RESEARCH ARTICLE

RICE – *RHIZOBIUM* INTERACTIONS FOR BIOLOGICAL NITROGEN FIXATION: TECHNICAL CHALLENGES

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Abstract: Nitrogen is the most important nutrient input required for rice production. As most of the soil is deficient of N, N-fertilizers are needed. But, instead of chemical fertilisers, biological nitrogen fixation (BNF) is preferred. In that too, conventional BNF has limited capacity to render rice independent of external sources of N. Therefore, a major goal of BNF research has been to extend the nitrogen fixing capacity to rice. In this context, recent advances in understanding symbiotic *Rhizobium*-legume interactions at the molecular level, the discovery of natural endophytic interactions of rhizobacteria with rice, potentiality of rice nodulation, as well as potentiality of introduction / expression of *nif* genes in (to) rice has offered exciting opportunities to stretch rice research horizons, though there are technological challenges. These aspects have been reviewed in this article.

Keywords: Rice-*Rhizobium* interactions, Biological nitrogen fixation, Endophytic association, Nodulation in rice

INTRODUCTION

Soil bacteria are very important in biogeochemical cycles. Among them, certain bacteria exert beneficial effects on plant development, which are defined as plant growth-promoting rhizobacteria (PGPR). They exert beneficial effects via direct and indirect mechanisms. The direct mechanisms include nitrogen fixation, production of hormones, production of enzymes and mobilization of nutrients (Khalid *et al*, 2004; Lucy *et al.,* 2004; Gray and Smith, 2005). The indirect mechanisms include increasing efficiency of fertilizers uptake, increasing the plant's tolerance towards stress, inducing host resistance or producing pathogen-suppressing substances (Raj *et al.,* 2003; Van Loon, 2007). These can work independently or simultaneously with each other (Keyeo *et al.,* 2011). The said PGPR can be divided into two groups, *viz.,* symbiotic bacteria and free-living associative bacteria considering their relationship with the plants (Khan, 2005), as well as according to their residing sites (Gray and Smith, 2005). The symbiotic bacteria live inside the plant cells in specialised structure forming nodules and free-living rhizobacteria live outside the plant cells and do not produce nodule, but still promote plant growth.

Among direct mechanisms, nitrogen fixation is considered to be most important, in which inert N_2 is converted to NH_3, a form that plants can use. As the conversion is by and in living organisms, the process is defined as biological nitrogen fixation (BNF). Again, BNF by symbiotic system has got advantage over associative nitrogen fixation. In associative nitrogen fixation, the involved bacteria in the rhizosphere of plants utilise the products of nitrogen fixation for their own growth, but release little while they are alive (Van Berkum and Bohlool, 1980). On measurement of associative nitrogen fixation using ^{15}N in rice, as well as in wheat, Okon (1985) confirmed that, the majority of the fixed nitrogen remain in the bacteria within the root environment. On the other hand, in symbiotic systems, fixed nitrogen becomes directly available to the plants due to more intimate metabolic exchange with host plants. Hence, the nitrogen-fixing bacteria colonize the plant internally and become endophytic, thus, insulated from competition with other rhizosphere microorganisms too (Reddy *et al.,* 1997; Webster *et al.,* 1997).

But, symbiotic system is confined in leguminous plants, and the best known symbiotic bacteria belongs to the genera *Rhizobium* (Hayat *et al.,* 2010). For BNF, the non-leguminous plants including cereal plants such as rice, there is dependency on free-living nitrogen-fixing bacteria. However, several researchers observed natural association of rice with rhizobia and also reported certain interactions in laboratory as well as in field experiments (Yanni *et al.,* 1997). As a result, possibilities of enhancement of natural association of rice with rhizobia (Yanni *et al.,* 1997) and even to the extent of extension of nodulation characteristics (Reddy *et al.,*1997) have been envisaged. Development of biotechnological tools have also generated optimism of incorporation / expression of nitrogen fixing genes in (to) rice (Stoltzfus *et al.,* 1997). However, inspite of potentialities, there are certain technological challenges.

