Luteolin absorption in *Rhizobium meliloti* wild-type and mutant strains

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Luteolin is a flavonoid produced by plants which is required for induction of *nod* genes in *Rhizobium meliloti*. *R. meliloti* absorbed luteolin at higher rate than all other bacteria tested, including *R. leguminosarum*. The flavonoids naringenin and quercetin, which do not induce the expression of nodulation genes of *R. meliloti*, were absorbed at a lower rate by this species, suggesting a certain degree of species specificity of flavonoid absorption. Luteolin accumulated preferentially in the outer membrane, but a small amount was always found in the inner membrane. Luteolin strongly inhibited NADH oxidase, an enzyme of the respiratory chain, raising the possibility that the site of luteolin absorption in the outer membrane allows the protection of the respiratory chain located in the inner membrane from an excess of flavonoids. The incorporation of luteolin was found to be affected in some *exo* or *nod* mutants of *R. meliloti*. The *exoB* mutant, which does not produce exopolysaccharides, accumulated lower amounts of luteolin in the outer membrane than the *exo*⁺ parent. Among the *nod* mutants affected in nodulation genes, those not expressing any of the three *nodD* genes accumulated luteolin at a significantly lower level in both the outer and the inner membrane. A strain overexpressing the *nod* genes, particularly the *nodD* genes, absorbed luteolin at a higher level in both membranes. These results indicate that absorption of luteolin by *R. meliloti* involves several gene products, including the NodD protein.

Introduction

In rhizobia the expression of nodulation (*nod*) genes is induced by the regulatory NodD protein in conjunction with flavonoids produced by the host plants (for a review see Kondorosi, 1992). In general, the *nodD* genes are expressed constitutively, and genetic data indicate that upon binding of the flavonoids to the NodD proteins the activated NodD can induce the expression of the *nod* genes. However, the specific binding of flavonoids to the NodD proteins has not yet been directly demonstrated.

The specific interaction between flavonoids of the host plant and NodD ensures the host-specific control of *nod* gene expression. For example, luteolin (3',4',5,7-tetrahydroxyflavone) isolated from *Medicago sativa* seed extracts was shown to be a potent *nod* gene inducer in *Rhizobium meliloti* (Peters *et al.*, 1986), while naringenin (5,7,4'-trihydroxyflavanone) isolated from *Vicia sativa* root exudate is an important *nod* gene inducer in *Rhizobium leguminosarum* biovar *viciae* (Zaat *et al.*,

1988). This latter compound was shown to have no *nod* gene inducing ability in *R. meliloti* (Györgypal *et al.*, 1991*a*). Isoflavonoids were found to be potent *nod* gene inducers in *Bradyrhizobium* but had no inducing activity in most *Rhizobium* species, or even inhibited induction of *nod* genes (for a review see Györgypal *et al.*, 1991*a*).

Using antibodies against a *lacZ*–*nodD* gene fusion product, Schlaman *et al.* (1989) found that the NodD protein is located exclusively in the cytoplasmic membrane of wild-type *R. leguminosarum* biovar *viciae* cells. In a *R. leguminosarum* strain overproducing NodD, the protein was found both in the cytoplasmic membrane and in the cytosol, indicating an influence of the protein abundance on its subcellular localization. A model for *nod* gene induction was presented which combines the localization and the DNA-binding properties of the NodD protein as well as the observed association of flavonoids with the cytoplasmic membrane.

The accumulation of naringenin in the cytoplasmic membrane of *R. leguminosarum* biovar *viciae*, without apparent metabolic conversion, was reported by Recourt *et al.* (1989). The binding of naringenin was highly reversible and *in vitro* experiments showed that narin-

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Table 1. Genetic and symbiotic characteristics of strains used in this study

