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Flavonoids Induce *Rhizobium leguminosarum* To Produce *nodDABC* Gene-Related Factors That Cause Thick, Short Roots and Root Hair Responses on Common Vetch

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Rhizobium leguminosarum **produced a factor(s) that caused thick, short roots (Tsr phenotype) as well as root hair induction (Hai phenotype) and deformation (Had phenotype) in *Vicia sativa* plants upon incubation with root exudate or with one of the *nod* gene inducers naringenin or apigenin; this was a *nodDABC* gene-dependent process. Detection of the Hai and Had phenotypes was much more sensitive than that of the Tsr phenotype.**

Rhizobium leguminosarum forms nitrogen-fixing root nodules on plants of the pea cross-inoculation group. The bacteria invade the host via infection threads formed by the plant in root hairs, which are curled under the influence of the bacteria (18). This curling process requires the *nodDABC* genes of the bacteria, which are located on the Sym (symbiosis) plasmid (5, 19). Activation of the *nodABC* promoter is mediated by the *nodD* regulatory gene product and requires a flavonoid inducer (7, 9–11, 21). Mutations in these genes abolish the ability of the bacteria to induce both root hair curling and the "thick and short roots" (Tsr) phenotype in *Vicia sativa* subsp. *nigra* test plants (14, 19), which is caused by a soluble factor(s) that is produced by *R. leguminosarum* in response to a factor(s) in sterile *V. sativa* root exudate (15). As naringenin, apigenin, and some other flavonoids can replace exudate for the induction of the *nodABC* promoter (21), we investigated whether naringenin or apigenin alone is sufficient to replace total root exudate in the process of Tsr factor synthesis by *R. leguminosarum*.

The flavonoids induced the production of Tsr factor only in a strain harboring a Sym plasmid, with maximal effects at concentrations of 700 nM and higher (Table 1). Production of Tsr factor was not detected when the Sym plasmid-cured strain RBL1387 or strains carrying mutations in either *nodD*, *nodA*, *nodB*, or *nodC* (RBL1402, RBL1409, RBL1410, and RBL1412, respectively) were used in otherwise identical experiments, showing that naringenin- or apigenin-induced Tsr factor synthesis followed the same requirements as defined previously for exudate-induced Tsr factor production (19). Quercetin, whose structure closely resembles that of naringenin but which does not induce the *R. leguminosarum nodABC* promoter (21), did not significantly induce Tsr factor production (Table 1). The growth of the bacterial strains was not affected by the flavonoids in the tested concentrations. Inocula of 5×10^5 CFU/ml reached concentrations of 2×10^6 to 5×10^6 CFU/ml after incubation, which is comparable to the growth observed in exudate.

The fact that only one flavonoid, either naringenin or apigenin, is sufficient for induction of the *nodABC* promoter (21) as well as for the production of Tsr factor (Table 1) eliminates models of Tsr factor synthesis in which one compound in root exudate induces the *nodABC* genes and one or more other compounds function as substrates for Tsr factor synthesis. However, activation of the *nodABC* promoter may not be the only role of naringenin or apigenin, since a naringenin concentration as high as 700 nM is required for optimal Tsr factor production (Table 1), whereas 100 nM is sufficient for optimal activation of the *nodABC* promoter (21). Therefore, Tsr factor synthesis could require the additional activation of less sensitive

TABLE 1. Induction of Tsr factor production in *R. leguminosarum* supernatant fluids by incubation with sterile root exudate or flavonoid compounds[a]

