"*In silico* analysis, cloning and overexpression of *miR172* in Potato (*Solanum tuberosum* L. ssp andigena)"

A thesis submitted towards partial fulfilment of

BS-MS duel degree programme

by

Stanzin Dadul

Under the guidance of

Dr. Anjan K. Banerjee, Asst. Professor, IISER Pune

Department of Biology,

Indian Institute of Science Education and Research Pune

(IISER Pune)

Certificate

This is to certify that this dissertation entitled "*In silico* analysis, cloning and overexpression of *miR172* in Potato (*Solanum tuberosum* L. ssp andigena)" towards the partial fulfilment of the BS-MS duel degree programme at the Indian Institute of Science Education and Research Pune, represents original research carried out by Stanzin Dadul at PMB (Plant Molecular Biology) Lab, IISER Pune under the supervision of Dr. Anjan K. Banerjee, Asst. Professor, during the academic year 2010-2011.

Stanzin Dadul

Name and signature of the student

Supervisor	**Head (Biological sciences)**
Date:	**Date:**
Place:	**Place:**

"*In silico* analysis, cloning and overexpression of *miR172* in Potato (*Solanum tuberosum* L. ssp andigena)"

Stanzin Dadul and Anjan K. Banerjee*

Dept of Biology, Indian Institute of Science Education & Research (IISER) Pune,
First Floor, Sai Trinity, Garware Circle, Pashan, Pune, Maharashtra, India. PIN 411008.

Abstract

MicroRNA172 (*miR172*), one of the most well studied microRNAs (miRNAs) in plants, mediates cleavage of *APETALLA2 (AP2)*/*APETALLA2 like* (*AP2 like*) mRNAs. *AP2*/*AP2 like* genes are involved in fruit ripening, meristem identity, flowering and many other functions in plants. In addition, *AP2 like* genes are also reported to be involved in stress response. In potato (*Solanum tuberosum*), only one *AP2 like* gene, called *RAP1* is reported so far. RAP1 inhibits tuberization in potato by repressing *StBEL5*, the mobile signal for tuberization. *miR172* known to enhance tuberization mechanism by down-regulating its target *RAP1*. In this study, a detail *In* analysis of eight putative *miR172* target genes has been performed using various softwares and databases. Our results suggest that four of them are *AP2* like genes, one has a b-Zip transcription factor domain, one codes for BTF3 like transcription factor whereas the rest two are coding for proteins having unknown functions. Unigene sequences for seven of them were also retrieved. All the predicted target mRNAs of *miR172* show transcript cleavage as their mode of inhibition and have one *miR172* binding site. In order to understand the *miR172* and target gene regulation, 35S::miR172 overexpression transgenic potato plants have been generated and putative clones have been validated by RT-PCR analysis. Further experiments are in progress.

'

Keywords *miRNA, miR172*, Potato (Solanum tuberosum), Abiotic stress, Transformation

Abbreviations

miRNA	*microRNA*
miR172	*microRNA* 172
AP2	*APETALA2*
AP2- like	*APETALA2 LIKE*

Introduction

Reproductive success in flowering plants is directly dependent on the timing of the switch from vegetative to reproductive growth phase and coincides with optimal environmental and developmental cues. The phenomenon of flowering is very complex in which even the roles of a small molecule, such as small RNAs are extremely crucial (Aukerman et al. 2003; Glazin´ska et al. 2009). It is now well known that small RNAs (19-25 bp in length), present in thousands in number, both in animals and plants, regulate various metabolic and developmental processes at transcriptional and post transcriptional levels. Lack or over-expression of just one small RNA in an organism leads to a haywire (Sunkar and Zhu 2004; Flynt and Lai 2008) in the system and in many cases are found lethal (Shan et al. 2009). Numerous reports suggest that these small RNAs control various biological processes such as; stress response (Zhang et al. 2010), flowering (Chen et al. 2004), fruit ripening (Moxon et al. 2008), germ cells growth (Kotaja et al. 2006) and development of seeds as well (Wu et al. 2009). Many of these small RNAs are conserved across the species of a kingdom, for example, *miR156* is found in plants belonging to different species including moss, which supports their early origin (Voinet 2009).

Small RNAs mediate gene regulation by translational arrest or cleavage of their target mRNAs. This is achieved by the binding of ARGONAUTE/RNA induced silencing complex (AGO/RISC) to their target mRNAs (Rand et al. 2004; Okamura et al. 2004). How small RNAs mediate gene silencing through AGO, an endonuclease, has been studied in great detail. Small RNAs bind to their target by Watson and Crick base-pairing. In animals, small RNAs bind to their target mRNA with a weak complimentarity, bringing about transcript destabilization through decapping and deadenylation pathways (Carthew and Sontheimer 2009) which leads to translational arrest of the target mRNAs. Whereas, plant small RNAs have near perfect complimentarity with their targets leading to the cleavage of target mRNAs (Millar et al. 2005).