Rhizobium : the obligate symbionts in leguminous plants

Rhizobia were first classified on the basis of cross-inoculation capability, *i.e.,* the ability to nodulate a specific group of hosts (Fred *et al.,* 1932). The seven cross-inoculation groups identified were :

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Sinorhizobium meliloti which nodulate alfalfa group, *Rhizobium trifolii* which nodulate clover group, *Rhizobium legumenosarum* which nodulate pea, *Rhizobium phaseoli* which nodulate bean, *Rhizobium lupine* which nodulate lupine group, *Bradyrhizobium japonicum* which nodulate soyabean and *Rhizobium* sp. which nodule cowpea (Fred *et al.*,1932). But, this method most variably failed in classifying different species (Coutinho *et al.,* 2000) as classification of rhizobia on the basis of host range and physiological properties does not reflect the true phylogeny of the group (Sahgal and Johri, 2003). Later molecular systematic provided a breakthrough and helped to evaluate *Rhizobia* diversity in different environments (Patel and Sinha, 2011). The present taxonomy of *Rhizobia* comprises of 98 species found in 13 genera (Weir, 2012). Most of these bacterial species are in genera either *Rhizobium*, *Mesorhizobium*, *Ensifer* and *Bradysrhizobium* (Annapurna and Govindasamy, 2009). Among them, the best known PGPR is the genera *Rhizobium*, unlike many other soil microorganisms, produce no spores and are aerobic and mobile (Baset Mia and Shamsuddin, 2010). Though, they live freely in the soil in the root region of both leguminous and non-leguminous plants, can enter into symbiosis only with leguminous plants (Subha Rao,1982; Bottomley and Dughri,1989; Bottomley and Maggard,1990).

In symbiotic association with legume, the *Rhizobium* form morphologically defined structure called nodules by responding chemotactically to flavonoid molecules released as signals by the legume host. These plant compounds induce the expression of nodulation (nod) genes, which in turn produce lipo-chitooligosaccharide signals that trigger mitotic cell division in roots, leading to nodule formation (Dakora,1995; Lhuissier *et al.,* 2001; Matiru and Dakora, 2004).

The nodulated legumes contribute a good deal to the amount of nitrogen fixed in the biosphere. BNF is estimated to contribute 180 X 106 metric tonnes per year globally (Postgate, 1998), of which 80 per cent comes from symbiotic associations and the rest from free-living or associative systems (Graham,1988). However, although, the value of cultivating legumes for BNF was known through centuries, experimental evidence in support of it came only in the later half of the nineteenth century from the results of a classical experiment conducted by Boussingault in 1883 (Fred *et al.*,1932). In another classical experiment, Wilforth and Hellriegal in 1888 compared the performance of oats and peas and concluded that, the root nodules are responsible for the special ability of pea plants to use atmospheric nitrogen (Fred *et al.,* 1932). Hellriegal and Wilfarth in 1888 also demonstrated that an increase in nitrogen content in soil is due to the presence of small tumer-like outgrowth on the roots of leguminous plants (Sharma, 2003). It was also found that, in absence of root nodules, growth of succeeding plant group was retarded. The results of various experiments revealed that, root nodule forming plant can feed upon atmospheric nitrogen and this capacity of plants can be observed only in those plants, which are growing in non-sterilized or bacteria containing soils (Sharma, 2003). These symbiotic bacteria utilize the free atmospheric nitrogen and synthesize it into new nitrogenous compounds, which are utilized by plants for their growth; and bacteria get their food from these plants. Such a mutual beneficial association of bacteria and plants is referred to symbiosis.

Natural association between *Rhizobium* and rice

In nature, *Rhizobium* is normally viewed as a microbe that survives saprophytically in soil between periods in which the host legume is absent. However, studies in Egypt have shown that clover (*Trifolium alexandrinum*) rhizobia also occupy another endophytic niche inside rice plant. Yanni *et al.* (1997) isolated *Rhizobium leguminosarum* bv. *trifolii* as a natural endophyte from roots of rice in the Nile delta, where rice has been grown in rotation with berseem clover (*Trifolium alexandrinum*) for about seven centuries. This probably promoted closer rhizobial affinity to this cereal as a 'host plant'. This hypothesis is re-enforced by the fact that population of clover-nodulating *Rhizobium* isolated from rice could occur up to 2.5 x 10^7 cell g^{-1} fresh weight of root, concentrations similar to those obtained for bacteroids in legume root nodules (Hayat *et al.,* 2010), and have potential to promote rice growth and productivity (Yanni *et al.*,1997).

