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Prospects of Nitrogen Fixation in Rice

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Abstract: Global agriculture relies heavily on fertilizers which are ecologically as well as economically expensive. Nitrogen which is undoubtedly the most important nutrient input required for rice production, is most frequently also a limiting factor. In a world facing acute energy crisis at global level and unpredictable spurs in world crude oil process due to political turmoil's and lack of dependable alternative energy resources, it is imperative to develop the system of rice production which, without compromising on yield out-put, lowers dependability on chemical N-fertilizers besides being ecologically compatible. Nitrogen fixation in rice seems to be an efficient prospective system that is compatible with principles of resource conservation and ecological security. The dream project of BNF rice was started in 1992 based on expert recommendations which involve improving endophytic associations between rice and N_2 fixing bacteria, engineering of rice plants capable of forming legume like symbiosis and nodules with rhizobia, transforming rice to ensure expression of nitrogenase and protect nitrogenase system from oxygen damage and enhancing N-use efficiency of rice. A large number of diazotropic microorganisms have been found to be associated with rice roots. Among these *endophytic diazotropes*, *Alcaligenes*, *Azoarcus*, *Serratia marcesscens* and *Azorhizobium caulinodons* have received major attention. Though most of the aspects of rice-diazotroph interaction and nitrogen fixation have been elucidated both at genetic as well as molecular level, the engineering of an autonomous nitrogen fixing rice plants is undoubtedly a long term endeavor. A large number of endophytic diazotrophs have been found to be associated with rice and factors encouraging bacterial colonization have been characterized but certain critical differences in rice-rhizobial interaction relative to root nodule symbiosis in legumes have to become a reality. It will require a series of genetic manipulation of nodulation genes from plants and *nif* genes from bacteria to realize the dream of developing a biologically nitrogen fixing rice.

Key words: Rice, nitrogen fixation, *nod*, *nif*, *fix*

INTRODUCTION

Nitrogen which is undoubtedly the most important nutrient input required for rice production, is most frequently also a limiting factor. Global agriculture relies heavily on fertilizers which are ecologically as well as economically expensive. Currently Nitrogen is being manufactured by Haber-bosch process (Burgess and Newton, 1977) which is largely an energy dependent process. Petroleum is the major energy source for fertilizer sector and thus fertilizers are vulnerable to political uncertainties and economic fluctuations in global petroleum market (Ladha and Reddy, 2000). It has been estimated that fertilizer production for global needs may require fossil fuel energy equivalent to 100 million tones per year. Due to its finite reserves and being a non-renewable energy resource, sustaining current level of fertilizer production will not be practically feasible on long term basis.

Currently the rice production globally consumes about 10 million tones of nitrogenous fertilizers. In order to boost up rice production to about 13 t na^{-1}, fertilizer inputs will have to be appropriately enhanced, from current level of 220 kg ha^{-1} to 400 kg ha^{-1} (assuming 50% fertilizer use efficiency). The super rice series cultivars developed by IRRI require higher fertilizer requirements in order to realize their optimum genetic yield potential.

In a world facing acute energy crisis at global level and unpredictable spurs in world crude oil prices due to political turmoil's and lack of dependable alternative energy resources, it is imperative to develop the system of rice production which, without compromising on yield out-put, lowers dependability on chemical N-fertilizers besides being ecologically compatible. Britto and Kronzuiker (2004) proposed four areas of emphasis in this regard.

- Nitrogen fixation in rice.
- Primary nitrogen acquisition.
- Manipulation of nitrogen metabolism.
- Interaction of Nitrogen and photosynthesis.

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Among these four areas, nitrogen fixation in rice seems to be an efficient prospective system that is compatible with principles of resource conservation and ecological security. Nitrogen, even though present as a major component of atmosphere, is however used mainly by lower organisms, such as bacteria, algae etc. these organisms are called as N-eaters or Diazotropes. Among plants, some 12000 have ability to use nitrogen by virtue of symbiotic relationship with such diazotropes. In such a symbiotic association, the soil bacteria (*Rhizobium, Bradyrhizobium* and *Azorhizobium*) infect the root hairs or emerging lateral or adventitious roots and induce formation of morphologically defined structures called as nodules. The nodulation occurs mostly in case of plants belonging to Leguminoseae. The only non-leguminous known to naturally form modules with either *Rhizobium* or *Bradyrhizobium* belongs to Genus Parasponia. (Cocking *et al*., 1996). The bacterial colonies infect wounded root tips. The final structure from such infections is a root swollen on either side of central vascular tissue by cortical cells filled with infection threads containing bacteria actively engaged in nitrogen fixation. One of important studies have revealed that some of Rhizobia strains are capable of nodulating both legumes and Parasponia

