Symbiosis between Nitrogen-Fixing Cyanobacteria and Plants

The establishment of symbiosis causes dramatic morphological and physiological changes in the cyanobacterium

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The cyanobacteria (blue-green algae) are a morphologically diverse group of Gram-negative prokaryotes within the domain Bacteria (Giavannoni et al. 1988). This group includes unicellular forms, filamentous forms that contain only vegetative cells, and filamentous forms that differentiate specialized cells (Castenholz and Waterbury 1989). Cyanobacteria are photoautotrophs with an oxygen-evolving photosynthetic mechanism that is identical to that of chloroplasts of algae and higher plants. Indeed, molecular genetic evidence strongly supports the idea that a recent ancestor(s) in the cyanobacterial evolutionary line gave rise to chloroplasts (Douglas 1994). Many cyanobacteria fix atmospheric nitrogen, which is an oxygen-sensitive process; they fix nitrogen either under low-oxygen growth conditions or, when in air, in specialized cells called heterocysts (Fay 1992). As a group, oxygenic photoautotrophic cyanobacteria, especially those species that fix nitrogen, are the most nutritionally independent organisms in the biosphere. Because of this nutritional independence, as well as their tolerance to physicochemical extremes, cyanobacteria are ubiquitous in global aquatic and terrestrial habitats that are illuminated.

Many unicellular and filamentous cyanobacteria grow in association with other prokaryotes, eukaryotic protists, metazoans, or plants (Schenk 1992). All of these associations can be considered symbiotic according to de Bary’s (1879) broad definition of the term; that is, they are long-lasting associations of two or more different organisms. The best studied cyanobacterial symbiotic associations are those that nitrogen-fixing genera of the order Nostocales (Anabaena, Calothrix, Nostoc, and Scytonema) form with fungi, primarily in the establishment of lichens, and those that they form with representatives of four of the major phylogenetic groups of plants. The plant groups include the spore-producing, nonvascular bryophytes, especially the hornworts, such as Anthoceros punctatus, and the liverwort Blasia pusilla; the spore-producing, vascularized ferns, specifically the aquatic genus Azolla; the cycads, such as Macrozamia spp., which are gymnosperms; and the angiosperm herbaceous genus Gunnera (Rai 1990a). In all of these associations, the cyanobacteria remain extracellular, except for the associations with Gunnera and with the nonlichen fungus Geosiphon pyriforme, in both of which the cyanobacterium is intracellular. The selective pressure on the formation of these associations is presumably the provision of fixed nitrogen by the cyanobacterium for growth of the plant partner.

The plant partner in a symbiotic association is a unique habitat with specific physicochemical factors that influence the growth and development of its cyanobacterial partner. When nitrogen-fixing cyanobacteria enter into a symbiotic association, their morphology and physiology change dramatically. These changes primarily involve a 5- to 10-fold enhancement in the degree of differentiation of motile filaments, called hormogonia, and of heterocysts in the vegetative filaments that develop from the hormogonia. The hormogonia serve as the infective units in the establishment of an association, whereas nitrogen is fixed in the heterocysts and made available to both cyanobacterial and plant partners in the functional association.

In this article, I emphasize the association between Nostoc species and the gametophyte stage of the hornwort A. punctatus (Figure 1) as a context for exploring these changes (Nostoc species do not infect the sporophyte stage of bryophytes; Rodgers and Stewart 1977). My research
uses the A. punctatus–Nostoc association because the symbiotic partners can be cultured separately in the laboratory. A. punctatus gametophyte tissue grows rapidly and can be maintained in liquid suspension pure culture much like plant tissue cultures, and the association can be reconstituted under liquid culture conditions with a variety of Nostoc isolates and mutant strains (Enderlin and Meeks 1983). After colonization of preexisting cavities in the gametophyte tissue and growth, the Nostoc appear as dark, macroscopic colonies of 0.4–1.0 mm in diameter (Figure 1b) that are highly active in nitrogen fixation (Steinberg and Meeks 1991). One particular isolate, Nostoc punctiforme, which was originally cultured from a coralloid root of the cycad Macrozamia sp. (Rippka et al. 1979) and can reconstitute both the A. punctatus (Enderlin and Meeks 1983) and Gunnera manicata (Johansson and Bergman 1994) associations, is amenable to genetic analysis and now serves as our experimental organism (Cohen et al. 1994).