Inducer	Length of main root (mm; mean ± SD) (% of control values)[b] with products of strain[c]:	
	248 (Sym$^+$)	RBL1387 (Sym$^-$)
None	79.4 ± 6.9 (103)	76.1 ± 5.6 (98)
Exudate (undiluted)	45.1 ± 6.1 (58)	73.0 ± 5.4 (94)
Exudate (10%)[d]	80.2 ± 8.4 (104)	76.3 ± 6.5 (99)
Naringenin (40)[e]	67.1 ± 11.2 (87)	76.1 ± 5.6 (98)
Naringenin (200)	56.3 ± 10.6 (73)	76.9 ± 6.3 (99)
Naringenin (400)	53.4 ± 6.4 (69)	69.8 ± 4.8 (90)
Naringenin (700)	46.5 ± 4.6 (60)	74.1 ± 4.9 (96)
Naringenin (1,000)	46.8 ± 3.0 (60)	72.2 ± 7.1 (93)
Naringenin (3,000)	47.2 ± 4.4 (61)	70.2 ± 4.3 (91)
Apigenin (700)	50.3 ± 3.8 (65)	71.7 ± 3.7 (92)
Quercetin (3,000)	80.3 ± 5.5 (104)	81.7 ± 5.5 (105)

[a] Bacteria were incubated at 5×10^5 CFU/ml in root exudate or deposit-free Jensen medium (17) supplemented with 0.1% (vol/vol) thiamine-free medium (13) and, as indicated, with naringenin, apigenin, or quercetin (Sigma Chemical Co., St. Louis, Mo.) for 24 h. After centrifugation and filter sterilization, the resulting fluids were tested on at least 12 *V. sativa* seedlings as described previously (15), and main root lengths were measured to quantify the Tsr phenotype. Inocula and root exudate were prepared as described previously (15).

[b] Plants grown in medium not incubated with bacteria and without inducer served as a control (length, 100%). Plants grown in undiluted exudate and in medium not incubated with bacteria but containing 3,000 nM naringenin reached lengths of 73.5 ± 6.2 and 79.6 ± 8.6 mm, respectively.

[c] Strain 248 is a wild-type *R. leguminosarum* strain harboring Sym plasmid pRL1JI (8). Strain RBL1387 is strain 248 cured of pRL1JI (15). Control strains RBL1402, RBL1409, RBL1410, and RBL1412 are RBL1387 harboring pRL1JI mutant plasmids pRL602 (*nodD*::Tn*5*), pRL610 (*nodA*::Tn*5*), pRL611 (*nodB*::Tn*5*), and pRL615 (*nodC*::Tn*5*), respectively (19) (see text).

[d] One-tenth volume of exudate in deposit-free Jensen medium.

[e] Final concentrations (nanomolar) of flavonoids are given in parentheses.

