Chapter 6: Summary and Conclusions
The ubiquitous glyoxalase system consists of two enzymes: glyoxalase I and glyoxalase II which act coordinately to convert 2-oxoaldehydes into 2-hydroxyacids using GSH as a cofactor. Glyoxalase I catalyses the formation of S-D-lactoylglutathione from hemithioacetal formed spontaneously from methylglyoxal and GSH which is further metabolized to D-lactate and GSH by glyoxalase II. Methylglyoxal is a primary physiological substrate for glyoxalase system which is produced non-enzymatically or enzymatically under physiological conditions from the triose phosphates (GA, GAP and DHAP) generated from Embden-Meyerhof and polyol pathways, and during metabolism of amino acids and acetone.

Although glyoxalase system was discovered in early nineteenth century, its function in the biological system is still a subject of debate. The present understanding of the role of glyoxalase system is mostly based on the studies from animal and microbial systems, which propose its involvement in the protection against α-oxoaldehyde cytotoxicity, regulation of cell division/proliferation, microtubular assembly, vesicle mobilization, tumor growth, clinical complications associated with diabetes mellitus and various diseases.

The presence of the glyoxalase system has been shown in plants. The functional significance of this system in plants is not yet clear, however modulation in the activity of glyoxalase I has been reported during cell division/proliferation and in differentiated cells and is proposed to be correlated with mitotic index. A number of studies on differentiated and proliferating tissue in plants have pointed towards an inverse correlation between tissue differentiation and the activity of glyoxalase I. Based on its regulation during cell division/proliferation, this system has been often regarded as a 'marker for cell growth'. However, the causal relationship between glyoxalase activity and rapid cell growth has not yet been established. In fact, glyoxalase I from tomato was shown to be upregulated in response to salt stress, osmotic and phytohormonal stimuli. High expression of glyoxalase I in metabolically active cells/tissues such as meristematic, newly dividing cells or cells undergoing stress indicate the fundamental importance of this system in plants.

Gene encoding glyoxalase I enzyme has been isolated and characterized from Pseudomonas, human, yeast, E. coli and Salmonella. The gene for Glyoxalase II has
been cloned from human and yeast. In comparison to the studies on microorganisms and human, very little is known about the genes encoding glyoxalase I and glyoxalase II from plants. The only published cDNA sequence of glyoxalase I from plants is of tomato and two genes for glyoxalase II from Arabidopsis.

The aim of the present work was to understand the physiological role of glyoxalase system in plants. In an effort to achieve this, we have cloned and characterized a glyoxalase I gene from B. juncea. The regulation of glyoxalase I was studied during developmental stages and environmental stresses. The Brassica glyoxalase I gene was introduced in tobacco in sense and antisense orientation to understand the role of glyoxalase I in plants. The following results were obtained:

• Glyoxalase I was purified to electrophoretic homogeneity from the cotyledonary leaves of 7-day old B. juncea seedlings using ammonium sulphate precipitation followed by affinity (S-hexyl Sepharose 4B) and gel filtration (Sephadex G-75) column chromatography. The purified glyoxalase I showed an apparent mol mass of 27 kDa on 10% SDS-PAGE.

• Polyclonal antibodies against the purified glyoxalase I was raised in New Zealand white rabbit. These antibodies identified a ~27 kDa protein on Western blots of crude protein extract from B. juncea which corresponds to glyoxalase I. The antibodies were highly specific to glyoxalase I protein as cross-reactivity with other proteins was not detected. These antibodies were able to identify the glyoxalase I protein from 2 µg of crude protein extract. Besides, antibodies precipitated the activity of glyoxalase I from the crude protein extract of Brassica.

• A cDNA expression library was constructed in Lambda ZAP II vector (Stratagene) using poly(A)+ RNA isolated from cotyledonary leaves of water stressed (400 mM mannitol) B. juncea seedlings.

• Eight immunopositive clones were identified by immunological screening of cDNA library with glyoxalase I antibodies. These clones were further purified till a homogenous population was obtained. The pBluescript SK(−) phagemid containing the cDNA clone was excised from all the Lambda ZAP II clones using ExAssist
Two sets of clones were identified with insert size of ~0.6 kb and ~0.8 kb.

- One of the clone, pBGLYIVA3, with a insert size of ~0.8 kb was sequenced by Sanger dideoxy chain termination method from both the ends using T3 and T7 primers. The cDNA was 784 bp in length with an ORF of 558 bp, a 5' non coding region of 44 bp and a 3' non coding region of 163 bp including a 19 bp poly(A) tail. The ORF encodes for 185 aminoacids with a calculated mol. mass of 20.782 kDa and a pl of 5.46 (Genbank Accession No. Y13239).

