INTRODUCTION

Seeds of higher plants harbour dormant embryonic plantlet and packed up nutrients. They are also one of the major food resources in world's diet. Around 90% of all cultivated seeds put in up to half of the world per capita energy intake. Seeds such as the cereals and legumes are major food sources whose importance exists in the storage of protein, oil and starch accumulated during seed development and maturation. The seed, with the enclosed embryo as the future plant in miniature, is physiologically and structurally well facilitated to perform as a dispersal unit and is well equipped with food stocks in order to provide the support to emergent seedling until it installs itself as an autotrophic, self-contained organism. Therefore, seeds constitute a significant position in the life cycle of the higher plants. The success with which the new individual is established the place, the time and the vigor of the young seedling is largely dependent upon by the physiological and biochemical features of the seed.

In angiosperms or flowering plants, seeds develop inside the ovary of flower and mainly consist of three morphologically and genetically distinct parts: a seed coat, derived from maternal tissue, the new embryo, and endosperm (usually triploid) derived from second fertilization. In other seed plants the remains of the haploid female gametophyte are present and there is no endosperm. Seeds are extremely variable in size, shape and structure, from coconuts weighing several kilograms to the microscopic seeds of orchids. An embryo consists of an embryonic axis bearing one or more cotyledons, or seedling leaves (Bewley and Black, 1994). At one end of the embryonic axis is the plumule, which will form the shoot (stems and leaves). At the other end there is the radicle, which gives rise to the root. Seeds of monocots have single cotyledon, called the scutellum (Bewley and Black, 1994).The function of the scutellum is primarily to mobilize and absorb nutrients during germination. Seeds of dicots have two cotyledons that supply the germinating seed with nutrients. Seed physiology involves one of the most two important physiological components namely germination and dormancy (Bewley, 1997). Seed germination starts with imbibition of water and terminates with the emergence of the embryonic axis, usually the radicle. It therefore involves numerous biochemical events like sub cellular structural changes, protein hydration, macromolecular synthesis, respiration and cell elongation, any of these events aren't unique itself to germination (Bewley, 1997). But their mutual effects act as a driving force to transform a dry as well as dehydrated embryo whose metabolism events are hardly detectable to a metabolically active growing plantlet of a germinating seed. It was earlier suggested that

germination event mainly include three several phases. During phase-I, the interesting event occur is quick leakage of solutes which is responsible for further cellular events like respiration and protein synthesis. Basically, a speedy initial uptake of water occur during phase-I, which is followed by a flat or plateau phase called phase-II. During phase-II, new mRNAs and proteins are synthesized. As mitochondria is the power house of a cell, the collection of mitochondria also happen in this stage to supply required energy intake. The second round of water uptake occurs just after completion of germination when the embryonic axes extend. Interestingly, dormant seeds cannot enter into phase-III as they couldn't complete germination since radicle growth is not possible for them due to some unknown reason. During phase-III, rapid synthesis of DNA occurs along with mobilization of stored reserves. On the contrary, seed dormancy is regarded as the inability of a normal metabolically active seed to germinate after imbibition under favourable environmental conditions. There are mainly two kind of dormancy which varies among the species. First one is termed as coat enhanced dormancy where the surrounding structure of an embryo restricts it in completion of germination. Another one is embryo dormancy where embryo is itself dormant.

Different atmospheric conditions such as oxygen, light, soil, temperature, humidity, moisture stress etc and some physiological factors such as dormancy period, viability of seeds, thickness of seed coat, etc also play significant roles in seed germination stages (Martin *et al.*, 2010; Weitbrecht *et al.*, 2011). Several recent studies have implicated that the interactions between different phyto hormones like auxin, gibberellins (GAs), abscisic acid (ABA), cytokinins (CKs), brassinosteroids (BRs) and ethylene take part in a significant role in regulating the interrelated molecular events which control liberation from dormancy and subsequently the activation stages during seed germination (Finkelstein *et al.*, 2008; Liu *et al.*, 2007). As some phyto hormones exert crucial but contrasting influence on the process, the activity of plant hormones needs to be precisely regulated. Studies had shown that ABA acts as a negative regulator of seed germination, but it acts as an enhancer of dormancy and its maintenance (Finkelstein *et al.*, 2008). The insensitivity or absence of ABA during later stages of seed development gives rise to viviparous or precociously germinating seeds; such as *Arabidopsis* ABA-deficient (*aba*), ABA-insensitive (*abi*), maize viviparous (*vp*) and tomato sitiens (*sit*) mutant seeds etc (Finkelstein et al., 2008). GA helps to release from dormancy and its role is quite similar to ethylene and BR in promoting germination by counteracting ABA effects. The crosstalk between ABA and auxin has also been come into

