Release and Modification of nod-Gene-Inducing Flavonoids from Alfalfa Seeds

Ueli A. Hartwig and Donald A. Phillips
Department of Agronomy and Range Science, University of California, Davis, California 95616

ABSTRACT

Traces of luteolin, an important rhizobial nod gene inducer in Rhizobium meliloti, are released by alfalfa (Medicago sativa L.) seeds, but most luteolin in the seed exudate is conjugated as luteolin-7-O-glucoside (L7G). Processes affecting the production of luteolin from L7G in seed exudate are poorly understood. Results from this study establish that (a) seed coats are the primary source of flavonoids, including L7G, in seed exudate; (b) these flavonoids exist in seeds before imbibition; and (c) both the host plant and the symbiotic R. meliloti probably can hydrolyze L7G to luteolin. Glycolytic cleavage of L7G is promoted by glucosidase activity released from sterile seeds during the first 4 hours of imbibition. Thus, L7G from imbibing alfalfa seeds may serve as a source of the nod-gene-inducing luteolin and thereby facilitate root nodulation by R. meliloti.

One early event in the formation of alfalfa root nodules by the soil bacterium Rhizobium meliloti involves expression of the bacterial nodulation genes nodABC (10). Transcription of those genes occurs through the cooperative action of a constitutive bacterial nodD gene product and plant flavonoids (14, 15). Five nod-gene-inducing flavonoids in alfalfa seed and root exudates have been identified (6, 11, 15), but factors within the plant that regulate the quantities of nod-gene inducers exuded into the soil are poorly understood.

Because roots and seeds are physiologically dissimilar organs, the processes controlling exudation of their nod-gene-inducing flavonoids may differ. In alfalfa seedling roots, the release of three nod-gene-inducing flavonoids at rates of 1 to 3 pmol . plant⁻¹ . h⁻¹ is closely coupled to their synthesis (12). By contrast, during the first 4 h of imbibition, the nod-gene inducer luteolin is discharged from alfalfa seeds at a rate of 70 pmol . plant⁻¹ . h⁻¹ (6). The particular seed tissues that release luteolin and whether concurrent synthesis is required for the process are not known.

L7G², which is released from alfalfa seeds at a rate of 800 pmol . plant⁻¹ . h⁻¹, comprises about 40% of the luteolin derivatives discharged during the first 4 h of imbibition (6). Although L7G, like luteolin, shows activity in nod-gene-inducing assays with R. meliloti, several orders of magnitude higher concentration are required. One interpretation of such results is that L7G itself is inactive, and rhizobia are induced by luteolin, which they hydrolyze from L7G. Although rhizobial hydrolysis of L7G in the soil may be an ecologically significant process, it is equally possible that alfalfa seeds release glucosidases that break down L7G.

The objectives of this study were to identify the seed tissues required for exudation of nod-gene-inducing flavonoids, to assess whether concurrent flavonoid synthesis is required for that process, and to investigate whether plant or rhizobial factors may be active in releasing luteolin from L7G as one step toward the establishment of root nodules and symbiotic N₂ fixation.

MATERIALS AND METHODS

Organisms

Seeds of alfalfa (Medicago sativa L.) cv ’Moapa 69’ were routinely sterilized for 3 min in 70% ethanol and rinsed three times with sterile distilled water. Rhizobium meliloti strain 1021 (13) was maintained on a defined minimal medium (5) supplemented to contain 20 to 30 μM L7G for metabolism experiments.

Flavonoids

Flavonoid concentrations were determined spectrophotometrically with the integrator function in a Waters 990 HPLC system (Millipore Corp., Milford, MA) using known quantities of authentic standards. The HPLC was equipped with a 250 × 4.6-mm Lichrosorb 5RP18 column (Phenomenex, Rancho Palos Verdes, CA) and was eluted at 0.5 mL . min⁻¹ with a 30-min linear gradient from 97.5:0.2:2.5 to 0:97.5:2.5, water:methanol:acetic acid (v:v:v). Flavonoid release from different seed tissues was assessed by removing seed coats from dry seeds with forceps and incubating the two seed parts separately in distilled water at room temperature for 4 h with gentle shaking. HPLC chromatograms were produced with a methanol:water:acetic acid elution profile described previously (6).

