MATERIALS AND METHODS

3.1 Materials:

3.1.1 Plant materials:

Arabidopsis seeds (Wt Col), mutant seed *sgs3-11* were from ABRC and the seeds of *pMIR390b::GUS* was generated as mentioned in (Montgomery *et al.*, 2008).

3.1.2 Bacterial Strains

Material	Genotype	Source
Escherichia coli (DH5a)	Φ8dlacZ Δ M15, recA1, endA1,gyr A96, thi-1, hsd17 (rk-mk+)supE44,relA1,deoR,(LacZY AargF)U19	Stratagene, (USA)
Agrobacterium tumefaciens (GV3101)	Carries pMP90 Ti-plasmid with gentamicin selection and rifampicin as chromosomal Selection	Novagen, (Germany)

3.1.3 Plasmids and vectors

Vector	Company
pGEM-T Easy	Promega Life Sciences, USA
pJet CLONING KIT	Thermo Fischer Scientific, USA
pCAMBIA1301	Stratagene, USA

3.1.4 Concentrations of various antibiotics

Antibiotics	Abbreviation	Concentration(µg/mL)
Ampicillin	Amp	100
Kanamycin	Kan	50
Rifampicin	Rif	25
Hygromicine	Hyg	25

3.1.5 General Chemicals and other materials

Category	Materials	Company
Chemicals for general purpose	Methanol, Chloroform, Iso-amyl alcohol,Iso-propanol, Formaldehyde, Glycerol, Sodium Chloride, Potassium Chloride, Sodium acetate,Sucrose, Ethylenediamene tetraacetic acid (EDTA), Acetic acid, Hydrochloric acid, Triton-X-100, Diethyl pyrocarbonate(DEPC),LithiumChloride(LiCl), *N*-Lauroylsarcosine sodium salt, Tris Base, Guanidin hydrochloride, DTT, MES hydrate, 2-Marcaptoethanol (or, β-Mercaptoethanol), Silwet L-77, Formamide, Sodium hydroxide, Acetic acid, Hydrochloric acid, X-gluc, DMSO, Potassium ferrocyanide, potassium ferricianide,	SigmaAldrich (USA), Himedia(India), Amresco (USA)
Markers and dNTPs	1 kb ladder, 100bp ladder, low range ladder dATPs, dGTPs, dCTPs, dTTPs	Fermentus (USA), New England Bio labs (USA), Gene Direx (India)

Dyes	Ethidium bromide, Xylene cyanol, Bromophenol blue,	Sigma (USA)
Antibiotics	Antibiotics Ampicillin, Kanamycin, Rifampicin, Hygromycin	Sigma Aldrich (USA), Himedia (India)
Filter papers	0.22 micron filters, Whatman sheet	Amersham(UK)

3.1.6 Enzymes

Enzyme	**Company**
Restriction enzymes	NEB, USA; Thermo Fisher scientific, USA
T4 DNA ligase	Thermo Fisher scientific, USA
T4 DNA Polymerase	Thermo Fisher scientific, USA
DNase I	Fermentus, Canada; Promega Life Science, USA
RNase A	Sigma Aldrich, USA
Calf intestinal alkaline phosphatase	Thermo Fisher Scientific, USA
Taq DNA Polymerase	Himedia, India
Superscript III RT	Invitrogen (Life technologies), USA
RNaseH	Invitrogen (Life technologies), USA
RNase OUT	Invitrogen (Life technologies), USA; NEB, USA

3.1.7 Plastic wares and Glasswares

Plastic wares and Glasswares	Company
Microcentrifuge tubes, micro tips, PCR tubes, reagent bottles, 96 well PCR plates, sterilization unit, oak-ridge and falcon tubes, Petri plates.	Tarsons, India; Axygen, India,Corning USA.
Reagent bottles, flasks, measuring cylinders, trays, beakers, culture tubes	Borosil, India; Schott Duran, India

3.1.8 Buffers and solutions -as described in (Sambrook *et al.*, 1989)

3.1.8.1 pH buffers

1x Phosphate buffer saline

8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO4 and 0.24 g of KCl was dissolved in 800 mL of distilled water. pH was adjusted to 7.4 with HCl and volume was made upto 1000 mL.

3.1.8.2 TE buffer (pH 8.0)

10 mM Tris-HCl, pH 8.0; 1 mM EDTA, pH 8.0

3.1.8.3 Tris-Cl (1M)

121.1 g of Tris base was dissolved in 800 mL distilled water and pH was adjusted to desirable value with HCl. Final volume was made upto 1000 mL with distilled water.

3.1.8.4 EDTA (0.5M, pH 8.0)

Added 186.1 g of EDTA to 800 mL of distilled water and stirred vigorously. The pH of the solution was adjusted with NaOH pellets to pH 8.0. EDTA is soluble at pH 8.0.

3.1.8.5 SDS (20%)

200 g of SDS powder was dissolved in 1000 mL of water and heated at 68 °C to assist dissolution.

