Reviews

Nitrogen fixation in agriculture: perspectives

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The importance of nitrogen in plants is shown by the fact that it is the fourth, most aboundant element in plants after carbon, oxygen and hydrogen. Nitrogen is present in nature in several forms. A continuous interconversion of these forms via physical and biological processes constitutes the nitrogen cycle. Most plants obtain their cellular nitrogen from nitrate and ammonium in the soil or water. In order to maintain a high agricultural productivity millions of tons of nitrogen fertilizer are used annually. Apart from being expensive, the overuse of fertilizer nitrogen gives grounds for to concern because of its polluting effects on the environment. The increased use of chemical fertilizer in developed countries has led to leaching of toxic nitrates into groundwaters and volatilization of nitrogen oxides into the atmosphere.

To be incorporated into organic matter, nitrogen must be converted to ammonium. The ability to directly utilize atmospheric nitrogen is restricted to a few groups of prokaryotic organisms. Among the principal N_2-fixers are a few groups of prokaryotic organisms (diazotrophs) including certain free-living soil bacteria, free-living cyanobacteria (blue-green algae) on soil surfaces or on water, cyanobacteria in symbiotic associations with fungi in lichens or with ferns, mosses and liverworts. Legume plant species have overcome this limitation by virtue of a highly organized interaction developed with nitrogen-fixing bacteria of the family *Rhizobiaceae*. The plants developed specialized organs, root or stem nodules, for housing the bacteroids (symbiotic forms of rhizobia) and providing the appropriate environment as well as nutrients to support biological fixation of atmospheric nitrogen (Mylona et al., 1995). In agronomical terms, a potential benefit of symbiotic nitrogen fixation is enormous since it accounts for 60 – 65% of nitrogen currently used in agriculture. It is estimated that this value might even be larger in the future when new, efficient systems of sustainable agriculture will be introduced.

To maintain a present — day protein consumption by the Earth's population (about 70 g of protein per day, i.e. 23 million tons of pure nitrogen per annum for the population of 5.3 billion people) it will be necessary to double or triple crop production over the next 30-50 years. Such increase of production will require development of more productive agricultural systems and utilization of large areas now considered as marginal for meaningful production. This will need to be achieved despite significant deterioration of prime agricultural land.

1. Genetic improvement of the host

Most legume plant species studied so far exhibit genetic variability to fix nitrogen. These have included differences in root nodule number and mass, host restriction of nodulation, seasonal accumulation of nitrogen and different enzyme function associated with nitrogen assimilation. Some researchers suggest that an increase in biological fixation of nitrogen could be achieved through plant selection and rational crop management.

It is estimated that only about 15% of nearly 20 000 species in the *Leguminoseae* family have been examined for N_2 fixation and approximately 90% of these have root nodules in which fixation occurs (Allen, Allen, 1981). Important nonlegumes that fix nitrogen are primarily trees and shrubs, including members of the genera *Alnus* (alder), *Myrica* (e.g. *M. gale*, the bog myrtle), *Shepherdia, Ceanothus, Eleagnus, Coriaria, Casuarina* and others. There is a considerable interest among the foresters to select and breed nitrogen-fixing nonlegume trees that can be planted together with or before more economical important trees.

Plant breeders have been very successful in selection of the most effective plant species by using phenotypic observation or measurements. Sometimes, however, this approach is difficult due to strong interactions with the environment, genetic complexity of the trait and delayed expression of the trait often postponed to the next generation. It is therefore so important to include into genetic analysis modern diagnostic tools such as RFLP markers, microsatellite markers simple repeats based on PCR amplification etc.

Plant genetic analysis of nodulation and the ability to form effective symbiosis has been restricted by the absence of sufficient genetic variation. The task of analyzing a legume genome is also difficult because legume plants usually have large genomes (diploid or tetraploid) with high content of repeated gene sequences and duplicated biochemical pathways.

The studies of plant genes involved in symbiotic nitrogen fixation are now based on the analysis of cDNA and genomic libraries and identification of sequences coding for nodulins (nodule-specific proteins). The analysis of this group of genes addresses fundamental questions of general biology such as:

— plant differentiation and development,
— plant-microbe interaction and communication signalling,
— gene expression and regulation of cell division,
— plant immune response.

Selection of induced mutants of legume plants with well defined genotypes is now one of the most important lines of research. In 1985, plant mutations were discovered in soybean that either increased (supernodulation) or decreased the nodule number on plant root (Carrol et al., 1985). Genetic analysis of these mutations induced by chemical mutagens suggests that they are located at separate loci and are inherited as single recessive Mendelian genes.

