Investigating the Role of miRNA160 in Local and Systemic Defense Responses of Potato against *Phytophthora infestans* Infection

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By

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CERTIFICATE

Certified that the work incorporated in the thesis entitled, **"Investigating the Role of miRNA160 in Local and Systemic Defense Responses of Potato against *Phytophthora infestans* Infection"** submitted by Ms. Bhavani Natarajan 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.

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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 have 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

Abscisic acid	ABA
AGD2-LIKE DEFENCE RESPONSE 1	ald1
Argonaute 1	AGO 1
Artificial Target Mimicry	MIM
Auxin Response Factor	ARF
AUXIN SIGNALLING F-BOX	AFB
Avirulence gene	avr
Azelaic acid	AzA
base pair	bp
Beta-Glucuronidase	GUS
Cauliflower Mosaic Virus	CaMV
Chromosome	Chr
Dluster of differentiation	CD2
Coiled-coil neucleotide-binding site leucine-rich repeat	CC-NBS-LRR
Colony forming units	CFU
Copper/Zinc superoxide Dismutase gene	CSD
Cortex	co
Days post inoculation	dpi
Defective in Induced Resistance 1	DIR1
Dehydroabietinal	DA
Dichrloromethane	DCM
Effector triggered susceptibility	ETS
Effector-triggered immunity	ETI
Electrophoretic Mobility Shift Assay	EMSA
Electrospray Ionization Mass Spectrometry	ESI-MS
Endogenous microRNA Target Mimic	eTM
Femto-mole	fmol
Flagellin 22	flg22
Flagellin Sensing	FLS
FLAVIN-DEPENDENT MONOOXYGENASE	FMO1
Forward Primer	FP
Fourier Transform Mass Spectrometry	FTMS
Gas Chromatography – Mass Spectrometry	GC-MS
Glyceraldehyde-3-phosphate dehydrogenase	GAPDH

Glycerol-3-Phosphate	G3P
Glycerol-3-Phosphate dehydrogenase	G3Pdh
Gretchen Hagen	GH3.5
Growth regulating factor	GRF
Histidine	His
Hours post inoculation	hpi
Hours post treatment	hpt
HUA ENHANCER 1	HEN 1
Hypersensitive Response	HR
Indole-3-Acetic Acid	IAA
Induced Systemic Response	ISR
Azelaic acid Insensitive 1	AZI1
Internal Standard	IS
Internal Transcribed Spacer 2	ITS2
Jasmonic Acid	JA
Knock Down	KD
Laser Capture Microdissection	LCM
Long day	LD
Mass by charge ratio	m/z
Mass Selective Detector	MSD
Messenger RNA	mRNA
Methyl Salicylate	MeSA
micro RNAs	miRNAs
Mildew resistance locus a	Mla
Mitogen Activated Protein Kinase	MAPK
Moderately Resistant	MR
Natural resistance associated macrophage protein 6	Nramp 6
Nicotiana benthamiana	nb
Nitrate Transporter	NT
Nitric Oxide	NO
No Template Control	NTC
Nonexpressor of Pathogensis Related Gene	NPR1
Nottingham Arabidopsis Stock Center	NASC
Nucleotide	nt
Nucleotide Binding	NB
Optical Density	OD

Oryza saiva	os
Over expression	OE
PAMP Triggered Immunity	PTI
Pathogen Associated Molecular Patterns	PAMP
Pathogenesis Related	PR
Pattern Recognition Receptors	PRR
Phloem	ph
Phloem Enriched Exudates	PEX
Pipecolic acid	Pip
Plant Genome Sequencing Consortium	PGSC
Poly(deoxyinosinic-deoxycytidylic)	Poly(dI-dC)
Poly-Acrylamide Gel Electrophoresis	PAGE
Pseudomonas syringe pv. tabaci	Pst
Quantitative Real Time PCR	qRT-PCR
Rapid Amplification of cDNA Ends	RACE
Reactive Oxygen Species	ROS
Resistance protein	R-protein
Reverse Primer	RP
RNA Induced Silencing Complex	RISC
RNA Polymerase II	RNA Pol II
RPM 1 Interacting Protein 4	RIN4
SA – BINDING PROTEIN 2	SABP 2
SA – METHYL TRANSFERASE 1	SAMT 1
Salicylic Acid	SA
Salicylic Acid Binding Protein	SABP2
Short Tandem Repeat	STR
SKP-CULLIN-F-BOX E3 ubiquitin ligase complex	SCF
SQUAMOSA PROMTER BINDING-LIKE	SPL
Standard deviation	SD
Super Oxide Dismutase	SOD
suppressor of fatty acid desaturase deficiency 1	Sfd 1
Susceptible	SUS
Systemic Acquired Resistance	SAR
Target prediction for plant microRNAs	TAPIR
TGACG Sequence-specific binding protein	TGA
Tobacco Mosaic Virus	TMV

Trans-acting SiRNA	TAS
TRANSPORT INHIBITOR RESISTANT 1	TIR1
Type 3 Secretory System	T3SS
Ultra Violet	UV
Vector Control	VC
Wild type	WT
Xylem	xy

Synopsis

Investigating the Role of miRNA160 in Local and Systemic Defense Responses of Potato against *Phytophthora infestans* Infection

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Introduction

Being sessile, plants are constantly exposed to various pathogens. To combat pathogens, plants employ multiple layers of defence responses in both local-infected and systemic-uninfected leaves (Chisholm et al., 2006; Jones and Dangl, 2006). In local leaves, plants can recognize pathogen-associated molecular patterns (PAMPs) using their membrane bound pattern recognition receptors (PRRs) leading to the activation of PAMP-triggered immunity (PTI) (Chisholm et al., 2006). Some pathogens can evade such detection by releasing PTI-suppressing 'effector protein' into the plant cell. In this arms race of plant defences and pathogen counter-defences, some plants have evolved resistance (R) proteins that can recognize these effectors and activate effector-triggered immunity (ETI) (Dodds and Rathjen, 2010). In addition to these local responses, PTI and ETI can induce systemic defences in the un-infected parts of the plant, resulting in a broad-spectrum, long-lasting resistance known as systemic acquired resistance (SAR) (Shah, 2009; Dempsey and Klessig, 2012). SAR is activated by the transport of a mobile SAR signal from the local infected tissues to the systemic tissues mostly via phloem (Guedes et al., 1980; Tuzun and Kuc, 1985). Extensive research has led to the identification of several potential SAR signals, most promising being (i) Methyl salicylate (MeSA) (Park et al., 2007), (ii) Azelaic acid (AzA) (Jung et al., 2009), (iii) Glycerol-3-Phosphate (G3P) or its derivatives (Chanda et al., 2011), (iv) dehydroabietinal (DA) (Chaturvedi et al., 2012) and (v) pipecolic acid (Pip) (Návarová et al., 2012). The process of PTI, ETI and SAR accompany mainly the increased accumulation

of phytohormone salicylic acid (SA) and induction of PATHOGENESIS-RELATED (PR) family of genes, along with several other molecular changes (Cameron et al., 1999).

Apart from these studies, many other reports have recently shown the role of microRNAs (miRNAs) in plant immunity including PTI and ETI responses (Ruiz-Ferrer and Voinnet, 2009; Sunkar et al., 2012; Seo et al., 2013). miRNAs are endogenous small ~21-nt non-coding RNAs that act as negative regulators of gene expression (Bartel and Bartel, 2003; Dugas and Bartel, 2004). Since their discovery, hundreds of miRNAs have been identified in plants and are shown to regulate transcription factors and proteins that play important functions throughout the plant life beginning from embryo development and seed germination to 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). The first report of miRNA's role in plant immunity was from the studies conducted by Navarro et al. (2006), wherein, the authors showed that treatment of Col-0 *Arabidopsis* seedlings with flg22 (a 22 amino acid N-terminal part of flagellin that acts as PAMP) leads to an induced expression of miR393 and triggers PTI (Navarro et al., 2006). The authors further elucidated the role miR393 in maintaining a balanced antagonistic relationship between SA-mediated defence responses and auxin-mediated growth (Navarro et al., 2006; Sunkar et al., 2012). Recent reviews have discussed the antagonistic crosstalk of auxin and SA as one of the mechanisms adopted by plants to mediate growth-defence trade-offs (Kazan and Manners, 2009; Denancé et al., 2013; Huot et al., 2014; Verma et al., 2016). Though several miRNAs have been shown to play role in PTI and ETI responses, no previous studies have yet described the role of miRNAs in SAR development to the best of our knowledge. Because miRNAs are very important regulatory molecules, we hypothesised that miRNAs could play significant role in establishment of SAR as well. Using potato – *Phytophthora infestans* interaction as a model system, we laid out a number of objectives to test our hypothesis. It is noteworthy that role of miRNAs in potato – *P infestans* interaction is also not investigated before.

Objectives:

1. To investigate the role of potential miRNAs and their targets in potato-*Phytophthora infestans* interaction.
2. To characterize the role of miR160 in local defence and SAR responses of potato.
3. To explore the mechanistic link of miR160 in defence response of potato.

1. Investigating the role of potential miRNAs and their targets in potato-*Phytophthora infestans* interaction

Earlier, several miRNA families have been predicted and validated in potato (Zhang et al., 2009; Yang et al., 2010b; Kim et al., 2011; Zhang et al., 2013; Martin et al., 2009; Bhogale et al., 2014). However, only two reports have so far described the role of potato miRNAs in biotic (Yang et al., 2010a) and abiotic stress responses (Kitazumi et al., 2015). One of our aims was to identify the miRNAs involved in potato-*P. infestans* interaction with a potential role in SAR. In this regard, we shortlisted 11 different miRNA family members based on the prior knowledge of miRNA's role in other plant-pathogen interaction as well as its presence in phloem. Out of the 11 shortlisted miRNAs, 10 were found to be expressed in potato. Upon *P. infestans* infection, 5 miRNAs (miR159, miR160, miR166, miR169 and miR172), exhibited differential expression indicating their potential role in potato-*P. infestans* interaction. Even though all these miRNAs showed promise towards their role in regulation of potato - *P. infestans* interaction, the present study was restricted to decipher the role of miR160 in this interaction.

To elucidate the role of miR160 in local (basal) and SAR responses, expression analysis of miR160 was carried out in local and systemic leaves as well as in the phloem enriched exudates (PEX) of potato plants upon *P. infestans* infection. miR160 expression was found to be induced in both local and systemic leaves at different time-points post infection. Increased accumulation of miR160 was also observed in the PEX of infected potato plants suggesting its potential role as a mobile SAR signal. Further, 7 different target genes were predicted for miR160 in potato. Off this, *StARF10 and StARF16* were validated as true targets of miR160. Upon *P. infestans* infection, expression of *StARF10* was also observed to be induced in local and systemic leaves of potato. Overall, this study indicated that miR160 has a potential role in local and SAR responses of potato.

2. Characterization of *miR160* and its role in local defence and SAR responses of potato

miR160 is a conserved plant miRNA whose role in plant growth and development and auxin signalling has been unequivocally established through elaborate studies in *Arabidopsis*, tomato, soybean and rice (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). Recently, the defence related functions of miR160 have also been

elucidated. Li and co-workers (2010) have shown that miR160 is involved in PAMP-induced callose deposition and PTI responses in *Arabidopsis* (Li et al., 2010). miR160 is also proposed to function as a positive defence regulator during rice-*Magnaporthe* interaction (Li et al., 2014). To test if miR160 plays role in local and SAR responses during potato-P. infestans interaction, both overexpression (OE) and knockdown (KD) transgenic lines of miR160 were generated. Basal defence and SAR response of these lines were analysed using local infection, SAR assays and grafting analysis.

In our analysis, miR160 OE and KD transgenic lines did not show any drastic morphological changes, except that miR160 OE line had a slight curled leafy phenotype suggesting the role of miR160 in leaf development. In addition, miR160 KD lines exhibited increased tuber yield, whereas OE lines had a reduced tuber yield indicating its potential role in potato tuberization. When infected with *P. infestans*, both miR160 OE and KD transgenic lines exhibited enhanced susceptibility compared to wild-type (WT) plants. This suggested that an optimal level of miR160 is possibly required for mounting a proper local defence response in potato. Further, SAR assays on these lines revealed that miR160 KD lines exhibit compromised SAR, whereas miR160 OE lines can elicit an effective SAR response. These results suggest that miR160 plays role in SAR responses of potato. Our grafting studies further indicated that miR160 KD lines were unable to both (a) generate and/or transport the SAR signal, and (b) perceive and/or process the SAR signal. These findings indicated that threshold levels of miR160 is possibly required in both local and systemic leaves of potato for establishment of an effective SAR response. Overall, this study showed that miR160 indeed plays a role in local and SAR responses of potato.

3. Exploring the mechanistic link of *miR160* in defence response of potato

It is well known that plants actively suppress auxin signalling processes to mount salicylic acid (SA)-mediated defence responses (Kazan and Manners, 2009; Denancé et al., 2013; Huot et al., 2014; Verma et al., 2016). As miR160 is shown to be an important component of auxin signalling pathway, we examined the enhanced susceptibility phenotype of miR160 OE and KD lines in light of auxin-SA signalling crosstalk. In this regard, expression of several genes (*StYUCCA1*, *StLAX4*, *StTIR1*, *StIAA16* and *StGH3*.6) involved in different aspects of auxin pathway were analysed. We observed that, upon *P. infestans* infection, the WT potato plants were able to successfully suppress the auxin signalling, however, miR160 OE and KD transgenic lines failed to exhibit the same. This suggests that

failure to attenuate auxin signalling could be one of the reasons for the enhanced susceptibility of miR160 KD and OE lines.

Additionally, to understand the reason for the compromised SAR response exhibited by miR160 KD lines, SAR-associated signals (SA and MeSA) and genes (*StPR1*, *StNPR1*, *StBSMT1*, *StMES1*, *StGH3.6*) were also examined. All these major SAR-associated signals and genes were found to be dysregulated in miR160 KD lines and hence explains the possible cause for compromised SAR response observed in miR160 KD lines. One of the common mediators in this auxin-SA crosstalk is the auxin-conjugator, *StGH3.6*. Based on the expression pattern of *StGH3.6* in miR160 OE and KD lines, we hypothesised that *StGH3.6* could be regulated by *StARF10* (the target gene of miR160) and this hypothesis was further tested with EMSA and Y1-H assays. Our results revealed that *St*ARF10 directly binds to the promoter of *StGH3.6.* and hence provides a mechanistic link between miR160 and the defence-related pathways involved in potato- *P. infestans* interaction.

Summary

In this investigation, we show that the potato miRNAs, miR159, miR160, miR166, miR169 and miR172, respond to *P. infestans* infection. Elaborate studies on miR160 showed its induced expression in local and systemic leaves as well as increased accumulation in PEX of infected plants. Expression of miR160 target gene, *StARF10*, was also induced upon infection. Further, local infection assays using miR160 OE and KD lines revealed the enhanced susceptibility of both these transgenic lines and suggested that optimal levels of miR160 is required for mounting proper basal response. Failure to attenuate auxin signalling pathway appears to be one of the reasons for the enhanced susceptibility observed in these lines. Additionally, SAR assays showed that miR160 KD lines, but not OE lines, are compromised in SAR response and thus implicated the role of miR160 in SAR development of potato. Our grafting studies revealed that compromised SAR response of miR160 KD lines could be because of the defects in SAR signalling at both local and systemic leaves. The major SAR-associated signals and genes were also found to be dysregulated in miR160 KD lines, which further explains its SAR-deficient phenotype. Through EMSA and Y1-H analysis we showed that there is a mechanistic link between StARF10 and *StGH3.6.* To the best of our knowledge, ours is the first study that implicates the role of miRNAs in potato-*P. infestans* interaction and demonstrates miRNA-mediated regulation of SAR in any plant species.

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Chapter 1

Introduction

1.1. Plant-pathogen interaction

Plants encounter millions of pathogens in their lifetime including bacteria, viruses, fungi, oomycetes, nematodes and herbivorous insects. These organisms feed on plant nutrients and their lifestyle can be categorised based on how they gain these nutrients (Dangl and Jones, 2001; Freeman and Beattie, 2008). Pathogens that keep their host alive for acquiring nutrients are called biotrophs. Some examples are *Xanthomonas oryzae*, the causative agent of bacterial blight of rice (Ryan et al., 2011; Zhang and Wang, 2013) and *Blumeria graminis*, the fungus that causes powdery mildew of barley (Zhang et al., 2005). In contrast, pathogens that kill their host to obtain the nutrients are called necrotrophs. The bacterial soft-rot pathogen *Erwinia carotovora* (Bhat et al., 2010) and the gray mold fungus *Botrytis cinerea* (Kan, 2006) belong to this category. However, there are some pathogens that follow a lifestyle intermediary of biotrophs and necrotrophs. These are called hemi-biotrophs and follow biotrophic phase in the early infection stages and necrotrophic phase in the later stages. The oomycete pathogen, *Phytophthora infestans* that causes late blight of potato and tomato is one of the notorious hemi-biotrophs (Akino et al., 2014; Fry, 2016).

Though myriads of pathogens attack plants, only few can cause a diseased state in a small group of plants called the 'host plants'(Freeman and Beattie, 2008). Host plants mount defence responses called the 'host resistance' which is pathogen-specific and is restricted to a particular pathogen species (Heath, 2000). In contrast, plants that do not develop disease when infected with a pathogen are called 'non-host plants' and the resistance exhibited by them is called 'non-host resistance'(Heath, 2000; Freeman and Beattie, 2008). Non-host resistance is the most common form of resistance exhibited by plants against majority of the potential pathogens (Mysore and Ryu, 2004; Hammond-kosack and Parker, 2003). Several excellent reviews have summarized the non-host responses in plants (Heath, 2000; Mysore and Ryu, 2004; Cell, 2006; Niks and Marcel, 2009; Senthil-kumar, 2013; Gill et al., 2015). In the current study, host resistance has been reviewed elaborately.

1.2. Host defence responses

Broadly, plant defence mechanisms can be classified as (i) *constitutive* and (ii) *induced*. Physical barriers like wax, suberin and cuticle as well as chemical barriers

such as secondary metabolites (phenolics, terpenoids and alkaloids) are constantly present in the plant system and comprise the constitutive level of defence (Wittstock and Gershenzon, 2002; Taiz and Zeiger, 2006). In order to gain access to the plant interior, pathogen needs to break such constitutive barriers. Some bacterial and oomycete pathogens manage to enter through wounds or natural openings like stomata (Freeman and Beattie, 2008). Few other pathogens secrete molecules that dissolve physical barriers and facilitate their direct entry into plants. For instance, fungi, such as *Botrytis cinerea* and *Fusarium oxysporum* secrete the enzyme cutinase which degrades the protective cuticle of the cell wall (Serrano et al., 2014). As a next level of immunity, plants possess the ability to perceive these invading pathogens and initiate the so called 'induced defence response'. The induced defence response in plants is accomplished by adopting two-tier surveillance system of pathogen perception which finally results in 'pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI)' and 'effector-triggered immunity (ETI)' in the host plants (Figure 1.1) (Chisholm et al., 2006; McDowell and Simon, 2008; Dodds and Rathjen, 2010; Bernoux et al., 2011; Asaf and Marg, 2013; Andolfo and Ercolano, 2015; Kushalappa et al., 2016). Apart from the induced defence responses at a localized site, plants also trigger systemic level responses called as systemic acquired resistance (SAR) and induced systemic response (ISR). Both of these resistances prime the plants for future infections (Vallad and Goodman, 2004; Gozzo and Faoro, 2013). Aspects of PTI, ETI and SAR defence responses have been discussed further.

1.2.1. PAMP-triggered immunity (PTI)

Certain structures or epitopes are conserved over a wide range of plant pathogens and are known as pathogen-associated molecular patterns (PAMPs) (Millet et al., 2010; Nicaise et al., 2009; Halim et al., 2009). Identification of such conserved patterns is a smart way to recognize and induce defence responses against a broad range of pathogens. As a consequence, plants carry an array of membrane bound receptors called pattern recognition receptors (PRRs), whose function is to recognize and bind to PAMPs. Highly conserved structures such as bacterial flagellin (Gómez-Gómez and Boller, 2002) and lipopolysaccharide (Desaki et al., 2006) and fungal chitin (Felix et al., 1993) and ergosterol (Granado et al., 1995) act as PAMPs and are promptly recognized by plant PRRs. Perception of PAMPs by PRRs is followed by

elicitation of defence responses and induction of PTI which then restricts further invasion of pathogen (Figure 1.1 A). Hence, PTI represents the first line of pathogen perception (McDowell and Simon, 2008).

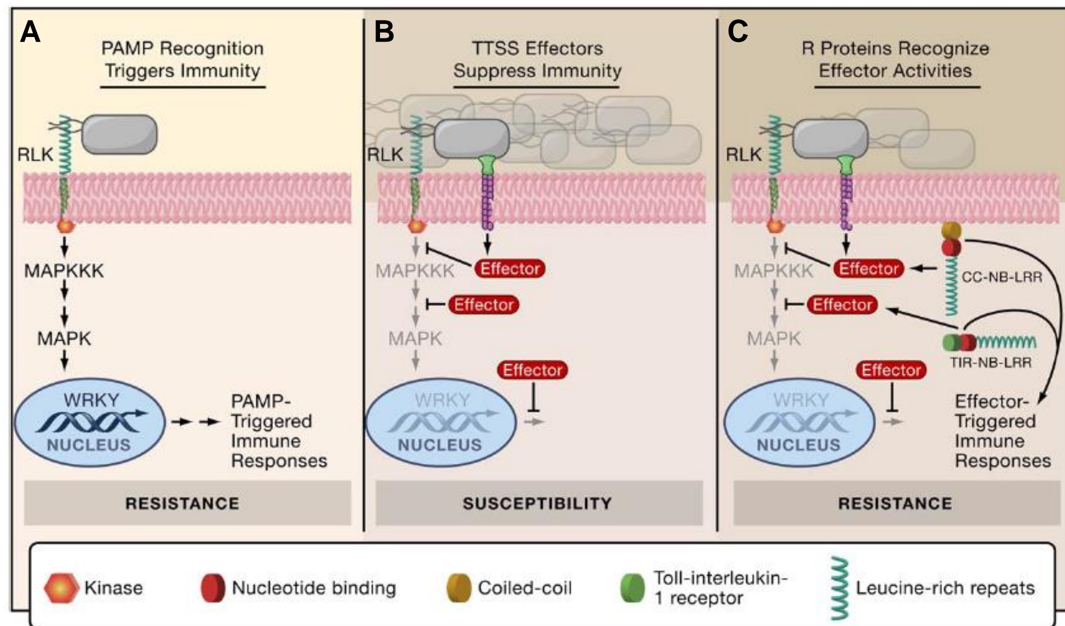


Figure 1.1. Model for plant-bacteria co-evolution. Left to right: Recognition of the bacterial flagellin (a pathogen-associated molecular pattern, PAMP), by plant transmembrane RECEPTOR-LIKE KINASE (RLK) (a pattern recognition receptor, PRR) results in the activation of MAPK signalling cascade and induction WRKY and other response genes. As a result, resistance is induced due to PAMP-Triggered-Immune (PTI) responses. However, bacteria have evolved mechanisms, like, secretion of effector molecules that interfere with the PTI responses via their Type 3 secretory system (T3SS) into the plant system. This result in a condition called effector-triggered susceptibility (ETS). Plants at their end have also evolved receptors that can detect these effectors. The R-proteins, CC-NB-LRR and TIR-NB-LRR, bring about the effector-triggered immune (ETI) responses *(Chisholm et al., 2006) - Reproduced with permission from Elsevier.*

PTI constraints pathogen growth and reproduction. However, certain pathogens have evolved strategies to release such constraints and escape PTI. Pathogens achieve this by either modifying or discarding the molecules that were recognised as PAMPs by PRRs (Attard et al., 2008). Often times PAMPs prove to be essential for pathogen survival and fitness and cannot be lost without penalty. In such cases, pathogens have evolved an alternative mechanism of PTI-suppression by secreting “effector” proteins into the apoplast and cytoplasm of the host plants

(Chisholm et al., 2006; Talbot, 2004). These pathogen-secreted effectors are products of *avirulence (Avr)* genes and are known to interact with components of PTI and interfere with the defence signalling pathways. By manipulating and blocking the defence signals, effectors promote disease progression in plants; such a state is called effector triggered susceptibility (ETS) (Figure 1.1 B). AvrPto and AvrPtoB are well studied *Pseudomonas syringae* pv. *tomato* effector proteins that target the components of PTI responses thereby rendering the plant susceptible (Mansfield and Elicitors, 2009; Dodds and Rathjen, 2010). In contrast, the effector fails to promote diseased state when delivered into a plant carrying corresponding resistance (R) protein (Xing et al., 2007). R-proteins are the products of *R-genes* and comprise the main players of second line of pathogen perception leading to ETI (McDowell and Simon, 2008).

1.2.2. Effector-triggered immunity (ETI)

It is evident that the interaction between a plant and its pathogen is an arms race and both try to co-evolve in the process (Chisholm et al., 2006). Unlike PRRs perceiving conserved motifs in all pathogens, R-protein recognizes its corresponding effector only. The induction of defence related genes on effector recognition by R-proteins contributes to ETI (Figure 1.1 C). Because of the one-to-one correlation between an *R-gene* and its *Avr-gene*, ETI is also known as gene-for-gene resistance. Also, the magnitude of defence response elicited in ETI is higher than that elicited during PTI (van Ooijen et al., 2007; Dodds and Rathjen, 2010). About 60 R-genes have been isolated from different plant species (Xiao et al., 2008) since the isolation of 1st R-gene in 1992 (Johal and Briggs, 1992). Kushalappa and co-workers (2016) have excellently summarized different R-genes and their products from various plant-pathogen interactions in their recent review (Kushalappa et al., 2016).

R-proteins can recognize effectors either directly or indirectly (Figure 1.2). In direct recognition, R-proteins recognize and bind to the effectors directly by physical association (Chisholm et al., 2006). For example, it has been shown by yeast two-hybrid and in vitro binding assays that the rice R-protein Pi-ta directly binds to the fungal effector AvrPita (Jia et al., 2000). In case of indirect recognition, R-proteins recognize the activity of the effectors. Here, the R-protein guards an accessory protein which is modified by the effector activity; therefore, it indirectly perceives the

effector (Mackey et al., 2002; Dodds and Rathjen, 2010). The R-protein can either identify a modified accessory protein (guard and decoy model) or the interaction of the effector to the R-protein associated accessory protein may guide in recognition of the effector by the R-protein (bait model) (Figure 1.2) (Dodds and Rathjen, 2010; Xiao et al., 2008; Chisholm et al., 2006). Finally, these interactions result in detection of the pathogen and activation of defence responses.

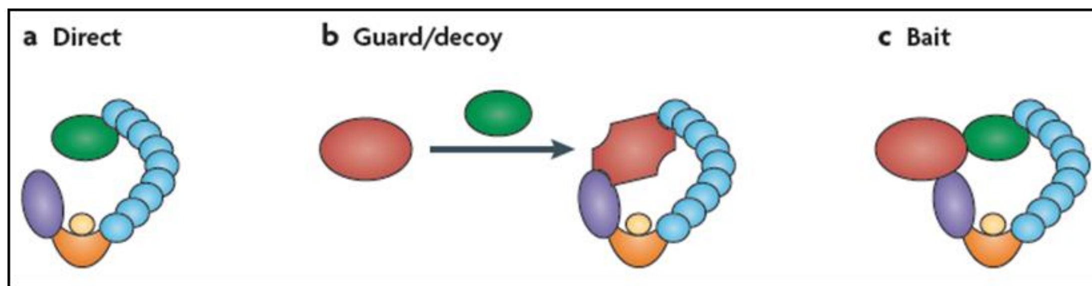


Figure 1.2: Different models of R-protein and effector interactions. a. In direct interaction, the R-protein recognizes the effector (green) by physically interacting with it. **b.** While according to guard/decoy model, activity of effector is perceived by R-protein. R-protein guards an accessory protein (red) which is modified by the effector. The modified accessory protein is recognized by the R-protein, leading to the activation of defence signalling. **c.** Bait model suggests that interaction of effector with the accessory protein that is physically associated with R-protein leads to the recognition of effector *(Dodds and Rathjen, 2010) - Reproduced with permission from Nature Publishing Group.*

1.2.3. Systemic acquired resistance (SAR)

Apart from a localized defence response, a systemic level response also operates in plants. Two kinds of systemic responses are observed in plants (i) induced systemic response (ISR) which is induced by non-pathogenic soil bacteria, and (ii) systemic acquired resistance (SAR), which is activated by pathogenic microbes (Vallad and Goodman, 2004; Gozzo and Faoro, 2013). Many interesting and informative reviews on ISR can be found elsewhere (van Loon et al., 1998; Heil and Bostock, 2002; Choudhary et al., 2007; Shoresh et al., 2010; Bakker et al., 2013; Pieterse et al., 2014). For the purposes of this study, only the concept of SAR has been elaborated. The process of SAR was first recognized in 1901 and was termed as “systemic acquired resistance” in 1961 by A. Frank Ross (Smith, 2000; Ryals et

al., 1994). SAR is a mechanism of induced defence response at distant site of a locally infected plant. It is long lasting and is known to operate against a broad spectrum of pathogens (Vlot et al., 2008; Shah, 2009; Durrant and Dong, 2004). It was long considered that SAR pathway is activated after the formation of either necrotic disease lesion or hypersensitive response (HR) (Ryals et al., 1996). However, recent studies suggest that SAR activation occurs even without the necrosis/HR and might be involved in non-host specific resistance as well (Mishina, 2007; Liu et al., 2010b). Mishina et al (2007) also argued that SAR induced by PTI and ETI are mechanistically identical (Mishina and Zeier, 2007). During SAR, several changes in gene expression occur at systemic-uninfected sites in response to a local pathogen infection (Ward et al., 1991; Uknes et al., 1992; Summermatter et al., 1995; Maleck et al., 2000; Gruner et al., 2013). In *Arabidopsis*, about 10% of the genes are transcriptionally activated upon SAR (Mukhtar et al., 2009). For example, accumulation of phytohormone salicylic acid (SA) and increased expression of PATHOGENESIS-RELATED (PR) family of genes are associated with the induction of SAR in plants (Cameron et al., 1999). The transcriptional co-activator, NPR1 (Nonexpressor of Pathogenesis-Related Genes 1) is a key SAR regulator. Function of NPR1 is discussed in the later section of this chapter.

SAR is established by transport of a systemic signal from the site of infection to the distant site (Jenns and Kuc, 1979; Guedes et al., 1980; Tuzun and Kuc, 1985). Several studies demonstrated the significant role of SA in SAR development and considered SA as the mobile SAR signal (Malamy et al., 1990; Yalpani et al., 1991, 1993; Gaffney et al., 1993; Shulaev et al., 1995). However, grafting studies in tobacco demonstrated that SAR was not impaired in wild-type (WT) scion grafted on SA-deficient rootstock (Vernooij et al., 1994; Pallas et al., 1996). In contrast, SAR was abolished in grafts containing WT rootstock and SA-deficient scion. This indicated that SA is not a mobile SAR signal and *de novo* SA accumulation is required at the systemic sites to establish an effective SAR response. This initiated the quest for new SAR signals. Extensive research thereafter has led to the identification of multiple potential SAR-associated genes and mobile signals apart from SA and PR genes (Figure 1.3). Among the most promising signals are (i) the methylated derivative of SA (MeSA) (Park et al., 2007b), (ii) a nine carbon (C9) dicarboxylic acid azelaic acid (AzA) (Jung et al., 2009), (iii) the phosphorylated sugar glycerol-3-phosphate (G3P) or its derivatives (Chanda et al., 2011), (iv) a

diterpenoid dehydroabietinal (DA) (Chaturvedi et al., 2012) and (v) an amino acid derivative pipecolic acid (Pip) (Návarová et al., 2012). Apart from these, few other molecules are also shown to be strongly associated with the process of SAR. Some examples are the lipid transfer proteins (LTPs): DEFECTIVE IN INDUCED RESISTANCE (DIR1) (Maldonado et al., 2002) and AzA INSENSITIVE (AZI1) (Jung et al., 2009), auxin (Truman et al., 2010), the factors involved in cuticle formation (Xia et al., 2009, 2010) and the free radicals: nitric oxide (NO) and reactive oxygen species (ROS) (Wendehenne et al., 2014). Interestingly, increasing evidences suggests that all these signals function in an interconnected fashion to establish an effective SAR response (Shah and Zeier, 2013). Here, we have reviewed some of these SAR-related genes and mobile signals (Figure 1.3). Some excellent reviews on SAR can also be found elsewhere (Heil and Ton, 2008; Dempsey and Klessig, 2012; Shah and Zeier, 2013; Kachroo and Robin, 2013; Gozzo and Faoro, 2013; Fu and Dong, 2013; Gao et al., 2015).

1.2.3.1. Methyl salicylate (MeSA)

Seskar and co-workers (1998) were the first to show that tobacco plants infected with tobacco mosaic virus (TMV) results in increased accumulation of MeSA in both local and systemic leaves (Seskar et al., 1998). Later, studies by Park and co-workers (2007) established that MeSA gets accumulated in phloem enriched exudates (PEX) of TMV-infected tobacco plants and is a mobile SAR signal (Park et al., 2007b). By generating grafts using WT plants and plants silenced for SA-BINDING PROTEIN 2 (SABP2, the protein that converts MeSA to SA), these authors showed that SAR is not established whenever SABP2-silenced plants were used as scion (Figure 1.4 A) suggesting that SABP2 activity is required only in systemic leaves (Park et al., 2007b; Heil and Ton, 2008). In contrast, when grafts were generated with WT plants and plants silenced for SA-METHYL TRANSFERASE 1 (SAMT1, the protein that converts SA to MeSA), no SAR was observed when SAMT1-silenced plants were used as rootstock (Figure 1.4 B). This suggested that SAMT1 activity is required only in local leaves (Park et al., 2007b; Heil and Ton, 2008; Liu et al., 2010a). Hence, it was proposed that MeSA is produced from SA by the activity of SAMT1 in local leaves. MeSA is then transported to systemic leaves, where it is converted into biologically active SA by

SABP2. Increasing SA levels then activate multiple downstream processes to induce SAR (Figure 1.4 C).

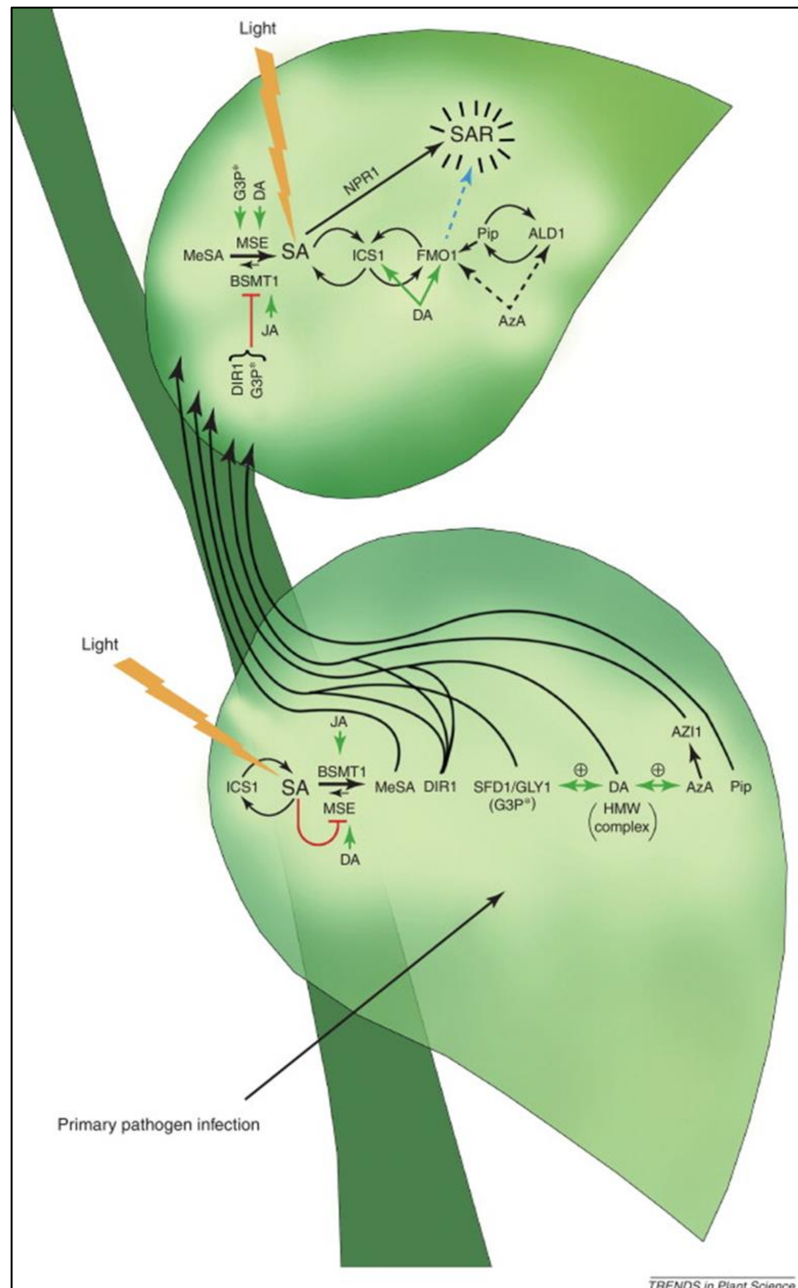


Figure 1.3. Summary of potential mobile signals in SAR. Upon primary pathogen infection, multiple mobile signals such as MeSA, DIR1, AzA, Pip, DA, G3P-derivative are thought to move from local site to systemic site through the phloem. In systemic site, activation of defence responsive gene such as ICS1, NPR1, FMO1 and ALD1 leads to SAR establishment. Detailed description of the molecules involved is provided in the text *(Dempsey and Klessig, 2012) - Reproduced with permission from Elsevier.*

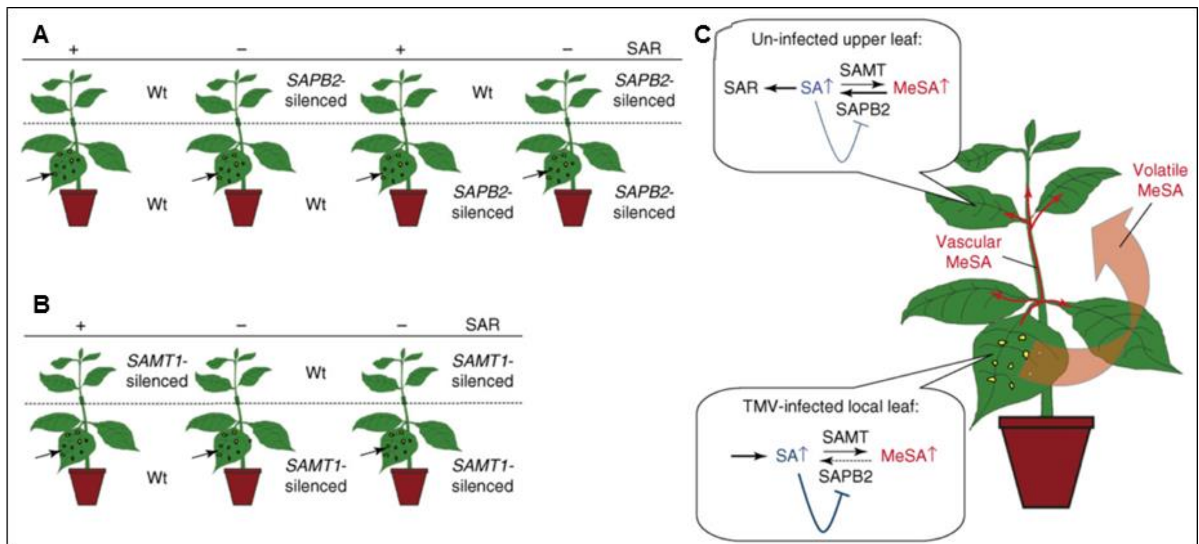


Figure 1.4. The role of MeSA as a mobile SAR signal. A. Grafting experiments with plants silenced for SA-binding protein 2 (SABP2) gene demonstrated that SAR is successfully induced in the WT scions of the grafts where SABP2–silenced plants (which cannot convert MeSA into SA) were used as rootstock. However, SAR was not observed in the grafts where SABP2-silenced plants were used as scions. **B.** Conversely, SAR was not observed in grafts were SAMT1-silenced plants (which cannot produce MeSA from SA) were used as rootstock. (+) SAR induced, (-) no SAR. **C.** SAMT1 is involved in conversion of SA to MeSA in local leaves. Here, high SA levels inhibit the activity of SABP2. The MeSA moves to systemic tissue through phloem and/or as airborne signal. In systemic site, MeSA is converted to SA by SABP2 activity. As SA levels are not very high in systemic leaves, the inhibition on SABP2 activity is lifted *(Heil and Ton, 2008) - Reproduced with permission from Elsevier*

Though MeSA was shown to act as a mobile signal in tobacco (Park et al., 2007b), Attaran and co-workers (2009) demonstrated that, in *Arabidopsis*, MeSA is not a mobile SAR signal (Attaran et al., 2009). This created a debate regarding the status of MeSA as a mobile signal. However, Liu et al (2011) showed that the extent to which MeSA is used as a mobile signal depends on the amount of light received by plants post primary infection (Liu et al., 2011a). These authors also addressed that differences in experimental design such as, plant age, time of infection and light conditions could have led to the contradictory results obtained by Park et al. (2007) and Attaran et al. (2009) (Liu et al., 2011a).

