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Review article

Cyanobacterial-Plant Symbioses

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Abstract

Diazotrophic cyanobacteria form symbioses with representatives from several plant divisions, but for unknown reasons, only one or a few plant groups in each division are involved. Representatives are found among lower plants, such as algae (diatoms), fungi (lichens), bryophytes and ferns, as well as among higher plants, such as the gymnosperm cycad and the angiosperm *Gunnera*. Thus, the host plants constitute a highly heterogenic group with apparently little in common besides being capable of using a nitrogen-fixing cyanobacterium to support their total need of nitrogen. In contrast, it appears that the cyanobacterial genera involved are limited and almost exclusively of a filamentous non-branching type capable of differentiating heterocysts. The purpose of this paper is to summarize some aspects of our current knowledge of cyanobacterial-plant symbioses with emphasis on bryophytes, *Azolla* and *Gunnera*. For a more comprehensive review the reader is referred to Rai (1990a).

Keywords: cyanobacteria, symbiosis, N_2-fixation, infection process, N-assimilation, heterocysts

1. The Host Plants

Bryophytes

In the division Bryophyta only six genera (out of a total of about 340) are documented to harbor cyanobacteria endophytically (Table 1; Rodgers and Stewart, 1977; Meeks, 1990). These are *Blasia, Cavicularia, Anthoceros,*

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Table 1. Plant structures involved and location of reported cyanobionts in cyanobacterial-plant symbioses

Symbiosis	Plant structure	Cyanobiont
Bryophytes	Cavities in the gametophyte	Intercellular; *Nostoc*
Water fern *Azolla*	Cavities in each dorsal leaf	Intercellular; *Nostoc*, *Trichormus*
Cycads	Root zone	Intercellular; *Nostoc*, *Calothrix*
Gunneras	Stem glands	Intracellular; *Nostoc*

Dendroceros, Notothylas and *Phaeoceros.* Such symbioses are found in two classes, the liverworts and the hornworts. Typically, the nitrogen-fixing bryophytes form small photosynthetic gametophyte thalli, a few cm in length. The cyanobacterium may be seen as dark "dots", less than 1 mm in diameter, scattered over the thalli (Fig. 1A). In nature, the small plants grow primarily on moist soils and rocks to which they are attached by rhizoids. This is the only group among the cyanobacterial-plant symbioses which occurs in temperate climates.

Ferns

Only one genus, with seven species within the division Pteridophyta, is known to form symbiosis with cyanobacteria, namely the free-floating aquatic fern *Azolla* (Fig. 1B; Table 1; Shi and Hall, 1988; Peters and Meeks, 1989; Braun-Howland and Nierzwicki-Bauer, 1990). These are small chlorophyllous plants like the bryophytes, but *Azolla* occupy water surfaces in their natural habitat. The plants are widely spread in tropical and subtropical areas but their distribution also includes some milder temperate regions. Considerable attention has been given to this symbiosis during the last decade since *Azolla* is successfully used as a green manure in rice farming areas of the world and therefore of considerable economic significance (Nierzwicki-Bauer, 1990).

Cycads

All members, about 150 species, within the division Cycadophyta, form symbioses with diazotrophic cyanobacteria (Table 1; Halliday and Pate, 1976; Grobbelaar et al., 1986; Lindblad and Bergman, 1990). This capacity makes