light recently (Finkelstein *et al.*, 2008). The study of microRNA (miRNA), a class of small non-coding RNAs (of 19–24 nucleotides length) has added a new dimension to the understanding of the regulation of cellular environment (Axtell *et al.*, 2007; Bartel, 2004). It has been shown that in both plants and animals they play multiple significant roles in case of growth, development, morphogenesis and various stress responses (Chen, 2012). Mature and functional miRNAs are produced from double stranded RNA precursors with the help of RNA-dependent RNA Polymerase (RDR), DICER-like (DCL), and ARGONAUTE (AGO) proteins (Allen *et al.*, 2005; Axtell, 2013; Mallory *et al.*, 2008; Rajagopalan *et al.*, 2006). miRNAs bind to the complementary sequences of their target genes and thus negatively regulate them. At the mRNA level, the small RNAs may be involved in chromatin remodeling (Huettel *et al.*, 2007; Pontier *et al.*, 2012; Xie and Yu, 2015) while at the post-transcriptional level, they can bring about the cleavage of the target mRNA (Rajagopalan *et al.*, 2006; Vaucheret, 2006) or can block their translation depending upon the nature of homology (Bartel, 2009; Poethig *et al.*, 2006; Vaucheret, 2006). The molecular functions and biosynthesis pathway of many of these small RNA genes are also regulated by various phyto hormones and environmental stresses (Khraiwesh *et al.*, 2012; Mallory *et al.*, 2005; Martin *et al.*, 2010; Reyes and Chua, 2007; Sanan-Mishra *et al.*, 2013; Shukla *et al.*, 2008; Sunkar *et al.*, 2007). Detailed characterization and study of multiple small RNA biogenesis pathway genes like *DCL1, HEN1, AGO1* and *HYL1* had shown that they exert severe developmental defects in embryo during embryogenesis and overall seed development (Willmann *et al.*, 2011). One fine example is *dcl1* mutant which produces early seed maturation phenotype than its control normal wild type seeds. The concept of the implication of small RNAs during seed germination and dormancy also has been focused following these studies. Leafy cotyledon (*LEC*) genes like *LEC2* and *FUS3* are the positive regulator of *DCL1*. Whereas *ASIL1, ASIL2* and *HDA6/SIL1* are considered as the repressor or negative regulator for early embryo maturation *(Willmann et al., 2011).*

Through the findings of two ABA supersensitive mutants for germination like *absg1* and *absg2* as the alleles of *dcl1* and *hen1*, the complex regulatory crosstalk between the hormones and the small RNAs was come into light (Willmann *et al.*, 2011). These two mutants showed positive regulation of the expression of ABA responsive genes (Zhang *et al.*, 2008). miR159 is one of the efficient players in the regulation of seed germination process by modulating GA and ABA hormone signaling. Another way we can say that the expression of miR159 is maintained by both GA and ABA (Martin *et al.*, 2010). Earlier reports suggested that

