Growth rates

Data on growth rate (Table 3) show that growth of 'transgenic' fish was faster in F_0 and F_1 generations but slower in F_2 generation than that of control fish. Secondly, there were indications that the accelerated growth observed in 'transgenic' fish of the F_0 and F_1 generations was at the cost of reproductive growth. It appears that the accelerated growth observed in the F_0 and F_1 generations may be correlated to the presence of extrachromosomal DNA in these individuals. Since it has already been demonstrated that extrachromosomal DNA is involved in transient expression[4], a similar effect may explain the accelerated growth rate.

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ACKNOWLEDGEMENTS. We gratefully acknowledge financial assistance from the Department of Biotechnology, New Delhi. S. K. received a senior research fellowship from CSIR, New Delhi.

Received 30 November 1990; revised accepted 20 February 1991

Imparting hydrogen-recycling capability to *Cicer*-rhizobial strains by plasmid pIJ1008 transfer

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Conjugal transfer of plasmid pIJ1008, which carries determinants for hydrogen uptake (Hup) activity, from *Rhizobium leguminosarum* to two Hup⁻ *Cicer*-rhizobial strains G 36–84 and BG4 conferred Hup activity in the free-living state as well as under symbiotic conditions. The acquired capability of the rhizobial strains to recycle hydrogen evolved by nitrogenase improved their relative efficiency of nitrogen fixation. This transfer and expression of *hup* genes suggests the possibility of improving the symbiotic energy efficiency of *Cicer-Rhizobium*, which lacks *hup* genes.

THE ATP-dependent evolution of hydrogen catalysed by the enzyme nitrogenase is a source of inefficiency in the legume–*Rhizobium* symbiotic system as nitrogen fixation is limited by the supply of photosynthate[1]. Nodule bacteroids that have a hydrogen-uptake (Hup) system can recycle this hydrogen and generate ATP[2,3] and reductant[4,5]. It has been suggested that an efficient hydrogen-recycling capability under symbiotic conditions is a desirable characteristic for *Rhizobium* strains[6]. The nitrogen-fixation efficiency of any Hup⁻ *Rhizobium* species may be improved if a functional Hup system can be transferred into it and be stably maintained. No strain of *Cicer-Rhizobium* that possesses Hup activity has been identified[7,8]. Presumably these strains do not contain *hup* genes[8]. In the present paper we report experiments aimed at interspecies transfer of Hup determinants located on the *Rhizobium leguminosarum* recombinant plasmid pIJ1008 into the Hup⁻ strains of *Cicer-Rhizobium*. Expression of Hup activity in the resulting transconjugants has been demonstrated under free-living as well as symbiotic conditions.

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Methods

Cicer-Rhizobium strain G 36–84 was obtained from the Division of Microbiology, IARI, New Delhi, while strain BG4 was isolated from the root nodules of chickpea in our laboratory. Plasmid pIJ1008 in *R. leguminosarum* strain B164 was kindly supplied by Dr N. J. Brewin of the John Innes Institute, UK. Cosmid pHU52 in *Escherichia coli* strain HB101 was kindly supplied by Prof. H. J. Evans of the Oregon State University, USA.

Yeast extract mannitol agar medium[9] was used for culturing *Rhizobium* strains. For bacterial matings, TY medium[10] was used. Luria–Bertani (LB) medium[11] was used for culturing *E. coli*. H_2-uptake medium as described by Maier *et al.*[12] was used to induce uptake-hydrogenase activity in rhizobial strains.

Plasmid transfer and Hup activity

Plasmid pIJ1008 was transferred from *R. leguminosarum* B164 to *Cicer-Rhizobium* strains by the biparental plate-mating system of Ditta *et al.*[13] The stability of plasmid pIJ1008 in the recipient strains was assayed by subculturing them for about 15 generations under non-selective conditions, i.e. in TY plates without kanamycin

(Kan), and checking resistance to Kan after every five generations.

Plasmids were isolated by the *in situ* lysis method[14], and plasmid composition was analysed in Eckhardt-type gels with minor modifications[15]. Electrophoresis was carried out in 0.6% agarose gel at 50 V for 22 h using a Crosspower 150 power supply (Atto Corp., Japan).