Strains and plasmids	Relevant genotype	Symbiotic phenotype	Reference/source
<i>R. meliloti</i>			
Rm41		Nod ⁺ Fix ⁺	Original isolate by K. Szende & F. Ordógh
AK631	Rm41 <i>exoB</i>	Nod ⁺ Fix ⁺	Kondorosi <i>et al.</i> (1989)
EK698	AK631 <i>nolR::Tn5</i>	Nod ⁺ * Fix ⁺	Kondorosi <i>et al.</i> (1989)
EK1261	EK698(pEK327)		Obtained from E. Kondorosi
Rm1021	<i>nolR</i>	Nod ⁺ * Fix ⁺	Meade <i>et al.</i> (1982)
AK1672	AK631 <i>nodC::Tn5</i>	Nod ⁻	Kondorosi <i>et al.</i> (1984)
ZB138	<i>nod-nif</i> deletion derivative of AK631	Nod ⁻	Banfálvi <i>et al.</i> (1981)
A2105	Rm1021 <i>nodC-lacZ</i> <i>nodD1D2D3</i>	Nod ⁻	Honma <i>et al.</i> (1990)
Plasmid pEK327	pLAFRI carrying <i>nodFEGHPQD3</i> and <i>syrM</i>		Schultze <i>et al.</i> (1992)
<i>R. leguminosarum</i> 248	Wild-type strain	Nod ⁺ Fix ⁺	Josey <i>et al.</i> (1979)
<i>A. tumefaciens</i> 82139	Wild-type strain		Michel <i>et al.</i> (1990)
<i>A. rhizogenes</i> A4TC24	C58C1		Petit <i>et al.</i> (1983)
<i>P. syringae</i> pv. <i>pisi</i>	Wild-type strain		Schmit (1991)
<i>E. coli</i> K12	C600		Appleyard (1954)

* About 2 d delay in nodulation.

genin had a high, pH-dependent affinity for the cytoplasmic membrane.

The flavonoids may have effects on rhizobia other than *nod* gene induction. The growth rate of *R. meliloti* was enhanced by flavonoids released naturally from *M. sativa* seeds, but only in a minimal medium that allowed rather poor growth and not in the presence of yeast extract and tryptone (Hartwig *et al.*, 1991). It was suggested that the mechanism which increases growth is distinct from its capacity to induce rhizobial *nod* genes and that luteolin may create ecochemical zones for controlling soil microbes.

We have undertaken a more extensive study of the mechanisms involved in luteolin absorption by *R. meliloti*. As a first step, we studied how luteolin was absorbed by *R. meliloti* and *R. leguminosarum* and by other Gram-negative bacteria, including *Agrobacterium tumefaciens*, *A. rhizogenes*, *Pseudomonas syringae* and *Escherichia coli*. In addition, absorption of two flavones not inducing the *nod* genes in *R. meliloti*, namely naringenin and quercetin, was investigated. By incubating live bacteria with luteolin, the amount of flavonoid absorbed in the inner and outer membranes of wild-type and mutant strains affected in the *nod* region was determined.

Methods

Bacterial strains and plasmids. These are listed in Table 1.

Media and culture conditions. Liquid cultures were grown at 30 °C with aeration on different media for each bacterium: TA medium

(Tryptone-yeast) (Kondorosi *et al.*, 1977) for *R. meliloti* and *E. coli*, TY medium (Beringer, 1974) for *R. leguminosarum*, YEB medium (Alt-Moerbe *et al.*, 1989) for *Agrobacterium*, YAD medium (Meng & Wang, 1987) for *P. syringae*. Growth of bacteria was followed spectrophotometrically by measuring the optical density at 540 nm. Samples were taken during the exponential phase of growth (OD₅₄₀ = 0.9).

Fractionation of membranes. Overnight bacterial cultures were harvested by centrifugation at 5000 g for 30 min at 4 °C, washed twice in 0.9% NaCl. The cells were broken by two passages through a French press cell (about 70 MPa). Membranes were fractionated on a discontinuous sucrose density gradient (Hubac *et al.*, 1992). The protein content of different fractions of the gradient was determined by the method of Bradford (1976). Two membrane markers, the KDO (2-ketodeoxyoctonate) content for the outer membrane and the NADH oxidase activity for the inner membrane (de Maagd & Lugtenberg, 1986), were used. By following the profile of the protein content, the outer membrane was found in the 55% (w/v) sucrose fractions, and the inner membrane in the 35% sucrose fractions (Hubac *et al.*, 1992).

