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Stimulation of indole-3-acetic acid production in *Rhizobium* by flavonoids

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Flavonoids activate *nod* gene expression in *Rhizobium* resulting in the synthesis of Nod signals which trigger organogenesis in the host plant. This paper shows that *nod*-inducers also stimulate the production of the phytohormone IAA (indole-3-acetic acid).

Indole acetic acid (IAA); Flavonoid

1. INTRODUCTION

The symbiotic bacterium *Rhizobium* releases signal molecules that control growth and differentiation in the leguminous host plant leading to the formation of root nodules in which the bacteria fix nitrogen. For the production of these bacterial signals the common *nodABC* genes and the host-range genes are required. The expression of these genes is regulated by the *nodD* activator proteins and requires the presence of flavonoids which are exuded from the roots of the host plant (for a review see [1]). Recently, a Nod signal molecule has been identified as a lipooligosaccharide which elicits nodule organogenesis on alfalfa [2,3]. Nodule-like structures on alfalfa roots were also induced by artificial auxin transport inhibitors [4] and by *R. meliloti* Nod$^-$ mutants synthesizing constitutively cytokinins [5]. These data suggest that an alteration of the phytohormone balance is required to elicit nodule formation. Moreover, the phytohormone IAA (indole-3-acetic acid) has been detected in culture filtrates of *Rhizobium* [6–11] and in relatively high amounts in root nodules [12].

Here we show that flavonoids activate in *Rhizobium* not only the expression of nodulation genes and thereby the synthesis of Nod signal molecules but also cause a significant increase in the production of IAA.

2. MATERIAL AND METHODS

2.1. *Bacterial growth conditions*

R. meliloti AK631 (Nod$^+$, Fix$^+$) is a compact colony variant of the wild-type *R. meliloti* 41 [13]. *R. meliloti* ZB138 (*nod*$^-$, *nif*$^-$, *fix*$^-$) carries a large deletion in the symbiotic region of the R. meliloti megaplasmid pRme41 [14]. These strains were grown at 28°C in M9 salts [15] supplemented with 0.2% casamino acids and 0.4% glycerol. *R. leguminosarum* 248 [16] was grown in a modified Bergersen minimal medium [17]. Induction experiments were carried out with 5 μM of daidzein, hesperitin, luteolin or naringenin (Roth, Karlsruhe, Germany). At the times indicated in the text the OD_{600} of the bacterial cultures was measured and after centrifugation the bacterial cells and the supernatants were stored at −20°C.

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2.2. *Extraction of IAA*

The supernatant (5 ml) was mixed with an equal amount of 0.1 N HCl and 3-[5(n)-^{3}H]IAA (250 Bq, 777 GBq/mmol, Amersham) was added. For solid phase extraction of IAA the acidified sample was applied to a C_{18} Bond-Elut (Analitichem Int.) column equilibrated with 50 mM HCl. The retained IAA was eluted with 5 ml diethyl ether. The ether was evaporated in vacuo and the residue was dissolved in 100% MeOH and dried in speed-vac (Hetovac, Ankersmit) prior to HPLC.

The results were confirmed after immunoaffinity purification. For this purpose, the methanol extract was evaporated in vacuo. The residue was taken up in 5 ml phosphate-buffered saline (10 mM potassium phosphate, pH 7.2, 0.9% NaCl) and purified consecutively over a pre-immune and an IAA-specific immunoaffinity column. IAA was recovered using 100% ice-cold methanol and dried prior to HPLC.

2.3. *HPLC analysis*

After a preparative ion suppression-reversed phase-HPLC run (50/49.5/0.5 ; H_2O/MeOH/HAc ; 0.5 ml·min^{-1} ; Rosil C_{18}, 3 μm, 10 cm, Alltech-RSL), IAA was analyzed by an analytical ion pairing-reversed phase-HPLC run (60/40 ; 1 mM phosphate, 10 mM Tetra Butyl Ammonium Hydroxide pH 6.6/MeOH, 0.5 ml·min^{-1}, same column) and measured on line with a Schimadzu RF 530 fluorescence detector (excitation at 285 nm, emission at 360 nm) [18]. The endogenous IAA content was calculated following the principles of isotopic dilution. Concentrations were expressed as p- or nmol IAA in 10 ml bacterial culture supernatant per OD_{600}.

2.4. *Experiments with radiolabelled IAA*

R. meliloti AK631 was grown at 28°C in 10 ml minimal medium in the presence of [1-^{14}C]IAA (71 kBq, 2.2 Bq/pmol, Amersham) with and without 5 μM luteolin as inducer. Samples were collected at intervals indicated in the text and to each sample 14 kBq ^{3}H-IAA was added. An aliquot of each fraction was counted for the initial ^{14}C/^{3}H

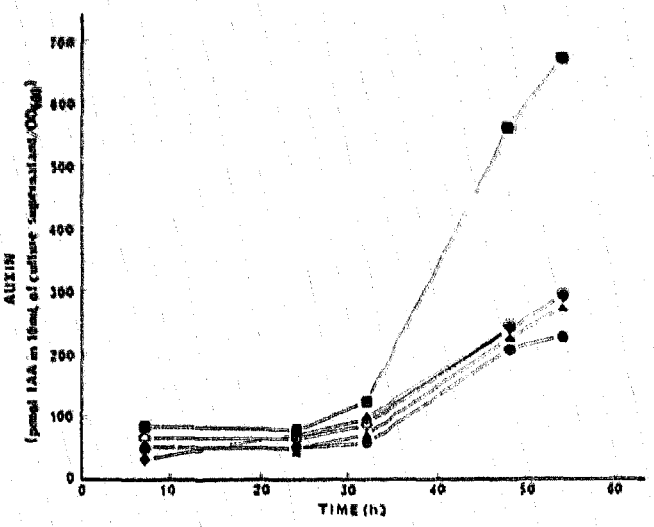

Fig. 1. Effect of different flavonoids on the IAA production of *R. meliloti*. Daidzein (▲); hesperitin (◆); luteolin (■); naringenin (●); no flavonoid added (○).

ratio (R_i). The remainder of each fraction was purified by ion pairing-reversed phase-HPLC and the $^{14}C/^{3}H$ ratio (R_e) was determined at the IAA specific retention time.

