (C-D) upon treatment with G3P. Data represents mean ± SD of at least three biological replicates having three technical replicates each. Statistical analysis was carried out with Student's t-test. Asterisks indicate values that were significantly different from the control plants, $P < 0.05$ (*). 'ns' indicates difference is non-significant.

3.4. Discussion

3.4.1. Role of G3P in regulation of potato defence

In this study, we established that known SAR elicitors, namely, SA, AzA, G3P, AA, and DA can confer SAR in potato plants (Figure 3.1), similar to that of *Arabidopsis* and tobacco plants. We have also confirmed that AA and SA induced SAR response is dose dependent in potato plants (Figure 3.2), and that the results are similar to previous reports published by Manslova et al., (2010). In Natarajan et al., (2018), authors have reported that, upon infection with *P. infestans,* miR160 expression is induced in the systemic leaves. Authors performed a SAR assay, using *P. infestans* as a primary pathogen, followed by *R. solanarecum* as a secondary pathogen, and showed that miR160 KD lines failed to establish SAR response. We had reconfirmed this result in our study as well (Figure 3.3 and 3.5 A). The study suggested that miR160 KD lines could be deficient in the synthesis of systemic signals upon infection. Thus, we treated the WT and miR160 KD lines of potato with various systemic signal elicitors, SA, G3P, DA, and AzA (Figure 3.1 and 3.3 B). It was observed that except G3P, none of the SAR elicitors could induce SAR response in the miR160 KD transgenic lines (Figure 3.5 B). Previously, it was attributed that the miR160 KD lines were not able to mount SAR response upon treatment with AA because of low accumulation of SA at both local and systemic sites, as compared to WT (Natarajan et al., 2018). The amount of mobile signal, MeSA, was also found to be similar at local site in both the WT and miR160 KD lines but, WT accumulated MeSA in significantly higher amount at later time points in systemic site. These results suggest that the potato plants require an intact NO/ROS-AzA-G3P branch to induce SAR response, upon infection with *P. infestans*. When miR160 KD line was treated with G3P, we observed the establishment of a robust SAR response (Figure 3.5). We treated the miR160 KD lines with different concentrations of G3P, and found that 100µM G3P application resulted in robust SAR development (Figure 3.5). Similar to these results, the Arabidopsis mutant *gly1/gli1*, deficient in synthesis of G3P and hence, accumulation of G3P, could only elicit SAR response upon treatment with exogenous G3P or WT phloem exudate containing G3P (Chanda et. al., 2011). Even the application of AzA, which is known to act upstream to G3P, could not rescue the compromised SAR response in potato miR160 KD lines (Figure 3.4 B). Our findings collectively suggest that the miR160 KD lines are deficient in G3P mediated SAR establishment, and could develop SAR only when G3P was exogenously provided.

3.4.2. G3P biosynthesis is reduced in miR160 KD lines

Since G3P is required to mount a SAR response in miR160 KD lines (Figure 3.4 and 3.5), we tried to understand which genes are involved in SAR regulation. Previous studies have reported the importance of various genes involved in the NO-ROS-AzA-G3P derived SAR response (Shine et al., 2019). It has been reported that AzA triggers the G3P biosynthesis in plants, and if the plant is deficient in G3P synthesis, then plant cannot mount SAR (Wang et. al., 2014). It proves that AzA alone cannot induce SAR but requires G3P to induce SAR response. In Arabidopsis, *gly1/gli1* mutant defective in G3P synthesis is also defective in SAR response and can be restored upon exogenous application of G3P (Chanda et al., 2011). Upon pathogen interaction, G3P induces lipid transport proteins (LTPs), DIR1 and AzI1 accumulation at the site of infection, and induces the synthesis of G3P (Yu et al., 2013). It has been shown that G3P stabilizes DIR1/AZI1 complex and interacts with them to get transported to the systemic sites, through symplastic movement (Lim et al., 2016). Thus, G3P and DIR1/AZI1 works as a positive feedback regulator loop. In our study, we found the expression of *DIR1* and *AZI1* is upregulated in the WT potato plants treated with G3P, when compared to untreated plants (Figure 3.7). In miR160 KD lines, we found that *DIR1* and *AZI1* genes were downregulated, and upon treatment with G3P, their transcript level increases (Figure 3.9). These results show that the miR160 KD lines are defective in G3P synthesis but, its signalling is not affected.

It has been shown that G3P application in SA-deficient plants, *sid2*, does not induce SAR, indicating that G3P requires SA-dependent signaling to mount a successful SAR response (Chanda et al., 2011). Thus, we analyzed the expression profile of *StNPR1*, and its newly found regulator, *StARF16 (Our own findings from Chapter # 2),* in miR160 KD lines, upon treatment with G3P. We found that miR160 KD lines have a high expression of *StARF16*; consistent to the results published previously (Natarajan and Banerjee, 2020) and described in Chapter 2. The transcript levels of *StNPR1* in both WT and miR160 KD lines were found to be lower in untreated plants, but upon G3P treatment, we observed that its level increased. Downstream targets of *NPR1, PR1* was found to be induced upon G3P treatment. These results suggest that upon G3P treatment, NPR1-dependent pathway gets activated and could be essential for mounting a SAR response. Unlike the previous study (Chanda et al., 2011), we show that *NPR1*

is upregulated at 48 hpt with G3P (Figure 3.7) treatment in potato suggesting a potential cross-talk between the two branches of SAR.

Additionally, Hu et al., (2014) have demonstrated that endogenous levels of G3P increase upon glycerol treatment. The increased levels of G3P have been shown to reduce the auxin transport genes *PIN1* and *PIN7* in roots, resulting in shorter primary root length and an increased number of lateral roots. This study suggests that G3P could regulate auxin pathway and, Natarajan et al., (2018) have demonstrated that miR160 KD lines are defective in auxin signaling. Authors reported that the auxin biosynthesis gene, *StYUCCA1*, and auxin signaling gene, *StTIR1*, were found to be significantly reduced in miR160 KD lines. Even upon infection, auxin responsive genes were found to be dysregulated in miR160 KD lines (Natarajan et al., 2018). We analyzed three auxin responsive genes in the current study, namely *StARF16, StYUCCA1* and *StTIR1*, which were shown to be attenuated during *P. infestans* infection in potato. We found that upon G3P treatment, the levels of *StYUCCA1, StARF16, and StTIR1* decreased (Figure 3.9), whose pattern is similar to that of WT (Figure 3.9), suggesting a potential regulation of auxin responsive genes mediated by G3P signaling. Here, we observed that G3P attenuates auxin signaling to mount a defence response in miR160 KD line. Meanwhile, we also analyzed the response of miR160 OE lines after G3P application, and found that the defence response at the local site is induced, as observed from the accumulation of *StNPR1, StAZI1, and StDIR1*; and the decreased levels of *StARF16, StTIR1, and StYUCCA1* (Figure 3.9).

Our findings suggest that upon treatment with *G3P* the miR160 KD lines could elicit SAR response. miR160 OE and KD lines both showed dysregulation of defence response due to mis-regulation in G3P-mediated defence response. We found that G3P application regulates NPR1-dependent defence response, either directly or indirectly, and attenuates auxin signaling to mount an effective defence response.

A manuscript is currently under preparation based on the findings from this study.

Kalsi H. S., & Banerjee A. K. (2022*). MiR160 regulates G3P dependent SAR response in potato (Under preparation).*

Chapter 4

Investigating the role of miR166 in regulating the defence response of potato during *P. infestans* infection

4.1. Introduction

In past decade various studies have shown the importance of microRNA (miRs) in controlling plant development and response to abiotic stress (De Lima et al., 2012). Even though, numerous studies have reported the accumulation of miRs upon infection with pathogen infections, in various plant systems, knowledge about their role towards the defence response is still limited. (Baldrich and San Segundo, 2016; Boccara et al., 2014; Campo et al., 2013; Li et al., 2014; Shivaprasad et al., 2012; Soto-Suárez et al., 2017). Furthermore, the mechanistic understanding of how miRs could regulate the plant pathogen defence is derived from the interactions between Arabidopsis and the bacterial pathogen *Psuedomonas syringae (*Fei et al., 2016; Kuan et al., 2016; Staiger et al., 2013; Weiberg et al., 2014;).

We have demonstrated the role of miR160 in potato local and systemic defence response. In this study, we show that miR166 is involved in plant defence response. The miR166 family comprises multiple members in monocotyledonous and dicotyledonous plants that are transcribed as monoscistronically as well as polycistronically (Salvador-Guirao R et al., 2018). miR166 is a highly conserved family of miRNAs that is known to target homeodomain-leucine zipper Class III (HD-ZIP III) transcription factors. These transcription factors, *REVOLUTA (REV), INCURVATA (ICU), PHABULOSA (PHB) and PHABOLUTA (PHV),* are involved in diverse developmental processes like; leaf development, root development to name a few (Emery et al., 2003; Itoh et al., 2008). miR166 is altered during abiotic stress, suggesting its role in abiotic stress response. Recently, it has been demonstrated that miR166 knockdown triggers drought resistance in rice (Zhang et al., 2018). Also, Salvador-Guirao R et al., (2018) for the first time have established the importance of miR166 in regulating defence response, during *Magnoporthe oryzae* infection in rice. A few other studies have confirmed differential defence response upon infection of *M. oryzae* in various rice varieties (Li et al., 2014, 2016).

4.1.1. Role of miR166 in development and abiotic stress

miR166 has been known to regulate various development process and plays extensive role in leaf development and root architecture. The architectural changes in the roots are driven by miR166. Further, Zhang et al. (2018) provided molecular and genetic evidence that miR166 targets *OsHB4,* that regulates leaf morphology and vascular development. Leaf rolling in drought exposed plants is a result of vascular constriction by miR166 on the xylem. In Acacia, miR166

was reported to regulate HD (Homeo Domain)-ZIP transcript genes for xylem development (Ong et al., 2012). In addition, miR166 has been implicated in the development of leaf polarity by targeting HD-ZIP genes where their expression is differential in abaxial and adaxial regions of leaves (Rubio-Somoza et al., 2011). The miR166 family has a greater tendency to regulate lateral root development under drought stress rather than under drought signalling. Further, miR166 via the posttranscriptional regulation of HD-ZIP contributes to cell development of roots, meristem, and leaves in *M. truncatula* (Boualem et. al., 2008). Liu et al. (2007) reported that HD-ZIP III positively regulates lateral root formation (Hawker et. al., 2004). However, when miR166 levels are elevated, HD-ZIP III is down regulated resulting in a reduction of lateral root formation under drought (Hawker et al., 2004). The knockdown of miRNA166 in rice led to morphological changes associated with drought tolerance, such as leaf rolling and constriction of xylem (Zhang et al., 2018). Overall, miR166 has been depicted to show major morphological changes upon altering miR166 levels either during development or during abiotic stress response.

4.1.2. Role of miR166 in defence response

There are only few recent reports describing the role of miR166 in defence response in rice. MiR166 accumulates at the infection site (Salvador-Guirao R et al., 2018; Wang et al., 2013). These studies indicate that upon infection, rice plants utilize miR166 to mount a PTI response though an EIN2 mediated pathway but it still remains unknown how the complete defence response is initiated (Salvador-Guirao R et al., 2018; Wong et al., 201). During further investigations, Salvador-Guirao R et al., (2018) for the first time showed that miR166-h/k works in a polycistronic manner to mount a defence response against the invading pathogen, and targets genes other than the members of HD ZIP III family. Along with this regulation, authors also reported an upregulation of genes belonging to EIN2-like family through an unknown mechanism resulting in an activation of ethylene dependent pathway, to regulate the plant defence response. This response has been hypothesized to be as a result of post translational repression and thus maintains an optimal level of *EIN2* and *EIN3* in the plant, rather than abolishing the possibility of EIN2 translation by targeting their transcripts. These results show that different miR166 forms have different role in regulating defence genes.

