Symbiosis, 19 (1995) 131–140
Balaban, Philadelphia/Rehovot

Xanthone Induction of *Nod* Gene Expression in *Bradyrhizobium japonicum*

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Received June 15, 1995; Accepted August 17, 1995

Abstract

Forty aromatic compounds were tested for their ability to induce *nod* gene expression in *Bradyrhizobium japonicum*. Of these, genistein, biochanin A, and formononetin have previously been shown to be inducers of both *nodD1-lacZ* and *nodY-lacZ* expression. In addition, two xanthones – 1,3,7-trihydroxyxanthone and 1,6-dihydroxy-2,8-dimethoxyxanthone, which were isolated from *Haploclathra* species – were found to be inducers of *nod* gene expression. Indeed, 1,6-dihydroxy-2,8-dimethoxyxanthone was as effective as genistein in inducing *nodD1*- and *nodY-lacZ* expression. These xanthone molecules were capable of inducing *nod* gene expression in *B. japonicum* mutant strain Δ1267, lacking the *nodD1*, *nodD2*, and *nolA* genes. These results suggest that the xanthone inducers are capable of activating the NodV/W two-component regulatory system known to be essential for *nod* gene expression in *B. japonicum*.

Keywords: *Bradyrhizobium*, nodulation gene induction, xanthones, isoflavones

Presented at the 10th International Congress on Nitrogen Fixation, May 28 – June 3, 1995, St. Petersburg, Russia

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1. Introduction

Nodulation of leguminous plants by *Rhizobium*, *Azorhizobium*, and *Bradyrhizobium* requires the *nod* gene protein products. Some of the *nod* genes encode enzymes that biosynthesize lipo-chitin nodulation signals that elicit *de novo* nodule formation on host roots (Carlson et al., 1994). Transcription of the *nod* genes requires the activity of the positive regulator, NodD, and the presence of specific flavonoid molecules. The NodD of a particular *Rhizobium* species is adapted to recognize the flavonoids produced by the compatible host species. Indeed, flavonoid specificity and nodulation host range can be changed by conjugation of the *nodD* gene from one *Rhizobium* species into another species (Spaink et al., 1987; Bender et al., 1988).

Although nodulation specificity is determined in part by NodD-flavonoid interaction, a surprising variety of additional compounds have been shown to act as inducers (Le Strange et al., 1990; Maxwell et al., 1989; Peters and Long, 1988). For example, anthocyanin compounds extracted from bean seed coats are important inducers of *nod* gene expression in strains of *Rhizobium leguminosarum* bv. phaseoli (Hungria et al., 1991). Surprisingly, the betaines, trigonelline, and stachydrine, have been shown to induce *nod* gene expression in *R. meliloti* (Phillips et al., 1992). *R. meliloti* possesses three separate *nodD* genes. Trigonelline and stachydrine appear to activate *nod* gene expression specifically through the action of $NodD_2$.

In *B. japonicum*, the inducers of *nod* gene expression produced by soybean roots are the isoflavones: genistein, daidzein, and the corresponding glycosides (Kosslak et al., 1987; Göttfert et al., 1988; Banfalvi et al., 1988; Smit et al., 1992). Previously, we screened over 1,000 compounds for their ability to act as inducers or as inhibitors of isoflavone-mediated *nodY-lacZ* induction in *B. japonicum* (Cunningham et al., 1991). The compounds tested included representatives of coumarins, benzoic acid analogs, benzo-phenones, chalcones, aurones, isoflavones, and flavonoids (i.e., flavones, flavonols, flavanones, and dihydroflavonols). Each of these chemical classes was found to contain molecules that act as either an inducer and/or inhibitor of *nod* gene expression.

Since these original experiments, we made the surprising discovery that the $nodD_1$ and $nodD_2$ genes of *B. japonicum* are dispensable for *nod* gene expression (Sanjuan et al., 1994). In a mutant strain (Δ1267) lacking $nodD_1$, $nodD_2$, and *nolA* (*nolA* encodes a repressor protein; Dockendorff et al., 1994), *nod* gene expression can be activated through the action of NodW. NodV and NodW are members or the two-component regulatory protein family (Göttfert et al., 1990).

