CHAPTER IV:

EXPRESSION ANALYSIS AND FUNCTIONAL VALIDATION OF \textit{BjERD4}
4.1. Introduction

The interaction between a plant’s genome and its environment determines it’s growth and development. Expression profiling has become an important tool to investigate responses of an organism to environmental changes at the transcriptional level [96, 195]. Sometimes these transcriptional changes are successful adaptations leading to tolerance while at other times, the gene expression is merely a response to stress and the plant fails to adapt to the new environment and is considered sensitive to that condition. Expression profiling can define both tolerant and sensitive genotype and is useful tool for studying regulatory genetic circuitry, which has an application in biotechnological approaches to improve stress tolerance. Beyond transcript profiling, genomics also facilitates the functional analysis of genes. As signaling cascades and metabolic pathways are elucidated in model systems and crop plants, key regulatory genes can be targeted for silencing or over-expression to study the role of these pathways in plant responses to stress.

The ultimate goal of any genomics studies is to identify the biological function of every gene in the genome. Molecular and genomic analyses using model plants facilitated the resolution of complex networks and led to the discovery of additional mechanism(s) of stress tolerance [196]. By employing molecular biology tools and genetic approaches, several abiotic stress-inducible genes were isolated and their functions have been precisely characterized in transgenic plants [84]. The functions of some of the genes have been identified directly by the appropriate assay, or have been inferred by homology to gene of known function in the other organisms [197]. Loss of function has been very informative about the role of some of these genes. Transformation of dsRNA can trigger specific RNA degradation, in a process known as RNA-interference [198]. This process
facilitates targeted post-transcriptional gene silencing (PTGS). Using hairpin RNA (hpRNA) constructs containing sense/antisense arms ranging from 98 to 853 nt can give efficient silencing in a wide range of plant species and inclusion of an intron in these construct has a consistently enhancing effect.

Further several genes that are involved in signaling and regulatory pathways or genes that encode proteins conferring stress tolerance or enzymes present in pathways leading to the synthesis of functional and structural metabolites have been transferred into crop plants to improve their tolerance against specific stress conditions. Over expression studies using a variety of genes associated with stress tolerance pathways has been employed to generate transgenic plants. Overexpression of the cold-induced plasma membrane protein gene (MpRCI) of plantain (Musa paradisiaca) in tobacco resulted in increased tolerance to low temperature [199]. A NAC-type transcription factor (OsNAC5) of rice, when overexpressed in transgenic rice, caused increased tolerance to salinity [200].

In this chapter, study related to changes in transcript expression of ERD4 gene under salt, PEG and other abiotic stress in Brassica juncea cv. Pusa Bold are presented. The role of Brassica juncea ERD4 protein in abiotic stress tolerance has been elucidated using knockdown and overexpression approaches.

4.2. Materials and methods

4.2.1. Plant materials, growth conditions and stress treatments:

Brassica juncea cv. Pusa Bold was used in all the experiments. Seeds were soaked in distilled water for 0.5 h, and then germinated in plastic Petri dishes containing filter paper saturated with distilled water in darkness at 22°C for 2 days. Seedlings were then transferred to hydroponic containers containing continuously aerated 1/2 Murashige
and Skoog (MS) liquid solution (pH 5.8, without agar and sugar). The 1/2 MS liquid solution was changed once every 3 days.

Basal expression of ERD4 during different stages (germinating seed, root, shoot, young leaves, mature leaves and pod) of Brassica juncea “Pusa Bold” was checked with semi quantitative PCR. For stress treatments (salinity and osmotic) three-week-old seedlings were transferred to new 1/2 MS liquid solution (pH 5.8, without agar and sugar) under a continuous time course (0, 0.5, 1, 2, 4, 8 and 16 h). For salt and osmotic treatments, seedlings were exposed to 1/2 MS solution (pH 5.8) containing 200 mM NaCl and 20 % (w/v) polyethylene glycol (PEG). For cold and heat treatments, seedlings were exposed to the 4°C and 37°C conditions in 1/2 MS solution (pH 5.8) for one hr. For mannitol, ABA and salicylic acid treatments, seedlings were exposed for 1 hr to 1/2 MS solution (pH 5.8) containing 200 mM mannitol, 100 μM ABA and 100 μM salicylic acid. All seedlings were placed under the same growth conditions, except for the different treatment factors, and exposed to 1/2 MS solution at 25°C as controls. The root and shoot samples were harvested in three biological replicates for RNA preparation.

