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**Review**

# Hydrogenase in *Bradyrhizobium japonicum*: genetics, regulation and effect on plant growth

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**A region of 15 kbp DNA located on the chromosome of *Bradyrhizobium japonicum* is essential for a $Hup^+$ phenotype and contains the hydrogenase structural genes. During the last few years, other genes with various functions related to hydrogenase oxidation, such as $Ni^{2+}$ incorporation into the hydrogenase enzyme, electron transport from hydrogenase to $O_2$, and regulation of hydrogenase expression in free-living conditions, have been identified in this region. A region in front of the hydrogenase structural genes is necessary for transcriptional regulation of hydrogenase expression by $O_2$, $H_2$ and $Ni^{2+}$. In addition, the $N_2$ fixation system and the tertiary structure of the chromosome seem to be involved in the expression of the hydrogenase genes. The effect of legume inoculation with $Hup^+$ rhizobia has been evaluated. The possible benefits of a $Hup^+$ phenotype, such as the regeneration of chemical energy in the form of ATP or reductants, and the removal of $O_2$ and $H_2$ from the active site of the nitrogenase enzyme where they might inhibit the nitrogenase reaction, are discussed. The data indicate that a $H_2$-uptake system is beneficial in soybean nodules, but host plant and environmental factors may interfere with the effects of $H_2$ cycling in the plant.**

*Key words: Bradyrhizobium*, hydrogenase, regulation, symbiosis.

During biological $N_2$ fixation, $N_2$ is reduced to $NH_3$ by the nitrogenase enzyme complex. As a by-product of this reaction, nitrogenase also evolves $H_2$, which adds considerably to the energy costs of the fixation. Some rhizobia possess an $O_2$-dependent enzyme system, termed 'uptake hydrogenase' (Hup), that is capable of recycling the $H_2$ released. The uptake-hydrogenase is a $Ni^{2+}$-containing enzyme, located in the periplasmic space, that re-oxidizes $H_2$ and transfers the electrons to $O_2$ through the electron transport chain. The enzyme is comprised of two subunits and is immunologically and structurally related to $Ni^{2+}$-containing hydrogenases in other bacteria.

Several reviews have been devoted to $H_2$ metabolism and recycling in the *Rhizobium*–legume symbiosis (Eisbrenner & Evans 1983; Maier 1986; Evans *et al.* 1987; Arp 1992). This review focuses on the more recent developments in research on the genetics, regulation and effect on plant growth of the hydrogenase in *Bradyrhizobium japonicum*.

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## Genetics of the Hup System

*Isolation of Hydrogenase Structural Genes*

The *hup* genes were first isolated by genetic complementation of a $Hup^-$ mutant for $H_2$ oxidation and chemolithotrophic growth. The complementing cosmid pHU1 contains chromosomal DNA from the $Hup^+$ strain *B. japonicum* USDA122DES (Cantrell *et al.* 1983; Haugland *et al.* 1984). Tn*5* mutagenesis of the insert DNA followed by marker exchange into the *B. japonicum* USDA122DES chromosome and phenotypic analysis indicated that about 15 kbp of pHU1 DNA contains *hup*-specific sequences, organized into at least two and probably three transcriptional units (Haugland *et al.* 1984). However, to confer Hup activity to free-living wildtype $Hup^-$ strains of *B. japonicum* and *Rhizobium meliloti*, a more extensive DNA region, present on cosmid pHU52, appeared to be necessary (Lambert *et al.*

1985). In *B. japonicum* and *R. meliloti* transconjugants harbouring pHU52, the 65.9 and 34.5 kDa polypeptide components of the uptake hydrogenase are synthesized under *hup*-derepressing conditions (Harker *et al.* 1985). Nevertheless, pHU1 on its own is sufficient for symbiotic hydrogenase activity in nodules formed by *B. japonicum* and *R. meliloti* transconjugants on soybean and alfalfa, respectively (Lambert *et al.* 1987). This indicates a difference in hydrogenase gene expression under free-living and symbiotic conditions.

