CHAPTER 1
Introduction and Review of Literature

1.1 Introduction

As per the requirement of continuously increasing world population which is estimated to increase by 35% by 2050 (U.S. Census Bureau, 2015), 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 (Parry and Hawkesford, 2010; Ort et al., 2015). It is expected that developing nations will experience the most aggravated challenges if the food security issues do not get resolved at the earliest (Cornish, 2017). In order to achieve this goal, improving C_3 carboxylation machinery is an open avenue for increasing photosynthesis and finally crop yield. Hence, there is an urgent need to understand and improve the crop photosynthesis by genetic engineering approaches and to fill the gap between these approaches and final crop yield (Long et al., 2015). Whether the improvement in photosynthesis will lead to enhanced crop yield or not has long been a debatable issue (Zelitch, 1982). In a theoretical correlation which is being extrapolated on the basis of a study (Ford et al., 1982) which showed enhanced leaf photosynthesis in soybean, it has been postulated that enhanced photosynthesis at leaf level does not assure an increase in the grain yield because at each level the impact of enhancement tends to reduce by 50% (Sinclair et al., 2004). Several other studies also support the fact that there is lack of correlation between the leaf photosynthesis improvement and increment in grain yield (reviewed by Long et al., 2006).

Contrary to the above, the effects of increased CO_2 studied in *Glycine max* by analyzing CO_2 assimilation rate, RuBisCO activity and stomatal conductance etc., indicate a direct correlation between photosynthesis and crop yield (Ainsworth et al., 2002). Kimball et al., (2002) also reported that enhanced CO_2 levels increased the photosynthesis and eventually biomass and yield in various C_3 species *viz.* wheat, rice, sorghum etc. In a recent study, plant photoprotection machinery was modified to enhance the photosynthesis and elevated plant productivity was checked under field conditions by analyzing total dry weight, leaf area, plant height and the weight of various plant parts (Kromdijk et al., 2016). This aspect has been reviewed intensely (Long et al., 2006; Murchie et al., 2009; Zhu et al., 2010) and 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 (*Oryza sativa*), wheat (*Triticum aestivum*), barley (*Hordeum vulgare*) and many more plant species possess C_3 pathway for CO_2 fixation (Price et al., 2011).

Ribulose-1, 5-Bisphosphate Carboxylase/Oxygenase (RuBisCO) is the primary enzyme involved in the process of photosynthesis. Being the initial CO_2 capturer, it catalyzes the rate-limiting step in carbon fixation. RuBisCO, however, possesses dual substrate specificity for CO_2 and O_2 and the ensuing photorespiration leads to loss of fixed carbon molecules (Parry et al., 2003; Rothschild, 2008; von Caemmerer and Evans, 2010; Price et al., 2013). C_3 species are devoid of any sort of efficient mechanism, that can prevent or reduce the oxygenation of RuBP, while the other photosynthetic organisms *viz.* cyanobacteria, algae, proteobacteria and few land plants (with C_4 and Crassulacean Acid Metabolism) have naturally developed carbon concentrating mechanisms (CCM) (Badger and Bek, 2008; von Caemmerer and Evans, 2010). CCM aids to overcome the dual specificity of RuBisCO by increasing the CO_2 concentration around the enzyme. The cyanobacterial CCM is comprised of two main components i.e., inorganic carbon (Ci) transporters (for CO_2 and HCO_3^-) and carboxysomes (Badger and Price, 2003; Mangan and Brenner, 2014) where the entry of oxygen is prohibited and carboxylation is preferentially carried out over the oxygenation reaction (Price and Howitt, 2014a). The algal CCM is mainly comprised of inorganic carbon transporter, carbonic anhydyrases and in some cases pyrenoid structures while the proteobacterial CCM is less characterized with several species containing various RuBisCO forms and carboxysome (Sidhu et al., 2014). C_4 and CAM pathway are more efficient than C_3 due to their spatial and/or temporal regulatory mechanisms for CO_2 fixation (Zabaleta et al., 2012). Apart from incorporation of specific CCM components in C_3 plants, the other possible aspects could be alterations in the existing C_3 pathway such as modifications in the key enzymes RuBisCO and/or RuBisCO activase, improving stomatal conductance for CO_2 diffusion, enhancing the availability of substrate RuBP and reducing the oxygenation reactions by manipulating photorespiration (von Caemmerer and Evans, 2010; Singh et al., 2014).

In order to enhance photosynthetic efficiency, C_3 plants could be engineered with the existing CCMs in such a way that RuBisCO becomes capable of working efficiently under CO_2 limiting conditions or it should utilize the available CO_2 more efficiently (Evans et al., 2009; von Caemmerer and Evans, 2010). In this context, the establishment of cyanobacterial CCM is assumed to be most fruitful and promising approach. The transfer of whole CCM assembly into the plant system is challenging as it requires establishment and coordination of multiple components. However, if successful, the

strategy holds great potential to significantly improve the RuBisCO's performance. In order to provide high Ci environment, the very first and simplest approach would be the introduction of HCO_3^- transporters into C_3 plant inner chloroplast membrane (Rolland et al., 2016; Uehara et al., 2016). BicA and SbtA are the inorganic carbon transporters found in cyanobacteria and are considered to be most suitable candidates for plant transformation as these are encoded by single genes and hence presumed to be favourable for genetic integration into a foreign genome.

In the present work, attempts have been made to introduce the BicA transporter into model C_3 plant systems. BicA transporter being a hydrophobic membrane protein becomes more difficult to express in a heterologous host hence the selection of suitable targeting sequence, generation of effective constructs along with selection of appropriate host plant is the prerequisite goal. The genetic constructs prepared and plant transformation techniques used in the present study were tested for successful expression and localization in host plant. We are anticipating that this work will be helpful in understanding the various aspects and possibilities for incorporating the cyanobacterial CCM in model plants followed by the crop plant species. Further, we have also looked for closely and distantly related homologous proteins of BicA transporter in cyanobacteria and other lower organisms which might provide an insight in understanding the evolutionary trajectories of the transporter.

1.2 C_3 photosynthesis

Photosynthesis is the vital process which produces energy using solar radiations by fixing atmospheric CO_2 into organic compounds and the Calvin-Benson-Bassham cycle (CBB) is the most widely used pathway for fixation of CO_2 in the majority of autotrophs such as plants, cyanobacteria and algae etc. C_3 photosynthesis is the most ancient pathway for CO_2 fixation, found in approximately 95% of plants (Rothschild, 2008). In this pathway, the first product of photosynthesis is two molecules of phosphoglyceric acid (3PGA), which is a three-carbon compound and is hence named as C_3 photosynthesis (Ehleringer and Cerling, 2002; Taiz and Zeiger, 2010). The PGA is the product of carboxylation of ribulose bis-phosphate (RuBP) in the presence of key enzyme ribulose 1, 5-bisphosphate carboxylase oxygenase (RuBisCO) and is used for generation of sugars by the downstream metabolic pathways. RuBisCO is capable of using two different substrates, i.e., CO_2 and O_2 which leads to carboxylation and

oxygenation, respectively of RuBP, thereby decreasing its specificity toward CO_2. In the ancient atmospheric conditions, CO_2 was abundant with very low O_2 levels hence RuBisCO did not have to face the dual substrate specificity but with the evolution of oxygenic photosynthesis, atmospheric O_2 levels have drastically increased and CO_2 has become extremely low (Badger and Price, 2003).

Thus, in the present atmosphere, RuBisCO also acts as an oxygenase and further catalyzes the photorespiration reaction resulting in less carbon fixation because it leads to generation of one, three-carbon molecule 3PGA and one, two-carbon molecule phosphoglycolate (2PG) (von Caemmerer and Evans, 2010). Although C_3 plant RuBisCO has evolved to have a better affinity for CO_2 but this led to a three-fold decrease in carboxylation rate (Tcherkez et al., 2006). As a consequence of the fickle specificity of RuBisCO and low catalytic turnover, several photosynthetic organisms (C_3 plants) developed RuBisCO with a better specificity for CO_2, while others developed a CCM (C_4 plants, CAM plants, algae and cyanobacteria etc.) to improve photosynthetic performance.