In this study, we screened 40 aromatic compounds for their ability to induce *nod* gene expression in *B. japonicum*. The results obtained add xanthone molecules to the family of chemicals known to induce both $nodD_1$- and *nodY*-

lacZ expression in *B. japonicum*. Interestingly, xanthones induce *nod* gene expression in mutant strain Δ1267 suggesting that these molecules can activate the NodV/W regulatory system.

2. Materials and Methods

Bacteria and plasmids

Bradyrhizobium japonicum strain ZB977 carries plasmid pZB32 containing a *nodY::lacZ* fusion (Banfalvi et al., 1988). Strain 573 carries a chromosomally integrated *nodC::lacZ* fusion (Göttfert et al., 1992). Strain ZB977 and 573 were derived from wildtype strain USDA110. *B. japonicum* strain LB101 was derived from wildtype stain USDA135 and was used because of the higher expression of $nodD_1$ in this strain, relative to strain USDA110 (Banfalvi et al., 1988). All of these strains are resistant to tetracycline. Mutant strain Δ1267 carries a deletion that removes the $nodD_1$, $nodD_2$, and *nolA* genes, as well as some additional DNA (Dockendorff et al., 1994). Strain Δ1267 contains plasmid pZB32 (i.e., *nodY::lacZ* fusion) and is resistant to kanamycin, spectinomycin, and tetracycline.

Media and chemicals

All strains were maintained on RDY (Rhizobium Defined Yeast Medium) agar and grown in RDY broth with addition of the suitable antibiotics at 100 μg/ml. RDY consists of 1 g/L yeast extract, 0.12 g/l KH_2PO_4, 0.12 g/l K_2HPO_4, 0.1 g/l $MgSO_4$, 1 ml/l of 1,000-fold concentrated trace element solution, 1 g/l sodium L-glutamate, and 5 g/l sodium D-gluconate, pH = 7.0 (So et al., 1987). Cells were induced for *nod* gene expression in MM (Minimal Medium) (Bergersen, 1961). MM consists of 0.3 g/l KH_2PO_4, 0.3 g/l K_2HPO_4, 0.1 g/l $MgSO_4 \cdot 7H_2O$, 1 ml/l of 1,000-fold concentrated trace element solution, 0.5 g/l NH_4NO_3, 0.4% glycerol, 5.8 g/l MOPS, 0.002 g/l biotin, 0.001 g/l thiamine, and 0.001 g/l calcium pantothenate added, pH = 7.0. Genistein was obtained from ICN Biomedicals (Cleveland, Ohio).

All xanthone samples tested in this work were isolated from leaves, wood, and roots of shrubs and trees collected in the Brazilian rain forest that belong to several different plant genera including *Kielmeyra*, *Haploclathra*, and *Vismia* species. These genera all belong to the Guttiferae family. The isolation procedure was described by Nagem et al. (1988a, 1988b, and 1989). The flavonoid samples were isolated from seeds of different cultivars of soybean (*Glycine max*) according to Nagem et al. (1993).

Nod gene induction assays

Cells were grown in RDY broth to log-phase (i.e., OD_{600nm} = 0.2 to 0.8). The cultures were diluted to 2 ml with MM to OD_{600nm} = 0.025. The 40 compounds tested, as well as genistein, were dissolved in ethanol and stored at 4°C until use. Each compound was added into the induction medium at a suitable concentration of 1–5 μM. As a control, an identical volume of ethanol was added (as "no addition"). Induction cultures were incubated at 28-30°C with shaking at 200 rpm for 10-14 hours. The level of *nod* gene expression was determined by the level of β-galactosidase activity as previously described (Banfalvi et al., 1988) using 0.5 ml of each culture. The substrate used was chlorophenol red-β-D-galactopyranoside (CPRG).

3. Results

Survey of 40 aromatic compounds for their ability to induce nodD_1- or nodY-lacZ expression

The 40 chemicals shown in Table 1 were tested at a concentration of 1 μM for their ability to induce *nod* gene expression in strains ZB977, 573, and LB101. In addition to the positive control genistein, 1,6-dihydroxy-2,8-dimethoxy-xanthone (compound 19), 1,3,7-trihydroxyxanthone (compound 26), biochanin A (compound 29), and formononetin (compound 39) were able to induce significant *nod* gene expression in all strains examined (Table 1). The relative levels of expression by all four compounds were approximately the same in these three strains. The structures of the various molecules tested are shown in Fig. 1.

