Aldonic Acids: A Novel Family of *nod* Gene Inducers of *Mesorhizobium loti*, *Rhizobium lupini*, and *Sinorhizobium meliloti*

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Molecular signals, such as flavonoids (or nonflavonoid type), *nod* gene-inducers, and bacterial lipochitin oligosaccharides (LCOs) act as modulators of species specificity during early stages of infection in *Rhizobium* spp.-legume interactions. The fact that signaling in *Lupinus albus* remains to be determined prompted us to investigate the flavonoid signal responsible for *nod* gene induction in *Rhizobium lupini*. A screening method was used based on the measurement of β -galactosidase activity of *R. lupini* strains harboring *nodC::lacZ* fusions in the presence of (i) authentic lupin isoflavones, (ii) carbohydrate-like inducers, and (iii) high-pressure liquid chromatography (HPLC)-fractionated lupin seed effusates and root exudates, as putative *nod* gene inducers. The results indicate that both erythronic and tetronic acids (4-C sugar acids) led to low but significant increases in β -galactosidase activities, compared with the controls. In addition, lupiwighteone, a monoprenylated isoflavone, exerts a synergistic effect with the carbohydrate-like inducers, compared with other isoflavone treatments. The natural occurrence of aldonic acids in *L. albus* root exudates and seed effusates has been demonstrated by HPLC analysis. When tested with *nodC::lacZ* fusions, tetronic acid resulted in *nod* gene induction in *Sinorhizobium meliloti*. In addition, a combination of luteolin and tetronic acid promotes further increases in *S. meliloti* *nod* gene expression, as shown by β -galactosidase assays. Incorporation studies with [^{14}C]LCO precursors confirmed the inductive role of both erythronic and tetronic acids in promoting LCO biosynthesis in *R. lupini* cultures, and of tetronic acid in *Mesorhizobium loti* and *S. meliloti*. Hydrolysis of the LCOs with various enzymes substantiated their putative identities. These results are discussed in relation to the impact of these unusual signal molecules on our knowledge of flavonoid signaling in *Rhizobium*-legume symbiosis.

chemically specialized in nitrogen fixation (Long 1989). Such symbiotic interactions depend primarily on the ability of the host to form root nodules (Phillips 1992); since these are mostly restricted to legumes, it would be of interest to extend the *Rhizobium* host range to other important crop plants.

The molecular basis of symbiotic interactions has become an intensive field of research during the last decade. One important finding of this research has been the recognition that some flavonoid compounds (Fig. 1) that are synthesized by the host plant and secreted in the rhizosphere act as molecular signals, thereby activating bacterial infection (Long 1989; Phillips 1992). These signals have been reported to act as chemoattractants of *Rhizobium* spp. (Caetano-Anollés et al. 1988) and as inducers of bacterial nodulation (*nod*) genes (Peters et al. 1986). It should be noted, however, that other nonflavonoid-type molecules such as betaines (Fig. 1) also act as *nod* gene inducers in *Sinorhizobium meliloti* (Phillips et al. 1994).

Fig. 1. Molecular structures of flavonoids (refer to Table 1 for common and generic names), aldonic acids, and stachydrine.

Symbiosis between legumes and *Rhizobium* spp. results in the formation of root nodules that are structurally and bio-

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Flavonoid compounds that are secreted in the rhizosphere by a compatible host activate the bacterial NodD protein, a transcriptional activator, thus resulting in the expression of all other bacterial *nod* genes (Fisher and Long 1992). These genes code for enzymes involved in the biosynthesis or secretion of lipochitin oligosaccharides (LCOs) that act as bacterial nodulation signals (e.g., Nod factors; Carlson et al. 1994; Dénarié et al. 1996). Therefore, both flavonoids (as well as some betaines) and LCOs are considered signal molecules that determine species specificity in these symbioses (Fisher and Long 1992; Phillips 1992), and this specificity is achieved by structural variations of either the flavonoids or LCOs.

In order to screen for unknown flavonoid inducers, induction of *nod* gene expression has been examined with reporter genes such as *lacZ* inserted downstream of the common *nodABC* operon (Zaat et al. 1989). As well, the biosynthesis of LCOs can be used as a screening method to recognize the putative *nod* gene inducer(s) (Lortet et al. 1996), and a clear link has been established between *nod* gene expression and LCO biosynthesis (Dénarié et al. 1996). In addition, the flavonoid-independent transcriptional activator (FITA) *nodD* (Spaink et al. 1989) has been successfully used to bypass the need for characterization of the *nod* gene inducer (Lopez-Lara et al. 1995).

Lupinus albus L. (white lupin), a member of the Leguminosae, is capable of establishing a symbiosis with *Rhizobium lupini*. The roots of white lupin synthesize a variety of isoflavonoid aglycones and glucosides, as well as their mono- and diprenylated derivatives (Fig. 1; Table 1), and the phytochemistry of these compounds has been well established (Gagnon and Ibrahim 1997 and references cited therein). Most of these compounds are secreted in the rhizosphere of intact roots (Gagnon et al. 1995b), and act as indicators of various stress responses in the plant (Gagnon and Ibrahim 1997). Unlike the majority of leguminous species, white lupin tissues constitutively synthesize and accumulate prenylated isoflavones (Harborne et al. 1976; Gagnon et al. 1992, 1995b). In addition, white lupin roots contain glycoproteins whose glycosidic moiety consists of 30% erythronic acid (Fig. 1C), an uncommon aldonic acid (H. Gagnon and R. K. Ibrahim, unpublished results).

Table 1. Flavonoid compounds tested, their trivial and generic names

Compound	Generic name
Flavones	
Luteolin	5,7,3',4'-tetrahydroxyflavone
Naringenin	5,7,4'-trihydroxyflavanone
Isoflavone aglycones	
Genistein	5,7,4'-trihydroxyisoflavone
2'-hydroxygenistein	5,7,2',4'-tetrahydroxyisoflavone
Lupinalbin A	5,7,4'-trihydroxycomaronochromone
Monoprenylated isoflavones^a	
Wighteone	6-prenyl-5,7,4'-trihydroxyisoflavone
Luteone	6-prenyl-5,7,2',4'-tetrahydroxyisoflavone
Licoisoflavone A	3'-prenyl-5,7,2',4'-tetrahydroxyisoflavone
Isowighteone	3'-prenyl-5,7,4'-trihydroxyisoflavone
Lupiwighteone	8-prenyl-5,7,4'-trihydroxyisoflavone
Derrone	7,8-hydroxypyran-5,4'-dihydroxyisoflavone
Diprenylated isoflavones	
Lupalbigenin	6,3'-diprenyl-5,7,4'-trihydroxyisoflavone
2'-hydroxylupalbigenin	6,3'-diprenyl-5,7,2',4'-tetrahydroxyisoflavone

^a Prenyl = 3,3-dimethylallyl-.