1.3 Carbon Concentrating Mechanisms (CCM)

These mechanisms accumulate CO_2 in the vicinity of enzyme RuBisCO and hence help RuBisCO enhance its catalytic rate and carboxylation efficiency. Additionally, CCMs suppresses RuBisCO's oxygenase activity and eventually loss of energy by photorespiration. These mechanisms improve the survival and photosynthetic efficiency of the organism even in low level of CO_2. Different variants of CCMs have developed in higher plants, algae, cyanobacteria etc., (reviewed by Sidhu et al., 2014). These mechanisms are basically the adaptations which have evolved to help organisms with the changing environment through the course of evolution.

1.3.1 CCM in higher plants

Higher plant CCM includes four-carbon (C_4) pathway and Crassulacean Acid Metabolism (CAM) pathway (Zabaleta et al., 2012).

(a) C_4 pathway

C_4 photosynthesis is more common among monocot plants such as maize, sugarcane and various types of grasses etc., than in dicot plants. These plants bear huge biochemical

and anatomical differences when compared to C_3 plants, which facilitates reduction in RuBisCO's oxygenation activity. Further, in this pathway, the first product is a four-carbon molecule (malate or aspartate) and hence, the pathway is termed as C_4 photosynthesis (Dengler and Nelson, 1999; Ehleringer and Cerling, 2002). Under the conditions of low CO_2 and high temperature, C_4 plants are more efficient than C_3 plants due to reduced photorespiration rates. While studying cross sections of leaves, it was observed that C_3 plants contain chloroplasts only in mesophyll cells while C_4 plants have chloroplast in both mesophyll and bundle sheath cells which are arranged in a distinct way (Taiz and Zeiger, 2010). The peculiar leaf structure of C_4 plant species is called as kranz anatomy in which undifferentiated mesophyll cells are present in form of the concentric layer around bundle sheath cells, although both are connected by plasmodesmata (Leegood, 2002). The initial carboxylation reaction occurs in outer mesophyll cells, catalyzed by the enzyme phosphoenolpyruvate (PEP) carboxylase. Malate or aspartate is formed which is further translocated to the interior of bundle sheath cells, where CO_2 released from the four-carbon compound is again fixed by RuBisCO enzyme to form two molecules of 3PGA. In this way, RuBisCO remains separated from atmospheric O_2 in the bundle sheath cells and CO_2 is continuously concentrated around RuBisCO, leading to increased photosynthetic efficiency. Hence, the C_4 pathway is the way of spatial separation of atmospheric O_2 from RuBisCO (Ghannoum et al., 2011). Some plant genera have been found to show the mixed characteristics of C_3 and C_4 plants hence they are known as C_3-C_4 intermediates, one example being *Flaveri*a (Monson et al., 2000).

(b) CAM pathway

In CAM plants also, various adaptations are found that help to cope up with the hot arid conditions. CAM pathway is found in many epiphytes and succulent plants of the arid regions such as cacti, pineapple etc. These plants close their stomata during the hot daytime, to reduce water loss through transpiration and open during the cold night time to collect CO_2. Thus, they temporally regulate the efficiency of CO_2 fixation pathway (Lüttge, 2002; Black and Osmond, 2003). At the same time, in CAM plants also, a spatial regulation similar to that of C_4 plants is found. During the night, CO_2 is taken up by PEP carboxylase present in the cytosol to form malate and then it is stored in vacuoles that is further transported to chloroplast during the day and where it gets decarboxylated

and the released CO_2 is fixed by RuBisCO. This way, CAM plants are able to work efficiently in water and CO_2 stressed conditions (Taiz and Zeiger, 2010).

1.3.2 CCM in algae

Algal CCMs are mainly comprised of inorganic carbon transporters and carbonic anhydrases. Although, specific inorganic transporters have not yet been characterized but LCI1 and HLA3 are probable candidates located on plasma membrane while LCIA, CCP1 and CCP2 are supposedly found on the chloroplast envelope (Atkinson et al., 2016). The second essential component is carbonic anhydrase (CAH) which catalyses the interconversion of carbon dioxide and bicarbonate and they are found in various locations of the cell such as periplasmic space (CAH1), thylakoid lumen (CAH3) and chloroplast stroma (CAH6) (Atkinson et al., 2016). Another important part of CCM is pyrenoid compartments which are not necessarily found in all CCM containing algae (Giordano et al., 2005). Reports suggest that all those algae which have pyrenoids, also have CCM in them (Atkinson et al., 2016) and it has been estimated that algal CCM which lack pyrenoid also exist but might be less efficient (Wise, 2006). Pyrenoids are part of chloroplast stroma where the majority of RuBisCO is accumulated and it has been observed that CO_2 is concentrated only in the pyrenoid region of chloroplast (Sinetova et al., 2012). These pyrenoid structures are analogous to cyanobacterial carboxysomes although the mechanism and structural components of algal CCM are different and less characterized in comparison to cyanobacterial CCM (Raven et al., 2008).

1.3.3 CCM in cyanobacteria

Cyanobacterial CCMs are most efficient and well studied mechanisms among the lower photosynthetic organisms which is being able to accumulate up to thousand fold CO_2 in vicinity of RuBisCO (Badger and Price, 2003). These CCMs are composed of two major elements i.e., inorganic carbon transporters (Ci) and carboxysome (Figure 1-1). Transporters allow the sufficient uptake of inorganic carbon which further gets accumulated inside carboxysome which harbors all the RuBisCO in it and hence enhances the carboxylation.

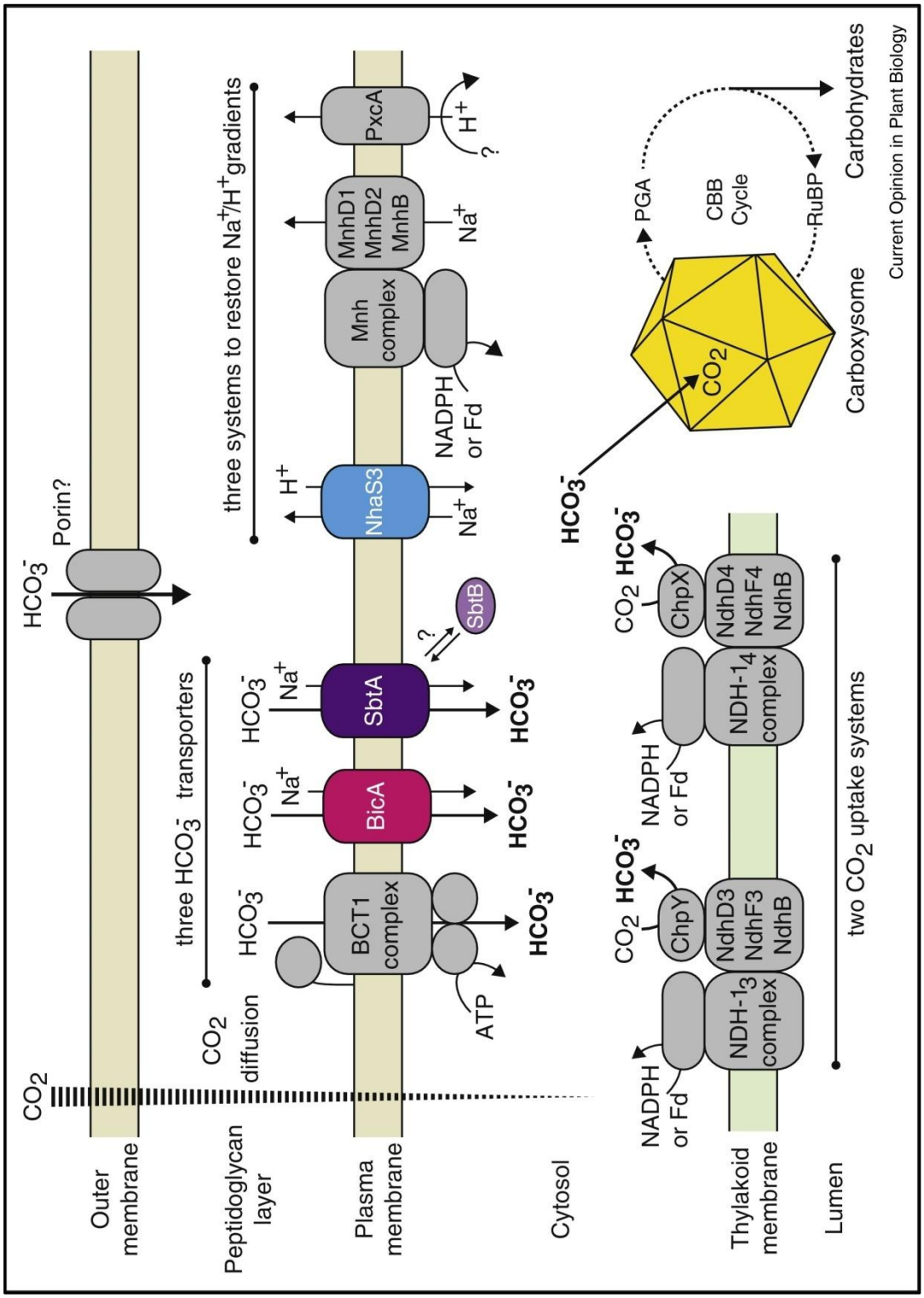

Figure 1-1: The representation of cyanobacterial carbon concentrating mechanism (CCM) components (Long et al., 2016)

