Efforts to Introduce Nitrogenase Gene into Chloroplast Genome in Non-Legume Plant

Since more than three decades ago when the *nif* genes were successfully transferred into *Escherichia coli* from the nitrogen fixing bacterium, *Klebsiella pneumoniae* (Dixon and Postgate 1972), the transfer of *nif* genes directly into plant cell to create diazotrophic plants has also been considered (Earl and Ausubel 1982). Several possible locations for expression of nitrogenase in the plant cell have been suggested (Merrick and Dixon 1984) and attempts to express *nif* genes in higher plants was achieved by targeting NifH or NifH plus NifM into tobacco chloroplasts, although in both cases NifH was found in the chloroplast stromal fraction at a very low level (Dowson-Day et al. 1991). *nif* genes not possible to express without *ntrA* gene because *ntrA* codes for sigma factor required for the recognition of *nif* genes promoter.

Expression of *nif* genes in chloroplast

There are advantages for the chloroplast being a potential environment for nitrogen fixation:

(i) the developed plastid carries out photosynthesis and thus provides a major source of ATP.

(ii) The chloroplast is also a major site for ammonia assimilation because both the glutamine synthetase (GS) and the glutamine oxoglutarate aminotransferase (GOGAT, also called glutamate synthase) pathways exist in the chloroplast.

(iii) From an engineering point of view, the plastid provides a convenient location for the introduction of *nif* genes because chloroplast genes are expressed in a prokaryotic-like fashion, allowing translation of polycistronic messages.

Chlamydomonas possesses a light-independent pathway for chlorophyll biosynthesis, with one of the enzymes in the pathway having potential structural and functional homologies to nitrogenase. The products of the *Chlamydomonas reinhardtii chlL*, *N* and *B* genes are structurally similar to the three subunits of nitrogenase, with the strongest sequence identity between *nifH* and *chlL* (nucleotide sequence 43%; putative amino acid sequence 35%). Therefore, the genes required for *chlL* protein activity might activate the *nifH* gene product to obtain Fe protein activity without the requirement for additional *nif* genes. Although regulation of the *chlL, N* and *B* genes is not yet clear, using the native *chl* system may

provide a strategy for the expression of *nifH* in an active form. In addition, the *nifH* gene product might substitute for the function of *chlL*. The first step was to precisely replace the coding region of *chlL* gene with that of *nifH* gene, keeping the untranslated regulatory regions intact.

One of the next possible approaches would be the replacement of the *chlN* and *chlB* genes with that of Mo-nitrogenase structural *nifDK* genes. Based on the similar assumption that the ChlNB complex may resemble the NifDK complex harboring a similar metal scaffold, provided by yet unknown DPOR biogenesis proteins in chloroplasts, the expectation is to develop a functional nitrogenase *in vivo*.

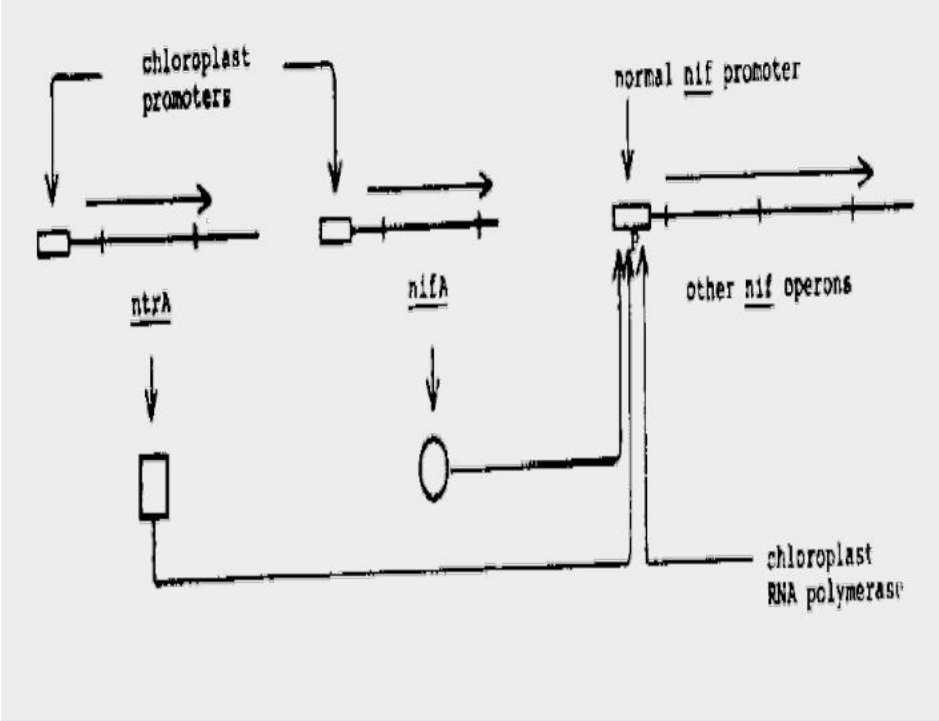

Figure 1. Strategy of *nif* genes transfer in plant chloroplast

But the major problems for doing so are:

(i) lack of chloroplast transferring techniques

(ii) protection of nitrogenase from O_2 evolved during photosynthesis

Expression of *nif* genes in mitochondria

As an alternative strategy, one might consider introducing nitrogenase proteins into mitochondria which may provide a suitable energy-rich, reducing environment to support nitrogenase. However, this introduces more complexity because each gene would have to be specifically modified to allow targeting each of the *nif* -encoded proteins into this organelle in the appropriate stoichiometry. Paradoxically, while the technology is available now to introduce and express nitrogenase component proteins in plant cells, substantial progress is limited by gaps in our fundamental knowledge of both plant and microbial physiology.