NITROGEN FIXATION AND ITS IMPROVEMENT THROUGH GENETIC ENGINEERING

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Abstract

The process of conversion of nitrogen to a combined form is catalyzed by a complex metalloenzyme nitrogenase. It catalyzes the reduction of dinitrogen to ammonia in an ATP-dependent manner. Recently, though in limited extent, evidences have been found regarding the nitrogen fixation in non leguminous crops. The total understanding of both leguminous and non-leguminous nitrogen fixation in the crops and the cause of limited nitrogen fixation is reviewed here. The various achievements in manipulating the host plant and the microorganism to improve the yield of nitrogen fixation are also discussed in this review.
Key words: nitrogenase, nif genes, St nitrogenase, uptake hydrogenase, chloroplast, protoplast, para nodules, ureide

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INTRODUCTION

Nitrogen is an abundant element found in the atmosphere. It was discovered by Daniel Rutherford in 1772 (Cheng, 2008). Most organisms including plants cannot access the atmospheric dinitrogen for metabolic processes. Plants can utilize only the reduced forms of the nitrogen, hence, nitrogen first must be fixed and converted to a combined form (either ammonia/nitrate) and then trapped by the plants (Smil, 2001). The process of conversion of nitrogen to a combined form by prokaryotes is referred as biological nitrogen fixation (BNF). It was first discovered by Beijerinck in 1901 (Wagner, 2012). In atmosphere the amount of free nitrogen present accounts to $4x10^{21}gN$ (Schlesinger, 1991) out of which $3x10^{24}gN$ is fixed to ammonia annually. Around, $2.5x10^{11}kg$ NH3 is fixed annually by biological means which is a huge amount in comparison to the physical nitrogen fixation. Plants utilize, assimilate the produced ammonia and generate various amino acids rapidly to avoid ammonium toxicity.

BNF is carried out by a special class of prokaryotes. These prokaryotes include aquatic organisms like cyanobacteria. Most of the nitrogen fixing prokaryotes found in soil is free living in nature. They fix the nitrogen without the direct interaction with other organisms. The examples in this category include *Azotobacter, Bacillus, Clostridium* and *Klebsiella*. Free living prokaryotes often behave as anaerobes during nitrogen fixation. They contribute to a very less amount of global nitrogen fixation due to scarcity of appropriate carbon sources **(**Wagner, 2012**).**

Associative types of microorganisms remain in close association with the rhizosphere region of members of family Poaceae (Rice, wheat, corn, oats, barley etc.). These bacteria fix an appreciable amount of nitrogen. Associative nitrogen fixation can supply 20–25% of total nitrogen requirements in rice and maize (Montanez et al., 2012). The most important examples exhibiting the associative nitrogen fixation are the species of *Azospirillum* (Saikia and Jain, 2007).

Symbiosis is a naturally occurring phenomenon where the microbes and higher plants manually and beneficially contribute to the process of nitrogen fixation. Such an association requires that bacteria provide the host plant with fixed nitrogen for the growth of plant in exchange of nutrients and carbohydrates that are utilized by them to fulfill the energy requirement of the process of nitrogen fixation.

Most commonly, the symbiotic associations occur between the plants of family Leguminosae and bacteria of genera *Azorhizobium, Bradyrhizobium, Photorhizobium, Rhizobium* and *Sinorhizobium*. These bacteria are collectively referred as rhizobia. Another common type of symbiosis occurs

between water fern *Azolla* with a cyanobacterium *Anabaena azollae. Anabaena* colonizes cavities of *Azolla* fronds where the cyanobacteria fix sufficient amount of nitrogen in heterocysts, the specialized cells meant for nitrogen fixation **(**Wagner, 2012**).** The amount of nitrogen fixation by cyanobacteria is comparatively more in symbiotic state than on free living condition (Santi et al., 2013).

Another example is the symbiosis between several woody plant species such as alder trees (*Alnus sp*.) with the soil bacteria of genus *Frankia*, an actinomycete (Wagner, 2012).

Nitrogen fixation is an "old" topic in scientific terms since it is over a century ago that scientists experimentally proved that some "unique" species of plants with the help of microbes that were later found bearing nitrogenase, are capable of utilizing atmospheric nitrogen. The thought of engineering major crops to adapt such a capability, either by association/symbiosis with microbes or by introducing nitrogenase directly into the plant, was proposed several decades ago but only limited experimental approaches were carried out due to the complexity of the nature of the biological nitrogen fixation process. This review summarizes some achievements in revealing the biochemical mechanism of nitrogenase, discovery of non conventional nitrogenase in *Streptomyces thermoautotrophicus*, initial efforts of introducing nitrogenase into chloroplast and the role of protoplast in the improvement of nitrogen fixation. In addition, this review also highlights the effectiveness of para nodules in nitrogen fixation.

Nitrogenase- key enzyme in nitrogen fixation

The fixation and reduction of atmospheric nitrogen is a complex phenomenon which requires a huge amount of energy (Postgate, 1982). The nitrogen molecule consists of two nitrogen atoms joined by a triple covalent bond which renders the molecule almost chemically inert and nonreactive (Wagner, 2012). It does not combine with other elements easily. The breaking of this bond is catalyzed by the microbial enzyme nitrogenase. Nitrogenase is the complex metalloenzyme that plays a crucial role in the reduction of biological nitrogen. Four principal classes of nitrogenases have been characterized (Eady RR, 1996) out of which three have nearly similar characteristic features which differs only by the heterometal atom present in the active site metal cluster (Mo, V or Fe). The fourth class isolated from *Streptomyces thermoautotrophicus* (Hoffman et al., 2000) is a superoxide dependent nitrogenase. The Mo-dependent nitrogenases are the most important and best studied enzyme. These are most widely distributed (Burgess et al., 1996).

