Generation of miR156 knockdown lines and expression analysis of putative miRNAs involved in potato tuberization.

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By

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TITLE **PAGE**

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भारतीय विज्ञान शिक्षा एवं अनुसंधान संस्थान, पुणे

INDIAN INSTITUTE OF SCIENCE EDUCATION AND RESEARCH (IISER) PUNE

(An Autonomous Institution of Ministry of Human Resource Development, Govt. of India)

Dr. Homi Bhabha Road, Pune - 411 008.

CERTIFICATE

This is to certify that this dissertation entitled "**Generation of *miR156* knockdown lines and expression analysis of putative miRNAs involved in potato tuberization.**" 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 **Ms. Neeraja Revi** at IISER Pune under the supervision of Dr. Anjan K Banerjee, Associate Professor, Biology Division, IISER Pune during the academic year 2014-2015. No part of this work presented in this report has been produced by her for any other degree from other institutions.

Signature of the Supervisor

Date: 25th March, 2015.

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DECLARATION

I hereby declare that the information provided in the research project titled "Generation of *miR156* knockdown lines and expression analysis of putative miRNAs involved in potato tuberisation" is result of an original work carried out by me in the Biology division of IISER Pune and is true to the best of my knowledge. No part of the work presented in this report has been produced by me for any other degree from any other institution.

Neeraja Revi
20101003

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ABSTRACT

Micro RNAs (miRNAs) are small, non-coding RNAs that regulate different developmental pathways in plants including tuberisation in potato. Previous reports have demonstrated the role of *miR156* and *miR172* in regulating tuberisation pathway in potato, where *miR156* and *miR172* facilitate tuber formation under tuber inductive Short Day (SD) conditions. Functional studies of these miRNAs in potato were carried out earlier by employing over expression strategies. To gain more insights in *miR156* function in potato development, we have attempted *miR156* knockdown approach. In this regard, we have cloned and generated mimicry-*miR156* (*MIM156*) lines in potato and tobacco. MIM-*miR156* transgenic plants were successfully generated and were confirmed by RT-qPCR. Currently, we are analyzing and comparing the *miR156* knockdown phenotype of potato and tobacco transgenics.

Apart from the reports of *miR156* and *miR172* in potato, many reports have demonstrated the presence of numerous miRNAs in potato by employing bioinformatic and deep sequencing approaches. However, their function in potato tuberisation pathway is not known. Based on the previous reports, we have shortlisted few miRNAs with an aim to identify other miRNAs possibly involved in tuberisation pathway. Tuberization being a photoperiod dependent phenomenon, we have carried out SD-LD photoperiod dependent expression pattern of these shortlisted miRNAs, where *miR126, miR394, miR171, miR157* and *miR159* showed differential expression. These miRNAs could potentially be involved in tuberisation pathway awaiting future investigations.

In this report, we aim to understand the miRNA mediated regulation of tuberisation pathway by (i) understanding the role of *miR156* using *MIM156* transgenic approach and (ii) SD-LD dependent expression pattern of different miRNAs in potato. Overall, this work will help to identify the role of other miRNA candidates potentially involved in tuberisation pathway.

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1. INTRODUCTION

Potatoes (Solanum tuberosum) are economically important food crops cultivated mainly in Europe and Asia. It is classified in to two different cultivar groups, Andigenum and Chilotanum. Andigenum is a photoperiod dependent variety and has been found originally in Andes. Chilotanum or the European potato is considered to be originated in Andes and then introduced to Europe (www.potato2008.org). A mature potato plant grows an average of 100cm in height. The plant reproduces through vegetative propagation and is mainly cultivated in cold climates as the optimum temperature for growth is 18-20 degree Celsius(Gregory, 1986).

India is the second largest producer of potatoes in the global market. Also it is the fifth highly cultivated food crop in India after Rice, Wheat, Sugarcane and Mango (FAOSTATS, http://www.faostat.fao.org). The edible part of the plant is a modified stem called tuber, commonly called as potato. It is rich in starch, proteins and fibres (www.potato2008.org). Due it to its nutritional values and the increasing food crisis, potato is considered to be a potential staple diet for poor and developing countries

History on the research on potato plants extends back to almost 7000 years, as early Andean civilization observed wild potatoes and started domesticating them. But extensive plant breeding and crossing for new varieties happened after the famous Irish famine, caused by the blight disease in potato (www.potato2008.org). Over the past few decades potato developed in to a model system for understanding tuber development, flowering, compound leaf development and signal transduction in plants.

The process by which a tuber is developed in a potato plant from a swollen stolon is called tuberisation. It is an ideal model system for studying how gene expressions are regulated in plants during organ development(Raices et al., 2003).It is affected by both exogenous (photoperiod) and endogenous factors (phytohormones, transcription factors, protein kinases etc). Changes in the external environment like day length is perceived by the leaves and is then relayed to stolons as signals for tuberisation. Corresponding changes in the endogenous factors are then brought about by various signal transducers like protein kinases. As a result,

stolons will elongate and swell due to rapid cell division and starch deposition leading to the formation of tubers(Raices et al., 2003).

Most of the commercially available potato varieties are photoperiod insensitive, but wild type potato plants like *Solanum tuberosum ssp andigena* depends strictly on photoperiod for tuberisation(Amador et al., 2001). These plants produce tubers only under short day conditions. It is also called as tuber inductive conditions. Tubers are not observed under long day conditions or short day conditions with a night break (Ewing and Struik.,1992) which are the tuber non inductive conditions. Studies have revealed the role of a phytochrome (PHYB) and gibberellins (GA) to act as negative regulators of tuberisation process in potato(Amador et al., 2001). It was observed that plant containing low levels of PHYB and a biogenesis mutant of Gibberellin promoted tuberisation in both tuber inductive and non inductive conditions. Also upon induced by short day photoperiod, BEL5 transcription factor along with its knox partner POTH1 moves from leaf veins to stolons. It forms a dimer and binds on a specific TTGAC motif in the *ga20oxl* promoter. This down regulates Gibberellin biosynthesis and promotes tuberisation (Mahajan et al., 2012).

FLOWERING LOCUS T (FT) protein is the main component of flowering hormone signal in *Arabidopsis thaliana.* A paralogue of this is present in *Solanum tuberosum ssp andigena*, *SP6A* and is known to have a role in regulating tuberisation. The expression of this gene is strongly up-regulated in leaves and stolons in plants under short day conditions prior to tuberisation. Under long day conditions the expression of *SP6A* is downregulated by the activity of CONSTANS protein mediated by PHYB by an unknown mechanism(Navarro et al., 2011). This was proved by the tuberisation of antisense PHYB plants under long day conditions. Furthermore it is supported by the absence/late induction of tubers in a short day plant over expressing CONSTANS protein.

Sucrose and intracellular calcium levels are also necessary for tuber development. Sucrose is an inducer for tuberisation and it interacts with the phytohormone pathway which down regulates the process of tuberisation. It leads to the influx of calcium ions to the cytoplasm of potato cell (Raices et al., 2003).

Calcium levels are considered to be intracellular responses against changes in the external environment. They are transduced by Calcium dependent protein kinases (CDPKs) as signals for tuberisation. Thus CDPK activities are necessary for the onset of morphological changes associated with a stolon during tuber formation in potato. In potato, CDPKs 1 and 2 are well studied(Gargantini et al., 2009; Giammaria et al., 2011). CDPK 1 is expressed in stolons and sprouting tubers where as CDPK 2 is expressed in leaves and green sprouts.