NADH oxidase activity measurement. The pellet containing the membranes was suspended in 50 mM-Tris/HCl (pH 8) and incubated for 5 min at 30 °C in 0.2 mM-dithiothreitol (DTT) and 0.12 mM-NADH as substrate. Luteolin at different concentrations was added to the membrane suspension and the NADH oxidase activity was measured spectrophotometrically (Ravanel *et al.*, 1982).

Luteolin analysis. Samples (40 ml) of *R. meliloti* cultures growing in the presence of luteolin were taken at different time intervals and centrifuged at 5000 g for 30 min at 4 °C. The bacterial pellet was washed in 0.9% NaCl (pH 7.4), suspended, and boiled in 2 ml ethanol, then 2 ml chloroform was added. After a second centrifugation at 5000 g for 30 min, the supernatant was collected, evaporated under a nitrogen stream and resuspended in 1 ml ethanol. This extract was then analysed for luteolin content by TLC, spectrophotometry or HPLC. For the fractionation of membranes, the analyses were made from 500 ml of bacterial culture containing luteolin.

For TLC, the solvent used was chloroform/acetone/methanol/acetic acid/water (10:4:2:2:1, by vol.; Trémolières & Lepage, 1971).

Luteolin gave a yellow spot in the first third of the chromatogram ($R_F = 0.72$).

For spectrophotometric measurement, the absorbance of luteolin (purchased from Carl Roth) was measured in ethanol at 351 nm against the same bacterial extract which had not been treated with luteolin. The amount of luteolin was calculated from a standard curve obtained with standard solutions. The absorbance of quercetin and naringenin (purchased from Extrasynthese) were measured at 373 nm and at 288 nm respectively in ethanol.

HPLC analysis was performed using a method adapted from Peters *et al.* (1986). Using a C18 ODS column (220 × 4.6 mm) a linear gradient was performed at 1 ml min⁻¹ starting from methanol (20%) and water (80%) with acetic acid (2%) to 100% (v/v) methanol. Luteolin was eluted at about 20 min and read at 351 nm; quercetin read at the same wavelength was eluted just before luteolin; naringenin eluted at 30 min was read at 280 nm. For quantitative measurements, standard flavonoids were used as references.

Results

Characteristics of luteolin absorption by whole bacteria

We first verified that luteolin did not affect the growth of bacteria in TA medium. The kinetics of luteolin absorption by *R. meliloti* strain Rm41 was then followed at different concentrations of luteolin in the culture medium. Fig. 1 shows that the uptake of luteolin increased with increasing concentrations of the flavonoid in the medium. The luteolin absorption was rapid; when a bacterial culture was mixed with luteolin-containing medium, a high level of absorption was recorded immediately. It was verified that this rapid absorption did not result from contamination during the centrifugation of the bacterial pellet with the culture medium by

Fig. 1. Kinetics of luteolin absorption by *R. meliloti* strain Rm41. Bacteria were incubated in a medium containing different luteolin concentrations (1 µM, 5 µM, 10 µM and 20 µM) and for different times. In order to take into account the growth rate of the bacteria, luteolin was added 360, 240, 180, 120, 60, 30, 20 and 10 min before the end of the experiment. Bacteria were pelleted and luteolin content was determined at the same culture OD for each time. Luteolin absorption is expressed in µg (g fresh bacterial pellet)⁻¹. Values are means of five experiments ± SE.

washing the pellet several times in 0.9% NaCl without significant loss of luteolin. At 10 and 20 µM, the two highest concentrations of luteolin in the medium, a transitory decrease of the bacterial flavonoid content was observed after the first rapid incorporation step (Fig. 1). This leakage of luteolin was observable only for high concentrations of luteolin (10 µM). The significance of this decrease is unknown. In further experiments, we always used luteolin at a concentration of 10 µM. When the concentration of luteolin was measured in the culture medium, only a very small decrease of this concentration (from 2.5 to 2.0 mg ml⁻¹) was observed at the beginning of the incubation, and after that it remained constant (data not shown). Under the conditions used, luteolin always remained in excess in the medium.

