Interactions among Flavonoid nod Gene Inducers Released from Alfalfa Seeds and Roots

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ABSTRACT

Alfalfa (Medicago sativa L.) seeds and roots can create complex rhizosphere effects by releasing flavonoids that induce nodulation (nod) genes in Rhizobium melliloti. Previous reports identified luteolin and 4',7-dihydroxy-2'-methoxychalcone as strong inducers that are released from seeds and roots, respectively, and 4',7-dihydroxyflavone and 4',7-dihydroxyflavanone as weaker inducers which are exuded by roots. As a first step toward identifying flavonoid interactions that may occur in the rhizosphere, combinations of these molecules were tested for transcriptional effects on a nodABC-lacZ fusion in R. melliloti. At low concentrations (e.g. 8.4 nanomolar), interactions of the three nod gene inducers from root exudate were additive. When the strong inducers 4',7-dihydroxy-2'-methoxychalcone and luteolin were present separately at higher concentrations (e.g. 21 nanomolar), their effect could be decreased significantly by the weaker inducers 4',7-dihydroxyflavone and 4',7-dihydroxyflavanone. In contrast, when low concentrations of luteolin from seed rinses and 4',4'-dihydroxy-2'-methoxychalcone from root exudate were present together, they produced synergistic increases in nod gene transcription. Tests with mixtures of the three nod gene inducers from root exudate indicated that alfalfa seedlings might easily decrease the strong inductive effect of the chalcone by releasing modest amounts of the weaker inducers. In addition, mixtures of luteolin and the nod gene inducer in root exudate suggested that interactions between nod gene inducers from seeds and roots may create a zone highly favorable to root nodule formation near the top of the primary root.

Development of the N2-fixing symbiosis between Rhizobium melliloti and alfalfa (Medicago sativa L.) requires several interactions between the two organisms. First the plant must release a signal that induces transcription of the rhizobial nodulation genes (nodABC) (16), then nod gene products induce root hair curling (12) and cortical cell division (5). Peters et al. (17) identified the first nod gene-inducing factor from an alfalfa seed extract as 3',4',5,7-tetrahydroxyflavone, a flavonoid with the trivial name luteolin. Maxwell et al. (14) found three other nod gene-inducing flavonoids, but not luteolin, in root exudates of 72-h-old 'Moapa 69' alfalfa seedlings. Those compounds included the weak nod gene inducer 4',7-dihydroxyflavanone, the moderate nod gene inducer 4',7-dihydroxyflavone, and the strong nod gene inducer 4,4'-dihydroxy-2'-methoxychalcone. Data showed that 4,4'-dihydroxy-2'-methoxychalcone fully induced the nodABC-lacZ fusion pRmM57 at 10% of the concentration required for full induction by luteolin. Quantitative studies of nod gene-inducing flavonoids in seed rinses of the same alfalfa cultivar have established the importance of luteolin and several inactive derivatives that probably are converted to luteolin in the soil (8). Thus, root nodule formation on this alfalfa cultivar involves potential interactions among at least four different nod gene-inducing flavonoids.

Several types of interactions involving nod gene-inducing flavonoids have been documented. Studies with compounds not exuded by the host legume have shown that weak nod gene inducers can restrict the activity of strong inducers in Rhizobium leguminosarum biovar vicieae (6), R. leguminosarum biovar trifolii (4), and in R. melliloti (18, 19). Other commercially available flavonoids without detectable nod gene-inducing activity inhibited activities of strong nod gene inducers in several systems (4, 6, 18). In one case, formononetin and umbelliferone, compounds with no nod gene-inducing activity, were identified in clover root extracts (20) and shown to inhibit nod gene induction by 4',7-dihydroxyflavone in R. leguminosarum biovar trifolii (4). Interactions among naturally exuded nod gene-inducing flavonoids have not been reported for any legume.

R. melliloti cells in the rhizosphere of alfalfa seedlings presumably respond to all nod gene-inducing flavonoids present in that environment. How the three nod gene inducers exuded by alfalfa roots interact among themselves and with any luteolin from the seed zone has not been reported. The present study was conducted as a first step toward defining how naturally occurring nod gene-inducing flavonoids can interact in the rhizosphere to influence alfalfa root nodule formation by R. melliloti.