1.2.3.2. Azelaic acid (AzA)

Azelaic acid (AzA), a nine carbon (C9) dicarboxylic acid, was first identified by Jung et al. (2009) from the PEX of *Arabidopsis* plants infected with avirulent bacteria (Jung et al., 2009). Recovery of radiolabelled AzA from PEX and the systemic leaves suggested its ability to move systemically. Further, these authors showed that application of AzA induced local as well as systemic resistance. This systemic resistance was not induced by direct increase of SA and PR1 levels in the systemic leaves, rather by priming the leaves for enhanced resistance during the subsequent infection (Jung et al., 2009). It was also observed that AzA-mediated SAR induction requires the lipid transfer proteins, AZI1 and DIR1 (Jung et al., 2009). The involvement of DIR1 in AzA and MeSA signalling (Liu et al., 2011b) suggests that different signals might be mediating the SAR process through common molecular players (Dempsey and Klessig, 2012).

1.2.3.3. Glycerol-3-phosphate (G3P)- dependent signal

Characterization of the SAR-defective mutant, *sfd1/gly1* (suppressor of fatty acid desaturase deficiency 1), revealed that it encodes for glycerol-3-phosphate dehydrogenase (G3Pdh) (Nandi et al., 2004). G3Pdh is involved in generation of G3P which is the precursor for all glycerolipids essential for growth and defences (Chanda et al., 2008). Hence, G3P or its derivatives were suspected to be responsible for the defective SAR response of *sfd1/gly1* (Chanda et al., 2011) . Chanda et al. (2011) showed that G3P increases in both local and systemic leaves during SAR and the combined application of G3P and avirulent pathogen restored SAR in *gly1* mutant plants. However, radiolabelled G3P could not be detected in systemic leaves suggesting that G3P itself is not phloem-mobile, whereas its derivatives could be. Further, these authors showed that G3P and DIR1 required each other for phloem translocation in *Arabidopsis* (Chanda et al., 2011).

1.2.3.4. Dehydroabietinal (DA)

Another scan of PEX from *Arabidopsis* plants infected with avirulent pathogen revealed an abietane diterpenoid, Dehydroabietinal (DA), as a potent SAR inducer (Chaturvedi et al., 2012). These authors demonstrated that exogenous

application of DA induces SAR in *Arabidopsis*, tobacco and tomato. Further, locally applied DA was rapidly translocated to systemic leaves and resulted in an induced SA accumulation and PR1 expression. It was observed that gene that are critical for biologically-activated SAR, such as, NPR1 (NON-EXPRESSOR OF PR GENES1), FMO1 (FLAVIN-DEPENDENT MONOOXYGENASE1) and DIR1 were also required for the DA-induced SAR. (Chaturvedi et al., 2012). However, whether DA is a long-distance SAR signal remains to be investigated.

1.2.3.5. Pipecolic acid (Pip)

Recently, a non-protein amino acid pipecolic acid (Pip) was also suggested to play important role in SAR (Návarová et al., 2012). Pip was demonstrated to be accumulated in local and systemic leaves as well as in PEX after infection. Further, SAR-defect observed in *ald1* mutants (AGD2-LIKE DEFENSE RESPONSE PROTEIN1) was attributed to lack of Pip production and exogenous Pip application was able to rescue the SAR-defect of *ald1* plants (Návarová et al., 2012). These results indicated Pip as a critical player in SAR response in *Arabidopsis*.

Undoubtedly, an enormous knowledge has been generated with respect to our understanding of PTI, ETI and SAR. However, the recent discoveries have also brought forward novel questions that would be worth investigating to better understand the plant-pathogen interaction.

1.3. Role of hormones in plant-pathogen interaction

Plant hormones or phytohormones play essential role in plant growth and development as well as in response to environmental cues such as biotic and abiotic stresses (Denancé et al., 2013). It has long been known that phytohormones salicylic acid (SA), jasmonic acid (JA) and ethylene have vital role in plant defence responses (Glazebrook, 2005; Erb et al., 2012; Pieterse et al., 2012; Wasternack, 2014). However, recently the growth-mediating hormones auxin, abscisic acid (ABA), cytokinins, gibberellins, and brassinosteroids, also have emerged as key regulators of plant immunity (Santner and Estelle, 2009; Sun, 2011; Zhao and Li, 2012). These studies have revealed involvement of complex networks of phytohormone interaction in mediating a balance between plant growth and defence. Recent reviews have elegantly summarized several aspects of such phytohormone cross-talk (Robert-Seilaniantz et al., 2011; Naseem and Dandekar, 2012; Denancé et al., 2013; Huot et

al., 2014; Naseem et al., 2015; Verma et al., 2016). It has been long acknowledged that plants redistribute their energy reserves between growth and fitness during stress conditions. Allocation of resources to defence responses often leads to reduced growth. For e.g. the drastically reduced growth observed in plants expressing constitutive defence responses may be due unnecessary diversion of energy from growth in the absence of a pathogen attack (Huot et al., 2014). Hence, plants need to maintain a growth-defence tradeoff in order to remain healthy and phytohormones crosstalk has fundamental role in fine-tuning this tradeoff (Huot et al., 2014). Additionally, several studies have shown that certain pathogens have the ability to modify plant hormone signalling, especially auxin signalling, to facilitate infection (Yamada, 1993; Glickmann et al., 1998; Donnell et al., 2003; Chen et al., 2007; Kidd et al., 2011). This further highlights the importance of phytohormones in plant defence. For the purposes of the present study, only SA and auxin and their cross-talk has been reviewed in the following section.

1.3.1.1. Salicylic acid (SA)

SA is known for its central role in plant-pathogen interaction involving biotrophic or hemi-biotrophic pathogens (Denancé et al., 2013). Levels of SA increase in the local-infected as well as systemic-uninfected sites after a pathogen attack (Malamy et al., 1990; Yalpani et al., 1991; Cameron et al., 1999). Spraying leaves with SA or its analogs have resulted in enhanced resistance in various plants against biotrophic pathogens. In contrast, SA-deficient *nahG* transgenic tobacco and *Arabidopsis* plants (expressing the SA hydrolysing enzyme) are extremely susceptible to pathogen infection (Gaffney et al., 1993; Delaney et al., 1994). The role of SA in SAR via MeSA pathway has been discussed in the previous section. Though role of SA in plant defence is widely studied, the identity of SA-receptor is still debated. Recently in 2012, Fu and co-workers identified NPR3 (NON-EXPRESSOR OF PR GENES 3) and NPR4, the paralogs of NPR1, as the long sought after receptors of SA (Fu et al., 2012). As mentioned before, NPR1 acts as a transcriptional co-activator and is a key SAR regulator. Several studies have elaborated the role of NPR1 in SA signalling (Dong, 2004; Wang et al., 2006; Mukhtar et al., 2009; Fu and Dong, 2013; Kaltdorf and Naseem, 2013). Under, normal conditions NPR1 is present in the cytoplasm as an oligomer (Mou et al., 2003). When SA levels increase after an infection, NPR1 monomers are released

from the oligomer state, which are free to enter the nucleus. In the nucleus, along with TGA transcription factors, NPR1 controls the expression of several defence related genes including PR genes and WRKY transcription factors (Zhang et al., 1999; Després et al., 2000; Zhou et al., 2000; Kinkema et al., 2000; Wang et al., 2006).

It has been shown previously that for proper functioning of this process, NPR1 needs to be constantly removed from the nucleus by the proteosomal degradation pathway (Spoel et al., 2009). As transgenic plants overexpressing NPR1 exhibit constitutively active defence (CAO et al., 1998; Chern et al., 2001; Friedrich et al., 2001; Lin et al., 2004), it is believed that constant removal and turnover of NPR1 is necessary for the fitness of the plants. According to the recent study by Fu et al (2012), NPR1 degradation is mediated by NPR3 and NPR4 that function as substrate-adaptors for CULLIN3-proteosomal complex. As NPR3 and NPR4 have different affinities for SA, they were proposed to mediate NPR1 degradation under high and low SA conditions respectively, thereby regulating basal defence, ETI and SAR responses (Fu et al., 2012). These authors also demonstrated that NPR1 does not have considerable SA binding activity. In contrast, Wu et al (2012) showed that NPR1 functions as SA receptor (Wu et al., 2012) and binds to SA with higher affinity than NPR3 and NPR4 (Kuai et al., 2015). While the quest for SA receptors remains, Manohar and co-workers (2015) have identified around 80 potential SA-binding proteins (SABPs) using three different assays (Manohar et al., 2015). Similar to Wu et al (2012), these authors also found that NPR1 is a potential SABP. In view of these studies, Kuai et al (2015) have raised questions about the conclusions of Fu et al (2012) regarding NPR3 and NPR4 as the SA receptors (Kuai et al., 2015). Only future studies can bring a clear picture regarding the status and number of SA receptors.

1.3.1.2. Auxin

Auxin is one of the important phytohormones that controls many fundamental aspects of plant growth and development especially stem and petiole elongation and root development (Vanneste, 2009; Leyser, 2010; Gallavotti, 2013; ENDERS and STRADER, 2015). Several studies have dissected the players involved in auxin biosynthesis, transport, signalling and conjugation (Teale et al., 2006; Zhao, 2010; Ljung, 2013). AUXIN RESPONSE FACTORS (ARFs) are the transcription factors

that mediate the expression of auxin responsive genes by binding to auxin response elements (AREs) in their promoter (Hagen and Guilfoyle, 2002; Chandler, 2016; Li et al., 2016). Under low auxin conditions, the AUX/IAA repressors form heterodimers with ARFs and inhibit expression of auxin responsive genes (Ulmasov et al., 1999; Tiwari et al., 2001; Liscum and Jw, 2002; Tiwari et al., 2004). The F-box protein, TRANSPORT INHIBITOR RESISTANT 1 (TIR1) is the receptor for auxin and TIR1/ AFB (AUXIN SIGNALING F-BOX) act as the substrate-recognition component of an SKP–Cullin–F-box (SCF) E3 ubiquitin ligase complex ($SCF^{TIR1/AFB}$) (Gray et al., 1999). During high auxin conditions, auxin facilitates the binding of $SCF^{TIR1/AFB}$ to AUX/IAA repressors, resulting in their degradation by 26S proteasome pathway (Kepinski and Leyser, 2004, 2005; Dharmasiri et al., 2005). This releases the repression on ARFs and leads to increased expression of auxin responsive genes. Among the genes that get activated by auxin are Aux/IAAs and GH3 family genes, which encode auxin-conjugating enzymes that inactivate auxin (Hagen et al., 1984; Abel et al., 1994). This, therefore, replenishes the repressor pool and hence a negative feedback loop is maintained.

1.3.1.3. Crosstalk between SA and auxin signalling

Recent studies have shown that plants actively suppress auxin signalling processes to mount SA-mediated defence responses, thereby implicating the importance of antagonistic crosstalk between SA and auxin signalling pathways (Figure 1.5) (Kazan and Manners, 2009; Denancé et al., 2013; Huot et al., 2014; Verma et al., 2016). A microarray based study by Wang et al. (2006) demonstrated that treatment of *Arabidopsis* plants with BTH (benzo(1,2,3)thiadiazole-7-carbothioic acid *S*-methyl ester is an SA analog) represses the genes involved in auxin signalling, import and export, whereas upregulates GH3 family of genes (Wang et al., 2006). A follow-up study by the authors showed that treatment of plants with SA results in stabilization of AUX/IAA repressors through the suppression of auxin receptor TIR1, thereby inhibiting the expression of auxin responsive genes (Figure 1.5) (Wang et al., 2007). These authors also observed that SAR induction leads to suppression of majority of the auxin responsive genes in *Arabidopsis* (Wang et al., 2007). Correspondingly, plants with constitutively active SA signalling showed highly retarded growth (Zhang et al., 2003) because of the suppression of auxin-mediated growth. On the other hand, SA-deficient plants

(expressing the salicylate hydroxylase) exhibit increased levels of endogenous auxin, IAA (indole-3-acetic acid) (Abreu and Munné-Bosch, 2009). Studies showed that *Arabidopsis* plants treated with auxin resulted in suppression of SA-mediated PR1 expression (Figure 1.5) (Wang et al., 2007; Park et al., 2007a). Consistent with this antagonistic relationship, auxin signalling mutants *axr1*, *axr2* and *axr6* are observed to be more susceptible to the necrotrophic fungi *B. cinerea* and *Plectosphaerella cucumerina* (Llorente et al., 2008).

One of genes that mediate crosstalk between SA and auxin signalling is *Arabidopsis* GH3.5 (*wes1* gene) (Figure 1.5). The GH3 family of genes encode proteins that adenylate phytohormones IAA, JA and SA, which in some cases also catalyse their conjugation to amino acid (Staswick et al., 2002, 2005). Zhang and co-workers (2007) showed that *Arabidopsis* GH3.5 possesses *in vitro* adenylation activity on both IAA and SA (Zhang et al., 2007). Further, these authors showed that *Arabidopsis* activation-tagged mutants overexpressing GH3.5 were more resistant to infection than WT plants and exhibit increased SA and *PR1* levels in both local and systemic leaves. However, the GH3.5 T-DNA insertional mutants were partially compromised in SAR response along with reduced *PR1* expression in systemic leaves. This study suggested the role of GH3.5 as a bifunctional modulator in both auxin and SA signalling during pathogen infection (Zhang et al., 2007).

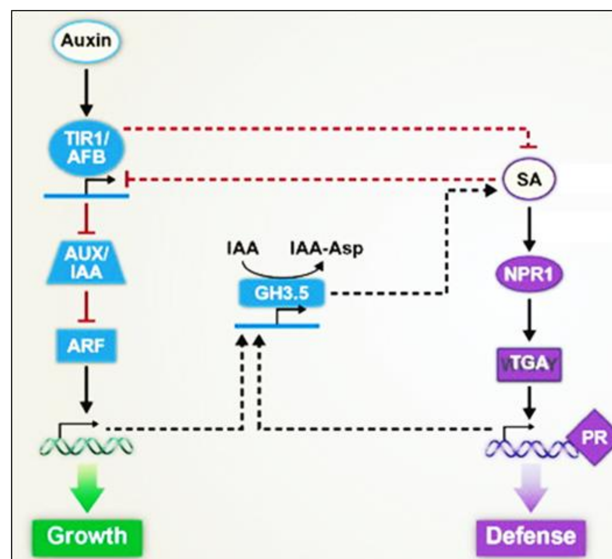


Figure 1.5 Antagonistic crosstalk between auxin-mediated growth and salicylic acid-mediated defence signalling pathways. Black arrows represent positive regulation and red, blunted lines represent negative regulation. Double helices and solid lines with bent arrows represent global transcriptional reprogramming and expression of TIR1/AFB and GH3.5

genes respectively. Known connections between two components are represented by solid lines, whereas unknown connections or missing steps in between two components are represented by dashed lines. TIR1, Transport Inhibitor Response 1; AFB, Auxin Signalling F-Box; AUX/IAA, Auxin-Inducible/IAA Inducible; ARF, Auxin Response Factor; IAA, Indole 3-Acetic Acid; Asp, Aspartate; NPR1, Nonexpressor of PR Genes 1; TGA, TGACG Sequence-Specific Binding Protein; PR, Pathogenesis Related *(Huot et al., 2014) - Reproduced with permission from Elsevier*

1.4. Plant microRNAs

MicroRNAs (miRNAs) are endogenous small ~21-nt non-coding RNAs that act as negative regulators of gene expression (Bartel and Bartel, 2003; Dugas and Bartel, 2004). miRNAs are generated by transcription of noncoding genes by RNA Pol II (Figure 1.6) (Katiyar-Agarwal and Jin, 2010). The primary miRNA transcript forms a stem-loop structure that is processed by the Dicer-like protein complex (DCL1-HYL1-SE) along with the DAWDLE (DDL) to produce precursor miRNA (pre-miRNA) (Yu et al., 2008). The pre-miRNA is further processed by DCL1-HYL1 complex to generate 21-nucleotide (nt) miRNAs. The newly synthesised miRNA (miRNA:miRNA∗) duplex is methylated at the 3'-ends by HUA ENHANCER 1 (HEN1) (Yu et al., 2005). These methylated miRNAs are then transported into cytoplasm by an exportin homolog, HASTY (HST) (Ruiz-Ferrer and Voinnet, 2009). The mature miRNA is 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 miRNA and the target mRNA (Vazquez et al., 2004). AGO1 then represses gene expression by either degrading the target mRNA or by repressing its translation.

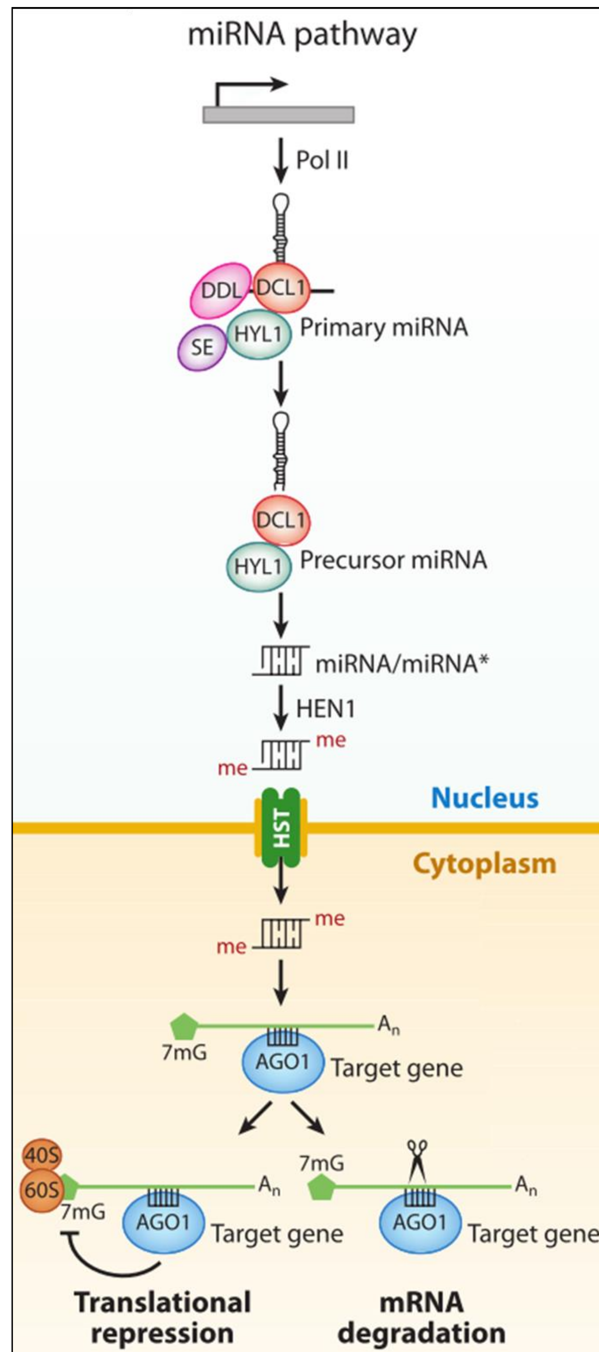


Figure 1.6. Biogenesis pathway of plant microRNAs. miRNAs are generated by transcription of noncoding genes by RNA Pol II. DCL1-HYL1-SE protein complex processes the primary miRNAs into precursor miRNA. This is further processed to generate 21-nt miRNAs by DCL1-HYL1 complex. The miRNA duplex is methylated at 3' ends by HEN1 and transported into cytoplasm by HST. Mature miRNA is then incorporated into RNA-induced silencing complex (RISC) containing AGO1 protein. The RISC is recruited to the target gene on the basis of sequence complementarity with incorporated miRNA leading to gene repression by either mRNA degradation or translational arrest. *Reproduced from (Katiyar-Agarwal and Jin, 2010)*

Since their discovery, hundreds of miRNAs have been identified in plants and are shown to regulate transcription factors and proteins that play important functions throughout the plant life beginning from embryo development and seed germination to reproduction and immunity (Figure 1.7) (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). For example, miRNA156 (miR156) and miR172 are shown to have important role in vegetative to reproductive phase transition in *Arabidopsis*. The expression patterns of these two miRNAs display some degree of opposite correlation. The expression of miR156 is high in the juvenile phase and keeps decreasing as the plant matures. In contrast, expression of miR172 is undetectable in juvenile phase and increases as the plant enters the reproductive phase. miR156 overexpression plants have a prolonged juvenile phase, whereas plants overexpressing miR172 show early flowering. *miR156* targets SQUAMOSA PROMOTER BINDING-LIKE (SPL) genes and one the SPL is shown to bind to the promoter of miR172. Hence, the sequential action of miR156 and miR172 is important for regulating developmental timing in *Arabidopsis* (Wu et al., 2009).

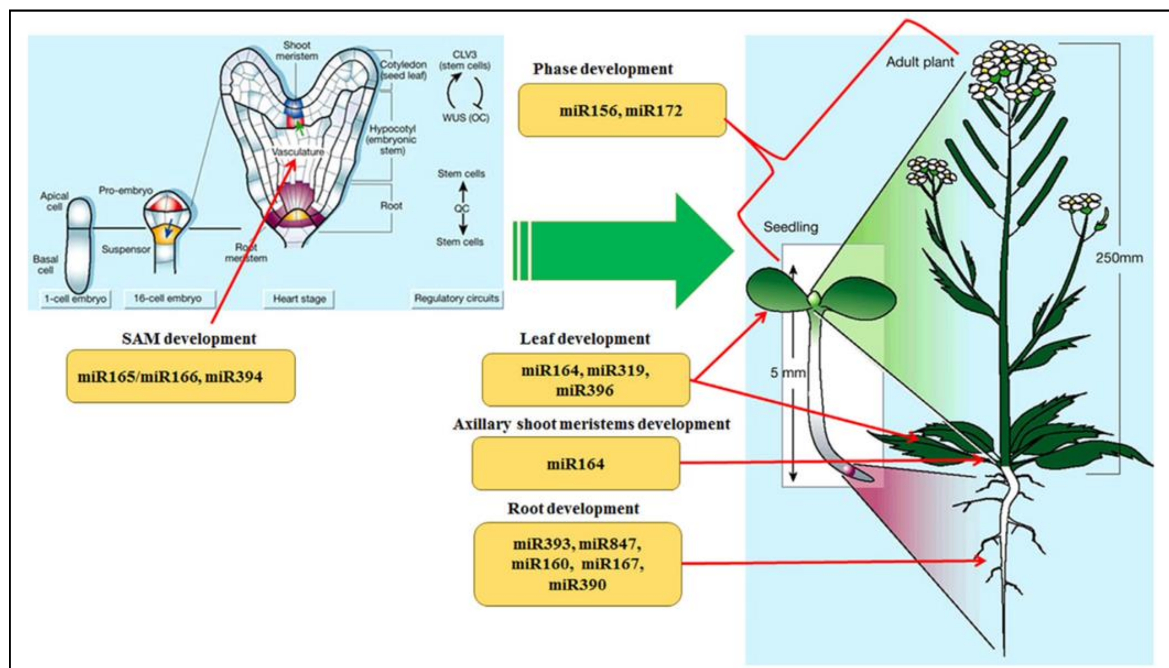


Figure 1.7 Role of miRNAs in plant growth and development. miRNAs play important roles in different aspects of plant growth and development. The figure depicts some of these miRNAs *(Li and Zhang, 2016; Weigel and Jürgens, 2002) - Reproduced with permission from John Wiley and Sons and Nature Publishing Group.*

1.4.1. miRNAs in plant – pathogen interaction

Recent studies suggest that miRNAs also play significant role during plant – pathogen interaction (Ruiz-Ferrer and Voinnet, 2009; Sunkar et al., 2012; Seo et al., 2013). The *Arabidopsis* miR393 was one of the first miRNAs to be demonstrated that has role in plant immunity. Navarro et al. (2006) found that treatment of *Arabidopsis* Col-0 seedlings with flg22 (a 22 amino acid N-terminal part of flagellin that acts as PAMP) leads to increased expression of miR393 (Navarro et al., 2006). These authors also showed that miR393 targets TIR1, the auxin receptor, that de-represses the inhibition on ARFs. As miR393 targets TIR1, it was proposed that miR393 is involved in the growth-defence tradeoff response and mediates the induction of PTI (Figure 1.8). Under non-infective conditions, miR393 levels are low, as a result TIR1 levels are high (Figure 1.8 A). This increased TIR1 levels inhibits 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 normal growth of plant. However, upon a pathogen attack, perception of pathogen by PRRs (like FLS2) induces expression of miR393 through unknown mechanisms. miR393 targets TIR1 for degradation and releases the inhibition on AUX/IAA. The ARFs are sequestered by AUX/IAA and the transcription of auxin responsive genes is reduced leading to enhanced PTI (Figure 1.8 B) (Navarro et al., 2006; Ruiz-Ferrer and Voinnet, 2009; Sunkar et al., 2012).

Some miRNAs are shown to target nucleotide-binding site (NBS) leucine-rich repeat (LRR) R-genes, which are involved in ETI, and trigger generation of secondary siRNAs (Zhai et al., 2011; Li et al., 2012; Shivaprasad et al., 2012). The tomato miRNAs, miR482 and miR2118, belongs to this category. It is observed that miR482-mediated silencing of R-genes are actively supressed by viral and bacterial infection (Shivaprasad et al., 2012) suggesting a counter-counter-defence strategy adopted by pathogens. Similar to above studies, multiple reports further unveiled the role of miRNAs in various plant-pathogen interactions. A summary of these reports is provided in Table 1.1.

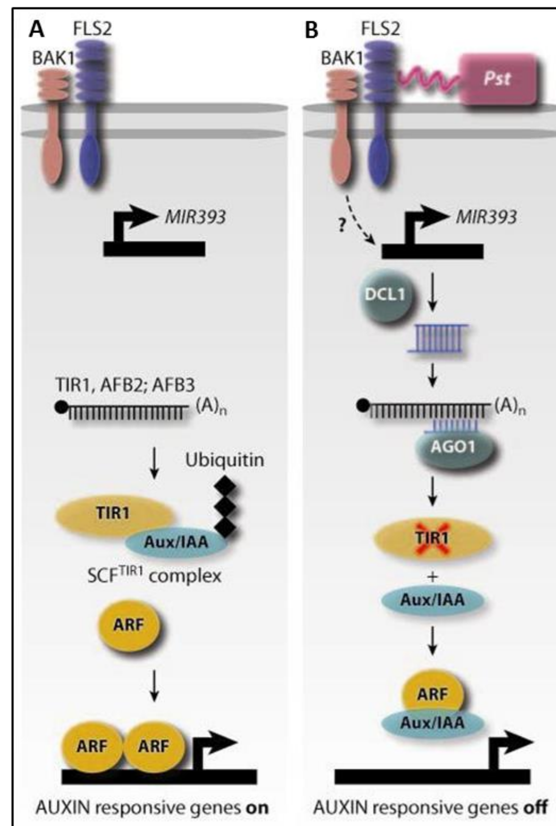


Figure 1.8 miR393-mediated balance of auxin-dependent growth and plant stress. A. Under low miR393 levels, TIR1 and related F-box proteins mark Aux/IAA factors for degradation, promoting auxin-responsive gene expression and suppression of defence. **B.** Upon flagellin perception by FLS2, *MIR393* is transcriptionally activated, resulting in suppression of *TIR1* mRNA and protein synthesis. The subsequent Aux/IAA accumulation reduces auxin-responsive gene expression, enhancing PTI. *Reproduced from (Ruiz-Ferrer and Voinnet, 2009).*

Table 1.1. List of miRNAs involved in plant-pathogen interaction. *Adapted and modified from (Huang et al., 2016) - with permission from John Wiley and Sons.*

miRNA	Plant	Pathogen	Target genes	miRNA Express-ion	Roles in plant-pathogen interaction	References
miR156	Wheat	Fungus *B. graminis f. sp. tritici*	Ta3711, Ta7012	Down	-	(Xin et al., 2010)
miR156	Loblolly pine	Fungus *C. quercuum f.sp.fusiforme*	NBS/LRR /TIR R-protein	Down	-	(Lu et al., 2007)
miR156	Tobacco	Virus, TMV	-	Up	-	(Bazzini et al., 2007)
miR159	*Arabidopsis*	Bacteria *P. syringae*	MYB33, MYB65, MYC101	Up	Affects GA and ABA signalling	(Zhang et al., 2011a)
miR159	Wheat	Fungus *B. graminis f. sp. tritici*		Down		(Xin et al., 2010)
miR160	*Arabidopsis*	Bacteria *P. syringae*	ARF10, ARF16, ARF17	Up	Involved in PTI response	(Li et al., 2010)
miR160	*M. esculenta*	Fungus *C. gloeosporio-ides*	ARF10	Up	Affects auxin signalling	(Pinweha et al., 2015)
miR160	*O. sativa*	Fungus *M. oryzae*	ARF16	Up	Affects accumulation of H_2O_2	(Li et al., 2014)
miR164	Wheat	Fungus *B. graminis f. sp. tritici*	-	Down	-	(Xin et al., 2010)
miR164	Tobacco	Virus, TMV	-	Up	-	(Bazzini et al., 2007)
miR166	Wheat	Fungus *B. graminis f. sp. tritici*	-	Down	-	(Xin et al., 2010)
miR167	*Arabidopsis*	Bacteria *P. syringae*	ARF8, ARF6	Up	Affects auxin signalling	(Fahlgren et al., 2007; Zhang et al., 2011a)
miR168	*O. sativa*	Viruses RSV and RDV	AGO1	-	Affects miRNA biogenesis	(Wu et al., 2015)
miR169	Loblolly pine	Fungus *C. quercuum f.sp.fusiforme*	Strictosidine synthase	-	-	(Lu et al., 2007)
miR169	Wheat	Fungus *B. graminis f. sp. tritici*	-	Down	-	(Xin et al., 2010)

miRNA	Plant	Pathogen	Target genes	miRNA Express-ion	Roles in plant-pathogen interaction	References
miR171	Loblolly pine	Fungus *C. quercuum f.sp.fusiforme*	Hypersensitivity-related protein	-	-	(Lu et al., 2007)
miR172	Loblolly pine	Fungus *C. quercuum f.sp.fusiforme*	AP2 transcription factor LRR protein kinase	-	-	(Lu et al., 2007)
miR390	*Arabidopsis*	Bacteria *P. syringae*	TAS3	Down	Affects auxin signalling	(Zhang et al., 2011a)
miR393	*Arabidopsis*	Bacteria *P. syringae*	TIR1, AFB2, AFB3	Up	Negatively regulates auxin signalling	(Navarro et al., 2006; Fahlgren et al., 2007)
miR394	*M. esculenta*	Fungus *C. gloeosporio-ides*	TIR1	Up	Affects auxin signalling	(Pinweha et al., 2015)
miR393b*	*Arabidopsis* and *Nicotiana benthamiana*	Bacteria *P. syringae*	MEMB12	Up	Increases PR1secretion	(Zhang et al., 2011b)
miR396	Wheat	Fungus *B. graminis f. sp. tritici*	-	Down	-	(Xin et al., 2010)
miR396a-5p	Tobacco	Oomycete *P. nicotianae*	GRF	Down	Negatively regulates resistance	(Chen et al., 2015)
miR398	*Arabidopsis*	Bacteria *P. syringae*	COX5b.1, CSD1 and CSD2	Down	Negatively regulates callose deposition	(Jagadeeswaran et al., 2009; Li et al., 2010)
miR398	*O. sativa*	Fungus *M. oryzae*	SOD2	Up	Affects accumulation of H_2O_2	(Li et al., 2014)
miR399	*Citrus sinensis*	Bacteria *C. liberibacter*	PHO2	Up	Affects Phosphorus homeostasis and signalling	(Zhao et al., 2013)
miR408	*Arabidopsis*	Bacteria *P. syringae*	Copper protein plantacyanin	Up/Down	-	(Zhang et al., 2011a)
miR408	Wheat	Fungus *Puccinia striiformis* f. sp. tritici	TaCLP1	Up/Down	Negatively regulates resistance	(Feng et al., 2013)
miR472	*Arabidopsis*	Bacteria *P. syringae*	CC-NBS-LRR	-	Negatively regulates resistance.	(Boccara et al., 2014)

miRNA	Plant	Pathogen	Target genes	miRNA Express-ion	Roles in plant-pathogen interaction	References
miR482	*S. lycopersicum*	Viruses TCV, CMV and TRV	NBS-LRR	Down	Affects expression of R protein.	(Shivaprasad et al., 2012)
miR482	*G. raimondii*	Fungus *V. dahliae*	NBS-LRR	Down	Affects expression of R protein	(Zhu et al., 2013)
miR482	*S. lycopersicum*	Fungus *F. oxysporum*	Solyc08g075630, Solyc08g076000	Down	Affects expression of R protein	(Ouyang et al., 2014)
miR773	*Arabidopsis*	Bacteria *P. syringae*	MET2	Down	Negatively regulate callose deposition	(Li et al., 2010)
miR825	*Arabidopsis*	Bacteria *P. syringae*	Remorin, zinc finger homeobox family	Up	-	(Fahlgren et al., 2007)
miR1507	*M. truncatula*	-	NBS-LRR	-	-	(Zhai et al., 2011)
miR1885	*Brassica napus*	Virus 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*	Viruses TCV, CMV and TRV	NBS-LRR	Down	Affects expression of R protein	(Shivaprasad et al., 2012)
miR5300	*S. lycopersicum*	Fungus *F. oxysporum*	*Solyc05g008650*, *tm-2*	Down	Affects expression of R protein	(Ouyang et al., 2014)
miR6019 /miR6020	*N. tabacum*	Virus TMV	TIR-NBS-LRR	-	Affects N-gene mediated resistance	(Li et al., 2012)
miR7695	*O. sativa*	Fungus *M. oryzae*	OsNramp6	-	Affects plant resistance.	(Campo et al., 2013)
miR9863	*Hordeum vulgare L.*	Fungus *Blumeria graminis* f. sp. *hordei*	Mla1	-	Affects resistance and cell-death signaling.	(Liu et al., 2014)

Undeniably, all these reports have established the role of miRNAs in PTI and ETI responses of various plants. However, role of miRNAs in the process of SAR remains unknown to the extent of our knowledge. As miRNAs are very important regulatory molecules, their role in SAR responses is worth investigating and can bring added dimension to the current knowledge of SAR.

1.5. Potato and *Phytophthora infestans* interaction

Late blight is one of the severe and devastating diseases of potato. The causal agent of late blight, a notorious oomycete pathogen *Phytophthora infestans*, was discovered by Anton deBary after the Irish Potato Famine in 1845 which lead to the death and emigration of millions of people (Sparrow, 1978). *P. infestans* is a hemi-biotroph with a genome size of 240 Mb, is considered to be a very sophisticated pathogen with a repertoire of effector genes employed to infect its host (Haas et al., 2009). Since, potato is the third-most important crop of the world, raising *P. infestans* resistant potato varieties has been the primary interest of plant breeders for several decades. The breeding programs involve the transfer of "resistance gene (R-gene)" from the wild relatives of potato to the cultivated varieties by the conventional crossing techniques (Kumar et al., 2006; Sliwka et al., 2010). Many R-genes have also been cloned from *Solanaceae* family in past two decades (Ooijen et al., 2007) and strategies have been developed to transfer them to cultivated crop varieties. However, the highly evolving nature of *P. infestans* consistently defeats the resistance gained by such strategies. It is worth noticing that few strains of *P. infestans* are now resistant to a number of fungicides such as metalaxyl (Rekanović et al., 2012). These alarming informations motivate researchers to find better strategies for developing *P. infestans* resistance potato varieties.