Mo-dependent nitrogenases consist of two component proteins called as component I and component II. These proteins are soluble in nature. Component I is also known as Mo Fe protein or dinitrogenase and component II is referred as Fe protein or dinitrogenase reductase (Burris RH., 1991). The structure of the nitrogenase has been shown in figure 1. The Mo Fe protein contains the active site for substrate reduction. It has a molecular weight of approximately 250kDa (Christiansen et al., 2001). The protein is associated with two Mo atoms, 30 non-heme Fe atoms and 32 acid labile sulfides (Rees et al., 2005). Polyacrylamide gel electrophoresis of Mo Fe protein revealed its structure which indicated that it is organized as an $\alpha2\beta2$ heterotetramer (where α=Nif D and β= Nif K protein) (Raymond et al., 2003). The Mo, Fe and S proteins of component I are organized into two unique metalloclusters called P (or [8Fe-7S]) cluster and iron-molybdenum cofactor (Fe-Mo cofactor) (Dos Santos et al., 2004). The actual reduction occurs in the Fe-Mo centre (Rees et al., 2005). The composition of Fe-Mo cofactor is MoFe7S9 homocitrate and it is present in two copies per molecule of nitrogenase. Some organisms possess non- molybdenum nitrogenase where Mo is replaced by vanadium. These so called alternative nitrogenase are found in few diazotrophs. The specificity & efficiency of Fe-Mo nitrogenase in binding nitrogenase is more than the alternative nitrogenase (Raymond et al., 2003). The auxillary P cluster mediates the transfer of electron from Fe protein to FeMo-co (Hoffmann et al., 2009).

Component II has a molecular weight of 55000-65000Da (Christiansen et al., 2001). The Fe protein is a homodimer of consisting of γ subunits (Nif H protein). It contains four non-heme Fe atoms and four acid labile sulfides referred as [4Fe-4S] cluster which is bridged between the dimer (Howard et al., 1989). Two nucleotide binding sites (one on each subunit) are also present in the Fe protein. The Fe protein delivers electrons one at a time to component I (Hoffmann et al., 2009).

A comparative account of the two components is depicted in the table below:

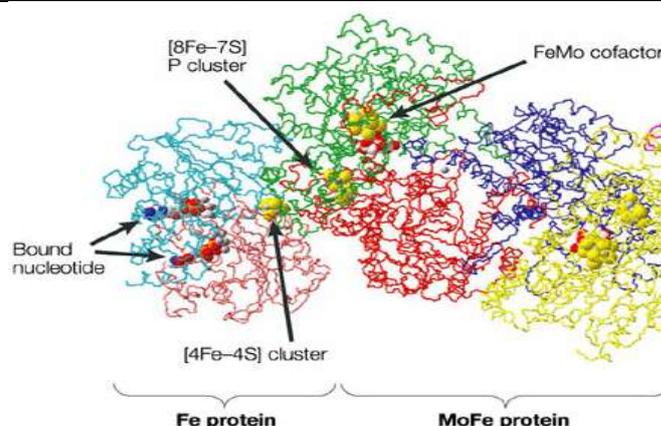| COMPOSITION	COMPONENT I	COMPONENT II
Molecular weight	200000-250000	55000-73000
Number of units	4	2
Molecular weight of subunits	595000 and 52000	Both with 27500
Fe per molecule	30-34	4
$S2^-$ per molecule	18-19	4
Mo per molecule	2	Nil
Mg^{2+}, Ca^{2+}	Present	Absent

Figure 1: Complex of the nitrogenase proteins (Fe protein and MoFe protein). The subunits of the protein dimers are coloured in cyan, brown, magenta and grey. The α subunits of the MoFe protein are coloured in green and yellow, with β subunits in red and blue. The oxygen-sensitive metalloclusters and bound nucleotides are shown in space-fill (Dixon and Kahn, 2004).

Electron flow in nitrogen fixation

The electrons for the reduction of nitrogen must be transferred to dinitrogenase reductase. Experiments conducted in vitro have shown that the electron donor transferring the electron to dinitrogenase reductase is a flavin mononucleotide containing flavoprotein which is synthesized only when nitrogenase is synthesized.

The process of nitrogen reduction is very complex. The reduction of nitrogen to ammonia is an exothermic reaction yet it requires energy in the form of ATP due to high activation energy (Brill, 1980). The schematic representation of the electron flow through various intermediates is depicted in figure 2. The Fe protein delivers one electron at a time to Mo Fe protein which requires the hydrolysis of two MgATP molecules (Seefeldt and Dean, 1997). Two MgATP molecules are bound to the reduced form of Fe protein. Conformation of dinitrogenase reductase (Fe protein) is altered upon ATP binding which lower its reduction potential and permits it to interact with dinitrogenase (Brill, 1980). In this state it forms a transient association with αβ unit of MoFe protein. Two MgATP molecules are hydrolyzed to two MgADP molecules during the association event and a single electron is transferred from the 4Fe-4S cluster of Fe protein to MoFe protein. The oxidized Fe protein ($[4Fe-4S]^{2+}$) with Mg-ADP bound to it then dissociates from the Mo Fe protein. The release of Fe protein is the rate limiting step for nitrogenase catalysis (Renner and Howard, 1996). Two or more electrons are required for the reduction of substrate by nitrogenase; hence, the cycle must be repeated several times for the reduction of substrate.

