Generation of *miR160* and *miR166* Endogenous Target Mimicry (eTM) Transgenic Lines in Potato and their Assessment against *Phytophthora* Interactions.

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भारतीय विज्ञान शिक्षा एवं अनुसंधान संस्थान, पुणे
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CERTIFICATE

This is to certify that this dissertation entitled "**Generation of *miR160* and *miR166* Endogenous Target Mimicry (eTM) Transgenic Lines in Potato and their Assessment against *Phytophthora* Interactions**" towards the partial fulfillment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research (IISER), Pune represents original research carried out by Supreeth K S at IISER Pune under the supervision of Dr. Anjan K Banerjee, Associate Professor, Biology Division, IISER Pune during the academic year 2014-2015.

Signature of the Supervisor

Date: 25th March, 2015.

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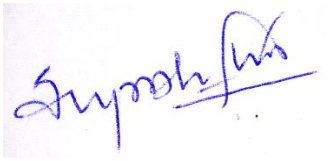
DECLARATION

I hereby declare that the matter embodied in the thesis entitled "**Generation of *miR160* and *miR166* Endogenous Target Mimicry (eTM) Transgenic Lines in Potato and their Assessment against *Phytophthora* Interactions** "are the results of the investigations carried out by me at the Biology Division, IISER Pune under the supervision of Dr. Anjan K Banerjee, Assistant Professor, Biology Division, IISER Pune and the same has not been submitted elsewhere for any other degree.



Signature of the Student

(Supreeth K S)

Date: 25th March, 2015.

ABSTRACT

One of the great challenges for researchers is to overcome the late blight disease of potato caused by the oomycete pathogen, *Phytophthora infestans*. Recent studies suggest involvement of several microRNAs during plant-pathogen interaction. microRNAs 160 (*miR160*) and *miR166* are highly conserved miRNAs among different plant species that are known to play various developmental roles. Studies in our lab indicate involvement of *miR160* and *miR166* during potato – *Phytophthora* interaction. In order to further investigate the role of these miRNAs in plant immunity, we generated knockdown lines of *miR160* and *miR166* using endogenous target mimicry (eTM) approach.

miR160, *miR166* and their target genes were successfully detected in our model system *Solanum tuberosum* spp. *Desiree*. Using *Agrobacterium*-mediated transformation technique, we successfully generated endogenous target mimicry (eTM) lines for *miR160* and *miR166*. Representative eTM lines of both these miRNAs have been confirmed by RT-PCR analysis. To assess the level of knockdown in these lines, real-time PCR based approach was attempted. Our results indicate successful reduction in levels of *miR160* by ~ 66% in eTM160 clone-1 and by ~91% in eTM160 clone-2, whereas reduction of *miR166* level was ~ 96% in eTM166 clone 1 and by ~91% in eTM166 clone 2. All the four clones were subsequently transferred to soil and were gradually hardened. These lines are presently grown in growth chamber conditions and are being subjected to *Phytophthora* infection to understand their role in immunity.

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I would like to take this opportunity to express deep regards to my mentor, Dr Anjan K Banerjee, for his faultless guidance, constant support and encouragement throughout the course of this project. His invocation and guidance shall carry me a long way in the journey of life which I am about to commence. I would like to thank IISER Pune Biology department for providing me a much better research experience.

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1. INTRODUCTION:

Plants encounter millions of pathogens in their lifetime which causes devastating diseases that have adverse effects in crop production and food safety. One of the classic examples is the case of Irish potato famine caused by oomycete pathogen *Phytophthora infestans* which did result in the demise of millions of people. Even though plant pathogen interactions are well studied, adding newer insights help us to understand how plants survive in an environment where there is increasing number of pathogens.

1.1 Plant-pathogen interactions:

Plants have evolved various defense mechanisms against their pathogens. Pathogen-associated molecular pattern (PAMP) and effector triggered immunity (ETI) are two inducible defense mechanisms in plants (Jones and Dangl, 2006). Pathogen recognition results in the activation of large number of signaling cascades which reprogram the expression of defense genes and pathogenesis associated proteins that help plants to counteract the infection.

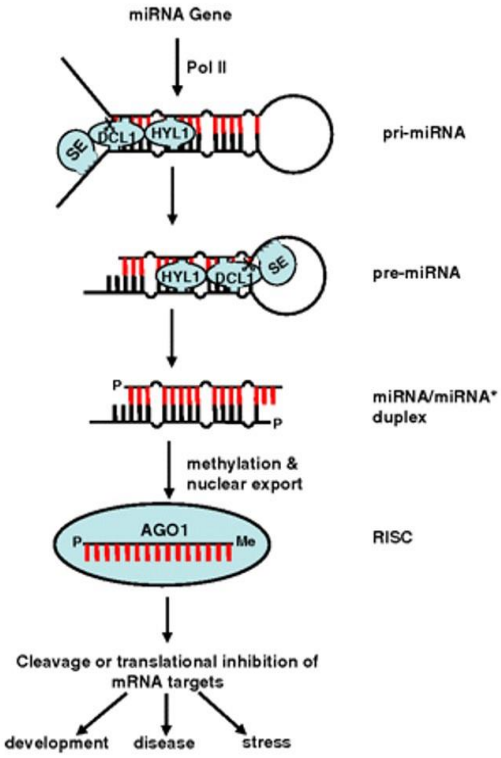

Figure 1: Biogenesis and functions of miRNA in plants

1.2 miRNAs in Plant-Pathogen interactions:

miRNAs are small non-coding RNA molecules(~20-22 nucleotides) which regulate post transcriptional gene expression resulting in RNA silencing via translational inhibition or cleavage of target mRNA. The Primary miRNA transcripts are processed by RNase III enzyme and its associated co factors. The miRNA is later methylated and transported to cytoplasm where it incorporates into the RISC complex which silence mRNA targets (Zhu, 2008).

A large number of miRNAs are involved in regulation of defense related mRNAs and in fine-tuning of hormone signaling pathways and thereby play an important role in plant defense. High-throughput deep sequencing studies helped in identifying more miRNAs involved in plant immunity (Padmanabhan et al., 2009).

One of the well studied microRNAs with a role in plant immunity is miRNA393. In *Arabidopsis*, overexpression of *miR393* enhanced the resistance against bacterial pathogen (Navarro et al., 2006). *miR393* under optimal conditions helps in normal homeostasis of auxin hence resulting in normal growth. Under biotic stress, the levels of *miR393* go up and thereby the auxin pathway is attenuated. This helps the plants to redirect their resources from development to defense related pathways.

In addition to *miR393*, recent studies have shown that *miR160*, *miR167* and *miR390* also regulate gene expression of components in the auxin signaling pathway. *miR159* and *miR319* suppress components of jasmonic acid (JA) and abscisic acid (ABA) signaling pathways and thereby regulate plant defense.(Zhang et al., 2011). Also, *miR166* have been shown to get upregulated in soyabean upon infection with *Phytophthora sojae*.

1.3 *miR160* and *miR166* in plant immunity:

MicroRNA 160 (*miR160*) and 166 (*miR166*) are highly conserved microRNAs among different plant species. *miR160* targets the auxin response factors 10 (ARF10), ARF16 and ARF17 in *Arabidopsis*. *miR160* play roles in seed germination and root development whereas *miR166* has roles in leaf development and vascularization. Few studies have also indicated the role of *miR160* and *miR166* in plant defense responses. Transgenic lines produced by over expression of *miR160* showed enhanced pathogen resistance via the down regulation of auxin responsive genes. *miR160* suppresses ARF16 and thereby down regulates auxin responsive genes which acts as negative regulators of plant resistance . Results from another study showed that the overexpression lines of *miR160* in *Oryzae sativa* resulted in a decrease in the amount of the target gene Os04g43910 which codes for auxin responsive factor (Li et al., 2013).

