RESULTS AND DISCUSSION

Chapter4.1- Identification of miRNAs related to seed physiology or germination.

4.1.1 Introduction

Seed germination process, which marks the transition from seed to seedling stage, plays a crucial role in the complete life cycle of higher plants. Dormant seeds germinate when the environmental conditions, such as temperature, water or humidity are favorable (Willmann *et al.*, 2011). The contrasting physiological event of seed germination is dormancy, which is regarded as the temporary failure or block of a viable seed to complete germination under seemingly unfavorable conditions and is an adaptive feature for optimizing the timing of germination (Das *et al.*, 2015). A complex co-ordination of different molecular, physiological and environmental factors governs the dynamic and triphasic process of seed germination (Das *et al.*, 2015; Weitbrecht *et al.*, 2011).

Small non-coding RNAs (of 19–24 nucleotides length) play diverse roles in growth, development, morphogenesis, and stress responses in both plants and animals (Axtell *et al.*, 2006; Bartel, 2004; Chen, 2012a; Sanan-Mishra and Mukherjee, 2007; Sanan-Mishra *et al.*, 2013). Recently, it has been shown that proteins involved in small RNA biogenesis such as DCL1 plays significant role in embryogenesis and seed development in *Arabidopsis* (Willmann *et al.*, 2011). Previous study reported 115 known miRNAs and 167 novel miRNAs in maize seeds imbibed for 24h, which is very early stages of seed germination (Wang *et al.*, 2011). They identified 24 conserved miRNA families in both dry and imbibed maize seeds through deep sequencing. Few novel and known miRNAs and some of their targets were validated (Li *et al.*, 2013). Deep sequencing of small RNAs from rice seed embryos identified 59 known and 230 novel miRNAs differentially regulated in the early stages of seed germination (He *et al.*, 2015). Moreover, in eudicot (*Nelumbo nucifera)* seed germination, 145 known and 78 novel miRNAs were identified (Hu *et al.*, 2016). These reports indicate the association of more number of small RNAs in the dynamic seed germination procedure. However, little is known about the condition specific regulation of small RNAs and their targets, which are potentially important contributors to the early stages of *Arabidopsis* seed germination.

In this study, we have identified both conserved and non-conserved small RNAs in both dry and imbibed *Arabidopsis* seeds under stratified (4˚C) and non-stratified conditions. We used miRNA-microarray approach to identify miRNAs functionally relevant in early stages of seed germination.

4.1.2 Results:

4.1.2.1 *Expression of miRNAs are differentially regulated in seeds under germination conditions*

In order to find out the microRNAs (miRNAs) involved in seed germination in *Arabidopsis* we have isolated the total RNAs by seed specific guanidine hydrochloride method (Singh *et al*, 2003) from the Wild type Columbia (Col-0) *Arabidopsis* seeds at different spatio-temporal conditions. The conditions were dry seed (zero hour of imbibition) and imbibed seed at 12 h, 24 h and 48 h at room temperature and 4˚C each. We have pulled out the total RNAs isolated from room temperature at different time points and the total RNAs isolated at cold condition (4˚C) at different time points. Quality of the total RNA was checked by Agilent bio-analyzer (**Fig 4.1.3**) and also in 1.2% TAE agarose gel (**Fig 4.1.1**) and in MOPS-Formaldehyde gel (**Fig 4.1.2**). Good quality total RNA was used for *Arabidopsis* miRNA chip miRNA v1.0 array (Affymetrix, USA), and a three way comparison was made to identify differently expressed miRNAs. A total of 58 differently expressed miRNAs were found to be either upregulated or downregulated in *Arabidopsis* in comparison to dry seed. Among the list of differentially expressed miRNAs, 15 miRNA genes were selected based on their p-values($\leq$ 0.05) and fold change values($\geq$ 2) for further validation, using stem loop qRT-PCR.

We identified a total of 58 miRNAs differently expressed in (i) IS-4˚C vs. DS (**Fig 4.1.4a**), (ii) IS-RT vs. DS (**Fig 4.1.4b**) and lastly, (iii) IS-4˚C vs. IS-RT (**Fig 4.1.4c**). We found that specific miRNAs were upregulated and down regulated in each of the three cases. The results identified 29 miRNA genes in IS- 4˚C vs. DS (**Fig 4.1.4a**), 28 miRNA genes in IS-RT vs. DS (**Fig 4.1.4b**) and 23 miRNA genes in IS-4˚C vs. IS-RT (**Fig 4.1.4c**). Among the 29 miRNAs in IS- 4˚C vs. DS (**Fig 4.1.4a**), only two miRNAs were upregulated and rest were downregulated. In case of 28 miRNAs in IS-RT vs. DS (**Fig 4.1.4b**), five miRNAs were upregulated and rest were downregulated, whereas among 23 miRNAs in IS-4˚C vs. IS-RT (**Fig 4.1.4c**), only eight miRNA genes were upregulated and rest were downregulated.

Figure 4.1.1 Total RNAs from *Arabidopsis* Wt Col seeds in TAE Agarose Gel (1.2%). [1- **DS**, 2-**12h/RT**, 3- **12h/4˚C**, 4-**24h/RT**, 5-**24h/4˚C**, 6- **48h/RT**, 7- **48h/4˚C** , (M)- **marker]**.

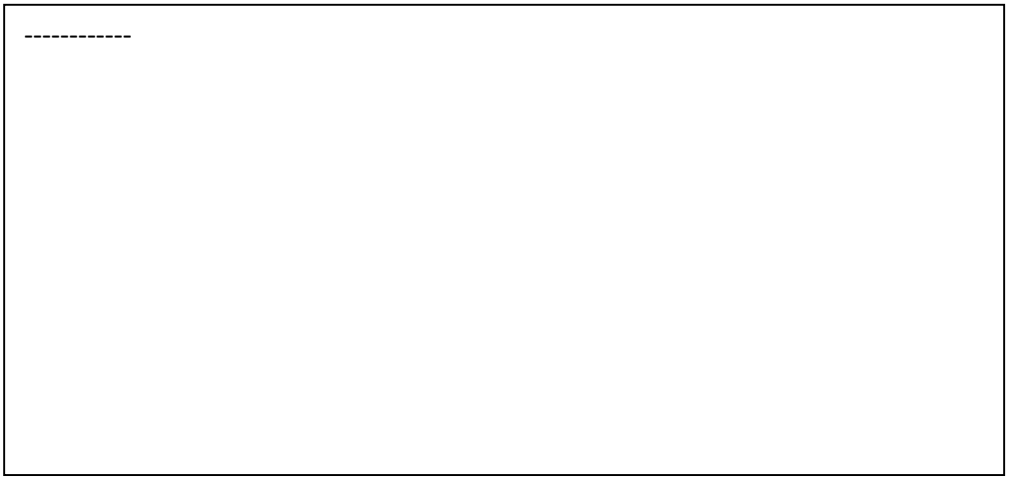

Figure 4.1.2 Total RNAs from *Arabidopsis* Wt Col seeds in MOPS-formaldehyde gel. [(A) and **(B):** Positive control RNAs from leaf and shoot tissues, (**C**): Empty lane having some RNA diffused into it from neighbouring well/s. **1- DS, 2-12h/RT, 3- 12h/4˚C, 4-24h/RT, 5-24h/4˚C, 6- 48h/RT, 7- 48h/4˚C**

Figure 4.1.3 Quality of the RNA was checked by Bio-analyzer, showing RIN value-7.1 (almost each of the 7 above mentioned spatio-temporal conditions we obtained the same quality of RNAs).

Table 4.1.1. List of miRNAs differentially expressed in Arabidopsis seeds in imbibed seeds compare to dry seed (0 hr imbibitions) in a miRNA microarray analysis.

Probe ID	P value	Fold change	Regulation
ath-miR165a_st	0.031280305	1.89	up
ath-miR165b_st	0.03036148	1.85	up
ath-miR172a_st	0.008049283	-2.95	down
ath-miR390b_st	0.026625743	-1.68	down
ath-miR160a_st	0.03898194	-1.34	down
ath-miR156h_st	0.013480782	1.36	up
ath-miR157a_st	0.02468334	-1.45	down
ath-miR157c_st	0.032628458	1.36	up
ath-miR157d_st	0.04986837	-1.53	down
ath-miR164a_st	0.027664782	-2.04	down
ath-miR169b_st	0.00931509	-2.39	down
ath-miR161.1_st	0.021728164	1.32	up
ath-miR399a_st	0.017388277	-1.66	down
ath-miR399b_st	0.00114095	-6.97	down
ath-miR399c_st	0.034463275	-4.65	down
ath-miR824_st	0.00269617	-1.66	down
ath-miR834_st	0.009251686	-3.06	down
ath-miR854a_st	0.019733708	-4.12	down
ath-miR2112-5p_st	0.005841855	-1.20	down

Through Venn diagram representation (**Fig. 4.1.4d**), we observed that 10 miRNA genes were common in both IS-4˚C vs DS and IS-RT vs. DS and they are miR161.2; miR169b, d, e; miR399a, b; miR824 and miR854a, c, e. Similarly, 8 miRNA genes were common in IS-4˚C vs. DS and IS-4˚C vs. RT and they are miR160b, c; miR161.2; miR164a, miR395a; miR447a and miR854a; while 6 miRNA genes were common in IS-RT vs. DS and 4˚C vs. RT and they are miR157c; miR161.2; miR834 and miR854a, b, d. Only two miRNAs, miR161.2 and miR854a, were common in all three cases here. Analysis of the data showed that amongst the differently expressed miRNAs, most abundant families were miR169 and miR854, with 11

and 5 family members, respectively (**Fig. 4.1.4e**). Among these miRNAs, eighteen precursor miRNA families were selected based on their p-values (≤ 0.05) and fold change values (≥ 2.0) across the three data sets for validation. Among these eighteen precursor miRNA, miR157a, miR157c, miR157d consist of same mature sequence; so we took only one mature sequence of miR157 (**Fig. 4.2.1f**) among the three. Also miR399b and miR399c contain the same mature sequence in *Arabidopsis*, therefore we had chosen only one among them (**Fig. 4.2.2e)**.

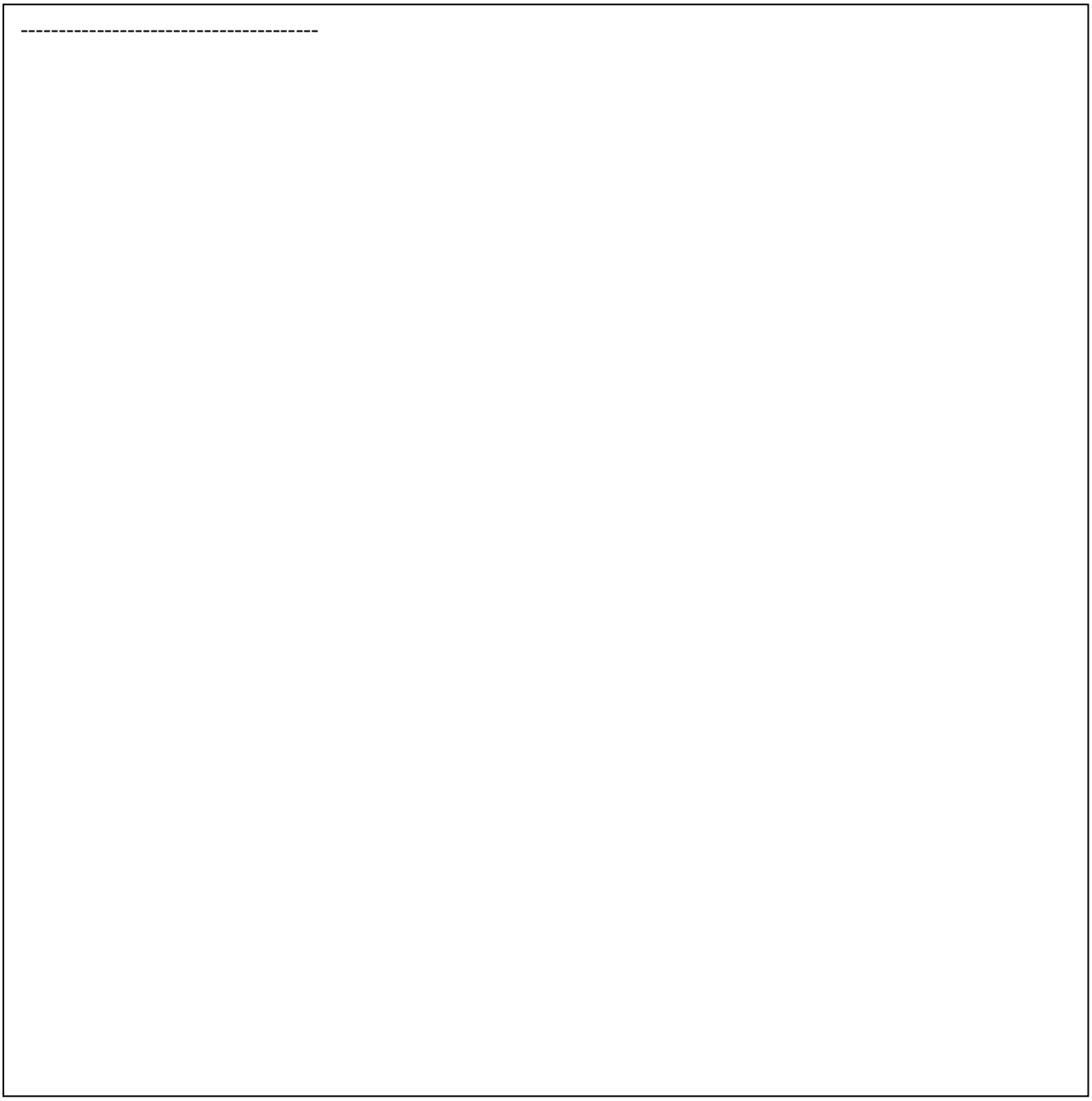

Figure 4.1.4 Expression patterns of miRNAs at different seed germination conditions in *Arabidopsis thaliana based on* Microarray. (a) **Heat map** analysis at Cold imbibitions (**4˚C**) vs. Dry

seeds (**DS**). (b) **Heat map** analysis at Room Temperature (**RT**) vs. Dry seed (**DS**). (c) Heat map analysis at Cold imbibitions **(4˚C**) vs. Room temperature (**RT**).The bars in the heat map represent the scale of expression levels of the miRNAs. During **Microarray** we have pooled all the Cold imbibed (**IS-4˚C**) and Room Temp imbibed (**IS-RT**) total RNAs separately, and the microarray was performed using two biological replicates for each individual samples. **(**d) The **Venn diagram** represents the comparison of the known miRNAs in between three different conditions (**DS, IS-4˚C and IS-RT**) used in the Microarray Experiment. (e) The graph represents the miRNA families and their respective family members which were detected in our Microarray analysis. [For generating heat map we used **MeV**(**Multiple Experiment Viewer) (http://mev.tm4.org**/)].