There are two major classes of small RNAs, named as (a) short interfering RNAs (siRNAs), and (b) micro RNAs (miRNA*s*). While the mode of action, for both the class of small RNAs on their target is the same, they differ in their biogenesis. Transposons, dsRNA from viruses and other bidirectionlly transcribed repetitive sequences and genes gives rise to siRNAs (Matzke et al. 2005). On the other hand, miRNAs are generated from miRNA genes (Axtell et al. 2008) whose transcript form a stem loop double stranded RNA structure, which are further processed into double stranded mature *miRNAs* (Axtell et al. 2008). However, the mode of action of gene silencing for both the class of small RNAs, is mediated by target cleavage or translational arrest with AGO/RISC complex mechanisms (Yu et al. 2010).

Micro RNAs *(*miRNA*s)* in plants originate from sequences present in the intergenic/intronic region which are transcribed by RNA polymerase II/III (Lu et al. 2008, Voinet 2009). The transcript, a stem loop structured primary miRNA, called the pri-miRNA, is stabilized by DAWDLE (DDL) and HYPNOSTIC LEAVES (HYL) (Tagami et al, 2009). It is further processed into a precursor of *miRNA* called *pre-miRNA* by an enzyme called DICER LIKE (DCL). DCL then cleaves the *pre-miRNA* into double stranded mature miRNA (Macrae et al. 2006) which is finally exported by HASTY, a plant homologue of Exportin, into the cytoplasm. The double stranded mature *miRNA* is then methylated by HEN1 at 2' hydroxyl group of the 3' terminal (Yang et al. 2006) of the *miRNA*. The process of methylation protects the 3' end of the small RNAs from exonucleases and probably helps in the detection by AGO proteins (Yu et al. 2005).

One of the earliest discovered and most well studied *miRNAs* in plants is *miR172* (Park et al. 2002). In many plants studied so far, more than one *miR172* loci are found. In *Arabidopsis*, five loci code for *miR172* while in rice there are four loci reported so far (2011). *miR172* regulates various processes in plants by targeting *APETALA2* (*AP2*) (Aukerman et al. 2003) or AP2-like transcription factors (Chuck et al. 2008) which play a major role in flowering. The process of flowering in *Arabidopsis* is regulated by three classes of organ identity genes: *A, B* and *C*. *AP2* belongs to the class A, which is antagonistic to *AGAMOUS* (*AG*), a B class gene (Bomblies et al. 1999). Literature reports suggest that mutation in *AP2* leads to over expression of *AG*, subsequently, the first whorl of the flower turns into a carpel and rosette leaves phenotype as reported earlier (Chen et al. 2004). Considering the deformed (rosette) leaves phenotype in AP2 mutants, it is logical to conclude that *AP2* and/or *AP2-like* genes are involved in processes other than flowering as well.

In fact, there are reports about the role of *AP2* in development of seeds (Jofuku et al. 1994), fruit development (Karlova et al. 2011) and regulation of leaf epidermal cell identity (Moose et al. 1996). In *Antirrhinum*, two *AP2 like* genes such as *LIPLESS1* and *LIPLESS2* (*LIP1* & *LIP2*) determine the sepal and petal formation in the plant but unlike *AP2*, none of the two is antagonistic to *AG*, showing that there has been a major evolutionary change in the genetic basis of the *AP2* sequence of *Antirrhinum* (Keck et al. 2003). In *Petunia hybrida*, three *AP2-like* genes *PHAP2A*, *PHAP2B* and *PHAP2C* have been discovered, out of which *PHAP2A* is most closely related to *AP2* of Arabidopsis (Maes et al. 2001). From various other studies, it is now quite clear that *AP2* and *AP2-like* genes have diverged not just in the structure, but in functions as well with the AP2 domains being conserved in all the AP2 proteins. *AP2-like* genes including *AP2* itself can be divided into two major families: euAP2 and *ANT* (*AINTAGUMENTA*) lineage (Shigyo et al. 2006;). Out of these two families, *euAP2* has the *miR172* binding site and lacks the ten amino acid motifs in R1domain and one amino acid motif in R2 domain (Kim et al. 2006). Considering the various paralogues of the *AP2* gene in the same organism, for example *LIP1* and *LIP2* in *Antirhinum* and *PHAP2A/B/C* in *Petunia*, it is not hard to imagine the various roles *AP2 like* genes play in plant development.

In potato (*Solanum tuberosum*), only one *AP2 like* gene, called *RAP1* is reported so far. RAP1 in potato inhibits tuberization by repressing the *StBEL5*, which acts as a positive mobile signal for tuberization in *Solanum tuberosum* (Banerjee et al. 2006a). It is now known that *miR172* enhances tuberization in *Solanum tuberosum* by down-regulating its target RAP1 (Martin et al. 2009). On the basis of the above information on *AP2* and *AP2 like* genes and their multiple functions, we hypothesise that there could be other paralogous genes present in potato apart from *RAP1* and that potentially be involved in plant growth and development under various environmental ques. No such literature is available in this regard.

To address this hypothesis, in the present study, we conducted a detail *In silico* analysis of *miR172* targets in potato by various softwares. Our *In silico* analysis suggests that out of the eight putative targets we analyzed; four of them are *AP2* like genes, one target has a b-Zip transcription factor domain, one codes for BTF3 like transcription factor whereas two code for proteins having unknown functions. Potato unigene sequences for most of these targets were also retrieved. This was followed by cloning and generation of over-expression of 35S::*miR172* transgenic lines of potato in order to understand the target gene regulations.