Colony hybridization was done according to the method described by Maniatis *et al.*[16] The *hup* gene probe labelled with [^{35}S]dATP (BARC; sp. activity 650 Ci mmol^{-1}) was prepared using *hup*-complementing cosmid pHU52 by a nick-translation procedure as described by Vasudev *et al.*[8]

Induction and assay of H_2-uptake (Hup) activity in free-living cultures of *Cicer*-rhizobial strains were done as described by Thimmaiah *et al.*[17]

Plant inoculation and growth

Seeds of chickpea (*Cicer arietinum* L. cv. Pusa 209) were surface-sterilized, sown (4 per jar) in Leonard-jar assemblies[9] containing washed river sand, and inoculated with rhizobial strains (13–20 × 10^9 cells per seed). Plants were grown in a PERCIVAL growth chamber maintained at 22°C during 14-h day period with light intensity of 8500 lux and at 14°C during night. Hoagland's N-free nutrient solution[18] was added to the jars as and when required.

Hydrogen evolution and acetylene reduction assays

Hydrogen evolution in air and reduction of acetylene to ethylene (nitrogenase activity) were determined by gas chromatography as described by Lodha *et al.*[19] using intact nodules from 60-day-old plants. Relative efficiency (RE) of N_2 fixation [1 − (Rate of H_2 evolution in air/Rate of C_2H_2 reduction)] was calculated as described by Schubert and Evans[20]. The RE estimate has been used to find out whether nodule bacteroids contain an uptake hydrogenase. If RE is greater than 0.8, the bacteroids probably possess hydrogenase.

Transfer of plasmid pIJ1008

Recombinant plasmid pIJ1008 (pRL4JI :: Tn5, pRL6JI) of *R. leguminosarum*, which not only carries *hup* genes but is also conjugatively transmissible, was transferred to two Hup$^-$ *Cicer*-rhizobial strains G 36–84 and BG4 by the biparental mating method. Since plasmid pIJ1008 carries transposon Tn5 which specifies resistance to Kan, transfer of the plasmid to recipient strains was monitored by looking for Kan resistance. The donor *R. leguminosarum* strain B164 was counter-selected using nalidixic acid, to which the recipient strains were resistant. The frequencies of transfer of Kan^r to strains G 36–84 and BG4 were, respectively, 15 × 10^{-9} and 3 × 10^{-9} per recipient cell.

The stability of plasmid pIJ1008 in *Cicer*-rhizobial strain G 36–84 was assessed as described above. All of the 24 colonies grown under non-selective conditions were found to retain Kan^r phenotype even after subculturing for 15 generations.

Since the basis of selection of the transconjugants was transposon-associated phenotype, it was possible that the Kan^r phenotype could have arisen by the transposition of Tn5 rather than by the stable maintenance of plasmid pIJ1008. To rule out this possibility, the presence of plasmid pIJ1008 in the transconjugants was verified by electrophoretic analysis of the plasmids on agarose gels. The results are presented in Figure 1. The donor strain *R. leguminosarum* B164 harbours two plasmids, of 350 kb and 280 kb, the latter being pIJ1008. The results show the presence of plasmid bands corresponding to both these plasmids in the transconjugants, while no such plasmid bands are seen in unmated cells of the recipient strains G 36–84 and BG4. Transfer of the 350-kb plasmid to *Cicer*-rhizobia was unexpected.

The presence of *hup* genes in the transconjugants was further tested by colony hybridization. Colonies of the transconjugants transferred to nitrocellulose sheets were hybridized to ^{35}S-labelled *hup* gene probe. Figure 2 shows positive hybridization signals for the transconjugants, and no such signals for unmated cells of the recipient strains.