From Senegal and Guinea, Chaintreuil *et al.* (2000) similarly isolated photosynthetic *Bradyrhizobia* from roots of *Oryza breviligulata*, an ancestor of African brown rice *Oryza glaberrima*, which generally grows in the same wetland where *Aeschymene sensitiva* (a stem-nodulated legume, associated with photosynthetic strains of *Bradyrhizobium*) grows. This suggest co-evolution of *Aeschynomene*, *Bradyrhizobia* and wild genotype of African brown rice (*Oryza glaberrima*), though whether the *Bradyrhizobia* affect growth of *Oryza glaberrima* plant has not been determined (Hayat *et al.,* 2010). Natural associations of endophytic diazotrophs in rice roots under rice-*Sesbania* rotation in the Phillipines has also been reported (Ladha *et al.,* 1989, 1996;Yanni *et al.*,1997). In Nepal too, endophytic bacteria of native races of rice *azorhizobia* have been reported (Engelhard *et al.,* 2000), but there was no report on clear association of the rhizobial endophytes with legumes.

Above evidences suggest that, there may be natural endophytic association between *Rhizobium* and rice, as well as stimulation of plant growth, but there is no conclusive evidence that the benefits involves symbiotic nitrogen fixation (James, 2000;Yanni *et al.,* 2001). Though there is no conclusive evidence, if it happens, there is question, how efficiently the endophytes can actually function for nitrogen fixation, when no obvious ‘symbiotic’ structures appear to be present.

Rhizobium as bioinoculum in rice cultivation

From time immemorial rhizobia have been used as bioinoculants for increasing the yield of legume crops. Recent trials shown that, it also play role in enhancement of production of rice. In field inoculation experiment, Yanni *et al.* (1997) observed that, two rice endophytes *Rhizobium leguminosarum* bv. *trifolii* E11 and E12 significantly increased shoot and root growth, as well as grain yield by 46 and 42 per cent respectively. Under green house conditions, a 20 per cent increase in shoot growth and grain yield of the wild rice *Oryza breviligulata* was obtained by inoculation with photosynthetic endophytic *bradyrhizobia* (Chaintreuil *et al.,* 2000). Yanni and Dazzo (2010) has conducted large-scale field experiments in Egypt and evaluated 5 rice (*Oryza sativa*) varieties inoculated with 7 endophytic rhizobial strains during 5 growing seasons, including at sites ranked as the world’s highest in rice production. Inoculation with single strains or multi-strain consortia significantly increased grain yield in 19 of the 24 trials. By combining superior rhizobial inoculants with agricultural extension training, grain yield increased up to 47 per cent in farmers field, with an average increase of 19.5 per cent. Data on rice straw production, harvested index and the agronomic fertilizer N-use efficiency also indicated positive agronomic benefits of rhizobial inoculation. Studies conducted at the IRRI too showed that its inoculation increased growth and yield of rice, and N, P and K uptake by rice plants significantly (Biswas *et al.,* 2000, 2000a). Dey and Srivastava (2001) opined that, the *Rhizobium* can fix N in the soil as free-living nitrogen fixer and there are reports that, *Rhizobium* utilizes the N from soil by promoting physiological growth response generating changes to the root morphology of the rice plant that favours its uptake (Biswas *et al.,* 2000; Yanni *et al.,* 2001;Kennedy *et al.,* 2004; Askary *et al.,* 2009).

Present authors also observed enhanced growth, enhanced metabolic and enzymatic activity in two high yielding varieties of rice plants (cv. IR – 64 and cv. NDR – 359 of *Oryza sativa* L.) in paddy field in West Tripura, India (Roy and Srivastava, 2010; Roy, 2013). For basal inoculation of rice plants, the *Rhizobium* spp. was isolated from the root nodules of *Mimosa pudica*, a local herbaceous weed. While positive response in regards to growth, soluble protein and *in vivo* nitrate reductase (NR) activity have been observed in both the cultivars, inoculation result found to be even better than *Azospirillum brasilense*, *Azotobacter chroococcum*, *Clostridium pasteurianum* and *Pseudomonous denitrificans* in IR – 64.