Analysis of plasmid-encoded proteins and immunoblotting (Zuber *et al.* 1986) provided evidence for the presence of hydrogenase structural genes on a 5.9 kbp *Hin*dIII fragment, which is present in pHU52 and pHU1 (Figure 1). This was confirmed by Sayavedra-Soto *et al.* (1988), who published the nucleotide sequence of the *B. japonicum* hydrogenase structural genes. On the 5.9 kbp *Hin*dIII fragment, two open reading frames were identified, corresponding in size and deduced amino acid sequence to the hydrogenase subunits. The gene encoding the small subunit, *hupS*, is located upstream from the gene encoding the large subunit, *hupL*, and the two genes probably constitute an operon (Figure 1). A similar arrangement is found in a wide variety of hydrogenase-containing bacteria (Pryzbyla *et al.* 1992). The deduced amino acid sequence of the gene coding for the small subunit contains a leader peptide of 46 amino acids, in accordance with its localization in the cytoplasmic membrane. The deduced amino acid sequences of the *B. japonicum* hydrogenase structural genes display significant sequence similarity with the structural subunits of the hydrogenases from *Desulfovibrio gigas*, *Desulfovibrio baculatus* and *Rhodobacter capsulatus* (Sayavedra-Soto *et al.* 1988). Recently, homology with the hydrogenases of *Rhizobium leguminosarum* (Hidalgo *et al.* 1990), *Azotobacter chroococcum*, *Azotobacter vinelandii* and *Rhodococcus gelatinosus* (Pryzbyla *et al.* 1992) has also been reported.

In *Rhod. capsulatus* (Richaud *et al.* 1990), *Escherichia coli* (Menon *et al.* 1990b), *Az. vinelandii* (Menon *et al.* 1990a), *Wolinella succinogenes* (Dross *et al.* 1992) and *Rhiz. leguminosarum* (Hidalgo *et al.* 1992), the genes encoding the small and large subunits of the hydrogenase are followed by a third open reading frame, highly homologous in the different bacteria and encoding a hydrophobic polypeptide with four potential transmembrane regions. Partial sequence analysis of the region downstream of the *B. japonicum* gene encoding the large subunit reveals the start of an open reading frame that is strikingly homologous to this third open reading frame (Sayavedra-Soto *et al.* 1988). Very recently, the nucleotide sequence downstream of this region was determined further in *B. japonicum* CB1809, a derivative of strain USDA122DES (C. Van Soom, unpublished results). This confirmed the presence of a complete third gene, called *hupC* (Figure 1), which can encode a highly hydrophobic polypeptide with the four conserved potential membrane-spanning regions. In *Rhod. capsulatus* (Cauvin *et al.* 1991), *W. succinogenes* (Dross *et al.* 1992) and *Rhiz. leguminosarum* (Hidalgo *et al.* 1992), there are several indications that the product of this third open reading frame is a *b*-type cytochrome that is involved in the electron transport from the hydrogenase enzyme to the respiratory chain. In *B. japonicum*, it was proposed that a *b*-type cytochrome (component $b_{559}$) is specifically reduced by $H_2$ in vegetative and symbiotic cells, and functions as an electron carrier between hydrogenase and ubiquinone (Eisbrenner & Evans 1982a,b). Later, this hypothesis was questioned by the observation that cytochrome $b_{558}$ (cytocrome *b'* or cytochrome *o*) located after ubiquinone is not specifically reduced by $H_2$ in *B. japonicum* cells, and that no cytochrome component is located between hydrogenase and ubiquinone (O'Brian & Maier, 1982, 1985). Although the exact location of the presumed electron carrier in the electron transport chain that is reduced by $H_2$ remains unclear, the gene product of the third open reading frame probably encodes a *b*-type cytochrome needed to transfer electrons from the hydrogenase enzyme to $O_2$ (Hidalgo *et al.* 1992). Nucleotide sequence analysis downstream of *hupC* identified three additional genes, named *hupD*, *hupF* and *hupG*, (C. Van Soom, unpublished results) which share significant homology with genes downstream of the hydrogenase structural genes in *Rhiz. leguminosarum* (Hidalgo *et al.* 1992; Rey *et al.* 1992), *Alcaligenes eutrophus* (Kortlüke *et al.* 1992) and *E. coli* (Menon *et al.* 1990b; Lutz *et al.* 1991). Fu & Maier (1993) demonstrated that a *B. japonicum* deletion mutant of the 2.9 kbp *Eco*RI fragment, which is located to the right of the 13.2 kbp *Eco*RI fragment (Figure 1), is Hup$^-$ and produces unprocessed forms of the hydrogenase subunits. This fragment contains almost the entire *hupG* gene and possibly more downstream located genes (C. Van Soom, unpublished results). Together with the results of complementation analysis of this mutant with different restriction fragments (Fu & Maier 1993), these data support a role for the *hupG* gene product, and eventually for other genes further downstream, in the processing of the *B. japonicum* hydrogenase subunits.

