Assembling BNF system in rice plant: frontier areas of research

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Current knowledge of the genetics of biological nitrogen fixation (BNF) and recent advances of the knowledge of the natural diversity among the diazotrophs kindled interest among concerned scientists in assembling BNF system in rice plant to fix its own nitrogen. Though the task is complex, emerging knowledge of functional genomics and new biotechnological tools opened up a number of research endeavours. Advanced laboratories around the globe in rice research are pursuing studies mainly in three well-defined approaches, viz. forced association between rice and diazotrophs, i.e. non-nodular symbiosis; forcing nodulation in rice plant to establish nodular symbiosis and constructing functional nitrogenase system in rice plants through transferring *nif* genes. Frontier areas of these research endeavours have been reviewed in this article.

Keywords: Biological nitrogen fixation, genetic manipulation, non-nodular and nodular symbiosis, *nif* genes, rice.

In agriculture, soil fertility and soil nitrogen have become almost synonymous as the crop productivity is largely determined by them. The nitrogen deduced by the plant parts is incorporated as protein, subsequently degraded, mobilized and metabolized to other developing parts. When nitrogen is deficient, their growth is markedly inhibited. The effect of nitrogen deficiency is strongest on the development of leaves, and also inhibited the formation and development of reproductive organs and seeds[1].

The rice plants require large amounts of mineral nutrients, including nitrogen for their growth, development and grain production. According to Ladha and Reddy[2], rice plants need 1 kg of nitrogen to produce 15–20 kg grain. As most of the paddy soils of the world are deficient in nitrogen, supply of nitrogen to paddy soil is important. Due to the development of high-yielding varieties of rice which are more nitrogen-responsive, continuous supply of nitrogen to paddy soil has become the trend of the day. Present trends suggest that tomorrow's rice land will be under even more pressure than today and as a result future technologies and management practices

shall have to ensure the production of more rice on less land, which will demand input of more nitrogen. Based on a model with the components of population, gross domestic product (GDP), the proportion of GDP to agriculture and nitrogen use per unit of agricultural output, Frink *et al.*[3] projected that worldwide nitrogen use by 2070 would be nearly 2.5 times the level of 1990s when it was around 80 million metric tonnes.

But, under the current methods of growing rice, more than half, and often as much as 70% of the nitrogen applied is lost, thus causing economic and environmental costs[4]. Long-term application of nitrogen depletes the soil organic matter content[5]. On the other hand, excess use does not give better productivity[6]. Moreover, excess nitrogen in the global system in its various forms augments greenhouse effects, diminishes ozone levels, promotes smog, contaminates drinking water, acidifies rain, results in eutrophication of rivers and stresses the ecosystem[7].

Because of the adverse impact of excess nitrogen on the global system, Socolow[7] argues that management of food and nitrogen connection should be given as much importance as carbon and energy connection undertaken at the global level due to climate change. In this context three approaches are advocated to address riskiness while satisfying the need to produce more rice from limited soil. The first of these approaches is to increase the yield of rice per unit of nitrogen uptake by the plant by improvements of variety through plant breeding[8]. Second is to regulate the timing of nitrogen application based on the plant's need, i.e. precision farming, thus increasing the efficieny of the plant's use of applied nitrogen[9]. Third is biological nitrogen fixation (BNF).

BNF was exploited for centuries without recognition of how it was functioning before the demonstration by Boussingault[10]. Naturally in the soil, there are a number of aerobic and anaerobic bacteria as well as blue-green algae or cyanobacteria which have ideal biological system for nitrogen fixation. Therefore, the idea was to inoculate these microorganisms, especially diazotrophic bacteria to ensure better nitrogen fixation for better yield. But, after several years of key research, it appears that non-nodular, conventional BNF is likely to have low potential in terms of the amount of nitrogen fixation[11]. On the other hand, advancement of the knowledge of nitrogen-fixing apparatus, particularly the genetics of BNF as well as advances

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in our knowledge of natural diversity among diazotrophs, encouraged the scientists to think beyond 'conventional' BNF.

According to James et al.[12], in this context, the best way would be to increase the ability of rice to fix its own nitrogen, which will ensure little or no loss. However, emerging knowledge of functional genomics and biotechnological tools has opened up a number of research endeavours.