Auxin acts as a negative regulator of plant resistance. As *miR160* cleaved the target mRNA (Auxin response factor) and thereby downregulated Auxin signaling, there was an increase in the resistance of rice plants against the fungus *Magnaporthe oryzae* (Li et al., 2013).

Studies on soybean have shown that *miR166* expression increases upon infection by *Phytophtera sojae*. The expression of *miR166* was induced upon the infection by heat killed *P. sojae* cells (Wong et al., 2014). *miR166* targets homeodomain leucine zipper transcription factors (HD-Zip III) and regulate processes namely organ polarity, vascular development and lateral root development. Upon induction of *miR166*, there was a reduction in the target genes in the *P. sojae* infected roots which may contribute to reduced growth of soybean to conserve energy and use it for defense purposes (Wong et al., 2014).

In agreement with these reports, previous studies in our lab have also indicated the involvement of *miR160* and *miR166* during potato-*Phytophthora* interaction.

1.4 Potato-*Phytophthora* interaction:

One of the devastating diseases of potato is the late blight caused by an oomycete pathogen named *Phytophthora infestans*. Identifying the resistance genes in the potato against *Phytophthora infestans* is a way by which plant breeders can develop *Phytophthora infestans* resistant plant varieties. Resistance genes were transferred from the wild relatives to the cultivated varieties by the conventional crossing techniques (Kumar et al., 2006; Sliwka et al., 2010).

1.5 miRNA knockdown by Endogenous Target Mimicry (eTM):

Endogenous Target Mimicry is an approach in which decoy RNAs bind to miRNAs via complementary sequences thus blocking the interaction between both the miRNAs and their authentic targets (Wu et al., 2013). Both engineered artificial RNAs and miRNA target mimics can induce target mimicry effects. Only one RNA (IPS1) is functional endogenous microRNA target mimic (eTM). eTM binds to its specific miRNA

as it is complementary to it. This prevents miRNA to bind to its targets, hence knocking down the miRNA. They also proved that the eTMs of *miR160* and *miR166* are functional target mimics in *Oryza sativa.* They also have found out their role in regulation of plant development. Therefore using this approach, we wanted to knock down these two miRNAs and find out the role of these two miRNAs by raising the transgenics and infecting them with *Phytophthora infestans*.

1.6 Objectives:

To understand the role of *miR160* and *miR166* during Potato-*Phytophthora* interaction; we are developing target mimicry lines (eTM) in our model *Solanum tuberosum* ssp *Desiree.*

1. Raising the transgenics.
2. Confirmation of the transgenics.
3. Estimation of the expression levels of *miR160* and *miR166* in knock down lines.
4. Assessment of phenotypical changes with respect to wild type.

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2. MATERIALS AND METHODS

Chemicals were obtained from Invitrogen and SIGMA. DNA polymerases and ligase were acquired from Geneii and New England Biolabs (NEB) respectively.

TABLE 1 : LIST OF PRIMERS USED

NAME	Sequence 5' to 3'
miRNA Knock down	
Ath-eTM160-FP	TCTTCAGAGATGGCCTGAC
Ath-eTM160-RP	AATCGTAATCCTAATCAGTGTT
Ath-eTM166-FP	GTTTATCACGATGAAATTGTTG
Ath-eTM166-RP	TTAGTTCGACGATACATTCCA
Targets of miRNAs	
ARF10-FP	GTCCAGCAGTCCTTTCTGTTGTTT
ARF10-RP2	GCTGCAACACGCTGGAAACTT
ARF16-FP	GGCAACCCCCTCAGGTCTAG
ARF16-RP2	TGCAACTTTTCGCTACGGTGGA
ICU-FP	CAAAGGCTACTGGAACTGCTGTT
ICU1-RP2	CAACCCTCGTTGGATCTAAACCAA
ICU2-RP2	GACCCTCGTTGGCTCTAGACC
PHV1-FP	GAAAGGCTACTGGAACTGCTGTC
PHV1-RP2	CAACCTTCGTGGGCTCTAGAC
PHV2-FP	GCTACTGGAACTGCTGTCGACTG
PHV2-RP2	CTCAGCAACCTTCATGGGCTCT
REV-FP	GGCTACAGGAACTGCTGTCGATT
REV-RP2	TAACAAGACCACATGCTCGGGC
Mature miRNA detection and qPCR	
miR160-STP	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGG ATACGACGGCATA
miR166-STP	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGG ATACGACGGGAAT
miR160-FP	TGGAGTTTGCCTGGCTCCCTG
miR166-FP	TGGAGGTTCGGACCAGGCTTC
Univ-miR-RP	AGTGCAGGGTCCGAGGT
Reference genes	
GAPDH-FP	GAAGGACTGGAGAGGTGGA
GAPDH-RP	GACAACAGAAACATCAGCAGT

2.1 Detection of *miR160* and *miR166* and their targets in *Desiree.*

2.1.1 RNA Isolation:

RNA was isolated using the entire *in vitro* grown plant tissue of *Desiree*.

- Plant tissue was homogenized with mortar and pestle using liquid nitrogen.
- 1ml of TRIzol reagent was added to 100mg of tissue and the components were mixed thoroughly by applying vortex for 2 to 5 min, and then were incubated at room temperature for 5 min.
- 200µl of chloroform was added to each sample and the tubes were inverted 5-6 times. Samples were incubated at room temperature for 10 min.
- For phase separation, samples were centrifuged at 12000 rcf at 4°C for 15min. Upper aqueous phase was collected in new tube.
- RNA was precipitated by adding isopropanol (500µl) and the tubes were thoroughly mixed by inverting. Samples were incubated at room temperature for 10 min followed by centrifugation at 12000 rcf at 4^0C for 10min. Supernatant was discarded.
- The obtained RNA pellet was washed using 1ml of 75% ethanol and centrifuged at 7500 rcf at 4^0C for 5 min. Supernatant was discarded. Excess of ethanol was removed by incubating it at 37°C for not more than 10min.
- RNA pellet was dissolved in DEPC water (30µl) by incubating it for 15min at 55°C.
- RNA concentration was estimated using Nanodrop 2000 Spectrophotometer (Thermo Scientific, Wilmington, USA). RNA was then stored at -80^0C until further use.

2.1.2 cDNA synthesis:

1µg of RNA was used for cDNA synthesis of both miRNAs and their mRNA targets. cDNA preparation for target detection was performed using oligodT_{-16} primer (Invitrogen). cDNA preparation for microRNAs were carried out using specific stem-

loop primers for *miR160* (*miR160*-STP) and *miR166* (*miR166*-STP) (refer to primer table). cDNA reactions for both cases were set up as below:

20 µL Reaction:

5x First strand buffer	4µL
0.1 M DTT	2µL
Rnase Out	0.2µL
SS III Reverse Transcriptase	0.3µL
50 µM OligodT Primer / 1 µM miRNA STP primers	1µL
10 mM dNTP (2.5 mM each)	0.5µL
RNA(1µg)	XµL
Water	12-X µL

Reactions conditions:

1. cDNA synthesis using OligodT primer for targets.

50°C	1hour
85°C	5min
4°C	Hold

2. cDNA synthesis using miRNA 160 and miRNA 166 specific stem-loop primers

16°C	30min
30°C	30sec
42°C	30sec
50°C	1sec
Repeat step 2-4 (60 times)	
85°C	5min
4°C	Hold

2.1.3 Detection of *miR160*, *miR166* and their targets using RT PCR :

cDNA prepared previously were used for performing PCR. Detection of *miR160* was done using primer sets – *miR160*-FP and Univ-miR-RP and *miR166* was done using primer sets – *miR166*-FP and Univ-miR-RP.

