Conclusions

Specific conclusions

Concise and specific conclusions have been drawn as mentioned below from the entire work presented in the thesis.

I. The BicA transporter was found to be present in a large number of cyanobacterial species specifically in several strains of *Synechococcus, Synechocystis, Microcystis, Prochlorococcus, Arthrospira* etc. *Synechococcus* 7002 and *Synechocystis* 6803 however are the only species with verified bicarbonate uptake activity by BicA transporter. Several types of proteins from sulfate transporter family *viz.* low/high affinity sulphate transporters, sulphate permeases etc. and MFS superfamily transporters have been identified as the closest homologues of BicA.

II. The presence of common conserved domains and the positions in phylogenetic tree showed that the majority of the closest BicA homologues were sulphate transporters from the phyla eubacteria, archaea and few from eukaryota. The study also confirmed the ancestral relatedness of SulP family member BicA with archaeal NCS-2 family proteins which are involved in nucleotide metabolism.

III. Distant homologues (with percent identity 21%-65%) of BicA with diverse substrate preferences and functionalities were identified in several eubacterial and archaeal species. BicA belongs to autotrophic organisms but interestingly its evolutionary relationship was observed with transporter proteins of both autotrophic as well as heterotrophic organisms. Therefore it can be hypothesized that autotrophic transporters (*viz.* BicA) might have diverged from rather simpler proteins (NCS-2 family members) involved in nutrient assimilation in the primitive heterotrophic species.

IV. In order to establish the BicA transporter of *Synechococcus* 7002 into the chloroplasts inner envelope membrane of model C_3 plants, specific targeting sequences (transit peptides) were selected from native proteins of plant origin. Therefore, *A. thaliana* proteome was screened and two transporter proteins with accession number AT2G24820 and AT5G17520 were chosen as a source of N-terminal transit peptides and their TPs were named as TICTP and MEXTP respectively.

V. A comparative codon usage analysis was carried out to ensure the compatibility of plant hosts (*Arabidopsis and Nicotiana*) as a heterologous expression system for cyanobacterial BicA transporter. The analysis showed that the mean difference in relative adaptiveness of codon usage in *bicA* gene was approximately 18% in cyanobacteria while 34-35% in plants. The analysis of low frequency codons confirmed that none of the codon's frequency was <10% and appearance of rare codon clusters was also not observed. Apart from these facts, the presence of plant sequence (transit peptide form *A. thaliana* proteins) at the N-terminus also enhances the possibility that the fusion proteins should express in the plant system.

VI. The TICTP and MEXTP transit peptides were successfully PCR amplified and then fused/cloned upstream of the *bicA* gene and further subcloned into *gus* reporter gene containing plant expression vector *pRI101*. The recombinant *pRI101* constructs (TICTP/MEXTP-*bicA-gus-pRI101*) were transformed into *N. tabacum* by particle bombardment which was followed by transient expression analysis of BicA constructs by GUS fluorometric assay. The assay shows a significantly higher expression level of GUS protein in both the constructs in comparison to the control sample which confirmed that fusion genes were transformed successfully into the plant system.

VII. The transient protein expression was also examined in *N. tabacum* and *A. thaliana* upon transformation with recombinant *pRI101* constructs by *Agrobacterium* mediated co-culture method. *A. thaliana* was found to be poorly responding and hence recalcitrant towards this method of transformation and hence the transformed tissue couldn't regenerate and survive further. On the other hand, *N. tabacum* explants regenerated into the transformed callus tissue which was further analyzed by GUS histochemical assay. The assay revealed the presence of blue colour only in transformed tissue which is the indication of *gus* gene expression which again assured the expression of fusion genes in the plant system.

VIII. For the development of stable plants, *N. tabacum* explants were transformed with recombinant *pRI101* constructs by co-culture method and multiples shoots were regenerated. Unfortunately, the gene specific PCR analysis revealed that the

integration of full length gene has not happened in the plant genome which may be due to truncation rearrangements in the T-DNA. Thereafter, efforts were made to regenerate stable plants by floral dip transformation method using *A. thaliana* host. But the survival and growth of transgenic plants were severely hampered and therefore sufficient transgenic material could not be regenerated for any further experiment.

IX. To perform sub-cellular localization studies fusion genes were cloned into *mgfp5* reporter gene containing plant expression vector *pCAMBIA-1302*. The recombinant *pCAMBIA* constructs were transformed into *N. benthamiana* by *Agrobacterium* mediated agroinfiltration method which confirmed the integration and expression of full length transgene at DNA and mRNA level by gene specific PCR analysis. Protein expression was proved by western blotting using anti-GFP antibodies which revealed the presence of near full length GFP fusions in total protein extracts.