1.3.3.1 Ci transporters

There are five major transporters which have been characterized so far and they can be further divided into two subgroups i.e., bicarbonate transporters (HCO_3^- as a substrate) which are located on plasma membrane and carbon dioxide uptake system (CO_2 as a substrate) which are supposedly located on thylakoid membrane (Price et al., 2007). Three of them act as HCO_3^- transporters i.e., BCT1, SbtA and BicA while remaining two are the CO_2 uptake systems i.e., NDH-I_3 and NDH-I_4 complexes which are based on NADPH dehydrogenase of respiratory complexes (Ogawa and Mi, 2007; Price, 2011). The additional transporters represented in the Figure 1-1 are NhaS3 which functions either as Na^+/H^+ antiporter or as Na^+ pump, Mnh complex which also acts as a Na^+/H^+ antiporter and PxcA which is H^+ pump. All of them are involved in maintaining Na^+/H^+ at both sides of the plasma membrane and the Na^+ gradient generated by these transporters energize the transport of HCO_3^- ions by BicA and SbtA transporters (Price, 2011; Long et al., 2016).

(a) BCT1 transporter

The BCT1 transporter is a multi-subunit membrane protein which was for the first time characterized in *Synechococcus elongatus* 7942 and found to be involved in HCO_3^- transport. The transporter gene is encoded by an operon which possesses *cmpA*, *cmpB*, *cmpC* and *cmpD* genes and belongs to traffic ATPase family of ATP binding cassette (ABC) type transporters (Omata et al., 1999). The presence of *cmpABCD* operon was observed in several other cyanobacterial species as well by sequence analysis studies (Tomar et al., 2016).

The CmpA protein is located on the periplasmic side of the plasma membrane where it binds with the substrate i.e., HCO_3^- and further transport it to the other transporter components. Recent studies (Koropatkin et al., 2007) have suggested that substrate binding site is also involved in binding of Ca^{2+} which is probably acting as a cofactor. CmpB is integral transmembrane (TM) protein, occurring as a dimer which basically forms the passage for transport of substrate through the plasma membrane. CmpC and CmpD are involved in ATP binding, although they are structurally different. Hence, BCT1 transporter acts as a uniporter which utilizes ATP for active transport of substrate and it is inducible, medium to low flux, high affinity transporter (Price et al.,

2007). BCT1 is found to be inducible under limiting substrate concentration and it shows high affinity for approximately 15 µM HCO_3^- concentration (Omata et al., 2002).

(b) SbtA transporter

The SbtA transporter is a single subunit, sodium dependent transporter which is found to be involved in HCO_3^- transport and it was first time identified in *Synechocystis* PCC 6803 (Shibata et al., 2002). The close homologues of SbtA were observed majorly in cyanobacteria and few proteobacterial species while no eukaryotic homologues have been identified till date (Price et al., 2011a). The *sbtA* gene is present in the genome as independent gene which encodes for the SbtA transporter but in certain organisms there is another gene named as *sbtB* which is sometimes present either in operon form with *sbtA* gene or it is present in the vicinity of *sbtA* gene (Rae et al., 2011). The genomic arrangement of both the genes suggests that they might be functionally related and as reported in a recent study (Du et al., 2014) it has been experimentally proved that both the proteins interact with each other where SbtB acts as a regulator of SbtA at transcriptional or translational level.

Topology analysis studies have revealed the presence of 10 TM helices which are arranged in a duplicated 5+5 structure with both N- and C- termini being periplasmic (Price et al., 2011a) (Figure 1-2).

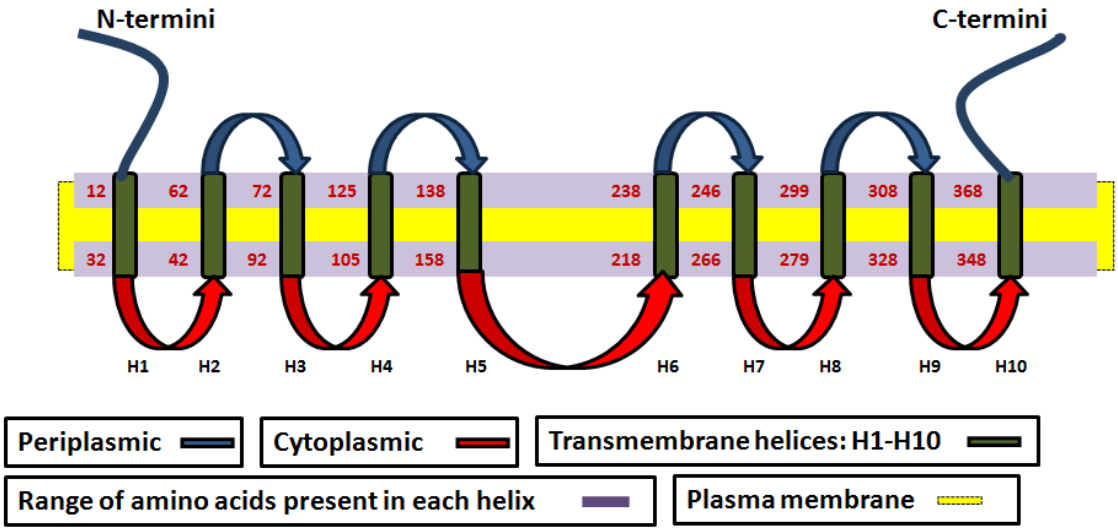

Figure 1-2: Diagrammatic representation of topology map of SbtA transporter; sodium dependent bicarbonate transporter from *Synechocystis* PCC 6803

The TM helices of both the duplicated halves are present in an inverted orientation and this inverted pattern has been found to be a specific feature of sodium coupled transporter families (Krishnamurthy et al., 2009). Hence, SbtA transporter is a Na^+/HCO_3^- symporter which utilizes periplasmic sodium gradient for transport of HCO_3^-. SbtA belongs to sodium solute symporter (SSS) family and it is an inducible, low flux and high affinity transporter. SbtA is found to be inducible under low substrate concentration and it shows high affinity for approximately 2-5 µM HCO_3^- concentration (Price and Howitt, 2014).

(c) BicA transporter

BicA is a single subunit, sodium dependent transporter which is found to be involved in HCO_3^- transport and was discovered in marine cyanobacterium *Synechococcus* PCC 7002 (Price et al., 2004). BicA homologues are widely spread in several cyanobacterial species as well as in various other organisms such as bacteria, plants, mammals etc. The reason behind the wide abundance of BicA transporter is that it belongs to SulP/SLC26 family which is an anion transport family with diverse substrate specificities and its members are found in the majority of prokaryotic and eukaryotic organisms (Shelden et al., 2010). The C-terminus of BicA protein possesses sulphate transporter anti-sigma factor-like domain (STAS) which is the characteristic feature of SulP/SLC26 family and it is possibly involved in regulation of transport function (Sharma et al., 2011).

Topology analysis studies have revealed the presence of 12 TM helices with both N- and C- termini being cytoplasmic (Figure 1-3). This analysis was theoretically performed using TOPCONS software and experimentally proved by PhoA-LacZ mapping (Shelden et al., 2010) and the results obtained in both cases confirmed the presence of 12 TM helices along with a conserved cytoplasmic domain at C-terminus. However, it is still controversial to determine the exact topology of BicA because a recent study (Price and Howitt, 2014) suggests the presence of 14 TM helices instead of 12 with a 7+7 inverted repeat structure. This conclusion was drawn on the basis of analogy in topologies of two different members of SulP/SLC26 family (Gorbunov et al., 2014) i.e., Ura A which is a bacterial uracil transporter (Lu et al., 2011) and rPRES which a transmembrane molecular motor protein of rat (Zheng et al., 2000) with that of BicA transporter.