In contrast to other *Rhizobium*-legume associations, both the flavonoid and LCO signals that confer specificity of lupin symbiosis with *R. lupini* are yet to be identified (Phillips 1992; Lopez-Lara et al. 1995; Stafford 1997). On the other hand, the structure of the LCOs produced by *Mesorhizobium loti*, a species closely related to *R. lupini*, has been defined by FITA-harboring *nodD*, even though no flavonoid inducer has yet been identified in this species (Lopez-Lara et al. 1995). In the search for a host-specific inducer molecule of *R. lupini*, we tested a number of naturally occurring lupin root isoflavonoids for *nod* gene induction and Nod factor biosynthesis by contemporary screening methods. Of the isoflavonoids tested (Table 1), only lupiwighteone exhibited low *nod*-inducing activity on its own. However, a naturally occurring lupin root constituent, erythronic acid, and its analog tetronic acid, acted as *nod* gene inducers. Since this type of molecule represents a new class of *nod* gene inducers, we also investigated its capacity to act as a signal molecule in two other species, *M. loti* and *S. meliloti*. Interestingly, however, the results indicate that tetronic acid induces *nod* gene expression in both species. Furthermore, a synergistic effect seems to take place in *S. meliloti* and *R. lupini* between aldonic acids and either lupiwighteone or luteolin, the natural flavonoid constituents of the respective host roots.

RESULTS

Transformation of *R. lupini* and β -galactosidase assays with *R. lupini* and *S. meliloti*.

Purified pRmM57 cosmid was used to transform competent cells of *R. lupini* by a freeze-thaw method. The efficiency of transformation was approximately 60 transformants per μ g of cosmid DNA, and the transformants were resistant to both spectinomycin and tetracycline. In addition, a dose-dependent response was observed after treatment with a range of luteolin concentrations (50 nM to 20 μ M), as indicated by the net relative β -galactosidase activity (Table 2). Therefore, it can be concluded that the pRmM57 cosmid is functional in an *R. lupini* background, and that this transformed strain harboring *nodC::lacZ* fusion can be used in β -galactosidase assays after treatment of the bacteria with the putative *nod* gene inducers. It should be noted, however, that the levels of β -galactosidase activity achieved with *R. lupini*/pRmM57 were low, compared with those of *S. meliloti*/pRmM57 (see below).

Table 2. β -Galactosidase activity of *Rhizobium lupini*/pRmM57 after induction with different concentrations luteolin^a

Luteolin concentration (μ M)	Miller units ^b	SE (%) ^c
Control	16.0	*
0.05	27.3	10
0.5	85.1	*
5	257.3	*
20	548.1	6

^a Cultures of pRmM57 transformants grown in yeast-mannitol medium were diluted to an OD₆₀₀ of 0.1 and induced with various concentrations of luteolin for a period of 20 h. Following induction, triplicate 100- μ l aliquots from each culture were assayed for β -galactosidase activity. These experiments were repeated and both results showed similar β -galactosidase activities. Control cultures were treated with 40% aq MeOH.

^b Averages of triplicate β -galactosidase assays.

^c Standard error of averaged β -galactosidase assays; *, SE < 5%.

Activation of *nod* genes in *R. lupini*/pRmM57 after treatment with various putative inducers.

In order to screen for putative isoflavonoid inducers, both seed effusates and root exudates of *L. albus* were fractionated by high-pressure liquid chromatography (HPLC) gradient elution and the lipophilic fractions (containing isoflavones) were used for the induction of *R. lupini*/pRmM57 cultures before β -galactosidase assays. None of the latter fractions induced levels of β -galactosidase activity higher than those of the controls (data not shown). However, of the authentic isoflavone standards tested (Table 1), lupiwighteone resulted in low β -galactosidase activities (Table 3).

Molecules other than isoflavones, such as aldonic acids, were used for the induction of *R. lupini*/pRmM57 transformant cultures. Betaines (Fig. 1) have been reported to induce *S. meliloti nod* gene expression at a 10^3 -fold higher concentration than that required for similar levels of induction with luteolin (Phillips et al. 1992); therefore, aldonic acids were used in this experiment at millimolar concentrations. Figure 2 shows that incubation of *R. lupini*/pRmM57 with different concentrations of aldonic acids (100 μ M to 80 mM) resulted in a dose-dependent increase in the relative β -galactosidase activities; but not with succinic acid, a structural analog of aldonic acids. It is probable, however, that the lower β -galactosidase activity observed with tetronic acid treatments, higher than 10 mM, may be ascribed to the fact that this compound lowers the pH of the medium at these concentrations. Nonetheless, both erythronic and tetronic acids may be considered *nod* gene inducers in *R. lupini*.

In order to further investigate this finding, the possible synergistic effect between erythronic acid and lupin isoflavones was tested with combinations of 20 mM erythronic acid and 5 μ M each of a number of lupin isoflavones. Treatment of *R. lupini*/pRmM57 with different combinations of lupiwighteone (0.05 to 5 μ M), together with erythronic acid (5 to 20 mM) or 10 mM tetronic acid resulted in the highest relative β -galactosidase activities when a mixture of 5 μ M lupiwighteone with either 20 mM erythronic acid or 10 mM tetronic acid was used (Table 3). Therefore, these results indicate that lupiwighteone, a genistein monoprenyl, exerts a synergistic effect with either of the aldonic acids in promoting β -galactosidase activity, and hence of *nod* gene induction.

Inducibility of *nod* genes in *S. meliloti*/pRmM57 after treatment with aldonic acids and/or luteolin.

In order to verify that the observed inducibility by aldonic acids of *lacZ* fusions in *R. lupini* was not due to the presence of the heterologous *S. meliloti nodD1* in pRmM57, a dose-response induction by aldonic acids was established with an *S. meliloti* 1021/pRmM57 transformant. A dose dependency between luteolin concentration and β -galactosidase activity was first carried out with *S. meliloti*/pRmM57 in order to assess its functionality under the assay conditions used, and as a positive control (Table 4). Treatment with aldonic acids showed a dose-response relation-

Fig. 2. Effect of various aldonic acid concentrations on β -galactosidase activity of *Rhizobium lupini*/pRmM57. Duplicate *R. lupini*/pRmM57 cultures, grown in yeast-mannitol medium, were induced for 20 h with different concentrations of erythronic acid (solid circles), tetronic acid (solid squares), and succinic acid (solid triangles). Following induction, triplicate β -galactosidase assays were carried out on each culture. β -Galactosidase activity of noninduced controls (approximately 16 U) was subtracted from those obtained after aldonic acid treatments, and net values were plotted. Standard errors are indicated by bars above each plotted value. These experiments were repeated and both results showed similar β -galactosidase activities.