Conventional BNF strategy in rice: limitations

BNF is the conversion of atmospheric nitrogen by living organisms into forms that plants can use. It involves, reduction of atmospheric nitrogen to ammonia by a metabolic process. This process was exploited for centuries without recognition of how it was functioning. Probably the first person to demonstrate clearly what was going on was Boussingault[10]. However, it was not convincing till the report of Hellreigel and Wilfarth[13]. Subsequently, for increased production, dependence on large quantities of exogenous inputs of industrially produced nitrogen enhanced tremendously. It was due to the demand of high yielding varieties of crop to produce more grain for increased population. Hence, the scientists were compelled to think of utilizing BNF benefit with human intervention. In this direction, research on conventional BNF system in rice started in a major way and from mid-1970s with the initiative of the International Rice Research Institute (IRRI), the Philippines. Thereafter, human intervention started worldwide for inoculation or introducing desired diazobacteria, cyanobacteria, azollae and aquatic green manure legume in paddy field for nitrogen fixation.

Among them, though cyanobacteria inoculation is a low-cost technology, it has many constrains, which resulted in the restriction of its success[2]. Factors such as phosphate deficiency, presence of high concentrations of nitrogen in flood water, low pH and arthropod grazer population limit the growth and BNF activities of cyanobacteria in rice fields[14,15]. Moreover, cyanobacterial nitrogen fixation is closely linked with their growth and fixed nitrogen is not made available to the plants until the organisms die and their organic nitrogen is mineralized[12]. Another factor is that the inoculated organisms are to compete with native microbial populations[16]. With regard to aquatic plants such as the water fern Azolla and semi-aquatic legumes, Sesbania, Ladha and Reddy[2] opined that these are too uneconomical to farmers because of additional costs of labour, land opportunity, irrigation, seed/inoculums, phosphate and pesticides.

In nature, there are two ways of biological fixation of nitrogen, viz. symbiotic and non-symbiotic. Symbiotic is the most important mechanism by which most of the atmospheric nitrogen is fixed, but it is limited to legume plant species and various trees and shrubs that form acti-norrhizal roots such as Frankia. Non-symbiotic BNF is carried out by free-living diazotrophs and this can stimulate growth of non-legume plants[17]. There are studies showing that nitrogen-fixing bacteria, free-living as well as Rhizobium strains, can stimulate the growth of non-legumes such as radish[17] and rice[18], thus contributing to reduced dependence of nitrogen-based fertilizers[19]. Hence, rice being non-legume, a number of technologies have been developed for inoculation of free-living diazotrophic bacteria in paddy fields, which were found to be effective in reducing the dependence on exogenous nitrogen supply for grain production in rice[20–46]. Though the free-living diazotrophic bacteria have advantages, they also suffer from certain limitations.

Due to high energy requirement for nitrogen fixation and relatively low metabolic activity, the ability of non-symbiotic bacteria to fix significant quantities of nitrogen is limited[47]. According to Ladha et al.[48], in rice, at best 20–25% of total nitrogen is available from BNF. There seems to be little scope to substantially increase the nitrogen supply from free-living diazotrophs, both phototrophic and heterotrophic, as the nitrogen that they fix is outside the plant and therefore subject to losses[49]. Moreover, for introduced diazotrophs it is also a difficult task to compete with the native microbial population[16]. Hence, Stoltzfus and de Bruijin[50] reviewing the colonization of plant roots by bacteria and nutrient transfer opined that in the rhizosphere, the bacteria use most of the nitrogen they fix for their own growth, rather than supplying nitrogen to the plant. Considering this, James et al.[12] argued to go beyond 'conventional' BNF.