When electrons are supplied to nitrogenase they combine with H^+ and produce hydrogen. Under normal growing conditions ~50-60% of electrons are lost as hydrogen. Besides hydrogen and

nitrogen many low molecular weight compounds can also be reduced to $NH4^+$ (Brill, 1980). The overall stoichiometric reaction for nitrogen reduction can be represented by the following equation-

$$N2 + 8e^- + 16MgATP + 8H^+ \longrightarrow 2NH3 + H2 + 16\ MgADP + 16\ Pi \text{ (Saikia and Jain, 2007)}$$
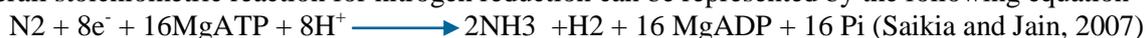
Although only six electrons are consumed in the reduction of N2 to NH3, two of these are wasted through the evolution of hydrogen which thus forms an intimate part of reaction mechanism of nitrogenase.
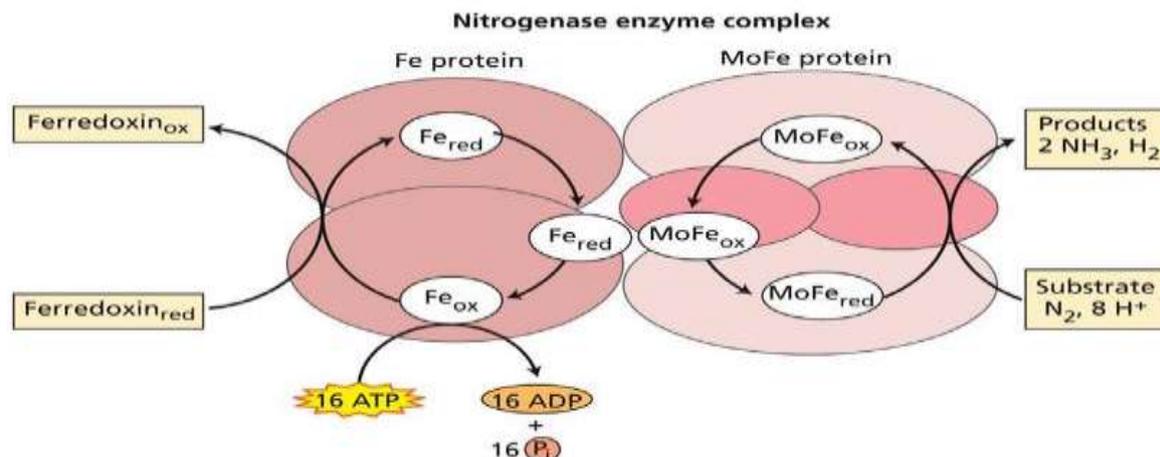

Figure 2: Schematic representation of the electron flow in nitrogen fixation (Taiz and Zeiger, 2006)

Biochemistry of nitrogen fixation in nodule

Symbiotic nitrogen fixation in legumes takes place in specialized organs called nodules that result from rhizobial infection (Krusell, 2005). As discussed earlier, the enzyme complex nitrogenase comprising the Fe and Mo subunits is responsible for the fixation of nitrogenase in root nodules. This nitrogenase exhibits similarity with the nitrogenase found in free living bacteria. The photosynthetic product such as sucrose, fructose and glucose play a part in the process of nitrogen fixation (Dommergues and Krupa, 1978) when nodules are attached to roots but these were rapidly exhausted from the root nodules which provide ATP to the nitrogen fixing system through the mechanism of oxidative phosphorylation. But the enzyme nitrogenase possesses a high degree of sensitivity towards oxygen (Downie, 2005) thus requiring an oxygen protecting system. An oxygen binding protein leghaemoglobin, LHb (red pigment of legume root nodule) acts as an oxygen buffer that cycle between oxidized Fe^{3+} and reduced Fe^{2+} and helps to maintain oxygen level sufficiently low inside the nodule (White et al., 2007). Ratio of LHb bound oxygen to the free oxygen is about 70000:1 (Downie, 2005). The vesicles, the site of nitrogen fixation in *Frankia* are surrounded by a laminated envelope composed of hopanoid lipids which provide the O2 protection to prevent nitrogenase inactivation (Harriott et al., 1991).

The nitrogen fixing microorganisms such as *Rhizobium* multiply within the plant cell and form a swollen structure called bacteroids. A portion of plant cell membrane surrounds the bacteroids to form symbiosome (Krusell, 2005). LHb lies within the membrane envelope that surrounds the bacteroids. The process of nitrogen fixation begins only after formation of symbiosome. The enzyme nitrogenase is confined to the bacteroid and is not released to cytoplasmic space. Membrane envelopes are the site of primary reaction of nitrogen fixation. Photosynthetic products translocated from the leaves to nodules are comprised of glucose, sucrose and organic compounds. Sucrose serves as the real substrate that support nitrogen fixation by bacteria. β hydroxybutyrate dehydrogenase present in bacteria helps to generate the reducing power of ATP for the support of nitrogen fixation (Trainer, 2006). Experimental work done by Keele, Hamilton and Elkan (Bergersen, 1971) gave the idea about TCA cycle in being the active pathway for pyruvate utilization. Oxidative phosphorylation is the major source of ATP for nitrogen fixation (Resendis-Antonio et al., 2007). The major organic compounds that are transported across the symbiosome membrane and into the bacteroid proper are the intermediates of TCA cycle (Lodwig, 2003) and the C4 organic acids such as succinate, malate and fumarate. These acids are used as electron donors for production of ATP and converted to pyruvate which serves as the ultimate source of electron for nitrogen reduction. The biochemical reactions occurring in the bacteroid are shown in figure 3. The first stable product in nitrogen fixation

is ammonia which accounted upto 90% of the total nitrogen fixed which is then rapidly assimilated to organic nitrogen compound. Bacteroids have the capability to assimilate a few amount of ammonia into organic compounds in the form of amino acids as the level of ammonia assimilating enzymes accounted in bacteroid is very low. Unlike bacteroid the enzymes present in plant's cell cytoplasm is quite high. This ammonia must be transported to plant cell (Franche et al., 2009) where the assimilation of ammonia is carried out by the assimilating enzymes. After the transamination reactions the products of nitrogen fixation are translocated to growing parts of the plant. The incorporation of ammonia into glutamic acid mediated by glutamate synthase (Ott et al., 2005) is the primary assimilation step.
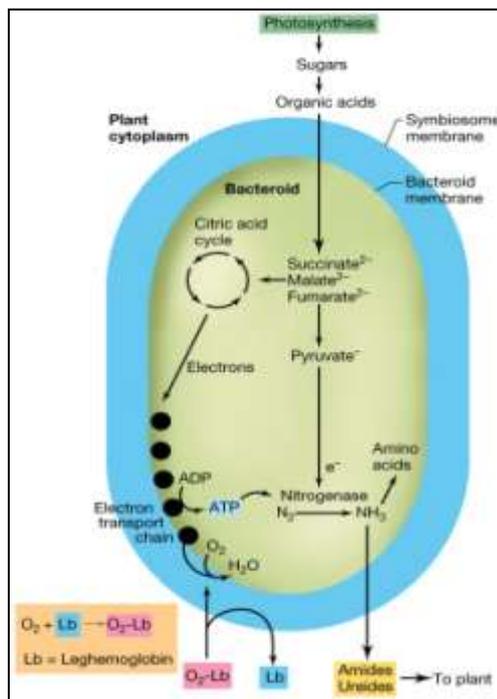

