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Flavonoid-Induced Expression of a Symbiosis-Related Gene in the Cyanobacterium *Nostoc punctiforme*

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The flavonoid naringin was found to induce the expression of *hrmA*, a gene with a symbiotic phenotype in the cyanobacterium *Nostoc punctiforme*. A comparative analysis of several flavonoids revealed the 7-*O*-neohesperidoside, 4′-OH, and C-2–C-3 double bond in naringin as structural determinants of its *hrmA*-inducing activity.

Cyanobacteria of the genus *Nostoc* can form N_2-fixing symbiotic associations with a diverse variety of plants (10). Under the influence of the plant partner, motile units of *Nostoc*, termed hormogonia, infect the plant tissue. Once inside, hormogonia dedifferentiate into filaments composed of both vegetative cells and N_2-fixing cells, called heterocysts. Symbiotically associated *Nostoc* form heterocysts at frequencies significantly higher than those found in free-living filaments (10). These heterocysts release the majority of their fixed nitrogen to the plant (10). Various extracts and exudates from both symbiotic and nonsymbiotic plants have been shown to influence the development of *Nostoc* (2, 3, 8, 11). Aqueous ground extract of the symbiotically competent bryophte *Anthoceros punctatus* increases transcript levels of the *hrm* locus in wild-type *Nostoc punctiforme* (E. L. Campbell and J. C. Meeks, personal communication) and in strains containing *luxAB* fusions within the locus (3). Mutants in *hrmU* and *hrmA* have a phenotype of increased infection frequency of *A. punctatus* which correlates with an increased sensitivity to plant-activated hormogonium formation (3, 4). Although the role of these genes in hormogonium formation has not been fully elucidated, sequence analysis shows that the *hrmU* gene belongs to a family of NAD(P)H-dependent oxidoreductases, while *hrmA* has no identifiable sequence motifs (3).

In contrast to our extensive knowledge of the signaling mechanisms in the more specific *Rhizobium*-legume symbioses, none of the signaling molecules involved in *Nostoc* symbioses have been identified. Flavonoids secreted by legumes establish communication with *Rhizobium* by binding the transcriptional activator protein NodD (6), and flavonoids are also likely to have a role in plant symbioses with mycorrhizal fungi (13–15). Seed rinse from *Gunnera*, a symbiotic angiosperm host of *Nostoc*, has been shown capable of inducing expression of *nod* genes in *Rhizobium*, thus raising the possibility of common chemical signals among host plants (12). For this study, several flavonoids were screened for the ability to increase expression of luciferase from a *hrmA-luxAB* transcriptional fusion in mutant strain UCD 328, formed by transposition of Tn*5*-1063 into *hrmA* of *N. punctiforme* (3, 4).

Strain UCD 328 was cultivated in a *Nostoc* basal growth medium at 23°C under light with continuous shaking (5). In test tubes, cells were suspended to a concentration of 0.6 μg of chlorophyll (Chl) *a* in 2,475 μl of medium and incubated under growth conditions. After 30 min, a 25-μl solution of 95% ethanol with or, for controls, without dissolved flavonoid was added to each cell suspension. To assay for LuxAB luciferase activity at various time intervals, 1 ml of cell suspension was combined with 111 μl of a 3 mM decylaldehyde–1.4% ethanol solution in a 12- by 55-mm test tube, vortexed, and placed in a luminometer (Compactlumi VS500; Microtek Nichion, Shizuoka, Japan). Luminescence (relative light units) from the cells was monitored for 90 s following a 50-s delay. To normalize the values, the Chl *a* content of each assay tube was determined. Luciferase activities are reported relative to the activity of cells from parallel control experiments.

Flavanones, flavan-3-ols, kaempferol, and myricetin were purchased from Sigma Chemical Co. (St. Louis, Mo.); anthocyanins, rutin, and rhoifolin were from Extrasynthèse (Genay, France); quercitin and flavone were from Nacalai Tesque (Kyoto, Japan).

Induction of *hrmA-luxAB* expression by naringin. Cells of strain UCD 328 incubated with 0.4 mM naringin showed a peak 16.1 ± 1.1-fold (mean ± standard error; $n = 7$) increase in luciferase activity 9 h after addition of the flavonoid (Fig. 1). Near-maximal induction was reached with 0.6 mM naringin (Fig. 1). Compared to the reported induction of luciferase activity by *A. punctatus* extract in strain UCD 328 and other *hrmA-luxAB* fusion strains (3), the response induced by naringin peaks a few hours earlier at nearly double the intensity.

Under the incubation conditions used for the assay, naringin had no discernible effect on the growth of strain UCD 328. Over a 2-day period, cells incubated with 0.4 mM naringin had a growth rate of 0.62 ± 0.03 d^{-1} ($n = 5$), identical to that of control cells, 0.62 ± 0.03 d^{-1} ($n = 6$).