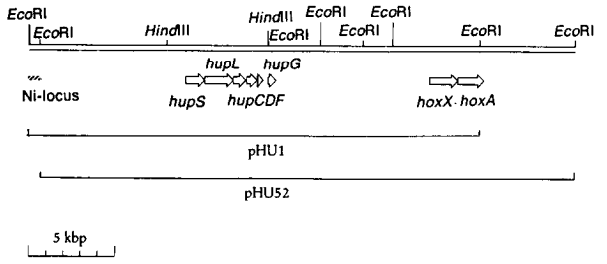


**Figure 1.** The *hup* region of *B. japonicum*, with indication of the identified genetic loci and the cosmids pHU1 and pHU52. For explanation and references see text.

Genes involved in hydrogenase oxidation in *B. japonicum* have also been isolated from other strains. From a *B. japonicum* I-110 genome library, Hom *et al.* (1985) isolated cosmid pSH22, which is able to complement a $Nif^-Hup^-$ mutant and a $Hup^-$ mutant strain. This indicates that the cosmid contains a gene involved in both nitrogenase and hydrogenase activities and at least one gene that is exclusively involved in $H_2$-uptake activity. Interestingly, the restriction patterns of the *nif/hup* cosmid pSH22 and the *hup* cosmid pHU1 are similar, but not identical (Hom *et al.* 1985). In order to characterize cosmid pSH22, different subclones were conjugated into various Tn5-induced *B. japonicum* mutants that were unable to grow chemolithotrophically (Hom *et al.* 1988). Two subclones of pSH22, one containing a 3.0 kbp *Eco*RI fragment and one a 13.2 kbp *Eco*RI fragment, could complement those mutants that were $Hup^-$ $Nif^+$, both in free-living and symbiotic conditions, to a $Hup^+$ phenotype. Thus, pSH22 must encode at least two separate diffusible products involved exclusively in the $Hup^+$ phenotype, one encoded by the 3.0 kbp *Eco*RI fragment and one encoded by the 13.2 kbp *Eco*RI fragment. Although the hydrogenase structural genes are located on the 13.2 kbp *Eco*RI fragment (Sayavedra-Soto *et al.* 1988), the mutation that is complemented by this fragment does not necessarily lie within the hydrogenase structural genes.

Recently, *hup*-containing cosmids were isolated from a *B. japonicum* CB1809 genome library by colony hydridization with synthetic oligonucleotides specific for the small and large subunits of the *B. japonicum* hydrogenase (Van Soom *et al.* 1993). The physical organization of the isolated cosmids pFAJ1002 and pFAJ1010 is identical to the maps of pHU1 and pHU52 (Van Soom *et al.* 1993). This is not surprising, since strain CB1809 is a derivative of strain USDA122 (P. Van Berkum, personal communication Cancun, 6–12 December 1992). The presence of *hup*-specific DNA on a 13.2 kb *Eco*RI fragment and a 5.9 kb *Hin*dIII fragment was confirmed by Southern hybridization with *Rhiz. leguminosarum hup* structural genes (Van Soom *et al.* 1993).

*Regulation of Hydrogenase Activity in Free-living Conditions*

Studies of the regulation of $H_2$ oxidation have predominantly relied upon the ability of $Hup^+$ strains to express hydrogenase activity in free-living culture, which requires incubation in a $H_2$-containing microaerobic atmosphere in the presence of $Ni^{2+}$ (Maier *et al.* 1978; Stults *et al.* 1986; Van Berkum 1987). Under these conditions, $Hup^+$ bradyrhizobia are able to grow chemolithotrophically (Hanus *et al.* 1979). Data on the ecological significance of free-living hydrogenase activity are limited. Viteri & Schmidt (1989) found that the soil serogroup 123 of *B. japonicum* outgrows serogroup 110 when the soil containing them is incubated under a $H_2$-containing microaerobic atmosphere. Since the $Hup^+$ phenotype is more common among isolates of serogroup 110 (Carter *et al.* 1978) than among those of serogroup 123 (Keyser *et al.* 1984), these data demonstrate that results obtained in the laboratory cannot always be used to predict behaviour in the field. All the results on the regulation of free-living hydrogenase activity discussed here were obtained under laboratory conditions. In studies on the regulation of hydrogenase activity, several classes of mutants have proven to be very useful. These mutants were reviewed by Maier (1986) and will not be discussed here.