The other miRNAs for validation were miR165/166 (**Fig 4.2.1a**), miR172a (**Fig 4.2.1b**), miR390b (**Fig 4.2.1c**), miR160a (**Fig 4.2.1d**), miR156h (**Fig 4.2.1e**), miR164a (**Fig 4.2.2a**), miR169b **(Fig 4.2.2b**), miR161.1 (**Fig 4.2.2c**), miR399a (**Fig 4.2.2d**), miR824 (**Fig 4.2.2f**), miR834 (**Fig 4.2.2g**), miR854 (**Fig 4.2.2h**) and miR2112-5p (**Fig 4.2.2i**).

Chapter 4.2- Validation of miRNA expression, identification of targets,functional annotation and tissue specific expression studies.

4.2.1 Introduction

Seed germination paves the way for the dormant embryo to establish itself as a new plant marking the first critical step in postembryonic plant growth and development. Germination starts with the uptake of water (imbibition), followed by induction of transcription, translation, energy metabolism, and cell division processes. Although small RNAs have been implicated in many developmental processes, their role during seed germination stages and conditions remained elusive. Here we show that seed germination conditions, like imbibition and temperature, dynamically regulate the expression of many developmentally important miRNAs and their targets.

We have identified 58 miRNAs belonging to 30 different families at different seed germination conditions. Amongst these, we validated the expression of 15 such miRNAs that we have selected based on P-value and fold change values, and their 27 selectd targets based on their expectation values at 12h, 24h and 48h imbibition at both temperatures. Interestingly, differential expression of miR390, which targets trans-acting siRNA locus (*TAS3)* derived transcripts, resulted in alteration of tasiR-*ARF* mediated regulation of expression of target *AUXIN RESPONSE FACTORs (ARF2/3/4).* Our results suggest that the dynamic expression of several miRNAs, their targets, and a crosstalk between miRNA and tasiRNA pathways contribute to the regulation of seed germination in *Arabidopsis thaliana*.

4.2.2 Results

4.2.2.1 Validation of expression reflects dynamic spatiotemporal regulation of miRNAs during seed germination.

The different time points used for validation of both the miRNAs were 0 h (DS), 12h/ RT, 12h/ 4˚C, 24h/ RT, 24h/ 4˚C, 48h/ RT and 48h/ 4˚C. The highest expression of miR165/166 was observed in case of 12h/ RT (**Fig 4.2.1a**), which drastically changed in case of 12h/ 4˚C. The lowest expressions were observed in 24h/ RT and 48h/ RT (**Fig 4.2.1a**). But it showed high expression at 24h/ 4˚C and nearly equal expression to DS at 48h/ 4˚C (**Fig 4.2.1a**). We observed highest expression level of miR172a (**Fig 4.2.1b**) during 24h/ 4˚C and then 12h/ 4˚C compare to DS (**Fig 4.2.1b**), and the level reduced at the later stage of germination at 48h/ 4˚C (**Fig 4.2.1b**). Interestingly, its expression reduced at RT. We

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Figure 4.2.1 Quantitative RT-PCR based validation of the Expression of Mature miRNAs in Imbibed seeds, in comparison to Dry seeds, in *Arabidopsis thaliana*. (The Expression profiles generated upon **qRT-PCR** slightly varied with the miRNA expressions depending upon Microarray analysis, probably due to the pooling of total RNAs we used during Microarray experiment). The **qRT-PCR** validation of miRNAs were done at six different germination condition as **12h/RT, 12h/4˚C, 24h/RT, 24h/4˚C, 48h/RT** and **48h/4˚C ,** each compared to **Dry seed**. The Expression values showed here representing the means of **three biological replicates ± standard deviation (sd**). The *Arabidopsis **ACTIN7*** was used for each samples as an **Endogenous control**. **(a) ath-miR165/166; (b) ath-miR172a; (c) ath-miR390b; (d) ath-miR160a; (e) ath-miR156h; (f) ath-miR157a/or, c/or, d**. In *Arabidopsis,* miR157a, c and d have the same mature sequence. **Asterisks indicate significant statistical differences: ***P < 0.001, **P < 0.01, *P <0.05 [One-way ANOVA].**

observed high level of expression of miR390 (**Fig 4.2.1c**) compared to DS in almost all the conditions that we used. We found higher expression of miR390 (**Fig 4.2.1c**) in both 24h/ RT and 24h/ 4˚C conditions, and the amount of this expression decreases at later stages of germination like 48h (RT/ 4˚C) (**Fig 4.2.1c**). The highest expression of miR160a was observed at 24h/ 4˚C(**Fig 4.2.1d**), then gradually in decreasing order at 24h/ RT, 12h/ 4˚C, 12h/ RT compared to DS.We observed the lowest and almost same expression in case of 48h/ RT and 48h/ 4˚C (**Fig 4.2.1d**). Similar expression patterns were observed in case of both miR156h (**Fig 4.2.1e**) and miR157a/c or, d (**Fig 4.2.1f**). The miR157 families a, c and d constitute the same mature miRNA sequences. In both of the cases, we observed highest level of expression at 24h/ 4˚C (2 fold in miR156 and ~5 fold in miR157). During cold imbibition, these miRNAs showed higher expressions compared to RT (**Fig 4.2.1e, f**). Both the miRNAs showed reduced expression pattern with increasing time.

Figure 4.2.2 The validation of Expression of Mature miRNAs in imbibed seeds, in comparison to Dry seeds, in *Arabidopsis thaliana* by quantitative RT-PCR method. The qRT-PCR validation of miRNAs were done at six different **Germination conditions** as **12h/RT, 12h/4˚C, 24h/RT, 24h/4˚C, 48h/RT and 48h/4˚C** each compared to **Dry seed**. The Expression values showed here representing the means of **three biological replicates ± standard deviation (sd**). The *Arabidopsis **ACTIN7*** was used for each samples as an **Endogenous control**. (a**) ath-miR164a; (**b**) ath-miR169b; (**c**) ath-miR161.1; (**d**) ath-miR399a; (**e**) ath-miR399b/or, c** (in *Arabidopsis,* miR399b and c have the same mature sequences); (f**) ath-miR824; (**g**) ath-miR834; (**h**) ath-miR854; (**i) **ath-miR2112-5p. Here Asterisks indicate significant statistical differences: ***P < 0.001, **P < 0.01, *P <0.05 [One-way ANOVA].**

We found maximum expression of miR164a (~ 4.5 fold) at 24h/ RT (**Fig 4.2.2a**) and lowest expression at 12h/ RT (**Fig 4.2.2a**). Also during 24h cold imbibed condition, miR164a was showing upregulation compare to DS (**Fig 4.2.2a**). Mainly, during very early cold imbibition stages (12h, 24h) miR164a showed high expression (**Fig 4.2.2a**) but it gradually decreases at the later stages (48h). Except 12h/ RT, in other germination conditions we got upregulation of miR169b (**Fig 4.2.2b**). The maximum expression of this miRNA was observed at 24h/ RT (**Fig 4.2.2b**) like miR164a (**Fig 4.2.2a**). The highest expression of miR161.1 was observed at 24h/ 4˚C and then 12h/ 4˚C (**Fig 4.2.2c**), and the lower expressions were observed at 48h/ RT and 12h/ RT, which were almost same (**Fig. 3c**). At 48h/ 4˚C, almost similar expression of miR169b was observed in both dry and imbibed seeds. Interestingly, in spite of single nucleotide difference in miR399a and miR399b/c, we observed a significant variation in their expression levels at different germination stages. Highest expression of miR399a (**Fig 4.2.2d**) was at 24h/ RT and then 48h/ 4˚C, whereas highest expression of miR399b/c (**Fig. 3e**) was at 12h/ 4˚C and then 24h/ RT. But expression of both of the miRNAs was less at 12h/ RT and 48h/ RT compared to DS. Interestingly, all the miR399 (a, b and c) were induced in case of cold imbibition (4˚C) rather than RT. This indicates that cold imbibition plays significant role in miR399 expression during seed germination. In this study, we found higher expressions of miR824 (**Fig 4.2.2f**) in almost every stages, especially in cold imbibed conditions. We observed the highest expression of this miRNA at 24h/ 4˚C (**Fig 4.2.2f**). Although miR834 was highly up regulated in every stages of germination compared to DS (**Fig 4.2.2g**) and the maximum expression level (~40 fold) was observed at 24h/ RT (**Fig 4.2.2g**). In case of miR854 (Fig 4.2.2h) and miR2112-5p (**Fig 4.2.2i**), highest expression of ~275 fold and ~130 fold respectively were observed at 24h/ RT. Over all, the expression pattern of most mature miRNAs were dynamically differentially regulated at different time points under various

germination conditions. Since miRNAs negatively regulate their targets, these results indicated possible differential regulation of their targets as well, which we further tested.

4.2.2.2 Differential expressions of miRNAs negatively regulate the expression of target transcripts during seed germination conditions.

The targets of the validated miRNAs were identified through "psRNATarget" tool (Dai and Zhao, 2011). Our prediction indicated more than one targets for each miRNA under standard settings of prediction tool. Some of the targets were novel and not indicated earlier. For experimental validation of miRNA-target expression correlation, we chose the targets for each miRNA considering its respective expectation

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Figure 4.2.3 Validation of the Expression of Mature miRNAs in imbibed seeds, in comparison to Dry seeds, in *Arabidopsis thaliana* by quantitative RT-PCR method. The qRT-PCR validation of miRNAs were done at six different **Germination conditions** as **12h/RT, 12h/4˚C, 24h/RT, 24h/4˚C, 48h/RT and 48h/4˚C** each compared to **Dry seed**. The Expression values showed here representing the means of the **three biological replicates ± standard deviation (sd)**. The *Arabidopsis **ACTIN7*** was used for each and every samples as an **Endogenous control**. **(**a-d**) targets of miR165/166; (**a**) *PHB*; (**b**) *PHV*; (**c**) *ATHB8*; (**d**) *ATHB15*.** (e-g**) targets of miR160; (**e**) *ARF10*; (**f**) *ARF16*; (**g**) *ARF17*. (**h-i**) targets of miR156/ or, miR157; (**h**) *SPL3;* (**i**) *SPL9*. Here Asterisks indicate significant statistical differences, ***P < 0.001, **P < 0.01, *P <0.05 [One-way ANOVA].**

value of 0.5 to 2.5 range. In cases where more than two significant targets were identified, all of them were used in the validation experiments. Target gene specific primers were designed in the region flanking to miRNA binding site. The list of selected targets and the target gene specific primers used in this study were provided as (table A1 and A2).We analyzed the expression of total 27 targets of 15 differentially expressed (dE) miRNAs using fast SYBR green fluorescent based qRT-PCR. Before doing qRT-PCRS, we have checked the expressions of the targets by semi quantitative qRT-PCRs in high percentage (3.5% to 4%) gel (**Fig 4.2.9**).