Xanthones induce nod gene expression in strain Δ1267

Previously, we reported that *nod* gene induction in strain Δ1267 was dependent on the presence of an active NodW. This protein is a member of the two-component regulatory protein family (Göttfert et al., 1990). We postulated that NodW, in conjunction with NodV (the response-regulator), responded to isoflavones and activated expression of the *nod* genes. As shown in Table 1, 1,6-dihydroxy-2,8-dimethoxyxanthone, 1,3,7-trihydroxyxanthone, biochanin A, formononetin, and genistein induce *nodY-lacZ* expression in strain Δ1267. These data suggest that the NodV/W regulatory system recognizes these chemicals.

Table 1. *nodY::lacZ* expression of *Bradyrhizobium japonicum* USDA110 strain ZB977, *nodC::lacZ* expression of *B. j.* USDA110 strain 573, *nodY::lacZ* expression of *B. j.* USDA110 strain Δ1267, and *nodD*$_1$*::lacZ* expression of *B. j.* USDA135 strain LB101 in the presence of 1 μM of compounds isolated from plants collected in Brazilian rain forest.

No.	Compounds		β-galactosidase activity (U)[a]			
		M.W.	ZB977	573	Δ1267	LB101
1	2-Hydroxyxanthone	212	3	1	1	2
2	3-Hydroxy-1,5,6-trimethoxyxanthone	302	3	1	2	1
3	1,3-Dimethoxyxanthone	256	3	1	1	1
4	4-Hydroxyxanthone	212	3	1	1	1
5	1,3-Dihydroxy-5,6-dimethoxyxanthone	288	3	1	1	1
6	4-Hydroxy-2,3-dimethoxyxanthone	272	2	1	1	1
7	2,8-Dihydroxy-1-methoxyxanthone	258	8	1	5	2
8	2,6-Dihydroxy-1,8-dimethoxyxanthone	288	3	1	2	1
9	2,8-Dihydroxy-1,6-dimethoxyxanthone	288	3	1	2	1
10	3-Hydroxy-2,4-dimethoxyxanthone	272	3	1	2	1
11	1-Hydroxy-7,8-dimethoxyxanthone	272	9	1	12	3
12	2,3-Dimethoxyxanthone	256	2	1	2	1
13	1,3,5-Trimethoxyxanthone	286	3	1	2	1
14	3-Hydroxyxanthone	212	2	1	2	1
15	1,5-Dihydroxy-3-methoxyxanthone	258	2	1	2	1
16	2,3,4-Trimethoxyxanthone	286	2	1	2	1
17	1,8-Dimethoxyxanthone	256	2	1	2	1
18	5,6-Dihydroxy-1,3-dimethoxyxanthone	288	2	1	4	1
19	1,6-Dihydroxy-2,8-dimethoxyxanthone	288	1840	398	1651	67
20	4-Methoxyxanthone	226	6	1	N.D.	3
21	2-Methoxyxanthone	226	7	1	1	2
22	1,8-Dihydroxyxanthone	228	2	1	1	1
23	1,7-Dihydroxyxanthone	228	3	1	2	1
24	1-Hydroxyxanthone	212	2	1	2	1
25	8-Hydroxy-1,2-dimethoxyxanthone	272	3	1	2	1
26	1,3,7-Trihydroxyxanthone	244	524	140	523	9
27	2,3-Dihydroxy-4-methoxyxanthone	258	2	1	2	1
28	2,5-Dihydroxy-1-methoxyxanthone	258	3	1	2	2
29	Biochanin A	284	2121	604	1918	46
30	Quercetin	302	2	1	2	1
31	Morin	302	2	1	2	2
32	Acetilated Rutin	792	3	1	3	2
33	Acetilated Morin	512	2	1	1	1
34	Acetilated Naringenin	398	2	1	3	1
35	Naringenin	272	5	1	5	2
36	Quercetrin	448	2	1	3	1
37	Kaempherol	286	4	1	4	2
38	Isoquercetrin	464	2	1	3	2
39	Formononetin	268	1042	48	1479	18
40	Modified xanthone (nucleus)	196	2	1	3	1
	Genistein	270	2270	866	2885	85
	No addition		2	1	2	1

[a]Values of β-galactosidase activity are the average of two or more assays with a deviation less than or equal to 20%.