Table 3. β -Galactosidase activity of *Rhizobium lupini*/pRmM57 after induction with different concentrations of lupiwighteone and aldonic acids^a

Lupiwighteone concentration (μ M)	Control ^b	SE ^c (%)	EA ^d 5 mM	SE (%)	EA 10 mM	SE (%)	EA 20 mM	SE (%)	TA ^d 10 mM	SE (%)
Control	15.7	*	34.7	7	42.4	8	46.8	*	48.7	9
0.05	17.9	*	34.8	*	42.4	9	47.3	*	49.4	*
0.1	14.4	8	36.1	*	44.2	3	48.3	*	48.2	*
0.5	19.2	6	35.7	*	42.7	*	48.9	*	49.8	*
1	14.2	6	34.1	6	40.0	11	50.2	7	47.1	7
5	15.6	*	37.0	*	41.0	6	51.5	6	52.6	10

^a Cultures of pRmM57 transformants grown in yeast-mannitol medium were diluted to an OD₆₀₀ of 0.1 and induced with lupiwighteone in the absence (control) or presence of aldonic acids for a period of 20 h. Following induction, triplicate 100- μ l aliquots from each culture were assayed for β -galactosidase activity. These experiments were repeated and both results showed similar β -galactosidase activities. Control cultures were treated with 40% aq MeOH.

^b Averages of triplicate β -galactosidase assays.

^c Standard error of averaged β -galactosidase assays; *, SE < 5%.

^d EA, erythronic acid; TA, tetronic acid.

ship between the concentration of tetronic acid used and an increase in relative β -galactosidase activity (Table 4, top row), but not with either erythronic acid or succinic acid (data not shown). In addition, the possible synergistic effect of tetronic acid and the well-known inducer luteolin was tested for *nod* gene inducibility with a range of concentrations (0.1, 1, and 10 mM tetronic acid and 0.05, 0.5, and 5 μ M luteolin, respectively). Table 4 shows that combinations of 0.5 μ M luteolin and 10 mM tetronic acid, as well as 0.5 or 5 μ M luteolin and 0.1 or 1 mM tetronic acid treatments, resulted in a significant increase of β -galactosidase activity, compared with the values of those treatments when each compound was tested alone.

Taken together, these results confirm that erythronic acid induces by two- to threefold, and without heterologous bias, the *nod* genes in *R. lupini*, whereas the observed *lacZ* expression by tetronic acid cannot be solely attributed to the endogenous *nodDs* of *R. lupini*. These results also demonstrate that tetronic acid acts as an inducer of *nod* genes in *S. meliloti*. In addition, a synergistic effect on *nod* gene expression is observed in *S. meliloti* after treatment with a combination of tetronic acid and luteolin, and in *R. lupini* after treatment with lupiwighteone and either of the aldonic acids.

Radiolabeling of *nod* metabolites (LCOs) and hydrolytic analyses.

Preliminary labeling experiments indicated that treatment with 5 μ M naringenin, an induction period of 3 h prior to label administration, and a metabolic period of 19 h (approximately 2.8 doubling time; OD₆₀₀ of cultures at 0.7) were optimum for LCO biosynthesis in *M. loti*/pMp2112. Naringenin-induced *M. loti*/pMp2112 incorporated the label of ¹⁴C-glucosamine (GlcN) and ¹⁴C-HOAc into two putative LCO bands, having similar relative mobilities on TLC (Fig. 3A), that were not observed in their respective noninduced controls. The fact that additional minor bands appeared in both induced and non-induced treatments administered ¹⁴C-HOAc may be attributed to some label incorporation into fatty acids, either with different chain lengths or with different degrees of unsaturation. As would be expected, acetate is a less specific precursor of LCO biosynthesis than is glucosamine (Fig. 3A). In fact, ¹⁴C-GlcN gave the best relative LCO yield with *M. loti*/pMp2112 (approximately 32% of administered label), whereas ¹⁴C-HOAc resulted in only 4% labeled product when used as a precursor (data not shown).

The putative radiolabeled LCO bands obtained from naringenin-induced *M. loti*/pMp2112 (Fig. 3A) were further ana-

lyzed by HPLC with an LCO mixture derived from naringenin-induced *M. loti*/pMp2112 as a reference. The relative activity profile obtained with a mixture of ¹⁴C-HOAc and ¹⁴C-GlcN shows that most of the radioactivity eluted at Rt 20.75 to 22.25 min (Fig. 4A), a retention time that corresponds to that observed with the *M. loti* LCO standards (data not shown). Hydrolysis of the radiolabeled LCO bands recovered from the ¹⁴C-GlcN feeding with either *N*-acylase, chitinase, *N*-acetylglucosaminidase, or egg white lysozyme gave additional radiolabeled bands (Fig. 3B), and different relative activity profiles (Fig. 4B), but not with proteinase K (Fig. 4B). These results show that hydrolysis of LCOs has been achieved with LCO-specific enzymes, and demonstrate that the hydrolyzed molecules are indeed LCOs. In addition, treatment with proteinase K is not expected to alter the HPLC elution profile of the radiolabeled LCOs, since they are not proteins. Considering that the *M. loti* LCO structures have been well characterized (Lopez-Lara et al. 1995), that their identity has been verified, and because naringenin-induction is known to promote an efficient LCO yield in FITA transformants of *M. loti*, compared with that of *R. lupini* (see below), therefore, the *M. loti*/pMp2112 LCOs were used as positive controls in further experiments (Fig. 3). In addition, it is expected the *R. lupini* LCOs would be similar to those of *M. loti* since the species are closely related.

Metabolic radiolabeling experiments were also carried out with FITA-harboring strains of naringenin-induced *R. lupini* and *M. loti* in order to compare the relative efficiency of the two strains for the incorporation of different enantiomeric amino sugars. Figure 3C shows that incorporation of ¹⁴C-GlcN into *M. loti*/pMp2112 LCOs is approximately 27-fold higher than that of ¹⁴C-galactosamine (GalN). On the other hand, galactosamine serves as a better precursor of LCOs than GlcN in the *R. lupini*/pMp2112, but with a much lower product yield (data not shown), compared with *M. loti*/pMp2112 when administered ¹⁴C-GlcN (Fig. 3C). HPLC analysis of a mixture of the ¹⁴C-GlcN and ¹⁴C-GalN labeled LCO bands produced by *R. lupini*/pMp2112 gave an elution pattern with relative activities similar to that of the FITA-harboring *M. loti* (Fig. 4A), which is consistent with their being LCOs. Of the amino sugar enantiomers used (¹⁴C-GlcN, ¹⁴C-AcGlcN, ¹⁴C-GalN, ¹⁴C-AcGalN, and ¹⁴C-ManN), both glucosamine and galactosamine were better LCO precursors in either naringenin-induced or -noninduced *R. lupini*/pMp2112 cultures, compared with the other radiolabeled compounds used (data not shown). However, these incorporation studies did not reveal which is the best precursor of LCOs in *R. lupini*/