4.2.2. Isolation of RNA from plants:

100 mg leaf and root tissue of treated samples were ground into a fine powder in liquid nitrogen. The powder was transferred to tubes containing 2 ml Trizol and 400 μl of chloroform, vortexed vigorously, and then spun down at 13000 rpm for 20 minutes at 4°C. The supernatant was transferred to a fresh tube and an equal amount of ice cold isopropyl alcohol was added. The contents were mixed by gentle inversion and kept at room temperature for 10 minutes to aid RNA precipitation. The samples were spun at
13000 rpm for 20 minutes at 4 ºC to collect the RNA pellet. The pellet was washed with ice cold 70% ethanol, dried in air and dissolved in 25µl of DEPC water.

For checking the RNA integrity, the samples were loaded on 2% agarose gel with 1 kb ladder. Two intact bands representing 28S and 18S RNA was visualized. The concentration of RNA was determined by measuring the absorbance at 260nm in a spectrophotometer by diluting 5µl of RNA sample with 995µl of DEPC autoclaved water. The ratio of 260nm and 280nm (A$_{260}$/A$_{280}$) was recorded as an estimate of RNA purity with respect to contaminants that absorb in the U.V spectra such as protein. Pure RNA has an A$_{260}$/A$_{280}$ ratio of 1.8-1.9. RNA concentration (µg/ml) was calculated using formula: 40 x Dilution factor x OD$_{260}$.

4.2.3. cDNA Synthesis:

First strand cDNA was synthesized from 2.5µg total RNA. The oligo (dT) primer was used for cDNA synthesis so that the same cDNA pool could be used for internal control and the target gene transcript expression analysis. To minimize the potential effects of the efficiency of synthesis during the reverse transcription reaction, three separate cDNA syntheses were performed and pooled for each RNA preparation. The cDNAs were then stored at −20°C until used for real-time PCR. In order to check DNA contamination, −RT control reaction was kept. The forward (BEEF: 5’TCCGTGAAGCTTTCACTTCC3’) and reverse (BEER: 5’GTTGGCTAAAGGTTGTTG 3’) primers for ERD4 and, forward (AraActF: 5’GGCTCCTTACACCCAAGG 3’) and reverse (AraActR: 5’ CAGTAAGGTTCGCTCAGCA3’) primers for β actin were designed using Primer3 software [201]. Real-time quantitative RT-PCR was carried out. The *Brassica juncea* β-actin gene was amplified in parallel with the target gene
ERD4, for gene expression normalization and providing relative quantification. Detection of real-time RT-PCR products was done using a SYBR Green master Mix kit. The quantity of cDNA used as a template for PCR was 2.0μl (the equivalent of 250ng of total RNA).

4.2.4. Real time PCR for transcript quantification:

The 2μl of diluted cDNA from each sample was taken for the real time RT PCR quantification of ERD4 gene. Arabidopsis thaliana actin gene was also run in parallel with the target gene which allowed the gene expression normalization. The detection of real time PCR product was done using the Sybr Green 2X Master Mix kit (Sigma 048K6272). the quantity of cDNA used as a template for PCR was 2μl and the PCR cycling condition was comprised of an initial cycle at 94⁰C for 5min, followed by at 94⁰C for 30 sec; 55⁰C for 30 sec and 72⁰C for 20 sec. For each sample, reactions were set up in triplicates to ensure the reproducibility of the results. At the end of each PCR run, melting curve was generated and analyzed with the dissociation curve software. The melt curve obtained depends on the GC/ AT ratio and the overall length of the amplicon. This analysis allowed products to be distinguished from one another and also to be identified primer dimmers and other erroneous dsDNA. The exact quantification was done using the software REST-MCS.

4.2.5A. Preparation of siRNA construct for the ERD4 gene:

A 442 nt piece of ERD4 gene coding region was amplified from Brassica juncea cDNA using a primer that added XhoI and KpnI site on the ends of one product (sense forward: CTGAGAGGGCTTGTGAGCGAAAACCA, reverse: GGTACCACAGAAAAC ACGCCGCTAGT ) and ClaI and BamHI site on the ends of the other product
(antisense, forward: GGATCCAGGCTCTTGAGACGAAACA, reverse: ATCGATACAGAAACAACGCGCTAGT). These two amplified products were cloned separately in TA cloning vector. After restriction digestion these two products were further directionally cloned into Hannibal (EHSA) and transformed in \textit{E. coli} XL1 blue strain. Double digestion with \textit{XhoI} and \textit{KpnI} and \textit{BamHI} and \textit{ClaI} confirmed the presence of sense and antisense arm. EHSA clone was digested with \textit{NotI} and upper band of \textit{NotI}-digested product of EHSA clone was then ligated with plant expression vector pART27 (digested with \textit{NotI}). Plant expression vector with expression cassette was transformed into \textit{E. coli} XL1 blue strain. Blue white screening method was used for selection and 10 colonies were double digested with \textit{XhoI} and \textit{BamHI} for confirmation of expression cassette. The siRNA construct was then transformed in \textit{Agrobacterium Eha105}. Presence of siRNA construct was confirmed by PCR using primers for \textit{nptII} gene and CaMv promoter region.