MATERIAL AND METHODS

Bacteria

Rhizobium melliloti strain 1021 containing plasmid pRmM57, which supplies an extra copy of nodD1 and a nodABC-lacZ fusion (16), was used as a bioassay system to measure nod gene-inducing activity of flavonoids released from alfalfa seedlings. To exclude the possibility that compounds tested in the assays affected β-galactosidase activity through a mechanism other than induction of nodABC-lacZ, control assays were done (a) with R. melliloti strain 1021, lacking pRmM57 and therefore without lacZ-dependent β-
galactosidase activity and (b) with *R. meliloti* strain 1021, containing plasmid pRmM61 (16) with a nodD1-lacZ fusion that produces β-galactosidase constitutively in the absence of inducers. All strains were generously provided by Dr. S. R. Long (Stanford University). The extent of nodABC-lacZ expression was determined as units of β-galactosidase activity (15) in an assay system (16) with modifications described previously (14). In this study, uninduced background activities were not subtracted from the reported values.

**Flavonoid nod Gene Inducers**

Compounds were obtained from the following sources: Spectrum MFG Corp., Gardena, CA, for luteolin, 4',7-dihydroxyflavone, and 4',7-dihydroxyflavanone. Dr. R. E. Carlson, Ecochem Research Inc., Chaska, MN, generously donated a sample of 4,4'-dihydroxy-2'-methoxychalcone.

Stock solution aliquots of nod gene inducers were prepared on the basis of the following spectrophotometric extinction coefficients (log ε): luteolin, 4.17 at 350 nm in 95% ethanol (13); 4,4'-dihydroxy-2'-methoxychalcone, 4.25 at 349 nm in methanol (3); 4',7-dihydroxyflavone, 4.52 at 328 nm in methanol (11); 4',7-dihydroxyflavanone 4.14 at 275 nm in methanol (10). Thereafter, the aliquots were dried under vacuum and stored at -20°C. For the assays, all compounds were initially dissolved in methanol and then suspended in the phosphate buffer; methanol was removed under vacuum before the bacteria were added to the assay mixtures.

All experiments were repeated at least twice, and every treatment in each assay was replicated three times. Results presented on a single graph are from the same assay; in addition, the three graphs in Fig. 1 (i.e., A, B, and C) are from a single assay. Data were examined by analysis of variance to calculate LSD0.01 values for each experiment.

**RESULTS**

Absolute β-galactosidase activities at full nodABC-lacZ induction declined gradually for unknown reasons during the period this study was made, but relative responses of individual nod gene inducers and combinations of nod gene inducers were consistent in all experiments. The relative induction patterns for 4,4'-dihydroxy-2'-methoxychalcone, 4',7-dihydroxyflavone, 4',7-dihydroxyflavanone, and luteolin were similar to those reported previously (14). The nod gene inducers used in this study had no effect on the β-galactosidase activities in the parent *R. meliloti* strain 1021 or in strain 1021pRmM61, which contains the constitutively expressed nodD1-lacZ fusion (data not shown).

To investigate possible interactions among the flavonoids exuded from roots and luteolin from seeds, compounds were first tested in pair-wise combinations. At the lowest concentrations tested, interactions among all nod gene inducers were usually additive (Figs. 1 and 2). Even the weakest inducer, 4',7-dihydroxyflavanone, in a concentration of 8.4 nM increased the activities of all three stronger inducers significantly (P ≤ 0.01) at their lowest test concentrations (Fig. 1). Supplementing 2.1 nM 4,4'-dihydroxy-2'-methoxychalcone with 4',7-dihydroxyflavone did not produce a strictly additive increase in nod gene induction, but the overall effect was significantly positive (P ≤ 0.01) (Fig. 2).

Both weaker inducers inhibited nod gene induction under certain conditions. If sufficient 4,4'-dihydroxy-2'-methoxychalcone (21 nM), luteolin (21 nM), or 4',7-dihydroxyflavone (84 nM) was present to induce a higher level of β-galactosidase than the saturating concentration of the weakest inducer, 4',7-dihydroxyflavanone (21 nM), then nod gene induction could be decreased by an equimolar concentration of that weak inducer (Fig. 1). Thus, 21 nM 4',7-dihydroxyflavanone significantly (P ≤ 0.01) inhibited induction by 21 nM 4,4'-dihydroxy-2'-methoxychalcone (Fig. 1A) or 21 nM luteolin (Fig. 1B), and 84 nM 4',7-dihydroxyflavone decreased the inducing effect of 84 nM 4',7-dihydroxyflavone (Fig. 1C). In no case was β-galactosidase activity decreased below that associated with a saturating concentration of 4',7-dihydroxyflavone.