Though, enormous data has been generated regarding various R-genes in potato, the knowledge of SAR in potato is still rudimentary. Also, unlike other plant-pathogen systems (Table 1.1), role of miRNAs in potato-*P. infestans* interaction has not been investigated yet. From the few available reports, it can be gathered that, potato has high endogenous levels of SA and possibly operates a different SA signalling mechanism than *Arabidopsis* and tobacco (Coquoz et al., 1995; Yu et al., 1997; Navarre and Mayo, 2004). Yu et al (1997) showed that this high endogenous

levels of SA in potato, however, do not lead to constitutively active defence (Yu et al., 1997). These authors also concluded that SA is important for the SAR induced by treatment of arachidonic acid (AA, a PAMP of *P. infestans*), as the SA-deficient plants failed to induce a SAR response (Yu et al., 1997). Though AA induces SAR in potato, the increase in SA levels were observed only in the local treated leaves and not in the systemic untreated leaves (Coquoz et al., 1995; Yu et al., 1997). The function of SA in potato defence was debated in the earlier days, nevertheless, recent reports suggest its indispensable role in defence against AA, *P. infestans*, Potato virus X and Potato virus Y (Halim et al., 2007, 2009; Sánchez et al., 2010; Baebler et al., 2014). Additionally, studies by Manosalva et al. (2010) showed that AA-induced SAR is indeed accompanied by increased SA levels in systemic leaves as opposed to the observations of Yu et al (1997) (Manosalva et al., 2010; Yu et al., 1997). These authors also demonstrated that similar to tobacco and *Arabidopsis*, MeSA is a mobile signal in potato and methyl esterase, *StMES1* (the ortholog of tobacco SABP2), is involved in the conversion of SA to MeSA in the local leaves treated with AA (Manosalva et al., 2010). Undoubtedly, further studies are needed to understand the process of SAR in potato as well as role of miRNAs in potato-*P. infestans* interaction.

1.6. Hypothesis and Objectives

Based on the role of miRNAs in several important aspects of plant growth and development as well as their functions in immune responses such as PTI and ETI, we hypothesised that miRNAs could play significant role in SAR responses of plants too. Also, the role of miRNAs in potato-*P. infestans* interaction is not known. Hence, following objectives were considered to understand the role of miRNAs in local defence and in SAR responses using potato-*P. infestans* interaction as a model system:

i. To investigate the role of potential miRNAs and their targets in potato-*P.infestans* interaction.
ii. To characterize the role of miR160 in local defence and SAR responses of potato.
iii. To explore the mechanistic link of miR160 in defence response of potato.

Chapter 2

Investigating the role of potential microRNAs and their targets in potato-*Phytophthora infestans* interaction

2.1. Introduction

This chapter describes approaches used to identify potential miRNAs and targets that could be involved in potato-*Phytophthora infestans* interaction.

2.1.1. Plant microRNAs and their targets

MicroRNAs (miRNAs) are endogenous small ~21-nt non-coding RNAs that act as negative regulators of gene expression (Bartel and Bartel, 2003; Dugas and Bartel, 2004). Since their discovery in plants, miRNAs have been attributed to play crucial role in multiple processes of plant growth such as seed germination (Liu et al., 2007), vegetative to reproductive phase transition (Wu et al., 2009), leaf morphology and shoot apical meristem regulation (Jung and Park, 2007; Nogueira et al., 2009), root development (Wang et al., 2005), plant immunity (Navarro et al., 2006) and many other developmental processes (Kidner and Martienssen, 2005; Jones-Rhoades et al., 2006; Borges and Martienssen, 2015; Li and Zhang, 2016). A detailed description of plant miRNAs and their functions have been discussed in Chapter 1. Plant miRNAs are also shown to function in non-cell autonomous manner and move long distance as mobile signals (Pant et al., 2008; Buhtz et al., 2010; Kasai et al., 2010; Bhogale et al., 2014; Sarkies and Miska, 2014). All these functions are mediated by miRNAs through repression of their target genes. The repression could either occur by cleavage of the target mRNAs or by inhibition of translation process (Jones-Rhoades et al., 2006). Due to high complementarity observed between plant miRNAs and their targets, many earlier studies have suggested cleavage of target mRNA as a preferred mode of repression in plants (Jones-Rhoades et al., 2006). However, more recent evidences indicate that translational repression is also common in plants (Brodersen et al., 2008; Li et al., 2013; Xie et al., 2015).

2.1.2. Role of miRNAs in plant – pathogen interaction

The process of plant – pathogen interaction is highly complex and involves many molecular players ranging from mRNAs and proteins to hormones. Recent studies suggest that miRNAs also play significant role during plant – pathogen interaction (Ruiz-Ferrer and Voinnet, 2009; Sunkar et al., 2012; Seo et al., 2013). The

first report was in *Arabidopsis*, wherein treatment of Col-0 seedlings with flg22 (a 22 amino acid N-terminal part of flagellin that acts as PAMP) resulted in an induced expression of miR393 (Navarro et al., 2006). These authors further concluded that miR393 functions as a positive regulator of defence by repressing auxin signalling pathway. Following this, several small-RNA microarray and transcriptomic studies implicated differential regulation of miRNAs in different plant-pathogen interactions, such as powdery mildew infection of wheat (Xin et al., 2010), viral infection of tomato (Lang et al., 2011), blast and sheath blight disease of rice (Baldrich et al., 2015; Li et al., 2016; Lin et al., 2016). A detailed description of these studies is provided in chapter 1. Further, a number of reports revealed the role of specific miRNAs and their potential association in plant-pathogen interaction. For example, defence related role has been demonstrated for miR160 and miR398 in PAMP-triggered immunity (PTI) of *Arabidopsis* as well as in rice-*Magnaporthe* interaction (Li et al., 2010, 2014), miR6019 and miR6020 in *R*-gene regulation in *Solanaceae* family (Li et al., 2012) and miR9863 in powdery mildew of barley (Liu et al., 2014). All these studies substantiate the significant role of host endogenous small RNAs in plant immune responses. Seo and co-workers have also summarized similar proposition in their recent review (Seo et al., 2013). Although the above studies showed the role of miRNAs in local (basal) defence responses in various plant-pathogen interaction, no previous study has investigated the role of miRNAs in systemic acquired resistance (SAR).

One of the devastating plant diseases is the late blight of potato caused by oomycete pathogen *Phytophthora infestans*. Though several miRNAs were bio-informatically predicted in potato in the past decade, (Zhang et al., 2009; Yang et al., 2010b; Kim et al., 2011; Zhang et al., 2013), their functional characterisation have been limited to tuber development (Martin et al., 2009; Bhogale et al., 2014; Lakhotia et al., 2014). Only two reports have so far described the role of potato miRNAs in biotic (Yang et al., 2010a) and abiotic stress responses (Kitazumi et al., 2015). No report yet describes the role of miRNAs in potato- *P. infestans* interaction. In light of the recent knowledge that effector proteins of *Phytophthora* s*sp.* can suppress host small RNA-mediated defence response resulting in increased pathogenicity (Qiao et

al., 2013, 2015; Ye and Ma, 2016), studying the role of potato miRNAs in defence against *P. infestans* could be of immense importance.

In order to decipher the role of miRNAs in potato – *P. infestans* interaction, the following analyses were undertaken:

i. Shortlisting and validation of the candidate miRNAs with potential role in potato – *P. infestans* interaction.
ii. Expression analysis of candidate miRNAs at the local-infected and systemic-uninfected leaves to understand their involvement in basal defence as well as SAR responses, respectively.
iii. Prediction, validation and expression analysis of select target genes of miRNAs.

2.2. Materials and methods

2.2.1. Plant and pathogen materials

Susceptible (SUS) and moderately resistant (MR) wild potato (*Solanum chacoense*) varieties were obtained from Central Potato Research Institute (CPRI, Shimla, India). *In vitro* potato plants were grown and maintained at 25°C under long day conditions (LD; Long day, 16 hrs light: 8 hrs dark) in a tissue culture incubator (Percival Scientific). Plants were later transferred and grown in a soil mixture (equal parts of soil: perlite: soil rite: coco peat) and were maintained at 22°C under LD conditions in plant growth chambers (Percival Scientific).

Oomycete pathogen, *Phytophthora infestans* strain A2, was obtained from CPRI and maintained in corn media, pea agar media and potato slices throughout the study. The strain was confirmed by amplifying a part of Internal Transcribed Spacer 2 (ITS2) ribosomal DNA using primer sets PINF and ITS5 (Trout et al., 1997). For all the infection assays, *P. infestans* hyphae from 7-10 days old pea agar plate were scrapped and transferred to sterile water in a Petri-dish. To ascertain the viability of the sporangia, hyphae were teased apart and incubated at 4ºC to induce release of zoospores. Sporangia concentration was then adjusted to $2x10^5$ sporangia per ml and plants are infected by applying desired volume of sporangia solution to the abaxial side of leaves.

2.2.2. Detached leaf infection experiment and Trypan blue staining

To compare the basal resistance of SUS and MR varieties, detached leaves from 8 weeks old plants were infected with 100 µl of *P. infestans* sporangia (concentration of 2×10^5). Infection was monitored for 4 days (96 hrs) and disease progression was assessed by performing microscopic examination. Trypan blue staining was performed after 96 hrs as per previous protocol with minor modifications of using ethanol as de-staining solution instead of chloral hydrate (van Wees, 2008).

2.2.3. Detection of candidate miRNAs in wild-type potato

Total RNA was isolated using TRIzol reagent (Invitrogen) from whole plant tissue of wild type potato as per manufacturer's instructions. One microgram (1 µg) of total RNA was used for cDNA preparation of selected eleven miRNAs using respective stem-loop primers (STP) followed by their end-point PCRs using miRNA-specific forward and universal reverse primer as described earlier (Varkonyi-Gasic et al., 2007).

2.2.4. *P. infestans* infection experiment

P. infestans sporangia concentration of $2x10^5$ sporangia per ml was used and treated plants were incubated at 18°C with a 90% humidity. Time-course expression analysis of miRNAs and their targets in SUS and MR varieties were performed by inoculating 10 µl of sporangia solution on the abaxial side of 8th to 11th leaves (counted from the top of the plant). Inoculated local leaves (leaf no. 8, 9, 10 and 11) and non-inoculated systemic leaves (leaf no. 5, 6 and 7) were harvested at 0, 6, 12, 24, 48 and 96 hours of post inoculation (hpi). Tissues were frozen immediately in liquid nitrogen and stored at -80°C until further use.

2.2.5. Quantitative real-time PCR (qRT-PCR) analysis of miRNAs

To analyse miR160 levels upon *P. infestans* infection, total RNA was isolated from local and systemic leaves harvested after 0, 6, 12, 24, 48 and 96 hpi by TRIzol method. One microgram (1µg) of total RNA was used for reverse transcription reaction using both miRNA stem-loop (STP) and oligo(dT) primers. All the quantitative RT-PCR (qRT-PCR) reactions were set using the KAPA SYBR Green Mix (Kapa Biosystems) in Eppendorf Mastercycler ep Realplex 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, *GAPDH* was used and qRT-PCR was performed using oligo(dT) cDNA and gene-specific primers (GAPDH-FP and GAPDH-RP) with PCR conditions as 95°C for 3 min followed by

40 cycles of 95°C for 5 sec, 60°C for 20 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).

2.2.6. Northern blot analysis of miR160

The total RNA isolated previously for qRT-PCR, was used for northern blot analysis. Fifteen µg (15 µg) of total RNA was fractionated on a 12.5% 8M urea PAGE gel and transferred to nylon membrane overnight (Amersham Hybond™-N+ GE Healthcare). Post transfer, RNA was UV cross-linked to nylon membrane for 2 mins, followed by a 2 hrs pre-hybridization and an overnight hybridization at 30°C. For hybridization, 21-nt of miR160 reverse complement sequence (miR160-RC) and 21-nt of U6 splicisomal RNA reverse complement sequence (U6-RC) (Hendelman et al., 2016) were labelled with γ-^{32}P-ATP using KinaseMax™ 5' End-Labelling Kit (Ambion®). Membranes were exposed to storage phosphor screen for a period of ~ 24-120 hrs and imaged using Typhoon imager (GE Healthcare Life Sciences). Images were analysed and quantified using ImageJ software (Schneider et al., 2012).

2.2.7. Bioinformatics-based prediction of miR160 targets

For prediction of miR160 targets, three different target prediction softwares namely psRNATarget (plantgrn.noble.org/psRNATarget/) (Dai and Zhao, 2011), TargetAlign (leonxie.com/targetAlign.php) (Xie and Zhang, 2010) and TAPIR (http://bioinformatics. psb.ugent.be/webtools/tapir/) (Bonnet et al., 2010) were used. For all the target predictions, *Solanum tuberosum* transcript library from the Potato Genome Sequencing Consortium (PGSC) (http://solanaceae.plantbiology.msu.edu/ pgsc_download. shtml) was used as target database and default parameters were used.

2.2.8. Cleavage site mapping analysis of miR160 targets

For *in planta* validations of miR160 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 (ARF10-RP1 and ARF16-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 (ARF10-RP2 and ARF16-RP2). The amplicons were cloned in the sub-cloning vector pGEM-T Easy (Promega) and sequence verified to identify the miRNA cleavage sites.

2.2.9. qRT-PCR analysis of *StARF10*

Total RNA isolated previously from local and systemic leaves (0, 6, 12, 24, 48 and 96 hpi) were used for analysis of *StARF10* levels. Oligo(dT) cDNA was prepared from 1 µg of total RNA using Superscript III Reverse Transcriptase (Invitrogen). qRT-PCRs were performed for *StARF10* and *GAPDH* (normalization gene) using the primer sets ARF10-FP_ARF10-RP2 and GAPDH-FP_GAPDH-RP respectively. Reactions were set using KAPA SYBR Green Mix (Kapa Biosystems) and performed on the Eppendorf Mastercycler ep Realplex system using the program 95°C for 2 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 20 sec. Melting curve analysis was included in the program to check the PCR specificity and the data was analysed by using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

2.2.10. Detection of miR160 precursor, *St-pre160*

For miR160 precursor detection, cDNA was prepared from one microgram (1 µg) of total RNA using oligodT primer and Superscript III Reverse Transcriptase (Invitrogen) as per manufacturer's instructions. PCR was performed using primers pre160-FP and pre160-RP and reactions were carried out with following conditions: 94˚C for 3 min, 40 cycles of 94˚C for 10 sec, 61˚C for 15 sec and 72˚C for 20 sec and final extension at 72˚C for 5 min. The isolated precursor was finally cloned into the sub-cloning vector pGEM-T Easy (Promega) and sequence verified.

2.2.11. Histology and Laser capture microdissection of phloem cells

To detect the presence of miR160 in phloem cells of SUS and MR plants, histology was performed as described previously (Bhogale et al., 2014; Cai and Lashbrook, 2006). Briefly, 0.5 cm stem pieces were rinsed in RNAse-free pre-chilled water. Stem pieces were then fixed in chilled ethanol: acetic acid (3:1) solution and vacuum infiltrated by applying 500 mmHg pressure for 4 hrs on ice, followed by incubating at 4˚C overnight. Fixed samples were dehydrated in the following series of ethanol solutions: 75% ethanol for 30 min at 4°C; 75% ethanol for 30 min at 4°C; 95% ethanol for 30 min at RT; 100% ethanol for 45 min at RT; 100% ethanol for 45 min at RT and 100% ethanol for 45 min at RT. The samples were then cleared with increasing gradient of xylene: 1:1 ethanol: Xylene for 45 min at RT; 100% Xylene for 45 min at RT; 100% Xylene for 45 min at RT and 100% Xylene for 45 min at RT. Paraplast infiltration and embedding was performed by transferring samples to oven set at 58°C and replacing 5 ml of xylene with 5 ml of molten paraplast at an interval of 4-5 hrs until xylene is completely removed from the samples. Tissue blocks were prepared and 10 µm sections were cut using microtome (Leica). Prepared sections were stretched by floating in a water bath maintained at 54°C, followed by recovering the sections of membrane slides and drying at 42°C for 30-45 mins. Slides were stored at 4°C for maximum 2-3 days before laser capture microdissection (LCM). Phloem cell isolation was performed using Laser Capture Microdissection System (Carl Zeiss) by marking phloem cell of the microtome sections using the PALM Robo software. The phloem cell harvest was carried out at 20X magnification with the parameter settings as: 45-60 laser energy, 70-80 laser focus and 50 speed of laser. RNA was isolated from LCM-harvested phloem cells using Arcturus Picopure RNA isolation kit (Arcturus).

2.2.12. Isolation of phloem enriched exudate (PEX) and analysis of miR160 levels

To analyse the levels of miR160 in phloem enriched exudates (PEX) of wild type potato, *P. infestans* infection of SUS and MR plants were performed as described earlier. PEX was collected at 0, 6, 12, 24, 48 and 96 hpi from *P. infestans* and mock (sterile water) inoculated plants as per pervious protocol (Campbell et al., 2008). A

precise incision was made in stem portion 1-2 cm above the axillary node of the inoculated leaves. Initial drops of exudates were discarded to eliminate contamination from neighbouring non-phloem cells. PEX were collected for a period of ~2 hrs in TRIzol reagent and stored at -80°C until further use. Total RNA was isolated from all the PEX samples using TRIzol reagent. Purity of PEX was confirmed by performing RT-PCR to analyse the presence of G2-like transcription factor (G2-FP and G2-RP) and absence of root-specific nitrate transporter, NT (NT-FP and NT-RP) transcripts as described previously (Banerjee et al., 2006). Hundred nanogram (100 ng) of total RNA was further processed to detect the mature and precursor forms of miR160 from uninfected plants. Whereas, for quantitative analysis of miR160, PEX collected at different time points from infected plants were used. Conditions for reverse transcription, end-point PCR and qRT-PCR were similar as mentioned before. Levels of miR160 in PEX were quantified and plotted using qRT-PCR cycle threshold (Ct) value differences as described previously (Pant et al., 2008).

2.2.13. Accession numbers

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

Table 2.1 Accession numbers

Name	**Accession**	**Source***
StARF10	PGSC0003DMT400020874	PGSC
StARF16	PGSC0003DMT400062489	PGSC
StCD2	PGSC0003DMT400071376	PGSC
StCCR4	PGSC0003DMT400077914	PGSC
G2-like TF	PGSC0003DMT400025001	PGSC
NT	XM_006340157	NCBI
GAPDH	PGSC0003DMT400044944	PGSC
U6	X60506	NCBI

* PGSC – Potato Genome Sequencing Consortium: http://solanaceae.plantbiology.msu.edu/integrated_searches.shtml

* NCBI – National Centre for Biotechnology Information: https://www.ncbi.nlm.nih.gov/

2.2.14. Primer sequences

Primers used in this chapter are listed in Table 2.2

Table 2.2 List of Primer

Primer Name	Sequence 5' - 3'
Mature miRNA cDNA preparation	
miR156_STP	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGTGCTC
miR159_STP	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTAGAGC
miR160_STP	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGGCATA
miR164_STP	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGCCACG
miR166_STP	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGGGAAT
miR169_STP	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTAGGCA
miR171_STP	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGATATT
miR172_STP	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTGCAGC
miR396_STP	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAGTTCA
miR414_STP	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTGACGA
miR1533_STP	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCATAA
Mature miRNA end-point PCR and quantitative real-time PCR	
miR156_FP	GCGGCGGTGACAGAAGAGAGT

miR159_FP	CGGCGGTTTGGATTGAAGGGA
miR160_FP	TGGAGTTTGCCTGGCTCCCTG
miR164_FP	CGGAGGTTGGAGAAGCAGTGCA
miR166_FP	TGGAGGTTCGGACCAGGCTTC
miR169_FP	CGGCGGTTAGCCAAGGATGACT
miR171_FP	TGTGGATTGATTGAGCCGCGCC
miR172_FP	CGGCGGCAGAATCTTGATGAT
miR396_FP	CGGCGGTTTCCACAGCTTTCT
miR414_FP	CGGCGGCTCATCTAGATCATCA
miR1533_FP	GCGGCGGCATAAAAAAAATAAT
Univ-miR_RP	AGTGCAGGGTCCGAGGT
miRNA precursor detection	
pre160_FP	GAGATCTAGACACGTCGTGTACACGTATA
pre160_RP	GAGAGAGCTCCAACATCATATACACGATATCGG
Northern blot analysis	
miR160_RC	GGCATACAGGGAGCCAGGCA
U6_RC	AGGGGCCATGCTAATCTTCTC
miRNA target detection, Cleavage site mapping and qRT-PCR	
ARF10_FP	GTCCAGCAGTCCTTTCTGTTGTTT
ARF10_RP1	GGCTGACCGAAGAGTAAGAACC
ARF10_RP2	GCTGCAACACGCTGGAAACTT
ARF16_FP	GGCAACCCCCTCAGGTCTAG
ARF16_RP1	GCATCAACTTGTTGGGAAGCGG
ARF16_RP2	TGCAACTTTTCGCTACGGTGGA
CD2_FP1	CCAGGGAGACAGGCATGGTTAT
CD2_RP2	TCTGGTTCCACCCATACCACTTGA
CCR4_FP1	AATCTCAATATCGCCTGCCGC
CCR4_RP2	CCAAGTGCAAGAGAAGTCAGATCC
Phloem-enriched exudate (PEX) analysis	
G2_FP	ACAACCGCACAAAGAATTTAATG
G2_RP	TGTTCTCCACATATGTTCAAAT

NT_FP	TGGTGTTACTGGTAGAGAA
NT_RP	TCTGTAAAGAAGCGAGGT
***P. infestans* confirmation**	
PINF	CTCGCTACAATAGGAGGGTC
ITS5	GGAAGTAAAAGTCGTAACAAGG
Reference Genes	
GAPDH_FP	GAAGGACTGGAGAGGTGGA
GAPDH_RP	GACAACAGAAACATCAGCAGT

2.3. Results

2.3.1. Potato *(Solanum chacoense)* and *Phytophthora infestans* as plant-pathogen model system

To decipher the role of miRNAs in potato - *Phytophthora infestans* infection, interaction between the susceptible (SUS) and moderately resistant (MR) varieties of wild potato *Solanum chacoense* and A2 strain of *P. infestans* were studied. *S. chacoense*, is widely used in potato breeding programs in India. SUS and MR varieties were obtained from Central Potato Research Institute (CPRI, Shimla, India) and successfully established at IISER Pune under *in vitro* conditions (Figure 2.1 A & B) as well as grown in green house conditions (Figure 2.1 C & D). *P. infestans* strain A2, was also obtained from CPRI and was maintained in corn media, pea agar media and potato slices throughout the study (Figure 2.1 E to H). Further, the *P. infestans* strain was confirmed by amplifying a part of Internal Transcribed Spacer 2 (ITS2) ribosomal DNA using primer sets PINF and ITS5 (Trout et al., 1997) (Figure 2.1 I). To understand and confirm the basal resistance of SUS and MR varieties, detached leaf infection experiments were performed (Figure 2.2) (María et al., 2001). In SUS leaves, massive growth of *P. infestans* hyphae was observed by 94 hpi (hours post inoculation) as indicated by Trypan blue staining (Figure 2.2 D). However, in MR leaves, hypersensitivity response (HR) was observed by 48 hpi (Figure 2.2 E) and Trypan blue staining revealed lesser growth of *P. infestans* (Figure 2.2 H) compared to SUS leaves.

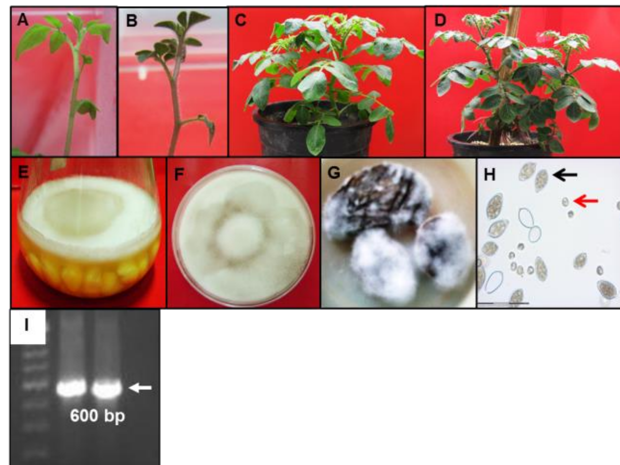


Figure 2.1. Maintenance of *Solanum chacoense* and *Phytophthora infestans*. A-B. One-week old *in vitro* culture of *S. chacoense* susceptible (SUS) (A) and moderately resistant (MR) (B) variety; **C-D.** Two-months old soil grown plants of SUS (C) and MR (D) varieties; **E-G.** Cultures of *P. infestans* maintained in corn media (E), pea agar (F) and potato slices (G). **H.** Released zoospores (red arrow) from *P. infestans* sporangia (black arrow) after cold incubation (4ºC) for 2 hours. **I.** PCR based detection and confirmation of A2 Strain of *P. infestans* used in all the experiments in this study. A part of Internal Transcribed Spacer 2 (ITS2) ribosomal DNA was amplified using PINF (forward Primer) and ITS5 (reverse primer) giving an product of ~600 bp (Trout et al., 1997).

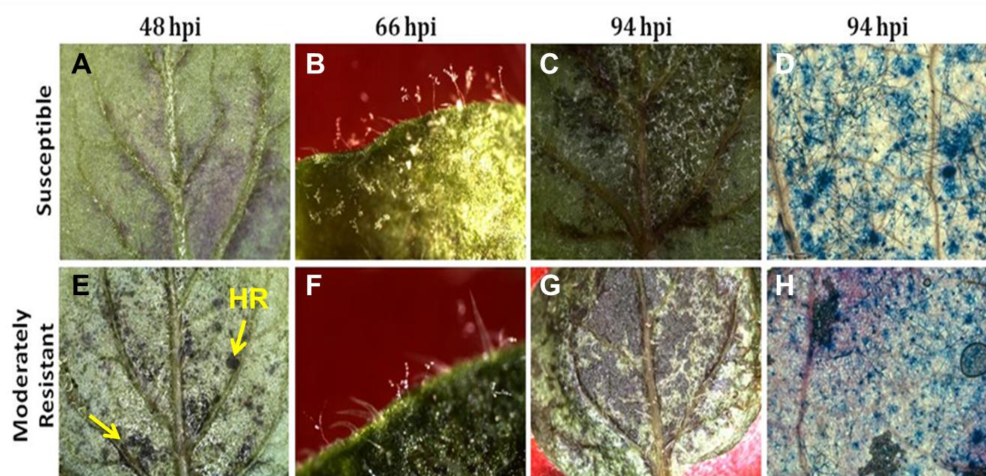


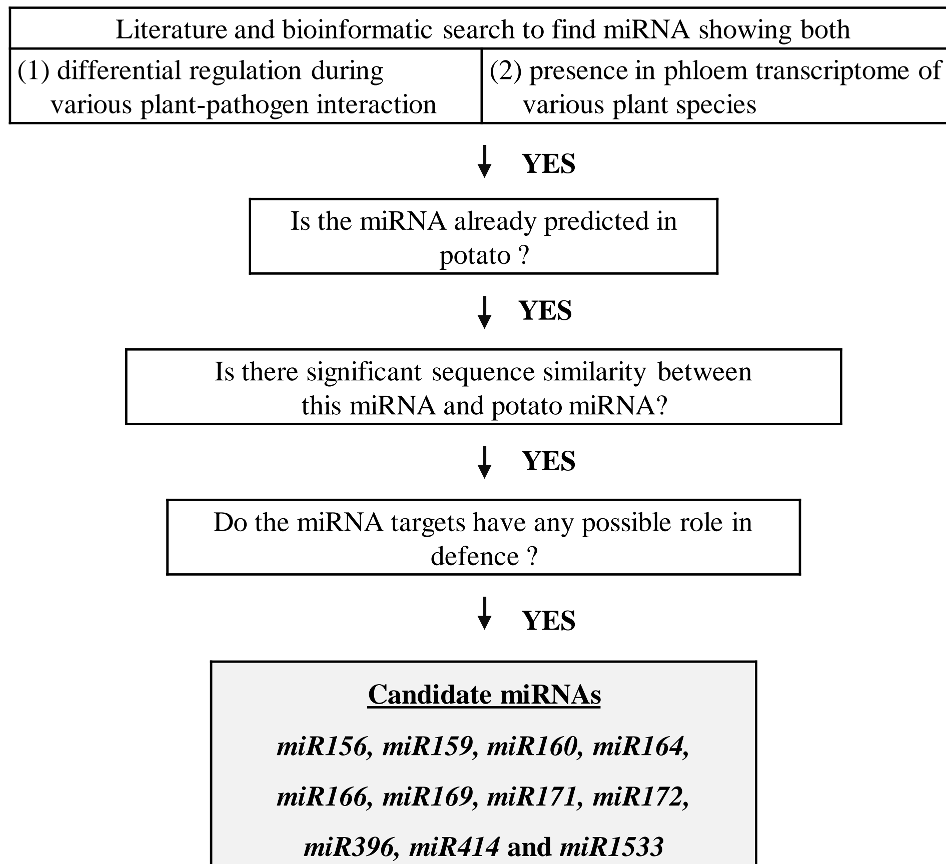
Figure 2.2. Progression of *P. infestans* infection in the detached leaves of SUS and MR varieties. A-C. *P. infestans* disease development in the detached leaves of SUS variety as observed at 48, 66 and 94 hpi (hours post inoculation); **D.** Trypan blue staining of infected SUS leaf at 94 hpi indicating extensive growth of *P. infestans* hyphae. **E-G.** *P. infestans* disease development in the detached leaves of MR variety over time; yellow arrows in (E)

point at Hypersensitive Response (HR) exhibited by MR variety; **H.** Trypan blue staining of infected MR leaf at 94 hpi indicating lesser growth of *P. infestans* hyphae than observed in SUS variety.

2.3.2. Shortlisting of candidate miRNAs and *in planta* detection in potato

Extensive literature survey was carried out to identify miRNAs that were previously shown to be involved in variety of plant-pathogen interaction (Detailed list is provided in Chapter 1, Table No. 1.1). Further, a strategy was adopted to select the candidate miRNAs to study their potential role in potato-*Phytophthora* interaction. A detailed flow diagram and the criteria for shortlisting of miRNAs is presented in Table 2.3. Since, the role of miRNAs in Systemic Acquired Resistance (SAR) was also one of our objectives, presence of miRNAs in the phloem was considered as a selection criterion (Buhtz et al., 2008; Varkonyi-gasic et al., 2010; Rodriguez-Medina et al., 2011). In brief, miRNAs which showed significant upregulation or downregulation upon an infection and earlier shown to be present in phloem were chosen. Further, we investigated if any of these selected miRNAs were already predicted in potato and whether they have sequence homology with potato miRNAs (Zhang et al., 2009; Yang et al., 2010b; Xie et al., 2011; Kim et al., 2011). Additionally, miRNAs whose target genes had been shown to have defence related role were considered as a selection criterion for narrowing the list of candidate miRNAs. Finally, eleven miRNAs (miR156, miR159, miR160, miR164, miR166, miR169, miR171, miR172, miR396, miR414 and miR1533) were shortlisted for studying their potential role in potato-*Phytophthora* interaction. Except miR164, all the candidate miRNAs were detected in SUS and MR varieties by stem-loop RT-PCR analysis (Figure 2.3).

Table 2.3 Schematic flow diagram for selection of candidate miRNAs



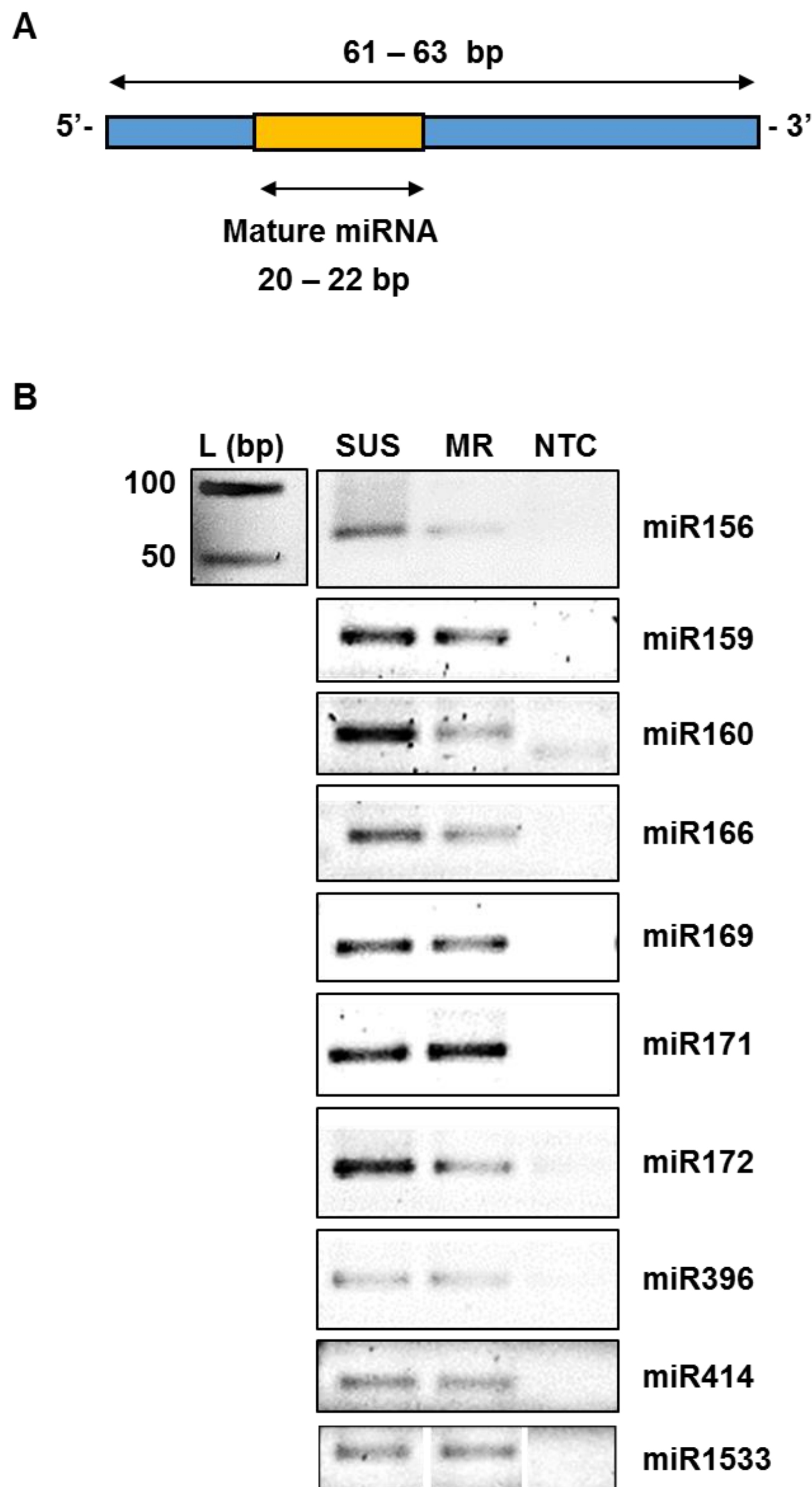


Figure 2.3. Detection of candidate miRNAs in potato. A The 61-63 bp Stem-loop RT-PCR product with 20-22 bp miRNA sequence within it. **B.** Stem-loop RT-PCR based detection of candidate miRNAs in the RNA isolated from whole tissue of SUS and MR varieties of *S. chacoense*. L (bp) is ladder in base pairs. NTC is no template control.

2.3.3. Potato miRNAs respond to *P. infestans* infection

To check if selected candidate miRNAs respond to *P. infestans* attack, infection experiment was performed with SUS and MR potato varieties (Figure 2.4). Infected leaf samples were collected at 0, 12, 24 and 96 hpi (hours post inoculation) and expression levels of the miRNAs were quantitated by qRT-PCR. While we detected ten miRNAs in potato, we could establish the association of only six (miR159, miR160, miR166, miR169, miR172 and miR396) in the potato-*P. infestans* interaction (Figure 2.5).

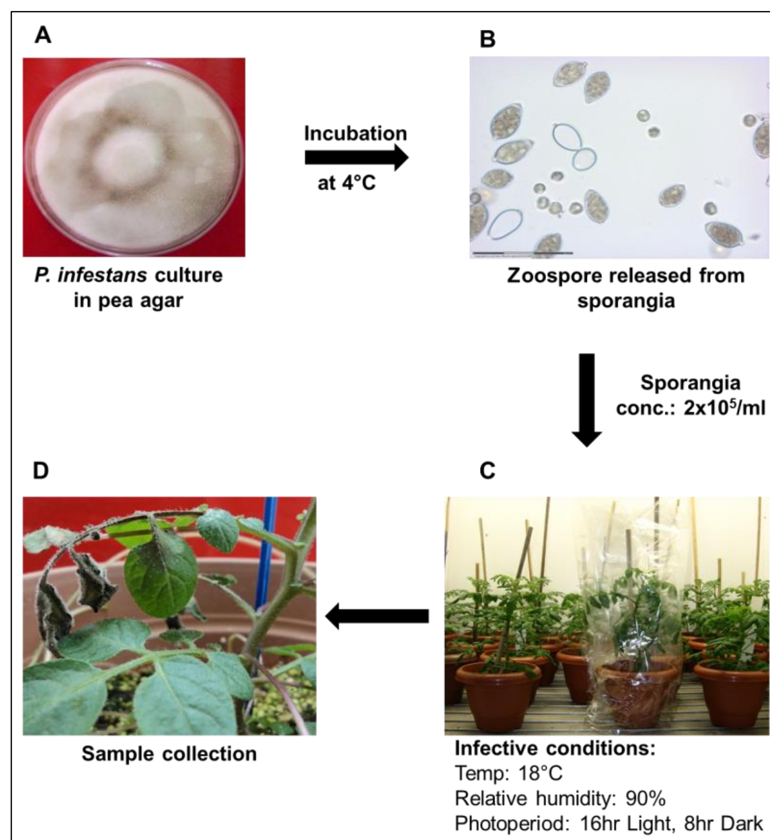


Figure 2.4. *In planta* infection experiment. A. From a 7-10 days old fully grown *P. infestans* pea agar plate hyphae are scrapped and transferred to sterile water in a Petri-dish. **B.** To check the viability of the sporangia, hyphae are teased apart and incubated at 4ºC to induce release of zoospores. **C.** Sporangia concentration is then adjusted to $2x10^5$ sporangia per ml and plants were infected by applying desired volume of sporangia solution to the abaxial side of leaves. Infected plants were incubated in the conditions mentioned for disease progression. **D.** Samples were collected after different time intervals for molecular and microscopic analysis.

Expression levels of miR159 increased at 24 hpi and decreased at 96 hpi in both SUS and MR varieties, however, the difference was significant only for SUS variety (Figure 2.5 A). More than 7-fold increase was observed for miR160 as early as 12 hpi in SUS variety, while the expression levels decreased significantly at 96 hpi (Figure 2.5 B). miR160 also showed a significantly increased expression at 12 hpi and a reduced expression by 96 hpi in MR plants. In case of miR172, expression levels were significantly reduced at all the time-points tested for SUS variety, however in MR plants, significant reduction of levels were observed only at 12 hpi (Figure 2.5 C). Similar to miR172, levels of miR169 were reduced at all the time-points in SUS variety, whereas for MR variety no significant difference was observed (Figure 2.5 D). A 20-fold increase was observed in miR166 levels at 12 hpi for SUS variety followed by a significant reduction at 96 hpi (Figure 2.5 E). In MR variety, significant reduction of miR166 was observed only at 24 hpi. No significant differential expression was observed for miR396 in either of the varieties (Figure 2.5 F) tested. Amongst all the miRNAs tested, highest differential expression was observed for miR160 and miR166 against *P.infestans* infection. Even though all these miRNAs showed promise towards their role in regulation of potato - *P. infestans* interaction, the present study was restricted to decipher the role of miR160 in this interaction.