Expression of *hup* genes in transconjugants

To find out whether *hup* genes are expressed in the new

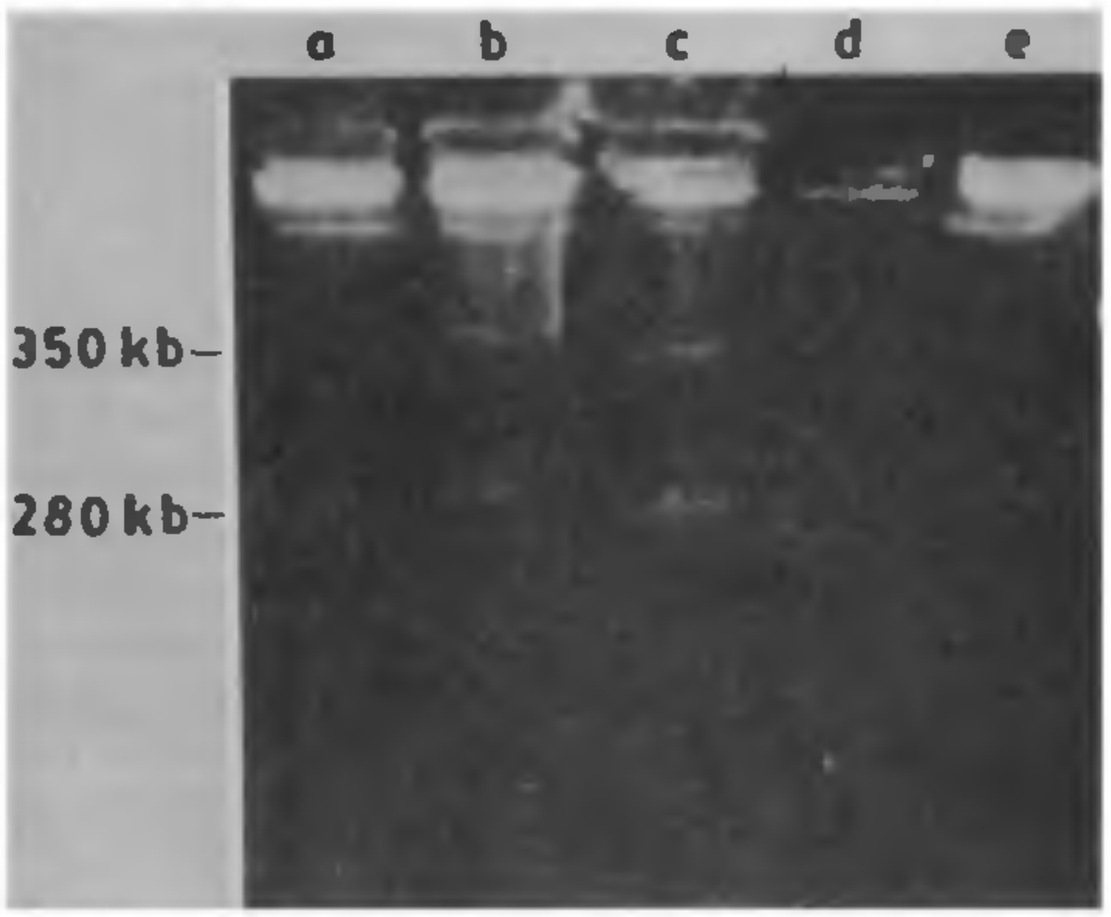

Figure 1. Plasmid-composition analysis of transconjugants of rhizobial strains. Lysates of bacterial cells prepared by *in situ* lysis were run on a 0.6% agarose gel for 22 h at 50 V. Lane a, donor *R. leguminosarum* B164 (pIJ1008); lanes b and c, transconjugants G 36-84 (pIJ1008) and BG4 (pIJ1008) respectively; lanes d and e, unmated cells of recipient strains G 36-84 and BG4 respectively.