- The deduced amino acid sequence of pBGLYIVA3 showed significant homology with the known glyoxalase I sequences from other organisms. This sequence has ~88% similarity with tomato, ~70% with human and 40-60% with microbial glyoxalase I sequences.

- Dendrogram prepared from all the available sequences for glyoxalase I suggested a segregation of glyoxalase I gene into prokaryotic and eukaryotic type. According to this, glyoxalase I sequences from Brassica, tomato, human and yeast are closely related to each other than that of Salmonella or E. coli. However, the Gly I from P. putida was found to be similar to eukaryotic type of glyoxalase I.

- Secondary structure of the Brassica glyoxalase I was very much similar to the human glyoxalase I. Regions involved in the formation of active site, zinc and glutathione binding sites are well conserved in Brassica glyoxalase I. Brassica glyoxalase I was found to contain two domains (αβαβαβαβ and βαββαβα motifs separated by an inter-domain region of 21 amino acids) which might have evolved due to gene duplication and 3-D domain swapping events as proposed for the human glyoxalase I.

- Hydrophilicity plot for the glyoxalase I protein suggest it to be a highly soluble in nature and therefore it could be localized in the soluble fractions.

- Southern blot analysis of genomic DNA and Western blot analysis of crude protein extract on native and denaturing PAGE suggested that glyoxalase I in B. juncea could be a member of multigene family.
• The isolated *Brassica Gly I* gene was over-expressed in *E. coli*. A plasmid construct pRSETA-*Gly I* was prepared by cloning the entire coding region of the glyoxalase I gene into pRSETA vector and used to transform the *E. coli* cells. The maximum induction of glyoxalase I protein occurred at 4 h of IPTG induction whereas maximum specific activity of glyoxalase I was observed at 2 h of IPTG induction (1200-fold higher as compared to the control cells).

• *E. coli* cells over-expressing glyoxalase I were tolerant to high concentration of exogenously added methylglyoxal (20 mM). In contrast the growth of control cells, which are not expressing glyoxalase I, were sensitive and failed to grow in methyglyoxal concentrations higher than 4 mM.

• Over-expressed protein in *E. coli* had an apparent mol mass of 24 kDa and showed cross-reactivity with glyoxalase I antibodies raised against purified glyoxalase I protein from *B. juncea*. The apparent mol mass of the induced glyoxalase I expressed in *E. coli* was higher than the expected size (20.782 kDa) calculated from the deduced amino acid sequence of the isolated cDNA clone but lower than the glyoxalase I protein (27 kDa) of *B. juncea*. This difference in mol mass could be due to post-translational modifications as computer analysis of the sequence showed five phosphorylation and two N-myristolation sites.

• Distribution of glyoxalase I was studied in six different species of *Brassica*. These species showed differences in the protein, activity and transcript level of glyoxalase I. Results of Southern and Western blot indicated the presence of different isoforms of glyoxalase I in different species of *Brassica*.

• Expression of glyoxalase I gene in *B. juncea* was regulated in response to environmental stresses. An increase of upto 2-3 fold in the level of glyoxalase I transcript was observed in response to sodium chloride (800 mM), mannitol (400 mM) and zinc chloride (20 mM) treatments.

• Study on time kinetics of the glyoxalase I expression in response to salt and heavy metal stress suggested that maximum induction occurred at 72 h of stress treatments (which is around 2-3 fold for salt and heavy metal stress). The increase in transcript level also resulted in an increase in protein level and specific activity.
Summary and Conclusions

These results suggested that glyoxalase I expression during stress conditions is regulated at the transcriptional level.

• Expression of the glyoxalase I was also regulated during developmental stages. The level of Gly I transcript increased with the age of seedlings from 0-5 days and thereafter it declined. The specific activity and level of the glyoxalase I protein followed the same trend suggesting that the expression of glyoxalase I during developmental stages is also regulated at the transcriptional level. Moreover, the regulation of glyoxalase I expression was not tissue specific as cotyledonary leaves and root showed similar patterns.