2.3.4. miR160 showed altered expression in both local and systemic leaves post infection

To examine the role of miR160 in both local and systemic defence responses during potato - *P. infestans* interaction, infection experiments were carried out with SUS and MR varieties and samples were harvested at different time-points (0, 6, 12, 24, 48, and 96 hpi) from local-inoculated and systemic-non-inoculated leaves. The expression levels of miR160 were analysed by both real-time PCR and northern blot analysis and results were mostly similar (Figure 2.6). In local leaves of both SUS and MR variety, a significant increase of miR160 expression was observed as early as 6 hpi and later at 96 hpi (Figure 2.6 A to D). Even though an increased expression was observed in other time-points (12, 24, and 48 hpi), the difference was not significant.

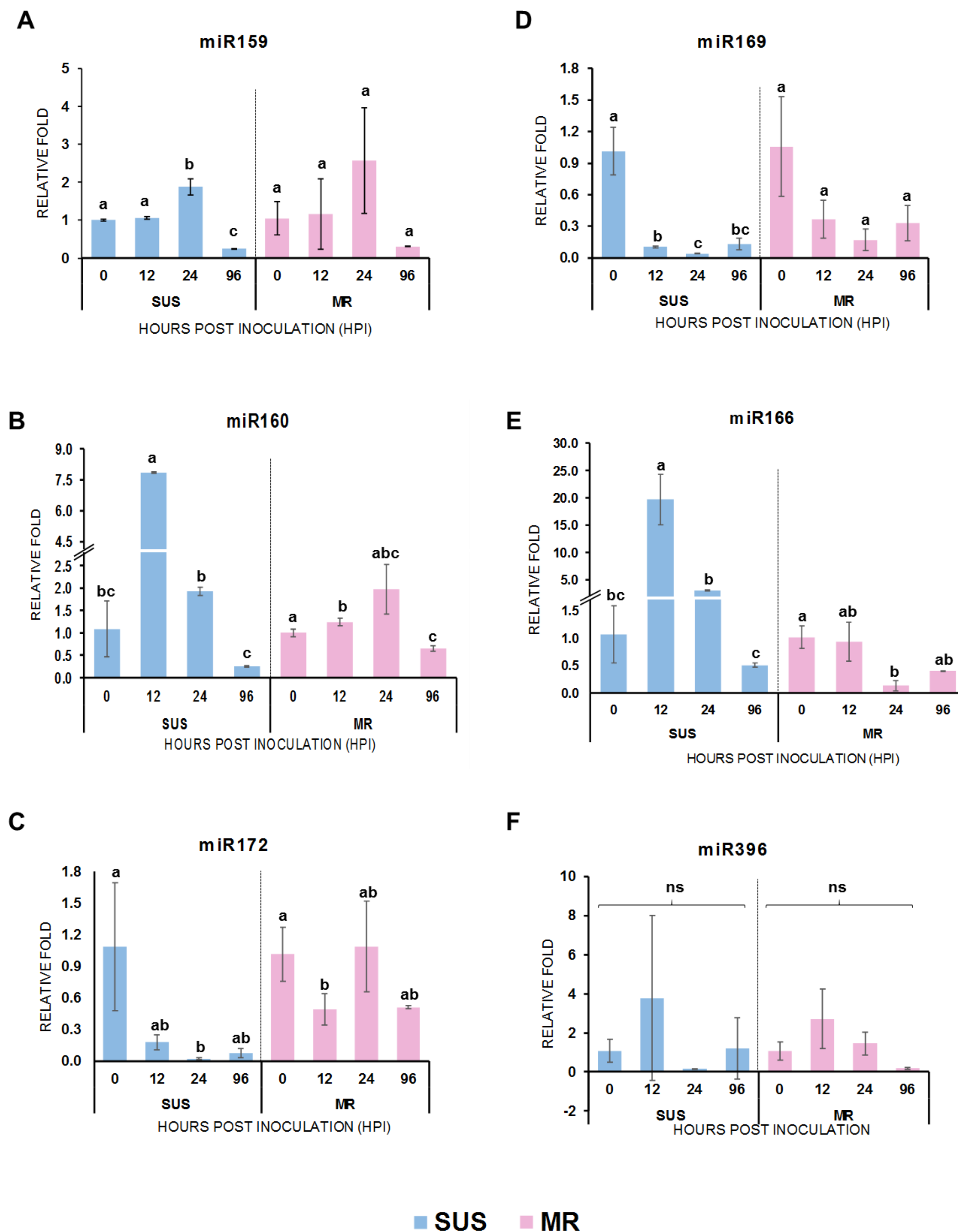


Figure 2.5. Quantitative real-time PCR of selected miRNAs. Effect of *P. infestans* infection in the expression of miR159 **(A)**, miR160 **(B)**, miR172 **(C)**, miR169 **(D)**, miR166 **(E)** and miR396 **(F)** over time (0, 12, 24, 96 hpi) in SUS (blue bars) and MR (pink bars) varieties. GAPDH was used as normalization gene and all time-points are normalized to 0 hpi. Each bar is the mean of two biological replicates each with at least two technical replicates. All the bars were compared to each other and different alphabets indicate significant difference between the two bars being compared as per Student's t-test ($p<0.05$). ns means not significant.

In systemic leaves, miR160 levels significantly increased as early as 6 hpi in SUS (Figure 2.6 E and F) and at 6 and 96 hpi in MR variety (Figure 2.6 G and H). The expression pattern of miR160 was not strikingly different when SUS and MR varieties were compared indicating that miR160 might not be the reason for differences in their basal resistance against *P. infestans*. Altogether, this analysis showed that miR160 levels were altered in both local and systemic leaves of potato post *P. infestans* infection, suggesting that miR160 is possibly involved in local and systemic defense responses during potato - *P. infestans* interaction.

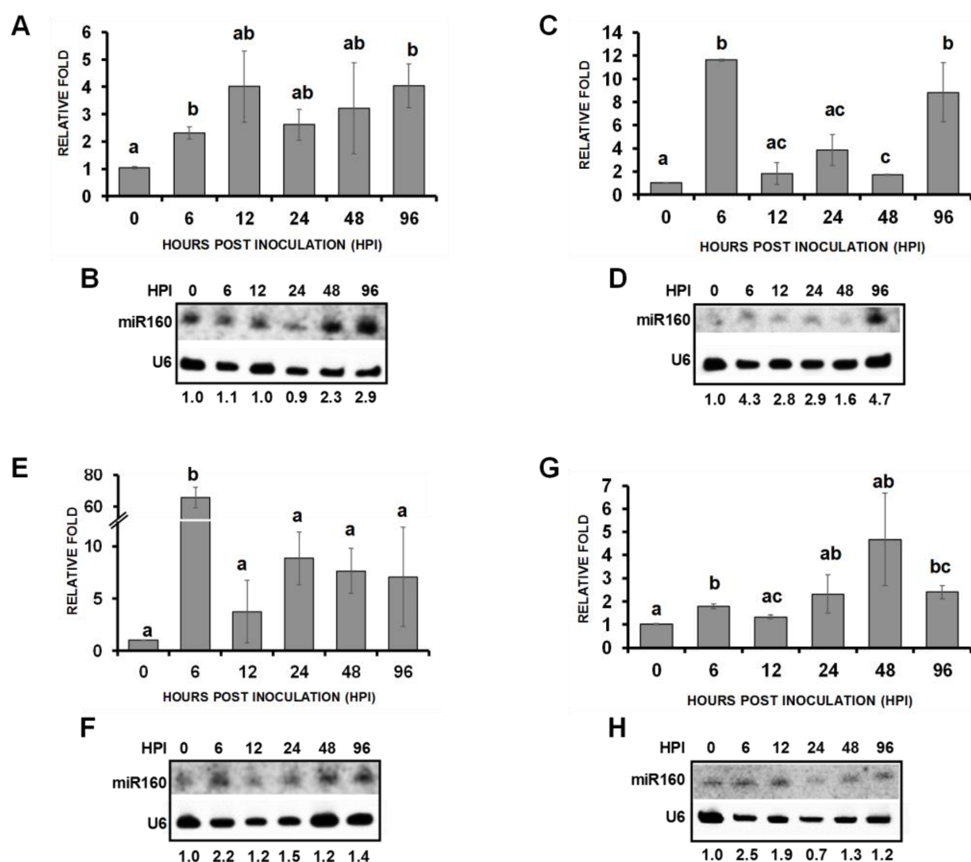


Figure 2.6. Differential expression of miR160 in local and systemic leaves upon *P. infestans* infection. A-B. qRT-PCR (A) and northern blot (B) based expression analysis of miR160 in the local leaves of SUS variety. **C-D.** qRT-PCR (C) and northern blot (D) based expression analysis of miR160 in the local leaves of MR variety. **E-F.** qRT-PCR (E) and northern blot (F) based expression analysis of miR160 in the systemic leaves of SUS variety. **G-H.** qRT-PCR (G) and northern blot (H) based expression analysis of miR160 in the systemic leaves of MR variety. GAPDH and U6 were used as normalization genes for qRT-PCR and northern blot analysis respectively. All time-points are normalized to 0 hpi. Each bar

in qRT-PCR analysis is the mean of two biological replicates each with at least two technical replicates. All the bars were compared to each other and different alphabets indicate significant difference between the two bars being compared as per Student's t-test ($p<0.05$). The northern blot image is the representative of two biological replicates.

2.3.5. Prediction and detection of miR160 target genes

Using *in silico* target prediction softwares such as psRNATarget (plantgrn.noble.org/psRNATarget/) (Dai and Zhao, 2011), TargetAlign (leonxie.com/target Align.php) (Xie and Zhang, 2010) and TAPIR (http://bioinformatics. psb.ugent.be/webtools/ tapir/) (Bonnet et al., 2010), seven putative target genes were predicted for miR160 in potato (Table 2.3).

Out of the seven targets, we could detect only four targets (*StARF10*, *StARF16*, *StCCR4* and *StCD2*) in the RNA of SUS and MR varieties (Figure 2.7 A). However, only *StARF10* and *StARF16* could be further validated as true targets of miR160 through cleavage site mapping assay (Figure 2.7 B to E). Based on cloning frequency of the miR160 cleaved products, ARF10 (11/11) appeared to be a stronger targets than ARF16 (4/10) and was studied in this investigation.

Table 2.3 List of putative targets of miR160. Targets of miR160 were predicted by three different target prediction software: psRNATarget, TargetAlign and TAPIR.

S. No.	PGSC Transcript ID	PGSC Transcript Name	Expectation value			Inhibition	Arabidopsis Homolog
			psRNA Target	Target align	TAPIR		
1	PGSC0003DMT400020874	Auxin response factor 10 (*StARF10*)	0	0	0	Cleavage	Auxin response factor 10 (AT2G28350.1)
2	PGSC0003DMT400062489	Auxin response factor 16 (*StARF16*)	0	0	0	Cleavage	Auxin response factor 16 (AT4G30080.1)
3	PGSC0003DMT400045323	Auxin response factor 16 (*StARF16-2*)	0.5	0.5	0.5	Cleavage	Auxin response factor 16 (AT4G30080.1)
4	PGSC0003DMT400055614	Auxin response factor 10 (*StARF10-2*)	1.5	1.5	1.5	Cleavage	No hit found
5	PGSC0003DMT400015264	Mitogen-activated protein kinase 14 (*StMAPK14*)	3	3	3	Translation	Mitogen-activated protein kinase 9 (AT3G18040.1)
6	PGSC0003DMT400077914	CCR4-NOT transcription complex subunit (*StCCR4*)	3.5	4	4	Cleavage	NOT transcription complex subunit VIP2 (AT5G59710.1)
7	PGSC0003DMT400071376	Cutin deficient 2 (*StCD2*)	3.5	3	4	Cleavage	Homeobox-leucine zipper protein ANTHOCYANINLESS 2 (AT4G00730.1)

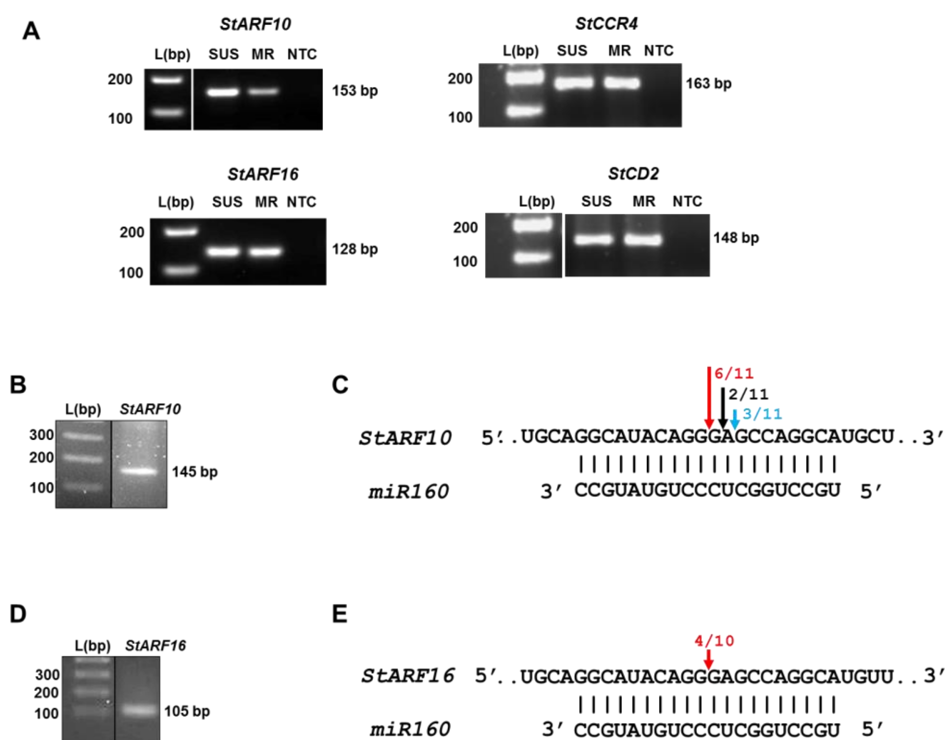


Figure 2.7. miR160 target gene detection and cleavage site mapping analysis. A. RT-PCR based detection of *StARF10*, *StARF16*, *StCCR4* and *StCD2* in the whole tissue RNA of SUS and MR varieties. L(bp) is ladder in base pairs and NTC is no template control. **B-E.** *In planta* confirmation of *StARF10* and *StARF16* as the true target of miR160. RLM-RACE based isolation of miR160 cleavage product of *StARF10* (B) and *StARF16* (D). Partial mRNA sequence of *StARF10* (C) and *StARF16* (E) aligned with miR160 and numbers denote the fraction of cloned cleavage products that terminates at different positions (arrows).

2.3.6. *StARF10***, target gene of miR160** ***also exhibited*** **altered expression levels upon** ***P. infestans*** **infection**

To investigate the effect of *P. infestans* infection on *St*ARF10, expression levels were analysed in local and systemic leaves of both SUS and MR varieties. It was observed that similar to miR160, *StARF10* transcript levels were also altered at both local and systemic leaves upon *P. infestans* infection (Figure 2.8). When the expression patterns of both miR160 and *StARF10* were compared, an inverse relationship was observed at most of the time-points (Figure 2.9) tested. *StARF10* expression peaked at 12 hpi for both local and systemic leaves of MR variety,

whereas, for SUS variety, highest expression was observed at 12 and 24 hpi in local and systemic leaves respectively. Overall, these results indicated that similar to miR160, the expression of its target gene *StARF10* was also altered in both local and systemic leaves upon *P. infestans* infection.

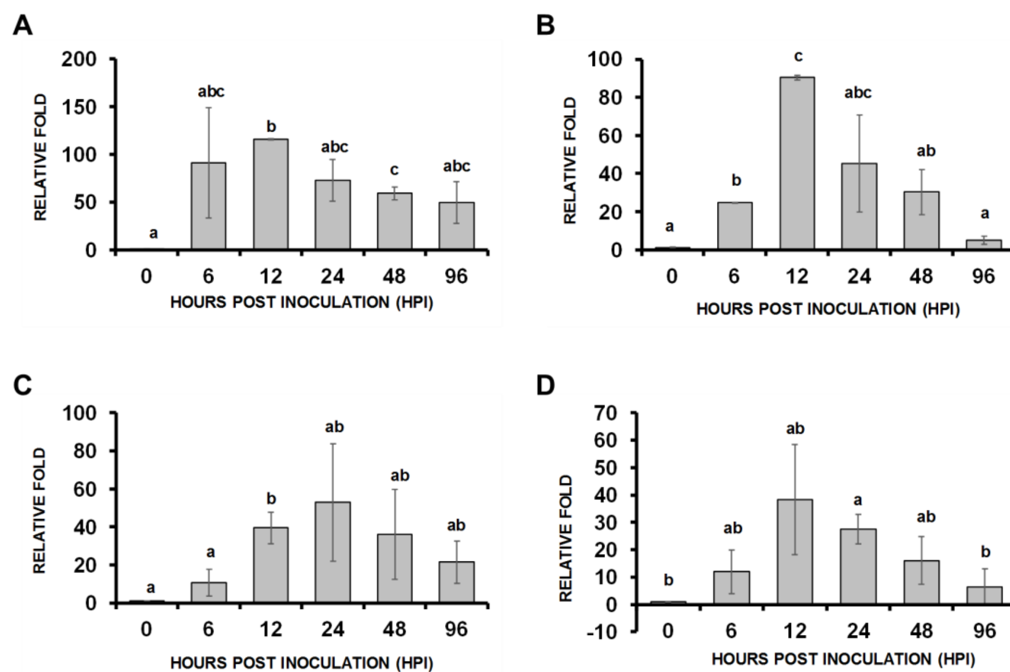


Figure 2.8. Differential expression of *StARF10* in local and systemic leaves upon *P. infestans* infection. A-B. qRT-PCR based expression analysis of *StARF10* in the local leaves of SUS (A) and MR (B) varieties. **C-D** qRT-PCR based expression analysis of *StARF10* in the systemic leaves of SUS (C) and MR (D) varieties. GAPDH was used as normalization gene and all time-points were normalized to 0 hpi. All data are the mean of two biological replicates having at least two technical replicates each. All the bars were compared to each other and different alphabets indicate significant difference between the two bars being compared as per Student's t-test ($p<0.05$).

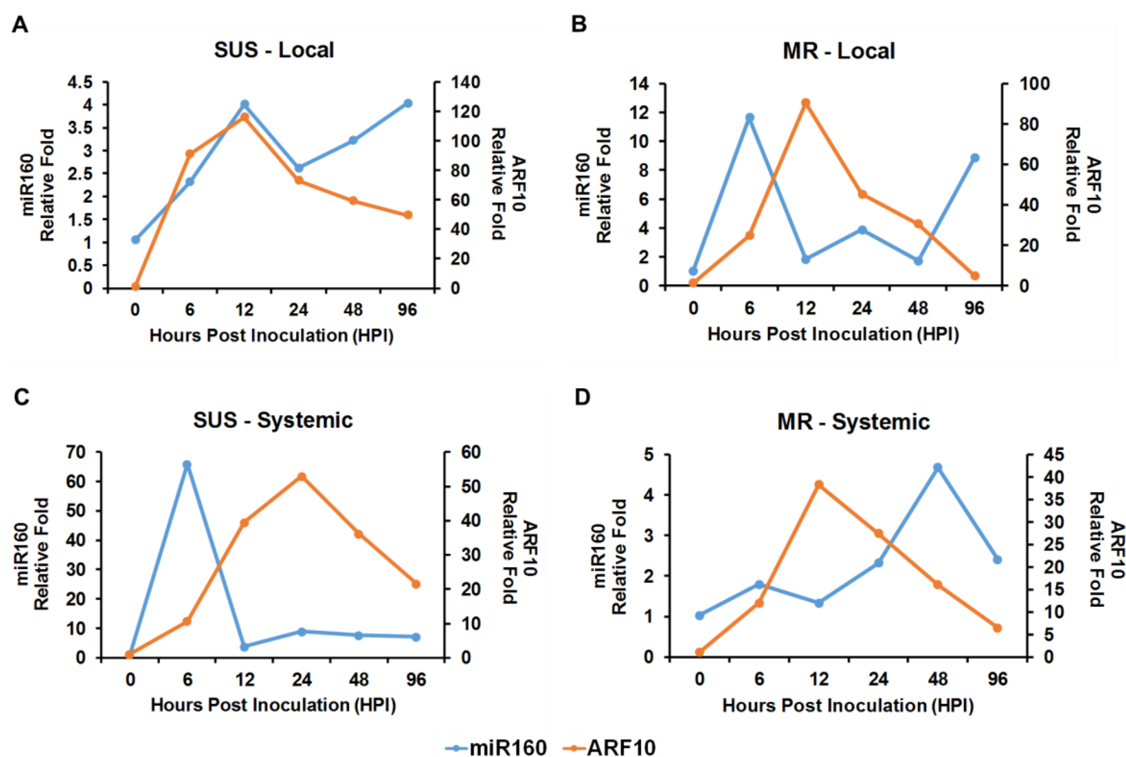
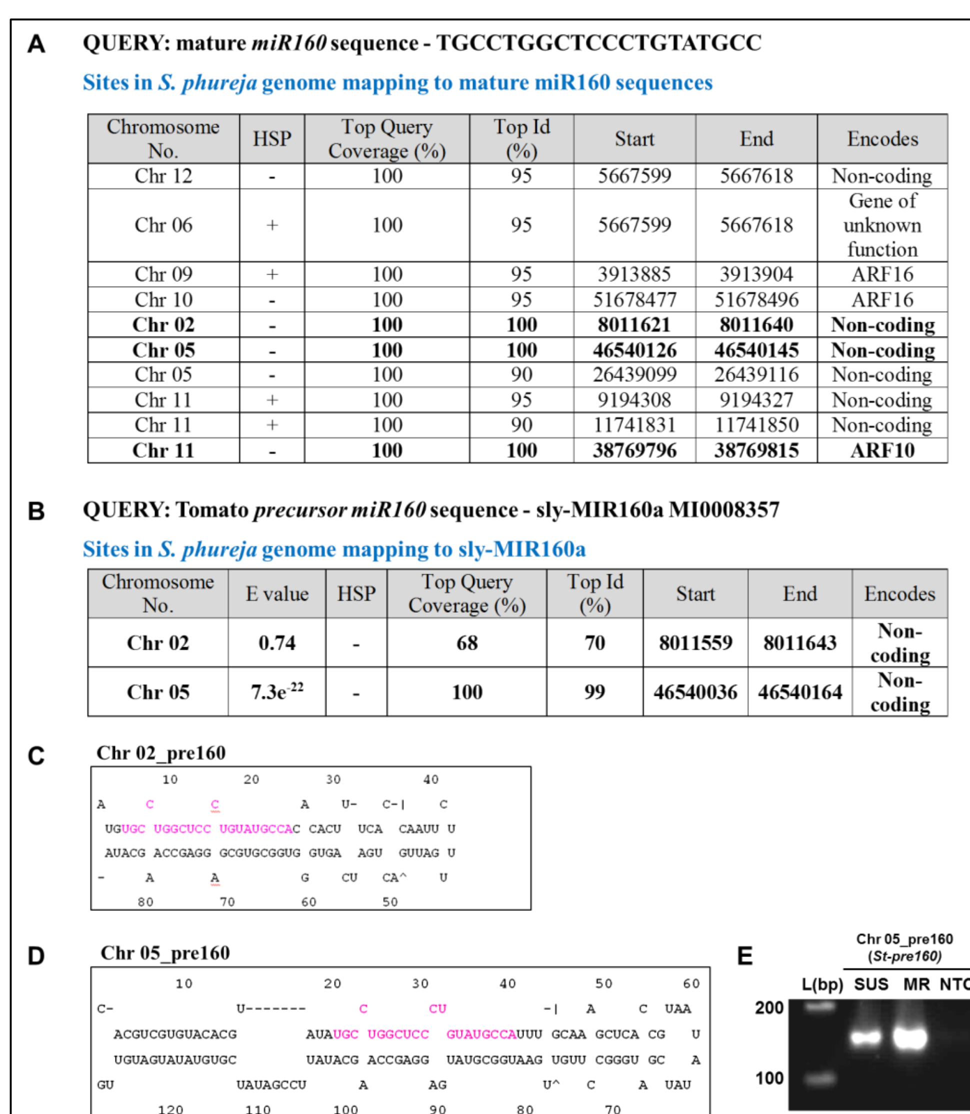


Figure 2.9. Inverse relationship of miR160 and *StARF10* expression patterns. A-B. Comparison of miR160 and *StARF10* expression levels at different time-points in local leaves of SUS (A) and MR (B) varieties. **C-D.** Comparison of miR160 and *StARF10* expression levels at different time-points in systemic leaves of SUS (C) and MR (D) varieties. qRT-PCR values of miR160 and *StARF10* expression are the same as that plotted in Figure 2.6 and Figure 2.8 respectively. *StARF10* is plotted in secondary y-axis for clarity.

2.3.7. Prediction and detection of miR160 precursor in potato

Two different approaches were used to identify precursors of miR160 from potato. In the first approach, the mature 21-nt sequence of miR160 was used as a query to search against the potato genome sequence (PGSC, Pseudomolecules 2.1.11 database) with lowest stringency parameter settings. Out of the ten hits obtained, three showed 100% match and were positioned on chromosome 02 (Chr 02), Chr 05 and Chr 11 respectively (Figure 2.10 A). For the second approach, precursor sequence of miR160 from tomato, sly-MIR160a (MI0008357), was used as a query to search against the above mentioned database with default parameter settings. Only two matches were obtained each from Chr 02 and Chr 05, pertaining to the same positions

as the matches obtained with the first search (Figure 2.10 B). These two sequences were fetched from the PGSC genome database and were named Chr02_pre160 and Chr05_pre160. The mfold (http://unafold.rna.albany.edu/?q=mfold) structure predictions of these two sequences were ideal to be accepted as a putative miR160 precursor (Figure 2.10 C and D). Though, we predicted two precursors of miR160, only Chr05_pre160 (hereafter *St-pre160*) could be detected in the RNA of SUS and MR varieties (Figure 2.10 E).



Chromosome No.	HSP	Top Query Coverage (%)	Top Id (%)	Start	End	Encodes
Chr 12	-	100	95	5667599	5667618	Non-coding
Chr 06	+	100	95	5667599	5667618	Gene of unknown function
Chr 09	+	100	95	3913885	3913904	ARF16
Chr 10	-	100	95	51678477	51678496	ARF16
Chr 02	**-**	**100**	**100**	**8011621**	**8011640**	**Non-coding**
Chr 05	**-**	**100**	**100**	**46540126**	**46540145**	**Non-coding**
Chr 05	-	100	90	26439099	26439116	Non-coding
Chr 11	+	100	95	9194308	9194327	Non-coding
Chr 11	+	100	90	11741831	11741850	Non-coding
Chr 11	**-**	**100**	**100**	**38769796**	**38769815**	**ARF10**

Chromosome No.	E value	HSP	Top Query Coverage (%)	Top Id (%)	Start	End	Encodes
Chr 02	**0.74**	**-**	**68**	**70**	**8011559**	**8011643**	**Non-coding**
Chr 05	**$7.3e^{-22}$**	**-**	**100**	**99**	**46540036**	**46540164**	**Non-coding**

Figure 2.10. Prediction, detection and validation of miR160 precursor. A. List of hits for potential precursors of miR160 from different chromosome locations of potato genome when mature miR160 sequence was used as query. The chromosomal positions highlighted in bold

showed highest similarity. **B.** List of hits for potential precursor of miR160 from different chromosome locations of potato genome when tomato miR160 precursor (sly-MIR160a) was used as query. The chromosomal positions obtained were same as the ones highlighted in bold in (A). **C-D.** *mfold* structures of the two potato miR160 precursors obtained from chromosome 02 (Chr02_pre160) (C) and chromosome 05 (Chr05_pre160) (D). Mature miR160 sequence is highlighted in pink **E.** Only the presence of Chr05_pre160 (from here on *St-pre160*) could be validated in SUS and MR variety by RT-PCR analysis. L(bp) is ladder in base pair and NTC is no template control.

2.3.8. miR160 is present in phloem and exhibit altered levels upon infection

Earlier, miR160 is shown to be present in the phloem of *Brassica* (Buhtz et al., 2008, 2010) and apple (Varkonyi-gasic et al., 2010). To test if miR160 is present in the phloem of potato, LCM (Laser Capture Microdissection) harvested phloem cells (PC) (Figure 2.11) as well as phloem enriched exudates (PEX) (Figure 2.12) were analysed from SUS and MR varieties. Purity of PC and PEX derived RNA were further assessed by RT-PCR mediated detection for the presence of phloem-specific G2-like transcription factor and absence of the root-specific nitrate transporter (Figure 2.11 D and 2.12 B & C). The mature miR160 was detected and sequence validated from both PC (Figure 2.11 E) and PEX (Figure 2.12 D) derived RNA. However, precursor of miR160 (*St-pre160*) could not be detected in the PEX RNA (Figure 2.12 E) indicating the possibility of non-phloem origin of mature miR160. As miR160 expression levels were found to be altered in the systemic leaves of both SUS and MR varieties (Figure 2.6 E to H), accumulation pattern of miR160 was analysed in the PEX of these varieties post infection. qRT-PCR analysis revealed a significant increase in accumulation of miR160 in the PEX collected at 6 hpi (~9 fold) for SUS (Figure 2.12 F) and 6 hpi (~9 fold), 12 hpi (~4.5 fold), and 24 hpi (~7.5 fold) for MR (Figure 2.12 G) variety suggesting the potential role of miR160 in regulating systemic defence response in potato - *P. infestans* interaction.

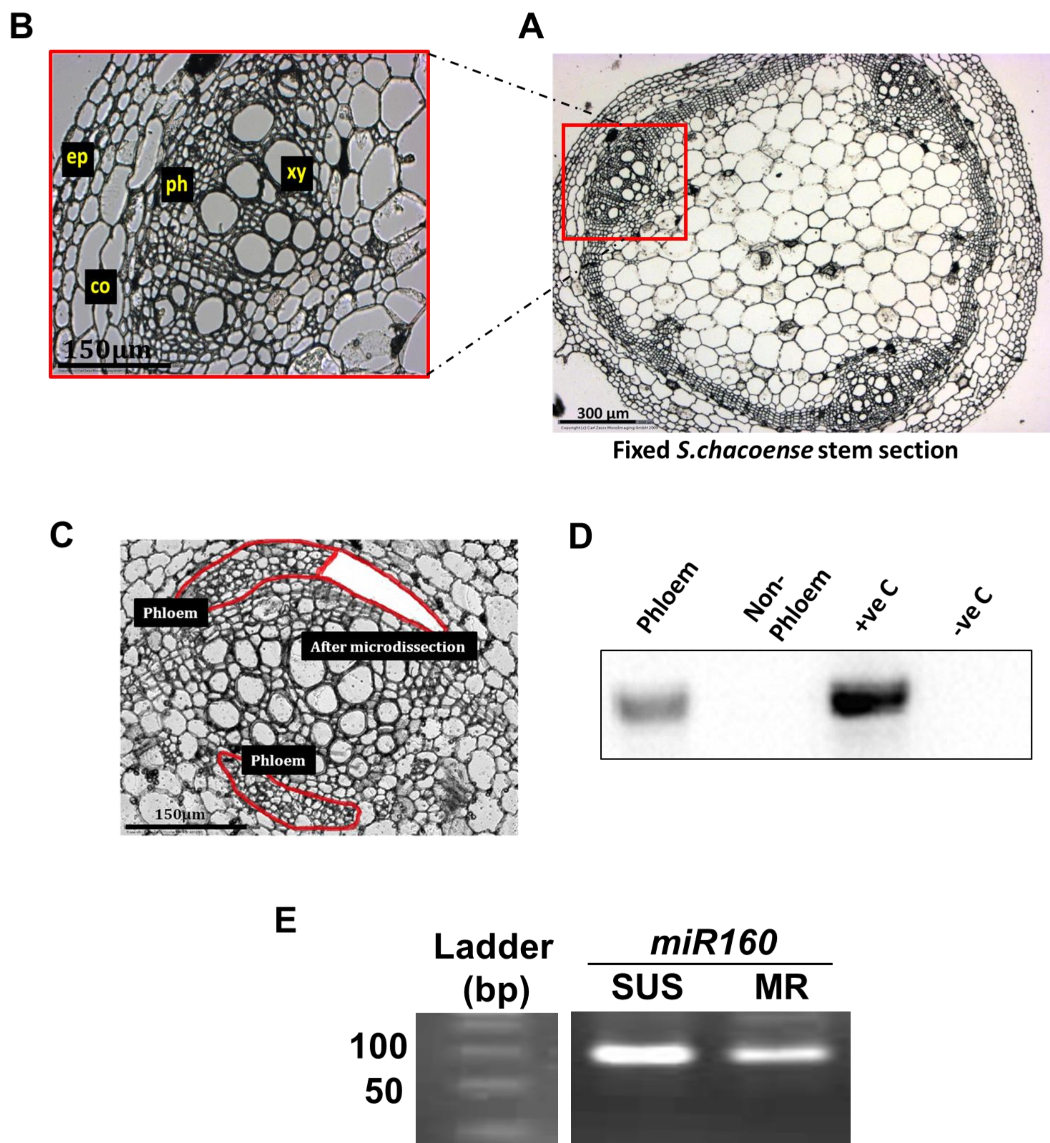


Figure 2.11. Laser capture microdissection (LCM) based isolation of phloem cells and detection of miR160. A-B. Ethanol-acetic acid fixed microtome-sectioned stem of potato (A) with one of the vascular bundle zoomed in (B), ph-phloem, xy-xylem, co-cortex and ep-epidermis. **C.** LCM-based isolation of phloem cells from the stem sections. **D.** Detection of phloem-specific G2-like transcription factor in the RNA from phloem cells and absence in RNA from non-phloem cells both harvested by LCM technique. +ve C is positive control reaction carried out with leaf RNA and –ve C is negative control reaction with no template. **E.** Detection of mature 21-bp miR160 in the LCM-isolated phloem cell RNA of SUS and MR variety.

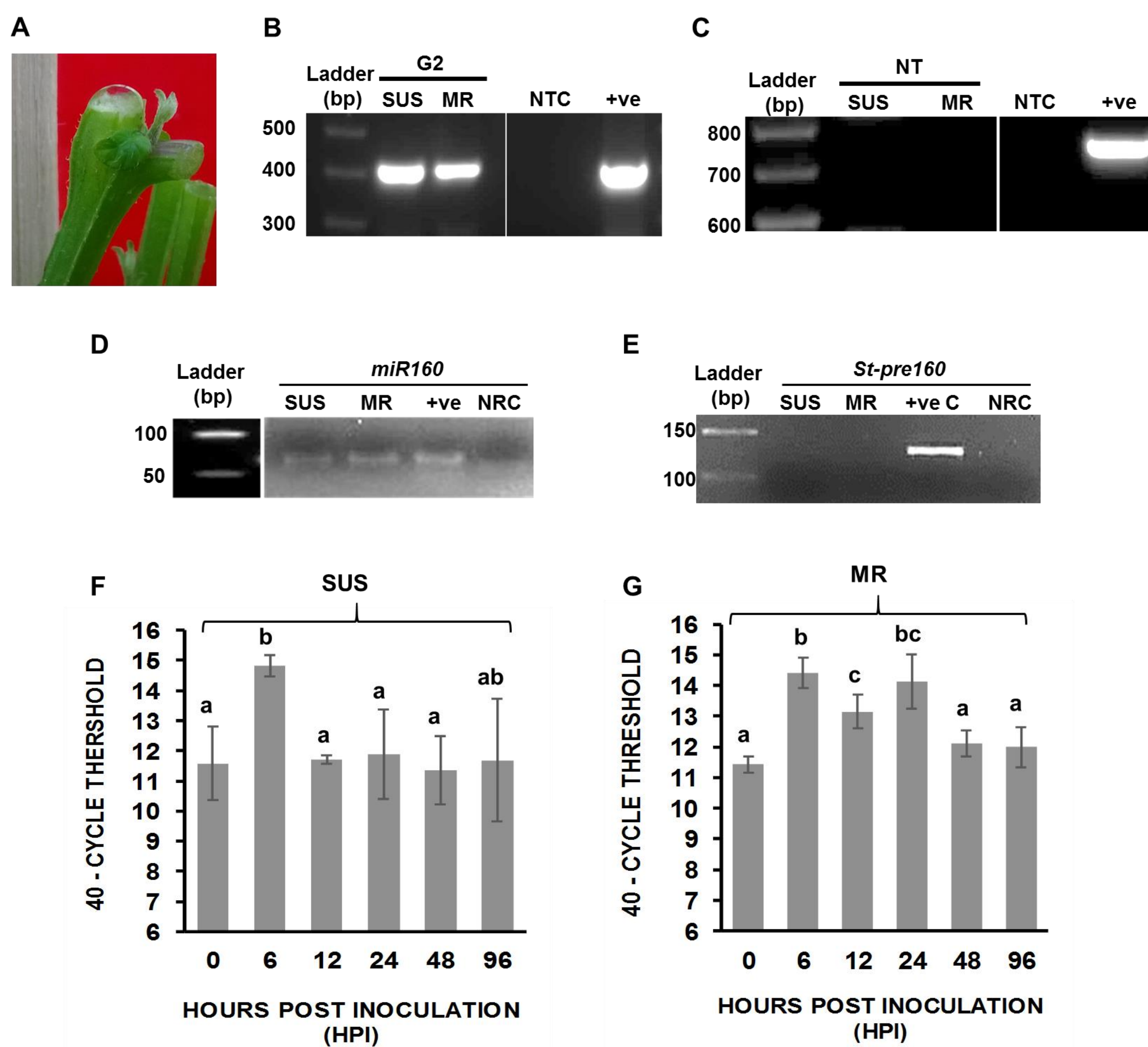


Figure 2.12. Phloem enriched exudate (PEX) isolation and analysis of miR160 and its precursor in PEX. A. Phloem enriched exudate (PEX) isolation from the stem of SUS and MR varieties. **B-C.** Detection of the presence of phloem-specific G2-like transcription factor (B) and absence root-specific nitrate transporter (NT) in the RNA isolated from the PEX of SUS and MR varieties. **D.** Detection of mature 21-bp miR160 in the PEX of SUS and MR varieties. **E.** *St-pre160*, precursor of miR160, is absent in the PEX of SUS and MR varieties. NTC is no template control and +ve C is positive control reaction carried out with leaf RNA. **F-G.** qRT-PCR based analysis of mature miR160 accumulation pattern in the PEX of SUS (F) and MR (G) variety at different time-points post *P. infestans* infection. Each bar is the mean of three biological replicates each with three technical replicates. Different alphabets indicate significant difference between the two bars being compared as per Student's t-test ($p<0.05$).