3.1.8.6 Sodium acetate (3M, pH 5.2)

408.3 g of sodium acetate was dissolved in 800 mL of water. pH was adjusted to 5.2 with glacial acetic acid. Final volume was made upto 1000 mL with water.

3.1.8.7 Ethidium Bromide (EtBr) (10mg/mL)

1 g of EtBr was dissolved in 100 mL of distilled water. Solution was kept in dark bottle as EtBr is light sensitive.

3.1.8.8 Extraction buffers for DNA mini preparation

3.1.8.8.1 Alkaline lysis solution I

Chemical	Concentration
Glucose	50mM
Tris-Cl (pH 8.0)	25mM
EDTA (pH 8.0)	10mM

3.1.8.8.2 Alkaline lysis solution II

Chemical	Concentration
SDS	1% (w/v)
NaOH	0.2 N

3.1.8.8.3 Alkaline lysis solution III

Chemical	Concentration
Potassium acetate (5M)	60 mL
Glacial acetic acid	11.5 mL
MQ water	28.5 mL

3.1.8.9 Gel loading buffers

3.1.8.9.1 Tris-Acetate EDTA buffer (50X)

Components	Amounts/L
Tris-base	242gm
Glacial-acetic acid	57.1gm
0.5M EDTA (pH 8.0)	100mL
MQ water	to 1 litre

3.1.8.9.2 MOPS [3-(N-morpholino) propanesulfonic acid] buffer (10X)

Components	Amounts/L
MOPS (200mM)	41.9gm
Sodium-acetate (pH 5.2) (50mM)	8.2gm
EDTA (pH 8.0, 10mM)	3.72gm
DEPC water	to 1 litre

Adjust the pH to 7.0 with concentrated NaOH.

3.1.8.10 Loading Dyes

3.1.8.10.1 6X gel loading buffer (DNA)

Components	Concentrations
Bromophenol blue	0.25%
Xylene cyanol FF	0.25%
Glycerol in water	30%

3.1.8.10.2 1.5X RNA loading dye

Components	Amount/ 1mL	Concentration

Formamide	960µL	95%
Bromophenol blue (2.5% w/v)	10µL	0.025%
Xylene Cyanol FF (2.5% w/v)	10µL	0.025%
EDTA (0.25 M, pH 8.0)	20µL	5mM

3.1.8.11 Extraction buffer and GH-buffer for isolation of RNA from seeds

3.1.8.11.1 Extraction buffer

Components	Amount/ 5mL	Concentration
DTT (100mM)	250µL	5mM
NaCl (2M)	375µL	150mM
Tris-Cl (1M, pH 9.0)	500µL	100mM
EDTA (0.5M, pH 8.0)	200µL	20mM
SLS (2%)		to 5mL

3.1.8.11.2 GH-buffer

Components	Amount/ 6.5mL	Concentration
Guanidine hydrochloride	5.2 gm	8M
MES hydrate	0.03gm	20mM
EDTA (0.5M, pH 8.0)	260 µL	20mM
β-Mercaptoethanol	26 µL	50mM

Adjusted the volume to 6.5mL with DEPC water.(β- Mercaptoethanol should be added freshly before use).

3.1.9 Growth medium

3.1.9.1 MS medium for plant growth (Murashige and Skoog, 1962) (1000 mL)

Constituents	Amount
MS Salt	4.40gm
Sucrose	10gm
Agar extra pure	8gm
Adjusted pH to 5.6-5.8 with 10N KOH	

3.1.9.2 Luria Bertini (LB) medium for bacterial growth

Constituents	Amount
Tryptone	10gm
Yeast extract	5gm
NaCl	5gm
Agar (optional)	16gm
Adjust the volume to 1000mL/or, 1L with MQ water	

3.1.10 List of software used for in *silico* analysis

Purpose	Program used	Website	References
miRNA database	miRbase	http://www.mirbase.org/	
Arabidopsis data search TAIR	TAIR	http://www.arabidopsis.org/	
Homology search	BlastN; BlastX; BlastP	http://www.ncbi.nlm.nih.gov/BLA ST	
Primer Tm calculator	Sigma Tm calculator	https://www.sigmaaldrich.com	
Expression analysis	Multi experiment viewer (MeV)	http://www.tm4.org/mev.htmL	
Gene expression	Affymetrix	http://www.affymetrix.com	

microarray		
miRNA target prediction	psRNATarget	http://plantgrn.noble.org/psRNATarget/
Real time PCR primer Deisign	Primer Exress 3.0	http://www.lifetechnologies.com/ protein domain structures
Real time primer Designing	IDT Oligo tools	http://eu.idtdna.com/scitools/Applications/RealTimePCR/
Reverse Complementation	Reverse complement	http://www.bioinformatics.org/sms/rev_comp.htmL
Restriction mapping	NEB cutter V2.0	http://tools.neb.com/NEBcutter2/
Tm calculator	oligo calculator	http://mbcf.dfci.harvard.edu/docs/ oligocalc.htmL