Figure 3: Schematic diagram of major metabolic reactions and nutrient exchanges occurring in bacteroid. The symbiosome is a collection of bacteroid surrounded by a single membrane originating from the plant (Madigan et al., 2000).

Genetics of nitrogen fixation

The genetics of nitrogen fixation was initially studied in *Klebsiella pneumoniae.* The genes that encode the enzymes involved in the nitrogen fixation are referred as nif genes. The primary enzyme encoded by nif genes is nitrogenase complex. Although the structure of nif gene has been studied by many workers in several microorganisms but the structural components nif gene of *K. pneumoniae* is well studied. The genes of *K. pneumoniae* are a part of a complex regulon called nif gene regulon. The nif gene of *K. pneumoniae* spans 24kb of DNA and contains about 20 genes arranged in several transcriptional units. A diverse array of structural genes and regulatory proteins are found in the nif gene regulon. Dinitrogenase is a complex protein consisting of α and β subunits. α subunit is the product of *nifD* gene while β subunit that of *nifK* gene (Beringer, 1984). The *nifH* gene encodes for the protein dinitrogenase reductase which is a protein dimer made up of two identical subunits (Roberts et al., 1978). Besides these three structural genes the complete assembly of nitrogenase requires products of nif genes that are involved in the synthesis of FeMo-co, in formation of FeS clusters and maturation of nitrogenase components. Fe-Mo cofactor is synthesized through participation of several genes including *nifN, nifV, nifB, nifQ, nifE, nifX, nifU,* nifS and *nifY*. Gene *nifS* and *nifU* play a part in the assemblage of Fe-S clusters (Hu et al., 2007). Products of *nifH, nifM, nifU* and *nifS* are required for maturation of Fe protein. *nifE* and *nifN* products have been proposed to function as scaffold for FeMo-co biosynthesis. The *nifB* gene product acts as iron and sulphur containing precursor of FeMo-co. The gene *nifQ* is a molybdenum-sulfur containing precursor of FeMo-co. The gene *nifV* encodes homocitrate synthase and is required for the synthesis of FeMo-co. The gene *nifW* is involved in stability of dinitrogenase and protect the protein from oxygen inactivation (Cheng, 2008). *Klebsiella* also contains the genes that mediate electron transport

to nitrogenase. *nifF* encodes flavodoxin that transfers electrons to nitrogenase and *nifJ* encodes pyruvate oxidoreductase that transfers electrons to flavodoxin from the pyruvate. *nif A* encodes positive regulatory protein that serves to activate transcription of other genes while *nifL* acts as repressor of nitrogenase (Beringer, 1984).

The nif cluster of *Klebsiella pneumoniae* and the roles of individual nif genes are represented in Figure.4.
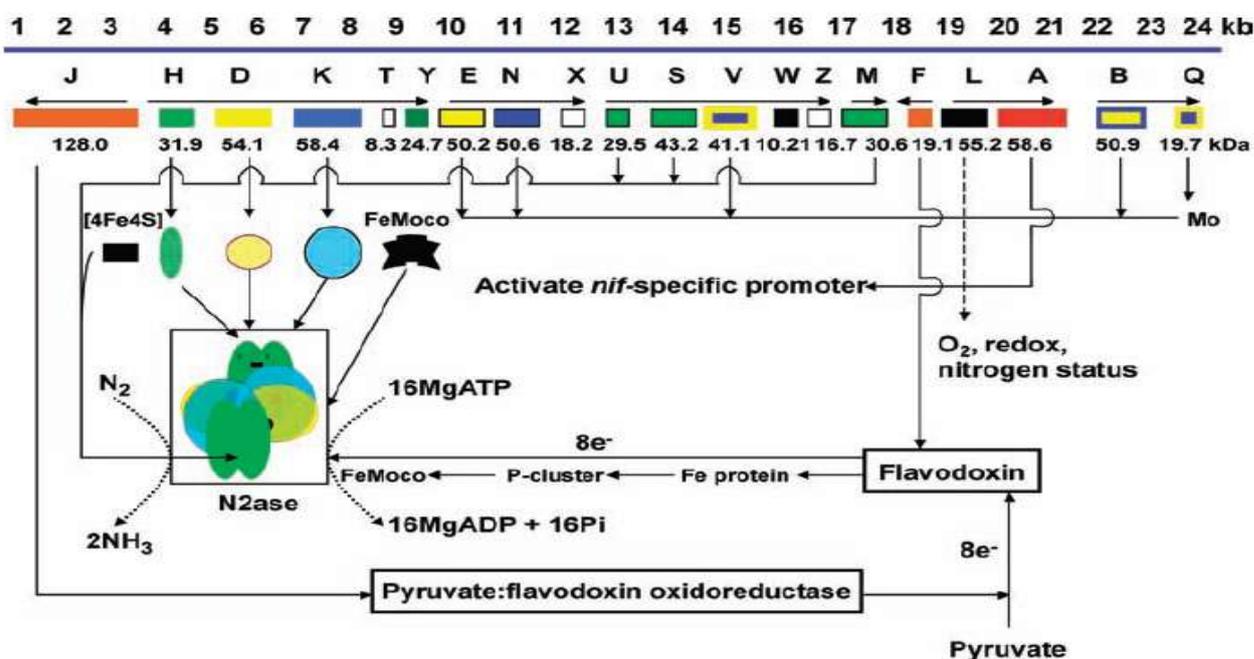

Figure 4: *Klebsiella pneumoniae nif* cluster including 20 genes (~24kb). Transcriptional orientation, protein product molecular weights and known functions are indicated (Cheng, 2008).