The establishment of a symbiosis between cyanobacteria and their plant partners is less complex than that between the Proteobacteria genera Bradyrhizobium and Rhizobium (Rhizobia) and leguminous plants. The Rhizobia–legume symbiosis involves an extensive series of reciprocal exchanges of chemical signals between the partners (Stacey et al. 1992). An exchange of signals that is essential for recognition first occurs during infection of the plant by the Rhizobia. Formation of an effective nitrogen-fixing association involves signal exchange in the regulation of growth, metabolism, and cellular differentiation of both the Rhizobia and legume partners, leading to the formation of nodules. In plants that associate with cyanobacteria, by contrast, the morphological changes are much less pronounced. In addition to elaborating plant hair filaments that function in nutrient transfer, the gametophyte and leaf cavity in bryophytes and Azolla sp., the coralloid root in cycads, and the specialized cells of the stem gland in Gunnera sp. all increase in size. Unlike legume nodules, however, which form only in the presence of the Rhizobia symbiont, all of these structures preexist in the absence of the cyanobacterial symbiont (Peters and Meeks 1989, Braun-Howland and Nierzwick-Bauer 1990, Bergman et al. 1992a).

The interactions with A. punctatus that influence the morphology and physiology of Nostoc and lead to a stable symbiotic association appear to be primarily unidirectional, from plant to cyanobacterium. They can be defined as two distinct stages, with parallels to the Rhizobia–legume symbiosis. The first stage is the initial establishment of the association (i.e., formation of hormogonia); the second is its development into a functional association (i.e., regulation of growth and metabolism, and stimulation of heterocyst differentiation coupled with release of fixed nitrogen). The existence of two distinct stages suggests that Nostoc respond to chemical signals produced by A. punctatus, with different signals activating different symbiotic-specific regulatory pathways. Similar stages of interactions and activation of regulatory pathways presumably occur in all other plant–cyanobacterial associations.

Figure 1. Photomicrographs of field-collected Anthoceros punctatus. (a) Gametophytes (G) grow in clusters; each typically bears a single sporophyte (S). Bar = 1 cm. (b) Ventral surface of gametophyte tissue with macroscopic dark large and small colonies of Nostoc (N) located randomly throughout the tissue. Bar = 0.25 cm.

Initial establishment of a cyanobacterial symbiosis

Interactions that result in the establishment of a symbiotic association can be characterized as Nostoc responding to chemical signals from A. punctatus that affect the differentiation and behavior of hormogonia, leading to infection of the plant tissue. The infection process is monitored by counting macroscopically visible Nostoc colonies in A. punctatus gametophyte tissue.

Formation of Nostoc infective units. Motile hormogonia are essential for establishment of plant–cyanobacterial symbiotic associations—that is, they appear to function as infective units (Campbell and Meeks 1989, Rasmussen et al. 1994). Hormogonia form transiently as part of a natural life cycle in some filamentous cyanobacteria, including some genera that differentiate heterocytes and others that do not; they are not unique to symbiotic strains (Rippka et al. 1979). Hormogonia are proposed to function as units of dispersal for colonization of new habitats (Tandeau de Marsac 1994). Hormogonia of Nostocales are morphologically distinct from vegetative filaments: They have a different cell shape, the cells are smaller, and the filaments lack heterocytes (Figure 2). Hormogonia move by a gliding mechanism, but motility is often lost in culture (Castenholz and Waterbury 1989). The differentiation of hormogonia is characterized by the immediate cessation of DNA replication, of protein synthesis (most notably of phycobiliproteins; Figure 2b), and of biomass increase, and by rapid septation (Tandeau de Marsac 1994).
Many environmental factors increase the frequency of hormogonium differentiation, in particular transfer to fresh medium, transfer from liquid to solid medium, and, for some *Calothrix* and *Nostoc* strains, exposure to red light (Tandeau de Marsac 1994). Nitrogen-limited *A. punctatus* stimulates hormogonium differentiation through an excreted hormogonium-inducing factor (HIF; Campbell and Meeks 1989). Similar inducing activity appears to be present in the mucilage secreted by *Gunniera* stem glands (Rasmussen et al. 1994) and in the root exudate of a traditionally non-symbiotic host, wheat (Gantar et al. 1993). An active factor has not been isolated and characterized from any of these plants.

The transient hormogonium cycle is best observed in nitrogen-fixing cultures. When heterocyst-containing filaments differentiate into hormogonia, the filaments fragment at the junction between the vegetative cells and heterocysts, detaching the heterocysts, which are now nonfunctional (Campbell and Meeks 1989). Hormogonia, therefore, do not fix nitrogen. Within 12 hours of exposure to *A. punctatus*-produced HIF, essentially 100% of vegetative *Nostoc* filaments are converted to hormogonia. The hormogonia remain in an actively gliding state for approximately 48 hours, after which they differentiate heterocysts, beginning with the end cells of the filaments, and begin to fix nitrogen. Reversion, or dedifferentiation, of hormogonia to vegetative nitrogen-fixing filaments is complete within 96 hours after exposure to HIF. Once the *Nostoc* culture has completed a cycle of HIF-induced hormogonium formation and reversion back to vegetative filaments, there is a lengthy delay before extensive hormogonium formation can occur again in response to the presence of HIF—that is, there appears to be a period of immunity to HIF (Elsie Campbell and John C. Meeks, unpublished results). Thus, the initial infection window for the *A. punctatus* association is approximately 48 hours, and secondary infection of the growing gametophyte tissue is delayed. An initial infection window of the same duration was observed microscopically in the liverwort *B. pusilla*—*Nostoc* association (Kimura and Nakano 1990).