Though there are several reports about the presence of miRNAs in regulating plant growth and development, it was not extensively studied in potato. miRNAs are non protein coding RNAs which are found in both plants and animals. They are found to have regulatory role in plants where they take part in a variety of functions ranging from basic development of the plant body to defence mechanisms and stress fight. They were first discovered in *C.elegans* in the year 1993 (Lee et al., 1993).After that almost ten years later the first plant miRNA was discovered in Arabidopsis thaliana(Jung, Jae-Hoon, 2009). miRNAs acts on targets by the mechanism of base pairing. Hence they and their target sequences are evolutionarily conserved (Willmann and Poethig, 2007). miRNAs are transcribed from miRNA genes mainly by RNA polymerase II and sometimes by RNA polymerase III (Sun, 2012)**.** The transcription process leads to the formation of primary miRNAs which have a cap and a poly adenyl tail. After the formation of primary miRNAs a protein called DAWDLE (DDL) binds to it and stabilises the whole structure. Mean while another protein called dicer-like 1(DCL1) cuts the ends of primary miRNAs to give rise precursor miRNAs with stem-loop hairpin secondary structures. These are then cleaved by DCL1 and Hyponasty leaves 1(HYL1) into a miRNA duplex in nucleus. Another protein called Hua Enhancer 1 (HEN1) protects this duplex from degradation by methylating its terminal sequences. This duplex is then transported via the nuclear pore to the cytoplasm, where the miRNA is loaded on to the RNA Induced Silencing complex (RISC). At the RISC complex the target mRNA-miRNA pair is cleaved in the middle (somewhere around 11 or 12 th base pair as mature miRNAs are 22 -24 base pair long) by a protein called ARGONAUTE 1 (AGO 1)(Sun, 2012). However the actual mechanism of miRNA duplex transition to mature miRNA is not well understood.

miRNAs act on target mRNAs by base pairing to them. miRNAs mediate target gene regulation via two mechanisms, by cleavage of the target mRNA or by translational inhibition. High levels of complementary base pairing between target mRNA and miRNA is needed for cleavage of the target mRNA to occur. On the other hand if there are any mismatches at the middle region or the 5' region of the miRNA to the target mRNA, target mRNA will not be cleaved by the miRNA(Fahlgren and Carrington, 2010). Instead the target mRNA regulation will be by means of translational inhibition. In that case protein product of the target gene will not be formed as the binding of miRNA prevents translational machinery to operate on it. But the mRNA will still be intact.

Few reports are available on the role of miRNAs in potato development. Recently, *miR156*, one of the most conserved miRNA across plant kingdom, was shown to be involved in the regulation of tuberisation in potato (*Solanum tuberosum*) (Bhogale et al., 2014). In *Arabidopsis*, it is involved in regulating the flowering pathway. Also it was recently discovered that *miR156* plays a role in rendering tolerance against recurring heat stress in Arabidopsis(Stief et al., 2014). In 2013 Eviatar- Ribak et al observed aerial tubers upon overexpression of Arabidopsis *miR156* in Desiree. This was an important discovery as it revealed the role of *miR156* in tuberisation and paved basis for later studies involving the role of *miR156* and tuberisation.

In *Solanum tuberosum ssp andigena,* it is shown to regulate the expression of another miRNA, *miR172* via its target SQUAMOUSA PROMOTER LIKE 9 (SPL9) transcription factor in long day conditions. *miR172* has been demonstrated to act as a positive siggnal for tuberization (Martin et al., 2009). By doing so, it prevents tuberisation in long day conditions (tuber non-inductive). In short day condition (tuber inductive), the accumulation of both *miR156* and *miR172* increases in stolons and facilitates tuberization(Bhogale et al., 2013).

So far, *miR156* studies in potato (*Solanum tuberosum ssp andigena)* have been restricted to generation of over expression lines and characterisation of their phenotype. An obvious question remains unanswered that in knockdown background of *miR156* in potato, what would be the phenotype of the plant? We are interested to

test this question in potato and we have attempted a knockdown approach using target mimicry of *miR156* (*MIM156*).

In plants, most of the miRNAs act on its target by means of cleavage mechanism which requires near perfect matching between target mRNA and miRNA. In target mimicry approach this concept is utilized, where a mismatch between10th-11th base pair is introduced artificially in a sequence, keeping rest of it to be complementary to the miRNA of interest. This leads to the sequestering of miRNA, creating a knock down effect. The artificial sequence is a miRNA complementary sequence except the central bulge, made in between IPS1(Induced Phosphate Starvation1) gene from *Arabidopsis thaliana*(Franco-Zorrilla et al., 2007).

Using the above method, we generated *miR156* knockdown lines in *Solanum tuberosum ssp andigena* (photoperiod sensitive), *Solanum tuberosum ssp andigena cv Desiree* (photoperiod neutral) and in *Nicotiana tabaccum (*a related species), in order to characterize and compare various morphological traits across photoperiod-dependent and related species.

Even though several insilico analysis were successful in predicting potential miRNAs in potato (Xie et al., 2011; Zhang et al., 2009; Yang et al., 2010), extensive research to validate and understand their function in the plant was not widely pursued. But recently, by employing high throughput sequencing, numerous conserved and potato specific miRNAs were discovered.(Zhang et al., 2013; Lakhotia et al., 2014). It was found that in *Solanum tuberosum ssp andigena*, 28 families of conserved miRNAs and 120 potato specific miRNAs are present.(Zhang et al., 2013). Also, in another cultivar of potato*,* 33 families of conserved miRNAs and 147 potato specific miRNAs were identified(Lakhotia et al., 2014). The authors were able to identify miRNAs and generate their expression profiles in the model plant, *Solanum tuberosum cv* Kufri Chandramukhi, in three vegetative tissue types and four stages of tuber development using high throughput sequencing. However, *Solanum tuberosum cv* Kufri Chandramukhi being a photoperiod neutral plant, it was interesting to screen for miRNAs in a photoperiod dependent cultivar (*Solanum tuberosum ssp andigena*) in same tissue types to understand their functions if any.

Since potato tuberisation is photoperiod dependent, we screened miRNAs in an SD-LD background.

To shortlist the miRNA potentially involved in tuberisation pathway, we selected miRNAs based on several factors including their expression and relative abundance in different stages of tuber transition as mentioned in previous report (Lakhotia et al., 2014). For example we screened miR164 due to its higher levels of expression in leaves and stem and little or no expression in stolons and during tuberisation. Table 1 describes selected miRNAs and their expression pattern in Solanum tuberosum cv chandramukhi.

miRNA	Criteria for selection
miR164_1	Higher levels of expression in leaves and stem and little or no expression in stolons.
miR171_2	Higher abundance in leaves and lower abundance in roots
miR396_1	Slightly increased expression in stolons during initiation of tuberisation and decreased expression in later stages.
miR394_1	Abundant in stem but decreased expression in tuber stages.
miR399_1	Abundant in root and found as graft transmissible.
miR398_1	Higher level of expression in root and moderate levels of expression in leaves and stem.
miR53	Potato specific miRNA with higher sequencing abundance
miR34	Decreased expression in all the stages of tuber development.
miR72	Displayed highest sequencing frequencies in stem and significant change in expression level during tuberisatio
miR111	Slightly increased expression in stolons during initiation of tuberisation and decreased expression in later stages.
miR152	Relatively high expression during tuberisation in stolons
miR18	Potato specific miRNA
miR19	Potato specific miRNA
miR126	Potato specific miRNA
miR100	Potato specific miRNA
miR102	Potato specific miRNA

miR157	Displayed relatively low abundance in roots but higher abundance in stolons and leaves.
miR159	Highest expression in stem and relatively low expression in leaves and root.