Flavonoid Metabolism

Seed exudates for ammonium sulfate precipitation experiments were prepared by imbibing 1 g seeds in 10 mL of filter-sterilized buffer (25 mM oxalic-malate buffer at pH 5.0, 3 mM
EDTA, 2 mM DTT, and 1 mM PMSF) for 4 h with gentle shaking at room temperature. After removing seeds, the exudate was centrifuged 30 min at 4000g. The supernatant was split into two fractions, one in which proteins were precipitated with 80% (w/v) ammonium sulfate and a second untreated control fraction. Both fractions were incubated at room temperature with gentle shaking.

Metabolism of L7G by *R. meliloti* was tested by suspending 1 mg of L7G in 100 mL of warm (40°C) minimal medium, stirring for 10 min, and filter-sterilizing with a 0.2 μm polycarbonate membrane (Nuclepore Corp., Pleasanton, CA). Bacterial cells were obtained from a culture in mid-logarithmic growth phase, and both the sterile control and the rhizobial treatment were incubated at 28°C with shaking.

**Protein Studies**

Studies of total protein released from seeds were done with seeds imbibed and shaken gently in sterile distilled water at room temperature. The solution surrounding seeds was sampled hourly, and proteins were quantified by a Bio-Rad protein microassay (Bio-Rad, Richmond, CA) with BSA as a standard.

Protein purification studies were initiated by imbibing 50 g seeds in 100 mL of buffer (100 mM sodium citrate buffer at pH 5.0, 3 mM EDTA, and 2 mM DTT) at room temperature with gentle shaking. After 4 h, the solution was decanted, cooled on ice, centrifuged at 4°C for 60 min at 40,000g, and concentrated overnight by dialysis against solid PEG in ServaPore (2.0 nm) dialysis tubing (Serva, Heidelberg). The protein solution was desalted on Sephadex G-25 (Pharmacia, Uppsala) at 4°C. Proteins were separated by chromatography on Bio-Gel P-150 (Bio-Rad, Richmond, CA) at 4°C and quantified with the Bio-Rad protein microassay.

Glucosidase activity of proteins in seed exudate was assayed by incubating 400 μL of the enzyme solution with 100 μL of 10 mM *p*-nitrophenyl glucopyranoside; 1, L7G; 2, quercetin-3-O-galactoside; 3, a conjugate of quercetin; 4, 5-methoxy-luteolin; 5, 3',5-dimethoxy-luteolin; 6, luteolin.

**Figure 1.** HPLC chromatogram of compounds removed from intact alfalfa seeds (5 mg) during a 4-h rinse with 50% methanol. The sample was run on a reverse-phase C₁₈ column in a methanol:water:acetic acid gradient (6). Regions indicated with numbers were identified by UV/visible spectra and relative retention time as containing the previously studied (5, 6) compounds: 1, L7G; 2, quercetin-3-O-galactoside; 3, a conjugate of quercetin; 4, 5-methoxy-luteolin; 5, 3',5-dimethoxy-luteolin; 6, luteolin.

**Figure 2.** HPLC chromatograms of 4-h aqueous rinses from (A) intact alfalfa seeds (10 mg), (B) isolated seed coats (2 mg), and (C) the remaining seed contents (3 mg). Regions indicated with numbers correspond to those in Figure 1. Chromatographic conditions were comparable to those in Figure 1.