Materials and methods

Plant material and growth conditions

Solanum tuberosum L. subspecies *andigena* line 7540 was used as the wild type potato plant for this study. All the plants were propagated and maintained in plant tissue culture medium as reported earlier (Banerjee et al. 2006b).

In silico analysis of targets of *miR172* in *Solanum tuberosum*

Since the role of *miR172* was reported to be involved in tuberization mechanism by Martin et al (2009), we carried out a literature survey and found that another report by Zhang et al. (2009) has provided a list of twelve *miR172* putative targets. Our analysis suggests that nine of which have TC annotations from DFCI (http://compbio.dfci.harvard.edu/tgi/) and the rest are from NCBI database. However, limited information was available regarding the name and functions of all *miR172* targets. We carried out an extensive *In Silico* analysis of *miR172* followed by the BLASTN search in multiple databases such as NCBI and SGN (www.**sgn**.cornell.edu), tomato and potato gene databases. Unigene sequences were searched wherever available. Open reading frames (ORF) for all the sequences were looked at NEB Cutter (http://tools.neb.com/NEBcutter2/) online software. Similar sequences were aligned and longer ESTs were obtained. Putative translated protein sequences were designed from the longest ORFs. We have used following databases /online softwares for studying the *miR172* targets analysis:

a. http://bioinfo3.noble.org/miRNA/miRU.htm (miRU)
b. http://biocomp5.noble.org/psRNATarget/ (psRNATarget)
c. http://www.leonxie.com/targetAlign.php (Target align)

miRU and psRNATarget softwares analyze the targets of a specific *miRNA* from various databases for any species of interest. In addition, psRNATarget and target align softwares provides information whether any transcript is a target of a particular *miRNA* (miRNA-target one on one analysis). Any target having a score less than or equal to 4, with default parameters, could be a target of that *miRNA* as suggested by the softwares. In our analysis, we used the sequence of *miR172* (5'-AGAAUCUUGAUGAUGCUGCAU-3') as metioned in Martin et al. (2009). In both, target align and psRNATarget software, the GU wobble is considered a score/mismatch point of 0.5, while other mismatches are given a score of 1.0. Lower the score better is the binding of miRNA and its target as recommended by all the softwares.

Flow chart for the Bioinformatics analysis is as follows

Accession numbers of target sequences (Zhang et al. 2009)
↓
Retrieval of sequences from respective Databases (TC annotations are from DFCI and CV annotations are from NCBI)
↓
Sequence alignment on NCBI to check for redundancy if any
↓
Verification of the targets by using miRU target prediction software
↓
miRNA 172 binding site prediction using Target Align/ psRNATARGET
↓
NCBI blast analysis of the positive hits

↓

ESTs assembly and recovery of potato unigenes in order to get the full coding sequence of the predicted targets (www.solgenet.org)

↓

In silico translation of the unigenes/ESTs on NEB cutter for longest ORF

↓

NCBI blast of the ORFs to identify putative protein and its functions

miR172 precursor sequence amplification and vector constructions

Genomic DNA from potato plant was isolated using Qiagen DNAeasy Plant Mini kit (Qiagen, Germany). A 133 bp *miR172* precursor (*miR172*pre) sequence was amplified using both forward, 5′GGTCTAGACATACAGTTGTTGCTTGCTA-3′, and reverse 5′-GGGTCGACATCAAGTCAT CAATTTGCCA-3′ primers as mentioned in Martin et al. (2009). Conditions for PCR amplifications were as follows: Initial denaturation at 94^0C for 5 min, 30 cycles of 94^0C for 30 sec, 52^0C for 30 sec, 72^0C for 30 sec and final extension at 72^0C for 5min. The final PCR product was subcloned into pGEMT easy vector (Promega,USA) and subsequently cloned into binary vector pBI121. The insert was digested with restriction enzymes *XbaI* and *SacI* specific to binary vector pBI121 (Fig.1 G). Recombinant clone of 35S::miR172pre-pBI21 was mobilized to *Agrobacterium tumefaciens* strain GV2260 for plant transformation purpose.

Agrobacterium mediated transformation of *Solanum tuberosum* ssp *andigena* (7540)

miR172 overexpression transgenic plants were generated using the protocol by using Banerjee et al (2006b). The cultures were incubated at a temperature of 27^0C. All other culture conditions remain same for transgenic plant maintenance and propagation. Micro shoots were rooted, hardened and transferred to soil for further phenotypic analysis.

Plant tissue harvest and RNA isolation

In vitro shoots were harvested in liquid nitrogen and stored at -80^0C for analysis. RNA was isolated using 'Qiagen RNAeasy plant mini kit' (Qiagen, USA). Aliquoted RNA was stored at -80^0C for RT-PCR analysis.

