SUMMARY

A seed of angiosperm contains a dormant embryonic plant, which resume its post embryonic life upon germination of the seed under favourable environmental conditions (Toole *et al.*, 1956). Some seeds germinate as soon as water is absorbed (imbibitions), whereas in others, germination requires additional factors. Three distinct stages are evident in germinating seeds, which are (i) imbibition of water, (ii) cell elongation, and (iii) increase in cell number. Seed germination is a dynamic process and involves various factors including transcription factors, environmental factors like soil, water, light, temperature, stress etc., plant hormones, and also small RNAs (Das *et al.*, 2015). However, the role of small RNAs in seed germination remained elusive. The aim of our current study was to identify the miRNAs that are potentially involved in seed germination, validation of their expression in a different conditions of germination, identification and expression pattern of the targets (of miRNAs) and its correlation with the miRNAs expression, probable crosstalk between different classes of small RNAs (miRNAs and ta-siRNAs), and functional characterization of selected miRNA (/s) for their potential role in the dynamic seed germination process in *Arabidopsis thaliana*.

Firstly, we chose the early stages of seed germination like 0h (dry seed), 12h, 24h and 48h of imbibition conditions at both room temperature and cold condition (4˚C), and compared them with dry seeds. We have isolated total RNAs from seeds using seed specific guanidine hydrochloride method (Singh *et al.*, 2003) at those above said germination conditions, checked the quality and integrity of the total RNAs by bioanalyzer and gel electrophoresis. Thereafter, we performed miRNA microarray analysis. We have identified 58 miRNA precursors (pre-miRNAs) belonging to 30 miRNA families to be differently expressed in the comparative study of three different sets of germination 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**). Differential expression of these miRNAs, potential regulators of seed germination, have been illustrated with Venn diagram (**Fig 4.1.4.d**).Among these, 15 miRNA precursors belonging to 14 families were under the cut off value of $P \leq 0.05$ and fold change ≥ 2.0 and were 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 have identified the targets of the selected miRNAs using psRNATarget tool. 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 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. We validated 27 different targets for 15 different miRNAs. The expression of the targets was determined using the same germination conditions as those for the miRNAs. We found a significant correlation between the miRNAs and its respective targets.

Since miR390 is required for *TAS3* transcript and tasiRNA production, we chose to characterize it further to understand possible involvement of miRNA-*tasiR-ARF* crosstalk in seed germination. We have found maximum expression of miR390b at 24h/ RT following 24h/ 4˚C (**Fig 4.2.1c**) using qRT-PCR method. We observed high level of expressions of miR390b (using *pMIR*390b::GUS seeds) at 24h/ RT (**Fig 4.2.7a**) and then 24h/ 4˚C (**Fig. 4.2.7b**) of germinating seeds, which was comparable with the expression data using the qRT-PCR method (**Fig 4.2.1c**). Our results indicate potential role of miR390 in the seed germination process.

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. 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. This result indicates 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 (**Fig 4.3.1a, b**)

Further we observed that the expression of *ARF2, ARF3* and *ARF4* were downregulated in conditions in which miR390 was up regulated, indicating expected correlation. To test the hypothesis whether miR390–*tasiR-ARF* module might play role in seed germination, we

performed seed germination assay 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 germinated, when only 35% of Wt Col seeds germinated under normal (no stress) condition (**Fig. 4.3.3f**). This indicates 15% higher germination rate of *sgs3-11* seeds in comparison to its wt counterpart. We observed higher rate of germination in *sgs3-11* in comparison to its control Wt Col under most of the stress conditions studied. Since *sgs3-11* is defective in *tasiR-ARF* production, the enhanced seed viability and germination of *sgs3-11* seeds indicates the role of *tasiR-ARF-ARF2/3/4* module in seed germination. Although we can't rule out additional involvement of other ta-siRNAs.Thus, our study uncovers the role of miR390-ta-siRNA-*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 thaliana*.

For functional characterization of miRNA, we selected miR165/166, and performed the germination assay of seeds from its target mimic line *eTM-miR165/166* (that overexpress target *HD-ZIP IIIs*) and control under different stress conditions. We observed higher rate of germination efficiency of the homozygous seeds of target mimic line, which indicates a significant contribution of miR165/166 in seed germination. Further functional characterization of the other miRNAs identified in this study will help to unravel the molecular network of miRNA mediated gene regulation underlying the dynamic seed germination process. The hypothetic model provided below shows the significant miRNAs and their targets we have identified and indicates their functions.