4.2.5B. Construction of overexpression cassette of \textit{ERD4} gene:

For construction of overexpression cassette of \textit{ERD4} gene, pART27-ERD4-GFP construct prepared for the subcellular localization study was used (details given in section 2.2.9A). pART27-ERD4-GFP construct was digested with \textit{KpnI} and \textit{HindIII} restriction enzyme and \textit{GFP} gene was released. The digested construct was blunted and ligation was performed. The resulted product named as pART27-ERD4 consisting of CaMV-\textit{ERD4}-OCS expression cassette in pART27 plant expression vector was used for overexpression study.
4.2.6. Transformation of *Agrobacterium tumefaciens* EHA105:

*Agrobacterium tumefaciens* Eha105 competent cells were prepared. To an aliquot of 100 µL of the resuspended cells, 2 µg of the transformation vector DNA was added. Electroporation was performed using multiporator (Eppendorf make). One ml LB was added to each tube and incubated at 28 ºC for 2 hours and then plated on LB plates (rifampicin 100 µg/ml, Spectinomycin 100 µg/ml). Transformed colonies appeared in 2-3 days after the plates were incubated at 28 ºC.

4.2.7. *Agrobacterium* mediated Arabidopsis transformation of *ERD4* siRNA and overexpression constructs:

The floral dip method developed by Clough and Bent was adapted [202]. Wild type Columbia was used to transform with knockdown transformation vectors. Plants were sown in soil that was sterilized by presoaking it in fertilizer for 10 minutes. The seeds were stratified for 5 days and then moved under constant light at room temperature. Once the first two true leaves were formed, they were pruned so that the number of plants per pot was ~25. The first bolts were cut to encourage the formation of secondary bolts. At this stage plants that did not produce the first bolts were removed. When most plants had produced inflorescences, they were once again pruned such that none of the plants possessed fruits or flowers that might have been pollinated. The plants were then bound at the base with elastic bands to keep the soil from dropping into the transformation solution. The transformation solution was made of 5 % sucrose solution and resuspended *Agrobacterium* cells. It was subjected to gentle stirring until the detergent was mixed completely. The plants were dipped into the transformation solution by inverting the pot into it and swirled gently for exactly 10 seconds. The dripping sucrose solution was
carefully drained off from the plants. The plants were placed on slanting trays, covered with plastic wrap and kept under low light for 24 hours. The wrap was slit to allow slow entry of air for the next 24 hours. After a total of 48 hours, the plants were returned to their erect positions and allowed to continue to grow until the maximum amount of seeds were harvested.

4.2.8. Screening of transgenic plants:

The bulk harvested T1 seeds were surface sterilized by vortexing them in 1 ml of 70% alcohol twice for 10 minutes each. Then seeds were thoroughly washed five times with autoclaved distilled water by vortexing them for 10 minutes each time. The sterilized T1 seeds were spread evenly on MS plates with kanamycin (100 µg/ml). The seeds were stratified at 4 ºC for 7 days, moved to room temperature under low light to induce germination. Resistant plants that grew on kanamycin-MS plates were considered as T1 transformants. They were transplanted into sterilized soil, allowed to self pollinate and individually harvested to collect T2 seeds. The T2 seeds were surface sterilized as described above and plated on kanamycin-MS plates. The resistant vs self pollinate and individually harvested to collect T2 seeds. The T2 seeds were surface sterilized as described above and plated on kanamycin-MS plates. The resistant Vs sensitive segregation ratio of T2 seeds on kanamycin-MS plates was used to identify single (3:1). The resistant T2 plants were transplanted into sterile soil, allowed to self pollinate, and individually harvested to collect T3 seeds. The T3 seeds that gave a 100% kanamycin resistance when grown on kanamycin-MS plantes were considered homozygous for single insertion of the gene-of-interest. These lines were used for further analysis.
4.2.9. Characterization of *BjERD4* knockdown and overexpressed lines

4.2.9.1. Molecular characterization of knockdown lines:

Based upon kanamycin selection, six knockdown and overexpressed lines were selected separately. Genomic DNA was isolated from all these lines. PCR was performed using primer for *nptII* gene (forward: TGTTCCGGCTGTCAGCGCAG, reverse: GATCCTCGCCGTCGGGCATG) and the PCR cycling condition was comprised of an initial cycle at 94°C for 5 min, followed by at 94°C for 1 min; 55°C for 1 min and 72°C for 30 sec. To see the change in transcript level of *ERD4* gene in knock down and overexpressed lines, quantitative real time PCR method was used. Total RNA isolation, cDNA preparation and real time PCR was performed as explained under expression analysis studies (sections 5.2.2 and 5.2.3).