3.2 Methods

3.2.1 Plant growth conditions

Arabidopsis thaliana (ecotype Col-0) and homozygous *sgs3-11, pMIR390b*::*GUS* seeds were sterilized with 70 % ethanol containing 0.1 % (v/v) Triton X-100 for 10 mins, followed by four washes of sterile water. Sterilized seeds were kept for stratification for 3 days at 4^{o}C. Sterilized seeds were sown on half-strength Murashige and Skoog (MS) agar medium in square petri plates (at 22 ± 2 ^{o}C under 16:8 hrs (light:dark) photoperiodic cycle; white light intensity was around 200 µmoles/m^2/s) for germination.

3.2.2 Sterilization protocols

All the glasswares, tissue culture tools and culture media were sterilized by autoclaving at 121.6 ^{o}C under 15 psi pressures for 15 mins. The antibiotics and other heat labile components were filter sterilized with PVDF syringe filter unit of 0.22 µm pore size (Millipore, USA). Before commencing RNA work, mortar, pestle, glassware, spatula and other required materials were baked at 180 ^{o}C for 6 hrs. Gel electrophoresis assembly and other plastic wares were overnight treated with 3% H_2O_2. All the reagents were prepared in DEPC (SIGMA-ALDRICH, USA) treated sterile Milli-Q water. RNase-free conditions were maintained by using RNase-OUT reagent (G-Biosciences, USA).

3.2.3 RNA isolation

Total RNA was extracted from germinating wild type Col-0 *Arabidopsis* seeds at 0 (dry seeds),12, 24 and 48 h after imbibition both at room temperature (RT) and 4˚C, using by slightly modified seed specific guanidine hydrochloride method (Singh *et al.*, 2003) from the wild type Col-0 *Arabidopsis* seeds. Approximately 50mg *Arabidopsis* seeds were crushed to fine powder in liquid nitrogen using mortar and pestle and homogenized in 500µL extraction buffer. To ensure complete dissociation of nucleoprotein complexes, homogenized samples were kept at room temperature for 5 min. After homozenisation, equal volume i.e. 500µL of phenol: Chloroform: Isoamylalcohol (25:24:1) was added to the homozenised sample. The homogenate was transferred to a microcentrifuge tube and vigorously mixed for 15 sec, then centrifuged for 15 min at 13000 rpm at 4 °C for 10 mins.. The mixture was separated into 3 phases, the upper aqueous phase (containing RNA) was light yellow in color. The aqueous phase was transferred to a 1.5 mL MCT and 350µL phenol: cholorofom: isoamylalcohol (25:24:1) and 650µL of GH buffer was added to it. Then centrifugation was done at 13,000

rpm for 10 mins at 4˚C. Again the supernatant was transferred in a fresh MCT and 500µL chloroform was added to it and mixed vigorously then centrifuged again at 13,000 rpm for 10 mins at 4˚C. The upper aqueous phase was transferred to two fresh MCTs and added 1/10th volume of 3M sodium acetate and two volume of100% ethanol. After proper mixing, the mixture was kept at -80˚C for minimum 1 hour followed by centrifugation at 13,000 rpm at 4˚C for 45 min. Then discarded the supernatant and washed the RNA pellet with 70% pre-chilled ethanol. The RNA pellet was then dried in room temperature and dissolved in 50µL sterile DEPC water.

3.2.4 Removal of genomic DNA from RNA preparation

DNase I is an endonuclease that digests single and double stranded DNA. It hydrolyzes phosphodiester bonds producing mono- and oligo deoxyribo nucleotides with 5'- phosphate and 3'- OH groups. The treatment of RNase free DNase I (Thermo Scientific, USA) to remove genomic DNA contamination from RNA was performed as described in manufacturer's guidelines. The reaction mixture consisted of total RNA (1 µg), 10 x reaction buffer with MgCl2 (1 µL), DNase I (1 µL/µg of RNA) and the volume of reaction was made up to 10 µL. The reaction mixture was incubated at 37 °C for 1 h. 1 µL of 50 mM EDTA was added to the reaction mixture to inactivate the DNase I and the sample was incubated at 65 °C for 15 min. DNase I treated RNA was used for reverse transcription (RT) reaction for cDNA synthesis.