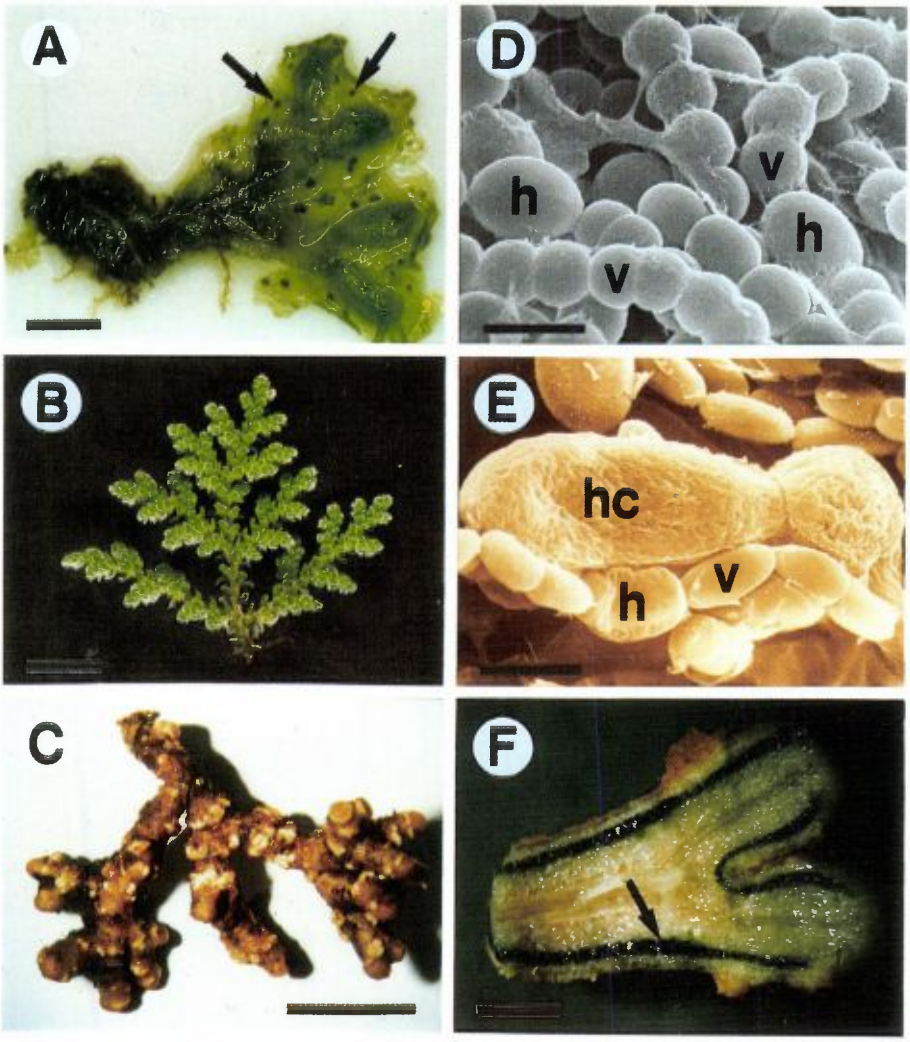

Figure 1. Three cyanobacterial-plant symbioses.
(A) In *Anthoceros*, the cyanobacteria are harbored within cavities in the thallus, and are readily seen as dark patches (arrows) through the surface. (Bar = 5 mm).
(B) In the *Azolla* sporophyte, the dorsal leaves contain cavities which are filled with symbiotic cyanobacteria and bacteria. (Bar = 5 mm).
(C) A coralloid root of the cycad *Zamia*. This is the organ where the symbiotic cyanobacteria dwell. (From Lindblad et al., 1985). With permission. Bar = 5 mm).
(D) The filamentous, heterocystous cyanobacterium *Nostoc*, which is commonly found in symbiosis with plants. When combined nitrogen is scarce, some of the vegetative cells (v) differentiate into heterocysts (h), which are specialized for nitrogen fixation. (Bar = 5 μm).
(E) The *Azolla* leaf cavity hair (hc) consists of two cells with transfer cell morphology. The cyanobacteria, with both heterocysts (h) and vegetative cells (v), are often located close to this. (From Neumüller and Bergman, 1981. With permission. Bar = 10 μm).
(F) A longitudinal section through a cycad coralloid root. The zone which lies between the inner and outer cortex and contains the symbiotic cyanobacteria is clearly revealed (arrow). (Photo by P. Lindblad; bar = 1 mm).

cycads unique among the gymnosperms. Cycads are large terrestrial plants growing in tropical and subtropical areas. They may for instance form dense understores in eucalyptous forests in Australia. Their geographical distribution is now very limited compared to a warmer period in the history of earth about 150 million years ago, when they were very common.

Angiosperms

Only one monogeneric family, Gunneraceae, among all the angiosperms, forms a diazotrophic symbiosis with cyanobacteria (Table 1; Bonnett, 1990; Osborne et al., 1991; Bergman et al., 1992). The genus *Gunnera* has about 50 species with a tropical/subtropical distribution within the southern hemisphere. The plants show a preference for extremely wet areas and high altitudes, and may colonize nutrient-poor soils. The size of the rhubarb-like plants varies enormously, from a few cm up to several meters in height.

2. Plant Structures Involved

The plant structures engaged in cyanobacterial-plant symbioses also comprise a heterogenic group and involve all major plant parts; leaves, stems and roots (Table 1). This is in contrast to the situation in rhizobial and actinorhizal symbioses where infection is restricted to the non-photosynthetic roots.

For instance, in the bryophytes, the cyanobacterium occupies cavities in the thin gametophyte thallus and is easily detected through the surface. Entrance of the cyanobacterium is via a pore on the ventral side of the thallus (Rodgers and Stewart, 1977). In the vegetative sporophyte of *Azolla* the cyanobacterium also inhabits cavities, in this case located in the chlorophyllous dorsal leaves (Calvert and Peters, 1981). Besides cyanobacteria, a number of bacteria are found in the same cavities (Petro and Gates, 1987; Grilli Caiola et al., 1988; Nierzwicki-Bauer and Aulfinger, 1990). Some of these bacteria are potentially diazotrophic (Lindblad et al., 1991). In both bryophytes and *Azolla*, several branched filamentous protrusions extend from the host cell layer lining the cavity into areas of the cavities occupied by the cyanobacteria (Fig. 1E; Stewart and Rodgers, 1977; Calvert et al., 1985). These show a distinct transfer cell morphology and are supposed to be involved in the exchange of metabolites between the host and the cyanobiont.