For target detection, the following primer sets were used.

Targets of *miR160*	**Primers used**
Auxin Response Factor-10, ARF10	ARF10-FP and ARF10-RP2
Auxin Response Factor-16, ARF16	ARF16-FP and ARF16-RP2
Targets of *miR166*	
Incurvata-1, ICU-1	ICU-FP and ICU1-RP2
Incurvata-2, ICU-2	ICU-FP and ICU2-RP2
Phavoluta-1, PHV-1	PHV1-FP and PHV1-RP2
Phavoluta-2, PHV-2	PHV2-FP and PHV2-RP2
Revoluta, Rev	REV-FP and REV-RP2

The following PCR reaction set up was used to perform the reaction with each set of primers for the seven targets.

10 µL Reaction:

10x Taq Buffer A	1µL
10mM dNTPs (2.5 mM each)	0.2 µL
10µM Forward Primer	0.1 µL
10µM Reverse Primer	0.1 µL
cDNA	1 µL
Taq Polymerase	0.15 µL
MQ water	(10-2.55) µL

Reaction conditions for the PCR:

94°C	5min
94°C	10sec
60°C	10sec
72°C	15sec
Repeat steps 2-4 (35 cycles)	
72°C	5min
4°C	Hold

2.2 Construct design of endogenous target mimics:

Endogenous target mimics of *miR160* (eTM-160) and *miR166* (eTM-166) were previously cloned in pCAMBIA 1300 under sites XbaI and SacI by Wu et al (Plant phys, 2013). For our study, both the eTM constructs were kindly provided by Professor Wang (Institute of Genetics and Developmental Biology, Beijing).

2.3 *Agrobacterium* transformation and confirmation of *Agro* competent cells:

- The eTM constructs were mobilized into GV2260 Agrobacterium cells by the process of freeze-thaw transformation technique as below (Rainer Hofgen and Lothar Willmitzer, 1988).
- The *Agrobacterium* competent cells were thawed and 0.5-1 µg of DNA was added.
- Mixture was incubated on ice for 5 min.
- Mixture was transferred to liquid nitrogen and incubated for 5 min.
- Mixture was further incubated at 37°C for 5 min.
- 1mL of Luria-Bertani (LB) broth was added to each tube and the mixture was incubated at 28°C for 3 hours under constant shaking.
- The cells were collected by spinning them briefly in a microcentrifuge and were spread on the LB agar plates containing the antibiotics Kanamycin (50mg/mL) and Rifamycin (100mg/mL**)**.

- The plates were incubated at 28°C for 2 days for transformed colonies to develop.

2.3.1 Plasmid Isolation from Agrobacterium colonies:

Plasmids were isolated from *Agrobacterium* colonies by Alkaline Lysis technique:

(I) Components in Alkaline lysis based plasmid isolation:

a) Alkaline Lysis Solution – I (ALSI):
   - 50mM glucose
   - 25mM Tris HCl
   - 10mM EDTA (pH 8.0)

b) Alkaline Lysis Solution – II (ALSII): (Freshly prepared)
   - 1ml of 2M NaOH
   - 1 ml of 10% Sodium dodecyl sulphate
   - 8 ml of Distilled water

c) Alkaline Lysis Solution – III (ALSIII):
   - 60 ml of Potassium acetate
   - 11.5 ml of Acetic acid
   - 28.5 ml of Distilled water

(II) Protocol for plasmid isolation by Alkaline lysis:

- Agrobacterial colony was grown in a 3 ml LB media with kanamycin and rifampicin at 28°C overnight.
- The cells were collected by centrifuging at 10000 rpm for 2min and the supernatant was discarded.
- 200 µL of ALSI and 5µL of lysozyme were added to the pellet and components were thoroughly mixed by applying vortex.
- 400 µL of freshly prepared ALSII was added. Components were mixed thoroughly and placed on ice for 2 mins.
- 300 µL of ALSIII was added and samples were mixed gently by inverting.

- Tubes were centrifuged at maximum speed at 4°C for 5min and supernatant was transferred to fresh tube.
- 5 µL RNase was added and was incubated at 37°C for 30 min.
- Equal amount of Phenyl Chloroform was added and did vortex.
- The tubes were later centrifuged at maximum speed at 4°C for 5min.
- Top aqueous layer were collected in new tubes and equal amount of chilled Isopropanol was added.
- Tubes were incubated at -20°C for 20min.
- The tubes were centrifuged at maximum speed at 4°C for 5min and the supernatant was discarded.
- 1 mL of 70% ethanol was added to the tubes.
- Tubes were centrifuged at maximum speed at 4°C for 2 min.
- Ethanol was removed and the tubes were air dried.
- Plasmids were dissolved in 30 µL of TE buffer.
- Plasmids were checked by performing gel electrophoresis.

2.3.2 Clone confirmation by PCR:

- The positive clones of Agrobacterium were identified by PCR using the isolated plasmids. 150ng of plasmid DNA was used to perform PCR using these primers - Ath-eTM160-FP and Ath-eTM160-RP as well as Ath-eTM166-FP and Ath-eTM166-RP.

Reaction (10µL):

10x Taq Buffer A	1µL
10mM dNTPs (2.5 mM each)	0.2 µL
Ath-eTM160/166-FP	0.1 µL
Ath-eTM160/166-RP	0.1 µL
cDNA	1 µL
Taq Polymerase	0.15 µL
MQ water	(10-2.55) µL

Program:

94°C	5min
94°C	10sec
48°C	15sec
72°C	30sec
Repeat steps 2-4 (40 cycles)	
72°C	5min
4°C	Hold

- Glycerol stocks were made from the confirmed Agrobacterium cells by mixing 700 ul of overgrown culture and 300 ul of 50% glycerol. The stocks were flash frozen using liquid nitrogen and stored in -80°C until further use.

2.4 Subculturing *Solanum tuberosum* ssp. *Desiree:*

Desiree was subcultured in Murashige and Skoog (MS) basal media.

MS basal media components (SIGMA):

M-1 stock solution: (vol 1L)

Ammonium nitrate	16.5g
Potassium nitrate	19g

M-2 stock solution: (vol 1L)

Magnesium sulphate	3.7g
Potassium dihydrogen Orthophosphate	1.7g

M-3 stock solution: (vol 1L)

Calcium chloride	4.4g

M-4 stock solution: (vol 100mL)

Boric acid	620mg
Potassium iodide	83mg
Manganese sulphate	2230mg
Zinc sulphate	860mg
Sodium molybdate	25mg
Copper sulphate	2.5mg
Cobalt chloride	2.5mg

M-5 stock solution: (vol 100mL)

Nicotinic acid	150mg
Thyamine HC	10mg
Pyridoxine HCl	50mg
Myoinositol	10000mg

M-6 stock solution: (vol 100mL)

Ferrous sulphate	556mg
Na_2EDTA	740mg

The following components were added to prepare 1L of MS basal agar media:

M1	100mL
M2	100mL
M3	100mL
M4	1mL
M5	1mL
M6	5mL

Sucrose (2%)	20g
Fill the volume to 1L	

pH of the media was adjusted to 5.8. 0.2% phytagel (2g) was added. Media was autoclaved and poured in the boxes.

2.5 Transformation of *Solanum tuberosum* L. ssp. *tuberosum* cv *Desiree*:

Transformation was performed according Banerjee et al, 2006. Briefly, the transformation was performed as following:

2.5.1 Preparation of *Agrobacterium* culture:

- Glycerol stock of transformed *Agrobacterium* cells of both eTM160 and eTM166 were revived by streaking on ~~a~~ LB agar with kanamycin and rifampicin and incubated at 28°C for two days.
- A single *Agrobacterium* colony was inoculated into 5mL LB broth (with Kanamycin and Rifampicin) as a primary culture and incubated overnight at 28°C.
- A 10ml secondary culture in plain LB broth (No antibiotics) was inoculated from the primary culture by setting the OD_{600} around 0.4.
- Secondary culture was allowed to grow till the OD_{600} was around 0.8-1. After this the culture was kept on ice.