3.2.5 Purification of RNA

Prepared a reaction mixture of 50µL containing 4-6µg of total RNA, 10X DNaseI buffer, DNaseI endonucease, and made the volume with DEPC treated water. After giving 37˚C incubation for 30 mins followed by 1µL addition of 0.25mM EDTA to the reaction mix and kept at 65˚C for about 10mins as described above. After chilling it on ice for several mins added 75µL of DEPC treated water. After gentle mixing, the equal volume (125µL) of chloroform: isoamyl alcohol (25:24) was added to the sample. Mix gently by tapping and then centrifuged at 13000 rpm at 4˚C for 20 mins.Transferred the aqueous phase to a fresh MCT. Then added 1/10th volume of 2.5M LiCl and 2 volume of pre-chilled absolute alcohol (100% ethanol).After proper mixing, kept the reaction at -20˚C for at least 1hour. Then it was centrifuged at 13,000rpm at 4˚C for 45 mins. Discarded the supernatant and take out the tiny trace of ethanol by pipetting after giving a short spin. The pellet was washed with 500µL-

1mL pre-chilled 70% ethanol. The supernatant was discarded. The pellet was dried under room temperature and then dissolved the pellet in 30µl of DEPC treated or nuclease free Milli-Q water.

3.2.6 Denaturing formaldehyde gel for RNA electrophoresis

Total RNA was resolved in 1.2 % denaturing formaldehyde gel. For preparation of gel, 1.2 g agarose was added to 72 mL DEPC treated water and was boiled till the agarose gets dissolved. Upon cooling, 18 mL of formaldehyde and 10 mL of 10X MOPS buffer were added. The contents were mixed by gentle swirling. The molten gel was poured in a casting tray with combs already fitted in. Meanwhile, RNA samples were prepared by mixing about 20 µg of total RNA, 2 µL of 10X MOPS buffer, 4 µL of formaldehyde, 1 µL of 10 mg/mL EtBr and 10 µL of formamide. Samples were heat denatured at 65ºC for 10 minutes and were immediately chilled on ice for 2 minutes. In the mean time 4X RNA loading dye was prepared. For 1 mL 4X loading dye: 100 µL 10X MOPS, 100 µL glycerol, 0.25% BPB were used. For loading the samples, 2 µl of RNA loading dye was added to each RNA sample. The gel was run at 80V for 1-2 hours in 1X MOPS buffer.

3.2.7 Stem-loop quantitative RT-PCR and semi –quantitative RT-PCR

The expression level of mature miRNAs was analysed by stem-loop quantitative reverse transcription- PCR (RT-PCR). Stem-loop reverse transcription was performed with stem-loop primers(SLP) using Super Script III reverse transcriptase (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Approximately, 80–150 ng aliquots of total purified RNA was used for each of the reverse transcription reactions. The reaction mixture contained (80-150) ng of total purified RNA, 10mM dNTPs and required RNase free water to make the total volume 7.5µL and kept the reaction at 65˚C incubation for 5mins and quickly chilled on ice for few mins. Further components were added to the reaction mix in following order such as 5X reaction buffer (4 µL), DTT(0.1M), RNase inhibitor (or, RNase OUT; 0.25µL/ 40U), Super script III reverse transcriptase (0.25µL/200U), stem loop primers and endogenous control *ACTIN7* in order to make the total reaction mixture 20µL. The stem-loop reverse transcription primers were designed such that its 3' end binds to the 3' sequences of mature miRNAs, and a stem-loop is formed with rest of its 5' sequence (Varkonyi-Gasic *et al.*, 2007), Chen et al 2005). A pulsed RT reaction was performed in a thermal cycler with the following modification as follows: 16 ˚C for 30 min: 1 cycle; 30˚C for 10 s, 42˚C for 10 s,

50˚C for 1 s: 60 cycles; 85˚C for 5 min: 1 cycle; holding at 4˚C. The prepared cDNAs were further diluted in 1:2 ratio and used 2µL of that for a single qRT-PCR reaction. The indivisual forward primers were miRNA specific and that excludes the last six nucleotides of the mature miRNA sequence in order to increase its efficiency (Varkonyi-Gasic *et al.*, 2007). The 3'end of the forward primer binds to the 5'end sequence of the specific miRNA. A 5' adapter sequence of around 6-7 nucleotides was added to each forward primers to increase its melting temperature (Varkonyi-Gasic *et al.*, 2007) and also to match the melting temperature of the universal reverse primer (UVRP). The randomly chosen adapter sequences were GC-riched and the qualityof the forward primers were checked by primer design software (IDT). The combination of universal reverse primer (UvRP), which is specific to SLP, and the forward primer was used for PCR amplification of reverse transcribed mature miRNAs. Real-time quantitative PCR was performed with ‘‘Step One Plus’’ real-time PCR system (Applied Biosystems, Foster City, CA, USA) using a SYBR green (Fast) based assay. The primers sequences used for the reaction were given in Table A1. The expression levels of mature miRNAs at different spatio temporal condition in imbibed seeds compare to dry seeds were quantified with respect to the internal / endogenous control *ACTIN7*. Fold change values were calculated using formula FC = $2^{-\Delta\Delta CT}$ (Pfaffl, 2001). Primer sequences used for qRT-PCR for miRNAs are listed in Table A1. For semi quantitative RT-PCR, 1 µL of cDNA was directly used for further PCR amplification using miR specific forward primers, UvRPs and with taq DNA polymerase to get ~ (60-70) bp amplified product with the following PCR program: 95C for 2mins, followed by 28 cycles of 95˚C for 30 s and 64˚C for 60 s and 74˚C, followed by 74˚C for 8mins, followed by 4˚C hold. 3.5% TAE agarose gel was used to visualize the RT amplified products. *ACTIN-7* was used as an endogenous control. Low range/ultra low range /100bp ladder was used to identify the proper band size.