**Figure 3.** Total protein released from sterile alfalfa seeds during imbibition in distilled water. Data points represent mean ± se of four replicates.
RESULTS

Source of Flavonoids

Normal hydration of seed tissues during imbibition was not required for flavonoid release, because a 4-h rinse with 50% methanol contained the flavonoids identified previously in aqueous rinses (Fig. 1) (6). When separate seed tissues were tested as sources of flavonoids present in an aqueous rinse after 4 h, HPLC analyses indicated that compounds released from intact seeds (Fig. 2A) also were discharged from isolated seed coats (Fig. 2B). Compounds were present in rinses of the remaining seed parts (Fig. 2C), but none of the peaks in Figure 2C had UV/visible spectra comparable to those of peaks in Figure 2A.

Modification of Flavonoids

Significant quantities of protein are released by seeds within a few hours after water is added (Fig. 3). A portion of that protein which is precipitated by 80% ammonium sulfate can produce luteolin aglycone from endogenous substrates in the seed rinse (Table I). When endogenous flavonoids were removed from a seed rinse by gel filtration, β-glucosidase activity that hydrolyzed p-nitrophenylglucose and L7G was detected (Table II). Subsequent separation of proteins on Bio-Gel P-150 increased specific glucosidase activity on both substrates (Table II), and the presence of those activities in the earliest fraction from the Bio-gel P-150 suggests a mol wt of 100,000 to 150,000 for the glucosidase(s).

Free-living cells of R. meliloti 1021 apparently also hydrolyze L7G to luteolin (Table III). Those data, which are representative of four experiments, show that the commercial L7G contained about 1.5% luteolin as a contaminant. Decreases in L7G and luteolin in sterile control flasks may have reflected adhesion of these poorly soluble compounds to glass surfaces. Although decreases in L7G were not matched by equimolar increases in luteolin in the presence of bacteria, in every case the amount of free luteolin increased during the 24 h incubation period despite apparent losses of the compound in sterile controls. This fact, coupled with possible adhesion of luteolin to bacterial cells, suggests the total luteolin released by R. meliloti activity was underestimated.

DISCUSSION

Results from this study indicate that biochemical processes responsible for transmitting a nod-gene-inducing signal from alfalfa to Rhizobium meliloti do not cease with release of flavonoids from the seed. In contrast to the concurrent synthesis and release of nod-gene-inducing flavonoid aglycones from alfalfa seedling roots (12), the present data show that most flavonoids released from seeds do not require seed hydration (Fig. 1 versus 2A) and, thus, must have been synthesized in the previous generation. Although some flavonoids may be stored in cotyledons (Fig. 2C), most apparently are present in the seed coat (Fig. 2B) and are discharged as glycosides during imbibition. The data do not prove that flavonoids are released passively into aqueous solutions, because the methanol rinse may have extracted compounds from storage pools. Although it is possible that no plant metabolic events are required for release of L7G, the compound apparently can be modified into the active nod gene inducer luteolin by glucosidase(s) from seeds (Tables I and II) and rhizobia (Table III). Glucosidases from other soil microorganisms (1, 19) may also contribute to this process. Whether the glucosidase(s) active in these alfalfa seed exudates are similar to glucosidases reported from other seeds (2, 4, 16, 17) remains to be determined.

Table II. Partial Purification of β-Glucosidase from Sterile Alfalfa Seed Exudate

<table>
<thead>
<tr>
<th>Stage of Purification</th>
<th>Volume</th>
<th>Total Protein</th>
<th>Specific Activity</th>
<th>Protein Yield</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mL</td>
<td>mg</td>
<td>pmol·g⁻¹·h⁻¹</td>
<td>%</td>
<td>-fold</td>
</tr>
<tr>
<td>Crude exudate</td>
<td>48</td>
<td>7.7</td>
<td>nd*</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>PEG dialysis</td>
<td>3.5</td>
<td>1.8</td>
<td>nd†</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>Sephadex G-25</td>
<td>6.4</td>
<td>0.5</td>
<td>0.75b</td>
<td>6.2</td>
<td>1b</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>237b</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bio-Gel P-150</td>
<td>5.0</td>
<td>0.05</td>
<td>3.6b</td>
<td>0.7</td>
<td>4.8b</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>348b</td>
<td></td>
<td>1.5b</td>
</tr>
</tbody>
</table>