2.5.2 Preparation of plant material for transformation:

- Big fully grown leaves were collected from the subcultured Desiree plants and petiole was removed from the leaves by very sharp and clean cut.
- These leaves were then injured by making small cuts 1mm apart on the mid vein and side vein.
- The cut leaves were then placed in 30 ml of liquid MS basal media in a petridish. Around 30 leaves were kept in one petridish and likewise two petridish were prepared for each of the eTM160 and eTM166 constructs.

2.5.3 Transformation event:

- 80µL of secondary culture of Agrobacterium eTM160 and eTM166 were added to the respective petridishes with the cut leaves and kept under shaking at 80rpm for 30 mins to facilitate constant distribution of the Agrobacterial culture.
- Plates were later incubated at room temperature in dark for two days for Agrobacterium infection to happen.

2.5.4 Callus, Shoot and Root Induction:

- After two days, the leaves were blotted and transferred into the Callus induction media (MGC) with specific antibiotics and hormones [MS basal media + NAA (5 mg/L) + BAP (0.1 mg/L) + Cefotaxime (250mg/L) + Hygromycin (3 mg/L)].
- The MGC plates were incubated in tissue culture growth chamber (22°C, 16hr light and 8 hr dark) for 7 to 10 days for callus induction to occur.
- The leaves with callus mass were transferred to the Shoot induction media (MGS) with specific antibiotics and hormones [MS basal media + Zeatin Riboside (2.2 mg/L) + NAA (0.02 mg/L) + GA3 (0.15 mg/L) + Cefotaxime (250 mg/L) + Hygromycin (3 mg/L)] after 7 to 10 days and incubated in tissue culture incubator with specific conditions.
- The leaves were transferred to the new MGS media every week unless and until shoots of specific length appeared.
- The shoots were transferred to rooting media (RM) with specific antibiotics and hormones [MS basal media + Cefotaxime (250 mg/L) + IBA (0.25 mg/L) and Hygromycin (3 mg/L)] for roots to develop.

2.6 Confirmation of clones:

For clone confirmations, total RNA was isolated from the rooted clones of eTM160, eTM166 and wild-type Desiree plants by the Trizol technique as described previously. For eTM160 clones, cDNA was synthesized from 500ng of total RNA

using specific reverse primers for eTM160. 1µl of this cDNA was used for PCR using eTM160-FP and eTM160-RP as primers. For eTM166 clones, one-step RT PCR was performed using 500 ng of total RNA and eTM166-FP and eTM166-RP as the primers.

cDNA reaction: (10µL)

5x First strand buffer	2µL
0.1 M DTT	1µL
Rnase Out	0.1µL
SS III Reverse Transcriptase	0.15µL
Univ-miR-RP	1µL
10 mM dNTP (2.5 mM each)	0.25µL
RNA(1µg)	XµL
Water	6-X µL

Reaction conditions:

50°C	1hour
85°C	5min
4°C	Hold

10µL PCR:

Taq Buffer A	1µL
dNTPs	0.2 µL
eTM160-FP	0.1 µL
eTM160-RP	0.1 µL
cDNA	2 µL
Taq Polymerase	0.15 µL
MQ water	(10-3.55) µL

Reaction conditions:

94°C	5min
94°C	10sec
48°C	15sec
72°C	30sec
Repeat steps 2-4 (40 cycles)	
72°C	5min
4°C	Hold

- One Step RT PCR was carried out for the confirmation of eTM 166 clones. (15 µL reaction)

2X reaction Mix	7.5 µL
eTM166 FP	0.3 µL
eTM166 RP	0.3 µL
Desiree RNA	X µL
SS III RT Platinum Taq Polymerase (Invitrogen)	0.6 µL
MQ water	(15-{8.7+X}) µL

Reaction conditions:

cDNA synthesis :

cDNA synthesis :	
48°C	15min
50°C	15min
PCR conditions:	
94°C	2min
94°C	15sec
48°C	15sec

68°C	30sec
Repeat steps 2-4 (40 cycles)	
68°C	5min
4°C	Hold

2.7 Checking the levels of miRNAs in confirmed clones:

The levels of *miR160* and *miR166* in confirmed lines were checked using Real Time PCR. cDNA was prepared using 500 ng of total RNA from eTM160, eTM166 lines and wild-type Desiree plants. Stem-loop primers, *miR160*-STP and *miR166*-STP, were used for synthesis of cDNA from eTM160 and eTM166 lines. OligodT cDNA was prepared from both lines using 500 ng total RNA for reference gene quantification. cDNA reaction wer set up as described previously. Real-time PCR was performed using 2X SyBr Green Mix (SafLabs) and the reaction was set up as described below. GAPDH was used as a reference gene in this experiment.

Reaction (10µL) for *miR160* and *miR166*:

2X SYBR reaction Mix	5 µL
miR160-FP / *miR166*-FP	0.3 µL /0.2 µL
Univ-miR-RP	0.3 µL/0.2 µL
cDNA (diluted 1:10 ratio)	1 µL
Nuclease free water	3.4 µL/3.6 µL

Reaction (10 µL) for the GAPDH reference gene:

2X SYBR reaction Mix	5 µL
GAPDH Forward Primer	0.2µL
GAPDH Reverse Primer	0.2 µL

cDNA (diluted 1:10 ratio)	1 µL
Nuclease free water	3.6 µL

Reaction conditions for *miR160*/*miR166* qPCR:

95°C	5min
95°C	5sec
60°C	10sec
72°C	8sec
Repeat steps 2-4 (40 cycles)	

Melting curve.

Increment of 0.5°C periodically from 65°C to 95°C for each 5sec.

Reaction conditions for GAPDH: (2 Step program)

95°C	3min
95°C	15sec
60°C	20sec
Repeat steps 2-3 (40 cycles)	

Melting curve.

Increment of 0.5°C periodically from 65°C to 95°C for each 5sec.

2.8 Transferring confirmed clones to soil and hardening:

The confirmed clones in the rooting media were transferred from *in vitro* to soil for hardening. Hardening is the process of acclimatizing the plants grown in the culture conditions with the outer environments. The plants were gently removed from the rooting media and washed thoroughly under running water to completely remove the

media. The plants were then treated with bavistin, a broad spectrum fungicide for 5 minutes. Further the plants were transferred to small pots containing soil mixture and covered using a plastic cover/glass plate and kept in growth chamber (22°C, 16 hr light and 8 hr dark) for 3 days. Small holes were made initially which was later increased in size as time progressed to ensure that the plants habituate with the outer environment. After 10 days, plants were transferred to bigger pots.

2.9 Subculturing and maintenance of *Phytophthora infestans*:

The A2 strain of Phytophthora infestans was maintained in pea agar media. For preparing 100 ml of pea agar media, 15 grams of frozen peas was crushed using mortar and pestle with 50 ml of distilled water. The mixture was boiled in microwave for 15 mins and filtered through a sieve. 500 mg of glucose and 2 g of agar was added to the mixture and the volume was made to 100 ml. After autoclaving, ampicillin (200 mg/L) and rifampicin (20 mg/L) were added and petridishes were prepared. *P.infestans* was sub-cultured into these pea agar plates by transferring a small block of *P.infestans* containing agar blocks from the old plates. The culture was incubated at 18°C for *P.infestans* growth to occur. After every 15 days the *P.infestans* culture was transferred to fresh pea agar media.