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Table 1: List of short listed miRNAs and their criteria for selection for miRNA screening work in *Solanum tuberosum ssp andigena*

Considering tissue specific differential expression of miRNAs, we hypothesized their possible involvement in tuberisation pathway. To understand the miRNA mediated regulation of tuberisation, the present study includes (i) *miR156* function in potato using knock down lines of *miR156*, an important regulator of tuberisation and (ii) the differential expression of some of the novel and conserved miRNAs in potato under SD-LD photoperiod.

In this regard, following are the objectives of our study:

- Cloning and generation of *MIM156* lines in *Solanum tuberosum ssp andigena* (photoperiod sensitive), *Solanum tuberosum cv Desiree* (photoperiod neutral) and *Nicotiana tabaccum* (a related species in the same family).

- Analysis of *miR156* expression levels across *MIM156* transgenic clones for their confirmation.

- Validation of shortlisted novel and conserved miRNAs in *Solanum tuberosum ssp andigena.*

- Generation of SD-LD photoperiod dependent expression profile of shortlisted novel and conserved miRNAs in *Solanum tuberosum ssp andigena.*

2. MATERIALS AND METHODS

2.1) Plant material

Solanum tuberosum ssp andigena 7540, Solanum tuberosum cv Desiree and *Nicotiana tabaccum* invitro plants were used for this study.

2.2) Plant growth conditions

Plants used for *miR156* knockdown study was grown under long day conditions (LD, 16 hours light and 8 hours dark) inside growth incubators from Percival scientific at 25±1ºC. Plants were propagated from single node stem cuttings on Murashige and Skoog basal Medium for 4 weeks before collecting explants for *Agrobacterium* mediated transformation.

For the miRNA expression analysis study, *Solanum tuberosum ssp andigena* plants were induced under both short day and long day conditions in environmental chambers for 15 days. Different tissues (leaf, stem, stolon) were collected after 15 days post induction.

2.3) Cloning of *MIM156*

2.3.1) Amplification of full length *MIM156*

MIM156 (target mimicry construct for *miR156*) construct cloned in pGREEN was obtained from National Arabidopsis Stock Centre. In order to clone *MIM156* in binary vector pBI121, the following gene specific primers were used for generating appropriate restriction sites.

Name	Sequence 5’ – 3’	Restriction sites (in red)
MIMpBI_FP	GAGACCCGGGAAAACACCACAAAAACAAAAGA	*XmaI*
MIMpBI_RP	GAGAGAGCTCAAGAGGAATTCACTATAAAGAG	*SacI*

Table 2: List of gene specific primers degined to amplify *MIM156*. MIMpBI_FP:

Forward primer with *XmaI* restriction sites and MIMpBI_RP: Reverse primer with *SacI* restriction sites.

Using the above primers, Polymerase Chain Reaction (PCR) amplification was performed with the following program: initial denaturation at 94^0C for 30sec, 40 cycles of denaturation at 94^0C for 30sec, annealing at 57^0C for 30sec and extension at 68^0C for 30sec, and final extension at 68^0C for 5min. The reaction mixtures were held at 4^0C. The PCR products were then resolved on a 2% agarose gel by electrophoresis. PCR products were then digested with *SacI* and *XmaI* restriction enzymes and subsequently PCR purified using PCR purification kit (Qiagen).

2.3.2) Preparation of binary vector**.**

In order to prepare vector backbone, the pBI121 plasmid was extracted using Alkaline lysis method. The plasmid was then double digested with *SacI* and *XmaI* restriction enzymes, and the reaction was resolved using 1.5% agarose gel by electrophoresis.

2.3.3) Generation of *MIM156* over-expression construct

The PCR purified product of *MIM156* was ligated to the linearised pBI121 vector backbone using T4 DNA ligase (NEB Labs). The ligated product was later used to transform DH5α *E.coli* competent cells. And it was confirmed by colony PCR, using a single colony as template DNA per reaction. The 35S::*MIM156* in pBI121 binary vector was then used to transform *Agrobacterium* GV2260 competent cells. This construct was confirmed by PCR using the same primers mentioned in Table 1. PCR amplification was performed with the following PCR program: initial denaturation at 94^0C for 30sec, 40 cycles of denaturation at 94^0C for 30sec, annealing at 57^0C for 30sec and extension at 68^0C for 30sec, and final extension at 68^0C for 5min. The reaction mixtures were held at 4^0C. The PCR products were then resolved on 1.5% agarose gel by electrophoresis. The construct was also confirmed by sequencing

2.4) Generation of transgenic plants

In order to generate *MIM156* transgenic lines, *in vitro* plants of *Nicotiana tabaccum, Solanum tuberosum ssp andigena and Solanum tuberosum cv Desiree* were raised at 25 ±1°C under long day conditions (16 hours light and hours dark).
N. tabaccum leaves were transformed as described by Horsch et al., 1985
Solanum tuberosum cv Desiree and *Solanum tuberosum ssp andigena* were transformed as described by Banerjee et al., 2006.

2.5) Validation of *MIM156* transgenic lines

MIM156 transgenic lines were validated using one step reverse transcription PCR using Superscript III RT platinum taq mix enzyme. Kan R_FP and NOSTR_Scr primers (Table 2) were used to amplify the kanamycin gene from pBI121 binary vector using the following PCR program. The reaction mixture was kept at 50ºC for 30 min followed by initial denaturation of 94ºC for 5 min. Then 35 cycles of 94ºC for 15 min, 50ºC for 15 sec and 68ºC for 30 sec was followed. After a final extension of 68ºC for 5 min, the reaction mixture was held at 4ºC.

NOSTR_ Scr	GCAACAGGATTCAATCTTAAG
Kan R_FP	GGATTGCACGCAGGTTCT

Table 3: Primers used to amplify kanamycin gene in pBI121 vector

2.6 Analysis of *miR156* levels in *MIM156* transgenic lines

Levels of *miR156* in the transgenic lines were determined by stem-loop qRT-PCR. Total RNA was isolated from *MIM156* lines using Invitrogen trizol reagent as per manufacturer's instruction. Mature *miR156* was detected by stem-loop RT-PCR as described earlier (Varkonyi-Gasic et al., 2007) using *miR156*STP. Quantitative PCR (qPCR) for *miR156* was performed using *miR156* forward primer and universal reverse primer in a Mastercycler ep realplex machine (Eppendorf). 5S ribosomal RNA levels were also analysed and used as an internal control. All the PCRs were performed at 95°C for 5 min followed by 40 cycles of 95°C for 5 s, 60°C for 10 s, and 68°C for 8 s followed by a melting curve. qRT-PCR data was then analysed using

2^(-ΔΔCt) method described by Livak and Schmittgen in 2001.