GAMYB acts as an activator and *DELLA* acts as a repressor for the GA signaling cascade (Finkelstein *et al.*, 2008; Peng and Harberd, 2002; Weitbrecht *et al.*, 2011). The *GAMYB* transcripts are governed by miR159 during the development of flower, fertility and seed germination (Reyes and Chua, 2007). Recent studies had revealed that alurone vacuolation, a GA-mediated programmed cell death (PCD) process in alurone layer of seed is essential for seed germination (Alonso-Peral *et al.*, 2010; Finkelstein *et al.*, 2008; Peng and Harberd, 2002). miR159 targets *MYB33* and *MYB101* which act as an upregulator of seed germination and dormancy (Martin *et al.*, 2010; Reyes and Chua, 2007). The miR159 upregulation was observed in case of *rdr2* and *dcl2 dcl3 dcl4* triple mutants. Interestingly *DCL2, 3, 4 and RDR2* are the critical factors for small RNA biogenesis, especially for heterochromatic siRNA biogenesis pathway (Allen and Howell, 2010; Axtell, 2013). These findings suggest that besides miRNAs, different kind of other small RNAs also could essentially play significant role in seed germination and dormancy. The role of the plant hormone auxin in seed germination came into light when (Liu *et al.*, 2007) showed that ARF10 is repressed by miR160 which plays actually very significant role in seed germination (Liu *et al.*, 2007). Auxin response factors (ARFs) play very important role in auxin signaling pathway during many plant growth and development. The miR160 is likely to act as the converging point of auxin and ABA regulated cross-talk during seed germination, as mutation in *ARF10* gives rise to developmental defects and over expression of ABA responsive genes (Liu *et al.*, 2007). In the same way it was found that over expression of miR160 caused hyposensitivity to ABA during germination (Liu *et al.*, 2007). Previous reports suggest that auxin homeostasis is essential for proper embryo development which is mediated by multiple miRNA actions like miR158, miR160, miR164, miR165/166 and miR167 (Martin *et al.*, 2010). This observation implies that miRNAs play a significant role during embryo and seed development by mediating suitable auxin signaling. Therefore, it could be inferred that several miRNAs not only act as an essential factor for maintaining dormancy but also actively helps in breaking from dormancy to promote embryo into seedling stage through seed germination (Huang *et al.*, 2013; Martin *et al.*, 2010; Zhang *et al.*, 2013). Ethylene, a gaseous hormone promotes seed germination through interaction with ABA signaling (Finkelstein *et al.*, 2008). The two mutants namely *ethylene resistant1* (*etr1*) and *ethylene insensitive2* (*ein2*) or*, enhanced response to aba3* (*era3*) show up regulation of ABA responsive genes and delay in seed germination (Finkelstein *et al.*, 2008).Whereas wild type seeds treated with ethylene precursor ACC (1-aminocyclopropane -1-carboxylic acid)

showed down regulation of ABA response factors (Finkelstein *et al.*, 2008). Further, *etr1-2* mutant showed over accumulation of GA content, which could be a compensation to over accumulation of ABA (Finkelstein *et al.*, 2008). As miR159 and miR160 both have regulatory effects on ABA and GA, and ethylene has a cross talk with ABA and GA, therefore, it could be presumed that these miRNAs may have direct or indirect control over ethylene mediated regulation during seed germination and dormancy. Plant steroid hormone brassinosteroid(BRs) that mainly effect stem elongation and leaf unfurling also effect seed germination. Mutational analysis has revealed that BR biosynthetic and signaling pathway are sensitive to ABA leading to decrement in the germination potential (Finkelstein *et al.*, 2008). For the activation of miR160, the possibility of a cross talk between BR and ABA signaling cannot be ruled out in seed germination (Liu *et al.*, 2007). Again, BRs induce the expression of distinct EXPANSIN (EXP) family members, which are cell wall loosening proteins that can indirectly influence seed germination (Bewley, 1997). Parallel studies indicate that those small RNA biogenesis pathway mutants which exposes higher expression of ABA, are strongly sensitive towards osmotic and salt stresses (Zhang *et al.*, 2008), thereby indicating the overlap with the environmental cues. miR395 acts both as an activator and repressor in case of seed germination under abiotic stress conditions (Kim *et al.*, 2010b). In *Arabidopsis* genome miR395 has six family members and they target *ATP Sulfurylases1, 3, 4(APS1, APS3, APS4)* and *Sulfate transporter (SULTR)* which are involved in sulfate assimilation and transport. In spite of single nucleotide difference between miR395e and miR395c, miR395e cannot target *APS1* and *APS4* (Kim *et al.*, 2010b). In *Arabidopsis,* these miRNAs also have several effects on seed germination under dehydration and high salinity stress conditions. Over expression of miR395c reduces the germination potential under high salt or dehydration stress condition; whereas over expression of miR395e enhances the germination potential under the same stress condition in *Arabidopsis thaliana (Kim et al., 2010b)*. Likewise, over expression of miR402 increases the seed germination efficiency in *Arabidopsis* under dehydration, salinity and cold stress conditions (Kim *et al.*, 2010a). Another conserved miRNA, miR402 down regulates its target gene *DML3* (DEMETER-LIKE protein3), which is an epigenetic modifier involved in DNA demethylation in plants, under various stress conditions (Kim *et al.*, 2010a). miR417 also exhibits a negative regulation over seed germination under salinity stress condition (Jung and Kang, 2007). However, the molecular mechanism of its action is not clear till date.