Forced association between rice and diazotrophs: possibilities of non-nodular symbiosis

The plant root system offers an excellent microhabitat for bacterial growth[51]. A number of endophytic diazotrophs have been found to be associated with different crops such as sugarcane and kallar grass. Other studies also revealed that a large number of nitrogen-fixing bacteria were present in root interiors of certain grasses[52,53]. These studies encouraged the research community to look for the development of efficient rice–endophyte system for conferring in plants the ability to fix atmosphere nitrogen. Initially the techniques for accurate enumeration and isolation of putative endophytes were standardized[54]. This was followed by isolation and characterization by PCR-based techniques of various prospective endophytes from wild as well as cultivated rice cultivars[55]. Azospirillum is one of the most common genus isolated from the rhizosphere of rice. Interestingly, although it is generally regarded as a rhizosphere bacterium, it has often been reported that the best results obtained with inoculation are with those strains that actually penetrate the roots[56],

suggesting that some strains of *Azospirillum* are also endophytic within grasses[57,58]. Microscopic evidence for the endophytic nature of *Azospirillum* in rice has also been presented[59,60]. Baldani *et al.*[59] used immunogold labelling to detect the bacteria in intercellular spaces and within dead cells in the leaves and roots.

Both *A. lipoferum* and *A. brasilense* have been isolated from roots and stems[61,62]. Pereira *et al.*[63] reported isolation of *A. amazonense* from the roots of rice. Stoltzfus and de Bruijn[50] isolated several putative endophytes of rice that are likely to be members of the genus *Azospirillum*, although they do not appear to be closely related to any of the presently described species. Christiansen-Weniger[64] inoculated 2,4-D-induced para-nodulated rice plants with an ammonium-excreting mutant of *A. brasilense* and suggested that the bacteria colonized the para-nodules in large numbers. James *et al.*[12] opined that the organisms may have initially entered the plants via cracks that had formed when the tumours emerged from the epidermis.

Diazotrophic *Alcaligenes* strains have been consistently isolated from the rhizosphere of rice[65–67], and the dominant strain appears to be *A. faecalis* A15 (refs 68–70). You and Zhou[68] claimed that A15 was an endophyte on evidence from micrographs of fixed and embedded material that putatively showed bacteria both outside and within the root cortex cell. Though there were controversies[12,58,71,72], more reliable evidence of the endophytic occurrence of A15 has come from the study of Vermeiren *et al.*[60], which has shown, using resin-embedded material, that gus A-marked A15 will colonize the root surface, particularly at the root tips and lateral root junctions, and will also enter the epidermal cells. Within the latter cells, the bacteria have been shown to express *nzfH* via *gus* A.

Azoarcus spp. were until recently known as endophytes of kallar grass[72]. But recently, its natural occurrence has also been found in association with wetland rice root in Japan[73] and within wild rice in the Philippines and Nepal[74]. In DNA retrieved from field-grown Japanese rice, a *nifH* gene fragment has been detected[73], which clustered with *nifH* sequences of *Azoarcus* spp. on phylogenetic sequence analysis[75]. Therefore, it now appears that *Azoarcus* is a genuine endophyte to rice as well as of kallar grass[12].

Diazotrophic strains of *Burkholderia* (e.g. strains of M130 and M209) have been isolated from the interior of rice roots, stems and leaves in Brazil[22]. Preliminary results with optical microscopy suggest that they can enter rice roots via cracks in the root epidermis, particularly at the points of emergence of secondary roots, and subsequently 'massively' colonize their interior[22]. Strains similar to M130 have also been isolated from surface-sterilized plants by Stoltzfus and de Bruijn[50].

There is not much evidence as yet that *Enterobacter* spp. are endophytic in rice, though there are reports that the diazotrophic enterobacters are frequently isolated from the rhizosphere of rice[12]. According to Achouak *et al.*[76], there is only colonization of the root surface by them. However, Quadt-Hallmann and Kloepper[77] have suggested that, enterobacters can be endophytic per se. The reason for this is that they have micrographic confirmation of *Enterobacter asburiae* strain JM22 within non-legumes, though not yet in rice. Hurek *et al.*[74] have also shown that a diazotrophic strain of *Serratia marcescens* (IRBG 500) can be isolated from surface-sterilized wetland rice. A *nifH–gus A* fusion of this bacterium was constructed and inoculated into gnotobiotically grown rice seedlings. The bacteria, expressing *nifH*, colonized the surfaces of the roots and were also observed associated with stems and leaves. Examination using light microscopy and TEM (coupled with immunogold labelling) showed that the bacteria colonized the root aerenchyma and inert cellular spaces and were particularly abundant within the stem aerenchyma and xylem vessels.