The expression of the targets was determined using the same germination conditions as those for the miRNAs, and in most of the cases we obtained opposite expression of the targets compare to their corresponding miRNAs. This inverse correlation partially validated the miRNA targets, since miRNAs mostly post-transcriptionally cleave their respective target gene transcripts. However, the inverse relationship of expression pattern between miRNAs and their targets were limited in some cases during specific stages of germination, which might be because of condition induced transcriptional regulation. We validated *PHB (PHABULOSA)* (**Fig 4.2.3a**), *PHV (PHAVOLUTA)* (**Fig 4.2.3b**), *ATHB8 (Arabidopsis thaliana HOMEOBOX GENE8)* (**Fig 4.2.3c**) and *ATHB15 (Arabidopsis thaliana HOMEOBOX GENE15)* (**Fig 4.2.3d**) as the targets of miR165/166. Whereas at cold imbibitions (4˚C) condition in 12h, miR165 expression was less compared to DS, it was become high in *PHB* (**Fig 4.2.3a**). At 48h/ 4˚C, miR165 expression was upregulated compared to DS but the expression of *PHB* was severely decreased in 48h/ 4˚C (**Fig 4.2.3a**). We found maximum expression of *PHB* in 24h/ 4˚C. The expression of *PHB* was slightly different compare to *PHV*, *ATHB8* and *ATHB15,* since both *PHV, ATHB8* and *ATHB15* showed high expressions at 12h/RT whereas PHV expression was reduced (**Fig. 4.2.3a, b, c, d**).

Figure 4.2.4 Validation of the Expression of Mature miRNAs in imbibed seeds, in comparison to Dry seeds, in *Arabidopsis thaliana* by quantitative RT-PCR method. The qRT-PCR validation of miRNAs were done at six different **Germination conditions** as **12h/RT, 12h/4˚C, 24h/RT, 24h/4˚C, 48h/RT and 48h/4˚C** each compared to **Dry seed**. The Expression values showed here representing the means of the **three biological replicates ± standard deviation (sd)**. The *Arabidopsis **ACTIN7*** was used for each and every samples as an **Endogenous control**. (a) **SPL10- target of miR156/or, miR157. (**b–e**): targets of miR172; (**b**) AP2; (**c**) TOE1; (**d**) TOE2; (**e**) TOE3. (**f–h**): targets of miR164; (**f**) NAC1; (**g**) CUC1; (**h**) CUC2; (**i**) NF-YA5- target of miR169. Here Asterisks indicate significant statistical differences; ***P < 0.001, **P < 0.01, *P < 0.05 [One-way ANOVA].**

The expressions of *ARF10* (**Fig 4.2.3e**), *ARF 16* (**Fig 4.2.3f**) and *ARF 17* (**Fig 4.2.3g**) as the target of miR160 were highly downregulated in all germination stages compare to DS. We analysed the expression of *SPL*3 (*SQUAMOSA PROMOTER BINDING PROTEIN LIKE3*) (**Fig 4.2.3h**), *SPL*9 (**Fig 4.2.3i**) and *SPL*10 (**Fig 4.2.4a**), which are the targets of both miR156/ 157. We observed low transcript level of the target genes *SPL*3, 9 and 10 in all the germination conditions compare to DS. But the transcript level started to increase in the later stage of germination at 48h/ 4˚C. We validated the expression of 4 targets of miR172, namely *AP2 (APETALA2), TOE1 (TARGET OF EARLY ACTIVATION TAGGED*1*), TOE2, TOE3* out of 6. We observed highest expression of the targets at 48h/ 4˚C (**Fig. 4.2.4b, c, d, e**). Comparatively, high expression of these targets was observed in case of RT, rather than cold imbibition (4˚C), which inversely correlates with increased expression of miR172 under cold imbibitions. We validated expression of three different targets of miR164, such as *NAC1 (NO APICAL MERISTEM)* (**Fig 4.2.4f**), *CUC1 (CUP SHAPED COTYLEDON1)* (**Fig 4.2.4g**) and *CUC2* (**Fig 4.2.4h**). The maximum transcript level of these targets was observed in 48h/ 4˚C and followed by expression in 12h/ RT. In these conditions, the expression level of *NAC1* was ~430 fold, whereas *CUC1* and *CUC2* expression was induced by ~450 and ~190 folds respectively. In other conditions, we found low expression of these targets. For miR169, we validated the expression of two targets *NF-YA5 (NUCLEAR FACTOR Y, SUBUNIT A5)* and *NF-YA8.* Like the targets of miR164a, we mostly observed upregulation of expression of the targets of miR169b in 48h/ 4˚C and 12h/ RT (**Fig 4.2.4i, 4.2.5a**), and highest transcript level was observed in 48h/ 4˚C (**Fig 4.2.4i, 4.2.5a**).

Figure 4.2.5 Validation of the Expression of Mature miRNAs in imbibed seeds, in comparison to Dry seeds, in *Arabidopsis thaliana* by quantitative RT-PCR method. The qRT-PCR validation of miRNAs were done at six different **Germination conditions** as **12h/RT, 12h/4˚C, 24h/RT, 24h/4˚C, 48h/RT and 48h/4˚C** each compared to **Dry seed**. The Expression values showed here representing the means of the **three biological replicates ± standard deviation (sd)**. The *Arabidopsis **ACTIN7*** was used for each and every samples as an **Endogenous control**. **(**a**) *NF-YA8*-target of miR169;** (b**) *PPR superfamily*- target of miR161.1; (**c**) *PHO2*- target of miR399; (**d**) *AGL16*- target of miR824; (**e**) *CIP4.1 or CIP4*- target of miR834; (**f**) *R3H*- target of miR854. Here Asterisks indicate significant statistical differences; ***P < 0.001, **P < 0.01, *P <0.05 [One-way ANOVA].**

The significantly high transcript level at 48h/ 4˚C in case of *NF-YA5* was 160 fold, and for *NF-YA8* it was ~750 fold. We validated expression of miR161 target *PPR (PENTATRICOPEPTIDE REPEAT)* super family (**Fig 4.2.5b**). The highest expression of the target was observed at 48h/ 4˚C (**Fig 4.2.5b**), and the transcript level was observed as ~190 fold. The second upregulation of this target was observed at 12h/ RT (~30 fold). In other cases were observed either same or downregulation of the targets compared to DS. *PHO2 (PHOSPHATE 2)* expression correlation was validated as the target of miR399 (**Fig 4.2.5c**). The highest transcript level of the target was observed at 48h/ 4˚C (~37 fold). The second upregulation was observed at 12h/ RT (~6 fold) and then 12h/ 4˚C (~2.5 fold). Expression in other conditions showed either downregulation or almost similar transcript level w.r.t. DS (**Fig 4.2.5c).** We validated the expression correlation *AGL16 (AGAMOUS-like 16)* (**Fig 4.2.5d**), *CIP4.1 (COP1-interacting protein4.1)* (**Fig 4.2.5e**) and *R3H* (**Fig 4.2.5f**) as the targets of miR824, miR834 and miR854 respectively. The maximum target transcript level of *AGL16* was observed at 48h/ 4˚C (~41 fold) and then 12h/ RT (~5 fold). In other cases the downregulation of the targets were observed. The highest transcript level for *CIP4.1* and *R3H* were observed at 48h/ 4˚C (**Fig 4.2.5e, f**). It was ~70 fold for *CIP4.1* and ~12 fold for *R3H*. We observed upregulation of the target w.r.t. DS in almost all conditions except 48h/ RT.

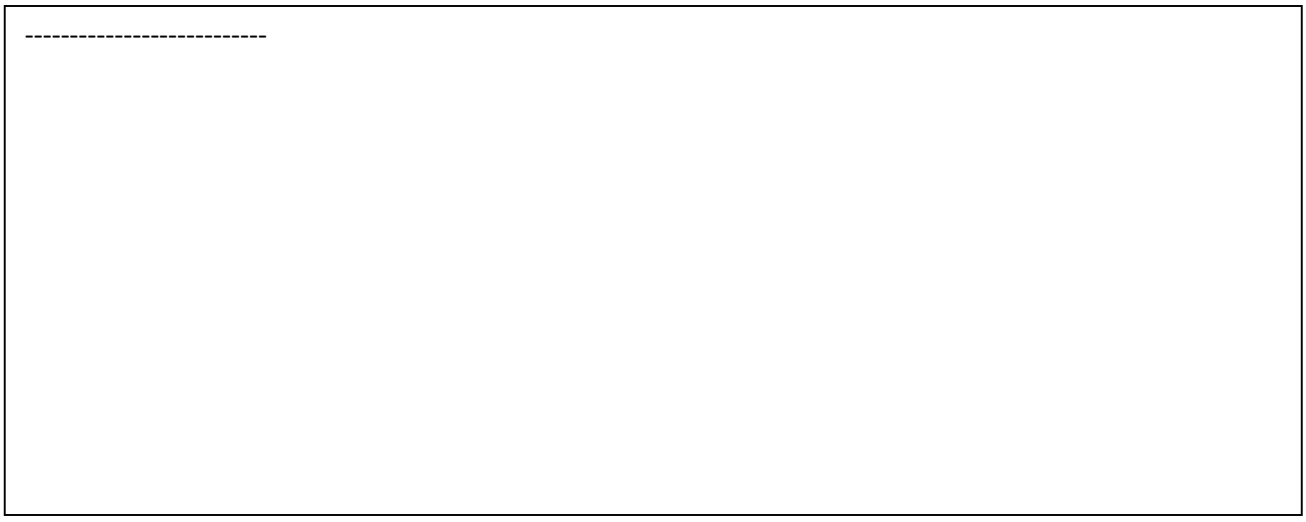


Figure 4.2.6. . ***pMIR*390b::*GUS* and *pMIR*160a::*GUS* conconstruction**

Figure 4.2.7 The Spatial Expression of *pMIR390b::GUS* in Germinating Seeds, the Function of miR390, and the Expression of the targets *ARF2, ARF3,* and *ARF4* during Seed Germination. (a**) *GUS* Expression** of ***pMIR390b::GUS* at 24h/RT** Imbibed condition; **(**b**) *GUS* Expression** of ***pMIR390b::GUS* at 24h/4˚C** Imbibed condition; **(**c**) Negative control** of *GUS* assay at **24h/ 4˚C** Imbibed Col-0 seed. **24h/RT-**Imbibed seeds were showing the higher expression of miR390b compare to **24h/4˚C**-Imbibed seeds, which is matching or similar to the Expression pattern of the qRT-PCR based validation result of **miR390b**; **(**d**)**The model representing the role of miR390 in the biogenesis of tasiR-*ARFs* and regulation of *ARF2/3/4* [According to **(Marin *et al.*, 2010)**]. **(**e-g**)** Expression pattern of *ARF2/3/4* (the targets of tasiR-*ARF)* by qRT-PCR method*;* **(**e**)** Transcript level of **target *ARF2***; **(**f**)** Transcript level of **target *ARF3*** and **(**g**)** Transcript level of **target *ARF4.***

4.2.2.3 Expression pattern of miR390b in seeds correlates with the expression of tasiR-*ARF* target *ARF2/3/4,* and indicates its role in seed germination.

Since miR390 is required for *TAS3* transcript and tasiRNA production, we chose to chracterize it further to understand possible involvement of miRNA-*tasiR-ARF* module in seed germination. We have found maximum expression of miR390b at 24h/ RT following 24h/ 4˚C through qRT PCR assay (**Fig 4.2.1c**). To verify the expression in the tissue level, we performed histochemical *GUS* assay with seeds of *pMIR390b::GUS* homozygous line along with the WT (Col-0) seeds negative control. We observed high level of expressions of miR390b at 24h/ RT (**Fig 4.2.7a**) and then 24h/ 4˚C (**Fig 4.2.7b**) of germinating seeds, where as Col-0 as a negative control, showing no expressions (**Fig 4.2.7c**). Since miR390 is essential for maturation of *TAS3* and regulate the production of functional tasiR-*ARF*, its expression should regulate transcript level of tasiR-*ARF* targets *ARF2, ARF3* and *ARF4* through different feedback mechanisms *(Marin et al., 2010)*. We observed that the transcript levels of targets *ARF2/3/4* were low at the early stages of germination and became high in later stage at 48h/ 4˚C (**Fig. 4.2.7e, f and g**). However, interestingly, at 48h/ RT, the expression of the targets *ARF2/3/4* was not high. Expression of *ARF3* (**Fig 4.2.7f**) and *ARF4* (**Fig 4.2.7g)** transcript level increased significantly at 48h/ 4˚C (for *ARF3* it was 4 fold, and for *ARF4* it was ~2.75 fold).

In the previous study it was reported that first 24 h (Phase-II of triphasic seed germination events) of imbibition is very critical for seed germination, because, maximum cellular repairments, RNA transcription and metabolism resumptions occur in this phase (Han *et al.*, 2014); (Galland *et al.*, 2014; Holdsworth *et al.*, 2008). Our results indicate potential role of miR390-tasiR-*ARF* module in seed germination process.

4.2.2.4 Expression pattern of miR160a in germinating seeds

We have also found the expression of miR160a at 12h/RT through qRT-PCR (**Fig 4.2.1d**). By histochemical *GUS* assay, we had verified the expression of miR160a at the tissue specific level with the homozygous seeds of *pMIR*160a::*GUS* line (**Fig. 4.2.8a,b**); and we observed a profound expression of miR160a in radicle and endosperm tissues at 12h/RT in germinating seeds. **Fig 4.2.6** is depicting the construct of *pMIR*160a::*GUS* we generated and used in this study.