Genetic basis of rhizobium-legume interaction: The induction of nodules harbouring nitrogen-fixing bacteria is result of complex interaction between *Rhizobia* and plant. It involves several sets of genes from both partners in a coordinated expression. The plant genes involved in different aspects of symbiosis are called as Nodulin genes. Nodulin genes are of two types; C-Nodulins which are common to all legumes and S-Nodulins, present in specific species (Lodha and Nainawatie, 1993). These nodulin genes are either constitutive or inducible or repressible. They are numbered in order of size. N-1 being the largest and N-30 (globin) the smallest one.

Almost 30 Nodulin genes have been identified, leghaemoglobin being the most well characterized. Nodulin-35 of Soybean has a uricase activity. A root nodulin of *Phaseolus vulgaris* is glutamine synthetase. The nodulin from *Dolichos biflors*, apyrase (*Nucleotide phosphohydrolase*) was isolated by Etzler *et al.* (1999). A similar ortholog of such apyrase (Gs-50) was isolated from *Glycine soja* by Stacey *et al.* (1999). Nodulin genes affect number and size of nodules, nodule morphogenesis and rate of nitrogen fixation activity. A detailed analysis of nucleotide sequence, mechanism of regulation and function of all nodulin genes is essential for genetic manipulation of biological nitrogen fixation.

A number of bacterial gene sets which control the events leading to nodule formation are collectively called as *Sym* genes, which consist of *nod*, *nif* and *fix* genes. In fast growing strains of rhizobia, *sym* genes are present on a large plasmid whereas in slow growing strains they are present on bacterial chromosome.

The nod genes are bacterial genes involved in nodule formation. Based on function various nod genes are:-

Gene	Function
roc	Root colonization
roa	Root adhesion
hab	Hair branching
had	Hair deformation
hac	Hair curling
hsn	Host specificity
inf	Infection
noi	Nodule initiation
inb	Infection thread branching
bar	Bacterial release
bad	Bacterial development

The *nod* genes are also classified on the basis of complementation or non-complementation by their heterologous strains. The *nod* A, B and C belong to first category and *nod* D belongs to latter category. The *nod* D is a regulatory gene which activates other *nod* genes. A number of such gene sets have been isolated and characterized. One such gene cluster is *nod* ABCD (Kondorsi, 1984) which controls general nodulation function, such as root hair curling and induction of meristematic cell division (Jacobs *et al*., 1985). Another set of genes *nod* EFGH also called as *hsn* ABCD conditions the host specificity of nodulation (Debelle and Sharma, 1986; Fisher *et al*., 1987). A number of other *nod* genes have been identified, some of which act in a manner similar to *hsn* ABCD genes (Renalier *et al*., 1987).

Apart from *nod* D which is expressed constitutively, other nod genes are induced by plant signals (Mulligan and Long, 1985). The analysis of root exudates revealed that certain flavones are potential *nod* inducers. In *R. trifolii* and *R. melilotus*, luteolin has been found to be major inducer (Peters *et al*., 1986). However, in addition to such inducers, product of nod D is also essential for their expression. Gettfert *et al*. (1986) found three different versions of *nod* D viz., D_1, D_2 and D_3 which regulate host-specific *nod* gene expression, by corresponding to specific root exudates (Gyorgypal *et al*. 1988). Thus it can be considered that D_1, D_2 and D_3 are allelic forms of *nod* D.