**Infection.** The actual infection process involves *Nostoc* hormogonia gliding to the site of entry into the plant structure that will be colonized (except in associations with *Azolla* spp.); the site of entry is most likely identified by a chemotactic signaling mechanism. In the bryophytes *A. punctatus* and *B. pusilla*, the hormogonia enter a preexisting slime-filled cavity in the gametophyte tissue (Rodgers and Stewart 1977). The actual infection process has not been observed in *A. punctatus*, but hormogonia have been observed by light microscopy to glide on the surface of *B. pusilla* gametophyte tissue before entering the cavity through a pore (Kimura and Nakano 1990). Although there is no evidence of chemotaxis to the cavity pore in any bryophyte, hormogonia of a *Nostoc* strain exhibit chemotactic behavior in response to exudate from *B. pusilla* (Knight and Adams 1996).

Associations with *Azolla* spp. are characterized by a cyanobacterial inoculant that is carried within the fern tissue throughout its life cycle, and the cyanobacterium may not have a free-living growth state (Peters and Meeks 1989). The cyanobacteria present in the fern apical meristem enter the leaf cavity by attaching hormogonium-like filaments to fern hair filaments. Growth of the leaf tissue subsequently encloses the inoculum (Peters and Meeks 1989).

The mechanism by which cyanobacteria enter the coralloid roots of cycads is unknown. Because the *Gunniera* association is intracellular and occurs several cell layers deep in the stem gland tissue, the infection process is more complex than it is for the other associations. The *Nostoc* hormogonia glide on *Gunniera* gland tissue that forms an intercellular channel from the exterior of the plant stem to the channel base (Johansson and Bergman 1992). Hormogonia of most cyanobacterial strains are positively phototactic; because the hormogonia move against a light gradient in the channel of *Gunniera* sp., Johansson and Bergman (1992) proposed that a chemotactic mechanism attracts the hormogonia to the channel base and that this mechanism must override any phototactic response. Once hormogonia reach the channel base, some *Gunniera* sp. gland cell walls dissolve by an unknown mechanism and are released into these nonphotosynthetic cells, although they remain separated from the host cell cytoplasm by a mem-
Repression of hormogonium formation. Because hormogonia are in a nongrowing state, continued entry into the hormogonium cycle is presumably lethal. One might predict that Nostoc would have a mechanism to block either the sensing of the continual presence of HIF (or an equivalent signal) or the initiation of hormogonium formation. A possible hormogonium repression system was identified by analysis of a transposon-induced mutant of N. punctiforme that is approximately 50-fold more infective of A. punctatus than its parental culture (Cohen et al. 1994). From this mutant, two genes were identified as part of an operon in N. punctiforme: one gene (hrmA) has no similarity in major databases, whereas the second gene (hrmU) has a signature sequence belonging to a family of NAD(P)H-dependent oxidoreductases. Reporter gene constructs in hrmU and hrmA are induced by an aqueous extract of A. punctatus tissue, but not by HIF; indeed, the presence of the aqueous extract eliminates HIF-stimulated hormogonium formation in the wildtype strain. The mutant strain may be more infective of A. punctatus because it immediately reenters the hormogonium state in the presence of HIF, thereby extending the initial infection window (Cohen and Meeks 1997). These observations suggest that the gene products of the hrmUA operon may block hormogonium formation by producing an inhibitor or by catabolizing an activator.

Although hormogonia are essential in establishment of a Nostoc...
symbiotic association, their continued formation prevents the accumulation of vegetative filaments containing heterocysts. This behavior is counterproductive to the formation of a functional nitrogen-fixing association. The hormogonium repressive mechanism that is activated by A. punctatus may, therefore, be important in symbiosis by shifting the developmental direction of the associated Nostoc toward heterocyst differentiation and nitrogen fixation simply by preventing hormogonium differentiation.

Development of a functional association

Interactions that lead to the development of a functional symbiotic association involve the provision by the cyanobacterium of nitrogen gas-derived ammonium or organic nitrogen for growth of the plant partner. The fixed nitrogen that is made available to the plant partner is in excess of that required for growth of the associated Nostoc species. Indeed, in its symbiotic state, Nostoc undergoes a metabolic decoupling such that higher rates of heterocyst differentiation and nitrogen fixation are accompanied by lower, rather than higher, rates of vegetative growth, photosynthetic carbon dioxide fixation, and ammonium assimilation. Because the plant partners themselves are photosynthetic and can assimilate ammonium, in most experiments the Nostoc sp. is physically separated from the association and its physiological and biochemical characteristics determined by in vivo or in vitro assays. Nitrogen fixation, which is unique to the associated Nostoc, can be monitored in the intact association (in situ).