Experimental trials on forced nodulation of rice

Though the molecular and cell biology of the *Rhizobium*-rice association differs in many respects from the biology underlying the development of root nodules in the *Rhizobium*-legume symbiosis (Reddy *et al.,* 1997), due to the findings of natural endophytic association between *Rhizobium* and rice, and positive result on inoculation on rice plants as mentioned above, it has been felt that ‘functional *Rhizobium*-rice association’ could be enhanced or created through forced interactions. In this context, several researchers have made experimental trials and reported a variety of responses, such as the ability of rhizobia to attach to rice roots (Terouchi and Syono,1990), elicit the deformation of root hairs (Plazinski *et al.,*1985), and to form nodule-like structures / hypertrophies (All-Mallah *et al.,*1989; Bender *et al.,* 1990; de Bruijn *et al.,*1995; Jing *et al.,*1990,1992; Li *et al.,*1991; Rolfe and Bender,1990) or development of thick short lateral roots on rice plants (Cocking *et al.,*1993). Though, the nodulation or nodule-like structure of rice by rhizobia has been reported, it either occurred at very low frequency (Bender *et al.,* 1990) or required an enzymatic pre-treatment of the roots (Al-Mallah *et al.,* 1989) and nitrogen fixation reported by only one group (Jing *et al.,*1990), in that case too, adequate controls were not presented (Spaink and Lugetenberg,1992). It also appears that, the nodules on rice roots (Bender *et al.,* 1990; Cocking *et al.,* 1990) does not possess highly organised internal structure of legume nodules, and hence, will not necessary provide the anaerobic environment essential for nitrogenase activity (Schell *et al.,* 1992) which possibly explain why the amounts of N_2 fixed are very low (Bender *et al.,* 1990; Cocking *et al.,* 1990; Jing *et al.,*1990). According to Reddy *et al.* (1997) it is unlikely that, a monocot plant such as rice would possess the complete complement of genes involved in the nodule ontogeny programme, and the rhizobial strains could induce the formation of genuine nodules on these plants.

Need of genetic modifications of rice plants

Interestingly, rice appears capable of perceiving Nod factors coded for by bacterial *nod* genes, and several homologues to legume *ENOD*s are present in rice (Reddy *et al.,* 1997). These (nodulin) genes specifically expressed in legumes during early events in infection and nodule formation (Reddy *et al.,* 1996; 1996a). Moreover, the promoter activity of rice *ENOD40* in soybean revealed that, its tissue-specific expression was identical to that of the endogenous soybean promoter, indicating that key regulatory features of these genes may be conserved in rice (Reddy *et al.* 2000). These results suggest that, rice may have at least part of the genetic program involved in a functional symbiosis with *Rhizobium*.

Rice also possesses the capacity to form symbiotic (mycorrhizal) associations with fungi (Secilia and

Bagyaraj, 1992; Khan and Belik, 1995). Genetic links between the processes involved in nodulation and arbuscular mycorrhiza have also been found in legumes (Gianinazzi-Pearson, 1996; Cook *et al.*, 1997). Thus Reddy *et al.* (1997) opined that, rice may possess part of the genetic programme necessary for entering into mutually beneficial, endosymbiotic associations with other soil microorganisms. Therefore, according to him, the rice would need to be genetically modified to respond to the appropriate rhizobial morphogenic triggers and subsequent *Rhizobium*-modulated nodule ontogeny requirement.

Technical options and challenges

There are three options to enhance / create 'functional BNF' in rice plants. These are : (i) enhancement of natural endophytic association of *Rhizobium* and rice (Yanni *et al.*,1997); (ii) development of novel symbiotic interactions resulting in the formation of nitrogen forming nodules or nodule like structures on rice root (Spaink and Lugtenberg,1992); and, (iii) direct incorporation / expression of the required complement of nitrogen fixation genes in (to) rice (de Bruijn *et al.*,1995; Ladha and Peoples,1995; Ladha *et al.*,1997; Dixon *et al.*, 2000; Sofi and Wani, 2007).

(i) Enhancement of natural endophytic association: The term 'endophytic' is used with various meanings in the literature on plant-microbe interactions (Saikia and Jain, 2007). Here, the term 'endophyte' is used to describe microbes that have colonized living plant tissue, but no formation of nodules and / or no harm to the host.

Existence of natural endophytic association of *Rhizobium* and rice (Ladha *et al.*,1989,1996;Yanni *et al.*,1997;Chaintreuil *et al.*, 2000; Engelhard *et al.*, 2000; Hayat *et al.*, 2010) suggest that, natural evolution itself have done some work for us, which, we can bank on. Therefore, Saikia and Jain (2007) advocated, it is advisable to observe and learn the natural phenomenon and interpret the finding in the laboratory.