Another set of *Sym* genes called as *nif* genes one highly conserved in various diazotrophs. Various types of

Table 1: Different *nif* genes isolated and characterized

Gene	Structure	Function
nif H0	Fe-portion submit	FeMo biosynthesis
nif D	Mo-Fe α-submit	Cofactor of holoprotein α 2 β_2 tetramer
nif K	Mo-Fe β-submit	Mofe cofactor biosyntheis
nif B	---	FeMo cofactor biosynthesis
nif E	---	FeMo cofactor biosynthesis. Forms α2β2 tetramer with *nif* N gene product
nif N	Fe Mo protein	Processing of Mo.
nif V	Fe MO protein	Encodes a homocitrate.
nif A	---	Positively regulates the activity of *nif* transcription
nif L	---	Negatively regulates the activity of *nif* transcription.
nif F	Flavodoxin	Phsiological reductant of fe-protein.
nif J	Pyruvate flavodoxin oxidoreductase	Reductio of flavodoxin
nif M	---	Activity of Fe-protein
nif S	---	Processing of MoFe protein
nif U	---	--do--
nif Y	---	Processing of MoFe protein but not required for diazotropic growth.
nif T	Unknown	Not required for diazotropic growth
nif W	--do--	Required for full activity of MoFe protein.
nif Z	--do--	--do--
nif X	--do--	Involved in FeMo cofactor biosynthesis.

nif genes are *nif* H, D and K, which code for nitrogenase polypeptides. The use of *nif* DHK probe of *Klebsiella pneumonia* and DNA hybridization studies have revealed that *nif* genes of *Rhizobium* and *Bradyrhizobium* have strong homology with *Klebsiella*. Despite such homology, there are certain differences in structural organization of *nif* genes in bacterial genome. In case of *R. melilotus* and *R. leguminoserum*, they are a part of a single operon while as in case of *B. japonicum* and *R. phaseoli* they reside on different operons. The regulatory mechanism of *nif* gene expression has been extensively studied in *R. melitotus*. The expression of *nif* A gene is regulated by *ntr* gene system (Ow *et al*., 1985) in case of *Klebiella* but in *Rhizobium ntr* has no role. The most important function of *nif* gene is activation of another gene set called as *fix* genes. An important aspect of *nif* genes which is of paramount significance is that *nif* gene transfer between bacteria has been observed in direct cell contact. The localization and characterization of *nif* genes on plasmids offers opportunity of transferring such genes to plants in near future. A number of *nif* genes have been identified and characterized structurally as well as functionally. Table 1 shows various such nif genes and their structural and functional details (Dean and Jacbs, 1992; Galton and Smith, 1993).

The other class of *sym* genes which govern the ability of rhizobia to *fix* nitrogen are called as *fix* genes. The activity of *fix* genes is regulated by *nif* genes. Various *fix* genes such as *fix* A, B and C have been characterized. Different *fix* genes share stringer homology in *rhizobia* than *Bradyrhizobium*. Even their genetic organization determine the late nodule developmental activities (Kondorosi *et al*., 1989), by expression of late nodulin genes. Verma *et al*. (1988) proposed that there may be several such late nodulin genes which may require different bacterial signals for expression. In an earlier study, it has been found that *fix* genes may also control the expression of various *nif* genes (Putnoky *et al*., 1988) using several *fix* mutants, (Putnoky *et al*., 1988) found that *fix* mutants induced nodules but do not express *nif* structural genes probably by impairing expression of *nif* A regulatory gene.

Biologically nitrogen fixing rice (bnf-rice): Plants take nitrogen mostly in form of nitrate and ammonia. By far, no green plant has been found to be capable of obtaining diatomic nitrogen directly from atmosphere. Thus biological nitrogen fixing rice is a non-natural existence. But, if brought into existence by genetic manipulations, it would amplify the potential for nitrogen supply to rice plant as the fixed nitrogen would be available to plant directly with no loss (Ladha and Reddy, 2000). However, things are always easier said than done. It will require assembly of complex enzyme and provision of appropriate physiological conditions in the absence of environment normally provided by a prokaryotic cell (Dixon *et al*., 2000). Thus putting all the essential units of such a complex biochemical system in appropriate order is both a challenge as well as opportunity to develop rice with inherent capacity to fix atmospheric nitrogen. This is all the more important because it has been estimated that if only half of N_2 applied to lowland rice could be obtained from biologically fixed nitrogen, it would save about 7.6 million tones of oil annually. Ladha *et al*. (1997) proposed two possible approaches to this end.

- Genetic manipulation of nitrogen fixing (*nif*) genes.
- Genetic manipulation of nodulation (*nod*) genes.