2.7) Screening for novel and conserved miRNAs in *Solanum tuberosum ssp andigena.*

For screening novel miRNAs, a list of evolutionarily conserved and potato specific miRNAs were made based on the findings of Lakhotia et al., 2014. In order to obtain expression profile of these miRNAs in *Solanum tuberosum ssp andigena ,* two vegetative tissues (stem, leaves) and stolons of plants, grown in short day and long day conditions, were collected , grounded and stored at -80^{0}C. RNA was isolated from these tissue samples (using Invitrogen trizol reagent as per manufacturer's instruction). It was followed by cDNA synthesis using a Reverse transcription PCR (RT-PCR) with stem loop primers of potential miRNAs (Varkonyi-Gasic et al., 2007). An end point PCR with respective forward and universal reverse primer was then performed and the products were resolved on a 3% agarose gel by gel electrophoresis.Primer details are given in Table 3 and 4 respectively. Expression analysis of all the validated miRNAs were then carried out using real time quantitative PCR. All the PCRs were performed at 95°C for 5 min followed by 40 cycles of 95°C for 5 s, 60°C for 10 s, and 68°C for 8 s. qRT-PCR data was then analysed using 2^(-ΔΔCt) method described by Livak and Schmittgen in 2001. 5SrRNA and *miR172* were used as internal and positive controls respectively.

Details of the primers used for reverse transcription PCR and endpoint PCR is given below in Table 4 and Table 5 respectively.

miRNAs	Stem loop primer sequence (5'-3')
miR164_1	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTGCACG
miR171_2	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGATATT
miR396_1	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCAGTTC
miR394_1	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGGAGGT
miR19	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCAATCA
miR126	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTTGAGT
miR157	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGTGCTCTC

miR159	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTAGAGC
miR100	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTTGAAG
miR102	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAGAGTC
miR34	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCTTCCA
miR53	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACACCCAA
miR72	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCTTAGA
miR399_1	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCGGGGC
miR398_1	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCAGGGG
miR111	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCAAAT
miR18	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCAATCA
miR152	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAATTCC

Table 4: List of stem loop primers used for detecting miRNAs by stem loop RT-PCR.

Primer	Primer sequence (5’-3’)
miR164_1 FP	CGGCGTGGAGAAGCAGGGCA
miR171_2 FP	CGGCGTGATTGAGCCGTGCC
miR396_1FP	GCGGCGTTCCACAGCTTTCTT
miR394_1FP	GCGGCGTTGGCATTCTGTCC
miR19 FP	GCGGCGGATCCCCTGGAGAAT
miR126 FP	GCGGCGTATCTAGGTGTGCAT
miR157 FP	CGGCGGCTTGACAGAAGATA
miR159 FP	CGGCGGTGGATTGAAGGGA
miR100 FP	GCGGCGTATGTTCCTCGGACT
miR102 FP	GCGCCGAAGCTATGTTGCACG
miR34 FP	GCGGCGTCTGTGACAGGATAA
miR53 FP	GCGGCGTTCCATGAGACTGTTT
miR72 FP	GCGGCGTTGGTTGAGTGAGCA
miR399_1FP	GCGGCGTGCCAAAGAAGATTT
miR398_1FP	GCGGCGTATGTTCTCAGGTCG
miR111 FP	GCGGCGAACATCTCCAGCCAT
miR18 FP	GCGGCGGATCCCCTGGAGAAT
miR152 FP	GCGGCGTATCTGAGTAGCATAG

Univ RP	AGTGCAGGGTCCGAGGT

Table 5: List of forward and reverse primers used for end point PCR and qRT-PCR

3. RESULTS

3.1) Amplification of full length *MIM156*.

MIM156 (target mimicry construct for *miR156*) is the IPS1 gene containing an artificial motif complementary to *miR156* with a central mismatch. This construct cloned in pGREEN was obtained from National Arabidopsis Stock Centre. Full length *MIM156* was then amplified and appropriate restriction sites were incorporated using MIMpBI_ FP and MIMpBI_RP.

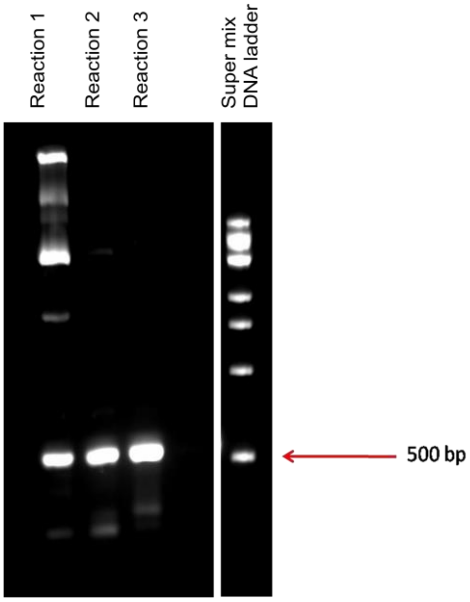


Figure 1: PCR amplification of full length *MIM156* from pGREEN construct. The arrow indicates the 500 base pair band in a super mix DNA ladder

3.2) Preparation of binary vector.

In order to prepare vector backbone, the pBI121 plasmid was extracted and linearised using double digestion with *SacI* and *XmaI* restriction enzymes. The reaction was resolved on 1.5% agarose gel by electrophoresis shown in Figure 2.

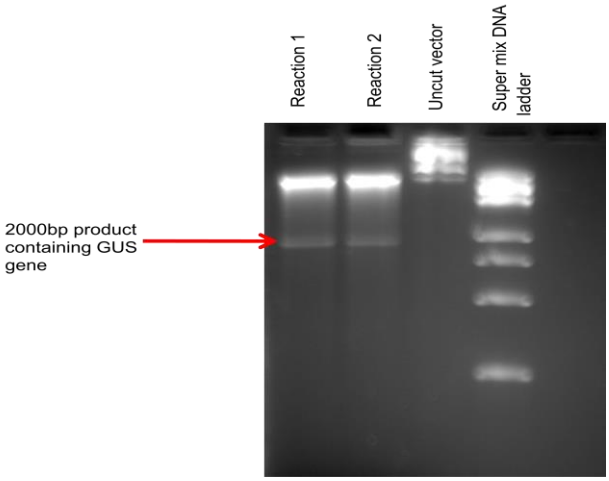


Figure 2: Restriction digestion reactions (Reaction 1 and Reaction 2) of pBI121 binary vector with *SacI* and *XmaI* restriction enzymes to develop vector backbone. Uncut vector is used as a positive control.

3.3) Generation of *MIM156* over-expression construct

The PCR purified product of *MIM156* (Figure 3) was then ligated to the digested pBI121 vector backbone using T4 DNA ligase (NEB Labs). The ligated product was used to transform DH5α *E.coli* competent cells. And it was confirmed by colony PCR, gel image of which is shown in Figure 4. Later *Agro* GV 2260 cells competent cells were transformed using the same vector (*MIM156* in pBI121) and were confirmed by a PCR amplification of *MIM156* (Figure 5) and restriction digestion (Figure 6) of the vector. It was later confirmed by sequencing also.

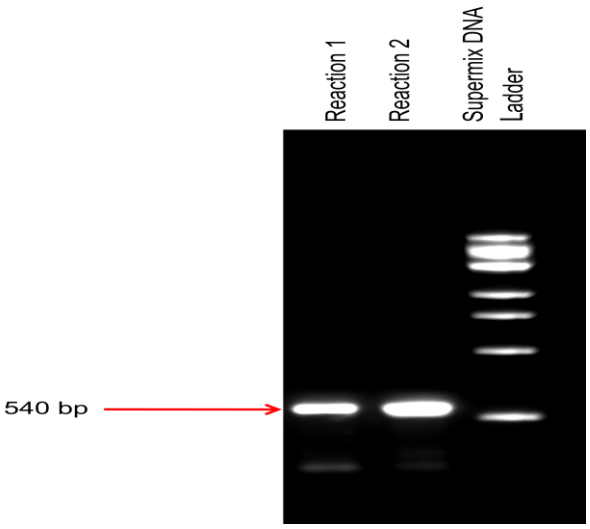


Figure 3: PCR purified *MIM156* construct (Reaction 1 and Reaction 2) using PCR purification kit, Qiagen as per manufacturer's instruction.