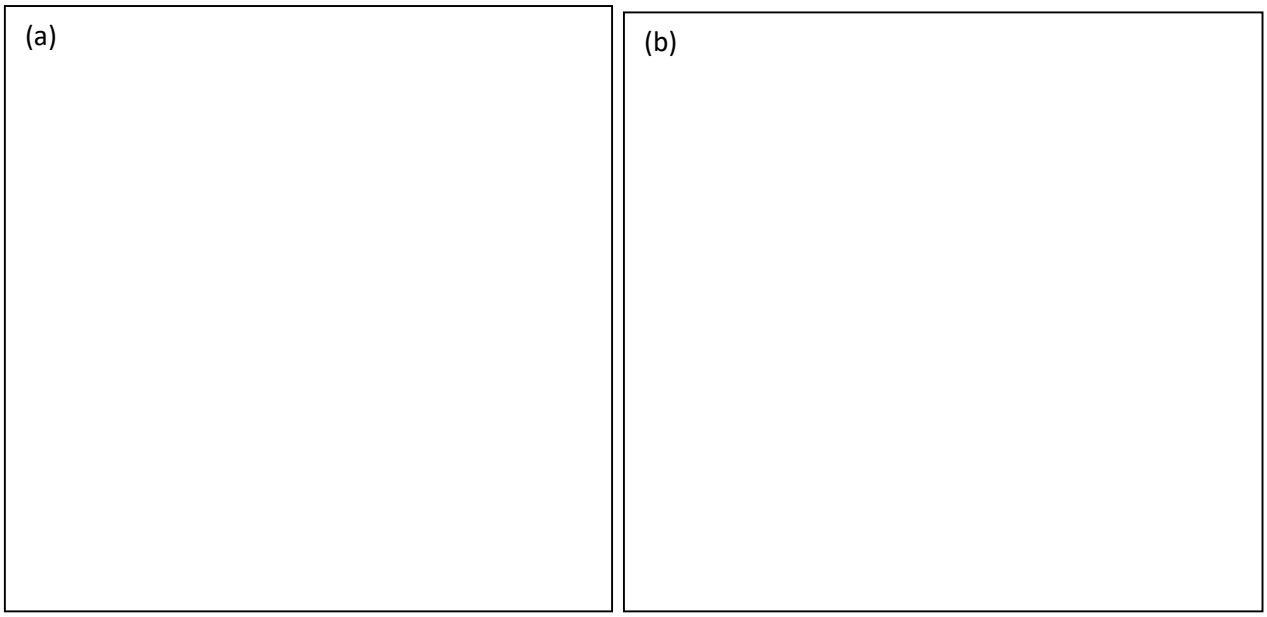


Figure 4.2.8. Spatial expression of *pMIR160a::GUS* in germinating seeds . (a). *GUS* expression in radicle at 12h/RT ; **(b).** *GUS* expression in endosperm at 12h/RT.

(a). **C1 C2 C3 C4 C5 C6 C7 M**

(b). **C1 C2 C3 C4 C5 C6 C7 M**

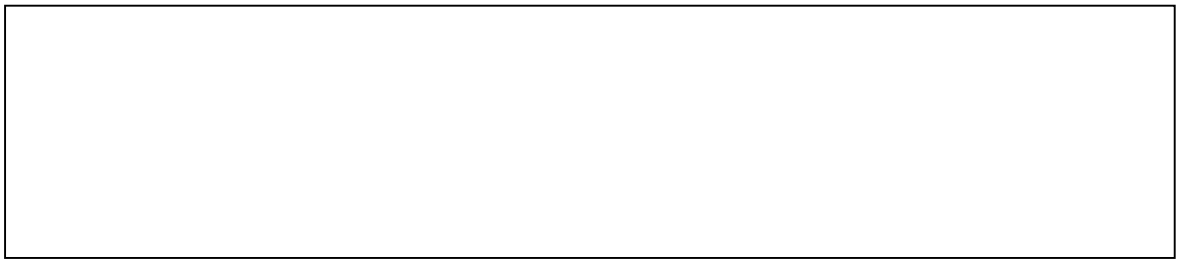
(c). **C1 C2 C3 C4 C5 C6 C7 M**

(d). **C1 C2 C3 C4 C5 C6 C7 M**

(e). **C1 C2 C3 C4 C5 C6 C7**

(f). **C1 C2 C3 C4 C5 C6 C7 M**

(g). **C1 C2 C3 C4 C5 C6 C7**



(h).

C1 C2 C3 C4 C5 C6 C7 M

(i).

C1 C2 C3 C4 C5 C6 C7

Figure 4.2.9 (a-i). Semi quantitative RT-PCR of the targets and miRNAs (all are not shown). *ACT7* was an endogenous control. C1-Dry seed, **C2**- 12h/RT, **C3**-12h/4˚C, **C4**-24h/RT, **C5**-24h/4˚C, **C6**-48h/RT, **C7**-48h/4˚C, **M**-marker

Table 4.2.1. The locus-ID and descriptions of the candidate validated targets of known miRNAs during *Arabidopsis* seed germination.

Serial no.	miRNA	Target gene no.(Locus ID)	Description of the target gene	Target annotation	Validated by
1	165/166	AT2G34710	Homeobox-leucine zipper protein PHB	PHB	qRT-PCR
2		AT1G30490	Homeobox-leucine zipper protein PHV	PHV	qRT-PCR
3		AT4G32880	Homeobox-leucine zipper protein ATHB-8	ATHB-8	qRT-PCR
4		AT1G52150	Homeobox-leucine zipper protein ATHB-15	ATHB-15	qRT-PCR
5	172	AT4G36920	APETALA2	AP2	qRT-PCR
6		AT2G28550	Target of early activation tagged(EAT)1	TOE1	qRT-PCR
7		AT5G60120	Target of early activation tagged(EAT)2	TOE2	qRT-PCR
8		AT5G67180	Target of early activation tagged(EAT)3	TOE3	qRT-PCR
9	390	AT5G62000	Auxin response factor 2	ARF2	qRT-PCR
10		AT2G33860	Auxin response factor 3	ARF3	qRT-PCR

11		AT5G60450	Auxin response factor 4	ARF4	qRT-PCR
12	160	AT2G28350	Auxin response factor 10	ARF10	qRT-PCR
		AT4G30080	Auxin response factor 16	ARF16	qRT-PCR
13		AT1G77850	Auxin response factor 17	ARF17	qRT-PCR
14	156/157	AT2G33810	Squamosa promoter binding protein-like 3	SPL3	qRT-PCR
15	156/157	AT2G42200	Squamosa promoter binding protein-like 9	SPL9	qRT-PCR
16		AT1G27370	Squamosa promoter binding protein-like 10	SPL10	qRT-PCR
17	164	AT3G15170	Cup-shaped cotyledon1(ANAC054, Arabidopsis NAC domain containing protein 54).	CUC1	qRT-PCR
18		AT5G53950	Cup-shaped cotyledon2(ANAC098, Arabidopsis NAC domain containing protein 98).	CUC2	qRT-PCR
19		AT1G56010	NAC domain proteins(ANAC022, Arabidopsis NAC domain containing protein 22).	NAC1	qRT-PCR
20	169	AT1G17590	Nuclear factor Y, subunit A8	NF-YA8	qRT-PCR
21		AT1G54160	Nuclear factor Y, subunit A5	NF-YA5	qRT-PCR
22	161	AT5G16640	Pentatricopeptide repeat (PPR) superfamily protein	(PPR) superfamily protein	qRT-PCR
23	399	AT2G33770	Phosphate 2	PHO2	qRT-PCR
24	824	AT3G57230	AGAMOUS-like 16	AGL16	qRT-PCR
25	834	AT4G00930	COP1-interacting protein 4.1	CIP4.1	qRT-PCR
26	854	AT5G05100	Single-stranded nucleic acid binding R3H protein	R3H	qRT-PCR

4.2.2.5 Discussions

In higher plants, seed germination is one of the most vital phase transition from seed to seedling stage (Das *et al.*, 2015). Although small RNAs have been implicated in various aspects of plant development, the regulatory role of small RNAs in seed germination is less explored area till date. However, few recent reports indicated involvement of miRNAs in different plant species such as rice, maize and other monocot but in *Arabidopsis,* whether and how small RNAs might be regulating dynamic process of seed germination is largely unknown to us. Our present study focused on the

identification and characterization of miRNAs mediated gene regulation in early stages of seed germination in *Arabidopsis thaliana*.

4.2.2.5.1 miRNAs and their targets are dynamically regulated at different conditions during germination.

We have identified 58 miRNA precursors (pre-miRNAs) belonging to 30 miRNA families to be differently expressed in comparative study of three different conditions – (1) IS-4˚C vs. DS (Fig 4.1.**4a**), (2) IS-RT vs. DS (**Fig 4.1.4b**) and (3) IS-4˚C vs. IS-RT (**Fig 4.1.4c**) during germination. Among these, 15 miRNA precursors belonging to 14 families were of $P \leq 0.05$ and fold change ≥ 2.0 and considered to be significant. Since mature miRNAs are the main functional molecule that regulates their targets, we observed their dynamic regulation using SL-qRT-PCR at three different time points (12h, 24h and 48h) of germination conditions. Expression of miR399a and miR399b/c were analyzed independently, since their sequence differed.

We observed significant upregulaion of miR165/ 166 in 12h/ RT and downregulation at 24h/ RT and 48h/ RT (**Fig 4.2.1a**). The expression of target *PHB* was downregulated at 12h/ RT (**Fig 4.2.3a**), where as it was upregulated at 24h/ RT and 48h/ RT (**Fig 4.2.3a**), indicating their post-transcriptional regulation by miR165/ 166 in these conditions. Additionally, *ATHB15* was also slightly upregulated in 24h/ RT (**Fig 4.2.3d**). Previous reports implicated miR165/166 module in leaf, shoot, root vascular patterning (Zhou *et al.*, 2007) and also in seed germination in maize (Li *et al.*, 2013) and rice (He *et al.*, 2015). Previous reports indicated role of miR172 in regulation of vegetative to reproductive phase change and cold stress induced response affecting root growth (Wu *et al.*, 2009; Zhao *et al.*, 2007). We observed that miR172a was significantly upregulated in 24h/ 4˚C (**Fig 4.2.1b**) and downregulated in 24h/ RT and 48h/ 4˚C (**Fig 4.2.1b**), whereas its target *AP2* (**Fig 4.2.4b**)*, TOE1* (**Fig 4.2.4c***), TOE2* (**Fig 4.2.4d***)* and *TOE3* (**Fig 4.2.4e***)* were significantly downregulated at 24h/ 4˚C. This inverse correlation of expression of miR172a and its targets indicate their post-transcriptional regulation by miRNAs. Our results indicate the role of miR172-target *AP2/ TOE*s module in seed germination process. miR160 is involved in auxin signalling pathway during various plant growth and development, such as root, shoot and proper formation of the aerial organs of roots (Liu *et al.*, 2007; Mallory *et al.*, 2005; Wang *et al.*, 2005) by negatively regulating its target transcription factors *ARF10*, *ARF16* and *ARF17* via hormonal crosstalk (Chen, 2009). We observed the significant upregulation of miR160 during all of the germination conditions we used and found maximum upregulation at 24h/ 4˚C followed by 24h/ RT (**Fig 4.2.1d**). This consistent upregulation

of miR160 led to the consistent downregulation of its targets *ARF10/ 16/ 17* (**Fig. 4.2.3e, f, g**) during all germination conditions. This indicates the potential significant role of miR160 during early stages of seed germination irrespective of their stratification status. The downregulation of *ARFs* by miR160 during imbibition indicates possible auxin-ABA crosstalk during germination since ABA became downregulated in over expressing miR160 plants (Liu *et al.*, 2007) and *ARFs* are known to be involved in auxin signalling. Earlier findings also indicate the role of miR160 in seed germination in rice (He *et al.*, 2015) and *Nelumbo nucifera* (Hu *et al.*, 2016). According to the previous report, miR156 and its closely related miR157 (Naya *et al.*, 2010) are the principal regulators of transition from juvenile to adult phase. They are also involved in shoot development, floral induction, initiation of leaf etc (Xu *et al.*, 2016) by negatively regulating their target *SPL.* Among the ten *SPL*s in *Arabidopsis*, we chose *SPL3* (**Fig 4.2.3h**), *SPL9* (**Fig 4.2.3i**) and *SPL10* (**Fig 4.2.4a**), since they are the targets of both miR156 and miR157. We observed significant upregulation of these two miRNAs at 24h/ 4˚C, followed by 12h/ 4˚C (**Fig 4.2.1e, f**); and the significant downregulation of their targets in the same above said germination conditions. This inverse correlation of miR156/ 157 and its target *SPLs* indicate their post-transcriptional regulation. Earlier studies also indicated role of miR156 in the dynamic seed germination process of maize (Li *et al.*, 2013) and *Nelumbo nucifera* (Hu *et al.*, 2016) . miR164 plays significant role in formation of proper organ boundaries (Mallory *et al.*, 2004), floral patterning (Sieber *et al.*, 2007), leaf morphogenesis (Nikovics *et al.*, 2006) and lateral root development (Guo *et al.*, 2005) by negative regulation of its target *NAC1*, *CUC1* and *CUC2*. We observed the maximum significant upregulation of miR164a at 24h/ RT (**Fig 4.2.2a**), followed by 24h/ 4˚C (**Fig 4.2.2a**), which was further followed by 12h/ 4˚C (**Fig 4.2.2a**). We also observed the inverse correlation of miRNA–target here. We found maximum significant upregulation of the targets at 48h/ 4˚C, followed by 12h/ RT (**Fig 4.2.4f, g, h**). The inverse correlation of target- miRNA indicates the post-transcriptional regulation by miRNAs. In the previous report, there is the indication of the involvement of miR164 in seed germination in maize (Li *et al.*, 2013). The miR169 family is the largest miRNA family in *Arabidopsis* and is encoded by 14 members (Xu *et al.*, 2013); however, only a few members have been annotated with specific functions. The miR169 targets members of the *Arabidopsis NF-YA* gene family (Jones-Rhoades *et al.*, 2006). *NF-Y* encodes a CCAAT-binding transcription factor, which participates in transcriptional regulation of a large number of genes (Zhao *et al.*, 2011). In *Arabidopsis*, there are 10 genes coding for the *AtNF-YA* subunit (Sorin *et al.*, 2014). It was reported that over expression of *NF-YA5* caused hypersensitivity to ABA during seed germination (Li *et al.*, 2008; Mu *et al.*, 2013). We observed the maximum significant upregulation of miR169b at 24h/ RT (**Fig 4.2.2b**), followed by 48h/ RT (**Fig 4.2.2b**); and