Genetic manipulation of nitrogen fixation

The subunits of nitrogenase from different nitrogen fixing microorganisms can be mixed to produce functional system (Emerich and Burris, 1978). Structural genes found in nitrogen fixing species are conservative in nature and the distribution results due to the transfer of genes. Genes can be manipulated to improve the fixation of dinitrogen. The microorganisms are modified in the host so that they are unable to assimilate the fixed nitrogen until the function of the nitrogenase is over and release $NH4^+$ directly to the plants (Peters et al., 1982). The nitrogen fixation can be regulated by leguminous plants by reducing the number of root nodule formation and by regulating the carbon flow to the microorganisms (Beringer, 1984). The well known nitrogen fixing bacteria *K. pneumoniae* resembles closely to non-nitrogen fixing bacterium *E.coli*. The genes of these two species could be transferred and expressed in either of the organisms. Nif- mutants of *K. pneumoniae* that are deficient in fixing nitrogen are located between genes for histidine biosynthesis (his) and shikimik acid uptake (shi A). The *his* and *nif* regions can be actively transferred from a strain of *K. pneumoniae* to an *E.coli* strain which require histidine. *E.coli* cells that do not require histidine anymore have acquired the ability to fix nitrogen. The conjugative plasmid pRDI that picked up the nif and his genes was selected and transferred to other bacterial genome (Dixon et al., 1976). The gene, *nifL*, that serves as the repressor of nitrogen fixation can be deleted thus allowing constitutive expression of nif promoter mediated by *nifA* and *ntrC* (nitrogen regulator) gene products (Beringer, 1984).

The nitrogenase activity has been observed in *E.coli* that carries the nif plasmid pRDI which was very much similar to that of *K. pneumoniae* strain. *Agrobacterium tumefaciens,* an obligate aerobe not resembling *Klebsiella* did not result in nitrogen fixing recombinants upon transfer of pRDI to itself. Mutants of *Azotobacter vinelandii* that lack either of the components of nitrogenase regained the nitrogenase activity when pRDI was transferred to them (Dixon et al., 1976). As described earlier, nitrogenase can be protected from the inhibitory action of oxygen by a protein leghaemoglobin. The

genes encoding these proteins can be isolated and transferred to other nitrogen fixing systems so as to protect the nitrogenase from oxygen activity (Beringer, 1984).

Nitrogenase activity is correlated with the hydrogenase activity that evolve hydrogen hence it requires more energy. The energy can be saved if the evolved hydrogen is further reduced to water releasing electrons. Many nitrogen fixing bacteria possess 'uptake hydrogenases' (Figure 5; Sajid et al., 1992) which consists of two subunits HupS and HupL and it is advantageous to introduce it together with the nif genes into hosts that do not possess uptake hydrogenase system.
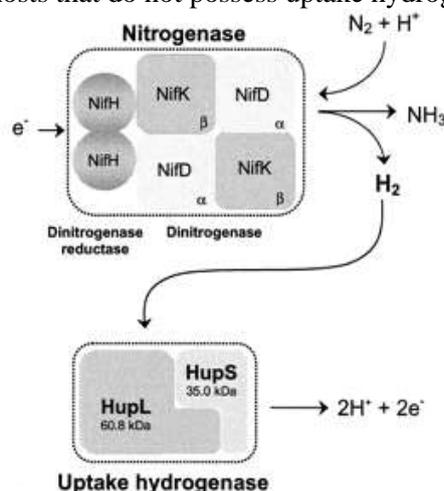

Figure 5: Schematic representation of the activity of uptake hydrogenase

Many attempts have been made to introduce the nif genes in eukaryotic cells. Unlike prokaryotes, eukaryotes have monocistronic mRNAs with a binding site at 5' end. Thus, if nif genes from prokaryotes were to be expressed in eukaryotes it is necessary to fuse the coding sequence of each genes to the promoters of eukaryotes so as to produce a suitable ribosome binding site at 5' end. The enzymes responsible for chlorophyll biosynthesis in *Clamydomonas reinhardtii* resemble structural and functional homology with nitrogenase (Figure 6). The products of *Clamydomonas reinhardtii* *chlL, N* and *B* genes are similar to the subunits of nitrogenase but the sequence identity is strongest between *nifH* and *chlL*. Hence the genes required for *chlL* protein activity can activate the *nifH* gene product without any additional requirement of genes. Furthermore *nifH* gene can substitute *chlL* in its function. The coding region of *chlL* can be replaced with *nifH* gene. By creating a petB::aadA insertional mutation and then converting it back to wild type petB, *nifH* can be introduced into the chloroplast genome. The diagrammatic representation of the process of the transfer of *nifH* gene is shown in the figure 7**.** However it is still a major challenge to introduce the nif gene as there is an uncertainty whether the physiology of plastid can face the requirements for nitrogenase.