Stoltzfus *et al.* (1997) also made suggestion that, first some basic knowledge about the presence, predominance and stability of endophytic bacteria in different rice tissues be obtained. The objectives of such endeavour be (i) isolating putative endophytic bacteria from diverse rice varieties grown in different soil types and assessing their diversity, (ii) developing molecular probes for the detection of putative N_2-fixing endophytes, and, (iii) studying the internal colonization of rice tissue by putative endophytic bacteria.

Saikia and Jain (2007) further opined that, the study can not be confined only to such a narrow space as the relationship between a certain host plant and a certain single strain microbe. Relationship of a plant with other plants and microbes, of the microbes with microbes and organisms, and sunlight, air, moisture, etc. are factors to be considered in the study (Saikia and Jain, 2007), which sometimes can even become crucial deciding factors under certain circumstances (G-Laboratory, 2002).

(ii) Search for nodulation in rice: Does nodulation occur naturally in rice? In investigating the feasibility of forcing nodulation in rice, it would be prudent to ascertain if nodulation occurs naturally in rice but at such a low frequency or under such unusual conditions that, it has so far escaped detection in the field (Bennett and Ladha, 1992). Bennett and Ladha (1992) also postulated that, if this is correct, then our efforts should be directed towards designing a rational search for the phenomenon and then attempting to increase its frequency through genetics and management.

But, the question is, how should we proceed? Bennet and Ladha (1992) proposed three approaches. These are: (i) to examine the roots of land races and wild species growing under N-limiting conditions, where nodulation would be an advantage and where the amount of mineralized N would be insufficient to repress nodulation, (ii) to examine the capacity of rice to support nodulation by rhizobia and other micro-symbionts derived from other plant studies, and (iii) to examine the rice genome itself for the presence of genes required for N_2-fixing symbiosis. If searches results in sufficient knowledge to design strategies for efficient nodulation and perhaps also N_2 fixation in rice, would it work in the field? This is not an unreasonable doubt as it has been observed that (i) often the rhizobia modified for superior performance in laboratory or in green house fail to establish themselves in nature (Bennet and Ladha, 1992), and (ii) the problems of achieving high levels of nodulation and N_2-fixation in legumes themselves (Keysar and Li, 1992).

Further, Saikia and Jain (2007) postulated that, no matter whether on leguminous plants or non-leguminous plants, root nodules once formed, may be either effective or ineffective, this depends on: (i) whether or not the microbes living inside the nodules are capable of fixing N; (ii) whether or not the microbes and the host plant can form a highly harmonious symbiotic N-fixing system; and (iii) the environmental condition in which the host plant is growing.

Another concern aroused by the idea of nodulation in rice relates to competition between nodules and the rest of the plant for photosynthate (Bennette and Ladha, 1992).

(iii) Incorporation / Expression of N_2-fixation genes in rice: The possibility of directly engineering nitrogen fixation in rice lies in the successful incorporation of the essential *nif* genes for nitrogenase activity into the rice genome (Britto and Kronzucker, 2004). But, the question is which is the appropriate location, where *nif* genes to be introduced? Parakaran (1997) proposed two approaches, *viz.*, transformation of rice leaf and transformation of rice root. Merrick and Dixon (1984) and Dixon *et al.* (1997) suggested that, root plastids or chloroplasts be the most suitable intracellular locations for foreign *nif* genes, rather than cell nucleus. This hypothesis is based on the fact that, plastid genetics most closely resemble that of N_2-fixing prokaryotes (Whitfeld and Bottomley, 1983).

According to Fischer (2000), the components of action for directly engineering nitrogen fixation in rice also includes protection of nitrogenase from inactivation by oxygen and to ensure energy supply for nitrogen fixation without compromising on the yield.

The activity of the nitrogenase enzyme complex is typically suppressed by oxygen, which is virtually present in all plant cells (Dixon *et al.,* 1997). In legume nodules it is sequestered by the leghaemoglobin protein. Possible solution to this problem in rice may be limiting *nif* genes expression to rice plastids in rice, where photosynthetically produced oxygen is not present, or diurnally regulating expression in chloroplasts such that nitrogen fixation only occurs at night (Britto and Kronzucker, 2004). Ribbe *et al.* (1997) envisaged another solution like expression of oxygen-tolerant nitrogenase found in the bacterium *Streptomyces thermoautotrophicus*. But, the process of use of such unusual oxygen tolerant nitrogenase fixation in chloroplast (Ribbe *et al.*, 1997) has problems of dependence on superoxide stress (Roy and Srivastava, 2010).