2.10 Disease assessment of transgenics upon infection with *Phytophthora infestans*:

As a further work, eTM160 and eTM166 lines will be infected with *P. infestans* and their disease symptoms would be compared to those developed by the wild-type plants. Briefly, sporangia would be released from *P.infestans* and set to a concentration of 10^5 sporangia/ml. The leaves eTM160, eTM166 and wild-type plants would be treated with the *P.infestans* sporangia. The control group of plants would be treated with water. All the plants would be covered with polybags and incubated at 18°C and 90% relative humidity for the infection to occur. As the disease progresses, the symptoms would assessed in the transgenics compared to the wild-type.

3. RESULTS AND DISCUSSIONS:

3.1 Detection of miRNA and their targets:

In order to confirm the presence of miRNA 160 and *miR166* and their respective targets in Solanum tuberosum Desiree, total RNA was isolated from wild-type Desiree plants by Trizol method (Fig 2 A). As described in materials and methods section, RT-PCR reactions were performed using specific primers to detect *miR160* and its two targets, Auxin Response Factor 10 (ARF10) and ARF16 and *miR166* and its five targets, Incurvata 1 (ICU1), ICU2, Phavoluta 1(PHV1), PHV2, Revoluta (REV). We have successfully detected the two miRNAs in potato (Fig 2B). Also the figure 2C shows that two *miR160* targets and the five *miR166* targets were successfully detected in potato.

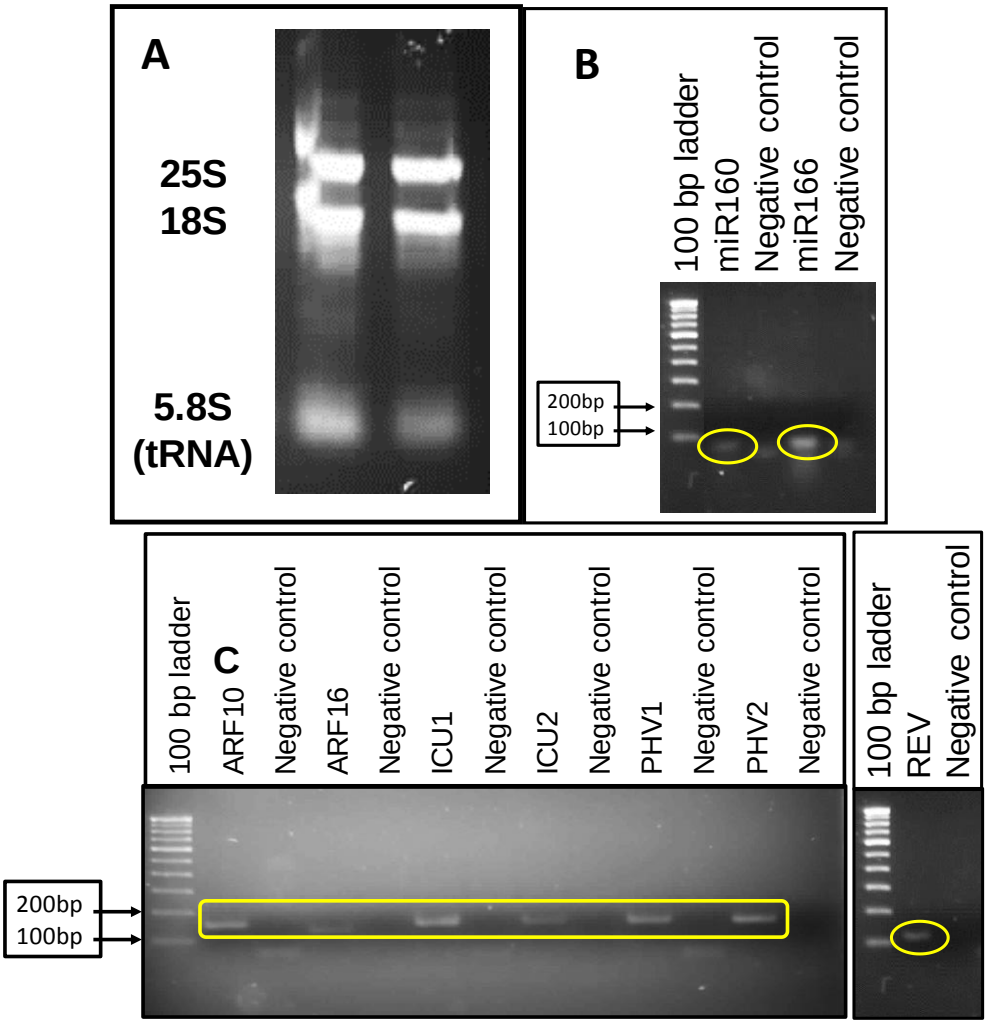


Figure 2: Detection of *miRNA* and their targets. A. RNA isolation from *Desiree*. B. Detection of *miRNA160* and *miRNA166* in wild-type *Desiree* leaves. C. Detection of *miRNA* targets in wild-type *Desiree* leaves. ARF10 and ARF16 are the targets of *miR160* and ICU1, ICU2, PHV1, PHV2 and REV are the targets of *miR166*.

3.2 Confirmation of *Agrobacterium* clones:

eTM160 and eTM166 were previously cloned into pCAMBIA 1300 by Wu et al, 2013. The two constructs were obtained from the authors and mobilized into *Agrobacterium* strain GV2260. In order to confirm the Agrobacterial clones, plasmids were isolated from the colonies that appeared after transformation and PCR reaction was performed using eTM specific primers. Amplicons of desired size were obtained for both eTM160 (180 bp) and for eTM166 (248 bp).

Bands were observed in the gel picture indicating the cloning was successful. Later, Glycerol stock of these constructs prepared and stored in -80°C.

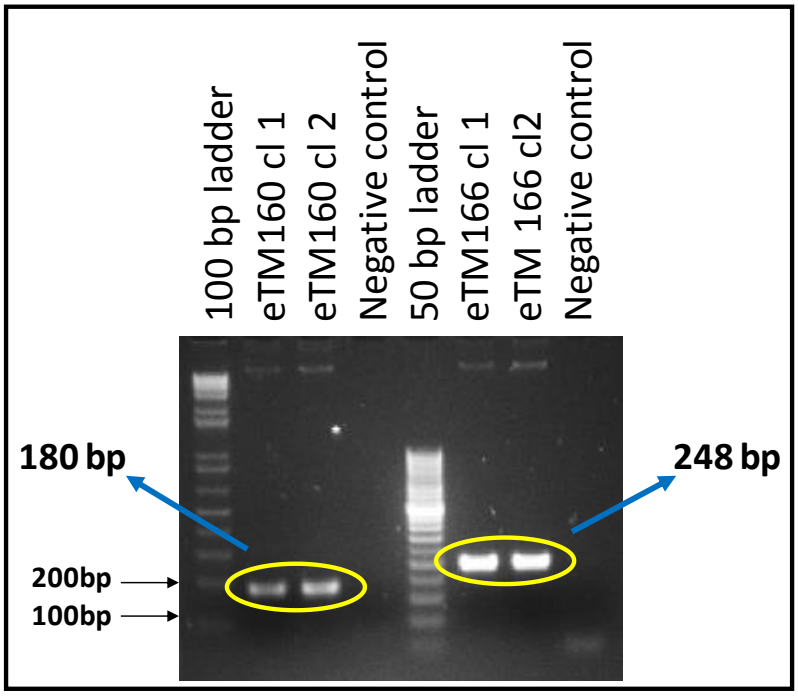


Figure 3: Confirmation of *Agrobacterium* clones by PCR, having eTM160 and eTM166 constructs. eTM160 cl1,2 - ~180bp and eTM166 cl1,2- ~248bp.