BicA is also a Na^+/HCO_3^- symporter with low affinity and high flux rate. It is inducible under limiting substrate concentration but it expresses at a low level even in high Ci conditions and being a low affinity transporter it requires approximately 38 µM HCO_3^- concentrations (Price, 2011).

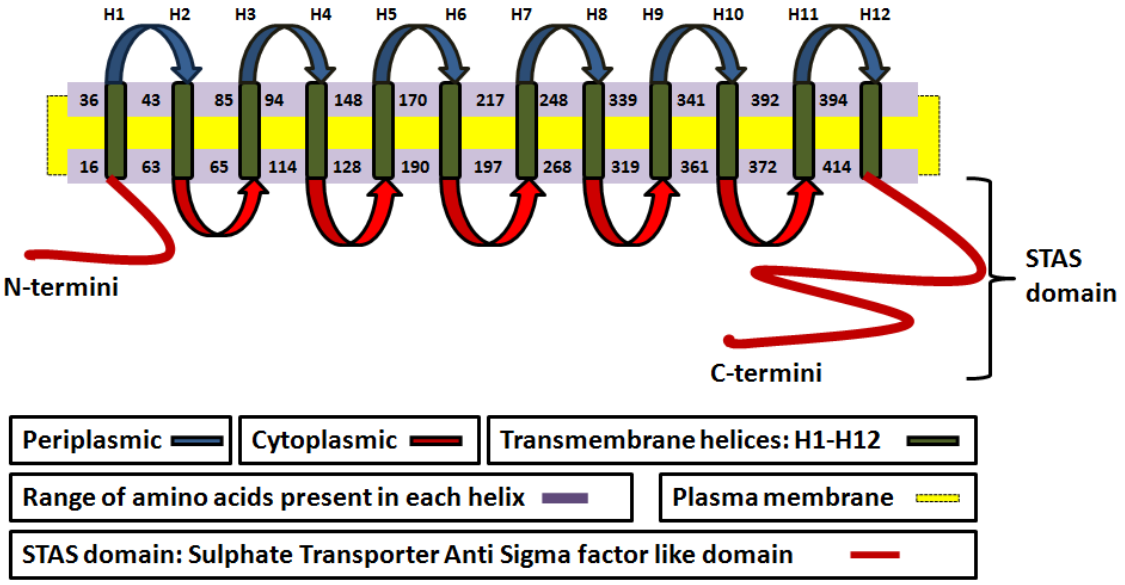

Figure 1-3: Diagrammatic representation of topology map of BicA transporter; sodium dependent bicarbonate transporter from *Synechococcus* PCC 7002

(d) NDH-I_3 complex

NDH-I_3 is a high affinity CO_2 uptake system which requires 1-2 µM of CO_2 and is inducible under Ci limiting conditions (Price, 2011). It is located on thylakoid membrane of cyanobacteria (Herranen et al., 2004) while remaining all the Ci transporters are found on the plasma membrane. NDH-I_3 complex proteins are encoded by *ndhF3/ndhD3/chpY* genes.

(e) NDH-I_4 complex

NDH-I_4 is a low affinity CO_2 uptake system which requires approximately 10 µM of CO_2 and is constitutively expressed (Price, 2011). Although it is believed to be located on the plasma membrane (Maeda et al., 2002; Price, 2011) but due to its functional analogy with NDH-I_3 it is being assumed that it might be located on thylakoid (Price et al., 2013) for which further confirmation is needed (Price, 2011). NDH-I_4 complex proteins are encoded by *ndhF4/ndhD4/chpX* genes.

The ChpX and ChpY are CO_2 hydration complexes which form an important part of CO_2 uptake systems. The CO_2 taken up through diffusion is further converted into HCO_3^- in the cell cytosol via these hydration complexes. Both the CO_2 uptake systems are widely present in various β-cyanobacterial species while α-cyanobacteria possesses only NDH-I_4 complex (Badger and Price, 2003) and further majority of the *Prochlorococcus marinus* species (α-cyanobacteria) are not found to contain any of the CO_2 uptake system (Badger et al., 2002)

1.3.3.2 Carboxysome

Carboxysome is a cytosolic organelle involved in enhancement of CO_2 fixation and is found in cyanobacteria as well as in various chemoautotrophs (Kerfeld et al., 2010). The carboxysome microcompartments are comprised of large polyhedral bodies that are surrounded by proteinaceous shell which selectively restrict the movement of various molecules through pores present in carboxysome shell (Espie and Kimber, 2011). The majority of cellular RuBisCO and carbonic anhydrases (CA) are accumulated inside the carboxysomes which determines it to act as the site for photosynthesis. The Ci transporters take up the HCO_3^- and CO_2 inside the cell, amongst which HCO_3^- molecules are capable of entering directly inside the carboxysome while CO_2 is converted into HCO_3^- by the CO_2 hydration complexes and then it enters the carboxysome via the pores.

The nature of carboxysome shell proteins is such that it only allows the entry of polar molecules like HCO_3^-, RuBP and 3PGA while the passage of CO_2 and O_2 is not efficient through this barrier. Hence, it helps in reducing CO_2 leakage and RuBisCO oxygenase activity. Once inside the carboxysome the HCO_3^- is converted back to CO_2 by carbonic anhydrases and then it is not able to escape carboxysome hence its concentration increases in the vicinity of RuBisCO, leading to increased carboxylation. Additionally, the carboxysomal entry of O_2 is also restricted which help minimize photorespiration, thus helping RuBisCO improve its carboxylation rate (Rae et al., 2013).

There are two different types of carboxysomes which are found in cyanobacteria i.e., α-carboxysome and β-carboxysome which contain type 1A RuBisCO and 1B RuBisCO, respectively. For the structural and functional characterization of α-carboxysome, *Halothiobacillus neapolitanus* is most studied organism while *Synechocytis* PCC 6803 and *Synechoccoccus* PCC 7942 were used as the model organism for the study of β-carboxysome (Espie and Kimber, 2011). Both types of

carboxysomes are found to contain a different set of carbonic anhydrases i.e., CcaA/IcfA/CcmM (gamma type CA) in α-carboxysome and CsoS3 (alpha type CA) in β-carboxysome (Price, 2011; Kerfeld and Melnicki, 2016). The structural proteins of carboxysome shell are also different in both of them *viz.* CsoS1A, CsoS1B, CsoS1C, CsoS1D, CsoS1E, CsoS2, CsoS4A and CsoS4B are found in α-carboxysome while in β-carboxysome, CcmK1, CcmK2, CcmK3, CcmK4, CcmL, CcmM, CcmO and CcmP are the major components (Kinney et al., 2011; Rae et al., 2013).

1.3.3.3 Regulatory mechanisms of cyanobacterial CCM

(a) At transcriptional level (Figure 1-4)

The basal level expression of cyanobacterial CCM is even found in the non limiting Ci conditions. This suggests that various CCM components *viz.* genes encoding for low affinity transporter NDH-I_4, RuBisCO and carboxysome proteins are found to be constitutively expressed irrespective of the Ci concentration in their surroundings (Kaplan and Reinhold, 1999). However, certain components of CCM are inducible under Ci limiting conditions such as the three bicarbonate uptake systems i.e., BCT1, SbtA and BicA and high affinity CO_2 transporter NDH-I_3. CcmR/NdhR and CmpR are the major transcription regulatory proteins which are involved in the induction of these Ci transporters (Wang et al., 2004). They belong to LysR-type transcriptional regulators (LTTRs) family which possess the most abundant and conserved transcription regulators in prokaryotic organisms (Maddocks and Oyston, 2008).

The experimental studies conducted in *Synechococcus* PCC 7002 suggest that CcmR acts as a negative regulator of the *ndhF3/ndhD3/chpY*, *bicA* and s*btA* genes. Under normal conditions CcmR activates the repressor by binding to it hence transcription of genes does not take place while the low Ci conditions induce the unbinding of CcmR from repressor leads to the activation of the transcription of genes encoding NDH-I_3, BicA and SbtA transporters (Woodger et al., 2007). In *Synechocystis* PCC 6803 also the CcmR regulator control the expression of *ndhF3/ndhD3/chpY* and *sbtA/sbtB* genes while the regulator CmpR is found to act as an activator/positive regulator for *cmpABCD* operon. Under limiting Ci conditions CmpR binds with the activator and hence starts the transcription of genes encoding for BCT1 transporter (Omata et al., 2001; Omata et al., 2002).