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**Figure 1.** Effects of 4',7-dihydroxyflavanone on the nod gene-inducing activities of 4,4'-dihydroxy-2'-methoxychalcone (MCh) (A), luteolin (B), and 4',7-dihydroxyflavone (DHF). Induction of nodABC was measured as β-galactosidase activity from a nodABC-lacZ translational fusion in *R. meliloti*. Means of three replicates and LSD0.01 are shown.
The moderate inducer, 4',7-dihydroxyflavone, also decreased β-galactosidase induction by the chalcone and luteolin, but a higher concentration was required to show a significant (P ≤ 0.01) effect (Fig. 2). Thus, 84 nM 4',7-dihydroxyflavone decreased the inducing effects of 21 nM 4',4'-dihydroxy-2'-methoxychalcone (Fig. 2A) and of 21 nM luteolin (Fig. 2B).

Experiments in which luteolin and 4,4'-dihydroxy-2'-methoxychalcone were tested separately and together showed an unexpected synergistic promotion of nod gene induction at low concentrations (Fig. 3). If one assumes an additive model, then assays supplemented to contain 8.4 nM luteolin as well as nonsaturating concentrations of 4,4'-dihydroxy-2'-methoxychalcone should have produced an additional 32.4 units of β-galactosidase activity, because that was the increase over the 0.0 control (8.8 units) caused by 8.4 nM luteolin alone. However, in the 2.1 nM 4,4'-dihydroxy-2'-methoxychalcone treatment, the presence of 8.4 nM luteolin increased β-galactosidase activity by 86.7 units to a value of 116.9 units, 54.3 units more than predicted by an additive model and slightly greater than the activity produced by 21 nM 4,4'-dihydroxy-2'-methoxychalcone alone. Likewise, in the 8.4 nM chalcone test, 8.4 nM luteolin increased β-galactosidase activity by 69.0 units, a total of 36.6 units more than the 32.4 unit increase predicted by an additive model. Both unexplained increases far exceeded the LSD0.01 value of 17.2 units.

To establish the relative importance of the three flavonoids from the root exudates as nod gene inducers, mixtures of these compounds were tested. Ratios between various compounds in mixtures were selected on the basis of data from Maxwell et al. (14). In that study, a 72-h-old alfalfa seedling root exudate contained 4,4'-dihydroxy-2'-methoxychalcone, 4',7-dihydroxyflavone, and 4',7-dihydroxyflavanone in a 1:2.5:1 ratio. The results showed that the inhibitory effects of weak inducers also occurred in a root exudate model system (Table I). For example, when the ratio of 4,4'-dihydroxy-2'-methoxychalcone to 4',7-dihydroxyflavone to 4',7-dihydroxyflavanone, was maintained at 1:2.5:1 over three different concentration ranges, the presence of the flavone and the flavanone inhibited nod gene-inducing activity of the 4,4'-dihydroxy-2'-methoxychalcone significantly (P ≤ 0.01) relative to the chalcone alone.

The possible relevance of synergistic interactions between...
luteolin and 4,4'-dihydroxy-2'-methoxylchalcone (Fig. 3) to complex systems was supported by the result from tests in which luteolin was added to mixtures of the three nod gene inducers from roots (Fig. 4). Those experiments showed that even very low concentrations of luteolin (0.84 and 2.1 nM), which did not induce β-galactosidase activity above the background level (Fig. 4), gave a very significant (P ≤ 0.01) synergistic increase in nodABC-lacZ transcription when added to the 8.4:21.8:8.4 nM mixture of the 4,4'-dihydroxy-2'-methoxylchalcone to 4',7-dihydroxylavone to 4',7-dihydroxylavonone (Fig. 4). Mixtures of root nod gene inducers in the medium (2.1:5.2:2.1 nM) and (0.8:21.8:0.8 nM) concentration ranges showed similar synergistic responses only when luteolin was added at concentrations of 2.1 and 8.4 nM or 8.4 nM, respectively.

