

Towards nitrogen autotrophic rice

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Nitrogen nutrition most frequently limits the crop productivity. Chemical nitrogen fertilizers being ecologically and economically expensive, interest in exploitation of alternative or supplementary nitrogen sources has been renewed to encourage sustainable agriculture. Reducing the fertilizer nitrogen use while maintaining the native soil nitrogen resources and enhancing crop nitrogen output by biological nitrogen-fixation systems is desirable from both environmental and economic perspectives. Such a production system would enhance agricultural production in the long term in a way that is economically viable and ecologically sound, as well as socially acceptable. Towards the sustainable rice production, rice cultivar with better nitrogen use efficiency is one of the options. In the case of nitrogen inputs into rice production systems, it may be the judicious use of nitrogen fertilizers and/or nitrogen derived from atmospheric nitrogen via biological nitrogen fixation. Developing rice with biological nitrogen fixation capacity, i.e. autotrophy for nitrogen, could help farmers overcome the nitrogen nutrition limitations and increase the productivity, reducing dependency on chemical nitrogen fertilizers. The various options and the pros and cons of such options are discussed herein, as a part of an approach to the prospect of achieving nitrogen autotrophic rice.

CEREALS are the world's major source of food for human nutrition. Among these, rice is very important and represents the staple diet for more than two-fifths (2.4 billion) of the world's population, making it the most important food crop of the developing world¹. The challenge of feeding the growing population has to be met with in a sustainable as well as equitable basis. Sustainable production of rice and hence the global food security, depends on reaching even higher levels of production which is not possible without additional nutritional input. In future, more rice is to be produced from less land, at a minimum cost, with reduced use of environmentally adverse inputs.

Nitrogen (N) is the nutrient that most frequently limits the agricultural production. Global agriculture now relies heavily on N fertilizers derived at the expense of petroleum that is vulnerable to the political and economic fluctuations in the oil markets. N fertilizers, therefore, are an expensive input, costing agriculture more than US \$ 45 billion per year². The demand for N fertilizers in world agriculture is increasing at a rate approximately equivalent to the rate of increase in the world population, i.e. about two per cent per annum³. In rice, it takes about 1 kg of N to produce 15 ~ 20 kg of grain⁴. Low input efficiency of N fertilizer, decline in crop yield under conti-

nuous cropping, nitrate pollution, acidification of soils and emission of greenhouse gases such as ammonia and nitrous oxide, are some economic and environmental factors that must be considered when using chemical N fertilizers⁵. In addition, run-down of N supply capacity of agricultural soils, lack of purchase power of impoverished farmers, gap between the researchers and farmers in N management of crops and soils for maximum production efficiency, have confounded the situation still further⁶. These concerns have renewed interest in exploiting alternative or supplementary N sources to encourage sustainable agriculture.

A fundamental shift has taken place in agricultural research and world food production. In the past, the principal driving force was to increase the yield potential of food crops and to maximize productivity. Today, the drive for productivity is increasingly combined with a desire for environmentally sustainable development, with use of renewable resources. For farming systems to remain productive and to be sustainable, it will be necessary to replenish the resources of nutrients which are removed or lost from the soil. Nowhere is this challenge more intense than in supplying rice with nitrogen nutrition².

Producing rice cultivars with better N-use efficiency is one of the options towards the sustainable rice production. In the case of nitrogen inputs into rice production (Figure 1), it may be the judicious use of N fertilizers or N derived from atmospheric N via biological nitrogen fixation (BNF).

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Rice genotypes with high N-use efficiency

The green revolution in agriculture of the developing world, which resulted in large increases in cereal grain production since the 1960s, has been a result of the development of plant genotypes highly responsive to chemical fertilizers, especially N fertilizers. The efficiency of N acquisition from soil solution, i.e. N acquisition efficiency (NAE) and N utilization efficiency (NUE) in rice plant are central to its overall yielding potential⁶. Improvement in these efficiencies can reduce N fertilizer requirement of the crop.