Resorption of luteolin

Bacteria incubated for 4 h in the presence of luteolin were pelleted, washed with NaCl (0.9%) and transferred into fresh medium without luteolin. Leakage of luteolin from the bacteria was observed immediately (Fig. 2) and only a low but significant amount of luteolin (about 10% of the original content) remained absorbed by the bacteria. After 10 min, the bacterial luteolin content slowly increased for several hours, probably because newly formed bacteria incorporated the luteolin leaked out into the medium. This rapid leakage could be attributed to a partitioning effect between the aqueous phase of the medium (where ionized luteolin can be dissolved) and the lipid phase of the bacterial membrane (where luteolin in non-ionized form remains absorbed), as proposed by Recourt *et al.* (1989). In our conditions (9.5 mg fresh bacteria per ml incubation medium), bacteria occupied less than 1% of the volume; in this case, the simple equilibrium process in a new medium devoid of luteolin would normally lead to a bacterial luteolin concentration ten times lower than that found in our experiment. Partitioning alone cannot explain such a result.

Absorption of luteolin by other Gram-negative bacteria and absorption of other flavonoids by *R. meliloti*

For all Gram-negative bacteria studied other than *R. meliloti*, a low rate of luteolin absorption was observed (Table 2). For *A. tumefaciens* and *A. rhizogenes*, *P. syringae* and *E. coli*, the absorption was about 10-fold less than for *R. meliloti*. With *R. leguminosarum* biovar *viciae*, a *Rhizobium* species where the *nod* genes are not induced by luteolin but by other flavones, such as naringenin (Recourt *et al.*, 1989), the absorption was also significantly lower than in *R. meliloti*. In this case, there

Fig. 2. Kinetics of luteolin resorption by *R. meliloti* strain Rm41. Bacteria were incubated with luteolin (10 μM) for 4 h, washed and then reincubated in a fresh medium without luteolin (arrow). Luteolin absorption is expressed as $\mu\text{g (g fresh bacterial pellet)}^{-1}$. Values are means of five experiments $\pm$ SE.

Table 2. Rate of luteolin absorption

R. meliloti (Rm41), *R. leguminosarum* biovar *viciae*, *A. rhizogenes*, *A. tumefaciens*, *P. syringae* and *E. coli* were incubated for 4 h in a medium containing 10 μM -luteolin. Values are means of five experiments $\pm$ SE.

	Bacterial luteolin content [$\mu\text{g (g fresh bacterial pellet)}^{-1}$]
<i>R. meliloti</i> Rm41	18.8 $\pm$ 1
<i>R. leguminosarum</i> biovar <i>viciae</i>	10.9 $\pm$ 0.6
<i>A. rhizogenes</i>	4.1 $\pm$ 0.4
<i>A. tumefaciens</i>	0.9 $\pm$ 0.2
<i>P. syringae</i>	2.2 $\pm$ 0.2
<i>E. coli</i>	2.2 $\pm$ 0.2

Table 3. Rate of luteolin absorption by *R. meliloti* (Rm41) and *R. leguminosarum* biovar *viciae*

Bacteria were incubated for 30 s, 10 min and 240 min with 10 mM-luteolin. Values are means of five experiments $\pm$ SE.

	Bacterial luteolin content [$\mu\text{g (g fresh bacterial pellet)}^{-1}$]		
	30 s	10 min	240 min
<i>R. meliloti</i>	26.5 $\pm$ 1	11.4 $\pm$ 0.4	18.8 $\pm$ 1
<i>R. leguminosarum</i> biovar <i>viciae</i>	13.5 $\pm$ 0.5	10.9 $\pm$ 0.3	10.9 $\pm$ 0.3

was a rapid and weaker incorporation which always remained at the same level and did not exhibit kinetics similar to that of *R. meliloti* (Table 3). When *R. meliloti* was grown in the presence of naringenin or quercetin,

Table 4. Rate of flavonoid absorption by *R. meliloti* (Rm41)

Bacteria were incubated for 4 h with luteolin, naringenin or quercetin, each at a concentration of 10 μM . Values are means of five experiments $\pm$ SE.