Barraquio *et al.*[55] reported infection of rice variety IR 42 by a *gus A* fusion of *Herbaspirillum seropedicae* strain 267, which was originally isolated by Baldani *et al.*[22] from Brazil. In the study, at 28 days after inoculation, surface-disinfested roots of the seedlings were only sparsely colonized by the bacteria, although there was some staining of the subepidermal region, suggesting the presence of the bacteria. Similar results have also been presented by Baldani *et al.*[59] with rice infected with *H. rubrisubalbicans*.

There is no direct evidence that diazotrophic strains of *Pseudomonas* are endophytic. It is also a fact that surface sterilization appears not to have been used in most of the studies and so far no microscopic evidence has been presented. But, among the putatively endophytic diazotrophs isolated by Stoltzfus *et al.*[54], one was closely related to *P. cepacia*. Recent evidence suggests that there may be natural association between rhizobia and rice. Among the putatively endophytic bacteria isolated by Stoltzfus *et al.*[54] and Stoltzfus and de Bruijn[50], some showed similarity to *Azorhizobium caulinodans*. Yanni *et al.*[78] have described in detail the isolation of *Rhizobium leguminosarum* bv. *trifolii* from wetland rice in Egypt and shown conclusively that it is endophytic.

Hallmann *et al.*[79], and James and Olivares[58] have suggested that some endophytic bacteria may be transmitted from generation to generation via seeds. Mukhopadhyay *et al.*[80] isolated strains of *Enterobacter*, *Serratia* and *Bacillus* spp. and showed that *E. agglomerans* could be transmitted to the next generation through seeds. Though they did not test the strains for nitrogen-fixing ability, they reported that the bacteria were associated with the seed hull and husk, and within the embryonic tissue. Moreover, diazotrophic strains of *Alcaligenes*, *Pseudomonas* and *Bacillus* have been isolated from rice seedlings germinated from surface-sterilized dehulled seeds, and hence could be seed-borne endophytes of rice[12].

From these and other similar observations, there are evidences of endophytic association between rice or grass and diazotrophs. This can be improved or extended and can be considered as symbiosis. But, the fundamental question remains; how efficiently can the endophytes actually function within grass or rice when no obvious 'symbiotic' structures appear to be present? There are reports of random distribution of bacteria within intercellular spaces, parenchyma, dead cells and xylem vessels, which typifies endophytic association[58]. But the question remains about how they perform functions analogous to those of highly evolved organs. To improve or extend non-nodular symbiosis for effective BNF in rice or in other grasses, the answer to these questions needs to be found. According to Olivares et al.[81] and James et al.[82], if we want to improve endophytic associations between rice or grasses and diazotrophs, it will presumably be necessary to increase the number of bacteria within the plants. How can this be done without provoking a host-defence response? Success of non-nodular symbiosis for BNF in rice or grasses depends on whether or how researchers in the field make it possible to answer these fundamental questions in coming days. James et al.[12] opined that until these fundamental questions are answered, it must be assumed that endophytic diazotrophs either do not make a significant contribution to grasses such as rice, or they do so via a means other than symbiotic nitrogen fixation. On the other hand, in spite of the observations of James et al.[12] that the number of diazotrophs living within rice appears to be relatively trivial while considering the size of the total population of diazotrophic bacteria isolated from the rhizosphere of rice, Sofi and Wani[51] opined that 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 plant N-fixing capability in rice.

Therefore, presently research initiatives are being pursued broadly using three approaches, viz. (i) identification of specific and predominant diazotrophs from rice, (ii) determination of mode of invasion and extent of colonization and (iii) assessment of contribution of diazotrophs to rice growth and yield through nitrogen fixation[51].

Forcing nodulation in rice: feasibility of nodular symbiosis

Rice being a non-leguminous plant, is it absurd to think of nodular symbiosis in rice? Given the comparatively recent discoveries of rhizobial nodulation of the non-legume *Parasponia* and non-rhizobial nodulation by Frankia in a range of non legumes, it appears that the notion is not absurd[83].

