J. Microbiol. Biotechnol. (2005), **15**(6), 1280–1285

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Comparative Analyses of Flavonoids for *nod* Gene Induction in *Bradyrhizobium japonicum* USDA110

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Received: March 5, 2005
Accepted: April 31, 2005

Abstract Using the *nodY::lacZ* fusion system in *Bradyrhizobium japonicum* USDA110, 22 flavonoids, which have structurally different features, were tested to define the role of the substituted functional groups as an inducer or inhibitor for the *nod* gene expression. A functional group of 4'-OH on the B-ring and the double bond between 2-C and 3-C on the C ring were required to induce the *nod* gene expression in *B. japonicum* USDA110. In the case of isoflavones, the 4'-methoxyl group, which blocks the open 4'-OH functional group, did not significantly lower inducing activity, as compared with isoflavones with 4'-OH. However, all flavonols tested, which have a 3-OH functional group on the C-ring, did not induce, but inhibited the *nod* gene expression. Flavone, 7-hydroxyflavone, and kaempferol (5,7,4'-trihydroxyflavonol) at 1 μM concentration significantly inhibited the *nod* gene expression induced by 7,4'-dihydroxyflavone. However, 7-hydroxy-4'-methoxyflavone at 1 μM concentration showed a synergistic effect with genistein and 7,4'-dihydroxyflavone on the induction activity.

Key words: Nodulation, *Bradyrhizobium*, isoflavonoid, flavonoid, induction

Rhizobia can make nitrogen-fixing nodules on the host plants in the sequential consequences of chemical communication between both partners [7]. The mutual interactions between the symbiotic partners determine the early stage of nodulation processes prior to infection, which are fairly specific [3, 4, 13, 18, 23]. The chemical compounds responsible for the early stage signals to rhizobia have been known to be flavonoids [14, 18, 19] which are synthesized through the phenylpropanoid pathway present in the host plants. Once released into the root zone, flavonoids serve as chemoattractants and regulate expression of nodulation (*nod*) genes of symbiotic rhizobia at the transcriptional level [14, 16, 21]. For the expression of *nod* genes, regulatory protein NodD, which is constitutively expressed in the rhizobia [13], is necessary to control the expression of the inducible *nod* genes via interaction with the *cis*-acting promoter element *nod* box. When NodD recognizes appropriate flavonoids on its C-terminal residue, deformation of the DNA helix in the vicinity of the *nod* box occurs, and transcription of adjacent *nod* genes starts [5, 9, 16]. As consequences of the expression of *nod* genes, vast biochemical reactions, including synthesis of Nod factors, are carried out for the formation of nodules on the host plants [9, 11, 17].

Since Redmond *et al.* [18] elucidated that flavones induce the expression of nodulation genes in *Rhizobium*, an extensive variety of additional compounds such as anthocyanin derivatives, betaines, trigonelline, and stachydrine have been revealed as inducers [1, 8, 15]. In addition, isoflavones, genistein, daidzein, and their glycoside derivatives have been found to work as *nod* gene inducers in *Bradyrhizobium japonicum* [1, 9, 22]. Using a *nodY::lacZ* fusion system in *B. japonicum*, Cunningham *et al.* [2] tested over 1,000 compounds for their induction or inhibition activity to regulate *nod* gene expression. The compounds tested included representatives of coumarines, benzoic acids, benzophenones, chalcones, aurones, xanthones, isoflavones, and flavones. Although extensive studies revealed that a rather broad range of derivates of plant exudates influence induction or inhibition of *nod* gene expression in the corresponding host bacteria, there is little information available about the role of a chemical functional group(s) in the flavonoids for the *nod* gene induction. In this study, 22 representative flavonoids, which contain a hydroxyl or methoxyl group(s) differently located on the carbon skeleton

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of the compounds, were selected to test their induction activity, using a *nodY::lacZ* fusion system in *B. japonicum* USDA110.