3.2.8 Reverse transcription (RT) PCR and semi- quantitative RT PCR reaction for target genes

For the reverse transcription of the target genes 500ng of total purified RNA was taken. To prepare the first strand cDNA, superscript III reverse transcriptase was used according to the manufacturer’s instruction. The reaction mixture contained 500ng total RNA, Oligo dT (5µM), Random hexamer (10 µM), dNTPs (10mM) and the volume was made upto 10µl with RNase free Milli Q- water. After proper mixing, kept the reaction mix at 65˚C for 5mins and quickly chilled on ice for few mins. In the mean time another reaction mix of 10 µl was

prepared that contains 2µl of 10X RT buffer, 4µl of $MgCl_2$ (25mM), 2µl of DTT(0.1M), RNase inhibitor(1µl/40U) and super script III (1µl/200U). Mixed properly and then mixed the second reaction mix to the first reaction mix so that the total volume would be 20µL. Gave a short spin after proper mixing and transferred the 20µL reaction mix from MCT to PCR tube and put it in a thermal cycler with the following programmes: 25˚C for 10mins, 50˚C for 50 mins, 85˚C for 5 mins and 4˚C for hold. Next added 1µL RNaseH, mixed by gentle tapping and kept it in 37˚C for 20 mins. The cDNAs then diluted in 1:2 ratio and used 2µl of that to a single qRT-PCR reaction. The relative expression level of the target transcripts was measured by performing qRT-PCR with the target gene specific forward and reverse primers, fast SYBR green dye and with nuclease free water. *ACTIN 7* was used as an endogenous control and the fold change values were calculated as described above. Primer sequences used for qRT-PCR for target genes are listed in Table 2. For semi quantitative RT-PCR, 1µl direct cDNAs was used together with target gene specific forward and reverse primers and taq DNA polymerase to get an amplicon length (cleavage product of the target gene) of approx 100-250 bp length with the following PCR program: 95˚C for 2mins, followed by 30 cycles of 95˚C for 30 s and 64˚C for 60 s and 74˚C, followed by 74˚C for 8mins, followed by 4˚C hold. 3.5% TAE agarose gel was used to visualize the RT amplified products. *ACTIN-7* was used as an endogenous control. 100bp marker was used to identify the exact required band.

3.2.9 Genomic DNA isolation

Small piece of young clean tissue was crushed with plastic pestle (treated with 0.25 N HCl for 1 hr and washed properly) in microcentrifuge tube. 500 µL of DNA Mini prep buffer (1 M Tris-Cl, pH7.5, 5 M NaCl, 0.5 M EDTA pH 8; 20 % SDS) was added to the tube and tissue was further homogenised properly. Homogenate was centrifuged at 13,000 x g, 10 mins, 4°C. Supernatant was transferred to fresh microcentrifuge tube and the DNA was precipitated with equal volume of chilled isopropanol. The tube was centrifuged at 13,000 x g, 20 mins, 4°C. DNA pellet was washed with 70 % ethanol (v/v), air dried and dissolved in 50 µL of sterile water or TE buffer. This protocol was a modification of earlier one (Neff *et al.*, 1998).

3.2.10 Spectrophotometric estimation of nucleic acid

The quantity and purity of nucleic acid was determined by measuring the absorbance at 230, 260 and 280 nm using Nano Drop 1000 Spectrophotometer (Thermo Scientific, USA). The

purity of nucleic acid was determined by $A_{260/280}$ and $A_{260/230}$ ratio. DNA and RNA were further analyzed on agarose gel.

3.2.11 Polymerase Chain Reaction (PCR)

Specific DNA fragments were amplified with PCR using Taq DNA polymerase. The PCR mixture consisted of 10 X PCR buffer (10 mM Tris-Cl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$), 200 µM each dNTP, 0.2 µM each forward and reverse primer, 200 pg/µL template, 0.05 U/µL of *Taq* polymerase. The amplification reaction was done in a thermocycler (Applied Biosystems, CA, USA). The amplification reaction was performed with modification of the Taq polymerase supplier company manual and according to our requirement. Below is an example of PCR with Himedia Taq polymerase.