3. RESULTS

It has been shown that luteolin which is present in alfalfa exudates is an active flavonoid compound for the activation of *nod* gene expression in *R. meliloti* [19]. We followed the production of IAA by *R. meliloti* in the presence of different flavonoids and found that only the addition of luteolin affects the synthesis of IAA. When *R. meliloti* cells were cultured in the presence of 5 μM luteolin the exogenous IAA levels increased after late logarithmic phase of growth (Fig. 1). Other flavonoids which have weak or no activity as *nod* gene inducers do not cause an increase in IAA synthesis compared to the control (Fig. 1).

Moreover, the *R. meliloti* mutant ZB138 in which the *nod-nif-fix*-region was deleted also showed enhanced IAA levels in the presence of luteolin (data not shown), which indicates that the genes involved in IAA biosynthesis are not located on the symbiotic region of the megaplasmid.

When we used the flavanone naringenin as a strong inducer of *nod* genes in *R. leguminosarum* an increase in IAA production with this bacterium was also observed (Fig. 2). The amount of IAA in the culture supernatant of the pea nodulating bacterium *R. leguminosarum* was considerably higher than with the alfalfa-microsymbiont *R. meliloti*. Other flavonoids tested (daidzein, hesperitin, luteolin) showed an IAA production by *R. leguminosarum* similar to the non-induced strain (data not shown).

To test whether luteolin itself may exert a protective

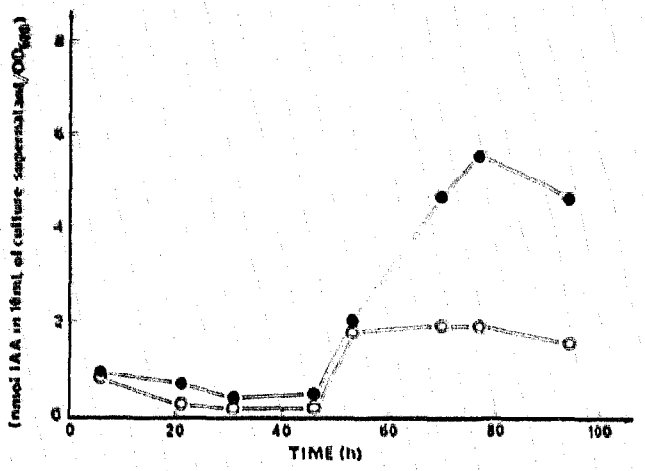

Fig. 2. Effect of naringenin on the IAA production of *R. leguminosarum*. Naringenin (●); no flavonoid added (○).

(anti-oxidant) effect on the IAA excreted by *R. meliloti* into the medium, ^{14}C-labelled IAA was added to the culture medium in the presence or absence of luteolin (see section 2). Constant R_e/R_i ratios indicate that no significant degradation or modification of the ^{14}C-IAA occurred during the growth period (Fig. 3).

4. DISCUSSION

It is well known that flavonoids activate *nod* gene expression in rhizobia resulting in the synthesis of Nod signals which trigger organogenesis in the host plant [19,20]. Here we show that the *nod*-inducers also stimulate the production of the phytohormone IAA. Changes in the phytohormone balance could be a necessary requirement to elicit nodule formation [4,5]. The exogenous supply of auxin by *Rhizobium* may further increase the phytohormone imbalance in the plant possibly caused by the Nod signal molecules [21].

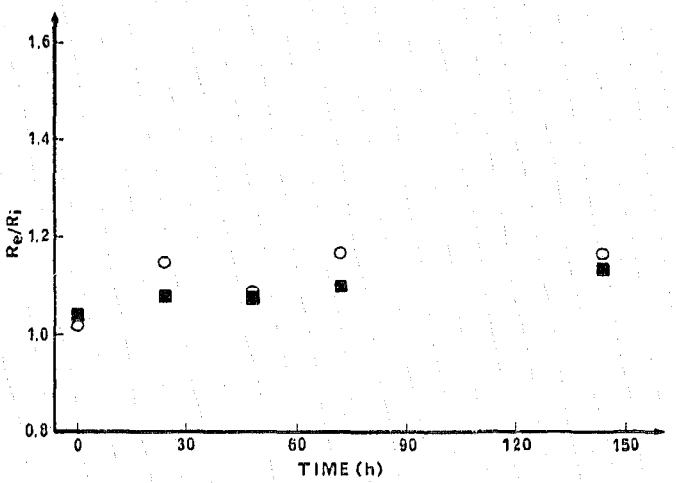

Fig. 3. R_e/R_i ratio of radiolabelled IAA in the culture supernatant of *R. meliloti*. Luteolin (■) ; no flavonoid added (○).

Nodule morphogenesis may be controlled by the highly specific Nod signal in combination with phytohormones like auxin released by *Rhizobium*.

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