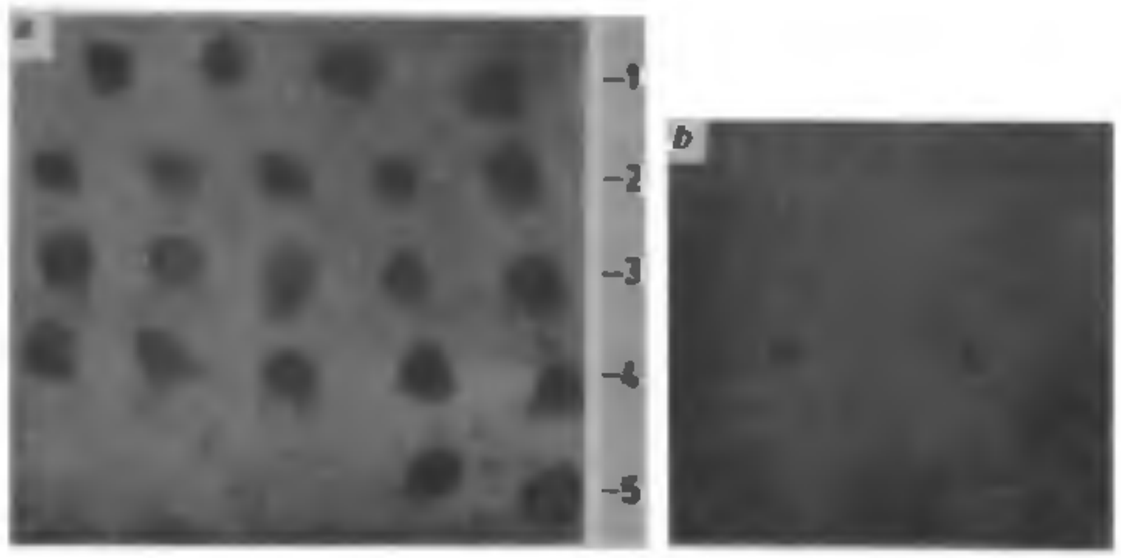

Figure 2. Autoradiograms showing colony hybridization of (*a*) *Cicer*-rhizobial transconjugants carrying plasmid pIJ1008 to nick-translated pHU52 row 1, BG4 (pIJ1008); rows 2–4, G 36–84 (pIJ1008), row 5, *E coli* HB101 (pHU52) used as positive control, and (*b*) unmated cells of *Cicer*-rhizobial recipient strains to nick-translated pHU52 spots a and b, BG4 and G 36–84 respectively, c and d, *E. coli* HB101 (pHU52) used as positive control.

genetic background of *Cicer*-rhizobia, derepression was carried out under free-living conditions and Hup activity measured. The results presented in Table 1 show a maximum activity of 62 nmol H_2 taken up per hour per agar slant after 6 days of incubation in the case of transconjugant G 36–84 (pIJ1008). In transconjugant BG4 (pIJ1008), though the maximum activity of 37 nmol H_2 taken up per hour per slant was observed after 8 days of incubation, there was not much change in the activity with increase in incubation time. Unmated cells of the recipient strains showed negligible activity.

The expression of plasmid-borne *hup* genes in chickpea nodules induced by the Hup^+ transconjugants was studied by calculating the RE of N_2 fixation. It is evident from Table 2 that, with the acquisition of *hup*-gene plasmid pIJ1008, RE increased from 0.73 to 0.91 for strain G 36–84 and from 0.70 to 0.84 for strain BG4. However, with the acquisition of pIJ1008 the specific nodule nitrogenase activity (nmol C_2H_2 reduced h^{-1} g^{-1} fr wt nodule) decreased in the case of strain G 36–84.

Discussion

The recombinant plasmid pIJ1008 (Kan^r, Tra^+, Hup^+, Nod^+, Fix^+) was constructed by Brewin *et al.*[21] and transferred to several Hup^- strains of *R. leguminosarum* to produce symbiotically superior strains[22]. Subsequently interspecies transfer of Hup determinants located on this *R. leguminosarum* plasmid to *R. meliloti* and expression of Hup activity in *Medicago sativa* nodules was demonstrated[15,23]. The present results also demonstrate that plasmid pIJ1008 of *R. leguminosarum* could be transferred to *Cicer*-rhizobial strains. The presence of the plasmid in the transconjugants was verified by electrophoretic analysis, and hybridization studies using radioactively labelled probe verified the transfer of Hup determinants into them. Higher RE of N_2 fixation (>0.8) of chickpea nodules formed by the transconjugants indicate expression of introduced *hup* genes under symbiotic conditions.