Other than in rice, miR166 was found in the cross-kingdom RNAi, where pathogens utilize sRNAs to help in successful establishment of infection. It has been reported that cotton (*Gossypium hirsutum*) and Arabidopsis roots accumulate miR166, upon infection with *V. dahliae*

(Zhang et al., 2016). Even in soybean, miR166 was observed to be upregulated upon infection with *P. sojae* (Wong et al., 2014). In the same study, they have shown that miR166 expression increases upon treatment with heat-inactivated *P. sojae*. In contrast, miR166 is shown to be down-regulated in response to *C. graminicola* (Balmer et al., 2017). These results indicate a possible role of miR166 in defence response, especially towards the PTI response of the plant.

4.1.3. Objectives

With several reports suggesting the role of miR166 in defence response against the infection with several hemi-biotrophic pathogens (Salvador-Guirao R et al., 2018; Wong et al., 2014), we hypothesised that upon infection with *P. infestans*, miR166 could contribute to potato defence response. Previous study from our lab has shown that upon *P. infestans* infection miR166 accumulates in potato local infected tissue but its role was not studied in potato defence response. Thus we laid down following objectives to understand the role of miR166 in defence response:

1. To investigate the miR166 defence response against *P. infestans* infection.
2. To generate OE and KD lines of miR166 in potato plants.
3. Investigate the plants' systemic defence response against *P. infestans.*

4.2. Material and methods

4.2.1. Plant and pathogen material

The wild-type (WT) and transgenic potato (*Solanum tuberosum* L. cv. Désirée) plants were maintained *in vitro* at 25°C under long day conditions (LD, 16 hours light: 8 hours dark) in tissue culture. Post two weeks, the plants were transferred to soil and were maintained at 22°C under LD conditions in greenhouse for three weeks. On fourth week, the plants were transferred to the environmental plant growth chambers (Percival Scientific) for experimentations.

Oomycete pathogen, *Phytophthora infestans* was maintained on a pea agar at 18°C in petri-dish, and prior to infection the fungal hyphae were scrapped and transferred to sterile water Petri-dish at incubated at 4°C, to induce the release of viable zoospores. Sporangia concentration was then adjusted to $1x10^6$ per ml, and plants were infected with $1x10^4$ spores on the abaxial side of the leaves.

4.2.2. *P. infestans* infection experiment

P. infestans at the concentration of $2x10^5$ sporangia per ml was used and treated plants were incubated at 18°C with 90% humidity. Time-course expression analysis of miRNAs and their targets in WT plants was performed by inoculating 10^4 sporangia on the abaxial side of 5th and 6th leaves (counted from the top of the plant). Inoculated local leaves and non-inoculated systemic leaves (leaf no. 5, 6 and 7) were harvested at 3, 6, 9, 12, 24, 48, 72, 96, and 120 hours post infection (hpi). Tissues were frozen immediately in liquid nitrogen and stored at -80°C until further use.

4.2.3. Quantitative real-time PCR (qRT-PCR) analysis of miR166

To analyse miR160 levels upon *P. infestans* infection, total RNA was isolated from local and systemic leaves harvested at select time points by RNA IsoPlus method. Two microgram (2µg) of total RNA was used for reverse transcription reaction using miRNA stem-loop (STP) primers. All the quantitative RT-PCR (qRT-PCR) reactions were set using the SYBR Green Mix (TAKARA) in Bio-Rad CFX 1000 system. For miRNAs, qRT-PCR was carried out using miRNA specific forward and universal reverse primers, and reactions were carried out at 95°C for 5 min followed by 40 cycles of 95°C for 5 sec, 60°C for 10 sec, and 68°C for 8 sec. For normalization, U6

was used and, qRT-PCR was performed using same miRNA, cDNA and gene-specific primers (U6-FP and U6-RP) with PCR conditions as 95°C for 5 min followed by 40 cycles of 95°C for 5 sec, 60°C for 10 sec, and 68°C for 8 sec. Melting curve analysis was included in the program to check PCR specificity and data was analysed by using $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

4.2.4. Cleavage site mapping analysis of miR166 targets

For prediction of miR166 targets, psRNATarget (plantgrn.noble.org/psRNATarget/) (Dai and Zhao, 2011) was used. For all the target predictions, *Solanum tuberosum* transcript library from the Potato Genome Sequencing Consortium (PGSC) (http://solanaceae.plantbiology.msu.edu/pgsc_downloads.html) was used as target database and default parameters were used.

For in planta validations of miR166 targets, a modified 5'-RNA ligase mediated rapid amplification of cDNA ends (RLM RACE) technique was performed using First Choice RLM-RACE kit (Ambion). RNA adapter ligation was performed using 3 to 4 µg of total RNA without any enzymatic pre-treatment. cDNA was prepared using gene specific reverse primers (ICU1-RP1, ICU2-RP1, REV-RP1, PHV1-RP1, and PHV2-RP1) with Superscript III Reverse Transcriptase (Invitrogen). A primary PCR was performed using adaptor specific outer primer and gene specific reverse primers (same as the primers used for cDNA preparation), followed by a secondary PCR using adaptor specific inner primer and a second gene-specific reverse primers (ICU1-RP2, ICU2-RP2, REV-RP2, PHV1-RP2, and PHV2-RP2). The amplicons were cloned in the sub-cloning vector pGEM-T Easy (Promega) and sequence verified to identify the miRNA cleavage sites.

4.2.5. Construct design and plant transformation

miR160 overexpression (OE) construct, 35S::St-pre166-pBI121, was generated by amplifying miR160 precursor (St-pre166-c), from potato RNA using primers Pre166-FP and Pre166-RP. The amplified product (129 bp) was finally cloned into binary vector pBI121 under 35S CaMV constitutive promoter. For knockdown (KD) construct, we used artificial target mimicry (MIM) where KD construct was obtained from European Arabidopsis Stock Centre (NASC) (Todesco et al., 2010). The MIM166 insert (542 bp) which was originally cloned into pGREEN vector, was re-cloned to pBI121 binary vector (to generate the construct 35S::MIM166-pBI121) and used for plant transformation.

For generation of miR166 resistant *StICU1,* we amplified *StICU1* from potato RNA using Oligo(dT) cDNA with specific primers. We sub-cloned the amplified product in pGEM-T-Easy vector (Promega) and sequence verified the construct. After that we amplified the whole sub-cloned vector using 5'mStICU1-FP and 5'mStICU1-RP containing site directed silent mutations and then treated the samples with *DpnI.* At this staged we verified *5'mStICU1*:*pGEM-T* construct through sequence analysis and then transferred the *5'mStICU1* insert (2.7KB) to pBI121 to generate 35S::5'mStICU1-pBI121 and used it for plant transformation.

All potato transformations were performed as described previously (Banerjee et al., 2006). The transgenic plants were raised in vitro and maintained under the selection of kanamycin (50 mg/l) for St-pre166 and MIM166. Transgenic lines were confirmed by performing gene specific PCR of pre166 (pre166-FP & NosT-RP) and MIM166 (MIM-FP and NosT-RP), respectively using the genomic DNA as template.

4.2.6. Systemic Acquired Resistance assay

Four-week old potato (WT, miR166 OE and miR166 KD) plants were given a primary infection with *P. infestans* followed by secondary infection with *Ralstonia solanacearum*. For primary infection, $1x10^4$ spores of *P. infestans* was swabbed on the abaxial side of the 5th and 6th mature leaf from the apex, and plants were incubated at 18°C for four days. On fifth day, 2nd and 3rd mature leaves from the top were infiltrated with 1x106 C.F.U. per ml (OD600 = 0.1) of *R. solanacearum* were incubated in growth chamber at 28°C for secondary infection. After five days of secondary infection one sq.cm leaf piece was removed from *R. solanacearum* infected leaves and crushed in sterile water. The sample was serially diluted and plated on nutrient agar medium and bacterial count was recorded. Bacterial population from the sampled leaves were confirmed by performing *R. solanacearum* specific PCR primers Rs_BP4R and Rs_BP4L as mentioned previously in Lee and Wang, 2000. Local (*P. infestans* treated) and systemic (non-infected) leaves were collected at the end of the experiment, and flash frozen in liquid nitrogen until further use.

4.2.7. RNA isolation and cDNA preparation

Total RNA was isolated using RNAIsoPlus (TAKARA) from the infected and control leaf samples of WT, miR166 OE and miR166 KD potato plants as per manufacturer's protocol. Two

microgram (2 µg) of total RNA was used for cDNAs preparation using oligo(dT) primers with MMLV Reverse Transcriptase (Promega) following manufacturer's instructions.

4.2.8. Quantitative real-time (RT-PCR) analysis

For analysis of transgenic lines, we selected, *StNPR1*, *StAZI1*, *StALD1*, *StDIR1*, and *StPR1* were selected; whereas *StTIR1*, *StYUCCA1*, and *StARF16* were selected for analysis of auxin pathway genes. For normalization, *SteIF3e* gene was used. qRT-PCRs were carried out using SYBR green mix (Takara). The reaction conditions for *StICU1, StICU2, StPHV1, StREV, StPHV2, StPR1* were 95°C for min, 40 cycles of 95°C 15 sec and 60°C 20 sec and for *StNPR1,* 40 cycles of 95°C 15sec, followed by 40 cycles of 95°C 15 sec, 50°C 15 sec and 68°C 20 sec. Melting curve analysis was included in all the programs to check the PCR specificity and the data was analysed using 2^{-ddCt} method (Livak and Schmittgen, 2001).