Table 2: List of institutions and their research priorities for collaborative efforts on BNF rice (Ladha and Reddy, 2000)

Country	Institution	Research Priority
Australia	Australian National University	Endophyte/ rhizobial interaction
	University of Sydney	Association diazotroph interaction
Belgium	Katholeik Universtiet Leuven	--do--
Brazil	EMBARAPA	Endophyte interaction.
China	Chinese Academy of Agr. Sciences.	Endophyte/Rhizobial interaction.
	University of Shandong	--do--
Egypt	Sakha Agricultural Research Station	Rhizobial interaction.
Germany	Max-Plant Institute	Endophyte interaction.
India	Banaras Hindu University	--do--
Japan	National Institute of Agrobiological resources.	Rice *ENOD* homologues, Chitin recognition and reference response.
Pakistan	National Institute for Biotechnology and Genetic Engineering	Endophyte interaction
Phillippines	IRRI	E/R interaction, legume *ENOD* gene expression in rice, rice *ENOD* homologues. Endophyte interaction.
	University of Philippines	
Switzerland	ETH	*ENOD* gene expression in rice.
UK	John Innes center	Expression of *nif* genes in rice.
	University of Dundee	E/R interaction
	University of Nottingham	Rhizobial interactions
USA	Michigan State University	E/R interaction
	University of Arizona	E/R interaction
	UCD University of Tennessee	Legume *ENOD* gene expression inrice.

The dream project of BNF rice was started in 1992 based on expert recommendations at the workshop on the feasibility of nitrogen-fixing capability in rice organized by IRRI. Four major approaches recommended at workshop were:-

- Improving endophytic associations between rice and N_2 fixing bacteria.
- Engineering of rice plants capable of forming legume like symbiosis and nodules with rhizobia.
- Transforming rice to ensure expression of nitrogenase and protect nitrogenase system from oxygen damage.
- Enhancing N-use efficiency of rice.

A number of institutions are working independently or in a collaborative framework to achieve the goal of identifying and improving endophytic diazotrophic associations in rice and determining the predisposition of rice for developing an endosymbiotic relationship with rhizobia (Table 2).

Identification of endophyte diazotrops in association with rice: Plant root system offers an excellent micro habitat for bacterial growth. A number of endophytic diazotrops have been found to be associated with different crops such as sugarcane (*Acetobacter diazotrophicus* and *Herbaspirillum*) and Kallar grass (*Azoarcus*). Other studies revealed that a large number of N_2-fixing bacteria were present in root interiors of certain grasses (Remhold-Hurcek and Fendrik, 1986; Olivares *et al*., 1996). These studied encouraged the research community looking for development of efficient rice-endophyte system for conferring in plant ability to fix atmosphere nitrogen. Initially the techniques for accurate enumeration and isolation of putative endophytes were standardized (Stoltzfus *et al*., 1997). This was followed by isolation and characterization, by PCR based techniques, of various prospective endophytes from various wild as well as cultivated rice cultivars (Barraquo *et al*., 1997). Various prospective diazotrophic endophytes isolated from rice are:

Species	Reference
Agrobacterium. tumifaciens	Bennet and Ladha (1992)
Alcaligene faccalis	-do-
Azospirillum caulinodans	Ladha *et al.* (1997)
Azospirillum lipoferum	-do-
Enterobacter coloacae	-do-
Klebsiela oxytoca	-do-
K. Planticola	-do-
Pseudomonas sp.	-do-
Azoarcus sp.	Hurek *et al.* (1994)
Rhizobium leguminoserum	Yanni *et al.* (1997)
Herbaspirillum seopedicae	Euan *et al.* (2000)
Sphingomonas paucimbolis	Hurek *et al.* (2000)
Burkholderia sp.	-do-
Psuedomonas stutzeri A15	Euan *et al.* (2000)
Serratia marcesscens	-do-

Among these endophytic diazotropes, *Alcaligenes, Azoarcus, Serratia marcesscens* and *Azorhizobium caulinodons* have received major attention. They are aggressive colonizers. *Alcaligenes* are seed-borne endophytes, whereas *Serratia* appears to infect rice grown in soil. *Azoarcus* is a promising prospect because they have been found to be capable to nitrogenase gene expression in gnotobiotic culture inside rice. In this regard *Azoarcus* sp. BH72 which is an endophyte of Kaller grass (*Leptochloa fusca*) has been studied as a model

endophyte because of its ability to invade rice. Strong evidence of presence of microcolonies of *Azoarcus* containing *nif* H expressing bacteria was reported in *japonica* cultivar *Nipponbare*. The bacteria colonized *Aerenchyma* tissue of rice roots, with strong evidence of nitrogenase gene expression using a reporter strain of *Azoarcus* carrying *nif* H: *gfp* fusion. An important finding was that bacteria were located between cell walls and not inside living cells (*Apoplastic expression*). Euen *et al.* (2000) also reported *Azoarcus* in root apoplasts but did not find any symbiotic organs. In modern cultivars; however *Klebsiella*, *Sphigomonas*, *Azorhizobium* and *Burkholderia* predominated.