2.4 Discussion

2.4.1. Multiple miRNAs respond to *P. infestans* infection in potato

Though miRNAs have been shown to play critical role in variety of plant – pathogen interaction (Ruiz-Ferrer and Voinnet, 2009; Sunkar et al., 2012; Seo et al., 2013), their role in potato-*Phytophthora* interaction have not been explored yet. Our effort is the first attempt in this regard.

In this study, we carried out expression analysis of six potato miRNAs (out of eleven shortlisted miRNAs) upon *P. infestans* infection. Out of these, five miRNAs showed differential expression upon infection in our analysis. For example, an overall induction of miR159 expression was observed upon infection in both SUS and MR plants (Figure 2.5 A). Similar to our observations, miR159 was earlier shown to be induced during bacterial infection of *Arabidopsis* (Zhang et al., 2011), leaf curl disease of tomato (Naqvi et al., 2010) and blast disease of rice (Li et al., 2016). In contrast, few other studies have also shown downregulation of miR159 during powdery mildew disease of wheat (Xin et al., 2010) and in phytoplasma-infected Mexican Lime trees (Ehya et al., 2013). These reports indicate that miR159 expression can vary based on the plant-pathogen interaction.

We also observed a high induction of miR160 and miR166 in SUS variety upon *P. infestans* infection as early as 12 hpi (Figure 2.5 B & E). These two miRNAs were previously shown to be induced during TMV infection in tobacco (Bazzini et al., 2011) and bacterial infection in *Arabidopsis* (Zhang et al., 2011). They also seem to play potential role in PTI responses of *Arabidopsis* and soybean (Li et al., 2010; Wong et al., 2014). Our results indicate that miR160 and miR166 could also play role in potato-*phytophthora* interaction. Unlike the above mentioned miRNAs, two other miRNAs, miR169 and miR172 exhibited an overall reduction in expression upon *P. infestans* infection (Figure 2.5 C & D). In contrast to this, miR169 levels were demonstrated to increase upon infection of *Phaseolus vulgaris* by *Rhizobium* (Arenas-Huertero et al., 2009). Similarly, Naqvi et al (2010) have also showed that miR172 levels were induced during leaf curl infection of tomato (Naqvi et al., 2010). However, no differences were observed in expression of both miR169 and miR172

during TMV infection of tobacco (Bazzini et al., 2011). Review of previous literatures as well as our own findings indicates that function of miRNAs could vary based on the kind of plant-pathogen interaction. Taken together, our results suggest the potential role of miR159, miR160, miR166, miR169 and miR172 in mediating defence responses during potato – *P. infestans* interaction. Though, all these five miRNAs appeared to be promising candidates, the present study, however, was restricted to the detailed investigation of miR160 in potato - *P. infestans* infection.

2.4.2. Potential role of miR160 in local and systemic leaves during potato – *P. infestans* interaction

As mentioned above, several studies have earlier shown the induction of miR160 during different plant pathogen interaction (Li et al., 2010; Bazzini et al., 2011; Zhang et al., 2011). However, it is important to note that these analyses were limited to the site of infection (local tissues). No studies were carried out to analyse the expression changes in systemic-non-infected tissues. In our analysis, we observed that miR160 expression is induced in both local and systemic leaves (Figure 2.6) of potato against *P. infestans* infection. Previous studies (Li et al., 2010; Bazzini et al., 2011; Zhang et al., 2011) and our findings support the premise that miR160 play potential role in the local (basal / PTI) defence responses of plants. Additionally, the induced expression of miR160 in systemic leaves as observed in our study, further indicates its possible role in systemic defence or systemic acquired resistance (SAR) response.

Interestingly, the expression of *StARF10*, target gene of miR160, was also induced in both local and systemic leaves of potato upon *P. infestans* infection (Figure 2.8). There could be two possible explanations for this correlated increase of both miR160 and *StARF10* expression. First, though our cleavage site mapping assay showed that miR160 can regulate *StARF10* mRNA via cleavage, it is also possible that *St*ARF10 is regulated at protein level by miR160-mediated translational repression. This could be the reason, why reduction in *StARF10* mRNA was not observed even though miR160 levels were induced. Second possible explanation could be both miR160 and *StARF10* induction is required during *P. infestans*

infection, however the optimal levels of *StARF10* is maintained by miR160. Overall, our analyses revealed a potential role miR160 and its target *StARF10* in local and systemic defence responses of potato against *P. infestans* infection.

2.4.3. miR160: possible role as mobile signal?

Consistent with previous reports from *Brassica* and apple (Buhtz et al., 2008, 2010; Varkonyi-gasic et al., 2010), our analyses also revealed the presence of miR160 in the phloem of potato (Figure 2.11 E and 2.12 D & E). Presence of miRNAs in phloem has been reported in various plant species (Yoo et al., 2004; Buhtz et al., 2008; Varkonyi-gasic et al., 2010), however, only *miR399*, *miR395*, miR172 and miR156 have so far been demonstrated to act as phloem mobile signals during abiotic stress and developmental phase change (Pant et al., 2008; Buhtz et al., 2010; Kasai et al., 2010; Bhogale et al., 2014). We observed a significant increase of miR160 (~9 fold) in the PEX (phloem enriched exudate) of both SUS and MR variety upon *P. infestans* infection (Figure 2.12 F) at 6 hpi. Furthermore, the absence of miR160 precursor (*Stpre160)* in PEX is an indication of non-phloem origin of mature miR160. This possibly suggests an active loading of miR160 into the potato phloem during infective conditions. As miR160 levels were also observed to be induced in systemic leaves, the possibility of miR160 as a mobile signal during infective conditions cannot be ruled out. This could be an important investigation for future studies, however, is not in the scope of present investigation.

In conclusion, our results demonstrate that multiple potato miRNAs (miR159, miR160, miR166, miR169 and miR172) respond to *P. infestans* infection and exhibit varied expression patterns. Detailed analysis of miR160 and its target *StARF10* revealed their induced expression in both local and systemic leaves. Also, increased accumulation of miR160 in phloem under infective conditions suggests its potential role as phloem mobile signal. To further decipher the function of miR160 in local and systemic defence responses of potato to *P. infestans* infection, transgenic approaches (overexpression and knock-down) were undertaken in the study described in chapter 3.

Chapter 3

Characterization of miR160 and its role in local defence and SAR responses of potato

3.1. Introduction

Since miR160 levels were found to be induced in both local and systemic leaves of potato upon *P. infestans* infection, we investigated the detail of function of miR160 in basal defence as well as systemic acquired resistance (SAR) in potato. To achieve this, miR160 overexpression and knockdown plants were generated and their local and SAR defence responses were analysed in the present study.

3.1.1. Local and systemic defence responses in plants

Upon infection by pathogens, plants mount defence responses in local infected site as well as in systemic non-infected sites (Stael et al., 2015; Cook et al., 2015). At local site, the activation of defence responses is established by the perception of pathogens through plant receptor molecules. Plants have evolved two different levels of pathogen perception mechanisms, (I) Pathogen-associated molecular pattern (PAMP)-perception and (II) effector protein perception, leading to PAMP-triggered immunity (PTI) and effector-triggered immunity (ETI) respectively (Chisholm et al., 2006; Jones and Dangl, 2006; Dodds and Rathjen, 2010; Zhang and Zhou, 2010; Bernoux et al., 2011). During PTI, membrane-bound pattern recognition receptors (PRRs) recognize conserved structures on pathogens and initiate defence responses (Felix et al., 1999; Gómez-Gómez and Boller, 2000, 2002; Zipfel et al., 2006; Chinchilla et al., 2006). One of the best understood examples of PAMP-PRR interaction is the recognition of flagellin (or flg22) by the *Arabidopsis* receptor kinase FLAGELLIN SENSING 2 (FLS2) (Gómez-Gómez and Boller, 2002; Chinchilla et al., 2006). However, certain pathogens have evolved mechanisms to suppress PTI by releasing proteins called “effectors” into plant cells. Effectors essentially interfere with defence pathways involved in PTI, hence restoring the susceptibility (Hauck et al., 2003; Keshavarzi et al., 2004). Plants, in turn, employ resistance (R)-proteins to detect these host manipulating effector proteins thereby inducing ETI responses as demonstrated by several reports (Jones et al., 1994; Song et al., 1995; Dangl and Jones, 2001; Fritz-Laylin et al., 2005). *Arabidopsis* protein RIN4, targeted by three *P. syringae* effectors AvrRpm1, AvrB and AvrRpt2, is the best characterised example of how R-proteins monitor the activity of effectors (Mackey et al., 2002; Axtell and Staskawicz, 2003; Kim et al., 2005). Recent studies also demonstrated the role

miRNAs in regulating PTI and ETI responses revealing a new layer of plant defence regulation (Ruiz-Ferrer and Voinnet, 2009; Boccara et al., 2014) A detailed description of PTI and ETI defence responses has been discussed in chapter 1.

Apart from the elicitation of such local responses, induction of systemic resistance is also observed in plants. Systemic acquired resistance (SAR) has been investigated for several decades (Fu and Dong, 2013; Shah and Zeier, 2013; Gozzo and Faoro, 2013; Wendehenne et al., 2014; Gao et al., 2015). The plant hormone salicylic acid (SA) is one of the well-studied and an important component in the process of SAR response (Shah, 2003; Durrant and Dong, 2004; Loake and Grant, 2007; Vlot et al., 2009; Gozzo and Faoro, 2013; Gao et al., 2015). In *Arabidopsis* and tobacco, SA levels are increased both locally and systemically upon SAR induction (Yalpani et al., 1991; Enyedi et al., 1992; Summermatter et al., 1995). Accompanying this is an increased expression of anti-microbial pathogenesis-related (PR) proteins in the systemic tissue (Ward et al., 1991; Uknes et al., 1992; Alexander et al., 1993). Apart from SA and PR genes, number of studies have revealed new molecular players in SAR process. For example, DIR1 (Defective in Induced Resistance) (Maldonado et al., 2002), 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). A detailed description of signals and genes involved in SAR pathway have been discussed in chapter 1. Increasing number of studies have revealed the complexity of SAR pathway along with raising several new questions, one of them being the role of miRNAs in SAR.

3.1.2. Role of miR160 in plant development and defence

miR160 is a conserved plant miRNA whose role in plant growth and development has been unequivocally established through elaborate studies in *Arabidopsis*, tomato, soybean and rice (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). The major targets of miR160 are Auxin responses factors, ARF10, ARF16 and ARF17. Proper functioning of all these targets are critical for regulation of auxin-mediated processes in plants. Studies show

that abolishing the activity of miR160 on these targets leads to several developmental abnormalities. For example, Mallory and co-workers (2005) have demonstrated that disrupting the regulation of miR160 on ARF17 leads to severe developmental defects in embryonic, root, vegetative and floral development suggesting the critical role of miR160-directed regulation of ARF17 in *Arabidopsis* (Mallory et al., 2005). Similarly, two studies in tomato have showed that knockdown of *Sl*-miR160 or overexpression of *Sl*-miR160-resistant *Sl*-ARF10 results in plants with abnormal leaves, sepals, petals and fruit shape (Hendelman et al., 2012; Damodharan et al., 2016). Another recent report in rice by Huang and co-workers (2016), indicated the role of *Os*-miR160 in regulation of *Os*-ARF18 (rice homolog of *Arabidopsis* ARF16) (Huang et al., 2016). The authors demonstrated that plants overexpressing *Os*-miR160-resistant version of *Os*-ARF18 exhibit pleiotropic defects such as dwarf stature, rolled leaves, small seeds with reduced starch accumulation. All these studies clearly establish the vital role of miR160 and its targets in plant (both dicots and monocots) growth and development.

Until recently, miR160 was known only for its role in plant development. The defence-related role of miR160 emerged after several studies reported its differential regulation during a variety of plant –pathogen interactions (Li et al., 2010; Bazzini et al., 2011; Zhang et al., 2011; Li et al., 2014). So far, only two detailed studies from *Arabidopsis* (Li et al., 2010) and rice (Li et al., 2014) validated the role of miR160 in plant biotic stress. Li and co-workers (2010) showed that treatment of *Arabidopsis* leaves with flg22 (bacterial PAMP) results in increased expression of miR160 (Li et al., 2010). Flg22 treatment also led to increased callose deposition in miR160 overexpression lines compared to WT plants, suggesting a role for miR160 in flg22-mediated PTI responses. These plants, however, did not exhibit any increase in resistance to bacterial infection (Li et al., 2010). Contrast to this, another recent report showed that overexpression of miR160 in rice leads to enhanced resistance to the rice blast fungus *Magnaporthe oryzae* (Li et al., 2014) suggesting a possible positive regulation of defence by miR160.

To address if miR160 has any role in local and SAR response of potato, following three approaches were undertaken in this study:

i. To generate overexpression (OE) and knockdown (KD) lines of miR160 in potato.
ii. To investigate the basal defence response of miR160 OE and KD lines to *P. infestans* infection.
iii. To analyses the SAR response of miR160 OE and KD lines to *P. infestans* infection.

3.2. Materials and Methods

3.2.1. Plant and pathogen material

The wild-type (WT) and transgenic potato *(S. tuberosum* cv. Désirée) and tobacco (*Nicotiana benthamiana*) plants were grown and maintained *in vitro* at 25°C under long day conditions (LD, long day, 16 hrs light : 8 hrs dark) in a tissue culture incubator (Percival Scientific). Plants were later transferred to soil mixture (soil : perlite : soilrite : coco peat) and were maintained at 22°C under LD conditions in environmental plant growth chambers (Percival Scientific). Oomycete pathogen, *Phytophthora infestans* was maintained as described in chapter 2 (materials and methods section). The bacterial pathogen *Ralstonia solanacearum* was maintained in nutrient agar medium.

3.2.2. Construct design and plant transformation

miR160 overexpression (OE) construct, 35S::*St*-pre160-pBI121 (Figure 3.1 A), was generated by amplifying miR160 precursor (*St-pre160*), from potato RNA using primers Pre160-FP and Pre160-RP. The amplified product (129 bp) was finally cloned into binary vector pBI121 under 35S CaMV constitutive promoter. For knockdown (KD) construct, two different approaches, namely endogenous target mimicry (eTM) (Wu et al., 2013) and artificial target mimicry (MIM) (Todesco et al., 2010) were considered (Figure 3.1 B). The KD construct 35S::ath-eTM160-pCAMBIA1300 was a kind gift from Prof. Wang (Institute of Genetics and Developmental Biology, Beijing, China) (Wu et al., 2013). The artificial target mimicry KD construct MIM160 was obtained from European Arabidopsis Stock Centre (NASC) (Todesco et al., 2010). The MIM160 insert (542 bp) which was originally cloned into pGREEN vector, was re-cloned to pBI121 binary vector (to generate the construct 35S::MIM160-pBI121) and used for plant transformation.

All the potato and tobacco transformation (Figure 3.1 C and D) were performed as described previously (Banerjee et al., 2006b; Horsch et al., 1985). The transgenic plants were raised *in vitro* and maintained under the selection of kanamycin (50 mg/l) for *St*-pre160 and MIM160 and hygromycin (3 mg/l) for eTM160. Transgenic lines were confirmed by performing gene specific PCR of

pre160 (pre160-FP & NosT-RP), eTM160 (eTM160-FP and eTM160-RP) and MIM160 (MIM-FP and NosT-RP), respectively using the genomic DNA as template. Further, miR160 and *StARF10* transcript levels were estimated by qRT-PCR analysis of transgenic lines as described in chapter 2.

3.2.3. Quantitative real-time PCR (qRT-PCR) analysis

For all the reactions, cDNA was prepared from one microgram (1 µg) of total RNA using oligo(dT) reverse primer and Superscript III Reverse Transcriptase (Invitrogen). qRT-PCR reactions were carried out using KAPA SYBR Green Mix (Kapa Biosystems). For *StPR1*, reaction conditions were 95°C 2 min, 40 cycles of 95°C 15 sec and 60°C 20 sec, whereas, for eTM160 and *St-pre160*, reaction conditions were 95°C 2 min, 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 by using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Analysis of miR160 and *StARF10* was performed as mentioned in chapter 2. *GAPDH* was used as normalization gene for potato and L23 (ribosomal RNA) was used as normalization gene in tobacco.

3.2.4. Northern blot analysis

Northern blot analysis of miR160 expression levels in transgenic potato KD and OE lines were performed as described previously in chapter 2 (materials and methods).

3.2.5. *P. infestans* infection experiment

To test the basal resistance of miR160 transgenic lines, infection experiment was carried out by spraying plants with *P. infestans* sporangia (concentration of $2x10^5$ /ml). For potato plants, *Désirée* WT, miR160-KD and miR160-OE lines, disease progression was monitored till 14 days post inoculation (dpi). For tobacco WT and KD lines, disease progression was followed till 9 dpi. Samples were collected on 0, 2, 5, 7, 9, 11 and 14 dpi for various molecular and microscopic analysis and stored until further use.

3.2.6. *P. infestans* DNA quantification

Infected leaf samples were collected from WT, vector control (VC i.e. pBI121), miR160 KD and miR160 OE lines 14 days post *P. infestans* inoculation. Genomic DNA was isolated from these samples using DNeasy Plant Mini Kit (Qiagen). Fifty picograms (50 pg) of DNA was used for qRT-PCR amplification of *P. infestans* specific O8 sequence using primers O8-3 and O8-4 (Judelson and Tooley, 2000). For absolute quantification, a standard curve for O8 was generated using different concentrations of *P. infestans* genomic DNA. The qRT-PCR reactions conditions were 95°C 2 min 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.

3.2.7. Systemic Acquired Resistance (SAR) assay

Four weeks old potato (WT, VC, miR160 OE & miR160 KD) and two weeks old tobacco plants (WT & miR160 KD) were subjected to primary infection with *P. infestans* followed by secondary infection with *Ralstonia solanacearum*. For primary infection, 50 µl of $2x10^5$ sporangia/ml *P. infestans* was swabbed on two lower-most leaves and plants were incubated in growth chamber at 18°C. Mock inoculation was carried with sterile water. Four days post primary infection, 2-3 upper leaves were syringe infiltrated with 10^6 CFU/ml (OD_{600} ~0.1) of *Ralstonia* and plants were incubated in growth chamber at 28°C for secondary infection. After five days of secondary infection, one cm^2 leaf piece from the *Ralstonia* inoculated leaves was excised 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 *Ralstonia* specific PCR using primers Rs_BP4R and Rs_BP4L as described previously (Lee and Wang, 2000).

3.2.8. Grafting assay

To assess the role of miR160 in affecting SAR response, homo and hetero-grafts were generated with WT plants and miR160 KD lines (eTM160-26) of potato. Three weeks old tissue culture plants were transferred to soil and hardened in plant growth chamber. Two weeks post transfer to soil, grafts were generated and incubated in growth chamber for another seven days. Two types of each homo-graft (WT / WT

and eTM160-26 / eTM160-26) and hetero-grafts (WT / eTM160-26 and eTM160-26 / WT) were generated. Altogether 32 -40 grafts (8-10 grafts for each combination) were made following a previously described protocol (Banerjee et al., 2006a). SAR assay were performed as described previously. In brief, two leaves of grafted stock plants were either inoculated with *P. infestans* or sterile water (mock treatment). Four days post-primary inoculations, the systemic scion leaves of all the grafts were inoculated with *R. solanacearum*. After five days of secondary infections, systemic scion leaves were harvested and bacterial count was recorded as described above.

3.2.9. Accession numbers

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

Table 3.1 Accession numbers

Name	Accession	Source*
StARF10	PGSC0003DMT400020874	PGSC
StPR1	AY050221	NCBI
GAPDH	PGSC0003DMT400044944	PGSC
U6	X60506	NCBI
L23	XM_016629168	NCBI
* PGSC – Potato Genome Sequencing Consortium: http://solanaceae.plantbiology.msu.edu/integrated_searches.shtml * NCBI – National Centre for Biotechnology Information: https://www.ncbi.nlm.nih.gov/		

3.2.10. Primer sequences

Primers used in this chapter are listed in Table 3.2

Table 3.2 List of primers

Primer Name	Sequence 5' - 3'
miR160 transgenic generation and clone confirmation	
pre160_FP	GAGATCTAGACACGTCGTGTACACGTATA
pre160_RP	GAGAGAGCTCCAACATCATATACACGATATCGG
eTM160_FP	TCTTCAGAGATGGCCTGAC

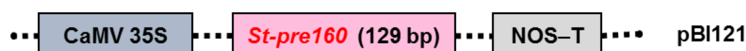
eTM160_RP	AATCGTAATCCTAATCAGTGTT
MIM_FP	GAGACCCGGGAAAACACCACAAAAACAAAAGA
MIM_RP	GAGAGAGCTCAAGAGGAATTCACTATAAAGAG
NosT_RP	GCAACAGGATTCAATCTTAAG
qRT-PCR analysis	
miR160_STP	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATAC GACGGCATA
miR160_FP	TGGAGTTTGCCTGGCTCCCTG
Univ-miR_RP	AGTGCAGGGTCCGAGGT
ARF10_FP	GTCCAGCAGTCCTTTCTGTTGTTT
ARF10_RP2	GCTGCAACACGCTGGAAACTT
PR1_FP	GTACCAACCAATGTGCAAGCG
PR1_RP	TGTCCGACCCAGTTTCCAAC
pre160_FP	GAGATCTAGACACGTCGTGTACACGTATA
pre160_RP	GAGAGAGCTCCAACATCATATACACGATATCGG
eTM160_FP	TCTTCAGAGATGGCCTGAC
eTM160_RP	AATCGTAATCCTAATCAGTGTT
Northern Analysis	
miR160_RC	GGCATACAGGGAGCCAGGCA
U6_RC	AGGGGCCATGCTAATCTTCTC
***Ralstonia* confirmation**	
Rs_BP4R	GACGACATCATTTCCACCGGGCG
Rs_BP4L	GGGTGAGATCGATTGTCTCCTTG
Reference Genes	
GAPDH_FP	GAAGGACTGGAGAGGTGGA
GAPDH_RP	GACAACAGAAACATCAGCAGT
L23_FP	AAGGATGCCGTGAAGAAGATGT
L23_RP	GCATCGTAGTCAGGAGTCAACC

3.3. Results

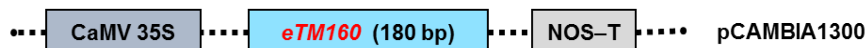
3.3.1. Generation of miR160 overexpression and knockdown transgenic lines

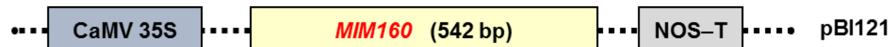
To further uncover the functions of miR160 during potato - *P. infestans* interaction, overexpression (OE) and knockdown (KD) transgenic lines of miR160 were raised in potato as well as tobacco. As SUS and MR potato varieties were not amenable for transformation, wild-type potato, *S. tuberosum* cv. Désirée was used for generation of transgenic lines. For tobacco, *Nicotiana benthamiana* plants were used for generation of transgenic lines (Figure 3.1). In potato, based on the expression patterns of *St-pre160*, miR160 and *StARF10*, OE lines pre160-L17C1 and pre160-L17-D1 were selected for further analysis from seven independent transgenic lines (Figure 3.2). Similarly, from ten independent KD lines of potato, eTM160-L24-2 and eTM160-26 KD lines were selected based on the expression patterns of eTM160, miR160 and *StARF10* (Figure 3.3). Selected OE and KD lines were further validated by qRT-PCR and northern analysis (Figure 3.4). In tobacco, two KD lines (MIM160-15 and eTM160-7) were chosen based on the expression patterns of miR160 and *NbARF10* (Figure 3.5).

A Overexpression construct



B Knockdown constructs





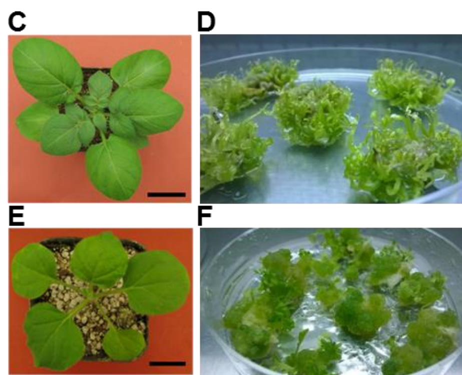


Figure 3.1. Generation *miR160* overexpression and knockdown transgenic lines in potato and tobacco. A. Overexpression construct; **B.** eTM and MIM Knockdown constructs; **C-D.** *S. tuberosum cv. Désirée* soil grown plant and transgenic lines**; E-F.** *N. benthamiana* soil grown plant and transgenic lines**.**

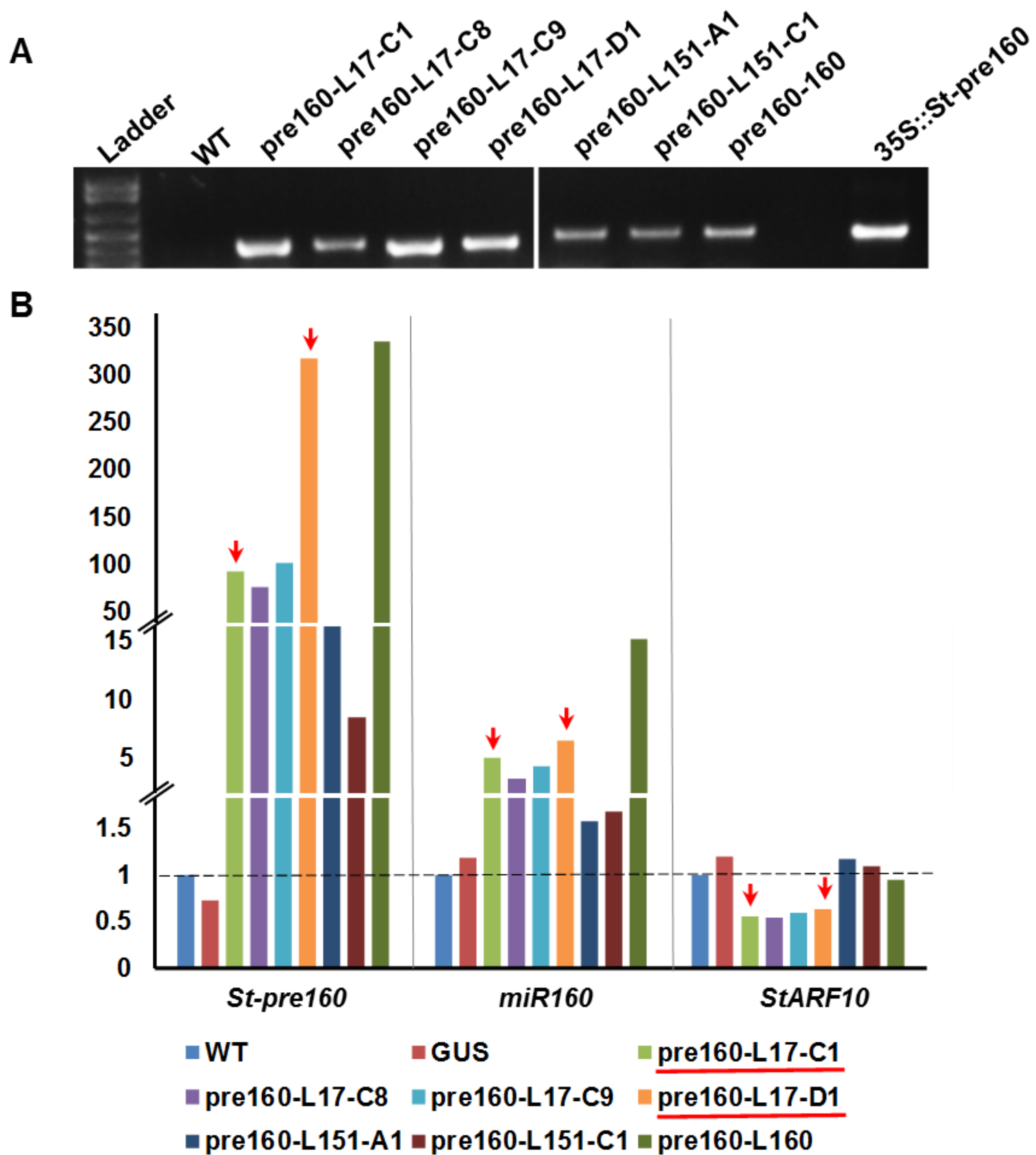


Figure 3.2. Generation of miR160 overexpression (OE) lines in potato. A. Confirmation of multiple OE lines by performing genomic DNA PCR using primers pre160-FP and NOS-T-RP. The genomic DNA from wild type (WT) plants was used as negative control and the plasmid, 35S::St-pre160-pBI121, was used as positive control in the reaction. **B.** qRT-PCR based analysis *St-pre160*, miR160 and *StARF10* levels in the OE lines. The OE lines, pre160-L17-C1 and pre160-L17D1 (underlined in red and red arrows), were selected for further analysis based on their high-expression of *St-pre160* and miR160 as well as reduced expression of the target *StARF10*. The data is mean of one biological replicate from three technical replicates.

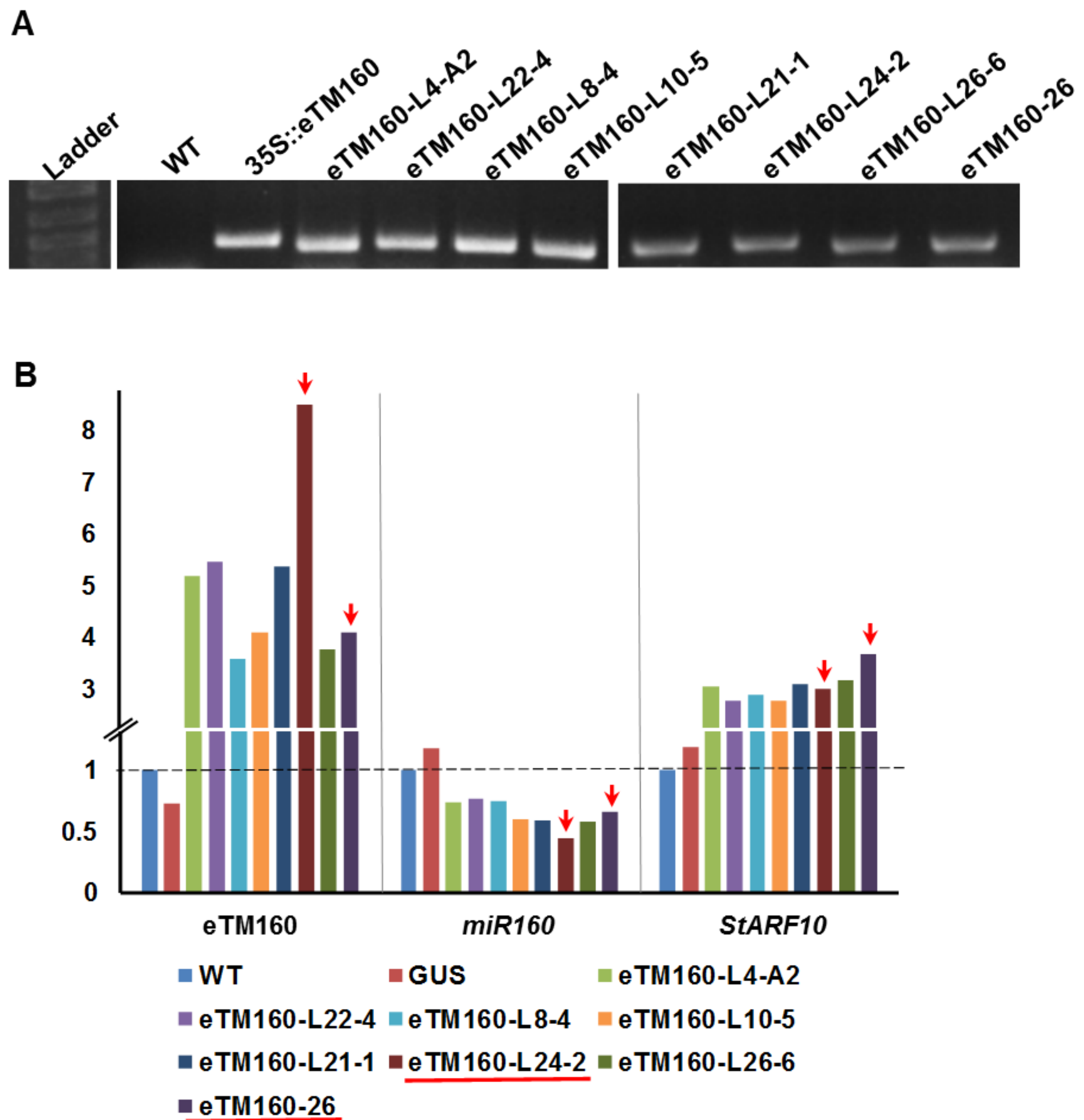


Figure 3.3 Generation of miR160 eTM knockdown (KD) lines in potato. A. Confirmation of multiple KD lines by performing genomic DNA PCR using primers eTM160-FP and eTM160-RP. The genomic DNA from wild type (WT) plants was used as negative control and the plasmid, 35S::eTM160-pCAMBIA1300, was used as positive control in the reaction. **B.** qRT-PCR based analysis eTM160, miR160 and *StARF10* levels in the KD lines. The KD lines, eTM160-L24-2 and eTM160-26 (underlined in red and red arrows), were selected for further analysis based on their high-expression of eTM160 and the target *StARF10* as well as reduced expression of miR160. The data is mean of one biological replicate from three technical replicates.

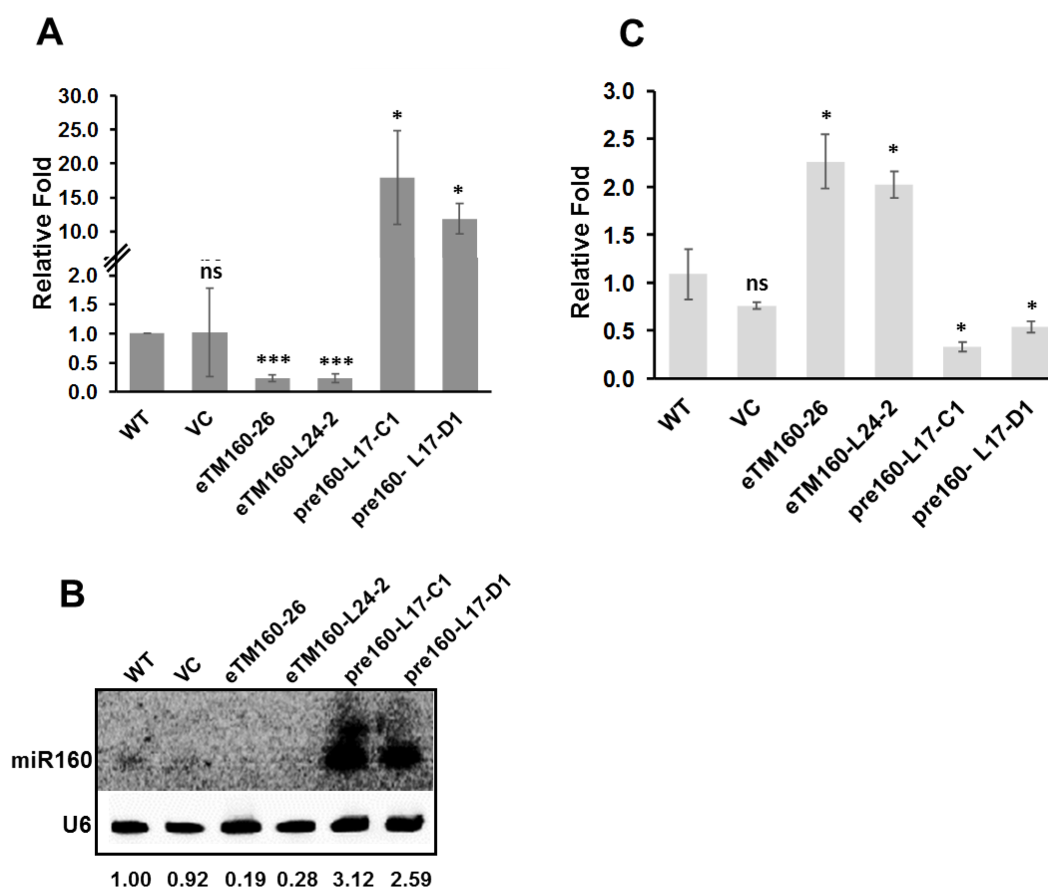


Figure 3.4 Validation of selected miR160 OE and KD lines of potato. A-B. Levels of miR160 in OE (pre160-L17-C1 and pre160-L17-D1) and KD (eTM160-26 and eTM160-L24-2) lines as analyzed by qRT-PCR (A) and northern blot (B). **C.** Levels of *StARF10* in OE and KD as analyzed by qRT-PCR. All the data from qRT-PCR analysis are plotted as mean ± standard deviation of three biological replicates with three technical replicates each. Northern blot is a representative of two biological replicates with similar results. WT is wild-type potato and VC is vector control plants transgenic for pBI121 vector. Asterisk indicate statistical significance where * is $p<0.05$ and *** is $p<0.005$ as per Student's t-test. 'ns' indicates not significant.