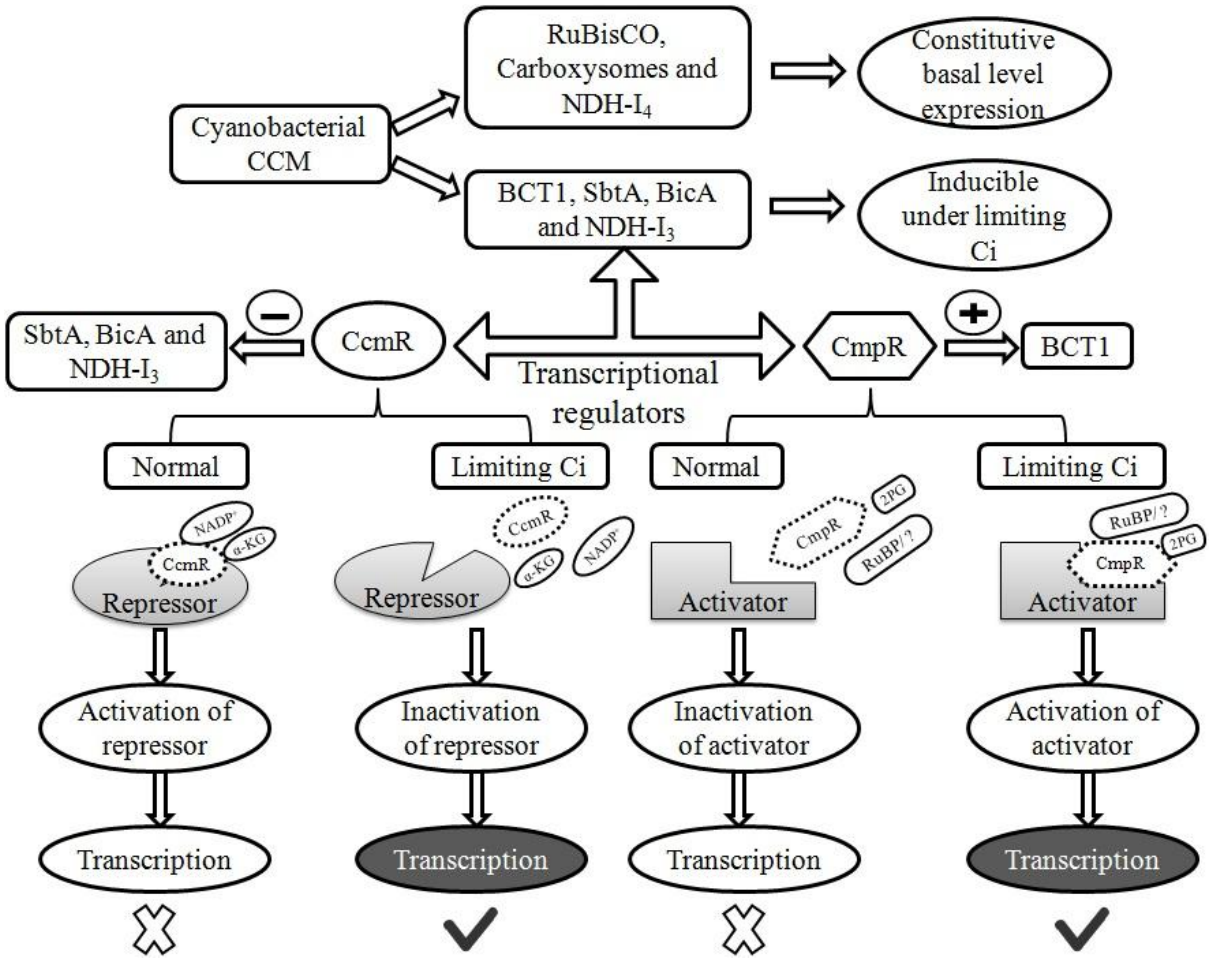

Figure 1-4: Regulation of cyanobacterial CCM at transcriptional level

The regulatory function of CmpR has been experimentally studied in *Synechocystis* PCC 6803 and *Synechococcus* PCC 7942 species which suggests that probable reason behind the activation by Ci limitation is the interaction between the CmpR and some co-inducer molecules (Takahashi et al., 2004). The low Ci conditions tends to reduce the photosynthesis that eventually leads to accumulation of RuBP, a substrate in the CO_2 fixation cycle and 2PG which is the product of photorespiration (The pathway which RuBisCO starts catalyzing if there is low CO_2). Although the exact mechanism is not clear but *in vitro* studies suggest that the low Ci conditions change the concentration of internal metabolite pool and few of the most abundant metabolites such as RuBP and 2PG may act as co-inducer for CmpR (Burnap et al., 2015) which will further lead to the transcriptional activation of *cmpABCD* operon. Although the RuBP accumulation in the cell is difficult to explain because under Ci limiting conditions RuBP is consumed in oxygenation reactions hence it does not really accumulated therefore 2PG

is the primary signal for activation of CmpR (Eisenhut et al., 2008; Nishimura et al., 2008; Daley et al., 2012).

In case of CcmR regulator, under normal conditions, it interacts with certain co-repressor molecules such as $NADP^+$ (Nicotinamide adenine dinucleotide phosphate) and α-ketoglutarate (α-KG) which are abundant in the cellular pool and this leads to the strong binding of CcmR onto the repressor region of the promoter (Daley et al., 2012). While in low Ci condition, the concentration of these co-repressor molecules decreases in the cell due to reduced photosynthetic activity and further transcriptional activation of *ndhF3/ndhD3/chpY*, *bicA* and *sbtA* genes occur due to derepression of CcmR. The reduction in the concentration of $NADP^+$ and α-KG can be explained by the fact that these are the molecules which are being produced in the Calvin cycle which becomes significantly slow in low Ci condition (Burnap et al., 2015).

(b) At post transcriptional level (Figure 1-5)

Although carboxysome shell proteins are constitutively expressed hence their expression is not regulated at transcriptional level but the number of carboxysomes in a cyanobacterial cell has been found to increase in response to low Ci conditions (Eisenhut et al., 2007). On the other hand, a change in the composition of carboxysomal shell proteins has been observed which is proposed to have an important role in making the shell selectively permeable, in order to allow the movement of specific metabolites (Kerfeld et al., 2005; Eisenhut et al., 2007). Similarly, in *Synechococcus* PCC 7002, the RuBisCO content (specifically RbcL protein) was also found to increase in the low Ci conditions and one of the probable reasons could be the enhanced half-life of RbcL protein (Eisenhut et al., 2007). A recent study (Georg and Hess, 2011) also suggests the role played by various *cis*-antisense RNA molecules in controlling the stability of mRNA transcript and translational machinery as well. Therefore, it has been proposed that specific regulatory RNA molecules may act differently in varying Ci concentrations, hence affecting the carbon metabolism in the cell.

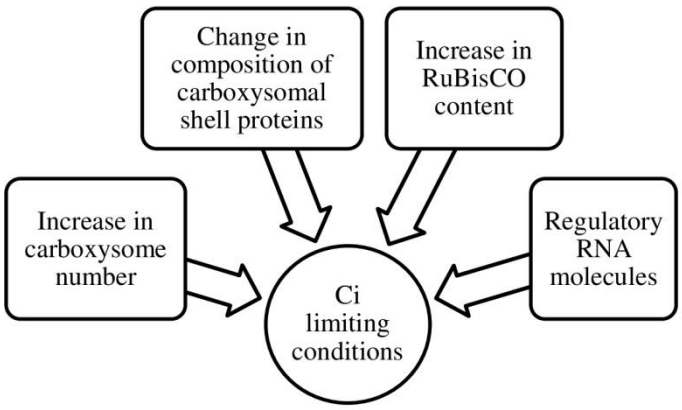

Figure 1-5: Regulation of cyanobacterial CCM at post transcriptional level

(c) At post translational level (Figure 1-6)

Apparently, it has been observed that all the Ci uptake systems remain inactive in dark, hence, for their energization light illumination need to be provided which will first activate the CO_2 pumps followed by the activation of bicarbonate transporters (Price et al., 2007). The possible mechanisms behind the rapid activation of transporters could be a redox regulatory signal (Kaplan et al., 1987) or phosphorylation-dephosphorylation mechanism (most probably in BicA and SbtA transporters) which act specifically on the serine or threonine residues present in the regulatory loops of transporter proteins.