4.2.9.2. Determination of germination:

Wild-type and erd4 mutant seeds sown on 1/2 MS were cold-stratified at 4°C for 2 days in the dark and then incubated at 22°C under the long-day condition for 5 days. Each day, the number of germinated seeds with protruding radicals was counted (Oh et al., 2006).

4.2.9.3. Assessment of Knockdown and overexpressed lines under stress treatment:

Wild-type, *Bjerd4* and overexpressed lines seeds sown on 1/2 MS were cold-stratified at 4°C for 2 days in the dark and then incubated at 22°C under the long-day condition for 5 days. Ten days old knockdown and wild type seedlings were transferred to different treatment conditions (Control-MS, Salinity- MS+100mM NaCl and Drought- 10% PEG or 150mM Mannitol) for 7 days. Performance of knockdown and
overexpressed lines were compared with wild type in terms of chlorophyll content, lipid peroxidation, GSH content and ROS production using DAB and NBT dye.

A. Determination of chlorophyll content:

Total chlorophyll content was determined spectrophotometrically according to the method described by Arnon [203]. The 300 mg leaves were grinded into powder with liquid nitrogen and then were transferred to a 15 ml Falcon tube. 5 ml of 80% acetone was added to the tube and mixed thoroughly. Centrifugation was performed at 4°C for 15 min (3,000 rpm). Supernatant was transferred to a new centrifuge tube and the absorbance of chlorophyll was measure using spectrophotometer. The chlorophyll concentrations are calculated as follows (80% acetone as a blank control was used as blank). 

\[
Ca+b (mg/g) = [8.02 \times A_{663} + 20.20 \times A_{645}] \times \frac{V}{1000} \times W.
\]

Where \( V \) = volume of the extract (ml); \( W \) = Weight of fresh leaves (g).

B. Estimation of lipid peroxidation (LP):

The LP level in plant tissues was determined by measuring the malondialdehyde (MDA) content via the 2-thiobarbituric acid (TBA) reaction [204]. Leaf tissue (100 mg) was homogenized in 1 ml of 10 mM sodium phosphate buffer (pH 7.4) and centrifuged at 4000 g for 5 min at room temperature. A 200 µl aliquot of the supernatant was added to a reaction mixture containing 100 µl of 8.1% (w/v) SDS, 750 µl of 20% (w/v) acetic acid (pH 3.5), 750 µl of 0.8% (w/v) aqueous TBA, and 200 µl of Milli-Q water. An identical reaction mixture in which 200 µl of supernatant was substituted by an equal volume of buffer was simultaneously set up as a blank. Both reaction mixtures were then incubated at 98 °C for 1 h. After cooling to room temperature the mixtures were centrifuged for 5 min. Absorbance at 535 nm was measured and corrected for non-specific absorbance at
600 nm. The level of LP was expressed as µmol of MDA formed derived from the difference in absorbance at 535 nm and 600 nm using an extinction coefficient of 156 mM⁻¹ cm⁻¹.

C. Measurement of glutathione content:

For estimation of reduced (GSH) plant material (500 mg) was frozen in liquid nitrogen and homogenized in 0.1 M phosphate-EDTA buffer (pH 8.0) containing 25% meta-phosphoric acid. The homogenate was centrifuged at 20,000 g for 20 min at 4°C. GSH content was determined fluorometrically in the supernatant after 15 min incubation with o-phthaldialdehyde (OPT) [205]. Fluorescence intensity was recorded at 420 nm after excitation at 350 nm on a fluorescence spectrophotometer.

D. Qualitative Assay of \( \text{H}_2\text{O}_2 \):

Detection of \( \text{H}_2\text{O}_2 \) was performed by infiltrating leaves with a solution of 1 mg mL⁻¹ DAB in MES buffer (pH 6.5) as described by Thordal-Christensen et al. [206]. \( \text{H}_2\text{O}_2 \) was visualized as a reddish-brown coloration. Prior to imaging, chlorophyll was removed from leaves with 70% (v/v) ethanol.