The results obtained from regulation studies in free-living conditions cannot be extrapolated to symbiotic conditions, since the regulatory pathways seem to be different. As mentioned previously, cosmid pHU52 confers Hup activity in both free-living bacteria and nodules on different plant hosts, whereas pHU1 does not confer Hup activity in free-living bacteria but yields a $Hup^+$ phenotype in nodules on alfalfa and soybean (Lambert *et al.* 1987). Also, two Tn5 insertions within a 5.0 kbp *Eco*RI fragment in the chromosome of *B. japonicum* USDA122DES obtained by marker exchange with Tn5-mutagenized pHU1 abolished Hup activity in free-living bacteria but not in nodules. Based on the physical maps of pHU1 and pHU52, Lambert *et al.* (1987) suggested that this 5.0 kbp *Eco*RI fragment, which is located at the right-hand end of pHU1 (Figure 1), contains a gene or part of a gene that is required for Hup activity in free-living bacteria but not in nodules, and which extends into the neighbouring 5.5 kb *Eco*RI fragment of pHU52. Recently, this region was sequenced and it appears to contain open reading frames homologous with genes involved in the regulation of hydrogenase expression in other bacteria (Van Soom *et al.* 1993). The homology was most striking with the *hoxA* gene of *Alcaligenes eutrophus*, which encodes a transcriptional regulator of the NtrC family (Eberz & Friedrich 1991). Probably, this region in *B. japonicum* encodes a transcriptional regulator that is necessary for Hup activity in free-living conditions but not during symbiosis. The interaction between host plant and hydrogenase expression is discussed below.

*Regulation by Oxygen and Hydrogen.* In *B. japonicum*, hydrogenase activity is repressed by $O_2$ (Maier *et al.* 1979; Maier & Merberg 1982), whereas hydrogenase expression requires depression of cells in a $H_2$-containing atmosphere (Maier *et al.* 1979; Van Berkum 1987). Although similar observations have been made in other bacteria, only in *B. japonicum* has it been demonstrated that this regulation occurs at the transcriptional level. Kim *et al.* (1991) showed that a *cis*-acting region upstream of the hydrogenase structural genes is necessary for transcriptional regulation of hydrogenase expression by $O_2$, $H_2$ and $Ni^{2+}$.

*Regulation by Nickel.* Nickel has been shown to be a necessary trace element for chemolithotrophic growth and

for expression of hydrogenase activity in $Hup^+$ bradyrhizobia (Klucas *et al.* 1983). The *B. japonicum* hydrogenase, like many other hydrogenases, is a $Ni^{2+}$-containing enzyme (Stults *et al.* 1984; Arp 1985). In addition to its role in catalysis, presumably at the active site of $Ni^{2+}$-containing hydrogenases (Lancaster 1988), $Ni^{2+}$ appears to play a role in regulating the expression of hydrogenase synthesis in free-living *B. japonicum*, since the amount of antigenically detectable hydrogenase increases with the amount of $Ni^{2+}$ supplied to the cells (Stults *et al.* 1986). By means of expression studies with a *hup-lacZ* transcriptional-fusion-construct and quantitative RNAse protection experiments with hydrogenase-specific mRNA, Kim & Maier (1990) demonstrated that $Ni^{2+}$ is required for the synthesis of the hydrogenase mRNA in *B. japonicum*, and that a region upstream of the hydrogenase structural genes is responsible for transcriptional regulation. Moreover, this *cis*-acting region, extending up to −118 and −168 with respect to the transcriptional initiation site of the hydrogenase structural genes, was later also proven to be essential for transcriptional regulation of hydrogenase expression by $O_2$ and $H_2$ (Kim *et al.* 1991). Since a specific *cis*-acting region is shared by three components, a regulatory mechanism mediated by a single DNA-binding component can be assumed. Kim *et al.* (1991) proposed a hypothetical signal-transduction pathway, with a membrane-bound sensor-protein to detect $Ni^{2+}$, and another intracellular component to detect the redox condition in the cell, which is determined by the $O_2$ and $H_2$ concentrations. The hydrogenase protein itself cannot be the sensor since the regulatory effect was also observed in a $Hup^-$ mutant with a Tn*5* insertion in the hydrogenase structural genes (Kim *et al.* 1991). With these observations in mind, the recent identification of a potential transcriptional activator of hydrogenase activity in free-living *B. japonicum* is of interest (Van Soom *et al.* 1993). A region necessary for Hup activity in free-living conditions but not in symbiotic conditions was sequenced. Two open reading frames, homologous with the *Alc. eutrophus hoxX* and *hoxA* genes (Eberz & Friedrich 1991), were identified. The structural characteristics of both gene products indicate that they form a sensor-effector couple involved in free-living *hup* gene expression (Van Soom *et al.* 1993).