Cyanobacteria only occupy non-chlorophyllous tissues in the higher plant symbioses. In the cycads for instance, specialized roots are formed (coralloid roots; Fig. 1C) which have an open zone between the inner and outer cortex in which cyanobacterial filaments reside (Fig. 1F; Wittman et al., 1965;

Neumann, 1977; Lindblad et al., 1985). This zone is transversed by elongated cells with transfer cell morphology interconnecting the two cortical zones (Lindblad et al., 1985; Lindblad and Bergman, 1990). In *Gunnera* spp., specific mucus-secreting glands develop on the stem at the base of each petiole (Fig. 2A; Jönsson, 1894; Bonnett and Silvester, 1981; Osborne et al., 1991; Schmidt, 1991; Johansson and Bergman, unpublished). These attract cyanobacteria which eventually invade the *Gunnera* host in a multistage fashion as described later.

In only one case the cyanobacterium is found intracellularly, namely in *Gunnera* (Fig. 2C; Table 1; Silvester and McNamara, 1976; Towata, 1985). In this respect it resembles the other diazotrophic angiosperm symbioses, with *Rhizobium* and *Frankia*. In the other three cyanobacterial symbioses, the cyanobacterium dwells in more or less mucus-filled extracellular spaces (Braun-Howland and Nierzwicki-Bauer, 1990; Lindblad and Bergman, 1990; Meeks, 1990). Why *Gunnera* have evolved the most intimate symbiosis is not known. Being about 95 million years old (Jarzen, 1980), it is from an evolutionary point of view the most recently developed plant group among those forming symbiosis with cyanobacteria.

The plant structures occupied by the cyanobacterium are in all cases formed by the plant in the absence of the cyanobacterium. This is unlike the situation in other diazotrophic plant symbioses. In these, the development of the root nodules is induced by the microsymbiont through an activation of specific plant genes (Long, 1989; Huss-Danell, 1990).

3. The Cyanobionts

The cyanobacteria which enter into symbiosis with plants almost exclusively have been ascribed to the genus *Nostoc* (Fig. 1D; Table 1). This genus is filamentous and when grown in the absence of combined nitrogen, a limited number of photosynthesizing vegetative cells differentiate into heterocysts. The heterocysts are formed at regular intervals along the filaments in free-living cyanobacteria. Heterocysts serve as nitrogen-fixing entities which receive fixed carbon from the vegetative cells and give fixed nitrogen, in the form of glutamine, in return. In all the cyanobacterial-plant symbioses the nitrogen-fixing enzyme nitrogenase is confined to the heterocysts only (Bergman et al., 1986; Braun-Howland et al., 1988; Bergman and Rai, 1989; Rai et al., 1989; Söderbäck et al., 1990). These structures show several structural, biochemical and genetic modifications, including a microaerobic environment, conducive to