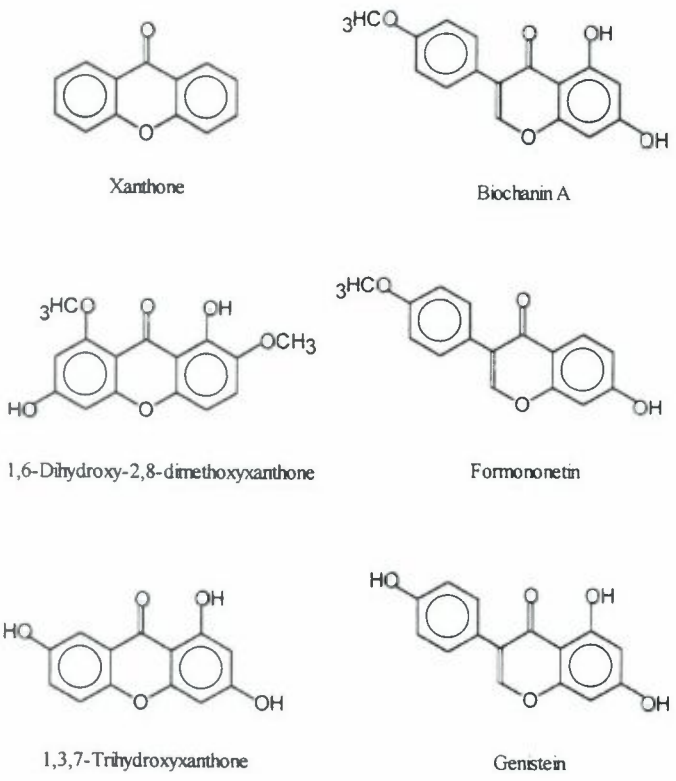

Figure 1. Structure of xanthone and tested compounds which were found to be able to induce *nod* gene expression.

Relative effectiveness of xanthones as inducers of nod gene expression

In order to gauge the effectiveness of 1,6-dihydroxy-2,8-dimethoxyxanthone and 1,3,7-trihydroxyxanthone as *nod* gene inducers, these compounds were tested at concentrations ranging from 0.005 to 5 µM (Fig. 2). For comparison, the known *nod* gene inducers genistein and formononetin were also tested at the same concentrations. Genistein is the primary, natural inducer of *nod* gene expression while formononetin has been previously shown to be a weaker inducer (Banfalvi et al., 1988; Smit et al., 1992). As shown in Fig. 2, 1,3,7-trihydroxyxanthone and formononetin showed a similar pattern of *nod* gene induction. The level of expression was maximal at 18-fold and 25-fold, respectively, at a level of 2 µM. Surprisingly, 1,6-dihydroxy-2,8-dimethoxy-xanthone induced a high level of *nodY-lacZ* expression at relatively low concentration (i.e., 6-, 18-, and 40-fold at 0.005, 0.01, and 0.025 µM, respect-

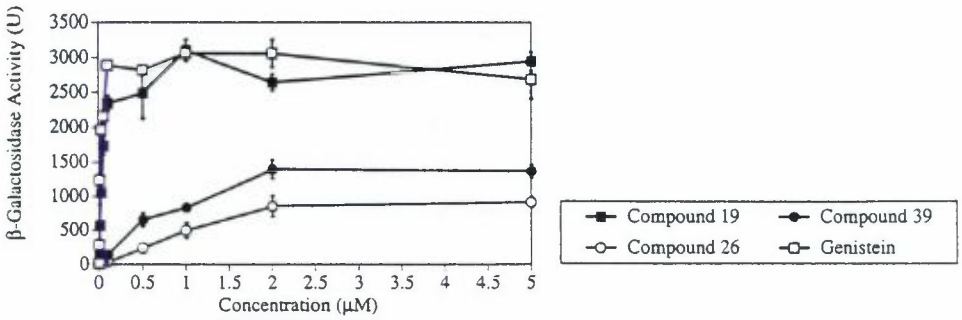

Figure 2. *nodY::lacZ* expression of *Bradyrhizobium japonicum* USDA110 strain ZB977 in the presence of compound 19 (1,6-dihydroxy-2,8-dimethoxyxanthone), compound 26 (1,3,7-trihydroxyxanthone), compound 39 (formononetin), and genistein at different concentrations. (The values of β-galactosidase activity are the average of two assays.)