X. The localization studies demonstrated the chloroplastic expression of both the constructs but some amount of cytosolic expression was also observed which may be due to the mislocalization of some fraction of targeted protein in plant cytosol or other cellular organelles. The exact location inside the chloroplast remains undetermined.

Future prospectives

There are several aspects and ideas relevant to the work presented in this thesis which could be explored further and/or verified by experimentation as mentioned below:

I. The BicA transporter's evolutionary relationship with NCS-2 family proteins could be further analyzed by generating 3D structural model of both types of proteins and comparing their substrate specificities.

II. The targeting sequences could be improved and/or modified to enhance the level of chloroplastic expression of the proteins.

III. The recombinant *pCAMBIA* constructs prepared in this work could be further used to generate stable transgenic plants which might perform better than previously used *pRI101* constructs due to relatively smaller size and the presence of a native reporter protein mGFP5 in the vector (*mgfp5* is smaller in size than *gus* reporter gene).

IV. Stable transgenic plants could be checked for enhanced growth and photosynthetic yield. In order to find out exact location of fusion proteins inside the cell, the use of organelle marker and chloroplast fractionation studies might also be carried out.

V. The studies performed on model plants could be replicated using agronomically important C_3 crops *viz.* rice, wheat etc., in field conditions.

VI. The installation of other transporters from cyanobacteria along with BicA or transfer of the whole CCM assembly which includes transporters and carboxysome would definitely lead to enhanced photosynthesis though the successful incorporation and functional activation of these many multi subunit CCM components are presumed to be extremely challenging.

Summary

The complete research work presented in this thesis can be summarized in the form of following sections:

Introduction and Review of Literature: As per the requirement of continuously increasing world population the current methods of crop productivity enhancement will soon loose pace and hence new strategies need to be devised to face the issues of food shortage. Recent studies have established the fact that the improved photosynthesis would serve to enhance the crop yields. It is important to target the C_3 photosynthesis as most grain crops *viz.* rice, wheat, barley and many more plant species possess C_3 pathway for the fixation of atmospheric CO_2. The primary enzyme i.e. Ribulose-1, 5-Bisphosphate Carboxylase/Oxygenase (RuBisCO) which is involved in the process of photosynthesis suffers from dual substrate specificity for both CO_2 and O_2 and low catalytic turn over which makes the C_3 pathway inefficient. Hence to improve net photosynthetic efficiency, C_3 plants could be engineered with the existing carbon concentrating mechanisms (CCMs) in such a way that RuBisCO becomes capable of working efficiently in the present environmental conditions. Cyanobacteria, algae, proteobacteria and few land plants (with C_4 and Crassulacean Acid Metabolism) have naturally developed carbon concentrating mechanisms (CCM) which aid to overcome the dual specificity of RuBisCO by increasing the CO_2 concentration around the enzyme. In this context, the establishment of cyanobacterial CCM which comprises of inorganic carbon (Ci) transporters (for CO_2 and HCO_3^-) and carboxysomes (has RuBisCO and carbonic anhydrases in it), is assumed to be most fruitful and promising approach. According to modelling studies, the very first and simplest yet efficient scheme would be the introduction of HCO_3^- transporters (BicA and SbtA) into inner envelope membrane of C_3 plant chloroplast. In the present work, attempts have been made to introduce, express and localize the BicA transporter of *Synechococcus* 7002 into model C_3 plant systems. It is believed that the understanding of BicA transporter will serve as a baseline towards achieving the long term goal of enhanced crop productivity.

Materials and Methods: Firstly the identification of BicA transporter protein was performed in several cyanobacterial species using Cyanobase and NCBI database by the protein-protein blast. Further, distant homologues of BicA protein were searched in various lower organisms by PSI blast, conserved domain search and phylogenetic analysis.

Furthermore, the chloroplast proteome was screened from AT_CHLORO database to select transit peptide sequences for targeting of BicA protein into C_3 plant chloroplast. The selected transit peptides were fused with *bicA* gene using sticky end ligation and the fused segments were eventually cloned into plant expression vectors *viz. pRI101-AN* and *pCAMBIA-1302*. Thereafter the recombinant plasmids were transformed into *Agrobacterium* GV3101 strain which was further used for transformation of *Nicotiana tabacum* and *Nicotiana benthamiana*. In the complete process various plant transformation techniques were utilized for transient expression studies. The transgene integration and expression were examined by reporter gene assays, diagnostic PCRs and western blotting along with the determination of sub-cellular localization by confocal microscopy.