Rice plants can absorb fertilizer N from flood water at a very high rate. The superficial root mat in the top few millimetres of soil captures a high proportion of ammonium ions (NH₄⁺), but most, if not all, of the root length is active in the uptake. Breeders selecting for N fertilizer responsiveness involving improving the acquisition of N from soil have favoured a well-developed root-mat⁶, a crucial trait in relation to the NAE. It is also possible that significant quantities of N are absorbed from the soil as amino acids, as indicated by the existence of amino acid transportation systems in plant roots⁷. To increase the acquisition of N, root systems can be genetically modified for better absorption and assimilation⁶.

Improving NUE involves enhancing the internal utilization of N by the plant. Internal utilization of N can be enhanced by better distribution of N between canopy (leaves, stem) and roots; better photosynthetic rate/unit leaf N, reduced leaf senescence, higher N content in grain, better sink capacity, higher harvest index and reduction in unproductive tillers⁸.

The ability of non-leguminous plants to stimulate N fixation in their rhizosphere is known as N fixation supportive (NFS) trait⁹. Genetic variability for NFS trait exists in rice^{8,10}. The trait is heritable¹¹, selectable⁶ and

can be used in breeding rice genotypes with high BNF. However, only the genotypes with appropriate NUE traits can utilize N supply from the stimulated associative N fixation⁶.

Restriction fragment length polymorphism (RFLP) analysis in rice indicates that the NFS trait in rhizosphere is controlled by multiple genes or the quantitative trait loci (QTLs) with recessive effects, on chromosomes 1, 3, 6 and 11 (ref. 12). Some of the rice genotypes with high NFS also had significantly higher N uptake and grain yield; but some genotypes with superior NFS were not superior for grain yield⁶. So, it is important to consider grain yield in addition to NFS for selection of genotypes. Differences in both N acquisition and physiological efficiency exist within rice genotypes with similar growth duration. Efficient lines achieve a maximum yield with less N acquisition compared to inefficient lines. N acquisition (better NAE) seems to be more important than physiological efficiency (i.e. NUE). A genotype possessing high NFS would diminish the need for further N, but would have no further impact on other cultural practices. Adoption of such cultivars involves no additional cost, is rapid and existing cropping and soil-water management systems are not affected⁶.

Chemical N fertilizers

The role of chemical N fertilizers in the green revolution, which more than tripled the rice production in India, is beyond dispute. Over the past four decades, farmers have become increasingly dependent on chemical sources of N for obtaining high grain yields. Nevertheless, a large number of farmers still use very little or no N fertilizer because of its non-availability when needed, lack of finances, and poor yield response due to adverse condi-