RT PCR analysis of the transgenic plants

Three microgram (3µg) of isolated RNA from each putative transgenic clone was reverse transcribed with Moloney Murine Leukemia Virus (MMLV) reverse transcriptase using the user's protocol (Promega, USA). The cDNA, thus generated, was amplified by PCR with initial denaturation at 95^0C for 2 min, followed by 35 cycles of 98^0C for 20 sec, 52^0C for 15 sec,72^0C for 30sec and final extension at 72^0C. The RT-PCR analysis was carried out for four putative clones. Appropriate negative and positive controls were also used. The primers for kanamycin resistance gene kanRF (GGATTGCACGCAGGTTCT) and kanRR (CGTCAAGAAGGCGATAGAA), were used respectively in order to amplify the ~ 800 bp *KanR* gene (Fig.1 H).

Results

In silico analysis of targets of *miR172*

In order to identify more targets of *miR172*; miRU, psRNATarget and Target align softwares were used. Our *In Silico* analysis of eight putative *miR172* targets using various softwares shows that four of them are *AP2* like genes; one target has a b-Zip transcription factor domain, one codes for BTF3 like transcription factor whereas rest two are coding for proteins having unknown functions. Unigene sequences for seven targets, except TC101620, were also retrieved. All the predicted targets of *miR172* show mRNA cleavage as their mode of inhibition and have one *miR172* binding site. Predicted functions of the targets are given in detail in a tabular form (Table 1).

Our investigations also reveal that out of twelve targets, as mentioned in Zhang et al (2009) report, TC94807 and TC208546 are the same sequences and have now been assembled into the latter sequence. Also, TC109385 is a part of BQ114970 and latter has the longer sequence. In addition, two more accessions such as TC94449 and TC217078 from Zhang et al (2009) report, now assembled in to TC199918 We also observed that two other accessions, TC94939 and CV496840, are the same sequences (Table 1). Our detailed bio informatics survey pointed that out of the twelve targets of *miR172* mentioned previously, some contain redundant sequences. Hence, analysis was continued for only eight targets as mentioned in Table 1. Appropriate scores to determine the level of binding between targets and *miR172* were followed as per software guidelines. Our prediction analysis suggests that all the *miRNA172*-target pairs have an expectation score (E) less than or equal to 4, least being the score of 0.

In silico translation of all four *AP2 like* target sequences suggest that all the AP2 Like proteins contains two AP2 conserved domains. Of the four AP2 targets of *miR172,* one accession TC101399 shows high degree of similarity with ESTs from potato abiotic stress cDNA libraries at NCBI database. Also, another accession, TC208546 which codes for a b-Zip domain transcription factor shows match with the potato abiotic stress cDNA libraries at NCBI search result. Our present observation is also similar with the previous reports (Chinnusamy et al. 2004) where authors showed

that *AP2* like genes and b-Zip transcription factors being involved in biotic and abiotic stress response mechanism.

Generation of 35S::*miR172* transgenic potato (*Solanum tuberosum*, ssp andigena line) plants

miR172 pre subcloned into pGEMT easy vector was verified by restriction digestions before being cloned into pBI121 binary vector driven by 35S promoter. Binary vector pBI121 carrying 35S*::miR172pre* was further validated by restriction digestions and by sequencing analysis of *miR172* precursor. *Agrobacterium tumefaciens* strain GV2260 containing the recombinant binary construct 35S::miR172pre-pBI121 was also validated before being used for plant transformation experiments. From the two independent transgenic events, altogether 20 putative clones were generated for the 35S::miR172pre-pBI121 binary vector harbouring the kanamycin resistance gene. Upon transfer to rooting medium, 80% of tissue culture raised shoots had successful root induction and good growth in culture medium. All transgenic clones were maintained in tissue culture medium as per the protocol. In vitro grown plants were subjected to RT-PCR analysis for validation of the presence of kanamycin resistance gene. Four putative clones (4B-miR172, 5B-miR172, 6B-miR172 & 9B-miR172) were analyzed. RT-PCR analysis revealed that three out of the four clones had a strong band of ~ 800 bp for Kanamycin resistance gene (Fig. 1H). The fourth clone (4B-miR172, lane 1, Fig 1J) had a similar sized faint signal specific to the gene of interest. This suggests *that* 35S*:miR172* precursor *T –DNA* cassette has been integrated in all clones with a varied degree of expression. RT-PCR validations for all the other putative transgenic clones and qPCR analysis for *miR172* over expression in clones are in progress. Clones having high expression of *miR172* would be taken further for studies of target gene validations.

Discussions

In potato, only one *AP2* like transcription factor, *RAP1* is reported as a target of *miR172*. *RAP1* acts as an inhibitor of tuberization where it down regulates *StBEL5*. Under tuber inducing conditions, *miR172* targets *RAP1* and relieves the inhibition on *StBEL5*, a tuber inducing signal. (Martin et al. 2009). A number of *AP2 and AP2* like transcription factors have been reported in different plants carrying out multiple functions (Kim et al. 2006). To identify paralogous genes present in potato apart from *RAP1*, bioinformatic analysis was performed. It is evident from our *in silico* analysis, that *miR172* has other targets in potato apart from *RAP1*; four of them being *AP2-like*, one containing b-Zip transcription factor, one being BTF3 like transcription factor and the rest two proteins having unknown functions.