MATERIALS AND METHODS

The bacterial strain was *B. japonicum* USDA110 (pZB32) [1], which has the *nod*-box and *nodY* gene fused with the *lacZ* gene on the plasmid. All the flavonoids were purchased from Indofine Chemical Co. (Somerville, NJ, U.S.A.). The *nod* gene induction activity of 22 flavonoids (Table 1) were determined by β-galactosidase assay, which has previously been described [13]. The flavonoid stock solutions were dissolved in methanol at a concentration of 1 mM. *B. japonicum* USDA110 (pZB32) was grown on AG medium [20] containing 30 μg/ml tetracycline. For the assay, the initial turbitiy of the culture was adjusted to $O.D._{600}$=0.05 in 5 ml of AG medium containing 2 μM flavonoids or solvents as controls. The bacterial cultures were incubated for 24 h at 27°C with shaking at 200 rpm. Assays were performed at 27°C, and replicated more than twice. To examine the synergistic or inhibitory effect of flavonoids, 1 μM each of genistein, 7,4'-dihydroxyflavone, flavone,. 7-hydroxyflavone, 7,4'-dihydroxyflavonol, apigenin (5,7,4'-trihydroxyflavone), kaempferol (5,7,4'-trihydroxyflavonol), and 7-hydroxy-4'-methoxyflavone was added to the culture medium of *B. japonicum* USDA110 (pZB32) containing either 1 μM of genistein or 7,4'-dihydroxyflavone.

RESULTS AND DISCUSSION

Current study was undertaken to define the important features of the functional groups necessary for the flavonoids to be an active inducer or inhibitor to express the *nod* gene expression in *B. japonicum* USDA110. Table 1 shows the effect of 22 representative flavonoids at 2 μM concentration on the induction of *nod* gene expression. Isoflavones with a 4'-OH functional group on the B-ring, genistein and daidzein, showed strong induction activity, as previously reported [2]. Comparing these two isoflavones, genistein with an additional 5-OH functional group on the A-ring induced the *nod* gene expression more significantly than daidzein with its additional 7-OH functional group on the A-ring. Formononetin and biochanin A, in which the 4'-OH functional group is blocked with a methyl functional group, showed relatively weak induction activity, compared with those of genistein and daidzein. Flavones with both 4'-OH functional groups on the B-ring induced *nod* gene expression. 4'-Hydroxyflavone showed strong induction activity, comparable to genistein. Interestingly, the hydroxyl functional group on the A-ring of the flavones had an effect to lower the induction activity. In addition, multiple hydroxyl functional groups on the A-ring of apigenin (5,7,4'-trihydroxyflavone) significantly lowered the induction activity, compared with 5,4'-, 6,4'-, and 7,4'-dihydroxyflavones. Surprisingly, flavonols 7,4'-dihydroxyflavonol, kaempferol (5,7,4'-trihydroxyflavonol), fisetin (7,3',4'-trihydroxyflavonol), and quercetin (5,7,3',4'-tetrahydroxyflavonol), which have both 4'-OH and 3-OH functional groups on the rings, did not show the *nod* gene induction activity. Blocking of the 4'-OH functional group of flavones with the methyl group, such as 7-hydroxy-4'-methoxyflavone, acacetin (5,7-dihydroxy-4'-methoxyflavone), and kaempferide (5,7-dihydroxy-4'-methoxyflavonol), dramatically lowered the *nod* gene induction activity. Flavones with no 4'-OH functional group, such as flavone, 5-hydroxyflavone, and 7-hydroxyflavone, did not induce *nod* gene expression. The hydroxyl group located on 2'- or 3'-C of the flavone on the B-ring, instead of 4'-C, could not induce *nod* gene expression. In addition, naringenin (5,7,4'-trihydroxyflavanone), which has a single bond between 2-C and 3-C on the C-ring, did not induce *nod* gene expression, although it contains a 4'-OH functional group.

Taken together, a general rule for the flavonoids to act as an effective inducing compound to express the *nod* genes in *B. japonicum* USDA110 could be summarized as follows: (1) the double bond between 2-C and 3-C on the C-ring of (iso)flavones should be present; (2) the 4'-OH functional group is required; (3) the 3-OH functional group should not be present; and (4) in the case of flavones, the 4'-OH functional group should not be blocked with the methyl group (Fig. 1). However, this contention is not applied for the induction of the *nodA* promoter of *Rhizobium legminosarum* Sym plasmid pRL1JI [23]. As the (iso)flavones induced *nod* genes in *B. japonicum* USDA110, some of them could induce the *nodA* promoter. The known (iso)flavones able to induce the *nodA* promoter are flavanones (naringenin and eriodictyol), flavones (apigenin, luteolin, and 7-hydroxyflavone), and flavonol (kaempferol). However, isoflavone genistein did not act as an inducing compound for the *nodA* promoter. The general structural features required to induce the *nodA* promoter of *Rhizobium legminosarum*