Table 4. β -Galactosidase activity of *Sinorhizobium meliloti*/pRmM57 after induction with different concentrations of luteolin and tetronic acid^a

Luteolin concentration (μ M)	Control ^b	SE (%) ^c	TA ^d 0.1 mM	SE (%)	TA 1 mM	SE (%)	TA 10 mM	SE (%)
Control	33.9	7	47.5	*	53.3	8	141.3	11
0.05	153.2	*	158.4	7	170.7	*	327.2	7
0.5	1,540.5	*	1,738.2	6	1,724.2	*	1,602.5	*
5	1,529.7	*	1,646.9	*	1,706.8	*	1,651.4	8
20	796.2	15	ND		ND		ND	

^a Cultures of pRmM57 transformants grown in yeast-mannitol medium were diluted to an OD₆₀₀ of 0.2 and induced with luteolin in the absence (control) or presence of tetronic acid for a period of 4 h. Following induction, triplicate 100- μ l aliquots from each culture were assayed for β -galactosidase activity. These experiments were repeated and both results showed similar β -galactosidase activities. Control cultures were treated with 40% aq MeOH.

^b Averages of triplicate β -galactosidase assays.

^c Standard error of averaged β -galactosidase assays; *, SE < 5%.

^d TA, tetronic acid.

pMp2112 transformants, mainly because of the low incorporation of radioactivity.

Metabolic radiolabeling LCOs in wild-type *R. lupini*.

Preliminary incorporation studies with wild-type *R. lupini* treated individually with 5 μ M concentrations of each of the 11 different lupin isoflavones shown in Table 1, or in combination with 2.5 mM tetronic or erythronic acids, were carried out with 0.5 μ Ci of 14 C-HOAc, 14 C-GlcN, or 14 C-GalN as

LCO precursors. None of these 108 different radiolabeling treatments resulted, after autoradiography, in any LCO incorporation, compared with the positive controls (data shown only for 14 C-GalN in Figure 3D). Surprisingly, however, the highest background incorporation level was observed with the negative control (Fig. 3D, lane 1a), which decreased with increasing lipophilicity of the isoflavone used, whereas it was not observed in any of the aldonic acid treatments. Since both aldonic acids, in combination with lupiwighteone, act as *nod*

gene inducers (as shown with *nodC::lacZ* fusions), these compounds are expected to promote LCO biosynthesis, although the reverse takes place in wild-type *R. lupini*. The only possible explanation may be that in this species the level of background incorporation into LCOs similar to those of *M. loti* is high, and that when *R. lupini* is treated with putative *nod* gene inducer it increases the synthesis of putative LCOs at the expense of LCOs similar to those of *M. loti*.

Use of an optimized extraction procedure (see Materials and Methods) and 2 μ Ci of 14 C-GlcN with aldonic acid-treated *R. lupini* cultures resulted in radiolabeled LCOs with relative mobilities similar to those of the positive control, although the intensity of the LCO band with fast mobility decreased with increasing concentration of aldonic acid (Fig. 3E). However, additional radiolabeled bands with a lower mobility on C18 silica were also observed (Fig. 3E). The latter increased with higher concentrations of either erythronic acid or tetronic acid, although a low but significant incorporation of label with succinic acid was also observed (see later). Therefore, it can be concluded that both aldonic acids promote LCO biosynthesis in wild-type *R. lupini*. HPLC analysis of an aliquot (approximately 1,200 disintegrations per min [dpm]) of the putative LCO bands (Fig. 3E) shows that most of the radioactivity was eluted at retention time (Rt) 22.5 to 23.75 min, a retention time that is slightly delayed by approximately 1 to 2 fractions, compared with that of the *M. loti*/pMp2112 LCOs (Fig. 4A). This elution behavior further suggests that these inducible, radiolabeled bands are less polar than those of *M. loti*. Enzyme hydrolysis of the remainder of the LCO bands, followed by HPLC analysis, gave an elution-radioactivity pattern that is different from that of either the control or proteinase K (Fig. 4C), which suggests that the hydrolyzed molecules are indeed LCOs. Similar inductions with aldonic acids, with 14 C-GalN (2 μ Ci) as a precursor, did not exhibit LCOs of lower polarities (data not shown).

Metabolic radiolabeling of LCOs in other *Rhizobium* spp.

Since aldonic acids promoted the metabolic radiolabeling of LCOs in *R. lupini*, the possibility that this phenomenon takes place in wild-type *M. loti* was investigated, in spite of the fact that the signal molecule(s) in this species remain to be determined (Phillips 1992). Radiolabeling of LCOs was carried out with 0.5 μ Ci of 14 C-GlcN and *M. loti* cultures that were induced with different concentrations of aldonic acids. The results show that, compared with the positive control, the production of radiolabeled LCOs increased with increasing concentrations of tetronic acid (10 to 15 mM) but not with either erythronic or

succinic acids, except at 20 mM (Fig. 3F). Therefore, tetronic acid acts as a *nod* gene inducer in wild-type *M. loti*, since a dose dependency exists between tetronic acid and LCO biosynthesis, although treatment with 20 mM succinate promotes such an effect. However, the latter result should be interpreted with caution due to the fact that succinate is a common metabolite that may not be involved in the induction process.

Since tetronic acid induced *lacZ* expression and, hence, *nod* gene induction in *S. meliloti*/pRmM57 (Table 4), LCO biosynthesis with 0.5 μ Ci 14 C-GlcN was carried out in wild-type *S. meliloti* after induction with either 20 mM erythronic acid, 10 mM tetronic acid, or 5 μ M luteolin, the natural flavonoid inducer. The results show that radiolabeled LCOs were produced after induction with tetronic acid but not with erythronic acid, based on the relative mobilities of the radiolabeled spots that were similar to those of the luteolin-induced positive control (Fig. 3G). Enzyme hydrolysis and HPLC analysis of aliquots (approximately 2,800 dpms each) of the radiolabeled bands (Fig. 3G) indicate that the LCO-specific enzyme hydrolysis products are different from those of both the control and proteinase K (Fig. 4D), and support the notion that the hydrolyzed molecules are indeed LCOs.

Natural occurrence of erythronic and tetronic acids in *L. albus*.