A class of small interfering RNAs known as trans-acting siRNA (ta-siRNA), is synthesized through a pathway involving specific *miRNAs* (like *miR173*, *miR390* and *miR828*) and other genes like *SUPPRESSOR OF GENE SILENCING 3* (*SGS3*), *RNA-DEPENDENT RNA POLYMERASE 6* (*RDR6*), *DICER-LIKE 4* (*DCL4*) and *ARGONAUTE 7* (*AGO7*) (Chen, 2009). It was previously reported that these ta-siRNA pathway genes are known to affect vegetative phase transition (Peragine *et al.*, 2004). *SGS3* encodes a novel plant-specific protein and is required for preventing ssRNA from degradation through stabilization and *RDR6* encodes a RNA-dependent RNA polymerase (RdRP), which is essential for the conversion of ssRNA to double stranded RNA (Dalmay *et al.*, 2000; Mourrain *et al.*, 2000; Peragine *et al.*, 2004). Mutations in these two genes provide sensitivity to cucumber mosaic virus and cabbage leaf curl virus, indicating their involvement in providing resistance to the plant against viral infection (Beclin *et al.*, 2002; Muangsan *et al.*, 2004). Mutations in *SGS3* and *RDR6* caused accelerated vegetative phase change and elongated and downwardly curled leaves (Marin *et al.*, 2010b). However, the role of *SGS3* in seed germination has not yet been studied. siRNA biogenesis is different from that of *miRNA*, since *miRNA* itself is required to initiate ta-siRNA biogenesis. Few specific *miRNAs* recognize and cleave *TAS* loci derived precursor transcripts to initiate ta-siRNA biogenesis (Allen *et al.*, 2005). It has been shown that *miR173*, *miR390* and *miR828* cleave precursor transcripts derived from the *TAS1, TAS2, TAS3* and *TAS4,* respectively. ta-siRNA derived from *TAS3* locus, targeting *AUXIN RESPONSE FACTOR*s (*ARF*s*)* are known as tasiR-*ARF* (Allen *et al.*, 2005). tasiR- *ARF*s downregulate its target *ARF2*, *ARF3,* and *ARF4* transcripts (Marin *et al.*, 2010a). Seed germination is considered as one of the most significant phase transition in higher plants where seedling appears from seed. Although small RNAs are known to be involved in several plant developmental or physiological processes, its regulatory effects on dynamic stages of seed germination are less explored area till date and poorly understood. Recent studies indicated involvement of some miRNAs in seed germination of different plant species like rice, maize and other monocot. However, in *Arabidopsis,* the regulation of small RNAs, especially miRNAs and its possible crosstalk to ta-siRNA, during the dynamic process and stages of seed germination is yet to be properly understood. Moreover, the impact of environmental conditions or stresses on miRNA mediated regulation of seed germination remains poorly understood. In current study, we have identified miRNAs and their target genes, which are potentially involved in the seed germination under normal and stressed condition. Our study also uncovers the role of

miR390–tasiR-*ARF2/3/4* module in seed viability and germination and suggests a crosstalk of miRNA and ta-siRNA in the dynamic process of seed germination in *Arabidopsis thaliana*.

The objectives of the thesis are:

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

2. Validation of miRNA expression, identification of targets, functional annotation and tissue specific expression studies (*in silico*).

3. Functional analysis of the selected miRNA(s) using transgenic/ molecular genetics approach.

Significance of work

Agriculture largely depends upon the cultivation of plants specifically crops, and seed production. Therefore, the overall success of food productivity includes seed viability, seed germination property and of course the efficiency of seed development. Only a small fraction of known small RNAs have been indicated for their role in developing and germinating seeds (Das *et al.*, 2015). Moreover, regulation and developmental or physiological role of miRNAs and other small RNAs in seed germination remain poorly understood. Our proposed study would identify small RNAs mainly miRNAs regulated nodes, as potential regulator of seed germination in different stages and under different conditions, like phytohormones and abiotic stresses. Thus, our study should identify miRNAs and underscore their regulation and function during the dynamic process of seed germination in model plant *Arabidopsis thaliana.* It should enrich our understanding on small RNA mediated gene regulation, in relation to hormone signaling and abiotic stress responses during the process of seed germination. Our work should help to understand the regulation of small RNAs and their interaction with other small RNA and downstream target genes in seed germination. Besides basic understanding, knowledge of functionally crucial miRNAs may be used to engineer the miRNA-target mediated regulatory pathway in crop to improve seed vigor and germination efficiency.