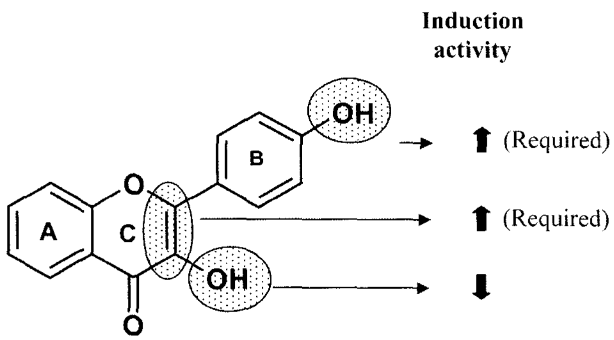


Fig. 1. Effect of the functional group of flavonoids on the induction of *nod* genes in *Bradyrhizobium japonicum* USDA110.

Table 1. β-Galactosidase activity of the *nodY*::*lacZ* fusion gene induced by (iso)flavones, flavonols, and flavanone at 2 μM concentration.

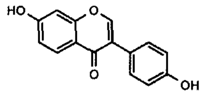
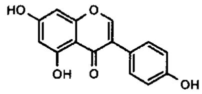
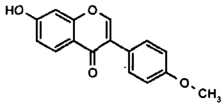
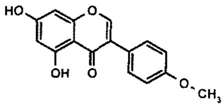
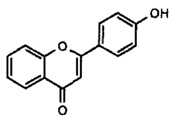
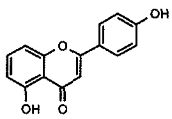
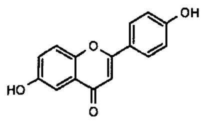
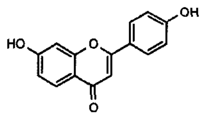
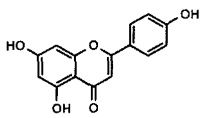
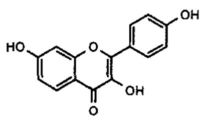
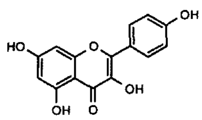
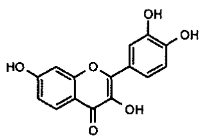
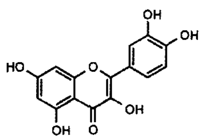
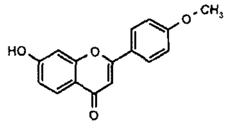
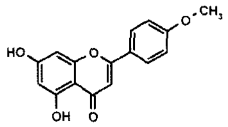
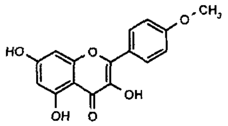
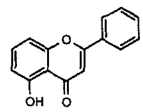
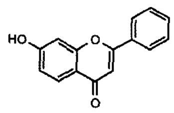
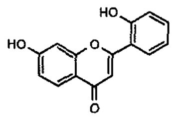
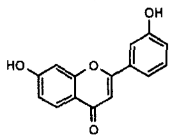
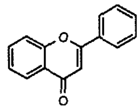
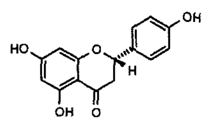
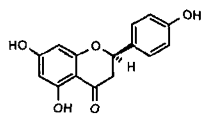
Flavonoids[a]	Chemical structure	Unit of β-galactosidase activity[b]
1) *No Flavonoids*	-	8.6±0.5
2) *Isoflavones with 4'-OH*		
Daidzein (7,4'-dihydroxyisoflavone)		121.0±3.0
Genistein (5,7,4'-trihydroxyisoflavone)		256.8±39.0
3) *Isoflavones with 4'-OCH$_3$*		
Formononetin (7-hydroxy-4'-methoxyisoflavone)		110.7±24.8
Biochanin A (5,7-dihydroxy-4'-methoxyisoflavone)		196.4±14.4
4) *Flavones with 4'-OH*		
4'-Hydroxyflavone		200.1±10.9
5,4'-Dihydroxyflavone		151.3±32.9
6,4'-Dihydoxyflavone		160.0±26.9
7,4'-Dihydroxyflavone		168.0±35.5
Apigenin (5,7,4'-trihydroxyflavone)		86.3±17.5
5) *Flavonol with 4'-OH*		
7,4'-Trihydroxyflavonol		9.2±1.4
Kaempferol (5,7,4'-trihydroxyflavone)		12.9±5.3
Fisetin (3,7,3',4'-trihydroxyflavonol)		11.6±3.0
Quercetin (5,7,3',4'-Tetrahydroxyflavonol)		8.7±0.4