E. Qualitative assay for superoxide radicals:

For visualization of superoxide generation as a result of abiotic stress, 2-week-old seedlings treated with mannitol (200mM), NaCl (150mM), for 7 d, along with unstressed plants were employed for NBT staining. Stained samples were transferred to 80% ethanol and incubated at 70°C for 10min to remove the chlorophyll. The NBT staining method described by Wang et al. [207] was used for superoxide detection.
4.3. Results

4.3.1. RNA isolation and spatial expression analysis:

The quality of the isolated total RNA was assessed in terms of spectrophotometric absorbance ($A_{260}/A_{280}$). These values ranged 1.9 to 2.0, indicating good purification. Further, the total RNA samples were separated on formaldehyde denatured agarose gel. The gel electrophoresis revealed integrity of the extracted RNA and suitability for further transcript expression analysis (Fig 4.3.1A).

Basal expression of $ERD4$ was checked in different stages of plant growth of $Brassica$ juncea using semi quantitative method. $ERD4$ transcript expression was detected in germinating seeds, root, young and mature leaves, shoot, and in pod. Maximum basal expression was observed in young leaves (Fig. 4.3.1B).

![RNA isolation from different plant parts (A) and ERD4 gene expression using semiquantitative PCR (B). Gel was run for the PCR product after 28 cycles. M. Marker, 1.Germinating seed, 2.Root, 3.Shot, 4.Young leaves, 5. Mature leaves and, 6. Pod.](image)

**Fig. 4.3.1.A & B:** RNA isolation from different plant parts (A) and ERD4 gene expression using semiquantitative PCR (B). Gel was run for the PCR product after 28 cycles. M. Marker, 1.Germinating seed, 2.Root, 3.Shot, 4.Young leaves, 5. Mature leaves and, 6. Pod.
4.3.2. Temporal expression analysis of ERD4 gene under different abiotic stress conditions:

To investigate the effect of different stress conditions on the expression of the BrassicaERD4 gene, real time PCR was performed using total RNA isolated from stressed and non stressed leaves and roots of Brassica juncea cv. Pusa bold. In case of salinity treatment, significant increase in BjERD4 gene expression was detected as early as within 0.5 hr of treatment (2.165 fold) which reached maximum after 4 hr of treatment (6 fold) in roots, while in shoots maximum expression was observed only after 2 hr of treatment (2.7 fold induction). With increasing time of treatment, down expression of this gene was observed (Fig.45.3.2A).

In case of PEG treatment, early induction of BjERD4 gene was observed in root (2.75 fold) after 0.5 hr of treatment, which reached maximum after 1hr of treatment (4.491 fold). In shoots also, significant fold increase was observed within 0.5 hr of treatment whereas maximum induction was seen after 4 hr of treatment (1.4 fold) (Fig 5.3.2B). Significant increase in BjERD4 gene expression was also detected in root and shoot within 1hr when treated with mannitol (100mM), ABA (100µM), SA(100µM), cold (4°C) and heat (37°C) (Fig. 4.3.2C).
4.3.2: Expression analysis of *ERD4* gene under abiotic stress conditions. Change in *ERD4* transcript under drought (A), salinity (B) at different time periods (temporal expression) and other abiotic stress after 1 hr (C).

4.3.3. Preparation of Knock-down and overexpression constructs, transformation and screening of *erd4* and *OERD4* lines:

To understand the functional role of *BjERD4*, knockdown and overexpressed Arabidopsis lines were generated. For knockdown construct, a 442 bp long fragment was
cloned in sense and antisense orientation in Hannibal vector under CaMV promoter. This was further subcloned in pART27 plant expression vector (Fig. 4.3.3.A, B & C). For overexpression construct full length ERD4 gene was cloned under CaMV promoter and subcloned in pART27 plant expression vector (4.3.3.D).

Knockdown and overexpression constructs were transformed in Arabidopsis using vacuum infiltration method and screening was performed on Kanamycin antibiotic for transformed lines. Six independent transgenic lines were selected based on the survival on kanamycin. All the transformed lines (T4 generation) were selected on kanamycin to attain homozygosity. The homozygous nature of these lines was also confirmed by the segregation ratio of kanamycin in all the generations (Fig45.3.3E).
Fig. 4.3.3.A, B, C & D: Preparation of knockdown and overexpression construct. (A)

Schematic representation of the construct used for the knockdown of ERD4 in Arabidopsis. Confirmation of knockdown clone in Haniibal (B) and in pART27 vector with NotI releases 1.6 Kb expression cartridges, (C) knockdown in Arabidopsis by restriction digestion. (D) Confirmation of overexpression cassette 1.1Kb ladder, 2. pART27 (NotI digestion) & 3. pART27-BjERD4 (EcoRI/KpnI double digestion).
4.3.3. E: Selection of transformed lines of Arabidopsis. Transgenic lines were selected on kanamycin (100 mg/lit) on MS plate based screening.