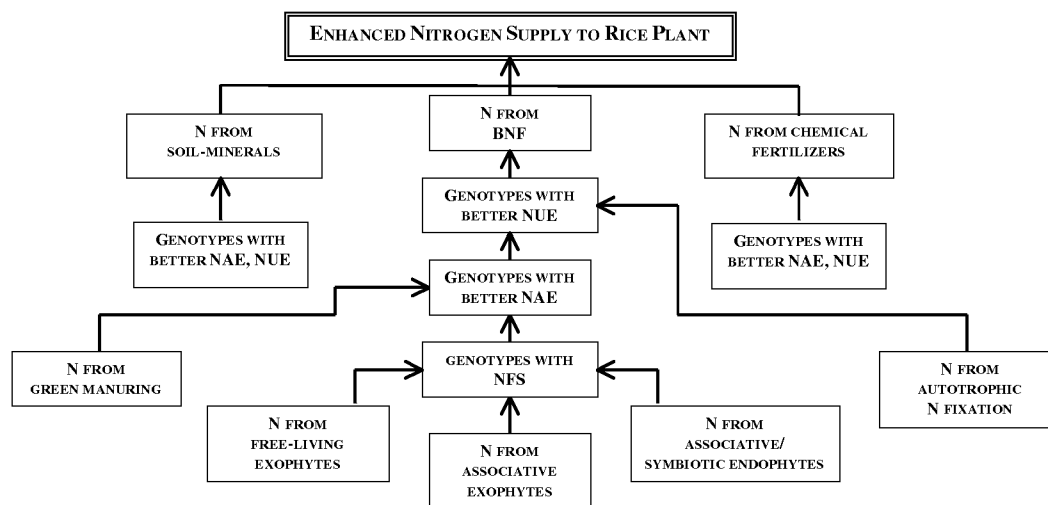


Figure 1. Schematic representation of the possible sources of nitrogen (N) nutrient to the rice plant and the genotypic factors involved. NAE, N acquisition efficiency traits; NFS, N fixation stimulation traits; NUE, N utilization efficiency traits (see the text for details).

tions². The use of N fertilizers though, has increased with time; the yields have often remained constant, implying a decline in yield response to N⁶. Therefore, it is essential to increase the efficiency of N use. The fertilizer use efficiency can be enhanced to a certain extent by improved technology of fertilizer forms such as super-granules, control release pellets, etc. and application technologies such as deep placement, multiple-split applications based on leaf chlorophyll levels, etc.

Currently, 10 ~ 12 million tonnes of chemical N fertilizers per year are used and in the next decade, it is expected that this figure would increase to 20 ~ 24 million tonnes¹³. In spite of an unlimited supply of N in the air, manufacture of 1 kg of N fertilizer would need six times more energy than that needed for the production of either phosphorous (P) or potassium (K) fertilizers¹⁴. Annual production of $\sim 77 \times 10^6$ t of ammoniacal N requires $\sim 0.1 \times 10^9$ t of oil equivalent per year¹⁵ at the rate of an average of 1.3 t of oil (or equivalent of that much energy) to fix 1 t of ammonia⁴, amounting to $\sim 544 \times 10^9$ MJ of fossil fuel energy per year for N fertilizers^{16,17}.

The major concerns about chemical N fertilizers are the resource availability and sustainability, which are seriously threatened by possible increase in oil price and the estimation that current oil resources would last only for about the next 50 years¹⁵. Currently, the level of recovery efficiency for N fertilizers is only 34–40 per cent¹³, leading to loss of 50–70 per cent applied chemical N fertilizers through leaching and runoff, denitrification and ammonia volatilization, because rice is grown under an environment conducive for N losses². These losses lead to environmental pollution, greenhouse effect and even destruction of stratospheric ozone layer. Thus, chemical N fertilizers not only deplete non-renewable resources, but also pose human and environmental hazards.

As the fossil fuel prices are not likely to remain at their present low levels for more than a decade, with ever increasing demand for food, fibre and energy from the growing human population of the tropics, high dependence on and continuous use of chemical N fertilizers may not be a sustainable option. In addition, it may pose a great risk to the Third World in future¹⁸.

Biological nitrogen fixation

Reducing N fertilizer use while maintaining the native soil N resources and enhancing crop N output by N fixation systems is desirable from both environmental and economic perspectives. Biological nitrogen fixation (BNF)-based farming systems would enhance agricultural production in the long term in a way that is economically viable and socially acceptable.

BNF is the principal mechanism of N supply in natural ecosystems and to a large extent for agriculture in

the tropics¹⁹. In many tropical soils, owing to low ion exchange and water-holding capacity of the mineral fraction, soil fertility is closely associated with soil organic content. As N is the element most easily lost when mineralization of soil organic matter is stimulated by land clearing and ploughing, it is often this effect that controls the organic matter content of the soil and hence its fertility and need for fertilization¹⁸.

Rice suffers from mismatch of its N demand and N supplied as fertilizer, resulting in severe loss of applied N fertilizer. In case of rice crop, N sources other than chemical fertilizers could be in the form of green manuring, free-living, associative exophytic, associative/symbiotic endophytic systems, and N autotrophic rice plant.

Green manuring

Green manuring is the most attractive and simplest alternative. The green manuring systems relevant to rice are the aquatic fern *Azolla* and semi-aquatic legumes such as *Sesbania*, *Aeschynomene*, *Astragalus*, etc. These are either grown during the 45–60 d fallow period between rice crops or supplied *ex situ*.