3.3 Transformation of *Desiree*:

eTM 160 and 166 constructs were transformed to *Desiree.* Leaves were kept in callus induction media (MGC plates). Fig 4A and 4B indicate the plates (MGC) after 15 days. Callus is observed everywhere on the leaves. Shoots do start appearing. After 15 days, leaves were transferred to shoot induction media (MGS). Fig 4C and 4D indicate the plates (MGS) after 30 days. More shoots were coming up and the increase in their length was observed. Fig 4E and 4F indicate the plates (MGS) after 45 days long enough to subculture in test tubes. Shoots of both the constructs were subcultured in rooting media with specific antibiotic. Fig 5A and 5C indicate the subcultured shoots in rooting media after 25days. Growth of both shoot and root was observed. Later, these clones were subcultured and multiplied in Magenta boxes in order to transfer them to soil (Fig 5B and 5D).

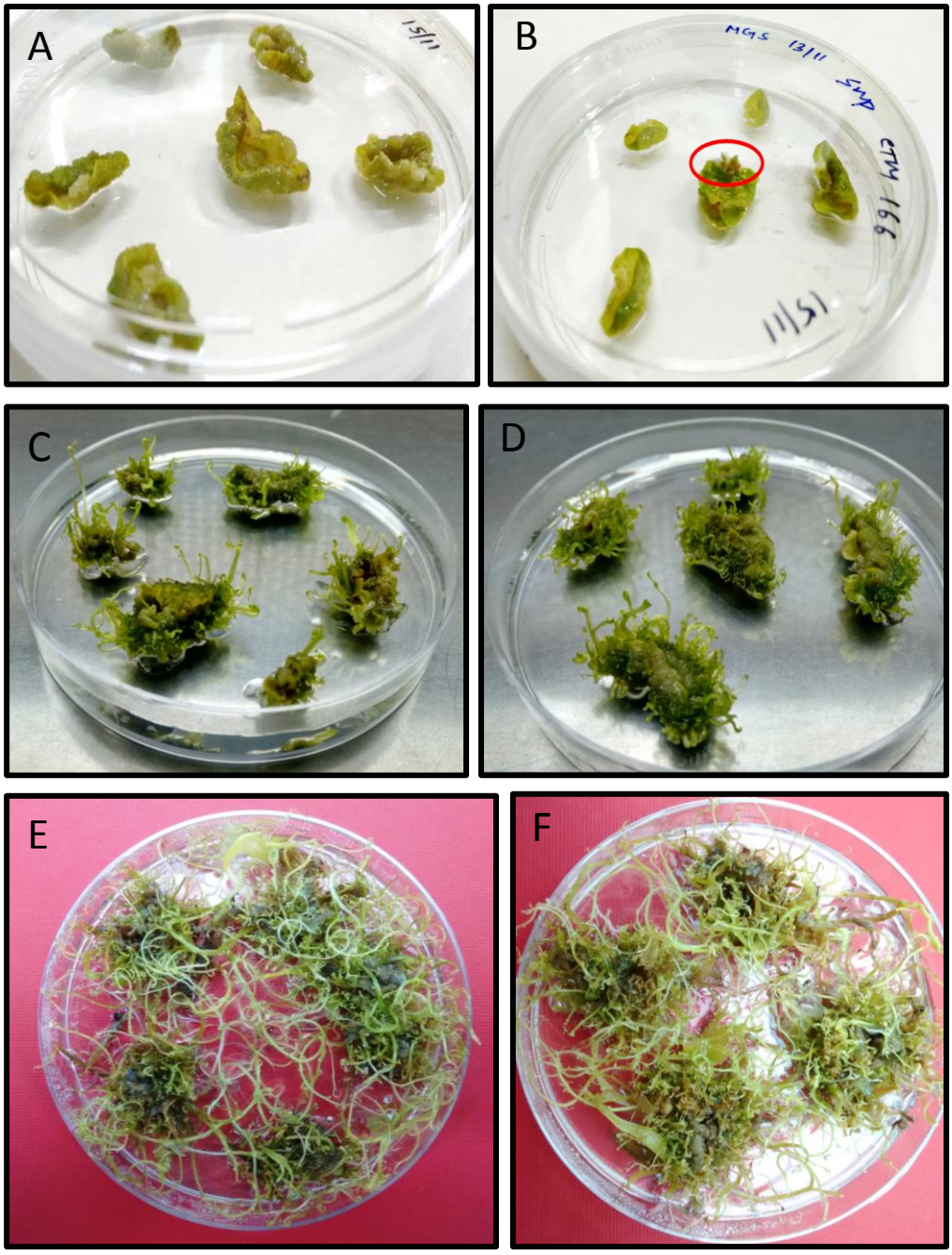


Figure 4: Callus and shoot induction of eTM160 and eTM166 constructs. (A,B)- Leaves in callus induction media after 15 days (A- eTM160, B- eTM166). (C,D)- Leaves in shoot induction media after 30 days (C- eTM160, D- eTM166). (E,F)- Leaves in shoot induction media after 45 days (E- eTM160, F- eTM166).

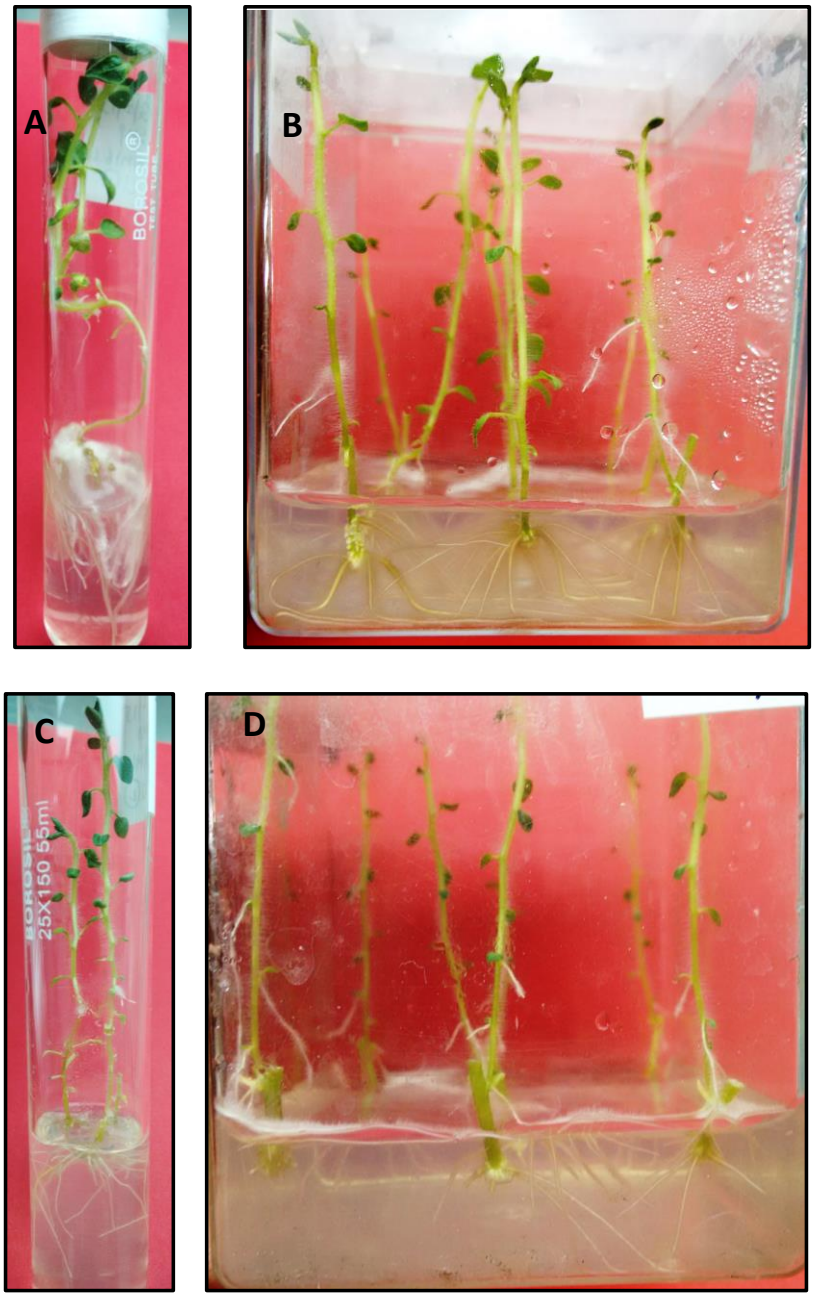


Figure 5: Subcultured shoots in rooting media (testubes) and then multiplied them in magenta boxes. (A,B) – eTM160 clones and (C,D) – eTM166 clones subcultured both in testube and magenta boxes.