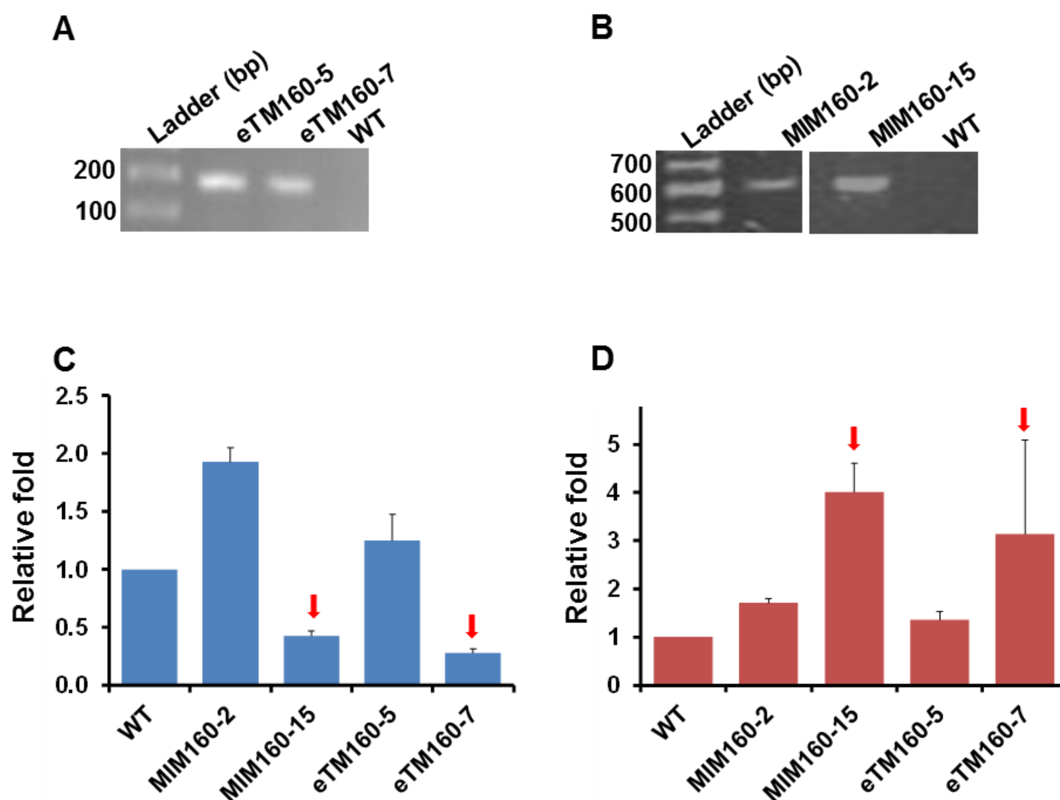


Figure 3.5 Generation of miR160 eTM and MIM knockdown (KD) lines in tobacco. A. Validation of tobacco KD lines generated by eTM (A) and MIM (b) approaches by performing RT-PCR using primers eTM160-FP & eTM160-RP and MIM160-FP & NosT-RP respectively. The RNA from wild type (WT) plants was used as negative control. **C-D.** qRT-PCR based analysis miR160 (C) and *NbARF10* (D) levels in the KD lines. The KD lines, MIM160-15 and eTM160-7 (red arrows), were selected for further analysis based on their reduced expression of miR160 and high-expression of *NbARF10*. The data is mean of three biological replicate from three technical replicates each.

3.3.2. Overexpression and knockdown of miR160 does not drastically alter plant morphology

As miR160 is known to play an important role in plant development, we analyzed miR160 OE and KD lines for any morphological changes. No drastic changes were observed in any of the miR160 OE and KD lines of potato (Figure 3.6 A & B) as well as KD lines of tobacco (Figure 3.6 C), except that miR160 OE lines of potato exhibited a slight downward curled leafy phenotype (Figure 3.6 D). Though

none of the transgenic lines showed any morphological changes, tuberization was significantly affected in both OE and KD potato lines. It was observed that miR160 KD lines showed increased tuber yield, whereas, in OE lines, tuber yield was drastically reduced (Figure 3.7). These results suggest the possible role of miR160 in tuberization pathway in potato.

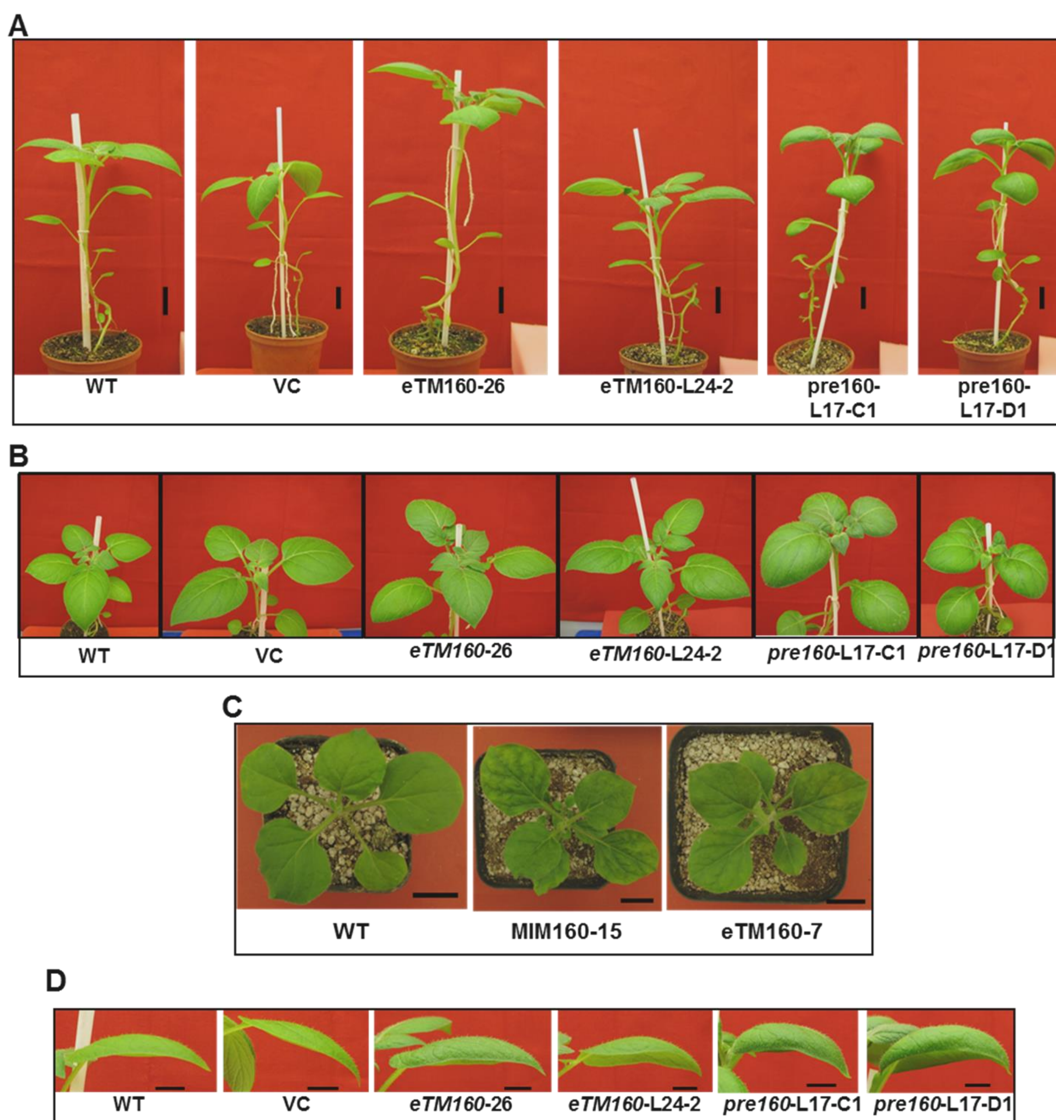


Figure 3.6 Morphological phenotype of potato and tobacco miR160 transgenic lines. A-B. Morphology of the potato plants were not severely affected due to miR160 OE and KD. Black bar represents scale of 2 cm. **C.** miR160 KD lines of tobacco also did not show drastic morphological changes compared to WT plants. Black bar represents scale of 5 cm. WT is wild-plants, VC is transgenic vector control plants. **D.** Downward curled leafs were present only in the miR160 OE lines, pre160-L17-C1 and pre160-L17-D1. Black bar represents scale of 1 cm.

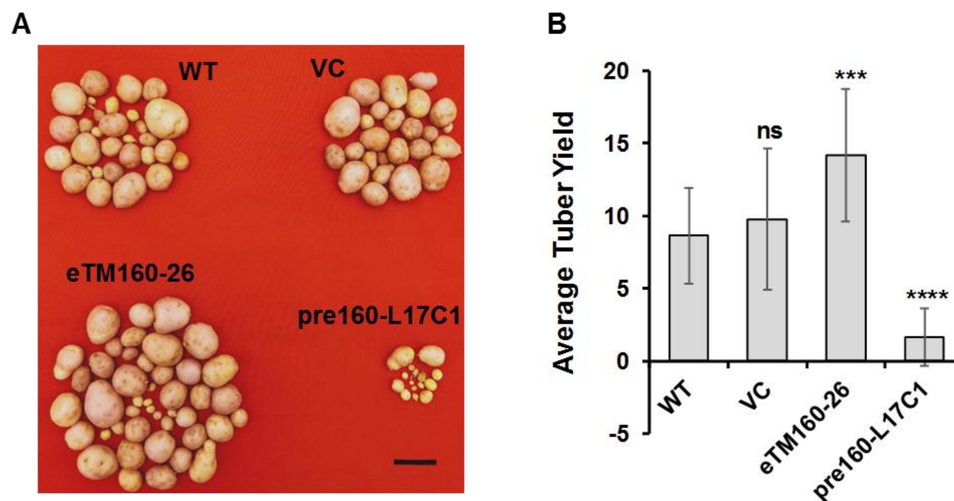


Figure 3.7 Tuber yield is affected in miR160 potato transgenic lines. B. miR160 KD line eTM160-26 developed more tubers and OE line pre160-L17C1 developed lesser tubers compared to WT and VC plants. Tuber picture is the data from six independent plants for each line. Black bar represents scale of 2 cm. **C.** At least six plants were used for quantification and average tuber yield was plotted as mean tuber weight (gm) per plant. Asterisk indicates statistical significance where *** is $p<0.01$ and **** is $p<0.0005$ as per Student's t-test. 'ns' indicates not significant.

3.3.3. miR160 OE and KD lines exhibit enhanced susceptibility to *P. infestans* infection

To assess the role of miR160 in local (basal) defence response, transgenic lines (OE & KD) were challenged with *P. infestans* and disease progression was monitored over a period of 14 days. Disease symptoms appeared as early as 8 dpi (days post inoculation) in OE lines of potato and by 11 dpi, both KD and OE lines developed severe disease symptoms as opposed to WT and vector control (VC) plants (Figure 3.8 A). Similarly, tobacco KD lines also showed reduced basal defence compared to WT plants (Figure 3.8 B).

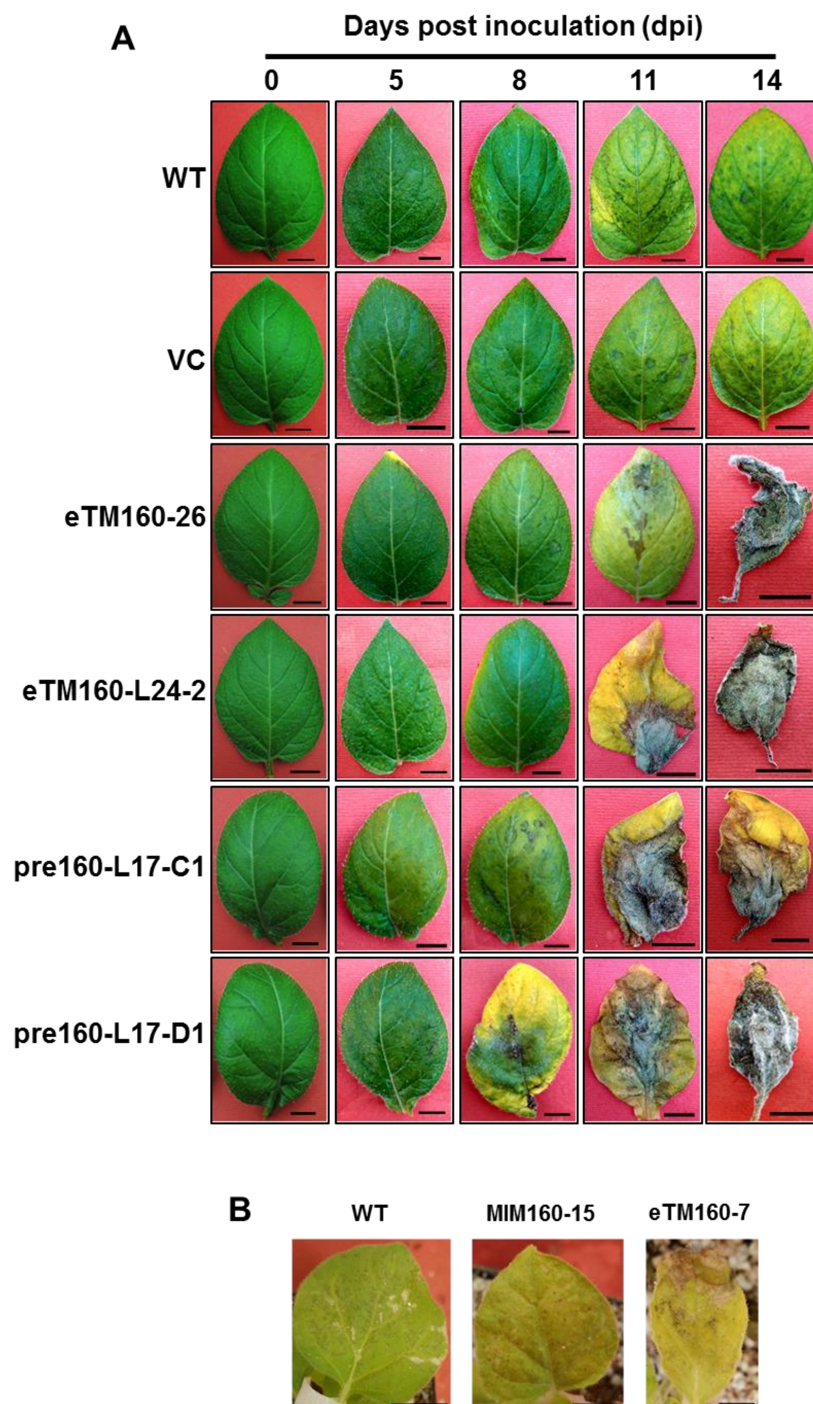


Figure 3.8 Disease progression in miR160 transgenic lines of potato and tobacco. A. *P. infestans* infected potato plants were monitored for a period of 14 days. Leaves from the infected plants showed that both miR160 KD (eTM160-26 & eTM160-L24-2) and OE (pre160-L17C1 and pre160-L17D1) results in reduced basal resistance and thereby increased susceptibility to *P. infestans.* **B.** miR160 KD lines of tobacco show increased susceptibility to *P. infestans* compared to WT plants as observed on 9 dpi.

By 14 dpi, majority of the OE and KD lines exhibited severe disease symptoms (Figure 3.9 A). The *P. infestans* load (biomass) was also significantly higher in these lines compared to WT and VC plants. (Figure 3.9 B & C). Though, OE and KD lines induced *StPR1* expression, the magnitude of induction was highly reduced compared to WT plants (Figure 3.9 D). Altogether, our findings indicated that both overexpression and knockdown of miR160 results in increased susceptibility and optimal levels of miR160 may be required for maintaining the basal resistance in potato against *P. infestans*.

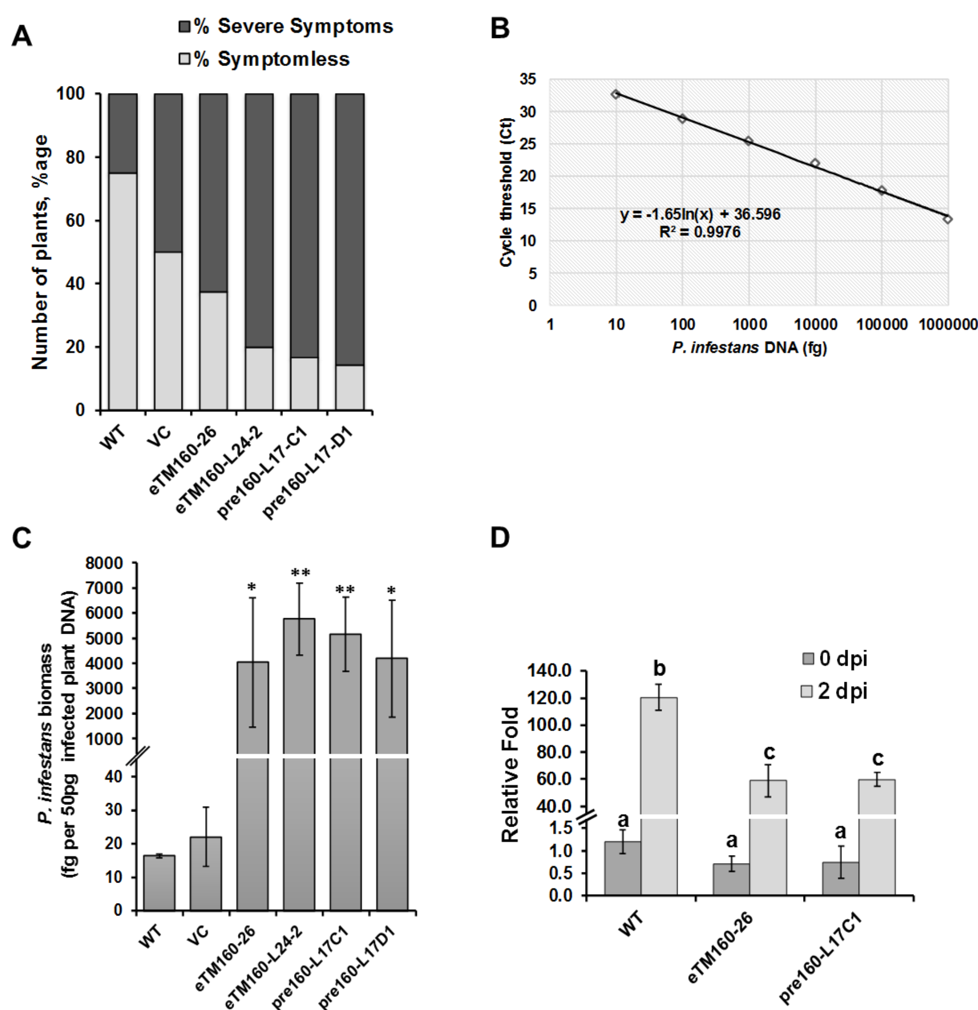


Figure 3.9 miR160 OE and KD lines are highly susceptible to *P. infestans* infection. A. By 14 dpi, majority of miR160 KD (eTM160-26 & eTM160-L24-2) and OE (pre160-L17C1 and pre160-L17D1) plants showed severe symptoms of *P. infestans* infection. Four to ten infected plants were assessed for each plant type. **B-C.** For absolute quantification of *P.*

infestans biomass, standard curve was prepared using different concentrations of *P. infestans* genomic DNA (B); qRT-PCR analysis of genomic DNA from infected plants (14 dpi) indicate increased *P. infestans* biomass in KD and OE lines (C). Data represented is mean ± standard deviation of three biological replicates. Asterisk indicates statistical significance where * is $p<0.05$ and ** is $p<0.01$ as per Student's t-test. **D.** qRT-PCR analysis of *StPR1* levels. At 2 dpi, *StPR1* levels increase in WT as well as eTM160-26 (KD line) and pre160-L17C1 (OE line), however the magnitude of increase is greatest in WT plants. Data represented is mean ± standard deviation of three biological replicates. Different alphabets indicate statistical significance with $p<0.05$ as per Student's t-test.

3.3.4. miR160 knockdown, not the overexpression, leads to compromised SAR response

Because miR160 overexpression and knockdown both resulted in susceptibility (Figure 3.8 and 3.9), we wanted to further understand if miR160 plays any role in SAR response as well. Using *P. infestans* as primary pathogen and *Ralstonia solanacearum* as secondary pathogen, we demonstrated that potato miR160 OE lines triggers significant SAR response similar to WT and VC (vector control) plants, however miR160 KD lines failed to mount an effective SAR (Figure 3.10 A). Additionally, we observed that SAR was compromised in miR160 KD lines of tobacco (Figure 3.10 B). Overall, our SAR analysis revealed that miR160 KD lines of both potato and tobacco are compromised in eliciting an effective SAR response.

This compromised SAR response exhibited by KD lines could be because of SAR signalling defects associated either with local or systemic leaves or both. To address this, SAR assays were performed on homo-grafts (WT/WT and eTM160-26/eTM160-26) and hetero-grafts (eTM160-26/WT and WT/eTM160-26) generated with WT plant and KD line eTM160-26 of potato (Figure 3.11 A). Consistent with our previous results (Figure 3.10 A), homo-grafts of WT/WT showed significant SAR development and homo-grafts of KD line (eTM160-26/eTM160-26) did not exhibit any SAR response (Figure 3.11 B). Also, none of the hetero-grafts showed a significant SAR response. To further analyse the defective SAR response, expression levels of *StPR1* was measured in the *P. infestans* inoculated local stock leaves as well as in non-inoculated systemic scion leaves at 4 dpi (of primary infection). Although *StPR1* expression was induced in local stock leaves of all grafted plants, WT/WT homo-grafts showed highest magnitude of induction (Figure 3.12 A). In systemic

scion leaves, induced *StPR1* expression was observed only in WT/WT homo-graft (Figure 3.12 B). Altogether, these results suggest that miR160 levels equivalent to WT or even more is perhaps required in both local and systemic leaves for mounting an effective SAR response.

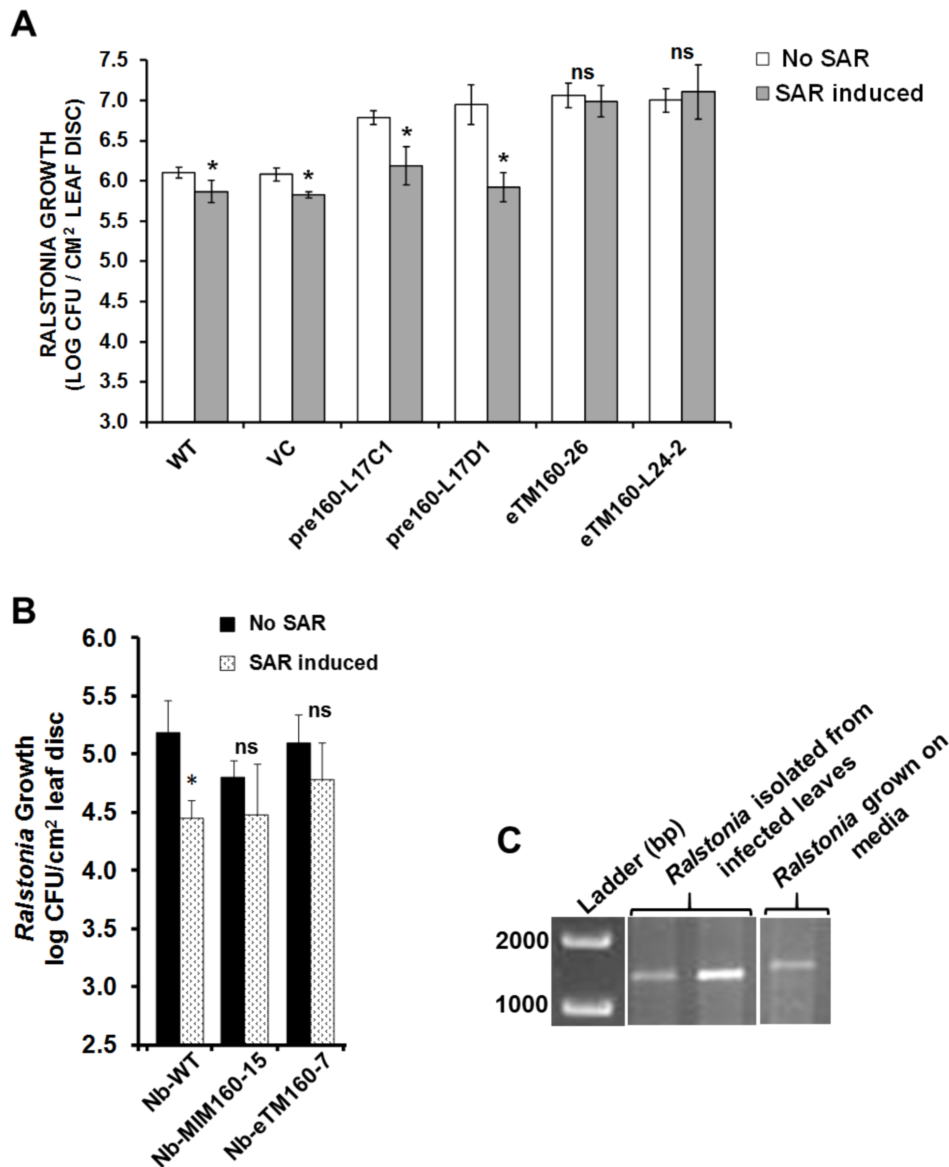


Figure 3.10 Knockdown of miR160 in both potato and tobacco leads to compromised SAR response. A-B. SAR assays performed with miR160 KD and OE lines of potato (A) and KD lines of tobacco (B). For primary infection, local leaves were either treated with sterile water (referred as 'no SAR') or *P. infestans* (referred as 'SAR induced'). After 4 days, systemic leaves of all the plants were given secondary infection with the bacterium, *Ralstonia*

solanacearum (OD_{600} 0.1). *In planta* bacterial titers were determined 5 days post *Ralstonia* infiltration in systemic leaves. In potato, OE lines were able to induce significant SAR response similar to WT to VC plants as evident by the reduced *Ralstonia* growth (A). However, KD lines of both potato (A) and tobacco (B) were unable to elicit effective 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 'No SAR' plants for each plant type, $P < 0.05$ (*). 'ns' indicates not significant. **C.** PCR based detection of *Ralstonia* isolated from potato plants infected during SAR experiment. DNA from *Ralstonia* grown on nutrient agar medium was used as positive control. Detection was performed using primers Rs-BP4-R and Rs-BP4-L as described by Lee and Wang, (2000).

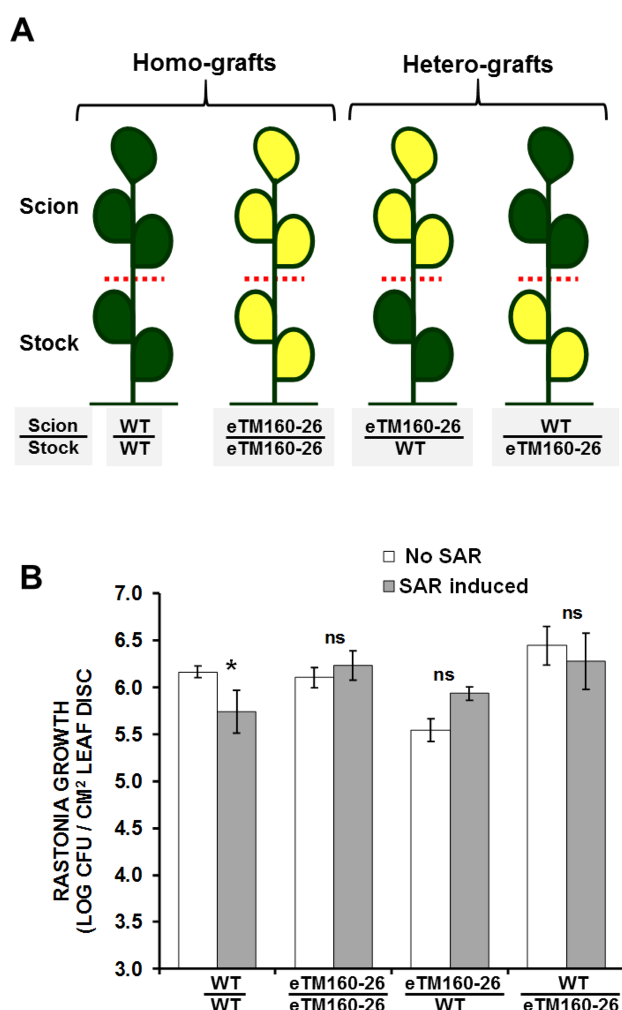


Figure 3.11 SAR analysis in homo- and hetero-grafts of WT and eTM160-26 KD line of potato. A. Schematics of the different combination of grafts developed in potato: homo-grafts (WT/WT and eTM160-26/eTM160-26) and hetero-grafts (WT/eTM160-26 and eTM160-

26/WT). **B.** Two lower leaves on the stock of each grafted plant were either treated with sterile water (No SAR) or *P. infestans* (SAR induced). After 4 days, untreated systemic leaves from the scion of all the grafted plants were treated with *Ralstonia solanacearum* (OD_{600} 0.1). *In planta* bacterial titers were determined 5 days post *Ralstonia* infiltration in systemic scion 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 'No SAR' plants for each plant type, $P < 0.05$ (*). 'ns' indicates not significant.

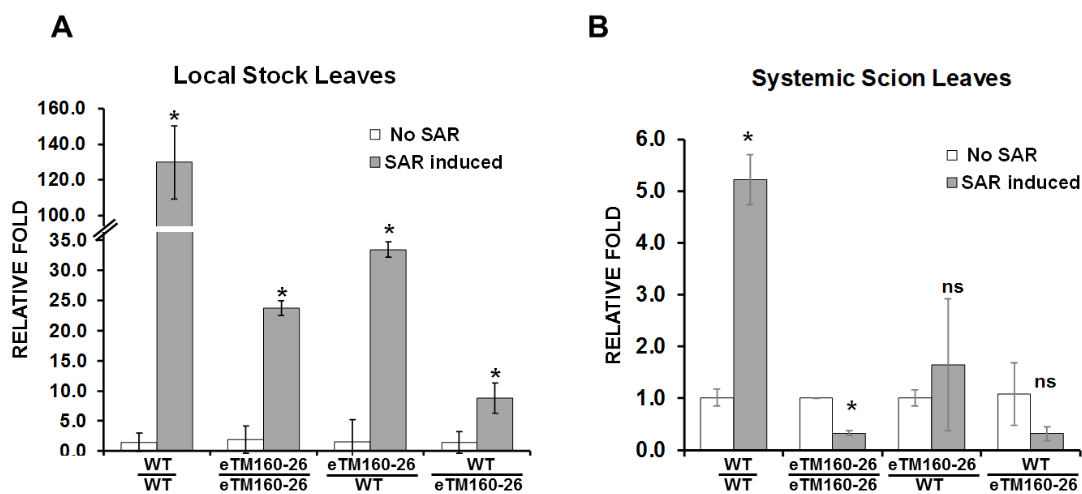


Figure 3.12 Analysis of *StPR1* expression in homo- and hetero-grafts of WT and eTM160-26 KD line of potato. A-B. qRT-PCR analysis of *StPR1* expression levels in the inoculated local stock leaves (A) and non-inoculated systemic scion leaves (B) of all the grafted plants after 4 days of primary infection with *P. infestans* (before giving the secondary infection with *Ralstonia*). Data represents mean ± SD of two 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 'No SAR' plants for each graft type, $P < 0.05$ (*). 'ns' indicates not significant.

3.4. Discussion

In this chapter, we have described the overall role of miR160 in potato development followed by its role in local and systemic defence responses against *P.infestans* infection.

3.4.1. Role of miR160 in potato development

Though our primary objective was to study the role of miR160 in potato- *P. infestans* interaction, we also analysed few morphological traits and tuberization phenotype of our miR160 overexpression (OE) and knockdown (KD) transgenic potato lines. Previously, miR160 is shown to regulate auxin response factors (ARF10/16/17) and its involvement in mediating auxin signalling pathway (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). Auxin is one of the major hormones involved in plant growth and development. Studies have shown that over-expressing or under-expressing miR160 and its targets lead to multiple developmental defects in plants. For example, in *Arabidopsis*, ARF10 and ARF16 have been identified as controller of root cap cell formation (Wang et al., 2005). Mallory et al (2005) have shown that overexpression of miR160 resistant ARF17 has dramatic developmental effects such as leaf shape defects, premature inflorescence, altered phyllotaxy etc. (Mallory et al., 2005). Another report by Liu et al. (2007) showed that repression of ARF10 by miR160 affects germination and post germination of seeds in Arabidopsis (Liu et al., 2007). Role of miR160 in symbiotic nodule development in soybean has also been demonstrated by Turner and coworkers (2013) (Turner et al., 2013).

Whether, miR160 has any role in potato development was not studied before. In our analyses, we observed no developmental defects in miR160 OE and KD lines of potato and tobacco as they grew similar to WT plants. Only miR160 potato OE line had a slight downward curling leaf phenotype (Figure 3.6) suggesting a potential role of miR160 in potato leaf development. The most striking phenotype was the increased tuberization of miR160 KD lines and complementary reduction of tuber yield in miR160 OE lines (Figure 3.7). This further suggests a negative regulation of tuberization by miR160 in potato. Until now, only *miR172* and *miR156* were studied

in detail for their role in potato tuberization (Martin et al., 2009; Bhogale et al., 2014). From our findings, it is now imperative that miR160 could also have a significant role in tuberization, however, elaborate studies are needed in order to conclusively understand the role of miR160 in potato tuberization.

3.4.2. miR160 plays significant role in local defence during potato – *P. infestans* interaction

As miR160 expression was induced in the *P. infestans* infected potato leaves (discussed in chapter 2), we were interested to further investigate its role in local defence responses of potato. For this, miR160 OE and KD lines were infected with *P. infestans* and disease progression was monitored. Though miR160 OE and KD lines showed a complementary tuberization phenotype, to our surprise, the disease symptom development was similar in both these transgenic lines. Upon infection, both miR160 OE and KD lines of potato showed enhanced susceptibility compared to wild-type (WT) and vector control (VC) plants (Figure 3.8 & 3.9). Similarly, tobacco miR160 KD lines also showed increased susceptibility in our study. This enhanced susceptibility observed in our miR160 OE lines was in contrast to the previous report in rice (Li et al., 2014). Li et al. (2014) showed that overexpression of miR160 in rice results in enhanced resistance to rice blast fungus *Magnaporthe oryzae*. Similarly, another study from *Arabidopsis* suggested a possible role of miR160 as a positive regulator of PTI responses (Li et al., 2010). These authors showed that flg22 treatment of plants overexpressing miR160 leads to increased callose deposition. Nevertheless, when infected with bacterial strain Pst DC3000, these miR160 overexpressing plants did not show any difference in resistance compared to WT plants (Li et al., 2010). Comparison of our results with these two previous reports suggests that miR160 might play different role based on the kind of plant-pathogen interaction. Apart from this, the inability of our miR160 OE and KD lines to induce *StPR1* expression (similar to WT plants), suggest that miR160 possibly regulates the defence pathway upstream of *StPR1*. Overall, our results indicate that optimal levels of miR160 (neither too high nor too low) might be required in potato for mounting a proper basal defence response against *P. infestans.*

3.4.3. Role of miR160 in SAR of potato

In our earlier experiments (as discussed in chapter 2), we observed an induced expression of miR160 in the systemic leaves and also an increased accumulation in phloem enriched exudates (PEX) upon *P. infestans* infection. This prompted us to investigate the role of miR160 in systemic defence responses of potato. For this, we performed SAR assays and grafting experiments on our miR160 OE and KD transgenic lines. So far, all the previous studies of SAR analysis in potato plants used arachidonic acid (a PAMP of *P. infestans*) as the SAR inducing agent. These studies analysed SAR response in systemic leaves based on the secondary infection of *P. infestans* (Coquoz et al., 1995; Yu et al., 1997; Manosalva et al., 2010). However, our study is the first report that successfully demonstrates SAR assay in potato using the biological organism, *P. infestans* as a primary pathogen and the bacterium *Ralstonia solanacearum* as a secondary pathogen. Results from our assays showed that miR160 KD lines were compromised in SAR responses, whereas miR160 OE lines exhibited SAR response equivalent to WT and VC plants (Figure 3.10). Similar to miR160 Potato KD line, the tobacco miR160 KD lines also exhibited a compromised SAR response. Most of the previously characterized SAR-deficient mutants, such as *npr1* (Cao et al., 1997), *pad4* (Jirage et al., 1999) and *sid2* (Wildermuth et al., 2001), are also affected in basal (local) defence responses. This is similar to our miR160 KD lines that exhibited both compromised local defence as well as defective SAR response. However, miR160 OE lines of potato, though showed defective local defence, was successful in mounting a proper SAR response. Similar to our miR160 potato OE lines, the *Arabidopsis* mutant, *eds5*, is the only plant that has so far been shown to be affected in local defence but capable of mounting a partial SAR response (Rogers and Ausubel, 1997). All these results indicate that a threshold level of miR160 could be required for mounting a successful SAR response against *P. infestans*.

As miR160 KD lines were unable to elicit SAR, we argued that this compromised SAR response could be because of one or both of the following reasons, (i) the local leaves of KD lines have failed to generate and/or transport the SAR signal to systemic leaves; (ii) the systemic leaves have failed to perceive and/or process the

SAR signal transported by local leaves. We addressed these possibilities through SAR assays on homo- and hetero-grafts of wild type (WT) and miR160 KD (eTM160-26) lines (Figure 3.11). We observed that, WT scions grafted on KD lines (hetero-grafts, WT/eTM160-26) were unable to mount SAR confirming the inability of KD stocks to generate or transport the signals to systemic scion. Similarly, KD scions grafted on WT stocks (hetero-grafts, eTM160-26/WT) also could not mount SAR response which confirms that KD scions failed to perceive and/or process the SAR signals transported by WT stock. Additionally, effective SAR response was not observed in homo-grafts of KD lines (eTM160-26/eTM160-26), which is consistent with our previous observation (Figure 3.10). However, all the homo-grafts of WT plants (WT/WT) exhibited a clear SAR response as well as an induced *StPR1* expression in both stock and scion leaves (Figure 3.12). This negates the possibility of grafting as a cause for impaired SAR response in hetero-grafts. Overall, our analysis suggests that function of miR160 is crucial at both local and systemic leaves for establishment of SAR in potato against *P. infestans* infection.

In conclusion, our study demonstrates the importance of miR160 in local and systemic defence responses of potato against *P. infestans* infection. Both OE and KD lines of miR160 showed enhanced susceptibility suggesting that an optimal level of miR160 could be necessary for mounting a proper local (basal) defence response in potato against *P. infestans* infection. Also, our SAR assays and grafting studies suggest that threshold levels of miR160 are perhaps required at both local and systemic leaves for exhibiting an effective SAR response. This could be because of the fact that miR160 lines failed to generate or transport proper SAR signals to systemic leaves and systemic leaves have failed to perceive or process the SAR signal. Overall, it also appears that miR160 could play differential roles in local defence and SAR response. For better understanding of this enhanced susceptibility and compromised SAR response exhibited by miR160 transgenic lines, our next aim was to analyse different auxin pathway genes, SAR associated signals and defence-related genes in this background.

Chapter 4

Exploring the mechanistic link of miR160 in defence response of potato

4.1. Introduction

Based on the response of miR160 OE and KD lines in both basal resistance and SAR, we were interested to understand if there is any mechanistic link of miR160 with known defence related genes and SAR associated signals in potato. Because miR160 has been previously shown to have role in auxin signalling and auxin being a critical player in defence response, we explored auxin pathway genes and SAR signals in this study.

