Rhizobium nodulation gene nod as a determinant of host specificity

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peratures results in low foraminiferal production and flux during the winter.

A simultaneous increase in primary productivity and zooplankton abundance occurs during the spring (Fig. 3), along with the development of a thermocline (Fig. 1). The increase in foraminiferal food source, both in terms of copepod abundance and necessary light intensity levels for symbiotic algae, together with a thick 5–10 °C habitat (surface to 100 m depth) results in increased production and flux of planktonic foraminifera during the spring.

Although food availability remains high, there is a sharp decrease in foraminiferal flux during most of the summer—probably because the surface layer becomes very stratified and temperatures in the upper 40 m exceed the tolerance range for subpolar species (Fig. 1). During the summer, the thickness of the 5–10 °C habitat is reduced and is restricted to the lower half of the thermocline. As previously stated, *O. universa* is the only non-subpolar species present at Ocean Station P[21], and it accounts for much of the summer flux. This subtropical–transitional species most probably lives in the surface mixed layer during this period. Zooplankton grazing may also contribute to the low foraminiferal fluxes during the summer. Zooplankton abundance is greatest at this time of year, so grazing pressure should also be at a maximum, but the data required to assess the impact of grazing on foraminiferal flux are not available.

During the autumn, the thermocline begins to decay and mixed layer temperatures decline back into the optimal range for subpolar species. This results in a vertical expansion of the 5–10 °C habitat for these species. These changes in thermal structure combined with a relatively high food availability result in an increase in foraminiferal production and flux.

Significantly more interannual variability exists in the foraminiferal flux record than in the carbonate flux record (Fig. 2*a* and *b*). In fact, the carbonate flux values are remarkably similar from year to year. The greatest amount of interannual variability in foraminiferal flux is associated with the spring and autumn periods of high flux. The variability in foraminiferal flux does not appear to be directly related to anomalous sea-surface temperatures (Fig. 1*a*). For example, the spring flux peaks range from a low of 5 mg m^{-2} d^{-1} in May 1984 to a high of 40 mg m^{-2} d^{-1} in May 1985. However, sea-surface temperatures in May of all three years differ by less than 0.4 °C. Year-to-year differences in the sizes of the flux peaks may be a function of the level of food availability. Unfortunately, the data necessary to test this possibility are not available.

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Rhizobium nodulation gene *nodD* as a determinant of host specificity

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The symbiosis between bacteria of the genus *Rhizobium* and their leguminous host plants results in the formation of root nodules in a species-specific way, in that a particular bacterial species can nodulate only a limited number of host species. In fast-growing *Rhizobium* species many nodulation (*nod*) genes, including the functional interchangeable or common *nod* genes *nodA,B,C,I,J* and *nodD* and the host-specificity genes *nodE,F*, are localized on large Sym (for symbiosis) plasmids[1–5]. On the *Rhizobium meliloti* Sym plasmid three *nodD* genes have been localized. Two of these, *nodD1* and *nodD2*, are required for efficient nodulation of the host plant (ref 6 and M. Honma, personal communication). Exudates of leguminous plants induce the expression of several Sym-plasmid-localized *nod* operons[7–10], a process in which the constitutively expressed *nodD* product is supposed to act as a positive regulator[8,9]. The inducing compounds found in these exudates have been identified as flavones, flavanones or closely related compounds[11–14]. We report here that the *nodD* products of the various fast-growing *Rhizobium* species differ from each other in that they confer different responsiveness, in a species-specific way, to different sets of flavonoids and exudates. Moreover, in one case, the *nodD* gene was shown to be a determinant of host-specific nodulation.