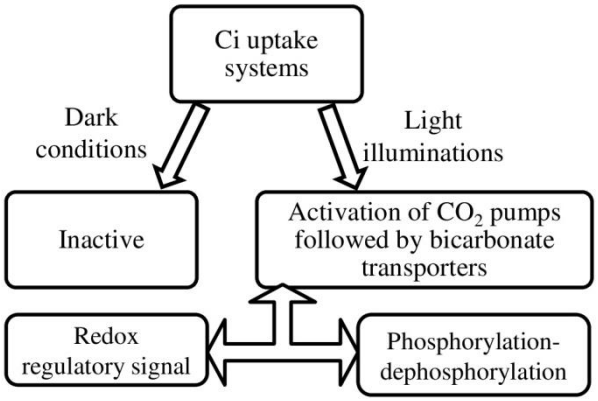

Figure 1-6: Regulation of cyanobacterial CCM at post translational level

Note: All these studies which are described in subsection (b) and (c) suggest both post transcriptional and post translational regulation of cyanobacterial CCMs for which the exact detailed mechanisms are yet to be explored.

As mentioned in section 1.2, C_3 plants have RuBisCOs with comparatively better substrate specificity but still the net carboxylation is low because they do not contain any form of CCM. Hence for improving C_3 photosynthesis, various approaches have been suggested as well as implemented, the details of which are mentioned in further subsections.

1.3.4 Various approaches for improving C_3 photosynthesis

1.3.4.1 Manipulation/Modification in the RuBisCO

RuBisCO is the major enzyme of the C_3 photosynthetic pathway which fixes CO_2 into the organic molecules but the imperfection lies in the fact that it can catalyze oxygenation reactions as well which eventually compromises its carboxylation efficiency and CO_2 fixation. Apart from having fickle specificity for CO_2 and O_2, RuBisCO has a low catalytic turnover rate (K_{cat}) which makes it an extremely slow enzyme and hence plant needs to invest huge amount of nitrogen (Carmo-Silva et al., 2015) for its synthesis to support the sufficient photosynthetic rate (Parry et al., 2013). RuBisCO is estimated to have evolved around 3000 million years ago but the imperfections in the design of the protein arose around 400 million years ago (Badger and Price, 2003) when the atmospheric composition changed with drastic increase in O_2 which almost doubled its concentration while there was a large fall off in CO_2 levels (Badger and Bek, 2008). However, the enzyme RuBisCO could not evolve in a better way to combat these changes and thus became slow and inefficient. Thus, manipulation in RuBisCO catalysis, specificity and regulation by human intervention is being attempted around the world for the improvement of C_3 photosynthesis (Whitney et al., 2011). One possibility could be the transfer of naturally existing efficient forms of RuBisCO into C_3 plants. It has been found that RuBisCO from red alga *Galdieria partita* shows better specificity (Uemura et al., 1996) (specificity value 238 which is almost thrice the crop plant's specificity factor) while C_4 plants have RuBisCOs with high turnover rate which makes them suitable for engineering into C_3 plant systems (von Caemmerer and Evans, 2010).

In the process of CO_2 fixation, RuBisCO first binds with the substrate RuBP and then forms an intermediate enediol which reacts with both CO_2 and O_2. Therefore it is difficult to increase its specificity towards CO_2 by manipulating the catalytic site because these gases are not directly interacting with it. Although, specific residues are found to affect the specificity but a direct correlation is difficult to draw (Parry et al., 2003).

Several efforts have been made to identify critical residues which can be used for manipulating the specificity and turnover of RuBisCO catalysis. A study conducted in *Chlamydomonas reinhardtii*, which made changes at the C-terminus by replacing valine (at position 331) with alanine residue suggested a drastic decrease in specificity by approximately 40% (Chen and Spreitzer, 1989; Andersson and Backlund, 2008) and it has been observed by structural studies that various C-terminus and N-terminus residues are involved in maintaining the favorable conformation of enzyme (Knight et al., 1990; Newman and Gutteridge, 1993).

Another study conducted in spinach and *Chlamydomonas* revealed the important role of C-terminus of RuBisCO large subunit in the catalytic turnover (Portis, 1990). There have been a few studies in which the overall RuBisCO content of the cell was increased by over expressing the *rbcS* gene but eventually, it led to a decrease in the net content and the probable reason was the silencing of native *rbcS* gene (Parry et al., 2003). A hybrid RuBisCO enzyme was also generated in which the genes encoding large subunit (cyanobacterial) and small subunit (eukaryotic) were taken from two different organisms and expressed in *E. coli*. Although the hybrid enzyme showed a reduced carboxylation rate, an enhancement in CO_2/O_2 specificity was observed (Read and Tabita, 1992).

Apart from modifications in the RuBisCO itself, the other possible approach could be the manipulation of its regulatory components. One of the main regulatory enzymes is RuBisCO activase, which play a role in activating the RuBisCO active site by removing the inhibitory sugar phosphates (Andersson, 2008) from the active site but unfortunately, it strongly suffers from high temperature sensitivity. Hence incorporation of thermostable RuBisCO activase should prevent the reduction in photosynthetic efficiencies in high temperature (>42°C) atmosphere (Crafts-Brandner and Salvucci, 2000; Parry et al., 2013). Even, after the extensive gain in knowledge of RuBisCO's structural and regulatory components, the manipulation strategies have not proven successful enough to significantly enhance the crop productivity.

1.3.4.2 Increasing RuBP regeneration

In addition to CO_2, RuBP is used as a substrate for synthesizing organic acids by the enzyme RuBisCO hence either of them if limiting, causes a reduction in overall photosynthetic efficiency (Parry et al., 2007). Even when CO_2 is enough but there is a

decrease in irradiance, the RuBP concentration becomes the limiting factor for photosynthesis. The possible reasons behind this are drop down in either electron transport capacity of chloroplast or enzymes which are involved in RuBP regeneration (von Caemmerer and Evans, 2010). Two such enzymes, fructose-1, 6-bisphosphatase (FBPase) and sedoheptulose-1, 7-bisphosphatase (SBPase) catalyze RuBP regeneration reactions in Calvin cycle and they are regulated in the light dependent manner by ferredoxin/thioredoxin system (Buchanan, 1991).

Furthermore, unlike other enzymes of Calvin cycle these two are present in extremely low levels. Thus it seems like an improvement in the performance of these enzymes could help enhance the RuBP regeneration and eventually net photosynthesis. The cyanobacterial fructose-1, 6-sedoheptulose-1, 7-bisphosphatase which can perform both the reactions catalyzed by plant FBPase and SBPase was expressed in *Nicotiana tabacum* chloroplast and showed enhanced growth, carbohydrate accumulation and CO_2 fixation (Miyagawa et al., 2001). Several other studies have reported the increased growth and photosynthesis either by over expressing the enzymes of plant origin or by incorporating efficient enzymes from cyanobacteria and/or algae (Lefebvre et al., 2005; Tamoi et al., 2006; Ding et al., 2016).

1.3.4.3 Reducing/bypassing photorespiratory pathway

RuBisCO is capable of performing oxygenation reactions in presence of high O_2 concentrations, thereby reducing the overall carboxylation rate. In the carboxylation reaction, there is production of two molecules of 3PGA while in oxygenation reaction RuBisCO reacts with RuBP and produces one molecule of 2PG and one molecule of 3PGA, leading to the loss of one carbon molecule which could have been fixed by carboxylation. 3PGA is used either for the regeneration of RuBP or for the synthesis of organic substances while 2PG is considered to be toxic metabolite which needs to be converted into non toxic forms. Consequently, 2PG is converted into 3PGA by a set of chemical reactions which are termed as photorespiratory pathway (Bauwe et al., 2010). This pathway involves three major organelles i.e., chloroplast, peroxisome and mitochondria in which two molecules of 2PG get converted into one molecule of 3PGA at the cost of one carbon which releases as CO_2 in mitochondria (Dalal et al., 2015). This conversion is an extremely energy demanding and inefficient chemical reaction for the cell (Peterhansel et al., 2010). Hence the manipulation of this pathway has great scope in

reducing the cell's ATP demand and the loss of carbon can be prevented and fixed by carboxylation.