3.4 Confirmation of clones:

In order to confirm the transgenics, RNA was isolated from the tissue of both the constructs (Fig 6). RT-PCR reactions were performed to identify the true clones. Out of

six clones tested for each eTM construct, two clones per eTM gave positive results. (Fig 7A, 7B and 7C). The positive clones were named as eTM160-clone 1 and eTM160-clone 2 for knockdown lines on *miR160* and eTM166-clone 1 and eTM166-clone 2 for knockdown lines on *miR166*.

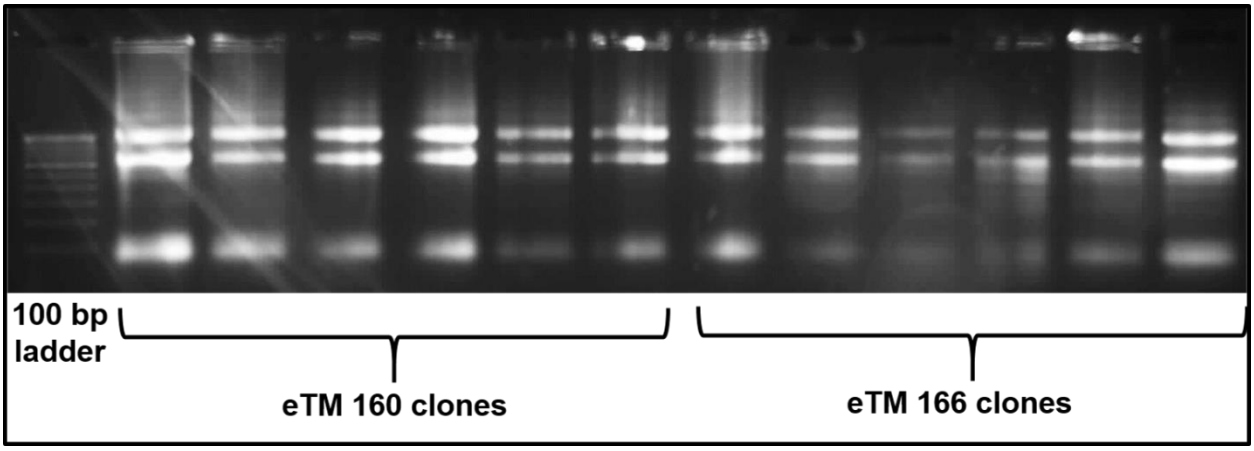
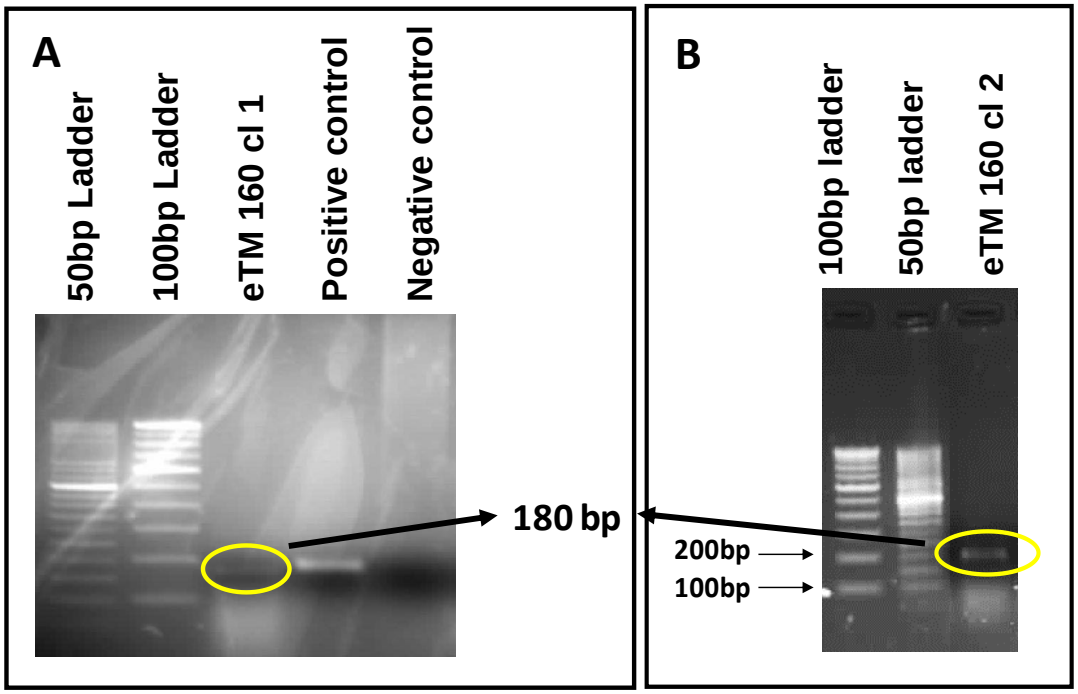


Figure 6: RNA isolation from eTM160 and eTM166 clones. Six clones each were used in the isolation.



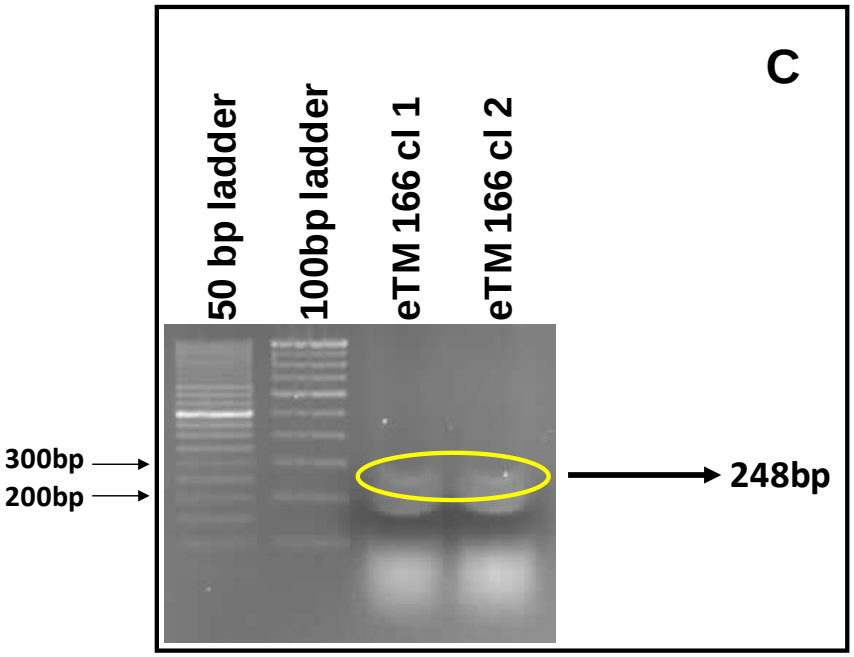


Figure 7: Conformation of the clones. A. Confirmation of eTM160 cl 1 using gene specific PCR. B. Confirmation of eTM160 cl 2 using gene specific PCR. C. Confirmation of eTM166 cl 1, 2 using one Step RT PCR.