we observed maximum significant downregulation in the above said germination conditions and maximum upregulation of the targets *NF-YA5* and *NF-YA8* at 48h/4˚C, followed by 12h/ RT (**Fig 4.2.4i, 4.2.5a**). This inverse correlation of target-miRNA indicates the post-transcriptional regulation of miRNA. The miR161 is a non conserved miRNA, since it is represented by single genes rather than multigene families (Allen *et al.*, 2004). The miR161 locus is unusual in that it encodes overlapping miRNAs (miR161.1 and miR161.2) from a single precursor sequence (Allen *et al.*, 2004). miR161.1 targets *PPR* superfamily through negative regulation, which has a major impact on evolutionary background (Barkan and Small, 2014). We observed the maximum significant expression of miR161.1 during 24h/ 4˚C (**Fig 4.2.2c**), followed by 12h/ 4˚C (**Fig 4.2.2c**); and we observed the maximum significant downregulation of the target *PPR* superfamily at 24h/ 4˚C (**Fig 4.2.5b**), then 12h/ 4˚C (**Fig 4.2.5b**). This inverse correlation of miR161.1 and its target PPR superfamily indicates the post- transcriptional regulation of miRNA. Our result indicates the role of miR161.1– target *PPR* superfamily module in seed germination process. The *Arabidopsis* genome encodes six miR399 genes (miR399a to -f), which are all induced by Phosphorus starvation to different extents (Kuo and Chiou, 2011). miR399 is involved in orthophosphate (Pi) deficiency signalling pathways target in the *PHOSPHATE2 (PHO2)* gene encoding E2 enzyme that negatively regulates phosphate uptake and root-to-shoot allocation (Hackenberg *et al.*, 2013; Kuo and Chiou, 2011). The miR399a and miR399b/c (miR399b and miR399c have same mature sequence in *Arabidopsis*) has single nucleotide difference in the 13th position from 5′ end. Throughout the germination stages miR399a and miR399b/c expression level was upregulated in comparison to DS and the target *PHO2* expression level was downregulated, except 48h/ 4˚C (**Fig 4.2.5c**). Previous reports also indicated the expression of miR399 in seeds of maize (Li *et al.*, 2013) and *Nelumbo nucifera* (Hu *et al.*, 2016). Availability of phytate and orthophosphate (Pi) in germinating seeds may regulate miR399 expression, which is known to be regulated by Pi availability, during germination (Kuo and Chiou, 2011). miR824 is *Brassicaceae*-specific miRNA (Fahlgren *et al.*, 2007; Kutter *et al.*, 2007; Rajagopalan *et al.*, 2006). miR824 has function in rosette and cauline leaves, shoots, inflorescence and roots (Alvarez-Buylla *et al.*, 2000; Kutter *et al.*, 2007) by negative regulation of its target *AGAMOUS-LIKE16 (AGL16),* which encodes a MADS box transcription factor (de Meaux *et al.*, 2008; Fahlgren *et al.*, 2007). We observed the maximum significant upregulation of miR824 during 24h/ 4˚C (**Fig 4.2.2f**), followed by 24h/RT (**Fig 4.2.2f**), which is further followed by 12h/ 4˚C (**Fig 4.2.2f**). We observed the significant downregulation of the target also. At 48h/ 4˚C, followed by 12h/ RT (**Fig 4.2.5d**), we observed the significant upregulation of the target. The inverse correlation of miR824 and its target indicates their post-transcriptional regulation by miRNA. Our results

indicate the role of miR824-target *AGL16* module in seed germination process. miR834 is a non-conserved miRNA . The predicted targets of miR834 are *DEMETER-LIKE 2 (DML2)* and *COP1-INTERACTING PROTEIN1 (CIP1)*. In this study we have validated *CIP4.1* (AT4G00930.1). *CIP1* was the first reported interacting protein for *CONSTITUTIVE PHOTOMORPHOGENIC 1 (COP1)* of *Arabidopsis* (Ren *et al.*, 2016). *CIP1* is a positive regulator of abscissic acid (ABA) response (Ren *et al.*, 2016). *CIP4,* a homologue of *CIP4.1,* is *COP1* interactive partner and acts downstream of *COP1*. We observed the significant upregulation of miR834 in all of the germination conditions that we used irrespective of stratification status; and found max significant upregulation during 24h/ RT (**Fig 4.2.2g**), followed by 12h/ 4˚C (**Fig 4.2.2g**). We found maximum significant downregulation of target *CIP4.1* during 48h/ RT, followed by 12h/ 4˚C (**Fig 4.2.5e**). The inverse correlation of miRNA-target also indicates the post-transcriptional regulation by miRNA and the miR834-target *CIP4.1* module in the dynamic seed germination process. miR854 is a conserved and stress responsive (Srivastava *et al.*, 2012) miRNA. Plants and animals share miRNAs of the miR854 family, suggesting a common origin of these miRNAs as regulators of basal transcriptional mechanisms (Arteaga-Vazquez *et al.*, 2006). Recently, it was shown that miR854 regulated the rhizome development and the essential oil biosynthesis in ginger (Singh *et al.*, 2015). We observed significant downregulation of miR854 expression in most of the germination conditions, except 24h/ RT (**Fig 4.2.2h**), we observed the inverse correlation of miRNA and its target *R3H* (**Fig 4.2.5f**), which indicate the post-transcriptional regulation by miRNA and miR854-*R3H* module in seed germination. miR2112- 5p is a non-conserved miRNA. We observed the maximum significant expression of this miRNA during 24h/ RT (**Fig 4.2.2i**), followed by 48h/ 4˚C (**Fig 4.2.2i**). According to psRNATarget tool, we found two targets of miR2112-5p. One is ergosterol biosynthesis ERG4/ ERG24 family and another is pentatricopeptide repeat PPR superfamily protein. miR2112- 5p targets both of the target proteins through translational inhibition.

We observed upregulation of most of the miRNAs in 24h imbibition, which indicates their role in seed germination during imbibition onwards in *Arabidopsis thaliana*. Some of the miRNAs showed the minor discrepancies between microarray data and the qRT-PCR results. The reason behind that is, during microarray we had pulled the RNAs of both RT and 4˚C imbibed seeds individually. Another reason could be the differences in the specificity, sensitivity and algorithm used between the two techniques. In most of the cases we found higher accumulation of the targets during 48h and 12h of germination conditions. In some conditions we didn't observe the inverse correlation between miRNAs and their targets, this indicates seed germination condition induced transcriptional regulation of these targets, besides their post-transcriptional regulation by miRNAs. This further

implies that both transcriptional and post-transcriptional regulation of miRNA targets play important role in seed germination process.

4.2.2.5.2 Expression pattern of miR390 and downstream *ARF2/3/4* indicates potential role of miRNA-tasiRNA crosstalk in seed germination process

Spatial expression pattern of *pMIR390b::GUS* in embryonic root, cotyledon and endosperm of germinating seeds (**Fig 4.2.6a, b**) indicates its potential role in seed germination. We observed that the induced expression of miR390b (**Fig 4.2.1c**) correlates downregulation of *tasiR-ARF* targets *ARF2, ARF3* and *ARF4* in early stages of seed germination (**Fig. 4.2.6e, f and g**). Upregulation of miR390 should enhance the production of *tasiR-ARF* leading to the down regulation of its target *ARF2/3/4*, which we observed in most of the germination conditions we used. This result indicates the role of miR390-tasiR-*ARF* mediated post-transcriptional regulation and a crosstalk of two classes of small RNAs (miRNA and ta-siRNA) in seed germination. Although we cann't rule out additional involvement of other ta-siRNAs. Thus, our study indicates that the miR390 – tasiR-*ARF* - *ARF2/3/4* module and crosstalk of miRNA and ta-siRNA pathways (**Fig 4.2.6d**) contributes to the regulation of the dynamic process of seed germination in *Arabidopsis,* besides role of other miRNAs and their targets.

Chapter4.3- Functional analysis of the selected small RNA pathway gene *SGS3* for role in seed germination

4.3.1. Introduction

The two major classes of small RNAs miRNA and ta-siRNA are involved in various aspects of plant developmental processes through the negative regulation of gene expression by acting as a developmental switch of gene expressions (Marin *et al.*, 2010). The classical examples include shoot and root development, reproductive development (Chen, 2012b; Guo *et al.*, 2005), maintenance of leaf polarity, developmental phase transitions etc (Chen, 2012b; Wu *et al.*, 2009). The ta-siRNA have also been implicated in plant development (Axtell, 2013; Nogueira *et al.*, 2007), such as vegetative to reproductive phase changes, leaf polarity and lateral root development in *Arabidopsis* (Allen and Howell, 2010; Chitwood *et al.*, 2007; Marin *et al.*, 2010; Peragine *et al.*, 2004). DCL4 is suggested to redundantly regulate processing of some miRNAs, besides role in ta-siRNA biogenesis (Rajagopalan *et al.*, 2006). ABA signaling is shown to be, at least partially, affect RDR6 accumulation (Zhang *et al.*, 2013). Although ta-siRNA has not directly been implicated in seed germination, their crosstalk with miRNA and hormone signaling in feed-back loops (Chen, 2012b; Marin *et al.*, 2010) as well as role in seed development (Zhang *et al.*, 2013) indicate their potential function in seed maturation and germination. This remains to be an interesting area to be explored in the complex process of seed germination. The function of miRNAs has been shown to be affected by hormones and stress responses (Liu *et al.*, 2007; Mallory *et al.*, 2005; Reyes and Chua, 2007). The miRNA mediated, ta-siRNA production is also significantly altered in drought, salinity and hypoxia stresses, besides their regulation by auxin and other hormones (Matsui *et al.*, 2014; Moldovan *et al.*, 2010). ta-siRNAs are generated from *TAS* (Trans-Acting SiRNA locus) gene derived non-coding transcripts through specific miRNA guided cleavage. The cleaved precursors of ta-siRNAs are bounded and stabilized by *SUPPRESSOR of GENE SILENCING3(SGS3*) and further synthesized into double stranded RNAs by *RDR6* (Allen and Howell, 2010; Axtell, 2013; Chen, 2009).The double stranded RNAs are cleaved several times by DCL4 from the miRNA mediated cleavage sites, so that 21nt long phased ta-siRNAs are produced. Similar to miRNAs, the ta-siRNAs are incorporated into RISCs, where they cleave the target mRNAs or repress translation (Allen and Howell, 2010; Allen *et al.*, 2005). There are four families of *TAS* gene in *Arabidopsis*, namely*TAS1, TAS2, TAS3, TAS4* (Allen and Howell, 2010; Rajagopalan *et al.*, 2006). For the initial processing *TAS1* and *2* require miR173 whereas *TAS3* and *TAS4* require miR390 and miR828, respectively for initial processing (Allen and Howell, 2010; Axtell, 2013; Chen, 2009). *TAS3* derived ta-siR-*ARF* that target different *AUXIN RESPONSE FACTOR2, 3 and 4 (ARF2, 3, 4*), that regulate various aspects of plant development.

Mutations in ta-siRNA biogenesis pathway lead to the upregulation of target mRNAs and affect the aforesaid aspects of plant development. The rice and maize ta-siRNA biogenesis mutants have been shown to have severely affected shoot and leaf development (Douglas *et al.*, 2010; Itoh *et al.*, 2006; Nagasaki *et al.*, 2007; Nogueira *et al.*, 2006). Mutants of the small RNA biogenesis pathway genes such as *DCL1, HYL1, HEN1* and *AGO1* that display severe defects in embryogenesis and seed development(Willmann *et al.*, 2011). The ta-siRNA biogenesis pathway mutants in monocots like rice show severe developmental defects compared to dicots like *Arabidopsis* (Abe et al., 2010). For example, loss-of-function mutants of *RDR6*, *DCL4* and *AGO7* in rice, like *shootless 2 (shl2), shoot organization1 (sho1) and shootless4 (shl4)* etc., leads to the severe developmental defects in shoot apical meristem (SAM) and in embryonic shoot (Abe *et al.*, 2010). Mutant *ago1* is responsible for radicleized leaves, lack of inflorescence branching and sterile as well as abnormal flowering phenotypes (Bohmert *et al.*, 1998). In maize also, a homolog of *Arabidopsis SGS3,* involves in maintaining leaf polarity and SAM development and maintenance has a severe leaf phenotypes in comparison to *Arabidopsis thaliana* (Juarez *et al.*, 2004; Nogueira *et al.*, 2007; Timmermans *et al.*, 1998). In *Arabidopsis*, *rdr6-11* and *sgs3-11* , that are defective in tasiR-*ARF* production, show elongated and downwardly curled leaves (Kumakura *et al.*, 2009; Peragine *et al.*, 2004), precociously formed abaxial trichomes and lower seed sets, and accelerated juvenile to adult phase change (Peragine *et al.*, 2004).