4.2.9. Accession number

Table 4.1 Accession numbers

Name	Accession	Source
StPR1	AY050221	NCBI
StNPR1	XM_006357647	NCBI
StICU1	PGSC0003DMT400068037	PGSC
StICU2	PGSC0003DMT400006735	PGSC
StPHV1	PGSC0003DMT400030829	PGSC
StPHV2	PGSC0003DMT400054421	PGSC
StREV	PGSC0003DMT400020801	PGSC
U6	*X60506*	NCBI

4.2.10. Primer Details

Table 4.2 List of primers

Primer Name	Sequence (bp; 5'- 3')

ElF3e qFP	GGAGCACAGGAGAAGATGAAGGAG
ElF3e qRP	CGTTGGTGAATGCGGCAGTAGG
NPR1_FP	AAGAGGCTCACTAGGCTT
NPR1_RP	GCTTCATACGCAAATCATCG
PR1_FP	GTACCAACCAATGTGCAAGCG
PR1_RP	TGTCCGACCCAGTTTCCAAC
StICU1_FP	AAAGGCTACTGGAACTGCTGT
StICU1_RP2	CAACCCTCGTTGGATCTAAACCAA
StICU1_RP1	AACAGCCCGGCAGTCACGATA
StICU2_FP	AAAGGCTACTGGAACTGCTGTT
StICU2_RP2	GACCCTCGTTGGCTCTAGACC
StICU2_RP1	AACCCGGCAGTCACGATACC
StPHV1_FP	GAAAGGCTACTGGAACTGCTGT
StPHV1_RP2	CAACCTTCGTGGGCTCTAGAC
StPHV1_RP1	AAGGCAACGGCAGTCGCGATA
StPHV2_FP	GCTACTGGAACTGCTGTCGACTG
StPHV2_RP2	CTCAGCAACCTTCATGGGCTCT
StPHV2_RP1	ATCAAGGCAACGGCAATCGC
StREV_FP	GGCTACAGGAACTGCTGTCGATT
StREV_RP2	TAACAAGACCACATGCTCGGGC
StREV_RP1	TCAACATTCCGGCAGTCCCG
miR166 STP	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGGGAAT
miR Universal RP	AGTGCAGGGTCCGAGGT
miR166_FP	TGGAGGTTCGGACCAGGCTTC
Pre166_FP	GAGATCTAGAGAGAGAGACATGTAATATTGTTGA
Pre166_RP	GAGAGAGCTCGTTGAGGGGAATGAAGCC
MIM-FP	GAGACCCGGGAAAACACCACAAAAACAAAAGA
MIM-RP	GAGAGAGCTCAAGAGGAATTCACTATAAAGAG
NosT-RP	GCAACAGGATTCAATCTTAAG
5'mStICU1_FP	GAGTGGGTCCAAATGCCTGGtATGAAaCCgGGcCCcGATTCCATTGGAATCATT GCTATTTCTCATGG

5’mStICU1_RP	CCATGAGAAATAGCAATGATTCCAATGGAATCgGGgCCcGGtTTCATaCCAGGC ATTTGGACCCACTC
StICU1full FP	AAAAAAGGATCCTGTCAATGTCCTGCAAGG
StICU2full_RP	AAAAAAGAGCTGTTAAACAAACGACCAATTCACA

4.3. Results

4.3.1. MiR166 accumulates in both local and systemic leaves post infection

To understand the role of miR166 in potato defence responses, infection experiment was carried out with WT Desiree plants and samples were harvested at early time points (viz 3, 6, 9 and 12 hours post infection (hpi)), and later time points (viz. 24, 48, 72, 98, and 120 hpi). The expression levels for miR166 were analysed by quantitative real time PCR (Figure 4.1). In local leaves, we observe that the plants accumulated miR166 as early as 3 hpi and later showed a dynamic decrease in expression profile till 12 hpi, after which the expression of miR166 at later time points reverted back to the control level as infection progressed (Figure 4.1 A and D). This expression profile of miR166 suggests that it could contribute to early plant defence response, upon infection with *P. infestans* in the local leaves. Alongside, we also analysed the expression profile of *StNPR1* and *StPR1* at the local site after infection and found them to be significantly accumulated as early as 12 hpi (Figure 4.1 C), and as the infection progressed the levels of these genes increased significantly (Figure 4.1 D). Additionally, in systemic leaves, miR166 levels increased greatly at 6 hpi in the WT plants (Figure 4.2) and then as the infection progressed, miR166 accumulated during early infection but at later time points levels reverted to control levels. These results show us that upon infection of potato plants with *P*. *infestans*, miR166 accumulates significantly at both local and systemic site during early infection time points.

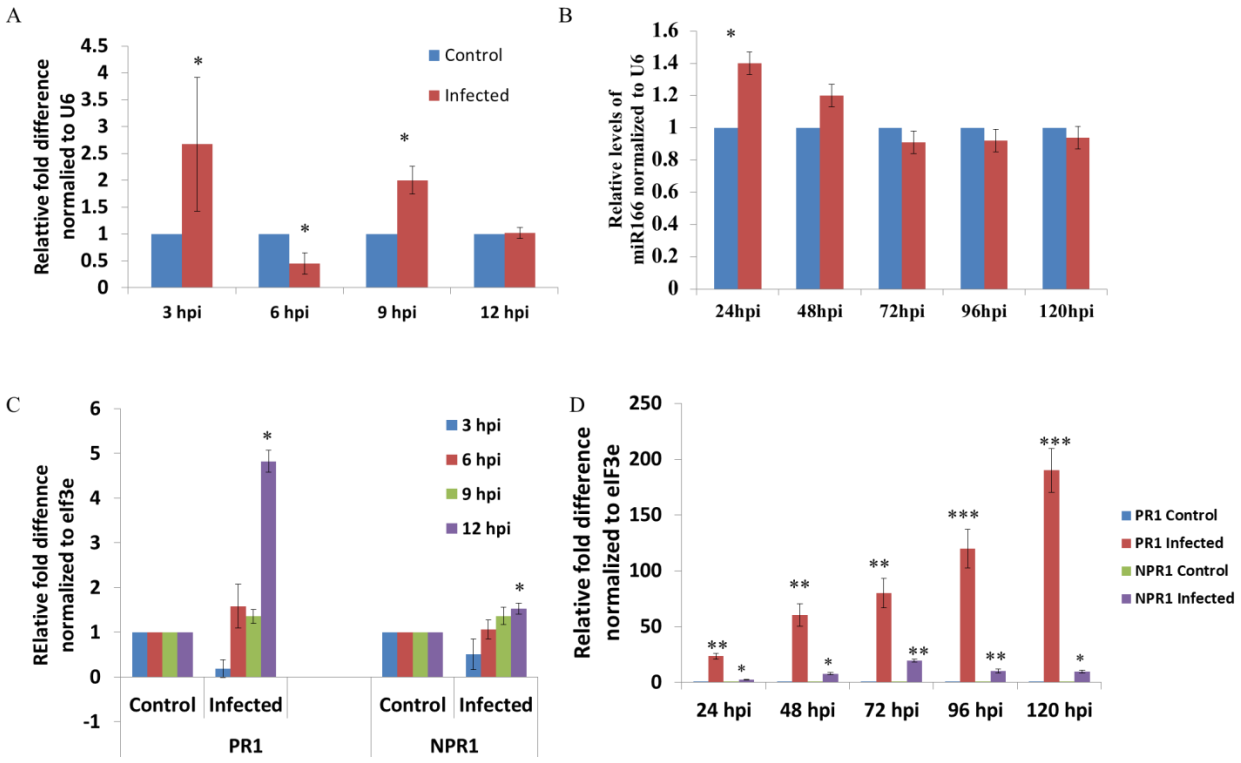


Figure 4.1 Expression analysis of miR166 upon infection with *P. infestans* at local infection site. Expression profile of miR166 upon infection with *P. infestans,* at time points 3, 6, 9, and 12 hpi (A) and at 24, 48, 72, 96, and 120 hpi (B) at local site of infection. Defence response gene *StNPR1* (C) *and PR1* (D) was analysed at 3, 6, 9, and 12 hpi and at 24, 48, 72, 96, and 120 hpi. Data represents mean ± SD of at least three biological replicates having three technical replicates each. Statistical analysis was carried out with Student's t-test. Asterisks indicate values that were significantly different from the control plants, P< 0.01 (**) P < 0.05 (*).

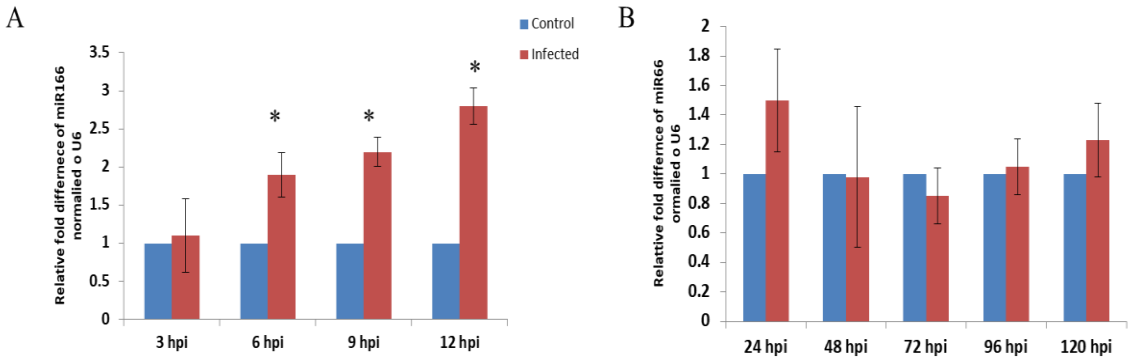


Figure 4.2 Expression analysis of miR166 upon infection with *P. infestans*. Expression profile of miR166 upon infection with *P. infestans,* at time points 3, 6, 9, and 12 hpi (A) and at 24, 48, 72, 96, and 120 hpi (B) at systemic site of infection. Data represents mean ± SD of at least three biological replicates having three technical replicates each. Statistical analysis was carried out with Student's t-test. Asterisks indicate values that were significantly different from the control plants, P< 0.01 (**) P < 0.05 (*).

4.3.2. Prediction and confirmation of miR166 target genes

As previously mentioned, miR166 is known to target HD ZIP III class members in various plant species. We used *in silico* target prediction software psRNATarget to find five putative transcript targets of miR166 in potato (Table 4.3). We have detected all five miR166 targets in WT potato plants and using cleavage site mapping assay confirmed them to be cleaved by miR166 (Figure 4.3). Thereafter, we analysed the expression profiles of St*ICU1*, St*ICU2*, St*REV,* St*PHV1* and St*PHV2* and investigated their role in potato's defence response.

Table 4.3 List of predicted targets of miR166. Targets of miR166 predicted using psRNATarget.

PGSC ID	**Description of the gene**	**Expectation value from psRNATarget**
PGSC0003DMT400068037	DNA binding protein Incurvata-like StICU1	1.5
PGSC0003DMT400006735	DNA binding protein Incurvata like StICU2	1.5
PGSC0003DMT400030829	PHAVOLUTA-like HD-ZIPIII protein StPHV1	2
PGSC0003DMT400054421	PHAVOLUTA-like HD-ZIPIII protein StPHV2	2
PGSC0003DMT400020801	StREV HD-ZipIII	2

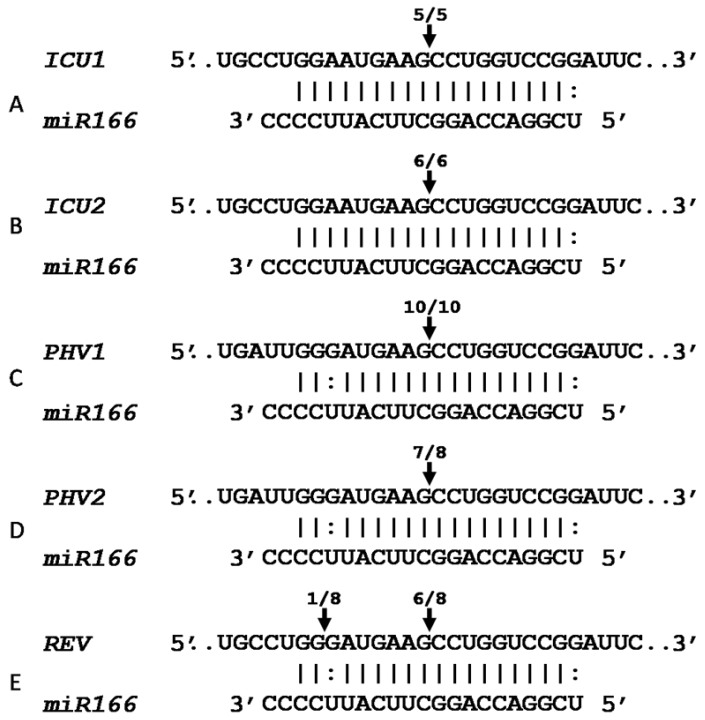


Figure 4.3 miR166 cleavage site mapping analysis. *In planta* confirmation of *StICU1, StICU2, StPHV1, StPHV2, and StREV* as true targets of miR66 in potato. Partial mRNA sequence *StICU1, StICU2, StPHV1, StPHV2, and StREV (A-E)* of aligned with miR166 and numbers denote the fraction of cloned cleavage products that terminates at different positions (arrows).