Though the conditions for the derepression of *hup* genes have not been defined for free-living *R. leguminosarum*[24], this study suggests that these genes could be derepressed in the free-living *Cicer*-rhizobial transconjugants G 36–84 (pIJ1008) and BG4 (pIJ1008) when grown on H_2-uptake medium. However, there

Table 1. H_2-uptake activity in transconjugants of *Cicer*-rhizobial strains under free-living conditions

Growth stage	Hydrogenase activity (nmol H_2 h^{-1} $slant^{-1}$)			
(days)	G 36–84	G 36–84 (pIJ1008)	BG4	BG4 (pIJ1008)
4	ND	32 ± 11	ND	29 ± 10
5	6 ± 2	20 ± 10	5 ± 1	19 ± 5
6	ND	62 ± 14	ND	28 ± 12
7	ND	41 ± 22	ND	20 ± 8
8	ND	39 ± 3	ND	37 ± 11

ND, not detected.
Values are mean ± SD of 6 assays

Table 2. Acetylene reduction activity, H_2 evolution rate and relative efficiency of N_2 fixation in chickpea nodules induced by *Cicer*-rhizobial strains carrying pIJ1008

Strain	C_2H_2 reduction rate (nmol h^{-1} g^{-1} fr wt nodule)	H_2 evolution rate (nmol h^{-1} g^{-1} fr wt nodule)	RE
G 36–84	2802 ± 883	703 ± 218	0 73
G 36–84 (pIJ1008)	1210 ± 487	104 ± 35	0 91
BG4	5791 ± 1158	1722 ± 369	0 70
BG4 (pIJ1008)	6961 ± 1701	1112 ± 223	0 84

Values are mean ± SD for a minimum of 3 assays, each assay contained 3–4 plants

was a difference in the pattern of derepression of *hup* genes, suggesting a role for the genetic make-up of the host strain.

On acquisition of plasmid pIJ1008, the symbiotic energy efficiency of *Cicer*-rhizobial strains was improved, as suggested by RE estimates (Table 2). Energy losses due to H_2 evolution from nodules were reduced by 14 and 18% respectively in Hup^+ transconjugants compared to their respective parental strains. However, in transconjugant G 36–84 (pIJ1008) specific nodule nitrogenase activity was significantly reduced. This may be due to antagonistic effects of pIJ1008, which also carries genetic determinants for nodulation capability as well as for symbiotic N_2 fixation[21].

This study thus opens up the possibility of improving symbiotic efficiency of *Cicer-Rhizobium* by incorporating H_2-recycling ability, which the *Cicer*-rhizobia, as a whole, normally lack.

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ACKNOWLEDGEMENTS. We are grateful to Dr N. J. Brewin, John Innes Institute, UK, for providing plasmid pIJ1008, and to Prof. H. J. Evans, Oregon State University, USA, for providing cosmid pHU52. S.V. is thankful to Post-Graduate School, AIRI, for a fellowship.

Received 11 April 1990, revised accepted 1 August 1990

Transient expression of GUS and CAT genes in electroporated rice protoplasts

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Bacterial β-glucuronidase (GUS) and chloramphenicol acetyltransferase (CAT) genes were introduced into rice protoplasts by electroporation. Detection of significant levels of GUS and CAT enzyme activity indicated that both genes are expressed in transformed rice protoplasts. GUS and CAT genes can therefore be used as reporter genes in transformation experiments in rice.

DIRECT introduction of DNA as a means of transformation of plant protoplasts has provided a system for monitoring gene expression within hours of transformation and for evaluation of the recombinant-DNA constructs, promoters and marker genes used[1]. In rice, direct gene-transfer methods have also been used to obtain transgenic plants from protoplasts[2–4]. Generally a strong promoter is fused to a gene whose product is required in increased amount to enhance expression of the gene *in vivo*. In studying regulation of gene expression, the experimental approach of fusing the promoter and various segments of upstream regulatory sequences of the gene of interest to the coding region of a 'reporter' gene whose protein product can be easily detected, introducing the constructs into a recipient plant or plant protoplasts, and monitoring expression of the reporter gene is a well-established one. The present communication shows that bacterial β-glucuronidase (GUS) and chloramphenicol acetyltransferase (CAT) genes introduced by electroporation into rice protoplasts are expressed.

The transforming DNA was in the form of plasmids. The plasmid constructs used were: (i) pBI121, carrying the GUS gene fused with the cauliflower mosaic virus (CaMV) 35S promoter; (ii) pDW2, carrying the CAT gene fused with the CaMV 35S promoter; (iii) pNOSCAT, carrying the CAT gene fused with the *Agrobacterium tumefaciens* nopaline synthetase (NOS)