Of all the *miR172* targets, AP2 and AP2 like transcription factors are very well studied. There are two major classes of *AP2 like* genes, *euAP2* and *ANT* lineage, former having roles in floral (Aukerman et al. 2003), leaves (Shukla et al. 2006) and seed development (Okamuro et al. 1997) and latter found to be involved in both biotic and abiotic stress response (Liu et al. 1998). Numerous reports (Kim et al. 2006; Chuck et al. 2008; Nair et al. 2009) have shown that *miR172* target euAP2 lineage of genes. As the *ANT* lineage of genes, which are involved in stress response, do not have the *miR172* binding site, they are not directly targeted by *miR172* (Kim et al. 2006). Thus, the *ANT* lineage of genes is involved in stress but do not show *miR172* binding site. However, one AP2 like gene similar to TC101399 on NCBI was identified as ESTs from potato abiotic stress cDNA libraries. This gives a rare case of *AP2* like transcription factor which has *miR172* binding site and appears to have a putative role in abiotic stress, suggesting other possible mechanism of gene regulation.

In plants, gene numbers are expanded by segmental and tandem duplication in gene families. The combination of a range of processes such as gene duplication, nucleotide substitution and domain duplication and exon/intron shuffling can generate complex set of related genes that may differ considerably in expression in both space and time (Lynch 2000). In the course of evolution of gene duplication, there can be three functions that can appear in the gene(s); neofunctionalization, subfunctionalization and nonfunctionalization. Presence of various *AP2 like* genes in plants perhaps could be the result of subfuntionalization. Thus, it can be anticipated that a gene having a role in stress response and also has a *miR172* binding site for gene regulation.

If there is a stress induced *AP2 like* gene having a *miR172* binding site, then it is logical to anticipate that when the *miRNA* expression is downregulated, the target mRNA expression would be upregulated. For example, in Rice (*Oryza Sativa*), *miR172* expression is downregulated under drought conditions. (Zhou et al. 2010). Apart from its role in flowering, *AP2 like* genes are required for maintenance of the juvenile phase of the plant and its expression is down regulated in Arabidopsis as the plant matures (Wu et al. 2009). Question obviously arises that is it under abiotic stress; a plant has to give attention to its own survival rather than its reproduction in order to maintain the cost benefit ratio? Or is it that the process of gene duplication and subfuncionalization has given rise to a different *AP2* like gene, different from EREBP/AP2 and DREB (*ANT* lineage *AP2* genes) (Kim et al. 2006), but having the AP2 domains, at least in potato, and perhaps in other plants like Rice (Zhau et al. 2010) and Japanese Larch (Zhang et al. 2010), which is not just involved in stress response but has an *miR172* binding site as well. Recent literature revealed that ethylene up-regulates *AP2* in tomato but when *AP2* is down-regulated by an RNAi mechanism the level of ethylene goes up as if in a negative feedback mechanism (Karlova et al. 2011). Ethylene production helps the plants to deal with both abiotic and biotic stress (Ludwig et al. 2005) and now we know that it can also regulate the levels of

AP2 or *AP2 like* genes as well (Karlova et al. 2011). Hence, the relationship of *miR172*, *AP2* and ethylene cannot be neglected and requires more experimentation.

To validate our hypothesis of *miR172* target being involved in stress response, over expression lines of 35S::miR172-pBI121 potato plants are generated (Fig 1 A-F) in order to study the relation of *miR172* and *AP2 like* genes under various abiotic stress conditions. Further experiment would include validation of all target genes in *miR172* over expression lines, plants response to various stress induction and establishment of the relation between *AP2 like* genes and *miR172* in potato.

Acknowledgements

This work was supported by the Dept of Biology, Indian Institute of Science Education and Research (IISER) Pune, India. We thank Ms. Sneha Bhogale and Mr. Ameya Mahajan (Ph.D scholars of AKB Lab) for their support in bioinformatics analysis and critical reading of the manuscript.

References

Aukerman MJ, Sakai H (2003) Regulation of Flowering Time and Floral Organ Identity by a MicroRNA and Its APETALA2 -Like Target Genes. Plant cell 15: 2730-2741

Axtell MJ, Bowman JL (2008) Evolution of plant microRNAs and their targets. Trends in plant sci 13: 343-9

Banerjee AK, Chatterjee M, Yu Y, Suh SG, Miller WA, Hannapel DJ (2006a) Dynamics of a mobile RNA of potato involved in a long-distance signaling pathway. Plant cell 18:3443-57

Banerjee AK, Prat S, Hannapel D (2006b) Efficient production of transgenic potato (S. tuberosum L. ssp. andigena) plants via Agrobacterium tumefaciens-mediated transformation. Plant Sci 170:732-738

Bomblies K, Dagenais N, Weigel D (1999) Redundant Enhacers Mediate Transcriptional Repression of AGAMOUS by APETALA2. Dev Biol 216:260 -264

Carthew RW, Sontheimer EJ (2009) Origins and mechanism of miRNAs and siRNAs. Cell 136:642-655

Chen X (2004) A MicroRNA as a Translational Repressor of APETALA2 in Arabidopsis Flower Development. Science 2022:2023-2025

Chinnusamy V, Schumaker K, Zhu J (2004) Molecular genetics perspectives on cross-talk and specificity in abiotic stress signalling in plants. J Exp Bot 55:225-236