Regulation of cyanobacterial growth and metabolism. Under optimal laboratory conditions, most heterocyst-forming cyanobacteria have doubling times in the free-living growth state that are substantially more rapid than those of their symbiotic plant partners (Enderlin and Meeks 1983, Peters and Meeks 1989, Braun-Howland and Haselkorn 1986). Thus, for a stable symbiotic association to endure, the growth rate of the previously symbiotically associated Nostoc must be slowed. Although slower growth of symbiotically associated Nostoc has been documented (Enderlin and Meeks 1983), nothing is known about the mechanisms of cyanobacterial growth control in any association.

In addition to a decreased growth rate, the vegetative cells of symbiotically associated Nostoc increase two- to threefold in circumference (compare Figures 3a and 3c). The reasons
Table 2. Nitrogen metabolic characteristics of cyanobacteria in symbiotic associations. Because more data are available for nitrogen than for carbon metabolism, quantitative values are reported as measured in the specific study, unless otherwise noted. For clarity and comparison, the glutamine synthetase (GS) values are reported as a percentage of the free-living values.\(^a\)

<table>
<thead>
<tr>
<th>Association</th>
<th>Heterocyst frequency</th>
<th>Nitrogenase specific activity(^b)</th>
<th>Nitrogen released as percentage of fixed and form in which released</th>
<th>Glutamine synthetase Activity</th>
<th>Glutamine synthetase Protein</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free-living Nostoc/Anabaena</td>
<td>3-10%</td>
<td>2.7-6.3</td>
<td>Less than 10% as organic nitrogen</td>
<td>100%</td>
<td>100%</td>
<td>Sampaio et al. 1979, Rai et al. 1983, Rai 1990b</td>
</tr>
<tr>
<td>Lichen, Peltigera canina(^d)</td>
<td>4.7%</td>
<td>5.3(^c)</td>
<td>55% as ammonium</td>
<td>5%</td>
<td>100%</td>
<td>Stewart and Rowell 1977, Sampaio et al. 1979, Rai et al. 1981b, Rai 1990b</td>
</tr>
<tr>
<td>Lichen, Peltigera aphthiod(^d)</td>
<td>21.2%</td>
<td>14.0(^c)</td>
<td>95% as ammonium</td>
<td>5%</td>
<td>100%</td>
<td>Hill 1975, Rodgers and Stewart 1977, Meeks et al. 1985, Joseph and Meeks 1987, Steinberg and Meeks 1991</td>
</tr>
<tr>
<td>Hornwort, Anthoceros punctatus</td>
<td>43-45%</td>
<td>23.5-185.7(^e)</td>
<td>80% as ammonium</td>
<td>15%</td>
<td>100%</td>
<td>Stewart and Rowell 1977, Sampaio et al. 1979, Rai et al. 1981b, Rai 1990b</td>
</tr>
<tr>
<td>Fern, Azolla spp.</td>
<td>30%</td>
<td>6.2(^c)</td>
<td>40% as ammonium</td>
<td>30%</td>
<td>38%</td>
<td>Hill 1975, Ray et al. 1978, Meeks et al. 1987, Lee et al. 1988</td>
</tr>
<tr>
<td>Cycad, Zamia, Macrozamia spp.</td>
<td>17-46%</td>
<td>26.7(^c)</td>
<td>Unknown amount as organic nitrogen</td>
<td>100%</td>
<td>100%</td>
<td>Lindblad et al. 1985, 1987, Lindblad and Bergman 1986, Perraju et al. 1986, Hate et al. 1988</td>
</tr>
<tr>
<td>Angiosperm, Gunnera spp.</td>
<td>20-60%</td>
<td>24.8</td>
<td>90% as ammonium</td>
<td>70%</td>
<td>100%</td>
<td>Silvester 1976, Soderback et al. 1990, Bergman et al. 1992b, Silvester et al. 1996</td>
</tr>
</tbody>
</table>

\(^a\)The values for GS activity and protein levels in free-living Nostoc/Anabaena are as follows: GS activity, measured as \(\text{\gamma-glutamyl hydroxamate formation in a transferase assay} = 0.85-1.8 \times 10^{-4} \text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}\); GS protein (milligrams GS per gram cellular protein) = 6.8-7.6. From a compilation of various control experiments in the cited references.

\(^b\)Nitrogenase specific activity (C\(_2\)H\(_4\) reduction) is given as \(\text{nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}\).

\(^c\)From a compilation of various control experiments in the cited references.

\(^d\)Bipartite association.

\(^e\)Converted from nanomoles per hour per microgram chlorophyll, assuming that chlorophyll makes up 4% of total cellular protein.

\(^f\)Tripartite association with an additional green alga, Coccocyma.