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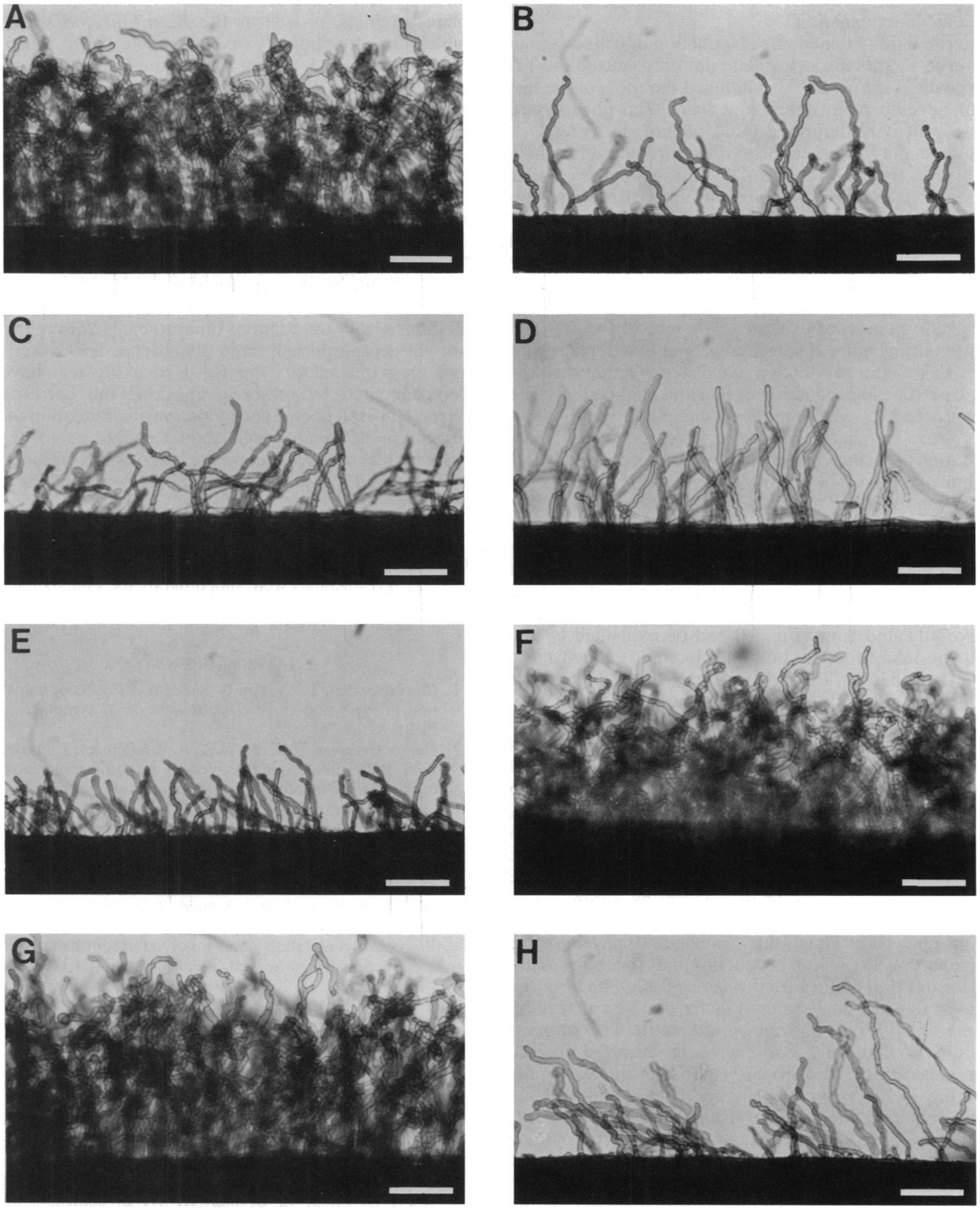

FIG. 1. Induction and deformation of root hairs of *V. sativa* test plants as caused by *R. leguminosarum* products. The plants were grown in test solutions as described previously (15). Hai and Had phenotypes of 12 plants were investigated microscopically on the entire main root after staining of the root hairs with methylene blue (16). (A) Sterile fluid obtained after incubation of strain 248 (Sym^+) in exudate. (B) Fluid of strain 248 incubated in medium without added inducers. (C) Root exudate in which no bacteria were incubated. (D) Medium. (E) Fluid of the Sym plasmidless strain RBL1387 incubated in exudate. (F) Hundredfold dilution of the fluid used in panel A. (G) Fluid of strain 248 incubated in medium supplemented with 200 nM naringenin. Similar results were obtained when 200 nM apigenin was used as an inducer. (H) Fluid of strain RBL1387 incubated in medium supplemented with 200 nM naringenin. Bars, 100 μm.

promoters or the presence of naringenin as a substrate. Alternatively, differences in experimental conditions, e.g., bacterial concentrations and incubation volume, cannot be excluded as an explanation.

The sterile fluid obtained after incubation of cells of strain 248 in sterile *V. sativa* root exudate not only caused the Tsr phenotype on *V. sativa* but also induced the roots of the test plants to develop many more root hairs (Hai phenotype), which were heavily deformed (Had phenotype) (Fig. 1A). Both exudate and bacteria appeared to be required to obtain active filtrates (Fig. 1A through D). Filtrates obtained after incubation of the Sym plasmidless strain RBL1387 in exudate induced neither the Tsr phenotype (Table 1) nor the Hai or Had phenotype (Fig. 1E). Similar results were observed when strain RBL1402, RBL1409, RBL1410, or RBL1412 was used, indicating that the *nodD*, *nodA*, *nodB*, and *nodC* genes are required for the production of Hai and Had factors in response to plant root exudate.