* Not determined because exudate flavonoids interfered with assay.
† L7G as substrate.
‡ p-Nitrophenylglucose as substrate.
Table III. Hydrolysis of L7G to Luteolin by R. meliloti 1021

Bacteria in mid-logarithmic growth were suspended to a density of $A_{600} = 0.075$ (about $3 \times 10^8$ viable cells mL$^{-1}$) in a defined minimal medium with 1.0 g L$^{-1}$ mannitol and L7G. Flavonoid concentrations were measured by HPLC. Values are means ± se of three replicates.

<table>
<thead>
<tr>
<th>Time of Incubation (h)</th>
<th>Sterile Control $\mu$M</th>
<th>L7G</th>
<th>Luteolin $\mu$M</th>
<th>+ R. meliloti L7G</th>
<th>Luteolin $\mu$M</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>28.7 ± 2.9</td>
<td>0.39 ± 0.08</td>
<td>23.2 ± 0.7</td>
<td>0.31 ± 0.05</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>20.1 ± 2.3</td>
<td>0.14 ± 0.02</td>
<td>21.4 ± 0.6</td>
<td>0.22 ± 0.09</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>22.6 ± 2.0</td>
<td>0.19 ± 0.10</td>
<td>10.5 ± 0.1</td>
<td>0.88 ± 0.24</td>
<td></td>
</tr>
</tbody>
</table>

Data from this study together with other recent findings suggest the following model of how conditions for the establishment of alfalfa root nodules are produced in the rhizosphere. Seeds release flavonoid glycosides that are more soluble in water than aglycones but less active as nod gene inducers in R. meliloti (6). These glycosides have the potential to diffuse away from the germinating seed, and L7G, which comprises a significant portion of the total flavonoid fraction, can be hydrolyzed to active nod-gene-inducing luteolin by seed exoenzymes (Tables I and II), by R. meliloti (Table III), or possibly by other soil microorganisms (1, 19). Populations of R. meliloti are increased around the imbibing seed by the positive chemotactic effect of luteolin (3) and by the capacity of luteolin, L7G, quercetin, and quercetin-3-O-galactoside to enhance growth rates of R. meliloti (5). The nod genes controlled by nodD in those rhizobial cells are then induced by luteolin and chrysoeriol from the seed (6). As roots develop, 4,4'-dihydroxy-2'-methoxychalcone, 4', 7-dihydroxylavone, and 4', 7-dihydroxyflavone are exuded into the seedling environment and also promote nod gene induction (11). In the region where 4,4'-dihydroxy-2'-methoxychalcone from the root occurs with luteolin from the seed, there may be a synergistic promotion of nod gene induction (7, 8). As a result, root hairs curl and rhizobia invade the plant root (10).

Individual components of the preceding model are supported by laboratory data, but the relative importance of separate steps in soil is unknown. Classical studies show that root nodule formation is favored by large numbers of effective rhizobial cells (18). Processes affecting release and modification of nod-gene-inducing flavonoids from alfalfa seeds could alter the size of the rhizobial population and the fraction of those cells transcribing nod genes. In that sense, these processes play a potentially important role in root nodulation and, in some cases (9), may limit nodule development and subsequent N$_2$ fixation.

ACKNOWLEDGMENTS

We thank M. Luscher, C. M. Joseph, and C. A. Maxwell for helpful discussions.

LITERATURE CITED

taxis of Rhizobium meliloti to the plant flavone luteolin requires functional nodulation genes. J Bacteriol 170: 3164–3169
sidases—their properties and applications. Enzyme Microb Technol 4: 73–79