Seed effusates and root exudates of *L. albus* (cv. Kievskij), as well as the cotyledon and seed coat tissues soaked in water for 24 h, were analyzed by HPLC for their aldonic acid content. HPLC analysis of the different fractions (Table 5) shows that both seed effusates and root exudates contain significant amounts of tetronic and erythronic acids, with a predominance of the former compound over the latter. In fact, the amounts of tetronic acid are three- to fourfold higher in the seed effusates, and an impressive 130-fold higher in root exudates, than those of erythronic acid (Table 5). Such predominance of tetronic over erythronic acid is also observed in both seed parts analyzed, although with a correspondingly lower magnitude. The total amounts of both aldonic acids are approximately fourfold higher in the cotyledons than in the seed coats, which suggests that the cotyledons act as a reservoir of these molecules (Table 5). Therefore, it appears that the aldonic acid content of the seed effusates, root exudates, and cotyledons is, on a molar basis, much higher than that of isoflavonoids, and amounts to 44-, 240- and 4,000-fold higher than total isoflavones, respectively (Gagnon et al. 1995b).

Similar analyses carried out with seed effusates of another lupin cultivar (Primovsky) indicated the presence of two- to

Fig. 3. Metabolic radiolabeling of lipochitin oligosaccharides (LCOs) with various 14 C-labeled LCO precursors in flavonoid-independent transcriptional activator (FITA)-harboring or wild-type *Rhizobium* strains. Six-milliliter cultures (OD₆₀₀ of 0.1) of pMp2112 transformants or of wild-type strains, grown in yeast-mannitol medium, were induced with various compounds, administered a 14 C-labeled precursor, and incubated for 21 h. After pre-purification of the culture supernatants, the LCO-containing extracts were chromatographed on thin-layer chromatography and autoradiographed. **A**, *Mesorhizobium loti*/pMp2112 transformants were induced with (+) or without (–) 5 μ M naringenin and administered 14 C-GlcN or 14 C-HOAc. **B**, The 14 C-GlcN bands (**A**) were subjected to hydrolysis with various enzymes; *N*-Ac-Glc-ase, *N*-acetyl glucosaminidase. **C**, Naringenin-induced *M. loti*/pMp2112 or *Rhizobium lupini*/pMp2112 transformants were administered either 14 C-GlcN or 14 C-GalN. **D**, Induction of wild-type *R. lupini* with combinations of 2.5 mM aldonic acids and/or 5 μ M isoflavones, followed by a 14 C-GalN feeding: a, water control; b, erythronic acid; c, tetronic acid; 1, 40% MeOH control; 2, genistein; 3, 2'-hydroxygenistein; 4, lupinalbin A; 5, luteone; 6, licoisoflavone A; 7, wighteone; 8, lupiwighteone; 9, isowighteone; 10, derrone; 11, 2'-hydroxylupalbigenin; 12, lupalbigenin. Wild-type *R. lupini* (**E**), *M. loti* (**F**), and *Sinorhizobium meliloti* (**G**) were treated with various concentrations of aldonic acids and administered 14 C-GlcN. Abbreviations: Ac, acetyl; EA, erythronic acid; Gal, galactose; Glc, glucose; HOAc, acetic acid; N, amine; SA, succinic acid; TA, tetronic acid; *, only one-sixth of the extract was chromatographed. These experiments were repeated and both results showed similar incorporation patterns.

threefold higher amounts of both aldonic acids than are present in Kievskij (data not shown). These results suggest that the natural occurrence of aldonic acids in other *Lupinus* spp. may be a common phenomenon in legume species.

DISCUSSION

Of the several *Rhizobium*-legume interactions that have so far been reported (Long 1989; Phillips 1992), signaling in *L. albus* and *R. lupini* symbiosis remains to be determined. This symbiotic association may be characterized by the following features: *R. lupini* is an "exceptional" symbiont due to the physical location of its *nod* genes, which are spread over the genome and a megaplasmid (Scott et al. 1996), and its copious secretion of exopolysaccharides, compared with other *Rhizo-*

bium spp. In addition, the host (*L. albus*) exhibits a marginal symbiotic behavior; its *Rhizobium*-infected nodules are neither determinate nor indeterminate, unlike most other legume species, and they are referred to as the "crown nodules" of lupin (Hirsch 1992). In addition, *L. albus* is one of the rare legumes that does not require mycorrhizae for optimal phosphorous uptake (Koide 1991). But, above all, the bacterial symbiont must cope with the capability of lupin roots, which constitutively synthesize a variety of prenylated isoflavones (Gagnon et al. 1995b, and references therein). These metabolites act as antimicrobial compounds, rather than as *nod* gene inducers. However, several indirect lines of evidence (Gagnon et al. 1995a; Gagnon and Ibrahim 1997), as well as others derived from the approaches described in this work, tend to support a role for some prenylated isoflavones as signaling molecules.

Fig. 4. High-pressure liquid chromatography (HPLC) fractionation of radiolabeled lipochitin oligosaccharides (LCOs) from various *Rhizobium* strains (A) or after hydrolysis with various enzymes (B, C, and D), and the relative radioactivity of individual fractions. After induction for 3 h with 5 μ M naringenin (open and light shaded bars, A) or aldonic acids (dark shaded and solid bars, A), *Rhizobium* cultures grown in yeast-mannitol medium were administered either 14 C-GlcN, 14 C-GalN, or 14 C-HOAc for 18 h. After extraction and thin-layer chromatography (TLC) analysis, radiolabeled bands were recovered from autoradiographed TLCs (Fig. 3), and chromatographed by gradient HPLC. Fractions were collected every 45 s, and relative radioactivity of each was monitored by liquid scintillation counting. HPLC profiles are omitted for ease of presentation. B, C, and D, HPLC analysis of radiolabeled LCOs from *Mesorhizobium loti*/pMp2112, wild-type *Rhizobium lupini*, and wild-type *Sinorhizobium meliloti*, respectively, after hydrolysis with various enzymes. No fractions contained significant activities outside of the region shown. GlcN-ase, glucosaminidase; D, E, and F, *M. loti*/pMp2112 LCOs positive control.

A clear link has been established between *nod* gene induction and Nod factor biosynthesis (Carlson et al. 1994). Therefore, the inducibility of *nod* genes in *Rhizobium* by *nodC::lacZ* fusions is adequate to use as an experimental approach, since most inducers have been discovered with this as a complement to other approaches. Of the symbiotic systems in which the flavonoid inducer has been determined, metabolic radiolabeling and accumulation of LCOs were achieved with the natural inducer. Both experimental approaches are complementary since they are used to investigate the same parameter, i.e., the induction of genes that encode the enzymes required for biosynthesis of LCOs (Carlson et al. 1994). Alternatively, FITA constructs have been used to bypass the need to identify the natural inducer, while they allow the determination of the structure of the LCO produced. A good example is *M. loti*: the structure of its Nod factor has been elucidated, but its *nod* gene inducer remains to be determined (Lopez-Lara et al. 1995).