As the role of tasi-RNA have been implicated in various developmental stages in plants and its role in seed germination is not studied so far, therefore we have addressed the functional analysis of one of the most vital protein in the ta-siRNA biogenesis pathway called SGS3. We observed the seed viability and then its seed germination efficiency of the mutant *sgs3-11* compare to age matched control Wt-Col; and we found that *sgs3-11* have higher seed viability and germination efficiency in both normal growing condition and in stress studed condition compare to control Wt Col. Both miR390 and *SUPPRESSOR OF GENE SILENCING3 (SGS3)* are involved in the processing of *TAS3* transcript into functional ta-siRNAs that targets and negatively regulate the *ARF* family members *ARF2, ARF3,* and *ARF4* (Mallory *et al.*, 2005; Marin *et al.*, 2010). We show that the germination efficiency and seed viability of *sgs3-11* mutant seeds are significantly altered under abiotic stress conditions, suggesting that both miRNAs, ta-siRNAs and their targets contribute to seed germination process under normal and stress conditions.

4.3.2. Results

4.3.2.1 Tetrazolium (TZ) assay indicate altered seed viability of ta-siRNA biogenesis mutant sgs3-11

The production of functional *tasiR-ARF* involves miR390 mediated cleavage of *TAS3* transcripts. Since *sgs3-11* mutant is impaired in *tasiR-ARF* production and leads to the upregulation of target *ARF2/3/4* transcripts (Marin *et al.*, 2010; Peragine *et al.*, 2004), we addressed if miR390 mediated regulation of *tasiR-ARF* production affects seed germination. First, we analyzed the seed viability and then the germination efficiency of *sgs3-11*. The seeds of mutant *sgs3-11* and control Wt Col of same age (~1year old) were compared for their viability (**Fig 4.3.1a, b, c**). After performing commonly used TZ assay, we found that average of 93% (**Fig. 4.3.2**) of *sgs3-11* seeds were viable compared to the 83% (**Fig. 4.3.2**) of control Wt Col. These results indicate that seed viability of Wt Col is 10% less than that of *sgs3-11* seeds of same age and condition. This result was further satisfied with observation in the germination assay, as described below.

Figure 4.3.1. Tetrazolium assay of small RNA biogenesis pathway mutant *sgs3-11* seeds along with its age matched control Wt-Col seeds. The assay was performed in triplicate in both the cases. The Brown/or, red/or, dark brown colored seeds were viable seeds, less bright colored seeds were comparatively less viable, and the yellow colored seeds were non-viable(heat killed); **(a).** *sgs3* mutant seeds, **(b).** Possitive control wt-col seeds; **(c).** Negative control (heat killed wt-col seeds).

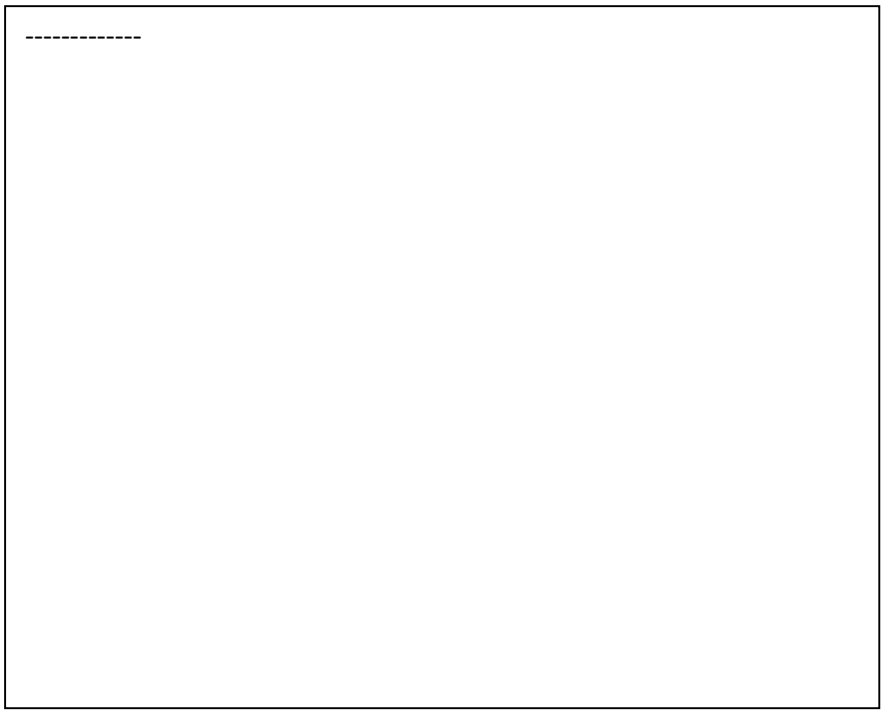


Figure 4.3.2. Relative percentage level of the viable seeds in both *sgs3-11* and Wt-Col ; the percentage was calculated based on the viable seeds of tetrazolium assay. The whole experiment was repeated at least three times along with the three set of independent lines of seeds.

4.3.2.2 Seed germination is affected in tasiR-ARF biogenesis pathway mutant *sgs3-11*

It is known that *ARF3/4* transcripts over accumulate in *sgs3-11* mutant (Peragine *et al.*, 2004) and these *ARF*s, like others, are regulated by auxin (Marin *et al.*, 2010). In *Arabidopsis,* auxin plays a significant role in seed dormancy and germination through its crosstalk with other hormones such as abscissic acid (ABA) (Liu *et al.*, 2007). In this study, we have validated the expression correlation of miR390 and the target of tasiR-*ARF ARF2, ARF3* and *ARF4* in different stages or conditions of seed germination. We found that *ARF2, ARF3* and *ARF4* were downregulated in conditions in which miR390 was up regulated. To test the hypothesis whether miR390–*tasiR-ARF* module might play role in seed germination, we performed seed germination assay of with seeds of *sgs3-11* and wt col under various conditions like salt, dehydration, ABA treatment, cold and heat stress (**Fig. 4.3.3**.). The germination assay **(Fig. 4.3.3.**) showed that at day 1, 50% of the *sgs3-11* seeds were germinated, when only 35% of wt col seeds germinated under normal (no stress) condition (**Fig. 4.3.3.f**). This indicates 15% higher germination rate of *sgs3-11* seeds in comparison to its wt counterpart. In case of

exogenous ABA (5µM) (**Fig. 4.3.3.c**), NaCl (150mM) (**Fig. 4.3.3.a**), Mannitol (200mM) (**Fig. 4.3.3.b**) and heat stress (**Fig. 4.3.3.d**) condition, we observed high rate of germination in *sgs3-11* in comparison to control (**Fig. 4.3.3.d**.) condition, we observed high rate of germination in

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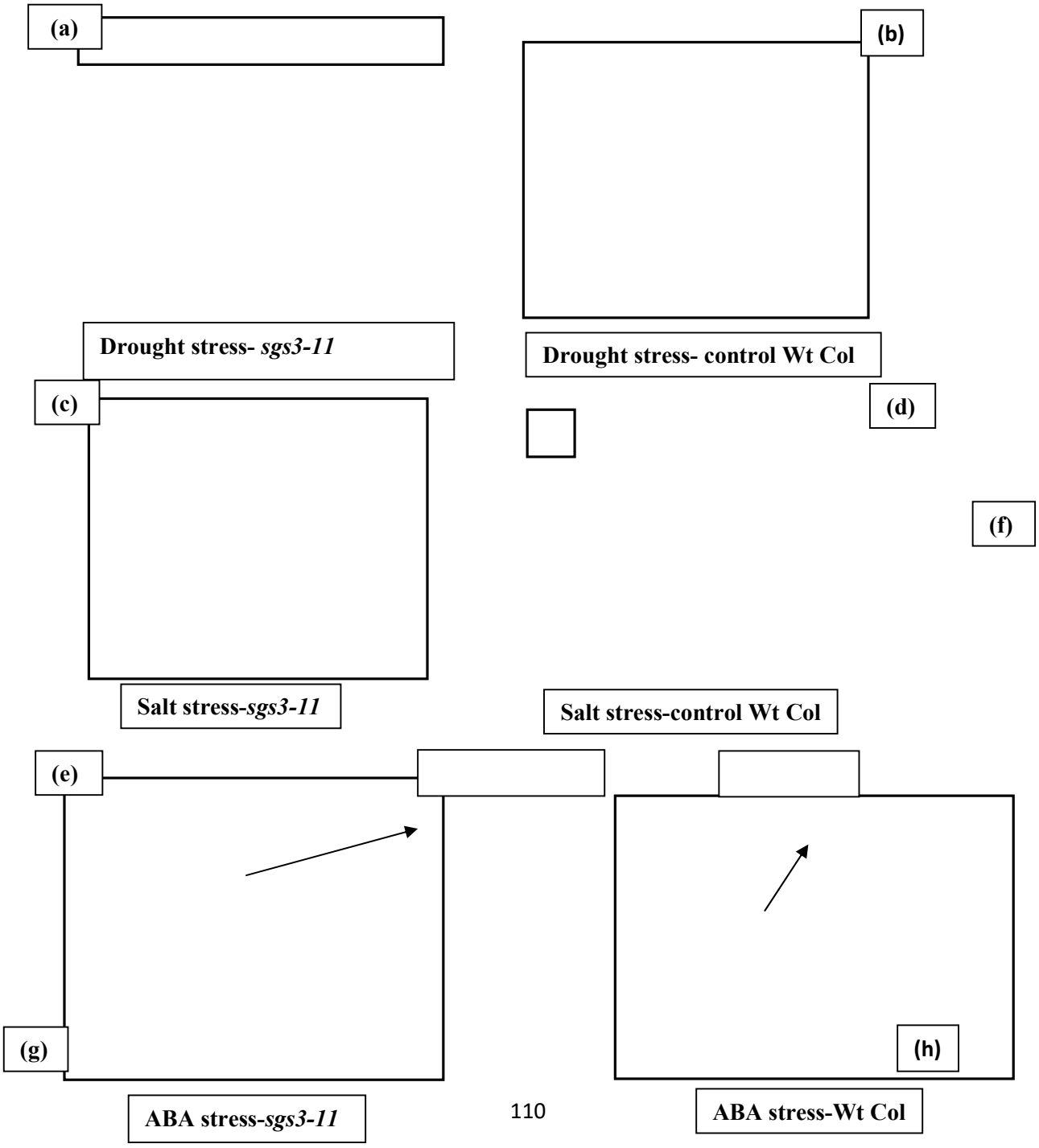
Figure 4.3.3 Germination assay of small RNA biogenesis pathway mutant *sgs3-11* seeds under abiotic stresses. (a). Salt (150mM NaCl), **(b).** Dehydration (mannitol, 200mM), **(c).** ABA z(5µM), **(d).** Heat(45˚C), **(e).** Cold(4˚C), **(f).** Control condition (without stress). Age matched seeds were surface sterilized and plated on either 1/2 MS or 1/2 MS supplemented with various stresses. Plates were stratified at 4˚C for 3 days and transferred to growth chamber at 22±2˚C. For cold stress, after stratification plates were transferred to cold room (4˚C) under 16/8h light/dark cycle till 4 days. Then transferred to again growth chamber at 22±2◦C. Values are mean ±SD of three independent sets (n=30).Asterisks indicate significant statistical differences, ***P < 0.001, **P < 0.01, *P <0.05 (One-way ANOVA).

sgs3-11 in comparison to control wt col (**Fig. 4.3.3.f**). On the contrary, we observed low rate of germination in *sgs3-11* compared to its counterpart wt col in case of cold stress (**Fig. 4.3.3.e**). These results indcate that *SGS3*/miR390 mediated regulation of *tasiR-ARF-ARF2/3/4* module play important role in seed germination in normal and stress conditions such as heat, cold and ABA treatment.