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                                   motif A
NIFH_AZOCH      MAMRQCAIYGKGGIGKSTTTQNLVAALAEMGKKVMIVGCDPKADSTRLILHSKAQNTIME 60
NIFH_AZOVI      MAMRQCAIYGKGGIGKSTTTQNLVAALAEMGKKVMIVGCDPKADSTRLILHSKAQNTIME 59
NIFH_KLEPN      -TMRQCAIYGKGGIGKSTTTQNLVAALAEMGKKVMIVGCDPKADSTRLILHAKAQNTIME 59
CHLL_CHLRE      ---MKLAVYGKGGIGKSTTSCNISIALRKRGKKVLQIGCDPKHDSTFTLTGFLIPTIIDT 57
                  : *:***********: *:  ** : ****: :***** ***   :      . *

NIFH_AZOCH      MAAEAGTVEDLELEDVLKVGYGGVKCVESGGPEPGVGCAGRGVITAINFLEEEGAYEDDL 120
NIFH_AZOVI      MAAEAGTVEDLELEDVLKAGYGGVKCVESGGPEPGVGCAGRGVITAINFLEEEGAYEDDL 119
NIFH_KLEPN      MAAEVGSVEDLELEDVLQIGYGDVRCAESGGPEPGVGCAGRGVITAINFLEEEGAYEDDL 119
CHLL_CHLRE      LSSKDYHYEDIWPEDVIYGGYGGVDCVEAGGPPAGAGCGGYVVGETVKLLKELNAFF-EY 116
                ::::    **:  ***: ***.* *.*:*** .*.**.*  *  :::::*:* .*:  :

                                   motif B
NIFH_AZOCH      DFVFYDVLGDVVCGGFAMPIRENKAQEIYIVCSGEMMAMYAANNISKGIVKYANSGSVRL 180
NIFH_AZOVI      DFVFYDVLGDVVCGGFAMPIRENKAQEIYIVCSGEMMAMYAANNISKGIVKYANSGSVRL 179
NIFH_KLEPN      DFVFYDVLGDVVCGGFAMPIRENKAQEIYIVCSGEMMAMYAANNISKGIVKYAKSGKVRL 179
CHLL_CHLRE      DVILFDVLGDVVCGGFAAPLN--YADYCIIVTDNGFDALFAANRIAASVREKARTHPLRL 174
                *.:::***********  *:.    *:   **  .. :  *::***.*: .: :  *.:   :**

NIFH_AZOCH      GGLICNSRNTDREDELIIALAAKLGTQMIHFVPRDNVVQRAEIRRMTVIEYDPTAKQADE 240
NIFH_AZOVI      GGLICNSRNTDREDELIIALANKLGTQMIHFVPRDNVVQRAEIRRMTVIEYDPKAKQADE 239
NIFH_KLEPN      GGLICNSRQTDREDELIIALAEKLGTQMIHFVPRDNIVQRAEIRRMTVIEYDPACKQANE 239
CHLL_CHLRE      AGLIGN-RTSKRD--LIDKYVEACPMPVLEVLPLIEEIRISRVKGKTLFEMSNKNNMTSA 231
                .*** * * :.*:  **    .         ::..:*  : :: :.:: *::* .    : :.

NIFH_AZOCH      YRTLARKVVENKMLIIPNPITMDELEALLMEFGVMEEEDESIVGKAAAAEE--------- 291
NIFH_AZOVI      YRALARKVVDNKLLVIPNPITMDELEELLMEFGIMEVEDESIVGKTAEEV--------- 289
NIFH_KLEPN      YRTLAQKIVNNTMKVVPTPCTMDELESLLMEFGIMEEEDTSIIGKTAAEENAA------- 292
CHLL_CHLRE      HMDGS-KGDNSTVGVSETPSEDYICNFYLNIADQLLTEPEGVIPRELADKELFTLLSDFY 290
                :   : *  :..: :  .*      :  *   . :  *  .:: :

NIFH_AZOCH      ---
NIFH_AZOVI      ---
NIFH_KLEPN      ---
CHLL_CHLRE      LKI 293
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Figure 6: Amino acid sequence alignment between ChlL and NifH.
Similar nucleotide binding motif A (GXXXXGK^{15}S), motif B (D^{125}XXG) and conserved cysteins for liganding the (4Fe-4S) cluster (Cys^{97} and Cys^{132}) are found. The identical residues are indicated by asterisk. CHL_CHLRE, *Chlamydomonas reinhardtii* ChlL; NIFH_KLEPN, *Klebsiella pneumoniae* NifH; NIFH_AZOCH, *Azotobacter chroococcim* NifH; NIFH_AZOVI, *Azotobacter vinelandii* NifH (Cheng, 2008)
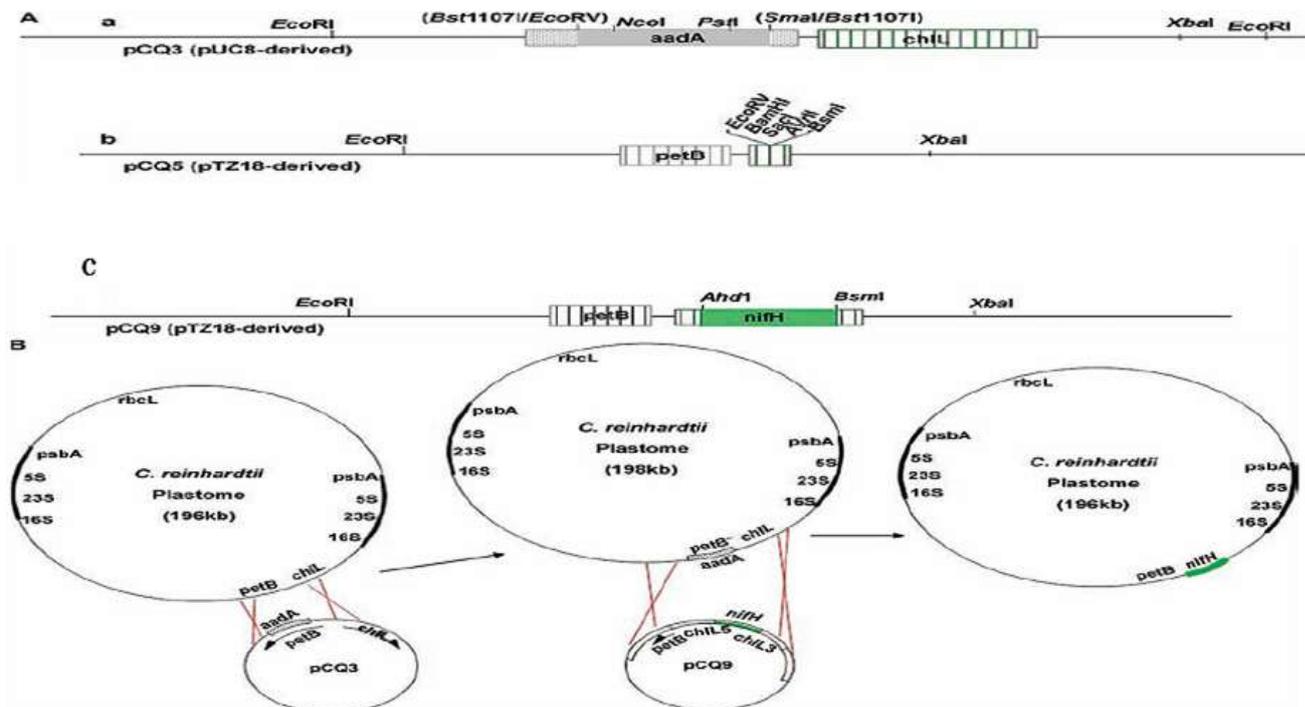
Figure 7: Schematic diagrams of constructs and two step chloroplast transformation, introduction of nitrogenase Fe protein (NifH) into chloroplast genome of *Clamydomonas reinhardtii* by replacing its native ChlL.