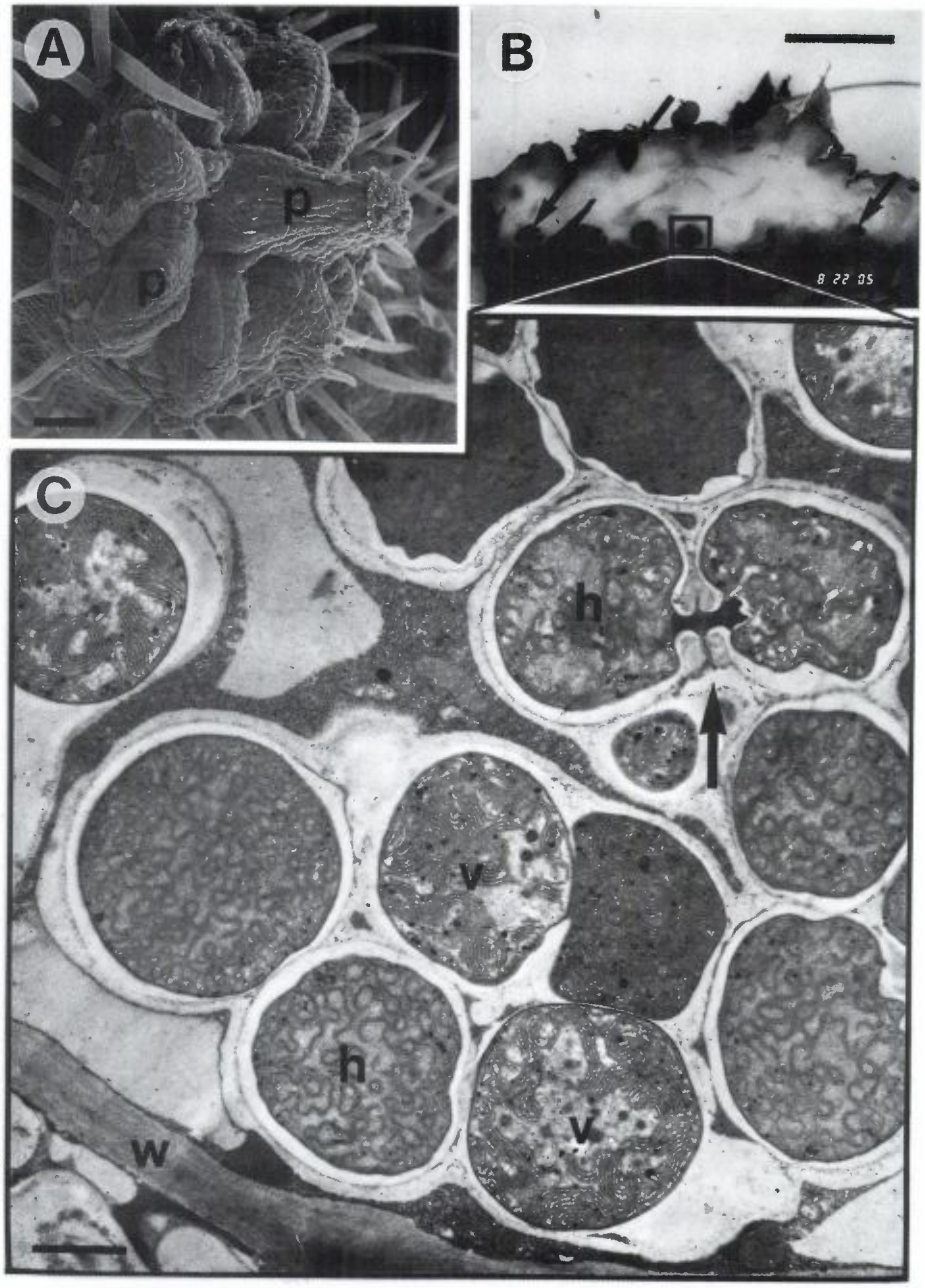

Figure 2. *Gunnera*, the only angiosperm which forms symbiosis with cyanobacteria.
(A) The stem gland of a young *Gunnera* seedling is composed of several multicellular papillae (p), between which the cyanobacteria enter the *Gunnera* tissue. (Bar = 200 μm).
(B) A longitudinal section through the mature *Gunnera* stem. The green patches (arrows) are clusters of cells filled with symbiotic cyanobacteria. (Bar = 5 mm).
(C) Large magnification of a *Gunnera* cell filled with cyanobacteria. Both vegetative cells (v) and heterocysts (h) are found, although the heterocysts are more common. Even double heterocysts (arrow) are commonly found, a feature which is very rare in free-living *Nostoc*. Part of the *Gunnera* cell wall (w) is also seen. (Bar = 2 μm).

nitrogenase functioning (Wolk, 1982). Therefore other means such as leghemoglobin synthesized by the plant are not required for the protection of nitrogenase against oxygen in cyanobacterial symbiosis. During certain circumstances and as a part of its life cycle, *Nostoc* filaments may also be differentiated into small-celled, filamentous hormogonia. This is a motile stage and probably of importance during the establishment of most of the cyanobacterial-plant symbioses.

However, a considerable number of *Nostoc* species (strains) are apparently involved in these symbioses, and indeed, a few cyanobionts do not belong to the genus *Nostoc*. There is for instance, a report on the occurrence of *Calothrix* in several species of the cycad *Encephalartos* (Grobbelaar et al., 1987) and lichens are known to harbor a few other cyanobacterial genera, including unicellular ones (Rai, 1990c). Like *Nostoc*, *Calothrix* also forms hormogonia. The classification of the *Azolla* symbiont has varied. First it was assigned to the genus *Anabaena*, as *Anabaena azollae* (Strasburger, 1873). However, this assignment has later been questioned and the genus *Nostoc* proposed (Meeks et al., 1988). During this meeting an assignment of the *Azolla* symbiont to the genus *Trichormus* was put forward (Grilli Caiola, personal communication). The difficulty in classifying the *Azolla* symbiont is mainly attributed to problems with isolating and growing the cyanobiont outside the host (Braun-Howland and Nierzwicki-Bauer, 1990). In *Azolla* an undifferentiated colony of the cyanobiont is carried along inside the sporocarps during the sexual reproduction of the plant (Lin et al., 1988) and hence reinfection is not necessary.

Definite proof for the occurrence of several different *Nostoc* strins in these systems has been obtained through reconstitution of the symbiosis with several cyanobacterial strains (Rodgers and Stewart, 1977; Bonnett and Silvester, 1981; Enderlin and Meeks, 1983; Johansson and Bergman, unpublished) and examination of DNA restriction fragment length polymorphism of freshly isolated cyanobionts from cycads, *Azolla* and *Gunnera* (Lindblad et al., 1989; Braun-Howland and Nierzwicki-Bauer, 1990; Zimmermann and Bergman, 1990). In addition, the pronounced differences seen in colony morphology and pigmentation among cyanobacterial isolates from a variety of *Gunnera* spp. also support this concept (Söderbäck, 1992; Bergman et al., 1992). Such data clearly show that several species are detected although perhaps only one (or a few) in each individual plant and that the same plant species may be infected by different cyanobacterial strains if growing in dissimilar habitats.