Chuck G, Meeley R, Hake S (2008) Floral meristem initiation and meristem cell fate are regulated by the maize AP2 genes ids1 and sid1. Development 3019:3013-3019

Flynt AS., Lai EC (2008) Biological principles of microRNA-mediated regulation: shared themes amid diversity. Nat Genet *9*:831-842

Glazinska PZA, Wojciechowski W, Kopcewicz J (2009) The putative miR172 target gene InAPETALA2-like is involved in the photoperiodic flower induction of Ipomoea nil. J Plant Phys 166:1801-1813

Jofuku KD, Boer BG, den Van Montagu M, Okamuro JK (1994) Control of Arabidopsis flower and seed development by the homeotic gene APETALA2. Plant Cell, *6*:1211-25

Karlova R, Rosin FM, Busscher-lange J, Parapunova V, Do PT, Fernie AR et al (2011) Transcriptome and Metabolite Profiling Show That APETALA2a Is a Major Regulator of Tomato Fruit Ripening. Plant Cell 10:1-20

Keck E, Mcsteen P, Carpenter R, Coen E (2003) Separation of genetic functions controlling organ identity in flowers. EMBO J *22*:1058-1066

Kim S, Soltis PS, Wall K, Soltis DE (2006) Phylogeny and domain evolution in the APETALA2-like gene family. Mol Biol Evo 23:107-20

Kotaja N, Bhattacharyya SN, Jaskiewicz L, Kimmins S, Parvinen M, Filipowicz W, et al. (2006) The chromatoid body of male germ cells: Similarity with processing bodies and presence of Dicer and microRNA pathway components. Proc Natl Acad Sci USA 103:2647-2652.

Liu Q, Kasuga M, Sakuma Y, Abe H, Miura S, Yamaguchi-shinozaki K (1998) Two Transcription Factors, DREB1 and DREB2, with an EREBP/AP2 DNA Binding Domain Separate Two Cellular Signal Transduction Pathways in Drought- and Low-Temperature-Responsive Gene Expression, Respectively, in Arabidopsis. Plant cell 10:1391-1406

Lu C, Jeong D, Kulkarni K, Pillay M, Nobuta K, German R et al (2008) Genome-wide analysis for discovery of rice microRNAs reveals natural antisense microRNAs (nat-miRNAs). Proc Natl Acad Sci USA 105:4951-4956

Ludwig AA, Saitoh H, Felix G, Freymark G, Miersch O, Wasternack C et al (2005) Calcium-dependent protein kinase and MAPK signaling controls stress responses in plants. Proc Natl Acad Sci USA 102:10736-10741

Lynch M (2000) The Evolutionary Fate and Consequences of Duplicate Genes. Science 290:1151-1155

Macrae IJ, Zhou K, Li F, Repic A, Brooks AN, Cande WZ, et al (2006) Structural Basis for Double-Stranded RNA Processing by Dicer. Science 311:195-198

Maes T, Steene NVD., Zethof J, Karimi M, Hauw MD, Mares G et al (2001) Petunia Ap2 -like Genes and Their Role in Flower and Seed Development. Plant cell 13:229-244

Martin A, Adam H, Díaz-Mendoza M, Zurczak M, González-Schain ND, Suárez-López, P (2009) Graft-transmissible induction of potato tuberization by the microRNA miR172. Development 136:2873-81

Matzke MA, Birchler JA (2005) RNAi-Mediated Pathways in the Nucleus. Nature 6:24-35.

Millar AA, Waterhouse PM (2005) Plant and animal microRNAs: similarities and differences. Func Int Genom 5:129-35

Moose SP, Sisco PH (1996) Glossy15, an APETALA2-like gene from maize that regulates leaf epidermal cell identity. Genes Dev 10:3018-3027

Moxon S, Jing R, Szittya G, et al. (2008) Deep sequencing of tomato short RNAs identifies microRNAs targeting genes involved in fruit ripening Deep sequencing of tomato short RNAs identifies microRNAs targeting genes involved in fruit ripening. Genom Res 18:1602-1609

Nair SK, Wang N, Turuspekov Y, Pourkheirandish M, Sinsuwongwat S (2009) Cleistogamous flowering in barley arises from the suppression of microRNA-guided HvAP2 mRNA cleavage. Proc Natl Acad Sci USA 107:1-6

Okamura K, Ishizuka A, Siomi, H, Siomi MC (2004) Distinct roles for Argonaute proteins in small RNA-dircted RNA cleavage pathways. Genes Dev 18:1655-1666

Okamuro JK, Caster B, Villarroel R, Van Montagu M, Jofuku KD (1997) The AP2 domain of APETALA2 defines a large new family of DNA binding proteins in Arabidopsis. Proc Natl Acad Sci USA 94:7076-81

Park W, Li J, Song R, Messing J, Chen, X (2002) CARPEL FACTORY, a Dicer Homolog, and HEN1, a Novel Protein , Act in microRNA Metabolism in Arabidopsis thaliana. Current Biol 12:1484-1495

Rand TA, Ginalski K, Grishin NV, Wang X (2004) Biochemical identification of Argonaute 2 as the sole protein required for RNA-induced silencing complex activity. Proc Natl Acad Sci USA 101:14385-14389