\(^g\)Tripartite association with an additional green alga, Coccocyma.

\(^h\)Rate of 185.7 from laboratory tissue grown with approximately one-half the intensity of full sunlight and 5% (by volume) CO\(_2\); because these high light and CO\(_2\) values are not steady state laboratory culture conditions, this rate was not used in the general comparisons.

for, and consequences of, this decrease in the surface-to-volume ratio are unknown.

Photosynthetic metabolism. In most plant-cyanobacterial associations, photosynthetic carbon dioxide fixation and nitrogen-derived ammonium assimilation capacities decrease in the associated Nostoc (Tables 1 and 2). Both the extent and mechanism of the declines vary among the associations. Only in the association with Azolla does the rate of light-dependent carbon dioxide fixation by the immediately separated symbiotic cyanobacterium appear to approach that of free-living cultures. However, like most other cyanobacterial symbionts, Anabaena maintains an insignificant photosynthetic rate in the intact Azolla association (Peters and Meeks 1989). These in vivo and in situ differences have not been reconciled. In other associations, the rate of photosynthetic carbon dioxide fixation by immediately separated symbiotic cyanobacteria ranges from essentially undetectable in the cycad and Gunnera associations to between 11% and 12% of free-living cultures in the A. punctatus and tripartite (cyanobacterium-eukaryotic alga-fungus) lichen associations (Table 1). In both bipartite (cyanobacterium-fungus) and tripartite lichen associations, the cyanobacterium is assumed to contribute photosynthate to the fungus (Rai 1990b). Nevertheless, the rate of photosynthetic carbon dioxide fixation by the symbiotic Nostoc in these lichens is lower than that in the free-living growth state.

The symbiotic growth state does not appear to lead to regulation of
the transcription of components of the photosynthetic machinery in associated Nostoc, despite their diminished photosynthetic capacity in symbiosis. There is no marked decrease in the synthesis of light-harvesting phycobiliproteins in vegetative cells of cyanobacteria in any association (Table 1). Synthesis of the primary carbon dioxide fixation enzyme, ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), is similar in symbiotic and free-living Nostoc cultures except, perhaps, in the Azolla association. Rubisco protein levels have not been quantified in the Anabaena associated with Azolla; however, mRNA encoding Rubisco in the separated symbiont accumulates to only 10% of the level in free-living cultures (Nierzwicki-Bauer and Haselkorn 1989), which is not consistent with the high rate of in vivo carbon dioxide fixation.

In the A. punctatus (Steinberg and Meeks 1989) and Peltigera aphthosa (Rai et al. 1981a) associations, regulation of carbon dioxide assimilation occurs in part by inhibition of Rubisco activity of the Nostoc partner. However, crude extracts of Nostoc associated with cycads (Lindblad et al. 1987) and with Gunnera sp. (Soderback and Bergman 1993) have as much Rubisco activity as free-living cultures. The insignificant light-dependent in vivo carbon dioxide assimilation by Nostoc in association with cycads and with Gunnera sp. may, therefore, be a consequence of impaired photochemical reactions, of limited substrates in the reductive pentose phosphate cycle, or of a reversibly bound inhibitor. Additional components of the photosynthetic machinery of symbiotically associated cyanobacteria have not been examined.

Nitrogen fixation and metabolism. Symbiotically associated cyanobacteria tend to have an enhanced rate of nitrogen fixation (Table 2). The electrons to reduce atmospheric nitrogen via nitrogenase in heterocysts come from photosynthate provided by adjacent vegetative cells, while the ATP can be derived in heterocysts from oxidative phosphorylation or photophosphorylation (Wolk et al. 1994). In the free-living growth state, the rate of nitrogen fixation by heterocyst-forming cyanobacteria is relatively low under dark heterotrophic conditions and is stimulated by light. (The role of light is to generate photosynthetic energy in vegetative cells and ATP in heterocysts.) The diminished photosynthetic capacity of symbiotic cyanobacteria implies that the enhanced rate of symbiotic nitrogen fixation probably depends on reduced carbon supplied by the plant tissues and that symbiotic cyanobacteria have a high potential for heterotrophic metabolism. These conclusions are supported by the high rate of dark heterotrophic nitrogen fixation, which is also equal to the light-dependent rate, in the A. punctatus-Nostoc association (Steinberg and Meeks 1991).