In order to evade the photorespiratory losses, three alternate bypass pathways have been incorporated in model plants for the recycling of 2PG which are comparatively less energy demanding. The first bypass pathway is the glycolate catabolic pathway of *E. coli* which is incorporated in the *Arabidopsis thaliana* chloroplast (Kebeish et al., 2007). Thus, the additional steps in peroxisomes and mitochondria were reduced to a certain extent, although not completely omitted resulting in enhancement of plant growth, biomass and chloroplastic CO_2 concentration. In the second bypass strategy, hydroxy pyruvate isomerase and glyoxylate carbo ligase enzymes of *E. coli* were attempted to express in peroxisome of tobacco. Even though this was presumed to be energy efficient for recycling of 2PG, hydroxy pyruvate isomerase in the transgenic tobacco line could not be detected. Thus the impact of this approach remains questionable (Carvalho et al., 2011). In the third bypass strategy, a complete glycolate catabolic cycle which was comprised of multiple enzymes was incorporated in the chloroplasts of *Arabidopsis thaliana*. Although, this pathway could successfully oxidize 2PG into CO_2 and the biomass and photosynthetic rate were reported to have increased (Maier et al., 2012), it was found to be less energy efficient.

Apart from the advancement of bypassing strategies, efforts have been made for reducing the photorespiration, which include incorporation of natural mechanisms with reduced photorespiration and refixing the CO_2 released. Given the natural plant anatomy, the CO_2, which is released by photorespiration does not enter into chloroplast, rather it tend to escape from the cell because the chloroplast envelope is less permeable to CO_2 than plasma membrane (Uehlein et al., 2008). Hence, the refixing strategy would involve 2PG recycling by modifying the plant anatomy in such a way that the most of the CO_2 evolved is made to pass into the chloroplast which can be fixed by RuBisCO (Busch et al., 2013). Another strategy to reduce photorespiration is incorporation of C_4 mechanisms which exhibit a special leaf anatomy with spatial regulation of CO_2 fixation mechanism, or the other CCMs into C_3 plants in a way that it enhances CO_2 concentration around RuBisCO (Betti et al., 2016).

In a nutshell, it can be said that various studies have reported an increased CO_2 fixation, plant growth and yield after reducing or bypassing the photorespiratory losses

but in several cases, the benefits were not consistent or they were applicable only in some specific conditions (Xin et al., 2015). On the other hand, these changes may adversely affect the various regulatory metabolic pathways of plants such as nitrogen assimilation which is linked with photorespiration by reducing the nitrogen availability for the plant which may in turn affect plant productivity (Bloom, 2015). Additionally, photorespiration is involved in synthesis of glycine and serine amino acids and also helps plant in excess light stress conditions by acting as an alternative electron sink. Both these functions, however, can be performed even in the absence of photorespiration (Sharkey, 2001). It also maintains redox homeostasis in the plant cells and activate pathogen defense mechanisms and responses towards abiotic stresses (Bloom, 2015). Thus it is extremely controversial to decide upon the complete removal of this pathway from the plant system.

1.3.4.4 Incorporating C_4 pathway into C_3 plants

The plants performing C_4 photosynthesis have a kranz leaf anatomy which makes them perform better than C_3 plants in high temperature conditions. It has been observed that under high temperature, photosynthesis can be reduced by up to 40% (Ehleringer et al., 1997) and hence the evolution of C_4 mechanisms was considered as an adaptation to cope up with this problem. Further, the C_4 photosynthesis exhibits high CO_2 fixation (increase by 50%) along with better use of nitrogen and water resources which make it a viable target for the improvement of C_3 plant photosynthesis. There have been attempts going on for the incorporation of C_4 mechanism in major C_3 crop plants i.e., rice (http://c4rice.irri.org/) (Sheehy et al., 2007; Hibberd et al., 2008; von Caemmerer et al., 2012). Since the typical C_4 leaf anatomy (dual cell kranz anatomy) is exceptionally difficult to be incorporated and made functional in other host systems, initially it was believed that the C_4 pathway that occurs in single cell system (Voznesenskaya et al., 2001) would be easier to establish. Irrespective of the above facts, understanding the different metabolic pathways in both the cases remains challenging (Hibberd et al., 2008).

Oryza barthii and *Oryza australiensis* have been reported to accumulate larger amounts of PEP carboxylase (Yeo et al., 1994) which is the main enzyme of C_4 pathway and hence these two rice varieties are assumed to be suitable for engineering C_4 pathway in them. These species also have distinct arrangement of mesophyll cells in them which

suggests that at least the morphological features of C_4 anatomy would be easy to integrate in them (Hibberd et al., 2008). Apparently, it is not easy to transfer the whole C_4 mechanism in the C_3 plants and thus attempts have been made to express only certain specific enzymes of the C_4 pathway. One such example is expression of phosphoenolpyruvate carboxykinase (PCK) gene which is found in a C_4 plant i.e., *Urochloa panicoides* into the rice chloroplast (Suzuki et al., 2000), which led to fixation of CO_2 into four carbon compounds with no significant gain in photosynthetic rate. Lastly, now it has been well studied and accepted that engineering C_3 plants with C_4 mechanisms are very complex phenomena to understand and implement (Hibberd and Covshoff, 2010; Driever and Kromdijk, 2013). A recent breakthrough was the identification of C_4 pathway specific genes in the wheat genome which is known to be a typical C_3 plant, extending the scope of improving C_3 photosynthesis by incorporating C_4 pathway with existing C_4 specific genes (Rangan et al., 2016).

1.3.4.5 Incorporating CAM mechanisms into C_3 plants

Like C_4 plants, CAM plants have also developed mechanisms to combat the hot and dry atmospheric conditions and utilize PEP carboxylase as the primary CO_2 acceptor. CAM pathway relies on the temporal regulation of CO_2 fixation in a way that it subsequently reduces the loss of water and hence photorespiration with increased concentration of CO_2 around RuBisCO. Lately, engineering C_3 pathway with CAM photosynthesis has become an attention seeking research area for enhancing biomass and better resource use efficiency (www.cambiodesign.org) and the vital understanding of regulatory mechanisms for this transition of C_3 to CAM is still under study (Borland and Yang, 2013). Attempts have been made to study the transcriptomics, proteomics and metabolomics by systems and computational biology approaches (DePaoli et al., 2014) and a detailed analysis of mRNA profiling in C_3-CAM plant i.e., *Mesembryanthemum crystallinum* were also conducted to find out the CAM specific gene expression patterns (Cushman et al., 2008). This is a C_3 plant which can be induced to act as CAM plant under specific stress conditions (Winter and Holtum, 2005) thus aids in understanding the comparative analysis of C_3 and CAM metabolism.

In a recent study, PEP carboxylase enzyme of a C_3 plant *Solanum tuberosum* was expressed in *Arabidopsis thaliana* under the control of dark inducible promoter. The transformed plants which were incubated in dark conditions showed improved CO_2

fixation, stomatal conductance and transpiration. Thus this model helps to understand the CAM-like temporal (day/night or light/dark) regulation (Kebeish et al., 2012). Another study had analyzed various parameters and mechanisms which will be required for efficient working of CAM mechanism in C_3 plants and suggested that *Arabidopsis* and *Populus* are potential host species for the same and the candidate CAM species could be *Agave* and *Opuntia* due to their high biomass yield (Borland et al., 2015). Even after all the efforts which have been made in this context, there is still a further need to understand the complex temporal regulation and its correlation with anatomical features of plants before engineering this pathway in C_3 plants.

1.3.4.6 Incorporating algal CCM into C_3 plants

In order to achieve enhanced crop productivity, engineering algal CCM into C_3 plants is also an attractive option (Sharwood, 2017). The green alga *Chlamydomonas reinhardtii* has been used as a model system for studying the algal CCM. The algal RuBisCO was used to create a hybrid in which native algal *rbc*L was co-expressed with plant *rbc*S cDNA in a mutant *Chlamydomonas* lacking rbcS (Genkov et al., 2010). The hybrid enzyme showed a marginal increase in specificity (discrimination between CO_2 and O_2) but the net photosynthesis had reduced and it could not regenerate pyrenoids which are the main components of algal CCM and required for enhanced CO_2 accumulation and fixation (Genkov et al., 2010). The study revealed that the RbcS of the *Chlamydomonas* is essential for the formation of the pyrenoids. Various other components of algal CCM *viz.* Ci transporters and carbonic anhydrases were transiently expressed in *Nicotiana benthamiana* leaves and two of the putative Ci transporters i.e., LCIA and HLA3 were stably expressed in *Arabidopsis thaliana* (Atkinson et al., 2016). The expression of these components could not promote any gain in plant growth and photosynthesis which point out towards the necessity of co-expressing additional CCM components. Another type of hybrid RuBisCO was generated by co-expressing native RbcL protein of *Arabidopsis* with SSU α-helices from *Chlamydomonas* in a *rbcs* mutant of *Arabidopsis* and the hybrid enzyme was found to be functionally active and could regenerate pyrenoids, although the efficient aggregation of RuBisCO in pyrenoids was still lacking (Atkinson et al., 2017). The plant growth and photosynthesis were nearly comparable with that of wild type plants.