The function of the *nodD* product in the recognition of the plant flavonoid inducer of the *nod* genes is unknown. It is striking that, although *nodD* is considered to be a common *nod* gene, the structural requirements for inducers of *nod* genes of *R. leguminosarum*[13,14], *R. trifolii*[12] and *R. meliloti*[11] seem to be different. Therefore, the *nodD* genes of the fast-growing *Rhizobium* species *R. leguminosarum*, *R. trifolii* and *R. meliloti* (the *nodD1* gene) were cloned (Fig. 1) in a broad host range IncP class vector and the resulting plasmids pMP280, pMP283 and pMP284 were subsequently transferred to *Rhizobium* strain LPR5045 which lacks a Sym plasmid. The inserts contain a complete *nodD* gene, including its own constitutively expressed promoter and some surrounding sequences, but no other complete *nod* gene[2,7–9,15–18] (Fig. 1). To measure the expression of inducible *nod* promoters, plasmid pMP154 (Fig. 1) was used, an IncQ transcriptional fusion vector in which a 114-base pair (bp) fragment containing the upstream region (including the presumed promoter[19]) of the *nodA,B,C,I,J* operon of the *R. leguminosarum* Sym plasmid pRL1JI was cloned. In pMP154, *nod* promoter activity could be detected as β-galactosidase activity. The upstream regions with inducible promoter activity of the *nodA,B,C* operons of *R. trifolii* and *R. meliloti* were cloned in the IncQ transcriptional fusion vector resulting in pMP193 and pMP194, respectively (Fig. 1) and promoter activity could be detected as with construct pMP154. Plasmid pMP154 was mobilized to the *Rhizobium* LPR5045 derivatives harbouring the *nodD*-containing plasmids, yielding an isogenic set of strains which differ only in the source of the *nodD* genes.

The results of the induction experiments (Table 1, lines 1–3) show that luteolin can induce the *nod* promoter to a substantial level regardless of the *nodD* source. For the other flavonoids the source of *nodD* determines which substances efficiently

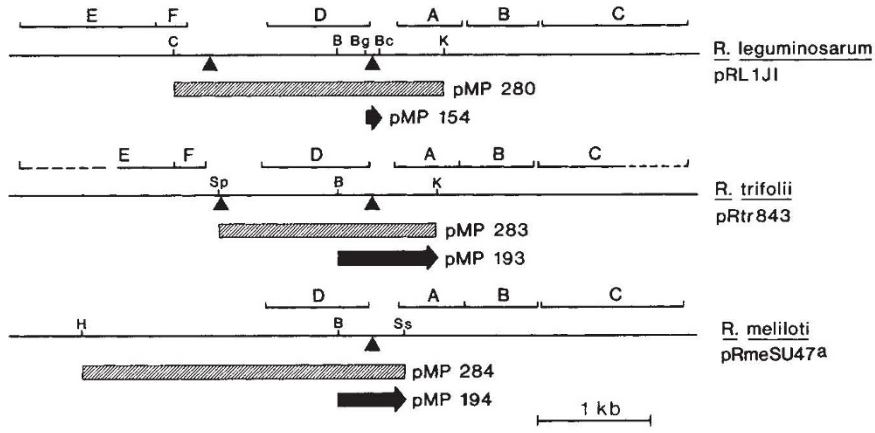

Fig. 1 Cloning of *nodD* genes from various *Rhizobium* species and of *nodA,B,C* upstream regions. The *nodD* genes of the *R. leguminosarum* Sym plasmid pRL1JI (ref. 25), the *R. trifolii* Sym plasmid pRtr843 and the *R. meliloti* Sym plasmid pRmeSU47a, present in the plasmids pMP104 (ref. 5), pRt308 (ref. 4) and pRmeSL26 (ref. 30), respectively, were subcloned (hatched bars) in the vector pMP92 (ref. 5). This IncP vector is 7 kilobases (kb) in size, codes for tetracycline resistance and contains a polylinker. The resulting constructs pMP280, pMP283 and pMP284 were mobilized to the *Rhizobium* strains used in this study. A 114-basepai (bp) *Bgl*II–*Bcl*I fragment, containing inducible promoter activity and the *nod*-box (defined according to ref. 24) of the *nodA,B,C,I,J* operon of pRL1JI, was cloned in the transcriptional fusion vector pMP190 (ref. 5) with the indicated direction towards *lacZ*, resulting in the plasmid pMP154 (ref. 5). The indicated fragments, which contain inducible promoter activity of the *nodA,B,C* operons of pRtr843 and pRmeSu47a were also cloned in pMP190 and resulted in the constructs pMP193 and pMP194, respectively. Vector pMP190, of the IncQ class, codes for chloramphenicol and streptomycin resistance and contains the *Escherichia coli lacZ* gene, without its promoter and operator region, as an indicator gene for transcriptional expression. Part of the *nod* regions, the restriction sites used, the conserved *Bam*HI site, and the localization of the *nod* genes in these regions, are indicated. Size and position of the *nod* genes, indicated by solid lines, are according to refs 2 and 15–18 except that the *nodA* translational start of pRL1JI is drawn 91 bp closer to the *Bcl*I site (unpublished results) than indicated in other studies[2,15], which makes this *nodA* product more homologous to the *nodA* products of other fast-growing *Rhizobium* species. The parts of *nodC* and *nodE* of *R. trifolii*, for which nucleotide sequences have not yet been reported, are indicated tentatively by dotted lines. *Nod*-boxes, strongly conserved DNA sequences which precede every inducible nod-operon[2,5,17,19,24,31], are indicated by triangles. Restriction enzyme sites are shown as follows: B, *Bam*HI; Bc, *Bcl*I; Bg, *Bgl*II; C, *Cla*I; H, *Hind*III; K, *Kpn*I; Sp, *Sph*I; Ss, *Sst*I.