Flavonoid	Bacterial flavone content [$\mu\text{g (g fresh bacterial pellet)}^{-1}$]
Luteolin	18.8 $\pm$ 1
Naringenin	8.3 $\pm$ 0.5
Quercetin	7.3 $\pm$ 0.5

Table 5. Rate of luteolin absorption by wild-type and mutant *R. meliloti* strains

Bacteria were incubated with 10 μM -luteolin for 4 h. Strains: Rm41 and Rm1021 are wild-type isolates; AK631, *exoB* derivative of Rm41; EK1261, overexpresses the *nod* genes; AK1672, *nodC*; A2105, *nodD1,D2,D3*, ZB138, derivative of AK631 carrying deletion in the *nod-nif* region of the megaplasmid. Values are means of five experiments $\pm$ SE.

Strain	Bacterial luteolin content [$\mu\text{g (g fresh bacterial pellet)}^{-1}$]
Rm41	18.8 $\pm$ 1
Rm1021	18.4 $\pm$ 1
EK1261	24.1 $\pm$ 2
AK631	13.7 $\pm$ 1.5
AK1672	14.5 $\pm$ 1.4
A2105	9.6 $\pm$ 1
ZB138	5.8 $\pm$ 0.6

which allow a normal rate of growth at the concentration used, but do not induce the expression of the nodulation genes in *R. meliloti* (Györgypal *et al.*, 1991b), only weak absorption was observed (Table 4).

Comparison of luteolin absorption between wild-type strains and mutants of *R. meliloti*

Using wild-type and mutant *R. meliloti* strains, luteolin absorption was measured for 4 h at a luteolin concentration of 10 μM for a starting bacterial OD₅₄₀ of 0.3, increasing to a final OD of 0.7. As shown in Table 5, the exopolysaccharide-producing wild-type strains Rm41 and Rm1021 had high levels of absorption. In strain AK631, an *exoB* derivative of Rm41, the absorption was reduced to about 30% of that of Rm41. In AK1672, a *nodC* mutant derivative of AK631, the luteolin absorption did not differ from AK631, while in a *nodD1D2D3* mutant (A2105) and in a derivative carrying a large deletion in the megaplasmid (ZB138), the absorption of luteolin was much lower. On the other hand, strain EK1261, which overexpresses the *nod* genes, showed a higher level of luteolin absorption than the

Fig. 3. Determination of the luteolin content in outer and inner membranes of different wild-type and mutant strains of *R. meliloti* after incubation for 8 h in 10 μ M luteolin-containing medium. Luteolin content is expressed in μ g (mg protein) $^{-1}$. ■, Rm41; ▨, EK1261; ▩, AK631; ▤, AK1672; □, A2105; ▒, ZB138.

wild-type strain Rm41. These data indicate that several genes (*exo* and *nod*) influence (directly or indirectly) the overall level of luteolin absorption by *R. meliloti*.

Comparison of luteolin incorporation in outer and inner membranes of the wild-type strain

After incubation for different times in the presence of luteolin, bacteria were pelleted, then disrupted in the French press, and outer and inner membranes were isolated as previously described (Hubac *et al.*, 1992). The luteolin content was determined by HPLC and spectrophotometric analysis. The luteolin content was also determined in the supernatant of the first centrifugation after the breakage of the cells. The luteolin found in the supernatant could represent both the cytoplasmic luteolin pool and the fraction of luteolin weakly attached to the external side of the bacterial envelope which could be lost during the disruption of the cells.

After 8 h of bacterial growth in the presence of luteolin (10 μ M), most of the bound flavonoids were found in the outer membrane, and only 10–20% was found in the inner membrane (Fig. 3). After short incubation times, most of the luteolin was found in the supernatants (Table 6). Then, the luteolin content decreased in the supernatant and increased progressively in the two membranes. The ratio of luteolin absorbed to the outer and inner membranes remained almost constant during the incubation period. These characteristics of luteolin binding were consistent with a two-step process: first, a rapid fixation on the external site of the bacterial envelope and then a slower accumulation in the membranes themselves and especially in the outer one. Nevertheless, it is difficult to be sure that the luteolin distribution found between isolated membranes repre-

sents the true situation in the living bacteria and that no cross-contamination occurred during the isolation process.