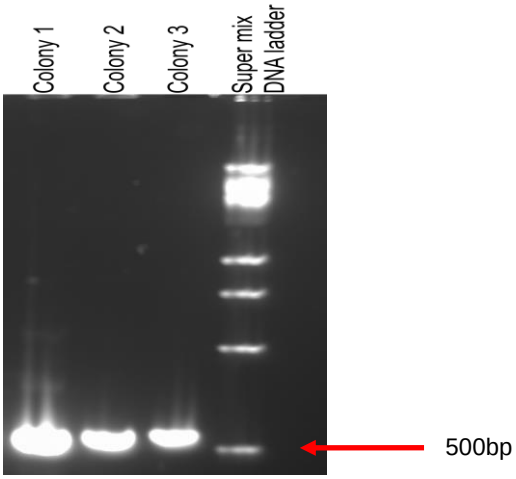


Figure 4: Gel image of confirmation of DH5α *E.coli* clones containing *MIM156* by colony PCR using single colony as a template per PCR reaction.

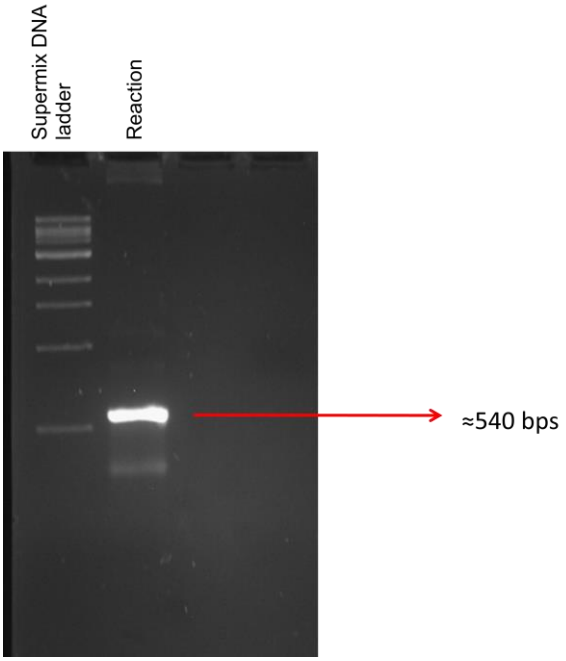


Figure 5: Confirmation of *MIM156* construct in *Agro* GV2260 cells using PCR amplification.

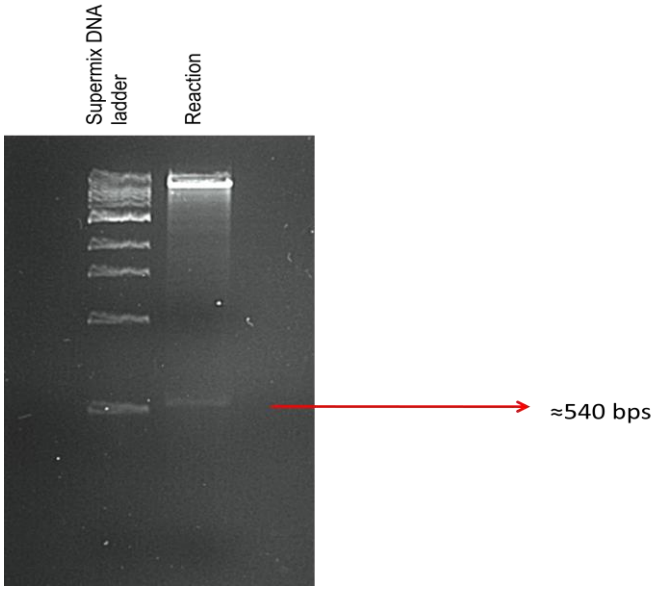


Figure 6: Confirmation of *MIM156* construct in *Agro* GV2260 cells using double digestion with restriction enzymes *SacI* and *XmaI*.

3.4) Generation of *MIM156* transgenic lines.

N. tabaccum leaves were transformed as described by Horsch et al., 1985. The leaves were maintained in regeneration medium for 4-6 weeks till shoots started coming from the leaf discs. These shoots were then transferred to root inducing medium. After 4 weeks the transferred shoots started rooting in the root inducing medium.

Figure 7: Transformed shoots regenerated from *N. tabaccum* leaves in selection media with *Agrobacterium tumefaciens* GV2260 containing *MIM156* construct.

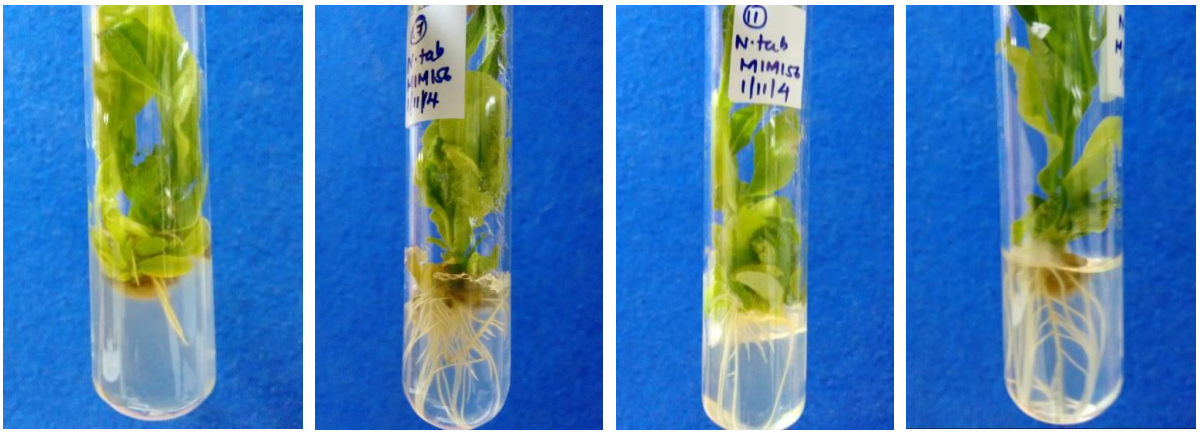


Figure 8: Regenerated shoots transferred to root inducing media after 4 weeks. Four clones, clone 11, clone 15, clone 17 and clone 18 displayed enormous.

Desiree and *Solanum tuberosum ssp andigena* were transformed as described by Banerjee et al., 2006. The *Desiree* transgenic lines are currently in shoot inducing media and will be soon transferred to the rooting media.

Transformation of *Solanum tuberosum ssp andigena Agrobacterium tumefaciens* GV2260 containing *MIM156* construct is in progress.