Table 1 Expression of *R. leguminosarum nodABCIJ* promoter by commercially available flavonoids and natural inducers in the presence of *nodD* genes from various sources

	Units β-galactosidase ($\times 10^{-3}$)							
nodD-containing plasmid	No inducer	Luteolin	Apigenin	Naringenin	Eriodictyol	7-Hydroxy-flavone	*Trifolium pratense* exudate	*T. repens* exudate
pMP280 (*R. leguminosarum*)	0.26	19	24	21	21	7.6	0.41	14
pMP283 (*R. trifolii*)	0.84	22	29	28	4.6	29	8.0	30
pMP284 (*R. meliloti*)	0.30	6.0	2.6	0.54	0.90	0.55	0.40	7.5
pRL1JI (*R. leguminosarum*)	0.30	18	20	19	19	7.3	0.43	9.3
pSyml (*R. leguminosarum*)	0.61	13	13	14	13	4.8	0.69	10
pPRE (*R. leguminosarum*)	0.43	14	14	16	12	5.2	0.62	14
HIM (*R. leguminosarum*)	0.40	19	17	17	16	7.2	0.90	17
pRtr843 (*R. trifolii*)	0.90	24	29	26	4.8	29	9.2	24
pSym5 (*R. trifolii*)	1.9	14	19	17	6.3	10	10	15

Rhizobium derivatives that contain an IncP plasmid, with one of the *nodD* genes, as well as the IncQ plasmid pMP154 (Fig. 1) were constructed by introducing these plasmids into strain LPR5045 which has been cured for its own Sym plasmid[23]. When intact Sym plasmids were to be used as the source of *nodD*, Tn5-marked Sym plasmids were transferred to LPR5045 either by direct conjugation (pRL1JI, pHIM and pSym5) or by mobilization with the helper plasmid R180 (ref. 23), and pMP154 was introduced subsequently. The Sym plasmids pRL1J1 (ref. 25) pSym1 (ref. 23) and pPRE (ref. 26) confer nodulation ability on *Pisum sativum* cv Rondo, *Vicia sativa* ssp. nigra and *V. hirsuta*, whereas pHIM (ref. 26) confers the ability to nodulate these *R. leguminosarum* hosts as well as *P. sativum* cv Afghanistan (a Middle East variety). The Sym plasmids pRtr843 (ref. 4) and pSym5 (ref. 23) allow nodulation on *Trifolium pratense* and *T. repens*. *Nod* promoter induction assays were performed as described previously[14] after incubation for 12 h (maximal induction), if applicable in the presence of 400 nM flavonoid or undiluted exudate, and the measured activities are indicated as units of β-galactosidase[27]. The induction assays were performed in triplicate and variation from the given value was within 10%. Exudates of *Melilotus alba*, *P. sativum*, *V. hirsuta*, *T. repens* and *T. pratense* were prepared as described previously[10]. The former four exudates had similar characteristics in that they induced the *nod* promoter in each strain to at least 50% of the induction level observed with luteolin. Only representative results (with *T. repens* exudate) are shown. The following control experiments were performed. To test whether the absence of inducing capacity of *T. pratense* exudate with several strains was due to the presence of inhibitors, the level of induction by 400 nM luteolin was compared with that of a mixture of 400 nM luteolin and undiluted *T. pratense* exudate. Induction levels were found to be identical. *Rhizobium* LPR5045 containing pMP154 (with no *nodD* gene present) showed 150 units β-galactosidase with all tested compounds. Apigenin, eriodictyol, and luteolin were from Carl Roth, Karlsruhe. Naringenin was from Sigma and 7-hydroxyflavone from EGA, Steinheim, FRG.