4.3.3. MiR166 regulates its targets upon infection

To investigate how miR66 could be regulating defence response we analyzed the expression profile of all the five miR66 targets in WT plants. It was observed that miR166 showed negative correlation with all of its five targets at 3 hpi at local site but as the infection progresses only *ICU1*, *ICU2* and *REV* maintained this negative correlation, whereas *PHV1* and *PHV2* maintained the negative correlation only at 3, and 9 hpi for local leaves (Figure 4.4 and 4.1). As we did not see consistent negative correlation of PHV1 and PHV2 at local site, we observed that even at systemic sites, we do not observe any significant changes (Figure 4.2 and Figure 4.5). Whereas we observed that *ICU1, ICU2 and REV* transcript levels decreased post infection at 6, 9 and 12 hpi (Figure 4.5). As the infection progressed, we did not see any significant alteration in the target levels of miR166. These results suggest that miR166 is involved in the early defence response against *P. infestans.*

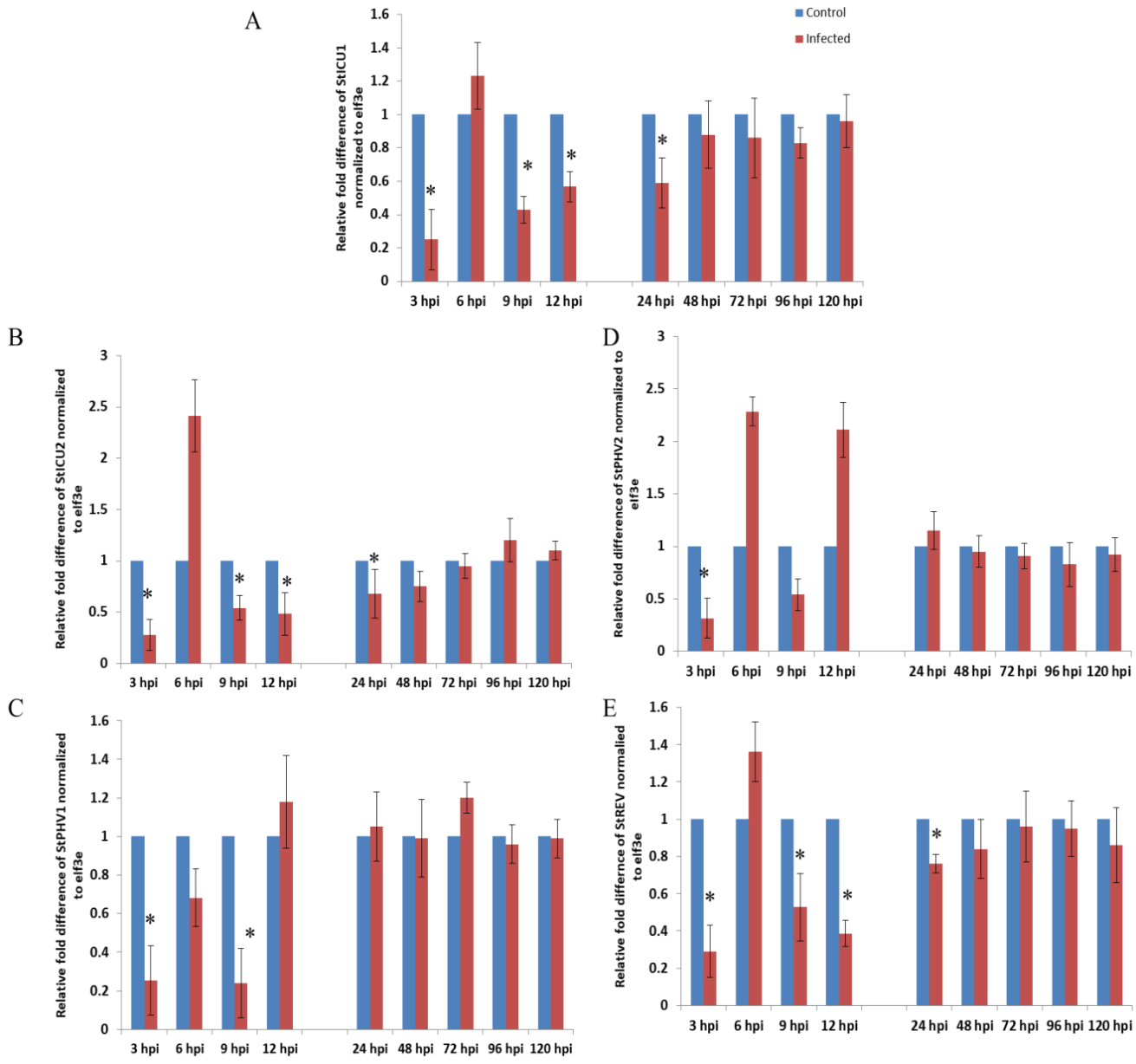


Figure 4.4 Differential expression of miR166 targets at local site upon infection with *P. infestans.* A-E expression profile of miR166 targets *StICU1* (A), *StICU2* (B), *StPHV1* (C), *StPHV2* (D), and *StREV* (E) at time points from 3 to 120 hpi in WT potato plants. All the data from qRT-PCR analysis are plotted as mean ± standard deviation of three biological replicates with three technical replicates each. Asterisk indicate statistical significance where * is $p<0.05$ and ** is $p<0.01$ as per Student's t-test.

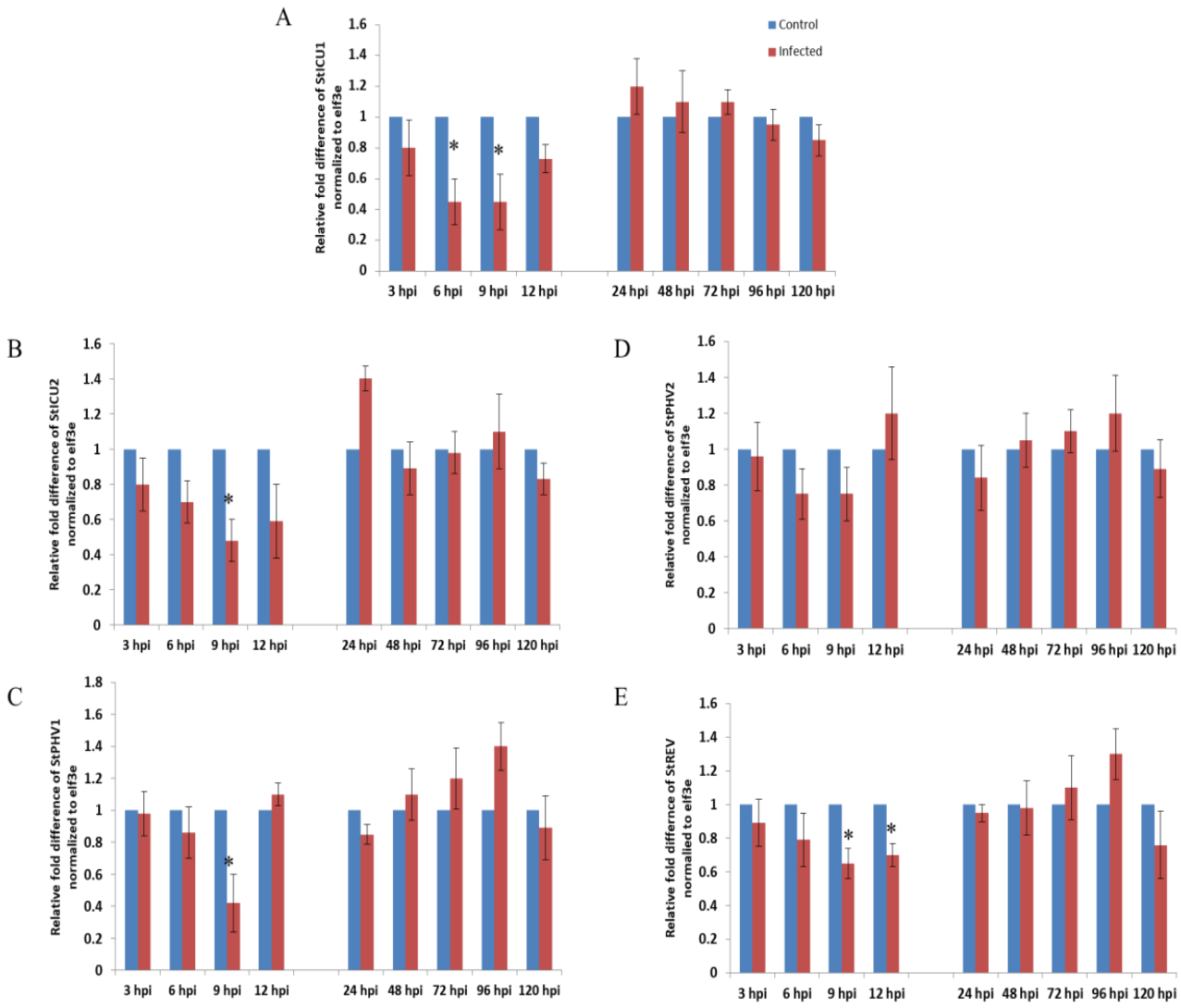


Figure 4.5 Differential expression of miR166 targets at systemic site upon infection with *P. infestans.* A-E expression profile of miR166 targets *StICU1* (A) *StICU2* (B), *StPHV1* (C), *StPHV2* (D), and *StREV* (E) at time points from 3 to 120 hpi in WT potato plants. All the data from qRT-PCR analysis are plotted as mean ± standard deviation of three biological replicates with three technical replicates each. Asterisk indicate statistical significance where * is $P < 0.05$ and ** is $P < 0.01$ as per Student's t-test.

4.3.4. MiR166 accumulates upon treatment with *P. infestans* PAMP treatment

As we observed that miR166 levels increased upon treatment with *P. infestans* at early time points, we explored the possibility of miR166 to be involved in the PTI response of the plant. We then treated the plants with *P. infestans* elicitor Arachidonic acid (AA) and found that

miR166 levels start accumulating within 90 minutes post treatment (Figure 4.6). Alongside, we even analysed the expression profile of miR166 targets *ICU1, ICU2 and REV* upon treatment with AA and found them to have strong negative correlation with the increasing miR166 levels (Figure 4.6). From these results, we concluded that both *P. infestans* and its elicitor upregulates miR166 levels, which suggests that miR166 is involve in the PTI response of the plant.