DAG4



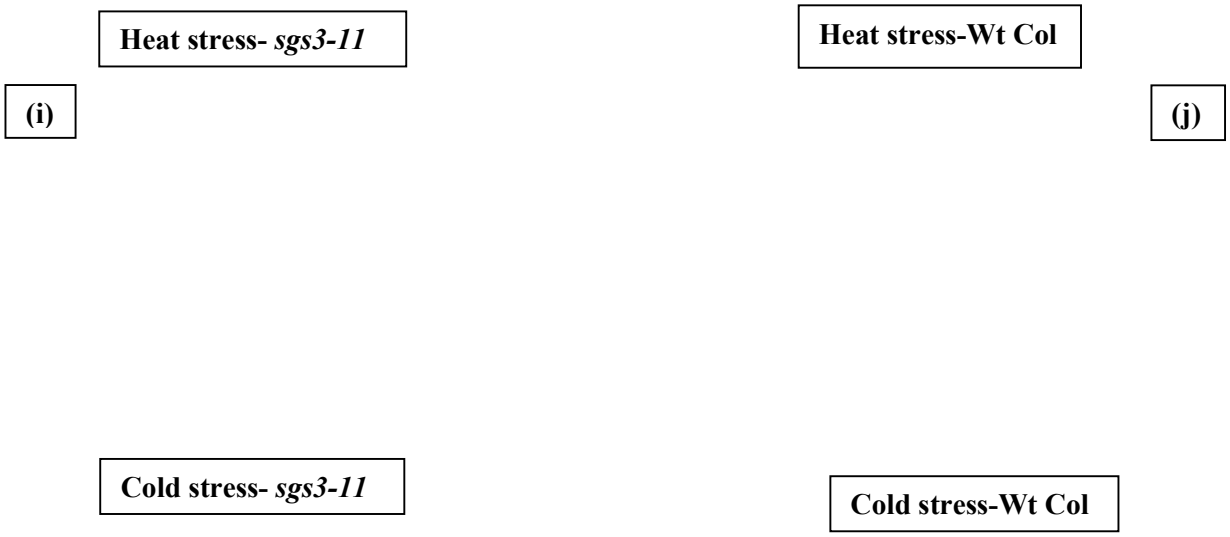


Figure 4.3.4. Effect of different stresses on germination of *sgs3-11* mutant seeds along with its control Wt Col on 4th day of germination (DAG4). (a, b) – Drought stress (mannitol (200mM), **(c, d)-** Salt stress (NaCl (150mM), **(e, f)-** Hormonal stress (ABA (5µM), (**g, h**)- Heat stress (45˚C), (i, j)- Cold stress. Rate of germination in the respective stress conditions are calculated in Fig. **4.3.2.3**.

4.3.3 Discussions

4.3.3.1 Analysis of *sgs3-11* suggests the role of ta-siRNA in viability and germination in *Arabidopsis*

In TZ assay we found that *sgs3-11* seeds, which are defective in ta-siRNA or tasiR-*ARF* production, were 10% more viable compare to the control wt col seeds. This result was further satisfied by the germination assay, where 15% *sgs3-11* seeds showed higher rate of germination compare to control wild type. These two results collectively indicate that *sgs3-11* seeds have higher seed viability leading to better seed germination, as compare to its counter wt col seeds. In TZ assay, high temperature induced dead (nonviable negative control) seeds showed bright yellow color, where as viable seeds were dark brown/ redish (**Fig 4.3.2.1**). This presence of less-viable (affected) seeds, as indicated by yellowish/light brown color, were more in number in wild type than in *sgs3-11* mutant, however, seeds were not completely dead, which helped them to recover to germination at later time points, as we observed at the day 5 of germination, where seeds from both the varieties have been germinated (**Fig. 4.3.2.3f**). Seed germination process is affected by phytohormone ABA and several other environmental factors such as, temperature, stress, dehydration/water availability and salinity (Jung and Kang, 2007; Kaur *et al.*, 2015), which may also affect ta-siRNA production. We observed that the germination of *sgs3-11* seeds significantly differed in salt stress (**Fig. 4.3.2.3. a**), dehydration stress (**Fig. 4.3.2.3.b**), heat stress (**Fig. 4.3.2.3.d**) and in exogenous ABA stress (**Fig. 4.3.2.3.c**) conditions in comparison to wild type (**Fig. 4.3.2.3.f**). These results suggest that ABA and stress conditions affect ta-siRNA mediated seed germination process.

We observed induced expression of miR390 (**Fig 4.2.1c**) and downregulation of *tasiR-ARF* targets *ARF2, ARF3* and *ARF4* in early stages of seed germination (**Fig 4.2.7e, Fig 4.2.7f** and **Fig 4.2.7g**). Upregulation of miR390 should enhance the production of *tasiR-ARF* leading to the down regulation of its target *ARF2/3/4*, which was observed in most of the germination conditions we used. This result indicates the role of miR390-tasiR-*ARF* mediated post-transcriptional regulation and a crosstalk of two classes of small RNA in seed germination. This hypothesis was further supported by *pMIR390b::GUS* expression in embryonic root, cotyledon and endosperm of germinating seeds. In contrast, *sgs3-11* is defective in *tasiR-ARF* production. Therefore, enhanced seed viability and germination of *sgs3-11* indicates the role of *tasiR-ARF2/3/4* module in seed germination. Although we cann't rule out additional

involvement of other ta-siRNAs. Thus, our study uncovers the role of miR390–tasiR-*ARF2/3/4* module in seed viability and germination. This also suggests the involvement of a crosstalk of miRNA and ta-siRNA pathways in the dynamic process of seed germination in *Arabidopsis*.

Chapter4.4- Functional analysis of the selected miRNA miR165/166 for its role in seed germination

4.4.1. Introduction

miR165/166 is one of the most extensively studied miRNAs, which have been shown to be involved in plant development (Carlsbecker *et al.*, 2010; Miyashima *et al.*, 2011; Zhou *et al.*, 2007). miR165 and miR166 has only single nucleotide difference in their mature sequences, miR165 consists of only two family member genes, a and b; whereas miR166 consists of 7 family members of likely a to g. Five members of the *HD-ZIP III* gene family [*PHABULOSA (PHB), PHAVOLUTA(PHV), REVOLUTA(REV), ARABIDOPSIS THALIANA HOMEOBOX 8 and 15(ATHB8 and ATHB 15)*] are targeted and negatively regulated by miR165/ 166 (Rhoades *et al.*, 2002). Earlier studies have shown that miR165/166 regulate meristematic activity, leaf polarity, vascular patterning and lateral organ formation (Zhou *et al.*, 2007). In recent studies, it was reported that miR166 was expressed during seed germination in monocots like maize (Li *et al.*, 2013) and rice (He *et al.*, 2015). We found that miR165/166 plays a significant role in seed germination under different abiotic stress conditions like salt, draught, heat or cold and hormones like ABA and GAs (**Fig 4.4.1 to 4.4.7**). During our study, another group from China (Yan *et al.*, 2016), has also performed similar experiment, using seeds of STTM (short tandem target mimic) transgenic plants of miR165/166 with moderate phenotype and indicated the role of miR165/166 in seed germination under ABA in *Arabidopsis* (Yan *et al.*, 2016). However, we observed significant role of miR165/166 in seed germination under other different abiotic stress conditions, besides ABA treatment. This suggests important role of miR165/166-target *HD-ZIP III*s module in *Arabidopsis* seed germination, under normal and various stress condition.

In our validation experiments of both miRNA 165/666 and its targets, we observed a significant correlation of them which implies that this miRNA plays regulatory role in seed germination. To further check this in genetic level, we used the target mimic line of miR165/166. The target mimic line over express all the *HD –ZIPIII*s by blocking the activity of miR165/166. Therefore it is denoted as *eTM-miR165/166* (Wu *et al.*, 2013). Therefore *eTm-miR165/166* which is constitutively expressed under the 35S promoter was supposed to quench miR165/166 activity by mimicking its targets and leading to the over expression of all the target *HD-ZIP IIIs* (Wu *et al.*, 2013). Transgenic plants were further analyzed over next generations to test Mendelian segregations and homozygous plants were confirmed further.

4.4.2 Results

4.4.2.1 Effect of salt stress in seed germination of *eTM-miR165/166*

We found reduced level of germination efficiency upon salt treatment (NaCl-150mM) in the target mimic line of miR165/166 compare to the Wt Col. But we observed a quicker radicle growth in *eTM-miR165/166* seeds (**Fig 4.4.1.d**) at the early stage of germination (DAG2) compare to Wt Col (**Fig 4.4.1.e**).

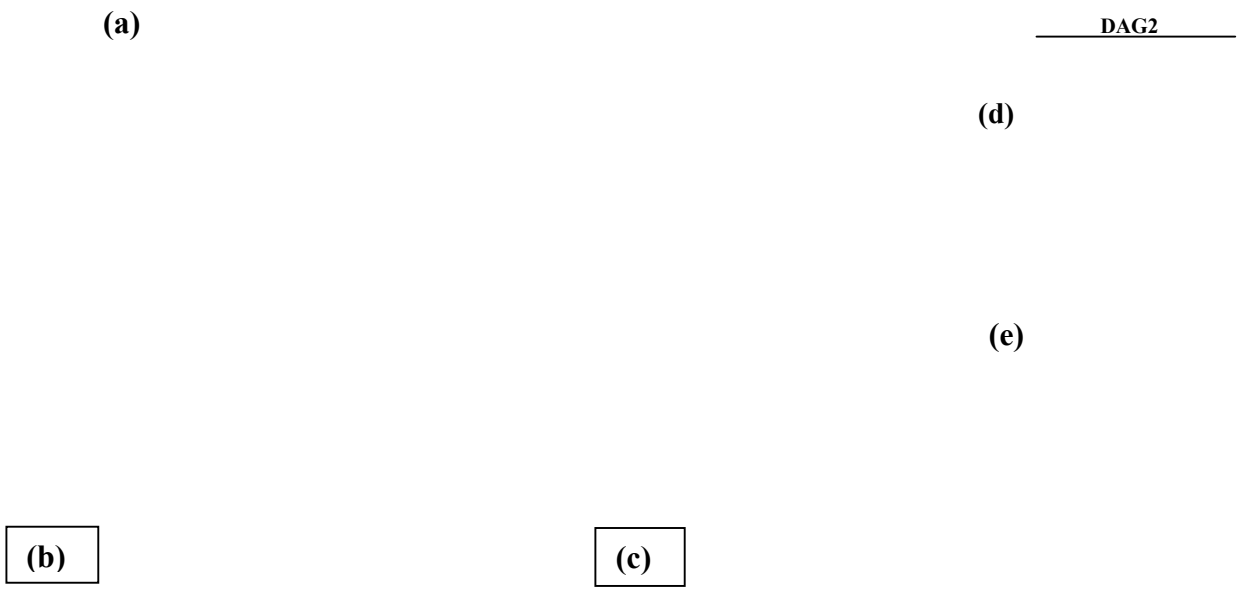


Figure 4.4.1. Effect of salt treatment on seed germination of the *eTM-miR165/166* and wild type plants. (a). Rate of germination of the *eTM-miR165/166* and wild type was measured in the medium supplemented with 150mM NaCl. And the germination scored at indicated days. (b), (c) Germination phenotype of *eTM-miR165/166* and wild type seeds in the media supplemented with salt respectively 3rd day after germination (DAG3). (d),(e) Radicle growth of *eTM-miR165/166* and Wt Col respectively after 3rd day of germination under steriozoom microscope.

4.4.2.2 Effect of draught stress in seed germination of *eTM-miR165/166*

We found high rate of germination in *eTM-miR165/166* seeds upon mannitol treatment (200mM), compare to control Wt Col. At day 1, while approximately 77% of *eTM-miR165/166* seeds have been germinated, only around 26% of Wt Col seeds have germinated (**Fig. 4.4.2**). In day 2 also, where almost all (96%) seeds of *eTM-miR165/166* line were germinated, only 67% Wt Col seeds have been germinated there (**Fig 4.4.2**). Upon mannitol treatment, the seeds of both of the cases germinated early (in day1) compare to other stresses.