Table 2 Nodulation of *T. pratense* and *T. repens* by *R. trifolii* derivatives containing *nodD* genes of various *Rhizobium* species

Rhizobium strain (and *nodD* source)	Nodulated plants (%)		Average number of nodules per nodulated plant	
	T. pratense	*T. repens*	*T. pratense*	*T. repens*
ANU851.pMP280 (*R. leguminosarum*)	3	100	1	4
ANU851.pMP283 (*R. trifolii*)	90	100	2	4
ANU851.pMP284 (*R. meliloti*)	0	70	—	2
ANU851 (*nodD*::Tn*5*)	0	0	—	—
ANU843 (*R. trifolii* wild type)	90	100	2	4

The plasmids pMP280, pMP283 and pMP284 (Fig. 1) were mobilized to the *R. trifolii* strain ANU851, which contains a Tn*5* insertion in the *nodD* of its Sym plasmid pRt843 (ref. 28). The resulting strains, as well as ANU851 and the wild-type strain ANU843, were tested for nodulation ability on *T. repens* (white clover) and *T. pratense* (red clover). For each strain, 60 plants were infected and nodulation assays were performed as described previously[29]. All nodulated plants were able to fix nitrogen. Plant seeds were from Kieft, Blokker (Netherlands).

activate the *nod*-inducible promoter. The strains containing the *nodD* gene of *R. leguminosarum* or *R. trifolii* can be most clearly distinguished by their reactions with eriodictyol and 7-hydroxyflavone in that eriodictyol is the better inducer in the presence of *R. leguminosarum nodD* (ratio eriodictyol/7-hydroxyflavone is 2.8) whereas 7-hydroxyflavone is a much better inducer in the presence of *R. trifolii nodD* (ratio eriodictyol/7-hydroxyflavone is 0.16). In contrast, neither these two substances nor naringenin were efficient inducers for the strain containing the *R. meliloti nodD* gene (Table 1). In the case of *R. leguminosarum* and *R. trifolii* the same differences in induction were also observed when the complete Sym plasmids pRL1JI and pRtr843 were used as the source of *nodD* (Table 1, lines 4 and 8). As a control, the strains containing the combinations pMP283 plus pMP193 and pMP284 plus pMP194, representing combinations of *nodD* clones with their own inducible promoters, were tested with the same inducers (Table 1). Almost similar results were obtained as with the inducible promoter in pMP154, although the combination pMP283 plus pMP154 showed a higher level of induction than the combination pMP283 plus pMP193 (data not shown).

To decide whether the observed differences in induction patterns could have species-specific basis, the Sym plasmids of three other *R. leguminosarum* strains and one other *R. trifolii* strain were tested as the *nodD* source (Table 1, lines 5, 6, 7 and 9). The three other *R. leguminosarum* Sym plasmids yielded essentially the same results as observed with pRL1JI as the *nodD* source. Similarly, the results obtained with the other *R. trifolii* Sym plasmid were comparable with that of pRtr843. The data in Table 1 therefore indicate that the *nodD* products of the *Rhizobium* species tested differ in their response to flavonoid inducers in a species-specific way.