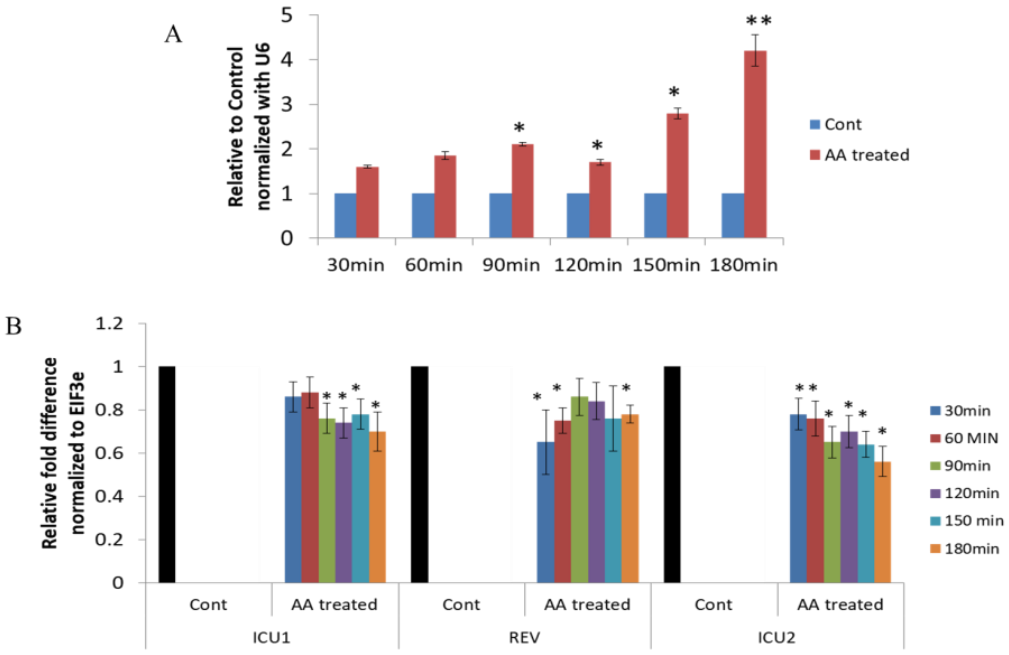


Figure 4.6 miR166 accumulates upon treatment with AA in WT plants. (A) miR166 expression upon treatment with AA at 30, 60, 90, 12, 150 and 180 minutes post treatment in WT potato plants. (B) Expression analysis of miR166 targets *StICU1, StICU2, and StREV* at the local site upon treatment with AA. Data represents mean ± SD of at least three biological replicates having three technical replicates each. Statistical analysis was carried out with Student's t-test. Asterisks indicate values that were significantly different from the control plants, P< 0.01 (**) P < 0.05 (*).

4.3.5. Generation of miR166 overexpression and knockdown transgenic lines

To decipher the role of miR166 during potato-*P. infestans* interaction, we generated OE and KD transgenic lines in potato using the constructs represented in Figure 4.7. The miR166 OE and KD transgenic lines were confirmed through PCR from cDNA using primers specific to pre166 and MIM166 constructs, respectively (Figure 4.8). Based on the expression patterns of

miR166 in all the confirmed miR166 OE, we selected L32 and L62 for further investigation from ten individual lines (Figure 4.8). Similarly from six independent KD lines L1-4 and L3-1 were selected for further investigations (Figure 4.8). Selected OE and KD lines were further validated by looking at miR166 targets expression profiles (Figure 4.8).

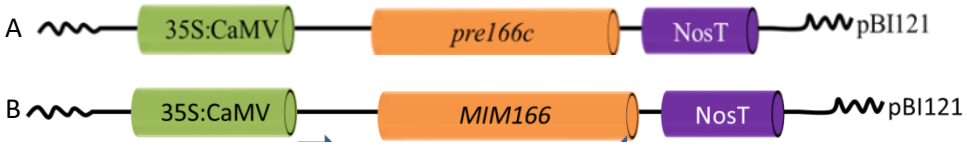
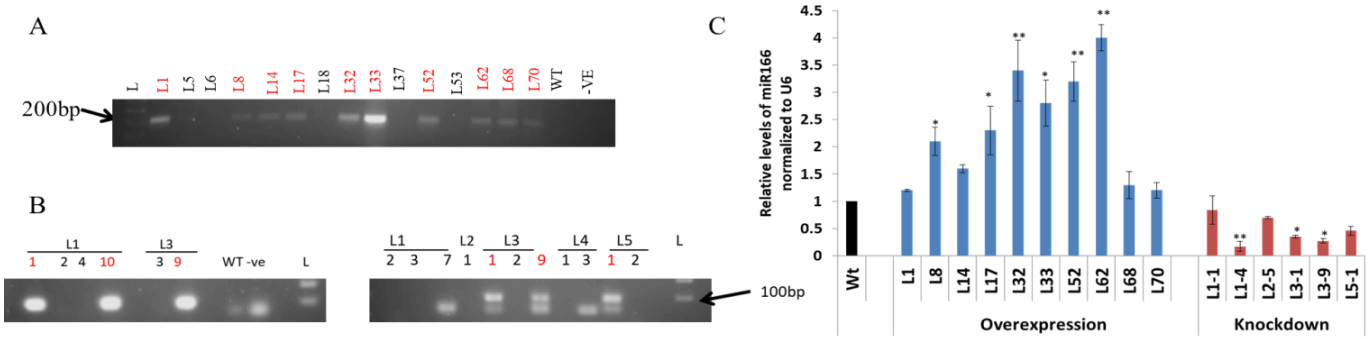


Figure 4.7 Constructs used in generation of miR166 OE and KD lines. (A) Shows the construct used in generation of miR166 OE expression using pre166c from potato. (B) Diagrammatic representation of miR166 KD construct used to generate miR166 KD lines in potato.



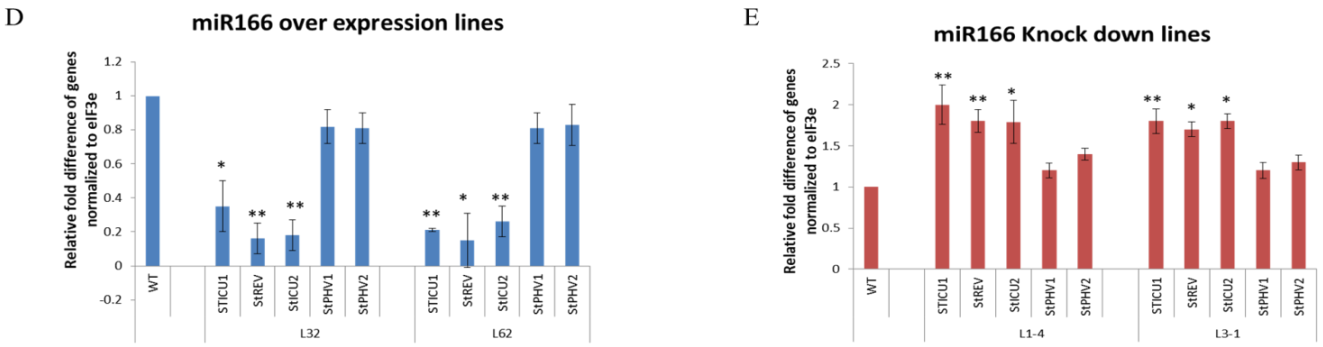


Figure 4.8 Confirmation of miR166 OE and KD lines in potato. (A) Shows PCR confirmation fo miR166 OE lines using Pre166-FP and NosT primers. (B) Shows PCR confirmation of miR166 KD lines using MIM166 primers**.** (C) Levels of miR166 in OE and KD lines analysed by qRT-PCR. (B-C) Levels of *StICU1, StICU2, StPHV1, StPHV2* and *StREV* in OE (B) and KD (C) as analysed by qRT-PCR. All the data from qRT-PCR analysis are plotted as mean ± standard deviation of three biological

replicates with three technical replicates each. Asterisk indicate statistical significance where * is $p<0.05$ and ** is $p<0.01$ as per Student's t-test.

4.3.6. Overexpression and knockdown of miR166 does not alter plant morphology

As miR166 is known to play role in several plant development processes, we examined miR166 OE and KD lines for any morphological changes. We observed no major changes in the any of the miR166 OE and KD lines of potato (Figure 4.9). miR166 is essential in leaf patterning, however we did not observe any drastic changes in leaf development of potato plants which is contrary to the established role of miR166.

4.3.7. MiR166 KD lines are SAR deficient

As miR166 is induced at both local and systemic sites upon infection with *P. infestans,* we wanted to understand if miR166 plays any role in SAR response. Using *P. infestans* as primary pathogen and *Ralstonia solanacearum* as secondary pathogen, we carried out a SAR assay in the transgenic lines of potato. We observed that the plants of miR166 OE lines and WT plants were able to mount a SAR response in response to SAR assay. But, miR166 KD lines were found to be deficient in establishing SAR response (Figure 4.10). Overall, our SAR analysis revealed that miR166 KD lines of potato were compromised in mounting an effective SAR response.

4.3.8. Overexpression of StICU1, miR166 target, leads to compromised SAR

To understand miR166 mode of action in miR166 KD lines which were deficient in SAR response, we focused our study towards StICU1. *StICU1* was found to play significant role in plant defence response as it consistently showed negative correlation with the miR166 expression. We aimed to generate transgenic lines overexpressing the miR166 resistant *StICU1* in potato. We generated miR166 resistant *StICU1 (5'mStICU1),* using site direct silent mutagenesis to overexpresses *StICU1* in potato plants. (Figure 4.11). We generated three transgenic lines, and based on its expression profile of *StICU1,* we confirmed these transgenic lines in potato. *StICU1* appeared to be highly expressing in the three transgenic lines; L32-1, L56-1 and L5-2 (Figure 4.11). We performed a SAR assay for all these three transgenic lines, and found that these lines were unable to mount a successful response to the primary infection of *P. infestans,* and secondary infection of *Ralstonia solanacearum* (Figure 4.12).

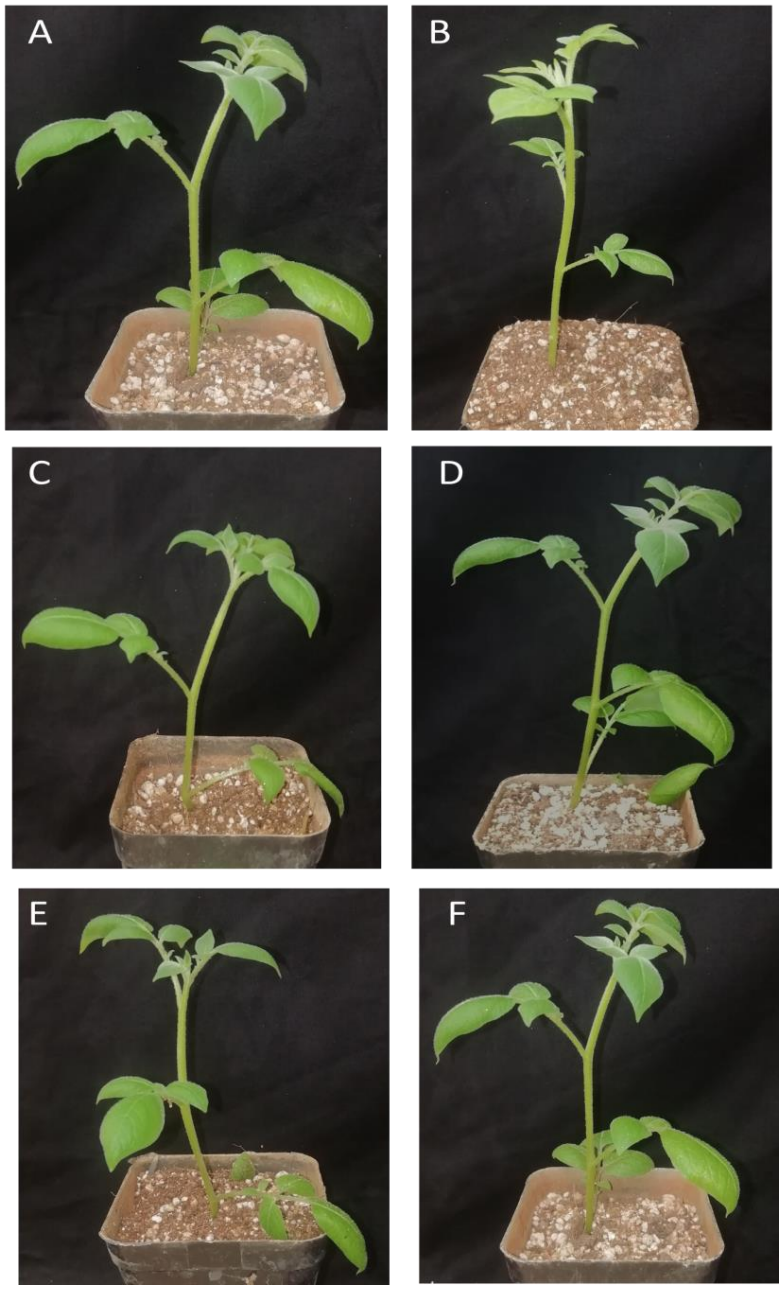


Figure 4.9 Morphological phenotype of miR166 OE and KD lines in potato. Morphology of the potato plants was not affected in miR166 OE (C (L32) and D (L62)) and D) and miR166 KD (E (L1-4) and F (L3-1)) and was comparable to Wild Type and vector control.