4. Reconstitution of the Symbiosis

Cyanobionts from all cyanobacterial-plant symbioses except *Azolla* have been successfully isolated and cultured in the laboratory (Rai, 1990c; Meeks, 1990; Lindblad and Bergman, 1990; Bonnett, 1990; Söderbäck, 1992). Using such isolated strains, as well as some non-symbiotic forms, symbioses have been reconstituted. Various *Nostoc* strains were found to be able to reconstitute *Anthoceros, Phaeoceros* and *Blasia* symbioses, although many cyanobacterial strains, including some *Nostoc* strains were not (Ridgway, 1967; Rodgers and Stewart, 1977). Enderlin and Meeks (1983) found 14 different *Nostoc* strains to be competent in reconstituting the *Anthoceros* symbiosis but found some *Nostoc* and all *Anabaena* strains to be incompetent. Similarly, six different *Nostoc* isolates, both symbiotic and free-living, were found to infect *Gunnera* while others, including *Anabaena*, failed to infect (von Neumann et al., 1970; Bonnett and Silvester, 1981). In the case of *Azolla*, there are several claims of successful culturing of the cyanobiont but none have been able to reinfect *Azolla* in reconstitution experiments (Braun-Howland and Nierzwicki-Bauer, 1990). However, reconstitution of the *Azolla* symbiosis has been successfully achieved by placing the apical cap from a fertile megasporocarp containing the cyanobiont onto an excised megasporocarp (Lin et al., 1988). From the reconstitution experiments, it is obvious that the cyanobiont always belongs to the genus *Nostoc*, although the strains involved may vary. Similar conclusions have been drawn from DNA restriction mapping and hybridization experiments on cyanobionts from *Anthoceros, Azolla*, cycads and *Gunnera* (Meeks et al., 1988; Lindblad et al., 1989; Zimmerman and Bergman, 1990).

The reconstituted systems are of great value in studying the sequence of events during the development of the cyanobacterial-plant symbioses. Among the cyanobacterial-higher plant symbioses, the *Gunnera-Nostoc* symbiosis would be especially suitable for identifying symbiosis-specific plant genes, on a pattern similar to the *Rhizobium*-legume symbioses, due to its ease in reconstitution, relatively fast growth and the angiosperm nature of the host plant. The *Gunnera-Nostoc* symbiosis is currently being reconstituted in our laboratory under axenic conditions and the infection process followed.

Little is known about the recognition processes in cyanobacterial-higher plant symbioses. However, in lichens, bryophytes and *Azolla* symbioses, the host plant has been shown to synthesize lectins, i.e. proteins which specifically recognize sugar residues on the cyanobiont cell surfaces (Lockhart et al., 1978; Mellor et al., 1982; Kobiler et al., 1982; Stewart et al., 1983; Kardish et al., 1991).

5. The Infection Process in *Gunnera*

Our knowledge about the establishment of cyanobacterial plant symbioses is scant. This is probably due to the fact that only two of the symbioses are easily re-established under laboratory conditions, namely those involving bryophytes and *Gunnera*. Still, the infection process has only been studied in the *Gunnera-Nostoc* symbioses in any detail, starting with the pioneering work of Reinke at the end of the last century (Reinke, 1872, 1873).

In brief, the following is known (for reviews see Bonnett, 1990; Osborne et al., 1991; Bergman et al., 1992): the infection process starts by the attraction of the cyanobacterium to specific glands located on the *Gunnera* stems. The glands are formed at the base of each petiole. Their development is controlled by the plant and the first glands that develop are amenable for infection already at the cotyledonary stage (Fig. 2A). A typical characteristic of the glands is the secretion of a carbohydrate-rich acidic mucus which completely covers the surface. The cyanobacteria gather in the mucus, as do bacteria and fungi. The cyanobacteria enter the hormogonial stage, i.e. they differentiate into small-celled filaments, lacking heterocysts and with the ability to glide. The formation of hormogonia is probably a prerequisite for compatible strains (Bonnett and Silvester, 1981). Next, the hormogonia immigrate towards the interior of the gland through one of the several narrow channels which are formed through cell wall dissolutions at an early stage of gland development (Jönsson, 1894). The cyanobacteria are still accompanied by bacteria and fungi. Eventually, the hormogonia reach the cavity at the bottom of each channel. The cells lining the cavity are characterized by a dense cytoplasm, a large nucleus, numerous mitochondria and Golgi bodies and are possibly the origin of the mucus pouring out of the gland. In addition, the cells contain polyphenols (in small vacuoles), show mitotic activity, and are most likely the source of the hypothetical chemoattractant which causes the normally photoautotrophic cyanobacteria to glide towards the dark interior of the glands. The walls of these cells often appear highly folded, even in the absence of cyanobacteria (Schmidt, 1991). The *Gunnera* cell walls are subsequently dissolved in the proximity to cyanobacterial cells (Johansson and Bergman, unpublished). Whether the actual dissolution of the cell wall is induced by the plant itself, the cyanobacterium or the accompanying bacteria is still an open question. The cyanobacteria enter the "opened" *Gunnera* cells, but do not penetrate the host plasmalemma. New cell walls soon form around the infected cells. Since only cyanobacteria are seen intracellularly, a mechanism to discriminate between the cyanobacterium and other microorganisms must be involved, but is as yet not identified. Once inside the *Gunnera* cells, the

cyanobacterium divides and the hormogonia revert back to the aseriate stage with considerably enlarged, more spherical vegetative cells, and soon a rapid heterocyst differentiation commences (Fig. 2C). Meanwhile, the cyanobacterial infection spreads within the gland, and the infected tissue eventually reaches its ultimate size (Fig. 2B). The channels disappear, the surface of the gland turns brown and infection is no longer possible. The size of the infected tissue varies depending on the *Gunnera* species but always constitutes a minor fraction of the total *Gunnera* biomass.