Step	Temperature	Time	
Initial denaturation	94-95 ˚C	2 min	
Denaturation	94-95 ˚C	30 sec	25-35 cycles
Annealing	56-68 ˚C	1 min	
Extension	72/74 ˚C	1 min per kb	
Final extension	72/74 ˚C	5-10 min	
Hold	4-16˚ C		

3.2.12 Agarose gel electrophoresis

Agarose gel electrophoresis was performed for qualitative and quantitative analysis of the DNA / RNA samples. The gel was prepared by dissolving agarose in 1 x Trisacetate EDTA (TAE). The concentration of the gel ranged between 0.8 - 2 % (w/v) depending on the size of DNA fragment to be resolved (shorter the fragment higher the agarose concentration used for analysis). 6 x DNA loading dye was added to sample prior to loading into the gels. Ethidium bromide solution (0.5 µg/ mL) was added to the gel to stain the DNA / RNA fragment. The gel was rinsed briefly with water to reduce the background staining. The DNA detection was done on Benchtop 3UV transilluminator (UVP, USA).

3.2.13 Elution of DNA from agarose gel

The PCR amplified required DNA fragment was electrophoresed on agarose gel. The DNA band was cut using sterile blade and collected in a 1.5 mL sterile microcentrifuge tube. The extra agarose gel was removed to minimize the size of gel slice. Gel elution was performed using Qiaquick gel extraction kit (Qiagen) using manufactures protocol. The excised pieces of agarose gel containing the desired DNA fragment were weighed and accordingly 3 volumes of QG buffer was added. The mixture was incubated at 55°C for 10 min, with random mixing, till the gel dissolves completely to give a yellow color solution. The mixture was loaded onto QIA quick spin column and column binding was performed by centrifugation at 10,000 rpm, 1 min at room temperature. This step was repeated 3-4 times to ensure high yield. The flow through was discarded and the column was washed twice with 750 µL buffer PE. 40 µL of Elution Buffer was added to the center of the column and centrifuged for 2 mins to elute the DNA. The eluted DNA was stored until further use at -20 ^{o}C.

3.2.14 Ligation reaction

100 ng plasmid vector was ligated to insert DNA in a molar ratio of 1:3. The reaction mixture of ligation contained:

Vector	100ng
Insert	3X in molar ratio
Ligation Buffer (10X)	5 µL
T4 DNA Ligase	10U
Milli Q Water	up to 10 µL

Ligation mixture was gently mixed with pipette, briefly centrifuged and incubated at 22 ^{o}C overnight. The *E. coli* competent cells were transformed with 5 µL of ligation mixture.

3.2.15 Preparation of Escherichia coli DH5α competent cells

E. coli (DH5α) competent cells were prepared by $CaCl_2$ method as mentioned in (Sambrook *et al.*, 1989). All the steps were performed aseptically in laminar flow hood. *E.coli* cells were streaked on LBA plate from 25 % glycerol stock (without antibiotics) and incubated (16 hrs, 37°C). A single bacterial colony was inoculated in 5 mL LB medium and kept in an incubator shaker for 16 h at 37 ˚C with vigorous agitation (200 rpm). 1 % of the overnight grown primary culture was inoculated in 100 mL fresh LB medium in 1 L flask. The culture was incubated for 3 h at 37˚C with vigorous agitation (200 rpm) and the growth of the culture was

monitored. It is important that the density of the culture does not exceed 10^8cells/mL (OD_{600} ≤ 0.5). Bacterial cells were transferred to sterile, ice cold 50 mL bottles. Culture was cooled to 0˚C by keeping the bottles on ice for 10 min. Bacterial cells were harvested by centrifugation at 5000 rpm for 10 min at 4˚C. Bacterial pellet was gently resuspended in 20 mL of ice cold $MgCl_2$-$CaCl_2$ solution. Cells were pelleted at 5000 rpm for 10 min at 4˚C. Bacterial cells were gently re-suspended in 2 mL of ice cold 0.1 M $CaCl_2$ containing 50 % glycerol.The suspension was dispensed in aliquots of 100 mL in sterile, pre-chilled1.5 mL microcentrifuge tubes and immediately freezed in liquid nitrogen and were stored at -80˚C until further use and transformation efficiency of competent cells was verified time to time with known quantity of plasmid DNA. The cells were used until their transformation efficiency was maintained up to 10^6 colony/µg of plasmid DNA.

3.2.16 Transformation in DH5 α E. coli competent cells

E. coli (DH5α) competent cells were transformed with ligation mixture by heat shock method as described in (Sambrook *et al.*, 1989). Plasmid DNA (20 pg – 50 ng) or ligated DNA mixture was added to 100 µL of competent cells and incubated on ice for 30 min with intermittent mixing. Cells were given heat shock at 42˚C for 90 sec and then chilled on ice for 5 min. 1 mL LB medium (without any antibiotic) was added to the cells and incubated on shaker for 45 min at 37˚C with shaking at 200 rpm. Transferred the appropriate volume (~100 µL per 90 mm plate) of transformed competent cells on LBA plate containing appropriate antibiotic and the plate was incubated at 37˚C for 16 h.