To see whether this specificity could also be reflected in nature, exudates of *Melilotus alba*, *Pisum sativum*, *Vicia hirsuta*, *Trifolium repens* and *T. pratense* were tested for their ability to induce the *nod* promoter of pMP154 in the presence of the various *nodD* clones. The former four exudates induced in each *nodD* strain the *nod* promoter to at least 50% of the induction level of luteolin (Table 1). In contrast, exudate of *T. pratense* (red clover) was only a good inducer in the presence of the *nodD* gene of *R. trifolii* (Table 1). We have also shown that the absence of induction with red clover exudate was not due to inhibitors (Table 1 legend). Because the control with *T. repens* exudate shows that induction with clover exudate is possible in all cases, these results confirm that the *nodD* genes of the tested plasmids have cross-inoculation group specific characteristics.

The results with the *T. pratense* exudate cast doubt on the general assumption that *nodD* is a common *nod* gene. We therefore investigated whether the *nodD* gene of either *R. leguminosarum* or *R. meliloti* can function in the nodulation of red clover. Therefore the plasmids with the different *nodD* genes were mobilized into *R. trifolii* strain ANU851 (pRtr843 *nodD*::Tn*5*). When the resulting strains were tested for nodulation ability on red and white clover, it appeared that the *nodD* genes of *R. leguminosarum* and *R. meliloti* are only able to complement the mutation of strain ANU851 for nodulation on white clover (and the *R. meliloti nodD* not completely) whereas the (control) *nodD* gene of *R. trifolii* can complement for nodulation on both *Trifolium* species (Table 2). We conclude that the host range of *R. trifolii* is narrowed when its own *nodD* gene is replaced by that of *R. leguminosarum* or *R. meliloti* and that therefore *nodD* cannot be considered as a common *nod* gene.

Although the *nodD* products of *R. leguminosarum*, *R. trifolii* and *R. meliloti* have a homology with each other of at least 75% (refs 2, 16 and 17), they are strikingly different in a number of properties. In an isogenic series of strains which differ only in the source of their *nodD* gene, it was found that these strains differ in their response to a set of flavonoid inducers (Table 1). For the *R. leguminosarum* and *R. trifolii* Sym plasmids tested, this response appears to be species specific (Table 1). In at least one case, namely the symbiosis between *R. trifolii* and *T. pratense*, the *nodD* gene does not behave as a common *nod* gene (Table 2). It has been reported that the *nodD* genes of fast-growing *Rhizobium* species can complement each other in nodulation assays on several legume host plants. Therefore *nodD* has been designated as a common *nod* gene[1,20–22]. Our results show that this notion should be revised. The results indicate that the interaction between flavonoid inducer and *nodD* product is crucial in the nodulation process. To extend the host range of nodulation, even to non-leguminous plants, strategies based upon manipulation of the interaction between these molecules could be successful.

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Targeting of bacterial chloramphenicol acetyltransferase to mitochondria in transgenic plants

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Most mitochondrial proteins are encoded by nuclear genes and are synthesized as precursors containing a presequence at the N terminus. In yeast and in mammalian cells, the function of the presequence in mitochondrial targeting has been revealed by chimaeric gene studies. Fusion of a mitochondrial presequence to a foreign protein coding sequence enables the protein to be imported into mitochondria *in vitro* as well as *in vivo*[1-4]. Whether plant mitochondrial presequences function in the same way has been unknown. We have previously isolated and characterized a nuclear gene (*atp2-1*) from *Nicotiana plumbaginifolia* that encodes the β-subunit of the mitochondrial ATP synthase[5]. We have constructed a chimaeric gene comprising a putative *atp2-1* presequence fused to the bacterial chloramphenicol acetyltransferase (CAT) coding sequence and introduced it into the tobacco genome. We report here that a segment of 90 amino acids of the N terminus of the β-subunit precursor is sufficient for the specific targeting of the CAT protein to mitochondria in transgenic plants. Our results demonstrate a high specificity for organelle targeting in plant cells.

Nucleotide sequence analysis revealed that the β-subunit of *N. plumbaginifolia* mitochondrial ATP synthase is synthesized as a precursor of relative molecular mass 59,000 (M_r 59K). Because the mature β-subunit is of M_r 50K, these results together suggest a presequence of ~9K (ref. 5).