Energy requirements for nitrogenase reaction need to come from cellular metabolic cycles in the form of adenosine triphosphate or ATP (Subha Rao, 1982) and for reducing every N_2 molecule 36 molecules of ATP are required (Shantharam and Mattoo,1997). Hence, localization of introduced *nif* genes within plastids / chloroplasts may have additional advantage, as the substantial energetic cost of nitrogen fixation be met directly through photosynthates (Merrick and Dixon,1984).

Considering above, it may be inferred that, the engineering of BNF in rice, though appears to be surmountable, remains a distant possibility till date. On the other hand, by contrast, simultaneous expression of a group of related enzymes that influence both nitrogen and carbon metabolisms appears to be more promising (Britto and Kronzucker, 2004). This optimism is partly because of recent successes that have been achieved in rice and other plant species through the overexpression of glutamine synthetase (GS), which catalyzes the incorporation of NH_4^+ into amino acids (Gallardo *et al.*, 1999; Habash *et al.*,2001) and glutamate dehydrognase (GDH), another enzyme that brings ammonium nitrogen into the amino acid pool (Ameziane *et al.*, 2000). Further, the enhancement of specific links between nitrogen metabolism and photosynthetic functions (Britto and Kronzucker, s2001, 2004), provides an intriguing and potential beneficial opportunity.

CONCLUSION

The natural endophytic association of rice with rhizobia (Ladha *et al.*, 1989, 1996; Yanni *et al.*,1997; Engelhard *et al.*,2000) and better growth of rice plant on inoculation of rhizobia (Yanni *et al.*,1997; Chaintreuil *et al.,* 2000; Roy and Srivastava, 2010;Yanni and Dazzo, 2010; Roy, 2013) represent major step forward in achieving the technically challenging goal of reducing dependence on the need for fertilizer-N without requiring highly developed system as the root nodule *Rhizobium*-legume symbiosis. However, this understanding only is beginning, which needed to be explored (Yanni *et al.,* 1997). The idea of nodulation in rice is also considered not as an entirely absurd notion, given the comparatively recent discoveries of rhizobial nodulation of the non-legume *Parasponia* and nonrhizobial nodulation by *Frankia* (Bennet and Ladha, 1992). Inspite of great progress has been made toward characterization of the genes involved in this process and the functions of the protein they express (Stougaard, 2001; Geurts and Bisseling, 2002; Trevaskis *et al.,* 2002), there is still large gaps in our knowledge and extensive hurdles (de Bruijn *et al.*,1995) and therefore, number of researchers opined that, more knowledge about the plant genes involved in nodulation and the symbiotic nitrogen fixation and the function of their gene products is needed before the development of true nodulation of cereals (de Bruijn *et al.*,1995; Kennedy *et al.*,1997; Reddy *et al.*,1997; Webster *et al.,* 1997). In comparison, possibly larger challenge is directly engineering nitrogen fixation in rice (Britto and Knonzucker, 2004). de Bruijn *et al.* (1995) and Dixon *et al.* (1997) too considered it even more complex. According to Stoltzfus *et al.*(1997), though most of the genes necessary for nitrogen fixation in bacteria are well characterized, the transfer of the genes to the plant genome, along with the appropriate expression of all these genes is beyond the current technical ability. In addition, the creation of the proper environment, in terms of oxygen concentration / supply, energy provision to the bacteria, and efficient ammonia assimilation within the plant cells present serious problems (de Bruijn, *et al.,* 1995; Dixon *et al.,* 1997). Therefore, it will likely require many years of intensive research and development before a useful product making for field trial is possible (Britto and Kronzucker, 2004). On the other hand, among the three choices, it appears that, employment of endophytic nitrogen fixing bacteria involve fewest technical challenges (Stoltzfus *et al.*, 1997). Hence, in the near future, enhancing rice nitrogen status by optimizing associations between rice and naturally colonizing endophytic bacteria may be more promising (Britto and Kronzucker, 2004). Even if an identified stably endophytic microbe do not have the capacity to fix nitrogen, the process of introducing and expressing the *nif* gene complement in such an endophyte would be significantly easier than engineering the rice plant itself to fix nitrogen (Stoltzfus *et al.*, 1997).

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