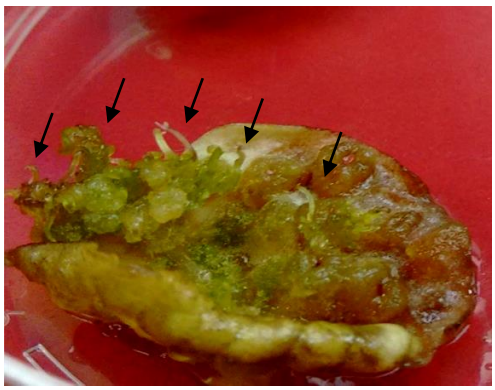
(A)  (B) 

Figure 9: *Solanum tuberosum ssp andigena* leaves after 3 weeks (A) 5 weeks (B) in shoot inducing medium. Regenerated shoots rising from leaf disc is pointed with black arrows (B)

3.5) Confirmation of *MIM156* transgenic Tobacco lines

Four *MIM156* clones which displayed rooting upon transferred to a root inducing medium was confirmed to be true knockdowns of *miR156* by one step RT PCR of kanamycin transgene. The PCR program followed is mentioned in section 2.5 of materials and methods.

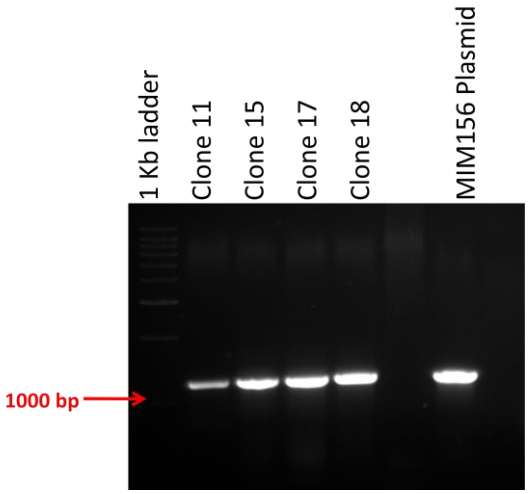


Figure 10: One step reverse transcription PCR amplification of Kanamycin transgene confirming positive *MIM156* clones.*MIM156* plasmid is used as a positive control.

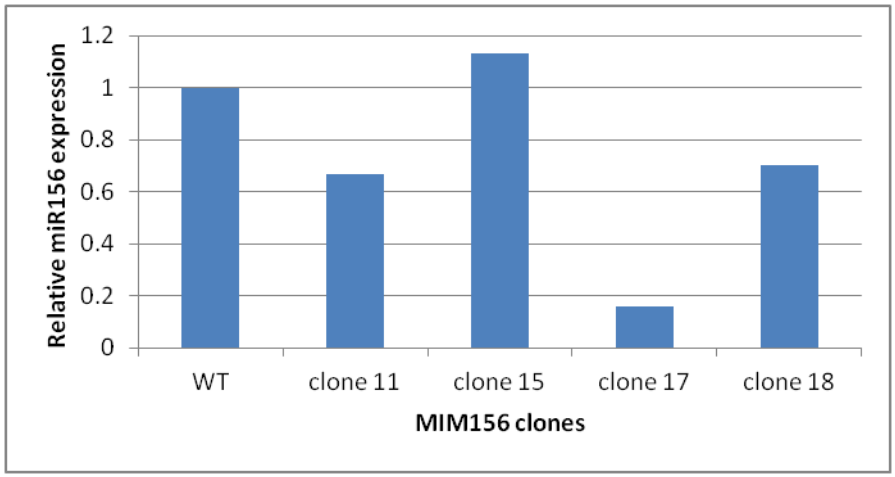


Figure 11: Preliminary analysis of *miR156* expression levels in *MIM156* clones using qRT-PCR. A single biological replicate and three technical replicates per clone were used for this study

3.6) Validation of shortlisted miRNAs in potato

For primary screening of potential miRNAs, total RNA was isolated using trizol reagent and used for reverse transcription PCR. An endpoint PCR was later carried out to validate the presence of miRNAs.

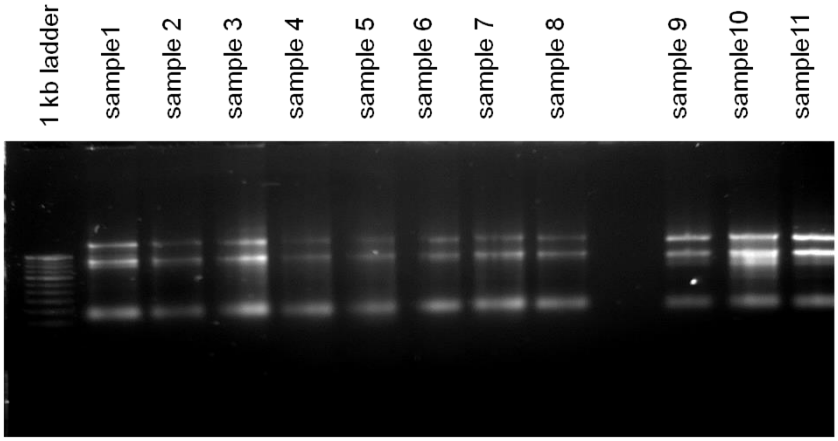


Figure 12: Gel image showing total RNA isolation from whole plant tissue of *Solanum tuberosum ssp andigena.*

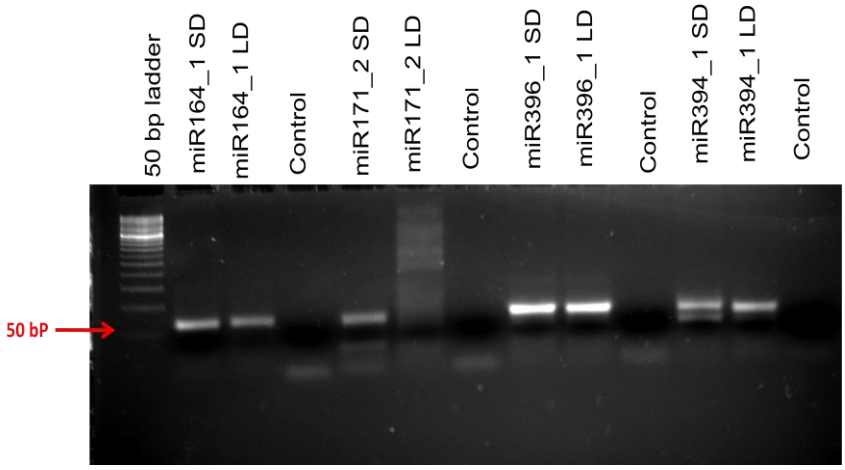


Figure 13: Detection of miRNAs miR164_1, miR171_2, miR396_1 and miR394_1 using end point PCR in Soalnum tuberosum ssp andigena plants grown under shortday (SD) and Long day (LD) conditions

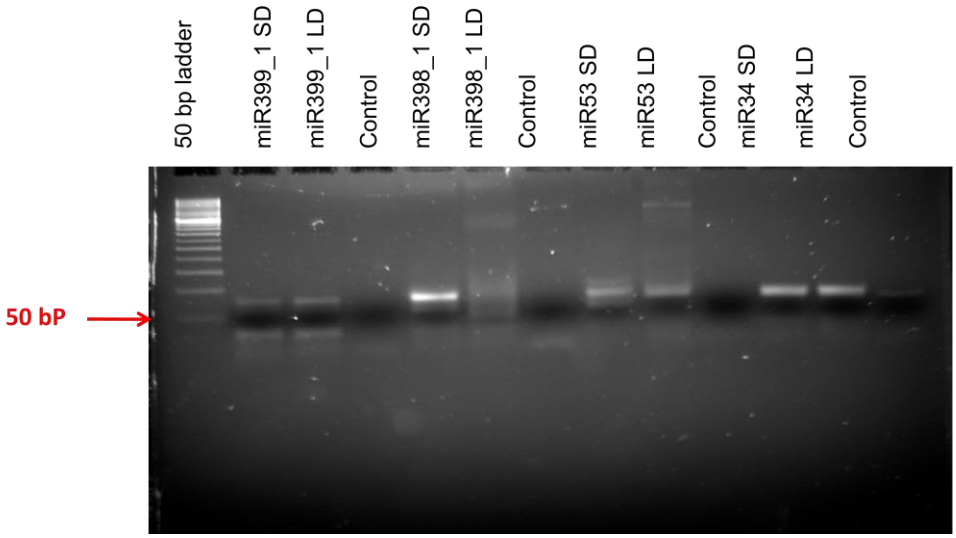


Figure 14: Detection of miRNAs miR399_1, miR398_1, miR53 and miR34 using end point PCR in *Soalnum tuberosum ssp andigena* plants grown under Short Day (SD) and Long day (LD) conditions.