ively). As a comparison, genistein induced 12-, 62-, and 98-fold expression at equivalent concentrations. β-Galactosidase expression in response to either 1,6-dihydroxy-2,8-dimethoxyxanthone or genistein leveled off at approximately 0.025 μM and was maximal at 1 μM. Thus, xanthone molecules can be very effective inducers of *nod* gene expression in *B. japonicum*.

4. Discussion

The repertoire of reported chemical compounds that induce *nod* gene expression in various rhizobial species now includes representatives of flavonoids, coumarins, chalcones, benzoic acid analogs, betaines, aurones, benzophenones, anthocyanins and xanthones (Bender et al., 1990; Cunningham et al., 1991; Hungria et al., 1991; Maxwell et al., 1989; Peters and Long, 1988). This is an amazing list of structurally diverse compounds. Xanthones are secondary metabolites which are C_{13} polyphenolic compounds normally produced in the plants of Guttiferae family. Their hydroxylation patterns are similar to the C_{15} flavonoids. However, their occurrence is not as common as flavonoids (Harborne and Simmonds, 1962).

It is generally thought that the NodD protein recognizes the specific chemical inducer, and this recognition is essential for activation of *nod* gene transcription. However, a direct interaction of NodD with an inducer molecule has not been shown. The best evidence in favor of a direct interaction is data

showing that a change in the primary sequence of NodD leads to a concomitant change in inducer specificity (Mclver et al., 1989).

An initial goal of our earlier study (Cunningham et al., 1991) on inducer specificity in *B. japonicum* was to define the important structural features necessary for a molecule to be an active inducer. Unfortunately, the data obtained did not allow such conclusions and, indeed, there appeared to be little consistent similarity between the various inducer or inhibitor molecules. One possible explanation for the variety of inducer molecules is that *B. japonicum*, as well as some *Rhizobium* species, possesses two *nodD* genes. In the case of *R. meliloti*, Phillips and colleagues (1992) have shown that the three NodDs in this species have different inducer specificities. For example, the betaines act primarily via $NodD_2$. In the case of *B. japonicum*, $nodD_2$ does not appear to be critical for *nod* gene induction (Göttfert et al., 1992) in that only mutations in $nodD_1$ affect *nod* gene expression. However, such experiments have not been done with a wide variety of inducer compounds.

An apparently unique feature of *nod* gene induction in *B. japonicum* is the involvement of the NodV/W two-component regulatory system. The action of this regulatory pathway, in conjunction with $NodD_1$ and $NodD_2$, may further explain the wide range of inducer molecules recognized by *B. japonicum*. Present data would suggest that the NodV/W system can recognize isoflavones and xanthones as inducer molecules.

A goal of rhizobial research is the development of superior inoculant strains for increased crop production. However, such inoculant strains usually compete poorly against indigenous soil rhizobia. Therefore, a chemical means to modify strain-strain competition would show considerable promise for increased legume production. In our previous study (Cunningham et al., 1991), we showed that the addition of inhibitors of *nod* gene expression could be used to modify interstrain competition for nodulation. However, one problem with this approach was the observation that different *B. japonicum* strains responded to different inhibitors; that is, no single inhibitor compound was found to have broad efficacy. This study was performed before the full complexity of *nod* gene regulation in *B. japonicum* was apparent. Further information concerning the range of inducer compounds recognized by *B. japonicum* and a full understanding of the molecular basis of *nod* gene regulation may eventually lead to the practical application of these chemical strategies for increased agricultural productivity.

Acknowledgements

This work was supported by USDA grant 92-37305-7814 (to GS).

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