(A) (a) Chloroplast transformation vector pCQ3. The *aadA* cassette is inserted in the opposite direction in the *petB* coding region; (b) expression vector pCQ5, containing multiple cloning site between *chlL* 5' and 3' untranslated regulatory sequences for insertion of foreign target genes; (c) secondary chloroplast transformation vector pCQ9, containing *nifH* gene driven by *chlL* promoter.

(B) Two-step chloroplast transformation via homologous recombination by bombardment vector pCQ3 to obtain *petB* mutant which was used as a recipient for the secondary transformation by delivering vector pCQ9 bearing *nifH* gene to obtain *C. reinhardtii nifH* transplatomic line (Cheng, 2008).

Though expression of some prokaryotic genes is achieved in some eukaryotes such as in yeast *Saccharomyces cerevisiae*, the expression of nif genes has yet not been reported. The nif genes are unable to express due to several reasons. Even though the promoters could be transcribed the enzymes would not function if the physiological requirements are not met (Beringer, 1984). A plasmid PJB5JI has facilitated transfer to many *Rhizobium sp*. thus converting mutants of *Rhizobium leguminosarum* that lack the ability to fix nitrogen (Fix⁻) to Fix⁺ (Johnston et al., 1978). A conjugative plasmid possessing the genes for *Trifolium* nodulation and nitrogen fixation has been found in *R. trifolli* strain. These genes when transferred to *A. tumefaciens* can nodulate the clovers but cannot carry out the process of nitrogen fixation. (Hooykaas et al., 1981)

A non nodulating line of soybean (T201) can be nodulated by inoculation of 2,4-D. These nodules were able to fix nitrogen when infected with *Bradyrhizobium japonicum* (Akao et al., 1991).

When the gene *dnf1-1* is transferred from *M. truncatula* (with indeterminate nodules) to *Lotus japonicus* (with determinate nodules), the recipient plant became capable to produce monobacterial symbiosomes with highly differentiated bacteroids (Velde et al., 2010).

Casuarina glauca, a non legume contains a gene *Cg12*. In transgenic *Casuarina glauca* containing *Cg12* promoter–reporter gene fusions expression of *Cg12* gene starts very early when infected by *Frankia* (Svistoonoff et al., 2003). When *Cg12*-reporter gene fusions were introduced in the legume *M. truncatula*, the expression pattern was found to be similar as observed during the nodulation process with *Mesorhizobium meliloti* (Svistoonoff et al., 2004). This suggests that a signalling pathway independent of Nod factors (responsible for nodulation during rhizobial infection) is activated specifically in cells infected by symbiotic bacteria.

Cyanobacteria can be used to create new symbioses with agricultural plants. The associative competence of symbiotic *Nostoc* strains was studied in rice roots which were isolated from *Gunnera* and *Anthocero*s (Nilsson et al., 2005). When associated with rice roots, the *Nostoc* strains increased their nitrogen fixation (Nilsson et al., 2002).

Protoplast play an important role in enhancing both nodulation and production capacity of *Rhizobium sp*. When protoplasts of two weak strains of *Rhizobium* (Rt11 and Rt12) and one efficient strain (RtAI) were fused it resulted in 1.93 to 5.67 fold increase in nodulation number compared to that of parental strains (Sabir and El-Bestawy, 2009). The ability to form nitrogen fixing nodules is species dependent (Morris and Djordjevic, 2006) while the effectiveness to produce nodules is determined by host (Miller et al., 2007). Achievement of high nodulation could fail due to inappropriate strains or some environmental conditions (Mhadhbi et al., 2008). The activity of nitrate reductase is more enhanced in the plants nodulated by *Azorhizobium* than the non nodulated ones (Saikia et al., 2006). Several strategies have been made developed for the construction of improved strains of *Rhizobium* and *Bradyrhizobium* that can enhance the nodulation and nitrogen fixation (Sabir and El-Bestawy, 2009). Protoplast fusion is one of such technologies that can improve the genetic traits of Gram positive (Hotchkiss and Gabor, 1980) and Gram negative (Atallah and Abd-El-Aal, 2007) bacteria as well as nodulation efficiencies. Fusing the protoplasts of *Frankia* with the fast growing actinomycete *Streptomyces griseofuscus* has resulted in production of fast growing nitrogen fixing actinomycete (Prakash, 1988).