• Three constructs were prepared for plant transformation by cloning the Gly I cDNA into pBI121 vector in the both sense and antisense orientations under the control of strong constitutive CaMV 35S promoter. In PBI-S1 vector, the Gly I and uidA genes were expressed under the control of separate CaMV 35S promoter and NOS terminator. In PBI-S2 vector, both Gly I and uidA genes were under the control of single CaMV 35S promoter. The cDNA fragment containing Gly I cloned in these vectors contained its own transcription initiation (ATG), termination and poly(A) signal sequences. A third plasmid construct (PBI-A) was prepared which is very similar to pBI-S2 except that the Gly I gene was placed in antisense orientation to down regulate the tobacco Gly I expression.

• Agrobacterium cells were transformed with sense (pBI-S1 and pBI-S2) and antisense (pBI-A) constructs and recombinant colonies were selected on medium containing kanamycin. The presence of recombinant plasmids in the kanamycin resistant colonies of Agrobacterium was further confirmed by restriction digestion.

• Transgenic plants were obtained in N. tabacum cv. Petit Havana, using Agrobacterium mediated leaf disc transformation method. A total of 150 kanamycin resistant plants were analyzed, all of them were GUS positive. Out of these, few plants were further analyzed by Southern blotting using Gly I gene as a probe. The intensity of the hybridized signal in each lane was variable suggesting multiple insertion of the Gly I gene in tobacco genome. The estimated number of the genes inserted in various plants ranged from 1 to 6 copies.
• Northern blot analysis of total RNAs isolated from plants transformed with sense and antisense constructs showed the presence of Gly \( I \) and \( uidA \) transcripts as expected. Transgenic plants with high copy of Gly \( I \) gene insertion had higher level of Gly \( I \) transcript and protein, whereas plants with low copy insertion had lower expression of Gly \( I \) transcript and protein. As expected, plants expressing Gly \( I \) in antisense direction had lower level of glyoxalase \( I \) protein and enzyme activity as compared to wild type tobacco plants suggesting that the decreased level of glyoxalase \( I \) protein is due to presence of antisense Gly \( I \) transcript.

• All the transgenic tobacco plants expressing Gly \( I \) in sense or antisense grew and matured normally under controlled growth conditions. Moreover, the seed setting in these transgenic plants were similar to that of untransformed tobacco plants.

• Transgenic plants over-expressing glyoxalase \( I \) are tolerant to salt and heavy metal stresses. Leaf discs of sense transgenic plants were tolerant to sodium chloride (400 mM and 800 mM) and zinc chloride (20 mM) treatment as senescence/bleaching was significantly less and they have a high Chl content as compared to the sodium chloride and zinc chloride treated leaf discs of wild type plants.

• Transgenic plants over-expressing glyoxalase \( I \) gene in sense orientation were also tolerant to high concentration of methylglyoxal (20 mM) whereas plants expressing glyoxalase \( I \) gene in antisense were more sensitive as compared to untransformed tobacco plants. As leaf discs of antisense plants showed early bleaching/senescence in response to methylglyoxal treatment as compared to wild type tobacco plants. Under similar conditions, leaf discs of sense transgenic plants showed no significant bleaching. Chlorophyll content in these leaf discs after 24 h treatment also confirmed the tolerant behaviour of sense transgenic plants.

• Besides glyoxalase \( I \), another clone, pBGSTVA4, containing insert size of ~0.6 kb was obtained after immunologically screening of Brassica cDNA expression library. It was sequenced by Sanger dideoxy chain termination method from both the ends using T3 and T7 primers. The cDNA was 588 bp in length with an ORF of 401 bp, a 3' non coding region of 177 bp including a 17 bp poly(A) tail. This ORF encodes a partial protein of 133 amino acids (Accession No. Y13839). The sequence showed
significant homology with glutathione S-transferases especially with type III class of GSTs which are known to be stress inducible in nature.

- Glyoxalase I antibodies from \textit{B. juncea} cross-reacted with a protein of 27 kDa in Western blot of crude protein extract from \textit{Arabidopsis}. These antibodies also precipitated the glyoxalase I activity in the crude protein extract from \textit{Arabidopsis}. Screening of a cDNA expression library constructed in Lambda gt11 vector from \textit{A. thaliana} resulted in identification of six immunopositive clones which were further screened to get a homogenous population. One of the positive recombinants (pAGSTV13) that contained the longest insert was sequenced using Sanger dideoxy chain termination method. The cDNA was 843 bp in length. The clone has an ORF of 675 bp with a 5' non coding region of 46 bp and a 3' non coding region of 122 bp. The sequence showed 100% identity with a \textit{Arabidopsis} cDNA clone (D44465) encoding glutathione S-transferase.