Chapter 6: Summary
SUMMARY

The family of nucleic acid strand separating enzymes known as helicases are found in all living organisms and participate in a wide variety of cellular processes. The central reaction catalysed is always the same; hydrolysis of nucleoside triphosphate (NTP; usually ATP) is coupled to the separation of nucleic acid duplexes, be a DNA-DNA, DNA-RNA, or RNA-RNA. This activity is almost essential for every aspect of nucleic acid metabolism in the cell, including chromosomal, and plasmid replication, transcription translation, RNA processing, and DNA recombination and repair (Schmid et al., 1992; Mason et al., 1994; Tuteja et al., 2004a). These pathways are very sensitive to many abiotic stresses that are responsible for reducing plant growth and productivity. However, the molecular targets responsible for this sensitivity are not well studied. These enzymes have not been well studied in plants in relation to stress tolerance. Keeping the above mentioned points in mind, a novel stress induced helicase gene was isolated from *Pisum sativum* and the encoded protein was biochemically characterized. The results based on these studies are summarized below.

1. Isolation and Sequence Analysis of Stress-Induced Helicase Gene from *Pisum sativum*

To isolate partial stress induced helicase clone cold stress cDNA isolated from cold stress treated pea plants was PCR amplified using degenerate primers from conserved helicase motifs. This partial clone was used to isolate full length gene by cDNA library screening. Two cDNA clones pBS-PDH47-1 cDNA (Accession no. AY167670) which is 1.646 kb in size with an ORF of 1.239 kb, and pBS-PDH47-2 cDNA (Accession no. AY167671) which is 1.638 kb in size with an ORF of 1.239 kb were obtained. These two clones were 92% identical to each other. Therefore, further work was continued with only clone pBS-PDH47-1(AY167670). The deduced amino acid sequence revealed a protein consisting of 413 amino acid residues with a calculated molecular mass of 47 kDa and pI 5.39. The gene was named PDH47 (pea DNA helicase 47 kDa). It belongs to DEAD-box protein family and shows remarkable identity of 93% identity to tobacco eIF4A. All the characteristic helicase domains are present in this protein.
2. Genomic Organization of PDH47
In order to study the genomic organization, genomic clone of PDH47 was isolated by PCR method. Sequence analysis of the genomic clone reveals that the PDH47 gene spans 2.2 kb (Accession no. AY513583). The PDH47 gene consists of four exons (68, 106, 430 and 638 bp in sizes) and three introns (147, 287 and 540 bp in sizes) that are distributed throughout the ORF. Most of the 3' and 5' splice junctions follow the typical canonical consensus dinucleotide sequence GU-AG found in other plant introns. The number of introns and exons, sizes of exons and introns/exon boundaries are highly conserved between the PDH47 and Arabidopsis elF4A (Accession no. gi 15293046) genomic clones.

3. Tissue Distribution of PDH47 by Transcript Analysis and Copy Number Determination by Southern Blot
The transcript levels of PDH47 in different organs of pea were studied by northern hybridization. It showed a single transcript of approximately 1.6 kb size in pea shoot and root but not in flower and tendril. The transcript level was found to be more in shoot as compared to roots. Genomic Southern blot analysis showed that PDH47 exists as a single copy gene in the pea genome.

4. Upregulation of Transcript Levels of PDH47 in Response to Various Abiotic Stresses
The transcript of PDH47 was induced in both shoot and root in response to cold (4°C) and salinity (300 mM NaCl) stress but there was no change in response to drought stress. In case of heat (37°C) stress the transcript level was higher in root than the shoot. ABA treatment showed increased expression in root but not in shoot, indicating the role of PDH47 in both the ABA-independent and ABA-dependent pathways in abiotic stress signalling.

5. Expression, Purification and Sub-Cellular Localization of PDH47 Protein
The pea cDNA encoding PDH47 was cloned into a bacterial expression vector pET28a and the recombinant six-his-tagged protein was purified in the soluble form through Ni^{2+}-
NTA-Agarose and heparin sepharose column chromatography. In western blotting, the antibody raised against the PDH47 protein detected a single band of 47 kDa in IPTG-induced, Ni\(^{2+}\)-NTA-Agarose and heparine sepharose purified fractions. The anti-PDH47 antiserum detected a protein band of about 47 kDa in the cytosol and in the purified nuclear extract but not in the purified chloroplast lysate.

6. In vivo Localization by Immunofluorescence Labeling and Confocal Microscopy

In vivo localization of PDH47 was done to have an insight into possible biological role. Immunofluorescence labeling of tobacco BY (Bright Yellow) 2 cells with anti-PDH47 antibodies followed by confocal microscopy showed that PDH47 protein was localized in the nucleus and cytosol. The dual localization showed that it may be a multifunctional protein.