The use of the cosmid pRmM57 for the inducibility of *nodC::lacZ* fusions in *R. lupini* has not revealed a significant role for any of the lupin isoflavonoids when tested alone as *nod* gene inducers, although there was some activity with 0.05 and 0.5 μ M lupiwighteone. However, the fact that erythronic acid occurs naturally in lupin tissues (H. Gagnon and R. Ibrahim, unpublished results), prompted us to test two isomeric aldonic acids (erythronic and tetronic) as inducers of *nod* genes in *R. lupini*. Similar molecules, such as the 4-C heterocyclic compounds, betaine, and stachydrine, act as *nod* gene inducers of *S. meliloti* (Phillips et al. 1992). There was a dose-dependent response of β -galactosidase activity for the erythronic acid- and tetronic acid-induced *R. lupini*/pRmM57, but not after induction with succinic acid. Furthermore, lupiwighteone, a monoprenylated derivative of genistein, acts in synergy with erythronic acid, albeit with low but significant β -galactosidase activity. These results strongly suggest that erythronic acid is a *nod* gene inducer of *R. lupini*. In addition, the synergistic effect of the latter compound with lupiwighteone constitutes the first reported evidence of *nod* gene induction in *R. lupini* that is promoted by two structurally different molecules. However, further investigation of the inducibility of *nod* genes by tetronic acid will be required in order to account for any heterologous bias. The natural occurrence of aldonic acids in seed parts, seed effusates, and root exudates provides further evidence for their role as co-inducers of *nod* genes in this symbiosis. Furthermore, the amounts of aldonic acids observed in these tissues are in the same order of magnitude as those used for β -galactosidase assays and LCO biosynthesis with *R. lupini*.

The requirement for the betaine stachydrine in the activation of the transcriptional regulator *nodD2* of *S. meliloti* has

been established (Phillips et al. 1992). As well, the activation of *nodD1* and *nodD3* by flavonoid inducers, such as luteolin, has been thoroughly investigated (Mulligan and Long 1989), although that of *nodD2* is independent of luteolin (Honma et al. 1990). However, the induction of *nod* genes by a combination of betaines and flavonoids has not yet been investigated in *S. meliloti*. Considering the fact that *nodDs*, as transcriptional regulators, are homologous to the LysR family, which act as heterodimers in their binding with the DNA promoter sequences, *S. meliloti* qualifies as a prime example to study the combined effects of betaines and flavonoids on the induction of this species. In this study, we provide evidence for a novel, synthetic *nod* gene inducer, tetronic acid, and demonstrate its synergistic effect with luteolin on *nod* gene induction in *S. meliloti*. This finding may, therefore, offer a rationale to reconcile the heterodimeric requirements known for LysR family members and the *Rhizobium* NodDs functional response to metabolites of the legume rhizosphere. It is expected that the betaine inducers, which are *nodD1* independent and *nodD3* independent, and luteolin, which is *nodD2* independent, would as well promote a synergy in the activation of *S. meliloti nodDs*. However, attempts to demonstrate the latter phenomenon were unsuccessful, possibly because of the low concentrations (μ M range) of betaine used (Phillips et al. 1993).

In contrast to the ease of discovering an inducer molecule for *M. loti* (tetronic acid), the incorporation of labeled precursors into LCOs of *R. lupini* has proven to be elusive. In fact, the various labeling experiments carried out with FITA-harboring *R. lupini* did not enable us to determine an unequivocally good LCO precursor. It has previously been reported that both 14 C-acetate and 14 C-glucosamine act as good precursors for the biosynthesis of LCOs in *Rhizobium* spp. (Spaink et al. 1992; Lopez-Lara et al. 1995). It has also been shown that metabolic radiolabeling of LCOs takes place more preferentially with 14 C-glucosamine than with 14 C-acetate (Price and Carlson 1995). However, we used every radiolabeled precursor molecule available commercially, and our results suggest that the LCOs of *R. lupini* may not be based on either glucosamine, galactosamine, or mannosamine oligomers, like other Nod factors isolated to date that are not based on these sugar residues (Dénarié et al. 1996). In contrast to *M. loti*, where incorporation of GlcN into LCOs is several-fold higher than that of GalN, the reverse is observed with *R. lupini*. It is, therefore, possible that a *nod*-dependent encoded epimerase, acting on the amino sugars in *R. lupini*, may be responsible for the low levels of 14 C-GlcN incorporation into *R. lupini* LCOs. Nonetheless, a relatively high level of LCO labeling has been achieved with higher amounts of labeled GlcN, coupled with a more efficient extraction method, which resulted in a dose dependency between the level of aldonic acid induction and LCO biosynthesis in wild-type *R. lupini*. In addition, the identity of the inducible LCOs has been shown by hydrolysis with specific enzymes, including two additional ones (cf. Price and Carlson 1995). These are *N*-acetylglucosaminidase, which acts on both glucosamine and galactosamine oligomers, and proteinase K, which serves as a negative control for hydrolysis, since LCOs do not contain peptide bonds. Furthermore, we have demonstrated, for the first time, the induction of *nod* genes by tetronic acid in both *S. meliloti* and *M. loti*, as well as its ability to promote the metabolic radiolabeling of

Table 5. Erythronic acid and tetronic acid content in seed effusates, root exudates, and seed parts of *Lupinus albus* cv. Kievskij

Fraction	Erythronic acid	SE (%) ^a	Tetronic acid	SE (%)
Seed effusates (nmol · seed ⁻¹)	30.8	6	90.2	6
Root exudates (nmol · seed ⁻¹)	8.9	11	1,175.7	*
Cotyledons (nmol · mg f.wt ⁻¹) ^b	98.3	*	137.9	11
Seed coats (nmol · mg f.wt ⁻¹)	14.3	15	34.4	29

^a Standard errors of duplicate high-pressure liquid chromatography determinations; *, SE < 5%.

^b f.wt, fresh weight.

LCOs, whose nature was established by hydrolysis with specific enzymes. Taken together, these results provide strong evidence for the role of tetronic acid as a signal molecule.

In conclusion, our results demonstrate, for the first time, that tetronic acid acts as an inducer of *nod* genes in two related *Rhizobium* spp. (*R. lupini* and *M. loti*), as well as in *S. meliloti*, although the natural occurrence of this inducer has yet to be investigated in the compatible hosts of *S. meliloti* and *M. loti* (*Medicago* and *Lotus* spp., respectively). These results may, therefore, render the generally accepted rationale of species specificity by flavonoids in *Rhizobium*-legume interactions a "questionable" phenomenon, at this point. In view of extending the host range of *Rhizobium* to other nonlegume hosts that are important crops, this work may contribute to the design of simpler bioengineering approaches for the production of *Rhizobium* strains that are less dependent upon host infection. Furthermore, the synergy between aldonic acids and flavonoids may be used to improve LCO yield in other legume symbionts.

MATERIALS AND METHODS

Chemicals.