3.2.17 Confirmation of the presence of insert by colony PCR

The presence of the insert in the clone was confirmed by the colony PCR using gene specific primers. Individual colonies were picked from the plate and mixed in 10 µL of sterile water in 0.2 mL microcentrifuge tubes. The cells were lysed by heating at 98˚C for 10 min. 2 µL of lysed cells was used as the template for PCR. Number of PCR cycles and cycling conditions were adjusted according to the annealing temperature of primers and the amplicon size.

3.2.18 Preparation of glycerol stocks

To make the glycerol stocks, bacterial cultures from single recombinant colony was inoculated in 5 mL LB media and incubated overnight at 37˚C with vigorous shaking (200

rpm). In cryovials, equal volumes of bacterial culture and 50 % sterile glycerol was added. The glycerol stocks were stored at -80˚C.

3.2.19 Plasmid DNA isolation by alkaline lysis method (Mini Prep)

Alkaline lysis method (Birnboim and Doly, 1979) was used for small-scale isolation of plasmid DNA. A single isolated colony was inoculated in 2 mL LB medium containing appropriate antibiotic and grown overnight at 37˚C with vigorous shaking. Bacterial cells were harvested by centrifugation at maximum speed for 30 sec at 4˚C in a microfuge. The supernatant was removed and the cells were resuspended in 100 µL of ice-cold alkaline solution I by vigorous vortexing. 200 µL of freshly prepared alkaline solution II (1 % SDS and 0.2 N NaOH) was added to the bacterial suspension and the cells were lysed by gently inverting the tube 5 times. The lysate was kept at room temperature for 2 min. 150 µL of ice-cold alkaline solution III to the lysed cells and the contents were mixed by inverting the tube several times. The lysate was kept on ice for 5 min. Lysate was centrifuged at maximum speed for 5 min at 4˚C in a microfuge and the supernatant was transferred to a fresh microcentrifuge tube. Equal volume of phenol: chloroform: isoamyl alcohol (25: 24: 1 v/v) solution was added to the supernatant. The aqueous and organic phases were mixed by vortexing. The emulsion was centrifuged at maximum speed for 5 min at 4˚C. The upper aqueous phase was transferred to a fresh tube. The nucleic acid in aqueous phase was precipitated with equal volume of isopropanol at room temperature. The precipitated nucleic acid was collected by centrifugation at maximum speed for 10 min at 4˚C in a microfuge. The pellet was washed with 1 mL of ice cold 70 % ethanol and the DNA was recovered by centrifugation at maximaum speed for 5 min at 4˚C in a microfuge. The traces of ethanol were removed from the tube by evaporation. The DNA was dissolved in 50 µL of TE (pH 8.0) containing DNase free RNase A (20 µg/mL). The DNA was stored at -20˚C.

3.2.20 Purification of plasmid DNA by PEG precipitation

For purification of plasmid DNA 8 µL of 4 M NaCl and 40 µL of 13 % (w/v) polyethylene glycol (PEG 8000) was added to the 32 µL plasmid DNA. The mixture was incubated on ice for 30 min. DNA was pelleted by centrifugation at maximum speed for 20 min at 4ºC. The pellet was washed with 500 µL of 70 % ethanol and dissolved in 30 µL of sterile water.

3.2.21 Sequencing and sequence analysis

Sequencing of cloned DNA was performed on ABI 3730 XL 96 capillary sequencer. DNA sequences were analysed using bioinformatics tools like reverse complement (http://reverse-complement.com/) nucleotide Blast (http://blast.ncbi.nlm. nih.gov/Blast. cgi) and CLUSTALW.

3.2.22 Transformation in Agrobacterium competent cells

The recombinant plasmid was transferred into *Agrobacterium tumefaciens* by freeze thaw method (Holsters *et al.*, 1978). For the preparation of competent cells, *A. tumefaciens* (GV3101) strain was grown in 50 mL LB medium at 28˚C with vigorous shaking until the OD_{600} reached approximately 0.5. The culture was chilled on ice and centrifuged at 3000 × g for 5 min at 4˚C. The pellet was resuspended in 1 mL of ice cold $CaCl_2$ (20 mM) and 0.1 mL aliquots were dispensed in pre-chilled centrifuge tubes and stored at -80˚C. Transformation of Agrobacterium with various plasmid constructs was done by mixing 1 µg of DNA with competent cells followed by immediate freezing in liquid nitrogen. Subsequently, cells were thawed by incubating at 37˚C for 5 min. Thereafter, 1 mL of LB medium was added to the tube and incubated at 28˚C for 3 hrs with shaking. Approximately 100 µL bacterial cells were spread on a LB agar plate supplemented with 50 µg/mL kanamycin and 25 µg/mL rifampicin and incubated at 28˚C. Transformed colonies that appeared after 2-3 days were analyzed by colony PCR. The positive clones were confirmed by restriction digestion.