In all but the cycad associations, the symbiotic Nostoc species release from 40% to 95% of their fixed nitrogen as ammonium into the symbiotic cavity, where it is taken up by the plant (Table 2). In the cycad association, the Nostoc releases organic nitrogen, not ammonium (Pate et al. 1988). The release of nitrogen-derived ammonium by symbiotic cyanobacteria inversely parallels a decrease in activity of glutamine synthetase (GS), the primary ammonium-assimilating enzyme in cyanobacteria. However, the rate of nitrogen fixation, the catalytic activity of GS, and the amount of nitrogen-derived ammonium released are not strongly correlated (Table 2). The regulation of GS expression by Nostoc appears to be variable in the different associations. In all but the cycad and Gunnera associations, the in vitro GS catalytic activity is markedly less than in free-living cultures, but it is still sufficient to assimilate all of the nitrogen-derived ammonium. The relatively higher GS activity of Nostoc species in the cycad association is consistent with their release of organic nitrogen to the plant. In all but the association with Azolla sp., the cellular concentration of GS in symbiotic cyanobacteria is similar to its concentration in the free-living growth state (Table 2).

Anabaena in association with Azolla synthesizes less GS protein than free-living cultures (Nierzwicki-Bauer and Haselkorn 1986, Lee et al. 1988). Because the Anabaena associated with Azolla may not have a free-living growth state (Peters and Meeks 1989), its reduced synthesis of GS may reflect the selection of a constitutively low GS level, which would be consistent with its slower symbiotic growth rate, rather than a transcriptional or translational regulatory mechanism.

Generalities of symbiotic growth and metabolism. In general, the rates of carbon dioxide and ammonium assimilation appear to decrease in symbiotic cyanobacteria approximately in proportion to the decreased rates of growth. Moreover, the enzymes catalyzing their assimilation are negatively regulated by post-translational mechanisms, or by secondary photochemical or substrate-producing reactions, rather than by control of enzyme synthesis. Whether the lower catalytic activities cause the lower growth rates, or the converse, is not known. The catalytic inactivations are presumably the consequence of biochemical reactions (i.e., modification systems) that could regulate other metabolic processes in the free-living growth state but that are activated differently or have altered substrate specificity in the symbiotic growth state. These unknown modification systems could act as sensors of the plant symbiotic signals.

Heterocyst differentiation and nitrogenase expression. In all symbiotic associations involving a eukaryotic photosynthetic partner, the heterocyst frequency of the symbiotic cyanobacterium is increased from twofold to more than sixfold relative to the free-living growth state (Table 2). The increased heterocyst frequency parallels an elevated rate of nitrogen fixation. The regulatory mechanism(s) responsible for the initiation of heterocyst differentiation in symbiosis may not be identical to those operative in the free-living growth state.

Heterocyst differentiation in the free-living growth state. When laboratory cultures of Nostoc and related genera are grown in the presence of combined inorganic nitrogen, such as ammonium or nitrate, heterocysts do not form. By contrast, when cultures are deprived of combined nitrogen, heterocysts differentiate. These observations imply that
a combined nitrogen sensing and signalating pathway is involved in the initiation of heterocyst differentiation. As many as 1000 genes could be involved in heterocyst formation and maintenance (Lynn et al. 1986); some of the regulatory and structural genes have been isolated and characterized and their transcription has been placed in epistatic order, primarily in the nonsymbiotic isolate Anabaena strain PCC 7120 (Buikema and Haselkorn 1993, Wolk et al. 1994, Wolk 1996).

The differentiation of a vegetative cell results in a heterocyst that is specialized for oxygen-sensitive nitrogen fixation in anoxic environment. Heterocysts lack the photosystem II oxygen-producing reaction; instead, they have an increased rate of respiratory oxygen uptake, and they synthesize an outer envelope of polysaccharide and glycolipid, which collectively impede the entry and accumulation of oxygen (Wolk et al. 1994). Although there is an exception in which nitrogenase genes are expressed in vegetative cells in the absence of oxygen (Theil et al. 1995), nitrogenase synthesis and its expression are generally confined to heterocysts in all free-living species that form them (Wolk et al. 1994). Immuno-electron microscopic experiments also show that the nitrogenase is confined to heterocysts of Nostoc in various symbiotic associations (Bergman et al. 1992a, 1992b), thus indicating that nitrogenase activity correlates with heterocyst frequency in symbiosis.

An immediate physiological response to deprivation of combined nitrogen in cyanobacteria is the mobilization of stored nitrogen. Cyanobacteria store nitrogen in a unique copolymer of aspartate and arginine called cyanophycin and in phycobiliproteins, which are photosynthetic accessory pigments, as well as in other proteins that are present at lower cellular concentrations. Specific proteases cleave amino acids from these polymers that are presumably used for synthesis of new proteins that are characteristic of heterocysts (Wolk et al. 1994). Synthesis of cyanophycin and phycobiliproteins ceases during nitrogen deprivation; thus, a vegetative cell that differentiates into a heterocyst in the free-living growth state initially has little or no phycobiliproteins. Such cells can be detected by fluorescence microscopy as being nonfluorescent when using excitation light absorbed by the phycobiliproteins. The phycobiliproteins are resynthesized in vegetative cells that do not differentiate into heterocysts, but not in heterocysts of free-living cultures (Figure 3c; for exceptions, see Wolk et al. 1994).