Another putative endophyte which holds good promise is *Serratia macrcesscens*. At IRRI various modern varieties were screened and about six different isolates of *Serratia* were identifies (Hurek *et al.*, 2000). However no N_2-fixing capability in terms of Acetylene reduction assay was detected. An advantage of using *Serratia marcesscens* is that it can be a potential bio-control agent owing to its chitinase activity (Mc Inroy and Kloepper, 1995) and can also induce systemic resistance in plants (Press *et al.*, 1997).

It has been found that *Accrenchyma* of rice roots may act as a suitable microenvironment for *diazotropic endophytes* for introgenase gene expression. The collaborative studies have provided evidences of a large number of diazotrophic and non-diazotrophic bacteria which are capable of colonizing rice seedlings after germination. Thus presently we have a workable pool of bacteria which can be further studied in terms of endophytic interactions with rice which is essential for beneficial rice-endophyte association for in plant N_2-fixing capability in rice. In this regard IRRI and collaborating centers are pursuing following research initiatives to explore and evolve strategies for identification and improving rice-endophytied diazotroph association.

- Identification of specific and predominant diazotroph form rice.
- Determination of mode of invasion and extent of colonization.
- Assessment of contribution of diazotrophs to rice growth and yield through nitrogen fixation.

Improving genetic predisposition of rice for symbiotic nitrogen fixation: In symbiotic association between legume and soil bacteria, the rhizobia infect plants via root hairs or cracks caused by emerging lateral roots. This is followed by formation of morphologically defined structures called nodules. In these nodules the bacteria fix nitrogen which becomes directly available to plants. In our endeavor to produce BNF rice, we have to ensure that the diazotropic endophytes develop such association with rice plant where by they can develop reports defined structures. There have been many reports about such nodule like structures at a low frequency (0.1-0.2%) upon inoculation of rhizobia to normal roots (Bender *et al.*, 1990; Jing *et al.*, 1992; Li *et al.*, 1991; De Bruijin *et al.*, 1995) or enzyme treated roots in presence of PEG and $CaCl_2$ (Al-Mallah *et al.*, 1989). Reddy *et al.* (1997) studied the interaction between various rhizobial strains and rice and found that:-

- Root exudates of certain rice cultivars do not activate nodulation genes such as *nod* SU of *Rhizobium* strain NGR 234, *nod* A and R of *R leguminoserum* and *nod* Y of *Bradyrhizobium japonicum*.
- Neither wild type rhiizobia nor purified Chitolooligoaccharides (CLOS) Nod factor elicit root deformation.
- Rhizobia produced Indole-3-acetic acid but neither CLOS nor Trans-zeatin Nod factor promoted formation of thick lateral roots.
- Rhizobia neither promoted symbiont specific pattern of hair attachment nor extensive cellulose microfibril production.
- Rhizobia primarily got entry by cracks of epidermal cells or fissures caused by developing lateral roots.
- Infection by rhizobia is *nod*-gene independent, non-specific and does not involve formation of infection threads.
- Endophytic colonization is restricted to intercellular spaces only.
- Cortical sclerenchymatons cells appear to be primary barriers to infection.

Cocking *et al.* (1996) inoculated rice seedlings treated with enzyme cellulase and pectolyase (which dissolve cell wall) with *Azorhizobium caulinodans* (ORS571) and both the varieties Lemont and IR42 developed N_2-fixing activity as evidenced by acetylene reduction assay. Reddy *et al.* (1998) stated that predisposition of rice is essential for symbiosis. They found that like legumes, certain exudates from rice roots induce transcription of *nod* genes of *Rhizobium* sp. NGR 234 but neither observed any root deformation not true nodule formation. they also reported that certain genes in rice c DNA were similar to legume Early Nodulation genes (*ENOD* 93 and *ENOD* 40) and are important for early developments for nodule formation. The legume *ENOD* 12 gene can be expressed in response to rhizobial Nod factors in rice (Reddy *et al.*, 1998). Rice genome was found to posses varied levels of homology to *ENOD* 40 (Kouchi *et al.*, 1999). Recent results with

ENOD 12 transgenic plants have shown that rice not only possesses the mechanism to recognize rhizobial Nod signals but also the signal transduction chain to activate *ENOD* gene transcription and more so the expression is restricted to vascular bundles as in legumes. Thus it can be assumed that *ENOD*-40 genes in both legumes and rice have a similar regularly mechanism.