3.2.23 Transformation of *A. thaliana*: Floral dip method

The transformation of *A. thaliana* was performed using floral dip method as described by (Clough and Bent, 1998) with some modifications. Approximately 20-22 flowering *Arabidopsis* plants were used in each transformation event. Seeds containing pods were manually cut before floral dip transformation. *Agrobacterium* culture was grown with appropriate antibiotics [rifampicin (25 µg/mL) and kanamycin (50 µg/mL)] upto OD_{600} 0.9 - 1.0. 250 mL of *Agrobacterium* culture was centrifuged at 6000 rpm for 15 min at room temperature. The supernatant was discarded and cells were resuspended in 500 mL of 5 % sucrose solution containing 120 µL of Silwet L-77 (Lehle Seeds, USA). The unopened buds of *Arabidopsis* were dipped into the bacterial suspension. Plants were covered to maintain humidity overnight. Plants were grown at 21°C under long day conditions. T1 seeds obtained

after floral dip method were used for hygromycin (25 µg/mL) selection and positive plants were transferred to soil for further growth.

3.2.24 β-glucosidase (GUS) histochemical analysis

GUS histochemical analysis was done as described by (Jefferson *et al.*, 1987; Rai *et al.*, 2015). During germination, the highest expression of miR390b was observed at 24h germination condition after imbibition both at room temperature and 4˚C. To check this *in vitro*, we imbibed *pMIR390b::GUS* transgenic homozygous seeds 24h in room temperature and 4˚C. After that, the germinating seeds were transferred to GUS staining buffer (100 mM sodium phosphate buffer pH 7.2, 10 mM EDTA, 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, 2 mM X-Gluc (5-bromo-4-chloro-3indolyl-β-D-glucuronic acid), 0.1% tritonX-100, 20% methanol) in sterile MCTs and incubated at 37^{o}C for 16-20 hrs. A blue coloured end product was produced and accumulated as GUS enzyme cleaves the substrate, X-Gluc. Samples were cleared with a chloral hydrate: glycerol: water solution (8:1:2, v/v/v) and mounted on slides with cover slips and checked the gus histochemical reactions under stereomicroscope.

3.2.25 miRNA microarray

For miRNA microarray, total RNA was isolated, and the quality of RNA was tested as described above. Approximately 250 ng of total RNA was used from each sample in two biological replicates for setting up *in-vitro* transcription reaction (Affymetrix, USA). The RNA purification, fragmentation and hybridization reactions were done using *Arabidopsis* miRNA chip miRNA v1.0 array (Affymetrix, USA). Washing and scanning were performed as suggested in Affymetrix Gene Chip total RNA procedure. Results obtained after scanning were analyzed using Gene spring GX software v11.5. The processed raw signal intensities were subjected to normalization using the same software. Expression of differentially expressed and selected miRNA genes in microarray were further validated by real time stem-loop quantitative RT-PCR (qRT-PCR).

3.2.26 Tetrazolium(TZ) assay

The Tetrazolium (TZ) assay was performed as described by (Verma and Majee, 2013; Wharton, 1955). Seeds were soaked in a 1% solution of 2, 3, 5-triphenyl tetrazolium chloride (Sigma) and incubated in darkness at 30°C for 48h. The colourless TZ becomes carmine red

coloured water-insoluble 2, 3, 5-triphenyl formazan by hydrogen transfer reaction catalysed by the cellular dehydrogenases present in the living cells (Verma and Majee, 2013). As a consequence of that, viable seeds containing living cells stain red and non viable or dead seeds remain unstained. Thus, the viability of seeds can be estimated by the staining pattern and the color intensity Heat-killed seeds (incubated at 100°C for 1 h) were used as a negative control.

3.2.27 Germination assay under stress conditions

Small RNA biogenesis pathway mutant allels *sgs3-11* homozygous seeds along with its control Wt Col seed lines were grown in identical controlled conditions as described earlier. Mature seeds were harvested on the same day from all individual plants along with its control and then stored in the dark under dry conditions at room temperature (22 ± 2 °C) for further use. The germination assay was carried out in triplicate samples of 50 age-matched seeds of *Arabidopsis* in each cases. To evaluate germination under stress conditions, seeds were surface-sterilized, plated on to half-strength Murashige and Skoog (MS) agar media (Himedia) supplemented with NaCl (150 mM), Mannitol (200mM), ABA (5µM) and kept in the dark at 4°C for 3 days stratification before being allowed to germinate in controlled culture room conditions (22 ± 2ºC) with a 16/8 h light/dark cycle. For heat stress, sterilized and stratified seeds were incubated at 45ºC for1h and then plated onto half strength MS agar plates. For cold stress, surface sterilised and stratified seeds were kept at 4°C with a 16/8 h light/dark cycle in cold room for four days and then transferred to normal growth condition (as described above). Seed germination was determined to be completed when the radicle protruded beyond the testa and was assessed for 5 to 6 days. In all experiments, at least three independent biological replicate were evaluated. The rate of germination was calculated according to the following (Kaur *et al.*, 2015a).

$$\text{Germination percentage (\%)} = \frac{\text{Number of germinated seeds}}{\text{Number of total seeds}} \times 100$$