**Heterocyst differentiation in the symbiotic growth state.** The enhanced level of heterocyst differentiation and nitrogenase expression by Nostoc in association with A. punctatus may be induced in response to a symbiotic sensing and signaling system. This hypothetical system could be activated by a plant product in a mechanism that is distinct from the combined nitrogen deprivation sensing and signaling pathway that operates in the free-living growth state. Several lines of evidence support this hypothesis. First, electron micrographs show distinctly the presence of cyanophycin and phycobiliproteins in vegetative cells of cyanobacteria in associations with the hornwort A. punctatus, the water fern Azolla caroliniana, the cycad Zamia skinneri, and the angiosperm G. manicata (see Braun-Howland and Nierzwicki-Bauer 1990, Meeks 1990, Bergman et al. 1992a, 1992b for reviews). These polymers are characteristic of nitrogen-replete, not nitrogen-starved, cells, implying that the vegetative cells in symbiotic cyanobacteria that differentiate into heterocysts are not extremely nitrogen starved and may be responding to a different signal in the initiation of differentiation.

Second, heterocysts of cyanobacteria in association with Azolla species (Kaplan et al. 1986), and possibly with A. punctatus (Meeks 1990), contain phycobiliproteins, in contrast to their near-universal absence in heterocysts of free-living cultures. There is some disagreement as to the presence of phycobiliproteins in heterocysts of Nostoc associated with A. punctatus, in which it is difficult to distinguish heterocysts and vegetative cells by light microscopy (Figure 3c). More than 95% of the cells of a symbiotic nitrogen-fixing Nostoc colony associated with laboratory cultures of A. punctatus fluoresce orange to red (more than 600 nm) when excited with green (510–550 nm) light, which is absorbed by the phycobiliprotein phycocerythrin; all vegetative cells of free-living cultures fluoresce in a similar fashion (Figures 3c, 3d). Electron micrographs of parallel preparations, however, show heterocyst frequencies ranging from 25% to 45% of the total cyanobacterial cells (Meeks 1990). The difference between the less than 3% nonfluorescent cells and the 25–45% frequency of identified heterocysts may reflect the presence of phycobiliproteins in heterocysts of symbiotic Nostoc. However, Rai et al. (1989) were unable to detect phycocerythrin by immunoelectron microscopy in heterocysts of Nostoc in association with field samples of A. punctatus that had an unknown physiological history.

These conflicting results can be reconciled if the vegetative cells differentiating into heterocysts in symbiosis initially contain phycobiliproteins that have not yet been degraded in response to a nitrogen-starvation signal. Except perhaps in the Azolla association, there is little or no synthesis of phycobiliproteins in heterocysts; therefore, in the course of normal turnover, phycobiliproteins decrease to low or undetectable levels in aging heterocysts of symbiotically associated Nostoc. The presence of phycobiliproteins in developing heterocysts in symbionts is consistent with initiation of their differentiation through a sensing and signaling system that does not activate the nitrogen-starvation response characteristic of free-living cultures.

The third line of evidence for a symbiotic sensing and signaling pathway is that heterocysts of symbiotically associated cultures are physiologically different from those formed in the free-living growth state. In particular, the linkage of carbon catabolism to nitrogen assimilation is different. In all but the cycad associations, heterocysts of symbiotic Nostoc primarily translocate ammonium to adjacent vegetative cells (Table 2), whereas heterocysts of free-living cultures translocate glutamine to adjacent vegetative cells (Thomas et al. 1977). Therefore, heterocysts of symbiotic cyanobacteria have a
lower demand than heterocysts of
free-living cyanobacteria for glutamate, or its precursor α-ketoglutarate,
in the assimilation of nitrogen-derived ammonium, but they also maintain a
high demand for hexose-derived reductant to support the high rate of
heterotrophic nitrogenase activity and oxidative phosphorylation.

As a consequence of the different metabolic demands, free-living and
symbiotic cyanobacteria must differ substantially in the end products of
the oxidative pentose phosphate pathway, which is the primary route of
hexose catabolism and nonphoto-synthetic reductant generation in
cyanobacteria (Summers et al. 1995). Cyanobacteria have an incomplete
citric acid cycle, lacking α-ketoglutarate dehydrogenase (Smith 1982);
thus, when hexose is catabolized in a linear oxidative pentose phosphate
pathway coupled to the citric acid cycle, the end product is α-ketoglutarate,
in addition to reductant as NADPH. To avoid the accumulation of α-ketoglutarate
in excess of that required for ammonium assimilation while generating reductant, the symbiont
must shift to a cyclic pentose phosphate pathway, whose end products
are carbon dioxide and NADPH. The extent of this change in carbon
flux in symbiotically associated Nostoc is not known, nor has the mechanism that controls it been identified. Such a metabolic change would imply
that different genes or gene products are activated following initiation
of heterocyst differentiation in free-living and symbiotic growth states,
perhaps as a consequence of different sensing and signaling systems.