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 the plants. If we are to force nodulation in rice for effective BNF, i.e. if our endeavour is to produce nodular symbiotic association between rice and diazotrophs, we have to ensure that the diazotrophic endophytes can have such an association with rice plants and develop that kind of defined structure.

There have been many reports about such nodule-like structure at a low frequency (0.1 to 0.2%) upon inoculation of rhizobia to normal roots[84–87] or enzyme-treated roots in the presence of PEG and $CaCl_2$ (ref. 88). According to Saikia and Jain[89], rhizobia have the ability to attach themselves to rice root hairs, elicite deformation of rice root hairs and to form nodule-like structures. There are also several reports[89] on induction of nodule-like structures termed as para-nodules on cereal roots using different plant hormones like 2,4-D, NAA, BAP and zeatin.

The BNF phenomenon already existed in nature before it was understood by man[89]. It is therefore advisable to observe and learn about the natural phenomenon and interpret the findings in the laboratory. In this context, from recent evidences, it can be said that natural association between rhizobia and rice exists[12], though, as such rice does not enter into a symbiotic association with rhizobia. However, a rhizobial strain *R. leguminosarum* bv. *trifoli* isolated from wetland rice in Egypt was also found to be an endophyte of rice and capable of nodulating berseem clover (*Trifolium alexandrium*), which is commonly grown in rotation with Egyptian rice as a green manure.[78] On the other hand, it has been observed that rice is able to interact symbiotically with certain mycorrhizal fungi[90].

In legume and rhizobium symbiosis, the host plant provides the genetic information for the development of nodules. The role of rhizobia is to trigger the host plant genes to facilitate infection and help create a nodular niche for nitrogen fixation[91,92]. According to Reddy et al.[93], a monocot plant such as rice would unlikely possess the complete complement of genes or genetic programmes involved in the nodule ontogeny programme that could be induced by rhizobial strains. But, the reason for optimism is that although rice does not develop a symbiotic association with rhizobia, it is able to enter into symbiotic associations with mycorrhizal fungi. Albrecht et al.[94] have also provided evidence of a genetic link between processes involved in nodule formation and arbuscular mycorrhizae in legumes, which is supported by subsequent studies too[95–97]. 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 mycorrhizal development[98]. Now, since rice is associated symbiotically with micorrhizae 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[99], which are instrumental in initiating nodule development; but rice is unlikely to possess all such genetic framework. Thus, according to Sofi and Wani[51], now it is our endeavour to find the missing links.

If the hypothesis that rice does possess some if not all of such traits (nodules) found in legumes that are important for nodulation holds, then reconstituting the nod signal recognition in rice could allow this plant to interact more intimately with rhizobia[51]. This line of thought gives more support to the idea put forth by Mylona *et al.*[100] and Carol *et al.*[101] that legume plants acquire the ability to form symbiotic nitrogen-fixing nodules by recruiting genes that have common functions in all plants. Sofi and Wani[51] thus opined that it is indeed a challenge whether a genetic engineering can re-recruit the counterparts of these genes in rice to form functional nodules[51].

Construction of functional nitrogenase system in plants: transferring *nif* genes

The advances in transformation of rice, characterization of various genes from both plant (nodule genes) and diazotrophic 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 atmospheric nitrogen, not only reasonable but also realizable[51]. According to Fischer[4], the components of actions in this direction could be to transform rice with *nif* genes to ensure the expression of nitrogenase, protection of nitrogenase from inactivation by oxygen and to ensure energy supply for nitrogen fixation without compromising on the yield. For this, Parakaran[102] proposed two approaches, viz. transformation of rice leaf and transformation of rice roots.

Transformation of rice leaf

Though 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 eukaryotes as well[103], it now appears that this is more complicated as in BNF system both the Fe protein (dinitrogen reductase) and the MoFe protein (dinitrogen) are involved in fixation as well as in the auxiliary system. According to Burris[104], there are more than 20 genes involved in the nitrogenase system; the role of many of these for nitrogen fixation has now been defined. Among them the structural genes are *H*, *D* and *K*, with *H* defining dinitrogenase reductase, and *D* and *K* coding for dinitrogenase. Sofi and Wani[51] postulated that there are certain genetic and physical consi-