*Bradyrhizobium japonicum* is able to accumulate $Ni^{2+}$, mostly bound to soluble proteins. These proteins are neither identical nor related to hydrogenase peptides (Stults *et al.* 1987; Maier *et al.* 1990). A $Ni^{2+}$-metabolism related locus, located 8.3 kbp upstream of the hydrogenase structural genes, was isolated from *B. japonicum* strain JH (Figure 1) (Fu & Maier 1991a). A mutant with a gene-directed mutation at this locus requires a much higher concentration of $Ni^{2+}$ during hydrogenase derepression than the wild-type strain. It was suggested that the gene product of the $Ni^{2+}$ locus might be involved in intracellular $Ni^{2+}$ incorporation into hydrogenase and possibly into a *trans*-acting regulator that is responsive to $Ni^{2+}$ concentration (Fu & Maier 1991a). To investigate the possible role of the locus in $Ni^{2+}$ insertion into hydrogenase, a hydrogenase constitutive mutant ($Hup^C$) that expresses hydrogenase activity under heterotrophic, aerobic growth conditions was mutated at the $Ni^{2+}$ locus (Fu & Maier 1992). In the presence of $Ni^{2+}$, the amount of hydrogenase apoprotein synthesis in the double mutant was the same as in the $Hup^C$ strain, but whole cell hydrogenase uptake activity was only half that seen in the $Hup^C$ strain. This supports the hypothesis that the lower hydrogenase activity in the double mutant is due to a defect in a factor responsible for incorporation of $Ni^{2+}$ into the hydrogenase apoenzyme. It is also possible to activate hydrogenase apoprotein *in vivo* by the addition of $Ni^{2+}$ to previously $Ni^{2+}$-starved and chloramphenicol-treated $Hup^C$ mutants (Fu & Maier 1992).

Reports on $Ni^{2+}$ transport are contradictory for different *B. japonicum* strains. In *B. japonicum* SR, a derivative of USDA122DES, and in the hydrogenase-constitutive strain derived from SR, strain SR470, $Ni^{2+}$ transport was shown to be energy-dependent and could not be inhibited by $Mg^{2+}$ (Stults *et al.* 1987). In contrast, in *B. japonicum* JH, a strain derived from USDA110, $Ni^{2+}$ is mainly transported by an energy-dependent system that also transports $Mg^{2+}$ and is strongly inhibited by $Mg^{2+}$ (Fu & Maier 1991b). So, it appears that significantly different mechanisms for $Ni^{2+}$ transport exist for two *B. japonicum* strains of different serogroups.

*Regulation by Carbon and Ribulose Bisphosphate Carboxylase.* A variety of complex carbon substrates has been shown to repress Hup activity in free-living *B. japonicum* USDA122 (Maier *et al.* 1979). However, bradyrhizobia can be induced to express hydrogenase activity under heterotrophic growth conditions in an induction medium with adequate buffering capacity and/or under conditions of increased $O_2$ availability (Van Berkum 1987; Van Berkum & Maier 1988). The previously reported repression of Hup activity by carbon substrates must therefore be attributed to changes in the pH of the induction medium or to limitations in the supply of $O_2$ to the cells caused by carbon substrate metabolism (Van Berkum & Maier 1988).

Under heterotrophic conditions, no co-ordinate expression of hydrogenase and ribulose-bisphosphate carboxylase (rubisco) can be measured (Van Berkum 1987). Co-ordinate expression of hydrogenase activity and rubisco activity in free-living $Hup^+$ bradyrhizobia may occur only under conditions of chemoautotrophy or when carbon substrates are limited (Simpson *et al.* 1979; Maier 1981; Van Berkum 1987). Since all $Hup^-$ mutants isolated so far and the $Hup^C$ mutants have very low rubisco activities, there must be a common step in regulation that can be uncoupled during symbiosis and heterotrophic growth (Maier 1986).

*Hydrogenase/Nitrogenase Relationships.* The high reversion rate of the $Nif^-Hup^-$ mutant SR139 and the ubiquity of reversion simultaneously to a $Nif^+$ $Hup^+$ phenotype indicate that this mutation is the result of a single lesion, probably in a regulatory gene (Moshiri *et al.* 1983; Hom *et al.* 1985). Cosmid pSH22, isolated from *B. japonicum* USDA110, has been shown to complement the defects of both nitrogenase and hydrogenase activities of SR139 (Hom *et al.* 1985). Tn*5*-induced mutagenesis, mapping and complementation analysis enabled the region within pSH22 that is necessary for complementation of the mutant phenotype to a $Hup^+$ phenotype to be located: its right border is less than 0.5 kbp upstream of the hydrogenase structural genes, and its left border is between 1 and 2.5 kbp upstream of these structural genes (Novak & Maier 1989b). The involvement of this locus in nitrogenase activity could not be proven unambiguously due to problems of plasmid instability.