An important finding in rhizobia-rice interaction was identification of the specific flavonoi compound. Ladha *et al.* (2003) found that Naringenin (A flavone) acts as a signal molecule for colonization of rice by *Azorhizobium caulinodans* ORS571. Similarly an apyrase (*Nucleotide phosphohydrolase*) was isolated from *Dolichos bifloris* and has the ability to bind to rhizobial Nod signals (Etzler *et al.*, 1999). Another ortholog of apyrase (GS50) was isolated from soybean (Stacey *et al.*, 1999). These proteins can be prospective receptors of Nod signals. In fact Dey *et al.* (2002) introduced a plasmid containing GS50 apyrase driven by CaMV35S promoter into rice cells by biolistic method of Taipei-309 which stably integrated and expressed this gene.

Rice as such does not enter into symbiotic association with rhizobia but is able to interact symbiotically with certain mycrorhizal fungi (Khan and Belik, 1995). Two important evidences have been gathered to safely conclude that rice can be engineered to trigger rhizobial nodulation. One of the evidence was provided by Albrecht *et al.* (1998), who found genetic links between processes of nodule formation and arbuscular mycorrhizae. Another study with nodulation mutants of pea revealed that the early nodulation genes in pea (*ENOD* 2, *ENOD* 11, *ENOD* 12 and *ENOD* 40) controlling early events of nodule development, also govern the early events of mycorrinzal development (Van Rhijn *et al.*, 1997). Now since rice is able to associate symbiotically with mycorrhizae and in both cases *ENOD* genes play a key role, it can be inferred that at least some, if not all, genetic mechanisms do exist and function in rice which are instrumental in initiating nodule development, but rice is unlikely to possess all such genetic framework. It is now our endeavour to gather the missing links and complement the existing genetic factors, so that rice acquires such unique traits that are necessary for rhizobial nodulation. This is an essential consideration while working for developing rice cultivars with in plant capacity of N_2-fixation in association with endophytic diazotrophs.

Engineering rice for inherent n_2-fixing capacity: Engineering rice to develop nitrogen fixing capacity will be one of the most crucial contributions of biotechnology to agriculture. The advances in transformation of rice, characterization of various genes from both plant (nodulin genes) and diazotropic endophyte (*nod*, *nif* and *fix* genes), identification of various rice specific promoters that can efficiently drive the foreign genes have made the dream, of having a rice plant with inherent ability to fix atmosphere nitrogen, not only reasonable but also realizable. Parakaran (1997) proposed two approaches for engineering rice plant.

- Transformation of rice leaf
- Transformation of rice roots.

Transformation of rice leaf: The transfer of nitrogen fixing (*nif*) genes from *Klebsiella pneumoniae* to *Escherichia coli* by conjugation and localization of *nif* genes on bacterial plasmids raised hopes of transferring them to eukoarytoes as well (Schlegal, 1993). However, there are certain genetic and physiological considerations which have, most obviously, put breaks on progress in engineering rice plant as one would expect given the rapid advances that have been made in efficient transformation of rice. These considerations are :

- Engineering of rice plant capable of N_2-fixing requires coordinated and regulated expression of almost 16 *nif* genes; 8 core *nif* genes (B, E, D, H, M,N,K,V) and 8 house keeping *nif*-genes (A, S,T, Q, U,Q,W, X,Y,Z) assembled in an appropriate cellular location (Dixon *et al.*, 1997).
- Additional genes to keep nitrogenase in an active form may also be needed.
- To optimize the expression of all 16 *nif* genes appropriate promoters will need to be put in place to drive these genes in eukaryotic genetic background as they are expressed normally in a prokaryotic system.
- Even though mitochondria would ideally offer an energy rich location for *nif* gene localization but targeting all 16 *nif* genes in this organelle is practically impossible.
- Nitrogenase is very sensitive to oxygen thus appropriate mechanisms for protection of this vital enzyme have to be developed well within eukaryotic cell structure.