Luteolin incorporation in outer and inner membranes of mutant strains

After 8 h growth of different mutant strains in the presence of luteolin (10 μ M), the bacteria were pelleted, disrupted in the French press, the outer and inner membranes were separated and the luteolin content was determined. As shown in Fig. 3, the luteolin content was reduced to about 30% in both the outer and inner membranes of AK631 not producing exopolysaccharides, but in those of the *nodC* mutant derivative of AK631 (AK1672) it was not further affected. In the two mutant strains which produce no NodD protein (A2105, ZB138) the luteolin accumulation was reduced in the two membranes, although a small amount of luteolin was always found in the inner membrane. The inner membrane was never completely devoid of luteolin in these two latter mutants; nevertheless contamination by the outer membrane cannot be completely ruled out. Strain EK1261, which overexpresses the *nod* genes, contained high amounts of luteolin in the two membranes.

The differences in luteolin content between the different strains were greater at the membrane level than when the luteolin content of whole cells was measured. This difference can be explained by the fact that luteolin found in the supernatant fraction was not taken into account when luteolin content in the isolated membranes was determined.

Effect of luteolin on NADH oxidase activity

Flavonoids can inhibit photosynthetic or respiratory electron transfer in chloroplasts or in plant mitochondria (Moreland & Novitzky, 1988; Ravelin *et al.*, 1982, 1990). Preferential accumulation of luteolin in the outer membrane might allow the protection of the respiratory chain located in the inner membrane against an excess of flavonoid. In order to test this possibility, we measured the effect of luteolin on NADH oxidase, one of the main enzymes of the respiratory chain.

When total membrane fractions of strain Rm41 were treated with luteolin, a significant inhibition of NADH oxidase activity was observed (Fig. 4a). The same result was obtained when isolated inner membranes containing NADH oxidase were treated with luteolin: 50 μ M-luteolin inhibited NADH oxidase activity by 65% and this activity was totally suppressed with luteolin at 100 μ M (Fig. 4b). No NADH oxidase activity was found in the outer membrane.

Table 6. Repartition of luteolin in outer and inner membranes and in the supernatant fraction after different times of incubation of *R. meliloti* (Rm41) in medium containing 10 μ M-luteolin

Washed bacteria were resuspended in Tris/HCl, broken by passage through French press, then centrifuged at 130000 *g* for 2 h. The luteolin content was determined in the supernatant. The membrane pellet was resuspended in 1 ml Tris/HCl and outer and inner membranes were separated on sucrose gradient. Luteolin content was then determined in the separated membranes.

Incubation time	Luteolin absorbed [μ g (mg protein) ⁻¹]		
	Supernatant	Outer membrane	Inner membrane
10 min	0.29	0.45	0.048
2 h	0.21	1.07	0.090
4 h	0.04	2.90	0.220

Fig. 4. Effect of luteolin on the activity of NADH oxidase. (a) Total membranes; (b) inner membranes from Rm41 isolated by centrifugation (130000 *g*, 2 h) of the cells broken by French press. Membranes were fractionated on a discontinuous sucrose density gradient. Samples (25 μ g) of membrane protein were incubated at 30 °C in Tris (pH 8.0), 0.2 mM-DTT and 0.12 mM-NADH with or without luteolin: 1, 0 μ M; 2, 50 μ M; 3, 100 μ M. Oxidation of NADH was followed at 340 nm for 16 min.