Table 1. Continued.

Flavonoids[a]	Chemical structure	Unit of β-galactosidase activity[b]
6) *Flavones and flavonol with 4'-OCH$_3$*		
7-Hydroxy-4'-methoxyflavone		8.9±1.4
Acacetin (5,7-dihydroxy-4'methoxyflavone)		8.5±0.2
Kaempferide (5,7-dihydroxy-4'-methoxyflavonol)		15.9±2.0
7) *Flavones with no 4'-OH*		
5-Hydroxyflavone		3.5±0.4
7-Hydroxyflavone		8.4±0.4
7,2'-Dihydroxyflavone		5.2±0.7
7,3'-Dihydroxyflavone		6.6±0.7
8) *Flavone*		
Flavone		10.2±3.2
9) *Flavanone*		
(*R*) and (*S*)-Naringenin mixture (5,7,4'-Trihydroxyflavanone)	 and 	12.4±1.4

[a]Final concentration of each chemical was 2 μM.
[b]The activity is expressed as the Miller unit. Values are means and standard deviations of duplicated or more assays.

are likely to have flavone or flavanone structures with the 4'-OH functional group.

In order to examine the role of flavones as an inhibitor of *nod* gene expression, certain flavones, which did not increase *nod* gene expression (Table 1), were added at 1 μM concentration to *B. japonicum* USDA110 broth culture containing a relatively strong inducer, 1 μM genistein. The *nod* gene expression was likely to be saturated at 1 μM genistein concentration, because the level of β-galactosidases activity was almost the same at both 1 and 2 μM concentration (data not shown). Smit *et al.* [22] reported the same pattern of induction in which β-galactosidase activity was

Table 2. β-Galactosidase activity of the *nod*Y::*lac*Z fusion gene induced by combinations of 7,4'-dihydroxyflavone and selected flavones or flavonols.

7,4'-Dihydroxyflavone+ Selected flavones or flavonols[a]	Unit of β-galactosidase activity[b]
No Flavones	8.5±1.1
7,4'-Dihydroxyflavone	85.6±42.0[c] 168.0±35.5[d]
Flavone	11.5±2.1
7-Hydroxyflavone	22.5±15.7
7,4'-Dihydroxyflavonol	81.7±18.5
Apigenin (5,7,4'-trihydroxyflavone)	125.3±17.5
Kaempferol (5,7,4'-trihydroxyflavonol)	40.6±12.7
7-Hydroxy-4'-methoxyflavone	103.9±44.3

[a]7,4'-Dihydroxyflavone and selected flavone were added to media at a concentration of 1 μM, respectively. The combined final concentration of 7,4'-dihydroxyflavone and selected flavone was 2 μM.
[b]The activity is expressed as the Miller unit. Values are means and standard deviations of duplicated or more assays.
[c,d]β-Galactosidase activities at the concentration of 1 μM and 2 μM of 7,4'-dihydroxyflavone, respectively.

saturated at around 1 μM of genistein, using the *nodY::lacZ* system in *B. japonicum* USDA135. Flavone and kaempferol (5,7,4'-trihydroxyflavonol), which did not induce *nod* gene expression, weakly decreased the level of the *nod* gene expression induced by 1 μM genistein. However, non-inducing compounds, such as 7-hydroxyflavone and 7,4'-dihydroxyflavonol, did not affect the induction activity of genistein. Interestingly, 7-hydroxy-4'-methoxyflavone showed a slightly synergistic effect with genistein to increase the induction level of *nod* gene expression, although it itself did not increase *nod* gene expression (data not shown).