These considerations have been thrust areas of research towards constructing a BNF rice. Dixon *et al.* (1997) proposed that plastids can be a suitable location for targeting *nif* genes because chloroplast genes are expressed in a prokaryotic like fashion and polycistronic m-RNA's are translated. The cluster of *nif* genes may be introduced simultaneously because now techniques are

available where many genes can be transferred into rice (Kohli *et al*., 1998; Agarwal *et al*., 2001). The process of photosynthesis and nitrogen fixation can be made to co-exist provided a mechanism to protect nitrogenase from oxygen damage is put in place. A possible mechanism can be temporal separation of oxygen-evolving process (photosynthesis) and oxygen-sensitive process (Nitrogen fixation) by restricting nitrogenase synthesis to dark period and supported by ATP and reductant to be provided by breakdown of endogenous glucan. The light period ensures photosynthesis and replenishment of glucan, (Dixon *et al*., 2000). However, the ATP and reductant available during dark period in plastids may not suffice the requirements of nitrogen fixation process. Besides, there is no efficient system of O_2 removal for protection of nitrogenase. A proposed mechanism of removal of oxygen is that nitrogenase itself could help remove oxygen. The nitrogenase Fe-protein would be oxidised without loss of activity (Thornely and Ashlay, 1989). However, this leads to problem of cleavage of a major by product i.e., hydrogen peroxide which would need another set of enzymes to be put in system in chloroplasts such as ascorbate peroxidase, monodehydro abcorbate reductase and dehydroascorbate reductase. Photo-respiration has also been found to prevent oxygen damage to nitrogenase but this mechanism eats up much of reductant produced during photosynthesis by photosystem-I.

Ribbe *et al*. (1997) studied the N_2 fixation in *Streptomyces thermoantotrophicus* and observed that the nitrogenase was tolerant to superoxide produced from oxygen. The process involves molybednum dinitrogenase and a manganese superoxide oxidoreductase. Such a system if put in chloroplasts can allow expression of active nitrogenase under oxic conditions. However, this system has problems of dependence on superoxide stress. Even if such a conditional expression is corrected by disabling superoxide dismutase, it will again lead to the same problem of increased levels of hydrogen peroxide. Besides such a system is not practically efficient as it has only 10% of activity of conventional nitrogenases at 25°C.

Transformation of rice roots: There are two aspects of root transformation of rice plant. One of the possible cases is to allow expression of *nif* genes in plastids of non-photosynthetic cells of roots, thus overcoming problems of separating photosynthesis from nitrogen fixation and since the roots of flooded rice are usually under anaerobic conditions. Oxygen damage to the nitrogenase is taken care of. But there are few practical limitations of this approach.

- The energy for driving nitrogenase system would have to be imported in form of ATP, in a non-photosynthetic tissue like root.
- The introduction of nitrogenase would trigger competition with other bio-synthetic process which need reductants, such as GOGAT enzyme for Ammonia assimilation which requires ferrodoxin (Browhec *et al*., 1996).

Another aspect of root transformation is to enact mechanism for development of nodule like structure which provide a competition free habitat for endophytic diazotrophs. Even though a number of diaziotrophs have been found to be associated with rice. There are certain obvious dissimilarities between the rice-diazotroph association and the root-nodule symbiosis in legumes. Engineering rice roots will have to mainly focus on effecting more intimate association between rice and the rhizobia. This will mainly rely on manipulation of *nod* genes and nodulin genes. the factors in legumes which ensure rhizobial infection and colonization (Flavones) will have to be put in place in rice root system. Such a system of rice root transformation should ensure expression of unique traits in rice which improve their pre-disposition to rhizobia and consequently development of nodules. Various such factors such as flavonoids (Luteolin, Naringenin) and certain enzymes like apyrase have been identified which act as receptors for Nod signals and have been successfully expressed in rice. The genes governing such traits or factors can be candidate genes for introduction into rice preferably driven by root specific promoters to favorably engineer rice to develop nodule like structure and eventually the rice plant having its own mechanism of nitrogen fixation. This is indeed a long term endeavour. Dey *et al*. (2002) stated that rice does possess some if not all of such traits (Nodulins) found in legumes that are important for nodulation. If this hypothesis is correct then reconstituting the nod signal recognition in rice could allow this plant to interact more intimately with rhizobia. This line of thought gives more support to idea put forth by Mylona *et al*. (1995) and Carol *et al*. (1996) that legume plants acquired the ability to form symbiotic nitrogen fixing nodules by recruiting genes that have common functions in all plants. Now it is indeed a daunting challenge whether the genetic engineering can re-recruit the counterparts of these genes in rice to form functional nodules.