Discussion

This paper demonstrates that luteolin absorption by *R. meliloti* is a far more complex process than previously postulated. We demonstrated some species-specificity of luteolin absorption in that another *Rhizobium* species, *R. leguminosarum* biovar *viciae*, or other Gram-negative bacteria, *Pseudomonas*, *Agrobacterium* or *E. coli* do not incorporate this flavonoid at the same level. Moreover, two other flavonoids which do not induce the nodulation process in *R. meliloti* are poorly absorbed by this bacterium. The biochemical basis of this specificity remains unknown. Nevertheless it can be supposed that some specific sites for this binding may exist in *R. meliloti* which are not found in the other bacteria.

The first step of luteolin absorption appears to be a rapid and reversible process of equilibrium of the

flavonoids between the medium and the bacterial cell surface as previously suggested for the absorption of naringenin by *R. leguminosarum* biovar *viciae* (Recourt *et al.*, 1989). The second step, allowing the accumulation of luteolin in the two membranes of *Rhizobium*, seems to be more progressive and we could not prove that this step is purely passive. Much work has to be done to establish the energetic basis of this accumulation. The binding of luteolin is only partly reversible since, after transfer of the bacteria into a medium without luteolin, a small but significant amount of the flavonoid remained firmly bound to the bacteria.

Studying absorption of naringenin to isolated membranes of *R. leguminosarum* biovar *viciae* *in vitro*, Recourt *et al.* (1989) reported that naringenin could be bound to the membrane only at acidic pH and, in this condition, the flavonoid accumulated exclusively in the inner membrane. Our studies on the absorption *in vivo* of luteolin by *R. meliloti* cells led to different observations. Accumulation of luteolin was detected principally in the outer membrane, while only low (but significant) amounts were bound to the inner membrane. According to previous reports (Ravanel *et al.*, 1982, 1990; Moreland & Novitzky, 1988) flavonoids can inhibit photosynthetic or respiratory electron transfer in plant chloroplasts or mitochondria. We found that luteolin at a concentration of around 50 μ M inhibited the activity of NADH oxidase, which is located in the inner membrane. According to our results, *R. meliloti* can incorporate around 20 μ g luteolin (g of fresh bacteria)⁻¹. Thus the molar concentration of luteolin in the whole bacteria can be estimated as 70 μ M. If we consider that most of this luteolin remained located in the membranes, that means that a concentration higher than 100 μ M (which is sufficient to totally inhibit NADH oxidase activity), can be found in this compartment. Based on these results, we can speculate that in *R. meliloti* there might be a specific system for the binding of large amounts of luteolin in the outer membrane, allowing the protection of the respiratory chain against this flavonoid.

Measurements of luteolin absorption by different mutant strains of *R. meliloti* have led to several conclusions. First, the incorporation into the *exoB* derivative of Rm41 was reduced to about 30%. This strain is Nod⁺ and was able to absorb luteolin and to incorporate it into the two membranes. The *nodC* mutant had a normal level of luteolin absorption. However, in the mutants where the three *nodD* genes are inactivated or deleted, the incorporation rate was significantly reduced. In contrast, the strain overexpressing the *nod* genes bound large amounts of luteolin.

The work of Horvath *et al.* (1987) and Spaink *et al.* (1989), and several further studies (Györgypal *et al.*, 1991a; Kondorosi, 1992), suggested direct interaction

between inducing compounds and the *nodD* gene product. Schlaman *et al.* (1989) presented a model for *nod* gene regulation based on the amphipathic NodD protein localized in the cytoplasmic membrane and its interaction with *nod* box DNA and flavonoids. From our results, it is difficult to firmly conclude that the NodD protein itself is the only site of binding for luteolin in the inner membrane. Luteolin was always found in the inner membrane of the triple *nodD* mutants and absorption by the outer membrane was also drastically reduced. This low level of absorption might be due to insufficient stabilization of luteolin in the bacterial membrane when NodD protein is lacking.

It is probable that only a small fraction of the luteolin pool interacts with the NodD protein. At present, nothing is known about the kinetic parameters and the stability of this interaction. It is likely that a relatively high level of luteolin per bacterium might be necessary to maintain the activity of the *nod* regulon. For the better understanding of the interactions between luteolin and NodD more suitable methods have to be developed which could allow the measurement of very small amounts of luteolin and also to determine the cytosolic luteolin content.

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