Table 2 shows the result of the inhibitory effect of flavones on *nod* gene expression, when 7,4'-dihydroxyflavone was used instead of genistein. 7,4'-Dihydroxyflavone induced stoichiometric expression of the *nod* gene as the concentration of the compound increased from 1 μM to 2 μM. However, flavone, 7-hydroxyflavone, and kaempferol (3,5,7,4'-tetrahydroxyflavone) significantly inhibited the induction effect of 7,4'-dihydroxyflavone on *nod* gene expression by 8-, 4-, and 2-fold, respectively. 3,7,4'-Trihydroxyflavone did not affect the *nod* gene expression induced by 7,4'-dihydroxyflavone. Induction activity by 7,4'-dihydroxyflavone on *nod* gene expression slightly increased with the addition of 7,3'-dihydroxyflavone, apigenin (5,7,4'-trihydroxyflavone), and 7-hydroxy-4'-methoxyflavone. With 1 μM genistein, there was no significant influence of other (iso)flavones tested on the *nod* gene expression in *B. japonicum* USDA110. This is likely to be due to a competitive effect of genistein against the (iso)flavones tested in binding the *nodD* gene product or an uptake system [3, 4, 21]. However, the inhibitory effect of other selected flavonoids on the *nod* gene induction activity was observed with 7,4'-dihydroxyflavone at 1 μM concentration. The inhibitory activity decreased in the following order: flavone>7-hydroxyflavone>kaempferol (5,7,4'-trihydroxyflavonol)>7,4'-trihydroxyflavonol. In general, the inhibitory activity was shown with flavones that have no 4'-OH functional group and/or have the 3-OH functional group. A similar result was also reported by Cunningham *et al.* [4], who suggested inhibitory activity with flavonol (3-OH), flavones (no 4'-OH), and flavanones (no double bond in 2-C and 3-C) in decreasing order.

In conclusion, using the *nodY::lacZ* expression system in *B. japonicum*, we were able to define certain functional features for the flavonoids to act as inducing or inhibitory compounds. This information could contribute to understanding rhizobia-soybean interaction and strain-strain competition as a way to increase legume production [4, 9].

Acknowledgments

We thank Professor G. Stacey at the University of Tennessee for providing the *B. japonicum* USDA110 (pZB32). This work was supported by a grant (No. R08-2003-000-10199-0) from the Basic Research Program of the Korea Science & Engineering Foundation.

REFERENCES

1. Banfalvi, Z., A. Nieuwkoop, M. Schell, L. Besl, and G. Stacey. 1988. Regulation of *nod* gene expression in *Bradyrhizobium japonicum*. *Mol. Gen. Genet.* **214:** 420–424.
2. Cunningham, S., W. D. Kollmeyer, and G. Stacey. 1991. Chemical control of interstrain competition for soybean nodulation by *Bradyrhizobium japonicum*. *Appl. Environ. Microbiol.* **57:** 1886–1892.
3. Djordjevic, M. A., J. W. Redmond, M. Batley, and B. G. Rolfe. 1987. Clovers secrete specific phenolic compounds which either stimulate or repress *nod* gene expression in *Rhizobium trifolii*. *EMBO J.* **6:** 1173–1179.
4. Firmin, J. L., K. E. Wilson, L. Rossen, and A. W. B. Johnston. 1986. Flavonoid activation of nodulation genes in *Rhizobium* reversed by other compounds present in plants. *Nature* **324:** 90–92.
5. Fisher, R. F. and S. R. Long. 1992. *Rhizobium*-plant signal exchange. *Nature* **357:** 655–660.
6. Hartwig, U. A., C. A. Maxwell, C. M. Joseph, and D. A. Phillips. 1990. Effects of alfalfa *nod* gene-inducing flavonoids on *nodABC* transcription in *Rhizobium meliloti* strains containing different *nodD* genes. *J. Bacteriol.* **172:** 2769–2773.
7. Hartwig, U. A., C. A. Maxwell, and D. A. Phillips. 1991. Flavonoids released naturally from alfalfa seeds enhance growth rate of *Rhizobium meliloti*. *Plant. Physiol.* **95:** 797–803.
8. Hungria, M., C. M. Joseph, and D. A. Phillips. 1991. Anthocyanidins and flavonols, major *nod* gene inducers from

seeds of back-seeded common bean (*Phaseolus vulgaris* L.). *Plant Physiol.* **97:** 751–758.