4.1.1. Role of auxin in defence signalling

Auxin has long been recognised for its role in plant development and in past two decades, its function in plant defence also began to emerge (Kazan and Manners, 2009). Recent reviews have discussed the antagonistic crosstalk of auxin and the defence hormone salicylic acid (SA) as one of the mechanisms adopted by plants to mediate growth-defence trade-offs (Kazan and Manners, 2009; Denancé et al., 2013; Huot et al., 2014; Verma et al., 2016). Studies by Wang et al. (2007) showed that treatment of *Arabidopsis* seedlings with SA causes repression of the *TRANSPORT INHIBITOR RESISTANT 1 (TIR1)/ AUXIN SIGNALING F-BOX (AFB)* genes and stabilization of auxin repressor protein AUX/IAA leading to repression of auxin responses (Wang et al., 2007). These authors have also observed that SAR induction suppresses majority of the auxin responsive genes in *Arabidopsis* (Wang et al., 2007). miR393 is also shown to attenuate auxin signalling by directly targeting TIR1 upon flg22 (PAMP) treatment (Navarro et al., 2006). Similarly, IAA has been shown to suppress SA-dependent expression of PR1 (Wang et al., 2007; Park et al., 2007) and the plants expressing the salicylate hydroxylase (SA-deficient plants) exhibit increased levels of endogenous IAA (Abreu and Munné-Bosch, 2009). These reports suggest that such antagonistic crosstalk might be important for plants to modulate defence and development. A detailed description of SA and auxin cross-talk is provided in chapter 1.

One of the ways in which plants maintain auxin homeostasis is by conjugating IAA with amino acids using the enzymes encoded by *GH3* gene family (Woodward and Bartel, 2005). The *Arabidopsis* GH3.5 (*wes1* gene), however, shows in vitro adenylation activity on IAA as well as SA, and is implicated in local and systemic

defence responses during avirulent pathogen infection (Park et al., 2007; Zhang et al., 2007, 2008). Another study showed that the levels of *GH3.5* are significantly reduced in plants overexpressing miR160-resistant ARF17, suggesting that miR160-directed regulation of ARF17 is important for proper expression of *GH3.5* in *Arabidopsis* (Mallory et al., 2005). As miR160 also targets ARF10 and ARF16 along with ARF17, it would be interesting to understand if any of the miR160 target genes directly regulate the expression of *GH3.5*.

4.1.2. SA signalling and Systemic Acquired Resistance (SAR) in potato

SA has been shown to play major role in various plant-pathogen interaction, however, SA signalling and SAR responses have not been well studied in potato. Unlike *Arabidopsis* and tobacco, potato has high endogenous levels of SA and is insensitive to external SA application. Various report suggested that SA signalling mechanism is different in potato (Coquoz et al., 1995; Yu et al., 1997; Navarre and Mayo, 2004). Yu et al (1997) showed that high endogenous levels of SA in potato do not lead to constitutively active defence as SA-deficient (*nah*G) plants do not show any increase in *P. infestans* growth (Yu et al., 1997). However, these authors also showed that SA is important for arachidonic acid (AA)-induced SAR since *nah*G plants failed to induce a SAR response (Yu et al., 1997). Though AA induces SAR in potato, the increase in SA levels were observed only in the local treated leaves and not in the systemic untreated leaves, unlike *Arabidopsis* and tobacco as shown by previous reports (Coquoz et al., 1995; Yu et al., 1997).

The function of SA in potato defence was debated in the earlier days, nevertheless, recent reports suggest its indispensable role in defence against AA, *P. infestans*, Potato virus X and Potato virus Y (Halim et al., 2007, 2009; Sánchez et al., 2010; Baebler et al., 2014). Additionally, studies by Manosalva et al. (2010) showed that AA-induced SAR is indeed accompanied by increased SA levels in systemic leaves as opposed to the observations of Yu et al (1997) (Manosalva et al., 2010; Yu et al., 1997). These authors also demonstrated that similar to tobacco and *Arabidopsis*, methyl salicylate (MeSA) is a mobile signal in potato and methyl esterase, *StMES1* (the ortholog of tobacco SABP2), is involved in the conversion of SA to MeSA in the local leaves treated with AA (Manosalva et al., 2010). In

conclusion, the similarities observed between SA and SAR signalling in potato, tobacco, and *Arabidopsis* suggest that regardless of their endogenous SA levels, certain SAR signalling components seems to be conserved among these plants.

Since, our miR160 KD and OE lines exhibited highly reduced basal resistance and miR160 KD lines also showed compromised SAR response, in this study, we investigated:

i. the expression of various auxin pathway genes (*StYUCCA1*, *StLAX4*, *StTIR1*, *StIAA16* and *StGH3*.6) in miR160 KD and OE lines to understand their role in basal resistance of potato.
ii. the levels of SAR associated signals (SA and MeSA), as well as expression of SAR related genes (*StPR1*, *StNPR1*, *StBSMT1*, *StMES1*, *StGH3.6*) to understand the compromised SAR response exhibited by miR160 KD lines.
iii. if *StARF10* (target gene of miR160) binds to the promoter of *StGH3.6* to regulate its gene expression.

4.2. Materials and Methods

4.2.1. Plant and pathogen materials

The wild-type and transgenic plants of potato (*S. tuberosum* cv. Désirée) were maintained as described previously in chapter 3. The pathogen, *Phytophthora infestans,* was maintained and infection experiments were carried out as described previously in chapter 2.

4.2.2. Arachidonic acid (AA) treatment

To find out if SAR associated signals and genes are affected, four-weeks old WT potato plants and eTM160-26 KD lines were treated with 30 µl of 0.05 mM arachidonic acid (AA) on two-three local leaves to trigger SAR response. In order to avoid any cross volatile signalling, WT plants and KD lines were incubated in separate growth chambers at 18°C under 90% relative humidity. Local (AA-treated) and systemic (AA-untreated) leaves were collected at 0, 24, 48, 72 and 96 hpt (hours post-treatment) and frozen with liquid nitrogen for storage until further use.

4.2.3. Expression analysis of auxin pathway and defence related genes

For analysis of auxin pathway genes, *StYUCCA1*, *StLAX4*, *StTIR1*, *StIAA16* and *StGH3*.6 were selected, whereas *StPR1, StNPR1, StMES1 and StBSMT1* were chosen for analysis of genes associated with defence. For all the expression analysis, cDNA was prepared with 1 µg of total RNA using Oligo(dT) reverse primer and Superscript III Reverse Transcriptase (Invitrogen) and qRT-PCR were carried out using KAPA SYBR Green Mix (Kapa Biosystems). The reaction conditions for *GAPDH*, *StPR1*, *StYUCCA1*, *StLAX4*, *StTIR1*, *StIAA16* and *StGH3*.6 were 95°C 2 min, 40 cycles of 95°C 15 sec and 60°C 20 sec and for *StNPR1, StMES1 and StBSMT1* were 95°C 2 min, 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 by using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) for auxin pathway genes and by $2^{-\Delta Ct}$ method for defence related genes. GAPDH was used as normalization gene in all the reactions.

4.2.4. Quantification of salicylic acid (SA) levels

For quantification of salicylic acid (SA) levels, modified protocol of Forcat et al. (2008) was followed (Forcat et al., 2008). Fifty milligram (50 mg) of ground leaf tissue was used for extraction in 400 µl of 10% methanol containing 1% glacial acetic acid. This mixture was vigorously vortexed and incubated on ice for 30 mins, followed by centrifugation to obtain the supernatant. This was repeated once and supernatant volume was adjusted to 1 ml using a volumetric flask. Samples were resolved through a Thermo Scientific Hypersil Gold column of particle size 1.9 µm and dimension 60 x 2.1 mm with a flow rate of 0.2 ml/min and a gradient solvent program of 10 min (0.0 min, 10 % methanol/water; 0.5 min, 10.0 % methanol/water; 3.0 min, 50 % methanol/water; 10 min, 50 % methanol/water). Formic acid (0.1 % LC-MS grade) was also added to water. MS and MS/MS experiments were performed in ESI-negative ion mode using the tune method as follows: sheath gas flow rate 45, auxiliary gas flow rate 10, sweep gas flow rate 2, spray voltage (|KV|) 3.60, spray current (µA) 3.70, capillary temperature (ºC) 320, s-lens RF level 50, heater temperature (ºC) 350. ESI-MS data were recorded in full scan mode within the mass range *m/z* 100 to 1000.

4.2.5. Quantification of methyl salicylate (MeSA) levels

Quantification of methyl salicylate (MeSA) was carried out with minor modification of earlier protocol (Schmelz et al., 2004). Approximately 100 mg of leaf tissue was ground in liquid nitrogen and 800 ul of extraction buffer (1-propanol: water: hydrochloric acid in 2:1:0.005 ratio) was added. Ten ng of 3'-methylacetophenone [m/z=134, 10 µl from 1000 ng/ml solution in dichloromethane (DCM)] was added as internal standard to the mixture. Post homogenization, 1 ml of DCM was added and samples were re-homogenized. DCM layer was separated by centrifugation and collected in 2 ml glass vial, which was further concentrated to approximately 100 ul using inert nitrogen gas. One µl of sample was injected in single quadrapole GC-MS system (Agilent 7890A GC and Agilent 5975-Inert XL EL/CL MSD MS) manually in inlet injector port held at a temperature of 250°C. Compounds were separated on SUPELCOWAX® 10 Capillary GC column (30 m x 0.20 m x 0.20 µm) (Sigma-Aldrich) with initial column temperature set as 60 °C followed by raising

of temperature till 220 °C using the following program: initial temperature 60 °C - 3 min, 60 °C to 100 °C at 2 °C/min, 100 °C to 150 °C at 5 °C/min and from 150 °C to 220 °C at 15 °C/min and held there for 5 min. Helium was used as carrier gas with 1 ml/min flow rate. Finally, area of internal standard methylacetophenone and MeSA were calculated by extracting peak for m/z=134 and m/z=152, respectively. Amount of MeSA per gram ground tissue was calculated using per unit area obtained of internal standard.

4.2.6. Yeast One-Hybrid (Y1-H) Assay

The coding sequence of *St*ARF10 and promoter sequences of *StGH3.6* (~2.4 kb upstream) and *AtGH3.5* (~3.0 kb upstream) were cloned into pGEM-T Easy vector (Promega). All the constructs for Y-1H were generated by Gateway cloning technology (Thermo Fisher Scientific) (Deplancke et al., 2004). For preparation of bait expression vectors, promoters were transferred to the destination vector pMW#2 (Addgene) through donor vector pDONRP4-P1r. Further, the yeast strain Y1H-aS2 (*his3 Δ1*) was transformed with bait expression vectors and selected in SD -His media. The prey expression vector was prepared by transferring coding sequence of *St*ARF10 to the destination vector pDEST-2μ-Gal4-AD via the donor vector pDONR221. The yeast strain Yα1867 was transformed with prey expression vector and selected in SD -Trp media. To study the interaction between the promoters and *St*ARF10, the prey yeast (Yα1867-*St*ARF10) and either of the bait yeast [Y1H-aS2 (*his3 Δ1*)-prom-*StGH3.6* or Y1H-aS2 (*his3 Δ1*)-prom-*AtGH3.5*] were mated by mixing the yeast culture in 1:1 ratio and growning in YPDA media. The mated yeast clones were then selected on SD -His -Trp media. The interaction was confirmed by growing the mated yeast clones on SD -His -Trp media supplemented with increasing concentrations of 3-AT (0, 2.5, 5, 10, 15, 20, 40 and 80 mM). Vectors pDONRP4-P1r, pDONR221 and pDEST-2μ-Gal4-AD and the yeast mating strains Y1H-aS2 (*his3-Δ1*) and Yα1867 were kind gifts from Prof. Walhout, University of Massachusetts Medical School, USA (Reece-Hoyes et al., 2011; Gaudinier et al., 2011).

4.2.7. Electrophoretic Mobility Shift Assay (EMSA)

For *St*ARF10-6xHis protein preparation, coding sequence of *St*ARF10 was PCR amplified and cloned into pET28a$^+$ vector (Novagen). Protein expression was performed using *E. coli* BL21(DE3) cells as host followed by Ni-NTA affinity column based protein purification. For bait DNA preparation, promoter fragments P1 (-1191 to -1607), P2(-620 to -1204), P3(-1 to -639) of *StGH3.6* were PCR amplified from potato genomic DNA and promoter fragment P4 (-624 to -1278) of *AtGH3.5* was PCR amplified from Arabidopsis genomic DNA. All the fragments were cloned in pGEM-T Easy sub-cloning vector (Promega) and sequence verified. For EMSA, probes were prepared by labelling promoter fragments with γ-P^{32}-ATP using KinaseMax End-Labelling kit (Ambion). The binding reactions were carried out as mentioned previously with minor modifications (Chen et al., 2004). Briefly, the reaction consisted of 10 mM Tris-HCl pH 7.5, 5% glycerol, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, 25 ng Poly(dI-dC).Poly(dI-dC), 0.5 µg *St*ARF10 and 1 fmol labelled promoter fragment. In case of cold competition analysis, increasing molar concentrations (10, 100, 500 mM) of unlabelled *St*GH3.6-P2 or *At*GH3.5-P4 were added to the reaction along with above mentioned components. All reactions were incubated at room temperature for 20 min followed by fractionating on 6% native polyacrylamide gel for 2-3 hrs. After fractionation, the gel was fixed in 20% methanol:10% acetic acid solution for 10 min followed by vacuum drying for 30 min (BioRad Vacuum Gel Dryer). Gel was then exposed to storage phosphor screen overnight and imaged using Typhoon imager (GE Healthcare Life Sciences).

4.2.8. Accession numbers

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

Table 4.1 Accession numbers

Name	Accession	Source*
StARF10	PGSC0003DMT400020874	PGSC
StPR1	AY050221	NCBI
StNPR1	XM_006357647	NCBI
StMES1	PGSC0003DMT400019806	PGSC

StBSMT1	XM_006354611	NCBI
StYUCCA1	PGSC0003DMT400067103	PGSC
StLAX4	PGSC0003DMT400049377	PGSC
StTIR1	PGSC0003DMT400029517	PGSC
StIAA16	PGSC0003DMT400050101	PGSC
StGH3.6	PGSC0003DMT400049613	PGSC
AtGH3.5	AT4G27260	TAIR
GAPDH	PGSC0003DMT400044944	PGSC
*PGSC – Potato Genome Sequencing Consortium: http://solanaceae.plantbiology.msu.edu/integrated_searches.shtml * NCBI – National Centre for Biotechnology Information: https://www.ncbi.nlm.nih.gov/ * TAIR – The Arabidopsis Information Resource: https://www.arabidopsis.org/		

4.2.9. Primer Sequences

Primers used in this chapter are listed in Table 4.2

Table 4.2 List of primers

Primer Name	**Sequence 5' - 3'**
Auxin pathway genes	
LAX4_FP	TTAACTCTGCTGTTGGGGCTCT
LAX4_RP	CTTGGCATGAAGGATGGTGG
TIR1_FP	AACCCTGAGCTTGGCAAGC
TIR1_RP	GGCCTTGCTCCGTCAAGGTT
YUCCA1_FP	AAATTAGGTCTCCGGCGA
YUCCA1_RP	TTTCCTTCACACCTGGCAT
IAA16_FP	GAAGACAAGGATGGTGATTGGA
IAA16_RP	TCCACTGCTCTTGGTGCTA
GH3.6_FP	AAGTCCATCGGGCCATTGGA
GH3.6_RP	CCATTTTGGGCATTTGGGGCT
Defence pathway genes	
PR1_FP	GTACCAACCAATGTGCAAGCG

PR1_RP	TGTCCGACCCAGTTTCCAAC
MES1_FP	CATCATTGGTGAGACCAAGCTC
MES1_RP	TGGTATGCCTTTGTCCTCAGT
BSMT_FP	GAGTGCCTGGTTCATTTTATAC
BSMT_RP	GGACTTGTACTTGCCATGTAA
NPR1_FP	AAGAGGCTCACTAGGCTT
NPR1_RP	GCTTCATACGCAAATCATCG
Y1-H analysis	
ARF10_Y1H_FP	AAAAAGCAGGCTTCATGAAGGAGGTTTTGGAGAAGT
ARF10_Y1H_RP	CAAGAAAGCTGGGTTCTATGCAAAGATGCTAAGAGG
ATTB1_Y1H_FP	GGGGACAAGTTTGTACAAAAAAGCAGGCT
ATTB2_Y1H_RP	GGGGACCACTTTGTACAAGAAAGCTGGGT
Prom-StGH3.6_Y1H_F	TATAGAAAAGTTGTCAATGGTAGTACCCCACG
Prom-StGH3.6_Y1H_R	TTTGTACAAACTTGCGGTTTCTTTTAATTAACAAAAGTGAACA
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
EMSA	
*St*ARF10_F_FP	GGATCCATGAAGGAGGTTTTGGAGAAGTGT
*St*ARF10_F_RP	AAGCTTTGCAAAGATGCTAAGAGGTCCA
*St*GH3.6-P1_FP	GGATCCGCAGGTAACGTGTCTATTT
*St*GH3.6-P1_RP	GAATTCGCGACTTAGAGTACGTATT
*St*GH3.6-P2_FP	GGATCCAATACGTACTCTAAGTCGC
*St*GH3.6-P2_RP	GAATTCTAGTTGGTGAGTTAGATCG
*St*GH3.6-P3_FP	GGATCCCGATCTAACTCACCAACTA
*St*GH3.6-P3_RP	GAATTCTGTGAAGAAAAAGAGAGAGTTTG
*At*GH3.5-P4_FP	GGATCCCTATCAAGTTTGGAGTCCA
*At*GH3.5-P4_RP	CCCGGGATTGCAGTGTAGTTGGTAC
Reference Genes	
GAPDH_FP	GAAGGACTGGAGAGGTGGA
GAPDH_RP	GACAACAGAAACATCAGCAGT

4.3. Results

4.3.1. Auxin pathway genes are affected in miR160 OE and KD lines

Auxin plays critical role in plant defense response and miR160 is known to be associated with auxin response pathways to regulate growth and development (Wang et al., 2007; Kazan and Manners, 2009; Mallory et al., 2005; Liu et al., 2007; Turner et al., 2013). To elucidate if auxin pathway is affected in miR160 OE and KD lines, basal expression levels of genes (*StYUCCA1*, *StLAX4*, *StTIR1*, *StIAA16* and *StGH3*.6) involved in this pathway were analyzed (Figure 4.1). Expression of *StYUCCA1,* the auxin biosynthesis gene, was significantly reduced in miR160 KD line eTM160-26 and not in the OE line pre160-L17C1. The levels of IAA influx carrier, *StLAX4* (Like auxin resistant 4), were not found to be altered in either of the transgenic lines. However, expression of the auxin receptor *StTIR1* (Transport inhibitor response 1), was significantly reduced in both KD and OE lines of miR160. The repressor *StIAA16* (Indole acetic acid induced protein), had increased expression only in pre160-L17C1 (OE) lines. Unlike any other genes tested, the amino acid conjugator *StGH3.6*, showed opposite expression pattern in OE and KD lines. *StGH3.6* had reduced levels in eTM160-26 (KD) plants and an increased expression in pre160-L17C1 (OE) plants. These results suggest that both overexpression and knockdown of miR160 affects the basal expression of multiple genes involved in auxin pathway (Figure 4.1).

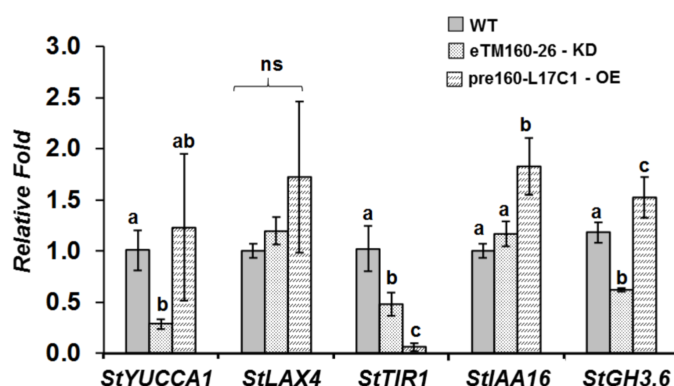


Figure 4.1 Effect of miR160 overexpression and knockdown on expression of auxin pathway genes. For each gene, relative fold of expression in eTM160-26 (KD) and pre160-L17C1 (OE) lines were normalized to expression in WT plants. Values represent mean ± SD of three biological replicate with three technical replicates each. Different alphabets indicate statistically significant difference as analysed by Student's T-test ($p<0.05$).

The responses of all these auxin pathway genes were also evaluated against *P. infestans* infection at 2 dpi (Figure 4.2 A to C). WT plants showed significant reduction in the levels of *StYUCCA1* and *StTIR1*, whereas, levels of *StIAA16* and *StGH3*.6 were significantly elevated upon infection. The expression of *StLAX4*, however, remained unchanged in WT plants (Figure 4.2 A). Though, eTM160-26 (KD) line showed similar expression pattern like that of WT plants, the magnitude of fold change was highly reduced (Figure 4.2 B). However, expression levels of *StYUCCA1* and *StLAX4* were not affected in this line upon infection. In contrast, no significant changes in gene expression were observed for any of these genes in the OE line (pre160-L17C1) upon infection (Figure 4.2 C).

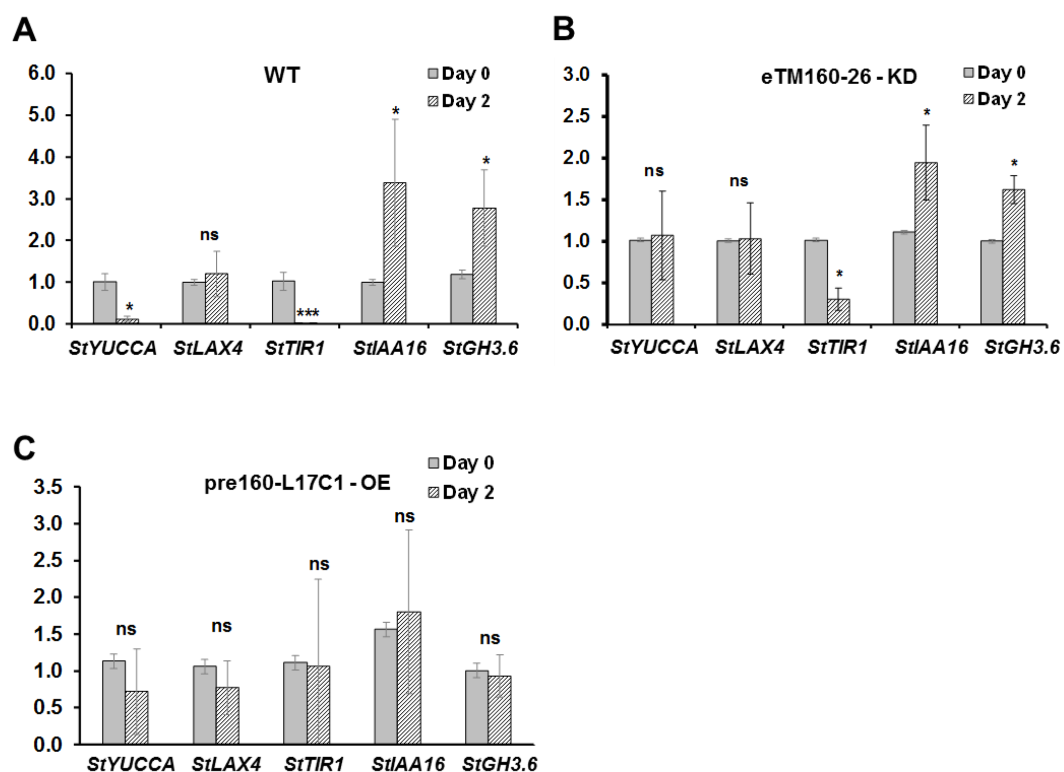


Figure 4.2 Effect of *P. infestans* infection on expression of auxin pathway genes in WT, miR160 OE and KD plants. Expression of different auxin pathway genes in WT (**A**), eTM160-26 KD (**B**) and pre160-L17C1 OE (**C**) plants analysed on 0 and 2 dpi (days post inoculation). Values represent mean ± SD of three biological replicate with three technical replicate each. Different alphabets indicate statistically significant difference as analysed by Student's T-test ($p<0.05$).

4.3.2. Salicylic acid (SA) and methyl salicylate (MeSA) levels are altered in miR160 KD lines

To understand the defective SAR response observed in miR160 KD lines as described previously in Chapter 3 (Figure 3.10), we have estimated the levels of SAR associated signals such as SA and MeSA in eTM160-26 (KD) and WT plants post arachidonic acid (AA) treatment. In potato, AA, a *P. infestans* PAMP, is well known to induce SAR response (Coquoz et al., 1995; Yu et al., 1997; Manosalva et al., 2010) that is equivalent to SAR induced by *P. infestans* treatment. Both result in an increased SA and *PR1* accumulation in local and systemic leaves (Manosalva et al., 2010). In this regard, High Resolution Mass Spectroscopy (HRMS) and Gas Chromatography-Mass Spectroscopy (GC-MS) were used for the quantification of SA and MeSA respectively (Figure 4.3 to 4.7).

The amount of SA peaked in local and systemic leaves at 24 and 72 hpt (hours post treatment) respectively for both WT and eTM160-26 plants. However, SA accumulation was comparatively lower than the WT level in eTM160-26 plants at these time-points (Figure 4.8). MeSA, the only known mobile SAR signal in potato (Manosalva et al., 2010), showed no differences in local leaves of eTM160-26, but in systemic leaves, MeSA had reduced levels at 72 and 96 hpt compared to WT plants (Figure 4.9). These results indicate that SAR signals, SA and MeSA, are significantly affected in miR160 KD plants.

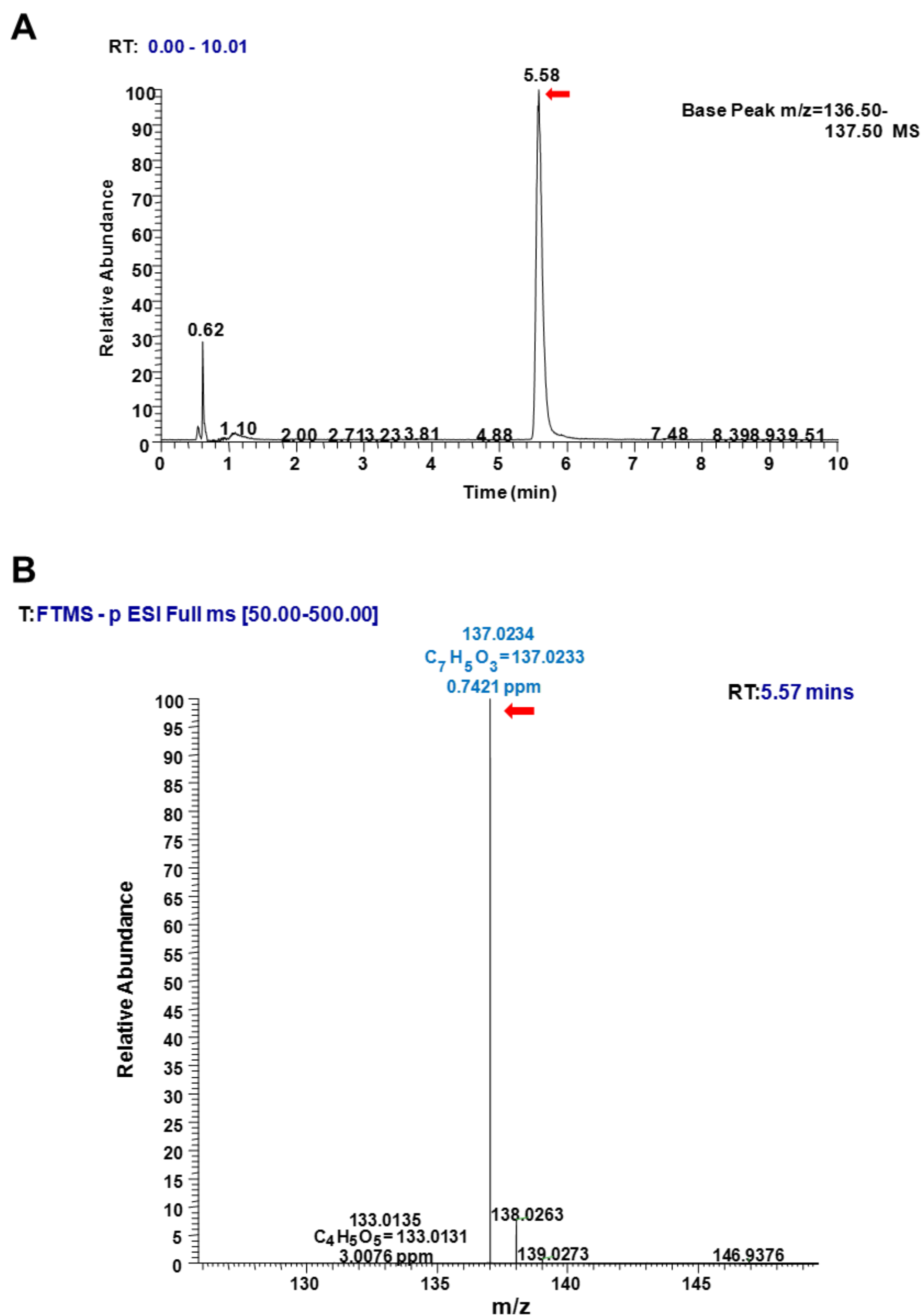


Figure 4.3 HR-MS based analysis of SA. A. HR-MS Chromatogram of SA (m/z: 137 g/mol) showing the retention time (RT) as ~5.58 min. **B.** HR-MS Mass Spectrum of SA ($C_7H_5O_3$).

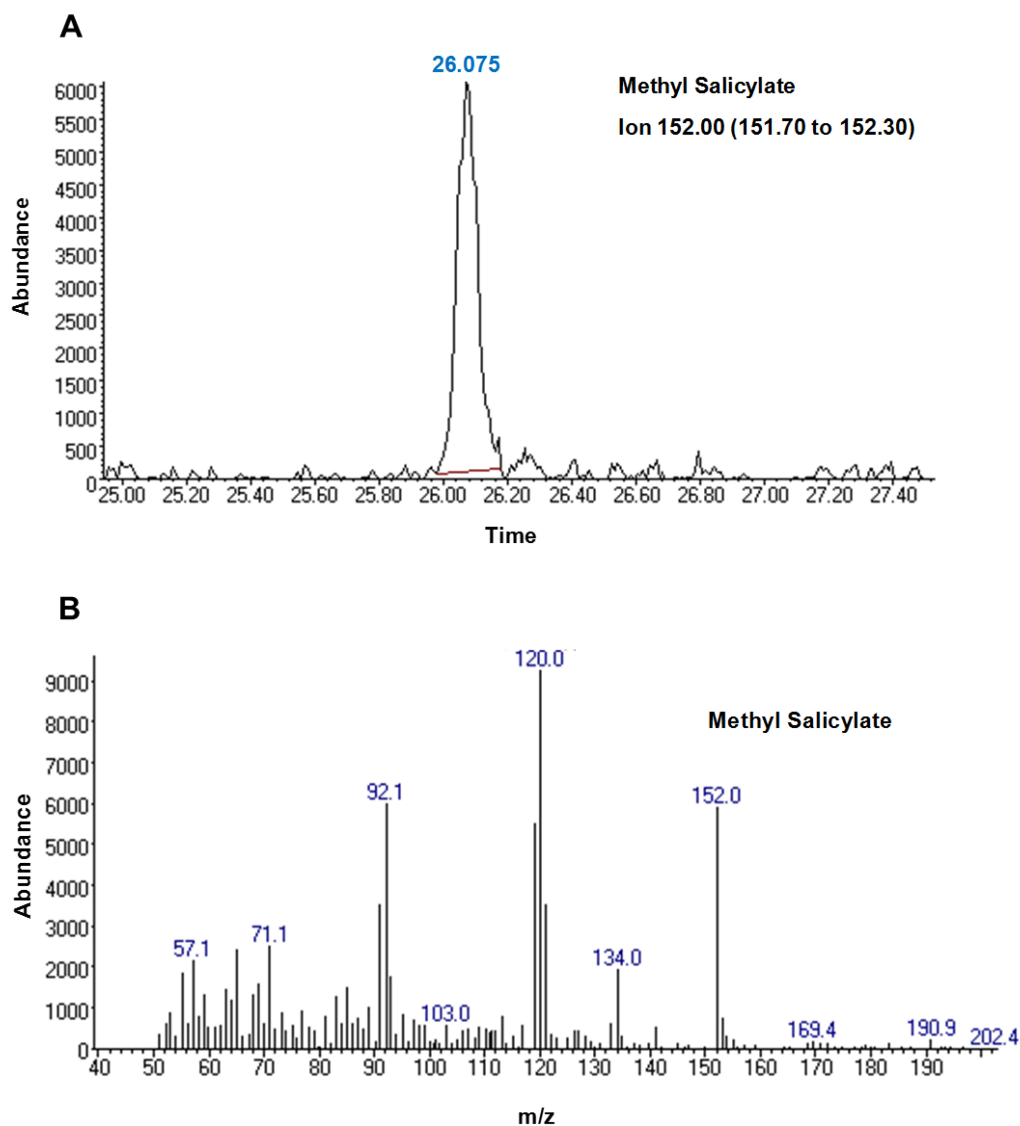


Figure 4.4 GC-MS analysis of MeSA. A. GC-MS chromatogram of MeSA (m/z: 152 g/mol) with a retention time (RT) of ~26.075 min. **B.** GC-MS mass spectrum of MeSA ($C_8H_8O_3$).

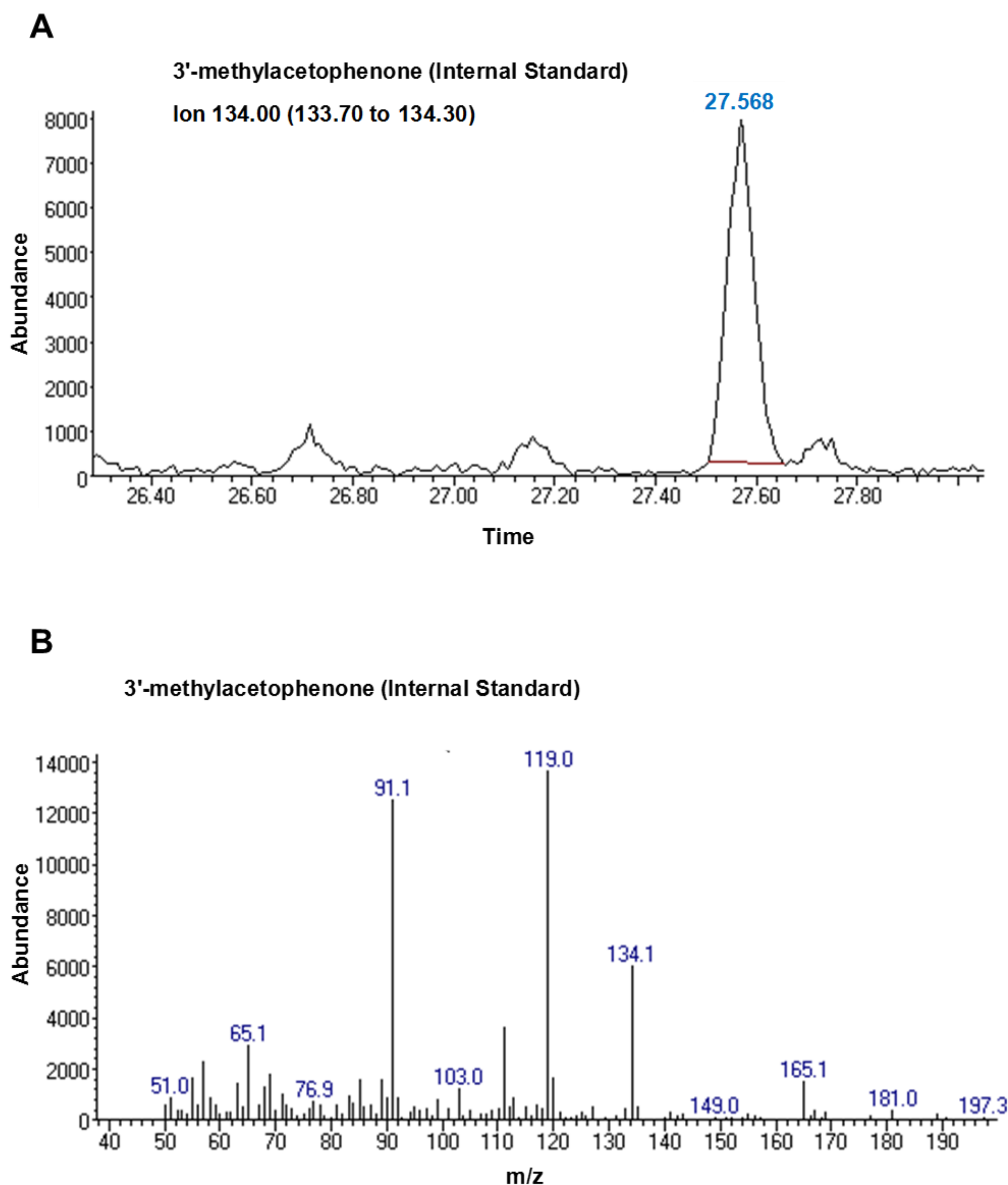


Figure 4.5 GC-MS analysis of 3'-methylacetophenone that was used as internal standard (IS) for the quantification of MeSA levels. A. GC-MS chromatogram of 3'-methylacetophenone (m/z: 134 g/mol) with a retention time (RT) of ~27.568 min that is at ~ 1 min later than the RT of MeSA (~26.075 min) and hence allowed a better separation of the IS from MeSA. **B.** GC-MS mass spectrum of 3'-methylacetophenone ($C_9H_{10}O$).

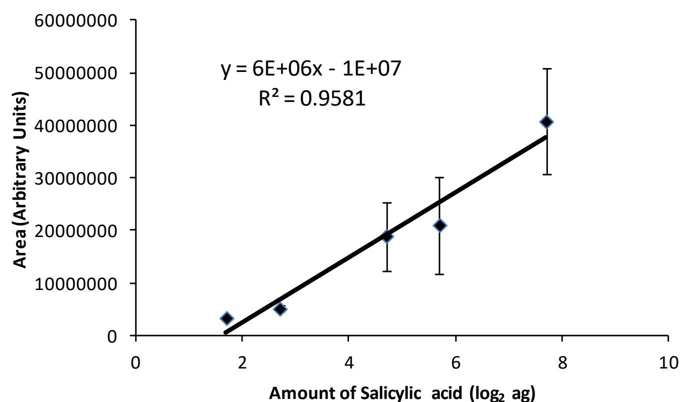


Figure 4.6 Standard Graph used for SA Quantification. Data points represents mean ± SD of three replicates.

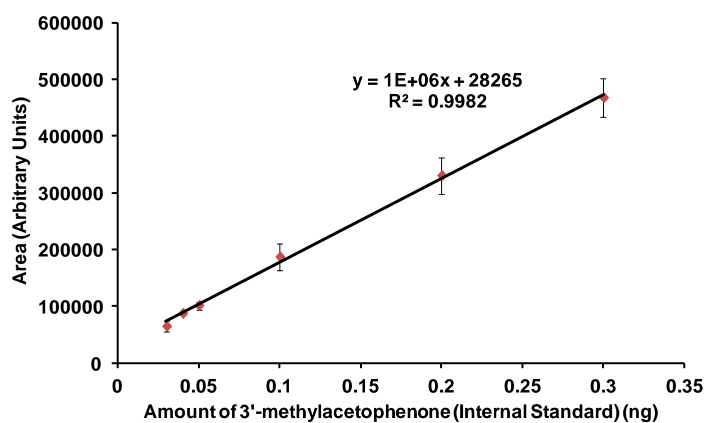


Figure 4.7 Standard Graph of the internal standard, 3'-methylacetophenone used for MeSA quantification. Data points represents mean ± SD of three replicates.