6. Nitrogen Fixation and Transfer of Fixed Nitrogen

Cyanobionts in all the cyanobacterial-plant symbiosis studied so far, are known to fix nitrogen and transfer such fixed nitrogen to the host plant, meeting the full nitrogen requirements for its growth (Rai, 1990a). Nitrogen fixation by the cyanobionts in such symbioses is at a considerably higher rate than that in their free-living counterparts due to a higher heterocyst frequency (see below). However, the nitrogenase activity varies from younger to older symbiotic tissues of bryophytes (Rodgers and Stewart, 1977), *Azolla* (Hill, 1975, 1977), cycads (Lindblad et al., 1985) and *Gunnera* (Söderbäck et al., 1990). In several *Azolla* species, the nitrogenase activity increases with increasing heterocyst frequency, from nearly zero at the shoot apex to a maximum at about leaf number seven, and then declines in older leaves (Braun-Howland and Nierzwicki-Bauer, 1990). In cycads and *Gunnera* a similar pattern occurs with the nitrogenase activity increasing from the youngest to more mature parts but then declining in old parts (Lindblad et al., 1985; Söderbäck et al., 1990).

The increased nitrogenase activity of the cyanobionts, however, is not linear with the increase in heterocyst frequency (Rodgers and Stewart, 1977; Lindblad et al., 1985; Söderbäck et al., 1990). The explanation seems to be that in these symbioses there are numerous double and multiple heterocysts (Fig. 2C) which may restrict efficient photosynthate movement from vegetative cells to the heterocysts (Bergman et al., 1986). Indeed the rates of nitrogen fixation do seem to correlate with the frequency of single heterocysts (Lindblad et al., 1985). The lowering in activity in older symbiotic tissues is not due to reduced levels of nitrogenase (Söderbäck et al., 1990).

Detailed studies on *Anthoceros* (Meeks et al., 1985), *Blasia* (Stewart and Rodgers, 1977) and *Azolla* (Peters et al., 1980) have shown that the cyanobionts in these symbioses release fixed nitrogen in the form of ammonia (Table 2). Such ammonia release is due to decreased levels of glutamine synthetase (GS) in the cyanobiont, particularly in heterocysts (Table 2; Meeks,

Table 2. Heterocyst frequencies and glutamine synthetase protein levels and activities in the cyanobionts

Host	Heterocyst frequency[a]	Glutamine synthetase			N-compound released
		Amount of protein[b]	Relative distribution[c]	Specific activity[d]	
bryophytes	30–50%	~ 86%	reduced in heterocysts	~ 38%	NH_4^+
Azolla	30–40%	5–10%	reduced in heterocysts	5–10%	NH_4^+
cycads	40–45%	100%	normal	100%	n.d.[e]
Gunnera	60–80%	100%	reduced in heterocysts	70%	n.d.

n.d. = not determined.

[a] heterocyst frequency, % of total cells

[b] Percentage of the GS protein levels in free-living cyanobacteria

[c] normal = a two-fold higher GS protein level in heterocysts compared to in vegetative cells

[d] Percentage of GS activities in free-living cyanobacteria

[e] Possibly citrulline and/or glutamine (see text and Pate et al., 1991).

1990; Braun-Howland and Nierzwicki-Bauer, 1990; Rai et al., 1989). However, in cycads and *Gunnera*, the form in which fixed nitrogen is released by the cyanobiont, and the reasons for such a release, are not known. Pathways of ammonia assimilation have been studied in *Azolla* and *Anthoceros* symbioses. In both cases, the glutamine synthetase-glutamate synthase (GS-GOGAT) pathway is the route of primary ammonia assimilation in the cyanobiont as well as in the host plant (Meeks et al., 1983, 1985; Peters et al., 1985).

In *Gunnera*, the intracellular location of the cyanobiont means that the nitrogen released by the cyanobiont would be directly available for assimilation in the host cell cytoplasm, enabling a more efficient utilization than that in the symbioses where the cyanobiont is extracellular. However, in the latter cases, special structures produced by the host plants may facilitate an equally efficient uptake of the released nitrogen. Such structures include finger-like protrusions in bryophytes (Rodgers and Stewart, 1977; Duckett et al., 1977), hair cells in *Azolla* (Fig. 1E; Duckett et al., 1977; Peters et al., 1978; Calvert et al., 1985) and elongated cortical cells traversing the cyanobiont zone in cycads (Wittman et al., 1965; Lindblad et al., 1985), all of which show transfer cell characteristics.