The water fern *Azolla* that harbours N fixing blue-green alga *Anabaena* can provide an average of 70 kg N/ha/crop, ranging from 20 to 150 kg N/ha²⁰. The semi-aquatic legumes can provide 40–230 kg N/ha, roughly sufficient to produce rice yield of 6–8 t grains/ha²¹. However, green manuring is not popular due to additional costs of labour, land opportunity, irrigation, inoculum/seed, additional P fertilizing, pesticides, etc. In addition, green manuring also enhances the emission of methane, contributing to the greenhouse effect².

Diazotrophic systems

The microbes capable of BNF are termed as 'diazotrophs', as these convert atmospheric di-nitrogen (N₂) to ammonia (NH₃) by electron reduction and protonation of gaseous N₂. The nitrogenase enzyme complex in diazotrophs is mainly responsible for such N fixation activity. These diazotrophs comprise of diverse N-fixing microbes, envisaging aerobes (e.g. *Azotobacter*, *Beijerinckia*, *Derrxia*, etc.), facultative anaerobes (e.g. *Clostridium*, *Pseudomonas*, *Rhizobium* etc.), heterotrophs (e.g. *Klebsiella*, *Enterobacter*, etc.) and phototrophs (e.g. *Anabaena*, *Azospirillum*, *Nostock*, etc.).

Based on the spatial and functional relationship between these diazotrophs and the host plant, the diazotrophic systems are broadly grouped into exophytic diazotrophic systems (when the diazotrophs remain outside the host plant) and endophytic diazotrophic systems (when the diazotrophs are found within the host plant). Further, the exophytic systems could be free-living (when the diazotrophs are not in direct contact with the host plant), associative (when the diazotrophs reside mostly

on the external surface, but at times within the host plant, in the intercellular space), or symbiotic (when the diazotrophs reside intracellularly, i.e. within the cells of the host plant) and the hypothesized autotrophic system (wherein the cells of the host plant are capable of diazotrophy). Figure 2 presents the classification of relevant diazotrophic systems. However, it should be kept in mind that the classification scheme is not very rigid.

Free-living diazotrophic systems

The major free-living BNF systems in rice fields are cyanobacteria and photosynthetic bacteria that inhabit water and soil and heterotrophic bacteria in the root zone. These have low to moderate N fixation potential, but the N fixed being outside the plant, is subjected to losses and also not immediately available to the plant²².

The cyanobacterial BNF may contribute from 10–80 kg N/ha/crop with an average of 30 kg N/ha/crop²³. The heterotrophic bacterial BNF can contribute 7 kg N/ha/crop, ranging from 11–16 kg N/ha¹⁰, contributing only 16–21 per cent of total N need of rice crop²⁴. Benign organisms such as free-living diazotrophs, in addition to fixing atmospheric N, compete for space with pathogens, thereby reducing the diseases. Such organisms, which are also capable of dissolving forms of P otherwise unavailable for plants, and cellulolytic organisms, are currently under-utilized and can have a potential role in rice crop production systems²⁵.

Associative exophytic diazotrophic systems

Some of the diazotrophs in the rhizosphere tend to associate with roots in response to a specific attractant excreted by the roots and favourable environmental conditions in the rhizosphere. Their association being limited to the root surface, these are known as exophytic diazotrophic systems. Unlike the C₄ plants where malic acid is the most important attractant to diazotrophs²⁶, in the C₃ rice plant, the main components of root exudates are citric and oxalic acids²⁷. However, 80 per cent of the bacteria associated with rice roots are found to be N fixing²⁸, leading to the questions, what component/s of rice root exudates is/are responsible? Whether there exists any genotypic variation for this? What type of diazotrophs proliferate on/in rice roots? What is the genetic basis of the mechanism¹²?