All supernodulation mutants in soybean share essential features such as shoot control of the phenotype and apparent tolerance to nodulation inhibiting nitrate levels (*nts*). Although not proven yet, it appears that all supernodulation mutants are mutated at the same locus. Since it seems difficult to approach *nts* locus by biochemical analysis involving the isolation of a diagnostic protein, or the use of homologous clones from other plants, a set of genetic steps has been proposed to identify the *nts* locus. The strategy is called "positional cloning" and can also be applied to other legumes (Gresshoff, Landau-Ellis, 1994). Necessary steps in the positional cloning of a desired legume gene involve are the following steps:

— detection of phenotype and determination of inheritance;
— connection with molecular markers using RFLP (restriction fragment length polymorphism) and MAAP (multiple arbitrary amplicon profiling);
— determination of genetic and physical distances;
— isolation of YAC (yeast artificial chromosome) clone;
— ordering of regional YACs, end clones;
— functional complementation by transformation into mutant cells or embryos and isolation of causative DNA sequence with characterization.

At present, nodulation clones of legumes have not been obtained by positional cloning, but certain markers close to genes of interest have been identified. The availability of several RFLP and amplification markers in the region of supernodulation locus of soybean makes this region the first target for a detailed molecular map which should facilitate the isolation of the *nts* gene.

The described above and similar molecular approaches have quite significant value for plant breeding and agriculture and might overlap with the approaches applied by classical plant genetics to the analysis of legume plant genomes.

As far as plant improvement to enlarge nitrogen fixation is concerned, the following strategies in general terms could be considered:

— recognition and introduction into agriculture of new varieties fixing as much nitrogen as the best N_2-fixing germplasms currently available. Enzymes of C/N pathway are often used as indirect markers of biological effectiveness;

— appropriate estimation of productivity of naturally occurring symbiotic systems including trees based on adequate balance of nodule mass and number, xylem ureide/amide content, duration of the activity and nitrate tolerance;

— introduction of supernodulating mutants into agronomically important cultivars.

2. Genetic improvement of the microsymbiont

When legumes are planted into soil containing indigenous rhizobia, the numerical superiority and more uniform distribution of the residing microorganisms affect the probability of effective symbiotic interactions. This is usually attributed to competition for nodulation sites on legume roots. Rhizobial strain competitiveness *per sé* can be studied within a limited time period using mixtures of strains distributed uniformly throughout the test soil. The infection events observed for efficient strains are usually compared to the infections reported for inefficient strains producing fewer nodules or nodules less effective when tested for nitrogen binding, reduction and assimilation.

Genetic improvement of the microsymbiont involves various lines of research including:

— identification of physiological as well as molecular basis for specific preferences for microsymbiont and restrictions in nodulation;

— development of alternate inoculation procedures aimed at nodulation also lateral roots;

— increased duration and improved efficiency of nitrogen fixation in crown nodules formed on the main root;

— recognition of the origin of non-ineffective rhizobia in soil and determination of factors which regulate the frequency of certain *sym*-plasmids in soil rhizobia.

3. Effect of environmental stress on the efficiency of nitrogen fixation in naturally occurring symbiotic systems

There are many questions about stress tolerance in the symbiotic relationships between legumes and rhizobia which need to be resolved. They are particularly important for establishing of new systems of sustainable agriculture which will utilize the areas of more marginal production where legume crops are exposed to additional abiotic as well as biotic stresses. It is well recognized that rhizobia vary significantly in acid tolerance. For example, many alfalfa rhizobia do not grow below pH 5.6, while the majority of cowpea rhizobia and soybean isolates grow at pH 4.5 and *R. tropici* even at pH 4.0. To explain acid pH tolerance, various mechanisms including regulation of cytoplasmic pH and differences in the cell membrane permeability to hydrogen ions have been proposed. It should be mentioned here that acid pH tolerance increases the competitiveness of the rhizobia and their ability to nodulation in acid soils. It has not been recognized how acid pH affects the communication between host plant and rhizobia and how this is modified when either or both symbionts are pH tolerant. Most likely, general mechanisms of abiotic stress tolerance of symbiotic models are related to those in other nonlegume systems. This includes tolerance to various cations and anions content in the soil as well as the presence of different chemical agents.

Research performed on molecular, cellular as well as physiological levels clearly demonstrates the economic and environmental benefits from using biologically fixed nitrogen as a replacement for nitrogen supplied with chemical fertilizers (see Palacios et al., 1993).

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Summary

To understand a process as complex as biological nitrogen fixation investigations are now performed in many different areas: such as genetic improvement of the host plants and microsymbionts, organ differentiation, chemical communication between organisms, etc. Further studies are necessary to understand the regulatory mechanisms and environmental effects. New horizons for nitrogen fixation research include selection, identification and introduction of organisms with novel arrangements of genetic information into the environment. The strategy of transferring legume genes involved in providing the nitrogen-fixing capability to non-leguminous crops still appears to be a long way off.

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biological nitrogen fixation, symbiotic systems, genetic analysis, sustainable agriculture.

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