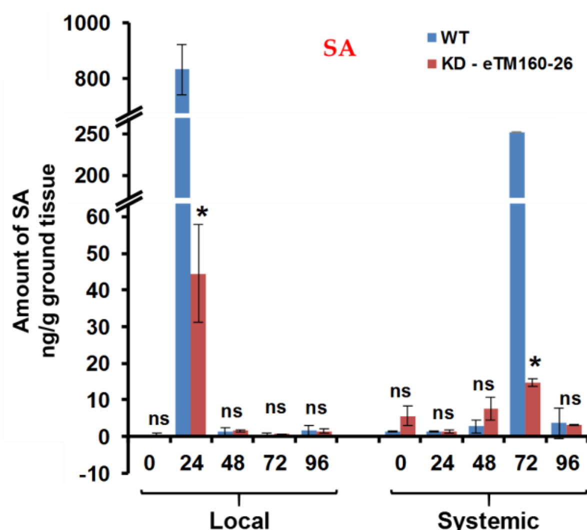


Figure 4.8 Analysis of SA levels in WT and eTM160-26 KD lines post arachidonic acid (AA) treatment. Sample were analysed at 0, 24, 48, 72 and 96 hours post treatment in both local leaves (AA-treated) and systemic (AA-untreated) leaves. Values represent mean ± SD of three biological replicate with three technical replicate each. Asterisks represent statistically significant difference as analysed by Student's T-test ($p<0.05$). 'ns' indicates not significant.

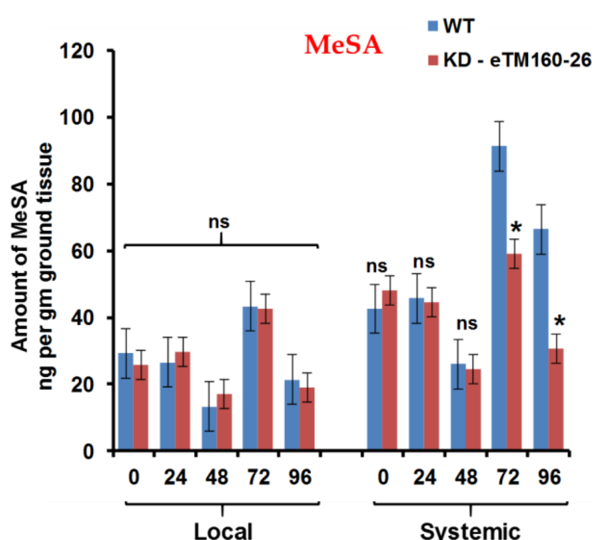


Figure 4.9 Analysis of MeSA levels in WT and eTM160-26 KD lines post arachidonic acid (AA) treatment. Sample were analysed at 0, 24, 48, 72 and 96 hpt (hours post treatment) in both local AA-treated leaves and systemic AA-untreated leaves. Values represent mean ± SD of three biological replicate with three technical replicate each. Asterisks represent statistically significant difference as analysed by Student's T-test ($p<0.05$). 'ns' indicates not significant.

4.3.3. Various defence related genes are affected in miR160 KD lines

Apart from the analysis of SA and MeSA levels, expression of SAR related genes *StPR1*, *StNPR1*, *StBSMT1, StMES1* and *StGH3*.6 were also analysed in miR160 KD lines post AA treatment. Similar to our previous observations (Chapter 3, Figure 3.9 D and Figure 3.12), expression of *StPR1* (a SAR marker gene) was significantly reduced in both local and systemic leaves of eTM160-26 (KD) plants post AA treatment (Figure 4.10 A & B). The other important SAR gene, *StNPR1*, also showed reduced levels in local and systemic leaves of eTM160-26 lines when compared with WT plants (Figure 4.10 C & D). Levels of *StBSMT1*, the gene involved in conversion of SA to MeSA (Chen et al., 2003; Song et al., 2009), was found to be reduced in the local leaves of eTM160-26 plants, however, no significant changes of mRNA levels were observed in the systemic leaves (Figure 4.10 E & F). On the other hand, expression of *StMES1*, the gene that converts MeSA to SA (Forouhar et al., 2004), was higher in eTM160-26 lines in both local and systemic leaves at 24 hpt (Figure 4.10 G & H).

Studies by Jagadeeswaran et al. (2007) and Zhang et al (2007,2008) showed that *AtGH3.5*, an amino acid conjugator of both auxin and SA, play an important role in local defence and SAR responses in *Arabidopsis* (Jagadeeswaran et al., 2007; Zhang et al., 2007, 2008). Expression of its potato homolog, *StGH3*.6, was found to be reduced in the local leaves of eTM160-26 at all the time-points tested (Figure 4.10 I). However, in systemic leaves of eTM160-26, *StGH3*.6 levels were low at 24 hpt and high at 72 & 96 hpt (Figure 4.10 J). Unlike all the other genes tested, the expression pattern of *StGH3*.6 in systemic leaves was opposite to that of WT plants, suggesting a possible dysregulation of this gene specifically in systemic leaves of eTM160-26 plants. Taken together, our analyses indicate that knockdown of miR160 not only affects SA and MeSA in potato, it also affects some of the major genes involved in SAR signalling pathway.

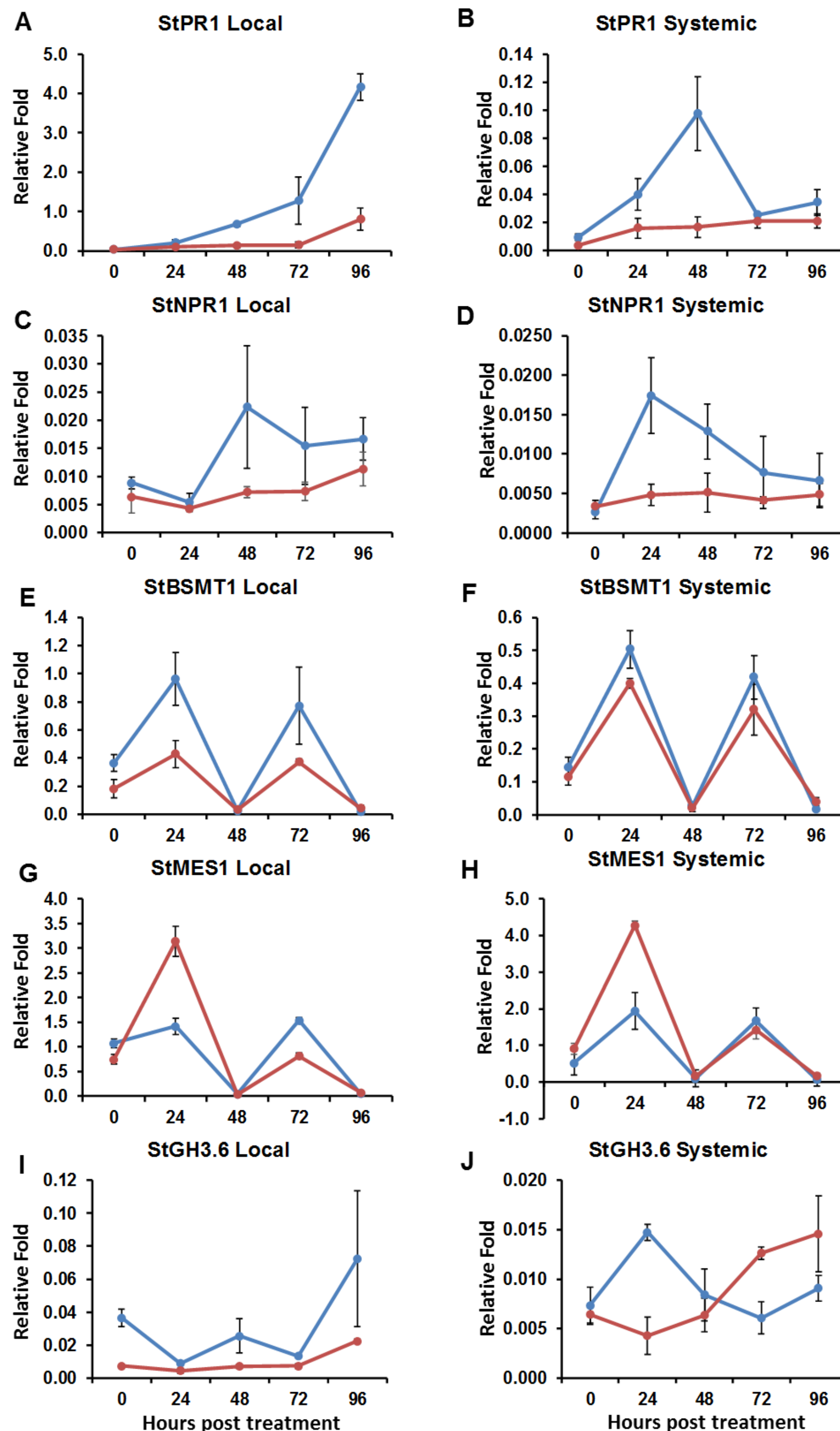


Figure 4.10 qRT-PCR based analysis of SAR related genes. The local and systemic leaves of WT and eTM160-26 KD lines were analysed for the expression levels of *StPR1* (**A** & **B**), *StNPR1* (**C** & **D**), *StBSMT1* (**E** & **F**), *StMES1* (**G** & **H**) and *StGH3.6* (**I** & **J**) post arachidonic acid treatment. Values represent mean ± SD of three biological replicate with three technical replicate each.

4.3.4. *St*ARF10 directly binds to the promoter of *StGH3.6*

It is well known that miRNAs regulate gene expression through their targets (Dugas and Bartel, 2004; Zhang et al., 2006; Borges and Martienssen, 2015). *StARF10*, the target of miR160, belongs to the family of auxin response factors that either activate or repress gene expression by directly binding to their target DNA (Guilfoyle and Hagen, 2007; Chandler, 2016; Li et al., 2016). Our study shows that expression of several genes are affected when miR160 is over-expressed or under-expressed (Figure 4.1 & 4.10). Presumably, miR160 mediates such regulation through *StARF10* or any of its targets. Out of all the genes that we analyzed, *StGH3*.6 emerged as a possible candidate, as it showed opposite expression patterns in miR160 KD and OE transgenic lines (Figure 4.1). This has led us to hypothesize that miR160 could be regulating *StGH3*.6 through its target *StARF10* in potato. Moreover, the differential expression pattern of *StGH3*.6 observed in local and systemic leaves of AA treated potato plants (Figure 4.10 I & J) further supports our hypothesis.

To test this, Yeast one-hybrid (Y1-H) analysis and Electrophoretic Mobility Shift Assays (EMSA) were performed using *St*ARF10 protein and promoters of potato *StGH3.6* and *Arabidopsis AtGH3*.5. For Y1-H assays, the interaction of *StARF10* protein were explored with ~2.4 kb and ~ 3.0 kb promoter of *StGH3.6* and *AtGH3*.5 respectively (Figure 4.11 A & B). As depicted in Figure 4.11 C, mated yeast colonies containing *St*ARF10 either with promoter of *StGH3.6* or *AtGH3.5* grew robustly on the selection media (Sd -His –Trp) with an increasing concentration of 3AT (Figure 4.11 C) suggesting the binding of *St*ARF10 to both promoter sequences. For EMSA, the promoter fragments P1 (-1191 to -1607), P2 (-620 to -1204) and P3 (-1 to -639) of *StGH3.6* and P4 (-624 to -1278) of *AtGH3*.5 were explored for potential binding with *StARF10* protein (Figure 4.12 A & B). The consensus ARF-binding motif (TGTCTC) or its variants (Mironova et al., 2014) were present only in P1 of *StGH3.6* and P4 of *AtGH3.5*. Our EMSA results concluded that *StARF10* protein was able to bind P2 (with higher affinity) and P3 promoter fragments of *StGH3.6* and P4 fragment of *AtGH3*.5 as evident with shifted band (Figure 4.12 C). No binding was observed in P1 of *StGH3.6*. Cold competition assay with increasing molar concentrations of unlabeled P2 (*StGH3.6*) and P4 (*AtGH3*.5) further confirmed our results (Figure 4.12

D). Finally, it can be concluded that potato ARF10 (*St*ARF10) directly binds to *St*GH3.6 promoter and possibly regulates its expression.

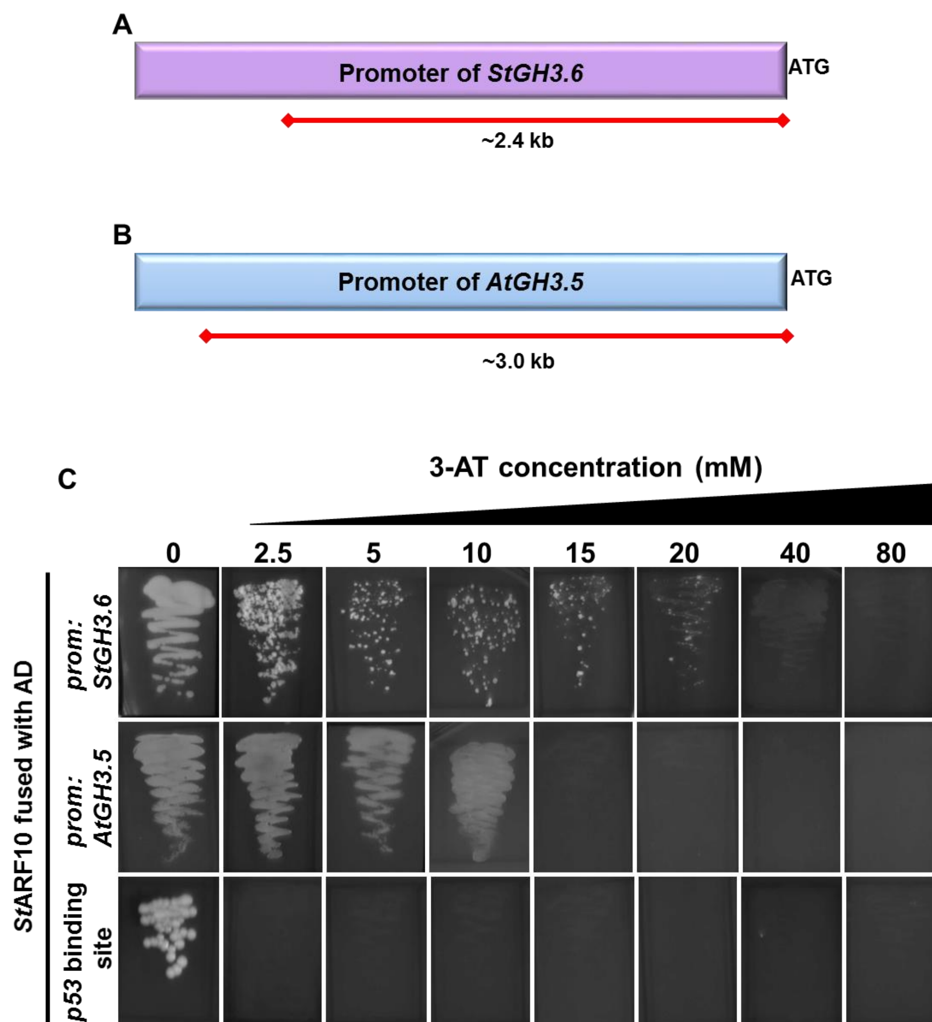


Figure 4.11. Yeast one-hybrid assay of the interaction between *St*ARF10 protein and promoters of *StGH3.6* and *AtGH3.5*. A-B. Diagrammatic representation of promoter used in this assay: ~2.4 kb upstream region of *StGH3.6* (A) and ~3.0 kb upstream region of *AtGH3*.5 (B). **C.** *St*ARF10 was used as prey protein and promoter sequences of *StGH3.6* and *AtGH3.5* were used as DNA baits. Growth of yeast strains containing *St*ARF10 prey and *StGH3.6* promoter bait till 20 mM of 3-AT and strains containing *St*ARF10 prey and *AtGH3.5* promoter bait till 10 mM of 3-AT, indicates the binding of *St*ARF10 to both *StGH3.6* and *AtGH3.5* promoters. Inhibition of yeast growth in all the concentrations of 3-AT suggests no interaction between p53 binding site and *St*ARF10 protein. ATG indicates 'start codon'; AD represents activation domain.

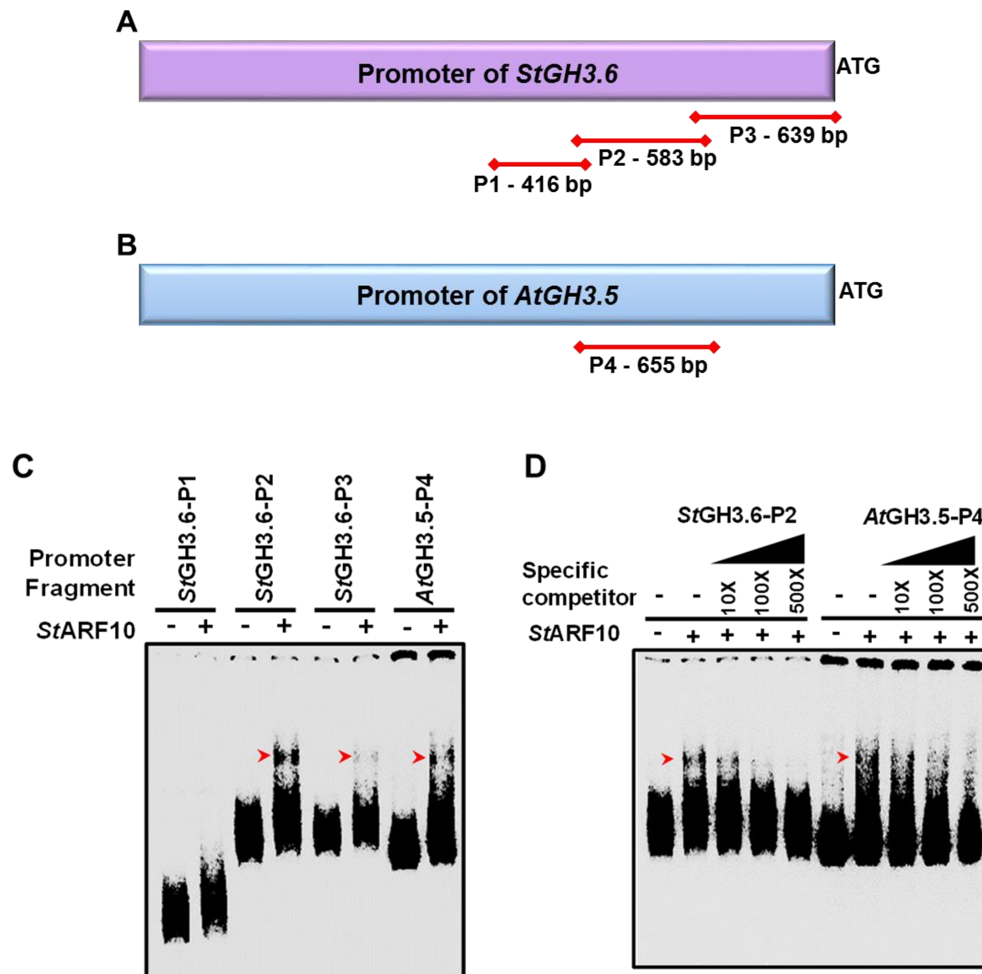


Figure 4.12 Electrophoretic mobility shift assay shows that *St*ARF10 can directly bind to the promoter fragments of *StGH3*.6 and *AtGH3.5*. A-B. Diagrammatic representation of promoter fragments P1 (416 bp, -1191 to -1607), P2 (583 bp, -620 to -1204) and P3 (639 bp, -1 to -639) of *StGH3.6* promoter (A) and P4 (655 bp, -624 to -1278) fragment of *AtGH3*.5 (B). **C.** Binding assays showing the interaction (red arrows pointing the shifted bands) of recombinant *StARF10* with P2 and P3 fragments of *StGH3*.6 promoter and P4 fragment of *AtGH3*.5 promoter. **D.** Cold competition assay performed with increasing molar concentrations of unlabelled P2 (of *StGH3*.6) and P4 (of *AtGH3*.5) gradually vanished the shifted band (red arrow) showing the interaction.

4.4. Discussion

4.4.1. Dysregulation of miR160 affects auxin signalling in potato

miR160 and its target auxin response factors (ARF10/16/17) have been shown to play important role in auxin signaling pathway in *Arabidopsis* and various other plants. Studies elucidate their role in root development (Ding and Friml, 2010; Gutierrez et al., 2012) and seed dormancy (Liu et al., 2013) in *Arabidopsis*, nodule development in soybean (Turner et al., 2013), rhizobial infection in *Medicago* (Breakspear et al., 2014) as well as ovary patterning, floral organ abscission and lamina outgrowth in tomato (Hendelman et al., 2012; Damodharan et al., 2016). Similarly, in potato, our results suggest that miR160 possibly has role in leaf and tuber development (Chapter 3). We also observed that the basal expression of genes involved in auxin biosynthesis (*StYUCCA1*), perception (*StTIR1*) and signaling (*StIAA16* and *StGH3.6*) were affected in miR160 KD and/or miR160 OE lines of potato (Figure 4.1). Similar expression patterns were also observed in other plants, where miR160 or its targets were dysregulated (Mallory et al., 2005; Huang et al., 2016). This suggests that miR160 plays intricate role in the interconnected and complex pathway of auxin signaling in plants.

Several earlier reports supported antagonistic crosstalk of auxin and SA signaling in *Arabidopsis* (Wang et al., 2007; Park et al., 2007; Abreu and Munné-Bosch, 2009; Truman et al., 2010). Similarly, a recent report suggested that auxin signal transduction pathway is strongly inhibited in potato infected with Potato virus Y (Baebler et al., 2014). We also observed that upon *P. infestans* infection of WT potato plants, the expression of auxin biosynthesis gene *StYUCCA1* and auxin receptor *StTIR1* were highly reduced whereas, the expression of AUX/IAA repressor *StIAA16* and the auxin conjugator *StGH3*.6 was significantly increased (Figure 4.2 A). All these changes are characteristics of attenuated auxin signaling and might help the WT plants to mount effective defense response by enhancing SA signaling. Such a regulation of auxin pathway genes were not observed in miR160 KD (eTM160-26) and OE (pre160-L17C1) lines (Figure 4.2 B & C) of potato. This suggests that failure to effectively attenuate the auxin pathway could be one of the reason for enhanced susceptibility exhibited by miR160 OE and KD lines (Chapter 3, Figure 3.8 A).

4.4.2. Knockdown of miR160 affects major SAR associated signals and genes

Earlier reports showed that SA and PR1 levels were induced only in AA (arachidonic acid, PAMP of *P. infestans*) treated leaves of potato (Coquoz et al., 1995; Yu et al., 1997) . In contrast, Manosalva et al. (2010) showed that AA treatment leads to SA and PR1 accumulation in both local and systemic leaves of potato (Manosalva et al., 2010). Our findings were consistent with that of Manosalva et al. (2010) (Figure 4.8). The discrepancy between our results and earlier reports of Coquoz et al. (1995) and Yu et al. (1997) could be because of the differences in time-points analysed for SA quantification. Hence, our results support the notion that SA plays role in AA-induced SAR response in potato. However, in our study, when miR160 KD lines were treated with AA, levels of SA and *StPR1* were not effectively changed (Figure 4.8 & 4.11 B. This perhaps explains why the miR160 KD lines exhibited compromised SAR response (Chapter 3, Figure 3.10 A).

NPR1 (Non-expresser of pathogenesis related 1) is known as the master regulator of SAR and involved in mediating SA signalling by directly regulating expression of several PR genes in *Arabidopsis* (Mukhtar et al., 2009; Pajerowska-Mukhtar et al., 2013). However, the role of NPR1 is not known in potato. *Arabidopsis* plants lacking functional NPR1 do not express PR1 and exhibit defective SAR response (Pajerowska-Mukhtar et al., 2013). Though this signalling works mainly by monomerization of oligomerized NPR1 protein (Mou et al., 2003; Spoel et al., 2009), increase in *NPR1* transcript levels also accompany this process (Yu et al., 2001). Our analyses showed that AA-treatment leads to increased expression of *StNPR1* in both local and systemic leaves of WT potato plants (Figure 4.10 C & D). However, equivalent increase of *StNPR1* transcription was not observed in eTM160-26 plants suggesting that reduced SA levels could be the cause for less-effective *StNPR1* signalling resulting in reduced *StPR1* levels. These results indicate that the potato *St*NPR1 might function similar to its *Arabidopsis* counterpart (*At*NPR1) in SAR development.

Manosalva et al. (2010) demonstrated that the potato *StMES1* is involved in conversion of MeSA to SA in the systemic leaves and MeSA potentially acts as a mobile signal during SAR development (Manosalva et al., 2010). In *Arabidopsis,*

AtBSMT1 is involved in conversion of SA to MeSA in local leaves (Chen et al., 2003; Song et al., 2009), however, role of BSMT1 in potato has not been demonstrated yet. We observed that, in WT potato plants, *StBSMT1* and *StMES1* have oscillating expression pattern in both local and systemic leaves, which corresponded to the accumulation patterns of MeSA. Though, local leaves of eTM160-26 plants had low levels of SA and reduced expression of *StBSMT1*, the accumulation of MeSA was comparable to WT plants suggesting that these low levels of SA were sufficient to produce optimum amount of MeSA and/or the conversion was possibly carried out by homologs of *StBSMT1*. Though, MeSA accumulation in local leaves was equivalent to WT plants, reduced levels observed in the systemic leaves in the later time-points suggested that MeSA transport is possibly affected in eTM160-26 plants thus leading to reduced SA levels. However, the reason for increased *StMES1* levels in eTM160-26 plants is not interpretable.

In *Arabidopsis*, overexpression of auxin amino acid conjugator, *AtGH3.5*, results in increased SA levels and PR1 expression in both local and systemic leaves (Zhang et al., 2007). However, these authors have also showed that loss-of-function mutation of this gene can results in partially compromised SAR response associated with decreased PR1 expression in the systemic leaves (Zhang et al., 2007). Our analysis revealed that expression of its potato homolog, *StGH3*.6, is also affected in local and systemic leaves during *P. infestans* infection (Figure 4.10 I & J) in WT plants, indicating its potential role in potato defence responses. However, in our miR160 knockdown lines, *StGH3.6* had a reduced expression suggesting the miR160 mediated possible regulation of *StGH3*.6. Taken together, our analyses indicate that knockdown of miR160 affects major SAR associated signals and genes, which is possibly the reason for defective SAR response exhibited by these plants.

4.4.3. *StARF10* mediated regulation of *StGH3.6*

One of genes that mediate crosstalk between SA and auxin signalling is *Arabidopsis* GH3.5 (Huot et al., 2014). The GH3 family of genes encode proteins that adenylate phytohormones IAA, JA and SA, which in some cases also catalyse their conjugation to amino acid (Staswick et al., 2002, 2005). A number of reports have shown that ARFs regulate GH3 family of gene by binding to the AREs (auxin

responsive elements) present in their promoters (Ulmasov et al., 1997; Hagen and Guilfoyle, 2002; Zhang et al., 2015). Further, Mallory et al. (2005) speculated that one of the GH3 family members, *AtGH3.5*/*GH3.6*, could be regulated by miR160 targeted ARFs (Mallory et al., 2005). Our experiments showed that overexpression and knockdown of miR160 results in dysregulation of *StGH3.6*, a homolog of *AtGH3.5* (Figure 4.1 and 4.10 I & J) suggesting its possible regulation by targets of miR160 in potato. Here, we have conclusively showed that potato protein *St*ARF10 directly binds to the promoter of both potato *StGH3.6* and *Arabidopsis AtGH3.5* (Figure 4.11 and 4.12). Though P2 and P3 promoter fragments of *StGH3.6* had no consensus AREs, binding was still observed with these fragments (Figure 4.12 C), suggesting that *StARF10* might recognize different DNA motifs. *AtGH3.5* has previously been shown to play role in local and SAR responses and its overexpression resulted in increased SA and PR1 levels in both local and systemic leaves (Zhang et al., 2007). The direct regulation of *AtGH3.5* and *StGH3.6* by *St*ARF10 as evident in our study provides a mechanistic link between miR160 and its targets in mediating both local as well SAR defence response in potato.

In conclusion, our findings showed that miR160 OE and KD transgenic lines failed to attenuate auxin signalling which resulted in enhanced susceptibility. Also, compromised SAR response observed in KD line could be because of the dysregulation of multiple SAR related signals and genes. *StGH3.6,* which is involved in both auxin and defence/SAR pathway is directly regulated by miR160 target gene *St*ARF10 in potato.

Summary

Being sessile, plants are constantly exposed to various pathogens. To ward off infection by pathogens, plants employ multiple layers of defence responses (Chisholm et al., 2006; Jones and Dangl, 2006) in both local and systemic leaves. In the pathogen infected local leaves, plants can recognize pathogen-associated molecular patterns (PAMPs) using their membrane bound pattern recognition receptors (PRRs) leading to the activation of PAMP-triggered immunity (PTI) (Chisholm et al., 2006). Some pathogens can evade such detection by releasing PTI-suppressing 'effector protein' into the plant cell. In this arms race of plant defences and pathogen counter-defences, some plants have evolved resistance (R) proteins that can recognize these effectors and activate effector- triggered immunity (ETI) (Dodds and Rathjen, 2010). In addition to these local responses, PTI and ETI can induce systemic defences in the un-infected parts of the plant, resulting in a broad-spectrum, long-lasting resistance known as systemic acquired resistance (SAR) (Shah, 2009; Dempsey and Klessig, 2012). SAR is activated by the transport of a mobile SAR signal from the local infected tissues to the systemic tissues mostly via phloem (Guedes et al., 1980; Tuzun and Kuc, 1985). Extensive research has led to the identification of several potential SAR signals, most promising being (i) Methyl salicylate (MeSA) (Park et al., 2007), (ii) Azelaic acid (AzA) (Jung et al., 2009), (iii) Glycerol-3-Phosphate (G3P) or its derivatives (Chanda et al., 2011), (iv) dehydroabietinal (DA) (Chaturvedi et al., 2012) and (v) pipecolic acid (Pip) (Návarová et al., 2012). Apart from these studies, many other reports have also unequivocally shown the role of microRNAs (miRNAs, 21-nt non-coding RNAs) in plant immunity including PTI and ETI responses (Ruiz-Ferrer and Voinnet, 2009; Sunkar et al., 2012; Seo et al., 2013) in addition to their functions in plant growth and development (Nogueira et al., 2009; Borges and Martienssen, 2015). To the best of our knowledge, no previous studies have yet described the role of miRNAs in SAR development. Because miRNAs are very important regulatory molecules, we hypothesised that miRNAs could play significant role in establishment of SAR as well. Using potato – *Phytophthora infestans* interaction as a model system, we laid out a number of objectives to test our hypothesis. It is noteworthy that role of miRNAs in potato – *P infestans* interaction is also not investigated before.

Objectives:

i. To investigate the role of potential miRNAs and their targets in potato-*Phytophthora infestans* interaction.
ii. To characterize the role of miR160 in local defence and SAR responses of potato.
iii. To explore the mechanistic link of miR160 in defence response of potato.

Chapter 1: Introduction

A thorough literature survey was carried out regarding (i) different types of defence responses (PTI, ETI and SAR) exhibited by plants and (ii) role of miRNAs in plant-pathogen interaction. This survey revealed that several miRNAs play role in the local defence responses of PTI and ETI. However, to the extent of our knowledge, there was no literature that describes the role of miRNAs in SAR. Further, in this chapter, we have also summarized the current knowledge of potato-*P. infestans* interaction. Finally, we have proposed a number of objectives to investigate the role of miRNAs in local and SAR defence responses using potato- *P. infestans* interaction as a model system.

Chapter 2: Investigating the role of potential microRNAs and their targets in potato-*Phytophthora infestans* interaction

Earlier, several miRNA families have been predicted and validated in potato (Zhang et al., 2009; Yang et al., 2010b; Kim et al., 2011; Zhang et al., 2013; Martin et al., 2009; Bhogale et al., 2014). However, only two reports have so far described the role of potato miRNAs in biotic (Yang et al., 2010a) and abiotic stress responses (Kitazumi et al., 2015). One of our aims was to identify the miRNAs involved in potato-*P. infestans* interaction with a potential role in SAR. In this regard, a number of candidate miRNAs were shortlisted based on the prior knowledge of miRNA's role in other plant-pathogen interaction as well as its presence in phloem. Out of the 11 shortlisted miRNAs, 10 were found to be expressed in potato and 5 showed differential expression upon *P. infestans* infection in our study. Off these 5 differentially expressed miRNAs, miR160 was chosen for elaborate studies. Expression of miR160 was analysed in local, systemic leaves and phloem enriched exudates (PEX) of potato plants upon *P. infestans* infection. We also predicted and validated targets of miR160 in potato followed by analysing the expression profile of *StARF10*, one of the miR160 targets, in local and systemic leaves upon *P. infestans* infection.

Following were the important findings from these analyses:

i. Upon *P. infestans* infection, potato miRNAs, miR159, miR160, miR166, miR169 and miR172, exhibited differential expression indicating their potential role in potato-*P. infestans* interaction.

ii. miR160 expression was found to be induced in both local and systemic leaves upon *P. infestans* infection suggesting its possible role in basal defence and SAR responses of potato.
iii. *StARF10 and StARF16* were validated as true targets of miR160.
iv. Upon *P. infestans* infection, expression of *StARF10* was also observed to be induced in local and systemic leaves of potato.
v. In our study, miR160 levels increased in the PEX of infected potato plants suggesting its potential role as a mobile SAR signal.

Chapter 3: Characterization of miR160 and its role in local defence and SAR responses of potato

miR160 is known for its crucial role in plant development and auxin signalling (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). Recently, the defence related functions of miR160 have also been elucidated. Li and co-workers (2010) have shown that miR160 is involved in PAMP-induced callose deposition and PTI responses in *Arabidopsis* (Li et al., 2010). miR160 is also proposed to function as a positive defence regulator during rice-*Magnaporthe* interaction (Li et al., 2014). Our results described in chapter 2 showed that miR160 is induced in both local and systemic leaves of potato post *P. infestans* infection. Hence, we hypothesized that miR160 could play a role in local and SAR defense response of potato. To test this, both overexpression (OE) and knockdown (KD) transgenic lines of miR160 were generated. Basal defense and SAR response of these lines were analysed using local infection, SAR assays and grafting analysis. Our results indicated that miR160 plays important role in development of both local defences and SAR responses in potato.

Following were the important findings from these analyses:

i. We have observed that overexpression and knockdown of miR160 renders the potato plants highly susceptible to *P. infestans* infection. This suggests that an optimal level of miR160 is possibly required for mounting a proper local (basal) defence response in potato.
ii. miR160 KD lines were found to be SAR-deficient, whereas miR160 OE lines were SAR-competent implicating that miR160 play role in SAR responses of potato.

iii. Our grafting studies further indicated that miR160 KD lines were unable to both (a) generate and/or transport the SAR signal, and (b) perceive and/or process the SAR signal. These findings suggested that threshold levels of miR160 is possibly required in both local and systemic leaves of potato for establishment of an effective SAR response.

Additionally, following development-related roles of miR160 also has emerged in our study:

iv. miR160 OE and KD transgenic lines did not show any drastic morphological changes, except that miR160 OE line had a slight curled leafy phenotype suggesting its role in leaf development.

v. miR160 KD lines exhibited increased tuber yield, whereas OE lines had a reduced tuber yield indicating its potential role in potato tuberization.

Chapter 4: Exploring the mechanistic link of miR160 in defence response of potato

Plants actively suppress auxin signalling processes to mount salicylic acid (SA)-mediated defence responses during a biotrophic or hemi-biotrophic pathogen attack. Several reports have suggested the importance of such an antagonistic crosstalk between auxin-mediated growth and SA-mediated defence pathways (Kazan and Manners, 2009; Denancé et al., 2013; Huot et al., 2014; Verma et al., 2016). As miR160 is shown to be an important component of auxin signalling pathway, we examined the enhanced susceptibility phenotype of miR160 OE and KD lines in light of auxin-SA signalling crosstalk. Additionally, SAR-associated signals (SA and MeSA) and genes (*StPR1*, *StNPR1*, *StBSMT1*, *StMES1*, *StGH3.6*) were also examined to understand the reason for the compromised SAR response exhibited by miR160 KD lines. One of the common mediators in this auxin-SA crosstalk is the auxin-conjugator, *StGH3.6*. Based on the expression pattern of *StGH3.6* in miR160 OE and KD lines, we hypothesised that *StGH3.6* could be regulated by *StARF10* (the target gene of miR160) and this hypothesis was further tested with EMSA and Y1-H assays.

Following were the important findings from these analyses:

i. Upon *P. infestans* infection, the WT potato plants were able to successfully suppress the auxin signalling, however, miR160 OE and KD transgenic lines failed to exhibit the same. This suggests that failure to attenuate auxin signalling could be one of the reasons for the enhanced susceptibility of miR160 KD and OE lines.

ii. We observed that major SAR-associated signals and genes were found to be dysregulated in miR160 KD lines. This further explains the possible cause for compromised SAR response observed in miR160 KD lines.

iii. Our EMSA and YI-H analysis showed that *St*ARF10 directly binds to the promoter of *StGH3.6.* This provides a mechanistic link between miR160 and the defence-related pathways involved in potato- *P. infestans* interaction

Future directions:

Our study is the first to establish the role of a miRNA in SAR responses of plants. The aforementioned findings indicate that miR160 is involved in both local defence and SAR responses of potato against *P. infestans* infection. To have further insight into the role of miRNAs in SAR development as well as potato-*P. infestans* interaction, following directions of work could be undertaken as part of future studies:

i. As increased levels of miR160 were observed in PEX of potato post *P. infestans* infection, the potential role of miR160 as a phloem-mobile signal can be explored.

ii. We have observed that miR160 expression is induced in both local and systemic leaves post *P. infestans* infection. It would be interesting to discover the upstream regulators involved in this induction.

iii. Apart from *StARF10*, miR160 also regulates many other targets. The role of these target genes in plant-pathogen interaction and SAR can be studied.

iv. From our studies, it appears that miR160 has different roles in local leaves and systemic leaves of potato. It would be interesting to examine the purpose and consequence of this differential role as part of future investigation.

v. Other candidate miRNAs (from the shortlist) can also be tested for their potential role in potato-*P. infestans* interaction and SAR development.

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