Several attempts have been made to introduce the nif genes to protoplasts of non-leguminous plants (Shanmugan and Valentine, 1975). Whole cells of nitrogen fixing bacterium *Azotobacter vinelandii* can be induced into plants of fungus *Rhizopogon* which is mycorrhizal in *Pinus radiata*. Small degree of fixation can occur by mycelia of the modified fungus that is associated with *P.radiata* (Pandey, 1978). The cowpea strain of *Rhizobium* produce nodules on roots of non-leguminous tree *Trema aspera* (Trinick, 1973) but these cannot fix nitrogen due to lack of leghaemoglobin (Dilworth, 1974). Rice seedlings inoculated with strains of *Burkholderia* sp. contribute high amount of nitrogen to rice through associative nitrogen fixation (Baldani et al., 2000).

Future prospects

Many methods have been proposed to nodulate the roots of cereal crops such as wheat and rice but significant nitrogen fixation has yet not been achieved. It may be due to poor internal colonization by the bacterium *Azospirilla* (New et al., 1991). Only a low proportion of *Azospirilla* can directly access to the energy substrates released by wheat roots. Only one nonlegume, the woody plant *Parasponia* sp., can be nodulated by rhizobia and utilize nitrogen fixed by the bacteria (Franche et al., 2009). More research is required to explain the method of lateral root development by rhizobia. This could be a clue to initiate the formation of a nodule primordium in a non-legume crop (Santi et al., 2013). By the treatment of enzymes 10% of rice seedlings develop nodules. Non-legumes can be nodulated by enzymatic treatment coupled with polyethylene glycol treatment. The strain of *Rhizobium* that was able to nodulate the non-legume *Parasponia* when inserted into the rice did not mark any sign of nodulation or nitrogen fixation (Kennedy and Tchan, 1992).

When 2,4-D was induced on wheat plant some lateral roots became modified that resembles the nodules of legumes. These are referred as para nodules (Figure 8; Kennedy and Islam, 2001). The para nodules emerged when the legumes are inoculated with *Azospirillum* along with the application of 2,4-D (Saikia et al., 2004). The para nodules of wheat have high level of C_2H_2 reduction but it is still to be discovered whether the C_2H_2 reduction is restricted to *Azospirilla.* In rice, 1 in 400-1000 rice plants are able to produce nodules. Rice can be colonized and nodulated by *Azospirillum caulinodans* but in neither case nitrogenase activity is found in nodules (Kennedy and Tchan, 1992). Though 3% of nitrogen fixation occurs but it is reported in free living state without the bacteroid differentiation (Kitts and Ludwig, 1994). Wheat para nodules to a little extent are colonized by *Nostoc* sp. strain 2S9B which provides favourable condition for nitrogen fixation (Saikia and Jain, 2007).

To what extent para nodules are effective in fixing nitrogen is still to be evaluated. Further progress is based on genetic manipulation of either microsymbiont, host plant or both. Though the rhizobia are able to colonize the roots of non legumes the basis of association between then is yet not

known. More research works must be done to extend the host range of rhizobia to non legumes. Suitable measures must be taken to allow expression of nitrogenase activity in non-leguminous crops.

Figure 8: Scanning electron micrograph showing a para-nodule of wheat inoculated with *Rhizobium leguminosarum*

In tribe Phaseoleae the restriction in efficient symbiotic nitrogen fixation is overcome by incorporation of products of nitrogen fixation into ureides (Provorov, 2013). Ureide assimilation is superior to amide assimilation because of two important advantages: (a) the N/C ratio in ureides is greater than in amides, which reduces carbon expenditures for nitrogen transport into aboveground organs; (b) the synthesis of ureides reduces accumulation of glutamine and asparagine in the roots and nodules that can suppress symbiotic nitrogen fixation (Romanov et al., 1987). The process of nitrogen fixation in which the bacteroids are specialized to assimilate the nitrogenous products into ureides will make it possible to produce new forms of plants highly efficient in symbiotic nitrogen fixation. The method of incorporation of uricase into the amide legume species will be a promising approach.

The fourth class of nitrogenase isolated from *Streptomyces thermoautotrophicus* unlike other nitrogenases is completely insensitive to oxygen. The dinitrogenase component of this class of nitrogenase is called St1 which is a heterotrimer and contains molybdenum and that of dinitrogenase reductase is a homodimeric manganese-containing superoxide oxidoreductase, referred as St2 (Madigan et al., 2000). Besides these two components it also contains an additional component known as carbon monoxide dehydrogenase (St3) which is also a molybdenum-containing heterotrimer. The St nitrogenase requires less energy in comparison to conventional type of nitrogenase. The schematic representation of nitrogen fixation in *S. thermoautotrophicus* is depicted in Figure 9.

Figure 9: Schematic representation of nitrogen fixation in *Streptomyces thermoautotrophicus*

Superoxide is produced by CO dehydrogenase through the oxidation of CO and the transfer of the electrons to O2. Subsequently, the superoxide is reoxidized by a superoxide oxidoreductase that

delivers the electrons to a dinitrogenase. The dinitrogenase is capable of reducing N2 and H^+, but not ethane (Ribbe et al., 1997; Cheng 2008).

Recently it has been shown to fix nitrogen but whether its primary function in the cell is to fix nitrogen is yet unknown. The discovery of the *Streptomyces thermoautotrophicus* nitrogenase system has renewed our hopes for the eventual genetic engineering of a nitrogenase system and this could turn the dream of nitrogen fixing row crops into a reality one day.

CONCLUSION

Recent advances in the understanding of endosymbiotic and endophytic nitrogen fixation with non-legume plants may represent original and alternative new avenues for engineering non-legume nitrogen-fixing crops. Understanding the molecular mechanism of BNF outside the legume-rhizobium symbiosis could have important agronomic implications and enable the use of N-fertilizers to be reduced or even avoided. Indeed, in the short term, improved understanding could lead to more sustainable exploitation of the biodiversity of nitrogen-fixing organisms and, in the longer term, to the transfer of endosymbiotic nitrogen-fixation capacities to major non-legume crops. The task of engineering the non legumes is a complex method but a concerted effort is required to fill the gaps of our knowledge.

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