Lupin isoflavonoids (Fig. 1; Table 1) were a generous gift from Satoshi Tahara, Hokkaido University, Japan. Luteolin and naringenin were purchased from Spectrum Chemicals (Gardena, CA) and Roth (Karlsruhe, Germany), respectively. Erythronic, succinic, and tetronic acids were obtained from Sigma-Aldrich (St. Louis, MO). Standard flavonoids and aldonic acids were solubilized in 80% aq MeOH and water respectively, then filter sterilized by centrifugation in microspin filters (CanadaWide Scientific, Ottawa, Ontario) prior to use in bioassays with rhizobia. [1-¹⁴C]sodium acetate (Spec. Act. 57.2 mCi · mmol⁻¹), and D-[1-¹⁴C]glucosamine hydrochloride, [1-¹⁴C]galactosamine hydrochloride, [1-¹⁴C]mannosamine hydrochloride, [1-¹⁴C]acetylglucosamine hydrochloride, and [1-¹⁴C]acetylgalactosamine hydrochloride (Spec. Act. 55 mCi · mmol⁻¹) were purchased from American Radiolabeled Chemicals (St. Louis, MO). Reversed phase, high performance thin layer chromatography plates (HPTLC; C18 derivatized silica, 100% silanized, 250 μm thick, 10 × 10 cm) were purchased from Sigma-Aldrich. HPLC-grade MeOH, AcN, and HOAc were used wherever mentioned, and purchased from Fisher Scientific (Mississauga, Ontario). Proteinase K and egg white lysozyme were obtained from Boehringer Mannheim (Laval, Quebec), and the other enzymes from Sigma-Aldrich.

Plant material, seed effusates, and root exudates.

Seeds of white lupin, *Lupinus albus* L. 'Kievskij', were generously supplied by Satoshi Tahara, and seeds of cv. Primovsky were purchased from Pelletier (Montréal, Quebec). They were surface sterilized as described by Gagnon and Ibrahim (1997) and allowed to imbibe water for 24 h while shaking (175 rpm; G10 Gyrotary Shaker, New Brunswick Scientific, Edison, NJ). Seeds were placed on sterilized vermiculite for another 24 h, then mounted aseptically on stainless steel grids over nutrient media-containing beakers and incubated under the following conditions: 16-h light (approximately 400 μE · s⁻¹ · m⁻²) and 8-h dark, at 26/20°C and 50% relative humidity. Seed effusates and root exudates were adjusted to pH 4.5, before being extracted twice with an

equal volume of EtOAc. Seed parts were extracted first with 80%, followed by 100% MeOH, and the combined extracts were lyophilized and redissolved in a measured volume of 80% aq MeOH.

HPLC analysis of isoflavonoids, aldonic acids, and LCOs.

HPLC analyses were carried out on silica C18 columns, either semipreparative Lichrospher (Merck, Darmstadt, Germany) or analytical Nova-Pack (Waters Millipore, Gilford, MA). Data acquisition based on photodiode array detection from 200 to 400 nm and data processing were achieved with the Millennium workstation (Waters Millipore). All HPLC gradient elutions were carried out with aq MeOH for isoflavone and aldonic acid analyses, or aq AcN for LCO analyses. The UV absorbances of flavonoids and aldonic acids were monitored and calibrated at 280 and 220 nm, respectively, whereas LCOs were monitored at 206 nm. For quantification of compounds, various concentrations of standard solutions of isoflavones (in 80% aq MeOH) and of aldonic acids (in H₂O) were chromatographed, and their absorbance monitored. The eluted peaks exhibited linear signal responses (i.e., $r^2 > 0.995$) in the range of 0.5 to 40 nmol (for flavonoids) or mmol (for aldonic acids), and their extinction coefficients were determined at 280 and 220 nm, respectively. LCOs were not calibrated.

HPLC analysis of lupin seed effusates and root exudates was carried out at a flow rate of 1 ml · min⁻¹ with 45% solvent A (0.5% methanolic HOAc) in 55% solvent B (0.5% aq HOAc) for 2 min, followed by a gradient increase to 100% solvent A in 23 min (Gagnon and Ibrahim 1997). Sixty-drop fractions (approximately 1.5 ml) from HPLC eluates, correlated to precise retention times, were collected in Eppendorf tubes and lyophilized in a Speed-Vac concentrator. HPLC protocols for LCOs and aldonic acid analyses are described in their respective sections.

Growth and transformation of bacterial strains.

Yeast-mannitol (YM) liquid broth was used as a growth medium for *Rhizobium* at 28°C, and consisted of 0.4 g · liter⁻¹ yeast extract, 10 g · liter⁻¹ mannitol, 0.05% KH₂PO₄ (pH 6.8), 0.02% MgSO₄ · 7H₂O and 0.01% NaCl (wt/vol) (Vincent 1970). The wild-type *Rhizobium* strains and cosmid-harboring strains utilized in this study are listed in Table 6. The pRmM57 cosmid (*nodC::lacZ* fusion; Spec^r, Tet^r) used to study *nod* gene inducibility was generously supplied by S. Long (Stanford University, CA) in an *Escherichia coli* background (Mulligan and Long 1985). The FITA *nodD* harboring cosmid pMp2112 (Spec^r) was obtained from H. P. Spaink (Leiden University, The Netherlands) and used as a positive

Table 6. Bacterial strains and their source

Bacterial strain	Source
<i>Mesorhizobium loti</i> ^a	NZP 2235
<i>M. loti</i> /pMp2112 ^a	Lopez-Lara et al. 1995
<i>Rhizobium lupini</i>	ATCC 35173
<i>R. lupini</i> /pMp2112	This study
<i>R. lupini</i> /pRmM57	This study
<i>Sinorhizobium meliloti</i>	ATCC 9930
<i>S. meliloti</i> /pRmM57 ^b	Mulligan and Long 1985

^a Supplied by H. P. Spaink, Leiden University, Netherlands.

^b Supplied by D. A. Phillips, University of California, Davis.

control in the LCO-incorporation studies. *S. meliloti* 1021 harboring cosmid pRmM57 was kindly supplied by D. A. Phillips (University of California, Davis). *E. coli* cells containing pRmM57 were used as a source of cosmid DNA for *nodC::lacZ* fusions, and *M. loti* cells containing pMp2112 as a source of FITA *nodD* containing cosmid. All cosmid purifications were achieved by the alkaline lysis method described by Sambrook et al. (1989). The transformation of competent *R. lupini* cells was achieved by the freeze-thaw method (Hofgen and Willmitzer 1988). *Rhizobium* transformation mixtures were grown in a rescue medium (20 mM glucose, 5 mM MgCl₂, 2% bacto-tryptone, 0.5% yeast extract, 0.05% NaCl; Sambrook et al. 1989) for 6 h, and plated on a selective yeast-mannitol-agar medium containing 50 µg · ml⁻¹ spectinomycin. Resistance to tetracycline (50 µg · ml⁻¹) was also tested with pRmM57 transformants.