The form in which nitrogen moves from the symbiont tissue to the rest of the plant has been studied in cycads and *Azolla* symbioses only. In cycads, glutamine and citrulline are the compounds moving from the roots to the

stem, as shown by analysis of the xylem sap (Pate et al., 1988). In *Azolla*, ^{13}N experiments suggest movement of glutamate, glutamine, ammonia and a glutamate derivative from the nitrogen-fixing cavities to the stem apex (Peters et al., 1985).

7. Structural-Functional Modifications

In all the cyanobacterial-plant symbioses a balance between the symbionts is always maintained during growth, preventing one partner from outgrowing the other (Stewart et al., 1983). For example, in several species of *Azolla* the cyanobionts' growth is synchronized with the host (Hill, 1977; Braun-Howland and Nierzwicki-Bauer, 1990). In the cycad *Zamia* and the liverwort *Blasia* a linear relationship exists between the biomass of the symbiotic tissue and the plant biomass (Rodgers and Stewart, 1977; Halliday and Pate, 1976). Similarly, in *Gunnera magellanica*, a progressive increase in cyanobiont biomass occurs from younger to older stem parts (Söderbäck et al., 1990).

In symbiosis, the cyanobiont undergoes structural and metabolic modifications which enable a close interaction between the partners (Rai, 1990b). Such changes may vary among different symbioses as well as in different parts within the same symbiosis, depending on the age of the symbiotic tissue. Some of these are discussed in more detail below.

Heterocysts and nitrogenase

In free-living *Nostoc* strains, the heterocyst frequency is 5–6% (Stewart, 1980). However, in symbiosis the heterocyst frequency increases remarkably (Table 2). In the cyanobionts of the bryophytes *Anthoceros* and *Blasia* heterocyst frequencies of up to 40% are common (Rodgers and Stewart, 1977; Duckett et al., 1977; Enderlin and Meeks, 1983). There is evidence that the extent of increase in heterocyst frequency varies, depending on the colony age. For example, in *Blasia pusilla*, cyanobionts in two-week old colonies had a 20% heterocyst frequency while those in six-week old colonies had 48% (Rodgers and Stewart, 1977). Similarly, in *Azolla*, the heterocyst frequency increases from zero in non-heterocystous hormogonia in young leaves to 30–40% in mature leaves (Hill, 1975). Heterocyst frequencies of up to 46% and about 65–80%, including changed spacing pattern and the formation of double and multiple heterocysts, occur in cycads and *Gunnera*, respectively (Fig. 2C; Lindblad and Bergman, 1990; Bonnett and Silvester, 1981; Söderbäck et al., 1990). As in other symbioses, a gradient in heterocyst frequency exists in cycads and *Gunnera*, being lowest at the growing tip and higher in older parts (Lindblad et al., 1985; Silvester, 1976; Söderbäck et al., 1990).

The precise reason for the increase in heterocyst frequencies of the cyanobionts is not known. Since the cyanobionts do not show nitrogen limitation, as evident from the presence of cyanophycin granules and phycobiliproteins (see below), other explanations are needed. It is interesting to note here that higher heterocyst frequencies (13–20% as against 4–6%) can be induced in free-living cyanobacteria using various altered culture conditions. These include immobilization (Shi et al., 1987), green light illumination (Guoliang et al., 1982), fructose supplementation (Braun-Howland and Nierzwicki-Bauer, 1990), calcium limitation (Zhao et al., 1991) and phosphate limitation (Fredriksson and Bergman, unpublished results). Although such increases do not match the increases in heterocyst fequencies of cyanobionts, further studies may be worthwhile in mimicking the symbiotic state of the cyanobiont.

Heterocysts are the sites of nitrogen fixation in heterocystous cyanobacteria (Stewart, 1980). Consistent with this view, nitrogenase has been found to be located only in heterocysts of the cyanobionts (Bergman et al., 1986; Braun-Howland et al., 1988; Rai et al., 1989; Söderbäck et al., 1990). All heterocysts examined have been found to contain nitrogenase protein, including the multiple heterocysts (Bergman et al., 1986; Rai et al., 1989). Although the levels of nitrogenase protein in heterocysts of cyanobionts from younger and older parts of the symbioses have not been compared in earlier studies, Söderbäck et al. (1990) have only found a small decrease in nitrogenase levels of heterocysts in older parts.

Glutamine synthetase

GS is the primary ammonia-assimilating enzyme in cyanobacteria (Stewart, 1980). In cyanobacterial-lower plant symbioses (bryophytes and *Azolla*), the GS activity is greatly decreased in the cyanobiont (Table 2; Ray et al., 1978; Joseph and Meeks, 1987; Rai et al., 1989). It is likely that the maximal reduction in GS in the cyanobiont may coincide with maximal nitrogenase activities and ammonia production in the middle parts.