7. Biochemical Characterization of PDH47

PDH47 protein sequence contained all the characteristic helicase domains. This protein was analysed for ATP binding, ATP hydrolysis and DNA-DNA, DNA-RNA, and RNA-RNA unwinding activities and found to be competent for the same.

(i) ATP Binding of PDH47: The purified PDH47 protein showed α\(^{32}\)P ATP binding.

(ii) ATPase Activity: PDH47 showed ssDNA- and Mg\(^{2+}\)-dependent ATPase activity.

(iii) DNA-DNA, DNA-RNA, RNA-RNA Unwinding Activity: PDH47 was able to unwind DNA-DNA, DNA-RNA, and RNA-RNA duplexes in an ATP and Mg\(^{2+}\) dependent fashion, suggesting its possible role in various aspect of nucleic acid metabolism during stress conditions. The enzyme did not show any specificity for overhanging tails or replication fork-like structures within the substrate. This is a unique helicase in being active even at acidic pH.

(iv) Bidirectional DNA Unwinding Activity of PDH47: PDH47 exhibited bipolar helicase activity. This is the first report of a plant homologue of eIF4A showing bidirectional helicase activity.
8. *In vitro* Phosphorylation of PDH47 by Protein Kinase C and Phospho Amino Acid Analysis

The protein kinase C phosphorylated PDH47 and phosphorylation occurred on Ser and Thr residues. Phosphorylated protein showed enhanced ATPase and helicase activity. PKC phosphorylation suggested that PDH47 may be involved in signal transduction pathways in regulating the cellular functions of the DNA helicase and ATPase activities of the protein.

9. Immunodepletion of DNA Unwinding and ATPase Activities of PDH47 by Antibodies

Immunodepletion results showed that both the DNA helicase and ATPase activities were depleted by anti-PDH47 IgG and anti-His antibodies respectively, whereas there was no reduction in activity by the pre-immune IgG treated PDH47 protein. This experiment proved that the enzymic activities are due to the PDH47 polypeptide and not due to contamination of any bacterial helicase in the protein preparation.

10. Function of PDH47 in Translation initiation of Proteins

The IgG of anti-PDH47 was used in an *in vitro* transcription-translation coupled assay. There was no effect of pre-immune IgG on the translational activity of the lysate. The increasing concentration of purified anti PDH47 antibody (IgG) inhibited *in vitro* translation. The anti PDH47 antibodies inhibited the activities of wheat germ eIF4A as well as PDH47. Furthermore, the addition of the purified PDH47 protein in standard *in vitro* translation reaction resulted in an enhancement of the translation.

11. Effect of DNA-interacting Ligands on DNA Unwinding and ATPase Activity of PDH47 and Kinetics of Inhibition

Various nucleic acid interacting ligands were used in the study to see their effect on the helicase and ATPase activities of PDH47. The ssDNA-dependent ATPase activity of PDH47 was effectively inhibited at 50 μM concentrations of nogalamycin, daunorubicin, ethidium bromide, mitoxantrone, actinomycin D and cisplatin. The apparent Ki values for inhibition of ATPase activity of PDH47 by actinomycin D, ethidium bromide, cisplatin,
daunorubicin, nogalamycin, and mitoxantrone were 4.0, 8.0, 8.0, 5.0, 5.0, and 8.0 μM, respectively. The DNA unwinding activity of PDH47 was effectively inhibited by nogalamycin, daunorubicin, netropsin, ethidium bromide, mitoxantrone, distamycinA, actinomycin D and cisplatin. The most effective inhibitors were noglamycin, distamycin, daunorubicin, netropsin, actinomycin D, and cisplatin with apparent Ki values of 0.5, 0.5, 2, 2, 2, and 2 μM, respectively. The apparent Ki values for other inhibitors, such as ethidium bromide and mitoxantrone are 8.0 and 6.0 μM, respectively. Noglamycin was the best inhibitor because it had the minimum Ki value both for ATPase and helicase activities. The inhibition might be due to the intercalation of inhibitors into duplex DNA, which can impede the translocation of the PDH47.

12. Production of Transgenic Plants Overexpressing Stress Induced Helicase (PDH47) Gene

The tobacco plants overexpressing stress induced helicase gene (PDH47) have been produced. These plants have been confirmed for the trans gene integration by PCR and southern blot analysis. The leaf disc senescence assay under salinity stress revealed the early bleaching of wild type and antisense leaf discs as compared to sense transgenic plants up to 72 hours in 150 mM NaCl. Further analysis of PDH47 transgenic plant is underway.

The isolation of such DEAD-box helicase might help in understanding of stress signalling and nucleic acid metabolism in plants under stress conditions. This study confirms the role of PDH47 in protein synthesis and may also help in understanding of mechanism of DNA unwinding in plants. Its bipolar helicase activities may be involved in distinct cellular processes during unfavourable conditions. Study on expression of these helicase during stress may help us to understand their role in such conditions. The production of transgenic plants for PDH47 may help in providing the stress tolerance.