3.5 Analyzing the levels of *miR160* and *miR166* in transgenics by qPCR:

To check the relative expression level of *miR160* and *miR166* knock down lines with respect to the wild type of *Desiree*, Real time PCR was performed. The levels of miRNAs were reduced in both the clones of eTM. Around 66% decrease in eTM160 cl 1 and ~91% decrease in eTM160 cl 2 was observed for *miR160* compared to wild type. *miR166* levels were reduced by ~ 96% in eTM166 cl 1,~91% decrease in eTM 166 cl 2 compared to wild type. (Fig 8 and 9).

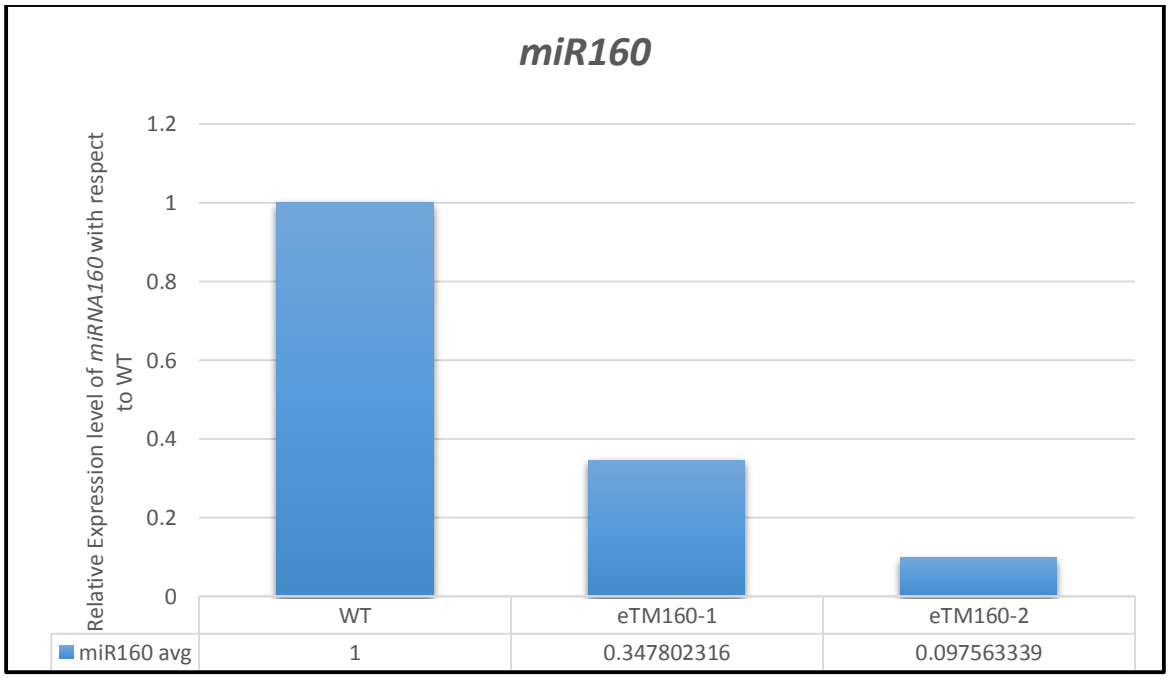


Figure 8: Expression level of *miR160* (cl 1, 2) with respect to WT.

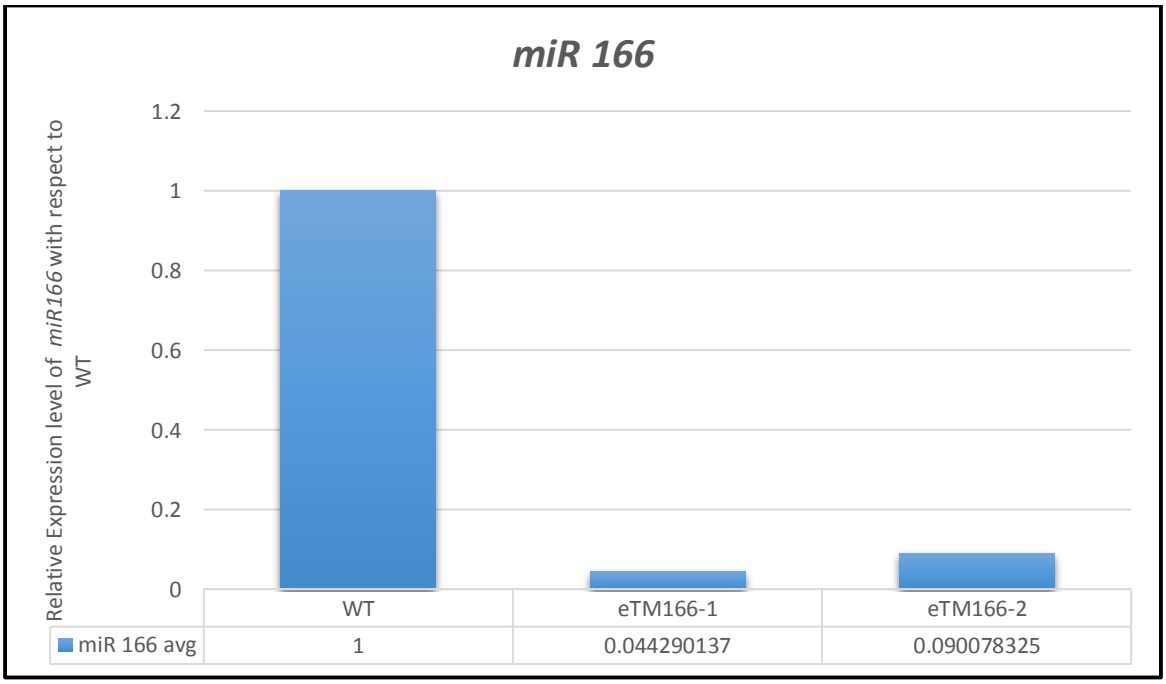


Figure 9: Expression level of *miR166* (cl 1, 2) with respect to WT.

3.6 Hardening of clones in soil:

Confirmed clones of eTM 160 and eTM 166 constructs were hardened by transferring them to soil (Fig 10). The pots were closed with plastic or glass plate and were made to meet the natural environment periodically. These clones are being grown in growth chamber currently and will be subjected to *P. infestans* infection. The disease progression of eTM160 and eTM166 lines will be compared to that of the wild-type plants in order to understand the function of *miR160* and *miR166* during potato-*Phytophthora* interaction.

Figure 10: Clones are being hardened in growth chamber and will be subjected to *P.infestans.*

Not much difference between the phenotypes was seen, but the root pattern in eTM160 cl 1 was different. Root pattern was much more organized with respect to wild type. Further phenotypical changes might occur after we transfer the transgenics to bigger pots (soil) for specific period of time.

SUMMARY:

Salient features of the present investigations are

- *miR160* and *miR166* and their target genes were detected in potato cultivar *Desiree*.

- Both the eTM constructs were mobilized to *Agrobacterium* before the plant transformation were carried out.
- Transgenic lines of eTM160 and eTM166 were developed by *Agrobacterium* mediated transformation technique.
- The clones were confirmed using RT-PCR and successful knock down of eTM160 and eTM166 was achieved in transgenic clones.
- The confirmed lines were transferred to the soil for hardening.
- The transgenic clones are presently being grown in growth chamber conditions.
- These transgenic clones are being subjected to *Phytophthora* infection to understand their role in immunity.

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