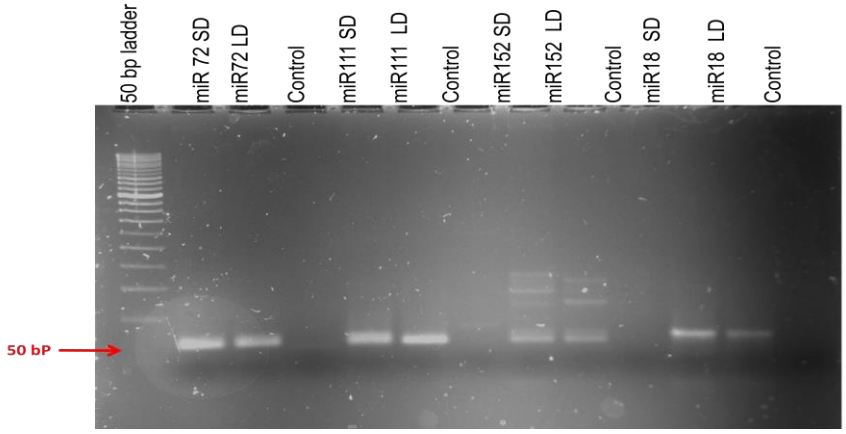


Figure 15: Detection of miRNAs miR72, miR111, miR152 and miR18 using end point PCR in *Soalnum tuberosum ssp andigena* plants grown under short day (SD) and Long day (LD) conditions.

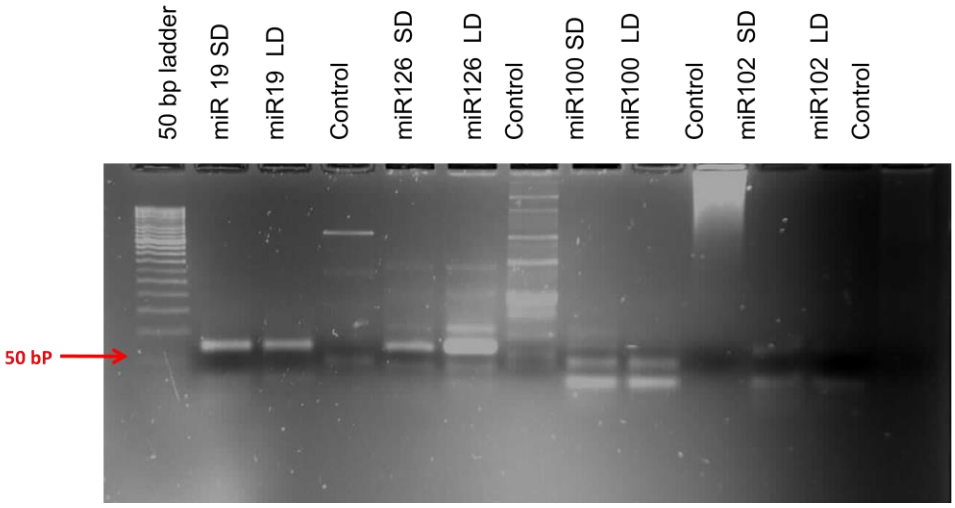


Figure 16: Screening for miRNAs miR19, miR16, miR100 and miR102 using end point PCR in *Soalnum tuberosum ssp andigena* plants grown under short day (SD) and Long day (LD) conditions.

3.7) SD-LD photoperiod dependent expression profile of miRNAs in *Solanum tuberosum ssp andigena*

The efficiency analysis of qRT-PCR amplification for all the shortlisted miRNAs was carried out. The standardization efficiencies are given in Table 6

miRNA	Efficiency	R^2
miR164_1	83.6%	0.876
miR171_2	106.1%	0.968
miR396	103%	0.88
miR394	73.33%	0.992
miR19	104.7%	0.80
miR126	98.3%	0.82

Table 6: Standardisation of efficiency of amplification for all the miRNAs validated in *Solanum tuberosum ssp andigena.*

A preliminary miRNA expression profile of shortlisted miRNAS were generated on an SD-LD basis in *Solanum tuberosum ssp andigena* leave tissue samples. Experiments using more biological replicates and generation of expression profile in stolon tissue are currently under progress.