4.3.4. Molecular characterization of Knockdown lines:

Six Arabidopsis *ERD4* knockdown and overexpressed lines were confirmed by PCR using nptII primer which showed amplification of nptII gene in transformed lines as in positive control, whereas no amplification product was observed in the Wild type (Fig. 4.3.4 A &C). The Real time PCR analysis was done for all these lines using primers designed to amplify specific gene sequence cloned in RNAi construct. Significant reduction of transcript in two lines was observed. Transcript reduction was up to 3.6 fold (Fig 4.3.4B). In case of overexpressed lines significant increase in transcript was observed in all lines but maximum fold increase in transcript was up to 4 times. (Fig 4.3.4D)
Fig. 4.3.4 A&B: Molecular characterization of transformed lines. PCR amplification with nptII primers of Knockdown (A) & overexpressed (C) lines. Lane 1: 1 kb ladder; Lanes 2 to 7: knockdown lines 1-6, Lane 8: Plasmid and Lane 9: Wild type. Real time PCR with ERD4 knockdown (B) & overexpressed (D) lines.

4.3.5. Phenotypic analysis of ERD4 RNAi lines:

Germination was scored following seed imbibition and subsequent development of roots and shoots was monitored. Germination time was delayed by two days in most of the ERD4RNAi seeds. When roots and shoots were examined, the RNAi transgenic Arabidopsis seedlings exhibited marked dwarf phenotype compared to the wild type seedlings (4.3.5A&B).
4.3.5A&B: Germination and phenotypic difference of ERD4 knockdown and wild type Arabidopsis

4.3.6. Performance of Knockdown lines under salinity and PEG treatment:

Ten days old Wt, RNAiL-2 and RNAiL-5 seedlings were transferred to different treatment conditions (Control-MS, Salinity- MS+100mM NaCl and Drought- 10% PEG/150mM mannitol) for 7 days.

**Root growth:** Root growth was restricted under drought and salinity stress. But reduction was more in the knockdown lines as compared to wild type plant (Fig. 4.3.6A).

**Chlorophyll content:** In ERD4RNAi plants, chlorophyll content and the carotenoid pigment per fresh wt was significantly decreased compared to control, while the Chl a/Chl b ratio was slightly higher in knockdown plants compared to WT. Salinity and drought stresses showed significant decrease in total chlorophyll content and the carotenoid pigment in both wild type and knockdown lines (Fig 4.3.4B).
MDA assay: As a marker of oxidative damage under abiotic stress, lipid peroxidation was measured by the TBARS assays. On the 7th day of exposure to salt and PEG, lipid peroxidation showed drastic increase of 225% and 145% in the shoot of knockdown seedlings compared to the WT seedlings (Fig 4.3.4C).

Redox state of ERD4 RNAi lines: High cellular GSH is crucial for the redox state of the cell which determines the survival of cells under any adverse condition and thought to act as redox sensor. When the concentration of GSH was measured in WT and ERD4 knockdown Arabidopsis plants, it was found that sodium chloride treatment did not perturb the GSH content in WT plants whereas, significant decrease (53%) was observed in knockdown lines (Fig 4.3.4D).

Qualitative Assay of H$_2$O$_2$: To test whether down expression of ERD4 gene leads to ROS production under stress conditions, wild type and knockdown lines leaves were stained with diaminobenzidine (DAB) for the detection of H$_2$O$_2$. As compared to wild type, intense staining was observed in knockdown lines compared to control implying that H$_2$O$_2$ level was high in knockdown lines under salt and drought stress (Fig 4.3.4 E).
Fig 4.3.6: Performance of knockdown lines under salinity and drought. Change in root length (A), Chlorophyll content (B), MDA content, (GSH content) and Qualitative Assay of H$_2$O$_2$ (E).
4.3.7. Phenotypic analysis of *ERD4* overexpressed lines:

Overexpressed lines showed significant change in plant morphology i.e enhanced plant size with more number of branches and increased leaf and pod size as compared to wild type when grown in MS medium (Fig 4.3.7).

![Morphological changes in overexpressed lines](image)

**Fig. 4.3.7: Morphological changes in overexpressed lines**

4.3.8. Assessing the performance of overexpressed lines under salinity and mannitol treatment:

Ten days old Wt, EO-1 and EO-4 seedlings were transferred to different treatment conditions (Control-MS, Salinity- MS+150 mM NaCl and Drought- 150mM mannitol) for 7 days.

**Chlorophyll content:** In *ERD4* overexpressed plants, chlorophyll content per fresh wt was significantly increased compared to wild type under control condition. Salinity and
drought stresses showed significant decrease in total chlorophyll content in both wild type and overexpressed lines (Fig 4.3.8A).