Although the contribution of associative N fixation to the N requirement of rice plant is small, it occurs mostly during heading stage when the availability of both soil and fertilizer N is low⁸. One major limitation of associative N fixation is that the diazotrophs in rhizo/phyllosphere of plants utilize the products of N fixation for their own growth, but release only a little while they are alive²⁹. Another important limitation is that the diazotrophs colonize mainly the surface, remaining vulnerable to competition from other rhizo/phyllospheric microbes³⁰. The ‘biased rhizosphere’⁴ approach is an attempt to ameliorate this situation by the use of transgenic host plant secreting a specific nutrient in its rhizosphere that can be utilized

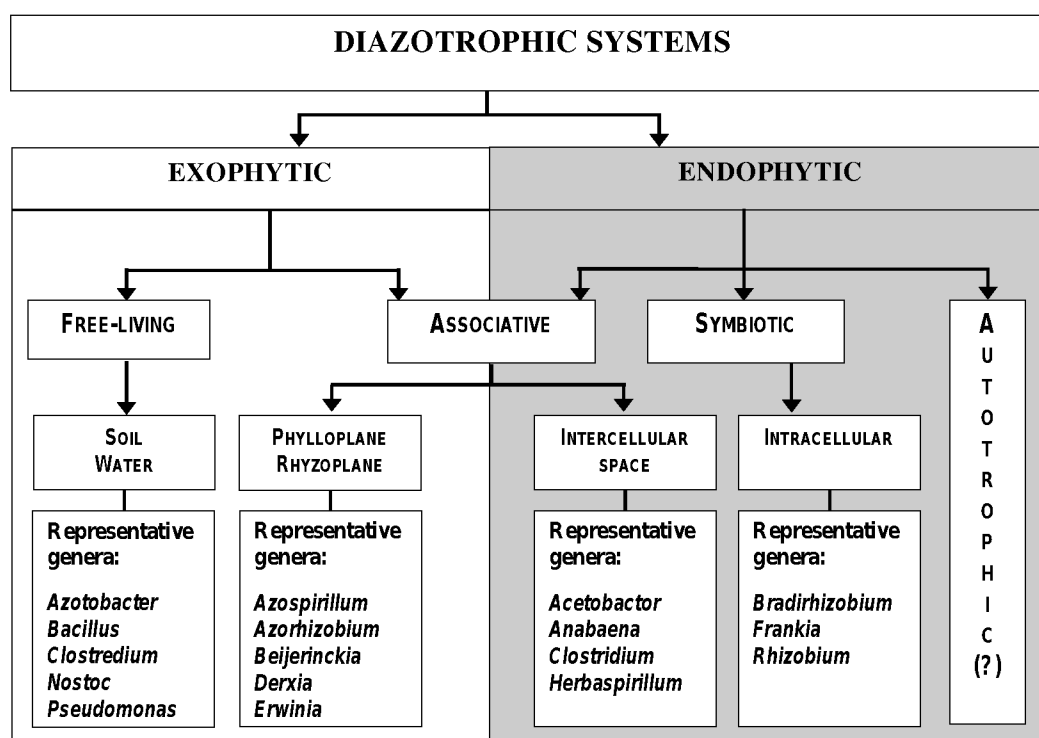


Figure 2. Diazotrophic systems (see the text for details).

only by the engineered microbes containing the genes to metabolize the specific nutrient.

Associative/symbiotic endophytic diazotrophic systems

If a BNF system is assembled within rice plant, fixed N would be available directly to the plant with little or no loss, leading to production of grain yield more than 8 t/ha, in rice genotypes with effective N utilization, besides ensuing no additional economic burden on farmers⁶.

Roots of healthy plants grown in natural soil eventually develop a continuum of root-associated micro-organisms extending from the rhizosphere to the rhizoplane, and even deeper into the epidermis, cortex, endodermis and vascular system²⁵. Typically, the presence of these microbes within roots does not induce obvious symptoms of disease. This endophytic habitat has already been identified as an important reservoir for isolation of N-fixing plant growth-promoting rhizobacteria (PGPR). Presumably, nature selects endophytes that are competitively fit to occupy compatible niches within this nutritionally-enriched and protected habitat of the root interior, without causing pathological symptoms on the host plant³¹.