Structural determinants of *hrmA-luxAB*-inducing activity. We assayed several other flavonoids in order to ascertain the structural specificity required for induction of *hrmA*. Flavonoids are characterized by a common three-ring structure whose A, B, and C rings are shown in Fig. 2. Subtle differences in the ring substitution pattern and localization of a flavonoid can have major effects on its function (9, 13, 16). Our survey included representatives from four flavonoid subclasses: the flavanones, the flavan-3-ols, the anthocyanins, and the flavones and flavonols, distinguished by differences in their basic structures (Fig. 2). Cells of strain UCD 328 were incubated for 7 h under growth conditions in 0.1 mM each compound before determination of luciferase activity. Rhoifolin, neohesperidin, and prunin were found to induce (Table 1) but at levels significantly lower than naringin (Fig. 3).

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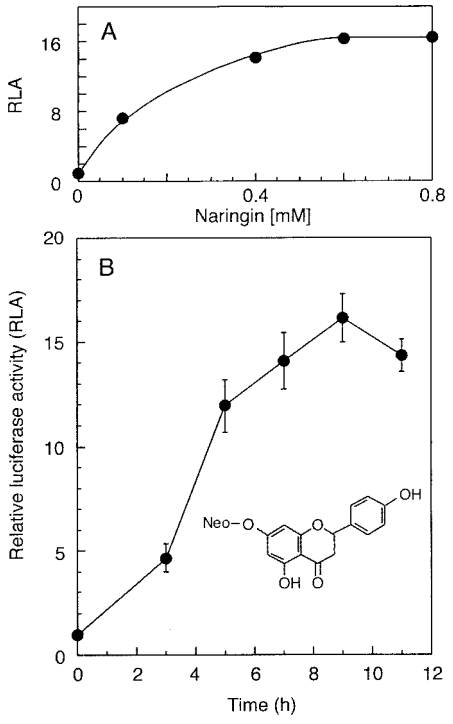

FIG. 1. Effect of naringin on *hrmA-luxAB* in strain UCD 328. (A) Luciferase activity of cells incubated 7 h in various concentrations of naringin relative to the value for control cells incubated without naringin (means from at least three experiments). (B) Cells incubated in 0.4 mM naringin were assayed for luciferase activity at various time intervals; values are the means ± standard errors from at least four experiments. A two-dimensional structure of naringin is presented in the inset. Neo, neohesperidose.

A comparison of naringin to rhoifolin shows the importance the C-ring conformation for *hrmA*-inducing activity. Naringin is a flavanone and therefore differs from rhoifolin, a flavone, in having a saturated C-2–C-3 bond. This confers a 37.9° difference in the C-2–C-3–C-4–C-10 tetrahedral bond angle of the otherwise identical compounds (Fig. 3). Rhoifolin gave a 1.27 ± 0.07-fold (n = 8) increase in luciferase activity, substantially lower than the 7.04 ± 0.26-fold (n = 4) increase

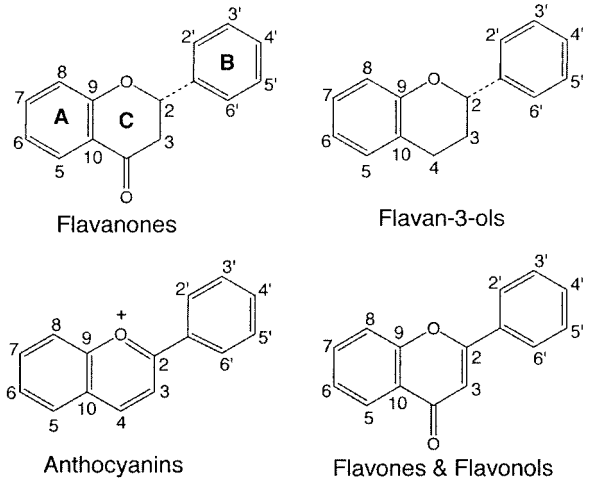

FIG. 2. The basic three-ring structures of the flavonoids examined in this study. Positions of the A, B, and C rings are indicated.

TABLE 1. Flavonoids assayed for *hrmA-luxAB* activity

Flavonoid	Structure[a]	Induction[b]
Flavanones		
Narigenin	5,7,4′-OH	–
Prunin	Naringenin-7-*O*Glc	+
Naringin	Naringenin-7-*O*Neo	+
Hesperitin	5,7,3′-OH, 4′-*O*Me	–
Neohesperidin	Hesperitin-7-*O*Neo	+
Hesperidin	Hesperitin-7-*O*Rut	–
Flavan-3-ols		
(+)Catechin	(+)3,5,7,3′,4′-OH	–
(–)Epigallocatechingallate	5,7,3′,4′,5′-(–)3-*O*-gallate	–
Anthocyanins		
Pelargonidin	3,5,7,4′-OH	–
Pelargonin-3-glucoside	Pelargonidin-3-*O*Glc	–
Cyandin	3,5,7,3′,4′-OH	–
Flavones and flavonols		
Flavone		–
Rhoifolin	5,4′-OH, 7-*O*Neo	+
Kaempferol	3,5,7,4′-OH	–
Quercitin	3,5,7,3′,4′-OH	–
Rutin	Quercitin-3-*O*Rut	–
Myricetin	3,5,7,3′,4′,5′-OH	–