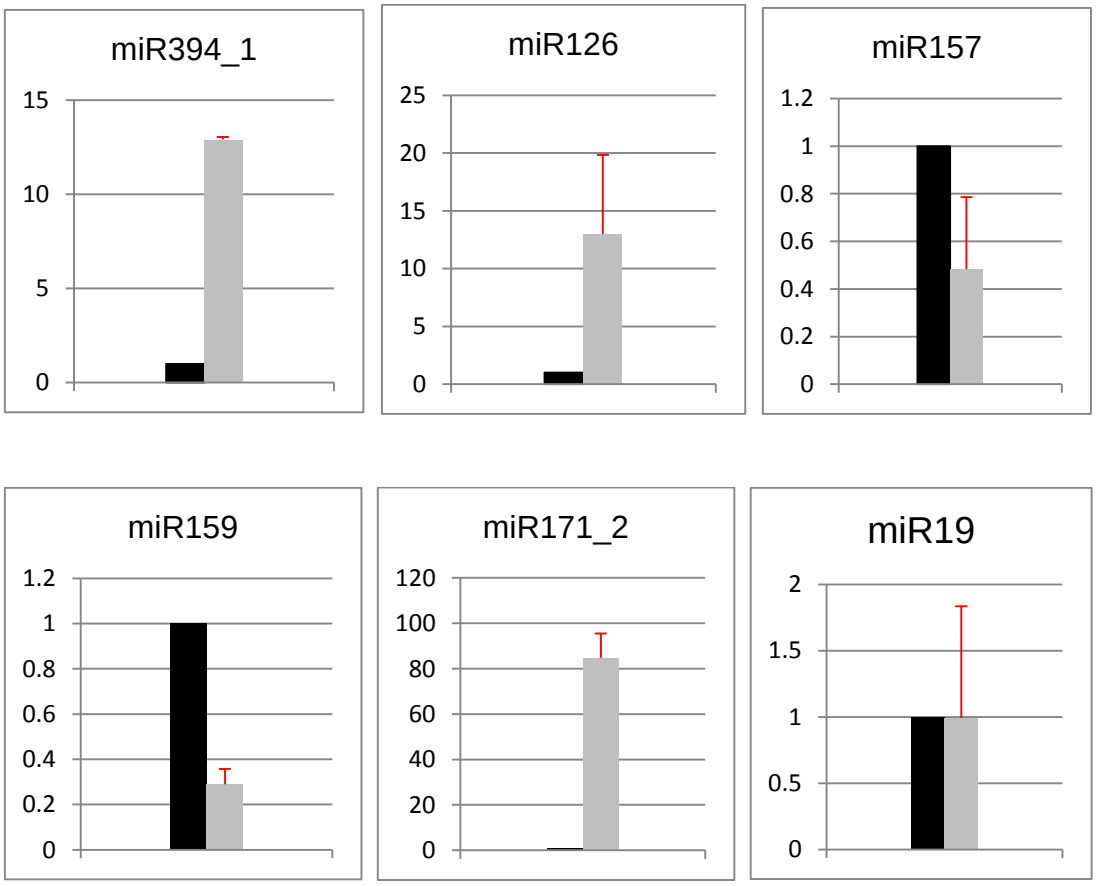


Figure 17: miRNA expression profile of *Solanum tuberosum ssp andigena* SD-LD leaf samples collected after 15 days post induction. Preliminary results shown here are from two biological replicates with four technical replicates per miRNA and the error bar shown is the standard deviation

4. DISCUSSION

miR156 is one of the most conserved miRNA across plant kingdom (Stief et al., 2014) . It is also one of the most well studied miRNA. It has roles in regulating diverse plant phenomenas like temporal regulation of shoot maturation to heat stress tolerance in plants (Wu and Poethig, 2006; Stief et al., 2014).

Earlier in 2014, Bhogale et al demonstrated the role of *miR156* in regulating the process of tuberisation in *Solanum tuberosum ssp andigena* 7540 plant. The authors were able to generate over expression lines of *miR156* in *Solanum tuberosum ssp andigena* and observed drastic phenotypes like aerial tubers, change in leaf morphology and shoot branching pattern etc. However, there is no information on *miR156* knockdown in *andigena* plants. Such a study would possibly lead to the identification of any parallel players/ pathways for tuberisation in potato.

Thus one of our aim was to generate knock down lines of *miR156* in *Solanum tuberosum ssp andigena 7540* (photoperiod sensitive), *Solanum tuberosum cv Desiree* (photoperiod neutral) and *Nicotiana tabaccum* (photoperiod neutral) and to understand and compare their phenotypes under different photoperiods.

miRNAs are used to knockdown target genes due to their ability to cleave/translationally inhibit target mRNAs by means of base pairing. So in order to conduct miRNA knockdown studies, standard knockdown approaches like RNA interference (RNAi) cannot be employed as there are high chances that the interfering RNA will be cleaved by the miRNA of our interest. But a complementary sequence of the miRNA with central/5' mismatches can sequester the miRNA and thereby prevent it from going and binding to the target sequence. This approach generates a knock down effect of the miRNA of our interest. But it is equally possible that the sequence which we are using to sequester the miRNA, could lead to the generation of some phenotypes in the plant due to its own expression. In order to avoid this drawback, we have used target mimicry aproach(Franco-Zorrilla et al., 2007).

We overexpressed a non-coding, non cleavable RNA-*Mimic of miR156* (*MIM156)* construct in *Solanum tuberosum ssp andigena* to obtain *miR156* Knockdown. This construct was cloned under 35S promoter in binary vector and used for generating *miR156* knockdown lines.

We were successful in generating *miR156* knock down lines in *N.tabaccum* using *MIM156* construct. Shoots emerged from explants after 3 weeks in regeneration medium (Figure 5A) and were then transferred to root inducing medium. Extensive root growth was observed after transferring to root inducing medium (Figure 5B).

From the transgenic plants selected after root induction, four were confirmed to be true *MIM156* clones after checking the amplification of kanamycin transgene (Figure). These clones, clone 11, 15, 17 and 18 were then propagated invitro and the levels of *miR156* were measured. Out of the four clones, clone 17 displayed maximum inhibition of *miR156*. Comparing to the wild type, *miR156* levels in clone 17 was lowered by more than 80%. In clone 11 and 18, the level of inhibition was approximately 30% as compared to wild type. In Clone 15, *miR156* levels were comparable to the wildtype and is not to be considered as a *MIM156* transgenic.

Using *MIM156* construct*, Desiree* and *Solanum tuberosum ssp andigena* were also transformed as described by Banerjee et al., 2006. The *Desiree* transgenic lines are currently in shoot inducing medium and will be soon transferred to the rooting media. Transformation of *Solanum tuberosum ssp andigena* using *Agrobacterium tumefaciens* GV2260 containing *MIM156* construct is currently under progress.

We were also interested in screening some potato specific and conserved miRNAs in *Solanum tuberosum ssp andigena* in a photperiod dependent and tissue specific manner. We did primary analysis of 16 potential miRNAs and were able to validate the presence of 14 miRNAs in *Solanum tuberosum ssp andigena*. Our findings indicated that most of the miRNAs were expressing under both short day and long day conditions except a few which only expressed under short day conditions. Out of the total 14 miRNAs detected in *Solanum tuberosum ssp andigena*, 12 miRNAs were expressing under both short day and long day

conditions. The other two miRNAs, miR171_2 and miR398_1 were detected only under short day conditions.

Relative expressions of these miRNAs were studied using a qRT-PCR analysis and an expression profile was generated based on tissue specific manner. We chose to analyse the expression levels of different miRNAs mainly in leaf samples and stolons. Leaf is where the signals are received and stolons are where, the signals are processed to form tubers. Thus understanding the levels of miRNAs in both the tissue types is important.

From the preliminary results we were able to conclude that miR171_2, miR394_1 and miR126 are highly expressed under short day conditions comparing to their expression under long day conditions in leaf samples. While miR157 and miR159 expression levels are higher in long day conditions as compared to short day conditions. miR19 shows almost similar levels of expression in both short day and long day leaves. However the expression levels of miRNAs in stolons is currently being investigated. A comparison between the expression levels of miRNAs in leaves and stolons on an SD-LD background will be followed.

Overall, this work will help to understand more aspects of role of *miR156* in tuber development and identify more miRNA candidates potentially involved in tuberisation pathway.

5. SUMMARY

The findings of the present investigations are summarized below.

1. *MIM156* was originally obtained from TAIR resources in pGREEN vector. In order to clone in pBI121 binary vector, we incorporated suitable restriction sites and successfully constructed MIM 156 and mobilized it to E. coli (DH 5 α) as well as in *Agrobacterium* strain GV2260.
2. Mimicry lines for tobacco and potato were developed using *Agrobacterium* mediated plant transformation.
3. All the transgenic mimicry lines were further confirmed by single step RT-PCR analysis.
4. Levels of micro RNA 156 inhibition in the mimicry lines were quantified using qRT-PCR analysis and our analysis indicate the almost 80% knockdown was achieved in one of the MIM clone.
5. In a separate investigation, a list of potential micro RNAs were made through a literature survey for their role in tuberization process.
6. These micro RNAs were detected in photoperiod sensitive cultivar *S. tuberosum* ssp andigena.
7. The differential expression profiles of the detected micro RNAs were carried out in leaf tissues using qRT-PCR analysis.
8. Presently, we are exploring the qRT-PCR analysis for stolon tissues from the same cultivar grown under photoperiod conditions in order to understand the micro RNA funcions.

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