Although no free-living expression of hydrogenase can be found in fast-growing rhizobia, some *hup*-related genes in *Rhiz. leguminosarum* are expressed microaerobically. This microaerobic expression was shown to be mediated by *fixK* through *fixLJ* in *Rhiz. meliloti*, and to be *nifA* and *ntrA* independent (Palacios *et al.* 1990). This points to a link between hydrogenase and nitrogenase expression, at least in *Rhiz. meliloti*. The function of the microaerobically expressed genes is not clear, but they are not involved in regulating the expression of the other *hup* genes (Palacios *et al.* 1990).

*Selenium.* Besides $Ni^{2+}$, Se seems to be involved in hydrogenase expression and autotrophic growth of *B. japonicum*. The hydrogenase activity of autotrophically grown *B. japonicum* increases upon addition of radioactively-labelled Se into the growth medium. Also, the final chromatographic purification of the hydrogenase resulted in a striking coincidence in peaks of protein content, hydrogenase activity and Se radioactivity (Boursier *et al.* 1988). Co-purification of Se and hydrogenase activity confirmed that the *B. japonicum* hydrogenase is a seleno-enzyme, with the metal bound in a labile form to the hydrogenase enzyme (Hsu *et al.* 1990).

*Supercoiling and Hydrogenase Expression.* Certain genes that are regulated in response to environmental stimuli, like anaerobiosis, show an altered expression that is dependent on DNA tertiary structure (Novak & Maier 1989a). Synthesis of the *B. japonicum* hydrogenase is repressed by high concentrations of $O_2$ (Maier *et al.* 1979; Van Berkum 1987), and enzyme synthesis has been shown to be prevented by inhibitors of DNA gyrase (Novak & Maier 1987). $Hup^C$ mutants display a pleiotropically altered phenotype; several proteins normally specific to a microaerobic environment during derepression for hydrogenase are expressed constitutively, including hydrogenase itself, rubisco, cytochrome *o* and some unidentified polypeptides. The frequency of occurrence of this mutation suggests that a single regulatory locus is altered. Interestingly, the $Hup^C$ mutants appear insensitive to the previously demonstrated inhibition of hydrogenase synthesis by DNA gyrase inhibitors, while sensitivity to growth inhibition by the inhibitors of the mutant strain itself was not changed (Novak & Maier 1989a). The $Hup^C$ mutant may be affected in two ways. Firstly, the tertiary structure of the DNA could be altered so that the mutant senses a lower $O_2$ tension than the actual tension, which could in turn affect the expression of a regulatory gene normally only active under anaerobic conditions. Alternatively, the regulatory gene itself could be altered, so that its product recognizes genes that are normally of the correct tertiary structure under anaerobic conditions (Novak & Maier 1989a).

## Effect of Hydrogenase Phenotype on Plant Growth

*Is Hydrogenase of Benefit to the Legume–*Rhizobium *Symbiosis?* Production of $H_2$ by nitrogenase represents an inefficiency of the $N_2$-fixing systems because the energy available for $N_2$ reduction is wasted in $H_2$ evolution. The presence of an active Hup system allows greater efficiency in biological $N_2$ fixation (BNF), as has been shown for many free-living diazotrophs (Arp 1992). In legume–rhizobia associations, the role of a Hup system seems more complex and considerable effort has been dedicated to the evaluation of the benefits as well as to the understanding of the intricate relationship between $H_2$ and $N_2$ metabolism in nodules.

One mechanism by which a Hup system might enhance the overall efficiency of BNF is by increasing the ATP or reductant availability to bacteroids (Dixon 1972), assuming that BNF is energy- and/or reductant-limited. This aspect has been intensively studied and reviewed (Evans *et al.* 1987; Neves & Hungria 1987; Arp 1992).

Dixon (1972) suggested that the presence of a Hup system in nodules may represent an auxiliary mechanism for $O_2$ protection, by scavenging $O_2$ from the immediate environment of nitrogenase. However, results of more recent research on the mechanism of $O_2$ diffusion in the root nodule favour a model in which the $O_2$ supply to bacteroids is controlled by a variable diffusion barrier (Witty *et al.* 1987). This barrier maintains a balance between the flow of $O_2$ into the bacteroids and their respiratory requirement (Minchin *et al.* 1988) so that the bacteroid nitrogenase is not inactivated by a surplus of $O_2$. A respiratory protection role for the Hup system is also put into doubt by the finding that the resistance of the $O_2$ diffusion barrier is altered by various environmental and

physiological factors, and that the rate of response differs with the host plant (Minchin *et al.* 1985). So, in cases where the $O_2$ concentration outside the nodule is too low to provide sufficient $O_2$ diffusion into the cell, the presence of a Hup system in a nodule may even be a disadvantage (Godfroy & Drevon 1991). This could explain some detrimental effects observed by Drevon *et al.* (1987) in $Hup^+$ strains in hydroponically-grown soybean, the growth of which is limited to a large extent by the level of aeration. In this study, which used isogenic $Hup^+$ and $Hup^-$ mutants, a deleterious effect of the $Hup^+$ phenotype on plant growth was observed for up to 75 days post-germination. The effect was attributed to $O_2$ limitation to the $Hup^+$ bacteroids. For unstressed nodules of peas and soybeans, $O_2$ is probably not a major limiting factor for $N_2$ fixation (Minchin *et al.* 1985) although this has been disputed (Minchin *et al.* 1988).