1.3.4.7 Incorporating cyanobacterial CCM components into C_3 plants

The cyanobacterial CCM has emerged as one of the most promising targets for improving C_3 photosynthesis. It should be noted that constant efforts are under progress for incorporating various components of cyanobacterial CCM into model plant species so that efficient strategies could be developed for expressing them in the chloroplast of crop plants such as wheat and rice. The long term and most effective approach would be the transfer of whole cyanobacterial CCM comprised of carboxysome and Ci transporters. However, their accurate protein folding, assembly, localization and functioning are the major issues to be addressed (Price et al., 2013). Recently, the successful expression of α-carboxysome was demonstrated in *E. coli*. which showed the correct assembly and function *in-vivo* (Bonacci et al., 2012). The chimeric carboxysomes have also been generated by combining shell proteins of α-carboxysome and β-carboxysome (Cai et al., 2015) and various studies are under progress for incorporating cyanobacterial RuBisCO or other components of carboxysome into plant chloroplast (Hanson et al., 2016). So far, no trials have been done for incorporating the multi subunit transporters (BCT1, NDH-I_3 and NDH-I_4) of cyanoabcaterial CCM into the plant system.

The possibility of incorporating one or two Ci transporters seems to be an early achievable target and would act as a beginning step for the incorporation of all the remaining components. BicA and SbtA are the simplest components among the Ci transporters of cyanobacterial CCM. Both are encoded by single genes each and present as a single subunit protein which indicates that their assembly and transformation would be comparatively easy and this makes them potential candidate for the beginning experiments in the area. Theoretical modeling studies have suggested that the installation of BicA and/or SbtA transporters into the inner envelope of C_3 plant chloroplast could attain almost 5-15% increase in the net CO_2 fixation (Price et al., 2011), although experimental validation is yet to be done. On the basis of simulation studies, a recent report by McGrath and Long (2014) also stated that incorporation of BicA transporter alone would show an increment of 9% in C_3 plant photosynthesis which can further enhance up to 16% if other bicarbonate transporters *viz.* BCT1 and SbtA are also added.

BicA and SbtA are bicarbonate transporters which will take up the cytosolic bicarbonate inside stroma where it will be converted back into CO_2 by stromal carbonic anhydrases which will lead to enhancement of CO_2 concentration in the vicinity of

RuBisCO. Now the important point to be noted here is that the leaf cytosol should have enough amount of bicarbonate for these transporters to be functional. At high light, ambient CO_2 and 25˚C temperature, the concentration of leaf cytosolic HCO_3^- is estimated to be about 250 µM (Evans and Von Caemmerer, 1996; Price et al., 2013) which seems to be sufficient as the HCO_3^- uptake affinities for BicA and SbtA are approximately 90-170 µM and 5-15 µM, respectively (Shibata et al., 2002; Price et al., 2004).

BicA and SbtA are the sodium dependent bicarbonate transporters which indicate that the leaf cytosol should have sufficient sodium ion concentration for generating the gradient to energize the transporters and drive the transport of bicarbonate. Like in the cyanobacterial plasma membrane, *Arabidospis thaliana* chloroplast envelope also possesses certain Na^+/H^+ antiporters (Rolland et al., 2003) and the leaf cytosol maintains Na^+ concentration of about 1-3 mM (Karley et al., 2000) which is suitable for BicA and SbtA since they require about 1 mM Na^+ to show their half maximum activity. If at all, the naturally existing Na^+/H^+ antiporters are not able to form sufficient gradient; then incorporation of any such transporter from cyanobacteria has to be done (Price et al., 2011). It is being assumed that these transporters will make the CO_2 fixation more energy efficient as the transport by BicA and SbtA requires only 0.25 and 0.5 ATP per HCO_3^- respectively and the water loss will also be reduced by minimizing the requirement of the opening of stomata (Price et al., 2011; Price et al., 2013).

In a recent study (Pengelly et al., 2014) BicA transporter of *Synechococcus* PCC 7002 was expressed in *Nicotiana tabacum* by plastid transformation but the majority of the protein was targeted to thylakoid membranes instead of chloroplast envelope. Although there was no negative effect on plant growth and photosynthesis, an increase in CO_2 fixation was also not observed. Therefore, to achieve the appropriate targeting other options need to be explored such as nuclear transformation using specific transit peptide sequences. In nuclear transformation method, the most important aspect is to choose the correct targeting sequences. Although chloroplast targeting sequences are known to contain high level of hydroxylated serine and threonine residues, positively charged and hydrophobic amino acids with lesser content of acidic residues (Bhushan et al., 2006) but there is no conserved sequence pattern available which is specifically known to target chloroplast proteins (Bruce, 2000). The studies by Rolland et al., (2016) and Uehara et al., (2016) demonstrated the successful expression and localization of BicA and SbtA

transporters in *Nicotiana benthamiana* and *Arabidopsis thaliana* respectively by nuclear transformation method. However, they have not yet reported the effect on photosynthesis after incorporation of these transporters.

1.4 Gaps in existing research

C_3 pathway being most established and prevalent mode of performing photosynthesis in the majority of crop plants, is still not efficient enough with present environmental conditions such as low CO_2 and high temperature. The major reason behind these inefficiencies is the fickle substrate specificity and low catalytic turnover of RuBisCO. Hence, there is an urgent need to develop strategies for improvement in C_3 photosynthesis and eventually the crop yield, in order to meet the expected rise in demands of food security. Since long, constant efforts are going on for manipulating C_3 photosynthesis either by modifying the critical enzymes or by incorporating various CCMs in the C_3 plants but till date the attainment of the desired objective is a dream.

Information which is available in the literature and from previous studies suggests that the introduction of cyanobacterial bicarbonate transporters seems to be effective in improving C_3 photosynthesis which is also being supported by modelling studies. Although a few aspects of this strategy have been explored but still it happens to be challenging because it is not very much clear as to what type of targeting sequences, genetic constructs and plant transformation techniques would be most suitable. In the present study, efforts were made to establish the BicA transporter in various model C_3 plant species. The understanding of BicA transporter will serve as a baseline towards achieving the long term goal of enhanced crop productivity. Although a beginning step, it will surely provide a platform for the more challenging task of incorporating the whole CCM in C_3 plant. The objectives as mentioned below were established in order to attain the target of BicA transformation into the model C_3 plant systems.

1.5 Objectives of proposed research

Based upon the existing knowledge and gaps in research, following objectives were designed.

1. Identification of bicarbonate transporter BicA (*Synechococcus* PCC 7002) in various cyanobacterial species and mining its distant homologues in other lower organisms.

2. Design and synthesis of fusion constructs of BicA transporter

- Screening and selection of candidate proteins from *Arabidopsis thaliana* proteome which can be used as a source of targeting sequences (transit peptides).
- Preparation of chimeric genetic constructs by fusing an appropriate transit peptide upstream of the *bicA* transporter gene. Cloning of chimeric constructs in an appropriate plant expression vector containing *gus* or *mgfp5* as a reporter gene.

3. Genetic transformation of host plant for transient expression analysis

- Transformation of recombinant constructs (containing *gus* reporter gene) into *Nicotiana tabacum* through particle bombardment and *Agrobacterium* mediated co-culture method followed by analysis of transient transformants via GUS reporter gene assay.

4. Genetic transformation of host plant for transient expression and localization studies

- Transformation of recombinant constructs (containing *mgfp5* reporter gene) into *Nicotiana benthamiana* through *Agrobacterium* mediated agroinfiltration method and detection of transgene integration and expression at DNA, mRNA and protein level.
- Determing subcellular localization of GFP tagged fusion proteins using confocal microscopy.