derations which have, most obviously, put breaks in the progress of transferring *nif* genes in rice plant. These considerations are: (i) engineering of rice plant capable of nitrogen fixing requires coordinated and regulated expression of *nif* genes assembled in an appropriate cellular location[105]; (ii) additional genes to keep nitrogenase in an active form may also be needed; (iii) to optimize the expression of *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; (iv) even though mitochondria would ideally offer an energy-rich location for *nif* gene, this idea can be ruled out, at least in short term, because of the complexity of targeting *nif* genes in this organelle[106], and (v) nitrogenase is sensitive to oxygen; thus appropriate mechanisms for protection of this vital enzyme have to be developed well within the eukaryotic cell structure. These considerations have been the thrust areas of research toward constructing functional nitrogenase system in rice plants, i.e. BNF rice.

According to Dixon *et al.*[106], the plastids may provide a more suitable location for introducing the *nif* genes because chloroplast genes are expressed in a prokaryotic-like fashion and translation of polycistronic messages occurs. The cluster of *nif* genes may be introduced simultaneously because now techniques are available where many genes can be transferred into rice[107,108]. However, nitrogenase enzyme needs to be protected from oxygen damage. A possible mechanism can be temporal separation of the 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[51,106]. The light period ensures photosynthesis and replenishment of glucan[106–109]. However, according to Dixon *et al.*[106], the levels of ATP and reductant available during the dark period are probably insufficient to support nitrogen fixation. Moreover, there is no efficient respiratory system in plastids under these conditions that could remove oxygen. Under conditions of illumination, reducing power is generated by photosystem I and the chloroplast stroma provide a reducing environment, as evidenced by the reduced state of thioredoxin, which acts as a signal for redox status[110]. Under these reducing conditions, it is possible that nitrogenase itself could help remove oxygen, when due the oxygen concentration, the Fe protein is oxidized without loss of activity[111]. However, this leads to a problem of cleavage of a major byproduct, i.e. hydrogen peroxide which would need another set of enzymes to be put in the system in chloroplasts such as ascorbate peroxidase, monodehydro ascorbate reductase and dehydroascorbate reductase. Photorespiration might also help protect nitrogenase from oxygen damage, but at the expense of photosynthetically generated reductant. It may also be possible to use the unusual oxygen-tolerant nitrogenase from nitrogen fixa-

tion in chloroplasts[112]. The process involves molybdenum dinitrogenase and a manganese superoxide oxidoreductase. Such a system, if put in the 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, it will again lead to the same problem of increased levels of hydrogen peroxide.

Transformation of rice roots

This is possible by allowing expression of *nif* genes in plastids of non-photosynthetic cells of roots, thus overcoming problems of separating photosynthesis from nitrogen fixation. Since the roots of flooded rice are usually under anaerobic conditions, oxygen damage to the nitrogenase is taken care of[51]. However, there are few practical limitations of this approach, viz. (i) the energy for driving nitrogenase system would have to be imported in form of ATP, in a non-photosynthetic tissue like the root, and (ii) the introduction of nitrogenase would trigger competition with other biosynthetic processes which need reductants, such as GOGAT enzyme for ammonia assimilation, which requires ferrodoxin[113].

Conclusion

The engineering of BNF in rice is no doubt an ardous challenge, because of a number of extensive enzymatic and developmental steps involved[114]. Hence, it appears to be a distant dream as of now. In this context, there is stress on conventional BNF, especially identification of appropriate diazotrophs and plant variety combination. On the other hand, searching some simpler ways is also going on, such as ways in which nature itself has evolved a successful system – through better nutrition of crops, which includes two options – indirect feeding of plant through transgenic diazotrophs and direct feeding of plant through artificial symbiosis or through transgenic diazotrophs[115].

Thus, BNF in rice plants opened a new frontier of science, offering exciting opportunities in the rice research horizon. Dixon *et al.*[106] 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'. According to Sofi and Wani[51], the surprise may well be an working prototype of BNF – rice, which may be able to complement if not totally replace the N-supply of rice plant. If achieved, this could enhance rice productivity, resource conservation and environmental security. The rate of obtaining success would, of course, benefit tremendously from concerted efforts from a critical mass of committed scientists around the world, as well as constant and continued funding support from the 'donor' community[116].

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