Advantages of endophytic association are that it protects the diazotrophs from competition; it offers more intimate metabolic exchange with the host plant in terms of reliable supply of metabolites (mostly the products of photosynthesis by the host), protection against very high oxygen concentration and transportation of fixed N to the host³².

Endophytic associations in rice have a N-fixing potential of ~ 150 kg N/ha/yr³³, but are at times unreliable. Some of the common endophytes of rice are: *Acetobacter diazotrophicus*³⁴, *Alkaligenes faecalis*³⁵, *Azoarcus*³⁶, *Azorhizobium caulinodans*³⁰, *Azospirillum brasilense*³⁷, *Enterobacter*³⁸, *Herbaspirillum*³⁹, *Pseudomonas diazotrophicus*³⁸, etc.

Inoculation with *Herbaspirillum* indicates that the endophytic diazotrophs can readily be introduced by application of bacterial culture on seed prior to germination⁴⁰. Infection occurs through roots as well as via stomata and the diazotrophs are translocated through the xylem to all parts of the plant. However, no potential N fixing endophyte has been conclusively shown to benefit the plant via fixed N alone².

Symbiotic N fixation system, as exemplified by the celebrated legume–rhizobium symbiosis envisages a dinitrogen-fixing microbe going into partnership with a provider of ideal niche for efficient BNF, in the form of nodules. In legumes, a sophisticated mechanism of nodulation has been evolved. Nodulation begins with secretion of flavonoids from the roots that trigger *nod* gene expression in diazotrophs, leading to production of Nod factors by the diazotrophs. The Nod factors induce root-hair deformation and promote the processes involved in initia-

tion of infection threads and cortical cell division during early nodule development. Nodules take sucrose from the phloem, convert it to succinate and through bacterial respiration generate the ATP and reduced ferredoxin required for conversion of N₂ to ammonia. The plant component of the nodule takes up the ammonia and assimilates it into glutamine and asparagine in temperate legumes or into the ureides allantoic acid and allantoin in tropical legumes⁴¹. The assimilates are then exported to the rest of the plant via the xylem.

Since the report of nodulation in non-legumes *Parasponia*⁴², *Casurina*, *Alnus*, *Myrica*, oil-seed rape⁴³, etc. it has been a cherished goal in the field of BNF to extend this type of symbiosis to presently non-nodulating cereals. There are several reports of induction of nodule-like structures, designated as paranodules⁴⁴, on the roots of wheat, rice, maize and barley⁴⁵. Interestingly, these paranodules have been shown to be colonized by bacteria^{45,46} and also that the colonizers fix N within the paranodules⁴⁷.

Paranodules can be induced by the use of plant growth regulators^{45,48}, cellulose degrading enzymes followed by inoculation with microbes⁴⁹, inoculation with engineered rhizobial strains⁵⁰, inoculation with microbial strains assisted with bio-flavonoids such as acetosyringone⁵¹, narenginin⁵², etc. or simply by microbial inoculation^{53,54}. Nodule-like structures/hypertrophies are induced on rice roots at low frequency (0.1 to 0.2% of the plants). These structures originate from the lateral root-primordia^{48,55,56}.

Paranodulation response in rice has been shown to be a heritable trait with lot of genetic variability⁵⁶. In rice genotypes, paranodulation is under a digenic complementary interaction⁵⁷. Also, there seems to be preferential colonization of the paranodules when different strains of diazotrophs are used for inoculation of the paranodules⁵⁷. The utility of the paranodulation would hinge upon the agronomic utility of the trait that remains to be established yet. This leads to a pertinent question about competition between the nodules and the rest of the plant for photosynthate. Competition of this nature exists in legumes⁵⁸. However, rice being a low storage protein crop, much lower rates of N₂ fixation than legumes would suffice, with less drain on the plant's photosynthates. So, it may not result in reduction in grain yield, if rice plants nodulate to support N fixation²².