**MDA assay:** Lipid peroxidation was measured by the TBARS assays. On the 7th day of exposure to salt and mannitol, MDA content showed drastic increase of 110% in the WT seedlings whereas the increase in MDA content observed in overexpressed lines was not significant as compared to wild type. (Fig 4..3.8 B).

**Qualitative Assay of hydrogen peroxide and superoxide radical:** To test whether down expression of *ERD4* gene leads to ROS production under stress conditions, wild type and overexpressed lines seedlings were stained with diaminobenzidine (DAB) for the detection of H$_2$O$_2$ and NBT for superoxide radical. As compared to wild type, intense staining was observed in wild type compared to overexpressed lines implying that H$_2$O$_2$ and superoxide radical level were high in wild type under salt and drought stress (Fig. 4.3.8 C& D).

**4.4. Discussion:**

To study the gene function and role in plant development, *ERD4* gene was amplified from *Brassica juncea*. The organ specific expression of *BjERD4* in *Brassica juncea* was detected by semiquantitative PCR. This expression pattern in germinating seeds, root, young and mature leaves, shoots, and in pod indicated that BjERD4 may also function in the normal programme of the plant growth and development. The early induction of gene expression was observed as early as 0.5 hr under drought and salinity treatments in *Brassica juncea*. Significant increase in *ERD4* transcript under other stress factors like mannitol, heat and cold was also observed. The early induction of *ERD4* at transcript level under these abiotic conditions was also observed in Arabidopsis [208],
Fig. 4.3.8: Performance of overexpressed lines under salinity and drought. Change in (A) Chlorophyll content, (B) MDA content, and Qualitative Assay of H$_2$O$_2$ (C) & superoxide radical (D).
sugarcane [127] and maize [126]. Different subsets of ERD family members have been shown to be up-regulated or downregulated by various other environmental stimuli, such as cold [209], light [210], excessive arsenate [211] or a transient increase in cytoplasmic Ca²⁺ [212].

Rapid adaptation to changing environmental conditions is essential for plant survival and for the development of tolerance to both abiotic and biotic stresses. Such tolerances can be achieved by distinct metabolic and physiological adjustments, which are mediated by a number of plant hormones, and are often specific to a certain type of stress [52]. As a central regulator of plants’ adaptation to environmental stress, ABA plays a crucial role in the regulation of transpirational water loss [31, 213]. The early responsive to dehydration (ERD) gene is one of the key negative regulators of ABA responses in plants. Changes to the abundance of ERD transcript abundance modulate ABA responsiveness in Arabidopsis. Song et al. demonstrated that the expression of ERD3, ERD4, and ERD7 responded rapidly to water deficit [214]. This implies that ERD genes are rapid drought responsive genes, as are the ERD genes in Arabidopsis. The ERD gene family has at least 21 members. The ERD3, ERD4, and ERD7 showed different expression patterns, indicating that various members of the ERD gene family may have separate functions in the water stress response.

In Arabidopsis ERD4 gene induction was reported before the accumulation of ABA, but in B. juncea we noted a significant increase in ERD4 transcript when exogenous ABA and SA treatment were given. Similarly the ZmERD4 was also induced in the presence of ABA [126]. This suggests that ERD4 gene expression is also modulated by hormones like ABA which is mainly a stress hormone [21]. Thus based
upon the expression analysis, it can be concluded that *BjERD4* expression is constitutive as well as inducible.

To further confirm the role of *BjERD4*, *ERD4* knockdown and overexpression lines were generated in Arabidopsis. In knockdown lines, seed germination was delayed compared to wild type and at a later stage (at 10 days) the developmental difference was also visible with dwarf plant type in case of knock down mutant. But in case of overexpressed lines the enhanced growth was observed in terms of leaf size, pod size, and branching under control condition itself. This suggests that this gene has pleiotropic effect and may be playing important role in plant development.