The decrease in GS activity has been found to be due to the repression of GS synthesis in lichen and *Azolla* cyanobionts (Stewart et al., 1983; Orr and Haselkorn, 1982; Nierzwicki-Bauer and Haselkorn, 1986) but due to a post-translational modification of the synthesized enzyme in the cyanobionts of *Anthoceros* (Joseph and Meeks, 1987; Rai et al., 1989). However, in all these symbioses, the common pattern has been the reduction in GS protein levels of the heterocysts, making it similar to that in vegetative cells (Table 2; Hällbom et al., 1986; Rai et al., 1989; Bergman and Rai, 1989). This is in contrast to the pattern found in free-living cyanobacteria, where heterocysts have twice as high

GS activities and protein levels as the vegetative cells (Wolk, 1982; Bergman et al., 1985). Such enhanced levels of GS in heterocysts are correlated to nitrogenase expression and necessary for assimilation of N_2-derived ammonia in heterocysts (Renström-Kellner et al., 1990). Similar levels of GS in heterocysts and vegetative cells of the cyanobiont therefore suggests an uncoupling of the expression of nitrogenase-encoding *nif* and GS-encoding *gln*A genes, and may explain the cyanobiont's inability in assimilating all the ammonia produced during nitrogen fixation which is then released to the host plant. This aspect needs further study to understand how the two processes are uncoupled.

In contrast to the above observations, GS levels and activities in the cycad cyanobionts have been found to be unaffected with GS activities, protein levels, and patterns of distribution between heterocysts and vegetative cells, being similar in the cyanobionts and their free-living counterparts (Table 2; Lindblad and Bergman, 1986).

RuBisCO and phycobiliproteins

Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) is the primary carboxylating enzyme in cyanobacteria (Stewart, 1980). In bryophytes (Rodgers and Stewart, 1977; Steinberg and Meeks, 1991) and *Azolla* (Peters et al. 1985; Braun-Howland and Nierzwicki-Bauer, 1990) the cyanobiont receives fixed carbon from the host plant, probably in the form of sucrose, its own carbon fixation being able to satisfy its needs only partially. Similar suggestions have been made in the case of cycads (Lindblad and Bergman, 1990) and *Gunnera* (Silvester, 1976; Bonnett, 1990), although the form in which the fixed carbon moves from the host to the cyanobiont is not known. While it is understandable that in cycads and *Gunnera*, where the cyanobiont occurs deep inside the tissue, lack of illumination may explain the need for photosynthate from host, in *Azolla* and bryophytes, the reduced carbon fixation ability of the cyanobiont is as yet unexplained, since RuBisCO apparently occurs in all the cyanobionts (Lindblad et al., 1987; Rai et al., 1989; Söderbäck and Bergman, 1992; Bergman et al., unpublished results). Studies by Söderbäck and Bergman (1992) show a reduction in the levels of carboxysomal RuBisCO in the cyanobiont from older parts of the *Gunnera* stem as compared to that from the younger parts. However, similar studies are lacking in other symbioses.

The phycobiliproteins, phycoerythrin (PE) and phycocyanin (PC), which are photosynthetic accessory pigments and also represent nitrogen reserves in cyanobacteria, are also present in the cyanobionts (Rai et al., 1989; Meeks, 1990; Braun-Howland and Nierzwicki-Bauer, 1990, Lindblad and

Bergman, 1989; Söderbäck and Bergman, 1992). This together with the fact that cyanophycin granules, another nitrogen reserve polymer, also occurs in cyanobionts (Rai et al., 1989; Söderbäck et al., 1990; Lindblad et al., 1985; Hill, 1977; Neumuller and Bergman, 1981) suggests that the cyanobionts are not nitrogen-starved in the symbiosis. However, it is not known whether such nitrogen reserves vary in different parts of the symbiosis except in *Gunnera magellanica*. Here, PE as well as PC levels in the cyanobionts were found to be similar to those in their free-living counterparts, and the levels did not vary in cyanobionts from different parts of the *Gunnera* stem (Söderbäck and Bergman, 1992).

8. Future Research

Although considerable progress has been made in physiological and biochemical characterization of cyanobacterial-plant symbioses, several key questions remain to be answered. In addition, genetic characterization of these symbioses, especially of the host plant, have not even begun. We list below some of the aspects which in our view deserve immediate attention.

1. Identification and analysis of symbiosis-specific plant and cyanobiont genes.
2. Recognition mechanisms involved in the selection of cyanobiont by the host.
3. Mechanisms involved in the development of a high heterocyst frequency in the cyanobiont.
4. Forms and mechanisms of photosynthate translocation from the host plant to the cyanobiont.
5. Forms of fixed nitrogen being transferred from the cyanobiont to the host plant in cycads and *Gunnera* symbioses.
6. Development of a method to isolate clean cyanobiont cells in sufficient quantity from the *Gunnera* glands to enable biochemical and genetic analysis.
7. Regulatory aspects of sporocarp development and germination in *Azolla* to enable development of improved *Azolla* strains for large scale application in rice cultivation.
8. Mechanisms involved in the uncoupling of *nif* expression from *gln*A in heterocysts of lower plant cyanobionts to enable manipulation of free-living cyanobacteria for photobiological production of ammonia and for agricultural applications such as biofertilizers.

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