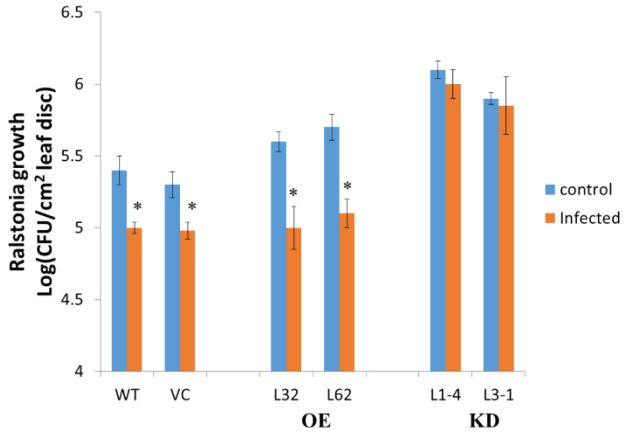


Figure 4.10 Knockdown of miR66 in potato leads to compromised SAR response. Transgenic lines of miR166 were subjected to SAR assay. Upon infection with *P. infestans* for fours days at 18°C followed by a secondary infection on distal site with *R. solanacearum* incubated at 28°C for five days. miR160 OE lines shows a robust SAR response. (lines do not show SAR response upon infection with *P. infestans* for fours days at 18°C followed by a secondary infection on distal site with *R. solanacearum* incubated at 28°C for five days. miR160 OE lines shows a robust SAR response. Data represents mean ± SD of at least three biological replicates having three technical replicates each. Statistical analysis was carried out with Student's t-test. Asterisks indicate values that were significantly different from the control plants, P < 0.05 (*).

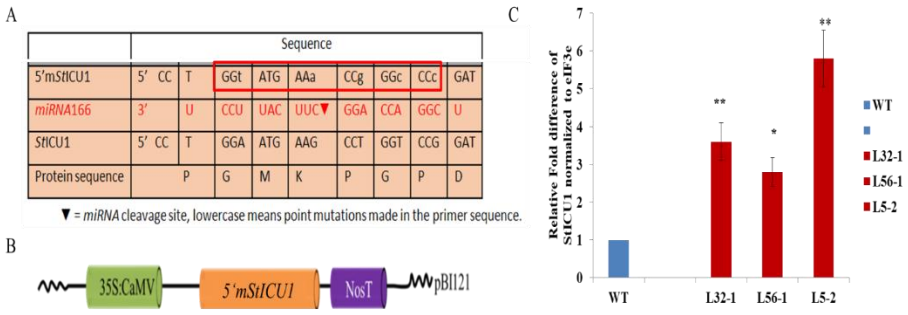


Figure 4.11 Confirmation of Transgenic lines over expressing miR166 resistant StICU1 in potato plants. (A) partial sequence of StICU11, showing the silent mutations made in the in the primers to amplify 5;'mStICU1, miR166 resistant form of StICU1. (B) Diagrammatic representation of StICU1 construct generate transgenic lines in potato. (C) qRT-PCR analysis of SttICU1 in the transgenic lines generated in the potato. qRT-PCR analysis is plotted as mean ± standard deviation of three biological replicates with three technical replicates each. Asterisk indicate statistical significance where * is p<0.05 and ** is p<0.01

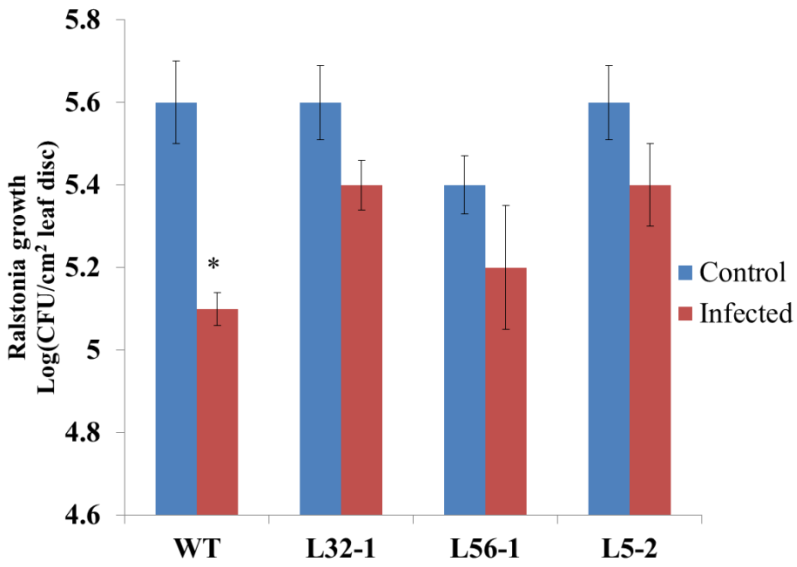


Figure 4.12 Overexpression of *StICU1* in potato leads to compromised SAR response. *5'mStICU1* lines overexpression miR66 resistant StICU1 lines do not shows SAR response upon infection with *P. infestans* for fours days at 18°C, followed by a secondary infection on distal site with *R. solanacearum* incubated at 28°C for five days. MiR160 OE lines show a robust SAR response. (lines do not shows SAR response, upon infection with *P. infestans,* for four days at 18°C, followed by a secondary infection on distal site with *R. solanacearum* incubated at 28°C for five days. miR160 OE lines shows a robust SAR response). Data represents mean ± SD of at least three biological replicates having three technical replicates each. Statistical analysis was carried out with Student's t-test. Asterisks indicate values that were significantly different from the control plants, $P < 0.05$ (*).

Our findings suggest that miR166 contributes in potato PTI, and systemic defense responses during *P. infestans* infection. These results are similar to rice where upon infection with *M. oryzae*, rice plants accumulate miR166 early to mount a PTI response in plants. But, our study demonstatres the involvement of miR166 in SAR response of potato.

4.4. Discussion

4.4.1. MiR166 accumulates upon infection of *P. infestans*

Though miRNAs have been shown to play critical role in variety of plant – pathogen interactions (Ruiz-Ferrer and Voinnet, 2009; Sunkar et al., 2012; Seo et al., 2013), their role in potato-*Phytophthora* interaction have still remain unexplored. Previous study from our lab, is one of the few studies that attempts to understand the role of miRs, miR160, in defence response of the potato plants against *P. infestans* (Natarajan et. al., 2018). In this study it was demonstrated that miR160 was necessary to mount a successful local and systemic defence response.

In this study, we carried out expression analysis of miR166 at early and late infection stages. We observed an induction of miR166 in the infected leaves (Figure 4.1), similar to the previous studies carried out in rice and soybean (Salvador-Guirao R et al., 2018; Wang et al., 2013). These studies were in a contrast to miR166 response to *C. graminicola* in maize, where upon infection, miR166 has been shown to be down-regulated (Balmer et al., 2017). These studies have shown induction of miR166 during different plant pathogen interaction, but these studies are limited to analysing the defence response at the site of infection. Natarajan et al., (2018), have carried out an expression profile of miR160 at the systemic non-infected site and found that upon infection miR160 accumulates at systemic site as well. In a similar analysis, we observed the induction of miR166 at the systemic site as early as 6 hpi (Figure 4.2), miR166 was found to accumulate significantly up to 12 hpi (Figure 4.2). Additionally, we analysed the expression profile of StNPR1 and StPR1 at the local site of infection. We found that during early time points of infection, especially at 3 hpi, there is a decrease of StNPR1 and StPR1 compared to control plants, even though insignificant in our study but could be biologically significant for *P. infestans* colonization (Figure 4.2 C). As the infection progressed to later time points, *StNPR1* and St*PR1 transcripts* levels were found to be significantly induced (Figure 4.2 D). These results point towards a potential role of miR166 in defence response at both local and systemic site, upon infection with the *P. infestans.*

MiR166 targets are well studied for their role in development processes. We found that miR166 in potato also targets HD ZIP-III class family members, namely *StICU1, StICU2, StPHV1, StPHV2, and StREV* (Emery et al., 2003; Itoh et al., 2008). Transcription regulation analysis of

miR166 through cleavage site mapping, indicated that all the predicted targets are cleaved by miR166 (Figure 4.3). We further carried out expression profile analysis of each of the targets at both local and systemic site of infection, and found that their expression profiles exhibit negative correlation with miR166 at 3 hpi. As the infection progressed, we observed that miR166 targets *StICU1, StICU2, and StREV*, all showed negative correlation with miR166. Interestingly, both *StPHV1 and StPHV2,* fails to show negative correlation at later time points of infection (Figure 4.4 and 4.5). In rice, it was shown that miR166 targets EIN family genes and upregulated EIN2.3 and EIN2.4 but suppressed its target EIN2.1 and EIN2.2 (Salvador-Guirao R et al., 2018). These results suggest that even though miR166 has a strict regulation over S*tICU1, StICU2 and StREV*, other two targets that are not strictly regulated could be responsible for activation of another branch to induce a miR166 mediated response. Thus, this study shows that miR166 and its target might be involved in a complex mechanism to establish local and systemic defence response in potato plants upon infection with *P. infestans.*

4.4.2. miR166 is involved in the PTI response of potato defence response

In a number of reports, miR166 has been shown to be upregulated upon infection *with V. dahliae* (Zhang et al., 2016) in Arabidopsis and cotton, and with heat inactivated *P. sojae* in soybean (Wong et al., 2014). Interestingly, it was observed that in response to *C. graminicola* in maize, miR166 was found to be down-regulated (Balmer et al., 2017). These results indicate a possible role of miR166 in early defence response, especially towards the PTI response of the plant. To investigate whether miR166 could contribute to PTI response in potato as well, treated WT potato plants with Arachidonic Acid (AA), a known PAMP of *P. infestan*s, and found that miR166 started to accumulate as early as 90 minutes post treatment. This observation suggests that miR166 could be involved in the PTI response of the potato, upon infection with *P. infestans* and a similar to previous studies in soybean and rice (Salvador-Guirao R et al., 2018; Wong et al., 2014). When we analysed the expression profile of three selected miR166 target, namely *StICU1, StICU2, and StREV,* (these miR166 target showed consistent negative correlation with miR166) we observed that gene expression of these targets was negatively regulated by miR166 (Figure 4.6). Interestingly, at 3 hours post treatment with AA we find that miR166 levels were high, and were comparable to miR166 upon *P. infestans* infection, justifying the fact that miR166 might be involved in PTI response of the plant.