It has been suggested that the Hup system protects $Hup^+$ nodules against $H_2$ inhibition of nitrogenase, although this has not been fully investigated. This hypothesis is supported by modelling studies of gas diffusion through soybean nodules (Hunt *et al.* 1988) and by measurements of $K_i$ for $H_2$ inhibition of nitrogenase in isolated bradyrhizobia bacteroids (Rasche & Arp 1989). The major benefit of $H_2$ oxidation in nodules may be to scavenge $H_2$ from the nitrogenase site (Hunt *et al.* 1988; see also Arp 1992). Direct measurement of $H_2$ concentration within detached soybean nodules using micro-electrodes indicated that $H_2$ does not reach inhibitory concentrations under the experimental conditions tested (Witty 1991). However, no data are available for the $K_i$ of $H_2$ in intact nodules, which may differ from the value for bacteroids *in vitro* (Jensen 1985). In addition, $H_2$ concentrations within nodules are likely to be determined by the rate of nitrogenase activity and by other physiological processes (e.g. electron-allocation ratio) that may be altered by many environmental factors (Neves & Hungria 1987). The interaction of $H_2$ production and oxidation and the mechanisms of $O_2$ control in the nodules remains to be investigated.

*Effect on Plant Growth*

The effect of a $Hup^+$ phenotype on growth, yield and nitrogen content of nodulated legumes has been addressed by several authors (see review by Eisbrenner & Evans 1983; Neves & Hungria 1987). Their results are quite conflicting, probably because most of the comparisons were made between strains that were not isogenic (except for the presence of a Hup system) and other characteristics could therefore have influenced the observed effects. However, use of isogenic $Hup^+$ and $Hup^-$ mutants has not eliminated conflicting results, even when only data on soybean, the most studied legume, are taken into account; variation in experimental conditions, from test tubes to field conditions, must then be largely to blame.

Most long-term experiments (for exceptions see Drevon *et al.* 1988) show significant increases in dry matter and/or total N fixed as a result of the $Hup^+$ phenotype. Under field conditions in a sandy soil, inoculation of soybeans with strains showing a high relative efficiency [RE = 1 − ($H_2$ evolution/acetylene reduction)] (Schubert & Evans 1976) resulted in an increased seed yield and nitrogen harvest index compared with plants inoculated with strains of low RE (Neves *et al.* 1985). In *Phaseolus vulgaris*, a very high correlation was found between the RE and the nitrogen content of pods of 30 symbiotic systems (Hungria & Neves 1986). Another isogenic *Bradyrhizobium* pair was tested in soybeans grown to maturity in large cylinders (Evans *et al.* 1985). Plants inoculated with the constructed $Hup^+$ mutant showed higher dry matter accumulation (9%) and N content (11%) than plants inoculated with the $Hup^-$ revertant. In these long-term experiments, plants were raised under $N_2$-limiting conditions and were, therefore, entirely dependent on BNF.

In another study, with soybeans grown to the mid-pod-fill stage, inoculation with an isogenic $Hup^+$ revertant increased specific nodule activity, percentage ureide-N transported in xylem sap, and harvest index (Hungria *et al.* 1989). These results corroborated those of previous experiments with soybeans, in which differences in seed yield and harvest index were correlated with a greater percentage of ureide-N transported in the xylem of plants inoculated with the $Hup^+$ strains (Neves *et al.* 1985). Hungria & Neves (1986, 1987) also reported a positive correlation ($r = 0.903$) between percentage N-ureide in the sap of *Phaseolus vulgaris* and the $H_2$ recycling capability of the inoculated strains. There is some evidence to indicate that the capacity for $H_2$ recycling within the nodules affects other metabolic processes in the nodules, but more investigation is definitely required. Increased rates of $N_2$ transport in soybeans inoculated with $Hup^+$ strains were reported by Minamisawa *et al.* (1983) and similar results were also obtained with several cultivars of *P. vulgaris* inoculated with a group of $Hup^+$ and $Hup^-$ strains (Hungria & Neves 1987). A possible relationship between the Hup system and carbon utilization has also been suggested (Minamisawa *et al.* 1981) and an intriguing accumulation of serinol in soybean nodules formed by $Hup^-$ strains has been linked to rhizobitoxine production (Minamisawa 1988).