**DISCUSSION**

Results from these experiments indicate that rhizosphere interactions among nod gene-inducing flavonoids actually released from alfalfa seeds and roots offer one mechanism by which the plant may control the extent and location of root nodule formation. There are two key observations: (a) the weak nod gene inducers 4',7-dihydroxylavone and 4',7-dihydroxylavonone can decrease the activity of the strong nod gene inducers 4,4'-dihydroxy-2'-methoxylchalcone and luteolin (Figs. 1 and 2), and (b) 4',4'-dihydroxy-2'-methoxylchalcone, released from the roots (14) interacts synergistically with luteolin from the seeds (8, 17) (Fig. 3). The fact that these phenomena occur in simple systems involving two nod gene inducers (Figs. 1–3) as well as in mixtures of three or four nod gene inducers (Table I, Fig. 4), suggests that the effects may be robust enough to function in the soil. Although previous results with flavonoids which were not necessarily isolated or released from the appropriate host legume indicated that weak nod gene inducers can inhibit strong inducers (4, 6, 18, 19), the effects shown here for a flavone and a flavanone, which are actually released from alfalfa roots, suggest that such interactions may occur in the rhizosphere of alfalfa.

The unexpected, synergistic interactions of luteolin and 4,4'-dihydroxy-2'-methoxylchalcone at low concentrations (Fig. 3) suggest one possible explanation for the commonly observed presence of many root nodules near the top of the primary root. Because luteolin is associated with the alfalfa seeds (8, 17) but is not observed in alfalfa root exudates between 48 and 72 h after imbibition (14), it probably occurs in a zone surrounding the germinating seed. During the initial stages of germination the young root will be growing through this luteolin-containing zone as it simultaneously exudes 4,4'-dihydroxy-2'-methoxylchalcone. Effective rhizobial cells present in that soil region, or those attracted to it by their chemotactic movement toward luteolin (2), will be exposed to a combination of these two strong nod gene inducers, and a synergistic transcription of nod genes similar to that shown in Figure 4 may occur. Although the molecular basis of such interactions is unknown, some published results suggest that various functional copies of nodD in R. meliloti (9) interact with different unknown factors (7). Thus, luteolin and 4,4'-dihydroxy-2'-methoxylchalcone may influence rhizobial cells through different nodD alleles. A direct test of this hypothesis can be conducted using genetically defined R. meliloti cells with the newly identified flavonoid nod gene inducers actually released by alfalfa (8, 14) and nod gene inducers yet to be identified in other hosts of R. meliloti. The results in this study were obtained with R. meliloti 1021pRM57, which contains at least twice as many copies of nodD1 as the parent strain 1021 (16). Therefore, it is not yet possible to extend these data in any quantitative manner to wild-type R. meliloti.

The concentration of nod gene-inducing flavonoids actually present in a soil rhizosphere has not been reported for any legume. In Medicago sativa (18) and Trifolium repens (20), nod gene-inducing flavonoids are released between the root tip and the emerging root hair zone, i.e. presumably near the site of infection (1). If 4,4'-dihydroxy-2'-methoxylchalcone, 4',7-dihydroxylavone, and 4',7-dihydroxyflavonone are all released from the same cells, then the 1:2.5:1 ratio reported in a hydroponic medium (14) might remain relatively constant near the site of infection. Over greater distances in the rhizosphere, the different solubilities of these molecules in the soil solution could alter the ratio. However, if those compounds are released from different root zones, then the 1:2.5:1 ratio would not remain constant. The approach used in this study of altering the concentrations of the three nod gene inducers from the root while maintaining the same 1:2.5:1 ratio is supported by observations of Peters and Long (18) who saw no evidence for a zone where nod gene inhibitors were released from alfalfa roots.

Several mechanisms may exist for controlling the amount of nod gene inducing activity in the alfalfa rhizosphere. Clearly, the synthesis and/or release of active flavonoids from the plant could be one of these mechanisms. The results from this study indicate, however, that interactions among nod gene-inducing flavonoids after release from the plant can influence rhizobial cells and, presumably, subsequent nodule
formation. Examining the details of such interactive control mechanisms will provide a new understanding of how plants affect rhizosphere events.

LITERATURE CITED