Results and Discussions: This section can be further divided into several subsections which are described below:

Identification of bicarbonate transporter BicA (*Synechococcus* PCC 7002) in various cyanobacterial species and mining its distant homologues in other lower organisms: The BicA transporter was identified in a large number of cyanobacterial species specifically in several strains of *Synechococcus, Synechocystis, Microcystis, Prochlorococcus, Arthrospira* etc., by protein-protein blast and literature search. The evolutionary trajectories of BicA transporter were looked upon by analyzing the presence of common conserved domains and phylogenetic relationship with various transporters from the phyla eubacteria, archaea and eukaryota. Distant homologues of BicA with diverse substrate preferences and functionalities were majorly identified in several eubacterial and archaeal species. The study revealed the ancestral relatedness of SulP family member BicA with archaeal NCS-2 family proteins. BicA belongs to autotrophic organisms but interestingly its evolutionary relationship was observed with transporter proteins of both autotrophic as well as heterotrophic organisms. Therefore it can be hypothesized that autotrophic transporters (*viz.* BicA) might have diverged from rather simpler proteins (NCS-2 family members) involved in nutrient assimilation in the primitive heterotrophic species.

Screening and selection of candidate proteins from *Arabidopsis thaliana* proteome which can be used as a source of chloroplast targeting sequences (transit peptides): An extensive *in-silico* analysis of *A. thaliana* envelope proteins was done in order to select most suitable protein candidates that can be used as the source of transit peptides (TP) for BicA transporter of cyanobacteria for targeting it into chloroplast inner envelope membrane of model C_3 plants. AT_CHLORO database was used to procure all the information of chloroplast proteome short listing of most relevant protein candidates was done by using various software. Finally, two transporter proteins with accession number AT2G24820 (TIC55 transporter) and AT5G17520 (maltose transporter) were chosen as a source of N-terminal transit peptides and their TPs were named as TICTP and MEXTP respectively.

Preparation of chimeric genetic constructs by fusing TPs upstream of the *bicA* gene and cloning of fusion constructs in plant expression vector (*pRI101-AN*) containing *gus* reporter gene followed by transformation of *N. tabacum* plants: The TICTP and MEXTP transit peptides were successfully PCR amplified from *A. thaliana* genomic DNA and then fused/cloned upstream of the *bicA* gene into a cloning vector and further subcloned into *gus* reporter gene containing plant expression vector *pRI101*. Thereafter the recombinant *pRI101* constructs (TICTP/MEXTP-*bicA-gus-pRI101*) were transformed into leaves of *N. tabacum* by particle bombardment which was followed by transient expression analysis by GUS fluorometric assay which further confirmed that fusion genes were transformed successfully into the plant system. The transient protein expression was also examined in calli of *N. tabacum* by *Agrobacterium* mediated co-culture method which was further analyzed by GUS histochemical assay. The assay revealed the presence of blue colour only in transformed tissue which again assured the expression of fusion genes in the plant system.

Cloning of fusion constructs in plant expression vector (*pCAMBIA-1302*) containing *mgfp5* reporter gene followed by transformation of *N. benthamiana* plants: The recombinant *pCAMBIA* constructs were transformed into *N. benthamiana* by *Agrobacterium* mediated agroinfiltration method which confirmed the integration and expression of full length transgene at DNA and mRNA level by gene specific PCR analysis using genomic DNA and cDNA as template. Moreover, transient protein expression was further proved by performing western blotting using anti-GFP antibodies which revealed the presence of near full length GFP fusions in total protein extracts.

Further, the localization studies confirmed the chloroplastic expression of both the constructs by the overlapping of GFP fluorescence and chlorophyll autofluorescence in mesophyll protoplasts. This observation indicates the targeting of fusion proteins in the chloroplasts of *N. benthamiana*, although the exact location inside the chloroplast remains undetermined.

Conclusions: The present research work was done with the intention of addressing the issues of global food scarcity and it is believed that this piece of work will act as a tiny, yet significant contribution in achieving the long term target of crop productivity enhancement. In this study, attempts were made to trace the evolutionary path of cyanobacterial bicarbonate transporter BicA and efforts have been made to install the transporter into various model C_3 plants. The transient expression of the targeted transporter protein was assured and its chloroplastic localization was confirmed indicating the success of our targeting strategies.