4.4.3. MiR166 regulates SAR response in potato

In our experiments, we have reported that miR166 is induced at both local and systemic sites upon infection with *P. infestans*. This prompted us to check miR166 dependent systemic defence response in potato. We generated several miR166 OE and KD lines and found that miR166 KD and OE lines do not show any morphological changes despite its well-known role in development responses (Figure 4.9). We performed SAR assay for miR166 transgenic lines, where we observed that WT and OE lines were able to mount a successful SAR response, but the KD lines were not able to mount a successful SAR response. This result indicates that miR166 is involved in eliciting systemic defence response in potato plants. To investigate the importance of miR166 targets in this response, we generated transgenic lines overexpressing miR166-resistant *StICU1.* We found that when the *StICU1* OE line was subjected to the SAR assay, the lines failed to mount a SAR response suggesting that miR166 dependent SAR response is negatively regulated by StICU1 in potato.

Overall our findings showed that upon infection of *P. infestans,* miR166 accumulates at both local and systemic leaves in potato. Further analysis revealed negative correlation between miR166 and its targets *StICU1, StICU2, and StREV2* but, not with *StPHV1 and StPHV2*. These results indicate a strict regulation of miR166 over *StICU1*, *StICU2* and *StREV* and that they have a role in defence response. Moreover, treatment of WT plants with PAMP of *P. infestans* showed that the plants mount a PTI response as early as 90 minutes post infection. We generated transgenic lines to explore the role of miR166 in defence response and upon performing SAR assays and we found that miR166 KD transgenic lines failed to elicit a SAR response, whereas miR166 OE lines showed a normal SAR response. For understanding the underlying mechanism on how miR166 regulate defence response, we generated transgenic lines overexpressing miR166 resistant *StICU1* in plants. SAR response was observed to be compromised upon *P. infestans* infection. In conclusion, our findings suggests that, in potato, miR166 and its targets could play a crucial role in mounting a defence response against P*. infestans*.

Summary

Plants being sessile are exposed to many pathogens. To combat the pathogen attacks, plants employ various mechanisms to defend themselves at both local and in systemic sites (Chisholm et al., 2006; Jones and Dangl, 2006). Plants recognize various pathogen-associated molecular patterns (PAMPs) using pattern recognition receptors (PRRs) to activate PAMP triggered immunity (PTI) (Chisholm et al., 2006). Some pathogens release effector molecules in plant cells to deter activation of defence response by suppressing PTI response of the plant, this type of susceptibility is called effector triggered susceptibility (ETS). To respond to ETS, plants have evolved Nucleotide binding leucine rich repeats receptors (NB-LRR) called Resistance (R) protein, that can recognize these effector molecules and activate effector –triggered immunity (ETI) (Dodds and Rathje, 2010). The PTI and ETI responses protect the plants at the site of infection, but plants also employ a systemic defence response, called systemic acquired resistance (SAR) in the uninfected distant tissue parts to protect them from future pathogen attacks (Shah, 2009; Dempsey and Klessig, 2012). Plants elicit SAR in the systemic tissue by translocating mobile SAR signal/s from the infected tissue to the systemic sites (Guedes et al., 1980; Tuzun and Kuc, 1985). In past two decades, researchers have uncovered various SAR mobile signals, namely Methyl salicylate (MeSA) (Park et al., 2007), Azealic acid (AzA) (Jung et al., 2009), Glycerol-3-Phosphate (G3P) (Chanda et al., 2011), dehydroabietinal (DA) (Chaturvedi et al., 2012) and pipecolic acid (Pip) (Návarová et al., 2012). Recently, we have demonstrated the importance of miRNA160 (miR160) in regulating plant's local and systemic defence response (Natarajan et al 2018). The study unravelled various questions as to how miRs could regulate the plant's systemic response, while regulating the local defence response as well. We found that *StARF10*, a target of miR160, could regulate *StGH3*.6 (a homolog of *AtGH3*.5) and maintain the balance between SA mediated defence response and auxin signalling in plant growth and development (Natarajan et al., 2018). But the authors did not explore the role of other miR160 targets, viz., *StARF16 and StARF17,* in regulation of the plant defence response. The study also demonstrated that miR160 & miR166 both are differentially regulated upon infection with *Phytophthora infestans.* Notably, we observed that miR160 knockdown lines failed to elicit SAR response, however, the molecular basis for compromised SAR response was rudimentary. Hence, using potato-*Phytophthora infestans* as a model system, the following objectives were considered to elucidate the underlying molecular mechanism of plant defence response modulation by miR160 and miR166 in potato.

Objectives:

1. Understanding the mechanistic link between miR160 target, StARF16 and defence response.

2. To investigate compromised SAR response in miR160 knockdown lines.

3. Investigating the potential role of miR166 in the defence response of potato.

Chapter 1: Introduction

A literature survey was carried out to understand the (i) defence responses of plants, (ii) mechanistic interaction involved in various defence responses and (iii) the role of SA and JA in regulating defence response. This survey revealed that there are numerous reports available that can extend our understanding of PTI and ETI response. However, there is only one study at present to explore how miRNA could regulate SAR response in plants. In this chapter, we have summarized the current knowledge of plant-pathogen interactions. Furthermore, based on this survey, we have proposed a number of objectives to investigate the role of miRs in plant defence response using potato-*P. infestans* as a model system.

Chapter 2: MiRNA160 target, *StARF16,* regulates *StNPR1* during plant pathogen interactions

There are several studies demonstrating the role of miR160 in the development process in various plants species (Wang et al., 2005; Mallory et al., 2005; Liu et al., 2007; Gutierrez et al., 2012; Hendelman et al., 2012; Liu et al., 2013; Turner et al., 2013; Huang et al., 2016; Damodharan et al., 2016). Moreover, the role of miR160 in defence response has also been elucidated in Arabidopsis and rice (Li et al., 2010; Li et al., 2014). miR160 has been reported to target various Auxin Response Factors (ARFs) plants, namely ARF10, ARF16 and ARF17. Previous study from our lab has implicated the role of miR160 target *StARF10* and its potential role in regulating *StGH3.6*, a homolog of *AtGH3.5* in potato (Natarajan et al., 2018). *AtGH3*.5 has been implicated to maintain the balance between auxin and SA signalling in plants. In one of our objectives, we tried to understand whether *StARF16*, another miR160 target, could regulate any known defence response. We used *P. infestans* (a hemi-biotroph) and *A.*

solani (a necrotroph) to decipher the role of specific role of *StARF16*, in defence response. From our *in silico* analysis, we found that *StARF16* could most likely bind to the promoter of *StNPR1*. We focused our study to explore under what conditions do *StARF16* regulates *StNPR1.* We employed yeast one hybrid (Y1H), co-transfection and chromatin immune precipitation assays (ChIP) to understand the regulatory mechanism of StARF16 over StNPR1.

Salient findings:

1. Upon infection with *P. infestans* (hemi-biotroph) and *A. solani* (necrotroph), potato miR160 was found to be differentially regulated at early time points (*viz*., 3, 6, 9, 12 hours post infection) of infection (Figure 2.3).
2. We noticed that during infection with *P. infestans* and *A. solani,* miR160-*StARF10* exhibited a negative correlation with each other. But *StARF16*, another target of miR160*,* showed positive correlation with its regulatory miR (Figure 2.3).
3. Upon further expression analysis, we found that during early infection with *P. infestans*, several biotrophic defence genes such as S*tNPR1, StPR1, StWRKY*6 were accumulated suggesting the activation of SA dependent pathway. However, during *A. solani* infection, we observed the accumulation of *StMYC2,* indicating the activation of JA-mediated defence pathway (Figure 2.5).
4. We noticed that *StARF16 and StNPR1* exhibit negative correlation upon infection with hemi-biotroph and necrotroph (Figure 2.3 and 2.5).
5. Further, when SA and JA are exogenously applied to plants in *in vitro* and *in soil, StARF16* and *StNPR1* showed negative correlation with each other (Figure 2.7).
6. Our *in silico* analysis revealed that there were eight ARF binding sites present upstream to 3.3 kb region of *StNPR1*. We further analysed the promoters of *NPR1* in several members of *Solanaceae* and *Brassicaceae* and observed the presence of high sequence similarities within the *Solanaceae* family members only (Figure 2.8 and 2.9).
7. Through Y1H, we showed that out of the two miR160 targets, viz., *StARF10* and *StARF16,* only StARF16 could strongly bind to the promoter of StNPR1 (Figure 2.9).
8. Our co-transfection assay in potato and tobacco protoplasts, demonstrate that miR160 target, *StARF16* binds to the promoter of *StNPR1* and represses the gene expression (Figure 2.13).
9. When multiple ARF binding sites were sequentially truncated from the promoter of StNPR1, we observed that a minimum of six ARF binding sites present within 2.6 kb region upstream of *StNPR1* is required to regulate its expression (Figure 2.14 and 2.15).

10. Through ChIP-quantitative Real Time Polymerase Reaction (qRT-PCR) analysis in potato protoplasts transfected with miR160 resistant form of *StARF16 (5'mStARF16),* we found that *StARF16* gets enriched over 4th, 5th, and 6th ARF binding sites in endogenous StNPR1 promoter. These binding sites are present within 2.6 kb region upstream of *StNPR1* gene, and are crucial in regulating its expression (Figure 2.14).
11. Finally, in this study, we demonstrate the importance of *StARF16* (a well-known development related gene), in the regulation of *StNPR1,* and thus SA pathway, during JA-mediated defence response upon necrotrophic pathogen interaction.
12. Also, we have proposed a working model, where miR160 target, StARF16, regulate the expression of *StNPR1* upon induction of JA (Figure 2.15).

Chapter 3: MiRNA160 knockdown (KD) lines are deficient in G3P induced SAR response

miR160 has been depicted to contribute to plant's basal defence response in potato, Arabidopsis, and rice (Li et al., 2010; Li et al., 2014, Natarajan et al., 2018). Previous study from our lab, showed that miR160 is associated in establishment of SAR response in the plant (Natarajan et al., 2018). In this study, we observed that the miR160 knockdown (KD) lines showed compromised SAR upon infection with *P. infestans* (Natarajan et al 2018). The study did not explore the underlying mechanism that could result in compromised SAR. To further expand the knowledge as how miR160 could regulate SAR response in plants, we preceded with establishing the fact that the known SAR signal molecules are capable of eliciting SAR response in WT potato plants. We exogenously applied various SAR signals to miR160 KD lines in an attempt to mount a successful SA response in miR160 KD lines. We noticed that miR160 KD lines were able to mount a SAR response upon treatment with G3P. Through further gene expression analysis, we observed that miR160 KD lines were unable to mount a G3P dependent SAR response.