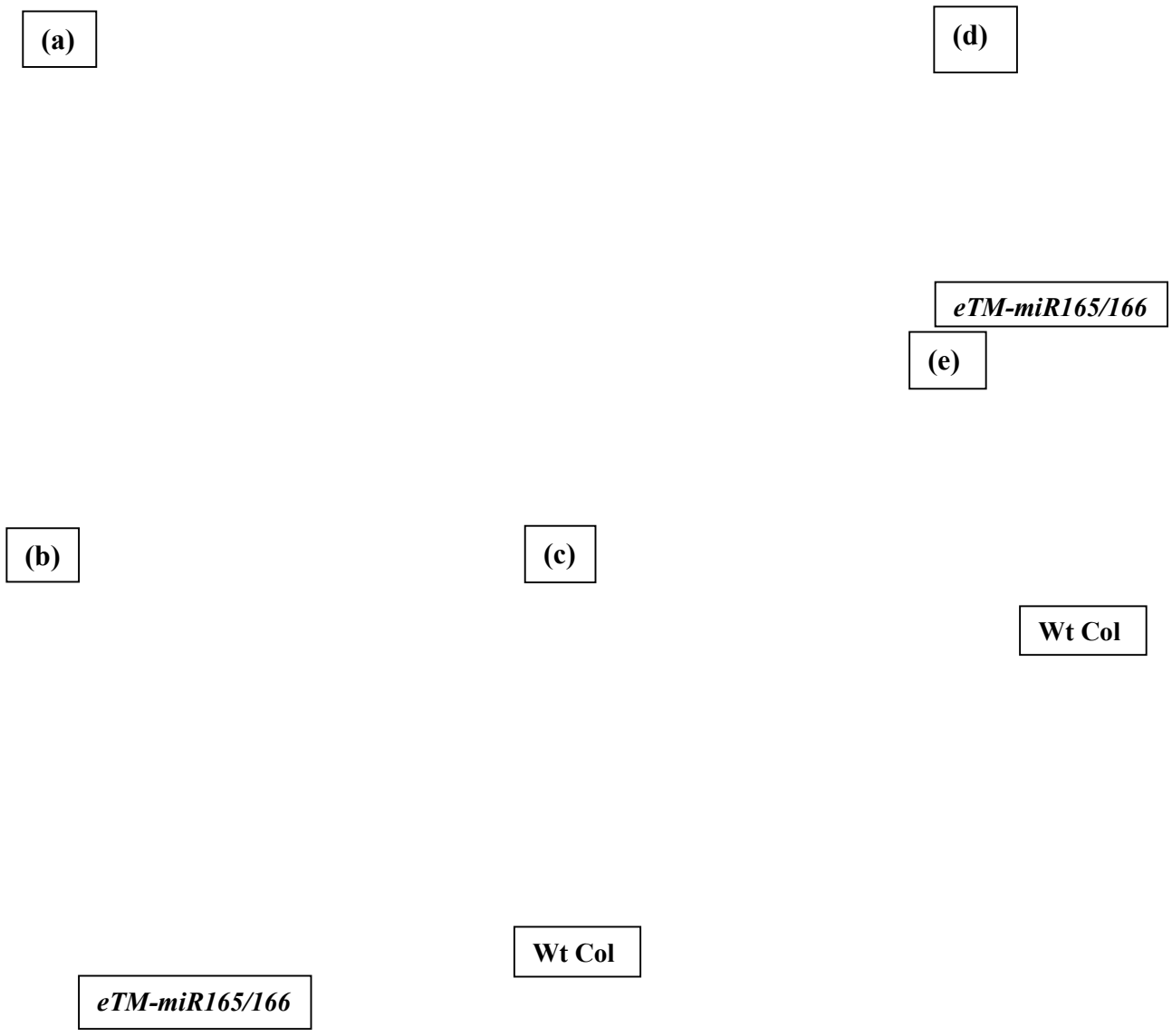
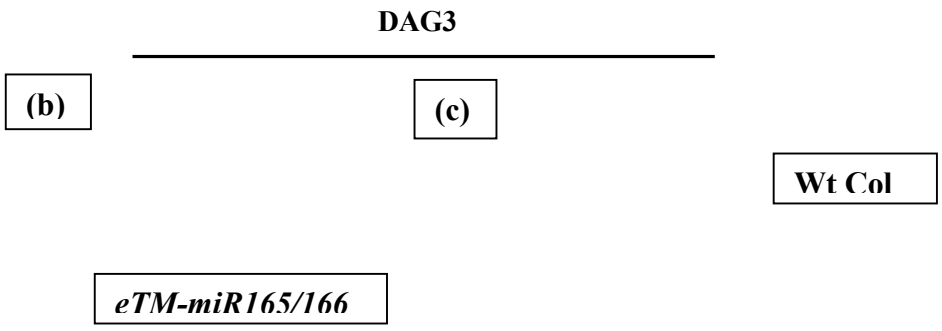


Figure 4.4.2 Effect of draught stress on seed germination of the *eTM-miR165/166* and wild type plants. (a). Rate of germination of the *eTM-miR165/166* and wild type was measured in the medium supplemented with 200mM mannitol. And the germination scored at indicated days. (b) and (c). Germination phenotype of *eTM-miR165/166* and wild type seeds in the media supplemented with mannitol respectively 3rd day after germination (DAG3). (d) and (e). Growth of *eTM-miR165/166* and Wt Col germinated seeds respectively after 3rd day of germination under steriozoom microscope.

We also observed that rate of growth in case of *eTM-miR165/166* seeds is higher compared to control Wt Col.

4.4.2.3 Effect of heat stress in seed germination of *eTM-miR165/166*

For heat stress, sterilized seeds were incubated at 45˚C for 1hr. and then plated on half strength MS plates (Silva-Correia et al., 2014). We observed high rate of germination in *eTM-miR165/166*, compare to Wt Col control. At day 2, where 88% of *eTM-miR165/166* seeds germinated, there only 61% of Wt Col seeds were germinated. There was no significant difference in growth was observed for both the lines (**Fig. 4.4.3b,c**)



(d)

(e)

eTM-miR165/166

Wt Col

Figure 4.4.3. **Effect of heat stress on seed germination of the *eTM-miR165/166* and wild type plants**. (a). Rate of germination of the *eTM-miR165/166* and wild type was measured under heat stress. (b) and (c). Growth of *eTM-miR165/166* and Wt Col germinated seeds respectively after 3rd day of germination upon heat treatment under steriozoom microscope. (d) and (e). Germination phenotype of *eTM-miR165/166* and wild type seeds upon heat stress after 3 day of germination (DAG3).

4.4.2.4 Effect of ABA in seed germination of *eTM-miR165/166*

(a)

In case of ABA stress also we found higher rate of germination in *eTM-miR165/166* seeds compare to its control Wt Col seeds. In day1, around 76% of *eTM-miR165/166* seeds were germinated, and only 18% of Wt Col seeds were germinated. Interestingly we observed that growth rate of target mimic line is higher compare to control Wt Col upon ABA (5µM) stress.

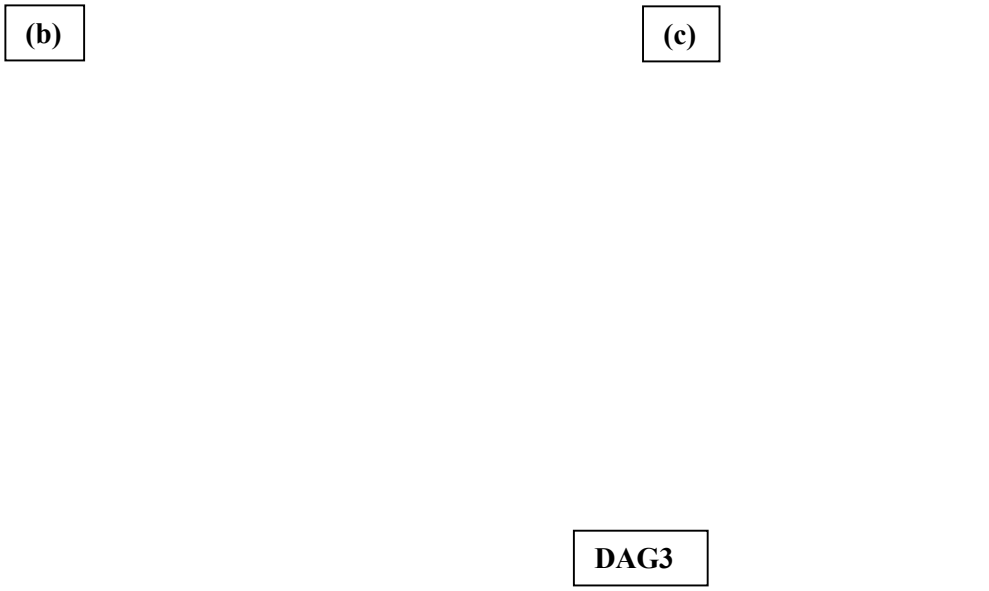
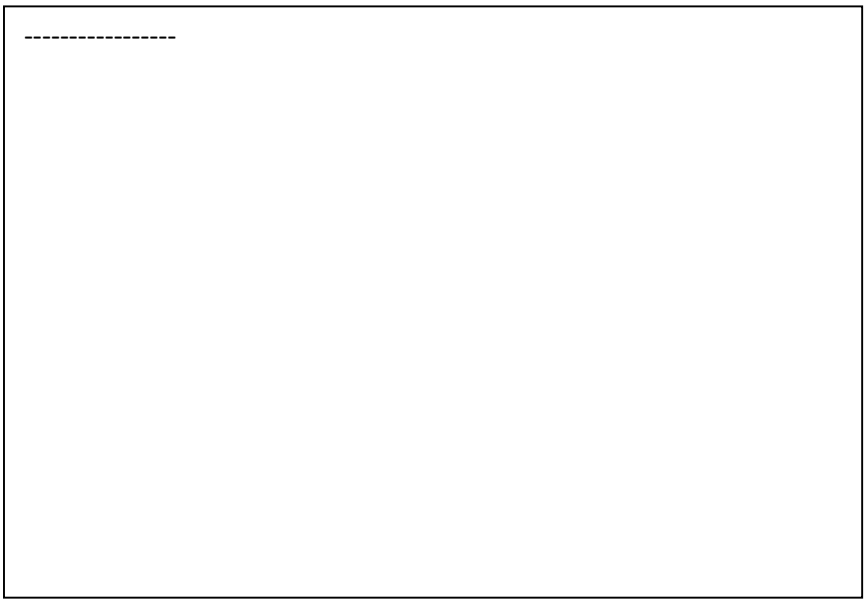


Figure 4.4.4. Effect of ABA(5µM) stress on seed germination of the *eTM-miR165/166* and wild type plants. (a). Rate of germination of the *eTM-miR165/166* and wild type was measured under ABA stress. (b) and (c). Germination phenotype of *eTM-miR165/166* and wild type seeds upon heat stress after 3 day of germination (DAG3). (d) and (e). Growth of *eTM-miR165/166* and Wt Col germinated seeds respectively after 3rd day of germination upon heat treatment under steriozoom microscope.

4.4.2.5 Effect of GA in seed germination of *eTM-miR165/166*

GA is a germination promoting hormone. We observed elevated level of germination upon GA (5µM) treatment in case of both *eTM-miR165/166* line and its control Wt col. But, interestingly the rate of germination was higher in *eTM-miR165/166* line. At first day of germination, where 88% seeds were germinated in target mimic line, 58% Wt Col seeds were germinated there (**Fig 4.4.5**). No any significant difference of growth was observed in case of both *eTM-miR165/166* and Wt Col (**Fig 4.4.5b** and **c**).

(a)



(b)

(c)

DAG3

eTM-miR165/166 **Wt Col**

(d) **(e)**

eTM-miR165/166 **Wt Col**

DAG3

Figure 4.4.5. Effect of GA (5µM) stress on seed germination of the *eTM-miR165/166* and wild type plants. (a). Rate of germination of the *eTM-miR165/166* and wild type was measured under GA stress.(b) and (c). Germination phenotype of *eTM-miR165/166* and wild type seeds upon GA(5µM) treatment after 3 day of germination (DAG3). (d) and (e). Growth of *eTM-miR165/166* and Wt Col germinated seeds respectively after 3rd day of germination upon GA treatment under steriozoom microscope.

4.4.2.6 Effect of cold stress in seed germination of *eTM-miR165/166*

In order to give cold stress, after cold stratification the plates were placed at cold room in 16h/8h light/dark cycle till 4th day after transferring to cold room. During this cold treatment no germination was observed in any seeds. At 5th day, the plates were transferred to normal growth condition, and observed germination. In day5, where 98% of *eTM-miR165/166* seeds were germinated, only 68% of control Wt Col seeds were germinated there. Therefore upon cold treatment also, eTM-miR165/166 confer comparatively high rate of germination. Interestingly, we observed a speedy growth of *eTM-miR165/166* seedlings comparative to Wt Col.

(a)

.....

(b) **(c)**

eTM-miR165/166 **Wt Col**

DAG1 (or, after 5th day from transferring to cold room).

Figure 4.4.6 Effect of cold stress on seed germination of the *eTM-miR165/166* and wild type plants. (a). Rate of germination of the *eTM-miR165/166* and wild type was measured upon cold treatment. (b) and (c). Growth of *eTM-miR165/166* and Wt Col germinated seeds respectively after 5th day of germination upon cold treatment under steriozoom microscope.

4.4.2.7 Seed germination of eTM-miR165/166 and control Wt Col in without stress conditions

Very interestingly, in the normal growth condition (22 ± 2˚C,16:8 h light:dark), we observed higher and early rate of seed germination efficiency in *eTM-miR165/166* line compared to its counterpart Wt Col. At 12h after germination, we found ~29% of seeds from target mimic line were germinated, while no Wt Col were germinated then (**Fig 4.4.8a**). During day1, 91% *eTM-miR165/166* line seeds were germinated, only 40% of Wt Col seeds were germinated then (**Fig 4.4.7a**).We couldn't observed any significant change in growth of germinating seedlings in both the cases (**Fig 4.4.7d** and **e**).

(a)

.....

(b) **(c)**

(d) **(e)**

Figure 4.4.7 Seed germination of *eTM-miR165/166* and wild type plants in normal half MS plate (without stress condition). (a). Rate of germination of the *eTM-miR165/166* and wild type was measured under normal growth condition. (b) and (c). Germination phenotype of *eTM-miR165/166* and wild type seeds in normal growth condition after 3 day of germination (DAG3). (d) and (e). Growth of *eTM-miR165/166* and Wt Col germinated seeds respectively after 3rd day of germination in without stress condition (normal growth) under steriozoom microscope.

4.4.3 Discussion

Although earlier studies have found different roles of miR165/166 in plant growth and developments like in the determination of leaf polarity, root development etc. but during seed germination as well as in overall seed physiology, the role of miR165/166 is poorly understood till date. In this study we tried to focus the role of this miRNA in seed germination and we found that under abiotic stress conditions miR165/166 plays significant role in seed germination process.