In non-legumes, less specialized mode of entry is observed where the diazotrophs enter the roots through epidermis or cracks created at the site of emerging lateral roots⁵⁹. The crack-entry mode facilitates bypassing the cellular machinery needed for sophisticated type of infection through root hairs. Use of *lacZ* or *uidA* (GUS)-tagged *Azorhizobium caulinodans* strain ORS571 (ref. 60) and *Herbaspirillum seropedicae* strain Z67 (ref. 61) shows colonization in the intercellular spaces in sub-epidermal and cortical cell zones of roots as detected by the expression of the GUS activity.

In legumes, the early nodulation (*ENOD*) genes accomplish the process of initiation of nodule induction. Sequences homologous to *ENOD* genes have been detected in rice^{60,62}. Unlike in legumes, Nod factors are not essential for rhizobial infection in rice. However, root exudates of a few rice genotypes could induce *nod* gene expression to a very low extent in few rhizobial strains²². Only few rhizobial strains could induce marginal deformation of root hair in few rice genotypes^{22,63}.

Efforts to induce preferred artificial sites of microbial colonization on rice roots (such as paranodes) now seem to be unnecessary as colonization of rice roots naturally occurs at the sites of lateral root emergence and the inoculum itself appears to be inducing the formation of lateral roots².

N autotrophic system

Achieving N autotrophy by rice plant is a highly ambitious prospect, needing probably at least a decade to realize. It demands extensive engineering of nuclear and extra-nuclear genes⁶⁴.

Transfer of N fixation (*nif*) genes into rice genome would involve transfer of at least 16 genes⁶⁴, viz. *nifD*, *nifH* and *nifK* genes encoding the three polypeptides of nitrogenase; *nifB*, *nifE*, *nifN*, *nifQ*, *nifV* and *nifY* involved in the synthesis of Fe–Mo co-protein; and *nifM*, *nifS* and *nifU* involved in the synthesis of active Fe protein; *nifF* and *nifJ* involved in electron transport; and *nifA* and *nifL* involved in activation and repression, respectively. The transfer should also take care of providing sufficient energy for their functioning and also protection of nitrogenase from being inactivated by oxygen.

Chloroplasts (cp) appear to provide the most suitable environment for *nif* gene expression in a plant cell⁶⁵, because the signals for transcription and translation in cp most closely resemble those of prokaryotes. The *nif*-encoded polypeptides are more stable when expressed in the cp compared to cytosol⁶. However, it may be necessary to substitute each of the promoters in the *nif* genes with appropriate cp promoter or some suitable analogue recognized by cpRNA-polymerase for proper expression and localization of the nitrogenase activity in such parts of cp where it is relatively anaerobic. N fixation in cp may also allow some of the energy costs of N assimilation to be met through the use of photosynthetically produced reductants⁴. Alternatively, N fixation may be temporally separated from photosynthesis by limiting its expression to dark periods, or spatially regulated by making the expression confined to the amyloplasts of roots, which are non-photosynthetic⁶⁴.

Stable cp transformation system in higher plants^{66,67} has heightened the expectations of introducing the *nif* genes directly into rice chloroplasts.

BNF is dependent upon physical, environmental, nutritional and biological factors. So mere inclusion of any N-fixing plant system does not guarantee increased contributions to the soil N pool. It would be pure speculation to predict what would be the fertilizer recommendation for a rice crop with BNF capacity. Whatever it may be, it would certainly not be nil¹⁵.

Thus, recent advances in understanding the symbiotic relationship as applicable to BNF and advent of powerful molecular engineering bio-techniques have created an excellent opportunity to investigate the possibilities of incorporating N fixation capability into rice. As early as 1992, a workshop was organized by the International Rice Research Institute (IRRI), which affirmed such opportunities do exist for cereals and recommended that rice be used as a model system⁶⁸. Subsequently, in 1994 IRRI developed a Frontier Project on assessing opportunities for nitrogen fixation in rice². The International Rice BNF group is given the responsibility to coordinate the worldwide collaborative efforts at reducing dependency of rice on mineral N resources. This certainly has marked the dawn of an epoch committed to N autotrophic rice.

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