Fourth, symbiotic nitrogen fixation is enhanced in proportion to the increase in heterocyst frequency, whereas the rate of assimilation of ammonium is decreased (Table 2). This observation indicates that vegetative cells of symbiotic Nostoc are exposed to a substantial concentration of nitrogen-derived ammonium before it is assimilated by the plant. Nevertheless, these cells continue to differentiate into heterocysts. Conversely, exogenously supplied ammonium or nitrate represses nitrogenase expression (and, presumably, heterocyst differentiation) of Nostoc associated with A. punctatus (Enderlin and Meeks 1983). Symbiotically associated Nostoc is unlikely to respond to exogenously supplied combined nitrogen by repression of differentiation; after all, it does not respond to its own excess of nitrogen-derived ammonium. Rather, the plant partner may sense the exogenous combined nitrogen and cease to produce the chemical signals that influence heterocyst differentiation of the associated Nostoc.

As an initial test of this suggestion, a chemically induced mutant of N. punctiforme that is defective in nitrate assimilation was isolated (Campbell and Meeks 1992). Nitrate neither supported growth nor repressed heterocyst differentiation and nitrogenase expression in the free-living growth state of the mutant strain, as it did in the wild-type strain; by contrast, ammonium was an effective repressor of both mutant and wild-type cultures, indicating the continued presence and operation of a combined nitrogen deprivation sensing and signaling pathway. The mutant strain also established a typical functional symbiotic association with A. punctatus. When nitrogen-fixing A. punctatus associations with either the wild-type N. punctiforme or the mutant strain were exposed to exogenous nitrate, nitrogenase expression (and, presumably, heterocyst differentiation) by the associated N. punctiforme strains was repressed. The response to exogenous nitrate by the symbiotically associated mutant strain is consistent with the plant partner ceasing to produce signals controlling cellular differentiation in the associated cyanobacterium; it is not consistent with a direct cyanobacterial response to the exogenous combined nitrogen. These results therefore support the presence of a combined nitrogen-independent sensing and signaling pathway in the initiation of symbiotic heterocyst differentiation.

This symbiotic sensing and signaling pathway may supersedes the combined nitrogen deprivation pathway operative in the free-living growth state but also converges into a common regulatory pathway of heterocyst development and maturation.

Physiological genetic techniques have recently been adapted to symbiotically competent N. punctiforme, leading to its molecular genetic characterization (Cohen et al. 1994). Future work on the A. punctatus association will focus on genetic analysis of N. punctiforme to identify genes involved in hormogonium formation and behavior and to test under nitrogen-fixing conditions in the free-living growth state and could infect the plants (i.e., retain infectiveness). However, such strains would fail to form a highly effective nitrogen-fixing symbiotic association because they could not respond to the plant signals that initiate an enhanced level of heterocyst differentiation.

Summary and future questions
The symbiotic interactions between cyanobacteria of the genus Nostoc and plants are characterized by a predominantly unidirectional signaling from the plant to control the activities of the cyanobacterium. At least five distinct steps in the interaction between the bryophytes B. pusilla or A. punctatus and Nostoc can now be identified in which specific chemical signals from the plant influence symbiotically competent Nostoc species. These plant-produced signals include hormogonium-inducing factors, which yield a high frequency of infective units and increase the probability of an infection; chemotactic factors, which influence the direction of hormogonium gliding for more efficient colonization of gametophyte tissue; hormogonium-repressing factors, which are released into the gametophyte symbiotic cavity and may shift developmental alternatives away from hormogonium and toward heterocyst differentiation; growth- and metabolism-inhibiting factors that are essential for the establishment of a stable association and that may also contribute to the distinct physiological characteristics of symbiotic heterocysts; and factors that activate a symbiotic-specific sensing and signaling pathway that not only supersedes the nitrogen deprivation pathway operative in the free-living growth state but also converges into a common regulatory pathway of heterocyst development and maturation.
the working hypothesis of a symbiotic sensing and signaling initiation pathway of heterocyst differentiation. Because of the broad symbiotic competence of N. punctatus, mutants and genes identified in this species can also be characterized in the cycad and Gunnera associations.

Essentially all of the research effort on symbioses between nitrogen-fixing cyanobacteria and the plants with which they associate has been on the cyanobacterial symbiont; little is known about how the plant partners respond to the symbiotic growth state. Identification of the plant-produced factors that act as signals has proven elusive. Assays for active factors would be simplified by the use of reporter gene fusions in the Nostoc target genes. Such studies will surely follow the identification of target genes; subsequently, the regulation of the synthesis and release of the factors from plant tissues can be examined.

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References cited

de Bary A. 1879. Die erscheinung der symbiose. Strassburg (Germany): Verlag van K. J. Trubner.

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