Because naringenin and apigenin were able to replace root exudate in Tsr factor production, we tested whether this was also the case for the production of Hai and Had factors. The latter two activities were produced under the same conditions as was Tsr factor but were detected when lower concentrations of inducer were used. Filtrates obtained after incubation of strain 248 cells with naringenin or apigenin concentrations of 200 nM or higher caused the same root hair responses as filtrates of this strain grown in the presence of exudate (Fig. 1G and A, respectively). The difference between the concentration of naringenin or apigenin required to induce optimal production of Tsr factor and that required for induction of Hai and Had factors (700 and 200 nM, respectively [Table 1 and Fig. 1]) can be explained by the fact that the assay for Hai and Had factors is much more sensitive than that for Tsr factor. Hundredfold dilutions of active fluids significantly induced Hai and Had factors (Fig. 1F), whereas a 10-fold dilution of the same preparation completely abolished the ability to induce the Tsr phenotype. Induction of significant production of Hai and Had factors by naringenin and apigenin concentrations of up to 3,000 nM was not observed with strain RBL1387, RBL1402, RBL1409, RBL1410, or RBL1412 (phenotypes similar to that shown in Fig. 1H). Quercetin, which was inactive in the induction of Tsr factor production (Table 1), was also unable to induce Hai and Had factor production by strain 248 in concentrations of up to 3,000 nM (phenotypes similar to that shown in Fig. 1H). Thus, the genetic and physiological requirements for Tsr factor production and for the production of Hai and Had factors are the same (Table 1 and Fig. 1). In all cases, exudate as a required factor can be replaced by naringenin or apigenin, but not by quercetin. The simplest explanation is that only one factor is excreted by the bacteria, generating a pleiotropic response of the test plants and resulting in Tsr as well as Hai and Had phenotypes.

Transposon insertions in *nodD*, *nodA*, *nodB*, and *nodC* abolish Tsr as well as Hai and Had factor production. Since *nodD* has a regulatory function (11, 21) and *nodABC* are part of the same operon, the *nodC* gene product and possibly the product of *nodA* or *nodB* (or both) are involved in the production of the factor(s) causing the Tsr, Hai, and Had phenotypes. Since they are part of the *nodABCIJ* operon (12), *nodI* and *nodJ* cannot be excluded from being involved in Tsr factor production, although no Tsr mutants with mutations in this region have been found (19).

The *R. leguminosarum* factor(s) causing Tsr, Hai, and Had phenotypes in *V. sativa* may be related to soluble factors in the *R. trifolii-Trifolium repens* symbiosis, which cause curling, branching, and other deformations of the root hairs (1, 6, 20) and which are also active on *V. sativa* (2). At physiological concentrations (10^5 to 10^6 bacteria per ml), plant products as well as the *R. trifolii nodDABC* genes, which are functionally exchangeable with those of *R. leguminosarum* (3, 4, 19), were required for the production of these factors. Remarkably, autoclaved supernatant fluids of very dense cultures of a Sym plasmid-cured *R. trifolii* strain induced similar plant responses. However, it was not shown whether the same or different factors were produced at high and low bacterial concentrations (2).

The substitution of exudate by the single compound naringenin allows us to control Tsr factor synthesis more precisely and to start the purification from a medium which lacks the complex exudate mixture. By replacing the assay for Tsr factor by an assay for Hai and Had factors, material can be saved, as the sensitivity of the latter is approximately 10 times higher than that of the former. However, since it is not yet certain that all three phenotypes are caused by one and the same factor, the Hai-Had assay will have to be complemented by routinely checking the correlation between Hai-Had phenotype induction and induction of the Tsr phenotype. This approach should enable us to determine whether one or more factors are responsible for inducing the Hai-Had and Tsr phenotypes and to purify this factor(s) at the same time.

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