A fragment containing the putative presequence of the *atp2-1* gene was obtained by 3′ *Bal*31 deletions. To retain the putative processing site, we chose a 3′-deleted fragment that encodes the amino-terminal 90 residues (M_r ~9K) of the β-subunit precursor as likely to contain the prebeta sequence as well as a few amino acids of the mature protein. After *Nco*I digestion at the first ATG[5], the DNA fragment was fused to the CAT coding sequence by appropriate *Hin*dIII linkers (Fig. 1*a*). To express this chimaeric coding sequence (prebeta-CAT) in plant cells, it was placed under the control of the constitutive 35S promoter of the cauliflower mosaic virus (CaMV)[6]. The CAT gene without the presequence of the *atp2-1* gene and driven by the same 35S promoter[7] was used as a negative control. Both constructs were cloned into the binary vector pMON505 (ref. 8) and introduced into tobacco cells.

Most of the transgenic plants containing either the prebeta-CAT or the control CAT construct showed CAT activity. The control CAT plant did not show any activity in organellar fractions. In contrast, CAT activity was highly enriched in the mitochondrial fraction and was barely detectable in the chloroplast fraction (Fig. 1*b*) of transgenic plants containing the prebeta-CAT construct.

To obtain further evidence that the prebeta-CAT product was localized in the mitochondria, we compared the CAT activity in different subcellular fractions with that of malate dehydrogenase, a marker enzyme for the mitochondrial matrix[9]. Table 1 shows that both enzymes behaved similarly during cell fractionation. About 30% of both activities in the homogenate was recovered in the crude mitochondrial fraction and nearly 50% was found in the crude supernatant. Further centrifugation of the supernatant at 100,000*g* for 30 min did not pellet any of the two enzyme activities (Table 1). We concluded that the soluble CAT activity could be attributed to enzymes released from mitochondria broken during homogenization.

We were unable to demonstrate directly that the prebeta-CAT product was sequestered by mitochondria because CAT was destroyed by proteolytic inactivation (not shown). However, washing mitochondria with high salt (1 M NaCl) failed to release the CAT activity (Fig. 1*c*) but repeated 'freeze and thaw' or sonication of the mitochondrial fraction solubilized the activity (Fig. 1*c*). Taken together, these results strongly suggest that the prebeta-CAT product was localized in the mitochondrial matrix.

To see whether the prebeta-CAT was processed to a mature form, we used an antiserum directed against CAT for Western blot analysis. The crude cytosolic fraction of control CAT plants showed a major band of 24K (Fig. 2*a*), an M_r consistent with that of the bacterial enzyme[10]. Distribution of the prebeta-CAT products (Fig. 2*b*) followed that of the CAT activity (Fig. 1*b*). Polypeptide bands decorated by the antiserum were detected in the mitochondrial but not the chloroplast fraction. The major band (M_r = 25.5K) was slightly larger than the normal CAT protein (Fig. 2*d*). This apparent difference (1.5 K) probably corresponds to the linker region and to beta polypeptide residues located downstream of the processing site (see Fig. 1*a*). We also observed minor bands that were larger (M_r = 29K and 30K) whose intensity was slightly variable among extracts from different transgenic plants (not shown). These polypeptides were smaller than the prebeta-CAT precursor (M_r = 34.5 K) as synthesized in an *in vitro* system (Fig. 2*d*) and probably resulted from

Table 1 Co-fractionation of CAT activity with malate dehydrogenase activity in a prebeta-CAT transgenic plant

		Homogenate	Supernatant	Crude chloroplasts	Purified chloroplasts	Crude mitochondria	Purified mitochondria
CAT	Specific activity	1.00	0.75	0.55	0.25	2.8	10.9
	Proportion of homogenate (%)	100	51.7	8.3	1.0	33.3	7.4
Malate dehydrogenase	Specific activity	1.00	0.85	0.54	0.25	2.4	9.1
	Proportion of homogenate (%)	100	59.1	8.2	1.0	28.8	6.2

CAT and malate hydrogenase activities were analysed in each subcellular fraction obtained from a prebeta-CAT transgenic plant as described in Fig. 1. Specific activities are expressed in arbitrary units and are given the value of 1 for the homogenate. The total activity of the fraction is given as a percentage of the total activity in the homogenate.