[a] *O*Glc, *O*-glucose; *O*Neo, *O*-neohesperidose; *O*Rut, *O*-rutinose.
[b] See Fig. 3 for relative activities of inducing compounds.

induced by the same concentration of naringin (Fig. 3). Thus, the bent confirmation of the C ring found in naringin appears to favor *hrmA-luxAB* induction.

The substitution pattern of the B ring also influences the *hrmA*-inducing activity of naringin. The flavanone neohesperidin differs from naringin only in the B ring; naringin contains a single -OH at C-4′, whereas neohesperidin has a -OCH_3 at C-4′ and a -OH at C-3′. Luciferase activity rose only 1.21 ± 0.07-fold in response to neohesperidin (Fig. 3). Therefore, since flavanones are known to interact with proteins (9), hy-

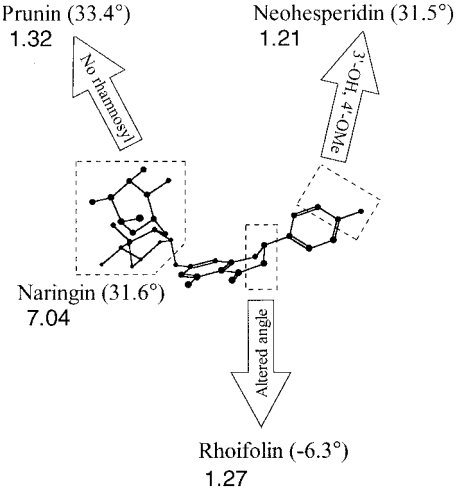

FIG. 3. Three-dimensional view of naringin contrasting its structural differences with the flavonoids that displayed low *hrmA-luxAB*-inducing activity. Relative inducing activity is indicated below the name of each compound. The C-2–C-3–C-4–C-10 tetrahedral bond angles, in parentheses, were determined from the MM2-minimal energy conformations of the structures (Chem3D; Cambridge Software, Cambridge, Mass.). Dashed lines enclose regions of contrast between the flavonoids.

drogen bond formation between the 4′ -OH of naringin and a putative target protein in *N. punctiforme* may be involved in the *hrmA* induction process.

Further comparisons illustrate the importance of the A-ring sugar. Neohesperidose, the 7-*O*-dissaccharide of naringin, is a rhamnosyl-glucose. Prunin and naringenin differ from naringin only in their 7-*O* linkage. Interestingly, prunin (having a 7-*O*-glucose) induced only a 1.32 ± 0.07-fold increase in luciferase activity (Fig. 3), while naringenin (having a 7-OH) did not induce at all. Therefore, the terminal rhamnosyl moiety on the 7-*O*-glucose is another *hrmA*-inducing determinant of naringin.

No induction of *hrmA-luxAB* by any of the other 12 flavonoids listed in Table 1 was observed, nor did we observe induction by 34 additional compounds found in plants, including hydroxycinnamic acids, caffeine, tannic acid, riboflavin, ascorbate, sucrose, and oxalate (data not shown).

Physiological implications. This report of *hrmA-luxAB* induction by naringin is the first direct evidence of a flavonoid influencing the gene expression of a cyanobacterium. Naringin is a relatively common flavonoid distributed among several plant families (1). It can accumulate in plants to near millimolar levels (7), concentrations that we have found to maximally induce *hrmA*. Although we have clearly shown strong *hrmA* induction by naringin, we do not consider that naringin will be the only molecule found to have this effect on *Nostoc*. Plants that establish symbioses with *Nostoc* might fine-tune the substitution pattern of their flavonoids in order to maximally exert control over their symbiont. However, knowledge of the flavonoid content in symbiotic hosts of *Nostoc* is limited. We have found that extract of *Azolla*, a symbiotically competent water fern, has a higher *hrmA-luxAB*-inducing activity than naringin (M. F. Cohen and H. Yamasaki, unpublished data). The identification of naringin as an inducer of *hrmA* should aid in elucidating the molecular mechanisms regulating *Nostoc* differentiation and provide important clues for the isolation of potential *hrmA* inducers from symbiotic plant partners including *Azolla* and *Anthoceros*. Such molecules could conceivably function in plant symbiotic cavities to prevent the formation hormogonia and thus permit higher frequencies of N_2-fixing heterocysts (3).

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