Salient Findings:

1. The known mobile SAR signals, namely SA, AzA, DA, G3P and Arachidonic Acid (AA), can establish SAR in WT *Solanum tuberosum* L. cv. Désirée (Figure 3.1).
2. We noticed that when these mobile SAR signals were exogenously provided to miR160 KD lines, only G3P was able to rescue the compromised SAR, indicating that miR160 KD lines were deficient in G3P induced SAR signalling (Figure 3.5).

3. Through dose dependent G3P treatments, we found that miR160 KD plants require an optimum level to mount a robust SAR response (Figure 3.6).
4. Our expression analysis in WT potato plants, revealed that G3P application could regulate several G3P dependent defence genes. But interestingly, upon G3P application, we observed the induction of *StNPR1* and *StPR1*. This indicates that G3P could cross talk with SA dependent SAR pathway in potato plants (Figure 3.7).
5. Quantitative real time PCR (qRT-PCR) expression analysis showed that miR160 KD lines that could not regulate auxin signalling upon treatment with *P. infestans,* could partially attenuate the auxin signalling in plants. Our current findings suggest that G3P could also regulate auxin mediated signalling in potato plants (Figure 3.9).
6. Furthermore, we found that miR160 KD lines could attenuate G3P dependent SAR genes upon treatment with G3P, indicating that miR160 KD lines are indeed deficient in G3P accumulation. Future investigations can prove the role of miR160 in regulating G3P dependent SAR response in potato plants (Figuure 3.9).

Chapter 4: Investigating the role of miR166 in regulating the defence responses in potato during *P. infestans* infection.

miR166 is well-studied for its role in development process (Boialem et. al., 2008; Hawer et. al., 2004; Ong et. al., 2012; Rubio-Somoza et. al., 2011). Recently, miR166 has been shown to contribute towards the defence response in plant species like soybean and rice against hemi-biotrophic pathogens, *Magnoporthe oryzae* and *Phytophthora sojae*, respectively (Salvador-Guirao R et al., 2018; Wong et al., 2014). In these studies, miR166 have been demonstrated to regulate the PTI response and shown to accumulate as early as 15 minutes upon recognition of PAMP molecules from the pathogen (Salvador-Guirao R et al., 2018). We explored a similar possibility in potato and analysed the expression profile of miR166 at local and systemic sites upon infection with a potato hemi-biotroph *P. infestans*. Hence, we generated miR166 KD and overexpression (OE) lines to validate the role of miR166 in potato defence response. Our results indicated that miR166 plays crucial role in defence response of plants upon infection with *P. infestans*.

Salient features:

1. Upon *P. infestans* infection, miR166 exhibited a dynamic expression profile suggesting it's potential role in potato- *P. infestans* interactions (Figure 4.1 and 4.2).

2. MiR166 was found to be induced as early as 3 hours post infection (hpi) at local site of infection, indicating its involvement in basal defence response of potato (Figure 4.1).
3. Notably, miR166 only accumulated till 24 hpi with *P. infestans* and after 48 hpi, the miR166 levels were comparable to that of control plants (Figure 4.1).
4. However, in systemic site, we observed high accumulation of miR166 at 6 hpi and then the levels started to decrease after 12 hpi, suggesting a potential role of miR166 in inducing SAR response at systemic site (Figure 4.2).
5. Through our *in silico* analysis, we found miR166 could target five transcripts in potato namely, homolog of INCURVATA (*StICU1 and StICU2*), PHAVOLUTA (S*tPHV1* and *StPHV2*), and REVOLUTA (*StREV*).
6. Using cleavage site mapping technique, we validated that miR166 can target *StICU1, StICU2, StREV, StPHV1 and StPHV* and cleave these transcripts of potato as predicted in our *in silico* analysis (Figure 4.3).
7. Upon infection of *P. infestans*, we observed that *StICU1, StICU2 and StREV* showed a negative correlation with miR66 at almost all the time points. However, *StPHV1 and StPHV2* fail to show a consistent negative correlation with miR166 (Figure 4.4 and 4.5).
8. Interestingly, upon infection with *P. infestans*, we found that the expression of both defence genes *StNPR1* and *StPR1* decreased at 3 hpi (Figure 4.1). Even though the decrease was found to be non-significant but it indicates that there could be an unknown mechanism required during colonization of *P. infestans.*
9. Further investigations revealed that upon treatment of WT potato plants with AA, a PAMP of *P. infestans,* miR166 started to accumulate as early as 90 minutes post treatment. This finding suggests the potential role of miR166 in potato PTI response against *P. infestans* (Figure 4.6).
10. The miR166 OE and KD lines did not exhibit any morphological changes (Figure 4.9).
11. When miR166 transgenic lines were treated with *P. infestans* to understand SAR response, miR166 OE lines elicited SAR comparable to that of WT but miR166 KD lines failed to elicit SAR. An early accumulation of miR166 in systemic site upon infection and the above findings together implicates the role of miR166 in SAR response of potato (Figure 4.10).
12. To investigate further the role of miR166 in SAR establishment, we generated transgenic lines overexpressing miR166 resistant *StICU1 (5'mStICU1)*. Upon SAR assay, *5'mStICU1* OE lines failed to establish a SAR response upon infection with *P. infestans* suggesting that miR166 target *StICU1,* and is a significant role player in regulating SAR response in potato (Figure 4.11).

Future directions:

Our study establishes that miR160 target, *StARF16,* regulates defence responses in WT potato plants through *StNPR1.* Furthermore, we also confirm that miR160 contribute to SAR response through the NO-ROS-G3P-AzA branch in response to *P. infestans* infection. Alongside, we also showed a positive role of miR166 in the defence response of potato against *P. infestans.* To have further insights into the role of microRNAs in defence response of potato against *P. infestans*, following points could be considered for future studies:

1. As miR160 and its target *StARF16* showed a positive correlation rather than the negative correlation, it would be interesting to explore how a positive correlation between microRNAs and its targets regulate the defence response or any other processes in plants.
2. As *StARF16* levels were differentially regulated upon infection with hemi-biotroph and necrotrophic pathogen interaction, exploring the upstream regulators involved in regulating miR160 and StARF16 could provide additional insights.
3. In our study, we found that miR160 KD lines were deficient in G3P dependent SAR response, but it would be fascinating to investigate the molecular mechanism how miR160 contributes toward the G3P dependent SAR response.
4. We observed that miR166 regulates potato defence response upon infection with *P. infestans* and is most likely involved in the PTI response in the plant defence response. It would be worthwhile to examine the importance of miR166 role in early defence response of the plant and how does the early induction of miR166 contribute towards the SAR response.
5. We demonstrated that miR166 resistant *StICU1* OE lines showed compromised SAR similar to that of miR166 KD lines. The underlying molecular mechanism of how *StICU1* regulates defence response in this regard remains to be investigated.

Annexure I

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Annexure II

Promoter sequence of ***StNPR1*** showing all the ARF binding sites (marked as grey) present upto 3.3kb region before transcription start site (highlighted as red). Sequence underlined mark the UTR region of gene.

>chr07:36288378..36291378 (reverse complemented)
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Promoter sequence of ***AtGH3*.5** showing all the ARF binding sites (marked as grey) present upto 3.3kb region before transcription start site (highlighted as red). Sequence underlined mark the UTR region of gene.

>AT4G27260 | chr4:13650609-13653608 FORWARD LENGTH=3000
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Promoter sequence of NPR1 in showing used in VISTA anlaysis

>ref|NC_015444.3|:c49523085-49520012 Solanum lycopersicum cultivar Heinz 1706 chromosome 7, SL3.0, whole genome shotgun sequence

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>ref|NW_008868824.1|:99918-103706 Nicotiana tomentosiformis unplaced genomic scaffold, Ntom_v01 Ntom_scaffold1363, whole genome shotgun sequence

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>ref|NW_015861707.1|:94391-97788 Nicotiana tabacum cultivar TN90 unplaced genomic scaffold, Ntab-TN90 Ntab-TN90_scaffold22046, whole genome shotgun sequence

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>ref|NC_025693.1|:c37852195-37849153 Camelina sativa cultivar DH55 chromosome 9, Cs, whole genome shotgun sequence

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> Arabidopsis thaliana chromosome 1 sequence

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>ref|NW_017353147.1|:27846682-27849767 Raphanus sativus cultivar WK10039 unplaced genomic scaffold, Rs1.0, whole genome shotgun sequence

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>ref|NC_024803.2|:c8002742-7999271 Brassica rapa cultivar Chiifu-401-42 chromosome A09, CAAS_Brap_v3.01, whole genome shotgun sequence

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>ref|NW_003302554.1|:361290-364737 Arabidopsis lyrata subsp. lyrata unplaced genomic scaffold ARALYscaffold_2, whole genome shotgun sequence

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TAAAA

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Cirriculum Vitae

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Academic details

Degree / Examination Passed	Subjects/Area of specilaization	Institution	Year of Passing	Average Marks/CGPA	Class
10th	None	St. Mary's Sr. Sec. School, Affiliated wiith C.B.S.E.	2008	83.5%	First
12th	Biology, physics, Chemistry and Maths	Harcouurt butler Sr. Sec. School, Affiliated wiith C.B.S.E.	2010	82.5%	First
B.Sc.	Life Sciences	Hans raj college, University of Delhi	2013	77.75%	First
M.S.(Part of Integrated PhD)	Biological Sciences	Indian Institute of Science Education and Research, Pune	Completed M.S. credits in 2015	8	First

Professional Skills

- Demonstrated experience in the standard techniques of molecular biology, cloning, genetics, biochemistry, and plant physiology
- Laboratory research experience in understanding plant pathogen interaction
- Previous experience in aseptic plant tissue culture and generation of transgenic lines
- Understanding of preparation dn handling of samples for HPLC and GC-MS
- Prior experience with confocal microscopy and sample preparation
- Proficiency in standard Microsoft Office software applications including Word, Excel, and PowerPoint
- Strong oral, written and presentation skills, and the ability to conduct research independently as well as being a team player

Research Publications

- **Kalsi H.**, Natarajan B. and Banerjee A. K. (2022). AUXIN RESPONSIVE FACTOR 16 (StARF16) regulates defense gene *StNPR1* upon infection with necrotrophic pathogen in potato. **Plant Molecular Biology** DOI:10.1007/s11103-022-01261-0.

- Kondhare K. R., Vetal P., **Kalsi H.,** and Banerjee A. K. (2019). StBEL5 regulates *CYCLING DOF FACTOR 1 (StCDF1)* through TGAC core motifs in potato. **Journal of Plant Physiology**, DOI:10.1016/j.jplph.2019.153014.

- Natarajan B., **Kalsi H.**, Godbole P., Malankar N., Thiagarayaselvam A., Siddappa S., Thulasiram H. V., Chakraborti S. and Banerjee A. K**.** (2018). MiRNA160 is associated with local defense and systemic acquired resistance of potato during Phytophthora infestans infection. **Journal of Experimental Botany**. DOI:10.1093/jxb/ery025

Conferences attended:

- Indian Society for Developmental Biologists Biennial meeting (InSDB2017): 24th - 27th June 2017, IISER Pune.
- International conference on Plant Developmental Biology & 3rd National Arabidopsis Meeting, 12th – 16th Decemeber, NISER, INDIA.
- IISER-WIS conference on Chemical Biology from January 17 to 19, 2018, IISER Pune.
- Molecular Intricacies of Plant associated Microorganisms (MIPAM) 1st -3rd February 2019, NIPGR, New Delhi.

Awards

- IISER Pune-JRF and SRF
- CSIR- SRF