Shan H, Xuelian L, Pan Z, Zhang L, Cai B, Zhang Y et al (2009) Tanshinone IIA protects against sudden cardiac death induced by lethal arrhythmias via repression of microRNA-1. British J Pharmacol 158:1227-35

Shigyo M, Hasebe M, Ito M (2006) Molecular evolution of the AP2 subfamily. Gene 366:256 - 265

Shukla RK, Raha S, Tripathi V, Chattopadhyay D (2006) Expression of CAP2, an APETALA2-Family Transcription Factor from Chickpea, Enhances Growth and Tolerance to Dehydration and Salt Stress in Transgenic Tobacco. Plant Physiol 142:113-123

Sunkar R, Zhu J-kang (2004) Novel and Stress-Regulated MicroRNAs and Other Small RNAs from Arabidopsis. Plant Cell 16:2001-2019.

Tagami Y, Motose H, Watanabe Y (2009) A dominant mutation in DCL1 suppresses the hyl1 mutant phenotype by promoting the processing of miRNA. *RNA (*New York, N.Y.), 15, 450-8.

Voinnet O (2009) Origin, biogenesis, and activity of plant microRNAs. Cell 136:669-87.

Wu G, Park MY, Conway SR, Wang J, Weigel D, Poethig RS. (2009) The Sequential Action of miR156 and miR172 Regulates Developmental Timing in Arabidopsis. Cell 138:750-759

Yang Z, Ebright YW, Yu B, Chen X (2006) HEN1 recognizes 21–24 nt small RNA duplexes and deposits a methyl group onto the 2’ OH of the 3' terminal nucleotide. Nucleic Acid Res USA 34:667-675.

Yu, B., Yang, M., Padgett, R. W., Steward, R, Chen-, X. (2005) Methylation as a Crucial Step in Plant microRNA Biogenesis. Science 932:932-935

Yu B, Bi L, Zhai J, Agarwal M, Li S, Wu Q et al (2010) siRNAs compete with miRNAs for methylation by HEN1 in Arabidopsis. Nucleic Acids Research USA 10:1-7

Zhang W, Luo Y, Gong X, Zeng W, Li S (2009) Computational identification of 48 potato microRNAs and their targets. Comp Biol Chem 33:84-93

Zhang S, Zhou J, Han S, Yang W, Li W, Wei H et al (2010) Four abiotic stress-induced miRNA families differentially regulated in the embryogenic and non-embryogenic callus tissues of *Larix leptolepis*. Biochem Biophys Res Comm 398:355-360

Zhou L, Liu Y, Liu Z, Kong D, Duan, M, Luo L (2010) Genome-wide identification and analysis of drought-responsive microRNAs in Oryza sativa. J Exp Bot 61:4157-4168

Zhu, QH, Helliwell CA (2011) Regulation of flowering time and floral patterning by miR172. J Exp Bot 62:487-495

S NO	Target accession no	Significant NCBI Hits (Accession No's)	SCORE/ MISMATCH PsRNA/ Target align (default parameters)	Translational Arrest/Cleavage	Sequence After EST Assembly (Bases)	Unigene sequences from *S.tuberosum* (length in bases) retrieved from SGN database	Putative ORF (a.a.)	BLASTp of ORF on NCBI	Putative function of The Target Gene
1	TC94939	CK256140.1 CK249227.1	1/2	Cleavage	849	U268527 (880)	210	AP2 LIKE IN Tobacco PHAP2B in *Petunia*	AP2 Like PHAP2B has role in flowering
2	TC100916	BM108496.1 BE472510.1	1/2	Cleavage	1119	U283811 (793)	165	AP2 Like (Tobccoo) Un-named protein product (*V. vinifera*)	Exact function not known
3	TC101399	CK267789 CK267790.1	1.5/2.5	Cleavage	1102	U295782 (633)	273	PHAP2A protein from *P.hybrida*	ESTs from NCBI are stress related , PHAP2A plays role in seed and flower development
4	TC199918	BQ516150.1 BQ113495	1.5/2.5	Cleavage	1426	U275544 (862)	239	AP2 Like	Homologues to PHAP2A
5	CV495900	Sequence from NCBI itself	0.5/1.5	Cleavage	642	U268526 (1390)	162	putative transcription factor BTF3-like *S.tuberosum*	Contains NAC domain. Few NAC TFs play role in regulating senescence and cell division
6	TC208546	CK277325.1 CK262296.1	4 .0 No result on psRNA Target	Cleavage	1723	U283737 (542)	327	Unknown protein Containing the b-zip transcription factor domain	ESTs from NCBI shows they are abiotic stress related
7	TC101620	CK267379.1 CK267380	3/3	Cleavage	1017	Unigene not found	234	A hypothetical protein from Ricinus cummunis	Not known
8	BQ114970	Sequence from NCBI itself	0/0	Cleavage	742	U285325 (767)	NA	No info availbale	Not known

Table 1. *In silico* analysis of miR172 targets in *Solanum tuberosum* (TF- Transcription Factor, aa – Amino Acid, SGN – Solanaceae Genomics Network)