9. Kobayashi, H., Y. Naciri-Graven, W. J. Broughton, and X. Perret. 2004. Flavonoids induce temporal shifts in gene-expression of nod-box controlled loci in *Rhizobium* sp. NGR234. *Mol. Microbiol.* **51:** 335–347.
10. Kosslak, R. M., R. S. Joshi, B. A. Bowen, H. E. Paaren, and E. R. Appelbaum. 1990. Strain-specific inhibition of *nod* gene induction in *Bradyrhizobium japonicum* by flavonoid compounds. *Appl. Environ. Microbiol.* **56:** 1333–1341.
11. Machado, D. and H. B. Krishnan. 2003. *nodD* alleles of *Sinorhizobium fredii* USDA191 differentially influence soybean nodulation, *nodC* expression, and production of exopolysaccharides. *Curr. Microbiol.* **47:** 134–137.
12. Miller, J. 1972. *Experiments in Molecular Genetics*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, U.S.A.
13. Mulligan, J. T. and S. R. Long. 1985. Induction of *Rhizobium meliloti nodC* expression by plant exudate requires *nodD*. *Proc. Natl. Acad. Sci. USA* **82:** 6609–6613.
14. Peters, N. K., J. W. Frost, and S. R. Long. 1986. A plant flavone, luteolin, induces expression of *Rhizobium meliloti* nodulation genes. *Science* **233:** 977–980.
15. Phillips, D. A., C. M. Joseph, and C. A. Maxwell. 1992. Trigonelline and stachydrine released from alfalfa seeds activate NodD2 protein in *Rhizobium meliloti*. *Plant Physiol.* **99:** 1526–1531.
16. Pueppke, S. G. 1996. The genetic and biochemical basis for nodulation of legumes by rhizobia. *Crit. Rev. Biotechnol.* **16:** 1–51.
17. Pueppke, S. G., M. C. Bolanos-Vasquez, D. Werner, M.-P. Bec-Ferte, J.-C. Prome, and H. B. Krishnan. 1998. Release of flavonoids by the soybean cultivars McCall and Peking and their perception as signals by the nitrogen-fixing symbiont *Sinorhizobium fredii*. *Plant Physiol.* **117:** 599–608.
18. Redmond, J. W., M. Batley, M. A. Djordjevic, R. W. Innes, P. L. Kuempel, and B. G. Rolfe. 1986. Flavones induce expression of nodulation genes in *Rhizobium*. *Nature* **323:** 632–635.
19. Sadowsky, M. J., E. R. Olson, V. E. Foster, R. M. Kosslak, and D. P. S. Verma. 1988. Two host-inducible genes of *Rhizobium fredii* and characterization of the inducing compound. *J. Bacteriol.* **170:** 171–178.
20. Sadowsky, M. J., R. E. Tully, P. B. Cregan, and H. H. Keyser. 1987. Genetic diversity in *Bradyrhizobium japonicum* serogroup 123 and its relation to genotype-specific nodulation of soybean. *Appl. Environ. Microbiol.* **53:** 2624–2630.
21. Schlaman, H. R. M., H. P. Spaink, R. J. H. Okker, and B. J. J. Lugtenberg. 1989. Subcellular localization of the *nodD* gene product in *Rhizobium leguminosarum*. *J. Bacteriol.* **171:** 4686–4693.
22. Smit, G., V. Puvanesarajah, R. W. Carlson, W. M. Barbour, and G. Stacey. 1992. *Bradyrhizobium japonicum nodD1* can be specifically induced by soybean flavonoids that do not induce the *nodYABCSUIJ* operon. *J. Biol. Chem.* **267:** 310–318.
23. Zaat, S. A. J., C. A. Wijffelman, H. P. Spaink, A. A. N. Van Brussel, R. J. H. Okker, and B. J. J. Lugtenberg. 1987. Induction of the *nodA* promoter of *Rhizobium leguminosarum* Sym plasmid pRL1JI by plant flavanones and flavones. *J. Bacteriol.* **169:** 198–204.