***nod* gene inducibility with *nodC::lacZ* fusions with *R. lupini*/pRmM57 and *S. meliloti*/pRmM57.**

The transformed strains were induced with isoflavone standards, aldonic acid standards, or HPLC-fractionated seed effusates or root exudates, and grown in YM medium for 4 h (*S. meliloti*, from OD₆₀₀ of 0.2 to 0.38), or 20 h (*R. lupini*, from OD₆₀₀ of 0.1 to 0.6). β-Galactosidase assays were carried out according to Sambrook et al. (1989), but with slight modification. Briefly, 100 µl-aliquots of these cultures were mixed with 900 µl of Z buffer (0.1 M KH₂PO₄, 0.01 M MgSO₄, 0.01 M KCl, pH 6.8), containing 30 µl of 0.5% (wt/vol) sodium dodecyl sulfate (SDS) and 30 µl of chloroform. A 200 µl-aliquot of *o*-nitrophenyl galactoside (4 mg · ml⁻¹, wt/vol; Boehringer Mannheim, Laval, QC) was added to the assay mixture prior to incubation at 28°C. At the end of the assay, 250 µl of 2.5 M sodium carbonate was added to the reaction mixture and centrifuged, and β-galactosidase activity was monitored at 420 nm. The latter values were calculated according to Sambrook et al. (1989) and expressed in Miller units.

It should be noted, however, that in contrast to erythronic acid, the pH of concentrated (approximately 1 M) standard solutions of tetronic acid is very low (approximately pH 2.9) and cannot be buffered, since it exists in either strongly acidic or basic forms (data not shown). In addition, whereas the addition of erythronic acid does not affect the pH of the growth media, tetronic acid leads to an extensive acidification, ranging from 0.1 pH unit at 10 mM to 1.2 pH unit at 15 mM concentration, with an apparent exponential shift.

Radiolabeling of Nod metabolites (LCOs), TLC and HPLC analyses, and enzyme hydrolysis.

Mid-log phase cultures grown in YM media were diluted to an OD₆₀₀ of 0.1, and 6-ml aliquots were treated for 3 h (Laeremans et al. 1996) with flavonoids and/or aldonic acids in the case of wild-type strains, or with naringenin for the pMp2112 transformants. The induced cultures were then administered either 1.25 or 2.8 µCi of [1-¹⁴C]sodium acetate, or 0.5 µCi of either D-[1-¹⁴C]glucosamine, D-[1-¹⁴C]galactosamine, D-[1-¹⁴C]mannosamine, D-[1-¹⁴C]acetylglucosamine, or D-[1-¹⁴C]acetylgalactosamine, and allowed to take up the radiolabeled precursors for 18 h (Laeremans et al. 1996). At the end of the metabolic period, the cultures were centrifuged in a clinical centrifuge (5,000 rpm, 90 min; IEC, Needham

Heights, MA), and the supernatants passed through a C18 RP Sep-Pack (Nihon Millipore, Yonezawa, Japan) pre-equilibrated with water, then washed with 10 ml of water. The labeled LCOs were eluted with 5 ml of absolute MeOH (Laeremans et al. 1996), lyophilized, and resuspended in 80% of aq MeOH before being applied onto HPTLC plates (Spaink et al. 1992; Laeremans et al. 1996). The latter were chromatographed for three successive runs in water-acetonitrile (1:1, vol/vol; Spaink et al. 1992), and autoradiographed on X-Omat AR film (Kodak, Rochester, NY), or placed in a Molecular Imager (Bio-Rad, Mississauga, Ontario). In order to assess the identity of the inducible radiolabeled LCOs, the spots were scraped off the TLC plates, and extracted twice, with 80 and 100% MeOH. The combined extracts were lyophilized and resuspended in a minimal volume of 80% aq MeOH, for either TLC or HPLC gradient analysis. The HPLC elution protocol consisted of 2 min in 15% aq AcN, followed by an increase to 40% aq AcN in 13 min, and to 100% AcN in 10 min; 750-µl fractions were collected for liquid scintillation counting.

For enzymatic hydrolysis of Nod factors, the recovered LCOs were resuspended in 30 µl of water, divided into 5-µl aliquots, and subjected to hydrolysis with one of the following enzymes: 250 U of porcine kidney acylase I, in 100 mM phosphate buffer, 120 mM NaCl, pH 7.0; 1.0 U of egg white lysozyme, in 100 mM Tris-HCl, pH 6.8; 0.1 U of chitinase from *S. marcescens*, in 50 mM citrate, 100 mM potassium phosphate, pH 6.0; 0.25 U of *N*-acetylglucosaminidase from jack beans, in 50 mM citrate, pH 5.0; and 0.2 U of proteinase K, in 10 mM Tris-HCl, pH 7.5. The enzyme reaction mixtures were incubated for 24 h at 30°C and the hydrolysates were lyophilized, resuspended in 80% aq MeOH, then subjected to HPLC (as described above), or loaded onto TLC plates for analysis. Due to the low yield of LCOs achieved with *R. lupini* (see Results), an improved extraction method was developed that involved washing the loaded Sep-Packs with 50% aq MeOH first, followed by elution with absolute MeOH to yield LCOs of a higher purity.

Aldonic acids in lupin seeds, seed effusates, and root exudates.

Cotyledons and seed coats, soaked for 24 h, were ground with a glass rod in a microfuge tube, and extracted twice with 80% aq MeOH and once with 100% MeOH. Aldonic acids were extracted with ethyl acetate from acidified seed effusates and root exudates. Ethyl acetate or methanolic extracts were lyophilized and resuspended in a minimal volume of 80% aq MeOH before being analyzed by gradient-HPLC with a semi-preparative RP-C18 column. The elution protocol consisted of 2 min in 5% methanolic phosphoric acid (pH 3), followed by an increase to 100% MeOH in 15 min, and maintained for a further 10 min. UV absorbance was recorded between 200 to 400 nm during elution, and the peak areas were integrated based on their absorbance at 220 nm. Under the optimized gradient-HPLC conditions used, erythronic and tetronic acids elute at 2.85 and 6.61 min, respectively, whereas the most polar compound of lupin isoflavones (2'-hydroxygenistein 7-*O*-glucoside) exhibits a retention time of 16.88 min. In addition, the spectral characteristics of the aldonic acids analyzed are similar to those of the standard solutions used for generating extinction coefficients of the acids. The extinction coefficients deduced from the calibration curves of erythronic and

tetronic acids, measured at 220 nm, amounted to 1.98 and 1.06 $\mu\text{mol} \cdot \text{V}^{-1} \cdot \text{s}^{-1}$, respectively.

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