*Host and Environmental Effect on $H_2$ Cycling*

Several studies report control of expression of the Hup system by the plant host (Keyser *et al.* 1982; Neves & Hungria 1987; Nautiyal *et al.* 1988; Phillips *et al.* 1990). Besides $Hup^+$ and $Hup^-$ bradyrhizobia, Van Berkum (1990) distinguished a third hydrogenase phenotype, the host-regulated (Hup-hr) phenotype. This phenotype is characterized by the expression of hydrogenase activity only in symbiosis with specific host plants, and it cannot be

expressed by free-living bacteria using the standard derepression methods developed for Hup$^+$ *B. japonicum*. Strains belonging to this Hup-hr phenotype contain DNA-sequences hybridizing with pHU1, but the hybridization pattern is different from that of Hup$^+$ strains. Nucleotide sequence analysis revealed the presence of an extra 1 kbp between the open reading frames encoding the small and large subunits of the hydrogenase enzyme in Hup-hr stains, compared with a region of 32 bp in Hup$^+$ strains (Salzwedel & Van Berkum 1992). Hup-hr strains predominate in the soil of soybean-producing regions in the southern United States and are highly competitive for nodulation, which inhibits the efficiency of using Hup$^+$ inocula to increase $H_2$ oxidation in soybean. Therefore, the identification of exotic soybean germ plasm which allows hydrogenase expression by Hup-hr strains could enhance BNF in soybean production (Van Berkum & Sloger 1991). Cultivar effects are the most likely cause of the conflicting results observed in these studies (see Drevon *et al.* 1988).

Environmental factors which alter the physiological processes of the host plant, such as temperature, plant age, shading and other carbon-limiting conditions, also alter $H_2$ production and/or recycling by the bacteroids (Neves & Hungria 1987). In soybean cv. Doko inoculated with isogenic Hup$^+$ and Hup$^-$ mutants, a significant increase in RE of nodules formed by the Hup$^-$ mutant was observed when plants were partially shaded (Table 1). This increase in efficiency was reflected in a greater proportion of fixed N being transported as ureides. The efficiency of the nodules formed by the Hup$^+$ mutant could not be further increased by the shading treatment, which reduced nodulation (data not shown) and acetylene reduction activity (ARA) (V. A. Santos & M. C. P. Neves, unpublished work). As a result, the significant differences in xylem sap composition (% ureide-N) observed in full daylight between the plants inoculated with Hup$^+$ and Hup$^-$ mutants were not observed in the shaded plants. In another experiment, these effects of shading on ARA and percentage ureide-N were confirmed and found to reflect similar changes in glutamine synthetase, glutamate dehydrogenase, and uricase activities.

Overall, the available data indicate a beneficial role for a Hup system in legume nodules, especially in *Bradyrhizobium* species. The presence of a Hup system in *Bradyrhizobium* allows recovery of all or nearly all of the $H_2$ produced by nitrogenase. However factors such as plant genotype, growth conditions, duration of experiments, and light intensity and quality may interfere with and/or mask the benefits the Hup$^+$ phenotype gives to the nodulated plant. Long-term experiments in conditions optimum for plant growth seem essential to show the beneficial effect the Hup$^+$ phenotype can have on plant growth.

**Table 1. Effect of shading on acetylene reduction activity (ARA), relative efficiency (RE) and % ureide-N in xylem sap of soybean cv. Doko inoculated with PJ17 (Hup$^-$) and PJ17-1 (Hup$^+$) isogenic mutants (V. A. Santos & M. C. P. Neves, unpublished work).**

| Inoculated strain | ARA ($\mu C_2H_2$/h) | RE* | % ureide-N |
|---|---|---|---|
| Shaded (75% full daylight) | | | |
| Hup$^+$ | 8.31 | 0.90$^a$ | 59.6$^{ab}$ |
| Hup$^-$ | 10.12 | 0.77$^a$ | 63.4$^{ab}$ |
| Unshaded control | | | |
| Hup$^+$ | 10.37 | 0.97$^a$ | 66.1$^a$ |
| Hup$^-$ | 11.12 | 0.33$^b$ | 49.0$^b$ |
| Coefficient of variation (%) | 33.89 | 14.56 | 12.19 |

* 1—($H_2$ evolution/acetylene reduction).

$^{a,b}$ Within columns, numbers followed by the same superscript letter are not significantly different ($P > 0.05$).

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