When knockdown mutant lines were tested for their performance under salinity and drought conditions, knockdown lines showed significant difference with the wild type plants (Fig 5.3.6). Significant increase in MDA content and ROS production was observed whereas chlorophyll content and GSH content were decreased. On the other hand, in case of overexpressed lines, increase in MDA content and ROS generation was not significant as compared to wild type plants. has It has been shown in Chapter III that the protein was localized in chloroplast (plastids) which is one of the major sites for reactive oxygen species production. Further, chloroplast is also the major site for the production of antioxidant compounds and enzyme like GSH, ascorbate, SOD etc [215]. In higher plants, pigments mainly accumulate in the thylakoid membrane of chloroplasts, where they function to harvest light and protect the photosynthetic apparatus from oxidative damage by quenching the triplet excited state of Chl (3Chl) and reactive singlet oxygen (\(^1\)O\(_2\)) and dissipating excess energy [216, 217]. There is a possibility that decrease in production of GSH, other antioxidants and enzymes may be because of
structural changes in chloroplasts in knockdown lines. This ultimately may lead to poor
defense against ROS and hence plants become more susceptible as compared to wild
type. Chloroplast localized RNA interference (RNAi) study of Ostrxm rice plants has also
shown developmental defects, including semi-dwarfism, pale-green leaves, abnormal
chloroplast structure, and reduced carotenoid and chlorophyll content. OstrxmRNAi
plants showed remarkably decreased Fv/Fm values under high irradiance conditions
(1,000 μmol m$^{-2}$ s$^{-1}$) with delayed recovery [218]. Lack of ERD10 protein accumulation
(Early responsive to dehydration 10) in late stage of seed maturation resulted in the
reduction of germination of erd10 mutant seeds. In addition ERD10 mutant also showed
reduced tolerance to drought stress [219]. In contrast, overexpression of BjERD4
enhanced tolerance in transgenic plants to both drought and salt stress as it was also
reported earlier for ZmERD4 [126].

ERD15 from Arabidopsis is functionally characterized as a common regulator of
the abscisic acid (ABA) response and the salicylic acid (SA)-dependent defense pathway
[52]. The overexpression of ERD15 reduced the sensitivity to ABA, as the transgenic
plants were less tolerant to drought and were impaired in increasing their freezing
tolerance in response to ABA. In contrast, the loss of the ERD15 function caused a
hypersensitivity to ABA, and the silenced plants displayed enhanced tolerance to both
drought and freezing [52]. A NAC-type transcription factor (OsNAC5) of rice, when
overexpressed in transgenic rice, caused increased tolerance to salinity [200].
Overexpression of OsLEA3-1 in rice resulted in enhanced tolerance to drought under
field conditions [220]. Similarly, expression of DREB1A of Arabidopsis, hybrid-proline-
rich protein (CcHyPRP) genes of pigeonpea (Cajanus cajan L.), and TaSnRK2.8 and
TaSnRK2.4 of wheat in Arabidopsis conferred tolerance to drought, salinity, and extreme temperatures [65, 221, 222].

Post-transcriptional metabolism of RNA involves both housekeeping and regulatory mechanisms. These processes require the interaction of RNA-binding proteins with specific RNA sequences [71, 75]. But at the primary sequence level RNA binding proteins are poorly conserved, making it difficult to detect any domain in ERD4 primary sequences, while the structural analysis is known to reveal the distant evolutionary links that could provide the first hypothesis about biological function of the uncharacterized domains [57]. Hence by using fold-prediction algorithms it was possible to identify the presence of two RNA-recognition motifs in its sequence [223]. For validation of this hypothesis the RNA EMSA was performed. Results suggested that ERD4-RRM domain binds to only RNA but not the ssDNA and binding is probably sequence specific.

Independent estimations predict the existence of about 60 chloroplast RNA-binding proteins (cpRBPs) in A. thaliana, based on the computer-assisted analysis of putative chloroplast-targeting signals. Different plastid RBPs possess differentially regulated RNA-binding activities [224]. Some of these exhibit sequence-specific and, thus, gene-specific binding affinities, while others represent more general RBPs that might establish a protein scaffold for chloroplast transcripts enhancing RNA stability and/or RNA-folding. Previous studies have supported translational roles of 46- and 47-kD RNA-binding chloroplastic membrane protein [225]. RB47 is associated with the second class of low density chloroplastic membranes, has been proposed to activate the translation of the chloroplast psbA mRNA [226]. Several previous reports suggest a role of the inner envelope membrane in chloroplast gene expression and thylakoid biogenesis.
Extensions of the inner envelope membrane and membrane vesicles in the stroma have been observed by electron microscopy in the chloroplasts of *C. reinhardtii* [227], tobacco, pea, soybean and spinach [228], and in the chromoplasts of red pepper fruits [229]. It has also demonstrated the presence of a homologue of the *Escherichia coli* ribosome releasing factor associated with the chloroplast envelope in spinach [225]. Thus, these evidences suggest that possibly translation of chloroplast mRNAs occurs at the chloroplast inner envelope membrane. Probably ERD4 protein could be one of the components involved in the protein biosynthesis or may participate in post-transcriptional modification of unidentified target RNAs or might hold a pool of mRNAs at the chloroplast envelope as a reserve to sustain protein synthesis during stress conditions of limiting transcription. Further studies are required to identify the target RNAs of ERD4 and to define how ERD4 regulates post-transcriptional modification during stress conditions.