Fig. 1

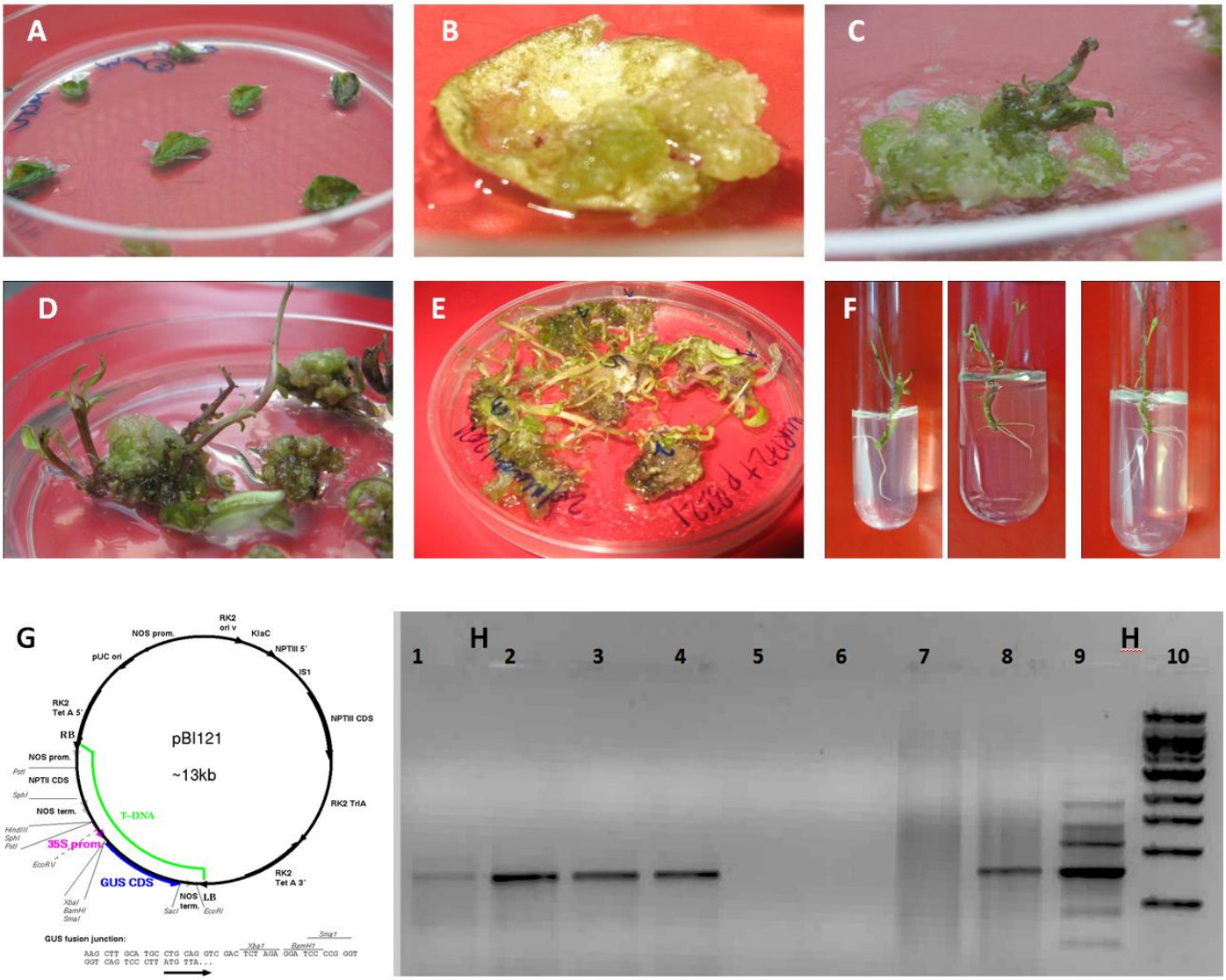

Fig. 1 (A-F) 35S::miR172-pBI121 overexpressing transgenic lines at various stages of culture. **A.** Leaf discs in callus induction medium after cocultivation.**B**. Same leaf discs in shoot induction medium after 2nd week of culture. **C.** Callus and shoot induction after 4-5 weeks. **D.** Elongated shoots in shoot induction medium after 6-7 weeks of growth **E**. Large number of elongated shoots from leaf discs explant after 8-10 weeks of growth. **F.** Microshoots in root induction medium ready for transfer to soil. **G.** Diagrammatic representation of pBI121 binary vector with GUS gene. The GUS fragment was removed from T-DNA cassette and replaced with 133bp *miR172* precursor gene using *XbaI* and *SacI* restriction enzymes**. H.** RT-PCR analysis of 4 putative transgenic clones with positive and negative control and supermix ladder, lane 1. Clone 4B-miR172, lane 2. Clone 5B- miR172, lane 3. Clone 6B- miR172, lane 4. Clone 9B- miR172, lane 5. RT negative control, lane 6. PCR negative (water) control, lane 7. Negative control with 7540 potato plant , lane 8. Positive control (35S::pPOTH1-His), lane 9. Positive vector control (35S::pBI121) and lane 10. Supermix ladder.