Benefits of BNF-rice: BNF-rice if developed can render following benefits to Agriculture especially in resource poor developing countries (Parakaran, 1997).

Table 3: Conventional and future BNF systems

BNF system	N-supply potential	Rice yield potential	Technology availability	Feasibility and adoption
Conventional BNF-Systems				
Free living or Associative	50-100 kg ha^{-1}	3-6 t ha^{-1}	3-5 years	High
Green manure (*Azolla, Anabena*)	100-200 kg ha^{-1}	5-8 t ha^{-1}	Available	Low
Future BNF-Systems				
Endophytic	?	?	3-5 years	High
Induced symbiosis (*Rhizobia, Frankia*)	>200 kg ha^{-1}	>8 t ha^{-1}	>5 years	High
Nif gene transfer	>200 kg ha^{-1}	>8 t na^{-1}	>5 years	High

Saves energy: The process of nitrogen fixation only requires 16 ATP's which is much less than the Haber-Bosch process used for chemical fixation of nitrogen. The Haber-Bosch process i.e

$$N_2 + H_2 \rightarrow NH_4$$

requires high pressure (100-200 atm) and high temperature (400-600°C). thus such high energy inputs can be saved if BNF rice is developed.

Saves money: It has been estimated that BNF rice can save almost 20 billion dollars annually (US agency for International development, 1994).

Saves environment: BNF rice is environment friendly technology. In chemical Nitrogen fixation carbon dioxide (CO_2) is released into environment. Carbon dioxide is a potential green house gas and causes global warming with severe implications on climatic patters of world. Similarly another toxic gas N_2O (Nitrous oxide) is released during denitrification bacteria (Galton and Smith, 1993).

Saves labour and soil: If rice is made to have in plant capacity to fix nitrogen for itself, a large amount of labour incurred in spreading fertilizer can be saved. Moreover, at the end of the season, BNF rice stubbles can be put into the soil to act as bio-fertilizers.

More productive: Engineering rice BNF system with either improved association with the rhizobia or by *nif* gene transfer has been found to be more productive Table 3 compares the potential and feasibility of various systems in rice aimed at nitrogen fixation (Papademerion, 2003).

Saves hunger: Rice feeds almost half of worlds populations. The increased pressure to increase rice production in view of ever increasing population means more requirement of nitrogen fertilizers. If BNF system can be put in place in rice, it will surely save millions of people from hunger given the declining energy resources of the world.

CONCLUSIONS

Since the inception of its idea, the development of BNF-rice not only poses a ardous challenge but a tremendous opportunity to bio-technologists and molecular biologists to realize this dream. Though most of the aspects of rice-diazotroph interaction and nitrogen fixation have been elucidated both at genetic as well as molecular level, the engineering of an autonomous nitrogen fixing rice plants is undoubtedly a long term endeavour. A large number of endophytic diazotrophs have been found to be associated with rice and factors encouraging bacterial colonization have been characterized but certain critical differences in rice-rhizobial interaction relative to root nodule symbiosis in legumes have to be worked out to make the rice-bacteria interaction so intimate that nodulation becomes a reality. The research towards developing a BNF rice has made remarkable progress in terms of elucidation of genetic and molecular mechanisms underlying this intricate biological process but the fact is that every newer insight takes us to newer complexities, hitherto unknown. The continued efforts in a collaborative framework will surely lead us to success provided the difficulties faced are overcome by consistent and problem oriented research. Some of the major problems identified in developing a BNF-rice include: large number of *nif* genes that need to be engineered into a rice plant, unpredictability of expression in a enkaryotic genetic background and lack of knowledge about differential oxygen concentrations in different plant tissues to identify most appropriate location for targeting *nif* genes. Thus as Dixon *et al.* (2000) stated, "In attempting to engineer nitrogen fixing plants we are taking a huge leap into unknown. Each step along the way may lead us to new difficulties or bring remarkable surprise". The surprise may well be a working prototype of a BNF-rice which may be able to compliment if not totally replace the N_2-supply of rice plant.

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